

Changes in the Composition of the Human Fecal Microbiome Following Bacteriotherapy for Recurrent *Clostridium difficile*-Associated Diarrhea

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ABSTRACT

CDAD is the major known cause of antibiotic-induced diarrhea and colitis, and the disease is thought to result from persistent disruption of commensal gut microbiota. Bacteriotherapy by way of fecal transplantation can be used to treat recurrent CDAD and is thought to re-establish the normal colonic microflora. However, limitations of conventional microbiologic techniques have until recently precluded testing of this idea. In this study we used T-RFLP and 16S rRNA gene sequencing approaches to characterize the bacterial composition of the colonic microflora in a patient suffering from recurrent CDAD, before and after treatment by fecal transplantation from a healthy donor. While the patient's residual colonic microbiota, prior to therapy, was deficient in members of the bacterial divisions-Firmicutes and Bacteriodetes, transplantation had a dramatic impact on the composition of the patient's gut microbiota. By 14 days post transplantation, the fecal bacterial composition of the recipient was highly similar to the donor and was dominated by *Bacteroides* spp. strains and an uncharacterized butyrate producing bacterium. The change in bacterial composition was accompanied by resolution of the patient's symptoms. The striking similarity of the recipient's and donor's intestinal microbiota following bacteriotherapy suggests that the donor's bacteria quickly occupied their requisite niches, resulting in restoration of both the structure and function of the microbial communities present.

Keywords: *Clostridium difficile*, Bacteriotherapy, T-RFLP, 16S rRNA

INTRODUCTION

Clostridium difficile associated disease (CDAD) is the major known cause of antibiotic-induced diarrhea and colitis, and is an increasing public health problem. In the last decade, the incidence of CDAD has increased several-fold, and the problem has been compounded further by emergence of newer more virulent strains, leading to more severe disease and increasing rates of fatality.¹ Major risk factors for CDAD include antibiotic exposure, age, and hospitalization. In addition, inflammatory bowel disease and pharmacologic gastric acid blockade have been recently identified as independent risk factors for contracting CDAD.²⁻⁶

Clinical presentation varies from mild and moderate cases characterized by watery diarrhea, to severe cases associated with signs of systemic inflammation such as fever, leukocytosis, and hypoalbuminemia. Only *C. difficile* strains producing exotoxins are pathogenic.^{1, 7, 8} The rising mortality of CDAD since 2000 has been associated with emergence of the BI/NAP-1/027 strain, which is characterized by markedly increased production of toxins A and B, resistance to fluoroquinolones, and production of binary toxin⁹. Most cases of CDAD respond to treatment with metronidazole or vancomycin.^{1, 10, 11} However, recurrence of the infection occurs in about 20% of cases, and it is typically caused by re-growth of vegetative *C. difficile* from residual spores, which are resistant to antibiotic treatments.¹¹ Factors that predispose patients to recurrence of CDAD include poor adaptive immune responses to the infection,¹² and a decreased diversity in colonic microflora, which normally limits expansion of *C. difficile*.^{13, 14} Moreover, CDAD is likely a dramatic consequence of a disruption, or imbalance, of the

commensal gut microbiota, which normally exists in a mutualistic relationship with the host.

Understanding of the microbial composition of human intestinal track has, in the past, been elusive, in large part due to limitations of standard microbiological techniques. However, several recent studies have used molecular approaches based on sequencing or fingerprinting of 16S rRNA genes to more deeply explore the composition of the gut microbiota, including non-cultured representatives.¹⁴⁻¹⁷ Taken together, these studies indicate that the intestinal tract of healthy humans is dominated by bacteria in the phyla Bacteroidetes and Firmicutes. However, the composition and species richness of intestinal tract microbiota in individuals with CDAD and recurrent-CDAD were found to be markedly different from control patients.¹⁴

The re-establishment of the normal composition of the intestinal flora has long been hypothesized to be a curative approach for recurrent CDAD when conventional treatment with antibiotics fails to clear the disease. Given the complexity of the human GI tract, it has been suggested that the best approach to do this is reestablish the patient's microbiome by transferring in the GI microbiota present in feces from a healthy donor via rectal or nasogastric infusion. To date, however, there are only a few published studies documenting this approach,¹⁸⁻²¹ and only limited information is available concerning whether this procedure results in a curative restoration of an ecologically- stable microbial population in the patient's intestine. An attempt to examine this treatment option in more detail was made in a small study by Tvede and Rask-Madsen in 1989.¹⁸ These investigators studied six patients with recurrent CDAD, and treated them with bacteriotherapy (fecal transplantation) by using fecal enemas or rectal instillation of

mixtures of colonic bacteria. This treatment resulted in detection of culturable *Bacteroides* sp. strains in fecal samples from patients, whereas the strains were not detected prior to bacteriotherapy, suggesting that the gut microbiota composition had been modified.

Little is currently known about the potential to alter the microbial composition in the human colon by introduction of exogenous colonic bacteria. The general composition of the colonic microbiota is relatively stable after the neonatal period²² and quite resilient to environmental impact, although fluctuations due to disease,¹⁶ diet,³⁰ and exposure to antibiotics^{23, 24} can occur. However, in patients who develop *C. difficile* colitis there is a dramatic alteration in the gut microbial composition that has important health consequences. In this report, we used molecular approaches to characterize the bacterial composition of the colonic microflora in a patient suffering from recurrent *C. difficile* colitis, before and after treatment of a fecal transplantation from a healthy donor. In addition, we aimed to document the ability of the donor bacteria to colonize the patient's GI tract and the curative effects of the treatment.

MATERIALS AND METHODS

Fecal sample collection and bacteriotherapy.

The patient was prepared for a colonoscopy using one-gallon of GoLyteley® (Braintree Laboratories, Braintree, MA). Fecal samples were collected from the patient by aspiration during unprepped lower endoscopy from the sigmoid colon 7 days before the procedure and 14 days after the procedure. Another sample was obtained from the patient on the day of the procedure (day 0) by colonoscopic aspiration of the residual liquid luminal contents throughout the colon. An excreted sample was also collected from the patient on day 33 by use of a toilet hat. The samples were immediately kept on ice and frozen at – 80°C within 1 hour of collection. For bacteriotherapy, about 25 g of fecal material obtained from the patient's husband (the healthy donor) was suspended in 300 mL of normal saline and homogenized using a pre-sterilized, stainless steel, laboratory-grade Waring blender (Waring Laboratory, Torrington, CT). A 250 mL aliquot of the suspension was injected into the cecum of the patient using a colonoscope. The donor fecal sample was also frozen at -80°C for subsequent analyses.

Terminal-restriction fragment length polymorphism (T-RFLP) analyses.

Bacteria were isolated from excreted, and endoscopic- and enema- obtained fecal samples as described previously.¹⁶ DNA was extracted from duplicate 250 mg samples from each sample using the MoBio Power Soil DNA Kit (MoBio, Solana Beach, CA), according to the manufacturer's instructions. The 16S rRNA genes were PCR amplified from each DNA extract (two technical replicates per extract) using the general bacterial primers Bact-8F (5'-AGAGTTGATCCTGGCTCAG-3') [25] 5' end-labeled with 6-

carboxyfluorescein (6-FAM), and 926r (5'-CCGTCAA TTCCTTTRAGTTT-3')²⁶ using conditions described elsewhere.¹⁵ DNA product amounts and sizes were confirmed by agarose gel electrophoresis using GeneRuler 100bp DNA ladder Plus (Fermentas Life Sciences, Burlington, Canada) as a size marker.

PCR products were digested with restriction enzyme *HaeIII* and the resulting fragments were separated on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA) as previously described.²⁷ The sizes of the fluorescently labeled fragments were determined by comparison with the internal GS ROX-500 size standard (Applied Biosystems). The T-RFLP electropherograms were imaged using the Peak scanner software (Applied Biosystems) and relative peak areas of each terminal restriction fragment (TRF) were determined by dividing the area of the peak of interest by the total area of peaks, using 50 and 500 bp lower and upper threshold values, respectively. Data was normalized by applying a threshold value for relative abundance at 0.5%, and only TRFs with higher relative abundances were included in the remaining analyses.

Cloning and sequencing.

To confirm the identities of the bacterial species corresponding to the dominant TRFs, cloning and sequencing of 16S rRNA genes from the fecal extracts were performed as described.¹⁶ DNA from samples were amplified by using the PCR and general bacterial primers Bact-8F²⁵ and 926R²⁶ using previously described reaction conditions.¹⁵ Three replicate PCRs and products from each sample were pooled and gel purified using the Qiagen gel extraction kit (Qiagen, Hilden, Germany). Clone libraries

were constructed by ligating PCR products into TOPO TA pCR 4.0 vectors (Invitrogen, Carlsbad, CA), followed by transformation into competent *E. coli* TOP 10 cells (Invitrogen). Clones were screened for inserts from each library with PCR using vector primers M13f and M13r (Invitrogen), using the same thermal cycling program as described above for amplification using general bacterial primers for T-RFLP. The PCR products were diluted 50-fold and used in a nested PCR reaction with primers 926r and fluorescently tagged primer Bact-8F for T-RFLP analysis of inserted clones, with the same running conditions as described previously for these primers (see above). Clones with unique TRF sizes were selected for sequencing, to determine which species comprised the TRFs. Several clones from redundant TRFs were also sequenced.

Sequences were aligned using the Silva SINA Webaligner subroutine of The Silva database (<http://www.arb-silva.de/aligner/>) and Fasta files were exported to the Fast Aligner subroutine of ARB (<http://www.arb-home.de/>). Closest reference sequences were selected for each clone from the 16S rRNA gene database of the PT server containing SSU Ref sequences (<http://www.arb-home.de/>). Sequences from the current study were phylogenetically assigned according to their best matches to sequences in the annotated tree, based on parsimony. Dendograms were constructed using the Neighbor-Joining algorithm. Bootstrap analysis was performed with 100 iterations using the ARB software.

Statistical analysis.

T-RFLP data from each sample was entered into a data matrix that consisted of the terminal restriction fragments as variables and the samples as objects. A consensus T-RFLP profile was constructed for each of the technical duplicates as previously

described.^{15, 16} Cluster analysis, based on Bray Curtis distances, and a dendrogram were created using the software PAST (URL: <http://folk.uio.no/ohammer/past/>).

RESULTS

Patient Case.

A 61 year old woman with chronic diarrhea that was associated with *C. difficile* infection was referred for evaluation to our institution. At initial encounter, she reported diarrhea of 8 months duration that originally started shortly following treatment with cephalosporin and quinolone antibiotics for back surgery and a pulmonary infection. During these eight months, she was repeatedly treated with metronidazole and vancomycin, and required several hospitalizations for intravenous hydration. The patient complained of loose small bowel movements every 15 minutes, accompanied by great urgency and rectal tenesmus. She wore diapers at all times, was confined to a wheelchair, and lost approximately 27 kg since symptoms onset. Flexible sigmoidoscopy performed at presentation in clinic demonstrated classic pseudomembranous colitis. Stool samples were positive for *C. difficile* toxins A and B, and stool culture confirmed heavy growth of this bacterium. The patient was again treated with vancomycin, but failed to respond. She was subsequently prescribed nitazoxanide (2-acetyloxy-N-(5-nitro-2-thiazolyl)benzamide), 500 mg orally, twice daily. Following antibiotic therapy, her bowel movement frequency decreased to six times per day, and flexible sigmoidoscopy demonstrated resolution of pseudomembranous colitis. Ten days after discontinuation of Nitazoxanide, however, the patient had recurrence of her original diarrheal symptoms. Endoscopic analyses indicated return of pseudomembranous colitis, and stool studies

were again positive for *C. difficile*. Despite two more cycles of Nitazoxanide, including one lasting a full month, and continuous administration of Florastor, a probiotic containing *Saccharomyces boulardii*, the *C. difficile*-induced colitis reoccurred within 10 days of stopping Nitazoxanide treatment.

Since conventional treatment failed to resolve the CDAD, fecal bacteriotherapy was offered to break the cycle of recurrences and achieve a potential cure. Informed consent was obtained from the patient and the donor following discussion with each of the potential risks, benefits, and alternative options. The patient was maintained on Nitazoxanide until the day before the procedure. The fecal donor material was taken from her husband of 44 years, who had no risk factors for blood-borne communicable diseases, had no recent exposure to antibiotics, had no gastrointestinal symptoms of any kind, and tested negative for common stool pathogens and *C. difficile*. Bacteriotherapy was delivered into the patient's right colon by way of a colonoscopy. The colon at the time of the procedure had no evidence of inflammation by endoscopic or histological examination, but was notable for mild diverticulosis, one of the factors proposed to be a possible risk factor for recurrent *C. difficile* infection.^{21, 28}

The patient had her first solid bowel movement on the second day following treatment, and developed constipation in the initial months following the procedure. Her abdominal pain gradually subsided, and at one month following bacteriotherapy her stool studies were culture negative for *C. difficile*. At about three months, the patient reported a transient episode of loose bowel movements during which she once again tested negative for *C. difficile* infection. The symptoms resolved without therapy within two weeks. At six months follow-up the patient reported once-daily formed stools.

Terminal restriction fragment length polymorphism analyses.

T-RFLP analyses of the 16S rRNA gene sequences of the intestinal microbiomes of the fecal donor and recipient patient, immediately before and after fecal transplantation, are shown in Figure 1A. The microbial fingerprints from the patient 7 days before, or on the day of, transplantation clustered separately from those of the donor on day 0. Moreover, the TRF pattern of fecal bacteria from the patient receiving fecal transplantation 14 or 33 days post inoculation clustered together. Results in Figure 1A also show that the patient's fecal flora retained characteristics of the donor 33 days post transplantation, although small changes can be seen relative to those of the patient 14 days after transplantation. This suggests that although microbiologically-mediated intestinal functionality was restored, the microbiome of the patient changed over time relative to the donor. There was low variability between the replicates at each time point.

The fecal and intestinal flora of the patient lacked TRFs representative of *Bacteroides* spp. strains, both 7 days prior to, and on the day of, transplantation (Fig. 1B). In contrast, *Clostridium* spp. strains were dominant in the fecal and biopsy samples from the patient (represented by TRF 222). Interestingly, the intestinal tract of the patient was dominated by *Veillonella* sp. strains (TRF 211), which were largely absent in the fecal donor. Moreover, the intestinal tract of the patient prior to treatment, contained TRFs corresponding to nsistent with *Lactobacillus* spp. (TRFs 326, 286, and 277), *Streptococcus* spp. (TRF 308), unclassified bacteria most similar to *Erysipelotrix* spp. strains (TRF 250), and *Lachnospiraceae incertae sedis* (TRF 285) and *Ruminococcaceae* (TRF 238). In contrast, the corresponding TRFs were entirely absent r not abundant in feces from the donor.

The transplantation had a dramatic impact on the composition of the patient's gut microbiota. By 14 days post transplantation, the fecal bacterial composition of the recipient was highly similar to the donor and was dominated by *Bacteroides* spp. strains (similar to *B. uniformis* spp.; TRF 262), *Bacteroides vulgatus* (TRF 83), and an uncharacterized butyrate producing bacterium (TRF 274). In addition, by Day 14 the patient, similar to the donor, contained TRFs representative of *Ruminococcaceae* (TRF 260).

At subsequent sampling periods some changes in the composition of the patient's gut microbiota were observed. While the patient's fecal microbiome was still dominated by *Bacteroides* sp. strains 33 days post transplantation, there were slight changes in fecal composition relative to Day 14. At the 33 day sampling period, the dominant bacteria detected were *Bacteroides* spp. (TRFs 262 and 257), *Bacteroides vulgates* (TRF 83), *Ruminococcaceae* spp. (TRFs 272 and 260), and *Anaerostipes* sp. strains (TRF 317). Overall, results from these studies indicated that the bacterial composition of the patient's feces mimicked that of the donors 14 days post transplantation and that this may have resulted in our inability to culture *C. difficile* following bacteriotherapy. This change in bacterial composition was, in turn, accompanied by restoration of normal bowel function shortly after therapy.

Results presented in Figure 2 show that the majority of clones recovered from DNA from patient intestinal bacteria (pool of the fecal and the mucosal samples) before transplantation (JD1) clustered with *Lactobacillus*, *Streptococcus*, *Veillonella*, and *Eubacterium* spp. strains, and were phylogenetically divergent from the other tested clones. In contrast, sequences recovered from libraries derived from feces from the

patient post transplantation (JD8) and the donor (JD4) clustered together in the dendrogram, and were primarily dominated by bacteria in the genus *Bacteroides*.

DISCUSSION

The recurrent form of *C. difficile* colitis is a particularly challenging problem associated with infection by this bacterium. In these cases patients are unable to cease repeated and prolonged courses of antibiotics, which can lead to significant morbidity and expense as illustrated by the patient in this study. An additional difficulty in our case reported here was the apparent failure of standard antibiotics, Metronidazole and Vancomycin, to treat CDAD. Although we were able to achieve temporary control of the infection by treatment with Nitazoxanide, complete resolution of the infection was not established. Unfortunately, this is a similar problem encountered by others treating this disease.¹

In the studies reported here, we used a molecular approach to gain a better understanding of the bacterial composition of the GI tract in a CDAD patient. While we used TRFLP and clone libraries to determine the bacterial populations present in the GI tract, other methods have also been used to study intestinal microbiota, including non-library based metagenomic analyses. Both of these methods are now routinely used in metagenomic analyses of the human intestinal microbiome.¹⁴⁻¹⁷ While the TRFLP approach used here is relatively inexpensive and rapid, and has been used to study the impact of lifestyle on the fecal microbiota of children in three European countries and the fecal microbiota of identical twins with Crohn's disease¹⁵⁻¹⁶, the method does not provide the same taxonomic resolving power as does sequence based approaches. Nevertheless,

our results clearly demonstrated that the patient's residual colonic microbiota, prior to therapy, was deficient in members of the normally-dominant bacterial divisions – Firmicutes and Bacterioidetes, and the data presented here is consistent with results obtained using both methods. These bacteria are well known dominant members of human fecal microbiota¹⁶ and these phyla are thought to play major ecological roles in establishment and maintenance of gut homeostasis.²⁹ A lack of *Bacteroides* sp. strains in patients suffering from CDAD has previously been noted by others,¹⁸ and members of this genus has been postulated to inhibit *C. difficile* proliferation.³⁰ It is likely that the atypical bacterial populations present, consisting mainly of species stains representatives of *Veillonella*, *Clostridium*, *Lactobacillus*, *Streptococcus*, and unclassified bacteria most similar to *Erysipelothrix*, were due, in part, to *C. difficile* infection and/or treatment with antibiotics. Significant differences were noted in patient's stool on days -7 and 0, suggesting that the intestinal microflora of the patient were evolving. Notably, the material collected on day 0 differed from the other samples as it represented washout by the purgative used to prepare the colon for the procedure and may have been enriched for bacteria carried from the upper GI tract. On day 0, the patient's GI tract was dominated by *Veillonella* sp., *Lachnospiraceae incertae sedis*, and *Ruminococcaceae* sp. strains. While some *Clostridium* sp. strains were also present on day 0, the patient tested negative for *C. difficile*, and this bacterium was not detected via subsequent T-RFLP and sequence analyses. This is consistent with pre-transplantation treatment of the patient with Nitazoxanide and the likelihood that the residual *C. difficile* spores were present below the threshold of detection by these molecular assays.

Dramatically, 14 days following bacteriotherapy, which was our first data point, the microbiome of the patient's GI tract (as assessed via mucosal washings and fecal sampling) changed to closely resemble that of the donor, and *Bacteroides* sp. strains and *B. vulgatus* became the dominant constituents. The patient's GI intestinal tract was still dominated by *Bacteroides* sp. strains 33 days post treatment, however, at later time periods other bacterial groups, including *Ruminococcaceae* and *Anaerostipes* sp. strains became more abundant. *Ruminococcaceae* and *Anaerostipes* species are a subgroup within the *Clostridium coccoides* known to produce butyrate.³¹ Another butyrate-producing bacterium (represented by TRF 274) also became more abundant 14 days post transplantation. Many Firmicutes produce butyrate and are postulated to be involved in obesity in humans and murine models.^{32, 33} Short chain length fatty acids (SCFAs), especially butyrate, have also been shown to play a critical role in maintaining integrity of the colonic epithelium and regulation of the mucosal immune responses.^{34, 35} Interestingly, rectal administration of SCFAs has been used successfully to treat antibiotic-associated diarrhea, including *C. difficile*-induced pseudomembranous colitis.³⁶ Limited SCFA availability to colonocytes has been proposed to play a role in pathogenesis in ulcerative colitis,³⁵ and administration of SCFAs has been proposed for a variety of inflammatory conditions affecting the intestine.³⁴

From a therapeutic standpoint, the patient in this study had a remarkably rapid and complete recovery from her diarrhea following colonic reconstitution with fecal microbiota from a healthy donor. This result dramatically demonstrated the benefits to the host from the specialized microbial communities that normally inhabit the colon. While fecal transplantation (bacteriotherapy) has previously been reported,¹⁸⁻²¹ there is

only limited information concerning whether this procedure results in restoration of beneficial intestinal microbiota. The striking similarity of the recipient's microbiota to that of the donor following bacteriotherapy strongly suggest that the donor's bacteria quickly occupied their requisite niches in the new host, resulting restoration of both the structure and function of microbial communities present. This case illustrates the importance and power of the mutualistic relationship between the eukaryotic host and its intestinal microbiome, and suggests that the gut microbiome can be reprogrammed to restore beneficial host functions. Of course, we are aware that the veracity of the conclusions are limited in a single case report; additional studies are under way using a larger number of patients and donors.

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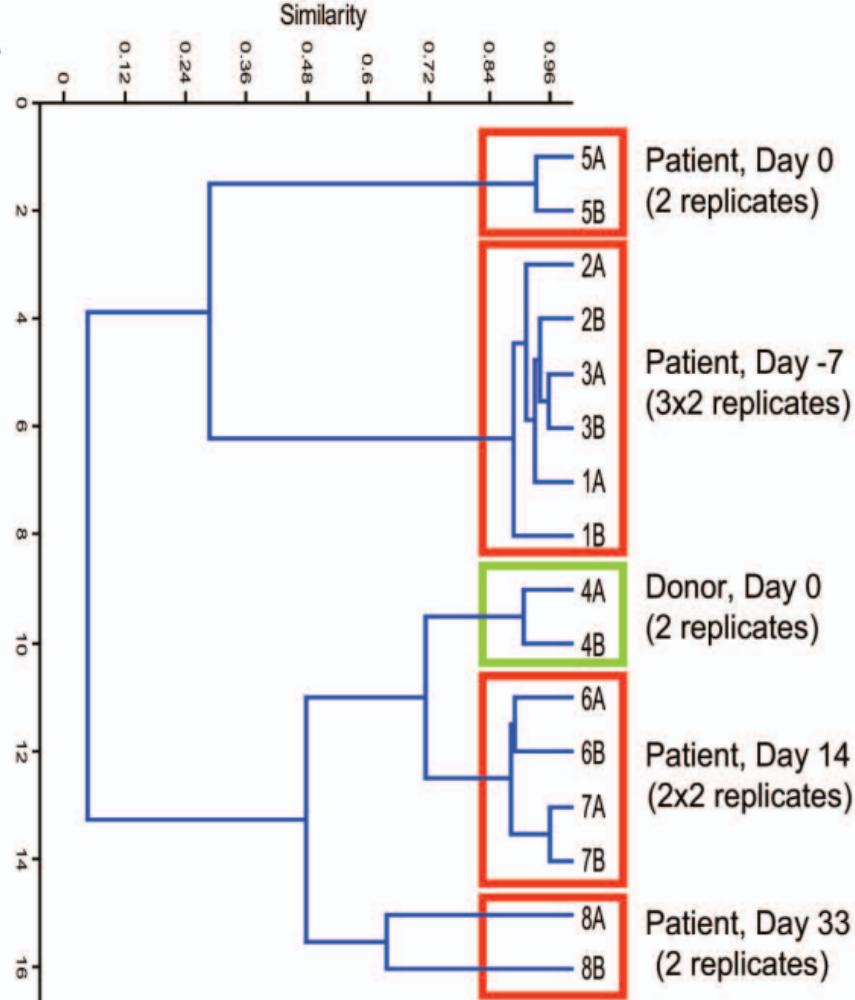
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FIGURE LEGENDS

Figure 1. (A) Dendrogram of 16S-based T-RFLPs obtained from fecal material from patient and the donor before and after fecal transplantation. (B) Distribution of bacterial species in feces of the donor and patient before and after fecal transplantation. The bacterial species represented by TRFs are color coded and are valid across columns. The purgative wash-out occurred on day 0, shortly before fecal transplantation.

Figure 2. Phylogenetic relatedness of 16S rDNA sequences recovered from clone libraries constructed from DNAs from donor and patient feces following fecal transplantation. Legend: JD1 - patient before fecal transplantation, JD4 - donor, and JD8 - patient after fecal transplantation. The number following JD designations represents clone number examined.

A



B Figure 1

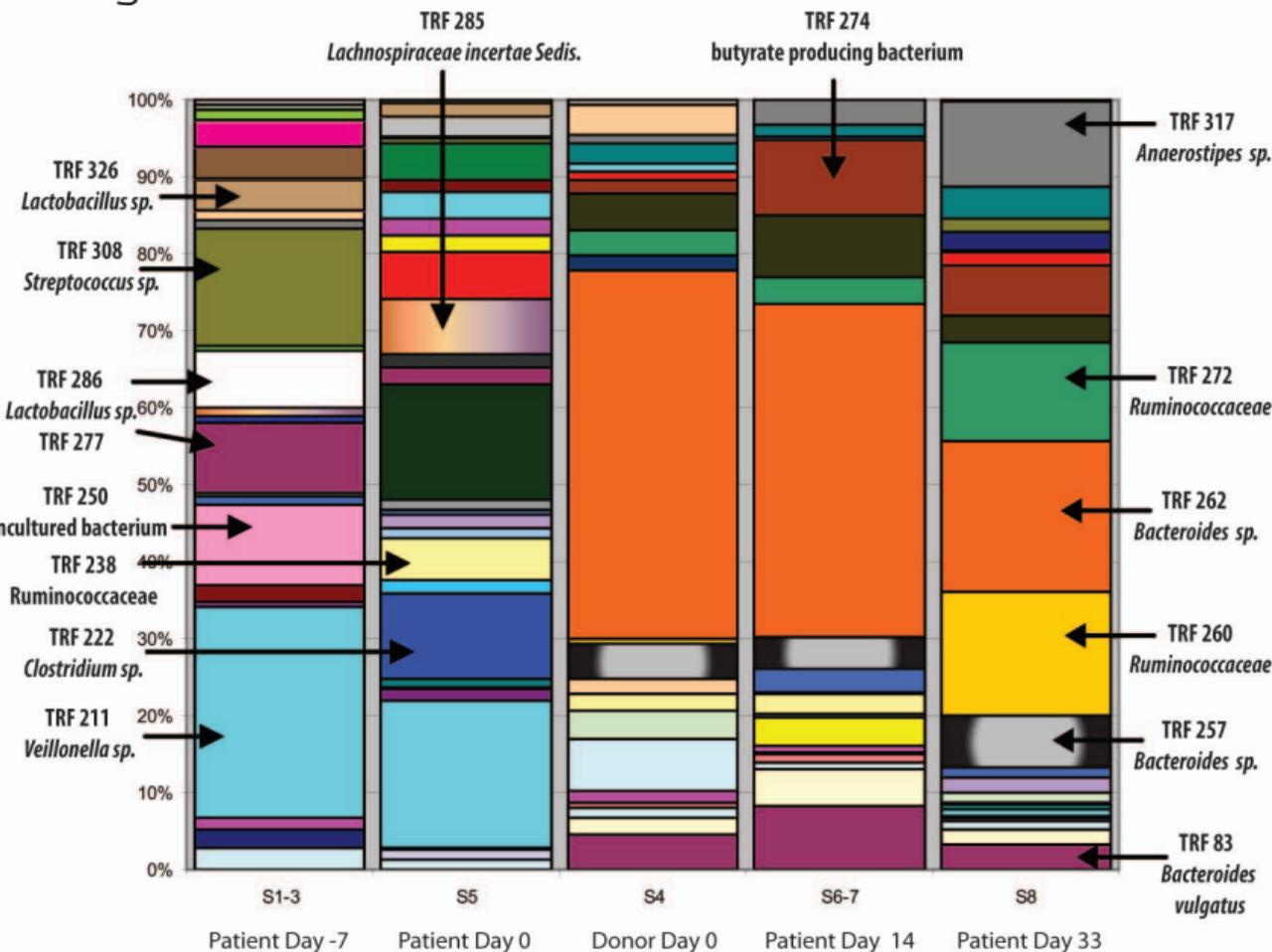


Figure 2

