Bifidobacterium bifidum reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis

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Khailova L., Mount Patrick SK, Arganbright KM, Halpern MD, Kinouchi T, Dvorak B. Bifidobacterium bifidum reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 299: G1118–G1127, 2010. First published August 12, 2010; doi:10.1152/ajpgi.00131.2010.—Necrotizing enterocolitis (NEC) is a devastating intestinal disease of neonates, and clinical studies suggest the beneficial effect of probiotics in NEC prevention. Recently, we have shown that administration of Bifidobacterium bifidum protects against NEC in a rat model. Intestinal apoptosis can be suppressed by activation of cyclooxygenase-2 (COX-2) and increased production of prostaglandin E2 (PGE2). The present study investigates the effect of B. bifidum on intestinal apoptosis in the rat NEC model and in an intestinal epithelial cell line (IEC-6), as a mechanism of protection against mucosal injury. Premature rats were divided into the following three groups: dam fed, hand fed with formula (NEC), or hand fed with formula supplemented with B. bifidum (NEC + B. bifidum). Intestinal Toll-like receptor-2 (TLR-2), COX-2, PGE2, and apoptotic regulators were measured. The effect of B. bifidum was verified in IEC-6 cells using a model of cytokine-induced apoptosis. Administration of B. bifidum increased expression of TLR-2, COX-2, and PGE2 and significantly reduced apoptosis in the intestinal epithelium of both in vivo and in vitro models. The Bax-to-Bcl-w ratio was shifted toward cell survival, and the number of cleaved caspase-3 positive cells was markedly decreased in B. bifidum-treated rats. Experiments in IEC-6 cells showed anti-apoptotic effect of B. bifidum. Inhibition of COX-2 signaling blocked the protective effect of B. bifidum treatment in both in vivo and in vitro models. In conclusion, oral administration of B. bifidum activates TLR-2 in the intestinal epithelium. B. bifidum increases expression of COX-2, which leads to higher production of PGE2 in the ileum and protects against intestinal apoptosis associated with NEC. This study indicates the ability of B. bifidum to downregulate apoptosis in the rat NEC model and in IEC-6 cells by a COX-2-dependent matter and suggests a molecular mechanism by which this probiotic reduces mucosal injury and preserves intestinal integrity.

epithelial homeostasis; enteral nutrition; mucosal inflammation; probiotics

NECROTIZING ENTEROCOLITIS (NEC) is the most common gastrointestinal emergency in prematurely born infants. The key risk factors for development of this disease are prematurity, the introduction of enteral feeding, and bacterial colonization (12, 25, 33). After birth, a sterile newborn’s gut is colonized within a few days. Prematurely born infants frequently experience postponed colonization caused by intestinal immaturity and exposure to broad-spectrum antibiotics (9). Probiotic bacteria (Bifidobacterium or Lactobacillus) dominate the intestinal microbiota of breast-fed babies, whereas formula-fed babies have more diverse microbiota (13, 16).

Three clinical studies indicate the beneficial effect of probiotics in the prevention of NEC (1, 19, 29). However, inconsistencies in probiotic mixtures and feeding protocols used make it difficult to address the molecular mechanisms of this protection. A recent report from our laboratory shows that oral administration of B. bifidum protects the small intestine against NEC in the neonatal rat model (24). This protective effect is associated with reduction of inflammation in the ileum, regulation of the mucus layer formation, and improvement of intestinal integrity (24).

Toll-like receptors (TLRs) are pattern-recognition receptors expressed on the surface of immune and intestinal epithelial cells (3). The interaction between enteric bacteria and TLRs is crucial for maintenance of intestinal epithelial homeostasis and essential for mucosal protection against gut injury (38). Because the microbial ligands detected by TLRs originate from both commensal and pathogenic microbes, TLRs are also responsible for protective signals allowing the intestine to tolerate the beneficial microflora (39). TLR-4 has been the most frequently studied in NEC models, and it is suggested that activation of TLR-4 signaling leads to increased intestinal injury (22, 27, 30). In colitis models, TLR-2 protects intestinal mucosa against injury via regulation of epithelial apoptosis (3, 4, 36), and, recently, a role for TLR-2 in NEC pathogenesis was also suggested (30, 41). However, the role of TLR-2 in probiotics-mediated protection against NEC is not known.

Upregulation of cyclooxygenase-2 (COX-2) is known to suppress apoptosis through prostaglandin E2 (PGE2) production in the gut (15, 31). Whereas high levels of intestinal COX-2 are reported in both human (6) and experimental (6, 17) NEC, the role of COX-2 and PGE2 in NEC pathogenesis is still controversial and not fully understood (31).

Intestinal epithelial homeostasis is maintained by balancing the rate between cell proliferation and cell loss, and apoptosis accounts for the majority of cell loss in the gut lumen (37). An important class of molecules that regulate enterocyte apoptosis is the Bcl-2 family (26). Members of the Bcl-2 family are involved in signaling pathways regulating caspase-3 activity necessary for chromatin condensation and DNA fragmentation that characterize apoptosis. The balance of pro- and anti-apoptotic Bcl-2 proteins is critical for cell survival (42). We (8) and others (21) showed that an uncontrolled increase of intestinal epithelial apoptosis leads to severe NEC injury.

The aim of this study was to further explore the molecular mechanisms underlying the protective effect of B. bifidum against experimental NEC, specifically, to determine if oral...
administration of *B. bifidum* in a rat model of NEC activates TLR-2, and whether increased COX-2 and PGE\(_2\) production is involved in downregulation of intestinal epithelial apoptosis in the site of injury. This hypothesis was then verified in an in vitro model using IEC-6 cells.

**MATERIALS AND METHODS**

**Animal Model**

This protocol was approved by the Animal Care and Use Committee of the University of Arizona (A-324801–95081). Seventy-six neonatal Sprague-Dawley rats (Charles River Labs, Pontage, MI) were collected by cesarian section 24 h before their scheduled birth, and the first feeding started 2 h after delivery. Animals were hand fed six times daily with a total volume of 850 µl of rat milk substitute/day (23). Experimental NEC was induced by asphyxia (breathing 100% nitrogen gas for 60 s) and cold stress (4°C for 10 min) twice daily (11). Neonatal rats were divided into the following experimental groups: hand fed with formula (NEC; \(n = 30\)); hand fed with formula containing 5 × 10\(^6\) colony-forming units (CFU) of *B. bifidum* OLB6378 in two feedings per day (NEC + *B. bifidum*; \(n = 30\)); and dam fed littersmates fed by surrogate mothers as a baseline control (DF, \(n = 16\)).

After 96 h, all surviving animals were terminated via decapitation. Animals that developed signs of distress or imminent death before 96 h were terminated and included in the study. Pathological changes in the intestinal architecture were evaluated using our previously described NEC scoring system (24).

**B. Bifidum Culture**

*B. bifidum* OLB6378 was incubated in MRS broth with 0.1% of sodium bicarbonate (Fisher, Wilmington, DE) and 0.1% of L-cysteine hydrochloride monohydrate (Sigma) for 48 h in anaerobic conditions at 37°C. Absorbance at 600 nm was measured to determine the number of CFU per 1 ml. *B. bifidum* was pelleted from the broth (10,000 rpm; 5 min) and washed with PBS before use.

**Cell Culture**

A rat intestinal epithelial cell line, IEC-6 (ATCC, Manassas, VA), was cultured in DMEM containing 5% FBS, 10 mg/l insulin, and 40 mg/ml gentamicin at a density of 1 × 10\(^6\) cells/ml and allowed to adhere overnight. Cells were then rinsed with PBS, and DMEM media containing 0.5% FBS only was used for the 15-min period of *B. bifidum* pretreatment (1 × 10\(^6\) CFU/ml). Cells were washed with PBS and incubated in DMEM media (containing 5% FBS, 10 mg/l insulin, and 40 mg/ml gentamicin) with tumor necrosis factor (TNF)-α and interferon (IFN)-γ at concentrations of 400 ng/ml each for an additional 4 h.

**In Vivo and In Vitro Experiments with COX-2 Inhibitor**

**In vivo study.** Neonatal rats were divided into the following groups in addition to previously described experimental groups: hand fed with formula and injected intraperitoneally one time a day with 5 mg/kg of the COX-2 inhibitor Celecoxib (Sigma, St. Louis, MO) (NEC + C; \(n = 8\)); or hand fed with formula containing 5 × 10\(^6\) CFU of *B. bifidum* OLB6378 in two feedings per day and injected intraperitoneally once a day with 5 mg/kg the COX-2 inhibitor Celecoxib (NEC + C + *B. bifidum*; \(n = 8\)).

**In vitro study.** IEC-6 cells were treated with Celecoxib (1 µM) for 1 h before *B. bifidum* pretreatment and exposure to the cytokine mixture as described above.

**Flow Cytometry**

The annexin V-FITC apoptosis detection kit (Calbiochem, Gibbstown, NJ) was used to identify cell membrane alterations that accompanied programmed cell death by labeling cells with annexin V-FITC and propidium iodide (PI). The procedure was carried out according to the manufacturer’s instructions. The percentage of apoptotic cells was determined using two-color flow cytomter analysis with a FACScan flow cytometer (BD Biosciences, San Jose, CA). Appropriate electronic compensation was adjusted by acquiring cell populations stained with each dye individually, as well as with an unstained control (AZCC/ARL-Division of Biotechnology Cytometry Core Facility http://cytometry.arl.arizona.edu).

**Immunohistology**

**Tissue staining.** A 2-cm section of distal ileum was collected from each animal and fixed overnight in 70% ethanol, paraffin-embedded, and sectioned at 4–6 µm. Serial sections were stained for TLR-2, COX-2, or cleaved caspase-3 (CC-3).

**COX-2 and CC-3.** After deparaffinization and rehydration, sections were blocked with 1.5% rabbit serum (Vector Laboratories, Burlingame, CA) in PBS for 30 min and then incubated with either rabbit polyclonal CC-3 (Cell Signaling, Danvers, MA) or COX-2 (Cayman Chemical, Ann Arbor, MI) antibody for 1 h, washed with PBS, and incubated with goat anti-rabbit biotinylated secondary antibody (Vector Laboratories) for 30 min. Vectastain Elite ABC reagent (Vector Laboratories) was then applied, followed by diaminobenzidine as a substrate. Sections were counterstained with hematoxylin, dehydrated, and covered with a cover slip.

**Cell staining.** IEC-6 cells were fixed with 3% formaldehyde for 30 min, rinsed with PBS, covered with methanol for 6 min, allowed to dry, and, after a PBS wash, were incubated with 1.5% rabbit serum (Vector Laboratories) in PBS for 1 h. Cells were then stained with CC-3 Alexa Fluor 488-conjugated primary antibody (Cell Signaling) at 4°C overnight, washed with PBS, and covered with cover slip with Prolong gold anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI).

**Cell staining.** IEC-6 cells were fixed with 3% formaldehyde for 30 min, rinsed with PBS, covered with methanol for 6 min, allowed to dry, and, after a PBS wash, were incubated with 1.5% rabbit serum (Vector Laboratories) in PBS for 1 h. Cells were then stained with CC-3 Alexa Fluor 488-conjugated primary antibody (Cell Signaling) at 4°C overnight, washed with PBS, and covered with cover slip with Prolong gold anti-fade reagent with DAPI. Goat anti-TLR-2 polyclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with Alexa Fluor 594-conjugated anti-goat secondary antibody (Molecular Probes, Eugene, OR) for 1 h and covered with a cover slip with Prolong gold anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI).

**Cell staining.** IEC-6 cells were fixed with 3% formaldehyde for 30 min, rinsed with PBS, covered with methanol for 6 min, allowed to dry, and, after a PBS wash, were incubated with 1.5% rabbit serum (Vector Laboratories) in PBS for 1 h. Cells were then stained with CC-3 Alexa Fluor 488-conjugated primary antibody (Cell Signaling) at 4°C overnight, washed with PBS, and covered with cover slip with Prolong gold anti-fade reagent with DAPI.

An Olympus IX-70 inverted fluorescent microscope equipped with a ×40 oil immersion objective was used to evaluate CC-3-positive cells and TLR-2 staining.

**Western Blot Analysis**

Individual frozen ileal samples were homogenized with a hand-held homogenizer (Pelec Pestle; Kimble/Kontes, Vineland, NJ) in a ×5 volume of ice-cold homogenization buffer (50 mM Tris·HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.1% SDS; 1% sodium deoxycholic acid; 1% Triton X-100; 50 mM dithiothreitol; 50 µg/ml aprotinin; 50 µg/ml leupeptin; and 5 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatant was collected. Cell lysates from IEC-6 cells were prepared after a wash with PBS using solubilization buffer (20 mM Tris, pH 8.0; 2 mM EDTA; 150 mM NaCl; 1% Triton X-100; and 10 mM SDS).

Total protein concentration was quantified using the Bradford protein assay. For protein analysis, 40 µg (tissue) or 15 µg (cells) of protein were added to an equal volume of ×2 Laemmli sample buffer and boiled for 5 min. The samples were run on 10% polyacrylamide gels at 110 V for 1.5 h. Protein was transferred to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad) at 100 mA for 1.5 h. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (Sigma) for 1 h at room temperature and then
incubated overnight at 4°C with one of the following rabbit polyclonal antibodies: anti-Bax (1:500; BD Pharmigen, San Diego, CA), anti-Bcl-w, (1:100; Stressgen, Ann Arbor, MI), anti-COX-2 (1:200; Abcam, Cambridge, MA), or a goat polyclonal antibody anti-TLR-2 (1:50; Santa Cruz Biotechnology). After extensive washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG (Santa Cruz Biotechnology). Proteins were visualized with a chemiluminescent system (Pierce, Rockford, IL) and exposed to X-ray film.

**PGE2 Enzyme Immunoassay**

The high-sensitivity PGE2 enzyme immunoassay kit (Assay Designs, Ann Arbor, MI) was used to determine the concentration of PGE2 in serum and in ileal tissue homogenates. Trunk blood was collected and centrifuged for 5 min at 5,000 rpm. Serum was collected and stored at −80°C until the assay was performed.

Individual frozen ileal samples were processed in the same manner as for Western blot analysis. Total protein concentration was quantified using the Bradford protein assay. Samples were diluted 1:10 with assay buffer before analysis. Results were calculated per 1 μg of protein.

**RNA Preparation, RT, and Real-Time PCR**

Total RNA was isolated from IEC-6 cells 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 (Thermo Fisher Scientific, Wilmington, DE).

RT and real-time PCR assays were performed to quantify steady-state mRNA levels of COX-2. cDNA was synthesized from 0.2 μg of total RNA. Predeveloped TaqMan primer and probe were used for the detection of COX-2 (Applied Biosystems, Foster City, CA). Reporter dye emission was detected by an automated sequence detector combined with ABI Prism 7700 Sequence Detection System software (Applied Biosystems). Real-time PCR quantification was performed with TaqMan 18S controls.

**Statistics**

Statistical analyses between experimental groups were performed using ANOVA followed by Fisher protected least-significant difference test. The χ2-test was used to analyze the difference in incidence of disease. All numerical data are expressed as means ± SE.

**RESULTS**

**Oral Administration of B. bifidum Reduces the Severity and Incidence of NEC**

As shown previously, ileal damage in rats administered *B. bifidum* was significantly reduced \((P < 0.01)\) to a median histological score of 1.0 compared with 2.0 in the NEC group, and the incidence of NEC was markedly decreased to 17% in the NEC *B. bifidum* group compared with the NEC group with an incidence of 57%. In DF rats, the median histological score was 0.5 and incidence of NEC 0% (24).

![Fig. 1](http://ajpgi.physiology.org/) Effect of oral administration of *Bifidobacterium bifidum* on expression and localization of Toll-like receptor-2 (TLR-2) in the ileum. *A*: representative TLR-2 (90-kDa) bands from Western blot analyses are shown for dam-fed littermates fed by surrogate mothers as a baseline control (DF, \(n = 5\)), littermates hand fed with formula (NEC, \(n = 5\)), and littermates hand fed with formula containing \(5 \times 10^9\) colony-forming units (CFU) of *B. bifidum* OLB6378 in two feedings per day (NEC + *B. bifidum*, \(n = 5\)). All samples were analyzed on the same gel. Mean relative density to β-actin was calculated for all groups. \(*P < 0.05\) vs. NEC and DF. *B*: representative slides from DF, NEC, and NEC + *B. bifidum* groups (\(n = 6\) animals/experimental group). Magnification: ×400.
B. Bifidum Increases Intestinal Expression of TLR-2 in a Rat Model of NEC

In the intestine, cross talk between enteric bacteria and the host is mediated in part by TLRs. Appropriate bacterial colonization and the activation of TLRs play a key role in the pathogenesis of NEC (30, 41), and administration of gram-positive probiotic bacteria increases the expression of TLR-2 in the intestinal epithelium (10). Thus we hypothesized that B. bifidum treatment of experimental NEC will stimulate expression of TLR-2 in the ileal epithelium.

Western blot analysis showed significantly higher levels of TLR-2 in the B. bifidum group compared with the DF and NEC groups (Fig. 1A). The intensity of TLR-2 staining was markedly elevated in ileal tissue of rats receiving B. bifidum. Animals in the NEC group expressed very low intensity of the staining, and, interestingly, staining in the DF group was barely detectable (Fig. 1B). These data suggest the response of the intestine to the presence of B. bifidum in the diet.

B. Bifidum Stimulates COX-2 and PGE2 Production in the Ileum

COX-2 is a rate-limiting enzyme for PGE2 biosynthesis. It is known that overproduction of PGE2 can suppress intestinal epithelial apoptosis (15). Therefore, we evaluated the expression of COX-2 protein in the ileum by immunohistochemistry. In addition, PGE2 production was evaluated in ileal homogenates and in serum using a high-sensitivity PGE2 immunoassay.

Staining for COX-2 revealed more intense staining in the ileum of animals receiving B. bifidum treatment compared with the NEC or DF groups (Fig. 2A). The signal for COX-2 in the NEC and NEC + B. bifidum groups was localized in epithelial cells at the top of the villi. In the DF group, the intensity of COX-2 staining was very low or not detected (Fig. 2A).

The ileal luminal concentration of PGE2 was significantly elevated in the B. bifidum-treated group compared with the DF and NEC groups (P ≤ 0.05 vs. DF or NEC; Fig. 2B). In contrast, the concentration of PGE2 in the serum did not show any significant changes between experimental groups (data not shown). These results suggest activation of COX-2 by B. bifidum administration, which results in increased production of PGE2 in the terminal ileum, the site of injury.

B. Bifidum Blocks Apoptosis in the Ileum of Neonatal Rats

Although severe NEC is associated with extensive bowel necrosis, accelerated apoptosis of the intestinal epithelium is observed in NEC patients (14) and in experimental NEC models (8, 21). To determine if B. bifidum treatment of NEC alters intestinal epithelial cell apoptosis, we evaluated protein levels of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2.

![Fig. 2. Effect of B. bifidum treatment on cyclooxygenase-2 (COX-2) expression and prostaglandin E2 (PGE2) production in the ileal tissue.](image-url)
protein Bcl-w in the ileum of DF, NEC, and NEC + \textit{B. bifidum} rats using Western blot analysis (Fig. 3A).

In the ileum of \textit{B. bifidum}-treated rats, protein levels of pro-apoptotic Bax were significantly decreased compared with the NEC group, whereas levels of anti-apoptotic Bcl-w were increased to levels found in the DF group (Fig. 3A). The Bax-to-Bcl-w ratio was shifted toward cell survival in the NEC + \textit{B. bifidum} group ($P \leq 0.05$; Fig. 3A).

Cleavage of procaspases to active caspases is a hallmark of most apoptotic systems, and the detection of CC-3 is a sensitive indicator of apoptosis (18). To confirm changes in apoptosis, CC-3 staining of ileal tissue was performed and evaluated (Fig. 3B). The number of CC-3-positive cells was significantly decreased in the NEC + \textit{B. bifidum} group compared with the NEC group, similar to values seen in the healthy DF group ($P < 0.05$). There were no positively labeled cells in the crypts.

Proliferation was also evaluated in the ileal sections by proliferating cell nuclear antigen staining. There were no statistically significant changes observed between the groups (data not shown). Thus alteration of intestinal epithelial proliferation can be excluded as a potential mechanism by which \textit{B. bifidum} treatment protects intestinal mucosa against NEC.

\textbf{Inhibition of COX-2 in the Rat Model of NEC Suppresses the Effect of B. Bifidum}

Administration of COX-2 inhibitor to animals treated with \textit{B. bifidum} (NEC + C + \textit{B. bifidum}) resulted in increased

\begin{table}[h]
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\begin{tabular}{|c|c|c|}
\hline
 & DF & NEC + \textit{B. bifidum} \\
\hline
Bax/Bcl-w ratio & 1 $\pm$ 0.4 & 744 $\pm$ 215 $^*$ & 55 $\pm$ 11 \\
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\end{tabular}
\caption{Relative expression of pro-apoptotic Bax and anti-apoptotic Bcl-w.}
\end{table}
incidence (75%) and severity of NEC (median histological score of 2) compared with NEC animals (57%) and animals treated with *B. bifidum* (17%). There was no difference in the incidence or severity of injury between animals in the NEC + C + *B. bifidum* group and the NEC + C group (75%, median histological score of 2).

To determine if inhibition of COX-2 affects changes in apoptosis seen in the ileum of neonatal rats treated with or without *B. bifidum*, CC-3 staining was performed and evaluated. The mean relative number of CC-3 positive cells was significantly increased in the NEC + C + *B. bifidum* (11.5 ± 3.0) and NEC + C (11.6 ± 3.6) groups compared with the NEC + *B. bifidum* group (1.3 ± 0.7) (*P* < 0.01). There was not a statistically significant difference between the NEC group and the NEC + C or NEC + C + *B. bifidum* group. Positively labeled cells were found only at the top of the villi and none in the crypts.

These results suggest not only an important role of COX-2 in NEC pathogenesis as previously shown (17) but also a possible mechanism by which *B. bifidum* protects against development of NEC through a decrease of apoptosis in the ileum.

**B. Bifidum Induces the Expression of TLR-2 in IEC-6 cells**

To verify the anti-apoptotic effect of *B. bifidum* on intestinal epithelium observed in the rat NEC model, we used a cytokine-induced apoptotic model in IEC-6 cells. This model mimics conditions in experimental NEC because TNF-α and IFN-γ are key regulators of inflammation in this disease. The presence of TLR-2 was detected only in cells pretreated with *B. bifidum* (Fig. 4). Control cells and cells exposed to the cytokine mixture only did not exhibit any detectable level of COX-2 signal.

**B. Bifidum Activates COX-2 in IEC-6 Cells**

To clarify the role of COX-2 in intestinal epithelium, we used Real-Time PCR and Western blot analysis to measure mRNA and protein levels of this enzyme in IEC-6 cells. Gene expression of COX-2 was significantly increased in IEC-6 cells pretreated with *B. bifidum* and then exposed to the cytokine mixture compared with control or *B. bifidum* groups (*P* ≤ 0.05; Fig. 5A). Quantification of COX-2 protein using Western blot analysis confirmed the gene expression results. In IEC-6 cells pretreated with *B. bifidum* followed by the exposure to the cytokine mixture, COX-2 protein levels were significantly increased compared with all other groups (*P* ≤ 0.05; Fig. 5B). These data show that the combination of proinflammatory cytokines and *B. bifidum* leads to increased levels of COX-2 in IEC-6 cells, which is similar to what was observed in the animal model.

**B. Bifidum Reduces Apoptosis in IEC-6 cells**

To test the hypothesis that *B. bifidum* reduces epithelial apoptosis, we labeled IEC-6 cells in all experimental groups with annexin V-FITC and PI and analyzed these by flow cytometry. The results revealed a significant decrease of apoptotic cells in IEC-6 cells pretreated with *B. bifidum* and exposed to the cytokine mixture in contrast to cells exposed to the cytokine mixture only (Fig. 6A). Cells pretreated with *B. bifidum* that were not exposed to cytokines did not show any differences from control cells.

Immunofluorescent staining for activated CC-3 was used to determine apoptotic changes in IEC-6 cells exposed to *B. bifidum* treatment (Fig. 6B). Positive cells were counted in 10 different fields, and the ratio of positive cells per total number of cells was plotted (Fig. 6C). Pretreatment of IEC-6 cells with *B. bifidum* before exposure to the cytokine mixture resulted in a significant decrease of CC-3 positive cells (*P* < 0.05) vs. cells treated with the cytokine mixture alone. The results showed a statistically significant increase in the number of CC-3 positive cells when treated with the cytokine mixture compared with control (*P* < 0.05). Thus *B. bifidum* pretreatment blocks cytokine-induced apoptosis in IEC-6 cells.

**TLR-2**

**DAPI**

**B. bifidum**

**TNF + IFN**

Fig. 4. TLR-2 expression in IEC-6 cells evaluated by fluorescent microscopy. Cells were treated with or without tumor necrosis factor (TNF)-α and interferon (IFN)-γ (400 ng/ml each) for 4 h and with or without a 15-min pretreatment with *B. bifidum*. Arrowheads indicate representative TLR-2-expressing cells and their nuclei [stained with 4',6-diamidino-2-phenylindole (DAPI)].
Inhibition of COX-2 Eliminates the Effect of B. Bifidum on Apoptosis in IEC-6 Cells

To further clarify the importance of COX-2 in regulation of apoptosis by B. bifidum, the COX-2 inhibitor Celecoxib was used to pretreat IEC-6 cells, which were labeled with annexin/PI and analyzed by flow cytometry. The number of apoptotic cells was significantly increased in the group pretreated with Celecoxib, treated with B. bifidum, and exposed to the cytokine mixture (31.2/H11006 5.2) compared with the control group pretreated with Celecoxib only (7.4/H11006 3.5). There was no statistically significant difference between the following groups: Celecoxib/H11001 cytokine mixture (18.0/H11006 0.5), Celecoxib/H11001 B. bifidum/H11001 cytokine mixture, Celecoxib/H11001 B. bifidum (28.6/H11006 2.8).

These results confirm the importance of COX-2 in the protection of intestinal epithelium against apoptosis by a B. bifidum treatment.

DISCUSSION

Recently, we reported that B. bifidum OL6378 reduces intestinal injury and improves intestinal integrity in the rat model of NEC (24). In the present study, we demonstrate that oral administration of B. bifidum to NEC rats stimulates TLR-2 expression in the ileal epithelium, enhances epithelial expression of COX-2, and increases intestinal production of PGE2. B. bifidum treatment of experimental NEC also results in a strong anti-apoptotic response in the site of intestinal injury. These results are confirmed in studies with intestinal epithelial cells, which mimic the point of first contact between bacteria and host. Indeed, pretreatment of IEC-6 cells with B. bifidum stimulates TLR-2 and COX-2 expression and blocks cytokine-induced apoptosis in these cells. These data suggest that the downregulation of intestinal apoptosis is a molecular mechanism by which B. bifidum reduces mucosal injury and preserves intestinal integrity.

Bacterial colonization of the gastrointestinal tract begins immediately after birth and is important for the development of immune and digestive functions (12). In contrast to pathogenic organisms such as Bacteroides, Clostridia, and Staphylococcus, probiotics such as Bifidobacterium and Lactobacillus are considered beneficial for the developing intestine (9). Because intestinal colonization can be altered nutritionally, oral administration of probiotic bacteria is a logical approach for establishing healthy intestinal microbiota in neonates. Indeed, clinical studies showed beneficial effects of probiotics in the prevention of NEC, but the molecular and cellular mechanisms were not studied in these trials (1, 19, 29).

The pathogenesis of NEC is unknown, but a breach in the intestinal mucosal barrier may allow bacterial translocation across the epithelium, resulting in an inflammatory response that leads to NEC (9). Previously, we have reported that the
intestinal epithelial barrier is compromised in the rat model of NEC (7) and *B. bifidum* treatment improves formation of epithelial tight junctions (TJ) in the ileum of NEC rats (24). A link between activation of TLR-2 signaling and preservation of intestinal TJ integrity was previously shown in other models of intestinal inflammation. For example, treatment of dextran sodium sulfate-induced colitis with TLR-2 agonist protects TJ integrity and decreases intestinal permeability (3, 4), and mucosal repair is delayed when colitis is induced in TLR-2-deficient mice (3, 38). Results from our study show increased expression of TLR-2 in rats treated with *B. bifidum*. Thus we speculate that elevated TLR-2 in *B. bifidum*-treated animals is responsible for protecting the intestine against acute injury through the formation of functional TJs, resulting in the improvement of intestinal integrity.

Upregulation of COX-2 can lead to suppression of intestinal epithelial apoptosis through production of PGE2 (5, 15). However, the role of COX-2 in NEC pathogenesis is still not clear. High levels of intestinal COX-2 are reported in animal models of NEC, suggesting its pathogenic effects during intestinal inflammation (6, 17). Conversely, administration of a COX-2 inhibitor results in a higher degree of intestinal inflammation in the rat NEC model (17), and studies with COX-2 knockout mice describe increased intestinal damage compared with wild-type controls (40). There have been only a few studies exploring the effect of different probiotics on COX-2 expression in intestinal cells or rat models of colitis. Both in vivo and in vitro studies show decreased COX-2 with probiotic treatment (28, 34, 35) except for *Lactobacillus acidophilus*, which significantly increased COX-2 expression in intestinal cells (34).

Fig. 6. Apoptosis in IEC-6 cells and the effect of *B. bifidum* pretreatment. Cells were treated with or without TNF-α and IFN-γ (400 ng/ml each) for 4 h and with or without a 15-min pretreatment with *B. bifidum*. A: percentage of annexin/propidium iodide (PI)-labeled cells analyzed by flow cytometry. *P ≤ 0.05 vs. control (no treatment); B. *bifidum* (+) TNF + INF (+); and B. *bifidum* (+) TNF + INF (-). B: CC-3-stained cells evaluated by fluorescent microscopy. Arrowheads indicate representative apoptotic cells/nuclei. Magnification: ×400. C: the percentage of CC-3-positive cells. *P ≤ 0.05 vs. control (no treatment); B. *bifidum* (+) TNF + INF (+); and B. *bifidum* (+) TNF + INF (-).
demonstrates how the effects of probiotics vary, and the protective mechanisms depend on each strain. Our results show increased expression of COX-2 in the villus epithelium in animals treated with *B. bifidum* compared with animals with NEC and an increased luminal concentration of PGE₂. Inhibition of COX-2 resulted in loss of *B. bifidum* protective effect against NEC injury in the neonatal rats. We conclude that an increase of COX-2 and consequently higher production of PGE₂ in the ileum plays an important role in protection against NEC. Because PGE₂ is a known anti-apoptotic regulator in epithelial cells (2, 43), we evaluated expression of major apoptotic regulators in the ileum.

Uncontrolled apoptosis may lead to a massive loss of cells in NEC (8, 21). The protective effect of *Lactobacillus* strains against NEC and their ability to regulate intestinal apoptosis was reported (20, 29). Several in vitro studies described the anti-apoptotic effect of lactobacilli and proteins produced by *Lactobacillus GG* in intestinal epithelial cells (20, 44, 45). Furthermore, the probiotic mixture VSL#3 prevented apoptosis in a murine model of colitis (32). Our results are the first to demonstrate that *B. bifidum* regulates apoptosis in the ileum of neonatal rats in favor of cell survival. The Bax-to-Bcl-w ratio clearly shows a decrease of apoptosis in the ileal tissue of the *B. bifidum* group, which is further confirmed with a reduced number of CC-3 positive cells. Taken together, these results indicate that *B. bifidum*-mediated reduction of NEC is associated with a reduction of epithelial apoptosis in the site of injury. Data from studies with COX-2 inhibitor clearly show that COX-2 is involved in apoptosis regulation in the ileum of neonatal rats subjected to the NEC protocol, and we can conclude that *B. bifidum* decreases apoptosis by a COX-2-dependent manner.

Cytokines are key regulators of inflammation in NEC, and TNF-α and IFN-γ are the major cytokines associated with intestinal inflammation. We used these cytokines to induce apoptosis in IEC-6 cells as a relevant condition to NEC and to confirm that *B. bifidum* prevents apoptosis in intestinal epithelial cells in an environment lacking additional stress factors that might be involved in the animal model. Indeed, our results show that TLR-2 was detected only in IEC-6 cells exposed to pretreatment with *B. bifidum*. These findings confirm activation of epithelial TLR-2 in the presence of the gram-positive bacteria. Pretreatment with *B. bifidum* leads to upregulation of COX-2 and reduction of apoptosis in IEC-6 cells. COX-2 inhibition results in an increase of apoptosis in cells treated with *B. bifidum*, confirming the role of COX-2 in the anti-apoptotic mechanism and supporting our findings in the rat NEC model.

In conclusion, oral administration of *B. bifidum* activates TLR-2, increases expression of COX-2, and leads to higher production of PGE₂ in the ileum of rat pups. *B. bifidum* treatment reduces the number of CC-3 positive cells and shifts the ratio between the pro-apoptotic protein Bax and the anti-apoptotic Bcl-w in favor of cell survival, and therefore provides a protection against intestinal apoptosis associated with NEC. Results from our experiments in IEC-6 cells confirm the protective effect of *B. bifidum* against cytokine-induced apoptosis. However, the definitive evidence of a direct link between TLR-2 activation and the upregulation of COX-2 in the intestinal epithelium has yet to be confirmed.

This study shows the ability of *B. bifidum* to downregulate apoptosis in both in vivo and in vitro models of NEC by a COX-2-dependent matter and suggests a molecular mechanism by which this probiotic reduces mucosal injury and preserves intestinal integrity.

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**DISCLOSURES**

No conflicts of interest are declared by the authors.

**REFERENCES**


