

# Cross-feeding between *Bifidobacterium infantis* and *Anaerostipes caccae* on lactose and human milk oligosaccharides

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## Abstract

The establishment of the gut microbiota immediately after birth is a dynamic process that may impact lifelong health. At this important developmental stage in early life, human milk oligosaccharides (HMOs) serve as specific substrates to shape the gut microbiota of the nursing infant. The well-orchestrated transition is important as an aberrant microbial composition and bacterial-derived metabolites are associated with colicky symptoms and atopic diseases in infants. Here, we study the trophic interactions between an HMO-degrader, *Bifidobacterium infantis* and the butyrogenic *Anaerostipes caccae* using carbohydrate substrates that are relevant in the early life period including lactose and total human milk carbohydrates. Mono- and co-cultures of these bacterial species were grown at pH 6.5 in anaerobic bioreactors supplemented with lactose or total human milk carbohydrates. *A. caccae* was not able to grow on these substrates except when grown in co-culture with *B. infantis*, leading to growth and concomitant butyrate production. Two levels of cross-feeding were observed, in which *A. caccae* utilised the liberated monosaccharides as well as lactate and acetate produced by *B. infantis*. This microbial cross-feeding points towards the key ecological role of bifidobacteria in providing substrates for other important species that will colonise the infant gut. The progressive shift of the gut microbiota composition that contributes to the gradual production of butyrate could be important for host-microbial crosstalk and gut maturation.

**Keywords:** bifidobacteria, butyrate, *Lachnospiraceae*, microbiome, pH

## 1. Introduction

The succession of microbial species in the infant gut microbiota is a profound process in early life (Backhed *et al.*, 2015; Koenig *et al.*, 2011), which coincides with the important development of the immune, metabolic and neurological systems (Arrieta *et al.*, 2014; Sherman *et al.*, 2015; Thompson, 2012). At this developmental stage, human milk is recognised as the best nourishment for infants (Neville *et al.*, 2012). Human milk contains a range of microbial active components and all human milk oligosaccharides (HMOs) play an important role in the development of the infant gut microbiota (Zivkovic *et al.*, 2011). HMOs are complex carbohydrates

composed of a lactose core, which may be elongated by N-acetylglucosamine (GlcNAc), galactose and/or decorated with fucose and/or sialic acid (Smilowitz *et al.*, 2014). The composition of HMOs in human milk is highly individual, driven by maternal genetic factors (Kunz *et al.*, 2017; McGuire *et al.*, 2017) and varies with the phases of lactation. The concentration of HMOs ranges from 23 g/l in colostrum to 7 g/l in matured human milk (Coppa *et al.*, 1993; Gabrielli *et al.*, 2011).

The majority of the HMOs escapes digestion by the host's enzymes in the upper gastrointestinal tract (Engfer *et al.*, 2000). HMOs confer important physiological traits by acting both as a decoy for the binding of pathogenic

bacteria and viruses, and as a prebiotic to stimulate the growth and activity of specific microbes in the infant gut (Bode, 2012). These complex carbohydrates exert a selective nutrient pressure to promote the HMO-utilising microbes, especially bifidobacteria belonging to the *Actinobacteria* phylum (Marcobal *et al.*, 2010). Specifically, infant-associated bifidobacteria are well adapted to utilise HMOs by employing an extensive range of glycosyl hydrolases and transporters, which lead to their dominance in the infant gut ecosystem (Sela and Mills, 2010). Upon weaning, the relative abundance of bifidobacteria decreases with the increase of *Firmicutes* and *Bacteroidetes* phyla whilst the gut microbial diversity increases (Laursen *et al.*, 2017).

The early dominance of bifidobacteria could be important for the maturation of the overall microbial community. In healthy children, the relative abundance of bifidobacteria is positively associated with the butyrate-producing *Firmicutes* from the family of *Lachnospiraceae* (also known as *Clostridium* cluster XIVa) and *Ruminococcaceae* (also known as *Clostridium* cluster IV) (Cheng *et al.*, 2015). This butyrogenic community is often present at much lower relative abundance in the gut of new-borns (Jost *et al.*, 2012). The subdominant butyrogenic species could however quickly become more dominant upon weaning as a result of the cessation of breast-feeding and the introduction of solid food (Backhed *et al.*, 2015; Laursen *et al.*, 2016). The expansion of the strict anaerobic, butyrate-producing bacteria could be a critical step for the gut and immune maturation (Arrieta *et al.*, 2015; Wopereis *et al.*, 2017). The interactions between lactate-producing bacteria (such as bifidobacteria) and lactate-utilising bacteria (such as *Ruminococcaceae* and *Lachnospiraceae*) are suggested to be associated with colicky symptoms and atopy disease in infants (Arrieta *et al.*, 2015; De Weerth *et al.*, 2013; Pham *et al.*, 2017; Wopereis *et al.*, 2017). To date, cross-feeding between glycan-degrading bifidobacteria and butyrate-producers using complex dietary carbohydrates (including starch, inulin, fructo-oligosaccharides, and arabinoxylan oligosaccharides) has been demonstrated in *in vitro* co-culturing experiments (Belenguer *et al.*, 2006; De Vuyst and Leroy, 2011; Falony *et al.*, 2006; Rios-Covian *et al.*, 2015; Riviere *et al.*, 2015). However, limited studies have shown the cross-feeding between these groups of bacteria on host-secreted glycans, such as HMOs (Schwab *et al.*, 2017) and mucins (Bunesova *et al.*, 2017).

In this study, we elucidated the trophic interaction between a versatile HMO-utiliser, *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) (Sela *et al.*, 2008) and a butyrogenic non-degrader. To this end the butyrate-producer *Anaerostipes caccae* was selected as the representative species for the *Lachnospiraceae* family as it is detected in the early life gut microbiota (Backhed *et al.*, 2015; Yatsunenکو *et al.*, 2012) with demonstrated *in vitro* cross-feeding interaction with adult-associated *Bifidobacterium*

*adolescentis* (Belenguer *et al.*, 2006). *A. caccae* is also recently identified as a key bacterium critical for regulating food allergy in early life (Feehley *et al.*, 2019). We showed that *B. infantis* metabolises HMOs into monosaccharides and metabolites including lactate and acetate, to support the growth and concomitant butyrate production by *A. caccae*. This butyrogenic cross-feeding postulates the role of bifidobacteria in driving both the maturation of microbial ecology and physiology of infant gut.

## 2. Materials and methods

### Screening of 16S rRNA gene amplicon libraries

16S rRNA gene amplicon sequencing datasets published by Yatsunenکو *et al.* (2012) were downloaded from European Nucleotide Archive (PRJEB3079). The sequencing data of 529 faecal samples with known age of the sample donors was analysed using the Quantitative Insights Into Microbial Ecology (QIIME) release version 1.9.0 package (Caporaso *et al.*, 2010). Sequences with mismatched primers, a mean sequence quality score <15 (five nucleotides window) or ambiguous bases were discarded. In total 1,036,929,139 sequences were retained with an average of 1,960,168.5 sequences per sample. The retained sequences were grouped into Operational Taxonomic Units with the USEARCH algorithm (Edgar, 2010) set at 97% sequence identity and subsequently, the Ribosomal Database Project Classifier (Cole *et al.*, 2009) was applied to assign taxonomy to the representative sequences by alignment to the SILVA ribosomal RNA database (release version 1.1.9) (Pruesse *et al.*, 2007).

### Bacterial strains and growth conditions

Bacterial pre-cultures were grown in anaerobic serum bottles filled with gas phase of N<sub>2</sub>/CO<sub>2</sub> (80/20 ratio) at 1.5 atm. Pre-cultures were prepared by overnight 37 °C incubation in basal minimal medium (Plugge, 2005) containing 0.5% (w/v) tryptone (Oxoid, Basingstoke, UK), supplemented with 30mM lactose (Oxoid) for *Bifidobacterium longum* subsp. *infantis* ATCC15697; and 30 mM glucose (Sigma-Aldrich, St. Louis, MO, USA) for *Anaerostipes caccae* L1-92 (DSM 14662) (Schwiertz *et al.*, 2002). Growth was measured by a spectrophotometer at an optical density of 600 nm (OD600) (OD600 DiluPhotometer™, IMPLLEN, München, Germany).

### Carbohydrate substrates

Lactose (Oxoid) and total human milk (HM) carbohydrates were tested as the carbohydrate substrates for bacterial growth. The usage and analysis of pooled HM samples described in this study were performed in accordance with ethical standards and guidelines as laid down in the Declaration of Helsinki. Ethical approval and written

consents of donors were given as stated in the publications (Thurl *et al.*, 1993). For preparation of total HM carbohydrates, a total carbohydrate mineral fraction was derived from pooled human milk after protein depletion by ethanol precipitation and removal of lipids by centrifugation as described by Stahl *et al.* (1994). Deviant from this workflow, no anion exchange chromatography was used to further separate neutral from acidic oligosaccharides present in the resulting total carbohydrate mineral fraction. The total HM carbohydrates contained approximately 90% of lactose, 10% of both acidic and neutral HMOs as well as traces of monosaccharides, as estimated by gel permeation chromatography (GPC) described below (Supplementary Figure S1).

### Anaerobic bioreactor

Fermentations were conducted in eight parallel minispinner bioreactors (DASGIP, Jülich, Germany) with 100 ml filling volume at 37 °C and a stirring rate of 150 rpm. Culturing experiments were performed in autoclaved basal minimal media (Plugge, 2005) containing 0.5% (w/v) tryptone (Oxoid), supplemented with 8 g/l of 0.2 µM filter-sterilised lactose or total HM carbohydrates. Anaerobic condition was achieved by overnight purging of anaerobic gas mixture containing 5% CO<sub>2</sub>, 5% H<sub>2</sub>, and 90% N<sub>2</sub>. Overnight pre-cultures were inoculated at starting OD<sub>600</sub> of 0.05 for each bacterial strain. Online signals of pH values and oxygen levels were monitored by the DASGIP control software. Cultures were maintained at pH 6.5 by the addition of 2 M NaOH.

### Gel permeation chromatography

Total HM carbohydrates were analysed using GPC. Glycans were separated by the GPC stationary phase and eluted according to size and charge. Neutral mono-, di-, and oligosaccharides, and acidic oligosaccharides with different degree of polymerisation (DP) could be detected. HM carbohydrate solution was prepared by dissolving 0.2 g/ml of total HM carbohydrates in ultrapure water (Sartorius Arium Pro, Goettingen, Germany) containing 2% (v/v) 2-propanol at 37 °C. 5 ml of 0.2 µM filter-sterilised HM carbohydrate solution was injected for each GPC run. The sample loop was cleaned by ultrapure water prior to analysis. Two connected Kronlab ECO50 columns (5×110 cm; YMC Europe, Dinslaken, Germany) packed with Toyopearl HW 40 (TOSOH Bioscience, Tokyo, Japan) were used. Milli-Q water was maintained at 50 °C using heating bath (Lauda, RE 206) for columns equilibration. Milli-Q (Milli-pore; Merck Millipore, Burlington, MA, USA) water containing 2% (v/v) of 2-propanol was used as the eluent. The flow rate of the eluent was set at 1.65 ml/min. Eluting glycans were monitored by refractive index detection (Shodex RI-101; Showa Denko America, Inc., New York, NY, USA). The resulting chromatograms

were analysed by using the Chromeleon® software (v 6.80; ThermoFisher Scientific, Waltham, MA, USA).

### High-performance liquid chromatography

For metabolite analysis, 1 ml of bacterial culture was centrifuged, and the supernatant was stored at -20 °C until high-performance liquid chromatography (HPLC) analysis. Crotonate was used as the internal standard, and external standards tested included lactose, glucose, galactose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose, malate, fumarate, succinate, citrate, formate, acetate, butyrate, isobutyrate, lactate, 1,2-propanediol, and propionate. Substrate and metabolite were measured with a Spectrasystem HPLC (Thermo Scientific, Breda, the Netherlands) equipped with a Hi-Plex-H column (Agilent, Amstelveen, the Netherlands) for the separation of carbohydrates and organic acids. A Hi-Plex-H column performs separation with diluted sulphuric acid on the basis of ion-exchange ligand-exchange chromatography. Measurements were conducted at a column temperature of 45 °C with an eluent flow of 0.8 ml/min flow of 0.01 N sulphuric acid. Metabolites were detected by refractive index (Spectrasystem RI 150; Thermo Scientific).

### Human milk oligosaccharide extraction

HMOs were recovered from 1 ml aliquots of bacterial cultures. Internal standard 1,5- $\alpha$ -L-arabinopentaose (Megazyme, Bray, Ireland) was added, at the volume of 10 µl per sample to minimise pipetting error, to reach a final concentration of 0.01 mmol/l. The solution was diluted 1:1 with ultrapure water and centrifuged at 4,000×g for 15 min at 4 °C. The supernatant was filtered through 0.2 µM syringe filter followed by subsequent centrifugation with a pre-washed ultra-filter (Amicon Ultra 0.5 Ultracel Membrane 3 kDa device; Merck Millipore) at 14,000×g for 1 h at room temperature. Finally, the filtrate was vortexed and stored at -20 °C until further targeted liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS<sup>2</sup>) analysis.

### Targeted liquid chromatography electrospray ionisation tandem mass spectrometry analysis

The identification and relative quantitation of HMOs were determined by LC-ESI-MS<sup>2</sup> as described in Mank *et al.* (2019). This method allowed the study of distinct HMOs structures differing in monosaccharide sequence, glycosidic linkage or the molecular conformation. Thereby even the HMOs isobaric isomers such as lacto-N-fucopentaose (LNFP) I, II, III and V could be distinguished. LC-ESI-MS<sup>2</sup> analysis was performed on a 1200/1260 series HPLC stack (Agilent, Waldbronn, Germany) consisting of solvent tray, degasser, binary pump, autosampler and DAD detector

coupled to a 3200 Qtrap mass spectrometer (ABSciex, Framingham, MA, USA). After HMOs extraction (see above) 5 µl of HMOs extract was injected into the LC-MS system. Oligosaccharides were separated by means of a 2.1×30 mm Hypercarb porous graphitized carbon (PGC) column with 2.1×10 mm PGC pre-column (ThermoFisher Scientific) using a 19 min water-ethanol (Merck, Darmstadt, Germany) gradient following 2 min pre-equilibrium according to Mank *et al.* (2019), with an additional 1 min for stabilisation at the end. Eluent flow was 400 µl/min and the columns were kept at 45 °C. The LC-effluent was infused online into the mass spectrometer and individual HMOs structures were analysed qualitatively and quantitatively by multiple reaction monitoring (MRM) in negative ion mode. Specific MRM transitions for neutral HMOs up to pentaoses and acidic HMOs up to trioses were included. The spray voltage was -4,500 V, declustering potential was at 44 V, and collision energy was set to 29 eV. Each MRM-transition was performed for 50 ms (except for LNT and lactose for 25 and 10 ms, respectively). The instrument was calibrated with polypropylene glycol according the instructions of the manufacturer. Unit resolution setting was used for precursor selection whereas low resolution setting was used to monitor fragment ions of the MRM transitions.

### Quantitative real-time PCR

The abundance of *B. infantis* and *A. caccae* in mono- and co-culture were determined by quantitative real-time PCR. Bacterial cultures were harvested at 16,100×g for 10 min. DNA extractions were performed using MasterPure™ Gram Positive DNA Purification Kit (Lucigen, Middleton, WI, USA). The DNA concentrations were determined fluorometrically (Qubit dsDNA HS assay; Invitrogen, Carlsbad, CA, USA) and adjusted to 1 ng/µl prior to use as the template in qPCR. Primers targeting the 16S rRNA gene of *Bifidobacterium* spp. (F-bifido 5'-CGCGTCYGGTGTGAAAG-3'; R-bifido 5'-CCCCACATCCAGCATCCA-3'; 244 bp product (Delroisse *et al.*, 2008)) and *A. caccae* (OFF2555 5'-GCGTAGGTGGCATGGTAAGT-3'; OFF2556 5'-CTGCACTCCAGCATGACAGT-3'; 83 bp product (Veiga *et al.*, 2010)) were used for quantification. Standard template DNA was prepared by amplifying genomic DNA of each bacterium using primer pairs of 35F (5'-CCTGGCTCAGGATGAACG-3' (Hayashi *et al.*, 2004)) and 1492R (5'-GGTTACCTTGTTACGACTT-3') for *B. infantis*; and 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R for *A. caccae*. Standard curves were prepared with nine standard concentrations of 10<sup>0</sup> to 10<sup>8</sup> gene copies/µl. PCRs were performed in triplicate with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a total volume of 10 µl with primers at 500 nM in 384-well plates sealed with optical sealing tape. Amplification was performed with an iCycler (Bio-Rad) with the following protocol: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 55 °C for 20 s, and 72 °C

for 30 s; 95 °C for 1 min and 60 °C for 1 min followed by a stepwise temperature increase from 60 to 95 °C (at 0.5 °C per 5 s) to obtain the melt curve data. Data was analysed using the Bio-Rad CFX Manager 3.0.

### Fluorescent *in situ* hybridisation

Bacterial cultures were fixated by adding 1.5 ml of 4% paraformaldehyde (PFA) to 0.5 ml of cultures followed by storage at -20 °C. Noted that for optimum fixation, at least 2 h to overnight incubation at 4 °C is recommended. Working stocks were prepared by harvesting bacterial cells by centrifugation at 8,000×g for 5 min of 4 °C, followed by re-suspension in ice-cold phosphate buffered saline (PBS) and 96% ethanol at a 1:1 (v/v) ratio. 3 µl of the PBS-ethanol working stocks were spotted on 18 wells (round, 6 mm diameter) gelatine-coated microscope slides. Hybridisation was performed using rRNA-targeted oligonucleotide probes specific for *Bifidobacterium* genus (Bif164m 5'-CATCCGGYATTACCACCC-3' [5']Cy3) (Dinoto *et al.*, 2006). 10 µl of hybridisation mixture containing 1 volume of 10 µM probe and 9 volumes of hybridisation buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.1% sodium dodecyl sulphate, pH 7.2-7.4) was applied on each well. The slides were hybridised for at least 3 h in a moist chamber at 50 °C; followed by 30 min incubation in washing buffer (20 mM Tris-HCl, 0.9 M NaCl, pH 7.2-7.4) at 50 °C for washing. The slides were rinsed briefly with Milli-Q water and air-dried. Slides were stained with 4,6-diamine-2-phenylindole dihydrochloride (DAPI) mix containing 200 µl of PBS and 1 µl of DAPI-dye at 100 ng/µl, for 5 min in the dark at room temperature followed by Milli-Q rinsing and air-drying. The slides were then covered with Citifluor AF1 (Hatfield, PA, USA) and a coverslip. The slides were enumerated using an Olympus MT ARC/HG epifluorescence microscope (Olympus, Tokyo, Japan). A total of 25 positions per well were automatically captured in two colour channels (Cy3 and DAPI) using a quadruple band filter. Images were analysed using Olympus ScanR Analysis software.

### Carbohydrate-active enzymes prediction

Carbohydrate-active enzymes (CAZymes) were predicted with dbCAN version 3.0 (Yin *et al.*, 2012), transmembrane domains with TMHMM version 2.0c (Krogh *et al.*, 2001) and signal peptides with signalP 4.1 (Petersen *et al.*, 2011).

## 3. Results

### Occurrence of *Bifidobacterium infantis* and *Anaerostipes caccae* across the life span

To understand the occurrence of *B. infantis* and *A. caccae* in the gut microbiota across life stages, a published dataset (Yatsunenکو *et al.*, 2012) was mined. The two infant-associated bacteria demonstrated opposite trajectories in

early life. *Bifidobacterium* genus showed high abundance at the first year followed by a sharp decline, with a negative correlation between age and relative abundance (Spearman  $\rho = -0.38$ ,  $P < 0.05$ ) (Figure 1), a similar finding as presented by Schwab *et al.* (2017). In contrast, *Anaerostipes* genus (Spearman  $\rho = 0.56$ ,  $P < 0.05$ ) and *Lachnospiraceae* family (Spearman  $\rho = 0.37$ ,  $P < 0.05$ ) were present at low abundance early in life and increased in relative abundance during the aging process (Figure 1).

### Model for *Bifidobacterium infantis* and *Anaerostipes caccae* co-occurrence

Mono- and co-cultures of *B. infantis* and *A. caccae* were setup to study the metabolism and interaction between a versatile HMO-utiliser and a butyrate producer. Bacterial

strains were cultured in anaerobic bioreactors controlled at pH 6.5 supplemented with either lactose or total human milk (HM) carbohydrates. *B. infantis* monoculture reached maximal cell density around 12 h at growth rate around  $0.33 \text{ h}^{-1}$  ( $\text{OD}_{\text{max}} = 1.40 \pm 0.38$  on lactose and  $\text{OD}_{\text{max}} = 1.37 \pm 0.25$  on total HM carbohydrates) (Figure 2). For *A. caccae* monoculture, no growth or substrate breakdown was detected in identical media ( $\text{OD}_{\text{max}} = 0.02 \pm 0.01$  on lactose and  $\text{OD}_{\text{max}} = 0.03 \pm 0.02$  on total HM carbohydrates) (Supplementary Table S1). The co-culture of *B. infantis* with *A. caccae* reached a growth rate of  $0.70 \text{ h}^{-1}$  on lactose and  $0.72 \text{ h}^{-1}$  on total HM carbohydrates, with maximal optical density at 11 h on lactose ( $\text{OD}_{\text{max}} = 3.63 \pm 0.61$ ) and at 9 h on total HM carbohydrates ( $\text{OD}_{\text{max}} = 3.54 \pm 0.60$ ). The cell density of the co-cultures was higher than the monocultures initially but significantly decreased after about 12 h of

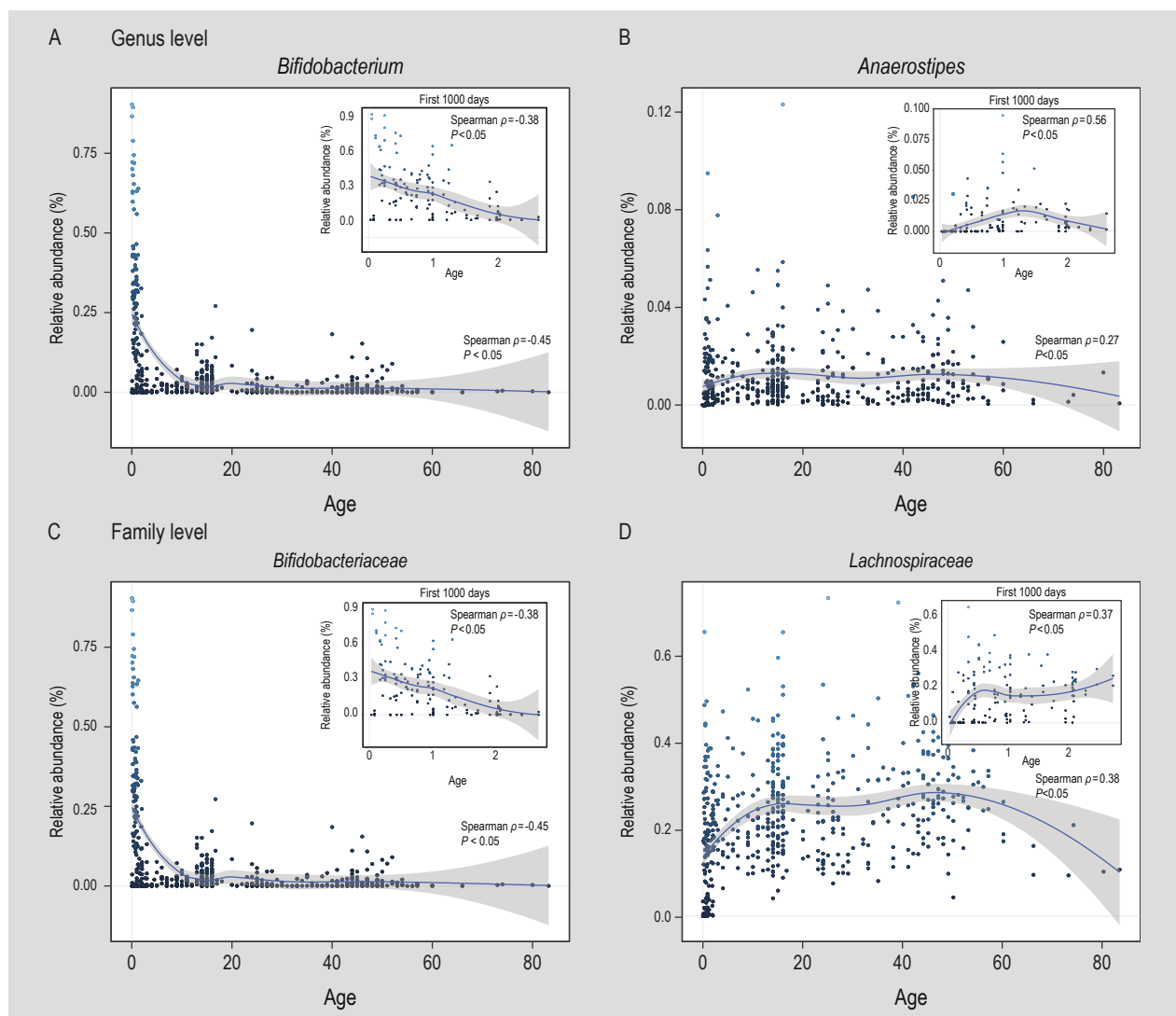


Figure 1. Occurrence of (A) *Bifidobacterium* and (B) *Anaerostipes* genus, as well as (C) *Bifidobacteriaceae* and (D) *Lachnospiraceae* family in the gut microbiota across age. The plot was generated from a published dataset (Yatsunenکو *et al.*, 2012) using R package ggplot2 version 2.2.1. The trend lines represent the smoothed conditional means using local polynomial regression fitting (Cleveland *et al.*, 1992).

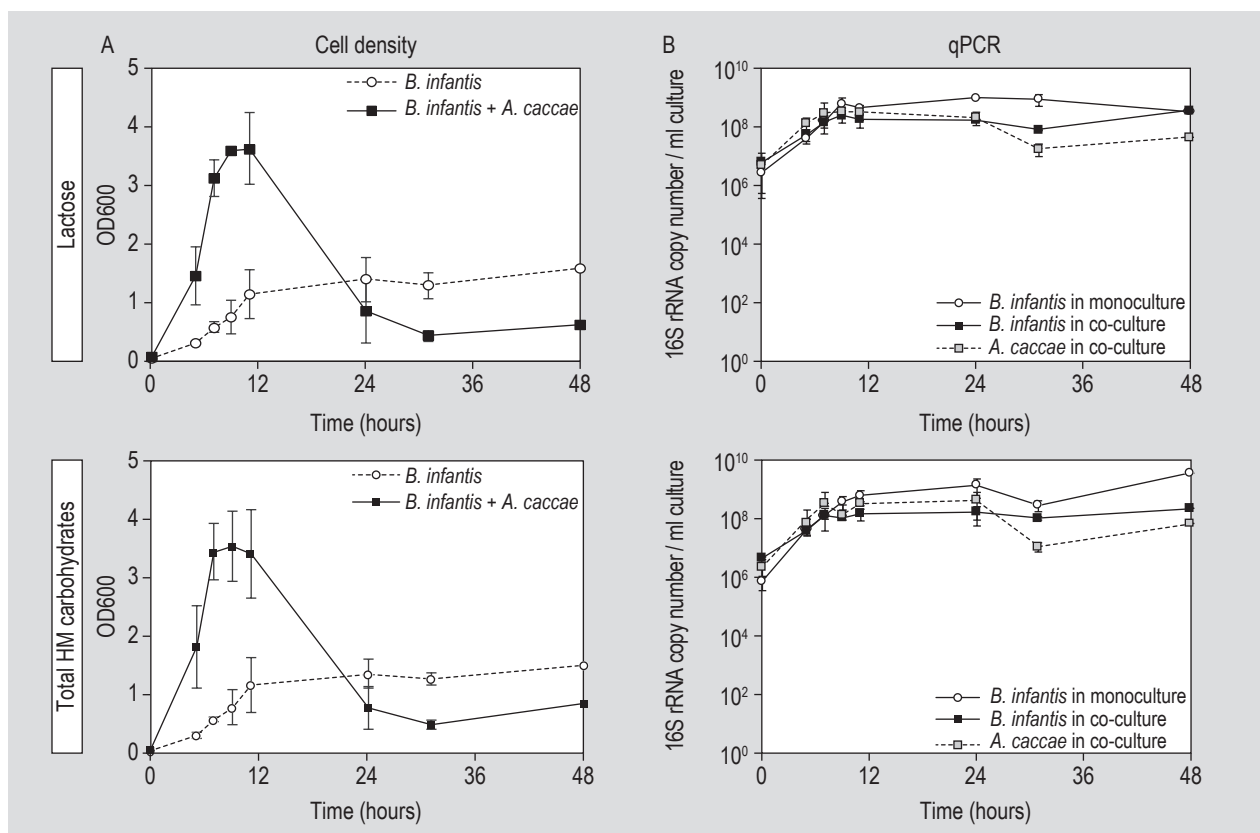
growth due to cell lysis. qPCR results showed that the number of *B. infantis* in the monocultures was about 2-fold higher than in the co-cultures from 9 h onwards. The community dynamic in the co-cultures was monitored over time by qPCR. An equal number of *B. infantis* and *A. caccae* (around  $10^6$  copy number/ml) was inoculated at the start of the fermentation. During the first 7 h, *B. infantis* and *A. caccae* increased 100-fold, after which growth halted. FISH analysis of samples harvested at 11 h showed a *B. infantis* to *A. caccae* ratio of 1:6 (Supplementary Figure S2 and Supplementary Table S1). These results accounted for both conditions either on lactose and total HM carbohydrates supplemented cultures.

### *Bifidobacterium infantis* supports the growth and metabolism of *Anaerostipes caccae* on lactose and HMOs

Substrate consumption and metabolite production were monitored over time (Figure 3). A similar profile was observed between the fermentation of lactose and total HM carbohydrates, probably because total HM carbohydrates was shown to consist of approximately 10% HMOs and 90% lactose (Supplementary Figure S1). On both substrates, the monoculture of *B. infantis* degraded the lactose present

into glucose and galactose resulting in the accumulation of monomeric sugars in the supernatant (Figure 3). Lactose was completely degraded at 9 h. At the same time point,  $17.49 \pm 1.83$  mM of glucose and  $15.24 \pm 2.06$  mM of galactose were detected in the media supplemented with lactose, whereas  $14.77 \pm 1.59$  mM of glucose and  $10.91 \pm 1.77$  mM of galactose were detected in the media supplemented with total HM carbohydrates. The monomeric sugars were fully consumed after 31 h. *B. infantis* produced acetate ( $56.96 \pm 4.48$  mM on lactose and  $50.76 \pm 3.23$  mM on total HM carbohydrates), lactate ( $22.73 \pm 3.02$  mM on lactose and  $17.69 \pm 1.21$  mM on total HM carbohydrates) and formate ( $6.56 \pm 0.09$  mM on lactose and  $8.04 \pm 0.21$  mM on total HM carbohydrates) as the major end metabolites. The final acetate to lactate ratio for *B. infantis* on lactose was 2.4:1 and 2.6:1 on total HM carbohydrates.

The co-culture of *B. infantis* with *A. caccae* also degraded lactose completely within 9 h. However, the co-cultures depleted glucose and galactose faster compared to the monocultures of *B. infantis*. The concentration of monomeric sugars peaked around 7 h in media supplemented with lactose, with  $4.62 \pm 1.21$  mM glucose and  $7.10 \pm 0.97$  mM galactose. In media supplemented with the total HM



**Figure 2.** *Bifidobacterium infantis* supported the growth of *Anaerostipes caccae* in human milk carbohydrates. (A) The optical density (OD600) indicating bacterial growth and (B) qPCR results showing the microbial composition in the mono- and co-cultures over time with lactose or with total human milk (HM) carbohydrates. Error bars represent the standard deviation for biological triplicates, except for time point 31 h (n=2) and 48 h (n=1).

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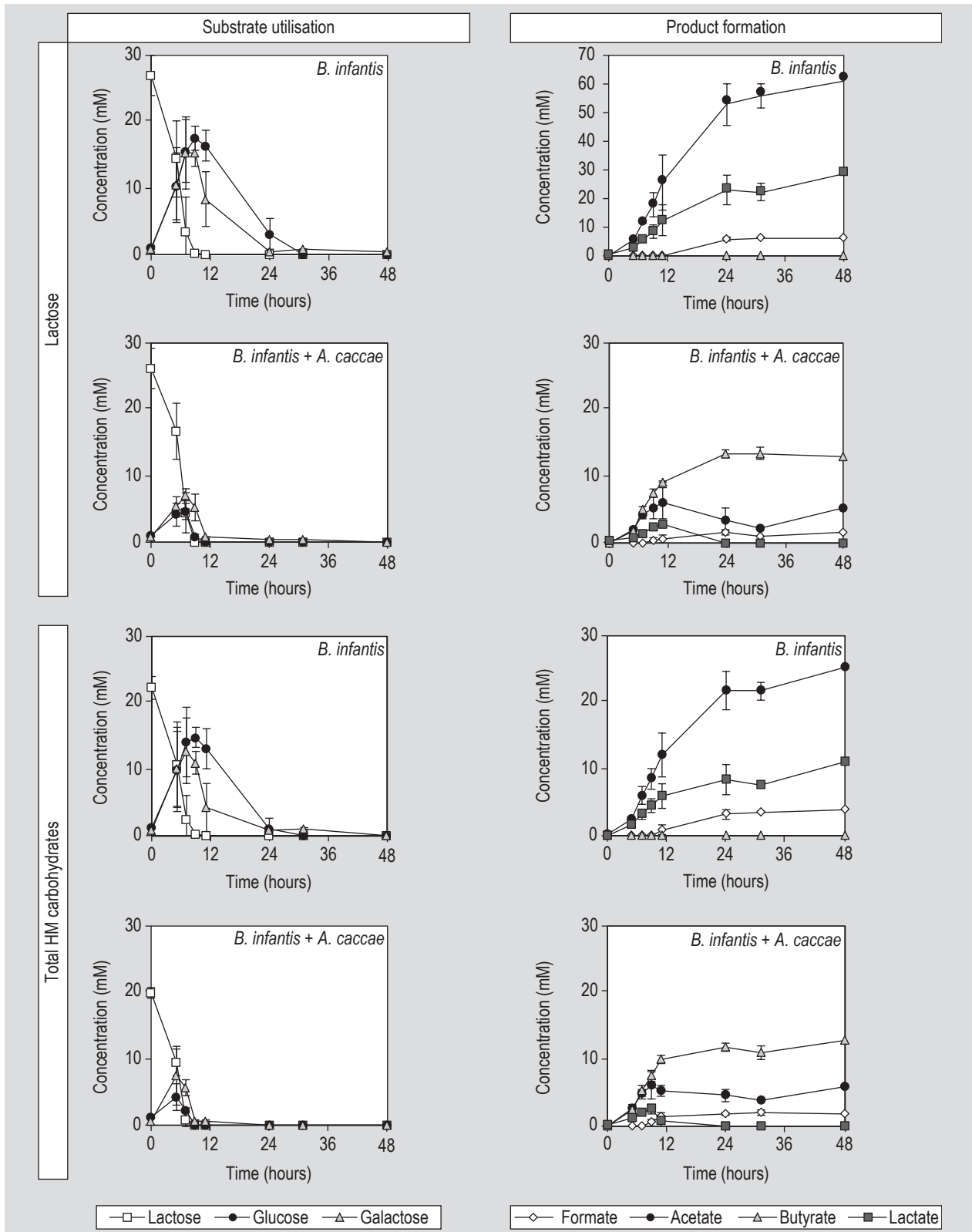


Figure 3. *Bifidobacterium infantis* supported butyrate production of *Anaerostipes caccae*. The substrate utilisation and product formation of co-cultures on lactose or total HM carbohydrates. Error bars represent the standard deviation for biological triplicates, except for time point 31 h (n=2) and 48 h (n=1).

carbohydrates, the maximum concentration for glucose ( $4.20 \pm 2.10$  mM) and galactose ( $7.39 \pm 4.45$  mM) was detected after 5 h. Only traces of monomeric sugars were still detectable after 9 h. The major end products of fermentation in the co-cultures were butyrate ( $31.39 \pm 2.15$  mM on lactose and  $25.80 \pm 2.45$  mM on total HM carbohydrates), acetate ( $5.44 \pm 0.30$  mM on lactose and  $9.05 \pm 0.71$  mM on total HM carbohydrates) and formate ( $2.53 \pm 0.16$  mM on lactose and  $4.78 \pm 1.16$  mM on total HM carbohydrates). Butyrate, the signature metabolic end product of *A. caccae* reached levels of  $31.39 \pm 1.36$  mM with lactose and  $27.68 \pm 1.38$  mM with total HM carbohydrates at 24 h. In contrast to the *B. infantis* monocultures, no lactate was detected after 11 h in the co-cultures.

The low molecular weight HMOs structures in the total HM carbohydrates were determined by LC-ESI-MS<sup>2</sup> for 0 and 24 h cultures in order to understand the specific glycan utilisation by these bacteria (Figure 4). The monoculture of *B. infantis* completely degraded the full range of neutral trioses (including 2'-fucosyllactose [2'-FL] and 3-fucosyllactose [3-FL]), tetraoses (including difucosyllactose [DFL], lacto-N-tetraose [LNT], lacto-N-neotetraose [LNnT]), pentaoses (lacto-N-fucopentaose I [LNFP I], lacto-N-fucopentaose II [LNFP II], lacto-N-fucopentaose III [LNFP III], lacto-N-fucopentaose V [LNFP V]), and acidic trioses (including 3'-sialyllactose [3'-SL] and 6'-sialyllactose [6'-SL]). No degradation of HMOs was observed in the *A. caccae* monoculture. On the other hand, the glycan utilisation pattern in the co-culture was identical to the profile of *B. infantis* monoculture indicative of the degrader role of *B. infantis* in the co-cultures. In addition, substrate quantification using HPLC showed that specific HMO-derived sugars, such as GlcNAc and fucose, were not detected, likely because these stay below the detection limit (0.5 mM) or due to overlap with other HPLC peaks.

#### Microbial cross-feeding results in a shift of acids pool

The cultures were maintained at pH 6.5 with the addition of 2 M NaOH. *B. infantis* monocultures required a higher amount of base addition compared to the co-culture with *A. caccae* (Figure 5A). The acidification of the cultures was reflected in the composition of acids. The total amount of acids at 31 h were higher in the monocultures ( $86.76 \pm 7.78$  mM on lactose and  $76.75 \pm 3.86$  mM on total HM carbohydrates) in comparison to the co-cultures ( $39.36 \pm 1.68$  mM on lactose and  $39.88 \pm 3.97$  mM on total HM carbohydrates). Furthermore, as a result of microbial cross-feeding in the co-cultures, lactate ( $pK_a=3.86$ ) produced by *B. infantis* monocultures was converted to butyrate ( $pK_a=4.82$ ). The  $pK_a$  value indicates the quantitative measurement of the strength of an acid in the solution with lower values for stronger acid. As the  $pK_a$  values are expressed in log scale, the decrease by one numerical value

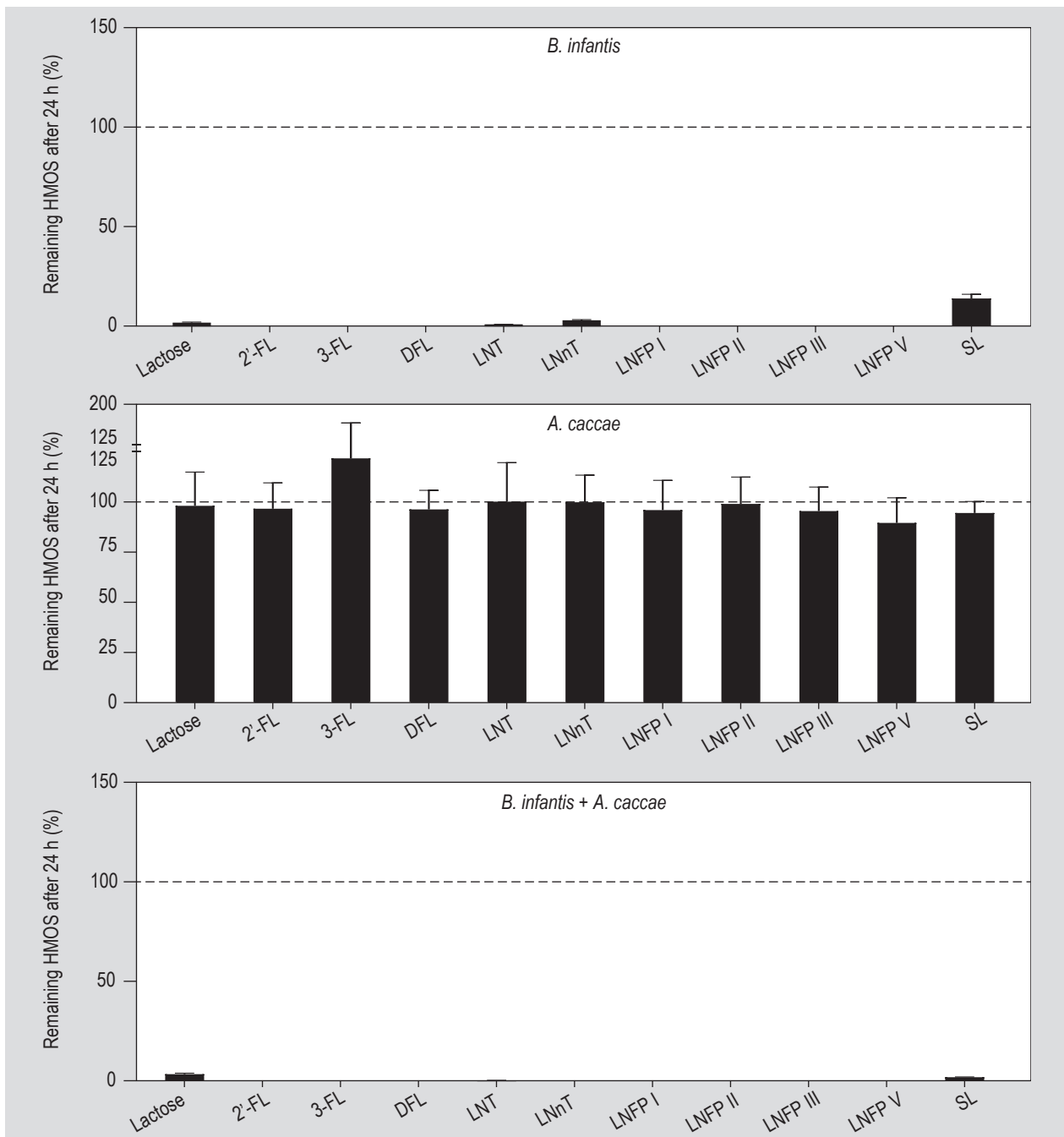
in lactate compared to butyrate may result in a 10-fold higher concentration of soluble protons. To investigate the dynamic of pH in early life, the data from Wopereis *et al.* (2017) was employed. We observed that the faecal pH for infants ( $n=138$ ) increased from pH 5.7 at 1 week to pH 6.0 at 6 months of life (Figure 5B).

#### 4. Discussion

The infant gut ecosystem is highly dynamic and marked by the succession of bacterial species (Backhed *et al.*, 2015). At this important window of growth and development, breast-feeding generally leads to the efficient colonisation of bifidobacteria in early life (Backhed *et al.*, 2015). Bifidobacteria could prime the development of gut barrier function and immune maturation (Ruiz *et al.*, 2017), as well as play an important ecological role in the establishment of the gut microbiota. Here, we showed that *B. infantis* could support the metabolism and growth of other important species in early life, such as the butyrate-producing *A. caccae* via cross-feeding. This microbial cross-feeding resulted in the shift of the acids pool and butyrate production. Physiologically, butyrate is associated with the enhancement of colonic barrier function and it could regulate host immune and metabolic state by signalling through G-protein-coupled receptors and by inhibiting histone deacetylase (Bolognini *et al.*, 2016; Fellows *et al.*, 2018; Geirnaert *et al.*, 2017; Koh *et al.*, 2016). Although the mechanistic evidences for butyrate are mostly generated from adult studies, a gradual shift in the ecosystem with slow induction of butyrate could be important for the maturation of the infant gut.

The dominance of bifidobacteria is often observed in the infant gut microbiota (Tannock *et al.*, 2016). Infant-associated bifidobacteria have evolved to be competitive in utilising human milk as substrate by employing a large arsenal of enzymes to metabolise HMOs (O'Callaghan and Van Sinderen, 2016). We showed that *B. infantis* effectively degrades the full range of the low molecular weight HMOs structures including neutral trioses, tetraoses, and pentaoses as well as acidic trioses. This is consistent with the unique HMOs utilisation capability of *B. infantis* by encoding a 43 kb gene cluster that carries the genes for different oligosaccharides transport proteins and glycosyl hydrolases (Underwood *et al.*, 2014). However, we did not detect the production of 1,2-propanediol from fucosylated HMOs, as demonstrated by Bunesova *et al.* (2016). No signal peptide or transmembrane domain was predicted for *B. infantis* enzymes involved in the cleavage of the monitored HMOs structures (Supplementary Table S2), indicating intracellular degradation of these substrates (Sela *et al.*, 2008). The monosaccharides including glucose and galactose were detected in the growth media of mono- and co-cultures (Figure 3) potentially released upon cell lysis. Furthermore, the distinct 'bifid shunt pathway' centred





**Figure 4.** *Bifidobacterium infantis* monoculture and co-culture with *Anaerostipes caccae* utilised the full range of low molecular weight HMOs. Error bars represent the error propagation for mean of three (for *A. caccae*) or four (for *B. infantis* and *B. infantis* + *A. caccae*) biological replicates measured in technical triplicates. The HMOs structures and glycosidic linkages are depicted according to Varki *et al.* (2015). 2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactose; DFL = difucosyllactose; LNT = lacto-N-tetraose; LNnT = lacto-N-neotetraose; LNFP I = lacto-N-fucopentaose I; LNFP II = lacto-N-fucopentaose II; LNFP III = lacto-N-fucopentaose III; LNFP V = lacto-N-fucopentaose V; SL = sialyllactose.

around the enzyme fructose-6-phosphate phosphoketolase (F6PPK) could also account for the competitiveness of bifidobacteria (O'Callaghan and Van Sinderen, 2016). The fermentation of sugars via F6PPK-dependent bifid shunt pathway yields more energy compared to the usual glycolysis or Emden-Meyerhof Parnas pathway which could

give bifidobacteria an additional advantage compared to other gut bacteria (Palframan *et al.*, 2003).

Lactose and HMOs fermentation by bifidobacteria results in acetate and lactate as major end products. Although majority of lactose from milk is expected to be

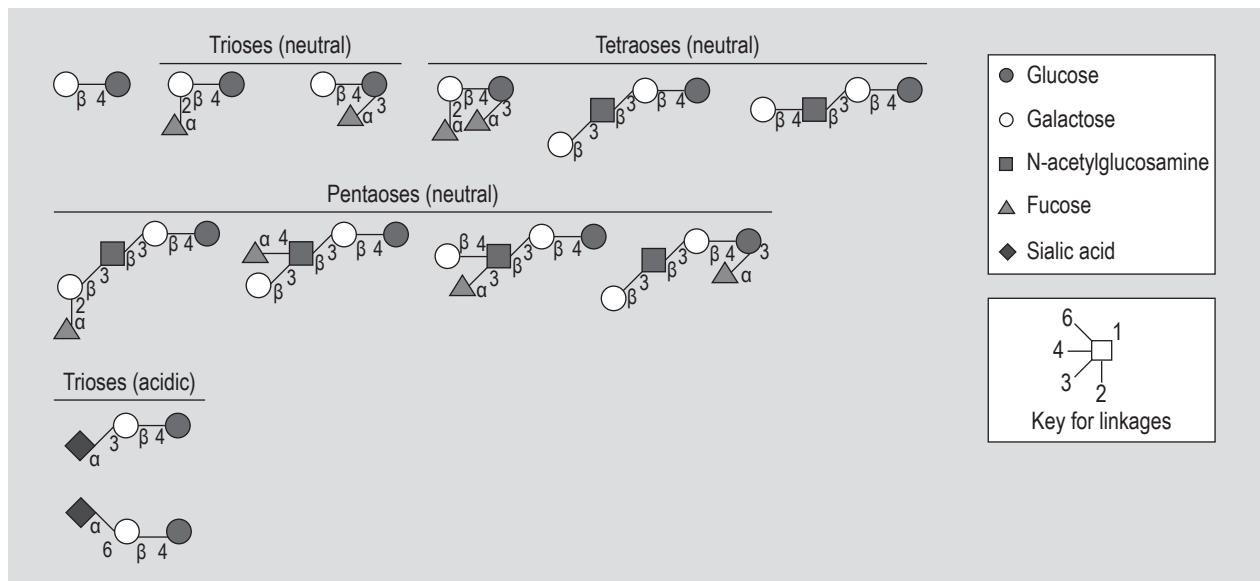
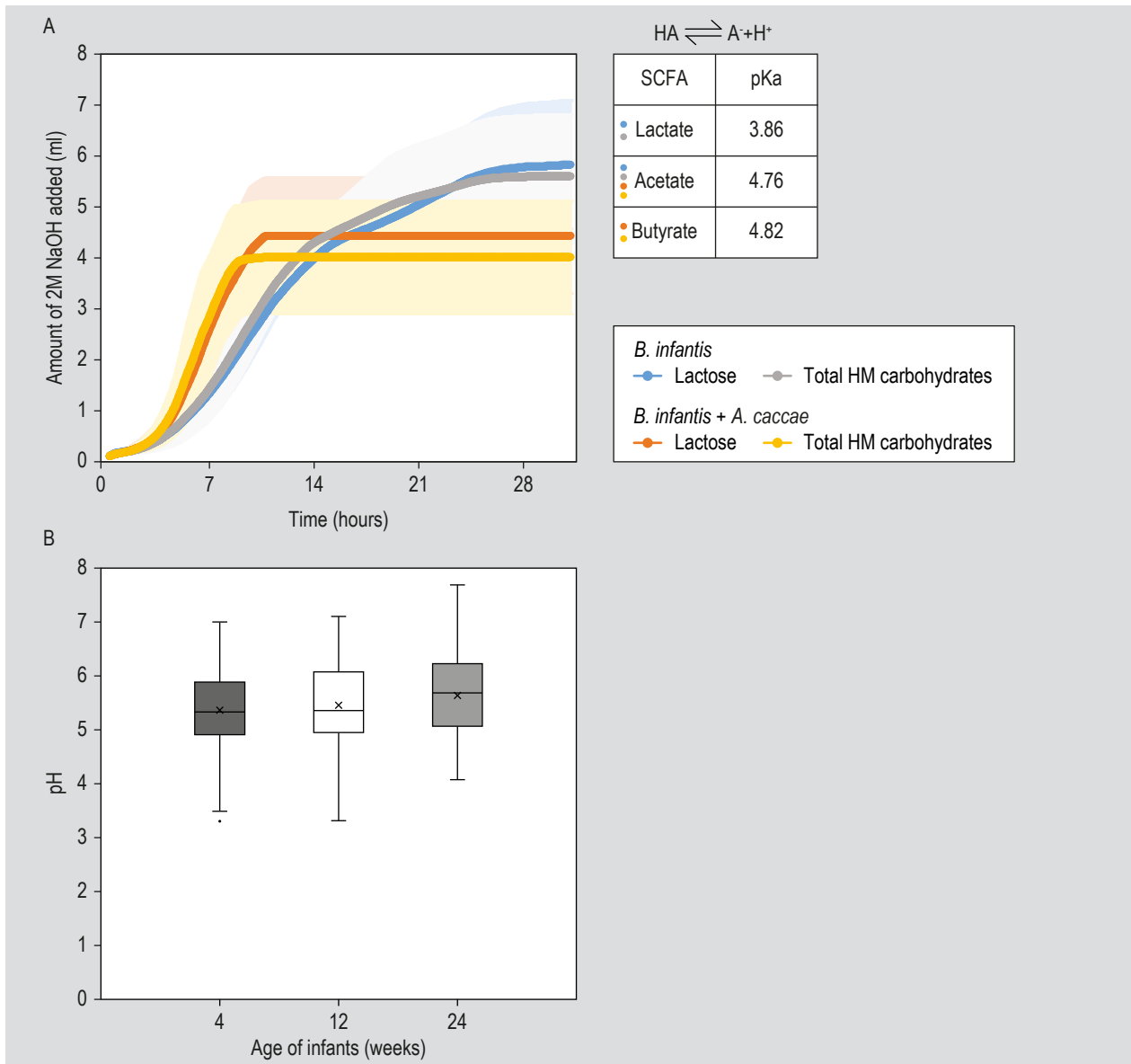


Figure 4. Continued.

degraded in the upper gastrointestinal tract of infants, colonic fermentation of lactose is still relevant especially for lactose-deficient subjects and lactose core could be liberated from HMOs-degradation (Venema, 2012). In addition to bifidobacteria, other primary colonisers like *Lactobacillus*, *Streptococcus*, *Staphylococcus*, and *Enterococcus* spp. also contribute to lactate production in the infant gut (Pham *et al.*, 2017). In the gut of breast-fed infants, the overall digestion and fermentation lead to a relatively high concentration of acetate and lactate with a low pH (Oozeer *et al.*, 2013; Pham *et al.*, 2016). The pH of the luminal content has a significant impact on the microbiota composition (Duncan *et al.*, 2009). Various bacterial groups have been shown to be inhibited by a low pH, such as opportunistic pathogens including *Salmonella* Typhimurium, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (Van Limpt *et al.*, 2004) as well as *Bacteroides* spp. (Duncan *et al.*, 2009; Walker *et al.*, 2005). In contrast, a low pH may promote butyrate production and the butyrogenic community (Reichardt *et al.*, 2017; Walker *et al.*, 2005). Given the above, the circumstances in the infant gut seems to be in favour of the colonisation of butyrate-producers.

In the first months of life butyrate levels in the faeces are generally low (Oozeer *et al.*, 2013; Pham *et al.*, 2016) and the major adult-type butyrate-producing population (*Roseburia* and *Faecalibacterium* spp.) remained undetectable up to 30 days postnatal (Jost *et al.*, 2012). We observed an increase of relative abundance for *Lachnospiraceae* family and *Anaerostipes* genus in the first year of life (Yatsunenko *et al.*, 2012). The majority of butyrate-producing bacteria from the *Lachnospiraceae* and *Ruminococcaceae* are not capable of utilising HMOs (Sheridan *et al.*, 2016). For

*A. caccae*, no growth or metabolism was detected in the media containing lactose and HMOs. These subdominant butyrogenic bacteria in the infant gut could depend on cross-feeding with species like bifidobacteria. Our results indicate that *A. caccae* could utilise the monomeric sugars and end products like acetate and lactate derived from *B. infantis* for metabolic activity and growth (Duncan *et al.*, 2004). *A. caccae* is known to convert 1 mol of acetate and 2 mol of lactate to yield 1.5 mol of butyrate (Duncan *et al.*, 2004). Our previous study (Belzer *et al.*, 2017) demonstrated that *A. caccae* in glucose has a specific growth rate around 0.2 h<sup>-1</sup>. Glucose supplemented with 10 mM of acetate enhanced the growth rate of *A. caccae* to around 0.6 h<sup>-1</sup>. In the monocultures of *B. infantis* (Figure 3), we showed that monosaccharides (glucose and galactose) were detected in the supernatant at the intermediary stage of fermentation, with acetate and lactate as end metabolites. We deduced that the growth rate for *A. caccae* in co-culture with *B. infantis* could be on par of the previously tested condition with glucose and acetate, due to the presence of key substrate to support the growth of *A. caccae*. This is supported by the observation that the total carbon in the end metabolites of the co-cultures was about 1.5-fold lower compared to the *B. infantis* mono-culture, indicating carbon utilisation for the growth of *A. caccae* to synthesise cell components. This metabolic interaction could also benefit the microbial community by reducing the metabolic burden (Seth and Taga, 2014), shown by the formation of a relatively weaker acid pool. The infant faecal pH showed an increasing trend with age (Figure 5) (Wopereis *et al.*, 2017), potentially due to the shift of colonic acids pool driven by changes in diet, microbiota composition and function, and physiological development. Faecal pH provides an indication on the acids pool although other factor such as the production of alkaline molecules, i.e. ammonia could



**Figure 5.** The acidification of cultures and faecal pH. (A) Base (2 M NaOH) added to maintain the anaerobic chemostat at pH 6.5. The shaded error bars indicate standard deviation for biological triplicates. (B) The faecal pH for infants (n=138). Data adapted from Wopereis *et al.* (2017).

affect the acidity level. Osuka *et al.* (2012) showed that the total organic acid was increased in acidic faeces (pH<6.0) and decreased in alkaline faeces (pH>7.2). Specifically, lactate, succinate, and formate were the main contributors to acidity in acidic faeces. Acetate and lactate as well as a small amount of propionate and butyrate can be detected in the faeces of infants (Pham *et al.*, 2016; Wopereis *et al.*, 2017). Whereas, the typical short chain fatty acids (SCFA) ratio in adult faeces is around 3:1:1 for acetate, propionate and butyrate respectively (Schwartz *et al.*, 2010; Scott *et al.*, 2011). The shift of the SCFA pool goes hand in hand with the transition of the gut microbiota, likely induced by dietary changes. Upon weaning, the diversification of indigestible fibres due to the introduction of solid foods

results in conditions leading to the decrease of the relative numbers of bifidobacteria and the relative increase of *Lachnospiraceae*, *Ruminococcaceae*, and *Bacteroides* spp. (Laursen *et al.*, 2017).

Although the contributing factors to the progression from bifidobacteria dominant community to *Firmicutes* and *Bacteroides* dominant community are not well understood, the well-orchestrated transition is important for health. An aberrant microbial composition and/or SCFA production are associated with colicky symptoms and atopy diseases in infants (Arrieta *et al.*, 2015; De Weerth *et al.*, 2013; Pham *et al.*, 2017; Stokholm *et al.*, 2018; Wopereis *et al.*, 2017). We demonstrated the role of *B. infantis* in driving

the butyrogenic trophic chain by metabolising human milk carbohydrates. This microbial cross-feeding is indicative of the key ecological role of bifidobacteria as substrates provider for subdominant butyrate-producing bacteria. The compromised health outcomes as a result of the delayed transition from bifidobacteria-dominant to butyrogenic microbial community highlight the importance of proper developmental stages in the infant gut.

## Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2020.0005>.

**Figure S1.** The GPC-RI chromatogram for total human milk carbohydrates showing a composition of approximately 90% of lactose, 10% of both acidic and neutral HMOs as well as traces of monosaccharides.

**Figure S2.** *Bifidobacterium infantis* supported the growth of *Anaerostipes caccae* in human milk carbohydrates: fluorescent *in situ* hybridisation of co-cultures at 11 h.

**Table S1.** The optical density, substrates and end products detected in *Anaerostipes caccae* monoculture on lactose and total HM carbohydrates and media blanks at the end of fermentation.

**Table S2.** Human milk oligosaccharide structures and prediction of degradation by *Bifidobacterium infantis* ATCC15697.

## Conflict of interest

This project is financially supported by Nutricia Research. MM, BB, RB, HW, KvL, ST, BS and JK are employed by Nutricia Research.

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