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RESEARCH ARTICLE

Faecalibacterium prausnitzii increases following fecal microbiota transplantation in recurrent *Clostridioides difficile* infection

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Abstract

Objective

Fecal microbiota transplantation (FMT) is a highly effective treatment for *Clostridioides difficile* infection (CDI). However, the fecal transplant's causal components translating into clearance of the CDI are yet to be identified. The commensal bacteria *Faecalibacterium prausnitzii* may be of great interest in this context, since it is one of the most common species of the healthy gut microbiota and produces metabolites with anti-inflammatory properties. Although there is mounting evidence that *F. prausnitzii* is an important regulator of intestinal homeostasis, data about its role in CDI and FMT are relatively scarce.

Methods

Stool samples from patients with recurrent CDI were collected to investigate the relative abundance of *F. prausnitzii* before and after FMT. Twenty-one patients provided fecal samples before the FMT procedure, at 2 weeks post-FMT, and at 2–4 months post-FMT. The relative abundance of *F. prausnitzii* was determined using quantitative polymerase chain reaction.

Results

The abundance of *F. prausnitzii* was elevated in samples (N = 9) from donors compared to pre-FMT samples (N = 15) from patients (adjusted P<0.001). No significant difference in the abundance of *F. prausnitzii* between responders (N = 11) and non-responders (N = 4) was found before FMT (P = 0.85). In patients with CDI, the abundance of *F. prausnitzii* significantly increased in the 2 weeks post-FMT samples (N = 14) compared to the pre-FMT

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samples (N = 15, adjusted P<0.001). The increase persisted 2–4 months post-FMT (N = 15) compared to pre-FMT samples (N = 15) (adjusted P<0.001).

Conclusions

FMT increases the relative abundance of *F. prausnitzii* in patients with recurrent CDI, and this microbial shift remains several months later. The baseline abundance of *F. prausnitzii* in donors or recipients was not associated with future treatment response, although a true predictive capacity cannot be excluded because of the limited sample size. Further studies are needed to discern whether *F. prausnitzii* plays an active role in the resolution of CDI.

Introduction

The annual incidence of *Clostridioides difficile* infection (CDI) in Sweden is 65/100,000 as compared with 147/100,000 in the United States, translating into nearly half a million cases and 29,000 deaths [1, 2]. Roughly 20% of patients experience recurrent disease after a period of an initial response to CDI treatment [2, 3]. Recurrent CDI is associated with an increased mortality risk compared to the index episode and most patients do not experience sustained cure after antibiotics [4, 5].

The onset of the CDI is most often preceded by a course of antibiotic treatment that translates into structural and functional disruptions of the normal host microbiome [6]. Consequently, colonization resistance is lost, allowing *C. difficile* spores to germinate into vegetative cells, resulting in clinical infection. Because dysbiosis of the gut microbiota plays a pivotal role in the pathogenesis of CDI [7], it is intuitive to treat the infection with bacteriotherapy. Three randomized controlled studies have demonstrated that fecal microbiota transplantation (FMT) is a highly effective treatment for recurrent CDI [8–10].

Metagenomic research has shown that FMT successfully reverses the dysbiosis [11], and after FMT, the microbiota of treated patients resembles that of the donor [12]. Differences between donors in terms of gut microbiota composition could in part explain the reported variation in post-FMT cure rates of 70–95% [13–15]. However, the fecal transplant's causal components translating into clearance of the CDI are yet to be identified. Identifying such mediators would be most helpful given that the information could be used to predict treatment response and identify suitable donors.

A reduction of the commensal bacteria *Faecalibacterium prausnitzii* has been reported in other diseases characterized by gut dysbiosis, including Crohn's disease [16], ulcerative colitis [17], celiac disease [18], obesity [19], diabetes [20], and psoriasis [21]. *F. prausnitzii* is one of the most common species of the healthy gut microbiota, representing >5% of the total bacterial count [22]. The bacteria produce at least two metabolites with anti-inflammatory properties, the short-chain fatty acid butyrate and the protein microbial anti-inflammatory molecule [23]. Although there is mounting evidence that *F. prausnitzii* is an important regulator of intestinal homeostasis, data about its role in CDI and FMT are relatively scarce. To examine the dynamics of *F. prausnitzii* in patients receiving FMT due to recurrent CDI, we conducted a longitudinal study to quantify the abundance of *F. prausnitzii* before and after treatment. We hypothesized that *F. prausnitzii* is depleted in patients with recurrent CDI and that the baseline abundance of the bacteria in donors or recipients may be used to predict treatment response.

Material and methods

Study design

In this dual-center study stool samples from patients with recurrent CDI were collected to investigate the relative abundance of *F. prausnitzii* before and after FMT. The patients were asked to provide fecal samples within 2 days before the FMT procedure, at 2 weeks (±7 days) post-FMT, and finally, 2–4 months (±5 days) post-FMT. Fecal samples were collected locally at each study site and stored at -80˚C until DNA extraction. The relative abundance of *F. prausnitzii* was determined using a quantitative polymerase chain reaction (qPCR) approach, targeting the 16S rRNA gene.

Two criteria were used to define treatment response: (1) resolution of diarrhea within 14 days of the FMT and (2) absence of symptoms associated with relapse during the follow-up of 2–4 months post-FMT. Patients had to meet both criteria to be classified as responders; otherwise, they were classified as non-responders.

Study population

We included consecutive patients treated with FMT for recurrent CDI at the Department of Infectious Diseases, Linköping University Hospital, Linköping (n = 15) and Ryhov Hospital, Jönköping (n = 6) between November 2015 and November 2017. All participants submitted written consent before inclusion. Patients who provided at least 2/3 of the requested fecal samples were included. In addition to this inclusion criterion, patients with underlying colonic comorbidity (colonic cancer, N = 1 and lymphocytic colitis, N = 1) were excluded, since it would have been difficult to evaluate the clinical response of the FMT in these patients.

Recurrent CDI was defined as a relapse of clinical symptoms combined with a positive laboratory test for *C. difficile* within 8 weeks of the previous episode of CDI. This definition is in accordance with the clinical guidelines from the European Society of Clinical Microbiology and Infectious Diseases [24]. Patients were treated based on local clinical guidelines and referred for FMT at the second relapse of CDI after failing previous treatment with metronidazole, vancomycin or fidaxomicin.

Medical records were reviewed by an experienced infectious disease specialist at each hospital to confirm the diagnosis of CDI and record information on symptoms and antibiotic use during the study.

Healthy, unrelated donors were recruited from the general population. All donors were screened through questionnaires, serologic testing and fecal culturing to avoid the transference of pathogenic microorganisms. The fecal transplants were kept in -80˚ C until the day of transplantation. The transplant was mixed with 0,25 liter of NaCl solution and delivered as a rectal enema. Patients were advised to stay in supine position for 30 minutes after administration. The pairing of donors and patients was determined by transplant availability only and no matching according to age or sex was applied. The study was approved by the Regional Ethics Committee in Linköping (DNR 2014/484-31).

DNA extraction

DNA was extracted from 100 mg of feces using QIAamp PowerFecal DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extraction started with 5 minutes bead beating at 30Hz in TissueLyser II (Qiagen) and further extraction was performed in QIAcube (Qiagen) automation. The DNA quantification was executed in a Qubit 2.0 fluorometer (Life Technologies) according to Qubit dsDNA high sensitivity, HS, assay (Thermo

Fisher Scientific). After quantification, the DNA samples were diluted to 5 ng/μL in Ultrapure water.

qPCR

Each PCR reaction was performed with 20 ng of template DNA and measured in triplicates. The HOT FIREPol® EvaGreen® qPCR was used to detect PCR products on an Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies). All samples were measured in triplicates. The ΔΔ-method was used to calculate the abundance of *F. prausnitzii* in relation to the total bacterial count [25], and the total bacterial abundance was measured using eubacterial primers. Thermal cycle conditions [26], primer sequences for *F. prausnitzii* (Willing B. et al.) and Eubacteria (Barman et al.) are described elsewhere [27, 28].

Statistics

The abundance of *F. prausnitzii* was expressed as the log-2 of the fold change related to the total bacterial count. To exclude the potential effect of anti-CDI antibiotics, analyses of follow-up samples were restricted to patients who had not been treated with metronidazole, vancomycin or fidaxomicin after FMT. Some patients in this study had incomplete data (e.g., only 2/3 requested fecal samples were provided). Because of this collection limitation, the overall difference in abundance of *F. prausnitzii* before and after FMT was primarily assessed using the Mann-Whitney U test (unpaired analysis). To account for the dependence between samples, the nonparametric Wilcoxon matched-pairs signed-rank test was used for the comparisions of samples that had been provided at two adjacent timepoints, e.g. before and after FMT (paired analysis). To adjust for multiple comparison between groups in the longitudinal analysis, the Benjamini-Hochberg method was applied with a false discovery rate of 0.05. Raw P values were adjusted for all six comparisons between groups. Pearson's correlation coefficient was used to evaluate the relation of the abundance of *F. prausnitzii* between donors and recipients. The Benjamini-Hochberg adjusted P values were computed in R-studio (version 1.3.1093) using the function "p.adjust". All other statistical calculations were performed in GraphPad Prism, version 8.

Results

Clinical cohort

In total, 21 patients met the inclusion criteria and formed the study population. Eleven patients provided only 2/3 of requested fecal samples, resulting in 55 samples collected during the study period. Eight unrelated donors contributed with 21 transplants to the study participants. Basic demographics and clinical characteristics of the study population are presented in Table 1. Of the 21 patients, 17 responded to the FMT treatment and four were non-responders. The four non-responders received CDI antibiotics (Vancomycin, N = 3; Fidaxomicin, N = 1) after the FMT and before the sampling 2 weeks later. Accordingly, the fecal samples (N = 8) from these four patients, collected after antibiotic prescription, were excluded from the analysis. Moreover, three samples from three patients were collected outside the predefined sampling intervals (7–21 days post-FMT and 55–125 days post-FMT) and therefore excluded from analysis (see Fig 1).

No association between abundance of *F. prausnitzii* and treatment response in patients and donors before FMT

The abundance of *F. prausnitzii* was elevated in donors (N = 9) compared to patients' pre-FMT samples (N = 15) (difference between medians = 15.6, adjusted $P < 0.001$ (Fig 2). No

Table 1. Basic demographics and clinical characteristics.

Total patients, N	21
Females, N (%)	11 (52.3)
Age in years, median (IQR)	76 (9)
Number of recurrences before FMT, median (IQR)	2 (1)
Treatment for the last recurrence before FMT	
Vancomycin, N (%)	21 (100)
Response to FMT	
Responder, N	17
Non-responder, N	4

IQR, interquartile range; FMT, fecal microbiota transplantation.

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significant difference in the abundance of *F. prausnitzii* between responders (N = 11) and non-responders (N = 4) was found before FMT (difference between medians = −3.2, P = 0.85), but the comparison was compromised by the limited number of samples. The abundance of *F. prausnitzii* in transplants provided to responders (N = 7) and non-responders (N = 2) appeared similar (difference between medians = −0.31), but the groups were too small to allow any statistically meaningful comparison.

The abundance of *F. prausnitzii* increases after FMT

Using all fecal samples in the unpaired analysis, the abundance of *F. prausnitzii* significantly increased in the patients' samples (N = 14) 2 weeks post-FMT compared to pre-FMT samples (N = 15) (difference between medians = 14.3, adjusted P < 0.001) (Fig 2). The increase persisted 2–4 months post-FMT (N = 15) compared to pre-FMT samples (N = 15) (difference between medians = 14.6, adjusted P < 0.001) (Fig 2). Based on the paired analysis, a similar increase in the abundance of *F. prausnitzii* was observed 2 weeks (adjusted P = 0.016) and 2–4 months (adjusted P = 0.012) post-FMT (Table 2).

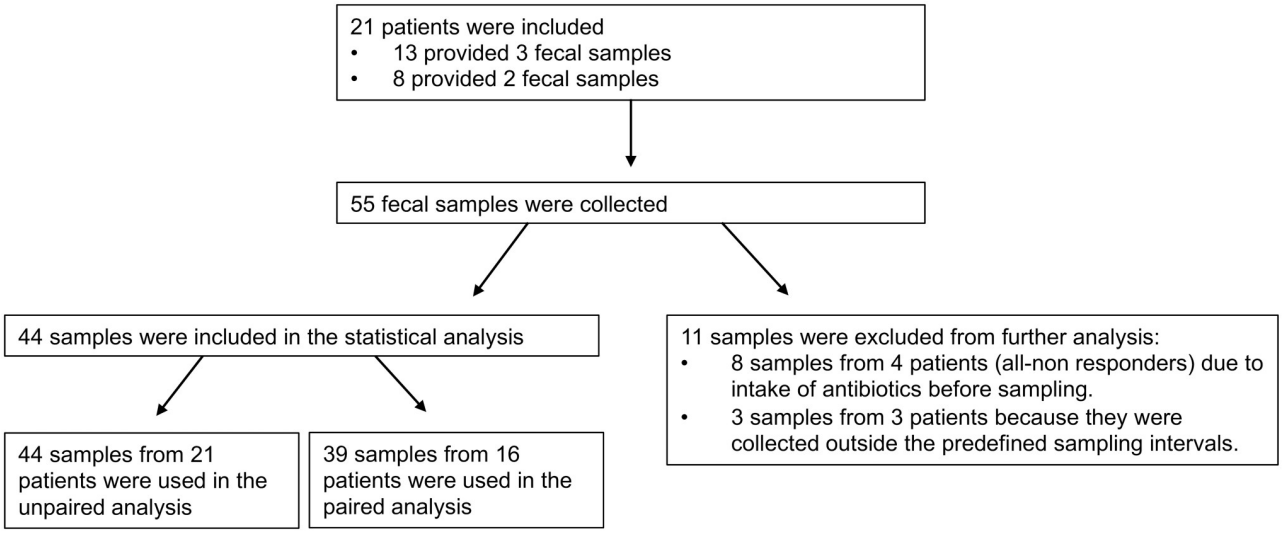

Fig 1. Flow chart of included patients and samples used in the statistical analysis.

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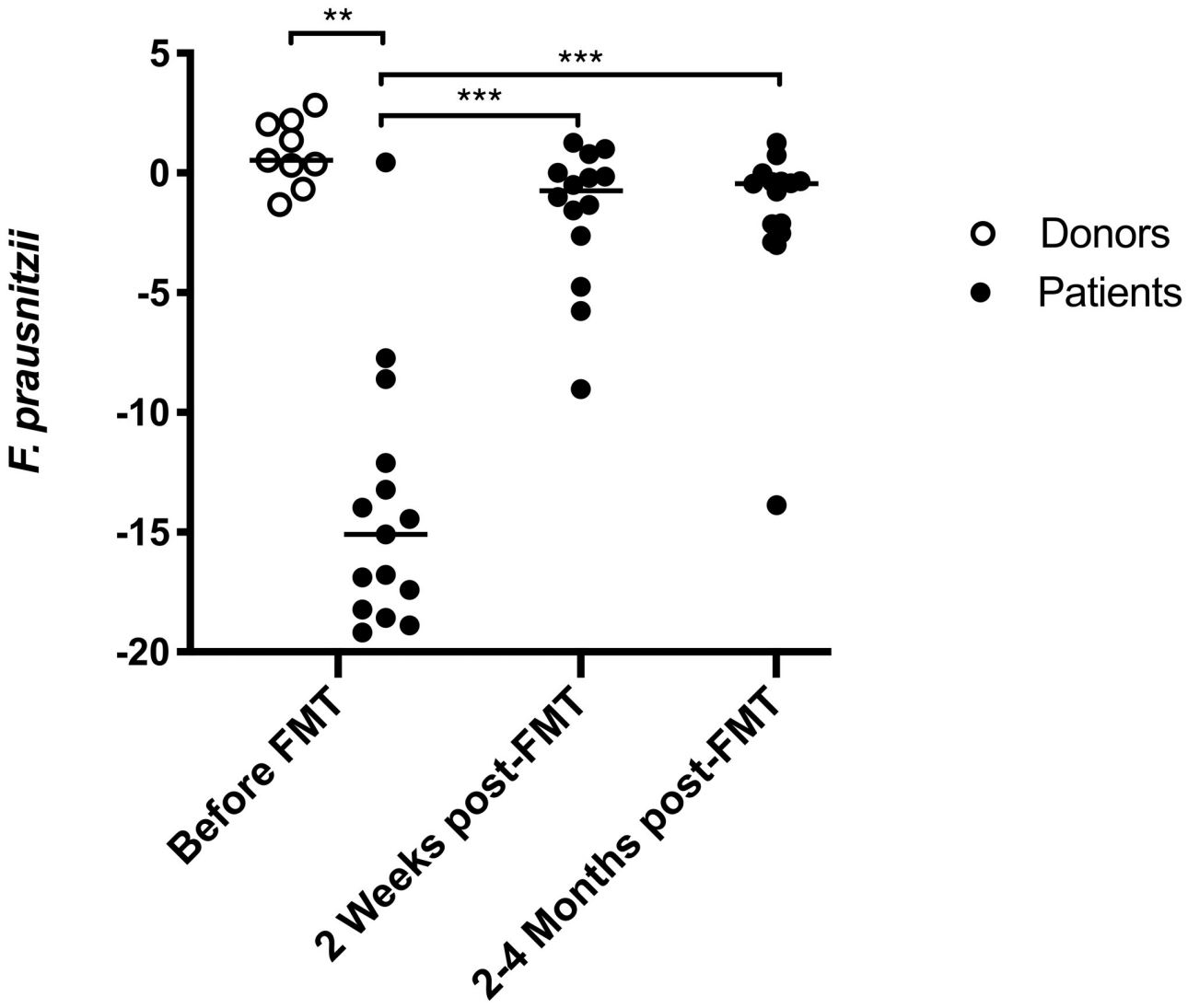

Fig 2. Abundance of *F. prausnitzii* before and after fecal microbiota transplantation (FMT) in 21 patients with *C. difficile* infection (CDI). The abundance of *F. prausnitzii* was elevated in donors compared with patients' pre-FMT samples. A significant increase in the abundance of *F. prausnitzii* in patients' samples was observed 2 weeks post-FMT, which was sustained at 2–4 months after FMT. Y-axis: The abundance of *F. prausnitzii* was expressed as the log-2 of the fold change related to the total bacterial count. The Mann-Whitney U test was used for comparisons between groups. Highly significant differences in the median abundance between groups are marked with *** (adjusted $P < 0.001$) or ** (adjusted $P < 0.01$). The horizontal lines indicate the group median.

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Although the abundance of *F. prausnitzii* increased substantially following FMT, some minor, but statistically significant, differences in the relative abundance of *F. prausnitzii* remained between patients and donors, both at 2 weeks (differences between medians = −1.3, adjusted P = 0.016) and 2–4 months post-FMT (differences between medians = −1.0; adjusted P = 0.010). In a linear correlation analysis of donor-recipient pairs, the abundance of *F. prausnitzii* in an individual donor did not correlate with the abundance in the recipient's sample two weeks post-FMT ($R^2 = 0.05$, P = 0.52).

Discussion

This study shows an increased relative abundance of *F. prausnitzii* after FMT in patients with recurrent CDI. The abundance of *F. prausnitzii* was notably higher in donors than in

Table 2. Results of the Wilcoxon matched-pairs signed-rank test.

Comparison	Number of pairs	Median of differences	P value	Adjusted p value
2 weeks post-FMT vs pre-FMT	9	15.9	0.012	0.016
2–4 months post-FMT vs pre-FMT	9	16.4	0.004	0.012
2–4 months post-FMT vs 2 weeks post-FMT	12	−0.49	0.57	0.57

FMT, fecal microbiota transplantation.

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recipients at baseline (i.e. pre-treatment). Already 2 weeks post-FMT, the levels of *F. prausnitzii* approached those of the donors and this shift remained at 2–4 months post-FMT. Although the observed increase in *F. prausnitzii* after FMT indicates that the bacteria could be important in resolving CDI, the baseline abundance of *F. prausnitzii* in recipients and donors was not predictive of future treatment response.

While the microbiota dynamics have been extensively studied in CDI patients receiving fecal transplantation, only a few previous study have reported a significant increase in *F. prausnitzii* after FMT [29, 30]. Other studies may have failed to detect *F. prausnitzii* as most of these studies used next-generation sequencing (NGS) techniques. Although NGS provides detailed information on gut microbiota composition, the method sometimes fails to provide comprehensive data at the species level due to insufficient sequencing depth. Of note, several studies have reported that CDI is characterized by a depletion of the family *Ruminococcaceae*, to which the species *F. prausnitzii* belongs [31, 32]. In the current study we used qPCR to measure the relative abundance of *F. prausnitzii*. This method may result in a more specific and reliable quantification of a single species compared to massively parallel sequencing techniques, including NGS.

Our data on the increase of *F. prausnitzii* after FMT is supported by some previous studies [29, 30, 33, 34]. Intriguingly, Mintz et al. also used qPCR to measure *F. prausnitzii* and observed an increased level after FMT in a cohort of 14 patients with recurrent CDI [29]. This finding was recently replicated in a study of 26 patients [30], who performed an in-depth characterization of the gut microbiota composition using an untargeted metagenomic sequencing approach. Additionally, expansion of *F. prausnitzii* post-FMT have also been reported in a case report, and in a study of nine pediatric patients with CDI, although the observed increase was not statistically significant in these studies [33, 34].

It is not yet clear whether FMT's effect is mediated by a few key species or by complex interactions between the donors and recipients' entire gut microbiome. An experimental mouse study indicates that a transfer of only six phylogenetically diverse species may be sufficient to trigger a shift in the gut environment that facilitates re-expansion of the recipient's commensal gut microbiota [35]. In 2013, Petrof et al. used a bacterial cocktail of 33 commensal species, including *F. prausnitzii*, as a successful treatment for two patients with antibiotic resistant CDI [36]. Selective bacteriotherapy has then been evaluated in a case series of 55 patients, and although conceptually successful, the remission rate reached only 64% [37], considerably lower compared to most FMT studies [15]. These findings indicates that although a few key species seem sufficient to inhibit *C. difficile* germination, transfer of an unfiltered microbiota with a higher diversity may provide a more robust and effective way to eradicate recurrent CDI.

Because this study is descriptive, it does not provide evidence that *F. prausnitzii* plays a causal role in resolving CDI. However, a previous study has shown that *C. difficile* induces inflammation by activating the nuclear factor κB (NF-κB) pathway [38]. In contrast, experimental studies have showed that *F. prausnitzii* is a major producer of short-chain fatty acids (e.g. butyrate), which in turn inhibits signaling through the (NF-κB) pathway [16, 39]. *F. prausnitzii* does also contribute to gut homeostasis by modulating the intestinal mucus barrier [40]. Moreover, increased levels of SCFA in feces have been reported after successful FMT [41].

Our study has several limitations. An important issue is whether the abundance of *F. prausnitzii* differs between responders and non-responders after FMT. *In vitro* studies suggest that *F. prausnitzii* is susceptible to vancomycin [42]. Because all four non-responders in our study were treated with CDI-antibiotics before a post-FMT fecal sample was secured, this issue could not be addressed. Ideally, future studies should collect fecal samples both before and after antibiotic exposure to determine the importance of dysbiosis in non-responders after FMT. Another limitation is that the qPCR-based approach in our study quantifies both viable and non-viable bacteria. Research shows that oxygen exposure during transplant preparation diminishes the proportion of viable *F. prausnitzii* [43]. Finally, eight patients provided only two of the three fecal samples sought. The missing data resulted in a decreased statistical power and reduced the possibility to identify potentially true alterations in the abundance of *F. prausnitzii*, including comparison of donors and recipients. However, the observed sizable increase in the abundance of *F. prausnitzii* after FMT is unlikely to be significantly altered due to missing data.

This paper demonstrates that *F. prausnitzii* increases after FMT, a finding that may be underreported in the literature owing to methodological limitations of sequencing studies. The baseline abundance of *F. prausnitzii* in donors or recipients was not associated with future treatment response, although a true predictive capacity cannot be excluded because of the limited sample size. Further studies are needed to discern whether *F. prausnitzii* plays an active role in the resolution of CDI.

Supporting information

S1 Table. Demographic data of donors.
(PDF)

S1 File. Source data for this study.
(XLSX)

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