Fecal Transplant in Children With *Clostridioides difficile* Gives Sustained Reduction in Antimicrobial Resistance and Potential Pathogen Burden

Suchitra K. Hourigan,1,2,3,4 Michelle Ahn,5 Keylie M. Gibson,5 Marcos Pérez-Losada,5,6,7 Grace Felix,3,4 Melissa Weidner,4 Ian Leibowitz,3 John E. Niederhuber,3,4,8 Cynthia L. Sears,3, Keith A. Crandall,5,6,a and Maria Oliva-Hemker4,8

1Inova Translational Medicine Institute, Falls Church, Virginia; 2Inova Children’s Hospital, Falls Church, Virginia; 3Pediatric Specialists of Virginia, Fairfax, Virginia; 4Johns Hopkins University School of Medicine, Baltimore, Maryland; 5Computational Biology Institute and 6Department of Biostatistics and Bioinformatics, Milken Institute School of Public Health, George Washington University, Washington, DC; 7CIBIO-InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, Vairão, Portugal; 8Public Health Sciences, School of Medicine, University of Virginia, Charlottesville, Virginia

**Background.** Fecal microbiota transplantation (FMT) treats *Clostridioides difficile* infection (CDI). Little is known regarding the changes in antimicrobial resistance (AMR) genes and potential pathogen burden that occur in pediatric recipients of FMT. The aim of this study was to investigate changes in AMR genes, potential pathogens, species, and functional pathways with FMT in children.

**Methods.** Nine children with recurrent CDI underwent FMT. Stool was collected from donor and recipient pre-FMT and longitudinally post-FMT for up to 24 weeks. Shotgun metagenomic sequencing was performed. Reads were analyzed using PathoScope 2.0.

**Results.** All children had resolution of CDI. AMR genes decreased post-FMT (P < .001), with a sustained decrease in multidrug resistance genes (P < .001). Tetracycline resistance genes increased post-FMT (P < .001). Very low levels of potential pathogens were identified in donors and recipients, with an overall decrease post-FMT (P < .001). *Prevotella* sp. 109 expanded in all recipients post-FMT, and no recipients had any clinical infection. Alpha diversity was lower in recipients vs donors pre-FMT (P < .001), with an increase post-FMT (P = .002) that was sustained. Beta diversity differed significantly in pre- vs post-FMT recipient samples (P < .001). Bacterial species *Faecalibacterium prausnitzii* and *Bacteroides ovatus* showed higher abundance in donors than recipients (P = .008 and P = .040, respectively), with expansion post-FMT. Biosynthetic pathways predominated in the donor and increased in the recipient post-FMT.

**Conclusions.** FMT for CDI in children decreases AMR genes and potential pathogens and changes microbiota composition and function. However, acquisition of certain AMR genes post-FMT combined with low levels of potential pathogens found in donors suggests that further study is warranted regarding screening donors using metagenomics sequencing before FMT.

**Keywords.** antimicrobial resistance; children; *Clostridioides difficile*; *Clostridium difficile*; fecal transplant; pathogen.

*Clostridioides difficile* infection (CDI), previously named *Clostridium difficile*, is the leading cause of nosocomial diarrhea in the United States and causes significant morbidity and mortality [1]. In both adults and children, CDI is increasing in prevalence in the hospitalized population and community [2–5]. The risk of CDI recurrence ranges from 20% after an initial episode to 60% after multiple prior recurrences [6, 7]. Fecal microbiota transplantation (FMT), the transfer of stool from a donor to a patient, has been shown to be an effective treatment for recurrent CDI in 2 randomized controlled trials in adults and in case series in children [8–10]. FMT has now also been included in recent practice guidelines for the treatment of recurrent CDI [11].

Dysbiosis of the intestinal microbiota has been well described in those with recurrent CDI, with markedly decreased fecal bacterial diversity [12]. Although the exact mechanisms have not been fully elucidated, FMT is hypothesized to be effective in the treatment of recurrent CDI by restoring intestinal microbiome composition and function. Indeed, through the use of 16S ribosomal RNA (rRNA) gene sequencing, FMT for recurrent CDI has been shown to increase bacterial diversity in recipients of all ages while shifting their microbiome composition toward their respective donors [13–15]. Given the success of FMT in CDI and the appeal and curiosity exhibited by providers and the lay public, it is not surprising that consideration of FMT for other conditions associated with disturbances of the microbiome is under active discussion [16]. Currently, there are ongoing trials in disorders including inflammatory bowel disease, irritable bowel syndrome, obesity, and even neurological disorders [16].

FMT generally appears to be clinically safe in short-term follow-up [17], although adverse events have been reported,
including infection [18] and even a recent death, which prompted a Food and Drug Administration (FDA) warning [19]. Currently, prospective donors for FMT are screened for infection by standard clinical culture and polymerase chain reaction (PCR) techniques. Screening protocols vary among institutions and providers, although many follow the minimum screening from previously published protocols [20]. However, on investigation of the human microbiome, low levels of potential disease-causing microorganisms, potential pathogens, have been shown to be present even in healthy individuals [21]. Moreover, antimicrobial resistance is an increasing global problem [22], and the human intestinal microbiome is well known for harboring antimicrobial resistance (AMR) genes [23, 24]. Therefore, in theory, FMT could transmit potential pathogens and AMR genes from fecal donor to recipient. Additionally, in a recipient with an altered microbiome or a suppressed immune system, potentially pathogenic bacteria, including those with AMR, may translocate from the gut to cause disseminated disease as demonstrated, for example, for vancomycin-resistant enterococci [25]. Conversely, as FMT is proposed to restore diversity and a “balanced” microbiome, it also has the potential to decrease the fecal potential pathogen burden and AMR resistance genes found in recipients by competitive exclusion or by changing the environment in a way that renders potentially pathogenic strains benign. The few studies to date looking at microbiome changes with FMT have mostly utilized 16S rRNA sequencing [13–15], which gives an overview of the bacterial composition of the microbiome but often lacks enough resolution to identify species or strains and does not identify bacterial virulence determinants, microbiota functional changes, or AMR genes. This information, however, can be attained by coupling shotgun metagenomic sequencing with a sophisticated analytic pipeline [26]. Moreover, existing differences between the developing gut microbiome in early childhood and the adult microbiome [27] raise additional concerns in pediatric recipients of FMT, who most often have an adult as the donor.

The aim of this study was to apply shotgun metagenomic sequencing and advanced bioinformatic tools to the analysis of fecal samples from pediatric recipients pre- and post-FMT as well as their adult donors to identify the prevalence and potential acquisition of potentially pathogenic microbial strains and AMR genes and to characterize their microbial composition and function.

**METHODS**

**Patients and Donors**

Pediatric patients (<21 years), without underlying inflammatory bowel disease (IBD), underwent FMT as clinically indicated for the treatment of recurrent CDI; no FMTs were conducted for research purposes. Subjects were recruited under institutional review board (IRB)–approved protocols both at Inova Fairfax Hospital (IRB#13-1441) and the Johns Hopkins Medical Institute (IRB#00096688). All study subjects required the following: (i) at least 3 recurrences of CDI, each with at least 3 episodes of diarrhea per day; (ii) positive results for toxigenic C. difficile for each recurrence on laboratory testing either by C. difficile toxin PCR or enzyme immunoassay (ELISA) of C. difficile toxins; and (iii) resolution or improvement of symptoms with CDI antibiotic treatment.

Healthy adult donors were used for the FMT. All donors had a health screening and had blood and stool screened for potential pathogens with testing at a minimum following previously published protocols [19]. Either donors were related to patients or prepared stool from donors was obtained from a commercially available stool bank (Openbiome, Cambridge, MA) (Table 1). There was a clinical change during the study period from using related donors to using stool banks in both participating centers.

**Fecal Microbiota Transplantation**

Patients stopped antibiotic treatment for CDI 48 hours before the FMT procedure. On the day before the procedure, fresh donor stool was collected from related donors within 12 hours of FMT. A total of 100 grams of donor stool was vortexed with 400 mL of nonbacteriostatic saline and filtered. Alternatively, prepared frozen donor stool from the stool bank intended for colonic delivery (colonoscopy) was thawed as per the stool bank instructions. Prepared stool was then delivered to the recipient via colonoscopy, primarily in the cecum, with a small amount of stool delivered through the rest of the colon as the colonoscope was withdrawn. After the FMT, recipients remained lying flat for 2 hours and received loperamide (weight-based standard dosing) to aid in stool retention.

Baseline information for FMT recipients was collected, including age, race, recent hospitalizations, antibiotic use, medications to suppress gastric acid suppression, and any diagnosed medical conditions (Table 1). Follow-up clinic visits and/or phone calls were conducted 1–4 days after FMT and at regular intervals for 6 months post-FMT. Adverse effects were screened for at each time point, including fever, chills, malaise, fatigue, anorexia, abdominal pain, diarrhea, constipation, nausea, and vomiting. Stool samples were collected longitudinally from recipients, usually corresponding with clinical visits for the recipients, up to 24 weeks post-FMT. Stool was also collected from both donor and recipient before FMT. All samples were frozen without additives at –80°C until analysis.

**DNA Extraction and Next-Generation Sequencing**

Microbial DNA from donor and patient stool samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Germantown, MD) for DNA isolation, following the manufacturer’s instructions. Quantification and quality of samples were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Subsequently, DNA libraries were prepared using the KAPA Hyper Prep Kit (Kapa Biosystems, Wilmington, MA), as per the manufacturer’s instructions. Prepared libraries
were subjected to paired-end sequencing (2×150 bp) on a single run using the Illumina HiSeq3000 platform (Illumina, San Diego, CA). Extractions and sequencing were conducted by Aperiomics, Inc. (Sterling, VA).

**Quality Assessment and Quality Control**

The quality of raw sequence reads was assessed using FastQC, version 0.11.5 [28]. Reads were then subjected to quality control using PRINSEQ, version 0.20.4 [29]. Reads with a length of <40 bp and a mean quality score ≤15 were filtered out. Additionally, the ends of reads were trimmed by quality score from the 3’- and 5’-end with a quality threshold score of 20. These cleaned reads were re-assessed with FastQC to ensure that low-quality regions and nucleotides had been properly and effectively removed.

**Microbial Diversity Analysis**

Bioinformatic techniques were applied to characterize and compare the microbial composition between donors and patients. PathoScope 2.0 [30] was used to map the metagenomic cleaned reads to the NCBI prokaryotic representative and reference sequence (RefSeq) database [31] for gut microbiome classification. Using the PathoMap module with its integrated Bowtie2 wrapper [32], reads were mapped to the NCBI target database, and any reads that aligned with a greater score to the human genome (hg38) and the PhiX174 sequencing control library were filtered out. After completion, the PathoID module was used to assign the mapped reads from the PathoMap module to the most likely genome of origin, acquiring accurate read counts for downstream analysis [33].

Singletons and taxa with a <1% relative abundance were filtered out. Phyloseq, version 1.20.0 [34], and ggplot2, version 2.2.1 [35], were used to visualize taxonomic composition for all donor and patient samples. Subsets of the data were also used to generate facet bar graphs that grouped (a) a recipient’s pre and post samples with their respective donor sample, (b) longitudinal collection time points, and (c) donors and recipients. In all analyses and figures, donor–recipient dyads can be identified by their assigned number (x); that is, x = donor–recipient pair; Dx = donor sample; RxA = recipient pre-FMT sample; RxB, RxC, and RxD = recipient stool samples collected longitudinally post-FMT.

Samples were normalized using negative binomial distribution [36], as implemented in the Bioconductor package DESeq2 [37]. This approach simultaneously accounts for library size differences and biological variability. Microbial normalized counts generated this way are referred to as taxon abundances throughout the text.

**Taxonomic alpha diversity** was estimated using the Shannon and Simpson diversity indices. These indices reflect both the

### Table 1. Recipient Demographics, Underlying Medical Problems, and Outcomes After FMT

| Study # | Age, y | Race | Possible Triggering Events for CDI | Medical Problems | Donor Stool Source | CDI Resolution During Study Follow-up | Side Effects |
|---------|-------|------|-----------------------------|------------------|----------------|----------------------------------------|--------------|
| 1       | 12    | Asian| Antibiotics                  | None             | Related         | Yes                                    | Prolonged diarrhea (C. difficile negative) |
| 6       | 16    | Caucasian | H2 receptor antagonist use | Postural orthostatic tachycardia syndrome | Related | Yes | No |
| 7       | 6     | Caucasian | Antibiotics Hospitalization Proton pump inhibitor use | Mitochondrial disease | Related | Yes | No |
| 10      | 2     | Caucasian | Antibiotics                  | None             | Stool bank      | Yes                                    | No |
| 13      | 12    | Caucasian | Unknown                      | Prematurity (32 wk) | Stool bank      | Yes                                    | No |
| 14      | 20    | Caucasian | Antibiotics Hospitalization Proton pump inhibitor use | Cardiofacialcutaneous syndrome | Stool bank      | Yes                                    | No |
| 15      | 5     | Caucasian | Proton pump inhibitor use    | Lennox-Gastaut syndrome | Stool bank      | Yes                                    | No |
| 16      | 16    | Caucasian | Antibiotics                  | Postural orthostatic tachycardia syndrome | Stool bank      | Yes                                    | No |
| 17      | 2     | Caucasian | Antibiotics Hospitalization H2 receptor antagonist use | Cystic fibrosis | Stool bank      | Yes                                    | No |

**Abbreviations:** CDI, Clostridioides difficile infection; FMT, fecal microbiota transplantation.
Antimicrobial Drug Resistance Analysis

Antimicrobial resistance (AMR) was characterized using AmrPlusPlus and the MEGARes database [42]. AmrPlusPlus, accessible with Galaxy [43], aligned the cleaned reads to the MEGARes database with its accompanying annotations. The AmrPlusPlus’ resistome identification pipeline tool was used to obtain read hits for AMR class, mechanism, group, and gene levels. The associations between calculated AMR abundances and samples categorized accordingly as “donor,” “pre-FMT,” and “post-FMT” were then analyzed using LME model analysis.

Potential Pathogen Identification

Potential pathogens within all samples were identified using the World Health Organization's global priority list of antibiotic-resistant bacteria [44] and Bode Science Center’s clinically relevant pathogens list [45]. Once identified, taxonomic information and read counts for these potential pathogens were isolated and graphically visualized. Abundances were calculated, and their associations with the donor, pre-FMT, and post-FMT groups were assessed using LME model analysis. The presence of potential pathogens does not necessarily indicate an “infection,” as pathogenicity is a dynamic between the host, the potential pathogen, and the environment. Nevertheless, we can quantify potential pathogens from these lists in both donors and recipients as important clinical information.

Functional Pathway Analysis

HUMAnN2: The HMP Unified Metabolic Analysis Network 2 was used for functional analysis. The HUMAnN2 pathway abundance output was used to determine key functional pathways. Pathways that had a total summed value of 0 were ultimately filtered out. The superclass of functional pathways was determined using the biocyc website (biocyc.org). A heatmap was created using ggplot2, and the Welch's 2-sample t-test was applied to assess significance. To correct for multiple hypothesis testing, the Benjamini-Hochberg false discovery rate correction method was applied at alpha = 0.05 [46].

RESULTS

Demographics, Stool Collection, and Clinical Results

Nine donor–recipient pairs were included in this study (Table 1), with a mean recipient age (range, median) of 10 (2–20, 12) years. Possible triggering events for CDI included antibiotic usage in 6 of the recipients, with 3 of these also having a hospitalization within 3 months of the first CDI occurrence. Five out of the 9 recipients also used medication to suppress gastric acid (3 used proton pump inhibitors, whereas 2 used H2 receptor antagonists). One recipient had no identified possible triggering events for CDI. Two recipients had no medical problems, whereas 7 recipients reported conditions including postural orthostatic tachycardia syndrome, gastroesophageal reflux disease, mitochondrial disease, autism, cardiofaciocutaneous syndrome, Lennox-Gastaut syndrome, and cystic fibrosis. No recipients had IBD or developed IBD during the follow-up period. With regards to antibiotic treatment for CDI, all recipients had failed a vancomycin taper. Three recipients received stool from related donors, and 6 received stool from a stool bank.

A recipient stool sample and a donor stool sample were collected from all dyads before FMT. Post-FMT, 7 recipients provided a stool sample between 2 and 7 weeks, 6 recipients between 8 and 13 weeks, 4 recipients between 14 and 19 weeks, and 5 recipients between 20 and 24 weeks. When sequenced, stool samples had an average (range) of 8 670 338 (6 187 488–9 732 386) reads. After quality assessment and quality control (QA/QC), there was an average of 8 607 521 quality reads per sample.

All recipients had resolution of CDI-related symptoms within 3 days of FMT, and none had recurrence of CDI during the study follow-up. One patient, recipient 1, had prolonged C. difficile–negative diarrhea, fecal urgency, and intermittent fecal incontinence after FMT, which was different in nature and less severe compared with their pre-FMT CDI symptoms. Extensive workup for infectious etiologies including multiple negative C. difficile toxin B PCR tests was conducted with no infectious cause found. The recipient’s symptoms gradually improved at
2 months after FMT with the use of behavioral psychology and cholestryramine. Six months post-treatment, this patient’s diarrhea had fully resolved and was no longer treated with medication. Interestingly, this patient’s donor sample (from a relative) looked very different from the other donor samples, with a high abundance of *Prevotella copri* (see below).

**Alpha and Beta Diversity**

Pre-FMT, the alpha diversity was significantly lower in recipients compared with donors (*P* < .001 Shannon index and Simpson index). After FMT, at 2–7 weeks, the alpha diversity increased in recipients (*P* < .001 Shannon index, *P* = .002 Simpson index). This diversity was sustained throughout all time points up to 24 weeks (Figure 1). Recipient samples collected pre- and post-FMT also showed significant differences in beta diversity for all measures (*P* < .001 Bray-Curtis, *P* < .001 Jaccard, *P* < .001 Jensen-Shannon divergence) (Figure 2).

**Relative Abundance of Bacterial Taxa**

Post-FMT, recipient samples more closely resembled donor samples than pre-FMT recipient samples throughout the study’s follow-up period. Recipient samples before FMT had a high level of Proteobacteria, primarily from the class Gammaproteobacteria and the family Enterobacteriaceae, which decreased with FMT and was sustained over time (Gammaproteobacteria recipient pre-FMT vs post-FMT, *P* < .001) (Figure 3A). Healthy donor samples consisted mainly of classes Clostridia (Figure 3A), primarily from the family Lachnospiraceae, and Bacteroidia, primarily from the family Bacteroidaceae, which expanded in FMT recipients after transplantation (Clostridia recipient pre-FMT vs post-FMT, *P* < .011; Bacteroidia recipient pre-FMT vs post-FMT, *P* < .031) (Figure 3A). Species *Faecalibacterium prausnitzii* and *Bacteroides ovatus* were significantly higher in donors than recipients (*P* = .008 and *P* = .040, respectively), with a sustained rise in recipients after FMT that reached significance for *Bacteroides ovatus* (*P* = .030) (Figure 3B). *Klebsiella pneumoniae*, specifically subspecies *K. pneumoniae HS11286*, was higher in recipients pre-FMT compared with donors (*P* = .045) and decreased in recipients after FMT (*P* = .017). A similar trend was also seen in *Klebsiella oxytoca*, with increased levels in recipients pre-FMT (*P* = .067) and a significant decrease after FMT (*P* = .027) (Figure 3B).

Recipient 1, who had persistent *C. difficile*-negative diarrhea post-FMT, had a bloom of the species *Prevotella copri*, specifically of the strain *P. copri DSM 18205*, that was sustained after

![Image](image.png)

**Figure 1.** Alpha diversity (Shannon and Simpson index) before and after fecal microbiota transplantation in donors and recipients.
FMT and differed from other FMT recipients. Recipient 1’s related donor also had a very high relative abundance of this *Prevotella copri* (Figure 3B). Donor fecal matter contained similar species composition, except for Donor 1 (Figure 3B), regardless of status as a related donor or commercial donor (Table 1). Unfortunately, because of the low sample size (6 commercial donors and 3 related donors), meaningful statistical testing for differences in donor material species composition could not be conducted. Nevertheless, it is clear from the species composition of the donor material that the major difference was with Donor 1, and this difference carried over to the recipient and persisted even to the last collection time point.

In initial analysis of recipient 14’s pre-FMT sample, only 960,471 reads mapped to the prokaryotic representative and RefSeq database compared with 2,236,806–3,951,266 reads for the remaining pre-FMT samples. Thus, the metagenomic cleaned reads were re-mapped to the NCBI viral, fungal, and protozoan RefSeq databases. In this new analysis of recipient 14’s pre-FMT sample, 99.52% of the reads mapped to the *Salmonella* phage Vi II-E1, which also comprised 96.11% of the viruses found in all samples. No clinical reason could be identified to explain this observation. In contrast, the composition of the remaining samples consisted primarily of bacteria (98.26% of all reads), with low proportions of viruses (1.36%), archaea (0.3%), and eukaryotes (0.08%).

**Antimicrobial Resistance Genes**

AMR genes were detected in all recipient and donor specimens. Generally, AMR gene proportions were significantly
Figure 3. Taxa plots showing the relative abundance of taxa at the class (A) and species (B) levels before and after fecal microbiota transplantation in donors and recipients.
higher in recipient pre-FMT samples (mean, 68,866) than in donor samples (mean, 11,193; \( P = .003 \)) and post-FMT samples (mean, 15,045; \( P < .001 \)). Pre-FMT recipient samples also had a higher relative abundance of multidrug resistance (MDR) genes (mean, 27,349) than donors (mean, 978; \( P < .001 \)), which decreased post-FMT and was sustained (mean, 1633; \( P < .001 \)) (Figure 4). MDR resistance mechanisms were mainly from multidrug efflux pumps (Supplementary Figure 1). Levels of tetracycline AMR genes were higher in donors (mean, 3225) than in recipients pre-FMT (mean, 1567; \( P < .001 \)) and increased in recipients post-FMT (mean, 3295; \( P = .003 \)) (Figure 4). No recipient developed any clinical infection with AMR organisms during the study follow-up. Again, due to low sample size, a statistical comparison between commercial and related donors could not be made, but the donors all had relatively low levels of AMR genes and similar relative abundance profiles, with some slight differences in the relative abundance of particular classes of AMR genes (Figure 4). In addition, due to the low sample size, the study was not powered to examine differences in AMR genes between recipients who had exposure vs no exposure to antibiotics (other than to treat CDI) pre-FMT.

**Potential Pathogens**

Overall, very low levels (mean, 1.46%) of potential pathogens were detected in both donors and recipients pre-FMT and post-FMT. After filtering to an abundance of \( >1\% \), 13 potential pathogenic strains remained, mainly from the phylum Proteobacteria: *Enterobacter cloacae* subsp. *cloacae* ATCC 13047, *Escherichia coli* O83:H1 str. NRG 857C, *Escherichia coli* IA139, *Escherichia coli* UMN026, *Klebsiella oxytoca*, *Escherichia coli* str. K-12 substr. MG1655, *Clostridium perfringens* ATCC 13124, *Prevotella* sp. 109, *Prevotella* sp. P4-76, *Prevotella* sp. S7 MS 2, *Prevotella* sp. S7-1–8, *Escherichia coli* O104:H4 str. 2011C-3493, and *Klebsiella pneumoniae* subsp. *pneumoniae* HS11286. Levels of potential pathogens significantly varied over time (Figure 5). A higher mean proportion of potential pathogens (determined by pathogen read counts/overall read counts) was detected in recipient pre-FMT samples (6.23%) compared with donors (1.22%; \( P = .002 \)). Potential pathogens in recipients significantly decreased after FMT (2.23%; \( P < .001 \)). However, in 1 recipient–donor pair, the donor had higher levels of potential pathogen reads (1383 reads) than the recipient pre-FMT sample (509 reads). Levels of potential pathogen reads among donors also varied from 1383 to 102,580 reads. *Prevotella* sp. 109 was found in all donor samples but only in 4 recipient samples pre-FMT. Post-FMT samples showed varying levels of *Prevotella* sp. 109, but it was found in all of these samples. No recipients developed infection with any of the detected potential pathogens during the study follow-up. Thus, although it is clear that both donors’ and recipients’ gut microbiomes have known potentially pathogenic strains of bacteria, these low-level pathogen frequencies fluctuate over time (Figure 5) and do not, in these cases, result in pathogenesis.

![Antimicrobial resistance drug classes](image)

**Figure 4.** Antimicrobial resistance gene relative abundances before and after fecal microbiota transplantation in donors and recipients. Abbreviation: MLS, macrolide, lincosamide and streptogramin.
Very low levels of *C. difficile* strains were detected in most samples: overall, 0.002%; donor, 0.001%; pre-FMT, 0.003%; and post-FMT, 0.001%. Only recipient 17 showed higher pre-FMT levels of *C. difficile* M120 (612 reads), which is ribotype 078 (Supplementary Figure 2).

**Functional Diversity**

Among identified functional pathways, after FDR correction for multiple testing, there were 82 pathways in which abundance varied significantly between donor and recipient pre-FMT samples (Figure 6; Supplementary Table 1). Similarly, 78 pathways also varied significantly between the recipient pre-FMT vs post-FMT samples (Welch’s 2 sample t test, Benjamini-Hochberg, \( P < .05 \)) (Supplementary Table 2a and b). Biosynthesis pathways (such as “cell structures biosynthesis,” “fatty acids and lipids biosynthesis,” and “cofactors, prosthetic groups, electron carriers biosynthesis”) predominated in the donor and increased in the recipient post-FMT. Although generally these pathways were identifiable in all recipients pre-FMT and increased post-FMT, there were some pathways that were not identifiable in certain recipients pre-FMT but were present in their corresponding donor and expanded post-FMT, suggesting “functional transmission.” Those included DTDP-RHAMSYN-PWY: dTDP-L-rhamnose biosynthesis I (not present in 1 recipient pre-FMT), and PWY-5177: glutaryl-CoA degradation (not present in 5 recipients pre-FMT). No differences in superclasses of functional pathways were seen between related donors and donors from the commercial stool bank. Moreover, functional changes seen in the first recipient stool sample post-FMT were most often sustained throughout the follow-up period.

**DISCUSSION**

Our study adds substantively to prior work by (1) using shotgun metagenomic sequencing (2) to examine longitudinal changes in (3) potential pathogen burden and (4) AMR gene frequency with FMT in (5) pediatric cases of recurrent CDI. FMT causes a decrease in AMR genes and potential pathogens that is sustained over time.

Notably, AMR gene abundance was higher in pre-FMT recipients than in donors, and the overall abundance of AMR genes decreased with FMT and was sustained over the follow-up period. Similar findings have been reported in adults using culture techniques to detect AMR as well as shotgun metagenomics [47–49]. It is not surprising that pre-FMT levels of AMR genes are higher in recipients compared with donors given that recipients have often received multiple courses of antibiotics for CDI and possibly other causes, whereas donors are excluded if they have received antibiotics within several months of their fecal donation. It is speculated that a decrease of microorganisms harboring AMR genes with FMT is a result of the ability of the transplanted microbiota to reestablish a healthy colonic environment, which provides colonization resistance against potential pathogens [50]. Our study showed a reduction in MDR genes with FMT, primarily from the multidrug efflux pump mechanism, also seen by Millan et al. [48]. Infection and colonization with MDR organisms have become major global health concerns, and FMT is being considered beyond recurrent CDI for the elimination of MDR organisms [51, 52], especially considering that organisms containing AMR genes may also precede systemic infection [24]. Although this shows great promise, caution must also be used. It has been shown...
that although overall depletion of AMR genes occurs, there is also acquisition of certain AMR genes in recipients with FMT, some of them with clinical significance [49]. In our study, most of the AMR genes acquired by recipients after FMT were tetracycline AMR genes, which are among the most abundant AMR genes and are present in most adults [53] and children [54]. Nevertheless, acquisition of clinically significant AMR genes is possible, and given that many recipients of FMT are immunosuppressed [55], this may be consequential. Therefore, it is worth considering whether FMT donors should be screened via metagenomics for AMR gene burden and characteristics before FMT, especially for select patients receiving FMT such as those with significant immunosuppression.

The incidence of severe infection was reported at 2.5% after FMT in 1 systematic review [18], with around 30% of these infections thought to be possibly or probably caused by FMT. A recent safety alert was released by the FDA, in recognition of the transfer of multidrug-resistant organisms by FMT causing invasive infections with extended-spectrum beta-lactamase (ESBL)–producing Escherichia coli in 2 immunocompromised recipients, 1 of whom died [19]. Thus, our study is exceptionally timely in that it not only provides insights into pediatric FMT, but also provides a clear and repeatable approach for characterizing microbial diversity, antibiotic resistance, and (importantly) potential pathogens through a combination of metagenomics and bioinformatics. Reassuringly in our study, potential pathogen levels were very low in both donors and recipients, with a sustained reduction in potential pathogens after FMT. After filtering at a 1% abundance threshold, most potential pathogens identified were gram-negative rods, many of which can be found in the healthy gut but could also be detrimental to immunocompromised patients. Future studies could employ metatranscriptomics to measure immune response in the host relative to potential pathogen gene expression in the microbial community to validate immune response to infection [56]. Low levels of Escherichia coli O104:H4 str. 2011C-3493, a producer of shiga toxin, were found in all recipients pre-FMT. This strain was also present in all donors except 1, and after FMT the recipient of this donor no longer carried this potential pathogen. It must be noted that although the group abundance of this strain was >1%, its abundance in most individual recipients and donors was far less. An adherent-invasive Escherichia coli (AIEC) strain, Escherichia coli O83:H1, was also identified in the majority of donors and recipients pre- and post-FMT and was acquired in 1 recipient post-FMT. AIEC strains are thought to play a pathogenic role in Crohn’s disease [57], and as FMT is often used in patients with recurrent CDI in Crohn’s disease, it could be hypothesized that acquisition of this strain may worsen the disease. Indeed, flares of inflammatory bowel disease have been reported after FMT [58]. Significant levels of C. difficile were only found in 1 recipient pre-FMT; this result is anticipated given that recipients receive antibiotics for the treatment of CDI up to 48 hours before transplantation. The persistent detection of C. difficile post-FMT in most recipients most likely reflects low-level colonization with nontoxigenic C. difficile, as also seen in the donors. It must be noted that although potential pathogens were identified, virulence determinants (eg, Shiga toxin presence or expression) were not assessed, and the samples were not cultured to identify the presence of viable species.

Although direct transfer cannot be proven, Prevotella sp. 109 did significantly expand in recipients post-FMT, possibly due to permissive changes in the microbiome post-FMT. Given the significantly higher levels of Prevotella sp. 109 in donors compared with recipients and the presence in all donors, it could be hypothesized that this strain is more prevalent in the gut microbiome of adults compared with that of children; therefore, using an adult donor for FMT may result in recipient microbiome changes that resemble the adult microbiome. Children, especially the very young, have a rapidly developing gut microbiome that correlates with the development of their immune system and other physiological functions and reaches a stage of stabilization within the first few years of childhood [26]. Animal models have shown that FMT may have the potential to transfer a pathogenic disease state from donor to recipient [59], which could be more significant in the developing microbiome [60]. In adults, the development of autoimmune disease and rapid weight gain has been reported after FMT [61, 62], raising the question as to whether the transfer of an adult microbiome to a developing microbiome in a child may predispose the child to quickening of immune-aging and development of immune-related complications [63]. Assessing whether age-matched donors may be more appropriate for FMT in children still needs to be further investigated.

Alpha diversity increased with FMT and was sustained, which concurs with findings from other studies [13–15]; however, few studies have described species-level changes with FMT because 16S rRNA gene sequencing data do not allow for the resolution of shotgun metagenomics. Our results showed that Faecalibacterium prausnitzii and Bacteroides ovatus, known to have high prevalence in the human gut [64], were significantly lower in recipients pre-FMT compared with donors and increased with FMT, whereas Klebsiella pneumonia and Klebsiella oxytoca decreased with FMT. An increase in F. prausnitzii and B. ovatus with FMT and high levels of K. pneumonia pre-FMT in recipients have been reported in adult studies of patients undergoing FMT [65, 66]. F. prausnitzii ferments dietary fiber, produces short-chain fatty acids, and is thought to have an integral role in intestinal health and amelioration of colitis [67, 68]. In the 1 patient who had adverse effects after FMT with prolonged C. difficile–negative diarrhea, a bloom of Prevotella copri after FMT was found; this differed from other FMT recipients. The donor for this recipient also had a very high relative abundance of this bacterium, which contrasted with other donors. When 16S rRNA gene sequencing was used, no differences in

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Table 1: Functional Pathways Comparing Donor and Recipient Pre- and Post-FMT

| Pathway                                | Donor Pre-FMT | Donor Post-FMT | Recipient Pre-FMT | Recipient Post-FMT |
|----------------------------------------|---------------|---------------|-------------------|-------------------|
| Amines and Polyamines Biosynthesis    |               |               |                   |                   |
| Amino Acids Biosynthesis               |               |               |                   |                   |
| Aromatic Compounds Biosynthesis       |               |               |                   |                   |
| Carbohydrate Biosynthesis             |               |               |                   |                   |
| Carbohydrate Degradation              |               |               |                   |                   |
| Carboxylates Degradation              |               |               |                   |                   |
| Cell Structures Biosynthesis          |               |               |                   |                   |
| Cofactors, Prosthetic Groups, Electron Carriers Biosynthesis |               |               |                   |                   |
| Fatty Acids and Lipids Biosynthesis   |               |               |                   |                   |
| Fermentation                          |               |               |                   |                   |
| Glycolysis                             |               |               |                   |                   |
| Nucleotide Biosynthesis                |               |               |                   |                   |
| Pentose Phosphate Pathway             |               |               |                   |                   |
| Secondary Metabolites Biosynthesis    |               |               |                   |                   |
| Superpathways                          |               |               |                   |                   |
| Antimicrobial-Chemistry                 |               |               |                   |                   |
| C.I. Compounds                        |               |               |                   |                   |
| Inorganic Nucleoside-Metabolism        |               |               |                   |                   |
| TCA Cycle                              |               |               |                   |                   |

**Figure 6.** Heatmap comparing functional pathways before and after fecal microbiota transplantation in donors and recipients. See Supplementary Table 1 for corresponding individual functional pathway list. Abbreviation: FMT, fecal microbiota transplantation.
the microbiome could be detected between this recipient and other FMT recipients [15], highlighting the power and resolution of the shotgun metagenomic approach.

Functional profiling showed significant differences in metabolic potential between donors and recipients pre-FMT and between recipients pre-FMT and post-FMT. This suggests that FMT not only changes the structure and composition of the microbiome but also its functional capability. Pathways within the core metabolic groups that predominated in the donor, “cell structures biosynthesis,” “fatty acids and lipids biosynthesis,” and “cofactors, prosthetic groups, electron carriers biosynthesis,” were underrepresented in the recipients pre-FMT and increased in the recipient post-FMT. Coenzyme A and adenine nucleotides biosynthesis pathways have been identified in the human microbiome project, which examines healthy volunteers, as core pathways with “housekeeping” functions [69]; indeed, in our study, these pathways were higher in donors than recipients pre-FMT and expanded after FMT, suggesting a shift toward a “healthier” microbiome function. Although some of our functional results (glycolysis pathways underrepresented pre-FMT, and arginine-related pathways overrepresented pre-FMT) concurred with those in a previous study that used 16S rRNA data to predict functional changes after FMT using the PICRUSt tool [14, 70], many of the functional changes described here are novel. This again highlights the resolution power of in-depth shotgun metagenomic techniques compared with 16S rRNA sequencing. However, larger studies with measurements of stool metabolites are needed to rigorously examine the functional changes that occur with FMT.

This study has several limitations: (i) This study had a relatively small sample size; however, this is the largest study to date looking at AMR genes, potential pathogens, and functional changes with FMT, as well as the only pediatric study. (ii) Not all recipients had stool samples for all time points, although all were clinically followed for 6 months and all provided a fecal sample up to 8–13 weeks post-FMT. (iii) As only DNA was extracted, we could not assess the presence of RNA viruses, which may play a role in the effect of FMT. Moreover, viruses could not be analyzed in detail due to the extraction technique, which was not designed to capture viruses. (iv) Donor stools were from either a related donor or a stool bank. Although the use of donors from 1 source would have been preferable, or even a universal donor, this was not possible in this study because over the study period there was a clinical change from using related donors to stool banks in both participating centers. Because of the small sample size, we could not reasonably test for differences between donor types.

CONCLUSIONS

FMT for recurrent CDI in children is clinically effective, and shotgun metagenomic sequencing reveals an overall decrease in AMR genes and potential pathogen burden with FMT. Given that FMT is being considered for diseases other than CDI and is often used in the immunocompromised, the risk of acquiring AMR genes and potential pathogens as a result of this procedure needs to be considered. Some of the changes seen in this study may also have resulted from the use of an adult donor to a child recipient. Additional study is warranted in terms of screening donors using metagenomics sequencing before FMT, particularly in significantly immunocompromised recipients and in consideration of age-matched donors for FMT in children.

Supplementary Data

Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copiededit and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Authors’ contributions. S.K.H. conceptualized and designed the study, participated in the acquisition of data and samples, analyzed and interpreted data, drafted the manuscript, and critically revised the manuscript. M.A. analyzed and interpreted data, provided statistical analysis, and critically revised the manuscript. K.M.G. analyzed and interpreted data, provided statistical analysis, and critically revised the manuscript. M.P.L. analyzed and interpreted data, provided statistical analysis, and critically revised the manuscript. G.F. participated in acquisition of data and samples and critically revised the manuscript. M.W. participated in acquisition of data and samples and critically revised the manuscript. I.L. participated in acquisition of data and samples and critically revised the manuscript. J.E.N. conceptualized and designed the study, supervised the study, and critically revised the manuscript. C.L.S. conceptualized and designed the study, supervised the study, and critically revised the manuscript. K.M.G. analyzed and interpreted data, provided statistical analysis, and critically revised the manuscript. M.O.H. conceptualized and designed the study, participated in acquisition of data and samples, and critically revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and material. The data sets generated and/or analyzed in the current study are available in the NCBI SRA repository under accession number PRJNA525458.
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