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The Acid-Dependent and Independent Effects of *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, and *Lacticaseibacillus rhamnosus* CLR2 on *Clostridioides difficile* R20291

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Abstract

Clostridioides difficile infections (CDI) result from antibiotic use and cause severe diarrhea which is life threatening and costly. A specific probiotic containing *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, and *Lacticaseibacillus rhamnosus* CLR2 has demonstrated a strong inhibitory effect on the growth of several nosocomial *C. difficile* strains by production of antimicrobial metabolites during fermentation. Though there are several lactobacilli shown to inhibit *C. difficile* growth by processes relying on acidification, this probiotic has demonstrated potency for CDI prevention among hospitalized patients. Here, we describe the acid-dependent and independent mechanisms by which these strains impair the cytotoxicity of a hypervirulent strain, *C. difficile* R20291 (CD). These bacteria were co-cultured in a series of experiments under anaerobic conditions in glucose-rich and no-sugar medium to inhibit or stimulate CD toxin production, respectively. In glucose-rich medium, there was low CD toxin production, but sufficient amounts to cause cytotoxic damage to human fibroblast cells. In co-culture, there was acidification by the lactobacilli resulting in growth inhibition as well as ≥ 99% reduced toxin A and B production and no observable cytotoxicity. In the absence of glucose, CD produced much more toxin. In co-culture, the lactobacilli did not acidify the medium and CD growth was unaffected; yet, the amount of detected toxin A and B was decreased by 20% and 41%, respectively. Despite the high concentration of toxin, cells exposed to the supernatant from the co-culture were able to survive. These results suggest that in addition to known acid-dependent effects, the combination of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 can interfere with CD pathogenesis without acidification: (1) reduced toxin A and B production and (2) toxin neutralization. This might explain the strain specificity of this probiotic in potently preventing *C. difficile*-associated diarrhea in antibiotic-treated patients compared with other probiotic formulae.

Keywords *C. difficile* · *Lactobacillus* · Probiotic · Antibiotic · Infection · Pathogenesis · microbiota · colonization resistance · Infection control · Preventive intervention · *L. acidophilus* CL1285 · *L. casei* LBC80R · *L. rhamnosus* CLR2

Introduction

In the USA, as well as in other industrialized countries, *Clostridioides* (*Clostridium*) *difficile* infection (CDI) is an important cause of nosocomial diarrhea in hospitals and long-term care facilities, which adds to the patient's length of stay and may even lead to death (22% mortality within 90 days) [1]. The high morbidity, mortality, and rate of recurrence highlight the need for strategies in primary prevention of the illness.

C. difficile is a strict anaerobe, spore-forming, Gram-positive bacillus found in the intestinal microbiota of 2 to 5% of healthy adults and in 10 to 20% of the elderly [2]. CDI mostly develops when patients undergo antibiotic therapy and experience dramatically decreased diversity of species of normal intestinal microbiota [3–6]. This alteration helps *C. difficile* establish an infection that involves colonization of the enteric epithelium. When the vegetative cells reach the stationary phase and glucose is scarce, they begin to secrete

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toxin A and toxin B, resulting in intestinal inflammation and colonic epithelial cell necrosis. Several factors are known to regulate toxin A and B production such as the regulator CCPA, the regulator CodY, *tcdC*, quorum sensing, or the CCR system [7]. CodY regulates negatively *C. difficile*'s toxin production when there is enough amino acids. Thus, when nutrients become limited, CodY will no longer be able to repress toxin production and *C. difficile* is able to secrete its toxins. Dineen et al. have demonstrated with the strain JIR8094:pSD21 in TY medium supplemented with 1% glucose that TcdA accumulation was significantly lower in the culture supernatant for this strain compared with the same strain grown in TY medium alone. These results suggest that glucose inhibition of toxin synthesis is at least partially independent of the effect of CodY as a repressor. [8].

Lactobacilli employ non-specific mechanisms, such as secretion of organic acids, bacteriocins, or hydrogen peroxide, to compete with pathogens like *C. difficile* [9]. Though several lactobacilli-based microbial preparations have been investigated in primary prevention of CDI, many have failed to demonstrate efficacy suggesting that not all lactobacilli are equal. A specific probiotic formulation containing *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, and *Lacticaseibacillus rhamnosus* CLR2 has been evaluated in three randomized double-blinded, placebo-controlled studies for the primary prevention of CDI [10–12]. In meta-analyses, there were 80% fewer cases of CDI when treated with these live bacteria compared with placebo [13, 14]. A very large study of 2,981 subjects randomized to receive a combination of 2 strains of *Lactobacillus* and 2 strains of *Bifidobacteria* failed to demonstrate a protective effect against CDI [15]. However, one microbial preparation composed of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 has reproducibly demonstrated potency for CDI prevention suggesting that strain-specific differences may result in differential clinical effects [16].

However, the mechanism by which the three lactobacilli formulation prevent infection is not fully elucidated. Co-culture experiments demonstrated that these specific lactobacilli are able to inhibit the growth of several hospital-acquired *C. difficile* strains [17]. It has been also demonstrated that antimicrobial metabolites are synthesized and secreted during fermentation [17]. Furthermore, *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2, individually and collectively, have the capacity to protect epithelial cells in vitro by neutralizing the toxins [17]. In each experiment, there was acidification of the medium, a possible explanation for the findings which is not specific to these *Lactobacillus* sp. strains [9].

The objective of these studies is to determine if these strains are able to minimize *C. difficile* virulence with and without acidification. To investigate this, we cultured the hypervirulent strain *C. difficile* R20291 alone and in co-culture with *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 in a glucose-rich medium that promotes lactic acidification, a modified brain heart infusion (BHI) with a higher concentration of glucose with modest acidification, and a sugar-free medium, tryptose yeast extract (TY) with no acidification.

Materials and Methods

Bacterial Strains

C. difficile strain R20291 (BI/NAP1/027) was kindly provided by Professor Louis-Charles Fortier, *Ph.D*. (University of Sherbrooke, Sherbrooke, Canada). The strains were stored at − 80 °C in Reinforced Clostridial Medium (RCM) broth (Oxoid, Nepean, Ontario, Canada) with 20% glycerol. *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, and *Lacticaseibacillus rhamnosus* CLR2 were graciously provided by Bio-K Plus International Inc. (Laval, Canada). They were stored in De Man, Rogosa, and Sharpe (MRS) broth (Sigma-Aldrich, Oakville, Ontario, Canada) enriched with 20% glycerol at − 80 °C until utilization.

Growth Kinetic

All growth kinetics were performed in an anaerobic chamber supplemented with a gas mix of 10% hydrogen, 5% carbon dioxide, and 85% nitrogen (MEGS Specialty Gases and Equipment, Quebec, Canada). In all the following growth kinetic experiments, each lactobacilli strain was thawed and transferred in 9 mL of MRS broth and incubated at 37 °C without oxygen for 24 h. The same procedure was repeated with the *C. difficile* strain in RCM. One *C. difficile* R20291 vial was thawed and cultured in 9 mL of pre-reduced RCM and incubated at 37 °C for 24 h. After two consecutive sub-cultures of all strains, bacteria were washed twice with phosphate buffered saline (PBS) and centrifuged 10 min at 3700×*g* (Legend RT Plus, ThermoFisher Scientific, St-Laurent, Quebec, Canada) in tightly screwed tubes. Thereafter, bacteria were suspended in 15 mL of the medium used in each of the experiment, modified Brain Heart Infusion (BHI) with 3 g/L of glucose (BD, Mississauga, Ontario, Canada), or tryptose yeast extract (TY) (pH of 7.4) (Oxoid, Nepean, Ontario, Canada; Fisher Scientific, Ottawa, Ontario, Canada). Optical density (OD) was then measured at 600 nm (Biomate Spectronic 3, ThermoScientific, Saint-Laurent, Quebec, Canada) and the culture was standardized to an OD of 0.05 for *C. difficile* and 0.5 for the *Lactobacillus* sp. strains in a final volume of 30 mL. Even though the TY medium without adjustment was set to pH 7.4 initially, after adding bacteria, the pH dropped. To run the assay at neutral pH, the solution was titrated to pH 7.0 with NaOH before

starting the experiment. Several parameters were followed for 24 h such as the bacterial count, pH, toxin quantification, and cytotoxicity on human fibroblast cells.

Microbial Analysis

Samples of 1 mL of *C. difficile* monoculture and co-culture of *C. difficile* with the probiotic strains were harvested at 0, 6, 12, and 24 h in order to evaluate the bacterial concentrations. After tenfold serial dilutions in pre-reduced peptone water, bacterial enumeration for *C. difficile* was carried out in a selective medium Cycloserine-Cefoxitin Fructose Agar (CCFA, Anaerobe System, California, USA) under anaerobic conditions and lactobacilli were enumerated in MRS agar under aerobic atmosphere. The CCFA plates were incubated at 37 °C for 24–48 h, whereas MRS plates were incubated 48 h at 37 °C. Bacterial identity confirmation steps, sterility controls and triplicates were executed for each experiment.

Quantification of Toxins A/B

The quantification of toxins A and B was performed using a commercial ELISA method (tgcBIOMICS GmbH, Mainz, Germany). According to the manufacturer's instructions, samples were added in microtiter plate coated with antibodies specific to toxin A and B and incubated for 60 min at 37 °C. Subsequent to three washes with the wash buffer, anti-toxins A and B were added and incubated at 37 °C for 30 min. Next, the substrate was added in each well and incubated at room temperature for 15 min. The color development was stopped with H_2SO_4. A standard curve was done with the inactivated pure toxin A and B samples provided by the manufacturer. The results were measured by a spectrophotometer (Vmax software, Molecular Devices, California, USA) at 450 and 650 nm. In all experiments, toxins A and B were quantified in supernatant from 24-h co-culture of *C. difficile* with and without the probiotic strains. Percentage of toxin reduction was calculated using the following equation:

$$\% = \left(\frac{\text{CD toxin A or B concentration } \left(\frac{\text{ng}}{\text{CFU}}\right) - (\text{CD} + \text{LB})\text{toxin A or B concentration } \left(\frac{\text{ng}}{\text{CFU}}\right)}{\text{CD toxin A or B concentration } \left(\frac{\text{ng}}{\text{CFU}}\right)} \right) \times 100$$

Cytotoxicity Assay

Cytotoxicity was evaluated with Bartels *Clostridium difficile* Cytotoxicity Assay (NovaCentury Scientific, A Trinity Biotech Company, Burlington, Ontario, Canada) according to the manufacturer's instructions. Cells were exposed to supernatant produced after 24 h of *C. difficile* cultured alone or in co-culture with the three lactobacilli strains in TY medium and in BHI medium. The culture supernatants were centrifuged for 10 min at 3700×*g* (Legend RT Plus, ThermoFisher Scientific, Saint-Laurent, Quebec, Canada), filtered, and added to the wells. As it is suggested by the manufacturer, 100 µL of the supernatant was diluted with 100 µL of the diluent. The cells were then incubated at 37 °C. After 24 h of incubation, it was possible to differentiate damaged cells from healthy cells using a microscope (EVOS XI Core Cell Imaging System, ThermoFisher Scientific, Saint-Laurent, Quebec, Canada) at a 20× magnification. Purified *C. difficile* toxin B was used as a positive control.

Titratable Acidity

Titratable acidity was conducted by adapting AOAC official method 942.15 with indicator method. Supernatants from 24-h culture of *C. difficile* cultured alone or in co-culture with the three lactobacilli strains in TY medium and in BHI medium were taken. The culture supernatants were centrifuged for 10 min at 3700×*g*, filtered, and 4 mL of the filtered supernatant was diluted with 4 mL of distilled water. Three to four drops of phenolphthalein indicator were added. The total titratable acidity was measured by titrating to just before end point with 0.2 M sodium hydroxide to a final pH at 8.0 ± 0.1 and a definite change to pink color persisting 30 s. The final burette reading (mL) was noted and results were reported as % lactic acid (g/100 g) as follows:

$$\%\ \text{Lactic acid} = [[(0.2\text{M NaOH} \times V_{\text{NaOH}}(\text{L}) \times 90.80 \times 2] / \text{wt test portion (mL)}] \times 100$$

(90.80 g/mol is a molecular mass of lactic acid ($C_3H_6O_3$) and 2 is the dilution factor used for sampling before titration).

Statistical Analysis

The experiment was done in triplicate, and for each replicate, three samples were analyzed. Concentration of toxins A and B were compared between the monoculture of *C. difficile* and the co-culture of *C. difficile* with the three lactobacilli. One-way ANOVA was employed for the toxin A and B analyses. Therefore, a Welch's ANOVA was performed for toxin A analysis. p values inferior or equal to 0.05 ($P \leq 0.05$) were considered as significantly different. The growth and pH were measured in each condition then compared by using ANOVA one-way where p values inferior or equal to 0.05 ($P \leq 0.05$) were considered as significantly different.

Results

Toxin Secretion Inhibition in Glucose-Rich Medium

Modified BHI broth containing 3 g/L of glucose was used in these series of assays in order to quantify the effect of acidification on toxin production. *C. difficile* grows well in this medium though toxin production is lower. The growth of *C. difficile* was quantified in co-culture with the specific probiotic and monoculture (Fig. 1). After 12 h of incubation, there was 2.28 $\log_{10}$ CFU/mL fewer CD observed in the co-culture compared with the monoculture of *C. difficile* and a pH drop of 1.1. Toxin A and B concentrations were 24 ± 0.4 ng/mL and 2 ± 0.06 ng/mL, respectively, in monoculture and ≤ 1.25 ng/mL in co-culture (Fig. 4a). Normalized toxin A and B concentration was at 3 ng/$\log_{10}$ CFU/mL and 0.3 ng/$\log_{10}$ CFU/mL in monoculture of *C. difficile* while in the co-culture, toxin A and B concentrations were lower than the limit of quantification of the ELISA kit (≤ 1.25 ng/mL). Similar results were observed in all three replicates. The concentration of toxins A and B in co-culture of *C. difficile* and probiotic strains after 24 h of incubation at 37 °C was reduced by ≥ 99 ± 0.5% and ≥ 99 ± 11%, respectively (Fig. 4b). The percentage of lactic acid present in the co-culture of *C. difficile* with the probiotic strains was about 0.14% higher than the monoculture of *C. difficile* (Fig. 3).

Toxin Secretion Inhibition in Sugar-Free Medium

C. difficile grew well in TY medium, with an increase of 1.04 $\log_{10}$ CFU/mL on average after 24 h (Fig. 2). In the co-culture with the three lactobacilli, with limited substrates to produce lactic acid, the growth of *C. difficile* also increased, by an average of 0.76 $\log_{10}$ CFU/mL although it is not significant (Fig. 3). After 24 h, the lactobacilli had no impact on the growth of *C. difficile*. Toxin A and B concentrations were higher in this medium, 127 ± 11 ng/mL and 202 ± 46 ng/mL, respectively, in monoculture and were 96 ± 16 ng/mL and 114 ± 32 ng/mL in co-culture (Fig. 4a). Normalized toxin A and B concentration values were, respectively, 18 ng/$\log_{10}$ CFU/mL and 30 ng/$\log_{10}$ CFU/mL in the monoculture of *C. difficile* while in the co-culture of *C. difficile* with the specific probiotic, toxin A and B concentrations were 14 ng/$\log_{10}$ CFU/mL and 18 ng/$\log_{10}$ CFU/mL, respectively. Thus, in the presence of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 strains, there was a statistically significant reduction of toxin A and toxin B

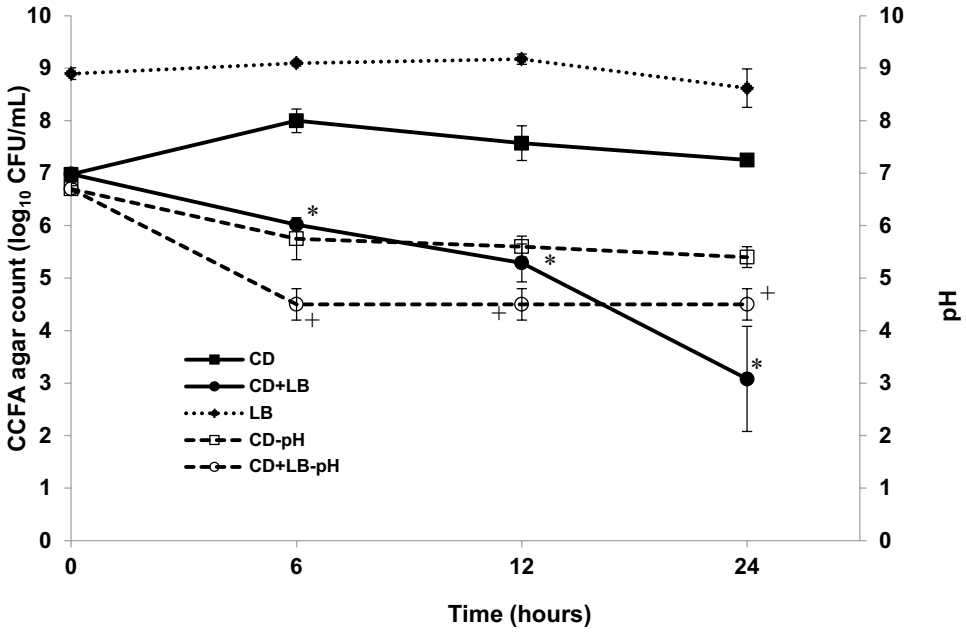

Fig. 1 In vitro model of *Clostridioides difficile* R20291 growth kinetic in co-culture with or without *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, and *Lacticaseibacillus rhamnosus* CLR2 in medium with glucose (BHI broth). (■) Growth and (□) pH of *C. difficile* monoculture; (●) growth and (○) pH of the culture of *C. difficile* with the three lactobacilli. Growth of *C. difficile* was measured by plate count on CCFA agar under anaerobic conditions after 48 h at 37 °C. This experiment was carried out in triplicate. *The values are significantly different from the monoculture of *C. difficile*. "+" indicates that the pH values are significantly different from the monoculture of *C. difficile*

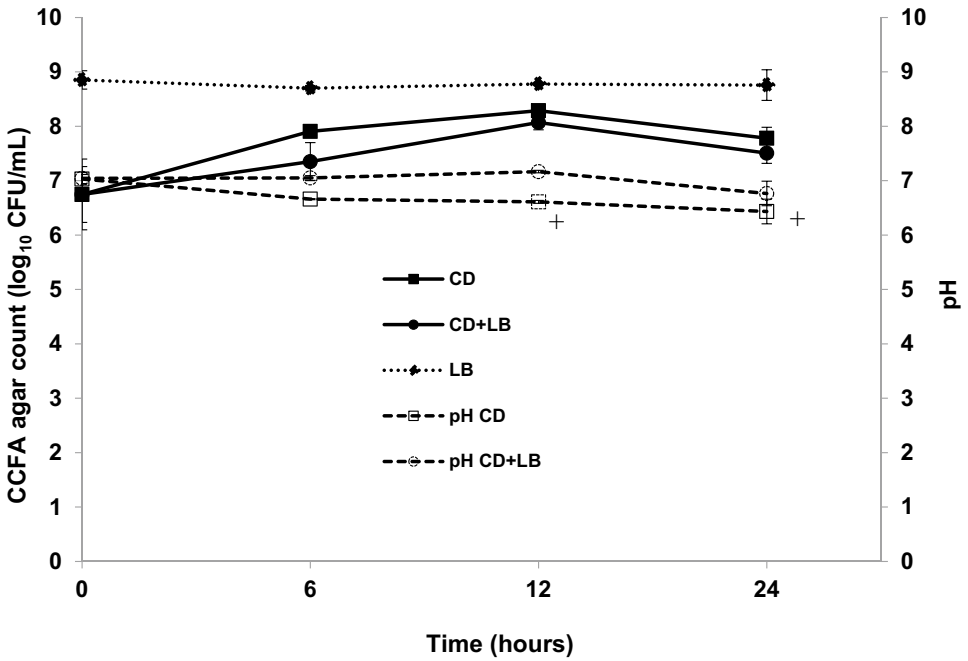

Fig. 2 In vitro model of *Clostridioides difficile* R20291 growth kinetic in co-culture with or without *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, and *Lacticaseibacillus rhamnosus* CLR2 in medium without glucose (TY broth) limiting acid production in the medium. (■) Growth and (□) pH of *C. difficile* monoculture; (●) growth and (○) pH of the culture of *C. difficile* with the three lactobacilli. Growth of *C. difficile* was measured by plate count on CCFA agar under anaerobic conditions after 48 h at 37 °C. This experiment was carried out in triplicate. "+" indicates that the pH values are significantly different from the monoculture of *C. difficile*

production, 20 ± 11% ($p \leq 0.001$) and 41 ± 5% ($p \leq 0.001$), respectively (Fig. 4c). In these experiments, the starting pH value was set to 7. The pH of *C. difficile* monoculture medium acidified significantly, ending up with a lower pH than the co-culture, 6.3 ± 0.0 ($p < 0.001$) and 6.6 ± 0.2 ($p = 0.01$) (Fig. 2). The percentage of lactic acid present in the co-culture of *C. difficile* with the probiotic strains was similar to the quantity of lactic acid in the *C. difficile* monoculture (Fig. 3).

Cytotoxicity Assay

Human fibroblast cells (HFC) are highly sensitive to intact *C. difficile* toxins. Even a minute amount of toxin should lead to potent cytotoxicity damage to the cells. The supernatant taken from CD culture alone in glucose-rich (Fig. 5b) and sugar-free medium (Fig. 5d) was cytotoxic to the cells. HFC became rounded and appeared refractile, as seen in the positive control with purified CD toxin (not shown). In contrast, cells exposed

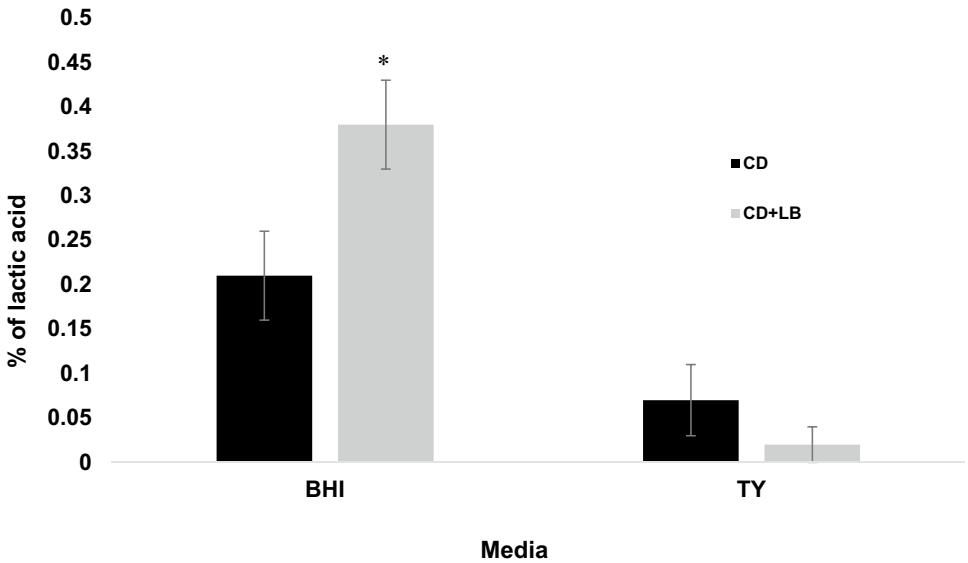

Fig. 3 Percentage of lactic acid measured in mono- and co-culture in BHI and TY medium. The black rectangles represent the supernatant after 24 h of monoculture of *C. difficile* and the gray rectangles represent the supernatant after 24 h from a co-culture of *C. difficile* cultivated with the specific probiotic strains *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, and *Lacticaseibacillus rhamnosus* CLR2

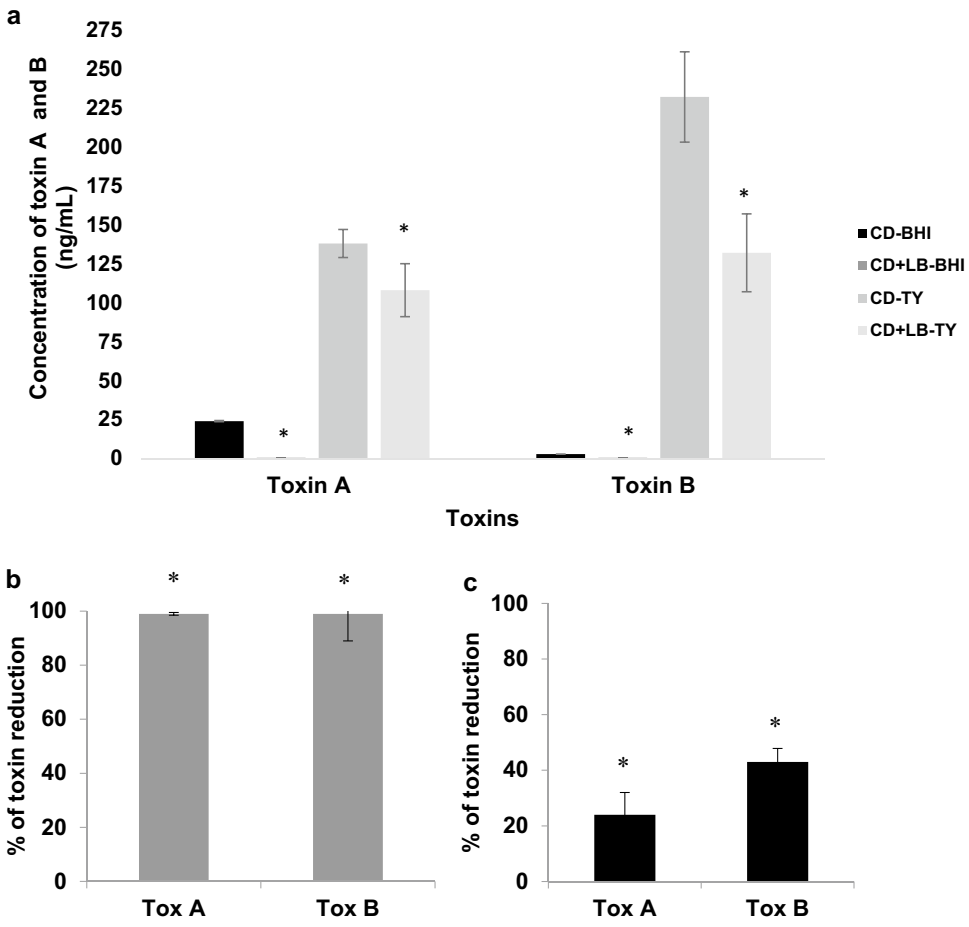

Fig. 4 ng/mL and % of toxin A and B reduction after 24 h in an in vitro model of *Clostridioides difficile* R20291 co-cultured with or without *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, and *Lacticaseibacillus rhamnosus* CLR2. Bacteria were cultivated in a medium with glucose and in a medium without glucose (BHI, TY broth, respectively). **a** Quantity of toxin A and B (ng/mL) in co-culture of *C. difficile* in BHI broth and TY broth (where CD represents *C. difficile* monoculture and CD + LB represents *C. difficile* cultured with the three strains of probiotic). **b** % of decrease in co-culture of *C. difficile* in BHI broth (). **c** % of toxin A and B decrease in co-culture of *C. difficile* in TY broth (■). This experiment was carried out in triplicate

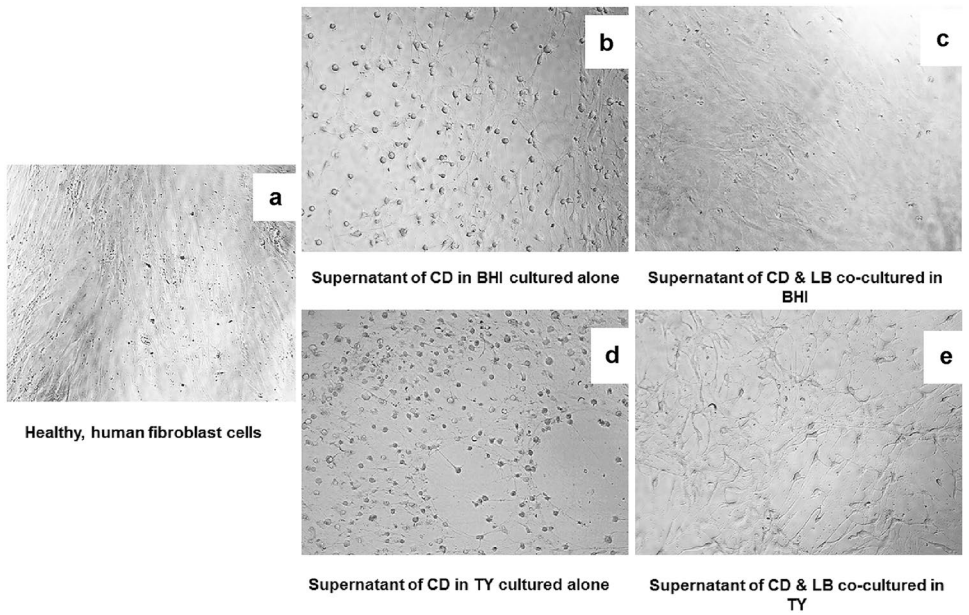

Fig. 5 Cytotoxicity assay of cell-free supernatant from 24 h of co-culture with or without *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, and *Lacticaseibacillus rhamnosus* CLR2 in BHI medium and TY medium on human fibroblasts. Supernatant was added onto the cells and photographs were taken after 24 h of incubation at 37 °C under CO_2-enriched atmosphere

to the supernatant from the co-culture of *C. difficile* with the three lactobacilli cultivated in glucose-rich medium remained healthy (Fig. 5c) and appeared similar to the negative control (Fig. 5a). Cells exposed to the supernatant from the co-culture of *C. difficile* with the three lactobacilli in sugar-free medium contained sufficient CD toxin to damage the cells, but the cells remained viable at 24 h, though they started to detach and adopt a stringy shape (Fig. 5e).

Discussion

Prevention of *Clostridioides* (*Clostridium*) *difficile* infections might be achieved by targeting one of the many stages of this pathogenic anaerobe's life cycle. Here, we focus on the stationary phase of the bacterium. Tejero-Sariñena et al. [9] investigated the inhibitory effects of 15 putative probiotic strains against *C. difficile* in its stationary phase, among other pathogens, and found that the fermentation of glucose to organic acids lowered the pH of the culture and inhibits *C. difficile* growth. The anti-*C. difficile* activity of the specific probiotic containing *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 strains observed in previous in vitro experiments may be associated to a non-specific mechanism available to many lactobacilli, the secretion of lactic acid, a major end-product of glucose fermentation [17]. However, very few lactobacilli-based probiotics have demonstrated an ability to reduce CDI in hospitalized patients. Therefore, lactic acidification is unlikely to be the only anti-*C. difficile* mechanism of action of this specific probiotic formulation (*L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2).

Previous experiments in glucose-rich medium have demonstrated that this specific probiotic protects cells from *C. difficile* toxin A and B cytotoxicity [17]. Here, again, we observed that when *C. difficile* and *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 strains are co-cultivated in a glucose-rich medium, there is potent acidification and a bactericidal effect on *C. difficile* occurs within 12 to 24 h, a 4 $\log_{10}$ CFU/mL decrease. The percentage of lactic acid present in the co-culture of *C. difficile* with the probiotics was higher by 0.14% than in the monoculture of *C. difficile*. *C. difficile* expectedly produced less toxin A and B in glucose-rich conditions, but this was enough to be cytotoxic. In co-culture, there was no detectable toxin A and B, a $\geq$ 99% reduction, and no cytotoxicity. Thus, these three lactobacilli employ at least two complimentary mechanisms for reducing *C. difficile* virulence: growth inhibition and toxin production reduction.

This finding corroborates clinical observations of this specific probiotic in primary CDAD prevention, but does not provide much clarity on how this might occur. Too many factors push the scales in favor of a non-specific protective effect. Running the same experiments in sugar-free medium, TY provides a unique window into conditions where there is high *C. difficile* toxin A and B production and no growth inhibition expected from the lactobacilli since there is no acidification. *C. difficile*, in fact, grew uninhibited with *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 strains. There was no significant difference between the percentage of lactic acid in the monoculture of *C. difficile* versus the co-culture of *C. difficile* with the probiotics. Toxin synthesis was more prolific in this growth medium, though there was an important decrease of toxin A and toxin B in co-culture measured by ELISA, 20% and 41%, respectively, relative to *C. difficile* alone. This suggests a partial inhibitory effect of the specific probiotic containing *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 on *C. difficile* cell toxin A and B production.

Toxin quantification by ELISA is only an estimate of the activated and inactivated toxins. Hence, we undertook cytotoxicity tests to confirm the potency of the detectable toxin. The qualitative cytotoxicity of each sample was assayed with human fibroblast cells as per the manufacturer. The supernatant of *C. difficile* grown in the sugar-free medium produced 202 ng/mL toxin B and expectedly rounded cells within 24 h. There was relatively less toxin B in the supernatant of the co-culture with the lactobacilli, 114 ng/mL, but more than enough to expect complete cell death. Yet, the cells were still viable after 24 h of incubation, showing only some signs of damage. Thus, *C. difficile* toxin A and B is mostly inactivated in the presence of the three probiotic strains, an effect unrelated to lactic acidification. For the first time, this probiotic was shown to provide a protective effect without lactic acidification. Compared with previous experiments, the protective effect to the cells occurred at high levels of toxin at neutral pH. Other lactic acid-producing bacteria decrease *C. difficile* viability and virulence when grown in glucose-rich medium, and this effect is not lost when the pH is neutralized after fermentation [9]. Recently, it was shown that certain strains of lactic acid-producing bacteria have pH-independent mechanisms of action against *C. difficile* [18, 19] related to bacteriocins or bacteriocin-like compounds [18, 20]. *L. acidophilus* La-5 showed reduced cytotoxicity and cytopathic effects of *C. difficile* on HT-29 and CaCo-2 cells, excluding lactic acidification [19].

In conclusion, this study has demonstrated that the presence of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 reduces toxin A and B production by *C. difficile* without lactic acidification and without inhibiting its growth. In addition, there was neutralization of the remaining toxin that conferred protection to human fibroblast cells. Thus, beyond the known acid-dependent protective effect of lactobacilli against CD pathogenesis, these findings suggest that there are also acid-independent protective effects, which may explain why these strains have demonstrated potency for primary CDI prevention when many other putative probiotics have not. However, further investigations will be needed to compare this activity to other lactobacilli.

Author contributions M.L. is responsible for the project. She was involved in the planning of the experiments, discussions of results with S.G, N.S, and M.M. M.M., P.D.P. and S.G contributed to the concept and experimental design. C.D and S.G carried out all the experiments. S.G. wrote the paper. S.G was supervised by M.L. and M.M. Funding

acquisition was obtained by M.L. All authors performed a critical revision of the manuscript and approved the final version.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Conflict of Interest Mathieu Millette, Patrick D. Paquette, Noam Ship are paid employees of Bio-K+ International Inc. Monique Lacroix, Sathursha Gunaratnam, and Carine Diarra have no conflict of interest.

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