

RESEARCH ARTICLE

Multistep mechanism of probiotic bacterium, the effect on innate immune system

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The etiology and pathogenesis of inflammatory bowel disease are still not fully understood. However, evidence from both animal models and clinical observations suggests luminal bacteria as the most probable inducer of this disease. The intestinal bacterial microbiota may be modified by dietary addition of viable probiotic bacteria, thereby constituting an alternative approach to disease prevention and treatment. The aim of this study was to evaluate and compare the effects of two probiotic regiments; *Lactobacillus GG* and a mixture of *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis* (YO-MIX™ Y 109 FRO 1000) in both normal and trinitrobenzenesulfonic acid colitis-induced rats. Colon morphology and damage were evaluated histologically; colonic tissues were used for mRNA analysis, using real-time PCR. Administration of both probiotics reduced the expression of proinflammatory cytokines tumor necrosis factor- α and IL-6 and increased the expression of mucin 2 in compared with colitis group and reduced the inflammatory response. These results provide additional support for the positive effect of probiotics in the gut and may shed light on the mechanism by which probiotic bacteria exert their action in an animal model.

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1 Introduction

There is mounting evidence from both animal models and clinical observations that luminal bacteria are the most probable inducers of inflammatory bowel disease (IBD) [1, 2]. Chronic IBD is believed to result from abnormal

immune responses to the enteric microbial environment. The precise identity of the bacterial stimuli that cause IBD remains unclear. However, the studies of experimental colitis in various animal models have shown the importance of the resident luminal flora in the initiation and perpetuation of intestinal inflammation [3, 4].

Over the last 10 years, focus has shifted to the dynamic balance between intestinal bacteria, particularly commensal flora, and host-defense mechanisms at the intestinal mucosa, and to their role in the initiation and maintenance of intestinal inflammation [5]. Increasing evidence suggests that some commensal bacteria enhance intestinal epithelial homeostasis and barrier integrity. Indeed, commensal bacteria regulate a number of host processes, including nutrition, development, and immune responses, that are relevant for both health and disease [6]. Several lines of evidence support the notion that dysregulation of the intestinal immune response to intestinal environmental

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Abbreviation: Ct, cycle threshold; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; IBD, inflammatory bowel disease; LGG, *Lactobacillus GG*; MUC, mucin; TLR, Toll-like receptor; TNBS, trinitrobenzenesulfonic acid; TNF- α , tumor necrosis factor- α ; Y 109, YO-MIX™ Y 109 FRO 1000

antigens, such as intestinal microbiota, is the main cause of intestinal inflammation [7, 8]. These studies imply that intestinal microbiota may play an important role in initiating and enabling colonic inflammation. Certain bacterial strains are more able to induce disease than others. The harmful role of some intestinal microorganisms has been established in murine models and is strongly suspected in humans [9]. However, other microorganisms seem to be protective [10].

IBD occurs more frequently in the terminal ileum and colon, which are the intestinal regions with the highest bacterial concentrations [11]. The studies have reported distinct characteristics that differentiate the microbiota colonizing the tract of patients with IBD from those in healthy people [12]; there is a high biodiversity of species in healthy subjects, whereas in IBD patients, biodiversity is lower [13]; human and animal studies have shown a significant decrease in the number of anaerobic bacteria and *Lactobacillus* in IBD groups compared with healthy subjects [14, 15].

Evidence has accumulated supporting the notion that microbiota modulates gut immunological function in IBD, and manipulation of intestinal bacterial flora has been used as an alternative health approach for disease prevention and treatment [9]. Administration of probiotics is one of the methods used to manipulate the intestinal microbiota. This therapeutic strategy aims to restore the balance of the gastrointestinal microbiota in order to reduce or prevent intestinal inflammation [16].

Probiotics are defined as living microorganisms that can survive stomach acid and bile, remain viable through extended periods of storage, and are safe for human consumption, and that, when ingested in sufficient amounts, exert a positive influence on host health or physiology [17]. The mode of action of probiotics is complex and not completely understood. Several mechanisms have been reported with respect to prevention and treatment of IBD, such as antimicrobial activity and suppression of bacterial growth, immunomodulation and initiation of an immune response, enhancement of barrier activity, and suppression of human T-cell proliferation [16, 18, 19].

We have previously shown the ability of both *Lactobacillus* GG (LGG) and a mixture of *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis* (YO-MIX™ Y 109 FRO 1000 (Y 109)) probiotics to promote the recovery of colonic tissue after trinitrobenzenesulfonic acid (TNBS) induction of colitis, and to modify the colonic microbiota which had been altered as a consequence of the TNBS-induced colitis [20]. However, restoration with respect to crypt damage and to the extent and severity of the inflamed tissue was more evident in rats that received LGG than in those that received Y 109. It was therefore of interest to evaluate and compare the effects of different probiotics using the same experimental model, in order to establish which microorganisms show the best profile as anti-inflammatory agents, and to determine whether different

probiotics may act synergistically to downregulate the intestinal inflammation by acting on different targets in the inflammatory response. In this article, we describe our two-stage study of the effects of two different probiotics, LGG and Y 109, on the rat's colon in terms of tissue damage and immune-related genes. The selection of these probiotics was based on our previous studies, which supported their potential beneficial effects.

In the last few years, several studies have been performed in order to evaluate probiotics effects on normal healthy subjects; [21, 22], hence, the first part of the study was carried out to examine the effects of LGG and Y 109 probiotics on normal rat colons. The second part of the study was aimed at testing the preventive effects of LGG and Y 109 probiotics in the TNBS model of rat colitis, a well-established model of intestinal inflammation that has some histological and biochemical features of the human disease [23].

2 Materials and methods

2.1 Animals

Male Wistar rats with an average weight of 375 g (range, 300–450 g) were obtained from the Harlan Laboratory at The Weizmann Institute of Science (Rehovot, Israel). They were housed in metal cages in a room with controlled temperature ($25 \pm 2^\circ\text{C}$), relative humidity ($65 \pm 5\%$), and light (0800–2000 h). Ethics approval was obtained for the study, and procedures were conducted in full compliance with the strict guidelines of the Hebrew University Policy on Animal Care and Use.

In the first part of the study, 18 rats were divided into three groups: one group served as a control and the other two received either YO-MIX™ Y 109 FRO 1000 probiotics (Y 109) or *Lactobacillus* GG probiotics (LGG) with the drinking water. The probiotics treatments were continued until rats were sacrificed, 10 days after the start of the experiment.

In the second part of the study, 24 rats were divided randomly into four groups: two of them received no probiotic treatment (control groups) and the other two received either Y 109 or LGG (treated groups). Probiotics (final amount 10^8 CFU/g) were administered orally with the drinking water each day. A week after starting the experiment, rats from one of the control groups and from both treated groups were administered TNBS to induce colitis.

2.2 Administration of probiotic bacteria

Probiotic strain LGG was from Valio (Helsinki, Finland) and YO-MIX™ Y 109 FRO 1000 probiotic bacterial mix was from Danisco Cultures (Niebull, Germany). This latter probiotic formulation consists of three live bacterial strains: *S. thermophilus*, *L. acidophilus*, and *B. lactis*.

2.3 Induction of colitis

A modification of the procedure developed by Morris *et al.* [24] was used to induce colitis. Rats were lightly anesthetized with ether and a rubber catheter was inserted through the anal canal for a distance of 8 cm into the colon just proximal to the splenic flexure. Colitis was induced by administering 0.3 mL 2,4,6-TNBS (Sigma Chemical, St. Louis, MO; 100 g/L dissolved in 50% ethanol).

2.4 Morphological examination

Fresh sections of colonic tissue were obtained from all rats and fixed overnight in 4% v/v buffered formaldehyde. Serial 5- μ m sections were prepared after sample dehydration in graded ethanol solutions, clearing in chloroform and embedding in paraffin. For morphological observations, sections were deparaffinized in xylene, rehydrated, stained with hematoxylin and eosin (H&E), and evaluated by light microscopy.

2.5 Assessment of colonic damage

Colitis severity was assessed by histological evaluation. Rats were sacrificed and the colon was removed aseptically and divided into three segments. Clonic segment was fixed in 4% buffered formaldehyde and the tissue was subsequently processed for histological evaluation. Equivalent colonic segments were also obtained from the noncolitic group. Inflammation and crypt damage were assessed on H&E-stained sections, using a modification of a validated scoring scheme described by Dieleman *et al.* [25] Each section was then scored for each feature separately and the total colonic histology scores were determined as the sum of the five different subscores. Using this scoring system, the minimum score was 0 and the maximum score was 18.

2.6 Total RNA isolation

Total RNA was isolated from the colon using the TRI-Reagent RNA/DNA/Protein Isolation Reagent 5 (1 mL/100 mg of tissue) according to the manufacturer's protocol (Sigma). The integrity of the RNA was verified by ethidium-bromide staining, and its concentration was determined spectrophotometrically.

2.7 Real-Time PCR

Total RNA was reverse-transcribed to produce cDNA in a 20- μ L volume containing 1 μ g of extracted RNA. Reverse transcription was carried out using the EZ-First Strand cDNA Synthesis Kit for RT-PCR according to the manufacturer's protocol (Biological Industries, Beit Haemek, Israel). The reaction was performed at 70°C for 10 min followed by 60 min at 42°C and 15 min at 70°C. Gene-specific primers were used for SYBR Green detection according to the published cDNA sequences for each of the studied genes (Table 1). Real-time PCR was performed on a Stratagene MX 3000P instrument (Stratagene Hogehilweg 15, Amsterdam, The Netherlands). The 20- μ L PCR mixture consisted of 10 μ L Platinum[®] SYBR Green qPCRSuperMix (Invitrogen[®], Carlsbad, CA), 5 μ L water, and 1 μ L of each primer which was added to 3 μ L of the cDNA diluted 1:25 v/v. All PCRs were performed in duplicate in ABgene PCR plates closed with Absolute QPCR seals (Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: 50°C for 2 min, 95°C for 2 min and 40 cycles of 95°C for 30 s, and 60°C for 1 min. In addition, a melting curve was determined under the following conditions: 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Each gene was amplified independently in duplicate within a single instrument run. Standard curves were also generated to determine the efficiency of amplification by pooling undiluted cDNA from each sample and diluting the pooled

Table 1. Real-time PCR primers used to amplify mRNA

Target gene	Sequence (5'-3')	Length (bp)	References
GAPDH	f-GTTACCAGGGCTGCCTCTC r-GGGTTTCCCGTTGATGACC	168	[38]
TNF- α	f-ATCCGAGATGTGGAAGTGGC r-CGATCACCCGAAGTTCAGTA	150	[39]
IL-6	f-GCCCTTCAGGAACAGCTATGA r-TGTCAACAACATCAGTCCCAAGA	79	[40]
β -Defensin 2	f-ATGAGGATCCATTACCTTCTCTTC r-TCATCCCATTGGTTCTTGGT	200	
MUC2	f-ATTACCCACAGTGGACAA r-GGGATGTCCACCACAAAGTT	244	[41]
TLR2	f-GTACGCAGTGAGTGGTGAAGT r-GGCCGCGTCATTGTTCTC	174	[42]
TLR4	f-AATCCCTGCATAGAGGTACTTCTAAT r-CTCAGATCTAGGTTCTTGGTTGAATAAG	106	[42]

cDNA to dilutions of 1:5, 1:25, 1:125, and 1:625 v/v. Cycle threshold (Ct) values were calculated for each sample automatically by MXpro software (Stratagene Hogehilweg 15, Amsterdam, The Netherlands). The Ct values of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were not significantly different ($p > 0.05$) among the different treatments and therefore gene expression was normalized for RNA loading using GAPDH as the internal control. Fold change was calculated relative to the control using the $\Delta\Delta Ct$ method of Pfaffl [26] including the efficiencies for all of the experimental genes and GAPDH (internal control).

2.8 Statistical analysis

Treatment-dependent changes were analyzed using one-way analysis of variance. Statistical differences among means were considered significant at $p < 0.05$. A *posthoc* test (Tukey–Kramer) was performed when the interaction between treatments was significant. Tests were carried out within segments and not between them. JMP version 6.0 (SAS Institute, Cary, NC, USA) was used for all analyses. Values are presented as means \pm SEM.

3 Results

3.1 Morphological observations

Histological assessment of colonic samples from the first part of the study revealed a normal architecture of the colon in all three groups. However, some colonic samples from the probiotics groups were characterized by neutrophil infiltration in the mucosal layer and submucosa (Fig. 1).

Histological assessment of colonic samples from the second part of the study revealed transmural disruption of the normal architecture of the colon in the colitis-induced groups, with extensive ulceration and inflammation involving all of the intestinal layers, giving a score of 15.88 ± 0.66 (mean \pm SEM). Colonic samples were characterized by neutrophil infiltration in the mucosal layer and submucosa. Most of the rats showed epithelial ulceration of the mucosa affecting over 85% of the surface. The inflammatory process was associated with crypt loss. Histological analysis of the colonic specimens from rats administered probiotics before

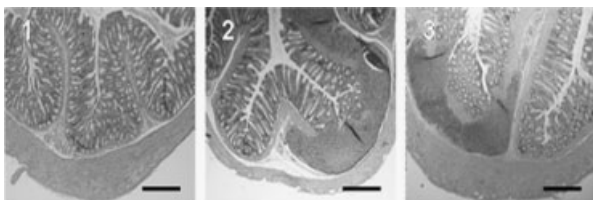


Figure 1. Effect of probiotic bacteria on normal rat colon. Representative light micrographs of control (1), LGG (2), and Y 109 (3) colons stained with H&E. Magnification X40; bar = 50 μ m.

induction of colitis revealed a more pronounced effect of Y 109 on colonic tissue, with a mean score that was significantly different from the colitis group (10.38 ± 2.90) in comparison to LGG administration with a mean score of 12.38 ± 2.58 . In addition, Y 109 improved colonic tissue architecture significantly relative to the colitis group; improvement in colonic histology was accompanied by a reduction in inflammation infiltrate.

3.2 Gene expression

We evaluated the expression of several genes related to the gut immune system and inflammation in both parts of this study. Gene-expression levels were determined by analyzing the resultant Ct values for each sample, normalized to the level of GAPDH expression for the same RNA sample.

In the first part of the study, the Ct ratio of the proinflammatory genes tumor necrosis factor- α (TNF- α) and IL-6 increased in the Y 109 probiotics groups compared with controls (indicating a decrease in fold change expression; Fig. 2). Administration of both Y 109 and LGG led to a decrease in the Ct ratio of mucin 2 (MUC2) mRNA; however, LGG probiotics had a more pronounced effect (increase in fold change expression, Fig. 2).

The Ct ratio of β -defensin 2 (β -def2) was increased by Y 109 probiotics administration relative to controls (decrease in fold change expression; Fig. 2). LGG administration had opposite effects on the Ct ratio of Toll-like receptors (TLRs): it increased the TLR2 Ct ratio (decrease in fold change expression; Fig. 2), and concomitantly decreased the TLR4 Ct ratio (increase in fold change expression; Fig. 2). In contrast, administration of Y 109 probiotics decreased both genes' Ct ratio compared with controls (increase in fold change expression, Fig. 2). None of the changes was statistically significant. The Ct ratios (gene Ct:GAPDH Ct) for each of the genes in each group are listed in Table 2: the lower the Ct value, the higher the expression level.

In the second part of the study, the Ct ratio of the proinflammatory genes TNF- α and IL-6 increased significantly in both probiotics groups compared with the colitis group (decrease in fold change expression; Fig. 3). Admin-

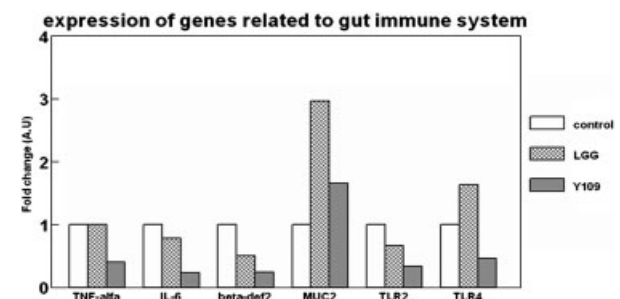
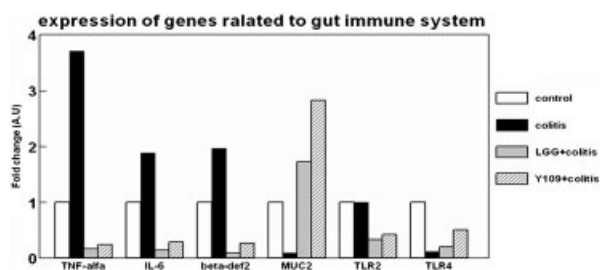


Figure 2. The effect of administration of probiotic bacteria on gene expression in the normal rat colon.

Table 2. The Ct ratios (gene Ct:GAPDH Ct) for examined genes in the colon of each group

	TNF- α	IL-6	β -Defensin 2	MUC2	TLR2	TLR4
Control	1.034 (\pm 0.023)	0.969 (\pm 0.021)	0.951 (\pm 0.006)	1.075 (\pm 0.046)	0.965 (\pm 0.012)	1.068 (\pm 0.017)
LGG	1.049 (\pm 0.026)	1.009 (\pm 0.025)	0.997 (\pm 0.026)	1.002 (\pm 0.014)	0.976 (\pm 0.033)	1.042 (\pm 0.020)
Y 109	1.051 (\pm 0.047)	1.030 (\pm 0.054)	1.015 (\pm 0.006)	1.033 (\pm 0.014)	0.986 (\pm 0.032)	1.12 (\pm 0.044)

**Figure 3.** The effect of TNBS colitis and probiotics administration on gene expression in the rat colon.

istration of both probiotics prior to induction of colitis each led to a significant decrease in the Ct ratio of MUC2 mRNA. However, Y109 probiotics had a more pronounced effect (increase in fold change expression, Fig. 3).

The Ct ratio of β -def2 was significantly increased by probiotics administration in comparison to the colitis group (decrease in fold change expression; Fig. 3). A differential effect was observed with the TLRs: colitis induction did not influence the TLR2 Ct ratio, but it increased TLR4 Ct ratio (decrease in fold change expression; Fig. 3). Administration of each of the probiotics before induction of colitis increased the TLR2 Ct ratio (decrease in fold change expression; Fig. 3), with a significant difference in Ct ratio between the probiotics and colitis groups. The Ct ratios among the LGG probiotics and colitis groups were not significantly different for TLR4. However, administration of Y 109 probiotics decreased this gene's Ct ratio compared with the colitis group (increase in fold change expression, Fig. 3). The Ct ratios (gene Ct:GAPDH Ct) for each of the genes in each group are listed in Table 3: the lower the Ct value, the higher the expression level.

4 Discussion

We conducted a two-part study to unravel the mechanism underlying probiotics' action in the gut epithelium. We compared the effects of two probiotics regimens on colonic tissue morphology and immune-related genes in the same rat colon model.

There is a well-established link between intestinal microbiota and the inflammation associated with IBD, with evidence of both proinflammatory and regulatory effects. Several studies have suggested that commensal microbiota

protect the mucosa from inflammation by decreasing intestinal permeability, increasing epithelial defense mechanisms, and promoting an immunoregulatory acquired immune response [27, 28]. There is evidence that metabolites produced by bacteria may interact directly with gut epithelial cells to enhance mucosal integrity [29]. In addition, commensal bacteria may compete with proinflammatory species to decrease access to the mucosa [30]. Thus, selective manipulation of the microbiota is an attractive therapeutic strategy for the treatment of disease and maintenance of remission.

The role of probiotics in disease has been the focus of many studies. However, only in the last few years studies focused on understanding the potential impact of probiotics on maintaining health has been carried out. Although it can be a challenge to demonstrate improved health in an already healthy population, some recent studies provide evidence that probiotics may reduce the risk of getting sick [31, 32]. Therefore, the first part of this study was conducted to examine the effect of two probiotics regimens on immune variables in normal rat gut. The approach included administration of probiotic bacteria for 10 days with the drinking water. Administration of probiotics led to nonsignificant changes in immune-related genes in the colon, this is probably due to the sample size which is smaller for reaching an optimal statistical power. The changes observed may have been significant, in a bigger sampling study. In addition, a longer period of probiotics administration may also increase the effect. Different responses were observed in both group that received probiotics. Both probiotics reduced the expression of TNF- α , IL-6, β -defensin 2, and TLR2; however, Y 109 effect was more pronounced. In addition, both probiotics increased the expression of MUC2 mRNA; nevertheless, LGG increment was bigger. TLR4 response to probiotics was opposite; Administration of LGG increased its expression, while Y109 decreased its expression. The different responses may probably be related to the different probiotics species in the examined regimens. Also LGG is one probiotic strain and Y109 is a mixture of three bacteria. In a mixture of bacteria, there are synergism effects in addition to the effect each strain has. Supplementation of probiotics led to some neutrophil infiltration in the mucosal layer and submucosa, which may reflect stimulation of the immune system.

Stimulation of the immune function has been observed in healthy subjects in other studies [33]. Olivares *et al.* [34] reported that probiotics induce an increase in the proportion

Table 3. The Ct ratios (gene Ct:GAPDH Ct) for examined genes in the colon of each treated group

	TNF- α	IL-6	β -Defensin 2	MUC2	TLR2	TLR4
Control	1.01066 (\pm 0.029) ^{a,b)}	0.969 (\pm 0.021) ^{b)}	0.951 (\pm 0.018) ^{b,c)}	1.095 (\pm 0.051) ^{a,b)}	0.965 (\pm 0.0125) ^{b)}	1.068 (\pm 0.017) ^{b)}
Colitis	0.9537 (\pm 0.0139) ^{b)}	0.936 (\pm 0.004) ^{b)}	0.917 (\pm 0.0087) ^{c)}	1.202 (\pm 0.031) ^{a)}	0.942 (\pm 0.0098) ^{b)}	1.165 (\pm 0.028) ^{a)}
LGG+colitis	1.137 (\pm 0.032) ^{a)}	1.11 (\pm 0.036) ^{a)}	1.064 (\pm 0.031) ^{a)}	1.003 (\pm 0.009) ^{b)}	1.036 (\pm 0.018) ^{a)}	1.134 (\pm 0.022) ^{a,b)}
Y 109+colitis	1.114 (\pm 0.0389) ^{a)}	1.074 (\pm 0.011) ^{a)}	1.032 (\pm 0.0299) ^{a,b)}	0.993 (\pm 0.034) ^{b)}	0.995 (\pm 0.016) ^{a,b)}	1.089 (\pm 0.019) ^{a,b)}

a-c) Significant changes are shown by different letters ($p < 0.05$).

of natural killer cells and in IgA concentrations. In their study, the effects were higher after 2 wk of treatment than after 4 wk, suggesting regulation of the immune system.

The second part of our study examined the preventive potential of probiotics *via* their administration with the drinking water for 1 wk prior to induction of colitis. Here, administration of probiotics decreased the expression of genes related to the inflammatory process and reduced the inflammatory response. Both probiotics assayed exhibited anti-inflammatory activities as evidenced histologically. However, each probiotic showed its own anti-inflammatory profile. Both probiotics regiments reduced the expression of TNF- α , IL-6, β -defensin 2, and TLR2; however, LGG administration affect was more prominent. On the other hand, while both probiotics increased MUC2 expression, Y109 effect was greater than that observed with LGG. Our results are in agreement with other studies, which have demonstrated the preventative effect of probiotics in the TNBS model [15].

The rationale for modulating gut microbiota with probiotics lies in the demonstration that the intestinal microbiota has an important influence on health [35]. Specific probiotic bacteria modulate intestinal and systemic immune responses [36]. Activation of immunological cells and tissues requires close contact of the probiotic with the immune cells and tissue on the intestinal surface.

Studies suggest that interaction of probiotic bacteria with the intestinal epithelium is a key determinant for cytokine production by enterocytes, and probably the initiating event in probiotics' immunomodulatory activity. The effects of probiotics can be direct, or indirect through modulation of the endogenous flora or of the immune system [31, 37]. In addition, probiotics may improve intestinal integrity.

The results obtained in this study provide additional support for the positive effect of probiotics in the gut. Furthermore, they confirm the intestinal anti-inflammatory activity of different probiotics. Our findings may shed light on the mechanism by which probiotics reinforce the mucosal barrier through MUC induction in an animal model. However, further studies need to be performed before these results can be extrapolated to humans.

We suggest that the effects/activities of probiotics are strain specific, and that a combination of probiotics could be beneficial. It is apparent from our studies that probiotics may be applied as preventive as well as therapeutic tools. Different pathways of bacterial-enterocyte crosstalk may

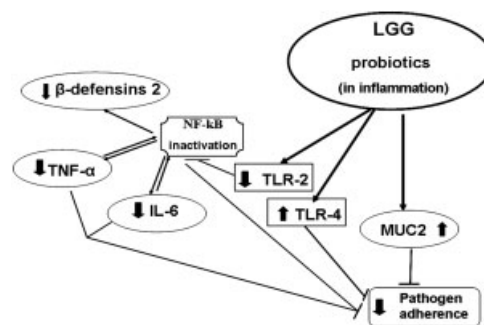


Figure 4. Schematic illustration of the proposed multistep mechanism of LGG's effect on the innate immune system and microbial ecology in rat intestine.

induce different immune responses in healthy and sick subjects.

We suggest here that LGG affects the innate immunity in rat intestines *via* a multistep mechanism (Fig. 4). LGG administration elevates MUC expression, which plays a fundamental role in the host's first line of defense. This, in turns, inhibits the adherence of pathogenic bacteria to intestinal epithelial cells. LGG also upregulated TLR4 expression, which implies enhanced activity of the epithelial barrier against pathogenic bacteria. Reduction in the amount of adhered bacteria results in downregulation of TLR2 expression, and as a result, nuclear factor kappa B is deactivated and IL-6, TNF α , and defensin expression is reduced. This reduction is also related to inhibition of the adhered pathogenic bacteria. Thus probiotics administration reinforces the mucosal barrier through MUC induction. This model should be validated through further studies using LGG and inflammatory markers.

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The authors have declared no conflict of interest.

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