Probiotic *Saccharomyces boulardii* CNCM I-745 prevents outbreak-associated *Clostridium difficile*-associated cecal inflammation in hamsters

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**Running head:** S.b protects against *C. difficile*

**Keywords:** probiotic, infection, and *C. difficile*

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Abstract:

Background: *C. difficile* infection (CDI) is a common debilitating nosocomial infection associated with high mortality. Several CDI outbreaks have been attributed to ribotypes 027, 017, and 078. Clinical and experimental evidence indicates that the non-pathogenic yeast *Saccharomyces boulardii* CNCM I-745 (*S.b*) is effective for the prevention of CDI. However, there is no current evidence suggesting this probiotic can protect from CDI caused by outbreak-associated strains. Methods: We used established hamster models infected with outbreak-associated *C. difficile* strains to determine whether oral administration of live or heat-inactivated *S.b* can prevent cecal tissue damage and inflammation. Results: Hamsters infected with *C. difficile* strain VPI10463 (ribotype 087) and outbreak-associated strains ribotype 017, 027 and 078 developed severe cecal inflammation with mucosal damage, neutrophil infiltration, edema, increased NF-kappaB phosphorylation, and increased pro-inflammatory cytokine tumor necrosis factor alpha (TNFalpha) protein expression. Oral gavage of live, but not heated, *S.b* starting 5 days before *C. difficile* infection significantly reduced cecal tissue damage, NF-kappaB phosphorylation, and TNFalpha protein expression caused by infection with all strains. Moreover, *S.b* conditioned medium reduced cell rounding caused by filtered supernatants from all *C. difficile* strains. *S.b* conditioned medium also inhibited toxin A- and B-mediated actin cytoskeleton disruption. Conclusions: *S.b* is effective in preventing *C. difficile* infection by outbreak-associated via inhibition of the cytotoxic effects of *C. difficile* toxins.
**New & Noteworthy:**

By utilizing a well-established hamster model of CDI we show that oral gavage of *Saccharomyces boulardii* CNCM I-745 (*S.b*) effectively prevented cecal tissue damage, NF-kappaB phosphorylation, and TNFalpha expression in hamsters infected with hypervirulent *C. difficile* strains. *S.b* culture supernatants also prevented toxin-mediated cellular actin disruption induced by conditioned medium from cultures of hypervirulent *C. difficile* strains. Thus, *S.b* may reduce the risk of infection of several outbreak-associated *C. difficile* strains.

**Introduction:**

*Clostridium difficile* infection (CDI) is one of the most common infectious diarrheas in hospitals and long-term care facilities in the United States related primarily to the use of antibiotics (46). *C. difficile* is an anaerobic bacterium that produces two toxins – toxin A and toxin B – that mediates diarrhea, inflammation and apoptosis of the mucosal epithelium in animals and humans (32). The effects of the toxin in target intestinal cells involve inactivation of the Rho family of GTPase, leading to cytoskeletal disorganization, epithelial cell apoptosis, and ultimately cell death (42, 54). *C. difficile* toxins also stimulate transcription of several pro-inflammatory genes, including tumor necrosis factor alpha (TNFα) (19) and activate transcription factors and mitogen-activated protein (MAP) kinases involved in their pro-inflammatory effects (18, 25). The mainstream CDI regimen includes the use of metronidazole, vancomycin, and fidaxomicin (55). However, many *C. difficile*-infected patients may also suffer from recurrent infections (13), while the recent epidemics that also involve new epidemic outbreak-associated strains (29) pose a major medical problem and epidemiologic concern. These challenges have been met with a broad range of preventive approaches against CDI, including the use of probiotics, most notably *Saccharomyces boulardii* (*S.b*) alone or in combination with established antibiotic treatment (23, 24).
S. b is a nonpathogenic yeast that represents one of the well-studied probiotics against CDI in both experimental and clinical settings (24, 40). Randomized double-blind placebo-controlled clinical trials have shown that S. b CNCM I-745 is an effective probiotic in the prophylaxis of antibiotic-associated diarrhea, and the most effective probiotic of prophylaxis against CDI (36, 49). Studies also indicate that S. b can be used in combination with vancomycin as therapy for relapsing CDI (37, 50). The putative mechanisms involved in the effectiveness of S. b in CDI include effects directed against the microbiome, the host, as well as C. difficile and its toxins (4, 6, 21, 40, 43). However, most of the experimental work examining the effects of S. b in CDI models has been limited to non-outbreak-associated C. difficile strains, mostly with strain VPI10463. However, the increased incidence and severity of the CDI global outbreaks has been associated with the emergence of outbreak-associated strains (20). The exact mechanism of the hypervirulence of these emerging strains is not fully understood (20). Moreover, studies with the use of probiotics in emerging CDI at both the preclinical and clinical levels have been limited.

Based on these considerations, we examined the effectiveness of oral S. b administration in preventing morbidity, cecal mucosal histology, and cytokine expression in response to C. difficile infection due to the outbreak-associated strains ribotype 017, 027, and 078 in a well-established model of hamsters. These strains were chosen based on their impact in global outbreaks (51). Lastly, the effect of the S. b-conditioned medium in cytotoxicity caused by C. difficile filtered supernatants in mouse fibroblasts was also evaluated.

**Materials & Methods:**

**C. difficile culture:**

C. difficile strains VPI10463 (ATCC stock 43255), ribotype 027 (ATCC BAA-1805), ribotype 078 (ATCC BAA-1875), and ribotype 017 (ATCC 43598) were cultured in Difco cooked meat media (#226730 BD, Fisher Scientific, Canoga Park, CA) at 37°C in anaerobic conditions as previously reported (19).
Hamster model of C. difficile infection:

Six weeks old Golden Syrian Hamsters (strain code 049) were purchased from Charles River Laboratories (San Diego, CA) and housed at the UCLA animal facility under standard conditions with a 12 hour light period and a 12 hour dark period per day at 25°C room temperature. Hamsters were housed in disposable plastic cages with HEPA filtered air circulation, autoclaved bedding, standard animal chow, and sterile water ad libitum. Average body weight of hamsters is 120 g.

Hamsters (n=5 per group) were orally administered with Clindamycin (30 mg/kg in 200 μL dissolved in water, #C5269, Sigma, St. Louis, MO) on day 1, followed by C. difficile (VPI10463, ribotype 017, 027, and 078, 10³ CFU in 100μL) infection via oral gavage on day 5 as previously described (10). Some hamsters were orally fed with live or heated S.b (3 g/kg body weight in 200 μL dissolved in water) twice a day from day 1 to day 7. The 3g/kg contains 3x10¹⁰ live yeast cells/kg, i.e. ~3.6x10⁹ per hamster. A positive control group received vancomycin (50 mg/kg, dissolved in water) orally once a day from day 1 to day 7 while a negative control group received live and heat-treated S.b without C. difficile infection.

Lyophilized S.b CNCM I-745 powder was provided by Biocodex (Gentilly, France). Lyophilized S.b was dissolved in water to produce live culture for immediate oral administration. Heat-treated S.b was prepared by heating the live culture in a microwave oven for 20 seconds, similar to previously used approaches (2, 9). The temperature of the heated S.b culture reached 95°C. Heated S.b cultures were then centrifuged and filtered (0.22μm filter) to generate cell-free supernatants. Heated S.b cell-free supernatants failed to prevent toxin A- and B-mediated cell rounding in 3T3-L1 cells. The details of cell rounding experiments are explained in the cell rounding experiments section below.
S.b protects against C. difficile

Details of C. difficile infection, vancomycin treatment protocol, and S.b treatment protocol were described in Figure 1A, 2A, and 3A. All animals were euthanized by carbon dioxide gas at day 7. Hamster cecal content, cecal tissue and serum samples were obtained at day 7 for further analysis. Animal experiments were approved by the UCLA Animal Research Committee (protocol 2007-116).

Histology scoring:

Cecal tissues in H&E staining were used for histology scoring. The severity of enteritis and colitis was graded using three parameters as previously published (41): (i) epithelial tissue damage; (ii) hemorrhagic congestion and mucosal edema; (iii) neutrophil infiltration. A score of 0-3 was assigned to each parameter. Total histology score was determined by the sum of all these three parameter scores (0-9). Four different locations per tissue section were observed. Each tissue section represented results from one hamster.

Cecal TNFα measurement:

The cecal levels of pro-inflammatory hamster TNFα was determined by ELISA kits (MBS2602630, Mybiosource.com, San Diego, CA) according to the manufacturer’s instructions.

Phosphorylated NF-κB immunohistochemistry:

Cecal tissues were fixed in 4% paraformaldehyde and embedded in paraffin. After incubation with blocking buffer, sections were incubated with a rabbit polyclonal anti-phosphorylated NF-κB p65 antibody (ab86299, Abcam, Cambridge, United Kingdom, 2 μg/ml dilution) overnight at 4°C. After washing, sections were incubated with donkey anti-rabbit IgG (19) and slides were stained with an ABC kit for color development (sc-2018, Santa Cruz, Dallas, Texas). Images were analyzed with a Zeiss AX10 microscope. Histology core facility of the University of California Los Angeles provided assistance in H&E staining and immunohistochemistry experiments. We have verified that the NF-κB signal was specific as the tissue slides
incubated with secondary antibody but without primary antibody had no detectable signal (data not shown). The quantitative difference of phosphorylated NF-κB signal was observed and scored: 0=normal; 1=mild; 2=moderate; and 3=strong. Four different locations per tissue section were observed and scored. Each tissue section represented results from one hamster.

Cell rounding experiments:

Mouse 3T3-L1 fibroblasts were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10^5 cells/well) in 6-well plates. Cells were grown in 2 ml of medium per well to around 70% confluence. Cells were serum starved overnight and then incubated with serum free DMEM media containing S.b-filtered supernatants for 4 hours, followed by toxin A or B (0.1 μg/ml) for additional 4 hours. The toxin A was purified from C. difficile VPI10463 as previously described (17, 45). After 4 hours, microphotographs to observe cell rounding were taken in a blinded manner as we previously described (28). The quantitative difference of cell rounding was observed and scored: 0=normal; 1=mild cell round; 2=moderate cell rounding; and 3=severe cell rounding. Four different locations were scored per cell culture wells.

Some of the cells were treated with cell-free supernatant of C. difficile-conditioned medium instead of toxin A or B. C. difficile (VPI10463, ribotype 017, 027, and 078) was cultured for one week at 37°C in the anaerobic condition as mentioned above. C. difficile culture suspension was centrifuged at 4000 rpm at 4°C for 15 minutes. The supernatant was transferred to a new tube and then filtered through 0.22 μm filter to remove cells. The cell-free supernatant was added to the 3T3-L1 fibroblast culture (1:10) and further incubated for 4 hours.
To prepare *S.b*-filtered supernatants, lyophilized *S.b* powder was dissolved in DMEM media (100mg/ml, i.e. 10%) overnight in 37°C with shaking as previously described (6). The culture was then centrifuged at 4000 rpm at 4°C, followed by a filtration through a 0.22 µm filter to remove the yeast cells. The filtered supernatants were then used to treat the serum-starved cells. The control group was incubated with serum-free DMEM without *S.b*-filtered supernatant.

**Actin cytoskeleton staining:**

Culture conditions of cells in the ActinGreen staining studies were the same as the cell rounding experiments. Two drops of ActinGreen reagent (R37110, ThermoFisher, Canoga Park, CA) were added to the culture (1 ml) during the last hour of the experiments. At the end of the incubation period, one drop of DAPI solution (Sc-24941, Santa Cruz) was added to the cell cultures and a coverslip was placed. Stained cells were observed under a confocal microscope: Green=actin; Blue=nuclei.

**PCR analysis of *C. difficile* toxin A and B genes in cecal content:**

To detect presence of *C. difficile* toxin A (*tcdA*) and toxin B (*tcdB*) genes in cecal contents, fresh cecal contents were collected into Eppendorf tubes and snapped frozen in dry ice, followed by storage in -80°C. 200 mg of cecal content per hamster was used to prepare bacterial DNA using QIAamp DNA stool Mini kit (#51504, Qiagen, Valencia, CA). The DNA concentration was measured by a Nanodrop machine and was then normalized to 100 ng per PCR reaction with water. The PCR detection of *tcdA* and *tcdB* were performed in separate reactions as previously described (34). All sample reactions were performed in duplicate. After 40 cycles of PCR amplification, the Ct values were used to evaluate the presence or absence of *tcdA* and *tcdB* genes. High Ct value (~35-40) or absence of Ct value indicates the absence of *tcdA* and *tcdB* in cecal content. Low Ct value below 35 indicates the presence of *tcdA* and *tcdB* in cecal content.

**Statistical analysis:**
Quantitative results were expressed with error bars as mean +/- standard error of the mean. Results were analyzed using Prism professional statistics software program (GraphPad, San Diego, CA). We used Mann-Whitney U two-tailed test for intergroup comparison unless otherwise specified. For all animal experiments, n=5 hamsters per group.

Results:

Live, but not heated S. boulardii, significantly inhibited C. difficile strain VPI10463-mediated cecal inflammation in hamsters.

As shown by a previous report, live S.b is effective in protecting from C. difficile infection in hamsters (5). To establish this model in our laboratory, we administered clindamycin via oral gavage five days before C. difficile inoculation as suggested by a previous report (10). Hamsters were then infected with C. difficile strain VPI10463 as described in Figure 1A. After 48 hours, we collected cecal content, cecal tissues, and serum samples for further analyses.

We observed that C. difficile-mediated intestinal inflammation was evident primarily in the cecum (Figure 1B). Tissue damage was characteristic of mild mucosal erosion/ulceration, severe neutrophil infiltration, and moderate mucosal edema. Images of C. difficile-infected hamsters are shown for evaluation (Figure 1B). These quantitative changes were assessed by a histologic score previously used by us (19, 41). Oral administration of live S.b improved C. difficile-mediated cecal tissue damage as shown by the reduced histology score (Figure 1B and 1D), consistent with a previous report (5). However, this improvement was not observed in hamsters fed with the same amount of heated S.b. This finding indicates that the anti-inflammatory effect of S.b was mediated by live S.b only.
To determine whether *S.b* alone alters cecal mucosal structure in hamsters, we treated hamsters with live and heated *S.b* without *C. difficile* infection. We did not observe any change in cecal tissue histology after live or heated *S.b* administration for seven days (Figure 1C-D).

In the clinical setting, *C. difficile* infection is commonly treated with antibiotics such as metronidazole, vancomycin, or fidaxomicin (46). Similar to a previous study using a hamster model (27), vancomycin treatment altered the severity of CDI, reflected by reduced cecal tissue damage and the corresponding histologic score in infected hamsters (Figure 2B & 2C).

Live, but not heated *S. boulardii*, significantly inhibited outbreak-associated *C. difficile* strains-mediated cecal inflammation in hamsters.

The incidence and severity of *C. difficile* outbreaks have been increasing with the emergence of outbreak-associated strains (20). We next infected hamsters with *C. difficile* strains ribotype 017, 027, 078, previously associated with several outbreaks (51), using the experimental protocol described in Figure 3A.

Our results show that ribotype 017-, 027-, and 078-mediated cecal tissue damage was similar to what VPI10463 did with comparable histology scores (Figure 3B-C). Live, but not heated *S.b* administration significantly reduced cecal tissue damage and corresponding histology score in hamsters infected with these outbreak-associated *C. difficile* strains (Figure 3B-C).

Live *S. boulardii*, but not heated *S. boulardii*, reduced outbreak-associated *C. difficile* strains-induced cecal pro-inflammatory TNFα protein expression and NF-κB phosphorylation.

As shown by our previous report (19), *C. difficile* infection results in an increased expression of the pro-inflammatory cytokine TNFα in the intestine. In our hamster model, infection with the common *C. difficile* strain VPI10463 leads to increased cecal TNFα protein expression, compared to cecal tissues without *C.
S. b protects against C. difficile infection (Figure 4A). Oral vancomycin administration significantly reduced cecal TNFα expression in C. difficile infected hamsters, suggesting successful suppression of C. difficile infection (Figure 4B). Live, but not heated S. b, significantly reduced cecal TNFα expression in all C. difficile-infected hamsters by approximately 30% (Figure 4A and 4C), while live or heated S. b did not affect basal level of cecal TNFα expression in hamsters without C. difficile infection (Figure 4A).

Also, our previous report showed that toxin A mediates TNFα expression via NF-κB activation (19). Colonic tissues from C. difficile infected mice and ileal tissues of toxin A-treated mice had increased expression of phosphorylated NF-κB (19). Here we detected NF-κB phosphorylation in hamster cecum using immunohistochemistry. Infection with all C. difficile strains (VPI10463, ribotype 017, 027, and 078) led to a stronger NF-κB phosphorylation in the cecal mucosa than those of uninfected hamsters (shown in brown, Figure 5A-E). Live, but not heated S. b substantially reduced NF-κB phosphorylation signal in the cecal mucosa of C. difficile infected hamsters (Figure 5A-D). The basal level of phosphorylated NF-κB signal in the cecal mucosa of uninfected hamsters was very low (Figure 5E). Quantitative assessment of phosphorylated NF-κB signal was shown in Figure 5F.

C. difficile toxins A and B cause apoptosis in intestinal tissues (19). We used TUNEL assay to detect apoptosis in cecal tissues as previously described (19). Infection of hamsters with all C. difficile strains (VPI10463, ribotype 017, 027, and 078) led to a small number of apoptotic cells in the cecal mucosa as shown by the intense brown spots (Figure 6A-D). Live or heated S. b did not alter the number of apoptotic cells in the cecal mucosa of C. difficile infected hamsters (Figure 6A-D). The apoptotic cells in the cecal mucosa of uninfected hamsters were almost undetectable (Figure 6E).
We detected whether \( S.b \) treatment affects the quantity of \( C. difficile \) in the cecum of hamsters using PCR. PCR results indicated the presence of \( tcdA \) in cecal contents of hamsters infected with VPI10463, and ribotype 027 and 078 (Figure 7A). \( tcdA \) was undetectable in ribotype 017-infected hamsters, consistent with the lack of expression of the \( tcdA \) in ribotype 017 (Figure 7A). \( tcdB \) was present in cecal contents of all \( C. difficile \)-infected hamsters (Figure 7B). Live and heated \( S.b \) did not affect the quantity of \( tcdA \) and \( tcdB \) in the cecal content of \( C. difficile \)-infected hamsters (Figure 7A-B). This finding suggests that the protective effect of \( S.b \) does not depend on an antibacterial effect against \( C. difficile \) (Figure 7C).

\( S. boulardii \)-filtered supernatant prevented toxin A-mediated cell rounding and prevented \( C. difficile \) supernatant-mediated cell rounding.

Toxin A and B disrupt the actin cytoskeleton that leads to cell rounding and then cell death (56). To better understand the protective mechanism of \( S.b \) against toxins produced by \( C. difficile \), we first examined whether cell-free \( S.b \) supernatant can prevent toxin A-associated cell rounding. Exposure of 3T3-L1 fibroblasts to purified toxin A for 4 hours resulted in cell rounding (Figure 8A). Consistent with a previous study (8), pre-treatment of cells with \( S.b \) cell-free filtered supernatant (10%) partially prevented the cell rounding effect of toxin A. \( S.b \)-filtered supernatants alone without toxin A did not affect cell morphology of 3T3-L1 cells. These results indicate that \( S.b \) protects cells against toxin A-mediated cytoskeletal disruption in 3T3-L1 cells.

On the other hand, toxin B also caused cell rounding in 3T3-L1 cells (Figure 8B). Pre-treatment of cells with cell-free \( S.b \) filtered supernatant (10%) partially prevented the cell rounding effect of toxin B (Figure 8B). This finding may provide an explanation for the protective effect of \( S.b \) on \( C. difficile \) ribotype 017 infection since this strain produces only toxin B (11, 47). Quantitative assessment of cell rounding is shown in Figure 8C.

To determine whether \( S.b \) protects cells from cell rounding due to outbreak-associated \( C. difficile \), we first cultured \( C. difficile \) (toxin A+B+ VPI10463, ribotype 027, 078 and toxin A-B+ ribotype 017) for one week.
and then prepared cell-free filtered supernatants. *C. difficile* supernatants caused cell rounding as observed with purified toxin A (Figure 9A-D). Pre-treatment of cells with cell-free filtered *S.b* supernatant reduced cell rounding caused by *C. difficile* supernatant (Figure 9A-D). Quantitative assessment of cell rounding is shown in Figure 9E.

The actin cytoskeleton is a known target of toxins A and B (14, 22). To visualize the distribution of intracellular actin fibers, we stained 3T3-L1 fibroblasts with the ActinGreen reagent. As shown in Figure 10, toxins A and B disrupted the actin cytoskeleton network in these cells. Addition of *S.b* supernatants partially inhibited toxin A- and B-mediated actin network disruption, providing a mechanism for the reduction of toxin A- and B-associated cell rounding.

**Discussion:**

This study demonstrated that prophylactic oral administration of *S.b* to hamsters significantly prevented cecal tissue damage, TNFα protein expression, and NF-κB phosphorylation caused by three well-established *C. difficile* outbreak-associated strains. We also show that conditioned media from *S.b*. CNCM I-745 cultures substantially inhibited cell rounding caused by supernatants from outbreak-associated ribotype 017, 027, and 078 *C. difficile* strains. *S.b* supernatants also prevented toxin A- and B-mediated cell rounding and actin cytoskeleton network disruption suggesting that this response is, at least in part, responsible for the *in vivo* protective effect seen in our study. Toxins A and B induce NF-κB phosphorylation that activates TNFα expression (19). TNFα may also cause cell death (33) and indirectly augments toxin A-mediated cytotoxicity (38). Thus, the anti-NF-κB *in vivo* response afforded by *S.b*. shown here may represent an additional mechanism of action of this probiotic yeast.
Actin disruption also causes downstream NF-κB activation (30), which is linked to inflammatory cytokine expression. We had demonstrated that NF-κB activation mediates toxin A-induced TNFα expression in monocytes and macrophages (19). The *S. b* mediated protection against toxin-mediated actin disruption may be pivotal in inhibiting cell death and inflammatory responses. *S. b* conditioned medium also prevented the cell rounding cytotoxic effects of cell-free filtered supernatants from ribotype 017, 027, and 078, suggesting an effect on the cellular effects of *C. difficile* toxins produced by these strains. Most importantly, however, our results suggest *S. b* CNCM I-745 administration may be effective in preventing CDI caused by outbreak-associated *C. difficile* strains.

Multiple additional mechanisms can mediate the *S. b*. CNCM I-745 effect observed in our study. For example, a previous report demonstrated that *S. b* increases anti-toxin A IgA secretion in the small intestine (43), suggesting that small intestinal IgA may protect the host from mucosal damage during *C. difficile* infection. *S. b* also modifies the human gut microbiome by enhancing short-chain fatty acid-producing bacteria, including *Lachnospiraceae* and *Ruminococcaceae* (39). Short chain fatty acids have known anti-inflammatory effects. *S. b* can also release a protease that digests toxin A and its receptor, thereby inhibiting the toxin effects (3, 4). It is not clear whether *S. b* can prevent CDI relapse or whether relapse will take place after withdrawal of *S. b*, but these issues worth further investigation in the future.

It is quite interesting that *S. b* had an inhibitory effect against all outbreak-associated *C. difficile* strains *in vivo* and *in vitro*, although the molecular and clinical characteristics of these strains are quite diverse. *C. difficile* ribotype 017 has been associated with several outbreaks CDI (11, 47) and is characterized by a toxin A negative and toxin B positive genotype (26). According to a mouse study, antibiotic treatment even promotes spore shedding of *C. difficile* ribotype 017 and subsequent animal-to-animal transmission (31). Since its tissue-
S. b protects against C. difficile

damaging effect is thought to be primarily mediated by toxin B (26), the anti-cell rounding activity of S. b in our study indicates its protective effects against toxin B (Figure 8 and 9).

Our results also show effective inhibition of C. difficile ribotype 027- induced intestinal inflammation mediated by S. b treatment. C. difficile ribotype 027 is one of the most serious outbreak-associated C. difficile strains responsible for multiple outbreaks in North America, Europe, and beyond (7, 53). It produces both toxin A and toxin B, and an additional toxin, the CDT binary toxin (12). Although the mechanism of its hypervirulence is not fully elucidated, this strain is fluoroquinolone-resistant (12) and appears to produce more toxin A and toxin B than any other C. difficile strains (35). The binary toxin also possesses cytotoxic activity, but its role in CDI is still not well understood (48). Based on these considerations, the in vivo and in vitro effects of S. b in C. difficile ribotype 027 may be directed against toxin A, toxin B, and/or the binary toxin.

Interestingly, another probiotic, Lactobacillus acidophilus GP1B, inhibited C. difficile growth and transcriptional activation of C. difficile toxin-related genes in vitro (57).

We present here evidence for effective inhibition of C. difficile ribotype 078-associated cecitis mediated by S. b exposure. C. difficile ribotype 078 used to be prevalent among cows and pigs (1). Recent ribotype 078 outbreak data showed that it was similar to C. difficile ribotype 027 in severity, but C. difficile ribotype 078 tends to affect young people and causes community-associated outbreaks (16). Like C. difficile ribotype 027, ribotype 078 expresses toxin A, toxin B, as well as a binary toxin (44). The effects of S. b against CDI ribotype 078 seen in our results can be directed against these toxins.

Filtered supernatants of S. b cultures exert protective effects against C. difficile toxin A via inhibition of pro-inflammatory chemokine interleukin-8 (IL-8) expression in cultured human NCM460 colonocytes via an ERK-dependent mechanism (6). In a mouse ileal loop model, injection of S. b-filtered supernatants into the loops prevented toxin A-induced tissue damage, ERK activation, and chemokine KC expression (6). This
finding is consistent with our results indicating that *S.b* reduced cecal expression of the pro-inflammatory cytokine TNFα expression in *C. difficile*-infected hamsters in response to all outbreak-associated strains.

The overall protective effect of *S.b* against *C. difficile* infection in our studies is preventive rather than therapeutic. Once the infection started, *S.b* was ineffective in suppressing pre-existing intestinal inflammation in hamsters (data not shown). It was necessary to start the *S.b* treatment before *C. difficile* infection and continue the treatment post-infection. This suggests that *S.b* requires some time to be established in the intestine in order to exert its protective effects. In our study, the three outbreak-associated strains are also hypervirulent strains and their enhanced toxicity was associated with a high mortality rate within a short period of time. The typical 10% *S.b* solution regimen in the drinking water used with regular *C. difficile* strains had no protective effect (data now shown) (52). We used the maximal dose of *S.b* within its solubility limit (3g/kg, twice per day) to achieve successful inhibition of *C. difficile*-mediated cecal damage and inflammation.

In summary, we demonstrated that the preventive effect of *S.b* against *C. difficile* in several outbreak-associated *C. difficile* strains was similarly effective. This study is consistent with a recent meta-analysis study that demonstrated a statistically significant efficacy in the prevention of *C. difficile* diarrhea (15). We suggest that *S.b* CNCM I-745 pre-treatment may reduce the risk of infection of several outbreak-associated *C. difficile* strains.

**Grants:**

This work was supported by a grant from Biocodex Inc. NIH K01 (DK084256) and R03 (DK103964) grants (HWK). The Blinder Research Foundation for Crohn’s Disease, NIH P30 DK 41301-26 (Animal Core), and the Eli and Edythe Broad Chair (CP) also provided financial support. C.P.K. is supported by NIH grant R01 AI095256, and X.C. by a Career Development Award from the Crohn’s and Colitis Foundation of America.
Disclosure:

Biocodex Inc sponsored this study.

Figure Legends:

**Figure 1**

*S. boulardii* reduced *C. difficile* VPI10463-induced cecal tissue damage in hamsters.

(A) Experimental design of *C. difficile* infection and *S. b* treatment. (B) H&E staining of cecal tissues. Infection of *C. difficile* VPI10463 caused severe cecal tissue damage that was reduced by oral gavage administration of live *S. b* from day 1-7. Administration of heated *S. b* had no protective effect. (C) H&E staining of cecal tissues from hamsters without *C. difficile* infection. The upper panel shows images with 100X magnification. The black bars indicate 200μm. (D) Histology score. Live *S. b* significantly reduced histology score of *C. difficile* VPI10463 infected hamsters.

**Figure 2**

Vancomycin reduced *C. difficile* VPI10463-induced cecal tissue damages in hamsters.

(A) Experimental design of *C. difficile* infection and vancomycin treatment. (B) H&E staining of cecal tissues. Infection of *C. difficile* VPI10463 caused severe cecal tissue damage that was reduced by oral gavage administration of vancomycin from day 1-7. Magnification 100X. The black bars indicate 200μm. (C) Histology score. Vancomycin significantly reduced histology score of *C. difficile* VPI10463-infected hamsters.

**Figure 3**

*S. boulardii* reduced cecal tissue damage in hamsters infected by outbreak-associated *C. difficile* ribotype 017, 027, and 078.
S. boulardii protects against *C. difficile*

(A) Experimental plan of outbreak-associated *C. difficile* infection and *S. boulardii* treatment. (B) H&E staining of cecal tissues. Infection of *C. difficile* ribotype 017, 027, and 078 caused severe cecal tissue damage that was reduced by oral gavage administration of live *S. boulardii* from day 1-7. Magnification 100X. The black bars indicate 200μm.

(C) Histology score. Live *S. boulardii* significantly reduced histology score of *C. difficile* ribotype 017, 027, and 078-infected hamsters, respectively. Administration of heated *S. boulardii* had no protective effect.

**Figure 4**

*S. boulardii* reduced cecal TNFα protein expression induced by multiple *C. difficile* strains.

(A) Cecal TNFα protein expression. Live *S. boulardii* but not heated *S. boulardii* reduced cecal TNFα protein expression in *C. difficile* strain VPI10463-infected hamsters. Cecal TNFα protein expression was low in normal hamsters without *C. difficile* infection and was not affected by live and heated *S. boulardii* administration. (B) Vancomycin administration reduced cecal TNFα protein expression in *C. difficile* VPI10463-infected hamsters. (C) Live *S. boulardii* but not heated *S. boulardii* reduced cecal TNFα protein expression in *C. difficile*-infected hamsters, ribotypes 017, 027, and 078, respectively. Administration of heated *S. boulardii* had no protective effect.

**Figure 5**

*S. boulardii* reduced cecal phosphorylated NF-κB expression in hamsters infected with multiple *C. difficile* strains.

(A-D) Phosphorylated NF-κB expression was heavily stained brown in the cecal mucosa of *C. difficile*-infected hamsters. Live *S. boulardii* but not heated *S. boulardii* reduced phosphorylated NF-κB expression in the cecal mucosa of infected hamsters, *C. difficile* strain VPI10463 and ribotypes 017, 027, and 078, respectively. (E) Cecal phosphorylated NF-κB expression in uninfected control hamsters. The black bars indicate 200μm. (F) Quantification of phosphorylated NK-κB signal intensity. Magnification 100X
Figure 6

*S. boulardii* did not alter apoptosis in cecal mucosa caused by multiple *C. difficile* strains.

(A) Apoptosis is absent in uninfected control hamsters. (B-E) Apoptotic cells are stained in intense brown spots in the cecal mucosa of *C. difficile*-infected hamsters. TUNEL staining shows that live and heated *S.b* did not alter the mild apoptosis in the cecal mucosa of infected hamsters, *C. difficile* strain VPI10463 and ribotypes 017, 027, and 078, respectively. Magnification 200X. The black bars indicate 200μm.

Figure 7

*S. boulardii* did not affect the presence of *C. difficile* bacteria in the cecum of the hamsters.

(A) Ct values of toxin A DNA in the hamster cecum detected by PCR. The high Ct value of uninfected control hamsters indicates the absence of *C. difficile* toxin A DNA in the cecum. The cecal content of hamsters infected with *C. difficile* VPI10463 and ribotype 027 and 078 had low Ct values that indicate the presence of *C. difficile* toxin A DNA. *C. difficile* toxin A DNA Ct values were not affected by live or heated *S.b* Toxin A DNA was undetectable in ribotype 017-infected hamsters, as represented by high Ct values. (B) Ct values of toxin B DNA in the hamster cecum detected by PCR. The high Ct value of uninfected control hamsters indicates the absence of *C. difficile* toxin B DNA in the cecum. The cecal content of hamsters infected with all *C. difficile* strains had low Ct values that indicate the presence of *C. difficile* toxin B DNA. (C) The presence of toxin A and toxin B in the cecal content of hamsters. All PCRs were performed with 100 ng cecal content DNA per well. All samples were assayed in duplicate wells.

Figure 8

*S. boulardii*-filtered supernatant prevented cell rounding caused by purified toxins A and B.

(A) Serum-starved 3T3-L1 preadipocytes were treated with DMEM or *S.b*-filtered supernatant (10%) in serum-free DMEM for 4 hours, followed by PBS or *C. difficile* toxin A (0.1 μg/ml) for an additional 4 hours. (B)
S. boulardii filtered supernatant prevented cell rounding caused the supernatant of multiple C. difficile strains.

(A-D) Serum-starved 3T3-L1 preadipocytes were treated with DMEM or S. b-filtered supernatant (10%) in serum-free DMEM for 4 hours, followed by filtered supernatant (10%) from the culture of C. difficile (strain VPI10463, ribotypes 017, 027, 078) for an additional 4 hours. (E) Cell rounding scores. Exposure to C. difficile-conditioned supernatant caused the loss of the spindle shape of 3T3-L1 cells, but co-incubation with the S. b-filtered supernatant partially prevented this change. The results are representative of two independent experiments.

Figure 9

S. boulardii-filtered supernatant prevented actin disruption caused by purified toxins A and B.

Serum-starved 3T3-L1 preadipocytes were treated with DMEM or S. b-filtered supernatant (10%) in serum-free DMEM for 4 hours, followed by PBS or C. difficile toxin A or B (0.1 μg/ml) for an additional 4 hours. The ActinGreen solution was added to the culture during the last hour of the experiment. Exposure to toxin A and B
caused the disruption of the intracellular actin network, but this change was prevented by co-incubation with the
S.b-filtered supernatant. The results are representative of two independent experiments.

References:


Reigadas E, Alcala L, Marin M, Martin A, Iglesias C, and Bouza E. Role of binary toxin in the outcome of Clostridium difficile infection in a non-027 ribotype setting. Epidemiol Infect 1-6, 2015.


S. b protects against C. difficile


Figure 1

A

- Clindamycin 30mg/kg
- Oral gavage

- Water, live SB 3g/kg or heat inactivated SB 3g/kg via oral gavage twice daily

- C. difficile VPI10463 10^2 cfu 100μL

- Euthanize
- Obtain blood and cecal tissues

B

VPI10463

C

water

D

p=0.0477

histology score

Water live SB heated SB

VPI10463 + live SB

live SB

VPI10463 + heated SB

heated SB

H&E staining cecum 100X
Figure 2

A

Clindamycin
30mg/kg
Oral gavage

Water, Vancomycin 50mg/kg
via oral gavage daily

C. difficile
VPI10463
10^3 cfu
100 μL

Euthanize
Obtain blood and cecal tissues

day

1 5 7

B

VPI10463

VPI10463 + vancomycin

H&E staining cecum 100X

C

<table>
<thead>
<tr>
<th>histology score</th>
<th>VPI10463 + water</th>
<th>VPI10463 + Vancomycin 50mg/kg</th>
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<td>p = 0.0175</td>
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200 μm
**Figure 3**

A. Clindamycin 30mg/kg Oral gavage

- Water, live SB 3g/kg or heat inactivated SB 3g/kg via oral gavage twice daily
- *C. difficile* Ribotype 027/017/078
- 10^3 cfu
- 100μL
- Euthanize
- Obtain blood and cecal tissues

B. Ribotype 027
- Ribotype 027 + live SB
- Ribotype 027 + heated SB

Ribotype 017
- Ribotype 017 + live SB
- Ribotype 017 + heated SB

Ribotype 078
- Ribotype 078 + live SB
- Ribotype 078 + heated SB

H&E staining cecum 100X

C. histology score

- Ribotype 027
- Ribotype 027 + live SB
- Ribotype 027 + heated SB
- Ribotype 017
- Ribotype 017 + live SB
- Ribotype 017 + heated SB
- Ribotype 078
- Ribotype 078 + live SB
- Ribotype 078 + heated SB

*p* = 0.0009, *p* = 0.0284, *p* = 0.0286
Figure 4

A

![Bar graph showing TNFα levels in CECUM with different treatments.](image)

- **p=0.0325**

B

![Bar graph showing TNFα levels in Cecum with VPI10463 + water and VPI10463 + Vancomycin 50mg/kg.](image)

- **p=0.0235**

C

![Bar graph showing TNFα levels in Cecum with different ribotypes and treatments.](image)

- **p=0.0406**
- **p=0.0499**
- **p=0.0281**
Figure 5

**VPI10463**  
Ribotype 017  
Ribotype 027  
Ribotype 078  
Uninfected control

**VPI10463 + live SB**  
Ribotype 017 + live SB  
Ribotype 027 + live SB  
Ribotype 078 + live SB

**VPI10463 + heated SB**  
Ribotype 017 + heated SB  
Ribotype 027 + heated SB  
Ribotype 078 + heated SB

**Phosphorylated NF-κB signal intensity**

- **p=0.0306**  
- **p=0.0316**  
- **p=0.0265**  
- **p=0.0284**  
- **p=0.0265**  
- **p=0.0265**  
- **p=0.0485**  
- **p=0.0485**
Figure 6

A  VPI10463  VPI10463 + live SB  VPI10463 + heated SB
   Ribotype 017

B  Ribotype 017  Ribotype 017 + live SB  Ribotype 017 + heated SB
   Ribotype 027

C  Ribotype 027  Ribotype 027 + live SB  Ribotype 027 + heated SB
   Ribotype 078

D  Ribotype 078  Ribotype 078 + live SB  Ribotype 078 + heated SB
   Uninfected control

E  TUNEL staining cecum 200X
Figure 7

A

Cecal content of hamsters

B

Cecal content of hamsters

C

Stool DNA PCR results

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<tr>
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<th>TcdB</th>
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<tr>
<td>uninfected control</td>
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</table>
Figure 8

A

PBS
toxin A 0.1μg/ml

3T3-L1 preadipocytes 4 hours 100X magnification

B

PBS
toxin B 0.1μg/ml

3T3-L1 preadipocytes 4 hours 100X magnification

C

Cell rounding score

0.00 0.00
toxin A 0.1μg/ml
toxin B 0.1μg/ml

3T3-L1 fibroblasts 4 hours

DMEM
S. boulardii 10%

p=0.0001
p=0.0001
p=0.0316
p=0.0405
Figure 9

A

VPI10463 supernatant

DMEM

S. boulardii 10%

B

ribotype 017 supernatant

C

ribotype 027 supernatant

D

ribotype 078 supernatant

3T3-L1 preadipocytes 4 hours 100X magnification

E

Cell rounding score

3T3-L1 fibroblasts 4 hours

VPI10463 supernatant

ribotype 017 supernatant

ribotype 027 supernatant

ribotype 078 supernatant

p=0.0485

p=0.0289

p=0.0289

p=0.0265

0.00

0.50

1.00

1.50

2.00

2.50

3.00
Figure 10

PBS + DMEM

toxin A 0.1 µg/ml + DMEM

toxin B 0.1 µg/ml + DMEM

PBS + S.b. 10%

toxin A 0.1 µg/ml + S.b. 10%

toxin B 0.1 µg/ml + S.b. 10%

ActinGreen staining 3T3-L1 fibroblasts 400X