Probiotic *Bifidobacterium* species stimulate human SLC26A3 gene function and expression in intestinal epithelial cells

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Kumar A, Hecht C, Priyamvada S, Anbazhagan AN, Alakkam A, Borthakur A, Alrefai WA, Gill RK, Dudeja PK. Probiotic *Bifidobacterium* species stimulate human SLC26A3 gene function and expression in intestinal epithelial cells. *Am J Physiol Cell Physiol* 307: C1084–C1092, 2014. First published August 20, 2014; doi:10.1152/ajpcell.00194.2014.—SLC26A3, or downregulated in adenoma (DRA), plays a major role in mediating Cl− absorption in the mammalian intestine. Disturbances in DRA function and expression have been implicated in intestinal disorders such as congenital Cl− diarrhea and gut inflammation. We previously showed that an increase in DRA function and expression by *Lactobacillus acidophilus* and its culture supernatant (CS) might underlie antidiarrheal effects of this probiotic strain. However, the effects of *Bifidobacterium* species, important inhabitants of the human colon, on intestinal Cl− /HCO3− exchange activity are not known. Our current results demonstrate that CS derived from *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Bifidobacterium bifidum* increased anion exchange activity in Caco-2 cells (~1.8- to 2.4-fold). Consistent with the increase in DRA function, CS also increased the protein, as well as the mRNA, level of DRA (but not putative anion transporter 1). CS of all three *Bifidobacterium* sp. increased DRA promoter activity (~1,183+/−114 bp) in Caco-2 cells (1.5- to 1.8-fold). Furthermore, the increase in DRA mRNA expression by CS of *B. breve* and *B. infantis* was blocked in the presence of the transcription inhibitor actinomycin D (5 μM) and the ERK1/2 MAPK pathway inhibitor U0126 (10 μM). Administration of live *B. breve*, *B. infantis*, and *B. bifidum* by oral gavage to mice for 24 h increased DRA mRNA and protein levels in the colon. These data demonstrate an upregulation of DRA via activation of the ERK1/2 pathway that may underlie potential antidiarrheal effects of *Bifidobacterium* sp.

Antidiarrheal; Caco-2; chloride absorption; downregulated in adenoma

**DIARRHEAL DISEASES** associated with infections by enteric pathogens or with inflammatory bowel disorders cause an enormous health care burden, accounting for ∼4% of overall deaths and ∼1.2 million deaths in children <5 yr of age (20). Diarrhea may result from increased secretion or decreased absorption of solutes and electrolytes. Thus, understanding the regulation of ion transport mechanisms involved in fluid absorption is of critical importance in defining the pathophysiology of diarrheal diseases, as well as developing better therapeutic interventions for treatment of these diseases. Previous studies from our laboratory utilizing purified plasma membranes from organ donor intestines, as well as molecular and cellular approaches, have shown that a major mechanism of electroneutral NaCl absorption in human ileum and colon involves coupling of Na+/H+ (NHE) and Cl− /HCO3− exchangers (7, 12). NHE3 and downregulated in adenoma (DRA) are two major apical ion exchangers implicated in NaCl absorption in the intestine. Putative anion transporter 1 (PAT-1), another apical anion exchanger, is also expressed in the apical membrane of intestinal epithelial cells (IECs); however, its role in diarrheal disorders is not well recognized. For example, PAT-1−/− mice showed a decrease in apical Cl−/HCO3− exchange activity but did not exhibit a diarrheal phenotype (42).

Increasing evidence indicates that disturbances in DRA function or expression play a major role in the pathophysiology of several diarrheal disorders. For example, mutations in DRA lead to a rare genetic disorder, congenital Cl− diarrhea, characterized by metabolic alkalosis, impaired Cl− /HCO3− exchange, and high fecal Cl− concentration (>90 mmol/l) (34). DRA−/− mice exhibit substantial diarrheal phenotype with serum electrolyte imbalances (34). Enteropathogenic *Escherichia coli*, an important food-borne pathogen causing diarrhea, decreases apical Cl−/HCO3− exchange activity concomitant with a decrease in DRA plasma membrane levels (14). Similarly, diarrhea caused by *Citrobacter rodentium* infection of mice is associated with a marked reduction in DRA gene expression (4). DRA expression has also been shown to be decreased in intestinal inflammation (2). Therefore, agents that increase DRA offer potential for utilization as antidiarrheal or proabsorptive agents. Probiotics have been shown to have beneficial effects as antidiarrheal agents (11, 17, 37), although the mechanisms underlying their effects are not fully understood.

Probiotics are viable microorganisms with beneficial effects on human health (26). Previous studies have reported that the culture supernatant (CS) of *Lactobacillus rhamnosus*, *Bifidobacterium infantis*, and VSL#3 (a mixture of *Lactobacillus* and *Bifidobacterium* strains) shows beneficial effects similar to those of live bacteria on intestinal epithelia (27). Multiple mechanisms of action, including suppression of growth or epithelial binding/invasion by pathogenic bacteria, decreased Cl− secretion, improved epithelial barrier function, and immunomodulation, have been suggested to explain the protective effects of probiotics (18, 26). However, studies investigating the mechanism of action of individual probiotic strains on epithelial functions are limited. Our previous studies demonstrated that live bacterium and CS of *Lactobacillus acidophilus* (LA) increased apical Cl−/HCO3− exchange activity, which may underlie the beneficial effects of LA in several diarrheal disorders (5, 30). The effects of *Bifidobacterium* species (important inhabitants of human colon) on DRA have not been investigated.

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Therefore, in vitro and in vivo mouse models were used to examine the effects of *Bifidobacterium* sp. on apical Cl⁻/HCO₃⁻ exchangers. Our findings demonstrate that treatment of Caco-2 cells with CS of *Bifidobacterium* sp. (*B. breve*, *B. infantis*, and *B. bifidum*) increases DRA expression in Caco-2 cells via the ERK1/2 MAPK pathway. DRA promoter activity was also stimulated by CS of *Bifidobacterium* sp., indicating the involvement of transcriptional mechanisms. Consistent with the in vitro results, in vivo data showed that *Bifidobacterium* sp. increased DRA mRNA and protein in mouse colon. Our studies suggest that the increase in DRA expression by *Bifidobacterium* may have therapeutic implications in diarrhea associated with ulcerative colitis or enteric infections.1

**MATERIALS AND METHODS**

**Materials.** Caco-2 cells, HT-29 cells, and probiotic *Bifidobacterium* sp. were obtained from American Type Culture Collection (ATCC, Manassas, VA), ¹²⁵I⁻ from Perkin Elmer, RNEnsy kits for RNA extraction from Qiagen (Valencia, CA), and real-time quantitative RT-PCR (qRT-PCR) kits from Stratagene (La Jolla, CA). 4,4'-Disothiocyanate-stibbene-2,2'-disulfonyl acid (DIDS) was purchased from Sigma Aldrich (St. Louis, MO), and ready-made SDS-polyacrylamide gels from Bio-Rad (Hercules, CA). DRA antibody was custom-synthesized against the COOH-terminal amino acid (745–764) sequence INTNGGLRNRVYEPVETKF of SLC26A3 (accession no. BC025671).

**Cell lines, cell culture, and treatments.** Caco-2 and HT-29 cells were grown at 37°C in an atmosphere of 5% CO₂ in a T-75 flask. Caco-2 cells were maintained in MEM supplemented with 20% fetal bovine serum, and HT-29 cells were maintained in McCoy’s medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum. Penicillin (100 IU/ml), streptomycin (100 µg/ml), and gentamicin (2 µg/ml) were added to HT-29 and Caco-2 media. Studies were performed in fully differentiated Caco-2 monolayers grown for 12–14 days post-plating on 24-well plastic supports or 12-well Transwell inserts between passages 25 and 45.

**Bifidobacterium culture and preparation of conditioned CS.** *B. breve* (product no. 15700, ATCC), *B. infantis* (product no. 15697, ATCC), and *B. bifidum* (product no. 15696, ATCC) were grown at 37°C in an anaerobic chamber, rather than the live bacterium, was used for treatment of cell monolayers. Caco-2 and HT-29 cells and mouse intestinal tissues using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. An equal amount of RNA for each sample was reverse-transcribed and amplified in a one-step reaction using the Brilliant SYBR Green QRT-PCR Master Mix kit (Stratagene) and Mx 3000 (Stratagene). The gene-specific primers for human or mouse DRA were used for the RT-PCR. ΔCt = DRA and ΔCt = GAPDH represent the difference between the threshold cycle of amplification of DRA and GAPDH, respectively.

**RESULTS**

*Bifidobacterium* CS increases Cl⁻/HCO₃⁻ activity in Caco-2 cells. Since *Bifidobacterium* is an obligate anaerobic bacterium, CS derived from overnight-grown culture medium in an anaerobic chamber, rather than the live bacterium, was used for treatment of cell monolayers. Caco-2 monolayers grown on Transwell inserts or regular plastic supports were treated apically with CS derived from *B. breve*, *B. infantis*, or *B. bifidum* and radioactivity was counted by a liquid scintillation analyzer (TRICARB 1600-TR, Packard Instruments, PerkinElmer, Boston, MA). Cl⁻/HCO₃⁻ exchange activity was assessed as DIDS-sensitive ¹²⁵I⁻ uptake, and values are expressed as nanomoles per milligram protein per 5 min.

**RNA extraction and mRNA expression.** RNA was isolated from Caco-2 and HT-29 cells and mouse intestinal tissues using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. An equal amount of RNA for each sample was reverse-transcribed and amplified in a one-step reaction using the Brilliant SYBR Green QRT-PCR Master Mix kit (Stratagene) and Mx 3000 (Stratagene). The gene-specific primers for human or mouse DRA were used for the RT-PCR. ΔCt = DRA and ΔCt = GAPDH represent the difference between the threshold cycle of amplification of DRA and GAPDH, respectively.

**Western blotting.** After treatment, Caco-2 cells were washed twice with ice-cold 1× PBS and lysed in 20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 1× protease cocktail inhibitor mixture. The cells were lysed by sonication, and the lysate was centrifuged at 7,000 rpm for 7 min at 4°C. Protein concentration was determined by the Bradford assay (6). Equal amounts (75 µg) of cell lysates were solubilized in gel loading buffer and boiled for 5 min. Samples were then loaded on ready-made 7.5% SDS-polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose membranes. The membranes were incubated in blocking buffer containing 1× PBS and 5% nonfat dry milk for 1 h and then with DRA antibody (1:100 dilution) in 1× PBS overnight at 4°C. The membranes were washed four times with the wash buffer containing 1× PBS and 0.1% Tween 20 for 5 min. Finally, the membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2,000 dilution), and the bands were visualized with enhanced chemiluminescence detection reagents.

**Assessment of promoter activity.** Caco-2 cells were transfected with DRA promoter (−1183/+114 bp) fragment cloned upstream of the luciferase reporter gene in pGL2-Basic and β-galactosidase expression vector by electroporation using the Amaxa Nucleofector System, as described previously (30). Activities of firefly luciferase and β-galactosidase were measured according to the manufacturer’s instructions (Promega, Madison, WI). DRA promoter activity is expressed in terms of relative luciferase activity normalized to β-galactosidase activity.

**In vivo studies.** In vivo studies performed in C57BL/6J mice were approved by the Animal Care Committee of the University of Illinois at Chicago and Jesse Brown Veterans Affairs Medical Center. Mice were gavaged with *Bifidobacterium* sp. (10⁹ colony-forming units) in 200 µl of sterile PBS as vehicle for 24 h. Intestines were resected, and mucosa was scraped for RNA and protein extraction. Sections (~2 cm) of the different regions of intestine (ileum and colon) were immediately snap-frozen in optimal cutting temperature embedding medium (Tissue-Tek OCT compound, Sakura) for immunofluorescence studies. RNA and protein were extracted, and real-time qRT-PCR and Western blotting were performed as described above.

**Statistical analysis.** Values are means ± SE of three to five independent experiments. Differences between controls and various treatments were analyzed using one-way analysis of variance with Tukey’s test. Differences were considered significant at P < 0.05.

1 This article is the topic of an Editorial Focus by Debolina Ray, Gianfranco Alpini, and Shannon Glaser (30a).

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Fig. 1. Long-term treatment with *Bifidobacterium* sp. culture supernatant (CS) stimulates Cl⁻/HCO₃⁻ exchange activity in Caco-2 cells. Fully differentiated Caco-2 cells were treated with a 1:2 dilution of *Bifidobacterium breve* *Bifidobacterium infantis*, or *Bifidobacterium bifidum* CS for 24 h (A) or with different dilutions of *B. breve* CS (B), and apical Cl⁻/HCO₃⁻ exchange activity (DIDS-sensitive ¹²⁵I⁻ uptake) was measured. Values are means ± SE of 3–4 separate experiments. *P < 0.05, **P < 0.01 vs. control.

(1:2 dilution) in DMEM-F12 medium for 24 h, and DIDS-sensitive ¹²⁵I⁻ uptake was measured after base loading the cells. Cl⁻/HCO₃⁻ exchange activity was significantly increased by CS obtained from all species of *Bifidobacterium* (Fig. 1A). Further dilution of CS to 1:5 or 1:10 diminished the stimulatory effect on DRA. These data indicate that the soluble effector molecules in the CS derived from *Bifidobacterium* sp. mediate the increase in Cl⁻/HCO₃⁻ exchange activity in Caco-2 cells (Fig. 1B).

*Fig. 2. Bifidobacterium* sp. CS increases DRA mRNA levels in IECs. Since long-term treatment with *Bifidobacterium* CS increased Cl⁻/HCO₃⁻ exchange activity, we next examined whether this stimulation occurs via an increase in expression levels of the apical anion exchangers DRA and/or PAT-1. Caco-2 and HT-29 monolayers were treated with *B. breve*, *B. infantis*, or *B. bifidum* CS for 24 h, and expression of DRA and PAT-1 was determined by real-time qRT-PCR. Consistent with the functional studies, treatment with CS (1:2 dilution) from all *Bifidobacterium* sp. increased DRA mRNA levels (Fig. 2A), mRNA levels of PAT-1, however, did not change significantly under these conditions (Fig. 2B), indicating that these effects were specific. Furthermore, these effects were not cell line-specific, as treatment of HT-29 cells with *Bifidobacterium* CS also increased DRA (Fig. 2C), but not PAT-1 (Fig. 2D), mRNA expression, similar to the effects in Caco-2 cells.
Bifidobacterium sp. CS increases DRA protein levels in Caco-2 cells. To examine whether the increase in DRA mRNA levels by Bifidobacterium CS translates into an increase in protein expression, Western blot studies were performed. Parallel to changes at the mRNA level, CS of Bifidobacterium sp. increased DRA protein levels in Caco-2 cells (Fig. 3A). Densitometric analysis of the protein band shows that CS treatment increases DRA protein levels (≈2-fold) compared with control (Fig. 3B).

Bifidobacterium sp. CS activates DRA promoter activity in Caco-2 cells. To elucidate the mechanisms underlying the increase in DRA function and expression, we further investigated the effects of Bifidobacterium sp. CS on DRA promoter activity in Caco-2 cells. Caco-2 cells were transiently cotransfected with DRA promoter construct along with pCMV-β-galactosidase vector as a control for transfection efficiency. At 24 h posttransfection, cells were treated with CS (1:2 dilution) derived from B. breve, B. infantis, or B. bifidum for 6 and 24 h, and DRA promoter activity was assessed. The results demonstrate an increase in DRA promoter activity in response to CS of all Bifidobacterium sp. at 6 and 24 h (Fig. 4). These data indicate that the Bifidobacterium sp. CS-mediated increase in DRA gene expression was via an increase in DRA promoter activity.

Actinomycin D blocks effects of B. breve and B. infantis CS on DRA mRNA expression. To further confirm whether the Bifidobacterium sp.-mediated increase in DRA mRNA expression occurs at a transcriptional level, we used actinomycin D (5 μM), which blocks the newly synthesized mRNA. Caco-2 cells were pretreated with actinomycin D for 1 h and then coincubated in the presence or absence of CS of B. breve or B. infantis (1:2 dilution) for 24 h. The increase in DRA mRNA levels in response to B. breve or B. infantis CS was abrogated in the presence of actinomycin D (Fig. 5). These data suggest that de novo synthesis of RNA is essential to elicit the effects of B. breve and B. infantis on DRA expression.

Bifidobacterium CS effects on DRA expression are ERK1/2 MAPK-dependent. Previous studies showed that soluble factors of Bifidobacterium sp. activate ERK1/2 MAPK-dependent signaling pathways (18, 31). We thus examined the role of the ERK1/2 MAPK pathway in upregulation of DRA by Bifidobacterium sp. The specific ERK1/2 MAPK inhibitor U0126 (10 μM) blocked the stimulatory effects of B. breve and B. infantis CS on DRA mRNA level in Caco-2 cells (Fig. 6), suggesting involvement of ERK1/2 MAPK in Bifidobacterium sp. effects on DRA expression. This was further confirmed by examining the phosphorylation levels of ERK1/2 in response to B. breve treatment of Caco-2 cells. Interestingly, activation of ERK1/2 occurred as early as 3 h and persisted for 6 h posttreatment (Fig. 6C).

Oral administration of live Bifidobacterium sp. enhances DRA expression. Because study of live Bifidobacterium is limited to in vitro studies due to oxygen sensitivity, we next examined the effects of Bifidobacterium sp. on DRA expression in an in vivo mouse model. Live B. breve, B. infantis, and B. bifidum (10⁹ colony-forming units in 200 μL of PBS)
or vehicle alone was administered by oral gavage to C57BL/6 mice. After 24 h, the mice were euthanized, the intestine was removed, and mucosa was scraped from ileal and colonic regions for RNA isolation and preparation of protein lysates. As shown in Fig. 7, colonic DRA mRNA and protein levels were significantly increased in response to *Bifidobacterium* sp. However, *Bifidobacterium* sp. did not change mRNA levels of DRA in the ileal region. *Bifidobacterium* sp. effects on DRA protein expression were further examined by immunofluorescence staining of colonic sections. As shown in Fig. 8, all three *Bifidobacterium* sp. increased DRA levels on the apical plasma membrane, as

Fig. 5. Actinomycin D blocks *B. breve-* and *B. infantis*-induced increases in DRA mRNA expression. Caco-2 cells were pretreated with the transcription inhibitor actinomycin D (ActD, 5 μM) for 1 h and then cotreated with *B. breve* (A) or *B. infantis* (B) CS (1:2 dilution) for 24 h. DRA mRNA levels were measured using gene-specific primers and normalized to GAPDH mRNA as the internal control. Values are means ± SE of 4 experiments performed in triplicate. **P < 0.01 vs. control. # P < 0.05 vs. *B. breve* (A) or *B. infantis* (B).

Fig. 6. *Bifidobacterium* CS effects on DRA expression are ERK1/2 MAPK-dependent. A and B: Caco-2 cells were pretreated with the specific ERK1/2 MAPK inhibitor U0126 (10 μM) for 1 h and then cotreated with *B. breve* (A) or *B. infantis* (B) CS (1:2 dilution) for 24 h. DRA mRNA levels were measured using gene-specific primers and normalized to GAPDH mRNA as the internal control. Values are means ± SE of 4 experiments. **P < 0.01 vs. control. # P < 0.05 vs. *B. breve* (A) or *B. infantis* (B). C: phosphorylation levels of ERK1/2 in response to *B. breve* CS for 1–6 h in Caco-2 cells. Levels of phosphorylated ERK1/2 (p-ERK1/2) protein were measured using specific antibodies and normalized to total ERK1/2 levels. Values are means ± SE of 4 separate experiments. *P < 0.05 vs. control (Con).
evidenced by increased colocalization (yellow) with villin compared with control.

DISCUSSION

Diarrhea generally occurs via dysregulation of normal intestinal electrolyte transport processes (19, 33, 36). The predominant route of Na\(^+\) and Cl\(^-\) absorption in mammalian ileum and colon involves coupled operation of Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\)\(^-\) exchangers (12, 13). The gene slc26a3, the mutations of which are linked to congenital Cl\(^-\) diarrhea, encodes a protein product, DRA, that mediates Cl\(^-\)/HCO\(_3\)\(^-\) exchange and, thus, plays a critical role in intestinal Cl\(^-\) absorption (22, 25). DRA\(^{-/-}\) mice exhibit substantial diarrheal phenotype with serum electrolyte imbalances (34). Recent genome-wide association studies and animal models of inflammation and infection linked the dysregulated DRA function or expression to diarrhea associated with infection and inflammatory bowel diseases (4, 8, 43, 44). Thus, DRA is emerging as an important target for intervention of diarrheal disorders and, therefore, could be a novel target for antidiarrheal therapy or interventions.

Accumulating evidence suggests that probiotics may provide an effective adjunct to the management of infectious and inflammatory diarrhea (16, 29). Several clinical trials attest to the efficacy of probiotics as a promising complementary approach for treatment of various forms of diarrheal disorders (11, 16, 17, 28, 29, 40). However, many studies showing the beneficial effects of probiotics have utilized a mixture of strains of probiotics, making interpretation of the data difficult, especially with regard to the mechanisms underlying their antidiarrheal effects. Thus, to fully exploit the potential of probiotics as antidiarrheals, a complete understanding of the cellular and molecular mechanisms of a given probiotic species is critical.

In the current report we show, for the first time, that the bacteria-free CS of the probiotic Bifidobacterium sp. stimulates DIDS-sensitive \(^{125}\)I\(^{-}\) uptake via upregulation of DRA expression in Caco-2 cells. Interestingly, all three species of Bifidobacterium (B. breve, B. infantis, and B. bifidum) had a similar effect on apical Cl\(^-\)/HCO\(_3\)\(^-\) exchange activity and DRA expression. In addition to Caco-2 monolayers, Bifidobacterium sp. exhibited stimulatory effects in another colonic cell line, HT-29, further ruling out the notion that these effects are not cell line-specific. It is important to note that the apical Cl\(^-\)/HCO\(_3\)\(^-\) exchange activity in IECs is contributed by two apical anion exchangers: DRA and PAT-1. The effects of Bifidobacterium sp. were specific to DRA, as the expression of SLC26A6 (PAT-1) remained unaltered in response to Bifidobacterium sp. treatment. It is worth mentioning here that upregulation of DRA by live Bifidobacterium sp. increases DRA expression in mouse colon.

Since a decrease in DRA function is implicated in infectious diarrhea, as well as in diarrhea associated with inflammatory bowel diseases, our studies demonstrating upregulation of DRA may underlie the potential beneficial effects of Bifidobacterium. The efficacy of Bifidobacterium in the prevention of antibiotic-associated diarrheal and Clostridium difficile diarrheal in older patients has recently been shown (1). Similarly, Bifidobacterium strains prevented the effects associated with C. difficile in a hamster model of enterocolitis (41). A very recent study showed that B. infantis modulates host inflammatory processes by reducing the TNF and IL-6 levels in ulcerative colitis (15). In addition, B. infantis was shown to suppress Peyer’s patch macrophage inflammatory protein-1α and -1β secretion during Salmonella infection (35). Bifidobacterium has also been shown to be protective against enteropathogenic infection through production of acetate and also via upregulation of the Toll-like receptor-negative regulators (10, 21). The soluble factor(s) in Bifidobacterium that upregulate DRA ex-
Expression is not known and will be subject of future investigations.

Previous studies from our laboratory have demonstrated that short-term treatment of IECs with the probiotic LA strain 4357 (ATCC) substantially increased Cl⁻/HCO₃⁻ exchange activity by increasing apical membrane DRA levels via a phosphatidylinositol 3-kinase-dependent pathway (5). Importantly, the bacteria-free CS of LA was equally effective in stimulating DRA function and apical membrane localization, indicating that LA-secreted soluble factor(s) mediates the effects on DRA trafficking and function. In addition to short-term modulation of DRA trafficking, long-term treatment of IECs with LA CS increased DRA expression and function via modulation of DRA gene transcription (30). Our studies show that long-term treatment of IECs with Bifidobacterium sp. CS increases DRA expression and function via modulation of DRA gene transcription (30). This was evident, as the transcriptional inhibitor actinomycin D was able to block the stimulatory effect of B. breve and B. infantis on DRA mRNA expression. These studies indicate that de novo RNA synthesis is involved in the effects of Bifidobacterium sp. CS on DRA expression. Whether the effects of Bifidobacterium sp. on DRA mRNA occur solely via transcriptional mechanisms is still unclear. DRA promoter activity was significantly increased as early as 6 h after treatment with Bifidobacterium sp. However, the effect on DRA promoter activity was less than the increase in DRA mRNA or function in response to Bifidobacterium. Other mechanisms, such as an increase in mRNA stability, may also be involved.

Bifidobacterium sp. have been shown to activate various signaling pathways or transcription factors in the host cytosol. For instance, administration of B. breve to preterm infants resulted in an upregulation of transforming growth factor-β1.

Fig. 8. Oral administration of Bifidobacterium sp. increases DRA immunostaining. Green, DRA; red, villin; blue, nuclei.
and Smad3 expression (9). *B. breve* has been shown to decrease proinflammatory cytokines in human dendritic cells challenged with *Salmonella typhi* through Toll-like receptor activation (3). *B. infantis* CS prevented cytokine-induced decrease in transepithelial resistance and rearrangement of tight junction proteins via the ERK1/2 MAPK pathway in colonic T84 cells (27). In the current study we used the specific ERK1/2 MAPK pathway inhibitor U0126 (10 μM), which blocked the stimulatory effect of *B. breve* and *B. infantis* CS on DRA mRNA expression in Caco-2 cells. Our data suggest that the effects of *B. breve* and *B. infantis* CS on DRA expression are ERK1/2 MAPK-dependent. This idea was further confirmed by examining phosphorylation levels of ERK1/2 in response to *B. breve* treatment of Caco-2 cells. Interestingly, activation of ERK1/2 occurred as early as 3 h and persisted to 6 h.

*B. infantis* and VSL#3 were shown to attenuate the degree of colitis in IL-10−/− mice (27). To validate the effects of *Bifidobacterium* sp. in an in vivo model, we further examined DRA expression in C57BL/6J mice gavaged with *B. breve*, *B. infantis*, or *B. bifidum*. DRA mRNA and protein expression was significantly enhanced in the colon of C57BL/6J mice gavaged with *B. breve*, *B. infantis*, or *B. bifidum*, whereas mRNA levels of PAT-1 were unchanged in the colon. No effect of *Bifidobacterium* sp. was observed in the ileum. This regional difference could be due to the higher relative expression of DRA and its functional role mainly in the colon compared with the small intestine (2, 23, 24). Another reason for the effects in the colon could be the rapid transit of probiotics from the small intestine compared with the colon and that *Bifidobacterium* sp. more predominantly colonize the colon than the ileum.

In summary, our findings, for the first time, demonstrate an upregulation of DRA function and expression by *Bifidobacterium* sp. via mechanisms involving ERK1/2 activation and increase in DRA promoter activity. Since DRA is one of the key transporters involved in coupled electroneutral NaCl absorption in the intestine, our findings represent a significant contribution to the study of molecular mechanisms underlying the beneficial effects of probiotics in treatment of diarrheal disorders. Further studies are needed to identify the bioactive soluble factors and study their effects on NaCl absorption, as well as to delineate mechanisms used by the bioactive factors of probiotics that produce these beneficial effects.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


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