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# Co-culture with potentially probiotic microorganisms antagonises virulence factors of *Clostridium difficile* in vitro

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**Abstract** Toxigenic strains of *Clostridium difficile* were co-cultured with different strains of bifidobacteria and lactobacilli. Spent culture supernatants were tested for biological activity on cultured Vero cells. Co-culture of *C. difficile* with some potentially probiotic strains lead to a reduction of the biological activity of spent culture supernatants. The observed effects cannot be ascribed either to secreted factors from the probiotic strains or to toxin adsorption by bacterial cells. Immunological assays showed that there was significant diminution of both clostridial toxins (TcdA and TcdB) in spent culture supernatants of co-cultures as compared with pure clostridial cultures. Even though co-cultured clostridial cells showed a slight increase of intracellular toxins, this increase did not completely explains the reduction of toxin concentration in culture supernatants. The evidence suggests that the antagonism could be due to the diminution of the synthesis and/or secretion of both clostridial toxins. Our findings provide new insights into the possible mechanisms involved in the protective effect of probiotics in the context of *C. difficile* infection.

**Keywords** *Bifidobacterium* · *Lactobacillus* · *Clostridium difficile* · Probiotics · Toxins · Virulence

**Abbreviations**

| | |
|---|---|
| SCS | Spent culture supernatants |
| TcdA | *C. difficile* toxin A |
| TcdB | *C. difficile* toxin B |
| CDAD | *C. difficile* associated diarrhea |
| $OD_{600\ nm}$ | Optical density at 600 nm |
| PBS | Phosphate buffered saline |
| DMEM | Dulbbeco's Modified Eagle's Medium |
| rd | Ratio of detached cells |
| NBT | Nitro-blue tetrazolium chloride |
| BCIP | 5-Bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt |
| DD50 | Dose of SCS that leads to the detachment of 50% of the cells |

## Introduction

*Clostridium difficile* is a Gram-positive spore forming bacterium that is carried asymptomatically in about 50% of neonates, 20% of hospitalized patients and 2% of healthy adults (Matsuki et al. 2005; Gursoy

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et al. 2007). In hospitalized individuals, administration of proton pump inhibitors or antibiotics such as clindamycin, cephalosporins, fluoroquinolones and ampicillin disturb the normal intestinal microbiota thus leading to the overgrowth of intestinal *C. difficile* or colonization with environmental clostridia that are normally present in healthcare centers (Schroeder 2005; Sunenshine and McDonald 2006; Dubberke et al. 2007). In this context, *C. difficile* is responsible for 90–100% of cases of pseudomembranous colitis (PMC), 60–75% of antibiotic-associated colitis and 30–60% of antibiotic-associated diarrhea (AAD) (Limaye et al. 2000). The main virulence factors of this microorganism are two large protein toxins: TcdA (308 kDa) and TcdB (260 kDa). These toxins act as glycosyltransferases that modify host cell small GTPases that are involved in actin polymerization and cytoskeleton assembly (Schirmer and Aktories 2004; Jank et al. 2007).

Evidence suggests that probiotics constitute an alternative approach to prevent/treat *C. difficile* associated diarrhea (CDAD). Indeed, there are several reports on the correction of microbiota unbalances by the administration of probiotics (Colombel et al. 1987; Wullt et al. 2003; Plummer et al. 2004; Kotowska et al. 2005; Segarra-Newnham 2007) or prebiotics (Lewis et al. 2005). Concerning mechanisms involved in the antagonism of *C. difficile* by probiotic microorganisms, evidence suggests that *Saccharomyces boulardii* could interfere with the virulence of *C. difficile* through different mechanisms such as toxin neutralization and inhibition of the inflammatory response (Chen et al. 2006). In addition, it has been demonstrated that pathogen adhesion to enterocyte-like cells and growth are significantly reduced by bifidobacterial extracellular factors (Trejo et al. 2006).

Given that the main virulence factors of *C. difficile* are TcdA and TcdB, we aimed to assess the effect of probiotic microorganisms on the production/secretion of these extracellular factors.

**Table 1** *Bifidobacterium* and *Lactobacillus* strains used in the present study

| *Bifidobacterium* and *Lactobacillus* strains | Strain | References |
|---|---|---|
| *B. bifidum* | NCC 235[a1] | I |
| *B. bifidum* | CIDCA 539[2] | I |
| *B. bifidum* | CIDCA 5310[2] | I |
| *B. bifidum* | CIDCA 5311[2] | I |
| *B. bifidum* | CIDCA 5313[2] | I |
| *B. bifidum* | CIDCA 5318[2] | I |
| *B. breve* | CIDCA 532[1] | I |
| *B. breve* | CIDCA 5312[2] | I |
| *B. breve* | CIDCA 5314[2] | I |
| *B. breve* | CIDCA 5315[2] | I |
| *B. infantis* | NCC200[b2] | I |
| *B. longum* | CIDCA 5316[2] | I |
| *B. longum* | CIDCA 5320[3] | I |
| *B. longum* | CIDCA 5325[4] | I |
| *B. longum* | CIDCA 5323[4] | I |
| *B. pseudolongum* | CIDCA 531 | I |
| *B. adolescentis* | CIDCA 5317[1] | I |
| *Bifidobacterium* spp. | CIDCA 5329[2] | II |
| *L. acidophilus* | ATCC 314[c] | III |
| *L. brevis* | ATCC 8287[c] | III |
| *L. casei* | DSMZ 20011[d] | III |
| *L. kefir* | CIDCA 8348[e] | III |
| *L. kefir* | CIDCA 8321[e] | III |
| *L kefir* | CIDCA 8344[e] | III |
| *L. plantarum* | CIDCA 83114[e] | III |

Strain CIDCA 531 was isolated from a fermented dairy product. Remaining strains were isolated from infant feces (aged from 6 days to 4 months)

Feeding: [1]Formula + Brest, [2]Breast, [3]Formula, [4]UHT milk

I: Gomez Zavaglia et al. 1998; Perez et al. 1998. II: Trejo et al. 2006. III: Garrote et al. 2001

[a] Nestlé Culture Collection. Formerly strain CIDCA 533

[b] Nestlé Culture Collection. Formerly strain CIDCA 538

[c] American Type Culture Collection

[d] DSMZ Deustche Smmlung von Mikroorganismen und Zellkulturen Gmbh, Germany

[e] Isolated from kefir grains

## Materials and methods

### Bacterial strains and growth conditions

Strains of bifidobacteria and lactobacilli included in the present study are shown in Table 1. Two strains of *C. difficile* were studied: ATCC 9689 (Bélanger et al. 2003) and the clinical isolate 117. Both strains are positive for TcdA and TcdB. Clostridia were grown for 20 h at 37°C in Brain Heart Infusion (BHI: Biokar Diagnostic, Beauvais, France) supplemented with 0.05% (w/v) cysteine chlorhydrate (BHI/cys) in

anaerobic conditions (AnaeroPak, Mitshubishi Gas Chemical Co, Inc). Plate counts were performed by plating serial dilutions of the cultures on Differential Reinforced *Clostridium* Medium (DRCM; Laboratorios Britania S.A., Argentina). Plates were incubated for 72 h at 37°C in anaerobic conditions.

Bifidobacteria and lactobacilli were grown in MRS broth (DIFCO, Becton–Dickinson and Company Sparks, MD 21252, USA) supplemented with 0.05% (w/v) cysteine chlorhydrate at 37°C for 20 h in anaerobic conditions. Plate counts were performed by plating serial dilutions on MRS agar (DIFCO). Plates were incubated for 48 h at 37°C in anaerobic conditions.

## Co-cultures

Five ml of BHI/cys were inoculated with 50 µl of 20 h old *Clostridium* culture ($10^8$ CFU $ml^{-1}$) and 50 µl of 20 h old *Bifidobacterium* ($5 \times 10^8$ CFU $ml^{-1}$) or *Lactobacillus* ($5 \times 10^8$ CFU $ml^{-1)}$ cultures. Co-cultures were incubated for 20 h at 37°C in anaerobic conditions. Spent culture supernatants were obtained by centrifugation at $13600 \times g$ for 15 min. Biological activity of co-cultures was compared with pure *Clostridium* cultures as described below.

## 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) fetal calf serum (BIOSER, Argentina, PAA Laboratories GmbH), 2 g $l^{-1}$ $NaHCO_3$, 10 mg $l^{-1}$ streptomycin and 10 IU $ml^{-1}$ penicillin G. Cells were inoculated ($7.5 \times 10^4$ 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.

## Biological effects on cultured cells

Cultured cells were washed twice with 1 ml of phosphate buffered saline (PBS; $KH_2PO_4$ 0.144 g $l^{-1}$, NaCl 9 g $l^{-1}$, $Na_2HPO_4$ 0.795 g $l^{-1}$, pH 7.5). Spent culture supernatants (SCS) were serially (two fold) diluted in DMEM without fetal calf serum. One hundred µl of diluted SCS were added per well and incubated at 37°C for 16 h in a 5% $CO_2$/95% air atmosphere. Biological activity was assessed by determination of cell rounding, labeling of F-actin cytoskeleton and evaluation of cell detachment (Minnaard et al. 2001, 2004)).

Cell rounding was evaluated by microscopy and the dose of SCS that leads to the rounding of 50% of the cells (Dr50) was determined. Staining of F-actin cytoskeleton was performed according to Minnaard et al. (2004). Briefly, Vero cells were grown on sterile glass coverslips (Assistant, Sondheim, Germany) in 24-well culture plates (Greiner Bio One, Germany). After incubation with SCS, cells were washed twice with PBS, fixed (2 min) with 3% (v/v) paraformaldehyde and washed again with PBS. Next, cells were treated with $NH_4Cl$ (50 mM) and then permeabilized with 0.2% (v/v) Triton × 100 solution in PBS. Cells were washed with PBS and then labeled with FITC-phalloidin (SIGMA, Inc., St. Louis, MO, USA) in PBS containing 0.2% (v/v) gelatin (SIGMA, Inc. St. Louis, MO, USA) for 45 min in the dark. Cells were observed by fluorescence microscopy.

To assay cellular detachment, after incubation with SCS, 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 0.013% (w/v) Crystal Violet 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 ratio of detached cells (rd), according to the following expression:

$$rd = 100 * (1 - (ODs - OD_0)/(ODc - OD_0))$$

where ODs, optical density of sample; $OD_0$, optical density of well without cells (control of stain adsorption by the well); ODc, optical density of untreated control cells.

The ratio of detached cells (rd) was modeled as a function of SCS concentration by using the following hyperbolic function:

$$rd = aC/(b + C),$$

where C is the concentration (% v/v) of SCS; a and b are parameters of the equation. By means of this model, the dose of SCS that leads to the detachment of 50% of the cells (DD50) can be calculated. This parameter is useful to compare biological effects of different SCS since DD50 inversely correlates with biological activity.

Effect of extracellular factors from bifidobacteria and lactobacilli

Two series of experiments were conducted. Firstly, cytotoxicity assays were performed with different ratios of SCS (*Clostridium/Bifidobacterium* or *Lactobacillus*). Ratios (volume of *Clostridium* SCS/ volume of *Lactobacillus* of *Bifidobacterium* SCS) tested were 0.5:1, 1:1 and 2:1. Secondly, *Clostridium* strains were grown in the presence of different concentration (25% and 50% v/v) of *Bifidobacterium* or *Lactobacillus* SCS. Afterwards, cytotoxicity assays were conducted as described above.

Determination of extracellular toxin concentration in spent culture supernatants

Bacterial cultures were centrifuged at 13600×*g* for 15 min and 2 µl of supernatants were spotted onto nitrocellulose membranes. Blocking was performed with 3% (w/v) skim milk in buffer TRIS/Tween: TRIS 50 mM (Hydroymethyl aminomethane Mallinckrodt, Baker Inc.), NaCl 150 mM pH 7.5, Tween 20 (Sigma–Aldrich, Inc., St. Louis, MO, USA) 0.05%. Afterwards, membranes were incubated for 40 min at 37°C with mouse anti-TcdA or anti-TcdB monoclonal antibodies (Meridian Life Science, Unc.). Next, membranes were incubated with biotin-goat anti mouse IgG and extravidin-alkaline phosphatase (Sigma–Aldrich, St. Louis, MO, USA). Finally, membranes were treated with a solution of NBT/BCIP (Sigma–Aldrich, St. Louis, MO, USA) until color development. After each incubation step, membranes were exhaustively washed with buffer TRIS/Tween**.** Toxin concentration was calculated by image analysis (TotalLab v2.01, Pharmacia, Orsay, France) and referred to that produced in pure cultures. Two independent assays were performed.

Determination of intracellular toxin concentration

One ml of bacterial culture (pure *Clostridium* or co-cultures) containing $10^8$ CFU $ml^{-1}$ *C. difficile* was centrifuged at 14000×*g* for 5 min and washed twice with PBS. Afterwards, the pellet was suspended in 1 ml of PBS and 5 pulses of sonication (6 min each)

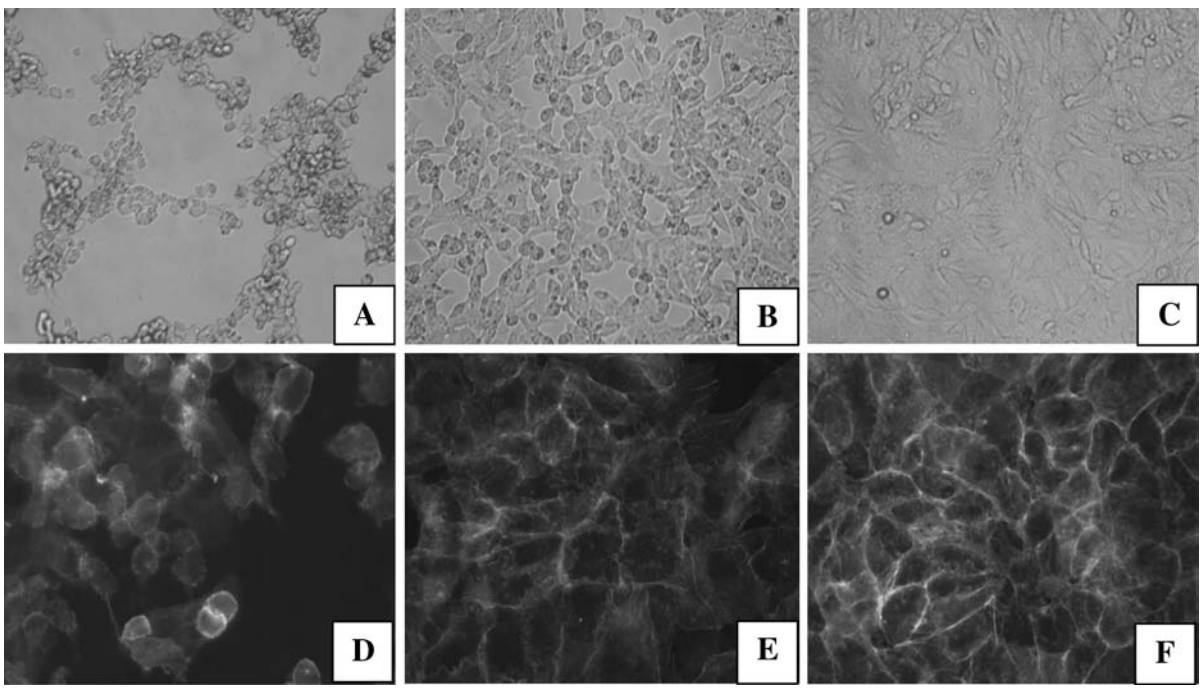


**Fig. 1** Effect on Vero cells of SCS from pure cultures of *C. difficile* or co-cultures with *Bifidobacterium bifidum* 5310. SCS were diluted in DMEM 1/16 **a** pure culture of *C. difficile* ATCC 9689, **b** co-culture of *C. difficile* and *B. bifidum* 5310, **c** control cells, **d** pure culture of *C. difficile* ATCC 9689, F-actin labeling, **e** co-culture of *C. difficile* and *B. bifidum* 5310, F-actin labeling and **f** control cells, F-actin labeling

were performed (20 kc, c:1/s, Ultrasonic Disintegrator, Model 60w, MSE Ltd.). Cell disruption was checked microscopically. After sonication, samples were centrifuged at 13600×*g* for 15 min and 2 µl of supernatants were spotted onto nitrocellulose membranes. Toxin detection was conducted as indicated above.

### Statistical analysis

Results were analysed by means of two-tailed Student's *t* test using the InfoStat software (InfoStat, Version 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). To assess the effects on toxin concentrations, the null hypothesis that toxin concentration in the co-cultures and toxin concentration of pure clostridial cultures were equal was tested.

## Results

### Cytopathic effects

Spent culture supernatants (SCS) of *C. difficile* ATCC 9689 lead to profound effects on cultured eukaryotic cells. As shown in Fig. 1, cytopathic effects encompass disorganization of the actin network that in turn leads to cell rounding and detachment (Fig. 1a, d). In contrast, when the same clostridial strain was co-cultured with *Bifidobacterium bifidum* strain CIDCA 5310, cytopathic effects were significantly diminished (Fig. 1b, e). Untreated control cells are shown in Fig. 1c and f.

Screening of the ability of several bifidobacteria and lactobacilli to antagonize cytopathic effects of *C. difficile* was performed by assessing the dose of SCS that leads to the rounding of 50% of the cells (Dr50) (Fig. 2). This allowed for the selection of strains able to antagonize cytopathic effects associated with *C. difficile* extracellular factors. High Dr50 indicates low biological activity. As shown in Fig. 2, the ability to antagonize cytopathic effects was strain-dependent. Interestingly, some strains (e.g. CIDCA 531, CIDCA 532, CIDCA 5310, ATCC 8287 and CIDCA 83114) were able to inhibit cytopathic effects from both clostridial strains under study.

On the basis of their ability to antagonize biological activity of both clostridial strains, *B. bifidum* CIDCA 5310 and *Lactobacillus plantarum* CIDCA 83114 were selected for further studies. Noteworthy, no inhibition of the growth of *C. difficile* was observed in co-cultures with strains CIDCA 5310 or CIDCA 83114 (Table 2A). In addition, growth of lactobacilli and bifidobacteria was not affected by the presence of clostridia in co-cultures (Table 2B).

### Cellular detachment

Biological activity, expressed as the ratio of detached cells (rd), was modeled as a function of SCS concentration and DD50 was calculated as indicated

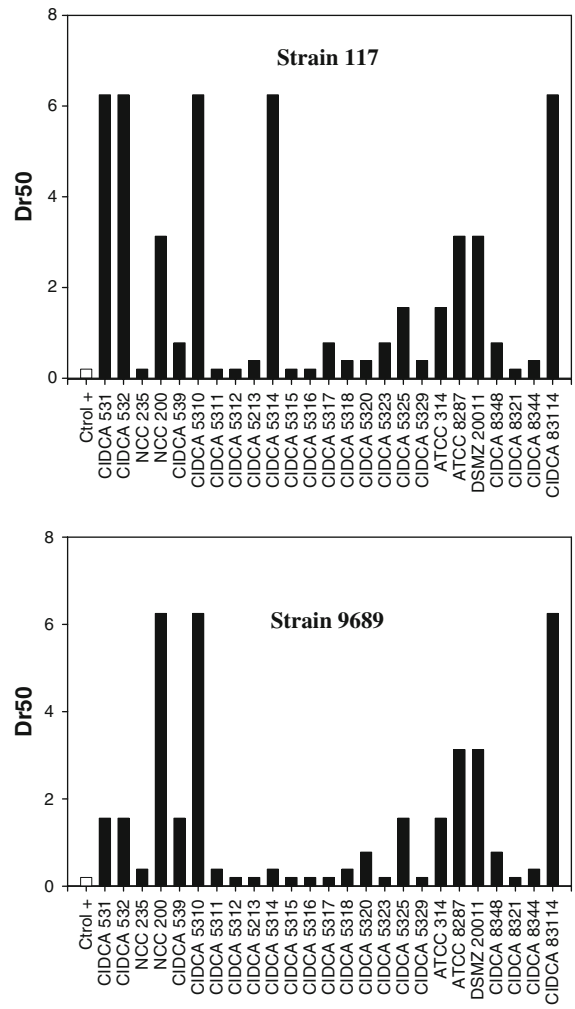


**Fig. 2** Effect of co-culture of clostridia with bifidobacteria or lactobacilli on the biological activity of SCS on Vero cells. Results are expressed as the dose that leads to the rounding or 50% of the cells (Dr50). Pure culture (*open square*); co-culture with *Bifidobacterium* or *Lactobacillus* strains (*filled square*)

**Table 2** (A) Growth of *C. difficile* in pure cultures and in co-cultures with *B. bifidum* strain CIDCA 5310 or *L. plantarum* strain CIDCA 83114; (B) Growth of *B. bifidum* strain CIDCA 5310 or *L. plantarum* strain CIDCA 83114 in pure cultures and in co-cultures with *C difficile* strains

| A | Pure culture (log CFU $ml^{-1}$) | Co-culture with *B. bifidum* 5310 (log CFU $ml^{-1}$) | Co-culture with *L. plantarum* 83114 (log CFU $ml^{-1}$) |
|---|---|---|---|
| *C. difficile* strain 117 | 8.32 ± 0.03 (pH 6.41 ± 0.12) | 8.21 ± 0.03 (pH 6.19 ± 0.12) | 7.80 ± 0.11 (pH 6.09 ± 0.10) |
| *C. difficile* strain 9689 | 7.83 ± 0.04 (pH 6.61 ± 0.12) | 7.73 ± 0.02 (pH 6.36 ± 0.18) | 7.50 ± 0.20 (pH 6.16 ± 0.13) |

| B | Pure culture (log CFU $ml^{-1}$) | Co-culture with *C. difficile* 117 (log CFU $ml^{-1}$) | Co-culture with *C. difficile* 9689 (log CFU $ml^{-1}$) |
|---|---|---|---|
| *Bifidobacterium* strain 5310 | 8.20 ± 0.20 | 7.79* ± 0.20 | 8.31 ± 0.50 |
| *Lactobacillus* 83114 | 8.66 ± 0.30 | 8.49 ± 0.20 | 8.18 ± 0.50 |

In brackets, pH ± standard deviation of cultures or co-cultures are indicated

* Significantly different from pure culture. Student *t* test ($P < 0.05$)

in the "Material and Methods" section (Fig. 3). Interestingly, when biological activity of SCS from co-cultures were analysed, DD50 values were from 5 to 20 times higher than those from pure clostridial cultures (Fig. 4).

### Effect of extracellular factors from *Bifidobacterium* and *Lactobacillus* cultures on the biological activity of *C. difficile*

Dilution of clostridial SCS with lactobacilli or bifidobacteria SCS did not antagonise the biological effects of strain 117 (Table 3). In contrast, biological activity of strain 9689 was not modified by SCS from *Lactobacillus* strain 83114 but a significant decrease of activity was observed when SCS from *Bifidobacterium* strain 5310 was used (Table 3). In addition, growth of clostridial strains in BHI medium with different concentrations of SCS from bifidobacteria or lactobacilli cultures did not abrogate the cytopathic effect (Table 3). Preincubation of clostridial SCS with high concentrations of either bifidobacteria or lactobacilli ($OD_{600} = 2$) did not modify the biological activity (data not shown). It is worth noting that 20 h old cultures of bifidobacteria or lactobacilli in BHI broth do not acidify below pH 6 (data not shown), suggesting that change in pH is not responsible for biological effects.

### Determination of toxin concentration

The above results prompted us to determine whether the diminution of the biological activity of SCS from co-cultures was due to the inhibition of toxin synthesis/activity or to the inhibition of toxin release from bacteria. To this end, we evaluated toxin concentration by immunoblotting (Fig. 5).

As shown in Fig. 6, TcdA concentration was dramatically reduced in co-cultures. Indeed, TcdA co-culture/TcdA pure culture ratios ranged from 0.1 to 0.3 (Fig. 6a) whereas TcdB co-cultures/TcdB pure culture ratios ranged from 0.1 to 0.5 (Fig. 6b).

The concentrations of the intracellular toxins of *C. difficile* strain 9689 were significantly lower when this strain was co-cultured with *B. bifidum* CIDCA 5310 (Table 4). There was a slight increase of intracellular TcdB of strain 117 when it was co-cultured with strain 5310.

## Discussion

Virulence of *C. difficile* is related to the production of two large (308–260 kDa) protein toxins: TcdA and TcdB. These extracellular factors have separate binding and enzymatic domains. The enzymatic domain is responsible for the glycosylation of proteins crucial for cytoskeleton assembly (i.e. Rho proteins). Even though both toxins have the same enzymatic activity, TcdA is mainly an enterotoxin whereas TcdB has cytotoxic activity (Lyerly et al. 1988). In vivo, the effect of these toxins leads to the recruitment of blood cells to the site of infection thus triggering a strong inflammatory response that is responsible for the clinical symptoms. It is known

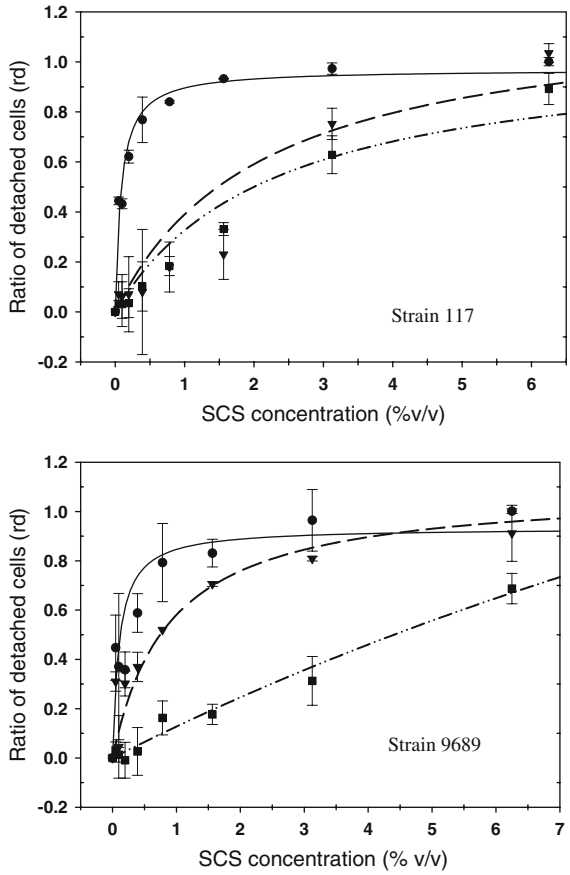


**Fig. 3** Dose response curves of the biological activity of SCS. Results of biological activity are expressed as the ratio of detached cells (rd). (*filled circle*) pure culture of *C. difficile*, (*filled square*) co-culture of *C. difficile* and *B. bifidum* 5310, (*filled inverted triangle*) co-culture of *C. difficile* and *L. plantarum* 83114. Results were adjusted to a hyperbolic model: rd = aC/(b + C), where rd: Ratio of detached cells and C: SCS concentration (% v/v). A representative experiment from three independent assays is shown

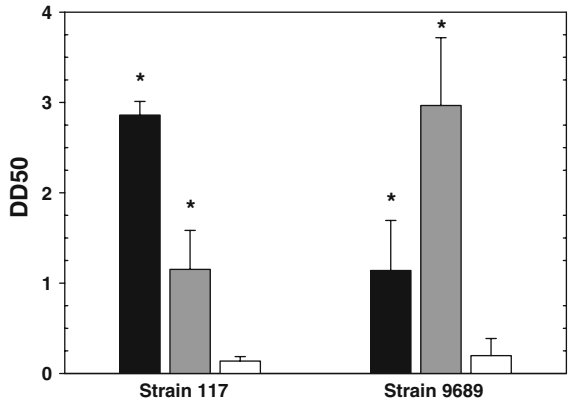


**Fig. 4** Biological activity (DD50) of pure cultures of *C. difficile* (*white bars*) or co-cultures with *B. bifidum* strain 5310 (*black bars*) or *L. plantarum* 83114 (*grey bars*). *Asterisks* indicate significant differences ($P < 0.01$) from the control (pure culture of *C. difficile*). Results are expressed as means ± standard deviation from three independent experiments

that TcdB exerts its biological effect at lower doses than TcdA (Castagliuolo et al. 1998).

In the present study we show that growth of *C. difficile* in the presence of some strains of bifidobacteria or lactobacilli leads to SCS with significantly lower biological activity as compared with pure clostridial cultures.

Antagonism of intestinal pathogens by potentially probiotic microorganisms has long been ascribed to growth inhibition (Trejo et al. 2006; Rönnqvist et al. 2007), competitive exclusion (Collado et al. 2005; Ruas-Madiedo et al. 2006) or immunomodulation (Benyacoub et al. 2005; Humen et al. 2005; Niers et al. 2007). Recently, novel mechanisms have been proposed. Indeed, inhibition of biological effect of *Bacillus cereus* by the polysaccharide kefiran (Medrano et al. 2008, 2009) and antagonism of virulence factors of enterohaemorrhagic *Escherichia coli* by probiotic lactobacilli have been demonstrated (Hugo et al. 2008).

Interestingly, the inhibition of the biological activity of *C. difficile* when co-cultured with lactobacilli has been reported (Banerjee et al.; 2009). In contrast with our observations, these authors suggest that SCS of lactobacilli contains some secreted factors that abolished biological activity of the *Clostridium* SCS. They hypothesize that proteolytic activity in the SCS would lead to toxin cleavage thus modifying biological activity. This hypothesis is in agreement with published results on the effect of *S. boulardii* on the activity of clostridial toxins (Castagliuolo et al. 1998).

Even though in the above-mentioned studies protective effects were clearly shown, the underlying mechanisms have not yet been elucidated. The present study gives further insight on the possible mechanisms involved. Indeed, we show that production of *C. difficile* toxins is significantly diminished in co-cultures. In addition, a slight effect on the toxin release was observed. Notably, growth of clostridial strains in co-cultures was similar to pure clostridial control cultures.

Toxin production by *C. difficile* is influenced by environmental conditions such as growth phase

**Table 3** Biological activity (DD50[a]) of spent culture supernatants from *C. difficile* in different experimental conditions

| *C. difficile* strain | *C. difficile* pure culture | DD50 | | | | |
|---|---|---|---|---|---|---|
| | | Ratio[b] $SCS_{Cd}/SCS_{Lp}$ | | | Percentage[c] (v/v) of $SCS_{Lp}$ added to the culture medium | |
| | | 0.5:1 | 1:1 | 2:1 | 25.0 | 50.0 |
| 117 | 0.11 ± 0.06 | 0.13 ± 0.05 | 0.13 ± 0.01 | 0.15 ± 0.03 | 0.17 ± 0.09 | 0.17 ± 0.06 |
| 9689 | 0.24 ± 0.21 | 0.19 ± 0.07 | 0.11 ± 0.05 | 0.12 ± 0.07 | 0.13 ± 0.17 | 0.08 ± 0.09 |
| *C. difficile* strain | *C. difficile* pure culture | DD50 | | | | |
| | | Ratio[b] $SCS_{Cd}/SCS_{Bb}$ | | | Percentage[c] (v/v) of $SCS_{Bb}$ added to the culture medium | |
| | | 0.5:1 | 1:1 | 2:1 | 25.0 | 50.0 |
| 117 | 0.11 ± 0.06 | 0.26 ± 0.22 | 0.19 ± 0.09 | 0.13 ± 0.03 | 0.07 ± 0.02 | 0.40 ± 0.40 |
| 9689 | 0.24 ± 0.21 | 0.02 ± 0.01 | 0.02 ± 0.01 | 0.03 ± 0.03 | ND | ND |

$SCS_{Cd}$ SCS of pure culture of *C. difficile*, $SCS_{Bb}$ SCS of pure culture of *B. bifidum* strain 5310, $SCS_{Cd}$ SCS of pure culture of *L. plantarum* strain 83114, *ND* not determined

[a] Dose of SCS that leads to 50% of cell detachment

[b] SCS of pure cultures of clostridia, bifidobacterias or lactobacilli were added to the cells at the indicated ratios

[c] Clostridia were cultured in BHI containing different percentages of SCS from bifidobacteria or lactobacilli cultures

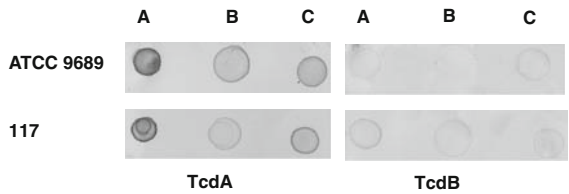


**Fig. 5** Blots for TcdA and TcdB present in SCS. (*A*) *C. difficile* pure culture, (*B*) Co-culture with *B. bifidum* strain 5310, (*C*) Co culture with *L. plantarum* strain 83114

(Hundsberger et al. 1997; Dupuy and Sonenshein 1998), aminoacids (Osgood et al. 1993), butyric acid (Karlsson et al. 2000), rapidly metabolizable carbon sources (Dupuy and Sonenshein 1998), antibiotics (Nakamura et al. 1982) and biotin (Yamakawa et al. 1998; Maegawa et al. 2002). Karlsson et al. (2008) demonstrated that during maximum toxin expression there is an increase of the enzymes involved in metabolic pathways related to succinate, folate and butyrate. Our findings demonstrate that co-culture with *Bifidobacterium* or *Lactobacillus* is able to inhibit biological activity of *C. difficile*. It can be hypothesized that during co-cultures, competition for nutrients would modify toxin production.

Genes related to *C. difficile* virulence are part of a pathogenicity locus positively regulated by an alternative sigma factor (TxeR) and negatively regulated by a membrane-associated protein encoded by the *tcdC* gene (Mani and Dupuy 2001; Govind et al. 2006; Dineen et al. 2007). The *tcdE* gene, coding for a holin that is involved in toxin release, is situated downstream of the *tcdB* gene. Expression of both *tcdC* and *tcdE* are under the control of the same promoter. Taking into account that nutrient-sensing regulators control the expression of toxins in *C. difficile* (Dineen et al. 2007), we hypothesize that co-culture of clostridia with lactobacilli or bifidobacteria leads to the modification of the environment thus leading to the repression of toxin synthesis/secretion perhaps through quorum sensing mechanisms.

Taken together, our results suggest that co-culture of toxigenic *C. difficile* with selected probiotic microorganisms lead to a modification of the *C. difficile* microenvironment that in turns modifies signaling pathways related to toxin production. This property was not due to the inhibition of the pathogen growth. Presence of viable probiotic microorganisms rather than their products of secretion was necessary for the inhibitory effect. In addition, given the low proteolytic activity of both bifidobacteria and lactobacilli strains under study, the effect cannot readily be ascribed to toxin degradation.

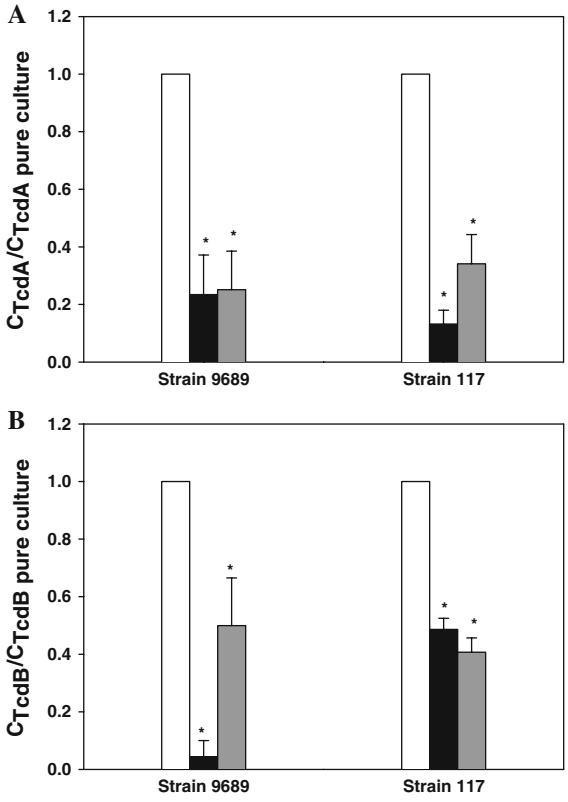


**Fig. 6** Toxin (TcdA and TcdB) concentrations in spent culture supernatants. Results represent the ratio between spot intensity of co-cultures and pure clostridial cultures. Pure clostridial culture (*white bars*), co-culture with *B. bifidum* strain 5310 (*black bars*), co-culture with *L. plantarum* strain 83114 (*grey bars*). Results are expressed as means ± standard deviation from three independent experiments

The results presented here provide evidence for novel protection mechanisms of probiotic microorganisms against intestinal pathogens. These results should contribute to understanding the mechanisms behind the protective effect of probiotics against *C. difficile*.

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**Table 4** Levels of intracellular TcdA and TcdB produced in pure culture of *C. difficile* or co-cultures with bifidobacteria or lactobacilli

| *C. difficile* strain | Pure culture | | Co-culture with *B. bifidum* strain 5310 | | Co-culture with *L. plantarum* strain 83114 | |
|---|---|---|---|---|---|---|
| | TcdA | TcdB | TcdA | TcdB | TcdA | TcdB |
| 117 | 1.01 ± 0.33 | 1.05 ± 0.02 | 3.05 ± 2.01 | 3.03* ± 0.31 | 3.01 ± 1.01 | 1.11 ± 0.91 |
| 9689 | 1.03 ± 0.21 | 1.01 ± 0.01 | 0.10* ± 0.05 | 0.11* ± 0.06 | 0.61 ± 0.30 | 1.15 ± 0.87 |

Values are expressed as the ratio between spot intensity from co-cultures and pure cultures

* Significant differences ($P < 0.05$) from the theoretical ratio = 1

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