Lactobacillus acidophilus attenuates downregulation of DRA function and expression in inflammatory models

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Singh V, Kumar A, Raheja G, Anbazhagan AN, Priyamvada S, Saksema S, Jhandier MN, Gill RK, Alrefai WA, Borthakur A, Dudeja PK. Lactobacillus acidophilus attenuates downregulation of DRA function and expression in inflammatory models. Am J Physiol Gastrointest Liver Physiol 307: G623–G631, 2014. First published July 24, 2014; doi:10.1152/ajpgi.00104.2014. —Probiotics, including Lactobacilli, are commensal bacteria that have been used in clinical trials and experimental models for the prevention and treatment of diarrheal disorders. Our previous studies have shown that Lactobacillus acidophilus (LA) and its culture supernatant (CS) stimulated Cl−/HCO3− exchange activity, acutely via an increase in the surface levels of downregulated in adenoma (DRA, SLC26A3) and in long-term treatments via increasing its expression involving transcriptional mechanisms. However, the role of LA in modulating DRA activity under inflammatory conditions is not known. Current in vitro studies using human intestinal epithelial Caco-2 cells examined the efficacy of LA or its CS in counteracting the inhibitory effects of interferon-γ (IFN-γ) on Cl−/HCO3− exchange activity. Pretreatment of cells with LA or LA-CS for 1 h followed by coincubation with IFN-γ significantly alleviated the inhibitory effects of IFN-γ on Cl−/HCO3− exchange activity. In the in vivo model of dextran sulfate sodium-induced experimental colitis (3% in drinking water for 7 days) in C57BL/6J mice, administration of live LA (3 × 109 colony-forming units) via oral gavage attenuated colonic inflammation. LA administration also counteracted the colitis-induced decrease in DRA mRNA and protein levels. Efficacy of LA or its secreted soluble factors in alleviating inflammation and inflammation-associated dysregulation of DRA activity could justify their therapeutic potential in inflammatory diarrheal diseases.

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Materials and Methods

Caco-2 cells were procured from ATCC. Radionucleotide 125I was obtained from PerkinElmer (specific activity 17.0 Ci/mg). RNeasy kits for RNA extraction were obtained from Qiagen (Frederick, MD), and the real-time qRT PCR kit was from Stratagene (La Jolla, CA).
4.4’-Disothiocyanate-stilbene-2,2’-disulfonic acid (DIDS) was pro-
cured from Sigma-Aldrich (St. Louis, MO). Human recombinant IFN-γ was obtained from Sigma. Common reagents for SDS-PAGE such as ammonium per sulfate, acrylamide, and bis-acrylamide were from Fisher Scientific (Pittsburgh, PA).

Cell Culture

Caco-2 cells were grown in T-75 cm² culture flasks at 37°C in a 5% CO₂-95% air incubator in Minimum Essential Medium containing 20% FBS, 20 mM HEPES, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Cells (2 × 10⁶) seeded per well in 12-well Transwell inserts were used between passages 25 and 45. Fully differentiated confluent monolayers were used for the experiments (10–12 days postplating).

Bacterial Culture

The following Lactobacilli species, with ATCC strain numbers given in parentheses, were used in this study: LA (4357), L. rham-
nosus (53103), L. plantarum (14917), and L. casei (393). These species were grown overnight in MRS broth (Difco, Detroit, MI) at 37°C without shaking. Bacteria were spun down by centrifuging at 3,000 rpm for 10 min. For in vitro studies, CS was separated from species were grown overnight in MRS broth (Difco, Detroit, MI) at 37°C without shaking. Bacteria were spun down by centrifuging at 3,000 rpm for 10 min. For in vitro studies, CS was separated from species were grown overnight in MRS broth (Difco, Detroit, MI) at 37°C without shaking. Bacteria were spun down by centrifuging at 3,000 rpm for 10 min. For in vitro studies, CS was separated from species were grown overnight in MRS broth (Difco, Detroit, MI) at 37°C without shaking. Bacteria were spun down by centrifuging at 3,000 rpm for 10 min. For in vitro studies, CS was separated from species were grown overnight in MRS broth (Difco, Detroit, MI) at 37°C without shaking. Bacteria were spun down by centrifuging at 3,000 rpm for 10 min. For in vitro studies, CS was separated from species were grown overnight in MRS broth (Difco, Detroit, MI) at 37°C without shaking. Bacteria were spun down by centrifuging at 3,000 rpm for 10 min. For in vitro studies, CS was separated from species were grown overnight in MRS broth (Difco, Detroit, MI) at 37°C without shaking. Bacteria were spun down by centrifuging at 3,000 rpm for 10 min. For in vitro studies, CS was separated from species were grown overnight in MRS broth (Difco, Detroit, MI) at 37°C without shaking.
Values are means ± SE of 3–8 independent experiments. Differences between control vs. treated were analyzed using one-way ANOVA with Tukey’s test. Differences were considered significant at P ≤ 0.05.

RESULTS

Long-Term Treatment with LA-CS Stimulates Cl−/HCO3− Exchange Activity in Caco-2 Cells

Our earlier studies showed that short-term treatments with LA and L. rhamnosus were effective in enhancing Cl−/HCO3− (OH−) exchange activity (5). In the current studies, the effects of long-term treatments with various Lactobacillus species on Cl−/HCO3− (OH−) exchange activity were evaluated. Postconfluent Caco-2 monolayers were treated with the CS (diluted 1:10 in DMEM) from different species of Lactobacillus for 24 h, and Cl−/HCO3− exchange activity was measured as DIDS-sensitive 125I uptake. As shown in Fig. 1, LA-CS significantly enhanced Cl−/HCO3− exchange activity (~2-fold) in Caco-2 cells after 24 h. The CS of L. rhamnosus, L. plantarum, and L. casei showed no significant effect. Therefore, all further in vitro or in vivo studies were carried out only with LA.

Live LA or CS blocks IFN-γ-Induced Inhibition of Cl−/HCO3− Exchange Activity in Caco-2 Cells

We next examined whether LA can counteract the inhibitory effects of IFN-γ on DRA function. Postconfluent Caco-2 monolayers were pretreated with live bacteria or CS (LA-CS) from the apical surface for 1 h, which was continued for another 24 h with and without IFN-γ (30 ng/ml) added basolaterally. The concentrations of IFN-γ used in this study are based upon our previous studies showing inhibition of NHE3 (2) and DRA (34) gene expression by IFN-γ in Caco-2 cells as well as other studies (15). Live LA or LA-CS (1:10) significantly stimulated activity, whereas IFN-γ treatment significantly (P < 0.05 vs. control) inhibited the Cl−/HCO3− exchange activity (~2-fold) in Caco-2 layers. Furthermore, both live LA (Fig. 2A) and LA-CS (Fig. 2B) completely blocked the IFN-γ-induced inhibition of Cl−/HCO3− exchange. These results show that either live bacteria or their secreted products in CS are enough to block the inhibition of Cl−/HCO3− exchange activity in response to IFN-γ treatment.

LA-CS Increases DRA mRNA Levels and Blocks IFN-γ-Induced Decrease in DRA

We next examined whether LA counteracts the IFN-γ-mediated decrease in Cl−/HCO3− exchange activity via alterations in DRA mRNA levels. For these studies, cells were pretreated with LA-CS (1:10) from the apical surface for 1 h, which was continued for an additional 24 h, with and without IFN-γ (30 ng/ml) added basolaterally in serum-reduced medium (1% FBS). As reported previously by us (28) DRA mRNA levels were significantly increased (~2.2-fold) in re-

Fig. 1. Long-term treatment with Lactobacillus acidophilus (LA)-culture supernatant (CS) stimulates Cl−/HCO3− exchange activity in Caco-2 cells. Overnight serum-starved postconfluent Caco-2 cells were treated with 1:10 dilution of CS of LA, L. rhamnosus (LR), L. plantarum (LP), and L. casei (LC) for 24 h, and apical Cl−/HCO3− exchange activity [4,4′-disothiocyanate-stilbene-2, 2′-disulfonic acid (DIDS)-sensitive 125I uptake] was measured. Values are means ± SE; n = 3 mice. *P < 0.05 compared with control.

Fig. 2. LA attenuates interferon-γ (IFN-γ)-mediated inhibition of apical Cl−/HCO3− exchange activity. Postconfluent Caco-2 monolayers were pretreated with live LA (A) or with LA-CS (B) from the apical surface for 1 h, which was continued for an additional 24 h, with and without IFN-γ (30 ng/ml) added basolaterally. DIDS-sensitive 125I uptake was then measured for 5 min. Values are means ± SE; n = 3. ***P < 0.01 and *P < 0.05 compared with untreated control. **P < 0.05 compared with IFN-γ.

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LA-CS attenuates IFN-γ-induced inhibition of DRA promoter activity in Caco-2 Cells

We have previously shown that the activity of DRA full-length promoter construct p-1183/+114 exhibited (~50%) inhibition in response to IFN-γ treatment compared with untreated controls (1, 34). In an attempt to examine whether LA-CS can reverse the effects of IFN-γ on DRA promoter, Caco-2 cells were transfected with DRA full-length promoter construct p-1183/+114. Twenty-four hours posttransfection, cells were treated with LA-CS (1:10 dilution) apically or IFN-γ basolaterally or coincubated with LA-CS and IFN-γ for 24 h, and DRA promoter activity was assessed. DRA promoter activity was markedly increased (~1.5-fold) in response to LA-CS; IFN-γ treatment exhibited a 50% decrease in the activity, whereas the inhibitory effects of IFN-γ on DRA promoter activity were blocked in cells pretreated with LA-CS (P < 0.05 vs. IFN-γ) as shown in Fig. 4.

LA-CS Attenuates IFN-γ-Induced Inhibition of DRA Promoter Activity in Caco-2 Cells

In Vivo Effects of LA on DSS-Induced Colitis in Mice

Our in vitro studies in Caco-2 cells clearly demonstrated that out of four Lactobacillus species tested, long-term treatment with LA showed maximum increase in Cl⁻/HCO₃⁻ exchange activity by increasing DRA expression. Also LA-CS was able to block the inhibition of Cl⁻/HCO₃⁻ exchange activity and repression of DRA promoter caused by IFN-γ in Caco-2 cells. Therefore, to get a comprehensive idea of the effect of this probiotic strain in a complex physiological setting, we next investigated the effects of LA on DRA expression in an in vivo mouse model of DSS-induced colitis.

**LA did not attenuate DSS-induced loss of body weight.** The DSS-induced colitis murine model is commonly used as a chemical injury model to address the pathogenesis of inflammatory bowel disease (14, 37). To test the efficacy of LA in ameliorating colitis, C57BL/6 mice were given 3% (wt/vol) DSS in drinking water for 7 days. As shown in Fig. 5A, significant weight loss was evident in the DSS-treated group compared with the control or LA-treated group. Whereas animals in the control group gained weight over a 1-wk period of observation, the mice in the DSS group showed significant reduction in weight over the same time, which was evident starting after the 5th day of DSS treatment and persisted until the 6th and 7th day compared with control. However, this weight loss was not attenuated in the LA + DSS mice group (Fig. 5A).

**LA reduces MPO activity in acute DSS-treated mice colon.** Because the DSS-induced colitis mostly affects the distal part of the colon (26), a part (2 cm) of distal colon was used for determination of MPO activity. Colonic MPO level, an index of neutrophil accumulation, was significantly increased in DSS mice compared with control or LA mice (P < 0.01 vs. control or LA) (Fig. 5B). This increase in MPO levels was attenuated by LA treatment in the LA + DSS group (Fig. 5B). These results suggested that LA exerts its protective effects in the colon of mice with colitis by decreasing inflammation caused by DSS.

Changes in colon weight-to-length ratio. Colon weight and length were recorded for each mouse immediately after death (8th day since the start of DSS treatment) (Fig. 5C). The length of the colon was significantly shortened (P < 0.05) in mice in the DSS group (Table 1). Although the colon length was greater in the LA group, compared with control, this was not significant (P = 0.26 vs. control). Furthermore, colon weight and weight-to-length ratio were significantly increased in the DSS group compared with the control group, whereas animals in the LA group showed a significant decrease in these values (Fig. 5D). These results suggest that LA reduces weight loss and weight gain associated with DSS-induced colitis.

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statistically significant. Also, the DSS group showed a significant increase in the colon weight-to-length ratio compared with control (Fig. 5C), an indication of fluid accumulation that, however, was not significantly attenuated in the LA + DSS mice group. It should be noted that the pelleted stool content (Fig. 5C) as examined visually (qualitative measurement) appeared to decrease in DSS colon compared with control. In contrast, in LA + DSS mice this phenotype was less prominent. These results indicate that LA may have beneficial effects in colons of mice, including potential antidiarrheal effects.

**LA Blocks DSS-Induced Decrease in DRA mRNA and Protein Expression in Distal Colon**

Our previous studies have already shown that DRA mRNA and protein expression in mice colonic tissue increases in response to live LA gavaged for 24 h (28). To examine whether LA could restore DSS-induced downregulation of DRA mRNA and protein expression, we examined DRA mRNA and protein levels in distal colonic mucosa of control, DSS, LA, and LA + DSS mice groups using real-time PCR and immunoblotting. As shown in Fig. 6, DRA mRNA levels in distal colon were significantly increased (~1.5-fold) in response to LA administration. DSS caused a significant decrease in DRA mRNA and protein expression (~50–60%) in distal colon. On the other hand, LA treatment to DSS mice significantly attenuated this decrease in the levels of DRA mRNA (Fig. 6) and protein (Fig. 7) in LA + DSS mice. LA-mediated reversal of DSS effects on DRA expression was also measured by immunofluorescence staining of colonic sections. As shown in Fig. 8, the apical membrane level of DRA was substantially reduced in response to DSS treatment. On the other hand, LA administration not only increased apical membrane DRA level compared with control but also almost completely blocked the inhibitory effects of DSS on DRA expression (Fig. 8). These results suggest that modulation of DRA protein expression by LA might be a major contributor to the observed antidiarrheal effects of LA in DSS colitis.

### Table 1. Colonic weight and colon length of different treatment groups

<table>
<thead>
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<th>Control</th>
<th>LA</th>
<th>DSS</th>
<th>LA + DSS</th>
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<tr>
<td>Length, cm</td>
<td>6.48 ± 0.18</td>
<td>6.75 ± 0.40</td>
<td>5.04 ± 0.09*</td>
<td>5.75 ± 0.24</td>
</tr>
<tr>
<td>Weight, g</td>
<td>0.38 ± 0.03</td>
<td>0.33 ± 0.02</td>
<td>0.41 ± 0.03</td>
<td>0.39 ± 0.03</td>
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Values are means ± SE. *LA, *Lactobacillus acidophilus*; DSS, dextran sulfate sodium. Full colonic length and weight (excluding cecum) were measured immediately after death. *P < 0.05 vs. control.

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Fig. 6. Oral administration of live LA in mice attenuates dextran sulfate sodium (DSS)-induced effects on body weight, myeloperoxidase (MPO) level, and the colon weight-to-length ratio. A: change in body weight during DSS treatment: mice were given 3% DSS in drinking water for 7 days. Control mice had only drinking water. LA (3 × 10^9 colony-forming units) were gavaged to control or DSS-treated mice for the first 2 days. Data were expressed as means ± SE of body weight in each animal (n = 4). B: MPO activity in distal colon during DSS treatment: measurement of colonic MPO activity, an index of neutrophil accumulation, was measured in the distal colonic mucosa of different groups, as described in MATERIALS AND METHODS. Data calculated as MPO units/g protein and are expressed as % of control (n = 4). ***P < 0.001 compared with untreated control. *P < 0.05 compared with DSS alone. C: changes in colon weight-to-length ratio in response to DSS treatment: C57BL/6 mice were administered 3% DSS in drinking water or LA as described above for 7 days. At the 8th day mice were killed, the entire colon was dissected out, and length and weight were recorded. Data were expressed as the means ± SE of the ratio of weight/length changes in each group (n = 8). *P < 0.05 compared with untreated control. Visual examination of colon from different groups suggests almost reversal of colon weight-to-length ratio in the LA + DSS group compared with DSS alone.

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**L. ACIDOPHILUS ATTENUATES COLITIS-INDUCED REPRESSION OF DRA**

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Diarrhea associated with intestinal inflammatory diseases, including colitis, is the result of a complex interaction of multiple inflammatory mediators and their effects on the intestinal epithelium. Diarrhea can result partly in response to enhanced secretion of high levels of proinflammatory cytokines such as IFN-γ and TNF-α (7, 13, 17). Studies have shown that Caco-2 cells treated with proinflammatory cytokines (IFN-γ, IL-1β) have diminished expression of DRA, and the same is true for patients suffering from ulcerative colitis (34, 36). SLC26A3 or DRA is the main candidate gene for luminal human intestinal Cl⁻/HCO₃⁻ exchange (12). Its importance is further emphasized by DRA knockout mice, which exhibit diarrheal phenotype owing to loss of luminal membrane Cl⁻/HCO₃⁻ exchange activity (35). We have previously shown in vitro and in vivo models that probiotics such as LA may have potential therapeutic value in treating diarrhea due to their efficacy in increasing Cl⁻/HCO₃⁻ exchange activity and DRA expression involving both short-term trafficking and long-term transcriptional changes, respectively (6, 28). Our group (4, 5) as well as others (30) have previously demonstrated that probiotics reverse the effects of inflammatory cytokines in human intestinal epithelial cells and inflammation in a mouse model (33). Experimental studies have implicated DRA repression as one of the potential events during intestinal inflammation (19, 38). However, the functional consequences of this repression have not been studied in detail. Also, nothing is known about the potential beneficial effects of probiotics on DRA (Cl⁻/HCO₃⁻ exchange) function and expression under inflammatory states such as colitis.

In the current report, we have shown that pretreatment with LA or bacteria-free CS of LA counteracts the inhibitory effects of IFN-γ on DIDS-sensitive apical Cl⁻/HCO₃⁻ exchange activity. Parallel to function, IFN-γ-mediated inhibitory effects on DRA mRNA levels and promoter activity were abrogated by LA-CS. Consistent with our data, earlier reports have shown that pretreatment with live probiotics reversed the effects of TNF-α or IFN-γ on cystic fibrosis transmembrane conductance regulator (30). These results suggest that LA-derived soluble factors exert distinct effects to alleviate/block the cytokine effects on DRA expression and function. Our functional studies are limited by lack of DRA-specific inhibitor; however, given that LA-CS treatment does not affect PAT-1 expression (28) and our functional read out is parallel to changes in DRA mRNA and protein, it is highly likely that LA-mediated effects on apical Cl⁻/HCO₃⁻ exchange activity are mediated by DRA.

Our promoter studies have shown that LA-CS enhanced the activity of the full-length (p-1183/1114) DRA promoter. We have previously shown that IFN-γ inhibition of DRA promoter activity involved the JAK/STAT1 pathway (34). Hence, it can be speculated that LA-secreted soluble factors in the CS could reverse the effects of inflammatory cytokines either by blocking the phosphorylation of STAT-1 signaling pathways induced by IFN-γ or via blocking the binding of cytokine to its receptor. However, because live LA or LA-CS were added from the apical side of cell monolayers, whereas IFN-γ receptors are localized to basolateral membranes of polarized Caco-2 cells, it is unlikely that live LA or its supernatant-derived soluble factors counteracted IFN-γ effects via blocking its binding to the receptors. In addition, LA could also act via an independent pathway to counteract inhibition of promoter activity by IFN-γ. For example, involvement of p38, ERK1/2 MAPK, and phosphatidylinositol 3-kinase (PI 3-kinase) (30) has been previously reported in LA-induced modulation of Cl⁻ secretion in Caco-2 and HT29/cl.19a cells (30). We previously showed that acute stimulation in DRA activity by LA and increase in DRA levels on the plasma membrane involved PI 3-kinase (but not MEK1, MEK2, and p38 MAPK pathway)(6). It is possible that the PI 3-kinase-mediated pathway may be involved in observed effects of LA in counteracting the effects of cytokines. Future studies may focus on delineating exact signaling mechanisms underlying the effects of LA.

We extended our in vitro studies showing efficacy of LA in reversing the effects of inflammatory cytokine on DRA activity using an in vivo model of DSS colitis. Based on our previous study (33), we used 3% DSS for 7 days to induce mild colitis in mice, since DSS at high concentrations (4–5%) is known to cause ulcerations and erosion of the epithelium (23). Two doses of LA were chosen based on our previous study showing that, under similar conditions, LA attenuated inflammation and reversed the reduced expression of MDR-1 transporter in DSS-treated mice. Also, multiple studies suggest that probiotic bacteria remain colonized in the gut for at least 7 days to exert beneficial effects (27, 33). In DSS-induced colitis in mice, the most affected segment of the gastrointestinal tract is known to be the distal colon showing inflammation, shortening of length and loose fecal pellet, compared with control mice (29). Our studies also showed that mice administered DSS showed significant weight loss and shortening of the colon. However, these effects were not significantly reversed by administration of live LA. Furthermore, weight loss, MPO activity, and colon weight-to-length ratio (a potential readout for diarrheal phenotype) were significantly greater in the DSS group compared

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**Fig. 7. LA increases DRA protein levels and blocks DSS-induced decrease in DRA protein expression.** DRA protein levels in distal colonic mucosal tissue lysates were measured by Western blot as described in MATERIALS AND METHODS. A representative blot is shown. Bottom, densitometric analysis of relative band intensities. Results represent means ± SE of 8 mice. *P < 0.05 and **P < 0.01 compared with control. *P < 0.05, LA + DSS compared with DSS alone.
with all other groups. Occurrence of solid fecal pellet in LA + DSS mice indicated potential beneficial effects of LA on diarrheal phenotype and on colonic inflammation caused by DSS. Also, the inhibition of enhanced colonic MPO activity in DSS mice by LA reflects a potent anti-inflammatory effect of LA against tissue injury. The anti-inflammatory/immunomodulatory properties of various *Lactobacillus* species have previously been described by our group and others in experimental models of colitis (10, 25, 33). Studies have shown that fecal excretion of HCO$_3^-$ is reduced in patients with active ulcerative colitis, which is caused by impairment of the colonic anion exchange process induced by inflammation (9). This is mainly due to a major decrease in the Cl$^-$/HCO$_3^-$ exchange activity in the luminal membrane, associated with a downregulation of the anion exchanger DRA (SLC26A3) in colitis (36). As expected, levels of DRA mRNA and protein expression were dramatically reduced in the distal colon of DSS-treated mice compared with control, whereas the other group where live LA was administrated along with DSS showed significantly less reduction in DRA mRNA and protein levels compared with the DSS-treated group alone. Our results are consistent with previous reports showing partial reversal of the effects of DSS in response to pretreatments with LA (11, 25). However, the mechanism of LA-mediated reduction in gut inflammation is not entirely clear. This could involve attenuation of effect of cytokines, beneficial effects on epithelial integrity, and improvement in barrier function (29).

Overall, our results demonstrated that the soluble factor(s) present in the CS of LA attenuate the IFN-$\gamma$-induced decrease in DRA function, mRNA and promoter activity. Also, live LA administration in vivo showed its effects by reversing the DSS-induced decrease in DRA mRNA and protein levels and appeared to have potential antidiarrheal effects in an experimental model of DSS colitis. These results are of critical importance in understanding the pathophysiology of inflammatory bowel disease and the potential therapeutic use of probiotics.

![Fig. 8. LA treatment blocks DSS-induced decrease in DRA immunostaining. Green, DRA; red, villin; blue, nuclei; scale bar = 20 µm.](http://ajpgi.physiology.org/)

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importance in increasing our understanding of the molecular basis of the beneficial effects of LA and L.A.-derived molecules. Additionally, efficacy of LA supernatant to counteract inflammation-induced downregulation of DRA expression and function highlights the novel therapeutic potentials of LA-secreted soluble factors for diarrheal disorders associated with gut inflammation where live bacteria may be counterindicated for therapeutic purposes due to compromised barrier function of the intestine. In future studies, it will also be important to examine efficacy of LA as a therapeutic (after DSS treatment) rather than preventive agent in reversing inflammation and DRA expression.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


