**MAIN TOPIC**

A. F. Mattar · R. A. Drongowski · A. G. Coran
C. M. Harmon

# Effect of probiotics on enterocyte bacterial translocation in vitro

**Abstract** Enteral probiotics such as *Lactobacillus casei GG* (LGG) have been used in the treatment of a variety of intestinal disorders in infants and children, including diarrhea, malabsorption, and *Clostridium difficile* colitis. We have previously demonstrated that the probiotic bacterium LGG has an inhibitory effect on bacterial translocation (BT) in a neonatal rabbit model. However, this in-vivo model is limited for investigating the cellular and molecular mechanisms responsible for probiotic inhibition of BT. The purpose of this study was to determine the efficacy of LGG in reducing the rate of *Escherichia coli C25* (*E. coli C25*) translocation using an in-vitro enterocyte cell-culture model. Human colonic carcinoma (Caco-2) enterocytes were seeded in porous filters in the apical chamber of a two-chamber cell-culture system and grown for 14 days to confluence. The monolayers were incubated at 37 °C with LGG for 180 min. Non-adherent LGG was washed away prior to a 120-min incubation period with $10^5$ CFU *E. coli C25*. *E. coli* that had translocated across the enterocyte monolayer were quantified by growing basal-chamber media samples on gram-negative bacteria-specific Mac-Conkey's agar. In order to determine monolayer integrity, transepithelial electrical resistance (TEER) was measured across Caco-2 cells treated with LGG and *E. coli*. Statistical analysis was by ANOVA with $P < 0.05$ considered significant. LGG inhibited *E. coli* translocation at all LGG concentrations tested. The TEER ratio was not significantly altered by addition of LGG or *E. coli* ($0.9 \pm 0.03$ vs $0.8 \pm 0.05$). These results demonstrate that the probiotic bacterium LGG inhibits BT of *E. coli C25* in a dose-dependent manner in an in-vitro cell-culture model. This model should be valuable in investigating the cellular and molecular mechanisms involved in the inhibition of pathological enteral bacteria by probiotic agents.

**Keywords** Bacterial translocation · Probiotics · *Lactobacillus casei* · Caco-2 cell

A. F. Mattar · R. A. Drongowski · A. G. Coran
C. M. Harmon (✉)
Section of Pediatric Surgery,
University of Michigan,
Mott Children's Hospital,
Ann Arbor, MI 48109-0245,
USA

## Introduction

More than 20 years ago, Fuller defined probiotics as: "A live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance" [8, 9]. Recently, probiotic organisms have been advocated for stabilizing the gut microflora in conditions where disturbances of the normal bacterial flora result in a wide range of gastrointestinal symptoms [2, 8, 9, 13]. *Lactobacillus casei Rhamnosus* (Lactobacillus GG, LGG) has been shown to be beneficial in the treatment of antibiotic-induced diarrheal disease, diarrhea, malabsorption, *Clostridium difficile* colitis, inflammatory bowel disease, *Helicobacter pylori* gastroenteritis, lactose intolerance, and irritable bowel syndrome [1, 2, 7, 8, 10, 14, 15, 20].

It has been reported that LGG adheres to cultured enterocytes and has an inhibitory effect on the pathogenesis of several bacteria like *Salmonella typhimurium*, diarrheogenic *Escherichia coli*, *Campylobacter jejuni*, and *Clostridium difficile* [1, 7, 11, 14, 15, 19, 20]. The ability to adhere to human colonic carcinoma (Caco-2) enterocytes in culture has provided one explanation for the ability of LGG to colonize the intestine [3–6]. However, the exact mechanism(s) by which LGG might inhibit pathogenic bacteria is unknown. We have previously demonstrated that LGG has an inhibitory effect on bacterial translocation (BT) in a neonatal rabbit model [16]. The aim of this study was to determine the efficacy of LGG in reducing the degree of *E. coli C25* translocation using an in-vitro enterocyte cell-culture model and to study possible mechanisms of this inhibition.

## Materials and methods

*E. coli C25*, a mild to moderately virulent, streptomycin-resistant strain originally isolated from a neonate with necrotizing entero-colitis, was kindly provided by Dr. Henri R. Ford (Pittsburgh, PA). The organisms were grown overnight in brain–heart infusion medium (BBL, Cockeysville, MD), washed two times with phosphate-buffered saline (PBS), and resuspended in Dulbecco's modified Eagle's medium (DMEM) (Fisher Scientific, Pittsburgh, PA) at a concentration of $10^5$ colony-forming units (CFU)/ml. The initial concentration of the bacteria was determined by spectrophotometry at 650 nm and the numbers of bacteria were verified by pour-plate assay using MacConkey's agar (Difco, Detroit, MI) and standard serial dilution techniques. LGG (ATCC 53103) obtained from the American Type Culture Collection (ATCC, Manassas, VA) was grown overnight in Ragosa SL broth (Difco), washed two times with PBS, and resuspended in DMEM at a concentration of $10^6$ CFU/ml. The initial concentration of LGG was determined by spectrophotometry at 600 nm and the numbers were verified by pour-plate assay as mentioned above.

Caco-2 cells were obtained from the American Type Culture Collection (No. HTB 37). Cell passages 25–37 were grown in DMEM supplemented with 10% fetal bovine serum, 1% non-essential amino-acid solution, 1% sodium pyruvate, penicillin G (100 IU/ml), and streptomycin (100 µg/ml) in a 5% $CO_2$ atmosphere at 37 °C. After the cells were grown to 70% confluence, they were harvested following trypsinization with trypsin-EDTA (Gibco, Grand Island, NY), washed, and resuspended in DMEM. The cells were seeded at a density of $1 \times 10^5$ per well into collagen-coated porous filters in the apical chamber of a two-chamber cell-culture system. Collagen coating of the plates was accomplished by incubating in 30 µl 1.0 mg/ml rat-tail type I collagen (Sigma, St Louis). The cells were grown for 14 days to confluence and full differentiation. Media were changed every other day. The formation of an intact, confluent monolayer of Caco-2 cells was monitored by measuring the transepithelial electrical resistance (TEER) with a Millicell electrical resistance system (Millipore EVOM-6: World Precision Instruments, Sarasota, FL). TEER reflects the integrity of the monolayer; as TEER increases, the tight junctions between the cells become stronger and the permeability of the monolayer decreases.

Caco-2 monolayers were washed three times with DMEM without fetal bovine serum or antibiotic supplements and "pre-TEER" was measured. The Caco-2 cells were then incubated at 37 °C for 180 min with $10^6$ CFU LGG for 180 min, followed by the addition of $10^5$ CFU *E. coli C25* for 120 min. The inner chamber containing the monolayer was then transferred to a new outer chamber and new medium was added to both the apical and basal chambers; post-BT TEER was then measured.

To evaluate the inhibitory effects of different LGG doses, the Caco-2 monolayers were pre-incubated at 37 °C with LGG at 0, $10^4$, $10^5$, $10^6$, $10^7$, and $10^8$ CFU/ml for 180 min. To remove the non-adherent LGG, the enterocyte monolayers were washed three times with PBS (the washes were cultured to rule out the presence of any free LGG) prior to a 120-min incubation period with $10^5$ CFU *E. coli C25*. The inner chamber containing the monolayer was then transferred to a new chamber and new medium was added to both apical and basal chambers, then post-BT TEER was measured.

During the experiment the cell-culture wells were maintained in a 5% $CO_2$ incubator at 37 °C. The integrity of the Caco-2 monolayers was monitored by measuring the TEER of each 12-mm transwell. In order to calculate the resistance, the blank-well TEER was subtracted from the subsequent TEER measurements. The *E. coli* that had translocated across the enterocyte monolayer were quantified by growing basal-chamber media samples on gram-negative bacteria-specific MacConkey's agar using standard serial dilution techniques. Colony counts were determined and statistical analysis between treatment groups was by ANOVA with $P$ below 0.5 considered significant.

## Results

### Effect of LGG on *E. coli* BT

*E. coli C25* BT in the standard Caco-2 cell in-vitro system was significantly inhibited across monolayers treated with LGG ($2.2 \pm 0.04$ log 10 CFU/ml) versus controls ($4.01 \pm 0.13$ log 10 CFU/ml, $P < 0.05$) (Fig. 1). This experiment demonstrates that LGG can inhibit BT when both organisms are simultaneously applied to the apical side of the transwell Caco-2 cell monolayer system, mimicking the in-vivo scenario of the gut lumen.

### Possible mechanism of LGG inhibition of BT

In order to determine whether LGG inhibition of BT in our in-vitro enterocyte model required direct LGG–*E. coli* interaction, LGG was washed away from the surface of the Caco-2 monolayer prior to the addition of *E. coli*. First, control experiments were conducted to determine the number of washes required to insure that LGG was adequately removed from the incubation media prior to adding *E. coli*. It was determined that three serial washes were required (Fig. 2). Based on this experiment, to remove all unbound LGG from the apical chamber three washes were used in the subsequent experiments. A significant inhibitory effect of LGG on *E. coli* translocation was observed after removal of un-bound LGG ($1.16 \pm 0.03$ log 10 CFU/ml) versus controls ($3.37 \pm 0.4$ log 10 CFU/ml; $P < 0.01$) (Fig. 3). The result of this experiment suggests that LGG acts on the enterocyte in order to inhibit BT.

### Dose-dependent effect of LGG inhibition of BT

*E. coli* translocation was inhibited by LGG in a dose-dependent manner as follows: 21.7% for $10^4$ LGG, 28.8% for $10^5$ LGG, 36.6% for $10^6$ LGG, and 50.2% for
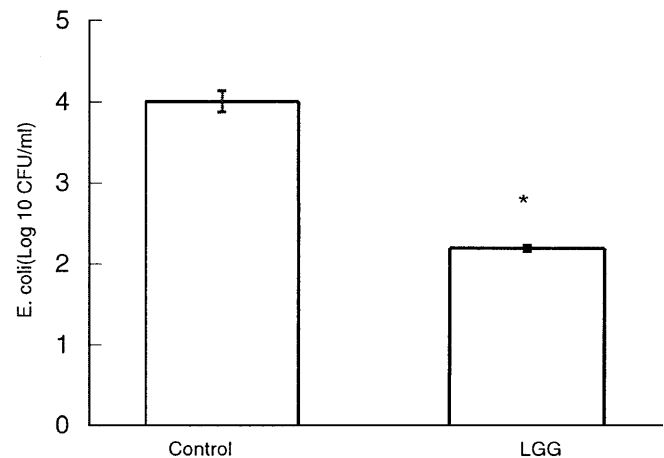
**Fig. 1** *E. coli C25* translocation through Caco-2 enterocyte monolayers challenged with LGG and *E. coli* or *E. coli* alone. *$P < 0.05$
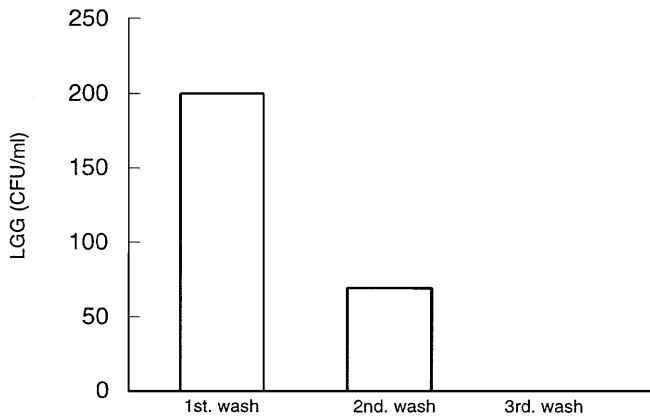
Fig. 2 Mean LGG concentrations (expressed as CFU/ml) in samples cultured from three consecutive serial washings
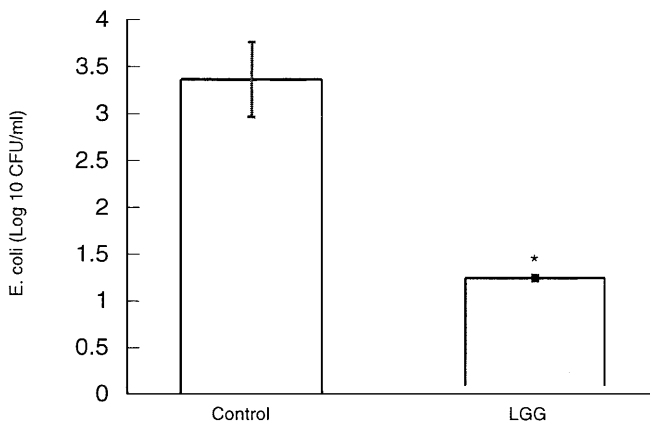


Fig. 3 *E. coli C25* translocation through Caco-2 enterocyte monolayers challenged with LGG and *E. coli* or *E. coli* alone after washing unbound LGG from apical chamber. *$P$ < 0.01

$10^7$ LGG concentrations. The highest concentration of LGG tested ($10^8$) resulted in complete inhibition (100%) of *E. coli* (Fig. 4).
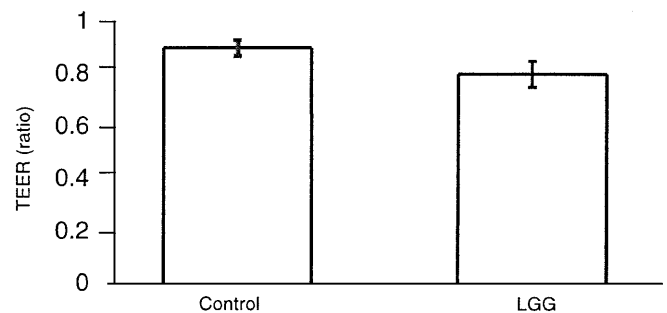
### Effect of LGG on transepithelial electrical resistance

The TEER ratio was measured before three serial washings (pre-TEER to remove unbound LGG and after addition and incubation of *E. coli* (post-TEER). TEER allows the assessment of monolayer integrity and tight-junction function. The mean TEER ratio (post-TEER/pre-TEER) across Caco-2 monolayers treated with *E. coli* alone (control) was $0.9 \pm 0.03$ versus $0.8 \pm 0.05$ for LGG (plus *E. coli*)-treated monolayers. These data indicate that serial washings as well as the addition of LGG had no significant effect on nonspecific monolayer permeability or integrity (Fig. 5).

### Discussion

Probiotics are defined as live organisms that, when ingested, result in health benefits including amelioration
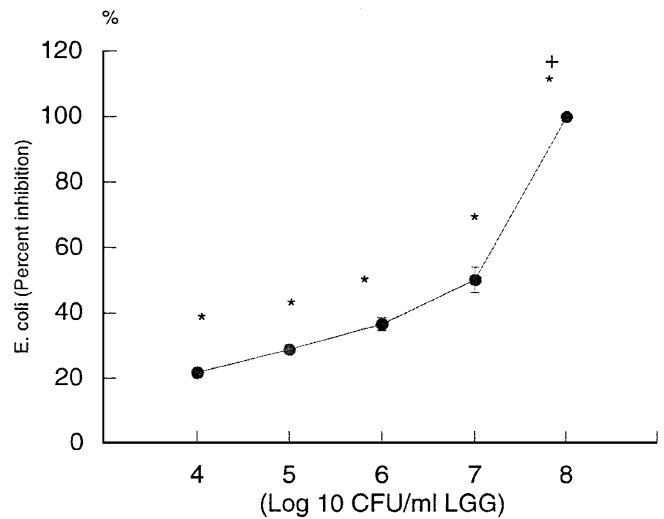


Fig. 4 *E. coli C25* translocation expressed as percent inhibition within Caco-2 enterocyte monolayers following dose-dependent LGG treatment. *$P$ < 0.05



Fig. 5 Mean transepithelial electrical resistance (TEER) ratios before three serial washings (pre-TEER) to remove unbound LGG and after addition and incubation of *E. coli* (post-TEER)

or prevention of a specific disease state. LGG has been used in vivo to treat a variety of conditions, including antibiotic-induced diarrhea, diarrhea, malabsorption, *C. difficile* colitis, inflammatory bowel disease, *H. pylori* gastroenteritis, lactose intolerance, and irritable bowel syndrome [10, 16, 18–20].

A number of mechanisms have been described that promote the health benefits of probiotics. For example, these organisms are known to enhance the intestinal microflora by replenishing suppressed bacteria and inhibiting the growth of pathogenic flora [8, 21, 22]. In addition, some probiotics, including LGG, actively secrete anti-microbial substances that have been shown to inhibit the growth of other organisms such as *S. typhimurium*, diarrheogenic *E. coli*, *C. jejuni*, and *C. difficile* [1, 7, 14, 15, 19, 20].

In this study, we describe an in-vitro enterocyte cell-culture model that has allowed investigation of the effects of LGG on *E. coli* translocation. We found that the addition of LGG to the enterocyte monolayer surface result in significant inhibition of BT. These results suggest that the inhibitory effect of LGG on BT is not a consequence of a generalized increase in tight-junction

permeability. However, our results do provide insight into possible mechanisms by which LGG might inhibit *E. coli* translocation. The inhibitor effect of LGG was not abolished by removing pre-incubated LGG prior to testing for BT. This indicates that there is a direct effect of LGG on enterocytes that persists after removal of LGG and results in diminished passage of *E. coli* across the epithelial monolayer.

LGG could bind to enterocyte cell-surface receptors (and not be removed by washing), thus competing with *E. coli* enterocyte attachment. Another possible mechanism is that LGG signals the enterocyte through direct binding or secreted substances to up-regulate epithelial barrier mechanisms. In our study, LGG did not significantly increase TEER, arguing against this as a possible mechanism of BT inhibition. Mack et al. have recently reported that LGG can inhibit BT in an HT-29 intestinal epithelial cell-culture model by up-regulating expression of several mucin molecules that are components of the epithelial barrier [12, 17]. It is possible that a similar mechanism is involved in the inhibition of *E. coli* translocation by LGG demonstrated in our current study.

In conclusion, we have shown that the probiotic LGG inhibits the translocation of *E. coli C25* across an enterocyte monolayer in vitro. This effect appears to be secondary to an interaction between probiotic and enterocyte. This model should prove helpful in further defining mechanisms by which probiotics such as LGG inhibit translocation of potentially pathogenic enteral micoorganisms that could contribute to sepsis.

## References

1. Aiba Y, Suzuki N, Kabir AM, Takagi A, Koga Y (1998) Lactic acid-mediated suppression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. Am J Gastroenterol 93: 2097–2101
2. Arvola T, Laiho K, Torkkeli S, Mykkänen H, Salminen S, Maunula L, Isolauri E (1999) Prophylactic *Lactobacillus GG* reduces antibiotic-associated diarrhea in children with respiratory infections: a randomized study. Pediatrics 104: 64
3. Barnet MF, Brassart D, Neeser JR, Servin AL (1994) *Lactobacillus acidophilus* LA 1 binds cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. Gut 35: 483–489
4. Charteris WP, Kelly PM, Morelli L, Collins JK (1997) Selective detection, enumeration and identification of potentially probi-
otic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. Int J Food Microbiol 35: 1–27
5. Chauviere G, Coconnier MH, Kerneis S, Fourniat J, Servin AL (1992) Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. J Gen Microbiol 138: 1689–1696
6. Darfeuille-Michaud A, Aubel D, Chauviere G, Rich C, Bourges M, Servin A, Joly B (1990) Adhesion of enterotoxigenic *Escherichia coli* to the human colon carcinoma cell line Caco-2 in culture. Infect Immun 58: 893–902
7. Fekety R, Shah AB (1993) Diagnosis and treatment of *Clostridium difficile* colitis. JAMA 269: 71–75
8. Fuller R (1989) Probiotics in man and animals. J Appl Bacteriol 66: 365–378
9. Fuller R (1990) Probiotics in human medicine. Ann Med 22: 37–41
10. Gibson GR, Roberfroid MD (1994) Dietary modulation of the human colonic microbiota: including the concept of probiotics. J Nutr 125: 1401–1412
11. Goldin BR, Gorbach SL, Saxelin M, Barakat S, Gualtieri L, Salminen S (1992) Survival of *Lactobacillus species* (strain GG) in human gastrointestinal tract. Dig Dis Sci 37: 121–125
12. Gork AS, Usui N, Ceriati E, Drongowski RA, Epitein M, Coran AG, Harmon CM (1999) The effect of mucin on bacterial translocation in I-407 fetal and Caco-2 adult enterocyte cultured cell line. Pediatr Surg Int 15: 155–159
13. Hamilton-Miller JMT, Shah S, Smith CT (1996) "Probiotic" remedies are not what they seem. Br Med J 312: 55–56
14. Kops SK, Lowe DK, Bement WM, West AB (1996) Migration of *Salmonella typhi* through intestinal epithelial monolayers: an in vitro study. Microbiol Immunol 40: 799–811
15. Kruis W, Schutz E, Fric P, Fixa B, Judmaier G, Stolte M (1997) Double-blind comparison of an oral *Escherichia coli* preparation and mesalazine in maintaining remission of ulcerative colitis. Aliment Pharmacol Ther 11: 853–858
16. Lee DJ, Drongowski RA, Coran AG, Harmon CM (2000) Evaluation of probiotic treatment in neonatal animal model. Pediatric Surg Int 16: 237–242
17. Mack DR, Michail S, Wei S, McDougall L, Hollingsworth MA (1999) Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. AJP 276: G941–G950
18. Majamaa H, Isolauri E, Saxelin M, Vesikari T (1995) Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. J Pediatr Gastroenterol Nutr 20: 333–338
19. McFarland LV, Mulligan ME, Kwok RY, Stamm WE (1989) Nosocomial acquisition of *Clostridium difficile* infection. N Engl J Med 320: 204–210
20. Meurman JH, Antila H, Korhonen A, Salminen S (1995) Effect of *Lactobacillus rhamnosus* strain GG (ATCC 51303) on the growth of *Streptococcus dobrinus* in vitro. Eur J Oral Sci 103: 253–258
21. Parker RB (1974) Probiotics, the other half of the antibiotic story. Anim Nutr Health 29: 4–8
22. Salminen S, Deighton M (1992) Lactic acid bacteria in the gut in normal and disordered states. Dig Dis Sci 10: 227–238