Bifidobacterium bifidum improves intestinal integrity in a rat model of necrotizing enterocolitis

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Khai lava L, Dvorak K, Arganbright KM, Halpern MD, Kinouchi T, Yajima M, Dvorak B. Bifidobacterium bifidum improves intestinal integrity in a rat model of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 297: G940–G949, 2009. First published August 27, 2009; doi:10.1152/ajpgi.00141.2009. Neonatal necrotizing enterocolitis (NEC) is a major cause of morbidity and mortality in premature infants. Oral administration of probiotics has been suggested as a promising strategy for prevention of NEC. However, little is known about the mechanism(s) of probiotic-mediated protection against NEC. The aim of this study was to evaluate the effects of Bifidobacterium bifidum treatment on development of NEC, cytokine regulation, and intestinal integrity in a rat model of NEC. Premature rats were divided into three groups: dam fed (DF), hand fed with formula (NEC), or hand fed with formula supplemented with 5 × 10⁹ CFU B. bifidum per day (B. bifidum). All groups were exposed to asphyxia and cold stress to develop NEC. Intestinal injury, mucin and trefoil factor 3 (TFF3) production, cytokine levels, and composition of tight junction (TJ) and adherens junction (AJ) proteins were evaluated in the terminal ileum. B. bifidum decreased the incidence of NEC from 57 to 17%. Increased levels of IL-6, mucin-3, and TFF3 in the ileum of NEC rats was normalized in B. bifidum treated rats. Reduced mucin-2 production in the NEC rats was not affected by B. bifidum. Administration of B. bifidum normalized the expression and localization of TJ and AJ proteins in the ileum compared with animals with NEC. In conclusion, administration of B. bifidum protects against NEC in the neonatal rat model. This protective effect is associated with reduction of inflammatory reaction in the ileum, regulation of main components of mucus layer, and improvement of intestinal integrity.

models does not occur prior to colonization of the intestine by bacteria or in germ-free animal models (6, 57).

Probiotics are living, nonpathogenic microorganisms that colonize the intestine and provide benefit to the host (28). Probiotics currently investigated in neonatal clinical practice are enterally fed normal commensals that do not translocate or cause mucosal injury to the host (21). Among probiotic organisms, Bifidobacterium predominates in the intestinal flora of breast-fed infants; other obligate anaerobes are rarely present (45). Recent clinical studies with NEC patients suggest that oral administration of probiotics is beneficial in the prevention of this disease (3, 39). However, selection of the optimal strain or a mixture of probiotic bacteria is critical to obtain desired protective effects, and the mechanisms of probiotic reduction of NEC are not well understood.

Cytokines are key regulators in inflammation in NEC, and several inflammatory and immune regulatory cytokines are dysregulated in this disease (16, 58). Among them, TNF-α, IL-10, and IL-6 are thought to have a diagnostic value in sepsis and in NEC (8, 14, 47). In vitro and in vivo studies with probiotics suggest their ability to decrease proinflammatory cytokines and activate the production of anti-inflammatory cytokines. Interestingly, not all probiotic strains have the same effect on the immune system, with differing immunological effects even within the same species of bacteria (18, 29).

It has been suggested that inflammation may initiate mucosal damage during NEC pathogenesis (49). There are several factors that contribute to intestinal barrier injury, such as the mucus coat, secretion of antimicrobial factors, and enterocyte cell junctions (24, 69). The intestinal epithelium protects tissue against oxidative stress and invasion by microbes through the production of mucins and trefoil factors (TFF). There are two main classes of mucins: 1) secreted gel-forming mucins and 2) membrane-bound mucins (54). In the rat small intestine, Muc2 is the predominant secretory mucin produced by goblet cells, whereas Muc3 is the major membrane-bound mucin detected in goblet cells and enterocytes (1, 2). Mucins are cosecreted with TFFs, small peptides exerting multiple biological effects on epithelium. Trefoil factor 3 (TFF3) is the most abundant in the intestine (65) contributing to the viscoelastic properties of the mucus layer (66) and modulating epithelial healing processes (64). Impaired production of MUC2 and TFF3 has been reported in clinical and experimental NEC (12, 67), and intraperitoneal administration of TFF3 reduced NEC-like injury in neonatal rats (59, 70).

Studies using intestinal epithelial cells (IEC) have suggested that some probiotic strains may stabilize protective responses, including enhancement of epithelial barrier functions (56),...
mucin secretion (43), and stabilization of tight junction (TJ) structure (51). Formation of functional TJs and adherens junctions (AJs) is critical for the maintenance of gut permeability and intestinal barrier function. TJs form continuous intercellular contacts between epithelial cells and create a dynamic barrier to the paracellular movement of water, solutes, and immune cells (17). Several TJ proteins have been identified; among them the transmembrane proteins occludin and claudins are considered crucial for creating functional TJs in neonatal intestine (12).

AJs are another type of cellular connection anchoring cells one to another and attaching to components of the intracellular matrix. AJs are composed of transmembrane and cytosolic components. Cadherins and catenins are the two major families of proteins involved in AJs structure (5).

The aims of this study were to determine whether oral administration of Bifidobacterium bifidum OLB6378 (NITE BP-31, Meiji Dairies, Odawara, Japan) protects against experimental NEC and what the possible mechanisms involved in this process are. In a rat model of NEC, we evaluated the efficacy of B. bifidum treatment on disease development, the effect on mucus and TFF3 expression, and inflammatory cytokine production. Intestinal protein expression and histological localization of the TJ and AJ proteins were also determined.

**MATERIALS AND METHODS**

**Experimental design.** 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 Laboratories, Pontage, MI) were collected by caesarian 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 per day (34). Experimental NEC was induced by asphyxia (breathing 100% nitrogen gas for 60 s) and cold stress (4°C for 10 min) twice daily (15). Caesarian section-delivered pups were divided into the following experimental groups: neonatal rats hand fed with formula (NEC; n = 30), neonatal rats hand fed with formula containing 5 × 10⁶ CFU per day of Bifidobacterium bifidum OLB6378 (B. bifidum; n = 30), and dam-fed littermates fed by surrogates 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.

**NEC evaluation.** After termination, a 2-cm piece of distal ileum was removed and fixed in 70% ethanol, paraffin embedded, sectioned at 4–6 μm, and stained with hematoxylin and eosin for histological evaluation of NEC. Pathological changes in intestinal architecture were evaluated by use of our previously published NEC scoring system (15). Histological changes in the ileum were scored by a blinded evaluator and graded as follows: 0 (normal), no damage; 1 (mild), slight submucosal and/or lamina propria separation; 2 (moderate), moderate separation of submucosa and/or lamina propria, and/or edema in submucosal and muscular layers; 3 (severe), severe separation of submucosa and/or lamina propria, and/or severe edema in submucosa and muscular layers, region villous sloughing; 4 (necrosis), loss of villi and necrosis (Fig. 1). Intermediate scores of 0.5, 1.5, 2.5, and 3.5 were also utilized to more accurately assess levels of ileal damage when necessary (15, 26). To determine incidence of NEC, animals with histological scores of less than 2 have not developed NEC; animals with histological scores of 2 or greater have developed NEC (Fig. 1).

**RNA preparation.** Total RNA was isolated from ileal tissue (snap frozen in liquid N₂) using the RNEasy Plus Mini Kit (Qiagen, Santa Clarita, CA) as described in the manufacturer’s protocol. RNA concentration was quantified by ultraviolet spectrophotometry at 260 nm, and the purity was determined by the A260/A280 ratio (SPECTRmax PLUS; Molecular Devices, Sunnydale, CA). The integrity of RNA was verified by electrophoresis on a 1.2% agarose gel (12).

**RT and real-time PCR.** RT real-time PCR assays were performed to quantify steady-state mRNA levels of Muc2, Muc3, Tff3, and...
selected cytokines (IL-1β, IL-6, IL-10, IL-12, IL-18, and TNF-α). cDNA was synthesized from 0.5 μg of total RNA. Real-time PCR amplification was performed using Primer Express Software (Applied Biosystems). Target probe was labeled with fluorescent reporter dye FAM. The following sequences were used: Muc2 (GenBank BC036170): sense primer 5′-actgggaattgacgtcactg-3′, antisense primer 5′-acccgtgttagtagtaacctgctc-3′, and probe 5′-aacaagttgtgctgcc-3′. TNF-α (GenBank X66539): sense primer 5′-gtgacgctggcaacacagga-3′, antisense primer 3′-gggccatgagctagtga-3′, and probe 5′-cctagggcaactc-3′.

Predevised TaqMan primers and probes were used for the detection of Muc3, Tff3, IL-1β, IL-6, IL-10, IL-12, and IL-18. Reporter dye emission was detected by an automated sequence detector with ABI Prism 7700 Sequence Detection System software (Applied Biosystems). Real-time PCR quantification was then performed with TaqMan 18S controls.

**Immunohistology and enumeration of Muc2- and Tff3-positive cells.** 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 either Muc2 or Tff3. Briefly, after deparaffinization and rehydration, sections were blocked with 1.5% goat serum (Vector Laboratories, Burlingame, CA) in PBS for 30 min, then incubated with either rabbit anti-Muc2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit polyclonal Tff3 antibody (63) for 30 min, washed with PBS three times, and incubated with a biotinylated goat anti-rabbit 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 mounted on coverslips. Muc2 and Tff3-positive cells were counted from nine animals per experimental group, and the total number of epithelial cells per crypt-villus unit was also enumerated. Ten crypt-villus units were counted for each animal.

There is currently no commercially available anti-rat Muc3 antibody. Anti-human Muc3 antibody (Santa Cruz Biotechnology) was unsuccessfully tested on our rat intestinal tissue samples.

**Western blot.** Individual frozen ileal samples were homogenized with a hand-held homogenizer (Pellet 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% Na-deoxycholic acid; 1% Triton X-100; 50 mM DTT; 50 μg/ml aprotinin; 50 μg/ml leupeptin; 5 mM PMSF). The homogenates were centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was collected. Total protein concentration was quantified by use of the Bradford protein assay (7). For protein analysis, 40 μg of protein was added to an equal volume of 2× Laemmli sample buffer and boiled for 5 min. The samples were run on a 10 or 12% polyacrylamide gel (Bio-Rad, Hercules, CA) at 110 V for 1.5 h. Protein was transferred to Immuno-Blot PVDF membranes (Bio-Rad) at 50–60 V for 1 h. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (Sigma, St. Louis, MO) for 1 h at room temperature and then incubated with one of the following rabbit polyclonal antibodies: anti-occludin, anti-claudin-1, anti-claudin-2, anti-claudin-3, anti-β-catenin (Zymed Laboratories, San Francisco, CA) or mouse monoclonal antibodies: anti-E-cadherin (BD Biosciences, San Jose, CA), anti-α-catenin (Zymed Laboratories) antibody overnight at 4°C. After extensive washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology). Proteins were visualized with a chemiluminescent system (Pierce, Rockford, IL) and exposed to X-ray film.

**Immunostaining of TJ and AJ proteins.** After deparaffinization and rehydration, sections were blocked in 5% BSA to prevent nonspecific staining and incubated with one of the following rabbit polyclonal antibodies: anti-occludin, anti-claudin-1, anti-claudin-2, anti-claudin-3, anti-β-catenin (Zymed Laboratories) or mouse monoclonal antibodies: anti-E-cadherin (BD Biosciences), anti-α-catenin (Zymed Laboratories), followed by incubation with Alexa-594 conjugated anti-rabbit or anti-mouse secondary antibody (Molecular Probes, Eugene, OR) and mounted with Vectashield Hard Set Mounting Medium containing DAPI as a nuclear counterstain (Vector Laboratories).

**Negative control sections** were treated with the same procedure in the absence of primary antibody; no immunostaining was observed in the controls (not shown). Sections from each experimental group were immunostained for a specific antigen at the same time. Confocal laser scanning microscope (Zeiss LSM 510 NLO/META) was used for imaging TJ proteins. Imaging of AJ proteins was performed with an inverted fluorescence microscope (Nikon TE-300).

**Statistics.** Statistical analyses between DF, NEC, and *B. bifidum* groups were performed by ANOVA followed by Fisher paired least significance test. The χ² test was utilized to analyze difference in incidence of disease. All statistical analyses were conducted by use of the statistical program StatView for Macintosh computers (Abacus Concepts, Berkeley, CA). All numerical data are expressed as means ± SE.

**RESULTS**

**Oral administration of *B. bifidum* reduces the severity and incidence of NEC.** The degree of intestinal injury and the incidence of NEC were evaluated in prematurely born rats treated with or without *B. bifidum*. Ileal damage in rats administered *B. bifidum* was significantly reduced (*P* ≤ 0.01) to a median histological NEC score of 1.0 compared with 2.0 in the NEC group (Fig. 2A). The incidence of NEC (Fig. 2B) was markedly decreased to 17% (5/29) in the *B. bifidum* group compared with the NEC group with NEC incidence of 57% (16/28). In DF rats, the median histological score was 0.5 and incidence of NEC was 0% (0/16). The survival rates for these studies were as follows: DF, 16/16; NEC, 28/30; and *B. bifidum*, 29/30.

**Evaluation of inflammatory response in the ileum.** Cytokines are key regulators in inflammation and several cytokines are dysregulated in this disease (58). Gene expression of selected inflammatory cytokines in the terminal ileum was determined by RT-PCR. Proinflammatory IL-1β, IL-6, IL-12, IL-18, and TNF-α, and anti-inflammatory IL-10 are the major cytokines associated with NEC pathogenesis and neonatal sepsis (25, 58). IL-6 expression was significantly increased in animals with NEC and decreased in the *B. bifidum* group to levels found in DF animals (Fig. 3). TNF-α expression was significantly increased in NEC animals compared to DF. There was no statistically significant change in TNF expression between the NEC and *B. bifidum* groups. Oral administration of *B. bifidum* did not have any significant effect on gene expression of IL-1β, IL-10, IL-12, and IL-18 (not shown) in the site of injury.

**Changes in gene expression of mucins and Tff3 in the ileum.** The mucus layer is an essential part of intestinal barrier function. Ileal gene expression of two major mucins (Muc2 and Muc3) and Tff3 was evaluated by real-time PCR (Table 1). Muc2 mRNA levels in the ileum were significantly decreased to 80% (4.58 ± 0.32) in the NEC group (*P* ≤ 0.01). In contrast, Muc3 mRNA levels were significantly increased in both the NEC and *B. bifidum* groups compared with the DF group (*P* ≤ 0.01). The changes in gene expression of Muc3 and Tff3 demonstrated significant differences between the NEC and *B. bifidum* groups. Oral administration of *B. bifidum* did not have any significant effect on gene expression of IL-1β, IL-10, IL-12, and IL-18 in the ileum.
Evaluation of Muc2 and Tff3 production. Ileal Muc2 production was evaluated by immunohistochemistry, and enumeration of Muc2-positive cells in the ileum was compared among all experimental groups (Fig. 4). Muc2 staining was significantly reduced \((P \leq 0.01)\) in both the NEC and \(B. \ bifidum\) groups compared with DF controls, but there was no difference between the NEC and \(B. \ bifidum\) groups (Fig. 4).

The production of ileal Tff3 was evaluated by enumeration of Tff3 positively stained cells. There was a significant increase of Tff3-positive cells in the ileal tissue from NEC animals compared with DF and \(B. \ bifidum\)-treated animals \((P \leq 0.01)\).

Occludin and claudin-3 expression and localization in the ileum. Major changes in the distribution and content of TJ proteins, such as occludin and claudin-3 in the ileum of NEC rats, correspond with increased intestinal permeability in these animals (12). To determine whether \(B. \ bifidum\) treatment of experimental NEC normalizes these changes, we quantified protein expression of occludin, claudin-1, -2, and -3 by Western blot analysis and evaluated their histological localization by immunofluorescence microscopy.

Western blot analysis of occludin showed significantly higher levels in the NEC group compared to the DF and \(B. \ bifidum\) groups (Fig. 5A). Confocal microscopy revealed that in NEC animals, occludin was localized in the cytoplasm of ileal enterocytes as well as associated with the membrane. Occludin was expressed at higher intensity throughout the entire length of villi (Fig. 5B, b and e). \(B. \ bifidum\) treatment decreased the intensity of the signal and cellular distribution was mainly in the crypt epithelial cells similar to that seen in DF animals (Fig. 5B, a and d).

Previous studies from our laboratory revealed that among the claudin family members, expression of claudin-3 was markedly altered during NEC pathogenesis (12). Western blot analysis revealed a significant increase of claudin-3 in NEC animals compared to DF and \(B. \ bifidum\) treated rats (Fig. 6A). In the ileal tissue of NEC animals, claudin-3 was localized mainly in the crypt epithelial cells similar to that seen in DF animals (Fig. 6B, c and f; arrows).

The signal was more pronounced at the top of the villi compared with the crypts. \(B. \ bifidum\) treatment resulted in reduction of claudin-3 signal as well as its association with the membrane of enterocytes in the crypts (Fig. 6B, f; arrows) compared with NEC animals. Interestingly, in the DF group, intensity of the claudin-3 staining was very low (Fig. 6B, a and d). These data suggest the influence of stress and hand feeding on the early formation of TJs in the NEC and \(B. \ bifidum\) animals. On the basis of the distribution pattern of claudin-3 in

Table 1. Effect of enteral Bidobacterium bifidum on Muc2, Muc3, and Tff3 mRNA expression

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<thead>
<tr>
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<th>DF NEC</th>
<th>B. bifidum</th>
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<tr>
<td>Muc2</td>
<td>1.00±0.11</td>
<td>0.70±0.07*</td>
</tr>
<tr>
<td>Muc3</td>
<td>1.00±0.08</td>
<td>2.51±0.33*</td>
</tr>
<tr>
<td>Tff3</td>
<td>1.00±0.12</td>
<td>1.06±0.06</td>
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Values are means ± SE; \(n = 8–10\) animals/experimental group. The mean steady-state mRNA level for the dam-fed (DF) group was assigned a value of 1.00, and mean mRNA levels from the necrotizing enterocolitis (NEC) and \(B. \ bifidum\) groups were determined relative to this number. \(*P \leq 0.01\) vs. DF. \(†P \leq 0.01\) vs. NEC.
low levels of these commensal bacteria may allow for colonization of the ileum of neonatal rats. A: Muc2-stained representative slides from DF, NEC, and B. bifidum groups are shown. Magnification: ×400. Enumeration of Muc2-positive cells in neonatal rat ileum is shown in the graph (n = 9 animals/experimental group). Data are expressed as mean Muc2-positive cells/100 epithelial cells ± SE. *P ≤ 0.01 vs. DF. B: Tff3-stained representative slides from DF, NEC, and B. bifidum groups are shown. Magnification: ×400. Enumeration of Tff3-positive cells in neonatal rat ileum is shown in the graph (n = 9 animals/experimental group). Numbers are expressed as mean Tff3-positive cells/100 epithelial cells ± SE. *P ≤ 0.01 vs. DF, *P ≤ 0.01 vs. DF.

DISCUSSION

Our study shows that oral administration of B. bifidum OLB6378 reduces the incidence and severity of NEC in a neonatal rat model. Mechanisms of bifidobacterial-mediated reduction of experimental NEC include reduced cytokine expression in the site of injury and improved development of cellular junctional proteins in the intestinal epithelium. Interestingly, treatment with B. bifidum does not affect ileal Muc2 production but normalizes the production of Tff3 in the site of injury.

NEC is a multifactorial disease involving three major risk factors: prematurity, enteral feeding, and bacterial colonization. Inappropriate intestinal colonization with unfavorable bacteria is likely a critical factor in NEC pathogenesis (13). Both clinical and experimental studies have shown that oral administration of probiotics has beneficial effects and results in decreased incidence of NEC (3, 10, 39, 61). However, not all probiotics have the same protective effects and the safety of administering live bacteria to premature babies must be considered. Selection of the right probiotic strain seems to be critical to achieve the desirable protective effect.

Bifidobacterium is the predominant organism among the intestinal flora found in breastfed infants (45). However, bifidobacteria and lactobacilli in the stool from extremely low-birth-weight infants during the first month of life represent only 5% of detected bacterial species (20). Thus low levels of these commensal bacteria may allow for colonization of the ileum of neonatal rats and may contribute to NEC pathogenesis. Further studies are needed to understand the mechanisms by which B. bifidum can protect against NEC in infants.
intestinal tract with other bacterial species, including pathogenetic bacteria (45). Bifidobacterium bifidum OLB6378 (NITE BP-31, Meiji Dairies) was originally isolated from human neonates. This strain induces strong IgA production in mouse Peyer’s patches and polymeric Ig receptor production in the human colon carcinoma cell line HT-29 (48). In addition, this strain is resistant to low gastric pH and bile acids and has anti-inflammatory properties (unpublished data). Therefore, we selected this probiotic strain for further evaluation of protective effects against intestinal injury in our neonatal rat model of NEC. Our results demonstrate that oral administration of live B. bifidum OLB6378 significantly reduced the incidence of NEC (from 57 to 17%) and severity of ileal damage in the rat NEC model.

The mechanisms underlying the protective effects of probiotics on the developing intestine are not fully understood. However, it has been suggested that bacterial endotoxin can trigger an innate immune response characterized by cytokine production (60). Our and other laboratories have shown that proinflammatory cytokines are important factors contributing to NEC pathogenesis (25, 27, 58). Clinical studies have reported increased serum levels of TNF-α and IL-6 in NEC patients (58). Interestingly, experimental studies indicate that administration of probiotics (such as Bifidobacterium) may alter intestinal production of proinflammatory cytokines (18, 29, 50) via the reduction of intestinal luminal pH, elevated production of antimicrobial substances, and consequently the inhibition of growth of pathogenic bacteria (29). In the present study, we have found that ileal gene expression of TNF-α and IL-6 was significantly increased in the ileum of NEC rats compared with healthy controls. In animals fed with formula supplemented with B. bifidum, ileal IL-6 levels but not TNF-α were normalized to values seen in controls. Thus bifidobacterium-mediated protection against NEC may be regulated via inflammatory mediators, such as IL-6.

Some probiotic strains may moderate a non-immune-related protection mechanism such as acting on barrier integrity (38). Studies using intestinal epithelial cells have suggested that probiotic treatment of IEC stimulates mucin secretion (43) and the enhancement of epithelial barrier functions (56), reduces enterocyte apoptosis (32, 40), and stabilizes formation of cellular TJs (51, 52). Despite recent advances in understanding probiotic actions on IEC, little is known about the mechanisms of protection from in vivo models of gastrointestinal diseases. Both in vitro and in vivo studies showed increased Muc2 and Muc3 gene expression in these cells and tissues after exposure to VSL#3 (a clinically tested probiotic formula consisting of three bacterial groups: Lactobacilli, Bifidobacteria, and Streptococci). Among these groups, only the Lactobacilli species were responsible for increased Muc2 secretion (not Bifidobacteria or Streptococci) (9).

Intestinal mucins and trefoil factors are part of a complex regulatory network that generates the first line of host defense against enteric pathogens. Alterations of this complex may play a role in the pathogenesis of NEC (46, 67). TFF3 is a key peptide in mucosal protection and repair, and its overprodu-
tion is observed in a variety of gastrointestinal inflammatory conditions (53). Intestinal MUC2 is secreted solely by goblet cells, whereas membrane-bound MUC3 is expressed in both goblet cells and enterocytes (11). Previously, we have reported the reduction of goblet cell density and abnormalities in goblet cell morphology in the ileum of rats with NEC (12). Results from the present study further elucidate major components of intestinal mucus layer. In the NEC group, a decrease in ileal Muc2 expression is concomitant with an increase in Muc3 expression and Tff3 production. A similar pattern has been recently shown in rats with colitis where in both the small and large intestine, Muc2 expression was significantly decreased and Muc3 expression was increased (1). The authors concluded that colitis-induced injury causes mucosal barrier dysfunction not only in the colon, but also in the adjoining ileum (1). In our study, we speculate that alteration in goblet cell function leads to the reduction of Muc2 layer that functions to protect the delicate neonatal epithelium against injury. Increased expression of nonsecretory Muc3 and Tff3 peptide suggest an activation of acute repair mechanisms in the site of injury: neonatal ileal epithelium.

The exposure to formula feeding, hypoxia, and stress leads to a delay in colonization by normal commensal bacteria and increases risk of colonization by pathogenic species (13). The presence of bacterial pathogens regulates mucin synthesis and secretion in adult intestine (41). However, little is known about the role of microbiota on goblet cell maturation, Tff3, and mucin production in neonatal gut. In our study, Muc2 gene expression and enumeration of Muc2-positive cells was reduced in the ileum of rats receiving B. bifidum compared with the controls. Muc3 mRNA levels were also significantly decreased in the B. bifidum group compared with the NEC group. In addition, B. bifidum treatment of NEC markedly reduced number of Tff3-positive cells to values seen in normal healthy controls. We speculate that oral administration of B. bifidum decreases colonization on neonatal gut by pathogenic bacterial species, leading to the reduction of inflammation and Tff3 to the levels seen in dam-fed healthy controls.

Probiotics were shown to maintain integrity of the mucosal barrier, reducing its permeability, and strengthening intestinal TJs (44, 62). Epithelial TJ proteins determine intestinal barrier function by creating a barrier to the paracellular movement of solutes. The primary proteins identified as TJ-specific integral transmembrane proteins are occludin and claudins. In our previous work, we showed that these TJs are altered during NEC pathogenesis and intestinal paracellular permeability is increased in NEC animals as a result of these changes at the TJ barrier (12). Occludin is considered to be the primary sealing and integral protein of the TJs, whereas claudins are thought to be the proteins regulating the size selectivity of the TJ barrier. Results from our study indicate that expression and localization of occludin in the ileum of B. bifidum-treated animals has a similar pattern as seen in healthy controls. The strongest signal is observed in crypt epithelial cells and is gradually decreased...
toward the tip of villi. In contrast, NEC animals have occludin distributed within the enterocyte cytoplasm throughout the villi. Similar distribution is observed with claudin-3. We speculate that altered distribution pattern of these two TJ proteins contributes to increased intestinal permeability and disassembly of TJ leading to a breach in mucosal barrier in NEC animals. Treatment with *B. bifidum* prevents these pathological changes by protecting intestinal barrier integrity, thus blocking translocation of luminal toxins into systemic circulation.

Although increased intestinal permeability is typically associated with decreased TJ protein expression, our data were obtained from neonatal intestinal epithelium in contrast to most of the studies done in adult intestinal tissue (19, 37, 55). In one study, using *Bifidobacterium infantis* as a treatment in neonatal rat model of NEC, there were no changes in mucosal permeability (10). However, there are no studies showing TJ distribution or their levels after probiotic treatment in this model. Our study is the first to describe the distribution pattern of epithelial TJ proteins in the intestine of neonatal rats after treatment with *B. bifidum*.

AJs are another type of cellular connection anchoring cells one to another and attaching to components of the intracellular matrix. AJs play an important role in organogenesis of epithelial tissue and are composed of transmembrane and cytosolic components. Cadherins and catenins are the two major families of proteins involved in AJ structure (5). The extracellular domain of E-cadherin is essential for connecting cells and the intracellular domain regulates cell-to-cell contact and is directly associated with the actin cytoskeleton via α-catenin and β-catenin (22, 35). The development of functional AJs during the early postnatal period is not fully understood. This study describes for the first time the localization of the major AJ proteins in the ileum of neonatal rats and shows dramatic changes between healthy DFs, animals with NEC, and rats treated with *B. bifidum*. When the intestinal epithelium is damaged, the process of immediate repair starts to preserve the epithelial barrier and involves cell migration and proliferation. During epithelial restitution, cell junctions must rapidly disassemble and reassemble, which means that catenin-cadherin-mediated cell-to-cell contacts are transiently dissociated (23). We found that AJ proteins are dispersed in the cytoplasm of ileal enterocytes of NEC animals compared with samples from healthy controls and animals treated with *B. bifidum*. Disassembled AJs, because of the ileal damage in the NEC group, can also contribute to the “leakiness” of the tissue. In the DF and *B. bifidum* groups, with no or much smaller damage to the tissue respectively, AJ proteins were clearly associated with the membrane of enterocytes.

In conclusion, this study shows the protective effect of orally administered live *B. bifidum* OLB6378 against NEC injury in

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**Fig. 7. Localization of adherens junction proteins (α-catenin, β-catenin, and E-cadherin) evaluated by inverted fluorescence microscopy. Representative slides from DF, NEC, and *B. bifidum* groups are shown (n = 6 animals/experimental group). No signal was observed in negative control sections (not shown). Magnification: ×400.**
the neonatal rat model. The molecular mechanisms associated with these protective effects include prevention of inflammation in the ileum and improvement of intestinal integrity. Alterations in the epithelial TJ and AJ structure may be a crucial factor by which probiotics protect intestinal barrier function. We are currently investigating the beneficial effects of various probiotic strains in this model.

GRANTS

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