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ORIGINAL ARTICLE

Functionality of exopolysaccharides produced by lactic acid bacteria in an *in vitro* gastric system

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Abstract

Aims: To evaluate whether slime-exopolysaccharides (EPS) or capsular-polysaccharide (CPS) production could protect the polymer-producing strains *Streptococcus thermophilus* CRL 1190 and *Lactobacillus casei* CRL 87 against the harsh conditions of an *in vitro* gastric system (GS). EPS stability on the GS was studied.

Methods and Results: An *in vitro* GS model containing human saliva and gastric juice was standardized. Polymer functionality on the cell viability and metabolic activity of the EPS-producing strains in the GS acidic conditions was evaluated. Two isogenic EPS/CPS deficient mutants were used for comparison. EPS or CPS conferred no significant protection on the cell viability of the studied strains after passage through the GS conditions. However, the phospho- and β-galactosidase activities of the EPS^+ strains were higher than those of the EPS^-. Cytoplasmic alterations in the wild-type and mutant strains and partial degradation of both EPS were detected.

Conclusions: The presence of EPS/CPS protected the metabolic activity of the assayed LAB strains, but had no effect on survival at low pH.

Significance and Impact of the Study: The presence of EPS/CPS as well as polymer resistance to the harsh conditions of the human GS could impact positively in probiotic strains to exert their properties in the host.

Introduction

The ability to produce exopolysaccharides (EPS) by micro-organisms is widespread in nature. Lactic acid bacteria (LAB) are able to produce EPS, either capsular polysaccharides (CPS) if they are tightly associated with the cell surface or slime-EPS if they are secreted into the extracellular environment. These biopolymers are composed of one type of monosaccharide (homopolysaccharides, HoPS) or formed by repeating units of two or more different monosaccharides (heteropolysaccharides, HePS) (De Vuyst and Degeest 1999). A large diversity of HePS from LAB exists regarding their chemical characteristics, yields and functionalities (Vaningelgem *et al.* 2004; Mozzi *et al.* 2006).

EPS from LAB have shown important rheological properties and are currently used in the food industry as a source of natural thickeners and stabilizing ingredients (De Vuyst and Degeest 1999; Ruas-Madiedo *et al.* 2002). The *in situ* production of EPS plays an important role in the manufacture of a diversity of fermented dairy products, such as yogurt, drinking yogurt, cheese, cultured cream and milk-based desserts (Doleyres *et al.* 2005).

Although their biological role is not well understood, EPS are believed to be involved in bacterial protection against adverse environmental conditions, such as desiccation, phagocytosis, antibiotics or bacteriophages, to sequester essential cations and to be involved in adhesion and biofilm formation (Looijesteijn *et al.* 2001; Ruas-Madiedo *et al.* 2002).

Additionally, beneficial effects for the human health have been assigned to these EPS, such as cholesterol-lowering, antitumoural or immunomodulating activities (Oda *et al.* 1983; Kitazawa *et al.* 1991a; Nakajima *et al.*

1992) as well as other immunological functions, such as proliferation of T-lymphocytes (Forsén *et al.* 1987), macrophage activation and induction of cytokine production (Kitazawa *et al.* 1991b, 1996). Also, Şengül *et al.* (2005) found that EPS-producing probiotic strains significantly attenuated experimental colitis in rats. Recently, Ruas-Madiedo *et al.* (2006) evaluated the functionality of EPS produced by probiotic LAB on bacterial adhesion properties; the competitive exclusion of probiotics in the presence of EPS suggested the involvement of these polymers in adhesion to mucus. Nagaoka *et al.* (1994) reported on the anti-ulcer effects of EPS from certain bifidobacteria, lactobacilli and streptococci strains. This effect was also observed with cell wall polysaccharides, particularly those with rhamnose content higher than 60%, suggesting that this monosaccharide might be involved in the anti-ulcer effect. The effect of food-associated EPS on human health depends on their digestive fate (Ruijssenaars *et al.* 2000). In some cases, EPS can act as prebiotics (Gibson and Roberfroid 1995; Korakli *et al.* 2002) if they are degraded by the beneficial microbiota of the colon resulting for example in short-chain fatty acids formation, which play a role in protection against colon rectal cancer. If the EPS are not degraded, they can offer protection against colorectal cancer by increasing the stool bulk or by specific adsorption of carcinogens (Harris and Ferguson 1993). Thus, the biodegradability of the EPS is an important feature in relation to its functional effect (Ruijssenaars *et al.* 2000).

To be able to function as a probiotic, bacteria have to survive the passage through the gastrointestinal tract. Several bacterial species are unable to survive the harsh conditions in the stomach, while others can survive the passage by using different defense mechanisms such as changes in gene expression and phenotype (Wall *et al.* 2007).

The aim of this study was to evaluate whether a specific phenotype such as the EPS or CPS production could protect the EPS-producing strains *Streptococcus thermophilus* CRL 1190 and *Lactobacillus casei* CRL 87 through the harsh conditions of an *in vitro* gastric system. The degradability of the biopolymers submitted to this system was also evaluated. This study attempts to provide valuable information on the functionality of EPS or CPS, linked or not to the producing-strains, to further exert their specific beneficial properties in the host.

Materials and methods

Micro-organisms, media and culture conditions

The CPS/EPS-producing strains *Strep. thermophilus* CRL 1190 and *Lact. casei* CRL 87 were obtained from the Culture Collection of the Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina). In milk cultures, *Strep. thermophilus* CRL 1190 synthesizes a high (arbitrarily defined as $>1 \times 10^6$ Da) molecular mass (MM) slime-EPS composed of galactose and glucose (1·5 : 1·0), and *Lact. casei* CRL 87 produces a low ($<1 \times 10^6$ Da) MM polymer composed of rhamnose, glucose and galactose (4·0 : 2·0 : 1·0) (Mozzi *et al.* 2006). The isogenic mutant strains *Lact. casei* M7 (CPS^-/EPS^-) and *Strep. thermophilus* M16 (CPS^-, reduced-EPS-producer) were isolated from the EPS/CPS-producing strains *Lact. casei* CRL 87 and *Strep. thermophilus* CRL 1190, respectively, according to Mozzi *et al.* (2001a). The mutant strain M16 produces a slime-EPS with similar MM and monomeric composition to that of the wild type strain (Rodríguez *et al.* 2008).

Strains were sub-cultured at least twice in MRS broth and transferred in reconstituted skim milk (RSM, 10% w/v) using 1% (v/v) inoculation prior to experimental use. Milk cultures were incubated at 37°C for 16 h. Strains were stored at −20°C in RSM (10% w/v), containing 1% (w/v) glucose, 0·5% (w/v) yeast extract and 10% (v/v) glycerol.

In vitro gastric system (GS) model

An *in vitro* gastric system model was adapted from those described by other authors (Mouécoucou *et al.* 2004; Versantvoort *et al.* 2004; Corcoran *et al.* 2005) to simulate the passage through the mouth and stomach. The following components were used: (i) human total saliva (6 ml), sterilized at 121°C for 15 min with human α-amylase (Sigma-Aldrich, St Louis, MO, USA) added to a final concentration of 0·76 mg ml^{-1} (the pH of this sterile mixture was 7·7). The exogenous addition of α-amylase was needed as no enzyme activity was detected (Amilasa 405 Test, Wiener Lab., Rosario, Argentina) after autoclaving the human saliva; (ii) artificial gastric juice (12 ml), composed of (g l^{-1}) KCl, 1·12; NaCl, 2·00; $CaCl_2$, 0·11; KH_2PO_4, 0·40; and mucin, 3·50, adjusted at a final pH of 2·0 with HCl and sterilized at 121°C for 15 min. Pepsin (Sigma-Aldrich), used at 0·1 mg ml^{-1} final concentration, was added prior to experimental use and (iii) sample (9 ml), either consisting of: (a) fermented RSM, by inoculating separately (1% v/v) the following strains: *Strep. thermophilus* CRL 1190, *Strep. thermophilus* M16 (cell count each: 3×10^8 CFU ml^{-1}), *Lact. casei* CRL 87 or *Lact. casei* M7 (cell count each: 3×10^9 CFU ml^{-1}) and incubated for 16 h at 37°C (final pH 4·4) or (b) 10 mg of the purified and freeze-dried EPS resuspended in 9 ml of RSM with a final pH of 4·4 adjusted with pure lactic acid (Sigma-Aldrich).

The sample (fermented RSM or EPS solution) was incubated with saliva at 37°C for 2 min (final pH 4·6), then gastric juice was added (final pH of 4·25) and the mixture was shaken for 30 min at 37°C to adapt the micro-organisms to the acidic conditions of the system. Finally, the mixture was acidified to different pH values in a range of 2·0–3·0 within 2 h by adding HCl (1·6 or 12 N). The acidification was performed either fast (by adding the acid at once or in four steps) or slowly (5·34 μl min^{-1}) by using a peristaltic pump (Peristaltic Pump P-3, Pharmacia Fine Chemicals, Uppsala, Sweden).

Effect of the GS on survival and metabolic activities of EPS/CPS-producing LAB strains from RSM cultures

Cell viability

Cell viability was determined by pour plating in MRS and LAPTg agar of appropriate dilutions of the cultures, made in peptone water (0·1%, w/v). Agar plates were incubated at 37°C for 48 h and the number of colony forming units (CFU) ml^{-1} were determined.

Metabolic activity

Preparation of cell-free extracts

Cells of LAB strains grown in milk at 37°C for 16 h, before and after being subjected to the *in vitro* GS, were harvested by centrifugation at 10 000 ***g*** for 10 min at 4°C, washed twice and resuspended in 0·1 mol l^{-1} sodium phosphate buffer (pH 7·0). Cells were disrupted with glass beads (0·10 mm, Glass beads; Biospec products Inc., Bartesville, OK), using a cell : phosphate buffer : beads ratio of 1 : 2 : 1, respectively, and mixed for 5 min with 1 min interval on ice after each minute of mixing. Glass beads and cell debris were removed by centrifugation (10 000 ***g*** 10 min, 4°C) and the supernatant fluid was used as cell-free extract (CE). The amount of protein in the CE was estimated according to Bradford (1976), using bovine serum albumin as standard. CE were maintained on ice and immediately used for enzyme activity determinations.

Enzyme activities

All *in vitro* assays were performed at 37°C in VersaxMax Tunable Microplate Reader (Molecular Devices, USA) using a final volume of 200 μl with freshly prepared CE as samples.

The activities of phospho-β-galactosidase (P-β-Gal) and β-galactosidase (β-Gal) were determined according to a modified method described by Miller (1972). For measuring the P-β-Gal activity, the reaction mixture containing 150 μl CE and 350 μl 50 mmol l^{-1} sodium phosphate buffer (pH 7·0) was incubated at 37°C for 10 min and 100 μl of the substrate *o*-nitrophenyl-α-D-galactopyranoside-6-phosphate (ONPG-6P; Sigma-Aldrich) (4 mg ml^{-1}) was added and incubated at 37°C for another 15 min. The reaction was stopped by adding 250 μl of 0·5 mol l^{-1} Na_2CO_3.

For determining β-Gal activity, the reaction mixture contained the following: 50 μl of CE plus 450 μl 50 mmol l^{-1} sodium phosphate buffer (pH 7·0) and was incubated at 37°C for 10 min and 100 μl of the substrate *o*-nitrophenyl-α-D-galactopyranoside (ONPG; Sigma-Aldrich) (4 mg ml^{-1}) was added and incubated at 37°C for 15 min. The reaction was stopped by adding 250 μl of 0·5 mol l^{-1} $NaCO_3$.

Samples were then centrifuged and the absorbance was determined (420 nm) in the supernatants. Enzyme activity was defined as nmoles of released ONP min^{-1} ml^{-1} and the specific enzyme activity was expressed as nmoles ONP min^{-1} mg of cell $protein^{-1}$.

All enzymatic measurements were carried out at least in triplicate and results were expressed as mean values with standard deviations.

Electronic microscopy determinations

Scanning electron microscopy (SEM)

Cells were harvested by centrifugation (10 000 ***g***, 10 min) and were resuspended in 1 v of 0·1 mol l^{-1} phosphate buffer (pH 7·4) containing 2·5% (v/v) glutaraldehyde and 1% (w/v) $CaCl_2$. The mixture was centrifuged (3000 ***g***, 5 min) and maintained at 4°C for 16 h. The prepared samples were included in agar and fixed in phosphate buffer containing 1% (wt/v) osmium tetraoxide at 20°C for 2 h. Dehydratation was performed using an ascendant concentration serial dilution of ethanol (30%, 50%, 70%, 85%, 90%, 100%) and dried with 100% acetone. The samples were set on metal supports and covered with a thin gold layer (200 Å) by using an ion-sputter. The electronic microscope observation was performed with JEOL 35 CF Microscope at the LAMENOA facilities (Instituto de Química Biológica, Facultad de Bqca., Qca. y Fcia., Universidad Nacional de Tucumán, Argentina).

Transmission electronic microscopy (TEM)

Cells were prepared and fixed in agar as described above. A second fixation was performed with 1 v of uranyl acetate for 40 min. Samples were dehydrated with alcohol and 100% acetone, included in Spurr medium (Spurr 1969) and stained according to Reynolds (1963). The electronic microscopy observation was carried out with a Zeiss EM 109 microscope at the LAMENOA facilities.

Stability of EPS produced by LAB submitted to the GS conditions

EPS isolation and purification

EPS isolation was carried out from two kind of samples submitted to the GS (see above): (i) 16 h-old RSM cultures (1%, v/v inoculum) incubated at 37°C and (ii) purified EPS (10 mg) resuspended in RSM. EPS formation was determined as CPS or slime-EPS. CPS formation was evaluated by the Indian ink negative staining technique (Mozzi *et al.* 2001b), and slime-EPS was isolated according to a modification of the method described by De Vuyst *et al.* (1998) in that three volume of ethanol were used instead of one volume of acetone. Further purification of the EPS was carried out by dissolving crude EPS in ultra pure water, adjusting to pH 7·0, and dialyzing against distilled water at 4°C for 3 days, with water replacement twice a day. For dialysis, cellulose membranes (Sigma-Aldrich) with a cut off of 12 000 Da were used. The purified EPS were freeze-dried and further characterized (see below).

Determination of the molecular mass (MM)

To evaluate the stability of the EPS through the acidic GS, purified and freeze-dried EPS were dissolved in ultra pure water at a concentration of 10 mg ml^{-1}. The MM of the EPS was determined by gel permeation chromatography (GPC) with a Knauer Wellchrom HPLC System (Knauer, Berlin, Germany), equipped with a Waters Ultrahydrogel column (within a range of 500 to $8{\cdot}0 \times 10^6$ Da) using 0·1 mol l^{-1} $NaNO_3$ (Sigma-Aldrich) as eluent at a flow rate of 0·6 ml min^{-1}. The column was previously calibrated with a series of dextran standards of different MM ($4{\cdot}9 \times 10^6$; $1{\cdot}4 \times 10^6$; $6{\cdot}7 \times 10^5$; $4{\cdot}1 \times 10^5$; $2{\cdot}7 \times 10^5$; $1{\cdot}5 \times 10^5$ and $8{\cdot}0 \times 10^4$ Da) (Sigma-Aldrich). The polysaccharide content was determined online with a Knauer refractive index detector at room temperature.

Statistics

All assays were carried out in triplicate and results were expressed as mean values with (±SD) standard deviations. Data were statistically evaluated by analysis of variance (ANOVA) and the Tukey comparison test with the INFOSTAT/Professional ver. 3 2007 software (Facultad de Cs. Agropecuarias, Estadìstica y Diseño, Universidad Nac. de Córdoba, Argentina). Differences were considered statistically significant at $P < 0{\cdot}05$. The MM determinations were performed in duplicate and results were presented as mean with error percentage bars.

Results

In vitro GS model

An *in vitro* GS model was standardized to evaluate whether the presence and the structural characteristics of the EPS and CPS could protect the cell viability and metabolic activity of the EPS-producing strains *Strep. thermophilus* CRL 1190 and *Lact. casei* CRL 87 under the acidic gastric conditions (Table 1); isogenic mutants derived from these LAB strains were included for comparison. The stability of the EPS in the GS model was also evaluated. The mixture composed of sample (fermented RSM or EPS solution), saliva and gastric juice had a final pH value of 4·25. To approximate the physiological conditions of the stomach after a meal (pH range within 2·0–3·5), the pH of the mixture was decreased until final values of 2·0–3·0 by adding HCl at different concentrations (1·6 or 12 N) either manually or by using

Table 1 Cell viability and enzyme activity of the lactic acid bacteria strains before (pH 4·4) and after (pH 3·0) being submitted to GS conditions

Viability and enzyme activity	*Streptococcus thermophilus*		*Lactobacillus casei*	
	CRL 1190	M16	CRL 87	M7
Cell viability*				
pH 4·4	8·59 ± 0·08	8·68 ± 0·13	9·50 ± 0·06	9·50 ± 0·02
pH 3·0	7·39 ± 0·08	8·04 ± 0·15	9·01 ± 0·09	8·81 ± 0·14
β-gal†				
pH 4·4	29·80 ± 3·02	31·05 ± 3·20	0·10 ± 0·01	0·37 ± 0·02
pH 3·0	13·20 ± 1·20	10·32 ± 0·98	0	0
P-*β*-gal†				
pH 4·4	ND	ND	0·32 ± 0·04	1·18 ± 0·13
pH 3·0	ND	ND	3·86 ± 0·45	2·81 ± 0·35

*Expressed as log CFU ml^{-1}.

†Expressed as ONP nanomoles min^{-1} mg $protein^{-1}$.

*,†Mean values from three independent experiments and standard deviations (±) are given.

ND, not detected.

a peristaltic pump. Survival of the assayed strains was only found at pH value of 3·0 by adding 1·6 N HCl with a peristaltic pump at a flow rate of 5·34 μl min^{-1}. The decrease in cell viability (14–6%) for each strain with respect to the initial cell count, because of the assayed acidic conditions of the GS model, was statistically significant ($P < 0{\cdot}05$). These conditions were further used to evaluate the acidic gastric environment on survival and metabolism of the EPS-producing strains as well as the degradability of their EPS.

Effect of the presence of EPS on cell viability and metabolic activity of the EPS-producing strains in the GS model

The *Lact. casei* strains showed a different behaviour than the *Strep. thermophilus* strains during the passage through the GS model (Table 1). *Lact. casei* CRL 87 (EPS$^+$/CPS$^+$) displayed cell viability similar to its mutant M7 after the passage through the GS while the mutant *Strep. thermophilus* M16 (CPS$^-$) displayed a higher cell viability (6%) than the parental strain. However, the observed differences for the wild type and mutant strains for each species were not significant ($P > 0{\cdot}05$). No differences between the two culture media assayed (MRS or LAPTg) were found with respect to cell viability.

The enzymes β-Gal and P-β-Gal, responsible for the hydrolysis of lactose, were used as markers to indicate the metabolic activity of the strains after passage through the gastric conditions. *Streptococcus thermophilus* CRL 1190 and M16 showed 44% and 32% of residual β-Gal activity, respectively, while no residual activity was detected for the strains of *Lact. casei* (Table 1). P-β-Gal activity, as expected, was only detected in the *Lact. casei* strains as this enzyme is not present in *Strep. thermophilus* species. The CPS$^+$/EPS$^+$ strains conserved the enzyme activity more than their mutant strains although the differences were not statistically significant ($P > 0{\cdot}05$). P-β-Gal activity values were higher after passage through the GS for both the wild type and mutant *Lact. casei* strains.

β-Gal and P-β-Gal activities were measured in culture supernatants before and after the submission to the GS. No detectable activity values were found in the supernatants suggesting that cell lysis did not occur.

Electronic microscopy determinations

Possible structural cell changes of the strains submitted to the GS were evaluated through SEM and TEM. The scanning electron micrographs of the strains *Strep. thermophilus* M16 and *Lact. casei* CRL 87 and M7 did not show any structural modifications after the gastric treatment. However, the *Strep. thermophilus* CRL 1190 cells showed irregular surfaces with respect to the cells before the treatment (Fig. 1a,b).

Remarkable differences between the treated and non treated cells of both species were detected by TEM (Fig. 2a–d). In all cases, independently of the presence of CPS or EPS, no cell wall damage, but cytoplasmic alterations such as light and dark zones and increase of membranous structures were observed.

Susceptibility of EPS to be degraded under the GS conditions

Degradability of the EPS produced by the strains *Strep. thermophilus* CRL 1190 and *Lact. casei* CRL 87 under the

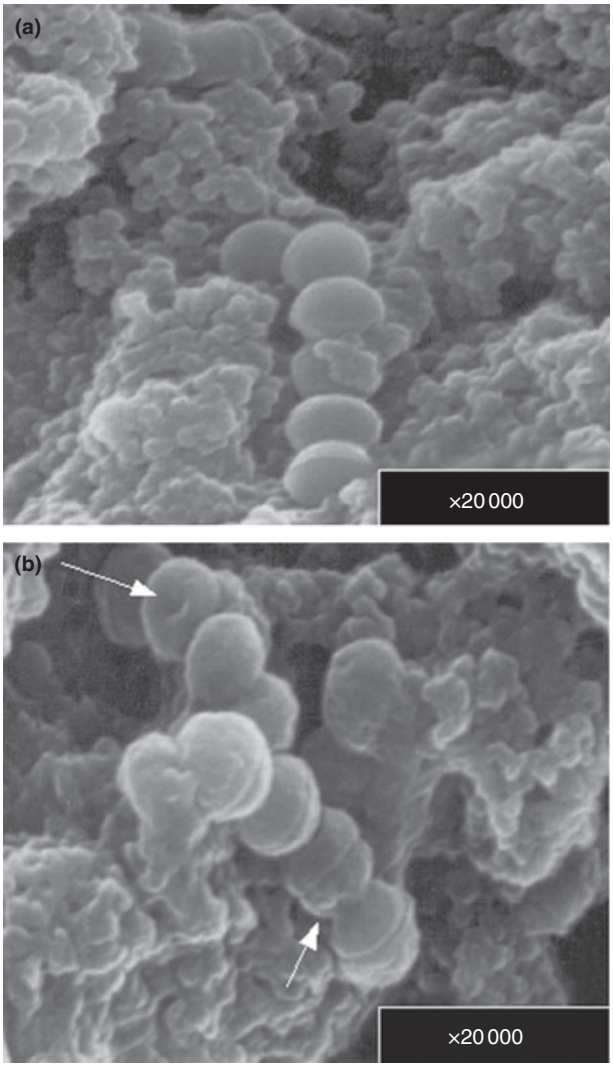

Figure 1 Scanning electron micrographs of *Streptococcus thermophilus* CRL 1190 in fermented RSM samples (a) before and (b) after passage through the GS model. Arrow in (b) indicates irregular cell surface.

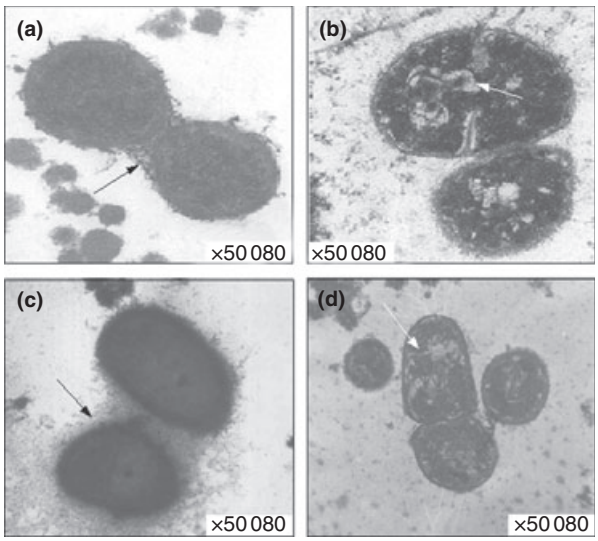

Figure 2 Transmission electron micrographs of LAB strains in fermented RSM samples: *Streptococcus thermophilus* CRL 1190 (EPS^+/CPS^+) (a) before and (b) after submitting the cells through the GS, and *Lactobacillus casei* CRL 87 (EPS^+/CPS^+) before (c) and after (d) the GS treatment. Arrows in (a, c) indicate CPS formation and in (b, d) increase of membranous structures at cytoplasmic level.

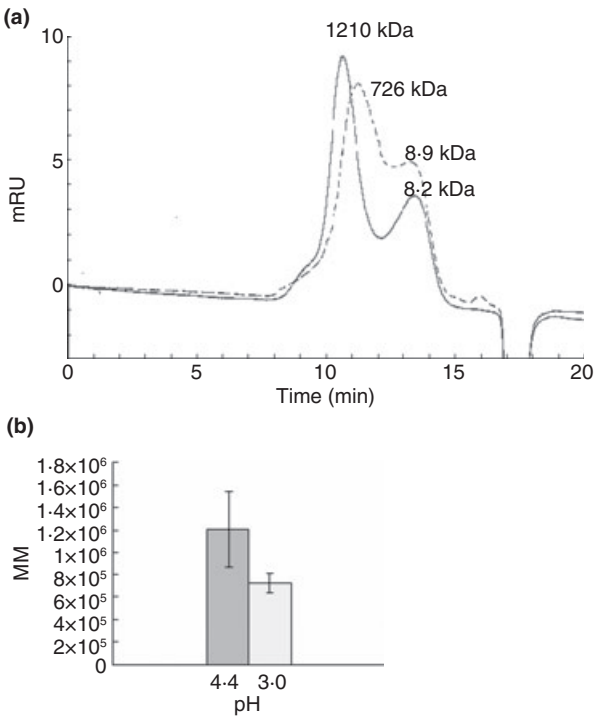

Figure 3 (a) Gel filtration chromatogram of the EPS produced by *Streptococcus thermophilus* CRL 1190 before (—) and after (- - - -) passage through the GS and (b) MM of the EPS expressed in Da before (pH 4·4) and after (pH 3·0) the passage through the GS.

harsh conditions of the GS employed was evaluated by possible modifications in their MM and/or gel filtration chromatogram profile obtained before and after the acidic treatment. Both EPS (isolated and purified as described earlier) were partially degraded after submission to the GS (Figs 3 and 4). The EPS from *Strep. thermophilus* CRL 1190 showed two peaks of 1210 and 8·9 KDa, which decreased to 726 and 8·2 KDa, respectively, after the acidic challenge while the MM of the EPS from *Lact. casei* CRL 87 was 442 and of 299 KDa before and after the GS treatment respectively. In the first case, the area of the highest peak decreased from 15·25 to 14·61 while the second one increased within the same range (from 7·61 to 8·27). The area of the EPS from the strain CRL 87 decreased from 28·44 to 24·30. Thus, the results showed a reduction of the MM of the EPS from both strains CRL 1190 and CRL 87 comprised in a range of 30–40%.

Discussion

The metabolic activity of the LAB strains assayed was measured through the β-gal and P-β-gal enzymes, which are responsible for the hydrolysis of lactose. Lactose uptake in *Strep. thermophilus* is mediated by an ATP-dependent permease or a galactose-lactose antiporter system; inside the cell lactose is further hydrolysed by β-gal into glucose and galactose. Generally, this LAB species lacks of the P-β-gal enzyme (Hutkins and Ponne 1991). In *Lact. casei*, however, lactose is mainly transported through a phosphoenolpyruvate-phosphotransferase system (PEP-PTS). In this case, the galactose moiety of lactose is phosphorylated at position 6 while entering into the cell (as lactose-P) and then is hydrolysed by a cytoplasmic P-β-gal (Bettenbrock and Alpert 1998). The activity of β-gal and P-β-gal was evaluated in both species. As expected, no P-β-gal activity was detected in the *Strep. thermophilus* strains. In contrast, the β-gal activity was lower than the P-β-gal in the *Lact. casei* strains. After submitting the cells to the harsh conditions of the GS model, no residual β-gal activity was found in the *Lact. casei* strains while the P-β-gal activity was higher, probably because of the induction of the enzyme activity under acid conditions. In previous studies (Torino *et al.* 2002), we have found that the *galE* gene, encoding the key enzyme UDP- galactose 4-epimerase in the sugar nucleotide synthesis for EPS formation, was induced in *Lact. casei* CRL 87 under acidic (constant pH = 5·0) culture conditions with respect to cells grown at free pH. Recently, Wall *et al.* (2007) found a differential expression of genes when examining the response of *L. reuteri* ATCC 55730 after a shift in environmental acidity to a pH close to the human stomach. The induced genes, encoding a

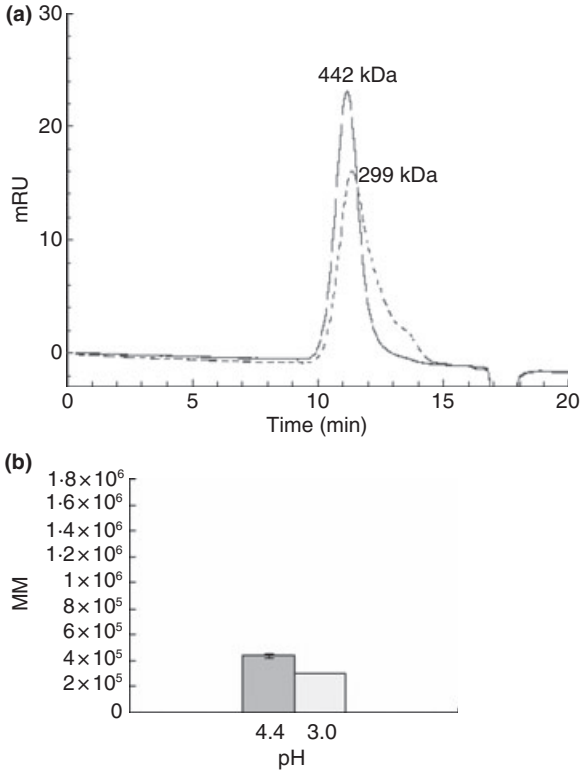

Figure 4 (a) Gel filtration chromatograms of the EPS produced by *Lactobacillus casei* CRL 87 before (—) and after (- - - -) the passage through the GS and (b) MM of the EPS expressed as Da before (pH 4·4) and after (pH 3·0) being submitting to the GS.

stress response gene and genes encoding putative cell envelope-altering proteins could have contributed to the survival of this strain in the gastrointestinal tract. In our study, the EPS^+/CPS^+ strains showed, in general, higher (although not statistically significant) enzyme activities (β-gal for *Strep. thermophilus* and P-β-gal for *Lact. casei*) than their corresponding EPS^- CPS^- mutants suggesting that the capsule and/or the slime-EPS, although not essential for surviving at low pH, could contribute to a better metabolic adaptation of the micro-organisms under the acidic stress conditions assayed. However, we do not discard that possibly other mutations than specifically for EPS or/and CPS production may have also occurred in the mutant strains as mutagenesis treatment with nitrosoguanidine leads to pleiotropic effects.

Electron microscopy studies were undertaken to evaluate whether the acid conditions of the GS altered the cell structure and to determine the potential protective effect of the CPS or EPS on the assayed micro-organisms. Even with the presence of CPS, cell wall alterations were observed for *Strep. thermophilus* CRL 1190 after passage through the GS, as revealed by SEM. This event may be linked to the decrease in the cell viability observed for this strain, which showed lower cell viability than its isogenic mutant strain (M16). Independently of the presence of CPS or EPS, the effect of the acid stress caused by the GS was more likely reflected at cytoplasmic level in all strains by using TEM. Similar findings were observed for bile-treated *Lact. reuteri* strains subjected to freeze-drying (Font de Valdez *et al.* 1997).

EPS from LAB have been claimed to have positive effect on human health. It is assumed that EPS can only exert a positive influence on the passage of bacteria through the intestine when EPS itself can resist digestion (Looijesteijn *et al.* 2001). The proposed protective function of EPS in the natural environment is also in accordance with the EPS resistance to biodegradation (Ruijssenaars *et al.* 2000). In this study, the degradability of two EPS (high- and low-MM) through the GS was evaluated by measuring possible variations in their MM. Both EPS produced by *Strep. thermophilus* CRL 1190 and *Lact. casei* CRL 87 showed partial degradation and showed no relationship with their physico-chemical characteristics; high and low MM and different monosaccharide content. EPS degradation could have been because of acid hydrolysis by the harsh conditions of the GS to which they were submitted. Ruijssenaars *et al.* (2000) evaluated the biodegradability of eight food-associated EPS (six of which were produced by LAB) by microorganisms isolated from human faeces or soil and found that four of them were degraded readily while the other ones remained unaltered. The EPS susceptibility to biological breakdown differed greatly and it was not possible to relate it directly to their EPS primary structure. In contrast to our results, Looijesteijn *et al.* (2001) reported that the EPS produced by *Lac. lactis* NZ4010 was stable during passage through the gastrointestinal tract performed *in vivo* and no changes in the EPS MM were found. Other authors (Pham *et al.* 2000; Degeest *et al.* 2002) observed degradation of the EPS produced by strains of *Strep. thermophilus* and *Lact. rhamnosus* upon prolonged fermentations by action of intracellular glycohydrolases liberated by cell lysis.

EPS produced by different organisms have different physiological functions, which are related to the natural habitat of the producing strains (Looijesteijn *et al.* 2001). The partial protection of the cells by EPS or CPS regarding their metabolic activity could be an advantage when the micro-organisms (e.g. probiotics) are submitted to the harsh conditions of the human GS to further exert their beneficial properties in the host. However, it is important to denote that probiotic strains should overpass other challenges in the gut, such as survival to high bile salts concentrations in the duodenum and in lower extent, along the small and large intestine. Further studies

are needed to have a better insight into the physiological functions of the large diversity of EPS produced by LAB.

Conclusions

The presence of EPS or CPS did not exert any significant protection on survival of *Strep. thermohilus* CRL 1190 and *Lact. casei* CRL 87 under the *in vitro* gastric conditions assayed in this study. However, the metabolic activity of these strains (EPS-producing) was more conserved than those of the non-EPS producing mutants. The EPS synthesized by both LAB species were partially degraded under the acidic conditions of the GS used.

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