



***Lactobacillus rhamnosus* GG Protects Cells from *Clostridium difficile* Toxins**

Eden Ephraim^{1,2*}, Ronald D. Schultz² and Nasia Safdar¹

¹*Division of Infectious Diseases, Department of Medicine, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin, USA.*

²*Department of Pathobiological Sciences, School of Veterinary Medicine, Madison, Wisconsin, U.S.A. *Current address: Kibow Biotech Inc. Newtown Square, PA, USA.*

Authors' contributions

This work was carried out in collaboration between all authors. Author NS designed the study and helped in execution and successful completion of the study. Author RDS supervised the cell culture studies. Author EE wrote the protocol, performed the experiments and the statistical analysis and prepared the first draft of the manuscript. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To determine the anti-cytotoxic effects of *Lactobacillus rhamnosus* GG (LGG) against extracellular and intracellular *Clostridium difficile* toxins.

Study Design: Co-culture system.

Place and Duration of Study: Division of Infectious Diseases, Department of Medicine, School of Medicine and Public Health and Department of Pathobiological Sciences, School of Veterinary Medicine, Madison, Wisconsin, between April 2010 and August 2011.

Methodology: In this study, we investigated the effects of a probiotic LGG (Culturelle®) against a toxigenic *C. difficile* strain (ATCC 9689) and a non-toxigenic *C. difficile* strain (ATCC 700057) in a co-culture system. Co-cultures were prepared with 3 ml of 1:10, 1:100 or 1:1000 dilution of an overnight culture of LGG and 2 ml of 1:100 dilution of either the toxigenic or the non-toxigenic strain. Cytotoxic effects of cell-free culture supernatants (CFS) and cell lysates of the toxigenic strain on Vero cells were evaluated after co-culturing. The relative abundance of toxin A (*TcdA*) and Toxin B (*TcdB*) genes in 72 h co-cultures were determined using real time PCR.

Results: In co-cultures with 1:10 or 1:100 dilution of LGG, counts of the toxigenic *C. difficile* strain were about one log unit lower than control pure cultures after incubation for

*Corresponding author: Email: edenephraim2002@yahoo.com;

48 h. In all co-cultures, counts of the non-toxicogenic strain were two log units lower than those of controls. Accordingly, LGG resulted in a significant decrease ($p < 0.05$) in the relative abundance of *TcdA* and *TcdB* in target DNA prepared from co-cultures containing the 1:10 or the 1:100 dilution of the probiotic. Co-culturing the toxigenic strain with the probiotic (1:10 and 1:100) decreased ($P < 0.05$) the cytotoxic effect of both extracellular and intracellular clostridial toxins resulting in up to 30% increase in cell viability.

Conclusion: LGG inhibits the growth of *C. difficile* in a dose-dependent manner and protects cells from *C. difficile* induced cytotoxicity.

Keywords: Probiotic; culturelle; LGG; *C. difficile*; anti-cytotoxic effect.

1. INTRODUCTION

Clostridium difficile is a pathogen of tremendous public health importance. According to a recent report by the Centers for Disease Control and Prevention (CDC), *C. difficile* infections (CDI) are at an all-time high [29]. CDI are linked to 14,000 deaths in the US each year. Between 2000 and 2007, deaths related to *C. difficile* increased by 400%. Almost half of the infections occur in people younger than 65 while more than 90% of deaths occur in older people. The risk factors associated with the increasing rate of CDI include exposure to antibiotics such as cephalosporins, clindamycin, fluoroquinolones and ampicillin [3].

Toxin A (*TcdA*) and toxin B (*TcdB*) are the causative agents of *C. difficile*-associated diarrhea and colitis [18]. In cultured cells, the two exotoxins are known to induce actin reorganization, cytopathic effects and cell death [20]. The binding and internalization of the toxins leads to a series of inflammatory cascades that involve the production of Interleukin (IL)-8 and increased permeability of the epithelial barrier [4]. Activation of *TcdR* gene triggers the induction of *TcdA* and *TcdB* gene transcription while *TcdC* is a negative regulator of *C. difficile* toxin synthesis [9,26].

The emergence of antibiotic resistant virulent *C. difficile* strains has warranted the assessment of non-antibiotic based novel treatment options. *TcdA* and *TcdB* are targets of CDI therapy. Antibodies specific for *TcdA* and *TcdB* have been shown to effectively treat CDI and prevent disease relapse in animal models and in humans [12]. Recently, Lowy et al. [19] reported reduction in CDI recurrence when human monoclonal antibodies developed against *TcdA* and *TcdB* were administered with antibiotics. However, antibody treatment is expensive and not commercially available at present.

There is emerging evidence that probiotics may be a promising approach to reduce the burden of CDI [21]. Probiotics are live microorganisms that are available over the counter and represent a low-cost, well tolerated, safe, non-antibiotic based strategy that may have efficacy as adjunctive treatment of infections without the attendant risks of promoting antimicrobial resistance. *Saccharomyces boulardii* is effective in preventing antibiotic-associated diarrhea and recurrent CDI in adults [16]. However, due to the potential risk of fungemia associated with this probiotic, it may be preferable to use other probiotics such as lactic acid bacteria to prevent CDI [13,28]. Some studies show the potential of *Lactobacillus rhamnosus* GG (LGG) to decrease the incidence of antibiotic-associated diarrhea caused by some antibiotics [16,23]. However, there is little evidence on the efficacy and mode of action of either probiotics to treat CDI [25].

The aim of this study was to investigate the effect of LGG (Culturelle®) against *C. difficile* and cytotoxicity induced by both its extra- and intra-cellular toxins using a co-culture system. Cytotoxic effects of the cell-free culture supernatants (CFS) and the cell lysates of the toxigenic strain were evaluated on Vero cells. The relative quantities of *TcdA* and *TcdB* in 72 h co-cultures were also determined using real time PCR.

2. MATERIALS AND METHODS

2.1 Bacteria and Cell Culture Conditions

A toxigenic *C. difficile* strain (ATCC 9689) and a non-toxigenic *C. difficile* strain (ATCC 70057) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Both were cultured in PRAS-Brain Heart Infusion (BHI) broth (Anaerobe Systems) and plate counts were performed by plating serial dilutions of cultures on Cycloserine Cefoxitin Fructose Agar (CCFA: Anaerobe Systems). LGG capsules obtained from i-Health, Inc. /Culturelle (Cromwell, CT) were cultured in MRS broth and plated on MRS agar. Both the *C. difficile* strains and the LGG cultures were incubated at 37°C in an anaerobic chamber.

For the cytotoxicity studies, Vero cells were cultured in Minimum Essential Medium (MEM) with 10% heat-deactivated fetal bovine serum (FBS), 1% v/v antibiotic antimycotic solution, 0.6% v/v tylosin and 1% glutamine. All incubations involving cell cultures were undertaken at 37°C in a humidified 5% CO₂ incubator.

2.2 Challenge Experiments

Overnight cultures of *C. difficile* and LGG at ca. 1×10^9 CFU were used for co-cultures. Three milliliters of 1:10 or 1:100 or 1:1000 dilutions of LGG were mixed with 2 ml of the 1:100 dilution of the toxigenic or the non-toxigenic *C. difficile* strain and incubated in an anaerobic chamber at 37°C for 48 h. Aliquots of the co-cultures were used to determine pH and counts of *C. difficile*. The rest of the co-cultures were used for determination of cytotoxic effects on Vero cells. The relative amounts of *TcdA* and *TcdB* in 72 h co-cultures were measured using real-time PCR.

2.3 Preparation of Cell-Free Culture Supernatants (CFS) and Cell Lysates

TcdA and *TcdB*, which have been implicated to be responsible for the cytotoxic effects of toxigenic *C. difficile* strains, are present both intra- and extra-cellularly [8,24]. Thus, CFS, for the extra-cellular toxins and cell lysates, for the intra-cellular toxins, were prepared as described below. After culturing *C. difficile* with the three different dilutions of LGG for 48 h, culture supernatants were collected and centrifuged at 13, 600 x g for 15 min. The supernatants were filtered through a 0.22 µm pore size filter membrane and used in the subsequent experiments. The residual bacterial cells were used to prepare the cell lysates. Cells were washed three times with PBS (1x) and were suspended in 3 ml of PBS. The suspension was subjected to a series of freeze-thaws (3x) and brought to 5 ml with PBS for sonication. The sonication was performed on ice at 30% duty cycle, output set between 3 and 4, for 10 min with 1 min interval. The sonicates were centrifuged (at 14, 000 rpm for 10 min) and filter-sterilized before used in the following cytotoxicity assays.

2.4 Cytotoxicity of CFS and Cell Lysates on Vero Cells

Monolayers of Vero cells (5×10^4 cells) were prepared in 96-well culture plates. Cells were washed with 1% PBS and incubated with the 1:2 dilution of the CFS or cell lysates of the co-cultures in MEM for 24 h. Positive control wells were incubated with the CFS or the cell lysates of the toxigenic *C. difficile* strain. Negative control wells were incubated with the CFS or the cell lysates of the non-toxigenic *C. difficile* cultures or that of LGG at the three different dilutions. In addition, some control wells were incubated in MEM without any CFS or cell lysate (untreated). After incubation, MTT assay, which serves as an index of living cells by measuring the mitochondrial function, was performed. The assay was carried out as previously described [10]. About 250 mg MTT was suspended in 50 ml of sterile 1% PBS to make 5 mg/ml MTT solution. Ten micro-liters of the MTT solution were added to each well and plates were further incubated at 37°C for 4 h. The media were replaced with 100 μ l of DMSO solution (94.6 ml DMSO, 0.6 ml acetic acid and 10 g SDS). Plates were then immediately read in a standard ELISA plate reader at 550 nm. The percent viability of Vero cells was calculated using the expression:

$$(\text{OD}_{550\text{nm}} \text{ of each treatment} / \text{OD}_{550\text{nm}} \text{ of control untreated cells}) \times 100$$

2.5 DNA Preparation

After incubation for 72 h, DNA samples were prepared from pellets of the co-cultures using QIAamp DNA mini kit (Qiagen) for Gram-positive bacteria. Cultures were centrifuged at 2,800 g for 25 min. Pellets were resuspended in distilled water and re-centrifuged. Following Qiagen's protocol, the bacteria were lysed and DNA samples were prepared from pure cultures of the toxigenic *C. difficile* strain and co-cultures of the toxigenic *C. difficile* strain with the three different dilutions of LGG. The final DNA extracts were suspended in ddH₂O and 2 μ l of each suspension was used as a target DNA for PCR analyses.

2.6 PCR Detection of Toxin A and Toxin B

Primer sequences specific for *TcdA* and *TcdB* were obtained from the literature [2]. Primers were synthesized (IDT; Carlsbad, CA) and diluted to 100 μ M with ddH₂O. The other components of the PCR reactions were obtained from Promega (Madison, WI). A 25 μ l PCR reaction mixture contained 11.13 μ l ddH₂O, 6.67 Buffer (5x), 1.25 μ l of each of the forward and the reverse primers, 2.5 μ l of dNTPs (2 mM), 2 μ l DNA template and 0.2 μ l Taq (5 U/ μ l). PCR was carried out according to the program: 95°C for 4 min, followed by 35 cycles of 95°C for 30 sec, 48°C (for *TcdA*) or 45°C (for *TcdB*) for 30 sec, 72°C for 1 min and a final extension at 72°C for 4 min. PCR products were ran on a pre-cast 1.2% Agarose FlashGel (Lonza, Rockland, ME) and images were visualized and documented using a Gel Doc2000 (BioRad; Hercules, CA).

2.7 Quantitative real-time PCR

To compare the relative abundance of *TcdA* and *TcdB* genes in the co-cultures and the pure toxigenic *C. difficile* strain, 25 μ l reaction mixtures containing 12.5 μ l of Sigma SYBR® Green JumpStart™ TaqReadyMix™ (Sigma–Aldrich Corp, St. Louis, MO), 0.3 μ l of 100 μ M of each of the forward and the reverse primer and 2.5 μ l of DNA were prepared. Quantitative real-time PCR was run with a Strata gene MX3005P™ quantitative PCR system (Agilent Technologies, LaJolla, CA) following the protocol: 94°C melt step for 4 min and 44 cycles of

94°C for 20 sec, 45°C (for *TcdA*) or 48°C (for *TcdB*) for 20 sec and 72°C for 30 sec. This was followed by a melting curve analysis, which ran for 1 cycle at 95°C for 1 min, 45°C for 30 min and 95°C for 30 sec. The relative abundance of *TcdA* and *TcdB* were related to the cycle threshold (Ct). DNA templates of pure cultures of LGG and the non-toxicogenic *C. difficile* strain were used as negative controls.

2.8 Data Analyses

All experiments were repeated at least three times allowing the mean and the standard deviations to be calculated. Statistical significances were determined by one-way repeated measures analysis of variance (ANOVA) (SPSS 12.0). A probability (P) value equal to or less than 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1 Counts of *C. difficile* in Co-cultures

After 48 h, counts of the non-toxicogenic *C. difficile* strain (CNT cont) in all co-cultures were about 2 logs lower than cultures without the probiotic (Fig. 1A). Relative to control pure cultures, counts of the toxicogenic strain (CT cont) were lower by one log unit when co-cultured with LGG at 1:10 and 1:100. There was no significant difference in the counts of the toxicogenic strain co-cultured with LGG at 1:1000 dilution (Fig. 1A). The pH of the co-cultures did not vary significantly from both the control pure toxicogenic and the non-toxicogenic *C. difficile* cultures and were higher than the pure LGG cultures at the three dilutions (Fig. 1B).

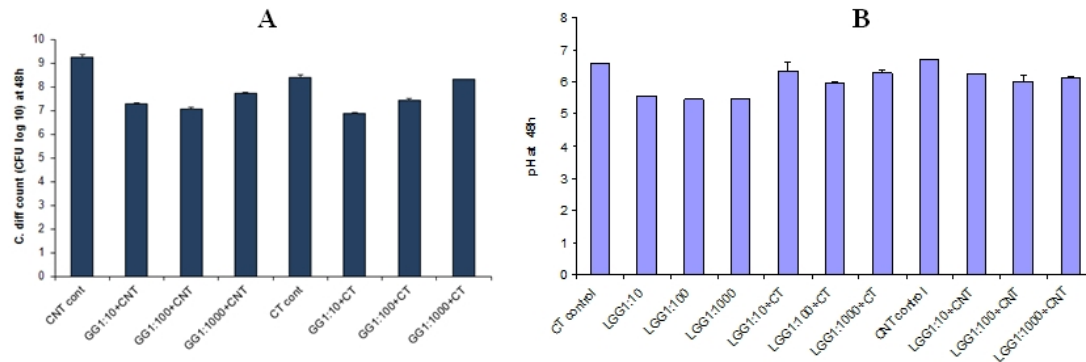


Fig. 1A. Mild growth inhibition of *C. difficile* by LGG in co-cultures. Counts of the non-toxicogenic *C. difficile* strain (CNT) after co-incubation with LGG at 1:10, 1:100 and 1:000 dilutions for 48 h decreased by about 2 logs. Only the 1:10 and the 1:100 dilutions of LGG had a mild growth inhibition on the toxicogenic strain (CT). Control pure *C. difficile* cultures (CNT cont and CT cont) did not contain the probiotic. Fig. 1B. PH profiles of pure and co-cultures of LGG with either the toxicogenic or the non-toxicogenic *C. difficile* strain after 48 h

Interestingly, *C. difficile* counts in co-cultures containing the 1:10 and 1:100 dilutions of LGG remained unchanged from the initial counts used to start the co-cultures (ca. 1×10^7 CFU/ml). This suggests that LGG suppressed the growth of *C. difficile*, which increased in control pure cultures by more than 1 (CT cont) or 2 logs (CNT cont) (Fig. 1A). Trejo et al.

[27] reported a significant decrease in *C. difficile* toxins in co-cultures containing strains of bifidobacteria while the growth of clostridial strains in co-cultures was similar to pure clostridial control cultures. Low pH conditions caused by organic acid secretion were shown to have inhibitory effects on the growth and cytotoxicity of *C. difficile* [30]. However, in our study the pH of co-cultures containing LGG was not significantly different from that of the pure clostridial cultures. This suggests the presence of a different mechanism by which LGG suppresses the growth of *C. difficile* and protects cells from its cytotoxic effects.

3.2 Cytotoxic Effect of Extracellular Toxin

CFS of 48 h old co-cultures and that of the pure *C. difficile* cultures were used to determine the effect of LGG on the cytotoxic effect of extracellular *C. difficile* toxins. Vero cells were incubated with either of the CFS for 24 h. The CFS of all cultures containing the toxigenic *C. difficile* strain caused cytopathic effects (cell rounding and detachment). Treatment of Vero cells with the CFS of the toxigenic *C. difficile* strain resulted in the most significant decrease in the number of viable cells (Fig. 2). There was significant ($p < 0.05$) protection by LGG resulting in 15-30% increase in the percent of viability when the toxigenic strain was co-incubated with LGG at the different dilutions (Fig. 2).

Co-cultures have been used to demonstrate the potential of various probiotic bacteria to decrease the number and virulence of *C. difficile* [11,27]. In our co-culture system, although the decrease in the number of the toxigenic *C. difficile* strain relative to the controls was only a log, LGG resulted in a significant decrease in the cytotoxic effects of clostridial toxins as compared to pure clostridial cultures. Similarly, Banerjee et al. [1] reported that the CFS of the probiotic *L. bulgaricus* LDB B-30892 reduced *C. difficile*-induced cytotoxicity. They hypothesized possible mechanisms of detoxification such as proteolytic cleavage of toxin or toxin receptors. In addition, *S. boulardii* has been shown to have proteolytic activity on *C. difficile* toxins [7]. In contrast, Carasi et al. [5] showed that proteolytic activity is not involved in the protective effect of kefir lactobacilli against clostridial toxins.

3.3 Cytotoxic Effects of Intracellular Toxin

The cell lysates of the co-cultures were used to study the cytotoxic effects of intracellular *C. difficile* toxins after co-culturing with LGG. As compared with the CFS, the cell lysates of the toxigenic *C. difficile* strain cultures had lesser cytopathic effects on Vero cells. When the cell lysates were added on monolayers of Vero cells, the viability of the cells decreased significantly after 24 h (Fig. 3). Co-incubating the toxigenic strain with culturelle had significant protective effect resulting in a 20%, 30% and 15% increase in cell viability with the 1:10, 1:100 and 1:1000 dilution of the probiotic, respectively (Fig. 3).

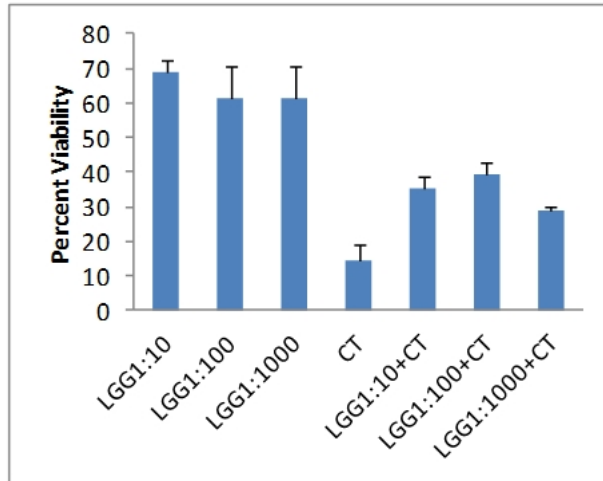


Fig. 2. Protective effect of LGG from extracellular *C. difficile* toxins in CFS. Vero cells were incubated with the CFS of LGG at 1:10, 1:100 or 1:1000 dilution, the CFS of the toxigenic *C. difficile* strain (CT) or the CSF of the co-cultures of the toxigenic *C. difficile* strain with either of the three different dilutions of LGG (LGG+CT) for 24 h. The absorbance at 550 nm was read after MTT test was performed. The percent viability of the cells was calculated as: $(OD_{550\text{ nm}} \text{ of each treatment} / OD_{550\text{ nm}} \text{ of the control untreated cells}) \times 100$

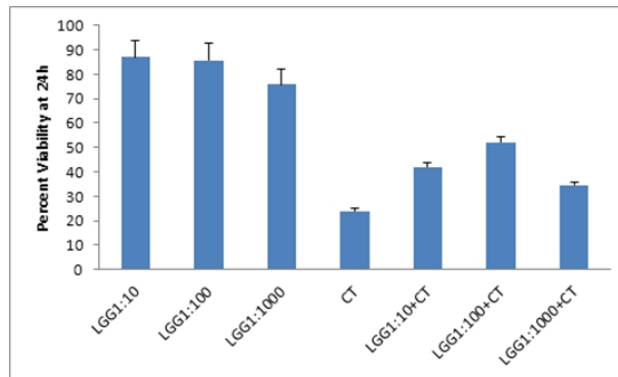


Fig. 3. Protective effect of LGG from intracellular *C. difficile* toxins in cell lysates. Vero cells were incubated with the cell lysate of LGG at 1:10, 1:100 or 1:1000 dilution, that of the toxigenic *C. difficile* strain (CT) or co-cultures of the toxigenic *C. difficile* strain with either of the three different dilutions of LGG (LGG+CT) for 24 h. The absorbance at 550 nm was read after MTT test was performed. The percent viability of the cells was calculated as: $(OD_{550\text{ nm}} \text{ of each treatment} / OD_{550\text{ nm}} \text{ of the control untreated cells}) \times 100$

Probiotics have many potential mechanisms of actions; including competitive exclusion, increasing host epithelial barrier function and modulation of host immune responses [14,17]; although in general the mechanisms of actions have not been adequately studied. Recently, different authors have reported protective effects of various probiotics against cytotoxic effects induced by cell-free culture supernatants (CFS) containing clostridial toxins [27,30].

In this study, we showed that the growth of *C. difficile* in the presence of LGG leads to CFS and cell lysates with significantly lower cytotoxic effect as compared with those from pure clostridial cultures. Most previous studies investigated the effect of probiotics only on the cytotoxicity of secreted *C. difficile* toxins in CFS. However, it is very important to study the protective effects on the intracellular clostridial toxins. Release of cytotoxins may be delayed by various factors such as the incubation period [24]. Furthermore, it has long been reported that the minimum dose of intracellular clostridial toxin causing cytotoxicity is lower than extracellular toxin [22]. In this regard, we were able to show that LGG decreased the cytopathic effect of not only the released toxins but also the cell-associated *C. difficile* toxins.

3.4 Relative Quantities of Toxin A and Toxin B Genes

After incubation for 72 h, *TcdA* (Fig. 4A) and *TcdB* (Fig. 4B) were detected in both the pure cultures of the toxigenic *C. difficile* strain (CT) and the co-cultures of the strain with the probiotic at the three different dilutions. The non-toxigenic *C. difficile* strain (CNT) was confirmed to be negative for both toxins (Fig. 4).

The relative abundance of *TcdA* and *TcdB* genes in DNA templates from the pure cultures of the toxigenic *C. difficile* strain (CT) and the co-cultures after 72 h was related to the Ct values (Fig. 5). The Ct values are inversely proportional with the number of each gene and the counts of the toxigenic strain. Co-cultures with LGG at 1:10 and 1:100 dilutions provided significantly higher Ct values ($P < 0.05$) than the pure *C. difficile* cultures (Fig. 5); indicating inhibition of the growth of *C. difficile* by the probiotic. LGG dilution at 1:1000 did not affect the relative amount of *TcdA* (Fig. 5A).

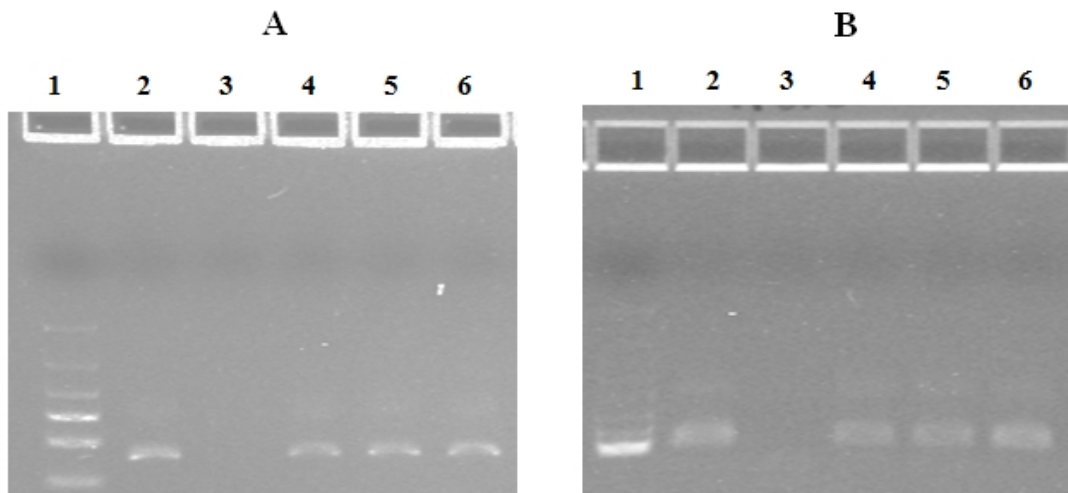


Fig. 4. Electrophoresis of conventional PCR amplification of Toxin A (A) and Toxin B (B). PCR was performed using target DNA from cultures containing the toxigenic *C. difficile* strain alone (2), the non-toxigenic *C. difficile* strain alone (3), the toxigenic *C. difficile* strain in co-culture with LGG at 1:10 dilution (4), at 1:100 dilution (5) and at 1:1000 dilution (6). DNA templates from all cultures containing the toxigenic *C. difficile* strain were positive for *TcdA* and *TcdB*. The non-toxigenic strain was confirmed to be negative for both toxins

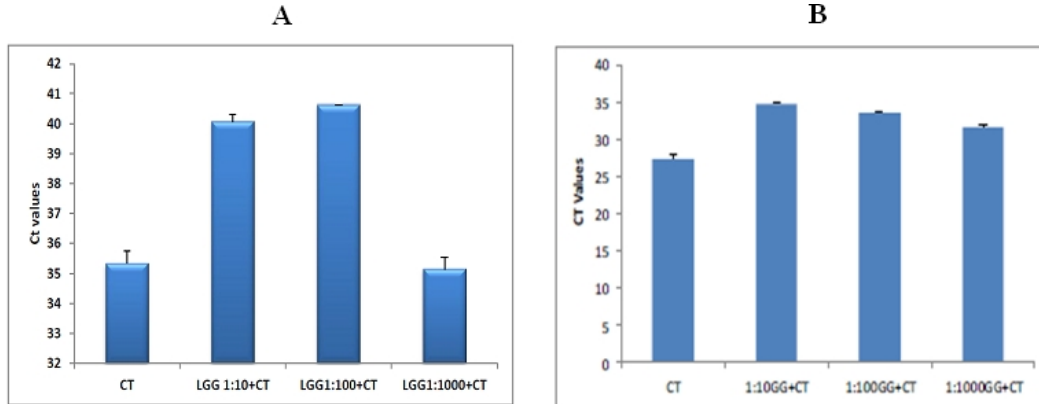


Fig. 5. Quantitative real-time PCR illustrating the effect of LGG on the relative quantities of *TcdA* (A) and *TcdB* (B). Co-incubation of the toxigenic *C. difficile* strain with the probiotic (LGG+CT) increased the Ct values of *TcdA* and *TcdB* significantly. The increase in the Ct values indicated the inhibition effect of the probiotic on the growth of *C. difficile*.

Our results showed LGG could inhibit the growth of *C. difficile* in co-cultures. This was supported by the lower quantities of *TcdA* and *TcdB* genes in co-cultures containing LGG at 1:10 and 1:100 dilutions (Fig. 5). In co-cultures with the 1:1000 dilution of LGG, the amount of Toxin A was almost the same as that in pure *C. difficile* cultures. This was in line with the counts of the toxigenic strain (Fig 1A) that were not affected by the probiotic at the lowest dilution. This suggests the ability of the probiotic to suppress the growth of *C. difficile* in a dose-dependent manner.

The mode of action of probiotics by which they antagonize gastrointestinal pathogens by decreasing the expression levels of their virulent genes has not been well investigated. Carey et al. [6] reported the down-regulation of Shiga toxin 2 subunit A in a Shiga toxin 2 subunit A producing *Escherichia coli* (STEC) strain after co-culturing with different probiotics. Various possible mechanisms of antagonism of *C. difficile* by lactic acid bacteria have been suggested [15,27]. Our study encourages further investigation on the effects of metabolic products of LGG on the regulation of clostridial toxins. In this study, the mRNA expression levels of Toxin A and Toxin B were not determined. This would have given more valuable information on the direct effect of LGG on the expression of the toxin genes. Furthermore, additional studies are warranted to determine the role of the probiotic in reducing levels of secreted and cell-associated clostridial toxins.

4. CONCLUSION

There is an increasing interest towards the use of probiotics in prevention and treatment of CDI [23]. In this study, we showed that Culturelle (LGG) has antagonistic effects against *C. difficile* by protecting eukaryotic cells from cytotoxicity induced both by intracellular and extracellular clostridial toxins. This was shown by the growth inhibition of *C. difficile* in co-cultures, which was confirmed by the decreased relative abundance of Toxin A and Toxin B genes in cultures containing the probiotic. Our study suggests further investigation on the role of the probiotic in regulating these genes and other genes in the pathogenicity locus of *C. difficile* that are involved in the synthesis of clostridial toxins.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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