



Fecal Microbial Profiling of Young Hirschsprung Disease Children After Pull-Through Operation

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Abstract

Introduction: This study aimed to characterize and compare the fecal microbial profiles between post pull-through Hirschsprung disease patients and healthy children aged younger than five years.

Method: Fresh fecal samples were collected from 10 post pull-through Hirschsprung disease patients and age range-matched 10 healthy children. Bacterial DNA obtained from fecal samples were analyzed using 16S rRNA Illumina MiSeq platform.

Results: Our findings demonstrated the significantly increased Firmicutes in Hirschsprung disease group compared to the healthy group (corrected p-value=0.007) at the phylum level. In addition, the Firmicutes/Bacteroidetes ratio in Hirschsprung disease group was 4.8 times higher than that of its control counterpart. Bacilli were also significantly increased (corrected p-value=0.004), while Erysipelotrichi and Actinobacteria were significantly decreased in Hirschsprung disease patients (corrected p-value=0.04 and 0.03, respectively) at the class level. Moreover, functional analysis demonstrated that 20 enzymes and 18 Kyoto Encyclopedia of Genes and Genomes pathways were significantly different between groups (corrected p-value<0.01).

Conclusion: A distinct dysbiosis even when the aganglionic segment had already been removed was remarkably evident in young children with Hirschsprung disease, with a significant increase in Firmicutes and a nearly five-fold increase in proportion of Firmicutes/Bacteroidetes which may potentially be employed as the dysbiosis-related biological indicator.

Keywords: Hirschsprung; Microbiome; Enterocolitis; Child

Introduction

The fundamental principle of Hirschsprung disease (HD) treatment is the removal of the aganglionic segment replacing with the ganglionic bowel while preserving the sphincter function [1]. However, after surgery, the patients still have a chance of Hirschsprung associated enterocolitis (HAEC) which ranges in severity from mild to life-threatening. The incidence of HAEC is approximately 17.3%- 35% [1-4]. The exact pathogenesis of HAEC remains unclear even though several causes have been proposed, including intestinal barrier dysfunction, abnormal innate immune response, and dysbiosis [5].

The microbiome refers to the full complement of microbiota, their genes, and genomes in a specific environment [6]. The human body harbors

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approximately 10-fold individual more microbes than our somatic and germ cells [7]. Most inhabit our gastrointestinal tract, which is estimated to contain 10 to 100 trillion microbial cells and over 1000 species [8]. The gut microbiota is a complex community of microbes. Most reside in the distal ileum and colon that possess the optimal niche with microbial nutrients (e.g., essential amino acids, vitamins) and indigestible compounds (e.g., plant polysaccharides) [9]. Although some microbes, bacteria, or viruses are reminiscent of pathogens, most intestinal microorganisms provide many benefits, including strengthening the gut barrier integrity, producing nutrients, promoting pathogen interception, and modulating host immunity [9,10]. A healthy intestinal environment is characterized by a diverse and abundant microbiota dominated by members of Bacteroidetes, Firmicutes, and Actinobacteria. In addition, other typical features include an intact mucosal barrier and high levels of short chain fatty acid (SCFA) production [11]. These balancing mechanisms can be disturbed as a result of an altered microbial composition and function or dysbiosis, often characterized by altering microbial community and its function, and disruption of mucus and epithelial barriers [12]. Dysbiosis is associated with many gastrointestinal disorders including HD [12-15].

In HD studies, the culture-dependent method has been used to show the association between HAEC and *Clostridium difficile* and rotavirus [16,17]. Unfortunately, only 1% of microbes can be cultured in laboratory conditions and this method cannot clarify the complexity of microbiomes and anaerobic bacteria. Therefore, many studies have employed other non-culture microbiome technologies, including polymerase chain reaction (PCR), amplified ribosomal DNA restriction analysis (ARDRA) and nucleotide sequencing to increase sensitivity and specificity [18-20]. Culture-independent studies in HD patients began around 2009 using a quantitative real-time PCR assay to quantify *Bifidobacterium* and *Lactobacillus* in feces of HD patients with or without HAEC and those of normal children [18]. Next-generation sequencing (NGS) or second-generation sequencing has been increasingly used to investigate microbial community in HD. The results demonstrated pathogenesis mechanisms and determined the relationships between the molecular biological patterns in HD and HAEC, suggesting that HAEC is more likely to be caused by dysbiosis rather than a single pathogen. Despite the fact that previous studies demonstrated the microbial differences between HD and normal children, results varied across different studies as microbial profiles can be significantly affected by age, birth route, geography, diet and drugs [13,21-23]. Therefore, we aimed to investigate the fecal microbial communities between post pull-through HD patients and normal children, with less than five years of age and similar ethnicity and geography.

Material and Methods

Patient recruitment

In this cross-sectional study, after providing information and receiving the written consent form, 10 Thai HD children (0-5 years old) who underwent the pull-through operation and followed up at Srinagarind Hospital, Khon Kaen University during October 2019 - September 2020 were enrolled. Ten Thai healthy children (0-5 years old) who were not diagnosed with gastrointestinal problems were included. All participants resided in the same geographical area. The diagnosis of HD was confirmed by histopathology. The location of the pulled-down ganglionic portion creating anastomosis was also confirmed by histopathology. Metadata collected in the current study included age, sex, weight, birth route, current diet, medical and surgical history, antibiotics, probiotic use, history of enterocolitis, and current problems. This study was approved by the Khon Kaen University Ethics Committee for Human Research (HE621496) and the written consent of the parents of all participants has been obtained and the blank consent form is in the supplemental file. Fresh stool was collected and stored frozen (-80 °C) until microbiome analysis.

Bacterial DNA extraction, amplicon preparation, and 16S rRNA gene sequencing

Bacterial DNA extraction was conducted using MoBio PowerSoil DNA isolation kit to isolate bacterial genomic DNA according to the manufacturer's protocol. A total weight of approximately 250 mg was transferred to the PowerBead Tubes followed by the addition of 750 µL of bead solution and 60 µL of solution C1 containing sodium dodecylsulphate (SDS) prior to homogenization and cell lysis. The supernatant was transferred to a clean 2 ml collection tube and 250 µL of solution C2 was added as lysis buffer. After the mixture was incubated at 4°C for 5 minutes and centrifuged, 200 µL of solution C3 was added to terminate decomposition. Then the supernatant was transferred into a clean collecting tube and 1,200 µL of solution C4 added for binding the DNA to a spin filter. After centrifugation at 10,000 x g, 500 µL of washing solution C5 was added followed by centrifugation at 10,000 x g for 30 seconds. Then, 100 µL of the elution solution C6 was added to the center of the white filter membrane and centrifuged at room temperature for 30 seconds at 10,000 x g. For quantification of the DNA extracted from different samples, a spectrophotometer (Nanodrop) was used with 1.5% agarose gel electrophoresis for visualization. Amplification and sequencing of the V1-V2 region were conducted. In brief, 7.5 µL of genomic DNA from fecal samples were amplified using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The polymerase chain reaction (PCR) was performed using a thermocycler (T100TM Thermal Cycler, Bio-Rad) and Hotstar Master

Mix (Qiagen, Germany). The PCR cycling conditions were as follows: initial denaturation at 95°C for 3 min; 25 cycles of denaturation at 95°C for 30 s; annealing at 55°C for 30 s; extension at 72°C for 30 s and the final extension step at 72°C for 5 min. The negative control (DNase free water) was applied in the DNA extraction and 16S amplification steps. Sequencing was performed on the Illumina MiSeq platform (Macrogen, Korea), with read length of 301 base pair, paired-end. Following standard quality control and demultiplexing, the reads were processed using the QIIME2™ version 2017.6.0 [24]. First, paired-end reads were joined, and size selected to reduce non-specific amplification. These reads were then grouped into operational taxonomic units (OTUs) based on sequence similarity using the Greengenes version 13.5 database and classified at $\geq 97\%$ identity of reads [25]. All relevant data can be found in Supporting information.

Statistical analysis

Microbiome analysis was performed using MicrobiomeAnalyst [26,27]. Data normalization was performed, and data scaling was using total sum scaling. The taxa abundance will be explored as relative abundance by stack bar chart. The differential abundance analysis was performed by edgeR algorithm with adjusted p-value cutoff equal or less than 0.05 for statistically significant. Clinical data analysis was conducted in STATA software version 14 using Fisher's exact test for categorical data comparison [28]. The normal distribution of continuous data was analyzed using a t-test, while a Mann-Whitney U-test was performed for non-parametric data. Data are expressed as mean \pm SD for normal distribution continuous data. A p-value ≤ 0.05 was considered statistically significant. The predicted functional profiling of the 16S rRNA gene sequence was conducted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [29-32]. Differences in the abundance of bacterial genera or KEGG pathways between groups were analyzed using STAMP software [33]. A p-value ≤ 0.05 was considered statistically significant.

Results

Ten young HD children were followed up in our institute during the study period. There were seven (70%) and six males (60%) in the HD and in children that did not have HD groups, respectively. Median age was 2.39 years (1.11 to 3.7 years) in the HD group and 1.05 years (0.76 to 1.61 years) in the children that did not have HD group (Table 1). All children resided in the same geographical area. Five HD rectosigmoid type children underwent transanal endorectal pull-through as the definitive surgery, while the other three patients with long segment aganglioneosis and two with total colonic aganglioneosis underwent abdominal assisted in

combination with endorectal pull-through. Five HD patients had recent antibiotics (within one week). Neither probiotics nor laxatives were administered in any patient. Six HD patients became ill with HAEC while collecting fecal samples (Table 2). For microbiome analysis, the microbial composition was assessed by comparing the relative abundances of taxa at phylum and class levels (Figure 1).

The phylum level analysis showed that Firmicutes was the most prevalent in both the HD and non-HD groups (83.76% and 62.88%, respectively). The HD group had a reduction of Bacteroidetes, Actinobacteria, Fusobacteria and TM7, while Firmicutes and Proteobacteria were increased compared to the healthy group. Moreover, the Firmicutes/Bacteroidetes ratio (F/B ratio) in the HD group (10.66) was 4.8 times higher than that in the non-HD group (2.22). At the class level, Bacilli was the most prevalent in the HD group (44.46%), whereas fecal Clostridia predominated in the children that did not have HD group (47.89%). Differential abundance analysis showed that Firmicutes was significantly increased in the HD group compared to the children that did not have HD group (corrected p-value=0.007) at the phylum level, and Bacilli was also significantly increased at the class level (corrected p-value=0.004). Erysipelotrichi and Actinobacteria were significantly decreased in HD children (corrected p-value 0.02 and 0.03, respectively) compared to the healthy children (figure 2).

Comparing between HD and HAEC group, the abundance at phylum level showed that Firmicutes was the most prevalent in both the HD with and without HAEC groups (85.47% and 82.77%, respectively). The HAEC group had a reduction of Bacteroidetes and increased Proteobacteria compared to the HD without HAEC patients (Figure 3). Moreover, the Firmicutes/Bacteroidetes ratio (F/B ratio) in the HAEC group was 1.89 times higher than in the HD group (11.22 vs. 5.95). At the class level, Bacilli was the most prevalent in the HAEC group (43.84%), whereas Clostridia was the most prevalent in the HD group (47.89%). Furthermore, the long segment aganglioneosis group had a reduction of Bacteroidetes and increased of Proteobacteria compared to the rectosigmoid aganglioneosis patients. At the class level, Clostridia was also the most prevalent in both rectosigmoid and long segment aganglioneosis groups (64.37% and 33.39%, respectively) (Figure 3).

In addition, differential abundance analysis between HD and HAEC group and between HD with rectosigmoid aganglioneosis and long segment aganglioneosis patients were also performed. There were no statistically significant differences in fecal bacterial analysis between HD and HAEC groups. Differential abundance analysis between each aganglionic segment showed that Bifidobacteriales and Pseudomonadales were significantly increased in the rectosigmoid aganglioneosis HD patients compared to

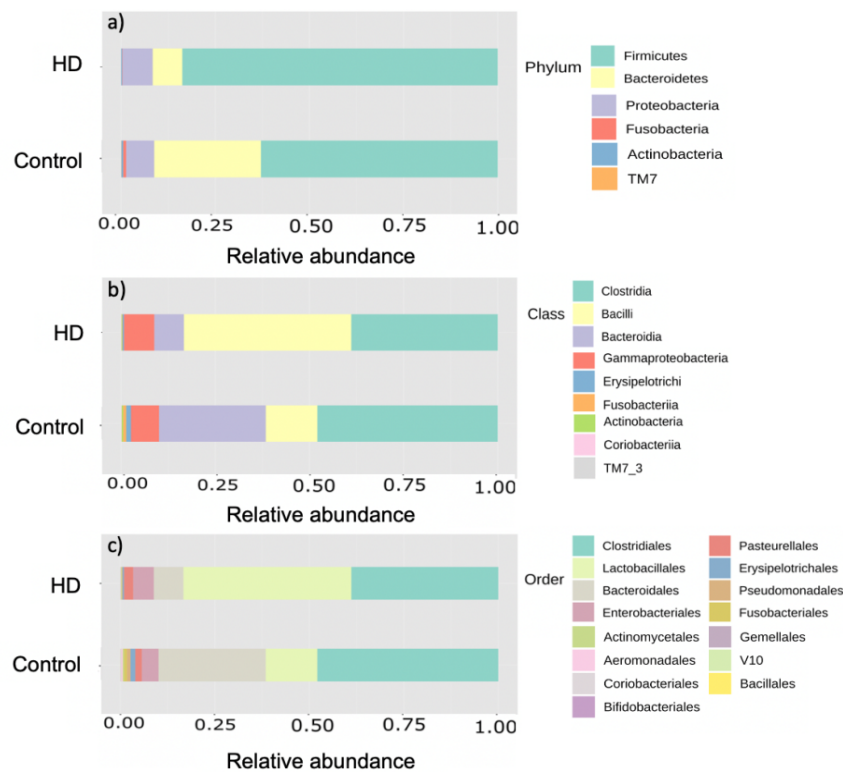


Figure 1: Taxonomic composition of fecal bacterial between Hirschsprung patients and normal control subjects. Stacked bar plot of taxonomic relative abundance at a) phylum b) class and c) order levels

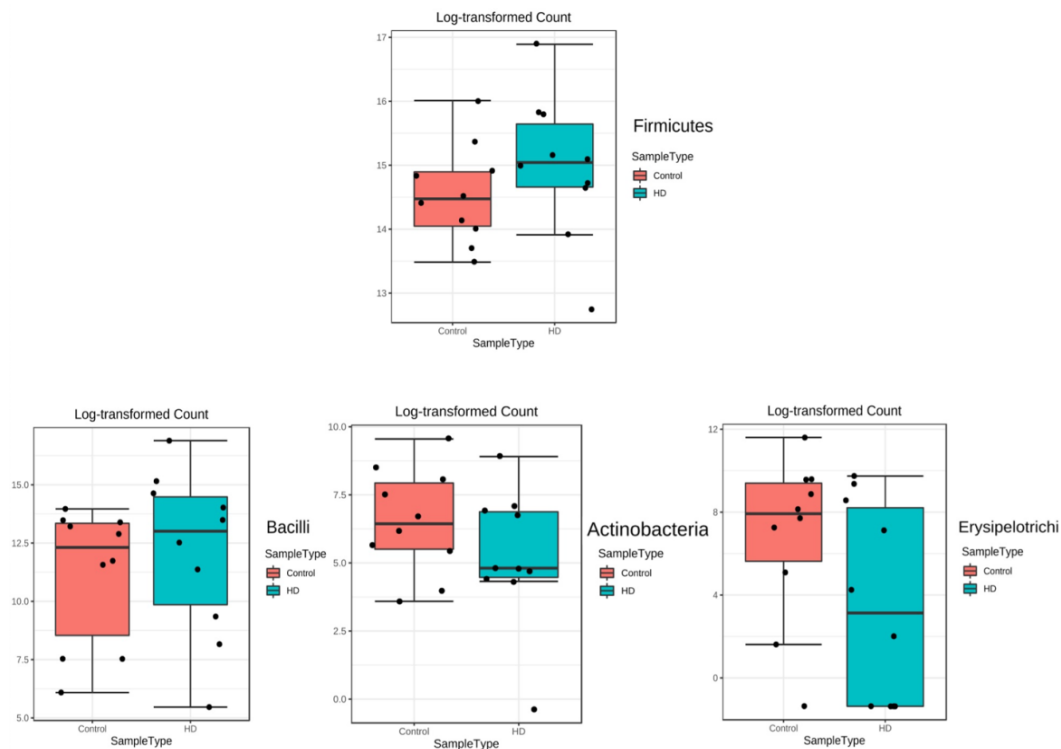


Figure 2: The box plots demonstrated the significantly different of fecal bacterial between Hirschsprung disease (HD) group and control group at the phylum and class level. A p-value less than 0.05 was considered as statistically significant.

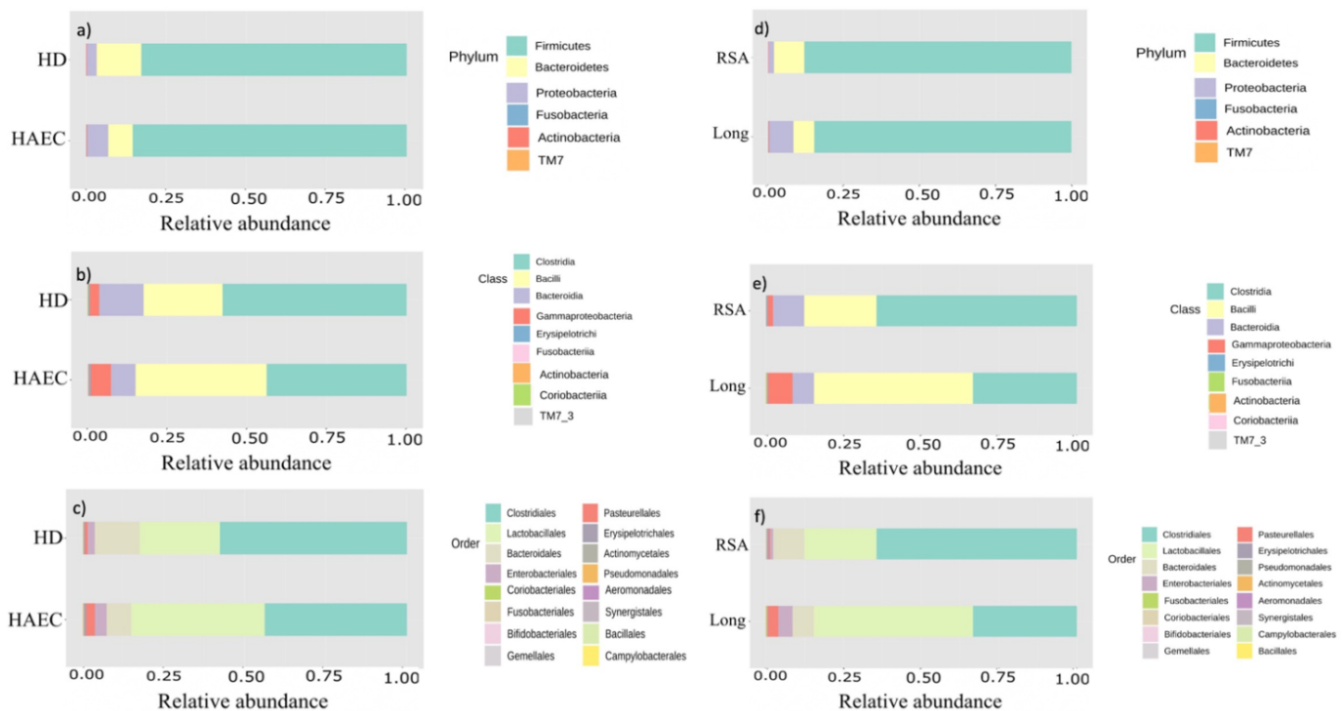


Figure 3: Taxonomic composition of fecal bacterial between Hirschsprung disease (HD) and Hirschsprung associated enterocolitis (HAEC) subjects, and between rectosigmoid (RSA) aganglionosis and long segment (Long) aganglionosis of HD patients. Stacked bar plot of taxonomic relative abundance at a) phylum b) class and c) order levels of fecal bacterial between HD and HAEC subjects. Stacked bar plot of taxonomic relative abundance at d) phylum e) class and f) order level of fecal bacterial between rectosigmoid (RSA) aganglionosis and long segment (Long) aganglionosis of HD patients.

the long segment aganglionosis HD patients (corrected p-value=0.045) at the order level. In contrast, Actinomycetales was significantly decreased in rectosigmoid aganglionosis HD patients compared to the long segment aganglionosis HD patients (corrected p-value=0.045) (Figure 4). Functional analysis showed that the abundance of 38 KEGG endpoints corrected p-value=0.045 which included 20 enzymes and 18 KEGG pathways, was significantly different between groups (corrected p-value<0.01) (Figure 5).

Two pathways including spermidine/putrescine transport system substrate-binding protein (K11069) and holing-like protein LrgB (K05339), were significantly upregulated in HD patients than that of non-HD group (corrected p-value<0.01 with 95% CI did not include zero). Conversely, the KEGG pathway IclR family transcriptional regulator, pca regulon regulatory protein (K02624), general secretion pathway protein S (K02465), selenoprotein W-related protein (K07401), general secretion pathway protein N (K02463), protein ImuB (K14161), twitching motility protein PilU (K02670), and putative proteasome-type protease (K07395) were significantly downregulated in HD patients compared with non-HD groups (corrected p-value<0.01 with 95% CI did not include zero). Moreover, there were six enzymes, polyphosphate glucokinase (EC:2.7.1.63; K00886), 3-oxoadipate CoA-transferase, beta subunit (EC:2.8.3.6;

K01032), 3-oxoadipate CoA-transferase, alpha subunit (EC:2.8.3.6; K01031), protocatechuate 3,4-dioxygenase, beta subunit (EC:1.13.11.3; K00449), protocatechuate 3,4-dioxygenase, alpha subunit (EC:1.13.11.3; K00448), inulin fructotransferase (DFA-I-forming) (EC:4.2.2.17; K10677), that were significantly predicted lower in HD group compared with non-HD group (corrected p-value<0.01 with 95% CI did not include zero).

Discussion

The gut microbiota is a complex community of microbes that are widely distributed in the gastrointestinal tract. Most microbes reside in the distal ileum and colon that contain the majority of microbial nutrients including essential amino acids and vitamins, together with indigestible components such as plant polysaccharides that can be microbially fermented into SCFAs [8]. An imbalance of the microbiota composition and its function, known as dysbiosis, could be involved with a number of gastrointestinal diseases, including HD [13-15,18]. After the advancement of analytical methods for microorganisms, HD-related microbiota has been studied although the results obtained from different studies varied [20,34-38]. Such different findings in the previous studies could result from the differences in age, geography, ethnicity and diet that are the important factors influencing

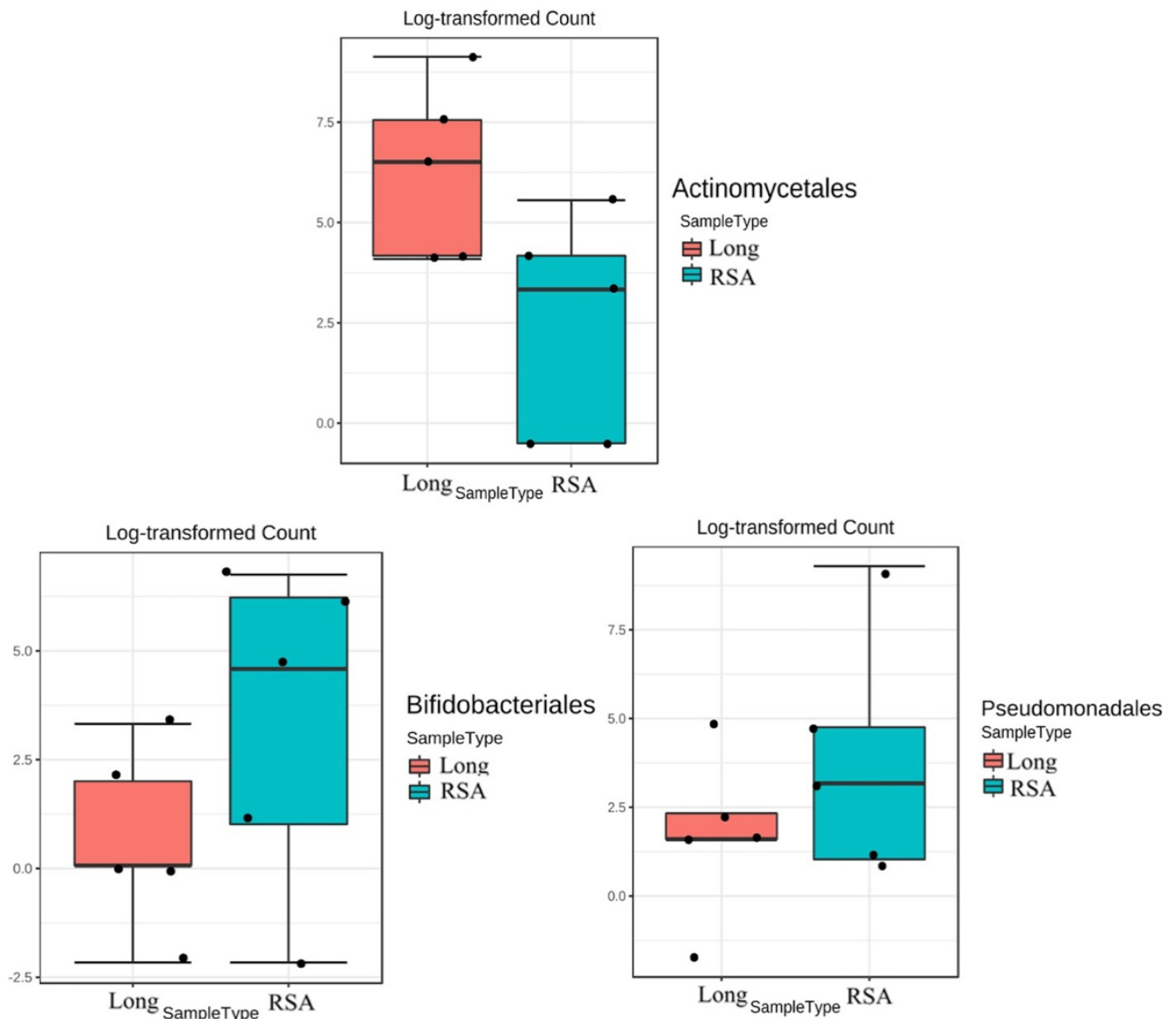


Figure 4: The box plots demonstrated the significantly different of fecal bacterial between rectosigmoid (RSA) and long segment aganglionosis (Long) in Hirschsprung disease patients at the order level. A p-value less than 0.05 was considered as statistically significant.

gut microbiota [7,13]. In addition, the children's microbial characteristics appear more diverse and converge towards the adult pattern after approximately 2.5 to 3 years of age, together with young post pull-through HD children being more susceptible to HAEC, leading to the age control of the sample collection in the current study [22,23]. In previous HD studies, the relative abundance of the phyla showed that Bacteroidetes predominated intraluminal content [20,35,37]. In contrast, the most predominant microbiota in both the HD and non-HD groups in our study was Firmicutes. This may be due to the differences in ethnicity, diet and the participant age range of our study compared with the others [20,34-38]. Even though Bacteroidetes was the second most abundant in the HD group, it was substantially decreased in the HD

group compared with children that did not have HD. Having considered the F/B ratio in the HD group (10.66), it was 4.8 times higher than that in the non-HD group (2.22). Firmicutes and Bacteroidetes are the two major bacterial phyla in the gastrointestinal tract; thus, the ratio between these two phyla was formerly reported to associate with gut homeostasis and the alteration of this ratio also demonstrated to be associated with various diseases [39-41]. More interestingly, this proportion was reported to vary with age. In Ukrainian population, the median of F/B ratio in children group (0-9 years) was 0.69 and tended to increase with age compared to the elderly [42]. In addition, in Brazilian young children, the average F/B ratio was between 1.47 to 2.0, which was close to the ratio of the children that did not have HD group in our findings [43].



Figure 5: The relative abundance of functional pathways in the gut microbiota between control subjects and Hirschsprung disease (HD) patients. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database functional categories are shown in the histograms. The corrected p-value determinations less than 0.01. Red and green denote the individual cases of control subjects and Hirschsprung disease patients.

Therefore, to interpret microbial community results, our findings suggested that it may be necessary to consider the age of the study group, besides the disease itself. Comparison and classification of the taxa between the non-HD and HD groups showed that Firmicutes was significantly increased in the HD group compared to the non-HD group at the phylum level. This is probably because most of our young children with HD had history of HAEC that is consistent with a study by Li et al., comparing the intestinal microbiome content between HAEC and non-HAEC patients. They found that the relative abundance of Firmicutes was increased in HD with HAEC compared to patients without HAEC [37]. In addition, the comparison of the fecal microbial profiling between the lengths of the aganglionic segment had been demonstrated that there were significantly higher relative abundances of Bacteroidetes in rectosigmoid aganglionosis compared to total colonic aganglionosis whereas Proteobacteria was significantly higher in total colonic aganglionosis group [38]. Likewise, this study found the reduction of Bacteroidetes and increase of Proteobacteria in the long segment aganglionosis group compared to the rectosigmoid aganglionosis group. Our study found that Bacilli significantly increased in the HD group, while Erysipelotrichi and Actinobacteria significantly decreased. Although Bacilli are usually abundant in infants and are among the most common groups of probiotic bacteria, dysbiosis with a higher abundance of Bacilli was reported to be linked to diseases including HD [36,44,45]. Bacteria belonging to class Erysipelotrichi are in the phylum Firmicutes. Previous studies demonstrated the association of an increased abundance of this taxon with colon cancer, obesity, and choline deficiency-induced fatty liver disease [14,15,46]. In contrast, the study by Labbé and co-workers exhibited that in IBD patients, Erysipelotrichaceae, a family of the class Erysipelotrichi, significantly reduced compared to children that did not have HD [47]. Significantly increased relative abundances of Actinomyces, a genus of the class Actinobacteria, in HD patients compared to healthy children were evident in the previous study [36]. The decrease in Actinobacteria is probably due to antibiotics used for HAEC treatment in HD patients in the study [48]. The functional analysis depicted that the spermidine/putrescine transport system substrate-binding protein (K11069) were significantly upregulated in HD patients compared with the children that did not have HD. Spermidine and putrescine are the main polyamines in human cells that are mainly derived from ingested food and absorbed in the upper parts of the intestine, whereas polyamines found in the lower part of intestine are considered to be synthesized by the gut microbiota [49]. In general, the availability of polyamines in human cells contributes to tissue homeostasis of the gastrointestinal mucosa [50]. Hence, the dysregulation of polyamines, either under- or overexpression, can affect growth, aging and several diseases such as gastrointestinal cancer [50]. In bacteria, spermidine/putrescine transport system substrate-binding

protein is in the ATP-binding cassette (ABC) transporters which include ABC importers for nutrient and micronutrient uptake, and ABC exporters for toxic substance excretion and drug resistance [30-32]. Holin-like protein LrgB appeared in hydrolases in a two-component system [51]. Hydrolysis activity regulates the growth rate by breaking apart the wall; thus, this activity plays an important role in many aspects of cell-wall growth, modification, and turnover in bacteria. It is, therefore, noteworthy to further investigate the host-microbial metabolic alteration in HD group. The limitations of this study included a small sample size and most HD patients, especially those who have a history of recurrent HAEC, require antibiotics for the treatment that may cause the microbial disturbance [36,38]. At present, from the current cross-sectional study, it is uneasy to determine whether the dysbiosis was altered by enterocolitis or by antibiotics. To tackle this challenge, further prospective study needs to be conducted.

Conclusion

Collectively, this study found significant differences in microbiota at both phylum and class levels between young Thai post pull-through HD and healthy children. There was a nearly five-fold increase in fecal F/B ratio of young post pull-through HD patients. Furthermore, the 38 KEGG endpoints of the functional analysis were found significantly altered between the two groups. Our findings revealed a distinct dysbiosis even when the aganglionic segment had already been removed in young HD children. Further longitudinal study with a larger sample size is still required to investigate the cause and effect of the gut microbiome in HD and HAEC patients.

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Financial interests

The authors declare they have no financial interests.

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