Original Paper

Acidic Conditions in the NHE2^{-/-} Mouse Intestine Result in an Altered Mucosa-Associated Bacterial Population with Changes in Mucus Oligosaccharides

Melinda A. Engevik^{a,c} Annelies Hickerson^a Gary E. Shull^{b,c} Roger T. Worrell^{a,c}

^aDepartment of Molecular and Cellular Physiology, ^bDepartment of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, ^cDigestive Health Center of Cincinnati Children's Hospital, Cincinnati, OH, USA

Key Words

Na⁺/H⁺-exchanger isoform 2 • Microbiome • Host-bacterial interaction • Colon

Abstract:

Background: The mechanisms bacteria use to proliferate and alter the normal bacterial composition remain unknown. The ability to link changes in the intestinal micro-environment, such as ion composition and pH, to bacterial proliferation is clinically advantageous for diseases that involve an altered gut microbiota, such as Inflammatory Bowel Disease, obesity and diabetes. In human and mouse intestine, the apical Na⁺/H⁺ exchangers NHE2 and NHE3 affect luminal Na⁺, water, and pH. Loss of NHE2 results in acidic luminal pH. Since acid resistance systems in gram-positive bacteria are well documented, we hypothesize that gram-positive bacteria would increase in representation in the acidic NHE2^{-/-} intestine. **Methods:** Intestinal ion composition was measured by flame photometry and chloridometry and pH measured electrochemically. DNA extracted from intestinal flushes or from mucosal scrapings was analyzed by qRT-PCR to examine luminal and mucosa-associated bacterial populations. Epithelial mucus oligosaccharide patterns were examined by histology with FIT-C labeled lectins. **Results:** Although total luminal and mucosa-associated bacteria were unchanged in NHE2^{-/-} intestine, gram-positive bacterial phyla were increased in the mucosa-associated bacterial population in a region-specific manner. The genera *Clostridium* and *Lactobacillus* were increased in the cecum and colon which corresponded to changes in NHE2^{-/-} mucus oligosaccharide composition of mannose, N-acetylglucosamine, N-acetylgalactosamine and galactose. **Conclusions:** Together these data indicate that changes in ion transport induce region-specific bacterial changes, which alter host mucus oligosaccharide patterns. These host-bacterial interactions provide a possible mechanism of niche-development and shed insight on how certain groups proliferate in changing environments and maintain their proliferation by altering the host.

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Introduction

Although it is well established that the gut microbiota can influence health and disease, the mechanism(s) which promote bacterial homeostasis or instability remain unknown. Alteration in the composition of gut microbiota, or microbial dysbiosis, has been implicated in the pathogenesis of various disease conditions including antibiotic-associated diarrhea (AAD) [1], *Clostridia difficile*-associated disease (CDAD) [2], inflammatory bowel disease (IBD) [3, 4], diabetes [5], and obesity [6]. A number of studies have demonstrated the effect of host genotype on fecal and cecal samples; however limited data exist on how bacteria inhabiting specific locations of the gut establish a niche and proliferate. Knowledge of how the intestinal environment affects specific bacteria will aid in the development of future therapies for disease with abnormal bacterial composition.

The fundamental mechanisms that govern the intestinal microbiota concentration and composition remain poorly understood. Antimicrobial peptides, mucus production, age, immune status, luminal pH, available fermentable materials and general living conditions all have been hypothesized to affect the gut microbiota [7]. Environmental factors and host genetics interact to control the acquisition, maintenance, and stability of a healthy gut microbiota. Changes in the microbiota, host genetics, or environment could result in dysbiosis and diseases [7]. Intestinal pH and ion concentration could be key factors in shaping the intestinal environment. All members of the GI microbiota have specific external pH growth ranges, but some members have developed mechanisms for adaptation to varying pH. Acid resistance systems in gram-positive bacteria have been well documented; these acid resistance systems allow these bacteria to proliferate in various acidic environments [8]. Gram-positive bacteria use a combination of constitutive and inducible strategies which result in the removal of protons (H⁺) by ATPase-dependent or ATPase-independent proton transport [9], changes in the composition of the cell envelope such as increased production of cyclopropane fatty acids (CFAs) [10], production of general shock proteins and chaperones, expression of transcriptional regulators, and responses to changes in cell density [8]. For example, gram-positive *E. hirae* (formerly *Streptococcus faecalis*) primarily uses the F1F0-ATPase to expend ATP to expel excess protons [9, 11-13] and low external pH increases the expression and activity of this ATPase [14]. The ATPase is also the key acid removal system for gram-positive *Lactococci* [15] and for gram-positive *Streptococci* [16], supplemented by catabolic mechanisms such as amino acid decarboxylases [9, 17]. In preference to more acidic conditions, gram-positive bacteria have been shown to produce more lactic acid than the gram-negative bacteria and to lower the ambient pH of their environment *in vitro* [18]. These studies indicate that gram-positive bacteria are capable of proliferating in more acidic environments.

In addition to reacting to the host environment, bacteria are capable of altering the host mucus oligosaccharide composition to suit their proliferation needs. Bacteria have been shown to interact with the outer loose layer of mucus composed primarily of highly glycosylated MUC2 mucin [19, 20]. Glycans make up approximately 80% of the mucin mass and contain different sugar residues: N-acetylglucosamine (GlcNAc), galactose (Gal), N-acetylgalactosamine (GalNAc), fucose (Fuc), N-acetylneuraminic acid (NeuNAc), sialic acid, mannose, glucose, and xylose. Only a small percentage of the gut microbiota are able to secrete glycan-degrading enzymes that sequentially release monosaccharides from mucus glycan chains [19, 21-23]. The genera *Ruminococcus*, *Bacteroides*, *Bifidobacterium*, *Lactobacillus* and *Clostridium* have been shown to degrade mucus oligosaccharides, which are then used as a bacterial carbon source [23-29]. Studies have demonstrated that mucin degradation often requires the participation of several bacterial species which expresses some or all of the required glycosidases [25]. Once oligosaccharides have been released, other resident bacteria which do not contain mucin-degrading enzymes are also able to use these released sugars, which further shapes the microbial composition [30, 31]. Cleavage of these oligosaccharides by the gut microbiota has been linked to subsequent host modulation of mucus glycosylation [27, 32-34] and mucin gene expression [35, 36].

Very few studies exist which combine altering the intestinal environment, monitoring the bacterial population and correlating microbiota changes with changes in mucus and oligosaccharide patterns. We hypothesized that mucosa-associated bacterium that prefer acidic pH (gram positive bacteria) will proliferate in the NHE2^{-/-} mouse intestine and will maintain their proliferation by increasing glycan oligosaccharide foraging options. Herein we show that the NHE2^{-/-} mouse displays acidic luminal pH in all intestinal segments, which correlates with increased mucosa-associated gram-positive bacteria. Increases in *Clostridium/Ruminococcus* and *Lactobacillus* are associated with changes in mannose, galactose, N-acetylgalactosamine and N-acetylglucosamine, but not fucose.

Materials and Methods

Mice

All experimental protocols were approved by the University of Cincinnati Animal Care and Use Committee and complied with National Institutes of Health guidelines. FVB/N NHE2^{-/-} mice were generated as previously described [37]. Littermate mice were co-housed in the same cage to ensure a common microbial environment. Mice were maintained on a normal mouse diet (7922 NIH-07 Mouse diet, Harlan Laboratories, Indianapolis, IN). At 6-8 weeks post weaning, terminal ileum, cecum, and colon (proximal and distal) segments were collected from WT and NHE2^{-/-} littermates. Individual intestinal segments were flushed with PBS (pH 7.4) and mucosal scrapings were collected for total DNA analysis as previously described [38-42]. Briefly, intestinal segments were flushed with 500 µl PBS. The segments were then opened lengthwise, washed thoroughly with PBS and glass slides were used to scrape the epithelia and mucus layer. Luminal flushes were processed for DNA and mucosal scrapings were processed either for DNA or for RNA. Sample wet weight was determined and homogenized with a Tissue Tearor homogenizer (Biospec Products Inc, Bartlesville, OK) for 1 min. RNA or DNA was extracted and stored at -80°C or -20°C until the samples were evaluated by quantitative real time PCR (qRT-PCR).

Histology

WT and NHE2^{-/-} mouse terminal ileum, cecum, proximal and distal colon segments were fixed for 4 h at 4°C in Carnoy's fixative and embedded in paraffin. Serial 6–7 µm thick sections were applied to glass slides and stained with hematoxylin and eosin (H&E) for intestine architecture or Periodic Acid-Schiff's/Alcian blue (PAS-AB) for goblet cells and mucus. Glycoconjugates on the mucosal surface was examined using a panel of FITC-conjugated lectins: Ulex europaeus agglutinin-1 (UEA-1) for terminal fucose; Concanavalin A (CONA) for mannose, *Dolichos biflorus* agglutinin (DBA) for N-Acetylgalactosamine, Peanut agglutinin (PNA) for galactose and Wheat Germ Agglutinin (WGA) for N-Acetylglucosamine (Vector Laboratories, Burlingame, CA) as previously described [42-44]. Briefly, sections were deparaffinized, blocked with PBS containing 10% BSA, and stained with FITC-labeled lectin (10 µg/ml) for 1 h at room temperature. Sections were then washed three times in PBS, mounted using Vectashield mounting medium with DAPI (Vector Laboratories), and analyzed by confocal laser scanning microscopy (Zeiss LSM Confocal 710, Carl Zeiss, Germany). Digital images of slides were evaluated by tabulating mean pixel intensity of the respective color channel on each image using Image J software (NIH). Five regions of interests of fixed size per slide, 3 slides per mouse, and n=4 mice were used for semi-quantitation of stain intensity.

qPCR amplification of 16S sequences

Total DNA was isolated with the QIAamp DNA Stool kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The lysis temperature was increased to 95°C and an incubation with lysozyme (10 mg/ml, 37°C for 30 min) was added to improve bacterial cell lysis as previously described [41, 42, 45-47]. The abundance of total bacteria and specific intestinal bacterial phyla, class, genus and species was measured by qPCR using a Step One Real Time PCR machine (Applied Biosystems, Carlsbad, California USA) with SYBR Green PCR master mix (Applied Biosystems) and bacteria-specific primers (Table 1) in a 20 µl final volume. Bacterial numbers were determined using standard curves from the pure bacterial cultures as previously described [47, 48] which correlated cycle of threshold values (C_t) to calculated bacteria number.

Table 1. Primer sequences for qRT-PCR of total bacteria and specific bacterial phyla and groups

Type	Bacteria	Forward	Reverse	Reference
Total	Universal (Total Bacteria)	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	[47, 114, 115]
Phyla	<i>Bacteroidetes</i>	GGCGACCGGCGCACGGG	GRCCTTCCTCTCAGAAGCC	[115]
Phyla	<i>Firmicutes</i>	GGAGYATGTGGTTTAATTGGAAGCA	AGCTGACGACAACCATGCAC	[114]
Phyla	<i>Actinobacteria</i>	CGCGGCCTATCAGCTTGTG	ATTACCGCGGCTGCTGG	[114]
Phyla	<i>α-proteobacteria</i>	ACTCCTACGGGAGGCAGCAG	TCTACGRATTCACCCTAC	[114]
Phyla	<i>β-proteobacteria</i>	CCGCACAGTTGGCGAGATGA	CGACAGTTATGACGCCCTCC	[114]
Phyla	<i>γ-Proteobacteria</i>	GAGTTTGATCATGGCTCA	GTATTACCGCGGCTGCTG	[116]
Class	<i>Clostridium coccoides</i> <i>cluster XIVa group</i>	ACTCCTACGGGAGGCAGC	GCTTCTTAGTCAGGTACCGTCAT	[46]
Class	<i>Clostridium leptum cluster</i> <i>IV group</i>	GTTGACAAAACGGAGGAAGG	GACGGGCGGTGTGTACAA	[46]
Class	<i>Lactobacillus/Enterococcus</i> <i>group</i>	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	[46]
Genus	<i>Bacteriodes</i>	GTTTCTGAGAGGAGGTCCC	CTGCCTCCCGTAGGAGT	[46]
Genus	<i>Prevotella</i>	CCAGCCAAGTAGCGTGCA	TGGACCTTCGTATTACCGC	[117]
Genus	<i>Mouse Intestinal Bacteria</i> <i>(MIB)</i>	CCAGCAGCCGCGTAATA	CGCATTCCGCATACTTCTC	[46]
Species	<i>Bacteriodes</i> <i>thetaiotaomicron</i>	GGTAGTCCACACAGTAAACGATGAA	CCCGTCAATTCTTTGAGTTTC	[118]

qRT-PCR of MUC mRNA

Total RNA was extracted from mucosal scrapings with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. 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 were performed with SYBR Green PCR master mix (Applied Biosystems), 200 ng sample cDNA in a 20 µl final volume on the Step One Machine (ABI). Data was reported as the delta delta C_T using GAPDH as the standard. Primers for MUC and GAPDH were used as previously described: MUC 1 Forward: CCAGACCCCTGCACTCTGAT; MUC 1 Reverse: CGCTTGACAAAGGCATGA; MUC 2 Forward: TGCCACCTCCTCAAAGAC; MUC 2 Reverse: TAGTTTCCGTTGGAACAGTGAA; MUC 3 Forward: TGGTCAACTGCGAGAATGGA; MUC 3 Reverse: TACGCTCTCCACCAGTTCTCT; MUC 4 Forward: CAATGCCCTCCACAAAAAGT; MUC 4 Reverse: CTGTGTGTTGGCAATTCTG; MUC 5ac Forward: TGGTTTGACACTGACTTCCC; MUC 5ac Reverse: TCCTCTCGGTGACAGAGTCT; GAPDH Forward: CATGGCCTTCGGTGTTCCTA and GAPDH Reverse: CCTGCTTACCACCTTCTTGAT [49-51].

Ion and pH measurements

Intestinal flushes of WT and NHE2^{-/-} mice were performed with 100 µl of double deionized water. Samples were weighed, centrifuged at 3,000 rpm for 10 min at 4°C to pellet the stool and the supernatant Na⁺ and K⁺ concentrations were determined using a digital Flame photometer (Single-Channel Digital Flame Photometer Model 02655-10; Cole-Parmer Instrument Company Vernon Hills, IL). Cl⁻ ion concentration was determined by a digital Chloridometer (Model 4425100, Labconco Kansas City, MO) and normalized to intestinal volume. pH measurements were performed electrochemically via an electronic pH meter (Orion Model 720A; Thermo Fisher Scientific Waltham, MA).

Statistics

The data are presented as the mean ± SEM. Comparisons between two groups were made with unpaired *t*-tests and comparisons between more than two groups were performed using ANOVA. A *P* < 0.05 value was considered significant while *n* is number of experiments.

Fig. 1. The intestinal tract of NHE2^{-/-} mice has normal histology. Mucosal morphology in WT and NHE2^{-/-} mouse ileum, cecum, proximal and distal colon demonstrating no gross alteration of the mucosal architecture in any segment. Similar villi number and length, crypt number and length, paneth cell number, and granules (observed in red) are present in WT and NHE2^{-/-} intestinal segments. Micrographs are representative H&E stained sections (n=4). Scale bar = 50 μ M.

Results

NHE2^{-/-} mice exhibit altered intestinal environment with regional changes in K⁺ and Cl⁻ and an acidic pH

Na⁺/H⁺ exchange across the apical membrane of enterocytes is critical for Na⁺ and fluid absorption and intestinal pH regulation [37, 52-54]. Na⁺/H⁺ Exchanger (NHE; gene locus symbol, Slc9aX) 1, 2, 3 and 8 are located in the intestine [55, 56]. NHE2 (Slc9a2) and NHE3 (Slc9a3) are located on the apical membranes of enterocytes in the intestine [57-59] but have unique expression patterns. NHE3 is located primarily in the absorptive intestinal cells, while NHE2 is located both in the absorptive cells and in the intestinal crypts [54]. NHE3 has been shown to be critical for both Na⁺ and fluid absorption in the intestine as demonstrated by chronic diarrhea in NHE3^{-/-} mice [42, 53]. Our previous work has shown NHE3^{-/-} mice have an alkaline intestinal lumen high in Na⁺ and a proliferation of gram-negative Bacteroidetes in the luminal and mucosa-associated bacterial populations [42]. In contrast to NHE3^{-/-} mice, NHE2^{-/-} mice lack diarrhea [60] and NHE3/NHE2 double-knockout mice do not have an aggravated diarrhea phenotype [61]. This may be due in part to upregulation by NHE3 [54, 62] and NHE8 [63] in NHE2^{-/-} mice. NHE2^{-/-} mice have no apparent changes in morphology as determined by H&E staining (Fig. 1).

In order to examine the ion and pH changes which may occur in the NHE2^{-/-} intestine, Na⁺ and K⁺ concentrations were examined by flame photometry and Cl⁻ concentration by chloridometry (Fig. 2). Consistent with other reports [60], NHE2^{-/-} intestinal pH was significantly decreased (more acidic) in terminal ileum, cecum, proximal and distal colon compared to WT littermates (Fig. 2A). NHE2^{-/-} mice had no significant change in Na⁺ concentration (Fig. 2B), which is consistent with NHE3 being the main apical Na⁺-absorbing NHE. K⁺ concentration was increased in NHE2^{-/-} ileum, but unchanged in the cecum and colon (Fig. 2C). Cl⁻ concentration was decreased only in cecum and distal colon of NHE2^{-/-} mice (Fig. 2D). We hypothesized that differences in intestinal ion composition and pH in NHE2^{-/-} intestine would cause region specific bacterial proliferation. Since gram-positive bacteria have been shown to be preferential to acidic conditions, we speculated that gram-positive bacteria would be generally increased in the NHE2^{-/-} mouse.

NHE2^{-/-} mice exhibit luminal and mucosa-associated MD at the phylum and subgroup level

In order to determine if total bacterial numbers were changed as a result of an altered intestinal environment, luminal and mucosa-associated bacterial gDNA was extracted

Fig. 2. NHE2^{-/-} mice have an acidic intestinal environment with regional changes in K⁺ and Cl⁻ ion concentrations in luminal fluid from WT (black bar) and NHE2^{-/-} (white bar) mouse intestinal segments. A) pH as determined by pH probe was significantly decreased in all NHE2^{-/-} intestinal segments. B) Sodium concentration as determined by flame photometry was not changed in any intestinal segment of NHE2^{-/-}

verses WT littermates. C) Potassium concentration as determined by flame photometry was significantly increased in ileum but not in cecum or colon of NHE2^{-/-} mice vs. WT littermates. D) Chloride concentration as determined by chloridometry was significantly decreased in the cecum and distal colon of NHE2^{-/-} mice vs. WT littermates. n=11 for WT and n=6 for NHE2^{-/-}. * P<0.05.

Fig. 3. NHE2^{-/-} mice show no significant change in total bacteria. Total bacteria quantified by qRT-PCR using a universal bacterial 16S DNA sequence and "calculated bacterial cell" number calculated from an

E. coli standard curve normalized to intestinal flush volume. A) Bacteria contained within the luminal flushes of WT (black bar) and NHE2^{-/-} (white bar) littermates. B) Mucosa-associated (adherent) bacterial levels between WT (black bar) and NHE2^{-/-} (white bar) littermates. NHE2^{-/-} luminal and mucosa-associated bacterial populations demonstrated no change in total bacteria compared to WT littermates. n=6, * P>0.005.

from luminal flushes and mucosal scrapings and analyzed by qPCR. As shown in Fig. 3A, no changes were observed in total luminal bacteria in the NHE2^{-/-} mouse ileum, cecum, proximal and distal colon. In addition no significant changes were observed in the total mucosa-associated bacteria (Fig. 3B) of the NHE2^{-/-} mouse. In contrast to the NHE3^{-/-} intestine, which demonstrates increased intestinal size and bacterial overgrowth, the NHE2^{-/-} intestine exhibits no changes in total bacteria.

Next we sought to determine if an alteration in the normal bacterial composition was occurring in NHE2^{-/-} luminal and mucosa-associated bacterial populations. The mouse and human gut microbiota is dominated by the phyla Firmicutes and Bacteroidetes and contains lesser amounts of Proteobacteria, Actinobacteria, Fusobacteria and Verruomicrobia [64,

Fig. 4. NHE2^{-/-} mice exhibit region-specific luminal and mucosa-associated MD. Relative abundance was calculated as the percentage of bacterial phyla in comparison to total bacteria for luminal and mucosa-associated bacteria. A) Luminal bacterial showed no changes in phyla composition except in terminal ileum which had increased Firmicutes. B) Mucosa-associated bacteria showed changes in bacterial phyla in every NHE2^{-/-} segment. C) Relative abundance of mucosa-associated bacterial subgroups of Firmicutes and Bacteroidetes phyla. Relative abundance was calculated as the percentage of bacterial subgroup in comparison to total bacteria. Regional changes were observed in the Firmicutes subgroup *C. coccoides* cluster XIVa, *C. leptum* cluster IV and *Lactobacillus/Enterococcus* group. Changes were also observed in the Bacteroidetes subgroup *Bacteroides* and *MIB* in the NHE2^{-/-} mouse mucosa-associated bacterial populations. n=6, * P>0.005.

65-70]. Firmicutes and Actinobacteria are gram-positive groups while Bacteroidetes and Proteobacteria are gram-negative groups. In order to examine if NHE2^{-/-} mice exhibit an altered microbiota composition, the major mouse intestinal bacterial phyla were compared as a percentage of total bacteria. As shown in the bacterial phyla representation in Fig. 4A, other than a slight increase of gram-positive Firmicutes and a decrease in gram-negative Proteobacteria in the terminal ileum, no changes occurred in the NHE2^{-/-} luminal bacterial population.

In contrast, large changes in the major phyla were observed in the NHE2^{-/-} mucosa-associated bacterial population as seen in Fig. 4B. In the NHE2^{-/-} terminal ileum, there was an increase in gram-positive Actinobacteria (39.1%) and a decrease in gram-positive Firmicutes (11.5%) and gram-negative Bacteroidetes (25.1%). In the NHE2^{-/-} cecum there was a decrease in Firmicutes (6.9%) and an increase in Proteobacteria (4.3%), Actinobacteria (1%) and unspecified bacteria (2.1%). In the NHE2^{-/-} proximal and distal colon there was an increase in Firmicutes (PC: 14.1%; DC: 6.8%), and a decrease in Bacteroidetes (14.9%; 6.8%). These data together indicate that the NHE2^{-/-} mucosa-associated bacterial population is significantly transformed in response to an altered ion transport status and that in general gram-positive bacteria are proliferating.

Since gram-positive Firmicutes and gram-negative Bacteroidetes compose the dominant microbial phyla, subgroups of these phyla were further examined in the mucosa-associated bacterial population to determine the groups responsible for shifts in the phyla (Fig. 4C). Firmicutes subgroups *Bacillales* (specifically *Lactobacillus/enterococcus*) and *Clostridium*

Fig. 5. Immunostain of fucose residues in intestinal segments. No significant change in fucosylation was observed in any NHE2^{-/-} segment. Fucosylation was determined by UEA-1-FITC lectin binding (red) and tissue sections counter stained with DAPI (blue, cell nuclei) A) Representative micrographs from n=4 mice of each genotype. Scale Bar = 50 μ M. B) Semi-quantitative analysis of oligosaccharide stains analyzed by Two Way ANOVA n=4, * P>0.005.

Fig. 6. Immunostain of N-acetylgalactosamine residues in intestinal segments. NHE2^{-/-} mice have increased N-acetylgalactosamine only in the cecum compared to WT littermates. N-acetylgalactosamine was determined by DBA-FITC lectin binding (purple) and tissue sections counter stained with DAPI (blue, cell nuclei). A) Representative micrographs from n=4 mice of each genotype. Scale Bar = 50 μ M. B) Semi-quantitative analysis of oligosaccharide stains analyzed by Two Way ANOVA n=4, * P>0.005.

(clusters XIVa and IV) were examined in addition to in Bacteroidetes genera *Bacteroides*, *Prevotella*, *Porphyromonas* and *Mouse Intestinal Bacteroidetes* (MIB) were analyzed by qPCR and presented as a percentage of total bacteria. In the mucosa-associated bacterial population (Fig. 4C), changes were observed at the subgroup level in every segments. Gram-positive Firmicutes members *C. coccoides* cluster XIVa was increased in the NHE2^{-/-} cecum (11.0%), proximal colon (4.2%) and distal colon (6.7%) while *C. leptum* cluster IV was increased in the proximal (29.4%) and distal (5.5%) colon. *Lactobacillus/Enterococcus* group was increased only in the cecum (4.9%) and proximal colon (4.8%). Interestingly, increases in the examined gram-positive Firmicutes subgroups were accompanied by decreases in other unspecified Firmicutes groups. The gram-negative Bacteroidetes member *Prevotella* was unchanged in all segments while *Bacteroides* was decreased in the NHE2^{-/-} ileum (4.2%) and increased in the cecum (3.9%), proximal colon (5.1%) and distal colon (7.1%). Additionally MIB was decreased in all the NHE2^{-/-} intestinal segments (I: 10.3%, C: 11.0%, PC 11.9%, DC: 9.7%). Decreases were also observed in unspecified Bacteroidetes subgroups. These data indicate that in the mucosa-associated bacterial population *Clostridium* clusters and *Lactobacillus/Enterococcus* group are responsible for the changes in the Firmicutes phyla in the NHE2^{-/-} cecum, proximal colon and distal colon

Fig. 7. Immunostain of N-acetylglucosamine residues in intestinal segments. NHE2^{-/-} mice have increased N-acetylglucosamine only in the cecum compared to WT littermates. N-acetylglucosamine was determined by WGA-FITC lectin binding (orange) and tissue sections were counter-stained with DAPI (blue, cell nuclei). A) Representative micrographs from n=4 mice of each genotype. Scale Bar = 50 μ M. B) Semi-quantitative analysis of oligosaccharide stains analyzed by Two Way ANOVA n=4, * P>0.005.

Fig. 8. Immunostain of mannose residues in intestinal segments. NHE2^{-/-} mice have increased mannose only in the cecum compared to WT littermates. Mannose was determined by ConA-FITC lectin binding (green) and tissue sections counter stained with DAPI (blue, cell nuclei). A) Representative micrographs from n=4 mice of each genotype. Scale Bar = 50 μ M. B) Semi-quantitative analysis of oligosaccharide stains analyzed by Two Way ANOVA n=4, * P>0.005.

NHE2^{-/-} mice exhibit altered mucus oligosaccharides with no change in MUC2

Bacteria that are able to metabolize mucus oligosaccharides display an adaptive advantage for survival and colonization in the intestine [26]. These bacteria have access to the host as an energy source and have the ability to recruit other monosaccharide-using bacteria. Together this interaction shapes the nutrient environment and the gut microbiota. Bacteria from the gram-positive genera *Ruminococcus*, *Clostridium*, *Lactobacillus* and *Enterococcus* have been shown to degrade mucin [23-26]. *Ruminococcus* species are found in both *C. coccoides* cluster XIVa and *C. leptum* cluster IV, *Lactobacillus* and *Clostridium* have all been shown to release a number of glycosidases [28, 40, 71, 72] releasing monosaccharides, such as galactose, mannose, N-acetylglucosamine, which then supplement bacterial growth. Gram-negative *Bacteroides* has also been shown to contain some mucin degrading enzymes [27, 32, 73], and to use intestinal fucose [27, 73, 74]. Since NHE2^{-/-} mice have increased *Clostridium*/*Ruminococcus* and *Lactobacillus*/*Enterococcus* groups in the cecum and colon we sought to determine host mucus oligosaccharides changes which may occur as the result of the altered microbiota.

Fig. 9. Immunostain of galactose residues in intestinal segments. NHE2^{-/-} mice have increased galactose in the cecum and decreased galactose in the distal colon compared to WT littermates. Galactose was determined by PNA-FITC lectin binding (yellow) and tissue sections counter stained with DAPI (blue, cell nuclei). A) Representative micrographs from n=4 mice of each genotype. Scale Bar = 50 μ M. B) Semi-quantitative analysis of oligosaccharide stains analyzed by Two Way ANOVA n=4, * P>0.005.

Fig. 10. PAS-AB stain of mucins in intestinal segments. NHE2^{-/-} mice have normal mucus production. No changes were observed in mucus morphology and production in WT and NHE2^{-/-} mouse ileum, cecum, proximal and distal colon. Micrographs are representative sections (n=4 mice of each genotype) stained with PAS-AB, which stains acidic mucins blue and neutral mucins magenta. There were no apparent differences in the expression of acidic and neutral mucins. Scale bar = 50 μ M.

Although gram-negative *Bacteroides* was increased in the cecum and colon, no changes were observed in host fucosylation in any of the NHE2^{-/-} intestinal segments (Fig. 5A and B). *Bacteroides* has been shown to have a culture-dependent induction of fucosylation [27, 73] and since the distal intestine has high levels of fucosylation it is possible that fucosylation is already at maximum and cannot be further induced. In NHE2^{-/-} cecum, gram-positive *C. coccoides* and *Lactobacillus* were increased while in NHE2^{-/-} distal colon *C. coccoides* and *C. leptum* were increased. Correlating to the changes in these known mucin degrading groups, we observed mucin changes in the NHE2^{-/-} cecum and distal colon (Fig. 6B-9B). Increases were observed in N-acetylgalactosamine (Fig. 6), N-acetylglucosamine (Fig. 7), mannose (Fig. 8) and galactose (Fig. 9) in the NHE2^{-/-} cecum. In addition, decreased terminal galactose was observed in the distal colon (Fig. 9A and B). No changes were observed in

Fig. 11. NHE2^{-/-} mice have decreased *MUC1* mRNA whereas normal *MUC2* mRNA is unchanged. *MUC* mRNA was analyzed by qRT-PCR and expressed as the $\Delta\Delta C_t$ relative fold difference. Decreased *MUC1* mRNA (A) was observed in NHE2^{-/-} (white bar) cecum and colon compared with WT (black bar) littermate mice. B) No significant change was observed in *MUC2* mRNA. n=8 for WT and n=6 for NHE2^{-/-}. * P<0.05.

NHE2^{-/-} terminal ileum or proximal colon. Galactose transporters and galactosidases have been found in *Clostridium* species [75-78]. Decreased galactose in the colon may be due in part to the increase in both *Clostridium* clusters, which may deplete the galactose as observed in Fig. 9.

Changes in mucus oligosaccharides can be either a direct result of changes in glycosyltransferases or mucus production. Mucin glycoproteins are encoded by *MUC* genes [79]. *MUC1*, *MUC3*, and *MUC4* mucins represent adherent mucus since their carboxy terminus contains transmembrane domains. In contrast *MUC2*, *MUC5AC*, and *MUC5B* represent secreted mucins, which comprise the loose outer layer of mucus which bacteria primarily interact [20, 80]. Mucus production was examined by PAS-AB Stains (Fig. 10) and *MUC* mRNA (Fig. 11). PAS-AB stains reveal no gross changes in mucus goblet cell number or mucus production. Likewise, no change was observed in *MUC2* mRNA (Fig. 11A). No change was also observed in *MUC3*, *MUC4* or *MUC5a* mRNAs (data not shown). However decreased *MUC1* mRNA was observed in the NHE2^{-/-} cecum and colon (Fig. 11A) with no changes in the terminal ileum. This indicates that although there does not appear to be any changes in the secreted mucus, there is a decrease in adherent mucus production. Adherent mucins have been hypothesized to play a role in mucosal defense since mice lacking the Muc1 cell surface mucin are predisposed to GI infection [81-83]. Altered *MUC1* expression could be a result of the altered bacterial composition of the NHE2^{-/-} mice since bacterial adhesion and bacterial products have been shown to stimulate mucus discharge [81, 84-86]. Despite the decreased *MUC1* mRNA levels, the NHE2^{-/-} mice do not have any neutrophil invasion (data not shown) or other overt signs of inflammation.

Together these data suggest that the acidic environment of the NHE2^{-/-} mouse intestine correlates to an increase in gram-positive bacteria in the mucosa-associated bacteria population, which in turn use mucus oligosaccharides to drive their proliferation. This information points to pH change as a driving force for shifting the bacterial composition and provides data on which groups may be pH sensitive under *in vivo* conditions. A better understanding of which groups are capable of proliferating in an altered intestine environment is key for developing better therapies for microbial-dysbiosis associated diseases.

Discussion

The factors involved in regional microbiota niche establishment and maintenance remain largely unknown. The NHE2^{-/-} mouse offers a simplified model of altered intestinal environment and alterations in the gut microbiota. In this study we have shown that in

general, gram-positive bacteria are increased in the NHE2^{-/-} intestine which correlate with acidic environmental conditions. Our data demonstrates that increased *Lactobacillus* and *Clostridium/Ruminococcus* groups correlate with acidic environmental conditions, which correlates to altered mucus glycoprotein patterns. Previous literature has shown that these gram-positive bacteria are pH sensitive. *Lactobacillus*, for example, contains genes and proteins involved in pH homeostasis and acid adaption [8]. *Lactobacillus* species have been found to participate in malolactic fermentation in which the dicarboxylic malic acid is converted to monocarboxylic lactic acid [87-89]. In malolactic fermentation, a lactate-malate antiporter or an electrogenic uniporter exports L-lactate, thereby allowing the synthesis of ATP at low pH [8]. *Clostridial* groups have been shown to have transmembrane proton transport which allows them to adapt to a range of pH [90]. Previous studies have shown that cell density plays a role in the acid resistance of gram positive bacteria [91]. Increases in gram-positive bacteria in the NHE2^{-/-} intestine may be responsible for the overall acid adaption of these organisms in the low intestinal pH environment. Interestingly, the changes in the NHE2^{-/-} bacteria are dramatically different from the NHE3^{-/-} mouse which has an alkaline intestinal environment. In the NHE3^{-/-} mouse, gram-negative Bacteroidetes proliferate, particularly in the mucosa-associated bacterial population. This proliferation of Bacteroidetes is primarily caused by a large increase in mucosa-associated *Mouse Intestinal Bacteroidetes (MIB)* in all the intestinal segments [42]. In contrast, mucosa-associated *MIB* is decreased in all the NHE2^{-/-} segments. *MIB* is found in both mouse and human intestine [47, 92] and from the data present herein it appears that *MIB* is influenced by pH.

Ion composition can also be used a mechanism of pH adaption. For gut bacteria which are normally exposed to a neutral environment, intracellular changes in bacterial pH typically require use of bacterial cation/proton antiporters. pH homeostasis in the model bacteria *E. coli* has revealed a major role in K⁺ extrusion and proton capture for pH regulation [9, 93-95]. The NHE2^{-/-} mice have increased K⁺ concentration in the terminal ileum compared to WT mice. This is also the only location where gram-positive Actinobacteria has increased to become the dominant phyla. Studies have shown that Actinobacteria member *Corynebacterium glutamicum* contains an active potassium channel which contributes to maintenance of internal pH and membrane potential of the bacteria, thereby allowing survival of the bacteria at low pH [96]. These results indicate that active potassium transport systems correlate with an improved potassium-dependent pH homeostasis in at least one Actinobacteria species and this may be why we observed both increased K⁺ and Actinobacteria in the NHE2^{-/-} ileum.

Bacteria have been shown to interact with the outer mucus layer and this interaction provides bacterial-host "cross talk" which allows the microbiota to modify the host, creating favorable bacterial niches for proliferation [19]. Oligosaccharides on mucus glycoproteins provide both a signaling mechanism for the host and a foraging option for bacteria. Mucus glycosylation differs depending on intestinal location. In the proximal and distal colon the mucus has increased sialylation (terminal sialic acid N acetyl neuraminic acid residues) and increased sulphation (terminal ester sulphate residues). Increased sialylation and sulphate increases the charge of the mucus, and thereby the tenacity, aiding in resistance of the mucus to bacterial enzymatic attack [79, 97]. As a result bacteria have to produce sialidase (neuraminidase) and sulphatase which can remove the sialic acid and ester sulphate before further oligosaccharide degradation can proceed [79, 98-101]. Gram positive gut bacteria from the genera *Clostridium* and *Streptococcus* have been found to produce more than one sialidase as isoenzymes [102]. Sialidases from *Clostridium* species *Clostridium septicum*, *Clostridium sordellii*, *Clostridium chauvoei*, and *Clostridium tertium* have a relatively high hydrolysis activity toward substrates with the α(2,3)-linkage [102, 103], which has been found to be the dominant intestinal linkage in cultured colonic epithelial cells [104]. It is possible that increased *Clostridium* in the NHE2^{-/-} distal colon produces more sialidases which in turn make the colonic mucus oligosaccharides more prone to enzymatic attack, resulting in decreased galactose residues. Interestingly oligosaccharides degradation by colonizing bacteria in the postnatal gut has been shown to stimulate increased oligosaccharide expression [73, 105-107]. If the cecum does not contain the same degree of sialylation and

sulphation bacteria may be able to directly interact with the mucus oligosaccharides, thereby triggering increased oligosaccharide production. Alteration in intestinal glycosylation has been observed in disease states such as Ulcerative Colitis (UC), Crohn's Disease (CD) and cancer [79, 108-111], [71, 112]. Although the exact mechanism by which these changes take place is unknown, the gut microbiota has been shown to be altered in IBD patients with a disproportionate increase in select mucolytic bacteria, particularly from the *Ruminococcus* genera [3, 4, 113], indicating a link between the oligosaccharide changes and the bacterial composition. Based on these studies and the study presented herein there is a correlation between the microbiota and the host glycosylation. Our study demonstrates that an altered intestinal environment is capable of disrupting the bacterial composition and increase mucolytic bacteria, which then affect the mucus oligosaccharide composition as well as the mucin gene expression.

Conflict of Interests

None.

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