Gastroenterology 2017;152:799–811

Efficacy of Sterile Fecal Filtrate Transfer for Treating Patients With *Clostridium difficile* Infection

Stephan J. Ott,[1,*] **Georg H. Waetzig**,[2,*] **Ateequr Rehman**,[3,*] Jacqueline Moltzau-Anderson,[3,4] Richa Bharti,[3] Juris A. Grasis,[5] Liam Cassidy,[6] Andreas Tholey,[6] Helmut Fickenscher,[7] Dirk Seegert,[2] Philip Rosenstiel,[3,§] and Stefan Schreiber[1,3,§]

[1]*Department of Internal Medicine I, University Hospital Schleswig-Holstein, Kiel, Germany;* [2]*CONARIS Research Institute AG, Kiel, Germany;* [3]*Institute of Clinical Molecular Biology, University of Kiel, Kiel, Germany;* [4]*Max Planck Institute for Evolutionary Biology, Plön, Germany;* [5]*Department of Biology, San Diego State University, San Diego, California;* [6]*Institute of Experimental Medicine, University of Kiel, Kiel, Germany;* [7]*Institute for Infection Medicine, University of Kiel and University Hospital Schleswig-Holstein, Kiel, Germany*

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BACKGROUND & AIMS: Fecal microbiota transplantation (FMT) is a highly effective therapy for recurrent *Clostridium difficile* infection (CDI). However, transferring undefined living bacteria entails uncontrollable risks for infectious and metabolic or malignant diseases, particularly in immunocompromised patients. We investigated whether sterile fecal filtrates (containing bacterial debris, proteins, antimicrobial compounds, metabolic products, and oligonucleotides/DNA), rather than intact microorganisms, are effective in patients with CDI. **METHODS:** We performed a clinical case series to investigate the effects of fecal filtrate transfer (FFT) in 5 patients with symptomatic chronic-relapsing CDI at the Department of Internal Medicine I at the University Hospital Schleswig-Holstein (Kiel, Germany). Patients were followed up for at least 6 months and for up to 33 months. Stool was collected from 5 donors selected by the patients, and fully characterized according to FMT standards. Stool was sterile-filtered to remove small particles and bacteria; the filtrate was transferred to patients in a single administration via nasojejunal tube. Fecal samples were collected from patients before and at 1 week and 6 weeks after FFT. Microbiome, virome, and proteome profiles of donors and patients were compared. **RESULTS:** In all 5 patients, FFT restored normal stool habits and eliminated symptoms of CDI for a minimum period of 6 months. Proteome analyses of selected FFT filtrates showed no obvious protein candidates associated with therapeutic efficacy. 16S ribosomal RNA gene sequencing detected diverse bacterial DNA signatures in the filtrates. Analysis of virus-like particles from a filtrate found to reduce symptoms of CDI showed a complex signature of bacteriophages. Bacterial phylogeny and virome profile analyses of fecal samples from recipients indicated longitudinal changes in microbial and viral community structures after FFT. **CONCLUSIONS:** A preliminary investigation of 5 patients with CDI shows that transfer of sterile filtrates from donor stool (FFT), rather than fecal microbiota, can be sufficient to restore normal stool habits and eliminate symptoms. This finding indicates that bacterial components, metabolites, or bacteriophages mediate many of the effects of FMT, and that FFT might be an alternative approach, particularly for immunocompromised patients.

Keywords: Fecal Transplant; Intestinal Microbiome; Feces; Microbe.

Fecal microbiota transplantation (FMT) is a highly effective therapy of recurrent *Clostridium difficile* infection (CDI) with consistent disease resolution rates of 85%–90% after 1 treatment and up to 100% after a second treatment, using either fresh or cryopreserved stool from healthy, well-characterized donors.[1–17] The paradigm of FMT is to improve intestinal dysbiosis by transferring stool preparations containing a stable, viable, diverse, and normal microbial community from a healthy donor or from defined intestinal bacterial strains.

Despite the superb efficacy and the good short-term safety profile of FMT, it bears major problems of standardization and is accompanied by potentially incalculable long-term risks, many of which are inherent to the transfer of living microorganisms.[6,18,19] Standardized recruiting of well-characterized healthy stool donor populations and the use of microbe-conserving freezing procedures for stool preparations is used to overcome the problem of availability and some of the safety issues.[1,4,7,9,12–14,16,20] Although FMT also has been shown to be safe and efficacious in immunocompromised patients,[21,22] it still would be highly desirable to reduce the risk of adverse events in patients with limited eligibility for FMT.[1,14,21] Moreover, even the most rigorous and costly donor screening procedures,[1,4,20] or defined panels of bacteria, cannot exclude the risk of transferring unknown pathogens or undetectable functional characteristics within the living microorganisms to the

*Authors share co-first authorship; §Authors share co-senior authorship.

Abbreviations used in this paper: **CDI, *Clostridium difficile* infection; FFT, fecal filtrate transfer; FMT, fecal microbiota transplantation; SDS, sodium dodecyl sulfate.**

Most current article

0016-5085
http://dx.doi.org/10.1053/j.gastro.2016.11.010

recipient, including bacterial or viral risk factors for metabolic diseases, cancer, atopy, or autoimmunity.[4,6,18–20,23–26]

The original concept of FMT is based on 2 main premises, namely that patients with dysbiosis have either completely lost their healthy microbiota or that their microbiota is unable to regain its normal functionality, and that these diverse pathologic states of the intestinal microbiota can be corrected by transferring a stable, viable, diverse, and healthy microbial community contained in stool preparations or defined bacterial groups from healthy donors. Classic FMT techniques therefore all aim to maintain as much of the microbial diversity and natural composition of the donor microbiota as possible, and techniques using defined sets of bacteria have the goal to transfer both the necessary and the sufficient microbial players. Interestingly, the results from recent clinical investigations, which successfully used different FMT-related techniques for the treatment of CDI, now allow determination of the intersection of the underlying therapeutic principles. A recent study clearly has shown the long-term co-existence of donor and host microbiota after FMT; however, whether a more efficient transfer (ie, higher similarity between donor and host) correlates with clinical success of FMT currently is unclear.[27]

In FMT using freshly prepared stool or cryopreserved stool containing living microbiota from either single or pooled donor feces,[1–17] the therapeutically active agent(s) theoretically could just as well be elements of the virome, other components of the fecal water, or even products of the donor's human cells. However, the similar success rates of therapies using defined panels of fecal bacteria (eg, rectal bacteriotherapy[28] or the microbiota suspension RBX2660 [Rebiotix, Roseville, MN][29]) or a selection of bacterial spores, such as the nontoxinogenic *C difficile* strain M3,[30] strongly suggest that the active agents are contained in the bacterial fraction, which may include, for example, specific structures, antimicrobial compounds, and/or metabolites produced by the transferred bacteria or also may be integrated bacteriophages (prophages) that may be activated and released under certain conditions.[31]

With FMT, considerable amounts of stool water containing dead bacteria, their debris, and their metabolites are transferred in addition to the living microbiota.[31] Given the diversity of effective therapies for CDI as outlined earlier, we examined whether the ingredients of fecal water alone (eg, bacterial debris, proteins, antimicrobial compounds, metabolic products, or oligonucleotides/DNA) had any clinical efficacy in patients with CDI. For this purpose, conventionally produced FMT preparations additionally were sterile-filtered to enable sterile fecal filtrate transfer (FFT) from healthy donors to 5 patients for the therapy of chronic-relapsing CDI-associated symptomatology.

Materials and Methods

Study Design

This was an open-label case series performed at the Department of Internal Medicine I of the University Hospital Schleswig-Holstein in Kiel, Germany. The application of FFT for the treatment of chronic-relapsing CDI-associated symptomatology received full and unconditional approval by the Institutional Review Board (medical governance commission) of the University Hospital Schleswig-Holstein. All participants provided written informed consent.

Study Population

Donors and patients were screened according to comprehensive guidelines.[1] According to German guidelines and corresponding standard operating procedures of our hospital, the following test plan was realized. All patients were tested for 3 diagnostic characteristics of *C difficile* infection: (1) enzyme-linked immunosorbent assay for *C difficile*–specific glutamate dehydrogenase (Techlab C. Diff Quik Chek Complete; Techlab, Blacksburg, VA), (2) *C difficile* toxin enzyme-linked immunosorbent assay (Techlab C. Diff Quik Chek Complete), and (3) detection of toxin-producing *C difficile* by culture. Any 2 positive results of these tests were considered a definitive diagnosis of *C difficile* infection. Patients were followed up clinically for a period of at least 6 months for no recurrence of symptoms; the first patient has been symptom-free since January 2014. The patient characteristics are summarized in Table 1.

Stool Preparation

Fresh donor stool was collected and stored in an airtight container at 4°C until processing. As a general rule, stool was processed no later than 2 hours after collection, and the transplantation to the recipient was performed no later than 6 hours after stool donation. In a dedicated biosafety cabinet, approximately 50 g of fecal material was weighed and transferred to a standard commercial blender (Moulinex Multi Moulinette 400 W; SEB, Écully, France). Subsequently, 500 mL of sterile normal saline (0.9% sodium chloride) was added, and the stool was homogenized for approximately 1 minute at the highest blending level. The resulting slurry then was distributed into ten 50-mL centrifugation tubes (Sarstedt, Nümbrecht, Germany) and centrifuged for 10 minutes at approximately 1800 $\times$ g to pellet large particles. The resulting supernatant was transferred to a beaker and filtered 3 times through disposable cellulose paper filters (pore size, 5–10 μm) placed in a stainless-steel sieve. For the conventional FMT treatment of patient 2, the resulting prefiltered slurry containing the fecal microbiota then was applied by nasojejunal tube as described later.

Preparation of FFT Filtrates

For preparation of the FFT filtrate, the prefiltered stool slurry was filtered further using a custom-built air pressure filtration system (PALL, Dreieich, Germany) including a 5.7 L (6 bar) UCON pressure unit (UCON, Hausach, Germany) and a WIKA 0.5-inch TC inline diaphragm seal (±6 bar, S/N; WIKA, Klingenberg, Germany) at a pressure of 1.5–2 bars and with the following filtration steps: (1) small-particle removal using 2 consecutive depth filters: Seitz K 700 P 60 D (retention rating, 6.0–15.0 μm; PALL) plus Seitz KS 50 P 60 D (retention rating, 0.4–0.8 μm; PALL); and (2) microbiota depletion using a SUPOR EKV Filter Mini Kleenpak 0.2-μm unit (PALL).

The resulting FFT filtrate was a light brown, clear liquid with a subjectively less unpleasant and intensive odor in comparison with the prefiltered, microbiota-rich slurry used in conventional stool preparations for FMT.

To validate the depletion of the donor's gut microbiota from the FFT filtrates, samples of the filtrates and of the original

Table 1. Baseline Characteristics and Treatment Results of the Patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age, *y*	59	73	72	49	75
Sex	Female	Female	Male	Male	Female
Prior CDI episodes	3	1	2	2	>2
Anamnesis before CDI	Life-threatening recurrent diverticulitis, sigma resection	Gastric carcinoma, gastrectomy, diverticulitis	Immunosuppressed since kidney transplant in 1990, multimorbid	Immunosuppressed, HIV infection under antiviral therapy, multimorbid	Colon cancer with colon surgery
Reported antibiotic use before CDI	Ciprofloxacin, metronidazole	Diverse antibiotics	Diverse antibiotics	Cefuroxime, clindamycin	Diverse antibiotics
Additional relevant diagnoses	Pseudomembranous colitis	None	Loss of kidney function	HIV infection, epilepsy	Chronic heart failure, coronary heart disease
Antibiotic(s) used for CDI therapy	Metronidazole, vancomycin	Metronidazole, vancomycin	Vancomycin, metronidazole, rifaximin	Metronidazole, vancomycin	Metronidazole, vancomycin
Type of CDI (refractory or recurrent)	Recurrent	Atypical (currently not detectable)	Recurrent	Recurrent	Recurrent
Additional relevant medications or treatments	None	FMT (donor: husband, March 28, 2014), recurrent symptoms	Cyclosporine, *Saccharomyces boulardii*	None	None
Donor	Son	Husband	Sister	Sister	Nonrelated donor
Date of FFT treatment	January 23, 2014	June 20, 2014	July 1, 2014	December 11, 2015	December 22, 2015
Diarrhea resolution after FFT	Yes	Yes	Yes	Yes	Yes
Days to discharge from hospital	1	1	1	1	1
Days to symptom-free status	3	3	3	2	4
Symptom-free until 30 June 2016 (end of study)	Yes	Yes	Yes	Yes	Yes

HIV, human immunodeficiency virus.

materials were subjected to bacterial culture. In brief, the filtrates and donor stools were transported to the accredited clinical microbiology laboratory of the University Hospital Schleswig-Holstein in Kiel (Germany) under anaerobic conditions. Here, filtrates and donor stool samples (dissolved in sterile saline) were streaked onto sterile blood agar plates (Columbia agar with 5% sheep blood: casein peptone 12.0 g, meat peptone 5.0 g, sodium chloride 5.0 g, beef extract 3.0 g, yeast extract 3.0 g, corn starch 1.0 g, sheep blood 5%, agar 13.5 g, and demineralized water up to 1000 mL; catalogue number PB5008A; Remel, Lenexa, KS) and cultured in parallel under anaerobic and aerobic conditions at 37°C for up to 48 hours. Donor stool was used as a positive control. Bacterial growth could not be observed from FFT filtrates, whereas the corresponding stool cultures yielded abundant colony numbers under both aerobic and anaerobic conditions.

Administration of FFT Filtrates

Before FFT administration, 1000 mL of Klean-Prep intestinal lavage solution (Norgine, Marburg, Germany) was administered to the patients via a nasojejunal tube inserted by gastroscopy using a fiberoptic system (Olympus, Hamburg, Germany) and controlled by an APPLIX Smart/Vision system (Fresenius KABI, Bad Homburg, Germany). Subsequently, the FFT filtrate was transferred to a plastic bag (APPLIX HydroBag; Fresenius KABI) and administered via the same nasojejunal tube within approximately 30 minutes.

Microbiota and Virus Analyses of Fecal Samples and FFT Filtrates

Genomic DNA was extracted from fecal samples using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions,[32] with some modifications as described previously.[32]

The 16S ribosomal RNA gene variable regions V4[33] or V3–V4[34] were amplified in duplicate reactions using 2 μL of fecal genomic DNA. The amplified products were run on agarose gels to assess the amplicon size and amplification performance. Amplicon quantities were normalized using the SequalPrep kit (Applied Biosystems/Thermo Fisher Scientific, Darmstadt, Germany). Equal amounts of polymerase chain reaction products were pooled in a single tube and sequenced on an Illumina MiSeq platform using 2 $\times$ 250 bp (for V4 regions) and 2 $\times$ 300 bp (for V3–V4 regions) sequencing kits (Illumina, San Diego, CA).

Sequencing reads were processed primarily for quality control using the software Mothur.[35] Forward and reverse reads were assembled to form contigs (herein referred to as *sequences*). Sequences with any ambiguous base or more than 6 homopolymers as well as sequences not perfectly matching with 16S-specific primers and/or barcode indices were removed from downstream analysis. This initial quality control enabled us to remove spurious sequencing artifacts. The remaining sequences were aligned to the Mothur-curated Silva reference alignment (release 123) and eliminated, if not aligned to a specific 16S variable region. The sequences remaining after this step were screened for chimeric origin by using the Uchime algorithm,[36] and chimeras were removed. Finally, sequences were assigned to taxonomic hierarchy using Greengenes reference training sets (13_8_99 release) with an 80% confidence threshold. This reference training data set contains approximately 10% species level classification. Sequences classified as eukaryotic, chloroplastic, mitochondrial, or of unknown origin also were removed from analysis. Sequences restricted to bacterial origins only were clustered into phylotypes (label = 1) using Greengenes reference taxonomy. Phylogenetic nominations of phylotypes were determined by using the complete taxonomy file generated as described earlier. Principle coordinate analysis was performed using PAST software[37] version 3.10 on distance matrices based on the presence/absence or the relative abundances of bacterial phylotypes in fecal microbiota. Bacterial estimated richness (Chao 1) and diversity (nonparametric Shannon index) were calculated on subsamples using shared phylotypes (4708 sequences per sample) to adjust the sampling depth. Subsampling and diversity estimation were performed using Mothur.[35]

Virus-like particle purification and DNA extraction were performed for patients 4 and 5, their donors, and the FFT filtrates as described previously,[38] with some modifications. The detailed methods are included in the Supplementary Materials and Methods section. Only the preparations from the samples of patient 4 fulfilled all quality criteria and could be analyzed adequately.

Liquid Chromatography-Mass Spectrometry–Based Proteome Analysis of FFT Filtrates

Samples of the FFT filtrates used to treat patient 4 (1 sample) and patient 5 (2 separate FFT filtrates prepared on different days; only the first was used for treating patient 5) were stored at -80°C. In brief, the resulting 3 samples were processed for liquid chromatography–mass spectrometry analysis after solid-phase extraction, stack gel sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, or 1-dimensional SDS–polyacrylamide gel electrophoresis followed by tryptic (in-gel) digestion. The detailed methods are included as Supplementary Materials and Methods section.

Results

Case Histories and Outcomes

Patient 1 was a 59-year-old Caucasian woman who presented with 3 episodes of recurrent CDI over a 6-month period (confirmed by positive testing for *C difficile* glutamate dehydrogenase and *C difficile* toxins A and B). The patient's first episode of CDI had occurred after sigmoid resection owing to life-threatening recurrent diverticulitis with severe bleeding, which had required therapy with ciprofloxacin and metronidazole (Table 1). At initial diagnosis, the patient suffered from pseudomembranous colitis. After comprehensive screening,[1] the patient was treated with sterile FFT filtrate from stool from the patient's son. The patient could be discharged on the next day and was symptom-free after 3 days. *C difficile* remained undetectable (culture, toxin) 4 weeks after the procedure. The patient had no further diarrhea, regained her normal weight, and has remained symptom-free to date (2 years and 5 months at the completion of the study on June 30, 2016) (Table 1).

Patient 2 was a 73-year-old Caucasian woman who had suffered for years from recurrent fetid diarrhea and

abdominal pain that developed after a CDI treated with antibiotics (Table 1). Although symptoms recurred, laboratory tests for *C difficile* toxins A and B or other pathogens performed during previous outside appointments remained negative. However, the symptoms responded to antibiotic therapy with metronidazole and vancomycin, suggesting an active role of *C difficile* in the pathogenesis of recurrent diarrhea in this patient. The further history comprised gastrectomy with postoperative chemotherapy 7 years previously and 1 episode of acute diverticulitis requiring antibiotic intervention 1 year before CDI. Three months after symptomatic recurrence, conventional FMT was performed (jejunal infusion of stool from the patient's husband) after comprehensive screening.[1] FMT resulted in a moderate increase in body temperature (up to 38°C) for approximately 2 hours after FMT and in 1 episode of watery diarrhea for 2 days after FMT. The patient reported a significant reduction of symptoms including normalization of stool frequency and consistency (≤2 formed stools/day). Diarrhea and abdominal pain recurred after 2 weeks, and 3 months after FMT the patient presented with similar symptoms as before. Tests for *C difficile* glutamate dehydrogenase were positive, however, results for toxin detection were inconclusive and culture failed; a polymerase chain reaction was not performed. The patient's husband also was used as a donor for FFT, which was well tolerated. Neither a change in body temperature nor diarrhea were observed, and the patient could be discharged the next day. The patient was symptom-free after 3 days and has been symptom-free since (2 years at the completion of the study) (Table 1).

Patient 3 was a 72-year-old Caucasian man hospitalized because of acute *C difficile*–associated diarrhea, which was his second relapse of CDI (Table 1). The history was significant for chronic therapy with cyclosporine after kidney transplant 24 years previously, with a series of severe infections, including septicemia resulting from an earlier CDI 3 years before the present episode. Comorbidities included severe coronary heart disease with multiple stent implants, syndrome X, and current nicotine abuse. The current CDI episode required hospitalization because of extensive diarrhea and fever leading to loss of kidney function (glomerular filtration rate, 48 mL/min). Therapy with vancomycin, rifaximin, and *Saccharomyces boulardii* failed, as documented by repeated detection of *C difficile* glutamate dehydrogenase and toxins A and B in stools. Because of the history of infectious complications, FFT was preferred over conventional FMT to avoid any infection risk through the transfer of living microbiota. After comprehensive screening,[1] the patient was treated with an FFT filtrate from the patient's sister. FFT was well tolerated without fever or any other side effects. The patient was discharged from the hospital on the day after FFT treatment, was symptom-free after 3 days, and has remained so until today (2 years at the completion of the study) (Table 1).

Patient 4 was a 49-year-old Caucasian man presenting with a third episode of acute *C difficile* infection with severe diarrhea and dehydration (Table 1). The patient was immunosuppressed because of chronic human immunodeficiency virus infection and received antiviral therapy. After treatment with cefuroxime and clindamycin for *Staphylococcus aureus* infection after bone surgery in July 2015, the patient developed severe diarrhea. The diagnosis of *C difficile* infection was established by positive tests as described earlier. Under antibiotic therapy with metronidazole, symptoms of *C difficile* infection ceased. Within a few weeks after initial treatment, however, symptoms reoccurred with positive test results for *C difficile* infection. A course of vancomycin stopped the symptoms, but *C difficile* infection recurred again after approximately 3 weeks. A second course of vancomycin over 10 days improved the clinical symptoms. After vancomycin, a therapy with FFT filtrate (from the patient's sister) was performed in December 2015. The FFT therapy was well tolerated without side effects. The patient was discharged on the next day, became symptom-free after 2 days, and has remained so to date (6 months at the completion of the study) (Table 1).

Patient 5 was a 75-year-old Caucasian woman with a diagnosis of colon cancer of the sigmoid (May 2015), which was removed surgically (Table 1). A few days after surgery, the patient developed sutural dehiscence with local infection. The patient was treated with diverse antibiotics for this local infection before severe diarrhea occurred and *C difficile* infection was detected by the tests described earlier. She received several courses of metronidazole and vancomycin, the last time before FFT therapy. We performed FFT therapy in December 2015 using fecal material from a nonrelated donor as a prophylaxis against further episodes of *C difficile* infection. The patient showed no side effects, could be discharged on the next day, was symptom-free after 4 days, and has remained so until today (6 months at the completion of the study) (Table 1).

Fecal Bacterial and Viral Profile Analysis in Donors and Patients

After the surprising treatment success with patient 1, 16S ribosomal RNA gene-based microbiota analyses were performed for the subsequent 4 patients using amplicon sequencing. When comparing samples from before, 1 week after, and 6 weeks after FFT treatment, we found that FFT led to substantial bacterial community shifts in all patients (Figures 1–4 and Supplementary Figures 1 and 2). β-diversity analyses (Supplementary Figure 3) showed that in none of the cases did the shifts represent a close phenocopy of the donor fecal microbiota community, as has been reported previously also for instances of normal FMT.[39,40] In many cases, the abundance shifts of bacterial phylotypes already were present at week 1 and remained stable until week 6, particularly in patient 3 (Figure 2 and Supplementary Figure 1*B*) and in patient 4 (Figure 3 and Supplementary Figure 2*A*). Formal Jaccard-based (shared phylotypes) and Bray–Curtis–based (phylotype abundance) β-diversity analyses confirmed major distance shifts between time points (Supplementary Figure 3). Detailed investigation of the most strongly decreasing or increasing bacterial phylotypes showed complex dynamic shifts, with the extent and direction of some phylotype changes also

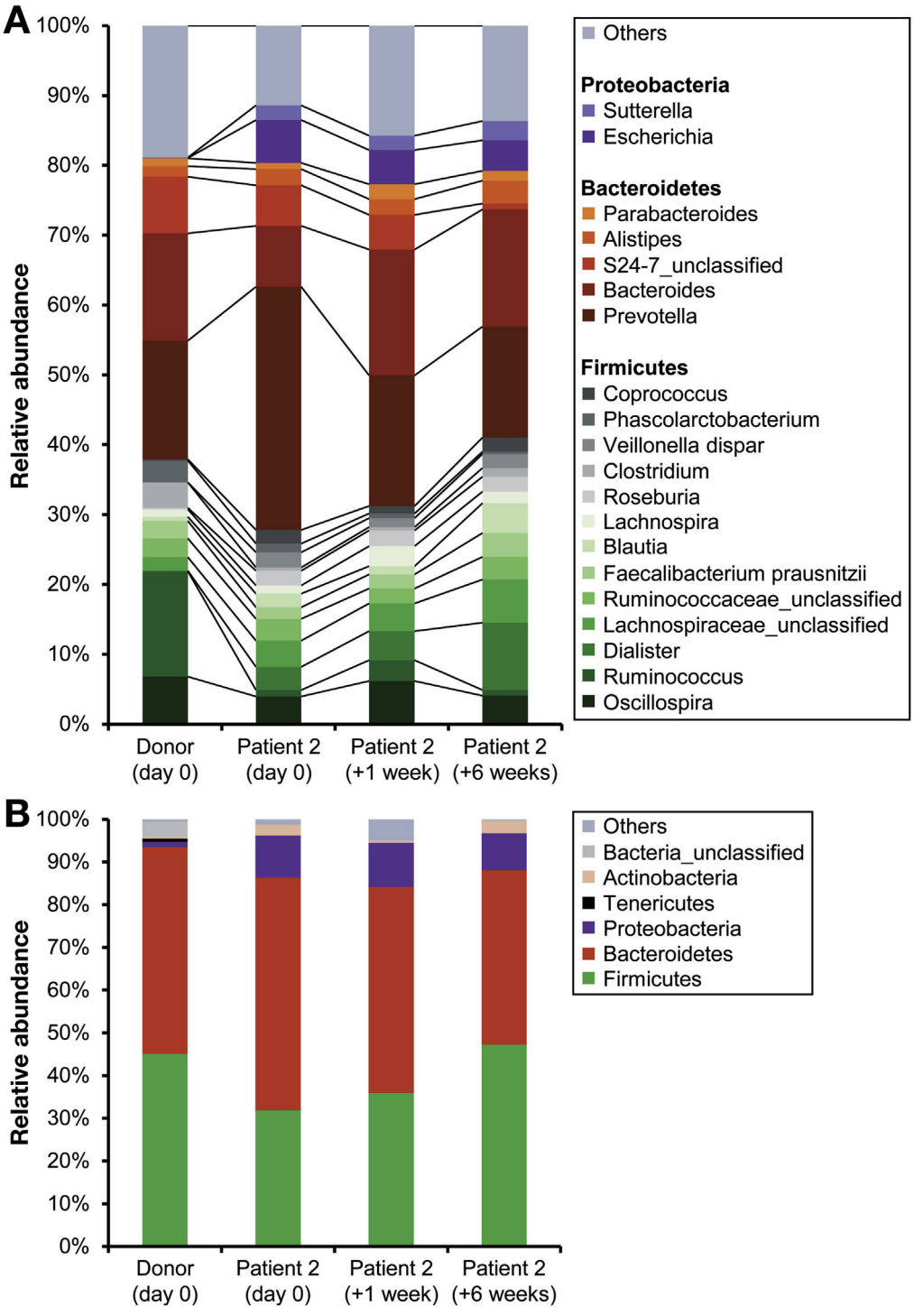

Figure 1. Relative abundances at the (*A*) genus or (*B*) phylum level in the fecal microbiota profiled by 16S ribosomal RNA detection through next-generation sequencing before (day 0) and after (week 1 or week 6) fecal filtrate transfer treatment in patient 2 and in her donor (day 0 only). Bacterial genera or phyla showing less than 5% cumulative abundance in all analyzed samples or not classified at the family level are represented as *Others*.

being substantially different between week 1 and week 6, for example, cyanobacteria and *Bifidobacterium* in patient 2 (Supplementary Figure 1*A*), or several changes in Bacteroidetes, particularly *Bacteroides eggerthii*, in patient 5 (Supplementary Figure 2*B*).

It is important to keep in mind that, in contrast to conventional FMT, transferring sterile FFT filtrates cannot be expected to establish a microbiota similar to that of the donor in the receiving patient. Accordingly, conventional comparisons of the stool microbiomes of the donor and patient before and after treatment as shown in Figures 1–4 may not appropriately reflect the relevant changes, but merely show a shift of α-diversity in the recipient after FFT treatment (Figure 5). Increases in the Chao 1 richness estimate (patient 5) and Shannon diversity index (patients 3 and 4) 6 weeks after FFT, and an interim dip after week 1 during microbiota re-establishment in patient 4 (Chao 1) and patient 5 (Shannon) suggested shifts of diversity, but

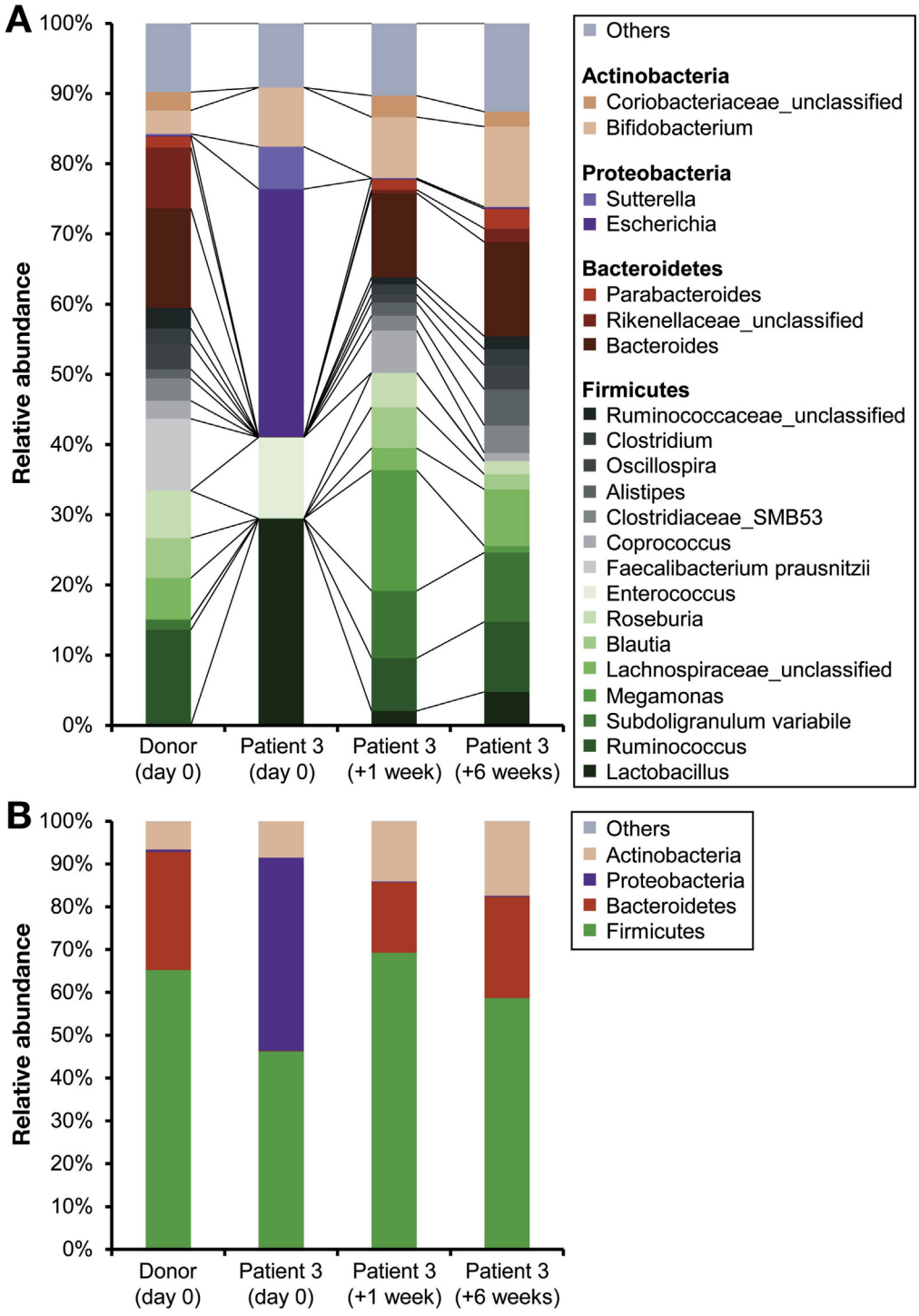

Figure 2. Relative abundances at the (*A*) genus or (*B*) phylum level in the fecal microbiota profiled by 16S ribosomal RNA detection through next-generation sequencing before (day 0) and after (week 1 or week 6) fecal filtrate transfer treatment in patient 3 and in his donor (day 0 only). Bacterial genera or phyla showing less than 5% cumulative abundance in all analyzed samples or not classified at the family level are represented as *Others*.

not universally in the same direction in all patients. With regard to microbial DNA as a potential stimulus for re-establishing the recipient's microbiome, the FFT filtrate contained significant amounts of bacterial DNA, which reflected at least in part the bacterial diversity from fecal DNA, as shown in Supplementary Figure 4 for the filtrate used in patient 4.

After the successful treatment of patients 1–3, we decided also to analyze viruses and the proteome (see later) contained in the FFT filtrates used to treat patients 4 and 5 to obtain an impression of the types and diversity of the transferred bacteriophages. We hypothesized that bacteriophages in the filtrate also could represent the mechanism of action of FFT, because the intersection of therapeutically effective FMT and FMT-like therapies reduces the candidates for viral efficacy factors to temperate phages (see Discussion section). A complete set of samples with sufficient quality for virus analyses could be obtained only for

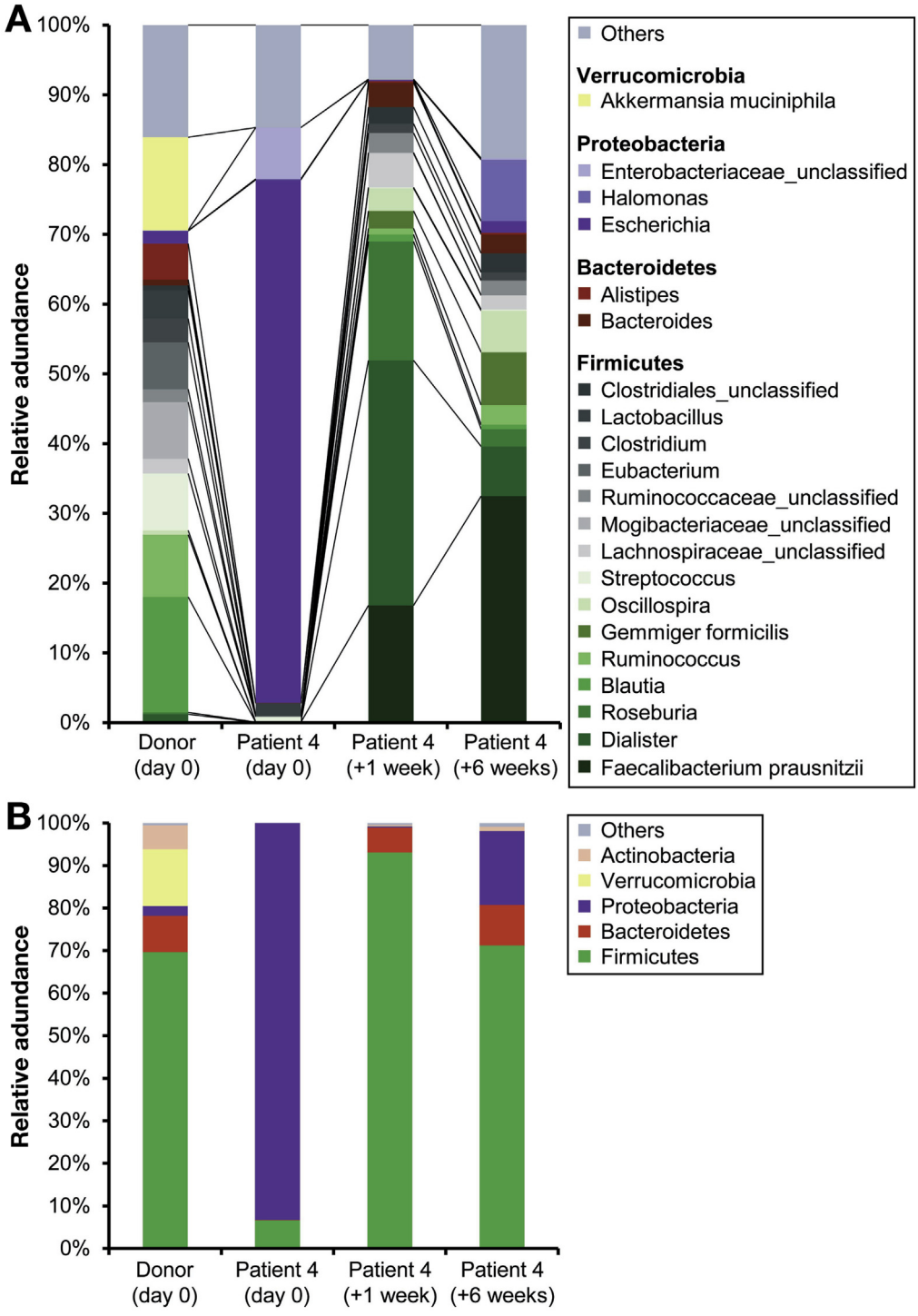

Figure 3. Relative abundances at the (*A*) genus or (*B*) phylum level in the fecal microbiota profiled by 16S ribosomal RNA detection through next-generation sequencing before (day 0) and after (week 1 or week 6) fecal filtrate transfer treatment in patient 4 and in his donor (day 0 only). Bacterial genera or phyla showing less than 5% cumulative abundance in all analyzed samples or not classified at the family level are represented as *Others*.

patient 4 (Figure 6). All samples were dominated by a rich variety of *Lactococcus* bacteriophages. Figure 6 shows that the phageome of the patient was altered substantially in response to FFT and tended to resemble the donor phageome after 6 weeks.

Proteome Analysis of FFT Filtrates

The proteome contained in the FFT filtrates used to treat patients 4 and 5 was analyzed to identify protein candidates that may be responsible, in part, for the therapeutic efficacy of FFT. From the donor feces for patient 5, there were 2 separate FFT filtrates prepared on different days to obtain a better impression of the reproducibility of the FFT filtrate's protein content.

The total numbers of proteins (or their degradation products) identified in the FFT filtrates using the stacked gel approach were 366 (filtrate for patient 4), 300 (filtrate for patient 5), and 267 (second preparation of FFT filtrate from

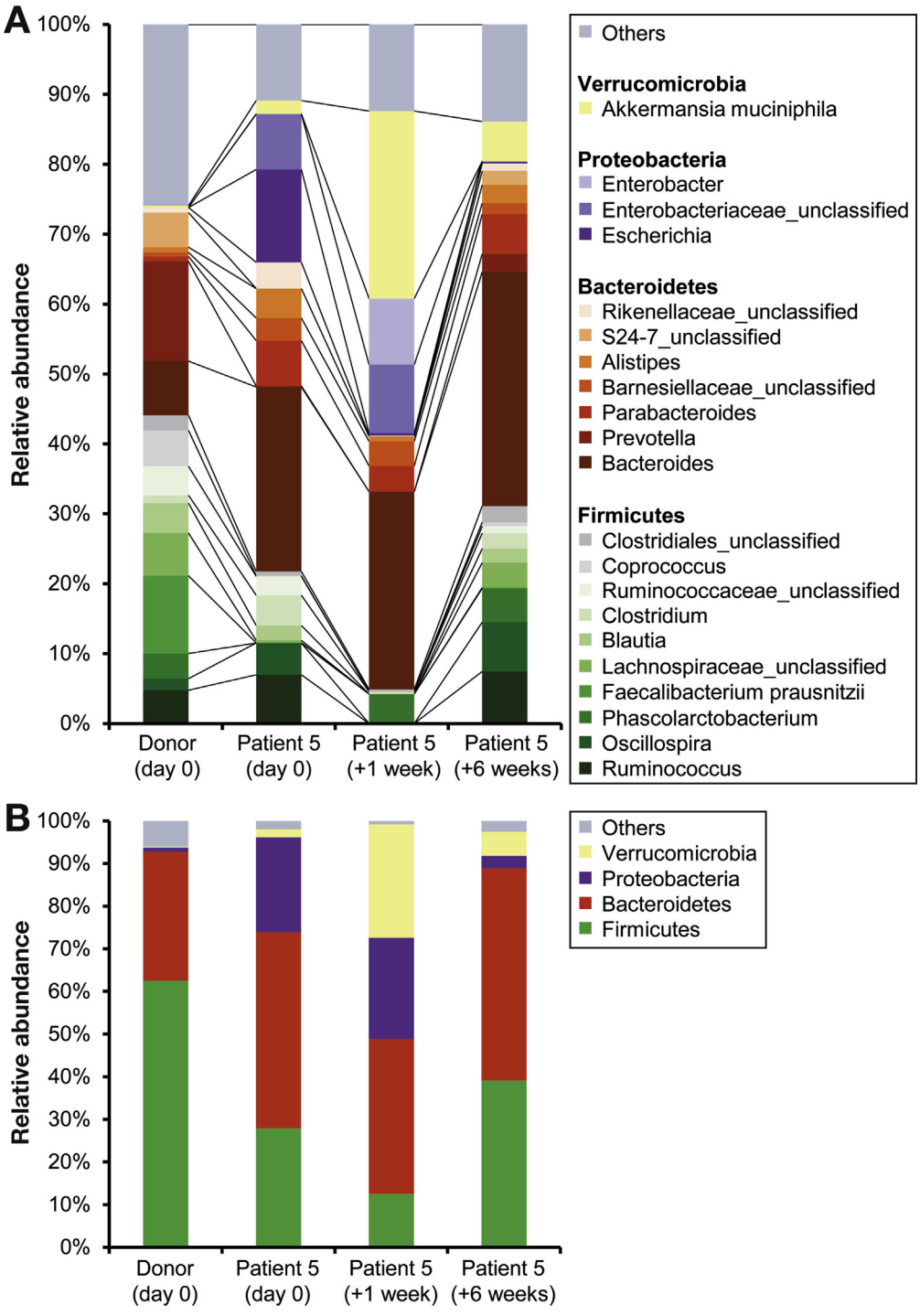

Figure 4. Relative abundances at the (*A*) genus or (*B*) phylum level in the fecal microbiota profiled by 16S ribosomal RNA detection through next-generation sequencing before (day 0) and after (week 1 or week 6) fecal filtrate transfer treatment in patient 5 and in her donor (day 0 only). Bacterial genera or phyla showing less than 5% cumulative abundance in all analyzed samples or not classified at the family level are represented as *Others*.

stool of the donor for patient 5). The majority of proteins identified in all 3 samples were of human origin (304, 259, and 245, respectively). Furthermore, a number of bacterial and fungal proteins were identified (62, 41, and 22, respectively). The solid-phase extraction approach yielded 10 additional proteins (3 of human origin and 7 of bacterial or fungal origin). Gel-based analysis (SDS–Coomassie, data not shown) and assessment of the peptide spectral matches as well as of the precursor ion signals showed significantly higher signals of all human matches compared with their bacterial and fungal counterparts. From the observed differences, we estimated the amount of human protein in the filtrates to be greater than 90%. The total number of proteins identified in the 3 samples was 496, with an overlap of approximately 50% between the FFT filtrates of the 2 different donors and approximately 70% between the 2 replicates from the donor for patient 5. The major components of the FFT filtrate proteome were human

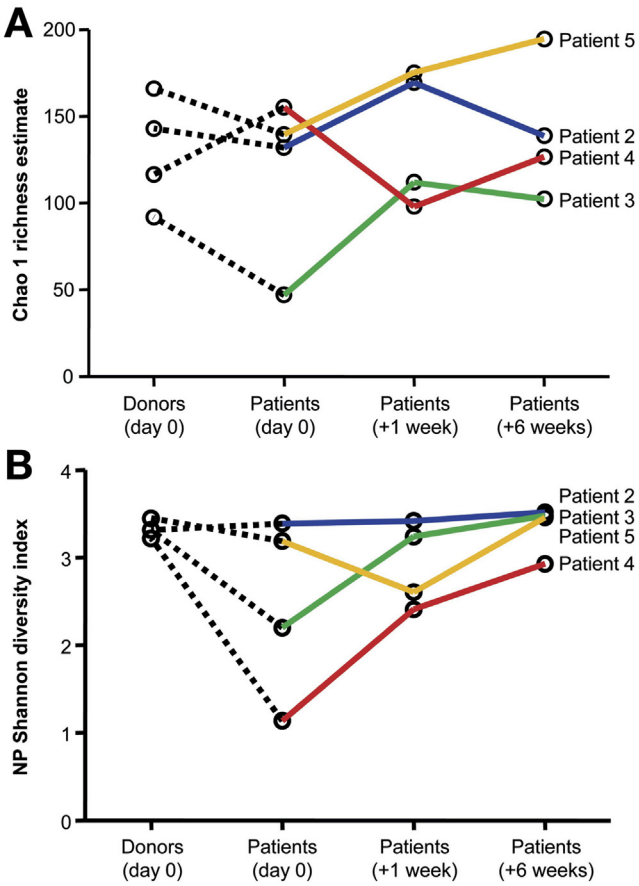

Figure 5. Bacterial diversity after fecal filtrate transfer treatment as determined by (*A*) Chao 1 richness estimation and by the (*B*) nonparametric (NP) Shannon index of diversity. The respective donor is connected to the patient graph by a *dotted line*.

enzymes such as intestinal-type alkaline phosphatase, chymotrypsin-like elastases, and α amylases. Only 11 bacterial hits were present in all 3 FFT filtrates investigated, consisting of metabolic enzymes and redox proteins without obvious microbiome-modifying properties, such as glyceraldehyde-3-phosphate dehydrogenase, phosphoenolpyruvate carboxykinase, glutaredoxin-1, or thioredoxin-1 (Supplementary Table 1). Complete lists of the identified proteins are presented in Supplementary Tables 2–4.

Discussion

This open-label series strongly suggests that FFT should be evaluated in a controlled setting in comparison with standard FMT. Source materials for such trials could be well-characterized standard donors, donor banks, artificial stool from bioreactors,[41,42] or defined bacteria.[28–30,43] A key advantage of FFT is the avoidance of all risks inherent to the transfer of living microorganisms. Further advantages include the potential for standardization and for the development of a robust, inexpensive, and patient-friendly formulation (ie, capsules filled with freeze-dried FFT preparations without the need to conserve living bacteria or spores). When contemplating the intersection of successful therapies related to FMT (classic FMT, spores, and FFT), it appears plausible that the active agent(s) of any FMT therapy are not living bacteria, but rather bacterial components, antimicrobial compounds of bacterial origin (eg, bacteriocins), or bacteriophages contributing to the normal intestinal microenvironment. These could be common to all successful FMT therapies and even rather unspecific regarding the bacterial strain(s) used for therapies.

Another important observation is the fast resolution of postinfectious clinical symptoms. Remaining diarrhea is a common clinical problem after CDI and also after FMT in CDI patients. In a significant percentage of patients, microbial tests to detect recurrent infection or re-infection are negative or inconclusive, pointing to postinfectious irritable bowel syndrome.[44] Both FMT and FFT appear to clear CDI, but also resolve the diarrhea, as particularly shown by patient 2. This may suggest that such diarrhea is related to remaining distortions of microbiome composition acting on the human host's mucosa and may point to a mechanistic interaction of FMT/FFT with epithelial function and/or motility that goes beyond the microbicidal effects of the procedure.

An interesting shift of the microbial community structures of patients 3 and 4 was observed. In both of these patients, expansion of the relative abundances of *Proteobacteria*, mostly attributable to *Escherichia* species, was a prominent component of the microbiome, which disappeared within 1 week after FFT. In all 4 patients investigated (Supplementary Figures 1 and 2), FFT was associated with a reduction of *E coli* and, in all but patient 2, also with a reduction of unclassified *Enterobacteriaceae*. This could suggest a potential for FFT to be applicable to other diseases with *Enterobacteriaceae*-driven dysbiosis such as inflammatory bowel diseases.

From our pilot study we cannot clearly define the exact mechanism of action. In particular, it cannot be decided whether the observed taxonomic shifts in the microbiota promoted the clearance of *C difficile* or rather reflect the resolution of disease with re-establishment of a health-associated microbiome after transfer of the active agent(s) from the donor. Two likely explanations include bacterial cell wall components or DNA fragments, which could stimulate host responses via pattern recognition receptors.[45,46] This in turn may lead to an alteration of the ecologic niches needed for outgrowth of existing beneficial bacteria or even successful novel colonization. Our findings lend a new interpretation to the results of a recent randomized, controlled, double-blind clinical trial for FMT in CDI that reported the frequently observed approximately 90% efficacy of FMT, but also showed that autologous stool transplantation was effective in more than 60% of patients.[47] The mechanism of efficacy of this procedure could be similar to that of FFT.

Another plausible explanation is the transfer of bacteriophages, which may act on the community dynamics of gut microbiota, thereby leading to a resolution of the initial dysbiosis.[31,48] In principle, this may be achieved by transfer of virus particles (ie, bacteriophages sensu

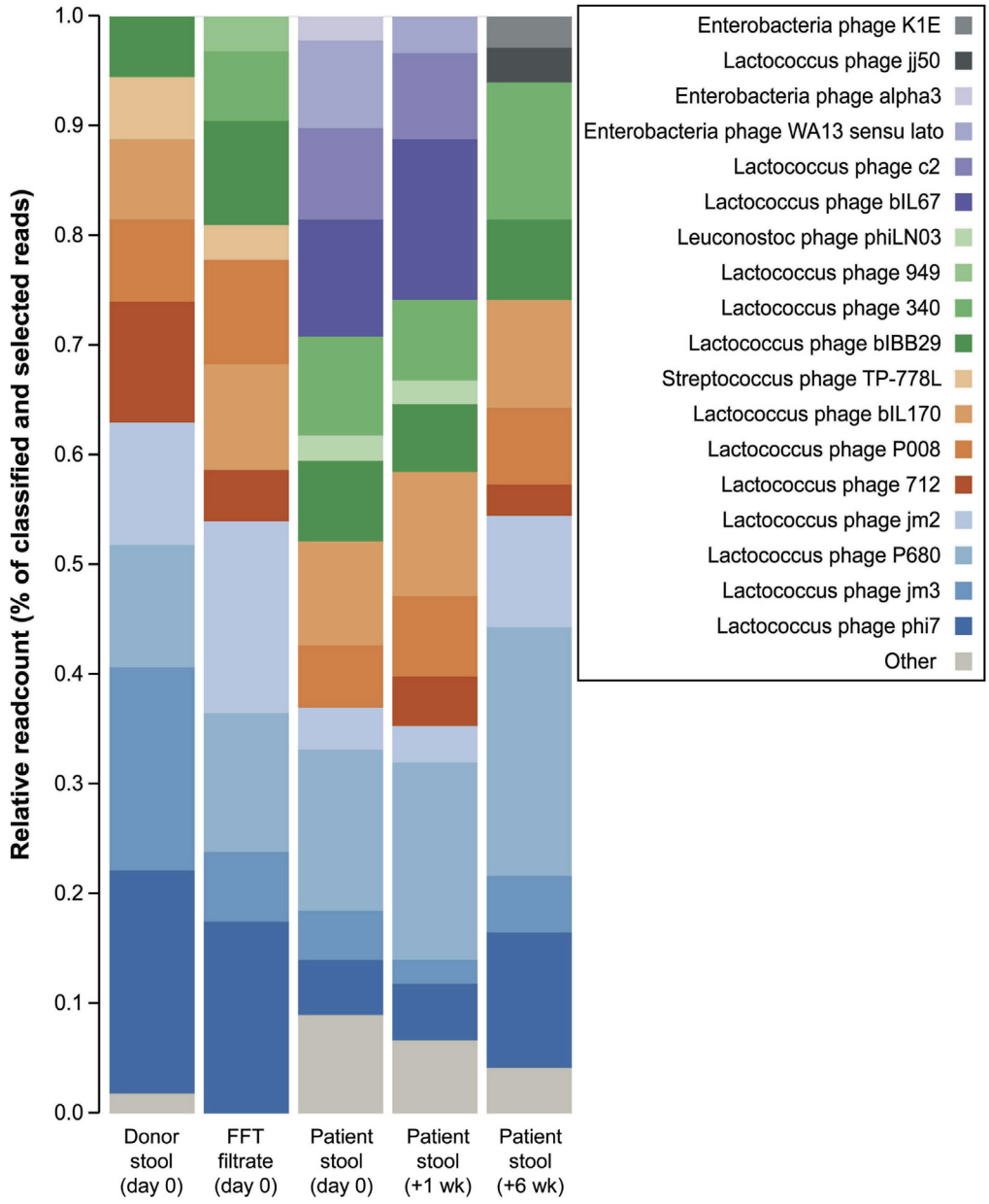

Figure 6. Virome analysis of the donor, filtrate, and patient sample sets for patient 4. Samples from donor stool (day 0), the resulting FFT filtrate and patient stool samples before FFT treatment (day 0), as well as at 1 or 6 weeks after treatment were compared for bacteriophage diversity.

stricto) or by transfer of specific bacteria harboring temperate phages in their genome, which may become active in a distinct microenvironment or condition. Two strong arguments for this hypothesis are provided by the therapeutic efficacy of bacterial cultures and spores (see earlier) and by our exemplary data showing that bacteriophages were detected in an FFT filtrate, which largely reflected the composition of the stool phageome of the donor. Moreover, some of these phages, which previously were absent, still were detectable in the recipient 6 weeks after therapy.

Future clinical studies and systematic microbiome and virome analyses should investigate whether FFT also is efficacious using filtrates of defined bacteria,[28–30,43] in particular in lyophilized formulations for oral administration.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2016.11.010.

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Author names in bold designate shared co-first authorship.

Received August 4, 2015. Accepted November 11, 2016.

Reprint requests
Address requests for reprints to: Stefan Schreiber, MD, Department of Internal Medicine I, University of Kiel and University Hospital Schleswig-Holstein, Arnold-Heller-Strasse 3, 24105 Kiel, Germany. e-mail: s.schreiber@mucosa.de; fax: (49) 431-500-22204.

Acknowledgments
The authors thank Karina Greve and Melanie Vollstedt for their expert technical assistance.

Conflicts of interest
These authors disclose the following: Georg H. Waetzig and Dirk Seegert are employees of CONARIS Research Institute AG; Stefan Schreiber is a shareholder of CONARIS, has been a consultant to Allergosan, Danone, and Nestlé, and has received lectureship compensation from Allergosan; and Stephan J. Ott has lectured for Allergosan. The remaining authors disclose no conflicts.

Funding
This study was supported by the German Excellence Cluster "Inflammation at Interfaces" (S.J.O, A.T., H.F., P.R., and S.S.), the SysINFLAME consortium of the German Ministry of Education and Research (P.R. and S.S.), the collaborative research center CRC1182 of the German Research Foundation (P.R.), and by CONARIS Research Institute AG.

Supplementary Materials and Methods

Methods for Virome Analyses

Sample processing. Virus-like particle purification and DNA extraction were performed as described previously,[1] with some modifications. Fecal samples were resuspended in 0.5 mL saline magnesium buffer and centrifuged 4 times at 2500 × *g* for 10 minutes, with the resulting supernatant passed sequentially through a 0.45-μm and a 0.22-μm pore diameter filter (Whatman/GE Healthcare, Munich, Germany). Each sample was treated twice with 0.2 volumes of chloroform and centrifuged at 5000 × *g* for 10 minutes at 4°C. The aqueous phase was treated with 1 U of DNase I and 70 μL of 10 × DNase buffer (Sigma-Aldrich, Taufkirchen, Germany) for 2 hours at room temperature the first time, and overnight at room temperature the second time. Enzyme activity was inactivated by incubation at 65°C for 10 minutes.

To extract viral DNA, 0.1 volumes of 2 mol/L Tris-Cl/0.2 mol/L EDTA, 1 volume of formamide, 1 μL of glycogen, and 10 μL of a 0.5 mol/L EDTA solution were added per 1 mL of sample. The sample subsequently was washed with 2 volumes of ethanol and centrifuged for 20 minutes at 13,800 × *g* at room temperature. The resulting pellet then was washed twice with 70% ethanol and resuspended in Tris-EDTA buffer followed by 10% SDS and 20 mg/mL solution of Proteinase K (Sigma-Aldrich) for 1 hour at 37°C, after which 5 mol/L NaCl and 10% cetyltrimethylammonium bromide/0.7 mol/L NaCl were added. Samples then were mixed with an equal volume of chloroform and centrifuged at 13,800 × *g* for 10 minutes at room temperature. To the resulting supernatant, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the mixture was centrifuged at 13,800 × *g* for 10 minutes. The aqueous phase was recovered, an equal volume of chloroform was added, and the resulting mixture was centrifuged again at 13,800 × *g* for 10 minutes, before adding 1 μL of glycogen with 0.7 volumes of isopropanol and precipitating overnight at 4°C. The next morning, samples were centrifuged at 13,800 × *g* for 30 minutes at 4°C, washed with 1 mL of 70% ethanol, air-dried, and resuspended in 50 μL of nuclease-free water.

Library construction. Libraries were generated using the TrueSeq Nano DNA kit (Illumina) according to the manufacturer's instructions. After quantification, normalized pools of all samples were sequenced on an Illumina MiSeq using the 2 × 150-bp sequencing kit (Illumina).

Quality control and metagenomic assembly. Truseq adaptors and low-quality reads (length, <55) were filtered from the Illumina paired-end reads (2 × 150 base) using Trimmomatic version 0.33.[2] These preprocessed paired-end reads were assembled using the SPAdes version 3.7.0 assembler, by implementing the "meta" function with a wide k-mer range of 21–77.[3]

Taxonomic assignment. The assembled contigs were classified at various taxonomic levels using the k-mer-based approach as described previously.[4] In brief, reads were subdivided into complete sets of 31-bp length-overlapping sequences (k-mers). Subsequently, these k-mers were compared against a large database (40,000 whole microbial genomes in the One Codex reference database) that contains information on known k-mers unique to specific taxonomic groups such as different bacterial clades or viruses. After comparison, each contig was assigned to a closely matched specific clade. Each sample then was summarized as a group of signature sequences depicting the presence of specific groups of organisms.[4]

Methods for Proteome Analyses

Sample Processing and Protein Digestion. *Bradford protein assay.* A protein assay (Bradford assay) was performed using 10 μL of each of the 3 FFT filtrate samples; the results from this test were below the limit of detection for the assay.

SDS–polyacrylamide gel electrophoresis stack gel. Aliquots of each sample (125 μL) were partially lyophilized and resuspended up to a volume of approximately 55 μL in ultrapure water (Arium 611VF; Sartorius, Göttingen, Germany). From each sample, 45 μL was transferred to a 500-μL centrifuge tube and 15 μL of Laemmli loading buffer was added; the remainder, approximately 10–15 μL, was used for solid phase extraction. The samples were heated at 95°C for 5 minutes and then loaded (20 μL in triplicate) onto a 12% SDS–polyacrylamide gel with a 4% stacking gel. Constant voltage (40 V) was applied to migrate the dye front into the stacking gel (15 min). Subsequently, the voltage was increased to 100 V for a further 15 minutes. The gel then was processed (fixed, washed, stained with Coomassie brilliant blue, and destained) and visualized. The triplicate protein-containing fractions of the gel were excised for each sample. This resulted in the collection of 9 gel pieces of approximately 10 × 8 mm in size.

One-dimensional SDS–polyacrylamide gel electrophoresis. The remaining 15 μL from each of the original samples was mixed with 5 μL of Laemmli loading buffer and heated at 95°C for 5 minutes before being loaded onto a 12% SDS–polyacrylamide gel with a 4% stacking gel. Constant voltage (40 V) was applied to migrate the dye front into the stacking gel (15 min). Subsequently, the voltage was increased to 100 V for a further 80 minutes. The gel then was processed (fixed, washed, stained with Coomassie brilliant blue, and destained) and visualized to assess the protein content of the samples. Four clearly visible protein bands from the FFT filtrate used for patient 4 and 2 faint bands from the duplicate FFT filtrates for patient 5 were excised.

Solid phase extraction. Peptides and small proteins from the remainder of resuspended samples (approximately 10–15 μL) were cleaned with a solution of 5% methanol + 0.1% trifluoroacetic acid on a Sep-Pak 1 cc (50 mg) C18 solid-phase extraction cartridge (Waters, Eschborn, Germany) before being eluted with increasing concentrations of acetonitrile, evaporated to dryness, and resuspended in loading buffer (3% acetonitrile + 0.1% trifluoroacetic acid) for mass spectrometry analysis.

In-gel digestion. The individual gel pieces from both the 1-dimensional SDS–polyacrylamide gel electrophoresis and

SDS–polyacrylamide gel electrophoresis stack gel approach were processed via a standard in-gel digestion protocol. Briefly, the gel pieces were diced into small fragments (approximately 1–2 mm^3), destained, and dehydrated. The samples were reduced (10 mmol/L dithiothreitol, 56°C, 60 min in 50 mmol/L triethylammonium bicarbonate buffer, pH 8), alkylated (55 mmol/L iodoacetamide, room temperature, 30 min in 50 mmol/L triethylammonium bicarbonate), and subjected to overnight trypsin digestion at 37°C in 50 mmol/L triethylammonium bicarbonate buffer, pH 8, with 100 ng of sequencing-grade trypsin. The resulting peptides were extracted from the gel pieces with increasing concentrations of acetonitrile (60%, 100%) and pooled with the supernatant from the overnight digestion, dried down in an Eppendorf Concentrator plus (Eppendorf, Hamburg, Germany), and resuspended in high performance liquid chromatography loading buffer (3% acetonitrile, 0.1% trifluoroacetic acid) for mass spectrometry analysis.

Liquid chromatography–mass spectrometry. *Procedures for liquid chromatography.* Chromatographic separation was performed on a Dionex U3000 ultra-high performance liquid chromatography system (Thermo Scientific Dionex, Idstein, Germany) equipped with an Acclaim PepMap 100 analytic column (2 μm, 75 μm $\times$ 500 mm; Thermo Fisher Scientific) coupled online to a mass spectrometer. The solvents used were as follows: buffer A (0.05% formic acid), buffer B (80% acetonitrile + 0.04% formic acid). The separation was performed over a programmed 86-minute run. Initial chromatographic conditions were 4% B for 2 minutes followed by a linear gradient from 4% to 40% B over 60 minutes before a 6-minute increase to 90% B and 3 minutes at 90% B. Subsequently, an inter-run equilibration of the column was achieved by 12 minutes at 4% B. A flow rate of 300 nL/min was used, and 3 μL of sample was injected per run. Injection repeats were performed for the stack gel-based samples.

Procedures for mass spectrometry. Acquisition runs were performed on a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany) coupled online to an ultra-high performance liquid chromatography. Ionization was performed with 1.5-kV spray voltage applied on a noncoated PicoTip emitter (10-μm tip size; New Objective, Woburn, MA) and the source temperature was set to 250°C. Mass spectrometry data were acquired from 1 to 80 minutes with mass spectrometer full scans between 300 and 2000 m/z at a resolution of 70,000 at m/z 400 (automatic gain control target of 3e6; maximum injection time of 100 ms). For each sample, independent liquid chromatography–mass spectrometry experiments were performed with the following settings: the 10 most intense precursors with charge states $\geq$2+ were subjected to fragmentation with higher-energy collisional dissociation (Fourier transform mass spectrometer; normalized collision energy of 27.5%; isolation width of 3 m/z; resolution, 17,500 at m/z 400; automatic gain control target of 1e5 and maximum injection time of 100 ms), dynamic exclusion for 20 seconds was applied with lockmass correction (445.120025 m/z).

Database searching and statistical validation. Mass spectrometry data were searched against a protein fasta database that contained all canonical human proteins, all bacterial taxonomy canonical reviewed proteins, all fungal canonical reviewed proteins, as well as common contaminant proteins listed in the common repository of adventitious proteins (cRAP). All protein fasta databases were downloaded from UniProtKB (access date: February 15, 2016). Database searches were performed with Proteome Discoverer 1.4.0.288 using the search algorithm SequestHT (both from Thermo Fisher). For in-gel digested samples, enzyme settings were trypsin (full) with a maximum of 2 missed cleavages. Carbamidomethylation of cysteine thiol groups was set as a static modification, and oxidation of methionine was set as a variable modification. For the 3 solid-phase extraction searches, no enzyme was specified, and oxidation of methionine was set as a variable modification. For all searches, precursor mass tolerance was 7 ppm and fragment mass tolerances were 0.02 Daltons. Peptide spectrum match validation was performed by Percolator (default values). Protein and peptide lists were exported as text files using only high confident matches with a false discovery rate of 0.01 or less, minimum of 1 unique peptide per protein.

In classic fecal metaproteomics studies, databases derived from metagenomic studies including predicted proteins often are used for protein identification.[5] In addition, steps to enrich bacterial fractions commonly are used to reduce the dominance of host/food proteins, which then allows for the identification of bacterial proteins.[6] Because of the total protein amounts and high complexity (in terms of numbers of detectable bacterial proteins), the use of databases derived from metagenomic studies, including predicted proteins, clearly is justified to allow deeper coverage of the bacterial (meta)proteome. In our study, we analyzed the free (extracellular) proteins from within the filtered aqueous extract; this, a priori, prevents the enrichment of bacterial proteins. Because the protein concentration in the extract was very low, with the exception of the few higher abundant proteins (see earlier), the obtained mass spectra were generally of low quality. By using a strict identification pipeline, the majority of the signals acquired with sufficient intensities that provided interpretable spectra could be assigned to protein identities using the annotated nonredundant database encompassing more than 282,000 bacterial, 20,000 human, and 28,000 fungal protein sequences. The identified proteins were mapped against previously validated proteins, thus providing the highest possible confidence in their identification when applying strict criteria (at least 2 independent peptides with 1 unique peptide) as performed herein.

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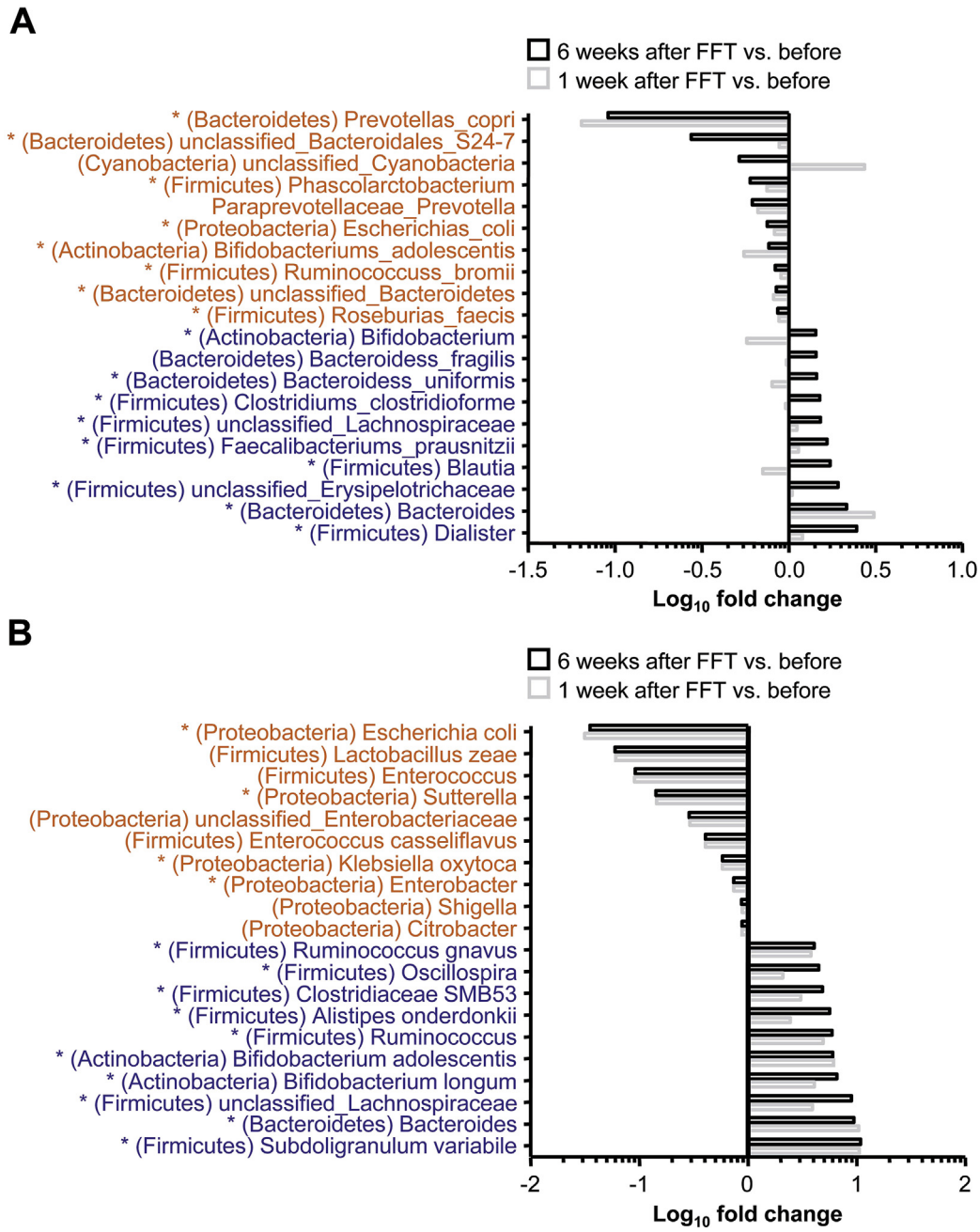
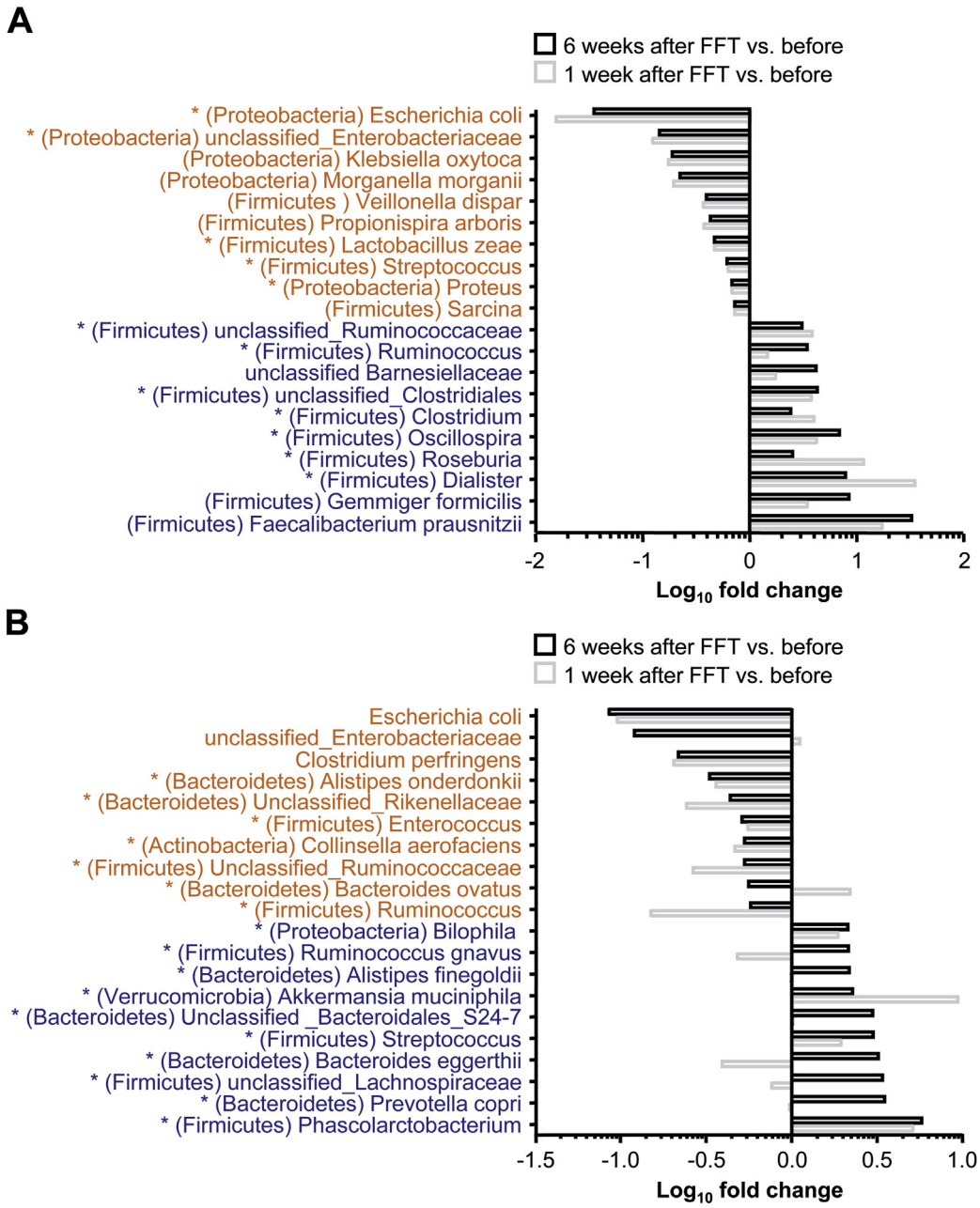
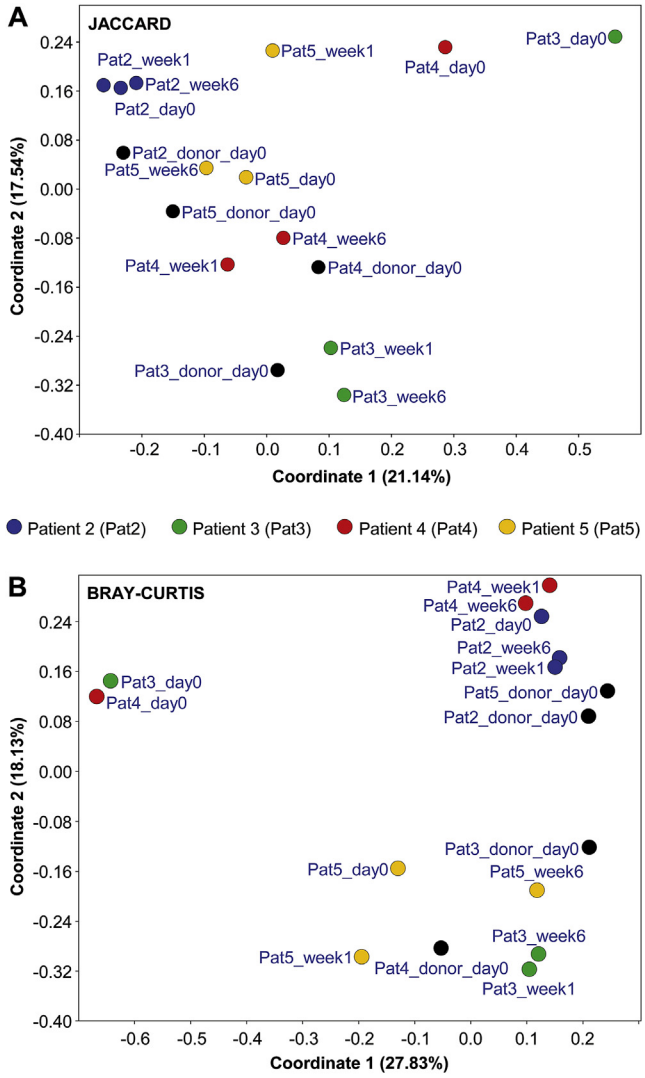


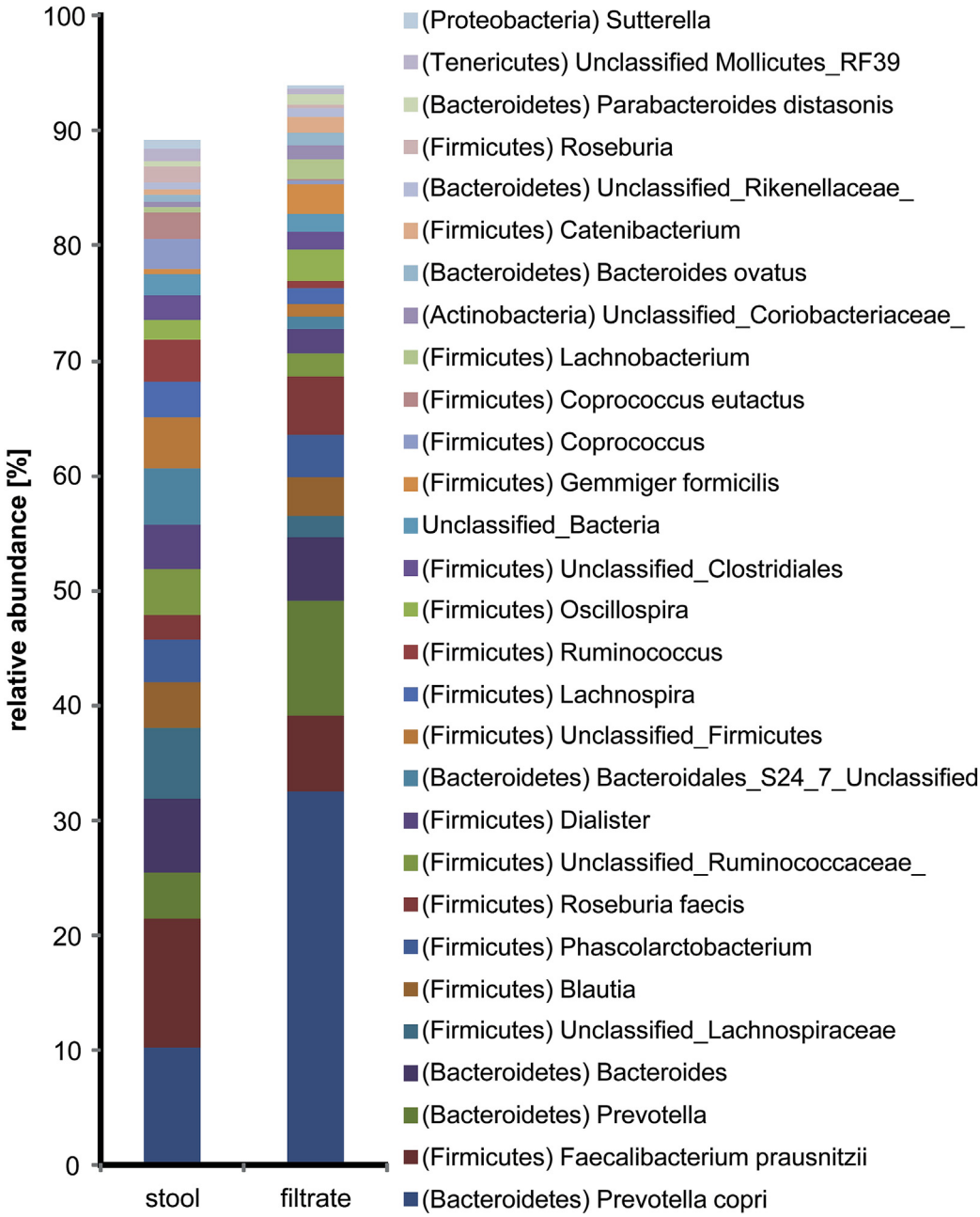
Supplementary Figure 1. Relative alteration of fecal bacterial composition after FFT treatment in (*A*) patient 2 and (*B*) patient 3. The figure represents the 10 most strongly decreasing (*red font*) or increasing (*blue font*) bacterial phylotypes after 6 weeks compared with their abundance before FFT treatment. Values for samples taken 1 week after FFT treatment were included for comparison (*grey bars*). *Bacterial phylotypes also detected in the feces of the donor.



Supplementary Figure 2. Relative alteration of fecal bacterial composition after FFT treatment in (*A*) patient 4 and (*B*) patient 5. The figure represents the 10 most strongly decreasing (*red font*) or increasing (*blue font*) bacterial phylotypes after 6 weeks compared with their abundance before FFT treatment. Values for samples taken 1 week after FFT treatment were included for comparison (*grey bars*). *Bacterial phylotypes also detected in the feces of the donor.



Supplementary Figure 3. Principal coordinate analysis of the bacterial community membership and structure using (*A*) Jaccard and (*B*) Bray–Curtis distances, respectively. Distances between *symbols* on the plot denote relative dissimilarities in bacterial community membership and structure in the patient samples before (day 0) and after FFT (week 1 or week 6) and to the respective donor samples.



Supplementary Figure 4. Relative distribution of dominant microbial 16S ribosomal RNA gene signatures in stool and filtrate samples from the donor for patient 5. Only bacterial phylotypes with at least 1% cumulative abundance in both samples are shown.