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Original Article

Faecal Bacteriome and Metabolome Profiles Associated with Decreased Mucosal Inflammatory Activity Upon Anti-TNF Therapy in Paediatric Crohn's Disease

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Abstract

Background and Aims: Treatment with anti-tumour necrosis factor α antibodies [anti-TNF] changes the dysbiotic faecal bacteriome in Crohn's disease [CD]. However, it is not known whether these changes are due to decreasing mucosal inflammatory activity or whether similar bacteriome reactions might be observed in gut-healthy subjects. Therefore, we explored changes in the faecal bacteriome and metabolome upon anti-TNF administration [and therapeutic response] in children with CD and contrasted those to anti-TNF-treated children with juvenile idiopathic arthritis [JIA].

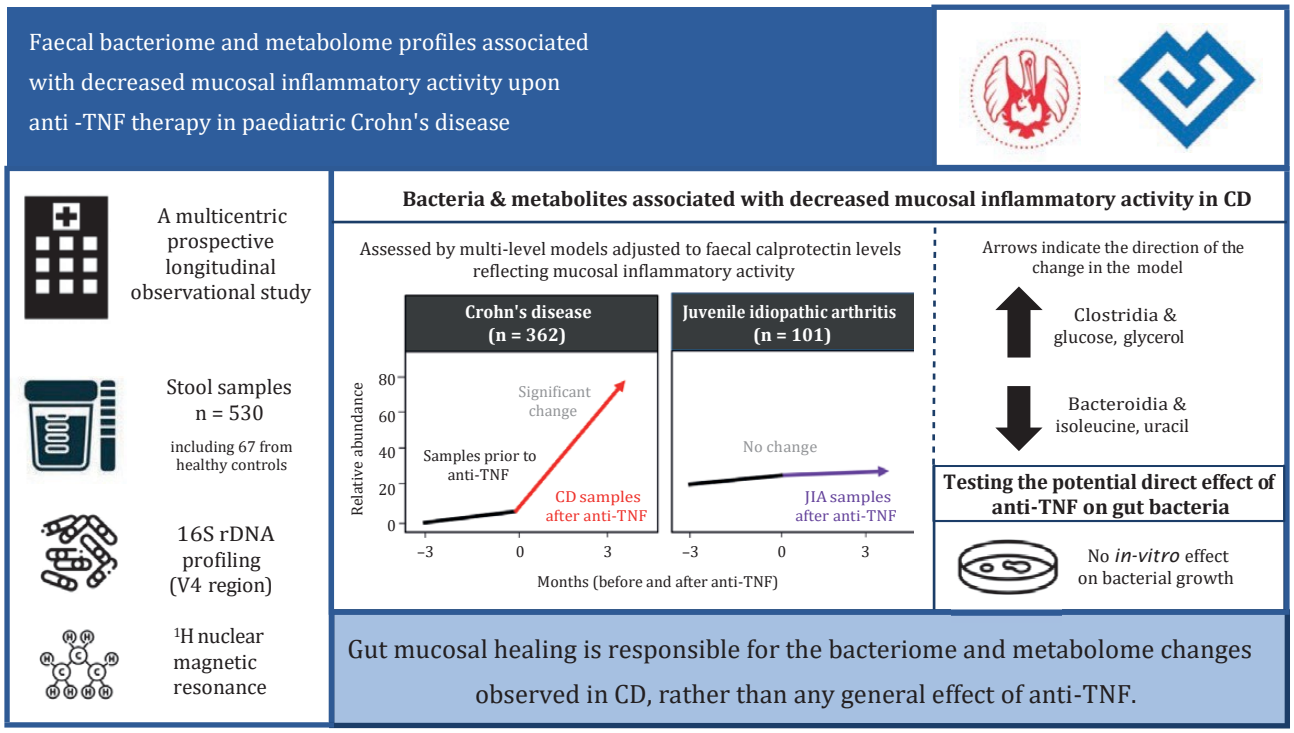
Methods: Faecal samples collected longitudinally before and during anti-TNF therapy were analysed with regard to the bacteriome by massively parallel sequencing of the 16S rDNA [V4 region] and the faecal metabolome by ^{1}H nuclear magnetic resonance imaging. The response to treatment by mucosal healing was assessed by the MINI index at 3 months after the treatment started. We also tested several representative gut bacterial strains for *in vitro* growth inhibition by infliximab.

Results: We analysed 530 stool samples from 121 children [CD 54, JIA 18, healthy 49]. Bacterial community composition changed on anti-TNF in CD: three members of the class Clostridia increased on anti-TNF, whereas the class Bacteroidia decreased. Among faecal metabolites, glucose and glycerol increased, whereas isoleucine and uracil decreased. Some of these changes differed by treatment response [mucosal healing] after anti-TNF. No significant changes in the bacteriome or metabolome were noted upon anti-TNF in JIA. Bacterial growth was not affected by infliximab in a disc diffusion test.

Conclusions: Our findings suggest that gut mucosal healing is responsible for the bacteriome and metabolome changes observed in CD, rather than any general effect of anti-TNF.

Key Words: IBD; Crohn's disease; anti-TNF; microbiome; metabolomics; children

Graphical Abstract



1. Introduction

Crohn's disease [CD] is an inflammatory bowel disease [IBD] characterized by chronic inflammation of any part of the gastrointestinal tract with a progressive and destructive course; its incidence is increasing worldwide.[1,2] Its aetiology remains unclear, with a strong contribution from the distorted microbiota.[3–13] The disease is incurable, with a multitude of therapeutic strategies available: in the last decade, tumour necrosis factor alpha blockers [anti-TNF] have been established as easily available and highly effective treatments.[14]

The body of knowledge on the effect of anti-TNF on the faecal microbiome and metabolome is limited. One of the first studies by Wang *et al*. in a population of 11 children with CD reported that anti-TNF blockade in CD is associated with an increased diversity and a shift towards the composition seen in healthy controls. Among others, they observed a significant decrease in the relative abundances of inflammation-promoting Enterobacteriaceae but without a significant increase in gut health-promoting taxa, such as short-chain fatty acid [SCFA] producers.[15] A subsequent investigation by the same team studied a population of 29 children with CD, of whom 18 received anti-TNF and 11 responded.[16] The authors described an enrichment in several SCFA producers following anti-TNF treatment and showed that infliximab responders were characterized by higher abundances at baseline of some non-SCFA-producing genera [*Methylobacterium*, *Sphingomonas*, *Staphylococcus* and *Streptococcus*]. Additionally, they reported a shift in the quantity of several amino acids as a potential marker of anti-TNF response.[16]

Several recent studies aimed to predict the response to anti-TNF [namely infliximab] in IBD using the microbiome composition. Ventin-Holmberg *et al*. reported a lower abundance of SCFA producers at baseline as a marker of non-response to anti-TNF in adults.[17] In a subsequent study in children, the response to anti-TNF was associated with several significant differences in bacterial abundance at baseline [namely, classes Clostridia and Bacilli vs class Gammaproteobacteria] and *Ruminococcus* [a known SCFA producer] combined with baseline calprotectin levels was a relatively good predictor of the response.[18] Höyhtyä *et al*. studied the baseline composition of the bacteriome in children and young adults—this was done based on absolute and relative abundances. They found modest differences in absolute abundances [more Bifidobacteriales and less Actinomycetales in the remission group]; however, the relative abundances on the order level were reported only in Bifidobacteriales.[19] The above study by Wang *et al*. also reported several borderline-significant associations of baseline bacterial abundance with the response to anti-TNF.[16]

Thus, there is compelling evidence that the gut microbiome of CD patients is altered upon anti-TNF therapy; however, it has not been established whether these microbiome shifts are due to decreasing mucosal inflammatory activity or whether anti-TNF may exert additional effects independent of the inflammatory activity and mucosal healing. A microbe specifically changing upon administering anti-TNF in CD but not reacting to anti-TNF in an extraintestinal diagnosis could indicate decreasing mucosal inflammatory activity. Therefore, in this study, we explored juvenile idiopathic arthritis [JIA] as such an extraintestinal paediatric diagnosis also treated with anti-TNF.

The aims of the present study were: [1] to characterize changes in the gut microbiome and metabolome associated with anti-TNF treatment, specifically in CD, but not in gut-healthy subjects with JIA—such changes would indicate decreasing mucosal inflammatory activity rather than a general effect of anti-TNF on immunity and the bacteriome; [2] to assess whether such bacteriome changes in CD differ by the indicators of mucosal healing 3 months after the start

of anti-TNF therapy; and [3] to assess whether common anaerobic human gut bacteria are susceptible *in vitro* to the anti-TNF drug infliximab applied alone or with serum complement—lack of such susceptibility would argue against a direct anti-TNF effect on bacterial flora.

2. Materials and Methods

2.1. Study cohort

Children diagnosed with CD or JIA were invited to participate in a multicentre prospective longitudinal observational study performed at the Departments of Paediatrics, Adult and Paediatric Rheumatology and Paediatric Surgery, Motol University Hospital, Prague, and Departments of Paediatrics at University Hospital Pilsen, University Hospital Olomouc, Thomayer University Hospital, Prague, Tomas Bata Regional Hospital, Zlin, and Masaryk Hospital, Usti nad Labem, all Czechia. The study was carried out between July 1, 2019 and February 25, 2022. Participants could enter the study at two-time points: either at diagnosis or at indication for anti-TNF. The inclusion criteria were age between 3 and 17 years [3–16 for JIA patients] at the study entry, diagnosis of CD [based on Porto criteria][20] or JIA [based on ILAR criteria],[21] and anti-TNF-naive state. Exclusion criteria in CD were ileocaecal stricture or co-occurrence of primary sclerosing cholangitis. In JIA, exclusion criteria were co-occurrence of diagnosed IBD or chronic diarrhoea with faecal calprotectin over 250 μg/g.[22,23] Stool samples were collected regularly once per month from entering the study until 3 months [between the 12th and 14th weeks] after commencing the therapy with anti-TNF or February 2022, whichever occurred first [the study scheme is shown in Supplementary Figure 1].

In addition, control faecal samples were obtained from healthy controls recruited among [1] children hospitalized at the same facility for benign surgeries without a need for antimicrobial pretreatment [e.g. hernia or circumcision] and [2] young adults [under 21 years of age] recruited as possible donors in another ongoing study with faecal microbiota transplantation in patients with irritable bowel syndrome registered at clinicaltrials.gov under NCT04899869.[24]

Written informed consent was provided by the patient's parents or guardians. The study was approved by the Ethics Committee of the Motol University Hospital on February 27, 2019 [EK-100/19]. The STROBE checklist was used for reporting the results of the study.[25]

2.2 Treatment with anti-TNFα antibodies

The anti-TNF therapy in CD was indicated either as a part of the step-up strategy or as a first-line therapy of the fistulizing perianal disease.[26] Indication was always based on clinical criteria, and laboratory and endoscopic findings, independently of the present observational study. As anti-TNF therapy, treatment with infliximab or adalimumab was performed according to current guidelines.[26,27] Infliximab was administered via an intravenous infusion of 5 mg/kg at weeks 0, 2, and 6, followed by maintenance intravenous infusions every 4–8 weeks; adalimumab was administered via subcutaneous injection at weeks 0, 2, and 4 in a scheme of 80–40–20 mg [up to 40 kg in weight] or 160–80–40 mg [above 40 kg in weight] followed by maintenance therapy with 25 mg/m^2 up to 40 mg every 2 weeks.

In JIA, only adalimumab was used as anti-TNF therapy. It was indicated after primary or secondary failure of disease-modifying antirheumatic drugs [usually methotrexate] according to European Alliance of Associations for Rheumatology [EULAR] recommendations.[28] Adalimumab was administered via subcutaneous injection every other week at a dose of 20 mg [up to 30 kg in weight] or 40 mg [above 30 kg in weight] without any induction scheme, as recommended by EULAR.[28]

2.3. Stool sample collection, faecal calprotectin analysis

Stool samples were collected using self-administered kits at the subject's home [or rarely during hospitalization], placed in a pre-frozen transport container and stored in a home freezer until transportation to the clinic. At the clinic, samples were stored at −20°C and transported to a deep freezer [−80°C] shortly afterwards. Faecal calprotectin levels were measured in an accredited laboratory [ISO 9001:2015; Supplementary Methods, section 1] and served to indirectly assess mucosal inflammatory activity within every sample.

2.4. Sequencing of 16S rDNA and bioinformatic analysis of the bacteriome

The stool samples were thawed on ice, and DNA was extracted from 50–100 mg via a DNeasy PowerSoil Kit according to the manufacturer's instructions on a QiaCube instrument [both Qiagen]. The quantity of bacterial DNA was then assessed using real-time PCR with primers and a hydrolysis probe targeting the V4 region of the 16S rRNA gene.

Bacteriome profiling was performed by sequencing the V4 region of the bacterial 16S rRNA gene according to Kozich *et al*.[29] Bioinformatic processing was based on the DADA2 pipeline [version 1.22] as advised by the software authors.[30] The procedures are described in detail in Supplementary Methods [sections 2.1 and 2.2].

2.5. Statistical analysis of the bacterial 16S rDNA profiles

2.5.1. Alpha diversity of the bacteriome

Alpha [within-sample] diversity was assessed from the unfiltered rarefied dataset using the observed species counts, and Chao1, Shannon and Simpson indices. Multi-level models were built to characterize the changes in alpha diversity during observation of the subjects, with a random intercept for the subject and time as one of the predictors. The other tested predictors were the diagnosis, the therapy with anti-TNF during the collection of samples, faecal calprotectin levels, and their meaningful interaction.

After assessment of alpha diversity, we eliminated amplicon sequence variants [ASVs] without assigned phyla, those with extremely rare phyla likely to be only passive travellers through the gut, and taxa outside the kingdom Bacteria. Then *phyloseq* objects were created by taxonomic agglomeration at several taxonomic levels: phylum, class, order, family, genus, and the fine-grained level of ASV. Finally, rare taxonomic units were eliminated, defined as those not present in at least two samples at more than 0.1% of reads.

2.5.2. Faecal calprotectin

Calprotectin concentrations were analysed using multi-level models similar to those employed in the alpha diversity

analyses [i.e. with a random intercept for the subject and time as one of the predictors—other predictors were the diagnosis and whether the patient had anti-TNF therapy]. The dependent variable was log-transformed calprotectin concentration.

2.5.3. Exploration of the community patterns

The bacteriome communities were first explored by Hellinger transformation-based principal component analysis [tb-PCA] ordination. Influential taxa in the ordination were found by inspection of the loadings and their formal testing using the *envfit* function. Associations between diagnosis, anti-TNF therapy, time since onset, alpha diversity measures and calprotectin levels were tested in principal component regression analysis models.

2.5.4. Changes in abundance of taxa on anti-TNF therapy

The effect of anti-TNF therapy was assessed using multi-level models constructed similarly as for the tests of alpha diversity. The models included random intercepts for individuals, while the fixed effects were the diagnosis, time since the start of observation, faecal calprotectin levels, whether the sample was taken while on anti-TNF, and meaningful two-way interactions of the terms. Taxa with a convincingly significant association with anti-TNF therapy [raw $p < 0.002$] or with a strong dependence on time were further inspected by plotting the abundances over time by diagnosis and anti-TNF therapy. At each taxonomic level, the p-values for the anti-TNF terms were corrected using Bonferroni correction for the count of meaningfully testable taxa, i.e. those taxa whose abundance exceeded 0.1% [10 reads per 10 000] in at least 3% of studied samples.

2.5.5. The bacteriome and response to anti-TNF therapy by mucosal healing

We analysed the associations of the gut bacteriome community with indicators of mucosal healing defined by using treatment effect data. The MINI index[31] was assessed in CD patients as a proxy for mucosal healing at 3 months since the start of the anti-TNF treatment [i.e. between the 12th and 14th weeks]. A score <8 was classified as mucosal healing after anti-TNF treatment, whereas a score of ≥8 indicated inflammatory activity in the mucosa.

Multi-level models with random intercept for subjects were then used to test whether the change in taxa abundance upon anti-TNF therapy differed by the response to this therapy and whether any appreciable differences were detectable already at baseline. For this analysis, baseline was defined as available stool samples collected up to 3 months before the first administration of anti-TNF.

Data from stool bacteriome profiling and metabolome profiling were combined to investigate possible associations [N-data integration] using multiblock sparse projection to latent structures – discriminant analysis, as implemented in the MixOmics R package 32.

2.6. Stool metabolome profiling

An aliquot of stool [200 mg] was processed [detailed in Supplementary Methods, section 3.1] to obtain a final supernatant used for nuclear magnetic resonance [NMR] analysis. ^{1}H NMR spectra were recorded on a 500-MHz Bruker Avance III spectrometer using a standard Bruker noesypr1d [90°-t1-90°-dmix-90°-FID] sequence as previously described by Jaimes *et al.*[32] Spectral intensities of the ^{1}H NMR spectra were pre-processed with an in-house built script under MATLAB R2020a [The MathWorks] consisting of multipoint baseline correction in user-defined segments, ensuring identical pre-processing for all the spectra (detailed in Supplementary Methods [section 3.2]). Spectra were reduced to predefined spectral bins, each bin representing a spin system or a part of a spin system that was ideally pure, distinct, and quantitative—in most cases, one bin for each metabolite. Ranges for the bins were chosen after the previous annotation of a subset of spectra in the software Chenomx version 8.6, using the built-in spectral library and our in-house database,[32] as shown in Supplementary Table—Metabolites [sheet RawData]. The bins represented 57 unique annotated compounds, which were corrected for dilution factor using probabilistic quotient normalization. Normalized data given are in Supplementary Table—Metabolites [sheet 'PQN-TSP normalised'].

Exploration of metabolome profiles used dimensionality reduction by principal component analysis [PCA] of the normalized and scaled metabolite levels. Multi-level models identical to those used above for bacteriome alpha diversity and taxon abundance were then used to test [1] the sample scores in the first four principal components [PCs], [2] individual metabolites, [3] the sum of SCFAs and their ratios to branched-chain fatty acids [BCFAs].

2.7. *In vitro* bacterial cultures with anti-TNF antibody

To test an unlikely direct effect of anti-TNF on the gut bacteria, we designed a simple *in vitro* disc diffusion assay. Isolates of bacteria commonly observed in the gut of healthy children were inoculated on Schaedler agar. Then, discs soaked with 20 µL of four different infliximab concentrations [increasing from 5 to 20 µg/mL] were applied on top of the inoculated agar and cultivated overnight under anaerobic conditions. These were used alone in one experiment and as a double-disc synergy test in another experiment—here, another disc was soaked with 20 µL of serum complement [concentration of 50 µg/mL] and placed near the infliximab disc to allow synergy between the antibody and complement action. Only Gram-negative bacteria were tested because complement activation is known to damage their cell walls, unlike Gram-positive bacteria, whose thick peptidoglycan layer makes them resistant to complement membrane attack complex.[33] The infliximab concentration was selected to mimic or exceed theoretical serum concentrations after infusion; the complement concentration reflected routine laboratory settings for purposes of the complement-fixation test. Plates were inspected and photographed after overnight anaerobic incubation for inhibition zones or growth enhancement.

3. Results

3.1. Subjects and their samples

A total of 121 subjects [CD 54, JIA 18, healthy controls 49] met the inclusion criteria, their parents signed the informed consent, and thus they entered the study. The basic characteristics of the studied subjects are shown in Table 1 and Supplementary Table 1. Of 54 analysed subjects with CD, 37 progressed to the need for anti-TNF therapy, whereas 17 remained on other therapeutic modalities. Among children with JIA, 14 received anti-TNF therapy during

Table 1. Characteristics of studied subjects and their samples.

	Crohn's disease [*n* = 54]			**Juvenile idiopathic arthritis [*n* = 18]**			**Healthy control [*n* = 49]**
Status	**Total**	**Received anti-TNF**	**Without anti-TNF**	**Total**	**Received anti-TNF**	**Without anti-TNF**	
No. of subjects	54	37	17	18	14	4	49
Age at diagnosis, median in years [IQR]	12.6 [9.2–15.0]	12.9 [9.1–15.0]	12.5 [10.2–14.9]	8.6 [3.8–10.7]	8.6 [3.8–10.8]	8.8 [6.8–10.0]	–
Female sex, *n* [%]	22 [41%]	15 [41%]	7 [41%]	11	9 [64%]	2 [50%]	20 [41%]
Caucasian ethnicity, *n* [%]	52 [96%]	35 [95%]	17 [100%]	18	14 [100%]	4 [100%]	49
Family history of IBD, *n* [%]	11 [20%]	9 [24%]	2 [12%]	–	–	–	–
No. of samples per patient, median [IQR]	6.0 [5.0–8.0]	6.0 [5.0–8.0]	6.0 [3.0–9.0]	6.0 [4.0–6.75]	6.0 [5.0–6.75]	4.0 [3.75–5.75]	1.0 [1.0–1.0]
Faecal calprotectin concentration [μg/g]; median [IQR] of patient means	1158 [219–1862]	1424 [655–1988]	219 [52–1807]	20 [10–55]	24 [10–56]	11 [6.4–16]	11 [7–21]
Treatment regimes[a]							
Immunomodulator monotherapy [azathioprine/methotrexate]	13 [24%]	–	13 [76%]	3 [17%]	–	3 [75%]	–
Anti-TNFα monotherapy[b]	19 [35%]	19 [51%]	–	4 [22%]	4 [29%]	–	–
Combined immunomodulatory + anti-TNFα	18 [33%]	18 [49%]	–	10 [56%]	10 [71%]	–	–
Other treatment[c]	4 [7%]	–	4 [24%]	1 [6%]	–	1 [25%]	

[a]Distribution of immunomodulatory agents: in CD 31/54 [57%] were treated with immunomodulatory agents [azathioprine 25, methotrexate 6]; in JIA 13/18 [72%] were immunomodulatory agents [all methotrexate].
[b]Distribution of anti-TNF agents in CD: infliximab 18/37 [49%]; adalimumab 19/37 [51%]; in JIA, all subjects received adalimumab as anti-TNF.
[c]In CD, three out of the four subjects provided samples only before the diagnosis and any treatment of CD; one subject was treated without immunomodulatory agents with supplementary enteral nutrition only; in JIA, this one patient was treated with non-steroid anti-inflammatory drugs only.
IQR = interquartile range.

the observation period, whereas four remained on other treatments.

Altogether, we collected and analysed 530 stool samples: 362 samples were from 54 patients with CD [203 samples taken while the patient was off anti-TNF, and 159 on anti-TNF], 101 samples were from 18 patients with JIA [63 samples before therapy and 38 on anti-TNF], and 67 samples were from 49 control subjects. Associations between the bacteriome or metabolome composition and the response of patients with CD to anti-TNF, assessed by the MINI index, were studied using subsets of samples taken up to 3 months before the first anti-TNF dose vs those taken up to 3 months after the initiation this therapy: there were 99 such stool samples from 23 CD patients who responded [49 before, 50 after the initiation of therapy], and 70 samples from 14 CD patients who did not respond [33 before, 37 after the initiation of therapy]. A flow chart of the study is shown in Figure 1; stool samples and the dynamics of their collection over time in each subject are shown in Supplementary Figure 2.

3.2. Mucosal inflammatory activity

In JIA, calprotectin levels were not different from controls [$p = 0.16$], and no association was noted for calprotectin with time or with anti-TNF therapy.

In patients with CD, faecal calprotectin levels were expectedly higher than in controls [~30-fold, $p < 10^{-16}$] as well as than in JIA [20-fold, $p < 10^{-12}$]. Those CD patients who eventually proceeded to anti-TNF therapy had 8.5-fold higher mean calprotectin levels over the whole study period as compared to those who remained on other therapies. When anti-TNF therapy was administered to CD patients, their calprotectin levels decreased by a mean of 67% as compared to the period off-therapy [$p = 1.2 \times 10^{-7}$; Supplementary Figure 3]. Analysis of calprotectin levels in stools from CD patients was then restricted to 3 months before commencing anti-TNF therapy and the following first 3 months of this therapy, with a multi-level model having a random intercept for subject and interaction terms for time and therapy: the results remained virtually unchanged, with the mean decrease in calprotectin with anti-TNF being 70% [$p = 0.0015$], and no appreciable independent effect of time.

In patients with CD receiving anti-TNF, 23 [62%] achieved mucosal healing as defined by MINI index <8, and 14 subjects [38%] did not.

3.3. Bacteriome profiles and anti-TNF therapy

3.3.1. Alpha diversity

The alpha [intra-sample] diversity of samples from CD patients was substantially lower than in those from control subjects [$p < 10^{-5}$ in Observed species, Chao1, and Shannon indices, and $p = 0.008$ for Simpson index in mixed-effect models with time, diagnosis, log-transformed calprotectin levels and anti-TNF therapy as fixed effects, and subject as a

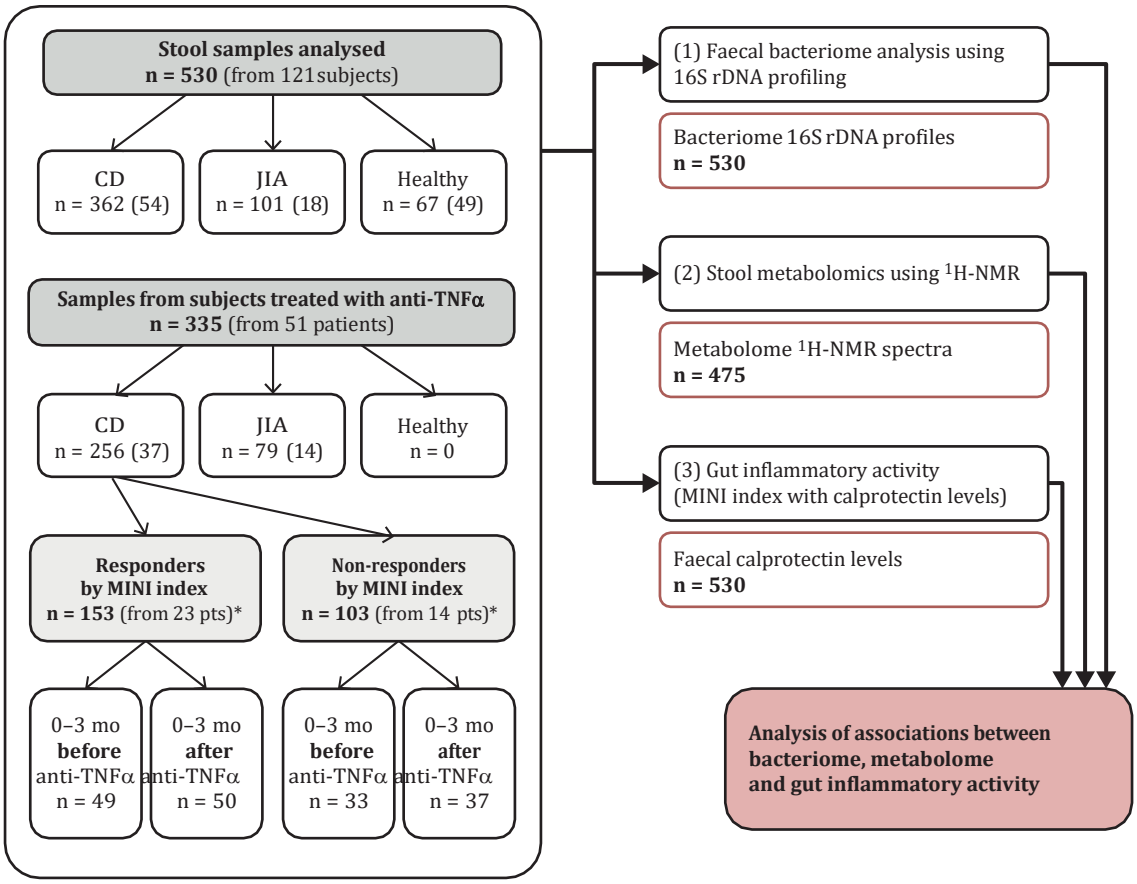


Figure 1. Flowchart of the study. *n* are counts of analysed samples [in parentheses are the numbers of subjects from whom the samples were obtained]. *Count also includes samples taken outside the 6-month window encompassing the start of anti-TNF.

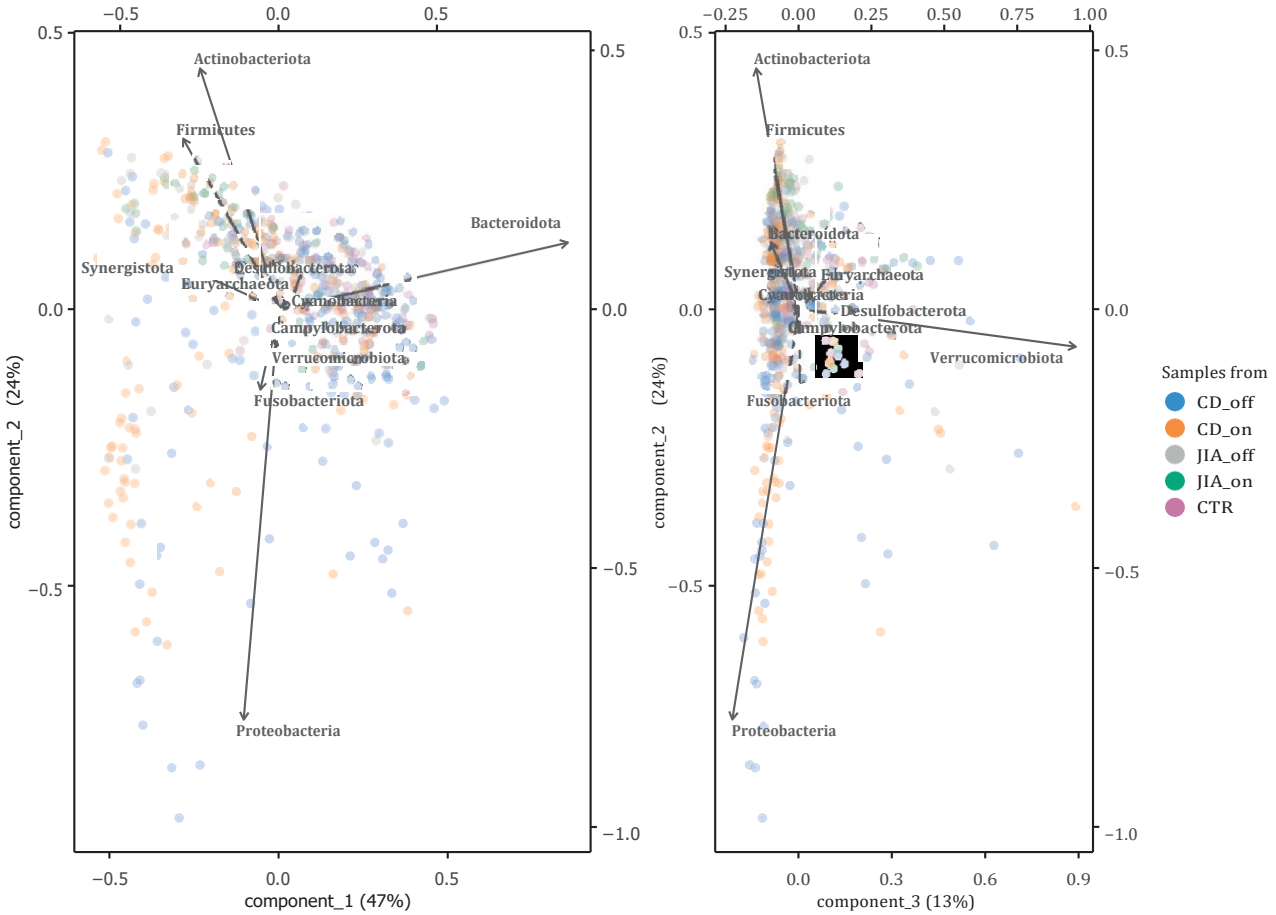


Figure 2. Bacteriome composition with the PCA showing the vectors for phyla. Samples from CD patients off therapy were driven mostly by the phylum Bacteroidota, including genera with colitis-inducing potential,[67] which are found more often in CD patients thanin healthy individuals,[40] and by the phylum Proteobacteria, driven mostly by genera associated with dysbiosis in CD patients.[37] The samples of CD on therapy were closer to the clusters of JIA patients, and healthy controls, driven mostly by the phylum Firmictues with genera known to be associated with better gut health.[37,40] The change in the bacteriome composition can be explained by both the gut health status and the bacteriome's alpha-diversity. Put simply, the more Bacteroidota [more negative on axis 1], the lower the alpha diversity, and similarly, the more Proteobacteria [the more negative on axis 2], the lower the diversity and the worse gut health.

random effect; alpha diversity index was the dependent variable]. Compared to JIA, samples from patients with CD had lower values of Shannon [$p = 0.001$] and Simpson indices [$p = 0.020$], but no significant difference was noted in Chao1 and Observed species indices [Supplementary Figure 4].

In patients with CD, all alpha diversity indices increased with time during the longitudinal observation [p-values between 0.003 and $<10^{-6}$ for the above indices]. The effect of time as a predictor is shown in Supplementary Figure 5. Some but not all indices showed suggestive negative associations with log-transformed calprotectin levels [$p = 0.042$ for the observed count of species, $p = 0.039$ for Chao1, $p = 0.094$ for Shannon, $p = 0.15$ for Simpson]. Ongoing anti-TNFα therapy was weakly independently associated with an increase in the Simpson index [$p = 0.024$] but not with the other tested indices. No significant association of the alpha diversity indices was noted with mucosal healing [assessed by MINI index 3 months after commencing anti-TNF] among CD patients on anti-TNF therapy.

No such differences or trends were observable in JIA: here, none of the alpha diversity indices changed with time [Supplementary Figure 5]; calprotectin levels were low, not appreciably different from control subjects, and were not associated with alpha diversity.

3.3.2. Bacteriome community exploration

In PCA ordination of the Hellinger-transformed abundance table at the phylum level, the first three dimensions were needed to explain >80% of the variance [47, 24, and 12% of the total variance; Figure 2]. PC1 captured the balance between Bacteroidota on one side and Firmicutes with Actinobacteriota on the other side. PC2 was driven by Proteobacteria and Fusobacteriota to negative values, counterbalanced by Actinobacteriota and Firmicutes. PC3 was driven by Verrucomicrobiota [for the abundance gradients of relevant phyla, see Supplementary Figure 6A and B]. Samples from control subjects and patients with JIA, as well as many samples from CD patients, were placed along the first PCs forming a cloud-like cluster. This largest variance component reflected the ratio between Bacteroidetes [right, with positive PC1 values] and Firmicutes with Actinobacteriota [left, negative PC1 values; Figure 2, left panel]. Numerous samples from CD patients and a few from JIA patients departed from the main cluster along the PC2 axis to negative values [i.e. towards an increasing abundance of Proteobacteria and/or Fusobacteriota]. Consequently, a significantly higher dispersion of the CD samples was noted compared to samples from control subjects or JIA patients [$p = 0.001$].

Table 2. Bacterial taxa whose quantity changed significantly with anti-TNF therapy in CD

Bacterial taxon	IBD	The change with anti-TNF therapy in patients with IBD		JIA	The change with anti-TNF therapy in patients with JIA		Notes
	Mean taxon abundance in IBD patients [% of total reads]	Mean abundance change in therapy [percentage points]	*p*-value: raw [corrected for number of tested taxa]	Mean taxon abundance in JIA patients [% of total reads]	Mean abundance change on therapy [percentage points]	Raw *p*-value [1]	
p. Bacteroidota └ c. Bacteroidia └ o. Bacteroidales	29%	−8.1	6.2×10^{-4} [0.0061]	22%	+4.1	0.34, NS	[2]
└ f. Rikenellaceae	2.6%	−1.9	2.3×10^{-5} [9.2×10^{-4}]	2.4%	+0.68	0.38, NS	
└ g. *Alistipes*	2.7%	−2.0	2.1×10^{-5} [0.0024]	2.4%	+0.71	0.37, NS	
p. Firmicutes	54%	+9.7	1.7×10^{-5} [1.7×10^{-4}]	64%	−4.8	0.19, NS	
└ c. Clostridia	43%	+11	2.0×10^{-6} [2.7×10^{-5}]	58%	−3.6	0.33, NS	
├ o. Peptostreptococcales	2.0%	+1.4	2.2×10^{-4} [0.0055]	1.8%	+0.29	0.53, NS	[3]
└ f. Peptostreptococcaceae	1.7%	+1.1	4.3×10^{-4} [0.017]	1.7%	+0.35	0.44, NS	
└ g. *Intestinibacter*	0.74%	+0.75	1.6×10^{-5} [0.0019]	0.56%	+0.13	0.47, NS	
├ o. Lachnospirales	26%	+9.4	1.5×10^{-7} [3.7×10^{-6}]	30%	−4.3	0.12, NS	
└ f. Lachnospiraceae	26%	+9.4	1.6×10^{-7} [6.4×10^{-6}]	31%	−4.1	0.14, NS	
└ g. *Ruminococcus*	2.3%	+2.7	1.4×10^{-4} [0.015]	0.2%	0.0	0.94, NS	[4]
└ ..... g. *Flavonifractor*	0.4%	+0.38	3.7×10^{-4} [0.042]	0.2%	−0.05	0.32, NS	[5]

[1] Neither of the tested taxa yielded a corrected *p*-value of the mean change after anti-TNF in JIA lower than 0.05. There were 13 taxa having a raw uncorrected *p*-value <0.05 in this test. Neither of them had a mean abundance >1% in either CD or JIA, and all these spurious *p*-values were caused by one or a few outliers, as confirmed by inspection of the abundance graphs.
[2] The three taxonomic categories completely overlapped in our human faecal dataset. There were no Bacteroidia other than Bacteroidales and no Bacteroidota other than Bacteroidia.
[3] The order is named Peptostreptococcales–Tissierellales in the SILVA database v. 138.
[4] Classified as *R. gnavus* group by SILVA database v. 138.
[5] Genus *Flavonifractor*, class Clostridia, order Oscillospirales, family Oscillospiraceae [SILVA database v. 138].
The regression coefficient corresponds to a mean increase or decrease associated with the anti-TNF therapy. Here it is expressed as a change in percentage points, as the bacterial abundance enters the model as a relative count of reads per 10 000.
At each taxonomic level, those taxa were tested whose abundance exceeded 0.1% [10 reads per 10 000] in at least 3% of studied samples. This resulted in a Bonferroni correction for 115 tests at the genus level, 40 tests at the family level, 26 at the order level, 13 at the class level, and 10 at the phylum level.
NS, not significant; p., phylum; c., class; o., order; f., family; g., genus.

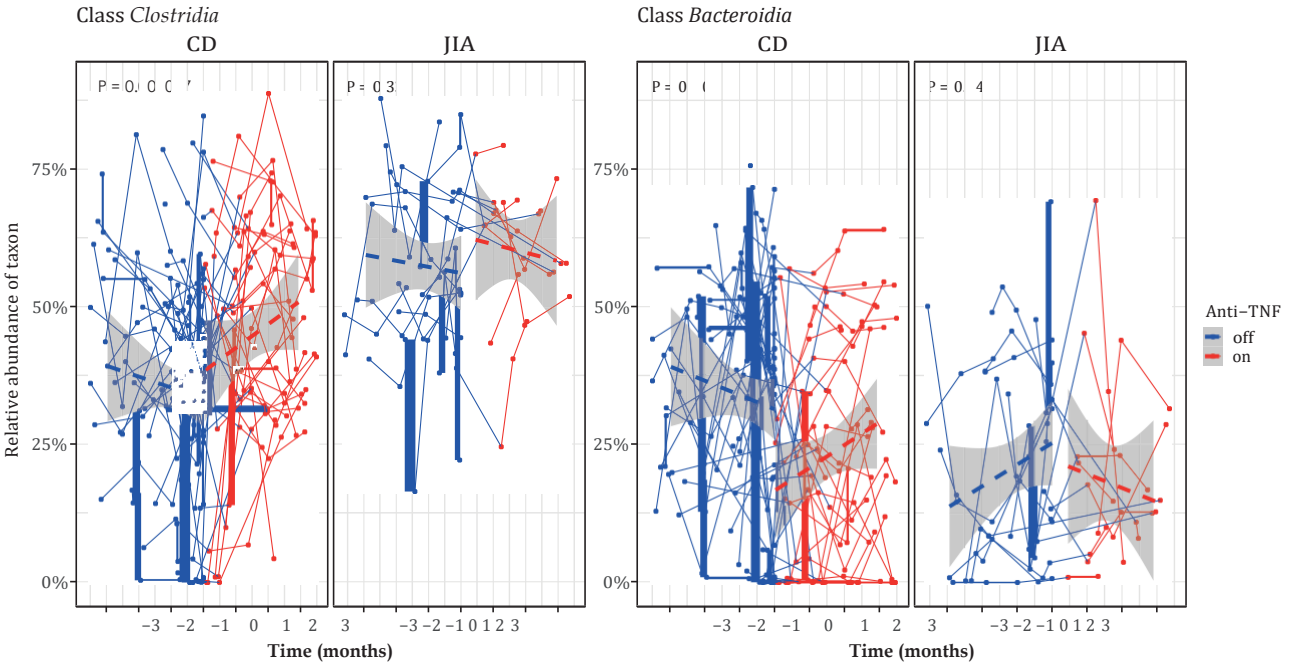


Figure 3. Relative abundance of classes Clostridia and Bacteroidia with time. Diagnosis: CD = Crohn's disease, JIA = juvenile idiopathic arthritis. Colours by therapy of anti-TNFα: red = stool sample taken while on anti-TNF; blue = stool sample taken when no anti-TNF was administered. The *p*-value is corrected for the number of tested taxa using multi-level modelling.

3.3.3. Testing associations of the bacteriome community composition using principal component regression

We explored putative associations of the PCA scores by multi-level models adjusted for known confounders. Community composition of the bacteriome was associated with alpha diversity: significant direct associations were noted for the first two PCs with all indices—i.e. the higher the score on the axis [towards Bacteroidota on PC1 and towards Firmicutes with Actinobacteriota on PC2], the higher the alpha diversity. Diversity scores were particularly low in samples with abundant Proteobacteria and Fusobacteria [Supplementary Figure 6C]. In addition, Simpson and Shannon indices were inversely associated [$p = 0.001$] with PC3 [i.e. these two indices decreased with increasing abundance of Verrucomicrobiota].

Faecal calprotectin in a stool sample was inversely associated with PC2 [$p = 1.3 \times 10^{-5}$] and PC3 [$p = 0.001$] of its bacteriome community composition, i.e. calprotectin was higher with increasing Proteobacteria [$p < 10^{-6}$] and/or Fusobacteriota [$p = 0.002$] abundnace, but marginally lower with increasing Verrucomicrobiota [$p = 0.042$] [Supplementary Figure 6D]. The association signal originated solely from subjects with CD. No such association was noted for PC1.

Anti-TNF therapy was associated with the faecal bacteriome community composition in patients with CD. There was an inverse association of anti-TNF therapy with PC1 [$p = 0.00017$] and PC2 [$p = 0.0012$, both in multi-level models adjusted for calprotectin]. Samples taken after anti-TNF therapy shifted from Bacteroidota towards an increased abundance of Firmicutes or Actinobacteriota. In JIA, neither of the PCs was associated with anti-TNF therapy.

Subjects with mucosal healing differed from those without in the reaction of the community composition to anti-TNF therapy. PC1 did not differ at baseline, but only samples from subjects without mucosal healing changed upon this treatment [towards negative values, $p = 3 \times 10^{-5}$]. PC2 differed already at baseline [patients with mucosal healing being higher, $p = 0.011$]; patients reacted to therapy with an increase in PC2 [$p = 0.0073$], regardless of the response by mucosal healing and with no appreciable interaction between the pace of increase and the response. In PC3, no baseline difference nor differential change was noted among categories of mucosal healing.

3.3.4. Abundance of individual bacterial taxa changes upon anti-TNFα therapy

We searched for taxa that were significantly changed in connection with the administration of anti-TNF therapy in CD but did not change upon TNFα blockade in JIA. No taxon changed significantly upon anti-TNF therapy in JIA [the smallest corrected $p = 0.38$]. In CD, significant changes upon anti-TNFα [corrected $p < 0.05$] were noted in two phyla, two classes, four families, and four genera [Table 2, Figure 3, Supplementary Table 2]. The phylum Bacteroidota, class Bacteroidia, order Bacteroidales decreased by 8.1 percentage points upon therapy [corrected $p = 0.0061$]. In Firmicutes, an increase was noted for the whole phylum, as well as in the class Clostridia and orders Peptostreptococcales and Lachnospirales. At the genus level, *Alistipes* was the only one which decreased upon anti-TNF in CD, whereas *Intestinibacter*, *Ruminococcus*, and *Flavonifractor* increased.

3.3.5. The bacteriome changes in responders and non-responders to anti-TNF therapy

Some of the above listed changes in bacterial taxa differed by whether the subject clinically responded to the therapy or not. Those patients responding with mucosal healing [defined by the composite MINI index being <8 at 3 months after commencing anti-TNF therapy] had a less pronounced increase in Firmicutes as compared to non-responders

[corrected $p < 0.05$; similarly to class Clostridia and order Lachnospiraceae], and a less pronounced decrease in the phylum Bacteroidota, its family Rikenellaceae and its genus *Alistipes* [corrected $p < 0.05$]. The pattern was rather complex. The differential response to anti-TNF therapy in Firmicutes is explainable by baseline differences: future responders had more Firmicutes at baseline [by 15 percentage points, $p = 5.3 \times 10^{-3}$], so the increase in responders was less pronounced [p of interaction 8.5×10^{-4}]. In contrast, Bacteroidota, its family Rikenellaceae and its genus *Alistipes* did not differ appreciably at baseline [$p = 0.72$], and only non-responders reacted to anti-TNF with a decrease [$p = 8.9 \times 10^{-4}$].

Having detected this effect modification, we performed an unplanned secondary exploration of whether the abundance of any taxon at baseline predicted the future responder status. For baseline we used taxon abundances in samples collected during the 3 months immediately preceding the start of anti-TNF. Several suggestive association signals emerged: at the phylum level [apart from the above reported higher abundance of Firmicutes], future responders had a lower abundance of Proteobacteria [by 14 percentahe points, uncorrected $p = 0.035$], propagating from the level of class Gammaproteobacteria and order Enterobacterales with similar difference and p values, as well as to the second principal component of the bacteriome ordination [Supplementary Table 3].

3.3.6. Disc diffusion testing potential direct effects of anti-TNF [infliximab] and/or serum complement on bacterial cells

We tested direct infliximab effects on six isolates of bacteria commonly observed in stool of healthy children: *Clostridium innocuum*, *Anaerococcus degeneri*, *Finegoldia magna*, *Prevotella nigrescens*, *Bacteroides fragilis*, and *Escherichia coli*. A disc-diffusion test with four different concentrations of infliximab assessed the potential effect of anti-TNF: *A. degeneri* had insufficient growth even after 48 h of incubation; *C. innocuum* and *F. magna* had a very discrete, <1-mm zone of irregular growth around some but not all discs with infliximab—this was deemed not to be consistent with susceptibility and concluded to be a mechanical effect or an effect of diluent diffusion; isolates of *P. nigrescens*, *B. fragilis*, and *E. coli* showed no visible zones around any of the infliximab discs. The Gram-negative bacteria *P. nigrescens*, *B. fragilis*, and *E. coli* were then subjected to a double-disc synergy test with complement, showing no visible inhibition zones around either infliximab or complement discs.

3.4. Metabolome profiling

Of 530 stool samples, 55 specimens mostly from control subjects had insufficient total quantity and were used up during processing for bacterial DNA extraction. The metabolome was thus analysed in 475 faecal specimens: 355 from patients with CD [194 before therapy and 161 after], 98 from patients with JIA [61 before therapy and 37 after], and 22 from control subjects. A total of 57 faecal metabolites were annotated, including primarily amino acids, SCFAs, BCFAs, other organic acids, carbohydrates, polyamines, choline metabolites, phenolic acids, nucleobase derivatives, and others.

3.4.1. Exploratory analysis of the metabolome

In PCA of the log-transformed scaled metabolome data, the first four PCs explained 42% of the total variance [16, 12, 7.4% and 7.0%], and the contributions declined thereafter. Of these axes, an increase in PC2 score was significantly associated with anti-TNF therapy in CD [$p = 1.6 \times 10^{-4}$ in a multilevel model adjusted for time, diagnosis, and calprotectin levels], i.e. towards higher concentrations of glucose, fucose, and glycerol, and lower concentrations of 4-aminobutyrate, glutamate, isoleucine, leucine, and valine [Supplementary Figure 7].

The above association was due to an increase in PC2 score in non-responders to anti-TNF among CD patients [interaction of the terms for anti-TNF treatment and being a responder $p = 0.010$], whereas no appreciable difference was present at baseline before anti-TNF treatment between CD patients who subsequently responded and those who did not.

In JIA, administration of anti-TNF was not associated with changes in any of the metabolome PC scores [uncorrected p values for the first four PC scores being 0.36, 0.56, 0.80, and 0.97].

Table 3. Faecal metabolites whose quantity changed significantly with anti-TNF therapy in CD.

	The change with anti-TNF therapy in patients with CD		Statistics for these metabolites in patients with JIA[b]	
	Metabolite abundance change on therapy[a]	*p*-value: raw [corrected for the number of tested metabolites]	Metabolite abundance change on therapy[a]	Raw *p*-value
Isoleucine	Decrease	1.6×10^{-4} [0.004]	Increase	0.84, NS
Uracil	Decrease	9.6×10^{-4} [0.002]	Increase	0.94, NS
Glucose	Increase	0.002 [0.03]	Decrease	0.38, NS
Glycerol	Increase	6.17×10^{-5} [0.003]	Decrease	0.94, NS

[a]Coefficients are not reported as they reflect the absolute change in units of the area under the peak, which is meaningless to interpret.
[b]In JIA, none of 57 tested metabolites showed a significant change with anti-TNF: the lowest raw p-value was 0.027, corresponding to a corrected p of 0.74.
NS, not significant.

3.4.2. Abundance of individual faecal metabolites upon anti-TNF therapy

The 57 annotated bins from the ^{1}H NMR spectra were tested by multi-level models similar to the bacteriome analysis. Four of the investigated metabolites changed significantly with anti-TNF therapy in CD [corrected $p < 0.05$, Table 3]: glucose and glycerol increased during anti-TNF therapy, whereas isoleucine and uracil decreased [Supplementary Figure 8].

There were no indications that individual metabolites at baseline could predict future response to anti-TNF in CD patients [lowest uncorrected p value 0.035].

Anti-TNF therapy in JIA was associated with no metabolite change [lowest uncorrected p value 0.027, corrected 0.996].

3.4.3. SCFAs upon anti-TNF therapy

Since SCFAs have been the subject of previous studies, we evaluated their estimated concentrations, the sum of their concentrations, and their ratios to BCFAs in a separate model: no appreciable association was noted for anti-TNF therapy [$p = 0.44$], calprotectin level [$p = 0.55$], or time [$p = 0.12$], and there was no difference between JIA and CD [$p = 0.19$]. Similarly, there was no appreciable association of anti-TNF therapy and the SCFA/BCFA ratio [data not shown]. No association with anti-TNF was found for individual SCFAs, either [in CD, all $p_{corr} = 1.0$].

3.5. Combined analysis of bacteriome and metabolome profiles

The datasets containing the bacteriomes [at the level of genus] and metabolomes were correlated, yielding correlation coefficients of 0.85, 0.77, and 0.68 for variates of the first components in partial least squares regression. The co-inertia plot is shown in Supplementary Figure 9. The permutation co-inertia test for the two tables was highly significant [$p < 10^{-4}$ by coinertia.randest of the package *ade4* in R].

In an analysis we then explored whether N-data multiomic integration [bacteriomes + metabolomes of the same samples] might perform better than bacteriomes only in simplified machine learning models [random forest and sparse projection to latent structures – discriminant analysis]. No improvement in accuracy was noted [data not shown].

4. Discussion

Our study describes changes in the faecal bacteriome and metabolome upon anti-TNF therapy in CD. It identified several bacterial taxa and metabolites that changed with anti-TNF, as well as taxa associated with the response to this therapy. Importantly, no such effects of anti-TNF on the faecal bacteriome or metabolome were observed in patients with JIA, who all were gut-healthy. Thus, this difference among two diagnoses treated with anti-TNF indicates that the observed changes in the bacteriome and metabolome reflect a reduction in mucosal inflammation and are not a general property of any anti-TNF administration [e.g. through a modification of the immune response to colonic bacteria]. This notion may be further supported by the absence of direct effects of infliximab in our *in vitro* growth experiments on anaerobic bacterial cultures.

4.1. Changes in the faecal bacteriome upon anti-TNF administration

Gut dysbiosis, in which the composition of the faecal bacteriome is very different from that considered normal, is a common feature of an anti-TNF-naive bacteriome in CD.[34,35] Its main signs are an overall decrease in diversity and a different composition compared to healthy subjects: a decrease in obligate anaerobes or bifidobacteria and an increase of facultative anaerobes [e.g. Enterobacteriaceae from Proteobacteria] or some Gram-negative anaerobes such as *Bacteroides*.[34,35] Consistent with multiple studies on the bacteriome in IBD of heterogenous design and methodology,[3–13] we also observed significant differences between CD and gut-healthy comparison groups [JIA and healthy controls] in both diversity and composition of the bacteriome. The alpha diversity in CD was remarkably reduced, gradually improving with time since entering the study. Interestingly, time remained its only strong predictor, with a weak contribution from calprotectin and none from anti-TNF. This suggests that the alpha diversity increase in time could be due to any type of therapy of CD, and not limited to anti-TNF. It is of note that a similar non-superiority of anti-TNF on alpha diversity course has been reported in relatively small longitudinal studies of Chinese children with IBD using stool[16] or biopsy samples.[36] The composition of the bacteriomes among children with CD was notably more diverse, with that od some samples being very different to compositions observed in children with JIA or in healthy controls. In particular, the samples of anti-TNF-naive CD patients were enriched in taxa among the phyla Proteobacteria and Fusobacteriota, a well-known sign of gut dysbiosis in CD, and which has been reported in multiple cross-sectional studies.[6,7,11,12,34,37] Altogether, these results confirm the dysbiotic patterns in CD even from the perspective of our longitudinal stool sampling.

The effect of anti-TNF on the human microbiota has been studied previously, primarily due to the infectious complications caused by these agents, as inhibiting the TNFα pathway increases the risk of several infectious diseases [tuberculosis, listeriosis, aspergillosis, coccidioidomycosis, histoplasmosis, or salmonellosis].[38] However, only a few studies have focused on changes following anti-TNF therapy in IBD. They reported a shift in towards a healthier composition , namely a decrease in Proteobacteria and/or increase of SCFA producers.[15,16,39] In our study, the bacteriome community composition upon anti-TNF in CD shifted from Bacteroidota towards Firmicutes. Such a shift could be considered a change towards a healthier gut bacteriome, as Bacteroidota are reported to be enriched among IBD patients in multiple studies,[7,8,12,40] whereas genera of the Firmicutes are associated with better gut health.[6,9–12,37,40] Thus, regarding the bacteriome composition, our results from a longitudinally collected sample series are consistent with previous studies on the paediatric population.[15,16,39]

Testing of individual taxa for the change upon anti-TNF administration revealed no shifts in JIA, but identified several taxa changing in CD: *Alistipes* [class Bacteroidia] decreased in abundance after therapy with anti-TNF, whereas *Intestinibacter*, *Flavonifractor*, and *Ruminococcus* [all class Clostridia] increased. Notable were *Ruminococcus*, from the family Lachnospiraceae, and *Flavonifractor*, from the family Oscillospiraceae. Both bacteria are known producers of SCFAs, critical nutrients for the gut epithelium.[41,42] *Ruminococcus* has been reported to be more abundant in a healthy population when compared to IBD,[7,9] and its

increase has been associated with a response to anti-TNF therapy in CD.[15,16] On the other hand, some species of *Ruminococcus* can produce pro-inflammatory molecules *in vitro*[43] and are associated with IBD[44]; and SCFA producers from the family Lachnospiracecae were found to decrease in responders to anti-TNF, which even the au- thors commented as being unexpected.[17] *Flavonifractor* is a phylogenetically diverse genus formerly classified as *Eubacterium*.[45] Its primary representative *F. plautii* [also known as *E. plautii*] is, with the other eubacteria, a well-recognized SCFA producer found in the healthy gut.[46] Of note, its close relative, *E. rectale*, was reported to be significantly increased at baseline in children responding to anti-TNF in CD.[39] A connection with CD has already been reported for the genus *Intestinibacter*[47] from the family Peptostreptococcaceae; however, disease activity [remission vs active disease] and gut inflammation status was not considered. More interestingly, an increase in an unknown genus from the Peptostreptococcaceae was reported to be a sign of anti-TNF response in children with IBD.[17] The role of *Alistipes*, belonging to the order Bacteroidales, remains unclear.[48] However, its connection with worse gut health seems probable based on recent studies,[49,50] and thus its decrease after therapy may be meaningful.

4.2. Response to anti-TNF therapy and gut bacteria

The reaction of the bacteriome differed between responders based on the MINI index and non-responders. The pattern was complex, also involving the baseline abundance of the microbe. Bacteroidota started at similar baseline levels in responders and non-responders, and only non-responders subsequently experienced an increase, possibly indicating deterioration of their bacteriome dysbiosis. In contrast, it appeared that Firmicutes in responders deviated less at baseline, and thus their increase was less pronounced. Differences at baseline among responders and non-responders were also noted in Proteobacteria, another candidate for dysbiosis. The above taxa are valid candidates for being causatively involved: Clostridia of Firmicutes are known SCFA producers, whereas Enterobacterales are known pro-inflammatory bacteria.[6]

Numerous previous studies have presented a heterogeneous spectrum of candidates for microbial predictors of response to biological therapies—both in children and in adults with IBD, on infliximab,[16,17,19,51] vedolizumab,[10] or a combination of agents.[52] The existence of such predictors questions the secondary origin of such changes. Given that there is a microbial predictor and its abundance increase is due to a reduction in mucosal inflammation, a third factor should exist [a mediator, confounder, or effect modifier]. Such a factor could be, for example, a CD susceptibility gene such as the interleukin 23 receptor [*IL23R*] previously found to be associated with a decreased diversity and abundance of beneficial taxa [e.g. *Faecalibacterium*].[53] However, studies of this relationship are currently lacking.

Although prokaryotes possess no receptors that could be blocked by anti-TNF therapy, and thus a direct effect of anti-TNF on bacterial cells is unlikely, it cannot be ruled out that the monoclonal antibody binds non-specifically to bac- terial structures. Therefore, we designed a series of simple experiments by exposing commonly occurring gut anaerobes to anti-TNF [infliximab] alone or together with serum complement in Gram-negative bacteria. Neither the disc diffusion test with discs soaked with infliximab on culture plates nor the double disc synergy test with infliximab and serum complement showed appreciable growth inhibition or growth enhancement. Given that commensal growth is not affected *in vitro*, that the purified therapeutic antibody is unlikely to cross-react with bacterial structures, and that there is no TNF-like pathway in prokaryotes, we concluded that a direct effect is most unlikely.

Overall, in the context of current the literature, we suggest that the changes in bacteriome indicate a meaningful connection between the associated taxa and healthier gut bacteriome composition and, thus, a decrease in mucosal inflammatory activity. Furthermore, the lack of such observations in gut-healthy JIA and the resistance of bacterial cultures to infliximab together suggest that bacteriome changes after anti-TNF are probably mediated by improved gut health in CD.

4.3. Changes in the faecal metabolome

Four faecal metabolites changed in concentration significantly with anti-TNF in CD patients, but no appreciable changes were noted in JIA [Table 3]. A decrease in isoleucine [and other amino acids prior to correcting for multiple comparisons] was observed. Excessive amounts of bacterial proteolytic enzymes were previously reported in faeces and biopsy samples from patients with active IBD.[54] Amino acids are also known to be enriched in stool samples of CD [or ulcerative colitis] patients during a disease flare compared to remission.[55,56] Moreover, a decrease in multiple amino acids [but not isoleucine] after therapy was described in children with CD.[16] Thus, the decrease in isoleucine seen in our study might be connected to mucosal healing or the establishment of bacterial homeostasis. Uracil, another metabolite showing a decrease, was previously shown to be actively secreted by *Escherichia coli* and *Pseudomonas aeruginosa*, playing a role in quorum sensing, biofilm formation, and pathogenicity.[54,57] Purine and pyrimidine pathways were recently reported to be down-regulated with diet-induced remission.[58]

The observed increase in glucose [Table 3] may relate to an increased capacity of microbiota to ferment dietary non-digestible fermentable carbohydrates and, thus, a shift from proteolytic towards saccharolytic fermentation. Glycerol can be derived from dietary fats and is commonly used as a food additive. It escapes absorption in the small intestinal, undergoes bacterial catabolism in the colon, and its rate varies in different microbial communities. Shifts in *Lactobacillus*–*Enterococcus* communities were associated with changes in glycerol, and their abundance was positively associated with SCFA production and negatively with BCFAs in batch fermentation studies.[59,60]

In general, all the changes among significant metabolites support the overall picture of the shift from proteolytic towards saccharolytic fermentation,[61,62] which is considered a healthier profile.[63] Interestingly, SCFAs are generally considered anti-inflammatory and are enriched in healthy people compared to patients with IBD,[55,56] but we have not seen their association with anti-TNF. A recent study came to a similar conclusion that SCFAs are not the main pathways associated with remission, in this case after intervention with exclusive enteral nutrition.[58]

4.4. Strengths of the study

To the best of our knowledge, our study design may be unique in the parallel observation of patients with JIA on anti-TNF therapy—this enabled us to filter out the potential direct general effect of anti-TNF on any gut microbiome, including that of gut-healthy individuals. Moreover, the absence of mucosal inflammation in JIA was verified by faecal calprotectin testing, although this was a priori very unlikely in the absence of gastrointestinal symptoms.[64,65] In accordance with recent trends, we used longitudinal observation to decrease the effects of short-term fluctuations in the abundance of individual microbial taxa.[66] Our multi-centre patient recruitment might increase the generalizability of our results. Furthermore, we meticulously checked the sample integrity throughout the cold chain: the temperature in the study subject's home freezers was checked, and shipping containers were frozen phase change cold packs [with holes for collection tubes] in a styropore box. Lastly, we obtained parallel calprotectin levels from all faecal samples, enabling us to adjust models for mucosal inflammation activity.

4.5. Limitations and their remedies

The foremost limitation is the limited number of JIA patients recruited into the study. In addition, the diagnosis itself is infrequent, and the children lacked the willingness to collect stool samples. The data, however, did not indicate any borderline effects of anti-TNF on either the JIA faecal metabolome or bacteriome: the difference in effect in contrast to CD is clear. To show that JIA patients do not differ in the overall faecal composition to control subjects, we used an older control dataset [age limit of 21 years, and we included first-year medical students in addition to paediatric controls]; as the goal was to document the absence of departure of JIA from the main cluster of control bacteriomes, this age difference is not likely to be important [Figure 2]. Additionally, entry to the study was not identical as two entry points were possible [Supplementary Figure 1].

4.6. Conclusions

We have described a shift in the faecal bacteriome and metabolome towards healthier compositions after anti-TNF therapy in CD, supported by a comprehensive longitudinal collection of stool samples. Most importantly, in a model adjusted for faecal calprotectin levels, we identified four bacterial taxa [notably SCFA producers *Flavonifractor* and *Ruminococcus*] and four faecal metabolites [especially isoleucine], whose abundance changed significantly after anti-TNF therapy only in CD and not in gut-healthy JIA. We observed no appreciable *in vitro* effect of infliximab on the growth of representative anaerobic bacteria, which, together with the above, implies that changes in the faecal bacteriome and metabolome are of secondary origin and that they probably reflect a decrease in mucosal inflammation rather than a general effect of anti-TNF. Consequently, the identified bacterial taxa and faecal metabolites could be seen as signs of a restored gut ecosystem, which might help to understand the nature of gut microbiome changes in during CD therapy.

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Conflict of Interest

OH received payment for lectures from MSD, Abbvie, Nestlé, Nutricia, Sandoz, and Takeda; neither of the lectures was related to the subject of the study. Other authors declare no conflicts of interest.

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Author Contributions

JHu performed the laboratory analysis, led the study, and wrote the manuscript. AMB performed the metabolome analysis and helped to interpret the metabolome data. LV contributed to the laboratory analysis. TL recruited the subjects and supervised the clinical metadata collection. AC performed the faecal calprotectin measurement. MK, HM, DC, JS, EK, TH, LT, KG, and VD recruited the subjects. RH, OH, and JB supervised the study at clinical departments and helped with subject recruitment. JHa contributed to the analysis of the metabolome data, supervised the metabolome analysis, and contributed to interpreting the metabolome data. OH contributed to the study design, and analysing and interpreting the bacteriome data. OC led the study as the main supervisor, designed the study, analysed the data, computed the statistics, created the models, and revised the manuscript as the senior author. All authors contributed to revising the manuscript.

Disclosure

No external assistance was used when writing the manuscript. The manuscript with its related data tables and figures has not been published elsewhere and is not under consideration elsewhere.

Data Availability

The data are available from authors upon reasonable request. Sequencing data along with essential metadata have been deposited in the Sequence Read Archive [SRA] at the National

Center for Biotechnology Information [NCBI] database under accession number PRJNA958468.

Supplementary Data

Supplementary data are available online at *ECCO-JCC* online.

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Parasites & Vectors

RESEARCH Open Access

Effects of *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* supplementation on the single-cell fecal parasitome in children with celiac disease autoimmunity: a randomized, double-blind placebo-controlled clinical trial

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Abstract

Background *Lactiplantibacillus plantarum* HEAL9 and Lacticaseibacillus *paracasei* 8700:2 positively affect the fecal bacteriome in children with celiac disease autoimmunity after 6 months of supplementation. The aim of the present investigation was to study the effects of *Lactiplantibacillus plantarum* HEAL9 and *Lacticaseibacillus paracasei* 8700:2 on the single-cell parasitome, with a primary focus on *Blastocystis*.

Methods Stool samples were collected from 78 Swedish children with celiac disease autoimmunity participating in a randomized, double-blind, placebo-controlled clinical trial to either receive a mixture of supplementation with *L. plantarum* HEAL9 and *L. paracasei* 8700:2 ($n=38$) or placebo ($n=40$). A total of 227 stool samples collected at baseline and after 3 and 6 months of intervention, respectively, were retrospectively analyzed for *Blastocystis* by quantitative real-time PCR and subtyped by massively parallel amplicon sequencing. Other single-cell parasites were detected by untargeted 18S rDNA amplicon sequencing and verified by real-time PCR. The relation between the parasites and the bacteriome community was characterized by using 16S rDNA profiling of the V3-V4 region.

Results Three different single-cell protists were identified, of which the highest prevalence was found for *Dientamoeba fragilis* (23.1%, 18/78 children), followed by *Blastocystis* (15.4%, 12/78) and *Entamoeba* spp. (2.6%, 2/78). The quantity of the protists was stable over time and not affected by probiotic intervention ($P=0.14$ for *Blastocystis*, $P = 0.10$ for *D. fragilis*). The positivity of the protists was associated with increased bacteriome diversity (measured by multiple indices, $P<0.03$). Bacterial composition was influenced by the presence of the protists: positivity of *Blastocystis* was inversely associated with *Akkermansia* (at the levels of the genus as well as its family, order, class and phylum); $P<0.002$), *Faecalibacterium* ($P=0.003$) and *Romboutsia* ($P=0.029$); positivity of *D. fragilis* was inversely associated with families Enterobacteriaceae ($P=0.016$) and Coriobacteriaceae ($P=0.022$) and genera *Flavonifractor* ($P<0.001$), *Faecalibacterium* ($P=0.009$), *Lachnoclostridium* ($P=0.029$), *Ruminococcus* ($P<0.001$) and *Granulicatella* ($P=0.018$).

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Conclusions The prevalence of single-cell protists is low in children with celiac disease autoimmunity. The colonization was stable regardless of the probiotic intervention and associated with increased diversity of the fecal bacteriome but inversely associated with some beneficial bacteria.

Keywords *Blastocystis*, *Dientamoeba fragilis*, Celiac disease, Probiotics, Gut microbiome

Background

The main protist of the human gut is *Blastocystis* sp. (hereafter referred to by its genus name *Blastocystis*). It is an anaerobic, non-flagellated single-cell and highly polymorphic organism with multiple forms. *Blastocystis* is proposed to be a marker of a balanced microbiome [1–3] and is believed to be the most common single-cell eukaryote in the gut of both adults [4] and children [5]. Being mainly transferred by the fecal-oral route, *Blastocystis* has a higher prevalence in developing countries with lower hygiene standards, usually between 40 and 84% [6–8], compared with high-income countries in Europe and the USA, where it commonly ranges between 7 and 56% [1, 9–15]. Among the genus *Blastocystis*, 26 genetic subtypes (ST) were identified [16] and proposed to be differing in biological properties. Of these subtypes, STs 1–17 are widely recognized as valid, whereas others may include molecular detection artifacts [17]. Of the ten subtypes in humans, ST1 to ST4 are the most prevalent [5, 18], from which ST4 is unique for Europe and ST3 is the most prevalent worldwide [10]. Another frequent protist is *Dientamoeba fragilis*, a flagellated trichomonad, surrounded by controversy about whether it is more a pathogen or a benign gut inhabitant [19–21]. Its prevalence varies between 0.4 and 71%, depending on the studied cohort and methods used [21–24]. Interestingly, unlike *Blastocystis*, its prevalence is lower in low-income countries, e.g. [25, 26], and higher in high-income countries, e.g. [22, 27].

Celiac disease is a chronic enteropathy due to an immune-mediated response to dietary gluten from wheat, rye and barley, arising in a small proportion of individuals who are genetically susceptible because carrying human leukocyte antigens (HLA) haplotypes DQ2 and/or DQ8 [28]. Although genetics and gluten are necessary for celiac disease development, it is most likely triggered by environmental factors such as gastrointestinal infectious episodes triggered by different microorganisms [29], 29. On the other hand, the prevalence of celiac disease has been reported to be significantly lower in populations of Russian Karelia with lower hygienic and socioeconomic standards with high exposures to microbes compared to populations living in Finland, despite the populations from the two geographically neighboring regions sharing the same genetic risk [31].

In contrast to the above study, recent prospective birth cohort studies on gastrointestinal infections have shown that patients who develop celiac disease have more frequent enterovirus [32] and parechovirus [33] infections prior to seroconversion of tissue transglutaminase autoantibodies (tTGA), a marker of celiac disease autoimmunity (CDA) [32].

The present study builds on the probiotic intervention trial in children with CDA [34], showing a moderate effect of probiotics on the gut bacteriome [35]. This study focuses on the fecal parasitome. The aim was to assess the effect of the intervention with two lactobacilli, probiotic strains *Lactiplantibacillus plantarum* HEAL9 and *Lacticaseibacillus paracasei* 8700:2, on the single-cell parasitome with specific emphasis on *Blastocystis* in a stool sample set of Swedish children with CDA. Our hypothesis was that the presumably beneficial probiotic bacteria might change the bacteriome composition, leading to an increased prevalence of *Blastocystis*.

Materials and methods

Study population

The Celiac Disease Prevention with Probiotics (CiPP) study, a double-blind placebo-controlled randomized clinical trial, was performed at the Department of Clinical Sciences, Unit of Celiac Disease and Diabetes, Lund University, Malmö, Sweden [34]. The trial was carried out between March 12, 2012, and August 25, 2015. A total of 118 children with CDA identified by screening were invited to participate in the CiPP study, of whom 89 accepted participation and 78 completed a 6-month follow-up of daily receiving either a mixture of *L. plantarum* HEAL9 and *L. paracasei 8700:2* (probiotic group, $n = 40$) or 1 g maltodextrin and yeast peptone (placebo group, $n = 38$) (the probiotic mixture is described in more detail in [34, 35]). Study participants were instructed to continue a gluten-containing diet and exclude other food products containing probiotics. During the intervention, six children in the probiotic group and four in the placebo group reported taking antibiotics ($P = 0.561$). Stool samples were collected at baseline and then 3 and 6 months after the intervention. Processing of the stool samples for DNA extraction was described earlier [35]. Briefly, 50 mg of thawed stool sample was used for the DNA extraction using the DNA tissue kit (Qiagen, Germany)

and run on the EZ1 DNA extraction robot (Qiagen, Germany). The study was approved by the Ethics Committee of the Medical Faculty, Lund University (Dnr 2011/335; Dnr 2021–04470) and registered at ClinicalTrials.gov (NCT03176095); 227 samples collected from the 78 children with CDA participating in the (CiPP) study were available for this retrospective analysis (Fig. 1).

Molecular methods

Blastocystis was tested and quantified by specific real-time PCR as the most common gut protist [18]. All positive samples for *Blastocystis* were then classified into genetic subtypes using massively parallel amplicon sequencing of a unique region of the 18S rDNA gene. The total parasitome was profiled by multiplex massively parallel amplicon sequencing of several amplicons of the 18S rDNA gene. Positive parasites from the 18S rDNA profiling (other than *Blastocystis*) were then confirmed and quantified by parasite-specific real-time PCR. After determining the single-cell parasitome content, the parasites were related to the intervention with studied probiotics and analyzed in the context of previously obtained bacteriome profiles [35].

Detection and quantification of blastocystis by specific real-time quantitative PCR

A real-time quantitative PCR assay with a specific probe for *Blastocystis* designed by Stensvold et al. [36] was used to test and quantify *Blastocystis*. For the calibration curve, DNA from a microscopically quantified xenic culture was used [37]. Platinum Taq Polymerase (Invitrogen, USA) from the Stensvold's original protocol was substituted by the HotStar Taq polymerase chemistry (Qiagen, Germany), and the PCR program was corrected accordingly with 15 min of initial denaturation at 95 °C followed by 45 cycles of 15 s denaturation at 94 °C and 60 s of combined annealing and synthesis at 60 °C [5]. Reactions were performed in duplicates throughout the study, and negative controls were included in the extraction as well as in the detection reactions. PCR tubes containing real-time PCR products were always discarded unopened. The detection PCR fragments do not overlap with those used for *Blastocystis* subtyping.

Subtyping of blastocystis by massively parallel amplicon sequencing

Samples positive for *Blastocystis* in the above-mentioned real-time PCR assay were thereafter subtyped by massively parallel amplicon sequencing using the protocol by Maloney et al. [38] with primers developed by Santin et al. [39] extended by Nextera tails. The amplicons were then purified and indexed by Nextera XT Index Kit v2 Set A and D (Illumina, USA) by a short eight-cycle PCR, enabling multiplex sequencing. Indexed products were purified and equalized using a SequalPrep Normalization plate (Invitrogen, USA) and pooled. The pool of libraries was sequenced on a MiSeq instrument using Reagent Kit v2, 2 × 250 bp (Illumina, USA), with the addition of 20% PhiX control to balance the amplicon signal.

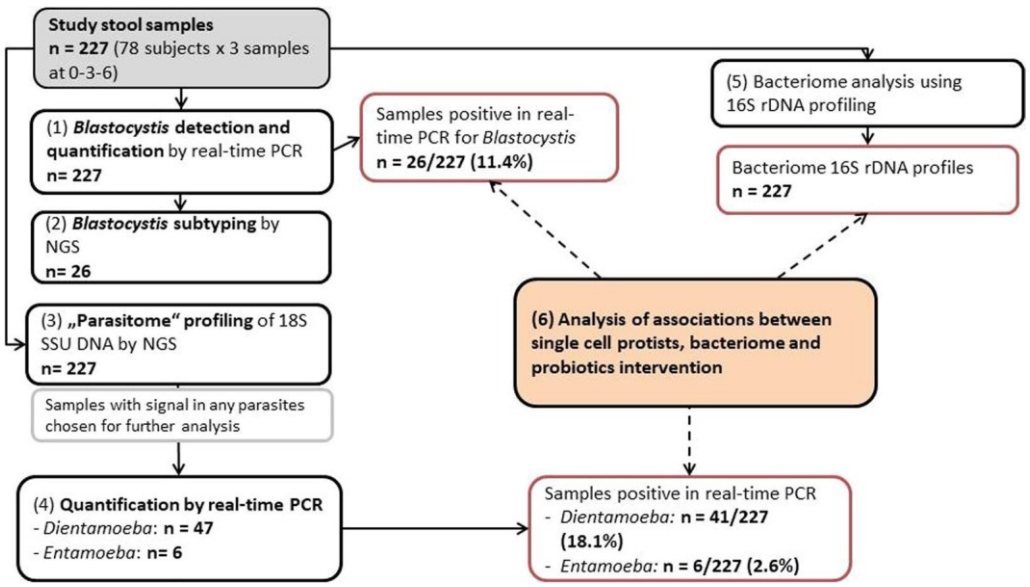

Fig. 1 Workflow of the study. *NGS* next-generation sequencing, *SSU* small subunit of the ribosome

Parasitome survey using massively parallel amplicon profiling of the 18S rDNA gene

The parasitome survey was performed using massively parallel sequencing with five different PCR amplicons of the 18S rDNA gene, as reported in detail previously [5]. The procedure identified 3546 unique sequence variants and zero-radius operational taxonomic units (ZOTUs); those with a relative frequency > 0.1% (n = 103) were manually classified by BLAST search to GenBank. Signals of the single-cell protists were expressed as the number of reads aligned to a particular organism divided by the count of rarefied reads per reaction.

Quantification of D. fragilis and Entamoeba spp. by quantitative real-time PCR

The single-cell protists with a signal in the parasitome survey (*D. fragilis* and *Entamoeba* spp. apart from already quantified *Blastocystis*) were then quantified by a specific probe-based real-time quantitative PCR assay. For *D. fragilis*, the primer and probe sequences came from the work of Verweij et al. [40]. A dilution series of DNA from a xenic culture of *D. fragilis* was used as a control in qPCR reactions, but its exact protist genomic equivalent content could not be established because of problems with the microscopic quantification of the culture. The PCR conditions for *D. fragilis* detection were published in [41]. As to *Entamoeba* spp. (*E. histolytica, dispar, moshkovskii, hartmanni, coli*), the primer and probe sequences were designed in the Geneious Prime software (version 2020) based on the 18S SSU rRNA gene sequence references (X64142, AB197936, Z49256, AF149906, AF149907, AF149915) retrieved from the GenBank database (see Additional file 2: Table S1 and Figure S1). The PCR program for *Entamoeba spp.* detection consisted of 15 min of initial denaturation at 95 °C followed by 35 cycles of 15 s denaturation at 94 °C and 60 s of combined annealing and synthesis at 60 °C. For a calibration curve, DNA from an axenic *Entamoeba* culture was used, and cell counts from the culture were calculated using a Bürker chamber and then serially diluted to obtain aliquots containing 1, 10, 10^2, 10^3, 10^4 and 10^5 cells per microliter, which were subsequently subjected to DNA extraction according to Lhotská et al. [15]. Negative controls were included in every PCR run, and the tubes with PCR products were discarded unopened.

Bacteriome analysis

The processing, DNA library preparation and sequencing had been done previously by massively parallel amplicon sequencing of the V3-V4 region of the bacterial 16S rRNA gene [35]. For this study, the previously generated *fastq* files with sequencing reads were downloaded and reprocessed using the DADA2 pipeline (version 1.22) [42], with taxonomic classification using the SILVA database (version 138) [43] instead of the now slightly outdated Greengenes database version 13.5 and the Qiime2 suite. Amplicon sequence variants (ASVs, analogous to operational taxonomic units, OTUs) were then further analyzed using vegan [44] and phyloseq [45] in the R programming language [46].

Statistical analysis

The prevalence of single-cell protists was determined by counting any PCR positivity at any level. Their presence and quantity were then modeled using generalized estimating equations (GEE) with the subject as the grouping variable, time point (factor with three levels—0, 3 and 6 months) and a first-order autoregressive covariance structure; the predictors were the study allocation (placebo or probiotics) and whether the sample was taken while on intervention with the live mixture.

Bacteriome alpha (within-sample) diversity was assessed from the unfiltered rarefied dataset, agglomerated at the genus level, using the observed counts, Chao1, ACE, Shannon, Simpson, inverse Simpson and Fisher indices. The association of alpha diversity indices with the presence or quantity of *Blastocystis* or *D. fragilis* was assessed in GEE models with the index as the dependent variable where predictors were the positivity or quantity of the parasites, and the study allocation (placebo or probiotics).

For the beta diversity (between samples) analysis, Bray-Curtis distance was calculated on Hellinger-transformed abundance data agglomerated at the taxonomic level of the genus, and ordination was performed using metric multi-dimensional scaling (MDS) and visually inspected. Associations of *Blastocystis* and/or *D. fragilis* positivity with fecal bacteriome composition were tested using constrained ordination, the redundancy analysis (RDA) on the Hellinger-transformed abundance data.

Using GEE, individual bacterial taxa were tested for associations with the positivity of the two abundant protists (*Blastocystis* or *D. fragilis*). Taxa having more than 20/15000 reads in at least two samples were considered. In this model, the outcome was the relative abundance of a bacterium, and the predictors were positivity for *Blastocystis* and/or *Dientamoeba*, time point (factor with three levels—0, 3 and 6 months) and intervention (on probiotics vs. on placebo). An autoregressive correlation matrix was used. The models were built for every taxonomic unit at the genus, family, order, class and phylum levels. Ensuing nominal P values were adjusted using the Bonferroni method for the number of taxonomic entities tested at the given level. In an additional model, the dependent variable was the fold difference of bacterium quantity from the ASV table after the centered log-ratio

transformation (CLR) with a small pseudo-count replacing zero values.

The commented R Markdown code and output of the statistical analysis are shown, along with the session information, as Additional file 1: Statistical analysis—R Markdown.

Results

Fecal parasitome analysis

The molecular survey using 18S rDNA parasitome profiling and/or specific quantitative PCR for the single-cell protists revealed positivity in widely varying quantities: in *Blastocystis* the threshold cycles (Ct) ranged from 17.9 to 41.2, corresponding to quantities of < 1/100 to > 10,000,000 genomic equivalents (g.e.) per µl DNA. In *D. fragilis*, the threshold cycles ranged from 19.6 to 42.6; the abundance in genomic equivalents is not known since the standard culture could not be microscopically quantified. In *Entamoeba*, the threshold cycles ranged from 23.2 to 30.3, corresponding to quantities of 1 to > 100 genomic equivalents per µl DNA.

The subject-wise prevalence of these three protists, calculated as at least once positive among the three time points, was highest in *D. fragilis* (18/78, 23.1%), slightly lower in *Blastocystis* (12/78, 15.4%) and the lowest in *Entamoeba* spp. (2/78, 2.6%) (Table 1 and Additional file 2: Fig. S2). *Dientamoeba fragilis* was more frequent in *Blastocystis*-positive samples ($P = 0.015$) but not the other way around ($P = 0.057$, both from GEE models). *Entamoeba* was only present in stable loads in one subject in each intervention group.

The protist frequency did not differ between the treatment groups (intervention vs. placebo) at baseline, and no significant effect on protist frequency was noted upon receiving the probiotic intervention (GEE with terms for the allocated treatment group, studied predictor being the ongoing intervention with lactobacilli: $P = 0.34$ for positivity of *Blastocystis*; $P = 0.14$ for *D. fragilis*; $P = 0.31$ for any of the three protists. Similarly, no influence of the intervention was noted on protist quantity—this was assessed in GEE models with the logarithm of quantity as the outcome (Fig. 2).

Blastocystis subtypes

Of 26 *Blastocystis*-positive samples, 25 had their subtype identified, while one failed in the sequencing (likely because of very low quantity, Ct 41.2). Four *Blastocystis* subtypes were identified: the most prevalent one was ST2 (10/25 identified, 40%), followed by ST4 (7/25, 28%), ST1 (5/25, 20%) and ST3 (3/25, 12%). None of the samples was positive for more than one subtype (Fig. 3A and B). Quantity did not differ by *Blastocystis* sequence type ($P = 0.46$), and there was no difference in the subject-wise prevalence of the four observed subtypes. The overview of each sample's results (qPCR, subtyping and parasitome survey) is summarized in the Additional file 3: Table—Parasites detection.

Protists and the fecal bacteriome

The presence of *Blastocystis* and/or *D. fragilis* was associated with a higher alpha diversity of the fecal bacteriome. This association was significant in a GEE model for the count of observed genera ($P = 0.018$), the Chao1 ($P = 0.013$), ACE ($P = 0.014$), Shannon ($P = 0.023$) and Fischer ($P = 0.017$) indices as well as for the Simpson ($P = 0.0057$) and inversed Simpson ($P = 0.026$) indices (Fig. 4). The difference in alpha diversity between protist-positive and -negative stools was most prominent in those collected at the second time point. Alpha diversity neither changed over time nor differed between treatment groups at baseline and was not associated with the probiotic intervention.

Redundancy analysis performed on Hellinger-transformed bacterial abundance data indicated no effect of the intervention ($P = 0.591$), but the bacteriome community composition was associated with the presence of *Blastocystis* ($P = 0.006$) and *D. fragilis* ($P = 0.001$) (Additional file 2: Figure S3). The proportion of overall community variance explained by the two protists was low (1.0% for *Blastocystis* and 1.95% for *D. fragilis*). The effects of the two protozoa were nearly orthogonal, i.e.

Table 1 Sample- and subject-wise prevalence of the protist among CiPP study participants

Protist species positivity	*Blastocystis*	*Dientamoeba fragilis*	*Entamoeba* spp.
By samples ($n = 227$)	26 (11.5%)	41 (18.1%)	6 (2.6%)
By subjects ($n = 78$)			
Ever positive	12 (15.4%)	18 (23.1%)	2 (2.6%)
Persistent positivity	8	10	2
Newly acquired positivity	1	2	0
Converted into negativity	3	5	0
Other pattern	0	1	0

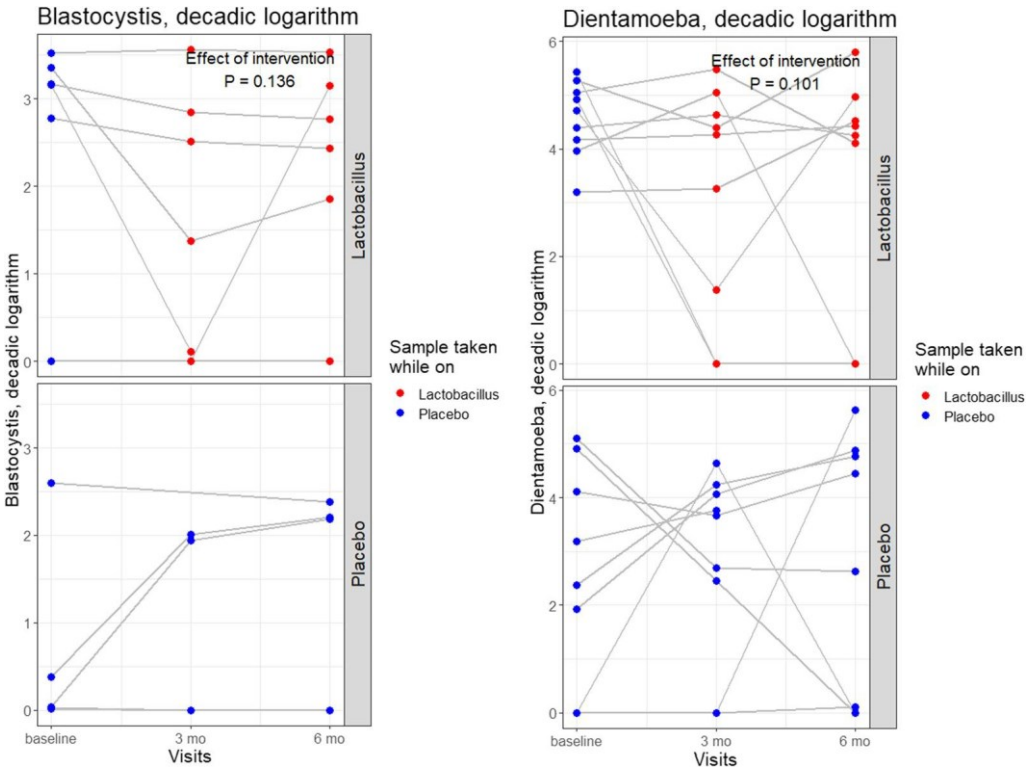

Fig. 2 Quantity of *Blastocystis* and *Dientamoeba fragilis* in children with celiac disease autoimmunity receiving *Lactobacillus* (*Lactobacillus* group) or placebo (Placebo group) over 6 months of intervention

the associations with individual microbes of the bacteriome differed. Also, upon an inspection of ordination plots of Bray–Curtis distance at the genus level, samples positive for either *Blastocystis* and/or *D. fragilis* showed a tendency towards moderate separation from those being negative for the parasites (Additional file 2: Figure S4). As the spread of the two categories significantly differed (function *vegan::betadisper*, $P < 0.001$), testing by permutational analysis of variance (PERMANOVA) was not meaningful—its significance ($P < 0.001$) reflected either the difference in centroid position or the above-demonstrated difference in the spread, or both.

When studying the bacterial taxa associated with positivity for parasites using adjusted GEE (Table 2), *Blastocystis* was inversely associated with the genera *Akkermansia* (and its taxonomic categories up to the level of phylum Verrucomicrobia), *Faecalibacterium* and *Romboutsia*, both from class Clostridia. The presence of *D. fragilis* was inversely associated with the genera *Flavonifractor, Faecalibacterium, Lachnoclostridium, Ruminococcus* and *Granulicatella*. Most of the above associations were apparent in both the linear and fold-difference models, except for *Akkermansia*, whose association was detectable only when linear quantity was used as the outcome.

Discussion

This study analyzed the single-cell parasitome in a randomized clinical trial of probiotic strain intervention in CDA and found a relatively stable protist colonization (*Blastocystis* and/or *D. fragilis*) regardless of the probiotic intervention. The intervention with the two lactobacilli caused moderate changes in the bacteriome [35]; however, we observed no effects on the parasitome composition. Rather, the opposite occurred: positivity for a parasite was stable over the observation period and associated with an increased richness of the bacteriome but inversely associated with some presumably beneficial bacteria.

By targeted molecular testing using quantitative real-time PCR, we first looked at the prevalence of *Blastocystis* as the prime representative of the fecal parasitome [4, 5]. Then, we explored the whole parasitome by 18S rDNA profiling and assessed the quantities of the positive ones by specific real-time PCR assays for each of the protists. The subject-wise prevalence of the single-cell protists among children with CDA was low (23.1% for *D. fragilis*, 15.4% for *Blastocystis* and only 2.6% for *Entamoeba* spp.),

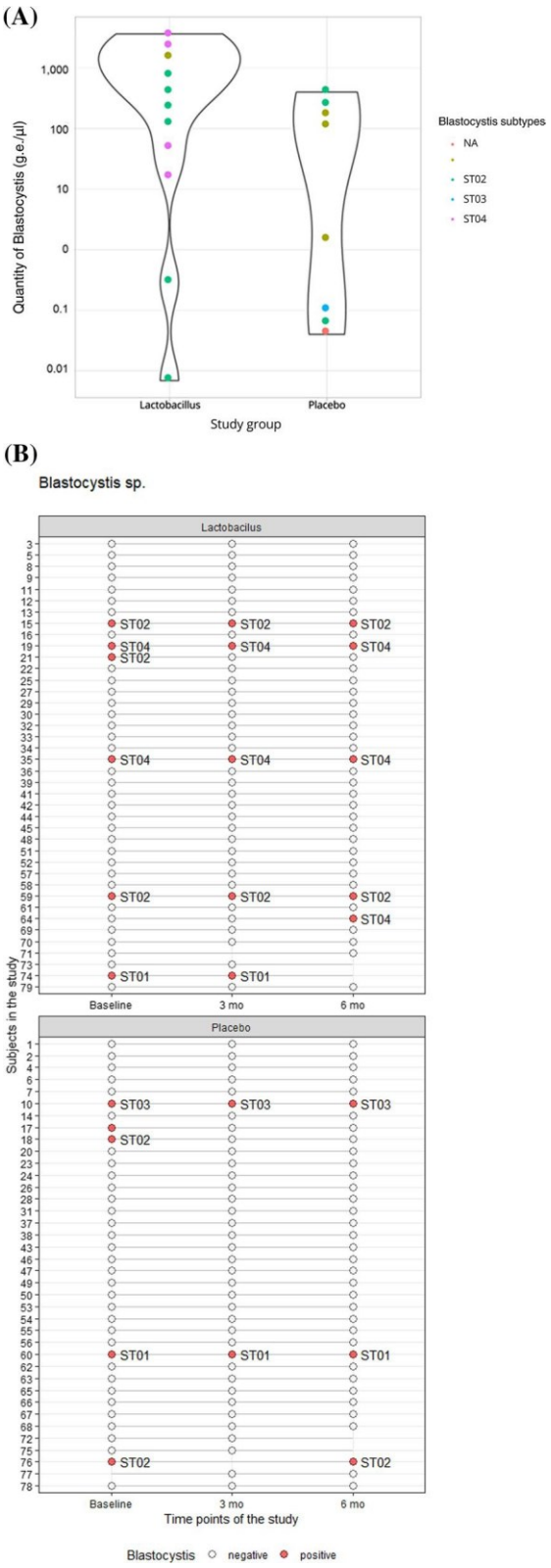

Fig. 3 Blastocystis subtypes. (**A**) *Blastocystis* quantity and subtype distribution; (**B**) stability of *Blastocystis* subtypes

in accordance with the hygiene hypothesis [47] and also to a recent report in celiac disease patients (regarding *Blastocystis*) [48].

The prevalence of *Blastocystis* is reported to be higher in lower-income countries and vice versa. Among the high-income countries, the lowest prevalence in a healthy population of only 7% was reported in Colorado, USA [13]. In continental Europe, it ranges around 20–30% (France 18%, The Netherlands 24%, Czechia 24%, Belgium 30%) [1, 11, 12, 15] and surprisingly higher prevalence of 56% was reported in Ireland [14]. On the other hand, two studies from rural populations of Nigeria [6] and Senegal [49] report a very high prevalence of 84% or 100%, respectively. Similarly, in underprivileged areas of Malaysia and Brazil, a higher prevalence of 41% and 47%, respectively, was reported [7, 8]. Thus, our results are in accord with the *Blastocystis* geographical gradient in healthy adults and children.

Although *Blastocystis* is often reported as the most common gut eukaryote [4], D. fragilis was more prevalent in the present dataset. This might be due to its higher prevalence in developed, urbanized countries [22, 27], but perhaps the location in the south of Sweden played a more prominent role, as supported by Jokelainen, who reported high prevalence in asymptomatic children under the age of 6 in day-care centeres in Copenhagen [20]. Additionally, a high prevalence of *D. fragilis* (compared to other intestinal parasites) was reported in urban areas of Copenhagen, Denmark [22, 50] (in adults) and Jönköping, Sweden [51] (in children) among patients with gastrointestinal issues suspected of parasitosis. Thus, the higher rates of *D. fragilis* than *Blastocystis* in our study seem meaningful regarding the geographic proximity to Copenhagen. Still, the high prevalence of *D. fragilis* in the region raises the question of its origin. It might be an endemic issue, but it might also be a simple reporting bias caused by the existence of several well-established parasite research groups in Denmark and Sweden. Similar isolated cases of a surprisingly high prevalence of *Dientamoeba* have been recently reported, e.g. in The Netherlands [52, 53] and Czechia [21], where groups studying single-cell protists also utilized molecular detection.

The colonization by these protists was stable over time and not influenced by the intervention of lactobacilli strains. The temporal stability in children was well described for *D. fragilis* [20]; however, for *Blastocystis* there is only one study in adults with a small number of subjects ($n = 10$) [14], making our study the first to report such stability of the parasitome among children.

The mixture of two lactobacilli strains was found to modulate the immune response [34] and change the fecal bacteriome towards a healthier composition [35].

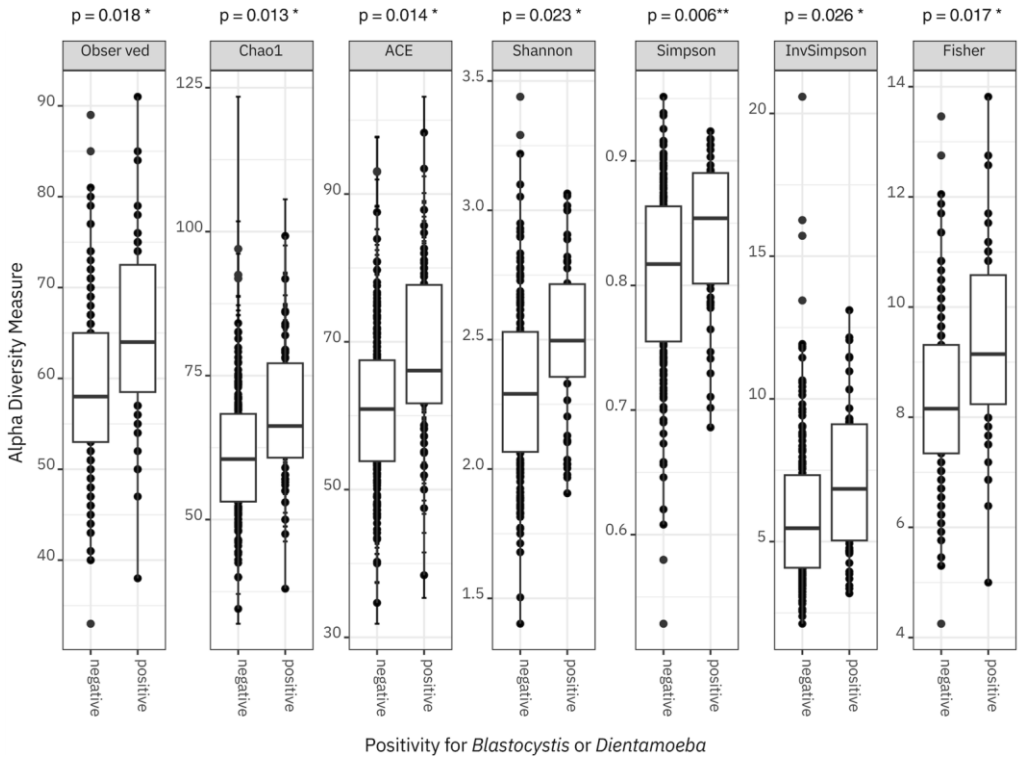

Fig. 4 Alpha diversity of the faecal bacteriome and associations with positivity for *Blastocystis* or *Dientamoeba fragilis*

However, it showed no detectable effect in modulating the fecal parasitome in presumably asymptomatic CDA patients. In contrast, probiotics may modulate parasite positivity in symptomatic patients: two studies investigated the effects of probiotics in treating symptomatic *Blastocystis hominis* infection. Dinleyici et al. showed a significant effect of *Saccharomyces boulardii* probiotics on *Blastocystis* eradication similar to metronidazole in vivo [54]; Lepczynska et al. reported an effect of a mixture of probiotics (*Lactobacillus rhamnosus, Lactococcus lactis* and *Enterococcus faecium*) on *Blastocystis* ST3 eradication in vitro [55].

In our study, ST2 was the most often identified subtype among study samples, albeit ST3 is reported as the most frequent worldwide [18]. ST3 was, on the contrary, found to be the least frequent. The occurrence of ST4, even as the second most common, is consistent with its strict European predilection [4]. Otherwise, the subtype distribution showed no association with the intervention.

The fecal bacteriome reaction to the mixture of *L. plantarum* HEAL9 and *L. paracasei* 8700:2 in our RCT was described earlier[35]. Briefly, the 6-month intervention with probiotics led to a shift in bacteriome composition in a healthier direction, most notably to an increase in *Akkermansia*, however, without any changes in alpha diversity indices. Celiac disease itself has been associated with changes in the gut bacteriome [56–60]. More specifically, it was associated with an increase in pro-inflammatory bacteria Enterobacteriaceae[56], *Bacteroides* [59] and *Fusobacterium* [56] and a decrease in *Akkermansia* [56], a beneficial bacterium for the gut epithelium [61]. Moreover, potential predictors of celiac disease were suggested, including an increase in *Porhyromonas*, *Dialister* and *Parabacteroides* and a decreased abundance of anti-inflammatory species [60] or decreased capacity to degrade gluten [57]. Importantly, 3-month probiotic supplementation shifted the bacteriome towards a healthier composition [58]. In contrast, recent work, with low patient count but innovative design, provided evidence that celiac disease might not be consistently associated with dysbiotic microbiome[62].

The probiotics did not influence the bacteriome's alpha diversity; however, we found evidence of protist positivity increasing the alpha diversity similar to what has previously been described in adults, e.g. [2], and children [5]. As speculated in our previous work, the relation between protist positivity and rich bacteriome ecosystem might be in the thriving of *Blastocystis* in distinct communities [5] or the protist itself modifying the ecosystem [63]. Moreover, based on our current findings, *D. fragilis*, not just *Blastocystis*, is associated with fecal bacteriome diversity.

Table 2 Bacterial taxa showing association with protist positivity: (A) with *Blastocystis* positivity, (B) with *D. fragilis* positivity

(A) with *Blastocystis* positivity

Variable	***Blastocystis* positivity**		Notes
Bacterial taxon	P value raw (corrected for the number of tested taxa)	Relation to the tested parameter	(1)
p. Verrucomicrobia	$6.3x10^{-6}$ ($5.7x10^{-5}$)	negative	(2)
└ c. Verrucomicrobiae	$7.2x10^{-6}$ ($1.2x10^{-4}$)		
└o. Verrucomicrobiales	$7.0x10^{-6}$ ($2.3x10^{-4}$)		
└f. Akkermansiaceae	$4.7x10^{-6}$ ($2.3x10^{-4}$)		
└ g. *Akkermansia*	$9.4x10^{-6}$ (0.0013)		
p. Firmicutes └ c. Clostridia └ o. Oscillospirales └ f. Ruminococcaceae └ g. *Faecalibacterium*	$2.0x10^{-5}$ (0.0028)	negative	(3, 4)
└ o. Peptostreptococcales └ f. Peptostreptococcaceae └ g. *Romboutsia*	$2.0x10^{-4}$ (0.029)	negative	

(B) with *D. fragilis* positivity

Variable	***D. fragilis* positivity**		Notes
Bacterial taxon	P value: raw (corrected for number of tested taxa)	Relation to the tested parameter	(1)
p .Proteobacteria └..... f. Enterobacteriaceae	$3.2x10^{-4}$ (0.016)	negative	(4)
p. Actinobacteriota └..... f. Coriobacteriaceae	$4.3x10^{-4}$ (0.022)	negative	(4)
p. Firmicutes └ c. Clostridia └ o. Oscillospirales └ f. Oscillospiraceae └ g. *Flavonifractor*	$3.4x10^{-7}$ ($4.8x10^{-5}$)	negative	(4,5)
└ f. Ruminococcaceae └ g. *Faecalibacterium*	$6.3x10^{-5}$ (0.0088)	negative	(4,6)
└ o. Lachnospirales └ f. Lachnospiraceae └ g. *Lachnoclostridium*	$2.1x10^{-4}$ (0.029)	negative	
└ g. *Ruminococcus*	$1.7x10^{-7}$ ($2.3x10^{-5}$)	negative	(4,7)
└c. Bacilli └ o. Lactobacillales └ f. Carnobacteriaceae └ g. *Granulicatella*	$2.5x10^{-4}$ (0.013) $1.3x10^{-4}$ (0.018)	negative	(4)

(1) Negative means an inverse correlation (and thus negative coefficient in the gee model) and vice versa
(2) *Akkermansia* was the only tested genus showing significance up to the level of phylum.
(3) Classified as *Faecalibacterium* sp. UBA1819 by SILVA database v. 138
(4) Significance noted also in the fold-change model with data after centered log-ratio transformation (adjusted $P < 0.05$)
(5) Significant only at genus level
(6) Classified as [*Ruminococcus*] torques group by SILVA database v. 138
Raw *P*-values were corrected using Bonferroni correction for each taxonomic level (9 phyla, 16 classes, 33 orders, 50 families and 119 genera)

The positivity of *Blastocystis* and/or *D. fragilis* was associated with a difference in bacterial community composition. Even though the protist occurrence explained only a small part of the bacteriome beta diversity, the association was highly significant. Of particular taxa associated with *Blastocystis*, a negative association with *Akkermansia* and its upward taxonomic categories stands out. *Akkermansia* is a known mucin degrader and producer of short-chain fatty acids (SCFA) [64] and is thus generally considered beneficial to the human gut ecosystem [61]. It was found to be depleted in celiac disease [56] and, on the contrary, enriched after receiving probiotics in the CiPP study [35]. Of note, here, the *Akkermansia* association was only significant in the linear model, and the absolute difference is not high. We speculate that the bacterium does not thrive in an ecosystem engineered by *Blastocystis*. Another beneficial bacterium, *Faecalibacterium*, a known producer of SCFA [65] having anti-inflammatory properties [66], was also inversely associated with *Blastocystis* positivity. *Romboutsia* has been previously associated with an increased risk of nonalcoholic fatty liver disease [67] and neurodevelopmental disorders in children [68]; however, recent rigorous work found its beneficial role in cardiometabolic health [69]. All this combined makes its weak inverse association with *Blastocystis* ambiguous to interpret.

The presence of *D. fragilis* was, among others, inversely associated with *Enterobacteriaceae*, an inflammation-promoting bacteria known to be enriched in the bacteriomes of celiac disease patients [56]. On the other hand, its presence was inversely associated with many beneficial bacteria producing beneficial SCFA (including *Flavonifractor*, *Faecalibacterium* or Lachnospiraceae family), leaving the interpretation of these associations also complicated.

Strengths and limitations

Our study provides a unique insight into a novel topic in celiac disease research as no study has yet investigated the gut parasitome by molecular techniques. The samples were taken longitudinally, which helps decrease the effects of short-term fluctuations in the abundance of individual microbial taxa [70]. Analysis was performed by several complementary methods: *Blastocystis* detection by real-time PCR assay was followed by its subtyping using amplicon sequencing of another rDNA region; in remaining parasites the positivity in the parasitome survey by massive parallel sequencing was then confirmed using real-time PCR. This, along with the inclusion of multiple negative controls, safeguarded against false positivity. Furthermore, the present study extended previous investigations to explore the whole parasitome by using multiple primer pairs [5]. Moreover, we reported the quantity of the protists, not only dichotomous positivity in analyses of subtypes but also in association with the intervention. Although absolute quantification from stool is cumbersome (as there is no internal standard for reference), we still believe that this quantitative aspect adds confidence to our findings, even though we did not find any association with the protist quantity.

The main limitation is the unavailability of a fresh fecal sample needed for direct morphological assessment by microscopy as only frozen samples were used for DNA extraction. Thus, we do not know what stages of the protist were present and whether morphology relates to the quantity of *Blastocystis* subtypes. However, such an interpretation of the microscopy result might still be difficult, given that *Blastocystis* possesses one or two nuclei [71]. Another limitation is that no background population without CDA was investigated for protist prevalence comparison as the trial was designed far earlier than this parasitome study. Thus, no control group of healthy children without CDA was used for establishing protist prevalence in the background population.

Conclusions

The prevalence of *Blastocystis* and *D. fragilis* in children with CDA is rather low, with *D. fragilis* being the more prevalent of the two protists. Their positivity or quantity did not appreciably change upon the probiotic intervention. The presence of *Blastocystis* and *D. fragilis* was linked with an increased bacteriome diversity, although inversely associated with the abundance of some beneficial bacteria, like e.g. *Akkermansia muciniphila*. Even though the probiotics may help children with CDA to modulate the immune response and positively affect the fecal bacteriome, the single-cell parasitome remains unaffected.

Abbreviations

CDA	Celiac disease autoimmunity
CiPP	Celiac disease Prevention with Probiotics
qPCR	quantitative real-time PCR
PCR	Polymerase chain reaction
SCFA	Short-chain fatty acids

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-023-06027-1.

Additional file 1. Statistical analysis–R markdown.

Additional file 2: **Figure S1.** The detection primers and hydrolysis probe annealing to the consensus of several sequences of *Entamoeba* sp. **Figure S2**. Positivity of *Blastocystis* sp., *Dieantamoeba fragilis* and *Entamoeba* sp. in individual study samples. **Figure S3.** Constrained ordination (redundancy analysis) of the bacteriome community composition by *Blastocystis* sp. positivity, *Dieantamoeba fragilis* positivity and intervention with lactobacilli. **Figure S4**. The dispersion of samples negative for *Blastocystis* sp.

or *Dieantamoeba fragilis* is significantly higher than that of their positive counterparts ($P < 0.001$), so testing by Permutational Multivariate Analysis of Variance would not be meaningful–its significant result may reflect not only the significant difference in centroid position but also the difference in spread. **Table S1**. Specific primers and probes for *Entamoeba* sp. quantitative PCR.

Additional file 3. Table–Parasites detection.

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Author contributions
JH performed laboratory analyses, analysed some of the data and wrote the manuscript; EO performed some laboratory analyses; AH supervised the laboratory analyses and co-lead the study; KJM and MJ contributed to the preparation of the detection assays, construction of quantitative calibrators and manuscript revision; CA designed and co-lead the study, including the subjects recruitment, metadata gathering and data management; OC designed the laboratory part of this study, analysed the data and revised the manuscript; DA conceived the CiPP study, participated in its organization, subject recruitment and data interpretation and revised the manuscript.

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Availability of data and materials
Data are available upon reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committee of the Medical Faculty, Lund University, on September 8th, 2011 (Dnr 2011/335) and on September 1st, 2021 (Dnr 2021-04470). The study is registered in ClinicalTrials.gov (NCT03176095).

Consent for publication
Not applicable.

Competing interests
DA is an inventor in a patent application based on the results of the clinical trial but has signed over all legal rights to the patent to Probi AB. Probi AB has developed and supplied the study material (active and placebo products) for the trial as well as financially supported the trial with minor costs for analyzing material. None of the authors are employed by Probi AB and no salaries, consultancy fees, etc., have been paid by Probi AB to the authors in connection with the trial. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Protocol for faecal microbiota transplantation in irritable bowel syndrome: the MISCEAT study – a randomised, double-blind cross-over study using mixed microbiota from healthy donors

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ABSTRACT

Introduction Several studies have demonstrated dysbiosis in irritable bowel syndrome (IBS). Therefore, faecal microbiota transplantation, whose effect and safety have been proven in *Clostridioides difficile* infections, may hold promise in other conditions, including IBS. Our study will examine the effectiveness of stool transfer with artificially increased microbial diversity in IBS treatment.

Methods and analysis A three-group, double-blind, randomised, cross-over, placebo-controlled study of two pairs of gut microbiota transfer will be conducted in 99 patients with diarrhoeal or mixed type of IBS. Patients aged 18–65 will be randomised into three equally sized groups: group A will first receive two enemas of study microbiota mixture (deep-frozen stored stool microbiota mixed from eight healthy donors); after 8 weeks, they will receive two enemas with placebo (autoclaved microbiota mixture), whereas group B will first receive placebo, then microbiota mixture. Finally, group C will receive placebos only. The IBS Severity Symptom Score (IBS-SSS) questionnaires will be collected at baseline and then at weeks 3, 5, 8, 11, 13, 32. Faecal bacteriome will be profiled before and regularly after interventions using 16S rDNA next-generation sequencing. Food records, dietary questionnaires, anthropometry, bioimpedance, biochemistry and haematology workup will be obtained at study visits during the follow-up period. The primary outcome is the change in the IBS-SSS between the baseline and 4 weeks after the intervention for each patient compared with placebo. Secondary outcomes are IBS-SSS at 2 weeks after the intervention and 32 weeks compared with placebo and changes in the number of loose stools, Bristol stool scale, abdominal pain and bloating, anthropometric parameters, psychological evaluation and the gut microbiome composition.

Ethics and dissemination The study was approved by the Ethics Committee of Thomayer University Hospital, Czechia (G-18-26); study results will be published in peer-reviewed journals and presented at international conferences and patient group meetings.

Trial registration number NCT04899869.

INTRODUCTION

Irritable bowel syndrome (IBS) is characterised as recurrent abdominal pain on average at least oneday/week in the last 3 months, associated with two or more of the following criteria: (1) related to defecation; (2) associated with a change in the frequency of stool and (3) associated with a change in the form (appearance) of stool.[1] It is common among the adult Europid population (approx. 10%[2]), but its aetiology is still unknown. It may, among other causes, include microinflammation, disturbance of the brain–gut axis, inadequate secretion of bile acids, increased permeability of the gut epithelial barrier, or gut dysbiosis. Dysbiosis in IBS has been suggested by several studies.[3] There are indications that Firmicutes may be disturbed, with *Dorea, Blautia* and *Roseburia* increased, whereas *Veillonella* and *Faecalibacterium* decreased. Among Actinobacteria, a

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Usage of mixed microbiota from multiple donors inflates the diversity of transferred microbiota by enriching it for numerous rare species.
⇒ All interventions will be carried out using the same active mixed microbiota or the same placebo.
⇒ Each intervention consists of two consecutive transfers, which increases the probability that the transferred microbiota engrafts.
⇒ Microbiome profiling, food records, anthropometry and bioimpedance data allow detailed monitoring of transfer effectiveness.
⇒ Mucosa-associated microbiota will not be assessed because the stool transfer will be performed by enema, not colonoscopy that would allow biopsies.

decrease in *Bifidobacterium* was noted, and among Proteobacteria, *Enterobacteriaceae* were increased. Conflicting and heterogeneous results were reported for Bacteroidetes. The major limitation of available studies is their cross-sectional character, which may not be enough in a disease where diarrhoeal episodes alternate with normal stool composition or constipation.

The faecal microbiota transplantation (FMT) has gained popularity by its remarkableeffect in recurrent *Clostridioides difficile* infections, where it has now become a recognised life-saving therapy.[4] The first published randomised, double-blinded study on FMT in IBS, published in 2018 when starting our study,[5] used stool intervention from an allogeneic donor or autologous stool. The intervention was centred on a well-defined group of IBS of predominantly diarrhoeal form. The stool was transferred by colonoscopy to the cecum. The primary outcome was an improvement in the IBS-Severity Symptom Score (IBS-SSS). The treatment was associated with a significant effect at 3 months but not at 12 months postintervention.[5] This study used single donors and did not assess stool microbiota. Thus, the transferred microbiota likely varied between transfers both in their composition and in their diversity. Since then, more studies focused on FMT in IBS have beencarried out.[6–11] They differed in design, but none of them used a mixed microbiota from multiple donors as the active substance. Furthermore, a recent meta-analysis of randomised control trials on FMT in IBS (including the above-mentioned articles) pointed out insufficient evidence quality to support recommending FMT in the treatment of IBS.[12]

Our study protocol aims to test whether FMT of mixed microbiota from several selected donors can alleviate symptoms of IBSmeasured by IBS-SSS 4 weeks after the intervention, as compared with autoclaved placebo. The secondary study aims to test the acute (after 2 weeks) and the long-term effect (after 6 months)on symptoms relief. We also focus on the number of loose stools, Bristol stool scale, abdominal pain and bloating, body mass index (BMI), fat content, waist circumference, skinfold thickness, psychological evaluation and the gut microbiome composition.

We hypothesisethat the transfer of active microbiota of high diversity can lead to changes in thepatient's gut microbiome composition and/or function toalleviate IBS symptoms.

METHODS AND ANALYSIS

Study design

This is a three-group, double-blind, placebo-controlled, randomised, cross-over study in adult patients diagnosed with IBS (diarrhoeal or mixed form) according to Rome IV criteria. Each study subject will undergo two pairs of FMT (a total of four enemas for each patient), with the pairs of transfers being 8 weeks apart. The active intervention substance is a mixed stool microbiota derived from healthy individuals who were preselected for high alpha diversity of their microbiome and distance in community ordination from IBS patient'smicrobiota. Placebo is the same mixture, inactivated by autoclaving.

The study subjects are randomly assigned to one of three groups: (A) enema with active substance first and with placebo second or (B) enema with placebo first and active substance second or (C) enemas of placebo only (detailed scheme in figure 1). Eligible participants will be followed up for 32 weeks after the first intervention to monitor symptom severity scoring of IBS (IBS-SSS), with regular profiling of their gut microbiome and other parameters like the number of loose stools, Bristol stool scale, abdominal pain and bloating, BMI, fat content, waist circumference, skinfold thickness and psychological evaluation.

The placebo group is planned because of the unknown onset and duration of the intervention effect: if the beginning of an effect is delayed, or if it persists for a long time, simple cross-over design would not have sufficient power due to the carry-over effect. In case the FMT was associated with significant but not durable amelioration of the status, the control group would still increase the statistical power.

This study protocol is reported as per the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidelines[13] (for the SPIRIT checklist see online supplemental appendix 1).

Study setting

The participants are recruited at a single centre, the Department of Internal Medicine, Thomayer University Hospital in Prague, Czech Republic. This hospital has approximately 1000 beds, including 80 in intensive care units, serves approximately 50 000 patients per year. The centre is experienced in treating patients with IBS and other functional gastrointestinal disorders, with about 200 such patients registered and further subjects coming for consultations from other workplaces to this tertiary referral centre.

Recruitment and eligibility criteria

Stool donors

Stool donor candidates were recruited among blood donors at Thomayer University Hospital and medical students in their first year of study (ie, preclinical) from the second Faculty of Medicine, Charles University, Prague. We obtained stool samples from 58 such candidates fulfilling the inclusion criteria (table 1). Based on their high bacterial alpha-diversity and by the position on the ordination plot of the weighted UniFrac distance against 46 patients with IBS-D (figure 2), 14 candidates proceeded to the safety screening,where by 8 passed it (for reasons of candidate's exclusion, see figure 3.

After 14 potential donors were selected based on the microbiota composition, they were screened for infectious diseases and clinically examined as indicated by the European consensus conference on FMT in clinical practice

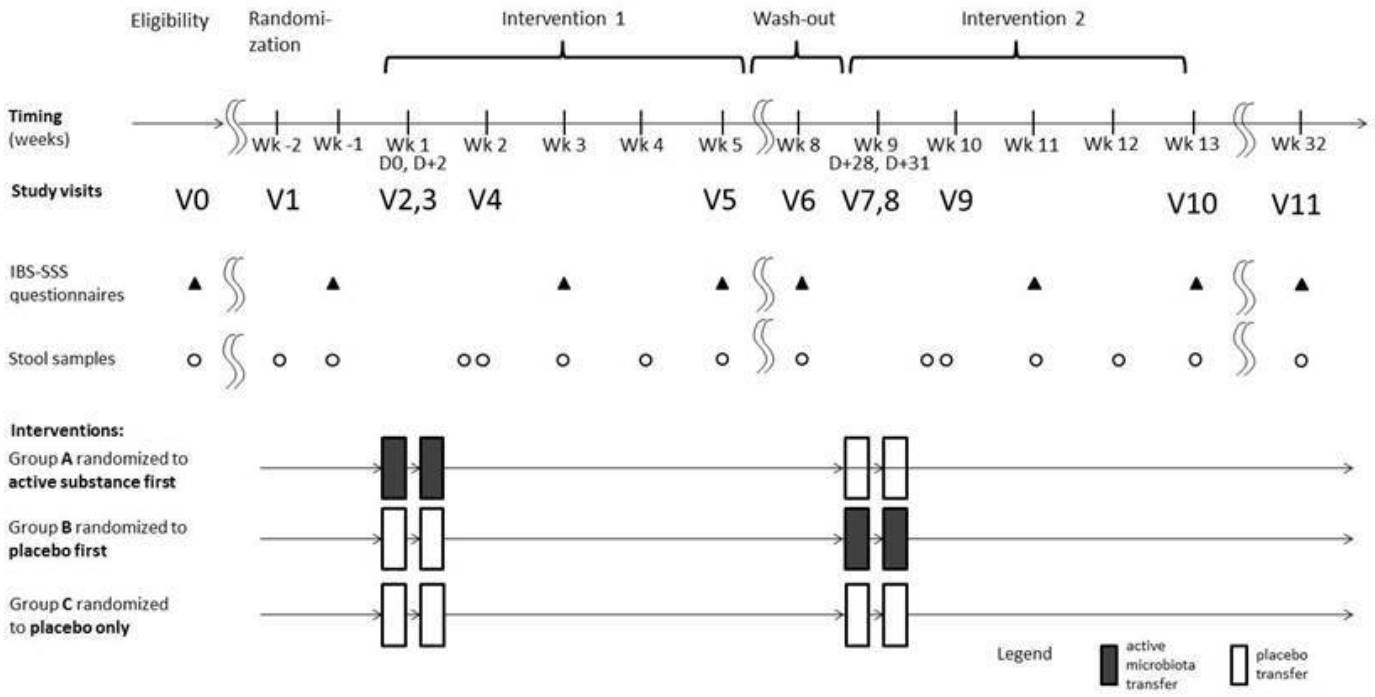

Figure 1 Per-protocol intervention scheme: the visits, questionnaires and samples. IBS-SSS, Irritable Bowel Syndrome Severity Symptom Score.

guidelines[14] (box 1). All subjects were also repeatedly tested for SARS-CoV-2 from both nasopharyngeal swab and stool. Six candidates were excluded (for reasons, see figure 3), whereas eight became regular stool donors. These eight donors were regularly investigated as follows:

- At every donation: by questionnaire for gastrointestinal symptoms, antibiotic usage, unprotected sex, travelling to exotic countries; clinical signs of COVID-19; the presence of SARS-CoV-2 in the donated stool.
- Every 4 weeks: for SARS-CoV-2 from nasopharyngeal swab.
- Every 8–12 weeks: for all other stool tests mentioned in box 1.

Table 1 Inclusion and exclusion criteria for FMT donors

Inclusion	Adults aged 18–65 years
	BMI 18.5–27 kg/m^2
	Lack of restrictive diets (diet discussed with experienced gastroenterologist)
	Bristol stool scale usually between 3 and 4
	High alpha diversity and significant difference in beta-diversity from patients (using 16S rDNA sequencing)
	Expected to donate regularly
	Consented in writing
Exclusion	Any chronic GI disease in patient's history (coeliac disease, inflammatory bowel disease, irritable bowel syndrome, colorectal carcinoma) or active acute GI issues (infectious gastroenteritis or enterocolitis, frequent bloating, diarrhoea or vomiting)
	Chronic disease in ' 'patient's history (cancer, autoimmune conditions, type 2 diabetes mellitus, coronary heart disease, hypertension, hypercholesterolaemia, gout)
	Clostridiodes difficile infection in patient's history
	Colorectal carcinoma in family history
	Any restrictive diet habits (raw-vegans, fruitarians, keto or carnivore)
	Any systemic antibiotics in the last 6 months
	Using proton-pump inhibitors in the last 6 months
	Regular unprotected sex with unknown persons

BMI, body mass index; FMT, faecal microbiota transplantation; GI, gastrointestinal.

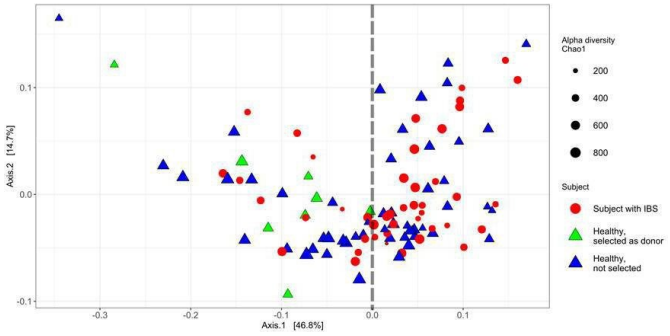

Figure 2 Ordination plot on the weighted UniFrac distance at the genus level for selectionof the donor candidates based on their gut microbiome alpha diversity and beta diversity. These are the results of a comparative microbiome case–control study which helped us to preselect 14 donor candidates. Alpha diversity calculation was based on Chao 1 index. The beta-diversity calculation was based on Non-Metric Dimensional Scaling (NMDS) with weighted UniFrac distance matrix for bacterial genus. NMDS axis 1 captured 46.8% of variability; NMDS axis 2 represents 14.7% of the variability. Healthy subjects were enriched in negative values of the first ordination axis; therefore, we selected donors among healthy subjects in this half of the graph and based on their microbiome's alpha diversity. The reason for concentrating healthy and enriched subjects in the left part of the plot could be their younger age. IBS, irritable bowel syndrome.

Prospective study participants

Patients diagnosed with IBS-D (diarrhoeal type) or IBS-M (mixed diarrhoeal and constipation type) who fulfil the inclusion and exclusion criteria listed in table 2 are recruited via regular' patient's check-ups at the Gastroenterological unit at Thomayer University Hospital, by referrals from their general practitioners, following our newspaper articles or word of mouth.

Study microbiota mixture for intervention

The intervention microbiotais a mixture of regular stool donations from the eight regular donors. The collection

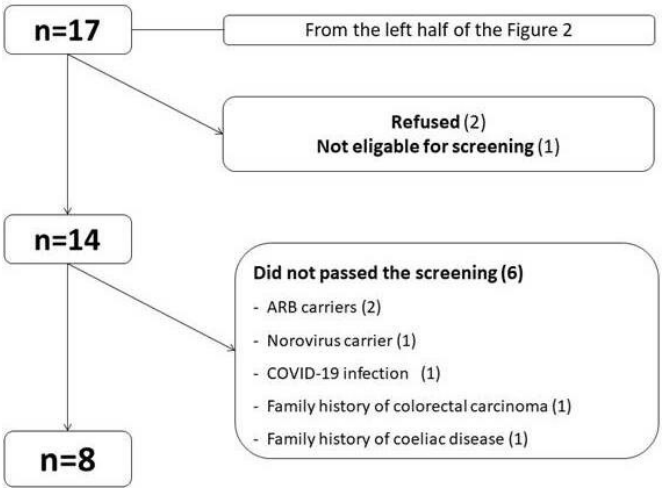

Figure 3 Process of donor selection and reasons for their excluding. ARB, antibiotic resistant bacteria.

Box 1 Laboratory screening of the FMT donors*

Blood testing
Hepatitis A, hepatitis B, hepatitis C and hepatitis E viruses (serology)
HIV-1 and HIV-2 (p24 antigen)
Treponema pallidum (serology)
Strongyloides stercoralis (serology)
Complete blood cell count with differential
Creatinine, aminotransferases, bilirubin

Stool testing
Clostridioides difficile (cultures, antigen testing)
Common enteric pathogens, including Salmonella, Shigella, Campylobacter, shiga toxin-producing *Escherichia coli*, Yersinia and *Vibrio cholerae* (cultures)
Antibiotic-resistant bacteria (ARB), including vancomycin-resistant Enterococci, meticillin-resistant *Staphylococcus aureus*
Gram-negative ARB including extended-spectrum β-lactamase-producing *Enterobacteriaceae*, and carbapenem-resistant *Enterobacteriaceae*/carbapenemase-producing *Enterobacteriaceae* (cultures)
Norovirus, rotavirus, adenovirus, sapovirus (PCR)
SARS-CoV-2 (reverse transcription-PCR)
Common intestinal parasites, including *Giardia intestinalis, Cryptosporidium parvum et hominis* (cultures and PCR), *Blastocystis hominis***, *Dientamoeba fragilis*** (both PCR only)

* The screening strategy is based on International consensus conference on stool banking for faecal microbiota transplantation in clinical practice[14]
** Based on the literature,[21] we decided to test both parasites but did not exclude the donors if they were tested positive and had no gastrointestinal symptoms. *Blastocystis* is believed to be commensal of the gut. *Dientamoeba's* status is not exact; however, based on our experiment, it does not survive freezing at −80°C and thawing to 5°C when mixing the study microbiota mixture.[22] Therefore, it cannot do any harm.

of stools for this purpose is already completed. The donors were advised to regularly defecate at their home toilet into a clean plastic bag placed in Fecotainer (Excretas Medical, NL) with an Anaerogen bag (Thermo Scientific, USA). This bag generated an anaerobic atmosphere during transport to ensure anaerobes survival. The stool was transported to the laboratory with the maximum allowable time until processing being 6 hours; the actual time was approximately 1.5 hours. The stool was weighed on arrival, inspected for blood admixture and immediately processed by blending with a solution consisting of sterile 0.9% saline (160 mL per 100 g of stool), sterile phosphate buffer saline at pH 7.4 (20 mL per 100 g of stool) and sterile 99.5% glycerol (20 mL per 100 g stool, which is approximately 10% of solution's volume; therefore, it is unlikely to have laxative properties on administration). From our experience, ~105 mL of the study mixture represents ~40 g of stool. The mixture was then filtered through a sterilestainless steel mesh of 0.8 mm pore size into a sterile plastic bottle, which was then immediately frozen at −80°C. Whenever possible (blending or postfiltration), the procedure was performed under a nitrogen atmosphere to protect obligate anaerobes. All stool portions were remixed together in a large stainless

Table 2 Inclusion and exclusion criteria for recipients of FMT

Inclusion	Adults 18–65 years
	Diagnosed with IBS-D or IBS-M according to the rome IV criteria
	Expected adherence to following the protocol
	Written consent to the study
Exclusion	The use of antibiotics and probiotics within 1 month prior to faecal microbiota transplantation
	History of inflammatory bowel disease or gastrointestinal malignancy, systemic autoimmune diseases (ongoing or in history)
	Previous abdominal surgery (other than appendectomy or cholecystectomyor hernioplasty or caesarean section)
	HIV infection or other active infection
	Renal or hepatic disease (both defined by biochemistry workup)
	Diabetes mellitus, abnormal thyroid functions not controlled by thyroid medications
	Bipolar disorder or schizophrenia (ongoing or history thereof), moderately severe depression defined by Patient Health Questionnaire-9 score >15
	Anxiety defined by a Generalised Anxiety Disorder 7 score >10, with any organic causes that can explain the symptoms of IBS
	Current pregnancy and lactation

FMT, faecal microbiota transplantation; IBS, irritable bowel syndrome.

steel bucket using an electric mortar mixer under anaerobic conditions and at low temperature (on ice).

Based on the recommendation from the Nanjing consensus,[15] the bacterial cell content of the study microbiota mixture was quantified. We performed a real-time PCR of the 16S rRNA gene with a standard curve derived from bacterial culture and controls from previously used stool transplants from another centre. It was estimated that the cell count in the transfer ranged between 2e+12 and 1e+13 (depending on the expected composition of the microbiota as to the 16S gene count per an average bacterial cell). Unfortunately, the Nanjing consensus[15] provides neither reference to the cell counting method (box 1 therein) nor to control materials. Therefore more exact direct comparison of the requested quantities is not possible.

The mixed microbiotasubstance wasdivided into aliquots of 13–14 g (which is ~35 mL). Two-thirds of the tubes served as a placebo: they wereimmediately autoclaved at 121°C for 30 min with slow cooling. Presterilised tubes were used to ensure that autoclaved placebos would not be visually distinguishable from tubes with the active substance. Assignation of tubes to the autoclave, numbering, sealing and labelling were done under the guidance of a statistical unit member (see below).

All aliquot tubes are kept frozen at −80°C in the same type of plastic tubes, labelled by codes. Three such aliquots represent one dose for FMT (~40 g of stool, in ~105 mL). Aliquoting into multiple 50 mL tubes instead of one larger volume was decided because of the availability of durable plastic, which must be both autoclavable and deep frost resistant.

Before administering, the study microbiota mixture will be thawed in a warm (37°C) water bath, with intermittent mixing by inverting the tubes.

Randomisation, allocation and blinding

At visit 1, each patient is randomised into one of three equally sized groups (figure 1) as described in the Study design section. Randomisation assignments is generated in advance in blocks of nine and stored in a protected database. For each patient, anonymous codes for tubes containing either active study microbiota mixture or placebo is received. Thus, the true assignment will remain concealed for the patients and the study staff until the end of the study observation period. The investigator is encouraged to maintain the blind as far as possible. The actual allocation must not be disclosed to the patient and/or other study personnel including other site personnel, monitors, corporate sponsors or project office staff; nor should there be any written or verbal disclosure of the code in any of the corresponding patient documents.

Study intervention

Study substance is administered during visit 2+3 and then again 7+8 as a retention colon enema and will be held optimally for at least 30 min. Bowel preparation is applied the day before the intervention (prior to visit 2 and visit 7) (natrii picosulfas 10 mg, magnesii oxidum leve 3, 5 g, acidum citricum 12 g). No preparation is performed before the second enema in the pair (visits 3 and 8).

A rectal tube is inserted into the rectum, and the enema is applied. Application kit (Irrigator PN 0462/E/93, Erilens, Czechia) is used. After the enema is applied, the patient position is changed to enable the study substance to be spread within the colon. The exact time of the enema completion is recorded as well as the enema retention time.

OUTCOMES

Primary outcome

The primary outcome is the change in the IBS-SSS in the active microbiota group relative to the placebo group. The change will be evaluated as the difference between the score at 4 weeks after the intervention (study weeks 5 or 13, respectively, see figure 1) and the baseline score (week −1 in group A or week 8 in group B).

Secondary outcomes

- The acute change in the IBS severity symptom score (IBS-SSS) between baseline and two weeks after intervention (study weeks 3 and 11, respectively, see Figure 1).
- The long-term change in the IBS severity symptom score (IBS-SSS) between baseline (week -1) and week 32 (see Figure 1). The long term change will compare group C (placebo only) to merged groups A+B (active study microbiota mixture).
- Following outcomes compare changes in the active microbiota group relative to the placebo group between baseline and study week 32:
  - Quantity of loose stools per day
  - Stool consistency evaluated by the Bristol Stool Scale.
  - Abdominal pain measured by the Visual Analogue Scale.
  - Frequency of bloating per week.
  - Body mass index in kg/m$^{2.}$
  - Body fat mass estimated by measuring combined skinfold thickness in millimetres at given locations (biceps, triceps, subscapular, suprailiac).
  - Percentage of body fat mass measured by bioelectrical impedance analysis.
  - Waist circumference in centimetres.
  - The psychological and well-being effects of the therapy scored by IBS-QoL questionnaires.
  - The faecal microbiome's alpha diversity measured by the Chao index.
  - The faecal microbiome's beta diversity assessed by the quantitative Bray-Curtis index ordinated by non-metric multidimensional scaling.
  - Quantity of *Blastocystis* sp. assessed by a specific quantitative PCR assay measured in genomic equivalents per µL DNA.

Data collection and follow-up

Timing of assessments

At visit 1 (the randomisation), the patient is given detailed instructions and thoroughly instructed by the study team. The patients are asked to keep the identical type of diet throughout the observation. They are asked to regularly (once a week) fill the study questionnaire. A study team member sends that via the Survey Monkey smartphone application, an online survey development cloud-based software. Relevant data are entered in a structured manner (frequency of defecation, Bristol stool scale, pain measures, other symptoms, dietary records, etc). This member also frequently communicate with study participants and answer any questions regarding the study to keep the patient's adherence. An overview of the examinations at each visit and the timing of the study visits could be seen in table 3.

Irritable Bowel Syndrome Severity Scale Score

The IBS-SSS is a five-question survey that reflects (1) the severity of abdominal pain, (2) frequency of abdominal pain, (3) severity of abdominal distention, (4) satisfaction with bowel habits and (5) interference with quality of life over the past 10 days. Subjects respond to each question on a 100-point Analogue Scale; thus, the score can range from 0 to 500, with higher scores indicating more severe symptoms.[16]

Table 3 The study visits with planned activities

Visit	0	*	X	2+3	4	X	5	6	7+8	9	X	10	11
Study week	?	−2	−1	*	†	3	5	8	9	10	11	13	32
Eligibility evaluation (E)/randomisation (R)/wrap-up visit (W)*	E	R											W
Colon enema with the study substance (active microbiota or placebo)				XX					XX				
Irritable bowel syndrome severity scale score		X	X			X	X	X			X	X	X
Weight, height, bioimpedance		X			X		X			X		X	X
Detailed anthropometry		X					X					X	X
Serum workup, archiving serum+plasma		X			X					X			X
Psychological evaluation		X											X
Dietary questionnaire and advice, evaluation of food records†					X					X			
Stool samples for microbiome analysis	X	X	X		X	X	X	X		X	X	X	X

*Here, the patient is offered a roll-over into an observational study with active microbiota administration. The patients will be informed of this option at the start of the study and regularly reminded.
†For IBS-SSS questionnaires assessing the primary outcome, please see the intervention scheme in figure 2. Their administering is not linked to study visits.
IBS-SSS, Irritable Bowel Syndrome Severity Symptom Score.

At eligibility screening, the patients is given instructions on how to fill the IBS-SSS questionnaires (via the Survey Monkey application). The questionnaires are filled in at eligibility screening and then at week −1, 3, 5 (before the first intervention, at the presumed peak of its effect, and after further 2 weeks), then at weeks 8, 11, 13 (similarly with the second intervention), and finally at week 32.

Weight, height, bioimpedance

Bodyweight, height and bioimpedance is examined during visit 0, 1, 4, 5, 9 and 11. Medical Body Composition Analyzer Seca mBCA 515, (Seca, Germany) is used to measure changes in body composition (8-point bioelectric impedance analysis at a frequency of 5–50 kHz with a current of 100 µA), scanning performed with three-pairs of hand electrodes and two pairs of leg electrodes, measurements performed with light clothing and without metal objects (jewellery, keys). The weight is determined in patients wearing underwear using the Seca mBCA 515. The height is determined by a standardised technique with a metal stadiometer with an accuracy of 1 mm. Seca analytics V.115 software is used to analyse the obtained data (Seca, Hamburg, Germany). The measurements is performed according to the NIHR (National Institute for Health and Care Research) Southampton Biomedical Research Centre standard protocol (Seca mBCA, NIHR Southampton Biomedical Research Centre, 2014).

Detailed anthropometry

It is performed by nutritional therapists in visit 1, 5, 10 and 11. It involvesweight, abdominal (waist) circumference, buttocks (hip) circumference, thigh circumference and skinfolds (thigh, triceps, subscapular, suprailiacal).

Serum workup, archiving serum +plasma

Blood is sampled at visits 0, 4, 9, 11 and will include: (A) serum+plasma archiving, (B) serum workup. Laboratory panel testing will comprise sodium, potassium, chloride, urea, creatinine, glucose, calcium, phosphate, total protein and albumin, AST (aspartate transaminase), ALT (alanine transaminase), ALP (alkaline phosphatase), GGT (gamma-glutamyl transferase), bilirubin, lipid panel, HS-CRP (high-sensitive C-reactive protein), blood cell count with differential count, INR (international normalized ratio), urine analysis (sediment and biochemistry). One plasma and one serum aliquots are made at these visits and frozen for forensic reasons.

Psychological evaluation

It is performed during visit 0 and visit 11 using a structured questionnaire evaluated by a qualified psychologist.

Dietary questionnaire and advice, evaluation of food records

It is performed by nutritional therapists at visit 4 and 9 and includes: evaluation of food records will include: overall daily energy intake, proteins, carbohydrates and lipids calculations and dietary fibre.

Gut microbiome composition

Faecal samples are collected at home by the subjects in the same way as described for donors above and at time points indicated in the sections above. If not immediately brought to the visit, the stool is frozen in a home freezer and then transported in a frozen tube container. DNA extraction is performed using the PowerSoil kit (Qiagen), and the bacteriome is characterised by 16S rDNA amplicon profiling using the tagged primers according to Schloss protocol[17] and sequencing on a MiSeq instrument with the 2×250 bases sequencing kit (both Illumina, USA).

The first steps of bioinformatic analysis will be performed in the DADA2 package.[18] Statistical analyses and visualisation will be then performed in R with its Phyloseq package. The functional potential of the bacteriome will be assessed using the PICRUST software, which predicts functional capabilities based on the 16S rDNA profiles.

The virome is assessed in a total of four stool samples per patient at visit 0, 4,9 and 11. The aim of this analysis is to assess the repertoire of major bacteriophages. The virome analysis is based on metagenomic sequencing of total DNA from a virus-enriched stool sample, according to the previously published protocol.[19]

Finally, a simple PCR-based semiquantitative parasite screening aims to identify several mostly benign unicellular parasites (eg, *Blastocystis*, *Dientamoeba*, *Entamoeba*, *Endolimax*).

Safety monitoring

All data are regularly monitored by the research team for any adverse events, and all potential adverse events are recorded. Contacts to study coordinators active 24/7 are provided in case adverse effects occur. If any concerns are identified during the screening or clinical assessment of donors or recipients, further clinical evaluation and/or examination is immediatelyrealised. All the concerns during the study are assessed, and the recipient will be withdrawn if this is thought to be in his best interest. A data monitoring and safety committee (DMSC) has been established and based on the data from the planned interim analysis, has the right to terminate the study if the frequency of severe adverse events crosses the 5% line (for a closer description of DMSC, its responsibilities and premature termination of the study see online supplemental appendix 2.

Sample size and power calculation

The study is powered to detect an absolute improvementof 62.5 points in IBS-SSS score over 8 weeks (which is 25% of the expected mean baseline score 250) between the active microbiota intervention compared with placebo. With asample size of 33 per group (99 total), the probability of detecting such an improvement is at least 0.9. This calculation assumes 20% drop-out rates, variance inIBS-SSS scores 100 (see the results in Palsson *et al*[20]), a correlation between the final and baseline IBS-SSS scores

0 (with a positive correlation, the power is higher), and no carry-over or temporal effect.

Data management

Data from IBS-SSS, frequency of urgent defecations, Bristol Stool Scale, abdominal pain and bloatingare collected and stored via the application Survey Monkey. All anthropometric data are entered and stored in password-protected platforms integrated within the hospital information system. Only the researchers involved in the study have access to the final study dataset (IBS-SSS, frequency of urgent defecations, Bristol stool scale, abdominal pain and bloating), which will be shared in an anonymised form via the Zenodo repository. The only data in this manuscript are bacteriome data; their anonymised form will be available on reasonable request.

Statistical analyses

The primary outcome analysis will be based on the difference in IBS-SSS scores over the second treatment period (week 14 vs week 8) minus the change over the first treatment period (week 5 vs week −1). This difference will be used as a response in a linear model, with intercept corresponding to the temporal effect (seen in the placebo group C), an indicator of group A corresponding to the cross-over effect (resulting from administration of placebo after active microbiota) and differences in indicators for groups A and B modelling the effect of active microbiota. A robust sandwich estimator of the variance matrix will be used to adjust for potentially unequal variances between the groups. Analyses of secondary outcomes will proceed by a similar methodology, comparing absolute or relative differences of the postintervention measure of each outcome relative to its baseline value. The Consolidated Standards of Reporting Trials 2010 guidelines will be followed in reporting the main trial results.

Study status

The first patient was recruited on 17 June 2021, and the first intervention was applied on 29 July 2021. As of 17 August 2021, 12 patients have signed the informed consent, and 6 interventions have been applied. It is expected that the study will be completed in December 2023.

Patient and public involvement

Information on the study has been spread at conferences, in newspapers and by local gastroenterologists contacted by researchers. Everyone interested got information material, which allowed the potential subjects to read about the study and reach the researchers if they wanted to participate. Participants were not involved in the development, recruitment of other participants or conduct of the study. All recipients are asked about any possible adverse effects of treatment at regular visits planned according to figure 1; a thorough investigation will be conducted if any occur. After completing the data analysis, all recipients will receive information about their results and be offered a roll-over (receiving an active study microbiota mixture).

ETHICS AND DISSEMINATION

Ethics approval for this study was granted in June 2018 by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital (Vídeňská 800, 140 59 Prague 4, Czech Republic). Involvement in this study is completely voluntary; donors and recipients are required to provide written informed consent prior to participation in the study (see online supplemental appendixs 3; 4). Recipients and their caregivers are informed of unexpected findings or unrecognised conditions and by possible future usage of their specimens in ancillary studies by trained physician or nurse; further medical care will be arranged. Study donors received financial compensation to pay for the required travelling costs when donating the stool. The patient will be offered a roll-over into an observational study with the administration of active microbiota. The patients are informed of this option at the start of the study and regularly reminded.

We aim to publish findings in impact peer-reviewed international journals. Gastroenterologists, internists and other careproviders will be informed through the national conference meetings, journals and patient groups meetings.

Protocol amendment number:

01. Modification of the study protocol will be communicated to the ethics committee.

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Freezing of faeces dramatically decreases the viability of *Blastocystis* sp. and *Dientamoeba fragilis*

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Dear Editor,

Faecal microbiota transplantation (FMT) is considered a well-tolerated method associated with only rare and mostly mild adverse effects [1]. However, the first transmission of *Blastocystis* via FMT to patients with recurrent *Clostridioides difficile* infection was reported by Terveer *et al*. [2].

They observed an apparent lack of *Blastocystis*-related complications, which led the authors to challenge the current guidelines that exclude *Blastocystis*-positive subjects from becoming FMT donors [3]. Moreover, *Blastocystis* and *Dientamoeba fragilis* seem to be associated with higher bacterial diversity, considered a marker of a healthy gut microbiome [4,5], and thus may be an asset for FMT donors rather than a risk. Regardless of function in the recipient's gut, more importantly, the protocol [2] used deep freezing of the donor stool, which we believe is one of the reasons for the favourable outcome of the recipient. The lack of symptoms after the transfer may have resulted not only from the nonpathogenicity of *Blastocystis* but also from its radically diminished viability following the deep freeze-thaw cycle.

To test our hypothesis of the decreased viability of *Blastocystis* and *D. fragilis* after deep freezing, we inoculated the positive donor's stool into Robinson and Dobell media [6] before (i.e. positive control sample) and after one cycle of overnight deep freezing at 80 °C and thawing (i.e. test sample). A positive stool sample was from a healthy FMT donor identified within our ongoing clinical trial on FMT (NCT04899869). This donor was repeatedly positive for *Blastocystis* and *D. fragilis* by cultivation followed by microscopy and real-time PCR (detailed protocols are available upon request). The experiment was repeated five times, each time with a different stool sample from the same donor. The positivity of the donor's stool for both parasites was always verified before culturing using real-time PCR.

In both culture media, all five control samples were consistently positive for both parasites. In contrast, after a single freeze-thaw cycle, *Dientamoeba* was no longer detectable by culture, and *Blastocystis* was found only once (Table 1). In addition, before inoculation into the media, pairs of fresh and freeze-thawed stool aliquots contained similar amounts of DNA of the respective protists (Table 1), indicating that viability, that is multiplication of the protist, is adversely affected by the freeze-thaw cycle.

We recorded a positive culture of *Blastocystis* in one of five frozen replicates, consistent with its previously reported transmissibility [2]. Given the unclear pathogenicity of *Blastocystis* and *Dientamoeba* [4] in parallel with our results of deep freezing, which seriously compromise the viability of both protists, a discussion on the current FMT recommendation should be opened [3]. Furthermore, if future studies confirm the safety of frozen-thawed FMT, the practice of excluding donors positive for *Blastocystis* sp., *D. fragilis* or both should be revised.

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Conflict of interest

There are no conflict of interest.

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Table 1. Detection of *Blastocystis* sp. and *Dientamoeba fragilis* from pairs of fresh[a] vs. frozen stool aliquots

Sample number, date of defaecation	Aliquot of stool inoculated into the culture	Content of protist DNA (Ct) in the stool before culture		Presence/absence of protists evaluated microscopically in cultures			
		Blastocystis sp.	*D.fragilis*	*Blastocystis sp.* Robinson medium	*Blastocystis sp.* Dobell medium	*D.fragilis* Robinson medium	*D. fragilis* Dobell medium
1: 17 Mar 2021	Fresh	17.75	13.56	++	++	++	+
	Frozen-thawed	16.53	14.49	Negative	Negative	Negative	Negative
2: 14 Apr 2021	Fresh	18.26	15.58	++	++	++	++
	Frozen-thawed	17.43	16.57	Negative	Negative	Negative	Negative
3: 21 Apr 2021	Fresh	16.78	14.24	++	++	++	+
	Frozen-thawed	18.00	16.01	Negative	Negative	Negative	Negative
4: 26 Apr 2021	Fresh	15.62	14.37	++	++	++	+
	Frozen-thawed	17.47	16.10	Negative	Negative	Negative	Negative
5: 12 May 2021	Fresh	16.76	13.17	+++	+++	++	++
	Frozen-thawed	17.37	15.64	+	++	Negative	Negative

Semiquantitative microscopy evaluation of the culture: +, detectable; ++, fair quantity; +++, massive quantity; Ct, threshold cycle of the PCR from the sample before culture. Lower Ct indicate higher content of the protist's DNA.
[a]fresh aliquot, a stool sample inoculated before freezing, i.e. control sample.

PLOS ONE

RESEARCH ARTICLE

Stool metabolome-microbiota evaluation among children and adolescents with obesity, overweight, and normal-weight using ^{1}H NMR and 16S rRNA gene profiling

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Abstract

Characterization of metabolites and microbiota composition from human stool provides powerful insight into the molecular phenotypic difference between subjects with normal weight and those with overweight/obesity. The aim of this study was to identify potential metabolic and bacterial signatures from stool that distinguish the overweight/obesity state in children/adolescents. Using ^{1}H NMR spectral analysis and 16S rRNA gene profiling, the fecal metabolic profile and bacterial composition from 52 children aged 7 to 16 was evaluated. The children were classified into three groups (16 with normal-weight, 17 with overweight, 19 with obesity). The metabolomic analysis identified four metabolites that were significantly different ($p < 0.05$) among the study groups based on one-way ANOVA testing: arabinose, butyrate, galactose, and trimethylamine. Significantly different ($p < 0.01$) genus-level taxa based on edgeR differential abundance tests were genus *Escherichia* and *Tyzzerella* subgroup 3. No significant difference in alpha-diversity was detected among the three study groups, and no significant correlations were found between the significant taxa and metabolites. The findings support the hypothesis of increased energy harvest in obesity by human gut bacteria through the growing observation of increased fecal butyrate in children with overweight/obesity, as well as an increase of certain monosaccharides in the stool. Also supported is the increase of trimethylamine as an indicator of an unhealthy state.

The items included there are: 1) S1 Table 1 (as referenced in the manuscript) containing the individual participant's gender, age, BMI, BMI z-score, as well as the kilocalorie and macronutrient daily percentage composition one and two days prior to sampling; 2) unrarefied source data for the 16S rRNA gene sequencing analysis; 3) Metabolite concentrations in mg/g derived from Chenomx NMR Suite version 7.5 for each of the 52 study participants; and 4) the 1H NMR spectra for the 52 study participants.

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Competing interests: The authors have declared that no competing interests exist.

Introduction

The proportion of children and adolescents aged 5 to 19 considered overweight has risen globally from approximately 1 in 10 in the mid-1970s to about to about 1 in 5 in 2016 [1]. There is strong evidence of a close relationship between childhood overweight/obesity and multiple comorbidities which, collectively, reduce life expectancy and increase mortality. This has become an emerging public health problem that has attracted the wide attention of researchers [2–4]. Identifying potential biomarkers in pediatric populations via metabolomics and 16S rDNA profiling can provide an opportunity not only to identify these conditions, but to find potential prevention and treatment approaches.

Despite inter-individual differences, approaches using 16S rRNA gene amplicon sequencing (16S rDNA profiling) of fecal samples have shown differing gut bacterial composition between children with obesity and those without [5]. For example, at the phylum level, a high Firmicutes/ Bacteroidetes ratio (decrease in Bacteroidetes, increase in Firmicutes) has been associated with obesity [6–8], and even specific strains such as *F. prausnitzii* have been positively correlated with BMI z-score [8]. Nevertheless, there is inconsistency in these observations and knowledge about the specific gut microbiota members relevant to the characterization of overweight/obesity remain elusive [6, 9, 10]. Furthermore, microbiome studies have thus far tended to focus on adult populations; consequently, compositional and functional differences between children and adult cohorts have not been reported [11].

Although powerful, 16S rDNA profiling falls short in telling us about the functional activities of the gut microbiota [12]. Another omics approach that helps fill this void is metabolomics. Fecal metabolomics in particular reports on the interaction between host, diet, and the microbiota, thus complementing 16S profiling by providing a functional readout of the microbiota [12, 13] and thus providing a characterization of the molecular phenotype [14–16]. Metabolomic studies have observed certain metabolic patterns and signatures as potential biomarkers of obesity. For example, studies using various biofluids have observed that an increased level of branched-chain amino acids (valine, leucine, isoleucine) and of aromatic amino acids (tyrosine, tryptophan, phenylalanine, methionine) appear to characterize the presence, and, in some cases, the propensity for obesity [2, 17, 18]. Nevertheless, further research is necessary to test whether proposed biomarker metabolites can be considered an established and specific metabolic signature [17]. Furthermore, it is important to differentiate metabolomic profile differences between children and adults; for example, one striking observed difference in childhood obesity in contrast to adult obesity is that impairment of fasting glucose levels is usually absent and, if present, it is a delayed finding [2]. A recent example integrating 16S rDNA profiling and metabolomics observed that 67.7% of the fecal metabolome variance was explained by the gut microbiota composition [12] and it has been widely observed that changes in metabolite levels are often associated with the microbiota [2, 5, 17, 19]. This high degree of association between the microbiota and the fecal metabolome makes integration of these two omics technologies a powerful investigative strategy.

This study integrates these two omics approaches. Using stool samples as the analytical matrix and an untargeted approach, we aimed to uncover potential metabolomic and gut bacterial biomarkers of childhood overweight/obesity in a group of 52 Czech children/adolescents aged 7 to 16 years. The analytical platforms used were ^{1}H NMR to evaluate the metabolomic profile and 16S rRNA gene sequencing to assess the gut bacteria composition. The significant results from each were then correlated to better define metabolome-gut bacteria relations.

Methods

Study participants

This is an observational study to characterize differences in the stool metabolome and bacteriome among 52 Czech youth (28 females, 24 males) aged 7 to 16 years classified into three comparison groups (normal-weight, overweight, and obese). The study was performed according to the Declaration of Helsinki and was approved by the Ethical Committee of the University Hospital Královské Vinohrady, reference number LEK-VP / 01/0/2018. All participants and their parents agreed to participate by signing appropriate written informed consents. These written informed consents were provided by the parents for the participation of their children in the study. The inpatient study took place during an eight-week period between late July to late September 2018.

Participant recruitment was carried out by the Olivova Children's Medical Institution (Olivova Dětská Léčebna) in Říčany, Czech Republic, from their then-present patient population. Recruitment criteria consisted of youth who were between 6 and 18 years of age, had no antibiotic intake for the past three months, were not currently taking any medication, and were physically healthy to take part in a physical activity program led by physiotherapists focused on aerobic activity, strengthening, and stretching twice a day on weekdays. The subjects considered overweight and obese were under treatment/rehabilitation of their overweight/obesity condition through diet and physical activity. The control group (normal weight) were youth with either a respiratory disease (chronic upper and lower respiratory cataracts, bronchial asthma, allergies) or with an orthopedic diagnosis (scoliosis, poor posture, patients after surgery). To minimize the potential of these conditions being confounding, those with a respiratory disease had to be in remission (not in an acute stage) whose only treatment was climatotherapy and respiratory physiotherapy. Those with an orthopedic diagnosis were also only receiving physiotherapy. Out of 121 identified youth who met these conditions, 52 of them ultimately agreed (along with their parents) and/or completed the entire study process. Once in the study, all participants were interned at Olivova for an eight-week period during which they underwent a similar physical activity regime and all received a similar diet (same ingredients, same dishes). The portion size for each participant was based on their age, gender, and weight. Those classified as overweight/obese received 30% less kilocalories than recommended for their age and gender, thus placing them on a caloric restriction. It is important to note that the diet was planned by a clinical dietitian, the meals and snacks were prepared by the Olivova cafeteria kitchen, and the composition of each meal (kilocalories, macronutrient composition) was known as determined by the disaggregated ingredients of each meal through the use of the Nutriservis Profi software (https://nutriservis.cz), a database of approximately 5000 ingredients, including over 900 Czech ones. There were three main meals provided (breakfast, lunch, dinner) plus two snacks throughout the day. The meals were based on recipes and foods typically eaten in a standard Czech school and home diet, thus, except for portion control, the meals did not represent an adjustment for most participants. Although the participants and their families agree to comply with the diet, potential leftover food and/or additional intake of other items outside the provided meals could not be accounted for. On average, the meals were composed of approximately 17% protein, 28% fat and 55% carbohydrates. This is a study period average, thus daily percentages were different. Participants were not all sampled at the same time, but throughout the study period with the earliest collections taking place after at least one week of habituation to the prescribed diet. As a result of the selection criteria, a similar physical activity regime, a homogenous diet among the participants, and the same ethnic and geographic background of the participants, the effects of cofounding variables were minimized.

Table 1. Characteristics of the participants per study group (N, OW, OB).

	N	OW	OB	p-value[a]
n	16	17	19	-
Gender	M = 7, F = 9	M = 6, F = 11	M = 11, F = 8	-
Age (years)	11.06 (± 2.46)	11.47 (± 2.24)	10.47 (± 2.37)	0.45
BMI (kg/m^2)	18.05 (± 2.45)	23.93 (± 3.10)	30.17 (± 4.28)	< 0.01
BMI z-score	0.07 (± 0.80)	1.53 (± 0.29)	2.37 (± 0.23)	< 0.01
% Carb. D-1	64.06 (± 5.09)	63.33 (± 3.93)	64.02 (± 4.32)	0.87
% Prot. D-1	18.51 (± 1.70)	19.55 (± 1.68)	19.12 (± 2.07)	0.29
% Fat D-1	17.43 (± 4.93)	17.11 (± 3.12)	16.86 (± 3.78)	0.92
% Carb. D-2	66.46 (± 3.18)	65.13 (± 6.64)	64.96 (± 5.89)	0.69
% Prot. D-2	18.29 (± 4.26)	21.00 (± 3.83)	19.43 (± 2.18)	0.10
% Fat D-2	15.25 (± 3.24)	13.88 (± 4.87)	15.61 (± 4.66)	0.48
kcal D-1	2224.80 (± 309.28)	1574.96 (± 154.58)	1565.42 (± 137.60)	< 0.01
kcal D-2	2066.15 (± 322.74)	1533.68 (± 129.93)	1553.61 (± 77.66)	< 0.01

M = male, F = female; values reported as mean (± standard deviation); D-1 = one day prior to sampling; D-2 = two days prior to sampling.

[a]. p-value based on one-way ANOVA test.

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The participants' body mass index (BMI) standard deviation score (z-score) was derived from age-specific and sex-specific parameters from the Czech National Institute of Public Health [20]. Based on World Health Organization's guidelines [21], these data were used to classify them into three categories: with obesity (OB) Z-scores > 2.00, with overweight (OW) Z-scores > 1.00, and with normal (N) Z-scores ≯ 1.00 and ≮ -2.00. In total, 16 classified with normal-weight (N), 17 with overweight (OW), and 19 with obesity (OB). Table 1 displays, per each of the three study groups, the size, gender, as well as the mean (± standard deviation) for the age, body mass index (BMI), BMI z-score, and the dietary percent macronutrient composition and kilocalorie content one day prior (D-1) and two days prior (D-2) the date of sampling. S1 Table provides similar data per each participant.

Sample collection

Stool sample collection was carried out by the child/adolescents themselves after proper instruction of the use of a disposable kit that consisted of a paper collection surface from which approximately 1 g of stool was collected with a plastic spoon and deposited into a plastic vial. The sample was then given to a nurse and stored at—20 ˚C until it was transported to the analysis lab where it was stored at -80 ˚C until the time of the analysis.

After thawing, three stool aliquots of approximately 200 mg were placed into three 1.5 mL microcentrifuge tubes. One aliquot was lyophilized to estimate the water content. No water content was reported for two N samples, and for three OB samples. These values were then used to normalize metabolite concentrations to water content as described in the statistics section. The other two aliquots were used for the NMR (metabolomic) and 16S rDNA sequencing analysis. Metabolomic analysis was applied to all 52 samples while 16S rDNA profiling was carried out in 47 samples due to insufficient sample amount (2 from the N and 3 from the OW group).

Metabolomic analysis

NMR sample preparation and processing. After thawing the aliquot from the 1.5 mL microcentrifuge tube, 600 μL of ultrapure water was added. This was then vortexed (3000 rpm,

10sec) and centrifuged (17000 x*g*, 10 min) using a fixed angle rotor. The resulting supernatant (540 μL) was transferred to another 1.5 mL microcentrifuge tube, and 60 μL of phosphate buffered saline (PBS, 1.5 M K_2HPO_4 / 1.5 M NaH_2PO_4, 5 mM 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid (TSP) + D_2O, 0.2% NaN_3, pH 7.4) solution was added. The sample was then centrifuged (17000 x*g*, 10 min) using a fixed angle rotor. The resulting supernatant (500 to 550 μL) was transferred to a 5 mm NMR tube and introduced into an NMR spectrometer for analysis.

NMR spectroscopy. ^{1}H NMR spectra were recorded on a 500.23 MHz Bruker Avance III spectrometer at a temperature of 298 K, equipped with a BBFO SmartProbe™ with Z-axis gradients and a 24 slot autosampler (Bruker Biospin, Germany). A standard Bruker noesypr1d (90˚-t1-90˚-dmix-90˚-FID) sequence was used to suppress signals from water molecules, where t1 is a 4 μs delay time and dmix is the mixing time (0.1 s). Acquisition parameters for the spectra were 128 scans, a 16 ppm spectral width collected into 32K data points, an acquisition time of 4 s, and an interscan relaxation delay of 5 s. Automatic routine including tuning, 3D shimming, 90˚pulse calibration and automatic receiver gain setting was run prior to each sample.

NMR data processing and analysis. The Free Induction Decay (FID) obtained were zero-filled to 64 k, Fourier-transformed, manually phased, and baseline corrected and referenced to TSP:0 ppm using TopSpin 3.1 software (Bruker Biospin, Rheinstetten, Germany).

Multivariate analysis (MVA) was carried out via a chemometric approach. The spectra were further manually phased and baseline corrected manually using Whittaker smoother algorithm in MestreNova NMR Suite software package (Ver. 6.0.2, Mestrelab Research, S.L., Spain). Spectra between δ 9.0–0.0 ppm (excluding the residual water region, δ 5.1–4.6 ppm) were reduced into consecutive, non-overlapping bins (buckets) of equal 0.04 ppm widths. Bins were integrated and normalized based on the total sum of the spectral integral. Unsupervised principal component analysis (PCA), and supervised partial least squares discriminant analysis (PLS-DA) were applied to the normalized bins using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca) [22, 23] under the following parameters: no data filtering, sum normalization, no data transformation, pareto scaling. The PLS-DA model was evaluated using a 10-fold cross-validation.

Univariate analysis (UVA) was carried out using a quantitative (deconvolution) approach. Using Chenomx NMR Suite (version 7.5, Chenomx Inc., Edmonton, Canada), fourier-transformed spectra were subject to line broadening of 0.3 Hz, followed by further phase and baseline manual correction. Metabolites were identified via the Chenomx Profiler library, the Human Metabolome Database (http://www.hmdb.ca), and the literature. The metabolite concentrations in mg/dL from Chenomx were adjusted for the sample dilution. Using the individual sample wet weight (g) the concentrations were converted to mg/g, then normalized by the mean water content of the entire data set, and finally Log2 transformed to prevent the dominance of higher abundance metabolites, to decrease the skewness of the data, and to approximate a more normal distribution. Under the hypothesis that there was no significant difference among the three study groups, a one-way analysis of variance (one-way ANOVA) was applied ($p < 0.05$, two-tailed) to the Log2 transformed concentrations. The test accounted for Levene's test for equality of variances and used Tukey's HSD as a post-hoc procedure. For comparison, the false discovery rate (FDR) was also evaluated. Afterwards, Log 2 transformed concentrations of the resulting significant metabolites, after removal of outliers using Tukey's method (above and below 1.5$^{\text{th}}$IQR), were evaluated for the presence and direction of a linear relationship between each metabolite pair and with the z-score through a Pearson correlation ($p < 0.05$). These analyses were carried using R statistical software version 3.6.3. [24].

16S rRNA gene profiling

DNA extraction and qPCR amplification. DNA for 16S rRNA sequencing was extracted from approximately 50–100 mg of unprocessed stool samples using the DNeasy PowerSoil Kit (Qiagen, Germany) per manufacturer's instructions. Extraction's control was performed by qPCR amplification targeting the V4 region of the 16S rRNA gene.

Library preparation and 16S rDNA sequencing. Samples were sequenced in duplicates in a single run. The V4 region of the 16S rDNA gene was amplified using tagged primers by Schloss et al. [25]. using the AccuPrime polymerase blend (Invitrogen, USA). The thermal protocol of the PCR reaction was composed of an initial denaturation at 95˚C for 5 minutes, followed by 30 cycles of 1) denaturation at 95˚C for 15 seconds, 2) primer annealing at 55˚C for 30 seconds and 3) elongation at 68˚C for 1 minute using slow amplification ramp of 1 ˚C per second to reduce chimera formation. A mock community, a mixture of known microbial DNA, was processed along with the research samples. The bacterial mock community was an in-house mixture comprising genomic DNA extracted from cultures of following bacteria, mixed in uneven ratios and frozen in suitably sized aliquots: *Actinomyces odontolyticus*, *Burkholderia cepacia*, *Clostridioides difficile*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Prevotella denticola*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*. The correct identification of the genera (or of species, wherever the V4 region is discriminative) was checked upon completion of the sequencing run. Amplicon size was checked by agarose gel electrophoresis. Amplified libraries of the 16S rDNA gene were purified with Ampure magnetic beads on a Biomek robot (both Beckman Coulter, USA). Purified libraries were then equalized, and pooled. Equalization was based on quantification by a real-time PCR assay using the KAPA library quantification kit (Kapa Biosystems, USA). Data from the qPCR machine were processed by a computer script calculating dilution ratios, and the equalization and pooling was run on a Biomek robot (Beckman Coulter, USA). The final concentration of the pools of 16S rRNA libraries was measured by Qubit dsDNA high-sensitivity assay (Thermo Fisher Scientific, USA). Sequencing was performed on a MiSeq instrument (Illumina, USA) with the sequencing kit for 2x 250 base pairs (Ilumina, USA).

16S rDNA data analysis. The ensuing demultiplexed sequencing reads were first trimmed and filtered by quality, dereplicated to remove redundancy, error rates were estimated, and true sequences inferred from the pooled sequencing reads of the whole run. Then the read pairs were merged, chimeras removed, and amplicon sequence variants (i.e. operational taxonomic units) tabulated by samples. Finally, taxonomic assignment was done using the Silva database version 132. These steps were performed using the DADA2 package [26]. The phylogenetic tree was constructed by the neighbour-joining method followed by generalized time-reversible distances with gamma rate variation implemented in the *phangorn* package [27]. Sequences classified as chloroplasts, archaea, or cyanobacteria were removed. Subsequently, the data were converted into a *phyloseq* object [28] and analyzed.

Alpha-diversity was compared among the N, OW, and OB groups using the Chao1, Simpson, Shannon, and ACE indices. Comparisons were carried out under the null assumption that there was no significant difference ($p < 0.05$) among the three groups. These were carried out at the phylum, family, and genus levels via MicrobiomeAnalyst (https://www.microbiomeanalyst.ca) [29, 30] and *phyloseq* by using the corresponding taxa total abundance after cumulative sum scaling. Due to previously observed association of a high Firmicutes/Bacteroidetes (F/B) ratio with obesity [6–8], the Ln-transformed F/B ratio derived from the relative abundance of these two phyla was tested under the hypothesis that there was no significant difference among the three study groups via a one-way ANOVA ($p < 0.05$) with

Tukey's HSD as a post-hoc procedure using R statistical software version 3.6.3. [24]. For the F/B ratio, samples 19 (N), 36 (OW), and 29 (OB) were excluded due to extreme values ($\pm$ 1.5$^{\flat}$IQR) in the Bacteroidetes counts.

Due to the controversial nature of differential abundance analysis in microbiome research [31, 32] and that no statistical method can fully capture biological phenomena, differential abundance testing was carried out using two techniques: 1) analysis of composition of microbiomes (ANCOM), which has been shown to provide lower false discovery rate (FDR) than comparable methods [32, 33]; and 2) edgeR, which has also displayed relatively lower FDR (although higher than ANCOM) and has been recommended for overall performance and smaller data sets [33–35]. ANCOM was applied to the total abundance table using a significance threshold of $\pm$ 0.8 and was carried out under the null assumption that among the three different groups there was no significant difference in the relative abundance proportion between each taxon pair at a specific taxonomic level. It was carried out at five taxonomic levels (genus to phylum) using the script ANCOM v2.1 [36] in R statistical software version 3.6.3. [24]. EdgeR (significance p $\pm$ 0.01 after FDR correction) was carried out under the null hypothesis that taxa were not differentially abundant among the three study groups using MicrobiomeAnalyst under the following parameters: at least 25% of values having a read count of 4 or greater, a variance > 10% by IQR throughout the experiment, and cumulative sum scaling without rarefaction or transformation.

Correlation of metabolomic and 16S rDNA analyses

Resulting significant genera from the differential abundance analysis were evaluated for the presence and direction of a relationship among themselves and with the significant metabolite concentrations and the z-score through a correlation test. This was also done globally for all the identified metabolites and genera. The Spearman correlation test ($p < 0.05$) was chosen due to the non-normal distribution of the genus level data despite attempted transformations. Due to the challenge of zero values in microbial composition [33, 37], a pseudo-count value of one was added to all read counts, then the relative abundance was derived for analysis. The analysis was carried out using R statistical software version 3.6.3. [24].

Results

Metabolomic analysis

Multivariate analysis (MVA). PCA did not display clear separation among the N, OW, and OB groups. The supervised approach using PLS-DA also failed to show clear separation. Cross-validation Q2 values were negative regardless of the number of principal components, strongly suggesting that the model lacked predictive power or that it was overfitted. This was attributed to noise in the data and a relative small sample size.

Univariate analysis (UVA). Sixty-three distinct metabolites were identified through compound deconvolution (Fig 1). One-way ANOVA identified five significantly different compounds among the three groups: butyrate ($p = 0.016$), arabinose ($p = 0.033$), galactose ($p = 0.036$), trimethylamine (TMA) ($p = 0.044$), and acetate ($p = 0.045$). After application of Tukey HSD post hoc test all compounds, except for acetate ($p = 0.063$), showed a significant difference between the N and OB groups and none showed significance between N and OW and between OW and OB groups. All of these compounds had a higher mean concentration in the OB group compared to the N group (Fig 2). Application of the false discovery rate (FDR) for multiple comparisons suggested that only 44% of these five metabolites would be expected to be significant. Given the study's sample size and not to discard potentially valuable

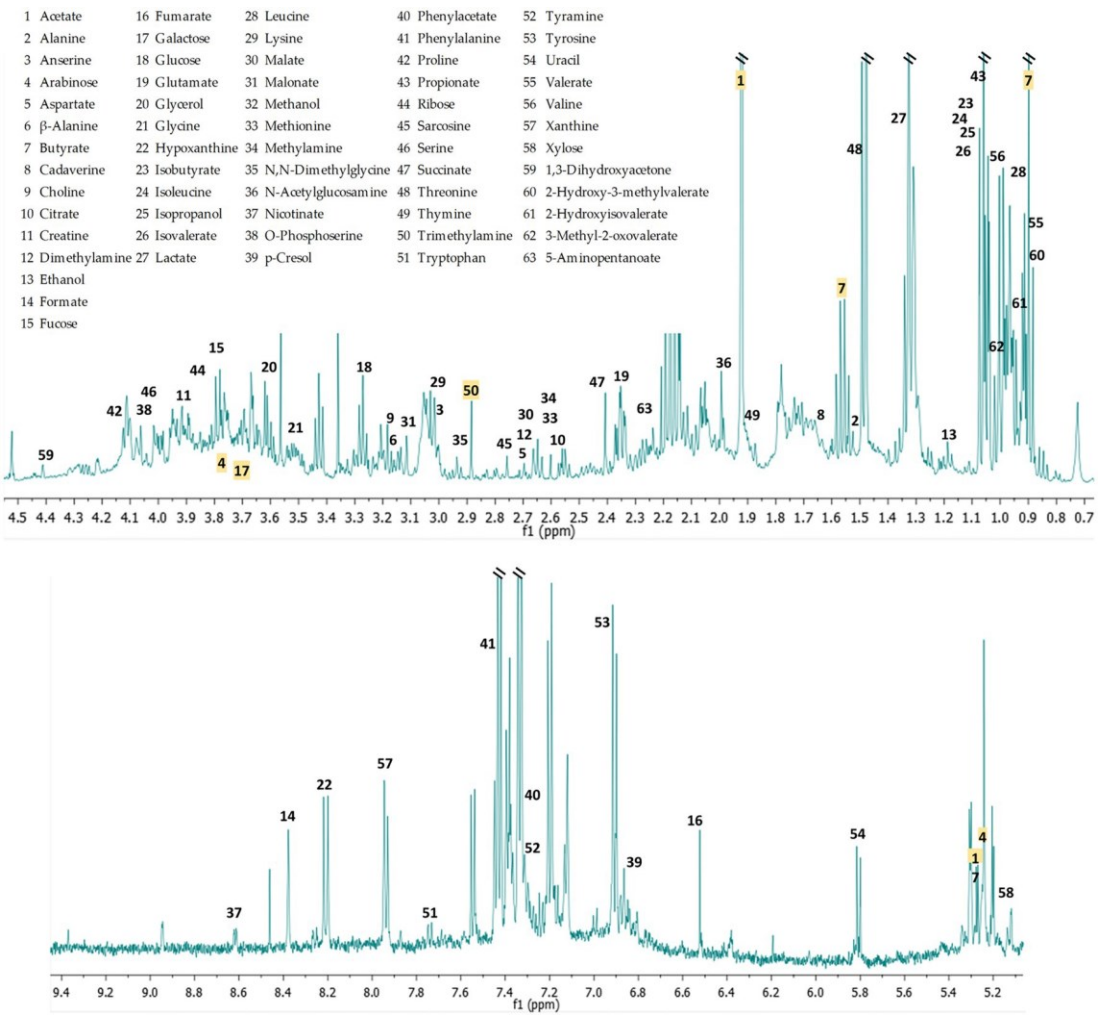

Fig 1. Representative ^{1}H NMR spectrum. 63 identified metabolites. Compounds in yellow are the five significantly different ($p < 0.05$) metabolites based on one-way ANOVA. For visual clarity, with the exception of the five significant metabolites, compounds are only listed once in the spectra regardless of their actual number of spectral peaks. The water region (4.5 to 5.2 ppm) has been excluded.

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metabolites that may be important for generating further hypotheses, we have included the metabolites identified through the Tukey HSD test in the Discussion section.

A Pearson correlation of these metabolites between themselves and the z-score showed the following as significant: z-score with arabinose ($p = 0.050$, correlation coefficient (cc) = 0.31), galactose ($p = 0.014$, cc = 0.38), and TMA ($p = 0.016$, cc = 0.34); acetate with butyrate ($p < 0.001$, cc = 0.73) and TMA ($p = 0.004$, cc = 0.40); and arabinose with galactose ($p < 0.001$, cc = 0.67). These all displayed a positive relationship with the strongest correlations between acetate with butyrate, and arabinose with galactose.

16S rDNA analysis

A total of 83 genus, 36 family, 19 order, 15 class, and 6 phylum level taxa were identified. Fig 3 is a heatmap based on the phyla's relative abundance among the participants in the three study groups. Alpha-diversity assessment at the genus, family, and phylum levels showed no

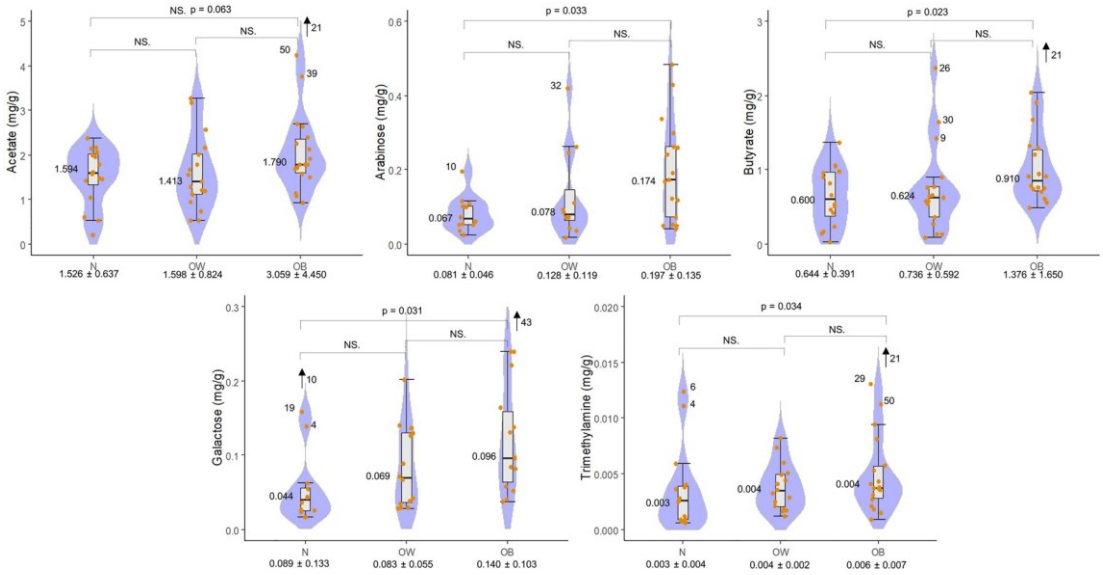

Fig 2. Metabolite concentration (mg/g) boxplots. Significantly different ($p < 0.05$) metabolites among the N, OW, and OB groups based on one-way ANOVA (significance found only between N and OB groups after post-hoc test. Acetate was not significant after application of post-hoc test). The x-axis shows the group name and the mean ± standard deviation. The numbers and text in the graphical area represent: the post-hoc p-value where significant, NS. = not significant, the median and the sample numbers that lie outside the visible rangearea.

https://doi.org/10.1371/journal.pone.0247378.g002

significant differences (all p-values > 0.3) among the N, OW, and OB groups. Likewise, the Firmicutes to Bacteroidetes (F/B) ratio was not significantly different among the three groups by one-way ANOVA testing.

Differential abundance analysis through ANCOM did not identify any significant taxa at any of the taxonomic levels, whereas the more permissive EdgeR identified the following suggestive associations: *Escherichia* (p = 0.005), and *Tyzzerella* subgroup 3 (p = 0.006) at the genus level; the signal from *Escherichia* was reflected also at the family level of *Enterobacteriaceae* (p = 0.009). No differential abundance was noted at the order, class, nor phylum levels. *Escherichia* is one of the main representatives of *Enterobateriaceae* [38], and both taxa displayed a similar relative abundance pattern by showing a decrease from the N to the OB group; in contrast, *Tyzzerella* subgroup 3 showed an increase. Fig 4 shows the genera with the highest

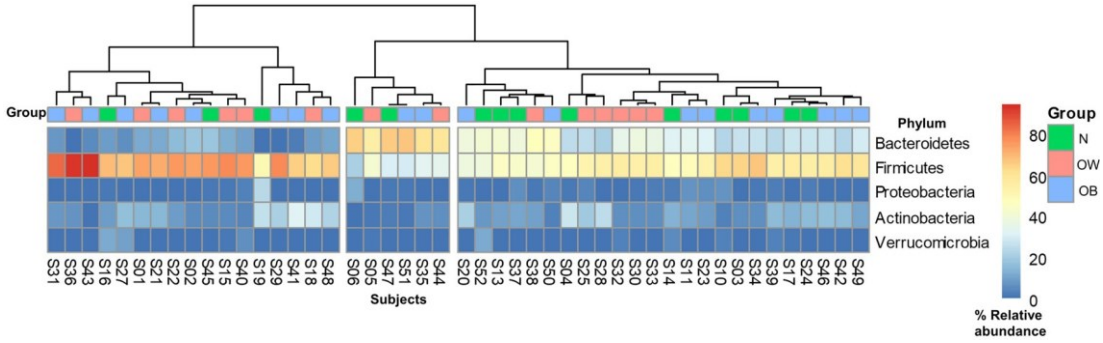

Fig 3. Heatmap based on phyla % relative abundance. Warmer colors indicate higher % relative abundance, which was exhibited by Bacteroidetes and Firmicutes. Cooler colors indicate lower % relative abundance. Inter-individual variability does not display clear clustering among the three study groups.

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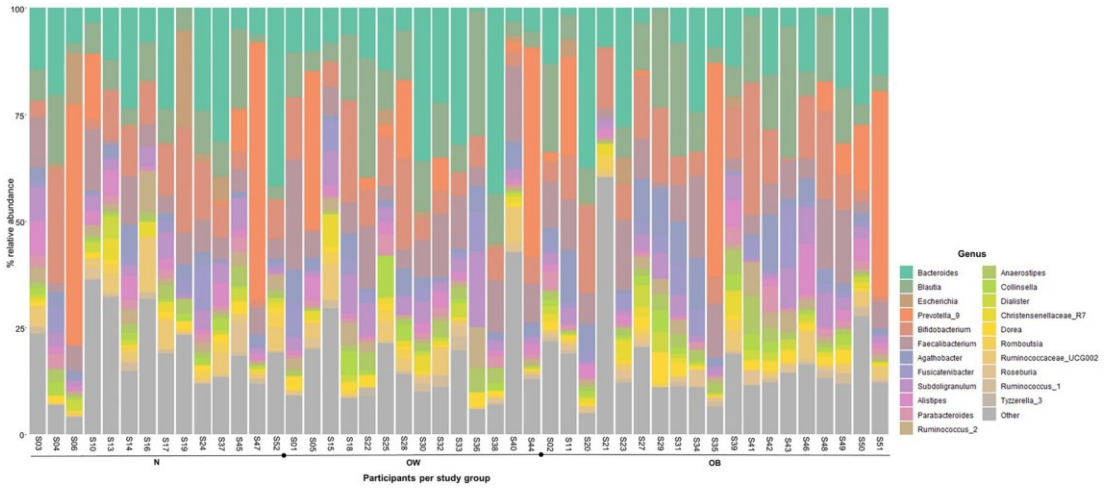

Fig 4. Stacked bar charts based on genera % relative abundance. Display of the 20 most abundant genera (by relative abundance), as well as the two significant genera (*Escherichia* and *Tyzzerella* subgroup 3) by study group. Genus Other represents the aggregate of the remaining 61 identified genera.

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relative abundances, as well as *Escherichia* and *Tyzzerella* subgroup 3. Fig 5 displays the log10 transformed relative abundance for the two significant genera in the three study groups.

Correlation of metabolomic and 16S rDNA analyses

Spearman correlation of the relative abundance of the significant taxa with the concentration of the significant metabolites and with the z-score did not show any positive significant correlations. Nevertheless, as shown in Table 2, strong positive and negative correlations were observed among other significant metabolites and non-significant genera, as well as the reverse. Fig 6 displays the strength of the positive and negative correlations among all identified genera and metabolites.

Discussion

Our results showed an increase of fecal butyrate in the OB compared to the N group, which lends support to previous observations of higher short-chain fatty acid (SCFA) concentrations

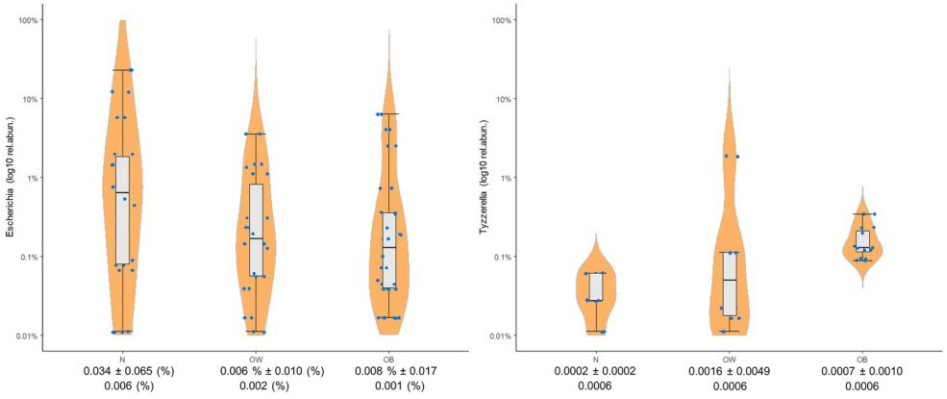

Fig 5. Genera relative abundance boxplots. The two genera that displayed significant difference among the N, OW, and OB groups. The x-axis shows the group name and the mean ± standard deviation, and underneath is the median.

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Table 2. Significant correlations of the significant metabolites with identified genera.

Genus	Butyrate	Arabinose	Galactose	TMA
Blautia			0.425	
Butyricicoccus			0.326	
Butyricimonas		- 0.346		
Catenibacterium				- 0.364
Coprococcus 1	- 0.481			
Coprococcus 3				- 0.303
Desulfovibrio	- 0.413			- 0.428
Eggerthella	- 0.290			
Erysipelotrichaceae UCG-003		0.457	0.450	
Fusinibacter			0.409	
Haemophilus	0.420			
Paraprevotella	- 0.300			- 0.311
Parasutterella				- 0.301
Romboutsia			- 0.339	
Roseburia	0.304			
Ruminoclostridium 5	- 0.477			- 0.398
Ruminoclostridium 6	- 0.317		- 0.327	
Ruminoclostridium 9			- 0.430	- 0.377
Ruminococcaceae NK4A214			- 0.472	- 0.360
Ruminococcaceae UCG-002			- 0.366	
Ruminococcaceae UCG-003			- 0.394	
Ruminococcaceae UCG-010			- 0.355	- 0.354
Slackia	- 0.349			

Values are Spearman correlation coefficients. TMA = trimethylamine.

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in children with overweight/obese compared to those who are normal-weight [8, 39–41]. An increased SCFA concentration, especially butyrate and acetate, has also been observed in obese mice when compared to their lean counterparts [42]. Two suggested reasons for this are: 1) higher substrate fermentation activity by gut microbiota, which translates into increased microbial energy harvest, and/or 2) decreased absorption due to either low-grade inflammation, more rapid gut transit time, or shifts in microbial cross-feeding patterns [8, 41, 43]. Given that the diet among the participants in our study was, except for portion size (OW and OB consumed, on average, 30% fewer kcal than N), approximately homogenous (approx. 17% protein, 28% fat and 55% carbohydrates), it does suggest higher microbial fermentation activity from fermentative substrates such as resistant starch and dietary fiber, the main sources of microbiota-derived SCFAs [5, 8, 44]. Despite consuming less kcal, the OW and OB groups showed significantly more fecal butyrate, which has been identified as the main energy supplier for colonic epithelial cells [8]. It is common for microbiota produced butyrate to end up in stool when not consumed by the colonic epithelium [8]. It has been estimated that SCFAs contribute about 60–70% of the energy requirements of colonic epithelial cells and 5–15% of the total caloric requirements of humans [45]. A proposed mechanism on how an increase in butyrate and other SCFAs may increase energy harvest is that SCFAs may serve as substrates for hepatic de novo lipogenesis (DNL) [42, 46]. The excess non-metabolized SCFAs reach the liver via the portal system, where they may serve as precursors for gluconeogenesis in case of propionate, and lipogenesis for acetate and butyrate [47, 48]. Goffredo et al. (2016) in a study of 84 youth ranging from non-obese to severely obese observed that the three major SCFAs

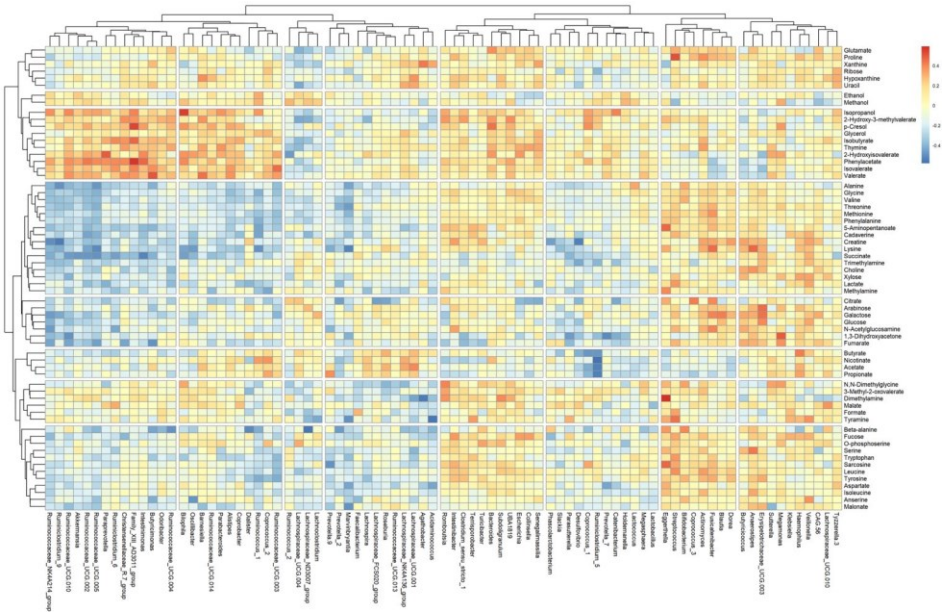

Fig 6. Metabolite-genus spearman correlation heatmap. The x-axis shows the genera and the y-axis the metabolites. Warmer colors indicate positive correlations. Cooler colors indicate negative correlations. The more intense the color, the closer the number is to the Spearman correlation value 1 or -1.

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were positively associated with body and visceral fat, and from these, butyrate was the only one significantly associated with hepatic fat; furthermore, when a subset of this group was tested for associations with DNL, butyrate was significantly associated with a delta increase in hepatic DNL after a controlled dietary carbohydrate load [46]. The same study, using an *in vitro* stool assay, also observed a higher fermentation capability of fructose by youth with obesity compared to nonobese, which further supports the concept of increased energy harvest from food in those youth with obesity [42, 46]. In looking for the potential instigators of these changes, the gut microbiota, it is important to keep in perspective that the SCFA-producing microbes are a phylogenetically diverse group [45], with a wide distribution of enteric bacteria producing acetate; a much more conservative distribution for butyrate, the most well-known being in the Firmicutes phylum (*Faecalibacterium*, *Eubacterium*, *Roseburia*); and for propionate several Firmicutes, Bacteroidetes, and Proteobacteria phyla such as families *Veillonellaceae* and *Lachnospiraceae* [49–51]. The two significant taxa in our study, *Escherichia* and *Tyzzerella* subgroup 3, were not significantly associated with butyrate; nevertheless, the SCFA was significantly associated with nine genera (Table 2). Butyrate's two positive correlations, with *Haemophilus* and *Roseburia*, are supported by several previous studies [7, 49, 50, 52].

Although the higher concentration of SCFAs in children with overweight/obese in several studies [8, 39–41], including butyrate in ours, could suggest them as an obesity biomarker, this is not without controversy given that SCFAs are attributed a myriad of health benefits such as, among others, improvement in blood lipid profiles, glucose homeostasis, and even reduced body weight [7, 53]. How does one reconcile this contradiction? A potential conceptual analogy is that of nutrient overload, a certain nutrient amount may confer benefits, but an excess of it could very well be detrimental. Also, it is important to keep in mind the limitations of each study; for example, the anti-inflammatory effects of butyrate have been studied mainly *in vitro* [45]. In addition, it may be more beneficial to look for biomarkers as part of a panel of biomarkers instead of individual ones as mentioned by Vignoli et al. (2019) [54].

In addition to SCFAs, monosaccharides arabinose and galactose also had higher concentrations in our OW and OB groups, and the two monosaccharides displayed a strong positive correlation with each other, and both showed a significant positive correlation with the BMI z-score. It appears that most monosaccharides in stool often originate from the non-absorbed breakdown of polysaccharides (resistant starches, dietary fiber), which are the main source of carbon and energy for the gut microbiota [55], or directly from the diet which can be used as nutrition by the host's enzymes [56]. A higher concentration of arabinose and galactose in the OW and OB groups may suggest an excess of saccharides from the diet or from the breakdown of polysaccharides which are not absorbed due to energy needs being met without them. Given the controlled diet of the participants, the results thus suggest the origin to be the polysaccharide breakdown. This would directly tie in with the concept of an increased energy harvest by the microbial dysbiosis in the obese state [8, 41, 43], which would result in an excess of monosaccharides as well as a higher load of SCFAs.

Trimethylamine (TMA) also showed higher concentrations in the OW and OB groups. It is known to be produced by various gut microbiota taxa from dietary quaternary amines, mainly choline and L-carnitine derived from eggs, milk, liver, red meat, poultry, shell fish and fish [57–59]. It's considered toxic due to its further oxidation into trimethylamine N-oxide (TMAO), which has been associated with atherosclerosis, cardiovascular diseases, and other ailments [57, 59, 60]. In a previous study, children with obesity showed a decrease of TMA in fecal water after a diet intervention consisting of rich amounts of non-digestible carbohydrates [60]. TMA was also shown to be downregulated in the urine of children supplemented with non-digestible carbohydrates [6, 61]. Other diet induced changes included significant weight loss, structural microbiota changes, a reduction of serum antigen load, and alleviation of inflammation [6]. The identified taxa involved in TMA production appear to constitute members of the core gut community, though at very low abundances and characterized by functional redundancies indicating that several taxa potentially contribute to the TMA pool [60]. The majority of these were members of the genus *Clostridium* XIVa and a specific *Eubacterium* [60]. This potential is further supported by TMA's significant positive correlation with acetate ($p = 0.004$) and the BMI z-score ($p = 0.016$).

Genera *Escherichia* (phylum Proteobacteria), and *Tyzzerella* subgroup 3 (phylum Firmicutes), were not significantly correlated with our significant metabolites, but they were significantly different among the three study groups. In our study, *Escherichia* decreased in relative abundance from the N to the OB group. The genus includes both commensal and pathogenic species, and although species *E.coli* has been observed to be increased in children with obesity compared those with normal-weight [62], a general pattern of this genus in relation to childhood obesity is still very open to investigation. With *Tyzzerella* subgroup 3 we observed a relative abundance increase from the N to the OB group. We could not find any associations between this taxon and obesity in the literature and, overall, this member of *Lachnospiraceae* appears to be of little medical relevance; nevertheless, it has been reported in connection to dietary variables [63], and one study did observe related genera *Tyzzerella* and *Tyzzerella* subgroup 4 to be enriched in a group of adults with higher cardiovascular disease risk when compared to lower-risk subjects [64]. Even though our 16S rDNA analysis only revealed two significantly different genera among the three study groups, it is important to point out, especially for future investigations, that the obese phenotype may be better characterized by the abundance of several distinct communities rather than by the presence of specific species [46]; furthermore, an alteration in efficiency of energy harvest produced by gut bacterial composition changes does not have to be great to contribute to obesity given that small changes in energy balance, over the course of a year, can result in significant changes in body weight [42, 65]. In addition, another possibility is that although inter-group compositional differences

may be minimal, the differences observed in our metabolite data could rather indicate differences in bacterial functional activity where metabolically versatile species adapt to changing nutritional circumstances by selectively metabolizing some substrates to the exclusion of others, thus affecting the types and amounts of fermentation products produced from substrates [55]. An apparent change in microbiota functionality, but not in composition, was observed in a study my Morales et al. (2016) where a high-fat diet accompanied by fiber supplementation induced inflammation while not altering gut microbiota composition [66].

In conclusion, our findings suggest support to the hypothesis of increased energy harvest in obesity by the human gut microbiota through the growing observation of increased fecal butyrate in children with overweight/obesity and an increase of certain monosaccharides in the stool. Also supported is butyrate's positive correlation with *Haemophilus* and *Roseburia*, as well as the increase of trimethylamine as an indicator of an unhealthy state.

Supporting information

S1 Table. Characteristics of the 52 participants. T1 and T2 refer to one and two days prior to the sampling date. The kcal amount is per the entire day.
(XLSX)

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Methodology: José Diógenes Jaimes, Andrea Slavíčková, Jakub Hurych, Ondřej Cinek, Jaroslav Havl´ık.

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Visualization: José Diógenes Jaimes.

Writing – original draft: José Diógenes Jaimes.

Writing – review & editing: Ondřej Cinek, Jaroslav Havl´ık.

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