

***Lactobacillus rhamnosus*GG (LGG) protects cells from *Clostridium difficile* toxins**

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ABSTRACT

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

Study Design: Co-culture system.

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 gene expression levels of *TcdA* and *TcdB* 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 decreased by about a log after incubation for 48 h. In all co-cultures, counts of the non-toxigenic strain decreased by about two logs. 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. In addition, LGG resulted in a significant decrease ($p < 0.05$) in gene expression levels of *TcdA* and *TcdB* in target DNA prepared from co-cultures containing the 1:10 or the 1:100 dilution of the probiotic.

Conclusion: LGG protects cells from *C. difficile* induced cytotoxicity and reduces the expression levels of *TcdA* and *TcdB* in a dose-dependent manner.

Keywords: Probiotics, 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) (VitalSigns CDC, 2012), *C. difficile* infections (CDI) are at an all-time high. 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 (Bartlett, 2010).

Toxin A (*TcdA*) and toxin B (*TcdB*) are the causative agents of *C. difficile*-associated diarrhea and colitis (Lica et al., 2011). In cultured cells, the two exotoxins are known to induce actin re-organization, cytopathic effects and cell death (Lyras et al., 2009). 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 (Canny et al., 2006). Activation of *TcdR* gene triggers the induction of *TcdA* and *TcdB* gene transcription while *TcdC* is a negative regulator of *C. difficile* toxin synthesis (Sun et al., 2010).

There is emerging evidence that probiotics may be a promising approach to reduce the burden of CDI (McFarland, 2009). 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 (Katz, 2006). 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 (Herbrecht and Nivoix, 2005; Venugopalan et al., 2010). Some studies show the potential of *Lactobacillus rhamnosus* GG (LGG) to decrease the incidence of antibiotic-associated diarrhea caused by some antibiotics (Katz, 2006; Nelson, 2008). However, there is little evidence on the efficacy and mode of action of either probiotics to treat CDI (Pochapin, 2000). In this study, we investigated the effect of LGG (Culturelle®) against both the extra- and intra-cellular *C. difficile* toxins in a co-culture system. Cytotoxic effects of the cell-free culture supernatants (CFS) and the cell lysates of the toxigenic strain on Vero cells were evaluated after co-culturing. The gene expression levels 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 700057) 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 CycloserineCefoxitin 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 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 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. Gene expression levels 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 (Chaves-Olarte et al., 1997; Osgood et al., 1993). 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 pore size filter membrane and used in the subsequent experiments. The residual bacterial cells were used to prepare the cell lysate. Cells were washed three times with PBS (1 x) and were suspended in 3 ml of PBS. The suspension was subjected to a series of freeze-thaws (3 x) 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. Control wells were incubated with the CFS or the cell lysates of the toxigenic or the non-toxigenic *C. difficile* cultures or that of LGG at the three different dilutions. 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 (Edmondson et al., 1988). About 250 mg MTT was suspended in 50ml 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 µl 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:

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

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 µl 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* (Table 1) were obtained from the literature (Belanger et al., 2003). Primers were synthesized (IDT; Carlsbad, CA) and diluted to 100µM with ddH₂O. The other components of the PCR reactions were obtained from Promega (Madison, WI). A 25 µl PCR reaction mixture contained 11.13 µl ddH₂O, 6.67 Buffer (5x), 1.25 µl of each of the forward and the reverse primers, 2.5 µl of dNTPs (2 mM), 2 µl DNA template and 0.2 µlTaq (5 U/µl). 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 run on a pre-cast 1.2 % AgaroseFlashGel (Lonza, Rockland, ME) and images were visualized and documented using a Gel Doc2000 (BioRad; Hercules, CA).

Table 1. Primers for *TcdA* and *TcdB* genes (Belanger et al., 2003)

Gene	Forward primer sequence	Reverse primer sequence	Product size
<i>TcdA</i>	5' TCT ACC ACT GAA GCA TTA C 3'	5' TAG GTA CTG TAG GTT TAT TG 3'	158 bp
<i>TcdB</i>	5' ATA TCA GAC ACT GAT GAG 3'	5'TAGCATATT CAGAGAATATTGT3'	108 bp

2.7 QUANTITATIVE REAL-TIME PCR

To compare the expression levels of *TcdA* and *TcdB* in the co-cultures and the pure toxigenic *C. difficile* strain, 25 µl reaction mixtures containing 12.5 µl of Sigma SYBR® Green JumpStart™ TaqReadyMix™ (Sigma–Aldrich Corp, St. Louis, MO), 0.3 µl of 100 µM of each of the forward and the reverse primer and 2.5 µl of DNA were prepared. Quantitative real-time PCR was run with a Stratagene 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-toxigenic *C. difficile* strain were used as negative controls.

2.8 DATA ANALYSIS

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 RESULTS

3.1.1 COUNTS OF *C. DIFFICILE* IN CO-CULTURES

Counts of the control non-toxicogenic *C. difficile* strain (CNT cont) decreased by about 2 logs after 48 h of co-incubation with all dilutions of LGG (Fig. 1). Counts of the toxigenic strain (CT cont) decreased by one log when co-cultured with LGG at 1:10 and 1:100; however, there was no significant decrease in the number of the toxigenic strain co-cultured with LGG at 1:1000 dilution (Fig. 1). The pH of the co-cultures did not vary significantly from both the control pure toxigenic and the non-toxicogenic *C. difficile* cultures (Data not shown).

3.1.2 CYTOTOXIC EFFECT OF EXTRACELLULAR TOXIN

CFS of the co-cultures and the pure *C. difficile* cultures were used to determine the effect of LGG on the cytotoxic effect of extracellular *C. difficile* toxins after co-incubation for 48 h. The CFS of all cultures containing the toxigenic *C. difficile* strain caused cytopathic effects (cell rounding and detachment) on Vero cells. 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).

3.1.3 CYTOTOXIC EFFECT 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 30 %, 20 % and 15 % increase in cell viability with the 1:10, 1:100 and 1:1000 dilution of the probiotic, respectively (Fig. 3).

3.1.4 DETECTION OF TOXIN A AND TOXIN B BY PCR

Co-culturing the toxigenic *C. difficile* strain with culturelle for 72 h did not eliminate *TcdA* (Fig. 4A) and *TcdB* (Fig. 4B). The toxins 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 (Fig. 4). The non-toxigenic *C. difficile* strain (CNT) was confirmed to be negative for both toxins (Fig. 4).

3.1.5 RELATIVE QUANTITIES OF TOXIN A AND TOXIN B GENES

The relative abundance of *TcdA* and *TcdB* gene copies 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 gene copies for each gene. 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 *TcdA* and *TcdB* expression by the probiotic. LGG dilution at 1:1000 did not affect the relative amount of *TcdA* (Fig. 5A).

3.2 DISCUSSION

The emergence of antibiotic resistant virulent *C. difficile* strains has warranted the assessment of non-antibiotic based novel treatment options. The two primary virulent factors of *C. difficile*, *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 (Giannasca et al., 1999). Recently, Lowy et al. (Lowy et al, 2010) 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 an increasing interest towards the use of probiotics in prevention and treatment of CDI (Nelson, 2008). Co-cultures have been used to demonstrate the potential of various probiotic bacteria to decrease the number and virulence of *C. difficile* (Folkers et al., 2010; Trejo et al., 2010). In our co-culture system, although the decrease in the number of the toxigenic *C. difficile* strain was only a log, the growth of *C. difficile* in the presence of LGG lead to a significant decrease in the cytotoxic effects of both its extra- and intracellular toxins as compared to pure clostridial cultures. Trejo et al. (Trejo et al., 2010) 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. Similarly, Woo et al. (2011) indicated that the slight decrease in the number of clostridia in co-cultures with different probiotics was not attributed to the decrease in the cytotoxic activities of *C. difficile* toxins. Low pH conditions caused by organic acid secretion were shown to have inhibitory effects on the growth and cytotoxicity of *C. difficile* (Woo et al., 2011). However, in our study the pH of co-cultures containing LGG was the same as the pure clostridial cultures.

Probiotics have many potential mechanisms of actions; including competitive exclusion, increasing host epithelial barrier function and modulation of host immune responses (Hugo et al., 2008; Lebeer et al., 2010), 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 (Trejo et al., 2010; Woo et al., 2011). In this study we show 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 CFS and cell lysates from pure clostridial cultures. Banerjee et al. (2009) 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. *S. boulardii* has been reported to have proteolytic activity on *C. difficile* toxins (Castagliuolo et al. 1999). In contrast, Carasi et al. (2012) showed that proteolytic

activity is not involved in the protective effect of kefir lactobacilli against clostridial toxins. 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 effect of probiotics on the intracellular clostridial toxins. Release of cytotoxins may be delayed by various factors such as the incubation period (Osgood et al., 1993). Furthermore, it has long been reported that the minimum dose of intracellular clostridial toxin causing cytotoxicity is lower than extracellular toxin (Meng et al., 1993). 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.

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. (2008) reported the down-regulation of Shiga toxin 2 subunit A in a Shiga toxin producing *Escherichia coli* (STEC) strain after co-culturing with different probiotics. Unlike the Japanese probiotic strain, *Clostridium butyricum* MIY AIRI 588 (Woo et al., 2011), our study showed LGG could decrease the gene expression levels of *TcdA* and *TcdB* in co-cultures. This is in line with the hypothesis given by Trejo et al. (2010) as a possible mechanism of antagonism of *C. difficile* by bifidobacteria. Certain amino acids and fatty acids can affect synthesis of *C. difficile* toxins (Karlsson et al., 2000). Our results encourage further study on the effects of metabolic products of LGG on the regulation of clostridial toxins. In a pathogenicity locus of *C. difficile*, the gene that has negative regulation on *C. difficile* toxin synthesis is *TcdC*[9]. Further studies are suggested to investigate the role of LGG in regulating *TcdC*.

4.CONCLUSION

In this study we showed that Culturelle (LGG) has antagonistic effects against *C. difficile* by protecting eukaryotic cells from cytotoxicity induced by both intracellular and extracellular clostridial toxins. The down-regulation of *TcdA* and *TcdB* by the probiotics suggests further investigation on the role of the probiotic in regulating other genes in the pathogenicity locus of *C. difficile*, such as *TcdC*, involved in the synthesis of clostridial toxins.

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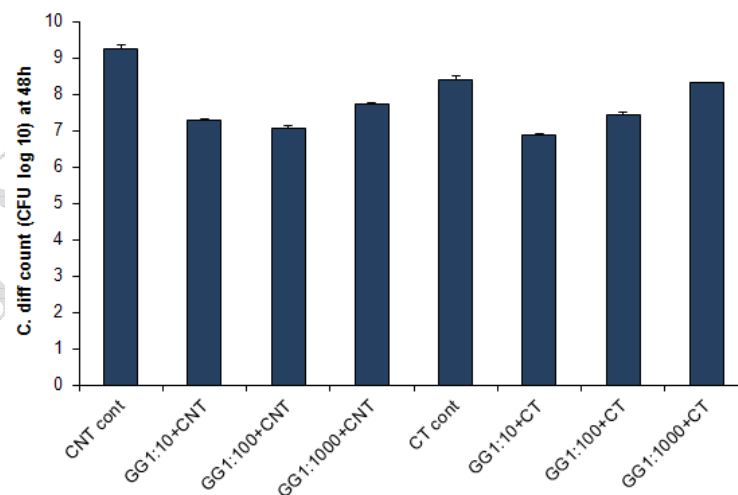
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Figures

Fig. 1.



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Fig. 2.

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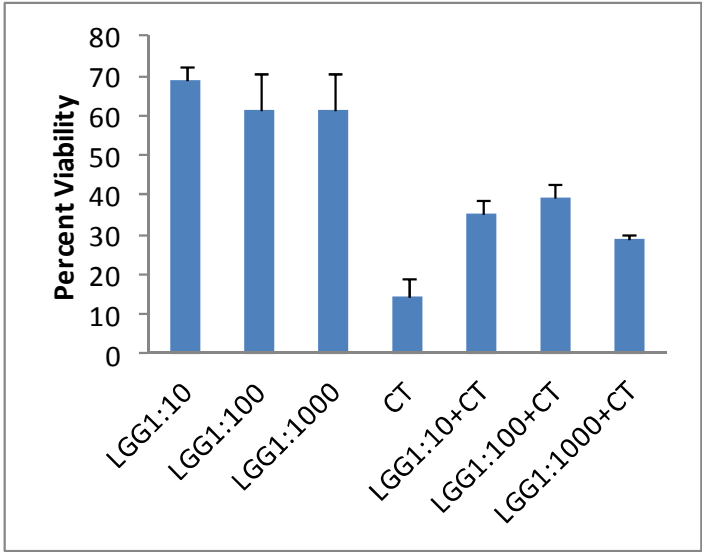


Fig. 3.

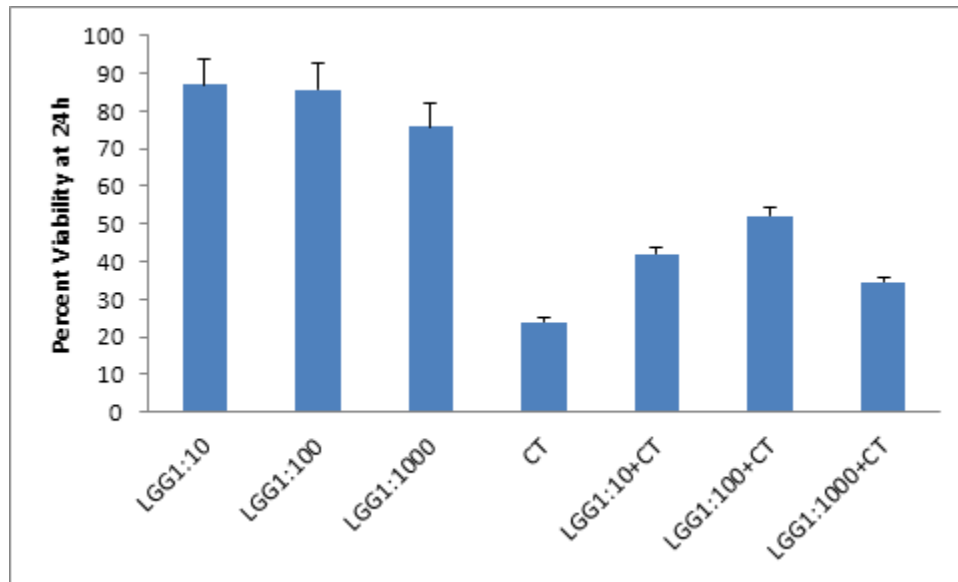


Fig. 4.

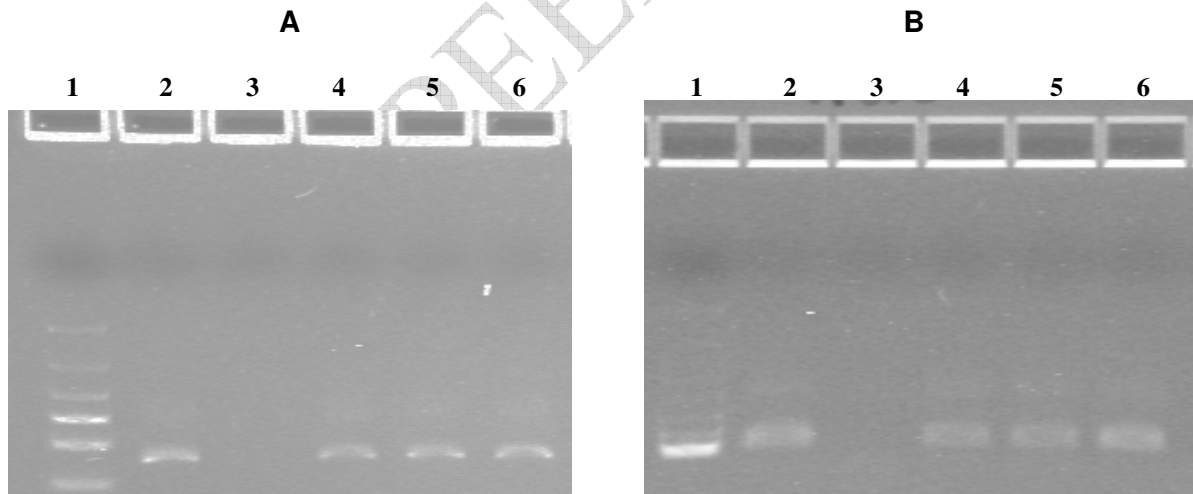


Fig. 5.

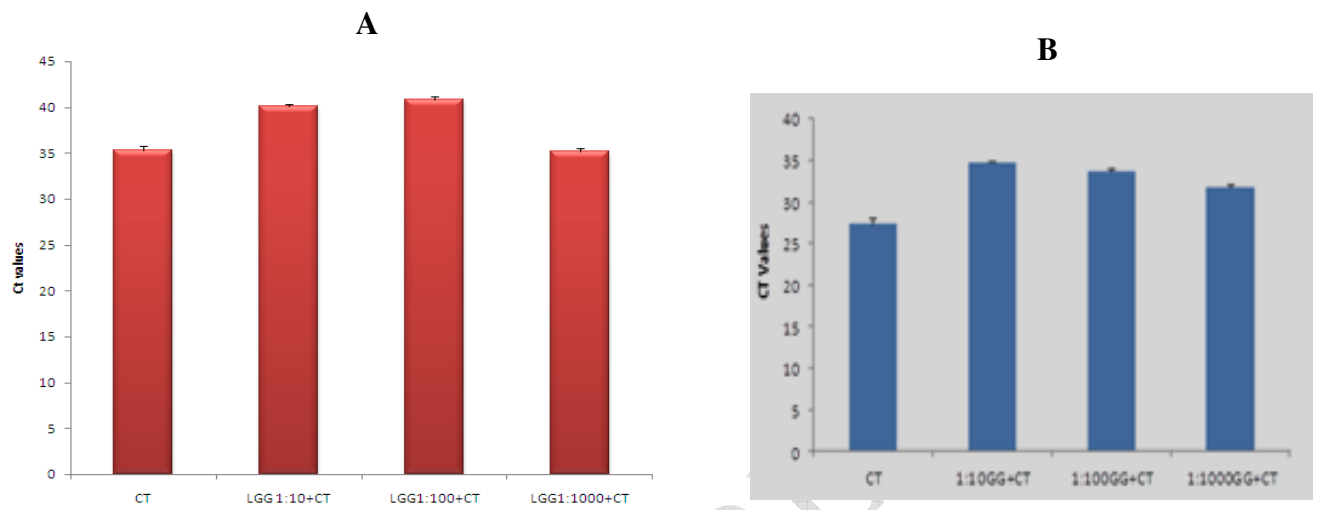


Figure captions

Fig. 1: 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 1:1000 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 toxigenic strain (CT). Control pure *C. difficile* cultures (CNT cont and CT cont) did not contain the probiotic.

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 (CT) with either of the three different dilutions of LGG for 24 h. The absorbance at 550 nm was read after MTT test was performed. The percent viability of the cells was then calculated as: $(OD_{550\text{ nm}} \text{ of each treatment} / OD_{550\text{ nm}} \text{ of the control cells}) \times 100$.

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 (CT) with either of the three different dilutions of LGG for 24 h. The absorbance at 550 nm was read after MTT test was performed. The percent viability of the cells was then calculated as: $(OD_{550\text{ nm}} \text{ of each treatment} / OD_{550\text{ nm}} \text{ of the control cells}) \times 100$.

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-toxicogenic *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 expression levels of toxin A (A) and toxin B (B).Co-incubation of the toxigenic *C. difficile* strain (CT) with the probiotic increased the Ct values of *TcdA* and *TcdB* significantly. The increase in the Ct values indicated the inhibition effect of the probiotic on the expression of *C. difficile* toxins. The 1:1000 dilution of the probiotic did not affect the relative amount of *TcdA* in the co-cultures (A).