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Original article

Gut microbiota analysis in patients with Hirschsprung's disease: A genomic pilot study

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Abstract. *Background and aim:* Hirschsprung's disease (HD) mortality is often caused by enterocolitis, specifically Hirschsprung's Associated Enterocolitis (HAEC). In the intestinal microbiota of patients with HD, Bacteroidetes constitute the majority of genomic sequences (46%), followed by Proteobacteria (21%). The incidence of HD is elevated in Makassar, with most cases referred to a central hospital due to high morbidity rates. This study aims to compare the microbiota profile of patients with and without HD. *Methods:* This study is a descriptive observational study, using samples from pediatric surgical patients diagnosed with HD or non-HD. The patients were divided into Group A (non-HD patients) and Group B (HD patients). Genomic DNA (gDNA) was extracted from samples and amplified with PCR using specific primers targeting the 16S V3-V4 region. The PCR results were used to generate genomic data, which was then sequenced using Illumina technology to find matches in a reference database. *Results:* This study included 10 patients, with an average age of 2 years and 2 months. The final genus-level taxonomic analysis revealed that the most prevalent microbiota genera across all samples were *Enterococcus, Escherichia-Shigella, Bacteroides, Akkermansia,* and *Pseudomonas*. In the HD group, there was considerable diversity among the genera identified, with *Escherichia-Shigella, Akkermansia,* and *Bacteroides* being the most consistently observed. In contrast, the non-HD group exhibited more uniformity, with *Bacteroides* and *Bifidobacterium* being predominant. *Conclusions*: While no specific microbiota pattern unique to HD was identified, microbiota composition in patients with the condition exhibited greater variability compared to individuals without HD, particularly at the taxonomic level. (www.actabiomedica.it)

Key words: intestinal microbiota, Hirschsprung's disease, gut dysbiosis, 16s rRNA sequencing, gut taxonomy, krona diagram, genomic analysis, microbial profiles.

Introduction

Hirschsprung's disease (HD) is a developmental disorder of the intrinsic enteric nervous system, characterized by the absence of ganglion cells in the myenteric and submucosal plexuses of the distal intestine. This condition results from a failure of cranio-caudal migration of ganglion cells around the 12th week of gestation, leading to aganglionosis in part or all of the colon. These ganglion cells are responsible for normal peristalsis (1). The global incidence of HD ranges from 1 in 4,400 to 1 in 7,000 live births. It is more common in boys, with a male-to-female ratio of 4:1. In Indonesia, the incidence is estimated at 1 in 5,000 live births. With a population exceeding 220 million and a birth rate of 35 per thousand, approximately 1,540 infants are expected to be born with HD annually in Indonesia (2). Diagnosis of HD involves a

systematic approach that begins with a medical history and physical examination, followed by supportive tests consisting of radiology, lab work, and histopathology. (1) Pre- and post-surgery complications can occur rapidly or gradually and include anastomotic leakage, stenosis, anal sphincter dysfunction, and enterocolitis. The mortality rate for untreated HD is approximately 80%, whereas with treatment, it decreases to around 30% (3). Mortality is often caused by enterocolitis, specifically Hirschsprung's Associated Enterocolitis (HAEC) (4). Using genomic analysis, researchers studied the bacterial microbiota in the colon of four infants with HD, of which two exhibited HAEC. Stool samples were collected from various segments of the colon during surgery, revealing greater bacterial diversity in the samples obtained from HAEC patients compared to those from patients with HD without HAEC (5). These preliminary results suggest that children with HD who develop HAEC experience a shift from a predominantly symbiotic microbiota to a potentially harmful pathobiotic microbiota, a condition known as dysbiosis. Dysbiosis has also been associated with other gastrointestinal disorders, such as inflammatory bowel disease (6)The gut microbiota of infants typically consists of lactic acid bacteria like *Bifidobacteria* and *Lactobacilli*. These bacteria produce lactate, acetate, and other acids, thereby reducing colon pH, which improves gut motility. These microbes and their byproducts also interact with the gastrointestinal lining, affecting the growth and differentiation of epithelial cells, and activating the immune system (7).

In patients with HD, Bacteroidetes have been found to constitute the largest proportion of genomic sequences (46%), followed by Proteobacteria (21%). However, in patients with HAEC, Proteobacteria account for 55%, followed by Firmicutes at 18%. The predominant genus in HAEC patients is *Enterobacteriaceae* (56%), while in HD patients, *Bacteroides* is the most common (47%). Other notable taxa in HAEC include *Enterococcus* (13%), *Acinetobacter* (6%), and *Eukaryota* (4%), whereas *Enterobacteriaceae* (24%) and *Fusobacterium* (4%) are prevalent in HD patients. Additionally, seven taxa are exclusive to HAEC patients, while 11 are uniquely found in HD patients (5). The prevalence of HD in Makassar is particularly high, with most cases being sent to a central referral hospital due to elevated morbidity rates, although the precise incidence remains unknown. Further, postoperative care and outpatient follow-up are often prolonged. Therefore, our study aims to investigate the microbiota profile of HD patients at the Dr. Wahidin Sudirohusodo Hospital, the central referral hospital for Eastern Indonesia in Makassar.

Materials and methods

The study utilized a descriptive observational approach with a cross-sectional design to compare the microbiota profile between patients with HD and those without it. Purposive sampling was used to select samples that met specific inclusion criteria. The inclusion criteria for the study were patients under 18 years old, patients who had been diagnosed with HD through biopsy and pediatric patients with non-HD without symptoms of gastrointestinal disorders. Patients with HD who had received antibiotic therapy in the last seven days, patients with non-HD accompanied by gastrointestinal disorders, and patients who were unwilling to participate in the study were excluded. The subjects were pediatric surgical patients with a diagnosis of HD or other non-HD conditions, who were hospitalized at the Dr. Wahidin Sudirohusodo Hospital in Makassar, Indonesia. The subjects were divided into two groups: Group A (non-HD patients) and Group B (HD patients), with participants randomly selected within these groups. The variables analyzed in this study include age, gender, source of infection, type of causative organism, bacterial classification, and outcome. HD patients are those whose clinical and histopathological tests reveal the presence of aganglionic tissue, as seen in HD, confirmed through either a rectal biopsy or a biopsy during laparotomy. Non-HD patients are individuals diagnosed with conditions other than HD and who exhibit no gastrointestinal symptoms.

Data collection occurred from January to December 2023. The study excluded patients with HD who had received antibiotic therapy within the week before sampling, non-HD patients with accompanying gastrointestinal disorders, and those unwilling to participate.

Identification of intestinal microbiota

The identification and detection of intestinal microbiota in HD patients were conducted at the Molecular Biology and Immunology Laboratory, Microbiology Section, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. In this study, genomic DNA (gDNA) was extracted from samples and amplified with polymerase chain reaction (PCR). We used 16S Amplicon Sequencing, a DNA sequencing technique that targets specific regions of the 16S or 18S ribosomal RNA (rRNA), referred to as amplicons, using universal primers. Next-generation sequencing of the 16S rRNA gene is widely used in clinical microbiota research to study the diversity of bacteria or archaea, while the 18S rRNA gene helps identify species in eukaryotic samples, such as those obtained from our patients. The 16S rRNA gene encompasses 9 hypervariable regions (V1–V9) that enable the differentiation of various taxonomies. Regions V3 to V4, spanning nucleotide positions 341 to 805 of the *E. Coli* 16S rRNA gene, are capable of distinguishing several taxonomies at different levels. Genomic data was sequenced using Illumina technology and a bioinformatic workflow, enabling us to visualize the microbiota profiles in samples from HD and non-HD patients and to categorize the microbiota into major taxonomic groups, including Phylum, Class, Order, Family, and Genus. Data analysis and visualization were conducted using the RStudio application (R version 4.2.3) and Krona Tools.

Data analysis

All research data were collected and tabulated using Microsoft Office Excel. Two types of data analysis were conducted, with the research results displayed in the form of tables and figures.

Ethics

Ethical recommendations were approved by the Ethics Commission of the Hasanuddin University Faculty of Medicine (No:440A/UN4.6.4.5.31 /PP36/2023)

Results

The demographic data for the patients in this study reveal an average age of 2 years and 2 months, with the youngest patient being 7 months old and the oldest being 5 years old. Regarding sex distribution, 8 out of the 10 patients (80%) were male, while 2 patients (20%) were female (Table 1).

According to histopathological examination, the data indicated that 3 samples (Samples 1 to 3) were derived from tissues of children without HD. The remaining 7 samples (Samples 4 to 10) were from biopsies demonstrating aganglionic tissue, confirming a diagnosis of HD. The microbiota profile for all samples was analyzed based on Phylum-level taxonomy, highlighting the five most abundant phyla: Firmicutes, Proteobacteria, Bacteroidota, Verrucomicrobiota, and Actinobacteriota (Figure 1). In the HD group, the three most prevalent phyla are Firmicutes, Proteobacteria, and Verrucomicrobiota. Conversely, in the non-HD group, the primary phyla are Firmicutes, Bacteroidota, and Actinobacteriota.

According to taxonomic class division, the microbiota profiles of all samples were primarily characterized within the classes Gammaproteobacteria, Clostridia, Bacilli, Bacteroidia, and Verrucomicrobiae (Figure 2). Among HD patients, the three most abundant classes are Gammaproteobacteria, Bacteroidia, and Clostridia. In contrast, in the non-HD group, the predominant classes are Clostridia, Bacteroidia, and Actinobacteria.

The analysis based on order taxonomy revealed that the five most abundant orders across all microbiota

Table 1. Demographic data of patients sampled for the study.

Characteristics	**n (%)**
Age (months)	***m = 54 (±40)***
Q1	27
Q3	81
Sex	
Male	8 (80)
Female	2 (20)
Histopathology	
Hirschsprung (HD)	7 (70)
Non-Hirschsprung (non-HD)	3 (30)

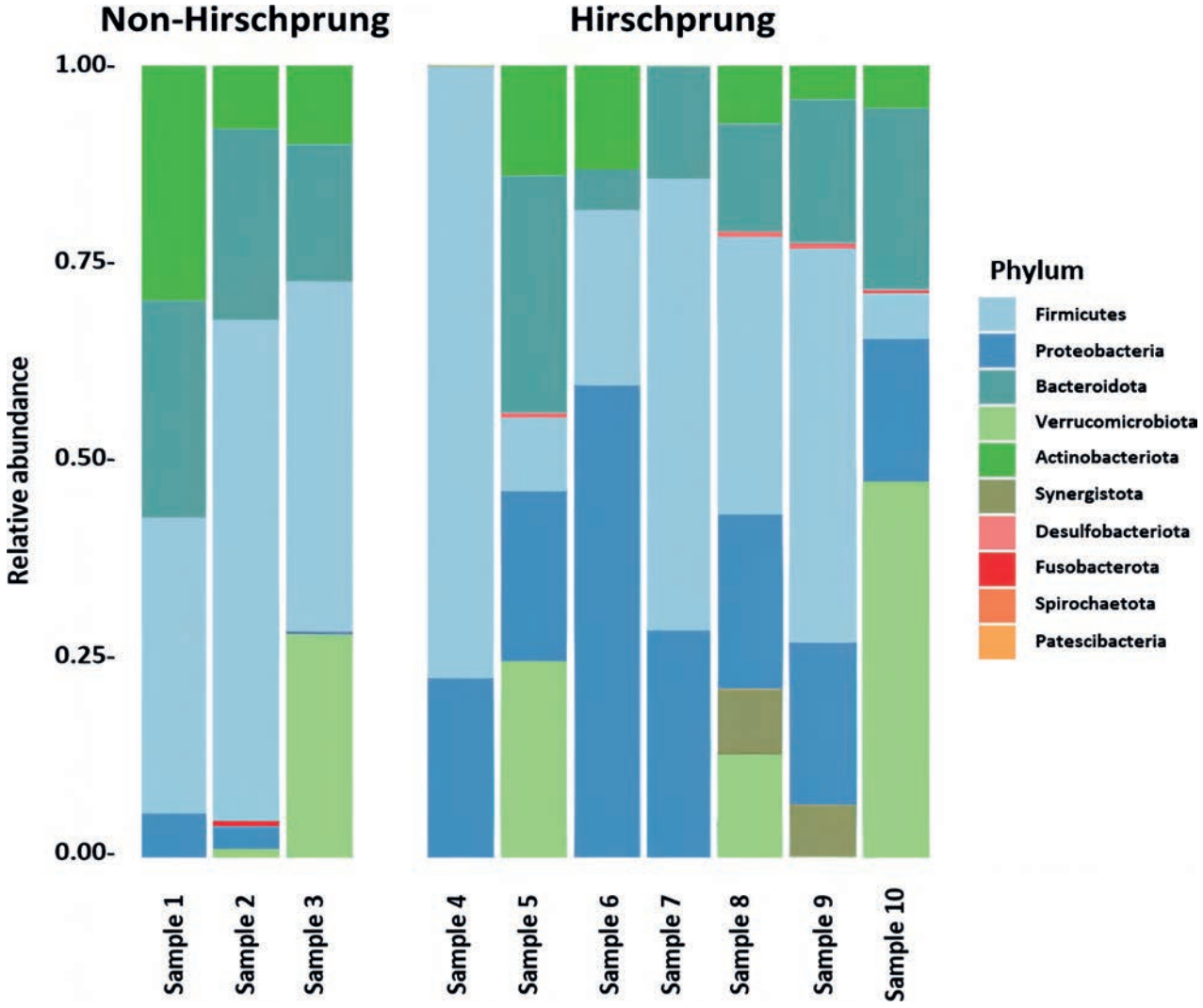

Figure 1. Phylum-level taxonomic identification of microbiota samples from Hirschsprung and non-Hirschsprung patients.

samples are Bacteroidales, Lactobacillales, Enterobacterales, Verrucomicrobiales, and Oscillospirales (Figure 3). Among HD patients, the highest-ranking order is Enterobacterales, followed by Bacteroidales and Verrucomicrobiales. Conversely, in non-HD individuals, the predominant order is Bacteroidales, followed by Bifidobacteriales and Lachnospirales, respectively.

Subsequently, a family-level taxonomic assessment revealed that the *Enterococcaceae, Enterobacteriaceae, Bacteroidaceae, Akkermansiaceae,* and *Pseudomonadaceae* families were the five most abundant among all microbiota samples (Figure 4). At this level, there is greater variability among the samples. In HD patient samples, three families were consistently identified: *Enterobacteriaceae, Bacteroidaceae*, and *Akkermansiaceae.* However, two samples were predominantly comprised of *Enterococcaceae*. In non-HD patient samples, the most prevalent families were *Bacteroidaceae, Lachnospiraceae,* and *Ruminococcaceae.*

The final genus-level taxonomic analysis revealed that the most prevalent microbiota genera across all samples were *Enterococcus, Escherichia-Shigella, Bacteroides, Akkermansia,* and *Pseudomonas,* in descending

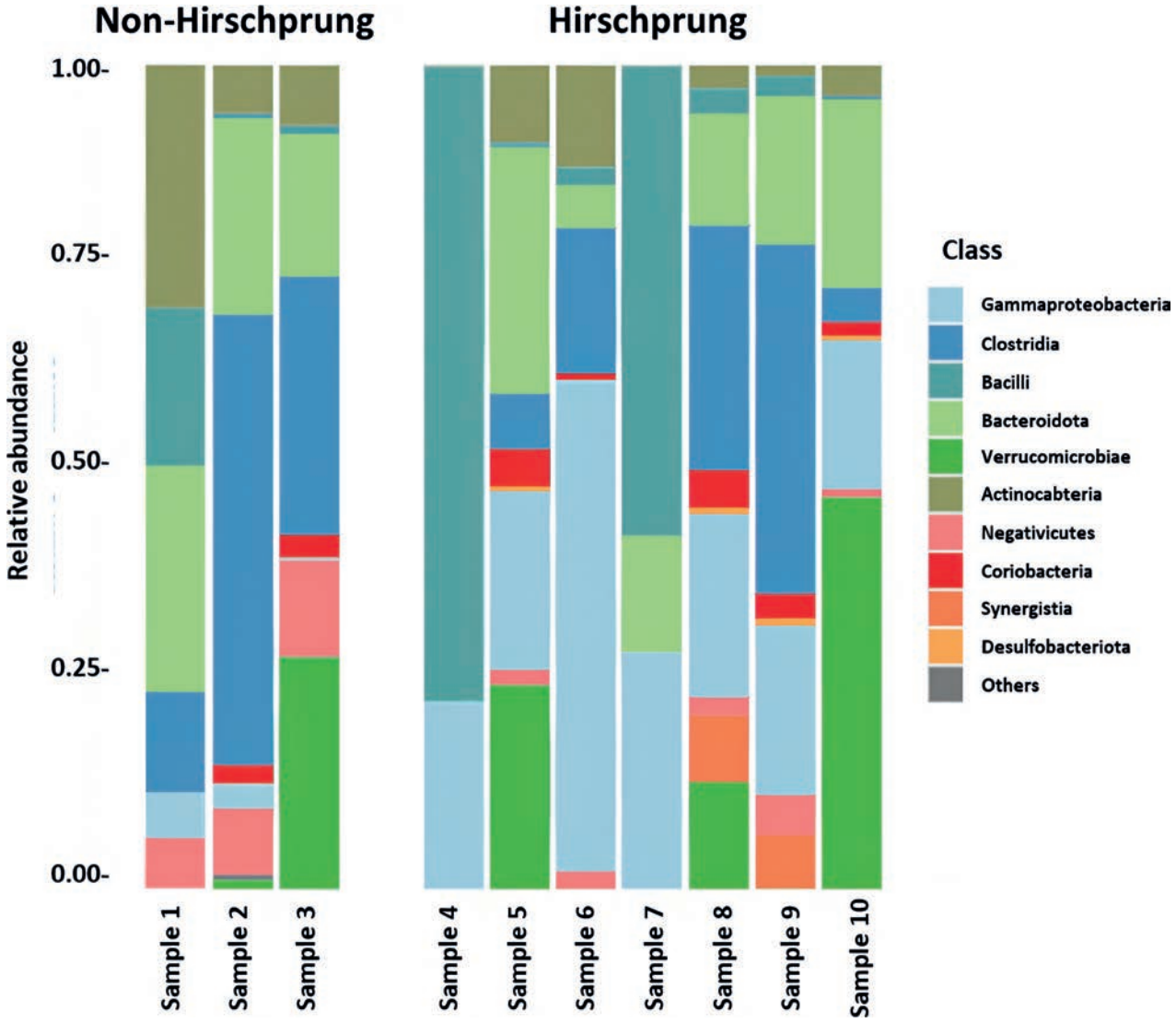

Figure 2. Class-level taxonomic identification of microbiota samples from Hirschsprung and non-Hirschsprung patients.

order of abundance (Figure 5). In the HD group, there was considerable diversity among the genera identified, with *Escherichia-Shigella*, *Akkermansia,* and *Bacteroides* being the most consistently observed. In contrast, the non-HD group exhibited more uniformity, with *Bacteroides* and *Bifidobacterium* being predominant, alongside a relatively even distribution of *Faecalibacterium* and *Akkermansia*.

Microbiota diversity analysis was conducted using the Krona Tools application, providing visualization of the distribution and profile of individual sample microbiota (supplemental figures S1-S10).

Discussion

While exploring microbiota across the human body is intriguing, the gut microbiota has garnered particular interest among researchers from diverse fields. Data on the gut microbiota are crucial for understanding a range of human gut-related diseases as well as homeostatic processes. An issue of *Nature Microbiology* in 2015 explored the landscape of microbiome research, defining the microbiome as a multi-species community of microorganisms inhabiting various environments, including the host, habitat, or ecosystem. One

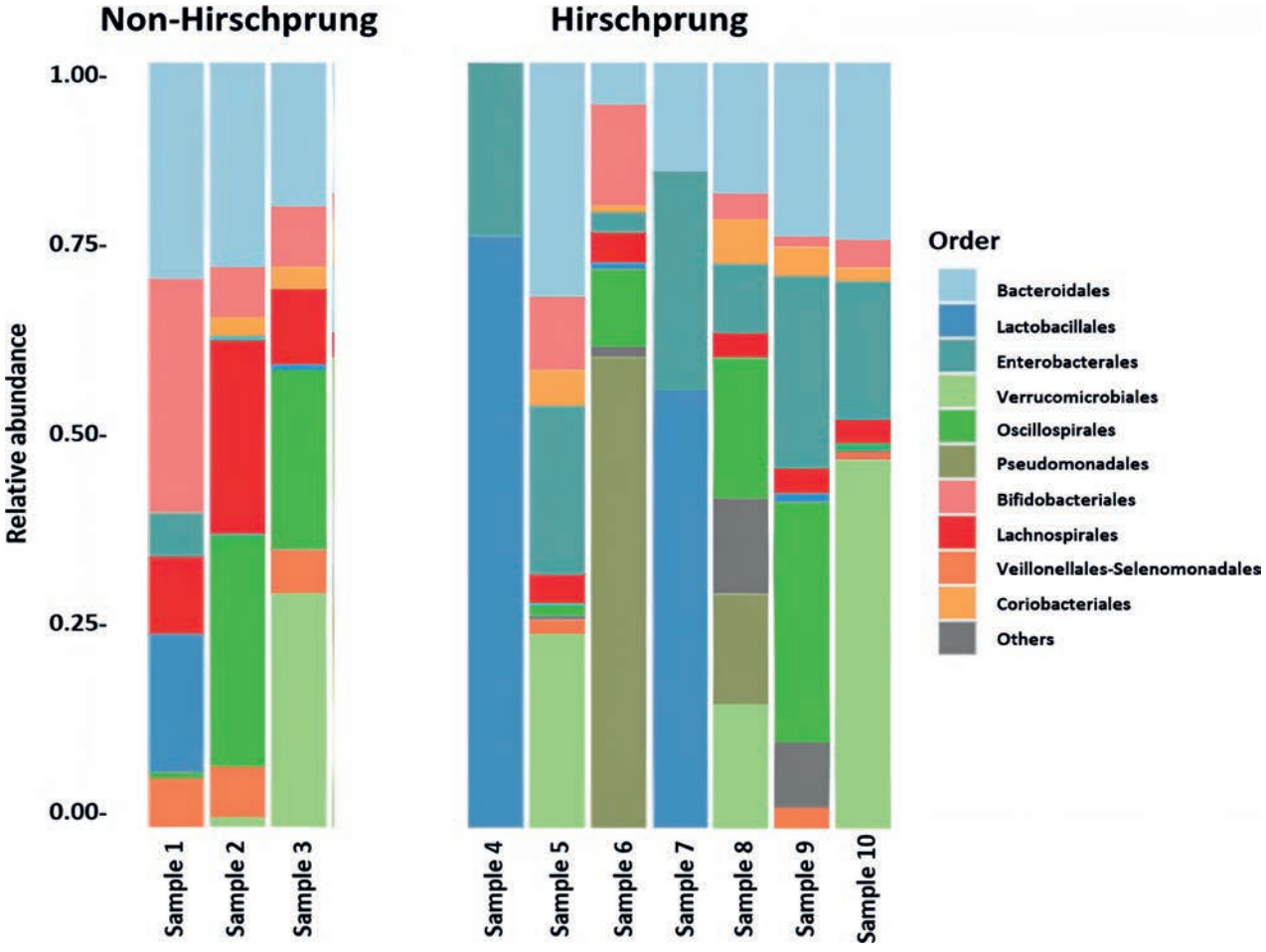

Figure 3. Order-level taxonomic identification of microbiota samples from Hirschsprung and non-Hirschsprung patients.

key conclusion drawn from these studies is the necessity for large-scale datasets, encompassing data from individuals across various ages, disease types, diverse populations, and for widespread dissemination of these data for meta-analyses (6). Therefore, in this study, we initiated a gene-based investigation into gut microbiota profiles among individuals with Hirschsprung's disease (HD) at our institution. Metagenomic analysis involves utilizing nucleotide sequences from curated sets of reference databases containing microorganisms or genes, followed by computational analysis using gene sequence reading algorithms to identify microorganism types and quantify their abundance (6). The genomic sequences of all observed organisms are individually displayed, facilitating comparisons between HD and non-HD groups. This research demonstrates that mapping microbiota profiles for various clinical needs—such as diagnostics, therapy planning, and treatment selection, as well as developing databases on the genomic and microbiota profiles of disease—can be achieved using gene sequencing methods. When applied on a larger scale with a more representative sample size, the data obtained will be invaluable for further microbiota analysis-based studies and clinical guidelines. Two previous *in vivo* studies have investigated alterations in gut microbiota in genetically modified mice with HD, revealing a greater microbiota diversity in mutant mice than in their wild-type counterparts ((8,9)). Another study by Hegde *et al.* examined the diversity of gut microbiota in mice with partial colon obstruction. This study found that obstruction in mice causes significant dysbiosis in the

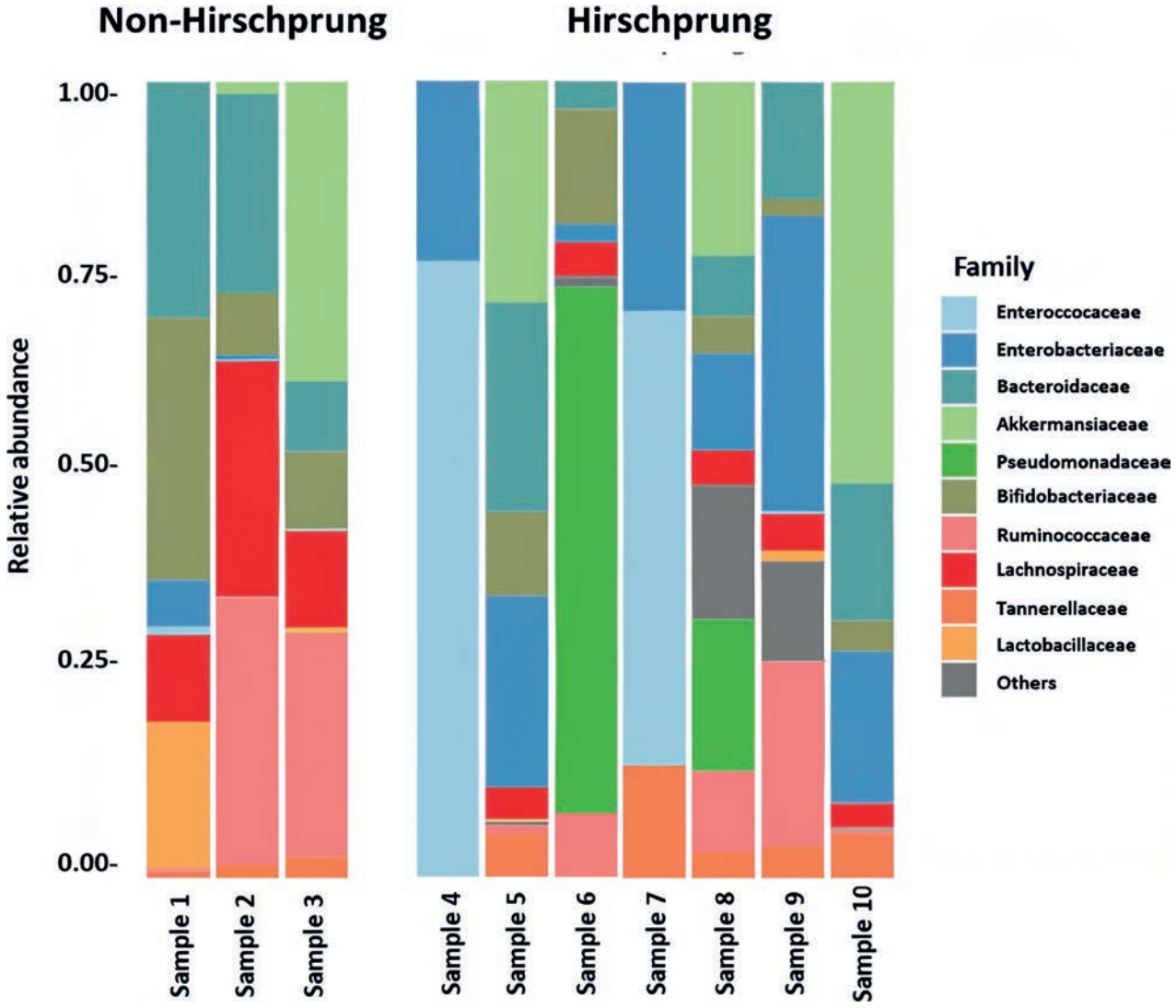

Figure 4. Family-level taxonomic identification of microbiota samples from Hirschsprung and non-Hirschsprung patients.

colon. The use of antibiotics to eliminate microbiota had minimal impact on the bacterial translocation, motility, or inflammation associated with obstruction (10). These findings are consistent with results obtained in functional gastrointestinal disorders such as constipation. This evidence suggests that increased variability in intestinal obstruction may result from slower transit rates in the obstructed bowel. Other research groups studying the gut microbiota in HD have observed a considerable increase in Proteobacteria and Bacteroidetes compared to non-HD individuals, while Firmicutes tend to decrease at the phylum level (11). However, in our study, Firmicutes remained the most common phylum in both HD and non-HD groups, followed by Bacteroidetes as the second most prevalent phylum among non-HD cases. When examining identifiable microbes in HD at more specific taxonomic levels, most studies report an increased presence of *Escherichia*, particularly *Escherichia coli*, from the Proteobacteria phylum, along with higher levels of *Bacteroides* and *Tannerella* from the Bacteroidetes phylum, and a noted reduction in *Lactobacillus* and *Staphylococcus* from the Firmicutes phylum (5). Additionally, a study investigating the gut microbiota of

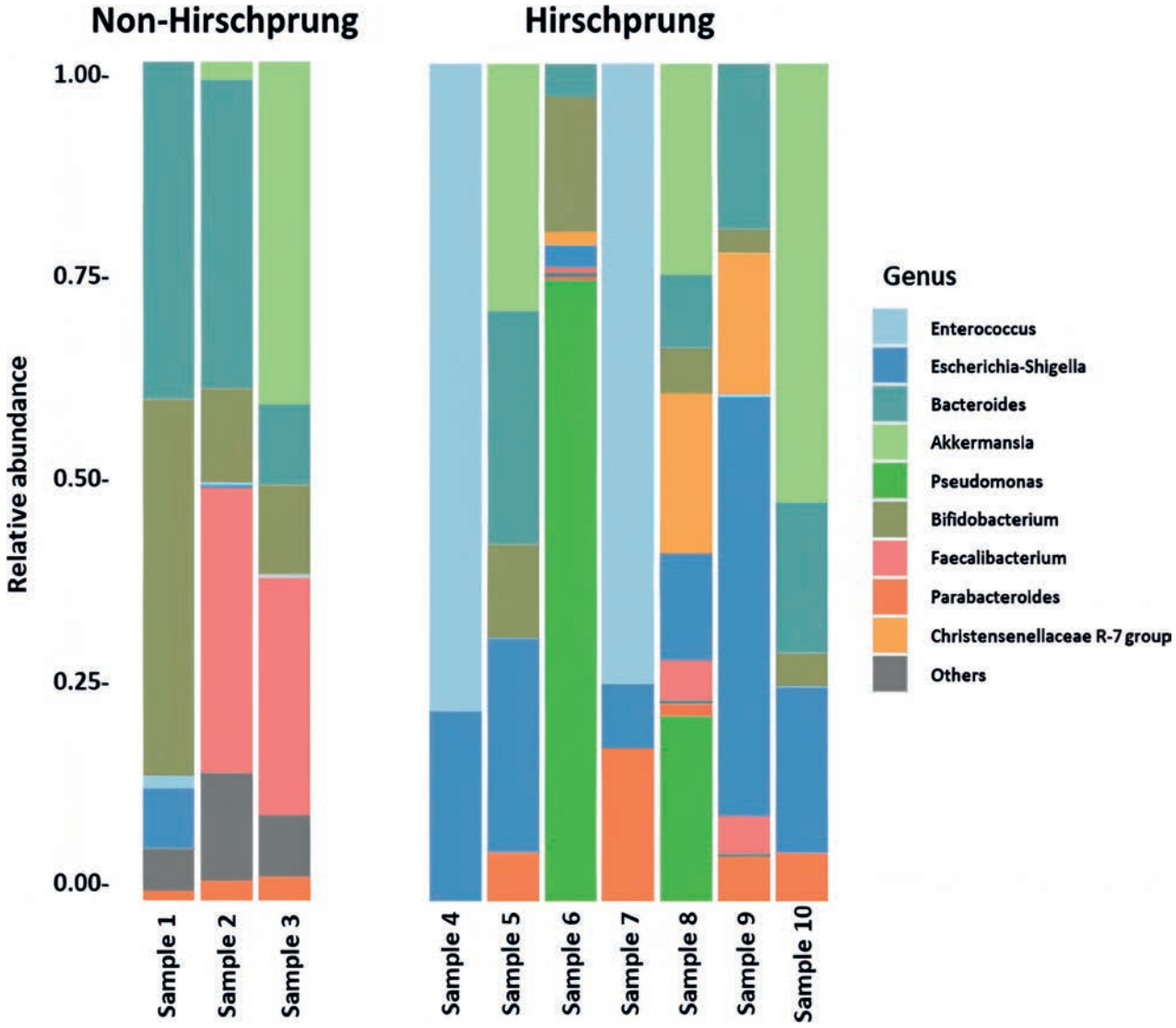

Figure 5. Genus-level taxonomic identification of microbiota samples from Hirschsprung and non-Hirschsprung patients.

children with HD found increased levels of *Escherichia* and *Pseudomonas* from the Proteobacteria phylum, as well as more *Prevotella* and *Actinomyces* from the Bacteroidetes and Actinobacteria phyla. These findings are consistent with the results of our study, where the most abundant genera of bacteria identified in HD patients were *Escherichia-Shigella*, *Akkermansia*, and *Bacteroides* (6,12,13). Previous studies have identified an association between gut microbiota dysbiosis and HD, although the exact cause remains unknown. In this research, we employed taxonomic tracing techniques to analyze the microbiota composition in patients with HD and compared it with that of non-HD individuals. The findings provided valuable insights into the nature of gut microbiota dysbiosis in gastrointestinal obstructive diseases, particularly HD (10). The gut dysbiosis observed in HD may be due to delayed bowel transit caused by intestinal obstruction or impaired motility. Some studies indicate that dysbiosis can persist even after surgery is performed to relieve the obstruction. Abnormalities in the mucosal barrier and impaired immune function in the intestines of patients with

HD likely contribute to this gut dysbiosis. Dysbiosis in HD compromises mucosal defense and gut immunity through various pathways, increasing the susceptibility to opportunistic colonization and invasion by infectious pathogens. Consequently, patients are at a heightened risk of developing enterocolitis and other complications associated with HD (13). The strength of this study lies in its pioneering nature as the first investigation in Indonesia and Southeast Asia to identify the microbiota profile of patients with or without HD. The study's limitations include a small sample size and based on a single institution. Further research involving a larger sample size and a more diverse multicenter study to comprehensively represent the microbiota profile of HD patients. In addition, gut microbiota profiles of patients without comorbidities could serve as an interesting comparison tool, shedding light on various types of gastrointestinal-related diseases prevalent in local populations in Indonesia and Southeast Asia. Further studies investigating the appropriate use of antibiotics and probiotics according to the patient's microbiota profile could provide valuable information for clinical applications, enhancing the effectiveness and efficiency of targeted antimicrobial interventions.

Conclusion

Together, these data indicate no distinctive microbiota pattern linked exclusively to HD. However, patients with HD exhibit greater variability in their microbiota composition compared to individuals without the disease, particularly when examining taxonomic details.

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Ethic Committee: All research designs were reviewed and approved by the Health Research Ethics Committee of Dr Wahidin Sudirohusodo Hospital, Faculty of Medicine, Hasanuddin University (No. 440A/UN4.6.4.5.31/PP36/2023) with protocol number UH23060392, on June 27, 2023.

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Authors Contribution: BB: Concept, Design, Resources, Materials, Data Collection and Processing, Analysis and Interpretation, Literature Search, Writing Manuscript; AW: Concept, Design, Supervision, Analysis and Interpretation, Literature Search; MM: Concept, Design, Supervision, Analysis and Interpretation, Literature Search), AQ: Concept, Design, Supervision, Analysis and Interpretation, Literature Search; SR: Concept, Design, Supervision, Analysis and Interpretation, Literature Search; MF: Concept, Design, Analysis and Interpretation, Critical Review. All authors read and approved the final version of the manuscript.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Appendix - Supplementary material

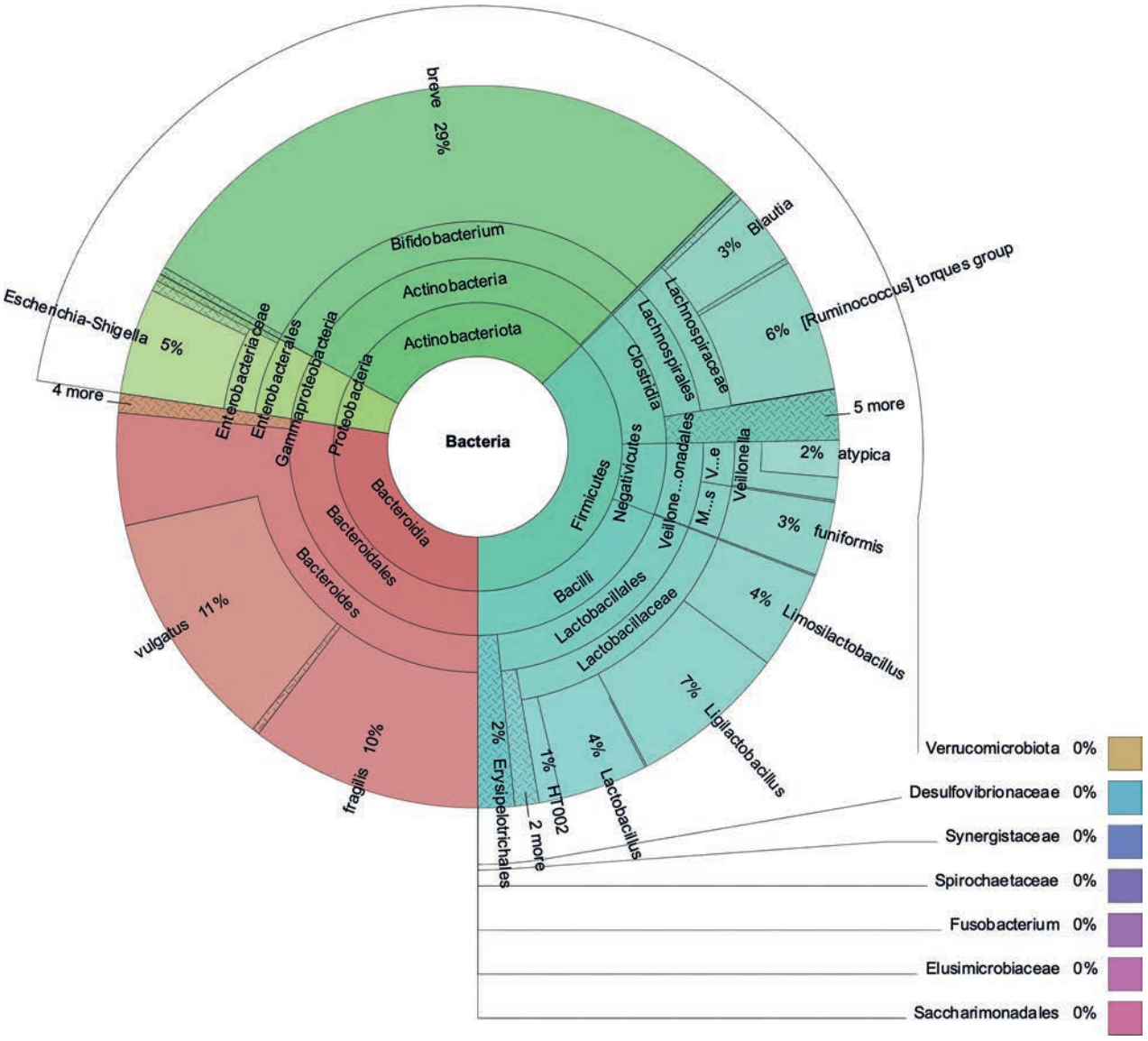

Figure S1. Krona diagram of a patient with non-Hirschsprung's disease (Sample 1).

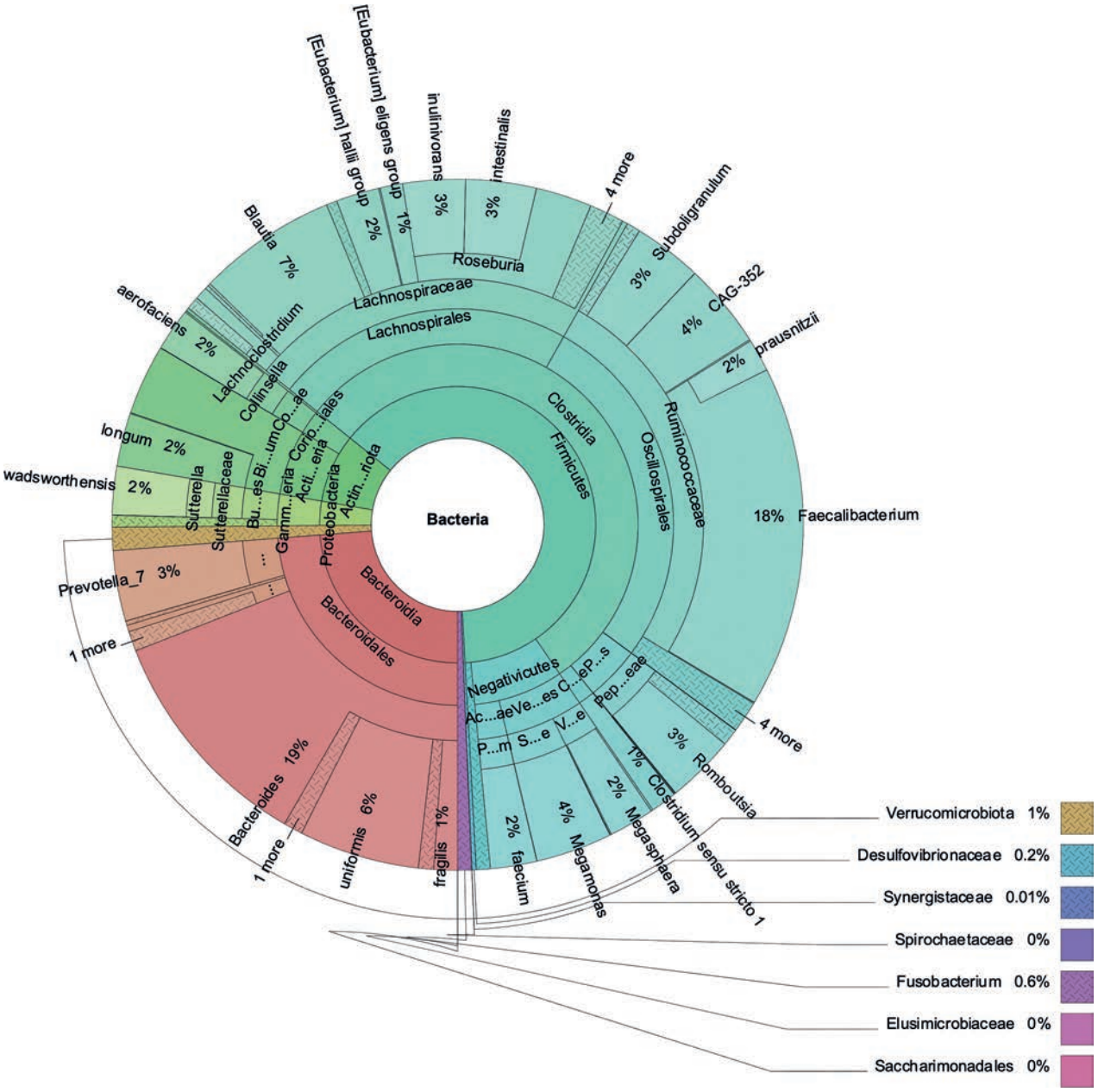

Figure S2. Krona diagram of a patient with non-Hirschsprung's disease (Sample 2).

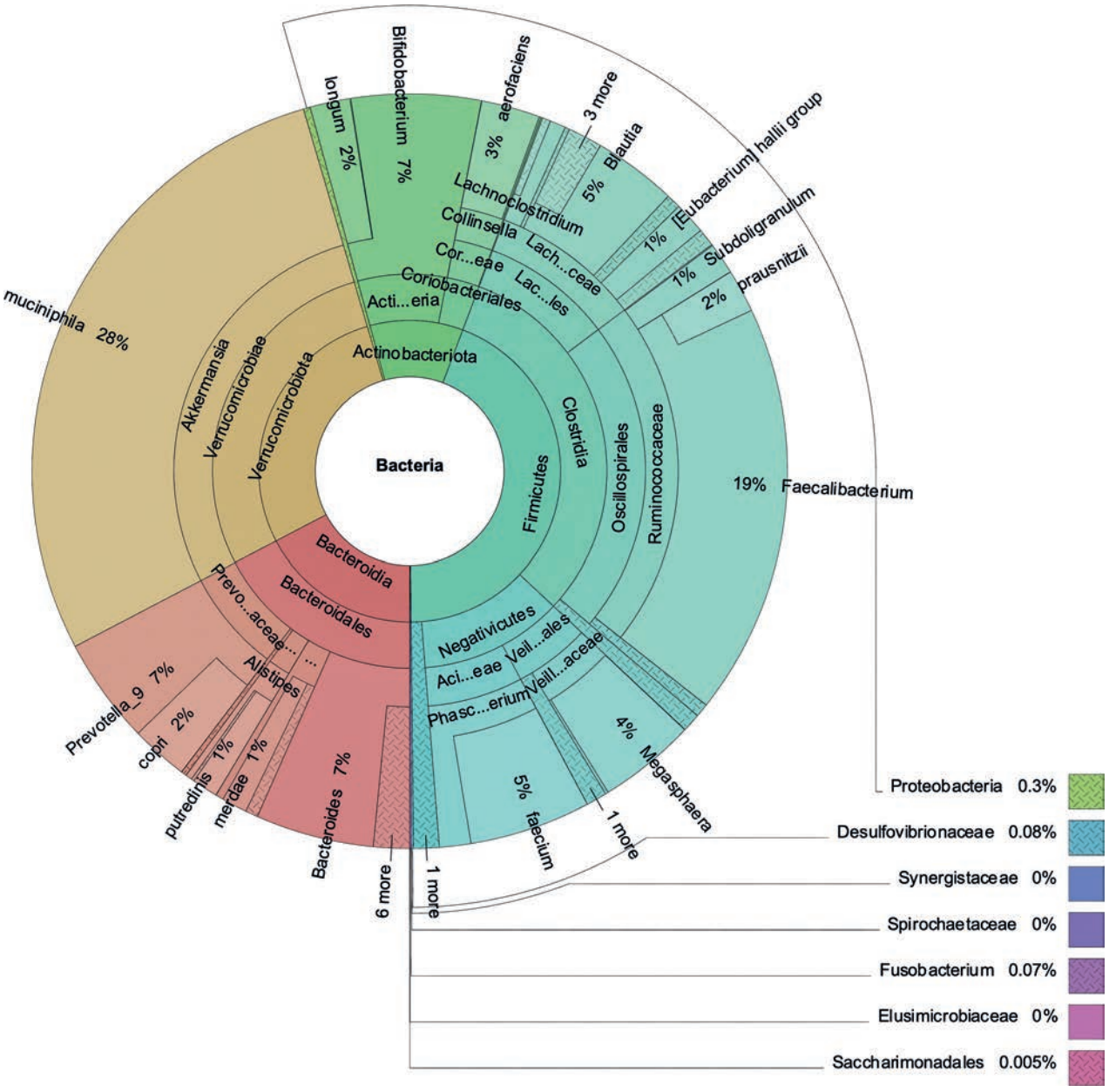

Figure S3. Krona diagram of a patient with non-Hirschsprung's disease (Sample 3).

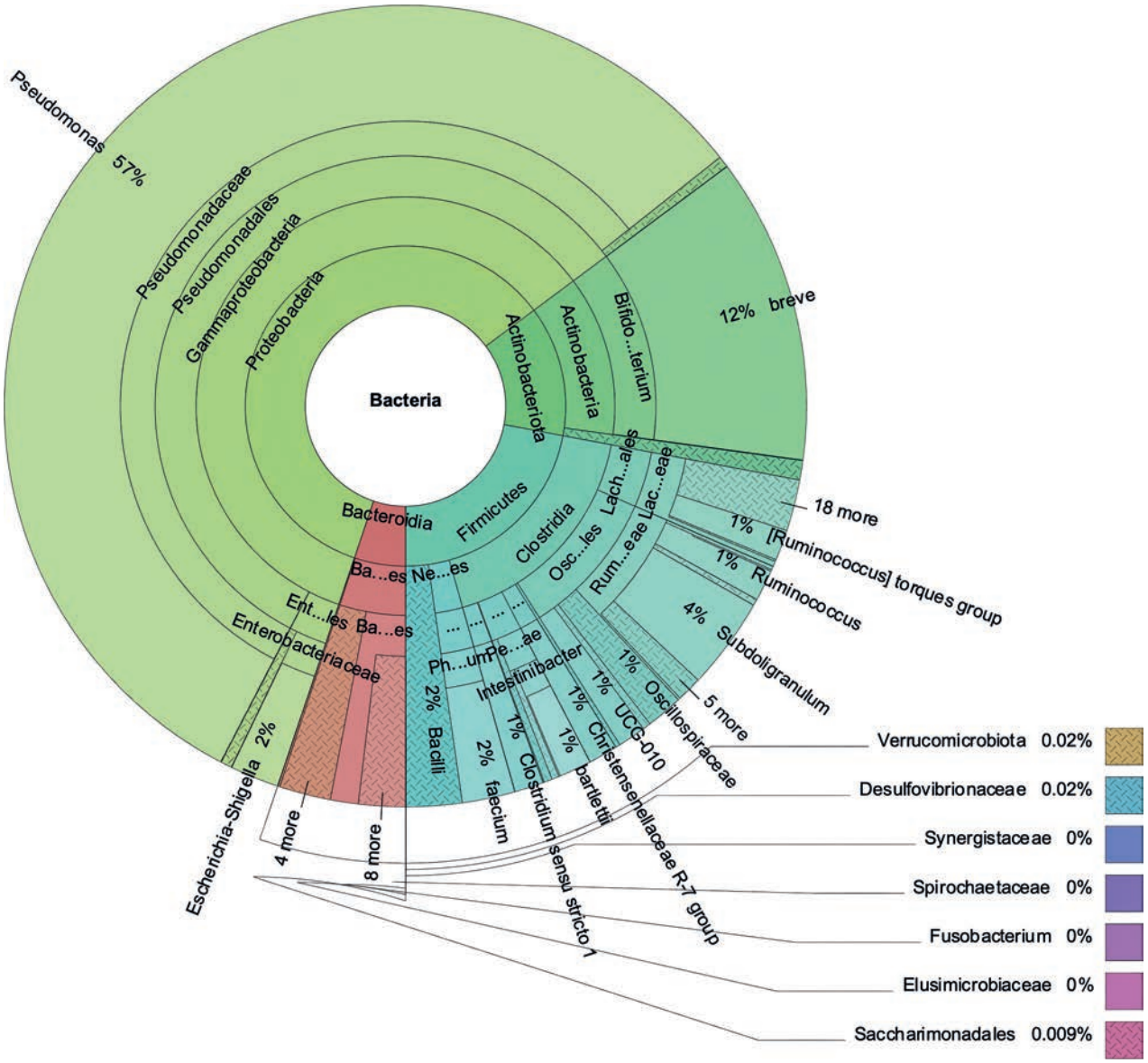

Figure S4. Krona diagram of a patient with Hirschsprung's disease (Sample 4).

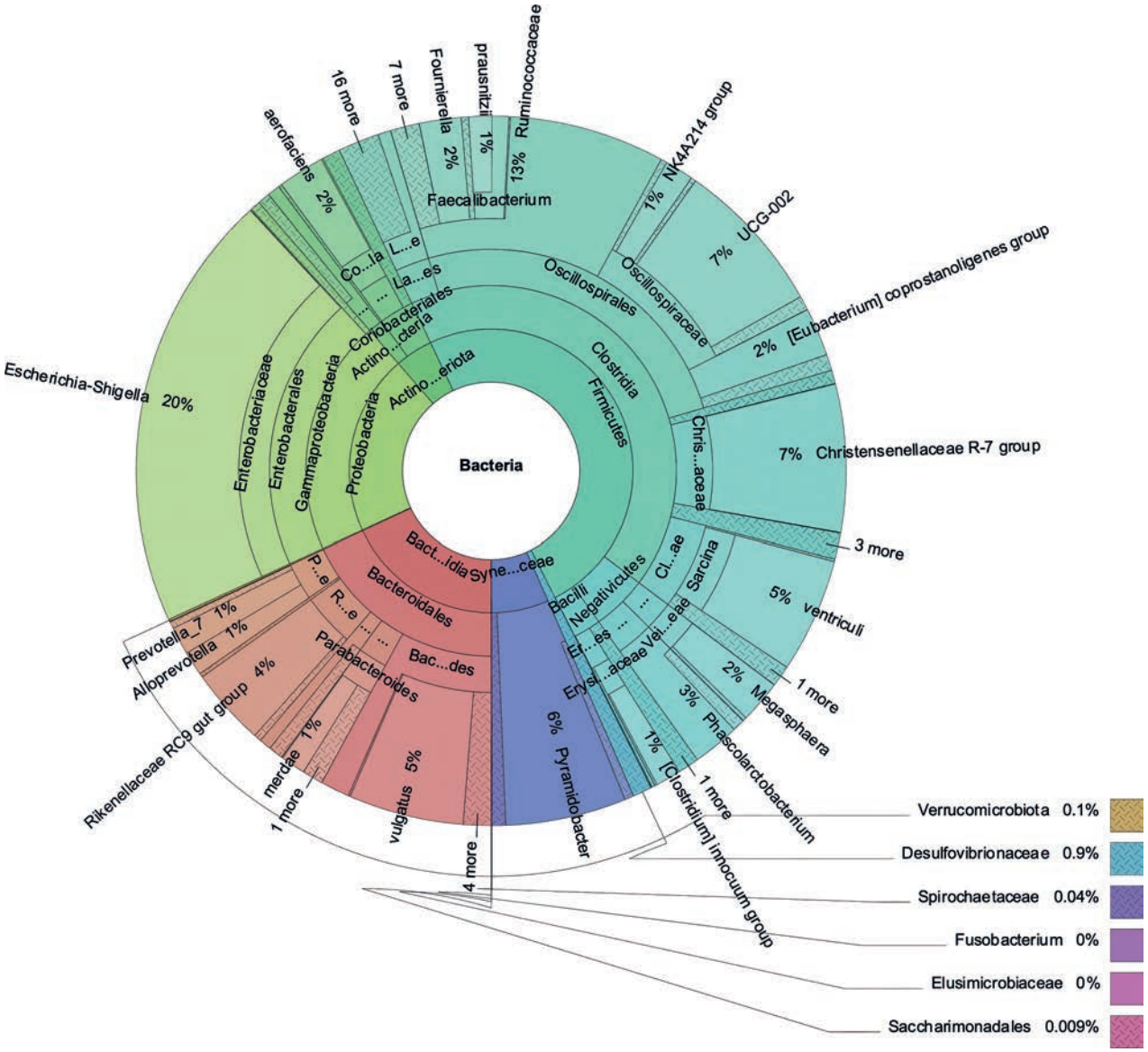

Figure S5. Krona diagram of a patient with Hirschsprung's disease (Sample 5).

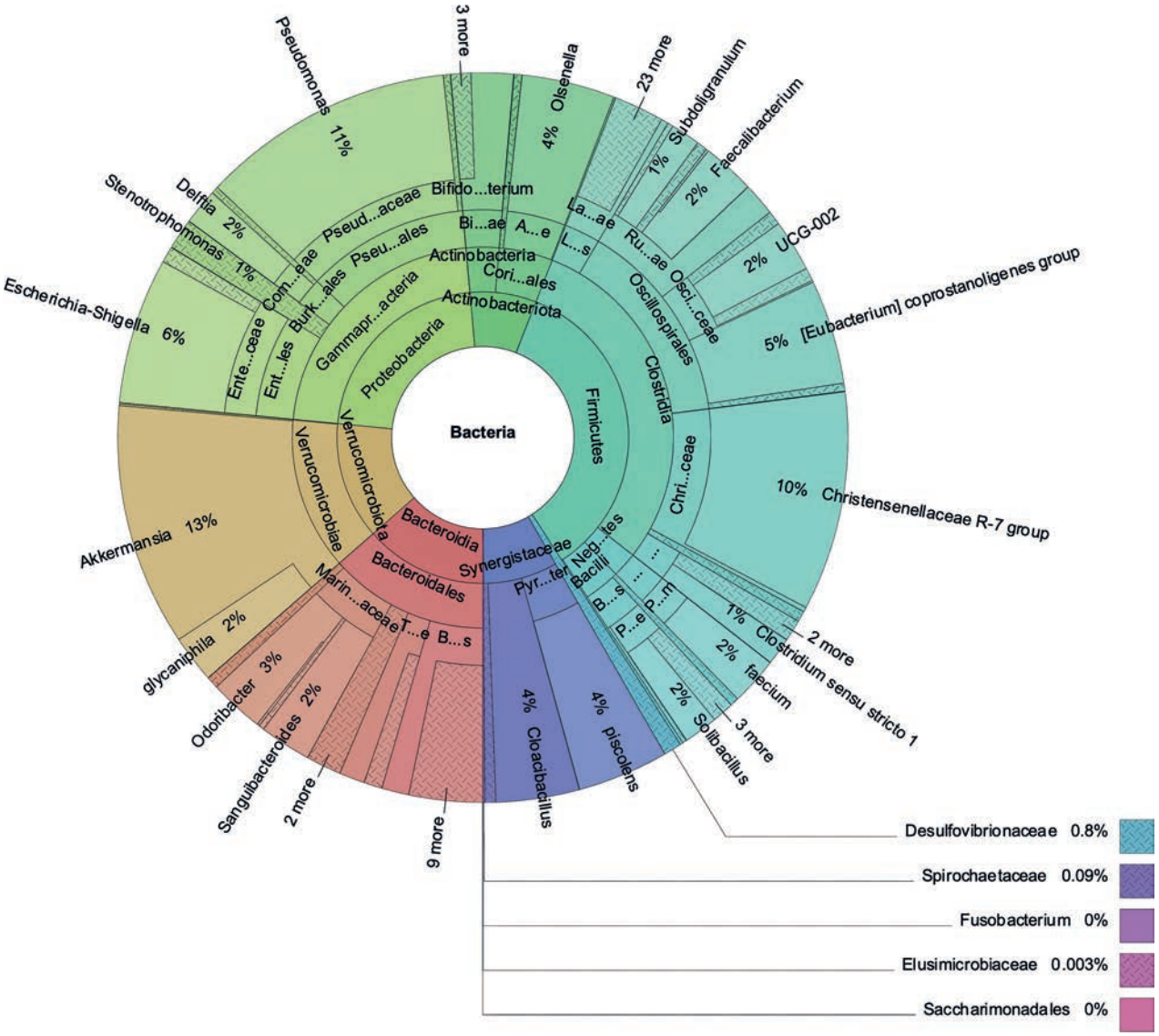

Figure S6. Krona diagram of a patient with Hirschsprung's disease (Sample 6).

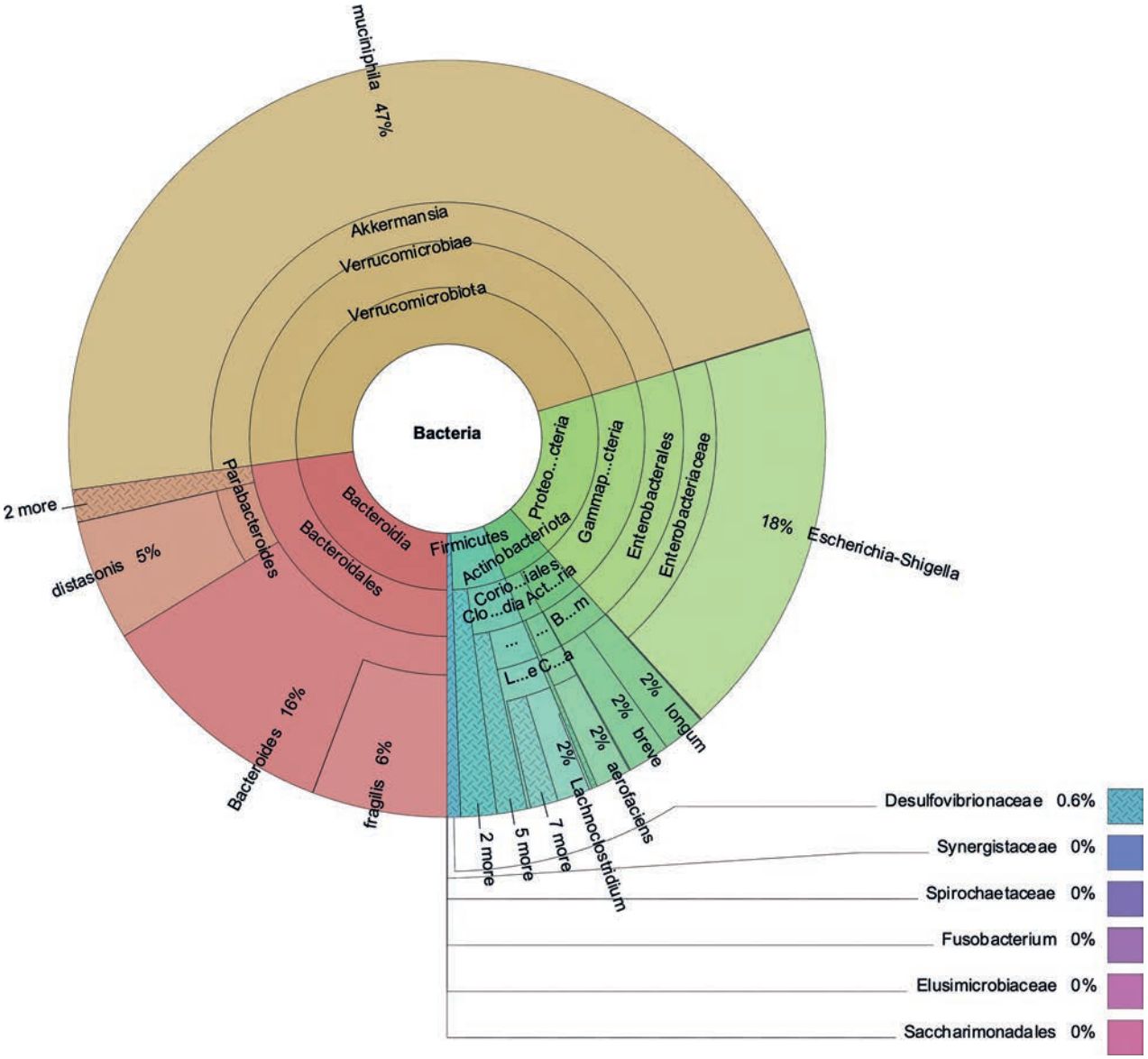

Figure S7. Krona diagram of a patient with Hirschsprung's disease (Sample 7).

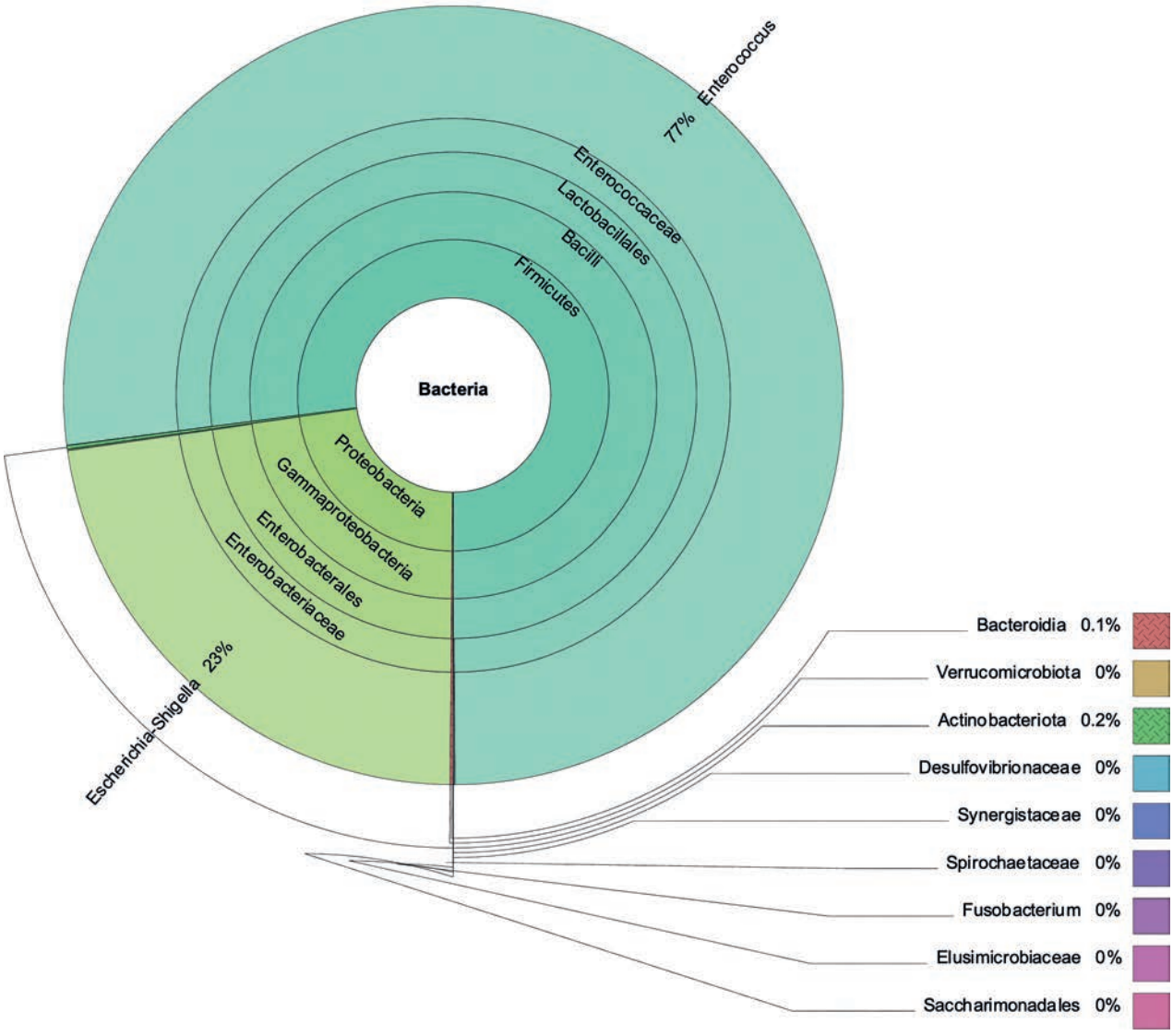

Figure S8. Krona diagram of a patient with Hirschsprung's disease (Sample 8).

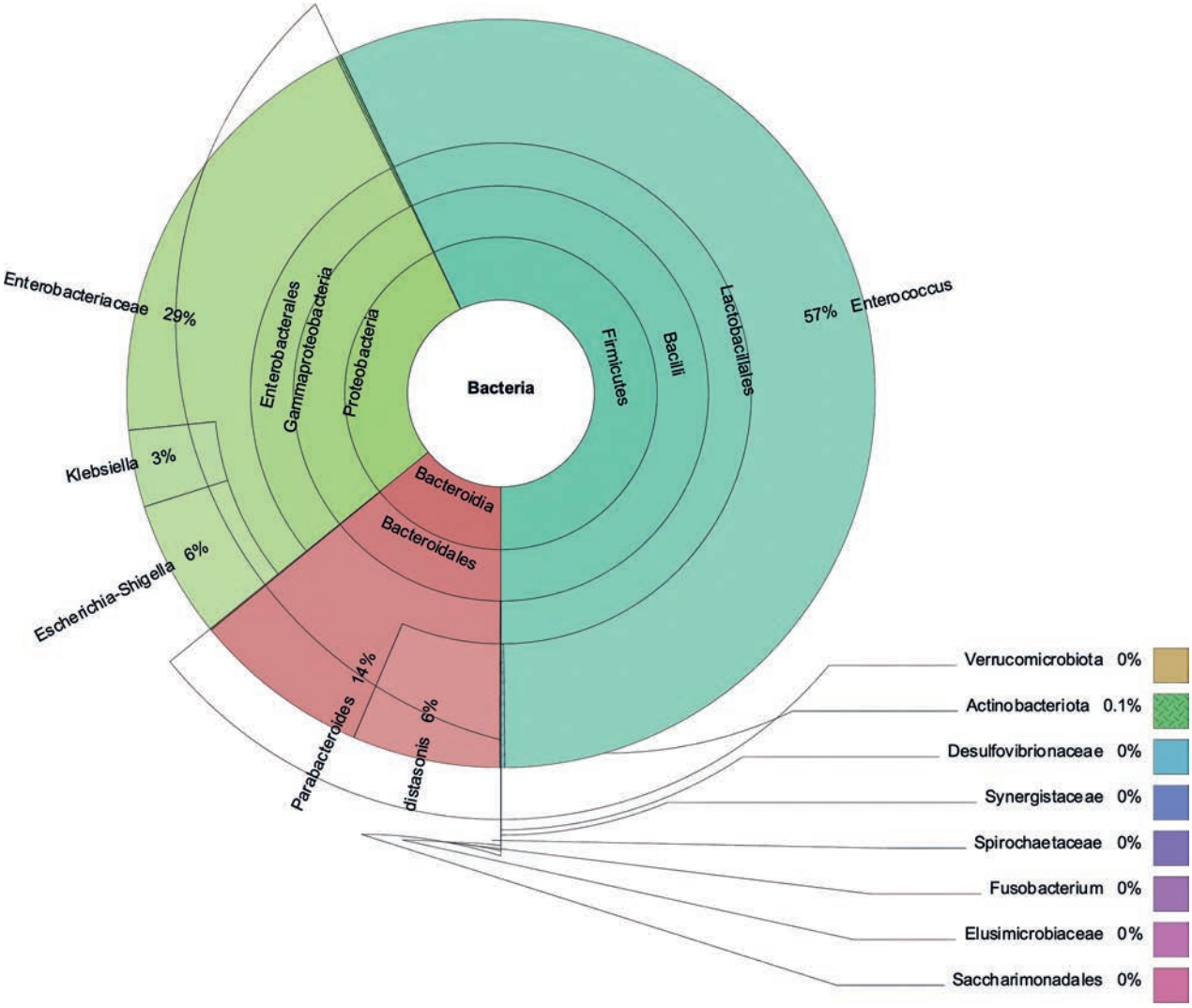

Figure S9. Krona diagram of a patient with Hirschsprung's disease (Sample 9).

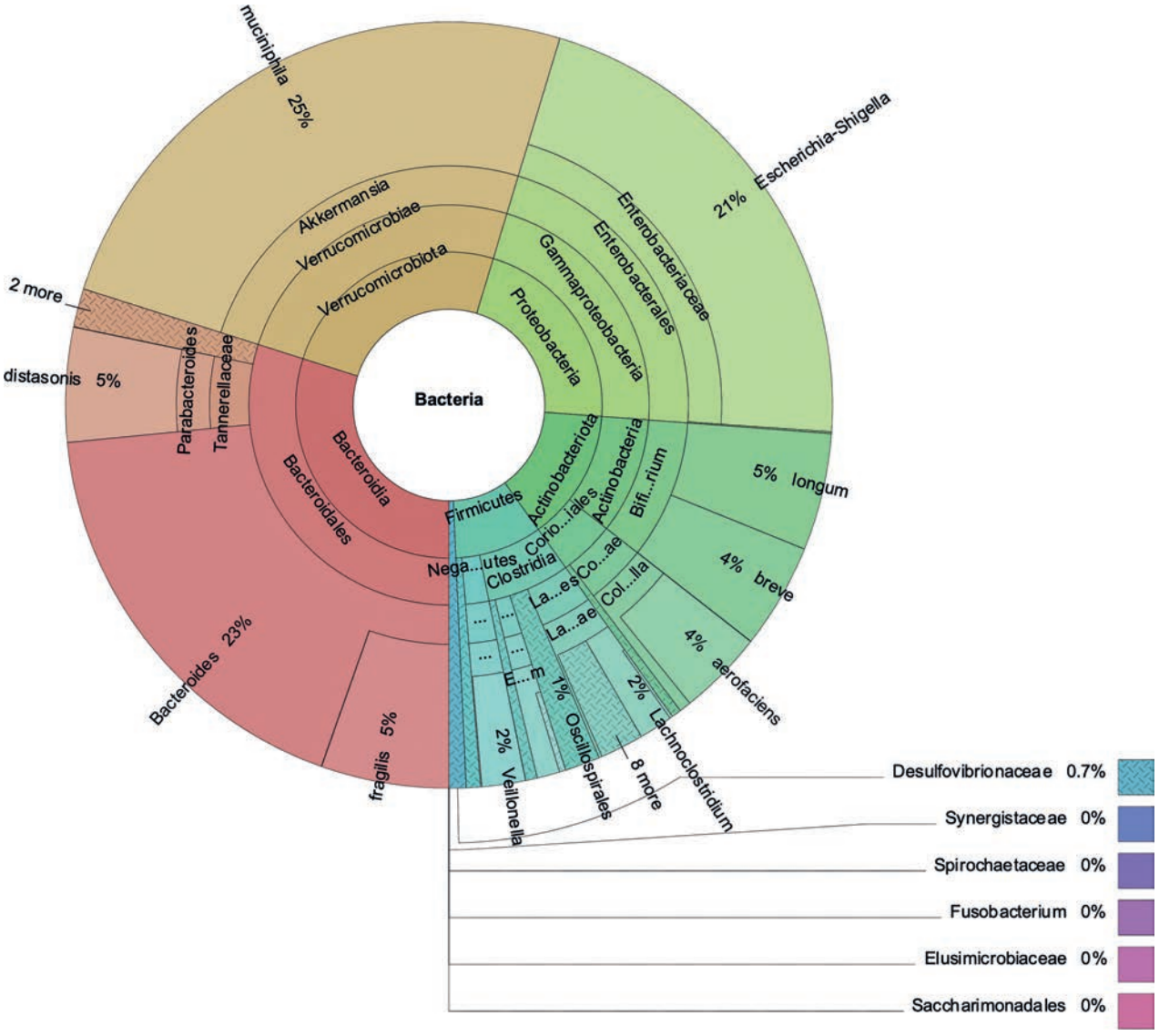

Figure S10. Krona diagram of a patient with Hirschsprung's disease (Sample 10).