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ORIGINAL ARTICLE

Exopolysaccharides produced by *Lactobacillus* and *Bifidobacterium* strains abrogate *in vitro* the cytotoxic effect of bacterial toxins on eukaryotic cells

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Keywords
Bacillus cereus, *Bifidobacterium*, Caco-2, erythrocytes, exopolysaccharides, *Lactobacillus*, streptolysin-O.

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Abstract

Aims: To evaluate the capability of the exopolysaccharides (EPS) produced by lactobacilli and bifidobacteria from human and dairy origin to antagonize the cytotoxic effect of bacterial toxins.
Methods and Results: The cytotoxicity of *Bacillus cereus* extracellular factors on Caco-2 colonocytes in the presence/absence of the EPS was determined by measuring the integrity of the tissue monolayer and the damage to the cell membrane (extracellular lactate dehydrogenase activity). Additionally, the protective effect of EPS against the haemolytic activity of the streptolysin-O was evaluated on rabbit erythrocytes. The EPS produced by *Bifidobacterium animalis* ssp. *lactis* A1 and IPLA-R1, *Bifidobacterium longum* NB667 and *Lactobacillus rhamnosus* GG were able to counteract the toxic effect of bacterial toxins on the eukaryotic cells at 1 mg ml^{-1} EPS concentration. The EPS A1 was the most effective in counteracting the effect of *B. cereus* toxins on colonocytes, even at lower doses (0·5 mg ml^{-1}), whereas EPS NB667 elicited the highest haemolysis reduction on erythrocytes.
Conclusions: The production of EPS by lactobacilli and bifidobacteria could antagonize the toxicity of bacterial pathogens, this effect being EPS and biological marker dependent.
Significance and Impact of the Study: This work allows gaining insight about the mechanisms that probiotics could exert to improve the host health.

Introduction

Exopolysaccharides (EPS) are extracellular biopolymers that are produced by many lactic acid bacteria (LAB) and other bacteria present in fermented foods and human environments, such as propionibacteria and bifidobacteria (Ruas-Madiedo *et al.* 2009b). In fact, the EPS-producing strains are used in the dairy industry because of the ability of their polymers to act as biological stabilizers, viscosifiers, emulsifiers and gelling agents (Abraham *et al.* 2009; Behare *et al.* 2009). In addition, in recent years, several health benefits have been attributed to EPS from LAB (Ruas-Madiedo *et al.* 2008).

The ecological role of EPS for the producing bacteria is not completely understood. It has been reported that they could be involved in cell recognition, adhesion to surfaces and biofilm formation, which would enhance the colonization of different ecosystems (Ruas-Madiedo *et al.* 2008). In this regard, it has been proposed that EPS produced by intestinal lactobacilli and bifidobacteria could be involved in the adherence to intestinal mucus and also in the interaction with enteropathogens (Ruas-Madiedo

et al. 2006). Also, EPS produced by bifidobacteria are able to modulate the intestinal microbiota by acting as fermentable substrates (Salazar *et al.* 2008, 2009b). On the other hand, bacterial EPS could counteract the activity of bacteriophages (Durlu-Özkaya *et al.* 2007) and of toxic compounds (Looijesteijn *et al.* 2001; Kim *et al.* 2006).

The synthesis of EPS in the gut ecosystem could confer a selective advantage for the survival and colonization of this niche by the producing bacteria. Indeed, EPS could improve the survival throughout the gastrointestinal tract of orally delivered bacteria given that bile is able to induce the synthesis of these polymers in *Bifidobacterium animalis* (Ruas-Madiedo *et al.* 2009a). In the gut environment, it has also been reported that kefiran, the EPS produced by LAB in the fermented milk kefir, is able to antagonize the biological activity of *Bacillus cereus* and also their extracellular factors on Caco-2 colonocytes (Medrano *et al.* 2008, 2009). However, there is no evidence as to whether EPS produced by lactobacilli and bifidobacteria from intestinal origin are able to exert similar protective effects against the activity of bacterial toxins.

The aim of this work was to evaluate the effect of the EPS produced by the strains *Lactobacillus rhamnosus* GG, *Bifidobacterium longum* NB667, *Bifidobacterium animalis* ssp. *lactis* A1 and IPLA-R1 on the biological activity triggered by bacterial toxins (extracellular factors of *B. cereus* and streptolysin-O (SLO) from *Streptococcus pyogenes*) on different eukaryotic cell models (Caco-2 cells and rabbit erythrocytes).

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study are shown in Table 1. Stock cultures of *B. cereus* B10502 were stored at −80°C in BHI (Brain Heart Infusion; Biokar Diagnostics, Beauvais, France) using glycerol 1% (v/v) as cryo-protective agent. The strain was re-activated in BHI supplemented with glucose 0·1% (w/v) for 16 h at 32°C in an orbital shaker, and these cultures were used to inoculate (4% v/v) 5 ml of fresh BHI-glucose that was incubated under the same conditions. Cell-free supernatants were obtained after centrifugation (900 ***g***, 10 min) and filtration (0·45 μm). Serial dilutions of supernatants were made in phosphate-buffered saline (PBS: KH_2PO_4 0·144 g l^{-1}, Na_2HPO_4 0·795 g l^{-1} and NaCl 9 g l^{-1}).

The four EPS-producing lactobacilli and bifidobacteria strains (Table 1) were maintained at −80°C in MRSC broth [MRS (Biokar) + 0·25% (w/v) L-cysteine (Sigma Chemical Co., St. Louis, MO, USA)] with 20% glycerol. For the isolation of EPS, frozen stocks were grown overnight at 37°C under anaerobic atmosphere (10% H_2, 10% CO_2, and 80% N_2) in a Mac500 chamber (Down Whitley Scientific, West Yorkshire, UK). Cultures (200 μl) were used to inoculate agar-MRSC plates using sterile glass beads. Plates were incubated for 5 days at 37°C in anaerobic conditions.

Isolation and characterization of the EPS

For the isolation of the EPS, cell biomass was collected from agar-MRSC plates using 2 ml ultrapure water per plate and the resulting volume was mixed with one volume of 2 mol l^{-1} NaOH. The suspension was gently stirred overnight at room temperature to promote polymer release, and afterwards cells were removed by centrifugation (8400 ***g***, 30 min). The EPS fraction was precipitated using two volumes of cold-absolute ethanol (4°C, 48 h), followed by centrifugation, resuspension in ultrapure water and dialysis for 3 days at 4°C with a daily change of water using dialysis tubes (Sigma) of 12–14 kDa molecular mass cut off. Finally, the dialysed EPS were freeze-dried (Ruas-Madiedo *et al.* 2006).

The protein content of the EPS fractions was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA). The molar mass (MM) and radius of gyration (Rg) of the EPS were measured by size-exclusion chromatography and multi-angle laser light scattering detection (SEC-MALLS, Salazar *et al.* 2009b). The monosaccharide composition was determined by GC–MS (Salazar *et al.* 2009a). The phosphate content of each EPS was directly measured in an atomic emission spectroscopy Perkin Elmer 5500 inductively coupled plasma (Perkin Elmer, Norwalk, CT, USA) at the 'Centro de Ciencias Medioambientales' (CCM-CSIC, Madrid).

Table 1 Micro-organism used in this study

Species	Strains	Origin*
Bacillus cereus	B10502	Food poisoning outbreak (Minnaard *et al.* 2004)
Lactobacillus rhamnosus	GG (LMG18243)	LMG Culture collection (human faeces)
Bifidobacterium animalis	A1	Isolated from commercial dairy product
ssp. *lactis*	IPLA-R1	Bile-adapted strain from A1 (IPLA collection)
Bifidobacterium longum	NB667	NIZO Culture collection (infant faeces)

*LMG: Belgian Co-ordinated Collections of Micro-organisms (Gent, Belgium) and NIZO Food Research Collection (Ede, the Netherlands).

Each EPS was resuspended in PBS solution at 20 mg ml^{-1}, and several dilutions ranging from 0·05 to 1 mg ml^{-1} were used for experiments.

Culture of human Caco-2 cell line

Caco-2 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol l^{-1} glucose (GIBCO BRL Life Technologies, Rockville, MD USA), supplemented with 15% (v/v) heat-inactivated (30 min at 56°C) foetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 1% (v/v) nonessential amino acids (PAA Laboratories GmbH), penicillin (12 UI ml^{-1}), streptomycin (12 μg ml^{-1}), gentamicin (50 μg ml^{-1}) and fungizone (1·25 μg ml^{-1}). For maintenance purposes, the cell line was passed weekly, using 0·02% (w/v) trypsin (Sigma) in PBS containing 3 mmol l^{-1} EDTA. Cell suspensions (1×10^5 cells per ml) were seeded in 6- or 24-well plates (Greiner Bio One, Frickenhausen, Germany) and incubated to form confluent-differentiated monolayers for 14 ± 1 days at 37°C in a 5% CO_2 atmosphere. The culture medium was changed every 2 days, and the experiments were performed with cells among passages 56 and 60.

Detachment of Caco-2 cells

The integrity of the Caco-2 monolayers was determined by measuring the cell detachment as previously reported by Minnaard *et al.* (2001). Briefly, differentiated cells grown in 24-well plates were co-incubated with several dilutions (from 1/2 to 1/64) of *B. cereus* B10502 supernatants at 37°C in 5% CO_2 atmosphere for 150 min in presence of different EPS concentrations (0, 0·05, 0·5 and 1 mg ml^{-1}). After incubation, Caco-2 cells were washed with PBS, fixed for 1 min with 2% (w/v) formaldehyde in PBS and washed again. Staining was performed with 500 μl of crystal violet solution [0·13% crystal violet, 5% ethanol and 2% formaldehyde (w/v/v) in PBS] by incubating for 20 min. Afterwards, stain excess was washed with PBS, and samples were treated for 1 h with 50% (v/v) ethanol. Absorbance of stained cells was measured at 590 nm in a Metrolab 330 spectrophotometer (Metrolab, Buenos Aires, Argentina). The percentage of attached cells was calculated as the absorbance of treated cells with respect to the absorbance of untreated-control cells (suspended in PBS and incubated in the absence of *B. cereus* supernatant and EPS).

Lactate dehydrogenase activity in Caco-2 cells

Caco-2 cell membrane integrity was determined by measuring the extracellular lactate dehydrogenase (LDH) activity using the LDH-P unitest kit (Weiner Lab, Rosario, Argentina), following the manufacturer's specifications. Differentiated Caco-2 cells grown in six-well plates were co-incubated with serial dilutions (1/16 to 1/32) of *B. cereus* B10502 cell-free supernatants in the presence (1 mg ml^{-1}) and absence of bacterial EPS at 37°C in 5% CO_2 atmosphere for 2 h. The extracellular LDH activity was determined by mixing 50 μl of Caco-2 supernatants with the enzyme substrate and incubating at 30°C. Absorbance at 340 nm was measured continuously during 120 s in a spectrophotometer Beckman DU650 (Beckman, Brea, CA, USA), and LDH activity was calculated as the decrease in absorbance for 60 s.

Haemolysis of erythrocytes

The putative protective effect of EPS against the toxic activity of SLO from *Strep. pyogenes* upon erythrocytes was assessed according to Promdonkoy and Ellar (2003). Commercial SLO (Sigma) was diluted in PBS containing 20 mmol l^{-1} L-cysteine, adjusted to pH 7·4, filtered and mixed with (2% v/v) rabbit erythrocytes suspended in PBS. Toxin units used in the experiments were around 300 UI enzyme per ml [one unit is defined as the amount of enzyme able to cause 50% lysis of 50 μl of 2% (v/v) red blood suspension in PBS, pH 7·4 at 37°C in 30 min]. Erythrocyte suspensions were co-incubated with SLO in the presence (1 mg ml^{-1}) and absence of the EPS at 37°C for 60 min. Two control samples were used: 0% lysis (erythrocytes in absence of SLO toxin) and 100% lysis [erythrocytes treated with 0·2% Triton X-100 (Sigma) in PBS]. Absorbance decrease in erythrocyte suspensions was measured at 600 nm in a Metrolab 330 spectrophotometer at different sampling points for 60 min. The percentage of remaining (not lysed) erythrocytes in the samples was calculated as follows: 100 × (absorbance of sample − absorbance of 100% lysis control)/(absorbance of 0% lysis control − absorbance of 100% lysis control).

Statistical analysis

All experiments have been carried out at least in triplicate. Statistical analysis was performed using the SPSS-PC 11.0 software (SPSS Inc., Chicago, IL, USA). Data were subjected to an one-way ANOVA, and the least significant difference test was used for the subsequent comparison of means. For the haemolysis data and within each type of EPS sample, a paired samples *t*-test was performed to compare the percentage of remaining erythrocytes after 60 min of incubation with respect to the initial values.

Results

Physico-chemical characteristics of the bacterial EPS

Table 2 shows the protein content, MM distribution and chemical composition of the EPS analysed. The protein content of the EPS was in general lower than 3%, indicating high-carbohydrate content and thus a good degree of purity of the polymer. *Lactobacillus rhamnosus* GG synthesized a unique peak of $2{\cdot}2 \times 10^5$ Da. However, the EPS synthesized by *Bifidobacterium* strains (A1, IPLA-R1 and NB667) presented more than one peak of different MM. The EPS produced by *Bif. longum* NB667 had two polymer fractions, one of low ($4{\cdot}3 \times 10^3$ Da) MM and another of high ($4{\cdot}4 \times 10^6$ Da) MM. *Bif. animalis* ssp. *lactis* A1 and IPLA-R1 are closely related strains. A bile-resistant derivative strain (A1dOx) was obtained from the parental A1 by gradual adaptation to bile salts. This bile-adapted strain converted spontaneously to a stable ropy phenotype by successive subcultivation, resulting in the strain IPLA-R1. The ropy phenotype of IPLA-R1 strain is absent in the parental A1, and these two strains produced polymers differing in MM and peak distribution (Table 2). Both strains share two EPS fractions of low MM (around 3×10^4 and 5×10^3 Da), but IPLA-R1 is able to additionally produce, in high amounts (56%), an EPS of high ($3{\cdot}5 \times 10^6$ Da) MM, probably being responsible for the ropy character. The EPS produced by these closely related strains also differed in their monosaccharide ratio composition. The EPS fraction A1 showed a higher glucose and galactose, and lower rhamnose, ratio than IPLA-R1. Although EPS NB667 and GG also have the same monomers in their composition, the ratio among the four polymers was different. It is worth noting that EPS A1 and IPLA-R1 also have phosphate in their composition, which was not detected in NB667 and GG. Finally, variations in the Rg of the highest MM peak of each EPS were also detected with values of 13, 17, 47 and 67 nm for EPS GG, A1, IPLA-R1 and NB667, respectively. These results show a high variability in the physico-chemical characteristics of the bacterial EPS used in this study, which could account for different biological effects.

Effect of bacterial EPS against *Bacillus cereus* cytotoxicity on Caco-2 cells

The protective effect of the bacterial EPS against the toxic effect of *B. cereus* extracellular factors on Caco-2 cells was determined by testing the integrity of Caco-2 cell monolayer. Figure 1 shows the Caco-2 cells that remained adhered to the surface of the well plates after co-incubation with serial dilutions (1/16 to 1/64) of *B. cereus* supernatants in the presence (1 mg ml^{-1}) and absence of EPS. Under these experimental conditions, all EPS were able to significantly ($P < 0{\cdot}05$) antagonize the cell detachment triggered by *B. cereus* B10502 supernatants at the highest toxin concentration (dilution 1/16), the EPS A1 being able to display the highest protection. At *B. cereus* supernatant 1/32 and 1/64 dilutions, the lower toxin concentration did not induce the detachment of the Caco-2 monolayers in the control (absence of EPS) samples, and no differences with respect to the presence of the EPS were detected. Higher toxin concentrations (dilutions 1/2 to 1/8, data not shown) caused the total detachment of the cells, and no protection was observed with any of the EPS assayed. Taking into account the previous results, the EPS with the highest (EPS A1) and one of the lowest (IPLA-R1) antagonism capability against *B. cereus* supernatant toxicity on Caco-2 cells were chosen to study a putative dose-response effect of these EPS (Fig. 2). For EPS A1, a significant ($P < 0{\cdot}05$) protection effect was detected at concentrations higher than 0·05 mg ml^{-1}, whereas for EPS IPLA-R1 was detected only at 1 mg ml^{-1}. In addition, at the highest EPS concentration tested (1 mg ml^{-1}), the percentage of cells that remained adhered was significantly higher ($P < 0{\cdot}001$) for EPS A1 than for EPS IPLA-R1. Thus, the differential EPS characteristics may have account for this variability in the protective capability between both EPS.

Table 2 Physico-chemical characteristics of the exopolysaccharides (EPS) used in this study

EPS	Protein (%)	Molar mass (Da) distribution (%)*			Monosaccharide ratio†			
		Peak 1	Peak 2	Peak 3	Glc	Gal	Rham	PO_4
A1	3·1	–	$2{\cdot}8 \times 10^4$ (44%)	$5{\cdot}0 \times 10^3$ (56%)	2·5	2	1	+
IPLA-R1	2·6	$3{\cdot}5 \times 10^6$ (56%)	$3{\cdot}0 \times 10^4$ (21%)	$4{\cdot}9 \times 10^3$ (23%)	1	1	1·5	+
NB667	2·2	$4{\cdot}4 \times 10^6$ (47%)	–	$4{\cdot}3 \times 10^3$ (53%)	1	2	1	–
GG	2·9	–	$2{\cdot}2 \times 10^5$ (100%)	–	1	4	1	–

*Percentage calculated with respect to the total amount (μg) of peaks measured by size-exclusion chromatography (SEC-MALLS).
†Glu, glucose; Gal, galactose; Rham, rhamnose.

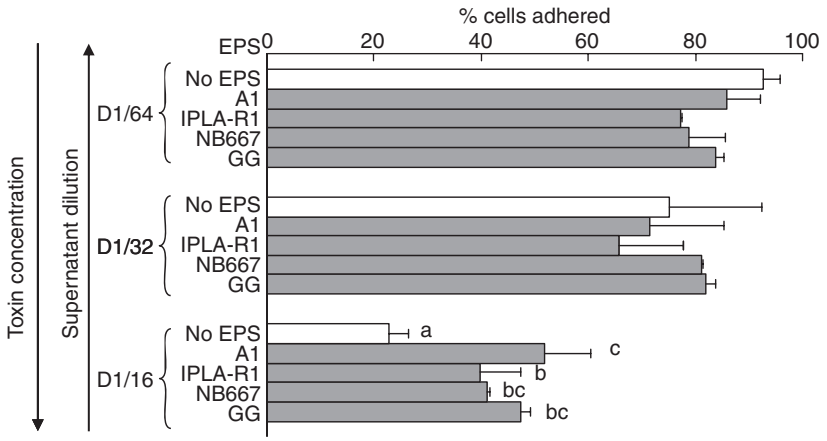

Figure 1 Percentage of Caco-2 cells adhered to the well plate after treatment with several dilutions (1/16, 1/32 and 1/64) of *Bacillus cereus* B10502 supernatants in the absence (white bars) and presence (1 mg ml^{-1}) of the exopolysaccharides isolated from the strains *Bifidobacterium animalis* ssp. *lactis* A1 and IPLA-R1, *Bifidobacterium longum* NB667 and *Lactobacillus rhamnosus* GG (grey bars). The percentage of cells that remained attached was calculated as follows: 100 × [590- nm absorbance of treated samples/absorbance of control cells (incubated in absence of *B. cereus* supernatant)]. Within each supernatant dilution, the columns with different letters are statistically different ($P < 0{\cdot}05$) according to the least significant difference mean comparison test.

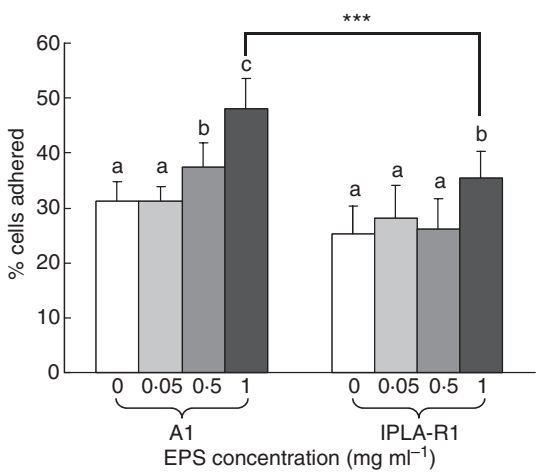

Figure 2 Percentage of Caco-2 cells that remained adhered to the well plate after the treatment with the dilution 1/16 of *Bacillus cereus* B10502 supernatant in the presence of several concentrations of the exopolysaccharides (EPS) isolated from the strains *Bifidobacterium animalis* ssp. *lactis* A1 and IPLA-R1. Concentrations: 0 (□), 0·05 (▨), 0·5 (▩) and 1 (■) mg ml^{-1}. Within each EPS, the columns that do not have a common superscript are statistically different ($P < 0{\cdot}05$) according to the least significant difference mean comparison test. At the highest concentration (1 mg ml^{-1}), an one-way anova test was performed to analyse differences between both polymers (***$P < 0{\cdot}001$).

To evaluate Caco-2 cell membrane integrity, the extracellular release of LDH activity (a cytoplasmatic enzyme) was measured in the milieu of Caco-2 co-cultivated with *B. cereus* supernatant dilutions 1/16 and 1/32 in the presence (1 mg ml^{-1}) and absence of bacterial EPS (Fig. 3). The four EPS tested showed significant differences ($P < 0{\cdot}05$) with respect to the control (absence EPS) sample at both toxin concentrations tested. The effect was also significant ($P < 0{\cdot}001$) at low-toxin concentration (dilution 1/32). As similarly occurred when cell detachment was analysed, the EPS fraction NB667 showed the lowest protective effect of the four EPS analysed. Thus, this result suggests that bacterial EPS exerted a protective effect on Caco-2 cells by avoiding the loss of membrane integrity and/or the release of cytoplasmatic content.

Effect of EPS against SLO toxin on erythrocyte cells

The results presented earlier prompted us to study the effect of these EPS on the biological activity of large-pore forming toxins. Rabbit erythrocytes were employed to evaluate the putative protection of bacterial EPS against the haemolytic activity of the commercial bacterial haemolysin SLO. Kinetics of haemolysis performed in the presence (1 mg ml^{-1}) and absence of the EPS under study are depicted in Fig. 4. The remaining erythrocytes of all samples significantly diminished with respect to the initial values after 60 min of erythrocyte co-incubation with the SLO toxin (paired *t*-test: $P < 0{\cdot}001$). However, when the EPS were added, the rate of haemolysis decreased and significant differences were found at the end of the incubation period with respect to the sample in absence of EPS ($P < 0{\cdot}05$). It is worth noting that in the case of haemolysis, the EPS A1 displayed the lowest protection capability of the four EPS tested, whereas the EPS NB667 showed the highest. This represents an opposite tendency

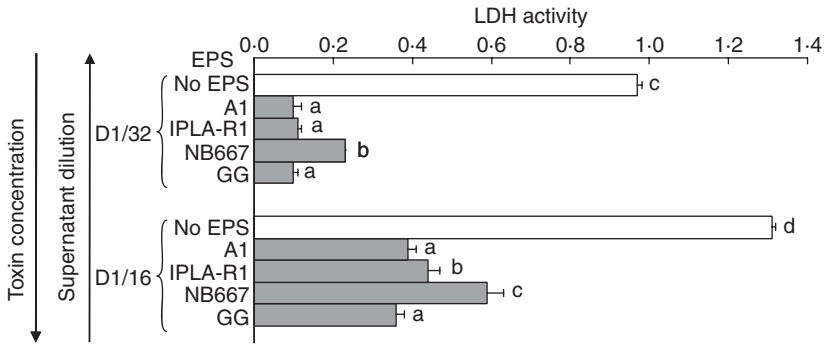

Figure 3 Extracellular lactate dehydrogenase (LDH) activity of Caco-2 cells after co-cultivation with *Bacillus cereus* B10502 supernatants (dilutions 1/16 and 1/32) in the absence (white bars) and presence (grey bars) of the exopolysaccharides (1 mg ml^{-1}) isolated from the strains *Bifidobacterium animalis* ssp. *lactis* A1 and IPLA-R1, *Bifidobacterium longum* NB667 and *Lactobacillus rhamnosus* GG. The LDH activity was calculated as the decrease in absorbance at 340 nm after 60 s of reaction. Within each *B. cereus* supernatant dilution, the columns that have not a common superscript are statistically different ($P < 0{\cdot}05$) according to the least significant difference mean comparison test.

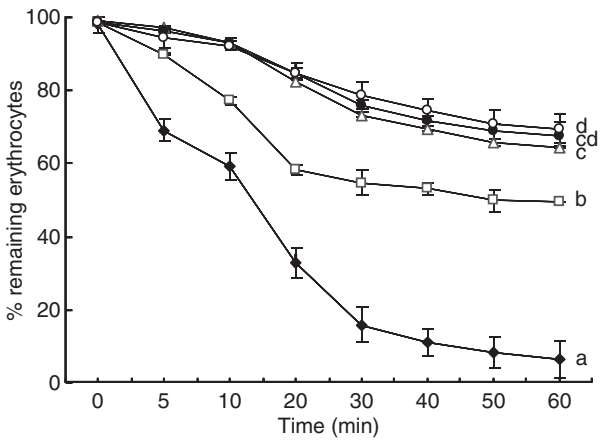

Figure 4 Lysis kinetics of rabbit erythrocytes co-incubated with streptolysin-O toxin (300 UI) without exopolysaccharides (EPS) (♦) or with 1 mg ml^{-1} of EPS A1 (□), EPS IPLA-R1 (△), EPS GG (●) and EPS NB667 (○). The percentage of remaining erythrocytes was calculated as follows: 100 × (600- nm absorbance of sample – absorbance of 100% lysis control)/(absorbance of 0% lysis control – absorbance of 100% lysis control). At the end of the reaction period (60 min), the symbols that do not share a common letter are statistically different ($P < 0{\cdot}05$) according to the least significant difference mean comparison test.

with respect to that observed for the model Caco-2 and extracellular toxins of *B. cereus*.

Discussion

The capability of bacterial EPS to antagonize the membrane eukaryotic cell damage triggered by *B. cereus* extracellular factors and by SLO from *Strep. pyogenes*, was evaluated in two complementary cell models: colonocyte-like cells (Caco-2 cellular line) and rabbit erythrocytes. These bacterial toxins produce different effects upon eukaryotic cells. *B. cereus* can trigger in humans diverse symptoms related to their adhesion to the intestinal epithelium and invasion of enterocytes (Minnaard *et al.* 2004) and/or because of the production of a wide range of extracellular factors such as cytolysins (cereolysin-O), phospholipases and haemolysins, among others (Alouf 2000; Minnaard *et al.* 2007; Stenfors-Arnesen *et al.* 2008). The SLO belongs to the large-pore forming family of toxins (Alouf 2000; Thelestam 2000), in which is included the cereolysin-O from *B. cereus*. In this study, we have shown that the EPS produced by strains of lactobacilli and bifidobacteria are able to counteract the toxic effect of bacterial toxins. However, the effect was different depending on the EPS considered and also on the biological (eukaryotic cell) marker studied.

In the case of the *B. cereus* supernatants, the EPS IPLA-R1 and NB667 showed the lowest protection capability by preventing the Caco-2 cell de-attachment and/or the LDH release. A common feature of these two polymers is the production of a high-MM EPS fraction (>10^6 Da). The high-MM fraction is not present in the EPS synthesized by strain A1 (parental strain of IPLA-R1), which exerted the highest protection, suggesting that the high-MM fraction is not necessary to exert the protective effect against *B. cereus* toxins. Additionally, the protective effect of EPS A1 was detected at lower doses (0·5 mg ml^{-1}) than EPS IPLA-R1, and at the concentration of 1 mg ml^{-1}, the EPS A1 elicited a significantly higher antagonic effect than IPLA-R1. The medium MM ($2{\cdot}2 \times 10^5$ Da) EPS produced by GG strains had a biological effect closer to EPS A1. However, the kefiran isolated from the kefir grains, which is a branched glucogalactan with an MM higher than 10^7 Da (Piermaría *et al.* 2008), was able to significantly abrogate the cytotoxic effect of

B. cereus toxins at similar concentrations (from 0·3 to 1 mg ml^{-1}, Medrano *et al.* 2008) than our EPS A1. Thus, the MM of a polymer is not the only determining factor involved in its protective capability. Also the chemical composition, type of union and the spatial structure of the EPS could be involved in the different biological effect. With respect to the putative mechanism against *B. cereus* toxins, the bacterial EPS could act by blocking the toxin receptors in the eukaryotic cell or by capturing the toxin, thus avoiding its interaction with the receptor. In both cases, the EPS would avoid the initiation of an intracellular cascade response that ends with the death, by necrosis or apoptosis, of the eukaryotic cell (Henderson *et al.* 1999).

Regarding the antagonism of bacterial EPS against the haemolytic activity of SLO on erythrocytes, our EPS were able to diminish the rate of haemolysis. However, in this case, the EPS A1 showed the lower percentage of protection and the EPS NB667 the highest; this is just the opposite behaviour of that detected for the Caco-2/*B. cereus* toxins system. This finding suggested that the mechanism involved in haemolysis protection might be different to that involved in cell detachment. One of the main mechanisms leading to haemolysis by haemolysins, such as SLO (and also the cereolysin-O from *B. cereus*), is the oligomerization and assembly of toxin monomers into the cytoplasmic membrane of eukaryotic cells, thus forming large pores (Palmer *et al.* 1998; Henderson *et al.* 1999). The EPS produced by our bifidobacteria and lactobacilli might display their antagonism by hindering the contact of the toxin with the erythrocyte membrane; this will avoid the toxin-subunits oligomerization and pore formation, thus EPS could exert osmotic protection. In this way, MM and Rg are important structural characteristic of EPS to be considered. We observed that the lowest protection on haemolysis was obtained with EPS with low MM and Rg, this is the EPS A1.

Thus, it seems that polymers having small Rg and low MM might be able to better abrogate the cytotoxic effect of *B. cereus* toxins, but are less effective for large-pore forming toxin such as SLO. The physical parameters and chemical composition of EPS are related to the structure of the repeating units that build the polymers. In our case, with the exception of EPS GG (Landersjö *et al.* 2002), the repeating units of EPS A1, IPLA-R1 and NB667 are still unknown. Thus, we do not have enough data to conclude that the MM and Rg are the unique parameters directly related to the anti-toxin effect of our EPS and to extend this conclusion to other bacterial EPS. However, it is well known that the physico-chemical characteristics of bacterial EPS are directly correlated with their technological and biological functionality (Ruijssenaars *et al.* 2000; Vaningelgem *et al.* 2004; Mozzi *et al.* 2009; Salazar *et al.* 2009a). Therefore, it seems that the physico-chemical properties of the EPS would also determine the specific interactions with eukaryotic cell receptors and/or with toxins.

Conclusions

The EPS synthesized by the human and dairy origin strains *Lact. rhamnosus* GG, *Bif. longum* NB667, *Bif. animalis* ssp. *lactis* A1, and its bile-adapted derivative IPLA-R1, are able to exert an *in vitro* antagonic effect against the cytophatic effect on Caco-2 cells triggered by *B. cereus* extracellular factors and against the haemolytic activity of a large-pore forming toxin, such as SLO, on rabbit erythrocytes. Differences among EPS depended on the biological marker studied and could be attributed to the variability of the physico-chemical characteristics of these biopolymers. Although the protective mechanisms are still not fully understood, it is tempting to hypothesize that the bacterial EPS could act by blocking receptors on the eukaryotic cell surface or by acting as toxin-scavenger agents. Nevertheless, the capability to abrogate the activity of bacterial toxins by EPS produced by lactobacilli and bifidobacteria is another mechanism involved in the antagonism against virulence factors of bacterial pathogens exerted by probiotic strains.

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