Research Article

Long-term beneficial effect of faecal microbiota transplantation on colonisation of multidrug-resistant bacteria and resistome abundance in patients with recurrent Clostridioides difficile infection

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Multidrug-resistant (MDR) bacteria are a growing global threat, especially in healthcare facilities. Faecal microbiota transplantation (FMT) is an effective prevention strategy for recurrences of *Clostridioides difficile* infections (rCDI) and can also be useful for other microbiota-related diseases. We study the effect of FMT in patients with rCDI on colonisation with MDR bacteria and antibiotic resistance genes (ARG) on the short (3 weeks) and long term (1-3 years), combining culture methods and faecal metagenomics. Based on MDR culture (n=87 patients), we see a decrease in the colonisation rate of MDR bacteria after FMT (20/87 before FMT = 23%, 10/87 three weeks after FMT = 11.5%). Metagenomic sequencing (n=63 patients) shows a reduction in relative abundances of ARGs in faeces, while the number of different resistance genes in patients remained higher compared to healthy donors (n=11 donors). Furthermore, plasmid predictions in metagenomic data indicate that rCDI patients harboured increased levels of resistance plasmids, which appear unaffected by FMT. In the long-term (n=22 patients), the recipients' resistomes became more donor-like, suggesting that microbiota restoration continues after 3 weeks post-FMT. Taken together, we hypothesise that FMT restores the gut microbiota to a composition that is similar to healthy donors, and potential pathogens are either lost or forced to very low abundances. This process, however, does not end in the days following FMT. It may take months for the gut microbiome to re-establish a balanced state. Even though a reservoir of resistance genes remains, FMT may lead to a more stable and resilient microbiota composition.

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Introduction

The discovery of antibiotics altered the natural course of infectious diseases and saved millions of lives. Antibiotics might be the most significant development in modern medicine, but there are important trade-offs to their use. Antibiotic resistant bacteria have emerged that are unaffected by standard therapies, which threatens effective prevention and treatment of infections. Antibiotic resistance is now considered a major threat to public health [1][2]. Besides, broad spectrum antibiotic therapy disrupts the human microbiota, paradoxically resulting in an increased susceptibility to infections, for example by *Clostridioides difficile* [3][4][5].

C. difficile can asymptomatically reside in the gut but thrives in an antibiotic-affected microbiota. *C. difficile* causes an infection (CDI) varying from self-limiting and mild diarrhoea to life-threatening pseudomembranous colitis. The disruption of the gut microbiota is essential in maintaining the recurrent nature of CDI, which is supported by the observation that replenishing the gut microbiota by faecal microbiota transplantation (FMT) results in prompt resolution of CDI recurrence (rCDI) [6][7]. It is thought that FMT restores the gut microbiota diversity after antibiotic treatment, thus preventing outgrowth of *C. difficile* spores [8], and possibly decreasing the risk of other infections as well. FMT has been mentioned in treatment guidelines for rCDI for years [9][10][11] and rCDI is currently the only disease that is routinely treated with FMT.

A gut microbiota disrupted by antibiotics is also more susceptible to colonisation with multidrug-resistant (MDR) bacteria [12], which in turn increases the risk of infection in critically ill patients [13]. A prominent and problematic group of MDR bacteria are extended spectrum beta-lactamase-producing (ESBL) Enterobacterales. Most infections with ESBL-producing Enterobacterales have high morbidity and mortality and are preceded by intestinal colonisation [14][15][16]. Hence, the prevention and eradication of ESBL-producing Enterobacterales from the intestinal tract is of global interest. Spontaneous decolonisation depends on comorbidities and type of species [17][18], and innovative strategies to promote decolonisation of MDR bacteria are desired. So far, there is no recommended decolonisation method [19]. However, Millan et al., found that FMT in patients with rCDI decreased the number and diversity of antimicrobial resistance genes in their faeces [20]. This observation was followed by various case reports of patients colonised with ESBL-producing Enterobacterales who were successfully treated with FMT [21][22][23][24][25][26][27][28][29][30]. Only a single, underpowered RCT has been conducted (n=39 patients) to assess decolonisation of MDR Enterobacterales by treatment with oral non-absorbable antibiotics and FMT [31]. No statistically significant advantage of FMT was found, although colonisation rates were slightly lower in FMT-treated patients compared to untreated control patients. Subsequently, questions were raised about the efficacy of FMT against MDR bacteria and experiments were suggested to further assess this [32].

To further explore the effects of FMT in rCDI patients on antibiotic resistance of the gut microbiota, we assess colonisation with MDR bacteria with both culture and metagenomics. We pay special attention to the resistome (collection of all antibiotic resistance genes (ARG) present). Additionally, we study the long-term effects on the microbiota up to three years after FMT.

Methods

In this cohort study, we use stool samples of rCDI patients treated with FMT provided by the Netherlands Donor Feces Bank (NDFB, Leiden, the Netherlands) to assess the presence of MDR bacteria and the resistome. The NDFB uses standardised procedures for the collection, screening, preparation and storage of donor faecal suspensions and treatment and follow-up of rCDI patients as described previously [33][34]. In short, patients are first treated with antibiotics against *C. difficile* for at least four days until 24 hours before FMT. The day before FMT, patients receive a bowel lavage with macrogol solution [7]. Pre-FMT samples are collected during or after antibiotic treatment and before bowel lavage. Approximately three weeks after FMT a short-term post-FMT sample is requested. Pre- and short-term post-FMT stool samples of rCDI patients and their corresponding donors were collected between May 2016 and March 2021. Additionally, in February 2021 we contacted 53 patients to obtain clinical information and request a long-term follow-up (LTFU), or long-term post-FMT, stool sample. (~2 years after FMT.) Clinical data, including recurrence of CDI after FMT, were recorded for further investigation. Stool samples were stored at -80°C until DNA extraction for metagenomics sequencing or stored in an end concentration of 10% glycerol until MDR culture testing. Based on availability, stool samples from the cohort were included for culture and/or sequencing.

Written informed consent was obtained from all patients and donors for use of their faecal samples and follow-up data. Ethical approval was granted for the protocols and practice of the NDFB by the local medical ethics committee at the Leiden University Medical Center (reference P15.145, and long-term follow-up: B21.49).

Definition of multidrug-resistant bacteria

Definitions and testing methods were used as described previously [35]. Multidrug-resistant (MDR) bacteria were defined according to the definitions of the Dutch Working Group on Infection Prevention [36]. This includes ESBL-producing Enterobacterales; Enterobacterales and *Acinetobacter* spp. that are resistant to both fluoroquinolones and an aminoglycoside or produce carbapenemases; *Pseudomonas aeruginosa* that produces carbapenemase or is resistant to at least three of the following antibiotic classes or agents: fluoroquinolones, aminoglycosides, ceftazidime or piperacillin, and carbapenems; co-trimoxazole resistant *Stenotrophomonas maltophilia*; penicillin and vancomycin-resistant *Enterococcus faecium* (VRE); or methicillin-resistant *Staphylococcus aureus* (MRSA).

Culture and antimicrobial susceptibility testing of multidrug-resistant bacteria

To identify MDR bacteria in stool, an inoculating loop was used to scrape 10 µL faeces from frozen faeces aliquots (containing 10% glycerol). The faeces was enriched in 15 mL of tryptic soy broth and incubated for 18h at 35°C prior to plating on ChromID ESBL, ChromID OXA-48 agar, MacConkey tobramycin (8 mg/L) plus ciprofloxacin (0.5 mg/L) agar and VRE agar (bioMérieux, Marcy l'Etoile, France). For MRSA detection a separate brain heart infusion enrichment broth was used which was supplemented with 2.5 sodium chloride and 10 mg/L colistin sulphate and inoculation on MRSA-ID agar plate. All suspected MDR colonies were identified by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF) Biotyper (Bruker Daltonik; Bremen, Germany). Antibiotic susceptibility was evaluated by VITEK2 (Card N199, bioMérieux) adhering to the European Committee of Antimicrobial Susceptibility Testing (EUCAST) breakpoints version 11.0 [37]. ESBL production was confirmed using the double disk method. Isolates with a meropenem minimum inhibitory concentration > 0.25 mg/L (ETEST, bioMérieux) were investigated for carbapenemase production with a carbapenem inactivation method (CIM) test and an in-house multiplex PCR to detect *KPC*, *VIM*, *NDM*, *OXA-48* and *IMP* genes. VRE were confirmed by an in-house PCR targeting the *vanA* and *vanB* genes, and MRSA with the BD MAX assay targeting the *MREJ, mecA/mecC* and *Nuc* genes (BD, New Jersey, USA). Six known MDR bacteria-positive and seven MDR bacteria-negative defrosted faeces aliquots (also stored in 10% glycerol) of the NDFB donor screening served as positive and negative controls.

Whole-genome sequencing of multidrug-resistant isolates

To assess the antibiotic resistance genotype of MDR isolates and persistence after FMT, 24 out of 30 cultured MDR bacteria (16 / 20 from pre-FMT stool samples, 8 / 10 from short-term post-FMT; Table 1) were subjected to whole-genome sequencing. Only isolates of which DNA was available in January 2021 were included for sequencing. DNA was isolated using the QIAsymphony DSP Virus/Pathogen Midi Kit (Qiagen, Hilden, Germany) and sent to GenomeScan B.V. (Leiden, Netherlands) to sequence on the Illumina NovaSeq6000 platform (Illumina, Inc., San Diego, California, USA) generating 150 bp paired-end reads. (Reads per bacterial isolate: 780k [258k-1.64M] (median [range]).) The raw sequencing reads were cleared of human-derived reads by mapping to the GRCh38 genome [38] using bowtie2 (version 2.4.2, option '--very-sensitive-local') [39] and samtools (version 1.11) [40] before adapter and low-complexity read removal and quality-trimming using fastp (version 0.20.1, parameters '--cut_right --cut_window_size 4 --cut_mean_quality 20 -l 50 --detect_adapter_for_pe -y') [41]. High-quality reads were assembled using SPAdes (version 3.15.2, option '--isolate') [42]. All scaffolds were screened for antibiotic resistance genes using ABRicate (version 0.8.13, https://github.com/tseemann/abricate) with both the CARD (from 25 March 2021) [43] and ResFinder (from 25 March 2021) [44] databases, only retaining hits of full-length genes (100% coverage) with at least 97% identity. These cut-offs were used to keep the method consistent with and comparable to the resistome analyses (see below). Furthermore, assembled genomes were taxonomically classified using GTDB-Tk (version 2.1.0) [45].

These classifications were used to verify or further specify classifications made by MALDI-TOF Biotyper as described above and are used as species identification for sequenced isolates. Sequence data have been deposited in the European Nucleotide Archive (ENA) under project number PRJEB64622.

Shotgun metagenomic sequencing

Samples collected before 2021 were stored and prepared for sequencing as previously described [46]. This resulted in metagenomes of 49 patients pre- and short-term post-FMT and 56 donor samples of 8 donors that have been deposited in the ENA under project number PRJEB44737. An additional 22 sets of patient pre-, short-term and now including long-term post-FMT samples, of which 7 were sequenced earlier, as well as 14 donor samples from 8 donors were sequenced at GenomeScan B.V. (Leiden, Netherlands) using the Illumina NovaSeq6000 platform generating a median of 42.6M 150bp paired-end reads per sample. Raw reads, excluding human-derived reads (see below), have been deposited in the ENA under project number PRJEB64621. DNA was extracted from 100 mg of unprocessed patient and donor faeces using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (ZymoResearch, Irvine, California, USA), with bead beating step on a Precellys 24 tissue homogeniser (Bertin Technologies, Montigny-le-Bretonneux, France) at 5.5 m/s for three times 1 min with short intervals, as described previously [47]. Libraries were constructed using the NEBNext Ultra II FS DNA kit and NEBNext Ultra II Ligation kit (New England Biolabs, Ipswich, Massachusetts, USA), producing DNA fragments of approximately 500-700 bp. Besides, control samples were included to verify successful DNA isolation and sequencing. These include blank (water) controls, and ZymoBiomics Community Standard (ZymoResearch). Negative controls returned no sequencing reads, while positive controls contained reads of all species present in the communities.

Metagenomic read processing

Human-derived reads were removed from raw metagenomic reads by mapping reads to the human reference genome (GRCh38, NCBI accession ID GCF_000001405.26) using bowtie2 (version 2.4.2, option '--very-sensitive-local') and samtools (version 1.11). Remaining non-human reads were then processed by fastp (version 0.20.1) to trim low-quality 3'-ends (parameters: '--cut_right --cut_window_size 4 --cut_mean_quality 20'), remove low-complexity sequences (parameter: '-y'), remove remaining adapter sequences (parameter: '--detect_adapter_for_pe') and remove reads shorter than 50 bases (parameter: '-l 50'). The resulting high-quality metagenomic reads were used in read-based taxonomic profiling and assembly-based ARG profiling.

Quantification of multidrug-resistant isolates in metagenomes

To identify and quantify cultured and whole-genome sequenced MDR bacteria, we mapped metagenomic reads derived from the same stool sample to the respective assembled whole genome using BWA-MEM (version 0.7.17) [48]. Mapped reads were counted and coverage was quantified using samtools coverage (version 1.10).

Taxonomic profiling

Taxonomic microbiota profiles were determined using MetaPhlAn (version 4.0.3) [49], which maps reads to its custom marker database. Resulting taxonomic profiles quantified as percentages of the total microbiota were imported as R phyloseq object to facilitate visualisation and statistical comparisons [50].

Resistome analysis

ARGs were detected using an assembly-based approach. Quality-trimmed reads were assembled into scaffolds using metaSPAdes (version 3.15.4, default parameters) [51]. Next, resistance genes were identified with ABRicate (version 0.8.13) using both the CARD (from 25 March 2021) and ResFinder (from 25 March 2021) databases, only retaining hits of full-length genes (100% coverage) with at least 97% identity. These criteria were selected based on visual inspection of the BLAST hits to balance high specificity and adequate sensitivity. As a control, we repeated the analyses using a coverage cut-off of 50% to include partial genes, which yielded equivalent results. ARGs were annotated with their respective target antibiotic and antibiotic class using the respective databases' annotation files. Scaffolds are quantified by mapping the metagenomic reads back to the scaffolds using BWA-MEM (version 0.7.17) and samtools (version 1.10). Quantifications were normalised to reads per kilobase per million (RPKM) by dividing the number of reads mapped to each contig by the length of the contig and the number of high-quality reads used for the assembly, multiplied by 1,000 * 1,000,000. To annotate scaffolds with additional information, they were taxonomically classified using the Genome Taxonomy Database Toolkit (GTDB-Tk; version 2.1.0) and the Contig Annotation Tool (CAT, version 5.2.3, parameters: '-r 10 -f 0.5', [52] – which uses Prodigal version 2.6.3 [53]; DIAMOND version 2.0.6 [54]; and the NCBI BLAST nr database from 7 January 2021, https://ftp.ncbi.nlm.nih.gov/blast/db/), using CAT as primary annotation and filling in gaps in classification using the result of GTDB-Tk. The genomic origin of scaffolds with ARGs (chromosome or plasmid) was estimated using viralVerify (version 1.1, option '-p', https://github.com/ablab/viralVerify). Finally, all the scaffold annotation data was loaded into R (version 4.0.2; https://www.R-project.org/) for further analyses. The source code of these analyses is available online at: https://doi.org/10.5281/zenodo.8256351.

Statistical analyses

The colonisation rate of MDR bacteria among patients was compared between pre- and short-term post-FMT and short-term and the long-term post-FMT using McNemar's chi-square test for paired data. Depth of

coverage of MDR bacteria in metagenomic data was compared between pre- and post-FMT with a paired t-test on log-transformed coverage values.

For comparing taxonomic compositions of metagenomes and resistomes between donors and patients, we selected one value for each donor as representative. For principal component analyses (PCA), we picked the middle sample for each donor based on donation date (number of samples / 2, rounded up). Aitchison distance was used as metric to describe differences between microbiota or resistome compositions, with resistomes we used a pseudocount of 0.001. For comparisons of diversity metrics using boxplots, we selected the median value for each donor. In PCA, donors and patients are compared using PERMANOVA and PERMDISP tests, considering the repeated measures in patients. Diversity metrics (richness, total abundance, Shannon index, and Simpson evenness) are compared between donors and patients using t-tests, or Wilcoxon rank sum tests when values were visually not normally distributed. Abundance values were log-transformed. Within patients, all pre- and short-term post-FMT measures are compared using a paired t-test, while within the subgroup of 22 patients of whom we have collected long-term post-FMT samples values are first compared using repeated measures ANOVA. If $p < 0.05$, paired t-tests were used as post-hoc test to determine differences between pre-FMT and long-term post-FMT and between short and long-term post-FMT.

To evaluate if antibiotic (vancomycin) treatment duration before FMT influenced the resistome, we compared the pre-treatment duration of patients (n=52) with their resistome richness (number of different ARGs), total abundance, Shannon diversity and Simpson evenness using Spearman correlation.

Colonisation rate of patients by Enterobacterales was determined using MetaPhlAn4. If the order Enterobacterales had abundance > 0%, we count it as present and otherwise absent. Colonisation rates between timepoints are compared using McNemar's test. Total abundances were compared using repeated measures ANOVA, followed by pairwise t-tests.

All statistical tests were done in R version 4.0.2, using the base, rstatix, vegan, and pairwiseAdonis packages. When multiple tests were conducted simultaneously, p-values were adjusted using Holm's method. A p-value below 0.05 was considered significant.

Data availability

Sequencing reads generated for this study are available in the European Nucleotide Archive under project numbers PRJEB64622, PRJEB44737 and PRJEB64621.

Results

Donor and patient population characteristics

During the sample collection period the NDFB provided faecal suspensions for 208 FMT treatments of 187 rCDI patients. Eighty-seven pairs of patient stool samples (median age: 73, interquartile range (IQR): 64-81 years, 56 females (64%)) from pre- and short-term post-FMT in 10% glycerol were available for testing for MDR bacteria by culture (Table 1). Sixty-three pairs of raw frozen patient stool samples (median age: 73 years, interquartile range (IQR) 65-81 years; 40 females (63%)) were available for shotgun metagenomic deep sequencing (Table 1). For 42 patients, samples were available for both culture and metagenomics. Twenty-two patients provided a long-term post-FMT sample (median age: 73, IQR 64-78 years; 14 females (64%)). Furthermore, a total of 70 donor stool samples from 11 different donors (median age: 31 years, IQR 27-42 years; 6 females (55%)) were included for metagenomics sequencing (Table 1). The resistome analysis includes only complete sample triads and sample tetrads with long-term post-FMT if both pre- and short-term post-FMT samples were available. That is, patients' pre-FMT and short-term post-FMT, and corresponding donor stool sample (63 patients short-term and 21 long-term post-FMT, and 52 donor samples from 11 donors). The median sampling times for patients are: 1 day pre-FMT (IQR 1-3 days), 27 days post-FMT (IQR 20-48 days; short-term), and 801 days post-FMT (IQR 447-1114 days; long-term).

Sample source	Faecal samples tested by culture for MDR bacteria	MDR isolates whole-genome sequenced / total cultured	Faecal samples used in metagenomic sequencing	Samples with both culture and metagenome data
Donor	76 (15 donors)	0 / 0	70 (11 donors)	43 (8 donors)
Pre-FMT	87	16 / 20	63	42
Short-term post-FMT (~3 wks)	87	8 / 10	63	44
Long-term post-FMT (~1-3 yrs)	22	0 / 2	22	22

Table 1. Overview of sample numbers and applied techniques

FMT: faecal microbiota transplantation; MDR: multidrug-resistant.

Prevalence of multidrug-resistant bacteria decreases after FMT

Stool samples of 87 patients were selectively cultured to assess carriership of MDR bacteria. Before FMT, 20/87 (23.0%) of patients carried an MDR bacterium (figure 1A, table 2). Three weeks after FMT, the colonisation rate decreased to 10/87 (11.5%; $p<0.0001$), of which 7 MDR bacteria were also cultured from stool samples of the same patient before the FMT. In the long-term, the colonisation rate decreased further to 2/22 (9.1%; $p = 0.0008$ compared to short-term post-FMT). Both were ESBL-producing *E.* coli also detected in the short-term post-FMT samples, thereby they appear to be long-term persisters. Within the subgroup of patients that provided long-term samples, colonisation rates and the shift after FMT were similar as in all 87 patients (figure 1B; pre-FMT 5/22 = 22.7%, post-FMT 4/22 = 18.2%; $p = 0.009$).

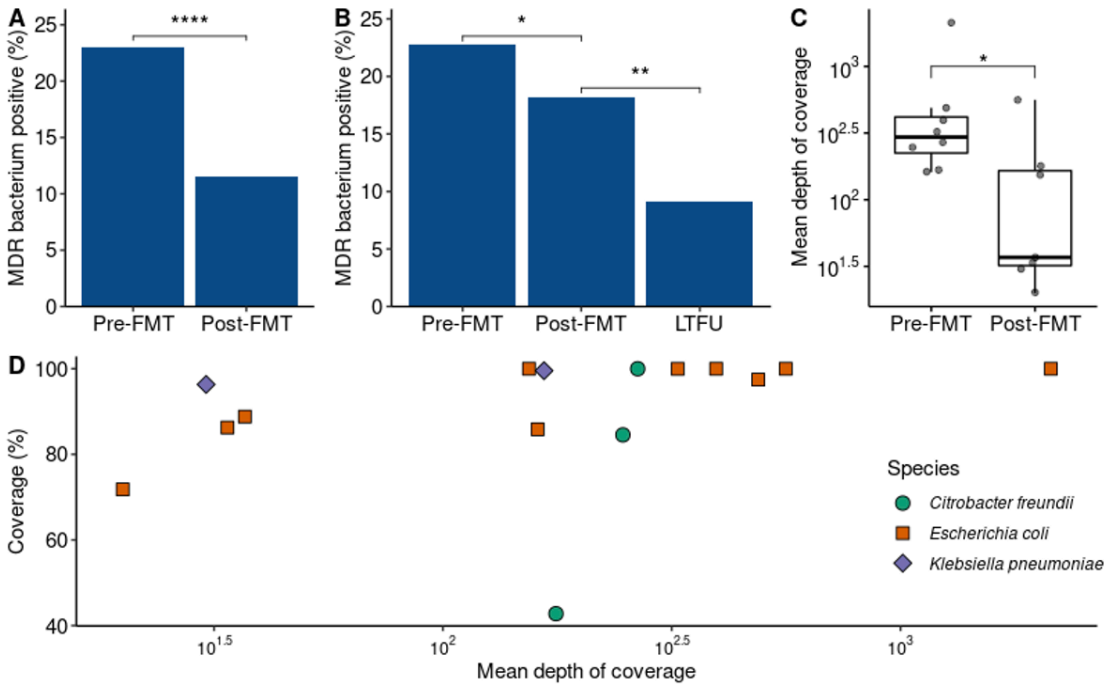

Figure 1. Effect of faecal microbiota transplantation on prevalence and abundance of cultured multidrug resistant bacteria. Stool samples of recurrent *C. difficile* infected (rCDI) patients were cultured to assess the prevalence of multidrug resistant (MDR) bacteria before and after faecal microbiota transplantation (FMT). For a subgroup of patients, long-term follow-up samples were obtained and tested. Cultured isolates were subjected to whole-genome sequencing and metagenomic sequencing data from the same stool samples were mapped to the assembled genomes to quantify the bacteria in the metagenomes. A) Prevalence of MDR bacteria in 87 rCDI patients. Before FMT, 20/87 patients were colonised by a MDR bacterium, after FMT 10/87 were colonised. The colonisation rate after FMT is significantly lower (McNemar's chi-square, $p < 0.0001$). B) Colonisation rates in 22 patients of whom long-term follow-up (~1-3 years after FMT) samples were collected. 5/22 had a MDR bacterium before FMT, 4/22 were colonized 3 weeks after FMT, and 2/22 were still colonized a few years later. In both time intervals, colonisation rates dropped ($p = 0.01$ and $p = 0.005$, respectively). C) To compare essay sensitivity, we mapped metagenomics reads to the assemblies of whole-genome sequenced cultured MDR isolates. In general, MDR bacteria had higher abundance in patients before FMT compared to 3 weeks after FMT (t-test, $p = 0.016$). D) Breadth of coverage and relative abundance of MDR bacteria in metagenomic sequencing data per species. Abundance is expressed as mean depth of coverage (nucleotides per position) as reported by samtools coverage. Asterisks indicate statistically significant differences, *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$.

MDR: multidrug resistant, FMT: faecal microbiota transplantation, LTFU: long-term follow-up.

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Patient	Sample timepoint	Species	Resistance phenotype	Genotype based on WGS	Detected in metagenome
P22	Post-FMT	*E. coli*	Aminoglycoside, fluoroquinolone, ampC	*APH(3')-Ia, APH(6)-Id, APH(3")-Ib, ANT(2")-Ia, acrD, ampC, QnrB5, emrR*	Yes
P30	Post-FMT	*E. coli*	Aminoglycoside, fluoroquinolone	*acrD, emrR, emrD*	Yes
P31	Pre-FMT	*E. coli*	Aminoglycoside, fluoroquinolone, ceftazidime	*acdD, emrR, emrB, ampC*	Yes
P33	Pre-FMT	*K. pneumoniae**	Aminoglycoside, fluoroquinolone, ESBL	*aadA2, aadA16, AAC(3-IId, TEM-1, SHV-119, CTX-M-14*	Yes
P33	Post-FMT	*K. pneumoniae**	Aminoglycoside, fluoroquinolone, ESBL	*aadA2, aadA16, AAC(3-IId, TEM-1, SHV-119, CTX-M-14*	Yes
P38	Pre-FMT	*E. coli**	Fluoroquinolone, ESBL	*CTX-M-27, ermR, emrB*	Yes
P38	Post-FMT	*E. coli**	Fluoroquinolone, ESBL	*CTX-M-27, ermR, emrB*	Yes
P39	Pre-FMT	*E. coli*	Aminoglycoside, fluoroquinolone, ESBL	*CTX-M-15, OXA-1, acrD, AAC(3)-IIe, emrA, emrB, emrR*	Yes
P44	Pre-FMT	*C. freundii**	Aminoglycoside, fluoroquinolone, ESBL	*CTX-M-15, OXA-1, AAC(3)-IIe, AAC(6')-Ib-cr, APH(6)-Id, APH(3")-Ib, QnrB6*	Yes
P44	Post-FMT	*C. freundii** + *E. coli*	Aminoglycoside, fluoroquinolone, ESBL + ESBL	*CTX-M-15, OXA-1, AAC(3)-IIe, AAC(6')-Ib-cr, APH(6)-Id, APH(3")-Ib, QnrB17*	Yes
P44	LTFU (3yr)	*E. coli*	ESBL	NA	NA
P51	Pre-FMT	*C. freundii*	ESBL	*CTX-M-9*	Yes

Patient	Sample timepoint	Species	Resistance phenotype	Genotype based on WGS	Detected in metagenome
P58	Pre-FMT	*E. coli*	Aminoglycoside, fluoroquinolone	*acrD, emrR*	Yes
P59	Pre-FMT	*E. coli**	Aminoglycoside, fluoroquinolone, ESBL	*CTX-M-14, acrD, AAC(3)-IIe, APH(3")-Ib, APH(6)-Id, emrR*	Yes
P59	Post-FMT	*E. coli**	Aminoglycoside, fluoroquinolone, ESBL	*CTX-M-14, acrD, AAC(3)-IIe, APH(3")-Ib, APH(6)-Id, emrR*	Yes
P59	LTFU (1yr)	*E. coli**	Aminoglycoside, fluoroquinolone, ESBL	NA	NA
P64	Pre-FMT	*E. coli**	Aminoglycoside, fluoroquinolone	*AAC(6')-Ib-cr, emrA, emrB, emrR*	NA
P64	Post-FMT	*E. coli**	Aminoglycoside, fluoroquinolone	*acrD, APH(3'')-Ib, APH(6)-Id, ampC, ampH, emrA, emrB, emrR*	NA
P65	Pre-FMT	*E. hormaechei_A (cloacae)*	Aminoglycoside, fluoroquinolone, ESBL	*ACT-27, CTX-M-15, OXA-1, TEM-1, AAC(3)-IIe, APH(6)-Id, APH(3'')-Ib, AAC(6')-Ib-cr, QnrB6*	NA
P66	Pre-FMT	*M. morganii*	ESBL	*DHA-18*	NA
P67	Pre-FMT	*P. mirabilis*	ESBL	*CTX-M-1*	NA
P68	Pre-FMT	*P. mirabilis_B (vulgaris/mirabilis)*	ESBL	(none)**	NA
P69	Pre-FMT	*C. freundii**	Aminoglycoside, fluoroquinolone, ESBL	*CTX-M-15, TEM-1, OXA-1, AAC(3)-IIe, APH(3")-Ib, APH(6')-Id, QnrB6*	NA
P69	Post-FMT	*C. freundii**	Aminoglycoside, fluoroquinolone, ESBL	*CTX-M-15, TEM-1, OXA-1, AAC(3)-IIe, APH(3")-Ib, APH(6')-Id, QnrB17*	NA
P70	Pre-FMT	*E. coli**	ESBL	*ampC, ampH, SHV-134*	NA
P70	Post-FMT	*E. coli**	ESBL	*ampC, ampH, SHV-134*	NA

Patient	Sample timepoint	Species	Resistance phenotype	Genotype based on WGS	Detected in metagenome
P71	Pre-FMT	*K. pneumoniae*	ESBL	NA	NA
P72	Pre-FMT	*P. hauseri*	ESBL	NA	NA
P73	Pre-FMT	*C. freundii*	ESBL	NA	NA
P74	Pre-FMT	*E. cloacae*	ESBL	NA	NA
P75	Pre-FMT	*E. cloacae**	ESBL	NA	NA
P75	Post-FMT	*E. cloacae**	ESBL	NA	NA

Table 2. Overview of cultured multidrug-resistant bacteria with genotype and phenotype.

**: same species before and after FMT, persistence is likely based on resistance genotype (supplementary figures 1-3) when available. **: no antibiotic resistance genes were detected in the genome sequence data. Species names are listed as in the Genome Taxonomy Database (GTDB), and the alias known by the National Center for Biotechnology Information (NCBI) is given in parentheses when different. When multiple multidrug-resistant bacteria were cultured from the same stool, isolate characteristics are separated by a plus ('+') sign.*

FMT: faecal microbiota transplantation, LTFU: long-term follow-up, ESBL: extended-spectrum beta-lactamase, NA: data not available (because the isolate and/or the metagenome were not sequenced)

Whole-genome sequencing of multidrug-resistant and comparison with metagenomics

Twenty-four cultured isolates of multidrug-resistant bacteria were subjected to whole-genome sequencing to study the ARGs and possible persistence after FMT. In all but one genome we were able to detect ARGs associated with the resistance phenotype; e.g., ESBL genes in isolates classified as ESBL-producing (table 2; supplementary figures 1-3). Furthermore, we have both cultured an MDR bacterium and sequenced total DNA from 16 patients' stool samples using shotgun metagenomics. We mapped metagenomic reads to the assembled isolate genome to compare essay sensitivity and determine relative abundances in the microbiota. As expected in patients pre-treated with antibiotics, we found that MDR bacteria had higher relative abundances in rCDI patients before FMT than after FMT (figure 1C; $p = 0.006$). We detected near-complete genomes of MDR isolates in the metagenomes, except one *Citrobacter freundii* (43%; figure 1D). We also compared resistance genes detected in the WGS data to those detected in metagenomic data to estimate the sensitivity of metagenomic sequencing compared to culturing. We found the relevant MDR bacterial resistance genes of cultured isolates back in their respective metagenomes (table 2). Besides, metagenomic data from P44 suggested the presence of an ESBL-producing *E. coli* in the pre-FMT sample, while culture only picked it up in the post-FMT faeces. These

data suggest that combining bacterial culture with metagenomic sequencing can be used synergistically and provide more detailed results than either method alone.

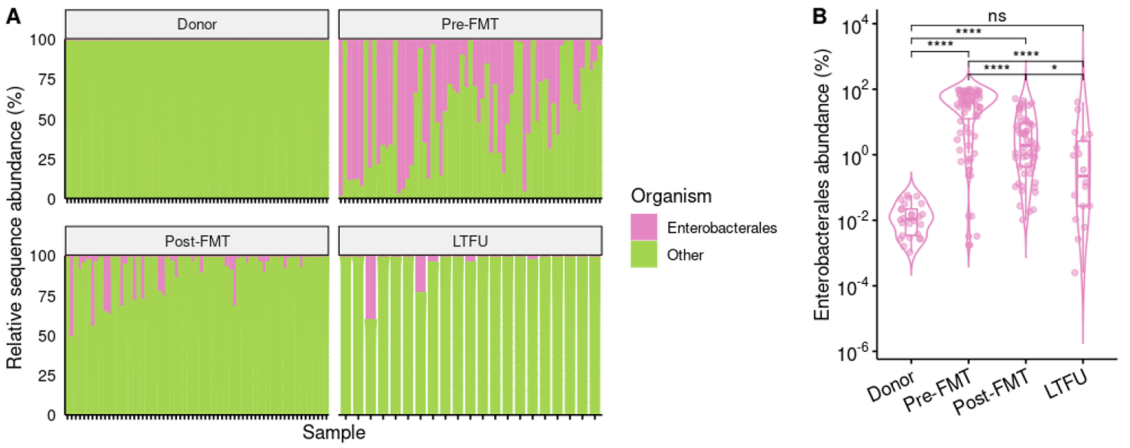

Figure 2. Prevalence and abundance of Enterobacterales in faecal donors and faecal microbiota transplantation recipients. A) Relative abundances of Enterobacterales in metagenomes as determined by MetaPhlAn4. B) Total abundances of Enterobacterales in stool donors and rCDI patients treated with FMT sampled one day before (Pre) FMT, three weeks after (Post) FMT and 1-3 years after FMT (long-term follow-up, LTFU). Statistically significant differences are indicates by asterisks, *: $p < 0.05$; ****: $p < 0.0001$.

FMT: faecal microbiota transplantation, LTFU: long-term follow-up.

Effect of FMT on gut microbiota composition and diversity

We used MetaPhlAn4 to study the gut microbiota compositions of faeces from healthy donors and FMT recipients before and after FMT. Donors had a stable microbiota, dominated by Firmicutes, Bacteroidetes and Actinobacteria (supplementary figure 4A-B). Enterobacterales were present in 26/70 donor stools (37%). In rCDI patients, that underwent anti-CDI treatment prior to FMT (53 x vancomycin, 6 x fidaxomicin, 1 x metronidazole 1 x metronidazole+vancomycin, 2 unknown), Actinobacteria and Bacteroidetes were much less present, while Proteobacteria (mostly *Escherichia coli* or *Klebsiella pneumoniae*) were often dominant (>50% abundance in 31/63 patients = 49%). Enterobacterales were present in all pre-FMT patient stools. After FMT, the patients' microbiota were mixed with their donors' and profiles were more donor-like. Enterobacterales prevalence dropped in the weeks after FMT (58/63 = 92%; $p < 0.0001$; figure 3A) and decreased further in the long-term (18/21 = 86%; $o = 0.012$). Abundance of Enterobacterales also decreased shortly after FMT ($p < 0.0001$; figure 3B) and continued lowering in the long-term ($p = 0.025$) to levels no longer different from those seen in donors ($p = 0.09$). We also compared the alpha and beta diversity between species profiles of donor and patient metagenomes to quantify differences (figure 3). PCA of species profiles showed differences between donors and patients (figure 3A; PERMANOVA, $p = 0.001$; PERMDISP, $p < 0.0001$). Differences in microbiota

profiles were most pronounced between donors and rCDI patients before FMT ($p = 0.003$). While still different, the microbiota profiles after FMT were slightly more donor-like ($p = 0.014$ for both short and long-term compared to donors). When comparing taxonomic profiles at the species rank, we see that richness and alpha diversity (Shannon index) were higher in donors than in rCDI patients (figure 3B-C; $p < 0.0001$) and increased dramatically in patients after FMT ($p < 0.0001$) to levels as seen in donors ($p > 0.1$). Richness and Shannon index remained high at the long-term. The Simpson evenness, also known as inverse Simpson index or Simpson's dominance, was not different between donors and patients (figure 3D; $p > 0.3$). Overall, our data show the expected pattern of lower diversity in rCDI patients, high diversity in FMT donors, and increased diversity in patients after FMT.

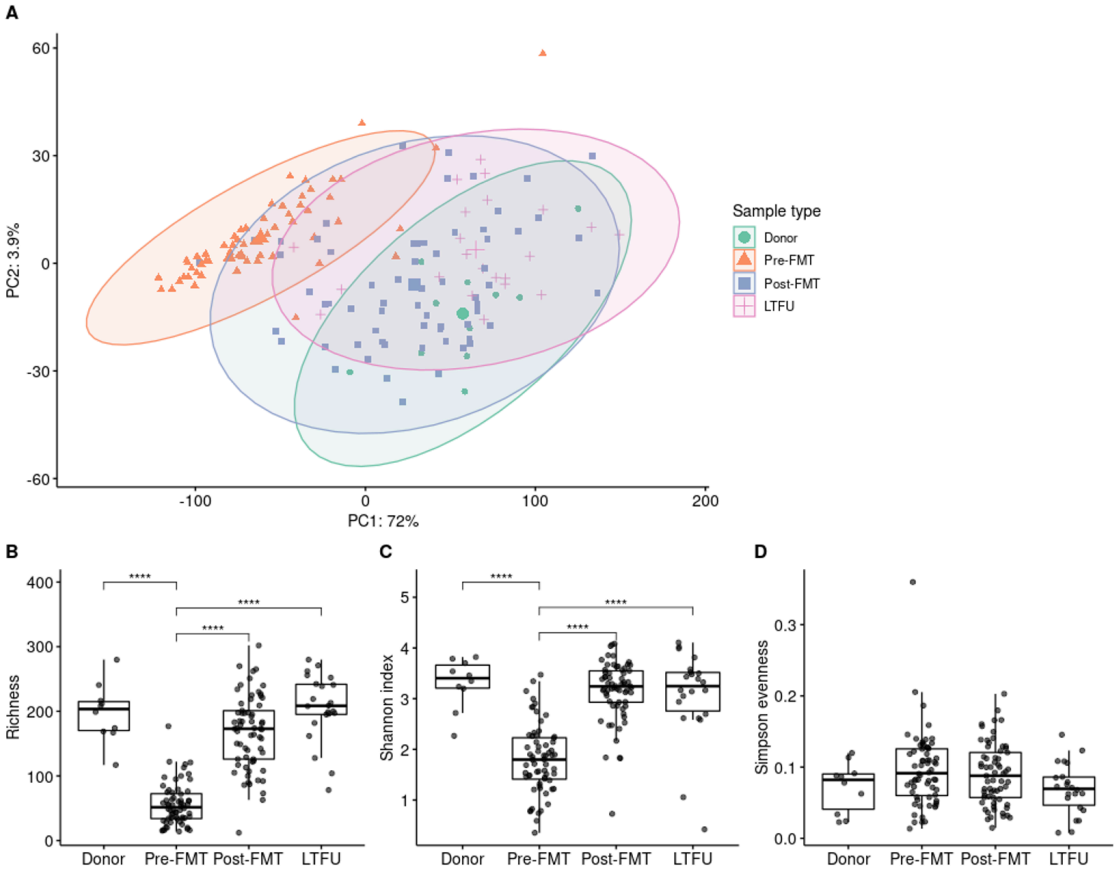

Figure 3. Comparison of gut microbiota composition and diversity. Stool samples ofhealthy donors and recipients of faecal microbiota transplantation (FMT) were sequenced using shotgun metagenomics. We used MetaPhlAn4 to determine the taxonomic composition of metagenomes and compared alpha and beta diversity at the species rank between donors and recipients before and after FMT, and at a long-term follow-up moment, roughly between 1-3 years after FMT. A) Beta diversity expressed as Aitchison distances in a principal component analysis (PCA). Percentages on the X- and Y-axis represent the variance explained by the first two components. B-D) Species richness, Shannon index and Simpson evenness compared between donors and recipients, respectively. Asterisks indicate statistically significant differences, ****: $p < 0.0001$.

FMT: faecal microbiota transplantation, LTFU: long-term follow-up.

FMT decreases resistance gene abundance, not diversity

Using the metagenomic sequencing data of stool samples from 52 donor stools from their respective 11 donors, we determined the resistome of 63 patients before and after FMT and (supplementary figures 5-6). Next, we quantified differences in resistome composition between donors and patients using PCA (figure 4A). Donors had similar resistomes and often had the same ARGs for aminoglycoside, diaminopyrimidine and tetracycline resistance (supplementary figure 5), while rCDI patients had a very different resistome (PERMANOVA, p =

0.003; PERMDISP, $p < 0.0001$), in which different ARGs for beta-lactam and fluoroquinolone resistance as well as multidrug efflux pumps were prevalent (supplementary figures 5-6). After FMT, a shift in the patients' resistome toward a donor-like composition is visible, although it remained different from the donors' ($p = 0.003$). At long-term follow-up (~1-3 years), the resistome appears visually more similar to the donor's, but was still statistically different ($p = 0.012$). To gain more insight in resistome changes after FMT, we visualised relative abundances of resistance genes by antibiotic class (supplementary figure 7A), and distances from each patient resistome to all donor resistomes (supplementary figure 7B). Both methods suggest that after FMT resistomes of patients become more like the donors'.

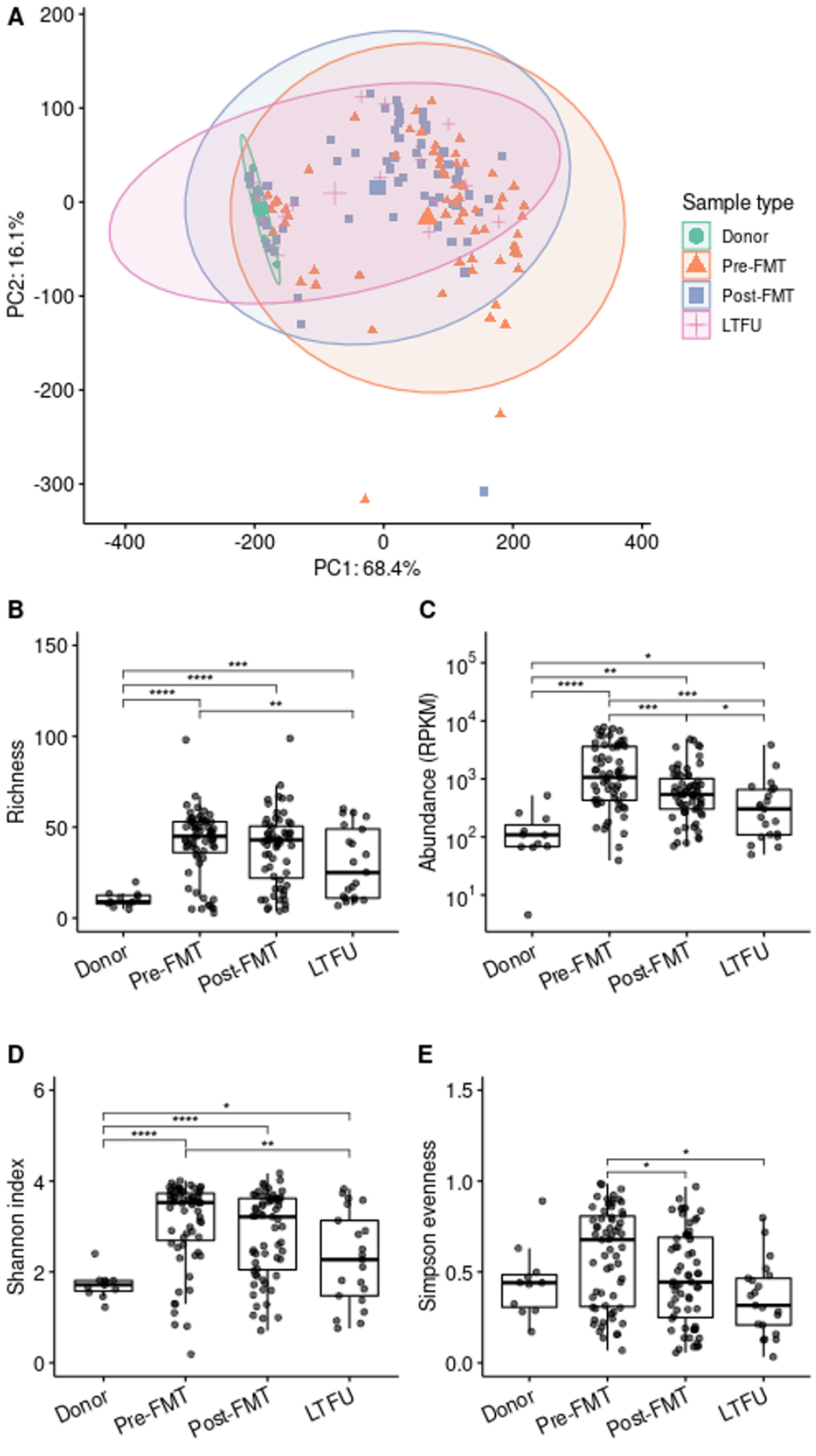

Figure 4. Overview of resistomes of faeces donors and faecal microbiota transplantation recipients. Following metagenomics sequencing of stool samples, we determined the

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resistome of faecal donors and faecal microbiota transplantation (FMT) recipients and looked for differences in these groups and between before and after FMT. A) Principal component analysis (PCA) of resistomes, based on Aitchison distances. Percentages on the X- and Y-axis represent the variance explained by the first two components. B-E) Antibiotic gene richness, total abundance, Shannon index and Simpson evenness compared between groups and between recipient timepoints, respectively. Asterisks indicate statistically significant differences, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

FMT: faecal microbiota transplantation, LTFU: long-term follow-up.

We find that patients before FMT had more different resistance genes (higher resistome richness) in their faecal metagenomes than donors ($p < 0.0001$; figure 4B). The duration of vancomycin pre-treatment did not significantly influence the resistome (supplementary figure 8). After FMT, resistome richness in patients did not change ($p > 0.1$) and remained higher than in donors (short-term post-FMT: $p < 0.0001$; long-term: $p = 0.0002$). The total abundance of resistance genes was also higher in patients pre-FMT than in donors (figure 4C; $p < 0.0001$), but in contrast to the resistome richness, abundance decreased in patients shortly after FMT ($p = 0.0003$). In the long term, the abundance lowered further ($p = 0.02$), although abundances remained higher than in donors ($p = 0.02$). The Shannon index combines richness and abundance and likewise showed a higher resistome diversity in rCDI patients compared to donors, and a decrease after FMT (figure 4D). The Simpson evenness shows no statistical difference between donors and patients (figure 4E; $p > 0.1$), but indicates a decrease of resistome diversity in patients after FMT ($p = 0.017$). In summary, FMT appears to alter the diversity of the resistome in recipients by lowering relative abundances of ARGs.

We observed different prevalence and abundance patterns of ARGs from different antibiotic classes (supplementary figures 5-6). To explore this further, we selected classes of which genes were present in both donors and patients and divided them in two groups. One group (beta-lactamase, fluoroquinolone, and multidrug efflux pump) consists of genes that are rare in donors and common and abundant in patients (supplementary figure 9 A-C and G-I). The abundance of genes in this group decreased shortly after FMT, while the resistome richness decreased only in the long-term. The second group (aminoglycoside, diaminopyrimidine and tetracycline) is common in donors (supplementary figure 9 D-F and J-L). Genes from this donor-associated group may have been transferred to the recipients, resulting in greater resistome richness after FMT and their abundance did not decrease after FMT. These results highlight that the effects of FMT on the resistome vary depending on type of antibiotic and the taxa that carry the genes.

Remarkable resistances

We found a number of ESBL genes in our resistome data, also in donor faeces. Furthermore, we found carbapenamase genes and one colistin resistance gene (mrc-10_1, predicted to be on a plasmid) only in rCDI patients before FMT. Vancomycin genes were detected by metagenomics in 7 out of 63 patients before FMT (11.1%) and 11 / 63 after FMT (17.5%; supplementary figure 10). Besides, our cultures picked up an vancomycin-resistant *Enterococcus faecalis* from short-term post-FMT stool, which is not listed as MDRO. These resistances as well as those predicted to be on plasmids are discussed in more detail in the supplementary results and supplementary figures 10-12.

Predicted plasmid-mediated antibiotic resistance remains high

Using the plasmid prediction algorithm from viralVerify, we assessed which contigs with resistance genes were likely to derive from chromosomes and which from plasmids. Most (4,567 / 6,662 or 68.6%) of the resistance genes were predicted to derive from chromosomes and 400 (6%) likely derived from plasmids. The remaining 1,695 (25.4%) contigs with ARGs could not confidently be classified to either plasmid or chromosome. Unlike chromosomal resistances, which follow the general resistome pattern and decrease after FMT (figure 5A-C), we find that the resistome richness, abundance, and diversity of ARGs derived from plasmids were higher in rCDI patients than in donors and stayed higher after FMT ($p \leq 0.01$; figure 5D-F). This effect persisted up to three years after the FMT, suggesting that FMT may not significantly influence plasmid-mediated antibiotic resistance.

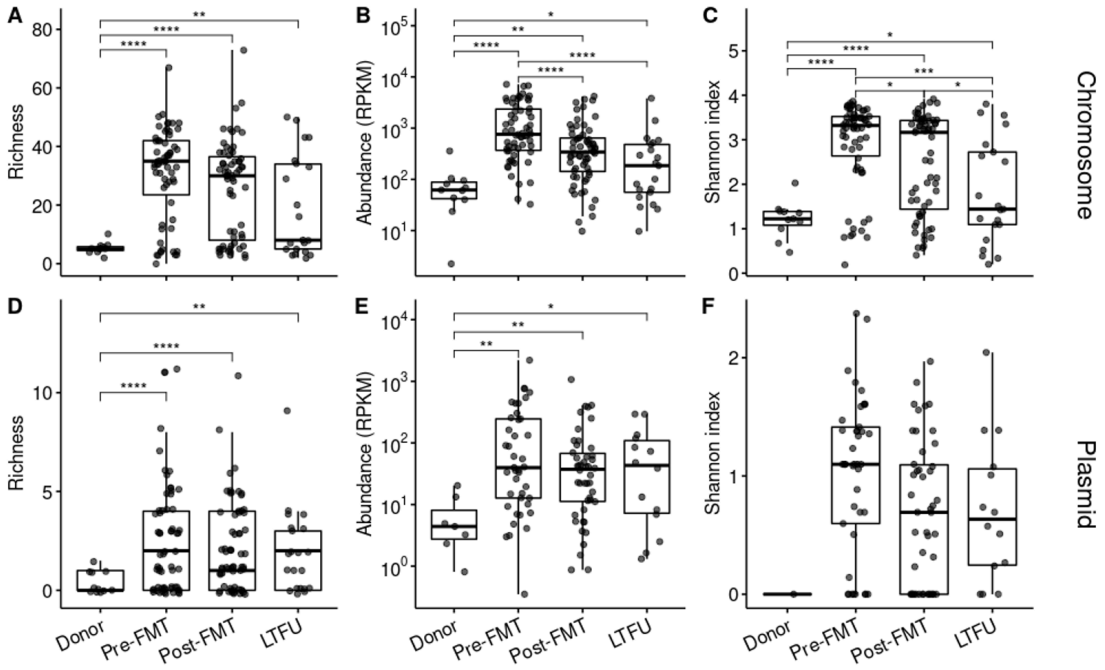

Figure 5. Resistome comparisons for chromosomal resistance genes and plasmid-associated resistance genes. To assess possible differences between antibiotic resistance genes encoded on the chromosome and plasmid-mediated resistance, we predicted the origin of assembled contigs using viralVerify. We then compared the parameters richness (number of resistance genes) total abundance and Shannon index between faecal donors and FMT recipients separately for resistances genes that are predicted to be on chromosomes (A-C) and genes that are predicted to derive from plasmids (D-F). Richness (A, D) is compared using Wilcoxon rank sum tests, while abundance (B, E) and Shannon index (C, F) are compared using t-tests. Statistically significant differences are indicates by asterisks, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

FMT: faecal microbiota transplantation, LTFU: long-term follow-up.

Discussion

Our current study leverages the strengths of validated culture techniques, metagenomic deep sequencing and long-term follow-up samples to provide the most detailed study on the effects of FMT on antibiotic resistance thus far. The FMT recipients had a more diverse and donor-like resistome and microbiota profile after FMT. The 1-3-year follow-up and plasmid predictions provide novel insights in microbiota dynamics and antibiotic resistance after FMT. We find strong correlations between culture and sequencing data, while each has its advantages. The plasmid predictions give insight into which resistances may be more easily transferred between bacteria, posing a risk when potentially pathogenic species are present.

We find effects of FMT on MDR bacteria and the resistome of rCDI patients that last for up to 3 years. The effect of FMT is more pronounced on the bacterial species composition than on ARGs. Already three weeks after FMT the recipients' taxonomic composition is similar to the donors', while the resistomes remain different even in the long-term. As most patients were pre-treated with vancomycin, Gram-negative bacteria such as Enterobacterales could thrive. Indeed, we see high prevalence and abundance of Enterobacterales before FMT and a significant decrease after FMT. Enterobacterales are well-studied and many ARGs and plasmids have been described in this group, leading to possible biases in reference databases. The high prevalence of Enterobacterales after FMT correlates with the persistently high resistome richness, while the abundance of both is reduced. This raises the hypothesis that FMT after antibiotic treatment primarily balances the bacterial species composition, lowering abundances of MDR bacteria. However, these bacteria are not eradicated and their ARGs remain present in low relative abundances. The outcome could then be that both *C. difficile* and MDR bacteria cause no symptoms after FMT, but as long as they are present in the patient, there is still a risk of infection [13][14][15].

There are vast differences in resistome composition between healthy donors and rCDI patients, which may relate to medical history and use of antibiotics [55]. More generally, it has been reported that anaerobic gut commensals frequently have aminoglycoside and tetracycline resistance genes [56], which we found to be common in donors and patients, although there were differences in the specific genes. These anaerobic and commensal bacteria may be transferred during FMT and cause a shift in the resistome composition. Consequently, the resistome of FMT recipients is complemented with a distinct donor resistome and this leads to different effects of FMT on different antibiotic classes, as was also shown before [57]. Furthermore, we find that most antibiotic resistance genes in the gut microbiota are chromosomally encoded and may not be as easily transferred as plasmids. Therefore, we hypothesise that most antibiotic resistances are bound to their host species. However, we also see that although the microbiota profile is restored after FMT, the resistome of recipients remains different, largely due to persistence of resistance genes and predicted plasmids. This may be associated with age and comorbidities. To further elucidate this discrepancy, techniques are needed that can link ARGs to their host organism, such as Meta-HiC [56]. This should provide clinicians extra information on the possible risk of infection and treatment options.

None of the patients in our cohort reported an infection with an MDR bacterium after FMT and we included no control group without FMT. Therefore, we have no data on risk of infections with MDR bacteria post-FMT. In other patient groups it has been found that FMT decreases the risk of infection [58][59]. In addition, FMT may reduce the number of infections with MDR bacteria even if the patients' guts are not decolonised with MDR bacteria [60]. The hypothesised mechanism is that the gut microbiota is restored by FMT to a balanced state that is resilient to MDR bacteria [58]. This situation may be described as reduced infection susceptibility or infection resistance. Our data give new details on the involved microbiota processes that play a role in this.

Microbiota therapies for the management of MDR bacteria are still in an experimental phase. To better assess the possible benefits, we need larger (randomised controlled) trials and multi-omics studies combined with classical microbiological methods that can link ARGs to bacterial taxa, and to the host's gut ecosystem. Additionally, the use of international registries for FMT can help collect long-term data to assess infection risks in different patient populations. And finally, studies with control patients and more diverse patients are needed to explain the resistome differences and obtain more generalisable results. Then we can evaluate the feasibility of FMT to control antibiotic resistance in infection-susceptible patients.

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Author contributions

SN, KEWV, RDZ, EJK and EMT conceptualised and designed the study. QRD, EJK, JJK and EMT supervised the study. KEWV, JJK, EJK and EMT supervised treatment of patients. KEWV collected clinical and microbiological data and performed analyses. SN performed genomics, metagenomics and statistical analyses and drafted the manuscript. All authors reviewed and edited the manuscript, and approved the final version.

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