Medical Research Archives

ESMED
European Society of Medicine

OPEN ACCESS

Published: November 30, 2022

Citation: Ramos RJF, Zhu C, et al., 2022. Metagenomic and Bile Acid Metabolomic Analysis of Fecal Microbiota Transplantation for Recurrent Clostridiodes Difficile and/or Inflammatory Bowel Diseases, Medical Research Archives, [online] 10(11). https://doi.org/10.18103/mra.v10i11.3318

DOI
https://doi.org/10.18103/mra.v10i11.3318

ISSN: 2375-1924

RESEARCH ARTICLE

Metagenomic and Bile Acid Metabolomic Analysis of Fecal Microbiota Transplantation for Recurrent Clostridiodes Difficile and/or Inflammatory Bowel Diseases

Ruben JF Ramos, Chencan Zhu, Dimitri F Joseph, Shubh Thaker, Joseph F LaComb, Katherine Markarian, Hannah J Lee, Jessica C Petrov, Farah Monzur, Jonathan M Buscaglia, Anupama Chawla, Leslie Small-Harary, Grace Gathungu, Jeffrey A Morganstern, Jie Yang, Jinyu Li, Eric G Pamer, Charles E Robertson, Daniel N Frank, Justin R Cross, Ellen Li*

*ellen.li@stonybrookmedicine.edu

ABSTRACT

Background. Fecal microbiota transplantation (FMT) is an effective treatment of recurrent *Clostridioides difficile* infections (rCDI), but has more limited efficacy in treating either ulcerative colitis (UC) or Crohn's disease (CD), two major forms of inflammatory bowel diseases (IBD). We hypothesize that FMT recipients with rCDI and/or IBD have baseline fecal bile acid (BA) compositions that differ significantly from that of their healthy donors and that FMT will normalize the BA compositions.

Aim. To study the effect of single colonoscopic FMT on microbial composition and function in four recipient groups: 1.) rCDI patients without IBD (rCDI-IBD); 2.) rCDI with IBD (rCDI+IBD); 3.) UC patients without rCDI (UC-rCDI); 4.) CD patients without rCDI (CD-rCDI).

Methods. We performed 16S rRNA gene sequence, shotgun DNA sequence and quantitative bile acid metabolomic analyses on stools collected from 55 pairs of subjects and donors enrolled in two prospective single arm FMT clinical trials (Clinical Trials.gov ID NCT03268213, 479696, UC no rCDI ≥ 2x IND 1564 and NCT03267238, IND 16795). Fitted linear mixed models were used to examine the effects of four recipient groups, FMT status (Donor, pre-FMT, 1-week post-FMT, 3-months post-FMT) and first order Group*FMT interactions on microbial diversity and composition, bile acid metabolites and bile acid metabolizing enzyme gene abundance.

Results. The pre-FMT stools collected from rCDI ± IBD recipients had reduced α-diversity compared to the healthy donor stools and was restored post-FMT. The α-diversity in the pre-FMT stools collected from UC-rCDI or CD-rCDI recipients did not differ significantly from donor stools. FMT normalized some recipient/donor ratios of genus level taxa abundance in the four groups. Fecal secondary BA levels, including some of the secondary BA epimers that exhibit in vitro immunomodulatory activities, were lower in rCDI±IBD and CD–rCDI but not UC-rCDI recipients compared to donors. FMT restored secondary BA levels. Metagenomic baiE gene and some of the eight bile salt hydrolase (BSH) phylotype abundances were significantly correlated with fecal BA levels.

Conclusion. Restoration of multiple secondary BA levels, including BA epimers implicated in immunoregulation, are associated with restoration of fecal baiE gene counts, suggesting that the 7-α-dehydroxylation step is rate-limiting.

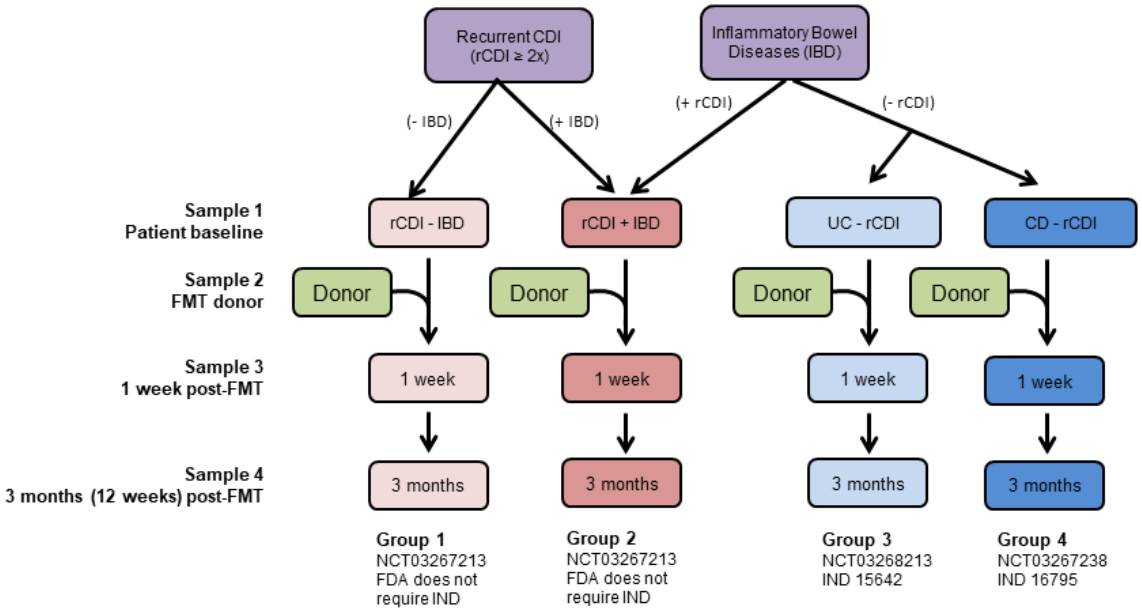
Introduction

Fecal microbiota transplantation (FMT) of healthy donor stool is a highly effective treatment of recurrent *Clostridioides difficile* infection (rCDI).[1-4] Patients with inflammatory bowel diseases (IBD), particularly ulcerative colitis (UC), indeterminate colitis (IC) and Crohn's colitis (CC), are at increased risk of developing CDI.[5-9] FMT is a very effective treatment of rCDI in these patients.[10-15] However, studies thus far on the effectiveness of FMT in the treatment of IBD, absent concomitant CDI, have utilized different protocols and have yielded mixed results.[16-27]

Restoration of bacterial metabolism of primary bile acid (BA) to secondary has been implicated in the mechanism by which FMT promotes resistance to CDI.[28] Primary conjugated BA synthesized in the liver are released into the gut lumen upon feeding and serve to emulsify dietary fats. Within the gut lumen, conjugated BA undergo deconjugation by bacterial bile salt hydrolases (BSH) in the small intestine.[29] Unconjugated primary BA undergo further conversion by bacterial enzymes, such as 7-alpha dehydroxylase, to form secondary BA. 7-alpha dehydroxylating bacteria, such as *Clostridium scindens*, have reduced abundance in rCDI patients and replenishment of these bacteria was demonstrated to inhibit CDI in an animal model.[28] More recently, epimers of deoxycholic and lithocholic acids (e.g. iso-deoxycholic, 3-oxo-lithocholic, isoallo-lithocholic acids) have been reported to have in vitro immunomodulatory activity.[30-32] Modified BA pool composition has been reported in IBD patients,[33-35] with altered levels of some BA epimers that exhibit potential immunomodulatory effects.

At Stony Brook University Medical Center (SBUMC), we conducted two prospective single arm clinical trials: 1.) ClinicalTrials.gov ID: NCT03268213, 479696, UC only Investigational New Drug (IND) 15642 and 2.) ClinicalTrials.gov ID: NCT03267238, CD only IND 16795, to examine the longitudinal microbiological and clinical effects of single donor FMT on the fecal microbiome in four groups of recipients: rCDI without IBD (rCDI–IBD); rCDI with IBD (rCDI+IBD); UC without rCDI (UC–rCDI); CD without rCDI (CD–rCDI, see **Figure 1**). Each FMT recipient selected their own donor, who was subsequently screened for pathogens and antibiotic resistant bacteria. This FMT study is unique in that it includes prospective collection of a relatively large set of healthy donor stools. The results of 16S rRNA sequence analysis of single donor colonoscopic FMT performed on a subset of 19 recipients was previously reported,[36] which included: 1) 11 rCD–IBD; 2) 3 rCDI+IBD (all with UC); and 3) 5 UC-rCDI. The previous results indicated marked changes in β diversity (i.e. overall microbiome composition differences between groups) in the rCDI+UC and rCDI–IBD groups (compared to donors). The rCDI-IBD and rCDI+UC recipients had a marked decreased abundances of anaerobic taxa in the Ruminococcaceae, (e.g. Faecalibacterium) and Lachnospiraceae groups, and marked increased abundances of microaerophilic taxa in the Bacilli group, and Proteobacteria and Fusobacteria phyla, which were restored post FMT. In contrast, the pre-FMT differences in β-diversity were much more subtle in the UC-rCDI group compared to their healthy donors. These differences were characterized by increased abundances of microaerophilic taxa in the Bacilli, Proteobacteria and Fusobacteria in the UC-rCDI group. With the onset of the COVID-19 pandemic in early 2020, the FDA held recruitment for all FMT clinical trials, because of concerns about the risk of transmitting COVID-19 by FMT. All SBUM, all elective inpatient and outpatient colonoscopic procedures were postponed until the late fall of 2020. Mainly because of budgetary funding constraints, this research team then ended these two clinical trials. We now report results of our multi-omic analysis of the fecal samples collected from 55 recipient-donor FMT through these two clinical trials that includes the 19 recipient-donor groups reported earlier,[36] as well as recipients in the CD-rCDI group. The multi-omic studies reported here include 16S rRNA sequence, quantitative BA liquid chromatography-mass spectrometry (LC-MS) and shotgun DNA metagenomic sequence analysis.

Figure 1. Schematic diagram of recipient group and donor fecal sample collection.



Materials and methods

Study protocols

The patient and donors were recruited through two open label study protocols. Clinical Trials.gov ID NCT03268213, 479696, UC no rCDI ≥ 2x IND 15642, was approved by the Stony Brook Institutional Review Board (479696) on 11/14/2013. Clinical Trials.gov ID NCT03267238, IND 16795, was approved by Stony Brook Institutional Review Board (973349) on 06/16/2017. The initial recipient/donor pair was recruited on 12/05/2013. The final recipient/donor included in this report were recruited 06/19/2019. There was a delay in the registration of NCT03268213 on Clinical Trials.gov relative to the initial recruitment of the first recipient/donor pairs as described previously [37], but no delay in the registration of NCT03267238 relative to the initial recruitment of patient/donor pairs. For the patients with rCDI (with or without IBD), the inclusion criteria were ≥ 2 recurrences despite treatment with antibiotics, documented by ≥ 3 positive stool tests for CDI (**Figure 1**). For the patients with UC without a history of rCDI ≥ 2x, the inclusion criterion was medication refractory UC, requiring step up therapy beyond mesalamine alone. Only one of the patients in this group had a distant prior history of CDI with the last episode 2 years prior to the procedure. For the patients with CD without a history of rCDI ≥ 2x, the inclusion criterion was medication refractory CD, requiring step up therapy beyond mesalamine alone. None of the CD patients had a history of previous CDI. Exclusion criteria for all of the recipients included: a) scheduled for abdominal surgery within 12 weeks of the study, b) pregnancy, c) Grade 4 anemia (Hemoglobin < 6 g/dL), d) Grade 1 neutropenia (Absolute Neutrophil Count <1500), e) known diagnosis of graft vs. host disease, f) major abdominal surgery within the past 3 months, g) administration of any investigational drug within the past 2 months., h) use of a TNF-α antagonist within 2 weeks of the proposed date of transplantation, i) bacteremia within past 4 weeks. (28 days), and j.) adults ≥18 years unable to or unwilling to give informed consent. The CONSORT flow diagram is outlined in **Figure 2**. Recruitment and collection of data and processing of patient samples were conducted as previously described in Mintz et al.[36] The IBD subphenotypes were classified using the Montreal classification in terms of disease location (L1, ileal CD; L2, Crohn's colitis; L3, ileocolitis; L4, upper gastrointestinal) for CD and extent of disease (E1, proctitis ; E2, left-sided UC; E3, extensive UC proximal to splenic flexure) for UC.[37] Mucosal disease activity in the colon and the terminal ileum at the time of the colonoscopic FMT was scored using the Mayo endoscopic subscore for UC-rCDI recipients and the simple endoscopic score for CD-rCDI (SES-CD).[38,39]

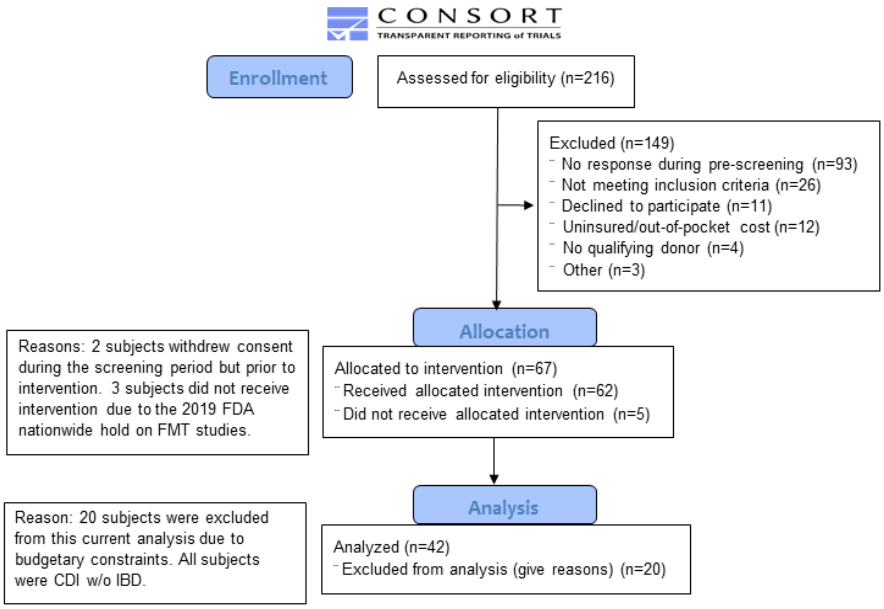


Figure 2. CONSORT 2010 flow diagram.

Dietary information

The recipients and donors were asked to record daily dietary intake on the Stony Brook University Medical Center Department of Pediatrics Food Diary/Calorie Count for 7 days prior to the collection of each stool sample. Patients were grouped into three categories on review of their dietary logs and interviews: 1.) animal protein-meat (mostly standard "western" diet); 2.) vegetarian (predominantly plant based but animal protein from eggs and dairy); and 3.) plant based protein source (vegan but could include honey).

Fecal calprotectin analysis

Levels of the neutrophilic fecal calprotectin protein, a clinical marker of gut mucosal inflammation, was measured using the PhiCal Test (Calpro AS, Norway) enzyme-linked immunosorbent assay (ELISA) following the manufacturer's recommended protocol as previously described.[36]

16S rRNA gene sequence analysis

16S rRNA gene sequencing targeting the V3V4 variable region of the 16S rRNA gene: primers 338F (5' ACTCCTACGGGAGGCAGCAG) and 806R (5' GGACTACHVGGGTWTCTAAT) was carried out by the Frank laboratory at the U of Colorado as previously described.[36] All de-multiplexed, paired-end 16S rRNA sequencing data along with the metadata were deposited into the NCBI Sequence Read Archive under BioProject accession number PRJNA739170.

BA metabolomics analysis by LC-MS

Human stool samples (~400 mg) were weighed into 7 mL bead tubes containing 1.4 mm ceramic beads (Omni International) and extracted using 80% methanol containing deuterated labeled internal standards [ISTDS: chenodeoxycholic acid–d4 (CDCA-d4),taurochenodeoxycholic acid-d4 (T-CDCA-d4), glycochenodeoxycholic acid-d4 (G-CDCA-d4), cholic acid-d4 (CA-d4), taurocholic acid-d4 (T-CA-d4), glycocholic acid-d4 (G-CA-d4), deoxycholic acid-d4 (DCA-d4), taurodeoxycholic acid-d4 (T-DCA-d4), glycodeoxycholic acid-d4 (G-DCA-d4), lithocholic acid-d4 (LCA-d4), tauro-lithocholic acid (T-LCA-d4), glycolithocholic acid-d4 (G-LCA-d4), ursodeoxycholic acid-d4 (UDCA-d4), T-UDCA-d4, and β-muricholic acid-d5 (β-MCA-d5)] for a final concentration of 100 mg/m. The ISTDs were purchased from Cambridge Isotope Laboratories (Tewksbury, MA); except β-MCA-d5 that was purchased from Isosciences (Ambler, PA). Samples were homogenized using a Bead Ruptor (Omni International, Kennesaw, GA) at 6 m/s for 6 cycles of 30 s at 4°C, then stored at -80°C overnight. Stool extracts were then vortexed, transferred to 5 mL Eppendorf tubes, centrifuged at 20,000 × g for 20 min at 4°C and diluted with Milli-Q water to 50% methanol content. Samples were then filtered using 96-well Acroprep Advance plates (Pall Corporation, Port Washington, NY) and analyzed by LC-MS analysis BA were separated using two chromatographic methods: 1.) Agilent 1290 Infinity LC system coupled to an Agilent 6550

iFunnel Q-TOF with a 1.6 µm, 2.1 × 50 mm CORTECS T3 column (Waters) and 2.) Agilent 1290 Infinity II LC system coupled to an Agilent 6546 Q-TOF with a 1.7 µm, 2.1 × 150 mm ACQUITY UPLC BEH Shield RP18 column (Waters). Active reference mass correction was used according to the manufacturer's instructions. Mobile phase A was 0.1% formic acid in water, mobile phase B was 0.1% formic acid in acetone. LC gradient for Method 1: 30% B to 65% B over 14 min (flow rate: 0.4 mL/min) and for Method 2: 30% B to 80% B over 30 min (flow rate: 0.3 mL/min). Both methods ended with 100% B for 2 min and re-equilibration for 6 min. Acquisition was from m/z 50-1,700 at 2 Hz. Mass spectrometer source conditions for both methods: gas temperature: 240°C; drying gas flow: 10 L/min; nebulizer: 60 psi; sheath gas temperature and flow: 400°C and 10 L/min, respectively. The voltages used were: fragmentor: 175 V; skimmer: 45 V; capillary: 4000 V; and nozzle: 500 V. Sample injection volume was 5 µL, and data was collected in negative ionization mode. A pooled sample was injected regularly throughout the batch to monitor instrument performance. Data files were converted to Agilent SureMass format and analyzed in Agilent MassHunter Quantitative Analysis software (version 10.1, Agilent Technologies, Santa Clara, CA). Concentrations were estimated by referencing peak areas to specified ISTDs of known concentration, except for sulfated-BAs and 3-oxo-LCA, which used an external calibration curve.

Shotgun DNA metagenomics analysis

Shotgun DNA metagenomics sequencing was carried out on fecal DNA samples at Novogene Corp Inc. (Beijing, China). DNA samples were assessed using Qubit Fluorimeter while agarose gel electrophoresis was carried out to check for DNA degradation. The NEBNext UltraTMII DNA Library PrepKit for Illumina® (Ipswich, MA) was used to prepare the sequencing libraries according to the manufacturer's protocols and Illumina 150 bp paired end (PE150) sequencing was conducted on the HiSeqX instruments with a targeted depth of 20 million. Quality filtering (e.g., removal of adapters, human sequences, low-quality sequences) was conducted using KneadData version 5.1 (http://huttenhower.sph.harvard.edu/kneaddata) as previously described.[33] All de-multiplexed, paired-end shotgun DNA metagenomic sequencing data along with the metadata were deposited into the Sequence Read Archive under BioProject accession number PRJNA739170. Reads corresponding to BA metabolizing enzymes were identified by a translated search with DIAMOND [40] and a manually curated protein sequence database, respectively at two threshold levels: 1.) 80% identity over 80% sequence and 2.) 50% identity over 80% sequence. Reads corresponding to antibiotic resistance genes were identified using the Comprehensive Antimicrobial Resistance Database (CARD).

Statistical analysis

No sample size power analysis was conducted for the two FMT clinical trials since they were both designed as exploratory observational single arm open label trials. Furthermore, both clinical trials were prematurely halted with the onset of the COVID-19 pandemic in 2020. The statistical anlysis described below is focused on analyzing the fecal samples collected during these two clinical trials.

Baseline patient characteristics corresponding to the analyzed fecal samples were compared between the donors and the four groups of FMT recipients Alpha diversity indices (e.g. Chao1, Shannon complexity H, Shannon Evenness H/Hmax) were calculated inferred through 1000 replicate resamplings using Explicet,[41] and β-diversity (Bray-Curtis distances) were calculated between the recipient samples and their paired donor samples as previously described.[36] The Sørensen similarity score is defined as 1- Bray-Curtis index, and 0.6 was selected as the threshold level for successful implantation of the donor microbiota.[27] P-values less than 0.05 were considered as statistically significant.

Because many of the 385 operational taxonomic units (OTUs) exhibited zero counts across multiple 16S rRNA gene libraries, generalized linear mixed models (GLMM) or generalized estimating equation (GEE) models were used to analyze 108 OTUs after eliminating OTUs with an average relative abundance of $< 0.001\%$ in the donor and recipient pre-FMT samples, and after discarding OTUs where more than 75% of the samples had a zero count. To compare the relative abundance of each OTU between donors and recipient time points before and after FMT (pre-transplant, 1 week post-FMT, 3 months post-FMT) within each disease group (rCDI - IBD, rCDI + IBD, UC - rCDI, CD - rCDI), GLMMs or GEEs were used by taking the actual counts of each OTU as the outcomes that were assumed to follow a negative binomial distribution.[36] The log-transformed overall sequence count for each individual at each time point was considered as an offset. Two-way interaction terms (Group*FMT) were used to estimate the difference between the time points within a specific disease group. Possible covariance

structures to model correlation among longitudinal measurement from the same patient and measurement in the corresponding donor were unstructured (UN) and compound symmetry (CS). In GEE, the dependence structure was chosen based on Quasi Information Criteria (QIC). Pair-wise P-values were based on the T-test for GLMM, and the Z-test for GEE. The P-values were adjusted for multiple comparisons by the Bonferroni correction (P threshold = 0.05/number of multiple comparison) or by the Benjamini-Hochberg method (false discovery rate or FDR < 0.05) as described further in the text.

Kruskal-Wallis tests were used to compare BA levels between donor and four pre-FMT recipient groups. Post-hoc Dunn's tests with Benjamini-Hochberg adjustments were then performed in comparison of recipient groups and donor. Linear mixed models were used to estimate the pairwise BA differences between pre-FMT recipient and donor as well as response post-FMT in each disease group. UN or CS covariance structures were selected to model the correlation among longitudinal measurements from the same patient and his/her corresponding donor based on (Aikaike Information Criteria (AIC). Log-transformation was applied if the normality assumption was not satisfied. If log-transformation was performed, pre-FMT recipient/donor ratios were shown; otherwise, donor- pre-FMT recipient differences were shown.

Kruskal-Wallis tests were also used to compare bacterial gene abundances (cpm) at 80% identity using the shotgun metagenomics sequencing data, between donor and four pre-FMT recipient groups. Estimated correlation coefficients were generated from linear mixed models treating FMT groups as clustering effect, in order to assess the correlations between bacterial gene abundances (cpm). Log transformation was applied to bacterial gene abundances if normality assumption was not met. All analysis was performed in SAS 9.4 (SAS institute Inc., Cary, NC) and R 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient characteristics of FMT donors and recipient samples analyzed

The characteristics of the 55 recipient/donor pairs, segregated according to donors and the four groups of recipients, are summarized in **Table 1**. The median age in the rCDI–IBD group was higher than the donor and the other three recipient groups. Differences in the proportion of males between groups was also observed, being particularly high in the UC-rCDI and CD-rCDI groups. Donors and recipients in all groups had predominantly White/European Ancestry (EA) and no Black/African Ancestry (AA) subjects. While only a minority of patients were current smokers, there were differences in the proportion of former smokers and never smokers between groups. All of the rCDI recipient groups received antibiotics within a month prior to the FMT in order to prevent recurrence of CDI, whereas none of the donors (within 3 months of donating the stool sample), UC-rCDI or CD-rCDI recipients (within one month of undergoing FMT) received antibiotics. 20% of rCDI-IBD recipients and 56-62% of the IBD recipients with or without rCDI had abnormal fecal calprotectin levels (> 120 µg/g). Of the 43 donors measured, only two had borderline values (> 50 µg/g, ≤ 120 µg/g) and the rest had normal values (≤ 50 µg/g).

Table 1. Baseline characteristics of donors and the four groups of FMT recipients. The median values and interquartile range (IQR) are listed for each group. Fecal calprotectin ≤ 50 µg/g, normal; 50.1-120 µg/g, borderline; > 120 µg/g, abnormal.

Variable	Donor n = 55	Group 1 rCDI–IBD n = 27	Group 2 rCDI+IBD n = 8	Group 3 UC-rCDI n = 11	Group 4 CD-rCDI n = 9
Age (y) ± IQR	43 ± 25	66 ± 21	37 ± 29	37 ± 18	18 ± 4
Sex n (%)					
Males	23 (42%)	8 (30%)	3 (38%)	6 (54%)	8 (89%)
Race n (%)					
White/EA	52 (95%)	27 (100%)	8 (100%)	10 (82%)	7 (78%)
Black/AA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Asian	3 (5%)	0 (0%)	0 (0%)	2 (18%)	2 (22%)
Ethnicity					
Hispanic n (%)	8 (15%)	2 (7%)	2 (25%)	2 (18%)	0 (0%)
Smoking n (%)					
Current	5 (9%)	4 (15%)	0 (0%)	1 (9%)	0 (0%)
Former	9 (16%)	13 (48%)	2 (25%)	4 (36%)	1 (11%)
Never	41 (75%)	10 (37%)	6 (75%)	6 (55%)	8 (89%)
Body mass index (BMI) kg/m² ± IQR	23.8 ± 7	27.1 ± 7.6	23 ± 7	27 ± 9	20 ± 5
Diet n (%)					
Meat	49 (89%)	27 (100%)	8 (100%)	10 (91%)	8 (89%)
Vegetarian (dairy)	5 (9%)	0 (0%)	0 (0%)	1 (9%)	1 (11%)
Plant based	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Antibiotics n (%) within 3 month	0 (0%)	27 (100%)	8 (100%)	1 (9%)	0 (0%)
IBD medications n (%)					
5-ASA	0 (0%)	0 (0%)	6 (75%)	7 (64%)	5 (56%)
Steroids	0 (0%)	0 (0%)	1 (12%)	4 (36%)	3 (33%)
Immunomodulators	0 (0%)	0 (0%)	5 (62%)	4 (36%)	4 (44%)
Biologics	0 (0%)	0 (0%)	2 (25%)	4 (36%)	4 (44%)
None	55 (100%)	27 (100%)	1 (12%)	1 (12%)	2 (22%)
Fecal calprotectin n (%)					
Abnormal	0 (0%)	6 (22%)	5 (62%)	7 (64%)	5 (56%)
Borderline	2 (4%)	6 (22%)	3 (38%)	2 (18%)	0 (0%)
Normal	41 (74%)	15 (56%)	0 (0%)	2 (18%)	3 (33%)
Missing	12 (22%)	0 (0%)	0 (0%)	0 (0%)	1 (11%)

Clinical outcomes for the four recipient groups

The primary endpoint for rCDI was defined as no further rCDI at 12 months after FMT in rCDI recipients with or without IBD. FMT was 92% effective in preventing rCDI in the rCD-IBD group and 75% effective in the rCDI+IBD group. These results compare favorably to the previously reported 91% efficacy in in donor FMT compared to 67% effectiveness of the sham FMT and previously reported uncontrolled FMT studies performed in rCDI patients with IBD.[2,10-15]

The primary endpoint for gut inflammation as measured by fecal calprotectin levels was one week after FMT for all recipients because many IBD patients were prescribed changes in their medications between one week and 3 month time points. We did not observe a clear effect of a single colonoscopic FMT on gut inflammation in IBD recipients as measured by decreased fecal calprotectin.

Serious adverse events (SAEs) were reported in six patients. The SAEs in three rCDI–IBD recipients and one UC-rCDI recipient, were deemed unrelated to the FMT procedure on review by the Data Safety Monitoring Board. The remaining two SAEs observed in one rCDI+IBD (CD+rCDI)) and one CD–rCDI recipient could have reflected progression of their underlying disease but could also have been related to the procedure, since

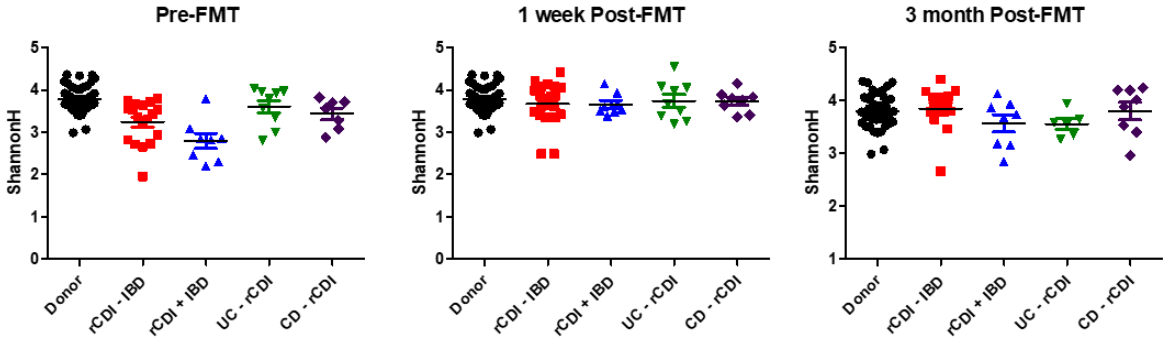
flares have been previously reported in other IBD patients post FMT.[12]

16S rRNA gene sequencing results

16S rRNA gene sequence profiling of fecal bacterial communities was accomplished for 165 fecal DNA samples, collected from 43 of 55 donors, 18 of 27 rCDI+IBD recipients, 8 of 8 rCDI+IBD recipients, 9 of 11 UC-rCDI recipients and 8 of 9 CD-rCDI recipients.

Linear mixed models comparing the ShannonH α-diversity index between donor and recipient pairs in each of the four groups (**Figure 3**) revealed significant differences between the donor group and both the rCDI-IBD (P = 0.0001) and rCDI+IBD (p <0.0001) groups at baseline (Pre-FMT), with resolution of these differences at the 1 week and three month time points. In contrast, differences in α-diversity between donor and recipient did not reach significance for either the UC-rCDI or CD-rCDI groups.

Figure 3. α-diversity (ShannonH) in the donor and four recipient groups Pre-FMT, 1 week Post-FMT and 3 month Post-FMT.



A recent report of a clinical FMT trial in CD patients set successful implantation of donor stools as achieving a Sørensen similarity index > 0.6 (the maximum value of 1 indicates identical communities) between paired donors and recipients.[27] We therefore adopted the same endpoint in analyzing our 16S rRNA gene sequencing results. The pre-FMT Sørensen similarity indices were below the endpoint in all of the rCDI recipients with and without IBD (**Table 2**), with overall increases in these indices post-FMT. Individually the endpoint of > 0.6 was reached post FMT in 73% of the rCDI recipients. Among the IBD-rCDI groups prior to FMT, 5 of 9 (56%) UC-rCDI recipients and 5 of 7 (71%) CD–rCDI recipients had donor-recipient pre-FMT Sørensen similarity indices below the endpoint. Three of these recipients had evidence of active disease based on their initial fecal calprotectin level and endoscopic score (Mayo or SES-CD). Three of the 5 UC–rCDI donor-recipient pairs and 3 of 5 CD-rCDI recipients reached the endpoint post-FMT at either the 1 week and/or 3 month time point. However, achieving this endpoint was not necessarily associated with reduction of gut inflammation measured using fecal calprotectin levels. Note that none of the CD recipients in the previously published sham controlled FMT study of CD recipients achieved a Sørensen similarity index > 0.6 at the 6 week post-FMT time point.[27]

Table 2. Median Sørensen similarity index ± IQR between paired donors and recipients at Pre-FMT, 1 week post FMT and 3 months post FMT

Group	Pre-FMT	1 week Post-FMT	3 month Post-FMT
rCDI-IBD (n =18)	0.26 ± 0.28	0.65 ± 0.09	0.66 ± 0.16 (3 missing)
rCDI+IBD (n = 8)	0.08 ± 0.26	0.55 ± 0.28	0.49 ± 0.15
UC-rCDI (n = 9)	0.54 ± 0.27	0.62 ± 0.14	0.61 ± 0.22 (3 missing)
CD-rCDI (n = 8)	0.52 ± 0.14 (1 missing)	0.62 ± 0.19	0.63 ± 0.17

We next analyzed how abundances of individual bacterial taxa (from 16S rRNA gene sequencing) differed between recipient groups (rCDI–IBD, rCDI+IBD, UC–rCDI, CD – rCDI), and healthy donors as previously described.[36] Pairwise comparisons of the estimated recipient/donor ratios were then carried out. 72 OTUs demonstrating significant estimated pre-FMT recipient/donor ratios with 95%CI in at least one of the four recipient groups with a FDR (q) <0.05 are shown in **Table 3**. FMT appeared to normalize the recipient/donor ratios of many of the OTUs. Notable exceptions included the *Escherichia-Shigella* OTU recipient/donor ratio in CD-rCDI recipients and the *Ruminococcus* OTU in the UC-rCDI recipient/donor ratio.

Table 3. Significant (FDR < 0.05) estimated pre-FMT recipient/donor ratios (95% CI) of the relative abundances of genus level OTUs for each of the four recipient groups. A ratio > 1 indicates ↑ relative abundance in recipient pre-FMT compared to donor samples. A ratio of < 1 indicated ↓ relative abundance in pre-FMT samples compared to donor samples. The 95% CI are shown in parenthesis. Only the estimated ratios with an FDR < 0.05 are listed. The * indicates those OTUs that also had significant estimated ratios of post-FMT/pre-FMT relative abundances at with 1 week and/ or 3 month time point, indicating that FMT had a significant effect.

Actinobacteria OTU	**rCDI-IBD**	**rCDI+IBD**	**UC-rCDI**	**CD-rCDI**
Actinomyces				4.15* (1.71,10.04)
Bifidobacterium	6.64 (1.98,22.29)			
Coriobacteriaceae/unspecified	0.01* (0.004,0.04)	0.02* (0.002,0.146)		
Collinsella	0.04* (0.006,0.22)	0.001* (0,0.002)		0.34* (0.18,0.62)
Gordonibacter		0.02* (0.01,0.03)		0.26 (0.09,0.79)
Bacteroidetes OTU	rCDI-IBD	rCDI+IBD	UC-rCDI	CD-rCDI
Unassigned	0.01* (0.002,0.05)	0.06* (0.03,0.14)	0.07 (0.008,0.52)	0.20* (0.11,0.37)
Bacteroides	0.15* (0.06,0.37)	0.08 (0.01,0.63)		
Barnesiella		0.002* (0.001,0.01)	4.91* (1.58,15.27)	
Butyricimonas	0.07* (0.02,0.27)	0.15* (0.04,0.65)		
Odoribacter	0.0014* (0.0006,0.003)	0.0001* (0.00001,0.001)		
Parabacteroides		0.002* (0.001,0.003)		
Prevotella		0.03* (0.005,0.19)		
Alistipes	0.002* (0.001,0.005)	0.0005* (0.0001,0.002)		0.13 (0.03,0.48)
S24-7	0.20* (0.05,0.75)			0.24* (0.09,0.64)
VC2.1-Bac22	0.01* (0.001,0.10)	0.03* (0.005,0.17)		
Ruminococcaceae OTU	rCDI-IBD	rCDI+IBD	UC-rCDI	CD-rCDI
Unspecified	0.23* (0.11,0.47)	0.07* (0.01,0.37)		0.28* (0.18,0.42)
Anaerofilum	0.15* (0.06,0.36)	0.37* (0.16,0.84)		0.43 (0.24,0.76)
Anaerotruncus				0.12 (0.03,0.47)
Faecalibacterium	0.004* (0.002,0.007)	0.003* (0.002,0.004)		
Ocillospira	0.04* (0.01,0.13)			
Ruminococcus	0.02* (0.003,0.14)	0.02* (0.003,0.07)	0.37 (0.23,0.61)	
Subdoligranum	0.01* (0.002,0.07)	0.003* (0.002,0.005)		0.07* (0.02,0.28)
Lachnospiraceae OTU	rCDI-IBD	rCDI+IBD	UC-rCDI	CDrCDI
Anaeorostipes	0.24* (0.08,0.74)			
Blautia	0.44* (0.25,0.78)			
Coprococcus		0.002* (0.001,0.004)		

Dorea	0.003* (0.001,0.008)	0.003* (0.001,0.005)		
Lachnospira		0.113 (0.024,0.527)		
Marvinbryantia	0.09* (0.02,0.47)	0.02* (0.001,0.22)		
Pseudobutyrivibrio	0.03* (0.01,0.16)			
Roseburia		0.17 (0.05,0.61)		
Bacilli OTU	rCDI-IBD	rCDI+IBD	UC-rCDI	CD-rCDI
Unspecified	138.75* (58.52,328.99)	44.62* (12.95,153.76)		
Planomicrobium	190.46* (24.01,1511.17)	120.81* (14.87,981.47)		
Staphylococcus	6.94* (2.37,20.31)		13.24* (2.61,67.31)	
Lactobacillales/ Unspecified	282.10* (99.02,803.69)	115.94* (35.08,383.18)	6.17* (1.84,20.73)	
Enterococcus	147.86* (32.49,672.95)		39.72 (12.42,127.00)	17.23* (5.33,55.67)
Lactobacillus	151.55* (40.69,564.42)	891.53* (300.81,2642.33)	94.61* (29.69,301.42)	
Streptococcacea/ Unspecified	13.50* (4.47,40.79)	9.05 (4.99,16.41)		
Lactococcus	3.42* (1.49,7.84)		18.71 (6.94,50.45)	0.03 (0.004,0.24)
Streptococcus			7.94 (1.95,32.36)	2.73* (1.67,4.44)
Proteobacteria OTU	rCDI-IBD	rCDI+IBD	UCrCDI	CD-rCDI
Unspecified	35.03* (12.71,96.54)	32.85 (8.35,129.20)		
Bilophila		0.004* (0.002,0.008)		0.037* (0.009,0.15)
Gammaproteobacteria/ Unspecified	99.54* (18.80,527.12)	300.78* (83.24,1086.85)	7.42* (2.23,24.66)	26.65 (3.95,179.90)
B38	16.86* (6.24,45.52)	214.04* (23.54,1946.14)		
Enterobacteriaceae/ Unspecified	57.60* (8.71,380.92)	1724.82* (517.90,5744.37)	5.01* (1.77,14.21)	
Citrobacter	14.20* (3.46, 58.35)	1022.05* (240.83,4337.51)		
Enterobacter	154.98* (25.78,931.71)	303.65* (30.73,3000.34)		0.00003* (0.000003, 0.00025)
Escherichia-Shigella	15.59* (4.29,56.68)	43.75* (12.06,158.73)		76.65 (9.39,625.70)
Klebsiella	895.51* (241.57,3319.72)	505.91* (115.21,2221.56)	5.85* (2.07,16.55)	
Other Taxa OTU	rCDI-IBD	rCDI+IBD	UC-rCDI	CD-rCDI
Other Firmicutes OTU				
Christensenellaceae/ Unspecified	0.059* (0.011,0.324)	0.002* (0.001,0.005)		
Anaerofustis		0.039* (0.006,0.263)		
Eubacterium	0.16* (0.07,0.39)			
Anaerococcus			514.3* (41.7,6343.2)	
Parvimonas			6.99* (5.38,9.07)	
Peptoniphilus			9.30 (3.42,25.27)	3.22* (1.61,6.45)
Family-XIII-Incertae-Sedis/ Unspecified		0.003* (0.001,0.01)	0.33* (0.15,0.69)	
Peptococcaceae/ Unspecified	0.003* (0.001,0.02)	0.002* (0.0001,0.038)		
Peptococcus	0.03* (0.02,0.06)	0.26* (0.10,0.63)		0.04* (0.01,0.14)
Turicibacter			5.35* (2.63,10.89)	
Catenibacterium	0.03* (0.005,0.13)	0.004* (0.001,0.013)		
Acidaminococcus	11.53* (2.23, 59.72)	0.023* (0.001,0.43)		
Phascolarctobacterium		0.005* (0.001,0.02)		0.19* (0.17,0.22)
Veillonellaceae/ Unspecified	1706.7* (527.4, 5522.9)	449.6* (152.3,1327.8)		
Megamonas	0.06* (0.01,0.32)	0.001* (0.0001,0.009)	0.002* (0.0001,0.02)	

Megasphaera			14.49* (2.61,80.33)	0.57* (0.42,0.77)
Veillonella	322.2* (125.3,828.6)	332.10* (144.7,762.0)		34.8 (9.36,129.4)
Fusobacteria OTU				
Fusobacteriales/ Unspecified	286.0* (78.1,1047.7)	1734.28* (473.9,6347.1)		
Fusobacterium	640.5* (147.2,2786.8)	3209.9* (875.9,11763)		13.51* (1.88,96.8)
Candidate-division-TM7 out				
Candidate-division-TM7		6.25* (2.18,17.89)		
Verrucomicrobia out				
Akkermansia	6.66* (2.89,15.34)			0.003* (0.0003,0.04)
Cyanobacteria OTU				
4C0d-2				0.07 (0.01,0.40)
Chloroplast				0.51 (0.39,0.67)
Bacteria/ Unassigned OTU				
Unassigned	0.29* (0.15,0.53)	0.09* (0.02,0.41)		0.19 (0.09,0.40)

The expanded dataset with 16S rRNA gene sequence data generated from 7 additional recipients in the rCDI–IBD group, confirmed 47 of 51 OTUs previously reported with a significant estimated pre-FMT recipient/donor ratio.[36] The expanded dataset for the rCDI+IBD recipients generated from 5 additional recipients, including 3 with underlying CD confirmed 54 of 71 OTUs previously reported with a significant estimated pre-FMT recipient/donor ratio.[36] The expanded dataset for the UC-rCDI recipients generated from 4 additional recipients confirmed only 15 of 29 OTUs previously reported with a significant estimated pre-FMT recipient/donor ratio.[36] Increased recipient/donor ratios for Bacilli, Proteobacteria and Fusobacteria OTUs were among those confirmed for the UC - rCDI group. The results of 16S rRNA gene sequence analysis for the fourth recipient group, CD-rCDI, detected significant estimated pre-FMT recipient/donor ratios in 27 OTUs, including decreased ratios in Actinobacteria, Bacteroides and Ruminococcaceae OTUs and increased ratios in Bacilli, Gamma-proteobacteria and Fusobacteria OTUs.

Quantitative targeted analysis of BA metabolites

We initially observed relative peak differences in BA on a subset of samples, which suggested that secondary BA metabolites were reduced not only in a subset of the rCDI–IBD and rCDI+IBD groups, but also in the CD-rCDI group (data not shown).[36] To further pursue these findings, 23 bile acid metabolites including secondary BA epimers (**Table 4**), which have recently been implicated in regulating immune function [30-32], were quantitatively measured in 212 samples in 55 donor/recipient pairs by targeted LC-MS. Prior to FMT, 19 BA metabolites differed significantly from the donor group by a post-hoc Dunns test with Benjamini-Hochberg adjustments (**Table 5**). The unconjugated secondary BA, lithocholic acid (LCA) and deoxycholic acid (DCA), were the predominant metabolites in the healthy donors, but low levels of secondary bile acid epimers, 3-oxo-lithocholic acid (3-oxo-LCA), iso-lithocholic acid (iso-LCA) and isoallo-lithocholic acid (isoallo-LCA) were also detected. In contrast, the unconjugated primary BA, chenodeoxycholic acid (CDCA) and cholic acid (CA), were the predominant metabolites detected in the rCDI-IBD recipients, with significant reduction of secondary BA and epimers. For the rCDI + IBD recipients, in addition to the increased levels of primary unconjugated BA (CDCA and CA) and reduced secondary BA levels, increased levels of conjugated primary BA, taurocholic acid (T-CA), glycochenodeoxycholic acid (G-CDCA) and glycocholic acid (G-CA), were also observed. The BA metabolite profile for the UC-rCDI recipients was very similar to that of the healthy donors, whereas a reduction of unconjugated secondary BA and epimers was observed for the CD-rCDI recipients.

Table 4. Median Pre-FMT BA levels (*pmol/g* wet weight of stool or *μM*) ± IQR in donor and four recipient groups.

BA (*μM*)	Donor n = 55 (2 missing)	rCDI-IBD n = 27 (4 missing)	rCDI+IBD n = 8	UC–rCDI n = 11	CD–rCDI n = 9	P-value
Primary conjugated						
T-CA	2.5 ± 0.0	2.5 ± 295.5	810.8 ±1354.5	2.5 ± 34.2	2.5 ± 36.5	0.0095
G-CDCA	51.3 ± 80.1	177.8 ± 230.5	1339.9 ± 2322.9	45.8 ± 84.7	59.8 ± 85.5	0.0002
G-CA	38.8 ± 77.6	136.5 ± 362.6	1554.6 ± 1797.4	31.1 ± 96.8	46.0 ± 62.1	0.0012
Primary unconjugated						
CDCA	140.1 ± 803.0	2802.2 ± 7396.5	1813.4 ± 2791.5	124.4 ± 258.7	2163.1 ± 5023.2	0.002
CA	74.3 ± 509.8	6194.4 ± 6964.8	3242.1 ± 5514.4	211.7 ± 395.0	4310.7 ± 4189.2	<.0001
CDCA-3-sulfate	29.3 ± 732.7	1949.4 ± 2547.4	803.7 ± 2111.9	515.4 ± 1036.8	341.1 ± 620.8	<.0001
CA-3-sulfate	2.7 ± 124.4	191.9 ± 328.9	30.5 ± 88.2	134.1 ± 299.5	33.0 ± 88.8	0.0002
Secondary conjugated						
G-DCA	40.3 ± 60.6	2.5 ± 45.9	2.5 ± 238.1	2.5 ± 52.9	2.5 ± 0.0	0.0034
Secondary unconjugated						
DCA	5722.2 ± 4678.1	2.6 ± 583.9	2.6 ± 5491.2	4771.9 ± 2523.6	247.7 ± 759.7	<.0001
LCA	10448.3 ± 7510.3	3.5 ± 642.0	3.5 ± 7034.7	7166.1 ± 9287.4	114.3 ± 1452.7	<.0001
UDCA	57.4 ± 302.5	2.5 ± 199.3	60.1 ± 599.7	70.5 ± 372.3	133.9 ± 270.8	0.4932
iso-CDCA	2.5 ± 39.8	35.6 ± 130.2	2.5 ± 36.7	2.5 ± 0.0	126.4 ± 477.9	0.0101
iso-DCA	1866.3 ± 1364.6	3.4 ± 37.4	3.4 ± 706.2	1226.7 ± 933.9	54.3 ± 252.3	<.0001
iso-LCA	1015.5 ± 1127.6	2.7 ± 71.0	2.7 ± 773.1	480.7 ± 750.4	2.7 ± 40.7	<.0001
isoallo-LCA	35.9 ± 119.6	2.5 ± 0.0	2.5 ± 0.0	2.5 ± 32.2	2.5 ± 0.0	<.0001
3-oxo-DCA	1307.0 ± 1041.2	4.5 ± 359.5	4.5 ± 2218.5	931.4 ± 1401.7	4.5 ± 69.1	<.0001
3-oxo-CA	2.5 ± 26.1	36.6 ± 289.7	2.5 ± 26.2	2.5 ± 31.3	77.6 ± 124.7	0.0013
3-oxo-LCA	1018.1 ± 1343.3	2.5 ± 281.5	2.5 ± 902.0	280.8 ± 933.7	2.5 ± 22.6	<.0001
UCA	28.8 ± 178.4	32.2 ± 2087.8	104.0 ± 2372.7	52.1 ± 737.2	114.7 ± 118.6	0.3837
3-oxo-CDCA	2.5 ± 0.0	40.1 ± 362.3	2.5 ± 0.0	2.5 ± 0.0	119.8 ± 476.5	0.0002
DCA-3-sulfate	2.5 ± 644.0	2.5 ± 281.6	2.5 ± 2040.9	921.9 ± 2205.7	55.9 ± 338.2	0.7952
LCA-3-sulfate	68.0 ± 363.2	189.9 ± 302.7	48.0 ± 1316.0	47.5 ± 1046.3	40.5 ± 460.1	0.7004
UCDA-3-sulfate	2.5 ± 266.9	439.9 ± 786.9	59.9 ± 844.0	212.3 ± 624.8	74.5 ± 74.7	0.0008

Table 5. Post-hoc Dunns test P-values with Benjamini–Hochberg adjustments, for 19 pre-FMT median BA metabolite levels in the four recipient groups compared to Donors.

BA	rCDI–IBD	rCDI+IBD	UC–rCDI	CD–rCDI
Primary conjugated				
T-CA	0.5366	0.0027	0.8094	0.7684
G-CDCA	0.0071	0.0038	0.6696	0.7098
G-CA	0.0304	0.0056	0.8355	0.8846
Primary unconjugated				
CDCA	0.0071	0.2396	0.7435	0.0845
CA	<.0001	0.0754	0.9117	0.0340
CDCA-3-sulfate	<.0001	0.1941	0.4037	0.4904
CA-3-sulfate	<.0001	0.9631	0.1090	0.9661
Secondary conjugated				
G-DCA	0.0128	0.5268	0.1825	0.0343
Secondary unconjugated				
DCA	<.0001	0.0153	0.5050	0.0070
iso-CDCA	0.1283	0.9285	0.4617	0.0156
LCA	<.0001	0.0036	0.4413	0.0042
iso-DCA	<.0001	0.0017	0.4614	0.0022
iso-LCA	<.0001	0.0111	0.2232	0.0004
isoallo-LCA	<.0001	0.0036	0.0867	0.0029
3-oxo-DCA	<.0001	0.0385	0.7059	0.0005
3-oxo-CA	0.0048	0.9269	0.8699	0.0382
3-oxo-LCA	<.0001	0.0162	0.1627	0.0004
3-oxo-CDCA	0.0060	0.3418	0.7394	0.0146
UDCA-3-sulfate	0.0002	0.3788	0.3962	0.5887

In order to measure the effect of FMT on the BA metabolites, fitted linear mixed models with pairwise comparisons were conducted for 16 BA metabolites that required log transformation to measure the estimated pre-FMT recipient donor ratios and response post-FMT (**Table 6**). Fitted linear mixed models with pair wise comparisons were conducted for the remaining three BA metabolites that did not require log transformation to measure the estimated donor-pre-FMT recipient differences and response post-FMT (**Table 7**). These results indicate that FMT has a significant effect on restoring secondary BA metabolite levels in rCDI – IBD, rCDI+IBD and CD-rCDI recipients.

Table 6. Estimated pre-FMT recipient/donor BA ratios (95% CI) in the four recipient groups. A ratio > 1 indicates ↑ recipient pre-FMT BA level compared to donor. A ratio of < 1 indicated ↓ recipient pre-FMT level compared to donor. Only the estimated ratios with an unadjusted p-value < 0.05 are listed. The * indicates those BA that also had a significant estimated ratios of post-FMT/pre-FMT ratios at the 1 week and/or 3 month time point, indicating that FMT had a significant effect.

BA	rCDI–IBD	rCDI+IBD	UC–rCDI	CD–rCDI
Primary conjugated				
T-CA		29* (3,333)		
G-CDCA	5* (2,12)	21* (4,100)		
G-CA	6* (2,20)	33* (4,250)		
Primary unconjugated				
CDCA	6* (1.4,29)			13 (1.03,143)
CA	24* (7,77)			26 (4,200)
CDCA-3-sulfate	43* (14,143)			12 (2,77)
CA-3-sulfate	15* (6,38)			
Secondary conjugated				
G-DCA	0.2* (0.07,0.4)			0.2 (0.05,0.85)

Secondary unconjugated			
iso-CDCA	3* (1.01,8)		20* (4,111)
iso-LCA	0.008* (0.003,0.027)	0.27* (0.004,0.207)	0.03* (0.005,0.22)
isoallo-LCA	0.06* (0.03,0.13)		0.11 (0.03,0.41)
3-oxo-DCA	0.014* (0.005,0.04)	0.047* (0.008,0.29)	0.04* (0.008,0.24)
3-oxo-CA	6.6* (2.4,18)		9.3 (1.9,48)
3-oxo-LCA	0.016* (0.004, 0.06)	0.04* (0.004,0.44)	0.017* (0.002,0.15)
3-oxo-CDCA	8.5* (3,23)		7.4 (1.4,38)
UCDA-3-sulfate	27* (9,77)		

Table 7. Estimated donor – pre-FMT recipient BA differences (pmol/g wet weight stool or µM, 95% CI) in the four recipient groups. A positive difference indicates that the donor secondary unconjugated BA level was greater than the pre-FMT recipient level. The (*) indicates significant paired end differences between the pre-FMT and the 1 week and/or 3 month post-FMT (p-value < 0.05).

Secondary unconjugated BA	rCDI–IBD	rCDI+IBD	UC–rCDI	CD–rCDI
DCA	6204* (4444,7694)	3749 (685,6813)		
LCA	10596* (7785,13408)	7968 (3063,12873)		5621 (996,10246)
iso-DCA	1871* (1491,2251)	1314 (656,1972)		715* (94,1335)

Shotgun DNA metagenomics data

Shotgun DNA metagenomics sequencing was accomplished on samples from 32 donor/recipient pairs (118 samples). After removing human sequences, an average of 21,513,616 sequences (range 220,028 - 59,164,588) was obtained for each sample. We initially screened the samples for extended-spectrum beta lactamase (ESBL) genes given the recent report of a fatality from transmission of an ESBL producing *E. coli* from donor to recipient.[42] OXA-137, an ESBL gene was detected in one rCDI no IBD recipient prior to the procedure and at one week post–FMT but not at three months post-FMT.[43] No ESBL genes were detected in the 32 donor stools sequenced.

Conversion of unconjugated primary BA to secondary BA (e.g. CA to DCA, and CDCA to LCA), is mediated by enzymes encoded by 8 genes in the BA-inducible (bai) operon (**Figure 4**), which is carried by a small population of commensal bacteria, including *Clostridium scindens*, *Clostridium hylemonae*, *Clostridium hiranonis*.[29] It has been reported that fecal baiCD gene copy numbers is negatively correlated with CDI in humans,[44] although concomitant presence of C. *scindens* and C. *difficile* has been reported in a metagenomics study.[45] The abundances of the baiCD gene, (coding the 5β-reductase), and the baiE gene, (which encodes the 7α-dehydroxylase), were estimated by translated searches of the shotgun metagenomics sequence data with DIAMOND[40] using the C. *scindens* protein sequence for the baiE and baiCD genes at two threshold levels: 1.) 80% identity over 80% sequence and 2.) 50% identity over 80% sequence to calculate the counts per million (cpm). There were high correlations between the baiE cpm and baiCD cpm at both homology thresholds, consistent with both genes being in the same operon. (**Table 8**). Lowering the threshold for identity from 80% to 50% markedly increased the baiCD gene cpm, but had only a modest effect on the baiE gene cpm. This suggested that a much larger number of bacterial genes, which may include genes not involved in the BA metabolism pathways, share protein sequence homology with the baiCD protein than the baiE encoded 7α-dehydroxylase.

Figure 4. BA metabolic pathways. Chenodeoxycholic acid (CDCA) is synthesized in the liver and conjugated with taurine and glycine before being secreted to the small intestine. Taurochenodeoxycholic acid (T-CDCA) and glycochenodeoxycholic acid (G-CDCA) are then deamidated to chenodeoxycholic acid (CDCA) (1) by gut bacteria containing the bile salt hydrolase (BSH) enzyme. CDCA can be further 7α-dehydroxylated to lithocholic acid (LCA) (2) by the concerted action of 6 enzymes in the bai operon (baiA2, baiB, baiCD, baiE, baiF, and baiH). LCA can be further transformed to 3-oxo-LCA (3) by action of 3α-hydroxysteroid dehydrogenases (3α-HSDH), and transformed to iso-LCA (4) if the ketone at position C3 of 3-oxo-LCA is reduced to a 3β-alcohol, by action of 3β-HSDH, or (5) isoallo-LCA by the concerted action of 5β/α-reductases and 3β-HSDH.

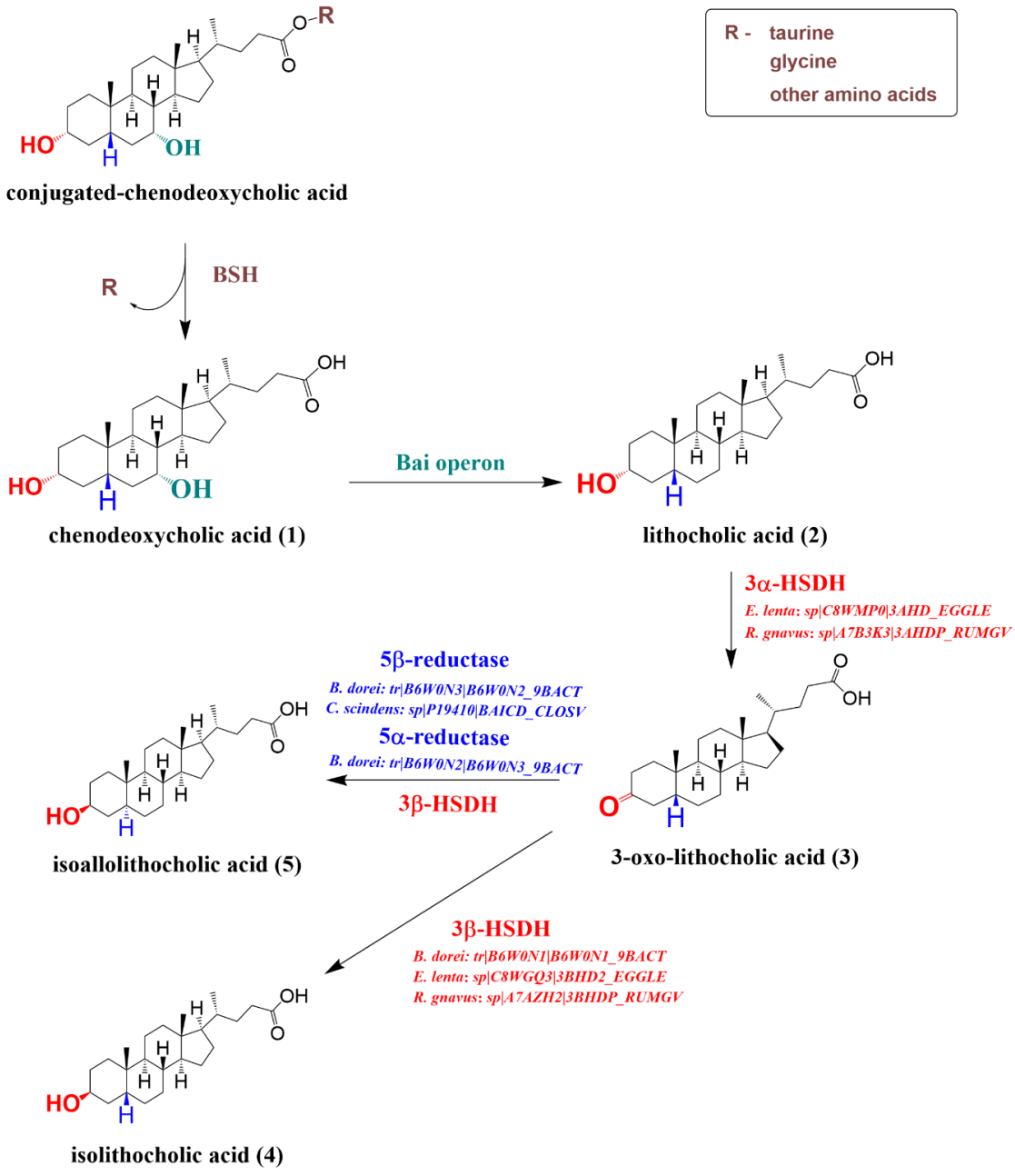
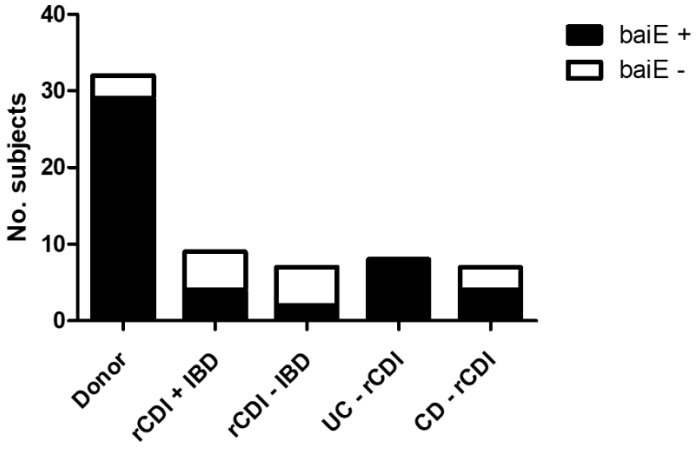


Table 8. Estimated coefficient (95% CI) with baiE cpm as covariate and baiCD cpm as outcome in linear mixed model treating FMT group as clustering effect.

	***baiCD* cpm 80% identity**		***baiCD* cpm 50% identity**	
	Estimated coefficient (95% CI)	P-value	Estimated coefficient (95% CI)	P-value
***baiE* cpm 80% identity**	5.01 (4.65, 5.36)	<.0001	1.18 (0.69, 1.68)	<.0001
***baiE* cpm 50% identity**	17.72 (12.77, 22.66)	<.0001	8.31 (5.49, 11.12)	<.0001

Previous studies have reported a correlation between baiCD gene counts measured using degenerate PCR primers and rCDI.[28,43] Using the same degenerate primers from these previous studieds, we observed multiple amplicons with a large range of sizes after analyzing the PCR reactions by gel electrophoresis. We only observed single amplicon bands using specific C. *scindens* and C. *hiranonis* primers. Consistent with previous studies, a higher proportion of rCDI±IBD recipients lacked detectable baiE cpm compared to donors (**Figure 5**).

Figure 5. Detection of baiE gene (50% identity) DIAMOND search of shotgun metagenomics data) in donors and the 4 recipient groups at baseline.



In contrast to the limited number of baiE 7α-dehydroxylase protein sequences in relatively few strains, there are hundreds of bacterial bile salt hydrolase (BSH) protein sequences distributed among 591 intestinal bacterial strains within 119 genera in the human microbiome.[46] Song et al. categorized these sequences into 8 BSH phylotypes (BSH-T0 to BSH-T7).[46] The custom DIAMOND search used in this study, included 31 BSH protein sequences from eight BSH phylotypes. The abundance of each BSH phlyotype was calculated as the sum of the individual BSH protein sequence cpms at 80% identity. The pattern of BSH phylotypes for the donors, UC-rCDI and CD-rCDI observed in this study was similar to the pattern described for the US population by Song et al. [46] with a relative prominence of the BSH-T6 phylotype. In contrast, the relative abundances of all BSH phylotypes were reduced in the rCDI–IBD and rCDI+IBD recipients prior to FMT.

The 3-α- and 3-β- hydroxysteroid dehydrogenases (HSDHs) from *R. gnavus* and *E. lenta*, metabolize LCA to 3-oxo-LCA and then to iso-LCA (**Figure 4**), which exhibit in vitro activity on T-cell regulation.[30-32] No significant differences in the median *R. gnavus* 3α-HSDH cpm or 3β-HSDH cpm were observed between the donors and the pre-FMT levels of the four recipient groups, although decreased estimated pre-FMT recipient/donor ratios for Ruminococcus genus rRNA gene was observed in the rCDI-IBD and rCDI+IBD groups (**Table 3**). However, a significant difference was observed for median *E. lenta* β-HSDH cpms between the donors and the pre-FMT levels of the four recipient groups (P = 0.0117), with significant difference between the rCDI-IBD and the UC rCDI

groups (P = 0.0184), and between the rCDI + IBD and UC-rCDI groups (P = 0.0200). Similarly, a significant difference was also observed for median *E. lenta* α-HSDH cpms between the donors and the pre-FMT levels of the four recipient groups (P = 0.0065), with significant difference between the rCDI-IBD and the UC-rCDI groups (P = 0.0252), and between the rCDI+IBD and UC-rCDI groups (P = 0.0157).

Because *B. dorei* has been reported to convert iso-LCA to isoallo-LCA, a DIAMOND search was conducted for the putative *B. dorei* 5β-reductase, 5α-reductase and 3β-HSDH enzymes identified by BLAST searches reported by Li et al.[32] The median cpms for each of these candidate genes were significantly decreased in the rCDI+IBD group compared to donors (P = 0.0003, 0.0003, <.0001 for 3β-HSDH, 5β-reductase and 5α-reductase respectively) and the UC-rCDI groups (P = 0.0076, 0.0059, 0.0056) and decreased in the rCDI - IBD groups compared to donors (P = 0.0002, 0.0007, <0.0001) and the UC-rCDI groups. This is consistent with the marked decrease in the estimated pre-FMT recipient donor ratios for the Bacteroides genus rRNA gene in the rCDI - IBD and rCDI + IBD groups (**Table 3**).

In order to integrate the shotgun metagenomics data with the BA metabolite data, we measured the correlation between the abundances of the BA metabolizing genes (cpms) and fecal BA levels using linear mixed models, which clustered values from each FMT group, to calculate the estimated coefficient and 95% confidence interval (CI). Log transformation was applied if the normality assumption was not satisfied. As shown in **Table 9**, significant negative relationships between the baiE gene abundance (cpm) were observed for fecal chenodeoxycholic acid and cholic acid levels with negative estimated coefficients and p-values < 0.05. Significant positive relationships between the baiE gene abundance were observed for fecal secondary BA (DCA and LCA) levels with positive estimated coefficients and p-values < 0.05. Significant positive relationships between the baiE gene abundance were also observed for fecal secondary BA epimer, (iso-LCA acid and isoallo-LCA) and the secondary BA intermediate (3-oxo-LCA) levels. No significant correlation was observed between any of the four HSDHs and either 3-ox-LCA or iso-LCA levels. No significant relationships were observed between the gene counts for any of the three candidate *B. dorea* enzymes (5β-reductase, 5α-reductase and 3β-HSDH) and fecal isoallo-LCA levels, which is present in very low levels across donors and all four recipient groups.

Table 9. Estimated coefficient and 95% CI for selected unconjugated BA for baiE cpm (50% identity threshold) in linear mixed models treating FMT group as clustering effect.

Unconjugated BA	Estimated coefficient (95% CI)	P-value
primary		
CDCA*	-0.57 (-0.84, -0.29)	< .0001
CA*	-0.64 (-0.92, -0.35)	< .0001
secondary		
DCA	455.63 (27.48, 883.77)	0.0373
LCA	1133.55 (329.45, 1937.65)	0.0063
secondary intermediate		
3-oxo-LCA*	0.57 (0.28, 0.86)	0.0002
secondary epimer		
iso-LCA	161.14 (36.43, 285.86)	0.0120
isoallo-LCA*	0.37 (0.16, 0.58)	0.0008

* Indicates that the value was log-transformed, since the normality assumption was not satisfied.

When we examined the correlation between the various BSH phylotype and conjugated primary BA levels, we excluded BSH-T3, which is composed predominantly of lactobacillus BSH genes, from the analysis because of multiple 0 counts in this phylotype. To correct for multiple comparisons between the remaining 7 BSH phylotype relative abundances (cpm) and the fecal levels of the three conjugated primary BAs, T-CA, G-CDCA and G-CA we applied the Bonferroni correction to set the threshold p-value as 0.05/7 = 0.007. Based on this threshold, we observed significantly negative relationships between BSH-T1, BSH-T5 BSH-T6 and BSH-T7 phylotype relative abundances (cpm) and fecal T-CA levels (**Table 10**). We observed significantly negative relationships between BSH-T2 and BSH-T4 phylotype abundances (cpm) and fecal G-CDCA and G-CA. These results may be consistent with varying substrate specificities of bacterial BSH enzymes for the taurine and glycine BA conjugates.[47, 48]

Table 10. Estimated coefficient and 95% CI for each conjugated primary BA for 7 of the 8 BSH phylotype cpm (80 % identity threshold) in linear mixed models treating FMT group as clustering effect. * Indicates that value was log-transformed.

BSH phylotype	Conjugated primary BA	Estimated coefficient (95% CI)	P-value
BSH-T0*	T-CA	0.0002 (-0.0004, 0.0007)	0.5513
	G-CDCA	-0.0001 (-0.0002.0.00001)	0.0674
	G-CA	-0.00006 (-0.0001, 0.00001)	0.0914
BSH-T1	T-CA	-0.06 (-0.09, -0.02)	**0.0064**
	G-CDCA	-0.0091 (-0.0161, -0.0021)	0.0123
	G-CA	-0.0096 (-0.0168, -0.0012)	0.0119
BSH-T2*	T-CA	-0.0004 (-0.0009, 0.00004)	0.0739
	G-CDCA	-0.00013 (-0.0002, -0.0001)	**0.0006**
	G-CA	-0.00014 (-0.0002, -0.0001)	**0.0008**
BSH-T4*	T-CA	-0.0004(-0.0014, 0.0002)	0.0104
	G-CDCA	-0.00013 (-0.0002, -0.0001)	**0.0006**
	G-CA	-0.00014 (-0.0002, -0.0001)	**0.0008**
BSH-T5	T-CA	-0.04 (-0.06, -0.01)	**0.0016**
	G-CDCA	-0.0052 (-0.0090, -0.0015)	0.0071
	G-CA	-0.0055 (-0.0094, -0.0016)	**0.0061**
BSH-T6	T-CA	-0.11 (-0.18,-0.04)	**0.0035**
	G-CDCA	-0.0154 (-0.0278, -0.0029)	0.0162
	G-CA	-0.0164 (-0.0292, -0.0035)	0.0133
BSH-T7	T-CA	-0.05 (-0.07, -0.02)	**0.0027**
	G-CDCA	-0.0063 (-0.0115, -0.0010)	0.0208
	G-CA	-0.0032 (-0.0057, -0.0007)	0.0135

Discussion

We report here the results of exploratory multi-omic analysis of human stools collected during two colonoscopic single donor FMT clinical trials conducted at SBUMC (2013-2020). These two colonoscopic single donor FMT trials were designed to collect four stools from each of four recipient FMT groups. The four stools were the donor stool used in the FMT and the pre-FMT recipient stool, the one week post-FMT recipient stool and the three month post-FMT stool. The four recipient groups were rCDI-IBD, rCDI+IBD, UC-rCDI and CD-rCDI. Our quantitative analysis of primary and secondary BA metabolites in human stool is novel in that secondary BA epimers, which have recently reported to exhibit immunoregulatory activity in vitro, were also measured in this study. Because each recipient selected their donor, who was subsequently screened for potentially pathogenic organisms, this study reports fecal BA levels on one of the larger sets of healthy donors.

Major limitations of this study include the small number of subjects in each of the three IBD recipient groups and that these studies were single arm in design. Unfortunately, the COVID pandemic has essentially forced a halt to these clinical trials. Nevertheless, the samples and multi-omic datasets generated from these samples will continue to provide an important resource for characterizing the effect of FMT on microbial function as further progress is made in identifying bacterial strains and enzymes involved in the biotransformation of BAs and other metabolites.

Decreases in secondary BA have been observed in rCDI and more recently in IBD.[28,49] In this study, we observed a general reduction in the levels of secondary BA including the immunomodulatory derivatives of LCA in the rCDI–IBD, rCDI+IBD and CD-rCDI groups but not the UC-rCDI group at baseline prior to transplant. FMT restored secondary BA levels in the rCDI–IBD group and to various extents in the rCDI+IBD and CD–rCDI groups.

Parallel estimation of the abundance of secondary BA pathway related protein/gene sequences by polymerase chain reaction assays or shotgun metagenomics sequence analysis and correlating these abundances with metabolite levels could potentially help us predict BA metabolic potential in the human gut.[50] Our approach has been to search the shotgun metagenomics data based on protein sequences of relevant BA-metabolizing enzymes. Because of the knowledge base accumulated on the bacterial metabolism of primary to secondary BA, we focused initially on the genes in the bai operon, which are present in only a select number of Clostridium species. Based on our analysis supplemented by qualitative PCR assays, we have identified the baiE gene cpm (measured at 50% identity over 80% sequence) as the biomarker associated with a rate-limiting step in the conversion of unconjugated primary BAs to secondary BAs. It is not clear why no significant correlations between the abundances of other bai operon genes, such as baiCD, and secondary BA metabolite levels were detected in this study. Based on the results of our gel electrophoresis analysis of previously reported

baiCD PCR assays, we suspect there may be significant homology between the baiCD gene sequence and other bacterial genes, which are unrelated to the BA transformation pathway. This could result in overestimation of the true relative abundance of the baiCD gene, due to contributions to the total gene sequence counts from genes outside of the baiE operon with close sequence homology.

Primary BA must be deconjugated prior to transformation to secondary BA. Consequently loss of BSH activity may also affect secondary BA levels. In contrast to the limited number of taxa involved in the initial conversion of unconjugated primary BA to secondary BA, multiple BSH genes from multiple taxa are implicated in deconjugation of conjugated primary BA. Furthermore some of the homologous gene sequences are pseudogenes that may not contribute to BSH activity.[46] Our approach to estimating BSH activity based on BSH gene abundances was to calculate the sum of selected BSH phylotype gene sequence abundances at 80% homology for each of 8 BSH phylotype described by Song et al.[6] This approach yielded donor profiles of BSH phylotype abundances that matched those reported for the US population.[46] BSH phylotype abundances were markedly reduced particularly in the rCDI+IBD recipients prior to FMT compared to healthy donors. Furthermore reduced BSH phylotype abundances were significantly correlated with increased levels of conjugated primary BA levels. These results suggest that rCDI+IBD patients have reduced secondary BA levels due to loss of both 7-α hydroxylase and BSH activities.

It has been reported that centenarians have microbiomes that are enriched for *B. dorei* and additional bacterial strain isolates capable of producing isoallo-LCA acid.[51] Both 5β-reductase, 5α-reductase and 3β-HSDH gene abundances, are decreased in rCDI-IBD and rCDI+IBD recipients compared to healthy donors. In this study, while no significant correlation was observed between these three gene abundances and levels of the isoallo-LCA epimer, significant correlations were observed between baiE gene abundance and both LCA and the isoallo-LCA epimer. Taken together the results suggest that the baiE encoded 7α-hydroxylase step may also be rate limiting for the generation of secondary BA epimers such as isoallo-LCA.

While FMT has proven to be very efficacious for reducing rCDI, it is associated with significant risks. We observed 2 SAEs that were possibly linked to FMT in our clinical trials. These SAEs occurred in one CD patient with rCDI (perianal abscess) and in one CD patient without rCDI (exacerbation of disease finally responding to biologics). Our clinical trials along with others were halted by the FDA after reports of transmission of ESBL to susceptible recipients, which resulted in ESBL bacteremia, and in one case death.[42] We were allowed to resume the clinical trials after developing an additional screen for ESBL in the SBUMC microbiology laboratory, although the screen was only estimated by the lab to be ~90% sensitive for ESBL. Fortunately, shotgun metagenomics analysis did not detect ESBL related genes in our donor stools. In order to reduce the risk of FMT, future clinical trials may shift towards measuring the effect of transplanting pure bacterial cultures rather the human stools to avoid the risk of transplanting potential pathogens.

Conclusion

Secondary BA metabolite levels, including epimers implicated in immune regulation, are reduced in rCDI–IBD, rCDI+IBD and CD–rCDI, but not UC-rCDI recipients compared to donors. Single donor colonoscopic FMT restored many of those levels in these recipients. Integration of shotgun metagenomics and quantitative targeted BA metabolomics analyses indicate that reduction of baiE gene abundance is correlated with reduction of secondary BA metabolite levels. In addition, BSH phylotype gene abundance is also reduced in particularly rCDI + IBD recipients and is negatively correlated with conjugated primary BA levels.

Conflicts of Interest Statement

None of the authors have a conflict of interest nor any financial disclosures with respect to this research or assistance with manuscript preparation.

Funding Statement

Supported by: Simons Foundation 415604 (Li, PI)

and the NIH R25 AI140472 (Cross, PI).

Acknowledgments

We want to thank the recipients and donors for participating in this study. We acknowledge the substantial contribution to the bioinformatics processing of the shotgun metagenomics sequencing data made by Eric Littman in Dr. Eric G. Pamer's laboratory at the U. of Chicago, and contributions of Drs. Ramona Rajapakse, Michael J Clores, Lionel D'Souza, Alexandra Guillaume, Bradley Morganstern and Juan Carlos Bucobo in the Division of Gastroenterology and Hepatology, for performing some of the FMT procedures. We acknowledge the biostatistical consultation

provided by the Biostatistical Consulting Core at School of Medicine, Stony Brook University. We thank Dr. Daniel Littman for his helpful comments on the manuscript.

References

1. Yoon SS, Brandt LJ. Treatment of refractory/recurrent C. difficile-associated disease by donated stool transplanted via colonoscopy: a case series of 12 patients. *J Clin Gastroenterol*. 2010;44(8):562-566. doi: 10.1097/MCG.0b013e3181dac035.

2. Kelly CR, Khoruts A, Staley C, Sadowsky MJ, Abd M, Alani M, et al. Effect of fecal microbiota transplantation on recurrence in multiply recurrent Clostridium difficile infection: A randomized trial. *Ann Intern Med*. 2016;165(9):609-616. doi: 10.7326/M16-0271.

3. Mattila E, Uusitalo-Seppälä R, Wuorela M, Lehtola L, Nurmi H, Ristikankare M, et al. Fecal transplantation, through colonoscopy, is effective therapy for recurrent Clostridium difficile infection. *Gastroenterology*. 2012;142(3):490-6. doi: 10.1053/j.gastro.2011.11.037.

4. Cammarota G, Ianiro G, Tilg H, Rajilić-Stojanović M, Kump P, Satokari R, et al. European consensus conference on faecal microbiota transplantation in clinical practice. *Gut*. 2017;66(4):569-580. doi: 10.1136/gutjnl-2016-313017.

5. Rodemann JF, Dubberke ER, Reske KA, Seo DH, Stone CD. Incidence of Clostridium difficile infection in inflammatory bowel disease. *Clin Gastroenterol Hepatol*. 2007;5(3):339-44. doi: 10.1016/j.cgh.2006.12.027.

6. Ananthakrishnan AN, Binion DG. Impact of Clostridium difficile on inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol*. 2010;4(5):589-600. doi: 10.1586/egh.10.55.

7. Pascarella F, Martinelli M, Miele E, Del Pezzo M, Roscetto E, Staiano A. Impact of Clostridium difficile infection on pediatric inflammatory bowel disease. *J Pediatr*. 2009;154(6):854-8. doi: 10.1016/j.jpeds.2008.12.039.

8. Martinelli M, Strisciuglio C, Veres G, Paerregaard A, Pavic AM, Aloi M, Martín-de-Carpi J, et al. Clostridium difficile and pediatric inflammatory bowel disease: a prospective, comparative, multicenter, ESPGHAN study. *Inflamm Bowel Dis*. 2014;20(12):2219-25. doi: 10.1097/MIB.0000000000000219.

9. Saffouri G, Gupta A, Loftus EV Jr, Baddour LM, Pardi DS, Khanna S. The incidence and outcomes from Clostridium difficile infection in hospitalized adults with inflammatory bowel disease. *Scand J Gastroenterol*. 2017;52(11):1240-1247. doi: 10.1080/00365521.2017.1362466.

10. Hourigan SK, Chen LA, Grigoryan Z, Laroche G, Weidner M, Sears CL, et al. Microbiome changes associated with sustained eradication of Clostridium difficile after single faecal microbiota transplantation in children with and without inflammatory bowel disease. *Aliment Pharmacol Ther*. 2015;42(6):741-52. doi: 10.1111/apt.13326.

11. Fischer M, Kao D, Kelly C, Kuchipudi A, Jafri SM, Blumenkehl M, et al. Fecal microbiota transplantation is safe and efficacious for recurrent or refractory Clostridium difficile infection in patients with inflammatory bowel disease. *Inflamm Bowel Dis*. 2016;22(10):2402-9. doi: 10.1097/MIB.0000000000000908.

12. Khoruts A, Rank KM, Newman KM, Viskocil K, Vaughn BP, Hamilton MJ, et al. MJ. Inflammatory Bowel Disease Affects the Outcome of Fecal Microbiota Transplantation for Recurrent Clostridium difficile Infection. Clin Gastroenterol Hepatol. 2016;14(10):1433-8. doi: 10.1016/j.cgh.2016.02.018.

13. Khanna S, Vazquez-Baeza Y, González A, Weiss S, Schmidt B, Muñiz-Pedrogo DA, et al. Changes in microbial ecology after fecal microbiota transplantation for recurrent C. difficile infection affected by underlying inflammatory bowel disease. *Microbiome*. 2017;5(1):55. doi: 10.1186/s40168-017-0269-3.

14. Tabbaa OM, Aboelsoud MM, Mattar MC. Long-term safety and efficacy of fecal microbiota transplantation in the treatment of Clostridium difficile infection in patients with and without inflammatory bowel disease: A tertiary care center's experience. *Gastroenterology Res*. 2018;11(6):397-403. doi: 10.14740/gr1091.

15. Chin SM, Sauk J, Mahabamunuge J, Kaplan JL, Hohmann EL, Khalili H. Fecal microbiota transplantation for recurrent Clostridium difficile infection in patients with inflammatory bowel disease: A single-center experience. *Clin Gastroenterol Hepatol*. 2017 Apr;15(4):597-599. doi: 10.1016/j.cgh.2016.11.028.

16. Kump PK, Gröchenig HP, Lackner S, Trajanoski S, Reicht G, Hoffmann KM, et al. Alteration of intestinal dysbiosis by fecal microbiota transplantation does not induce remission in patients with chronic active ulcerative colitis. *Inflamm Bowel*

Dis. 2013;19(10):2155-65. doi: 10.1097/MIB.0b013e31829ea325.

17. Kump P, Wurm P, Gröchenig HP, Wenzl H, Petritsch W, Halwachs B, et al. The taxonomic composition of the donor intestinal microbiota is a major factor influencing the efficacy of faecal microbiota transplantation in therapy refractory ulcerative colitis. *Aliment Pharmacol Ther*. 2018;47(1):67-77. doi: 10.1111/apt.14387.

18. Kunde S, Pham A, Bonczyk S, Crumb T, Duba M, Conrad H Jr, et al. Safety, tolerability, and clinical response after fecal transplantation in children and young adults with ulcerative colitis. *J Pediatr Gastroenterol Nutr*. 2013;56(6):597-601. doi: 10.1097/MPG.0b013e318292fa0d.

19. Moayyedi P, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, et al. Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial. *Gastroenterology*. 2015;149(1):102-109.e6. doi: 10.1053/j.gastro.2015.04.001.

20. Mizuno S, Nanki K, Matsuoka K, Saigusa K, Ono K, Arai M, et al. Single fecal microbiota transplantation failed to change intestinal microbiota and had limited effectiveness against ulcerative colitis in Japanese patients. *Intest Res*. 2017;15(1):68-74. doi: 10.5217/ir.2017.15.1.68.

21. Nishida A, Imaeda H, Ohno M, Inatomi O, Bamba S, Sugimoto M, et al. Efficacy and safety of single fecal microbiota transplantation for Japanese patients with mild to moderately active ulcerative colitis. *J Gastroenterol*. 2017;52(4):476-482. doi: 10.1007/s00535-016-1271-4.

22. Paramsothy S, Kamm MA, Kaakoush NO, Walsh AJ, van den Bogaerde J, Samuel D, Leong RWL, et al. Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet*. 2017;389(10075):1218-1228. doi: 10.1016/S0140-6736(17)30182-4.

23. Costello SP, Hughes PA, Waters O, Bryant RV, Vincent AD, Blatchford P, et al. Effect of fecal microbiota transplantation on 8-week remission in patients with ulcerative colitis: A randomized clinical trial. *JAMA*. 2019;321(2):156-164. doi: 10.1001/jama.2018.20046.

24. Fuentes S, Rossen NG, van der Spek MJ, Hartman JH, Huuskonen L, Korpela K, et al. Microbial shifts and signatures of long-term remission in ulcerative colitis after faecal microbiota transplantation. *ISME J*. 2017;11(8):1877-1889. doi: 10.1038/ismej.2017.44.

25. Vermeire S, Joossens M, Verbeke K, Wang J, Machiels K, Sabino J, et al. Donor species richness determines faecal microbiota transplantation success in inflammatory bowel disease. *J Crohns Colitis*. 2016;10(4):387-94. doi: 10.1093/ecco-jcc/jjv203.

26. Gutin L, Piceno Y, Fadrosh D, Lynch K, Zydek M, Kassam Z, et al. Fecal microbiota transplant for Crohn disease: A study evaluating safety, efficacy, and microbiome profile. *United European Gastroenterol J*. 2019;7(6):807-814. doi: 10.1177/2050640619845986.

27. Sokol H, Landman C, Seksik P, Berard L, Montil M, Nion-Larmurier I, et al. Fecal microbiota transplantation to maintain remission in Crohn's disease: a pilot randomized controlled study. *Microbiome*. 2020;8(1):12. doi: 10.1186/s40168-020-0792-5.

28. Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, et al. Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. *Nature*. 2015;517(7533):205-8. doi: 10.1038/nature13828.

29. Ridlon JM, Harris SC, Bhowmik S, Kang DJ, Hylemon PB. Consequences of bile salt biotransformations by intestinal bacteria. *Gut Microbes*. 2016;7(1):22-39. doi: 10.1080/19490976.2015.1127483.

30. Hang S, Paik D, Yao L, Kim E, Trinath J, Lu J, et al. Bile acid metabolites control TH17 and Treg cell differentiation. *Nature*. 2019;576(7785):143-148. doi: 10.1038/s41586-019-1785-z.

31. Campbell C, McKenney PT, Konstantinovsky D, Isaeva OI, Schizas M, Verter J, et al. Bacterial metabolism of bile acids promotes generation of peripheral regulatory T cells. *Nature*. 2020;581(7809):475-479. doi: 10.1038/s41586-020-2193-0.

32. Li W, Hang S, Fang Y, Bae S, Zhang Y, Zhang M, et al. A bacterial bile acid metabolite modulates Treg activity through the nuclear hormone receptor NR4A1. *Cell Host Microbe*. 2021;29(9):1366-1377.e9. doi: 10.1016/j.chom.2021.07.013.

33. Franzosa EA, Sirota-Madi A, Avila-Pacheco J, Fornelos N, Haiser HJ, Reinker S, et al. Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat Microbiol*. 2019;4(2):293-305. doi: 10.1038/s41564-018-0306-4.

34. Paik D, Yao L, Zhang Y, Bae S, D'Agostino GD, Zhang M, et al. Human gut bacteria produce TH17-modulating bile acid metabolites. *Nature*. 2022;603(7903):907-912. doi: 10.1038/s41586-022-04480-z.

35. Connors J, Dunn KA, Allott J, Bandsma R, Rashid M, Otley AR, et al. The relationship between fecal bile acids and microbiome community structure in pediatric Crohn's disease. *ISME J*.

2020;14(3):702-713. doi: 10.1038/s41396-019-0560-3.

36. Mintz M, Khair S, Grewal S, LaComb JF, Park J, Channer B, et al. Longitudinal microbiome analysis of single donor fecal microbiota transplantation in patients with recurrent Clostridium difficile infection and/or ulcerative colitis. *PLoS One*. 2018;13(1):e0190997. doi: 10.1371/journal.pone.0190997.

37. Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut*. 2006;55(6):749-53. doi: 10.1136/gut.2005.082909.

38. Mohammed Vashist N, Samaan M, Mosli MH, Parker CE, MacDonald JK, Nelson SA, et al. Endoscopic scoring indices for evaluation of disease activity in ulcerative colitis. *Cochrane Database Syst Rev*. 2018;1(1):CD011450. doi: 10.1002/14651858.CD011450.pub2.

39. Khanna R, Zou G, Stitt L, Feagan BG, Sandborn WJ, Rutgeerts P, et al. Responsiveness of endoscopic indices of disease activity for Crohn's disease. *Am J Gastroenterol*. 2017;112(10):1584-1592. doi: 10.1038/ajg.2016.580.

40. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. *Nat Methods*. 2015;12(1):59-60. doi: 10.1038/nmeth.3176. Epub 2014 Nov 17.

41. Robertson CE, Harris JK, Wagner BD, Granger D, Browne K, Tatem B, et al. Explicet: graphical user interface software for metadata-driven management, analysis and visualization of microbiome data. *Bioinformatics*. 2013;29(23):3100-1. doi: 10.1093/bioinformatics/btt526.

42. DeFilipp Z, Bloom PP, Torres Soto M, Mansour MK, Sater MRA, et al. Drug-resistant E. coli bacteremia transmitted by fecal microbiota transplant. *N Engl J Med*. 2019;381(21):2043-2050. doi: 10.1056/NEJMoa1910437.

43. Leung V, Vincent C, Edens TJ, Miller M, Manges AR. Antimicrobial resistance gene acquisition and depletion following fecal microbiota transplantation for recurrent Clostridium difficile infection. *Clin Infect Dis*. 2018;66(3):456-457. doi: 10.1093/cid/cix821.

44. Solbach P, Chhatwal P, Woltemate S, Tacconelli E, Buhl M, Gerhard M, et al. BaiCD gene cluster abundance is negatively correlated with Clostridium difficile infection. *PLoS One*. 2018;13(5):e0196977. doi: 10.1371/journal.pone.0196977.

45. Amrane S, Bachar D, Lagier JC, Raoult D. Clostridium scindens is present in the gut microbiota during Clostridium difficile infection: a metagenomic and culturomic analysis. *J Clin Microbiol*. 2018;56(5):e01663-17. doi: 10.1128/JCM.01663-17.

46. Song Z, Cai Y, Lao X, Wang X, Lin X, Cui Y, et al. Taxonomic profiling and populational patterns of bacterial bile salt hydrolase (BSH) genes based on worldwide human gut microbiome. *Microbiome*. 2019;7(1):9. doi: 10.1186/s40168-019-0628-3.

47. Dong Z, Lee BH. Bile salt hydrolases: Structure and function, substrate preference, and inhibitor development. *Protein Sci*. 2018;27(10):1742-1754. doi: 10.1002/pro.3484.

48. Foley MH, O'Flaherty S, Barrangou R, Theriot CM. Bile salt hydrolases: Gatekeepers of bile acid metabolism and host-microbiome crosstalk in the gastrointestinal tract. *PLoS Pathog*. 2019;15(3):e1007581. doi: 10.1371/journal.ppat.1007581.

49. Duboc H, Rajca S, Rainteau D, Benarous D, Maubert MA, Quervain E, et al. Connecting dysbiosis, bile-acid dysmetabolism and gut inflammation in inflammatory bowel diseases. *Gut*. 2013;62(4):531-9. doi: 10.1136/gutjnl-2012-302578.

50. Das P, Marcišauskas S, Ji B, Nielsen J. Metagenomic analysis of bile salt biotransformation in the human gut microbiome. *BMC Genomics*. 2019;20(1):517. doi: 10.1186/s12864-019-5899-3.

51. Sato Y, Atarashi K, Plichta DR, Arai Y, Sasajima S, Kearney SM, et al. Novel bile acid biosynthetic pathways are enriched in the microbiome of centenarians. *Nature*. 2021;599(7885):458-464. doi: 10.1038/s41586-021-03832-5.