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15-strain live biotherapeutic product or same donor fecal microbiota transplant for recurrent *Clostridioides difficile* infection: a randomized phase 1b trial

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Abstract

Fecal microbiota transplant (FMT) is an effective therapy for recurrent *Clostridioides difficile* infection (rCDI) but has undefined composition and poor scalability. *In vitro* manufactured live biotherapeutic products (LBP) enable both scalability and defined strain composition but with higher manufacturing complexity, resulting in few LBP clinical trials. Here we show how an accessible platform to produce human-grade LBPs could accelerate LBP development. We provide regulatory documentation and manufacturing protocols to facilitate translating microbiome

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Author contributions:

Lukas Bethlehem performed the manufacture of MTC01 with assistance from Joseph Eggers, Constantin Ruprecht, Gerold Bongers, Ilaria Mogno, and Zhihua Li. Lorenza Bartu performed the manufacture of the FMT and stability assessments of MTC01 and FMT with assistance from Gina Marke, and Lukas Bethlehem. Ari Grinspan, Phyu Mar, and Sari Feldman conducted the clinical trial. Jeremiah Faith, Gerold Bongers, Lorenza Bartu, Varun Aggarwala, and Constantin Ruprecht conducted the bioinformatics analysis. Graham J Britton performed preclinical testing. Nancy Yang and Elizabeth L Hohmann provided scientific and medical advice. Lukas Bethlehem, Jeremiah Faith, and Ari Grinspan wrote the manuscript, as well as the IND.

Inclusion and ethics statement:

The study included participants of both sexes and multiple age groups (≥ 18 years). Ethical approval was obtained from the (Icahn School of Medicine IRB, STUDY-23-00563), and all participants provided written informed consent prior to enrolment.

advances to human trials. With this platform, we conduct the first direct comparison of the same bacterial strains from donor sourced FMT compared to an *in vitro* manufactured 15-strain LBP drug product MTC01 for the treatment of recurrent *Clostridioides difficile* infection. In a phase 1b randomized controlled trial, 18 of 20 screened patients met eligibility and were randomized equally to one of four arms: low dose FMT (N=4), high dose FMT (N=5), low dose MTC01 (N=4), or high dose MTC01 (N=5) with a 5:1 female to male ratio. The primary outcome of safety was met with 10 adverse events across 8 patients, evenly spread across MTC01 (5 events) and FMT (5 events) recipients and no treatment-related adverse events across all four groups. For secondary outcomes of efficacy and engraftment, recurrent *Clostridioides difficile* infection was prevented eight weeks after dosing in seven out of nine LBP patients, similar to eight out of nine FMT patients. Strain engraftment was high and durable for both FMT and MTC01 with a dose effect for the LBP [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT05911997) identifier: [NCT05911997](https://clinicaltrials.gov/ct2/show/study/NCT05911997).

Introduction

The recent approvals of two stool-derived therapeutics, live-jslm (Rebyota)¹ and live-brpk (Vowst)², for the treatment of recurrent *C. difficile* infection (rCDI) mark a key milestone in the development of live microbial therapeutics. However, the undefined composition of these products also highlights our limited understanding of what is necessary and sufficient for success. Rebyota demonstrates that a complete fecal slurry is an effective rCDI therapy, as supported by numerous prior fecal microbiota transplantation trials^{3–6}, while Vowst demonstrates that the bacterial spore fraction remaining after ethanol treatment of stool is sufficient as an effective rCDI treatment. However, fecal-based drugs have little to no overlap in strain composition across the varied donors used in their production^{7–9}, and therefore the efficacy and safety of such products represent an average across many microbial compositions resulting in uncertainty for individual product batches.

Defined *in vitro* manufactured bacterial strains, known as live biotherapeutic products (LBPs), provide the potential to identify sets of bacterial strains that are sufficient to treat a given disease with a fixed *in vitro* manufactured composition. LBPs have the clear safety profile and consistent efficacy of a single defined product^{10–12} and enable biomanufacturing scaling rather than a linear scaling by the number of donor samples for stool-derived products. Five LBPs have been tested for rCDI to date including one positive phase 2 trial of an 8 strain consortium¹³, one negative phase 2 trial of a 12 strain consortium¹⁴, and three promising smaller trials with larger defined communities^{15–17}. Despite the inherent advantages of LBPs, FMT trials still far outnumber LBP trials as FMTs require only simple equipment to manufacture and have published manufacturing^{18,19} and regulatory²⁰ know-how. As a result, we lack advancement on several fundamental principles for designing LBPs such as 1) how to select the best engrafting strains, 2) whether *in vitro* manufactured strains engraft as well as donor-sourced materials, and 3) how to run safe, low-cost LBP trials.

We developed a novel cost- and space-efficient platform for manufacturing human-grade LBPs for clinical trials. To maximize community access and the therapeutic potential of the microbiome for human health, we share the regulatory documentation and protocols,

as a blueprint for other manufacturing efforts. The manufacturing platform introduces the concept of sterilizable anaerobic chambers with disposable culture flasks as an alternative to space-intensive clean rooms and costly bioreactors. Using our LBP platform, we manufactured two doses of a 15-strain obligate anaerobe defined consortium to address fundamental questions about LBP strain selection, engraftment, and dosing in a phase 1b clinical trial. The strains were isolated from a frequently successful rCDI FMT donor^{8,21}, and selected for LBP inclusion based on engraftment frequency in rCDI FMT patients. In parallel, we produced FMT, at the same doses as the LBP, from the same donor used to isolate the LBP strains. These shared donor-derived FMT and donor-isolated LBP drug products allowed us to compare the safety, efficacy, and engraftment of the same strains manufactured *in vitro* with the full spectrum donor sourced FMT fecal slurry at two matched doses. This first of its kind head-to-head comparison in eighteen subjects with rCDI treated with either FMT or LBP demonstrates that *in vitro* manufactured bacteria have similar efficacy and engraftment to the same microbes derived from their native human colon environment for FMT administration.

Results

Manufacturing of LBP MTC01 and an FMT product from the same donor

Drug candidate “Microbiome Translational Center drug 01” (MTC01) is composed of 15 obligate anaerobe bacterial strains isolated from a single FMT donor (D283) whose stool was used in 28 fecal transplantations to treat rCDI from 2014 to 2017 with 89% prevention of relapse at 8 weeks across all transplants²¹. From five donor stool samples, we isolated and sequenced bacterial strains using a high throughput culturing platform with selective media to enrich different bacterial groups yielding 67 candidate strains from 1387 total isolates across 49 species for a defined alternative to D283 FMT^{7,8} (Figure 1A).

Given prior observations that higher engraftment of FMT and LBP donor strains is predictive of relapse prevention in rCDI trials of microbial therapeutics^{8,13,22–25}, we hypothesized that an LBP comprised of strains with high FMT engraftment frequencies would facilitate high LBP engraftment and protection from relapse, while avoiding the manufacture and administration of non-engrafting strains that would not prevent relapse. Using the empirical engraftment data from recipients of D283 strains administered by FMT⁸, we identified a drug candidate MTC01 – comprised of 15 phylogenetically diverse strains from commonly isolated gut species that frequently engraft in non-relapsing rCDI recipients (Supplementary Table 1). During the design of the consortium, we performed several filtering steps to improve the safety profile and bring the defined consortium to a manageable size for manufacturing. For species with multiple strains isolated from D283 stool, we selected only a single strain to maximize species diversity. We excluded *Escherichia coli* based on its previous association with FMT adverse events^{11,26,27}. We eliminated a strain of *Bacteroides fragilis* as a result of its *in silico* antibiotic resistance profile and the species’ common role in abscesses. Last, we eliminated rare species, as we hypothesized that commonly occurring strains of bacteria are sufficient to treat rCDI given that many different donors have been successful in rCDI treatment with FMT. These criteria led to a consortium with representatives from the *Actinomycetota*, *Bacillota*, and

Bacteroidota phyla. MTC01 is particularly rich in *Bacteroidota*, aligned with previous demonstrations of their high engraftment rate^{8,23} and their potential role in CDI prevention through succinate-driven host immune modulation²⁸.

Prior to GMP manufacturing, we plated each MTC01 strain from the culture libraries onto solid agar to obtain individual bacterial colonies (Figure 1A). We inoculated a liquid culture with a single colony of each strain to generate research cell banks (RCB) for strain characterization. RCB vials were used to identify that all 15 strains were obligate anaerobes and did not interfere with microbiological test methods for non-sterile products, defined by the United States Pharmacopeia (USP), USP<61> (enumeration of total aerobic microbial, yeast and mold) or USP<62> (detection of specific pathogens) assays. Each strain has at least seven antibiotic susceptibilities, and all strains share a susceptibility to three of the tested antibiotics, allowing us to control MTC01 in case of adverse events in the clinical trial (Supplementary Table 1). We used an established manufacturing and banking system for producing MTC01, generating Master Cell Banks (MCB), Drug Substance (DS), and Drug Product (DP), to ensure traceability, genetic stability, and quality control following regulatory guidance²⁹ (Figure 1A). To shorten the time to the clinic, we did not generate Working Cell Banks for this production of MTC01. MCB, DS, and DP manufacturing was performed according to GMP standards in a dedicated vinyl anaerobic chamber with an integrated port to sterilize the chamber environment and the equipment with ionized hydrogen peroxide before each production run (Figure 1B). Strains were grown individually in either side of the two-sided anaerobic chamber to prevent internal cross-contamination, utilizing single-use, sterile consumables. The production of MCB, DS and DP was documented in batch records and concluded within 9 months (Extended Data Table 1). All GMP cell banks were stored in a dedicated -80°C freezer (Figure 1B). Since rCDI FMT trials have used widely varied doses, typically defined in grams of stool rather than a direct measure of microbial potency, a high dose and a low dose drug product were designed for both the LBP and FMT with colony forming units (CFU) as the potency measure.

Each MCB and DS strain was batch fermented in an animal product free complex medium for 12–54 hours at 37°C. After fermentation, cells were washed with USP-grade phosphate buffered saline before resuspension in USP-grade formulation buffer. To generate MTC01 DP, DS aliquots from each of the 15 strains were thawed and combined in a sterilized anaerobic chamber to reach a target cumulative high dose of 1×10^{11} CFU or a low dose of 1×10^{10} CFU. For twelve of the fifteen MTC01 strains, an equal potency of viable cells (CFU) was added to the DP to reach the desired cumulative target dose in a total volume of 250 mL. For *Bacteroides caccae*, *Eubacterium rectale* and *Dorea longicatena* with insufficient viable cells after DS production, the highest strain dose possible was used (Supplementary Table 2). Identity criteria for release of MCB and DS were met with over 48 high quality MALDI-TOF spectra (Bruker Biotyper, confidence score >1.8) from independent bacterial colonies matching the correct Bruker library entry for the MTC01 strain produced. DP identity release was met with over 200 high quality spectra matching to the 15 MTC01 strains. Acceptable potency range for high dose was 5×10^{11} - 6×10^{10} CFU and for low dose 5×10^{10} - 6×10^9 CFU. All MCB, DS, and DP passed USP<61> and

USP<62> testing (Extended Data Table 2). The Right First Time manufacturing percentage was 53%, 80%, and 100% for the MCB, DS, and DP respectively.

To generate FMT drug product, two fecal samples from D283 were individually homogenized and filtered (Seward Stomacher EVO 400) to generate two batches of concentrated fecal slurry that were frozen until FMT DP dilution. An aliquot of each concentrated fecal slurry was treated likewise, frozen and thawed, whereupon its potency was assessed by CFU. This potency was then used to dilute the concentrated fecal slurries into two FMT DP batches that met the same high dose and low dose CFU release criteria as MTC01 (Extended Data Table 3). Both the FMT and MTC01 DPs used the same formulation buffer in a volume of 250 mL and were stored at -80°C until the day of colonoscopic delivery. We found robust stability of cumulative DP potency at -80°C (Figure 1C). We also found MTC01 cumulative potency to be stable under the clinical application conditions that included up to 4 hours thaw time and up to 1 hour time in ambient aerobic conditions outside of the storage bottle prior to colonic infusion (Extended Data Figure 1B). We did, however, find that strains *D. longicatena* and *E. rectale* might decrease in potency after longer storage periods (Extended Data Figure 1A).

Regulatory considerations were discussed with the FDA via a pre-investigational new drug (preIND) application with an FDA written response. An IND application was subsequently submitted with all manufacturing details and a clinical study protocol with no hold. Pre-IND communications, the IND (29067), the master batch records (MBRs), the study protocol, and the statistical analysis plan are provided to facilitate other manufacturing efforts (supplementary files preIND, IND, MBR, Study Protocol, Statistical Analysis Plan). Institutional research board (IRB, STUDY-23-00563) approval was obtained for a single site trial at Mount Sinai Hospital registered on 2023-06-12 at [clinicaltrials.gov](https://clinicaltrials.gov/NCT05911997) (NCT05911997).

Phase-1b clinical trial of MTC01 and FMT for rCDI

MTC01 and FMT drug products from the same human donor were tested in a phase 1b clinical trial at Mount Sinai Hospital enrolling individuals ≥ 18 years old with 2 or more qualifying CDI episodes occurring within the prior 6 months, inclusive of the current episode. A qualifying episode was defined as CDI symptoms starting within 60 days of randomization with ≥ 3 unformed stools/day for ≥ 2 days that is clinically consistent with CDI, documented positive stool test for toxigenic *C. difficile* (toxin EIA or PCR) for the current episode within 60 days of randomization, and received standard-of-care CDI antibiotics for the current episode for 10–42 days with adequate clinical response to the antibiotics defined as ≤ 3 unformed stools/day defined as Bristol stool scale of 6–7 for ≥ 2 days. Subjects treated with LBP or FMT in the prior 6 months were excluded. All outcomes of the trial are presented here with no plans to present additional outcomes elsewhere.

Patients enrolled into the study were block randomized (block size = 4) into either low-dose FMT, high-dose FMT, low-dose MTC01, or high-dose MTC01 (Figure 2A and Supplementary Table 3). Prior to colonoscopic drug administration, subjects were taken off all antibiotics for 48 hours and underwent a standard colonoscopy bowel preparation within 12 hours of their procedure (Figure 2B). The microbial therapeutic was administered to the right colon by colonoscopy. The prespecified primary endpoint of safety was determined

by the incidence of serious adverse events (SAE) and adverse events (AE) up to 24 weeks. Prespecified secondary endpoints were 1) recurrence of CDI up to 8 weeks after treatment with recurrence defined as positive *C. difficile* toxin EIA as well as diarrhea (≥ 3 loose stools in 24h period, defined as Bristol stool scale 6–7, for 2 days), 2) the engraftment of MTC01 and FMT in the fecal microbiome 2 weeks after treatment, and 3) MTC01 and FMT related taxonomic changes in the fecal microbiome 2 weeks after treatment. Other prespecified outcome measures were recurrence of CDI up to 24 weeks and microbiome changes up to 26 weeks. An early version of the study protocol listed an ECG assessment. However, ECG was not included on the schedule assessments in the IND submission and subsequent trial due to its limited utility and to reduce patient burden. In error, it was not removed from the Outcomes section of the study protocol.

Twenty subjects were assessed for eligibility with 18 patients dosed (5 high dose FMT, 4 low dose FMT, 5 high dose MTC01, 4 low dose MTC01) and 2 screen failures. The first patient was enrolled January 8, 2024, and the last patient was enrolled June 4, 2025. The median patient age was 59.5 with 15 females and 3 males (Table 2, Supplementary Table 3, Extended Data Table 4). The higher enrolment of female participants reflects the higher incidence of rCDI in females^{30,31}, higher female recipients of FMT³², and high female proportion in prior trials^{1,2,13}. We did not pre-specify formal sex-stratified efficacy analyses given the small cohort size. For the primary endpoint of safety, one patient had a severe AE due to severe rCDI requiring hospitalization, and two patients had moderate AEs. In addition, there were 7 mild AEs observed in 6 total patients (Table 1). The 10 adverse events across 8 patients were evenly spread across MTC01 (5 events) and FMT (5 events) recipients. These events were considered unrelated to the study intervention due to the presence of comparable findings in the control group and their consistency with the expected post-infection course of recurrent CDI.

For the secondary endpoint of recurrence of CDI up to 8 weeks after treatment^{2,13,33,34}, three of the eighteen subjects had a recurrence with success rates of 100%, 75%, 80%, and 75% across high dose FMT (5 of 5), low dose FMT (3 of 4), high dose MTC01 (4 of 5), and low dose MTC01 (3 of 4) respectively (Figure 2A; Table 2; Table S4). Among the recurrences, one occurred in a high dose patient and two occurred in low dose patients for success rates of 90% and 75% in the high dose and low dose recipients respectively. The median age of the three patients that recurred was 72 compared to a median age of 59 in non-recurring patients. Both recurrences to MTC01 occurred in the first week, while the FMT recurrence occurred at week 8. No additional patients recurred out to the final assessment at week 24 for both the MTC01 and FMT treated individuals.

Engraftment of MTC01 and FMT strains in rCDI recipients

To assess the prespecified secondary outcome of engraftment of strains from D283 FMT product and the *in vitro* manufactured 15 strain consortium isolated from D283 in rCDI recipients, we collected recipient stool samples prior to drug administration and at 1, 8, 12, and 26 weeks after the single endoscopic dose (Figure 2B). Metagenomics sequencing was performed on both patient and donor samples. We assessed the presence or absence of all 15 MTC01 strains in each recipient and donor sample with the Strainer algorithm that identifies

strain-specific kmers in each strain and assesses their abundance in patient and donor stool metagenomes relative to a negative control database of unrelated metagenomes⁸. For FMT recipients and the donor, we also used Strainer to assess colonization of an additional 115 strains that were not in MTC01 but that were previously isolated and sequenced from donor D283, encompassing strains cultured from donor D283 at the original isolation of MTC01 strains and from additional samples from donor D283 including the new samples collected to produce the FMT drug product used in this trial. We used linear mixed effects models to assess differences in engraftment between groups using all samples and incorporating random effects to account for the multiple measures for each participant.

As expected, we detect MTC01 strains far more often in recipients after dosing than prior to dosing ($p < 1.0 \times 10^{-8}$ for all groups; linear mixed effects model) – with the few detections prior to dosing likely representing false positive detections of genetically similar strains (Figure 3A). High dose MTC01 had the highest overall number of the 15 strains colonized even when compared with high dose FMT ($\beta = 3.9$, 95%CI [1.8,6.0], $p = 0.004$; Figure 3A). These differences in colonization between high dose FMT and high dose MTC01 were largely driven by lower colonization of *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Eubacterium rectale*, and *Bifidobacterium pseudocatenulatum* suggesting that, between their initial isolation in 2015 and the resampling of the donor to produce the FMT product, these strains were lost from the donor microbiome and therefore unable to be transmitted to recipients or alternatively that they are too low in abundance in the stool of the donor for effective transmission compared to the higher doses attainable in MTC01 where each strain dose can be individually controlled (Supplementary Table 2). Interestingly, *Bifidobacterium longum*, *Bifidobacterium adolescentis*, and *Eubacterium rectale* had one or more additional non-MTC01 strains in the donor microbiota that preferentially transmitted to FMT recipients⁷.

Low dose MTC01 had the fewest number of strains colonized suggesting a likely dose effect in the LBP recipients ($\beta = -3.2$, 95%CI [-5.5,-0.9], $p = 0.019$; linear mixed effects model compared to high dose FMT). This dose effect was consistent across strains except for *B. pseudocatenulatum* that colonized with similar frequencies in both high and low dose MTC01 (Figure 3B). Notably, we did not find a significant dose effect in FMT recipients, and most MTC01 strains colonized well in FMT recipients despite generally being present at much higher relative abundance in MTC01 where they are not diluted out by the larger number of strains in the FMT drug product (Supplementary Table 2). While it was recently demonstrated that fecal filtrate is not non-inferior to FMT with live microbes³⁵, perhaps fecal metabolites or the broader diversity of a complete FMT community allow strains to engraft at lower doses. As we had previously observed for FMT⁸, we find that once colonized in recipients the strains dosed either via FMT or LBP remained durably colonized for the duration of the sampling period (Figure 3C).

To test our exploratory hypothesis that selection of frequently engrafting strains via empirical FMT results could focus LBP manufacturing on strains that engraft well rather than spending resources on strains that poorly engraft, we performed a post-hoc analysis to compare the engraftment frequency of MTC01 strains with that of strains from D283 that are not in the 15 MTC01 strains. We find the 15 MTC01 strains have significantly

higher engraftment frequencies compared to non-MTC01 strains both when administered by FMT or by LBP (FMT median difference=0.67, 95%CI=[0.20,0.87], $p=1.1\times 10^{-7}$; LBP median difference=0.92, 95% CI=[0.79,0.93], $p=4.7\times 10^{-11}$; Wilcoxon test; Figure 3D). Many of the non-MTC01 strains from D283 that were detected in $\geq 50\%$ of samples were strains of the same species that were not included due to our design criterion of including only one strain per species in the LBP. The only species with high engrafting strains without a representative in MTC01 were *Bifidobacterium bifidum*, *Bacteroides fragilis* (excluded from MTC01 as stated above), *Barnesiella intestinihominis*, *Bacteroides stercoris*, *Blautia massiliensis*, *Blautia wexlerae*, *Collinsella aerofaciens*, *Catenibacterium mitsuokai*, *Ruminococcus bicirculans*, and *Ruminococcus torques*.

Recipient gut microbiota restoration

An FMT administers a full spectrum microbial community whereas the 15-member MTC01 LBP contains a subset of highly transferable strains hypothesized to be sufficient to prevent *C. difficile* infection relapse. To assess the impact of these two intervention types on the broader recipient gut microbiota, we used metaphlan4³⁶ to determine the species-level composition of each recipient microbiota before and after administration of live microbes. As expected, FMT recipients had restoration of Shannon diversity to levels near the donor's alpha diversity by week 1 at the first post-FMT stool sample (Figure 4A), while the LBP recipients receiving only a partial microbial community did not reach the levels of the donor microbiota until week 8. Presumably, the 15-strains of MTC01 provide an initial seed community to prevent *C. difficile* relapse, which then allows a complete community to be restored from the environment or the subject's endogenous microbes. These changes are paralleled by observations in beta diversity (Figure 4B) where post-FMT recipients generally cluster with the donor, while MTC01 recipients only partially approach the donor microbiota beta diversity. If maintaining microbial population diversity across the human population is a priority³⁷, the LBP seed approach with the smallest possible effective community allows for the highest population diversity across recipients as they are not all colonized by nearly identical communities.

Discussion

The strong safety profile of LBPs^{14,15,38,39}, the growing set of tools to track their engraftment^{8,22,38,40–43}, and their promising results in clinical studies make them a compelling drug modality for future trials⁴⁴. Unlike FMT based discovery trials, LBPs allow testing of clearly defined hypotheses^{45–53} regarding the microbiome's influence on disease with tracking of each strain to identify associations with markers of disease response. Although numerous academic and commercial laboratories have the capacity to grow enough anaerobic bacteria for clinical trials with LBPs, the lack of know-how and added quality and regulatory burden for safely manufacturing them for human trials is a barrier that has limited the translation of numerous promising discoveries in animal models and human observational studies. To alleviate this barrier, we developed a manufacturing platform requiring only a hydrogen peroxide ionizer and a dedicated anaerobic chamber – both of which are straightforward to use and practical with most laboratory budgets.

We provide regulatory documentation and manufacturing batch records as blueprints to accelerate future exploratory trials.

A key limitation to the manufacturing process used here is the time restriction of only manufacturing two strains per week, which could be prohibitive to produce very large consortia. However, there is ample space in the anaerobic chamber to grow far more strains in parallel if adequate controls were established. A second limitation is the endoscopic formulation, where most patients would prefer an oral delivery. Although we chose a colonoscopic delivery in this study to compare FMT and LBP without confounding variables of enteric capsule delivery that might cloud interpretation, the drug substance could be formulated for oral delivery.

Manipulation of living cells or their protein constituents *in vitro* or *ex vivo* is fundamental to numerous therapies including *in vitro* fertilization, biologics, and CAR-T cells. These *ex vivo* and *in vitro* processes risk altering the chemical and structural composition in ways that reduce their efficacy relative to the same components generated in their native environment. Although the *in vitro* manufactured strains in MTC01 are grown in monoculture without many of the environmental constituents they would receive from a diverse microbiome and human host, we find *in vitro* manufactured monocultures colonize human recipients similarly to the same bacterial strains cultivated in a human gut with similar efficacy and safety in the treatment of rCDI, supporting the future use of *in vitro* manufactured LBPs. For therapeutic strains whose functional benefit requires durable colonization, we demonstrate that isolating bacterial strains from FMT donors and using those with the highest empirical engraftment frequency in human recipients provides a strong selection for strains that will engraft when administered as an LBP. Selection of consistent engrafting strains reduces the burden of manufacturing and clinically evaluating strains that poorly colonize the host and thus cannot realize their therapeutic potential. For other indications like inflammatory bowel disease where FMT has shown promise^{54–59} that has not been matched so far by LBPs, selection based purely on engraftment may not suffice to achieve therapeutic efficacy. In this context, engraftment data can be a starting point from which to narrow down on specific mechanisms of action, such as microbial modulation of bile acid metabolism, short-chain fatty acid production, ecological competition, effects on gut barrier integrity, and host immune responses. We hope that the platform and accompanying materials shared through this work will help empower the broader translation of microbiome science into human health by enabling more hypothesis-driven and foundational clinical studies.

Online Methods

Strain Isolation

Clarified and diluted donor stool was plated onto a variety of solid selective and non-selective media under anaerobic, microaerophilic, and aerobic conditions. Plates were incubated for 48–72 hours at 37°C. 384 single colonies from each donor microbiota sample were individually picked and regrown in liquid LYHBHiv4 media for 48 hours under anaerobic conditions (see supplementary files IND Appendix 9 for growth media details)^{7,8,60}. Regrown isolates were identified at the species level using a combination of MALDI-TOF mass spectrometry (Bruker Biotyper) and 16S rDNA amplicon sequencing.

All regrown isolates were stored in LYHBHiv4 with 15% glycerol at -80°C and strains for use in MTC01 were genome sequenced with paired-end 150nt reads on an Illumina platform. The original design of MTC01 included an additional strain of *Collinsella aerofaciens* as MTC01.06. This species had been found to have high strain engraftment but was not detectable in D283 at the time of consortium design. MTC01.06 was isolated from a different FMT donor that had been used for a single rCDI recipient. The strain was characterized and risk assessed but was removed from the consortium prior to GMP manufacturing in favour of a trial that only compared an LBP with strains from a single donor with FMT from the same donor.

Strain characterization and risk assessment

To produce RCBs, single colonies were selected after plating the relevant strains from the donor D283 culture library on rich solid agar (chocolate agar). Up to 20 freezer stocks were made of each strain (LYHBHiv4 with 15% glycerol) for use in strain characterization and the generation of master cell banks. The test for antibiotic resistance of each strain was performed in-house by e-test pre-screening on solid agar and through an external CRO (Micromyx) according to CLSI standards (Supplementary Table 1 and supplementary files IND Appendix 3 for full report). Definitive MICs and interpretations rely on CLSI-aligned methods performed at the external CRO. Differences between e-test and CLSI testing are expected and likely reflect methodological factors, like medium and redox conditions for strict anaerobes, inoculum density, incubation atmosphere/time, and e-test endpoint criteria.

For each strain, genome annotation was performed with Prokka v1.13.4⁶¹. The antibiotic resistome was predicted using the Resistance Gene Identifier (RGI) v5.1.0⁶² with the Comprehensive Antibiotic Resistance Database (CARD) v3.0.9. Virulence genes were identified using BLASTp v2.9.0⁶³ against the curated Virulence Factor Database (VFDB; downloaded from the Bacterial and Viral Bioinformatics Resource Center [bv-brc.org] on July 17, 2020⁶⁴). Genomic islands were predicted using Colombo with the SIGI-HMM plugin (v4.0) and IslandPath v1.0.4⁶⁵. All analyses were performed using default parameters, and BLAST hits were filtered using thresholds of $E\text{-value} < 1e-7$, sequence identity $> 80\%$, and query coverage $> 80\%$.

Manufacturing of MTC01

In addition to the methods described here, a detailed description can be found in the IND and MBRs (supplementary files IND, MBR).

A dedicated vinyl flexible film two-sided anaerobic chamber with a sterilization port on each side of the chamber was used for the manufacture of MCB, DS, and DP (see supplementary files IND Appendix 5 for facility design). Compared to a bioreactor, the chambers are relatively robust in operation, require little training and are quickly operational after installation. The confined space of the chamber allowed us to design a unique and simple sterilization process that grants easier microbial control than a more typical setup of gowned staff in a clean room. Prior to manufacturing of MCB, DS, or DP, almost all equipment and consumables are sterilized in place with ionized hydrogen peroxide. The few items that must be imported (e.g., the strains to be cultured) are surface sterilized with

ethanol and imported through the gas exchange port that uses a HEPA filtered air supply. The sterilization procedure was tested and confirmed by biological (Mesa Labs Stainless steel ribbons for VHP sterilization; ATCC#12980) and chemical indicators (LaMotte Iodine Test Papers 2948-BJ) during the design phase of the platform. During the sterilization for each cell-bank or DS production, the correct sterilization was confirmed using the chemical indicators.

All fermentations and culture manipulations were performed in single-use, disposable, sterilized plasticware with animal product free LYH_VIB media (vegitone infusion broth [Sigma-Aldrich], yeast extract, monosaccharide mix, disaccharide mix, cysteine, malic acid, sodium sulfate, MOPS, menadione, tween 80, vitamin-K; see supplementary files IND Appendix 9 for growth media details), and each strain was manufactured in a dedicated side of the anaerobic chamber with at most two strains manufactured in parallel. OD600 was used for in process monitoring of cell density. Colony forming units was the primary potency assessment for the cell banks and products. For MCBs, a 10 mL starter culture in a conical tube was inoculated with the total volume of the 2 mL RCB glycerol stock and varying potency of 2.0×10^4 - 2.0×10^9 CFU/mL. After reaching sufficient cell density, the starter culture was used to inoculate the primary MCB fermentation volume of 100 mL to OD600 0.1–0.2 in a single-use shake flask. In late exponential to early stationary growth phase, the 100 mL culture was mixed 1:1 with cryo-buffer (PBS pH 7.5, 0.5 g/L cysteine, 30% glycerol). Up to 192×1 mL MCB cryo-tubes were generated for each strain. The timepoint of harvest for each strain was estimated from liquid batch fermentations of RCB cultures utilizing the same growth medium.

For DS generation, three 100 mL starter cultures were each inoculated with 1–3 mL MCB glycerol stock depending on the stock potency (3.5×10^7 - 2.4×10^9 CFU/mL). After reaching sufficient cell density, starter cultures were used to inoculate 1.4 L DS primary fermentations to a target OD600 of 0.1–0.2 in a single-use shake flask.

After DS primary fermentation reached late exponential to early stationary growth phase, strains were centrifuged and washed with United States Pharmacopeia (USP) grade phosphate buffered saline (PBS, pH 7.5, 0.5 g/L cysteine) to remove non-USP grade components of the growth media and bacterial fermentation products. Centrifugation to pellet the bacterial cells was performed in sterile single-use 250 mL centrifuge tubes spun at $4000 \times g$ for 15 mins. After washing, each DS was centrifuged again and resuspended at approximately 1:10 the original volume (140ml) in USP grade formulation buffer (PBS, 0.5 g/L cysteine, 30% glycerol) to facilitate mixing of all 15 strains into a 250 mL DP. The DS was stored in 4×35 mL aliquots at -80°C in sterile 60 mL cryo-bottles along with 0.25 mL QC-aliquots in 10 cryo-tubes for downstream stability monitoring, although in retrospect 20 cryo-tubes would have been better to allow for retests and extended stability.

The DP was produced by simultaneously thawing one 35 mL DS aliquot for each strain in a sterile room temperature water bath in the anaerobic chamber. We generated DP at a high dose (1×10^{11} CFU) and a low dose (1×10^{10} CFU), with the high dose as close as practical to the average dose of FMT previously used with D283 (4.95×10^{11}) and the low dose targeting 10-fold lower than the high dose. DP was made in batches of 5–6 high doses per batch

(or an equivalent CFU combination of high doses and low doses; e.g., 5 high doses and 10 low doses). For strains with insufficient viable cells to provide 1:15 of the target dose for at least 15 high and 15 low doses using a single 35 mL DS aliquot, the full 35 mL was used to reach the highest strain dose possible. For the remaining strains, an equal potency of cells was added to the DP to reach the target dose in a total volume of 250 mL. The resulting strain-specific doses are in Supplementary Table 2. Three rounds of DP production (Extended Data Table 2) were sufficient to reach our target of 15 high doses and 15 low doses, leaving one aliquot of DS per strain for future use.

Quality assessment and stability of MTC01

The characterization of the MCB, DS and DP used colony forming units (CFU) as the primary potency metric. CFU was measured by spot-plating ten times 1 uL of four dilutions of an aliquot of a bacterial culture on at least 2 chocolate agar plates. MALDI-TOF biotyping (Bruker) was used as the primary identity metric for strain manufacturing characterization. The composition of the MCB, DS and DP, was analysed by spread plating dilutions from an aliquot of the individual strain (MCB and DS) or the mixture of all strains (DP) on chocolate agar plates followed by picking individual colonies for Biotyping. For MCB and DS at least 48 high quality spectra reads (confidence score >1.8) were analysed, and for the DP at least 200 high quality spectra reads were analysed. All high quality MALDI-TOF spectra had to match the correct MTC01 strain or strain mixture (i.e. no single colony mismatch was allowed). In the identification of strains in the DP mixture, 14 out of 15 strains of MTC01 were identified. Among the 1440 colonies picked for all three DP production runs, only MTC01.11 *E. rectale* was not detected by Biotyping. In monoculture, *E. rectale* has slower growth on chocolate agar compared to the remaining 14 bacterial strains, reducing chances of identification. For this DP produced for a phase 1b clinical trial the quantification of all viable bacterial strains within the mixed DP was not a release criterion, and it remains a challenge in the field of defined microbiota therapeutics⁶⁶.

For the microbiological examination of nonsterile products, USP<61> (microbial enumeration tests) and USP<62> (tests for specific microorganisms) are frequently used bioburden assays. Except for the specific test for *Clostridia* in USP<62>, all culture conditions for USP61 and USP62 use aerobic growth on rich agar. We cultured all individual MTC01 strains aerobically on rich agar and found all strains were obligate anaerobes with no aerobic growth. An external CRO (Focus Laboratories, Allentown, PA), subsequently performed USP<61> and USP<62> suitability assays on all strains individually as well as on the combined MTC01 community, demonstrating that none of the strains or communities inhibit the target bacteria and fungi in the USP<61> and USP<62> assays. By testing different temperature, media, atmosphere, and dilutions to discourage MTC01 strains, *Clostridium sporogenes*, which growth in anaerobic conditions like MTC01, was successfully isolated in suitability testing. All MCB, DS, and DP passed the USP bioburden assessments.

Since clinical trials take months or years to recruit and drugs need a defined shelf-life, monitoring the stability of the MCB, DS, and DP in long-term storage is critical. Based on the common storage duration of FMT at -80 °C for up to two years¹⁹, we would expect

individual bacterial strains and the defined LBP would likewise remain stable for extended periods. The CFU of the 15 individual strains (MCB, DS) and the 15 mixed strains (DP) stored at -80°C in cryo-buffer (PBS, 0.5 g/L cysteine, 15–30% glycerol) has been measured for at least 96wks (Extended Data Figure 1).

Given the need for flexible time windows when performing the clinical procedure of thawing and application of the DP, we quantified a range of times for these two clinical steps and found the total potency of DP is stable under aerobic conditions in the cryogenic storage bottle post thawing for at least 4 hours and in the clinical tray for at least 1 hour (Extended Data Figure 1).

Manufacturing of donor D283 FMT drug product

Fecal samples from D283 were collected, weighed, and split into 40–80 g subsamples in Seward Stomacher filter bags. Each sample was treated separately; the subsamples were weighed and stored at -80°C until production of concentrated fecal slurry (cFS). Each stool's subsamples were homogenized with 6 mL/g cryogenic media (PBS, 0.5 g/L cysteine, 30% glycerol) through the Seward Stomacher EVO 400. The homogenized cFS from the filtered side of the stomacher bags was combined into 1 L collection bottles and weighed. After combining all cFS from a stool's subsamples, the containers were weighed and stored at -80°C along with three 100 μL QC aliquots. QC vials were freeze-thawed after ≥ 24 hours, returned to -80°C for another 24 hours, then spot-plated ten times 1 μL from 10^{-3} – 10^{-6} dilutions across two chocolate agar plates to determine cFS CFU/mL potency.

The volume of cFS needed for high or low dose was calculated based on CFU potency of the QC vials. Cryo-media was prepared to normalize the cFS to 1×10^{10} for low doses and 1×10^{11} for high doses in a final volume of 250 mL FMT (Figure 1B). Ten 100 μL QC vials were each taken from a low and high dose product container for stability assessment (Figure 1C). The QC vials were spot-plated as the cFS QC with ten times 1 μL from 10^{-3} – 10^{-6} dilutions across two chocolate agar plates to determine FMT CFU/total 250 mL dose potency (Extended Data Table 3).

Trial design and ethics approval

This was a randomized, single-blind, parallel-group, phase 1b clinical trial with 1:1:1:1 allocation and individual participant randomisation. The trial was preregistered on Jun 6, 2023 at [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT05911997) identifier: [NCT05911997](https://clinicaltrials.gov/ct2/show/study/NCT05911997). There were 3 amendments and 4 versions to the study protocol throughout the trial. Version 1 was submitted with the IND. Version 2 (Oct 17, 2023; prior to first patient enrolled) adjusted the screening window from “Day -45 to Day 0” to “Day -30 to Day 0” per FDA feedback. Version 3 (Jan 30, 2024) proposed a reduced exclusion criteria to remove requirements of no inflammatory bowel disease, assessment of lab values for WBC, ANC, CBC, CRP, CMP, HIV, viral hepatitis, syphilis, and fecal calprotectin that are not used in rCDI standard of care. This version was never implemented as the FDA felt it was necessary to keep these assessments. Version 4 (April 11, 2024) reversed the changes back to that of Version 2.

Patients were consented at Mount Sinai Hospital under IRB STUDY-23–00563 ([NCT05911997](https://clinicaltrials.gov/ct2/show/study/NCT05911997)). The primary objective was to assess safety and tolerability of the

investigational product. Secondary objectives included recurrence of CDI up to 8 weeks and microbiota engraftment. The schedule of assessments included stool collections for microbiome analysis and CDI tests. ECG was not included on the schedule assessments in the IND submission due to its limited utility and to reduce patient burden. In error, it was not removed from the Outcomes section of the study protocol.

Inclusion criteria:

1. Ages eligible for study: 18 years and older
2. Able and willing to provide written informed consent
3. History of recurrent CDI defined as 2 episodes of CDI occurring within the previous 6 months (inclusive of the current episode)
4. Subjects with a qualifying recurrent CDI episode, defined as:
 - a. History of diarrhea (≥ 3 unformed stools per day for 2 or more consecutive days that is clinically consistent with CDI)
 - b. Documented positive stool test by local laboratory for toxigenic *C. difficile* (toxin EIA or PCR-based testing) for the current CDI episode within 60 days prior to randomization.
 - c. Received a course of standard-of-care (SOC) CDI antibiotics for the most recent CDI episode (for 10 to 42 days, with exact duration, antibiotic type and dose at the discretion of the Investigator)
 - d. Demonstrated adequate clinical response, defined as ≤ 3 unformed stools per day for 2 or more consecutive days during SOC CDI antibiotics prior to randomization.
5. CDI symptoms started within 60 days prior to randomization

Exclusion criteria:

1. Female subjects who are pregnant or breastfeeding or are planning to become pregnant during the study. Women with reproductive potential should use a reliable method of birth control:
 - a. Consistent use of an approved hormonal contraception (birth control pill/patches, rings)
 - b. An intrauterine device (IUD)
 - c. Contraceptive injection (Depo-Provera)
 - d. Double barrier methods (Diaphragm with spermicidal gel or condoms with contraceptive foam)
 - e. Sexual abstinence (no sexual intercourse) or
 - f. Sterilization

2. Known or suspected toxic megacolon, ileus or bowel obstruction at the time of enrollment.
3. Subjects with active gastroenteritis due to infectious causes other than CDI
4. Subjects with allergies to ingredients present in the investigational product
5. Prior participation in studies of investigational live biotherapeutic products or FMT within the last 6 months.
6. Major gastrointestinal surgery within the last 3 months before enrollment.
7. Use of drugs that alter gut motility.
8. History of acute leukemia or hematopoietic stem cell transplantation or myelosuppressive chemotherapy within 2 months prior to enrollment.
9. Unable or unwilling to undergo a colonoscopy
10. Inpatient status, though patients can be screened while inpatients, the must be outpatient for the planned colonoscopy.
11. Anticipated immediate or upcoming surgery within 30 days
12. Need for continued non-anti-CDI antibiotic therapy
13. History of total proctocolectomy
14. Patients who are unable to give informed consent
15. Participation in a clinical trial in the preceding 30 days or simultaneously during this trial
16. Severe food allergy (anaphylaxis or anaphylactoid-like reaction)
17. Life expectancy < 6 months
18. Unable to adhere to protocol requirements
19. Patient who have received an FMT in the past year
20. Any condition that the physician investigators deems unsafe, including other conditions or medications that the investigator determines that it will put the subject at greater risk from FMT
21. Clinically significant abnormal lab values including but not limited to WBC $>15 \times 10^3/\text{mm}^3$, ANC $<0.5 \times 10^3/\text{mm}^3$, or laboratory evidence of acute kidney injury at Investigator's discretion, at screening
22. If a patient is heavily immunosuppressed and is negative for CMV or EBV

Randomization, dosing, and safety assessment

Prior to colonoscopic drug administration, subjects were taken off all antibiotics for 48hr and underwent a standard colonoscopy bowel preparation within 12 hours of their procedure. In this open label trial, patients were block randomized (fixed block size = 4) without stratification to receive either high dose FMT, low dose FMT, high dose MTC01,

or low dose MTC01. The block randomization was performed in R using the blockrand package. Patient enrolment and group allocation were carried out by different personnel. An original sample size of 15 subjects per group was selected based off feasibility of manufacturing and the size of prior exploratory studies to assess safety and microbiome engraftment in rCDI. The study set in the present analysis are the first 18 patients dosed. The relevant microbial therapeutic was administered to the right colon by colonoscopy in a total volume of 250 mL. The study was open label with no concealment of the randomization from the clinical team.

Vital signs including heart rate, blood pressure, temperature and weight were collected at all in-person visits. We defined serious adverse events as any adverse event in the view of the investigator that resulted in any of the following outcomes: death, life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, congenital anomaly/birth defect, persistent or significant disability or incapacity or substantial disruption of the ability to conduct normal life function.

Detection of MTC01 and donor D283 FMT bacterial strain colonization

All sequenced bacterial strains from donor D283 were tracked using the open source Strainer algorithm^{8,67}. Although there are numerous strain tracking algorithms, Strainer is particularly well suited to achieve high sensitivity and specificity when a genome is available of the strain to be tracked⁴¹. Briefly the algorithm uses a database of bacterial strains and metagenomes not isolated from donor D283 to identify unique regions of the D283 strains. These unique regions are quantified in metagenomic samples of the donor and the recipients of FMT or MTC01. A strain is designated as detected if the informative kmers is above that estimated in a background database of unrelated metagenomes. Since the original publication, we have slightly modified the algorithm to 1) make it more streamlined for automation in high performance computing environments and 2) used the breadth of informative kmers covered in the metagenomes rather than the depth as the metric for scoring when comparing with the background database of metagenomes.

Strain-specific potencies (Supplementary Table 2) in the FMT drug product batches were estimated from the total product potency multiplied by the strain relative abundance determined by the Strainer algorithm. Strain-specific potencies in MTC01 were directly measured on individual drug substances by CFU plating and inferred in the drug product based on their mixing proportions in each batch.

Species-level assessment of recipient metagenomes

Metagenomic paired end reads were filtered and de-duplicated using default fastp settings (fastp -D -i R1 -I R2)⁶⁸. One sample with <500K reads was excluded, and the remaining samples all had >10M reads. Metaphlan4 was run in paired end mode with subsampling to 13 million reads³⁶. Species level metaphlan4 output was used to determine Shannon diversity index, quantify bray-curtis distance, and perform PcoA analysis using scikit-bio⁶⁹.

Statistical analyses

All statistical tests were two-sided. Linear mixed effects models, using the lme4 R package⁷⁰, were used to assess differences in engraftment before and after treatment with microbial therapeutics as well as between treatment modalities/doses, while accounting for non-independence between samples from the same subject. P-values for fixed effects were obtained using Satterthwaite's approximation for degrees of freedom with the lmerTest package. While the study comparison groups were prespecified prior to the trial, the exact linear mixed effect models below were defined after starting the trial. Given the size of the study population and the bias towards female patients, we did not perform sex-based analyses.

To assess if strain engraftment increased post-dosing, we used the following model:

$$\text{number_MTC01_strains_detected} \sim \text{baseline_drugDose} + (1 | \text{patient_id})$$

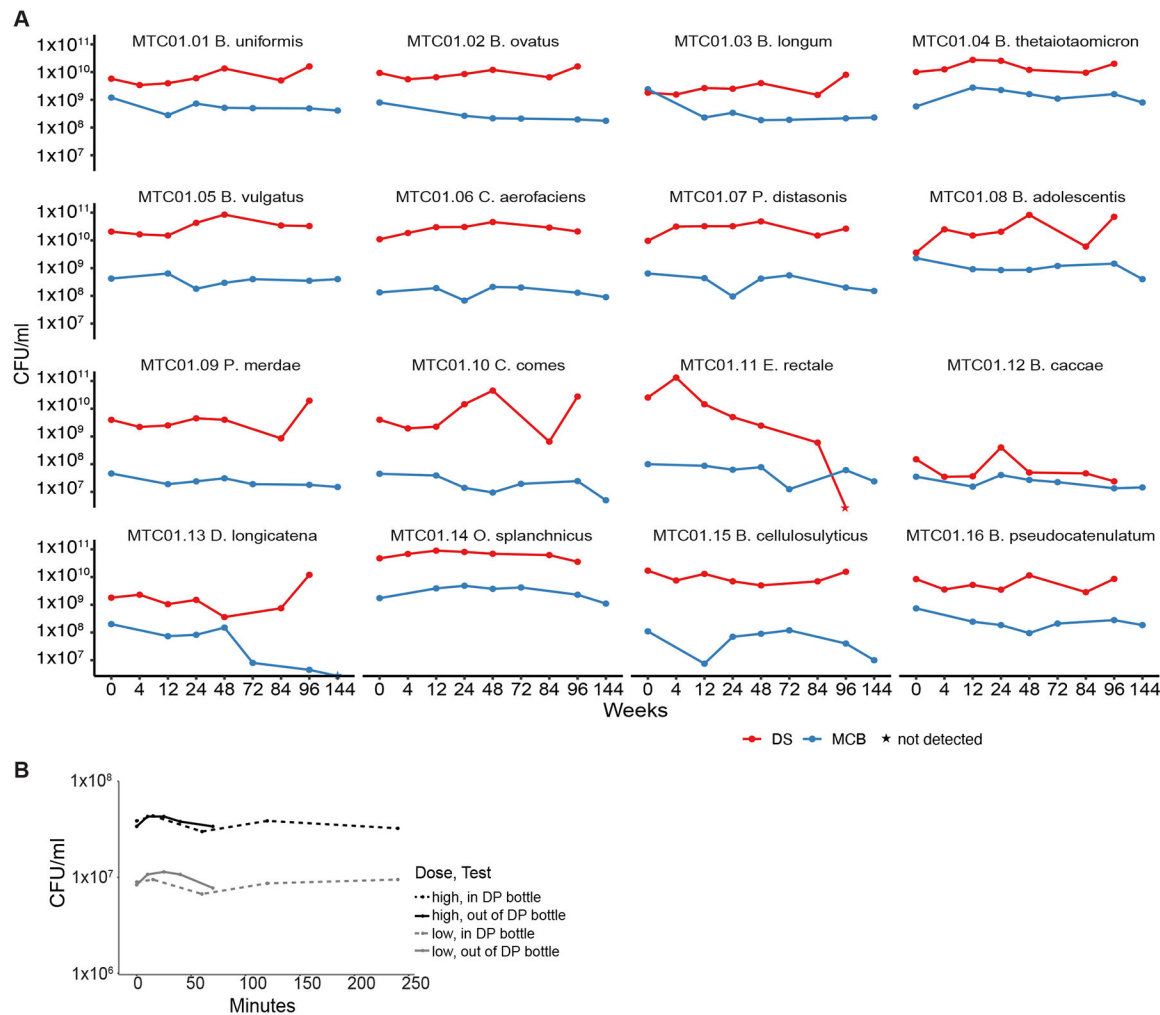
with a random intercept for patient that accounts for the non-independence between samples collected over time from the same individual, post-dosing. Baseline engraftment values were pooled across all drugs and doses. The model compared the pooled pre-treatment values with engraftment from FMT high dose, FMT low dose, MTC01 high dose, MTC01 low dose.

To assess if strain engraftment increased or decreased in any of the treatment groups relative to high dose FMT, we used the same model structure:

$$\text{number_MTC01_strains_detected} \sim \text{drugDose} + (1 | \text{patient_id})$$

To assess if strain engraftment frequencies were higher for the strains selected in MTC01 relative to the other strains in the D283 donor microbiota, we used a Wilcoxon rank-sum test where the inputs were the engraftment frequencies of the MTC01 strains in FMT or LBP recipients compared to the engraftment frequencies of all other D283 strains that were not in the set of 15 MTC01 strains.

Extended Data



Extended Data Figure 1. Strain specific stability.
(A) The MCB and DS cell banks were largely stable over the sampling period except for *Eubacterium rectale* and *Dorea longicatena* which showed a reduction in potency over time.
(B) DP potency was stable in the sealed bottle for at least 4 hours (typical thawing-time in clinic is about 1 hour) and outside the bottle for at least 1 hour (typical time outside the bottle in clinic is about 5 minutes).

Extended Data Table 1.
Manufacturing and trial timelines

PreIND (2 months)	
preIND meeting request	3-Sep-20
preIND submission	2-Oct-20
preIND feedback (written response only)	4-Nov-20
Manufacturing (2 years; including building facility)	

equipment purchase and installation	8 months
RCBs	1 month
QC	4 months
MCBs	4 months
DS	4 months
DP	1 month
drug stability prior to IND submission	1 month
IND 29067 (1 month)	
IND submission	4-Nov-22
IND feedback	29-Nov-22
IRB (7 months)	
IRB discussions	1-Dec-22
IRB submission	11-May-23
IRB approval	27-Jun-23
FMT donor sampling, and testing (3 months)	
	1-Aug-22
Trial recruitment (21 months)	
	1-Nov-23

Extended Data Table 2.

MTC01 release data.

		Potency	Identity	Purity	
Batch Number	Dose	CFU	MALDI	USP<61>	USP<62>
2022-08-12-MTC01-DP	low	6.9×10 ⁹	100% MTC01 (283 spectra)	passed (0 CFU)	passed (0 CFU)
2022-08-12-MTC01-DP	high	8.3×10 ¹⁰	100% MTC01 (269 spectra)	passed (0 CFU)	passed (0 CFU)
2023-11-24-MTC01-DP	high	1.5×10 ¹¹	100% MTC01 (278 spectra)	passed (0 CFU)	passed (0 CFU)
2024-01-05-MTC01-DP	low	1.8×10 ¹⁰	100% MTC01 (270 spectra)	passed (0 CFU)	passed (0 CFU)
2024-01-05-MTC01-DP	high	2.4×10 ¹¹	100% MTC01 (265 spectra)	passed (0 CFU)	passed (0 CFU)

Potency specification: 6×10⁹- 5×10¹⁰(low dose), 6×10¹⁰- 5×10¹¹ (high dose)

Identity specification: ≥200 high quality spectra matching MTC01 strains

Purity specification: USP<61> Total Aerobic Plate Count (<10³ CFU/ml), Total Yeast/Mold Count (<10² CFU/ml);

USP<62> absence of specific pathogens (bile tolerant gram-negative, Clostridia, P. aeruginosa, C. albicans, E. coli, S. aureus, Salmonella spp.)

Extended Data Table 3.
FMT release data.

		Potency
Batch Number	Dose	CFU
2023-09-30-FS.D283.02	low	1.9×10 ¹⁰
2023-09-30-FS.D283.02	high	1.9×10 ¹¹
2024-01-25-FS.D283.01	low	9.8×10 ⁹
2024-01-25-FS.D283.01	high	3.4×10 ¹¹

Potency specification: 6×10⁹- 5×10¹⁰ (low dose), 6×10¹⁰- 5×10¹¹ (high dose)

Extended Data Table 4.
Comorbidities

FMT		MTC01	
high-dose (N=5)	low-dose (N=4)	high-dose (N=5)	low-dose (N=4)
Chronic Cough, Constipation, Dyslipidemia, GERD, History of Hysterectomy, History of pelvic floor surgery, Hyperlipidemia (2), Hypertension (2), Hypothyroidism, Joint Pain, Left rotator cuff surgery, Peripheral neuropathy, Restless leg syndrome, Vitamin Deficiency, Weight Gain	Anxiety (2), Hemorrhoid, Hyperlipidemia, Overweight, Right Hip Tendon Rupture	ADHD, CAD, Chronic hip pain, Diabetes, GERD, History Herpes Genitalis, History of Leiomyoma, Hypertension (2), Hypomagnesemia, Hypothyroidism, history of myocardial infarction, OSA, Osteoarthritis, Vertigo	Atrial Fibrillation, Back Pain, Bladder Dysfunction, Breast cancer, GERD, Glaucoma, Hyperlipidemia, Hypertension, Hypothyroidism, IgG4 related disease, Insertion of right iliac artery stent, Lymphedema, Multiple thyroid nodules, MVP Repair

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing interests:

J.J.F. is on the scientific advisory board of Vedanta Biosciences, reports receiving research grants from Janssen Pharmaceuticals, and reports receiving consulting fees from Genfit, and Vedanta Biosciences. A patent is filed on MTC01 (J.J.F., L.B., J.E., V.A.). The remaining authors declare no conflicts with this study.

Data availability:

All data supporting the findings of this study are available within the paper and its supplementary files, including the pre-IND communications, the IND (29067), the master batch records (MBRs), the study protocol (SP), the statistical analysis plan, and the

CONSORT checklist. Individual clinical metadata are available in Supplementary Table 3. Metagenomic sequencing data are available at [PRJNA1322507](https://www.ncbi.nlm.nih.gov/sra/PRJNA1322507). Strain genome accessions are in Supplementary Table 2.

Code availability:

Custom code used for strain tracking is available at **GitHub** (<https://github.com/jeremiahfaith/strainer2>).

Material availability:

MTC01 strains are available via an MTA upon request.

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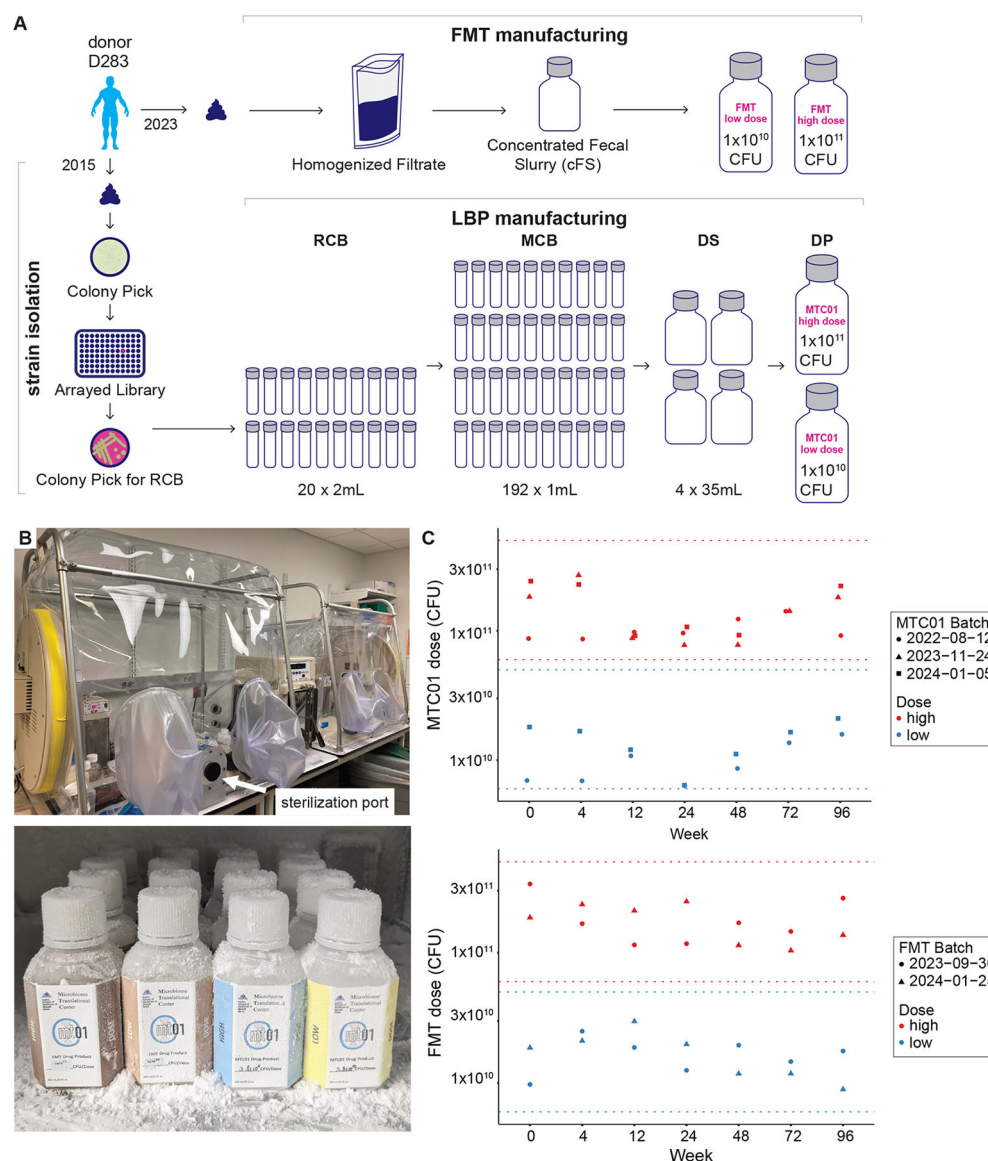


Figure 1. Manufacturing of FMT and LBP from the same donor's microbiome.

(A) Bacterial strains were isolated from FMT donor D283 stool samples collected in 2015 by plating on solid media and picking single colonies for cultivation and storage in 96-well plates with each well containing a single isolate confirmed by MALDI-TOF and genome sequencing. To manufacture MTC01 a Research Cell Bank (RCB, 20 vials per strain) was generated from a single colony isolated from plating each MTC01 strain. A Master Cell Bank (MCB, up to 192 vials per strain) was generated from each RCB strain, and Drug Substance (DS, 4 × 35 mL per strain) was generated from each MCB. The Drug Product (DP) was generated from the DS by combining all strains at even CFU per dose, where possible, for a total dose of approximately 1×10^{11} CFU (high dose) or 1×10^{10} CFU (low dose) in a volume of 250 mL. In 2023, additional stools were collected from donor D283, homogenized, coarse filtered, and used to generate FMT DPs at the same high and low dose potencies as MTC01. (B) Manufacturing was performed in a dedicated flexible film

anaerobic chamber equipped with a sterilization port to fog the chamber with sterilant. Final DPs were stored in a volume of 250 mL at -80°C until the time of colonoscopic delivery. (C) MTC01 and FMT DPs were stable within the specification bounds (horizontal dotted lines) of high dose and low dose for the duration of the assessment.

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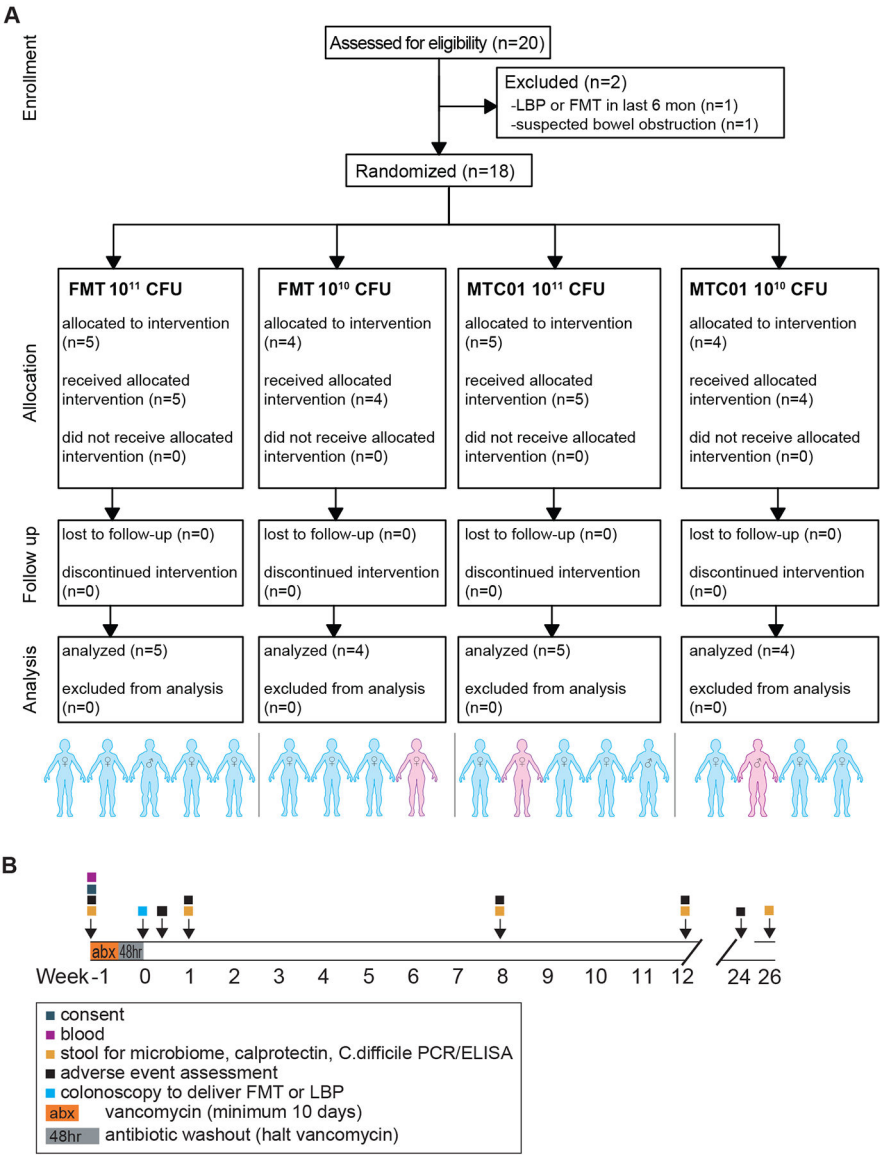


Figure 2. Clinical trial design and outcomes. (A) Patients were randomized to receive either low dose FMT, high dose FMT, low dose MTC01, or high dose MTC01. Patient outcomes for each arm are presented just below each assignment arm in the CONSORT diagram with patient sex indicated by the symbol on each individual and outcomes at week 8 indicated by the color (non-relapse=cyan; relapse=magenta). (B) Adverse events were assessed for up to 24 weeks. Stool samples were collected for up to 26 weeks for metagenomic sequencing to track FMT donor strains and MTC01 strains in recipients over time. Data included are from all 18 dosed patients in the trial.

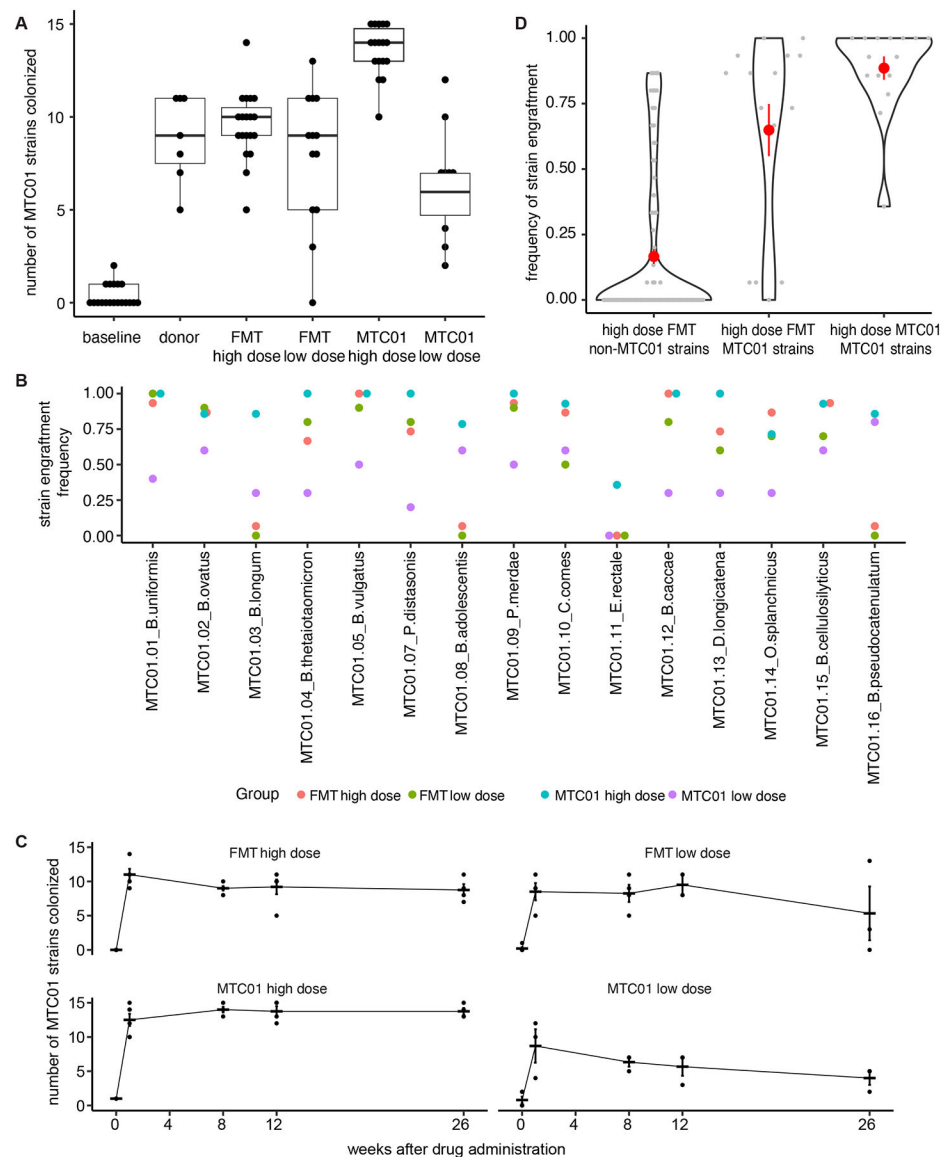


Figure 3. Engraftment of strains in FMT and MTC01 recipients.

(A) Few of the MTC01 strains were detected in baseline samples from all recipients prior to drug administration. After administration, up to all 15 MTC01 strains were detected in recipients with the most strains detected in recipients of the high dose MTC01. Boxplot center line indicates the median, while the box indicates the interquartile range. (B) Individual strains generally had high engraftment frequencies when administered in the high dose MTC01, lower engraftment frequencies when administered in the low dose MTC01, and varied engraftment when administered in the FMT drug products. (C) Strain engraftment was stable after the single dose administration of live microbial therapeutic. Center line = mean, error bars = standard error of the mean. (D) The 15 MTC01 strains had higher engraftment frequencies than the non-MTC01 strains in donor D283 both when administered via FMT or LBP. Each gray dot represents the engraftment frequency of an individual bacterial strain across all post-dosing recipient samples. The red dots and error

bars represent the mean $\pm$ standard error of the mean of the engraftment frequency of all strains in a group. Data included are from all 18 dosed patients in the trial.

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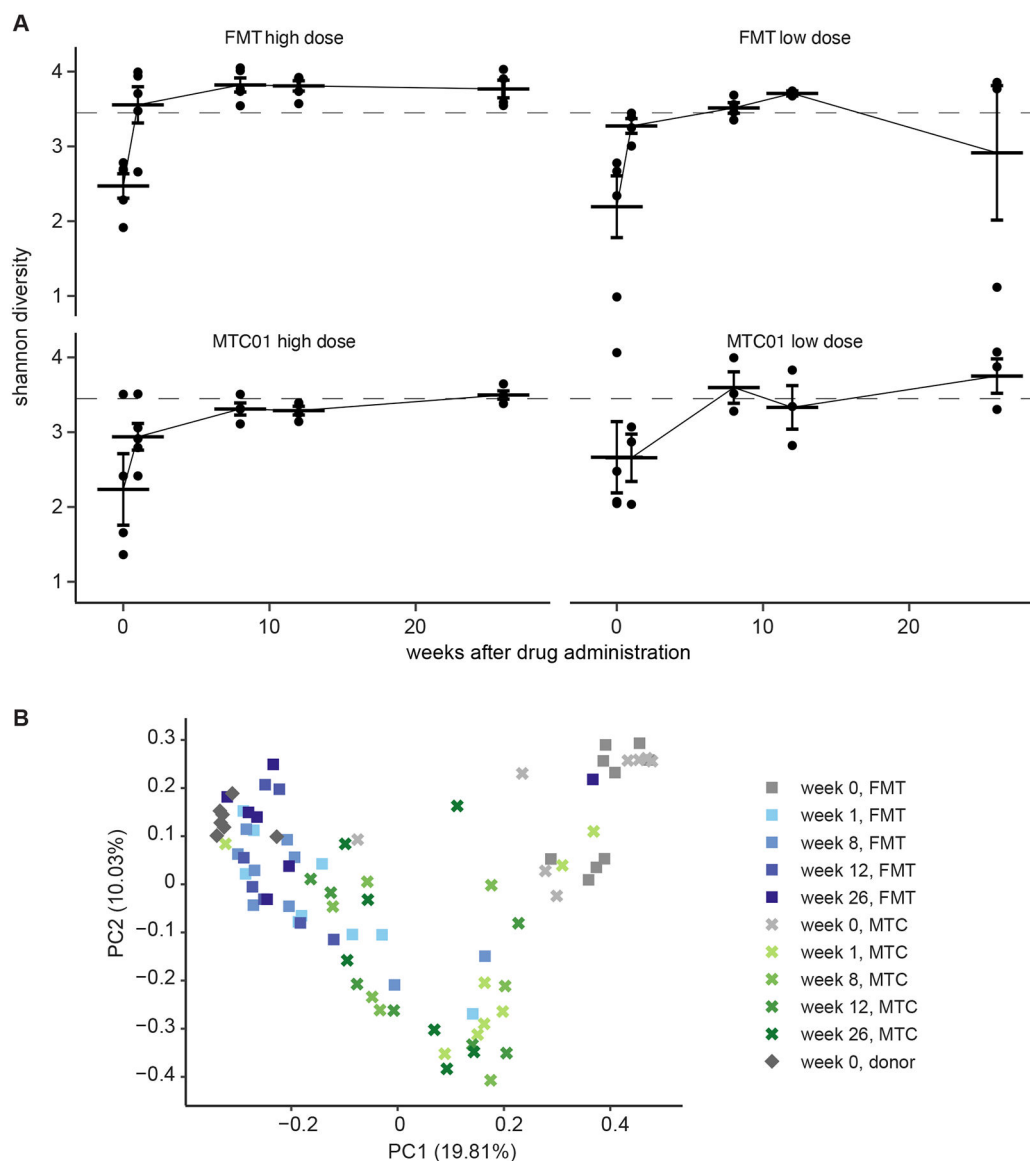


Figure 4. Recipient gut microbiota alpha and beta diversity.

(A) Recipients of FMT had an immediate increase of Shannon diversity to levels similar to the FMT donor (mean D283 diversity = dotted line), whereas MTC01 recipients arrived at similar levels to the donor by week 8. Center line = mean, error bars = standard error of the mean. (B) In recipient gut microbiota beta diversity using bray-curtis distance with PCoA, the D283 FMT recipients cluster very closely with the donor microbiota after FMT, whereas recipients of the MTC01 subject of D283 strains more distantly associate with the D283 gut microbiota. Data included are from all 18 dosed patients in the trial.

Table 1.
Safety data of adverse events across all four study groups.

high-dose FMT				
adverse event	any grade	mild	moderate	severe
<i>patients with an event</i>	1 (20%)	1 (20%)	0 (0%)	0 (0%)
worsening of constipation	1 (20%)	1 (20%)	0 (0%)	0 (0%)
low-dose FMT				
adverse event	any grade	mild	moderate	severe
<i>patients with an event</i>	2 (50%)	2 (50%)	1 (25%)	0 (0%)
diarrhea	1 (25%)	1 (25%)	0 (0%)	0 (0%)
GERD	1 (25%)	1 (25%)	0 (0%)	0 (0%)
hemorrhoidectomy	1 (25%)	1 (25%)	0 (0%)	0 (0%)
peritrochanteric infection	1 (25%)	0 (0%)	1 (25%)	0 (0%)
high-dose MTC01				
adverse event	any grade	mild	moderate	severe
<i>patients with an event</i>	4 (80%)	4 (80%)	0 (0%)	0 (0%)
<i>C. difficile</i>	1 (20%)	1 (20%)	0 (0%)	0 (0%)
diarrhea	1 (20%)	1 (20%)	0 (0%)	0 (0%)
irritable bowel syndrome	2 (40%)	2 (40%)	0 (0%)	0 (0%)
low-dose MTC01				
adverse event	any grade	mild	moderate	severe
<i>patients with an event</i>	1 (25%)	0 (0%)	0 (0%)	1 (25%)
<i>C. difficile</i>	1 (25%)	0 (0%)	0 (0%)	1 (25%)

Table 2.
Baseline characteristics and secondary outcome of recurrence at week 8 (no change in recurrence at week 24).

FMT			MTC01	
	high-dose (N=5)	low-dose (N=4)	high-dose (N=5)	low-dose (N=4)
recurrence	0 (0%)	1 (25%)	1 (20%)	1 (25%)
median age	71	53	45	66.5
hispanic	2 (40%)	0 (0%)	1 (20%)	0 (0%)
not-hispanic	3 (60%)	4 (100%)	4 (80%)	4 (100%)
male	1 (20%)	0 (0%)	1 (20%)	1 (25%)
female	4 (80%)	4 (100%)	4 (80%)	3 (75%)

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