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Fecal microbiota transplantation is safe and tolerable in patients with multiple sclerosis: A pilot randomized controlled trial

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Abstract

Background: Patients with MS have an altered gut microbiota compared to healthy individuals, as well as elevated small intestinal permeability, which may be contributing to the development and progression of the disease.

Objective: We sought to investigate if fecal microbiota transplantation was safe and tolerable in MS patients and if it could improve abnormal intestinal permeability.

Methods: Nine patients with MS were recruited and provided monthly FMTs for up to six months. The primary outcome investigated was change in peripheral blood cytokine concentrations. The secondary outcomes were gut microbiota composition, intestinal permeability, and safety (assessed with EDSS and MRI).

Results: The study was terminated early and was subsequently underpowered to assess whether peripheral blood cytokines were altered following FMTs. FMTs were safe in this group of patients. Two of five patients had elevated small intestinal permeability at baseline that improved to normal values following FMTs. Significant, donor-specific, beneficial alterations to the MS patient gut microbiota were observed following FMT.

Conclusion: FMT was safe and tolerable in this cohort of RRMS patients, may improve elevated small intestinal permeability, and has the potential to enrich for an MS-protective microbiota. Further studies with longer follow-up and larger sample sizes are required to determine if FMT is a suitable therapy for MS.

Keywords: Multiple sclerosis, fecal microbiota transplantation, microbiome, intestinal permeability

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Introduction

Multiple Sclerosis (MS) is an autoimmune, inflammatory, demyelinating disease of the central nervous system influenced by genetic susceptibility and environmental factors.[1] The gut microbiota is one such environmental factor that has been implicated in the development and progression of the disease.[2] Past studies have repeatedly demonstrated that MS patients have numerous taxonomic alterations in their gut microbiota composition, including (but not limited to) relative increases in *Pseudomonas*, *Blautia*, *Streptococcus*, and *Akkermansia spp.*, and decreases in *Prevotella*, *Bacteroides*, *Parabacteroides, and Clostridia spp.* compared to healthy individuals.[2] These findings suggest the microbiota as a potential interventional therapeutic target for MS.

The gut microbiota regulates intestinal permeability in a multifactorial manner.[3] Increased intestinal permeability due in part to a perturbed microbiota may

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allow microbial and dietary antigens to pass through the intestinal epithelium unregulated, trigger autoimmune responses in the host, and exacerbate MS pathophysiology.[4,5] Elevated intestinal permeability has been found in 20–73% of MS patients,[3,6] so modulating the gut microbiota in MS patients has the potential to subsequently improve this aspect of the disease.

Fecal microbiota transplantation (FMT) is gaining attention for its efficacy in treating both intestinal and extra-intestinal microbiota related diseases.[7–9] Indeed, it has been previously shown to improve elevated intestinal permeability in patients with non-alcoholic fatty liver disease[10] and may have the same beneficial effect in patients with MS. Due to the links between MS and the gut microbiota, this study aimed to investigate the potential therapeutic benefit of FMT for relapsing-remitting MS (RRMS) patients. It was hypothesized that FMTs from healthy donors without a personal or family history of autoimmune diseases would be safe and well tolerated, decrease elevated gut permeability, shift the balance of cytokines in the peripheral blood to an anti-inflammatory state, and beneficially alter the microbiota composition of RRMS patients. The findings from this study will provide insights into the future use of FMT in the clinical treatment strategy for MS.

Materials and methods

Patient recruitment and group randomization

The original study plan was to enroll 40 patients with RRMS with 20 patients randomized into the early intervention group and 20 patients randomized into the late intervention group. Early intervention patients received one FMT per month for six months in the first six months of the study and were to be monitored for six months post-FMTs. Late intervention patients were recruited to the study and were monitored for six months prior to intervention. They then were planned to receive one FMT per month for six months in the last six months of the study. The study was terminated early upon request of the Research Ethics Board following the unexpected death of the principal investigator (MK). Therefore, total recruitment was stopped early and the total number of FMTs performed per subject were reduced in the late intervention arm. Details of the number of FMTs performed are found in Figure 1 and detailed below. The duration of follow-up was decreased in some patients as detailed below.

Ten patients (three males and seven females) with RRMS were recruited to the study between October 2017 to May 2018 at a neurology clinic at University Hospital in London, ON, Canada (Figure 1). [ClinicalTrials.gov (NCT03183869)] The study was approved by Health Canada as well as Western University's Research Ethics Board (REB: 109306). All patients provided written informed consent. A single patient progressed to secondary progressive MS (SPMS) prior to the first FMT and thus was not included in the analysis. This patient tolerated the FMT and had no change in magnetic resonance imaging (MRI) activity following FMT; this patient's data are reported separately (Supplementary Table 1 and Supplementary Figure 1).

Patients were randomly assigned to either the early (n = 4) or late intervention group (n = 5). Patients were assigned to receive FMTs from either Donor 1 (n = 5) or Donor 2 (n = 4) based on donor availability. Patients 1, 2, 3, 4, 7, and 8 received 6 FMTs; Patient 5 received 5 FMTs; Patients 6 and 9 received 2 FMTs.

Study primary and secondary outcomes

The primary outcome was changes in peripheral blood cytokine concentrations; due to premature study termination and accompanying low sample size, this endpoint was not conclusively addressed, but the most complete data set (baseline vs. 4 weeks post-FMT) is reported here. The secondary outcomes were gut microbiota composition, intestinal permeability, and safety (assessed with EDSS and MRI). A summary of when each patient had each assessment performed can be found in Table 1.

FMT donor selection

Potential donors were screened using our previously published protocol.[11] Briefly, potential donors had their medical histories and physical examinations undertaken by a physician. Blood, stool, and urine were screened for 31 viral, bacterial, fungal, and protozoan agents in addition to biochemical characteristics. Donors were excluded if they engaged in behavior that was high risk for infectious disease transmission. Donors were also excluded if they had a personal and/or family history of malignancy, gastrointestinal disease, autoimmune disease, psychiatric disorder, metabolic syndrome, diabetes, early onset coronary disease, or liver disease as these conditions have been reported to have altered gut microbiome compositions. Two donors were selected for this study. Donor 1 provided FMTs to five patients, and Donor 2 provided FMTs to four patients.

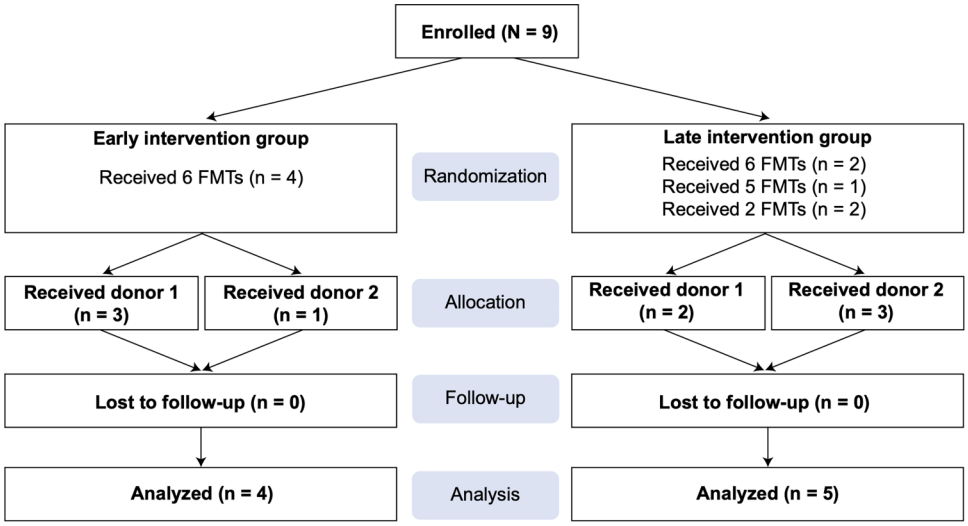

Figure 1. CONSORT diagram of progress through the phases of the pilot randomized controlled trial.

Fecal microbiota transplant

Stool samples of 50–70 g were collected from donors and stored as whole stool at −80 °C for up to three months. The stool samples were thawed in a 37 °C water bath for one hour prior to preparation of the rectal enema. Two hundred and twenty milliliters of saline and 50–70 g of donor stool were placed inside of a BA614/STR filter bag (Seward, Islandia, NY) and were mixed using the Stomacher® 400 Circulator (Seward, Islandia, NY) at 230 rpm for 30 s. The filtered material was then transferred into an AMSure® Enema Bag (Amsino, Pomona, CA). The enema was prepared thirty minutes before the scheduled FMT and was stored at room temperature until the procedure took place. Rectal enema was used because it was a feasible and safe delivery route for repeated FMTs.[12]

Blood, urine, vitals

Routine blood work, urinalysis, and vitals were taken at each visit (once per month for up to twelve months). Blood and urine were collected, and vitals were taken prior to administering the FMT at each monthly appointment. A summary of tests performed can be found in Supplementary Tables 2–4.

Cytokine analysis

Peripheral blood was collected into Ethylenediaminetetraacetic acid coated Vacutainer tubes (BD Biosciences, San Jose, CA) and spun immediately, and the plasma was collected and stored at −80 °C for later analysis.[13] Cytokine levels in plasma samples were quantified using multiplexed immunoassays (HTH17MOG-14 K; Millipore, MA, USA) using a Bio-Plex 200 system (Bio-Rad, CA, USA). The cytokine panel included both pro- and anti-inflammatory cytokines: Interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-31, IL-33, Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon (IFN) γ, macrophage inflammatory protein (MIP)-3α, tumor necrosis factor (TNF) α and TNFβ. Levels of cytokines in plasma samples were determined using a standard curve as per manufacturer's instructions (Millipore). As a comparator, 10 healthy control blood donors (2 males, 7 females, 1 sex not recorded; age 40.3 ± 11.7 years) were locally recruited for one blood draw at the initiation of the study and their plasma was analyzed alongside patient samples.

EDSS and MRI

Clinical and radiological signs of disease activity or progression were measured using the Expanded Disability Status Scale (EDSS)[14] once per month for up to twelve months and MRI at baseline, six months, and twelve months. Due to early study closure the actual number of MRI follow-ups was reduced.

Intestinal permeability

Patients were asked to drink a solution of 5 g of lactulose (Calbiochem®, EMD Millipore Corp., Billerica, MA), 2 g of mannitol powder (BDH®, VWR analytical, Mississauga, ON), 1.5 g of Kool Aid (Kraft

Table 1. Summary of patient assessments.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
Visit 1 (MRI, EDSS, Blood, Urine, Gut Permeability, Toilet Paper Sample)	X FMT	X	X	X FMT	X	X	X FMT	X FMT	X
Visit 2 (EDSS, Blood, Urine, Toilet Paper Sample)	X FMT	X	X	X FMT	X	X	X FMT	X FMT	X
Visit 3 (EDSS, Blood, Urine, Toilet Paper Sample)	X FMT	X	X	X FMT	X	X	X FMT	X FMT	X
Visit 4 (EDSS, Blood, Urine, Toilet Paper Sample)	X FMT	X	X	X FMT	X	X	X FMT	X FMT	X
Visit 5 (EDSS, Blood, Urine, Toilet Paper Sample)	X FMT	X	X	X FMT	X	X	X FMT	X FMT	X
Visit 6 (EDSS, Blood, Urine, Toilet Paper Sample)	X FMT	X	X	X FMT	X	X	X FMT	X FMT	X
Visit 7 (MRI, EDSS, Blood, Urine, Gut Permeability, Toilet Paper Sample)	X	X FMT	X FMT	X	X FMT	X FMT	X	X	X FMT
Visit 8 (EDSS, Blood, Urine, Toilet Paper Sample)	X	X FMT	X FMT	X	X FMT	X FMT	X		X FMT
Visit 9 (EDSS, Blood, Urine, Toilet Paper Sample)	X	X FMT	X FMT	X	X FMT	X			X
Visit 10 (EDSS, Blood, Urine, Toilet Paper Sample)	X	X FMT	X FMT	X	X FMT				
Visit 11 (EDSS, Blood, Urine, Toilet Paper Sample)	X	X FMT	X FMT	X	X FMT				
Visit 12 (MRI (early group) EDSS, Blood, Urine, Gut Permeability (early group) Toilet Paper Sample)	X	X FMT	X FMT		X				
Visit 13 (MRI (late group), EDSS, Blood, Urine, Gut Permeability (late group), Toilet Paper Sample		X	X						

X: indicates patient attended appointment; FMT: indicates patient received an FMT at this appointment.

Foods, Ingleside, ON), 100 g of sucrose, and 450 mL of tap water the evening before their baseline, six month, and twelve month follow-ups. The subjects were asked to collect all the urine that they passed throughout the night and morning of their appointment and store it in a urine collection bottle. This bottle was brought to the clinic, the total volume of urine was recorded and then aliquoted into 10 mL. Concentrations of lactulose, mannitol and sucrose were determined using high performance liquid chromatography.[15]

Fecal sample collection

Fecal samples were collected from patients at each time point using a validated toilet paper sampling method.[16] Briefly, patients collected a visibly soiled piece of toilet paper after passing a stool 1–3 days before their scheduled appointment. The subjects brought the fecal sample in a Fisherbrand™ Opaque Sterile Sampling Bag (Fischer Scientific, ThermoFisher Scientific, Mississauga, ON) to their appointments. The samples were then frozen at - 80 °C until DNA extraction took place. Fecal samples were collected once per month for up to twelve months.

DNA extraction

DNA from the toilet paper samples was extracted using the DNeasy® Powersoil® HTP 96 Kit (Qiagen, Toronto, Ontario, Canada), as per the manufacturer's instructions. Extracted DNA was stored at −20 °C until polymerase chain reaction (PCR) amplification.

DNA amplification

PCR amplification was completed using the Earth Microbiome universal primers, 515F and 806R, which are specific for the V4 variable region of the 16S rRNA gene. Primers and barcode sequences are listed in Supplementary Table 5. PCR reagent setup and amplification was performed as previously described.[17]

Sequencing and data analysis

Sequencing was carried out at the London Regional Genomics Center (http://www.lrgc.ca; London, ON, CAN). Amplicons were quantified using pico green and pooled at equimolar concentrations prior to cleanup using QIAquick (Qiagen, Germantown, Maryland, USA). Using the 600-cycle MiSeq Reagent Kit, paired-end sequencing was carried out as 2×260 cycles with the addition of 5% ϕX-174 at a cluster density of ~1100. Data were exported as raw fastq files (uploaded to NCBI Sequence Read Archive, BioProject ID: PRJNA703364).

The sequencing run yielded a total of 7,756,560 reads, ranging from 5325 to 233,072 reads per sample. After demultiplexing, an average of 13.9% of reads were removed from each sample following quality filtering performed utilizing the DADA2 pipeline (version 1.21.0).[18] Amplicon sequence variants (SVs) were assigned taxonomy with the SILVA (version 138) training set.[19] Samples and SVs were further pruned such that the final dataset used in all downstream analyses retained samples with >1000 reads, SVs present at > 1% abundance in any sample, and did not assign taxonomy to Eukaryota, Mitochondria, or Chloroplast. The final dataset included 236 SVs from 102 samples.

Functional potential of the gut microbiota was determined by inferencing gene content from taxonomic abundances with PICRUST2 software as previously described.[20] All SVs from the donor and MS patient samples were below the nearest-sequenced taxon index (NSTI) <2.0 cutoff recommended for a high level of confidence in predictions. Unstratified metagenome predictions for KEGG Ortholog (KO) and enzyme commission (EC) numbers were normalized to 16S rRNA copies, regrouped to MetaCyc reactions using the default mapping file, and then metabolic pathway abundances were inferenced with the MetaCyc pathway database.

Statistical analysis

Friedman test was used to compare EDSS at baseline to all subsequent time points. Wilcoxon matched-pairs signed rank test was used to compare biochemical test results in MS patients before and after FMT. 95% Confidence intervals were generated for each cytokine analyzed with GraphPad Prism (version 9.2.0). Wilcoxon matched-pairs signed rank test was used to compare cytokine concentrations at baseline to subsequent time points.

Microbiota analyses were performed conservatively in agreement with standards in the field, using CoDaSeq,[21] ALDEx2,[22] MaAsLin2,[23] Vegan[24] and core R packages.[25] All appropriate false-discovery rate corrections were employed, and P values, sample numbers, and names of statistical tests are provided in the main text and figure legends for Figures 2–5. Determination of data stratification, statistical tests, and figure preparation were performed in both GraphPad Prism and R (version 4.0.4). All tests of statistical significance utilized a P value of ≤ 0.05 or effect size of $\geq|1|$ as a cut-off. Custom scripts are available at (https://github.com/kait-al/MS_FMT).

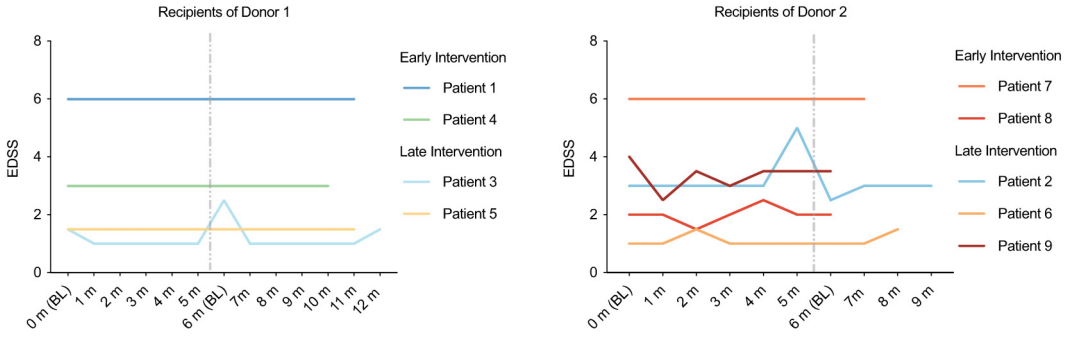

Figure 2. EDSS is stable following multiple FMTs. Patients received one FMT per month for six months and EDSS was measured at each visit. Six patients received all six FMTs and nine patients received at least one FMT. EDSS did not significantly differ at any timepoint compared to baseline (Friedman test).

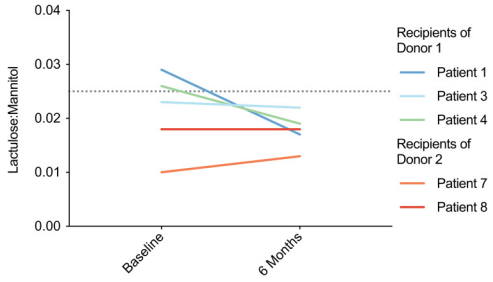

Figure 3. Abnormal small intestinal permeability normalized following six FMTs. Wilcoxon matched-pairs signed rank test was used to compare lactulose:mannitol at baseline and six months (p=0.375). Abnormal intestinal permeability is > 0.025 lactulose:mannitol, which is represented by the gray dotted line. Two patients had elevated small intestinal permeability at baseline and this improved in both post-FMT.

Results

FMT was safe and tolerable in MS patients

Nine patients (three males and six females) with RRMS were enrolled in this study. The patients had a mean age of 44 ± 8.2 years, the average age of diagnosis was 32.1 ± 8.5 years of age, and the average duration of MS was 14.6 ± 6.8 years. Patient demographics and medications can be found in Supplementary Table 6. Five of the nine patients recruited to this study were randomized to the late intervention group and observed for six months prior to receiving an FMT. Adverse events were documented and are summarized in Table 2. Only one adverse event was found to be related to FMT; a patient that developed hives after receiving an FMT. This resolved without treatment and did not recur after subsequent FMTs. Common adverse events included nausea, vomiting, and abdominal discomfort. Adverse events occurring during the observation period prior to FMT in the late intervention group are listed in Supplementary Table 7. No grade 3 or 4 adverse events occurred during the study.

Routine blood work and urinalysis were performed, and vitals were taken at each appointment. There were no significant differences in routine laboratory parameters following the administration of six FMTs (Supplementary Tables 2–4). At baseline the mean EDSS was 3.0 ± 1.9 (n = 9). EDSS was measured at every visit and there was no significant change in EDSS following repeated FMTs (Figure 2). MRI was performed as a safety measure since patients may have MRI activity even in the absence of clinical relapse. No new MRI activity developed when MRIs from baseline were compared to MRIs performed following FMTs (data not shown).

Using a multiplex assay, the levels of cytokines, including pro-inflammatory (e.g. IL-6, IL-15, GM-CSF), regulatory (IL-10), Th17 (e.g. IL-17A, IL-17F, IL-21, IL-22) and Th1 (e.g. INFγ, IL-2, TNFβ) cytokines were measured in the plasma of patients at baseline and 4 weeks post the first FMT (Post-FMT). We did not observe a significant change in the levels of any of the cytokines measured post FMT (n = 9) or compared to healthy controls (n = 10), although the sample size was underpowered to accurately assess this endpoint (Table 3). The n of additional monthly FMTs decreased, further underpowering the evaluation of this endpoint, but again no change in the cytokine levels was detected in the corresponding plasma sample collected (12 weeks (n = 7), 24 weeks (n = 4), 28 weeks (n = 4) and 32 weeks (n = 3); data not shown).

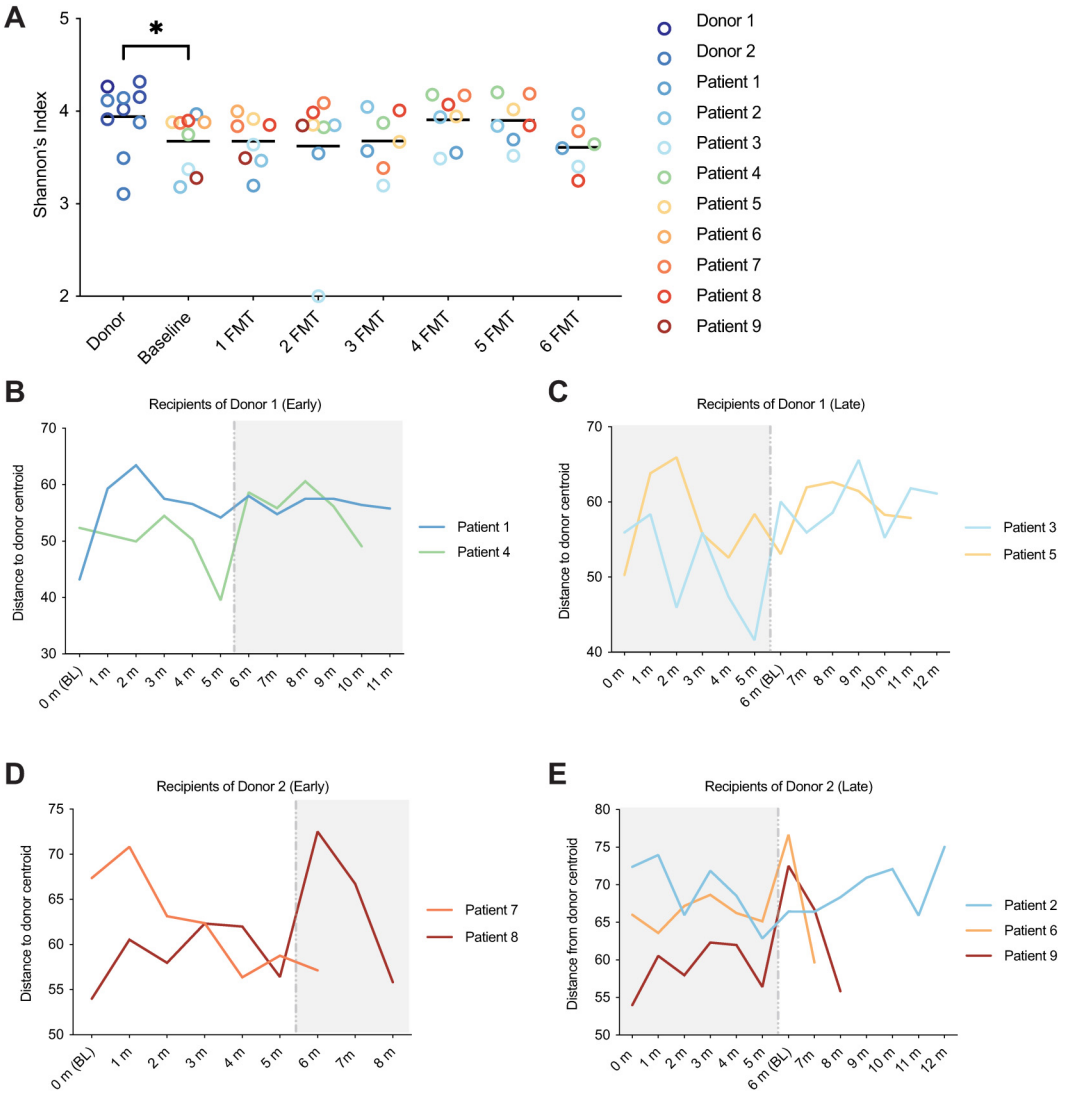

Figure 4. Microbiota diversity metrics of RRMS patients following multiple FMTs. A) Shannon's index of alpha diversity was higher in healthy donors than MS patients at baseline ($p = 0.036$, Mann-Whitney test), but did not significantly change in patients over time ($P = 0.399$, simple linear regression). B-E) Microbiota beta diversity was measured by Aitchison distance. Distance between MS patients and the centroid value of the Donor from which they received FMTs was not significantly different over time. White vs. grey portions of the plots represent FMT and non-FMT treatment periods, respectively.

Elevated small intestinal permeability was normalized following FMT

Six patients had their small intestinal permeability assessed at both baseline and following completion of six FMTs. One patient's urine sample had undetectable levels of lactulose and mannitol at baseline and the samples from this patient were excluded from intestinal permeability analysis, thus only five patients were included in the analysis of intestinal permeability. Two patients had abnormal small intestinal permeability at baseline (>0.025 lactulose:mannitol,[26] which normalized following six FMTs (Figure 3). Both patients with elevated intestinal permeability were randomized to receive FMTs from donor 1.

FMT was associated with significant alterations in the gut microbiota

The most abundant SVs present in the MS patients corresponded to the genera *Bacteroides*. At baseline, patients had significantly higher relative abundance of SVs 11, 30 and 15 (*Bacteroides*, *Blautia faecis*, and *Bacteroides uniformis*) than the healthy donors

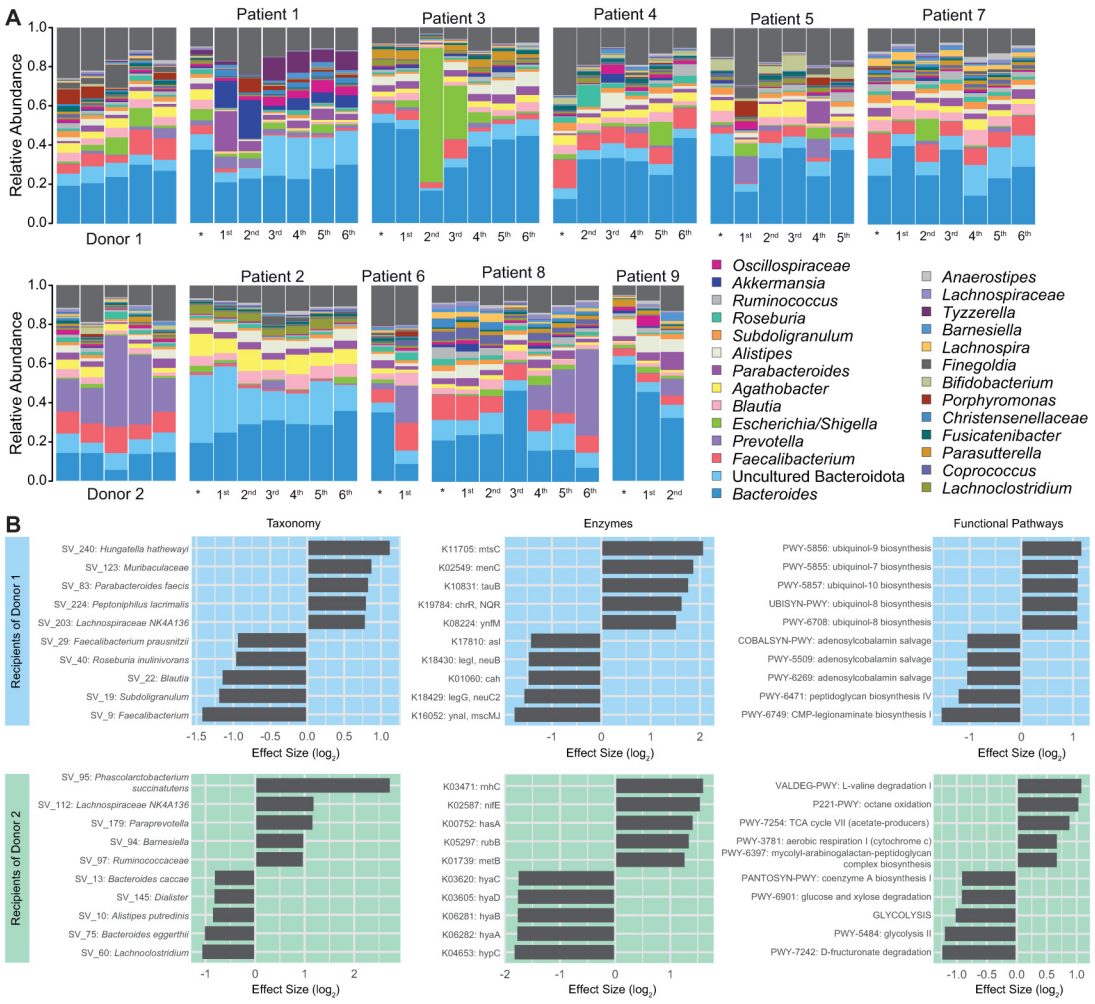

Figure 5. Gut microbiota composition of RRMS patients following multiple FMTs. A) Each vertical bar represents the relative SV abundance within a single sample. Samples are grouped by participant, and in rows corresponding to their respective donor. * Represents the sample taken at baseline before any FMTs were administered, followed by samples taken following the patient's 1st to 6th FMTs. Relative abundance of SVs is colored by genera, with common genera shown in the legend. B) Effect sizes showing differential features were determined with ALDEx2. Comparisons were made for MS patients following their final FMT relative to their baseline, where positive values represent increased relative abundance, and negative values represent decreased relative abundance at the patient's final FMT compared to their baseline. Patients were grouped based on donor, and the ten most divergent taxonomic and functional features are shown for each comparison.

(effect size >1), while donors trended towards higher relative abundance of SVs 102 and 179 (*Prevotella 9* and *Paraprevotella*, effect sizes of 0.96 and 0.92, respectively).

To determine whether patient and sample attributes (metadata) correlated with microbiota variation, CLR-transformed sample-wise Aitchison distances were evaluated using the *envfit* function from the vegan R package. With this approach, several metadata factors were determined to be microbiota confounders, including Patient, FMT donor and Early vs. Late randomization (Table 4). Adjusting for significant confounders with a general linear mixed model in MaAsLin2, several statistically significant multivariate associations were determined to occur over time (Table 5). These included an association with increased *Muribaculaceae* (family) in recipients of Donor 1, and increased peptidoglycan synthesis-associated *ddl* in recipients of Donor 2.

Alpha diversity as measured by Shannon's Index was calculated, and the donors were found to have a fecal

Table 2. Summary of adverse events.

	Treatment Group (n = 9)				Related to Treatment			
	Severity				Severity			
Adverse Event	1	2	3	4	1	2	3	4
Nausea	2	1	0	0	0	0	0	0
Cramping	1	0	0	0	0	0	0	0
Abdominal discomfort	2	0	0	0	0	0	0	0
Vomiting	0	1	0	0	0	0	0	0
Diarrhea	1	1	0	0	0	0	0	0
Difficulty swallowing	1	0	0	0	0	0	0	0
Yeast Infection	0	0	0	0	0	0	0	0
Influenza virus	0	0	0	0	0	0	0	0
Common cold	1	0	0	0	0	0	0	0
Hypertension	0	0	0	0	0	0	0	0
Kidney Stone	0	0	0	0	0	0	0	0
Ear infection	0	0	0	0	0	0	0	0
Extreme fatigue	1	0	0	0	0	0	0	0
Hives	1	0	0	0	1	0	0	0
Acne	1	0	0	0	0	0	0	0

microbiota diversity that was significantly higher than MS patients at baseline ($P = 0.036$, Mann-Whitney test), however, MS patients did not undergo a significant change in fecal microbiota diversity following repeated FMTs (Figure 4A). Microbiota beta diversity as measured by Aitchison distance (CLR-transformed Euclidian distance) was calculated and was compared between MS patients over time to the centroid value of their respective donor (Figure 4 B–E). Beta diversity between the MS patients and their donor did not significantly change over time, likely due to substantial intraindividual longitudinal variability ($P = 0.399$, simple linear regression).

Despite the substantial intraindividual longitudinal variability (Figure 5A), when comparing differentially abundant taxa and functional outputs between patients' baseline and following their final FMT, the groups demonstrated numerous statistically significant alterations which were donor-specific (Figure 5B). Following the MS patient's final FMT, Donor 1 recipients were significantly enriched for SV 240 (*Hungatella hathewayi*), metal cation transporter *mtsC*, menaquinone biosynthesis-associated *menC*, taurine transporter *tauB*, and ubiquinol biosynthesis. Among other features, these patients were depleted in SVs 9, 19, and 22 (*Faecalibacterium*, *Subdoligranulum*, and *Blautia*, respectively), peptidoglycan biosynthesis and adenosylcobalamin salvage. Donor 2 recipients were dramatically enriched in SV 95 (*Phascolarctobacterium succinatutens*) and hyaluronan synthase *hasA*. Among other features, these patients were depleted in glycolysis and Hydrogenase-I (*hyaABCD*) (ALDEx2 effect sizes all >|1|).

Discussion

This study demonstrated that FMT was a safe and tolerable intervention in this group of MS patients, with the potential to normalize intestinal permeability and produce beneficial alterations to the gut microbiota. There were no significant changes observed in patients' MRIs, EDSS, or plasma cytokine levels over the duration of the study, although the premature study termination and very low sample size due to the tragic death of the principal investigator is a significant limitation that may have hindered the detection of further beneficial outcomes of this intervention.

Previous studies identified an imbalance of cytokines in circulating blood in MS patients favoring a pro-inflammatory environment. This includes elevated levels of proinflammatory cytokines TNFα,[27] GM-CSF,[28] IL-1β,[29] and IL-6[30] compared to healthy controls, as well as a reduction in regulatory cytokines.[31] In the current study, the underpowered nature precluded accurate assessment of plasma cytokine levels, so although no significant changes were detected post-FMT or compared to healthy controls, this neither supports nor rejects the use of FMT in MS patients with respect to their cytokine profile.

Table 3. Cytokine concentrations are unchanged following FMT.

Cytokine	Concentration (pg/mL) Pre-FMT (n = 9) Mean ± SD	Concentration (pg/mL) Post-FMT (n = 9) Mean ± SD	P value	Confidence interval of median paired difference
IL-17F	90.0 ± 75.8	72.2 ± 80.28	0.52	−115 −35
GM-CSF	177.8 ± 195.9	211.1 ± 222.8	0.63	−137−137
INFγ	70.4 ± 45.1	53.1 ± 38.7	0.43	−68−25
IL-10	29.7 ± 24.2	20.1 ± 19.3	0.36	−28−11
MIP3a	54.0 ± 35.1	29.6 ± 20.5	0.20	−56−11
IL-12p70	28.5 ± 20.1	24.3 ± 20.0	0.82	−32−8
IL-13	100.4 ± 195.7	99.1 ± 192.3	>0.99	−33−33
IL-15	33.3 ± 39.2	26.7 ± 36.17	0.84	−25−25
IL-17A	20.04 ± 19.5	19.0 ± 23.4	0.94	−15−15
IL-22	451.1 ± 452.1	470.0 ± 511.4	0.94	−388−388
IL-9	39.9 ± 39.9	28.5 ± 34.4	0.50	−40−15
IL-1β	14.8 ± 11.9	10.4 ± 9.3	0.43	−16−3
IL-33	111.0 ± 59.2	73.9 ± 82.7	0.25	−103−28
IL-2	18.1 ± 18.1	14.5 ± 14.7	0.69	−12 −12
IL-21	46.2 ± 30.9	31.0 ± 21.9	0.30	−47−10
IL-4	474.4 ± 423.1	337.2 ± 379.4	0.74	−466−466
IL-23	9668 ± 8562	7787 ± 8744	0.57	−12,732−4252
IL-5	26.3 ± 21.5	21.1 ± 18.5	0.65	−26−10
IL-6	62.0 ± 123.1	66.3 ± 128.6	0.63	−13−13
IL-17E	158.3 ± 159.3	174.4 ± 190.7	0.63	−137−137
IL-27	1672 ± 704.4	1359 ± 919.5	0.52	−1177−597
IL-31	170.0 ± 228.0	240.6 ± 329.5	0.95	−323−303
TNFα	43.5 ± 35.9	51.5 ± 36.4	0.15	−8−18
TNFβ	346.1 ± 610.2	360.0 ± 636.4	0.63	−51−51
IL28A	2111 ± 2578	1668 ± 2569	0.43	−1506−626

Recent data suggesting that FMT can induce a therapeutically useful pro-inflammatory state in patients with metastatic malignancy indicates that future studies in MS patients will need to continue to monitor for safety to assure that proinflammatory processes are not activated.[32]

Current literature demonstrates that 20%-73% of MS patients have abnormal small intestinal permeability;[6] this is comparable to the 40% of patients in this study that had abnormal small intestinal permeability at baseline. Both patients with abnormal intestinal permeability improved to within the normal range (<0.025 lactulose:mannitol[26]) following FMT. The microbiota is known to play a major role in maintaining intestinal barrier integrity, so a study with a larger sample size is needed to determine what changes in the microbiota may have caused this effect.

In this cohort, the MS patients had lower bacterial alpha diversity than the healthy donors at baseline; however, the diversity did not significantly change in the MS patients over time following multiple FMTs. This is in agreement with some,[33] but not other previous studies,[34,35] which cite no difference in Shannon's Index between MS patients and healthy controls. It is therefore unlikely that bacterial alpha diversity is a significant indicator in MS, and the lack of change detected longitudinally in this study is unremarkable. Beta diversity as measured by Aitchison distance between the donor and the MS patients over time did not significantly decrease, meaning that the MS patients did not trend towards a more "donor like" microbiota. Thus, although significant changes were observed post-FMT, the donor microbiota engraftment did not increase following subsequent FMTs. However, longitudinal trends in patients 8 and 9 demonstrate persistent and cumulative effects of the repeated FMTs from Donor 2, indicating a patient- and donor-specific response to the intervention. Indeed, time was a significant covariate of microbiota variation from

Donor 2, indicating that perhaps FMTs from this donor engrafted further with additional treatments.

The microbiota is a major regulator of both the innate and adaptive immune responses,[36] and bacterial species and their metabolites have been shown to both ameliorate and exacerbate MS.[37,38] Here, potentially clinically relevant changes to the gut microbiota following FMT were conserved in MS patients in a donor-dependent manner. Specifically, post-FMT decreases in the MS-associated taxa *Blautia* and *Subdoligranulum* demonstrate that the intervention may exert beneficial taxonomic alterations, although a mechanistic relationship between these taxa and the disease is yet unknown.[2] *Parabacteroides* has previously been negatively associated with MS, and induced a protective, anti-inflammatory T cell response in mice;[2,37] this genus increased following FMT in our cohort. A dramatic enrichment of *Phascolarctobacterium succinatutens* was observed post FMT. This species utilizes succinate to produce the multifaceted health promoting short chain fatty acid propionate.[39,40] Propionate has repeatedly been shown to be depleted in both the plasma and stool of MS patients, and supplementation of propionate slows MS disease progression.[41] Health-associated *Hungatella hathewayi* was also enriched post-FMT.[42] Recent evidence suggests a causal link between *H. hathewayi* depletion in MS patients and decreased serum taurine levels.[42] Subsequent supplementation with *H. hathewayi* can increase circulating taurine and ameliorate MS in a mouse model.[38,42] Importantly, the taurine-transporter *tauB* was also increased in our cohort post-FMT, suggesting a potential role of FMT in modulating the bioavailability of this noteworthy metabolite.[43]

Table 4. Significant covariates of microbiota taxonomic and functional variation.

Microbiota Feature	Metadata	P value
Taxonomy	Patient	>0.001
	FMT Donor	>0.001
	Time	0.104
	Early vs. Late	>0.001
Enzymes	Patient	>0.001
	FMT Donor	>0.001
	Time	0.429
	Early vs. Late	0.863
Functional Pathways	Patient	>0.001
	FMT Donor	>0.001
	Time	0.295
	Early vs. Late	0.282

Further potentially advantageous, MS-ameliorating functional alterations occurred following FMT. Biosynthesis of the anti-inflammatory electron carrier ubiquinol was increased; oral ubiquinone supplementation has been previously shown to reduce oxidative stress, inflammation, fatigue and depression in MS patients.[44–46] The menaquinone (Vitamin K)-related gene *menC* was increased; Vitamin K is depleted in MS patients and its oral supplementation has demonstrated protective effects in MS patients.[47] *HasA*, a gene encoding hyaluronan synthesis was also increased post-FMT. Although hyaluronic acid in the brain is associated with demyelinated lesions,[48] intestinal hyaluronic acid is known to decrease intestinal permeability by upregulating tight junction protein expression.[49] Hyaluronan has also been shown to greatly enrich the abundance of *Akkermansia muciniphila,*[49] a bacterium which is associated with MS but has only recently been linked to lower disability, and the potentially compensatory decreased immune response and amelioration of experimental autoimmune encephalomyelitis.[50] These factors all point to the ability for FMT to potentially exert clinically significant protective and preventative functional alterations to the MS microbiota.

Strengths of this study include that the microbiota of MS patients was followed for six months without any microbiome intervention, as well as for up to six months following FMT. To our knowledge, this is

Table 5. Significant correlations in the microbiota over time after adjusting for confounders. Features are positively correlated with time (during FMT treatment period).

Sample Cohort	Metadata	Feature	Coefficient	FDR
All samples	Time	SV 123: *Muribaculaceae*	0.0005	>0.001
Donor 1 recipients	Time	SV 123: *Muribaculaceae*	0.0006	>0.001
Donor 1 recipients	Time	SV 152: *Muribaculaceae*	0.0005	>0.001
Donor 2 recipients	Time	K01921: *ddl*, D-alanine-D-alanine ligase	4.29×10^{-6}	0.029

the first longitudinal study of the microbiome of MS patients and shows that the gut microbiota composition of MS patients can fluctuate over time without intervention, as has been previously reported in healthy individuals.[51] Repeated FMTs were administered in this study to ensure that changes in the gut microbiome persisted over time. Maintenance FMTs have proved to be beneficial at improving the efficacy of FMT for treating recurrent *Clostridium difficile* infections[52] and were used in a previous FMT study involving three MS patients.[53]

The biggest limitation of this study was the small number of patients. While FMT did not cause any adverse events in these participants and two patients experienced an improvement in intestinal permeability, additional studies with larger more representative cohorts are required to determine the effect of FMT in MS patients. Another limitation was that patients did not receive a bowel prep or antibiotics prior to the initial FMT procedure, and this potentially limited the ability of the donor microbiota to colonize the MS patients. While antibiotics or bowel prep may have improved bacterial colonization after the first FMT, subsequent use of these methods could have had a deleterious effect on donor engraftment from previously administered FMTs. Dietary intake was not evaluated before or during this study and it is possible that changes in the gut microbiota composition could be a result of change in diet. Various MS treatments have been found to cause alterations in gut microbiota composition, and it is also possible that some of the changes observed in gut microbiota composition were caused by treatment instead of FMT.[54] However, we feel that treatment effects were very unlikely as patients did not have any MS treatment changes during the course of the study.

Overall, FMT was well tolerated in this group of MS patients, normalized elevated intestinal permeability, has the potential to beneficially alter the gut microbiota composition, and these alterations can persist with repeated FMT. Larger studies will be required to assess the efficacy of this intervention in the treatment of MS.

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Author contributions

The study was designed by MK, MSS, JBP, and SK. KFA, LJC, and SG prepared the manuscript. KFA, LJC, and SG performed the microbiota analysis. HW extracted DNA from fecal samples. LJC and SNP prepared FMT material. CG administered FMTs and assessed patients at each visit. AW and MK recruited and assessed patients. SM and CC reviewed patients' charts. JM performed the intestinal permeability analysis. MS analyzed MRI data. KP and SK performed cytokine analysis. All authors contributed to the revision of the manuscript.

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Supplemental material

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