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ORIGINAL PAPER

Kefiran protects Caco-2 cells from cytopathic effects induced by *Bacillus cereus* infection

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Abstract The aim of this work was to evaluate the ability of kefiran to antagonize cytopathic effects triggered by *Bacillus cereus* strain B10502 on cultured human enterocytes (Caco-2 cells). Cell damage was evaluated by F-actin labelling, scanning electron microscopy and determination of ratios of necrotic and detached cells. To assess the interaction between kefiran and bacteria or eukaryotic cells, flow cytometric analysis was conducted with FITC-labelled kefiran. Kefiran significantly protected infected cells from cytopathic effects induced by *B. cereus* such as cell necrosis, F-actin disorganisation and microvilli effacement, although presence of kefiran did not modify the adhesion of microorganisms to cultured human enterocytes. Results could be ascribed to the ability of kefiran to interact with both bacteria and eukaryotic cells thus antagonizing interactions necessary for maximal biological effects. Our findings encourage further research on the use of bacterial exopolysaccharides to antagonize virulence factors associated to direct bacteria–cell interactions.

Keywords *Bacillus cereus* · Caco-2 cells · Exopolysaccharide · Kefiran · Kefir · Virulence factors

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Introduction

Bacillus cereus is a spore forming gram-positive pathogen widely distributed in the environment. This microorganism can contaminate foods, and thermal resistance of spores makes difficult to inactivate them (Ghelardi et al. 2002; Lund and Granum 1997; McKillip 2000).

B. cereus is able to produce several virulence factors leading to two main syndromes: emetic and diarrheic. Emetic syndrome is due to the production of cereulide, a heat stable dodecadepsipeptide (Agata et al. 1994, 1995; Mikami et al. 1994). In contrast, the diarrheic syndrome is a multifactorial process that is related to several extracellular factors: (a) cereloysin-O, a thiol-activated cholesterol binding cytolysin

(Alouf 2000; Guignot et al. 2001); (b) phospholipases (Beecher et al. 1995b, 2000; Granum and Nissen 1993); (c) hemolysin BL, a tripartite enterotoxin that requires all the three components for maximal activity (Beecher et al. 1995a; Beecher and Lee Wong 2000; Callegan et al. 1999; Lund and Granum 1997); (d) the non haemolytic enterotoxin complex, Nhe, a complex with 3 subunits (Lund et al. 2000); (e) hemolysin IV (Hly), with a strong activity in plasma membranes with diverse compositions (Beecher et al. 2000) and (f) the cytotoxic protein CytK, associated with necrotic enteritis (Hardy et al. 2001; Lund et al. 2000). Extracellular factors can be produced during vegetative growth in the small intestine (Granum 2001). It has been shown that *B. cereus* spores (Andersson et al. 1998; Rönner et al. 1990) and vegetative cells (Minnaard et al. 2004) can adhere to epithelial cells. This direct prokaryote-eukaryote interaction constitutes an additional virulence factor of *B. cereus* leading to profound cytopathic effects (Minnaard et al. 2004, 2007).

In recent years, the use of exopolysaccharides from lactic acid bacteria as health promoters has gained interest. Those biomolecules could provide positive effects to the host through selective stimulation of the growth of beneficial intestinal microorganisms (prebiotic effect). However, the possible effect of exopolysaccharides on the interaction of intestinal microbiota with host's cells remains unknown.

Kefiran is the exopolysaccharide produced by microorganisms present in kefir grains (Abraham and De Antoni 1999; Garrote et al. 2001; Rimada and Abraham 2001). It is a branched hydrosoluble glucogalactan, composed by equal amounts of glucose and galactose with glycosidic bonds that are not hydrolyzed by digestive enzymes. Molecular weight, determined by HPLC, is higher than 10^7 Da (Micheli et al. 1999; Piermaría et al. 2008). Recently, it has been demonstrated that kefiran is able to antagonize biological effects of *B. cereus* extracellular factors (Medrano et al. 2008). In addition, evidence on its immunomodulating activity has been reported (Vinderola et al. 2006).

Above results along with the fact that direct interaction with enterocytes constitutes a key virulence factor for *B. cereus* pathogenesis, led us to look at the effect of kefiran on the infection of cultured human enterocytes by *B. cereus*.

Materials and methods

Bacterial strains and culture conditions

B. cereus strain B10502, from a food poisoning outbreak, was kindly provided by the Laboratorio Central de Salud Pública, Buenos Aires, Argentina (Minnaard et al. 2004). Stock cultures were preserved at −80°C in brain heart infusion (BHI) broth (BIOKAR Diagnostics, Beauvais, France) using glycerol 1% (v/v) as a cryoprotectant. Bacteria were reactivated at 32°C in BHI supplemented with 0.1% (w/v) glucose under orbital agitation. Microorganisms were then inoculated (4% v/v) into 5 ml of BHI–glucose 0.1% (w/v) and incubated with agitation at 32°C for 3 h. Bacteria were harvested by centrifugation (900*g* for 10 min), washed twice with PBS (KH_2PO_4: 0.144 g l^{-1}; Na_2HPO_4: 0.795 g l^{-1}; NaCl: 9 g l^{-1}; pH 7) and suspended in DMEM (Dulbecco's modified Eagle's minimum essential medium, GIBCO, BRL Life Technologies Rockville, MD, USA) containing 100 μg ml^{-1} chloramphenicol to prevent bacterial growth during the experiment. It has been demonstrated (Minnaard et al. 2004) that chloramphenicol does not affect neither viability nor biological activity of vegetative cells of *B. cereus*.

Cell densities were adjusted to $A = 1$ (600 nm) in a spectrophotometer (Metrolab 330 Spectrophotometer, Metrolab, Argentina). This optical density represents 10^8 CFU ml^{-1} as assessed by plate counts. pH was adjusted to 7 with 5N NaOH. Bacterial suspensions were conserved on ice until infection. Cells were infected with bacterial suspensions ranging from 1.25 to 5×10^7 CFU ml^{-1} (multiplicity of infection, MOI, from 12.5 to 50 microorganisms per cell).

Isolation and quantification of kefiran

A weighted amount of kefir grains CIDCA AGK1 was treated in boiling water (1:10 w/w) 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 left 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 once. Polysaccharide was then dissolved

in hot distilled water (kefiran solution) after which it was liophilized (Heto FD4. Heto-Holten, Denmark). Polysaccharide concentration was determined by the anthrone method with glucose solutions as standards (Southgate 1991). All the samples were tested for the absence of free 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 in kefiran solutions was tested by the Bradford's method (Bradford 1976). Anthrone, Bradford and TLC reagents were obtained from Sigma (St. Louis MO 63178 USA). Kefiran solutions were prepared by dissolving lyophilized kefiran in PBS or DMEM. Before experiments, solutions were sterilized by filtration (0.45 μm pore diameter).

Culture of human enterocytes

Caco-2 cells (Fogh et al. 1977; Pinto et al. 1983) were routinely grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 25 mM glucose (GIBCO BRL Life Technologies Rockville, MD, USA), 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 IU ml^{-1}), streptomycin (12 μg ml^{-1}) and gentamicin (50 μg ml^{-1}). Monolayers were prepared in 24-well tissue culture plates (Greiner Bio One, Frickenhausen, Germany). Experiments and cell maintenance were carried out at 37°C in a 5% CO_2–95% air atmosphere. Culture medium was changed every 2 days and assays were performed with cells at passages between 56 and 60. Fully differentiated cells (14 days in culture) were used throughout. For microscopy, cells were grown on round glass coverslips (Assistent, Sondheim, Germany).

Infection assays

Cell monolayers were washed three times with PBS before the infection assays. In some experiments, Caco-2 cells were preincubated for 1 h with kefiran solution (800 mg l^{-1}). Afterwards, polysaccharide was removed and replaced with an equal volume of DMEM. Bacterial suspensions ranging from 1×10^7 to 1×10^8 CFU ml^{-1} were added to the monolayers in the presence and absence of kefiran 800 mg l^{-1} and incubated for different time periods at 37°C in a CO_2 5%–95% air atmosphere. All the infections assays were performed in the presence of 100 μg ml^{-1} chloramphenicol to avoid bacterial growth. This in vitro model of *B. cereus* infection has been validated in our laboratory (Minnaard et al. 2004). To assess bacterial adherence to Caco-2 cells, after infection, monolayers were washed three times with PBS and then incubated for 1 h with 1 ml of distilled water per well to lyse the cells. Serial dilutions of the samples were plated onto nutrient agar (BIOKAR Diagnostics, Beauvais, France), and incubated for 16 h at 37°C.

Detachment of cells

Detachment of Caco-2 cells was assessed as previously reported by Minnaard et al. (2004). Briefly, enterocyte-like cells were incubated with bacterial suspensions ranging from 1×10^7 to 1×10^8 CFU ml^{-1} at 37°C for 3 h. The experiment was performed in the absence and presence of kefiran (800 mg l^{-1}). In some assays, cells were preincubated with kefiran 1 h prior to infection. Then, monolayers were washed with PBS and fixed for 1 min with 2% (v/v) formaldehyde in PBS and washed again with the same buffer. Afterwards cells were stained by incubating for 20 min with 500 μl of a solution containing 0.13% (w/v) crystal violet, 5% (v/v) ethanol and 2% (v/v) formaldehyde in PBS. After being washed with PBS to remove stain excess, samples were treated for 1 h with freshly prepared 50% ethanol (v/v) at room temperature. Absorbance was measured at 650 nm in a spectrophotometer (Metrolab 330, Argentina). Percentage of attached cells was calculated as: $100 \times A/A_c$, where A is the absorbance of infected cells and A_c is the absorbance of uninfected control cells.

Labelling of necrotic cells

Labelling was performed according to a previously published method (Minnaard et al. 2001). Briefly, after 60 min infection, cells were washed twice with buffer containing 25 mM HEPES, 125 mM NaCl, and

2.5 mM $CaCl_2$ and 0.2% (w/v) gelatin (pH 7.2). Then, 100 µl of buffer containing 1 µg ml^{-1} propidium iodide (Sigma, St. Louis, USA) and 100 µg ml^{-1} RNAase (Sigma, St. Louis, USA) were added per well. Cells were incubated at room temperature for 15 min, washed and mounted in glycerol 50% (v/v) in buffer. Micrographs were obtained with a fluorescence microscope LEICA DMLB (Leica Microsystems, Wetzlar GmbH, Germany) coupled to a DC100 camera (Leica Microscopy Systems Ltd., Heerbrugg, Switzerland). Analysis was conducted on 5 random microscopical fields.

F-actin cytoskeleton labelling

Labelling of F-actin cytoskeleton was performed as previously described (Minnaard et al. 2004). Briefly, after 2 h infection, Caco-2 cells were washed three times with PBS and fixed with 3% (w/v) paraformaldehyde in PBS for 15 min. Samples were then treated with 50 mM NH_4Cl for 10 min to block aldehyde functions, permeabilized for 4 min with Triton X-100 (Sigma Chemical Co., St. Louis, MO), incubated for 45 min with 1 µg ml^{-1} fluorescein-labelled phalloidin (Sigma Chemical Co., St. Louis, MO) in PBS and finally mounted in 50% (v/v) glycerol–0.1% (v/v) sodium azide in PBS. Samples were examined by conventional epifluorescence microscopy using a microscope LEICA DMLB (Leica Microsystems, Wetzlar GmbH, Germany) coupled to a DC 100 camera (Leica Microscopy Systems Ltd.).

Scanning electron microscopy

After incubation for 30 min at 37°C with bacterial suspensions (with or without kefiran 800 mg/L), cells were washed three times with PBS, fixed with 4% (v/v) glutaraldehyde (Riedel de Haen, Seelze, Germany) for 16 h at 4°C and 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.).

Flow cytometry

Vegetative cells of *B. cereus* B10502 and Caco-2 cells were incubated with kefiran labeled with fluorescein isothiocyanate (FITC) for 30 min. Afterwards, samples were centrifuged, fixed with paraformaldehyde 3% (w/v) and diluted to approximately 1×10^6 cells per ml. Flow cytometry (10,000 events) was performed in a FACScalibur flow cytometer (Becton Dickinson Biosciencies, San Jose, USA). Analysis was conducted with Cell Quest Pro (Becton Dickinson) and WinMDI (version 2.9; Joseph Trotter) softwares.

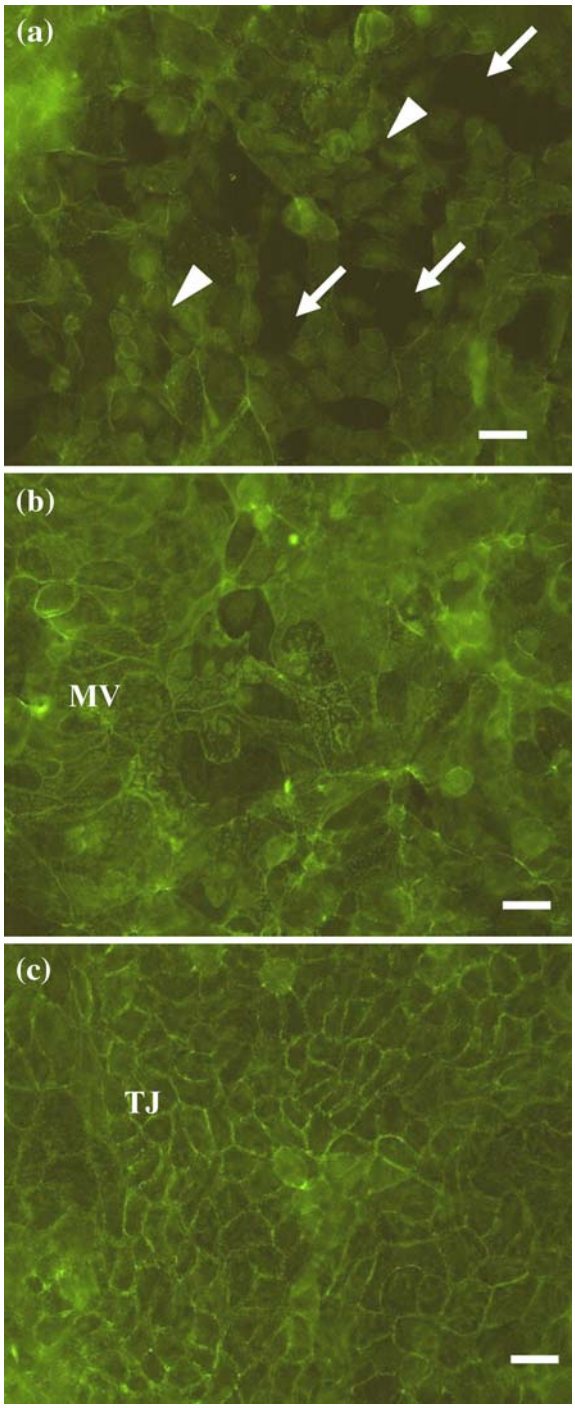
Statistical analysis

Results were compared by means of two-tailed Student's *t*-test and Mann–Whitney test (InfoStat version 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

Results

The cytoskeleton of infected enterocyte-like cells was studied in the presence or absence of kefiran. As shown in Fig. 1a, an evident damage of the cytoskeleton was observed when Caco-2 cells were infected with *B. cereus* B10502. There were several regions with no fluorescent labelling and cell rounding was also noted. In contrast, when monolayers were preincubated with kefiran prior to infection, both actin network and cell morphology were preserved (Fig. 1b). It is important to point out that cells incubated with kefiran alone did not show cytoskeleton alterations (data not shown) as compared with uninfected control cells (Fig. 1c).

To gain further insight on the protective effect of kefiran, we analyzed monolayers by scanning electron microscopy (SEM). Attachment of strain B10502 to Caco-2 cells led to microvilli effacement (Fig. 2a). In contrast, when infections were conducted in the presence of kefiran, cell surface was not modified (Fig. 2b) as compared with control uninfected cells (Fig. 2c). Noteworthy, total number of adhering bacteria was not modified by kefiran ($1.24 \pm 0.03 \times 10^5$ CFU ml^{-1} and $1.03 \pm 0.02 \times 10^5$ CFU ml^{-1} without and with kefiran, respectively). However, adhesion pattern was different when monolayers were incubated with kefiran. This finding is shown in Fig. 2 where patches of bacteria



◀ **Fig. 1** F-actin labelling of Caco-2 cells after 2 h infection with 2×10^7 CFU ml^{-1} *B. cereus* B10502 (MOI = 20) **a** without kefiran, **b** preincubated with kefiran 800 mg l^{-1} prior to infection, **c** uninfected control cells treated with DMEM. Regions with lack of fluorescent label (*arrows*) and cell rounding (*arrowheads*) are evident in infected cells with no kefiran added. MV: microvilli, TJ: tight junctions. Results show representative images from 3 independent experiments. *Bar*: 10 μm

attached to the enterocytes can be observed when no kefiran is added (Fig. 2a). In the presence of kefiran, bacteria were evenly distributed on the monolayer (Fig. 2b).

Infection of Caco-2 cells by strain B10502 not only modified cell morphology and F-actin network but also led to diminution of cell viability. Indeed, after 1 h infection, high numbers of necrotic cells were observed (Fig. 3). Necrotic cells per microscopic field diminished from 40, when no kefiran was added, to 24 when cells were preincubated with kefiran 800 mg ml^{-1} prior to infection ($P < 0.05$). When no preincubation was performed, there was a trend to the diminution of the number of necrotic cells in the presence of kefiran ($P = 0.17$) as compared with the effect observed in infected monolayers without kefiran. Uninfected cells showed around 3 necrotic cells per microscopic field (Fig. 3). Preincubation with kefiran alone did not increase the number of necrotic cells as compared with uninfected cells without kefiran (data not shown).

Infection of enterocytes for 3 h with strain B10502 led to cell detachment. Preincubation of cells with kefiran significantly preserved cell monolayers. As shown in Fig. 4, when cells were infected with 2.5×10^7 bacteria, around 60% of cells remained attached to the plastic surface when no kefiran was added, whereas this value rose to 90% when cells were preincubated with kefiran prior to infection (Fig. 4). The protective effect was more evident when higher infection doses (5×10^7 bacteria) were used. Under these conditions, around 15% of cells remained attached when no kefiran was added, whereas this value increased up to 85% when cells were incubated with kefiran prior to infection (Fig. 4). When kefiran was added together with bacteria, protection was not observed (Fig. 4).

Above results prompted us to study what structures kefiran was interacting with. To this end, flow cytometry analysis using FITC-labeled kefiran was performed. Histograms, shown in Fig. 5, demonstrate that kefiran is able to interact with both bacteria and eukaryotic cells (Fig. 5).

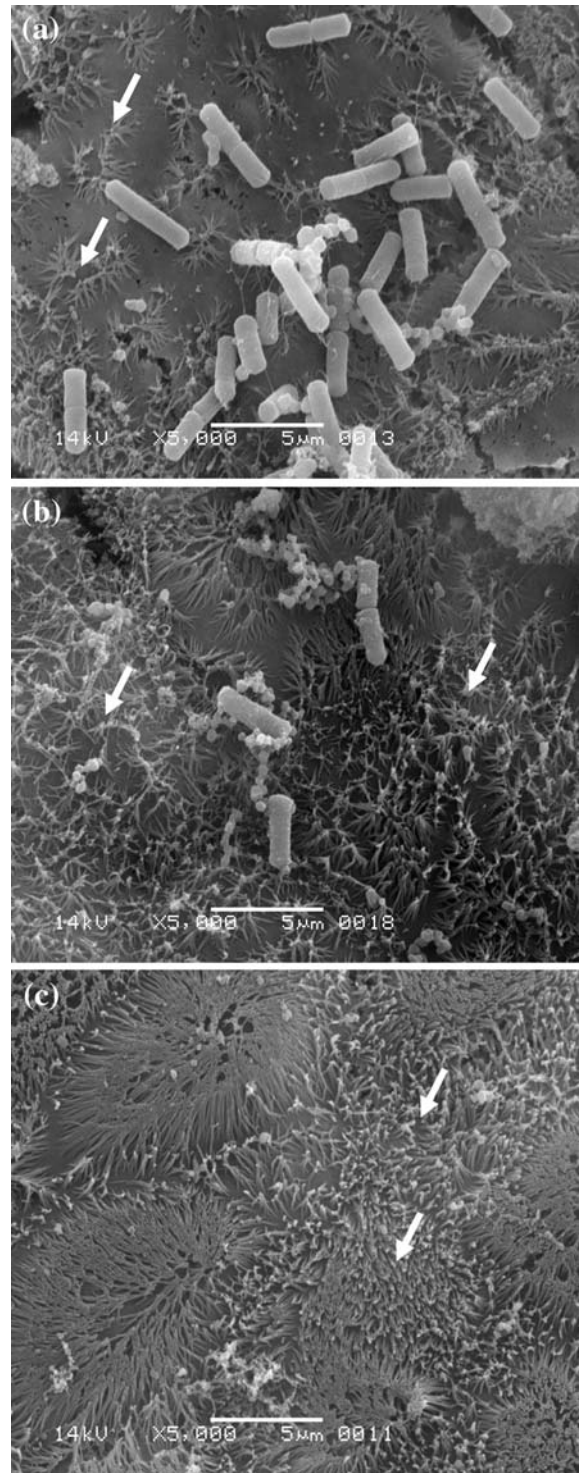


Fig. 2 Scanning electron microscopy of Caco-2 cells after 30 min infection with 5×10^7 CFU ml^{-1} *Bacillus cereus* strain B10502 (MOI = 50) **a** without kefiran, **b** in the presence of kefiran 800 mg l^{-1}, **c** uninfected cells treated with DMEM. Uninfected cells incubated with kefiran alone did not differ from cells incubated with DMEM (not shown). Results show representative images from 3 independent experiments. *Bar*: 5 μm. Microvilli are indicated by *white arrows*

Discussion

Virulence of *B. cereus* has long been ascribed to the production of extracellular factors (Drobniewski 1993; Kotiranta et al. 2000). Nevertheless, the possible role of adhesion/invasion events on the pathogenesis of this microorganism has been demonstrated (Andersson et al. 1998; Minnaard et al. 2004, 2007; Ramarao and Lereclus 2006).

Recently published results show the ability of kefiran to protect cultured cells from biological effects of *B. cereus* extracellular factors (Medrano et al. 2008). It is worth to note that the protective effect of kefiran against extracellular factors of *B. cereus* could not be mimicked by dextran (Medrano et al. 2008). In the present paper we show for the first time the ability of an exopolysaccharide from lactic acid bacteria (kefiran) to antagonize biological effects that follow direct interaction between *B. cereus* and enterocyte-like cells.

Previously published results show that some EPS from lactobacilli and bifidobacteria increase the adhesion of intestinal pathogens (Ruas-Madiedo et al. 2006). It is worth noting that, even though kefiran do not antagonize adhesion of *B. cereus* to enterocytes, increased adhesion in the presence of kefiran was not observed. Our results clearly show that kefiran significantly modifies the distribution of adhered bacteria on the monolayers. Indeed, aggregative adherence was observed when no kefiran was added whereas diffuse adherence was evident in the presence of kefiran (Fig. 2). It could be hypothesized that bacterial concentration in the patches reaches the necessary threshold for cytopathic effect. It is possible that kefiran is antagonizing cytopathic effects either by blocking interaction between *B. cereus* and receptors on the cell surface or by interfering signaling cascades related to virulence mechanisms. Interestingly, we demonstrated that kefiran can interact with both bacteria and enterocytes (Fig. 5). These findings could indicate that this polysaccharide recognizes equivalent receptors on both bacteria and eukaryotic cells or that the interaction is not specific.

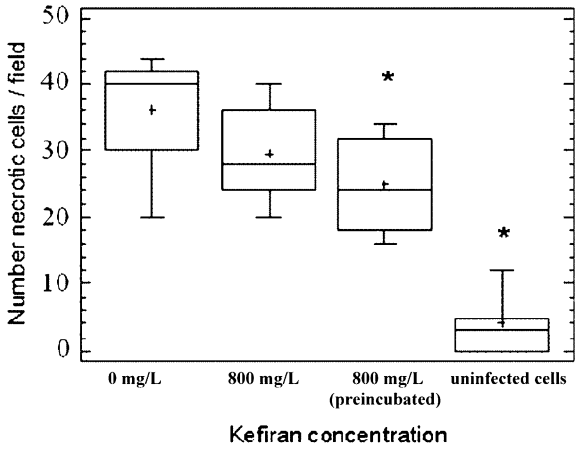


Fig. 3 Necrosis induced by infection of Caco-2 cells with *B. cereus* B10502. Cells were infected for 60 min with 10^7 CFU ml^{-1} (MOI = 20) Necrotic cells were determined from micrographs (160 total cells per image) on 5 random microscopic fields after staining with propidium iodide. Infections were performed in the presence or absence of kefiran 800 mg l^{-1} or after preincubation of cell monolayers with kefiran 800 mg l^{-1}. Necrotic cells in uninfected monolayers are also shown. Comparisons were done with infected cells without kefiran (0 mg l^{-1}) and significant differences ($P < 0.05$) are indicated by an asterisk. *Box plots* show representative results from 3 independent experiments

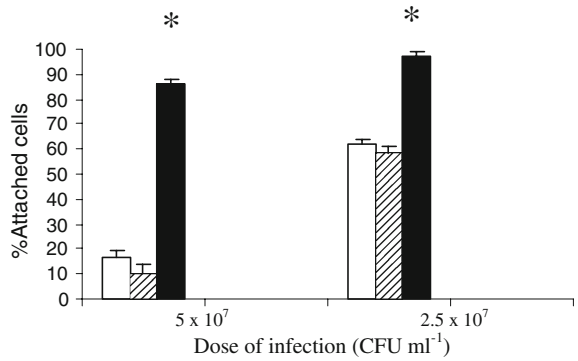


Fig. 4 Percentage of Caco-2 cells that remained attached after 3 h infection with 2.5×10^7 and 5.0×10^7 CFU ml^{-1} of *Bacillus cereus* strain B10502 (MOI: 25–50). *White bars*: without kefiran, hatched bars: infection in the presence of 800 mg l^{-1} kefiran, *black bars*: 1 h Preincubation with 800 mg l^{-1} prior to infection. Cell attachment was evaluated by crystal violet staining as stated in "Materials and methods" section. Results represent averages from a representative experiment from 3 independent experiments. *Error bars* indicate standard deviation. Asterisks represent significant differences ($P < 0.05$) with controls without kefiran

Biomolecules such as polysaccharides play a leading role in biological systems. Indeed, they are involved in cell-to-cell adhesion and in subsequent

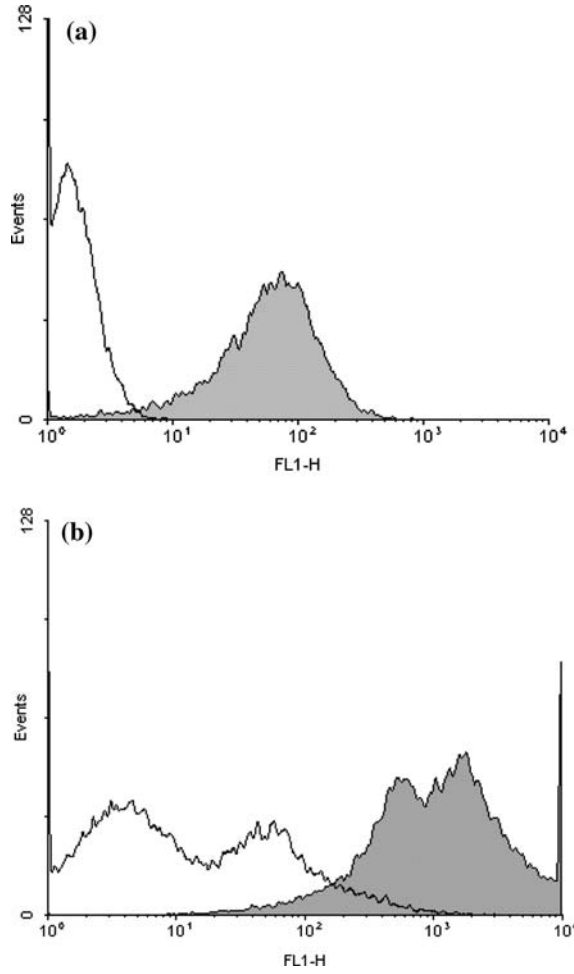


Fig. 5 Flow cytometry analysis of the interaction of kefiran with *B. cereus* B10502 and Caco-2 cells. Bacteria (**a**) and enterocyte-like cells (**b**) were incubated for 30 min with FITC-kefiran (*grey histograms*) or unlabeled kefiran (*open histograms*). Values of green fluorescence intensity are represented in the abscissa. Results are representative of 3 independent experiments

recognition, receptor activation and cell growth. In addition, carbohydrates assist in protein folding or improve their stability (Harris et al. 1998; Mimura et al. 2001; Varki 1993). In the context of microbial recognition by host cells, many host receptors are glucidic in nature (Feizi 2000) and interaction between bacterial exopolysaccharides and host cells has been demonstrated (Finlay and Caparon 2000; Henderson et al. 1999; McArthur and Ceri 1983). Furthermore, specific recognition of regions in the bacterial surface by glucidic molecules is also possible (Tarasenko et al. 2004). Kefiran exert a

protective effect on Caco-2 cells infected with *B. cereus*, that was not observed with dextran (data not shown), indicating that chemical composition and three-dimensional structure of the biopolymer play a crucial role in the interaction.

It is also known that one of the main protective mechanisms in the intestinal tract is the barrier effect due to the mucin layer (Forstner et al. 1995). Interestingly, sugar residues on the host's cell surface can act as "traitorous" signposts for microbial attack (Varki 1993). In contrast, some others have masking and "decoy" functions that abrogate detrimental reactions. Pathogenic microorganisms or their toxins could encounter their cognate glucidic ligand before they reach the natural target. This decoy function is well documented for mucins, and exogenous polysaccharides (e.g. kefiran) could also play a similar function. An alternative (or complementary) explanation to our findings could be the modulation of signaling cascades by interaction with structures situated in neighboring regions near the receptors (Bremer et al. 1984; Hakomori 1990; Zeller and Marchase 1992).

Taken together, our findings show the potential of bacterial exopolysaccharides such as kefiran to modulate key steps in the virulence of microorganisms in the context of intestinal infections. Even though underlying mechanisms are not known, the present paper encourages further research on the role of bacterial polysaccharides in functional foods.

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