The role of gut microbiota in human metabolism
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CHAPTER 4

DUODENAL INFUSION OF DONOR FECES FOR
RECURRENT CLOSTRIDIUM DIFFICILE

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Abstract

Background
Recurrent Clostridium difficile infection is difficult to treat, and failure rates for antibiotic therapy are high. We studied the effect of duodenal infusion of donor feces in patients with recurrent C. difficile infection.

Methods
We randomly assigned patients to receive one of three therapies: an initial vancomycin regimen (500 mg orally four times per day for 4 days), followed by bowel lavage and subsequent infusion of a solution of donor feces through a nasoduodenal tube; a standard vancomycin regimen (500mg orally four times per day for 14 days); or a standard vancomycin regimen with bowel lavage. The primary end point was the resolution of diarrhea associated with C. difficile infection without relapse after 10 weeks.

Results
The study was stopped after an interim analysis. Of 16 patients in the infusion group, 13 (81%) had resolution of C. difficile-associated diarrhea after the first infusion. The 3 remaining patients received a second infusion with feces from a different donor, with resolution in 2 patients. Resolution of C. difficile infection occurred in 4 of 13 patients (31%) receiving vancomycin alone and in 3 of 13 patients (23%) receiving vancomycin with bowel lavage (P<0.001 for both comparisons with the infusion group). No significant differences in adverse events among the three study groups were observed except for mild diarrhea and abdominal cramping in the infusion group on the infusion day. After donor-feces infusion, patients showed increased fecal bacterial diversity, similar to that in healthy donors, with an increase in Bacteroidetes species and clostridium clusters IV and XIVa and a decrease in Proteobacteria species.

Conclusion
The infusion of donor feces was significantly more effective for the treatment of recurrent C. difficile infection than the use of vancomycin. (Funded by the Netherlands Organization for Scientific Research; Netherlands, Netherlands Trial Register number, NTR1177)
Introduction

Antibiotic treatment for an initial *Clostridium difficile* infection typically does not induce a durable response in approximately 15 to 26% of patients (1-3). An effective treatment against recurrent *C. difficile* infection is not available. Generally, repeated and extended courses of vancomycin are prescribed (4). The estimated efficacy of antibiotic therapy for a first recurrence is 60%, a proportion that further declines in patients with multiple recurrences (2, 5-7). Mechanisms that have been proposed for recurrence include persistence of spores of *C. difficile*, diminished antibody response to clostridium toxins, and persistent disturbance with a reduced diversity of intestinal microbiota (8-12).

Infusion of feces from healthy donors has been reported as an effective treatment of recurrent *C. difficile* infection in more than 300 patients (13-18). However, experience with this procedure is limited by a lack of randomized trials supporting its efficacy and the unappealing nature of the treatment. In this study, donor feces were infused in patients with recurrent *C. difficile* infection and compared with conventional 14-day vancomycin treatment, with and without bowel lavage.

Methods

Study design

The complete methods are included in the Supplementary Appendix, which along with the research protocol is available with the full text of the article at NEJM.org. In this open-label, randomized, controlled trial, we compared three treatment regimens: the infusion of donor feces preceded by an abbreviated regimen of vancomycin and bowel lavage a standard vancomycin regimen, and a standard vancomycin regimen with bowel lavage.

The study was conducted at the Academic Medical Center in Amsterdam, the Netherlands. Patients who had been admitted to referring hospitals were visited by the study physicians, who performed the randomization. All participants provided written informed consent. A data and safety monitoring board monitored the trial.
on an ongoing basis. The research protocol was approved by the ethics committee at the Academic Medical Center. The first and last two authors vouch for the accuracy and completeness of the reported data and for the fidelity of the report to the study protocol.

**Study population**

Included in the study were patients who were at least 18 years of age and who had a life expectancy of at least 3 months and relapse of *C. difficile* infection after at least one course of adequate antibiotic therapy (≥ 10 days of vancomycin at a dose of ≥125 mg four times per day or ≥ 10 days of metronidazole at a dose of 500 mg three times a day). *C. difficile* infection was defined as diarrhea (≥ 3 loose or watery stools per day for at least 2 consecutive days or ≥ 8 loose stools in 48 hours) and a positive stool test for *C. difficile* toxin. Available isolates were characterized by polymerase-chain-reaction (PCR) ribotyping (19).

Exclusion criteria were prolonged compromised immunity because of recent chemotherapy, the presence of human immunodeficiency virus (HIV) infection with a CD4 count less than 240, or prolonged use of prednisolone at a dose of 60 mg per day); pregnancy; use of antibiotics other than for treatment of *C. difficile* infection at baseline; admission to an intensive care unit; or need for vasopressor medication.

**Treatments**

Patients received an abbreviated regimen of vancomycin (500 mg orally four times per day for 4 or 5 days), followed by bowel lavage with 4 liters of macrogol solution (Klean-Prep) on the last day of antibiotic treatment and the infusion of a suspension of donor feces through a nasoduodenal tube the next day; a standard vancomycin regimen (500 mg orally four times per day for 14 days); or a standard vancomycin regimen with bowel lavage at day 4 or 5. Patients in whom recurrent *C. difficile* infection developed after the first donor feces infusion were given a second infusion with feces from a different donor. Patients in whom antibiotic therapy failed were offered treatment with donor feces off protocol.
Infusion of donor feces for C. difficile

Infusion of donor feces
Donors (<60 years of age) were volunteers who were initially screened using a questionnaire addressing risk factors for potentially transmittable diseases. Donor feces were screened for parasites (including Blastocystis hominis and Dientamoeba fragilis), C. difficile, and enteropathogenic bacteria. Blood was screened for antibodies to HIV; human T-cell lymphotropic virus types 1 and 2; hepatitis A, B, and C; cytomegalovirus, Epstein-Barr virus, Treponema pallidum, Strongyloides stercoralis; and Entamoeba histolytica. A donor pool was created, and screening was repeated every 4 months. Before donation, another questionnaire was used to screen for recent illnesses.

Feces were collected by the donor on the day of infusion and immediately transported to the hospital. Feces were diluted with 500 ml of sterile saline (0.9%). This solution was stirred, and the supernatant strained and poured in a sterile bottle. Within 6 hours after collection of feces by the donor, the solution was infused through a nasoduodenal tube (2-3 minutes per 50 ml). The tube was removed 30 minutes after the infusion, and patients were monitored for 2 hours. For patients who had been admitted at referring hospitals, the donor feces solution was produced at the study center and immediately transported and infused by a study physician.

Outcomes
The primary end point was cure without relapse after 10 weeks after the initiation of therapy. For patients in the infusion group who required a second infusion of donor feces, follow up was extended to 10 weeks after the second infusion. The secondary end point was cure without relapse after 5 weeks. Cure was defined as an absence of diarrhea or persistent diarrhea that could be explained by other causes with three consecutive negative stool tests for C. difficile toxin. Relapse was defined as diarrhea with a positive stool test for C. difficile. An adjudication committee whose members were unaware of study-group assignments decided which patients were cured.

Patients kept a stool diary and were questioned about stool frequency and consistency, medication use, and adverse effects at days 7, 14, 21, 35, and 70 after the initiation of vancomycin. Stool tests for C. difficile toxin were performed in a central laboratory (Premier Toxins A&B, Meridian Bioscience) on days 14, 21, 35, and 70 and whenever diarrhea occurred.
Analysis of fecal microbiota

We analyzed the fecal microbiota for bacterial diversity by extracting DNA from samples from patients before and after donor-feces infusion and from the respective donors samples (20). We then characterized 16S ribosomal RNA gene amplicons using the Human Intestinal Tract Chip (HITChip), a phylogenetic microarray, as described previously (21). We estimated the diversity of the bacterial communities before and after donor-feces infusion using Simpson’s Reciprocal Index of diversity (22), on a scale ranging from 1 to 250, with higher values indicating greater diversity.

Statistical analysis

The objective was to determine the superiority of donor-feces infusion, as compared with vancomycin, both without and with bowel lavage. A cure rate of 90% for donor feces infusion (13;14) and 60% for antibiotic therapy (2;6) was assumed. Per group, 38 patients were needed to achieve a power of 80% to detect a difference between groups with a one-sided level of significance of 0.025. To account for drop-outs, we planned to enroll 40 patients per group. All analysis were performed on a modified intention-to-treat basis with the exclusion of one patient who required high-dose prednisolone treatment after randomization but before the study treatment was initiated. Differences in cure rates were assessed with Fisher’s exact probability test. Since the trial was terminated early according to the Haybittle-Peto’s rule (i.e. P < 0.001 for the primary end point), rate ratios for the primary end point (overall cure) were calculated with their exact 99.9% confidence interval.

On the basis of Simpson’s Reciprocal Index of diversity (22), the statistical significance of a change in microbiota diversity was assessed with the use of a paired samples Student t-test. A principal component analysis was performed on profiles derived from the HITChip phylogenetic microarray (21). Wilcoxon signed-rank tests were performed with the application of the Benjamini-Hochberg approach to determine microbial groups that were significantly different in matched pairs of fecal samples obtained from patients before and after infusion (23).
Results

Patients and termination of the trial

From January 2008 through April 2010, a total of 43 patients were randomly assigned to receive donor-feces infusion (17 patients), vancomycin (13), or vancomycin and bowel lavage (13). Initially, the inclusion of 40 patients per study group was planned. Because most patients in both control groups had a relapse, the data and safety monitoring board performed the interim efficacy analysis and advised termination of the trial, as described in the Supplementary Appendix. At that time, 43 patients were included, with one of the, subsequently excluded from further analysis (Table 1 and Fig.1). In 39 patients, a positive toxin test before inclusion was confirmed by a positive *C. difficile* culture. PCR ribotyping was performed on strains obtained from 34 patients (see the Supplementary Appendix).

Forty-one patients completed the study protocol. One patient in the vancomycin group was discharged home from the hospital after the initiation of vancomycin. At home, the patient decided to discontinue all medication because of severe heart failure and chronic obstructive pulmonary disease and died 13 days after randomization, without providing data on response. In the intention-to-treat analysis, vancomycin therapy was considered to have failed in this patient. Another patient in the infusion group required high-dose prednisolone because of a rapid decrease in renal graft function. The patient had received a renal transplant from an unrelated donor 11 months before study enrollment and graft dysfunction was noted immediately after randomization but before the study treatment was initiated. At that time, the nephrologist objected to treatment with donor feces. The patient was treated with vancomycin for 45 days, had a recurrence 41 days after cessation of vancomycin, and was subsequently cured by donor-feces infusion. This patient was excluded from the analysis because of a clinically driven protocol deviation, which meant that the patient’s response to treatment could not be evaluated.
## Table 1. Baseline Demographic and Clinical Characteristics of the Patients*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Donor-Feces Infusion (N=16)</th>
<th>Vancomycin Only (N=13)</th>
<th>Vancomycin and Bowel Lavage (N=13)</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age – yr</td>
<td>73±13</td>
<td>66±14</td>
<td>69±16</td>
<td>0.39</td>
</tr>
<tr>
<td>Body Mass Index‡</td>
<td>22±3</td>
<td>22±4</td>
<td>24±4</td>
<td>0.41</td>
</tr>
<tr>
<td>Female sex - no. (%)</td>
<td>8 (50)</td>
<td>7 (54)</td>
<td>3 (23)</td>
<td>0.22</td>
</tr>
<tr>
<td>Karnofsky performance status§</td>
<td>50±18</td>
<td>50±17</td>
<td>56±21</td>
<td>0.62</td>
</tr>
<tr>
<td>Median Charlson comorbidity index (range)-score¶</td>
<td>3 (0-4)</td>
<td>1.0 (0-8)</td>
<td>1.0 (0-6)</td>
<td>0.53</td>
</tr>
<tr>
<td>Median recurrences of CDI (range)-no.</td>
<td>3 (1-5)</td>
<td>3 (1-4)</td>
<td>2 (1-9)</td>
<td>0.69</td>
</tr>
<tr>
<td>Prior failure on vancomycin taper schedule – no. (%)</td>
<td>10 (63)</td>
<td>8 (62)</td>
<td>6 (46)</td>
<td>0.63</td>
</tr>
<tr>
<td>Previous failure of tapered vancomycin therapy - no. (%)</td>
<td>16 (100)</td>
<td>12 (92)</td>
<td>13 (100)</td>
<td>0.62</td>
</tr>
<tr>
<td>Hospital-acquired CDI infection - no. (%)</td>
<td>10 (63)</td>
<td>6 (46)</td>
<td>10 (77)</td>
<td>0.27</td>
</tr>
<tr>
<td>Admitted to a hospital at study inclusion - no. (%)</td>
<td>5 (31)</td>
<td>4 (31)</td>
<td>4 (31)</td>
<td>1.00</td>
</tr>
<tr>
<td>Days of antibiotic use for CDI since first diagnosis-no.</td>
<td></td>
<td>63±41</td>
<td>51±27</td>
<td>49±38</td>
</tr>
<tr>
<td>Use of proton-pump inhibitor – no. (%)</td>
<td>13 (81)</td>
<td>10 (77)</td>
<td>11 (85)</td>
<td>0.88</td>
</tr>
<tr>
<td>ICU admission in preceding month - no. (%)</td>
<td>1 (6)</td>
<td>0 (0)</td>
<td>1 (8)</td>
<td>1.00</td>
</tr>
<tr>
<td>Feeding tube present - no. (%)</td>
<td>3 (19)</td>
<td>2 (15)</td>
<td>2 (15)</td>
<td>0.96</td>
</tr>
<tr>
<td>Median stool frequency per 24 hrs (range)-no.</td>
<td>5 (3-20)</td>
<td>5 (3-12)</td>
<td>5 (3-10)</td>
<td>0.72</td>
</tr>
<tr>
<td>Leukocyte count– per mm±**</td>
<td>8,000</td>
<td>8,100</td>
<td>6,500</td>
<td>0.39</td>
</tr>
<tr>
<td>Range</td>
<td>4,000-15,000</td>
<td>4,000-23,000</td>
<td>3,000-14,000</td>
<td></td>
</tr>
<tr>
<td>Albumin - g/dl**</td>
<td>3.7±0.7</td>
<td>3.8±0.7</td>
<td>3.9±0.8</td>
<td>0.66</td>
</tr>
<tr>
<td>Median creatinine (range)-mg/dl**</td>
<td>1.3 (0.6-10.3)</td>
<td>1.0 (0.5-1.8)</td>
<td>0.9 (0.6-5.2)</td>
<td>0.26</td>
</tr>
<tr>
<td>Ribotype 027 in first sample- no. (%)††</td>
<td>3 (23)</td>
<td>1 (11)</td>
<td>0 (0)</td>
<td>0.28</td>
</tr>
</tbody>
</table>

* Plus-minus values are means ±SD. To convert the values for creatinine to micromoles per liter, multiply by 88.4. CDI denotes *Clostridium difficile* infection, and ICU intensive care unit.
† P values are for the overall comparisons among the three groups.
‡ The body-mass index is the weight in kilograms divided by the square of the height in meters.
§ The Karnofsky performance status ranges from 0 to 100, with higher scores indicating improved functional status.
¶ Scores on the Charlson comorbidity index range from 0 to 6 for each of 17 indicators, with higher scores indicating greater severity of illness.
|| Data were missing for one patient in the infusion group and one in the vancomycin-only group.
**Date were missing for one patient in the vancomycin-only group,
†† Data for ribotype 027 (a more virulent strain of *C. difficile*) were missing for three patients in the infusion group, four in the vancomycin-only group, and two in the group receiving vancomycin with bowel lavage.
Infusion of donor feces for *C. difficile*

**Figure 1.** Enrollment and Outcomes.
After randomization, one patient in infusion group required high-dose prednisolone because of a rapid decrease in renal-graft function, that was noted immediately after randomization but before the study treatment was initiated. This patient was excluded from the analysis. One patient in the vancomycin-only group died before the first stool sample could be tested for the presence of *Clostridium difficile* toxin.
**Donors**

Of 77 candidates, 25 donors were approved for donation (see the Supplementary Appendix for results of donor screening). Feces from 15 donors were used for 43 infusions in the infusion group and for patients who had a relapse after vancomycin treatment. A mean (±SD) of 141±71 g of feces was infused. The mean time from defecation to infusion was 3.1±1.9 hours.

**Study outcomes**

Of 16 patients in the infusion group, 13 (81%) were cured after their first infusion of donor feces. The 3 remaining patients received a second infusion with feces from a different donor at 14, 50, and 53 days after randomization; of these patients 2 were subsequently cured. Overall, donor feces cured 15 of 16 patients (94%). Resolution of infection occurred in 4 of 13 patients (31%) in the vancomycin-alone group and in 3 of 13 patients (23%) in the group receiving vancomycin with bowel lavage. Donor-feces infusion was statistically superior to both vancomycin regimens (P< 0.01 for both comparisions after the first infusion and P<0.001 for overall cure rates) (Fig. 2). The overall cure ratio of donor feces infusion was 3.05 relative to vancomycin alone (99.9% confidence interval [CI], 1.08 to 290.05) and 4.05 relative to vancomycin with bowel lavage (99.9% CI, 1.21 to 290.12).

The median time to recurrence was 23 days (range, 13-43 days) after initiation of vancomycin alone and 25 days (range, 18-70 days) after the initiation of vancomycin with bowel lavage. Fourteen patients who were cured reported having diarrhea during follow up; these episodes were short and self-limiting in 10 patients. Three patients had a pre-existent defecation frequency of ≥ 3 stools per day, a frequency that was markedly increased during episodes with *C. difficile* infection and returned to normal after donor feces infusion. In these patients, toxin tests were repeatedly negative and there was no clinical suspicion of recurrence. One patient in the vancomycin-only group had persistent diarrhea, with repeatedly negative toxin tests; this patients was considered to have had a response, although there was clinical suspicion of recurrence.

Eighteen patients who had a relapse after initial antibiotic treatment received off-protocol donor-feces infusions; of these patients, 15 (83%) were cured. Eleven patients were cured after one donor-feces infusion, and 4 patients were cured after a second infusion.
Infusion of donor feces for C. difficile

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(99.9% CI, 1.21 to 290.12).

(range, 13 to 43) after the initiation of vancomycin alone and 25 days (range, 18 to 70) after the initiation of vancomycin with bowel lavage. Five weeks after the initiation of therapy, there was a recurrence of infection in 1 of 16 patients (6%) in the infusion group, 8 of 13 (62%) in the vancomycin-alone group, and 7 of 13 (54%) in the group receiving vancomycin with bowel lavage. Fourteen patients who were cured reported having diarrhea during follow-up; these episodes were short and self-limited in 10 patients. Three patients had a preexistent defecation frequency (≥3 stools per day), a frequency that was markedly increased during episodes with C. difficile infection and returned to normal after donor-feces infusion. In these patients, toxin tests were repeatedly negative, and there was no clinical suspicion of recurrence. One patient in the vancomycin-only group had persistent diarrhea, with repeatedly negative toxin tests; this patient was considered to have had a response, although there was clinical suspicion of recurrence.

Figure 2. Cure Rates for Recurrent Clostridium difficile infection without Relapse

Shown are the proportions of patients who were cured by the infusion of donor feces (first infusion and overall results), by standard vancomycin therapy, and by standard vancomycin therapy plus bowel lavage.

Adverse events

A complete description of adverse events is included in the Supplementary Appendix. Immediately after donor-feces infusion, most patients (94%) had diarrhea. In addition, cramping (31%) and belching (19%) were reported (Table 2). In all patients, these symptoms resolved within 3 hours. During follow-up, three patients who were treated with donor feces (19%) had constipation. No other adverse events related to study treatment were reported. The death of one patient from severe heart failure and chronic obstructive pulmonary disease in the vancomycin-only group was considered to be unrelated to the study drug.
Table 2. Adverse events in 16 Patients in the Infusion Group.*

<table>
<thead>
<tr>
<th>Adverse Events</th>
<th>Day of Infusion of Donor Feces</th>
<th>During Follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belching</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Nausea</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Vomiting</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Abdominal cramps</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Constipation</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>2 (associated with cramping)</td>
<td>0</td>
</tr>
<tr>
<td>Infection</td>
<td>0</td>
<td>2†</td>
</tr>
<tr>
<td>Hospital admission</td>
<td>NA</td>
<td>1‡</td>
</tr>
<tr>
<td>Death</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other adverse events</td>
<td>1§</td>
<td>1‡</td>
</tr>
</tbody>
</table>

* Adverse events that were reported on the day of donor-feces infusion and those that were reported during follow-up are listed separately. NA denotes not applicable.
† During follow-up, one patient with recurrent urinary tract infections had a urinary tract infection for which antibiotics were prescribed. Another patient had fever during hemodialysis for which antibiotics were prescribed, cultures remained negative.
‡ On day 56, one patient with known choledocholithiasis was hospitalized for endoscopic retrograde cholangiopancreatography with stone extraction.
§ One patient with autonomic dysfunction had dizziness combined with diarrhea after donor-feces infusion.

Fecal microbiota

The Simpson’s Reciprocal Index of diversity of fecal microbiota obtained from nine patients who were evaluated before the donor-feces infusion was consistently low (mean, 57±26) and increased within 2 weeks after infusion to 179±42 (P<0.0001), becoming undistinguishable from the fecal microbiota diversity level of the donors (mean, 172±54) (Figure 3). In eight patients from whom samples were available, the diversity of fecal microbiota remained undistinguishable from that of the donor during follow-up.

In addition, a principal component analysis was performed on the phylogenetic microarray profiles of each sample. This unsupervised analysis showed that nearly 50% of the variation in the data was explained by the first two principal components, indicating a major shift in the patients’ microbiota after donor-feces infusion towards that of the donors (Fig. S2 in the Supplementary Appendix).
After donor feces-infusion, we observed quantitative changes in relevant groups of intestinal bacteria (P<0.05) (Table S2 in the Supplementary Appendix). These changes included increased numbers of Bacteroidetes species and of clostridium clusters IV and XIVa (by a factor of 2 to 4 for both groups) and decreased numbers of Proteobacteria (by a factor of up to 100).

**Figure 3.** Microbiota Diversity in Patients before and after Infusion of Donor Feces, as Compared with Diversity in Healthy Donors

Microbiota diversity is expressed as Simpson’s Reciprocal Index of diversity in fecal samples obtained from nine patients before and 14 days after the first infusion of donor feces, as compared with their donors. The index ranges from 1 to 250, with higher values indicating more diversity. The box-and-whisker plots indicate interquartile ranges (boxes), medians (dark horizontal lines in the boxes), and highest and lowest values (whiskers above and below the boxes).

**Discussion**

In this small, open-label, randomized, controlled trial, we found that the infusion of donor feces is a potential therapeutic strategy against recurrent *C. difficile* infection. Our study population of mainly elderly patients reflects the population in whom *C. difficile* infection develops in daily practice. However, we excluded three groups of patients at risk for recurrent *C. difficile* infection. Patients with prolonged immune deficiency were excluded to prevent the potential translocation of infused intestinal...
bacteria. Infectious complications were not observed after donor infusion in our study and have not been reported in the literature (15). Also, critically ill patients who were admitted to an Intensive Care Unit (ICU) were excluded. However, *C. difficile* infection in the ICU is associated with high death (24), and anecdotal reports have shown promising results of donor-feces infusion in critically ill patients (25,26). The third excluded group comprised patients required additional antibiotics to treat infections other than *C. difficile* infection because it seems reasonable to postpone donor-feces infusion until antibiotics can be stopped, enabling colonization of the bowel with healthy donor feces.

Although our study was designed for patients with any recurrence of *C. difficile* infection, only 8 of 43 patients were included after a first relapse, reflecting the reluctance of patients and physicians to choose donor-feces infusion at an early stage. The efficacy of antibiotic therapy decreases with subsequent recurrences, and it seems reasonable to initiate treatment with donor-feces infusion after the second or third relapse. It has yet to be established whether other promising treatment strategies, such as fidaxomycin or infusion of antibodies against clostridium toxins (3;27), are effective against recurrent *C. difficile* infection.

The power calculation of our study was based on the efficacy of vancomycin for a first recurrence of *C. difficile* infection. Because most patients had several relapses prior to inclusion (typically, after a failure of vancomycin treatment), the efficacy of vancomycin in our study was considerably lower than expected, which probably contributed to the findings of a difference between study groups. At study termination, 16 patients had been treated with donor-feces infusion. The success rate of donor-feces infusion was extended off protocol in another 18 patients who had initially been assigned to receive antibiotic therapy. A prolonged tapering schedule of vancomycin may be prescribed for recurrent *C. difficile* infection and was not incorporated in the trial for practical reasons. This may be a limitation of our study, although 56% of the patients were unsuccessfully treated with prolonged and tapering vancomycin schedules before inclusion.

Several questions remain unanswered. The optimal protocol for donor-feces infusion is unknown. We pretreated patients with vancomycin and bowel lavage, following a protocol that was effective in previously published case series (15;28). Bowel lavage
was incorporated to reduce the pathogenic bowel content, facilitating colonization of healthy donor microbiota. Whether bowel lavage indeed contributes to the efficacy of donor-feces infusion is not known (29). However, the possibility that bowel lavage itself cures *C. difficile* is unlikely, since no benefit was seen in the second control arm, in whom vancomycin was combined with bowel lavage. Furthermore, the amount of feces required and the optimal route of infusion (nasoduodenal tube, enema, or colonoscopy) are unknown since the literature reports many different treatment protocols (15;18;30). In our study, infusion of a relatively large amount of feces through a nasoduodenal tube had an acceptable adverse-event profile and was logistically manageable.

The mechanism underlying the efficacy of donor-feces infusion is probably the reestablishment of the normal microbiota as a host-defense against *C. difficile* (31). Changes in the gut bacterial phyla Firmicutes and Bacteroidetes were associated with *C. difficile* infection (31;32). We found that the fecal microbiota in patients with *C. difficile* infection had a reduced bacterial diversity, extending previous observations (12). Infusion of donor-feces resulted in improvement in the microbial diversity, which persisted over time. Also, there was an increase in Bacteroidetes species and clostridium clusters IV and XIVa (Firmicutes), whereas Proteobacteria species decreased.

In conclusion, in patients with recurrent *C. difficile* infection, this study showed increased efficacy for the infusion of donor feces, as compared with vancomycin therapy. In particular, patients with multiple relapses of *C. difficile* infection benefited from this unconventional approach.
Acknowledgments

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**References**

Supplementary appendix to
Duodenal infusion of donor feces for recurrent Clostridium difficile

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Appendix A: Complete description of methods

Trial design
This was an open label randomized trial comparing donor feces infusion to 14 days of vancomycin treatment for recurrent *C. difficile* infection. Patients were randomly allocated at a 1:1:1 ratio to three treatment options: (1) donor feces infusion, preceded by 4 days vancomycin and bowel lavage by 4 liters macrogol solution; (2) 14-day vancomycin, and (3) 14-day vancomycin with bowel lavage. The latter option was incorporated to exclude the possibility that the beneficial effect of donor feces infusion could be attributed to the bowel lavage. To achieve adequate allocation concealment, each patient was randomized by applying automated biased coin minimization in ALEA with stratification for hospitalization status (clinical or outpatient) and the number of previous recurrences (1, 2, >2). The coin bias factor was set at 3, the bias coin lower threshold at 2. Study physicians at the coordinating center in charge of randomization were unaware of the model specifications used.

The study was conducted from January 2008 to August 2010 at the Academic Medical Center in Amsterdam, the Netherlands. The study was announced on a national level enabling physicians from other hospitals in the Netherlands to refer patients for participation. Patients who were admitted in referring hospitals were visited, included and randomized by the study physicians. All participants provided written informed consent prior to randomization. A data safety monitoring board consisting of an internist and a biostatistician monitored the trial on an ongoing basis for patient safety. The research protocol was approved by the Ethics Committee of the Academic Medical Center. The study was registered at the Dutch trial register, NTR1177 (The FECAL trial, Fecal therapy to Eliminate *Clostridium difficile* Associated Longstanding diarrhea).

The study was designed by MN, EK, JB, PS, MD, and JK. The data were gathered by EvN, AV, SF, EZ, and CV. The data were analyzed by EvN, SF, EZ, WdeV, JT, MD, and JK. All authors vouch for the data and the analysis. The paper was written by EvN, WdeV, EK, MD and JK. The initial version of the manuscript was written by EvN and JK. All authors contributed to the manuscript. EvN, MD and JK decided to publish the paper. There were no agreements concerning confidentiality of the data between the sponsor and the authors.
**Study population**

Patients (≥18 years) with a life expectancy ≥ 3 months, and a microbiologically confirmed relapse of *C. difficile* infection after at least one course of adequate antibiotic therapy (≥ 10 days of vancomycin ≥ 125 mg q.i.d., or ≥ 10 days metronidazole 500 mg t.i.d.) were included. *C. difficile* infection was defined as (i) diarrhea (≥ 3 loose or watery stools per day for at least 2 consecutive days, or ≥ 8 loose stools in 48 hours) and (ii) a positive *C. difficile* toxin stool test. Toxin stool tests of patients from referring hospitals were repeated in the central laboratory at the Academic Medical Center in Amsterdam, The Netherlands. The Meridian A/B toxin premier test was used. All follow up samples were scheduled to be performed in the central laboratory at the AMC. Available isolates were further investigated by PCR-ribotyping (1). Exclusion criteria were an (expected) prolonged compromised immunity (due to recent chemotherapy, Human Immunodeficiency Virus (HIV) infection with a CD4 count < 240, or prolonged use of prednisolone ≥ 60 mg per day), pregnancy, use of antibiotics other than for *C. difficile* infection at the day of inclusion, admission to an Intensive Care Unit or need for vasopressive medication for maintenance of normal blood pressure.

**Treatments**

Patients received vancomycin 500 mg orally q.i.d. for four or five days, followed by bowel lavage with 4 liters macrogol solution (Klean-Prep®) on the last day of antibiotic treatment and infusion of fresh donor feces suspension through a nasoduodenal tube the next day; or vancomycin 500 mg orally q.i.d. for 14 days; or vancomycin 500 mg orally q.i.d. for 14 days with bowel lavage using 4 liters macrogol solution (Klean-Prep®) at day four or five. Patients who developed recurrent *C. difficile* infection following the first infusion with donor feces were given a second infusion with donor feces solution from a different donor. Patients who failed on antibiotic therapy were offered treatment with donor feces infusion off protocol.
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Donor feces infusion

Selection of donors

Donors (<60 years) were volunteers employed at our hospital without direct patient contact, or healthy blood donors from outside the hospital, or relatives of patients. Potential donors were not allowed to perform clinical work, which could increase their chance of contracting Clostridium difficile between screening and feces donation. Donors were not paid for donation. Candidates had to fill out a questionnaire (see below). The answers were evaluated and discussed with the donor to clarify if there was a potential risk for transmittable diseases. Reasons for exclusion of candidates were: age > 60 years; behaviour associated with an increased risk for (contracting) infectious diseases in the phase between screening and donation of feces (such as a recent visit to a tropical area in the last three months, risky sexual behaviour defined as a new sexual contact in the last six months, recent needle stick accident, receiving blood products, or getting a tattoo); any gastrointestinal illness or gastrointestinal complaints (abdominal discomfort, regularly loose stools, or constipation); a family history of intestinal cancer or inflammatory bowel disease; a general illness or use of medication that could be excreted in feces and pose a potential risk for recipients.

Following approval of the questionnaire, blood and feces samples of candidates were screened for potentially transmittable diseases. Donor feces were screened for parasites (including Blastocystis hominis and Dientamoeba fragilis), Clostridium difficile, and enteropathogenic bacteria (Salmonella, Shigella, Yersinia enterocolitica and Campylobacter species). Blood was screened for antibodies to Human Immunodeficiency Virus (HIV); Human T-lymphotropic virus Type I and II (HTLV-1 and II); Hepatitis A, B, C; Cytomegalovirus, Epstein-Barr virus, Strongyloides stercoralis; and Entamoeba histolytica. The specific tests are listed in table S1. If donors tested positive for one of the above mentioned pathogens, they were excluded. A resolved EBV or CMV infection was not an exclusion criterion, if the patient who was scheduled to receive the donor feces had suffered from the same infections. If a donor had antibodies against Hepatitis A (IgG), but was IgM negative and did not visit a tropical country in the past six months, he or she was not considered at risk for Hepatitis A and therefore not
excluded. Donors with a resolved Hepatitis B virus infection were excluded. Donors with Blastocystis hominis or Dientamoeba fragilis in their stool were excluded.

After approval, donors had to fill out a second questionnaire the day before donation (see below), concerning their stool frequency and pattern, general health, use of antibiotics and sexual behaviour. This was to screen for any acute (gastrointestinal) illness, newly contracted infectious diseases or other situations that could pose a risk for the patients. If donors answered yes on one of the questions of the second questionnaire, they were excluded until they had undergone complete new microbiological and serological screening.

A donor pool was created. Microbiological and serological screening was repeated every four months.

**Table S1.** Screening of blood and feces from candidate donors.

<table>
<thead>
<tr>
<th>Blood tests:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytomegalovirus (IgG and IgM)</td>
</tr>
<tr>
<td>Epstein-Barr Virus (VCA IgM, VCA IgG, VCA, antiEBNA)</td>
</tr>
<tr>
<td>Hepatitis A (total antibodies, and if positive also Hepatitis A IgM)</td>
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<tr>
<td>Hepatitis B (HbsAg, antiHbsAg)</td>
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<tr>
<td>Hepatitis C (anti HCV)</td>
</tr>
<tr>
<td>HIV-1 and HIV-2 (Combined HIV Antigen/Antibody test)</td>
</tr>
<tr>
<td>Human T-lymphotropic virus types I and II (HTLV) (antibodies)</td>
</tr>
<tr>
<td>Treponema pallidum (TPHA)</td>
</tr>
<tr>
<td>Entamoeba histolytica (agglutination and dipstick test)</td>
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<tr>
<td>Strongyloides stercoralis (ELISA)</td>
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<table>
<thead>
<tr>
<th>Fecal tests:</th>
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<tr>
<td>Bacteriological evaluation by local standards</td>
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<tr>
<td>Parasitological evaluation by local standards (triple feces test)</td>
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<tr>
<td>Test for Clostridium difficile (toxin ELISA and culture)</td>
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</table>

**Preparation of donor feces solution**

Feces were collected by the donor on the day of infusion and immediately transported to the hospital in a clean closed plastic container. For patients admitted in referring hospitals, donor feces solution was prepared at the study center (Academic Medical Center, Amsterdam) and immediately transported and infused by a study physician.

The donor feces solution was prepared in a laminar flow cabinet under semi-sterile conditions by one of the study physicians. Feces were weighed, and processing proceeded if > 50 gram was available. Feces were diluted with 500 cc sterile saline (NaCl 0.9%).
The feces were poured in a container with saline (NaCl 0.9%), approximately 100 cc at a time, and stirred with spatulas or a small rudder. The upper part (“supernatant”) of stirred feces was poured in a funnel, in which two unfolded gauzes (10x10 cm) served as a sieve and the solution was collected in a bottle that was closed after filling. This procedure was repeated until all saline was dissolved and a 500 cc bottle was filled.

**Preparation of the patient prior to infusion**

Patients were treated with vancomycin orally 500 q.i.d. 4 or 5 days before infusion of donor feces. Vancomycin was discontinued on the day of donor feces infusion. Bowel lavage using a standard four liter macrogol electrolyte suspension was followed by a light meal one day before donor feces infusion. Some patients did not succeed in drinking 4 liters, but all patients took at least 3 liters macrogol solution before donor feces infusion. On the day of donor feces infusion, patients were sober and a nasoduodenal tube (which fitted on a 50 cc luer-lock syringe) was placed using an electromagnetic sensing device (Cortrak<sup>™</sup>)(2), or through duodenoscopy. The position of the tube was confirmed by X-ray.

**Infusion of donor feces solution**

The donor feces solution was infused slowly with a 50 cc syringe (approximately 30 seconds per syringe) through the nasoduodenal tube. The first 4 or 5 syringes were infused in about 10 minutes. After a break of 10 minutes, the remaining 5 syringes were infused. Patients were allowed to drink during the procedure (to set them at ease). The tube was flushed with tap water after infusing the donor feces suspension, and left in situ for at least 30 minutes after infusion. Immediately after removal of the tube, lemonade was offered to the patient. Patients were clinically monitored for two hours. Patients were advised to visit the toilet before going home, because most patients had loose stools after infusion of donor feces solution.
Outcomes
The primary endpoint was cure without relapse after 10 weeks of initiation of therapy. For patients randomized to donor feces infusion who required a second donor feces infusion, follow up was extended to 10 weeks after the second infusion. The secondary endpoint was cure without relapse after 5 weeks. Cure was defined as absence of diarrhea, or persisting diarrhea explained by other causes with 3 consecutive negative stool toxin tests. Relapse was defined as diarrhea with a positive C. difficile toxin stool test. An adjudication committee blinded to treatment allocation decided which patients were cured without a relapse. The adjudication committee consisted of two internists: MM Levi, MD PhD and H Büller, MD PhD.

Patients kept a stool diary, and were questioned about stool frequency and consistency, medication use and adverse effects at day 7, 14, 21, 35 and 70 after initiation of vancomycin. C. difficile toxin stool tests were performed in a central laboratory (Meridian A/B toxin premier test) at day 14, 21, 35, and 70, and whenever diarrhea occurred.

Analysis of fecal microbiota
In available samples from patients before and after donor feces infusion, as well as the respective samples from the donors, the fecal microbiota was analyzed for bacterial diversity by extracting DNA (3), followed by the characterization of 16S rRNA gene amplicons using the Human Intestinal Tract Chip (HITChip), a phylogenetic microarray, as described previously (4).

DNA was isolated from fecal samples by mechanical disruption and subsequently used for microbiota diversity analysis using the Human Intestinal Tract Chip (HITChip) (4). The HITChip is a custom made Agilent-based microarray that enables studying the GI tract microbiota at high spatio-temporal resolution, and combines the power of 16S rRNA-based phylogenetic fingerprinting and relative quantification from phylum to species level for all currently known GI tract microbes. In short, 16S rRNA genes were PCR amplified using the fecal DNA samples as targets followed by in vitro transcription. After labeling with either Cy3 or Cy5 the samples were hybridized to the microarrays for 16h followed by washing and drying of the microarrays. Data were extracted from microarray images using the Agilent Feature Extraction software,
version 7.5 (www.agilent.com). Data were normalized using a set of R based scripts (http://www.r-project.org/), microarrays were analyzed in a custom designed relational database which runs under MySQL database management system (http://www.mysql.com/) using a series of custom R scripts as previously described\(^4\). The diversity of the microbiota expressed as Simpson index of the hybridization profiles on the HITChip. The Simpson’s reciprocal index of diversity (1/D) was calculated using the equation \(\lambda = 1/\Sigma Pi^2\) where \(Pi\) is the proportion of each probe signal compared to the total HITChip hybridization signal. Student t-tests were used to determine the significance of differences between microbiota diversities.

**Statistical analysis**

The objective was to determine superiority of the treatment with feces compared to the treatment with vancomycin, both without and with bowel lavage. Based on previous data, a cure rate of 90% for donor feces infusion \((5;6)\) and 60% for conventional antibiotic therapy \((7;8)\) was assumed. It was calculated that 38 patients per group were needed to achieve a power of 80% to detect a difference between the donor feces infusion group and each antibiotic therapy group, using two continuity corrected Chi-square tests with one-sided 0.025 levels of significance. To account for 5% drop-out, 40 patients per group were to be included, or 120 patients overall. Analysis was performed on an intention-to-treat basis. Differences in cure rates were assessed with Fisher’s exact probability test. As the trial had been terminated early according to Haybittle-Peto’s rule (i.e. with a P-value < 0.001 for the primary endpoint), rate ratios for the primary endpoint (overall cure) were calculated with their (exact) 99.9% confidence interval (CI).

Descriptive data are reported as means ± standard deviation or median with range depending on distributional properties, in case of continuous data based on Kolmogorov-Smirnov tests. Depending on distributional properties, statistical significance of differences between groups at baseline was assessed with analysis of variance (e.g. age) or Kruskal-Wallis tests (e.g. leukocyte count) for continuous data and with Fisher’s exact test (e.g. ICU admission) or Chi-square tests (e.g. sex) for categorical data, with a 0.05 two-sided significance level.
The diversity of the bacterial communities before and after donor feces infusion was estimated through Simpson’s Reciprocal Index of diversity (9), with statistical significance of a change in diversity assessed with a paired samples Student’s t-test. Multivariate statistical software Canoco 4.5 for Windows (10) (Biometris, Plant Research International, Wageningen, The Netherlands) was used to perform a Principal Component Analysis (PCA) on log transformed probe signal intensity profiles derived from the HITChip phylogenetic microarray (4). Wilcoxon signed-rank tests were performed - while correcting for false discovery rate using the Benjamini-Hochberg approach - to determine microbial groups that are significantly different in matched pairs of fecal samples from patients before and after infusion (11).
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Appendix B: Questionnaires used for screening of donors

1. Questionnaire for initial screening of donors, used in the FECAL trial

1. What is your sex?
2. What is your weight?
3. What is your height?
4. Have you ever been rejected as a (blood?) donor? If yes, why?
5. Have you ever donated blood? If yes, when?
6. Have you ever visited a medical specialist? If yes when, and for what reason?
7. Have you ever been tested for diabetes? If yes, what was the result?
8. Has Creuzfeldt Jakob’s disease ever occurred in your family?
9. Were you born in a country outside Europe, or have you ever resided in a country outside Europe for more than 5 years? If yes, when and where?
10. Were you a resident of the United Kingdom between 1980 and 1996 for 6 months or?
11. Do you have a profession that is associated with an elevated risk for blood-transmittable diseases? (e.g. daily contact with patients or inmates)
12. Have you ever had a “blood-incident” (e.g., an injury from a needle or another blood-stained object from someone else?). If yes, when?
13. Have you ever received blood products? If yes, when?
14. Have you ever used drugs intravenously?
15. Have you ever sniffed drugs?
16. Have you ever had a tattoo? If yes, when and in which country was the tattoo placed?
17. Have you ever had a piercing/earrings? If yes, when and in which country were the piercing/earrings placed?
18. Have you ever had acupuncture? If yes, when and in which country?
19. Have you ever undergone treatment with growth hormone?
20. Have you ever received a tissue transplantation? (e.g. cornea)
21. Have you undergone a hair transplantation?
22. Have you ever had an operation or undergone clinical treatment with poor hygienical conditions (e.g. in a developing country)? If yes, when and where?
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23. Have you been to a tropical country in the last two years? If yes, where and when?

24. Have you ever had malaria? If yes, in what year?

25. Have you ever had a rare infectious disease? (e.g., Trypanosomiasis, Tuberculosis, Herpes). If yes, which one?

26. Have you received vaccinations (not immunoglobulin’s)?
   - For Hepatitis A?
   - For Hepatitis B?
   - If yes, was your antibody response for hepatitis B vaccination measured and adequate?

27. While visiting another country (for work or vacation), have you ever had sexual contact with people originating from that country?
   - If yes, in what country?

28. Do you have a new sexual partner with whom you have commenced sexual relations within the last 12 months?

29. Have you ever had anonymous sexual contacts?

30. Have you ever had sexual contact with someone who uses IV drugs?

31. (for men) Have you ever had sexual contact with a man?

32. (for women) Have you ever had sexual contact with a bisexual or homosexual man?

33. In the last 12 months, have you had receptive anal sex with a new partner?

34. Have you ever had sexual contact with someone who received money from you for this contact?

35. Have you ever had sexual contact with someone who turned out to be infected with HIV, HTLV, Hepatitis, or Syphilis?

36. Have you ever had a sexually transmittable disease?

37. Have you ever worked as a prostitute?

38. Are certain inheritable diseases more prevalent in your family?
   - If yes, which one?

39. Do you have regular bowel movements?

40. On average, how many bowel movements do you have in a day? ......times

41. Are you, more than average, bothered by flatulence?
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42 Have you ever been treated for an intestinal infection?
43 Do you have a chronic intestinal condition? (e.g. Crohn’s disease or Ulcerative Colitis)
44 Do you ever use any products with the sole purpose of changing or influencing your defecation frequency?
45 If yes how often?
46 Do you often use products? (e.g. prunes, fibres or probiotics drinks)
47 If yes which products? (e.g. prunes, fibres or probiotics drinks)
48 Do you often (more than once a month) have difficulty defecating? (hard stools)
49 Do you often have abdominal cramps?
50 Do you have abdominal cramps?
51 Do you have any family members with intestinal diseases? If yes, which ones?
52 Do you have any family members with intestinal cancer or polyps? If yes, in which relatives?
53 Have you used antibiotics in the past two months?
54 Have you used antibiotics in the last year? If yes, when? What antibiotics?
55 Have you ever had blood in your stools? If yes, were additional investigations performed? What were the results?
56 Have you had a fever in the past two weeks?
2. Questionnaire for donors, used one day before donation of feces

1 Have you developed diarrhea since the last screening? (diarrhea is defined as: >3 bowel movements per day, unformed stool, or > 8 bowel movements in 48 hours)
   - If yes, when?
   - If yes, how many bowel movements a day?
   - If yes, how many days?
   - If yes, did you have other complaints? (fever, abdominal or pain, nausea or vomiting)
   - If yes, is there a possible explanation? (were other people ill, did you eat something that might have been the cause of the problems?)

2 Have you been ill since your last screening?
   - If yes, did you have a fever?
   - If yes, where you jaundiced?
   - If yes, did you notice swollen lymph glands?
   - If yes, did you notice throat pain?

3 Have you used antibiotics since your last screening?

4 Have you gone abroad since your last screening?
   - If yes, where did you go?

5 Have you had a new sexual partner since your last screening?

6 Have you had homosexual sexual contacts since your last screening?

1* if diarrhea had occurred, donors could not participate until stool was tested and negative for bacterial and parasitological pathogens

2* if patients had been ill, they could not participate until a new screening was performed
Appendix C: Donors (results)

Results of donor screening

Seventy-seven potential donors completed the initial questionnaire. Seven were excluded from further screening of blood and feces. This was because they performed clinical work (2), were considered too old (1), had risky sexual behaviour (2) or had visited a tropical country in the months prior to screening (2). Seventy were screened following approval of their questionnaire, of them, 25 subjects were approved for donation. Feces from 15 different donors were eventually used for treatment of patients randomised to donor feces infusion, or off protocol treatment of patients who initially failed on antibiotic therapy. Forty-five donors were excluded after screening of feces and blood because of the following reasons: two had a positive stool C. difficile toxin test, 23 tested positive for Blastocystis hominis, 4 patients tested positive for Dientamoeba fragilis, 3 donors carried both Dientamoeba and Blastocystis, one had positive Strongyloides antibodies, 10 patients failed to collect feces or blood, and 2 donors moved (see figure 1). The donors that had a positive toxin C. difficile stool test were asymptomatic, and not treated. The donors that carried Blastocystis hominis and/or Dientamoeba fragilis were asymptomatic, and not treated. The donor that had serologic evidence of a Strongyloides infection reported a visit to the tropics 8 years ago. He was empirically treated with Ivermectin. Follow up serology was not performed.

Donors, donations and outcome of treatment per donor

Fifteen donors were used for donation. Their mean age was 44 years (SD 18.1). A total of 43 donations were performed (19 donations in patients randomised to donor feces infusion and 24 donations in patients who relapsed after vancomycin or vancomycin with bowel lavage and received donor feces infusion off protocol). Thirteen donations failed; these were given to patients as first infusion (10 of 34 first infusions failed), or as a second infusion (3 of 9 second infusions failed). Seven donors donated once, 3 donors donated twice, 1 donor donated 3 times, 1 donor donated 4 times, 1 donor donated 5 times and two donors donated 9 times (43 donations in total, mean of 2.9 per donor). Of the 7 donors that donated once, one was not
Infusion of donor feces for *C. difficile*

successful. All three donors that donated twice had one successful donation and one failure. The donor that donated three times had one successful donation. The donor that donated four times had three successful donations. The donor that donated 5 times had 2 successful donations. The two donors that donated 9 times had 8 and 7 successful donations respectively.

77 questionnaires were evaluated

7 persons were excluded from screening blood and feces
- 2 performed clinical work
- 1 was considered too old (> 60 years)
- 2 had riskful sexual behaviour in the last months
- 2 recently visited a tropical country

70 persons were allowed to be screened for blood and feces

45 persons were excluded
- 2 had positive *C. difficile* stool toxin test
- 23 carried *Blastocystis hominis* in stool
- 4 carried *Dientamoeba fragilis* in stool
- 3 carried both *Dientamoeba fragilis* and *Blastocystis hominis* in stool
- 1 had positive *Strongyloides stercoralis* antibodies
- 10 failed to collect feces
- 2 approved testing of blood and feces but moved

25 persons were suitable for donation

15 persons donated stool for donor feces infusion during the course of the study

*Figure S1.* Results of donor screening.
Appendix D: Ad-hoc decision to perform an interim analysis for efficacy

In the course of 2009 the coordinating team consisting of the principal investigator (JJK), the study coordinator (EvN), the chair of the department of infectious diseases (PS) and the study statistician (MGWD) became aware of an (unexpected) extremely low response rate in the two control arms, which seemed much lower than the 60% used in the sample size calculation. The principal investigator subsequently requested the data safety monitoring board (DSMB) for advice. The DSMB consisted of an internist (J. van der Meer, MD PhD) and a biostatistician (J.G.P. Tijssen, PhD), and was granted a mandate to perform a formal interim analysis for efficacy when at least 40 patients (one third of the anticipated total sample size) had a complete follow-up (12). This singular interim analysis for efficacy was unforeseen and the biostatistician of the DSMB decided to apply the Haybittle-Peto stopping boundary (p<0.001).

At the time of the interim analysis complete follow-up-data were available for 43 patients. In addition, results of donor feces infusion in 17 patients who initially failed on vancomycin or vancomycin with bowel lavage were available. In preparation of the interim analysis, data on disease status of the included 43 patients were offered for endpoint assessment to an independent adjudication committee (MM Levi, internist; H Büller, internist) that was blinded for treatment allocation. One patient who was randomized to treatment with donor feces infusion required a course of high dose prednisolone because of a rapid decrease of renal graft function that was noted immediately after randomization but before study treatment was initiated. At that time, the nephrologist objected to treatment with donor feces infusion. The patient was treated with vancomycin (which was prescribed during 45 days on request of the nephrologist) and developed a recurrence 41 days after stopping vancomycin. This patient was subsequently cured by donor feces infusion that was given according to the protocol. This patient was included in the interim analysis as a responder on the (delayed) treatment with donor feces. In the final intention to treat analysis, however, this patient was excluded. Following this endpoint assessment procedure, the biostatistician of the DSMB applied the Fisher’s exact test twice to compare the experimental arm with each control arm for the primary outcome. The full DSMB advised the principal investigator to put the trial on hold.
Appendix E: Adverse events

In patients treated with donor feces infusion (n=16), only mild adverse events were encountered. Immediately after infusion, most patients experienced diarrhea (94%). Furthermore, cramping (31%) and belching (19%) were present in some patients. One patient experienced nausea (6%) without vomiting, two patients experienced abdominal pain that was associated with cramping (13%) and one patient known with autonomic dysfunction experienced dizziness combined with diarrhea following donor feces infusion.

During follow up, 3 patients (19%) had constipation for which laxatives were prescribed to two patients. Three patients reported adverse effects that were considered unrelated to donor feces infusion: one patient was hospitalized for choledocholithiasis on day 56 for which ERCP was performed; one patient had fever during hemodialysis for which this patient received antibiotics; and one patient known with recurrent urinary tract infections experienced an urinary tract infection during follow up.

In vancomycin treated patients (n=13), few and only mild adverse events were encountered. One patient experienced dyspeptic complaints, and one patient had constipation for which laxatives were prescribed. Two adverse events were considered unrelated to study therapy: one patient died 13 days after randomization after discontinuation of all his medication for known severe heart failure and chronic obstructive pulmonary disease; another patient had increased pain due to known rheumatoid arthritis during follow up, for which additional analgesics were required.

In patients treated with vancomycin with bowel lavage (n=13), few and only mild adverse events were encountered. Two patients had constipation, for which one received oral laxatives. Two patients had other gastrointestinal complaints: one patient reported excess gas and the other persistent diarrhea. The latter patient was eventually diagnosed with celiac disease. One patient had a urinary tract infection on day 10 for which ciprofloxacine was given for four days.
Appendix F: Additional results of Microbiological testing

_Clostridium difficile toxin testing (ELISA)_

All patients had repeated positive toxin tests prior to inclusion, performed at the local microbiological laboratories. At inclusion in the study, a baseline sample of all patients was collected and (re)tested in the central microbiology laboratory at the AMC (Amsterdam, The Netherlands) with the Meridian Premier toxin A/B test. However, many of these samples were obtained after initiation of vancomycin at the referral hospital, and 19/43 baseline samples that were tested at the reference laboratory remained negative.

The follow up samples were collected by patients and transported to our central hospital on the day of planned follow up visits. If patients were unable to travel to our hospital for follow up visits, they were visited by a study physician who collected the samples. Tests were performed at 171 of 179 planned time points (96%). Of these tests, 168 of 171 (98%) were performed in the central laboratory.

_Culture_

Of 43 patients included in the study, _C. difficile_ was cultured from 39 patient stool samples collected before inclusion. Negative cultures were found in 2 patients belonging to the donor feces group, 1 patient in the vancomycin treated group and 1 patient in the vancomycin and bowel lavage treated group.

In 13 of 20 patients who failed after study treatment, a positive toxin test was confirmed by a positive culture. One patient died and therefore did not provide follow up samples. In 6 patients with diarrhea and positive tested feces after study treatment, cultures were negative. Failure was not confirmed by a positive culture in the only patient who failed after donor feces infusion (culture was also negative before study entry of this patient), in 2 of 8 patients who failed after vancomycin, and in 3 of 10 patients who failed after vancomycin with bowel lavage.
Infusion of donor feces for *C. difficile*

**PCR ribotyping**

Of 39 patients with a positive *C. difficile* feces culture before inclusion, thirty-four isolates were characterized at the Netherlands reference laboratory at Leiden University Medical Centre by PCR ribotyping and the presence of toxin genes. From five patients that were diagnosed in referring hospitals with a positive culture prior to inclusion, no isolate was sent to the Netherlands reference laboratory. PCR ribotyping was not repeatedly performed after recurrence of *Clostridium difficile* infection. In the donor feces group, PCR ribotyping was performed in 14 of 17 patients. Twelve *C. difficile* isolates were classified as: Type 027 (n=3), Type 001 (n=4), Type 006 (n=1), Type 016 (n=1), Type 023 (n=1), Type 087(n=1), and “no 027” (not further specified) (n=1). Two patients were infected with *C. difficile* from which the PCR ribotype was not present in the library of the reference laboratory at the LUMC.

In the vancomycin treated group, PCR ribotyping was performed in 9 of 13 patients. Nine *C. difficile* isolates were classified as: Type 027 (n=1), Type 002 (n=1), Type 018 (n=1), Type 021(n=1), Type 029 (n=2), and “no 027” (not further specified)(n=1). From one patient, no isolate was available prior to inclusion, but *C. difficile* PCR ribotype 228 was identified during follow up. One patient was infected with *C. difficile* of which the PCR ribotype was not present in the library of the reference laboratory at the LUMC.

In the vancomycin with bowel lavage group, ribotyping was performed in 11 of 13 patients. Nine *C. difficile* isolates were classified as: PCR Type 001 (n=2), Type 002 (n=1), Type 014 (n=3), Type 044 (n=1), Type 076 (n=1), and Type 122 (n=1). Two patients were infected with *C. difficile* of which the PCR ribotype was not present in the library of the reference laboratory of the LUMC.

The percentage of the more virulent *C. difficile* Type 027 (9%) in our study is high compared to the sentinel surveillance data from the National Reference Laboratory, collected in the period January 2008 and March 2010. *C. difficile* Types 001 and 014 predominated and Type 027 was found in 3% only (13). It is likely that this increased incidence in our patients group reflects the association of Type 027 with frequent relapsing CDI. The other more virulent *C. difficile* type 078 was not identified in patients in our study.
Appendix G: Analysis of fecal microbiota

Figure S2: Principal Component Analysis (PCA) of the microbiota of patients based on the HITChip microarray probe signals. Samples from the nine patients before (Pxb) and after (Pxa) infusion, and from their infused donor samples (Dx) are indicated with different symbols. The two first principal components (PC1 and PC2) and the percentage of variation they respectively explain are presented. Six patients (P1-P3, P5, P7, P9) were initially randomized to donor feces infusion. Three patients received donor feces infusion off-protocol: two patients (P4, P8) in the vancomycin with bowel lavage group and one patient (P6) in the vancomycin only group.
Infusion of donor feces for *C. difficile*

Table S2

<table>
<thead>
<tr>
<th>Phylum Phylum (Class) / Genus-like</th>
<th>Relative abundance (%±SD)</th>
<th>Donor Before</th>
<th>After</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteroidetes</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Allistipes et rel.</td>
<td>1.15±0.88</td>
<td>0.41±0.87</td>
<td>2.30±2.46</td>
<td>0.03</td>
</tr>
<tr>
<td>Bacteroides intestinalis et rel.</td>
<td>0.47±0.51</td>
<td>0.12±0.36</td>
<td>0.52±0.54</td>
<td>0.03</td>
</tr>
<tr>
<td>Bacteroides ovatus et rel.</td>
<td>0.46±0.37</td>
<td>0.30±0.91</td>
<td>0.91±0.94</td>
<td>0.03</td>
</tr>
<tr>
<td>Bacteroides plebeius et rel.</td>
<td>0.87±0.81</td>
<td>0.20±0.46</td>
<td>0.96±1.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Bacteroides splachnicius et rel.</td>
<td>0.44±0.31</td>
<td>0.32±0.78</td>
<td>0.90±1.14</td>
<td>0.04</td>
</tr>
<tr>
<td>Bacteroides uniformis et rel.</td>
<td>0.64±0.68</td>
<td>0.31±0.73</td>
<td>0.98±1.16</td>
<td>0.04</td>
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<tr>
<td>Bacteroides vulgatus et rel.</td>
<td>0.93±1.17</td>
<td>0.09±0.25</td>
<td>1.21±1.61</td>
<td>0.02</td>
</tr>
<tr>
<td>Parabacteroides distasonis et rel.</td>
<td>1.00±0.80</td>
<td>0.46±1.28</td>
<td>1.77±2.22</td>
<td>0.04</td>
</tr>
<tr>
<td>Prevotella ruminicola et rel.</td>
<td>0.15±0.09</td>
<td>0.16±0.49</td>
<td>0.34±0.31</td>
<td>0.04</td>
</tr>
<tr>
<td>Prevotella tannerae et rel.</td>
<td>0.83±0.76</td>
<td>0.25±0.74</td>
<td>0.78±0.87</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacilli</td>
<td>2.69±2.71</td>
<td>41.46±27.69</td>
<td>8.11±6.54</td>
<td>0.01</td>
</tr>
<tr>
<td>Aerococcus</td>
<td>0.00±0.00</td>
<td>0.06±0.09</td>
<td>0.01±0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Granulicatella</td>
<td>0.00±0.00</td>
<td>0.10±0.12</td>
<td>0.02±0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>Streptococcus mitis et rel.</td>
<td>0.75±0.78</td>
<td>8.84±6.72</td>
<td>2.23±2.13</td>
<td>0.04</td>
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<tr>
<td>Clostridium cluster IV</td>
<td>25.60±10.74</td>
<td>3.43±3.25</td>
<td>14.66±7.19</td>
<td>0.0001</td>
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<tr>
<td>Anaerotruncus colihominis et rel.</td>
<td>0.20±0.11</td>
<td>0.10±0.24</td>
<td>0.37±0.46</td>
<td>0.04</td>
</tr>
<tr>
<td>Clostridium cellulosi et rel.</td>
<td>0.73±0.42</td>
<td>0.13±0.23</td>
<td>1.01±1.07</td>
<td>0.04</td>
</tr>
<tr>
<td>Clostridium leptum et rel.</td>
<td>0.37±0.27</td>
<td>0.05±0.05</td>
<td>0.59±0.81</td>
<td>0.01</td>
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<tr>
<td>Faecalibacterium prausnitzii et rel.</td>
<td>13.62±8.68</td>
<td>0.89±2.42</td>
<td>3.44±2.99</td>
<td>0.04</td>
</tr>
<tr>
<td>Oscillospira guillermondii et rel.</td>
<td>3.25±3.47</td>
<td>0.15±0.13</td>
<td>1.95±3.55</td>
<td>0.03</td>
</tr>
<tr>
<td>Ruminococcus bromii et rel.</td>
<td>0.44±0.36</td>
<td>0.07±0.21</td>
<td>0.41±0.42</td>
<td>0.04</td>
</tr>
<tr>
<td>Ruminococcus callidus et rel.</td>
<td>1.72±1.41</td>
<td>0.02±0.03</td>
<td>0.77±1.23</td>
<td>0.02</td>
</tr>
<tr>
<td>Sporobacter termitidis et rel.</td>
<td>0.72±0.53</td>
<td>0.06±0.10</td>
<td>1.10±1.60</td>
<td>0.02</td>
</tr>
<tr>
<td>Subdoligranulum variable et rel.</td>
<td>2.59±1.40</td>
<td>0.26±0.30</td>
<td>3.00±3.66</td>
<td>0.02</td>
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<tr>
<td>Clostridium cluster XIVa</td>
<td>53.75±14.68</td>
<td>27.97±27.22</td>
<td>54.92±18.46</td>
<td>0.01</td>
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<tr>
<td>Anaerostipes caccae et rel.</td>
<td>2.59±1.15</td>
<td>1.26±2.96</td>
<td>1.96±1.23</td>
<td>0.04</td>
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<tr>
<td>Clostridium colinum et rel.</td>
<td>0.42±0.33</td>
<td>0.02±0.02</td>
<td>0.30±0.19</td>
<td>0.02</td>
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<tr>
<td>Clostridium sphenoidei et rel.</td>
<td>2.96±1.73</td>
<td>0.94±0.91</td>
<td>2.45±1.45</td>
<td>0.04</td>
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<tr>
<td>Eubacterium rectale et rel.</td>
<td>3.49±1.53</td>
<td>0.92±1.52</td>
<td>2.31±1.47</td>
<td>0.04</td>
</tr>
<tr>
<td>Eubacterium ventriosum et rel.</td>
<td>2.10±0.48</td>
<td>0.63±1.43</td>
<td>1.22±0.67</td>
<td>0.04</td>
</tr>
<tr>
<td>Lachnocabillus bovis et rel.</td>
<td>2.16±1.09</td>
<td>0.33±0.53</td>
<td>1.33±0.79</td>
<td>0.03</td>
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<tr>
<td>Ruminococcus lactaris et rel.</td>
<td>0.84±0.57</td>
<td>0.25±0.42</td>
<td>0.79±0.38</td>
<td>0.04</td>
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<tr>
<td>Ruminococcus obeum et rel.</td>
<td>9.68±5.13</td>
<td>4.34±6.13</td>
<td>13.40±7.46</td>
<td>0.03</td>
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<tr>
<td>Uncultured Clostridiales</td>
<td>2.93±3.66</td>
<td>0.02±0.02</td>
<td>1.85±2.26</td>
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<td>Uncultured Clostridiales II</td>
<td>0.91±0.84</td>
<td>0.02±0.02</td>
<td>1.00±1.05</td>
<td>0.02</td>
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<td><strong>Proteobacteria</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Enterobacter aerogenes et rel.</td>
<td>0.01±0.01</td>
<td>1.36±2.30</td>
<td>0.01±0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Klebsiella pneumoniae et rel.</td>
<td>0.00±0.00</td>
<td>0.96±1.26</td>
<td>0.01±0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Proteus et rel.</td>
<td>0.00±0.00</td>
<td>0.19±0.36</td>
<td>0.00±0.00</td>
<td>0.04</td>
</tr>
<tr>
<td>Vibrio</td>
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<td>0.06±0.05</td>
<td>0.00±0.00</td>
<td>0.02</td>
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<tr>
<td>Yersinia et rel.</td>
<td>0.00±0.00</td>
<td>0.27±0.44</td>
<td>0.00±0.01</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Bacterial groups that significantly change in relative abundance (%) in microbiota of patients following donor feces infusion. Ten matched pairs of fecal samples from patients before and after donor feces infusion were used for this analysis with fecal samples from their donors (N=9) as reference. Comparisons were done using the Wilcoxon signed-rank test corrected for false discovery rate using the Benjamini & Hochberg approach. Corrected p values<0.05 were considered significant. Bacterial groups at phylum and genus-like levels are included that are present at a relative abundance of >0.5% and >0.05%, respectively.
Chapter 4

References