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# INTESTINAL MICROBIOTA IN HIRSCHSPRUNG DISEASE

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**ABSTACT**

*Objectives*

To characterize the microbiota profiles of patients with Hirschsprung's disease (HD), and to evaluate this in relation to postoperative bowel function and the incidence of Hirschsprung-associated enterocolitis (HAEC).

*Methods*

All patients operated on for HD at our center between 1987-2011 were invited to answer questionnaires on bowel function and to participate in a clinical follow-up for laboratory investigations, including fecal DNA extraction, fecal calprotectin (FC), and brush border lactase (*LCT)* genotyping. The microbiota compositions of HD patients were compared to those of healthy controls aged 2-7 years.

*Results*

The microbiota composition of eligible HD patients ($n$=34; median age 12 (range, 3-25) years) differed from the healthy controls ($n$=141), showing decreased overall microbial richness ($p$<0.005). 77% had experienced HAEC. Normal maturation of the intestinal flora was not observed, but patients had a significantly increased abundance of Proteobacteria among other taxa ($p$<0.005) resulting in a reduced carbohydrate degradation potential, as predicted by the taxonomic composition. Genetic lactase deficiency was present in 17% and did not correlate with bowel symptoms. No patients reported active HAEC at the time of sampling and FC was within the normal range in all samples.

*Conclusion*

Patients with HD and HAEC had a significantly altered intestinal microbiome compared to healthy individuals, characterized by a lack of richness and pathologic expansions of taxa, particularly Enterobacteria and Bacilli. Further evaluation is needed to identify whether these observations are intrinsic to HD or secondary to the recurrent use of antibiotics during early childhood.

Keywords: Hirschsprung-associated enterocolitis; bowel function impairments; stool consistency; fecal calprotectin; LCT genotype

**What is known:**

- Pathophysiology of postoperative enterocolitis and bowel functional impairments in patients with Hirschsprung's disease (HD) are not fully known, but associated disturbances of intestinal microbiota have recently been reported
- Sustaining a balanced intestinal microbial community is critical for maintaining intestinal health

**What is new:**

- HD patients showed decreased intestinal microbial richness with expansion of Enterobacteria, which associated with a history of enterocolitis episodes and loose stools.
- Intestinal microbiota in HD patients did not undergo normal age-related maturation
- Pathologic alterations of the intestinal microbiota may contribute to postoperative bowel dysfunction in HD patients

## INTRODUCTION

Hirschsprung's disease (HD) is characterized by the absence of intrinsic parasympathetic ganglia (aganglionosis) in varying length of the bowel, resulting in functional obstruction.(1) The incidence is 1-2 per 10 000 births.(2,3). Despite resection of the aganglionic bowel segment during infancy in most cases,(4) significant post-operative morbidity commonly occurs due to Hirschsprung-associated enterocolitis (HAEC) (5-7) and other disturbances in bowel function. Although the etiology of these has remained unclear (6,8-10), a role dysbiosis of the gut microbiota has been recently proposed (11-13). Dysbiosis comprises a disruption of the normal microbiota composition and function, including a significant reduction in diversity and an abnormal expansion of new bacterial groups of commensal species.(13,14) Gut micronial dysbiosis has already been associated with a number of pathological conditions including inflammatory bowel disease (14,15) and a role in HAEC has been implicated (16,17).

Food-allergies, particularly cow's milk allergy, have also been associated with an increased prevalence of HAEC (6) and postoperative bowel symptoms in HD (18-20). However, connections between the *LCT* genotype C/C (21), that leads to lactase deficiency (22), and HAEC alongside other bowel symptoms in HD has not been explored. Moreover, no studies exist of fecal calprotectin (FC) levels, which are a sensitive marker of intestinal inflammation (23,24), among patients with HD.

This study aimed to characterize the microbiota profiles of patients with HD, and to evaluate the microbiota composition in relation to postoperative bowel symptoms including HAEC. To further understand the pathophysiological basis of functional gastrointestinal problems in HD, we investigated FC levels and prevalence of the *LCT* genotype C/C in these patients.

## METHODS

### *Patients*

All patients with HD treated at our tertiary referral center between 1987-2011 were identified from records and contacted by post by an independent investigator who had not been involved in their care between 2012-2013. They were invited to answer a detailed questionnaire concerning bowel symptoms and medications. On a voluntary basis and after written informed consent, they were also asked to participate on a clinical follow-up, which included giving blood samples for the genotyping of the brush border lactase *(LCT)* gene, and stool samples for DNA isolation and calprotectin measurement. Patient characteristics, operative treatment, hospital attendances for enterocolitis and medication history were obtained retrospectively from records. The ethics committee of the Helsinki University Hospital approved the research protocol.

### *Operative principles*

All patients had undergone endorectal pull-through (EPT) as the definitive surgical treatment. EPT was performed completely transanally or in combination with transabdominal colonic mobilization via laparotomy or laparoscopy. Transanal mucosectomy was commenced 5mm above the dentate line, proceeding to full-thickness dissection approximately 3 cm thereafter (25). Total colonic aganglionosis was managed by restorative proctocolectomy with a short J-pouch ileoanal anastomosis with a protective temporary ileostomy (26). The same surgical team of consultant pediatric colorectal surgeons operated and followed the patients up until adulthood.

*Definitions of clinical symptoms*

Soiling was defined as small amounts of fecal staining on underclothing, which defined to be frequent as occurring at least once a week. Enterocolitis was defined on an intention-to-treat basis in the presence of clinical symptoms including abdominal distension, diarrhoea and vomiting, and, in some cases, bloody stools and/or fever (27). Enterocolitis was considered recurrent or persistent, when symptoms lead to two or more successive treatment episodes. Normal defecation frequency was defined as stooling every other day to twice per day (28).

*Postoperative bowel function and medication*

Patients without major cognitive impairment, an antegrade continence enema (ACE) or enterostomy ($n$ = 28), answered questions regarding soiling, fecal accidents and constipation from the Rintala Bowel Function Score (BFS)(28) that were assessed with reference to the microbiota composition. Patients were enquired about their current use of antibiotics due to enterocolitis and medication for bowel function. Additionally, stool consistency (formed/loose or liquid) was enquired from all patients without an ACE or enterostomy ($n$ = 34; including 6 with cognitive impairment) or their caregivers.

*Fecal DNA extraction and sequencing*

The patients with an ACE ($n$ = 3; bowel emptying with washouts) and a proximal small bowel enterostomy ($n$ = 1) were excluded from the microbiome analysis due to the invalid fecal sample from dilutional effects. Fecal samples were collected and frozen at -20°C within 4h of collection and stored at -70°C until analysis**.** Bacterial DNA was extracted from the fecal samples using the repeated bead beating method,(29) and 16S rDNA amplicon sequencing was used to analyze the microbiota composition. The V3-V4 variable region was sequenced using Illumina HiSeq using

the forward primer CTACGGGNGGCWGCAG and the reverse primer GACTACHVGGGTATCTAATCC. However, the analysis in this study is based on V3 region only (see below).

*Sequence processing*

The DNA amplicon sequences were processed using the R package mare (30). Only the forward reads were analysed. The reads were trimmed to 150 bases and reads representing less than 0.001% of all unique reads were discarded as potentially erroneous. For taxonomic annotation, the reads were not clustered into operational taxonomic units (OTUs) but, instead, mapped to the Silva reference database for taxonomic annotation, as OTU clustering would cause unnecessary errors to the taxonomic annotation. After annotation, the abundances reads were summarised at different taxonomic levels. OTU clustering, using USEARCH (31), was done only for the richness analysis, as it is not possible to estimate richness based only on the number of unique reads or taxonomic classification. Furthermore, taxonomic classification of 16S reads at the species level is not reliable. Therefore, microbial richness was defined as the number of species-like OTUs (97% similarity) present in the sample.

*The analysis of carbohydrate-active enzyme levels and carbohydrate degradation potential*

To further evaluate the function of the gut microbiome, carbohydrate-active enzymes and carbohydrate degradation potential using the CAZy database (32). Carbohydrate active enzyme abundances were predicted based on the observed taxonomic profiles.

*Fecal calprotectin (FC)*

The level of FC was measured in a routine clinical laboratory using a quantitative, enzyme-linked immunoassay (PhiCal Test, Calpro AS, Oslo, Norway; NovaTec Immunodiagnostica, Dietzenbach, GmBH, Germany) in routine analyses of the central laboratory services of the

Helsinki University Hospital. A level of 100 micrograms/g was taken as the upper limit of normal.(15,33)

*LCT genotype*

For the lactase gene *LCT* genotype (LCT-13910), genomic DNA was extracted from blood samples and genotyped using standard methods.(34) The *LCT* genotype C/C results in lactase deficiency and lactose intolerance.(22)

*Healthy controls*

Of a previously collected cohort of 236 Finnish children aged 2-7 years without serious chronic illnesses (35), 141 children had donated fecal samples, which served as control data for intestinal microbiota composition. Two independent samples per healthy control were analysed, resulting in 282 control samples. The control samples were analysed using a phylogenetic microarray, HITChip. Additionally, a small subset of the control samples was sequenced using the same methods as with the patient samples. To remove potential technical bias, the microarray data were standardized using the sequenced control samples as the reference. The standardization was done as previously described (36).

*Statistics*

Statistical analyses were conducted in R, using the package mare, including tools from packages vegan,(37) metacoder,(38) sp,(39) MASS,(40) and nlme (41). Data was presented as frequencies or medians ± range unless otherwise stated. Taxa occurring in less than 30% of the samples, that is, taxa occurring in 10 samples or less, were excluded from the taxon-specific analyses. However, the full control data was derived from a microarray experiment and did not contain zeros, and, thus, the tests comparing the patients and the control had no omitted taxa. Analysis of

the microbiota composition and relative abundances of bacterial taxa were examined by age, stool consistency, history of antibiotic treatment, symptoms of HAEC, *LCT* genotype and FC as appropriate, and associations between them were tested using negative binomial models. All analyses, except the patient-control comparisons, were adjusted for patient age, level of aganglionosis, and a history of HAEC. If these models did not fit the data due to heteroscedasticity, general least squares models were used. $P < 0.005$ were considered significant.

## RESULTS

### *Patients*

Of 89 patients responding to the survey questionnaire, 48 patients (54%) participated in the clinical follow-up of whom 38 (43%) gave stool and 37 (41%) blood samples for laboratory analysis. The baseline patient characteristics and surgical details of participants are presented in **Table 1**. After excluding patients with ACE (n=3) or small bowel enterostomy (n=1), 34 stool samples remained for the microbiota analysis, including 28 patients without major cognitive impairment who had also participated in the functional outcomes survey.

### *Bowel functional outcomes*

Any fecal soiling was reported by 50% ($n$ = 19/28), and in 33% it occurred frequently. Stooling frequency was abnormally increased in 36% ($n$ = 10/28), and 56% ($n$ = 19/34) experienced loose or liquid stools. No participants reported constipation. Six patients ($n$ = 6/28; 21%) currently used medication for bowel function, including loperamide ($n$ = 4), psyllium ($n$ = 1) and

macrogols combined with weekly enema of docusate sodium and sorbitol solution (*n* = 1) due to frequent soiling.

*Hirschsprungs' Associated Enterocolitis (HAEC)*

Two patients (*n* = 2/34; 6%) had experienced at least one episode of enterocolitis before definitive surgery, 77% of patients (*n* = 26/34) had experienced at least 1 episode after definitive surgery, and 53% (*n* = 18/34) had a history of recurrent or persistent HAEC. All patients with recurrent HAEC had received multiple courses of oral antibiotics, and 83% of them (*n* = 15/18) had previously or currently used prophylactic oral antibiotics long-term. At the time of the survey, two patients (*n* = 2/34; 6%) were currently taking prophylactic oral metronidazole, but none reported having HAEC at the time. Additionally, four patients (12%) were taking regular probiotics.

*Fecal calprotectin (FC) level*

The median FC was 9 (range 0-65) ug/g) among patients at the time of sampling and therefore within the normal range.

*LCT genotype*

Blood samples were available for *LCT* genotyping from 35/38 patients (1 refusal, 2 missing samples). The *LCT* genotype C/C was detected in six participants (*n* = 6/35; 17%). No associations were found between the C/C genotype and HAEC, fecal soiling, increased stooling frequency or abnormal stool consistency ($p < 0.05$ for all).

*Intestinal microbiota composition*

Compared to healthy controls, patients with HD had significantly increased relative abundances of *Escherichia, Pseudomonas, Dialister, Actinomyces,* Bacilli, and *Prevotella* (shown in red in **Figure 1**) and decreased abundances of most normally abundant taxa, including *Bacteroidales, Ruminococcaceae, and Lachnospiraceae* (shown in blue in **Figure 1**; $p < 0.005$ (FDR-corrected *p*-value <0.01 for all). The levels of major bacteria against different variables are listed in the supplementary tables (**Supplemental Digital Content 1-4, http://links.lww.com/MPG/B359, http://links.lww.com/MPG/B360, http://links.lww.com/MPG/B361, http://links.lww.com/MPG/B362)**. Patients with HD had a predicted lower abundances of carbohydrate-active enzymes compared to controls, based on the taxonomic data ($p < 0.005$).

*Effects of age on microbiota composition*

Among patients with HD, the microbiota composition showed some changes with age: the abundance of *Christensenella* decreased, and the abundance of Clostridia, *Asaccharobacter*, *Collinsella* and *Lactococcus* increased (**Supplemental Digital Content 2, http://links.lww.com/MPG/B360**); $p < 0.005$), but the DNA richness stayed constant up to the age of 25 years ($p = 0.72$).

*Intestinal microbiota and recurrent enterocolitis*

Patients with a history of recurrent HAEC had a significantly lower microbial richness (mean 112 OTUs) than the patients without a history of recurrent HAEC (mean 150 OTUs); $p = 0.004$). Abundances of Proteobacteria and *Lactobacillus* were significantly increased, and abundances of Clostridia and *Prevotella* were significantly decreased in patients with a history of recurrent HAEC ($p < 0.005$ for all**; Figure 2)** Moreover, regarding some taxa, the patients with a history of

recurrent HAEC had significantly differing abundances compared both to healthy controls and patients without HAEC ($p < 0.05$); *Lactococcus* and *Escherichia* were dramatically increased among the patients with HAEC, but only modestly in the patients without HAEC ($p < 0.05$). Conversely, the abundance of *Oscillospira* and *Holdemania* were significantly decreased in the patients with a history of HAEC compared to those without it ($p < 0.05$). Furthermore, a few taxa, such as *Roseburia,* were equally low in both patient groups compared to the healthy controls (**Figure 3**). The predicted enzyme-levels for carbohydrate degradation were decreased ($p < 0.005$). However, the microbiota compositions of both of these patient groups clearly differed from the controls, and we acknowledged that the changes of the microbiota in the patients with a history of HAEC are closely associated to the use of oral antibiotics (**Supplemental Digital Content 5, http://links.lww.com/MPG/B363**).

*Intestinal microbiota and bowel functional impairment*

Patients with normal stool consistency had a lower abundance of Proteobacteria and higher abundances of Actinomyces compared to those with abnormal stool consistency ($p < 0.005$) (**Supplemental Digital Content 6, http://links.lww.com/MPG/B364**). Additionally, patients with frequent soiling had decreased abundance of Clostridia in relation to controls ($p < 0.005$) No associations were found between increased stooling frequency and the microbiota composition ($p > 0.005$).

## DISCUSSION

Antibiotic usage, which is common in patients with HD, can modify the microbiota composition significantly (13,14). In children, a single course of oral antibiotics has shown to induce long-term, but largely reversible changes in the microbiota, including decrease in the abundances of

Firmicutes and Actinobacteria and increase in the abundances of Bacteroidetes and Proteobacteria (13,14). Established dysbiosis from repeated courses of oral antibiotics can lead to permanent disturbances in the metabolic and immunological health (13,42). In this study, all patients with a history of recurrent HAEC had taken multiple courses of oral antibiotics during childhood, and most also had a history of taking long-term prophylactic antibiotics. Another important finding was that the HD patients had an immature microbiome composition, as indicated by decreased microbial richness and high abundances of Bacilli and Enterobacteria. These bacteria are normally present at high abundance in infants, but not in adults (43). The immature type of microbiota composition could result from HD itself (44), antibiotics or dietary alterations. As microbial richness is essential for the stability and resilience of the intestinal microbiota (45,46), the lack of maturation and loss of richness found in the HD patients could increase the vulnerability of the gut microbiome to colonization by pathogenic bacteria and permit pathological outgrowth of selected taxa (45,46). Our previous work has shown that the tendency to HAEC symptoms decreases over time (47). Therefore, most antibiotic treatments are received by our patients during early childhood around the time that normal microbiome maturation should be taking place.

Among patients with recurrent HAEC, particularly the expansion of Proteobacteria was an important finding. Proteobacteria have been previously implicated in the pathogenesis of HAEC (11,16) and other inflammatory and diarrhoeal gastrointestinal diseases including IBD (48-52). Proteobacteria are Gram-negative opportunistic pathogens that produce potent inflammatory compounds, lipopolysaccharides (48). In healthy individuals, Proteobacteria comprise only a small proportion (1-2%) of the microbiota, and expanded abundances have been suggested to be a diagnostic microbial signature of epithelial dysfunction, dysbiosis and risk for disease

development (48,49,50). Consistent with this finding, patients with loose stools at the time of survey were also found to have increased abundances of Proteobacteria in their stool. Furthermore, the patients with frequent fecal soiling had less Clostridia in their stool. However, although transit time is known to determine the gut microbial habitat as it affects the nutrient and water absorption along the intestinal wall (51,52), we did not find an association between microbiota composition or increased stooling frequency. This could be due to the low sample size of our cohort and large variation caused by other factors, such as antibiotic use history.

The anaerobic bacteria of the colon normally ferment complex dietary carbohydrates to short chain fatty acids (SCFAs) (49). SCFAs may have a marked nutritional and functional value to the gut epithelium (49), and alterations in their levels may therefore impact on the bowel function. In this study, HD patients had reduced levels of carbohydrate-active enzymes in relation to controls, which may be a consequence of the low abundances of Clostridia and Bacteroidia, which are the dominant carbohydrate-degrading taxa. The expansion of Proteobacteria observed is likely to be another important etiologic factor, as Proteobacteria participate very little in the digestion and fermentation of carbohydrates. The degradation of complex carbohydrates is one of the most important digestive functions that the microbiota perform for the human host, who intrinsically has a very limited repertoire of carbohydrate-active enzymes. This can serve to explain why patients with HD commonly report gastrointestinal disturbances akin to lactose intolerance, although the actual prevalence of lactase deficiency (genotype C/C) was only 17% in our study and no correlation with bowel symptoms was found. No patients reported HAEC or active bowel inflammation at the time of calprotectin measurement, and consistent with this FC levels were within the normal range in all cases.

A limitation of this study is clearly the small number of patients. Also, it cannot be shown from the data whether HD itself causes intrinsic disturbances to the gut microbiome because most patients in this series had received antibiotics at the time of study. Additionally, we found that the patients without a history of recurrent HAEC had a microbiota that in some respects resembled more that of healthy controls compared to the patients with a history of recurrent HAEC. Further prospective evaluation is needed to establish the natural history and maturation of the gut microbiome in HD, the impact of this on HAEC and to re-evaluate the rationality of antibiotic treatment and prophylaxis of HAEC.

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FIGURE AND TABLE LEGENDS:

**Figure 1.** Comparison of the microbiota compositions of the patients (n=34) and the controls (n=141). The colours represent the strength of the association: blue colour indicates higher in controls and red higher abundance in patients. The size of the nodes represents the relative abundance of the taxonomic group.

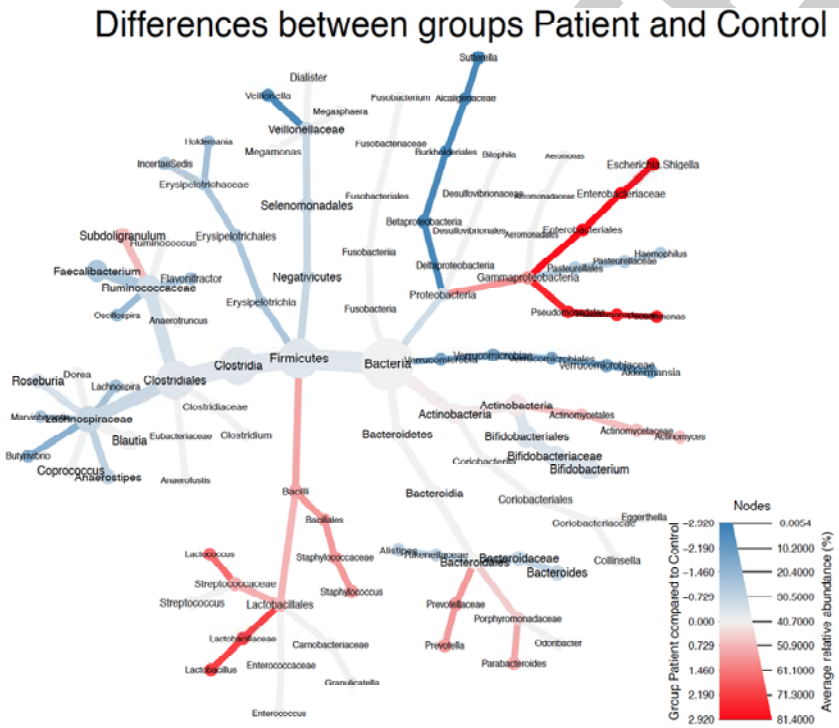

**Figure 2.** Comparison of microbiota compositions between patients with (n=18; Group 2 in the figure) and without a history of recurrent Hirschsprung-associated enterocolitis (HAEC; n=16; Group 1 in the figure) presented as a heat tree. Significantly ($p<0.005$) increased relative abundances of the bacteria in the HAEC group are shown as red, and significantly decreased relative abundances as blue.

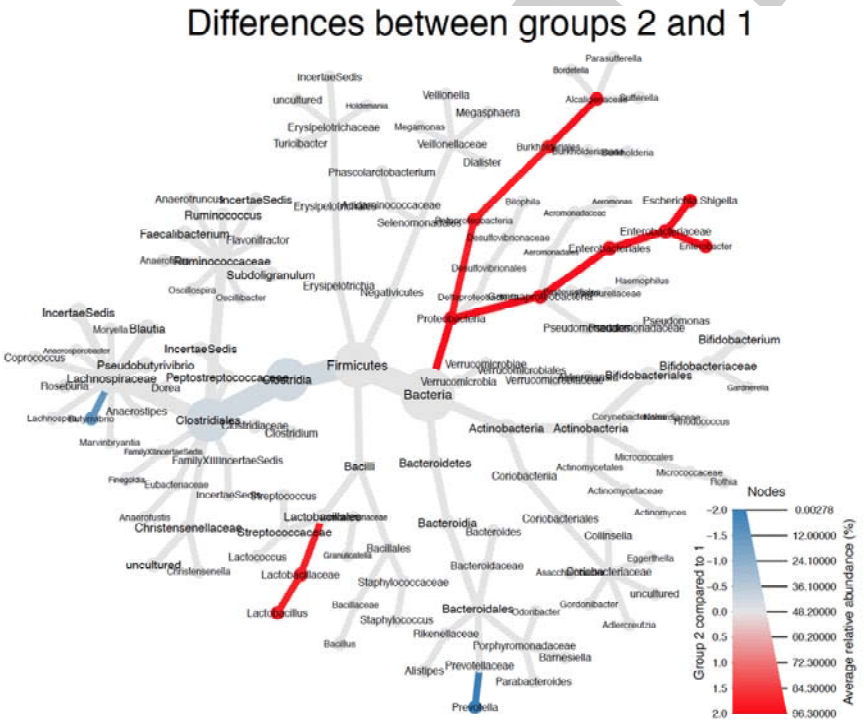

**Figure 3.** Significant (p<0.005) differences in the relative abundance of bacterial taxa between healthy controls and patients with (n=18) and without a history of recurrent Hirschsprung-associated enterocolitis (n=16,HAEC).

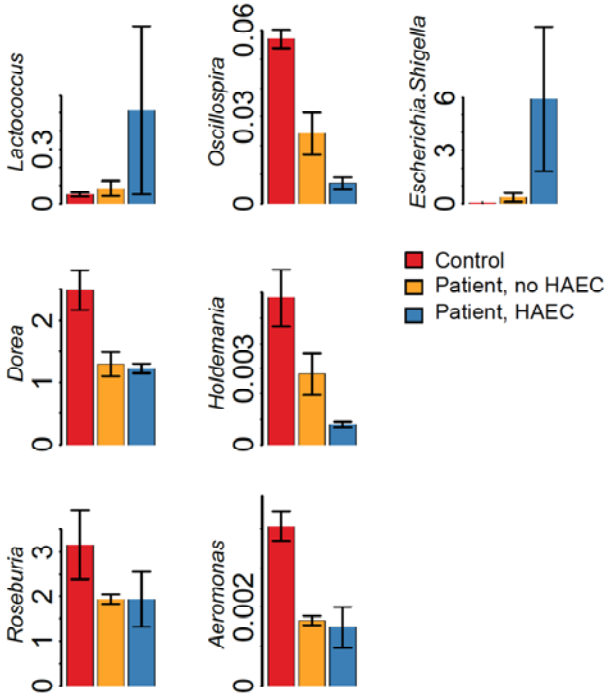

**Table 1.** Patient characteristics of the eligible participants for fecal microbiome analysis (n=34).

| | |
|---|---|
| Age (years) | 12 (3-25) |
| Sex (M:F) | 28:6 (5:1) |
| Age at surgery (weeks) | 5 (1-257) |
| HD in the family (%) | 3 (9) |
| Level of aganglionosis (%) | |
| Rectosigmoid | 30 (88) |
| Long segment | 3 (9) |
| Total colon | 1 (3) |
| An associated syndrome * | 6 (18) |

Data are frequencies (percentage) or medians (range).

* Down syndrome (*n* = 3), Mowat-Wilson syndrome (*n* = 2), cartilage-hair-hypoplasia (*n* = 1).