The effect of a microbial ecosystem therapeutic (MET-2) on recurrent *Clostridioides difficile* infection: a phase 1, open-label, single-group trial

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Summary

Background Faecal microbiota transplantation (FMT) is highly effective for recurrent *Clostridioides difficile* infection but has inherent risks. Microbial Ecosystem Therapeutic 2 (MET-2) is an oral encapsulated formulation of 40 lyophilised bacterial species initially isolated from stool of a healthy donor, but subsequently manufactured independently of donors, eliminating potential risks introduced by changes in donor health. The aim of this study was to determine MET-2 activity, safety, and tolerability.

Methods This phase 1, open-label, single-group feasibility study was done in Alberta, Canada. The main inclusion criteria were mild to moderate *C difficile* infection and at least one episode of *C difficile* infection recurrence (ie, two episodes of *C difficile* infection) within 12 months. Initial daily treatment was ten oral capsules for 2 days, then three capsules for 8 days. If *C difficile* infection recurred, a higher dose was offered: 20 capsules for 2 days, then three capsules for 8 days. Patients were followed for adverse events and *C difficile* infection recurrence up to day 130. The primary outcome was absence of *C difficile* infection recurrence (fewer than three unformed bowel movements in 24 h persisting for at least 2 days) at day 40 by intention-to-treat analysis. Secondary outcomes were mortality or hospitalisation due to *C difficile* infection, infections attributed to treatment, nausea, abdominal pain, vomiting, or diarrhoea during treatment, quality of life (*C difficile* Health Related Quality of Life Questionnaire) before and after treatment, and engrafted MET-2 bacteria in patient stool. Absence of *C difficile* infection recurrence at day 130 was an exploratory outcome. This study is registered with ClinicalTrials.gov, NCT02865616.

Findings Between Sept 19, 2018, and Feb 28, 2020, we enrolled 19 adult patients with at least two episodes of mild to moderate *C difficile* infection (median age 65 years [IQR 56–67]; 12 women [63%], seven men [37%]). Recurrent *C difficile* infection was absent at day 40 in 15 (79%) of 19 patients after initial treatment, increasing to 18 (95%) 40 days after retreatment. No mortality associated with *C difficile* infection, infections associated with MET-2 treatment, or other serious adverse events were observed. The most common self-limited, mild to moderate symptoms reported during treatment were diarrhoea in 12 (63%) of 19 patients and abdominal cramps in 12 (63%). After MET-2 treatment, quality of life improved significantly, as did alpha diversity in stool microbial composition ($p=1{\cdot}93\times10^{-6}$). MET-2 associated taxa were found in greater abundance in most patients after treatment compared with baseline. 16 (84%) of 19 patients did not have recurrence of *C difficile* infection by day 130.

Interpretation MET-2 appears to be safe, efficacious, and well tolerated among patients with recurrent *C difficile* infection. Results must be validated in controlled studies.

Funding NuBiyota.

Introduction

Clostridioides difficile infection is the most common cause of diarrhoea acquired in acute health-care settings. Hospital-acquired *C difficile* infection increases health-care costs by four times over matched hospitalisation, an added annual cost of US$1 billion in the US and CA$100 million in Canada.[1,2] Within 8 weeks of finishing treatment for *C difficile* infection, 20–30% of patients will have *C difficile* infection recurrence with vancomycin or metronidazole. Risk of recurrence increases with each subsequent episode, approaching 60% after a third episode.[3–6] Furthermore, incidence of multiply recurrent *C difficile* infection is increasing disproportionately to incidence of *C difficile* infection. Between 2001 and 2012, the incidence of *C difficile* infection increased by 46%, whereas the incidence of recurrent *C difficile* infection increased by 189%.[7] Although *C difficile* itself might be suppressed by vancomycin, patients with recurrent *C difficile* infection continue to experience fatigue, anorexia, weight loss, and reduced quality of life.[8] Managing recurrent *C difficile* infection is a major clinical challenge, because there are few therapeutic options. The result is increased health-care use, costs, and patient suffering.

Research in context

Evidence before this study

We searched MEDLINE for articles published between database inception and Sept 19, 2020, in English with search terms including "*Clostridium difficile*" or "*Clostridiodes difficile*", combined with "fecal microbiota transplant*", "fecal transplant*", "microbiome therapeutic*", "microbial therapeutic*", "microbiota therapeutic*", or "microbial ecosystem therapeutic*". This search yielded 574 results. Randomised controlled trials to date have focused on faecal microbiota transplantation (FMT) for recurrent *Clostridioides difficile* infection. Resolution of recurrent *C difficile* infection in randomised trials was lower (about 76%) than in open-label studies (about 83%). FMT is not without its clinical challenges, however, because it is undefined, variable, and requires dedicated stool donors with substantial screening of donors for known pathogens. Even with stringent donor selection, when screening and quarantine of donor material between two screening points is correctly done, there is still potential to transmit disease, including antibiotic-resistant organisms and emerging pathogens, such as SARS-CoV-2.

From our literature review, live biotherapeutic products that are under development include stool specimens treated with ethanol (SER-109), microbiota suspension manufactured using standardised processes (RBX2660), and sterile filtrate from donor stool. These products have shown possible effectiveness for recurrent *C difficile* infection in small phase 1 or 2 trials including 5–40 patients.

Microbial Ecosystem Therapeutic 2 (MET-2) consists of a proprietary consortium of 40 bacterial species derived from the stool of a screened, healthy donor. Strains were highly purified and combined as lyophilised material in capsules for oral delivery. This product does not require further stool donors.

Added value of this study

We assessed the safety, tolerability, and activity of MET-2 in the treatment of recurrent *C difficile* in a phase 1, single-group trial. This treatment was not associated with any serious adverse events, and 18 (95%) of 19 participants achieved clinical resolution of recurrent *C difficile* infection. Alpha diversity metrics increased in these patients, and abundances of taxa associated with MET-2 were shown to increase, indicating potential incorporation of MET-2 taxa into patient microbiomes. These findings are similar to those from FMT treatment. This study is also one of the few to assess *C difficile* ribotypes and toxinotypes throughout its 130-day study period. Patients were infected with a variety of ribotypes and toxinotypes, and ribotype switching occurred in two patients during follow-up. Recurrent *C difficile* infection resolution was not necessarily associated with clearance of *C difficile*.

Implications of all the available evidence

There is an unmet need for an effective and safe treatment for recurrent *C difficile* infection that does not require donor stool, which MET-2 might fulfil. Larger clinical trials are needed to validate these promising results.

Faecal microbiota transplantation (FMT) seems to be the most effective and cost-effective treatment for prevention of recurrent *C difficile* infection.[9,10] The first randomised trial[11] of FMT compared FMT by nasogastric tube with vancomycin and found that a single FMT treatment was 81% efficacious compared with 31% for vancomycin. Further randomised trials showed that FMT is highly efficacious through various delivery routes (colonoscopy 91%,[12] enema 85%,[13] and oral capsules 96%[14]) and in various formulations (fresh 100% and frozen-and-thawed 85%).[13] Lyophilised FMT capsules have similar efficacy to fresh or frozen FMT (80–90%).[15]

The short-term safety profile of FMT is favourable, but potential exists for transmission of disease, despite donor screening. In the USA, eight cases of transmission of serious infections through FMT have occurred: two cases of extended spectrum β-lactamase-producing *Escherichia coli*, two cases of enteropathogenic *E coli*, and four cases of Shiga toxin-producing *E coli*. Three patients died in association with these transmissions, prompting warnings from the US Food & Drug Administration (FDA).[16–20] Emergence of novel pathogens (eg, SARS-CoV-2) poses an additional safety hazard, and donor screening protocols must be constantly updated to reflect continually evolving threats. Although these eight cases of transmission could have been prevented with more thorough donor screening, a defined microbiome-derived therapy would mitigate these risks and improve safety.

Petrof and colleagues[21] developed a defined microbial consortium (RePOOPulate or Microbial Ecosystem Therapeutic 1 [MET-1]), comprised of 33 bacterial strains derived from stool of a healthy donor. This formulation (100 ml; $3 \cdot 5 \times 10^9$ colony forming units/mL), delivered by colonoscopy, prevented recurrence in two patients with recurrent *C difficile* infection and demonstrated the feasibility of MET-1 as an alternative to FMT. The latest iteration, MET-2, comprises strains of 40 bacterial species, lyophilised and encapsulated for oral administration. This study was done to determine the safety, activity, and tolerability of MET-2 in recurrent *C difficile* infection patients.

Methods

Study design and participants

In this phase 1, open-label, single-group feasibility study, participants aged 18 years or older with mild to moderate recurrent *C difficile* infection were recruited at the University of Alberta Hospital (Edmonton, AB, Canada).

C difficile infection severity was evaluated clinically in accordance with practice guidelines by the Infectious Diseases Society of America.[22] University of Alberta Hospital is a tertiary centre in Alberta providing health care to patients in central and northern Alberta and North West Territories, with a catchment area of more than 2 million people. Because the Edmonton FMT programme is one of two centres offering FMT in Alberta, all recurrent *C difficile* infection patients in northern Alberta are referred there, averaging between two and four referrals per week. Outpatients with at least one episode of *C difficile* infection recurrence (ie, two episodes of *C difficile* infection) within 12 months were included. An episode of recurrence was defined as recurrence of diarrhoea within 8 weeks of completing a previous course of treatment for *C difficile* infection, with detection of *C difficile* toxin and resolution of diarrhoea following appropriate treatment (for the purpose of primary outcome, clinical assessment at day 40 was used). Permitted treatments for *C difficile* infection were metronidazole, vancomycin, and fidaxomicin. Vancomycin and fidaxomicin were permitted to be used as suppressive regimen before enrolment. Initial screening for *C difficile* used the QuikChek Complete test (Techlab; Blacksburg, VA, USA); if negative for toxin but positive for glutamate dehydrogenase, PCR for toxin B-encoding *tcdB* (Cepheid; Sunnyvale, CA, USA) was done. Exclusion criteria were severe *C difficile* infection (defined as neutropenia [absolute neutrophil count of $<0{\cdot}1\times10^9$ cells per L or white blood cell count of $>30\times10^9$ cells per L], serum creatinine greater than two times baseline [defined as the screening visit, which occurred within 28 days before study day 1], presence of toxic megacolon or intestinal perforation, or admission to intensive care unit), chronic diarrhoeal illness, including inflammatory bowel disease and diarrhoea-predominant irritable bowel syndrome, life expectancy less than 6 months, colostomy, use of antibiotics for infection other than *C difficile* infection, need for regular use of drugs affecting intestinal motility, pregnant or planning to be pregnant in the next 6 months, or planned elective surgery with preoperative antibiotics within 6 months of enrolment. We obtained written informed consent from all patients before screening. This study was approved by Health Canada (control number 231649) and the University of Alberta Research Ethics Board (Pro0008113).

Procedures

Following 10 or more days of oral vancomycin (125 mg four times daily until diarrhoea resolution) to treat an active *C difficile* infection episode, each participant received oral vancomycin suppression at 125 mg two times daily up to 48 h before MET-2 treatment. The 40 MET-2 bacterial strains (with each strain representing a distinct bacterial species; appendix p 1) were chosen for favourable safety and antibiotic sensitivity profiles. Each capsule contains $0{\cdot}5$ g of MET-2 product (around $9{\cdot}0\times10^8$–$1{\cdot}3\times10^9$ colony forming units) and remains stable for up to 9 months at room temperature. Data detailing the exact composition of MET-2 and antimicrobial susceptibility testing are not fully reported in this manuscript but are on file with Health Canada. Both capsule content and stability were assessed by flow cytometric methods.[23] No bowel preparation was required. Initial daily treatment was ten MET-2 capsules per day (ie, 5 g per day) by mouth for 2 days, followed by three capsules per day for 8 days. No dose alterations were permitted. If *C difficile* infection recurred, a higher daily treatment course of 20 capsules per day (ie, 10 g per day) for 2 days, then three capsules per day for 8 days, was offered after completion of at least 10 days of vancomycin. If *C difficile* infection recurred after higher-dose treatment, patients were offered a third treatment with 30 capsules (ie, 15 g) dissolved in water and administered by colonoscopy (appendix p 2). Each patient was assessed clinically at screening and 30, 90, and 120 days after completion of 10 days of treatment (corresponding to study days 40, 100, and 130; appendix p 2). Patients completed a stool diary daily from the first to the 40th day after treatment. At each clinical assessment before the first treatment, patients completed the *C difficile* Health Related Quality of Life Questionnaire, a validated questionnaire for assessment of *C difficile* infection.[24] Patients were removed from the study if they withdrew consent, became pregnant, or developed adverse events as per opinion of the investigators. Adverse events were assessed at each visit. Complete blood count and differential, electrolytes, renal function, liver function test, and lipid panel were assessed at screening visits and at each follow-up visit. Stool samples were collected at screening during vancomycin suppression, then during treatment and 2–5, 30, and 140 days after treatment completion (corresponding to study days 4, 12–15, 40, and 130).

We did microbial composition analysis, *C difficile* ribotyping, and toxin quantitative PCR (qPCR) on stool samples (stored at –80°C) before and after MET-2 treatments. For microbial composition analysis, DNA was extracted from the patient cohort's frozen faecal material using the Quick-DNA Fecal/Soil Microbe Kits (Zymo Research; Irvine, CA, USA) and normalised by stool weight. Library generation and next generation sequencing were done at Mr DNA Molecular Research (Shallowater, TX, USA). The 16S rRNA gene V4 variable region was amplified with PCR using primers 515F (GTGYCAGCMGCCGCGGTTA) and 806R (GGACTACNVGGGTWTCTAAT), with the barcode on the forward primer, and HotStarTaq Plus Master Mix Kit (Qiagen; Germantown, MD, USA). PCR consisted of 30 cycles of 94°C for 3 min, then 30–35 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 60 s, and a final elongation step at 72°C for 5 min. After amplification, PCR products were resolved by electrophoresis on a 2% agarose gel to determine amplification and relative band intensity. Multiple samples were pooled in equal proportions, on the basis of their molecular weight and DNA concentrations, and purified with calibrated Ampure XP beads

See Online for appendix

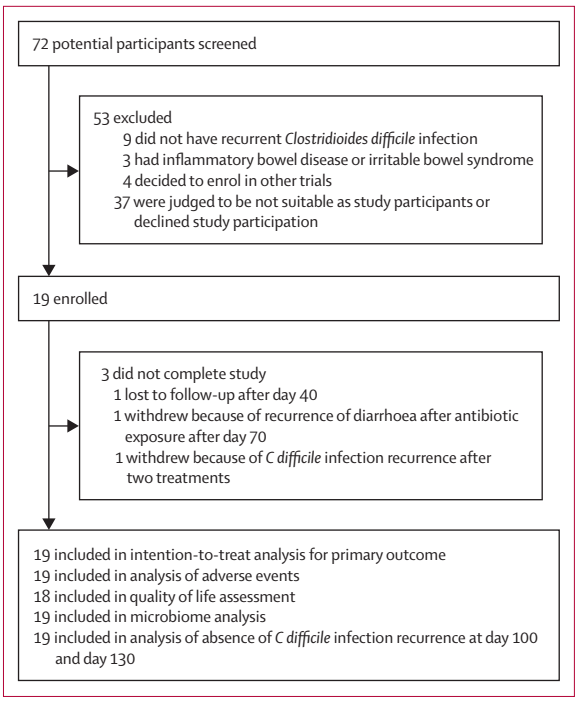

Figure 1: **Study profile**

	Value (N=19)
Age, years	65 (56–67)
Sex	
Female	12 (63%)
Male	7 (37%)
Charlson Comorbidity Index	2 (1·5–3·5)
Immunocompromised patients*	3 (16%)
Proton pump inhibitor use at screening	4 (21%)
Number of episodes of *Clostridioides difficile* infection at screening	3 (2–3)
Duration of recurrent *C difficile* infection before treatment, weeks	11·9 (8·6–17·9)
Number admissions associated with *C difficile* infection before treatment	0 (0–0)
Haemoglobin, g/L	135 (128–150)
White blood cell count, 10^9 cells per L	6·3 (5·4–9·6)
Albumin, g/L	44 (40–46)
Creatinine, µmol/L	78·7 (71·6–87·5)

Data are median (IQR) or n (%). *One had chronic lymphocytic anaemia and hypogamaglobulinaemia requiring ibrutinib and intravenous immunoglobulin, one had relapsing polychondritis on prednisone, and one had hairy cell leukaemia on rituximab.

Table 1: **Baseline characteristics**

(Beckman Coulter; Brea, CA, USA). Pooled and purified PCR product was used to prepare an Illumina (San Diego, CA, USA) Nextera DNA library. Sequencing was done by Mr DNA (Shallowater, TX, USA) using an Illumina (San Diego, CA, USA) MiSeq with version 3 reagents and generating 300-bp paired-end reads. Reads in which more than 70% of bases had a Phred score of 30 or more were retained and trimmed using DADA2 version 1.14. Faecal microbiome profiling of the patient cohort used sequence data from Mr DNA and the DADA2 open-source software package.[25] Taxonomy was assigned with a native implementation of the naive Bayesian classifier method and trained with the Silva database (from kingdom to genus taxonomic levels). Amplicon sequence variants were assigned and collated to the closest related species using NCBI BLAST. Microbial composition analysis used family-level taxonomic resolution.

For *C difficile* ribotyping and toxin qPCR, stool samples were treated with 95% ethanol to remove vegetative cells and then aerobically enriched in Hardy Diagnostics *C difficile* Banana Broth (Micronostyx; Ottawa, ON, Canada) for 24–72 h at 35°C. Aliquots were added to NucliSENS Lysis Buffer (bioMérieux; Montreal, QC, Canada) in Bertin Corp SK38 soil grinding tubes (ESBE Scientific; Saint-Laurent, QC, Canada), incubated under ambient conditions for 15 min, and centrifuged (5 min, 15·871×g). DNA was extracted from resulting lysates with the NucliSENS easyMAG extractor (bioMérieux) and used as template for toxinotyping and ribotyping. For toxinotyping, qPCR targeting the 16S rRNA genes and *tcdA*, *tcdB*,[26] and *cdtB*,[27] which encode toxins, was done using extracted DNA. Details of primers and probes (Integrated DNA Technologies; Coralville, IA, USA) are provided in the appendix (p 3). Assays were done with an Applied Biosystems 7500 Fast qPCR instrument using PrimeTime Gene Expression Master Mix (Integrated DNA Technologies) and HotStarTaq Polymerase (Qiagen). For ribotyping, template DNA was standardised using qPCR 16S rRNA gene cycle threshold values and amplified using primers developed by Janezic and colleagues.[28] Capillary electrophoresis was done as previously described using an Applied Biosystems 3130 Genetic Analyzer (ThermoFisher Scientific; Waltham, MA, USA).[29] Electropherograms were analysed using GeneMapper version 4.0 with a sizing table imported into BioNumerics version 6.01 for analysis and with adaptations to allow matching to bands used for generating the reference ribotype database.[30] Ribotypes were assigned visually by comparison to a national database (National Microbiology Laboratory; Winnipeg, MB, Canada) using international nomenclature where possible.

Outcomes

The primary outcome was absence of *C difficile* infection recurrence at day 40 after receiving at least one course of MET-2, assessed by the study investigators and reviewed by the data safety monitoring board. A *C difficile* infection recurrence was defined as at least three unformed bowel movements per 24 h persisting over 2 consecutive days, with a positive *C difficile* toxin test, requiring anti-*C difficile* infection therapy. Secondary outcomes were mortality or hospitalisation due to *C difficile* infection, infections attributed to treatment, nausea, abdominal pain, vomiting,

or diarrhoea during treatment, quality of life (*C difficile* Health Related Quality of Life Questionnaire) before and after treatment, and engrafted MET-2 bacteria in patient stool. The exploratory outcome was absence of *C difficile* infection recurrence at day 130. Participants who withdrew or were lost to follow-up before assessment at any study timepoint were assigned a treatment failure outcome. Participants who developed persistent diarrhoea for more than 2 consecutive days received a *C difficile* test, and if positive, were assigned a treatment failure outcome (ie, non-response). Post-hoc analyses included absence of *C difficile* infection recurrence at day 100 and *C difficile* toxin assays and ribotyping.

Statistical analysis

The primary outcome was analysed by intention-to-treat. Continuous demographic parameters (eg, age) were summarised for the intention-to-treat population using descriptive statistics. Categorical demographic parameters were summarised as a proportion of the intention-to-treat population. Non-normally distributed continuous variables were described with medians and IQRs. Normally distributed continuous variables were described with means and SDs. Categorical variables were described with numbers and percentages. Each *C difficile* Health Related Quality of Life Questionnaire item was transformed to a score between 0 (worst quality of life) and 100 (best quality of life).[25] A global score was the last questionnaire item. Items were aggregated by domain (physical, mental, and social).[26] Missing datapoints were replaced by the average of remaining domain items at the same timepoint. Global and domain scores at days 40 and 130 were compared with baseline using the paired Wilcoxon signed-rank test. Correction for multiple comparisons used a significance level of less than 0·025. Changes in stool microbial diversity, indicating MET-2 engraftment, were measured by the Shannon diversity index. Analyses used R version 3.4.3.

Sample size was calculated on the basis of the 30% recurrent infection resolution with vancomycin therapy in patients with recurrent *C difficile* infection reported by van Nood and colleagues,[11] although most of their patients had at least two recurrences and a third were hospitalised at the time of enrolment. Assuming 90% resolution of diarrhoea among study participants[11] and using a two-sided α of 0·05, we estimated sample size of 19 patients was needed to achieve at least 90% power ($1-\beta=0\cdot9$).

Additional diversity and statistical analyses of the microbiome taxonomic data used web-based MicrobiomeAnalyst software.[31] To remove low-quality features from the raw count dataset, low counts were filtered at a 20% prevalence with a minimum count of ten. This retains a feature if at least 20% of values contain

	Primary response at day 40	Day of diarrhoea recurrence*	Secondary response to 10 g MET-2†	Day of diarrhoea recurrence*	Faecal microbiota transplantation	MET-2 response at day 100	MET-2 response at day 130	Study completion	Outcome
Patient 1	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 2	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 3	No	16	Yes	..	NA	Yes	Yes	Yes	Success
Patient 4	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 5	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 6	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 7	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 8	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 9	No	10	Yes	..	NA	No	No	No	Lost to follow-up after completing day 40 follow-up
Patient 10	No	8	No	8	Yes	No	No	No	Withdrew from study before day 40 assessment
Patient 11	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 12	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 13	Yes	..	NA	..	Yes	No	No	No	Withdrew from study before day 100 follow-up
Patient 14	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 15	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 16	No	7	Yes	..	NA	Yes	Yes	Yes	Success
Patient 17	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 18	Yes	..	NA	..	NA	Yes	Yes	Yes	Success
Patient 19	Yes	..	NA	..	NA	Yes	Yes	Yes	Success

Success outcome was defined as the absence of *Clostridioides difficile* infection recurrence during the study. MET-2=Microbial Ecosystem Therapeutic 2. NA=not applicable. *Day of diarrhoea recurrence denotes the number of days since first MET-2 treatment. †30 days after the end of retreatment.

Table 2: **Study participant treatment response to MET-2**

at least ten counts. The low variance filter was set to 10% and measured using IQR. Data were further normalised by rarefying to the minimum library size and scaled using the total sum method. Alpha diversity scores were then assigned to each patient sample using the Shannon diversity measure at species taxonomic resolution.

This study is registered with ClinicalTrials.gov, NCT02865616.

Role of the funding source

NuBiyota designed the study but had no role in patient data collection and did not interact with patients. NuBiyota scientists were provided with samples of patient stool under a secondary site ethics approval (REB#18-11-018) from the University of Guelph's Natural, Physical, and Engineering Science Research Ethics Board. NuBiyota scientists contributed to data analysis and manuscript writing.

Results

19 patients who met inclusion and exclusion criteria were enrolled (figure 1) between Sept 19, 2018, and Oct 23, 2019, with follow-up to Feb 28, 2020, with 16 (84%) completing the entire study and 17 (90%) completing up to day 40. All 19 patients were included in the analyses. One patient was lost to follow-up after day 40, despite multiple telephone calls to schedule appointments, but this patient had no repeat *C difficile* testing, prescription for *C difficile* infection, emergency department visits, or hospitalisation for *C difficile* infection on the basis of review of provincial electronic medical records 120 days after treatment completion. This patient was assigned treatment success at day 40 but failure at day 100 and 130. Baseline characteristics are shown in table 1. Three patients were immunosuppressed. Detailed individual *C difficile* infection histories are provided in the appendix (p 4).

Of 19 patients, 15 (79%) achieved the primary outcome (absence of recurrent *C difficile* infection at day 40) with a single MET-2 treatment course (table 2). All four non-responders were retreated with a higher dose of MET-2 and *C difficile* infection recurrence was absent in three of these patients 30 days after the end of one retreatment. Overall response rate was 95% (18 of 19). One patient had *C difficile* infection recurrence on day 8 of retreatment, and withdrew from the study. This patient had one one hospital admission associated with *C difficile* infection before study enrolment and one hospital admission during the study, and stool culture revealed a novel *C difficile* ribotype that did not match any in the National Microbiology Laboratory's database. This patient declined MET-2 treatment by colonoscopy, and chose to have FMT, with 15 oral capsules given twice, 1 week apart and had no further *C difficile* infection recurrence.

No mortality associated with *C difficile* infection, MET-2-associated infection, or serious adverse events were observed in any study participants. No patient required dose reduction or discontinued the study for treatment-associated toxicity. Mild and self-limited symptoms were reported during the treatment period: vomiting (n=0 [0%]), nausea (n=6 [32%]), diarrhoea (<2 consecutive days; n=12 [63%]), and abdominal cramps (n=12 [63%]). Improvement in quality of life was statistically significant when comparing screening visit with day 40 and day 130 in all domains on the *C difficile* Health Related Quality of Life Questionnaire (figure 2). Comparison of faecal samples from before treatment and final faecal samples indicated a significant increase in Shannon alpha diversity in stool microbial composition after patients received MET-2 ($p=1\cdot93\times10^{-6}$; appendix p 5) and engraftment of MET-2 bacteria (figure 3).

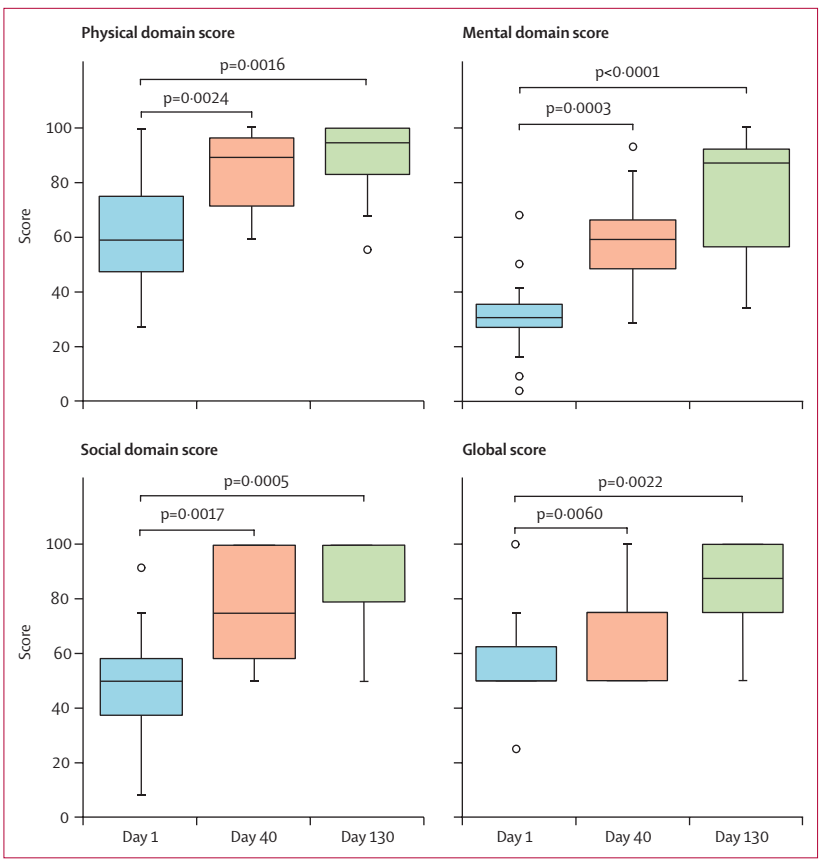

Figure 2: **Box plots of *Clostridioides difficile* Health Related Quality of Life Questionnaire score by domain**
Whiskers are 1·5 times IQR. If no points exceed this distance, whiskers are the minimum and maximum values.

Microbial composition data showed an increase in abundance of Lachnospiraceae, Bifidobacteriaceae, Ruminococcaceae, Erysipelotrichaceae, and Coriobacteriaceae families, all of which are represented by various members in MET-2, and a decrease in abundance of Enterobacteriaceae, Lactobacillaceae, Streptococcaceae, and Fusobacteriaceae families, several of which contain known opportunistic pathogens (appendix pp 6–7).

C difficile infection did not recur by day 100 or by day 130 in 16 (84%) of 19 patients (table 2). One patient was given a 5-day course of nitrofurantoin for a urinary tract

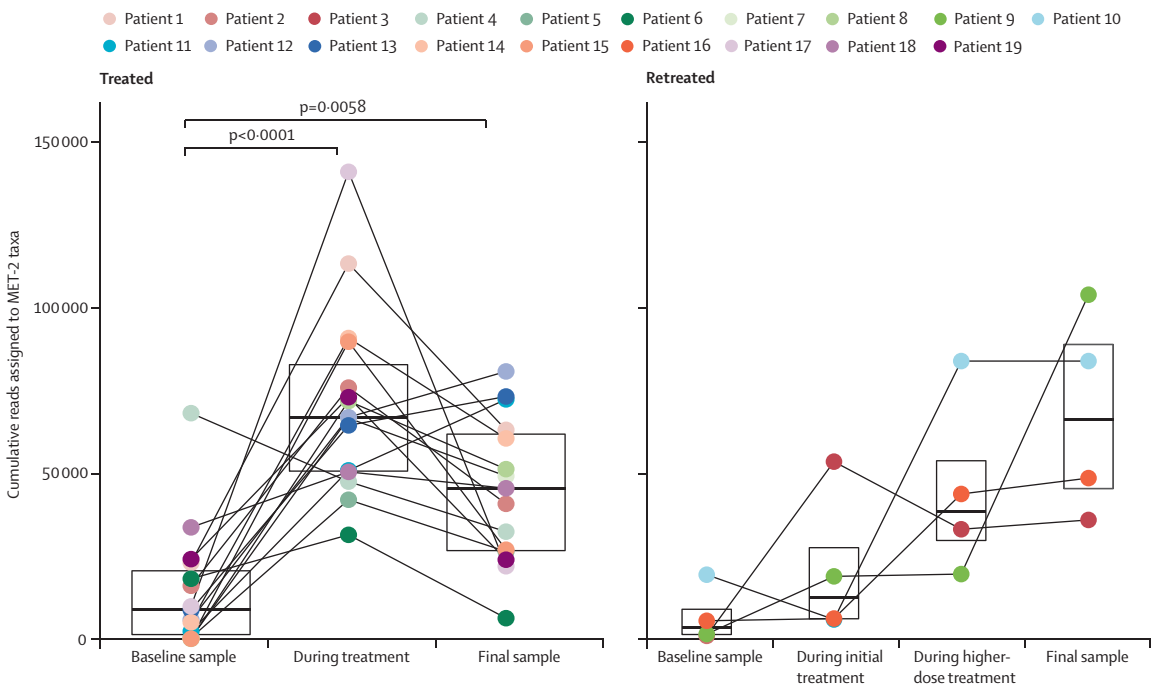

Figure 3: **Cumulative reads assigned to MET-2 taxa in patients with recurrent *Clostridioides difficile* infection**
Patients who received a single MET treatment are depicted in the "Treated" panel and patients who received two MET treatments are depicted in the "Retreated" panel. "Baseline samples" indicates those from before treatment, "during treatment" indicates samples from study days 8–15, and final sample indicates samples from study days 32–40 for patients 9, 13, and 16, and 120–138 for the remainder of the patients. In all cases, some taxa were present in samples that were also in the MET-2 consortium. Taxa distribution fluctuated within any given patient over the treatment time course. MET-2=Microbial Ecosystem Therapeutic 2.

	First treatment					Second treatment				
	Baseline	Day 4	Day 12	Day 40	Day 130	Baseline	Day 4	Day 12	Day 40	Day 130
Patient 1	No growth	385 $^{tcdA, tcdB, cdtB}$	385 $^{tcdA, tcdB, cdtB}$	385 $^{tcdA, tcdB, cdtB}$	014 $^{tcdA, tcdB}$	..	..	..	..	..
Patient 2	Not tested	014 $^{tcdA, tcdB}$	Not tested	Not tested	No growth	..	..	..	..	..
Patient 3	Not tested	No growth	328 $^{tcdA, tcdB, cdtB}$	..	..	328 $^{tcdA, tcdB, cdtB}$	No growth	328 $^{tcdA, tcdB, cdtB}$	328 $^{tcdA, tcdB, cdtB}$	No growth
Patient 4	Not tested	629 $^{tcdA, tcdB}$	Not tested	Not tested	No growth	..	..	..	..	..
Patient 5	Not tested	057 $^{tcdA, tcdB}$	Not tested	Not tested	No growth	..	..	..	..	..
Patient 6	Not tested	019 $^{tcdA, tcdB, cdtB}$	Not tested	Not tested	019 $^{tcdA, tcdB, cdtB}$	..	..	..	..	..
Patient 7	Not tested	Not tested	014 $^{tcdA, tcdB}$	014 $^{tcdA, tcdB}$	No growth	..	..	..	..	..
Patient 8	No growth	010 ^{negative}	010 ^{negative}	No growth	014 $^{tcdA, tcdB}$	..	..	..	..	..
Patient 9	Not tested	043 $^{tcdA, tcdB}$	Not tested	..	..	No growth	043 $^{tcdA, tcdB}$	No growth	No growth	..
Patient 10	No growth	New $^{tcdA, tcdB, cdtB}$	No growth	..	..	New $^{tcdA, tcdB, cdtB}$	New $^{tcdA, tcdB, cdtB}$	..	..	..
Patient 11	Not tested	027 $^{tcdA, tcdB}$	Not tested	Not tested	No growth	..	..	..	..	..
Patient 12	Not tested	No growth	Not tested	Not tested	629 $^{tcdA, tcdB}$	..	..	..	..	..
Patient 13	Not tested	027 $^{tcdA, tcdB, cdtB}$	Not tested	027 $^{tcdA, tcdB, cdtB}$	..	..	..	..	..	..
Patient 14	Not tested	104 $^{tcdA, tcdB}$	Not tested	104 $^{tcdA, tcdB}$	104 $^{tcdA, tcdB}$	..	..	..	..	..
Patient 15	Not tested	No growth	Not tested	Not tested	070 $^{tcdA, tcdB}$	..	..	..	..	..
Patient 16	Not tested	No growth	No growth	Not tested	..	..	No growth	..	020 $^{tcdA, tcdB}$	020 $^{tcdA, tcdB}$
Patient 17	Not tested	New $^{tcdA, tcdB, cdtB}$	..	New $^{tcdA, tcdB, cdtB}$	New $^{tcdA, tcdB, cdtB}$	..	..	..	..	..
Patient 18	No growth	No growth	New $^{tcdA, tcdB, cdtB}$	New $^{tcdA, tcdB, cdtB}$	No growth	..	..	..	..	..
Patient 19	Not tested	No growth	Not tested	Not tested	No growth	..	..	..	..	..

"No growth" indicates that *Clostridioides difficile* did not grow in the Banana Enrichment Broth. "New" infers a novel ribotype not yet defined in the National Microbiology Laboratory's ribotype database. Strains are shown with the corresponding toxin genes. MET-2=Microbial Ecosystem Therapeutic 2.

Table 3: **Results of ribotyping from stool samples collected after MET-2 treatment**

infection 60 days after completing initial MET-2 treatment. Diarrhoea recurred, but a stool sample collected when this patient presented to the emergency department for assessment was lost. Diarrhoea was presumed to be due to *C difficile* infection, because the patient responded to a course of vancomycin. This patient withdrew from the study to pursue FMT treatment, which prevented further *C difficile* infection recurrence.

Analysis of *C difficile* ribotypes showed that patients were infected with a variety of ribotypes (table 3). With the exception of one isolate that was toxin negative (a ribotype 010 strain recovered from patient 8), all recovered *C difficile* strains harboured *tcdA* and *tcdB*. Approximately half of the recovered ribotypes additionally carried *cdtB*, although the *cdtB*-positive genotype did not correlate with need for retreatment. Ribotype switching occurred in two patients (patients 1 and 8), suggesting new infection rather than relapse of old infection. There was no correlation between ribotype switching events and number of recurrent *C difficile* infection episodes. Three novel ribotypes (denoted A, B, and C) that did not match any listed in the National Microbiology Laboratory's database were isolated from the patient who developed *C difficile* infection despite higher-dose retreatment (patient 10) and from two other patients (patients 17 and 18). All three of these patients had a relatively high Charlson Comorbidity Index of 4 or more. Other treated patients with a Charlson Comorbidity Index of 4 or more (patients 11 and 13) carried ribotype 027. We could not detect *C difficile* in patient 19's stool samples, despite this patient's high Charlson Comorbidity Index. Toxin genes were still detected by PCR in eight patients (1, 6, 8, 12, 14, 15, 16, and 17), despite resolution of recurrent *C difficile* infection at day 130.

Discussion

In this preliminary study of 19 patients, we showed MET-2 to have activity for treating recurrent *C difficile* infection in 18 (95%) of 19 of patients, on par with FMT. 15 (79%) of 19 responded after a first treatment, and three (16%) of 19 responded after a second higher-dose treatment course. The one patient who did not respond to two courses of MET-2 opted to withdraw from the study and did not proceed with an offered option of a higher MET-2 dose delivered by colonoscopy. MET-2 is well tolerated with no short-term safety concerns. The microbial community during recurrent *C difficile* infection was characterised by an increased abundance of Proteobacteria, reduced abundance of Bacteroidetes and Firmicutes, and reduced microbial diversity. MET-2 treatment reversed these changes and restored the microbial community, similar to other clinical trials using encapsulated FMT.

Although FMT is a proven and widely accepted therapy for recurrent *C difficile* infection, its short-term and long-term safety is less well established. The initial enthusiasm for this highly effective therapy in treating a condition with no effective alternatives, coupled with early studies showing few adverse events during short-term follow-up, led to early and widespread adoption of FMT for recurrent *C difficile* infection. However, FDA warnings of transmission of extended spectrum β-lactamase-producing *E coli*, enteropathogenic *E coli*, and Shiga toxin-producing *E coli* and associated patient deaths (with some of these reports originating from well established stool banks) have given reason for pause.[16–20] The possibility of new emerging pathogens, including SARS-CoV-2, being transmitted by material from asymptomatic donors is also a potential concern.[32–34]

These recognised pitfalls of FMT have driven development of microbiome drugs to improve safety. Strategies include treating donor stool with ethanol to isolate spores of intestinal bacteria (SER-109),[35,36] which reduces the phylogenetic diversity of the therapeutic product to a single phylum of endospore-producers (Firmicutes), or filtering donor stool and using only the sterile filtrate,[37] which does not replace microbial diversity lost through antibiotic treatment owing to the absence of live organisms. Additionally, neither of these approaches obviates the need for stool donations. By contrast, MET-2 is a complex mixture representing several bacterial phyla cultured from the stool of an intensely screened, single healthy donor. Subsequent manufacturing is independent of stool donation, thus eliminating any potential risks introduced by changes in donor health. Additionally, MET-2 is a lyophilised consortium of bacterial strains, each of which has been fully sequenced and characterised, including antibiotic susceptibility testing. This selected microbial consortium might therefore confer safety benefits over traditional FMT.

Family-level data were used to depict the results of MET-2 treatment, rather than exact species composition. We postulate that live biotherapeutic products modulate the ecology of the gut and provide a scaffold for the recovery of other species already present in the colonic microbiome, rather than simply affecting colonisation by components of live biotherapeutic products. For example, following treatment with SER-109, recipients were found to have changes in microbial taxa which could not be directly attributed to SER-109 (which contains only Firmicutes), since recipients had changes in other bacterial phyla not present in SER-109, such as Bacteroidetes.[36] MET-2 may act similarly, as several patients demonstrated recovery of microbes associated with the Erysipelotrichaceae and Rikenellaceae families, neither of which are present in MET-2. These studies suggest that the details of individual species used are likely secondary to the microbiome-wide effects that these novel therapeutics provide to the recovery of ecosystem diversity. Future studies and advances in methodology will likely help explain this occurrence.

The non-responding patient who opted to withdraw from the study and pursue FMT had multiple comorbidities and was infected with a novel ribotype of *C difficile* that had not previously been reported in the National Microbiology Laboratory's database. Whether this uncharacterised ribotype is more virulent than other strains is unknown,

but patients with a Charlson Comorbidity Index of 4 or more were infected either with a novel ribotype or with ribotype 027. Additional studies are underway to determine the clinical significance of this finding. Furthermore, despite absence of *C difficile* infection recurrence after MET-2 treatement, eight of 18 patients still harboured toxigenic strains of *C difficile*, suggesting that resolution of recurrent *C difficile* infection might not require clearance of *C difficile*. Whether these patients will be *C difficile* carriers indefinitely or whether they are at a greater risk of future *C difficile* infection than those who have cleared this organism is unknown. The patient who developed recurrence of *C difficile* infection desptite two rounds of MET-2 responded well to FMT, attesting that FMT still remains a useful and overall safe treatment for a condition with few effective alternatives, when the appropriate donor screening process is strictly followed.[38]

This study has several strengths. To our knowledge, this is the first study to test a complex, defined, lyophilised, and fully characterised microbial ecosystem to treat recurrent *C difficile* infection patients.[39] The study included patients with immunosuppression, who are frequently excluded from this type of clinical study. *C difficile* toxin gene assays, ribotyping, and microbial composition analyses were done, providing some mechanistic insights into recurrent *C difficile* infection resolution. Limitations of this study include the absence of a control group, a small sample size, and recruitment of patients from a single centre. Furthermore, our primary outcome defined as absence of *C difficile* recurrence 30 days after completion of treatment is shorter than the 8–12-week timepoint used in most recurrent *C difficile* trials; however, we did a post-hoc analysis of outcome at 90 days.

In summary, this small pilot study shows that the activity, tolerability, and short-term safety of MET-2 in treating recurrent *C difficile* infection is comparable to that of FMT. Although FMT remains unrivalled in its ability to treat recurrent *C difficile* infection, we propose that MET-2 provides a valuable option at a time when emerging diseases, such as COVID-19, impose additional regulatory challenges to the use of FMT. A larger, randomised controlled trial is needed to confirm these encouraging results.

Contributors

NuBiyota designed the study. DK, KW, RF, and BR collected data, which were analysed and interpreted by DK, KW, RF, KC, KS, LC, CL, ADB, EOP and EA-V. DK, KW, EOP, and EA-V wrote the initial manuscript, and all authors revised the manuscript. DK, KW, and RF had full access to all the data in the study, and DK had final responsibility for the decision to submit for publication.

Declaration of interests

Funding for this study was provided by NuBiyota to DK. EAV and EOP are both cofounders of NuBioyota. KC and KS are employed by NuBiyota. All other authors declare no competing interests.

Data sharing

Deidentified individual participant data will be made available upon request to the corresponding author with investigator support after approval of a proposal with a signed data access agreement with the University of Alberta.

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