Human *Clostridium difficile* infection: Inhibition of NHE3 and microbiota profile

Melinda A. Engevik¹, Kristen A. Engevik¹, Mary Beth Yacyshyn³, Jiang Wang⁴, Daniel J. Hassett², Benjamin Darien⁵, Bruce R. Yacyshyn³,⁶, Roger T. Worrell¹,⁶

¹Department of Molecular and Cellular Physiology
²Department of Molecular Genetics, Biochemistry and Microbiology
³Department of Medicine Division of Digestive Diseases
⁴Department of Pathology and Lab Medicine
University of Cincinnati College of Medicine, Cincinnati, OH 45267
⁵University Wisconsin-Madison
Animal Health and Biomedical Sciences, Madison, WI 53706
⁶Digestive Health Center of Cincinnati Children’s Hospital, Cincinnati, OH 45229

*Corresponding Author:
Roger T. Worrell, Ph.D.
Department of Molecular and Cellular Physiology
University of Cincinnati College of Medicine
Cincinnati, OH 45267
Phone: 513-558-6489
Email: Roger.Worrell@uc.edu
ABSTRACT.

*Clostridium difficile* infection (CDI) is principally responsible for hospital acquired, antibiotic-induced diarrhea and colitis and represents a significant financial burden on our healthcare system. Little is known about *C. difficile* proliferation requirements and a better understanding of these parameters are critical for development of new therapeutic targets. In cell lines, *C. difficile* toxin B has been shown to inhibit Na\(^+\)/H\(^+\) Exchanger 3 (NHE3) and loss of NHE3 in mice results in an altered intestinal environment coupled with a transformed gut microbiota composition. However, this has yet to be established *in vivo* in humans. We hypothesize that *C. difficile* toxin inhibits NHE3, resulting in alteration of the intestinal environment and gut microbiota. Our results demonstrate that CDI patient biopsy specimens have decreased NHE3 expression and CDI stool has elevated Na\(^+\) and is more alkaline compared to stool from healthy individuals. CDI stool microbiota have increased Bacteroidetes and Proteobacteria and decreased Firmicutes phyla compared with healthy subjects. *In vitro*, *C. difficile* grows optimally in the presence of elevated Na\(^+\) and alkaline pH, conditions which correlate to changes observed in CDI patients. To confirm that inhibition of NHE3 was specific to *C. difficile*, human intestinal organoids (HIOs) were injected with *C. difficile* or healthy and CDI stool supernatant. Injection of *C. difficile* and CDI stool decreased NHE3 mRNA and protein expression compared with healthy stool and control HIOs. Together these data demonstrate that *C. difficile* inhibits NHE3 *in vivo* which creates an altered environment favored by *C. difficile*. 

ABSTRACT.
INTRODUCTION

_Clostridium difficile_ is a gram-positive anaerobic bacterium from the phylum Firmicutes that is responsible for the majority of antibiotic-associated diarrhea (17). _C. difficile_ infection (CDI) affects thousands of patients each year and treatment cost of over 1 billion dollars in the United States (11, 18, 49, 59). Furthermore, _C. difficile_-related deaths have been steadily rising since 1999 (54), and will likely remain a problem, especially in the face of current antibiotic regimens. CDI has been associated with a spectrum of symptoms ranging from mild to watery diarrhea and abdominal pain, to life-threatening pseudomembranous colitis (PMC) and toxic megacolon (8). Although most of the symptoms of CDI have been linked to _C. difficile_ toxin production (31, 37, 41), the mechanism of _C. difficile_ colonization is still unclear. Thus, a better understanding of _C. difficile_ pathogenesis is critical for developing new therapeutics.

_C. difficile_ pathogenesis has been hypothesized to be a three-step process: (1) antibiotic disruption of the normal gut microbiota provides a potential niche for growth from its normal gut spore form; (2) the colonization phase, which includes bacterial-host interaction and adhesion; and (3) multiplication which maintains high numbers of vegetative _C. difficile_ and toxin production, both of which exacerbate the infectious process (15, 34). Antibiotic use has been shown to decrease the dominant gut microbiota bacterial phyla Bacteroidetes and Firmicutes (40) and increase Proteobacteria (1, 14, 16, 30, 35, 43, 62), resulting in increased gut susceptibility to _C. difficile_ infection (2, 5, 36, 52, 55, 67, 78). Once _C. difficile_ binds to the gastrointestinal (GI) mucus layer (15, 69), the bacterium can deliver two exotoxins, toxin A (TcdA) and toxin B (TcdB) (17, 32, 74). The Tcd toxins bind to uncharacterized host receptors and are then internalized into the enterocyte cytoplasm, where they become enzymatically active and glycosylate the Rho family of GTPases (19, 29). Inhibition of such GTPases has been shown to
have several effects including: (1) disorganization of the host actin cytoskeleton, (2) loss of cellular tight junctions, (3) disruption of signaling cascades, and (4) arrest of cell cycle progression (3, 15, 19, 33). In addition, toxin B inhibition of Rho GTPase in cell lines leads to the internalization of the Na\(^+\)/H\(^+\) exchanger isoform 3 (NHE3) (29), but this has yet to be established \textit{in vivo} in animals or in humans. Inhibition of NHE3 in mice results in chronic diarrhea (25, 60), elevated Na\(^+\) and alkaline luminal fluid, and an altered microbiota composition with decreased members of Firmicutes and increased Bacteroidetes (20). It has been suggested that the diarrhea associated with CDI is a result of damage to the host epithelium or a response designed to “flush out” the pathogen. However, we hypothesize that \textit{C. difficile} toxin production inhibits NHE3, creating an altered intestinal micro-environment and gut microbiota composition which favor \textit{C. difficile} proliferation and colonization of the mucosal lining. In this study, we demonstrate that biopsy specimens from patients with CDI have decreased NHE3 with increased Bacteroidetes and decreased Firmicutes phyla in their stool. \textit{In vitro}, \textit{C. difficile} growth depends on the high [Na\(^+\)] and a more alkaline environment which can be caused by downregulation of NHE3. This study is the first to demonstrate downregulation of NHE3 and an altered luminal environment in patients with CDI.
METHODS

Patient information. All patients and healthy volunteers at the University of Cincinnati Medical Center Hospital, Cincinnati, OH provided informed consent approved by the University of Cincinnati IRB. Samples were evaluated from patients with recurrent CDI. Initial CDI cases, defined as only one *C. difficile* positive laboratory test with no prior history of CDI, were not included in this study. Recurrent CDI was defined as onset of new diarrhea after a symptom-free period of >3 days, more than one *C. difficile* positive laboratory test and completion of at least one round of antibiotic treatment. *C. difficile* infection (CDI) was defined as a new onset of diarrhea (> 3 loose stools/day for more than 24 hours) and at least one positive *C. difficile* laboratory test. Diagnosis of CDI was determined by at least one ELISA positive toxin test or a positive LAMP test. Over the course of fecal collections, two types of toxin tests were used. From November 2010 – August 2011, the EIA for toxins A and B was used. After August 2011, the Meridian Illumigene® LAMP test was used. This shift in toxin testing represents a switch to in house testing, lowering the cost, and an upgrade to a more sensitive method.

Fecal samples were collected from 12 recurrent CDI patients with an average age of 56, age range 32-76. This group included 8 females and 4 males. Selected patients did not have history of Inflammatory Bowel Disease (IBD), small bowel obstruction, diverticulosis, colostomy, or cancer. Fecal samples were also collected from 12 healthy volunteers with an average age of 41, age range 28-61. This group included 7 females and 5 males. To address antibiotic use and stool composition, fecal samples were collected from 8 patients with diarrhea, no antibiotics (age range: 34-67, mean age: 49, female: 3, males: 5), 5 patients with normal stool and antibiotics (vancomycin/clindamycin) but without CDI infection (age range: 29-56, mean age: 39, female: 4, males: 1) and 7 patients with diarrhea and antibiotics...
(vancomycin/clindamycin) but without CDI infection (age range: 38-70, mean age: 52, female 4, males: 3). Healthy volunteers and patients with diarrhea and/or antibiotics were without previous or current GI symptoms, history of chronic disease or cancer. All stool samples were processed for total DNA, ion concentration, pH and stored at −20°C.

Colon biopsy specimens collected from five healthy volunteers were obtained by consent and fixed in neutral buffered formalin and paraffin-embedded. Healthy subjects had an average age of 52, patient age range of 45-63 and included 3 females and 2 males. Healthy volunteers were without previous or current GI symptoms, history of chronic disease or cancer. Paraffin sections of biopsies and colon resections were obtained from 5 de-identified patients with current CDI diagnosis (C. difficile-positive toxin test) and no other known morbidity/disorder. The average patient age 44, patient age range of 28-65, and included 2 females and 3 males. Selected patients did not have history of Inflammatory Bowel Disease (IBD), small bowel obstruction, diverticulosis, colostomy, or cancer. Confirmation of C. difficile infection was performed by tissue staining with C. difficile specific antibody as described below.

Histology. Healthy and CDI biopsy and surgical resections were obtained from the transverse colon and fixed overnight at 4°C in neutral-buffered formalin and embedded in paraffin. Serial 6–7 μm thick sections were applied to glass slides and intestinal architecture was examined by H&E staining. Expression of NHE3 was examined with rabbit anti-human NHE3 antibody (dilution 1:100, NBP1-82574, Novus Biologicals, Littleton, CO) and C. difficile binding was examined with rabbit anti-C. difficile cell surface protein antibody (dilution 1:100, ab93728, ABCAM, Cambridge, MA). Briefly, sections were removed of paraffin and incubated for 40 min at 97°C with Tris–EDTA–SDS buffer as previously described (68). Sections were then blocked
with PBS containing 10% serum, and stained with primary antibody overnight at 4°C. Sections were then washed three times in PBS, incubated with goat-anti-rabbit IgG Alexa Fluor® secondary antibody (dilution 1:100) (Life Technologies, Grand Island, NY) for 1 hr at room temperature and counterstained with Hoechst (0.1 µg/ml) (Fisher Scientific). Sections were analyzed by confocal laser scanning microscopy (Zeiss LSM Confocal 710, Carl Zeiss). Digital images of slides were evaluated by tabulating mean pixel intensity of the respective color channel on each image using Image J software (NIH) and reported as relative fluorescence. Five regions of interest per image, four images per slide, and n=5 healthy and CDI patients were used for semi-quantification of stain intensity normalized to healthy subjects and referred to as relative fluorescence.

**Human intestinal organoids (HIOs) and microinjection.** Organoids resembling human proximal colon, hereafter referred to as HIOs, were generated by the Cincinnati Children’s Hospital Medical Center (CCHMC) Pluripotent Stem Cell Facility through directed differentiation of human pluripotent stem cells (hPSC). Differentiation of hPSCs from a single subject was obtained by culturing hPSCs for 3 days in ActivinA, followed by fibroblast growth factor 4 (FGF4) and Wnt3a. HIOs achieved 3D dimensional growth in matrigel with epidermal growth factor (EGF), R-spondin and Noggin as previously described (77). HIOs were obtained in matrigel from the CCHMC Puripotent Stem Cell core. These organoids have been previously shown to contain all major intestinal epithelial cell types: enterocytes (villin), goblet cells (mucin), paneth cells (lysozyme), and enteroendocrine cells (chromogranin A) (77). The luminal compartment of HIOs were microinjected with bacteria and stool supernatant to analyze host-microbe interactions as previously described (20). Injection needles were pulled on a horizontal
bed puller (Sutter Instruments) and the tip cut to a tip diameter of ~10-15 μm. HIOs were injected with *C. difficile* ATCC 1870 or stool from healthy or CDI patients. *C. difficile* ATCC 1870 was grown in Tryptone yeast TY broth as previously described (22). For stool, 0.5 g of healthy or CDI stool was added to 4.5 ml Tryptic Soy Broth (TSB) (Fisher Scientific) in an anaerobic hood. Samples were vortexed and centrifuged at 150 x g for 10 min to pellet solid materials. Stool supernatant, *C. difficile* or *C. butyricum* cultures or TSB broth were injected into HIOs using a Nanoject microinjector (Drummon Scientific Company, Broomall, PA). To minimize stretch effects on epithelial cells injection volumes of ~10% or less of the organoid luminal volume were used. Under these conditions no leakage of cultures from the HIOs was observed. HIOs were processed either for RNA or immuno-staining. For RNA, organoids were homogenized in Trizol and extracted with chloroform according the manufacturer’s instructions (Invitrogen). For staining, HIOs were incubated overnight after microinjection and fixed with 4% paraformaldehyde for 30 min at room temperature. HIOs were washed in PBS and transferred to sucrose (30% in PBS) and incubated overnight at 4°C. The next day, HIOs were placed in OCT embedding medium and frozen at -80°C for 1 day. 7 μm sections were cut on a cryostat. Slides were stained with rabbit anti-human NHE3 antibody (dilution 1:100, NBP1-82574, Novus Biologicals) and analyzed by confocal laser scanning microscopy (Zeiss LSM Confocal 710, Carl Zeiss).

**Bacterial strains and culture conditions.** *C. difficile* ATCC BAA-1870 and *Blautia producta* ATCC 27340D were purchased from ATCC (American Type Culture Collection, Manassas, VA). *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli*, *Burkholderia cepacia*, and *Faecalibacterium prausnitzii* were locally available (Hassett laboratory). *Bacteroidetes*
\textit{thetaiotaomicron} ATCC 29741 was purchased from Fisher Scientific (Thermo Fisher Scientific, Waltham, MA). \textit{Lactobacillus acidophilus}, \textit{Rhizobium leguminosarum} and \textit{C. butryicum} were purchased from Carolina Biological Supply Company (Carolina Biological Supply Company, Burlington, NC). \textit{S. aureus}, \textit{M. luteus}, \textit{L. acidophilus}, \textit{B. thetaiotaomicron}, \textit{E. coli}, \textit{B. cepacia}, and \textit{R. legaminsarum} were used to generate qPCR standard curves as previously described (20). \textit{E. coli}, \textit{S. aureus}, \textit{M. luteus}, \textit{B. cepacia}, \textit{L. acidophilus} and \textit{R. legaminsarum} were grown in Luria–Burtani (LB; Thermo Fisher Scientific) broth at 37°C in a shaking incubator. \textit{B. thetaiotaomicron} was grown in TSB (Fisher Scientific) and \textit{C. difficile} was grown in TYG (Tryptone-Yeast extract-Glucose broth; Thermo Fisher Scientific) at 37°C in a Coy Systems, dual-port anaerobic chamber (Coy Lab Products, Grass Lake, MI).

To determine the optimal [Na\textsuperscript{+}] for growth, \textit{C. difficile} (\textit{Cluster XI}), \textit{C. butryicum} (\textit{Cluster I}), \textit{Blautia producta} ATCC 27340D (\textit{C. coccoides Cluster XIVa}) and \textit{Faecalibacterium prausnitzii} (\textit{C. leptum Cluster IV}) were grown in media where sodium chloride (NaCl) was either removed or replaced with cesium chloride (CsCl) or potassium chloride (KCl) as previously described (9, 10, 20). Briefly, low Na\textsuperscript{+} media was mixed with normal media at various ratios to obtain varying concentrations of Na\textsuperscript{+} for bacterial growth measurements. Actual Na\textsuperscript{+} and K\textsuperscript{+} concentrations were confirmed by flame photometry (Single-Channel Digital Flame Photometer Model 02655-10; Cole-Parmer Instrument Company Vernon Hills, IL) and Cl\textsuperscript{-} concentration measured by chloridometry (Digital Chloridometer Model 4425100, Labconco Kansas City, MO). Bacteria were grown under anaerobic conditions at 37°C to early stationary phase in normal TYG media (12 hrs, O.D. 560nm ~1) and used to inoculate media containing varying [Na\textsuperscript{+}]. Growth was measured as the optical density (O.D. 560nm) with an Amersham Biosciences Ultospec 3100 Spectrophotometer (GE Healthcare Life Sciences, Pittsburgh, PA). \textit{Clostridial...}
titers were determined by bacterial cell counts using a Petroff-Hauser chamber (Hausser Scientific; Horsham, PA) and also by colony forming units (CFU) (9, 10). No differences in growth patterns were observed between 4 (early exponential phase), 12 (early stationary phase), 24 or 48 (stationary phase) hr time points (data not shown). As a result, all data are represented as the OD$_{560nm}$ and CFU at the 24 hr time point. To determine the optimal pH for growth, C. difficile, C. butyricum, Blautia producta ATCC 27340D (C. coccoides) and Faecalibacterium prausnitzii (C. leptum) were grown in TYG media containing either normal media or low Na$^+$ media adjusted to pH values ranging from 5.5 to 7.0 as determined electrochemically using a pH meter (Orion Model 720A; Thermo Fisher Scientific, Waltham, MA).

Quantitative real time-PCR amplification of 16S sequences. QIAamp DNA Stool kit (Qiagen, Valencia, CA, USA) was used to isolate total DNA from stool of healthy subjects or patients with recurrent CDI. To improve bacterial cell lysis, the temperature was increased to 95°C and incubation with lysozyme (10 mg/ml, 37°C for 30 min) was used as previously described (12, 20, 24, 48, 56, 57). qPCR was used to access the abundance of total bacteria and specific intestinal bacterial phyla using a Step One Real Time PCR machine (Applied Biosystems (ABI) Life Technologies) with SYBR Green PCR master mix (ABI) and bacteria-specific primers (Table 1) in a 20 µl final volume. Cycle of threshold values ($C_T$) were correlated to the calculated bacteria number using standard curves from the pure bacterial cultures as previously described (4, 20, 51, 56). Total bacteria were calculated using a universal bacterial primer that recognizes all bacterial groups and represents the total stool microbiota.
Quantitative real time-PCR amplification of mRNA. To examine NHE3 mRNA level, total RNA was extracted from HIOs with Trizol Reagent (Invitrogen Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Briefly the matrigel surrounding the HIOs was removed by the addition of ice-cold PBS and 400 µl of Trizol was added to the HIOs and homogenized. RNA was extracted by the addition of chloroform and reverse transcription was performed using 50 µg/ml oligo(dT) 20 primer and SuperScript reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Amplification reactions of NHE3 mRNA were performed using SYBR Green PCR master mix (ABI) on a Step One Real Time PCR Machine (ABI). The following gene specific qRT-PCR primers derived from previous literature were used: human NHE3 Forward 5'- GAGCTGAACCTGAAGGATGC -3', NHE3 Reverse 5'-AGCTTGGTCGACTTGAGGA -3', human GAPDH Forward 5'- TGCACCACCAACTGCTTAGC -3' and GAPDH Reverse 5'-GGCATGGAAGTGCTTACGTGAG -3' (53). Data were reported as the delta delta C\_T using GAPDH as the standard. Differences in mRNA expression were determined by qRT-PCR and expressed as the \( \Delta \Delta C_T \) relative fold difference.

Ion and pH measurements. Stool fluid ion composition was analyzed by flame photometry and chloridometry. Briefly 0.3 g of human stool/liquid was added to tubes and 300 µl of double deionized-water was added and vortexed thoroughly. The samples were centrifuged at 3,000 rpm for 10 min at 4°C to pellet solids and the supernatant Na\(^+\) and K\(^+\) concentrations determined using a digital Flame photometer (Single-Channel Digital Flame Photometer Model 02655-10; Cole-Parmer Instrument Company Vernon Hills, IL). Cl\(^-\) ion concentrations were determined by a digital Chloridometer (Model 4425100, Labconco Kansas City, MO). All values were
normalized to weight. pH measurements were performed electrochemically via a pH meter (Orion Model 720A; Thermo Fisher Scientific Waltham, MA).

Statistics. Data are presented as mean ± SEM. Comparisons between groups were made with either One or Two Way Analysis of Variance (ANOVA), and the Holm-Sidak post-hoc (parametric) test used to determine significance between pairwise comparisons using SigmaPlot (Systat Software, Inc., San Jose, CA). A $P < 0.05$ value was considered significant while $n$ is the number of experiments performed.

RESULTS

NHE3 has been shown to be essential for intestinal absorption of Na$^+$, and, therefore, water (25, 60). Work in cell lines (LLC-PK$_1$: pig kidney, OK: opossum kidney and BeWo: human placenta) have demonstrated that C. difficile toxin B inhibits NHE3 by dephosphorylation and redistribution of ezrin, which normally anchors NHE3 to the cytoskeleton, resulting in the loss of NHE3 from the apical membrane (29). To determine if NHE3 was inhibited in CDI patients, intestinal architecture was examined by H&E staining (Fig. 1A) and NHE3 expression was examined by immunofluorescence (Fig. 1B). Colonic biopsy specimens demonstrate normal healthy crypts in healthy subjects (Fig. 1A). Consistent with CDI pathology, colon segments demonstrated pronounced thickening of the colonic wall (black arrows) and pseudomembranes (grey arrows) as previously described (73). To confirm the presence of C. difficile in patients with CDI, slides were stained with an anti-C. difficile antibody. As expected, healthy colonic tissue and adjacent areas did not contain any C. difficile. In patients with CDI, C. difficile was found primarily in the expelled mucus layer ($89 \pm 3\%$) and occasionally in the crypts close to the host epithelium ($11 \pm 2\%$) ($n=5$) (Fig. 1A).
In healthy subjects, NHE3 is located along the apical membrane of absorptive enterocytes (Fig. 1B). In contrast, CDI patient biopsy specimens demonstrated varying levels of NHE3. In CDI biopsy and surgical resections, there are areas with intact NHE3 and areas with decreased or no NHE3; together CDI samples demonstrate a 48% decrease in NHE3 compared with healthy colon samples (Fig. 1C). Image J analysis of C. difficile and NHE3 immunofluorescence revealed decreased NHE3 expression correlated with C. difficile presence (P=0.042). This is the first study that demonstrates that NHE3 is inhibited in patients infected with C. difficile.

Mice lacking NHE3 have increased intestinal [Na\(^+\)] and an alkaline pH (20, 25, 60). We predicted that loss of NHE3 activity in patients would likewise result in increased [Na\(^+\)] and an altered intestinal pH. In order to determine if CDI patients had an altered ion composition, stool from healthy subjects with or without diarrhea and antibiotics and CDI patients were examined by flame photometry and chloridometry (Fig. 2A-D). Patients with CDI had higher [Na\(^+\)] (P=0.005) (Fig 2A), and [Cl\(^-\)] (P <0.001) (Fig 2C) with no change in [K\(^+\)] (P= 0.106) (Fig 2B) (Two WAY ANOVA) (Fig. 2). To account for differences in stool composition and antibiotic use, patients with normal stool and diarrhea with or without antibiotics were included for analysis. For [Na\(^+\)], ant interaction existed between the presence of diarrhea and antibiotics (P = 0.045) (Fig 2A). A significant difference was found between healthy and diarrhea patients without antibiotics (P<0.001) and with antibiotics (P=0.039). An interaction was also observed for [Cl\(^-\)] (P = 0.002) between healthy and diarrhea patients without antibiotics (P=0.025) and with antibiotics (P=0.015) (Fig 2C). Interestingly a difference was observed in [K\(^+\)] between patients with or without antibiotics (P=0.027) (Fig 2B).

Non-Cl anion gap calculations ([Na\(^+\)] + [K\(^+\)] − [Cl\(^-\)]) demonstrated an increase in bulk non-Cl\(^-\) anions (Fig 2D) for between patients with or with antibiotics (P = 0.012), patients with
and without diarrhea (P= 0.032) and patients with and without CDI (P<0.001). This suggests increase in bicarbonate or short chain fatty acids (SCFA). In addition, CDI patients had a more alkaline stool (pH 6.9 ± 0.3) compared with healthy subjects (pH 6.0 ± 0.1) (P=0.003) (Fig. 2E). Differences in pH were also found between patients with and without antibiotics (P=0.026). This data indicates that changes in ion composition and pH occur with antibiotic use, but infection with *C. difficile* alters these parameters further.

The altered intestinal environment observed in recurrent CDI patients (increased [Na+] and more alkaline pH) is similar to our observations in NHE3 null mice and may promote the growth of a different subset of gut microbiota. Mice lacking NHE3 exhibit an altered gut microbiota with increased Bacteroidetes and decreased Firmicutes phyla (20). To address whether patients with recurrent CDI exhibit a similar profile, stool microbiota extracted from nine healthy and nine CDI patients was examined by qPCR. Total stool bacteria density remained unchanged between the groups (Fig. 3A). In CDI patients *C. difficile* represented < 2% of total bacteria (data not shown). In healthy subjects, the gut bacterial phylum Firmicutes constituted the most abundant group, followed by Bacteroidetes (Fig. 3B), consistent with other reports (13). In contrast, patients with CDI had increased Bacteroidetes and decreased Firmicutes (P < 0.001, Two Way ANOVA) (Fig. 3C). CDI patients also had increased αβγ-Proteobacteria (P = 0.02) that may result from antibiotic use, since antibiotics have been shown to increase Proteobacteria titers (1, 14, 16, 30, 35, 43, 62). In order to determine if resident *Clostridial* groups (Firmicutes phylum) were changed in CDI, *C. coccoides* cluster XIVa and *C. leptum* cluster IV titers were examined (Fig. 3D) and both were decreased compared to healthy subjects. In a healthy patient, *C. coccoides* and *C. leptum* account in total for approximately 50% of the total stool bacteria (28, 38, 63) and decreases in these groups represents a significant decrease in
the Firmicutes phylum. These observations point to the proclivity of \textit{C. difficile} in generating an altered intestinal environment.

To address whether \textit{C. difficile} prefers the environmental conditions mediated by inhibition of NHE3, \textit{C. difficile} ATTC BAA-1870 was grown \textit{in vitro} anaerobically in TYG media containing various [Na$^+$] (8-106 mM Na$^+$). Growth was examined by OD$_{560nm}$ and CFU enumeration at 4 (early exponential phase), 12 (early stationary phase), 24 or 48 (stationary phase) hr time points as previously described (75). Growth patterns were found to be the same for all time points and the data are represented as CFU at the 24 hr time point. \textit{C. difficile} was found to grow optimally at $>$ 16 mM [Na$^+$] (media pH 6.0), conditions observed in stool of CDI patients (Fig. 4A). This experiment was repeated using KCl or CsCl replacement, and \textit{C. difficile} again was noted to grow more efficiently at higher [Na$^+$] (data not shown). \textit{C. difficile} was also grown \textit{in vitro} at [Na$^+$] and pH values designed to mimic human stool (healthy: Na$^+$ 8 mM, pH 6.0; CDI: Na$^+$ 75 mM pH 7.0, refer to Fig. 2). As shown in Fig. 4B, \textit{C. difficile} also grew better at pH 7.0 vs. pH 6.0 (P = 0.003) at both [Na$^+$], indicating that \textit{C. difficile} is also influenced by pH. The resident \textit{Clostridial} members \textit{C. butyricum} (Cluster I), \textit{Blautia producta} (\textit{C. coccoides Cluster XIVa}) and \textit{Faecalibacterium prausnitzii} (\textit{C. leptum Cluster IV}) were also grown in TYG media in high and low Na$^+$ at pH 6.0 and 7.0 to determine if all \textit{Clostridial} groups preferred the similar environment conditions as \textit{C. difficile} (Fig. 5). \textit{Clostridium butyricum} is a resident bacteria that has been used as a probiotic (58, 61) and has been shown to prevent experimental colitis via an IL-10-dependent mechanism (27). \textit{C. butyricum} grew well at lower [Na$^+$], but proliferation significantly dropped at high [Na$^+$] (Fig. 5A). \textit{B. productus} and \textit{F. prausnitzii} had similar growth preferences to \textit{C. butyricum} with decreased growth at high [Na$^+$] (Fig. 5B and C). These data demonstrate that \textit{C. difficile} is distinct in its preference for a high [Na$^+$], alkaline
pH environment, adding credence to the hypothesis that *C. difficile* prefers an altered intestinal environment which may be caused by the loss of NHE3 function. Of note, at low [Na\(^+\)], *C. butyricum* proliferated to much higher levels compared to *C. difficile*, even at the high [Na\(^+\)]. This suggests that under healthy conditions, *C. butyricum* would be able to out-compete *C. difficile* for a given niche.

Although CDI patients demonstrate decreased NHE3 expression, it could be argued that a number of different bacterial groups could be responsible for changes in NHE3 levels. We have previously used intestinal organoids to address microbial-host interaction (20). To determine if *C. difficile* alone was sufficient to decrease NHE3, human intestinal organoids (HIOs) were used. HIOs have been shown to mimic native tissue: the cellular diversity and architecture is similar to tissue; HIOs contain all the intestine cell lineages; secretory and absorptive functions are present; HIOs also contain a significant degree of epithelial and mesenchymal complexity, and secrete mucus (77). In order to confirm that decreases in NHE3 were *C. difficile*-specific, HIOs were injected with *C. difficile*, *C butyricum* and stool supernatant from healthy and CDI patients. mRNA levels of NHE3 ([Fig. 6A](#)) demonstrate that *C. butyricum* and healthy stool does not inhibit NHE3 expression. However, injection of *C. difficile* and CDI stool supernatant resulted in a substantial decrease in NHE3 mRNA compared to broth injected (control) organoids. This inhibition was also observed at the protein level ([Fig. 6B and C](#)), demonstrating that *C. difficile* is sufficient for NHE3 inhibition in patients with CDI. Taken together, these data indicate that *C. difficile* is capable decreasing NHE3 expression *in vivo* and *in vitro*. Stool from patients with CDI exhibit increased Na\(^+\) and Cl\(^-\) and are more alkaline in pH compared with healthy subjects and patients on antibiotics. Stool gut microbiota from patients with CDI are higher in Bacteroidetes and lower in Firmicutes compared to healthy subjects, with decreased resident
Clostridial members C. coccoides and C. leptum. In vitro high Na\(^+\) and pH conditions results in C. difficile proliferation and the suppression of resident Clostridial members. Injection of C. difficile into HIOs confirms that C. difficile alone is capable of eliciting changes in NHE3 at the level of protein and mRNA.

DISCUSSION

C. difficile represents an ever increasing public concern as the major cause of antibiotic-induced diarrhea and colitis. The incidence of CDI has increased in the last decade, and, with the emergence of more virulent strains, C. difficile will likely persist as a major health concern. Herein, we have demonstrated several novel aspects of CDI including: (1) CDI patients exhibit decreased NHE3 expression in the apical membrane of intestinal enterocytes and higher [Na\(^+\)] and alkaline stool pH compared to healthy subjects. The altered gut intestinal environment correlates with changes in the dominant bacterial phyla, Firmicutes and Bacteroidetes with decreased C. coccoides and C. leptum, (2) C. difficile has increased proliferation at Na\(^+\) concentrations greater than 16 mM and at more alkaline pH level in vitro, a pattern of proliferation that is not observed in resident C. butyricum, B. producta or F. prausnitzii; and (3) C. difficile alone and in combination with a complex microbiota (CDI stool) is capable of decreasing NHE3 expression in HIOs. These new findings shed light on several novel aspects of the C. difficile colonization phase. This study represents the first in vivo analysis of NHE3 inhibition in response to C. difficile infection. Targeted disruption of the normal intestinal environment via regulation of ion transport may help explain both the diarrhea phenotype and how C. difficile maintains a competitive advantage.
We propose that in healthy individuals, the luminal and mucosa-associated gut microbiota compete for a *C. difficile* niche (see model in Figure 7). In an antibiotic microbiota-depleted environment, *C. difficile* spores germinate and vegetative *C. difficile* likely use intestinal nutrients (such as cleaved mucus oligosaccharides) to enter a colonization phase. After a colonization phase, *C. difficile* enters a virulence phase and produces toxins which inhibit NHE3. *C. difficile* toxin inhibition of NHE3 alters the intestinal environment producing a high [Na$^+$], and a more alkaline fluid, which enhances *C. difficile* proliferation and inhibits competitive *Clostridial* groups proliferation. This altered intestinal environment further shapes the re-emerging gut microbiota (which is restructuring after antibiotic use), perhaps so that non-inhibitory Bacteroidetes members proliferate. Altered gut microbiota may also play a role in further shaping the intestinal environment, making it more favorable for *C. difficile* colonization. *C. difficile* toxin B inhibition of NHE3 was demonstrated in cells lines (29), but this is the first study that has demonstrated that inhibition of NHE3 occurs in infected human patients. Loss of NHE3 in mice appears to mimic the effects of *C. difficile* toxin production in humans as NHE3$^{-/}$ mice have higher [Na$^+$], alkaline intestinal fluid and a distal colon microbiota that are higher in Bacteroidetes and lower in Firmicutes (21). It should be noted that NHE3$^{-/}$ have higher Bacteroidetes and lower Firmicutes in both the luminal and mucosa-associated bacterial populations, with the mucosa-associated bacterial population being more dramatic than the luminal population. If the mucosa-associated bacteria of CDI patients likewise reflects the luminal bacterial composition, this indicates that this alter composition represents a non-competitive population to *C. difficile*.

NHE3$^{-/}$ mice also exhibited increased [Na$^+$], alkaline fluid and Bacteroidetes in the small intestine (terminal ileum) in addition to changes in the colon. This suggests that the altered
intestinal environment, due in part to the loss of NHE3, may occur upstream as well as in the
colon. Although C. difficile studies have focused on the colon, C. difficile infection has also been
reported in the small intestine (47, 70, 76) and can cause small bowel disease (66, 71, 72). These
studies suggest that C. difficile infection is not localized solely to the colon and may provide
keys areas for the initial pathogenesis of C. difficile. Knowledge of C. difficile colonizing in the
intestine (either small or large intestine) is critical for developing better therapies against CDI.

It has been speculated by some that the diarrhea observed in CDI patients is the direct
result of epithelial integrity or secretory diarrhea by the host to remove the pathogen. Our work
has demonstrated that patients with CDI have a dramatic increase in stool [Na\(^+\)] and a moderate
increase in [Cl\(^-\)]. This suggests that loss of Na\(^+\) absorption, and concomitant water absorption, in
combination with increased [Cl\(^-\)], may contribute to the diarrhea observed in CDI patients. A
caveat to such a study is the fact that it is unfeasible to collect samples before patients become
infected with C. difficile so it remains to be determined the extent of alterations in ion
composition and pH in each patient before the acquisition of CDI. However, this data
demonstrates that [Na\(^+\)] and [Cl\(^-\)] in CDI patients is significantly different from that of patients
with or without diarrhea on antibiotics, indicating that the large alterations in Na\(^+\) and Cl\(^-\) content
in CDI patients are likely due to the presence of C. difficile.

Hayashi et al. (29) demonstrated in placental and renal cell lines that exposure to C.
difficile toxin B resulted in decreased expression of NHE3 at the apical membrane and
translocation to a subapical endosome. This redistribution in NHE3 location was suggested to
contribute to loss of NHE3 activity. However, our work with the HIOs has shown that injection
with toxin-producing C. difficile decreased NHE3 expression at the level of the protein and
mRNA. This indicates that a dual mechanism of NHE3 inhibition may be occurring in patients
with CDI. In renal cell lines, NHE3 activity has also been shown to be diminished at the level of apical protein and mRNA by parathyroid hormone (PTH) (6). PTH addition correlated with significant decrease in promoter -152/+55 activity. This promoter segment contains putative binding sites for Sp1, AP2, and NF-Y, which seem to be essential for NHE3 gene transcription (42). \textit{C. difficile} toxin, similar to PTH, may also lead to decreased NHE3 promoter activity and thus transcription, but this remains to be determined. \textit{C. difficile} toxin production has been demonstrated to disrupt intestinal actin cytoskeleton, which is thought to lead to cell death (44). Loss of cell integrity could also contribute to decreased NHE3 in diseased segments of the intestine of CDI patients. However, decreased NHE3 mRNA may point to a selective inhibition of NHE3 to ensure a more favorable environment and re-emerging bacterial composition for \textit{C. difficile} growth.

\textit{In vitro} \textit{C. difficile} has optimal growth at higher sodium and more alkaline pH compared to healthy subjects. This might represent a biphasic event where \textit{C. difficile} is capable of using \([\text{Na}^+]\) until a more alkaline pH is obtained. Patients on antibiotics (either with or without diarrhea) have elevated \([\text{Na}^+]\) compared with patients not on antibiotics (Fig 2). Since patients who are on antibiotics and have diarrhea have a lower pH compared with CDI patients, \textit{C. difficile} may use \([\text{Na}^+]\) until the pH becomes more alkaline. Once the pH is alkaline, in vitro data suggests that \textit{C. difficile} is insensitive to \([\text{Na}^+]\). Additional experiments are likely needed to identify the exact conditions which stimulate \textit{C. difficile} growth \textit{in vivo}.

Ion transport now represents a new route for combating CDI. For example, were NHE3 to be upregulated, this may provide a means to re-establish the normal intestinal environment and thus shift the microbiota toward one that is considered normal. The effects of normal commensal microbiota on NHE3 expression and function may prove valuable in this regard. \textit{Lactobacillus}
has been used as a probiotic treatment for CDI (7, 45, 50) and *Lactobacillus acidophilus* has been shown to upregulate NHE3 (64). In addition, *Lactobacillus* has been shown to produce lactic acid that inhibits *C. difficile* growth (46). Since normal gut microbiota has been shown to out-compete and inhibit *C. difficile* growth, control of ion transport can provide a novel therapeutic for CDI.

**References:**


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Disclosures:

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Figure Legends:

Figure 1. CDI specimens exhibit altered intestinal structure and decreased apical NHE3 expression. A) H&E stains of healthy and CDI patient biopsies or surgical resections demonstrate that these CDI patients have regions of pseudomembranes (composed of inflammatory cells, necrotic epithelium and mucus) (grey arrows) and areas of thickened intestine wall (black arrows). Scale Bar = 500 μM Presence of C. difficile was confirmed with an anti-C. difficile antibody (purple). Healthy tissue contained no C. difficile stain, while CDI specimens contained C. difficile at the level of the mucus (89 ± 3%) and epithelium (11 ± 2%) (n=5). B) Confocal images from healthy and CDI patient biopsies or surgical resections depicting NHE3 (red) and nuclei (blue) stained with Hoechst. Scale Bar = 100 μM. Representative micrographs of observations from n = 5 healthy and CDI patient specimens. NHE3 was found to vary in degrees in CDI patients, representing a 48% decrease in NHE3 expression compared to healthy colon (arrows). C) Semi-quantitative analysis of surface NHE3 expression in healthy colon, CDI biopsy non-diseased adjacent tissue, and CDI biopsy diseased tissue. Data presented as relative fluorescence normalized healthy NHE3 expression. *P < 0.005 Two Way ANOVA. n = 5.
Figure 2. Stool from CDI patients have increased Na\(^+\) and a more alkaline pH compared to healthy subjects with or without antibiotics. Sodium (Na\(^+\)) concentration and potassium (K\(^+\)) as determined by flame photometry for patients with normal stool (n=12), diarrhea without antibiotics (n=8), for patients with normal stool on antibiotics (n=5) and diarrhea on antibiotics (n=7) and patients with recurrent CDI (n=12). A) Na\(^+\) was significantly increased in CDI compared to healthy patient stool (**P= 0.005). Na\(^+\) was also increased in patients with diarrhea (*P<0.001) compared to normal stool, between patients on antibiotics vs. no antibiotics (**P= 0.010). B) No changes were observed in [K\(^+\)] between CDI patients and healthy subjects, but differences were observed between patients with or without antibiotics (*P=0.027). C) Chloride concentration as determined by chloridometry was significantly increased in CDI compared to healthy patient stool samples (**P <0.001). Differences were also observed between healthy and diarrhea patients without antibiotics (*P=0.025) and with antibiotics (**P=0.015). D) Non-Cl anion gap was calculated with the following equation: [Na\(^+\)] + [K\(^+\)] − [Cl\(^−\)]. Differences were between patients with or with antibiotics (**P = 0.012), patients with and without diarrhea (*P= 0.032) and patients with and without CDI (**P<0.001). E) Stool pH was determined electrochemically and CDI patient stool was more alkaline compared to all other groups (**P=0.003) (Fig. 2E). Differences in pH were also found between patients with and without antibiotics (*P=0.026). * P < 0.05. TWO Way ANOVA. Holme-Sidak.

Figure 3. CDI patients have an altered gut microbiota compared to healthy subjects. qPCR was performed with total bacteria and bacterial phyla specific primers. (A) No differences are observed in total bacteria between patients with healthy subjects (black bar) and CDI patients (white bar). (B) Healthy and CDI stool microbiota bacterial phyla alterations in Firmicutes,
Bacteroidetes and Proteobacteria. (C) CDI patients also have decreased resident Firmicutes members *C. coccoides* (Δ 21%) and *C. leptum* (Δ 19%) levels. One Way ANOVA, Holme-Sidak

*P < 0.05. n = 9.

**Figure 4. In vitro** growth of *C. difficile* ATCC BAA-1870 in varying Na⁺ and pH conditions. [Na⁺] ranges for healthy and CDI stool are displayed as bars along the x-axis. A) Growth (CFU) of *C. difficile* in TYG broth at varying [Na⁺] which mimic those seen *in vivo* for healthy and CDI stool (*Fig. 2A*) at 24 hrs. *C. difficile* grew optimally > 16mM Na⁺ (pH 6.0) which is above the in vivo concentration of 3 mM Na⁺ for healthy patient stool. B) Growth of *C. difficile* in TYG broth at varying pH which mimics that seen *in vivo* for healthy and CDI stool (*Fig. 2B*). Growth was determined at 8 mM Na⁺ (●) mimicking healthy stool and 75 mM Na⁺ (○) mimicking CDI stool. There is a significant difference between 8mM Na⁺ and 75mM Na⁺ at pH 5.5 (P = 0.001), 6.0 (P < 0.001) and 6.3 (P = 0.001)*. In addition there is a significant difference between growth at pH 5.5 vs 6.0-6.5 (P < 0.001) and pH 6.0-6.5 and pH 7.0 (P < 0.001) for both 8 mM Na⁺ and 75 mM Na⁺. ** P < 0.05. Two Way ANOVA, Holme-Sidak.

**Figure 5. In vitro** growth of Clostridial members in a range of Na⁺ and pH conditions. Growth (CFU) of *Blautia producta* (*C. coccoides* Cluster XIVa) (A) *Faecalibacterium prausnitzii* (*C. leptum* Cluster IV) (B) and *C. butyricum* (Cluster I) (C), in TYG broth at varying [Na⁺] which mimic that seen *in vivo* for healthy (pH 6, ●) and CDI stool (pH 7, ○) at 24 hrs. All Clostridial groups grew optimally from 7-40 mM Na⁺ (pH 6.0 and 7.0) * P < 0.05. Two Way ANOVA, Holme-Sidak.
Figure 6. Human intestinal organoids (HIOs) grown in three-dimensional culture microinjected with bacterial or stool supernatant. Organoid culture in matrigel left hand panel with injection needle. A) NHE3 mRNA levels indicate decreased expression in HIOs injected with CDI stool and *C. difficile*. No changes were observed between healthy stool or *C. butyricum* culture. B). NHE3 protein expression as determined by immunofluorescence is decreased in HIOs injected with CDI stool and *C. difficile* compared to control, healthy and *C. butyricum* infected HIOs (White asterisk designates lumen). Side panel depicts widefield view of HIOP injection. C) Semi-quantitative analysis of NHE3 florescence. * P < 0.05. Two Way ANOVA, Holme-Sidak. n = 6 to 9 organoids.

Figure 7: Working model of *C. difficile* inhibition of NHE3. Under normal conditions, Firmicutes is the dominant member of the human colon at the level of the luminal (our data) and mucosa-associated bacterial populations (17). Antibiotic-use alters the gut microbiota and allows for the germination of *C. difficile* spores. Vegetative *C. difficile* uses fuel sources to proliferate and once in virulence phase, produces toxins which inhibit NHE3. Loss of functional NHE3 results in a high in Na\(^+\) and more alkaline pH luminal environment which shapes the gut microbiota to be increased in Bacteroidetes and decreased in Firmicutes. It remains unknown what changes are occurring in the mucosa-associated bacteria under these conditions. High Na\(^+\) and more alkaline pH favors *C. difficile* proliferation and prevents resident Clostridial groups from outcompeting *C. difficile*. 
Table 1. qPCR primer sequences for total bacteria, bacterial phyla and C. difficile.

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<th>Reverse</th>
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Figure 2

(A) Distribution of Na+ (mM) across Normal Stool, Diarrhea, Normal Stool, Diarrhea, and Recurrent CDI. 
(B) Distribution of K+ (mM) across Normal Stool, Diarrhea, Normal Stool, Diarrhea, and Recurrent CDI. 
(C) Distribution of Cl- (mM) across Normal Stool, Diarrhea, Normal Stool, Diarrhea, and Recurrent CDI. 
(D) Distribution of Non-Cl- Anion gap across Normal Stool, Diarrhea, Normal Stool, Diarrhea, and Recurrent CDI.
Figure 2

**pH**

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<td>Antibiotics</td>
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**Normal stool**

- pH: 5 - 6

**Diarrhea**

- pH: 5 - 7

**Recurrent CDI**

- pH: 7 - 8

Significance:

- *: p < 0.05
- **: p < 0.01
Figure 3

% Clostridial group/total bacteria

C. coccoides

C. leptum

*
Figure 4

A

B

C. difficile CFU

Healthy

CDI

Na+ mM

0 20 40 60 80 100 120

C. difficile CFU

pH

Healthy

CDI
Figure 5

A. B. producta CFU

B. F. prausnitzii CFU

C. C. butyricum CFU

Healthy CDI

pH 6.0

pH 7.0

Na+ mM
Figure 6

A

NHE3 mRNA fold difference

Control
Healthy Stool
CDI Stool
C. difficile
C. butyricum

*  
**