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Microbial taxonomic and metabolic alterations during faecal microbiota transplantation to treat *Clostridium difficile* infection

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Running title

FMT for rCDI alters microbiota structure and function

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Summary

Objectives: This study aimed to examine changes to the microbiota composition and metabolic profiles of seven patients with recurrent *Clostridium difficile* infection (rCDI), following treatment with faecal microbiota transplant (FMT).

Methods: 16S rDNA sequencing and ^{1}H NMR were performed on faecal samples from the patients (pre-, post-FMT, and follow-up) and the associated donor samples. Sparse partial-least-square analysis was used to identify correlations between the two datasets.

Results: The patients' microbiota post-FMT tended to shift towards the donor microbiota, specifically through proportional increases of *Bacteroides*, *Blautia*, and *Ruminococcus*, and proportional decreases of *Enterococcus*, *Escherichia*, and *Klebsiella*. However, although cured of infection, one patient, who suffers from chronic alcohol abuse, retained the compositional characteristics of the pre-FMT microbiota. Following FMT, increased levels of short-chain fatty acids, particularly butyrate and acetate, were observed in all patients. Sparse partial-least-square analysis confirmed a positive correlation between butyrate and *Bacteroides*, *Blautia*, and *Ruminococcus*, with a negative correlation between butyrate and *Klebsiella* and *Enterococcus*.

Conclusions: Clear differences were observed in the microbiota composition and metabolic profiles between donors and rCDI patients, which were largely resolved in patients following FMT. Increased levels of butyrate appear to be a factor associated with resolution of rCDI.

Keywords: *Clostridium difficile*; Faecal microbiota transplantation; Metataxonomics; Metabonomics; Alcohol abuse; Sparse partial-least-square analysis.

Introduction

Although *Clostridium difficile* is present in the intestines of ~3-5% of healthy adults,[1] the occurrence of *C. difficile* infection (CDI) in healthy individuals is relatively uncommon due to the protective effect of the gut microbiota. The incidents and severity of CDI has risen significantly over the last decade, and it is now recognised as the main causative agent of healthcare-associated infectious diarrhoea in hospitals worldwide.[2] The standard treatment for CDI is the administration of metronidazole for mild to moderate infections, and oral vancomycin or fidaxomicin for severe infections and relapses. The ability of *C. difficile* to form spores, coupled with the increase in antibiotic-resistant strains, can lead to persistence of infection, relapses, and the administration of more antibiotics, which further depletes the commensal bacteria. This creates an environment that is more favourable to *C. difficile*, thus setting up a cycle of relapse and re-infection. It is estimated that 20-30% of patients who develop a first episode of CDI go on to have at least one relapse, and of these, a further 60% develop further episodes of relapses.[3] This increases the need for further antibiotics, the risk of antibiotic-resistance in the gut commensal flora, and costs to the health service, with each episode of CDI estimated to cost approximately £7000 in 2010.[4]

Faecal microbiota transplants (FMT) represents an effective alternative to antibiotics to treat recurrent CDI (rCDI), with a primary cure rate as high as 91%.[5] The central tenet behind FMT is that the introduction of a healthy bacterial community into the intestines produces an environment that is less favourable to *C. difficile* by increasing colonisation resistance and reinstating a protective effect. The

advantages of this treatment are that it is quick, cost-effective, and could help to eradicate antibiotic resistant strains of *C. difficile*.

It is known that a dysbiotic gut microbiota increases the risk of developing CDI, however whether there is a common element within this community composition that could help to determine if a patient is at greater risk of rCDI is as yet unknown. The reduction in diversity within the dysbiotic gut microbiota would also suggest a reduction in metabolic potential through the loss of gene diversity. The functional redundancy[6] within the gut microbiota suggests, that metabolic function is more relevant than which species are present or absent. Whilst a number of studies have looked at the changes in microbiota composition due to FMT,[7-10] we know little about the changes to the metabolic capacities of the altered microbiota. The aim of this study was to assess FMT-induced changes in both the microbial community structure and metabolite profiles of the gut microbiomes of seven patients with rCDI, as well as those of their associated FMT donors.

Patients and methods

Patients

Patients were selected as candidates for the FMT procedure if they had at least two confirmed recurrences of CDI. *C. difficile* testing was based on a two stage algorithm in line with Public Health England recommendations.[11] This involves screening faecal samples by glutamate dehydrogenase enzyme immunoassay (Techlab, USA), followed by *C. difficile* toxin testing by enzyme immunoassay (Techlab, USA). Glutamate dehydrogenase positive, toxin negative samples were further tested for the presence of toxigenic genes by PCR. FMT exclusion criteria included immunocompromised patients, those aged less than 16, and those with severe

comorbidities which would make the patient unfit for endoscopy. FMT was introduced into clinical care at Norfolk and Norwich University Hospital following approval by the New Therapies committee, and was performed in accordance with the Helsinki Declaration of 1975. Patients were consented for the study by a clinician following a detailed discussion of the procedure with the patient or their next of kin. All patient data is fully anonymised.

Donor screening

The faecal donors used for the cohort of patients who underwent FMT in this study were both healthy Caucasian males with a BMI between 24-27 kg/m^2, aged 36 (D05) and 30 (D03) years of age, respectively. Potential donors were asked to complete a questionnaire adapted from van Nood *et al*[12] regarding their medical history and lifestyle habits to identify risk factors for potentially transmittable diseases. Eligible candidates provided blood and stool samples for laboratory screening tests. Blood samples were screened for hepatitis A, B, C, and E antibodies, HIV 1 & 2, human T-lymphotropic virus 1 & 2, Epstein-Barr virus, *Cytomegalovirus*, syphilis, *Entamoeba histolytica*, *Strongyloides stercoralis*, and *Treponema pallidum*. Stool samples were tested for the presence of *C. difficile* or its toxins, *Helicobacter pylori* antigen, *Norovirus*, methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, extended-spectrum β-lactamase-producing organisms, carbapenemase-producing *Enterobacteriaceae*, *Escherichia coli* O157, *Salmonella* spp., *Shigella spp.,* and *Campylobacter* species. In addition, microscopy was used to investigate for ova, cysts, and parasites. Prior to the donation of stool samples for each FMT procedure, donors were asked to refrain from eating peanuts and

shellfish, and to complete a short screening questionnaire to confirm no changes to health or lifestyle since the last donor screening that may put the patient at risk.

Faecal suspension preparation

Donor faeces were collected in a sterile container on the day of the procedure, and transferred to a sterilised class II safety cabinet (Walker Ltd, UK). A maximum of 80 g of donor stool was homogenised with sterile saline (0.9%), to a ratio of 5 ml saline per gram of stool, in a strainer bag (BA6141/STR; Seward Limited, UK) using a Stomacher® 400 Circulator (Seward Limited, UK) set to 230 RPM for a duration of 1 minute. The filtered faecal preparation was drawn up into labelled sterile 60 ml syringes using nasojejunal tubing connected to the Luer lock. The syringes were secured with sterile Luer lock caps and transported immediately to the hospital. Aliquots of the donor faecal sample were immediately stored at -20 ˚C until analysis.

Faecal suspension infusion

Patients were prescribed oral vancomycin 500 mg four times daily for 4 days, with the last dose received the night before the procedure. Also, on the day before the FMT procedure, a bowel lavage is performed using 4 litres of macrogol solution (Klean-Prep, Norgine). Patients were taken to the endoscopy unit for insertion of nasojejunal tube the night before the procedure. Our FMT protocol was adapted from that of van Nood *et al.*[12] On the day of FMT infusion, the patient's headrest was elevated to 45 degrees, patency of the nasojejunal tube was checked by flushing with water, and 420 ml of faecal suspension was delivered slowly by the patient's bedside in the isolation room via a nasojejunal tube using the prefilled syringes. This was performed at a rate of ~20 ml per minute with a break of 5-10 minutes applied

halfway through the procedure. Post-infusion instructions were to monitor observations, and record bowel motions. Patients could take on fluids one hour after the procedure, and were observed overnight before discharge the next day at the earliest. Although there are no agreed durations of follow-up post-FMT,[13] van Nood *et al*[12] used two endpoints to measure cure, namely no relapse after 5 weeks, and no relapse after 10 weeks. Resolution was defined as type 4 or less on the Bristol stool chart or stool normal for the patient e.g. in case of percutaneous endoscopic gastrostomy feeding. We followed patients up by telephone or in person if they were re-admitted into the hospital for an unrelated illness. Post-FMT samples were collected after a minimum of 10 days post-FMT, and postal kits were provided to patients who were willing to donate a 'follow-up' sample up to 2 weeks later.

Sample analysis

Faecal microbiota analysis

Faecal samples were collected from recipients within 9 days prior to FMT, however the pre-FMT sample for patient R13 was not collected within this timeframe, and a previously frozen sample obtained whilst the patient was suffering from the same episode of CDI was used. Further samples were collected for all recipients following the procedure ('post-FMT' range: 11-141 days; 'follow-up' range: 4-14 days after post-FMT sample), and stored at -20 ˚C until analysis. The DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, UK) with a bead-beating step.[14] DNA yield was quantified using the Qubit fluorometer prior to the samples being sent to the Earlham Institute (UK), where the V4 hypervariable region of the 16S rRNA genes were amplified using the 515F and 806R primers with built-in degeneracy.[15] The amplicons were sequenced using paired-end Illumina sequencing (2 x 250 bp)

on the MiSeq platform (Illumina, USA). Sequencing data, for the 21 samples that had an appropriate level of sequencing depth, were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) 1.9 software and RDP classifier 16S rRNA gene sequence database.[16, 17] The trimmed reads were filtered for chimeric sequences using ChimeraSlayer, bacterial taxonomy assignment with a confidence value threshold of 50% was performed with the RDP classifier (version 2.10), and trimmed reads clustered into operational taxonomic units at 97% identity level. Alpha diversity and rarefaction plots were computed using the Chao1 index. Weighted and unweighted UniFrac distances were used to generate beta diversity principal coordinates analysis plots, which were visualised using the Emperor tool.

Faecal metabolite analysis

A known mass (~ 100 mg) of thawed faecal samples were added to sterile tubes. The faecal waters were generated by adding the phosphate buffer (prepared in D_2O) to 8.3% w/v. Homogenised faecal waters were centrifuged at 16,200 x g at room temperature for 5 min. The supernatants were filter sterilised (0.2 µm) and placed in a 5 mm NMR tube. The ^{1}H NMR spectra were recorded at 600 MHz on a Bruker Avance spectrometer (Bruker BioSpin GmbH, Germany) running Topspin 2.0 software and fitted with a cryoprobe and a 60-slot autosampler. Each ^{1}H NMR spectrum was acquired with 1280 scans, a spectral width of 12,300 Hz, and an acquisition time of 2.67 seconds. The “noesypr1d” pre-saturation sequence was used to suppress the residual water signal with a low-power selective irradiation at the water frequency during the recycle delay and a mixing time of 10 ms. Spectra were transformed with a 0.3 Hz line broadening, and were manually phased,

baseline corrected, and referenced by setting the TSP methyl signal to 0 ppm. The metabolites were quantified using the software Chenomx® NMR Suite 7.0™.

Statistical analysis

Data for groups were expressed as mean ± standard deviation, with the exception of the microbiome relative abundance data, which is expressed as median ± standard deviation. Statistical comparisons of the alpha diversity measurements were performed using one-way analysis of variance followed by Tukey's Multiple Comparison post-test with an alpha value of 0.05. Multivariate statistical analysis (principal component analysis) of the ^{1}H NMR data was carried out using the PLS Toolbox v5.5 (Eigenvector Research Inc.,Wenatchee, WA) running within Matlab v7.6 (The MathWorks Inc., Natick, MA). Autoscaling was applied to the variates. ^{1}H NMR and sequencing datasets (the former comprising 100 metabolites and the latter the relative abundance of 271 bacterial taxa, for the same 21 samples) were also analysed jointly. Firstly, the two blocks of data were analysed by canonical correlation analysis (CCA), using the sPLS variant to cope with mathematical issues arising from the data being high-dimensional.[18] This analysis defines latent variables, or canonical axes, for each of the NMR and sequencing blocks of data; the data is then summarised by the position of the samples (scores) on these axes. The sample scores on corresponding pairs of canonical axes reflected the overall strength of the relationship between the two blocks of data. The original variables (NMR and sequencing) were then simultaneously displayed on 'correlation circle plots', in which the correlation between each variable and each canonical axis is projected.[19] This revealed possible correlations which were further explored by calculating the Pearson correlation coefficient between each bacterial taxa and each of the

metabolites measured by ^{1}H NMR. CCA was carried out using the R (v3.3.3) package mixOmics v6.1.2, and correlation coefficient maps produced in Matlab v9.1 (The MathWorks Inc., Natick, MA). Prior to CCA and calculation of the correlation coefficient, the sequencing data were subjected to the centered log-ratio transformation to avoid spurious results.[20-22]

Results

Patient demographics

Seven patients with rCDI were given FMT at the Norfolk and Norwich University Hospital. Metagenomic and metabonomic analyses were performed on stool samples collected before and after the FMT procedure, along with the associated donor stool samples. Patient demographics are shown in Table 1.

16S rRNA gene sequencing analysis

Faecal microbiota composition

The sequencing data generated 1,627,111 high-quality reads, with an average of 77,481 ± 10,888 reads per sample, which clustered into 9,818 operational taxonomic units at 97% identity. Diversity analyses were rarefied to 63,159 sequences per sample to match the lowest number of sequences obtained for a sample, and therefore avoid bias. The metataxonomic data (Supplemental figures 1 & 2) indicated that although the microbiota of many of the patients resembled more closely that of the donor microbiota following FMT (Figures 1A & 2A), the microbiota alteration in patient R44 did not follow this trend (Figures 1B & 2B). It transpired that this patient suffers from chronic alcohol abuse, and this may have greatly affected the ability of

the donor microbiota to successfully colonise this recipient's gastrointestinal tract. The results in Table 2 indicate that following FMT, there was a general trend towards an increase in members of the *Bacteroidetes* (pre-FMT 1.1%, post-FMT 6.5%, follow-up 12.4%), an initial decrease of *Firmicutes* (pre-FMT 57.8%, post-FMT 50.3%, follow-up 61.5%), and limited change in the relative proportions of *Proteobacteria* (pre-FMT 13.9%, post-FMT 16.3%, follow-up 3.6%). Comparison of the metataxonomic data at the genus level suggests an increase in the relative proportions of *Bacteroides* (pre-FMT 0.4%, post-FMT 4.6%, follow-up 9.6%), *Blautia* (pre-FMT 0.2%, post-FMT 3%, follow-up 7.3%), and members of *Ruminococcus* of the *Lachnospiraceae* family (pre-FMT 1.6%, post-FMT 4.9%, follow-up 11.1%). In contrast, the relative proportions of *Enterococcus* (pre-FMT 11.3%, post-FMT 1.1%, follow-up 0.4%), and *Escherichia* (pre-FMT 5.1%, post-FMT 3.5%, follow-up 1.1%) decreased after treatment. However, although cured of CDI, it is interesting to note that these data were skewed by the inclusion of the post-FMT sample obtained from patient R44. Omission of patient R44 samples significantly impacted on the median relative proportions of *Proteobacteria* (post-FMT 1.5% vs 16.3%), and *Klebsiella* (post-FMT 0.2% vs 0.02%).

Faecal microbiota diversity analyses

Alpha diversity analysis using the Chao1 index (Figure 3), and one-way ANOVA with Tukey's multiple comparisons test, indicated a significant reduction in bacterial diversity in the pre-FMT samples, compared to the donor samples ($P = 0.0091$). Although bacterial diversity was observed to increase following the FMT procedure, this was not statistically significantly different to the diversity within the pre-FMT samples. However, neither the post-FMT nor the follow-up samples were

significantly different from the donor samples, indicating an increase in bacterial diversity following FMT. Weighted beta diversity analysis indicated a clear separation between samples obtained from donors, which clustered to the right of the principle coordinates analysis plot, and those from recipients prior to FMT, which were located on the left side of the plot (Figure 4). In addition, it indicates that the faecal microbiota of R42, R05, and R23 were modified by the FMT procedure to closely resemble that of the healthy donors.

Faecal metabolite profile

A total of 22 samples obtained from 2 donors (*n* = 5) and 7 patients (pre-FMT *n* = 7, post-FMT *n* = 7, and follow-up samples *n* = 3) were prepared, ^{1}H NMR spectra recorded, and signals carefully characterised using information from 2-dimensional NMR experiments, such as HSQC, HMBC, and COSY, the literature data,[23-25] and the human metabolome database. A specialised Chenomx software® was used to quantify 100 different metabolites in an absolute manner (Table 3).

Donor profiles were characterised by relatively high levels of butyrate, propionate, acetate, succinate, glucose, and ethanol (30, 13, 108, 22, 8, and 6 mmol/kg, respectively), and lower levels of a diverse range of other metabolites. The presence of many compounds was sample-dependant but amino acids, their associated products (methylsuccinate, 2-piperidinone, β-alanine, and 5-aminovalerate), phenolic compounds (3-(3-hydroxyphenylpropionate), phenylpropionate, and phenylacetate), amines (methyl-, dimethyl- and trimethyl-amines, putrescine, cadaverine, and tyramine), nucleobases (adenine and uracil), sugars (glucose, arabinose, fucose, and ribose), and nicotinate derivates (nicotinate, NAD+, and nicotinamide ribotide) were detected in the ^{1}H NMR profiles of the donor samples (Supplemental figure 3).

As with the donor's profiles, the patient profiles displayed great intra- and inter-individual variability in the levels of many metabolites. Metabolic profiles of pre-FMT patient profiles contained, on average, more uncharacterised signals including those of analgesics (6.80, 6.85, and 6.93 ppm), higher levels of amines (dimethylamine, cadaverine, putrescine and tyramine), amino acids, branched chain fatty acids (BCFAs) (isobutyrate, isocaproate, isovalerate and 2-methylbutyrate), phenol, benzoate, and p-cresol, and lower levels of butyrate, alcohols (ethanol and methanol), osmolytes (choline), and bile acids (deoxycholate, lithocholate, and cholate).

Using patient R42 as an example of metabolic changes, it was found that butyrate, propionate, and acetate levels increased from 1.6, 2.8, and 46.6 mmol/kg, to 18.3, 26.2, and 110.6 mmol/kg, respectively. Conversely, putrescine, cadaverine, tyramine, 1-methylnicotinamide, and some unidentified NMR metabolite signals disappeared. A decrease of 3-hydroxybutyrate (from 9.6 mmol/kg to 0.8 mmol/kg) was also observed. Glucose levels increased from 0.3 mmol/kg to 12.2 mmol/kg, which is within the range observed from samples of the donor associated with patient R42. In contrast, there was little improvement in the levels of butyrate, propionate, and acetate in the samples from patient R44 (0, 0.8, 14.3 mmol/kg vs 0.2, 3.9, 23.3 mmol/kg), and although there was a decrease in putrescine levels, the amount of cadaverine and tyramine were largely unchanged. Interestingly, increased levels of lactate, 1,2-propanediol, glycerol, and glucose were observed in this patient following FMT (data not shown). The levels of butyrate (Figure 5) were relatively low in the pre-FMT patient samples (5.39 mmol/kg ± 7.46), compared to the donor samples (30.25 mmol/kg ± 5.07), and the concentration of butyrate increased in most patients following the FMT procedure (average 7.67 mmol/kg ± 6.66). For those patients that

provided a further post-FMT sample (follow-up sample; between 23 and 36 days after treatment), a higher concentration of faecal butyrate (17.67 mmol/kg ± 11.72) was detected compared to pre-FMT levels (Figure 5). The levels of butyrate, acetate, and fucose increased post-FMT converging towards those of the donors (Supplemental figure 4). Despite this improvement, the levels of butyrate, acetate, ethanol, methanol, galactose, arabinose, deoxycholate, lithocholate, and isovalerate remained significantly different compared to the donor samples, even after the FMT procedure (Table 3). Only the levels of galactose and arabinose significantly differed from the donors in the follow-up samples (Table 3). Principal component analysis indicated that a positive score on the second principal component (PC) may potentially correlate with a healthier metabolic profile, as the scores of all five of the donor samples were found in this half of the score plot (Supplemental figure 5). Interestingly, some of the patients' metabolic profiles (R44, R42, and R23) shifted towards the profiles of the donors to varying degrees after the FMT procedure.

Correlation analysis

The scores obtained by canonical correlation (samples coordinates on the latent variables defined for each of the two blocks of data, (Supplemental figure 6)) show that there is strong evidence of a relationship between ^{1}H NMR and 16S rRNA gene sequencing datasets ($r > 0.9$ for scores 1 to 10). Furthermore, they highlight the fact there are characteristics that are specific to donors and to recipients, as shown on the first canonical axis, both in terms of NMR measurements and bacterial taxa. The correlation circle plots (which show the correlation between the original variables in either the NMR or the 16S rRNA gene sequencing dataset, and their canonical axes) indicate which of the original variables contribute most of that, being situated towards

the outside edge of the circle corresponding to correlation = 1 on the first canonical axis (Supplemental figure 7). Two clusters of variables can be seen on the first axis, one with large negative, and the other with large positive coordinates. These two sets of variables are expected to be negatively correlated. Moreover, it is expected that variables within a cluster will be positively correlated.[19] Further examination of the map of Pearson correlations between these two clusters of variables confirms the high correlations between a number of metabolites and bacterial taxa (Figure 6). For instance, butyrate was found to positively correlate to the presence of *Bacteroides*, *Blautia*, and *Ruminococcus,* whilst *Klebsiella* and *Enterococcus* were negatively correlated with butyrate.

Discussion

In this study, we performed gut microbiota community composition and metabonomic analyses on the faecal samples of seven patients who underwent FMT to combat r-CDI. The symptoms associated with CDI were resolved for six of the seven patients, and no recurrences of CDI have been reported in the 22 - 27 months following the single administrations of FMT. It has been observed that CDI sufferers generally have a dysbiotic microbiota, with a larger proportion of *Proteobacteria* than is generally seen in a healthy microbiota.[7, 26] Our results based on the 16S rRNA gene analysis confirm the higher levels of *Proteobacteria* in rCDI patient samples before FMT. Post treatment there was a general increase in the relative proportions of *Firmicutes* and *Bacteroidetes*, with a decrease in members of the *Proteobacteria* as has been previously described.[7, 26] At a higher resolution, the relative proportions of *Bacteroides, Blautia,* and *Ruminococcus* from the *Lachnospiraceae* family increased,

whilst *Enterococcus* and the *Proteobacteria* *Escherichia* and *Klebsiella* decreased, which supports the findings of previous studies.[8-10, 26, 27] Interestingly these changes were not observed in patient R44, whose post-FMT sample, collected 11 days after the procedure, continued to resemble a dysbiotic community, and on the contrary, the levels of *Klebsiella* (*Proteobacteria*) increased from 5.8% to 84.6% (Supplemental figure 2) of the total bacterial population. Despite what appears to be an adverse effect on the gut microbiota composition, rCDI was resolved, and it is possible that the patient microbiota had changed after FMT but over the eleven days before a post-FMT sample was collected, a resumption of alcohol abuse had reversed this effect. It is known that chronic alcohol use can lead to a dysbiotic microbiota, with a predomination of *Gammaproteobacteria* including *Klebsiella* species, as was observed with patient R44.[28-30] Therefore, the long-term success of FMT procedures may be influenced by lifestyle, and it could be interesting to explore if chronic alcohol abuse increases the susceptibility of individuals to CDI.

As with some other gastrointestinal diseases, a reduction in the diversity of the colonic microbiota is commonly seen in CDI patients.[31, 32] Alpha diversity measurements (Figure 3) indicated that the donor's gut microbiota contained a higher species richness than the patients before FMT ($P = 0.0091$). The administration of FMT increased the diversity of patients' gut microbiota, and this remained stable over time, although this diversity did not reach the levels observed in the healthy donors. This is potentially due to the natural decline in bacterial diversity that has been reported in the gut microbiota as we age (mean age: FMT recipients 71.3 ± 15.9 years; donors 33.0 ± 4.2 years).[33, 34] Beta diversity analysis (Figure 4), which gives an indication of the similarities or differences between samples, shows a clear separation of the donor's and pre-FMT patient samples,

indicating substantial differences between the microbiota profiles of the two groups. As this separation was determined by principal coordinate 1, it suggests that the microbiota composition of the two groups represented the greatest variability. After treatment, the profiles shift towards the associated donor samples.

Metabonomic analysis was performed using ^{1}H NMR to observe any differences between the metabolic profiles of those suffering CDI and the donors, and whether FMT restored these metabolic functions. One hundred metabolites were identified and quantified in 22 faecal samples. Principal component analysis suggested that the metabolome of some of the patients shifted towards the profiles of the donors to varying degrees after FMT (Supplemental figure 5). Coupled with the alleviation of symptoms, this change in metabolite profile may reflect a shift towards a healthier metabolic state. It is difficult to discern which metabolites, if any, may be associated with this improvement, but an increase in the short-chain fatty acids acetate and butyrate were observed in most patients. Butyrate has been linked with multiple health benefits for the host, such as providing an energy source for colonocytes, inhibition of growth and virulence gene expression of pathogens,[35, 36] inflammation suppression,[37, 38] and the direct inhibition of *C. difficile* growth *in vivo*.[39] In addition, a recent study by Fuentes *et al*[40] observed that long-term remission of ulcerative colitis following FMT was associated with a restoration of the capacity of the gut microbiota to produce butyrate. Acetate offers host mucosal protection and healing [41, 42] by modulating host defence [43] and inflammatory responses.[44] Using sparse partial-least-square (sPLS) canonical correlation analysis, it was possible to separate donor and patient samples based on microbiota composition and metabolite profile (Supplemental figure 6). Furthermore, calculating the Pearson correlation coefficient enabled correlations between bacterial taxa and metabolites to be mapped (Figure

6). This identified a range of metabolites, including butyrate, which were positively correlated with the taxa (*Bacteroides*, *Blautia*, and *Ruminococcus*) that proportionally increased following FMT, and negatively correlated with *Klebsiella* (except for a largely neutral correlation to succinate), and *Enterococcus*, which exhibited proportional decreases. Many of the rCDI patients had relatively high levels of branched chain fatty acids (BCFAs), phenolic compounds, amino acids, and certain amines, compared to the donor samples. In general, the levels of some of these metabolites were found to decrease following the FMT procedure, and this may have also contributed to the improved health of the patients. Although the majority of FMT recipients showed an increase in short-chain fatty acids after treatment, this was not observed in patient R44, who suffered from alcoholism. The increased levels of lactate and glycerol could be an effect of alcohol abuse following ethanol metabolism.[45] It has been shown that *Klebsiella* can metabolise glycerol under anaerobic conditions to produce propanediols, which can then be used as an energy source for these bacteria.[46] As such, the chronic consumption of alcohol could lead to a dysregulation of hepatic processes, which may provide increased levels of these metabolites that would give bacteria, such as *Klebsiella* species, a competitive advantage over more beneficial bacteria.

It has previously been shown that chronic alcohol abusers often have increased proportions of *Gammaproteobacteria* in their gut microbiota,[29] which is consistent with the observations made here. However, further work is required to identify if elevated levels of faecal lactate, glycerol, and propanediols are potential biomarkers of this disorder.

The results of this study confirm the value of FMT as a viable alternative to the use of costly antibiotics for the treatment of rCDI. Although the modulation of the microbiota composition is considered to play a pivotal role in FMT, the resulting metabolic alteration of the gut microbiota should also be considered as an important factor in the resolution of rCDI. A larger study is in progress focusing on the microbial-based metabolites to identify biomarkers for identification of individuals susceptible to CDI based on both microbiota composition and their metabolic profiles.

Conclusions

Faecal microbiota transplantation has been shown to be a robust and cost-effective method to combat recurrent-*Clostridium difficile* infection (rCDI), however to increase the effectiveness of this treatment it is necessary to elucidate the mechanisms by which this works, and factors that may reduce its efficacy. Our results indicate that the resolution of rCDI was accompanied by an alteration to the microbiota composition towards that of the donor profile to varying degrees, with increased diversity that was maintained beyond seven weeks after the FMT procedure. We also demonstrated that the shift in bacterial composition also correlated with altered functional metabolism of the recipient microbiota, where the metabolic profile again shifted towards that of the donor. Correlation analysis enabled the identification of strong relationships between bacterial taxa and metabolites, including positive correlations between butyrate and taxa that proportionally increased after FMT. Finally, lifestyle factors, such as chronic alcohol consumption, could potentially

increase a person's susceptibility to CDI, and should be considered prior to FMT administration.

List of abbreviations

AKI: acute kidney infection, **BCFAs:** branched chain fatty acids, **CCA:** canonical correlation analysis, **CCF:** congestive cardiac failure, **CDI:** *Clostridium difficile* infection, **CKD2/4:** chronic kidney disease stage 2/4, **FMT:** faecal microbiota transplantation, **HAP:** hospital-acquired pneumonia, **HBP:** high blood pressure, **LoS:** length of stay, **NIDDM:** noninsulin-dependent diabetes mellitus, **PC:** principal component, **QIIME:** Quantitative Insights Into Microbial Ecology, **R.A:** rheumatoid arthritis, **rCDI:** recurrent *Clostridium difficile* infection, **sPLS:** sparse partial-least-square, **UC:** ulcerative colitis.

Declarations

Ethics approval and consent to participate

All participants provided informed written consent in accordance with protocols approved by the New Therapies committee at the Norfolk and Norwich University Hospital. All participants consented to provide stool samples, and to the use of the stored samples for research purposes.

Disclosure of interest

The authors report no conflict of interest and have no financial interests to disclose.

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Authors' contributions

NE and AN contributed to the study design. LK collected the samples, and performed the experimental work and microbial bioinformatic analysis. IB and NE performed the FMT procedures. GLG was responsible for all aspects of the ^{1}H NMR analysis. MD performed correlation and statistical analysis. LK, GLG, MD, NE, and AN contributed to the writing and editing of the manuscript prior to submission for publication. All authors approved the final manuscript.

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Figure legends

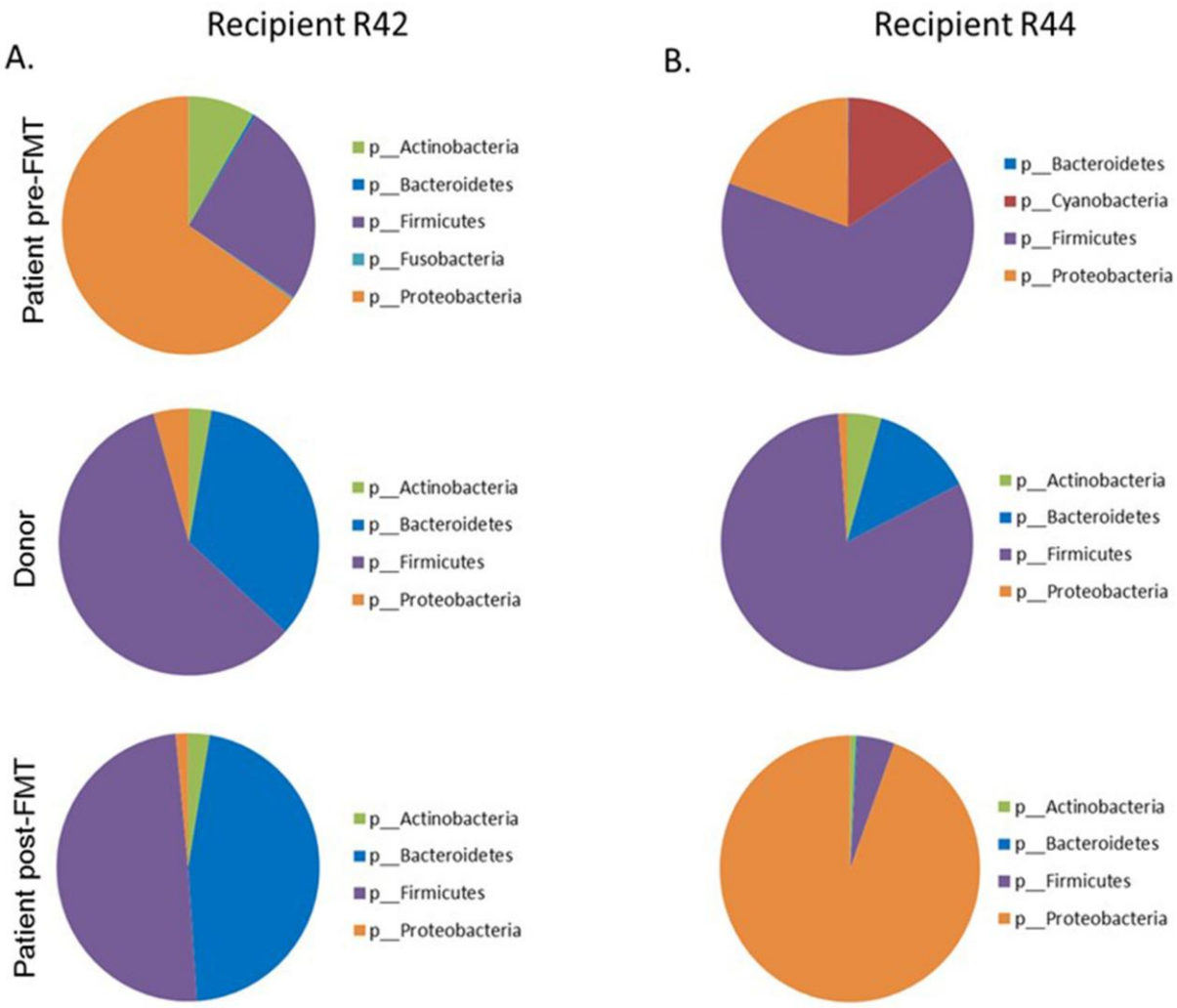

Figure 1. Post-FMT profiles resembled the associated donor profile, but this was not observed in all recipients. Proportions of bacterial phyla in pre- and post-FMT samples, as well as the associated donor samples. Bacterial DNA was extracted from faecal samples, and the 16S rRNA genes were sequenced using paired-end sequencing on an Illumina MiSeq platform. Bioinformatic analysis was performed using QIIME 1.9.0 and RDP classifier.

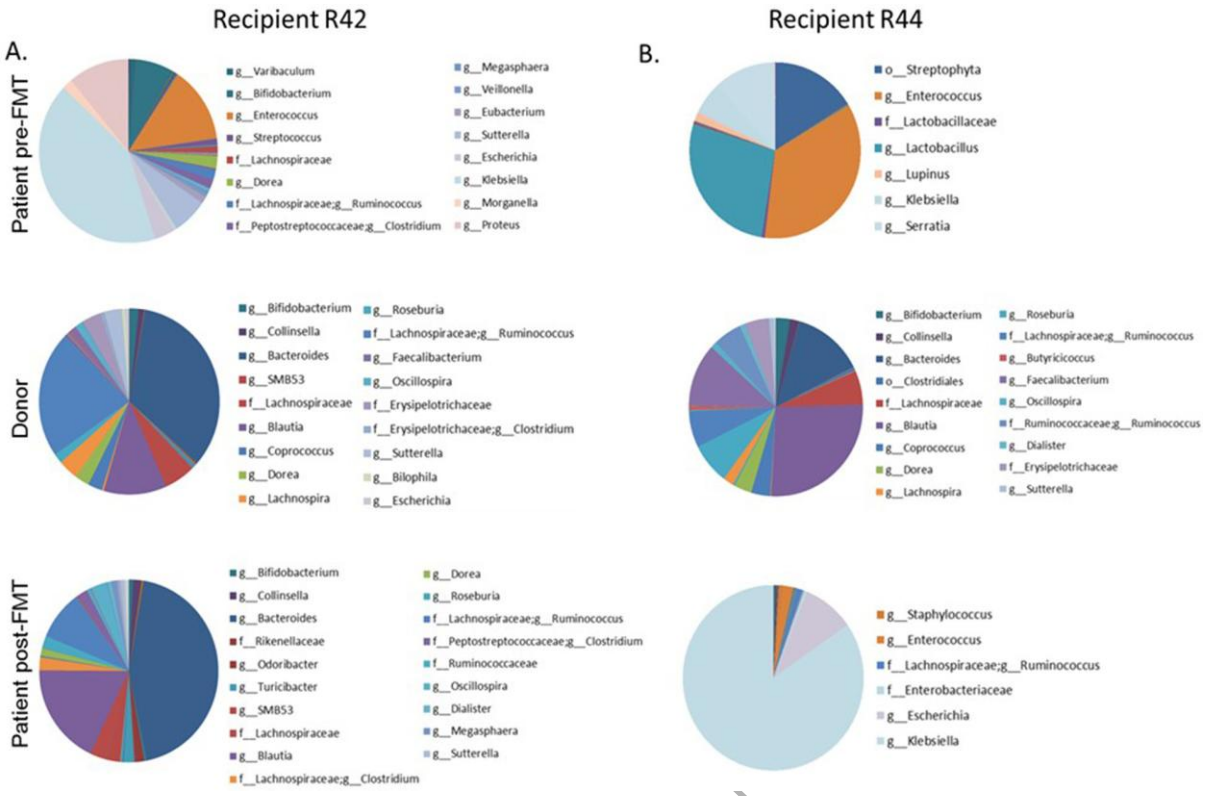

Figure 2. Proportions of bacterial genera in faeces of pre- and post-FMT patients and the corresponding donors. Bacterial DNA was extracted from faecal samples, and the 16S rRNA genes were sequenced using paired-end sequencing on an Illumina MiSeq platform. Bioinformatic analysis was performed using QIIME 1.9.0 and RDP classifier. The key refers to bacterial genera present at ≥0.5% of the population.

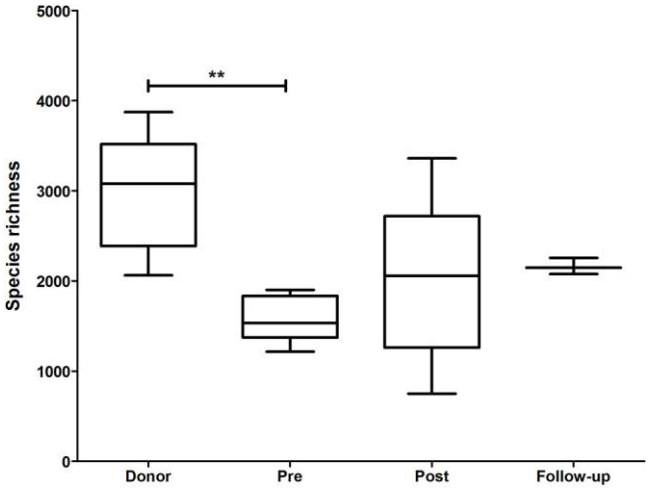

Figure 3. FMT treatment increased bacterial diversity. Alpha diversity rarefaction measures of faecal bacteria before (*n* = 7) and after FMT treatment (*n* = 6), follow-up samples (*n* = 3), and associated donor samples (*n* = 5) were calculated using QIIME 1.9.0. Data for the post-FMT sample for patient R48 was omitted due to poor sequencing depth. Mean ± SD, analysed using one-way ANOVA with Tukey's multiple comparisons test, and an alpha value of 0.05; ** *P* = 0.0091.

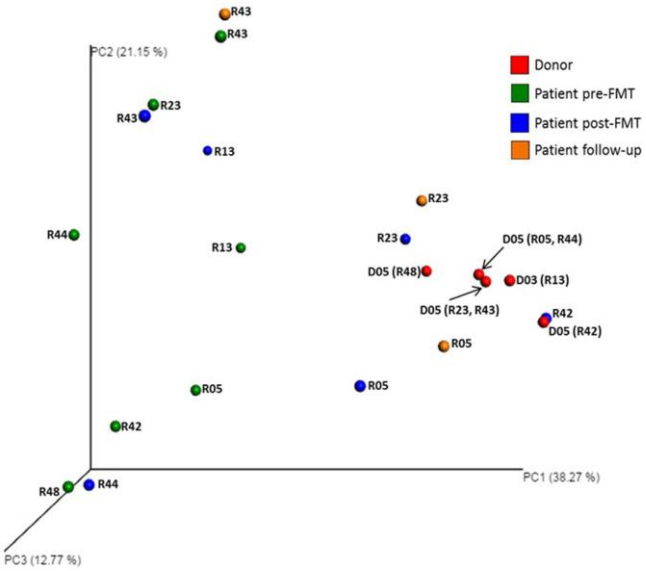

Figure 4. Beta diversity analysis shows a separation of faecal microbiota based on health status (pre-FMT vs donor). Weighted beta diversity analysis of faecal microbiota samples from patients (pre-FMT (*n* = 7), post-FMT (*n* = 6), and follow-up samples (*n* = 3)) and donors (*n* = 5). Beta diversity analysis was performed using the UniFrac metric using QIIME 1.9.0, and visualised as a 3D principal coordinates analysis plot using Emperor. Data for the post-FMT sample for patient R48 was omitted due to poor sequencing depth. The anonymised identifiers indicate which samples belong to each patient, and which donor samples were used for each patient.

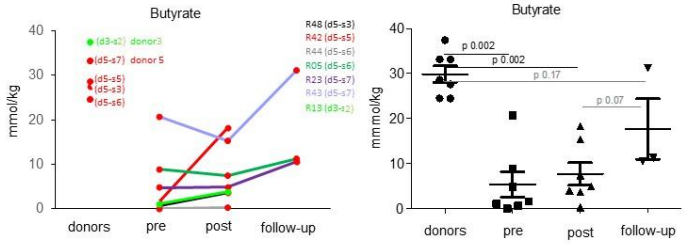

Figure 5. Butyrate levels for the 2 donors (5 samples) and 7 patients (17 samples consisting of 7 pre-FMT, 7 post-FMT and 3 follow-up samples). Patients were matched to a sample of either donor 5 (d5-s3, -s5, -s6 or –s7) or donor 3 (d3-s2). High levels of butyrate characterised the donors (between 24 and 37 mmol/kg) while the pre-FMT levels were disparate ranging from low (0) to high (21 mmol/kg). The butyrate levels for most patients were found to have increased in the post-FMT and/or follow-up samples. The levels for patient R44 remained low (increasing from 0 at pre-FMT to 0.2 mmol/kg post-FMT). The *P* values are the results of Mann-Whitney t-tests between two groups of samples.

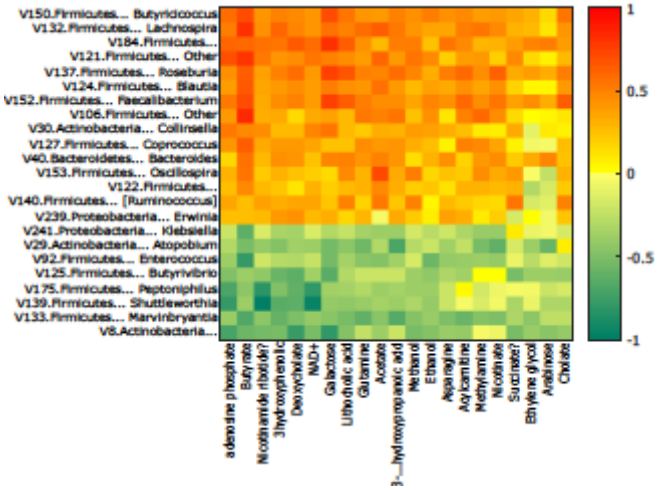

Figure 6. Correlation map for selected metabolites and bacterial taxa.

Metabolites and bacterial taxa were chosen based on having 0.5< | correlations | <1 on the first axis of the sPLS canonical correlation analysis.

Tables

Table 1. Patient demographics.

Patient code	Gender	Age (yrs)	LoS post FMT (days)	Bowel lavage administered?	Donor sample	Pre-FMT sample (days)	Post-FMT sample (days)	Documented Resolution of symptoms post-FMT	Symptoms at 5 weeks	Symptoms at 10 weeks	Co-morbidities
R48	Female	66	1	No	D05-S3	9	141	1 day	No	No	UC, HBP, diverticular disease
R42	Female	63	1	Yes	D05-S5	9	48	1 day	No	No	NIDDM, liver cirrhosis, pancreatic insufficiency
R44	Female	42	1	No	D05-S6	8	11	1 day	No	No	Alcohol abuse
R05	Male	79	63	No	D05-S6	1	30	No response due to concomitant carbapenem for HAP.	n/a	n/a	Cancer, CKD4, NIDDM
R23	Female	80	3	No	D05-S7	2	23	2 days	No	No	CKD4, renal dialysis, R.A, hypothyroidism
R43	Male	>90	43	No	D05-S7	2	21	8 days	Relapse of symptoms	Deceased	CKD2, CCF
R13	Male	78	6	No	D03-S2	45	88	6 days	No	No	Dementia, AKI

AKI – acute kidney injury; CCF – congestive cardiac failure; CKD2 and CKD4 – chronic kidney disease stage 2 and 4; HAP – hospital-acquired pneumonia; HBP – high blood pressure; LoS – length of stay; NIDDM – noninsulin-dependent diabetes mellitus; R.A – rheumatoid arthritis; UC – ulcerative colitis.

Table 2. Median (± SD) relative abundance of bacterial taxa in which the largest proportional changes were observed across the different groups.

	Including Patient R44				*Excluding Patient R44*		
Bacterial phyla	**Donor (%)**	**Pre-FMT (%)**	**Post-FMT (%)**	**Follow-up FMT (%)**	**Pre-FMT (%)**	**Post-FMT (%)**	**Follow-up FMT (%)**
Bacteroidetes	15 (±11)	1.1 (±4)	6.5 (±18)	12.4 (±15.6)	1.5 (±4.2)	7.1 (±18.5)	12.4 (±15.6)
Firmicutes	78.5 (±10.4)	57.8 (±17.4)	50.3 (±24.3)	61.5 (±9)	54.9 (±18.6)	51 (±14.6)	61.5 (±9)
Proteobacteria	1.1 (±1.4)	13.9 (±26)	16.3 (±36.6)	3.6 (±9.4)	8.4 (±28.7)	1.5 (±18.4)	3.6 (±9.4)
Bacterial genera							
Bacteroides	14.7 (±10.9)	0.4 (±4.1)	4.6 (±17.3)	9.6 (±15.2)	0.7 (±4.4)	5.4 (±18)	9.6 (±15.2)
Blautia	23.14 (±7.6)	0.2 (±1.6)	3 (±10.2)	7.3 (±9.5)	0.3 (±1.7)	3.1 (±10.4)	7.3 (±9.5)
Ruminococcus	10.4 (±7.5)	1.6 (±4.3)	4.9 (±4.9)	11.1 (±6.4)	1.8 (±4.5)	5.8 (±4.8)	11.1 (±6.4)
Enterococcus	0.01 (±0.1)	11.3 (±12)	1.1 (±9.5)	0.4 (±4.1)	9.1 (±5.9)	0.2 (±10.5)	0.4 (±4.1)
Escherichia	0.1 (±0.3)	5.1 (±16.4)	3.5 (±12.9)	1.1 (±8.5)	6 (±17.3)	0.7 (±14.4)	1.1 (±8.5)
Klebsiella	0.01 (±0)	4.4 (±16)	0.2 (±34.4)	0.3 (±0.3)	3 (±17.7)	0.02 (±0.7)	0.3 (±0.3)

Table 3. Mean values (± SD) of 100 metabolites (mmol/kg) identified in faecal samples obtained from donors and patients.

		mmol/kg			
Metabolite	**Index (ppm)**	**Donors (n = 5)**	**Pre-FMT (n = 7)**	**Post-FMT (n = 7)**	**Follow-up FMT (n = 3)**
Butyrate	0.89	30.25 (± 5.07)	5.39 (7.46) **	7.67 (± 6.66) **	17.67 (± 11.72)
Acetate	1.90	108.44 (± 21.43)	49.94 (± 45.14) *	48.81 (± 33.81) *	126.04 (± 27.76)
Propionate	1.05	13.20 (± 5.03)	12.46 (± 12.36)	10.57 (± 9.43)	26.92 (± 9.26)
Valerate	0.87	1.58 (± 1.69)	0.78 (± 1.69)	0.87 (± 0.88)	1.93 (± 3.07)
Isobutyrate	1.05	0.20 (± 0.34)	1.60 (± 2.14)	1.62 (± 2.05)	2.96 (± 2.47)
Isocaproate	0.87	0 (± 0)	1.04 (± 2.09)	0.71 (± 1.43)	0.22 (± 0.39)
Isovalerate	0.90	0.32 (± 0.44)	1.59 (± 1.97)	1.68 (± 1.45) *	2.84 (± 2.55)
2-Methylbutyrate	0.85	0.10 (± 0.17)	0.77 (± 1.20)	0.63 (± 0.65)	1.16 (± 1.11)
Lactate	1.33	1.98 (± 1.92)	1.23 (± 1.56)	7.73 (± 12.67)	16.08 (± 27.29)
Lactaldehyde	1.37	0 (± 0)	0.08 (± 0.22)	0.15 (± 0.36)	0.56 (± 0.96)
Formate	8.46	0.43 (± 0.62)	0.32 (± 0.60)	0.07 (± 0.04)	0.41 (± 0.53)
1,2-propanediol	1.13	0.22 (± 0.15)	0.13 (± 0.08)	1.14 (± 2.68)	0.77 (± 1.29)
Ethanol	1.17	5.86 (± 2.95)	1.07 (± 0.79) **	1.92 (± 1.53) *	5.55 (± 8.45)
Methanol	3.35	1.41 (± 0.36)	0.50 (± 0.97) *	0.25 (± 0.24) **	0.55 (± 0.81)
Ethylene glycol	3.70	1.87 (± 0.78)	1.27 (± 0.73)	0.98 (± 0.47)	1.10 (± 0.19)
Glycerol	3.55	1.07 (± 1.10)	1.70 (± 1.77)	1.64 (± 2.64)	0.85 (± 0.49)
Indole-3-lactate	7.50	0.01 (± 0.02)	0.01 (± 0.02)	0 (± 0)	0.03 (± 0.05)
Indoleacetate	7.62	0 (± 0)	0.01 (± 0.01)	0 (± 0.01)	0.07 (± 0.12)
Methylamine	2.59	0.57 (± 0.57)	0.16 (± 0.20)	0.17 (± 0.14)	0.33 (± 0.27)
Dimethylamine	2.71	0.03 (± 0.03)	0.19 (± 0.17)	0.08 (± 0.14)	0.13 (± 0.11)
Trimethylamine	2.89	0.24 (± 0.10)	0.29 (± 0.34)	0.24 (± 0.30)	0.35 (± 0.19)
Putrescine	3.04	0.15 (± 0.12)	2.39 (± 2.67)	1.06 (± 1.19)	2.29 (± 2.05)
Cadaverine	3.00	0.38 (± 0.52)	0.80 (± 1.33)	1.02 (± 0.74)	1.93 (± 1.00)
Tyramine	7.21	0.10 (± 0.20)	0.27 (± 0.34)	0.30 (± 0.70)	0.10 (± 0.17)
4-Hydroxyphenylacetate	6.85	0.05 (± 0.08)	0.21 (± 0.31)	0.53 (± 0.99)	1.94 (± 2.83)
3-hydroxyphenylpropionate	6.74	0.17 (± 0.20)	0 (± 0.01)	0.01 (± 0.03)	0 (± 0)
3-Hydroxyphenyl derivate	6.70	0.18 (± 0.18)	0 (± 0.01)	0.01 (± 0.02)	0.03 (± 0.06)
3-Phenylpropionate	7.26	0.19 (± 0.17)	0.11 (± 0.15)	0.20 (± 0.24)	0.11 (± 0.10)
Phenylacetate	7.29	0.08 (± 0.08)	0.79 (± 0.81)	0.62 (± 0.85)	1.00 (± 0.78)
Phenylacetylglycine	7.35	0 (± 0)	0.04 (± 0.08)	0.03 (± 0.04)	0.02 (± 0.04)
4-Aminohippurate	7.66	0 (± 0)	0.01 (± 0.01)	0.01 (± 0.02)	0.07 (± 0.12)
p-Cresol	7.14	0 (± 0)	0.06 (± 0.08)	0.06 (± 0.09)	0.10 (± 0.18)
Phenol	6.98	0 (± 0)	0.03 (± 0.05)	0.03 (± 0.05)	0 (± 0)

Benzoate	7.47	0 (± 0)	0.02 (± 0.05)	0 (± 0)	0.08 (± 0.14)
2-Hydroxyisovalerate	0.82	0.02 (± 0.02)	0.19 (± 0.11) *	0.14 (± 0.13)	0.17 (± 0.17)
3-Methyl-2-oxovalerate	1.09	1.14 (± 1.10)	2.81 (± 3.76)	1.45 (± 1.67)	3.55 (± 2.83)
ɣ-aminobutyobetaine	3.12	1.76 (± 1.72)	2.69 (± 2.21)	2.82 (± 5.46)	4.02 (± 2.48)
β-Alanine	2.55	0.45 (± 0.25)	0.28 (± 0.26)	0.41 (± 0.45)	1.07 (± 0.88)
2-Aminoadipate	2.26	0 (± 0)	0 (± 0)	0.74 (± 1.65)	0 (± 0)
2-Oxoisocaproate	0.92	0 (± 0)	0.07 (± 0.13)	0.01 (± 0.02)	0.02 (± 0.04)
3-Aminoisobutanoate	1.18	0 (± 0)	0.65 (± 1.20)	1.89 (± 4.83)	0 (± 0)
Methylsuccinate	1.09	0.24 (± 0.23)	0.08 (± 0.13)	0.06 (± 0.11)	0.22 (± 0.29)
2-Piperidinone	3.28	0.17 (± 0.15)	0.01 (± 0.03)	0.15 (± 0.22)	0.26 (± 0.31)
5-Aminopentanoate	2.22	2.45 (± 1.93)	5.71 (± 10.64)	1.51 (± 0.97)	1.19 (± 0.78)
Acetaminophen	7.24	0 (± 0.01)	0.12 (± 0.15)	0.08 (± 0.09)	0.22 (± 0.39)
Analgesic at 6.80 ppm	6.81	0 (± 0)	0.58 (± 1.53)	2.06 (± 5.42)	0 (± 0)
Analgesic at 6.85 ppm	6.85	0 (± 0)	0.14 (± 0.37)	0.21 (± 0.57)	0 (± 0)
Analgesic at 6.93 ppm	6.93	0 (± 0)	0.34 (± 0.91)	0.04 (± 0.06)	0.01 (± 0.02)
Arabinose	4.51	0.62 (± 0.09)	0.24 (± 0.59) *	0.14 (± 0.18) **	0.04 (± 0.06) *
Fucose	4.54	0.23 (± 0.15)	0.01 (± 0.04) *	0.22 (± 0.23)	0.31 (± 0.32)
Galactose	4.57	0.73 (± 0.19)	0.15 (± 0.16) **	0.22 (± 0.18) **	0.24 (± 0.17) *
Glucose	4.64	8.12 (± 5.15)	3.95 (± 3.30)	10.97 (± 17.91)	5.70 (± 3.27)
Ribose	4.92	0.70 (± 1.13)	0.59 (± 0.45)	0.55 (± 0.34)	1.64 (± 0.54)
Alanine	1.47	3.58 (± 2.39)	5.21 (± 3.60)	5.32 (± 3.72)	7.25 (± 4.76)
Asparagine	2.94	0.57 (± 0.16)	0.24 (± 0.25)	0.18 (± 0.26)	0.37 (± 0.64)
Aspartate	2.80	1.66 (± 1.23)	0.87 (± 0.59)	1.00 (± 0.34)	2.00 (± 1.07)
Glutamate	2.33	3.24 (± 2.90)	3.76 (± 4.81)	2.86 (± 2.57)	3.94 (± 2.76)
Glutamine	2.46	2.37 (± 1.82)	0.59 (± 0.41)	0.73 (± 0.48)	1.24 (± 0.49)
Glycine	3.54	2.21 (± 0.94)	5.79 (± 6.49)	2.92 (± 2.47)	2.74 (± 1.46)
Histidine	7.05	0.37 (± 0.21)	0.62 (± 0.79)	0.62 (± 0.53)	0.57 (± 0.24)
Isoleucine	1.00	1.00 (± 0.60)	1.47 (± 0.96)	1.31 (± 0.73)	1.91 (± 1.21)
Methionine	2.63	0.47 (± 0.43)	0.57 (± 0.32)	0.80 (± 0.44)	1.25 (± 0.37)
Valine	1.03	1.97 (± 1.15)	3.82 (± 2.39)	2.57 (± 2.04)	3.11 (± 1.67)
Leucine	0.94	1.44 (± 0.90)	2.56 (± 1.44)	2.53 (± 1.22)	3.68 (± 1.32)
Lysine	3.01	1.55 (± 0.10)	1.08 (± 0.11)	1.98 (± 0.13)	2.01 (± 0.06)
Serine	3.84	0.89 (± 0.34)	1.27 (± 0.69)	1.24 (± 0.86)	2.60 (± 1.02)
Threonine	4.25	1.07 (± 0.50)	0.58 (± 0.43)	1.15 (± 0.59)	2.16 (± 1.37)
Phenylalanine	7.32	0.67 (± 0.35)	1.57 (± 1.05)	1.18 (± 0.61)	1.63 (± 0.41)
Proline	4.12	0.48 (± 0.50)	1.55 (± 2.11)	0.90 (± 0.65)	0.96 (± 0.38)
Pyroglutamate	2.50	0.10 (± 0.23)	0.12 (± 0.33)	0.07 (± 0.18)	0 (± 0)
Tryptophan	7.73	0.18 (± 0.11)	0.39 (± 0.50)	0.30 (± 0.17)	0.38 (± 0.18)
Tyrosine	7.18	0.85 (± 0.44)	1.01 (± 0.97)	1.04 (± 0.65)	1.65 (± 0.64)
4-Aminobutyrate	2.27	0 (± 0)	0 (± 0)	0.42 (± 1.10)	0.94 (± 1.63)

Carnitine	3.22	0.02 (± 0.05)	0.38 (± 0.79)	0.05 (± 0.06)	0.02 (± 0.02)
Taurine	3.25	0.95 (± 0.80)	1.06 (± 1.21)	0.38 (± 0.54)	0.35 (± 0.07)
Choline	3.18	0.08 (± 0.07)	0.14 (± 0.18)	0.15 (± 0.15)	0.11 (± 0.08)
Betaine	3.25	0 (± 0)	0 (± 0)	0.04 (± 0.11)	0 (± 0)
Citrate	2.53	0.03 (± 0.05)	0.30 (± 0.42)	0.09 (± 0.10)	4.55 (± 7.33)
Fumarate	6.51	0.05 (± 0.02)	0.05 (± 0.08)	0.03 (± 0.03)	0.05 (± 0.02)
Succinate	2.39	21.85 (± 14.91)	8.80 (± 11.57)	9.79 (± 14.44)	9.23 (± 13.79)
Pyruvate	1.46	0.63 (± 1.41)	1.17 (± 0.88)	0.23 (± 0.30)	0.49 (± 0.51)
Creatine	3.02	0.23 (± 0.16)	1.02 (± 0.86)	0.13 (± 0.17)	0.24 (± 0.14)
Creatinine	3.03	0.13 (± 0.15)	0.15 (± 0.22)	0.03 (± 0.05)	0.01 (± 0.02)
3-Hydroxybutyrate	1.19	0.09 (± 0.12)	1.42 (± 3.67)	0.08 (± 0.22)	0 (± 0)
Acylcarnitine	3.18	0.33 (± 0.26)	0.06 (± 0.10)	0.05 (± 0.05)	0.07 (± 0.06)
Deoxycholate	0,71	0.35 (± 0.19)	0.10 (± 0.09) *	0.07 (± 0.13) *	0.10 (± 0.07)
Lithocholate	0.66	0.84 (± 0.31)	0.29 (± 0.37) *	0.31 (± 0.35) *	0.37 (± 0.34)
Cholate	0.71	0.52 (± 0.40)	0.14 (± 0.14)	0.37 (± 0.40)	0.24 (± 0.28)
Adenine	8.10	0.07 (± 0.10)	0.08 (± 0.17)	0.03 (± 0.04)	0.02 (± 0.02)
adenosine phosphate	8.60	0.03 (± 0.02)	0 (± 0.01)	0 (± 0)	0.01 (± 0.01)
Hypoxanthine	8.18	0.17 (± 0.16)	0.09 (± 0.08)	0.18 (± 0.13)	0.27 (± 0.20)
Inosine	6.09	0.02 (± 0.02)	0.01 (± 0.02)	0.01 (± 0.03)	0 (± 0.01)
Uracil	5.79	0.23 (± 0.28)	0.29 (± 0.14)	0.29 (± 0.18)	0.71 (± 0.43)
Uridine	5.89	0.03 (± 0.02)	0.01 (± 0.02)	0.03 (± 0.06)	0.04 (± 0.02)
Xanthine	7.92	0.15 (± 0.09)	0.06 (± 0.06)	0.19 (± 0.29)	0.87 (± 1.41)
Nicotinate	8.60	0.12 (± 0.12)	0.03 (± 0.03)	0.03 (± 0.03)	0.09 (± 0.01)
NAD+	9.32	<0.01 (± 0.01)	0 (± 0)	0 (± 0)	0 (± 0)
Nicotinamide ribotide	9.58	0.05 (± 0.07)	0 (± 0)	0 (± 0)	0 (± 0)
1-Methylnicotinamide	9.27	0 (± 0)	0.11 (± 0.29)	0 (± 0)	0 (± 0)
Trigonelline	9.11	0 (± 0)	0.02 (± 0.05)	0 (± 0)	0.01 (± 0.01)

The index in ppm indicates the chemical shifts of the most characteristic group of proton(s) for each metabolite. Mann-Whitney t-tests between two groups of samples (donors vs each of the other groups) * $P \leq 0.05$, ** $P \leq 0.01$