Probiotic Administration Reduces Mortality and Improves Intestinal Epithelial Homeostasis in Experimental Sepsis

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ABSTRACT

Background: Recent clinical trials indicate that probiotic administration in critical illness has potential to reduce nosocomial infections and improve clinical outcome. However, the mechanism(s) of probiotic-mediated protection against infection and sepsis remain elusive. The authors evaluated the effects of *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium longum* (BL) on mortality, bacterial translocation, intestinal epithelial homeostasis, and inflammatory response in experimental model of septic peritonitis.

Methods: Cecal ligation and puncture (n = 14 per group) or sham laparotomy (n = 8 per group) were performed on 3-week-old FVB/N weanling mice treated concomitantly with LGG, BL, or vehicle (orally gavaged). At 24 h, blood and colonic tissue were collected. In survival studies, mice were given probiotics every 24 h for 7 days (LGG, n = 14; BL, n = 10; or vehicle, n = 13; shams, n = 3 per group).

Results: Probiotics significantly improved mortality after sepsis (92 *vs.* 57% mortality for LGG and 92 *vs.* 50% mortality for BL; $P = 0.003$). Bacteremia was markedly reduced in septic mice treated with either probiotic compared with vehicle treatment (4.39 ± 0.56 *vs.* 1.07 ± 1.54; $P = 0.0001$ for LGG; *vs.* 2.70 ± 1.89; $P = 0.016$ for BL; data are expressed as mean ± SD). Sepsis in untreated mice increased colonic apoptosis and reduced colonic proliferation. Probiotics significantly reduced markers of colonic apoptosis and returned colonic proliferation to sham levels. Probiotics led to significant reductions in systemic and colonic inflammatory cytokine expression *versus* septic animals. Our data suggest that involvement of the protein kinase B pathway (*via* AKT) and down-regulation of Toll-like receptor 2/Toll-like receptor 4 *via* MyD88 in the colon may play mechanistic roles in the observed probiotic benefits.

Conclusions: Our data demonstrate that probiotic administration at initiation of sepsis can improve survival in pediatric experimental sepsis. The mechanism of this protection involves prevention of systemic bacteremia, perhaps *via* improved intestinal epithelial homeostasis, and attenuation of the local and systemic inflammatory responses.

What We Already Know about This Topic

- Probiotic therapy appears to be useful in prevention of hospital-acquired and antibiotic-associated infections, but the mechanisms underlying this are not well defined

What This Article Tells Us That Is New

- Probiotic administration in septic mice decreased mortality and systemic bacteremia and led to decreased apoptosis of colonic epithelium, increased colonic epithelial proliferation, and decreased systemic and colonic cytokine expression

SEPSIS is a common cause of death in children and adults despite advances in the supportive care for the critically ill patients. Centers for Disease Control data shows that death rates from sepsis have increased at a rate greater than from any other common cause of mortality in the last year, and sepsis is now one of the top 10 causes of death in the United States.[1] Annually, above 1 million deaths worldwide are associated with sepsis within the pediatric and neonatal population alone.[2,3] Even when pediatric and adult patients survive, they face substantial long-term adverse consequences after sepsis and critical illness.[4–6]

Critical illness and sepsis are systemic syndromes that lead to a hostile environment in the gut, resulting in an imbalance of the intestinal microbiota in favor of pathogenic species.[7] The intestine plays a central role in the pathogenesis of sepsis and has been referred to as the "motor" of the systemic inflammatory response.[8,9] Perturbations of intestinal epithelial

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homeostasis in sepsis result in increased proinflammatory cytokine production,[10] barrier dysfunction,[11–13] and increased apoptosis[14–17] which may lead to multiple organ failure.

Probiotic therapy represents a promising intervention for the treatment of nosocomial infections in the intensive care unit, which often lead to sepsis and multiple organ failure.[18] Probiotics are living nonpathogenic bacteria that colonize the intestine and provide a benefit to the host.[19] Results from recent randomized controlled trials in pediatric and adult populations suggest a benefit to the use of probiotics in the intensive care unit.[18,20–22] In an age of increasing antimicrobial resistance, the use of probiotics as a potential treatment for severe infections and subsequent septic shock may be a promising, cost-effective, preventative strategy.

The potential beneficial role(s) of probiotics on survival given in early systemic polymicrobial sepsis, and the mechanisms by which probiotics may function to protect against experimental sepsis, particularly pediatric sepsis, are currently not well understood. Therefore, we sought to delineate the clinical utility and potential mechanistic targets of acutely administered probiotic therapy at initiation of experimental pediatric sepsis *via* a weanling mouse peritonitis model. We selected the two commonly clinically used probiotic strains, *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium longum* (BL), given at clinically relevant doses, to evaluate possible strain-related differences in clinical outcome and mechanistic targets in the gut.

Lactobacillus spp. and *Bifidobacterium spp*., alone or in combination, are the most frequently used probiotic strains in the treatment of various gastrointestinal disorders[23–25] or as therapy for clinical conditions including antibiotic-associated diarrhea,[26,27] ventilator-associated pneumonia,[18,21,28] sepsis, and postoperative infections.[29,30] Although probiotics seem to be an effective treatment in various clinical conditions, the specific mechanisms responsible for their beneficial action are complex and not fully understood.[31] On the basis of the results from several *in vivo* and *in vitro* studies, we hypothesized that LGG and BL would prevent bacterial translocation,[32,33] reduce the overgrowth of pathogenic bacteria, decrease apoptosis in intestinal epithelial cells,[34–37] and reduce inflammation.[38–41]

Materials and Methods

Probiotic Treatment and Septic Peritonitis Model

The animal protocol used in these studies was approved by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus. Briefly, 3-week-old FVB/N mice were orally gavaged with 200 μl of either LGG (1×10^9 colony forming unit [CFU]/ml), BL (1×10^7 CFU/ml), or sterile water (vehicle) immediately before initiation of the cecal ligation and puncture (CLP) procedure.[42] Briefly, a small midline abdominal incision was made, the cecum was ligated just distal to the ileocecal valve, and was then punctured twice with a 23-gauge needle. The cecum was squeezed to extrude a small amount of stool, replaced in the abdomen, and the peritoneum and skin were closed in layers. Sham mice were treated identically, except the cecum was neither ligated nor punctured. All mice received 1.0 ml normal saline subcutaneously after the surgery to compensate for fluid loss. Animals were euthanized at either 24 h (for acute studies) or followed 7 days for survival (LGG, n = 14; BL, n = 10, or vehicle, n = 13; shams, n = 3 per group). For survival studies, mice were treated with probiotics daily for 7 days. For acute studies, mice received a single dosage of probiotics before tissue collection.

LGG and BL Culture

LGG (ATCC, Manassas, VA) was incubated in de Man, Rogosa, and Sharpe broth (Becton Dickinson, Sparks, MD) for 24 h at 37°C and 5% CO_2. BL (ATCC) was cultured in Trypticase soy broth (Becton Dickinson) for 72 h in an anaerobic chamber at 37°C. A_{600} was measured to determine the number of CFU per 1 ml. BL and LGG were pelleted from the broth (10,000 rpm; 10 min) and resuspended in distilled water.

Bacteremia and Bacterial Analysis of the Colon

Blood was collected at 24 h from the inferior vena cava of anesthetized mice, serially diluted in sterile 0.9% saline and cultured on Trypticase soy agar plates with 5% sheep blood (Becton Dickinson) for 24 h at 37°C/5% CO_2. CFU were then enumerated for each animal (shams, n = 8 per group; septic, n = 10; LGG, n = 7; and BL, n = 8).

DNA was extracted from collected frozen colon samples obtained from a separate study to ensure sufficient amount of tissue (shams, n = 4 per group; septic, n = 12; LGG, n = 10; and BL, n = 9; luminal and intestinal wall contents; 24-h time point) using UltraClean Fecal DNA kit (MO BIO Laboratories, Inc., Carlsbad, CA). The concentration, integrity, and purity of DNA were determined using a NanoDrop (Thermo Fisher Scientific, Wilmington, DE). Quantification was performed by quantitative polymerase chain reaction (PCR) using standard curves derived from cloned 16S ribosomal RNA genes[43] using the following primers: *Lactobacillus*[44]: LactoF (5′ TGGAAACAGRTGCTAATACCC) and LactoR (5′ GYCCATTGTGGAAGATTCCC). *Bifidobacterium*[45]: Bif1F (5′ TCG CGT CYG GTG TGA AAG) and Bif1R (5′ CCA CAT CCA GCR TCC AC). The following cycling protocol was used: denaturation at 95°C (10 min) and 40 cycles of 95°C (15 s), 60°C (30 s), and 65°C (1 min). Reporter dye emission (SYBR green, Applied Biosystems, Foster City, CA) was detected by an automated sequence detector combined with ABI Prism 7300 Real Time PCR System (Applied Biosystems).

Gram staining was performed on tissue sections and evaluated by microscopy for presence of Gram-positive or Gram-negative bacteria.

Immunohistology

Colon was collected from each animal at 24 h and fixed overnight in 10% formalin, paraffin-embedded, and sectioned at 4–6 μm. Serial sections were stained (shams, n = 5 per group; septic, n = 5; LGG, n = 4; and BL, n = 4). After deparaffinization and rehydration, sections were blocked with 1.5%

rabbit or goat serum (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline for 30 min, then incubated with either rabbit polyclonal cleaved caspase-3 (1:100; Cell Signaling, Danvers, MA) or mouse monoclonal proliferating cell nuclear antigen (1:100; Invitrogen, Camarillo, CA) antibody for 1 h, washed with phosphate-buffered saline, and incubated with either goat anti-rabbit or anti-mouse biotinylated secondary antibody (Vector Laboratories) for 30 min. Vectastain Elite ABC reagent (Vector Laboratories) was then applied, followed by diaminobenzidine as substrate. Sections were counterstained with hematoxylin, dehydrated, and cover-slipped.

To determine apoptosis in the colonic epithelium, apoptotic epithelial cells were quantified in 100 consecutive crypts by using two complimentary methods: morphological analysis of hematoxylin and eosin–stained sections where apoptotic cells were identified by characteristic morphology of nuclear fragmentation (karyorrhexis) and cell shrinkage with condensed nuclei (pyknosis)[46] and second by enumeration of cleaved caspase-3–positive cells. Colonic epithelial proliferation was determined by quantifying proliferating cell nuclear antigen-positive cells in 100 consecutive crypts. All counting was performed by a blinded evaluator.

RNA Preparation, Reverse Transcription, and Real-time PCR

Total RNA was isolated from colonic tissue (snap frozen in liquid nitrogen and collected at 24 h) using the RNeasy Plus Mini Kit (Qiagen, Santa Clarita, CA) as described in the manufacturer's protocol. RNA concentrations were quantified at 260 nm, and the purity and integrity were determined using a NanoDrop. Reverse transcription and real-time PCR assays were performed to quantify steady-state messenger ribonucleic acid (mRNA) levels of interleukin (IL)-6, tumor necrosis factor-α (TNF-α), IL-1β, IL-10, MyD88, Toll-like receptor (TLR)-4, and TLR-2 (shams, n = 4 per group; septic, n = 7; LGG, n = 6; and BL, n = 4). Complementary DNA was synthesized from 0.2 μg of total RNA. Predeveloped TaqMan primers and probes (Applied Biosystems) were used for detection. Reporter dye emission was detected by an automated sequence detector combined with ABI Prism 7300 Real Time PCR System (Applied Biosystems). Real-time PCR quantification was performed with TaqMan glyceraldehyde-3-phosphate dehydrogenase controls.

Western Blot Analysis

Individual frozen colon samples (shams, n = 3 per group; septic, n = 8; LGG, n = 4; and BL, n = 4; 24-h time point) were homogenized with a hand-held homogenizer in a 5x volume of ice-cold homogenization buffer (Tris HCl, 50 mm; pH, 7.4; NaCl, 100 mm; EDTA, 10 mm; Triton X-100, 0.5%) with added protease inhibitors (Roche Diagnostics, Mannheim, Germany). The homogenates were centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was collected. Total protein concentration was quantified using the Bradford protein assay.[47] For protein analysis, 40 μg of protein was added to an equal volume of 2x Laemmli sample buffer and boiled for 5 min. The samples were run on 4–15% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) at 200 V for 30 min. Protein was transferred to Immuno-Blot PVDF membranes (Bio-Rad Laboratories) at 65 V for 4 h. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO) for 1 h at room temperature and then incubated overnight at 4°C with one of the following rabbit polyclonal antibodies: Bax (1:1,000; Cell Signaling), Bcl-w (1:1,000; Cell Signaling), anti-Akt, (1:1,000; Cell Signaling), and anti-P-Akt (1:1,000; Cell Signaling). After extensive washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (Santa Cruz Biotechnology, Dallas, TX). Proteins were visualized with a chemiluminescent system (Pierce, Rockford, IL) by using Epi Chemi II Darkroom (UVP BioImaging System, Upland, CA).

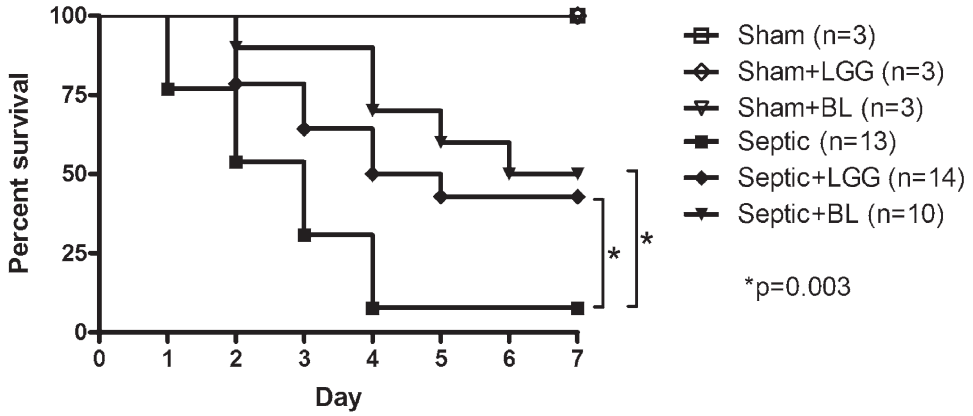

Fig. 1. Effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* on mortality in sepsis. Three-week-old FVB/N mice were subjected to 2 × 23-gauge cecal ligation and puncture. Control animals underwent sham laparotomy. All mice were followed for survival for 7 days. Septic mice treated with *L. rhamnosus* GG (LGG) or *B. longum* (BL) had significantly decreased mortality compared with untreated septic mice (57 *vs*. 92% mortality and 50 *vs*. 92% mortality, respectively; $P = 0.003$). Shams, n = 3 per group; septic, n = 13; LGG, n = 14; BL, n = 10. All sham mice survived.

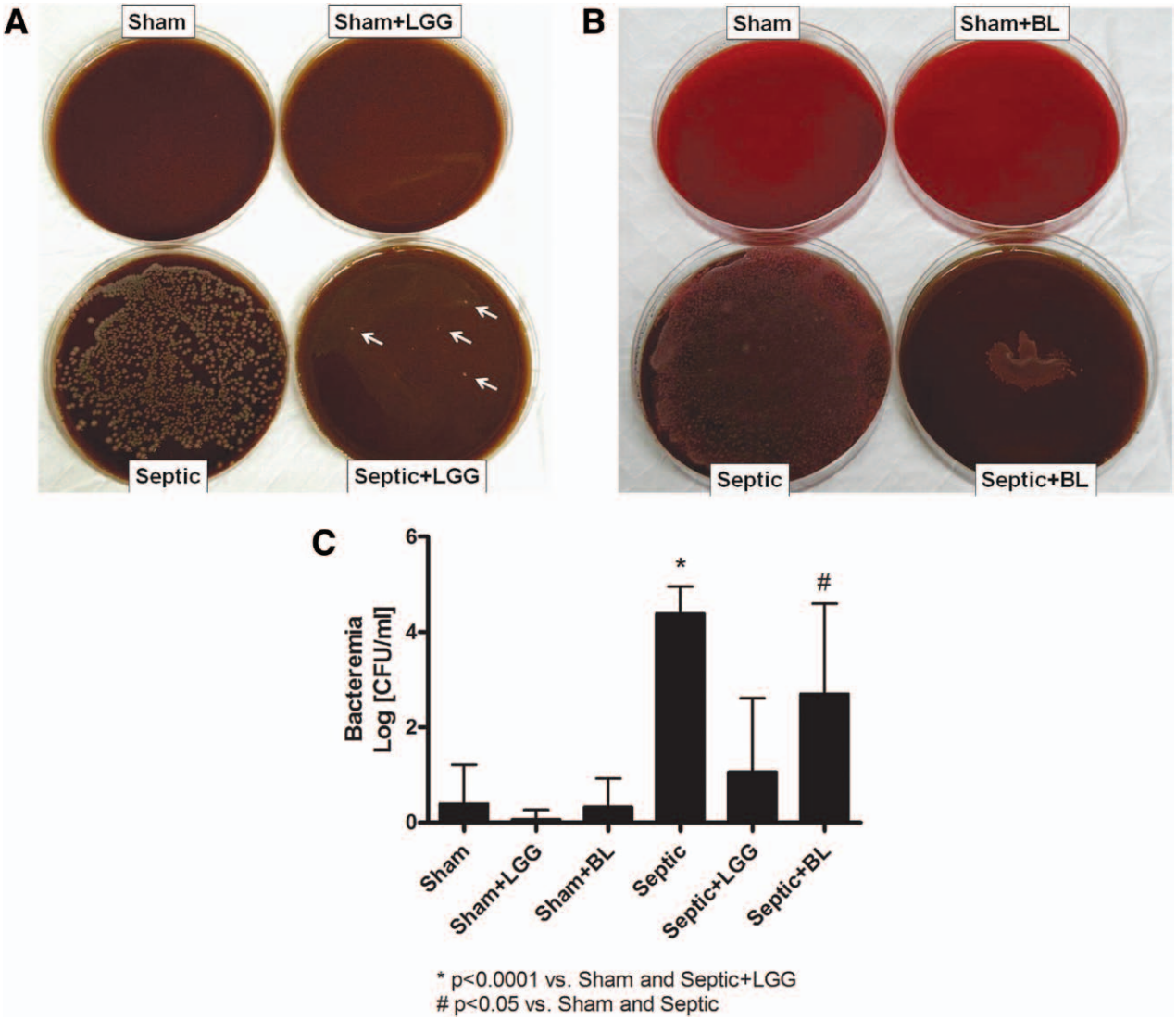

Fig. 2. Effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* treatment on bacteremia in sepsis. Bacterial growth on representative Trypticase soy agar plates with sheep blood (5%) are shown for each experimental group (*A*, *B*). Septic mice had increased bacteremia (*C*) compared with shams (4.39 ± 0.56 *vs*. 0.40 ± 0.81; $P = 0.0001$), whereas septic mice treated with *L. rhamnosus* GG (LGG) or *B. longum* (BL) had reduced bacterial load in the blood (1.07 ± 1.54, $P = 0.0001$ and 2.70 ± 1.89, $P = 0.016$, respectively). Shams, n = 8 per group; septic, n = 10; LGG, n = 7; and BL, n = 8. Data are expressed as the mean ± SD. CFU = colony forming unit.

Serum IL-6 and TNF-α Analysis

Enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) was used to determine the concentrations of TNF-α and IL-6 in serum (shams, n = 5 per group; septic, n = 7; LGG, n = 5; and BL, n = 6; 24-h time point) according to the manufacturer's instructions. Serum was collected after centrifuging blood for 10 min at 5,000 rpm and stored at −80°C until the assay was performed.

Statistics

Continuous data sets were tested for Gaussian distribution by using a Shapiro–Wilk Test for normality, and the Levene F-Test for equality of variances. Although results of these tests showed some departures from normality and equality of variances, it is well documented that the one-way ANOVA is robust with respect to violations of these assumptions. Thus, multiple group comparisons were performed with one-way ANOVA followed by the Newman–Keuls *post hoc* test. Survival studies were analyzed *via* the log-rank test. No measurements or animals were lost for observation or missing in the analysis. Data were analyzed using Prism 4.0 (GraphPad Software, San Diego, CA) and reported as means ± SD. A *P* value less than 0.05 was considered to be statistically significant.

Results

LGG and BL Improve Mortality and Prevent Bacteremia in Septic Peritonitis

To determine whether treatment with LGG or BL have an effect on mortality in peritonitis-induced sepsis, a separate cohort of mice (shams, n = 3 per group; septic, n = 13; LGG,

Table 1. *Lactobacillus* and *Bifidobacterium spp*. in the Colon of Septic Mice

	Sham (n = 4)	Sham + LGG (n = 4)	Sham + BL (n = 4)	Septic (n = 12)	Septic + LGG (n = 10)	Septic + BL (n = 9)
Lactobacillus spp.	$6.6\times10^3 \pm 2.5\times10^3$	$1.1\times10^4 \pm 1.3\times10^4$	—	$6.2\times10^3 \pm 6.6\times10^3$	$3.5\times10^4 \pm 4.1\times10^{4*}$	—
Bifidobacterium spp.	$4.2\times10^5 \pm 1.1\times10^5$	—	$1.7\times10^6 \pm 2.4\times10^6$	$2.0\times10^6 \pm 1.5\times10^6$	—	$2.4\times10^7 \pm 2.9\times10^{7*}$

PCR quantification data are expressed as the mean of cells ± SD.

* $P < 0.05$ *vs.* septic.

BL = *Bifidobacterium longum*; LGG = *Lactobacillus rhamnosus* GG; PCR = polymerase chain reaction.

n = 14; and BL, n = 10) were subjected to CLP and followed 7 days for survival (fig. 1). Mice treated with either LGG or BL had significantly improved 7-day survival compared with untreated septic mice. All sham animals survived.

To examine whether the probiotics LGG or BL could prevent bacteremia in sepsis, whole blood was cultured and bacteria were counted. The data are expressed as the Log of CFU per 1 ml (fig. 2, A–C). Septic animals had significantly increased bacteremia compared with shams. Although both probiotic strains reduced bacteremia, septic mice treated with LGG exhibited normalization to sham mouse blood bacterial counts whereas septic mice treated with BL exhibited significantly decreased bacteremia compared with untreated septic mice, but still had an increased bacterial load in the blood compared with shams.

After Oral Administration, LGG and BL Persist in the Intestine for at Least 24 h

DNA isolated from the colon, including luminal and intestinal wall content, was analyzed by PCR to quantify the presence of *Lactobacillus spp*. and *Bifidobacterium spp*. There was a significant increase in *Lactobacilli* in septic animals treated with LGG compared with untreated septic mice. Similarly, there was an increase in *Bifidobacteria* in septic mice treated with BL compared with untreated septic mice (table 1). These data demonstrate that LGG and BL are indeed able to survive and propagate in the gastrointestinal tract for at least 24 h after oral administration. Therefore, LGG and BL may help to prevent the overgrowth of pathogenic bacteria. This hypothesis was confirmed by Gram staining of intestinal wall specimens, which demonstrated an increased number of both Gram-positive and Gram-negative bacteria in the colon of septic mice compared with shams, and most importantly, less bacteria is seen in the colons of mice treated with either LGG or BL (fig. 3).

Cell Proliferation and Apoptosis in Colon Are Normalized in Septic Mice Treated with Probiotics

As measured by quantifying proliferating cell nuclear antigen-positive cells in 100 consecutive crypts, septic mice exhibited a significant decrease in proliferation of the colonic epithelium compared with sham mice. In contrast, the proliferative response in septic animals treated with either probiotic strain was normalized to levels observed in sham mice (fig. 4).

Colonic epithelial apoptosis was increased in untreated septic mice compared with shams, both when assayed by cleaved caspase-3 staining and also by morphological criteria in hematoxylin and eosin–stained sections. In contrast, septic mice treated with either LGG or BL exhibited decreased colonic apoptosis, with levels similar to those seen in the colons of sham mice (fig. 5, A–D). The ratio of proapoptotic to antiapoptotic molecules is often used as an indicator of sensitivity to apoptosis.[48] Untreated septic mice exhibited significantly increased ratio of Bax/Bcl-w protein expression compared with shams, suggesting a shift toward increased programmed cell death. In contrast, septic mice treated with either LGG or BL exhibited a decrease in the ratio of Bax/Bcl-w compared with untreated septic mice, suggesting a shift toward increased cell survival (fig. 5E).

Increased p-Akt/Akt Ratio in Probiotic-treated Animals Suggests Involvement of Protein Kinase B Pathway

Previously published data by Yan *et al.*[37] showed that LGG promotes survival of intestinal epithelial cells through regulation of the antiapoptotic Akt/protein kinase B signal transduction pathway. Likewise, our results show a significant increase in antiapoptotic Akt in the colonic tissue of both probiotic groups, LGG and BL, compared with the untreated septic group (fig. 6, A–B). This indicates a possible involvement of the pathway in the protective mechanism of both tested probiotic strains against sepsis.

LGG and BL Attenuate the Systemic and Local Inflammatory Response in the Colon during Sepsis

Reduction of inflammation as well as improvement of innate immunity are hypothesized to be protective mechanisms after probiotic administration.[38–41] To determine the effect of probiotics on these parameters, levels of the proinflammatory cytokines such as IL-6 and TNF-α in serum and colonic tissue were measured by enzyme-linked immunosorbent assay. In parallel, gene expression of IL-6, TNF-α, IL-1β, and IL-10 in the colon was quantified using real-time PCR. All cytokine levels were measured 24 h after CLP.

Systemic levels of IL-6 significantly increased in untreated septic mice compared with shams (fig. 7), whereas treatment with LGG or BL in septic mice led to significantly reduced systemic IL-6 levels compared with septic mice not treated with probiotics. Interestingly, systemic levels of TNF-α remained unchanged among all studied groups.

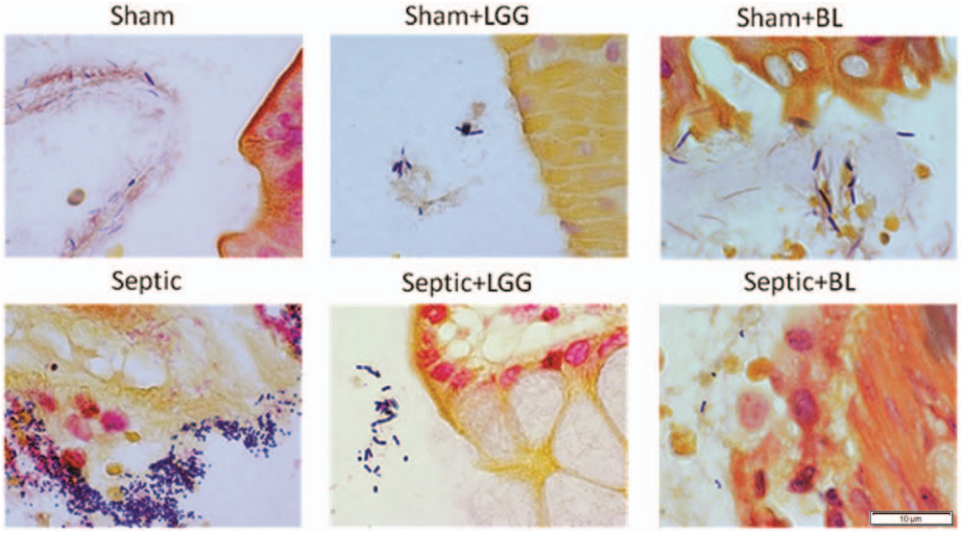

Fig. 3. Gram staining of colonic tissue. Increased numbers of Gram-positive and Gram-negative bacteria in the colon of septic mice compared with shams and mice treated with either *Lactobacillus rhamnosus* GG (LGG) or *Bifidobacterium longum* (BL).

Local, colonic gene expression of TNF-α, IL-1β, and IL-10 (fig. 8, A–C) was significantly increased in the colon of untreated septic animals, whereas levels of both were markedly decreased in colons of septic mice treated with LGG or BL. Colonic gene expression of IL-6 was increased in untreated septic mice and decreased in both probiotic-treated groups although the differences did not reach statistical significance (data not shown).

Probiotics Activate TLR Pathways in the Colon

TLRs signal through the MyD88 pathway, which includes NFκB transcriptional factors, activating various cytokines involved in the innate immunity response. MyD88 has an important role in early recruitment of inflammatory cells and in the control of bacterial infection.[49] Expression of the TLR-2, TLR-4, and MyD88 genes were markedly increased in the colon of untreated septic mice compared with shams, while LGG or BL treatment significantly reduced these levels (fig. 9, A–C).

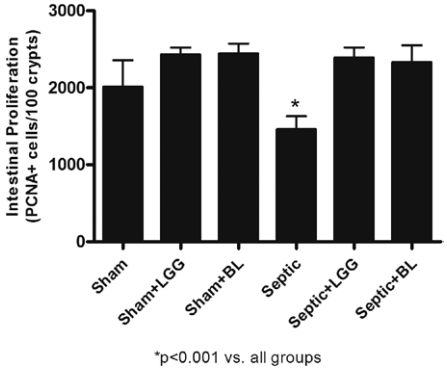

Fig. 4. Effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* treatment on colonic proliferation in sepsis. Colonic epithelial proliferation was determined by quantifying proliferating cell nuclear antigen (PCNA)-positive cells in 100 consecutive crypts. Septic mice had a significantly decreased number of proliferating cells compared with shams (1,456 ± 174 *vs.* 2,010 ± 347; P = 0.0128), whereas septic mice treated with *L. rhamnosus* GG (LGG) or *B. longum* (BL) showed normalization to sham levels (LGG: 2387 ± 137, P = 0.0001; BL: 2,326 ± 224 *vs.* septic 1,456 ± 174, P = 0.0003). Shams, n = 5 per group; septic, n = 5; LGG, n = 4; and BL, n = 4. Data are expressed as the mean ± SD.

Discussion

This study demonstrates for the first time that two different probiotic strains, LGG and BL, confer a significant survival benefit in weanling mice subjected to septic peritonitis. To our knowledge, this is the first description of improved survival after live probiotic therapy in a pediatric or adult polymicrobial sepsis model. This advantage of survival was associated with decreased bacteremia, decreased colonic apoptosis and increased colonic proliferation, decreased systemic and local expression of inflammatory cytokines, and reduced colonic expression of TLR-2/TLR-4 and MyD88.

Critical illness and its treatments (vasopressors, antibiotics, opiates, *etc.*) create a hostile environment in the gut by altering the microbiota, and thereby favor the growth of pathogens.[50] This is partially due to the loss of the beneficial lactic acid bacteria,[51] which can inhibit the overgrowth of pathogens by producing bacteriocins, hydrogen peroxide, organic acids, ammonia and by increasing the competition for adhesion sites on intestinal epithelia.[52,53] In critical illness, enhanced virulence gene expression in bacteria, called quorum sensing, leads to aggressive bacterial behavior, toxin expression, and ultimately translocation into the gut wall and/or gut barrier dysfunctions. This impairment of the gut barrier can lead to gut-derived sepsis, progression to organ failure, and ultimately mortality in the critically ill patients.[50] Interestingly, the administration of beneficial probiotic organisms has been shown to enhance gut epithelial

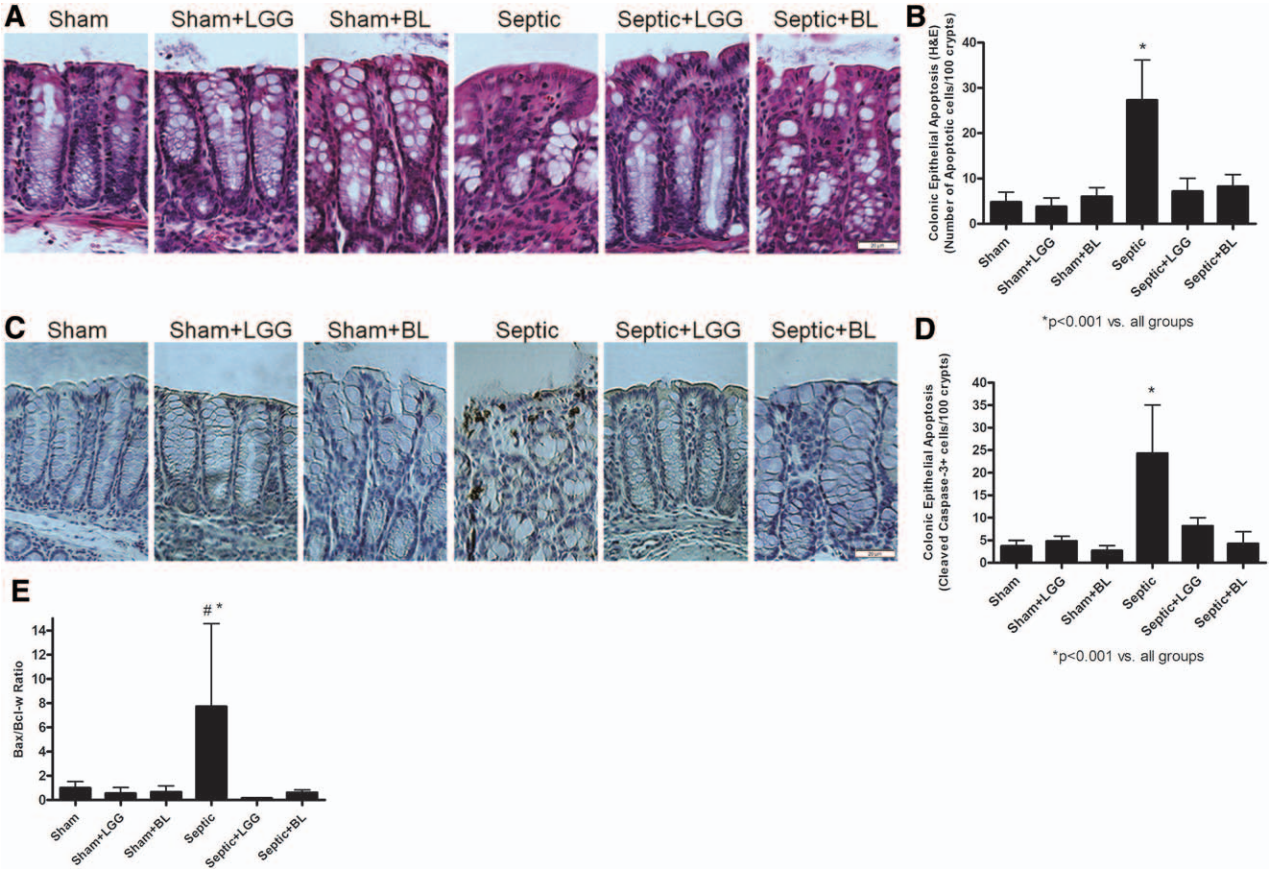

Fig. 5. Effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* treatment on colonic epithelial apoptosis. To determine apoptosis in the colonic epithelium, apoptotic epithelial cells were quantified in 100 consecutive crypts by using two complimentary methods: morphological analysis of hematoxylin and eosin (H&E) stained sections (*A*, *B*) and enumeration of cleaved caspase-3–positive cells (*C*, *D*). Both methods showed significantly increased colonic apoptosis in untreated septic mice compared with controls (CC3: 24.30 ± 10.74 *vs*. shams 3.67 ± 1.32, *P* = 0.0001; H&E: 27.30 ± 8.86 *vs*. shams 4.78 ± 2.17, *P* = 0.0001). *L. rhamnosus* GG (LGG) or *B. longum* (BL) treatment normalized apoptosis to levels seen in sham mice (for CC3, LGG: 8.20 ± 1.79 *vs*. septic, 24.30 ± 10.74, *P* = 0.006; BL: 4.25 ± 2.63 *vs*. septic, 24.30 ± 10.74, *P* = 0.0036 and for H&E, LGG: 7.11 ± 2.93 *vs*. septic, 27.30 ± 8.86, *P* = 0.0001; BL: 8.25 ± 2.63 *vs*. septic, 27.30 ± 8.86, *P* = 0.0014). Shams, n = 5 per group; septic, n = 5; LGG, n = 4; and BL, n = 4. (*E*) The ratio between proapoptotic Bax and antiapoptotic Bcl-w protein expression analyzed by Western blot showed shift toward cell death in untreated septic mice (septic, 7.73 ± 6.84 *vs*. shams, 1.00 ± 0.52, *P* = 0.036), whereas septic mice treated with LLG or BL exhibited a shift toward cell survival (LGG: 0.147 ± 0.049 *vs*. septic, 7.73 ± 6.84, *P* = 0.048; BL: 0.602 ± 0.24 *vs*. septic, 7.73 ± 6.84, *P* = 0.050). Shams, n = 3 per group; septic, n = 8; LGG, n = 4; BL, n = 4. Data are expressed as the mean ± SD.

resistance to injury in *in vitro* models.[50] Consistent with these previous findings, our data demonstrate statistically significant reduction of bacteremia, improvement of colonic epithelial homeostasis, and enhancement of survival in animals treated with either probiotic strain compared with the untreated septic animals.

Existing clinical trials indicate that currently used probiotic strains may not be administered early enough to optimize benefits on prevention and therapeutic efficacy against severe infections and sepsis.[54–57] In our mouse model of sepsis, probiotics were administered early, at the onset of infection, to better imitate the clinical setting of early treatment to prevent progressive infection and sepsis (*i.e.*, before or at onset of nosocomial pneumonia or postoperative abdominal sepsis).

The observed overgrowth of potentially pathogenic bacteria has been shown to cause intestinal cell apoptosis and disruption of epithelial tight junction permeability.[58] As previously shown, intestinal proliferation and intestinal epithelial apoptosis are altered in the CLP sepsis model.[14] Our data are consistent with previous reports of reduced apoptosis with probiotics in intestinal injury.[34,36]

TLRs play a central role in the initiation of innate immune responses and in the development of subsequent adaptive immune responses to microbial pathogens.[59] In existing data from murine CLP models, TLR-2 and TLR-4 expressions are significantly up-regulated in multiple organs[60,61] including the intestine[62] when compared with sham mice. Our data are consistent with the hypothesis that down-regulation of TLR-2/TLR-4 *via* MyD88 in the colon of LGG- or BL-treated mice may play a protective role in attenuating the local and systemic inflammatory response and ultimately the pathophysiology of polymicrobial sepsis.

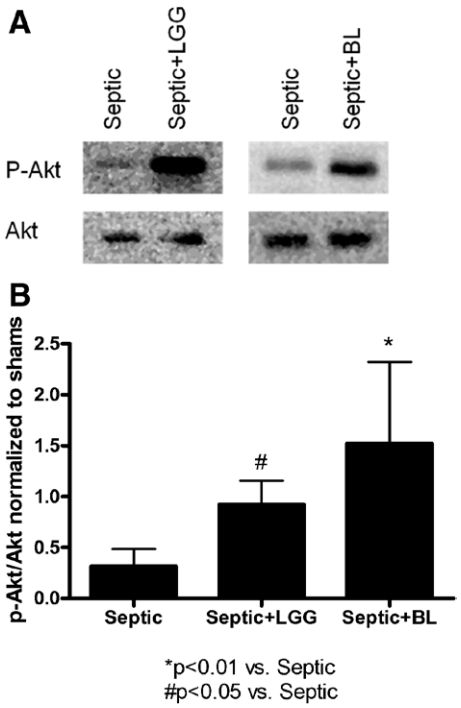

Fig. 6. Effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* treatment on expression of P-Akt/Akt. Representative Western blot for expression of p-AKT and Akt in the colon of all studied groups (*A*). P-Akt/Akt ratio for septic and probiotics-treated septic mice normalized to shams (*L. rhamnosus* GG [LGG], 0.922 ± 0.24 *vs*. septic, 0.3159 ± 0.17, *P* = 0.0004 and *B. longum* [BL], 1.521 ± 0.80 *vs*. septic, 0.3159 ± 0.17, *P* = 0.0017 (*B*). Shams, n = 3 per group; septic, n = 8; LGG, n = 4; BL, n = 4. Data are expressed as the mean ± SD.

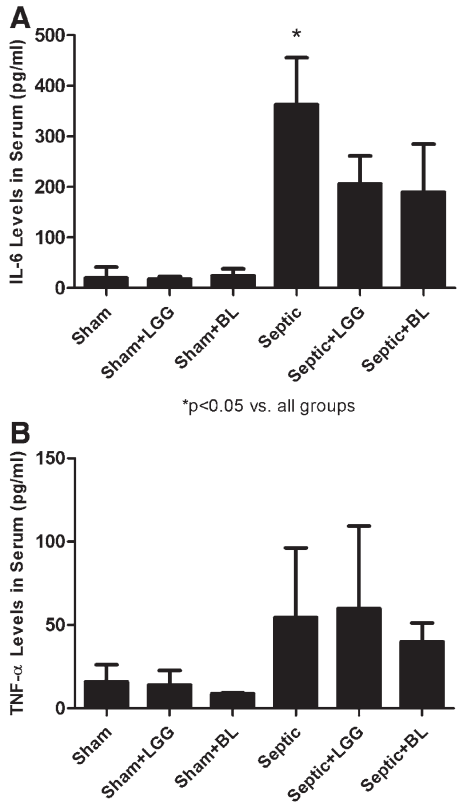

Fig. 7. Effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* treatment on the systemic inflammatory response. Enzyme-linked immunosorbent assay was used to determine the concentrations of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 in serum. (*A*) IL-6 was significantly increased in the serum of septic mice compared with shams (septic, 383.5 ± 93.1 *vs*. shams, 19.8 ± 21.4, *P* = 0.0001), whereas treatment with *L. rhamnosus* GG (LGG) or *B. longum* (BL) in septic mice led to significantly reduced systemic IL-6 levels (LGG, 206.1 ± 55.2 *vs*. septic, 383.5 ± 93.1, *P* = 0.0075 and BL, 189.4 ± 95.2 *vs*. septic, 383.5 ± 93.1, *P* = 0.0069) compared with septic mice not treated with probiotics. (*B*) There were no differences among groups for TNF-α in the serum. Shams, n = 5 per group; septic, n = 7; LGG, n = 5; and BL, n = 6. Data are expressed as the mean ± SD.

Limitations of this research include that we did not administer antibiotics as part of this initial evaluation of early probiotics to prevent progression of infection and sepsis. This was to ensure the adequate colonization of our probiotic therapy, and we plan to do future studies administering antibiotics after treatment with probiotics. Furthermore, time-point studies are being planned currently to examine the effects of both probiotic strains more closely and to explore other possible pathways involved in their protective mechanisms in sepsis. It is important to note that previous clinical trials that have administered antibiotics with probiotic therapy have still noted significant reduction of nosocomial infections (such as ventilator pneumonia) despite antibiotic therapy concomitant with probiotic therapy.[21] Furthermore, killed probiotic organisms have been shown to be as clinically effective in some studies as live probiotic therapy.[63] Finally, in our statistical analysis, we did not adjust our analysis for multiplicity (*i.e.*, multiple comparisons). Given our small sample sizes, and that these are the first experiments of this type reported, such adjustments would decrease the statistical power of our tests. This would lead to an increase in the probability of making a type II error, *i.e.,* the failure to reject a false null hypothesis. In any experimental setting, there is always a balance in type I and type II errors. Presently, for this line of research, we are more concerned with missing a potentially valuable finding that we hope will lead to further investigation.

In conclusion, our data show that early therapy with either LGG or BL can reduce mortality and systemic bacterial translocation in experimental sepsis in weanling mice. These results indicate that potential mechanistic explanations for this clinical benefit may include reduced intestinal epithelial apoptosis and restoration of colonic epithelial cell proliferation, which may be mediated by Akt/protein kinase B signal transduction pathway. Another potential mechanism may be probiotic-mediated attenuation of local and systemic inflammatory response, mediated by down-regulation of TLR-2/TLR-4 signaling pathway *via* MyD88 in the colon.

We believe our data adds to existing clinical trial data supporting the potential efficacy of probiotics in critical

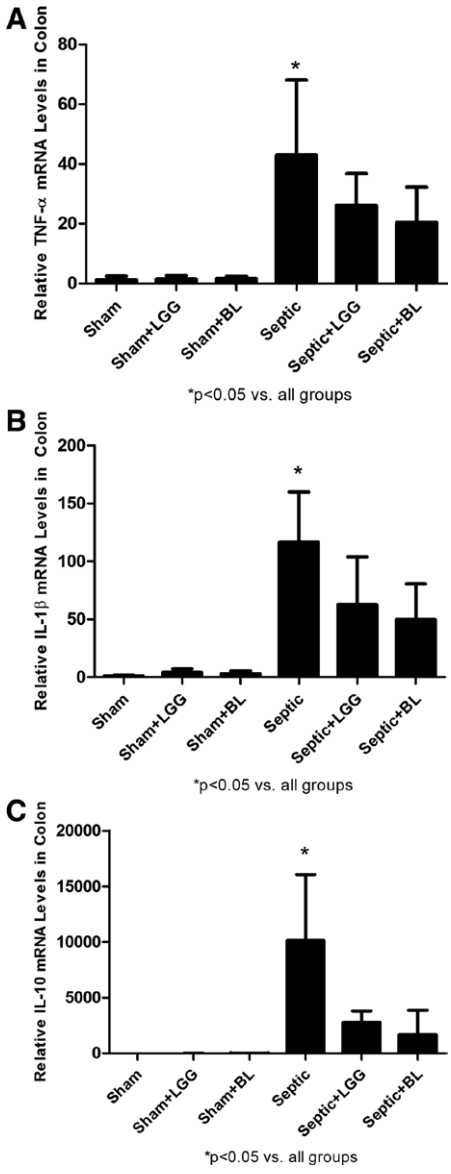

Fig. 8. Effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* treatment on the inflammatory response in the colon. Reverse transcription and real-time polymerase chain reaction assays were performed to quantify steady-state messenger ribonucleic acid (mRNA) levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-10. Gene expression of TNF-α (*A*), IL-1β (*B*), and IL-10 (*C*) was significantly increased in the colon of untreated septic animals (TNF-α: septic, 47.02±25.02 *vs*. shams, 1.31±1.11, $P = 0.019$; IL-1β: septic, 125.82±43.10 *vs*. shams, 1.10±0.57, $P = 0.003$; IL-10: septic, 9,303.45±5,926.29 *vs*. shams, 1.31±1.18, $P = 0.024$), whereas levels of both were markedly decreased in colons of septic mice treated with *L. rhamnosus* GG (LGG; TNF-α, 26.09±10.68 *vs*. septic, 47.02±25.02, $P = 0.049$; IL-1β, 62.53±41.46 *vs.* septic, 125.82±43.10, $P = 0.050$; IL-10: 2,766.34±1,038.60 *vs*. septic, 9,303.45±5,926.29, $P = 0.024$), or *B. longum* (BL; TNF-α: 20.50±11.72 *vs.* septic, 47.02±25.02, $P = 0.027$; IL-1β, 49.67±30.91 *vs.* septic, 125.82±43.10, $P = 0.018$; IL-10: 1,689.26±2,200.26 *vs*. septic, 9,303.45±5,926.29, $P = 0.008$) when compared with untreated septic mice. Shams, n = 4 per group; septic, n = 7; LGG, n = 6; BL, n = 4. Data are expressed as the mean ± SD.

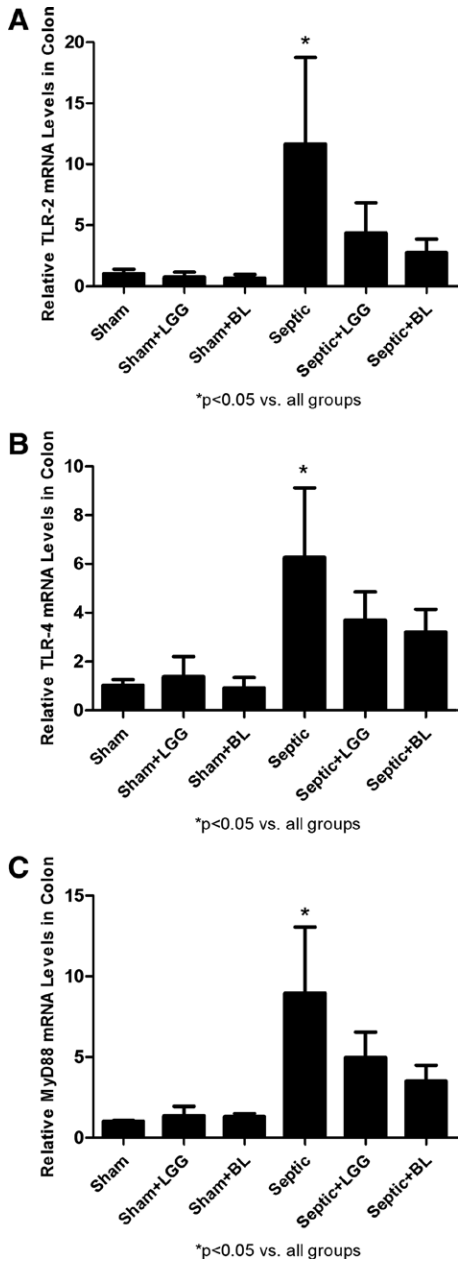

Fig. 9. Effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* treatment on Toll-like receptors (TLRs) and MyD88 gene expression in the colon. Reverse transcription and real-time polymerase chain reaction assays were performed to quantify steady-state messenger ribonucleic acid (mRNA) levels of TLR-2 (*A*), TLR-4 (*B*), and MyD88 (*C*). Levels of TLR-2 (*A*), TLR-4 (*B*), and MyD88 (*C*) were markedly increased in the colon of untreated septic mice compared with shams (TLR-2: 8.94±7.11 *vs*. 1.04±0.36, $P = 0.041$; TLR-4: 6.78±2.85 *vs*. 1.02±0.24, $P = 0.015$; MyD88: 7.85±4.09 *vs*. 1.00±0.07, $P = 0.012$), whereas *L. rhamnosus* GG (LGG; TLR-2: 4.37±2.47 *vs*. 8.94±7.11, $P = 0.039$; TLR-4: 3.69±1.17 *vs*. 6.78±2.85, $P = 0.047$; MyD88: 4.97±1.57 *vs*. 7.85±4.09, $P = 0.046$) or *B. longum* (BL; TLR-2: 2.75±1.11 *vs*. 8.94±7.11, $P = 0.023$; TLR-4: 3.20±0.94 *vs*. 6.78±2.85, $P = 0.029$; MyD88: 3.50±1.00 *vs*. 7.85±4.09, $P = 0.016$) treatment significantly reduced these levels. Shams, n = 4 per group; septic, n = 7; LGG, n = 6; and BL, n = 4. Data are expressed as the mean ± SD.

illness. To this point, the design of larger scale clinical trials and/or more routine clinical administration of live probiotics to children and adults have recently been limited by concerns regarding safety and potential increased risk of infection from the administered probiotics. Although we did not specifically evaluate for bacteremia from our administered probiotic strains, we did observe a statistically significant reduction in overall bacteremia and mortality in our pediatric mice treated with live probiotic bacteria. Furthermore, the results of the recent American Health Care Research and Quality report on the safety of probiotic therapy in over 600 published clinical trials and case reports are reassuring with regard to the safety of probiotic administration,[64] although isolated adverse effects of probiotic administration have been reported.[65] One recent clinical trial studying probiotics in severe pancreatitis (the PROPATRIA trial) found an unexpected increase in mortality in probiotic-treated patients.[66] This trial was unique as it administered multiple strains of probiotic bacteria and prebiotic-like fiber *via* a postpyloric feeding tube (placed in the small bowel). This postpyloric method of administration was associated with an increase in small bowel necrosis, which was subsequently associated with death in a number of patients receiving the prebiotic fiber/probiotic mixture. It is possible that the postpyloric administration of this fiber/multiple probiotic strain mixture in pancreatitis patients may carry significant risk and should likely be avoided.[67] In addition, methodological and safety concerns regarding the conduct of this trial have been raised.[67] In any case, careful and appropriate safety monitoring in all future probiotic clinical trials should be conducted. Perhaps the time has come to proceed with carefully designed, carefully monitored, multicenter randomized clinical trials of probiotic therapy to attempt to reduce the risk of infection, sepsis, and mortality in critically ill children and adults.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

De Jarnette's Optic Chromatic System of Anesthesia

Conceived (1926), marketed (1928), withdrawn (1931), and remarketed (1939), the "Optic Chromatic System of Anesthesia" was invented by Major Bertrand De Jarnette (1899–1992). An engineer, osteopathic physician, and chiropractor, he wrote that "each thalami" [*sic*] of the brain has large masses of sensory fibers [that] pass through." De Jarnette assumed that a "slow movement is never associated with red, while a fast stimuli [*sic*] is never associated with blue." By "the addition of red" color to "blue and deep violet" lenses, De Jarnette produced goggles (*right*) that he "designed for BLOODLESS SURGERY and not orthopedic work." For advertising purposes, the shipping box (*left*) depicts goggles with one lens blue, the other one red. By illuminating goggled patients with a 75 W white light that he first switched on-and-off at a 2:1 ratio and then followed with continuous white light, De Jarnette aimed to lessen pain from chiropractic manipulations involving the "abdomen and pelvis" but not the extremities. As for others' doubts, he noted that "science is never without scoffers." In 1940 De Jarnette filed a U.S. Patent application for his anesthetic goggles. It was declined. (Copyright © the American Society of Anesthesiologists, Inc.)

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