RESEARCH ARTICLE

Synbiotic *Lactobacillus acidophilus* NCFM and cellobiose does not affect human gut bacterial diversity but increases abundance of lactobacilli, bifidobacteria and branched-chain fatty acids: a randomized, double-blinded cross-over trial

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Abstract

Probiotics, prebiotics, and combinations thereof, that is synbiotics, have been reported to modulate gut microbiota of humans. In this study, effects of a novel synbiotic on the composition and metabolic activity of human gut microbiota were investigated. Healthy volunteers ($n = 18$) were enrolled in a double-blinded, randomized, and placebo-controlled cross-over study and received synbiotic [*Lactobacillus acidophilus* NCFM (10^9 CFU) and cellobiose (5 g)] or placebo daily for 3 weeks. Fecal samples were collected and lactobacilli numbers were quantified by qPCR. Furthermore, 454 tag-encoded amplicon pyrosequencing was used to monitor the effect of synbiotic on the composition of the microbiota. The synbiotic increased levels of *Lactobacillus* spp. and relative abundances of the genera *Bifidobacterium*, *Collinsella,* and *Eubacterium* while the genus *Dialister* was decreased ($P < 0.05$). No other effects were found on microbiota composition. Remarkably, however, the synbiotic increased concentrations of branched-chain fatty acids, measured by gas chromatography, while short-chain fatty acids were not affected.

Introduction

The human gut microbiota has gained enormous interest during the last couple of decades as it appears to play a key role in human health and disease. Altered composition of gut microbiota has been reported for several diseases including low-grade inflammatory diseases such as type 2 diabetes (Larsen *et al.*, 2010; Qin *et al.*, 2012) and obesity (Ley *et al.*, 2006; Schwiertz *et al.*, 2010) as well as metabolic disorder (Munukka *et al.*, 2012). Apart from the composition of the gut microbiota, also its metabolic activity is important, and especially production of short-chain fatty acids (SCFA), and possibly branched-chain fatty acids (BCFA), are of interest. The production of BCFA and SCFA is often used to gain knowledge on the metabolic activity of the gut microbiota as BCFA are produced solely through protein breakdown by the gut microbiota while SCFA are also produced from fermentation of carbohydrates (Wong *et al.*, 2006). While SCFA often are studied due to their beneficial effects, effects of BCFA are seldom studied and largely unknown, but a role has been indicated in Na^+ uptake, bowel disorders, and rotavirus infections (Brooks *et al.*, 1984; Zaharia *et al.*, 2001; van Nuenen *et al.*, 2004; Huda-Faujan *et al.*, 2010; Le Gall *et al.*, 2011). Modulation of gut microbiota and its activity is therefore a potential strategy for improving gut health and well-being and currently probiotics, prebiotics, and synbiotics receive attention for

their effects on gut microbiota (Roberfroid *et al.*, 2010; Quigley, 2011).

Several studies have reported probiotics to increase numbers of lactobacilli and bifidobacteria, which generally is accepted as being a desired effect (reviwed by Sanders, 2011). Recently, however, high-throughput profiling methods did not demonstrate probiotics to have significant effects on composition of the gut microbiota of healthy humans (McNulty *et al.*, 2011; Kim *et al.*, 2013; Lahti *et al.*, 2013). Few human studies investigate effect of probiotics on BCFA and SCFA, and most of these studies do not report changes in healthy humans (Goossen *et al.*, 2003; Koning *et al.*, 2008; Roessler *et al.*, 2011).

Like probiotics, prebiotics are also commonly found to increase numbers of lactobacilli and bifidobacteria as reviewed for a large number of *in vitro* and *in vivo* studies (Roberfroid *et al.*, 2010). Few human studies on how prebiotics affect the human microbiota using pyrosequencing have to our knowledge been published. A study of the effect of galacto-oligosaccharide intake on microbiota of healthy humans reported a specific enrichment of bifidobacteria, (Davis *et al.*, 2011) while another study of two types of resistant starch reported increase in the phyla *Actinobacteria* and *Bacteroidetes* and decrease in the phylum *Firmicutes* for a chemically modified starch (Martínez *et al.*, 2010). Furthermore, polydextrose and soluble corn fiber increased members of the families *Clostridiaceae*, *Veillonellaceae* as well as the genera *Faecalibacterium*, *Phascolactobacterium,* and *Dialister* while members of the family *Eubacteriaceae* were decreased (Hooda *et al.*, 2012). Effects of prebiotics on BCFA are not well investigated and both increase and decrease levels of fecal BCFA have been found (Gråsten *et al.*, 2003; Nilsson *et al.*, 2008) while increase in SCFA is often reported (Roberfroid *et al.*, 2010).

It has been suggested that by combining probiotics with prebiotics to synbiotics, beneficial effects of both may be gained. Although synbiotics have been reported to increase numbers of lactobacilli and bifidobacteria in healthy humans (Bartosch *et al.*, 2005; Shioiri *et al.*, 2006; Casiraghi *et al.*, 2007), few human studies have previously investigated the effects of synbiotics on composition of gut microbiota, BCFA and SCFA production in healthy humans. Using denaturing gradient gel electrophoresis Worthley *et al.* (2009) found a combination of *Bifidobacterium animalis* ssp. *lactis* and resistant starch to induce a shift in the bacterial community while Vitali *et al.* (2010) and De Preter *et al.* (2011)did not observe any changes for a combination of *Lactobacillus helveticus*, *B. longum* and fructo-oligosaccharides and a combination of *L. casei* Shirota and oligofructose-enriched inulin, respectively. However, a recent study reported a synbiotic consisting of *B. longum* and inulin-based prebiotic to affect composition of gut microbiota at phylum level by increasing *Actinobacteria* and *Firmicutes* as well as decreasing *Proteobacteria* (Macfarlane *et al.*, 2013). In healthy humans, BCFA is mostly unaffected by synbiotic intake (Shioiri *et al.*, 2006; Worthley *et al.*, 2009; De Preter *et al.*, 2011) however, recently a synbiotic of *B. longum* in combination with a inulin and oligofructose mixture was found to increase the concentration of isobutyrate (Macfarlane *et al.*, 2013). Human studies have moreover reported synbiotics to increase levels of SCFA (Shioiri *et al.*, 2006; Ndagijimana *et al.*, 2009; Vitali *et al.*, 2010; De Preter *et al.*, 2011; Macfarlane *et al.*, 2013).

Lactobacillus acidophilus NCFM (NCFM) is a well-studied probiotic bacterium which has been reported to increase lactobacilli numbers (Larsen *et al.*, 2011), and in combination with lactitol, increase in lactobacilli and bifidobacteria has been reported (Ouwehand *et al.*, 2009; Björklund *et al.*, 2011). Cellobiose is a nondigestible carbohydrate which has been reported to be fermented in the human intestine (Nakamura *et al.*, 2004). The prebiotic potential of cellobiose has been investigated by *in vitro* fermentations in the presence of a human fecal inoculum, where cellobiose increased bifidobacteria and SCFA (Sanz *et al.*, 2005). In combination with *L. rhamnosus*, cellobiose was found to increase lactic acid bacteria (Umeki *et al.*, 2005) as well as SCFA (Umeki *et al.*, 2004).

We have previously shown that cellobiose is able to stimulate growth of NCFM under laboratory conditions and provided a competitive advantage for NCFM in a model system of the human colon (van Zanten *et al.*, 2012). Furthermore, the combination of NCFM and cellobiose increased bifidobacteria by 10-fold and showed a tendency of decreasing the modified ratio of *Bacteroidetes/Firmicutes*. While BCFA (i.e. isobutyrate, 2-methylbutyrate and isovalerate) were significantly decreased, concentrations of total SCFA (i.e. acetate, propionate and butyrate) were increased fourfold. These findings indicate that NCFM in combination with cellobiose may be able to affect the composition and metabolic activity of the microbiota in humans.

The aim of this study was to investigate the effect of the synbiotic combination of NCFM and cellobiose on composition and metabolic activity of gut microbiota in healthy humans to substantiate our *in vitro* findings. In a randomized double-blinded placebo-controlled trial, the effect of the synbiotic on microbial composition was assessed by tag-encoded pyrosequencing of fecal samples, and the metabolic activity was examined by measuring levels of BCFA and SCFA using gas chromatography.

Materials and methods

Study design

The study was performed as a double-blind, randomized, placebo-controlled, and cross-over trial (Fig. 1). The

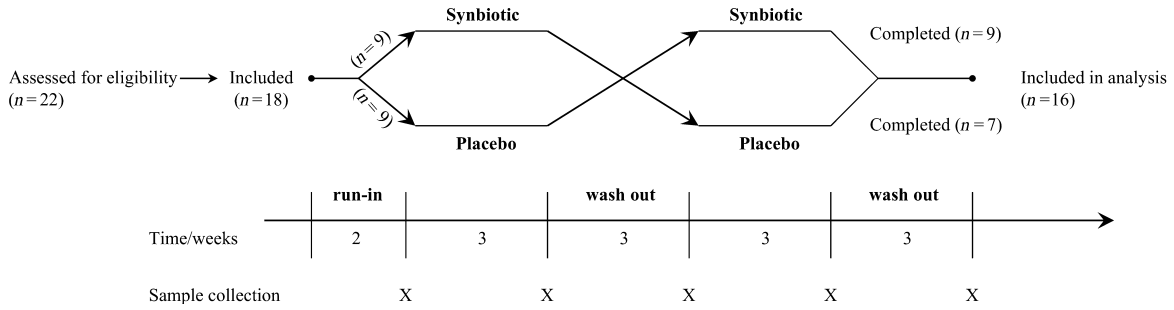

Fig. 1. Schematic presentation of the study design showing the timeline and the number of volunteers which were assessed and included in study and the number of volunteers which completes the study. Crosses indicate collection of fecal samples.

inclusion criteria for enrollment in the study were that volunteers were healthy, between 18 and 50 years and had not used antibiotics 3 months prior to the study. Exclusion criteria were chronic illness, regular intake of medication (birth control pills excepted), and postmenopausal women. Volunteers were excluded during the study in case of serious illness, long periods of illness, and antibiotic treatment. The study was approved by The Scientific Ethics Committee of Capital Region, Denmark (reference H-4-2010-137) and registered at ClinicalTrials.gov (NCT01716910). Written informed consent was obtained from volunteers prior to recruitment.

Two weeks prior to study start and throughout the study, the volunteers were instructed to avoid consumption of products containing probiotic or prebiotic formulations. No other dietary restrictions or observations were made. After randomization (1 : 1), volunteers received sachets containing either synbiotic, consisting of 5 g cellobiose (Matsutani Chemical Industry Co, Japan; 97% cellobiose as determined by High Performance Anion Exchange Chromatography with pulsed amperometric detection (Damager *et al.*, 2001) using authentic cellobiose and glucose as standards) and 1×10^9 CFU lyophilized *L. acidophilus* (NCFM; ATCC 700396) or 5 g placebo, consisting of maltodextrin (C*Dry A 01318, kind gift from Cargill Nordic A/S, Denmark). Sachets were marked with a numerical code and products were indistinguishable. The contents of the sachets were to be consumed daily. One group received the synbiotic and the other received placebo for a 3-week period which was followed by a 3-week washout period after which the intervention was crossed (Fig. 1) Fecal samples were collected prior to intervention and at the end of each period (synbiotic, placebo and washout). Randomization codes were kept unbroken until all samples were analyzed. Subsequently, codes were broken in a manner so the groups, but not the order of placebo and synbiotic intake, were known to the investigators and analyzed prior to breaking the code for intake periods.

Volunteers were instructed on collection of fecal samples and asked to immediately bring the sample to the Department of Food Science, while keeping it cold. Samples were stored at −80 °C until further analysis. Questionnaires for each period were used to collect information on overall health and well-being, defecation frequency and consistency, and gastrointestinal symptoms (Supporting Information, Table S1). Volunteers were instructed to keep a daily record consumption of synbiotics/placebo for compliance.

Analysis of fecal *L. acidophilus* and *Lactobacillus* spp. by quantitative PCR

Bacterial DNA was extracted from fecal samples using QIAamp® DNA Stool Mini Kit (Qiagen, Germany) and performed according to manufacturer's instructions with minor deviations. An additional step of mixing 30 s in the presence of 1 g 1000 μm glass beads (Sigma-Aldrich) prior to extraction was included, and lysis of bacterial cells was performed at 95 °C instead of 70 °C for 10 min. Concentration and quality of extracted DNA was determined using NanoDrop 1000 spectrophotometer Thermo Scientific (Saveen Werner ApS Denmark).

Numbers of *L. acidophilus* and *Lactobacillus* spp. were determined by quantitative real-time polymerase chain-reactions (qPCR). For quantification of *L. acidophilus*, Taqman® methodology using species specific primers and probe (Ouwehand *et al.*, 2009) was done at an annealing temperature of 61 °C. For *Lactobacillus* spp. quantification was done using SYBR green methodology and genus specific primers (Walter *et al.*, 2001; Heilig *et al.*, 2002) with an annealing temperature of 56 °C. Standard curves made by 10-fold dilutions series of target species DNA were done and assays were performed with ABI Prism® 7000 or 7500 FAST sequence Detection System (Applied Biosystems).

Analysis of fecal microbiota composition by tag-encoded amplicon pyrosequencing

Tag-encoded amplicon pyrosequencing for baseline, synbiotic and placebo samples of all volunteers, was performed

as described previously (Masoud *et al.*, 2011) using DNA extracted as described above. In brief, amplifications of the region flanking the V3 and V4 region of the 16S rRNA gene were done using modified primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNG GGTATCTAAT) (Yu *et al.*, 2005) After amplification, PCR products were purified and a second PCR amplification, under same conditions as the first, was done but using primer 341F with adapter-A and tag sequence and primer 806R with adaptor-B sequence. Tag-encoded amplicon pyrosequencing was performed using GS Titanium Plates and GS FLX sequencing system in accordance to manufacturer's instructions (Roche).

Sequence analysis of the microbial community

To ensure a comprehensive insight into the bacterial distribution within all 48 samples, 16S rRNA gene amplicons were sequenced using two Titanium Plates (two separate runs). Sequence quality control, sorting, trimming and clustering into operational taxonomic units (OTUs) as well as alpha and beta diversity analyses were conducted using Quantitative Insights Into Microbial Ecology pipeline (QIIME-pipeline http://qiime.org/index.html) (Caporaso *et al.*, 2010). Splitting libraries according to barcodes was done with split_libraries.py script [min sequence length $\geq$ 300 bp, quality score (QS) $\geq$ 25) (QIIME-pipeline]. The RDP classifier (Ribosomal Database Project release 10, http://rdp.cme.msu.edu/) was used for taxonomic annotations. Metrics, such as observed species and chao1 index, were calculated using alpha_rarefaction.py workflow. Data subjected to this analysis composed of the control group sequenced of each individual run, and the set of all merged control groups together. A raw data set was initially denoised with denoise_wrapper.py script (QIIME-pipeline) followed by chimera purging, performed with identify_chimeric_seqs.py (QIIME-pipeline) where ChimeraSlayer algorithm was employed. Two-dimensional Principal Coordinate Analysis (PCoA) plots were generated using jackknifed_beta_diversity.py workflow with the *–e* value equal to the 80% of the sequence number of the most poor sample. Group differences were analyzed with ANOSIM (Analysis of Similarities) test based on the weighted UNIFRAC distance matrix.

Analysis of fecal concentrations of volatile fatty acids by gas chromatography

For determination of BCFA and SCFA concentrations, samples were prepared by addition of 0.5 mL of internal standard [20 mM pivalic acid (Sigma)] and 4.0 mL of water to 0.5 g of feces and shaking for 5 min. After centrifugation (5000 ***g***, 10 min, 19 °C), 500 μL supernatant was mixed with 250 μL saturated oxalic acid (Fluka) and incubated for 1 h at 4 °C. The samples were centrifuged (3 min, 16 000 ***g***) and 500 μL supernatant was transferred to a sample vial and gas chromatography was performed as described previously (Holben *et al.*, 2002).

Statistical analysis

Statistical analysis of fatty acids and qPCR results was performed by paired sample *t*-test using MICROSOFT OFFICE EXCEL and pyrosequencing results were analyzed by Two Paired Sample Signed-Rank Test (Wilcoxon) using Statistics Online Computational Resource (SOCR, http://socr.ucla.edu/SOCR.html). Spearman Rank correlation between relative abundance of genera, above 0.001%, and the concentration of BCFA along with SCFA was done using MATLAB (http://www.mathworks.com). Results are expressed as median values with quartile ranges (QR) and presented by box plot and whisker charts. Differences with $P < 0.05$ were considered significant and samples obtained from washout periods were not included in the analysis.

Results

Characteristics of participants

Healthy volunteers ($n = 18$), 10 female and 8 male 20–30 years of age, fulfilling the inclusion criteria were enrolled in a randomized, placebo-controlled, and cross-over trial (Fig. 1). The volunteers daily consumed a synbiotic, consisting of 5.0 g cellobiose and 1×10^9 CFU lyophilized *L. acidophilus* (NCFM; ATCC 700396) or 5.0 g placebo, consisting of maltodextrin (Cargill Nordic A/S, Denmark). Two female volunteers withdrew during the study due to reasons unrelated to the intervention. The synbiotic was well tolerated by the 16 volunteers completing the study and the majority reported an overall well-being as 'neither good nor bad', 'good' or 'very good' in both placebo and synbiotic periods (Table S1). Self-reported gastrointestinal symptoms did not differ between groups. Product compliance (self-reported) for both intervention periods was 96% (data not shown).

Quantification of *L. acidophilus* NCFM and *Lactobacillus* spp. by qPCR

Quantification results of NCFM showed the majority of fecal samples to have low levels of NCFM below detection limit of the qPCR assay. Median values of lactobacilli detected by qPCR corresponded to 3.6 Log_{10} cells g^{-1} feces (QR 2.6–4.4) for baseline samples and 4.1 Log_{10} cells g^{-1} feces (QR 3.7–4.6) for placebo samples (Fig. 2).

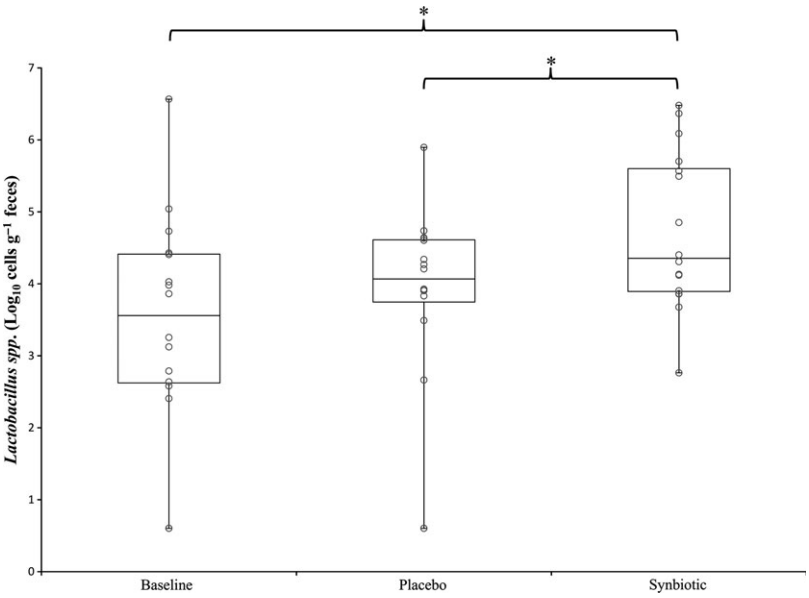

Fig. 2. Quantification of *Lactobacillus* spp. by qPCR in samples obtained from baseline, placebo, and synbiotic periods. Results are shown as median values with the upper (75%) and lower (25%) QR. Whiskers indicate the highest and lowest values, and asterisks indicate significance ($P < 0.05$). Circles represent individual measurements.

Intake of synbiotic significantly increased lactobacilli to 4.4 Log_{10} cells g^{-1} feces (QR 3.9–5.6) as compared both to baseline and placebo ($P = 0.01$ and $P = 0.04$, respectively).

Characterization of the effects of synbiotic on the intestinal microbiota by tag-encoded amplicon pyrosequencing

A total of 48 samples obtained before and after intervention were sequenced (NCBI accession number SRA058021). The number of pooled reads yielded 2 092 165 sequences and the number of sequences passing quality control (length $\geq$ 300 bp, QS $\geq$ 25) was 1 459 548, with an average of 30 407 sequences per individual (max = 47 136, min = 13 576, SD = 8235) and an average sequence length of 446 bp. Sequences from two independent runs were merged resulting in increased average numbers of estimated species (calculated per 2000 reads) for an average of 6% (max = 9.6, min = 1.8, SD = 5.5) when comparing with a single plate results. Correspondingly, the chao1 index increased by 11% (max = 22.4 min = −7.4, SD = 13.1). Using the Shannon and chao1 indexes, no differences in diversity was observed between synbiotic, placebo, and baseline (data not shown). Moreover, there were no differences found in the observed species between the synbiotic, placebo, and baseline samples (data not shown).

In samples obtained before intervention (baseline samples), *Firmicutes* (71%) and *Bacteroidetes* (19%) were identified as the dominating phyla (Table 1) while the remaining reads were from *Proteobacteria* (1.5%), *Actinobacteria* (0.5%), and *Verrucomicrobia* (0.1%). Approximately 2% of the sequences were classified as other bacteria. No differences in phyla, or in the ratio between *Bacteroidetes* and *Firmicutes*, were observed for intake of synbiotic compared with baseline and placebo. In the baseline samples, the most diverse phylum was *Firmicutes*, with the most abundant genera being unclassified *Lachnospiraceae* (16%), *Roseburia* (11%) and unclassified

Table 1. Relative abundances (%) of the main phyla identified for baseline, placebo and synbiotic periods

				P-values, synbiotic compared to	
	Baseline*	Placebo*	Synbiotic*	Baseline	Placebo
Phylum					
Actinobacteria	0.36 (0.15–1.08)	0.30 (0.10–0.70)	0.47 (0.18–1.05)	0.796	0.134
Bacteroidetes	18.54 (12.84–23.72)	16.42 (13.83–22.3)	17.21 (12.97–24.86)	0.918	0.535
Firmicutes	70.97 (67.02–78.09)	71.29 (61.78–78.48)	73.09 (67.21–79.26)	0.836	0.918
Other	1.96 (1.69–3.57)	2.73 (2.21–4.38)	2.67 (2.21–3.24)	0.352	0.756
Proteobacteria	1.27 (0.74–2.90)	1.14 (0.65–1.95)	0.96 (0.43–2.42)	0.438	0.796
Verrucomicrobia	0.10 (0.01–0.33)	0.16 (0.02–7.87)	0.05 (0.01–0.33)	0.438	0.134

*Median values with the upper and lower quartile range in parenthesis.

Clostridiales (10%) while the genera *Dialister*, *Oscillibacter*, *Coprococcus*, *Faecalibacterium*, *Subdoligrandilum,* and unclassified *Ruminococcaceae* accounted for 1% to 5% (data not shown). The most abundant genera in the phylum *Bacteroidetes* were *Bacteroides* (5%), *Alistipes* (1%), and unclassified *Bacteroidales* (1%) (Table S2).

Comparison of bacterial abundance before and after intervention is shown in Table 2. A decrease at class level for *Clostridia* and *Deltaproteobacteria* was seen. The orders *Clostridiales* and *Desulfovibrionales* were decreased, as was the family *Desulfovibrionaceae*, which was twofold lower compared with the baseline samples while unclassified *Bacteroidales* was increased (Table 2). At the genus level, unclassified *Bifidobacteriaceae* and *Parabacteriodes* were increased by threefold and twofold, respectively, while a twofold decrease was found for *Blautia* and *Roseburia*.

No changes on class, order, and family levels were observed between synbiotic and placebo (Table 2). However, threefold increases in the genera *Bifidobacteria* and *Collinsella*, a twofold increase in the genus *Eubacterium* and a fivefold decrease in the genus *Dialister* were observed when synbiotic intake was compared to placebo.

For further investigation of the effect of synbiotic intake on the composition of the gut microbiota, PCoA based on weighted UNIFRAC distance matrix was generated as shown in Fig. 3. The PCoA showed that volunteers did not cluster according to baseline or placebo and synbiotic intake (ANOSIM $P = 0.854$, $R = -0.025$). Rather, samples showed tendency to cluster according to volunteer (ANOSIM $P = 0.001$, $R = 0.420$). Moreover, the analysis showed that the pattern of each volunteer was highly individual.

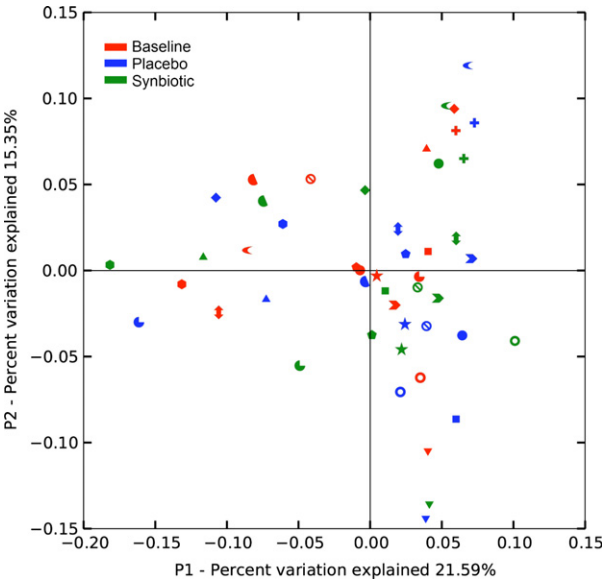

Fig. 3. PCoA based on weighted distance matrices each calculated from 10 rarefied OTU tables. Together the first two principal components explain 37% of the variations in microbial community. Different symbols represent different subjects and are colored according to baseline (red symbols), placebo (blue symbols), and synbiotic treatment (green symbols).

Table 2. Relative abundances (%) of bacteria for synbiotic intake as compared to placebo and baseline

	Baseline	Placebo	Synbiotic
Class			
Clostridia	75.35 (68.48–79.31)	69.82 (59.16–74.68)	72.21* (59.22–75.28)
Deltaproteobacteria	0.30 (0.11–0.53)	0.14 (0.07–0.29)	0.12* (0.05–0.20)
Order			
Clostridiales	73.08 (67.62–78.37)	68.51 (58.03–74.32)	68.73* (58.81–74.83)
Desulfovibrionales	0.29 (0.10–0.52)	0.14 (0.07–0.26)	0.12* (0.05–0.19)
Family			
Bacteroidales, unclassified	0.77 (0.45–1.07)	0.75 (0.65–1.48)	0.97* (0.58–1.63)
Desulfovibrionaceae	0.24 (0.09–0.45)	0.12 (0.06–0.22)	0.11* (0.05–0.16)
Genus			
Bifidobacterium	0.20 (0.08–0.60)	0.13 (0.06–0.35)	0.33† (0.06–0.80)
Bifidobacteriaceae, unclassified	0.04 (0.03–0.09)	0.04 (0.02–0.15)	0.10* (0.04–0.29)
Collinsella	0.04 (0.01–0.15)	0.02 (0.01–0.04)	0.05† (0.01–0.12)
Parabacteroides	0.30 (0.14–0.38)	0.41 (0.17–1.11)	0.58* (0.32–1.23)
Eubacterium	0.05 (0.02–0.11)	0.03 (0.01–0.08)	0.05† (0.01–0.10)
Blautia	0.52 (0.41–0.97)	0.26 (0.23–0.37)	0.30* (0.21–0.47)
Roseburia	11.23 (6.59–13.98)	7.46 (3.54–12.88)	4.83* (1.82–10.95)
Dialister	1.45 (0.06–8.53)	3.31 (0.01–12.43)	0.73† (0.01–4.11)

Numbers are presented as median values with the upper and lower quartile range in parenthesis, for baseline, placebo and synbiotic periods synbiotic.

*Significantly different as compared to baseline, $P < 0.05$.

†Significantly different as compared to placebo, $P < 0.05$.

The effect of synbiotic on production of branched- and SCFA

Figure 4 shows the concentrations of BCFA in samples obtained from baseline, placebo, and synbiotic samples. Levels of 2-methylbutyrate significantly increased from 0.8 (QR 0.5–1.3) and 0.7 (QR 0.5–1.0) mM g^{-1} feces for baseline and placebo, respectively, to 1.1 (QR 0.9–2.7) mM g^{-1} feces for synbiotic intake ($P = 0.006$ and $P = 0.01$, respectively; Fig. 4). Levels of total BCFA were increased from 3.5 (QR 1.7–5.0) in baseline to 4.1 (QR 3.4–5.2) for intake of synbiotic ($P = 0.02$). For placebo, levels of BCFA in placebo were 2.8 (QR 2.0–4.0) and synbiotic also showed a trend of increasing levels as compared to placebo ($P = 0.06$). Median values of isobutyrate and isovalerate in baseline were 1.2 (QR 0.9–2.0) and 1.3 (QR 0.5–1.9) mM g^{-1} feces, respectively. Synbiotic showed tendency of increasing isobutyrate ($P = 0.06$); however, no significant changes were observed as compared to baseline or placebo.

Figure 5 shows the concentrations of SCFA in samples obtained from baseline, placebo and synbiotic samples. Median levels of acetate, propionate, butyrate, and valerate in baseline samples were 54.1 (QR 38.5–65.0), 13.5 (QR 10.3–19.4), 14.7 (QR 8.6–21.4) and 1.37 (QR 0.0–2.4) mM g^{-1} feces, respectively. No effect was seen for synbiotic intake compared with baseline and placebo.

No correlations between any of the short-chain and branched-chain fatty acids and relative abundance of the bacteria (phylum, class, order, family, and genus level) were observed (data not shown). Investigation of individual effects revealed a high level of interindividual differences with regard to changes in bacterial abundances (also reflected in the PCoA plot in Fig. 3), BCFA, and SCFA concentrations as well as for correlations between relative abundance of bacteria and levels of BCFA and SCFA (data not shown).

Discussion

As the role of the human gut microbiota in health and disease has become of increasing interest, there is a growing interest in ways of modulating the composition and the microbial activity using, for example probiotics,

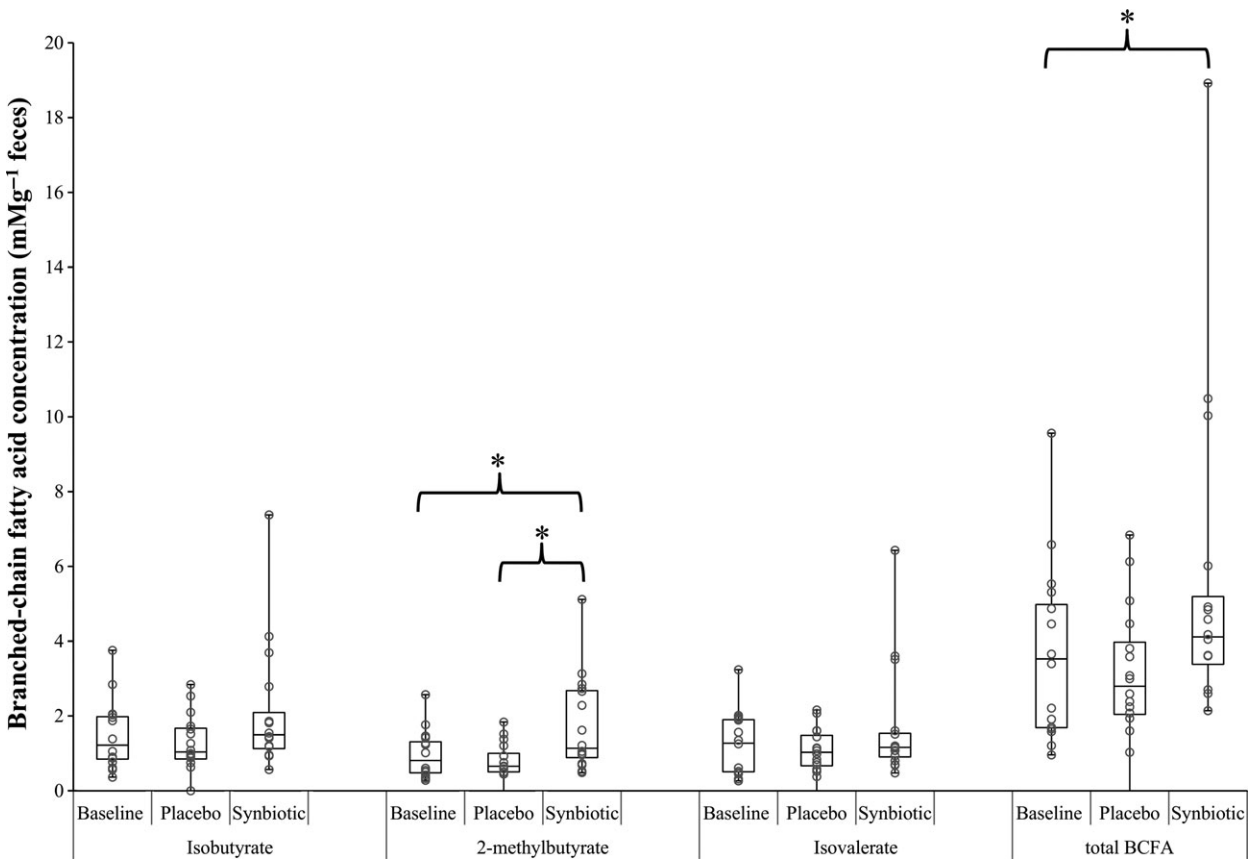

Fig. 4. Concentrations of BCFA detected by gas chromatography, in samples obtained from baseline, placebo, and synbiotic periods. Results are shown as median values with the upper (75%) and lower (25%) QR. Whiskers indicate the highest and lowest measured values, and asterisks indicate significance ($P < 0.05$). Circles represent individual measurements.

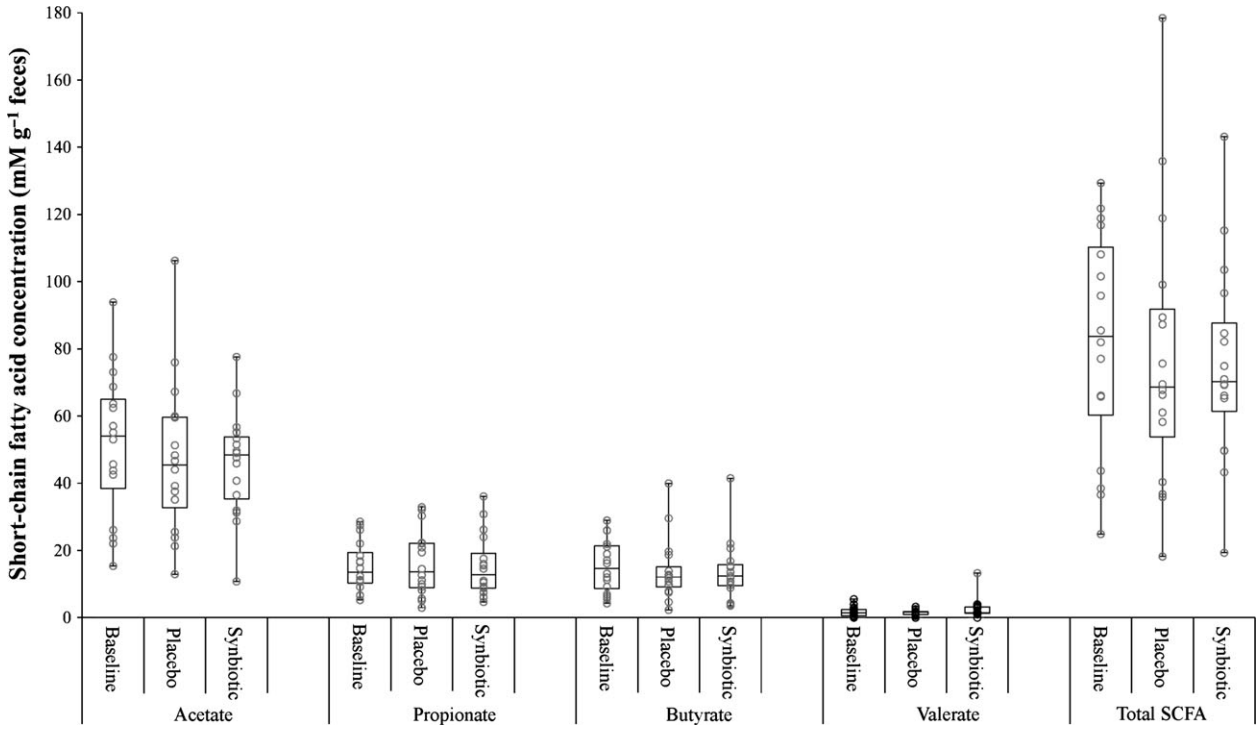

Fig. 5. Concentrations of SCFA detected by gas chromatography, in samples obtained from baseline, placebo and synbiotic periods. See Fig. 4 for definition of box and whisker plot.

prebiotics and synbiotics (Roberfroid *et al.*, 2010; Sekirov *et al.*, 2010; Quigley, 2011). The present study is the first to test the effects of a synbiotic, consisting of NCFM and cellobiose, in humans. Overall, this novel synbiotic was well tolerated by the volunteers and did not result in unwanted side effects (Table S1). The genome of NCFM contains genes involved in acid and bile tolerance as well as adhesion (Altermann *et al.*, 2005) and i*n vitro* investigations confirm survival of NCFM during passage of the gastrointestinal tract and that duodenal digestion upregulates genes involved in adhesion (Weiss & Jespersen, 2010). NCFM has been shown to adhere to intestinal cells (Sanders & Klaenhammer, 2001) and several human studies report intake of NCFM to increase *L. acidophilus* cell numbers in fecal samples (Ouwehand *et al.*, 2009; Gøbel *et al.*, 2010; Larsen *et al.*, 2011). However, in the present study, intake of synbiotic resulted in increase in NCFM numbers in only three of the 16 volunteers (data not shown). One explanation for this is that the amounts used were 2–10 times lower compared with other studies (Ouwehand *et al.*, 2009; Andreasen *et al.*, 2010; Larsen *et al.*, 2011). However, the synbiotic increased the genus *Lactobacillus* generally as well as induced a significant increase in the relative abundance of the genus *Bifidobacterium,* which is in line with our previous findings using a colonic model system (van Zanten *et al.*, 2012). Increases in lactobacilli and bifidobacteria are generally regarded as beneficial and although the beneficial effects may vary between species they possess traits that are desired, such as production of acids which decreases intestinal pH thereby inhibiting growth of potentially pathogenic bacteria (Servin, 2004).

Although the number of studies is limited, effects of probiotics and prebiotics on human gut microbiota have previously been investigated using pyrosequencing, but to our knowledge, this is the first study applying this technique to investigate the effect of a synbiotic on the composition of the gut microbiota of healthy humans. In the study conducted by Larsen *et al.* (2011) neither of the probiotic bacteria *L. acidophilus* NCFM nor *B. animalis* ssp. *lactis* Bi-07 altered the composition of the microbiota in children with atopic dermatitis. Likewise no effect on microbiota composition was found by McNulty *et al.* (2011) in gnotobiotic mice or healthy monozygotic twins consuming a fermented milk product or in healthy humans consuming a range of different probiotic lactobacilli and bifidobacteria as investigated by Kim *et al.* (2013).

Prebiotic galacto-oligosaccharide was found to induce a specific increase in relative abundance of bifidobacteria, while few other bacterial groups were affected (Davis *et al.*, 2011), whereas resistant starch, polydextrose and soluble corn fiber changed abundances of some of the

major phyla and families (Martínez *et al.*, 2010; Hooda *et al.*, 2012).

In the present study, the distribution of sequences was in general agreement with what has previously been reported for healthy humans, with the majority of reads belonging to *Firmicutes* and *Bacteroidetes* at phylum level and *Bacteroides*, *Faecalibacterium*, and *Roseburia* being among the predominant bacteria at genus level (Martínez *et al.*, 2010; Davis *et al.*, 2011). No changes at phylum level were observed in the present study (Table 1), only on genus level did the synbiotic intake increase relative abundance of the genera *Bifidobacterium*, *Collinsella* and *Eubacterium* and decrease the genus *Dialister* as compared to placebo (Table 2). Bifidobacteria are regarded as beneficial and an increase in their relative abundance is desired. Little is known about the role of *Collinsella*, *Eubacterium,* and *Dialister* in the microbiota; however, low abundance of these genera has been reported to be associated with irritable bowel syndrome (Kassinen *et al.*, 2007; Lyra, 2009) and Crohn's disease (Joossens *et al.*, 2011).

Using PCoA, the present study found samples to group according to individuals rather than according to placebo and synbiotic intake indicating huge differences in the individual microbiota composition (Fig. 3). Moreover, interindividual variations in the clustering of each volunteer in response to synbiotic and placebo intake were observed. It is known that the composition of the human gut microbiota is highly variable between individuals (Turnbaugh & Gordon, 2009) and Kim *et al.* (2013) found highly individual changes of gut microbiota of healthy humans in response to probiotic intake. Also individual responses to intake of galacto-oligosaccharides and resistant starch have been reported (Martínez *et al.*, 2010; Davis *et al.*, 2011). In the present study, differences between individuals with respect to effects of synbiotic intake on lactobacilli and bifidobacteria were also observed (data not shown), and it is possible that variability and individual responses of the microbiota in the volunteers may mask effects on other bacterial groups.

The gut microbiota plays an important role in metabolizing undigested protein and carbohydrates which results in production of BCFA and SCFA (Wong *et al.*, 2006). BCFA are solely formed during breakdown of branched-chain amino acids and while SCFA, especially butyrate, have received much attention for their beneficial effects, BCFA have not been the target of many studies (Macfarlane & Macfarlane, 2012). Previously, the NCFM in combination with cellobiose significantly decreased BCFA in a colonic model system (van Zanten *et al.*, 2012). But interestingly, the present study found increase in BCFA (Fig. 4). Although model systems are helpful in predicting the effects of probiotics, prebiotics, and synbiotics, interactions with the host are lacking (Venema & van den Abbeele, 2013). Intestinal epithelial cells are of importance as they, for example, absorb metabolites such as SCFA. Also, dead cells are broken down, producing BCFA. The lack of epithelial cells, along with a constant supply of cellobiose, may contribute to inhibition of BCFA production observed in the colonic model. The present study shows the importance of verification of *in vitro* observations in human trials.

Recently, another synbiotic was reported to increase isobutyrate, while other synbiotics do not affect levels of BCFA (Ouwehand *et al.*, 2009; Worthley *et al.*, 2009; Vitali *et al.*, 2010; Kekkonen *et al.*, 2011). BCFA have been used as markers of unwanted proteolytic activity which results in a number of potentially harmful compound such as ammonia, amines, phenols, and sulfides (reviewed by Windey *et al.*, 2012). The produced compounds have different fates and are, for example, further metabolized, absorbed, and excreted, however, the fate of BCFA is not thoroughly investigated and their role in host health is unclear. Although increases in BCFA are associated with protein intake, a previous study found β-glucan-enriched oat bran to increase concentrations of both SCFA and BCFA (Nilsson *et al.*, 2008).

Few studies have investigated the effects of BCFA, but as mentioned above isobutyrate has been reported to be involved in Na^+ absorption in rats (Zaharia *et al.*, 2001) and an *in vitro* study found the microbiota of patients with inflammatory bowel disease (IBD) to have increased levels of SCFA and BCFA (van Nuenen *et al.*, 2004). In humans, decreased levels of BCFA have been reported in IBD and IBS patients (Huda-Faujan *et al.*, 2010; Le Gall *et al.*, 2011), indicating that production of BCFA may be linked to host health.

Although the synbiotic previously was found to increase acetate, propionate and butyrate in a colonic model system (van Zanten *et al.*, 2012), the present study did not observe any effects on SCFA (Fig. 5).

In conclusion, the present study confirmed in an human intervention study that a novel synbiotic consisting of *L. acidophilus* NCFM in combination with cellobiose increase the levels of bifidobacteria as previously observed in a model system of the human colon (van Zanten *et al.*, 2012). Moreover, the synbiotic increased lactobacilli as well as the relative abundance of *Collinsella* and *Eubacterium* whereas it decreased *Dialister*. The synbiotic used in the human intervention study did additionally increase the concentrations of BCFA, in particular 2-methylbutyrate and isobutyrate. Thereby, the presence study could not confirm the decrease in BCFA and the increase in SCFA by *L. acidophilus* NCFM in combination with cellobiose as seen in the colonic model system. Generally, this study provides novel insight into how a synbiotics affects the complex human microbiota and highlights the complexity of

intervention studies as well as *in vitro* model studies. To increase the significance of the present findings, larger human intervention studies are required.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Self-reported overall well-being and gastrointestinal symptoms (a) and frequency of defecation and consistency of feces (b) of volunteers assessed by questionnaires following placebo and synbiotic intake.
Table S2. Relative distributions in individual volunteers in fecal samples from baseline, placebo, and synbiotic periods as determined by tag-encoded pyrosequencing.