Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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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 vouche 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.

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.

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

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^{TM})[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[3] 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].

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-t ransmittable 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-s tained 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?

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?

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?
If yes how often?
If yes which products? (e.g. prunes, fibres or probiotics drinks)

45 Do you often (more than once a month) have difficulty defecating? (hard stools)

46 Do you have haemorrhoids?

47 Do you often have abdominal cramps?

48 Do you have any family members with intestinal diseases? If yes, which ones?

49 Do you have any family members with intestinal cancer or polyps?
If yes, in which relatives?

50 Have you used antibiotics in the past two months?

51 Have you used antibiotics in the last year? If yes, when? What antibiotics?

52 Have you ever had blood in your stools? If yes, were additional investigations performed? What were the results?

53 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)

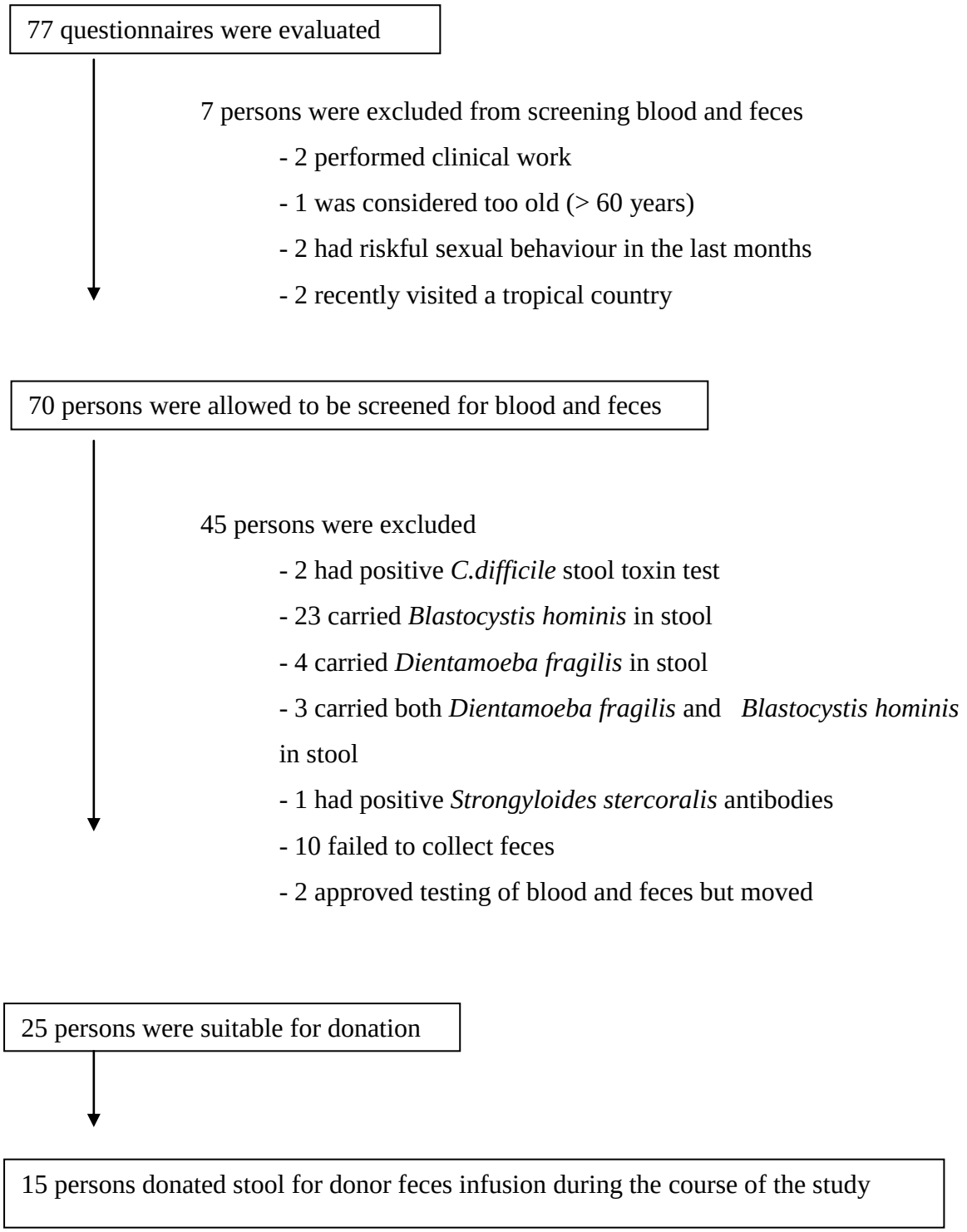
RESULTS OF DONOR SCREENING

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 riskful 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 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.

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[12]) had a complete follow-up. 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.

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[1]. 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.

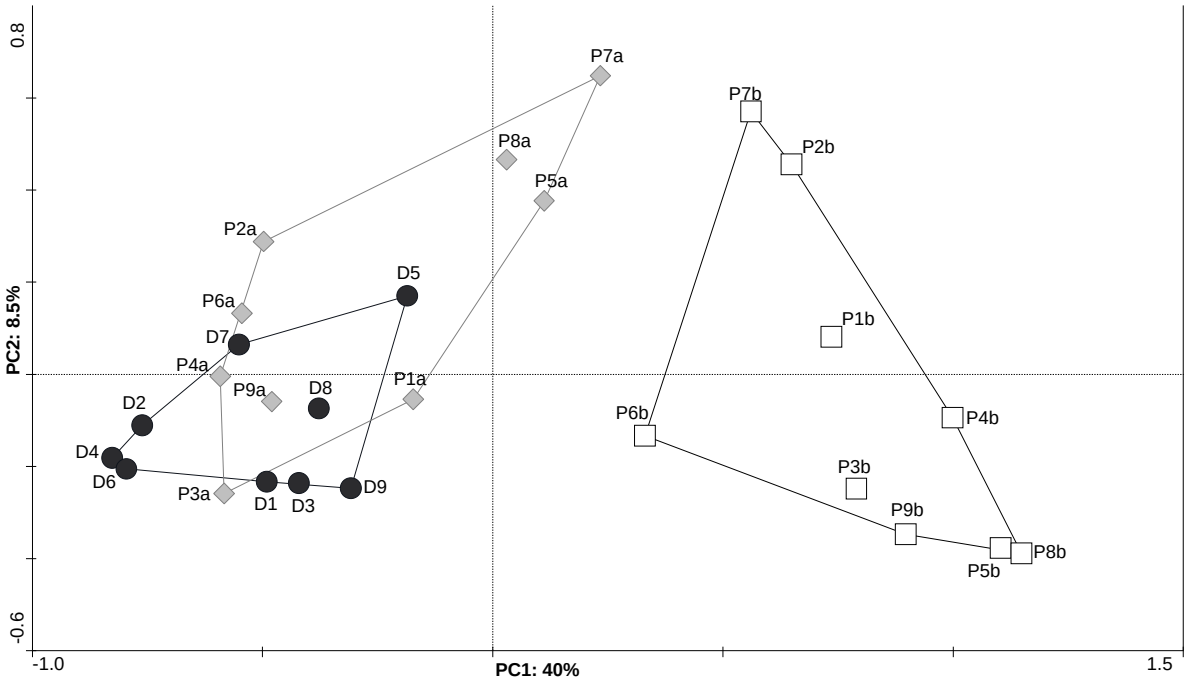


Table S2: Bacterial groups that significantly change in relative abundance (%) in microbiota of patients following donor feces infusion. Ten matched pairs of fecal samples from patients before and after donor feces infusion were used for this analysis with fecal samples from their donors (N=9) as reference. 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.

		Relative abundance (%±SD)			
Phylum	**Phylum (Class) /** Genus-like	**Donor**	**Before**	**After**	**p value**
Bacteroidetes	**Bacteroidetes**	10.56±8.29	5.27±9.04	13.69±14.42	0.04
	Allistipes et rel.	1.15±0.88	0.41±0.87	2.30±2.46	0.03
	Bacteroides intestinalis et rel.	0.47±0.51	0.12±0.36	0.52±0.54	0.03
	Bacteroides ovatus et rel.	0.46±0.37	0.30±0.91	0.91±0.94	0.03
	Bacteroides plebeius et rel.	0.87±0.81	0.20±0.46	0.96±1.02	0.04
	Bacteroides splachnicus et rel.	0.44±0.31	0.32±0.78	0.90±1.14	0.04
	Bacteroides uniformis et rel.	0.64±0.68	0.31±0.73	0.98±1.16	0.04
	Bacteroides vulgatus et rel.	0.93±1.17	0.09±0.25	1.21±1.61	0.02
	Parabacteroides distasonis et rel.	1.00±0.80	0.46±1.28	1.77±2.22	0.04
	Prevotella ruminicola et rel.	0.15±0.09	0.16±0.49	0.34±0.31	0.04
	Prevotella tannerae et rel.	0.83±0.76	0.25±0.74	0.78±0.87	0.03
Firmicutes	**Bacilli**	2.69±2.71	41.46±27.69	8.11±6.54	0.01
	Aerococcus	0.00±0.00	0.06±0.09	0.01±0.01	0.03
	Granulicatella	0.00±0.00	0.10±0.12	0.02±0.03	0.04
	Streptococcus mitis et rel.	0.75±0.78	8.84±6.72	2.23±2.13	0.04
	Clostridium cluster IV	25.60±10.74	3.43±3.25	14.66±7.19	0.0001
	Anaerotruncus colihominis et rel.	0.20±0.11	0.10±0.24	0.37±0.46	0.04
	Clostridium cellulosi et rel.	0.73±0.42	0.13±0.23	1.01±1.07	0.04
	Clostridium leptum et rel.	0.37±0.27	0.05±0.05	0.59±0.81	0.01
	Faecalibacterium prausnitzii et rel.	13.62±8.68	0.89±2.42	3.44±2.99	0.04
	Oscillospira guillermondii et rel.	3.25±3.47	0.15±0.13	1.95±3.55	0.03
	Ruminococcus bromii et rel.	0.44±0.36	0.07±0.21	0.41±0.42	0.04
	Ruminococcus callidus et rel.	1.72±1.41	0.02±0.03	0.77±1.23	0.02
	Sporobacter termitidis et rel.	0.72±0.53	0.06±0.10	1.10±1.60	0.02
	Subdoligranulum variable et rel.	2.59±1.40	0.26±0.30	3.00±3.66	0.02
	Clostridium cluster XIVa	53.75±14.68	27.97±27.22	54.92±18.46	0.01
	Anaerostipes caccae et rel.	2.59±1.15	1.26±2.96	1.96±1.23	0.04
	Clostridium colinum et rel.	0.42±0.33	0.02±0.02	0.30±0.19	0.02
	Clostridium sphenoides et rel.	2.96±1.73	0.94±0.91	2.45±1.45	0.04
	Eubacterium rectale et rel.	3.49±1.53	0.92±1.52	2.31±1.47	0.04
	Eubacterium ventriosum et rel.	2.10±0.48	0.63±1.43	1.22±0.67	0.04
	Lachnobacillus bovis et rel.	2.16±1.09	0.33±0.53	1.33±0.79	0.03
	Ruminococcus lactaris et rel.	0.84±0.57	0.25±0.42	0.79±0.38	0.04
	Ruminococcus obeum et rel.	9.68±5.13	4.34±6.13	13.40±7.46	0.03
	Uncultured Clostridiales	2.93±3.66	0.02±0.02	1.85±2.26	0.0005
	Uncultured Clostridiales II	0.91±0.84	0.02±0.02	1.00±1.05	0.02
Proteobacteria	*Enterobacter aerogenes* et rel.	0.01±0.01	1.36±2.30	0.01±0.01	0.02
	Klebisiella pneumoniae et rel.	0.00±0.00	0.96±1.26	0.01±0.01	0.02
	Proteus et rel.	0.00±0.00	0.19±0.36	0.00±0.00	0.04
	Vibrio	0.00±0.00	0.06±0.05	0.00±0.00	0.02
	Yersinia et rel.	0.00±0.00	0.27±0.44	0.00±0.01	0.03

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