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Kefir-isolated *Lactococcus lactis* subsp. *lactis* inhibits the cytotoxic effect of *Clostridium difficile in vitro*

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Kefir is a dairy product obtained by fermentation of milk with a complex microbial population and several health-promoting properties have been attributed to its consumption. In this work, we tested the ability of different kefir-isolated bacterial and yeast strains (*Lactobacillus kefir, Lb. plantarum, Lactococcus lactis* subps. *lactis, Saccharomyces cerevisiae* and *Kluyveromyces marxianus*) or a mixture of them (MM) to antagonise the cytopathic effect of toxins from *Clostridium difficile* (TcdA and TcdB). Cell detachment assays and F-actin network staining using Vero cell line were performed. Although incubation with microbial cells did not reduce the damage induced by *C. difficile* spent culture supernatant (SCS), *Lc. lactis* CIDCA 8221 and MM supernatants were able to inhibit the cytotoxicity of SCS to Vero cells. Fraction of *Lc. lactis* CIDCA 8221 supernatant containing components higher than 10 kDa were responsible for the inhibitory activity and heating of this fraction for 15 min at 100 °C completely abrogated this ability. By dot-blot assay with anti-TcdA or anti-TcdB antibodies, concentration of both toxins seems to be reduced in SCS treated with *Lc. lactis* CIDCA 8221 supernatant. However, protective effect was not affected by treatment with proteases or proteases-inhibitors tested. In conclusion, we demonstrated that kefir-isolated *Lc. lactis* CIDCA 8221 secreted heat-sensitive products able to protect eukaryotic cells from cytopathic effect of *C. difficile* toxins *in vitro*. Our findings provide new insights into the probiotic action of microorganisms isolated from kefir against virulence factors from intestinal pathogens.

Keywords: *Lactococcus lactis* subsp, *lactis*, kefir, *Clostridium difficile*, toxin.

Abbreviations: AAD, Antibiotic-associated diarrhoea; CDAD, *Clostridium difficile*-associated diarrhoea; SCS, Spent culture supernatant; MM, microbial mixture.

Introduction

The use of probiotics to prevent different gastrointestinal disorders is increasing, since it has been demonstrated that this kind of microorganism is capable of inhibiting the growth or invasion of pathogens, enhance the intestinal barrier, and modulate the mucosal and systemic immune response by regulating secretion of different cytokines (Servin 2004; Corthésy et al. 2007).

Diarrhoea is a common side effect of antibiotic treatment and *Clostridium difficile*-associated diarrhoea (CDAD) has become a major issue in many countries (Limaye et al. 2000). The predominant mechanism of virulence of *C. difficile* is associated with the production of two large toxins: TcdA (308 kDa) and TcdB (260 kDa). These toxins act as glycosyltransferases, modifying host cell small GTPases involved in actin polymerisation and cytoskeleton assembly (Schirmer & Aktories 2004; Jank et al. 2007). Castagliuolo et al. (1998) reported that TcdA is mainly an enterotoxin whereas TcdB has cytotoxic activity and exerts its biological effect at lower doses than TcdA, even though both toxins have the same enzymatic activity.

The search for the best way to prevent CDAD includes the administration of probiotic microorganisms. Several experimental trials employing either a single strain or a combination of different microorganisms for treatment of patients with CDAD have been performed (Hickson 2011) but currently the results obtained are inconclusive. To date, most evidence in this area involves *in vitro* and *in vivo*

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studies with *Saccharomyces boulardii* (Pothoulakis et al. 1993; Castagliuolo et al. 1999; Qamar et al. 2001) or *Lactobacillus rhamnosus* GG (Mack et al. 1999; Madsen, 2001). In particular, it has been reported that *Sac. boulardii* interferes with virulence of *C. difficile* through neutralisation or degradation of toxins (Castagliuolo et al. 1999) and inhibition of inflammatory immune response (Chen et al. 2006). In addition, Trejo et al. (2006) demonstrated that extracellular factors from bifidobacterial species significantly reduce growth of *C. difficile* and its adhesion to enterocyte-like Caco-2 cells. More recently, the same authors proposed that co-culture with potentially probiotic bacteria affects the synthesis and/or secretion of clostridial toxins reducing the biological activity of *C. difficile* culture supernatant (Trejo et al. 2010).

Kefir is a dairy product obtained by fermentation of milk with kefir grains and its consumption has been associated with several health-promoting properties such as antimicrobial, antitumoral, immunological and hypocholesterolemic effects (Farnworth, 2005; Vinderola et al. 2005, 2006). Kefir grains are clusters of microorganisms that include mainly lactic acid bacteria (lactobacilli, lactococci, leuconostoc), yeasts and acetic acid bacteria which coexist in a symbiotic association in a matrix of polysaccharides and proteins (Garrote et al. 2001; Hsi-Chia et al. 2008).

Our workgroup has isolated and characterised more than 100 bacterial and yeast strains from kefir grains which have showed differences in surface and probiotic properties (Garrote et al. 2004; Golowczyc et al. 2007, 2008). Among these kefir-isolated microorganisms, it has been demonstrated that yeasts are able to down regulate the pro-inflammatory response induced in intestinal epithelial cells *in vitro* and *in vivo* (Romanin et al. 2010), and that different lactobacilli strains show a high inhibitory power against *Salmonella* spp., *Shigella* spp. and *Escherichia coli* O157:H7 *in vitro* (Golowczyc et al. 2007, 2008; Hugo et al. 2008). Besides, our group demonstrated the ability of surface proteins (S-layer) from *Lb. kefir* strains to antagonise cytotoxic effects of clostridial toxins on cultured eukaryotic cells (Carasi et al. 2012).

Recently, we have developed a mixture constituted of three bacteria (*Lb. plantarum* CIDCA 83114, *Lb. kefir* CIDCA 8348 and *Lactococcus lactis* CIDCA 8221) and two yeasts (*Sac. cerevisieae* CIDCA 8112 and *Kluyveromyces marxianus* CIDCA 8154) isolated from kefir grains which was able to inhibit *Sh. sonnei* growth *in vitro* and preserved its viability, antimicrobial capacity and safety after freeze-drying procedure (Bolla et al. 2011). These properties and the potentiality of kefir-isolated microorganisms (or mixtures of them) as starters in probiotic fermented products encouraged us to evaluate the inhibitory effect of these selected strains against other pathogens. Since, as we mentioned above, the use of probiotics to prevent CDAD could be an interesting alternative to the antibiotic treatments, we decided to evaluate the ability of these bacterial and yeast strains isolated from kefir to antagonise the effect of *C. difficile* toxins on eukaryotic cells *in vitro*.

Materials and Methods

Bacterial strains and growth conditions

Pure cultures used in this study comprised *Lc. lactis* subsp. *lactis* CIDCA 8221, *Lb. plantarum* CIDCA 83114, *Lb. kefir* CIDCA 8348, *K. marxianus* CIDCA 8154 and *Sac. cerevisiae* CIDCA 8112. These strains were previously isolated from kefir grains and have been identified and characterised by Garrote et al. (2001) and Delfederico et al. (2006). The original reference cultures were maintained in milk at −80 °C. Both lactobacilli and yeasts were propagated in MRS-broth (DIFCO, Detroit, USA) for 48 h at 30 °C. *Lc. lactis* was grown in 1.1.1 growth media (10 g tryptone/l—Difco, Detroit, USA; 10 g yeast extract/l—Biokard Diagnostic, Beauvais, France and 10 galactose/l—Mann Research Laboratories, NY) Abraham et al. (1990) for 24 h at 30 °C. To obtain the microbial mixture (MM), the same volume of each microbial suspension was centrifuged at 10 000 ***g*** for 15 min and resuspended together in 1 ml sterile PBS (KH_2PO_4 0·144 g/l, NaCl 9 g/l, Na_2HPO_4 0·795 g/l, pH 7·4). The concentrations of bacteria and yeasts in MM were determined by plate counting using MRS agar for lactobacilli, YGC (Yeast extract Glucose Chloramphenicol Agar, Biokard Diagnostic, Beauvais, France) for yeast strains, and 1.1.1. agar for *Lc. lactis*. Final concentrations of bacteria and yeasts were 10^9 CFU/ml and 10^6 CFU/ml respectively.

The clinical isolate of *C. difficile* strain 117, obtained from the Hospital Dr Muñiz (Buenos Aires, Argentina) and previously characterised as positive for TcdA and TcdB production, was grown for 24 h at 37 °C in Brain Heart Infusion (BHI: Biokar Diagnostic, Beauvais, France) supplemented with 0·5 g cysteine chlorhydrate/l (BHI/cys) in anaerobic conditions (AnaeroPak, Mitshubishi Gas Chemical Co, Inc). Culture was centrifuged at 15 000 ***g*** for 15 min and the *C. difficile* spent culture supernatant (SCS) containing toxins was retained, then passed through a 0·22 μM filter and used in the experiments.

Incubation of SCS with microorganisms or their supernatants

For incubation of *C. difficile* SCS with each microorganism, microbial cells in stationary phase were harvested by centrifugation and washed three times with PBS. The pellet was resuspended in SCS at a final bacterial concentration of $OD_{550} = 1{\cdot}0$ and incubated for 60 min at 37 °C. Spent culture supernatants pre-incubated with each isolated microorganism were obtained by centrifugation at 10 000 ***g*** for 15 min. Similarly, SCS was pre-incubated for 60 min at 37 °C with MM at final concentrations of 10^9 CFU bacteria/ml and 10^6 CFU yeasts/ml.

For preparation of microbial supernatants, individual microbial cultures and MM were harvested by centrifugation at 10 000 ***g*** for 15 min, washed three times with PBS and resuspended in BHI/cys. After incubation for 1 h at 37 °C in aerobic conditions, microorganisms were centrifuged at

10 000 ***g*** for 15 min and the supernatants (SN) were passed through a 0·22 μM filter and conserved at −20 °C until used.

For some experiments, *Lc. lactis* CIDCA 8221 supernatant was fractionated through an Amicon ultrafiltration StirreCell 8050 Millipore Corporation US, equipped with a regenerated cellulose membrane (cut-off 10 kDa) or was heated at 100 °C for 15 min or was treated with different proteases (trypsin, pepsine, quimiotrypsine or proteinase K) (SIGMA, USA) at 2·5 mg/ml during 1 h at 37 °C based on previous protocols described by Trejo et al. (2006) and Golowczyc et al. (2009).

For the incubation of kefir-isolated microorganisms or MM culture supernatants with SCS, supernatants were added to SCS from *C. difficile* in a 1:1 ratio and biological activity of these samples was compared with SCS obtained from pure *C. difficile* cultures as described below. In each case, acidity of treated SCS did not show changes compared with control SCS (pH = 6·5).

Cell cultures

Vero cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL Life Technologies, Rockville, MD, USA) supplemented with 10% (v/v) inactivated (30 min/60 °C) foetal calf serum (BIOSER, Argentina, PAA Laboratories GmbH), 2 g $NaHCO_3$/l, 10 mg streptomycin/l and 10 IU penicillin G/ml. Cells were inoculated ($6{\cdot}25 \times 10^4$ cells per well) into 48-well tissue culture plates (Corning, NY) and incubated at 37 °C for 48 h in a 5% (v/v) CO_2, 95% (v/v) air atmosphere to allow the formation of a cell monolayer.

Biological effects on cultured cells

The assay was performed as described by Trejo et al. (2010). Briefly, cultured cells were washed twice with 1 ml PBS and 300 μl cell culture medium were added to each well. Control *C. difficile* SCS, SCS treated incubated with microorganisms (each strain or MM) and SCS treated with microbial supernatants (SCS + SN) were serially two fold diluted in DMEM without foetal calf serum. Two hundred μl of DMEM- diluted samples were added per well and incubated at 37 °C for 16 h in a 5% (v/v) CO_2, 95% (v/v) air atmosphere. Biological activity was assessed by evaluation of cell detachment and labelling of F-actin cytoskeleton (Minnaard et al. 2001, 2004). In some cases, cell detachment assays with SCS + SN *Lc. lactis* CIDCA 8221 were performed in presence of a cocktail of protease inhibitors (Roche, USA).

For cellular detachment assay, after incubation cells were washed twice with PBS and fixed with 2% (v/v) formaldehyde for 1 min (Minnaard et al. 2001). The remaining cells were stained with 0·13 g Crystal Violet/l in 5% (v/v) ethanol and 2% (v/v) formaldehyde. Next, an extraction with 50% (v/v) ethanol was performed and OD_{540} was determined. Biological activity was expressed as the percentage of detached cells (rd), according to the following expression:

$$rd = 100 \times (1 - (Am - Ao)/(Ab - Ao))$$

where Am, absorbance of sample; Ao, absorbance of well without cells (control of stain adsorption by the well); Ab, absorbance of untreated control cells.

The ratio of detached cells (rd) was modelled as a function of SCS concentration by using a hyperbolic function according to Trejo et al. (2010). This approach allows for the calculation of the dose of SCS that leads to the detachment of 50% of the cells (DD50). This parameter inversely correlates with biological activity of SCS.

Staining of F-actin cytoskeleton was performed using Vero cells grown on sterile glass coverslips (Assistant, Sondheim, Germany) in 24-well culture plates (Greiner Bio One, Germany). After incubation with SCS or SCS + SN *Lc. lactis* CIDCA 8221, cells were washed twice with PBS and fixed (2 min) with 3% (v/v) paraformaldehyde. Afterwards, cells were treated with NH_4Cl (50 mM) and then permeabilised with 0·2% (v/v) Triton X100 solution in PBS before labelling with FITC-phalloidin (SIGMA, Inc., St. Louis, MO, USA) in PBS containing 2 g gelatin/l (SIGMA, Inc. St. Louis, MO, USA) for 45 min in the dark (Minnaard et al. 2004). Cells were observed by fluorescence microscopy.

Dot-blot assay

To test the effect of *Lc. lactis* CIDCA 8221 SN on TcdA and TcdB present in SCS, a dot-blot assay was performed. After incubation of SN with *C. difficile* SCS for 60 min at 37 °C, 4 μl of the sample was spotted onto nitrocellulose membranes. After drying for 30 min at room temperature, strips were blocked with 30 g skim milk/l buffer T-TBS: 0·05 M Tris (hydroxymethyl aminomethane Mallinckrodt, Baker Inc.), 0·15 mol NaCl/l, 0·5% (v/v) Tween 20 (Sigma–Aldrich, Inc., St. Louis, MO, USA) pH 7·5, for 90 min at 37 °C. Afterwards, membrane strips were incubated 90 min at 37 °C with mouse monoclonal antibodies anti-TcdA (1/1000) or anti-TcdB (1/500) (Meridian Life Science, Unc.). After washing three times with T-TBS, strips were incubated for 60 min at 37 °C with peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Inc., USA) and the reaction was revealed using 4-chloro-1-naphthol (9 g/l) and 18 μl 30% (v/v) H_2O_2 (E. Merck, Germany) dissolved in 3 ml methanol and 15 ml TBS.

Statistical analysis

Data were analysed by InfoStat Software (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). Results were statistically tested using Student *t*-test to determine any significant difference.

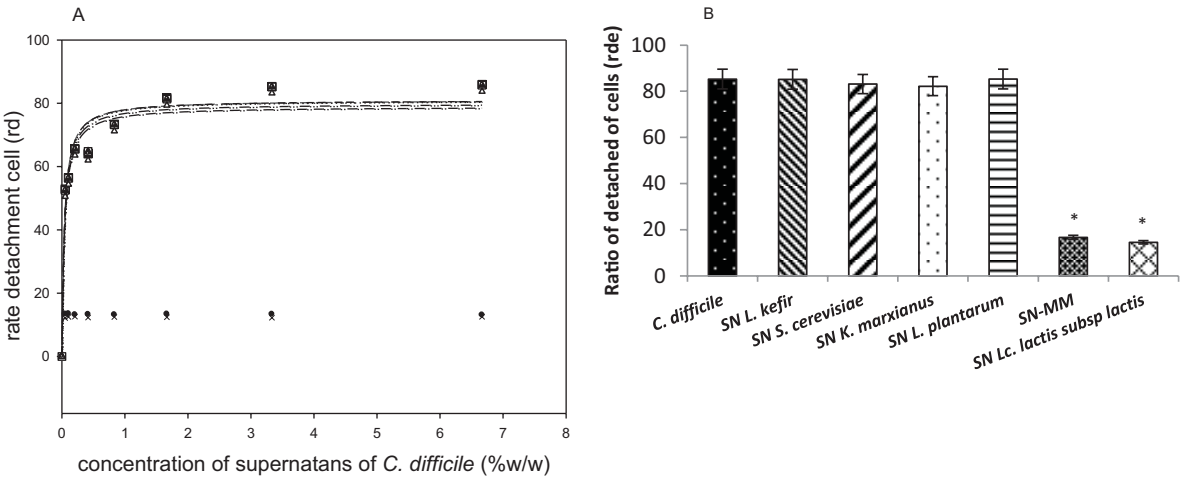

Fig. 1. Biological activity of *C. difficile* SCS on Vero cells. (a) Dose response curves of cell detachment assay (results expressed as ratio of detached cells) obtained for SCS -○- and SCS treated with microbial supernatants (SN) from *Lc. lactis* subsp *lactis* CIDCA 8221 -●-; *Lb. kefir* CIDCA8348 -△-; *Sac. cerevisiae* CIDCA 8112 -▲-; *K. marxianus* CIDCA 8154 -□-; *Lb. plantarum* CIDCA83114 -■-; MM -X-. (b) Percentage of detached cells at fixed concentration (3·5%) of SCS -fx1- and SCS treated with SN from *Lc. lactis* subsp *lactis* CIDCA 8221-fx2-; SN from *Lb. kefir* CIDCA8348 -fx3-; SN from *Sac. cerevisiae* CIDCA 8112 -fx4-; SN from *K. marxianus* CIDCA 8154 -fx5-; SN from *Lb. plantarum* CIDCA 83114 -fx6-; SN from MM -fx7-. **P* < 0·05 compared with SCS

Results and discussion

Effect of microorganisms or microbial supernatants on the biological activity of C. difficile *toxins on Vero cells*

The ability of kefir-isolated strains to antagonise cytopathic effects of *C. difficile* SCS was tested performing dose response curves in a cell detachment assay and by determining the dose of SCS that leads to the detachment of 50% of Vero cells (DD50). When Vero cells were incubated with SCS treated with each microorganism or MM (the mixture containing all strains under study), DD50 was not increased compared with control of treatment with SCS (0·063 ± 0·009 vs. 0·065 ± 0·009).

Then, a series of experiments were performed in order to test the effect of microbial supernatants on biological activity of SCS. Figure 1(a) shows the cell detachment curves obtained for SCS and SCS added to supernatants from each strain or MM (SCS + SN). Supernatants obtained from *Lc. lactis* CIDCA 8221 and MM had the ability to inhibit the cytotoxic effect of *C. difficile* SCS meanwhile SCS + SN from isolated strains *Lb. kefir* CIDCA 8348, *Lb. plantarum* CIDCA 83114, *Sac. cerevisiae* CIDCA 8112 and *K. marxianus* CIDCA 8154 did not protect Vero cells against SCS cytopathic effect. Figure 1(b) shows the highest percentage of detached cells determined for all the SCS + SN assessed in the study. There were no significant differences between the results obtained for SCS + SN *Lc. lactis* CIDCA 8221 and SCS + SN MM, suggesting that the products released by *Lc. lactis* CIDCA 8221 do not lose its inhibitory activity against clostridial toxins even in the presence of the other microorganisms. The DD50 determined for each SCS + SN is shown in Table 1. Besides, SCS + SN *Lc. lactis* CIDCA 8221 showed a dose-dependent effect, maintaining its inhibitory ability up to a 10-fold dilution (data not shown).

Table 1. DD50 determined for C. difficile spent culture supernatant (SCS) and SCS incubated with supernatants (SCS + SN) belonging to each strain under study or a mixture of them (MM)

Supernatants	DD50
SCS	0·063 ± 0·008
SCS + SN *Lb. plantarum* CIDCA 83114	0·063 ± 0·009
SCS + SN *Lb. kefir* CIDCA 8348	0·063 ± 0·009
SCS + SN *Lc. lactis* CIDCA 8221	> 6·7
SCS + SN *Sac. cerevisiae* CIDCA 8112	0·068 ± 0·009
SCS + SN *K. marxianus* CIDCA 8154	0·071 ± 0·008
SCS + SN MM	> 6·7

Figure 2 shows phalloidin-FITC labelled F-actin cytoskeleton of Vero cells after treatment with SCS and SCS + SN from *Lc. lactis* CIDCA 8221. The SCS from *C. difficile* 117 induced the disruption of the actin network, cell rounding and detachment of Vero cells. However, no damages were observed when cell monolayers were treated with SCS + SN *Lc. lactis* CIDCA 8221 as compared with untreated controls.

In this sense, different authors reported that *Lactobacillus* and *Bifidobacterium* strains are able to inhibit toxigenic effect of *C. difficile* on eukaryotic cell cultures (Trejo et al. 2006, 2010; Banerjee et al. 2009). More recently, Trejo et al. (2010) described that the synthesis and/or secretion of clostridial toxins is reduced by co-culture with potentially probiotic bacteria, including a lactobacilli strain isolated from kefir (*Lb. plantarum* CIDCA 83 114). Although several

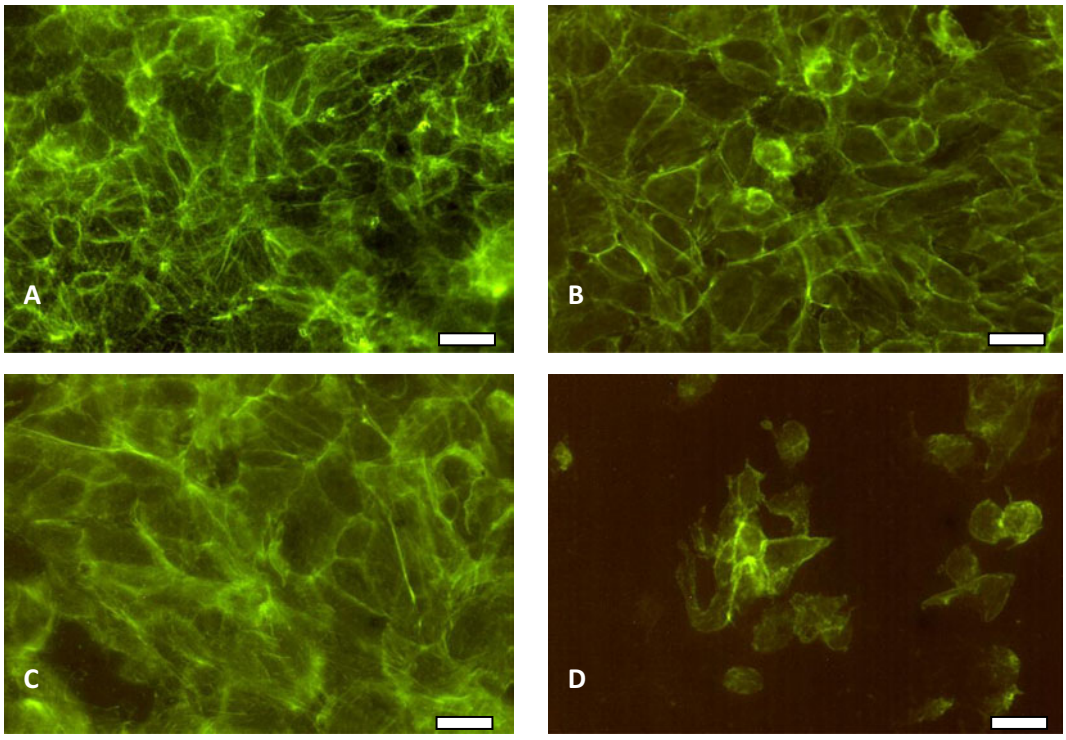

Fig. 2. (Colour online) Staining of F-actin cytoskeleton of Vero cells without treatment (panel A), and after treatment with SN from *Lc. lactis* CIDCA 8221 (panel B), SCS + SN from *Lc. lactis* CIDCA 8221 (panel C) or SCS (panel D). (Bar = 10 μM)

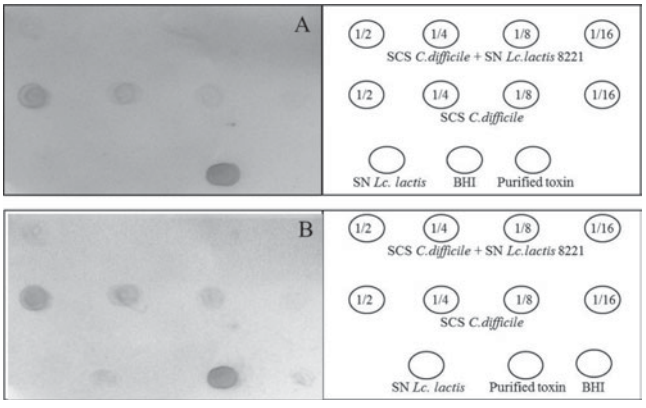

Fig. 3. Dot blot assay with specific monoclonal antibodies against TcdA (panel A) or TcdB (panel B). SCS was incubated with *Lc. lactis* CIDCA 8221 SN for 60 min at 37 °C, and serially two fold diluted samples were spotted onto nitrocellulose membranes (4 μl/spot). BHI medium, *Lc. lactis* CIDCA 8221 SN and purified toxins were spotted as controls

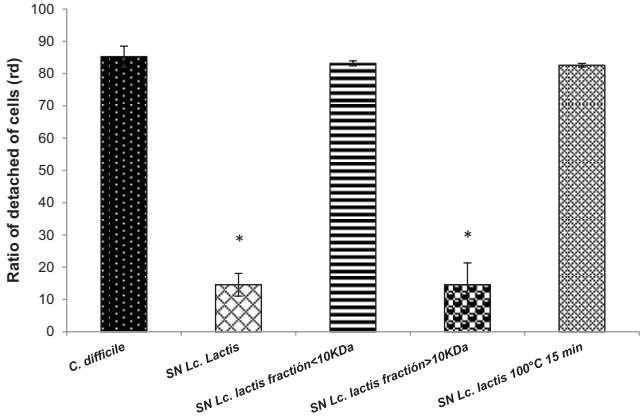

Fig. 4. Biological activity of *C. difficile* SCS on Vero cells. DD50 obtained for SCS -fx1- and SCS treated with supernatant (SCS + SN) from *Lc. lactis* CIDCA 8221-fx2- ; SCS + SN from *Lc. lactis* CIDCA 8221 fraction > 10 KDa -fx3-; SCS + SN from *Lc. lactis* CIDCA 8221 fraction < 10 KDa -fx4-; SCS + SN from *Lc. lactis* CIDCA 8221 heated at 100 °C for 15 min -fx5-. *P < 0·05 compared with SCS

authors have been reported the ability of kefir-derived products to antagonise virulence factors of intestinal pathogens *in vitro* (Garrote, 2000; Londero et al. 2011) and *in vivo* (Bekar et al. 2011), to our knowledge this is the first report of protective activity of secreted extracellular factors from kefir-isolated microorganisms against *C. difficile* toxins.

Effect of Lc. lactis CIDCA 8221 supernatant on the concentration of C. difficile *toxins*

We tested the effect of incubation of SCS with *Lc. lactis* CIDCA 8221 SN on concentration of clostridial toxins by dot-blot assay. As shown in Fig. 3, the intensity of the spots for both TcdA and TcdB was reduced after incubation

of SCS with *Lc. lactis* CIDCA 8221 SN for 60 min. These findings could suggest a decrease in the concentration of both toxins in SCS treated with *Lc. lactis* CIDCA 8221 SN.

Effect of different treatments on the inhibitory ability of Lc. lactis *CIDCA 8221 supernatant against* C. difficile *SCS*

To gain further insight into the characteristics of the released products involved in the inhibitory activity of the *Lc. lactis* CIDCA 8221, the supernatant was fractionated using a 10 kDa cut-off membrane before cell detachment assays. Only the fraction containing components with a molecular mass higher than 10 kDa was able to inhibit the toxigenic effect of SCS on eukaryotic cells (Fig. 4). Moreover, the heat treatment of this fraction at 100 °C for 15 min completely inactivated its inhibitory activity (Fig. 4). These results suggest the involvement of a thermo sensitive component in the inhibitory ability of *Lc. lactis* CIDCA 8221.

Based on these findings and taking into account the results obtained by dot-blot assay, we decided to test the presence of proteolytic activity. However, treatment of *Lc. lactis* CIDCA 8221 supernatant with different proteases (trypsin, chymotrypsin, pepsine and proteinase K) or the presence of protease-inhibitor cocktail did not abrogate the protective effect of *Lc. lactis* secreted products. There are many reports about the synthesis of different peptidases and serine proteases by *Lc. lactis* with molecular weights ranging from 40–180 KDa (Sasaki et al. 1995; Christensen et al. 1999; Poquet et al. 2000; Foucaud-Scheunemann & Poquet 2003). However, we have found no record of the activity of these enzymes against cytotoxic effects of pathogenic bacteria such as *C. difficile*. In this sense, Castagliuolo et al. (1996) described a 54-kDa serine protease from *Sac. boulardii* which digests TcdA and TcdB *in vitro*. More recently, Banerjee et al. (2009) reported that *Lb. delbrueckii* subsp. *bulgaricus* B-30892 secreted one or more bioactive component which neutralise cytotoxicity of *C. difficile* probably by inactivating its toxins. In this work, since no abrogation of protective effect was observed after treatment with different proteases or in presence of proteases-inhibitors, we have not been able to demonstrate a proteolytic activity against clostridial toxins in *Lc. lactis* CIDCA 8221 supernatant, although results of dot-blot assay showed that both the concentration of TcdA or TcdB seemed reduced in treated *C. difficile* SCS. In order to explain these findings, we hypothesise that there could be a non-covalent interaction between soluble molecules present in *Lc. lactis* CIDCA8221 supernatant and clostridial toxins which would be responsible for the inhibitory effect on cytotoxicity of *C. difficile* SCS. This interaction could also interfere with the epitope–paratope reaction, leading to a misreading of the dot-blot assay. Further experiments are needed to confirm the mechanism of action and to determine the nature of *Lc. lactis* CIDCA 8221 secreted-metabolites.

In conclusion, we demonstrated that kefir-isolated *Lc. lactis* CIDCA 8221 released heat-sensitive metabolites able to protect eukaryotic cells from cytopathic effect of *C. difficile* toxins *in vitro*. These findings provide new insights into the probiotic action of microorganisms isolated from kefir against virulence factors from intestinal pathogens.

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