In silico analysis of changes in predicted metabolic capabilities of intestinal microbiota after fecal microbial transplantation for treatment of recurrent *Clostridioides difficile* infection

by

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Abstract:

Importance: Fecal microbial transplantation (FMT) is effective in treating recurrent *Clostridioides difficile* (RCDI) infection. The therapeutic efficacy of FMT is thought to be related to restoration of intestinal microbial composition and diversity and key microbially derived compounds, such as short-chain fatty acids (SCFAs) and bile acids. However, it is unknown if other microbially derived metabolites or processes contribute to FMT efficacy.

Aim: To identify changes in KEGG orthology (KO) groups pre-FMT versus post-FMT to explore other potential microbial derived products that may be important for the therapeutic benefits of FMT in treatment of RCDI.

Methods: In-silico analyses of shotgun metagenomics sequencing data was used to examine the change in KO groups comparing pre-FMT to post-FMT stool samples in patients with RCDI. Statistical significance was determined using univariate linear mixed models analyses to estimate changes in KOs pre-FMT and at week 1, 4, and 12 post-FMT, with time (weeks) as the main variable. A literature review was completed to propose biological relevance.

Results: Of the 59,987 KO groups identified by shotgun metagenomics sequencing, only 27 demonstrated a statistically significant change after FMT. These KO groups are involved in many cellular processes including metabolism (10/27), DNA synthesis/replication/repair (8/27), cellular signaling (2/27), substrate transport (6/27), and other miscellaneous functions (1/27). Of interest, KO groups involved in iron homeostasis, glycerol metabolism, and arginine regulation, have been implicated to play an important role bacterial growth and virulence, in addition to modulating the intestinal microbial composition.

Conclusion: Our analyses demonstrate several biologically plausible KO groups that are potentially important in mediating the efficacy of FMT in RCDI, beyond microbial diversity, SCFAs, and bile acids.

Preface:

The raw data used for the basis of the data analysis for this thesis was obtained from a previous study completed by Dr. Kao et al., published as D. Kao, B. Roach, M. Silva, P. Beck, et al., "Effect of Oral Capsule- vs Colonoscopy-Derived Fecal Microbiota Transplantation on Recurrent *Clostridium difficile* Infection," *JAMA,* vol. 318, issue 20, 1985-1993[1]. The original study received research ethics approval from Health Canada (control No. 176567) and the ethic board at the University of Alberta., Secondary analyses of this data set does not require ethics approval.

I was responsible for the data analysis including statistical analyses, in addition to manuscript composition. D. Kao is the supervisory author and was involved in conceptualization and manuscript composition.

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1. Introduction:

Clostridioides difficile is an anaerobic, spore-forming bacterium that can colonize the human gastrointestinal (GI) system, and is the leading cause of hospital-acquired infectious diarrhea[2,3]. Risk factors of *C. difficile* infection (CDI) include antimicrobial use, advanced age, proton pump inhibitors, and inflammatory bowel disease[4,5]. Hospital-acquired *C. difficile* infection (CDI) imposes a huge financial burden to the healthcare system, adding costs upwards of $6.3 billion in the United States (US)[6]. In addition to the significant financial impact of CDI, the clinical burden is substantial, associated with high rates of patient morbidity and mortality[7].

Currently, first line therapy for initial CDI is oral vancomycin or oral fidaxomicin[8]. Vancomycin, as the oral formulation is not systemically absorbed, achieves high colonic concentrations and exhibits broad spectrum antimicrobial activity[9,10]. After an initial course of anti-CDI directed antimicrobial therapy, the rate of recurrence after a primary CDI episode range between 10-30%, reaching approximately 60% after a third episode[11,12]. Recurrence is defined as an episode within 8 weeks of a previous episode, provided the symptoms from the initial episode had completely resolved[13,14]. Fidaxomicin has been shown to reduce the risk of recurrence; however, studies to date have only included patients with a primary episode or first recurrent infection[15]. Limited treatment options exist for recurrent CDI (RCDI). Bezlotoxumab, a monoclonal antibody against *C. difficile* toxin B, has shown efficacy in lowering rates of recurrence compared to placebo, with sustained cure rates of 64% versus 54% with placebo, and the number needed to treat to prevent a recurrence is 10[16]. However, Bezlotoxumab is not approved for use in Canada. In other countries with approval, such as the US, the cost of Bezlotoxumab, is over $4500 USD per dose[17]. Currently, fecal microbial transplantation (FMT) is the most effective treatment for recurrent CDI (RCDI), with efficacy ranging from 60-90% after a single treatment[18,19].

Although highly effective in RCDI treatment, the mechanisms of action of FMT are not well established. The intestinal microbiota plays an important role in CDI pathogenesis where a healthy microbiome can prevent infection and reduce recurrence[20]. Commensal bacteria are involved in key metabolic processes that inhibit *C. difficile* growth, such as the conversion of primary to secondary bile acids and the formation of short chain fatty acids (SCFAs)[21,22]. Antibiotics drive compositional and functional changes to the intestinal microbiota leading to

intestinal dysbiosis, favoring *C. difficile* germination and propagation. This dysbiosis is exacerbated by antibiotics intended for CDI treatment, further increasing the risk of recurrence[23]. FMT is shown to restore fecal microbial composition and increase diversity, thus reestablishing colonization resistance and restoring key microbially derived compounds[21,24-26].

1.1 Effect of *C. Difficile* on Stool Microbial Composition:

Alterations in the gut microbial composition plays a significant role in *C. Difficile* colonization and infection. In CDI patients and asymptomatic carriers, microbial richness is reduced in comparison to healthy individuals[25]. Furthermore, bacterial taxa differences are observed between carriers and CDI patients, which may play a role in the progression to infection in carriers[27]. Stool samples of CDI patients have shown a significant change in microbial composition and diversity, where the relative abundance of *Bacteroidetes* and *Firmicutes* was significantly reduced and while the relative abundance of P*roteobactereia* was increased compared to healthy controls[27-28]. Similarly, dysbiosis is observed in asymptomatic carriers, however, to a lesser extent than CDI patients[27]. Bacterial families within *Bacteroidetes* (i.e., Bacteroidacae) and *Firmicutes* (i.e., *Lachnospiraceae*) play important roles in CDI resistance, maintenance of gut homeostasis, and inhibition of *C. difficile* germination[29]. Following FMT, intestinal microbial ecology is restored in the recipient, resembling that of the stool donor[30]. This transition from dysbiosis to restored microbial composition and diversity is thought to account for the efficacy of FMT. However, much remains unknown as to the specific mechanisms mediating its therapeutic efficacy.

1.2 Bile Acid Conversion and Implications in *C. difficile* Pathogenesis:

Healthy individuals, when compared to first occurrence of CDI and RCDI patients, have a higher ratio of secondary to primary bile acids in their stool[21,31]. Bile acids are produced in the liver, which is also the site of conjugation of bile acids to the amino acids glycine and taurine. Once secreted bile acids enter the small intestine as predominantly primary bile acids[32]. The transformation of tauro- and glycol- conjugated primary bile acids (cholic acid and chenodeoxycholic acid) to secondary bile acids (deoxycholic acid and lithocholic acid) occur in the small intestine and colon; this step is performed by key enzymes produced solely by the gut microbiota, and not humans[32]. The first step of the biotransformation of primary bile acids to secondary bile acids includes bile salt hydrolase (BSH), which hydrolyses the glycine or taurine

group, converting conjugated primary bile acids to their unconjugated form[32]. The second step involves bacterial 7- α-dehydroxylase, which converts the primary bile acids to secondary bile acids[33]. BSH expression has been widely observed amongst most major bacterial species of the gut microbiota[32]. In contrast, 7- α-dehydroxylase is only found in a small group of gut microbial species, predominantly Clostridium clusters XIVa and XI[33].

The stool samples of Patients with CDI exhibit a higher predominance of primary bile acids to secondary bile acids, in addition to reduced expression of BSH hydrolysis activity[21,34]. Bile acid composition is thought to play an important role in the ability of *C. difficile* to undergo various stages of its life cycle[32]. The conjugated primary bile taurocholic acid (TCA), and other cholic acid derivatives, promotes the in-vitro germination of *C. difficile*, where glycine acts as the co-germinant[32]. CspC has been shown to be the *C. difficle* bile acid germinant receptor[32]. Conversely, colonization resistance is promoted by secondary bile acids through their complex interaction with *C. difficile* at multiple stages of the bacterium's life cycle, mainly through the inhibition of germination and/or inhibiting growth of the vegetative form[21]. Particularly, secondary bile acids [(ω-muricholate (MCA), lithocholate (LCA), and ursodeoxycholate (UDCA)] inhibits TCA-mediated germination of *C. difficile* spores[32.] Additionally, murine models demonstrate that secondary bile acids (UDCA, LCA, hyodeoxycholate (HDCA), and deoxycholate (DCA)), inhibits *C. difficile* growth in a dose dependent manner[35]. Further studies have also demonstrated that secondary bile acids, more so than primary bile acids, are able to bind to *C. difficile* TcdB toxin causing significant conformational changes, inhibiting TcdB toxin function by inhibiting binding to the human intestinal surface receptors[36]. The efficacy of FMT is thought to be partially related to restoring this ratio of secondary bile acids to primary bile acids, better resembling healthy donor stool composition[21].

1.3 Short Chain Fatty Acids and Protection Against *C. Difficile* Pathogenesis:

Another well described class of compound, SCFAs, impact *C. difficile* pathogenesis. SCFAs are a product of a healthy microbiome. SCFAs are organic fatty acids produced by bacteria largely through the fermentation of non-digestible carbohydrates, and lesser through fermentation of branched-chain amino acids[37]. The most abundant SCFAs found in the human intestine are acetate, propionate, and butyrate[37]. The concentration of SCFAs in the human gut can be increased by increasing the number of SCFA-producing bacteria, utilizing dietary fiber, resistant

starches, and/or complex carbohydrates[38]. SCFAs play a vital role in maintaining intestinal epithelial integrity and function[22]. SCFAs can protect against damage caused by bacterial toxins by increasing tight junction expression and helps reduce intestinal permeability, thus reducing inflammation and bacterial translocation[22]. Through these mechanisms, SCFAs are likely playing a protective role against CDI, which have been supported by in-vivo murine models where animals with higher levels of SCFAs in their GI tract were less susceptible to CDI[26,31]. Arguably, the most important SCFA in the gut is butyrate. Butyrate enhances the innate antimicrobial immunity and cytokine-mediated immune responses, and plays a critical role in preventing epithelial damage against *C. difficile* toxins[39]. Butyrate can induce the expression of antimicrobial peptides, regenerating islet-derived protein 3 (Reg3) γ and β-defensins and exhibits a large role in preventing inflammation through inhibition of histone deacetylase and NF -κB[40]. Butyrate also promotes neutrophil recruitment to colonic lamina propria during CDI by mediating G protein-coupled receptor activation[40]. Specifically, butyrate binds to GPR43, which is expressed in a variety of epithelial cells and immune cells, and triggers directed neutrophil migration[40]. Intestinal epithelial cells use butyrate as their main carbon source[41]. Growth of facultative anaerobes is inhibited by PPAR γ signaling, where PPAR γ promote β-oxidation and suppress synthesis of inducible nitric oxide synthase in response to butyrate, thereby decreasing the bioavailability of oxygen in the colon and maintaining the colons anaerobic microbial environment[41].

In the gut, SCFA-producing bacteria belong to phyla *Bacteroidetes* and *Firmicutes*[22]. As a result of intestinal dysbiosis in CDI, there is a decreased relative abundance of SCFA-producing bacteria leading to reduced production of SCFAs, potentially contributing to CDI[22].

1.4 Role of Other Metabolites in *C. Difficile* Pathogenesis and Potential Targets of Action for Fecal Microbial Transplantation:

Growth of *C. difficile* and colonization is also highly influenced by the presence of certain amino acids and cholesterol-derived compounds crucial to *C. difficile* metabolism. The Stickland metabolic pathway is the major energy producing pathway utilized by *C. difficile* to generate ATP[42]. Stickland pathway couples anaerobic amino-acid fermentation reactions to sodium/proton gradient formation mediated by the Rnf-complex[43]. The amino acids used in the Stickland metabolic pathway act as either electron donors or acceptors in the coupled oxidation-reduction

reactions[42,43]. Many amino acids can be used in the oxidation pathway, where the electron-donating amino-acid is deaminated or decarboxylated to form NADH; however, only proline or glycine can be used for the reduction reaction to regenerate NAD+[41]. The reduction reactions are facilitated by proline reductase (PrdB, PrdA) and glycine reductase (GrdA, GrdB), where both enzymes are comprised of selenium[42]. Previous studies have demonstrated that proline is the preferred substrate for reduction, where in proline rich environments, glycine reductase expression is reduced; whereas when proline is limited, glycine reductase expression is increased as the NADH/NAD+ ratio increases[42]. The depletion of these essential aminos acids have been implicated in limiting *C. difficile* growth and contributing to CDI resistance.

Although the correlation between the depletion of metabolic substrates, and increased SCFAs and secondary bile acids, in colonization resistance and preventing CDI are well described, little research to date has explored the role of other microbially derived metabolites following successful FMT for RCDI. Furthermore, 16S rRNA sequencing has been more commonly used in prior microbiome studies. Unlike 16s sequencing, shotgun metagenomics sequencing can survey all genomic DNA in a sample, as opposed to being restricted to a specific region of DNA[44]. In this study, we use in-silico analysis of whole genome shotgun metagenomics sequencing data to compare KEGG orthology (KO) groups pre-FMT versus post-FMT to explore potential processes or pathways that may contribute to the therapeutic benefits of FMT in treatment of RCDI.

2. Methods:

2.1 Study Design:

We conducted in-silico analyses to examine the change in KO groups pre-FMT versus post-FMT to propose potential mechanistic pathways accounting for the therapeutic benefits of FMT for treatment of RCDI. This study analyzed previously obtained metagenomics sequencing data collected from the original Kao et al. (2017) study[1], where patients were treated with either oral capsule or colonoscopy delivered FMT. Treatment success post-FMT was defined as the absence of CDI recurrence at 12 weeks. Included patients were adult outpatients, between the ages of 18-90 years, and had at least 3 documented episodes of CDI within 8 weeks of completing a course of treatment. CDI was confirmed with either a. toxin positive by glutamate dehydrogenase and *C*

difficile toxins A/B (Cdiff QuikChek Complete; Techlab) or b. detection of glutamate dehydrogenase and *C difficile* cytotoxin B gene (Cepheid), with resolution of diarrhea for the current CDI episode. Patients were excluded if they had complicated CDI, chronic diarrheal illness, inflammatory bowel disease unless in clinical remission for 3 or more months prior to enrollment, cancer undergoing therapy, subtotal colectomy, colostomy, or ileostomy, dysphagia, life expectancy of less than 3 months, pregnancy, breastfeeding, and conditions requiring antibiotic therapy. Serial stool samples were collected before FMT and at week 1, 4, and 12 after FMT, and frozen at -80°C. Using the FastDNA Spin Kit for Feces (MP Biomedicals), stool microbial DNA was extracted to allow for whole-genome shotgun sequencing. Metagenome libraries were created using the Nextera XT (Illumina) protocol and then sequenced using a paired-end 300-cycle protocol. Due to cost limitations, of the 116 recruited patients (mean [SD] age, 58 [19] years; 79 females [68%]; 105 who completed the trial with 57 randomized to the capsule group and 59 to the colonoscopy group), 46 (23 oral capsule, 23 colonoscopy) patients stool samples were further analyzed by shotgun metagenomics sequencing. Of the 46 patients, 18 patients had complete samples (pre-FMT, weeks 1, 4, and 12 post-FMT) and were included for further analysis in our study. Using metagenomics sequencing data, we are able to map to KO groups (completed by Juan Jovel), using HuMAnN2 analysis pipline[45].

Subsequent statistical analyses were completed to determine which KO groups demonstrated a statistically significant change over time; of those KO groups with a statistically significant change, a literature search was performed to propose potential metabolic pathways involved. The literature search was conducted using PubMed, Google Scholar, and Ovid Medline databases. Peer-reviewed papers outlining gene pathways, role in intestinal dysbiosis, role in protection or propagation of infection, relation to FMT and/or role in *C. difficile* pathogenesis were included.

2.2 Statistical Analyses:

We used univariate linear mixed models to estimate changes in KOs pre-FMT and at week 1, 4, and 12 post-FMT, with time (weeks) as the main variable. The time variable assesses if the KO groups changed significantly, estimating X-unit of change (increase or decrease) per 1-unit of time (weeks). 95% confidence intervals (CI) for the change in biomarker per unit-time were calculated. KO groups that demonstrated an increase were separated from those that decreased. The Bonferroni correction was used to correct p values in both the increase and decrease group,

where bacterial genes with p values less than the corrected p-values were determined to have statistically significant change in expression. All statistical analyses were completed using R Core Team (v4.1.3, 2022) and Microsoft Excel (2019).

2.3 Ethical Considerations:

The study by Kao et al. (2017)[1] received approval from Health Canada (control No. 176567) and the ethic review board at the University of Alberta. The data used in this study has been obtained from the original study.

3. Results:

3.1 Patient demographics:

Of the 46 patients with stool shotgun metagenomics sequencing data, 18 (mean [SD] age, 56 [17.1] years; 14 [77.8%] female; 11 (61.1%) FMT by colonoscopy) had complete data sets at all time points: pre-FMT and at weeks 1, 4, and 12 post-FMT. Baseline characteristics are shown in Table 1.

None of the patients included in the study had a history of HIV/AIDs, inflammatory bowel disease, solid organ or bone marrow transplant, known antibiotic resistance, corticosteroid use, or immunosuppressant use (Table 1).

Table 1: Baseline characteristics of study population

Variable	Study Population (n=18)
Age, mean (SD), y	56.0 (17.1)
Female, No. (%)	14 (77.8)
History of inflammatory bowel disease (IBD), No. (%)	0 (0)
History of HIV/AIDs, No. (%)	0 (0)
History of solid organ or bone marrow transplant, No. (%)	0 (0)
Known antibiotic resistance, No. (%)	0 (0)
Corticosteroid use, No. (%)	0 (0)
Immunosuppressant use, No. (%)	0 (0)
Antibiotic use prior to first episode of CDI, No. (%)	17 (94.4)
FMT by oral capsule, No. (%)	11 (61.1)

3.2 Changes in KOs Following FMT:

Shotgun metagenomics sequencing mapped to 59,987 KO groups. Of these, 93 KO groups demonstrated either an increase or decrease post-FMT, and underwent further analysis to

determine if the change was statistically significant. Statistical significance was determined by the presence of p-values less than the corrected p-value, based on the Bonferroni correction (p-value <0.00053763). Of the 93 KO groups, 2 demonstrated a statistically significant increase post-FMT (Table 2), whereas 25 demonstrated a statistically significant decrease post-FMT (Table 3).

Of the KO groups that increased after FMT, one is involved in DNA replication (K02315), whereas the other is involved in signaling pathways in response to environmental stimuli (K07646). KO groups with an observed decrease post-FMT are implicated in a number of cellular and molecular processes, including metabolism (10/25), DNA synthesis/replication/repair (7/25), cellular signaling (1/25), substrate transport (6/25), and other miscellaneous functions (1/25).

Table 2: KO Groups which increased Post-FMT

Cellular Process	KO Group	Function	p-value	Time (95% CI)
DNA replication	K02315: DNA replication protein DnaC	Accessory protein that facilitates the interaction of DnaB with single-stranded DNA/duplex DNA to aid in DNA replication[46]	1.95 x 10E-8	6.64 (2.89 to 10.39)
Cellular Signaling	K07646: two-component system, OmpR family, sensor histidine kinase KdpD [EC:2.7.13.3]	Senses environmental signals, Regulatory role in potassium transport in combination with KdpE, Potassium homeostasis[47]	7.21E-4	2.45 (1.09 to 3.80)

Table 3: KO Groups which decreased Post-FMT

Cellular Process	KO Group	Function	p-value	Time (95% CI)
	K00925: acetate kinase [EC:2.7.2.1]	Catalyzes the conversion of acetyl phosphate and ADP into acetate and ATP; phosphoryltransferase[48]	0.000222	-4.64 (-6.95 to -2.35)
	K04042: bifunctional UDP-N-acetylglucosamine pyrophosphorylase / Glucosamine-1-phosphate N-acetyltransferase [EC:2.7.7.23 2.3.1.157]	Trimeric bifunctional enzyme responsible for CoA-dependent acetylation of Glc-1-PO(4) to GlcNAc-1-PO(4) and catalyzes uridyl transfer from UTP to GlcNAc-1-PO(4) to form the final products UDP-GlcNAc and pyrophosphate[49]	1.28E-4	-4.38 (-6.49 to -2.27)
	K01652: acetolactate synthase I/II/III large subunit [EC:2.2.1.6]	Catalyzes the conversion of 2 pyruvate molecules into acetolactate in the first common step to synthesize branch-chained amino acids (leucine, valine, and iso-leucine)[50]	0.000513	-6.01 (-9.24 to -2.78)
	K02495: oxygen-independent coproporphyrinogen III oxidase [EC:1.3.99.22]	Catalyzes the oxygen-independent conversion of coproporphyrinogen-III to protoporphyrinogen-IX[51]	0.000279	-4.48 (-6.77 to -2.19)
	K03402: transcriptional regulator of arginine metabolism	Regulates arginine metabolism through negative regulation of operons involved in arginine biosynthesis[52]	3.93E-5	-6.20 (-8.96 to -3.43)
	K02536: UDP-3-O-[3-hydroxymyristoyl]	First enzyme involved in the formation of	4.39E-4	-3.69 (-5.65 to -1.73)

	glucosamine N-acyltransferase [EC:2.3.1.191]	lipid A; lipopolysaccharide biosynthesis[53]		
Metabolism	K08591: glycerol-3-phosphate acyltransferase PlsY [EC:2.3.1.15]	Glycerophospholipid metabolism; catalyzes the transfer of an acyl group for acyl phosphate to glycerol-3-phosphate to form lysophosphatidic acid[54]	1.20E-5	-4.25 (-6.02 to -2.49)
	K00656: formate C-acetyltransferase [EC:2.3.1.54]	Pyruvate metabolism, propionate metabolism, butanoate metabolism; conversion of formate + CoA to pyruvate + CoA[55]	3.04E-4	-4.98 (-7.54 to -2.41)
	K01207: beta-N-acetylhexosaminidase [EC:3.2.1.52]	Lysosomal isoenzyme that releases N-acetylglucosamine and N-acetylgalactosamine from glycoproteins, glycolipids, and glycosaminoglycans[56]	1.77E-4	-2.87 (-4.29 to -1.45)
	K01439: succinyl-diaminopimelate desuccinylase [EC:3.5.1.18]	Catalyzes the hydrolysis of N-succinyl-L-diaminopimelic acid to produce L-diaminopimelic acid and succinate[57]	1.46E-4	-3.92 (-5.83 to -2.01)
	K02313: chromosomal replication initiator protein	ATP-dependent, binds to origin of replication (oriC) to initiate the formation of DNA replication initiation complex, key in initiating and regulation chromosomal replication[58]	8.96E-5	-3.85 (-5.67 to -2.04)

DNA synthesis /replication/repair	K01756: adenylosuccinate lyase [EC:4.3.2.2]	Purine biosynthesis; catalyzes the conversion of SAICA ribotide into aminoimidazole carboxamide ribotide and conversion of succinyladenosine monophospohate to adenosine monophosphate[59]	1.05E-4	-3.93 (-5.80 to -2.06)
	K02528: dimethyladenosine transferase	Ribsosomal maturation, DNA mismatch repair[60]	2.22E-4	-4.10 (-6.15 to -2.04)
	K04047: starvation-inducible DNA-binding protein	Protects bacteria against stressors (starvation, oxidative stress, metal toxicity, or thermal stress) through DNA binding and ferroxidase activity[61]	3.63E-5	-4.77 (-6.88 to -2.65)
	K00526: ribonucleoside-diphosphate reductase beta chain [EC:1.17.4.1]	Catalyzes the biosynthesis of deoxyribonucleotides[62]	1.79E-4	-4.91 (-7.34 to -2.48)
	K01524: exopolyphosphatase / guanosine-5'-triphosphate,3'-diphosphate pyrophosphatase [EC:3.6.1.11 3.6.1.40]	Catalyzes the conversion of guanosine 3'-diphosphate 5'-triphosphate + H2O to guanosine 3',5'-bis(diphosphate) + H^+ + phosphate[63]	4.48E-5	-4.13 (-5.98 to - 2.27)
	K06901: putative MFS transporter, AGZA family, xanthine/uracil permease	Pyrimidine metabolism[64]	3.60E-5	-9.13 (-13.18 to -5.08)

Cellular Signaling	K06207: GTP-binding protein	Signalling protein, binds GTP[65]	5.27E-4	-3.25 (-5.00 to -1.50)
Substrate Transport	K02440: glycerol uptake facilitator protein	Glycerol diffusion[66]	3.10E-4	-5.70 (-8.64 to -2.76)
	K02016: iron complex transport system substrate-binding protein	Part of ATP-Binding Cassette (ABC) family of transporters, iron transport[67]	0.000359	-6.65 (-10.11 to -3.18)
	K10441: ribose transport system ATP-binding protein [EC:3.6.3.17]	Part of ABC family of transporters, ribose import[68]	3.11E-5	-5.34 (-7.69 to -2.99)
	K02761: PTS system, cellobiose-specific IIC component	Recognizes and binds sugars and transports them across the cell membrane into the cytoplasm[69]	1.37E-4	-11.12 (-16.52 to -5.73)
	K02013: iron complex transport system ATP-binding protein [EC:3.6.3.34]	Part of ABC family of transporters, iron transport[67]	6.03E-5	-8.61 (-12.56 to -4.66)
	K02073: D-methionine transport system substrate-binding protein	D-methionine uptake[70]	4.28E-5	-5.92 (-8.57 to -3.26)
Miscellaneous	K00425: cytochrome d ubiquinol oxidase subunit I [EC:1.10.3.14]	Electron/proton transport, part of aerobic respiratory chain, oxidative phosphorylation[71]	2.46E-4	-4.32 (-6.51 to -2.13)

4. Discussion:

Although highly effective in treatment of RCDI, the mechanism of FMT is still unclear. Previous studies have identified the efficacy of FMT is related to restoration of intestinal microbial ecology and restored production of metabolic products of commensal bacteria, such as SCFAs and secondary bile acids. However, less is known on the impact of FMT on the other pathways and the roles they may play. Here we demonstrate changes in KO groups involved in a variety of

vital cellular processes (metabolism, DNA replication, cellular signalling, substrate transport) after FMT. Only two KO groups were found to have statistically significant increase post-FMT: DNA-replication protein DnaC and two-component system, OmpR family, sensor histidine kinase KdpD. DNA-replication protein DnaC has been most commonly documented to be associated with DNA replication in *E.coli* species[72]. DnaC, combined with DnaB, forming the replicative DNA helicase, aiding in the replication process[72]. Although most research associates DnaC with *E.coli*, DnaC has also been found to play a crucial role in replication in other bacterial species. For example, DnaC expression has also been noted to play a crucial role in DNA replication in *B. subtilis*, where *B. subtilis* DnaC functions similarly as *E.coli* DnaB[73]. Interestingly, *B. subtilis* has been found to play a protective role in digestive health by limiting inflammatory responses and strengthening the intestinal barrier[74]. Similarly*, B. subtilis* spores have been found to play a protective role against CDI in murine models[75]. The other KO groups increased post-FMT is two-component system (TCS), OmpR family, sensor histidine kinase KdpD. The two-component system, OmpR family, sensor histidine kinase KdpD is quite complex and is involved in cellular signaling, with an important role in potassium transport and homeostasis[76,77]. However, similarly to DnaC expression, KdpD histidine kinase has been identified in many bacterial phyla, including *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. Due to the non-specific expression of DNA-replication protein DnaC and sensor histidine kinase KdpD, the implication of increased presence of these two KEGG orthology groups post-FMT in either the prevention or promotion of RCDI remains unclear.

Interestingly, the KdpD/KdpE TCS has also been implicated in enhancing the virulence of pathogenic bacterial species[76]. Although not well studied in *C. difficile*, the KdpD/KdpE TCS plays a critical role in enhancing survival of other key pathogenic species, such as *Staphylococcus aureus*, entero-haemorrhagic *Escherichia coli*, *Yersenia petis*, *Salmonella typhimurium*, and mycobacteria[76]. The activity of the KdpD/KdpE TCS regulates gene expression of various virulence loci through direct promotor binding and regulates responses to a variety of virulence-related conditions such as phagocytosis, host hormones, antimicrobial stress, osmotic stress, oxidative stress, and other stress response pathways[76]. Although we demonstrate a change in KdpD sensor histidine kinase post-FMT, our in-silico analysis did not demonstrate a significant change in the KDP operon response regulator, KdpE; both KdpD/KpdE form the TCS

and so it is difficult to determine the significance of the increased KdpD sensor histidine kinase that we observe.

Decreased KO groups post-FMT can be grouped into 4 broad functional domains: metabolism, DNA synthesis/repair/replication, cellular signaling, and substrate transport, with the majority involved in metabolism (10/25). They are found in a wide variety of bacterial species, making it difficult to ascertain specific pathways. However, one KO group of interest is acetate kinase (AK). AK is involved in acetate metabolism, where depending on the metabolic requirements, AK can interconvert acetyl-phosphate and acetate to either generate or catabolize acetyl-CoA[77]. Although *C. difficile* heavily relies on reduction pathways through Stickland reactions, *C. difficile* harbours many diverse energy conserving and producing pathways. Interestingly, *C. difficile* is the only human pathogen with a complete set of genes encoding the Wood-Ljungdahl Pathway (WLP), which allows for reduction of CO2 to acetate through acetogenesis[43]. In the WLP, AK can be used to generate ATP from acetyl-phosphate and the utilization of the WLP is thought to provide a metabolic advantage for *C. difficile* pathogenesis in the human GI system[78,79]. The presence of acetate is particularly interesting because increased acetate is often associated with CDI resistance, where acetate has been found to enhance innate immune response through coordinating neutrophil and ILC3 responses by acetate-FFAR-2 signaling[80]. In addition, like other SCFAs, acetate strengthens the interface between the intestinal lumen and epithelial layer, contributing to host defenses[80]. Previous studies have identified sustained increases in acetate, in addition to other SCFAs such as butyrate, post-FMT and may contribute to the efficacy of FMT in the treatment of RCDI[26].Although we demonstrate a decrease in acetate kinase, that does not necessarily correlate with a decrease in acetate production due to potential alternate pathways involved in the synthesis of acetate.

For amino acid metabolism, we observed a decrease in the following KO groups post-FMT: K01652: acetolactate synthase I/II/III large subunit [EC:2.2.1.6], and K03402: transcriptional regulator of arginine metabolism. Acetolactate synthase I/II/III large subunit is involved in the biosynthesis of valine, isoleucine, and leucine. Previous studies have demonstrated that the presence of valine, isoleucine, leucine, and proline is required for the growth of *C. difficile*, and are commonly used in Stickland oxidative and reductive reactions[81]. Acetolactate synthase

I/II/III large subunit has been isolated in a number of micro-organisms including a wide variety of bacterial species spanning multiple phyla, fungal species, and archaea species. Similarly, K03402: transcriptional regulator of arginine is also expressed in many bacterial species. The transcriptional regulator ArgR inhibits the transcription of multiple genes involved in the biosynthesis and uptake of arginine[82,83]. Additionally, ArgR influences arginine catabolism by coactivating the *astCADBE* operon, which encodes the arginine succinyl transferase pathway involved in arginine catabolism[84].

Arginine is an important amino acid involved in protein synthesis, metabolic gene expression, and modulating cellular and bacterial responses to environmental stressors[85,86]. Additionally, arginine plays an important role in impacting the intestinal microbiome and activating intestinal innate immunity[87]. In colitis mice models increased dietary arginine reduced colitis and was thought to be related to restoring microbial diversity by increasing *Bacteroidetes* relative abundance[88]. Similarly, in nontuberculous mycobacterial pulmonary disease mice models, oral administration of arginine enhanced gut microbiota composition with *Bifidobacterium* species, boosting pulmonary immune defenses through the gut-lung axis[89]. The virulence of pathogenic bacteria, such as Enterohemorrhagic *E. coli* (EHEC), is directly impacted by the presence of arginine[90]. The pathogenicity of EHEC relies on the engagement of type-III secretion system (T3SS) allowing for the translocation of effectors into host cells, leading to colonization and disease[91]. In the presence of arginine, ArgR directly activates the expression of genes that encode the T3SS, inducing EHEC virulence[90]. Although well demonstrated in other pathogenic enteric bacteria, the role of arginine on *C. difficile* toxin production remains controversial and inconclusive. One study found that arginine may have an effect on toxin production in *C. difficile*, where the addition of arginine enhances toxin production in complex media[92]. Another study found that arginine has no effect on toxin production[93,94], and yet a third study showed that arginine insufficiency leads to poor growth but enhanced toxin production[94]. Another study suggested that arginine may contribute to enhanced *C. difficile* growth[81]. These findings, although controversial, highlights the importance of arginine regulation and sensing in the vulnerability of hosts to certain pathogens.

Other KO groups with decreased expression post-FMT are involved in other cellular processes such as DNA replication/repair, cellular signaling, and substrate transport. Although not previously explored in CDI, a number of the isolated KO groups have been explored in other pathogenic models, especially intestinal dysbiosis. Our in-silico analyses identified a decrease in K02440: glycerol uptake facilitator protein, which plays an important role in glycerol diffusion in the cell. Although the importance of glycerol uptake facilitator protein has not been specifically studied in many pathogenic models, glycerol, in itself, is thought to play an important role in modulating intestinal dysbiosis[95]. Glycerol containing probiotics have also been explored as a potential therapy for CDI. Spinler *et al.* (2017)[96] found that human-derived *Lactobacillus reuteri* co-delivered with glycerol was effective against *C. difficile* colonization, but ineffective when treated with either *L. reuteri* or glycerol alone. It is difficult to ascertain the significance of the observed decrease in glycerol uptake facilitator protein in isolation, as we did not observe a change in other enzymes that play a crucial role in the glycerol metabolic pathway, such as glycerol-3-phosphate dehydrogenase.

Similarly, to glycerol, iron concentrations in the gut can influence the composition of intestinal microbiota. Iron is an essential element for all organisms and is linked to many cellular processes, such as transport and storage of oxygen, hormone synthesis, DNA replication, electron transfer, nitrogen fixation, and control of the cell cycle[97,98]. In humans, iron deficiencies have been implicated in a number of disease states, most commonly iron deficiency anemia[99]. As a strategy to improve iron intake, many supplements are fortified with iron; however, typically the absorption of iron is low leading to large amounts of unabsorbed iron to pass into the colon[100]. Although iron oxidation and utilization can be identified across different bacterial phyla, the largest abundance of 'iron-oxidizers' belong to *Proteobacteria*[101]. Many pathogenic enteric bacteria (i.e., *Salmonella, Shigella, E. coli*) compete for unabsorbed dietary iron and largely impacts the growth and virulence of these species[102]. Some protective bacteria, such as *Lactobacilli*, have profound metabolic capabilities allowing them to successfully compete with iron-dependent bacteria in iron-rich environments, but also grow well in iron depletion[103]. Therefore, changes in the presence of unabsorbed iron in the gut can have a major influence on modulating the intestinal microbiota. In a study on Kenyan infants, supplementation with iron-fortified powders promoted intestinal dysbiosis, reducing the abundance of *Bifidobacteria* and

increasing the abundance of *Enterobacteriaceae* (i.e., *E. coli*, *Shigella*) and *Clostridium* species[104]. Similar findings have been demonstrated in other studies assessing the impact of iron fortified foods on intestinal microbiota[100,105]. Interestingly, a study conducted on young women in South India found that women with iron-deficiency had lower relative abundance of *Lactobacillus acidop*hilus in the gut[106]. Although studies have been limited, iron homeostasis is important in modulating the intestinal microbiota where both iron deficiency and iron excess can lead to changes in the composition of intestinal microbiota changes. Beyond influencing intestinal dysbiosis, iron homeostasis can also alter the concentration of SCFAs. Specifically, in rat models, lower levels of propionate and butyrate were observed in luminal iron deficient conditions, notably related to significantly modified gut microbiota composition[107].

In our in-silico analysis, we observe a decrease in ABC transporter groups involved in iron transport: K02016: iron complex transport system substrate-binding protein and K02013: iron complex transport system ATP-binding protein [EC:3.6.3.34]. These proteins play important roles in facilitating the uptake of iron from the periplasm to the cytoplasm of a variety of bacterial species[108]. The observed decrease in these two KO groups post-FMT may be related to the restored diversity of gut microbiota and reduced relative abundance of pathobionts that preferentially utilize iron for growth and virulence. Interestingly, although identified in our study, we did not observe significant changes in other KO groups that play important roles in bacterial iron transport, such as ferrous iron transport protein A and ferrous iron transport protein B. Nevertheless, as iron homeostasis plays an important role in impacting bacterial virulence and growth, and modulating intestinal microbiota, our findings prompt the need for further studies to evaluate how iron metabolism may contribute to FMT efficacy.

A number of other KO groups that we have identified also play a role in bacterial virulence and growth in non-CDI models, namely: K02528: dimethyladenosine transferase[60], K04047: starvation-inducible DNA-binding protein[61] and K01439: succinyl-diaminopimelate desuccinylase [EC:3.5.1.18][57]. It is interesting to note that some enzymes that play an important role in *C. difficile* pathogenesis, such as glutamate dehydrogenase, failed to produce statistically significant change pre- versus post-FMT in our in -silico study. The lack of statistical significance may possibly be more so related to our small patient size. Nevertheless, our study

has identified several biologically plausible KO groups which have not been previously explored as potentially important in mediating efficacy of FMT in RCDI, beyond microbial diversity, SCFAs, and bile acids.

5. Limitations:

Although our study presents interesting findings of potential changes in KO groups after FMT, and hypothesizes mechanisms of action of FMT in treatment of RCDI, we recognize that there are limitations to our study. First, our study is purely exploratory and inferring gene presence based off shotgun metagenomics sequencing data, as we, did not perform metabolomics to correlate the results. Second, shotgun metagenomics sequencing is only as good as the available database; similarly, KEGG orthology groups are only as good as the existing database. Therefore, there are likely many other important metabolites and pathways that will be missed with our in-silico approach. Furthermore, the metagenomics sequencing was done with shallow sequencing, and this will further limit what can be identified through in silico analysis. For example, bacterial genes, BSH, 7- α-dehydroxylase, that are important in RCDI and FMT was not identified in the shotgun metagenomics sequencing results. Third, our patient population (n=18) is small, making our study vulnerable to type II error and potentially suggesting or missing changes that otherwise would have been apparent with a larger population size. Furthermore, our cohort population did not include patients with higher predispositions to CDI, such as those with IBD, which could have implications on the presence of certain KO groups and related bacterial genes. Finally, due to the high efficacy rates of FMT in the treatment of RCDI, all of the patients included in this study were successfully treated for FMT, which means we cannot comment if these changes would also be absent in patients with failed FMT treatment.

To address these limitations, we suggest future studies to include a larger patient cohort, and to include metabolomics analyses. It would also be important to include both successful and failed RCDI cases following FMT. Animal models can also be considered for further validation work.

6. Conclusions:

FMT has emerged as the “standard of care" therapy for RCDI treatment. FMT is both cost-effective and therapeutically effective with high efficacy following a single treatment. Although

effective, the mechanism of action of FMT is not fully understood. Restored intestinal microbial ecology and metabolism of key microbially derived metabolites, such as SCFAs and bile acids, are thought to contribute to the therapeutic benefits of FMT. In this study we demonstrate several KO groups and metabolic pathways that may mediate the efficacy of FMT, such as arginine regulation, glycerol metabolism, and iron homeostasis, which warrant further investigation.

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