Cross-Sectional and Longitudinal Comparisons of the Predominant Fecal Microbiota Compositions of a Group of Pediatric Patients with Cystic Fibrosis and Their Healthy Siblings^{▽†}

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Although only poorly documented, it can be assumed that intensive antibiotic treatments of chronic lung infections in patients with cystic fibrosis (CF) also affect the diversity and metabolic functioning of the gastrointestinal microbiota and potentially lead to a state of dysbiosis. A better knowledge of the differences in gut microbiota composition and stability between patients with CF and healthy subjects could lead to optimization of current antibiotic therapies and/or development of add-on therapies. Using conventional culturing and population fingerprinting by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA amplicons, we compared the predominant fecal microbiota of 21 patients with CF and 24 healthy siblings in a cross-sectional study. General medium counts, as well as counts on media specific for lactic acid bacteria, clostridia, *Bifidobacterium* spp., *Veillonella* spp., and *Bacteroides-Prevotella* spp., were consistently higher in sibling samples than in CF samples, whereas the reverse was found for enterobacterial counts. DGGE fingerprinting uncovered large intersubject variations in both study groups. On the other hand, the cross-sectional data indicated that the predominant fecal microbiota of patients and siblings had comparable species richness. In addition, a longitudinal study was performed on 7 or 8 consecutive samples collected over a 2-year period from two patients and their respective siblings. For these samples, DGGE profiling indicated an overall trend toward lower temporal stability and lower species richness in the predominant fecal CF microbiota. The observed compositional and dynamic perturbations provide the first evidence of a general dysbiosis in children with CF compared to their siblings.

The human gastrointestinal tract is colonized by a highly dynamic and complex microbial community that offers several health benefits to the host, including metabolic fermentation of nondigestible dietary components, acquisition of vitamins and nutrients, storage of fat, regeneration of intestinal epithelium, resistance to colonization by pathogens, and development and homeostasis of the mucosal immune system (4, 14, 18, 20, 22, 52, 56). Although the underlying mechanisms are not fully understood, it is known that dietary alterations, changes in environment, physical and/or psychological stress, and antimicrobial agents can disrupt the natural colonization barrier, which may trigger overgrowth by autochthonous opportunistic pathogens and eventually contribute to the development of several disorders (13, 15, 21, 68). Particularly in very young infants, disturbance of the indigenous enteric microbiota could have a tremendous impact on the host's metabolic needs, as well as on the development of the intestine-associated immune system (65).

There is growing evidence showing that diseases primarily manifested at the extraintestinal level, such as obesity (31, 63)

and atopic diseases (5, 28, 45, 69), are associated with perturbations of the gastrointestinal microbial ecosystem (24, 61). Also, for cystic fibrosis (CF), a lethal hereditary disorder leading to respiratory infections and chronic inflammation, a possible association with intestinal dysbiosis has been assumed but never documented. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes an ion channel in the apical membranes of exocrine epithelial cells (10, 29). Not only do patients with CF have extremely viscous respiratory secretions, but also, abnormal mucus production in the small intestine is thought to contribute to gastrointestinal (GI) symptoms of malabsorption and obstruction (40, 50). Because of recurrent bronchopulmonary infections, patients with CF are treated with repeated courses of antibiotics. During antibiotic therapy, important bacterial groups may be radically reduced or eliminated from the gut, depending on the dosage of the antimicrobial agent and its pharmacokinetics (21, 42, 51). Although it is commonly accepted that the ecological balance is restored a few weeks after antibiotic administration, there is also evidence showing that antibiotic-associated disturbances can persist for a long period after treatment (25, 27, 49). Furthermore, overgrowth by antibiotic-resistant bacteria and fungi may emerge from prolonged exposure to antimicrobial agents (51). In patients with CF, antibiotic-associated infections with opportunistic pathogens, such as *Clostridium difficile*, may cause severe diarrhea and/or pseudomembranous colitis (23, 55).

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TABLE 1. Characteristics of children participating in the cross-sectional study

Patient with CF or sibling	Gender [no. (%)]			Age	Antibiotic history [no. (%)]
	Male	Female	Total		
Patient with CF	11 (61)	10 (37)	21 (47)	1–13 yr	21 (91) ^a
Sibling	7 (39)	17 (63)	24 (53)	9 mo–15 yr	2 (9)
Total	18 (100)	27 (100)	45 (100)	9 mo–15 yr	23 (100)

^a See Table S1 in the supplemental material.

The hypothesis that continuous impairment of digestive processes coupled with repeated use of high-dose antibiotics may lead to a permanent state of dysbiosis in patients with CF has not been thoroughly investigated (6, 7, 70, 71). To this end, improved insight into the diversity and stability of the intestinal CF microbiota is not only essential to better predict clinical outcomes, but also could trigger the development of alternative or supplementary therapies based on pro- and/or prebiotics (7, 70). The primary goals of this study were to compare the predominant fecal microbiota in patients with CF and their healthy siblings for compositional differences and to monitor the temporal stability of these bacterial populations.

MATERIALS AND METHODS

Subjects and samples. Twenty-one children with CF and one or two of their healthy siblings were included in a cross-sectional study (Table 1). For the longitudinal study, two patients were selected based on clinical history and stool morphology (Table 2). The Ethics Committee of the University of Leuven (Belgium) approved the study, and all volunteers gave informed consent. Fresh stools were collected in sterile plastic containers at the University Hospital of Leuven and frozen at -80°C .

Culture. One gram (wet weight) of thawed fecal sample was homogenized in 9 ml peptone-buffered saline (PBS) (0.1% [wt/vol] bacteriological peptone [L37; Oxoid, Basingstoke, United Kingdom], 0.85% [wt/vol] NaCl [106404; Merck, Darmstadt, Germany]). The resulting 10-fold serial dilutions (10^{-1} to 10^{-6}) in PBS were further processed anaerobically (8% [vol/vol] CO_2 , 8% H_2 , and 84% N_2). Aliquots (50 μl) were spread plated in triplicate onto the general medium for colon bacteria (MCB) (66) and six selective media, i.e., eosin methylene blue agar (EMB) (CM0069; Oxoid) for isolation of *Enterobacteriaceae*; deMan-Rogosa-Sharp (MRS) agar (CM0361; Oxoid) for isolation of lactic acid bacteria; *Veillonella* agar (VA) (53) for *Veillonella* spp.; reinforced clostridial medium (RCM) (CM0149; Oxoid) supplemented with 15 ml 60% Na-lactate (VWR International), 0.001 g resazurin sodium salt (R-7017; Sigma-Aldrich), and 0.25 g L-cysteine HCl (C6852; Sigma-Aldrich) for isolation of clostridia; BCCM/LMG modified Columbia agar (M144) for isolation of *Bifidobacterium* spp. (39); and medium for *Bacteroides-Prevotella* (MBP) (35). All plates were incubated anaerobically for 72 h at 37°C .

After incubation, the bacterial concentrations (CFU g^{-1}) in the original sample were determined, and one MCB plate per dilution was selected for harvesting. The resulting cultured fractions from MCB were homogenized in 500 μl resuspension (RS) buffer (0.15 M NaCl, 0.01 M EDTA [pH 8.0]), and the pellets were frozen at -20°C until DNA extraction.

Isolation of DNA from fecal samples and cultured fractions. DNA was prepared from fecal samples, as well as from the cultured fractions harvested from MCB. For fecal samples, DNA was obtained using a slightly modified version of the protocol of Pitcher and coworkers (48) as previously described (67). For cultured fractions from MCB, DNA was obtained according to the protocol for DNA extraction of Gram-positive bacteria of Pitcher and coworkers (48), with the addition of 40 μl mutanolysin per sample to the enzyme mixture. The DNA concentration, purity, and integrity were determined using 1% (wt/vol) agarose gels stained with ethidium bromide and by spectrophotometric measurement at 234, 260, and 280 nm. Total DNA solutions were 10-fold diluted, and DNA solutions obtained from cultured MCB fractions were diluted to 50 ng μl^{-1} (an optical density of 1).

Community PCR for denaturing gradient gel electrophoresis (DGGE). The variable V_3 region of the 16S rRNA gene was amplified using the universal bacterial primers F357-GC and R518 (62, 75). The PCR and temperature program used was previously described by Vanhoutte et al. (67).

DGGE analysis and gel processing. The resulting 16S rRNA amplicons were analyzed by DGGE fingerprinting analysis (D-Code System; Bio-Rad, Nazareth, Belgium) using 35 to 70% denaturing gels as previously described (62). Per lane, 30 μl of PCR product was loaded, and electrophoresis was performed at 70 V for 990 min. Afterward, the DGGE gels were stained for 30 min with 1 $\times$ SYBR Gold nucleic acid gel stain (S-11494; Invitrogen, Merelbeke, Belgium) in 1 $\times$ Tris-acetate-EDTA (TAE) buffer (Bio-Rad), and the band profiles were digitally visualized using a charge-coupled device (CCD) camera and the Bio-Rad Quantity One software program. A standard reference lane containing the V_3 -16S rRNA amplicons of 12 taxonomically well-characterized bacterial species was included every fifth or sixth lane for normalization of the fingerprint profiles using BioNumerics software version 5.10 (Applied Maths, St.-Martens-Latem, Belgium).

Data analysis. For each medium, total counts of the cross-sectional CF patient and sibling samples were compared with a paired t test (95% confidence interval [CI]). The counts obtained in the longitudinal study were explored using a linear mixed model, which accounts for the temporal variation between the consecutive samples from each subject nested within a patient-sibling pair.

Similarities between DGGE fingerprint profiles of fecal samples and cultured fractions were determined using the Pearson similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA) in BioNumerics version 5.10 (Applied Maths, St.-Martens-Latem, Belgium). For cross-sectional, as well as longitudinal, data sets, box plots were generated to compare the variable range of pairwise similarities between fecal samples from patients with CF and healthy siblings. Student's t test was used to explore statistically significant differences (95% CI). In addition, DGGE fingerprints from the longitudinal study were subjected to moving-window analysis to express the temporal variation between the eight consecutive time points (38). In that way, the percent change was plotted over 8 different time points with intervals of 3 months.

Three richness indices were defined to compare the overall taxonomic diversity of the predominant fecal microbiota between patients with CF and their siblings. The total richness index (TRI) corresponds to the total number of bands in a single DGGE fingerprint profile. The other two richness indices were

TABLE 2. Characteristics of children participating in the longitudinal study

Subject	Gender	Age (at baseline) (yr)	Sampling period	No. of samples	Antibiotic history
Patient sibling pair 1					
Patient with CF ^b	Female	13	October 2007–August 2009	7	Yes ^a
Sibling	Female	9	August 2007–August 2009	8	NA ^d
Patient sibling pair 2					
Patient with CF ^c	Female	2	August 2007–May 2009	8	Yes ^a
Sibling	Female	5	August 2007–May 2009	8	NA

^a See Table S2 in the supplemental material.^b Patient 1 is a female with persistent bronchopulmonary obstruction. The patient is chronically colonized with *Staphylococcus aureus* and *Achromobacter xylosoxidans* and has a history of intensive antibiotic treatment courses. Stool samples were yellow-brown appearance and had a Bristol stool score of 6.^c Patient 2 is a female with chronic *S. aureus* and *Haemophilus influenzae* infections. Stool samples were brown and had a Bristol stool score of 5.^d NA, not applicable.

TABLE 3. Total viable counts ($\log_{10}$ CFU g^{-1}) of fecal samples from the cross-sectional study on EMB agar, VA, MRS agar, M144, DRCM, MBP, and MCB^a

Medium	No. of samples		Total count ($\log_{10}$ CFU g^{-1}) (mean $\pm$ SE)		<i>P</i> value ^b
	Patients	Siblings	Patients	Siblings	
EMB agar (<i>Enterobacteriaceae</i>)	17	19	5.5 $\pm$ 0.6	5.0 $\pm$ 0.6	0.54
VA (<i>Veillonella</i> spp.)	17	18	4.2 $\pm$ 0.6	5.0 $\pm$ 0.4	0.32
MRS agar (lactic acid bacteria)	17	19	6.6 $\pm$ 0.3	6.6 $\pm$ 0.3	0.98
M144 (<i>Bifidobacterium</i> spp.)	17	19	6.9 $\pm$ 0.3	7.2 $\pm$ 0.3	0.88
DRCM (clostridia)	17	19	6.9 $\pm$ 0.3	6.7 $\pm$ 0.3	0.71
MBP (<i>Bacteroides-Prevotella</i> spp.)	16	17	3.5 $\pm$ 0.7	5.0 $\pm$ 0.5	0.07 ^c
MCB (general medium for colon bacteria)	17	18	6.9 $\pm$ 0.3	6.9 $\pm$ 0.3	0.93

^a Subjects from whom no or less than 1 g of fecal material was obtained were not included.^b The *P* values are based on paired *t* tests (95% CI).^c Borderline significant difference between CF and sibling samples based on paired *t* test (95% CI).

correlated with phylogenetic diversity by defining two percent GC (%GC) regions in the DGGE profile, representing two subpopulations of low- and high-%GC species. Whereas the low-%GC richness index (LRI) refers to the number of bands in the low-%GC region, the high-%GC richness index (HRI) is a measure of the number of bands in the high-%GC region. The delineation of these two regions was determined by the position of the reference strain, *Escherichia fergusonii* LMG 7866 (GenBank accession number NC_011740), which has a genomic %GC of 49.9 and a V₃-16S rRNA %GC of 50.8. The region covered by the LRI contains V₃-16S rRNA amplicons in the DGGE %GC range of 35.0 to 52.5 and thus mainly represents low-G+C bacteria (genomic %GC < 50.0). The region covered by the HRI contains V₃-16S rRNA amplicons in the DGGE %GC range of 52.5 to 70.0, which largely corresponds to the high-%GC bacteria (genomic %GC > 50.0). The results were represented as means $\pm$ standard errors (SE), and statistical analysis was performed using SPSS software version 17.0, with *P* values of less than 0.05 considered to be significant.

RESULTS

Culture-based enumeration of selected bacterial groups.

When comparing bacterial counts obtained from the 21 patient-sibling pairs included in the cross-sectional study (Table 3), the largest difference between the two subject groups was found on MBP, which is selective for *Bacteroides/Prevotella*. MBP counts were 1.5 mean log CFU g^{-1} higher in the sibling group, with borderline significance (Student's *t* test; *P* = 0.07). Samples from healthy siblings revealed equal to slightly higher counts on MCB and media selective for lactic acid bacteria, bifidobacteria, *Veillonella* spp. (differences ranging from 0 to 0.8 mean log CFU g^{-1}) compared to CF patient samples. The opposite was observed for counts on EMB agar, selective for

Enterobacteriaceae (0.5 mean log CFU g^{-1} difference), and counts on DRCM, selective for clostridia (0.2 mean log CFU g^{-1} difference), both of which were lower in samples from the sibling group. None of the differences in EMB agar or DRCM counts were statistically significant.

In the longitudinal study, all the medium counts but those for EMB agar were higher in sibling samples than in the CF patient samples, with the largest differences found on MBP and M144 (mean log CFU g^{-1} differences ranging from 0.4 to 2.7) (Table 4). On EMB medium, mean counts were generally higher in samples from patients with CF than in samples from healthy siblings. None of these differences between the two subject groups were statistically significant. However, comparison of the evolution of the medium counts in the longitudinal study revealed (borderline) significant differences in MCB, DRCM, M144, and MRS agar counts (Fig. 1). For these counts, the temporal variation during the sampling period was narrower for sibling samples than for CF patient samples.

DGGE analysis of cross-sectional samples. DGGE profiles obtained for 21 patient-sibling pairs were initially compared by hierarchical clustering using the Pearson correlation coefficient (see Fig. S1 in the supplemental material). Analysis of the cross-sectional DGGE data revealed considerable interindividual variation within both the patient and healthy sibling groups. As a result, DGGE profiles of patients with CF and healthy siblings did not group in two distinct clusters. The variation of the predominant bacterial diversity within these

TABLE 4. Total viable counts ($\log_{10}$ CFU g^{-1}) of fecal samples from patient-sibling pairs 1 and 2 (longitudinal study) on EMB agar, VA, MRS agar, M144, DRCM, MBP, and MCB

Medium	No. of samples ^a		Count (log ₁₀ CFU g ⁻¹) (mean ± SE)				Log difference ^b
			Total		Minimum–maximum		
	Patients	Siblings	Patients	Siblings	Patients	Siblings	
EMB agar (<i>Enterobacteriaceae</i>)	11	14	5.9 ± 0.7	5.5 ± 0.2	0.0–7.0	0.3–6.4	−0.4
VA (<i>Veillonella</i> spp.)	13	12	4.3 ± 0.9	5.9 ± 0.3	0.0–8.6	0.0–8.0	1.6
MRS agar (lactic acid bacteria)	12	11	5.8 ± 0.9	7.9 ± 0.2	0.0–8.2	0.3–8.8	2.1
M144 (<i>Bifidobacterium</i> spp.)	12	12	5.9 ± 0.9	8.3 ± 0.2	0.0–8.8	0.0–8.9	2.4
DRCM (clostridia)	12	12	5.9 ± 0.9	7.9 ± 0.3	0.0–8.7	0.0–9.0	2.0
MBP (<i>Bacteroides-Prevotella</i> spp.)	11	12	3.9 ± 0.8	6.6 ± 0.5	0.0–7.7	2.3–8.6	2.7
MCB (general medium for colon bacteria)	12	10	5.9 ± 0.9	7.8 ± 0.4	0.0–8.7	0.0–9.2	1.9

^a Only 7 samples were obtained from patient 2. Noncountable data were excluded.^b Difference between $\log_{10}$ mean CFU g^{-1} values for CF patients and siblings for each medium.

FIG. 1. Total viable counts from the longitudinal study on DRCM (a), EMB agar (b), M144 (c), MBP (d), MCB (e), MRS agar (f), and VA (g). Since no countable data were retrieved from the second sampling point for both patients with CF, a broken line was used to connect the first and the third sampling points. *a*, significant difference ($P < 0.05$); *b*, borderline significant difference ($P = 0.072$). The error bars indicate SD.

two subject groups was assessed by determining pairwise similarity values between all fecal fingerprints in each group. For the cross-sectional samples, this resulted in a data set of 190 pairwise combinations per subject group, which were further compared using box plot analysis (see Fig. 3a). Comparison of the mean percent pairwise similarity revealed that DGGE profiles of CF patient samples were overall significantly (Student's *t* test; $P < 0.01$) less similar ($23.80\% \pm 1.22\%$) and thus more variable in compositional complexity than the sibling samples ($32.23\% \pm 1.54\%$).

Analysis of the pooled data revealed that the fecal fingerprints of patients with CF together displayed 104 distinct band positions, whereas in the profiles of healthy siblings, 110 different bands were found. In the pooled sets of DGGE profiles from cultured MCB fractions, both subject groups displayed 68 distinct band positions. In the cross-sectional study, the total

species richness values as indicated by TRI values were comparable in sibling and CF patient samples for both total fecal DNA and cultured MCB fractions (Tables 5 and 6). Based on the TRI values, the profiles obtained from cultured fractions on MCB were generally less complex than those obtained from total DNA extracts, and both types of profiles contained common, as well as unique, bands within a given sample (for an example, see Fig. S2 in the supplemental material). In banding patterns of total fecal DNA, as well as MCB fractions, the LRI was found to be higher than the HRI in both subject groups. No significant (Student's *t* test; $P > 0.05$) difference in LRI and HRI values was found between CF patient samples and sibling samples.

DGGE analysis of longitudinal samples. Whereas no subject-specific clustering was observed for the first patient-sibling pair, DGGE fingerprints of the second pair clearly indicated

TABLE 5. LRI, HRI, and TRI values for DGGE fingerprint profiles of total fecal DNA

Fecal sample	No. of samples	LRI			HRI			TRI	
		Mean $\pm$ SE ^a	Minimum–maximum	P value	Mean $\pm$ SE ^b	Minimum–maximum	P value	Mean $\pm$ SE ^c	P value
Cross-sectional									
Patients with CF	20	13.0 $\pm$ 1.1	3.0–23.0	0.63	5.9 $\pm$ 0.6	0.0–11.0	0.42	19.2 $\pm$ 1.4	0.65
Siblings	22	13.8 $\pm$ 1.2	5.0–27.0		5.3 $\pm$ 0.5	5.0–27.0		20.6 $\pm$ 1.5	
Longitudinal patient									
sibling pair 1									
Patient 1	7	8.3 $\pm$ 1.0	6.0–14.0	0.02 ^d	5.4 $\pm$ 1.6	1.0–14.0	0.08 ^d	13.9 $\pm$ 1.9	0.28
Sibling 1	8	15.5 $\pm$ 2.8	6.0–26.0		2.4 $\pm$ 0.4	1.0–4.0		18.1 $\pm$ 3.1	
Longitudinal patient									
sibling pair 2									
Patient 2	8	7.0 $\pm$ 1.1	4.0–12.0	0.22	3.5 $\pm$ 0.8	1.0–8.0	0.08	11.0 $\pm$ 1.5	0.16
Sibling 2	8	10.0 $\pm$ 2.1	4.0–20.0		5.4 $\pm$ 0.6	4.0–9.0		15.6 $\pm$ 2.7	

^a Mean number of bands in the low-%GC region of the DGGE fingerprint profile.

^b Mean number of bands in the high-%GC region of the DGGE fingerprint profile.

^c Mean total number of bands in the DGGE fingerprint profile.

^d Significant difference between CF samples and sibling samples (95% CI).

subject-specific grouping (Fig. 2a and b). Twenty-one pairwise combinations were made for samples from patient 1 and 28 combinations for samples from sibling 1 (Fig. 3b). For the second patient-sibling pair, a set of 28 pairwise combinations was obtained for both subjects. In both pairs, box plot analysis revealed a significantly (Student's *t* test; $P < 0.0001$) higher similarity between the fecal fingerprints of the healthy siblings (55.00% $\pm$ 2.92% and 74.0% $\pm$ 2.59% for patient-sibling pairs 1 and 2, respectively) than for those of the patients (16.16% $\pm$ 4.70% and 20.34% $\pm$ 4.00% for patient-sibling pairs 1 and 2, respectively). Likewise, moving-window analysis (Fig. 4) illustrated that fluctuations between CF samples (between 75% and 95% change) over a 2-year period are more pronounced than for the sibling samples (between 5% and 60% change). The remarkable drop in percent change between samples 3 and 4 of the second patient indicates very high similarity between the two fingerprint profiles.

In the longitudinal study, values for the TRI, LRI, and HRI were obtained from pooled data sets of all consecutive samples

collected per subject (Tables 5 and 6). When comparing the fecal fingerprints of the two patient-sibling pairs, the number of distinct band positions was slightly lower in CF patient fingerprints (61 and 42, respectively) than in the fingerprints of both siblings (67 and 43, respectively). This difference was also observed in the overall banding patterns of MCB fractions of the second patient-sibling pair, displaying 53 and 58 distinct band positions, respectively. For the first pair, the fingerprints obtained from the patient revealed in total 21 distinct band positions, whereas 81 band positions were found in the sibling fingerprints. This large difference can be explained by the lower number of MCB fingerprints obtained for this patient. Analysis of the TRI in the fecal fingerprint profiles of both patient-sibling pairs revealed on average lower values for CF patient samples (13.9 $\pm$ 1.9 and 11.0 $\pm$ 1.5, respectively) than for both siblings (18.1 $\pm$ 3.1 and 15.6 $\pm$ 2.7, respectively), but this difference was not significant (Mann-Whitney; $P > 0.05$) (Table 5). Higher TRI values were also observed in the MCB fingerprints of both siblings compared to those of the two

TABLE 6. LRI, HRI, and TRI values for DGGE fingerprint profiles of cultured fractions on MCB

Cultured fraction on MCB	No. of samples	LRI			HRI			TRI	
		Mean $\pm$ SE ^a	Minimum–maximum	P value	Mean $\pm$ SE ^b	Minimum–maximum	P value	Mean $\pm$ SE ^c	P value
Cross-sectional									
Patients with CF	13	6.1 $\pm$ 0.7	1.0–14.0	0.53	3.0 $\pm$ 0.3	0.0–8.0	0.31	9.2 $\pm$ 0.8	0.54
Siblings	12	6.3 $\pm$ 0.6	1.0–14.0		4.1 $\pm$ 0.6	0.0–14.0		10.6 $\pm$ 1.0	
Longitudinal patient									
sibling pair 1									
Patient 1	3	6.0 $\pm$ 1.5	4.0–9.90	0.73	1.3 $\pm$ 0.7	0.0–2.0	0.23	7.3 $\pm$ 0.9	0.54
Sibling 1	5	6.4 $\pm$ 0.7	4.0–12.0		3.1 $\pm$ 0.7	0.0–8.0		9.6 $\pm$ 1.2	
Longitudinal patient									
sibling pair 2									
Patient 2	6	3.0 $\pm$ 0.3	0.0–6.0	0.0001 ^d	2.3 $\pm$ 0.4	0.0–7.0	0.002 ^d	5.4 $\pm$ 0.5	0.00007 ^d
Sibling 2	8	5.1 $\pm$ 0.3	1.0–9.0		3.8 $\pm$ 0.3	1.0–10.0		8.9 $\pm$ 0.6	

^a Mean number of bands in the low-%GC region of the DGGE fingerprint profile.

^b Mean number of bands in the high-%GC region of the DGGE fingerprint profile.

^c Mean total number of bands in the DGGE fingerprint profile.

^d Significant difference between CF samples and sibling samples (95% CI).

FIG. 2. Clustering of V_3 -DGGE fingerprint profiles of consecutive fecal samples from patient-sibling pair 1 (a) and patient-sibling pair 2 (b) using Pearson correlation and UPGMA. Numbers at nodes represent similarities, which are percentages of Pearson correlation coefficients.

patients, and in the second pair, this difference was statistically significant (Mann-Whitney; $P < 0.001$) (Table 6). In the total fecal fingerprints of both patient-sibling pairs, LRI values were higher than HRI values (Table 5). A significantly (Student's t test; $P < 0.05$) lower LRI value was detected in patient 1 than in his sibling. For both pairs, a borderline significant difference (Student's t test; $P = 0.08$) was found between HRI values of CF patient samples (5.4 ± 1.6 and 3.5 ± 0.8) and sibling samples (2.4 ± 0.4 and 5.4 ± 0.6). In contrast to the second pair, HRI values were slightly higher in samples from sibling 1 than in corresponding CF patient samples. Also, fingerprints from cultured fractions on MCB revealed higher LRI values than HRI values in both patient-sibling pairs (Table 6). LRI and HRI were lower for samples from both patients than for samples from their siblings, but only for the second pair were

these differences statistically significant (Mann-Whitney; $P < 0.01$).

DISCUSSION

Using a cross-sectional, as well as a longitudinal, sampling approach, the current study set out to investigate if the relative composition and temporal stability of the predominant fecal microbiota in patients with CF are significantly different from those in their healthy siblings. Although siblings cannot be considered controls for patients with CF, it can be assumed that the effects of several factors that may influence the composition of the gastrointestinal microbiota, such as genetic background, age, and environment, are to a certain extent comparable in the two subject groups. However, other factors, such as the effect of daily diet, certainly differ, since patients with CF are recommended to consume a high-fat diet to meet their energy needs. Provided that such cohorts can be recruited, comparison between patients with CF who are on frequent versus infrequent antibiotic therapy would be an alternative approach to assess the contribution of antibiotics to the development of dysbiosis. In addition to culture-independent community profiling, the study also made use of conventional culturing with the aim of establishing a culture collection of fecal isolates for future functional analyses, such as determination of specific metabolic properties and antimicrobial susceptibility profiles. However, by plating fecal samples onto a series of group-specific media for enumeration of metabolically important subpopulations, it is inevitable that nontarget groups will also be recovered on selective media and thus contribute to the counts. Taken together with the intrinsic biases of culture-dependent isolation of intestinal bacteria (43), the results of these enumerations are thus only indicative and should be further substantiated by other, more specific quantitative methods.

In both the cross-sectional and longitudinal studies, the greatest difference in bacterial counts was found on the medium selective for *Bacteroides* and *Prevotella*, showing higher mean counts for sibling samples than for CF patient samples. *Bacteroides* numbers tend to be highly variable among different individuals (14, 32, 58). Whether the observed reduction in MBP counts in CF samples involves the loss of one or more specific species or rather indicates the lower abundance of an entire functional subpopulation requires further investigation. Therefore, preliminary speculations concerning metabolic consequences for patients with CF are difficult to make. Although major quantitative shifts in *Bacteroides* members have been reported in several intestinal disorders, a causal link with any of these diseases is still not clear. Studies on fecal (54) and mucosal (9) samples from inflammatory bowel disease (IBD) patients revealed a functional reduction of the *Bacteroides fragilis* subgroup, which potentially shields the host against *Escherichia coli*-induced colitis. However, other studies have demonstrated a possible increase of *Bacteroidetes* group members in the mucosal microbiota of patients suffering from Crohn's disease (19, 60) and ulcerative colitis (33). Several studies have shown that orally administered antibiotics can significantly decrease *Bacteroides* counts (2, 3, 46). In fact, a decline in intestinal *Bacteroides* numbers has been reported after treatment

FIG. 3. (a) Box plot of pairwise comparisons of the DGGE fingerprints of the cross-sectional data set. (b) Box plot of pairwise comparisons of the DGGE fingerprints of patient sibling pair 1 and patient sibling pair 2. Outlier values are indicated by open circles.

with amoxicillin, a beta-lactam antibiotic that was also administered to many of the patients in the current study (41).

In the longitudinal study, mean bifidobacterial counts on M144 showed a trend toward lower abundance in samples from patients with CF than in their healthy siblings. Relative *Bifidobacterium* numbers are often used as a tentative indication of intestinal health status. In atopic dermatitis (5, 28, 69), type II diabetes (73), and Crohn's disease (17) patients, a reduction in bifidobacteria has also been demonstrated. In contrast, a molecular study assessing the fecal microbial populations of Crohn's disease patients revealed a relatively stable *Bifidobacterium* community (54). Many studies have reported an antibiotic-induced decrease in *Bifidobacterium* counts (3, 16, 41, 46). Several *Bifidobacterium* strains are used as probiotics in healthy and diseased populations (47, 64), but until now, only very few studies have assessed the effects of probiotics in patients with CF (6, 7, 70).

Counts on the EMB medium selective for *Enterobacteriaceae* were generally higher in CF samples than in samples from healthy siblings. It has been hypothesized that potentially

pathogenic *Gammaproteobacteria* can proliferate from inflamed intestinal tissue, triggering increased oxygen release, increased availability of pathogen receptors, and competitive advantage in the altered microenvironment (34, 59). Hence, intestinal inflammation in patients with CF could be correlated with increased enterobacterial counts. In addition, several studies concerning IBD pathology (1, 9, 34, 57) and celiac disease (8) have reported increased *Gammaproteobacteria* levels. Bennet and coworkers (3) examined fecal microbial samples from very young infants during antimicrobial treatment and observed an increase of *Enterobacteriaceae*.

In the current study, DGGE fingerprints from the cross-sectional data set revealed a largely subject-specific clustering. Samples from subjects belonging to the same patient-sibling pair did not group together, and no typical CF fingerprint was found. Besides CF-related characteristics, genotype, age, and/or environmental factors also may have contributed to the observation that the fingerprints were unique. In addition, the microbiota of different individuals may respond in unique ways to a single antibiotic (26). Analogous observations were re-

FIG. 4. Moving-window correlation of the longitudinal data set. Subjects 5 and 6 belong to patient-sibling pair 1, subjects 23 and 24 to patient-sibling pair 2. The dropout between sampling points 3 and 4 for subject 23 is due to the high similarity between the two samples.

ported when comparing stool samples from children with celiac disease with those from their siblings by DGGE (11). Comparison of mean pairwise similarity values from the cross-sectional study revealed significantly higher compositional variation among CF samples than between samples from healthy siblings. Possibly, this observation could reflect periods with changing rates of dysbiosis mediated by personalized antimicrobial treatment courses. Still, the average similarity appeared to be relatively low in both subject groups, which again indicates the large interindividual variations. Likewise, several comparative studies of the fecal microbiota in Crohn's disease have reported greater variability between samples from patients than between those from healthy controls (54, 57). Relatively low similarity values have been reported between DGGE banding patterns of patients suffering from atopic dermatitis, as well as between healthy controls (44). Unlike our findings, Seksik and coworkers observed relatively little variation between the temperature gradient gel electrophoresis (TGGE) fingerprints of the healthy controls (57).

In the longitudinal study, pairwise comparisons of fecal fingerprints revealed significant overall variation for both patients with CF compared to their siblings. In addition, moving-window analysis of banding patterns originating from patients with CF in both patient-sibling pairs revealed greater variation between consecutive sampling points than in those of healthy siblings, which is indicative of a considerable reduction in temporal stability. Interestingly, the culture-dependent study also revealed a remarkable decrease in temporal stability in CF samples (Fig. 1a, c, e, and f). Possibly, a combination of long-term antibiotic administration and intestinal inflammation in patients with CF could explain the observed fluctuations in their fecal bacterial communities. Several comparisons between fecal samples from IBD patients and healthy subjects have also described a highly dynamic bacterial community in the chronically inflamed intestine (12, 37, 54, 57). In addition, Jakobsson and coworkers (26) previously reported that fecal samples showed considerable variation in community structure over a 4-year period of antibiotic administration. Compared to previous population-fingerprinting studies (37, 67, 76), the present 2-year survey revealed less similarity between fecal fingerprints of healthy siblings, suggesting that the gastrointestinal microbiota may be more dynamic over a prolonged period. Also, in this case, dietary regimen, age, and/or environmental conditions are likely to play a role in the observed time-dependent variations of the healthy intestinal microbiota.

The species richness index is considered to be a valuable parameter for measuring the ecosystem's fitness and flexibility (72). A decrease in species richness is thought to negatively impact the ecosystem's functional stability because the presence of fewer species may result in lower productivity, and it would make the system less flexible to compensate for natural fluctuations (74). In the present study, species richness was measured by determining the total number of bands, as well as determining the band numbers in two defined regions in the DGGE profile representing low- and high-%GC organisms. For both sample types, the cross-sectional study revealed no significant differences in LRI, HRI, and TRI values between patients and their siblings, which again seems to indicate that the complexities of the predominant fecal microbiota in patients and healthy subjects are comparable. In contrast, pro-

nounced differences in species richness between CF patient and sibling samples were found in the longitudinal study. The fingerprints of both patients displayed lower mean LRI values than those of the corresponding siblings. The LRI is linked to the low-%GC region of the DGGE profiles, which mainly represents Gram-negative *Bacteroidetes* and Gram-positive *Firmicutes*, the two most abundant phyla in the human colon (14). In IBD patients (19, 36), as well as in patients undergoing antibiotic treatment (2, 3, 41, 46), an underrepresentation of fecal *Bacteroidetes* and *Firmicutes* has been reported. Except for the fecal fingerprints of the first patient-sibling pair, HRI values were lower for fecal and MCB fingerprints of both patients. The high-%GC region of the DGGE profiles, which corresponds to the HRI, mainly includes members of the *Actinobacteria*, such as bifidobacteria. Given the beneficial roles attributed to bifidobacteria in the human gut ecosystem (30), a decline in actinobacterial diversity may have a negative impact on the host's well-being. The fact that antibiotic regimens in patients with CF are often highly personalized, in combination with the subject specificity of the predominant colon microbiota, might explain the observed relative differences in species richness between patient-sibling pairs 1 and 2. On the other hand, the TRI, LRI, and HRI values might have been biased by the small sample size included in the longitudinal study.

In conclusion, the results from culturing and community profiling appear to indicate a continuous state of intestinal dysbiosis in patients with CF. Possibly, both the intrinsic characteristics of the disease (such as abnormal mucus secretions and pancreatic insufficiency) and the detrimental effects of intensive antimicrobial treatment courses play key roles in this dysbiosis. In addition, many of our findings are supported by insights obtained from other gut-related disorders. Furthermore, it can be speculated that some of the borderline significant differences observed between the study groups might further increase in significance if the sample size could be increased. Clearly, much better insight into the dynamics and homeostasis of the intestinal CF microbiota is needed in order to predict how CF dysbiosis may have dramatic functional consequences for the gut ecosystem and the host's well-being.

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