

Microbiota in Pediatric Inflammatory Bowel Disease

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Objective To test the hypothesis that compared with controls, children with inflammatory bowel disease (IBD) exhibit differences in the relationships between gut microbiota and disease activity.

Study design Children and adolescents (n = 69; median age, 14 years) with IBD and 25 healthy controls (median age, 14 years) were recruited for the study. The disease activity was determined according to the Pediatric Ulcerative Colitis Activity Index or the Pediatric Crohn Disease Activity Index. Cell counts of 9 bacterial groups and species in the fecal microbiota were monitored by real-time polymerase chain reaction analysis.

Results Although no major changes were observed in patients with ulcerative colitis, except for a decrease in bifidobacteria in the active state of IBD, children with active and inactive Crohn's disease (CD) had lower numbers of *Faecalibacterium prausnitzii* and bifidobacteria ($P < .05$), and patients with active CD had higher numbers of *Eshcherichia coli* ($P < .05$).

Conclusions The microbiota in children with CD is characterized by decreased numbers of *F prausnitzii* and increased numbers of *E coli*. (*J Pediatr* 2010; ■: ■-■).

The incidence of inflammatory bowel disease (IBD) in children living in North America was reported to be about 8 per 100 000 in 2003, with the incidence of Crohn's disease (CD) being more than twice that of ulcerative colitis (UC).¹ A study from France reported that in children and adults with IBD, the incidence of CD has increased significantly, but the incidence of UC has decreased, in recent years.² The pathogenesis of IBDs remains poorly understood. A specific question is why an increasing number of patients are acquiring the disease significantly earlier in life. Along with host genetic defects or defective host immunoregulation, an imbalance in the intestinal microbiota is considered crucial for the development of chronic intestinal inflammation.³ Thus, dysbiosis is thought to increase the vulnerability of the gut mucosa and possibly to be a factor in the development of IBD.⁴⁻⁷ Several studies have noted reductions in potentially beneficial microbes, such as *Bifidobacterium* species in CD and UC and the butyric acid-producing *Faecalibacterium prausnitzii* cluster in CD.^{5,8,9}

The *F prausnitzii* cluster represents a subgroup of the *Clostridium leptum* group, which comprises fibrolytic- and butyrate-producing microorganisms contributing to processes important to colonic health.^{10,11} *C leptum* and *C coccoides* groups comprise the majority of the phylum *Firmicutes*, one of the dominant bacteria phyla next to *Bacteroidetes* in the normal human fecal microbiota.¹²

To date, few studies have investigated the composition of the intestinal microbiota in children.^{13,14} The aim of the present study was to examine the composition of the gut microbiota of pediatric patients with IBD to determine whether any imbalances of the commensal microbiota exist and, if so, whether they are correlated with disease activity.

Methods

Sixty-nine patients (age range, 1-20 years; median, 14 years) with documented IBD were recruited in the University of Erlangen's Pediatric Gastroenterology Unit. Disease activity was determined according to the Pediatric Ulcerative Colitis Activity Index (PUCAI)¹⁵ or the Pediatric Crohn Disease Activity Index (PCDAI).¹⁶ Active UC (AUC) was defined as a PUCAI >10, active CD (ACD) was defined as PCDAI >10, CD in remission (CDR) was defined as a PCDAI ≤10, and UC in remission (UCR) was defined as a PUCAI ≤10. In addition, 25 healthy children (age range, 5-19 years; median, 14 years) were recruited as controls. Each participant or a parent, when appropriate, provided informed consent. The study was approved by the university's Ethics Committee. No antibiotic treatment was provided during the 4 weeks before the analysis.

ACD	Active Crohn's disease	PCDAI	Pediatric Crohn Disease Activity Index
AUC	Active ulcerative colitis		
CD	Crohn's disease	PUCAI	Pediatric Ulcerative Colitis Activity Index
CDR	Crohn's disease in remission		
FISH	Fluorescence in situ hybridization	qPCR	Quantitative polymerase chain reaction
GI	Gastrointestinal		
IBD	Inflammatory bowel disease	UC	Ulcerative colitis
IL	Interleukin	UCR	Ulcerative colitis in remission

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From the fresh stool sample provided by each subject, DNA was extracted using the Easy Mag DNA Isolation system (Bio-Merieux, Nuertingen, Germany) according to the manufacturer's instructions. Primers were selected to recognize similar bacterial groups as previously published 16S rRNA-targeting probes used for fluorescence in situ hybridization (FISH) analysis. A particular FISH-defined group containing the appropriate target sequence in the ARB program () was selected, and quantitative polymerase chain reaction (qPCR) primers were designed with the ARB program to amplify the same group of commensal bacteria.¹⁷ Discriminating nucleotides were chosen to be at the 3' end of the primer, and a specific primer was combined with a universal primer that does not exclude any members of that particular group. Primers for bacterial groups were stringently selected using the Primer Designer program () to avoid primer-dimer formation and yield 100- to 300-bp products (Table I; available at www.jpeds.com). The standard line was based on actual counting of cultured bacteria and correlated directly to the Ct values of the qPCR. We validated the qPCR data through comparison with actual bacterial counts as reported by Barman et al.¹⁸ The specificity of the various qPCR primer sets had been tested previously,¹⁹⁻²⁶ and several of the primers had been used in at least one other previous study.^{27,28}

Quantitative PCR amplification and detection were carried out using the primers listed in Table I. PCR amplification and detection was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Darmstadt, Germany) in optical-grade 96-well plates sealed with optical sealing tape. Each reaction mixture (25 μ L) comprised 12.5 μ L of QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 2 μ L of primer mixes (10 pmol/ μ L each), 9 μ L of sterile distilled water, and 1.5 μ L of stool DNA (10 ng/ μ L). For the negative control, 2 μ L of sterile distilled water instead of the template DNA solution was added to the reaction solution. A standard curve was produced using the appropriate reference organism to quantify the qPCR values into numbers of bacteria per gram. The standard curves were prepared using the same PCR assay as used for the samples. The fluorescent products were detected in the final step of each cycle. A melting curve analysis was carried out after amplification to distinguish the targeted PCR products from the nontargeted PCR products. The melting curves were obtained by slow heating at temperatures of 55°C-95°C at a rate of 0.2°C/second, with continuous fluorescence collection.

Real-time qPCR was performed in triplicate, and average values were used for enumeration. PCR conditions were optimized based on those described in the literature.¹⁹⁻²⁶ The amplification program used for all primers consisted of one cycle of 95°C for 15 minutes and then 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds.

In all assays, the amplification efficiency was >90%, and the standard curve showed a linear range across at least 5 logs of DNA concentrations with a correlation coefficient

>0.9. The lowest detection limit of all assays was as low as 10-100 copies of specific bacterial 16S rDNA per reaction, corresponding to 10⁴-10⁵ copies per gram of wet-weight feces. All data were analyzed using ABI Prism software ().

Statistical Analyses

All statistical analyses were performed using SPSS (SPSS Inc, Chicago, Illinois). The normality of the data was checked using the nonparametric Kolmogorov-Smirnov test with Lilliefors correction. Before analysis for treatment differences, data were subjected to the Levene test for homogeneity of variances. Depending on the normality of the underlying data, analysis of variance or the Mann-Whitney *U* test was used for statistical analyses. Differences were considered significant at *P* < .05.

Results

Diagnoses included 21 ACD, 19 CDR, 13 AUC, and 16 UCR (Table II). Median activity indexes were 24 for ACD (range, 13-60), 0 for CDR (range, 0-10), 25 for AUD (range, 20-70), and 0 for UCR (range, 0-5). The IBD and healthy control groups did not differ in terms of age or sex.

Quantification of Predominant Bacterial Groups in Stool

Quantitative PCR analyses were performed to quantify individual bacterial groups in stool samples collected from the study group. Based on the universal bacterial primer, total bacterial cell numbers (log count per gram of feces) did not differ between patients with UC or CD and the healthy controls. No changes in cell numbers between the various disease states were detected. The most abundant bacterial groups detected were members of the *Bacteroides* and *Prevotella* genus, which form the majority of the phylum *Bacteroidetes*, and gram-positive bacteria belonging to the clostridial cluster XIVa (*C coccoides* group) or the clostridial cluster IV (*C leptum* group), which represent the majority of the phylum *Firmicutes* (Table II; Figures 1 and 2). Two other groups detected were *Escherichia coli*, the major representative of the *Proteobacteria* within the human microbiota, and the genus *Bifidobacterium*, the major representative of the *Actinobacteria*. Overall, with the primers used herein, we were able to cover a median of 90% of the total microbiota detectable with the universal primer.

Changes in bacterial groups were detected in the patients diagnosed with CD and UC (Table II; Figures 1 and 2). *Bifidobacterium* cell counts were lower in patients with AUC compared with healthy controls. Interestingly, patients with ACD and patients in remission had significantly lower fecal concentrations of *Bifidobacterium* and *F prausnitzii* compared with healthy controls (*P* < .05), and patients with ACD had significantly higher concentrations of *E. coli* (*P* < .05) (Table II).

Compared with healthy controls, in patients with ACD, the median *E coli* count increased from 9.5 \times 10⁵ (log₁₀ 5.97) to 5.3 \times 10⁸ (log₁₀ 8.72) cells per gram of feces,

Table II. Median concentrations by range, log₁₀ cells/g of feces, of microbiota in patients with IBD and controls

	PUCAI		PCDAI		Controls 0 (n = 25)
	≤10 (n = 16)	>10 (n = 13)	≤10 (n = 19)	>10 (n = 21)	
<i>Firmicutes</i>	10.11 ± 0.40	10.19 ± 0.50	9.53 ± 0.92	10.02 ± 0.80	10.09 ± 0.34
<i>C leptum</i> group (clostridial cluster IV)	9.97 ± 0.41	9.96 ± 0.51	9.44 ± 0.99	9.84 ± 0.82	9.94 ± 0.29
<i>F prausnitzii</i>	9.52 ± 0.69	9.53 ± 0.53	8.93 ± 1.27*	8.89 ± 2.16*	9.59 ± 0.48
<i>C coccoides</i> group (clostridial cluster IVa)	9.24 ± 0.56	9.25 ± 0.65	8.87 ± 2.77	9.28 ± 0.90	9.56 ± 0.49
<i>Lactobacillus/Enterococcus</i>	5.12 ± 2.27	5.40 ± 2.33	5.53 ± 1.51	5.49 ± 2.45	5.24 ± 2.07
<i>Eubacterium cylindroids</i>	7.61 ± 1.35	7.49 ± 1.25	6.85 ± 1.90	7.41 ± 1.90	7.13 ± 1.10
<i>Bacteroidetes</i>	9.77 ± 2.39	9.93 ± 0.41	9.87 ± 1.28	9.92 ± 2.19	9.70 ± 0.65
<i>Bacteroides</i> spp	9.77 ± 2.39	9.93 ± 0.41	9.87 ± 1.28	9.92 ± 2.19	9.70 ± 0.65
<i>Prevotella</i> spp	6.49 ± 2.15	6.32 ± 2.50	6.14 ± 1.74	6.46 ± 1.91	7.41 ± 1.70
<i>Actinobacteria</i>					
<i>Bifidobacterium</i> spp	6.87 ± 1.15*	7.12 ± 1.31	6.98 ± 1.40*	7.01 ± 1.06*	7.51 ± 0.64
<i>Proteobacteria</i>					
<i>E coli</i>	7.01 ± 1.42	7.81 ± 1.46	7.68 ± 1.40	8.72 ± 1.62†	5.97 ± 2.07
Total cell count	10.31 ± 0.35	10.37 ± 0.23	10.06 ± 0.58	10.24 ± 0.55	10.30 ± 0.28

*Significant decrease compared with controls ($P < .05$).†Significant increase compared with controls ($P < .05$).

representing up to 3% of the total microbiota (Figure 2). Interestingly, the ratio of *Firmicutes* to *Bacteroidetes* was changed in favor of *Bacteroidetes* in the ACD group compared with healthy controls. In patients with CDR, the ratio increased further in favor of *Bacteroidetes* (Figure 1).

However, because the individual numbers of the various bacterial groups representing the large phyla *Firmicutes* (clostridial clusters IV and XIVa) and *Bacteroidetes* (*Bacteroides* spp) were so widely scattered, no significant changes in proportions could be detected.

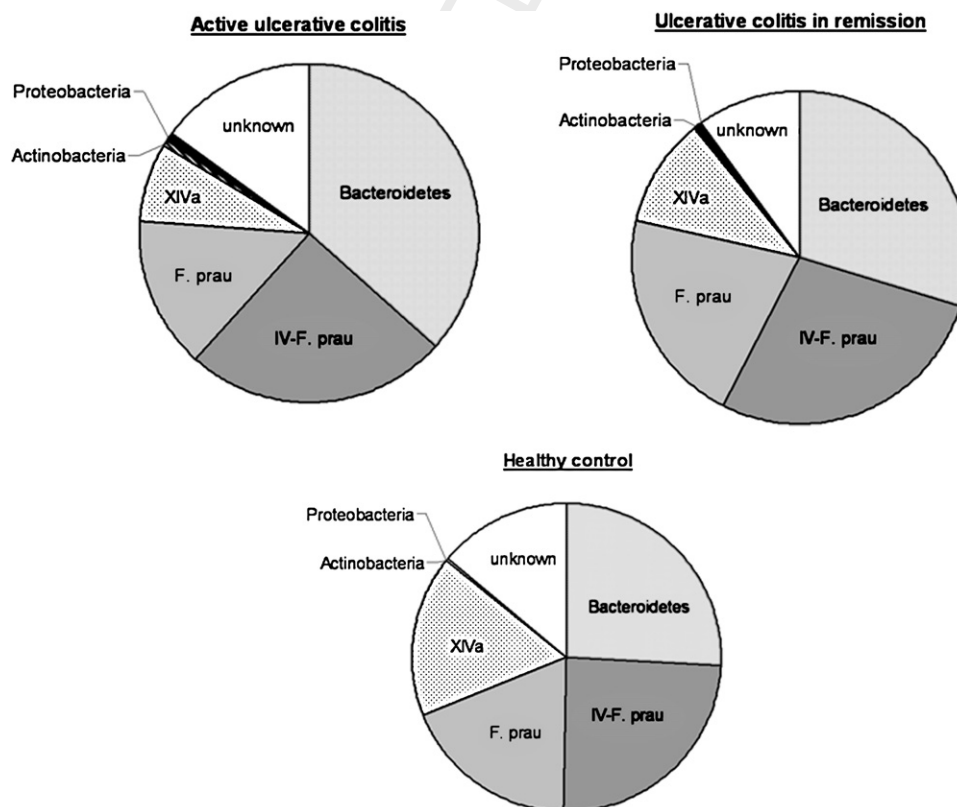


Figure 1. Mean proportions of different bacterial groups in feces of children diagnosed with ulcerative colitis compared with healthy controls (assessed by qPCR; Table II). IV, clostridial cluster IV; XIVa, clostridial cluster XIVa; F. prau, *F prausnitzii*. *F prausnitzii* is a member of clostridial cluster IV.

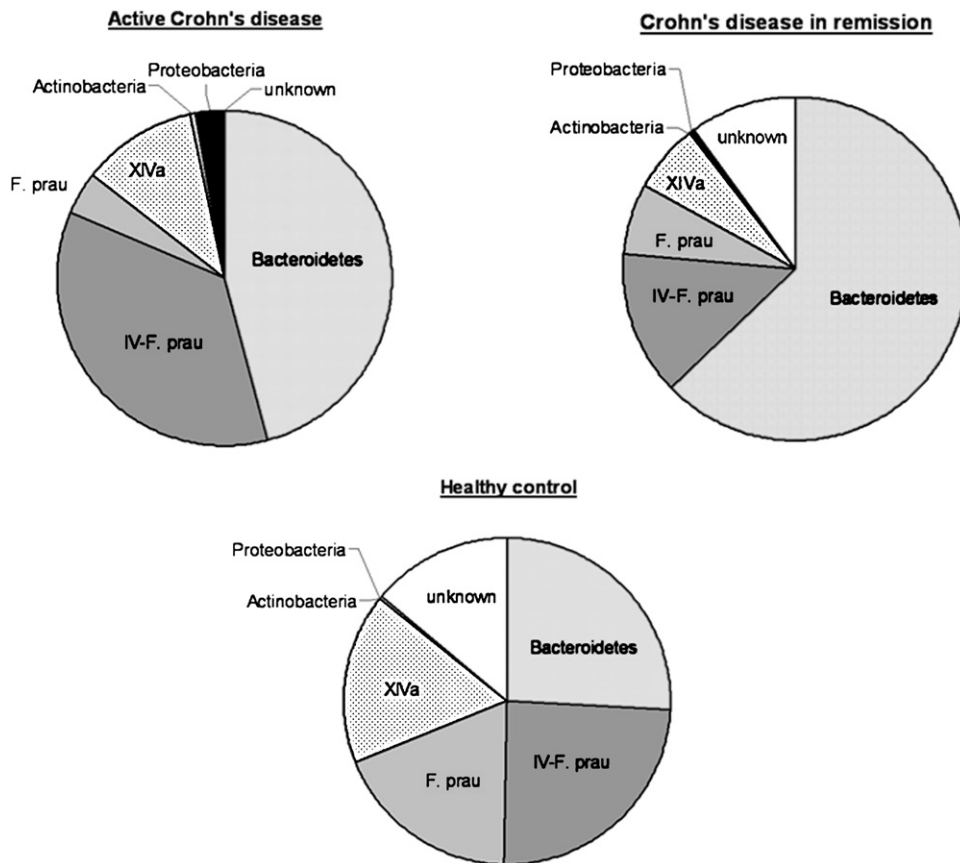


Figure 2. Mean proportions of different bacterial groups in feces of children diagnosed with Crohn's disease compared with healthy controls (assessed by qPCR; **Table II**). IV, clostridial luster IV; XIVa, clostridial cluster XIVa; F. prau, *F. prausnitzii*. *F. prausnitzii* is a member of clostridial cluster IV.

Discussion

CD and UC are thought to be the result of continuous microbial antigenic stimulation of pathogenic immune responses developing as a consequence of host genetic defects in mucosal barrier function, innate bacterial killing, or immunoregulation. The intestinal microbiota appears to be the target of immune reactivity, as has been demonstrated in various genetic studies and animal models of mucosal inflammation. Numerous studies have used molecular techniques to detect changes in the composition of fecal microbiota in patients with IBD;³ however, little data are available on the microbiota in children, which is a group distinct from adults in terms of disease onset and severity.^{13,29}

In contrast to previous results in adults,^{5,9} we detected no major changes in microbiota composition in patients with UC, except for decreased *Bifidobacterium* in patients with AUC (**Table II** and **Figure 1**). This might be because our study population was rather small. Because our study population was rather young, it also might be speculated that changes in microbiota composition in patients with UC may occur later in life. In contrast, our patients with CD exhibited changes in microbiota composition (**Table II** and

Figure 1). Decreased numbers of *Firmicutes*, particularly those of clostridial clusters XIVa and IV groups, has been reported in patients with CD.⁹ Other analyses have detected decreased concentrations of *F. prausnitzii*, a predominant species of the clostridial cluster IV, in patients with CD.^{8,30}

In our study group of children and adolescents with CD, *F. prausnitzii* likewise was decreased in the active and the remission phase of the disease (**Table II**). The median number of *F. prausnitzii* cells in healthy controls was 3×10^9 (log 9.59), which represented 18.62% of the total detectable bacterial species. In patients with ACD and CDR, the total number of *F. prausnitzii* cell was decreased to $<10^9$ cells per gram of feces and to proportions of $<5\%$ and $<8\%$ of the total microbiota, respectively (**Table II** and **Figure 2**). Interestingly, although the median total *F. prausnitzii* cell count was decreased in both groups, counts $<1 \times 10^6$ were more common in patients with CDR (data not shown).

It can be speculated that a dramatic reduction in the quantities of microbes that provide metabolic services to the host gastrointestinal (GI) tract exacerbate certain forms of IBD. Butyrate, which is produced exclusively by bacterial metabolism, is an important source of energy for colonic epithelial cells and may enhance the integrity of the epithelial barrier

and modulate the GI immune system. Butyrate also has been reported to modulate inflammation in IBD, possibly by down-regulating the production of proinflammatory cytokines. The *F prausnitzii* group of organisms is second to the *Roseburia* group as the most abundant group of butyrate-producing bacteria within the human gut.¹¹ *F prausnitzii* may be important not only for its provision of butyrate to the host, but also for its anti-inflammatory effects. *F prausnitzii* A2-165 has been shown to release high interleukin (IL)-10/IL-12 cytokine levels from peripheral blood mononuclear cells. It also can reduce IL-1 β -induced IL-8 secretion by Caco-2 cells, and its supernatant can abolish tumor necrosis factor- α -induced NF- κ B activity in HT-29 cells. Furthermore, both *F prausnitzii* A2-165 and its supernatant were found to reduce scores and blood measures of inflammation in TNBS-induced colitis in Balb/c mice, and, when administered intraperitoneally, its supernatant protected mice from death induced by TNBS.⁸ In addition, *Bifidobacterium* counts were decreased, even though *E coli* counts were increased, in patients with ACD. These results are in accordance with other studies in which increased *E coli* counts and increased proportion of *E coli* in the GI microbiota were associated with CD and lower bifidobacteria counts.^{9,31}

Although marked alterations in fecal and mucosal bacterial communities are seen in IBD, whether these alterations cause the disease or are due to changes in the gut environment resulting from inflammatory reactions and extensive tissue destruction is unclear. We have demonstrated that the microbiota changes in IBD are already present at a young age, at least in patients with CD. ■

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UNCORRECTED PROOF

Table 1. 16S rRNA gene-targeted group and species-specific primers used in this study

Target	Primer name	Primer sequence (5'-3')	Reference
Total bacteria	UniF340 UniR514	ACTCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGGC	32
<i>Firmicutes</i>			
<i>C leptum</i> group (clostridial cluster IV)	C-lept-F1123 C-lept-R1367	GTTGACAAAACGGAGGAAGG GACGGGCGGTGTGTACAA	20
<i>C coccoides</i> group (clostridial cluster XIVa)	Univ-F338 C.coc-R491	ACTCCTACGGGAGGCAGC GCTTCTTAGTCAGGTACCGTCAT	21
<i>E cylindroides</i> group	Univ-F338 E.cyl-R399	ACTCCTACGGGAGGCAGC CATTGCTCGTTCAGGGTTC	23
Lactobacilli/Enterococci	Lab-F362 Lab-R677	AGCAGTAGGGGAATCTCCA CACCCTACACATGGAG	22
<i>F prausnitzii</i>	PrausF480 PrausR631	CAGCAGCCGGTAAA CTACCTCTGCACTACTCAAGAAA	28
<i>Bacteroidetes</i>			
<i>Bacteroides</i>	Bact-F285 Univ-R338	GGTTCTGAGAGGAGGTCCC GCTGCCTCCCGTAGGAGT	19
<i>Prevotella</i>	Prevo-F449 PrevoR757	CAGCAGCCGGTAATA GGCATCCATCGTTACCGT	20
<i>Proteobacteria</i>			
<i>E coli</i>	EcoliF395 EcoliR470	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA	28
<i>Actinobacteria</i>			
<i>Bifidobacterium</i>	Bifido-F143 Univ-R338	CTCCTGGAAACGGGTGGT GCTGCCTCCCGTAGGAGT	25

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