ORIGINAL ARTICLE

Alterations in intestinal microbiota composition in children with hirschsprung disease: A comparative study with healthy controls

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ABSTRACT

Introduction: Hirschsprung disease (HSCR) is a congenital condition characterized by the absence of ganglion cells in the distal colon, resulting in bowel obstruction and motility disorders. Recent studies have highlighted the role of intestinal microbiota in intestinal health and disease, yet its specific alterations in HSCR patients remain unclear. This study aimed to investigate the intestinal microbiota profile of children with HSCR and compare it with healthy controls to identify potential microbial signatures associated with the disease.

Materials and Methods: This comparative cross-sectional study analyzed fecal samples from 7 preoperative HSCR patients and 3 healthy controls using 16S rRNA gene sequencing. Inclusion criteria required no antibiotic use within the previous two weeks. Clinical data, including nutritional status, medication history, bowel management methods, and history of HAEC, were recorded. Microbiota composition was compared at the phylum and family levels.

Results: HSCR patients exhibited significantly higher relative abundance of Enterobacteriaceae (mean 0.2582) compared to healthy controls (mean 0.0236), representing an approximately eleven-fold increase. HSCR patients also showed decreased proportions of Firmicutes, Actinobacteria, Bacilli, and Clostridia, while Bacteroidales were increased. Classification of taxa revealed a reduction in beneficial bacteria (e.g., *Lactobacillus*, *Bifidobacterium*) and enrichment of potentially pathogenic taxa (e.g., Escherichia-Shigella).

Conclusion: HSCR patients demonstrate a distinct dysbiotic microbiota profile, with reduced beneficial taxa and elevated Enterobacteriaceae levels. These findings highlight potential microbiota-targeted strategies for clinical management of HSCR.

KEYWORDS:
Hirschsprung disease, intestinal microbiota, dysbiosis, 16S rRNA sequencing

INTRODUCTION

Several studies have reported various alterations in the intestinal microbiota in both animal and clinical studies of Hirschsprung's disease (HSCR). Research indicates significant alterations in the intestinal microbiota associated with HSCR and its complications, particularly Hirschsprung-associated enterocolitis (HAEC). These changes are observed in both clinical and animal studies, highlighting the microbiota's role in disease pathogenesis and potential therapeutic avenues.[1-2]

In HAEC patients, a notable decrease in beneficial bacteria such as Bifidobacterium and Lactobacillus was observed, alongside an increase in Enterococcus. Studies in HSCR mice showed increased alpha diversity over time but a decrease post-surgery, indicating surgical impact on microbiota.[1]

The presence of Proteobacteria, particularly Escherichia, was linked to HAEC occurrences, suggesting a pathogenic role.[3] Functional analyses revealed that virulence factors associated with these bacteria may contribute to HAEC development.3 Fecal microbiota transplantation (FMT) has shown promise in enhancing cell therapy outcomes in related conditions, indicating potential for HSCR treatment.[3-4] While these findings underscore the microbiota's critical role in HSCR and HAEC, the variability in microbiota responses post-surgery suggests a complex interplay that warrants further investigation.

The lack of preliminary data on intestinal microbiota in HSCR patients compared to healthy children in Makassar, Indonesia, highlights a significant gap in understanding the disease's pathogenesis.

MATERIALS AND METHODS

The authors collected fresh fecal samples from 7 HSCR patients and 3 healthy children. This study utilized purposive sampling to select samples that met specific inclusion criteria. The subjects were pediatric surgical patients diagnosed with either HSCR or other non-HSCR conditions who were hospitalized at our center. The subjects were divided into two

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groups: Group 1 for HSCR and Group 2 for non-HSCR or healthy children, with participants selected randomly within these groups.

The authors extracted bacterial DNA from the fecal samples and then amplified it using target-specific primers (16S V3-V4). After amplification, we prepared a library using the PCR products and sequenced the final library on the Illumina platform to generate paired-end raw reads at the Molecular Biology and Immunology Laboratory, Microbiology Section, Faculty of Medicine, Hasanuddin University.

Data collection took place from January to August 2024. The study excluded patients with HSCR who had received antibiotic therapy within the last week, non-HSCR patients with accompanying gastrointestinal disorders, and those unwilling to participate.

The study processed various variables, including age, gender, source of infection, type of organism, bacterial classification, and outcome. HSCR are those whose clinical and histopathological tests revealing the presence of aganglionic tissue, as seen in HSCR, through either a rectal biopsy or a biopsy during laparotomy. Non-HSCR patients have diagnoses other than HSCR and show no signs of gastrointestinal symptoms. The ethical recommendations have been approved by the Hasanuddin University of Faculty of Medicine Ethics Commission (No:605/UN4.6.4.5.31/PP36/2024)

RESULTS

The demographic data for the patients in this study show that the youngest patient was 7 months and the oldest was 8 years. Regarding gender distribution, 6 out of the 10 patients (60%) were male, while 2 patients (40%) were female (Table I).

Based on histopathological examination, the data revealed that 7 samples were from biopsies that showed aganglionic tissue, confirming a diagnosis of HSCR. The remaining data pertained to healthy children.

Figure 1a illustrates the intestinal microbiota profile for all samples based on Phylum-level taxonomy, highlighting the five most abundant phyla: *Firmicutes, Proteobacteria, Bacteroidota, Verrucomicrobiota,* and *Actinobacteriota*. Our findings demonstrated that HSCR patients showed lower relative abundance of Firmicutes (mean 37.6% vs 55.4%) and Actinobacteria (mean 4.1% vs 9.8%), with higher Bacteroidetes (mean 28.9% vs 21.3%) and Proteobacteria (mean 29.4% vs 11.2%).

Based on the taxonomic class division shown in Figure 1b, the intestinal microbiota profiles of all samples were mainly characterized by the classes *Bacteroidia, Clostridia, Gammaprotobacteria, Bacilli,* and *Verrucomicrobiae*. In HSCR patients, there was a decrease in the presence of Bacilli and Actinobacteria at the class level.

The analysis based on order taxonomy reveals that the top five intestinal microbiota orders across all samples are Bacteroidales, Lactobacillales, Enterobacterales, Verrucomicrobiales, and Oscillospirales, in sequential order. Among HSCR patients, the highest-ranking order is Enterobacterales, followed by Bacteroidales and Verrucomicrobiales. Conversely, in non-HSCR individuals, the predominant order is Bacteroidales, followed by Bifidobacteriales and Lachnospirales, respectively, as illustrated in Figure 1c.

Following, a family-level ordered evaluation uncovered that the intestinal microbiota from the Enterococcaceae, Enterobacteriaceae, Bacteroidaceae, Akkermansiaceae, and Pseudomonadaceae families were the five most copious among all tests (Figure 1d). At this level, there's more inconstancy among the tests. In HSCR persistent tests, three families were reliably found: Enterobacteriaceae, Bacteroidaceae, and Akkermansiaceae. In any case, two tests appeared a dominance of the Enterococcaceae family. In non-HSCR quiet tests, the foremost predominant families were Bacteroidaceae, Lachnospiraceae, and Ruminococcaceae.

The analysis of the genus taxonomic (Figure 1e) revealed that the most common intestinal microbiota genera across all samples, in order, were Enterococcus, Escherichia-Shigella, Bacteroides, Akkermansia, and Pseudomonas. In the HSCR disease group, the identified genus showed considerable variety, with the most consistent sequences in the samples being Escherichia-Shigella, Akkermansia, and Bacteroides. On the other hand, the non-HSCR group displayed more uniformity, with Bacteroides, Bifidobacterium, and a relatively even distribution between Faecalibacterium and Akkermansia. There were increased relative abundances of Escherichia-Shigella and decreased abundances of Faecalibacterium in HSCR patients compared to healthy children.

An analysis of diversity was conducted to identify the presence of intestinal microbiota dysbiosis. Beta diversity, as shown in Figure 2, was used to visualize the distribution and profile of individual sample microbiota. The PCoA plots offer a clear visualization of the differences in intestinal microbial communities between samples. It is important to note that there is no distinct group clustering immediately apparent from this visualization, suggesting some overlap in microbial diversity between samples.

DISCUSSION

Whereas investigating microbiota from all parts of the human body is interesting, the intestinal microbiota has gathered specific intrigued among analysts from differing areas. Information on typical intestinal microbiota are significant for understanding a extend of human gut-related infections. A version of the diary Nature Microbiology in 2015 dove into the setting of microbiome investigate, characterizing the microbiome as a multi-species community of microorganisms in different situations, counting the have, environment, or biological system. One of the conclusions drawn from these considers is the require for large-scale data, including information from different ages, sorts of illnesses, assorted populaces, and broad dispersal of this information

Table I: Characteristics of the samples

Participant ID	Group	Age (months)	Sex status	Nutritional (WHO z-score)	Laxative use	Bowel management method	Stoma present	Antibiotic use (past 2 weeks)
HSCR-01	HSCR	12	M	-1.2 (normal)	No	Rectal washout	No	No
HSCR-02	HSCR	7	F	-0.8 (normal)	Yes	Rectal washout	No	No
HSCR-03	HSCR	15	M	-2.1 (moderate underweight)	Yes	Stoma	Yes	No
HSCR-04	HSCR	10	M	-1.5 (normal)	No	Stoma	Yes	No
HSCR-05	HSCR	14	M	-0.7 (normal)	No	Stoma	Yes	No
HSCR-06	HSCR	9	F	-1.0 (normal)	No	Stoma	Yes	No
HSCR-07	HSCR	11	M	-1.8 (mild underweight)	Yes	Rectal washout	Yes	No
CTRL-01	Healthy control	96	F	0.2 (normal)	No	N/A	No	No
CTRL-02	Healthy control	72	M	0.5 (normal)	No	N/A	No	No
CTRL-03	Healthy control	12	F	-0.1 (normal)	No	N/A	No	No

Notes: Nutritional status classified according to WHO z-scores: normal (> -2), mild underweight (-2 to -3), moderate underweight (< -3). HAEC = Hirschsprung-associated enterocolitis. N/A = Not applicable.

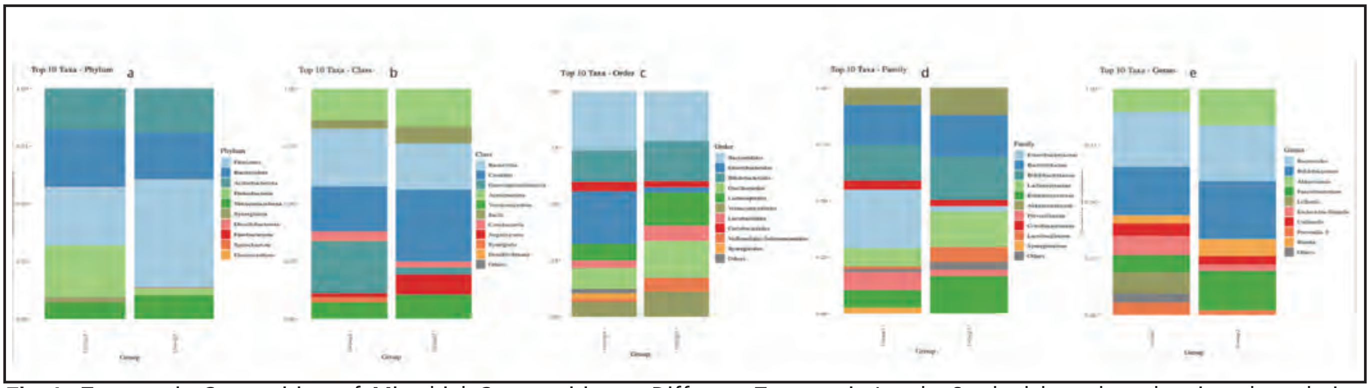

Fig. 1: Taxonomic Composition of Microbial Communities at Different Taxonomic Levels. Stacked bar plots showing the relative abundance of the top 10 microbial taxa in two different groups at five taxonomic levels: (a) Phylum, (b) Class, (c) Order, (d) Family, and (e) Genus. The y-axis represents the relative abundance of taxa, while the x-axis represents different groups under comparison

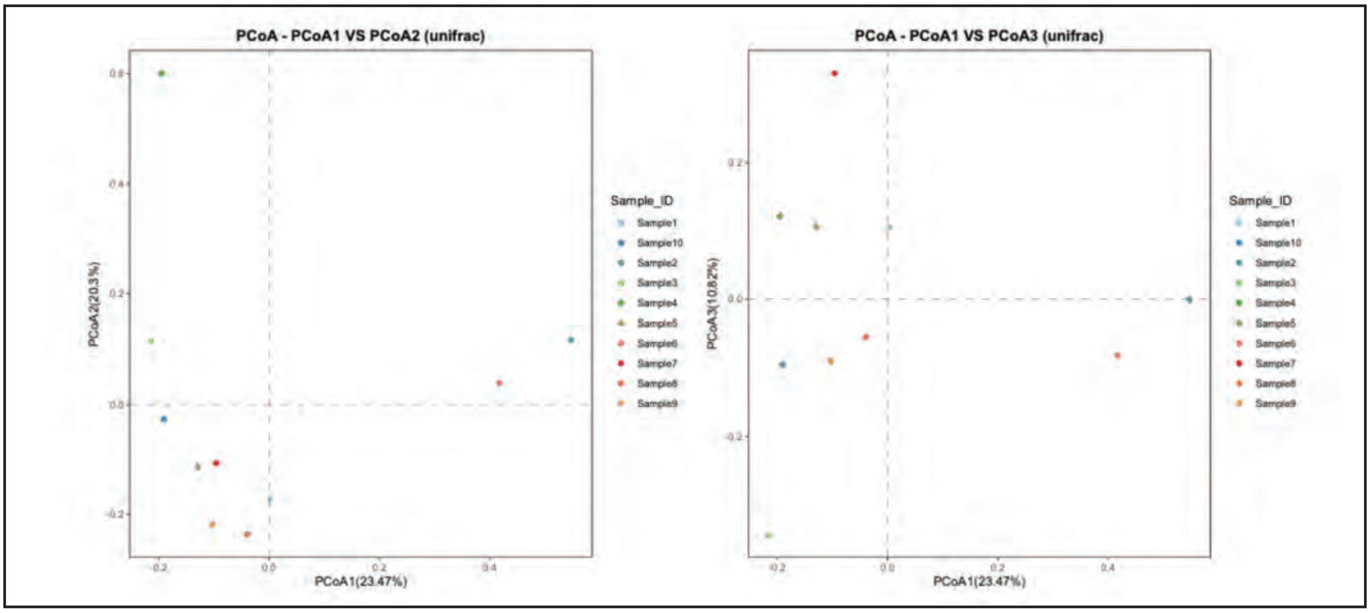

Fig. 2: PCoA on Weighted UniFrac Distance

for meta-analysis studies.6 Hence, in this think about we spearheaded a gene-based ponder in following intestinal microbiota profiles, particularly for sufferers of HSCR in our center.

Metagenomic study currently entails extracting nucleotide sequences from a collection of reference databases including microorganisms or genes, followed by computer analysis employing gene sequence reading algorithms to identify microbe kinds and measure their abundance.[6] The genetic sequences of all detected organisms are shown separately, allowing for comparisons between HSCR and non-HSCR groups. To investigate the particular intestinal microbiota profile in HSCR, we collected samples from two groups: HSCR and non- HSCR, and compared their intestinal microbiota profiles at the phylum, class, order, family, and genus levels. We then determined the top five groups in each category.[7-8]

In this investigation, we employed 16S Amplicon Sequencing, a DNA sequencing approach that targets particular portions of the 16S or 18S ribosomal RNA (rRNA), known as amplicons, using universal primers. Next-generation sequencing of the 16S rRNA gene is commonly used in clinical microbiota research to explore the diversity of bacteria and archaea, whereas the 18S rRNA gene aids in identifying various species in eukaryotic samples. The 16S rRNA gene has nine hypervariable regions (V1-V9) that allow for taxonomic distinction. Regions V3–V4, which encompass locations 341–805 of the E. coli 16S rRNA gene, can differentiate distinct taxonomies to varying degrees.[8-9]

We amplified genomic DNA (gDNA) from our samples using primers that targeted the 16SV3-V4 region. The PCR findings were utilized to create genomic data, which was subsequently sequenced using Illumina technology to identify matches in a reference database. The R Studio tool (R version 4.2.3) and PCoA were used to analyze and visualize the data. This technique allowed us to show bacterial profiles in samples from HSCR and non-HSCR patients, as well as categorize the microbiota into key taxonomic categories such as phylum, class, order, family, and genus.

This study concludes that mapping intestinal microbiota profiles for various clinical needs, such as diagnostics, therapy planning, and treatment method selection, as well as developing databases on a disease's genomic and microbiotic profiles, is possible using gene sequencing methods, as we demonstrated. If performed on a bigger scale and with a more representative number of samples, the results will be extremely beneficial for future microbiota analysis-based research and therapeutic guidelines.

Two animal studies looked at intestinal microbiome alterations in genetically engineered mice with HSCR.[9-10] Both investigations found that mutant mice had more intestinal microbiota diversity than wild-type mice. Another research by Hegde discovered the variety of intestinal bacteria in mice with partial colon blockage.[11] These findings are congruent with those reported in functional gastrointestinal diseases such as constipation. This study implies that higher variability in intestinal obstruction may be due to slower transit rates in the blocked gut.

The bulk of research on the intestinal microbiota in HSCR has found a considerable rise in Proteobacteria and Bacteroidetes, whereas Firmicutes have decreased at the phylum level. The same finding with our research, with Bacteroidetes being the second most common phylum among non-HSCR cases.[12-13]

When identifiable microbes in HSCR are examined at more specific taxonomic levels, most studies report an increase in the presence of Escherichia, particularly Escherichia coli, from the Proteobacteria phylum, as well as higher levels of Bacteroides and Tannerella from Bacteroidetes, while Lactobacillus and Staphylococcus from the Firmicutes phylum are noted to be reduced. Further research on intestinal microbiota in children with HSCR discovered higher amounts of Escherichia and Pseudomonas from the Proteobacteria phylum, as well as more Prevotella and Actinomyces from the Bacteroidetes and Actinobacteria phyla, respectively. These patterns are consistent with our findings, which showed that the most prevalent genera in HSCR illness were Escherichia-Shigella, Akkermansia, and Bacteroides [10-11,13]

Previous research has suggested a relationship between intestinal microbial dysbiosis and HSCR, albeit the specific etiology is uncertain. In this study, we used taxonomic tracing techniques to examine the intestinal microbiota makeup of HSCR patients. We then compared this intestinal microbiota profile to that of people without the disease. The findings provided important information on the nature of intestinal microbiota dysbiosis in gastrointestinal obstructive illnesses, specifically HSCR.[14]

Intestinal microbiota dysbiosis in HSCR may be caused by delayed bowel transit due to intestinal blockage or inadequate motility. Some studies imply that dysbiosis can remain even after surgery to remove the blockage. In individuals with HSCR, an aberrant mucosal barrier and decreased immunological activity in the intestines may lead to gut dysbiosis. Dysbiosis in HSCR impairs mucosal defense and gut immunity via many mechanisms, increasing the risk of colonization and invasion by pathogenic microorganisms. This increases the risk of individuals developing enterocolitis and other HSCR complications.[12-13]

This study demonstrated distinct gut microbiota alterations in HSCR patients compared to healthy controls, characterized by reduced beneficial taxa and increased potentially pathogenic bacteria. Several factors may contribute to these alterations: Dietary patterns: HSCR patients often have modified diets with lower fiber intake due to gastrointestinal symptoms, potentially reducing beneficial taxa. Bowel management: The presence of stomas or frequent rectal washouts can alter gut environment and microbiota composition. Chronic constipation: Prolonged intestinal transit time in HSCR creates a milieu favorable for Enterobacteriaceae proliferation. Comorbidities: Nutritional deficits and recurrent infections may further impact microbial diversity.

LIMITATIONS

The small sample size limits generalizability. The cross-sectional design precludes possible causal inference. Hospital-based recruitment and lack of dietary records may introduce potential biases.

CONCLUSION

HSCR patients exhibit a distinct dysbiotic microbiota profile, with significantly higher relative abundance of

Enterobacteriaceae and reduced beneficial taxa compared to healthy controls. These alterations may have clinical relevance for monitoring and preventing complications such as HAEC.

CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest to declare.

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