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Kefiran antagonizes cytopathic effects of *Bacillus cereus* extracellular factors

Micaela Medrano [a], Pablo Fernando Pérez [a,b], Analía Graciela Abraham [a,*]

[a] *Centro de Investigación y Desarrollo en Criotecnología de Alimentos – 47 y 116 (s/n) – La Plata (1900), Argentina*
[b] *Cátedra de Microbiología – Facultad de Ciencias Exactas – UNLP-47 y 115 – La Plata (1900), Argentina*

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Abstract

Kefiran, the polysaccharide produced by microorganisms present in kefir grains, is a water-soluble branched glucogalactan containing equal amounts of D-glucose and D-galactose. In this study, the effect of kefiran on the biological activity of *Bacillus cereus* strain B10502 extracellular factors was assessed by using cultured human enterocytes (Caco-2 cells) and human erythrocytes.

In the presence of kefiran concentrations ranging from 300 to 1000 mg/L, the ability of *B. cereus* B10502 spent culture supernatants to detach and damage cultured human enterocytes was significantly abrogated. In addition, mitochondrial dehydrogenase activity was higher when kefiran was present during the cell toxicity assays. Protection was also demonstrated in hemolysis and apoptosis/necrosis assays. Scanning electron microscopy showed the protective effect of kefiran against structural cell damages produced by factors synthesized by *B. cereus* strain B10502. Protective effect of kefiran depended on strain of *B. cereus*. Our findings demonstrate the ability of kefiran to antagonize key events of *B. cereus* B10502 virulence. This property, although strain-specific, gives new perspectives for the role of bacterial exopolysaccharides in functional foods.

Keywords: Kefiran; Bacterial exopolysaccharides; *Bacillus cereus*; Caco-2 cells; Virulence; Protein toxins

1. Introduction

Kefir is a fermented milk produced by the action of lactic acid bacteria, yeast and acetic acid bacteria, trapped in a complex matrix of polysaccharides and proteins.

Kefir has been traditionally consumed and many health promoting properties have been proposed (Otes and Cagindi, 2003; Farnworth, 2005). Beneficial effects of kefir milk have been ascribed to the presence of kefir microflora and their metabolic products. Kefir consumption has been associated to improvement of lactose digestion (Hertzler and Clancy, 2003), enhancement of antitumoral activity (Murofushi et al., 1983; Hosono et al., 1990) and modulation of the immune response (Thoreux and Schmucker, 2001; Vinderola et al., 2005). Furthermore, antibacterial (Garrote et al., 2000) and antifungal activities (Cevikbas et al., 1994) have been demonstrated.

Exopolysaccharides produced by lactic acid bacteria have gained the attention of food researchers because of its food grade status and the textural properties they impart to dairy products (Zubilaga et al., 2001; Laws and Marshall, 2001). Indeed, polysaccharides are used as thickeners, emulsifiers and gelling agents (De Vuyst and Degeest, 1999; Ruas-Madiedo et al., 2002).

Kefiran, the polysaccharide obtained from kefir, is a water-soluble branched glucogalactan containing equal amounts of D-glucose and D-galactose produced by microorganisms present in kefir grains (Mukai et al., 1988; Micheli et al., 1999; Rimada and Abraham, 2001). The exopolysaccharide is released into the media, reaching values of 218 mg L^{-1} and 247 mg L^{-1} in milk or whey respectively (Rimada and Abraham, 2003). Due to its physicochemical properties, kefiran improves texture of acid milk gels (Rimada and Abraham, 2006).

Bacillus cereus is a spore-forming gram-positive pathogen that is widely distributed in the environment. Its frequent occurrence in soil and on plant surfaces allows for easy dissemination into the food chain, and the spores are capable of

* Corresponding author. Tel./fax: +54 221 424 9287.
E-mail address: aga@biol.unlp.edu.ar (A.G. Abraham).

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surviving thermal processing conditions used in the food industry (Christiansson et al., 1999).

Concerning intestinal pathologies associated to *B. cereus*, two main syndromes have been reported: emetic and diarrhoeic. Whereas emetic syndrome is associated to cereulide, a thermostable dodecadepsipeptide (Agata et al., 1994; Horwood et al., 2004; Ehling-Schulz et al., 2005), the diarrhoeic syndrome has been related to a plethora of extracellular factors with different biological effects (Alouf, 2000; Beecher et al., 2000; Granum, 1997; Fagerlund et al., 2004).

B. cereus and its extracellular factors are able to produce different biological effects on cultured human enterocytes (Caco-2 cells) and erythrocytes. These experimental approaches constitute suitable models to study virulence factors of this microorganism (Minnaard et al., 2001; Minnaard et al., 2004; Minnaard et al., 2007).

Moreover, in recent years, the study of *B. cereus* virulence factors started to highlight the importance of direct bacteria–enterocyte interactions (Rowan et al., 2001; Minnaard et al., 2004). It is worth to note that production of exocellular factors by *B. cereus* is also responsible for extraintestinal pathologies such as postraumatic endophtalmitis (Callegan et al., 1999).

Taking into account that many bacterial toxins recognise glucidic receptors on cell surface, we decided to study the effect of kefiran on biological activity of *B. cereus* extracellular factors. For this purpose we used two different and complementary experimental models: cultured human enterocytes and red blood cells.

2. Materials and methods

2.1. Bacterial strains and culture conditions

B. cereus strain B10502, isolated from a food poisoning outbreak, was kindly provided by the Laboratorio Central de Salud Pública, Provincia de Buenos Aires, Argentina (Minnaard et al., 2004). Stock cultures were preserved at −80 °C using glycerol 1% (v/v) as cryoprotectant. Bacteria were reactivated for 16 h at 32 °C in BHI broth (BIOKAR Diagnostics, Beauvais, France) supplemented with 0.1% glucose (w/v). Microorganisms were then inoculated (4% v/v) in 5 ml of BHI broth supplemented with 0.1% (w/v) glucose and incubated at 32 °C for 16 h in an orbital shaker. Cell free spent culture supernatants were obtained after centrifugation (900 ×*g* for 10 min) and filtration (0.45 μm pore diameter). pH of supernatants was adjusted to 7 with 5 N NaOH to avoid cell damage on account of the acid environment (Minnaard et al., 2001). Serial dilutions of supernatants were done in PBS. BHI controls were run throughout. Titers of biological activity were determined for each assay (cell detachment, MTT assay, haemolysis, apoptosis–necrosis).

2.2. Isolation and quantification of kefiran

Kefir grains CIDCA AGK1 were treated in boiling water (grains:water ratio = 1:10 w/v) for 3 h with discontinuous stirring. The mixture was centrifuged (Sorvall RC-5B Plus centrifuge) at 10,000 ×*g* for 20 min at 20 °C. The polysaccharide in the supernatant was precipitated by addition of two volumes of cold ethanol and stored at −20 °C overnight. The mixture was centrifuged at 10,000 ×*g* for 20 min at 4 °C. Pellets were dissolved in hot water and the precipitation procedure was repeated twice. The precipitate was finally dissolved in hot distilled water (kefiran solution) and lyophilised (Heto FD4. Heto-Holten, Denmark). Polysaccharide concentration was determined by the anthrone method, measuring absorbance at 620 nm (Metrolab 330 Spectrophotometer, Metrolab, Argentina) and using glucose solutions as standards (Southgate, 1991). All the samples were tested for the absence of other sugars by qualitative thin layer chromatography (TLC) on Silica gel G type 60 plates (Merck D-64271 Darmstadt Germany) using *n*-propanol–acetic acid–water (70:20:10 v/v/v) as the mobile phase. TLC plates were developed with *p*-amino benzoic acid 7 g L^{-1} and *o*-phosphoric acid 30 g L^{-1} in methanol (Zweig and Sherma, 1978). Absence of proteins was assessed by the Bradford's method (Bradford, 1976). Reagents for anthrone, Bradford and TLC were obtained from Sigma (St. Louis, MO, USA). Kefiran was diluted properly in PBS to reach final concentrations in each assay between 300 to 1000 mg/L.

2.3. Culture of human enterocytes

Caco-2 cells (Fogh et al., 1977; Pinto et al., 1983) were grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 25 mM glucose (GIBCO BRL Life Technologies Rockville, MD. USA), supplemented with 15% (v/v) heat-inactivated (30 min at 56 °C) fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 1% (v/v) nonessential amino acids (PAA Laboratories GmbH), penicillin (12 UI/ml), streptomycin (12 μg/ml), gentamicin (50 μg/ml) and fungizone (1.25 μg/ml). For maintenance purposes, cells were passed weekly, using 0.02% (w/v) trypsin (Sigma, St. Louis, USA) in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS; KH_2PO_4 0.144 g/L; Na_2HPO_4: 0.795 g/L; NaCl: 9 g/L) containing 3 mM EDTA.

Monolayers were prepared in 24-well tissue culture plates (Greiner Bio One, Germany) by seeding 7×10^4 cells per well. Experiments and cell maintenance were carried out at 37 °C in a 5% CO_2–95% air atmosphere. The culture medium was changed every 2 days. Assays were performed with cells at passages between 56 and 60. Fully differentiated cells (14 days in culture) were used throughout.

2.4. Detachment of cells

Detachment of Caco-2 cells was measured as previously reported by Minnaard et al. (2001). Briefly, differentiated cells were co incubated with different dilutions of filter sterilized supernantants at 37 °C for 2 h in the presence of kefiran concentrations ranging from 0 to 1000 mg/L. After incubation, cells were washed with phosphate-buffered saline (PBS), fixed for 1 min with 2% (v/v) formaldehyde in PBS and washed again. Staining was performed by incubating for 20 min at room

temperature with 500 µl of crystal violet solution (0.13% crystal violet, 5% ethanol, and 2% formaldehyde in PBS w/v/v). After being exhaustively washed with PBS to remove stain excess, samples were treated for 1 h with freshly prepared 50% ethanol in PBS (v/v) at room temperature. Absorbance was measured at 650 nm in a Metrolab 330 Spectrophotometer. Percentage of attached cells was calculated as: $100 \times (A/A\text{c})$, where A is the absorbance of treated cells and Ac is the absorbance of untreated control cells.

2.5. Mitochondrial dehydrogenase activity

Caco-2 monolayers were incubated with serial two fold dilutions of *B. cereus* B10502 cell free supernatants in the presence and the absence of kefiran (800 mg/L). After incubation at 37 °C for 1 h, cells were detached by mechanical scraping and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium (MTT; Sigma St Louis, USA) for 4 h at 37 °C (final concentration 0.5 µg/ml in PBS). MTT assay is based on conversion of MTT to an insoluble purple formazan by mitochondrial dehydrogenase activity. Samples were centrifuged at 14,000 ×*g* for 1 min and stain was extracted with 0.1 N HCl in isopropanol. After centrifugation, absorbance was measured at 550 nm in a plate reader (SLT Spectra Rainbow, SLT LABinstruments FO39046, Austria). Percentage of remaining activity was calculated as: $100 \times A/A\text{c}$, where A is the absorbance of treated cells and Ac is the absorbance of untreated control cells.

2.6. Labeling with FITC-annexin V and propidium iodide

A modification of a previously published method was used (van Engeland et al., 1999). Briefly, after 1 h of incubation with *B. cereus* B10502 spent culture supernatants and different kefiran concentrations, cells were washed twice with binding buffer (25 mM HEPES, 125 mM NaCl, and 2.5 mM $CaCl_2$, gelatin 0.2%, pH 7.2). Then 5 µl of FITC-annexin V (Sigma St. Louis, USA), 1 µg of propidium iodide (Sigma St. Louis, USA) per ml, and 100 µg of RNAse (Sigma St. Louis, USA) per ml were added in 100 µl of binding buffer. Cells were incubated at room temperature for 15 min, washed 3 times with binding buffer, and mounted in 50% (v/v) glycerol in binding buffer (v/v). Micrographs were obtained in a fluorescence microscope LEICA DMLB (Leica Microsystems, Wetzlar GmbH, Germany) coupled to a DC100 camera (Leica Microscopy Systems Ltd., Heerbrugg, Switzerland).

2.7. Scanning electron microscopy

Caco-2 cells were cultured on round glass coverslips (Assistent, Sondheim, Germany). After coincubation for 2 h at 37 °C with *B. cereus* B10502 cell free supernatants with (800 mg/L) or without kefiran, specimens were washed 3 times with PBS, and fixed with 2.5% (v/v) glutaraldehyde (Riedel de Haen, Seelze, Germany). Smears were dehydrated in a graded series of ethanol solutions. Finally, samples were critical point dried using CO_2 (Model CP30, Baltec), gold coated (Jeol FineCoat Sputter JFC-1100, Jeol Ltd. Akishima Tokyo, Japan), and examined using a Jeol model JSM 6360 LV scanning electron microscope (Jeol Ltd. Akishima, Tokyo, Japan).

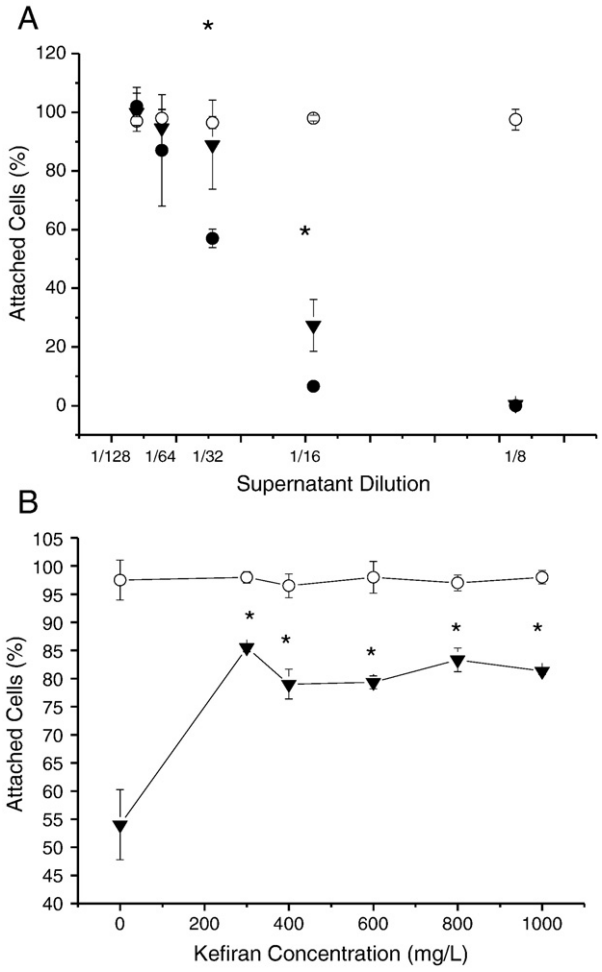

Fig. 1. Effect of *Bacillus cereus* B10502 spent culture supernatants on cultured human enterocytes (Caco-2 cells). (A) Dose response curves performed without (●) or with (▼) kefiran 800 mg/L. BHI dilution control (○). (B) Effect of the addition of different kefiran concentrations on cell detachment by 1/32 dilutions of spent culture supernatants (▼) or BHI (control) (○). Percentage of cells that remained attached was calculated as $(A\text{x}/A\text{c}) \times 100$, where $A\text{x}$ = Absorbance (450 nm) of the samples treated with spent culture supernatant and $A\text{c}$ = Absorbance (450 nm) of control cells (incubated with PBS). Results are averages of 3 values ± SD. (*) Indicates significant differences with the corresponding control ($p < 0.05$).

2.8. Hemoglobin-release assay

Hemolysis was assessed according to Promdonkoy and Ellar (2003). Briefly, serial dilutions of *B. cereus* B10502 cell free spent culture supernatants were added to human erythrocytes suspended in PBS (final concentration 0.66% v/v). Suspensions were incubated at 37 °C and absorbance at 600 nm was determined at different time points. The effect of kefiran was tested at 0, 500, 800 and 1000 mg/L.

Percentage of not lysed erythrocytes was calculated as % = $(A_{600}\text{sample}/A_{600}\text{ control}) \times 100$. Absorbance was measured at

600 nm in a Metrolab 330 Spectrophotometer. Control of 100% lysis was performed with red blood cells treated with 0.2% (v/v) Triton X-100 in PBS (Sigma St. Louis, USA).

2.9. Statistical analysis

Statistical analysis of the variations was performed by mean of two tailed Student's *t*-test.

3. Results

Spent culture supernatants of *B. cereus* B10502 tested on Caco-2 cells lead to a dose response effect. As shown in Fig. 1A, biological effects encompass total cell detachment when high concentrations of extracellular factors were employed. No detachment was observed when cells were incubated either with BHI alone or BHI with kefiran. It is worth to note that kefiran (800 mg/L) significantly protects cultured enterocytes from detaching activity of *B.cereus* B10502 supernatants. This protective effect was observed at dilutions of spent culture supernatants ranging from 1/16 to 1/32.

Fig. 1B shows the effect of different kefiran concentrations on the biological activity of *B. cereus* B10502 extracellular factors diluted 1/32. Protection against biological activity of the supernatant was equivalent at kefiran concentrations ranging from 300 mg/L up to 1000 mg/L. Indeed, only 55% of cell remained attached when no kefiran was added whereas this value was around 80% for all kefiran concentration assayed.

Biological effects of *B. cereus* extracellular factors include the impairment of mitochondrial dehydrogenase activity

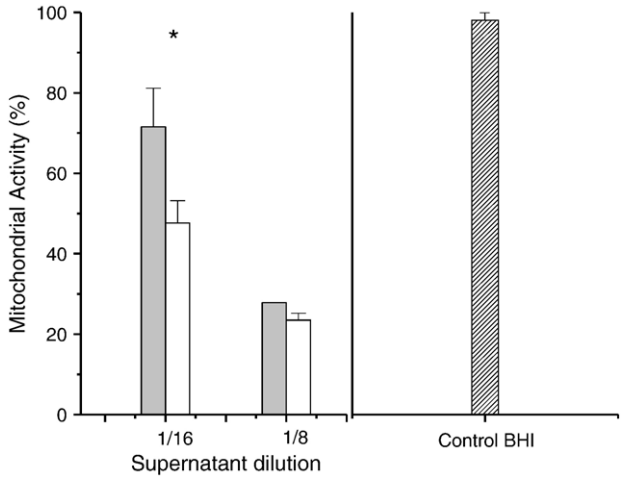

Fig. 2. Effect of *Bacillus cereus* B10502 supernatants on the mitochondrial dehydrogenase activity of Caco-2 cells in the presence and absence of kefiran. Bars represent the mitochondrial activity (MTT assay) of cells coincubated for 1 h with serial dilutions of spent culture supernatants of *Bacillus cereus* B10502 without (open white bars) and with (grey bars) kefiran 800 mg/L or with BHI (dashed bars). Percentage of remaining mitochondrial dehydrogenase activity was calculated as: (Ax/Ac)×100, where Ax=Absorbance (450 nm) after treatment with spent culture supernatant and Ac=Absorbance (450 nm) of control cells (PBS). Results are averages of 3 values±SD. (*) Indicates significant differences with the corresponding control ($p<0.05$)

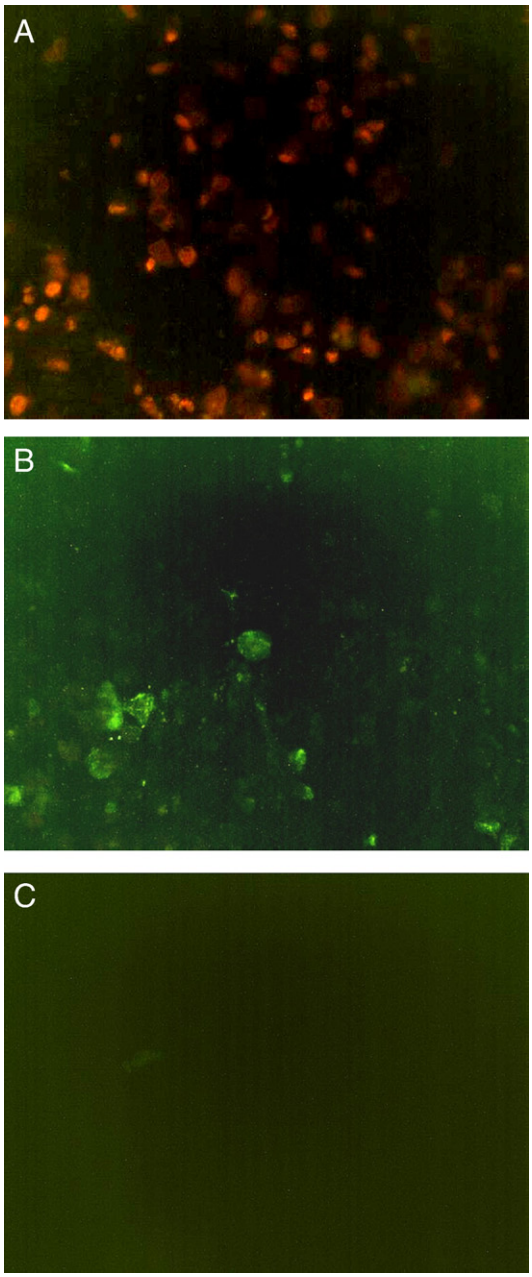

Fig. 3. Assessment of the number of necrotic and apoptotic cells by labelling with Annexin V-FITC-Propiduim Iodide of Caco-2 cells incubated for 1 h with spent culture supernatants (dilution 1/16) of *Bacillus cereus* B10502 without (A) or with (B) kefiran 600 mg/L. (C) BHI control. Magnification 40×.

(Fig. 2). Kefiran also protects against this cytopathic activity. Indeed, 1/16 dilutions of *B.cereus* B10502 spent culture supernatants lead to 50% of remaining mitochondrial dehydrogenase activity whereas around 70% was observed in the presence of kefiran ($p<0.05$).

Caco-2 monolayers incubated for 1 h with *B. cereus* B10502 supernatants evidenced high proportion of necrotic cells (Fig. 3A). In contrast, when kefiran was present during incubation, the necrotizing effect was significantly diminished and only a few apoptotic cells were observed (Fig. 3B,C). Studies performed by scanning electron microscopy were in

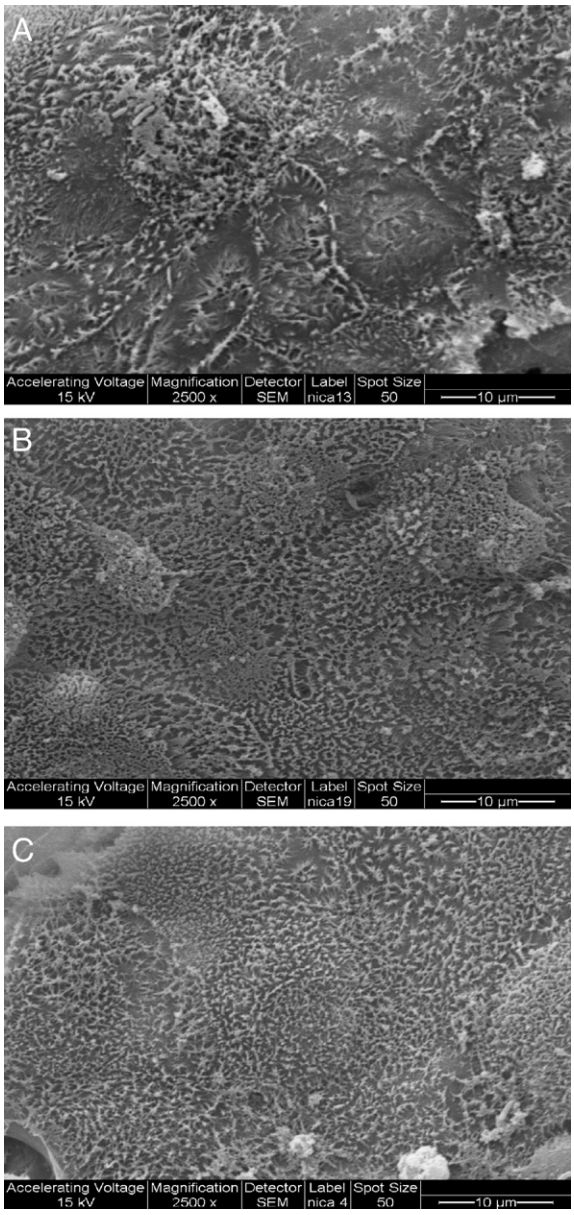

Fig. 4. Scanning electron microscopy of Caco-2 cells after 1 h incubation with spent culture supernatant of *Bacillus cereus* strain B10502 without (A) of with (B) kefiran 800 mg/L. (C) BHI control. Magnification 2500×.

agreement with the above findings. Enterocyte surface became dramatically modified after incubation with *B. cereus* spent culture supernatants. These modifications include both microvilli shortening and effacement (Fig. 4A). In contrast, in the presence of kefiran, cell surface was preserved (Fig. 4B) maintaining the same aspect as was observed in control experiments (Fig. 4C).

These results prompted us to study the haemolytic activity of the supernatants as a read out related to cytolysins. Kinetics of haemolysis performed in the presence and absence of kefiran are depicted in Fig. 5. High dilutions (1/192) of *B. cereus* B10502 spent culture supernatants lead to significant lysis of human red blood cells. The haemolytic activity was

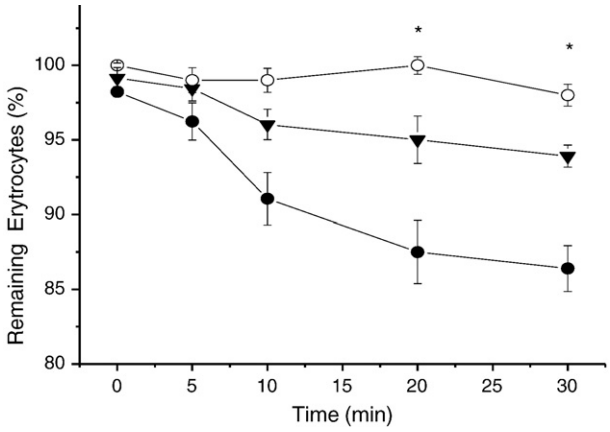

Fig. 5. Time course of the lysis of human red blood cells coincubated with spent culture supernatants (dilution 1/192) of *Bacillus cereus* B10502 without (●) or with (▼) kefiran 800 mg/L. BHI control (○). Results are the average of 3 values± standard deviation. Percent of haemolysis was calculated as: $(Ax/Ac)\times 100$, where Ax = Absorbance (600 nm) of the sample and Ac = Absorbance (600 nm) of the control (0% Haemolysis). (*) Indicates significant differences with the corresponding control ($p<0.05$).

almost completely abolished when 800 mg/L kefiran were added.

4. Discussion

Beneficial effects of kefir are supported by an increasing body of scientific evidence (Garrote et al., 2000; Hertzler and Clancy, 2003; Farnworth, 2005; Vinderola et al., 2005). In the present study we show for the first time the ability of the exopolysaccharide isolated from kefir (kefiran) to antagonize biological effects elicited by extracellular factors from *B. cereus* B10502 cultures.

Interaction between bacterial toxins and eukaryotic cells involves various domains and receptors on the cell surface. Following this interaction, signaling events trigger a cascade of cellular responses that in turn lead to structural and functional modifications. The study of the virulence of *B. cereus* revealed a multifactorial system that encompasses different bacteria-cell interactions (Minnaard et al., 2001; Minnaard et al., 2004).

Caco-2 cells provide a useful *in vitro* model for the study of the interaction between intestinal pathogens and human enterocytes. Extracellular factors of *B. cereus* elicit dramatical changes in cultured human enterocytes. These effects include dose-dependent cell detachment that is total when undiluted supernatants are tested (Minnaard et al., 2001). Here we show that this drastic cytopathic effect can be abrogated in the presence of at least 300 mg/L kefiran (Fig. 1B). This protective effect was confirmed by using other read outs such as measurement of mitochondrial dehydrogenase activity, double labeling with Annexin V/propidium iodide and scanning electron microscopy (Figs. 2, 3–4).

Interestingly the protective effect of kefiran was also demonstrated in a hemoglobin release assay with human red blood cells (Fig. 5). These results suggest antagonism of the activity of *B. cereus* hemolysins.

B. cereus factors having hemolytic activity include hemolysin BL, sphingomyelinase, phosphatidylinositol phospholipase C, cytolysin K and cereolysin O. There are two main mechanisms leading to hemolysis by protein toxins, i.e. oligomerization and assembly of toxin monomers into the plasma membrane (Palmer et al., 1998) and enzymatic disruption of the red blood cell envelopes (Beecher et al., 2000).

The larger pores that can be formed by pore forming toxins such as thiol-activated haemolysins (e.g. cereolysin O) are about 30–40 nm in diameter (Henderson et al., 1999). Interestingly, we have found that non-ionic polymers of hydrodynamic radius larger than 25 nm are unable to protect red blood cells from the haemolytic activity of *B. cereus* extracellular factors (data not shown). These findings suggest that, although kefiran hydrodynamic radius is larger than 100 nm (data not shown), mechanisms other than osmotic protection can be involved in the protective effect of kefiran. For example, kefiran could antagonize key events in the oligomerization and assembly of homotypic aggregates of toxin monomers necessary for large pore formation.

It is worth to note, that pore forming toxins can act not only as cytopathic factors by themselves but also in the delivery of bacterial effectors into the cell (Madden et al., 2001; Walev et al., 2001). By avoiding formation of large pores, kefiran could be also antagonizing some of the cytopathic effects elicited by bacteria–enterocyte interaction such as depolimerization of F-actin (Minnaard et al., 2004).

An alternative mechanism accounting for the antagonistic activity of kefiran against haemolytic activity of *B. cereus* B10502 could be related to the inhibition of toxins having enzymatic activity such as sphingomyelinase.

Protective effect of kefiran against extracellular factors depended on the strain studied. The effect observed against biological activity was also observed when *B. cereus* strain M2 was assayed but no protection was observed against neither strain 2 nor T1 (data not shown). These findings indicate a strain-specific protective activity of kefiran in agreement with the multifactorial character of *B. cereus* virulence factors (Minnaard et al., 2007). Indeed, different strains produce not only different virulence factors but also different ratios of the same factors. It is worth to note, that strains B10502 and M2 clustered together when multifactorial analysis of virulence factors was performed. In addition, strains T1 and 2 belong to another well defined group (Minnaard et al., 2007).

Our results show the ability of kefiran to antagonize key events involved in the virulence of a spore-forming intestinal pathogen. Even though the effect is strain-specific and the mechanisms responsible for the inhibition of the biological activity remain unknown, it is tempting to hypothesize that kefiran could block receptors on the cell surface, inhibit cytolytic factors or elicit some cellular response opposite to that triggered by *B. cereus*.

Taken together, our findings suggest a novel mechanism accounting for health promoting effects of probiotics thus enlarging the field of application of bacterial products as additives in functional foods.

Acknowledgments

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