Bioactive Carbohydrates and Dietary Fibre 24 (2020) 100235

Contents lists available at ScienceDirect

Bioactive Carbohydrates and Dietary Fibre

journal homepage: http://www.elsevier.com/locate/bcdf

Pooled faecal inoculum can predict infant fiber fermentability despite high inter-individual variability of microbiota composition

Madelon J. Logtenberg [a], Jolien C.M. Vink [a], Renske M. Serierse [a], Ran An [b], Gerben D. A. Hermes [b], Hauke Smidt [b], Henk A. Schols [a,*]

[a] Laboratory of Food Chemistry, Wageningen University & Research, Bornse Weilanden 9, 6708 WG, Wageningen, the Netherlands
[b] Laboratory of Microbiology, Wageningen University & Research, Stippeneng 4, 6708 WE, Wageningen, the Netherlands

ARTICLE INFO

Keywords:
In vitro fermentation
Galacto-oligosaccharides
Short-chain fatty acids
16S rRNA gene amplicon sequencing
Porous graphitic carbon chromatography

ABSTRACT

Infants are known for their high inter-individual variability in gut microbiota composition in the first months of life. This poses difficulties when predicting the fate of non-digestible carbohydrates (NDCs) in the infant gut using *in vitro* models, since numerous experiments with individual faecal inocula of different infants are required. In this study an *in vitro* fermentation experiment was performed with galacto-oligosaccharides (GOS) using both individual and pooled faecal inocula of five two-week-old infants. It was shown that pooled faecal inoculum can be used to judge the fermentability of GOS, as a similar trend in total organic acid production and relative increase in *Bifidobacterium* was observed for the pooled faecal inoculum and four out of five individual faecal inocula. An additional fermentation using pooled faecal inoculum of five other infants of the same age confirmed these findings. Additionally, we provided evidence for both size- and isomer-specific fermentation of GOS by infant microbiota, which reveals the potential for the production of tailored NDC mixtures to meet the needs of specific subgroups of infants. Hence, the use of pooled faecal inocula contributes to increasing our knowledge on structure-specific effects of NDCs in infants efficiently.

1. Introduction

There is a growing body of literature that recognizes the link between early life gut microbial dynamics and infant's health (Wang et al., 2016). Recent research suggests various associations between the abundance of specific bacteria and the likelihood of developing metabolic or inflammatory diseases in later life (de Goffau et al., 2014; Li, Wang, & Donovan, 2014).

In general clear evidence was found for the colonization upon birth by facultative anaerobic bacteria, which reduce the oxygen levels and in this way promote further colonization by strictly anaerobic microorganisms (Korpela & de Vos, 2018). During the first days after birth, members of the Proteobacteria predominate, after which members of the Clostridia and bifidobacteria become more prominent (Korpela & de Vos, 2018). Additionally, in the first months of life large inter-individual variation in microbiota composition and dynamics between infants has been observed. These differences are to a large extent caused by the mode of delivery, gestational age at birth, the infant feeding mode, maternal diet, environment and host genetics (Milani et al., 2017). Furthermore also large intra-individual differences exist during the first weeks after birth, as the infant microbiota is highly dynamic with more stable periods alternated with complete shifts in composition caused by e.g. fever, antibiotic therapy or changes in diet (Milani et al., 2017).

Diet shapes the infant microbiota and can prevent future health complaints. Previous research comparing the microbiota composition of formula- and breast-fed infants revealed profound differences caused by the presence of a complex mixture of human milk oligosaccharides (HMOs) in breast milk (Bezirtzoglou et al., 2011). HMOs are resistant to digestion in the small intestine and serve as prebiotics in the large intestine (Engfer et al., 2000). Furthermore HMOs have been shown to support the maturation of the gut barrier by promoting the differentiation of enterocytes (Holscher et al., 2017). To mimic the effects of HMOs, non-digestible carbohydrates (NDCs) can be added to cow's milk-based infant formulas. However, very little is known about the relation between specific HMOs and NDCs and the effect on the infant gut microbiota. Studies up to now have mainly focused on *in vitro* fermentation experiments with prevalent bacterial isolates. Such approaches ignore the importance of cross-feeding networks (Cockburn &

* Corresponding author.
E-mail address: henk.schols@wur.nl (H.A. Schols).

https://doi.org/10.1016/j.bcdf.2020.100235
Received 17 March 2020; Received in revised form 13 July 2020; Accepted 31 July 2020
Available online 4 August 2020

Koropatkin, 2016; Ruiz-Moyano et al., 2013; Valdés-Varela et al., 2017). However, the high inter-individual variability in microbiota composition requires *in vitro* experiments with individual faecal inocula of around ten infants, which makes the study of several structurally different NDCs laborious and costly (Dogra et al., 2015). An alternative approach could be the use of pooled infant faecal inoculum, which consists of the faecal material of multiple infants. In previous research the use of pooled faecal inoculum has already been validated for adults who generally show a more stable faecal microbiota composition than infants. The pooled adult faecal inoculum resulted in a comparable bacterial activity as the individual faecal inocula (Aguirre et al., 2014).

The objective of this study was to determine whether pooled infant faecal inoculum can be used as an alternative for individual infant faecal inoculum leading to a more efficient screening of the fermentability of NDCs. *In vitro* fermentations were performed with galacto-oligosaccharides (GOS) as carbohydrate source using both individual and pooled faecal inocula of two-week-old infants. The microbiota composition and production of organic acids were followed in time, as well as the fate of individual GOS compounds using ultra-high-performance liquid chromatography with porous graphitic carbon coupled to mass spectrometry (UHPLC-PGC-MS), which showed us that pooled faecal inoculum can be used to judge the fermentability of GOS and potentially also other NDCs.

2. Materials and methods

2.1. Materials

Purified Vivinal GOS with <3% (w/w dry matter) monomers and lactose was kindly provided by Friesland Campina DOMO (Borculo, The Netherlands). This GOS preparation was produced from Vivinal GOS by the hydrolysis of lactose, followed by nanofiltration to remove the monomers. An overview of the complexity of the Vivinal GOS mixture with the isomers present per degree of polymerization (DP) is shown in Fig. 1. Relative abundance of different DPs in purified Vivinal GOS was as follows: DP1, 2.5%; DP2, 10.9%, DP3, 46.2%, DP4, 25.4%, DP5, 9.6%, DP6, 3.7%, DP > 7, 1.7%. GOS DP3 standards β-3'-galactosyl-lactose, β-4'-galactosyl-lactose and β-6'-galactosyl-lactose were purchased from Carbosynth (Berkshire, UK).

2.2. Fermentation of GOS by infant faecal inoculum

2.2.1. Culture medium

Standard ileal efflux medium (SIEM; Tritium Microbiology, Veldhoven, The Netherlands) was prepared as described elsewhere with minor modifications (Gu et al., 2018). Low amount of carbohydrates was added to mimic the infant ileal environment while minimizing background fermentation. The carbohydrate medium component contained (g/L): pectin, 12; xylan, 12; arabinogalactan, 12; amylopectin, 12; and starch, 12 with a final concentration of only 0.24 g/L.The pH was adjusted to 5.8 using 2-(N-morpholino)ethanesulfonic acid (MES) buffer.

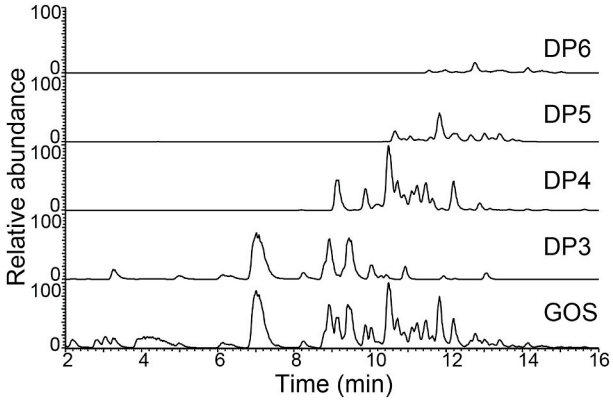

Fig. 1. UHPLC-PGC-MS profile of total reduced GOS and of DP 3-6 after selection of the appropriate *m/z* for that DP. Elution profiles were normalized to the real contribution of each DP to the total mixture.

2.2.2. Infants and infant faecal inoculum

Ten two-week-old infants were included in the current study. The solely breast-fed infants were born vaginally and did not show any health complaints. These infants did not receive antibiotic treatment in the first 2 weeks of life. Faecal inoculum was prepared as reported elsewhere with some minor modifications (Leijdekkers et al., 2014). Parents collected faecal material from the diaper of two-week-old infants directly after defecation. Faecal material was immediately transferred to tubes and stored at −20 °C. Afterwards tubes were stored at −80 °C.

After thawing, faecal material (±100 mg) was diluted with 6 mL sterilized 0.9% (w/v) NaCl solution in a laminar flow cabinet. In case of pooled faecal inocula, faecal material of five infants (5× ±100 mg) was combined and diluted to obtain a similar final concentration. An additional pool containing faecal material of five other infants was included in this study to determine reproducibility. The ten infants were randomly divided over the 2 pools. Homogenization was performed by the addition of sterile glass beads prior to shaking using a vortex.

The following steps were performed in an anaerobic chamber (gas phase: 81% N_2, 15% CO_2 and 4% H_2)(Bactron 300, Sheldon Manufacturing, Cornelius, USA). Faecal solutions were combined with SIEM in a ratio of 5:82 (v/v) in a sterile 20 mL serum bottle. The bottle was closed with a rubber stopper and secured with a metal crimp cap. The final faecal inoculum adapted to the new environment by overnight incubation in an incubator shaker (Innova 40, New Brunswick Scientific, Nijmegen, The Netherlands) (37 °C, 100 rpm) (Venema et al., 2015).

2.2.3. In vitro fermentation

The *in vitro* fermentation was performed in an anaerobic chamber. Activated faecal inoculum was combined with SIEM containing GOS (11.1 mg/ml) in sterile serum bottles in a ratio of 1:10 (v/v) with a total volume of 3.33 ml. Serum bottles were closed with a rubber stopper and secured with a metal crimp cap. Afterwards they were placed in an incubator shaker (37 °C, 100 rpm). At the start and after 6, 12, 24, 34 and 48 h, three aliquots of each 70 μl were taken from the same serum bottle with a syringe and put into separate tubes. One series of samples was immediately frozen in liquid nitrogen and stored at −80 °C to preserve the samples for later microbial analysis. The remaining two series were heated for 5 min in a boiling water bath to inactivate the enzymes. Subsequently they were stored at −20 °C until further analysis.

In vitro fermentations were performed in duplicate and blanks without faecal inoculum or substrate were included to monitor possible contamination of the substrate or background fermentation over time. Data on the duplicate fermentation of infant 5 after 12 h is missing due to a broken serum bottle.

2.3. Fate of GOS upon fermentation

2.3.1. GOS pretreatment before analysis

Prior to analysis fermentation samples were reduced to avoid anomerization of oligosaccharides. Freshly prepared 0.5 M sodium borohydride in water was added to 10 times diluted fermentation samples in a ratio of 1:1 (v/v) with a final volume of 400 μl. Samples were incubated overnight at room temperature.

Solid phase extraction was applied to remove salts in order to purify the reduced fermentation samples. First cartridges (Carbograph, Supelclean ENVI carb, bed weight: 250 mg; Sigma Aldrich) were activated with 1.5 ml of 80:20 (v/v) acetonitrile (ACN)/H_2O containing 0.1% (v/v) trifluoroacetic acid (TFA), followed by 1.5 ml H_2O (Milli-Q). The reduced sample was loaded on the column, after which the column was

washed 4 times with 1.5 mL H_2O (Milli-Q). Subsequently reduced GOS were eluted with 1.5 mL of 40:60 (v/v) ACN/H_2O containing 0.05% (v/v) TFA. Eluted reduced GOS were dried under nitrogen gas, solubilized in 400 µl of H_2O (ULC-MS grade) and centrifuged (5 min, 15 000g).

2.3.2. Characterization of GOS by UHPLC-PGC-MS

Reduced GOS were analyzed on an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA). The system was equipped with pump and autosampler. Samples (5 µl) were injected on a PGC column (3 µm particle size, 2.1 mm × 100 mm; Hypercarb, Thermo Scientific) in combination with a guard column (3 µm particle size, 2 mm × 10 mm; Hypercarb, Thermo Scientific).

As mobile phase A: ULC-MS water + 0.1% (v/v) formic acid + 1% (v/v) ACN was used. Mobile phase B consisted of ACN + 0.1% (v/v) formic acid. The flow rate was 300 µL/min. The solvents were eluted according to the following profile: 0–3.3 min, 3% B; 3.3-8 min, 3-10% B; 8-20 min, 10-20% B; 20–26.7 min, 20-40% B; 26.7–27.5 min, 40-100% B; 27.5–37.5 min, 100% B; 37.5–38.5 min, 100-3% B; 38.5–43.5 min, 3% B. The temperature of the autosampler and column oven was controlled at 10 and 45 °C, respectively. Needle wash solvent containing 3% ACN was used to wash the autosampler.

To obtain mass spectrometric data, the flow of the UHPLC was directed to a Thermo Scientific LTQ-Velos Pro equipped with an electrospray ionisation probe. Helium and nitrogen were used as sheath and auxiliary gas, respectively. The MS settings were set to a source voltage of 3.5 kV, a source heater temperature of 225 °C, a capillary temperature of 350 °C, a sheath gas flow of 38 and an auxiliary gas flow of 11.

MS data in negative mode were collected over an *m/z* range of 300–2000. Data dependent MS () analysis was performed with a normalized collision energy of 30%, activation Q of 0.25, activation time of 30 ms and isolation width of *m/z* 2. MS^2 fragmentation was performed on the 1st and 2nd most abundant ion in the MS chromatogram from a parent list containing *m/z* of both unreduced and reduced GOS.

DP3 standards β-3'-galactosyl-lactose, β-4'-galactosyl-lactose and β-6'-galactosyl-lactose were used for the identification of GOS isomers. Vivinal GOS was used in a concentration of 0.005–1 mg/ml to create calibration curves per DP. GOS DPs were quantified by selection of the specific mass range followed by integration of the peaks. Data acquisition and processing were performed using Xcalibur (version 2.2, Thermo Scientific).

2.4. Production of SCFAs and other organic acids

In order to quantify the production of volatile short-chain fatty acids (SCFAs) fermentation samples were analyzed using gas chromatography (GC). Fermentation samples were 10× diluted with water (ULC-MS grade). Standards containing acetic acid, butyric acid and propionic acid in concentration range 0.01–3 mg/mL were also included in the analysis. Standards and diluted fermentation samples were mixed in a ratio of 2:1 (v/v) with a solution containing hydrochloric acid (0.3M), oxalic acid (0.09M) and the internal standard 2-ethyl butyric acid (0.45 mg/mL). The mixture was kept for 30 min at room temperature and afterwards centrifuged (5 min, 15 000g).

Samples (1 µl) were injected in a CP-FFAP CB column (25 m × 0.53 mm x 1.00 µm) (Agilent Technologies, Santa Clara, CA, USA). The temperature profile during GC analysis was as follows: 100 °C, maintained for 0.5 min; raised to 180 °C at 8 °C/min, maintained for 1 min; raised to 200 °C at 20 °C/min, maintained for 5 min. Glass wool was inserted in the glass liner of the split injection port to protect the column from contamination (Zhao et al., 2006). Obtained data were integrated using Xcalibur software (Thermo Scientific).

Organic acids were analyzed using high-performance liquid chromatography (HPLC) on an Organic Acid column as described elsewhere (Ladirat et al., 2014). Besides 10× diluted fermentation samples, standards of lactic and succinic acid in a concentration range of 0.01–1 mg/mL were included in the analysis. Obtained data were integrated using Chromeleon 7.0 (Thermo Scientific).

2.5. Microbial composition analysis

2.5.1. DNA extraction

DNA was extracted from fermentation samples using repeated bead beating. Briefly each fermentation sample (70 µl) was added to a sterilized screwed cap tube containing 0.25 g of zirconia beads and 3 glass beads (2.5 mm). After the addition of 300 µl Stool Transport and Recovery Buffer (STAR; Roche Diagnostics Corporation, Indianapolis, IN), the samples were treated with a bead beater (room temperature, 5.5 m/s for 3 × 1 min) (Precellys 24, Bertin Technologies) and heated for 15 min (95 °C, 100 rpm). Subsequently the samples were centrifuged for 5 min (4 °C, 14000g) and supernatants were collected. Another cycle was performed with the pellet using 200 µl of STAR buffer. Supernatants of both cycles were combined, and 250 µl was purified using Maxwell 16 Tissue LEV Total RNA Purification Kit Cartridge (AS1220). DNA was eluted with 50 µl of Nuclease-Free water (Promega, Wisconsin, USA). Finally, DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and if needed diluted to a concentration of 20 ng/µl with Nuclease-Free water.

2.5.2. PCR amplification

Microbiota profiling was performed as described previously with some modifications (Ramiro-Garcia et al., 2016). The V5–V6 region of 16S ribosomal RNA (rRNA) genes was amplified in triplicate polymerase chain reactions (PCR) with a uniquely barcoded primer pair F784 (RGGATTAGATACCC) and 1064R (CGACRRCCATGCANACCT). For samples containing 4.8–7.4, 7.8-12 and 15-20 ng/µl DNA template respectively 5, 3 or 0.7 µl DNA template was used in each reaction. The samples were amplified with 0.7 µl of 10 µM stock solutions of the primer pair, 7 µl of 5× HF buffer (Finnzymes, Vantaa, Finland), 0.7 µl of 10 mM dNTPs (Roche, Diagnostics GmbH, Mannheim, Germany) and 0.35 µl of 2U/µl Phusion® Hot Start II High Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland). Nuclease-free water was added to reach a total reaction volume of 35 µl. The amplification program included a 30 s initial denaturation step at 98 °C, followed by 25 cycles of denaturation at 98 °C for 10 s, annealing at 42 °C for 10 s, elongation at 72 °C for 10 s, and a final extension at 72 °C for 7 min. The PCR product presence and size (≈290 bp) was confirmed with gel electrophoresis using the Lonza FlashGel System (Lonza, Cologne, Germany). Synthetic mock communities of known composition were included as positive control (Ramiro-Garcia et al., 2016).

2.5.3. Library preparation and sequencing

PCR products were purified using HighPrep PCR kit (MagBio Genomics, Alphen aan den Rijn, The Netherlands). Purified amplicons were quantified using Qubit dsDNA BR assay kit (Life Technologies, Leusden, The Netherlands). Seventy unique barcode tags were used in each library (Ramiro-Garcia et al., 2016). An amplicon pool was formed by combining 150 ng of each barcoded sample and afterwards concentrated to 40 µl volume using the HighPrep PCR kit. The resulting library was sent for adapter ligation and sequencing on an Illumina Hiseq2500 instrument (GATC-Biotech, Konstanz, Germany).

2.5.4. Data analysis

Processing and analysis of the 16S rRNA gene sequence data was carried out using the NG-Tax pipelineand R version 3.5.0 (Ramiro-Garcia et al., 2016).

Beta-diversity was calculated using weighted UniFrac distances based on relative abundance of bacteria at genus level present in the fermentation digesta using the functions *tax_glom* and *ordinate* in the *phyloseq* R package. The output was visualized using Principal Coordinate Analysis (PCoA) using the function *plot_ordination* in the *phyloseq* R package.

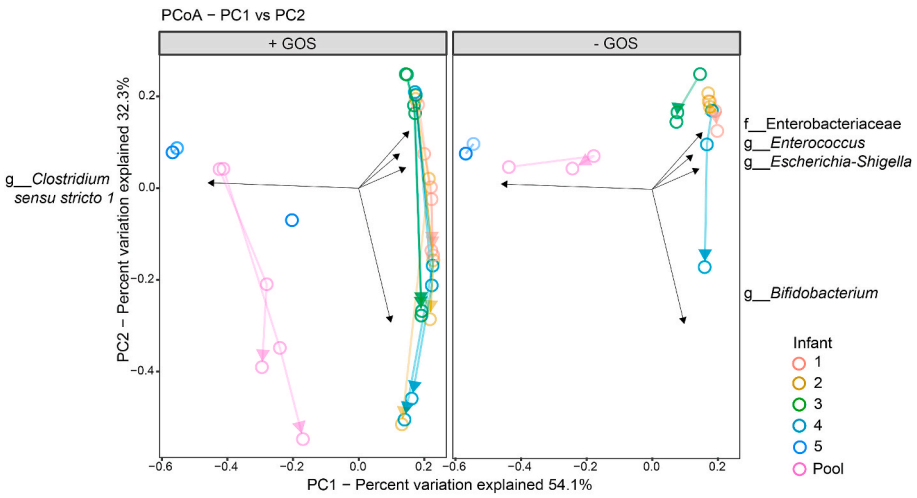

Fig. 2. PCoA using weighted UniFrac distances of microbiota composition in digesta collected at the start and after 12 and 24 h of *in vitro* fermentation with (left) and without (right) GOS using inoculum of two-week-old infants. Direction of changes in time is presented by arrows in the same colour. Direction and length of the taxa arrows (black) presents the contribution of selected taxa to PC1 and PC2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The contribution of taxa to the principle coordinates was determined from a PCoA plot of the taxa based on the same distances using the function *plot_ordination (type: taxa)* in the *phyloseq* R package. Taxa vectors with the extracted x- and y-coordinates were plotted on the first PCoA plot using the function *geom_segment* in the *ggplot2* R package.

A higher resolution analysis of *Bifidobacterium* at the level of individual sequences (Amplicon Sequencing Variants (ASVs)) was performed using the function subset_taxa in the *phyloseq* R package. Bifidobacterial ASVs with a relative abundance of less than 0.5% in each sample were removed using the functions *filterfun* and *filter_taxa* in the *genefilter* and *phyloseq R* package.

3. Results and discussion

3.1. Relative increase in Bifidobacterium upon fermentation of GOS using both individual and pooled infant faecal inoculum

The responsiveness of the microbiota to NDCs is dependent on the microbiota composition (Walker et al., 2011). As such, it is important to determine the effect of pooling of infant faecal inocula on the changes in microbiota composition upon fermentation of GOS. Fermentation samples were analyzed with 16S rRNA gene amplicon sequencing followed by PCoA using weighted UniFrac distances (Fig. 2). The contribution of most abundant taxa to PC1 and PC2 is visualized by taxa vectors. Together these taxa comprise more than 70% of each fermentation sample. Relative abundances of all bacterial taxa present in the fermentation samples are shown in Fig. S1.

First of all, it must be stated that the microbiota composition of the faecal inocula at 0h (after the overnight adaptation) does not relate to faecal microbiota of two-week-old infants previously reported in literature, where *Bifidobacterium* and *Bacteroides* are present at high levels (Hill et al., 2017). Instead, bacteria belonging to the genera *Clostridium, Enterococcus, Shigella* and *Escherichia* and the family Enterobacteriaceae were found to be present at high relative abundance at 0h with the fermentation of GOS using the individual and pooled faecal inocula as well as the background fermentation (Fig. 2). Growth of these facultative anaerobic bacteria could be explained by traces of oxygen still present during the overnight activation of the faecal inoculum. However, these bacteria were also reported to grow well on peptides and amino acids, which are present in high abundance in SIEM: bactopeptone (24 g/L), casein (24 g/L), ox-bile (0.4 g/L) and cysteine (0.16 g/L) (Richardson et al., 2013). Prior to fermentation faecal bacteria were activated overnight in SIEM, and the microbiota composition measured suggests the adaptation of the bacteria to the medium constituents (Maathuis et al., 2009). This also explains why the composition of the pooled faecal inoculum at 0h does not represent the average composition of the individual faecal inocula. The hypothesis is supported by previous *in vitro* study using an identical fermentation set-up, but without overnight adaptation of the infant faecal inoculum. Bacteria belonging to the genera *Escherichia, Shigella* and *Enterococcus* were enriched in control fermentations without added carbohydrates, confirming their stimulation by SIEM constituents (Logtenberg et al., 2020).

However, despite the aberrant microbiota composition at 0h still a clear compositional shift was observed upon fermentation of GOS for faecal inocula of infant 1-4 (Fig. 2). The shift was mainly characterized by a relative increase in *Bifidobacterium*, which is consistent with data obtained in previous *in vitro* (Watson et al., 2013) and *in vivo* studies (Matsuki et al., 2016). For infant 1 the relative abundance of *Bifidobacterium* at 0h was 2–11% and increased up to 24-29% in 24 h of fermentation. For faecal inocula of infant 2-4 this was lower than 1.3% at 0h but increased up to 43-68% in 24 h of fermentation of GOS. Differences in relative increase of *Bifidobacterium* between individual infant faecal inocula could possibly be ascribed to differences at the species level, since it has been reported that bifidobacterial species show different levels of cell reproduction upon fermentation of GOS (Barboza et al., 2009). Classification of bacteria at species level is hindered by sequence similarity of the 16S rRNA gene between different species within the same genus (Janda & Abbott, 2007). However, a higher resolution analysis at the level of ASVs showed a high variability in bifidobacterial ASVs between the individual faecal inocula suggesting the presence of multiple bifidobacterial species (Fig. S2). No significant changes in the less abundant bacteria were observed upon fermentation of GOS using individual faecal inocula of infant 1-4.

In contrast, faecal inoculum of infant 5 did not contain *Bifidobacterium* and was dominated by bacteria belonging to the genus *Clostridium sensu stricto* (Fig. 2). After 12 h of fermentation faecal inoculum of infant 5 even consisted exclusively of bacteria belonging to this genus. Additionally, the fermentation showed abberant behaviour after 12 h as organic acids were not detected and GOS concentrations seemed to increase (Figs. 3 and 4). Unfortunately, a missing duplicate fermentation of infant 5 prohibited confirmation of this outlier.

Clostridium sensu stricto was also present in the pooled faecal inoculum at 0h at high relative abundance (79-80%) (Fig. 2). Despite the presence of the aberrant faecal inoculum of infant 5, a clear increase in *Bifidobacterium* relative abundance was observed during the fermentation of GOS using pooled faecal inoculum (39-63%). Background fermentations without the addition of GOS did not show such shift in *Bifidobacterium* relative abundance. These findings indicate the potential of using pooled faecal inoculum to screen for bifidogenic potential of NDCs, although the microbiota composition of the pooled infant faecal inoculum does not mimic the microbiota composition of the individual infant faecal inocula.

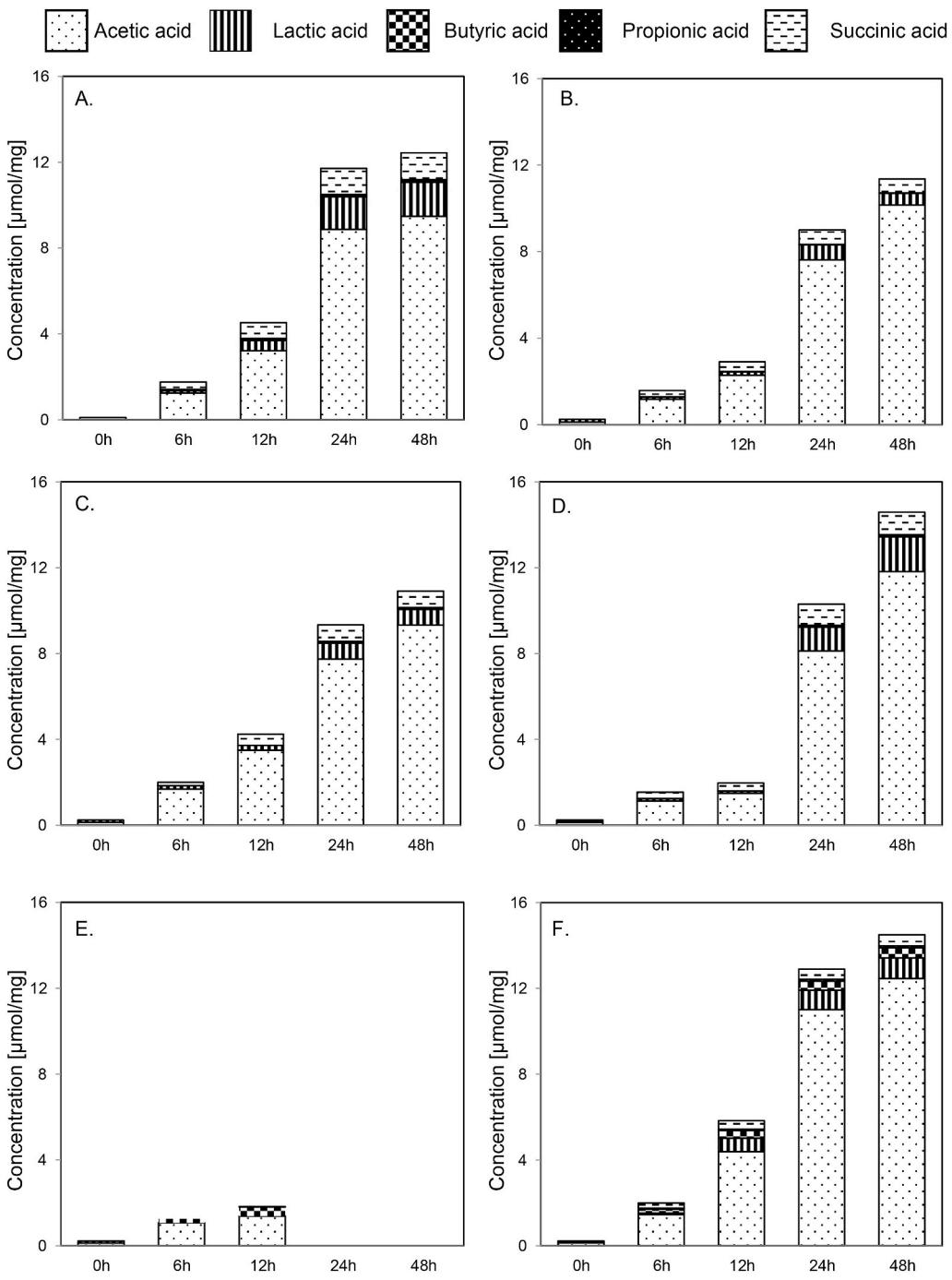

Fig. 3. Production of organic acids upon *in vitro* fermentation of GOS using faecal inoculum of individual infants 1: A, 2: B, 3: C, 4: D, 5: E, and the pool of inocula infant 1-5: F.

3.2. Production of SCFAs and other organic acids

Upon the fermentation of GOS, SCFAs and the intermediate fermentation products lactic and succinic acid were produced by the bacteria, which were analyzed by GC and HPLC (Fig. 3). The total amount of organic acids increased to 10.9–14.6 µmol/mg GOS after 48 h of fermentation using individual infant faecal inocula 1-4. Composition of the organic acids produced during the fermentation differed for the individual infant faecal inocula. Nevertheless, in all fermentations, acetate was produced at highest abundance followed by lactate.

Reproducibility was confirmed by duplicate fermentations (Table S1). In addition, both background fermentations without GOS or inoculum showed hardly any production of organic acids (Table S2). Taking into account both the organic acid production and the changes in microbiota composition, it could thus be suggested that the high abundance of acetate upon fermentation of GOS could be ascribed to a large extent to the growth of *Bifidobacterium*, members of which are known as acetate- and lactate-producing bacteria (Palframan et al., 2003).

Fermentation of GOS using pooled infant faecal inoculum resulted in similar amounts of organic acids after 48 h as the individual infant faecal inoculum that produced most organic acids (infant 4), respectively 14.5 and 14.6 µmol/mg GOS. Both faecal inoculum of infant 4 and the pool were dominated by *Bifidobacterium* after 24 h of fermentation, respectively 69% and 63%, while fermentation using faecal inoculum of infant

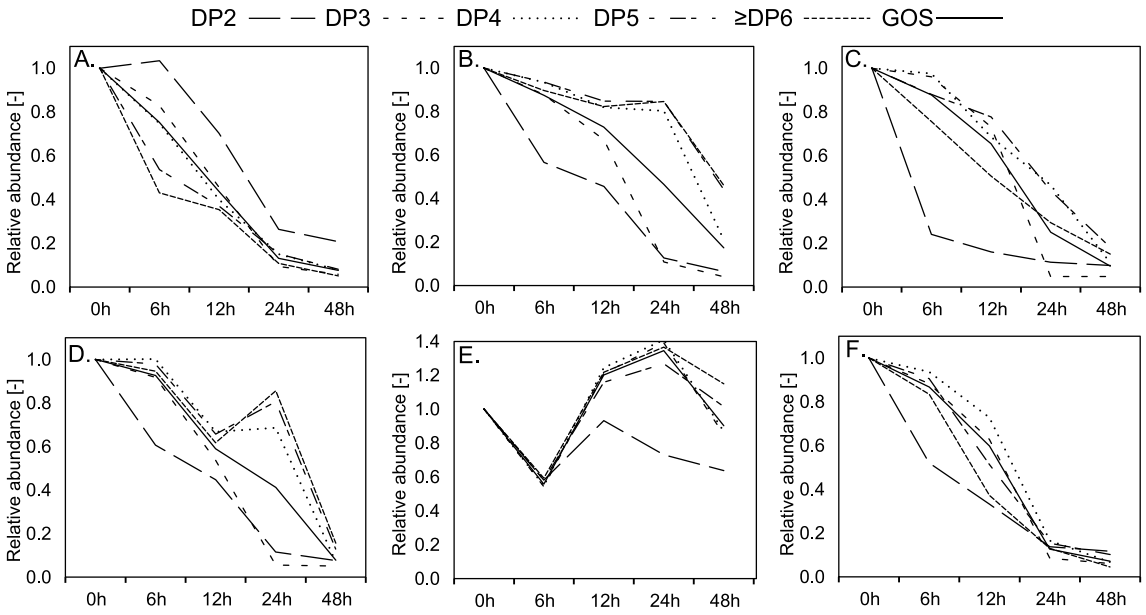

Fig. 4. Relative proportion of remaining oligosaccharides from GOS and individual DPs of GOS during *in vitro* fermentation using faecal inoculum of individual infants 1: A, 2: B, 3: C, 4: D, 5: E and the pool: F. Analysis was performed by RP-UHPLC-PGC-MS. Concentrations per DP in the original GOS mixture were set to 1.0.

1 and 3 individually resulted in a relative abundance of only 27 and 44% respectively. These findings suggests the overruling effect of *Bifidobacterium* species of faecal inoculum of infant 4 over other bacteria within the pool.

In contrast to faecal inoculum of infant 4, pooled faecal inoculum resulted in the production of minor amounts of butyrate (0.6 μmol/mg) after 24 h of fermentation. *Clostridium sensu stricto* is the 2nd most abundant genus-level group in the pool, but is not detected in the faecal inoculum of infant 4 (Fig. 2). Cross-feeding between *Bifidobacterium* and *Clostridium sensu stricto* could have resulted in the minor production of butyrate during fermentation of GOS using pooled infant faecal inoculum (Rivière et al., 2016).

Notwithstanding the aberrant microbiota and organic acid composition, it can be concluded that the fermentability of GOS is reflected by the total organic acid production upon fermentation using pooled infant faecal inoculum.

3.3. Degradation kinetics of GOS

Degradation of GOS was monitored by UHPLC-PGC-MS to determine the effect of pooling faecal inocula on the degradation capability of individual GOS structures. To visualize the degradation kinetics of GOS by individual and pooled faecal inocula, both the proportion of the remaining total GOS mixture and oligomers with different DPs present in the mixture are shown in Fig. 4. Individual inocula showed different speed of degradation of total GOS, with a slower degradation for individual inocula of infant 2 and 4 (>40% remaining) and faster degradation for infant 1 and 3 (<25% remaining) after 24 h of fermentation. As expected, a lower amount of GOS remaining coincided with a higher amount of organic acids produced (Figs. 3 and 4).

Zooming in on the degradation kinetics at DP level showed a high variability in preference for specific DPs of GOS between individual infant inocula. For the fermentation using faecal inocula of infant 2, 3 and 4, oligomers DP $\leq$ 3 were fermented faster than DP4 and DP5 oligomers. Contrarily, DP $\geq$ 6 oligomers were the first to be degraded during the fermentation using faecal inoculum of infant 1. These differences could be explained by the DP-specific oligomer utilization of GOS by different species, as exemplified for *Bifidobacterium* (Barboza et al., 2009). Degradation of total GOS was observed to be somewhat faster and more complete during fermentation using pooled faecal inocula compared to the individual faecal inocula, i.e. 7% of oligosaccharides were remaining after 48h of fermentation using pooled inocula, compared to 8-17% for individual inocula 1-4. This finding could possibly be explained by the overruling of specific *Bifidobacterium* species with the highest GOS fermentation capacity originating from the different individual faecal inocula, since previous study showed clear differences in GOS fermentation capacity between *Bifidobacterium* species (Barboza et al., 2009).

Since GOS consists of multiple isomers per DP, it is also of interest to zoom in at the isomer-level. In Fig. 5 the fate of GOS DP 3 with his constituent isomers is displayed for the individual and pooled faecal inocula. Individual isomers differed quite significantly in fermentation rate and extent. The isomer-specific degradation was consistent with previous research in which for example individual strains of *Bifidobacterium*, *Streptococcus* and *Lactobacillus* showed a general preference towards galactosyl residues having β(1-6) and β(1-1) linkages (Cardelle-Cobas et al., 2011). In future work the relation between structure and degradation should be further unraveled by using different LC/MS techniques and nuclear magnetic resonance analysis for the characterization of the various ((non)fermentable) GOS isomers (Logtenberg et al., unpublished data).

Comparison of the degradation kinetics of the individual and pooled faecal inocula reflected again the dominance of the *Bifidobacterium* species of the inoculum of infant 4 in the pooled faecal inoculum. DP3 isomer 2, 11, 12 and 13 were degraded almost completely in the first 12 h of fermentation by both inocula, whereas these isomers were still present upon the fermentation of GOS using inocula of infant 1, 2 and 3. Nevertheless, the degradation of GOS using pooled faecal inoculum was not solely caused by the bacteria originating from infant 4. The complete degradation of isomer 16 in the first 12 h of fermentation using pooled faecal inoculum could for example be ascribed to bacteria originating from infant 2 (Fig. 5).

The results on the degradation kinetics thus highlight again that a pooled faecal inoculum cannot predict the exact utilization of specific NDC structures by particular species present in the microbiota of a specific individual. However, fermentation using a pooled faecal inoculum clearly demonstrates the ability of infant microbiota in general to utilize specific oligosaccharides and facilitates the efficient screening of the potentially prebiotic NDC mixtures.

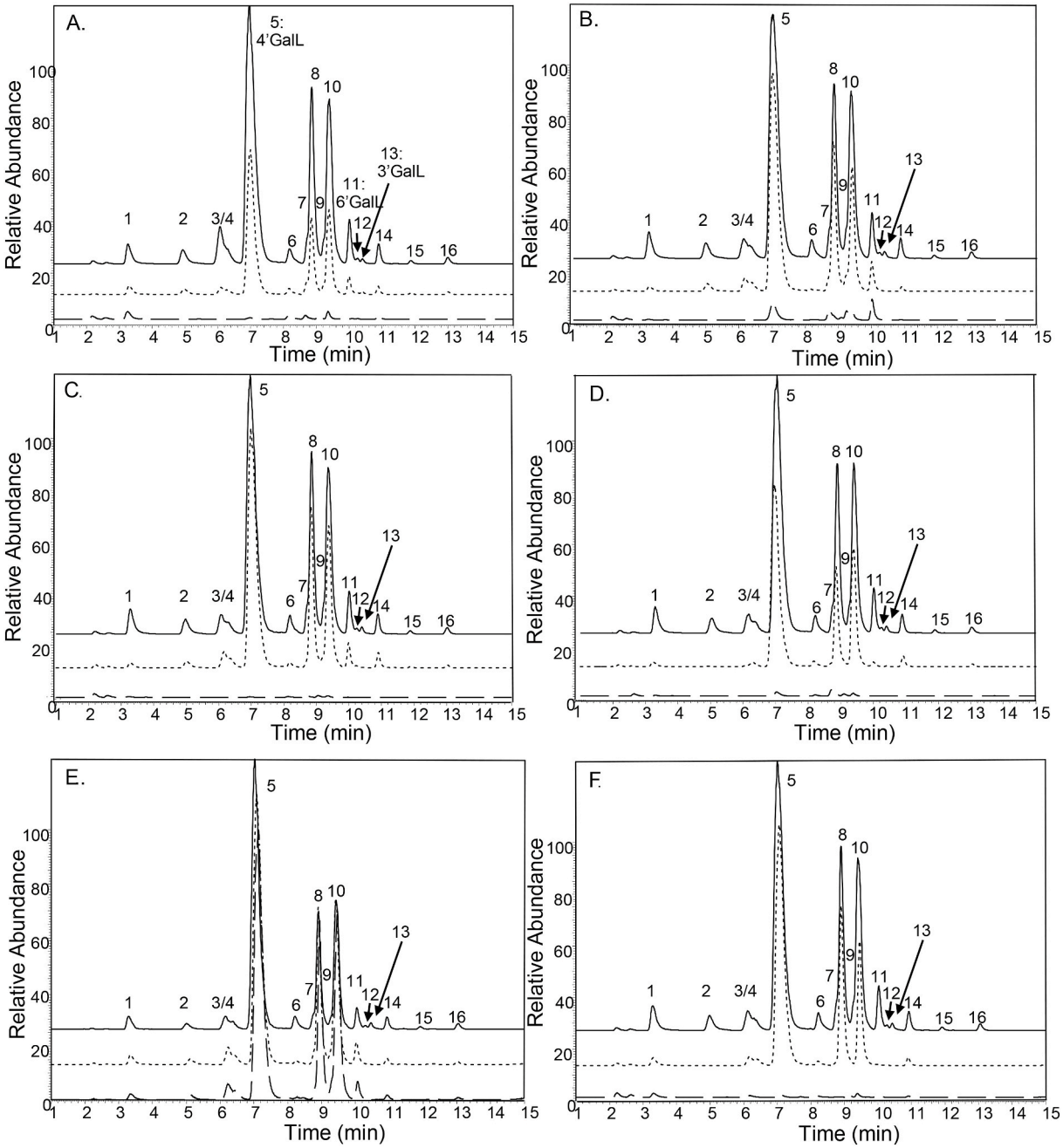

Fig. 5. UHPLC-PGC-MS profile of reduced GOS with selection of DP3 at the start (solid), after 12 (dotted) and 24 (dashed) h of fermentation using faecal inoculum of individual infants 1: A, 2: B, 3: C, 4: D, 5:E and the pool: F. All elution profiles were normalized to similar intensity.

3.4. Validation of pooled infant faecal inoculum

An additional fermentation using faecal material of 5 other infants of the same age (2 weeks) was performed to validate the use of pooled infant faecal inoculum. Faecal microbiota of these infants fermented GOS significantly faster than the primary pooled faecal inoculum described above. After 12 h of fermentation of the validation trial, only 7% of the total GOS mixture was remaining (Fig. S3), while still 60% was remaining for the primary pooled faecal inoculum. However, similar preference on DP level was observed as DP2 and DP $\geq$ 6 were fermented first for both pooled faecal inocula (Fig. S3). GOS DP 3 was degraded completely between 6 and 12 h of fermentation. As a consequence no information on preferential degradation of specific isomers could be obtained from the digesta taken at the given time points (Fig. S4).

Despite the differences in fermentation speed between the two pooled faecal inocula, a similar increase in *Bifidobacterium* was observed upon fermentation of GOS (Fig. 6). After 24 h 68-74% of the bacteria belonged to the genus *Bifidobacterium*. The fermentation coincided with a slightly higher total production of organic acids compared to the primary pool in this study, respectively 15.0 and 14.5 μmol/mg after 24 h of fermentation (Fig. S5). This validation experiment with the pooled inoculum containing faecal material of 5 other infants of the same age thus reinforced that the use of pooled infant faecal inoculum allows judgement of the fermentability of GOS efficiently.

4. Conclusions

During the last decade several studies have been published on the *in vitro* fermentation of GOS by individual bacterial strains, which neglect important cross-feeding relations between different bacteria. The use of

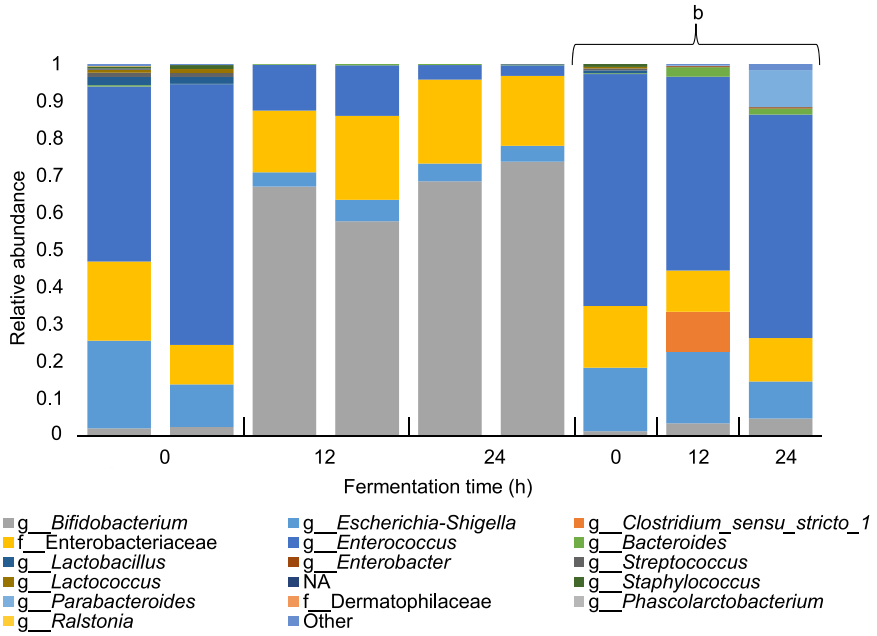

Fig. 6. Relative abundance of bacteria with highest known taxonomy in duplicate fermentation digesta collected at the start and after 12 and 24 h of an additional fermentation of GOS using pooled faecal inoculum of 5 other infants at the same age (2 weeks) for validation with b: background fermentation (only SIEM and inoculum). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

infant faecal inoculum results in a better view on the actual fate of GOS in the infant gut. However, it introduces some challenges due to high inter-individual variability of the infant microbiota requiring numerous *in vitro* experiments using individual faecal inocula of different infants. In this study it has been shown that pooled infant faecal inoculum can function as an alternative approach. Our data reinforced that, despite the divergent microbiota composition, the bacterial functionality of the pooled faecal inoculum was largely equivalent to that of the individual faecal inocula, as a similar trend in organic acid production and relative increase in *Bifidobacterium* was observed between the pooled faecal inoculum and 4 out of 5 individual faecal inocula. These findings were confirmed by an additional fermentation using pooled faecal inoculum of 5 other infants of the same age. The functionality of the pooled faecal inoculum was thus largely representative of the faecal inocula of the infant population in general. Although the current study is only based on the bifidogenic GOS, the findings suggest that pooled faecal inoculum is suitable for high throughput screening purposes of the fermentability of novel NDCs.

Additionally, the use of PGC-MS with their excellent separation of oligosaccharide isomers showed us some first insights on DP and isomer-specific fermentation of GOS by infant microbiota. Knowledge on the structure-specific effects of GOS and other NDCs could give potential for the production of tailored NDC mixtures to meet the needs of specific subgroups of infants. With this perspective in mind the use of pooled faecal inoculum will help us to move forward in future studies as it will make room for the incorporation of multiple NDCs in one study. Highly promising NDCs can be selected by *in vitro* fermentations using pooled inocula prior to further *in vitro* and *in vivo* experiments targeting responses of individuals. In this way our understanding of structure-specific effects of NDCs on infant microbiota will be increased efficiently.

Author contributions

Madelon J. Logtenberg: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing-original draft and Writing—review and editing; **Jolien C.M. Vink:** Investigation; **Renske M. Serierse:** Investigation; **Ran An:** Methodology and Writing—review and editing; **Gerben D.A. Hermes:** Software, Formal analysis and Writing—review and editing; **Hauke Smidt:** Conceptualization, Methodology, Resources and Writing—review and editing; **Henk A. Schols:** Conceptualization, Methodology, Resources, Writing-original draft, Writing—review and editing and Supervision. All authors have read and agreed to the published version of the manuscript.

Funding

Carbohydrate Competence Center (CCC, www.cccresearch.nl) CarboKinetics is financed by participating industrial partners Agrifirm Innovation Center B.V., Cooperatie Avebe U.A., DSM Food Specialties B.V., FrieslandCampina Nederland B.V., Nutrition Sciences N.V., VanDrie Holding N.V. and Sensus B.V., and allowances of The Netherlands Organisation for Scientific Research (NWO). The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

CRediT authorship contribution statement

Madelon J. Logtenberg: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Jolien C.M. Vink:** Investigation. **Renske M. Serierse:** Investigation. **Ran An:** Methodology, Writing - review & editing. **Gerben D.A. Hermes:** Software, Formal analysis, Writing - review & editing. **Hauke Smidt:** Conceptualization, Methodology, Resources, Writing - review & editing. **Henk A. Schols:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcdf.2020.100235.

References

Aguirre, M., Ramiro-Garcia, J., Koenen, M. E., & Venema, K. (2014). To pool or not to pool? Impact of the use of individual and pooled fecal samples for *in vitro* fermentation studies. *Journal of Microbiological Methods, 107*, 1–7.

Barboza, M., Sela, D. A., Pirim, C., LoCascio, R. G., Freeman, S. L., German, J. B., Mills, D. A., & Lebrilla, C. B. (2009). Glycoprofiling bifidobacterial consumption of galacto-oligosaccharides by mass spectrometry reveals strain-specific, preferential consumption of glycans. *Applied and Environmental Microbiology, 75*(23), 7319–7325.

Bezirtzoglou, E., Tsiotsias, A., & Welling, G. W. (2011). Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe, 17*(6), 478–482.

Cardelle-Cobas, A., Corzo, N., Olano, A., Pelaez, C., Requena, T., & Avila, M. (2011). Galactooligosaccharides derived from lactose and lactulose: Influence of structure on *Lactobacillus, Streptococcus* and *Bifidobacterium* growth. *International Journal of Food Microbiology, 149*(1), 81–87.

Cockburn, D. W., & Koropatkin, N. M. (2016). Polysaccharide degradation by the intestinal microbiota and its influence on human health and disease. *Journal of Molecular Biology, 428*(16), 3230–3252.

Dogra, S., Sakwinska, O., Soh, S.-E., Ngom-Bru, C., Brück, W. M., Berger, B., Brüssow, H., Lee, Y. S., Yap, F., Chong, Y.-S., Godfrey, K. M., & Holbrook, J. D. (2015). Dynamics of infant gut microbiota are influenced by delivery mode and gestational duration and are associated with subsequent adiposity. *mBio, 6*(1).

Engfer, M. B., Stahl, B., Finke, B., Sawatzki, G., & Daniel, H. (2000). Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *American Journal of Clinical Nutrition, 71*(6), 1589–1596.

de Goffau, M. C., Fuentes, S., van den Bogert, B., Honkanen, H., de Vos, W. M., Welling, G. W., Hyöty, H., & Harmsen, H. J. M. (2014). Aberrant gut microbiota composition at the onset of type 1 diabetes in young children. *Diabetologia, 57*(8), 1569–1577.

Gu, F., Borewicz, K., Richter, B., van der Zaal, P. H., Smidt, H., Buwalda, P. L., & Schols, H. A. (2018). *In vitro* fermentation behavior of isomalto/malto-polysaccharides using human fecal inoculum indicates prebiotic potential. *Molecular Nutrition & Food Research, 62*(12), Article e1800232.

Hill, C. J., Lynch, D. B., Murphy, K., Ulaszewska, M., Jeffery, I. B., O'Shea, C. A., Watkins, C., Dempsey, E., Mattivi, F., Tuohy, K., Ross, R. P., Ryan, C. A., O' Toole, P. W., & Stanton, C. (2017). Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. *Microbiome, 5*(1), 4.

Holscher, H. D., Bode, L., & Tappenden, K. A. (2017). Human milk oligosaccharides influence intestinal epithelial cell maturation *in vitro*. *Journal of Pediatric Gastroenterology and Nutrition, 64*(2), 296–301.

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology, 45*(9), 2761–2764.

Korpela, K., & de Vos, W. M. (2018). Early life colonization of the human gut: Microbes matter everywhere. *Current Opinion in Microbiology, 44*, 70–78.

Ladirat, S. E., Schuren, F. H., Schoterman, M. H., Nauta, A., Gruppen, H., & Schols, H. A. (2014). Impact of galacto-oligosaccharides on the gut microbiota composition and metabolic activity upon antibiotic treatment during *in vitro* fermentation. *FEMS Microbiology Ecology, 87*(1), 41–51.

Leijdekkers, A. G. M., Aguirre, M., Venema, K., Bosch, G., Gruppen, H., & Schols, H. A. (2014). *In vitro* fermentability of sugar beet pulp derived oligosaccharides using human and pig fecal inocula. *Journal of Agricultural and Food Chemistry, 62*(5), 1079–1087.

Li, M., Wang, M., & Donovan, S. M. (2014). Early development of the gut microbiome and immune-mediated childhood disorders. *Seminars in Reproductive Medicine, 32*(01), 074-086.

Logtenberg, M. J., Akkerman, R., An, R., Hermes, G. D. A., de Haan, B. J., Faas, M. M., Zoetendal, E. G., Schols, H. A., & de Vos, P. (2020). Fermentation of chicory fructo-oligosaccharides and native inulin by infant fecal microbiota attenuates pro-inflammatory responses in immature dendritic cells in an infant-age-dependent and fructan-specific way. *Molecular Nutrition & Food Research, 64*(13), 2000068.

Maathuis, A., Hoffman, A., Evans, A., Sanders, L., & Venema, K. (2009). The effect of the undigested fraction of maize products on the activity and composition of the microbiota determined in a dynamic *in vitro* model of the human proximal large intestine. *Journal of the American College of Nutrition, 28*(6), 657–666.

Matsuki, T., Tajima, S., Hara, T., Yahagi, K., Ogawa, E., & Kodama, H. (2016). Infant formula with galacto-oligosaccharides (OM55N) stimulates the growth of indigenous bifidobacteria in healthy term infants. *Beneficial Microbes, 7*(4), 453–461.

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., Belzer, C., Delgado Palacio, S., Arboleya Montes, S., Mancabelli, L., Lugli, G. A., Rodriguez, J. M., Bode, L., de Vos, W., Gueimonde, M., Margolles, A., van Sinderen, D., & Ventura, M. (2017). The first microbial colonizers of the human gut: Composition, activities, and health implications of the infant gut microbiota. *Microbiology and Molecular Biology Reviews, 81*(4). e00036-17.

Palframan, R. J., Gibson, G. R., & Rastall, R. A. (2003). Carbohydrate preferences of *Bifidobacterium* species isolated from the human gut. *Current Issues in Intestinal Microbiology, 4*(2), 71–75.

Ramiro-Garcia, J., Hermes, G. D. A., Giatsis, C., Sipkema, D., Zoetendal, E. G., Schaap, P. J., Smidt, H., & NG-Tax. (2016). A highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. *F1000Research, 5*, 1791-1791.

Richardson, A. J., McKain, N., & Wallace, R. J. (2013). Ammonia production by human faecal bacteria, and the enumeration, isolation and characterization of bacteria capable of growth on peptides and amino acids. *BMC Microbiology, 13*, 6-6.

Rivière, A., Selak, M., Lantin, D., Leroy, F., & De Vuyst, L. (2016). Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. *Frontiers in Microbiology, 7*, 979.

Ruiz-Moyano, S., Totten, S. M., Garrido, D. A., Smilowitz, J. T., German, J. B., Lebrilla, C. B., & Mills, D. A. (2013). Variation in consumption of human milk oligosaccharides by infant gut-associated strains of *Bifidobacterium breve*. *Applied and Environmental Microbiology, 79*(19), 6040–6049.

Valdés-Varela, L., Ruas-Madiedo, P., & Gueimonde, M. (2017). *In vitro* fermentation of different fructo-oligosaccharides by *Bifidobacterium* strains for the selection of synbiotic combinations. *International Journal of Food Microbiology, 242*, 19–23.

Venema, K. (2015). The TNO *in vitro* model of the colon (TIM-2). In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The impact of Food bioactives on health: In vitro and ex vivo models* (pp. 293–304). Cham: Springer International Publishing.

Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., Brown, D., Stares, M. D., Scott, P., Bergerat, A., Louis, P., McIntosh, F., Johnstone, A. M., Lobley, G. E., Parkhill, J., & Flint, H. J. (2011). Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *The ISME Journal, 5*(2), 220–230.

Wang, M., Monaco, M. H., & Donovan, S. M. (2016). Impact of early gut microbiota on immune and metabolic development and function. *Seminars in Fetal and Neonatal Medicine, 21*(6), 380–387.

Watson, D., O'Connell Motherway, M., Schoterman, M. H., van Neerven, R. J., Nauta, A., & van Sinderen, D. (2013). Selective carbohydrate utilization by lactobacilli and bifidobacteria. *Journal of Applied Microbiology, 114*(4), 1132–1146.

Zhao, G., Nyman, M., & Jonsson, J. A. (2006). Rapid determination of short-chain fatty acids in colonic contents and faeces of humans and rats by acidified water-extraction and direct-injection gas chromatography. *Biomedical Chromatography, 20*(8), 674–682.