# FECAL AND MUCOSAL MICROBIOTA PROFILING IN PEDIATRIC INFLAMMATORY BOWEL DISEASES

***Short title: Gut Microbiota in Pediatric IBD***

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Conflict of interest: none declared

Source of funding: Research Fund "Ateneo" Sapienza University of Rome and Finalized Research from Bambino Gesù Children's Hospital, IRCCS, Rome

**Abstract**

**Background**. An altered gut microbiota profile has been widely documented in inflammatory bowel diseases (IBD). Most studies are focused only on stool samples, and mainly in adult patients.

We aimed to define the gut microbiota profile either by assessing fecal and colonic mucosa samples (inflamed or not) of pediatric IBD patients,

**Patients and Methods**. Fecal and colonic samples from pediatric IBD (Crohn's Disease or Ulcerative Colitis) and controls (CTRLs) were selected. The relative abundance of bacteria, at phylum and genus/species levels, and bacterial diversity were determined through 16S rRNA sequence-based of fecal (FM) and mucosal microbiota (MM) analysis.

**Results**. 59 children with IBD (26 Crohn's disease, 33 ulcerative colitis) and 39 controls were investigated. A clear separation between IBD and CTRLs in the overall composition of FM and MM was found, as well as a reduced bacterial richness in FM of IBD. At phylum level, abundance of Proteobacteria and Actinobacteria occurred in FM of IBD, while species with anti-inflammatory properties (i.e. Ruminococcus) were reduced. Fusobacterium prevailed in inflamed IBD areas in comparison to non-inflamed and CTRLs samples.

**Conclusions**. Significant alterations in gut microbiota profile were shown in our IBD pediatric patients. Our data clearly indicate an abundance of species with a proinflammatory mucosal activity. An analysis of gut microbiota should be incorporated in designing future personalized treatment scenarios in IBD.

Key words: children, Crohn's disease, dysbiosis, inflammatory bowel disease, microbiota, ulcerative colitis

## Introduction

Over recent years, a wide range of studies have described disruptions in the gut microbiome profile and function in subjects with intestinal and extraintestinal disorders [1]. This deviation from regular microbial balance in the human gut, referred to as dysbiosis, has been reported in several chronic intestinal diseases, in particular in inflammatory bowel diseases (IBD). The latter include Crohn's disease (CD), ulcerative colitis (UC) and unclassified IBD (IBD-U) [2] and result from complex interactions between genetics, environment and the host immunity leading to an abnormal immune response towards intestinal microbes and, subsequently, to a chronic unremitting intestinal inflammation [3].

A healthy gut microbiota is necessary for intestinal immunological homeostasis and function. Normally, it is dominated by Bacteroidetes, Firmicutes and, less relevantly, by Proteobacteria and Actinobacteria. Numerous external factors, such as diet, probiotics, prebiotics, antibiotics, fecal microbiota transplantation, can affect structure and function of gut microbial community [4]. The greater prevalence of IBD in the Western countries, as well as in the newly industrialized geographic areas, has been related to detrimental diet components, acting through gut microbial changes [5]. The altered diversity and richness of the gut microbiota and the colonization with specific pathobionts might be significant triggers of IBD and their maintenance.

Investigating pathogenetic variables in pediatric IBD has recently attracted the interest of the scientific community, since pediatric IBD represent an evolutionary phase of the disease in which the underlying mechanisms are much less influenced by exogenous factors and comorbidities as compared to IBD in adulthood. Recently a research interest has increased toward the study of gut microbiota profile in pediatric IBD; Moreover, investigating differences between fecal and mucosal microbiota has gathered a special interest [6].

Thus, the aim of this cross-sectional study was to evaluate the gut microbiota profile from both fecal and mucosal specimens in pediatric IBD patients, compared to age-matched controls, by using a targeted-metagenomics approach. In addition, mucosal samples from inflamed and non-inflamed mucosa were collected and compared in IBD patients.

**Patients and Methods**

Pediatric patients (<18 years) with a diagnosis of IBD according to the recent Porto Criteria [7] were prospectively recruited at the Pediatric Gastroenterology and Liver Unit, Sapienza University of Rome. Clinical activity was defined by a PCDAI >10 [8] and a PUCAI >10 [9] for CD and UC, respectively.

In CD patients,, endoscopic activity was described with the Simple Endoscopic Score for Crohn's Disease SES-CD [10]: the size of mucosal ulcer, the surface involved by ulceration and by inflammation and the presence of stenosis were graded from 0 to 3 in each bowel segment; a total score of 0-2 indicated remission, 3-6 mild endoscopic activity, 7-15 moderate endoscopic activity, and >15 severe endoscopic activity. In UC patients the Mayo sub-score was used as follows [11]: grade 0 normal mucosa; grade 1 mild disease (erythema, decreased vascular pattern, mild friability); grade 2 moderate disease (marked erythema, erosions, absent vascular pattern, friability); grade 3 severe disease (spontaneous bleeding, ulcerations).

We included subjects as controls (CTRLs) if they fulfilled the following criteria: absence of chronic pain conditions, infectious or inflammatory disorders, neuro-behavioural illness, or administration of systemic-acting drugs.

Within the two weeks prior to the endoscopy, all patients followed an identical diet in term of oligo-fructose, lactulose, inulin-containing juices, and fibres to avoid microbiota and transit time alterations. A standardized questionnaire for the GI symptoms and the

quality of life was administered the day before the colonoscopy to all children or their parents [12].

The study was performed in accordance with the principles of the declaration of Helsinki and was approved by the Medical Ethics Committee of Sapienza University (CE: 4032, Prot n 281/16). All participants and their parents or caregivers signed the informed consent before the enrolment.

During the ileocolonoscopy mucosal specimens were obtained from the colonic mucosa of CTRLs and from inflamed (I) and non-inflamed (NI) colonic regions of IBD patients. The not washed biopsies were sent at controlled temperature (dry ice) to the Human Microbiome Research Unit of the Children's Hospital and Research Institute "Bambino Gesù" in Rome, Italy and immediately stored at -80 °C at the local BBMRI OPBG Microbiota biobank.

Fresh fecal samples were collected from IBD patients and CTRLs the day before the endoscopy. Once collected, faeces were transferred at controlled temperature, to OPBG biobank, in less than 1 hour, to strictly prevent bacterial overgrowth and stored at -80 °C before DNA extraction.

Targeted-Metagenomics. Fecal DNA was extracted by QIAamp DNA Stool Mini Kit, while mucosal DNA was extracted by using the Bio Robot EZ-1 (Qiagen, Germany), following manufacturer's procedures. The V1-V3 region of the 16S ribosomal RNA (rRNA) locus was amplified for pyrosequencing utilizing a 454-Junior Genome Sequencer (Roche 454 Life Sciences, Branford, USA). Primers and barcode sequences were previously described [13]. Reads were analysed by Quantitative Insights into Microbial Ecology (QIIME, v.1.8.0) [14], according to the pipeline described in [15]. After demultiplexing, sequences, with an average quality score upper than 25, length longer than 300 bp and without an ambiguous base calling were selected and denoised [14]. The chimeras were checked by *identify_chimeric_seqs.py* using either Blast fragments

and Chimera Slayer (http://qiime.org/scripts/identify_chimeric_seqs.html) approaches. The OTUs with a 97% of similarity were picked and the representative sequences were aligned by PyNAST [15]. The sequence clustering was performed by UCLUST [16] and the database used was greengenes (v 13.8). The ecological analyses (α and β-diversity) were performed by QIIME software, using *alpha_rarefaction.py*, *beta_diversity_through_plots.py*. The nonparametric test followed by Monte Carlo permutations was used to determine the *p*-value for α-diversity comparisons by compare_alpha_diversity.py. β-diversity statistical analyses were performed by compare_categories.py script, which applied a Permanova test determining a significance p value through permutations [17].

Kruskal-Wallis test was performed by the group_significance.py script to compare OTU relative abundances across samples [17]. Taxonomic levels phylum and genus/species were studied, and raw p value $p<0.05$ and false discovery rate adjusted P (p FDR) $<0.05$ were considered as statistically significant.

**Results**

Fifty-nine patients (26 with CD, 33 with UC) were enrolled. Thirty-nine asymptomatic subjects served as controls (CTRLs): 29 of them underwent colonoscopy for rectal bleeding in the absence of abdominal symptoms (i.e., juvenile polyps, anal fissure refractory to local therapy, screening for familial history of polyposis). Table 1 reports the demographic and clinical characteristics of children with IBD and CTRLs. Fifty-nine mucosal samples from IBD patients and 29 from CTRLs, and 37 stool samples from IBD and 39 from CTRLs were collected (Table 2).

*Gut microbiota profiles from mucosal and fecal samples*. A total of 280,253.60 sequencing reads was obtained from 88 mucosal samples, with a mean value of

3184.73 ± 1851.92 sequences per sample. From 76 stool samples a total of 331.803.31 sequences were obtained, with a mean value of 4365.83 ± 2452.36.

*Ecological analyses of fecal microbiota* (FM). The 37 IBD and 39 CTRLs stool samples were analysed in term of $\alpha$–diversity by Shannon and Chao1 indexes. This analysis revealed higher values of diversity for CTRLs compared to IBD samples ($p<0.05$) (Figure 1, panel A). Conversely, by comparing separately CD and UC patients with CTRLs, there was only a statistically significant difference for the Shannon index between CTRLs and CD patients (Figure 1, panels B and C). No statistically significant difference was observed between CD and UC patient indexes (Figure 1, panel D).

The Permanova analysis on β-diversity unweighted UniFrac matrix, visualized by Principal Coordinate Analysis (PCA), showed clear separation between IBD and CTRLs. Moreover, stratifying samples into CD and UC, a clearly defined cluster was identified in comparison with CTRLs, while no statistically significant separation was observed between CD and UC patients (Figure 2).

*Percent variation of stool bacteria*. The microbiota analysis of IBD stool samples showed at phylum level an increment of Actinobacteria, Fusobacteria and Proteobacteria and a decrease of Bacteroidetes, Tenericutes and Verrucomicrobia ($p < 0.05$) compared to CTRLs (Table 3). No statistically significant difference was observed between CD and UC. In comparison to CTRLs, CD patients showed an increase of Tenericutes, while UC patients showed a reduction of Bacteroidetes and Verrucomicrobia and an increase of Fusobacteria ($p <0.05$**)** (Table 3).

Moreover, at genus/species level, a predominant presence of *Haemophilus parainfluenzae* in IBD and *Oscillospira* and *Ruminococcus* in CTRLs ($p < 0.05$) was observed (Figure 3, panel A). By comparing UC and CD, CD samples were characterized by *Bacteroides fragilis*, UC samples by *H. parainfluenzae*, *Proteus*,

*Ruminococcus gnavus*. Between CD and CTRL, *Bacteroides ovatus* was incremented in CD, while *Oscillospira*, *Ruminococcus* and *Parabacteroides* were more abundant in CTRLs. Finally, *H. parainfluenzae* and *R.* gnavus were higher in UC than in CTRLs, in which *Ruminococcus* was higher (Figure 3, panels B-D).

*Alpha and beta diversity analysis of mucosal microbiota* (MM). For MM analysis, biopsies samples were taken from 29 CTRLs and 59 IBD (26 CD and 33 UC). Twenty-nine IBD biopsies were from inflamed areas (IBD-I) and 30 non-inflamed areas (IBD-NI) (Table 2). The comparison of Shannon and Chao1 indexes between CTRLs and IBD-I, between IBD-I and IBD-NI, between UC and CTRLs revealed not statistically significant differences. (Figure 4, panels A, B and D). Instead, the comparisons between CD and CTRLs and between UC and CD revealed a statistically significant decreasing for both indexes and for both comparisons in CD (Figure 4, panels C and E).

The β-diversity, visualized by PCA, showed clear separation between IBD and CTRLs (Permanova p value<0.05). Moreover, stratifying samples into CD and UC, a clearly defined cluster was identified in comparison with CTRLs and between each other (Permanova p value<0.05), while no separation was observed between IBD-I and IBD-NI (Permanova p value>0.05) (Figure 5, panels A-E).

*Percent variation of bacteria in gut mucosa*. At phylum level, the analysis showed an increment of Actinobacteria in IBD-I if compared to CTRLs, of Tenericutes in IBD-I compared to IBD-NI, of Firmicutes in CTRLs compared to IBD and of Actinobacteria in UC compared to CTRLs (Table 4). At genus/species level, mucosal samples IBD-I was enriched of *F. prausnitzii*, while *Dorea*, *Bacteroides fragilis* and *R. gnavus* were more abundant in CTRLs ($p < 0.05$) (Figure 6, panel A). *Fusobacterium* was more prevalent in IBD-I group compared to IBD-NI ($p < 0.05$) (Figure 6, panel B). CTRLs samples presented a higher presence of *R. gnavus*, *Dorea* and *Prevotella copri* in comparison to CD ($p < 0.05$) (Figure 6, panel C). UC samples were conversely characterized by a

higher presence of *F. prausnitzii* and a lower prevalence of *B. fragilis*, *Dorea* and *R. gnavus* in comparison to CTRLs ($p < 0.05$) (Figure 6, panel D). Finally, by comparing UC and CD, we observed that *B. ovatus* was associated with CD, while *P. copri* and *H. parainfluenzae* with UC ($p < 0.05$) (Figure 6, panel E).

## Discussion

An alteration of the gut microbiota, referred to as dysbiosis, has been widely described in IBD [18,19]. We found a clear separation between overall composition of both FM and MM between IBD groups and CTRLs and a significantly reduced bacterial richness in IBD stool specimens. These features are in line with similar previous data [18–21]. The α and β diversities of FM were instead similar in CD and UC groups.

At phylum level, in FM of IBD patients a reduced abundance of Bacteroidetes, Tenericutes and Verrucomicrobia and an increased presence of Proteobacteria and Actinobacteria were detected. At species level, we observed a predominance of *H. parainfluenzae* and a decreased presence of *Ruminococcus* and *Oscillospira* in IBD groups in comparison to CTRLs. Indeed, several studies have reported that IBD patients tend to have reduced abundance of Bacteroidetes and Firmicutes, along with an enrichment in Proteobacteria [2,22]. The increase of Proteobacteria has been often attributed to the presence of members of Enterobacteriaceae family, especially adherent-invasive *E. coli* strains, suggesting a role in disease pathogenesis [23,24]. Curiously, we found a predominant presence of H*. parainfluenzae* (phylum of Proteobacteria) in IBD, mainly in UC patients. These features confirm previous observations of an overrepresentation of *Haemophilus* in fecal samples from children with UC [25]. Recent data from a large prospective pediatric CD study show that *H. parainfluenzae* was significantly associated with newly diagnosed CD [26]. Interestingly, *Haemophilus* spp. are known as important causes of respiratory tract disease, due to

their mucosal inflammation properties [27], however their role in IBD pathogenesis is still unclear and deserves further investigation.

*Ruminococcus* spp. were more abundant in CTRLs than in IBD stools. Several species from this genus are normally found in the human gut microbiota and a decreased abundance in fecal samples isolated from IBD patients is well known [28]. Remarkably, these bacteria can degrade dietary cellulose and complex polysaccharides, in order to produce acetate, a short-chain fatty acid (SCFA), and other important energy sources for the gut microbiome [29]. Moreover, the lacking of *Oscillospira* in both CD and UC patients is already reported in a meta-analysis of microbiota studies in patients with IBD [30]. Interestingly *Oscillospira* has been significantly correlated with production of secondary stool bile acids, that are known to protect against infection from *Clostridium difficile* [31].

Analyses of α-diversity in MM samples revealed a decrease in CD compared to CTRLs and UC patients. Indeed, most studies that compared mucosal and fecal microbiota composition of IBD patients with controls have reported decreased microbial diversity and richness among IBD adult and pediatric patients [26]. We found a clear differentiation in the overall structure of MM between IBD and CTRLs, confirmed by subgroup comparisons CD-CTRLs, UC-CTRLs and UC-CD. Interestingly, no difference has been highlighted between inflamed and not inflamed mucosa.

Various studies have demonstrated no difference between IBD inflamed and non-inflamed tissues from adults, using different tools, such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) [32], temporal temperature gradient gel electrophoresis (TTGE) [33,34], fluorescence in situ hybridization (FISH) [33], microarray analysis [35] and 16S rRNA-targeted metagenomics [13]. Moreover, Kansal et al. recently studied the gut MM in a pediatric CD cohort with longitudinal

sampling, not finding significant differences between inflamed and non-inflamed samples [26].

At phylum level, an increment of Firmicutes in CTRLs compared to CD patients was observed in MM. Firmicutes phylum dominates the gut microbiota of healthy individuals along with Bacteroidetes [22,36]. Ruminococcaceae represent one of the most prevalent bacterial families of the human gut microbiota and plays a significant part in gut homeostasis, as mentioned above. Furthermore, the analysis showed an increment of Actinobacteria in UC compared to CTRLs as previously described [2,37].

Interestingly, IBD-I group showed a higher prevalence of phylum Fusobacteria and genus Fusobacterium, than IBD-NI. Fusobacterium spp. involvement in IBD is well known [20,26], due to the adherent and invasive abilities of these bacteria, in addition to their likely role in the progression of colorectal carcinoma, a long-term complication of IBD [38]. Moreover, we found a higher presence of *F. prausnitzii* in MM of IBD, particularly UC, compared to CTRLs, in marked contrast to numerous other studies that have showed its underrepresentation in IBD [20,39]. *F. prausnitzii* is a marker of a healthy gut microbiome and seems to have a protective role against mucosal inflammation, by stimulating the production of anti-inflammatory cytokines, such as IL-10, by down-regulating inflammatory pathways [40] and by producing butyrate, an anti-inflammatory member of SCFAs [41]. Our findings are instead in line with few studies showing a higher abundance of *F. prausnitzii* in pediatric CD [42], thus suggesting that further investigations are warranted.

Finally, we found an overall prevalence of *Ruminococcus*, *Dorea*, *Prevotella copri* and *Bacteroides fragilis* in MM of CTRLs compared to IBD patients. *Dorea*, such as the aforementioned *Ruminococcus*, is a producer of SCFAs in the gut [43]. In addition, *P. copri* and *B. fragilis* play an essential role in metabolic activities, such as the degradation of indigestible carbohydrates [44], with beneficial effects on the host immune system. *B.*

*fragilis*, in particular, produces polysaccharide A, which induces the secretion of the anti-inflammatory cytokine IL-10 and has a role in regulating pathogenic/Treg cell balance [45]. Remarkably, mice that are colonized by *B. fragilis* or treated with purified PSA seem to be protected from the induction of experimental IBD [46].

The present study has some limitations. Biopsies were collected from colonic mucosa only, excluding ileal tissue, which might have provided further information. However, we limited the number of mucosal samplings to reduce both the duration of ileocolonoscopy and risks for children. Since we wanted to compare MM and FM, we decided to focus on colonic biopsies to better compare the two microbial environments. Moreover, this is a cross-sectional study, which does not examine the long-term dynamics of the gut microbiome: however, repeating or keeping on with the same diet before the endoscopy for pediatric patients, making a longitudinal study difficult to perform.

Our data confirm that fecal and mucosal microbiota profiles in children with IBD can be discriminated from controls. This mainly results from increased or reduced abundance of certain bacterial communities, which typically colonize the healthy gut microbiome, rather than the presence of pathogenic species [47]. In the present study higher values of bacterial diversity from CTRLs was statistically evident in FM, but not in MM samples, while a clear separation between CTRLs and patients was evident at the analysis of β-diversity both in FM and MM samples. If these features are related to the young age of the patients or to an early stage of the disease in several of our patients is still unclear, but it surely suggests further investigations. It is also of exciting interest the abundance of species with anti-inflammatory properties in control subjects compared to patients, while abundance of species with proinflammatory species was detected in IBD samples, thus confirming that gut microbiota in implicated in immunological homeostasis of human intestine.

An intriguing feature was the abundance of *F. Prausnitzii* in inflammatory MM from UC patients compared to controls. This agent is traditionally viewed as an anti-inflammatory agent and has been correlated to improved outcome in adults with CD (40) [40]. Intriguingly, there are observations on the reduction of stool abundance of *F. prausnitzii* in adults with CD who have responded to enteral nutrition [48]. However, the presence of *F. prausnitzii* in stool samples depend from different variables, such as the diet content in fibers and the integrity of mucus layer of gut mucosa [49,50]. It is also conceivable that the increased abundance of *F. Prausnitzii* in UC MM can be related to the early phases of the disease, that characterizes the pediatric IBD, while most data on this agent come from adult patients with CD.

In conclusion, the investigation of gut microbiota in human health and disease has gained a great interest in the scientific community with a focus on factors that modulate its composition and function. Thanks to new technologies such as high-throughput “omic” approach, a deep investigation of the pathogenetic variables underlying IBD will be available, opening up new therapeutic avenues to the personalized therapy of these disorders. With the use of next generation sequencing to characterize gut microbiota, the latter will be incorporated in a personalized therapeutic program of IBD.

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**Figure Legends**

**Figure 1 -** Alpha diversity of fecal microbiota (FM). Box plots of Shannon and Chaol indexes for each sample group**.** The plot represents the average, minimum, maximum values, 25$^{th}$ and 75$^{th}$ percentiles calculated for CTRLs vs IBD (panel A), CTRLs vs CD (panel B), CTRLs vs UC (panel C) and CD vs UC (panel D).

**Figure 2 -** Beta diversity of fecal microbiota (FM). Principal coordinates analysis plot of CTRLs and IBD groups (panel A), of UC and CD groups (panel B), CD and CTRLs (panel C), CTRLs and UC (panel D). The plots show the first two principal coordinates

(axes) for principal coordinates analysis (PCoA) using unweighted UniFrac algorithm. P value is calculated by Permanova analysis.

**Figure 3** - Global distribution of operational taxonomic units (OTUs) of fecal microbiota (FM). Bar chart representing Kruskal–Wallis test results on operational taxonomic units (OTUs) in genus/species for the comparison between IBD vs CTRLs (panel A), UC vs CD (panel B), CD vs CTRLs (panel C) and UC vs CTRLs (panel D). Each histogram in the plot represents taxa. The ordinate axis reports the values of relative abundance for each taxon.

**Figure 4 -** Alpha diversity of mucosal microbiota (MM). Box plots of Shannon and ChaoI indexes for each sample group. The plot represents the average, minimum, maximum values, $25^{th}$ and $75^{th}$ percentiles calculated for CTRLs vs IBD-I (panel A), IBD-I vs IBD-NI (panel B), CD vs CTRLs (panel C), UC vs CTRLs (panel D) and CD vs UC (panel E).

**Figure 5 -** Beta diversity of mucosal microbiota (MM). Principal coordinates analysis plot of CTRLs and IBD-I groups (panel A), of IBD-I and IBD-NI (panel B), CD and CTRLs (panel C), CTRLs and UC (panel D), UC and CD groups (panel E). The plots show the first two principal coordinates (axes) for principal coordinates analysis (PCoA) using unweighted UniFrac algorithm. P value is calculated by Permanova analysis.

**Figure 6 -** Global distribution of operational taxonomic units (OTUs) of mucosal microbiota (MM).Bar chart representing Kruskal–Wallis test on operational taxonomic units (OTUs) at genus/species level. The comparison between IBD-I vs CTRLs (panel A), IBD-I vs IBD-NI (panel B), CD vs CTRLs (panel C), UC vs CTRLs (panel D) and UC vs CD (panel E). Each histogram in the plot represents taxa. The ordinate axis reports the values of relative abundance for each taxon.