Active Crohn's Disease and Ulcerative Colitis Can Be Specifically Diagnosed and Monitored Based on the Biostructure of the Fecal Flora

Alexander Swidsinski, MD, Vera Loening-Baucke, MD,* Mario Vaneechoutte, MD,† and Yvonne Doerffel, MD‡*

Background: The intestinal microflora is important in the pathogenesis of inflammatory bowel disease (IBD). The impact of its spatial organization on health and disease is unknown.

Methods: We investigated sections of paraffin-embedded punched fecal cylinders. Fluctuations in spatial distribution of 11 bacterial groups were monitored in healthy subjects ($n = 32$), patients with IBD ($n = 204$), and other gastrointestinal diseases ($n = 186$) using fluorescence in situ hybridization (FISH).

Results: The microbial structure differed in patients with Crohn's disease (CD), ulcerative colitis (UC), and healthy and disease controls. The profiles of CD and UC were distinctly opposite in 6 of 11 FISH probes used. Most prominent were a depletion of *Faecalibacterium prausnitzii* (Fprau$<1 \times 10^9$/mL) with a normal leukocyte count in CD and a massive increase of leukocytes in the fecal-mucus transition zone (>30 leukocytes/$10^4 \mu m^2$) with high Fprau in patients with UC. These 2 features alone enabled the recognition of active CD (Crohn's Disease Activity Index [CDAI] >150) or UC (Clinical Activity Index [CAI] >3) with 79%/80% sensitivity and 98%/100% specificity. The mismatch in the sensitivity was mainly due to overlap between single IBD entities, and the specificity was exclusively due to the similarity of Crohn's and celiac disease. When inflammatory bowel disease (IBD) patients were pooled the sensitivity was 100% for severe disease, 84% for moderate activity, 72% for IBD with ≤12 months remission, and 24% for IBD with >12 months remission.

Conclusions: The fecal flora is highly structured and spatially organized. Diagnosing IBD and monitoring disease activity can be performed based on analysis of punched fecal cylinders independent from the patient's complaints.

(*Inflamm Bowel Dis 2008;14:147–161)*

Key Words: intestinal flora, monitoring disease activity, CD, UC, IBD, FISH, celiac disease

Misbalance of the intestinal flora is thought to be crucial for the pathogenesis of inflammatory bowel disease (IBD). However, until now, neither the nature of this misbalance nor specific hazards or benefits of single shifts in bacterial composition could be defined.[1–3] The previous investigations of the fecal flora used smears, homogenates, or DNA isolates of stool.[1,3] The fecal flora is, however, unevenly composed. All natural-occurring microbiota are structured and spatially organized. The intestinal flora is probably the most sophisticated of all known bacterial communities. However, presently we do not know much about its biostructure.

We developed a new method using a punched-out fresh stool cylinder. After fixation and embedding the stool cylinder in paraffin, we were able to visualize bacteria on sections of the native feces in relation to each other and also in relation to core, surface, and mucus cover of feces using multicolor ribosomal rRNA fluorescence in situ hybridization (FISH). Our aim was to study the spatial structure of fecal microbiota and to correlate it to diagnosis and disease activity.

MATERIALS AND METHODS

Patients

The participating patients for this study were outpatients attending the Charité hospital between January and June 2007. All IBD and non-IBD patients had complete gastroenterological diagnostics including colonoscopy, gastroscopy, ultrasound, and laboratory investigation. Each participant delivered 3 fecal samples in ≈2-week intervals. Only stool samples of patients without change in the therapy 2 weeks prior to and during the study period were analyzed. The resulting groups are presented in Table 1. The healthy control group consisted of laboratory and medical staff and their relatives without intestinal complaints or known diseases.

Received for publication October 8, 2007; Accepted October 9, 2007.

From the *Humboldt University, Charité Hospital, Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms, Berlin, Germany, †Department Clinical Chemistry, Microbiology & Immunology, University Hospital Ghent Belgium, ‡University Poliklinik, Charité, Berlin, Germany.

Reprints: Alexander Swidsinski, *Humboldt University, Charité Hospital, Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms, 10098 Berlin, Germany (e-mail: alexander.swidsinski@charite.de).

DOI 10.1002/ibd.20330

Published online 29 November 2007 in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. Spatial Structure of Fecal Microbiota in Healthy, Disease Control Groups, and IBD (Mean ± SD)

		A	B	C	D	E	F	G	H	I	J	
		Healthy $n = 32$	IBS $n = 45$	Diarrhea $n = 33$	Upper gut disorder $n = 45$	Colonic disorder $n = 42$	Crohn's disease $n = 82$	UC $n = 105$	IC $n = 17$	Celiac disease $n = 12$	Slc $n = 9$	Differences
Age (year)	Mean	40.2	45.4	44.2	46.5	48.4	34.8	41.2	46.4	35	32	
	Range	18–60	24–72	20–82	26–66	38–85	17–78	18–84	19–80	22–68	19–66	
Mucus layer												F/G $P = 0.02$;
Thickness (μm)	Th	27 ± 43	58 ± 92	156 ± 337	68 ± 115	18 ± 29	19 ± 30	8.2 ± 23	12 ± 30	36 ± 95	30 ± 95	A/C, A/G $P < 0.001$
	O	66%	51%	45%	36%	57%	30%	22%	24%	42%	44%	AF ns.
Leukocytes	C	0	2	4–12	3–6	6–24	88 ± 281	358 ± 407	289 ± 399	0	30–48	F/G; FGH/other $P < 0.001$;
	O		1% (1)	6%	4%	7% (3)	23%	71%	53%		22%	
Erec	C	26 ± 6.1	20.1 ± 7.7	12.7 ± 6.8	20 ± 5.5	19.6 ± 7.9	15.3 ±	15.1 ± 7.8	13.5 ± 8.6	13.4 ± 8.6	21.5 ± 6.4	A/FG $P < 0.001$;
	O	100%	100%	100%	100%	100%	10.4 100%	100%	100%	100%	100%	C/FG $P < 0.01$.
Bac	C	19 ± 3.5	14.0 ± 11	7.6 ± 5.5	15 ± 5.2	17.9 ± 4.5	11.6 ± 6.8	9.1 ± 6.1	11.2 ± 5.2	10.1 ± 6.9	12.7 ± 7.1	A/FG $P < 0.001$;
	O	100%	92%	100%	100%	100%	96%	100%	100%	100%	100%	C/FG; F/G $P < 0.05$.
Fprau	C	14.9 ± 4.5	15.1 ± 10	9.1 ± 4.6	14.2 ± 5.2	16.3 ± 4.0	5.6 ± 5.9	13.9 ± 9.9	11.5 ± 5.6	5.5 ± 6.7	14.6 ± 5.2	F to all but I
	O	100%	100%	100%	100%	100%	49%	97%	58%	92%	100%	$P < 0.0001$.
Bif	C	0.7 ± 2.2	1.3 ± 2.2	1.6 ± 1.8	1.6 ± 2.4	0.9 ± 1.4	0.84 ± 2.9	2.8 ± 3.2	1.2 ± 1.7	1.3 ± 2.4	2.2 ± 2.9	G to all but J
	O	93%	74%	83%	84%	65%	44%	87%	74%	70%	82%	$P < 0.05$ (most < 0.001)
Bif $\geq 8 \times 10^9$bac/mL	O	3%	7%	7%	10%	0	3%	22%	25%	8%	6%	
Ebac fecal	C	0.1 ± 0.4	0.4 ± 1	2.0 ± 3.6	1.2 ± 2.5	1.5 ± 2.9	1.8 ± 3.0	0.5 ± 1.5	0.2 ± 0.5	0.5 ± 1.2	1.0 ± 2.3	A/BCDEF $P < 0.05$–0.001;
	O	31%	52%	54%	45%	66%	41%	67%	44%	56%	35%	F/G $P < 0.001$;CDEG ns.
Ebac mucotrop	C	0.6 ± 1.6	2.4 ± 3.7	4.7 ± 7.1	0.9 ± 1.6	1.3 ± 5.2	3.2 ± 6.5	5.9 ± 14	0.2 ± 2.7	2.7 ± 5.1	1.9 ± 3.2	CFG ns.
	O	71%	80%	75%	58%	71%	71%	70%	70%	65%	62%	CFG/ABDE $P < 0.05$–0.001;
Hel274 fecal	C	0.2 ± 0.6	0.14 ± 0.3	1.3 ± 1.1	0.2 ± 0.8	0.14 ± 0.5	0.4 ± 1.6	0.3 ± 0.8	0.17 ± 0.4	0.40 ± 1.4	0.4 ± 1.2	C to all $P < 0.01$–0.001.
	O	9%	8%	38%	20%	12%	13%	8%	6%	15%	18%	
Hel274 mucotrop	C	3.2 ± 7	4.2 ± 8.7	12.7 ± 16.2	3.7 ± 10.4	2.6 ± 6.2	0.9 ± 4.8	2.1 ± 7.1	0.9 ± 0.4	1.7 ± 4.5	4.6 ± 17.5	C to all $P < 0.01$–0.001;
	O	35%	34%	69%	33%	28%	12%	15%	10%	18%	28%	A/F $P < 0.05$; FG ns.
Ecyl	C	0.4 ± 1.7	0.4 ± 1.2	0.9 ± 1.2	0.5 ± 0.97	0.32 ± 1.1	0.6 ± 0.9	0.9 ± 1.40	0.6 ± 1.1	0.72 ± 0.84	0.4 ± 0.97	
	O	78%	85%	77%	86%	65%	42%	78%	76%	47%	50%	
Ehal	C	0.15 ± 0.8	0.3 ± 1.7	0.35 ± 1.3	0.1 ± 1.0	0.09 ± 1.1	0.15 ± 0.6	0.14 ± 0.4	0.4 ± 0.9	0.09 ± 0.4	0.1 ± 0.37	
	O	85%	90%	84%	71%	45%	39%	66%	76%	75%	68%	
Clit	C	0.09 ± 1.1	0.19 ± 1.5	0.1 ± 0.9	0.09 ± 1.1	0.04 ± 0.8	0.16 ± 0.4	0.08 ± 0.4	0.28 ± 0.6	0.1 ± 0.6	0.05 ± 0.09	
	O	73%	54%	50%	40%	50%	34%	57%	70%	24%	46%	
Chis	C	0.07 ± 1.4	0.12 ± 1.8	0.25 ± 1.9	0.09 ± 1.1	0.2 ± 0.49	0.16 ± 0.8	0.19 ± 0.6	0.41 ± 0.5	0.08 ± 0.11	0.02 ± 0.04	
	O	58%	55%	59%	45%	65%	56%	59%	63%	58%	63%	
Ato	C	0.8 ± 1.2	1.0 ± 1.0	0.8 ± 1.1	0.7 ± 1.2	1.1 ± 1.9	0.7 ± 1.6	1.4 ± 1.6	1.2 ± 1.2	1.2 ± 1.4	1.1 ± 1.4	FG $P = 0.02$;
	O	95%	96%	91%	93%	95%	78%	90%	85%	90%	93%	G/ABCD $P < 0.05$. to 0.001.

C, Concentrations of leukocytes as mean ± SD cells within a region of 100 × 100 μm. Concentrations of bacteria are expressed as mean ± SD × 10^9bacteria/mL. (Mean values are replaced by range if number of patients was less than 4.) O, Occurrence. Ns, not significant.

Inflammatory control groups were patients with self-limiting colitis and celiac disease. The noninflammatory control groups consisted of patients with irritable bowel syndrome (IBS), patients with diarrhea (idiopathic [$n = 22$], lactose intolerance [$n = 6$], and chronic pancreatitis [$n = 5$]), patients with upper gut disorders (liver cirrhosis [$n = 12$], NASH [$n = 11$], reflux esophagitis [$n = 9$], peptic ulcer [$n = 7$], and Barrett's esophagus [$n = 6$]), and patients with colonic disorders (diverticulosis [$n = 21$], colon polyps [$n = 11$], and constipation [$n = 10$]).

Crohn's disease (CD), ulcerative colitis (UC), and indeterminate colitis (IC) were diagnosed according to accepted criteria[4,5] prior to inclusion in the study and the diagnosis was left unchanged in the course of further investigations. Self-limiting colitis (Slc) was defined as acute unspecific colitis with unclear etiology that spontaneously healed within 3 months after the start of symptoms. CD and UC patients were further subdivided into those with high (Crohn's Disease Activity Index [CDAI] >300, Clinical Activity Index [CAI] >9), moderate ($150 \leq$ CDAI ≤ 300; $3 \leq$ CAI ≤ 9) activity, and those with remission of less than 12 months or more than 12 months. CDAI and CAI were calculated as previously published.[6,7] The groups of patients with indeterminate colitis and self-limiting colitis were too small to be further subdivided by disease activity.

Sample Collection and Handling

Stools were either dropped on cleansing tissue or on the dry flat surface part of the toilet, which is common in Germany. The 4–10 mm-long fecal cylinders were punched from the stool using a plastic drinking straw with a 3 mm inside diameter (Schlecker, Germany). The drinking straw was precut to 4 cm-long pieces and handed out to participants of the study together with a 50-mL Falcon tube filled with 30 mL of Carnoy solution (6/6/1 vol. ethanol/glacial acetic acid/chloroform). Participants were instructed how to puncture the stool cylinder. The pieces of drinking straw with the stool were put into the Falcon tube, fixated in Carnoy for 24 hours at room temperature, and then kept refrigerated at 4°C until delivered to the laboratory within 1 to 2 weeks. In the laboratory the straws with the enclosed fecal cylinder were removed and dipped in black ink to mark the internal portion of the stool cylinder. The fecal cylinder was then removed from the straw, embedded in paraffin using standard techniques, cut into 4-μm sections, and placed on SuperFrost slides (R. Langenbrinck, Emmendingen, Germany).

Light Microscopy

Alcian blue/PAS (Periodic Acid Schiff) stains were used for evaluation of mucus and leukocytes in the stool specimens and for orientation within cylinders. The mucus layer at 1 pole identified the surface of the cylinder, the ink stain on the opposite pole identified the deep or central portions of the feces.

FISH

Microscopy was performed with a Nikon e600 fluorescence microscope. The images were photo documented with a Nikon DXM 1200F color camera and software (Nikon, Tokyo, Japan). Hybridizations were performed in multicolor FISH according to previously described protocols for evaluation of tissue specimens and identification of bacteria.[8,9]

For each group-specific FISH probe, high-power ($\times$1000 magnification) images were made. High concentrated bacteria were counted within a 10×10 μm area of the microscopic field representative of the region of interest. Bacteria with uneven distribution or overall low concentrations were enumerated within larger areas of 100×100 μm, whole microscopic fields, or over the complete surface of the fecal cylinder.

The conversion of the numbers within defined microscopic areas to concentrations of bacteria per mL was based on the calculation that a 10-μL sample with a cell concentration of 10^7 cells per mL has 40 cells per average microscopic field at a magnification of 1000; the details of conversion were previously described.[9]

Leukocytes were enumerated in DAPI stain (large fluorescent blue nuclei) in regions of maximal expression, covering at least 100×100 μm ($10^4 \mu m^2$) and confirmed with PAS stain.

Selection of FISH Probes

Fecal cylinders of 40 IBD patients, 20 diarrhea patients, and 20 controls were hybridized with 86 FISH probes (Table 2), which were developed for identification of intestinal bacteria, medically relevant isolates, and waste water microorganisms. The FISH probes selected had to hybridize with more than 1% of the fecal population within at least 1 microscopic field of at least 5% of the tested subjects and to represent bacterial signals with unique morphology, distribution, and localization. Probes specific for related bacterial groups and partially covering the same fecal bacterial population (names of these FISH probes are successively ordered and underlined in Table 2). We chose those with the highest fluorescence signal by the lowest noise of the background fluorescence at conditions of optimal stringency (underlined twice in Table 2). This resulted in 11 group-specific FISH probes (bold in Table 2) being selected for use in all patients and samples in this study. The fluorescence signals of the Hel274 probe were in most cases not typical for the species and the concentrations were much higher than expected in humans. However, Hel274 hybridized with a spatially uniquely organized coccoid bacteria, which were not covered by any of the other 85 tested probes. We therefore left the Hel274 probe in the list despite concern regarding its specificity. However in the description of the findings we avoided the genus name of these bacteria (*Helicobacter*) and used the term Hel274-positive bacteria.

TABLE 2. FISH Probes

Ebac1790 (*Enterobacteriaceae*)
ECO1167 (*Escherichia coli*)
Ent (*Enterobacteriaceae*)
ENT183 (*Enterobacteriaceae*)
GAM42a (*Gammaproteobacteria*)
DSV687 (*Desulfovibrionales*)
ACA652/ACA23A (*Acinetobacter*)
ACAC (*Actinobacillus actinomycetemcomitans*)
AERO1244 (*Aeromonadaceae*)
Alc-476 (*Alcaligenes faecalis*)
ARC1430 (*Arcobacter*)
Ato291 (*Atopobium* cluster)
Hpy-1 (*Helicobacter pylori*)
Hel274 (*Helicobacter* sp., *Wolinella* sp.)
HEL717 (*Helicobacter* sp., *Wolinella* sp.)
GAN1237 (*Helicobacter ganmani*)
B(T)AFO (*Tannerella* forsythensis)
Bac303 (most *Bacteroidaceae*)
Bdis656 (*Bacteroides distasonis*)
Bfra602 (*Bacteroides fragilis* group)
CF319a (most *Flavobacteria*, some *Bacteroidetes*)
CFB560 (*Bacteroidetes*)
MIB724 (mouse intestinal bacteria)
MIB661 (mouse intestinal bacteria)
Chis150 (*Clostridium histolyticum*)
Clit135 (*Clostridium lituseburense* group)
CLOBU1022 (*Clostridium butyricum*)
Csac67 (*Clostridium* sp.)
CST440 (*Clostridium stercorarium*)
DSS658 (*Desulfobacteriaceae*)
E.bar1237 (*Eubacterium barkeri*)
E.bif462 (*Eubacterium biforme*)
E.con1122 (*Eubacterium contortum*)
E.cyl461 (*Eubacterium cylindroides*)
E.cyl466 (*Eubacterium cylindroides*)
E.dol183 (*Eubacterium dolichum*)
E.had579 (*Eubacterium hadrum*)
E.len194 (*Eubacterium lentum*)
E.lim1433 (*Eubacterium limosum*)
E.mon84 (*Eubacterium moniliformе*)
E.ven66 (*Eubacterium ventriosum*)
Ecyl387 (*Eubacterium cylindroides*)
Ehal1469 (*Eubacterium hallii*)
Erec482 (*Eubacterium rectale*, *Clostridium coccoides* group)
FUS664 (most *Fusobacterium* sp.)
FUSO (*Fusobacterium* sp.)
Lach571 (*Lachnospira multipara*)
Bcv13b (*Burkholderia vietnamensis*)
Pce (*Burkholderia* spp.)
Myc657 (*Mycobacterium*)
Pae997 (*Pseudomonas* spp.)
PBR2 (*Bifidobacterium breve*)
Pden654 (*Prevotella denticola*)
Pint649 (*Prevotella intermedia*)

TABLE 2. FISH Probes

Pnig657 (*Prevotella nigrescens*)
Phasco741 (*Phascolarctobacterium faecium*)
POGI (*Porphyromonas gingivalis*)
Ppu (*Pseudomonas* spp.)
Ppu56a (*Pseudomonas putida*, *P. mendocina*)
Ppu646 (*Pseudomonas* spp.)
PRIN (*Prevotella intermedia*)
ProCo1264 (*Ruminococcus* productus)
Rbro730 (*Clostridium sporosphaeroides*, *Ruminococcus bromii*, *Clostridium leptum*)
Rfla729 (*Ruminococcus albus*)
Urobe63a/Urobe63b (*Ruminococcus obeum*-like)
Veil223 (*Veillonella dispar*)
VEPA (*Veillonella parvula*)
VIB572a (Genus *Vibrio*)
Saga (*Streptococcus agalactiae*)
Sau (*Staphylococcus aureus*)
Spn (*Streptococcus pneumoniae*)
Spy (*Streptococcus pyogenes*)
Stemal (*Stenotrophomonas maltophilia*)
Str (*Streptococcus* spp.)
Strc493 (most *Streptococcus* spp.)
SUBU1237 (*Burkholderia* spp.)
SRB385Db (*Desulfobacterales*)
Sval428 (some *Desulfobulbaceae*)
Sita-649 (*Candidatus Sphaeronema italicum*)
SNA (*Sphaerotilus natans*)
SPH492 (*Sphingomonas*, *Erythrobacter*)
STEBA1426 (some members of the *Sterolibacterium* lineage)
EUB338 (most Bacteria)
EUB338 II (*Planctomycetales*)
EUB338 III (*Verrucomicrobiales*)
Bif164 (*Bifidobacteriaceae*)
Fprau (*Faecalibacterium prausnitzii*)[11]

The names of the FISH probes are listed according to abbreviations of the probeBase online resource for rRNA targeted oligonucleotide probes (http://www.microbial-ecology.net/probebase/credits.asp).[10] The Fprau probe is not mentioned in the probeBase and is described elsewere.[11]
Probes were synthesized with FITC, Cy3- or Cy5-reactive fluorescent dye at the 5′end (BioTeZ, Berlin Buch, Berlin, Germany).

Statistics

All statistical analyses were performed with SPSS software package v. 12 (Chicago, IL). Significant differences for parameters found using analysis of variance (ANOVA) were further compared group-to-group by Mann–Whitney *U*-test.

RESULTS

Patient Compliance

The overall compliance was higher in patients with gastrointestinal complaints than in healthy controls. Twelve of 44

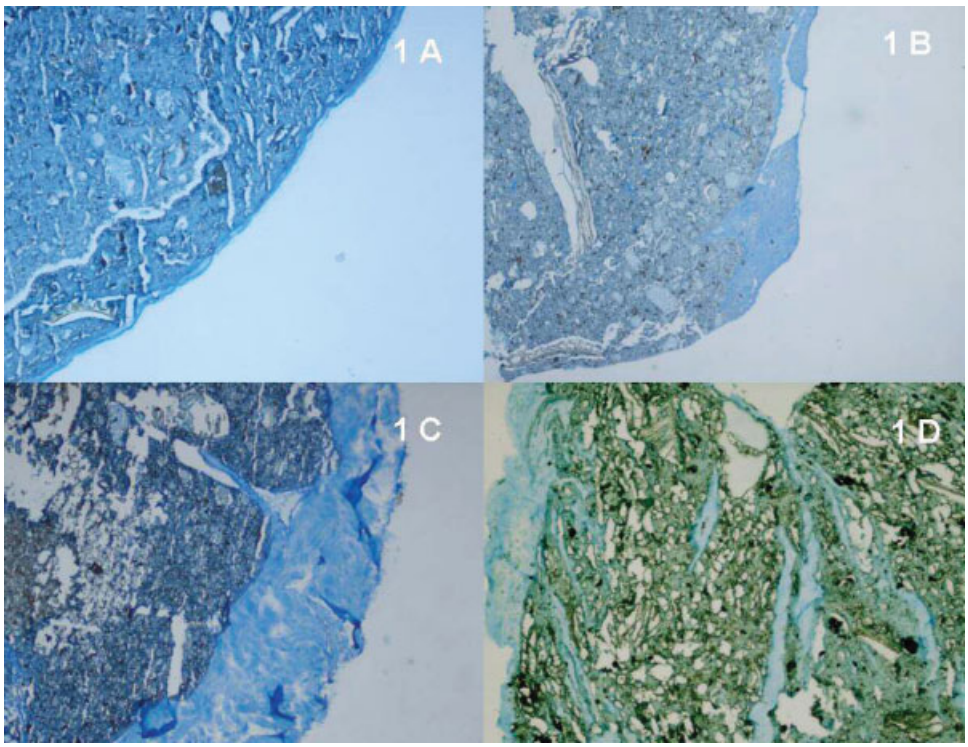

FIGURE 1. Examples of the mucus layer in a healthy subject (A) and patients with IBS (B) and diarrhea (C). The mucus is often enclosed in nonformed stool of patients with diarrhea, leading to septa irregularly traversing the feces (C,D). Photographs were made at a magnification of 100 using light microscopy and Alcian stain.

healthy controls stopped delivering probes after the first collected sample. In contrast, all but 4 IBD patients (3 of those in remission for >2 years) delivered 3 consecutive samples. Similar high compliance was observed in other disease groups. The reasons to stop participation of healthy subjects were discomfort with the handling of feces and a lack of time; the reasons in IBD patients were lack of an appropriate toilet and the inability to puncture the cylinder from loose stool. Although many patients with diarrhea had initial difficulties to collect fecal samples, after some retraining, they were able to deliver 3 stool cylinders despite mainly watery stools. The diarrhea symptoms are usually fluctuating, with portions of stool being more or less appropriate for investigation. The 2-week intervals between sample collections allowed all patients to gather samples, which was appropriate for investigation.

Light-microscopic Structure of the Fecal Cylinder

Mucus

The frequency of the presence of the mucus layer on the surface of the fecal cylinders was lower in all patient groups compared to healthy controls (Table 1; Fig. 1), indicating growing difficulties to preserve the mucus cover in unformed stools. However, the width of the mucus layer was independent of the stool consistency and significantly higher in patients with diarrhea than in healthy controls ($P < 0.001$; Fig. 1). In the noninflammatory control groups such as IBS and other gastrointestinal disorders, the mucus layer was broader than in healthy controls, but the differences were not significant. The lowest frequency of occurrence and width of the mucus layer was observed in patients with UC ($P < 0.02$, UC versus CD; $P < 0.001$, UC versus healthy controls).

Leukocytes

No leukocytes were detectable in the stool samples of healthy controls and rarely and sporadically seen in patients with noninflammatory disease (1 in IBS, 2 in diarrhea, 1 in liver cirrhosis, 1 in chronic pancreatitis, 2 in diverticulosis, and 1 in a patient with colon polyps). The number of leukocytes in noninflammatory controls never exceeded 30 cells per 10^4 μm^2. Two to 6 leukocytes per fecal cylinder (Fig. 2A) were found in most of these cases and leukocytes were never present in more than 1 of the 3 fecal samples.

The occurrence of leukocytes in UC, indeterminate colitis, and CD was 71%, 53%, and 23% accordingly. All 3 samples from the same patient were affected and the concentrations of leukocytes in mucus were similar in at least 2 of the samples.

In IBD patients, leukocytes were present in the mucus or at the surface of the fecal cylinder in high numbers condensed to layers of adherent rows or strings of inflammatory cells (Fig. 2B–F). No leukocytes were observed within the remainder of the fecal cylinder. Leukocytes were found within feces only in samples with completely disturbed ar-

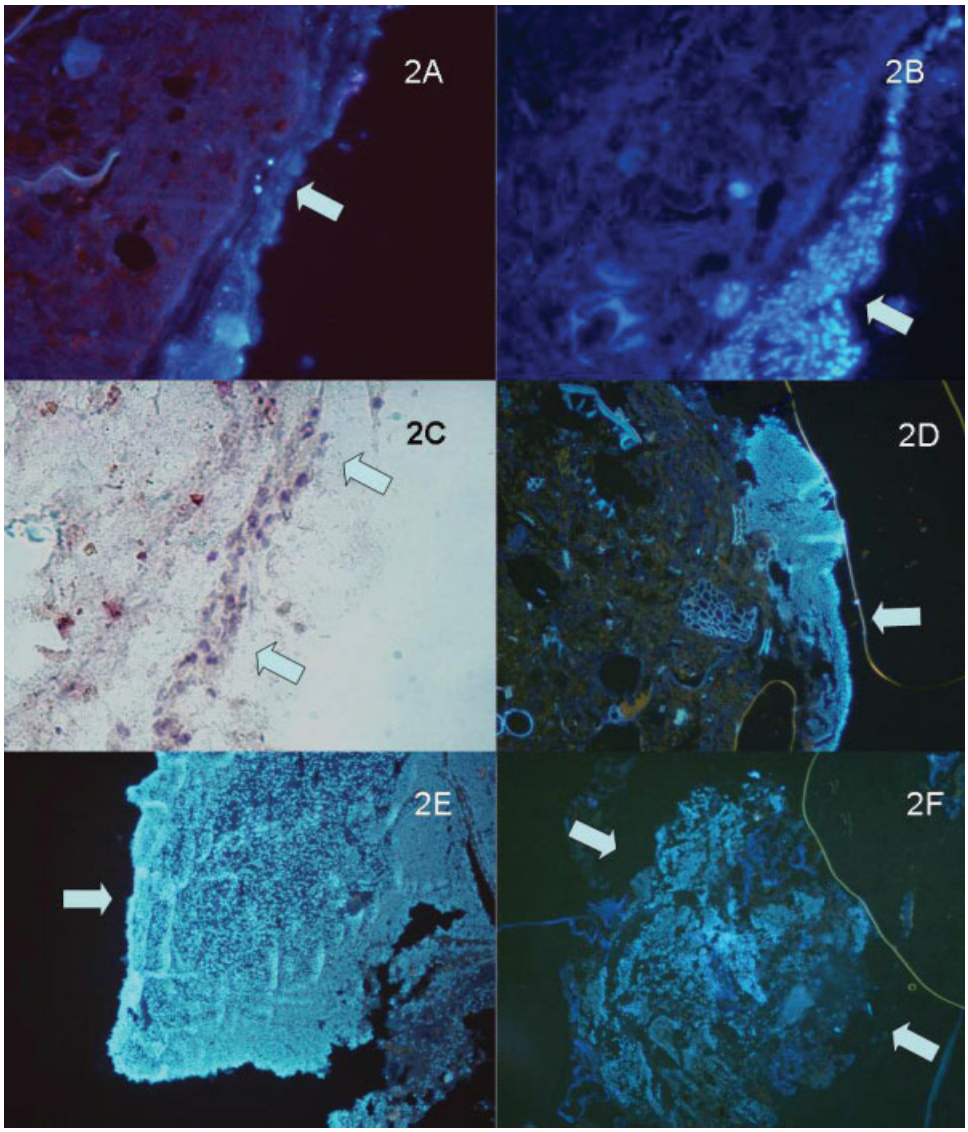

FIGURE 2. Leukocytes in the mucus of a fecal cylinder (blue arrows) in a patient with IBS (A), UC in remission for 6 months (B,C), moderately active UC (D), and severe UC (E,F). (C) A light microscopy of a PAS stain confirming that the large blue nuclei seen in DAPI stain are leukocytes. (B,C) Magnification of 400, all other figures at low magnification of 100 to give a better overview of proportions of regions including and omitting leukocytes. The leukocytes are located exclusively in the mucus (typical for self-limiting colitis and UC in remission) or the feces/mucus transition zone (typical for active UC and IC; B–E). The leukocytes were mixed with feces only after complete loss of the stool structure (F).

chitecture, but here it would be more appropriate to speak of feces within pus (Fig. 2F). The difference in the mean numbers of leukocytes between IBD and all other groups (Table 1) were significant ($P < 0.001$). The highest concentrations of leukocytes were seen in UC.

The occurrence of leukocytes in mucus was higher in patients with self-limiting colitis than in healthy controls but the mean concentrations were low and the leukocytes were never found in all 3 stool samples, with rapidly vanishing concentrations on follow-up investigation.

FISH Biostructure of the Normal Fecal Flora

Single bacterial groups could be divided in either habitual or occasional, diffusely spread or focally condensed, fecomucus, mucotrop, or mucophob (Figs. 3, 4).

Habitual Bacteria

Habitual bacteria were present in high concentrations in all stool samples of all healthy subjects. Single bacteria of the habitual groups contacted each other and were woven to a homogeneous carpet throughout the fecal cylinder (Fig. 3A,B). Sometimes the concentrations of habitual bacteria were higher on the surface of food remnants. Habitual bacteria were represented by *Eubacterium rectale* (Erec), *Bacteroides* (Bac), and *Faecalibacterium prausnitzii* (Fprau). Each of these groups composed 15%–40% of the total fecal population in single healthy control. Generally the concentrations of Erec were higher than that of Bac and concentrations of Bac higher than that of the Fprau groups (Table 1). Habitual bacteria were fecomucus, i.e., their concentrations were highest within feces but they also penetrated in low

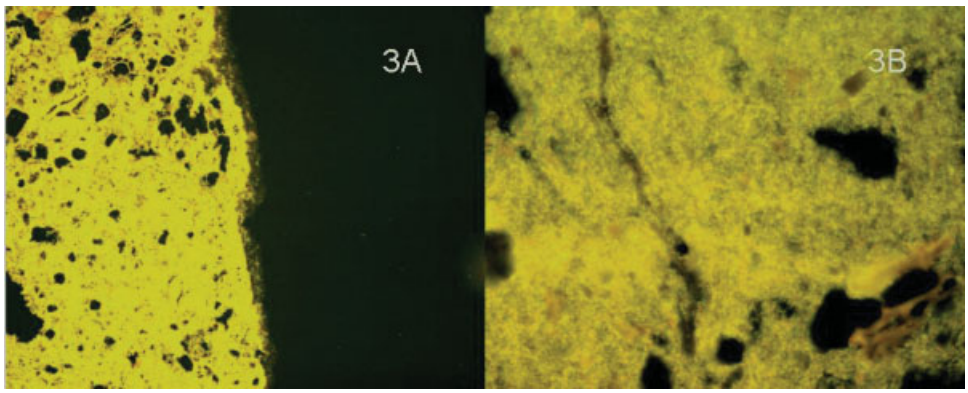

FIGURE 3. Distribution of *Bacteroides* in feces from a healthy control (A, ×100; B, ×400; Cy3 orange fluorescence). Bacteria are woven into a homogeneously carpet intercepted only by undigested food remnants. The fluorescence is excellent all over the stool cylinder.

numbers into adjacent portions of the mucus, with concentrations rapidly decreasing with increasing distance from the fecal surface (Fig. 4A).

Mucophob Bacteria

Bacteria that hybridized with the Bif (*Bifidobacteria*) probe were mucophob; they avoided mucus and were found mostly at a distance of 0–5 μm from the mucus layer or from the fecal surface (Fig. 4B). Bif were occasional and found in 93% of the healthy controls (Table 1). The concentrations of Bif were markedly lower than that of the habitual bacterial groups. In most cases Bif were diffusely scattered over the surface of the stool cylinder without contact with each other. Less often they were additionally condensed to isolated groups (islands) of bacteria. Bif were woven into a web-like structure in a single healthy control with a concentration higher than 8×10^9 bacteria/mL, similar to previously described observations for habitual bacteria.

Mucotrop Bacteria

Enterobacteriaceae (Ebac) and Hel274 bacteria were mucotrop. Their concentrations were highest in mucus adjacent to feces or in the transition zone from feces to mucus (Fig. 4C,D). The concentrations of mucotrop bacteria in feces were at least a power lower than in mucus or they were absent. To correctly quantify the mucotrop bacteria, the numbers and occurrence of Hel274 and Ebac groups were separately enumerated for the mucus/feces transition zone (mucotrop Ebac,

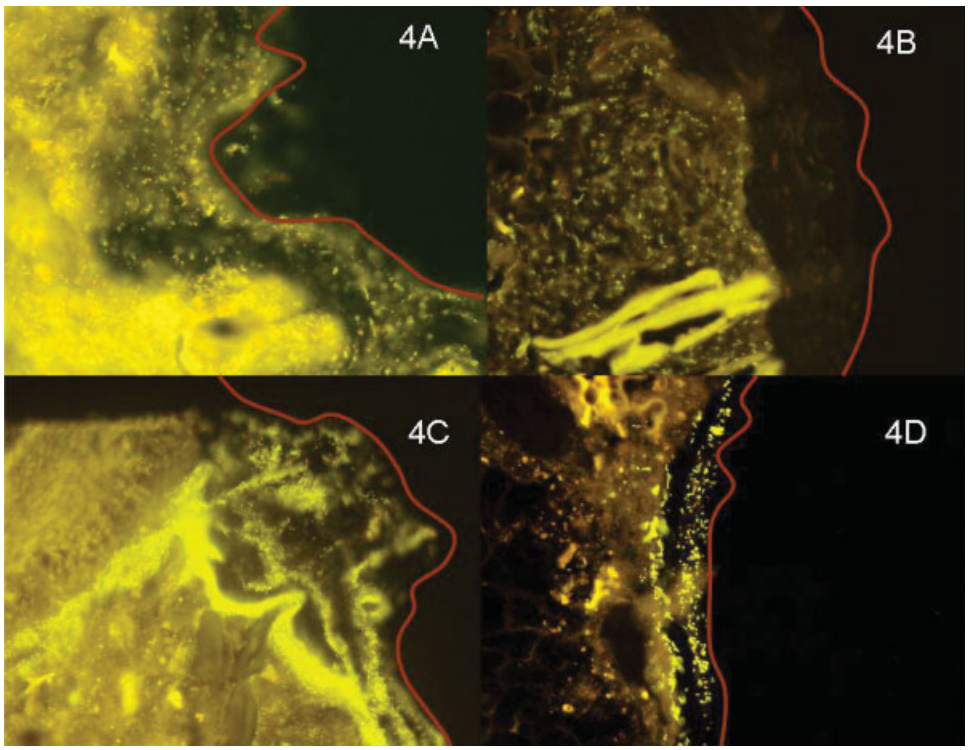

FIGURE 4. Bacteria in the transition zone from feces to mucus: Fecomucus bacteria (A; Fprau, ×400, Cy3, orange) are mainly located in feces; however, they can enter mucus in low concentrations. Mucophob bacteria (B; Bif, ×400, Cy3) avoid mucus and often even the edge of feces. Mucotrop bacteria (C; Hel274, ×400, Cy3) prevail in the transition zone between feces and mucus and may be completely absent in feces (D).

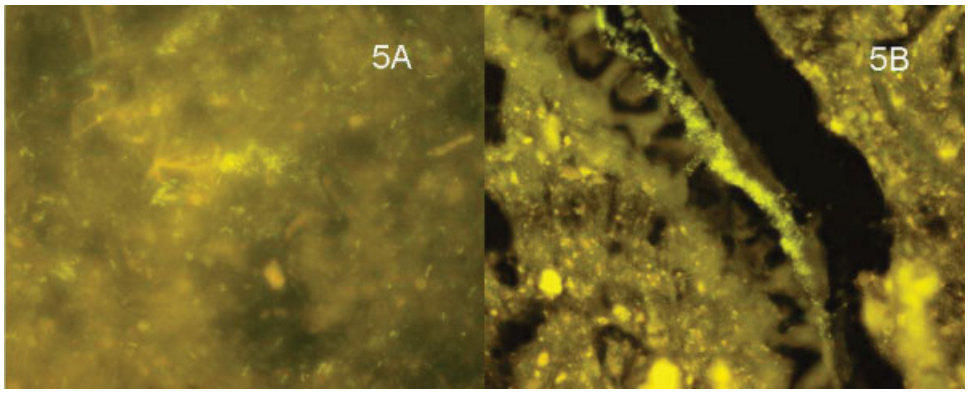

FIGURE 5. (A) Diffusely distributed *Eubacterium cylindroides* (×1000, Cy3) and additionally aggregates in single islands. (B) *Clostridium histolyticum* (×1000, Cy3) occurs only in some regions of the fecal cylinder forming layers and island or is diffusely scattered over a limited region. In B, Chis is seen as a dense biofilm on the surface of food residue.

mucotrop Hel274) and for fecal regions at least 100 μm below the fecal surface (fecal Ebac, fecal Hel274). The fecal Hel274 bacteria were always associated with mucotrop Hel274 and were obviously a part of the same population, which prefers mucus near region, but could or could not spread into the remainder of feces. Fecal Ebac occurred independently from mucotrop Ebac. No mucus Ebac could be detected in some patients with fecal Ebac. Fecal and mucotrop Ebac are phenotypically different species but hybridized with the same FISH probe.

Focally Distributed Occasional Bacteria

Clostridium histolyticum (Chis) and *Clostridium lituseburense* (Clit) were located in island or lawns (Fig. 5B). In some samples they were diffusely scattered; however, even then they were found only in restricted regions of less than 20% of the fecal cylinder. The shape and sizes of the Chis and Clit bacteria were highly variable, even between different samples of the same person or within different regions of the same fecal cylinder. Both bacterial groups were mucophob, but they often preferred regions next to the fecal surface, remaining at a clear distance from the mucus or the fecal surface. Often Chis and Clit were associated with food remnants in stool (Fig. 5B), where their concentrations could be very high.

Diffusely Distributed Occasional Bacteria

Atopobium (Ato), *Eubacterium hallii* (Ehal), and *E. cylindroides* (Ecyl) (Fig. 5A) were diffusely distributed occasional fecomucus bacteria. Typically, single bacterial cells occurred at more or less larger distances from each other but could be condensed to groups of 2 to 4 (Ehal, Ecyl) or more bacteria (Ato).

Disease-dependent Changes of the Fecal Biostructure

Habitual Bacteria

Changes typical for habitual bacteria in disease were: hybridization silence at the center of the feces, disintegration of the web structure, and general and selective bacterial depletion (Fig. 6).

Hybridization silence. The fluorescence intensity of habitual bacteria in most healthy controls was brilliant all over the surface of the fecal cylinder (Fig. 3A,B). In all other groups and especially in patients with diarrhea, some IBS, and CD patients, the fluorescence of habitual bacteria was reduced or even lost in the center of the feces but maintained at the periphery next to the stool surface (Fig. 6A). The portion of the cylinder with the suppressed fluorescence varied considerably in samples from the same patient. In especially distinct cases, bacteria could be detected exclusively in the mucus close regions of less than 5 μm width below the fecal surface or even within mucus alone. The loss of fluorescence in the transition zone from excellent to poor fluorescence was gradual, while the number of bacteria remained the same, indicating that the decreasing number of recognizable bacteria is not due to falling concentrations but rather due to worsening traceability. We called the decreasing fluorescence, despite constant bacterial numbers, hybridization silence. The hybridization silence could vary from 2%–98% between successive samples of the same patient, indicating a highly dynamic state of this phenomenon.

Because of the high lability of the hybridization silence and because the bacterial silence was mainly observed in diarrhea and IBS patients and, less often, in the IBD groups, we did not quantify it in this study. However, we evaluated the hybridization silence visually. The bacterial numbers of the habitual bacterial groups could be correctly enumerated only in regions of optimal fluorescence.

Bacterial depletion. The highest concentrations of habitual bacteria were observed in healthy controls (Fig. 3A,B). In all other groups the concentrations of habitual bacteria (Erec, Bac, Fprau) were reduced. The reduction involved single habitual groups unequally and disease-dependent (Table 1). Erec and Bac were lower in patients with diarrhea than in all other and the IBD groups ($P < 0.001$). Erec was similarly

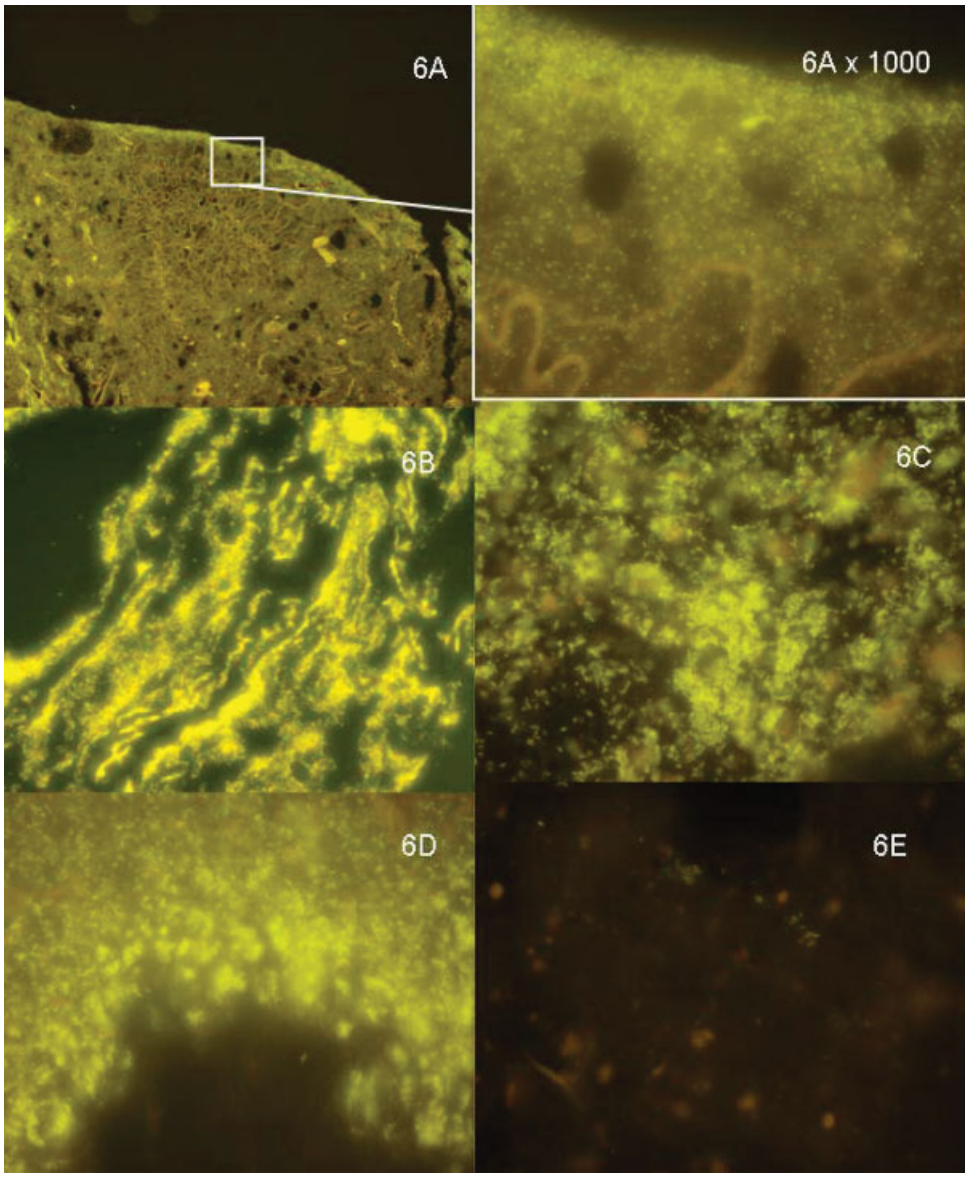

FIGURE 6. Examples of alteration within the web structure of habitual bacterial groups. (A) Hybridization silence (Cy3, ×100 left, ×1000 right micrograph) in an IBS patient. The fluorescence signals of *Bacteroides* gradually fade from the surface to the center of fecal cylinder. Bacterial counts remain unchanged in zones of high and intermediate fluorescence. (B) Mucus striae interrupting the web of the habitual bacteria in a patient with diarrhea (Bac, Cy3, ×400). (C,D) Spheroid precipitation of *Bacteroides* in a patient with active UC. (E) Subtotal depletion of *Faecalibacterium prausnitzii* in CD. B–E at magnification of 1000, Cy3.

reduced in UC and CD. The reduction of Bac was significantly more pronounced in UC than in CD ($P < 0.05$). Fprau was dramatically reduced in patients with CD ($P < 0.001$) and celiac disease. The concentration of habitual bacteria seldom fell below 2×10^9 bacteria/mL and the occurrence below 100%. A total or subtotal depletion (Fig. 6E) of single habitual groups was observed exceptionally for Fprau in patients with CD, celiac disease, and less often in UC, and for Bac in some patients with IBS and CD. Since habitual bacteria are normally present in each person in high numbers, their complete absence is a striking finding.

Disintegration of the web structure. The homogeneous web structure of the spatial arrangement of habitual bacteria disintegrated more and more with increasingly unformed stools. Typical for diarrhea were longitudinal striae, which completely or partially covered the surface of the stool cylinder (Fig. 6B). A precipitation of Bac to spheroid islands in regions close to the fecal surface was observed in 46% of patients with active UC (Fig. 6C,D). Different from striae, which could be found in all patients with loose stool, spheroid precipitation of Bac was observed exclusively in patients with UC, indeterminate colitis (34%), and CD CDAI >150 (8%) but not in any of the control groups.

Mucophob Bacteria

The occurrence of Bif was moderately reduced in most disease groups compared to healthy controls. Bif were completely absent in 56% of the patients with CD ($P < 0.05$). Despite less frequent occurrence, the mean concentrations of

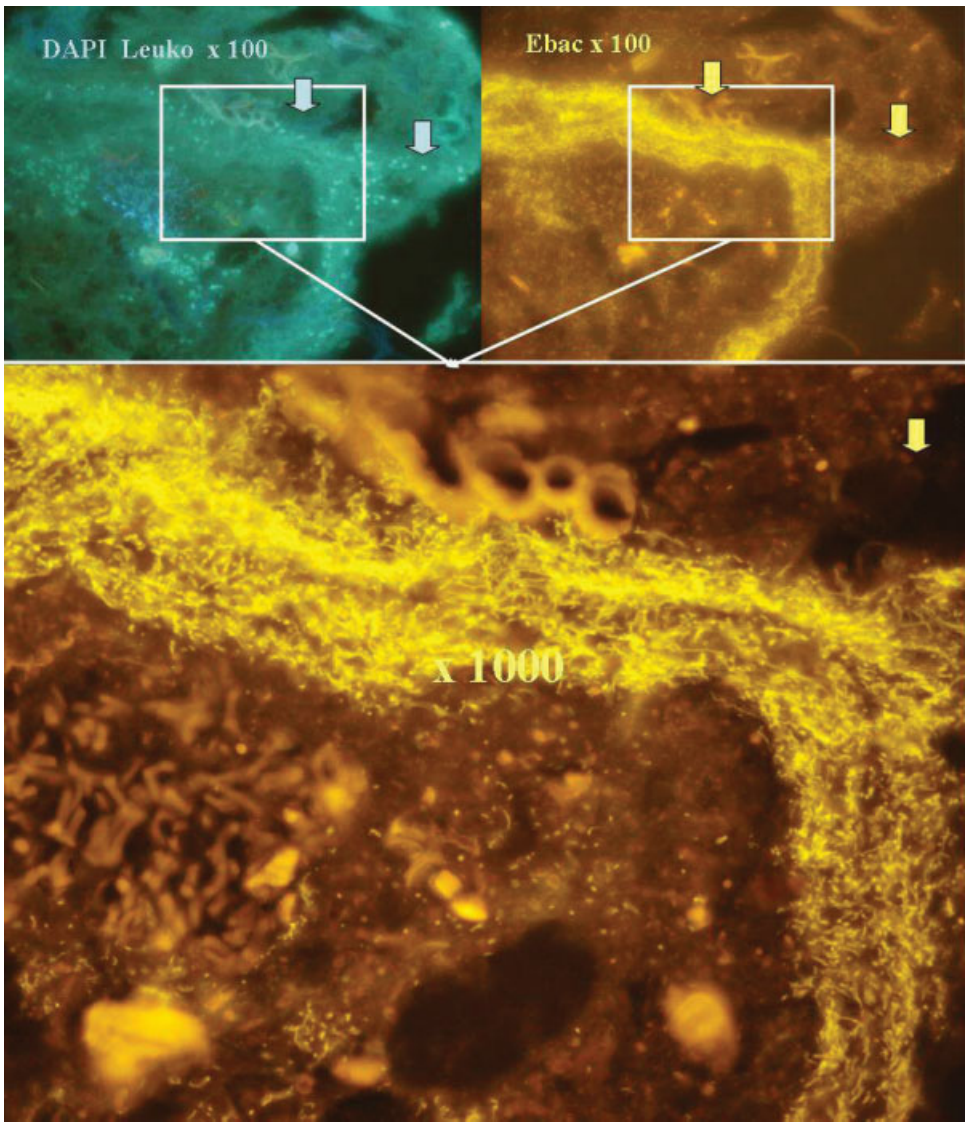

FIGURE 7. Mucotrop *Enterobacteriaceae* are elevated in UC and prefer a location between leukocytes or next to them (DAPI and Cy3 of the same microscopic field).

Bif were increased in all disease groups. The highest concentrations of Bif were observed in patients with UC and indeterminate colitis. The increase in the mean concentration of Bif in UC was due to the high proportion of patients with concentrations of Bif of $>8 \times 10^9$ bacteria/mL. Bif had an appearance of woven texture typical for habitual bacterial groups when $\geq 8 \times 10^9$ bacteria/mL. The proportion of patients with Bif $\geq 8 \times 10^9$ bacteria/mL was markedly higher in UC than in CD and other groups.

Mucotrop Bacteria

The mean concentrations of mucotrop Ebac and mucotrop Hel274 were significantly increased in patients with diarrhea correlating with thicker mucus. In IBD patients, where the mucus layer was reduced, the mean concentration of mucotrop Hel274 was even lower than in healthy controls ($P < 0.05$ for CD). However, the concentrations of mucotrop Ebac were independent from the mucus thickness and increased in patients with IBD compared to healthy controls ($P < 0.001$). Another peculiarity was that Ebac were seen in regions with leukocytes. The presence of leukocytes is usually associated with suppression of bacteria in their proximity, leading to zones completely omitting bacteria. Bacteria mixed with leukocytes only after loss of the fecal structure. In contrast, mucotrop Ebac were unrestricted by leukocytes, demonstrated excellent fluorescence, and occurred in high numbers next to leukocytes. It seems as if Ebac are attracted by leukocytes following the same route as the leukocytes do and being enriched in regions containing leukocytes (Fig. 7).

The mean concentrations of fecal Hel274 were unchanged in all groups with the exception of patients with diarrhea. In them, the occurrence and concentration were increased compared to all other groups ($P < 0.001$), probably

due to the high mucus proportion within diarrheal feces. The mean concentrations of fecal Ebac were increased in all disease groups. The highest values of fecal Ebac were observed in diarrhea and CD patients. In UC, the concentrations of fecal Ebac were comparatively low and statistically not different from healthy controls.

Focally Distributed Occasional Bacteria

Both Chis and Clit were slightly elevated in patients with diarrhea and in most other groups compared to healthy controls, but we could not detect any disease-specific changes, including local distribution, morphology of focal accumulation to islands, and lawns of these bacteria. The comparison of findings between different groups of patients was difficult and probably incomplete due to the polymorphism of the bacterial cell morphology and the extremely heterogeneous distribution within the stool cylinder. In a subset of UC patients with total disruption of the fecal structure, and massive reduction of all other bacterial groups, the Chis group could became predominant, composing more than 10% of the fecal bacterial population. However, this was observed only in 3 UC and 1 CD patients and had no impact on the mean concentration and occurrence of bacteria in the total group.

Diffusely Distributed Occasional Bacteria

The occurrence of Ehal and Ecyl was markedly reduced in patients with CD, but the mean concentrations were unchanged. The mean concentration of Ato was significantly increased in UC compared to most noninflammatory controls and CD.

Disease Activity and Biostructure of Fecal Microbiota in CD and UC

Leukocytes, mucus thickness, and the concentrations/occurrence of 6 of the 11 bacterial groups were dependent on disease activity (Table 3). The characteristics of these parameters were different or even opposite in CD and UC, except for mucotrop Ebac and Chis.

The occurrence and the concentrations of leukocytes grew progressively with the severity of the disease in both IBD groups ($P < 0.001$); however, the occurrence of leukocytes in patients with CD was low compared to UC patients.

The mucus layer was thin in UC even in patients in remission. The median mucus thickness decreased with increasing UC activity. In CD, no clear relationship of mucus thickness and disease activity was apparent, but the occurrence of the superficial mucus layer was similarly low as in UC.

The concentrations of all habitual bacterial groups decreased in general with increasing inflammation both in UC and CD. However, in UC the reduction of Bac and Erec concentrations was more pronounced than that of Fprau resulting in Fprau being the most predominant group in active UC. In all other patient groups, the concentrations of Erec or Bac were higher than that of Fprau (Table 1). The situation was the opposite in CD: increasing disease activity resulted in a reduction of Fprau that exceeded the reduction of all other habitual groups ($P < 0.001$) and led to complete loss of these bacteria in 87%–89% of patients (Table 3).

The occurrence and concentrations of Bif in CD were lower in active disease than in remission and significantly lower than in UC ($P < 0.05$). In UC the concentrations of Bif were high and independent of disease activity.

The concentrations of fecal Ebac were independent of disease activity in patients with UC and increased progressively with increasing CD activity. The concentrations of mucotrop Ebac increased progressively with the disease activity both in CD and UC.

Mucotrop Hel274 bacteria were gradually reduced with disease activity in patients with UC ($P<0.05$) and inconsistently changed in CD.

Chis concentrations were higher in active disease than in remission both in CD and UC.

The occurrence of Ehal and less of Ecyl were reduced in active CD.

No dependence of disease activity was observed in either IBD groups for Ato and Clit.

Diagnostic Value of *Faecalibacterium prausnitzii* and Leukocytes

The reduction of Fprau in CD and increase in leukocytes in UC are the most prominent features of IBD. Since Fprau is present, while leukocytes are absent, in all healthy and noninflammatory controls, the quantitative changes of these parameters can be universally applied for diagnostic purposes. To detect the predictive significance of Fprau and leukocytes, we choose 3 cutoffs for each value: Fprau = 0, 0<Fprau≤1, and Fprau>1, Leuko = 0, 0<Leuko≤30, and Leuko>30. The combination of cutoffs for Fprau and leukocytes results in 9 possibilities (upper part of Table 4). When patients with different disease activity were assigned to each possibility a clear segregation in just 4 recognition patterns occurs: CD pattern (Fprau≤1, Leuko≤30), ulcerative colitis pattern (Fprau>1, Leuko>30), intermediate IBD pattern (Fprau≤1, Leuko>30), and a noninflammatory pattern (Fprau>1, Leuko≤30). The patterns could not predict a disease as such, since less than 30% of patients in remission of more than 12 months have characteristic changes. The situation was different in active disease. Using the 4 recognition patterns, active CD and UC could be recognized with 79%/80% sensitivity and 98%/100% specificity (lower part of Table 4). The sensitivity decreased gradually with decreasing disease activity or duration of remission, indicating that the chosen patterns are activity criteria and not etiologic components. We do not present positive (negative) predictive values and sensitivity for each of the subgroups since these can be easily deduced from the data presented in Table 4.

TABLE 3. Spatial Structure of Fecal Microbiota and Disease Activity

		Crohn's Disease (A)				Ulcerative Colitis (B)				Differences
		CDAI>300	150≤CDAI ≤300	Rem ≤1 year	Rem >1 year	CAI 9	3≤CAI≤9	Rem ≤1 year	Rem >1 year	
		n = 19	n = 23	n = 18	n = 22	n = 27	n = 32	n = 14	n = 32	Active Disease versus Remission
Mucus		34 ± 68	4.7 ± 17	5 ± 12	21 ± 56	3 ± 12	2.7 ± 9	16 ± 42	13 ± 63	A ns
		42%	17%	27%	36%	15%	22%	29%	25%	B $P < 0.001$
Leukocytes		162 ± 350	103 ± 280	8 ± 35	19 ± 72	612 ± 467	273 ± 356	189 ± 224	17 ± 66	A,B $P < 0.001$
	O	32%	26%	22%	14%	100%	88%	79%	25%	
Erec	C	13 ± 6.9	14.3 ± 7.8	14.7 ± 8.6	18 ± 8.9	12 ± 7.8	14 ± 7	17 ± 7.8	18 ± 8.4	A,B $P < 0.001$
	O	100%	100%	100%	100%	100%	100%	100%	100%	
Bac	C	9.9 ± 6.5	11 ± 6.9	12.6 ± 6.7	13 ± 6.4	6.7 ± 6.2	8.3 ± 5.5	9.4 ± 5.3	12 ± 6.6	A,B $P < 0.001$
	O	100%	96%	100%	95%	100%	100%	100%	100%	
Fprau	C	1.4 ± 4.4	1.7 ± 4.3	4.0 ± 5.9	8.1 ± 7	13 ± 8	14 ± 13	13 ± 9	16 ± 11	A $P < 0.001$
	O	11%	22%	72%	91%	96%	100%	93%	96%	B ns
Bif	C	0.7 ± 1.4	0.2 ± 1.7	0.6 ± 1.3	1.6 ± 2.7	2.2 ± 3.0	3.2 ± 3.9	2.9 ± 3.0	2.5 ± 5.0	ns
	O	37%	43%	44%	54%	89%	84%	79%	88%	
Bif $\geq 8 \times 10^9$/mL	O	0%	4%	6%	6%	26%	22%	28%	16%	
Ebac fecal	C	2.5 ± 2.8	1.9 ± 4.1	1.6 ± 2.2	1.1 ± 2.8	0.5 ± 1.2	0.5 ± 1.7	0.6 ± 2.6	0.6 ± 1.6	A $P < 0.001$
	O	42%	48%	39%	41%	63%	72%	71%	66%	B ns
Ebac mucotrop	O	6.8 ± 11.7	3.4 ± 3.4	2.2 ± 3.4	0.6 ± 0.5	9.9 ± 2	8.4 ± 9	2.7 ± 4.8	1.1 ± 1.6	A,B $P < 0.001$
		74%	78%	67%	73%	74%	69%	71%	66%	
Hel274 fecal	C	0.6 ± 2.2	0.5 ± 1.6	0.17 ± 0.9	0.2 ± 1.1	0	0.3 ± 1.2	0.08 ± 0.4	0.34 ± 1.3	ns
	O	16%	22%	6%	9%	0%	13%	14%	6%	
Hel274 mucotrop	C	1.1 ± 3.2	0.6 ± 3.1	0.14 ± 0.5	1.6 ± 6.2	1.3 ± 6.9	2.2 ± 5.9	2.6 ± 9.2	2.9 ± 9.8	A ns
	O	11%	13%	11%	14%	7%	13%	14%	25%	B $P < 0.05$
Ecyl	C	0.7 ± 1.1	0.6 ± 1.0	0.6 ± 1.0	0.2 ± 0.6	0.5 ± 0.9	1.2 ± 2.0	0.7 ± 1.3	1.1 ± 1.7	ns
	O	26%	35%	44%	64%	77%	75%	71%	84%	
Ehal	C	0.24 ± 0.7	0.1 ± 0.5	0.04 ± 0.1	0.2 ± 0.8	0.05 ± 0.1	0.14 ± 0.2	0.20 ± 0.4	0.19 ± 0.5	ns
	O	21%	22%	39%	68%	66%	62%	43%	78%	
Clit	C	0.004 ± 0.01	0.19 ± 0.45	0.1 ± 0.3	0.28 ± 0.64	0.23 ± 0.48	0.02 ± 0.03	0.01 ± 0.04	0.013 ± 0.3	A $P < 0.05$
	O	16%	17%	55%	50%	66%	53%	64%	50%	B ns
Chis	C	0.35 ± 0.8	0.24 ± 0.39	0.06 ± 0.1	0.04 ± 0.1	0.41 ± 0.8	0.23 ± 0.4	0.02 ± 0.04	0.02 ± 0.5	A,B $P < 0.001$
	O	63%	40%	50%	73%	59%	66%	50%	56%	
Ato	C	0.8 ± 1.4	0.9 ± 2.8	0.6 ± 0.7	0.9 ± 0.9	1.4 ± 1.3	1.2 ± 1.4	1.6 ± 1.8	1.7 ± 1.8	ns
	O	68%	74%	89%	82%	88%	97%	71%	94%	

Table 3 is structured in the same way as Table 1. The values of single patients groups in Table 3 can be directly compared to the values of non-IBD subgroups in the Table 1. Rem, remission; ns, not significant.

More than 70% of patients with remission of less than 12 months still had clear signs of either active CD or UC. Of the non-IBD groups and healthy controls, only celiac disease overlapped with CD but not with UC. Indeterminate colitis was more similar to UC than to CD and not identical with an intermediate IBD recognition pattern. The intermediate IBD recognition pattern was found more often in UC and IC than in CD patients. The changes observed in self-limiting colitis were insufficient to allow assignment to any of the IBD entities. None of healthy controls or noninflammatory control patients had changes consistent with IBD.

DISCUSSION

Fecal flora was in the past intensively investigated using microscopy, microbial culture, DNA isolation, polymerase chain reaction (PCR) cloning, sequencing, and FISH.[1,3] None of the approaches revealed diagnostically relevant differences between CD, UC, or other intestinal disorders.[1–3] All studies were based on more or less sophisticated methods of bacterial (DNA) isolation and homogenization of material. This implies an even distribution of bacteria in stool. However, the latter was never shown. Our data demonstrate that the fecal flora is highly organized, with local concentrations of single

TABLE 4. Percent of Patients and Number of Occurrences Positive for Fprau/Leuko Cutoff Values Within Groups Related to Specific Diagnosis and Disease Activity

	Crohn's Disease				Ulcerative Colitis								
	CDAI >300	150≤CDAI ≤300	Rem. ≤1 year	Rem. >1 year	CAI >9	3≤CAI ≤9	Rem. ≤1 year	Rem >1 year	IC	Celiac Disease	Slc	Healthy	Noninflammatory Disease Controls
	n = 19	*n* = 23	*n* = 18	*n* = 22	*n* = 27	*n* = 32	*n* = 14	*n* = 32	*n* = 17	*n* = 12	*n* = 9	*n* = 32	*n* = 165
Fprau=0; Leuko=0	68%	60%	28%	9%				3%	18%	8%			
Fprau=0; 0<Leuko≤30	16%	9%											
Fprau=0; Leuko>30	5%	9%			4%		7%		24%				
Fprau <1; Leuko=0		4%	17%	9%		3%				50%			
Fprau <1; 0<Leuko≤30			5%					3%					
Fprau <1; Leuko<30					4%	7%	7%	3%					
Fprau >1; Leuko>30	11%	9%	17%	9%	92%	69%	50%	12%	29%				
Fprau >1; 0<Leuko≤30				5%		12	14%	6%		8%	22%		5%
Fprau >1; Leuko=0		9%	33%	68%		9%	21%	72%	29%	32%	78%	100%	93%
Recognition patterns of IBD													
Fprau <1; Leuko≤30 Crohn's disease	84%	73%	50%	18%		3%	7%	6%	18%	58%			
Fprau <1; Leuko >30 Intermediate IBD	5%	9%			7%	7%	14%	3%	24%				
Fprau >1; Leuko>30 UC	11%	9%	17%	10%	93%	69%	50%	13%	29%				
Leuko≤30; Fprau>1 Non-inflammatory		9%	33%	72%		21%	29%	78%	29%	42%	100%	100%	100%

bacterial groups ranging from undetectable to more than 10^{11} bacteria/mL. The homogenization of feces obscures the results, making the correct evaluation of the microbial community impossible. For example, habitual bacteria such as *Bacteroides, Eubacterium rectale,* and *Faecalibacterium prausnitzii* are usually numerically dominant and diffusely distributed in healthy feces. However, in patients with diarrhea, IBS, and IBD these bacteria are often suppressed at the center of feces and therefore purely or completely resistant to hybridization. We called this phenomenon hybridization silence. The detectable concentrations of habitual bacteria decreased from normal in the fecal core to zero at the fecal center. Homogenization of such samples prior to FISH analysis will lead to falsely low concentrations and nonreproducible results. The mucotrop bacteria such as *Enterobacteriaceae* and Hel274 are found mainly on the border between mucus and feces. The correct enumeration of these bacteria in fecal homogenates is impossible. Many bacterial groups are principally unevenly distributed in stool, located only in some portions of the stool cylinder, and even there condensed to isolated islands and lawns. The enumeration of focally distributed bacteria in homogenates of stool is highly accidental. Even leukocytes cannot be correctly enumerated in homogenates. Leukocytes in UC are located in the transition zone between feces and mucus, where they reach astronomic numbers and cannot be simply overlooked. In feces, leukocytes appear first after complete disintegration of the fecal structure, which is seen only in a subset of patients with high activity. We quantified parallel leukocytes in smears and in punched cylinders of the same stool probes. The detection success rate in smears was below 20% and the numbers were completely underestimated (data not presented).

On the other hand, the low consistency of stool proved to be no problem in the investigation of the fecal biostructure. All of the patients with gastrointestinal complaints eagerly participated and were able to deliver 3 samples at ≈2-week intervals. The high engagement of patients, the ease of collection, and the convenience of storage and delivery of stool samples allow screening of large cohorts of patients, if necessary even on a daily basis. The samples of unformed stool are often completely devoid of the mucus cover and even contain no portions of the stool surface; however, they regularly include mucus and pus within the fecal body forming alternating layers. As long as larger pieces of feces can be embedded in paraffin, the evaluation of the spatial arrangement of the fecal flora is possible and reliable for diagnostic purposes. The comparison of the microbial biostructure in healthy, noninflammatory controls, UC, and CD reveals many characteristic details, which enable discrimination between these conditions. The most prominent features in IBD were: reduction of mucus thickness especially in UC, progressive decrease in the concentrations of the habitual bacteria and disintegration of their web structure, spheroid precipitation of *Bacteroides* to isolated island in patients with UC, increased concentrations of leukocytes in the mucus and on the surface of feces in UC, reduction and loss of *Faecalibacterium prausnitzii* in CD, high concentrations by excellent fluorescence of *Faecalibacterium prausnitzii* in UC, increased concentrations and occurrence of mucotrop *Enterobacteriaceae* with decreased concentrations of mucotrop Hel274 bacteria in both CD and UC patients, increased concentrations of fecal *Enterobacteriaceae* in CD with low concentrations of fecal *Enterobacteriaceae* in patients with UC, reduced occurrence of *Eubacterium hallii* and *E. cylindroides* bacteria in CD, and elevated concentrations of *Bifidobacteria* and *Atopobium* in patients with UC.

The dynamics in concentrations and/or occurrence of *Faecalibacterium prausnitzii*, fecal *Enterobacteriaceae, Bifidobacteria, Atopobium, Eubacterium cylindroides, E. hallii*, and leukocytes were strikingly opposite in UC and CD, allowing differentiation between both and indicating that these diseases are distinctly different entities and not just different expressions of the same inflammatory process.

FISH probes in this study were selected for high fluorescence and legibility of signals within the complex texture of native feces. Although chosen mainly for reasons of microscopic convenience, 6 of the 11 FISH probes demonstrated disease-specific dependence in at least a subset of patients and 9 of 11 probes were numerically different from healthy controls. It is easy to imagine that with the widening of the FISH probe spectrum new details will emerge and allow us to refine the diagnostic possibilities and understanding of both diseases.

However, the quantitative assessment of 2 parameters: leukocytes at the feces/mucus border and *Faecalibacterium prausnitzii* concentrations, were sufficient to diagnose active CD and UC with a 79/80% sensitivity and 98/100% specificity. The lack of sensitivity in our study was due to overlap between CD and UC and IC, and the lack of specificity was due to overlap between CD and celiac disease. No overlap occurred between IBD and healthy controls, self-limiting colitis, and noninflammatory disease subjects. In fact, none of the subjects from the healthy or the noninflammatory control groups matched criteria for IBD.

We did not reevaluate the diagnoses of our patients after the start of the study, although the clear discrimination between CD and UC in clinical practice is often elusive and it is highly probable that some of the IBD patients were incorrectly labeled. The overlap between CD and UC is therefore not surprising. The overlap between CD and celiac disease, however, is unexpected and it is for future investigations to clarify whether these 2 diseases share a common pathogenesis or whether the inflammation of the small intestine is responsible for similarities in the fecal findings. Obviously, the depletion of *Faecalibacterium prausnitzii* in active Crohn's and celiac diseases is not an expression of

bacterial shortage or misbalance of intestinal flora, but a result of an activated immune response, which specifically eradicates selective groups of bacteria. High-dose cortisol therapy or infliximab are able to completely restore the *Faecalibacterium prausnitzii* concentrations from zero to levels higher than 14×10^9 bacteria/mL within days (data not shown), indicating that the bacteria are not deficient, just completely suppressed by the host. With the reduction of the cortisol dose or with increasing time after infliximab infusion, the concentrations of *Faecalibacterium prausnitzii* start to gradually decrease until they completely vanish. *Faecalibacterium prausnitzii* is a dominant component of the normal flora. It is difficult to imagine that it could be the primary target of the immune response. Much more probable is that a process linked to another pathogen or factor concomitantly affects it. The disappearance of *Faecalibacterium prausnitzii, Bifidobacteria, Eubacterium hallii,* and *E. cylindroides* from the bacterial spectrum is therefore not important per se. The lack of selective bacterial groups indicates like dark lines in the spectrophotometer the elements of activated innate immunity. The high sensitivity and specificity of these features enables us to monitor the IBD therapy based on criteria that are independent of the complaints of the patients.

It is commonplace that clinical symptoms of IBD are uncharacteristic, overlap broadly with other intestinal diseases, and are often misleading. For example, patients with IBD, IBS, and idiopathic diarrhea all complain about increased amounts of mucus. However, the Alcian/PAS stains show that the mucus production is increased exclusively in the last 2 groups. In patients with UC the amount of mucus is significantly reduced. What the UC patients interpret as mucus is in reality pure pus composed of leukocytes, principally different from mucus in IBS or diarrhea.

Not all changes observed in this study can be described in terms of bacterial suppression. Thus, *Bifidobacteria* and *Atopobium* concentrations are increased in UC but not in CD, and fecal *Enterobacteriaceae* concentrations increase with increasing activity of CD but stay unchanged in active UC. Concentrations of mucotrop *Enterobacteriaceae* are increased with increasing disease activity in both IBD entities. In UC patients where mucus is often completely absent, mucotrop *Enterobacteriaceae* are even enriched in regions of leukocyte accumulation. Obviously, some of bacterial groups profit from the ongoing inflammation. It is presently impossible to say whether the bacterial groups found to be increased in IBD are initiators or opportunists of the bacterial misbalance.

Although our data collection and analysis is still in progress and the investigated panel of bacterial groups is limited, it is already obvious that many common beliefs about IBD must be revised. The fact that more than 70% of the IBD patients in remission of less than 12 months have obvious signs of active disease is alarming and clearly demonstrates that the present therapy, which is mainly based on clinical symptoms, is inadequate, leading in most cases to suppression, but not to interruption of the inflammation. On the other hand, the fact that nearly 80% of the patients with remission of more than 12 months have a normal fecal biostructure indicates that the termination of the inflammation and probably even of the disease are wanted and achievable goals.

REFERENCES

1. Tamboli CP, Neut C, Desreumaux P, et al. Dysbiosis as a prerequisite for IBD. *Gut.* 2004;53:1057.
2. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature.* 2007;448:427–434.
3. Strober W, Ivan Fuss I. Mannon P. The fundamental basis of inflammatory bowel disease. *J Clin Invest.* 2007;117:514–521.
4. Chapman RW. Selby WS, Jewell DP. Controlled trial of intravenous metronidazole as an adjunct to corticosteroids in severe ulcerative colitis. *Gut*. 1986;27:1210–1212.
5. Malchow H, Ewe K, Brandes JW, et al. European Cooperative Crohn's Disease Study (ECCDS): results of drug treatment. *Gastroenterology*. 1984;86:249–266.
6. Rachmilewitz D. Coated mesalazine (5-aminosalicylic acid) versus sulphasalazine in the treatment of active ulcerative colitis: a randomised trial. *BMJ*. 1989;298:82–86.
7. Best WR, Becktel JM, Singleton JW, et al. Development of a Crohn's Disease Activity Index. National Cooperative Crohn's Disease Study. *Gastroenterology.* 1976;70:439–444.
8. Swidsinski A. Standards for bacterial identification by fluorescence in situ hybridization within eukaryotic tissue using ribosomal rRNA-based probes. *Inflamm Bowel Dis.* 2006;12:824–826.
9. Swidsinski A, Weber J, Loening-Baucke V, et al. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *JCM*. 2005;43:3380–3389.
10. Loy A, Maixner F, Wagner M, et al. probeBase — an online resource for rRNA-targeted oligonucleotide probes: new features 2007. *Nucleic Acids Res*. 2007;35:800–804.
11. Suau A, Rochet V, Sghir A, et al. Fusobacterium prausnitzii and related species represent a dominant group within the human fecal flora. *Syst Appl Microbiol.* 2001;24:139–145.