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# Altered fecal short chain fatty acid composition in children with a history of Hirschsprung-associated enterocolitis☆,☆☆

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ABSTRACT

*Purpose:* Children with Hirschsprung disease (HD) who have a history of enterocolitis (HAEC) have a shift in colonic microbiota, many of which are necessary for short chain fatty acid (SCFA) production. As SCFAs play a critical role in colonic mucosal preservation, we hypothesized that fecal SCFA composition is altered in children with HAEC.

*Methods:* A multicenter study enrolled 18 HD children, abstracting for history of feeding, antibiotic/probiotic use, and enterocolitis symptoms. HAEC status was determined per Pastor et al. criteria (12). Fresh feces were collected for microbial community analysis via 16S sequencing as well as SCFA analysis by gas chromatography–mass spectrometry.

*Results:* Nine patients had a history of HAEC, and nine had never had HAEC. Fecal samples from HAEC children showed a 4-fold decline in total SCFA concentration vs. non-HAEC HD patients. We then compared the relative composition of individual SCFAs and found reduced acetate and increased butyrate in HAEC children. Finally, we measured relative abundance of SCFA-producing fecal microbiota. Interestingly, 10 of 12 butyrate-producing genera as well as 3 of 4 acetate-producing genera demonstrated multi-fold expansion.

*Conclusion:* Children with HAEC history have reduced fecal SCFAs and altered SCFA profile. These findings suggest a complex interplay between the colonic metabolome and changes in microbiota, which may influence the pathogenesis of HAEC.



## 1. Background

Hirschsprung-associated enterocolitis (HAEC) is a life-threatening complication of congenital aganglionic megacolon, or Hirschsprung disease (HD). While surgical pull-through achieves satisfactory restoration of functional intestinal continuity in most patients, up to 40% of children experience at least one episode of HAEC after surgery [1]. Despite being the primary cause of morbidity for children with HD, the pathophysiology of HAEC is poorly understood. Several contributing factors have been identified, including dysfunctional host immunity, diminished epithelial barrier function, and altered gut microbiota, although no unifying causative agent has been identified [2].

A microbial etiology of HAEC has been postulated since initial reports of elevated *Clostridium difficile* toxin titers in children with HAEC [3], although carriage rates have since proven highly variable [4]. Analysis of changes in the gut microbiome using molecular microbiological techniques has shown decreased colonization of *Bifidobacterium* and *Lactobacillus* in children with HD who developed HAEC [5], while genomic approaches have found increased bacterial population diversity during HAEC episodes in children with HD [6], yet modest differences in children with a history of HAEC [7]. Similar findings have been noted in animal models of HD using neural crest cell specific endothelin receptor B (Ednrb) knockout mice [8]. At the genus level, Ednrb

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knockout mice demonstrated decreased *Lactobacillus* and increased *Bacteroides* and *Clostridium* prior to enterocolitis. These findings suggest that disequilibrium in the gut microbiome – or dysbiosis – may result in an altered microbial ecosystem that leads to HAEC development.

The mechanisms by which altered colonic microbiota relate to the development of HAEC is not known. One important physiologic role of gut bacteria is the production of short chain fatty acids (SCFAs). Complex oligosaccharides and other organic, indigestible fiber matter not absorbed in the upper intestinal tract are fermented by the anaerobic microbial community of the colon, producing SCFAs (primarily acetate, propionate, and butyrate) and gas [9]. These SCFAs, of which butyrate is the best-studied, play a key role in maintaining gut homeostasis and epithelial integrity [10]. Butyrate serves as a principal energy source for colonocytes, regulates host gene expression via histone deacetylase inhibition [11]. This latter role has led to both an increase in IL-10 production and an anti-inflammatory role by inhibiting NF-κB signaling [12]. We hypothesized that children with a history of HAEC would have altered fecal SCFA composition, as well as disequilibrium of SCFA-producing microbiota.

This study was designed to evaluate the fecal SCFA makeup of patients who had completed definitive surgical treatment for HD. We compared 9 children with a history of at least one episode of HAEC to 9 children with no episodes of HAEC. We first performed gas chromatography–mass spectrometry (GC-MS) based quantification of fecal SCFA contents, and then compared this to 16S-based microbiota analysis of the same samples to elucidate the relationship between an altered microbiome and SCFA production.

## 2. Methods

### 2.1. Patient selection

This was a multi-institution study of children younger than 18 years of age who had completed definitive pull-through surgical treatment for a histopathological diagnosis of HD. Twenty children were enrolled by four member institutions of the HAEC Collaborative Research Group (HCRG): Cedars-Sinai Medical Center (CSMC), Los Angeles, CA; Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Children's Hospital Los Angeles, Los Angeles, CA; Children's Hospital of Oakland, Oakland, CA. Of these, 10 children had a history of HD without documented enterocolitis, and 10 had at least one episode of HAEC as defined by Pastor et al. criteria [13]. Two of these 20 children were excluded from analysis owing to the presence of a diverting ileostomy and active HAEC at the time of stool collection, respectively. Thus, a total of 9 HD and 9 HAEC samples were analyzed. Medical records were reviewed and parent interview was performed using standardized questionnaires. Data collected included demographics, medical/surgical history, diet in the first year of life, medications including antibiotics, probiotic use, and enterocolitis symptoms. This study was approved by the CSMC institutional review board (IRB no. CR00008054) as a multicenter study, as well as individual approval by all participating sites, and the University of Michigan IRB (HUM00079878). Stool was collected within one week of enrollment and immediately snap-frozen at −80 °C in air-tight containers to prevent the loss of volatile SCFAs. Frozen sample aliquots were shipped to the University of Michigan for SCFA analysis and to CSMC for bacterial DNA isolation.

### 2.2. Fecal SCFA analysis

Sample extraction was performed using aqueous extraction solvent containing 3% 1 M HCl (v/v) and isotope-labeled internal standards (d7-buytric acid and d11-hexanoic acid). Samples were then homogenized and centrifuged. Supernatants were transferred to new Eppendorf tubes for extraction by diethyl ether. After layer separation, the upper layer was transferred to an autosampler vial for GC-MS analysis. GC (Agilent 6890, Wilmington, DE) separation was performed using a ZB-Wax plus column, 0.25 μm × 0.25 mm × 30 m (Phenomenex, Torrance, CA). A single quadrupole mass spectrometer (Agilent, 5973 inert MSD) was used to identify and quantify SCFAs using Agilent Masshunter software, version B.06 [14]. Absolute quantities of SCFAs were normalized to sample mass.

### 2.3. Bacterial DNA isolation and amplicon preparation

Fecal samples were suspended in 50 mM Tris buffer (pH 7.5) containing 1 mM EDTA, 0.2% β-mercaptoethanol (Sigma) and 1000 U/ml of lyticase (Sigma). The mix was incubated at 37 °C for 30 min, and DNA was isolated using QIAamp DNA Stool Mini Kit (Qiagen). Bacterial 16S rRNA gene amplicons spanning variable regions 1–4 were generated in 20 μL PCR reactions using 20 ng of fecal DNA with 25 cycles using high-fidelity Phusion Polymerase (New England Biolabs, Beverly, MA) at 52.7 °C annealing using with degenerate 8F (AGAGTTTGATCM TGGCTCAG) and R357 (CTGCTGCCTYCCGTA) primers. All PCR reactions were purified using Agencourt AmPure Magnetic Beads (Beckman), resuspended in 20 μL of nuclease-free water and quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA).

### 2.4. 16S sequencing

Paired-end adapters with unique indexes were ligated to 100 ng of 16S amplicons and used to generate Ion Torrent sequencing libraries using the Ion Xpress Library Kit (Life Technologies, Carlsbad, CA). Library enrichment was performed with 10 cycles of PCR and purified using Agencourt Ampure Magnetic Beads (Beckman). All libraries were subjected to quality control using qPCR, DNA 1000 Bioanalyzer (Agilent), and Qubit (Life Technologies, Carlsbad, CA). Pooled libraries were assayed on Agilent Bioanalyzer (Santa Clara, CA) to check final sizing and KAPA Biosciences qPCR for quantitation. 16S samples were multiplexed and sequenced on the Ion Torrent PGM on a 318 chip with 400 bp chemistry. 250 single-end sequencing-by-synthesis was performed using the MiSeq Illumina sequencer (Illumina, San Diego, CA). Torrent reads shorter than 200 bp, or not containing the designed 16S primers (>2 nt mismatches) were discarded. 300 bp sequences of remaining high-quality reads were aligned to the Greengenes reference database (February 2011 release) using BLAST v2.2.22 in QIIME v1.5 wrapper [15] with an identity percentage ≥97% to select the operational taxonomic units (OTUs). Taxonomy for each sequence was assigned using the Ribosomal Database Project (RDP) classifier v2.2.

### 2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). Comparison between two groups used the two-tailed, unpaired Student's T-test. All results are expressed as mean ± standard deviation unless otherwise specified. A p value of $<0.05$ was considered significant.

## 3. Results

### 3.1. Patient characteristics

The two groups (HD and HAEC) each included 9 patients, with an equal distribution of 8 males and 1 female (Table 1). Median age was 2.7 years (3 months to 8 years) for all children, 2.3 years for the HAEC group, and 3.5 years for the HD group ($p = 0.40$). There were no significant differences in length of aganglionosis, non-HAEC complications, early feeding type, or probiotics received. While no children in the HD group received antibiotics within 2 months of stool collection, three patients in the HAEC group had received antibiotics (two for prior HAEC treatment, and one for sickle cell prophylaxis). Trisomy 21 was present in one patient in the HD group and two patients in the HAEC group.

**Table 1**
Patient demographic characteristics.

| Demographics | HD | HAEC |
|---|---|---|
| N | 9 | 9 |
| Male | 8 | 8 |
| Median age (years) | 2.3 | 3.5 |
| Trisomy 21 | 1 | 2 |
| **HD characteristics** | | |
| Rectosigmoid transition zone | 8 | 8 |
| Postop complications[a] | 0 | 0 |
| **Diet**[b] | | |
| Breast milk | 5 | 5 |
| Formula | 7 | 7 |
| **Medications** | | |
| Probiotics[c] | 4 | 3 |
| Antibiotics[d] | 0 | 3 |

[a] Complications occurring within 30 days of pull-through operation, not including HAEC.
[b] Diet in the first year of life.
[c] Probiotics included *Lactobacillus* spp. and *Bifidobacterium* spp.
[d] Received within 2 months prior to stool collection.

### 3.2. Fecal SCFA composition

Total fecal SCFA composition of children with a history of HAEC was 4-fold lower than that of HD patients without a history of HAEC (56.98 ± 42.30 mM/g stool vs. 255.20 ± 259.70 mM/g; p = 0.038; Fig. 1A). The composition of individual SCFAs was then secondarily examined and was markedly altered in HAEC children. The concentration of each SCFA detected by GC/MS in each group is shown in Table 2. Fecal samples from the HAEC group had a significantly lower concentration of acetate (34.04 ± 40.40 μM/g vs. 224.59 ± 266.12 μM/g; p = 0.049) compared to the HD group, without a significant change in the absolute concentration of other detected SCFAs. When comparing pooled SCFA compositions (expressed as % of total SCFAs) between the two groups, a significant reduction in the proportion of acetate (46.70 ± 29.89% vs. 78.66 ± 19.08%; p = 0.016) and an increase in the proportion of butyrate (24.99 ± 15.64% vs. 6.75 ± 7.40%; p = 0.006; Fig. 1B) was found in samples from the HAEC group versus the HD group. While individual SCFA composition varied within the HD group, a preponderance of acetate was present, followed by propionate and butyrate (Fig. 1C). In children with a history of HAEC, however, the fecal SCFA profile became markedly more varied between individuals (Fig. 1D), reflecting a loss of acetate and increased proportion of other SCFAs.

### 3.3. SCFA-producing bacterial analysis

A detailed analysis of the broad changes in the fecal microbiome using 16S analyses has been reported [7]; however, a subanalysis of this dataset was used in the present paper to examine the anaerobic microbiota which produce fecal SCFAs. Therefore, we compared the populations of SCFA-producing bacterial strains between HAEC and HD group fecal samples. As the full fecal microbiome was dominated by non-SCFA-producing bacteria, we identified genera that are known to have butyrogenic [16] and acetogenic [17] strains. While no genus-level changes reached statistical significance (Table 3), 10 out of 12 butyrogenic genera, identified by 16S sequencing, demonstrated

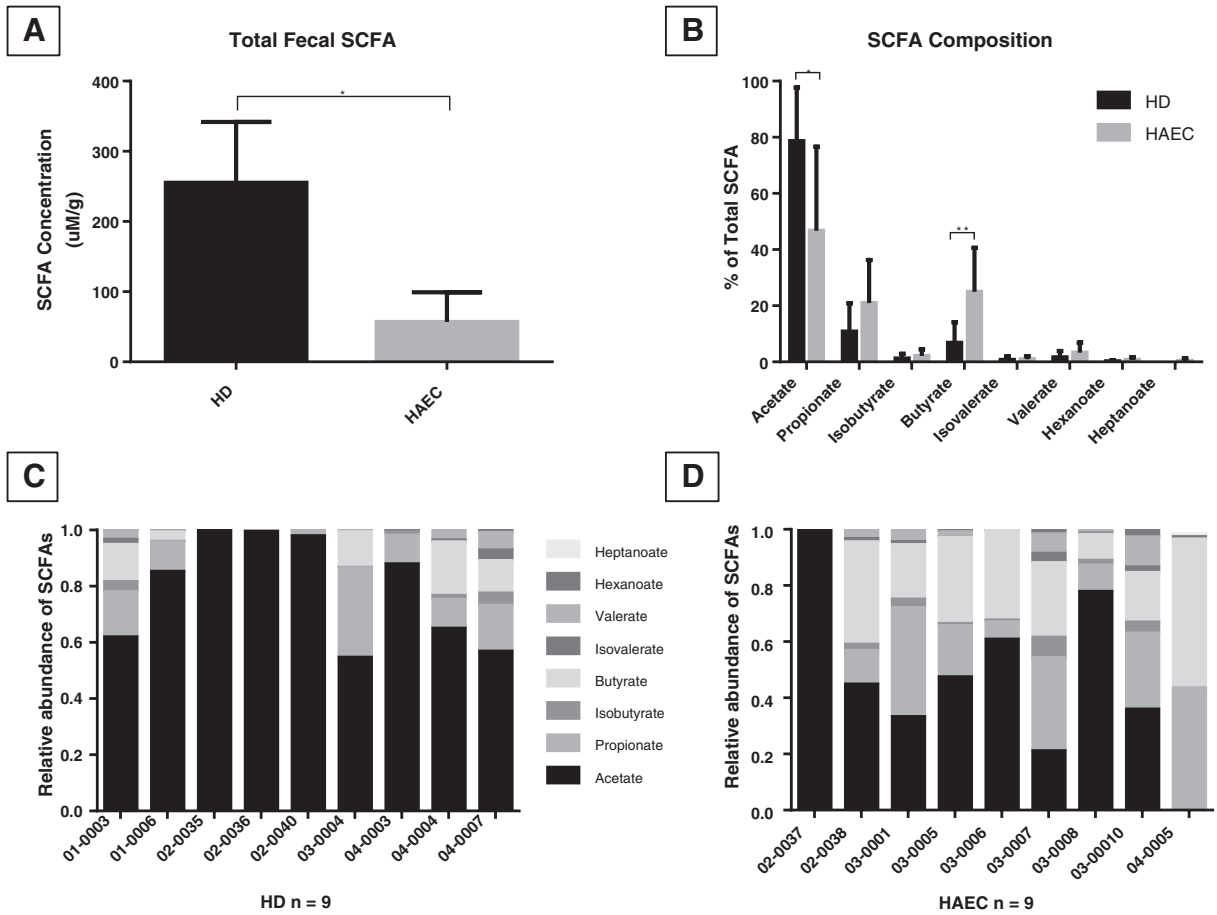


**Fig. 1.** (A) Fecal SCFA concentration was significantly reduced in HAEC children versus those with HD without HAEC. (B) Individual SCFAs expressed as % of total SCFAs from HD children with a history of HAEC and those with no history of HAEC. (C) Histograms demonstrating the fecal SCFA composition of individual subjects with HD and HAEC (D). Individual subject numbers are labeled on the X axis and expressed as relative SCFA abundance per each subject. *p < 0.05, *p < 0.01.

**Table 2**
SCFA concentration in feces (μM/g).

| | HD (mean ± SD; n = 9) | HAEC (mean ± SD; n = 9) | p value |
|---|---|---|---|
| **SCFAs (all)** | **255.20 ± 259.70** | **56.98 ± 42.30** | **0.038*** |
| Acetate | 224.59 ± 266.12 | 34.04 ± 40.40 | 0.049* |
| Propionate | 16.93 ± 16.07 | 7.54 ± 5.81 | 0.119 |
| Butyrate | 9.35 ± 8.33 | 11.98 ± 12.82 | 0.613 |
| Isobutyrate | 1.35 ± 1.76 | 0.99 ± 0.98 | 0.605 |
| Isovalerate | 0.77 ± 1.20 | 0.45 ± 0.53 | 0.481 |
| Valerate | 1.90 ± 2.08 | 1.65 ± 2.03 | 0.800 |
| Hexanoate | 0.25 ± 0.32 | 0.30 ± 0.51 | 0.827 |
| Heptanoate | 0.01 ± 0.04 | 0.03 ± 0.05 | 0.359 |

SCFA = short chain fatty acid.
* $p < 0.05$.

elevated OTUs (operational taxonomic units) with a mean increase of 640% in the HAEC group compared with the HD group (Fig. 2A). This mirrored the relative increase in butyrate found on SCFA analysis of HAEC samples. Interestingly, despite a decrease in the proportion of acetate in HAEC samples, 3 of 4 acetogenic genera had increased OTUs in the HAEC group, with a mean increase of 1710% (Fig. 2B).

## 4. Discussion

HAEC is a highly morbid, though poorly understood complication of HD. While a role for altered colonic microbiota has been suggested by prior studies [5–8,18], the mechanism by which changes in bacterial community characteristics might impact susceptibility to or influence the development of HAEC is not clear. In this study, we found an over 4-fold reduction in total fecal SCFA content in children with a history of HAEC compared to those who never had HAEC. This may suggest that the colonic environment associated with HAEC is associated with a microbial community that has reduced capacity to produce SCFAs. Given that SCFAs are critical nutrients in the maintenance of healthy colonic mucosa, the loss of SCFAs may adversely affect enterocyte homeostasis thereby contributing to HAEC development.

Specifically, acetate was found in this study to be the most markedly reduced among children with HAEC, although it has been less vigorously studied in the context of colitis pathophysiology. The SCFA butyrate is also a major source of energy for colonic epithelial cells and has both immunomodulatory and anti-inflammatory properties [19–21]. Interestingly, a reduction in butyrogenic bacteria has been shown in infectious and active inflammatory colitis versus healthy subjects, suggesting a role of intestinal dysbiosis and butyrate reduction in colitis development [22,23]. Butyrate has also been shown to ameliorate the impairment of mucosal immunity seen with total parenteral nutrition [24]. The trophic effect of mixed SCFAs (including acetate) has been shown to exceed that of butyrate alone [25], it is likely that these other SCFAs play an important role in colonic mucosal integrity.

**Table 3**
SCFA-producing bacterial genera in feces.

| | HD (mean[a] ± SD; n = 9) | HAEC (mean ± SD; n = 9) | p value |
|---|---|---|---|
| **Butyrogens (all)** | 4.9% ± 6.4% | 4.2% ± 3.6% | 0.783 |
| *Roseburia* | 4.0% ± 6.4% | 1.5% ± 1.9% | 0.282 |
| *Odoribacter* | 0.4% ± 0.9% | 1.0% ± 1.9% | 0.450 |
| *Faecalibacterium* | 0.2% ± 0.2% | 0.3% ± 0.5% | 0.499 |
| *Eubacterium* | 0.08% ± 0.2% | 0.4% ± 0.7% | 0.261 |
| *Subdoligranulum* | 0.05% ± 0.06% | 0.3% ± 0.6% | 0.221 |
| *Peptoniphilus* | 0.04% ± 0.1% | 0.001% ± 0.002% | 0.337 |
| *Coprococcus* | 0.03% ± 0.05% | 0.2% ± 0.3% | 0.135 |
| *Fusobacterium* | 0.01% ± 0.02% | 0.2% ± 0.7% | 0.354 |
| *Porphyromonas* | 0.01% ± 0.03% | 0.02% ± 0.05% | 0.750 |
| *Clostridium* | 0.008% ± 0.02% | 0.2% ± 0.5% | 0.303 |
| *Anaerotruncus* | 0.007% ± 0.01% | 0.01% ± 0.03% | 0.619 |
| *Megasphaera* | 0.002% ± 0.01% | 0.04% ± 0.1% | 0.337 |
| **Acetogens (all)** | 0.1% ± 0.2% | 0.7% ± 0.9% | 0.065 |
| *Eubacterium* | 0.08% ± 0.2% | 0.3% ± 0.7% | 0.261 |
| *Clostridium* | 0.008% ± 0.02% | 0.2% ± 0.5% | 0.303 |
| *Ruminococcus* | 0.003% ± 0.006% | 0.1% ± 0.2% | 0.053 |
| *Syntrophococcus* | 0.001% ± 0.003% | <0.0001% | 0.151 |

[a] Mean % of total operational taxonomic units (OTUs).

This study found a relative increase in the proportion of fecal butyrate in children with a history of HAEC, and that the net loss of fecal SCFAs found among these patients was primarily owing to a significant reduction in acetate. However, the absolute butyrate concentration was maintained, thereby making up a larger proportion of the remaining SCFA pool. The significant net loss of acetate may represent decreased acetate production via fermentation, or it may be the result of increased acetate absorption by the colonic mucosa versus increased acetate metabolism by colonic microbiota. Acetate is the most prevalent SCFA in the healthy colon [26]. Bacteria isolated from the human colon have been shown to utilize acetate for the production of butyrate [27]. This might provide an explanation for the reduction in acetate found in children with a history of HAEC. More metabolically active butyrogenic strains, as observed in our study, may have led to lower acetate levels in these patients, leading to a higher proportion of butyrate while decreasing the total concentration of SCFAs primarily via a reduction in remaining acetate.

While the etiology of the change in SCFA concentrations observed in this study is unknown, the relative increase in butyrate production may represent a compensatory mechanism to decrease the associated inflammatory state driven by other unknown factors. For example, epithelial inflammation may lead to elevated butyrate requirements by the colonic mucosa of children with a history of HAEC. This may drive the relative expansion of butyrogenic strains in these patients, which maintain butyrate concentrations, perhaps metabolizing acetate in this process. Future work to specifically examine the metabolic activity of the colonic microbiota and epithelium in children with HD might provide key insights into the pathogenesis of HAEC.

This is the first study to investigate SCFA changes in children with HD compared with those with a history of HAEC, Ward et al. performed a nuclear magnetic resonance (NMR)-based fecal metabolome analysis using a mouse model of HD [18]. This untargeted investigation found a significant reduction in formate, a minor SCFA product of bacterial fermentation, in endothelin receptor B knockout mice prior to onset of HAEC. However, no targeted SCFA metabolomic analysis was performed in this mouse study, and changes in other SCFAs were not characterized. We did not detect formate in the SCFA analysis which was not surprising, given that formate is not a major human fecal SCFA; although the parallel to the Ednrb-null mouse is noteworthy.

The types of SCFAs produced by bacterial fermentation are determined by the relative abundance of undigested carbohydrates, proteins, and amino acids in the colonic lumen [26]. We therefore used matched cohorts of patients with equal distributions of breast milk and formula feeding histories within the first year of life to minimize differences in nutrients on microbial community development. A more detailed nutrition assessment, quantifying caloric intake with protein:carbohydrate ratio at the time of sample collection was not performed, although this may have detected differences in oral intake between the two groups. Another variable which may impact SCFA production is gut motility – as shorter colonic transit has been shown to increase SCFA production [28]. While no patients had active HAEC at the time of sample collection, colonic transit time was not assessed in this study.

While probiotic prophylaxis has been shown to decrease the risk of other inflammatory diseases of the intestine such as necrotizing enterocolitis [29], such intervention has not been shown to reduce the risk of HAEC in children [30]. This study suggests that the altered microbiota seen with HAEC may mediate their influence on the colonic mucosa via dysfunctional SCFA metabolism. Future work will be required to better understand how the complex interplay between diet, colonic bacteria, and mucosal metabolism in the setting of HD leads to a unique SCFA profile in children with a history of HAEC. As butyrate enemas have shown promise in the treatment of colitis [26], a therapeutic

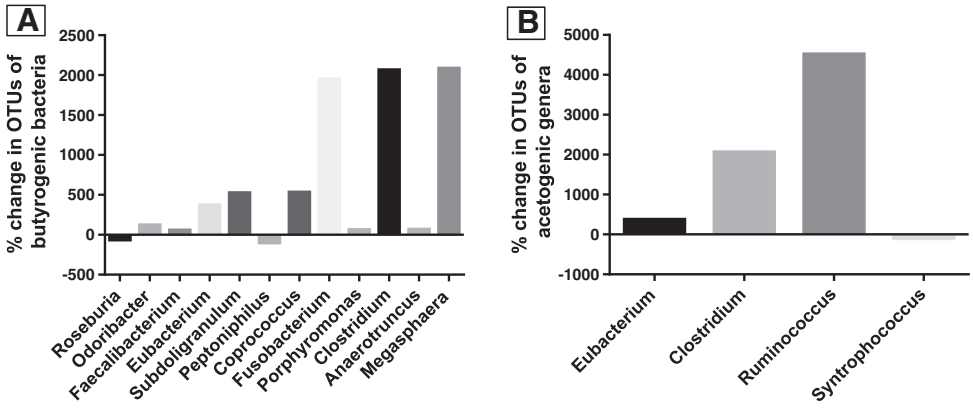


**Fig. 2.** Genus-level identification of fecal microbiota via 16s rRNA sequencing. Percent change in mean OTUs of (A) butyrogenic bacteria and (B) acetogenic bacteria in fecal samples from children with a history of HAEC versus HD children without HAEC.

role may exist for such therapy directed at normalization of the colonic SCFA profile in the prevention of HAEC, or perhaps by delivering substrate (nondigestible fibers) for increased butyrate production by this altered microbiome.

This study demonstrates a strong association between prior episodes of HAEC and an altered SCFA profile, however, it does not establish the initiating cause. It is possible that prior HAEC episodes lead to the altered microbiome and resultant SCFA changes, perhaps contributing to further episodes of HAEC. A prospective study of patients after surgical correction of HD without prior HAEC would address this question by comparing changes in the microbiome and SCFA profile between patients who do and do not later develop HAEC.

A limitation of this study is a lack of long-term antibiotic history, prior to the 2-month window before sample collection. Patients with a history of HAEC may have received several courses of antibiotics to treat prior HAEC episodes, which may have influenced the development of an altered colonic microbial community. Post-hoc analysis of SCFA profiles after exclusion of the three patients who had received antibiotics in the HAEC group demonstrated continued reduction in total SCFA content, as well as consistent changes in acetate and butyrate content. Another limitation of this study is the small sample size of each group. While this study is the first to date to investigate SCFA content and related microbiota in humans with HD, a more robustly powered study may have detected more significant changes in SCFA concentrations as well as genus-level microbial shifts. Additionally, this study evaluated fecal microbial changes as a proxy for direct measurement of colonic microbiota. Substantial variation has been reported between fecal and colonic mucosa-associated microbiomes [31]. Though more invasive specimen collection would be required for colonic microbiome and metabolome assessment, such an investigation might reveal more physiologically relevant data. Finally, bacterial 16S sequencing in this study did not allow identification beyond the genus level. The identification of specific species with known capacity to produce SCFAs was therefore not performed, but rather the genera including these strains were identified. Future work might be directed at complete genome sequencing to confirm the ability of specific strains to produce butyrate, acetate, and other SCFAs.

In summary, children with HAEC history were found to have markedly reduced fecal SCFAs, and an altered SCFA profile. These findings suggest a complex interplay between altered local environment and changes in intestinal microbiota, which may influence the pathogenesis of HAEC.

## Author contributions

FR Demehri, Z Cheng: Conceptualization, sample processing, data interpretation, and manuscript preparation.

C Ruan, V Funari: Sample processing, data interpretation, and manuscript preparation.

T Wester, A Nordenskjöld, A Kawaguchi, TT Hui, AL Granström: Conceptualization, specimen collection.

PK Frykman, DH Teitelbaum: Conceptualization, data interpretation, and manuscript preparation.

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## Appendix A. Discussions

Presented by Farokh R. Demehri, Ann Arbor, MI

UNIDENTIFIED SPEAKER This is really interesting. The thing that we worry about in the kids who have recurrent enterocolitis, we have always thought of it as a stasis problem. Which comes first? Do you have the altered microbiome because you have stasis, or do you have – which is the chicken and which is the egg here?

FAROKH DEMEHRI Right. That's a great question. I think this study is really a hypothesis-generating study because of these two groups there was only one patient in the enterocolitis group who demonstrated stricture and stasis and required a dilation. None of the others did, but you are right that is often thought to be a contributing factor. I think going forward we need to understand actually what is the motility of these patients and then what is the function of the bacteria that we are finding in these patients?

BRAD WARNER (St. Louis, MO) My question sort of gets to the metabolome and you focus on basically on short-chain fatty acids, but there are obviously a lot of other things that the bacteria are metabolizing to include amino acids and other things in carbohydrate metabolism that may play a role as well. If you focus specifically on short-chain fatty acids, which is an obvious first start – that is the fuel for the colonocytes. Have you looked at these other factors?

FAROKH DEMEHRI Thank you. That is an excellent question. Actually when we had these samples we had to decide are we going to do a targeted metabolomic analysis or untargeted. We decided to start with targeted because as you mentioned this is a good first step, but what we hope to do is an untargeted analysis and that might generate all kinds of other questions about not just what these bacteria are able to produce but also what the interplay is between the epithelium and the bacteria. Thank you.

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