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Gut microbiota and its metabolic activity in children with intestinal failure dependent on long-term parenteral nutrition

In preparation

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ABSTRACT

Introduction

This study aimed to characterise the gut microbiota composition and its metabolic activity in children with intestinal failure (IF) compared with healthy controls in a longitudinal way and to explore associations with clinical parameters.

Methods

Clinical data and serial fecal samples (n=68) were collected from 15 IF patients (median age 4.3y, dependent on parenteral nutrition (PN) for a median of 3.6y) and single control samples from 25 healthy children. The median time between the first and last sample of each patient was 14 months (IQR 10-21). Fecal microbiota using 16S rRNA gene amplicon sequencing, short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA), D and L isomers of lactate, were measured.

Results

At the first sample, IF patients had lower concentration of total SCFA ($p=0.008$), propionic acid and butyric acid ($p<0.001$) and a higher concentration of D- and L-lactate than healthy controls ($p<0.001$). Patients had a lower total bacterial load (16S rRNA gene copies/g, $p=0.003$); their microbial community was characterised by a lower α-diversity (Shannon index) and evenness (metric of species distribution, both $p<0.001$) and taxon richness (number of distinct species, $p=0.006$) than healthy controls. Patients with surgical IF had lower α-diversity ($p<0.039$) than patients with functional IF. When looking at all samples, the percentage of calories provided by PN (%PN) was negatively associated with microbial diversity. Duration of PN, %PN and fiber intake explained most of the variation in microbial community structure (respectively 6, 6 and 5%). At family level, patients had a significantly higher abundance of Enterobacteriaceae and Staphylococcaceae, and lower abundance of Bacteroidaceae and Bifidobacteriaceae. Two patients weaned off PN; after weaning their microbial structure moved closer to that of the healthy controls.

Conclusions

The microbiota of paediatric IF patients is distinct to that of healthy controls with altered production of SCFA/BCFA, lower bacterial diversity than healthy controls, loss of dominant microbial taxa and increased abundance of sub-dominant and potentially harmful species. Associations between microbial characteristics and clinical parameters associated with PN offer the potential to use the gut microbiota as a biomarker to guide clinical practice during intestinal adaptation.

INTRODUCTION

Patients with intestinal failure (IF) cannot absorb enough nutrients and fluids[1,2] because of a critical reduction of functional gut mass and are therefore dependent on parenteral nutrition (PN). The intestine is either too short, as a consequence of surgical resection or congenital conditions, or dysfunctional despite adequate length.

The gut microbiota plays a key role in fermentation and absorption of nutrients.[3,4] Previous studies have reported an altered gut microbiota composition in patients with IF, including a marked decrease in bacterial diversity[5-7] and an increase in the relative abundance of pathogenic bacteria.[5-10] These compositional shifts in the gut microbiota together with changes in luminal availability of the amount and type of nutrients are likely to influence microbiota metabolism and luminal microenvironment with subsequent consequences to the host. As a result, the metabolism of short-chain fatty acids (SCFA) may change. These are important end-products of fermentation of non-digestible dietary carbohydrates, indirectly contributing to energy for the host, stimulating vascular flow and motility, cell proliferation, differentiation and apoptosis of carcinogenic cells.[11-13] A previous study in children with IF showed that fecal concentration of SCFA acetate was lower in children with IF compared to healthy controls, while there was no difference in propionate, butyrate and total SCFA levels.[7]

Gut microbiota in the light of IF has been associated with adverse clinical outcomes such as bacterial translocation, onset of D-lactic acidosis, central-line associated bloodstream infection, poor growth, and liver disease.[6,7,10] However, most of the previous literature is based on cross-sectional data. In addition, most studies have focused on children with short bowel syndrome; only one study has included children with functional IF.[6] It would be of interest to compare the microbiota between functional IF patients and surgical IF patients, since they have different gastro-intestinal anatomy. Moreover, most functional IF patients are not expected to wean off PN, whereas surgical IF patients might be able to wean off because of the process of intestinal adaptation.

The aim of this study was to prospectively characterise the fecal microbiota of children with IF over time, including the measurement of SCFA, calprotectin and secretory IgA, and relate it with clinical characteristics.

METHODS

Study population

Children stable on home PN (> 3 months) attending the IF team of the Erasmus Medical Center – Sophia Children's Hospital were asked to participate in a prospective observational study. Additionally, 25 otherwise healthy Dutch children were recruited through word of mouth. None of them had undergone previous gastro-intestinal surgery and none of them had received antibiotics for at least two months prior to fecal sample collection. The study was approved by the local research ethical committees (MEC 2015-002, Dutch Trial Register NTR6080) and informed consent of the patients, healthy controls and/or their parents was obtained.

Clinical data

Demographic and clinical data (e.g. underlying disease, duration of PN) were obtained from the medical records. Height and body mass index (BMI) standard deviation score (SDS) were calculated using the latest available Dutch national reference standards.[14] Target height and target height range (±1.6 SDS) were calculated as described previously.[14-16] Percentage PN was used as a measure of PN dependency and was defined as the percentage of total energy intake provided by PN. In addition, we calculated the calories of PN provided, divided by the resting energy expenditure (REE), as calculated by the Schofield formula.[17] Oral nutrition was defined as a normal diet appropriate for age. Patients were considered to suffer from small intestinal bacterial overgrowth if they had associated symptoms (e.g. bloating, abdominal distension, diarrhea) requiring use of antibiotics.

Fecal sample collection

Fecal samples were collected directly from the diaper, the enterostomy, or using a 'feces hat' placed in the toilet and immediately transferred into a sterile tube. We collected samples longitudinally during 2 years, aiming at collecting samples every 3 months if patients were visiting the outpatient clinic. A single fecal sample was collected from the 25 healthy controls.

For microbiota analysis, samples were stored at -80°C and DNA was extracted within a maximum of 2 months of sample collection. For SCFA, fecal samples were homogenized in NaOH 1M w/v and stored at -20°C until analysis. Fecal water content was calculated after lyophilization of the samples.

Calprotectin

Fecal calprotectin concentration, a proxy marker of colonic inflammation, was measured with the Bühlmann ELISA, with normal values of 5-50 µg/g.[18]

Secretory IgA

Secretory IgA in feces was measured with the IDK® sIgA kit (K8870, Immundiagnostik, Bensheim, Germany) and according to the manufacturer specifications, with reported normal values of 510-2040 µg/ml.

Fecal lactate

D and L isomers of lactate were measured in freeze-dried fecal samples using an enzymatic commercial assay (D-lactic acid and L-lactic acid, Boehringer Mannheim Roche) scaled down for use to a 96 microtiter plate (see supplementary methods).

Short-chain and branched-chain fatty acids

Short-chain fatty acids (SCFA; C2-C8) and branched-chain fatty acids (BCFA; iC4-iC6) were measured by gas chromatography (see supplementary methods).[19] Results were presented per gram dry mass of fecal material (µmol/g) and as proportional ratio (%) to total SCFA.

Microbiota

The composition of the gut microbiota was characterised with amplicon sequencing of the V4 region of the 16S rRNA gene. Bacterial DNA was isolated using the bead-beating combined with the chaotropic method.[19,20] The concentration, purity and integrity of DNA were estimated visually by electrophoresis on 1% agarose gel and using Nanodrop™ and Qubit™. Quantification of total bacteria (16S rRNA gene copy number/g feces) was carried out with quantitative PCR.[19] Sequencing of the pooled libraries was performed on the MiSeq (Illumina) platform using 2x250 bp paired-end reads as described previously.[21]

Bioinformatics

Microbiota composition was analysed using operational taxonomic units (OTUs) obtained from the 16S rRNA sequencing data and clustered at a level of 97% similarity. OTUs were generated from the raw data using a modified version of the VSEARCH pipeline (https://github.com/torognes/vsearch/wiki/VSEARCH-pipeline).[22] The paired fastq files were merged together and quality filtering was performed with a fastq_maxee (maximum expected error value for merged sequences) parameter of 0.5. Sequences longer than 275bp and shorter than 225bp were filtered out. The files were then combined, dereplicated, and all singleton sequences were removed. Sequences were preclustered at 98%, and chimeras were identified and removed from the dataset using the VSEARCH implementation of the UCHIME de-novo algorithm.[23] A secondary chimera detection and removal step was carried out, this time using the UCHIME reference based method and the 'Gold' ChimeraSlayer reference dataset.[24] OTUs were then generated by cluster-

ing the remaining sequences at 97%. OTUs were taxonomically classified to genus level using the assignTaxonomy function in the dada2 R package.[25]

Data analysis and statistics

Descriptive statistics were expressed as median and interquartile range (IQR) or range, or as counts with percentages. In order to show raw data and give the opportunity to compare our data with other studies, we present the results of the first sample as well as all samples together correcting for repeated measurements. For group comparisons, Mann-Whitney U, Chi square and Fishers exact test were used. A p-value of < 0.05 was considered statistically significant. For microbiota data, NMDS analysis was carried out using the phyloseq package in R[26] and permutation ANOVA results were found using the Adonis function in the R vegan package.[27] Significantly different OTUs, genera and families were identified using t-tests on the log-proportional abundances of each OTU/genus. In the cases where the variables of comparison included different time points for the same subjects, paired t-tests were used. Benjamini-Hochberg corrections for multiple testing were applied to the resultant p-values.

Generalized linear mixed models (GLMs) were used to identify relationships between clinical parameters and microbial diversity measures; each model was generated using one variable of interest and the subject's age as explanatory variables with the subject ID included as a random effect. The GLM analysis was carried out using the lme4 package in R.[28] Significance thresholds were applied at 0.05 for unadjusted p-values and 0.1 for adjusted p-values. Adjusted p-values are mentioned in the text. All diversity, evenness and richness measures were found using the appropriate functions in vegan. Correlation tests were performed using the cor.test function in R for unpaired data and using the rmcorr function when analysing repeated measures. Statistics were performed using SPSS version 21 (SPSS, IBM, Armonk) and R version 3.4.3.

RESULTS

Participants' characteristics

Fifteen patients were included between June 2015 and September 2017, with a median age of 4.3 years (range 0.7 – 16.6 years) at study enrolment. The healthy controls were comparable to the patients regarding age, BMI SDS and the proportion of boys/girls. Participants' characteristics are shown in **Table 1.** Eight patients had surgical IF and 7 patients functional IF. Fourteen patients had (re-established) intestinal continuity, while 1 patient had an enterostomy due to chronic intestinal pseudo-obstruction syndrome. A significant higher proportion of patients with functional IF had their ileocecal valve in situ ($p = 0.003$). Four patients underwent surgical lengthening procedures; none in the year prior to sample collection.

All patients were PN dependent at the time of the first sample collection with a median PN duration of 3.6 years (IQR 2.0 - 5.0). Two patients (13%) weaned off PN during the study period after a total PN duration of 1.2 and 2.0 years, respectively; one of them had functional IF and one had surgical IF. Twelve (80%) patients had received antibiotics in the last 2 months before the first sample collection, of them 8/8 with surgical IF and 4/7 with functional IF ($p = 0.04$). Two patients received enteral/oral antibiotic treatment due to suspected bacterial overgrowth and three patients received amoxicillin/clavulanic acid as prokinetic agent. None of the patients received probiotics and none developed D-lactic acidosis during the study period, neither did patients develop intestinal-failure associated liver disease. The median follow-up time i.e. time between the first and last sample of each patient was 14 months (IQR 10 - 21, range 4 - 23).

Table 1. Participant's characteristics at first sample for intestinal failure patients, divided into surgical and functional intestinal failure, and healthy controls

Clinical characteristic	All IF patients n = 15	Surgical IF patients n = 8	Functional IF patients n = 7	Healthy controls n = 25
Sex: boys:girls	8:7 (53:47)	5:3 (63:38)	3:4 (43:57)	13:12 (52:48)
Age at first sample	4.3 (0.7-16.6)	6.1 (0.7-9.9)	3.7 (0.7-16.6)	6.6 (1.1-15.4)
Underlying diseases				
Intestinal atresia	3	3	0	
Gastroschisis (with apple peel atresia and volvulus)	2	2	0	
Necrotizing enterocolitis	2	2	0	
Esophageal atresia with motility problems	1	0	1	
Herniation and strangulation of small bowel	1	1	0	
Chronic intestinal pseudo-obstruction syndrome	1	0	1	
Microvillus inclusion disease	1	0	1	
Protein losing enteropathy based on primary intestinal lymphangiectasia	1	0	1	

Table 1. Participant's characteristics at first sample for intestinal failure patients, divided into surgical and functional intestinal failure, and healthy controls (continued)

Clinical characteristic	All IF patients n = 15	Surgical IF patients n = 8	Functional IF patients n = 7	Healthy controls n = 25
Tricho-hepato-enteric syndrome	1	0	1	
Filamin A mutation with pseudo-obstruction	1	0	1	
Unknown cause	1	0	1	
Whole small bowel in situ	5 (33)	0 (0)	5 (71)	
Remaining small bowel length in cm	65 (30-180)	63 (46-103)	180 (NA)*	
Ileocecal valve in situ	9 (60)	2 (25)	7 (100)	
Enterostomy at first sample	1 (7)	0 (0)	1 (14)	
Partial or total colectomy	5 (33)	4 (50)	1 (14)	
Duration of PN until first sample, years	3.6 (2.0–5.0)	4.4 (1.1-7.3)	3.2 (2.0-4.3)	
PN dependency in %	76 (40-100)	62 (38-87)	82 (67-100)	
Type of nutrition**				
PN only	4 (27)	1 (13)	3 (43)	
PN and tube feeding	7 (47)	4 (50)	3 (43)	
PN and oral nutrition	1 (7)	0 (0)	1 (14)	
PN and tube feeding/oral nutrition	3 (20)	3 (38)	0 (0)	
Mode of tube feeding**				
Continuous	6 (40)	3 (38)	3 (43)	
Bolus	3 (20)	3 (38)	0 (0)	
Combination of continuous and bolus	1 (7)	1 (13)	0 (0)	
Type of tube feeding				
Polymeric	2 (13)	2 (25)	0 (0)	
Semi-elemental	7 (47)	5 (63)	2 (29)	
Elemental	1 (7)	0 (0)	1 (14)	
Antibiotic use 2 months before 1st sample	12 (80)	8 (100)	4 (57)	0 (0)
Antibiotic use at sample because of suspected bacterial overgrowth	2 (13)	5 (63)	2 (29)	NA
Antibiotic use at sample as motility agent	3 (20)	2 (25)	1 (14)	NA
Proton pump inhibitor use	11 (73)	5 (63)	6 (86)	0 (0)
BMI SDS	0.34 (-0.11-1.29)	0.03 (-0.63-0.53)	1.14 (0.40-1.48)	0.07 (-0.67-0.77)

Legend: Values shown as median (IQR) or n (%) unless stated otherwise. * for one patient the small bowel length was not known. ** minimal enteral feeding not included.
Abbreviations: BMI, body mass index; IF, intestinal failure; IQR, interquartile range; PN, parenteral nutrition.

SCFA, BCFA, lactate, sIgA and calprotectin at first sample

A total of 68 fecal samples were collected (median of 3 samples per patient, range 1-10). At the first sample, IF patients had lower concentration of total SCFA (210 µmol/g versus 472 µmol/g per gram dry feces, p = 0.008), propionic acid (7.7 µmol/g versus 64 µmol/g, p < 0.001) and butyric acid (2.0 µmol/g versus 54.3 µmol/g, p < 0.001) than healthy controls (**Table 2, Figure 1**). The median acetic acid level was not different between IF patients and controls, but the proportion of acetic acid was higher in patients than in controls (p < 0.001). Patients had a higher concentration of D- and L-lactate than healthy controls (total lactate levels of 3739 µg/g versus 256 µg/g per gram dry feces; p < 0.001). Patients with surgical IF had median total lactate levels of 4577 µg/g, 2525 µg/g for L-lactate and 4578 µg/g for D-lactate, whereas these levels were 518 µg/g, 287 µg/g and 298 µg/g for functional IF respectively.

Since patients with IF had significant higher water content, we choose to express data per gram of dry feces. Expressing the same data per mass of wet feces, however, produced similar results (**Supplementary Table 1**). Likewise, when comparing the last sample collected from each participant with the healthy controls and when correcting for sex, age and BMI-SDS, results were generally the same (**Supplementary Table 2**).

Fecal secretory IgA and calprotectin concentrations were only measured in patients and not in the healthy controls. Due to sample availability, calprotectin values were available for 12 patients (80%) at the first sample. Median calprotectin value was 34.95 µg/g (IQR 19.5 - 221, range 19.5 - 814); 7 patients had a calprotectin level < 50 µg/g. The median secretory IgA level at the first sample was 3352 µg/mL and 10 out of 12 patients (83%) had secretory IgA levels above the manufacturer's normal range.

Gut microbiota

We extracted DNA from 66 fecal samples from 14 patients, since for 1 patient the sample amount was inadequate. Four samples could not be amplified, leading to a total of 62 included.

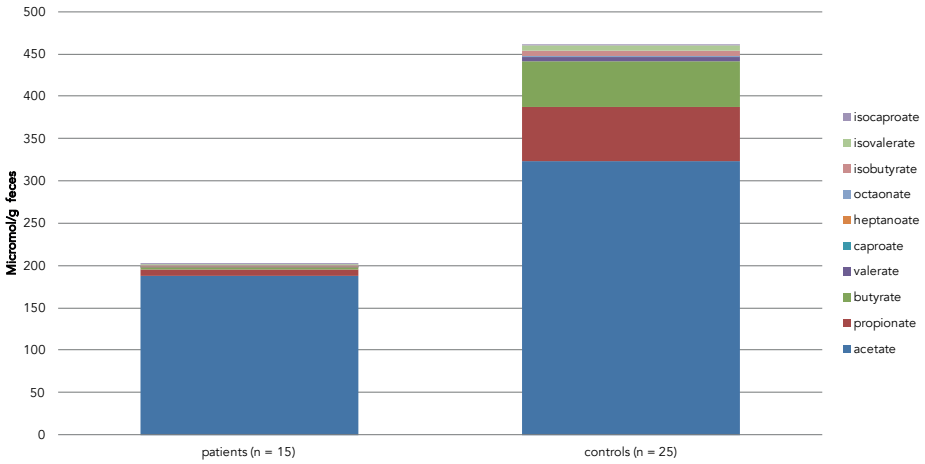
In addition, DNA was extracted from 25 healthy control samples. All these samples were sequenced, of which 7 twice. In total, this yielded 10,382,519 reads, an average of 110,452 reads per sample prior to quality filtering. Twenty percent of the reads were discarded during quality filtering leaving 8,306,581 reads in total. After repeated samples were combined, all samples had greater than 5000 reads and it was agreed that this was sufficient for all samples to be included in the downstream analysis. After OTU clustering, there were 1129 OTUs, 8,306,581 reads, and 87 samples.

Table 2. Fecal water content, concentration of SCFA, BCFA, lactate, secretory IgA, calprotectin and number of 16S rRNA gene copies for IF patients and healthy controls at first sample

	n	Patients with IF n = 15	n	Healthy controls n = 25	p-value
Fecal water content (%)	14	83 (66-87)	25	65 (62-74)	p = 0.011
SCFA (per gram dry feces)	15		25		
Acetic acid (C2), µmol/g		188 (86.8-515)		323 (266-370)	p = 0.074
%		91.8 (83.4-94.4)		67.6 (64.7-61.3)	p < 0.001
Propionic acid (C3), µmol/g		7.73 (1.03-18.6)		64.0 (47.5-85.1)	p < 0.001
%		3.64 (1.19-8.15)		13.7 (10.6-18.8)	p < 0.001
Butyric acid (C4), µmol/g		2.04 (1.08-18.4)		54.3 (36.6-71.0)	p < 0.001
%		0.96 (0.73-4.10)		11.6 (5.60-14.9)	p < 0.001
Valeric acid (C5), µmol/g		0.19 (0.11-5.66)		4.94 (2.04-9.56)	p = 0.001
%		0.18 (0.07-0.65)		1.39 (0.36-2.26)	p = 0.002
Caproic acid (C6), µmol/g		0.45 (0.33-0.62)		0.51 (0.26-3.72)	p = 0.046
%		0.21 (0.07-0.39)		0.12 (0.08-0.78)	p = 0.912
Heptanoic acid (C7), µmol/g		0.71 (0.44-0.87)		0.07 (0.04-0.17)	p = 0.001
%		0.39 (0.11-0.54)		0.01 (0.00-0.05)	p < 0.001
Octanoic acid (C8), µmol/g		0.09 (0.00-0.68)		0.17 (0.04-0.34)	p = 0.659
%		0.05 (0.00-0.37)		0.01 (0.01-0.08)	p = 0.761
Total, µmol/g		210 (103-618)		472 (397-592)	p = 0.008
BCFA (per gram dry feces)	15		25		
Iso-butyric acid (iC4), µmol/g		0.82 (0.18-3.67)		6.40 (3.73-10.2)	p < 0.001
%		0.21 (0.13-1.00)		1.42 (0.92-2.17)	p = 0.003
Iso-valeric acid (iC5), µmol/g		1.05 (0.11-5.66)		6.27 (3.48-10.2)	p < 0.001
%		0.44 (0.13-1.05)		1.18 (0.82-14.9)	p = 0.006
Iso-caproic acid (iC6), mol/g		0.46 (0.18-1.05)		0.35 (0.26-0.48)	p = 0.201
		0.18 (0.09-0.31)		0.07 (0.05-0.09)	p = 0.002
D-lactate, µg/g dry feces	8	1815 (485-7107)	24	79 (58-156)	p < 0.001
L-lactate, µg/g dry feces	8	1923 (464-3675)	24	211 (102-257)	p < 0.001
Total lactate, µg/g dry feces	8	3739 (898-11157)	24	256 (193-376)	p < 0.001
% D-lactate per gram dry feces	8	48 (42-57)	24	33 (19-50)	p = 0.023
Secretory IgA, µg/mL	12	3352 (2340 – 6183)	NA	NM	NA
Calprotectin, µg/g (per gram wet feces)	12	35.0 (19.5-222)	NA	NM	NA
Log of 16S rRNA gene copy per g dry feces (IQR, range)	14	10.7 (9.92-10.9, 0.53-11.5)	25	11.1 (10.9-11.3, 10.7-11.7)	p = 0.003
Log of 16S rRNA gene copy per g wet feces (IQR, range)	14	1.96 (1.60-3.87, 1.28-9.93)	25	3.82 (3.33-4.37, 2.6-18.4)	p = 0.015

Legend: Values shown ad median (IQR) or n (%) unless stated otherwise.
Abbreviations: BCFA, branched-chain fatty acids; IF, intestinal failure; IQR, interquartile range; NA, not applicable; NM, not measured; SCFA, short-chain fatty acids.

Figure 1. Stacked bar chart showing the median levels of short and branched-chain fatty acids (in µmol/g dry feces) for patients and healthy controls



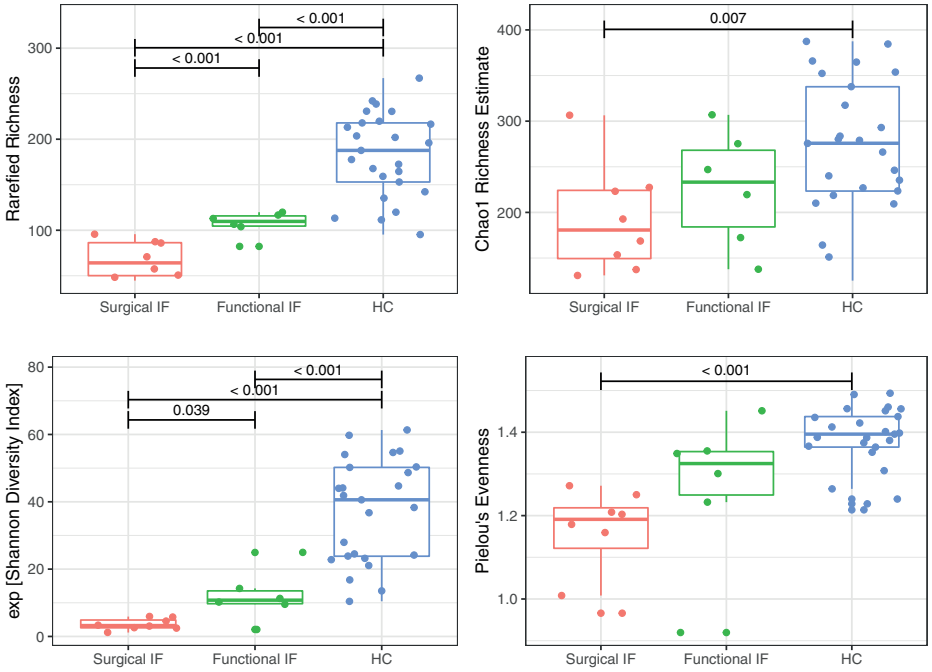
The total bacterial load (16S rRNA gene copies per gram of dry or wet feces) was lower in patients than healthy controls ($p = 0.003$ and $p = 0.015$ respectively). The microbial community structure of IF patients was characterised by a lower Shannon diversity ($p < 0.001$), taxon richness (Chao richness, $p = 0.006$) and evenness (Pielou's evenness, $p < 0.001$) than healthy controls (**Figure 2**). There was no difference in total bacterial load between surgical and functional IF patients. Patients with surgical IF had a lower rarefied richness ($p < 0.001$) and Shannon diversity ($p = 0.039$) than patients with functional IF.

The microbial community structure of IF patients was distinct, clustered separately and presented a higher degree of inter-individual variation from that of healthy controls ($p = 0.002$) (**Figure 3a and b**). Similar analysis was observed using weighted UniFrac distances (**Figure 3c**). Within the IF group, patients with surgical IF tended ($p = 0.009$) to cluster separately from those patients with functional disease whose community structure was less dissimilar and less distant to healthy controls (**Figure 3d**).

Bacteria identified in the fecal samples from patients and healthy controls included those from the 6 dominant phyla of the gut microbiota including Firmicutes, Proteobacteria, Bacteriodetes, Actinobacteria, Verrucomicrobia and Fusobacteria. However, the relative abundance of several phyla was different when compared to controls. IF patients had increased relative abundance of Proteobacteria, whereas they had decreased relative abundance of Bacteroidetes and Verrucomicrobia (**Figure 4**).

At the first sample, the microbiota of IF patients at OTU level was characterised by a higher abundance of taxa belonging to Escherichia-Shigella ($p = 0.006$), Cronobacter ($p = 0.001$) and Staphylococcus (OTU 14, $p < 0.001$) than healthy controls (**Figure 5**).

Figure 2. Alfa diversity in patients with surgical (n = 8) and functional (n = 7) intestinal failure and healthy controls (HC, n = 25)

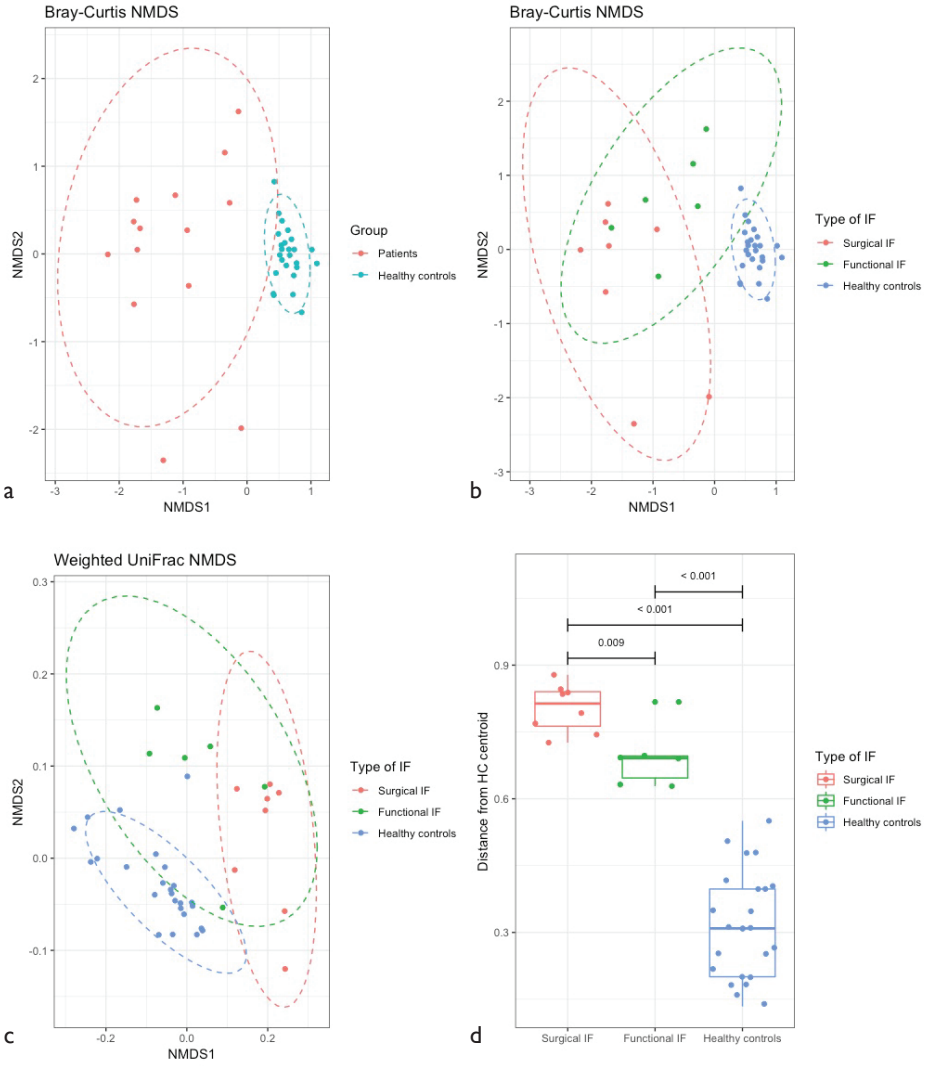


Abbreviations: HC, healthy controls; IF, intestinal failure.

IF patients had a lower abundance of taxa belonging to Faecalibacterium (OTU 114 and 31, p < 0.001) and Ruminococcus 1 and 2 (OTU 83, 167, 262, 42, 119 and 64, p < 0.001). At family level, patients had significant more Enterobacteriaceae (p = 0.001) and Staphylococcaceae (p = 0.001), whereas they had less Bacteroidaceae (p = 0.013) and Bifidobacteriaceae (p = 0.004) (**Supplementary Figure 1**). There were no significant differences in taxon relative abundance at OTU and family level between patients with surgical and functional IF.

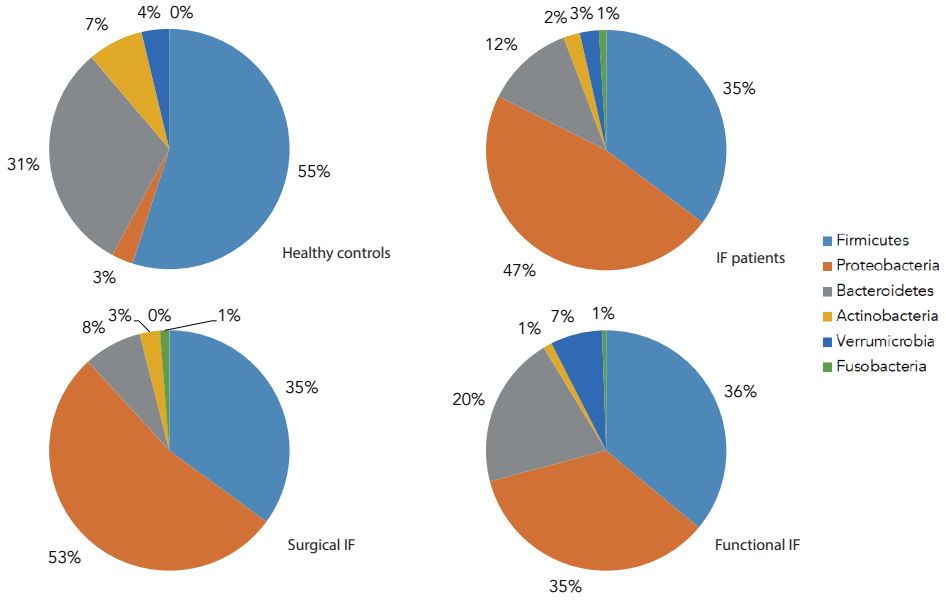
Analysing the data for all samples showed similar results (**Supplementary Table 3 and 4)**. When comparing surgical IF to functional IF patients, surgical IF patients had a higher abundance of taxa belonging to Lactobacillus (OTU 18, 166 and 201, p = 0.003; OTU 38, p = 0.019; OTU 17, p = 0.020; OTU 4, p = 0.037) and Cronobacter (p = 0.020), whereas functional IF patients had a higher abundance of taxa belonging to Lachnoclostridium (OTU 16, p = 0.035l; OTU 45, p = 0.009; OTU 84, p = 0.004 and OTU 22, p = 0.003), Ruminococcaceae (OTU 69, p = 0.012) and Blautia (OTU 93, p = 0.033; OTU 26 and 71, p = 0.031) (**Supplementary Table 5 and 6**).

Figure 3. Non-metric multidimensional scaling (NMDS) of operational taxonomic unit (OTU) community structures for a. intestinal failure patients (n = 15) and healthy controls (n = 25) at the first sample, and b. surgical (n = 8) and functional (n = 7) intestinal failure patients and healthy controls at the first sample. Samples that are clustered closely together are considered to be more similar in terms of microbial species composition than samples that are more separated. c. Weighted UniFrac NMDS of OTU community structures for surgical and functional IF patients and healthy controls at the first sample. d. Bray-Curtis distances from healthy centroid for surgical and functional intestinal failure patients and healthy controls at the first sample.



Abbreviations: IF, intestinal failure; NMDS, non-metric multidimensional scaling; OTU, operational taxonomic unit.

Figure 4. Pie charts representing the major bacterial phyla for surgical, functional and all intestinal failure patients and healthy controls (for all samples)



Abbreviation: IF, intestinal failure.

Gut microbiota and associations with clinical parameters

Using a univariate mixed model to account for the repeated measure design, we analysed associated measures of α-diversity in context with clinical metadata. In all samples analysis, the percentage of PN was negatively associated with Shannon diversity (**Table 3**), and having the whole small bowel in situ was positively associated with Chao richness and rarefied richness. The duration of PN was not significantly associated with % or absolute amounts SCFA and BCFA. The use of antibiotics at or between sample collection was negatively associated with absolute c3, ic4, c4 and c5 levels (**Supplementary Table 7**). The oral/enteral fibre intake was positively associated with absolute levels of c2, c3 and ic5 (**Supplementary Table 8**). Regarding D- and L-lactate, percentage of PN was positively associated with L-lactate (**Supplementary Table 9**). Duration of PN (y) and %PN explained respectively 5.5% and 6.3% of the variation in microbial community structure ($p < 0.01$), and fiber intake (g/kg) 4.8% ($p = 0.01$) (**Table 4**).

Clinical variables associated with OTUs are shown in **Supplementary Table 10**. OTUs belonging to the Genus Bacteroides were positively related to oral nutrition (OTU 13, $p = 0.015$ and OTU 28, $p = 0.032$) and oral/enteral fibre intake (OTU 99, $p < 0.001$, OTU 145 and 32, $p = 0.001$, OTU 13, $p = 0.008$).

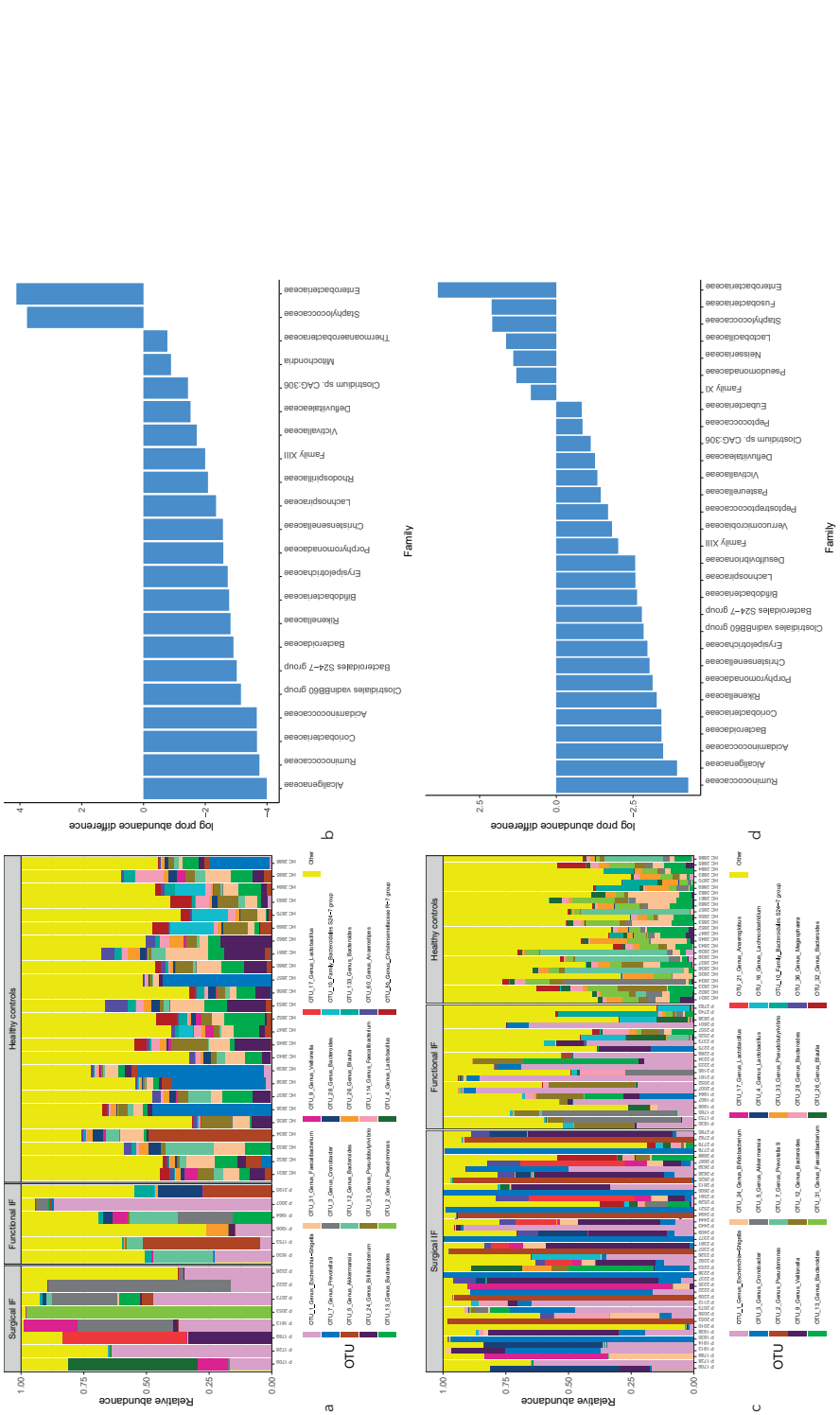


Figure 5.
a. Taxonomic composition of microbiota of paediatric IF patients (n = 15) and healthy controls (n = 25) at operational taxonomic unit (OTU) level for the 20 most abundant OTUs at the first sample
b. Microbial communities in children with IF showing relative abundance of most common taxonomic families at the first sample
c. Taxonomic composition of microbiota of paediatric IF patients and healthy controls at OTU level for the 20 most abundant OTUs for all samples
d. Microbial communities in children with IF showing relative abundance of most common taxonomic families for all samples
Abbreviations: IF, intestinal failure; OTU, operational taxonomic unit.

Table 3. General linear mixed model for clinical variables and Shannon diversity index, Chao richness, Pielou's evenness and rarefied richness

	Shannon diversity		Chao richness		Pielou's evenness		Rarefied richness	
	Beta coefficient	unadjusted p-value adjusted p-value	Beta coefficient	unadjusted p-value adjusted p-value	Beta coefficient	unadjusted p-value adjusted p-value	Beta coefficient	unadjusted p-value adjusted p-value
Nutrition								
Duration of PN (years)	-0.830	0.230 0.688	-4.121	0.306 0.729	0.005	0.729 0.807	-3.991	0.119 0.618
Type of nutrition (PN only, PN+tube feeding, PN+oral nutrition±tubefeeding, tubefeeding/oral nutrition)	0.027 1.855 17.556	0.000 0.001	-45.370 -4.393 40.439	0.021 0.079	0.103 0.059 0.167	0.213 0.300	-28.157 -2.264 44.126	0.001 0.016
Percentage of PN (%)	-0.127	0.001 0.026	-0.333	0.310 0.418	-0.001	0.087 0.180	-0.259	0.164 0.268
Calories of PN divided by REE (%)	-8.132	0.002 0.047	-24.672	0.192 0.298	-0.097	0.073 0.227	-18.620	0.122 0.265
Oral nutrition (yes/no)	6.099	0.021 0.318	37.645	0.046 0.427	.028	0.595 0.768	27.687	0.018 0.318
Oral/enteral fibre intake per kg (g/kg)	20.110	0.017 0.135	-85.295	0.083 0.251	.350	0.024 0.768	-18.263	0.616 0.318
Tube feeding (yes/no)	1.491	0.614 0.732	-39.406	0.045 0.349	.090	0.133 0.474	-18.077	0.153 0.474
Tube feeding type (polymeric, semi-elemental, elemental)	-8.885 8.067	0.009 0.103	10.142 96.316	0.015 0.103	-0.217 -0.005	0.160 0.552	-1.360 61.058	0.017 0.103
Mode of tube feeding (continuous, bolus, both)	-1.965 -3.15	0.806 0.957	-29.706 -35.070	0.290 0.786	-0.031 0.004	0.919 0.957	-23.968 -21.712	0.242 0.786
Gastro-intestinal characteristics								
Whole bowel in situ (yes/no)	7.470	0.052 0.146	69.990	0.000 0.002	0.091	0.282 0.514	43.646	0.000 0.003
Remaining small bowel length (cm)	-0.002	0.970 0.989	-0.011	0.956 0.989	0.001	0.516 0.842	-0.277	0.041 0.605

Ileocecal valve in situ (yes/no)	8.857	0.016 0.098	55.728	0.013 0.098	.048	0.565 0.802	40.515	0.005 0.051
Partial or total colectomy (yes/no)	-4.757	0.194 0.460	-45.038	0.014 0.224	-0.049	0.537 0.716	-31.319	0.008 0.224
Growth								
BMI SDS	-0.489	0.731 0.872	-17.236	0.157 0.852	0.022	0.463 0.852	-5.195	0.507 0.852
Height-for-age SDS < -2 (yes/no)	19.154	0.004 0.044	110.996	0.008 0.059	.134	0.370 0.704	76.935	0.003 0.044
Growing outside target height range (yes/no)	18.328 -4.221	0.013 0.136	112.661 9.482	0.028 0.215	.113 -0.121	0.489 0.722	74.499 -12.973	0.012 0.136
Medication use (yes/no)								
Proton pump inhibitor	-8.814	0.003 0.024	-0.212	0.991 0.991	-0.132	0.029 0.089	-14.412	0.261 0.427
Motility agents	-7.404	0.042 0.087	-14.523	0.501 0.648	-0.233	0.001 0.012	-19.686	0.172 0.267
Cholestyramine	16.201	0.000 0.000	15.310	0.542 0.647	.017	0.772 0.825	39.810	0.064 0.241
Ursochol	-21.685	0.000 0.001	-105.421	0.011 0.080	-0.056	0.543 0.836	-76.505	0.014 0.080
Treatment of bacterial overgrowth	-5.109	0.321 0.623	-28.663	0.321 0.623	-0.038	0.726 0.833	-13.549	0.486 0.696
Antibiotics at sample*	-1.510	0.566 0.784	1.890	0.913 0.967	-0.101	0.046 0.095	2.038	0.860 0.967
Antibiotics between samples	-4.532	0.040 0.089	5.623	0.749 0.844	-0.097	0.031 0.073	-4.612	0.688 0.844
Line sepsis (yes/no)**	-6.984	0.028 0.173	-27.209	0.260 0.537	-0.049	0.435 0.562	-12.810	0.413 0.562

Legend: *Due to bacterial overgrowth, line sepsis or another cause. **with a range of two months before and 2 months after sample collection.
A positive beta coefficient means that the two variables are positively associated, a negative coefficient means that they are negatively associated. For categorical variables the beta coefficients of all categories relative to the first category are mentioned; positive coefficients means that it is more positively associated with higher values of the response variable than the first category.
Abbreviations: BMI, body mass index; PN, parenteral nutrition; REE, resting energy expenditure; SDS, standard deviation score.

Intra-individual variation was very large (**Supplementary Figure 2**). However, when looking at the 2 patients (13%) who weaned off PN during the study period after a total PN duration of 1.2 and 2.0 years, respectively, their microbiota looks more similar to healthy controls (**Figure 6**). When looking at their microbiota, OTUs belonging to Bacteroidetes and Bifidobacteria seem to increase and more different OTUs are present. **Figure 7** shows the composition of the intestinal microbiota according to the proportion of enteral nutrition intake at time of the stool sample collection.

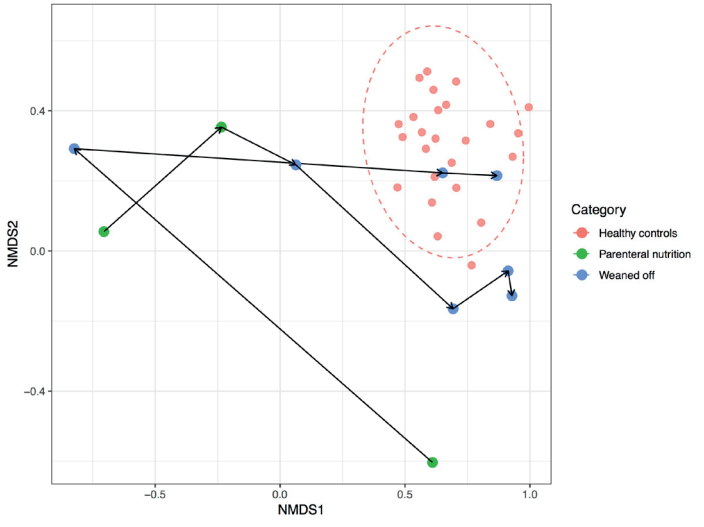
Table 4. Permutation ANOVA analysis for the inter-individual variation in microbiota community structure, attributed to different clinical variables

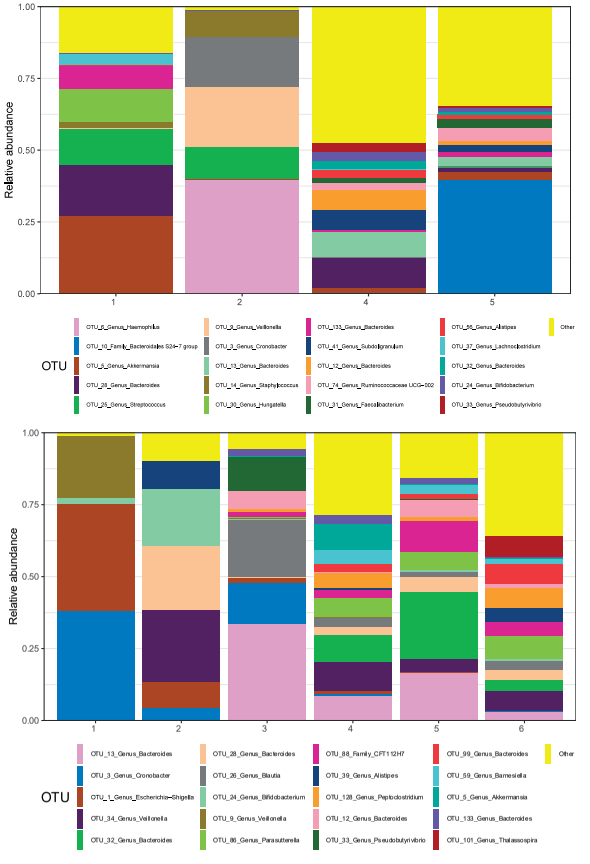
Variable	R^2	p-value
Nutrition		
Duration of PN (years)	0.06	0.005
Type of nutrition (PN, PN±tubefeeding, PN+oral nutrition±tube feeding, tube feeding/oral nutrition)	0.15	0.039
Percentage of PN (%)	0.06	0.005
Calories of PN divided by REE (%)	0.05	0.004
Oral nutrition (yes/no)	0.04	0.522
Oral/enteral fiber intake per kg (g/kg)	0.05	0.011
Tube feeding (yes/no)	0.07	0.221
Tube feeding type (polymeric, semi-elemental, elemental)	0.11	0.339
Mode of tube feeding (continuous, bolus, both)	0.09	0.474
Growth		
BMI (SDS)	0.03	0.965
Height-for-age SDS < -2	0.03	1.000
Growing outside target height range (yes/no)	0.04	1.000
Gastro-intestinal characteristics		
Whole small bowel in situ (yes/no)	0.06	1.000
Remaining small bowel length (cm)	0.06	1.000
Ileocecal valve in situ (yes/no)	0.08	1.000
Partial or total colectomy (yes/no)	0.07	0.308
Medication		
Proton pump inhibitor (yes/no)	0.07	0.209
Motility agents (yes/no)	0.08	0.098
Cholestyramine (yes/no)	0.04	0.869
Ursochol (yes/no)	0.02	0.673
Treatment of bacterial overgrowth (yes/no)	0.05	1.000
Antibiotics at sample (yes/no)*	0.05	0.005
Antibiotics between samples (yes/no)	0.04	0.020
Line sepsis**	0.02	0.134

Legend: *Due to bacterial overgrowth, line sepsis or another cause. **with a range of 2 months before and 2 months after sample collection

Abbreviations: BMI, body mass index; PN, parenteral nutrition; REE, resting energy expenditure; SDS, standard deviation score.

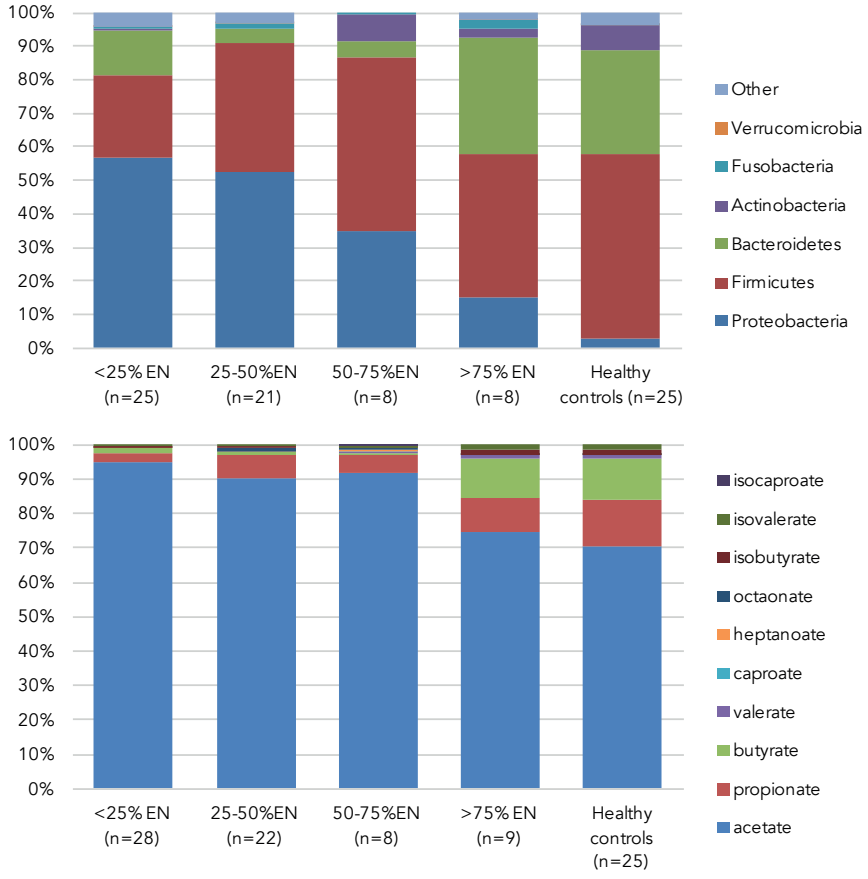
Figure 6.





a. Non-metric multidimensional scaling (NMDS) of operational taxonomic unit (OTU) community structures for the two patients who weaned off parenteral nutrition (PN) during the study period.
b. Taxonomic composition of patients who were able to wean off PN. The patient with functional IF on the upper right was on PN at the first sample, and after that weaned off. The patient on the lower right was on PN at the first two samples and after that weaned off and had surgical IF.

Figure 7. a) Composition of the intestinal microbiota and b) amount of short-chain fatty acids and branched-chain fatty acids according to the proportion of enteral nutrition intake at time of the stool sample collection



During the study period, three patients were suspected of small intestinal bacterial overgrowth and were treated with antibiotics. The microbial community structure was not different between the samples of patients suspected of bacterial overgrowth versus those not suspected of bacterial overgrowth.

DISCUSSION

The aim of this study was to prospectively characterise the fecal microbiota composition and its metabolic activity of paediatric IF patients and relate it with clinical characteristics in a longitudinal way. Similar to previous reports, the gut microbiota of children with IF presents distinct characteristics of microbial dysbiosis, both in terms of composition as well as diet-related functionality.[5-7,29,30] The bacterial diversity and richness, presumptive markers of optimal gut health, were markedly reduced in IF patients compared to healthy controls, and the microbial structure of the former was distinct to that of the latter group. When we looked at the taxon relative abundance of these two groups, patients with IF had a higher relative abundance of Proteobacteria, an observation which is consistent with previous studies.[5-7,9] A parallel decrease in the abundance of Firmicutes and Bacteroidetes was found, as described previously.[9,30,31]

Proteobacteria and other species whose relative abundance was increased in IF patients normally represent a very small fraction of the gut microbiota. Many species belonging to the Proteobacteria phylum are opportunistic pathogens, such as *E. coli*, *Klebsiella* and *Cronobacter*. The clinical significance of this observation is yet unclear. Their increased relative abundance and their metabolites in conjunction with a compromised gut barrier function and suppression of beneficial species may increase translocation of bacterial metabolites such as Lipopolysaccharides. This may induce an immune response, potentially affecting clinical outcomes and disease prognosis in this population.[32] More specifically, *Cronobacter,* which can invade intestinal cells and the blood-brain barrier, has been related to various infections including bacteremia and necrotizing enterocolitis.[33]

The features of microbial dysbiosis observed in this study are not unexpected and is in agreement with our hypothesis. Changes in normal gastro-intestinal anatomy and physiology are among the main contributing factors of the microbial dysbiosis. Extensive small bowel resection alters intestinal environment, including lowering of luminal pH, increasing oxygen concentration and disrupting the enterohepatic circulation of bile acids.[34-36] Other factors that might play a role are the rapid transit time and the large amount of undigested nutrients that are presented at the remaining colon for bacterial usage.[37] This may all lead to proliferation of aerobic bacteria at the expense of anaerobic bacteria. Indeed in this study we have observed differences in the microbial community structure between patients with surgical IF and those with IF owing to loss of gut function but having their gut in situ. Functional IF patients had a microbial community structure more similar to healthy controls than surgical IF patients. Moreover, patients with functional IF had a lower abundance of taxa belonging to Lactobacillus and Cronobacter. One previous study including both patients with surgical and functional IF[6] did not evaluate differences between these groups.

The lack of fermentable substrate necessary for anaerobic bacteria growth, such as fibre- and resistance starch, might explain the staggering decrease in fibre fermenting species belonging to Firmicutes and Bacteroidetes. This decrease of in main producers of SCFA has a significant impact, as shown by lower total as well as most individual SCFA levels. This is in contrast with one previous study including infants with short bowel syndrome, showing only differences in fecal acetate concentration.[7] SCFA stimulate vascular flow, motility, increase sodium absorption, affect cell proliferation and differentiation and enhance the immune system.[11-13] They inhibit the growth of potentially harmful bacteria and promote the growth of beneficial bacteria. In addition, acetate contributes to the energy requirements of the host by absorption by the colon.[38]

Next to a decrease of Firmicutes and Bacteroidetes, Proteobacteria can metabolise broader substrates and therefore are more resilient to changes in the diet of the host.[39] We showed that the higher amount of enteral nutrition patients received, the less Proteobacteria they had, in accordance with previous studies.[5,6,29,40] Also, when we looked at the microbiota of patients whose gut adapted, diversity increased and their overall microbial structure moved closer to that of the healthy controls. Moreover, selective species such as Bacteroides and Bifidobacterium appeared to bloom in patients whose gut adapts over time.

The dominance of lactate producing bacteria and the decreased abundance of lactate consuming bacteria[39 41,42], results in the production of both D- and L-lactate, as we observed in this study. We did observe higher values of lactate in surgical IF patients, in agreement with the fact that they had a higher relative abundance of Lactobacillus than functional IF patients. In contrast to previous studies[6,7], we were not able to relate this to D-lactic acidosis as none of the patients in our study developed this condition.

Murine studies have shown that PN results in decreased levels of secretory IgA. In contrast, most of our patients had high values of secretory IgA. In addition, most patients had normal calprotectin levels. A previous study showed that fecal secretory IgA and calprotectin did not differ between infants with short bowel syndrome and healthy controls.[7] Because of limited amount of feces available, we were not able to measure secretory IgA and calprotectin levels in healthy controls. The fact that most patients had normal calprotectin levels might be explained by the fact that there is no involvement of neutrophils or macrophages in small intestinal bacterial overgrowth, although previous studies reported conflicting results.[43,44]

The strengths of our study are the longitudinal nature of the study and the analysis we performed with prospectively collected clinical metadata. Another strength is the fact that all patients with IF including those with functional IF were included. However, one of the limitations was the relatively small sample size. Since IF is a rare condition, it is

difficult to perform single center studies with large sample sizes. Future research should therefore be preferably multi-center. Microbiota analysis was only performed on fecal samples and therefore may not reflect the mucosal microbiota. Obtaining biopsies for microbiota analyses, however, was not possible since we do not routinely perform endoscopies. Moreover, including endoscopies in our study protocol may be unacceptable for institutional review board approval. Another limitation was the fact that the majority of patients received antibiotics within 2 months prior to sample collection. Several previous studies have shown how antibiotics influence the microbiota.[45-47] However, this reflects clinical practice and the population typically treated by IF teams.

Changes in the gut microbiota may be used as biomarker to judge the optimal time of transition from PN to enteral nutrition. Since SCFA are altered in patients with IF, future studies following patients during the process of intestinal adaptation from the start of IF onwards should also include SCFA measurements as well as the species that are increased after weaning off PN.

Furthermore, the altered microbiota may be a therapeutic target. Currently, many IF patients receive broad-spectrum antibiotics because of suspected small intestinal bacterial overgrowth. However, this may further reduce the abundance and diversity of the normal beneficial microbiota and targeted antibiotics may be more beneficial. Prebiotics, probiotics or synbiotics may also be valuable, particularly during the active process of gut adaptation and transition from PN to enteral nutrition feeding.[48] However, cases of bacteremia due to probiotics have been reported too.[49] It is not well known how pro-/prebiotics act in a non-physiological environment after surgical resection. There are no clear guidelines if fiber should be supplemented and how much fiber should be used, but the findings of this study are supporting this practice.[50] Future studies should therefore evaluate the response to fiber therapy and also focus on type and dose of these fibers, especially since the composition of the microbiota also influences the fermentation of fiber.[42] Another therapeutic option might be fecal microbial transplantation, which has recently been performed in a pediatric IF patient with therapy a resistant D-lactic acidosis.[51] However, risks of fecal transplantation such as bacterial translocation and sepsis are currently not well understood, as well as the duration of the effect of transplantation in this specific population.

In summary, we have observed pronounced differences in the composition and metabolic activity of the fecal microbiota; not only between paediatric IF patients and healthy controls, but also within different subtypes of IF. The extent of dysbiosis appears to resolve as the patients adapt their gut and transit from PN to oral and tube feeding. Future research should explore whether these differences precede or follow gut adaptation; hence the role they may play in adjusting clinical practice based on the gut microbiome during this process. Association between dysbiosis features and

clinical outcomes, including small intestinal bacterial overgrowth and D-lactic acidosis and PN associated liver disease should be explored in future prospective research. In case of positive results, active manipulation of the gut microbiota during gut adaptation can improve patients' outcomes. These findings may offer new opportunities to use the microbiota and its metabolic aspects as a diagnostic marker and/or therapeutic target.

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