

## MAIN TOPIC

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## Evaluation of probiotic treatment in a neonatal animal model

**Abstract** The clinical use of probiotic agents such as enteral *Lactobacillus* to enhance intestinal defense against potential luminal pathogens has been tested in vivo; however, an understanding of the mechanisms responsible for the observed protection is lacking. The purpose of this study was to evaluate the effects of *Lactobacillus* on bacterial translocation (BT) in a neonatal animal model. Newborn New Zealand white rabbit pups were enterally fed a 10% Formulac solution inoculated with or without a  $10^8$  suspension of ampicillin-resistant *Escherichia coli* K1 (*E. coli* K1A) and/or *Lactobacillus casei* GG (*Lacto* GG). Pups received either no bacteria ( $n = 10$ ), *Lacto* GG ( $n = 8$ ), *E. coli* K1A ( $n = 26$ ), or a combination of *Lacto* GG and *E. coli* K1A ( $n = 33$ ). On day 3, representative tissue specimens from the mesenteric lymph nodes (MLN), spleen (SPL), and liver (LIV) were aseptically harvested in addition to a small-bowel (SB) sample that was rinsed to remove luminal contents. The specimens were then cultured in organism-specific media. Statistical analysis was by one-way ANOVA with  $P$  values less than 0.05 considered significant. Neonatal rabbits receiving *Lacto* GG-supplemented formula exhibited a 25% decrease ( $P < 0.05$ ) in small-bowel colonization by *E. coli* K1A. In addition, *Lacto* GG decreased the frequency of extraintestinal BT by 46% ( $P < 0.05$ ), 61% ( $P < 0.05$ ), and 23%, respectively, in the MLN, SPL, and LIV. We have shown that enterally-administered *Lacto* GG decreases the frequency of *E. coli* K1A translocation in a neonatal rabbit model. These results may have significant implications for the treatment of BT and sepsis in the human neonate and provide a model for further studies.

**Key words** Bacterial translocation · Probiotics · Neonate

### Introduction

Disruption of the gut mucosal barrier (GMB) can occur as a result of various forms of stress and trauma, including infection, hypoxia, starvation, malnutrition, and postsurgical stress. Because the intestinal lumen houses large numbers of indigenous bacteria, during these times of barrier dysfunction opportunistic pathogens may penetrate the impaired GMB and disseminate to extraintestinal sites through a process called bacterial translocation (BT) [41]. It is believed that BT, defined as the passage of viable or nonviable enteric bacteria and their by-products across the intestinal barrier [6], is the cause of sepsis and various bacterial diseases in cases in which there is no other identifiable focus of infection [9]. BT is especially problematic in the hospital setting among the neonatal population, who are considered to be at increased risk of infection by enteric bacteria because of their compromised immune system and incompletely developed GMB. Findings confirming the correlation between age and frequency of BT support this concern [37]. Sepsis has been suspected to contribute to the onset of gastroenteritis, necrotizing enterocolitis, Crohn's disease, and other gastrointestinal (GI) disorders [9, 15, 24].

Preserving the gut's protective ability against BT involves maintaining a physiologically normal intestinal microflora balance, gut and immunological function, and a healthy intestinal epithelium and mucosa [41]. Various treatment strategies have been proposed to help prevent the occurrence of BT, most of which involve a modification of one or more of these aspects. One such treatment involves the oral administration of probiotic bacteria, or probiotics, which are defined as viable bacteria that have a beneficial effect on the health of the host [30]. It has been suggested that these bacteria, when enterally administered, possess the ability to normalize

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altered GMB function. Probiotics are proposed to work through a number of different mechanisms including intestinal microflora modification, antagonistic activity against potentially pathogenic bacteria, stimulation of the immune system, and sustenance provision for the intestinal mucosa [4].

Clinical studies have suggested therapeutic effects of probiotic administration in infants and children [15, 19, 23, 24, 29, 34, 38]. Investigations utilizing animal models have also provided evidence for a probiotic capacity to stabilize the GMB [1, 10, 17, 25, 39]; however, adult animal models have been primarily utilized in these studies, with little focus on neonates. Wagner et al. assessed the growth and mortality of mice born to dams colonized with probiotic bacteria, but their results were inconclusive [39, 40]. Due to the basic differences that exist between adult and neonatal gut physiology, it is important to establish a neonatal model by which the efficacy and mechanisms of probiotic treatment in an immature gut can be further studied.

In this study, we investigated the effects of *Lactobacillus* probiotic treatment in a neonatal animal model. More specifically, we focused on the *Lactobacillus casei* GG (*Lacto* GG) strain and its enteric effect on BT of the exogenous pathogen *Escherichia coli* K1 (*E. coli* K1) in neonatal rabbit pups. Through a method similar to one developed by Kazantsev et al. [22], the ampicillin-sensitive *E. coli* K1 strain was transformed with a plasmid encoding for ampicillin resistance, allowing for a convenient method to isolate and identify the specific pathogen of interest (*E. coli* K1A). The therapeutic efficacy of *Lacto* GG has been extensively studied, and its strong candidacy as a probiotic has been well established. We hypothesized that *Lacto* GG would act as an antagonist against the transformed *E. coli* K1 in this neonatal model, and that this would be evident in the inhibition of the colonization and BT by the pathogen.

## Materials and methods

Formulac was obtained from the Unit for Laboratory Animal Medicine of the University of Michigan Medical Center. The pGEM-7Zf(+) plasmid DNA (pGEM-7, 1 µg/µl) was obtained from Promega (Madison, WI). *E. coli* K1 (ATCC 23503) and *Lacto* GG (ATCC 53103) were obtained from the American Type Culture Collection (Manassas, VA). Formamide and LB broth base were obtained from Gibco BRL (Grand Island, NY). The Select Agar used in combination with the LB broth base to make LB agar was obtained from Sigma (St. Louis, MO). Rogosa SL broth and agar were obtained from VWR Scientific Products (Detroit, MI). 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was obtained from Fisher Scientific (Pittsburgh, PA). Ampicillin was

obtained from the University of Michigan Medical Center (Ann Arbor, MI). A stock solution of X-Gal dissolved in formamide was prepared (40 mg/ml) and 1 ml of this solution was supplemented into 1 l LB agar medium so that the final concentration of X-Gal in the LB agar plates was 40 µg/ml.

On the day of delivery, newborn New Zealand white rabbits pups were separated from their mothers, placed in an Isolette infant incubator maintained at 28 °C, and given orogastric gavage feedings of 2 ml 10% Formulac twice daily via a 5 Fr premature infant feeding tube. The guidelines stated in NIH Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) were followed.

*E. coli* K1 were transformed with a non-transferable, 3,000 base-pair pGEM-7 plasmid encoding for ampicillin resistance. Non-transformed *E. coli* K1 displayed ampicillin sensitivity. Transformation was performed using the calcium chloride/heat shock method developed by Cohen et al. [8]. Briefly, *E. coli* K1 were rinsed twice with ice-cold 0.1 M CaCl<sub>2</sub> and kept on ice in order to make them competent for transformation. The pGEM-7 plasmid was then added to the competent bacteria, placed on ice for 30 min, heat shocked (42 °C) for 45 s to allow pGEM-7 transformation, and then placed back on ice for 2 min. The bacteria were then incubated in SOC medium for a 1 h recovery period, plated on LB agar plates supplemented with ampicillin (100 µg/ml) and X-Gal (40 µg/ml), and incubated for 24 h at 37 °C. The X-Gal in the LB agar serves as a substrate for the lacZ alpha-peptide, also encoded by the pGEM-7 plasmid. Through the expression of this gene, successfully transformed *E. coli* K1 appeared as blue colonies on the X-Gal-supplemented LB agar plates. Several blue colonies of transformed *E. coli* K1 were randomly selected using a flame-sterilized inoculating loop and used to start stock cultures that were grown in LB broth supplemented with ampicillin (100 µg/ml) at 37 °C. Transformed ampicillin-resistant *E. coli* K1 (*E. coli* K1A) served as our pathogen in the current model.

According to American Type Culture Collection (ATCC), a 5% CO<sub>2</sub> environment is the ideal culturing condition for *Lacto* GG, but we found that growth in the Rogosa broth was not inhibited by aerobic conditions. Stock cultures of *Lacto* GG were grown aerobically in a *Lactobacillus*-selective Rogosa broth at 37 °C and served as the probiotic.

Seventy-seven rabbit pups were randomly separated into four groups based on their respective feeding regimens: (1) controls (n = 10); (2) LGG (n = 8); (3) K1A (n = 26); and (4) LGG+K1A (n = 33). For the LGG, K1A, and LGG+K1A groups, the 10% Formulac solution was inoculated with a suspension of *Lacto* GG and/or a suspension of *E. coli* K1A depending on the group being fed (Table 1). Feedings were administered twice daily for 2 days. Each pup in these groups received 10<sup>8</sup> *Lacto* GG and/or 10<sup>8</sup> *E. coli* K1A per feeding. Rabbit pups were returned to the infant incubator (28 °C) between feedings, and were not allowed access to additional food or water.

On day 3, the pups were anesthetized with a subcutaneous dose of acepromazine (1 mg/kg) followed by an intramuscular dose of ketamine HCl (50 mg/kg). In preparation for tissue extraction, the anterior abdominal wall was prepped with betadine solution and a sterile laparotomy was performed. Representative tissue specimens were aseptically harvested from the mesenteric lymph nodes (MLN), spleen (SPL), liver (LIV), and jejunum of the small-bowel (SB), in this sequence, using a different set of sterile instruments for each tissue to minimize the possibility of bacterial contamination between samples. The small-bowel lumen was aseptically irrigated with 2 ml sterile saline prior to culturing in order to clear the luminal contents.

**Table 1** Inoculation of bacteria into feeding solutions

Group	Feeding 1	Feeding 2	Feeding 3	Feeding 4
1 Control	No bacteria	No bacteria	No bacteria	No bacteria
2 LGG	<i>Lactobacillus</i> GG	<i>Lactobacillus</i> GG	<i>Lactobacillus</i> GG	<i>Lactobacillus</i> GG
3 K1A	No bacteria	No bacteria	<i>E. coli</i> K1A	<i>E. coli</i> K1A
4 LGG + K1A	<i>Lactobacillus</i> GG	<i>Lactobacillus</i> GG	<i>Lactobacillus</i> GG + <i>E. coli</i> K1A	<i>Lactobacillus</i> GG + <i>E. coli</i> K1A

Tissue specimens were divided into two samples and aerobically incubated for 48 h at 37 °C in 3 ml LB broth supplemented with ampicillin (100 µg/ml) and 3 ml Rogosa broth. Due to the small size of the MLN, the specimen was homogenized in 0.5 ml sterile saline and then 0.2 ml homogenate was inoculated into both selective broths. After 48 h, broth aliquot samples were aseptically streaked onto selective agar plates and incubated for 24 h at 37 °C. Unlike the Rogosa broth, the Rogosa agar plates were incubated in an anaerobic chamber due to the poor ability of *Lacto* GG to grow on these plates in an aerobic environment. Detection of viable *E. coli* K1A was determined by the growth of blue streaks or blue colonies on the LB agar plates and detection of viable *Lacto* GG by any white, opaque streaks or colonies on the Rogosa agar plates. Data were recorded as either presence or absence of either bacterium in each tissue sample. Final frequencies of *E. coli* K1A and *Lacto* GG growth were calculated for the MLN, SPL, LIV, and SB data. The presence of either bacterium in the MLN, SPL, or LIV was interpreted as BT of that bacterium to those sites. The presence of either bacterium in the SB was interpreted as colonization by that bacterium.

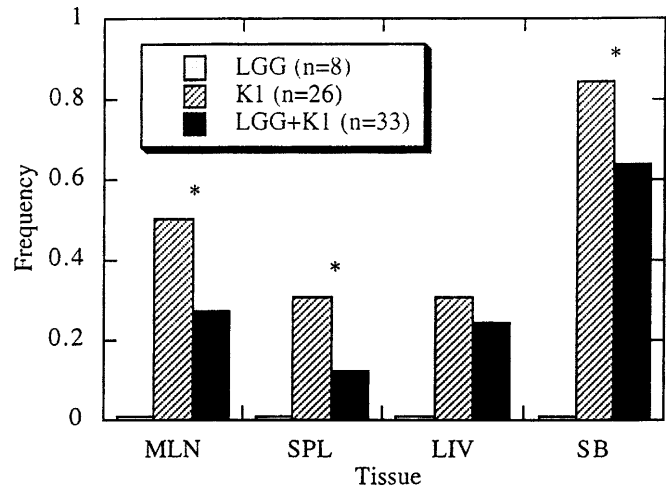
Representative samples of SB from all groups were stored in a 37% formaldehyde buffer solution and later subjected to hematoxylin and eosin (H&E) staining and light-microscopy analysis by a pathologist. The data were analyzed using one-way analysis of variance with Sheffe's post-hoc testing for multiple comparisons between groups. *P* values less than 0.05 were considered significant.

## Results

Although newborns are initially germ-free, the rabbit pups used in this study were not kept in a germ-free environment, and so establishing the selectivity of our model and methodology for the pathogen and probiotic was critical. No growth of *E. coli* K1A was detected in the MLN, SPL, LIV, and SB in the control or LGG group. Since both of these groups were not administered *E. coli* K1A, they served as negative controls with results establishing that the pathogen was not indigenous to the neonatal rabbit, and confirming the selectivity of the model for the pathogen. This enabled us to conclude with confidence that any *E. coli* K1A present in the tissues originated from the exogenous supply in the feedings, which allowed us to focus specifically on colonization and BT by this bacterium.

Rogosa broth and agar are only selective for *Lactobacillus* sp., not specifically for *Lacto* GG. However, no growth of any *Lactobacillus* sp. was detected in the MLN, SPL, LIV, and SB of the control and K1A groups. Neither group was administered *Lacto* GG, indicating that there were no lactobacilli indigenous to the neonatal rabbit, and hence, the selectivity of the model for the probiotic was confirmed. Observation of colonization and BT *Lacto* GG through detection of its presence in the tissue specimens could, therefore, also be done with confidence.

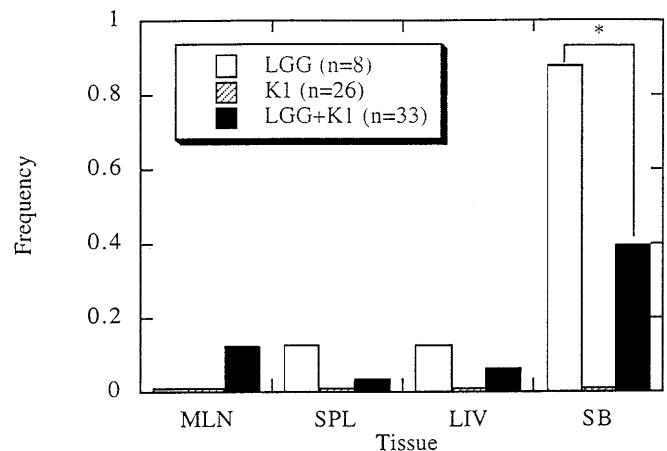
There was a 25% decrease in the frequency of *E. coli* K1A colonization of the SB (0.85 to 0.64,  $P < 0.05$ ) between the K1A and LGG + K1A groups (Fig. 1). The frequency of *E. coli* K1A translocation to the MLN decreased by 46% (0.50 to 0.37,  $P < 0.05$ ) between the K1A and LGG + K1A groups. BT to the SPL decreased by 61% (0.31 to 0.12,  $P < 0.05$ ) between the K1A and LGG + K1A groups. The LIV exhibited a



**Fig. 1** Frequency of *E. coli* K1A presence calculated for mesenteric lymph nodes (MLN), spleen (SPL), liver (LIV), and small bowel (SB). LGG pups displayed no presence of bacteria, while LGG + K1A pups exhibited decreased frequency in all tissues compared to K1A pups  
\* $P < 0.05$

23% decrease in BT frequency between these two groups (0.31 to 0.24), but the decrease was not statistically significant.

The frequency of *Lacto* GG colonization of the SB decreased by 56% comparing the LGG and LGG + K1A groups (0.88 to 0.39,  $P < 0.05$ , Fig. 2). No significant differences were detected in the frequency of *Lacto* GG BT to the extraintestinal sites between the LGG and LGG + K1A groups. Therefore, *Lacto* GG translocation to the MLN, SPL, and LIV did not seem to be affected by the presence of *E. coli* K1A, but was detected at low frequencies in both the LGG and LGG + K1A groups. In the LGG group, none of the



**Fig. 2** Frequency of *Lactobacillus* GG presence calculated for mesenteric lymph nodes (MLN), spleen (SPL), liver (LIV), and small bowel (SB). K1A pups displayed no bacteria, LGG + K1A pups exhibited decreased SB frequency compared to K1A pups, both groups exhibited low frequencies in the MLN, SPL, and LIV  
\* $P < 0.05$

pups showed signs of probiotic translocation to the MLN, while 1 of the 8 pups exhibited BT to the SPL and LIV (0.13). In the LGG + K1A groups, the frequency of *Lacto* GG BT to the MLN, SPL, and LIV was detected at 0.12, 0.03, and 0.06, respectively. Histologic examination of the SB (H&E) did not show any dissimilarities between the groups observable through light microscopy. Mucosal damage was not detected in the SB of any of the groups (data not shown).

Rabbit pups in any one group did not show clinical signs of being more healthy or ill than pups of any other group. A low mortality of 7% was observed across all groups. No group exhibited significantly higher mortality than the others (data not shown). Mortality seemed attributable to congenital factors of poor health or injuries suffered during gavage feedings rather than to pathogenesis by enteric bacteria. Rabbit pups who either died before tissue harvest or displayed bleeding of the esophageal lining due to excessive trauma during gavage feedings (2%), as evidenced by the presence of regurgitated blood in the mouth, were excluded from the data.

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## Discussion

It is believed that the GI tract of the neonate is physiologically incomplete in its development at the time of birth compared to that of the adult. Evidence for this immaturity was indicated by a study performed in our laboratory demonstrating that the neonatal rabbit was prone to a higher incidence of BT than the adult rabbit [37]. Neonates display a propensity toward immature immune function [16, 34], microfloral ecology [5], gut function (motility, gastric acid secretion, proteolytic activity) [18, 33, 35], mucosa and mucosal gel layer development [13, 28, 31], and intestinal epithelial development [31]. Since the normal neonate exhibits several of the factors that have been postulated to promote BT in the adult [9], it is not unreasonable to speculate that probiotics may provide similar benefits for the neonate.

This assertion has been suggested by various clinical studies involving infants and children with GI disorders [15, 19, 23, 24, 29, 36, 38]. Animal studies have also been performed in an attempt to identify proficient probiotic strains and ascertain the effects and mechanisms of probiotic treatment [1, 10, 17, 25, 39]. Most of these studies, however, utilized adult models with little attention given to probiotic treatment in a neonatal model. While studying the protective capacities of different probiotic strains in immunodeficient adult mice, Wagner et al. also assessed the effect on mice born to dams colonized with probiotic bacteria [39]. Specifically, they examined the growth and mortality of the mice at 4 and 8 to 12 weeks after delivery, but their results were inconclusive and no analysis of the probiotic effects on the GMB was performed. It seems that, due to the basic physiological differences in the GI tract of neonates and adults, a neonatal model must be established in which probiotics can be further studied. The results obtained in

the current study support the candidacy of the proposed neonatal rabbit model.

We evaluated the ability of the probiotic *Lacto* GG to inhibit BT and dissemination of *E. coli* K1A in a neonatal rabbit model. The human *Lacto* GG strain has exhibited characteristics that make it a good candidate for probiotic treatment: *Lacto* GG has been shown to survive in the GI tract of different animal models and humans [14, 17, 20], adhere to the human Caco-2 intestinal cell line [11], produce an antimicrobial substance [32], and has generally had a good safety record [29]. Use of this strain in clinical and laboratory studies has produced results verifying its probiotic ability. The *E. coli* K1 strain that was transformed with the pGEM-7 plasmid was isolated from human infants and has been known to be highly virulent in newborns, causing meningitis and bacteremia [12]. Transforming the *E. coli* K1 with the plasmid enabled us to easily isolate and identify the pathogen from the tissue samples. Further identification of *E. coli* K1A can be performed through plasmid DNA analysis, as was done in a previous study performed in our laboratory [26].

The finding of principal interest in this study was that *Lacto* GG inhibited the colonization of *E. coli* K1A and its translocation to extraintestinal sites. In previous studies, the incidences of BT and dissemination were used as indicators of the integrity of the GMB. Thus, our results imply that *Lacto* GG was able to stabilize the immature GMB, establishing its efficacy as a probiotic in the neonatal rabbit model. Histologic analysis of the small bowel did not show any mucosal damage inflicted by *E. coli* K1A, indicating that this was not a mechanism responsible for the translocation process in this animal. This is further supported by the observation that *Lacto* GG BT did not significantly increase in the LGG + K1A group. If the *E. coli* K1A were to cause mucosal damage, then one would expect to also see an increase in *Lacto* GG translocation. It seems that prevention of mucosal damage was not a mechanism by which *Lacto* GG inhibited BT of the pathogen in this model.

Colonization of the germ-free newborn GI tract normally does not begin until after exposure to the extrauterine environment. This immature microfloral ecology provides an opportunity for overgrowth of any single strain of bacteria in the gut, since it is able to proliferate without competition [5]. Hence, introduction of *E. coli* K1A or *Lacto* GG into the GI milieu would most likely lead to small-bowel colonization by these bacteria, as indicated by the high colonization frequency occurring in pups monoassociated with either *E. coli* K1A or *Lacto* GG in our data. However, the frequencies of colonization by *E. coli* K1A and *Lacto* GG declined in pups associated with both bacteria, possibly indicating luminal competition between the two bacteria and an established balance in the microfloral ecology.

As relatively nonpathogenic bacteria, probiotics have been shown to provide the resistance necessary to suppress the overgrowth of potentially pathogenic bacteria

in adult animals, presumably through competition for nutrients and adhesion sites along the GI lining [1, 17, 25, 39]. It has also been suggested that probiotics impede proliferation of potential pathogens through stimulation of the immune response and lymphocyte development [5, 7, 34, 39] and through the production of antimicrobial compounds and other inhibitory molecules [2, 27, 32], although it is questionable whether these substances are produced in vivo.

*Lactobacillus* sp. are considered relatively nonpathogenic bacteria in the general human population. In the current study, we observed a low frequency of BT by *Lacto* GG, less than 0.13, to extraintestinal sites. Although there was no apparent clinical distress among the pups infected with *Lacto* GG, evidence of any translocation of putative probiotic is a cause for concern, especially in immunodeficient hosts such as the neonate. In a study assessing the pathogenesis of probiotic bacteria in congenitally immunodeficient mice, Wagner et al. concluded that the probiotic species they tested were innocuous for adults but were associated with mortality and decreased growth rates in pups born to dams colonized with the bacteria [40]. These results, in conjunction with our own, suggest that further studies evaluating the safety of probiotic treatment in the neonate should be initiated.

Our results provide some evidence of the beneficial effects of probiotic administration in neonates and propose a neonatal animal model by which this treatment can be further studied. The *Lacto* GG strain used in this study appeared to display effects in the neonatal rabbit characteristic of an ideal probiotic; however, in previous studies, different strains have exhibited different probiotic capacities. The most promising probiotic strains that have emerged in clinical studies have been primarily lactic-acid strains, including *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, and *Bifidobacterium bifidum* [4]. The efficacy of these and other probiotic strains should be further tested in a neonatal model in order to identify those most suitable for probiotic therapy in newborns and infants. In addition, the use of prebiotics, substrates that are effective in modifying the microflora, can be evaluated as an indirect therapeutic approach capable of inducing the proliferation of indigenous probiotic bacteria within the GI tract [3, 21]. Future studies utilizing the neonatal model should provide insight into the mechanisms by which probiotics exert their beneficial effects.

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