Secreted bioactive factors from *Bifidobacterium infantis* enhance epithelial cell barrier function

Julia B. Ewaschuk, Hugo Diaz, Liisa Meddings, Brendan Diederichs, Andrea Dmytrash, Jody Backer, Mirjam Looijer-van Langen, and Karen L. Madsen

*Department of Medicine, University of Alberta, Edmonton, Alberta, Canada*

Submitted 10 March 2008; accepted in final form 1 September 2008

Ewaschuk JB, Diaz H, Meddings L, Diederichs B, Dmytrash A, Backer J, Looijer-van Langen M, Madsen KL. Secreted bioactive factors from *Bifidobacterium infantis* enhance epithelial cell barrier function. *Am J Physiol Gastrointest Liver Physiol* 295: G1025–G1034, 2008. First published September 11, 2008; doi:10.1152/ajpgi.90227.2008.—Live probiotic bacteria are effective in reducing gut permeability and inflammation. We have previously shown that probiotics release peptide bioactive factors that modulate epithelial resistance in vitro. The objectives of this study were to determine the impact of factors released from *Bifidobacterium infantis* on intestinal epithelial cell permeability and tight junction proteins and to assess whether these factors retain their bioactivity when administered to IL-10-deficient mice. *B. infantis* conditioned medium (BiCM) was applied to T84 human epithelial cells in the presence and absence of TNF-α and IFN-γ. Transepithelial resistance (TER), tight junction proteins [claudins 1, 2, 3, and 4, zonula occludens (ZO)-1, and occludin] and MAP kinase activity (p38 and ERK) were examined. Acute effects of BiCM on intestinal permeability were assessed in colons from IL-10-deficient mice in Ussing chambers. A separate group of IL-1-deficient mice was treated with BiCM for 4 wk and then assessed for intestinal histological injury, cytokine levels, epithelial permeability, and immune response to bacterial antigens. In T84 cells, BiCM increased TER, decreased claudin-2, and increased ZO-1 and occludin expression. This was associated with enhanced levels of phospho-ERK and decreased levels of phospho-p38. BiCM prevented TNF-α- and IFN-γ-induced drops in TER and rearrangement of tight junction proteins. Inhibition of ERK prevented the BiCM-induced increase in TER and attenuated the protection from TNF-α and IFN-γ. Oral BiCM administration acutely reduced colonic permeability in mice whereas long-term BiCM treatment in IL-10-deficient mice attenuated inflammation, normalized colonic permeability, and decreased colonic and splenic IFN-γ secretion. In conclusion, peptide bioactive factors from *B. infantis* retain their biological activity in vivo and are effective in normalizing gut permeability and improving disease in an animal model of colitis. The effects of BiCM are mediated in part by changes in MAP kinases and tight junction proteins.

interleukin-10; intestine; tight junctions; permeability; cytokine; probiotics; *Bifidobacterium* sp

Humans normally exist in a remarkably harmonious relationship with over 500 species of intestinal microbes (8). Aberrant immune responses to luminal microbes can result in chronic inflammation in the gut, and numerous lines of inquiry have implicated bacteria in the development of inflammatory bowel diseases (IBD) (10, 46). Identification of the link between intestinal microflora and IBD has led to an abundance of studies investigating the therapeutic potential of bacterial modification of the luminal environment by introducing probiotics to the gastrointestinal tract (11). Through these studies, it has become clear that certain strains of bacteria are integral in maintaining intestinal homeostasis. Various categories of activity have been identified, including enhanced barrier function, modulation of the mucosal immune system, production of antimicrobials, and alteration of the intestinal microflora (30). Despite much recent research in this field, the enormous complexity of the relationships between the prokaryotic and eukaryotic cells of the intestine indicates that numerous mechanisms likely remain undiscovered.

Many identified interactions between intestinal epithelial cells and microbes are related to signaling that occurs because of close contact and interaction between live cells. However, several probiotic and commensal bacteria, including *Lactobacillus GG* (LGG), *Bifidobacterium breve*, *Streptococcus thermophilus*, *B. bifidum*, and *Ruminococcus gnavus*, have been shown to secrete metabolites that are capable of eliciting responses in epithelial and other immune cells (25, 47). These metabolites are also capable of traversing the intestinal barrier and have been shown to contribute to intestinal homeostasis and barrier function. Two isolated and purified peptides secreted by LGG have been shown to promote intestinal homeostasis by inducing Akt activation, inhibiting cytokine-induced epithelial cell apoptosis, and promoting cell growth (47). Other low-molecular-weight peptides secreted from LGG induce expression of heat shock proteins and activate MAP kinases (40). In its live form, the probiotic compound VSL#3 attenuates inflammation in the IL-10-deficient mouse model of colitis (21) and has shown efficacy in several clinical trials for IBD (11). We have previously shown that epithelial barrier function and resistance to *Salmonella* invasion are enhanced by exposure to a proteinaceous soluble factor secreted by the bacteria found in the VSL#3 compound (21). VSL#3 conditioned medium has also been shown to inhibit proteasome activity, inhibit NF-κB signaling, and induce expression of cytoprotective heat shock proteins in intestinal epithelial cells (32).

Barrier function in the intestine is maintained via a number of structures. Epithelial cells comprise the largest amount of surface area and are sealed at paracellular interfaces by tight junctions (41). Tight junctions are made up of complex lipid-protein structures that form fibrils that traverse the lateral plasma membrane to interact with proteins from the adjacent cell. To date, the primary proteins identified as tight junction-specific integral transmembrane proteins are claudins and occludin (41). Claudins are a multigene family that have been...
shown to impart resistance and ion selectivity to epithelial paracellular pathways (41). Studies investigating the effects of probiotics on tight junction proteins have shown live *S. thermophilus* and *Lactobacillus acidophilus* to alter phosphorylation of several related tight junction proteins (34), whereas live *Escherichia coli* Nissle 1917 has been shown to increase zonula occludens (ZO)-1 production (49). In rats, administration of *L. plantarum* increased colonic occludin expression in rats with experimental abdominal infection (36).

The objectives of this study were, first, to determine which strain in the VSL#3 probiotic compound was the most efficacious in enhancing epithelial resistance and to examine whether these secreted bioactive factors retained their efficacy when given orally. Secondly, we carried out long-term feeding studies to determine whether reducing intestinal permeability would attenuate colonic inflammation in the IL-10-deficient mouse model of colitis.

**METHODS**

**Conditioned medium.** Individual strains of the VSL#3 probiotic cocktail (*Bifidobacteria breve*, *B. infantis*, *B. longum*, *L. acidophilus*, *B. delbrueckii* subsp. *Bulgaricus*, *L. casei*, *L. plantarum*, *S. salivarius* subsp. *Thermophilus*) (VSL#3 Pharmaceuticals, Ft. Lauderdale, FL) were inoculated at 0.18% (wt/vol) into 25 ml of Tryptone Soy Broth (Difco no. 0370-17-3) and grown statically overnight (18–20 h) at 37°C. For both in vitro and in vivo studies, *B. infantis* was incubated overnight in RPMI 1640 medium, then adjusted to pH 7.4 and filtered through a 0.2-μm syringe filter to remove live bacteria. To ensure the absence of any live bacteria, a sample of each preparation was plated on MRS agar and incubated anaerobically.

**T84 cell culture studies.** T84 cells at passages 30–34 were grown as monolayers in a 1:1 mixture of Dulbecco-Vogt modified Eagle’s medium and Ham’s F-12 medium supplemented with 15 mM Na+-HEPES buffer, pH 7.5, 14 mM NaHCO₃, and 5% newborn calf serum. For subculture, a cell suspension was obtained from confluent monolayers by exposing the monolayers to 0.25% trypsin and 0.9 mM EDTA in Ca²⁺- and Mg²⁺-free phosphate-buffered saline. Cells were seeded at a density of 1 × 10⁶ cells/1.13 cm² polycarbonate tissue culture-treated filter and maintained at 37°C in a 5% CO₂ atmosphere. Cultures were refed daily with fresh medium. To qualitatively determine whether the T₈₄ cells had reached confluence, formed tight junctions, and established cell polarity, the electrical conductance across the monolayer was determined by use of an EVOM voltohm-

---

**Table 1. PCR primer sequences**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α forward</td>
<td>5′-ATGAGGAGCACAGGATGATG-3′</td>
</tr>
<tr>
<td>TNF-α reverse</td>
<td>5′-TACAGCTTGACTGAATT-3′</td>
</tr>
<tr>
<td>IFN-γ forward</td>
<td>5′-TACTCCAGAGCCACAGCTTG-3′</td>
</tr>
<tr>
<td>IFN-γ reverse</td>
<td>5′-GGCCACACTCCTTTTGGTCTC-3′</td>
</tr>
<tr>
<td>TGF-β forward</td>
<td>5′-CCGGCCGACCTGGACGAC-3′</td>
</tr>
<tr>
<td>TGF-β reverse</td>
<td>5′-CTGTCGCACCTTGGTGCGACCAC-3′</td>
</tr>
<tr>
<td>Actin forward</td>
<td>5′-CTGGGCGCGGCTTAGGGGAAC-3′</td>
</tr>
<tr>
<td>Actin reverse</td>
<td>5′-TTGGGCGTTAGGGGAGGGG-3′</td>
</tr>
</tbody>
</table>

---

Fig. 1. Changes in transepithelial resistance in T84 cells in response to conditioned medium from the probiotic bacterial strains in VSL#3. Each strain from VSL#3 was incubated separately in RPMI 1640 medium overnight, and live cells were filtered out. Application of conditioned medium from 6 of the strains to a T84 monolayer resulted in varying increases in epithelial resistance.

---

Fig. 2. Effect of BiCM on mannitol flux and resistance in T84 cells. Application of BiCM to T84 cells in Transwells significantly decreased apical-to-basolateral mannitol flux and increased resistance within 6 h (A). This effect was maintained through 24 h. Values shown are calculated as % of control. Values are means ± SD of 2 experiments with 3 replicates each. *P < 0.05 relative to control.
Flux of mannitol was assessed by adding 5 μCi [3H]mannitol into the apical chamber and sampling from the apical chamber; 1 mmol/l mannitol was present in both apical and serosal chambers. Aliquots were counted in a scintillation counter. To determine whether BiCM was effective in preventing monolayer disruption by IFN-γ or TNF-α, monolayers were preincubated for 2 h with BiCM, followed by the addition of either IFN-γ (10 ng/ml) or TNF-α (10 ng/ml) to the serosal chamber. For experiments assessing the role of MAPK, the ERK inhibitor PD-98059 (25 μM) was added to the apical surface 15 min prior to the addition of BiCM. Resistance was measured with an EVOM voltohmeter following 24 h of incubation. Measurements are expressed as ohms per centimeter squared.

Western blotting. For Western blot analysis, T84 cells were lysed in Mono Q buffer (1.08 g β-glycerophosphate, 38.04 mg EGTA, 0.5 ml Triton X-100, 200 μl 1 M MgCl₂ per 100 ml), and 50 μg of protein was subjected to electrophoresis on 10% SDS-polyacrylamide gels as previously described (14). Antibodies used were anti-claudins-1, 2, 3, and 4, anti-ZO-1, and anti-occludin (Cell Signaling Technology, Beverly, MA). Secondary staining was conducted using horseradish peroxidase-conjugated goat anti-rabbit Ig (1:3,000, Amersham, Baie d’Urfé, QC, Canada) followed by chemiluminescent detection using a commercial reagent following manufacturer’s instructions (Lumiglit, Roche, Laval, QC, Canada). Equivalent loading was confirmed by Ponceau’s staining and multiple exposures were done to ensure that film was not overexposed.

Immunofluorescence labeling. T84 cells were seeded (~5 × 10⁵ cells/well) into four-chamber slides and incubated overnight and then pretreated with 100 μl BiCM for 4 h. Monolayers were then treated with 10 ng/ml TNF-α or 10 ng/ml IFN-γ for 24 h. Plates were washed twice with cold PBS and incubated in 2% paraformaldehyde for 1 h. After 1 h of blocking with 5% skim milk in PBS, cells were incubated with anti-claudin 1 or anti-occludin antibody (1/100) for 60 min and then with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody for claudin-1 (Invitrogen, Burlington, ON, Canada). Cells were mounted with coverslips in FluorSave. Slides were examined with a Zeiss Axiosvert 100 M confocal microscope coupled with a Zeiss LSM510 laser scanning system. Images were taken with a ×63 Plan-apochromat numerical aperture 1.4 with a zoom factor of 2. Alexafluor 488 was scanned with an argon laser (excitation wavelength, 488 nm; emission wavelength, 505 nm), and Cy5 was scanned with an HeNe laser (excitation wavelength, 543 nm; emission wavelength, 560 nm).

Animals and in vivo procedures. Wild-type and IL-10-deficient mice on a 129 Sv/Ev background had ad libitum access to 9% fat rodent blocks and water and were housed behind a barrier under pathogen-free conditions. The facility’s sanitation was verified by Health Sciences Laboratory Animal Services at the University of Alberta. For acute experiments, mice were gavaged with 30 μl of BiCM or vehicle and euthanized after 4 h for measurement of colonic permeability in vitro in Ussing chambers. For long-term studies, 8 wk-old mice were fed BiCM or vehicle (30 μl/2 × day) for 30 days. All experiments were performed according to the Canadian Council of Animal Care guidelines for the care and use of laboratory animals in research and with the approval and permission of the local ethics committees.

Epithelial function. For measurement of intestinal permeability, a colonic segment was mounted in Ussing chambers exposing mucosal and serosal surfaces to 10 ml of oxygenated Krebs buffer (in mM: 115 NaCl, 8 KCl, 1.25 CaCl₂, 1.2 MgCl₂, 2 KH₂PO₄, 225 NaHCO₃; pH 7.35). The buffers were maintained at 37°C by a heated water jacket and circulated by CO₂–O₂. Fructose (10 mM) was added to the serosal and mucosal sides. For measurement of basal mannitol fluxes, 1 mM of mannitol with 10 μCi ([3H]mannitol) was added to the mucosal

Fig. 3. BiCM increased phospho-ERK and decreased phospho-p38 in T84 cells. T84 cells were treated with BiCM for various times and whole cell extracts were prepared for Western blotting. Extracts were immunoblotted with antibodies specific for the phosphorylated (phospho) forms of ERK and p38. All gels were run 3 times and equal protein loading was confirmed by protein assay and by densitometric analysis of control Western blots by using antibodies specific for the nonphosphorylated (total) forms of these kinases. ERK1/2 was transiently phosphorylated following treatment with BiCM, with maximal phosphorylation evident by 10 min and decreasing thereafter whereas levels of the phosphorylated form of p38 decreased (A). B: densitometry analysis. C: Western blot confirming the absence of the phosphorylated form of ERK1/2 in the presence of PD-98059.
side. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current ($I_{sc}$; expressed as μA/cm²) with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT), except for 5–10 s every 5 min when PD was measured by removing the voltage clamp. Tissue ion conductance (G; expressed as mS/cm²) was calculated from PD and $I_{sc}$ according to Ohm’s Law (6). Baseline $I_{sc}$ and G were measured after a 20-min equilibration period, and increases in $I_{sc}$ were induced by addition of the adenylate cyclase-activating agent forskolin ($10^{-5}$ M) to the serosal surface. Epithelial responsiveness was defined as the maximal increase in $I_{sc}$ to occur within 5 min of exposure to the secretagogue.

Mucosal cytokine measurements. Mice were euthanized by cervical dislocation and whole colons were removed, flushed with phosphate-buffered saline, and resuspended in tissue culture plates (Falcon 3046; Becton Dickinson Labware, Lincoln Park, NJ) in RPMI 1640 medium supplemented with 50 mM 2-mercaptoethanol (2-ME), 10% fetal calf serum, streptomycin (100 U/ml) and penicillin (100 U/ml). Cultures were incubated at 37°C in 5% CO₂ for 24 h. Supernatants were harvested and stored at $-70°C$ for cytokine level quantification. Levels of TNF-$\alpha$ and IFN-$\gamma$ in culture supernatants were measured by using ELISA kits (Medicorp, Montreal, QC, Canada) according to manufacturer’s instructions. Total RNA was isolated from tissue samples by using Trizol (GIBCO, Burlington, ON, Canada) following manufacturer’s instructions. mRNA (1 μg) was reverse transcribed and amplified using the polymerase chain reaction (PCR) and standardized to β-actin (14). TNF-$\alpha$, IFN-$\gamma$, and β-actin sequences were obtained from GenBank and used to design intron-spanning primers. Primer sequences used for PCR are shown in Table 1.

Histopathology. Colonic tissue was obtained at the time of euthanasia for histological analysis. Formalin-fixed and paraffin-embedded sections were stained with hematoxylin and eosin. The slides were reviewed in a blinded fashion and were assigned an intestinal inflammation histological score according to the sum of four scoring criteria: mucosal ulceration, epithelial hyperplasia, lamina propria mononuclear infiltration, and lamina propria neutrophilic infiltration as previously described (21).

Preparation and activation of murine spleen cells. Spleens were removed aseptically from mice and teased into single-cell suspensions in RPMI 1640 medium with 10% fetal calf serum. The cell suspension was centrifuged at 400 g for 5 min, and the cell pellet was resuspended in lysis medium (1 volume of 0.17 M Tris, pH 7.6, 9 volumes of 0.16 M NH₄Cl) to remove red blood cells and repelleted. Cells were washed twice with RPMI 1640 medium and placed into 96-well plates at a concentration of $1 \times 10^6$ per well in the presence of bacterial sonicates at a protein concentration of 50 μg/ml according to the method of Sydora et al. (39). Bacterial sonicates included Clostridium...
sordelli, L. reuteri, and Bacteroides vulgatus (39). After 48-h incuba-
tion at 37°C in a humidified incubator at 5% CO2, plates were
centrifuged and IFN-γ in the supernatant quantified by ELISA.
Controls included plate-bound anti-CD3ε clone 145–2C1 (PharMingen
Canada, Mississauga, ON, Canada) and medium alone.

Statistical analysis. Data was tested for normality of distribution
and analyses were performed by use of the statistical software
SigmaStat (Jandel, San Rafael, CA). Differences between means were
evaluated by analysis of variance, paired t-tests, or a nonparametric
Mann-Whitney rank sum test where appropriate. Specific differences
were tested by the Student-Newman-Keuls test.

RESULTS

B. infantis exerts the greatest effect on monolayer resistance.
Live probiotics and commensals have been shown to improve
monolayer barrier function in cultured human epithelial cells
(34). We have previously shown that conditioned medium
from VSL#3, with no live cells, increases T84 cell monolayer
resistance (21). To minimize the complexity of the conditioned
medium utilized in further studies, we assessed the ability of
each strain from the VSL#3 probiotic cocktail to enhance
monolayer resistance in T84 cells. Of the eight probiotic strains
tested, BiCM had the greatest effect on TEP and was thus
selected for use in all further experiments performed in this
study (Fig. 1).

BiCM enhances T84 cell monolayer resistance via the para-
cellular pathway. To determine the nature of the increased
resistance induced by BiCM, we examined paracellular perme-
ability by measuring the movement of mannitol, a small,
hydrophilic macromolecule with a cross-sectional diameter of
6 Å. As shown in Fig. 2, application of BiCM to T84 cells
significantly decreased apical-to-basolateral mannitol flux and
increased resistance by 6 h as measured by an EVOM volto-
hmeters. This effect was maintained through 24 h. Figure 2B
confirms these findings by real-time electric cell-substrate
impedance sensing (26) and demonstrates that effects of BiCM
are long lasting.

MAPK phosphorylation is induced by BiCM. At least three
distinct groups of MAPKs have been identified in mammals,
including the extracellular signal-regulated kinases (ERKs),
the c-Jun NH2-terminal kinases (JNKs), and p38. ERK is
typically stimulated by growth-related signals, whereas the
JNK and p38 MAP kinase cascades are activated by various
stress stimuli (16). Barrier function and paracellular permeabil-
ity have been shown to be regulated by ERK expression (12)
whereas p38 activation contributes to the disruption of epithe-

dial barrier function (2). Activation of these kinases was as-
sayed by using antibodies specific for the phosphorylated,
active forms of these kinases by Western blotting. As shown in
Fig. 3A, ERK1/2 was transiently phosphorylated following treatment with BiCM, with maximal phosphorylation evident
by 10 min and decreasing thereafter. In contrast, an anti-
phospho-p38 blot of BiCM-treated cells showed levels of the
phosphorylated form of p38 to decrease (Fig. 3A). Densitome-
try analysis is shown in Fig. 3B. Inhibition of ERK1/2 with
PD-98059 abolished the BiCM-induced rise in resistance (Fig.
2B). A Western blot confirming the absence of the phosphor-
ylated form of ERK1/2 in the presence of PD-98059 is shown in
Fig. 3C.

BiCM alters tight junction protein expression. Although the
plasma membrane of intestinal epithelial cells is an effective
barrier to hydrophilic substances, the paracellular spaces must
also be sealed to form an intact barrier. To accomplish this,
intestinal epithelial cells are joined together by a series of
intercellular tight junction proteins along their lateral mem-
branes (29). To determine whether the decrease in paracellular
permeability induced by BiCM was associated with altered
tight junction protein expression, T84 cells were treated with
BiCM and expression of claudins 1, 2, 3, and 4, ZO-1, and
occludin was measured by Western blot (Fig. 4). Claudins
responded in a time-dependent differential manner to BiCM.
Treatment of monolayers to BiCM resulted in enhanced protein
expression of claudin-4, ZO-1, and occludin, whereas clau-
din-2 levels decreased over time.

BiCM protects against IFN-γ and TNF-α induced permeabil-
ity and tight junction disruption. Inflammatory cytokines
IFN-γ and TNF-α both cause a functional disruption of epipi-
theal tight junctions primarily by altering the localization of
tight junction proteins as well as by modulating the membrane
microdomain lipid composition (9, 19, 20). To determine
whether BiCM was effective in preventing a cytokine medi-
ated drop in resistance, T84 cells were preincubated with
BiCM followed by treatment with either IFN-γ or TNF-α.
Transepithelial resistance was measured, and claudin-1 and
occludin protein expression and organization were then as-
sessed by Western blot and immunofluorescence, respectively.
As seen in Fig. 5, TNF-α and IFN-γ both caused a drop in
transepithelial resistance by 24 h, which was prevented when
cells were preincubated with BiCM. Under normal conditions,
the tight junction proteins occludin and claudin-1 were local-
ized in a pattern consistent with their distribution in tight
junctions (Fig. 6A). IFN-γ and TNF-α induced a dramatic
redistribution of occludin and claudin-1 from the tight junction
membranes into the cytoplasm (Fig. 6A). This alteration was

![Fig. 5. Effect of BiCM on resistance in T84 cells in the presence of IFN-γ and TNF-α. T84 cells were treated with ERK1/2 inhibitor (PD-98059, 25 μM) for 15 min and then with BiCM or medium alone for 4 h prior to addition of IFN-γ (10 ng/ml) or TNF (10 ng/ml). Transepithelial resistance was measured 24 h after treatment by using a voltohmeter. Both TNF-α and IFN-γ reduced epithelial resistance compared with control cells. BiCM pretreatment prevented the cytokine-induced drop in epithelial resistance. The protective effect of BiCM on barrier function was abolished by the inhibition of ERK1/2 with PD-98059. Values are means ± SD of 2 experiments with 3 replicates. *P < 0.05 compared with control.](http://ajpgi.physiology.org/)

AJPGI-Gastrointest Liver Physiol • VOL 295 • NOVEMBER 2008 • www.ajpgi.org
characterized by discontinuities in membrane staining and submembranous internalization of these proteins (Fig. 6A).

Pretreatment with BiCM prevented the cytokine-induced tight junction protein disruption (Fig. 6A) and the drop in epithelial resistance (Fig. 5). As seen in Fig. 6B, BiCM had no effect on whole cellular levels of these tight junction proteins in the presence of IFN-γ or TNF-α. The ability of BiCM to protect against an IFN-γ- and TNF-α-induced drop in epithelial resistance was abolished by ERK1/2 inhibition (Fig. 5).

The biological activity of BiCM is maintained in vivo. IL-10-deficient mice spontaneously develop colitis at 6–8 wk of age and demonstrate increased colonic permeability prior to the onset of inflammation (15, 21). This enhanced gut permeability is believed to have a causal role in the onset and...

Fig. 6. Confocal micrographs of T84 monolayers with dual immunofluorescent staining with antibodies to claudin-1 and occludin (A) and the effects of TNF-α and IFN-γ on claudin and occludin expression at 24 h (B). IFN-γ and TNF-α induced a dramatic redistribution of occludin and claudin-1 from the tight junction membranes into the cytoplasm. Pretreatment with BiCM prevented the cytokine-induced tight junction protein disruption. B: Western blotting of whole cellular protein lysates shows increased occludin in response to BiCM. Protein expression of occludin and claudin-1 as determined by Western blotting was unchanged following cytokine incubation. Experiments were repeated 3 times with similar results.
perpetuation of inflammation in this model. To determine whether BiCM retained its ability to decrease permeability in vivo, adult IL-10-deficient 129 Sv/Ev mice (n = 7) were administered BiCM or vehicle and colonic permeability was assessed in Ussing chambers after 4 h. BiCM decreased mannositol permeability in the proximal colon of IL-10-deficient mice within 4 h of administration (A). This was accompanied by a decrease in overall tissue conductance (B). Values are means ± SE of duplicate samples for n = 7 animals per group. *P < 0.05 relative to colons from untreated IL-10-deficient mice.

BiCM improved clinical presentation and intestinal function in IL-10 mice. To assess whether the reduction in colonic permeability induced by BiCM would be effective in improving clinical signs of colitis if given long term, IL-10-deficient 129 Sv/Ev mice (n = 8) were treated with BiCM for 30 days. Mice receiving BiCM treatment showed enhanced weight gain, decreased colonic weight, and decreased colon weight-to-length ratio compared with control animals (Table 2). This was associated with a significant attenuation of histological inflammation and a significantly reduced mannitol flux compared with controls (Table 3).

**Epithelial ionic function.** In vitro and in vivo studies have demonstrated that proinflammatory cytokines can alter epithelial transport and barrier functions (4, 20). We have previously shown that colonic epithelium from IL-10 gene-deficient mice exhibit physiological alterations in colonic ionic function (24). To determine whether treatment with BiCM was associated with enhanced intestinal function, colons from IL-10-deficient mice were studied in Ussing chambers. IL-10 gene-deficient mice demonstrated significant reductions in basal I_sc and also demonstrated reduced short-circuit response to forskolin, suggesting impairment in cAMP-dependent active chloride secretion (Table 3). BiCM treatment normalized baseline I_sc and I_se response to forskolin. Physiological function of colonic tissue from control mice was not affected by BiCM treatment (data not shown).

**BiCM reduced mRNA levels of IFN-γ.** Colonic epithelium from IL-10 gene-deficient mice exhibit high levels of TNF-α and IFN-γ (21, 22). To assess whether the improvement in colonic histology and physiological function in mice treated with BiCM was associated with altered expression of cytokines, levels of colonic mRNA were measured. Mice receiving BiCM exhibited decreased levels of colonic IFN-γ mRNA in conjunction with increased TGF-β mRNA (Fig. 8). However, interestingly, levels of TNF-α were not altered. To determine whether this decrease in proinflammatory cytokine levels in the colon was due to a direct effect of BiCM on tissue, small intestine and colon were incubated for 24 h in the presence of BiCM and levels of secreted TNF-α and IFN-γ were measured. BiCM had no effect on secretion of these cytokines (Fig. 9), suggesting that the decrease in cytokine secretion was not due to a direct effect of BiCM on intestinal tissue.

**BiCM reduces systemic inflammatory response.** Figure 10 shows IFN-γ release from spleen cells stimulated with sonicates prepared from pure cultures of *C. sordelli*, *L. reuteri*, and *B. vulgatus*. As previously shown in this model (38), spleen cells from IL-10-deficient mice secrete significant amounts of IFN-γ in response to stimulation with bacterial sonicates compared with control mice. Spleen cells from IL-10-deficient mice treated with BiCM did not show any enhanced IFN-γ secretion compared with controls, demonstrating that the increase in gut barrier function induced by treatment with BiCM was associated with an altered systemic immune response to bacterial antigens in IL-10-deficient mice.

### Table 2. Morphological parameters and histological score of control mice, IL-10 deficient mice, and IL-10 deficient mice receiving BiCM for 30 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight Gain, g</th>
<th>Colon Length, cm</th>
<th>Colon Weight, g</th>
<th>Weight-to-Length Ratio, %</th>
<th>Histological Score (0–10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>2.4±0.4</td>
<td>8.8±0.02</td>
<td>0.29±0.02</td>
<td>3.3±0.2</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>IL-10 deficient (n = 9)</td>
<td>1.1±0.3*</td>
<td>8.3±0.3</td>
<td>0.57±0.06*</td>
<td>6.8±0.5*</td>
<td>3.9±0.4*</td>
</tr>
<tr>
<td>IL-10 deficient + BiCM (n = 8)</td>
<td>2.1±0.3</td>
<td>8.9±0.2</td>
<td>0.36±0.04</td>
<td>4.4±0.3</td>
<td>1.5±0.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE. BiCM, *Bifidobacteria infantis* constituted medium. *P < 0.05 compared with control and IL-10 gene-deficient + BiCM; †P < 0.05 compared with control.
Tight junction proteins selectively modulate the passage of molecules and ions via the paracellular pathway and also prevent the lateral diffusion of membrane proteins (43). In cell culture models, several claudins have been shown to decrease cation permeability (claudins 1, 4, 5, 7, 8, 10, 13, and 14), whereas others appear to express cation-selective activity (43). In particular, differential expression and properties of the claudin family of tight proteins appear to determine the tissue-specific variations in electrical resistance and paracellular ionic selectivity (43). In cell culture models, several claudins have been shown to decrease cation permeability (claudins 1, 4, 5, 7, 8, 10, 13, and 14), whereas others appear to express cation-selective pores (claudins 2 and 16) and increase permeability (43). Probiotics have become the subject of a great deal of investigation, particularly in the context of IBD. We previously showed that secreted factors from the probiotic cocktail VSL#3 increase transepithelial resistance in vitro (21). Here, we further assessed the actions of soluble secreted factors from *B. infantis* (a component of VSL#3) on epithelial barrier function both in vitro and in vivo. We determined that BiCM increases transepithelial resistance by altering tight junction protein expression. Barrier disruption induced by IFN-γ and TNF-α was also prevented by BiCM, and this activity appeared to be functioning through an MAP kinase-dependent pathway. We further demonstrated that oral treatment of IL-10-deficient mice with BiCM effectively decreases colonic permeability in conjunction with a reduction in mucosal levels of proinflammatory cytokines, a significant improvement in colitis, and an altered systemic response to bacterial antigens.

Tight junction proteins selectively modulate the passage of molecules and ions via the paracellular pathway and also prevent the lateral diffusion of membrane proteins (43). In particular, differential expression and properties of the claudin family of tight proteins appear to determine the tissue-specific variations in electrical resistance and paracellular ionic selectivity (43). In cell culture models, several claudins have been shown to decrease cation permeability (claudins 1, 4, 5, 7, 8, and 14), whereas others appear to express cation-selective pores (claudins 2 and 16) and increase permeability (43). In this study, treatment of T84 monolayers with BiCM resulted in a time-dependent increase in transepithelial resistance. This correlated with a decrease in the expression of claudin-2 and an increase in levels of ZO-1 and occludin. These findings of probiotic-induced increase in epithelial resistance and tight junction protein expression are in agreement with other previously published findings. Live *L. acidophilus*, *S. thermophilus*, and *E. coli* Nissle, and secreted products from VSL#3 are able to maintain or enhance tight junction protein expression under normal and physiological stressed conditions (27, 34, 42, 49).

Patients with IBD exhibit increased gut permeability (5). Furthermore, increases in permeability have been shown to precede clinical relapse in patients with Crohn’s disease (1). Distribution and expression levels of various tight junction components have been shown to be altered in Crohn’s disease patients (5). Increased expression of claudin-2 has been described in colonic epithelium from Crohn’s and ulcerative

### DISCUSSION

**Table 3. Electrical and permeability parameters of colons from control mice, IL-10-deficient mice, and IL-10-deficient mice receiving BiCM for 30 days**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mannitol Flux, nmol/cm²·h</th>
<th>ΔIsc, μA/cm²</th>
<th>Forskolin, A/cm²</th>
<th>G, mS/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>13.2±1.8</td>
<td>62±7</td>
<td>86±10</td>
<td>12.3±1.9</td>
</tr>
<tr>
<td>IL-10 deficient (n = 9)</td>
<td>19.5±2.1*</td>
<td>31±5y</td>
<td>50±6*</td>
<td>13.1±1.9</td>
</tr>
<tr>
<td>IL-10 deficient + BiCM</td>
<td>12.4±1.0</td>
<td>52±12</td>
<td>54±11</td>
<td>11.5±0.8</td>
</tr>
</tbody>
</table>

*Values are means ± SE. ΔIsc, short-circuit current; G, tissue ion conductance. *P* < 0.05 compared with control and IL-10 gene-deficient mice; †P < 0.05 relative to untreated IL-10-deficient mice.

**Fig. 8. Colonic cytokine mRNA levels in animals receiving BiCM. IL-10-deficient mice were treated with BiCM daily (30 μl) for 30 days and colons removed for measurement of IFN-γ, TNF-α, and TGF-β mRNA levels. After 30 days of treatment, mice receiving BiCM showed reduced mRNA levels of colonic IFN-γ and increased TGF-β levels. There was no difference in TNF-α mRNA levels. Values are means ± SE of duplicate samples for n = 8 animals per group. *P < 0.05 relative to untreated IL-10-deficient mice.**

**Fig. 9. Colonic cytokine secretion in vitro in response to BiCM. Whole colons were removed from untreated IL-10-deficient mice and resuspended in tissue culture plates in the presence and absence of BiCM. Cultures were incubated at 37°C in 5% CO2 for 24 h. Supernatants were harvested and TNF-α and IFN-γ levels measured by ELISA. BiCM had no effect on either TNF-α or IFN-γ secretion. Values are means ± SE of 6 animals per group.**

**Fig. 10. IFN-γ release from spleen cells. Spleens were removed aseptically from wild-type control mice, IL-10-deficient mice, and IL-10-deficient mice receiving BiCM for 30 days. Spleen cells were placed into 96-well plates at a concentration of 1 x 10⁶ per well in the presence of bacterial sonicates (50 μg/ml: *Clostridium sordelli*, *L. reuteri*, and *Bacteroides vulgatus*). After 48 h incubation at 37°C, plates were centrifuged and IFN-γ in the supernatant quantified by ELISA. Controls included plate bound anti-CD3 clone 145-2C1 and medium alone. IL-10-deficient mice had increased levels of IFN-γ release when stimulated with *C. sordelli*, *B. vulgaris*, and *L. reuteri*. BiCM treatment reduced the splenic response to all three bacterial antigens. Values are means ± SE of 5 animals per group. *P < 0.05 relative to wild-type and BiCM-treated IL-10-deficient mice.**
colitis patients (33, 48). Other studies have shown occludin and ZO-1 to be relocated to the basolateral membrane in Crohn’s disease patients (31) and claudin-5 and -8 to be redistributed from the tight junction region (48). These studies strongly suggest that alterations in tight junction composition and protein localization may have a key role in the pathogenesis of chronic inflammatory conditions. A link between altered levels of proinflammatory cytokines and intestinal permeability has been described (5). In particular, levels of TNF-α and IFN-γ have been shown to be upregulated in patients with Crohn’s disease (28, 37). Numerous studies have demonstrated that TNF-α and IFN-γ decrease transepithelial resistance by inducing redistribution of various tight junction proteins by internalization (4, 19) or by selectively activating different populations of paracellular pores (45). In contrast, other cytokines such as IL-13 have been shown to increase permeability by increasing levels of claudin-2 (33). In this study, conditioned medium from B. infantis was effective in attenuating both a TNF-α and IFN-γ-induced drop in epithelial resistance. Furthermore, BiCM also prevented the IFN-γ-induced rearrangement of occludin and claudin-1 in T84 cells. Inhibition of ERK abolished the BiCM-induced rise in resistance and ability to protect against TNF-α and IFN-γ-induced decreases in barrier function. This is in agreement with other studies showing that beneficial effects on epithelial barrier function exerted by probiotics are MAP kinase pathway-dependent (35, 40).

In addition to their ability to modulate epithelial responses to proinflammatory cytokines, probiotics have also been shown to prevent a hydrogen peroxide-induced disruption of tight junctions via a protein kinase C- and MAP kinase-dependent mechanism (35). This would suggest that probiotics are effective at maintaining epithelial barrier function in the presence of varied insults.

The IL-10 gene-deficient mouse demonstrates an increase in colonic permeability that is absent in mice raised under germ-free conditions (23). We have previously demonstrated that VSL#3 treatment reduced colonic permeability in IL-10 gene-deficient mice and significantly improved colitis (21). However, whether the improvement of colitis was due primarily to a bacterial-induced improvement in permeability or whether other effects of the probiotic bacteria found in VSL#3 on immune cell function contributed to the improvement of colitis is unknown. Results from this study indicate that bioactive factors released from B. infantis alone are also effective in attenuating colitis, while having no effect on TNF-α or IFN-γ secretion when applied directly to gut tissue, suggesting that modulation of gut permeability alone may be sufficient to improve inflammation in this model. However, the application of the bioactive factors could have had effects on other cytokines not measured in this study.

Probiotics have been consumed by humans in fermented dairy products for thousands of years and have an excellent safety record with virtually no known side effects in healthy populations (13). However, administration of probiotic bacteria to individuals with compromised immune systems, pancreatitis, and short gut syndrome may potentially lead to bacteremia (3, 7, 17). The risk of probiotic sepsis may also be higher in premature infants (18). In one study of athymic neonatal mice, colonization of L. reuteri led to sepsis and resulted in a high mortality rate (44). Administration of probiotic components, such as BiCM may be useful in patients at risk for bacteremia, as physiological efficacy is maintained without the accompanying risk of sepsis from live strains.

In conclusion, orally administered conditioned medium from B. infantis is effective in reducing colonic permeability and attenuating inflammation in a mouse model of colitis. Furthermore, this protection involves effects on tight junction proteins and is mediated through the MAPK pathway. In that altered gut permeability is thought to drive gut inflammation in many different autoimmune diseases, the use of probiotic-derived products rather than live bacteria to increase barrier resistance and maintain the intestinal barrier may have significant clinical implications.

GRANTS

This research was supported by grants from Alberta Heritage Foundation for Medical Research, Crohn’s and Colitis Foundation of Canada, and Canadian Institutes for Health Research.

REFERENCES