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Spatio-temporal dynamics of the human small intestinal microbiome and its response to a synbiotic

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ABSTRACT

Although fecal microbiota composition is considered to preserve relevant and representative information for distal colonic content, it is evident that it does not represent microbial communities inhabiting the small intestine. Nevertheless, studies investigating the human small intestinal microbiome and its response to dietary intervention are still scarce. The current study investigated the spatio-temporal dynamics of the small intestinal microbiome within a day and over 20 days, as well as its responses to a 14-day synbiotic or placebo control supplementation in 20 healthy subjects. Microbial composition and metabolome of luminal content of duodenum, jejunum, proximal ileum and feces differed significantly from each other. Additionally, differences in microbiota composition along the small intestine were most pronounced in the morning after overnight fasting, whereas differences in composition were not always measurable around noon or in the afternoon. Although overall small intestinal microbiota composition did not change significantly within 1 day and during 20 days, remarkable, individual-specific temporal dynamics were observed in individual subjects. In response to the synbiotic supplementation, only the microbial diversity in jejunum changed significantly. Increased metabolic activity of probiotic strains during intestinal passage, as assessed by metatranscriptome analysis, was not observed. Nevertheless, synbiotic supplementation led to a short-term spike in the relative abundance of genera included in the product in the small intestine approximately 2 hours post-ingestion. Collectively, small intestinal microbiota are highly dynamic. Ingested probiotic bacteria could lead to a transient spike in the relative abundance of corresponding genera and ASVs, suggesting their passage through the entire gastrointestinal tract. This study was registered to http://www.clinicaltrials.gov, NCT02018900.

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Small intestine; microbiota; metabolomics; synbiotic

Introduction

The human intestinal tract plays an important role in food digestion, nutrient absorption, and fermentation of unabsorbed components. Along the entire length of the intestinal tract, large numbers of microbes are present, collectively called intestinal microbiota, which plays an important role in human health.[1] In most studies, feces are used as a proxy for colonic microbiota due to ease of access. Although fecal microbiota composition is considered to preserve relevant and representative information for distal colonic content, it is evident that it does not represent microbial communities inhabiting the small intestine.[2–4]

The small intestinal microbiota interacts with its host through many ways, including immune modulation, production of antimicrobial compounds and metabolites (*e.g.* short-chain fatty acids (SCFAs) and

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vitamins).[1,5] The level of expression of SCFA receptors was found to be higher in the human ileum than in the distal colon.[6] Therefore, investigating factors that influence composition and functionality of the small intestinal microbiota can have important implications for our ability to understand its role in host health.

The human small intestine is much longer than the large intestine and is divided into different regions referred to as duodenum, jejunum and ileum. The small intestinal environment is characterized by a relatively short transit time and rapid luminal flux, as well as more gastric acid exposure and high concentrations of digestive enzymes, antimicrobial peptides and bile acids, which together act as selective forces for its inhabiting microbes.[7,8] Nevertheless, due to the complications associated with sample collection, available human studies investigating the spatial differences in small intestinal microbiota are often based on only one or a few subjects, or comparing samples from different locations that are derived from different subjects.[9–13] As an alternative, collection of effluent from people with an end-ileostomy, *i.e.* subjects without a colon, enables repeated sampling in a noninvasive way.[14] However, ileostoma effluent cannot be used to study the spatial differences within small intestinal microbial composition and functionality. To this end, the recent development of techniques for delivery of compounds to or aspirating intestinal fluids from the intestinal lumen,[15,16] including ingestible cathethers, now allows the study of dynamic processes in the intestinal lumen.

To date, the majority of dietary intervention studies investigated their impact on the fecal microbiota. In turn, the small intestinal microbiota that is more sensitive to dietary changes,[5,17–20] received only limited attention.

In the current study, we aimed to investigate the spatial and temporal dynamics of the microbial and metabolomic composition in the luminal content of duodenum, jejunum, proximal ileum and feces, as well as their response to a synbiotic supplementation. The synbiotic was chosen because the probiotic mixture was previously shown to convey positive effects on intestinal barrier function *in vitro* and *in vivo*,[21,22] being early intestinal colonizers,[23] and because the prebiotic has been shown to stimulate the activity of the probiotic mixture.[24] We hypothesized that microbiota composition differs per sampling site and that these location-specific microbial communities respond differently to the synbiotic supplementation.

Materials and methods

This study was approved by the Medical Ethics Committee of Maastricht University Medical Centre+ and registered at the US National Library of Medicine (http://www.clinicaltrials.gov, NCT02018900). All subjects gave written informed consent before screening. All authors had access to the study data and reviewed and approved the final manuscript.

Study overview

Details of the study design have been reported earlier.[24] This study followed a double-blind, randomized, controlled, parallel design. The 20 healthy adults (Figure S1) were randomly assigned to the placebo control ($n = 10$; P1 – P10) or synbiotic group ($n = 10$; S1 – S10) with no significant differences in age and BMI (Table S1). None of the participants took any medication 14 days prior to the study, nor antibiotics 90 days prior to the study. Subjects in the synbiotic group took 6 g/day of the probiotic mixture Ecologic®825 (1.5×10^{10} CFU/day, Winclove Probiotics BV, Amsterdam, the Netherlands) combined with 10 g/day FOS P6 (degree of polymerization 3–5) in two equal dosages. Ecologic®825 was composed of nine probiotic strains, namely *Lactococcus lactis*, *Lacticaseibacillus paracasei*, *Lactobacillus acidophilus*, *Ligilactobacillus salivarius*, *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, *Bifidobacterium bifidum*, *Bifidobacterium lactis* and *Bifidobacterium lactis*. The control group received 10 g/day maltodextrin and 6 g/day of the probiotic carrier material. Subjects ingested the supplements for 14 days. Subjects ingested the supplements after dissolving the contents of the duo sachets in 200 ml water every morning and evening at the same time for 14 days

Sample collection and storage

The luminal content (from duodenum, jejunum and proximal ileum) was sampled using a multilumen customized sampling catheter (Mui

Scientific, Mississauga, Ontario, Canada). The catheter was 270 cm long and contained four individual lumina, three of which contained side-holes at, respectively, 5, 65, and 125 cm proximal to the distal catheter tip, and an inflatable balloon near the distal tip. The catheter was introduced transnasally by a gastroenterologist. Under intermittent fluoroscopic control, when the distal tip passed the ligament of Treitz, the balloon was inflated with 10 ml of air to enhance catheter progression by peristalsis to the distal ileum. Appr. 3–5 hours afterward, the position of the cathether was checked by fluoroscopy. When the catheter was successfully positioned (catheter tip in the mid- to distal region of the ileum, according to the gastroenterologist), the balloon was deflated. The small intestinal luminal content was collected from side-holes of the catheter, *i.e.*, duodenum (side-hole 125 cm proximal to the distal catheter tip, jejunum (side-hole 65 cm proximal to the distal catheter tip) and proximal ileum (side-hole 5 cm proximal to the distal catheter tip). Six days prior to the start of the intervention (day −6) the luminal content was sampled in the morning, after an overnight fast. At day 14, the luminal content was sampled from the same locations at three time points, *i.e.* in the morning after overnight fast, around noon (*i.e.* approximately 2 hours after the supplements intake), and around 16:00, *i.e.* 3 hours after a standardized lunch (noodles). At the same day as luminal content collection, single fecal samples were collected at day −6 and 14. All samples were snap frozen and stored at −80 °C

Microbiota composition analysis

Microbiota composition was determined with barcoded 16S ribosomal RNA (rRNA) gene amplicon sequencing (Illumina Hiseq2500 (2 × 150 bp)). The intestinal fluid (≤1 ml) was immediately transferred to a screw cap tube containing 0.25 g of 0.1 mm zirconia beads and 3 glass beads (diameter 2.5 mm) to which 300 µl Stool Transport and Recovery (STAR) buffer (Roche Diagnostics, United States) was added. The mix was subjected to repeated bead beating (5.5 ms 3 × 60 s), followed by 15 min heating at 95°C at 1000 rpm. The lysate was then centrifuged for 5 min at 4°C (14000 g). The supernatant was collected and stored in a separate tube. The pellet was subjected to another cycle of cell disruption with 200 µl of STAR buffer. Supernatants from both cycles were pooled. Two times 250 µl of the supernatant was purified using a Maxwell extraction instrument (Promega, United States) with a Maxwell® 16Tissue LEV Total RNA purification Kit Cartridge customized for DNA extraction (XAS1220) and eluted in 40 µl of nuclease free water. For fecal material, 0.25 g feces were transferred to a screw cap tube containing 0.5 g of 0.1 mm zirconia beads and 5 glass beads (diameter 2.5 mm) to which 700 µl STAR buffer was added. Subsequent procedures were the same as described above for small intestinal samples, except for the second-round of cell disruption that used 300 µl STAR buffer and only one time 250 µl supernatant being used for purification. Obtained total DNA was then diluted to 20 ng/µl before amplification.

The V4 region of the 16S rRNA gene was amplified in triplicate using primers 515F[25] and 806 R[26] and extracted DNA as template. The amplification program was as described previously,[27] but with an annealing temperature of 50°C. Purified PCR products were mixed in equimolar amounts and sent for sequencing (Eurofins Genomics, Konstanz, Germany). Throughout the process, mock communities (*i.e.* mix of 16S rRNA gene sequences of known composition), biological replicates of random samples as well as no-template controls were included for quality control. Raw sequence reads were processed using the NG-Tax 1.0 pipeline using default settings.[28] Taxonomy assignment was based on the SILVA database (version 138).[29,30]

Metabolomic analysis

Small intestinal fluids collected with empty stomach and feces were subjected to metabolomic analysis. Specifically, for each sample, 100 µl of intestinal fluids were mixed with 200 µl of phosphate buffered saline that consists of 1.9 mM Na_2HPO_4, 8.1 mM NaH_2PO_4, 150 mM NaCl and 1 mM TSP (sodium 3-(trimethylsilyl)-propionate-d_4) in D_2O. After centrifugation (17000 g, 10 min), 300 µl of supernatant was transferred to a 3 mm NMR tube for analysis. Fecal extracts were prepared by mixing 20 mg of frozen fecal material with 1 ml of phosphate buffered saline mentioned above. After mixing thoroughly, samples were centrifuged (17000 g, 5 min). The supernatant

was filtered through a 0.2 µm membrane filter and 300 µl of the filtrate was transferred to a 3 mm NMR tube for analysis. The centrifugation time for the different sample types was determined based on visual inspection and the fact that a clear supernatant (for good NMR spectra) was obtained.

^{1}H-NMR spectra were recorded using 600 MHz Bruker spectroscopy, and the ^{1}H NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7; Bruker Biospin). Spectral intensities of ^{1}H NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region δ 10.00–0.2. The regions of δ 4.96–4.56 were excluded as it is remnant D_2O signal.

Metatranscriptomic analysis

Luminal content from two subjects in the synbiotic group (S8, S10) and one subject in the control group (P8) were used for metatranscriptomic analysis. Due to difficulties in sample collection and amount of samples used to study the microbial composition, we unfortunately had sufficient amounts of material left to be used for metatranscriptome analysis for these three subjects, allowing for exploratory analyses only. RNA from small intestinal fluid was isolated as described earlier,[31] with minor modifications. Briefly, about 1 ml intestinal fluid was equally split into four bead beating tubes (containing 0.8 g 0.1 mm zirconia beads each), and mixed with 500 µl ice-old 3×TE buffer, 0.18 g macaloid suspension, 50 µl 10% SDS and 500 µl acid phenol (4°C; pH 4.5), followed by repeated bead beating (5.5 m/s 3 × 45 s) with 90 s break in between on ice and centrifugation at 13,400 g 4°C for 15 min. The obtained aqueous phase (in a new tube) was mixed with 250 µl ice-old acid phenol and 250 µl chloroform:isoamylalcohol (24:1) by vortexing and centrifuged at 13,400 g at 4°C for 5 min. The obtained aqueous phase (in a new tube) was then mixed with 500 µl chloroform:isoamylalcohol (24:1), followed by vigorously shaking and centrifugation at 13,400 g at 4°C for 5 min. The supernatant was mixed with 1/10 volume 3 M NaAc (pH 5.2) and 3 volumes of 95% ethanol of −20°C, and stored at −80°C overnight. Afterwards, they were rehydrated by centrifugation at 13,400 g at 4°C for 15 min. The pellet was washed with 70% ethanol of −20°C, followed by centrifugation at 13,400 g at 4°C for 5 min, dried at room temperature for 15 min, and resuspended in 100 µl 1×TE buffer, followed by DNAse digestion using RNeasy mini kit (Qiagen Inc.) according to manufacturer's protocol. Obtained RNA was sent to Novogene (Cambridge, United Kingdom), where samples were further processed to remove rRNA using the Ribo-Zero rRNA Removal Kit (California, USA), to synthesize cDNA and double-stranded cDNA library, followed by paired-end sequencing on an Illumina NovaSeq instrument (150bp). The sequence data was trimmed with Trimmomatic[32] and potential host transcripts were tested against human genome database with bowtie2[33] before merged pair-end reads with PEAR.[34] The remaining ribosomal reads were removed with SortMeRNA[35] by default settings. Functional profiling of remaining reads (*i.e.* the mRNA) was performed using default settings of HUMAnN2[36] with the full ChocoPhlAn,[37] UniRef 90[38] and MetaCyc databases.[39]

Statistical analysis

The 16S rRNA gene sequence read counts were normalized to relative abundance. The microbial alpha diversity indices (Faith's phylogenetic diversity, Inverse Simpson and Pielou index) were calculated based on amplicon sequence variants (ASVs). Normality of the data was checked by Shapiro-Wilk test. As data were not normally distributed in the current study, the Friedman test and pairwise Wilcoxon signed-rank tests were used for paired measurements. At baseline, the number of samples per sample type, *i.e.* feces, jejunum, duodenum and proximal ileum, was unequal, due to unsuccessful sample collection and/or DNA isolation. Therefore, the pairwise Mann-Whitney U test was used as an alternative. Correlation analysis (Spearman) was conducted using the *rcorr* function in the Hmisc package.[40] Significant differences between groups were tested by permutational multivariate analysis of variance (PERMANOVA) based on pairwise weighted and unweighted UniFrac distances at ASV level. Intervention effects on individual genus-level taxa were tested using a linear mixed effect model, with subject as random factor, and time and treatment as fixed factors. For metabolomic analysis,

orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed and validated in SIMCA-P+ (Umetrics, Umeå, Sweden). OPLS-DA model was validated using the CV-ANOVA method in SIMCA-P+ software. *p* values were corrected for multiple testing by the false discovery rate (FDR) using the Benjamini-Hochberg procedure. Corrected *p* values (referred to as q values) < 0.05 were considered to indicate significance. All statistical analyses were conducted in R (*R*-3.5.0).

Results

Gradual change in microbial and metabolomic composition from upper, mid, to lower sections of the intestine

We first set out to assess spatial gradients in microbial and metabolomic composition based on baseline data obtained 6 days prior to the start of the intervention. PERMANOVA based on weighted UniFrac (considering relative abundance of ASVs and their position in the phylogenetic tree) and unweighted UniFrac (considering only presence or absence of ASVs and their phylogenetic position) distance matrices, revealed significant (all $q < 0.05$) differences between the microbiota of feces and that of small intestinal fluids at baseline, *i.e.*, day −6 of the trial (Figure 1). Within the small intestine, principal coordinate analysis (PCoA) based on weighted and unweighted UniFrac distance matrices indicated significant differences in microbial composition between duodenum and jejunum (q=0.0036 and q=0.0024), between jejunum and proximal ileum (q=0.0140 and q=0.0060), as well as between duodenum and proximal ileum (both q=0.0015) (Figure 1a,b). Overall, sample site explained 66.7% (based on weighted UniFrac distance) or 55.0% (based on unweighted UniFrac distance) of the observed variation in microbial composition. Observed variation in microbial composition within each sample site was not significantly explained by recorded subject characteristics (*i.e.* age, sex and BMI).

Microbial diversity (Inverse Simpson) changed gradually along the small intestine (Figure 1c). The fecal microbial diversity was significantly higher than that of jejunum (q=0.0029) and proximal ileum (q=0.0001). The fecal microbial richness was higher than that of jejunum (q=0.0124) and proximal ileum (q<0.0001), but not significantly different from that of duodenum (Figure 1d). Within the small intestine, no significant differences were found in microbial diversity between duodenum and jejunum, but the microbial diversity of duodenum and jejunum was significantly higher than that of ileum (q=0.0021 and q=0.0457, respectively). Differences between small intestinal locations were more pronounced with respect to phylogenetic richness (Figure 1d), with significant differences between duodenum and jejunum (q=0.0039), jejunum and proximal ileum (q=0.0030), as well as between duodenum and proximal ileum (q<0.0001). Moreover, in line with findings in microbial diversity, weighted UniFrac distance between the microbiota of feces and that of small intestinal fluids was significantly (all q<0.05) larger than that among small intestinal samples (Figure 1e).

Zooming into the microbial composition, in addition to a large inter-individual variation, at family level the fecal microbiota was in general predominated by members of *Lachnospiraceae* and *Ruminococcaceae*. In some subjects, however, we observed high relative abundance of *Bifidobacteriaceae*, and few subjects showed high relative abundance of *Prevotellaceae* (Figure 2a). Concurrently, *Streptococcaceae* predominated the duodenal and jejunal microbiota, with some subjects having high relative abundance of *Prevotellaceae* in duodenum, and *Veillonellaceae*, *Pasteurellaceae* or *Enterobacteriaceae* in jejunum and/or ileum. Furthermore, at the genus level, the relative abundance of eight out of 197 observed taxa was significantly (all q<0.05) higher in luminal duodenal microbiota compared to proximal ileum (Figure 2b). The relative abundance of four of these eight genus-level taxa, namely *Prevotella, Prevotella* 7, *Porphyromonas* and *Fusobacterium*, was also significantly (all q<0.05) higher in the duodenal microbiota comparing to that of jejunum (Figure 2b). In contrast, no significant differences were observed in the relative abundance of individual taxa between jejunum and proximal ileum. Collectively, these data indicate a gradual change in microbial composition along the small intestine

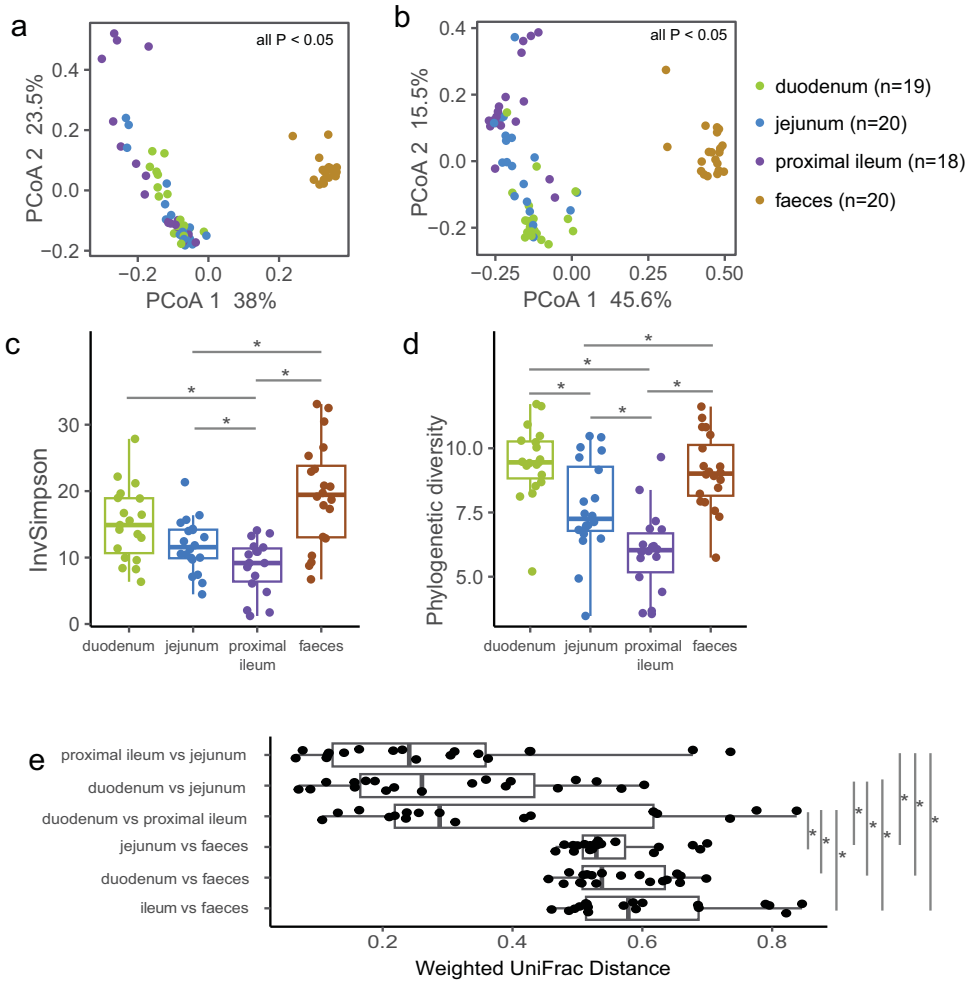


Figure 1. Gradual change in microbial composition from upper, to mid, to lower sections of the intestine at baseline at day – 6 of the trial. PCoA of microbial composition based on (a) weighted or (b) unweighted UniFrac distance matrices. Significant differences between samples based on pairwise weighted and unweighted UniFrac distances at ASV level were tested by PERMANOVA. The percentage of variation in microbial composition explained by the two principal coordinates is shown at the axes. (b) Microbial diversity and (d) phylogenetically weighted richness. (e) Differences in microbial composition between different sample types within each individual. Distance was calculated based on weighted UniFrac distance. Scale ranges from 0 to 1, with lower values indicating higher similarity. Comparison of distances between sample types was done using a linear mixed effect model with subject as random intercept. Numbers of samples per subject vary due to technical reasons (for details, please refer to table S2). * indicates significant differences ($q < 0.05$) between groups.

from upper, mid to lower sections, being in line with the biogeographical and physiological distances along the intestinal tract.[41]

OPLS-DA was applied to visualize the differences in the metabolome of intestinal fluids (Figure 3). The fecal metabolome was well separated (all $q<0.05$) from that observed in small intestinal content (Figure 3a). OPLS-DA without fecal samples revealed that the duodenal metabolome was well separated (all $q<0.05$) from that of jejunum and ileum, whereas the difference between the metabolome of jejunum and proximal ileum was not significant (Figure 3b). When evaluating individual metabolites, concentrations of uracil, adenine, ethanol and formate were found to be higher in feces compared to small intestinal fluids. Metabolomes in small intestinal fluids were less diverse than in feces and consisted mostly of amino acids (phenylalanine, tyrosine, threonine, alanine, valine, isoleucine), carbohydrates (mainly glucose), amines (choline) and glycerol. In addition, duodenum fluids had lower levels of glucose compared to all of the other parts.

Correlation analysis between relative abundances of observed genus-level taxa and metabolites, revealed significant correlations between microbial and

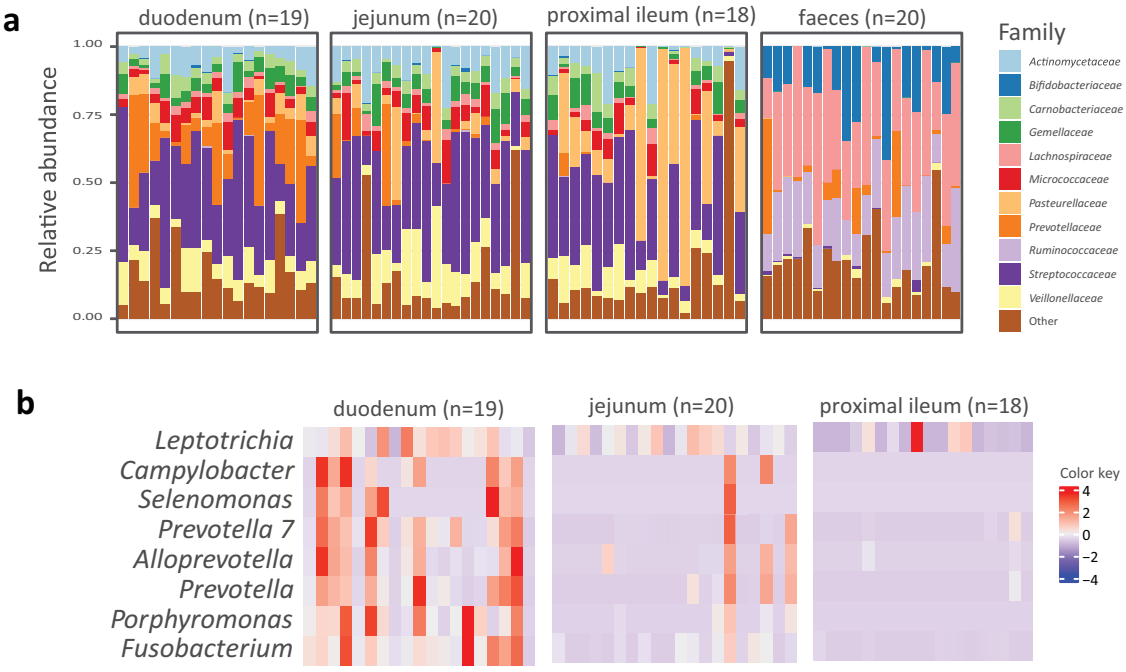


Figure 2. Microbial composition in different sample types at baseline at day – 6 of the trial. (a) Relative abundance of most predominant bacterial families (top 12, ranked base on the average relative abundance across the entire dataset). Top 12 microbial families are listed in the legend. Other families are summarized as "other". Each column represents a given type of sample from one subject. (b) Heatmap of microbial genus-level taxa that significantly differed between samples taken at different regions along the small intestine. Color in the heatmap reflects the relative abundance normalized per taxon, with blue color indicating lower than average relative abundance and red color indicating higher than average relative abundance. Scaling of colors in the heatmap was done by subtracting the overall mean of a given genus across the entire dataset from its value for a specific sample and dividing by the standard deviation of the given genus. Numbers of samples per subject vary due to technical reasons (for details, please refer to table S2).

metabolomic compositions with Spearman correlation coefficients ranging from −0.77 to 0.98 (Figure S2). Among those, the relative abundance of 37 and 9 out of the 197 observed genus-level taxa positively or negatively correlated with metabolomic data with absolute Spearman correlation coefficients bigger than 0.70 (Figure 3c). The relative abundance of three genus-level taxa (*i.e.*, *Dorea*, *Subdoligranulum* and *Lachnospiraceae NK4A136 group*) correlated with the amount of different metabolomic compounds, both positively and negatively.

Re-assessing microbial community composition along the small intestine at different time points

Microbial composition along the small intestine was reevaluated 20 days after the first evaluation (baseline: at day −6). Consistent with findings reported for baseline measurements, small intestinal samples collected with empty stomach, at noon before lunch and three hours after lunch showed the same trend with respect to microbial diversity and richness, *i.e.* being highest in duodenum followed by jejunum then proximal ileum (Figure S3). Nevertheless, within the small intestine, differences in microbial diversity and richness were only significant between the microbiota of duodenum and proximal ileum (all $q < 0.05$) and between duodenum and jejunum (all $q < 0.05$). No significant difference was detected between the microbiota of jejunum and proximal ileum.

PERMANOVA based on unweighted UniFrac distance matrices revealed significant differences between the different small intestinal segments after overnight fasting, whereas at noon and three hours after lunch, no significant difference was measurable between the different small intestinal segments. When using weighted UniFrac distance matrices, differences were only found significant ($q = 0.0091$) between the microbiota of duodenum and proximal ileum after overnight fasting (Figure S4).

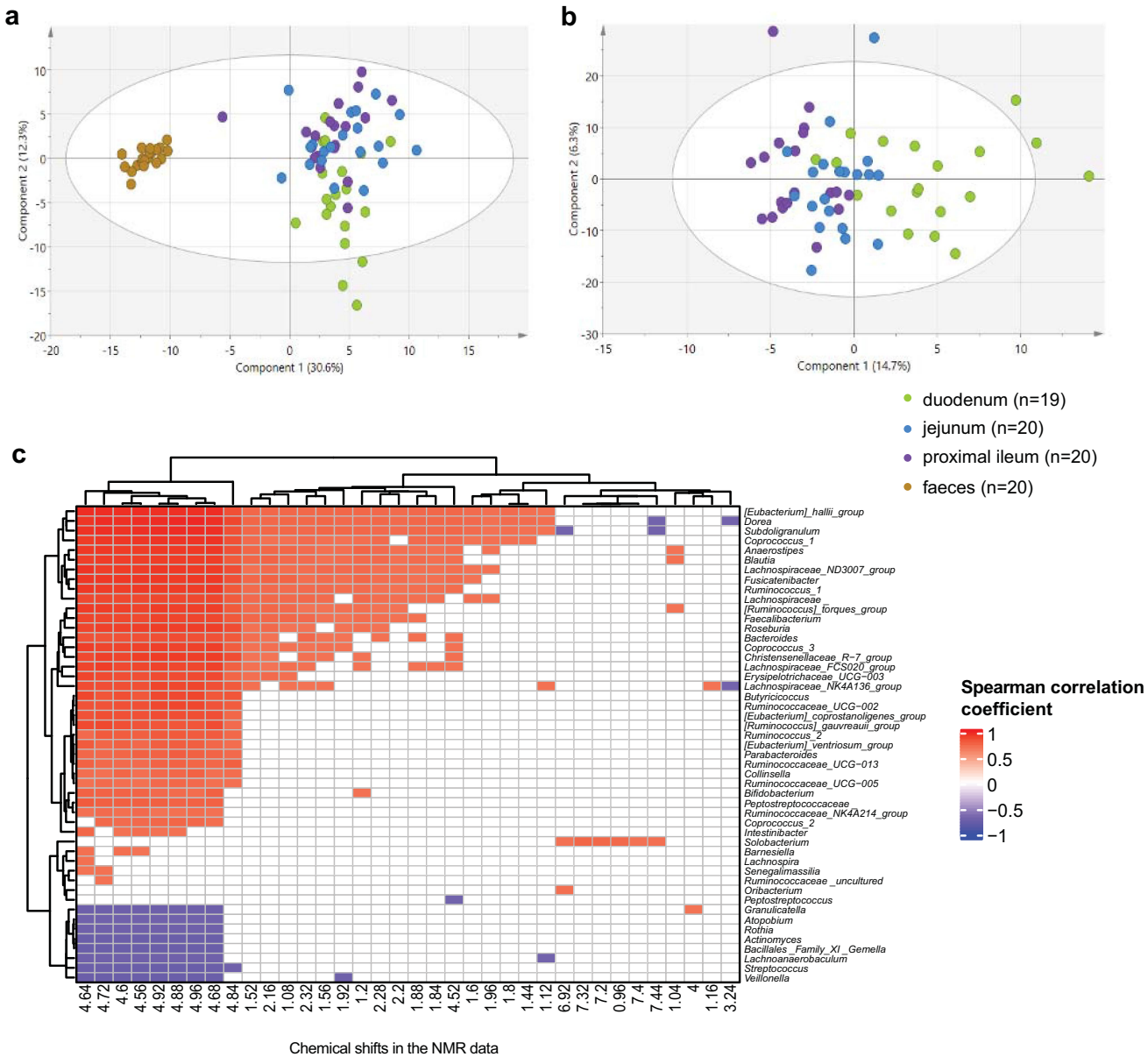
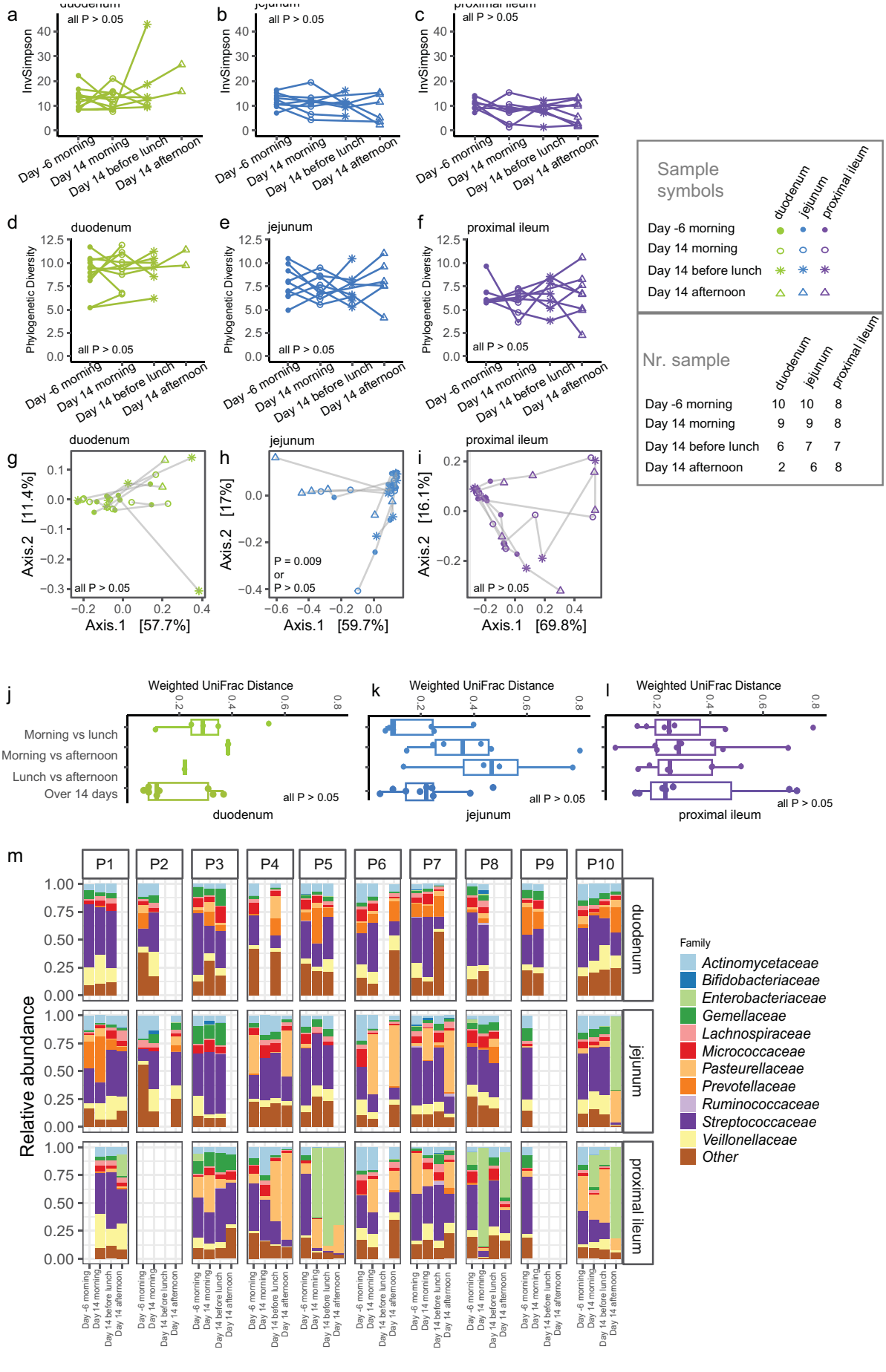


Figure 3. Metabolomic composition in different types of samples and their correlation coefficient with microbial composition. (a) Orthogonal partial least squares-discriminant analysis (OPLS-DA) of metabolomic data collected at the different sites. (b) OPLS-DA of metabolomic data in small intestinal fluids. Numbers of samples per subject vary due to technical reasons. Specifically, we failed in collection of one duodenum sample (for details, please refer to table S2). (c) Correlation analysis between the microbial and metabolomic composition. Color in the heatmap reflects Spearman correlation coefficients, with blue color indicating negative correlation and red color indicating positive correlation. Hereby only significant correlations with absolute correlation coefficients above 0.7 or below – 0.7 are included.

Dynamics of small intestinal microbiota within a day and over 20 days

In terms of microbial diversity (Figure 4a-c) and richness (Figure 4d-f), no significant changes were observed in time (hours within a day and over 20 days). PERMANOVA revealed that microbiota composition was only significantly different (p = .009) between jejunum microbiota at noon and three hours after lunch based on weighted UniFrac distance matrix. Except that, no significant changes were identified in microbiota composition within a day (Figure 4g-i), and over 20 days. Although overall, weighted UniFrac distances between the small intestinal microbiota at different time points showed no significant difference (Figure 4j-l), visual inspection of microbiota composition


a
duodenum
all P > 0.05
InvSimpson
b
jejunum
all P > 0.05
c
proximal ileum
all P > 0.05
Day -6 morning
Day 14 morning
Day 14 before lunch
Day 14 afternoon
d
duodenum
e
jejunum
f
proximal ileum
Phylogenetic Diversity
g
duodenum
h
jejunum
i
proximal ileum
Axis.2 [11.4%]
Axis.1 [57.7%]
Axis.2 [17%]
P = 0.009
or
P > 0.05
Axis.1 [59.7%]
Axis.2 [16.1%]
Axis.1 [69.8%]
Sample symbols
duodenum
jejunum
proximal ileum
Day -6 morning
Day 14 morning
Day 14 before lunch
Day 14 afternoon
Nr. sample
duodenum jejunum proximal ileum
Day -6 morning 10 10 8
Day 14 morning 9 9 8
Day 14 before lunch 6 7 7
Day 14 afternoon 2 6 8
j
Weighted UniFrac Distance
Morning vs lunch
Morning vs afternoon
Lunch vs afternoon
Over 14 days
duodenum
k
jejunum
l
proximal ileum
m
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
Relative abundance
Family
Actinomycetaceae
Bifidobacteriaceae
Enterobacteriaceae
Gemellaceae
Lachnospiraceae
Micrococcaceae
Pasteurellaceae
Prevotellaceae
Ruminococcaceae
Streptococcaceae
Veillonellaceae
Other

demonstrated remarkable intra-individual dynamics within a day and over 20 days (Figure 4m).

Changes in microbial and metabolomic composition after synbiotic supplementation

Comparative analysis before and after the synbiotic intervention, corrected using data obtained for the placebo control group, revealed neither significant changes in overall microbial composition in duodenum, jejunum, proximal ileum or feces (Figure 5a–d and Figure S5a-d), nor in the relative abundance of individual taxa. Microbial diversity in the jejunum decreased significantly ($q = 0.0206$) after synbiotic supplementation (Figure 5f), attributing to the significant decrease in microbial evenness ($q = 0.0072$, Figure 6). In contrast, neither the microbial diversity in duodenum, proximal ileum or feces (Figure 5f–h), nor phylogenetically weighted richness in duodenum, jejunum, proximal ileum and feces were affected significantly after synbiotic supplementation (Figure 5e-h). As such, the lower microbial diversity in jejunum can be attributed to the decrease in microbial evenness in the jejunum. Both in the placebo control group and in the synbiotic group, weighted UniFrac distance between samples taken at day −6 and day 14 was significantly ($p = .0043$ and $p = .0407$) higher in proximal ileum than that in feces (Figure 5i), indicating ileal microbiota had lower stability than fecal microbiota. Moreover, it is worth mentioning that small intestinal response to the synbiotic supplementation was highly personalized.

No significant intervention impact was identified on the metabolomic data of duodenum, jejunum, ileum or feces (Figure 6a-d). This result was line with findings of the microbiota data and highlighted the strong correlation between microbiota and metabolome data (Figures 5 & 6).

Kinetics of ingested microbes throughout the intervention period

Bacterial strains included in the synbiotic belong to *Lactococcus*, *Bifidobacterium,* *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus* and *Ligilactobacillus* genera. At day 14 (Figure 7, white area), four subjects in the synbiotic group showed a strong increase in the relative abundance of the genera included in the synbiotic formulation, in the duodenum (p values corrected and calculated for all participants, all $p > .05$), jejunum (all $Q > 0.05$) and proximal ileum (*Lactococcus* q=0.08, *Lacticaseibacillus* q=0.08, *Lactiplantibacillus* q=0.15, *Bifidobacterium* q=0.28) about two hours after ingestion of the synbiotic. However, these levels returned to baseline within three hours (Figure 7). Concurrently, subjects in the placebo control group did not show any significant alteration in the relative abundance of these genera, neither after two weeks of supplementation, nor during day 14.

A comparison of samples taken in the morning at baseline (day −6) and day 14 showed that 14 days of supplementation did not significantly affect the relative abundance of *Lactococcus*, *Bifidobacterium, Lactobacillus, Lacticaseibacillus, Lactiplantibacillus* or *Ligilactobacillus* in duodenum, jejunum, proximal ileum or feces when considering all subjects (Figure 7 beige background), albeit with inter-individual differences with respect to the observed variation at different locations. After synbiotic supplementation, one subject (subject S8) had increased levels of *Bifidobacterium* in jejunum. Another subject (subject S3) had increased relative abundance of *Lactobacillus* and *Lactiplantibacillus* in jejunum. Another subject (subject S10) showed an increased level of *Ligilactobacillus*. However, the relative abundance of these genera decreased again during the last intervention day (Figure 7 white background). This can likely be attributed to the

Figure 4. Longitudinal changes in small intestinal microbial composition within a day and over 20 days, based on subjects included in the placebo control group. Changes in microbial diversity of duodenum (a), jejunum (b) and proximal ileum (c) over time in individual subjects. Changes in microbial richness of duodenum (d), jejunum (e) and proximal ileum (f) over time in individual subjects. PCoA of microbial composition based on weighted UniFrac distance matrices of duodenum (g), jejunum (h) and proximal ileum (i). Percentages at the axes indicated the percentage of variation explained. Weighted UniFrac distances between samples collected at different timepoints in terms of duodenum (j), jejunum (k) and proximal ileum (l). (m) Relative abundance of different bacterial families (top 12, ranked base on the average relative abundance across the entire dataset, see legend for taxa) in different sample types. Each column represents a given sample from one subject at a specific timepoint. Missing columns are due to technical issues (for details, please refer to table S2). Subject ID was re-coded to adhere to privacy regulations.

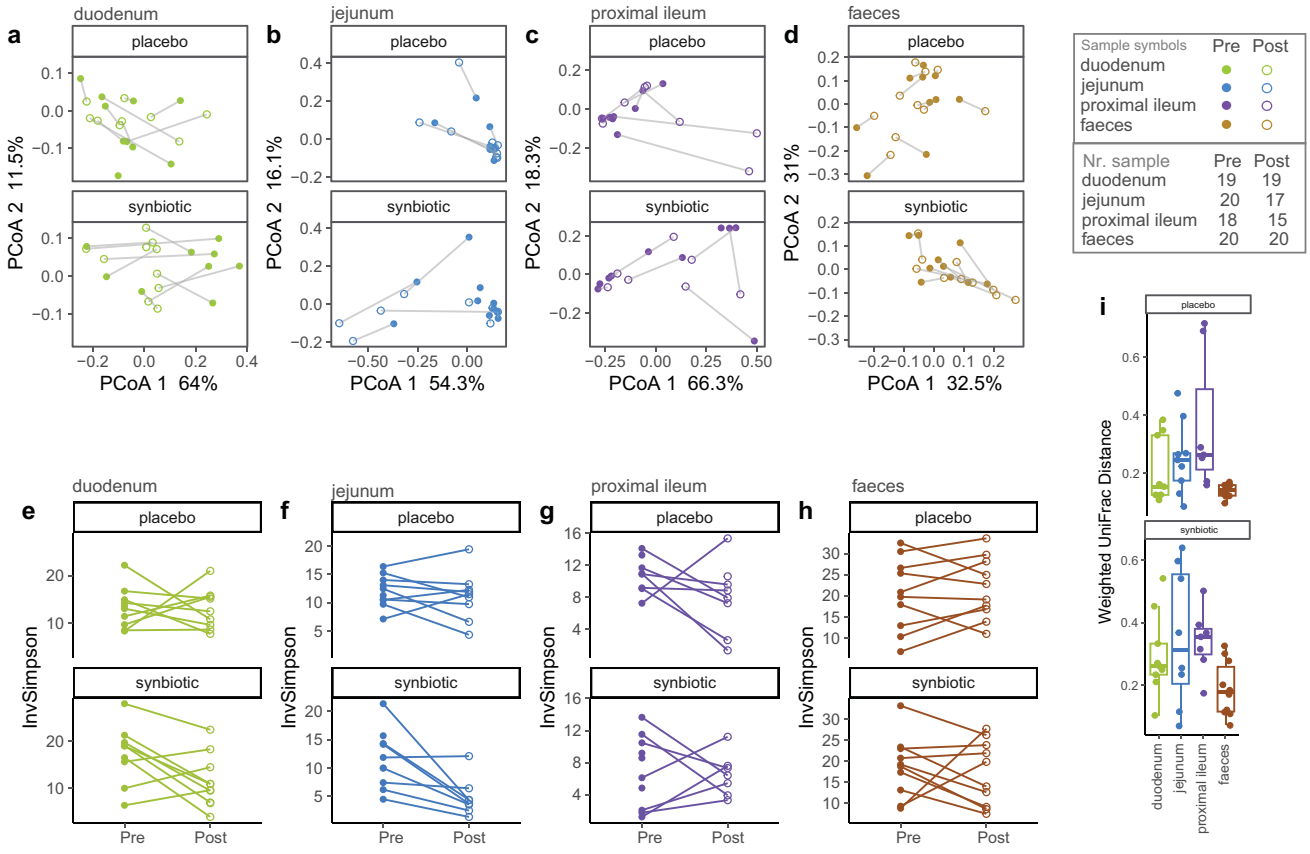


Figure 5. Comparison of microbial composition before and after the intervention (placebo control, synbiotic). PCoA of microbial composition pre vs post intervention in (a) duodenum, (b) jejunum, (c) proximal ileum and (d) feces, as well as corresponding changes in microbial diversity in (e) duodenum, (f) jejunum, (g) proximal ileum and (h) feces. (i) Weighted UniFrac distances between samples collected at day – 6 and day 14. PCoA is based on weighted UniFrac distances. Values are presented in scatter plots and linked per individual. Small intestinal samples were collected after overnight fasting. Pre: before Intervention; post: 14 days after start of the intervention. Percentages at the axes indicated the percentage of variation explained. Comparative analysis between timepoints within each sample type and supplementation group (e.g. duodenum pre synbiotic vs duodenum post synbiotic), was performed with PERMANOVA. Within each sample type, intervention effects on the microbial diversity were compared with variance components (random intercept) linear mixed models and correction for baseline values. Sample sizes vary due to technical reasons (for details, please refer to table S2).

transient presence of bacterial DNA from the synbiotic strains in the small intestine.

At ASV level, sequences corresponding to ingested probiotic species were observed in the 16S rRNA gene sequence data (Figure 7). Specifically, the changes in the relative abundance of ASVs classified as *Lactococcus lactis* and *Bifidobacterium* were in line with the observations at genus level, *i.e.* increased relative abundance about two hours after ingestion and rapid decrease afterward (Figure 7a,b). Moreover, we observed ASVs corresponding to all *Lactobacillus, Lacticaseibacillus, Lactiplantibacillus* and *Ligilactobacillus* species in the probiotic mixture, including *L. plantarum, L. acidophilus, L. salivarius, L. paracasei* and *L. casei*. The relative abundance of these *Lacticaseibacillus* and *Ligilactobacillus* species was highest about two hours after the ingestion at day 14, coinciding with the observation at genus level (Figure 7d,f). It should be noted, however, that the inherent limitations with respect to species-level resolution in V4 sequence data did not in all cases allow for unequivocal assignment of ASVs to specific species (Figure 7). Similarly, for *Bifidobacterium*, observed sequences corresponded to *B. bifidum*, while *B. lactis* was classified as *B. longum/B. lactis*, with an increase in relative abundance after synbiotic ingestion in all sample types. These data strongly suggest the transient passage of bacterial DNA from the ingested probiotic strains.

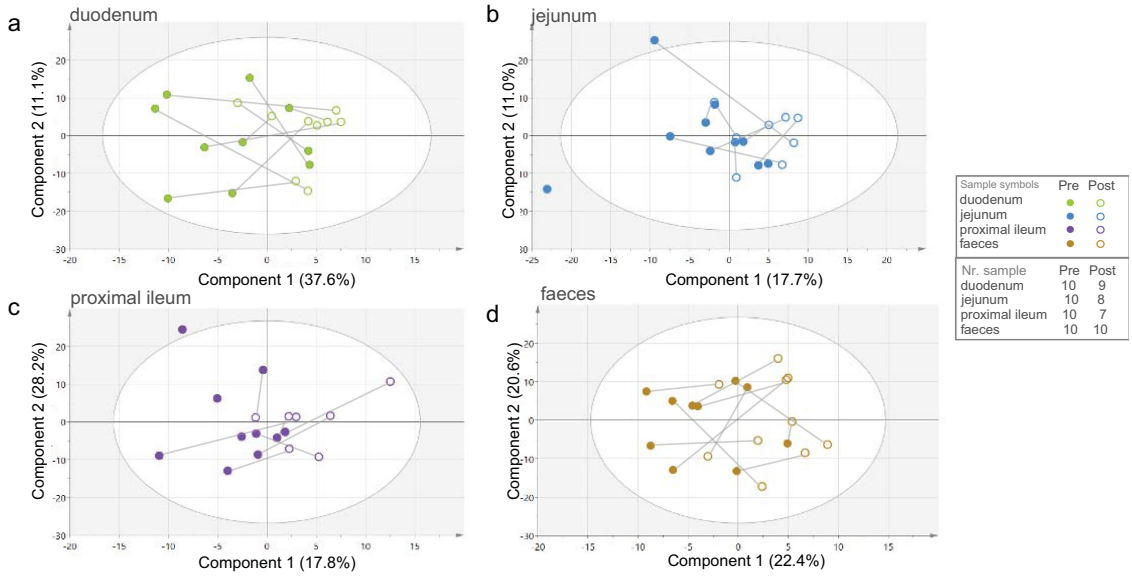


Figure 6. OPLS-DA of metabolomes pre vs post intervention in (a) duodenum, (b) jejunum, (c) proximal ileum and (d) feces. Sample sizes vary due to technical reasons.Specifically, one duodenum sample (post intervention) failed in obtaining NMR data. In addition, we failed in the sample collection of jejunum ($n = 2$) and ileum ($n = 3$) samples after over night fasting (for details, please refer to table S2).

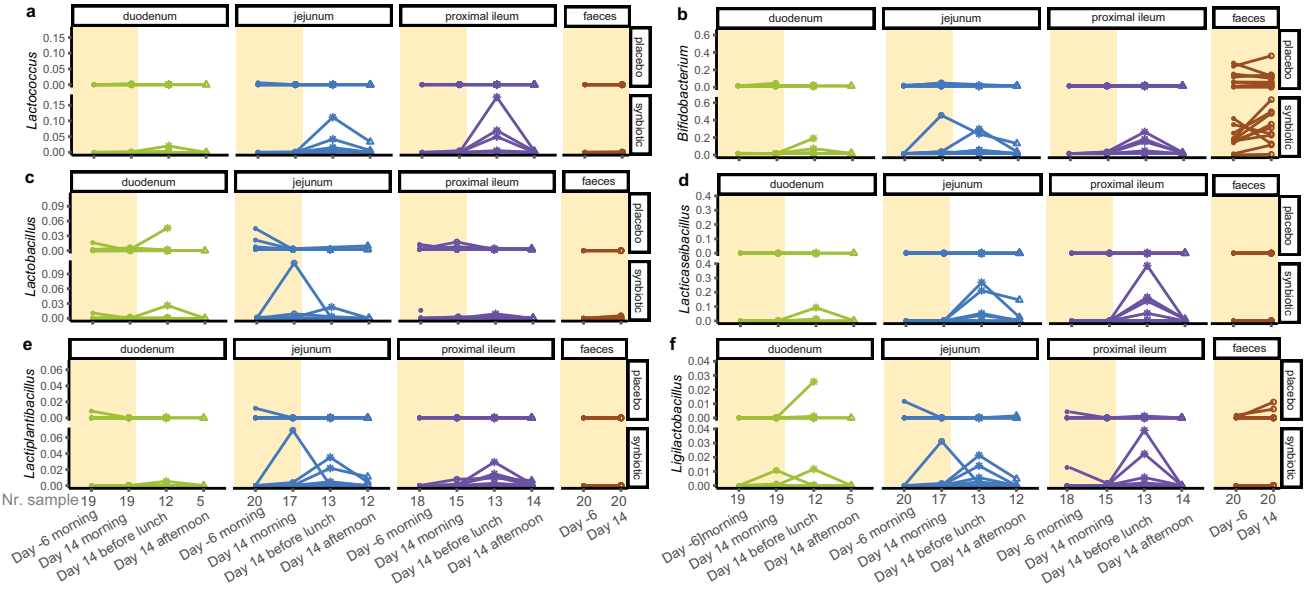


Figure 7. Kinetics of ingested bacteria. Relative abundance of (a) lactococcus, (b) bifidobacterium, (c) lactobacillus, (d) lacticaseibacillus, (e) lactiplantibacillus and (f) ligilactobacillus before and after supplementation of placebo (control) or synbiotic.Samples are linked per individual. Faeces was collected at the same day as small intestinal samples. Day − 6 morning and Day 14 morning: samples were collected with empty stomach. Day 14 before lunch: samples were collected about 2 hours after last ingestion. Day 14 afternoon: samples were collected about three hours after standardized lunch (noodles). Sample sizes vary due to technical reasons (for details, please refer to table S2).

With the increase in the relative abundance of ingested probiotic strains in the small intestine about two hours after consumption of supplements, we investigated the microbial activity while the ingested microbes were passing through the small intestine by metatranscriptomic analysis, albeit only

in a small number of subjects, owing to the limited availability of sufficient material. The microbial activity of small intestinal samples from two subjects (subject S8 and S10 in the synbiotic group) with large increase in the relative abundance of probiotic strains about two hours after the last supplementation was compared with that of one subject (subject P8) in the placebo control group. Despite the technical challenges due to the low biomass in these small intestinal samples (from about 1 ml intestinal fluid) and subsequent background noise, we obtained 1.89×10^5 to 1.38×10^7 mRNA reads per sample. Aside from the expression of housekeeping functions, like nucleotide biosynthesis pathways, glycolysis pathways and amino acid biosynthesis pathways *etc*, we observed considerable variation between the metatranscriptomes. However, we did not observe enrichment of metatranscriptomic reads that were specifically assigned to taxa corresponding to the probiotic strains in the samples taken from subjects that ingested the synbiotic formulation.

Discussion

In the current study, we investigated the spatio-temporal dynamics of the small intestinal microbiome within a day and over 20 days, as well as its responses to a 14-day synbiotic supplementation in 20 healthy subjects. We hypothesized that microbiota composition differs per sampling site and that these location-specific microbial communities respond differently to the synbiotic supplementation. We found significant differences between the microbiota and metabolome of duodenum, jejunum, proximal ileum content and feces. The significant difference along the small intestine was most pronounced in the morning after overnight fasting, whereas it became not always measurable around noon or in the afternoon. Over 20 days and over a few hours within a day, the small intestinal microbiota changed remarkably per individual, while this change demonstrated great heterogeneity between subjects. In addition, ingestion of probiotic bacteria induced a short-term spike in the relative abundance of corresponding genera approximately two hours after ingestion in some individuals. These ingested microbes were not active as based on metatranscriptome data, although it should be noted that this could only be measured in a small fraction (2 out of 10) of our study group, suggesting their passage through the small intestine. Technical limitations of this analysis (*i.e*. low biomass, time required for sample aspiration) however has to be considered. Except for decrease in jejunum microbial evenness, two weeks of synbiotic supplementation did not significantly affect the microbial and metabolomic composition in duodenum, jejunum, proximal ileum or feces. Nevertheless, their reponse to the supplemented synbiotic was highly personalized.

Although some subjects had high relative abundance of *Veilonellaceae*, *Enterobacteriaceae* or *Pasteurellaceae*, the small intestinal microbiota was generally predominated by *Streptococcaceae*, as found previously in ileostoma effluent from ileostomists and small intestinal fluid from healthy volunteers.[11,12,16,42] Consistent with earlier studies,[2] the spatial and physiological differences between upper, mid and lower regions of the small intestine and feces, were reflected in their microbial and metabolomic composition. Differences between different small intestinal microbial communities were in line with the corresponding physical distance between sampling sites, attributing to the gradual change in physiological conditions along the intestine, like pH and concentrations of oxygen and nutrients.[43] This could also explain, in part, the higher relative abundance of several taxa that are known members of oral microbiota (like *Leptotrichia*, *Campylobacter,* *Prevotella*, *Selenomonas*, *Fusobacterium*, *Porphyromonas* and *Alloprevotella*) in duodenum compared to proximal ileum ,[44–47]

Comparison of ^{1}H-NMR spectra showed that fecal metabolomes were more complex and diverse than those of small intestinal fluids. In most fecal samples, SCFAs such as acetate, propionate and butyrate were dominant metabolites.[48] Besides SCFAs, concentrations of uracil, adenine, ethanol and formate were higher in feces compared to intestinal fluids. Metabolomes in small intestinal fluids were less diverse and consisted mostly of amino acids (phenylalanine, tyrosine, threonine, alanine, valine, isoleucine), carbohydrates (mainly glucose), amines (choline) and glycerol. This reflects the main functions of the small intestine, which is digestion and absorption of nutrients from our diet. The most prominent difference in

duodenum fluids compared to that of the other parts of the small intestine was a lower level of glucose.

In contrast to previously reported increasing microbial diversity from the stomach to the colon,[49] comparative analyses in the current study showed that the microbial diversity and richness decreased from upper to lower sections of the small intestine. Early on, researchers investigated the small intestinal microbiota based on specimens of recently deceased subjects, which are often hard to obtain and are small in sample size, and doubts have been raised with respect to these samples being representative for the *in vivo* situation.[50] Wang *et al.*, compared the microbial diversity of jejunum and distal ileum in a single subject, and revealed lower microbial diversity in jejunum as compared to distal ileum.[10] Recently Seekatz *et al.* used a customized multichannel catheter and collected luminal content of duodenum and the proximal, mid- and distal jejunum from eight healthy subjects, demonstrating higher microbial diversity in duodenum compared to proximal and mid jejunum,[42] being in line with findings of the current study. Collectively, comparing to available studies, the current study is the largest study (based on 20 subjects) to date investigating spatio-temporal variation in small intestinal microbiota composition. In addition, we used a multichannel aspiration catheter which enabled comparative analysis among different sites of the small intestine within the same subject, therefore allowing to account for subject specificity, including subject- and location-specific responsiveness to a given intervention, such as the synbiotic supplement that was used here.

Many strains included in the supplemented synbiotic are known for their tolerance/resistance to low pH and bile,[51] as well as adhesion to mucus, epithelial cells or enterocyte-like Caco-2 cells,[52–54] Compared to single strain probiotics, multispecies probiotic mixtures are suggested to have potential advantages to convey additive or synergistic effects and exhibit better efficacy in health-related outcomes.[55] Two weeks of synbiotic supplementation of the healthy population in the current study did not alter microbial and metabolomic composition in the lumen of the small intestine and feces, except for a decrease of microbial evenness in jejunum. The cause and implication of the observed temporally decreased microbial evenness on health-related parameters in the jejunum remains to be determined. Nevertheless, Maxwell®825 was developed and studied in subjects with increased risk or even already developed chronic gastrointestinal disorders (*e.g.* IBD/IBS) and (low-grade) inflammatory conditions.[22,56] Additionally, the response of intestinal microbiota in healthy subjects could differ from that of unhealthy subjects.

Findings of the current study are in line with those described earlier in which eight weeks of Maxwell®825 intervention did not affect the diversity of mucosal pouch microbiota in patients with active pouchitis.[22] In another study, Moser and colleagues showed that supplementation with the probiotic mixture OMNi-Ecologic® Stress Repair, containing the same strains as Ecologic®825, increased the microbial diversity in the upper gastrointestinal tract (*i.e.* gastric corpus and duodenum mucosal specimen), but not in the lower intestinal tract (ascending colon biopsies or feces), of IBS-D patients.[56] These authors found a significant increase in the relative abundance of unclassified *Lactobacillaceae*, and a decrease in relative abundance of *Moraxella* and *Moryella* in feces. In the current study, no significant effects of two weeks intervention with respect to the relative abundance of bacterial groups were observed, including genera included in the synbiotic. It should be noted, however, that comparability of both studies is limited. Differences between the current study and the study of Moser and colleagues[56] with respect to target group (healthy subjects vs. IBS-D patients), sampling sites (lumen vs mucosal specimen), dosage of the probiotic (1.5×10^{10} CFU vs 6.75×10^{10} CFU) and prebiotic (10 g/day FOS P6 vs. inclusion in the carrier material only), and duration (two vs. four weeks) might at least in part have contributed to the observed differences in microbial data between studies.[57,58] To this end, it is of interest to note that a number of studies reported reduced mucosal and/or fecal microbial diversity and richness in IBS patients vs healthy controls,[59–61] and thus, the more diverse communities in healthy individuals might be more resilient, and thus less amenable to change. In part,

this might also contribute to explaining the previously observed lack of effects on intestinal permeability both under basal and under indomethacin-induced stressed conditions, immune function or intestinal symptoms in subjects receiving this synbiotic supplement.[24] In addition, the current study illustrated the passage of ingested probiotic microbes through the intestine, while cultivation of specified microbes is required to uneuivacally confirm their survival.

The metabolomes observed before and after synbiotic supplementation in the present study also showed a similar phenomenon. Two weeks intervention of synbiotic supplementation did not alter metabolomes in feces and in small intestinal fluids, the latter being in line with the observed limited influence on the microbiota composition in the small intestine. Fecal metabolites are the physiological product of microbial activity in the gut and therefore, it can likely be assumed that the fecal metabolome should reflect the current gut metabolic status and microbial activities.[62] Some studies showed that the luminal microbiota is altered during probiotic or synbiotic treatment and therefore leads to alterations in metabolic activity.[63] However, those studies were mostly based on long term intervention periods (3–6 months) and/or under different health conditions. Interestingly, a recent study showed activity of a probiotic strain in ileostomy effluent samples, which contrasts to the observations in the current study.[64] Whether differences in observations are due to site of sampling (effluent vs jejunum/ileum lumen), strain-specificity, or diminished probiotic strains' survival, remains to be determined.

Consistent with earlier observations that small intestinal microbiota is highly dynamic,[2] we also observed fluctuations in small intestinal microbial composition within one day. In the synbiotic group, the fluctuation of ingested microbes' relative abundance in the small intestine, albeit not in all subjects, could be partly attributed to individual differences in transit time[65] and differences in baseline microbiota composition (Figure 2). Up to now, although studies investigating the probiotic effect on mucosal transcriptomic responses exist, the short-term effect of synbiotic supplementation on small intestinal microbiota has not been addressed before. The implications of the observed elevated levels of probiotic strains approximately two hours after the ingestion on host gene expression remain to be addressed in follow-up studies.

Considering limitations of the current study, our metatranscriptome analysis was based on two subjects in the synbiotic group and one suject in the placebo group, only allowing for initial exploratory analyses. Although no metabolic activities of ingested probiotic strains were identified based on obtained metatransptome data, the conclusion on the activity of probiotic strains is unavoidably limited by the small sample-size used for the analysis. Another limitation of the current study was the use of healthy subjects, whose baseline microbiota might differ from that of unhealthy subjects also with respect to the small intestine. The intestinal microbiota response of unhealthy subjects might therefore also differ from that of healthy subjects.

Overall, the current study investigated the spatio-temporal dynamics of the small intestinal microbiome in healthy subjects, as well as its response to synbiotic supplementation, with a dedicated control panel of healthy individuals receiving placebo intervention. We demonstrated significant differences in microbial and metabolomic composition at baseline between duodenum, jejunum, proximal ileum content and feces. In addition, the significant difference in microbial composition along the small intestine was most pronounced in the morning after overnight fasting, while it was not always measurable around noon or in the afternoon. Two weeks of synbiotic supplementation did not affect the overall microbiota and metabolomic composition in small intestinal fluids and feces differently from placebo. Moreover, small intestinal microbiota is highly dynamic, and ingested probiotic bacteria were shown to lead to a transient spike in the relative abundance of corresponding genera and ASVs, suggesting their passage through the entire gastrointestinal tract.

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Disclosure statement

J.G. and I.B.V. are employees of Winclove Probiotics. They have no direct or additional financial interests, and moreover this does not alter our adherence to policies on open and transparent sharing of data and materials. All other authors disclose no conflicts of interest.

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Data availability statement

The raw sequence data of the microbiota composition has been deposited into European Nucleotide Archive with accession number PRJEB42262. The RNA-seq data generated within this study has been deposited into European Nucleotide Archive with accession number PRJEB55599.

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