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***Lactobacillus* GG and Tributyrin Supplementation Reduce Antibiotic-Induced Intestinal Injury**

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Abstract

Background—Antibiotic therapy negatively alters the gut microbiota. *Lactobacillus* GG (LGG) decreases antibiotic-associated diarrhea (AAD) symptoms, but the mechanisms are unknown. Butyrate has beneficial effects on gut health. Altered intestinal gene expression occurs in the absence of gut microbiota. We hypothesized that antibiotic-induced changes in gut microbiota reduce butyrate production, varying genes involved with gut barrier integrity and water and electrolyte absorption, leading to AAD, and that simultaneous supplementation with LGG and/or tributyrin would prevent these changes.

Methods—C57BL/6 mice aged 6–8 weeks received a chow diet while divided into 8 treatment groups ($\pm$ saline, $\pm$ LGG, $\pm$ tributyrin, or both). Mice received treatments orally for 7 days with $\pm$ broad-spectrum antibiotics. Water intake was recorded daily and body weight was measured. Intestine tissue samples were obtained and analyzed for expression of genes and proteins involved with water and electrolyte absorption, butyrate transport, and gut integrity via polymerase chain reaction and immunohistochemistry.

Results—Antibiotics decreased messenger RNA (mRNA) expression (butyrate transporter and receptor, Na⁺/H⁺ exchanger, Cl⁻/HCO₃⁻, and a water channel) and protein expression (butyrate transporter, Na⁺/H⁺ exchanger, and tight junction proteins) in the intestinal tract. LGG and/or tributyrin supplementation maintained intestinal mRNA expression to that of the control animals, and tributyrin maintained intestinal protein intensity expression to that of control animals.

Conclusion—Broad-spectrum antibiotics decrease expression of anion exchangers, butyrate transporter and receptor, and tight junction proteins in mouse intestine. Simultaneous oral supplementation with LGG and/or tributyrin minimizes these losses. Optimizing intestinal health with LGG and/or tributyrin may offer a preventative therapy for AAD.

Keywords

probiotics; tributyrin; antibiotics; diarrhea; intestine

Trillions of bacteria consisting of more than 800 different bacterial species and 7000 strains comprise the gut microbiota.¹ The gut microbiota markedly influences the biology of the host through several mechanisms, including energy balance, gene expression, immune function, and disease processes; thus, any disturbances in the microbiota can lead to a

variety of pathogenic states.^{1–3} Antibiotic therapy is believed to represent such a condition.⁴ Antibiotic-associated diarrhea (AAD), defined as diarrhea associated with the administration of antibiotics without another obvious cause, occurs in approximately 5%–25% of patients receiving antibiotics, varying with the class of antibiotics used and patient risk factors.⁵ Overgrowth by the toxigenic bacterium *Clostridium difficile* is responsible for virtually all cases of antibiotic-associated pseudomembranous colitis, which can lead to complications such as paralytic ileus and colonic dilatation and perforation.⁶ However, it is estimated that only 15%–25% of all cases of AAD are due to the overgrowth of *C difficile*.⁴ Alterations in the composition and quantity of gut microbiota leading to losses of beneficial metabolic activities of the normal colonic microbiota are associated with non-*C difficile* AAD.^{7–9}

Short-chain fatty acids (SCFAs) are fermentation by-products of undigested polysaccharides and some proteins by anaerobic bacteria formed in the intestinal tract of mammals.^{10,11} The metabolism of undigested fiber and starch by colonic anaerobes to SCFAs, particularly butyrate, is hypothesized to prevent osmotic diarrhea as well as provide a supply of the preferred carbon and energy source to the colonic enterocytes.^{12–14} Recent metabolomic studies have shown that depletion of Gram-positive bacteria by vancomycin disrupts carbohydrate fermentation in mice; these changes increase quantities of unfermented oligosaccharides in the feces and reduce concentrations of the SCFAs acetate, butyrate, propionate, and lactate.⁷ Distinct gut microbiota diversity, including a marked decrease in the prevalence of butyrate-producing bacteria, was found following administration of the antibiotic amoxicillin-clavulanate.⁸ This alteration is of significant concern because among the SCFAs, butyrate is highly important as it contributes to the differentiation of epithelial cells, enhancement of electrolyte and water absorption, promotion of angiogenesis, and modulation of the immune function.^{12–14}

It is estimated that the average intraluminal concentration of SCFA is between 100 and 170 mM, and acetate, propionate, and butyrate are present in a nearly constant molar ratio of 60:25:15.¹¹ Butyrate can reach concentrations up to 20 mM in the colon and feces of mammals with normal gut health.¹⁰ In the adult human with a fecal output of 80–230 g/d, SCFAs are excreted only at a rate of 5–20 mM/d, so most SCFAs (95%) are absorbed.¹⁵ Butyrate is also available in the diet with low levels in many fruits and vegetables, and milk fat, which contains 3%–4% butyrate in a complex of glycerides or esters of glycerol, is also a good source of butyrate.¹⁶ Another source of butyrate, glyceryl tributyrinate (tributylin), is a triglyceride with glycerol esterified with butyrate at the 1, 2, and 3 positions.¹⁰ Tributyrin is neutral, chemically stable, and rapidly hydrolyzed by pancreatic and gastric lipases to glycerol and 3 butyrate molecules.¹⁰ There are several mechanisms by which butyrate exits the gut lumen. Lipid-soluble protonated SCFAs diffuse readily across cell membranes, but ionized SCFAs do not and require various anion exchangers for diffusion.¹⁰ Recent studies have identified an Na⁺-coupled transporter for butyrate and other SCFAs.^{17,18} SLC5A8 is expressed in the apical membrane throughout the intestinal tract and most abundantly in the ileum and colon.¹⁸ SLC5A8 transports butyrate via an Na⁺-dependent electrogenic process, and the expression of the transporter is reduced markedly in colon cancer and germ-free (GF) mice.^{18,19} Other monocarboxylate transporters are also present throughout the intestinal tract.^{20,21}

Providing butyrate can be challenging for several reasons, including short metabolic half-life, toxicity, and patient intolerance. Butyrate has been provided via several routes: intravenously, rectally as enemas, and orally. There are limitations to providing butyrate intravenously (500 mg/kg body weight) in that large volumes are required, and the metabolic half-life is very short, with blood levels peaking about 6 minutes after delivery.¹⁰ Providing higher rates of intravenous (IV) butyrate infusion is undesirable due to risk of toxicity from sodium overload. Rectal enemas (100 mmol/L) have been successful in

reversing negative gastrointestinal (GI) effects in patients with inflammatory bowel disease; however, this mode of delivery lends to very poor patient compliance.¹⁰ Tributyrin overcomes many of the problems of the parent compound. Tributyrin delivered orally in animals has a plasma half-life of 40 minutes.¹⁶ In humans, oral delivery provided once daily for 3 weeks was without severe toxicity, and peak plasma butyrate concentrations occurred between 0.25 and 3 hours after dose and ranged from 0–0.45 mM, which is near those found to be effective in vitro (0.5–1 mM).²²

Probiotics, defined as “live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host,” have been used in the treatment and prevention of AAD as well as in the prevention of relapses of *C difficile*-associated diarrhea.²³ The exact mechanism of how probiotics prevent AAD is unknown, but they are believed to compete with pathogenic microbes for available nutrients and epithelial binding sites; decrease GI luminal pH, making it less favorable for pathogenic bacteria; modulate the immune response; and reestablish the intestinal barrier function.²⁴ A meta-analysis of randomized controlled trials found a moderate beneficial effect of *Lactobacillus* GG (LGG), *Saccharomyces boulardii*, and a combination of *Bifidobacterium lactis* and *Streptococcus thermophilus* in preventing AAD.²⁵ A Cochrane review of 10 randomized controlled trials with probiotics found a significant reduction in the incidence of AAD, confirming the efficacy of LGG and *S boulardii*.²⁶

The expression of genes in the ileum and colon is altered markedly in GF mice compared with mice raised under conventional conditions.^{19,27,28} DNA microarray analysis showed that ~700 genes were affected (increased or decreased) by more than 2-fold in the colon from GF mice compared with the colon from conventional mice, and these changes were completely reversed when the colon was recolonized.¹⁹ Most notable among the genes that were downregulated in GF mouse colon compared with conventional mouse colon were those involved in immune development and antimicrobial defense, with some downregulated more than 20-fold. Transporters involved with water and electrolyte exchange were also down-regulated, including SLC5A8 (sodium-coupled butyrate transporter), SLC26A3 (chloride-bicarbonate exchanger), aquaporin 4 (AQP4, water channel), NHE3 (sodium-hydrogen exchanger), and a butyrate receptor involved with inflammation (GPR109a).¹⁹ It is very interesting and potentially clinically relevant that genes involved with water and electrolyte absorption were downregulated in GF mouse ileum and colon, suggesting that conventional gut microbiota play an active role in the control of water and electrolyte absorption.

Since antibiotic usage can cause profound changes in gut microbiota, it is likely that there is a consequential reduction of butyrate produced in the GI tract. We hypothesize that altered gut microbiota from antibiotic therapy affects expression of genes involved with water and electrolyte absorption as seen in GF mice, as well as those dependent on butyrate for expression. The objective of this work was to explore the efficacy and mechanism of probiotics and/or tributyrin provision as a clinically feasible method for mitigating AAD through the preservation of physiologic responses in the intestine.

Materials and Methods

Materials

Antibiotics, sucrose, tributyrin, MRS broth and agar, and Mueller Hinton media were purchased from Sigma-Aldrich (St Louis, MO); *Lactobacillus rhamnosus* strain GG (LGG) was purchased from ATCC (ATCC, Rockville, MD); RNA extraction reagent (TRIzol) was from Invitrogen-GIBCO (Carlsbad, CA); GeneAmp reverse transcription polymerase chain reaction (RT-PCR) kit was from Applied Biosystems (Foster City, CA); and Taq polymerase

kit was from TaKaRa (Tokyo, Japan). All primers for real-time RT-PCR were synthesized by Integrated DNA Technologies (Coralville, IA). Primary antibodies were purchased from the following companies: Abcam (Cambridge, MA) for SLC5A8, NHE3, and zonula occludens 1 (ZO-1) and Hycult Biotech (Plymouth Meeting, PA) for occludin.

Animals

Studies were performed at 2 institutions: the Medical College of Georgia (Augusta, GA) and the Cleveland Clinic (Cleveland, OH). Female C57BL6 mice (6–8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME) or the National Cancer Institute (NCI, Frederick, MD). All mice were housed, maintained, and studied in accordance with approval from the National Institutes of Health (NIH), Medical College of Georgia, and/or the Cleveland Clinic Institutional Animal Care and Use Committee. Upon arrival, the animals were acclimated, and during this time, the animals had access ad libitum to tap water and regular unsterilized food. The animals were divided into 8 treatment groups and housed together with 4 mice per cage (see below). Feeding trials were repeated for adequate statistical power.

Antibiotic-Free Groups

- Group 1** control (provided with sodium bicarbonate, plain broth, or saline for 7 days)
- Group 2** LGG group (provided 10^6 colony-forming units [CFU] LGG for 7 days)
- Group 3** Tributyrin group (provided 5 mM tributyrin for 7 days)
- Group 4** LGG and tributyrin group (provided 10^6 CFU LGG and 5 mM tributyrin for 7 days)

Antibiotic Therapy Groups

- Group 5** metronidazole, neomycin sulfate, vancomycin (MNV) group only control (provided with sodium bicarbonate, plain broth, or saline for 7 days)
- Group 6** LGG group (provided 10^6 CFU LGG for 7 days)
- Group 7** Tributyrin group (provided 5 mM tributyrin for 7 days)
- Group 8** LGG and tributyrin group (provided 10^6 CFU LGG and 5 mM tributyrin for 7 days)

Fresh stool samples were obtained on days 0 and 7. Following antibiotic therapy and probiotic and tributyrin treatments (see below), mice were euthanized and the proximal jejunum, terminal ileum, and proximal and distal colon were removed for preparation of RNA and tissue sections.

Antibiotic Delivery

Antibiotics were provided as described previously.²⁹ For antibiotic treatment, mice were provided metronidazole (1 g/L), neomycin sulfate (500 mg/L), and vancomycin (1 gm/L) in their water supply daily for 7 days. The water supply for both the antibiotic-treated and control (antibiotic-free) groups contained 15% sucrose concentration to encourage consumption. The amount of water consumed was recorded daily, and animal weight was measured and recorded on days 0, 3, and 7 of the treatments.

Oral Inoculation of LGG and Assay for Fecal Excretion

The colonization of mice with LGG was performed as described previously for other bacteria.^{30,31} Briefly, 6- to 8-week-old C57BL/6 mice were inoculated orally daily for 7 days throughout the antibiotic therapy with LGG as follows. Single-colony LGG was cultured in MRS broth at 37°C in an atmosphere of 5% (v/v) CO₂ in air for 18–20 hours prior to the inoculation. Mice were given 0.15 mL of 5% sodium bicarbonate by oral gavage to buffer stomach acidity. The mice were then provided a dose of 1×10^6 CFU in 0.15 mL LGG broth by oral gavage. Control animals received sodium bicarbonate and LGG broth only. Tributyrin (5 mM/L) was provided in a similar manner as the LGG. One 0.15-mL bolus was provided for the mice that received both tributyrin and LGG. Colonization with LGG was determined by viable counts of LGG bacteria in fecal pellets, which was enumerated on selective media (MRS agar; Sigma-Aldrich). The presence of the most commonly encountered aerobic and facultative anaerobic bacteria was determined by viable counts of bacteria in fecal pellets enumerated on Mueller Hinton agar (Sigma-Aldrich).

RT-PCR

RNA was prepared as previously described from antibiotic-treated and control mouse ileum and colon, which were used for RT-PCR.³² The PCR primers for gene-specific products were designed based on the nucleotide sequences available in GenBank (Table 1). The level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) was used as the internal control in RT-PCR. PCR products were size fractionated on agarose gels. Bands were visualized by ethidium bromide signals quantified using the STORM phosphorimaging system (Global Medical Instrumentation, Inc, Ramsey, MN). RT-PCR was carried out with 3 or 4 biological replicates, and PCR was repeated at least twice with each RNA sample. The band intensity of each PCR product was normalized using GAPDH mRNA as an internal control.

Immunofluorescence

Cryosections of mouse intestinal sections were fixed at room temperature in 4% paraformaldehyde for 20 minutes at room temperature and then washed with phosphate-buffered saline (PBS). Sections were then blocked with 2% bovine serum albumin (diluted in PBS) containing 0.1% Triton X-100 for 1 hour, followed by overnight incubation at 4°C with the primary antibody (anti-SLC5A8, 1:2000; anti-NHE3, 1:1000; anti-occludin, 1:50; or anti-ZO-1, 1:100). Negative control sections were treated identically except that primary antibody was substituted with PBS for overnight incubation. All sections were rinsed with PBS (3 times for 5 minutes each), incubated with the fluorochrome-conjugated secondary antibody for 2 hours in the dark at room temperature (for detection of SLC5A8 labeling, sections were incubated with 1:250 goat anti-rabbit IgG Alexa Fluor 568; ZO-1 and NHE3 with 1:250 goat anti-rabbit IgG Alexa Fluor 488; occludin with 1:250 goat anti-guinea pig IgG Alexa Fluor 568; Invitrogen, Grand Island, NY), washed again in PBS, and mounted with VECTASHIELD containing DAPI and antifade reagent (Vector Laboratories, Burlingame, CA). Fluorescent images were acquired using an inverted fluorescent microscope (Leica, Cologne, Germany). No specific immunostaining was seen in sections incubated with PBS.

Statistical Analysis

All values presented represent means $\pm$ standard error of the mean (SEM), with $n = 4$ –12 experimental points (per site). Data were analyzed by analysis of variance using the general linear models procedure (SAS Institute, Cary, NC) and per experimental site. Data were log-transformed, if needed, to obtain a normal distribution. Follow-up comparisons were made by least squares means testing. A P value of <0.05 was considered statistically significant.

Results

Effects of Treatments on Mouse Health

The mice tolerated the supplemental oral gavage treatments well. The dose of LGG was sufficient to survive transit through the gut of mice; mice receiving LGG had 10^3 – 10^4 CFU LGG in their fecal pellets compared with no detectable LGG in animals receiving saline, broth, or only tributyrin (Table 2). All groups were colonized with bacteria, but the total number of bacteria in the antibiotic-saline group was minimal. At the study start (day 0), for animals enrolled at both study sites, mouse weights did not differ between treatment groups (Table 3). However, by day 3, the mice in antibiotic-treated groups weighed less than those not receiving antibiotics. Interestingly, despite consuming less water each day (Figure 1A), for experiments conducted at the Cleveland Clinic, tributyrin restored body weight to that of antibiotic-free mice by day 7. Mice mortality was highest in the antibiotic-treated broth/saline groups ($n = 2$ – 3) compared with any other treatment group ($n = 1$). Following 7 days of treatments, the gross appearance of the cecum in animals receiving antibiotics was visually markedly enlarged compared with the antibiotic-free groups (Figure 1B). Tributyrin and, to a lesser extent, LGG supplementation reduced the antibiotic enlargement of the cecum.

Expression of Butyrate Receptor GPR109A and Butyrate Transporter SLC5A8

We investigated whether the provision of antibiotics influences the expression of the butyrate transporter SLC5A8 and the butyrate receptor GPR109A in the intestinal tract and if LGG and/or tributyrin supplementation affects these changes. The steady-state levels of SLC5A8 and GPR109A mRNA in the colon and ileum were reduced markedly in mice receiving antibiotics compared with antibiotic-free mice (Figure 2A, B). In the antibiotic-saline-treated mice, there was an 80% and 53% reduction in mRNA expression for the butyrate receptor and transporter, respectively. Supplementation with LGG and/or tributyrin prevented the reduced butyrate receptor and transporter mRNA expression. Protein expression for the butyrate transporter SLC5A8 was predominantly seen on the lumen-facing apical membrane of the ileal and colonic epithelial cells in antibiotic-free mice (Figure 2C). The immunoreactive SLC5A8 was visibly reduced in antibiotic-saline-treated mice. These changes were absent in mice supplemented with tributyrin.

Expression of Ion Exchangers SLC26A3 and NHE3 and Water Channel AQP4

SLC26A3 is an anion exchanger, mediating chloride-bicarbonate exchange and thus serving an important role in electrolyte absorption in the intestinal tract. NHE3 is a sodium-hydrogen ion antiporter. AQP4 is a water channel responsible for water reabsorption in the gut. Both ion exchangers and water channel are expressed predominantly in the apical membrane of the intestinal enterocytes.³³ Steady-state levels of SLC26A3, AQP4, and NHE3 mRNA were reduced by 47%, 40%, and 20%, respectively, in antibiotic-saline-treated mice (Figure 3A–C). There was no difference in the mRNA levels of SLC26A3 and NHE3 in the antibiotic-treated mice that received any of the 3 supplement treatments—LGG, tributyrin, or LGG/tributyrin—compared with all the antibiotic-free mouse groups. AQP4 mRNA expression was protected in the antibiotic-treated animals receiving LGG but not the animals receiving tributyrin (Figure 3C). NHE3 protein was expressed in the apical membrane of ileal and colonic epithelial cells in antibiotic-free mice (Figure 3D). The staining intensity for NHE3 decreased in antibiotic-saline treated mice, but the intensity was not affected in antibiotic-treated mice receiving tributyrin supplementation.

Expression of Tight Junction Proteins Zonula Occludens and Occludin

Butyrate is known to have an important role in maintaining gut integrity.^{34,35} Since antibiotic therapy negatively alters butyrate-producing bacteria and likely butyrate levels in the gut lumen, as well as expression levels of genes and proteins involved with gut physiology, we tested the hypothesis that antibiotics also would alter proteins integral to maintaining gut integrity. Immunofluorescence localization of ZO-1 and occludin demonstrated an intact tight junctional protein network in the ileum, proximal colon, and jejunum (not shown) in antibiotic-free mice (Figure 4). The staining intensity for ZO-1 and occludin was reduced in the antibiotic-saline-treated mice. Interestingly, tight junction protein staining intensity was preserved in mice receiving antibiotics and supplemental tributyrin.

Discussion

The present study evaluated the effect of LGG and tributyrin oral supplementation on antibiotic therapy-induced changes in expression levels of water and electrolyte exchangers and intestinal epithelial cell permeability markers. Although probiotics, such as LGG, are known to decrease the duration and severity of symptoms of AAD, the mechanisms are not fully understood. We demonstrated that simultaneous treatment with LGG and tributyrin prevents antibiotic-induced downregulation of genes and proteins involved with intestinal fluid and electrolyte homeostasis and intestinal barrier function.

The suppression and elimination of microbial pathogens by antibiotics is a time-tested approach in medical management. Recent studies have highlighted the profound changes in microbial populations that result from applications of antimicrobial agents. AAD is a significant adverse effect of antimicrobial administration. A critical factor in the pathogenesis of AAD is believed to be an alteration in the normal GI microbiota.⁹ Changes in the human-associated microbiota are usually temporary, but long-term microbial population fluctuations have been reported in healthy adults.³⁶ Vancomycin, neomycin, and metronidazole eliminate Gram-positive, Gram-negative, and anaerobic commensal bacteria.²⁹ Administration of this antibiotic combination not only depletes and alters the gut microbiota community richness and structure,³⁷ thus providing space and nutrients for opportunistic pathogenic bacteria, but also impairs mucosal innate immune defenses.²⁹ This antibiotic combination is not reported to cause diarrhea in mice, but we chose this antibiotic combination due its ability to deplete the gut microbiota and therefore create a clinically relevant “germ-free” gut microenvironment. The antibiotic therapy in this study did deplete total bacteria in fecal pellets. Our results corroborate previous data in GF mice showing that altered gut microbiota by antibiotic therapy affects the expression levels of genes and proteins involved with water and electrolyte absorption in the gut.^{19,27–29}

Alterations in commensal gut microbiota impair the concentration and distribution of organic compounds such as carbohydrates, SCFAs, and bile acids.⁹ The most numerous butyrate-producing bacteria in the gut have been found to belong to the clostridial clusters IV and XIVa; absences of these commensals were identified following antibiotic treatment for sinusitis.⁹ Since butyrate is an important molecule for gut homeostasis, it is likely that antibiotic therapy compromises butyrate actions in the intestine by altering the levels of butyrate-producing bacteria, thus limiting the availability of luminal effects of butyrate. Although the literature supports provision of probiotics, specifically LGG, for mitigating AAD, the end products of most probiotics do not include butyrate, which raises questions about their effectiveness in promoting bowel health in adults.³⁸ There are no reports in the literature for providing tributyrin to improve symptoms of AAD caused by broad-spectrum antibiotics.

The experimental treatments were carefully selected for their significance to current literature and future clinical application. LGG was selected as a clinically relevant, commercially available probiotic that is well studied in AAD.^{23–26} LGG administration is safe and well tolerated; the dosage provided augmented colonization of gut microbiota in treated animals. Tributyrin has been provided at various dosages in vitro and in vivo, in animals and humans, with the goal end point of plasma butyrate levels being >0.5 mM.¹² The molar ratio of propionate and acetate in the blood is much higher than butyrate at physiologic conditions.¹¹ This is because butyrate is the primary energy source for the colonic mucosal enterocytes, accounting for 70% of their oxygen consumption; butyrate is preferentially oxidized over propionate and acetate in a ratio of 90:30:50.¹⁰ In vitro studies have shown beneficial effects of butyrate when provided at concentrations of 1–10 mM/L.^{11,32} Although the in vivo physiologic concentration is proposed to be 10–15 mM/L, knowing that tributyrin can have cytotoxic effects and that 1 mole of tributyrin yields 3 moles of butyrate, we chose to dose tributyrin at 5 mM/L initially to determine potential beneficial effects. Indeed, we found this dose of tributyrin to be well tolerated by mice and to have positive benefits.

SCFAs, including butyrate, are the end products of anaerobic bacterial fermentation of undigested carbohydrates in the distal intestine.¹⁴ The total concentration and relative molar concentrations of individual SCFAs are greatly influenced by the diet. The average intraluminal concentration of SCFAs is estimated to be between 100 and 170 mM, with butyrate representing approximately 15% of the colonic SCFAs.¹⁰ Although it is feasible to manipulate different dietary substrates to achieve desired ratios of SCFAs, the composition of the commensal microbiota is an important factor if a particular SCFA is desired to be present in the colon.¹⁴ Since a decreased number of total gut microbiota, particularly butyrate-producing bacteria, are noted with antibiotic therapy, we opted to provide butyrate directly to ensure its availability due to the uncertainty that modulation of dietary fiber/carbohydrate may or may not yield adequate butyrate during antibiotic therapy. Butyrate has been provided via several routes (eg, intravenously, rectally, and orally), all having limitations.¹⁰ Tributyrin overcomes many of the problems of the parent compound. Provided orally, tributyrin is hydrolyzed by pancreatic and gastric lipases, yielding glycerol and 3 butyrate molecules¹⁰; has a longer half-life compared with IV delivery¹⁶; and is safe when provided at lower doses but can be cytotoxic at higher doses (eg, in vivo, 10.3 g/kg; in vitro, >10 mM).^{10,22,32,39,40} Although the liberated butyrate molecules can exit the proximal intestinal lumen by passive diffusion, butyrate transporters are also present in the proximal intestinal tract and therefore available for active transport of butyrate across the apical membrane.^{17,18,20} Interestingly, although tributyrin is rapidly hydrolyzed in the proximal intestinal tract, we found beneficial effects on mRNA and protein expression in the distal intestine. Others have shown similar benefits of not only a direct trophic effect of butyrate provision but also trophic effects on unexposed adjacent intestinal tissue.^{11,41,42} Jejunal trophic effects of cecally infused SCFAs were mediated afferently by the autonomic nervous system and associated with increased jejunal gastrin.⁴³ It is also possible that, with tributyrin administration orally, butyrate reaches the colon at sufficient concentrations to elicit the changes on gene expression. Since the mechanisms of butyrate action on colonocytes at least partly involve inhibition of histone deacetylation, a process that is seen at micromolar concentrations of butyrate, such levels of butyrate can easily be reached in the colon with oral administration of tributyrin.

In these experiments, the animals tolerated oral tributyrin supplementation well without any adverse events. Antibiotic-treated animals consumed significantly less water than the antibiotic-free mice for experiments performed at both research sites, likely because the antibiotics are unpalatable and have a bitter taste. Adverse effects for oral delivery of these antibiotics include nausea, diarrhea, appetite loss, and stomach cramps. Although there was

no significant difference in body weight for antibiotic-treated mice supplemented with LGG and/or tributyrin for the experiments performed at the Medical College of Georgia, the mice treated at the Cleveland Clinic receiving tributyrin were comparable in body weight and activity level to the antibiotic-free animals at day 7. Similar effects have been noted in clinical studies in which cancer patients treated with tributyrin reported an improved sense of well-being, appetite, and pain control.³⁹

It is known that GF mice have striking abnormalities that interfere with normal histologic development of the intestinal epithelium, which brings about a gross enlargement of the cecum.^{44,45} These abnormalities are rapidly corrected when GF animals are associated with some components of the normal gut microbiota.⁴⁴ These components may be not just the bacteria but also fermentation end products (eg, SCFAs). The gut microbiota uses specific glycoconjugates on the enterocyte surface as receptors to colonize a region of the gut, lending these glycoconjugates to likely determine the colonization of gut microbiota.⁴⁶ Modification of glycosylation could feasibly result in an opportunity for pathogens to attach on the luminal surface, enabling colonization and invasion of the gut barrier, leading to inflammatory responses. Our data corroborate GF data in that an antibiotic treatment known to alter and deplete the gut microbiota enlarges the cecum of mice. Particularly noteworthy is that LGG and/or tributyrin supplementation diminishes this response, but the response is more dramatic with tributyrin. Original experiments with GF mice recolonized with lactobacilli and anaerobic streptococci corrected cecal enlargement, but slowly and imperfectly; the response was more rapid and remarkable when GF mice were associated with *Bacteroides* bacteria.⁴⁴ Specific strains of bacteria (eg, *Bacteroides thetaiotaomicron*) have been shown to modulate the expression of host genes related to important intestinal functions, including nutrient absorption, mucosal barrier function, and intestinal maturation.²⁷ Likewise, similar effects are known to occur with butyrate provision.¹² Our data show that tributyrin alone can exhibit positive effects on the cecum of antibiotic-treated mice. Butyrate is a major metabolic fuel for colonocytes and promotes a normal phenotype in these cells. Butyrate interacts not only with its transporters but also with butyrate receptors localized in the apical membrane of the intestine. GPR109a is a butyrate receptor known to have anti-inflammatory properties upon interaction with its ligand.³² It is unknown from these data whether butyrate provided during antibiotic therapy produces a less inflammatory environment through interaction with GPR109a, causing a pattern shift in glycoconjugate expression, thus decreasing the cecum size of antibiotic-treated mice, but this may warrant further investigation.

As an inhibitor of histone deacetylases, butyrate has the ability to influence gene expression in the colon. This can lead to hyperacetylation of histones that is followed by increased gene expression. Two potential mediators of the biologic effects of butyrate are SLC5A8, an Na⁺-coupled transporter for butyrate, and GPR109A, a G-protein-coupled receptor; both are expressed in the lumen-facing apical membrane of colonic epithelial cells.^{18,19,32} Prior work shows that the gut microbiota is obligatory for optimal expression of these 2 genes and their protein products as well as hundreds of others.¹⁹ Fascinating and clinically relevant is that our results corroborate this prior work; gene expression of the butyrate transporter and receptor, several ion exchangers, and a water channel were significantly downregulated in antibiotic-treated animals. Supplemental treatments, LGG, tributyrin, and their combination were able to preserve the expression of these genes and/or their protein products. The dose of LGG provided was able to survive and reach the colon as indicated by growth patterns in the fecal pellets of animals supplemented with LGG. It is thus likely that supplemental LGG contributed to a positive influence on gene and protein expression. Appealing is that provision of tributyrin alone was also able to maintain gene and protein expression. Others have reported transporter regulation via luminal nutrient sensing through interaction with cell surface receptors.^{21,47} GPR109A, which has a higher affinity for butyrate than SLC5A8,

was recently associated with the trafficking of monocarboxylate transporters to the apical membrane in response to the presence of butyrate.²¹ It is unknown from these data how much of the liberated butyrate from tributyrin supplementation was able to reach the colon. However, only micromolar concentrations of butyrate are needed to inhibit histone deacetylases, even though millimolar concentrations are needed to activate GPR109A. It is likely that butyrate liberated from tributyrin reaches the colon at least at levels sufficient to affect histone acetylation and hence gene expression.

During acute cholera, in addition to a decrease in colonic anaerobes, there is also reduced production of SCFAs and decreased absorption of electrolytes.³³ SLC26A3, NHE3, and AQP4 are important for water and electrolyte homeostasis. Intestinal ion transport and the pathophysiology of diarrhea are complex and reviewed elsewhere.³⁵ SLC5A8 functions as a Na⁺-coupled transporter for butyrate with an Na⁺/butyrate stoichiometry of 2:1; therefore, the transporter may promote Na⁺ absorption in the colon in the presence of the bacterial fermentation product butyrate. GPR109A is coupled to Gi, the inhibitory G-protein. Activation of the receptor by butyrate or other agonists leads to a decrease in intracellular levels of cAMP. This cyclic nucleotide is one of the major signaling molecules in the intestinal tract that controls electrolyte and water absorption; elevation of intracellular levels of cAMP in the intestinal tract causes secretory diarrhea.⁴⁸ Studies have investigated the effects of SCFAs on enterotoxin-induced electrolyte and fluid secretion. SCFAs, particularly butyrate, reduce cholera toxin-induced water and electrolyte secretion.⁴⁹ Our data are suggestive that benefits, such as decreased duration and severity of diarrheal symptoms, associated with LGG and/or butyrate supplementation during antibiotic therapy are linked with preservation of genes and proteins involved with electrolyte and water homeostasis.

In addition to its role in stimulating intestinal NaCl absorption and inhibiting the prosecretory action of several cAMP-generating secretagogues, butyrate is also known to improve the barrier function of the gut epithelia.³⁴ The barrier function of the intestinal mucosa is critical to maintain beneficial relationships between the host and the gut microbiota. The tight junction between the mucosal epithelial cells is the primary physical barrier in the intestines. The tight junction is composed of several transmembrane proteins such as claudins, zonula occludens, and occludin.³⁵ Butyrate promotes transepithelial resistance and reduced permeability, which is attributed to reorganization of the tight junction molecules ZO-1 and occludin.⁵⁰ Our data reveal that antibiotic therapy disrupts the organization and expression of tight junction proteins throughout the intestinal tract and that tributyrin supplementation preserves the epithelial barrier. Beneficial effects of butyrate have been suggested in acute gastroenteritis, cholera, congenital chloride diarrhea, and inflammatory bowel disease.³⁴ The link with butyrate and inflammatory bowel disease predominantly surrounds the involvement of tight junction protein alterations. Thus, preservation of tight junction proteins with tributyrin therapy during antibiotic therapy may also be clinically relevant.

In summary, this work indicates that oral supplementation with LGG and/or tributyrin counteracts the negative effects induced by antibiotic therapy on expression of genes and their protein products involved with water and electrolyte absorption and gut barrier function in the intestinal tract. The fact that tributyrin alone was able to exhibit these beneficial effects is intriguing as many factors can impair efficacy of probiotic provision (eg, viability, dosing, timing, colonization, storage temperature). These issues surrounding the efficacy of probiotic therapy should be considered if a treatment effect of probiotics is not found. If tributyrin supplementation alone achieves the desired outcomes of improved gut integrity and preservation of genes and proteins involved with water and electrolyte homeostasis, then this therapy may prove more attractive to clinicians and patients. Further

work investigating whether the same effects are found with various antibiotics and/or their dosing or whether benefits are found in humans would be interesting.

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Figure 1.

Treatment effects on mouse health. (A) Water consumed (mL) per day per mouse. Different letters over bars indicate a statistically significant difference ($P < .05$). (B) Representative photo of mouse cecum on day 7. Control and antibiotic $\pm$ tributyrin, *Lactobacillus* GG (LGG), and tributyrin/ LGG treatment groups. Data are expressed as mean $\pm$ SEM.

Figure 2.

Expression levels of butyrate receptor and transporter. (A, B) Levels of messenger RNA (mRNA) for GPR109A and SLC5A8 in the proximal colon of mice treated with or without antibiotics, *Lactobacillus* GG (LGG), tributyrin, or combined LGG and tributyrin. Different letters over bars indicate a statistically significant difference ($P < .0004$). (C) Levels of SLC5A8 protein (red) and its localization in the ileum and proximal colon of mice $\pm$ antibiotics and $\pm$ tributyrin. DAPI (blue) was used as a nuclear stain. Magnification of $\times 40$ for ileum and $\times 20$ for proximal colon.

Figure 3.

Expression levels of ion exchangers and water channel. (A–C) Levels of messenger RNA (mRNA) for SLC26A3, NHE3, and AQP4 in the ileum of mice treated with or without antibiotics, *Lactobacillus* GG (LGG), tributyrin, or combined LGG and tributyrin. Different letters over bars indicate a statistically significant difference ($P < .05$). (D) Levels of NHE3 protein (green) and its localization in the jejunum and ileum of mice $\pm$ antibiotics and $\pm$ tributyrin. DAPI (blue) was used as a nuclear stain. Magnification of $\times 40$ for jejunum and ileum. AQP4, aquaporin 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 4.

Expression of tight junction proteins. Levels of zonula occludens 1 (ZO-1; green) and occludin (red) proteins and their localization in the ileum (A) and proximal colon (B) of mice $\pm$ antibiotics and $\pm$ tributyrin. DAPI (blue) was used as a nuclear stain. Magnification of $\times 40$ for ileum and $\times 20$ for proximal colon.

Table 1

List of Primers Used in This Study

Gene (Genbank Accession No.)	Primer Sequence	Position	Product Size, bp	Annealing Temperature, Cycle No.
GPR109A (NM_177551)	Sense: 5 -CGAGGTGGCTGAGGCTGGAATTGGGT-3	325–347	646	60°C, 30
	Antisense: 5 -ATTGTCAGGGCCATTCTGGAT-3	950–970		
SLC5A8 (NM_145423)	Sense: 5 -GGGTGGTCTGCACATTCTACT-3	371–392	351	60°C, 30
	Antisense: 5 -GCCCAAGGTTGACATAGAG-3	700–721		
NHE3 (NM_009700)	Sense: 5 -TGG CCG GGC TTT CGA CCA CA-3	1425–1445	248	60°C, 30
	Antisense: 5 -GGG ACC CAC GGC GCT CTC CCT-3	1651–1672		
AQP4 (NM_009700)	Sense: 5 -ACTATTTTGGCCAGCTGTGATTCCAAACGA-3	517–547	423	61°C, 24
	Antisense: 5 -TTCCCCTTCTTCTTCTCCACGGTCA-3	912–939		
SLC26A3 (NM_021353)	Sense: 5 -CACAAATTCAGAAGACGAACATCGCAGACC-3	734–764	607	61°C, 24
	Antisense: 5 -GCATCAGCATTCCCTTTAAGTTTCCGAGTG-3	1310–1340		
GAPDH (NM_008084)	Sense: 5 -CTCTGGAAAGCTGTGGCGTGAT-3	567–589	122	61°C, 24
	Antisense: 5 -CATGCCAGTGAGCTTCCCGTTCAG-3	664–688		

Table 2

Final Fecal Bacterial Patterns

Treatment Groups	LGG	Total Bacteria
Antibiotic free, saline	ND	+++
Antibiotic free, LGG	1.3×10^4 CFU	+++
Antibiotic free, tributyrin	ND	+++
Antibiotic free, LGG/tributyrin	1.3×10^5 CFU	+++
Antibiotic, saline	ND	+
Antibiotic, LGG	2.5×10^4	++
Antibiotic, tributyrin	ND	++
Antibiotic, LGG/tributyrin	1×10^3 CFU	++

CFU, colony-forming units; LGG, *Lactobacillus* GG; ND, none detected; +, 10^2 CFU; ++, 10^3 CFU; +++, 10^4 CFU.

Table 3

Body Weight, g

Treatment Groups	Day 0	Day 3	Day 7
Medical College of Georgia			
Antibiotic free, broth	23.5 ± 0.5 ^a (n = 7)	24.0 ± 0.6 ^a (n = 7)	23.9 ± 0.7 ^a (n = 7)
Antibiotic free, LGG	25.8 ± 0.5 ^a (n = 8)	25.4 ± 0.6 ^a (n = 8)	25.1 ± 0.7 ^a (n = 8)
Antibiotic free, TB	24.9 ± 0.9 ^a (n = 8)	24.9 ± 0.7 ^a (n = 8)	25.5 ± 0.8 ^a (n = 8)
Antibiotic free, LGG/TB	24.8 ± 0.8 ^a (n = 8)	24.5 ± 0.7 ^a (n = 8)	22.5 ± 0.5 ^a (n = 8)
Antibiotic, broth	24.6 ± 0.5 ^a (n = 7)	21.0 ± 0.7 ^b (n = 4)	20.5 ± 0.5 ^b (n = 4)
Antibiotic, LGG	24.0 ± 1.0 ^a (n = 8)	18.8 ± 1.0 ^b (n = 8)	19.6 ± 1.4 ^b (n = 8)
Antibiotic, TB	24.3 ± 0.7 ^a (n = 8)	19.6 ± 0.8 ^b (n = 8)	19.9 ± 0.9 ^b (n = 7)
Antibiotic, LGG/TB	24.9 ± 0.8 ^a (n = 8)	20.1 ± 0.7 ^b (n = 8)	19.7 ± 0.6 ^b (n = 8)
Cleveland Clinic			
Antibiotic free, saline	16.2 ± 0.8 ^a (n = 6)	15.9 ± 0.5 ^a (n = 6)	16.7 ± 0.6 ^a (n = 6)
Antibiotic free, TB	16.5 ± 0.5 ^a (n = 6)	16.5 ± 0.4 ^a (n = 6)	16.8 ± 0.5 ^a (n = 6)
Antibiotic, saline	16.2 ± 0.3 ^a (n = 12)	14.2 ± 0.4 ^b (n = 11)	14.0 ± 0.5 ^b (n = 10)
Antibiotic, TB	16.0 ± 0.6 ^a (n = 10)	14.4 ± 0.6 ^b (n = 10)	14.6 ± 0.7 ^a (n = 9)

LGG, *Lactobacillus* GG; TB, tributyrin. Values with different superscripts at each time point are significantly different with $P < .05$.