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Correlations between microbiota and metabolites after faecal microbiota transfer in irritable bowel syndrome

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RESEARCH ARTICLE

Abstract

Faecal microbiota transfer (FMT) consists of the infusion of donor faecal material into the intestine of a patient with the aim to restore a disturbed gut microbiota. In this study, it was investigated whether FMT has an effect on faecal microbial composition, its functional capacity, faecal metabolite profiles and their interactions in 16 irritable bowel syndrome (IBS) patients. Faecal samples from eight different time points before and until six months after allogenic FMT (faecal material from a healthy donor) as well as autologous FMT (own faecal material) were analysed by 16S RNA gene amplicon sequencing and gas chromatography coupled to mass spectrometry (GS-MS). The results showed that the allogenic FMT resulted in alterations in the microbial composition that were detectable up to six months, whereas after autologous FMT this was not the case. Similar results were found for the functional profiles, which were predicted from the phylogenetic sequencing data. While both allogenic FMT as well as autologous FMT did not have an effect on the faecal metabolites measured in this study, correlations between the microbial composition and the metabolites showed that the microbe-metabolite interactions seemed to be disrupted after allogenic FMT compared to autologous FMT. This shows that FMT can lead to altered interactions between the gut microbiota and its metabolites in IBS patients. Further research should investigate if and how this affects efficacy of FMT treatments.

Keywords: microbe-metabolite interactions, colonic microbiota, metabolite profiles, faecal microbiota transplantation

1. Introduction

Faecal microbiota transfer (FMT) consists of the infusion of faecal material from a healthy donor into the intestine of a patient with the aim to restore the disturbed faecal microbiota of the recipient. FMT is found to be a highly effective treatment in patients with recurrent *Clostridioides difficile* infection (CDI) (Quraishi *et al.*, 2017). Additionally, it has been studied as a potential treatment for ulcerative colitis (UC) (Costello *et al.*, 2019; Moayyedi *et al.*, 2015; Paramsothy *et al.*, 2017; Rossen *et al.*, 2015), metabolomic syndrome (Kootte *et al.*, 2017; Vrieze *et al.*, 2012) and irritable bowel syndrome (IBS) (Halkjaer *et al.*, 2018; Holster *et al.*, 2019b; Johnsen *et al.*, 2018).

Microbes are the major component transferred in FMT (De Vos, 2013) and it has been shown that FMT results in changes of the microbial composition in the intestinal tract that can persist for months (Li *et al.*, 2016). While these new microbes may interact directly with the host, it is likely that it is their metabolic activity that has an impact on the gastrointestinal ecosystem. Hence, the produced metabolites are an important link between the gut microbiota, the colonic mucosal response and, potentially, the immune homoeostasis and health of the host (Levy *et al.*, 2016). Nevertheless, only few studies have investigated the impact of FMT on gut microbiota metabolites. The metabolites found in the highest amounts are short chain fatty acids (SCFAs) and branched chain fatty acids (BCFAs),

mainly derived from undigested dietary carbohydrates and proteins, respectively (Scott *et al.*, 2013). SCFAs (acetate, propionate and butyrate) are essential for the homeostasis of the host (Van der Beek *et al.*, 2017). For instance, butyrate is the main energy source for colonocytes and has shown to have anti-inflammatory (Correa-Oliveira *et al.*, 2016) and anti-carcinogenic effects (Wu *et al.*, 2018), as well as having the ability to decrease visceral sensitivity in healthy subjects (Vanhoutvin *et al.*, 2009). In addition, the gut microbiota can affect the metabolism of dietary amino acids and is able to synthesise amino acids *de novo* (Lin *et al.*, 2017). Faecal amino acids have been shown to be more abundant in colorectal cancer (Yang *et al.*, 2019), and amino acid profiles in serum and plasma could be used to distinguish between IBD and healthy subjects (Schicho *et al.*, 2012). The most abundant amino acids within the gut microbiota are branched chained amino acids (BCAA) (Dai *et al.*, 2011), and these have shown to be harmful when present in high amounts in the systemic circulation (Neis *et al.*, 2015). Serum levels of these amino acids were found to be associated with the aberrant microbiota of subjects with insulin resistance, possibly providing a link between the gut microbiota and insulin sensitivity (Pedersen *et al.*, 2016). Hence, profiling these gut microbiota-derived metabolites might give us an important insight into the function of the gut microbiota and their integration with the host environment.

We have previously reported the effects of allogenic FMT (faecal material from a healthy donor) as well as autologous FMT (own faecal material) on the symptoms and faecal microbiota of IBS patients based on phylogenetic microarray analysis two and eight weeks after FMT compared to baseline (– two weeks sample) (Holster *et al.*, 2019b). Here, we extend the faecal microbial composition data with samples from one day, three days, seven days, four weeks, eight weeks and six months using 16S rRNA gene amplicon sequencing. In parallel, faecal metabolites were assessed with gas chromatography coupled to mass spectrometry (GS-MS) in samples from the same time points. The aim of this study was to investigate the effect of FMT on both faecal microbiota composition and functional profiles as well as the associated microbiota-produced metabolites (e.g. SCFAs, BCFA and amino acids), and patterns of their interactions.

2. Methods

Study design

As described in our recent publication, we treated 17 IBS patients with either FMT from a healthy donor (allogenic FMT, n=8) or FMT with their own stool (autologous FMT, n=9) (Holster *et al.*, 2019a). The faecal transplant (30 g of stool in 150 ml sterile saline and 10% glycerol) was administered into the caecum by whole colonoscopy after bowel cleansing. One of the patients in the autologous FMT group decided not to continue with the study after receiving the FMT. Hence, faecal samples from 16 patients were collected two weeks and one day before FMT (baseline) and three days, seven days, two weeks, four weeks, eight weeks and six months after FMT. The study was conducted according to the principles of the Declaration of Helsinki and its revisions, and ethical approval was obtained from the Central Ethical Review Board of Uppsala, Sweden (registration number 2013/180). The trial has been registered at ClinicalTrials.gov (NCT02092402) on March 20, 2014.

Faecal microbial DNA extraction and microbiota analysis

Microbial DNA from faecal samples was isolated using repeated bead beating (Salonen *et al.*, 2010) and the QIAamp DNA stool extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was assessed using a Qubit spectrometer (Thermo Fisher Scientific, Boston, MA). The samples were subjected to 16S rRNA gene V3-V4 PCR amplification and the amplicons were indexed and characterised by next generation sequencing (NGS) using an Illumina HiSeq platform (Illumina, San Diego, CA, USA) as previously described (Virtanen *et al.*, 2019) in order to assess changes in faecal microbiota composition. To assess the microbiotic profiles sequencing reads were processed with Mothur v. 1.35.1 using Greengenes version 13.5, containing OTUs clustered at 99% sequence identity, as the reference database (Schloss *et al.*, 2009). Taxonomic classification on species level were used in further analyses.

Faecal metabolite extraction and quantification

One aliquot of 100 mg faecal material was used for dry weight determination and one for SCFAs and amino acid extraction. 1 ml of 5 mM aqueous NaOH containing internal standard (5 µg/ml caproic acid-d3 + 5 µg/ml valine-d) was added to the 100 mg aliquot for SCFAs and amino acid extraction. The sample was homogenised with a micropestle and mixed for 10 min on a shaker (300 rpm). After shaking, the sample was centrifuged for 20 min at 13,200×*g* at 4 °C. 300 µl of MQ water, 500 µl propanol/pyridine mixture solvent (v/v = 3:2), 100 µl of propyl-chloroformate were added to 500 µl of faecal water obtained after centrifugation. The sample was vortexed and ultrasonicated for 1 min. After adding 300 µl of hexane, the sample was vortexed and centrifuged for 5 min at 2,000×*g*. 300 µl from the hexane layer was collected in a GC vial and the sample was reextracted with 200 µl hexane, and retention index standards were added before analysis. The chemicals used for metabolites analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following metabolites in the faecal samples were measured by GC: acetic acid, propionic acid, isobutyric acid, butyric acid,

2-methylbutyric acid (2-MBA), isovaleric acid, valeric acid, methione, tyrosine, valine, leucine and isoleucine. Internal standards were used for quantification: valine D_8 and hexanoic acid D_4. Undecane and tridecane were used as retention time index standards (RI). The aliquots with 100 mg faecal sample for dry weight determination were freeze-dried overnight at -50 °C.

The measurements of metabolites were performed on a HP 6890 N gas chromatography coupled to HP 5973 single quadrupole mass spectrometry (GC-MS) (Agilent Technologies, Santa Clara, CA, USA). The GC oven temperature was as follows: 45 °C (4 min); 10 °C/min to 70 °C; 3 °C/min to 85 °C; 5 °C/min to 110 °C; 30 °C/min to 290 °C (8 min). Splitless injections of 1 µl of the final extract and using a single taper deactivated liner (Agilent Technologies), were carried out at 260 °C onto a DB-5MS (5% diphenyl-95% dimethylpolysiloxane), 30 m × 0.25 mm × 0.25 µm (Agilent Technologies). Helium was used as carrier gas at a constant flow rate of 1.0 ml/min. MS measurements were performed in the selective ion monitoring (SIM) mode. The ions monitored for the analytes are shown in Supplementary Table S1.

Gene expression colonic transporters

Expression of the colonic butyrate transporters (MCT1 and SMCT1), subunits of the large neutral amino acid transporter (LAT1), the serotonin transporter (SERT) and the folate carrier (RFC1) was measured with qPCR. Colonic biopsies were collected from the uncleansed sigmoid (20-25 cm from the anal verge, at the crossing with the *arteria iliaca communis*) at baseline, two weeks and eight weeks after FMT. RNA was isolated as previously described (Holster *et al.*, 2019a). cDNA was synthesised according to the protocol of the Superscript™ VILO™ cDNA synthesis kit (ThermoFisher Scientific, Waltham, MA, USA). The following Taqman primer-probe sets (Life Technologies, Carlsbad, CA, USA) were used for the amplification of each colonic transporter by RT PCR: SLC5A8 (SMCT1, Hs00377618_m1), SLC16A1 (MCT1, Hs01560299_m1), SLC3A2 (LAT1 heavy chain, Hs00374243_m1), SLC7A5 (LAT1 light chain, Hs01001189_m1), SLC3A2 (LAT1 heavy chain, Hs00374243_m1), SLC6A4 (SERT, Hs00984349_m1), SLC19A1 (RFC1, Hs00953344_m1) and GAPDH (Hs03929097_g1). The thermal cycling conditions of the Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies) were set to 95 °C for 20 s, followed by 40 cycles at 95 °C for 2 s, ended by one cycle of 60 °C for 20 s and then hold at 4 °C. GAPDH was used as reference gene for each sample. The relative gene expression of the colonic transporters was calculated using the comparative 2-ΔΔCt method (Livak and Schmittgen, 2001), and was normalised to GAPDH expression.

Data analysis

Mixed-effect analysis of the faecal metabolites

The faecal metabolites were analysed using a mixed-effects linear model analysis as implemented in Graphpad Prism 8.0. This mixed model uses a compound symmetry covariance matrix and Geisser-Greenhouse correction and is fitted using Restricted Maximum Likelihood (REML). The Sidak's multiple comparisons test was used to correct for multiple comparisons.

Principle Coordinate Analysis

Principle Coordinate Analysis (PCoA) has proven a flexible tool for coordination analysis of microbiota composition data (Hout *et al.*, 2013; Kenkel and Orloci, 1986; Navas-Molina *et al.*, 2013) to detect overall effects of treatments on composition of microbiota. We implemented it by using function 'cmdscale' as part of the R-language. For analysing composition data, as in the microbiota compositional profiles, a specific distance measure, Aitchison distance, has been shown to be especially appropriate (Aitchison and Greenacre, 2002). In our analysis, we implemented R-package 'coda.base' for using it (Comas-Cufi, 2019).

Microbial functional prediction

The NGS data was used to predict the functional capacity of the microbiota found at the time points before and after FMT. Functions were predicted using PICRUSt version 1.1.1 using Greengenes database version 13.5 and default settings (Langille *et al.*, 2013). The predicted functions are given as profiles of KEGG pathways (Kanehisa and Goto, 2000).

Trajectory analysis of microbiota composition, metabolites and functional prediction profiles

Sparse Partial Least Squares (sPLS) regression was employed for visualising the changes over time of treated individuals' microbiota composition, metabolites and functional prediction profiles across the two baseline and six follow-up time points. The sPLS model was used to describe how bacterial composition, metabolites and bacterial predicted functions varied over time, separately for subjects treated with allogenic and autologous FMT. The sPLS latent variables, hence, represent those parts of varying bacterial composition, metabolites or predicted functions which vary with time in a similar matter for all subjects in the respective group. Function 'spls' from R-package 'mixOmics' (Cao *et al.*, 2017; Rohart *et al.*, 2017) was used for this analysis, choosing ncomp=2 components and keepX=10 allowed variables in each component. For microbiota composition, metabolites and predicted functions, respective parts of the data (e.g. baseline versus later timepoints) were characterised by their mean values

(centre of ellipses) and variance-covariance structure (axes orientation and dimensions of the displayed ellipses). This allows comparing location and variance without claiming to give 95% confidence intervals of the means, as these are not as easily computed and interpreted for data with timeseries and repeated values structure.

Partial correlation analyses of interactions between microbiota and metabolite time courses

Pairwise shrunk partial correlations were computed for all pairs of microbiota compositional features, i.e. relative abundance of genera and species, and the faecal metabolite profiles (function 'cor2pcor' from R-package 'corpcor' (Schafer and Strimmer, 2005; Schafer *et al.*, 2017)). Using a cut-off of $\rho>0.4$, partial correlation network graphs for the interactions between microbiota species (genus) and metabolites were produced.

Multivariate correlation structures between microbiota and metabolites

Sparse canonical correlation analysis (function 'CCA' from R-package 'PMA' (Witten *et al.*, 2009)) was employed to characterise the crosstalk of microbiota species and faecal metabolites across the time series, for each of the individuals in our study. In order to maintain the impact of the large proportional differences in microbiota composition between the different microbiota species (genus) as well as metabolite levels, the analyses were run without prior standardisation of either microbiota or metabolite time series profiles. Multivariate correlations of the first components from the canonical correlation analysis are reported as single values together with boxplots. These analyses were performed for correlating all measured metabolites to either all microbiota composition (genus) profiles or to the predicted functional profiles (KEGG pathways). In addition, we focused on specific pathways which represent either mainly saccharolytic or proteolytic metabolic processes, and investigated their correlation pattern with those measured faecal metabolites in our study which can be grouped as to belong to these processes.

3. Results

Next generation sequencing microbiota composition data

Supplementary Figure S1 shows the top 10 most abundant genera in the faecal samples of patients before and after allogenic or autologous FMT. Microbiota compositional profiles obtained by NGS were analysed separately in the allogenic and in the autologous group using both unsupervised (PCoA, Figure 1) and supervised (sPLS, Figure 2) ordination methods. The PCoA analysis shows that the microbiota of each individual clusters together, even after receiving allogenic FMT. However, the samples after allogenic FMT are separated from the baseline samples in most of the individuals (Figure 1A), which is not the case after autologous FMT (Figure 1B). This indicates, as to be expected, that the gut microbiota in the IBS patients after FMT from a healthy donor changes more than after receiving their own stool back. When focusing on the differences between the time points (see the sPLS plots in Figure 2) it appears that the faecal microbiota of the subjects that received allogenic FMT (Figure 2A) separates more clearly from their baseline samples than is the case for the autologous FMT group (Figure 2B). This confirms further that the allogenic FMT causes a long-term colonisation of newly introduced microbes in the IBS subjects that lasts at least six months.

Some of the six-month samples in the autologous group show a very distinct separation from the other samples (Figure 2B). The autologous FMT did not result in improved symptoms (Holster *et al.*, 2019b), hence, subjects in this group were more likely to change their diet and/or medication, even if asked not to during the course of the study. As these changes could have had large effects on the microbiota composition, they were excluded in a separate analysis, however, similar results were obtained (see Supplementary Figure S2).

In addition, Figure 2 shows that there are differences in microbiota composition between subjects. Apart from known differences in microbiota composition in IBS patients (Pittayanon *et al.*, 2019), this could be due to the temporal instability of the microbiota in IBS patients (Durban *et al.*, 2013; Matto *et al.*, 2005) or a change in symptoms over time. However, in general, there does not seem to be a clear relationship between improvement of symptoms and change in microbiota after FMT (Halkjaer *et al.*, 2018; Lahtinen *et al.*, 2020).

Faecal metabolites

Figure 3 shows the concentrations of the metabolites measured in faecal samples before and after FMT in the allogenic and autologous FMT group, corrected for baseline values. No large differences between the allogenic and autologous FMT group were observed. Butyric acid was significantly increased three days after FMT in the autologous group (mixed-effect analysis with Sidak multiple comparisons test, $P<0.01$), which is probably due to the differences in the absolute values at baseline (see Supplementary Figure S3). Similar results were obtained when the metabolite concentrations were normalised to faecal dry weight (Supplementary Figure S4 and S5). Sparse PLS analyses were not able to separate the different time points from each other (Figure 4), contrary to what was shown for the microbiota composition data. These results show that although the microbiota changes after allogenic FMT, the metabolites measured in this study did not.

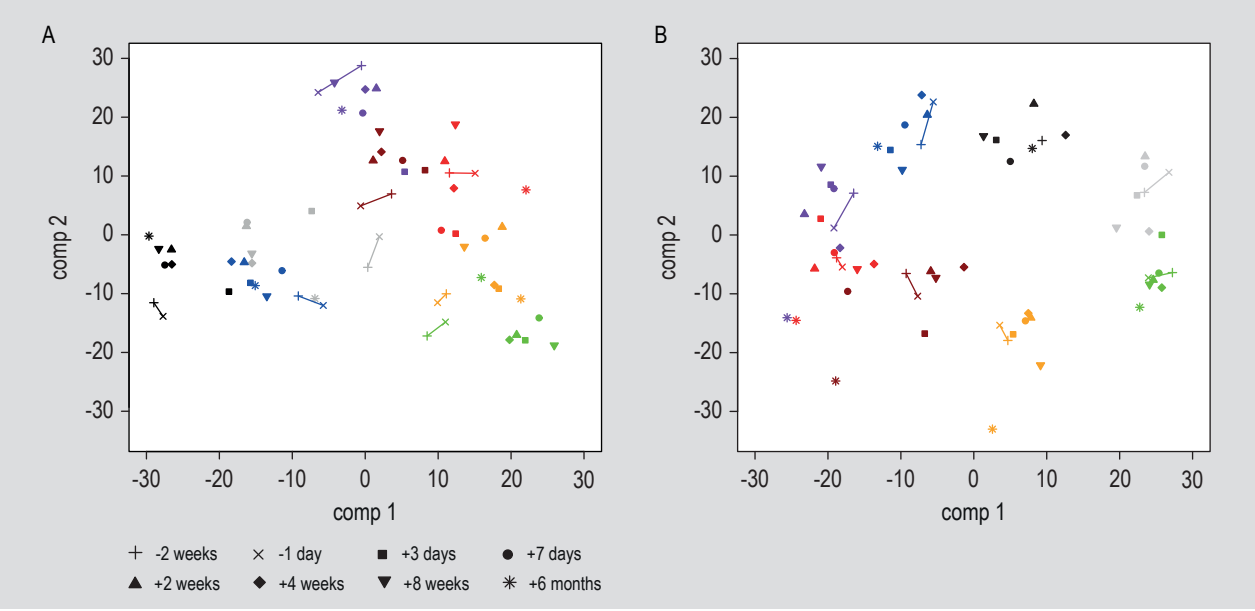

Figure 1. Principal component analysis score plots of faecal microbial composition within the allogenic (A) and autologous group (B) at different time points before and after faecal microbiota transfer (FMT). The first and second component are shown on the x- and y-axis. Colours indicate the different subjects and symbols represent time points. The time points before FMT (-2 weeks and -1 day) are connected with a line in order to visualise that the samples after allogenic FMT were separated from the baseline samples in most participants. In the autologous FMT group the samples after FMT were less separated from the corresponding baseline samples.

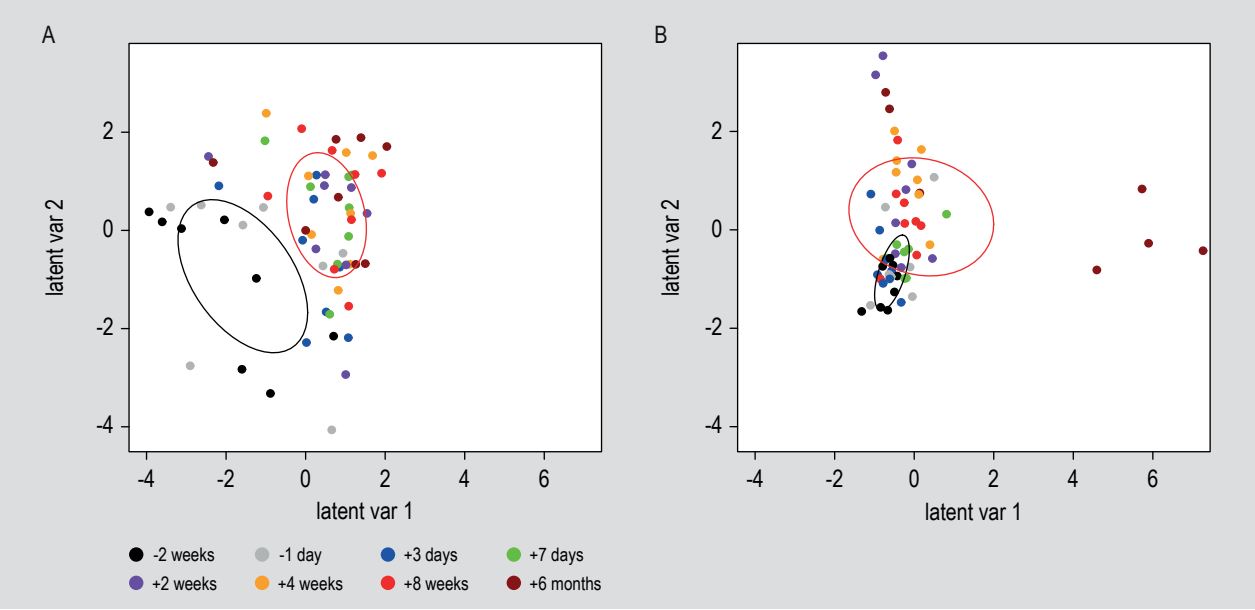

Figure 2. Sparse partial least squares score plots of the microbiota composition of faecal microbial composition within the allogenic (A) and autologous group (B) at different time points before and after faecal microbiota transfer (FMT). The first and second latent variable are shown on the x- and y-axis. Different colours indicate different time points. Ellipses represent location and dimensions of mean values and covariance structure of the respective part of the data. The black ellipse represents the samples before FMT (-2 weeks and -1 day samples) and the red ellipse represents the samples after FMT (3 days, 7 days, 2 weeks, 4 weeks, 8 weeks and 6 months). The ellipses show that the microbiota samples were clearly separated from the baseline samples after allogenic FMT, which was not the case after autologous FMT.

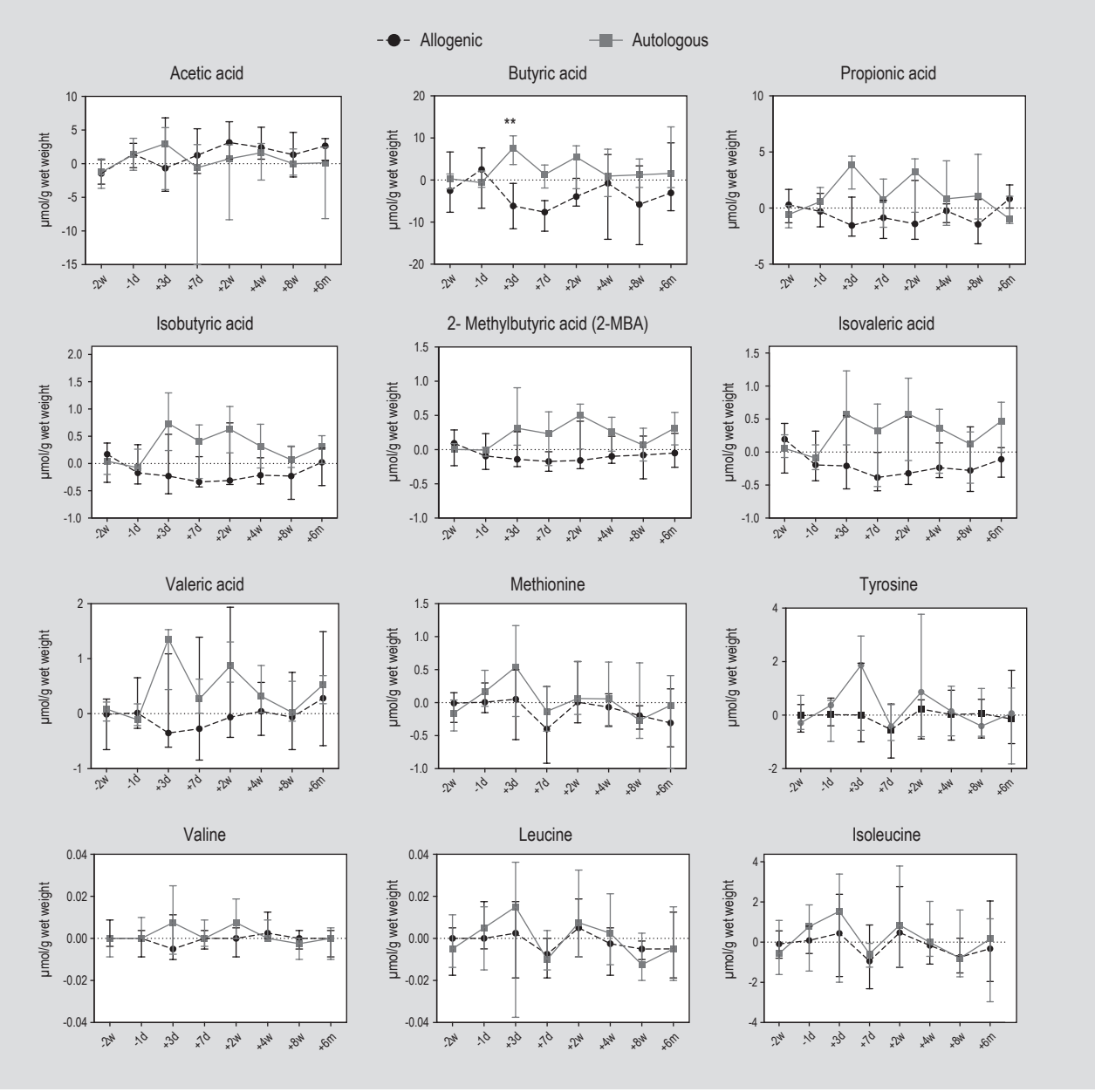

Figure 3. Baseline-corrected concentrations of different metabolites in faecal samples before and after allogenic or autologous faecal microbiota transfer in irritable bowel syndrome patients. The average of the concentrations from two weeks and one day before treatment were subtracted from the concentrations at all time points. ** indicates *P*<0.01. Median values with interquartile range are shown. The absolute data can be found in Supplementary Table S2. D=days, w=weeks, m=months.

Correlations between microbiota and metabolites in faecal samples

Shrunk partial correlations

In order to investigate the interactions between the microbiota composition and the metabolites measured in the faecal samples, correlation analyses were performed. Pairwise correlations between single faecal microbiota taxa profiles and specific metabolite profiles were assessed as shrunk partial correlations in order to overcome the imbalance of a large number of variables compared to a small number of samples, which does not allow for the classical approach of computing partial correlations. Analysis of shrunk partial correlations between individual faecal microbiota compositions and the measured faecal metabolites resulted in a smaller number of relevant correlations (a cut-off of $\rho>0.4$) in the allogenic FMT group compared to the autologous FMT group (Figure 5A, Supplementary Table S2). These results indicate that the correlations present after autologous FMT, which is considered the control group, are disrupted after

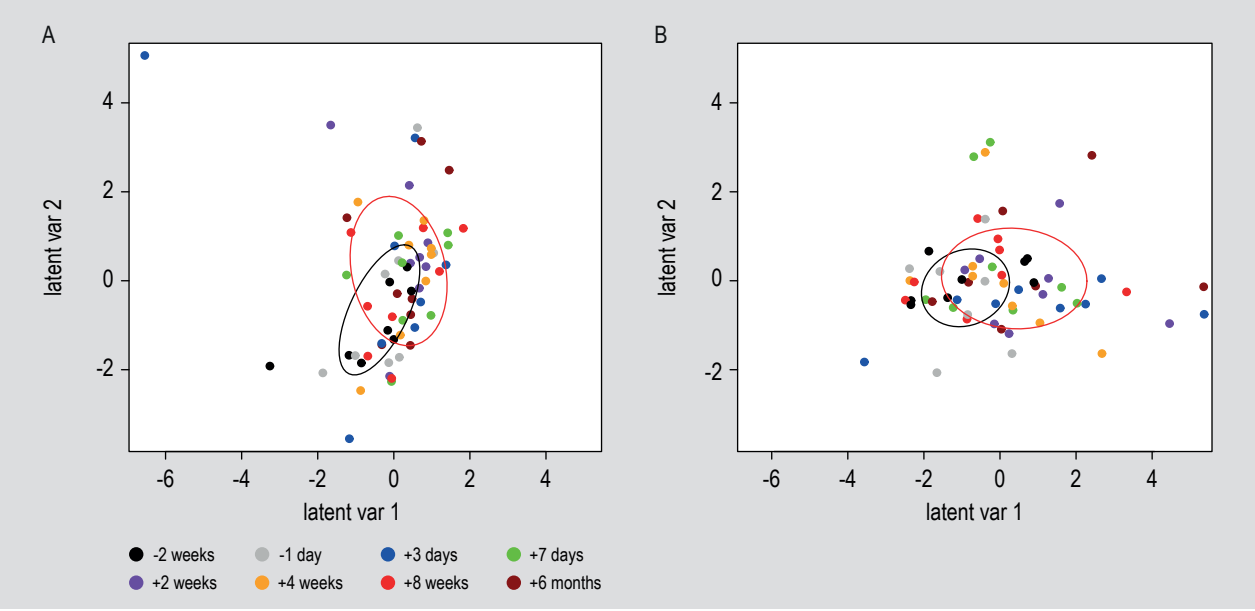

Figure 4. Sparse partial least squares score plots of measured metabolites within the allogenic group (A) and autologous group (B) at different time points before and after faecal microbiota transfer (FMT). The first and second latent variable are shown on the x- and y-axis. Different colours indicate different time points. Ellipses represent location and dimensions of mean values and covariance structure of the respective part of the data. The black ellipse represents the samples before FMT (-2 weeks and -1 day samples) and the red ellipse represents the samples after FMT (3 days, 7 days, 2 weeks, 4 weeks, 8 weeks and 6 months). No separation between the samples before and after FMT was observed in any of the groups.

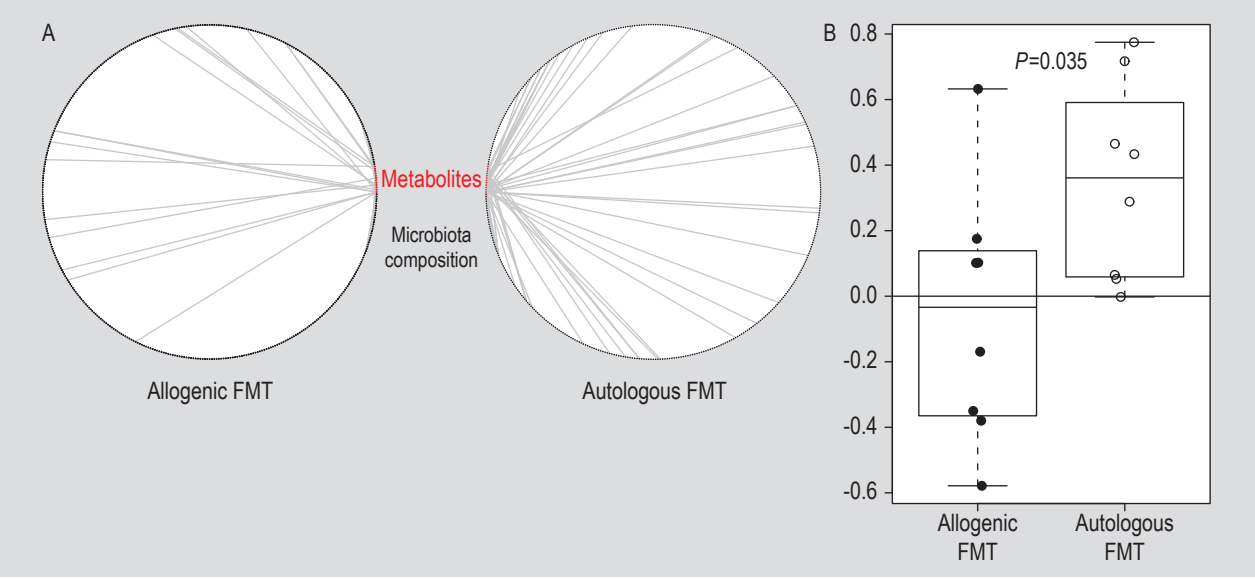

Figure 5. Correlation between metabolites and microbiota composition data. (A) Visualisation of partial correlations between 12 faecal metabolites and the microbiota composition on species level in the allogenic as well as the autologous faecal microbiota transfer (FMT) group. The average of all time points is taken for all individuals in the corresponding groups. The names of the metabolites and the microbial species that correlate can be found in Supplementary Table S2. (B) Canonical correlation analysis performed on the microbial composition data and the measured metabolites. Both analyses suggest that the interactions between microbiota and metabolites were disrupted after allogenic FMT.

allogenic FMT. Similar results were obtained when the measured metabolites were corrected for faecal dry weight (Supplementary Figure S6), and also if different relevance cut-offs for partial correlations were chosen before drawing an interaction line in these plots (data not shown).

Sparse canonical correlation analysis

As a multivariate approach to assess correlations between microbiota and specific metabolic profiles, we employed a sparse version of Canonical Correlation Analysis (sCCA), allowing us to deal with a large number of variables compared to samples in our datasets. sCCA analyses were conducted for each subject, in order to relate development of microbiota to changes in metabolite profiles during FMT treatments. Figure 5B shows the multivariate correlation values for each individual in the allogenic and autologous group, which were on group level significantly different from each other (P=0.035). The correlation values in the allogenic FMT group were distributed around zero whereas the values from the autologous FMT group were mostly larger than zero. These results show that the correlations found after allogenic FMT were less homogenous in direction than the correlations found after autologous FMT, indicating again that the interaction between microbiota and metabolites were disrupted after allogenic FMT. The faecal metabolites contributing most to these correlations that were shared by all subjects in the allogenic FMT group included acetic acid and butyric acid. The microbiota contributing the most to these correlations included *Clostridium bolteae, Hespellia porcina, Faecalibacterium prausnitzii* and *Gemmiger formicillis*. In the autologous group, acetic acid correlated with *Clostridium bolteae* and *Hespellia porcina*. Excluding the six-month samples showed similar results, however no statistically significant difference was reached (Supplementary Figure S7A). The sCCA for dry weight corrected faecal metabolites did not show any differences between allogenic and autologous FMT (Supplementary Figure S7B).

Functional prediction using next generation sequencing data

Microbiota composition can be used to predict the microbiota's functional potential, based on annotations of reference genomic sequences. We made use of this possibility to produce functional microbiota profiles for all faecal samples from allogenic and autologous FMT treated subjects in order to investigate whether the newly introduced microbiota took over a similar functional capacity as of the original microbiota (using PICRUSt). Figure 6A and B show sPLS analyses results for the functional prediction data. The plots show that the baseline samples separate more clearly from the samples after allogenic FMT compared to autologous FMT. This is similar to the results for the microbiota composition (Figure 1), although to a somewhat lesser extent, which indicates that additionally to the microbiota composition the functional capacity also changes after allogenic FMT. Finally, in order to study the relation between the predicted pathways involved in saccharolytic and proteolytic processes and their metabolites, these pathways and their respective metabolites were correlated (Supplementary Figure S8 and S9). The correlation patterns seem to differ between the allogenic and the autologous group but the correlations are weak and not statistically significant.

Gene expression of metabolite transporters

A reason for unaltered levels of faecal metabolites in parallel to an altered functional capacity of the microbiota could be an increased uptake of the produced faecal metabolites. FMT could potentially affect the uptake of these metabolites which is often substance-induced. Therefore, the gene expression of several transporters in colonic biopsies before and after FMT was measured with qPCR. While the relative expression of the butyrate and some other transporters was higher after the allogenic than the autologous FMT, no significant differences were observed (Supplementary Figure S10).

4. Discussion

FMT is the introduction of a new gut microbiota with the aim to restore a disturbed microbiota. This is the first study, to the best of our knowledge, that provides insight into the interaction between microbial composition and faecal metabolites after the introduction of a foreign microbiota. Especially allogenic FMT (from donor stool) resulted in an altered gut microbiota composition of the recipients even after six months, which is in line with our previously published results where both the faecal and the mucosal gut microbiota, analysed by HITChip, were altered two and eight weeks after FMT (Holster *et al.*, 2019b). However, the measured faecal bacterial metabolites in our study did not seem to be affected by neither allogenic nor autologous FMT. Interestingly though, our data indicate that the correlations between the microbiota and its metabolites appeared to be disrupted after allogenic FMT.

Partial correlations between faecal microbiota and its metabolites showed less correlations in the allogenic FMT group than in the autologous FMT group, which could indicate that the number of functional interactions between microbiota and metabolites is reduced after introducing a new microbiota. Assessing these correlations in a multivariate approach showed that the observed canonical correlations in the allogenic FMT group were less homogeneous in direction than observed in the autologous FMT. In addition to investigating the microbial composition, the 16S rRNA gene based NGS data was used to predict the functional capability of the microbes in the faecal samples. The supervised multivariate analysis showed that the functional capacity of the microbiota after allogenic FMT differed from baseline, similarly to the microbial composition which was altered after allogenic FMT, although to a lesser extent. This could suggest that the change of the microbial ecosystem induced by FMT also

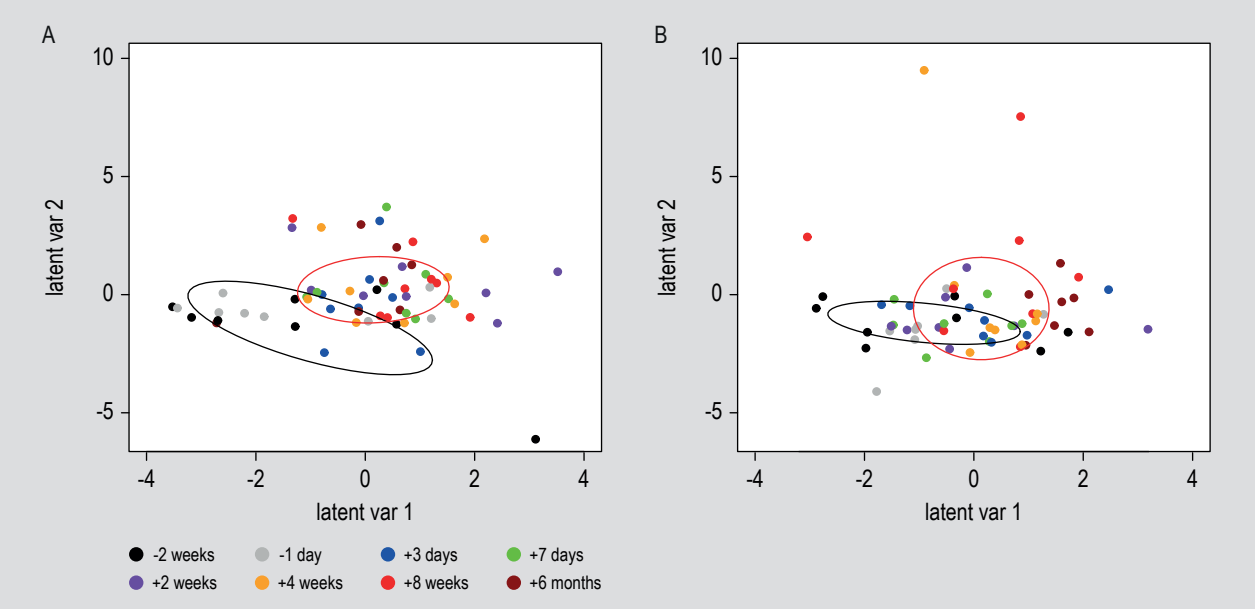

Figure 6. Sparse partial least squares score plots of the functional microbial prediction analysis within the allogenic (A) and autologous group (B) at different time points before and after faecal microbiota transfer (FMT). The first and second latent variable are shown on the x- and y-axis. Different colours indicate different time points. Ellipses represent location and dimensions of mean values and covariance structure of the respective part of the data. The black ellipse represents the samples before FMT (-2 weeks and -1 day samples) and the red ellipse represents the samples after FMT (3 days, 7 days, 2 weeks, 4 weeks, 8 weeks and 6 months). The ellipses show that the predicted functional capacity of the faecal microbiota samples were separated from the baseline samples after allogenic FMT, which was not the case after autologous FMT.

changed its overall metabolic capacity but had less impact on the restricted metabolites which were measured in the faecal samples of this study. An additional explanation could be that changes in other microorganisms such as viruses, eukaryotes and fungi had an effect on the metabolic capacity. Furthermore, the microbiota analysis was restricted to 16S rRNA-targeted sequencing. Whole genome sequencing could have provided a wider insight into the metabolic capacity of the microbiome.

Regularised methods of data analysis were implemented to assess both univariate and multivariate correlations between microbiota compositional profiles and specific metabolite profiles in the faecal samples during FMT treatment. This was a necessary choice to enable computation of partial correlations as well as canonical correlations, both of which otherwise could not be computed due to our data structure with an excess of variables compared to samples (and time points). Moreover, partial correlations are to be preferred over classical Pearson or Spearman correlations in this case, in order to avoid indirect correlations as far as possible. Partial correlations are frequently used for reverse engineering purposes, to develop hypotheses related to causal interactions (Werhli *et al.*, 2006). Regarding canonical correlation analysis, we looked for specific combinations of microbiota compositional profiles co-varying with combinations of the specific metabolite profiles in faecal samples after FMT. Such analyses allow to investigate more complex interactions between microbiota and metabolite profiles, which would be missed if assessing correlations on single-pairwise levels only.

In our previous study on the clinical effect of FMT in IBS patients, we showed that allogenic FMT did not have a significantly greater effect on symptoms than autologous FMT, although IBS symptoms improved compared to baseline only in the allogenic group (Holster *et al.*, 2019b). However, the mucosal gene expression response was clearly different after allogenic compared to autologous FMT, suggesting an altered microbe-host interaction (Holster *et al.*, 2019a). Bacterial metabolites are an important part of the microbe-host interaction. We hypothesised that changes in bacterial metabolites could have caused the large differences in mucosal response. This would possibly indicate a new mode of action as well as a novel possible therapeutic target. Contrary to our hypothesis, allogenic FMT did not result in an altered metabolite profile in our study.

While faecal SCFA seem to be altered in IBS (Sun *et al.*, 2019), studies investigating other metabolites in IBS are scarce. A study by Shankar *et al.* (2015) showed that children with IBS-D had a different faecal metabolite profile compared to healthy controls. Additionally, strong associations between microbes and metabolites were found in the healthy children, whereas in the IBS-D group only

weak associations were detected (Shankar *et al.*, 2015). It seems as if FMT, at least in our study, is not able to restore the weak associations between microbes and metabolites as found in the study by Shankar *et al.* (2015). However, FMT did result in altered correlations between microbiota composition and faecal metabolites, indicating a change in microbe-metabolite interactions.

A recent study investigated the metabolite profiles post-FMT in IBS patients and concluded that SCFAs normalised after allogenic FMT, however the study did not include a control group and therefore their results cannot be compared to autologous FMT or natural changes occurring over time in IBS patients (Mazzawi *et al.*, 2019). Additionally, in CDI increased levels of the SCFAs butyrate, propionate and acetate were found in six patients after treatment with FMT (Seekatz *et al.*, 2018). Untargeted metabolomics in four paediatric UC patients showed that several metabolites were altered after FMT and correlated with clinical outcomes, and that the patients' metabolomic profile shifted towards their respective donors (Nusbaum *et al.*, 2018). A larger study, which investigated the outcomes of FMT in adult UC patients, suggested that specific metabolomic functional pathways of the microbiota were associated with achieved remission after FMT (Paramsothy *et al.*, 2019).

There are several possible reasons why no changes in metabolites after introducing a foreign microbiota could be observed in our study. First of all, measuring the metabolites in faecal samples does not necessarily reflect the production of bacterial metabolites. Metabolites are taken up by the host, converted into other metabolites or excreted from the host. Although the gene expression of several host mucosal transporters in our study was not affected by FMT, it is still unclear if changes in measured metabolites reflect changes in bacterial production of these metabolites or the ability versus inability of the host to absorb and utilise these metabolites. Therefore, other measures, such as assessing the metabolome in urine and plasma, could give additional insights into the dynamics of the metabolome produced by the gut microbiota.

Another limitation of this study was that only a selective number of metabolites was measured and changes in other metabolites could have been missed. In addition, the autologous FMT group (receiving its own faecal material back) was regarded as a control group, while our earlier publications showed that also after autologous FMT microbial changes were observed and host gene sets were differently expressed. These changes were possibly due to the bowel cleansing before the FMT and/or the handling of the stool in preparation for the FMT. This could have also affected the outcomes measured in this study. In addition, both FMT groups had a limited number of patients not allowing for subgroup analyses. Furthermore, although we asked the participants to maintain their dietary habits while participating in the study and Food Frequency Questionnaire (FFQ) data did not show substantial changes during the study, already small changes in diet could have affected the participants' microbial composition and measured metabolites. However, we expect the effect of FMT to be larger than the effect of possible minor changes in food intake. Finally, although FMT is generally considered a safe treatment with a low incidence of serious adverse effects and the donors used in this were extensively tested, the risk that multi-drug resistant organisms and, possibly, other diseases are transferred seem to be present (DeFilipp *et al.*, 2019). In addition, the long-term risks are not yet fully investigated.

We used the tool PICRUSt for the prediction of functional profiles based on 16S rRNA gene date which can provide predicted functional profiles based on pathway or protein level. The small sample size of our study only allowed for such profiles on pathway level, while a larger samples size would have enabled more accurate profiles based on protein level. In addition, PICRUSt is one of several choices of methods for predicting functional profiles, and the use of alternative pipelines would very likely have resulted in differences in data analysis results. Given the limited sample size of our study population and the known large variation in microbiota composition between different individuals, we could not deliver detailed results on any specific predicted functional profile, but instead constrained our conclusions to overall measures of correlation patterns, such as total numbers of observed partial correlations, or intensities of observed canonical correlations, between measured faecal metabolites and microbiota composition or predicted functional profiles. In addition, at least in our study, it seems as if using prediction of metabolic function based on microbiota composition analysis does not seem to be a good surrogate marker for the faecal metabolites that were measured.

In conclusion, we could show that FMT resulted in disturbed interactions between the microbiota and its metabolites in IBS. Future studies need to elucidate how this affects treatment outcome in FMT therapy, especially with regards to personalised medicine.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2020.0010.

Table S1. The quantification ions in the GC-MS method.

Table S2. Partial correlations between metabolites and microbiota composition data in the allogenic FMT and autologous FMT group.

Figure S1. Top 10 most abundant genera in faecal samples of IBS patients included in this study.

Figure S2. sPLS figures of faecal microbial composition within the allogenic group and autologous group at different time points before and after FMT without six months sample.

Figure S3. Absolute data of the twelve metabolites measured in the faecal material before and after allogenic or autologous FMT in IBS patients.

Figure S4. Baseline-corrected data of the twelve metabolites measured in the faecal material before and after allogenic or autologous FMT in IBS patients.

Figure S5. sPLS figures of measured metabolites normalised to dry weight within the allogenic group and autologous group at different time points before and after FMT Ellipses represent location and dimensions of mean values and covariance structure of the respective part of the data.

Figure S6. Correlation between metabolites corrected for faecal dry weight and microbiota composition data.

Figure S7. Canonical correlation analysis performed on the microbial composition data and the measured metabolites and the microbial composition data and metabolites corrected for dry weight without the six-month sample.

Figure S8. Heatmap of correlations between the predicted pathways involved in saccharolytic processes and their metabolites in the allogenic group and autologous group.

Figure S9. Heatmap of correlations between the predicted pathways involved in proteolytic processes and their metabolites in the allogenic group and autologous group.

Figure S10. Relative expression of several transporters in biopsies of IBS patients after allogenic or autologous FMT, 2 and 8 weeks after FMT compared to baseline expression.

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Conflict of interest

The authors declare no conflict of interest.

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