Biostructure of Fecal Microbiota in Healthy Subjects and Patients With Chronic Idiopathic Diarrhea

ALEXANDER SWIDSINSKI,* VERA LOENING–BAUCKE,* HANS VERSTRAELEN,‡ SYLWIA OSOWSKA,* and YVONNE DOERFFEL§

*Gastroenterology, Humboldt University, Berlin, Germany; ‡Ghent University Hospital, Ghent, Belgium; and §University Poliklinik, Charité, Berlin, Germany

See Iwarzon M et al on page 893 in *CGH*.

Background & Aims: **Dysbiosis is a key component of intestinal disorders. Our aim was to quantitatively access the biostructure of fecal microbiota in healthy subjects and patients with chronic idiopathic diarrhea and evaluate the responses to *Saccharomyces boulardii* treatment.** ***Methods:*** **We investigated punched fecal cylinders from 20 patients with chronic idiopathic diarrhea and 20 healthy controls using fluorescence in situ hybridization. Fluctuations in assembly of 11 bacterial groups were monitored weekly for 3 weeks before, during, and after oral *S boulardii* supplementation.** ***Results:*** **The structural organization of fecal microbiota in healthy subjects was stable and unaffected by *S boulardii*. The assembly of fecal microbiota in idiopathic diarrhea was markedly different, characterized by mucus depositions within feces; mucus septa and striae; marked reduction in concentrations of habitual *Eubacterium rectale*, *Bacteroides*, and *Faecalibacterium prausnitzii* groups; suppression of bacterial fluorescence in the center of the feces; increased concentrations and spatial shift of mucotrop bacteria to the fecal core; and increased concentrations of occasional bacteria. Except for elevated concentrations of some occasional bacterial groups, all parameters typical for diarrhea improved significantly with *S boulardii* treatment and most changes persisted after cessation of therapy. The improvement of the fecal microbiota was accompanied by partial (40%) and complete normalization (30%) of the diarrheal symptoms.** ***Conclusions:*** **The fecal microbiota is highly structured. Fluorescence in situ hybridization analysis allowed us to quantitatively study the dysbiotic changes. *S boulardii* significantly improved the fecal biostructure in patients with diarrhea but had no influence on the feces in healthy subjects.**

Frequency, form, consistency, and composition of feces are changed in a variety of gastrointestinal diseases. Bacteria compose more than 90% of the fecal mass and are always involved in these alterations. In the past 150 years, intensive efforts have been undertaken to quantitatively characterize the normal fecal microbiota and to define the nature of their disturbances[1,2] in patients and healthy subjects of different age groups, cultural habits, diets, or residencies. Although the investigations were repeatedly performed with the introduction of each advanced microbiologic method, no clear cutoff between healthy and diseased fecal microbiota could be established. The fecal microbiota proved to be extremely complex and highly dynamic. The composition and concentrations of single bacterial groups overlapped broadly. Previous investigations of the fecal microbiota ignored its structure; microscopy, bacterial culture, polymerase chain reaction, sequencing, or fluorescence in situ hybridization (FISH) used either homogenized samples or smears. The homogenization implies the equal distribution of bacteria within feces. However, the last was never shown. Based on what we know, the fecal microbiota are not equally intermixed but spatially organized multicellular communities.[3] The term "dysbiosis" was introduced to describe the variety of pathologic changes of the fecal microbiota without naming any particular one.[1,2] An important point in the evaluation of dysbiosis is the stability, reproducibility, and vulnerability of findings in response to environmental, dietetic, or probiotic challenges.

Saccharomyces boulardii is a yeast probiotic with the clinically proven ability to positively influence acute diarrheal symptoms in humans. Its clinical effect is supposed to be due to changes in the composition of the enteric microbiota.[4] The morphologic appearance and ribosomal RNA sequences of *S boulardii* differ strongly from those of bacteria, and its oral supplementation does not interfere with the bacterial detection by FISH.

The aims of this study were to investigate the spatial organization of fecal microbiota in patients with chronic idiopathic diarrhea and to study the effects of *S boulardii* treatment using multicolor ribosomal FISH on punched stool cylinders.

Abbreviation used in this paper: FISH, fluorescence in situ hybridization.

0016-5085/08/$34.00
doi:10.1053/j.gastro.2008.04.017

Patients and Methods

Patients

Patients with chronic idiopathic diarrhea were recruited from the gastroenterology outpatient clinic of the Charité Hospital. All patients had a complete gastroenterological diagnostic investigation, including colonoscopy, gastroscopy, ultrasonography, and laboratory tests. The diagnosis of idiopathic (functional) diarrhea was made as described.[5,6] However, for this study, only patients with diarrhea of at least 12-month duration with a weekly average of ≥4 stools/day were included. Their ages ranged from 25 to 72 years (mean, 48.2 years; 11 men and 9 women). The healthy controls consisted of laboratory and medical staff and their relatives, with an age range of 18–60 years (mean age, 41.2 years; 8 men and 12 women), who had no intestinal complaints or known diseases. The biostructure of fecal microbiota in patients with idiopathic diarrhea and healthy controls was studied. In addition, we investigated the spontaneous variability and changes due to *S boulardii* treatment. In each group, 3 weeks of surveillance was followed by 3 weeks of oral supplementation with the yeast probiotic *S boulardii*. The microbiota was also followed up over 3 weeks after the end of probiotic supplementation. Compliance was an important inclusion feature. The study required weekly investigations of fecal cylinders over 9 weeks. Only subjects who provided all 9 stool cylinders were evaluated, and recruitment continued until each group had 20 subjects.

Sachets containing 250 mg of *S boulardii* were applied to meals twice a day for 3 weeks (Perenterol sachets; UCB GmbH, Kerpen, Germany). In subjects who disliked the taste of the probiotic, the application was continued with the same dosage but given as a capsule (Perenterol 250 mg). Patients with diarrhea recorded the number of stools and their complaints in diaries. The healthy participants were asked to record unusual events in a free written form. Each participant was interviewed at least 3 times: at inclusion in the study, after the end of *S boulardii* supplementation, and 3 weeks later.

The study protocol was approved by the institutional review board of Charité Hospital.

Sample Collection and Handling

Stools were either dropped on cleansing tissue or on the dry flat surface part of the toilet (common in Germany). The 4- to 10-mm-long fecal cylinders were punched from the stool using plastic drinking straws with an inside diameter of 3 mm (Schlecker, Ehingen, Germany). The drinking straws were precut to 4 cm in length and handed out to participants of the study together with 50-mL Falcon tubes filled with 30 mL of Carnoy's solution (6/6/1 vol ethanol/glacial acetic acid/chloroform). Participants were instructed on how to obtain the stool cylinders and given written instructions (see supplementary material online at www.gastrojournal.org). The pieces of drinking straw with the stool inside were put into the Falcon tube, fixated in Carnoy's solution for 24 hours at room temperature, and then kept refrigerated at 4°C in Carnoy's solution until delivered to the laboratory within 1–2 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 cylinders were then removed from the straws with a plastic rod, embedded in paraffin using standard techniques, cut longitudinally into 4-μm sections, and placed on SuperFrost slides (R. Langenbrinck, Emmendingen, Germany).

To test the reliability of the single measurements and the stability of stored material, multiple fecal cylinder samples were taken from different portions of the same stool, fixated in Carnoy's solution, and stored in a refrigerator. After different periods of time, the samples were embedded in paraffin. Except for occurrence and thickness of the mucus layer, the differences in concentration and distribution of single bacterial groups between samples of the same stool were negligible. The quality of fluorescence signals remained unchanged over 4 weeks of storage in Carnoy's solution at 4°C.

Light Microscopy

Alcian blue/periodic acid–Schiff stains were used for evaluation of mucus and leukocytes in the stool specimens and for the orientation within cylinders. The mucus layer at one pole identified the surface of the cylinder; the ink stain on the opposed pole identified the deep or more central portions of the feces.

FISH

A Nikon e600 fluorescence microscope was used. The images were photodocumented with a Nikon DXM 1200F color camera and software (Nikon, Tokyo, Japan). Hybridizations were performed in multicolor FISH according to previously described protocols.[3,7]

Bacteria were quantified using group-specific Cy3 probes. The fluorescein isothiocyanate–marked universal probe was used in each hybridization to evaluate the number of all bacteria; a Cy5-marked probe with a different Cy3 probe specificity was used to determine the spatial relation of different bacterial groups to each other. When bacteria occurred in high concentration, they 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, within whole microscopic fields, or using the complete surface of the fecal cylinder. All enumerations were performed at a magnification of 1000×. The conversion of the numbers within defined microscopic areas to concentrations of bacteria per milliliter was based on the calculation that a 10-μL sample with a cell concentration of 10^7 cells/mL has 40 cells per average microscopic

field. The details of this conversion were previously described.[7]

FISH Probes

To select FISH probes, which are most appropriate for longitudinal investigations, the first fecal cylinder from each subject participating in this study (N = 40) was hybridized with a total of 86 different probes, which were developed for identification of intestinal bacteria, medically relevant isolates, and waste water microbial communities (see Table 1), and with the universal Eub 338 probe. The *Faecalibacterium prausnitzii* probe is described by Suau et al.[8] The names of all other FISH probes are listed according to the abbreviations of the probeBase online resource for ribosomal RNA–targeted oligonucleotide probes (http://www.microbial-ecology.net/probebase/credits.asp).[9]

We selected FISH probes that hybridized with more than 1% of the fecal population within at least one microscopic field of at least 5% of the tested subjects and represented bacterial signals with unique morphology, distribution, and localization. We chose those with the highest fluorescence signal by the lowest noise of the background fluorescence at conditions of optimal stringency from probes specific for related bacterial groups and partially covering the same fecal bacterial population. As a result, 11 group-specific FISH probes (bold in the list) were selected for use in all patients and samples in this study. The fluorescence signals of bacteria that hybridized with the Hel274 probe were in most cases not typical for the species, and the concentrations of bacteria that positively hybridized with Hel274 were much higher than expected in humans. Because of this discrepancy, we attempted to culture the *Helicobacter* spp from the feces of patients with positive signals (data not presented) but were not able to confirm their presence. However, Hel274 hybridized with a unique subgroup of spatially organized coccoid bacteria, which were covered with no other probes. We therefore left the Hel274 probe in the list despite concern regarding its specificity; however, we avoided the genus name of these bacteria (*Helicobacter*) and used the term "Hel274-positive bacteria" instead.

Statistics

All statistical analyses were performed using the statistical software package SPSS version 15.0 (Chicago, IL). Details are described in the supplementary material (see supplementary material online at www.gastrojournal.org).

Results

Compliance

Nine healthy controls and 2 patients with idiopathic diarrhea who were enrolled in the study failed to complete the study protocol and hence were excluded from the study and replaced by compliant subjects. The

Table 1. 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* spp, *Wolinella* spp)
HEL717 (*Helicobacter* spp, *Wolinella* spp)
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* spp)
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 moniliforme*)
E.ven66 (*Eubacterium ventriosum*)
Ecyl387 (*Eubacterium cylindroides*)
Ehal1469 (*Eubacterium hallii*)
Erec482 (*Eubacterium rectale*, *Clostridium coccoides* group)
FUS664 (most *Fusobacterium* spp)
FUSO (*Fusobacterium* spp)
Lach571 (*Lachnospira multipara*)
Bcv13b (*Burkholderia vietnamiensis*)
Pce (*Burkholderia* spp)
Myc657 (*Mycobacterium*)
Pae997 (*Pseudomonas* spp)
PBR2 (*Bifidobacterium breve*)
Pden654 (*Prevotella denticola*)
Pint649 (*Prevotella intermedia*)
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*)

Table 1. Continued

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*)

NOTE. The probes chosen for hybridization with all samples are listed in **bold**.

inability to collect stools with very loose consistency apparently accounted for the noncompliance in patients with diarrhea, whereas healthy subjects attributed their noncompliance to discomfort with handling of feces and/or lack of time. The overall readiness to participate in the study was much higher in patients with chronic idiopathic diarrhea than in healthy controls.

Clinical Symptoms

Two of the healthy controls and 6 of the patients with diarrhea found the taste of the medication unpleasant and therefore continued the study with *S boulardii* capsules instead of powder. Eight subjects reported occasional belching with a sweet, julep yeast-like taste but without any self-reported discomfort. Two patients with diarrhea reported transient nausea that coincided with *S boulardii* supplementation, but considering the transient and mild nature of this adverse event it was not a reason to discontinue the study.

No healthy controls reported changes in general well-being, stool appearance, or stool frequency for the whole duration of the study. In the patient group, symptoms associated with chronic diarrhea persisted in the period before *S boulardii* supplementation. However, these symptoms improved in 14 patients (70%) during *S boulardii* supplementation, and in particular, the mean number of stools decreased significantly beginning in the first week of intervention (Table 2 and Figure 1*A*). In 6 of these patients, the improvement was complete and the stool frequency dropped to less than 3 stools a day. After the cessation of *S boulardii* supplementation, 5 patients with initial relief during supplementation (1 full response and 4 partial responses) reported a relapse of symptoms, although the symptoms never reached the initial severity. No change in symptoms was observed during the entire study period in 6 patients with diarrhea. Finally, no patients reported a worsened pattern of diarrhea and associated symptoms under *S boulardii* treatment.

Microscopic Structure of the Fecal Cylinder

Mucus. Mucus was observed either as a layer covering the surface of the stool (mucus layer; Figure 2*A* and *B*) or as broad mucus septa, irregularly intersecting the fecal mass (mucus septa; Figure 2*C* and *D*). In addition, in patients with diarrhea, mucus was also observed as multiple 5- to 20-μm-wide slender lines or striae, arranged in parallel (Figure 2*F*) and present in extended regions of the fecal cylinder surface. While the mucus layer and the broad mucus septa could be clearly visualized with alcian stain, the slender mucus striae were most apparent on FISH investigation, using the universal bacterial probe.

The amounts of mucus were massive and significantly increased in patients with diarrhea (Figure 1*B–D*) compared with healthy controls (Table 2, Figure 2*A/B* and *C/D*).

No changes in mucus-related characteristics were observed in the healthy controls during and after *S boulardii* supplementation (Table 2). In contrast, *S boulardii* supplementation significantly reduced the amount of mucus in feces of patients with diarrhea. The mucus-related changes included reduced surface and thickness of the mucus layer covering the feces, a decline in the number of mucus septa, and a significantly decreased number of mucus striae, including the proportion of the fecal surface covered with striae (Table 2 and Figure 1*B–D*).

Mucus layer. A mucus layer covering the stool surface was present in 108 of 180 samples obtained from healthy controls (60%) and in 86 of 180 samples from patients with idiopathic diarrhea (48%; $P = .019$). In each healthy control, at least 3 of 9 investigated stool samples had a mucus layer. Seven of the patients with diarrhea had no mucus layer at any time of the investigation. No subject in either group had a mucus layer in all stool samples. Despite the overall higher prevalence of a mucus layer in healthy controls, the mean thickness of the mucus layer was significantly lower in healthy controls than in patients with idiopathic diarrhea (36 $\pm$ 35 μm vs 176 $\pm$ 231 μm; $P < .001$).

The median values of the mucus layer thickness in patients with diarrhea decreased from 265 $\pm$ 266 μm before *S boulardii* supplementation to 96 $\pm$ 118 μm during supplementation ($P = .002$) and increased thereafter (Figure 1*B*). The occurrence of the superficial mucus layer in patients with diarrhea increased during *S boulardii* supplementation and remained unchanged in the period thereafter, indicating the higher consistency of stool, which helped in the preservation of the fecal cylinder.

Table 2. Spontaneous- and *S boulardii*-Induced Changes of Microbiota

		Before treatment			During treatment with *S boulardii*			After treatment		
Week		1	2	3	4	5	6	7	8	9
Mean no. of stools/wk[a]		40 ± 16	39 ± 15	39 ± 16	25 ± 18	26 ± 20	26 ± 20	29 ± 23	32 ± 23	32 ± 22
Mucus layer[b]	Occurrence (%)	60	60	55	40	55	60	60	75	60
	Maximal value/	50	80	120	200	120	100	80	120	120
	Width of mucus layer (μm), mean ± SD	23 ± 17	22 ± 24	35 ± 34	41 ± 64	39 ± 40	34 ± 30	34 ± 23	42 ± 36	49 ± 37
Mucus layer[a]	Occurrence (%)	45	30	45	60	60	35	45	45	55
	Maximal value	1000	800	700	300	500	300	1000	500	1000
	Width of mucus layer (μm), mean ± SD	244 ± 307	400 ± 253	197 ± 224	87 ± 80	122 ± 159	110 ± 109	192 ± 341	138 ± 157	198 ± 288
Mucus septa[b]	Occurrence (%)	5	0	10	0	5	10	20	15	5
	Total no. of septa	2	0	4	0	1	4	6	4	1
Mucus septa[a]	Occurrence (%)	35	45	35	75	89	75	60	55	60
	Total no. of septa	22	31	26	29	32	34	35	34	30
	Mean ± SD	4.4 ± 2.9	3.8 ± 2.2	4.1 ± 2.9	1.0 ± 1.0	1.9 ± 1.2	2.1 ± 1.2	2.7 ± 1.2	2.9 ± 1.6	2.2 ± 1.7
Mucus striae[b]	Occurrence (%)	0	0	0	0	0	0	0	0	0
Mucus striae[a]	Occurrence (%)	100	100	100	50	60	50	65	55	60
	No. of striae/ microscopic field at 1000×, mean ± SD	8.6 ± 1.7	8 ± 2.2	7.9 ± 2.2	3.9 ± 2.5	4.5 ± 2.2	3.6 ± 1.8	3.7 ± 2.1	4.3 ± 2.6	5.5 ± 2.4
	S	78 ± 23	85 ± 31	63 ± 33	41 ± 44	38 ± 43	40 ± 42	51 ± 40	49 ± 49	51 ± 42
E rectale[b]	C	24 ± 7.7	24.9 ± 6.4	25.3 ± 6.2	23.6 ± 6.1	24.2 ± 7.0	23.4 ± 6.8	23.4 ± 6.8	24.7 ± 6.7	23.7 ± 5.9
E rectale[a]	C	11.3 ± 4.9	11.8 ± 4.7	11.7 ± 5.9	15 ± 8.6	15.2 ± 8.2	14.5 ± 8.5	14.1 ± 8.5	14.6 ± 8.8	14.9 ± 9.7
HS partial[a]	S	10 ± 13	14 ± 12	6 ± 13	5 ± 9	3 ± 5	5 ± 10	8 ± 12	10 ± 16	8 ± 12
HS complete[a]	S	34 ± 37	38 ± 43	33 ± 39	6 ± 13	12 ± 20	12 ± 27	22 ± 32	21 ± 31	20 ± 26
Bacteroides[b]	C	183 ± 3.9	19.7 ± 4.7	19.3 ± 5.2	18.8 ± 5	19.5 ± 5.6	19.3 ± 4.9	20.1 ± 5.2	20 ± 4.9	18.4 ± 6.2
Bacteroides[a]	C	7.7 ± 5.1	6.2 ± 4.0	6.7 ± 4.2	12.3 ± 6.7	14 ± 6.3	11.5 ± 6.9	9.9 ± 6.9	8.3 ± 5	8.9 ± 6.3
HS partial[a]	S	12 ± 25	18 ± 26	13 ± 27	9 ± 23	12 ± 29	13 ± 24	13 ± 23	11 ± 23	8 ± 20
HS complete[a]	S	50 ± 47	33 ± 44	42 ± 46	9 ± 14	15 ± 24	18 ± 24	27 ± 27	28 ± 33	31 ± 34
F prausnitzii[b]	C	14.5 ± 4	14.3 ± 3.7	14.5 ± 4.2	13.7 ± 3.2	14.5 ± 4.0	14.2 ± 3.9	15.0 ± 4.1	12.7 ± 3.0	14.7 ± 4.6
F prausnitzii[a]	C	8.7 ± 4.1	9.9 ± 4.4	9.5 ± 3.7	10.22 ± 4.2	11.2 ± 4.7	10.7 ± 3.6	9.5 ± 4.2	10.1 ± 3.7	10.8 ± 4.9
HS partial[a]	S	29 ± 20	27 ± 12	24 ± 11	4 ± 7	6 ± 15	4 ± 10	6 ± 15	4 ± 10	7 ± 21
HS complete[a]	S	8 ± 12	5 ± 10	5 ± 9	4 ± 8	5 ± 12	3 ± 10	5 ± 12	3 ± 10	11 ± 15
Bifidobacterium[b]	C	0.72 ± 1.5	0.37 ± 0.66	0.45 ± 1.04	0.41 ± 0.72	0.42 ± 0.74	0.82 ± 1.13	0.25 ± 0.38	1.10 ± 2.44	0.72 ± 1.36
Bifidobacterium[a]	C	2.31 ± 2.2	1.78 ± 2.0	2.1 ± 1.91	1.25 ± 2.04	1.36 ± 1.99	1.76 ± 2.04	1.20 ± 1.18	2.06 ± 2.02	1.92 ± 2.06
Atopobium[b]	C	0.84 ± 1.1	0.55 ± 0.66	0.89 ± 1.14	0.88 ± 1.31	0.94 ± 0.70	0.73 ± 0.83	0.80 ± 0.72	0.65 ± 1.26	0.38 ± 0.97
Atopobium[a]	C	0.98 ± 1.0	0.94 ± 1.10	0.92 ± 1.10	0.90 ± 1.13	0.96 ± 0.94	1.02 ± 1.18	0.91 ± 1.39	0.99 ± 2.39	0.73 ± 1.33
E cylindroides[b]	C	0.48 ± 0.7	0.33 ± 0.42	0.53 ± 0.91	0.48 ± 0.71	0.64 ± 0.83	0.88 ± 1.00	0.57 ± 1.08	0.45 ± 0.98	0.44 ± 0.98
E cylindroides[a]	C	1.03 ± 1.5	0.94 ± 1.07	0.82 ± 1.11	0.90 ± 1.13	0.72 ± 0.80	077 ± 1.10	0.99 ± 1.39	0.50 ± 1.12	0.56 ± 1.08
E hallii[b]		0.24 ± 0.4	0.21 ± 0.47	0.19 ± 0.31	0.09 ± 0.24	0.19 ± 0.36	0.19 ± 0.43	0.06 ± 0.07	0.17 ± 0.45	0.14 ± 0.32
E hallii[a]		0.32 ± 1.5	0.24 ± 0.28	0.34 ± 0.89	0.37 ± 0.68	0.22 ± 0.55	0.18 ± 0.49	0.32 ± 0.37	0.11 ± 0.26	0.25 ± 0.27
C lituseburense[b]	C	0.057 ± 0.13	0.14 ± 0.45	0.06 ± 0.13	0.08 ± 0.23	0.064 ± 0.22	0.086 ± 0.23	0.031 ± 0.044	0.076 ± 0.22	0.06 ± 0.1
C lituseburense[a]	C	0.21 ± 0.52	0.29 ± 0.54	0.16 ± 0.36	0.21 ± 0.41	0.12 ± 0.30	0.16 ± 0.46	0.12 ± 0.45	0.15 ± 0.46	0.16 ± 0.26
C histolyticum[b]	C	0.021 ± 0.07	0.026 ± 0.07	0.058 ± 0.22	0.045 ± 0.12	0.19 ± 0.36	0.089 ± 0.24	0.06 ± 0.07	0.031 ± 0.043	0.04 ± 0.13
C histolyticum[a]	C	0.13 ± 0.30	0.14 ± 0.33	0.11 ± 0.46	0.10 ± 0.24	0.22 ± 0.55	0.18 ± 0.49	0.12 ± 0.27	0.12 ± 0.45	0.14 ± 0.13
Enterobacteriaceae mucotrope[b]	Occurrence (%)	70	70	50	80	55	80	50	55	70
Enterobacteriaceae mucotrope[a]	Occurrence (%)	70	90	70	70	90	70	90	90	75
Enterobacteriaceae mucotrope[b]	C	0.2 ± 0.33	1.4 ± 4.5	0.31 ± 0.8	1.3 ± 4.4	1.2 ± 2.7	0.7 ± 2.2	1.0 ± 1.7	0.7 ± 1.3	1.0 ± 1.6
Enterobacteriaceae mucotrope[a]	C	3.5 ± 7	5.1 ± 7	3.2 ± 5.4	2.7 ± 4.1	3.7 ± 6.9	5.8 ± 7.9	2.7 ± 3.7	7.5 ± 10.1	2.4 ± 5.9
Enterobacteriaceae fecal[b]	Occurrence (%)	45	40	25	25	30	30	30	45	45
Enterobacteriaceae fecal[a]	Occurrence (%)	65	60	50	50	55	50	50	55	65
Enterobacteriaceae fecal[b]	C	0.1 ± 0.22	0.05 ± 0.22	0.01 ± 0.04	0.005 ± 0.02	0.04 ± 0.18	0.03 ± 0.06	0.07 ± 0.02	0.07 ± 0.22	0.2 ± 0.4
Enterobacteriaceae fecal[a]	C	1.4 ± 3	2.3 ± 4	1.0 ± 2.4	0.3 ± 1.3	3.7 ± 6.9	1.6 ± 3.2	0.5 ± 2.2	2.2 ± 3.9	2.0 ± 3.7
Hel 274 mucotrope[b]	Occurrence (%)	40	50	50	50	25	55	35	35	30
Hel 274 mucotrope[a]	Occurrence (%)	85	80	70	55	65	70	50	55	50
Hel 274 mucotrope[b]	C	4.1 ± 9.4	3.7 ± 9	3.8 ± 7.9	4.5 ± 7	2.6 ± 6.0	3.4 ± 5.3	3.5 ± 8.2	3.5 ± 7.9	2.3 ± 8.7
Hel 274 mucotrope[a]	C	15.1 ± 16.5	14.6 ± 16	14.7 ± 21.5	7.6 ± 13.7	6.4 ± 9.0	5.8 ± 7.9	11.2 ± 20	9.7 ± 15.7	10.1 ± 19.4
Hel274 fecal[b]	Occurrence (%)	15	15	10	5	15	20	15	20	20
Hel274 fecal[a]	Occurrence (%)	30	45	45	50	50	55	45	45	45

Table 2. Continued

		Before treatment			During treatment with *S boulardii*			After treatment		
Week		1	2	3	4	5	6	7	8	9
Hel274 fecal[b]	C	0.21 ± 0.69	0.61 ± 1.8	0.04 ± 0.16	0.005 ± 0.02	0.19 ± 0.69	0.005 ± 0.02	0.14 ± 0.5	0.06 ± 0.22	0.02 ± 0.04
Hel274 fecal[a]	C	1.26 ± 2.69	1.15 ± 2.6	1.8 ± 2.9	0.6 ± 1.5	0.85 ± 1.5	0.99 ± 2.1	0.95 ± 2.3	1.2 ± 2.2	1.8 ± 3.0

NOTE. See supplementary material online at www.gastrojournal.org.
S, mean ± SD surface of the cylinder covered with striae; C, mean ± SD concentrations of bacteria; HS, hybridization silence.
[a]Data from patients with diarrhea.
[b]Data from controls.

Mucus septa. In firm feces, the mucus can be found exclusively as a layer on the fecal surface. In less firm but formed feces, the mucus layer can be incorporated in fecal masses and form mucus septa. In loose feces, no mucus layer can be found. Mucus striae dominate instead.

In healthy controls, singular mucus septa were observed in 12 of 180 samples (Figure 2*C*) but were independently seen apart from *S boulardii* supplementation. In patients with chronic diarrhea, mucus septa were frequent and often occurring in numbers higher than 2 (Table 2 and Figure 2*D*). Mucus septa were consistently present in all stool specimens in 5 patients with diarrhea over the whole investigation. They were observed in at least some of the stool samples in all other patients. Overall, the septa were observed in 38% of samples in the period before *S boulardii* supplementation. The occurrence of mucus septa then increased to 80% of all samples during *S boulardii* supplementation and remained high, in 63% of the samples, after cessation of *S boulardii* supplementation (Figure 1*C*).

Mucus striae. In healthy controls, structures resembling mucus striae were occasionally seen in regions adjacent to the mucus layer; however, they never occurred within the fecal core and never fully occupied one microscopic field in healthy controls. In untreated patients with diarrhea, mucus striae were present in all samples and covered at least 75% of the total fecal surface (Figure 2*F*). The occurrence of the striae and the proportion of the fecal surface occupied by mucus striae diminished significantly beginning in the first week of *S boulardii* supplementation and further decreased progressively during this therapy (Figure 1*D*). With *S boulardii* supplementation, striae were observed in 62% of the samples covering, on average, 39% of the total cylinder surface. Following *S boulardii* supplementation, 65% of the samples had striae and the total percent of surface covered with striae was 50%.

Leukocytes within mucus. No leukocytes were detected within mucus either in healthy controls or patients with diarrhea.

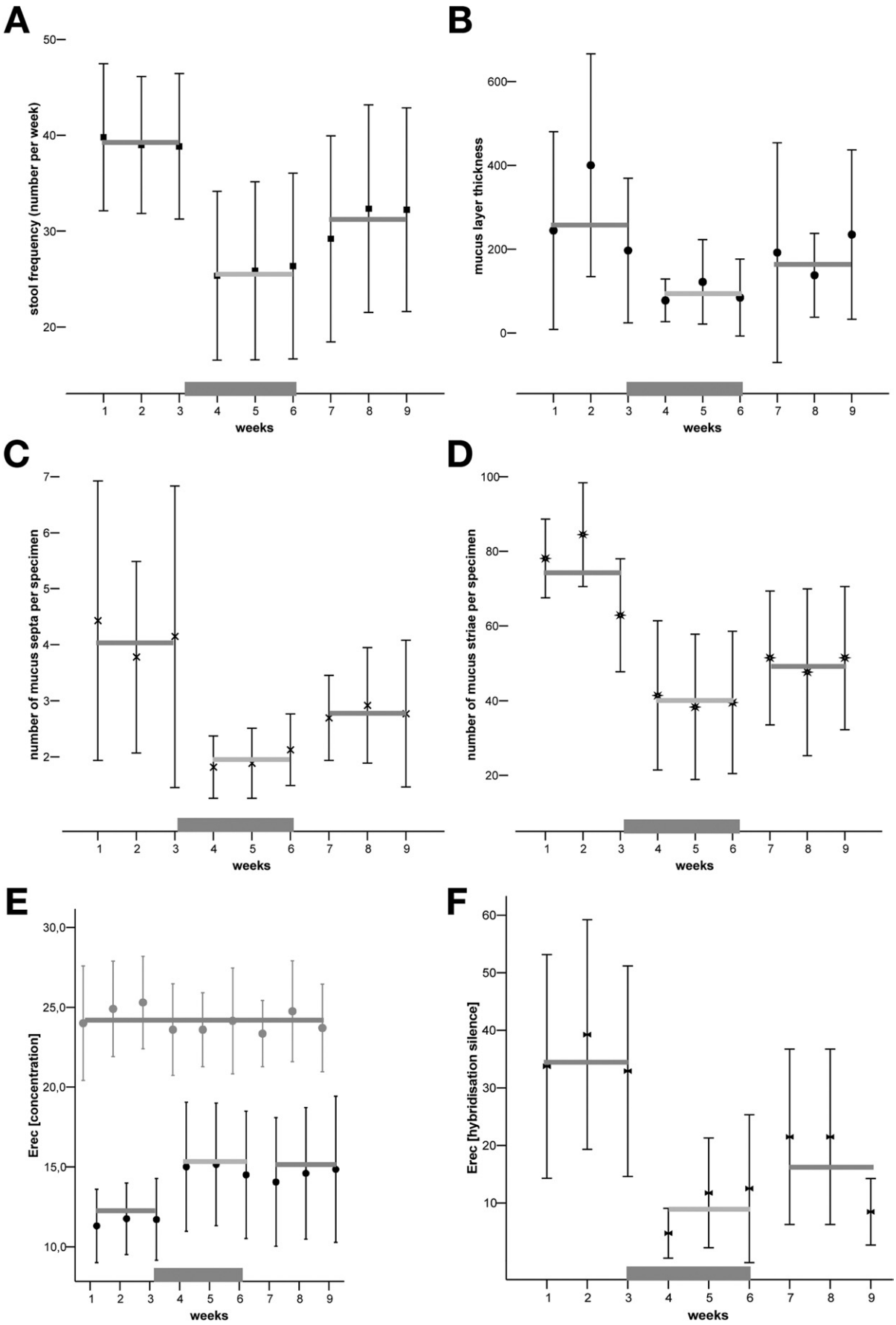
FISH Biostructure of the Normal Fecal Microbiota

Generally, bacteria could be divided either as habitual or occasional, diffusely spread or condensed locally. Typical for the healthy fecal biostructure was a predominance of habitual bacterial groups; each individual had a characteristic distribution and composition of occasional bacteria within the fecal cylinder, which allowed visual differentiation between samples of different subjects.

Habitual bacteria were represented by *Eubacterium rectale*, *Bacteroides*, and *F prausnitzii*. They were present in all samples in high concentrations of more than 10^{10} bacteria/mL and composed each 15%–50% (Figure 3) of the total fecal bacterial population. The concentrations of *E rectale* were usually higher than that of *Bacteroides* and concentrations of *Bacteroides* higher than those of *F prausnitzii*. Habitual bacteria were nearly evenly distributed within the fecal cylinder, with concentrations remaining high close to the inner surface of the mucus layer yet gradually decreasing from the inner to the outer surface of the mucus layer. The fluorescence of habitual bacteria was brilliant over the entire surface of the fecal cylinder in nearly all samples, and this pattern was very similar in all specimens (Figure 3).

All other investigated bacterial groups were occasional, that is, present only in some of the subjects. The concentrations of occasional bacteria were markedly lower than that of habitual bacteria. Occasional bacteria were either diffusely distributed (*Atopobium*, *Bifidobacterium*) or focally condensed. The differences in concentrations of the same occasional bacterial groups ranged from 10^{10} bacteria/mL to below detectable within the same sample or between different samples or patients (Figure 4).

Additionally, bacteria had a characteristic spatial distribution with regard to the mucus layer and the fecal surface and could be divided into mucotrop, mucophob, or fecomucus. Mucotrop bacteria were represented by 2 groups: *Enterobacteriaceae* and Hel274 (unclear taxonomy). The mucotrop bacteria were located mainly on the interface between feces and mucus and were either completely absent in feces or had at least markedly lower concentrations (Figure 5). To correctly quantify the mucotrop bacteria, the numbers and occurrence of Hel274 and *Enterobacteriaceae* groups were separately enumerated for the mucus/feces transition zone (mucotrop *Enterobacteriaceae*, mucotrop Hel274) and for the fecal regions at least 100 μm below the fecal surface (fecal *Enterobacteriaceae*, fecal Hel274). The fecal Hel274 bacteria were always associated with mucotrop Hel274. Fecal


A
stool frequency (number per week)
50
40
30
20
1 2 3 4 5 6 7 8 9
weeks
B
mucus layer thickness
600
400
200
0
1 2 3 4 5 6 7 8 9
weeks
C
number of mucus septa per specimen
7
6
5
4
3
2
1 2 3 4 5 6 7 8 9
weeks
D
number of mucus striae per specimen
100
80
60
40
20
1 2 3 4 5 6 7 8 9
weeks
E
Erec [concentration]
30,0
25,0
20,0
15,0
10,0
1 2 3 4 5 6 7 8 9
weeks
F
Erec [hybridisation silence]
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9
weeks

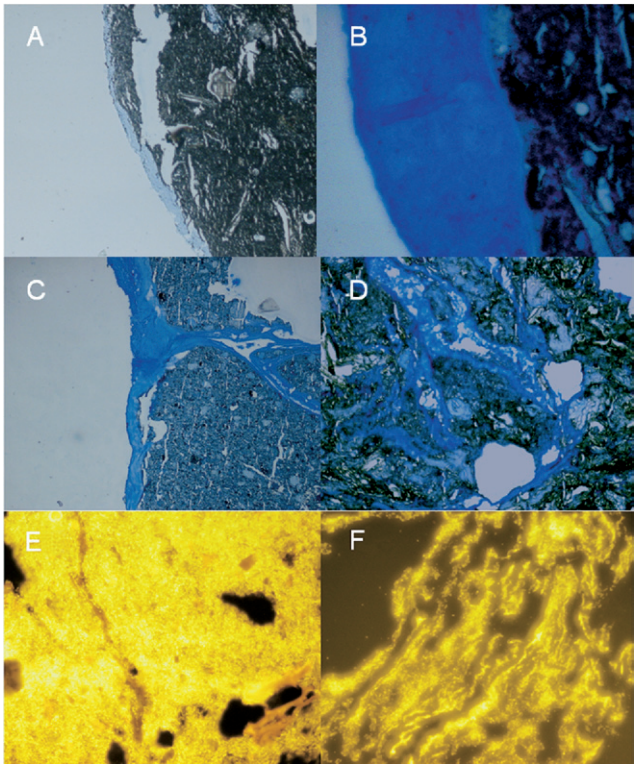


Figure 2. Mucus in feces of (*A*, *C*, and *E*) a healthy control and (*B*, *D*, and *F*) a patient with diarrhea. The superficial mucus layer is significantly thicker in patients with diarrhea than in healthy controls (*B* vs *A*), while the mucus inclusions in the feces leading to mucus septa are seldom observed in healthy controls (*C*) yet prominent in patients with diarrhea (*D*). A homogeneous web structure of the bacterial assembly in healthy controls (*E*) is disrupted by multiple striae in loose, diarrheal stools (*F*). (*A–D*) Original magnification 100× (alcian stain). (*E* and *F*) Universal Eub Cy3 probe, orange fluorescence at original magnification 400×.

Enterobacteriaceae occurred independently from mucotrop *Enterobacteriaceae*.

Bacteria that hybridized with the *Bifidobacterium* probe were mucophob that avoided mucus and were found mostly at a distance of 0–5 μm to the mucus layer or to the fecal surface. *Bifidobacterium*-positive bacteria were occasional and found in 90% of the healthy controls.

All other bacteria, *E rectale*, *F prausnitzii*, *Bacteroides*, *Eubacterium cylindroides*, *Clostridium histolyticum*, and *Clostridium lituseburense*, were fecomucus. Their concentrations were highest in feces, but they could also enter mucus in lower concentrations.

Fecal Microbiota in Patients With Diarrhea and Spontaneous and S boulardii*–Induced Changes*

The concentrations, occurrence, distribution, and fluorescence intensity of single bacterial groups differed markedly between healthy controls and patients with diarrhea (Table 2). In patients with diarrhea, we observed a significant reduction in the concentrations of habitual bacterial groups, suppression of the fluorescence signals of the habitual bacteria in the central regions of the stool cylinder (hybridization silence) with massive increase in the concentrations and spatial shift of mucotrop bacteria in the central regions of the stool cylinder, and increase in the relative concentrations of occasional bacterial groups. We observed high fluctuations in the occurrence and concentrations of single bacterial groups between consecutive investigations and a marked response to *S boulardii* supplementation.

Reduction in concentrations of habitual bacteria. Typical for diarrheal stools were markedly lower concentrations of the habitual bacteria. The differences compared with healthy controls were highly significant ($P < .001$) for each habitual bacterial group.

The decrease in concentrations could only be partially explained by dilution of feces through higher deposits of mucus (striae, septa). The concentrations of bacteria were diminished even in zones of maximal concentration. *S boulardii* supplementation significantly increased the concentrations of habitual bacteria (Figure 1*E*/*F*).

Hybridization silence. In patients with diarrhea, the fluorescence intensity of diffusely distributed bacteria was reduced or even lost in the center of the feces but maintained at the periphery close to the stool surface (Figure 6*A* and *B*). The phenomenon was especially well seen for habitual bacterial groups, which were present in each patient in high concentrations, but was also observed for diffusely distributed occasional bacteria such as *Bifidobacterium* and *Atopobium*. The portion of the cylinder with suppressed fluorescence varied considerably in samples from the same patient. In distinct cases, bacteria could be clearly detected exclusively in the regions (Figure 6*B*) of less than 5 μm below the fecal surface or even within the mucus alone. The loss of the 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 decreasing concentrations but rather due to worsening traceability. Because occasional bacteria were not present in each sample, we quantified the phenomenon of bacterial hybridization silence only for habitual bacteria. The extent of the hybridization silence was expressed as a percent of the fecal cylinder surface in which 10% (partial hybridization silence) or less than 0.1% (complete hybridization silence) of bacteria could be clearly identified. The concentrations of bacteria at the fecal surface were used as reference for assessment of the hybridization silence.

Figure 1. (*A*) Stool frequency, (*B–D*) mucus amounts in feces, (*E*) concentrations of habitual bacteria as mean ± SD × 10^{12}, and (*F*) mean ± SD percent of partial and complete hybridization silence (*Eubacterium rectale*) before, during, and after *S boulardii* supplementation in patients with diarrhea. (E) *E rectale* concentrations in healthy controls (*in gray*) and patients with diarrhea (*in black*).

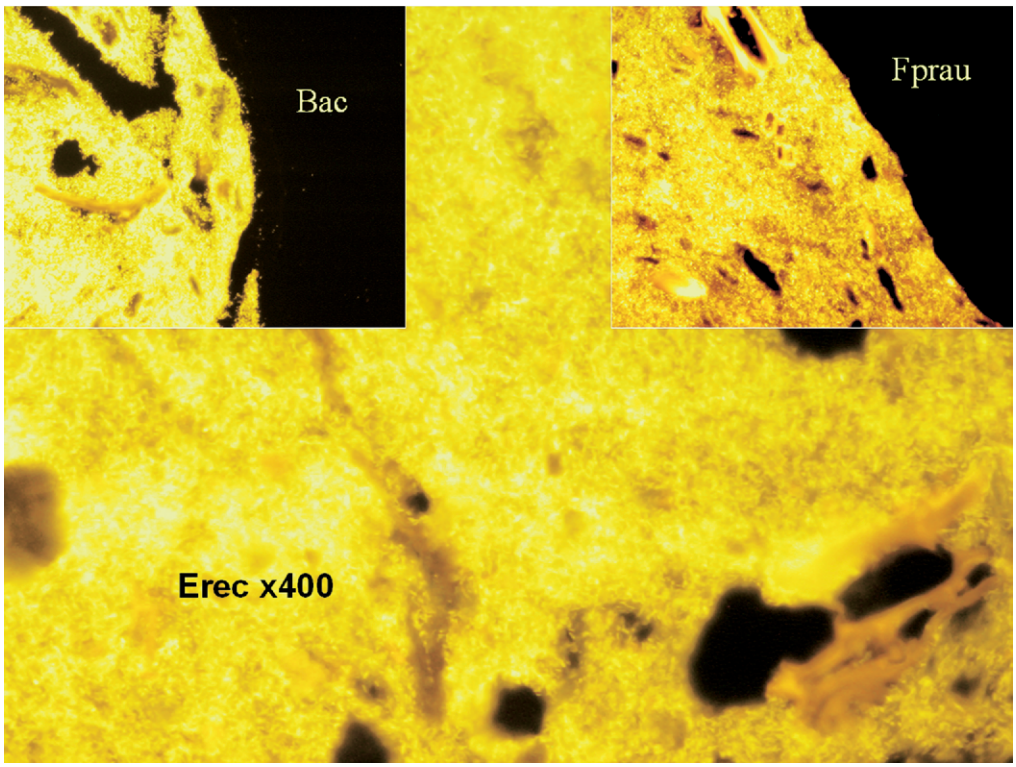


Figure 3. The distribution of habitual bacteria within the fecal cylinder in a healthy control is nearly uniform. Habitual bacteria are represented by 3 groups (*E rectale*, *F prausnitzii*, and *Bacteroides*), which are highly concentrated and contribute each about 15%–50% to the fecal bacterial population.

Hybridization silence was a feature pertaining to a particular sample, and dependent on selected bacterial groups. While some of the bacterial groups were heavily suppressed, others showed excellent fluorescence all over the cylinder. Commonly the fluorescence of *Bacteroides* was more suppressed than that of *F prausnitzii* and *F prausnitzii* more than that of *E rectale*. The hybridization silence was lowest in the *Atopobium* and *Bifidobacterium* groups. The extent of the hybridization silence for the same bacterial group varied from 2% to 98% between

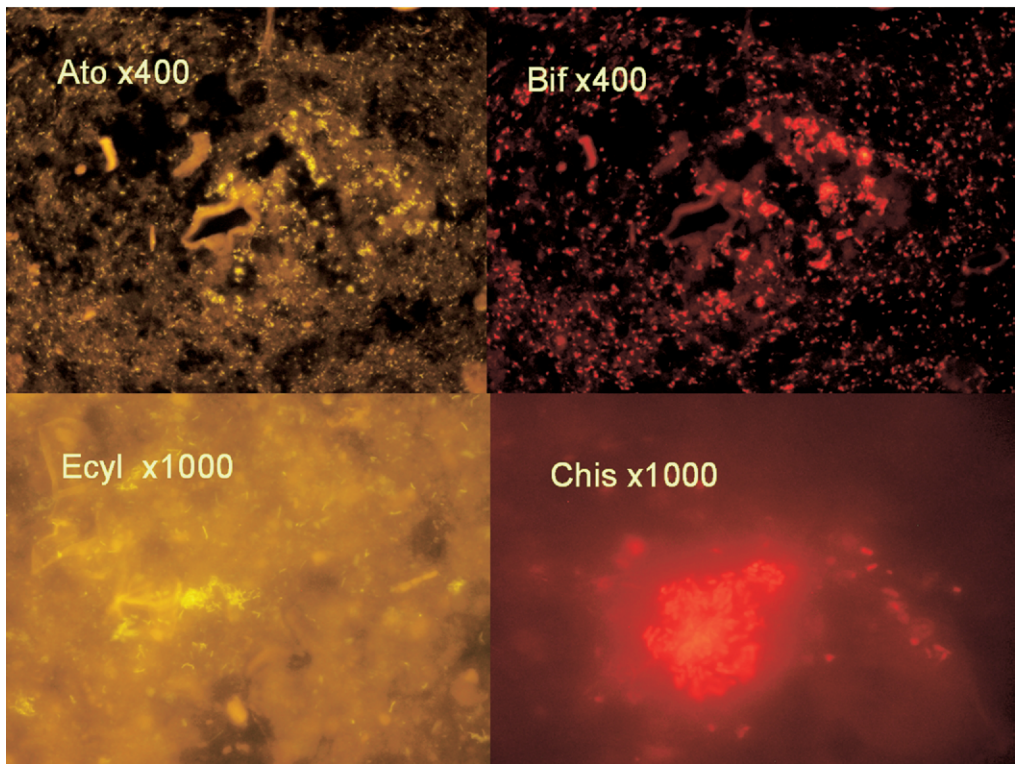


Figure 4. Some examples of occasional bacterial groups hybridized with Cy3-labeled (*orange*) or Cy5-labeled (*red*) probes.

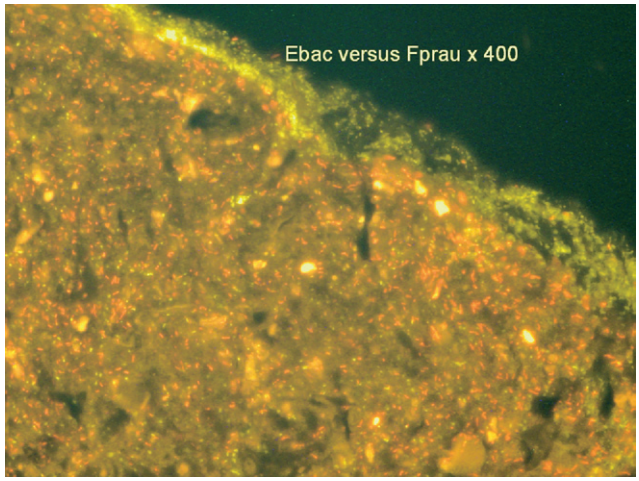


Figure 5. Multicolor FISH shows simultaneously habitual *F prausnitzii* (Cy5, *red fluorescence*) and mucotrop *Enterobacteriaceae* (Cy3, *orange fluorescence*) in a patient with diarrhea. While *F prausnitzii* is mainly located in feces and enters mucus in low concentrations, *Enterobacteriaceae* is located on the border between feces and mucus and readily enters mucus in significant numbers and has significantly lower concentrations in feces.

successive samples of the same patient, indicating a highly dynamic state of this phenomenon. Notwithstanding this variability, the involvement of single bacterial groups in hybridization silence was patient specific. In all probes from the same patients, one of the habitual bacterial groups was suppressed more than the other, leaving some bacterial groups completely unaffected.

The hybridization silence was nearly lacking in the healthy group with an exception of about 15% of samples irregularly demonstrating a partial hybridization silence. Less than 5% of the total surface of all stool cylinders from healthy subjects showed partial hybridization silence. Complete hybridization silence was not observed in any of the stool cylinders from healthy subjects. Because of the low incidence and surface involved, we did not quantify the hybridization silence in the healthy controls.

S boulardii supplementation had a profound effect both on concentrations and hybridization silence of habitual bacteria in patients with diarrhea, leading to improvement of both parameters for all habitual groups. The improvement was maximal in the first week of *S boulardii* supplementation.

Proliferation of mucotrop bacteria. In patients with diarrhea, the occurrence and concentrations of mucotrop bacterial groups were massively increased both in the fecal/mucus transition zone and in the fecal core ($P < .001$).

S boulardii supplementation significantly reduced the mucus in feces. This decrease was accompanied by a reduction in the concentration of Hel274, both in the fecal core and the feces/mucus transition zone. However, the concentrations of mucotrop *Enterobacteriaceae* were not perceptibly changed following *S boulardii* supplementation (Table 2), indicating that the increase of mucotrop *Enterobacteriaceae* in diarrhea must be for other reasons than the amounts of mucus.

Increase in Concentrations of Occasional Bacteria. The concentrations of all occasional bacteria in patients with diarrhea were increased when compared with healthy controls. The difference between patients with diarrhea and healthy controls was significant for *Bifidobacterium*, *E cylindroides*, *C histolyticum*, and *C litusebuense*. *S boulardii* supplementation reduced the difference for *Bifidobacterium* and *E cylindroides* but not for the other occasional bacteria.

Discussion

The analysis of the fecal microbiota in health and disease was generally regarded as highly unreliable, diffi-

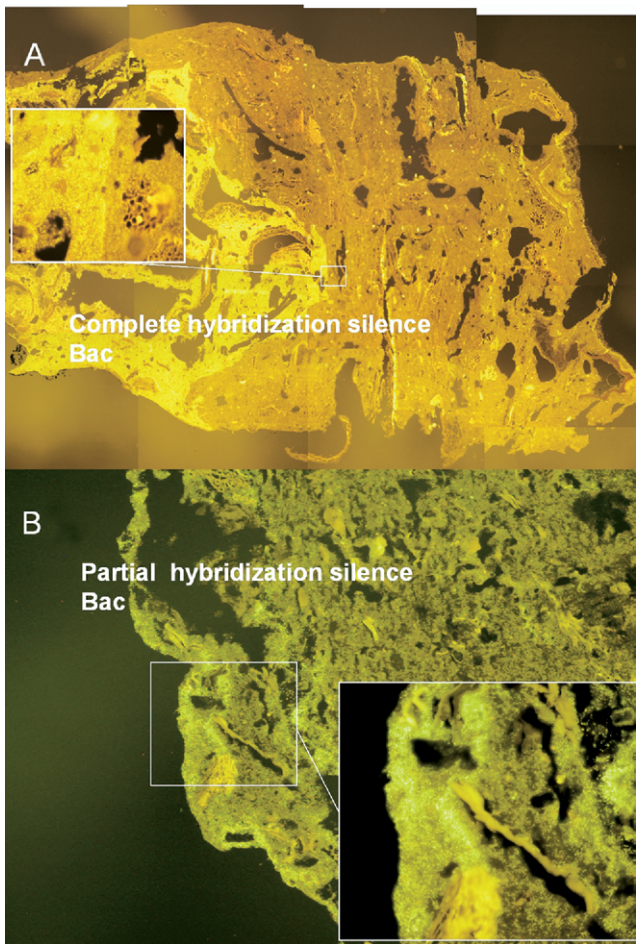


Figure 6. (*A*) The surface of a stool cylinder composed of many single microphotographs (original magnification 100×) demonstrates the loss of bacterial fluorescence in the center: complete hybridization silence (*Bacteroides,* Cy3, *orange*). (*B*) Partial hybridization silence involving the whole cylinder with the exception of regions close to the mucus layer. Bacteria at the fecal core are suppressed but not absent (*Bacteroides*, Cy3, *orange*, original magnification 400×, *inset* original magnification 1000×).

cult to perform, and useless for the clinic.[1,2] Our study and another previous publication[10] show that this is definitely not the case. Quantitative analysis of bacterial composition and fluorescence intensity in the spatial relation to the fecal core, surface, and mucus allow us to differentiate diseased and normal feces and to longitudinally follow-up the progression of the symptoms and change to the normal state. The reason for the failure of previous attempts to characterize the altered fecal microbiota was the lack of awareness for or disregard of the spatial structure of the stool. The FISH analysis of punched fecal cylinders revealed the highly organized fecal biostructure with local concentrations of bacteria that varied from 10^{10} bacteria/mL to levels below detectable.[10] The irregular distribution of bacteria within feces, the differences in fluorescence intensity, and the viability of the bacteria between core, surface, and mucus of the feces bias the results when homogenized fecal samples are used. The fecal microbiota are furthermore highly vulnerable and temperature sensitive. The main masses of fecal microbiota are obligate anaerobe, for which contact with air and decreasing temperature are detrimental. The unavoidable shifts in microbial composition occurring between collection, fixation, storage, and transport of the samples to the laboratory alter the results. A variation in stool consistency makes the standardization of findings with regard to weight, volume, and density uncertain. Of the vast variety of intestinal diseases in which the consistency and composition of stool are altered, the investigation of diarrhea is especially problematic. In the past, diarrhea was defined mainly in terms of stool frequency, consistency, volume, or weight, of which only stool frequency, volume, and weight could be quantified. However, the assessment of these parameters afforded the cooperation of patients in keeping a stool record and collecting fecal material. The data on stool frequency are highly subjective (the difference between drive, tenesm, and defecation are vague), and the collection of feces over extended periods, which is necessary for assessment of volume and weight, is odious and of low informative value. In contrast, analysis of the fecal biostructure using punched fecal cylinders allows a reproducible and dynamic quantitative assessment of the multiple features characterizing diarrhea, all of which are independent of subjective complaints and patient behavior. These include the thickness of the mucus layer covering the feces, the number of mucus septa per fecal cylinder, the number of mucus striae per microscopic field/percentage of surface covered with striae, bacterial concentrations of different bacterial groups, the spatial organization of bacteria (relative to the mucus layer, fecal core, and surface of the fecal cylinder), and hybridization silence.

The ease of collection, convenience of storage, and delivery of stool samples together with the high motivation of chronically ill patients leads to high patient compliance. The immediate fixation of the material in Carnoy's solution and succeeding embedment in paraffin preserves the microbiota in their native state, leads to high reliability of the results, and enables investigations of the samples in reference laboratories of choice even years later to answer emerging questions and to take advantage of newly developed FISH probes.

The number of probes used in this study was limited. Only 3 of them were habitual and present in each sample. It is imaginable that with the widening of the spectrum of FISH probes, idiopathic diarrhea could be further subdivided into specific syndromes based on patterns of bacterial distribution, occurrence, and hybridization silence. However, even 11 FISH probes were sufficient for conclusive evaluation.

The typical features of the healthy fecal biostructure were predominance of habitual bacterial groups, 3 of which compose up to 90% (at least 70%) of the whole fecal bacterial population; homogeneous distribution and high fluorescence of habitual bacteria over the surface of the fecal cylinder, leading to a web-like appearance of their texture; absence or very low concentrations of mucotrop Hel274 bacteria within the fecal core; and low amounts of mucus within the fecal core. Characteristic for diarrhea were massive increases of mucus secretion shown either by the thickness of the superficial mucus layer, massive mucus inclusion within the fecal core forming thick septa, or leading to zebra-like disintegration of architecture of the habitual bacteria by multiple slim mucus striae covering more or less extended regions of the fecal surface; increase in concentrations and spatial shift of mucotrop Hel274 bacteria to the fecal core; the massive reduction in concentration of dominant bacterial groups; hybridization silence of habitual bacteria at the fecal core; and relative increase in concentrations of occasional bacteria.

Except for hybridization silence, all mentioned changes were general for patients with diarrhea and can be explained as a result of impaired intestinal mucus secretion and water reabsorption. The origin of hybridization silence appears to be different. The hybridization silence involved single bacterial groups, differently in single patients, indicating the presence of substances in feces, which selectively suppressed intestinal bacterial groups. The epicenter of hybridization silence was located at the fecal core and was never observed in proximity to the mucus layer or fecal surface, indicating that the suppressive factor leading to hybridization silence is not controlled by the colonic wall but originates from the upper regions of the gastrointestinal tract and presumably small intestine. We assume that the high viscosity of mucus does not allow the "suppressive factor" to enter the compartment adjacent to the colonic mucus layer, where bacteria can survive the host response, building an intact stem zone for later regeneration of the fecal microbiota.

The parameters characterizing healthy and diarrheal stools were stable over the 3 weeks preceding *S boulardii*

supplementation. *S boulardii* had a marked influence on the structural organization of diarrheal feces but had no impact on healthy feces. The normalization of the fecal biostructure following *S boulardii* supplementation was accompanied by subjective improvement of the clinical symptoms.

At present, we can only speculate how Perenterol works. It may interfere with potential pathogens located in the small intestine. It can neutralize exaggerated immune response factors (eg, defensins), specifically or nonspecifically adsorbing them. It can also modulate the mucus barrier and secretion.[3] However, because the effects of *S boulardii* can now be quantified, all these mechanisms can be specifically addressed in future experiments.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2008.04.017.

References

1. Tamboli CP, Neut C, Desreumaux P, et al. Dysbiosis in inflammatory bowel disease. Gut 2004;53:1–4.
2. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007;448:427–434.
3. 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.
4. Czerucka D, Piche T, Rampal P. Review article: yeast as probiotics—*Saccharomyces boulardii*. Aliment Pharmacol Ther 2007; 26:767–778.
5. Afzalpurkar RG, Schiller LR, Little KH, et al. The self-limited nature of chronic idiopathic diarrhea. N Engl J Med 1992;327:1849–1852.
6. Habba SF. Chronic diarrhea: identifying a new syndrome. Am J Gastroenterol 2000;95:2140–2141.
7. 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.
8. 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.
9. 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.
10. Swidsinski A, Loening-Baucke V, Vaneechoutte M, et al. Active Crohn's disease and ulcerative colitis can be specifically diagnosed and monitored based on the biostructure of the fecal flora. Inflamm Bowel Dis 2008;14:147–161.

Received January 11, 2008. Accepted April 10, 2008.

Address requests for reprints to: Alexander Swidsinski, MD, PhD, Humboldt University, Charité, 10098 Berlin, Germany. e-mail: alexander.swidsinski@charite.de; fax: (49) 30 450 514 923.

The authors report that there is no conflict of interest to disclose.

Shortened Translation of the German Version of Instructions on Stool Sample Collection

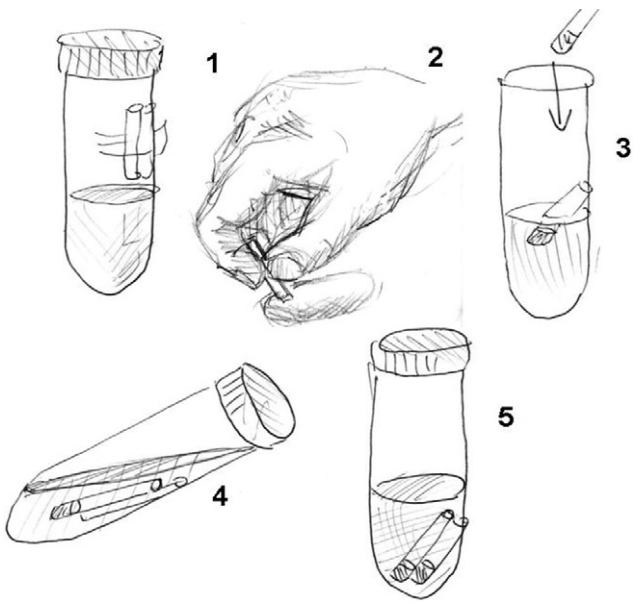
The fecal portion inside the straw represents the outer side of the feces. The fecal portion on the outer end of the straw represents an interior of the feces. Dipping the outer end in ink marks this part of the feces and allows orientation during light microscopy.

How to Collect the Stool Sample

The bottle contains a special solution for the conservation of the stool. Should you accidentally splash some of the fluid on your skin or in the eye, rinse with plenty of water. The solution is not harmful to you.

Each bottle has 2 pieces of a straw attached (see Figure 1). Stick each of the straws into the stool about 1/3 inch deep using a twisting movement (see Figure 2). Use 2 different areas of the stool for sampling. Then withdraw the straw with the stool sample inside and drop the straw into the fluid-filled glass tube. If the stool did not stay in the straw, try again. The straw helps to protect the stool from falling apart. After you screwed the lid tight, tip the bottle once so that the solution flows into the straw. Please do not shake; that would loosen the stool and it may fall out of the straw (Figures 3–5). Please do not put loose stool parts (not contained within the straw) in the bottle, because there is not enough solution to fix the stool.

If the stool that is sampled is very loose and falls out of the straw, please use some Vaseline to close the lower end of the straw. The upper end of the straw needs to stay open so the solution can reach the stool sample. The stool sample should stay at room temperature for 24 hours and then can be kept in the refrigerator for 2–4 weeks.



Statistics

All statistical analyses were performed using the statistical software package SPSS version 15.0 (Chicago, IL). Deviation of each continuous variable from a theoretical normal distribution was assessed through the one-sample Kolmogorov–Smirnov test procedure. Accordingly, because the P value to the Kolmogorov–Smirnov Z-statistic was consistently $<.05$, all analyses were performed under the nonparametric assumption. Differences in the distribution of a variable at subsequent points in time were first explored by comparing mean ranks of that variable for each point in time. If the latter comparison revealed a significant difference, then data were reanalyzed according to a repeated-measures design under the nonparametric assumption with the Friedman test. Statistical significance was accepted if the 2-tailed probability level was $<.05$.

Supplementary Material to Table 2 Describing Details of the Statistical Analysis

Stool Frequency

Distribution of stool frequency among patients with chronic idiopathic diarrhea was heavily skewed to the right and thereby deviated significantly from a theoretical parametric distribution (one-sample Kolmogorov–Smirnov test; $P = .010$). The mean rank of stool frequency per week differed significantly for each week when accounting for the entire study period ($\chi^2 = 19.59$; $P = .012$) and in particular when accounting for the pretreatment and treatment period, that is, up to 6 weeks ($\chi^2 = 20.76$; $P = .001$). When data were pooled as serial data according to pretreatment, treatment, and posttreatment status, respectively, a highly significant trend was observed (Friedman $\chi^2 = 72.66$; $P < .001$).

Proportion of Stool Specimen Surface Covered With a Mucus Layer

The proportion of the stool specimen surface covered with a mucus layer was non-normally distributed (one-sample Kolmogorov-Smirnov test; $P < .001$). When accounting for cases for which a mucus layer was observed, the mean rank of percentage surface covered with mucus differed significantly for each week during the first 6 weeks (Kruskal–Wallis test; $P = .027$) although not over the entire study period (Kruskal–Wallis test; $P = .108$) because the initial difference with treatment was apparently leveled off by the reappearance of the mucus layer following treatment cessation.

Mucus Septa

The number of mucus septa was non-normally distributed (one-sample Kolmogorov-Smirnov test; $P < .001$). When accounting for cases with chronic idiopathic diarrhea for which mucus septa were observed, the mean rank of the

number of septa varied significantly over time (Kruskal–Wallis test; $P = .014$) and especially during the first 6 weeks (Kruskal–Wallis test; $P = .014$). A similar trend was not observed with healthy control subjects (Kruskal–Wallis test; $P = .072$ for the first 6 weeks and $P = .353$ for the entire study period, respectively). Overall, when data were pooled as serial data according to pretreatment, treatment, and posttreatment status, respectively, a significant trend was observed in the number of mucus septa among patients (Friedman $\chi^2 = 9.27$; $P = .010$), although not with healthy controls (Friedman $\chi^2 = 4.32$; $P < .115$).

Mucus Striae

The number of mucus striae was non-normally distributed (one-sample Kolmogorov-Smirnov test; $P < .001$). The mean rank of number of mucus striae differed highly significantly per week throughout the study (Kruskal–Wallis $\chi^2 = 27.99$; $P < .001$). When data were pooled as serial data according to pretreatment, treatment, and posttreatment status, respectively, a highly significant trend was observed in the number of mucus septa among patients (Friedman $\chi^2 = 72.71$; $P < .001$).

E rectale

When data were pooled as serial data according to pretreatment, treatment, and posttreatment status, respectively, there was a highly significant trend over time in *E rectale* concentrations among healthy controls (Friedman $\chi^2 = 18.70$; $P < .001$) and among patients with chronic idiopathic diarrhea (Friedman $\chi^2 = 12.31$; $P < .002$) as well as a significant trend in average *E rectale* hybridization silence (Friedman $\chi^2 = 49.52$, $P < .001$ and $\chi^2 = 25.09$, $P < .001$, respectively).

Bacteroides

When data were pooled as serial data according to pretreatment, treatment, and posttreatment status, respectively, there was a highly significant trend over time in *Bacteroides* concentrations among healthy controls (Friedman $\chi^2 = 14.45$; $P = .001$) and among patients with chronic idiopathic diarrhea (Friedman $\chi^2 = 20.83$; $P < .001$).

F prausnitzii

When data were pooled as serial data according to pretreatment, treatment, and posttreatment status, respectively, there was a highly significant trend over time in *F prausnitzii* concentrations among patients with chronic idiopathic diarrhea (Friedman $\chi^2 = 8.08$; $P < .018$), although not among healthy controls (Friedman $\chi^2 = 0.13$; $P = .9$).

Bifidobacterium

The mean rank of *Bifidobacterium* concentrations did not differ significantly across subsequent sampling weeks or among healthy subjects ($P = .49$) or patients with chronic idiopathic diarrhea ($P = .46$).

Ato

The mean rank of *Ato* concentrations did not differ significantly across subsequent sampling weeks or among healthy subjects ($P = .43$) or patients with chronic idiopathic diarrhea ($P = .10$).

E cylindroides

The mean rank of *E cylindroides* concentrations did not differ significantly across subsequent sampling weeks or among healthy subjects ($P = .33$) or patients with chronic idiopathic diarrhea ($P = .17$).

Ehal

The mean rank of *Ehal* concentrations did not differ significantly across subsequent sampling weeks among healthy subjects ($P = .31$) but showed a marked difference over time among patients with chronic idiopathic diarrhea (Kruskal–Wallis $\chi^2 = 25.43$; $P = .001$). Hence, when data were pooled as serial data according to pretreatment, treatment, and posttreatment status, respectively, there was a highly significant trend over time in *Ehal* concentrations among patients with chronic idiopathic diarrhea (Friedman $\chi^2 = 16.18$; $P < .001$).

C lituseburense

The mean rank of *C lituseburense* concentrations did not differ significantly across subsequent sampling weeks or among healthy subjects ($P = .64$) or patients with chronic idiopathic diarrhea ($P = .21$).

C histolyticum

The mean rank of *C histolyticum* concentrations did not differ significantly across subsequent sampling weeks or among healthy subjects ($P = .81$) or patients with chronic idiopathic diarrhea ($P = .18$).

Enterobacteriaceae Surface

Mean rank of *Enterobacteriaceae* surface concentrations did not differ significantly across subsequent sampling weeks or among healthy subjects ($P = .51$) or patients with chronic idiopathic diarrhea ($P = .45$).

Enterobacteriaceae Deep

The mean rank of *Enterobacteriaceae* deep concentrations did not differ significantly across subsequent sampling weeks or among healthy subjects ($P = .46$) or patients with chronic idiopathic diarrhea ($P = .42$).

Hel Surface

The mean rank of *Hel* surface concentrations did not differ significantly across subsequent sampling weeks or among healthy subjects ($P = .55$) or patients with chronic idiopathic diarrhea ($P = .23$).