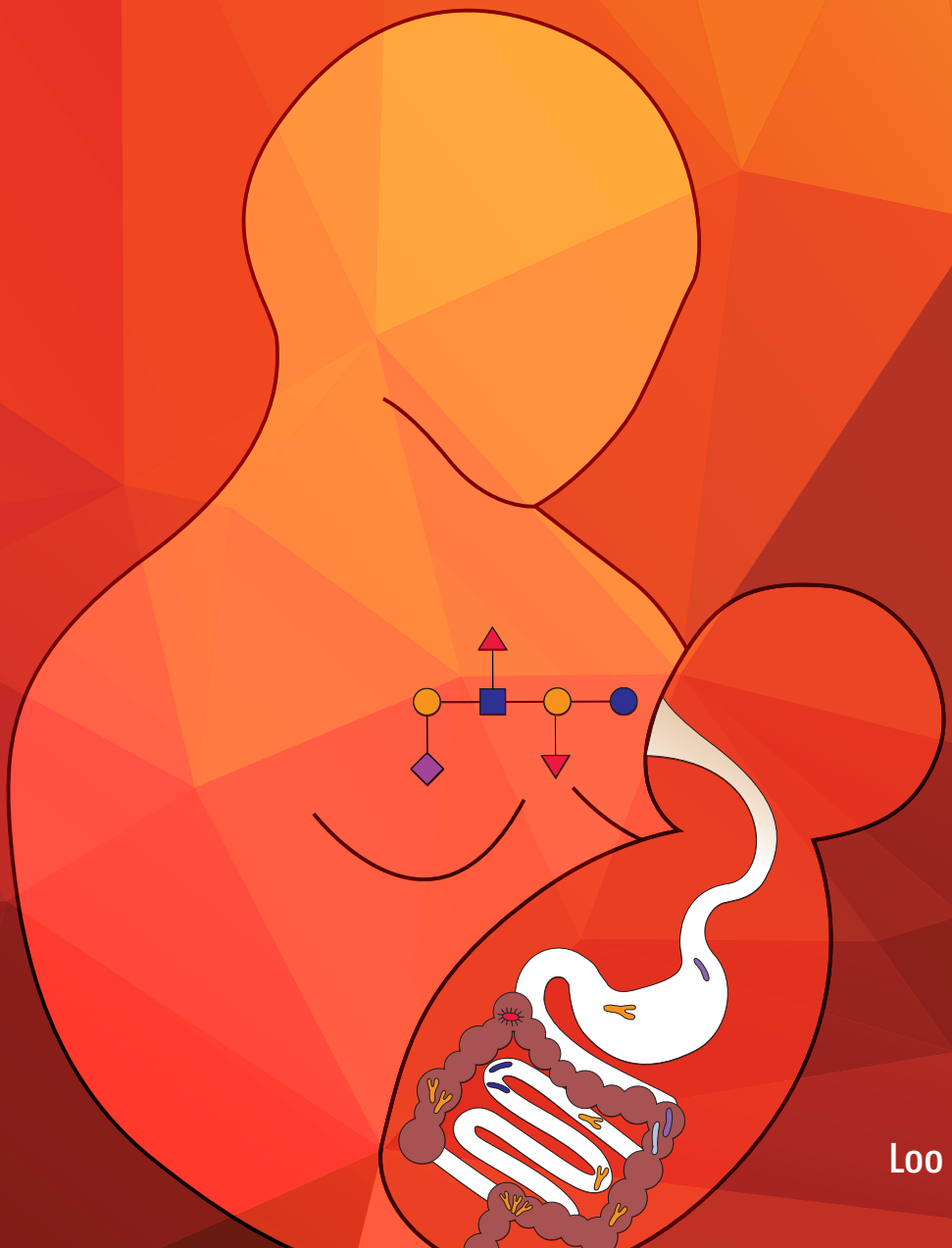
Cross-feeding interactions of gut symbionts driven by human milk oligosaccharides and mucins



Loo Wee Chia

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

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Thesis

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 20 June 2018
at 1:30 p.m. in the Aula.

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Cross-feeding interactions of gut symbionts driven by human milk oligosaccharides and mucins,
187 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2018)

With references, with summaries in English and Dutch

ISBN: 978-94-6343-847-6

DOI: 10.18174/444432

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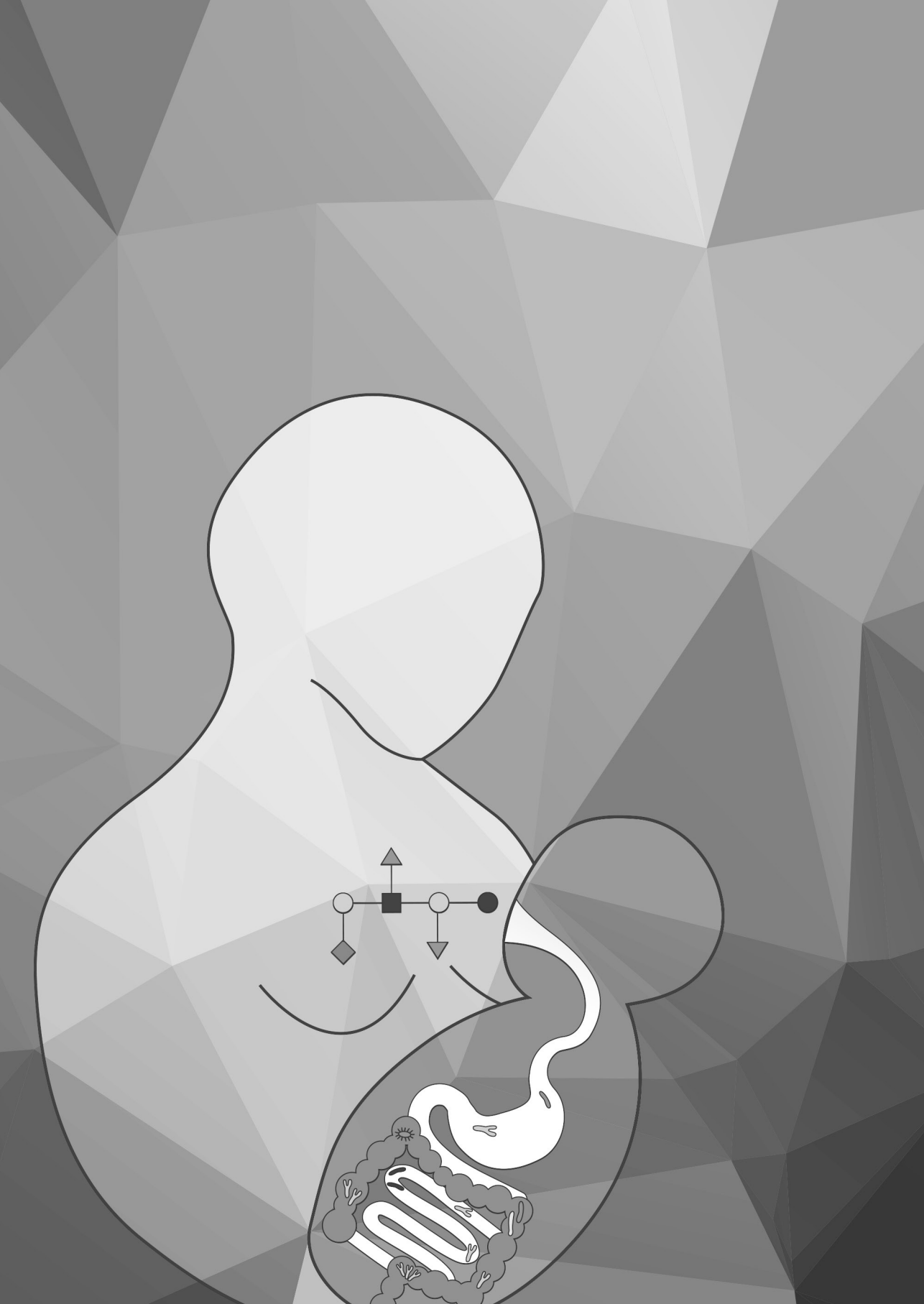


Chapter 1

General introduction and thesis outline

General introduction

Humans can be considered holobionts, consisting of cells from the host and an even larger number of microorganisms (Postler and Ghosh 2017). The majority of microbes are residing in the gut (Sender *et al* 2016). The human gut microbiota is a complex ecosystem consisting of bacteria, archaea, microeukaryotes and viruses (Clemente *et al* 2012). Over 2000 species-level phylotypes (prokaryotes including bacteria and archaea) have been detected for the gut microbiota, with each individual estimated to host at least 160 species (Qin *et al* 2010, Ritari *et al* 2015, Zoetendal *et al* 2008). The gut microbiota may impact the well-being of human and susceptibility for diseases by interacting with our metabolic, immune and neurological system (Honda and Littman 2016, Koh *et al* 2016, Rogers *et al* 2016). Furthermore, the gut microbiota contributes to a variety of metabolic functions and can be seen as an essential part of our digestive system (El Kaoutari *et al* 2013). Bacterial symbionts allow the human hosts to utilise inaccessible nutrients by converting complex substrates to short chain fatty acid (SCFA) and vitamins (Fisher *et al* 2017). Bacterial-derived SCFAs are estimated to contribute about 10% of our daily caloric requirement (Bergman 1990). While all SCFAs are vital for maintaining gut homeostasis, butyrate is of particular interest because it has been attributed to a range of health-promoting functions. Butyrate is the primary energy source for colonic epithelial cells and is associated with the enhancement of colonic barrier function, increase of satiety, pain relief, anti-inflammatory responses, and protection against colorectal cancer (Banasiewicz *et al* 2013, Bolognini *et al* 2016, Donohoe *et al* 2011, Furusawa *et al* 2013, Geirnaert *et al* 2017, Goncalves and Martel 2013).

Considering the physiological benefits of butyrate to human health, this thesis investigates the microbial interaction of gut symbionts that support butyrate production driven primarily by the host produced glycans in human milk and mucus. Glycans are compounds consisting of an array of glycosidically linked monosaccharides (monosaccharides and disaccharides are collectively termed sugars) such as human milk oligosaccharides (HMOS) and mucins (Varki and Kornfeld 2017). HMOS present in mother milk are the major compositional and functional driver of the infant gut microbiota (Backhed *et al* 2015). Mucin glycans covering the intestinal lining create a stable niche for bacterial colonisation (Ouwerkerk *et al* 2013, Tailford *et al* 2015). We hypothesise that the bacterial degradation of host glycans in human milk and mucus

could support the growth and butyrate production of butyrogenic bacteria. This introductory chapter gives context to the next chapters by reviewing the scientific literature on the role of human milk and mucus in shaping the gut microbiota composition. An overview is provided of the molecular structures of HMOS and mucins with potential associations to microbial strategies for glycan degradation. Furthermore, the microbial cross-feeding driven by glycan-degraders and the potential microbe-microbe interactions leading to butyrate production are discussed.

The establishment of the gut microbiota

The gut microbiota is essential to sustain health in humans throughout life (Kundu *et al* 2017, Scholtens *et al* 2012). The development of the microbiota in early life is of particular importance, as this time period could be critical to shape the host metabolic, immunological and neurological development with long-lasting effect (Kundu *et al* 2017, Thompson 2012). Recently, microbial signatures have been detected in the foetal environment suggesting that the colonisation of the gastrointestinal tract might begin even before birth (Aagaard *et al* 2014, Collado *et al* 2016, Gronlund *et al* 2011, Hornef and Penders 2017). Regardless of the exposure *in utero*, the birthing process marks an important event for microbial colonisation due to drastic change of the infant's environmental exposure. As such, different modes of delivery lead to compositional distinction in the microbiota of vaginal- versus caesarean-born infants (Dominguez-Bello *et al* 2010). A desirable transmission of vaginal and gut microbiota from mother to infant is observed in vaginally-delivered infants during birth, but not for caesarean-delivered infants (Dominguez-Bello *et al* 2016). Shortly after birth, microbes rapidly colonise the infant gut, although the composition and diversity only stabilize after several years in humans (Backhed *et al* 2015, Yatsunenko *et al* 2012).

Apart from birth mode, the establishment of the gut microbiota is driven by factors including gestational age, the mode of feeding, antibiotic use, hospitalization time, childcare or day-care, family environment, and exposure to household pets and siblings (Azad *et al* 2013b, Laursen *et al* 2015, Martin *et al* 2016, Penders *et al* 2006, Scholtens *et al* 2012, Thompson *et al* 2015, Yassour *et al* 2016). In general, the first microbial colonisers are facultative anaerobic bacteria, such as *Escherichia coli* and *Streptococcus* spp. due to the relatively high oxygen levels in the new-born intestines

(Mackie *et al* 1999). The growth of these species reduces the oxygen levels in the gut and gradually creates an environment for the colonisation of obligate anaerobes including bifidobacteria, *Bacteroides* spp. and *Clostridium* spp. (Dominguez-Bello *et al* 2011). During this first days and weeks of life, nutrition is a major driver for the development of the gut microbiota (Azad *et al* 2013a, Liu *et al* 2016). Distinct faecal microbial compositions are found between infants fed with human milk and infant fed with formula. The microbiota of breast-fed infants for example is characterised by high levels of *Actinobacteria* (mainly bifidobacteria), and low microbial diversity (Backhed *et al* 2015, Schwartz *et al* 2012, Tannock *et al* 2013).

One of the most prominent differences between human milk and formula milk is the presence of glycans in human milk. Human milk glycans include glycoprotein, glycolipids, oligosaccharides, and mucins, can serve as microbial substrates (Bode 2012, Liu and Newburg 2013, Smilowitz *et al* 2014, Zivkovic *et al* 2011). Milk glycans resist host digestion, as the human genome encodes only for a narrow range of digestive enzymes limited to the breakdown of sucrose, lactose and starch (El Kaoutari *et al* 2013). Hence, these complex carbohydrates from human milk reach the infant large intestine to selectively promote the growth and colonisation of glycan-degrading bacteria. The bacteria able to degrade milk glycans, such as bifidobacteria and *Bacteroides* spp. thus become dominant members of the microbial ecosystem in the infant gut (Backhed *et al* 2015, Marcobal *et al* 2010). Upon weaning, during which complementary food is introduced and (human) milk feeding is gradually decreased, the infant gut microbiota undergoes re-structuring in response to the diversification of substrates from diet (Backhed *et al* 2015, Fallani *et al* 2010, Favier *et al* 2002, Koenig *et al* 2011, Laursen *et al* 2017). This is generally marked by a decrease in the relative abundance of bifidobacteria and an increase of *Lachnospiraceae* (also known as *Clostridium* cluster *XIVa*), *Ruminococcaceae* (also known as *Clostridium* cluster *IV*) and *Bacteroides* spp. (Laursen *et al* 2017). Around three years of age, a more adult-like microbiota establishes, which is dominated by species from the *Firmicutes* and *Bacteroidetes* phyla (Guaraldi and Salvatori 2012, Koenig *et al* 2011).

The sugar code of human milk oligosaccharides (HMOS) and mucins

Host-secreted glycans such as HMOS and mucins are important drivers for the composition and functionality of the gut microbiota (Backhed *et al* 2015, Tailford *et al* 2015). HMOS and mucins show similar molecular characteristics as both are composed of comparable monosaccharide building blocks and linkages (Fig. 1). HMOS consist of a lactose core, which may be elongated by N-acetylglucosamine (GlcNAc), galactose and/or decorated with fucose and/or sialic acid (Fig. 1). HMOS are the third most abundant component in human milk after lactose and fat, with the concentration ranges from 23 g/L in colostrum to 7 g/L in matured milk (Coppa *et al* 1993, Gabrielli *et al* 2011). Currently, over 200 HMOS structures are detected with only 50 structures accounting for 99% of the total HMOS abundance in human milk (Ninonuevo *et al* 2006, Smilowitz *et al* 2014, Wu *et al* 2010, Wu *et al* 2011). The composition of HMOS is different between individuals and is determined by maternal genetic factors (McGuire *et al* 2017, Thurl *et al* 2017). The Secretor Status (Se) and Lewis (Le) genes, which determine the blood group of humans also determine the presence or absence of some fucosylated oligosaccharides in human milk (Bode 2012). On average, 30% of women worldwide are non-secretors (Ferrer-Admetlla *et al* 2009). Non-secretor mothers lack a functional 2-fucosyltransferase (FUT2) enzyme to synthesize α1,2-fucosylated oligosaccharides. This results in compositional changes and also around 40% reduction of total HMOS abundance in human milk (Kunz *et al* 2017). The absence of α1,2-fucosylated oligosaccharides in human milk mostly reduced the abundance of bifidobacteria in the infant gut (Table 1) and has been associated with a higher risk of diarrhoea (Morrow *et al* 2004, Newburg *et al* 2004) and allergic disease (Sprenger *et al* 2017).

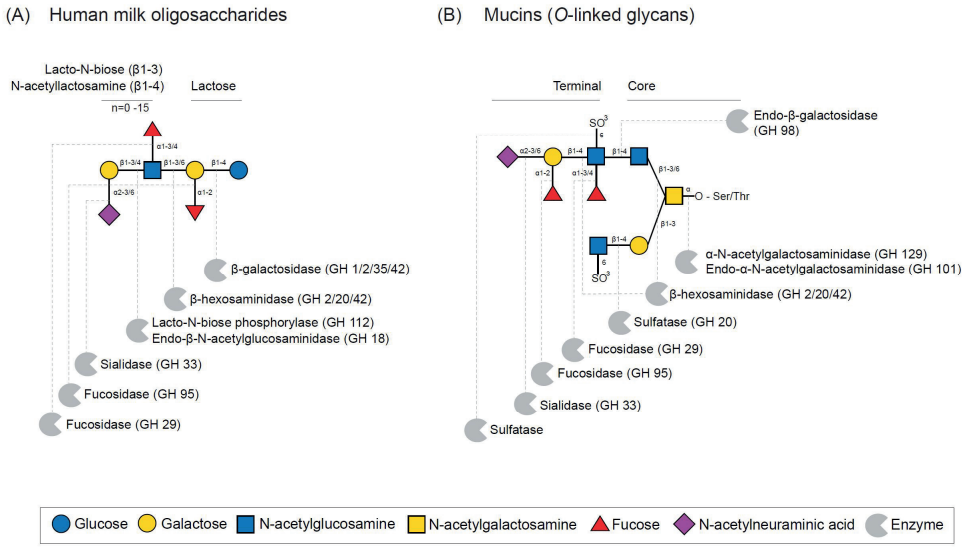


Figure 1. **The structure of HMOS and mucins show molecular similarities**. The schematic representation for (A) HMOS and (B) mucin *O*-glycans, summarising the sites of action and carbohydrate-active enzymes (CAZymes) (Lombard *et al* 2014) predicted to cleave the glycosidic linkages. N-acetylneuraminic acid (Neu5Ac), the predominant sialic acid in mammalian cells is depicted. HMOS may also carry α1-2 fucose linked to galactose, α1-3 fucose linked to glucose, and/or α2-6 Neu5Ac linked to GlcNAc. The depiction for specific HMOS structures are available at Thurl *et al*. (Thurl *et al* 2017). The hypothetical mucin *O*-glycan chain is adapted from Tailford *et al*. (Tailford *et al* 2015). Detailed structural representation for mucin *O*-glycans is available at Brockhausen and Stanley (Brockhausen and Stanley 2017). *Abbreviation*: GH, glycosyl hydrolase.

The colonic epithelial cell surface is covered by an inner mucus layer firmly adhered to the cells, and a loose layer consisting predominantly of mucin glycoproteins (Johansson *et al* 2011, Johansson *et al* 2014). Mucin *O*-glycans are large and complex glycoproteins consisting of a protein core that is rich in proline, threonine and serine moieties, to which oligosaccharides are attached (Fig. 1). The oligosaccharide chain is composed of N-acetylgalactosamine (GalNAc), GlcNAc, galactose, fucose, and sialic acid (Brockhausen and Stanley 2017). A gradient of increasing sialic acid and decreasing fucose from ileum to distal colon is observed in the adult intestine (Robbe *et al* 2004). However, this region-specific glycosylation is not observed in new-borns where the intestinal barrier function and the mucin secretion are not fully developed (Martin *et al* 2010, Robbe-Masselot *et al* 2009). It is speculated that HMOS mimic the structure of mucins as a means to stimulate colonisation of mucolytic gut commensals

until the mucus secretion process matures (Marcobal *et al* 2011). Furthermore, human milk contains mucins and antimicrobial proteins, which could prevent the binding of pathogenic microorganisms to the mucosal surface (Liu and Newburg 2013). Microbes colonising the gut mucosa early in life are particularly critical to prime mucosal immune response and tolerance (Wopereis *et al* 2014). The composition of the gut microbiota could also be affected by genetic makeup such as secretor status (Table 1). The secretor status determines the expression of blood group antigens on the mucosa secretions with reduced α1,2-fucosylated structures present in the mucins of non-secretors (Ferrer-Admetlla *et al* 2009, Magalhaes *et al* 2016). However, no consistent microbial group is shown to be affected by personal secretor status (Table 1) and a recent twin cohort study did not confirm the association between secretor status and the gut microbiota (Davenport *et al* 2016). Non-secretors are found to be less susceptible to acute gastroenteritis caused by *Helicobacter pylori*, rotaviruses and norovirus, which are suggested to target the blood group antigens as receptors (Lindesmith *et al* 2003, Magalhaes *et al* 2016, Nordgren *et al* 2014, Nordgren *et al* 2016).

Table 1. **Host secretor status affects the composition of the gut microbiota.** The effect of maternal secretor status on the infant/child faecal microbial composition is summarised in the upper panel. The effect of personal secretor status is provided in the lower panel. The bacterial changes for non-secretors are summarised, ↑ denotes higher abundance, and ↓ denotes lower abundance relative to secretors.

Subject	Country	Microbial composition for non-secretors	Reference
Effect of maternal secretor status on infant/child			
Premature infants (30 gestational weeks)	United States	↓ *Firmicutes* ↑ *Proteobacteria*	(Underwood *et al* 2015)
Infants (1 week – 4 months old)	United States	↓ *Bifidobacterium* ↑ *Streptococcus*	(Lewis *et al* 2015)
Infants (1 week – 3 months old)	Armenia and Georgia	↓ *Bifidobacterium*	(Lewis *et al* 2017)
Children (2 years old)	Australia	↓ *Bifidobacterium*	(Smith-Brown *et al* 2016)

Effect of personal secretor status			
Children (13-14 years old)	Finland and India	↓ *Bifidobacteriaceae* and *Verrucomicrobiaceae* ↑ *Veillonellaceae* and *Enterobacteriaceae*	(Kumbhare *et al* 2017)
Pregnant women	Finland	↓ *Clostridium coccoides* group, *Lactobacillus-Enterococcus* group and *Actinobacteria* ↑ *Proteobacteria*	(Kumar *et al* 2015)
Adults (31-61 years old)	Finland	↓ *Bifidobacterium*	(Wacklin *et al* 2011, Wacklin *et al* 2014)
Adults (29-47 years old)	Spain	↑ *Prevotellaceae* and *Paraprevotellaceae*	(Rodriguez-Diaz *et al* 2017)
Adults (28-78 years old)	United States	↓ *Lachnospiraceae* ↑ *Bacteroides*	(Gampa *et al* 2017)

Microbial adaptation strategies for glycan uptake and utilisation

A large repertoire of microbial carbohydrate-active enzymes (CAZymes) is required to fully degrade milk and mucin glycans (Fig. 1). However, many species within the gut microbiota are better adapted for the metabolism of diet-derived carbohydrates (Turroni *et al* 2017). Hence, milk and mucin glycans provide a selective nutrient advantage for bacteria equipped with glycan-degrading capacity (Flint *et al* 2012a). The glycan-foraging trait is likely acquired by a group of resident bacteria as a result of adaptation to the mammalian gut environment (Pereira and Berry 2016). Bacterial glycosyl hydrolases (GH) hydrolyse the glycosidic linkages between carbohydrates to breakdown glycans into fermentable monosaccharides (Koropatkin *et al* 2012). Several extracellular functional catalysts of either membrane-bound or secreted enzymes are required, as only short glycans can be transported into the bacterial cell. For Gram positive bacteria the secreted enzymes are exported into the extracellular environment, whilst the secreted enzymes for Gram negative bacteria can be located either at the periplasm or the extracellular compartment (El Kaoutari *et al* 2013). In this section, the glycan uptake and catabolism of the representative intestinal members from five major microbial phyla including *Actinobacteria*, *Firmicutes*, *Bacteroides*, *Verrucomicrobia*, and *Proteobacteria* is briefly described. The CAZymes that can

degrade HMOS and mucins are predicted to scatter among the taxonomic groups but concentrated in the genome of specialised microbes (Fig. 2).

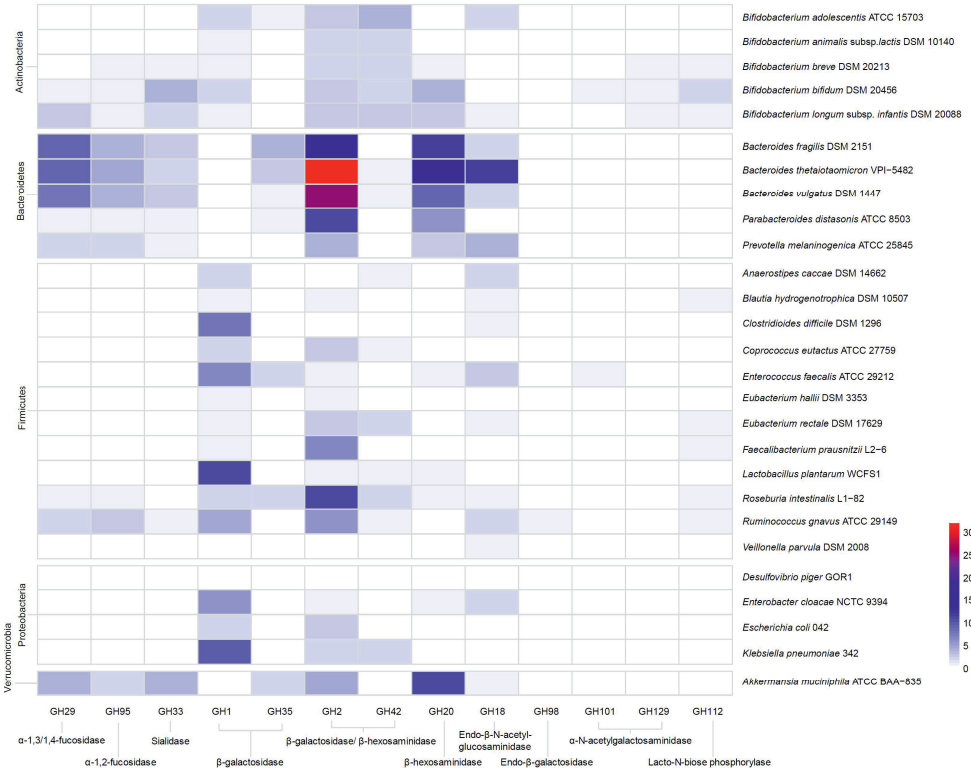


Figure 2. **Differential glycan-degrading capability among gut microbiota members.** Carbohydrate-active enzymes (CAZymes) involved in the catabolism of host-secreted glycans for representative species of the major bacterial phyla in the human gut. Colour key indicates the number of CAZymes predicted in the genome.

Bifidobacteria members from the phylum *Actinobacteria* often dominate the infant gut (Tannock *et al* 2016). *Bifidobacterium* species vary in their capability to metabolise host-secreted glycans. Infant-associated bifidobacteria including *Bifidobacterium longum* subsp. *infantis* (*Bifidobacterium infantis*), *Bifidobacterium breve*, and *Bifidobacterium bifidum* are adapted to utilise HMOS, but not the adult-associated species such as *Bifidobacterium adolescentis* (De Vuyst and Leroy 2011, Duranti *et al* 2016). *Bifidobacterium infantis*, a dominant member in the infant gut microbiota, is particularly effective in utilising HMOS. It employs a unique 43 kb gene

cluster that encodes enzymes required for HMOS internalisation including extracellular solute binding proteins and ATP-binding cassette (ABC) transporter systems, as well as enzymes for HMOS catabolism including fucosidase, sialidase, β-hexosaminidase, and β-galactosidase (Sela *et al* 2008). Moreover, different strategies are deployed by infant-associated bifidobacteria in utilising HMOS (Sela and Mills 2010a). Distinct modes of uptake and metabolism are demonstrated with the extracellular enzymatic digestion by *Bifidobacterium bifidum* and the internalisation of intact HMOS by *Bifidobacterium infantis* (Garrido *et al* 2013). This species-specific metabolic specialisation could contribute to the collective fitness of bifidobacteria via interspecies carbohydrate sharing (Egan *et al* 2014, Milani *et al* 2015, Turroni *et al* 2017). Despite the structural similarity between mucins and HMOS (Fig. 1), only some *Bifidobacterium* species, particularly *Bifidobacterium bifidum* strains can utilise mucins for growth (Marcobal *et al* 2013, Underwood *et al* 2014). Comparative genomics reveal that *Bifidobacterium bifidum* harbours an extensive range of CAZymes (including two distinct groups of carbohydrate-binding modules) required for mucin-degradation which are not detected in other members of bifidobacteria (Turroni *et al* 2011).

In addition to bifidobacteria, *Bacteroides* spp. from the *Bacteroidetes* phylum are often observed as dominant members of the infant gut microbiota (Backhed *et al* 2015, Lewis and Mills 2017). *Bacteroides* spp. are known to utilise a broad range of complex substrates including mucins and HMOS (Marcobal *et al* 2013). It harbours many paralogous of polysaccharides utilisation loci (PUL), that encode for the starch utilisation system (*sus*) comprised of several membrane-bound proteins and lipoproteins involved in substrate binding, degradation, and uptake (Martens *et al* 2009, Reeves *et al* 1997). One of the most well-studied gut symbionts *Bacteroides thetaiotaomicron* can breakdown most of the glycosidic linkages found in the human gut by employing its large repertoire of GHs (Xu *et al* 2004). The GHs are often organised in a modular manner adjacent to *Sus*C-like TonB-dependent transporters, *Sus*D-like outer membrane-binding proteins and sensor regulators such as hybrid two-component systems (HTCS) and extracytoplasmic function (ECF) σ-factors (Ravcheev *et al* 2013, Wexler 2007, Xu *et al* 2003). The sophisticated environmental sensing systems enable *Bacteroides thetaiotaomicron* to interpret the nutrient availability and fine-tune its carbohydrate-utilising capability (Xu *et al* 2004). The transcriptional response of *Bacteroides thetaiotaomicron* from the gut of suckling mice shows an

increase in gene expression levels for enzymes utilising monosaccharides and host glycans present in mother's milk, while a switch towards genes involved in the breakdown of plant-derived polysaccharides is observed after weaning (Bjursell *et al* 2006).

Upon weaning, members of *Bacteroidetes* and *Firmicutes* phyla outnumber the bifidobacteria community (Laursen *et al* 2017). Two families of *Firmicutes* i.e. *Lachnospiraceae* and *Ruminococcaceae*, are present at high numbers in the adult gut and have the key metabolic capacity to produce butyrate and propionate (Flint *et al* 2012a, Louis and Flint 2017). The degradation of complex carbohydrates by *Firmicutes* involves a modular enzyme system encoded by gene loci resembling the PUL in the Gram-negative *Bacteroides*, namely Gram-positive PUL (Sheridan *et al* 2016). Gram-positive PUL encode for an assortment of polysaccharide-degrading enzymes, carbohydrate transport systems and transcriptional regulators (Sheridan *et al* 2016). The majority of bacteria from the *Firmicutes* phylum cannot utilise milk and mucin glycans (Lopez-Siles *et al* 2012, Marcobal *et al* 2010, Martin *et al* 2017, Sheridan *et al* 2016) except some strains of *Ruminococcus gnavus* and *Ruminococcus torques* (Crost *et al* 2013, Hoskins 1993). However, *Lachnospiraceae* and *Ruminococcaceae* are observed to be enriched at the mucosal environment suggesting metabolic interaction with the mucus-degrading species (Nava *et al* 2011, Van den Abbeele *et al* 2013).

The symbiont *Akkermansia muciniphila* is the only cultured representative of the *Verrucomicrobia* phylum from the human intestine (de Vos 2017). *Akkermansia muciniphila* has adapted to the gut mucosal environment by using mucins as the sole carbon and nitrogen source (Derrien *et al* 2004). Its relatively small genome (2.6 MB) contains high proportion of genes (11%) to encode GHs, proteases and sulfatases required for mucins degradation (Belzer and de Vos 2012, van Passel *et al* 2011). The mucin-foraging lifestyle is shown by the growth of this bacterium in defined minimal medium supplemented with mucin-derived components i.e. L-threonine and either GlcNAc or GalNAc (van der Ark *et al* 2018). The dependency on mucins as growth substrate is also demonstrated in an *in vitro* gut model (SHIME) with over 4 log increase of *Akkermansia muciniphila* upon mucin addition (Van Herreweghen *et al* 2017). Despite the high mucolytic activity of *Akkermansia muciniphila*, it is not known to degrade or utilise HMOS. The genetic signature of *Akkermansia muciniphila* has

been detected in human breast tissue and colostrum (Aakko *et al* 2017, Urbaniak *et al* 2014). This bacterium is speculated to employ the mucin-degrading machinery to metabolise HMOS and other human milk components in order to facilitate its passage through the gastrointestinal tract for the infant gut colonisation (de Vos 2017, Ottman 2015). Low levels of *Akkermansia muciniphila* are detected in 1 month old infant faeces, with its relative abundance increases to adult levels after 1 year of age (Collado *et al* 2007). The high relative abundance of *Akkermansia muciniphila* is often associated with a healthy state in adults, which might indicate the importance of early colonisation with this bacterium. (Derrien *et al* 2016, Png *et al* 2010).

Low levels of *Proteobacteria* are generally found in the faeces of healthy adults and an outgrowth of species from *Enterobacteriaceae* family is associated with gut dysbiosis (Trosvik *et al* 2015, Winter and Baumler 2014). Besides, *Proteobacteria* are enriched in unstable ecosystems with low microbial diversity, for example the gut of new-borns, obese individuals, individuals suffering from intestinal inflammation, and those who have undergone antibiotic treatment and gastric bypass surgery (Fei and Zhao 2013, Hill *et al* 2010, Jakobsson *et al* 2014, Liou *et al* 2013, Morgan *et al* 2012, Shin *et al* 2015). Most of the *Proteobacteria* members are not capable of degrading complex milk and mucin glycans but rely on the of mono- and disaccharides for their nutrition (Kamada *et al* 2013). Therefore, in a glycan-rich niche, this bacterial group is often dependent on the simple sugars liberated from other microbiota members (Sicard *et al* 2017). Other than serving as a carbon source, the terminal fucose and sialic acid at mucin glycans are potential attachment targets for foodborne pathogens that belong to the *Proteobacteria* phylum such as *Salmonellae enterica* serotype Typhimurium (*Salmonella* Typhimurium) and *Helicobacter pylori* (Aspholm *et al* 2006, Chessa *et al* 2009).

The differential glycan utilisation of gut bacteria can be exploited to devise strategies to modulate the gut microbiota. As such, gut symbionts can be selectively promoted by introducing complex carbohydrate that confer growth advantage, in order to suppress the bloom of *Proteobacteria* via competitive exclusion. Several mechanisms of exclusion are proposed including competition among bacteria for nutrients and mucosal adhesion sites as well as the modification of chemical environment to inhibit the growth of pathogens via production of acids and antimicrobial compounds (Collado *et al* 2010).

Glycan-degraders as keystone species for microbial network formation

An environment enriched in milk or mucin glycans provides a selective nutrient source for the colonisation of glycan-degrading microbes. This is particularly apparent in the gut of breast-fed infants, and at the gastrointestinal lining that is covered with mucins. Cooperative behaviour is often observed among symbiotic gut bacteria by exploiting the complementary glycan-degrading capabilities of each other (Tuncil *et al* 2017, Turroni *et al* 2017). The primary glycan-degrading bacteria could shape the local bacterial community and functionality by cross-feeding. In essence, the glycan-degraders alter the chemical environment by converting glycans into simple carbohydrates and metabolites to sustain the growth and metabolism of bacterial community in their vicinity. The metabolic products or nutrients (such as co-factors, vitamins and amino acids) produced by one microbial species could support the growth of another microbe, often resulting in indirect benefits for all species involved (Box 1. Proposal for a definition of cross-feeding). Furthermore, the reciprocal exchange of metabolites among two or more microorganisms could drive biochemical functions that neither of them can perform alone (namely syntrophic interaction) (Morris *et al* 2013). On the other hand, antagonistic interactions are also taking place, for example the competition for limiting resources or the release of toxic compounds (Foster and Bell 2012, Fuller and Gibson 1997).

Box 1. Proposal for a definition of cross-feeding

The metabolic products or nutrients produced by one microbial species support growth of another microbe, often resulting in indirect benefits for all species involved.

Nutrient cross-feeding – the production of a molecule such as a vitamin or amino acid that is used by both the producing organism and other microbes in the environment that relaxes the metabolic burden on anyone microbe in the community (Seth and Taga 2014).

Cooperative interactions are typically unidirectional with no obvious benefit to the giver. However, within their natural communities, bacteria typically form close cooperative loops resulting in indirect benefit to all species involved (Freilich et al 2011).

Metabolic products produced from dietary prebiotics by one bacterial species may then provide substrates to support growth of other populations (Belenguer et al 2006).

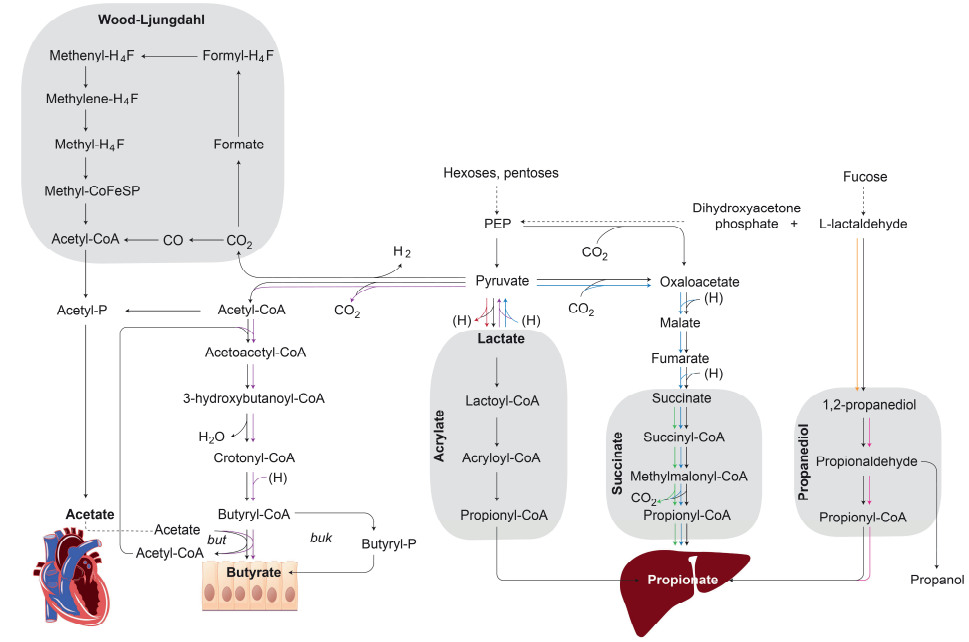
Intermediate carbohydrate breakdown products and certain fermentation products serve as carbon and energy sources for cross-feeding bacteria (Duncan et al 2004).

The catabolism of milk and mucin glycans by the degrader species can result in the release of free sugars and/or oligosaccharides into the environment. Subsequently,

other microbes in the vicinity could cross-feed on the simpler sugars for their metabolism and growth. The host-specific sugars i.e. fucose and sialic acid are shown to assist the colonisation of both commensal (Autieri *et al* 2007, Hooper *et al* 1999) and pathogenic bacteria (Ng *et al* 2013), and are involved in the regulation of bacterial pathogenicity (Pacheco *et al* 2012). Gut symbionts such as *Akkermansia muciniphila* and *Bifidobacterium infantis* can metabolise fucose to produce 1,2-propanediol (Bunesova *et al* 2016, Ottman *et al* 2017a, Reichardt *et al* 2014). Besides, fucose released by *Bacteroides thetaiotaomicron* from mucins, increases the colonisation of enterohemorrhagic *Escherichia coli* (EHEC) and represses its virulence (Pacheco *et al* 2012). Cross-feeding on sialic acid is observed between gut symbionts in which *Bifidobacterium breve* UCC2003 benefits from sialic acid released by *Bifidobacterium bifidum* PRL2010 from HMOS degradation (Egan *et al* 2014). On the other hand, free sialic acid released by *Bacteroides vulgatus* induces the outgrowth of *Escherichia coli* during inflammation (Huang *et al* 2015). Furthermore, an increase level of free sialic acid liberated by *Bacteroides thetaiotaomicron* after antibiotic treatment in mice facilitates the expansion of pathogenic *Salmonella* Typhimurium and *Clostridium difficile* (Ng *et al* 2013). Hence, the availability of fucose and sialic acid can be particularly important to maintain the symbiotic relationship between the gut microbiota and human host (Pickard and Chervonsky 2015, Tailford *et al* 2015).

The metabolic by-products of glycan degradation from milk and mucins, such as acetate, lactate, succinate, and 1,2-propanediol can be utilised by other members of the gut microbiota (Fig. 3). Further modification of these fermentation intermediates contributes to the SCFA composition in the gut (faecal samples are frequently studied as a proxy for the large intestine). Acetate and lactate as well as a small amount of propionate and butyrate can be detected in the faeces of infants (Fig. 4) (Pham *et al* 2016, Wopereis *et al* 2017). In adulthood, lactate is often converted to butyrate by the lactate-utilising butyrate-producing bacteria (LUB) resulting in a faecal SCFA composition ratio of 3:1:1 for acetate, propionate and butyrate respectively (Schwiertz *et al* 2010, Scott *et al* 2011). The intestinal SCFAs can contribute to the host caloric requirement as around 90% is re-absorbed in the large intestine (Backhed *et al* 2004, Wong *et al* 2006). Butyrate is consumed locally by the colonic epithelium as the preferred energy source (Donohoe *et al* 2011). Whilst, the other absorbed SCFAs

travel through the portal vein with propionate mostly metabolised in the liver and acetate reaching the systemic circulation (Fig. 3) (Cummings *et al* 1987).



Pathway

Species	Wood-Ljungdahl	*but*	*buk*	Acrylate	Succinate	Propanediol
	Blautia hydrogenotrophica	*Anaerostipes* spp.	*Coprococcus eutactus*	*Bifidobacterium* spp.	*Dialister succinatiphilus*	*Bifidobacterium infantis*
	Marvinbryantia formatexigens	*Eubacterium hallii*	*Coprococcus comes*	*Bacteroides* spp.	*Phascolarctobacterium succinatutens*	*Akkermansia muciniphila*
	Clostridium spp.	*Faecalibacterium prausnitzii*		*Lactobacillus* spp.	*Akkermansia muciniphila*	*Roseburia inulinivorans*
	Stroptococcus spp.	*Eubacterium rectale*		*Stroptococcus* spp.	*Bactoroides* spp.	*Blautia wexleri*
		Coprococcus catus		*Enterococcus* spp.	*Veillonella* spp.	*Ruminococcus obeum*
		Roseburia spp.		*Coprococcus catus*		*Eubacterium hallii*
				Megasphaera elsdenii		

Figure 3. **The major pathways for intestinal SCFA biosynthesis and routes of bacterial cross-feeding.** The representative species involving in the pathways are listed**.** Species shown in: purple can convert lactate to butyrate, red can convert pyruvate to lactate, green can convert succinate to propionate, blue can convert lactate to propionate, orange can convert fucose to 1,2-propanediol and pink can convert 1,2-propanediol to propionate. Butyrate can also be synthesized from proteins via glutarate, 4-aminobutyrate and lysine pathways (Vital *et al* 2017). Around 90% of the bacterial-derived SCFAs are absorbed in the gut. Butyrate is consumed locally by the colonic epithelium as the preferred energy source. Other absorbed SCFAs travel through the portal vein with propionate mostly metabolised in the liver and acetate reaching the systemic circulation. Figure is modified from Flint *et al.* and Koh *et al.* (Flint *et al* 2014, Koh *et al* 2016). *Abbreviations*: PEP, phosphoenolpyruvate; *but*, butyryl-CoA: acetate CoA transferase; *buk*, butyrate kinase.

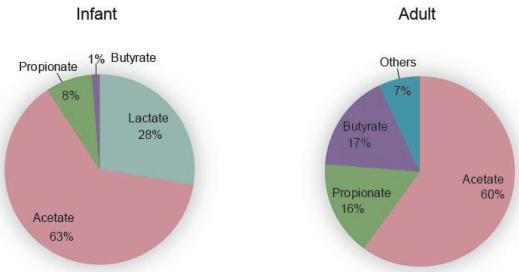


Figure 4. **Faecal SCFA composition differs between the gut of infants and adults.** The faecal SCFAs composition for 3 months old infants and adults is derived from Pham *et al.* and Schwiertz *et al*. (Pham *et al* 2016, Schwiertz *et al* 2010). Others in adult include iso-butyrate, iso-valerate, and valerate, which are mainly the products of protein fermentation.

The composition of SCFAs directly affects the ecophysiology of the gut environment by influencing the pH, transit time as well as the composition and functionality of the microbiota (Flint *et al* 2014). On top of this, SCFAs are involved in the regulation of host immune and metabolic response by acting as major signalling molecules for specific receptors in the gut (Koh *et al* 2016). Luminal SCFAs can stimulate G-protein-coupled receptor (GPR, also known as fatty acid receptor [FFAR]) triggering different downstream functions depending on cellular type (Tremaroli and Backhed 2012). In general, the activation of GPR41 (or FFAR3) and GPR43 (or FFAR2) releases peptide YY and glucagon-like peptide-1 (GLP-1), which affect host satiety and intestinal gluconeogenesis. Besides, SCFAs signalling through GPR109A exerts anti-inflammatory and anti-tumorigenic responses (Macia *et al* 2015). Butyrate and propionate to a lesser extent, can also be involved in the epigenetic regulation of host gene expression by inhibiting histone deacetylase (HDAC) (Fellows *et al* 2018). Furthermore, SCFAs reaching the systemic circulation, mostly acetate and a small amount of propionate, can induce beneficial metabolic effects on organs like lung, adipose tissue, brain, and liver (Koh *et al* 2016).

The intricate balance of keeping the mutualistic relationship between the gut microbiota and the host is important for health (Faust and Raes 2012). On the one hand, the intestinal bacteria contribute to host health by providing macro-and micro nutrients such as simple carbohydrates e.g. lactose (the core of the HMOS), amino

acids, and vitamins; the inhibition of pathogens by competitive exclusion; maintenance of gut immune function; maintenance of normal gut motility; and the prevention of cancer and cardiovascular disease (Brennan and Garrett 2016, Honda and Littman 2016, Kamada *et al* 2013, Quigley 2011, Rowland *et al* 2018, Tang *et al* 2017). On the other hand, an aberrant gut microbiota is associated with diseases including cardiovascular diseases, metabolic syndrome, non-alcoholic fatty liver diseases, obesity, type 2 diabetes, and inflammatory bowel diseases (de Vos and de Vos 2012). The understanding on how host-secreted glycans such as those from milk and mucus affect the microbiota composition and function could aid the development of novel therapeutic strategies via nutritional intervention that could support health. For instance, current research is investigating the feasibility to deliver the beneficial effects of human milk by supplementing infant formula with some structures of HMOS (Elison *et al* 2016, Goehring *et al* 2016, Marriage *et al* 2015, Puccio *et al* 2017).

Microbial networks leading to butyrate production

Intriguingly, the level of butyrate in the gut increases with age (Fig. 4). This metabolic outcome can be partially explained by the composition of the gut microbiota. Butyrate-producing bacteria are mainly belonging to the *Firmicutes* phylum, from the families of *Ruminococcaceae* and *Lachnospiraceae* (Louis and Flint 2017). Butyrogens are detected at low levels early in life (de Weerth *et al* 2013, Jost *et al* 2012). However, their abundancy increases during the first years of life reaching levels comparable to that of adults at around 3 years of age (Backhed *et al* 2015, Yatsunenko *et al* 2012). In healthy adults, the butyrate-producing bacteria comprise 10 to 20% of the total gut microbiota of which *Faecalibacterium* spp. (belonging to *Clostridium* cluster *IV* within *Ruminococcaceae* family), and *Anaerostipes* spp./ *Eubacterium* spp./ *Roseburia* spp. (belonging to *Clostridium* cluster *XIVa* within *Lachnospiraceae* family) are the prevalent genera (Arumugam *et al* 2011). The gradual increase of the butyrate levels and the emergence of the butyrogenic community in the gut could be important for gut maturation, as aberrant microbial composition and/or SCFA production is 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).

Despite the physiological importance of the butyrogenic community in the gut of breast-fed infants and the mucosal layer, most of the butyrate-producing bacteria are not able to degrade host-derived glycans (Sheridan *et al* 2016). Hence, the occurrence of the butyrogens could be sustained by the metabolic interdependencies with the primary glycan-degrading microbes. *In vitro* experiments showed that the butyrate-producing bacteria could metabolise sugars and intermediates such as lactate and acetate liberated by the microbial species from the breakdown of complex carbohydrates (Belenguer *et al* 2006, Chassard and Bernalier-Donadille 2006, De Vuyst and Leroy 2011, Louis and Flint 2009, Marquet *et al* 2009). For instance, *Eubacterium hallii* and *Anaerostipes caccae* were shown to produce butyrate by utilising bifidobacteria-derived lactate from dietary component such as starch and fructo-oligosaccharides (FOS) degradation (Belenguer *et al* 2006, De Vuyst and Leroy 2011, Falony *et al* 2006). Recent studies indicated that host-secreted glycans i.e. 2'-fucosyllactose (2'-FL, a HMOS) and mucins could also drive a similar cross-feeding interaction between *Bifidobacterium* spp. and *Eubacterium hallii* (Schwab *et al* 2017). Other butyrogens i.e. *Faecalibacterium prausnitzii*, *Eubacterium rectale* and *Roseburia* spp., on the other hand, are predicted to consume acetate but not lactate for butyrate production (Duncan *et al* 2002a, Duncan *et al* 2006, Heinken *et al* 2014, Louis and Flint 2009). This thesis sets forth to further unravel the metabolic interdependencies between the first-tier glycan-degraders and the second-tier butyrate-producing bacteria in the infant gut and at the mucosal niche.

Aim and thesis outline

The co-evolution between gut microbes and the human host has refined the capability of some resident bacteria to utilise host (milk and mucin) glycans. These glycan-foraging microbes could subsequently drive the microbial network via cross-feeding. The resulting glycan-enriched subpopulation could exert a disproportionally large influence on the host physiology, by influencing the immune, metabolic, and neurological development early in life, and by conferring colonisation resistance throughout life. Therefore, this thesis focuses on unravelling the interaction of gut symbionts in utilising HMOS or mucins, specifically on the cross-feeding between the key degrader species and butyrate-producing bacteria. We hypothesise that host-secreted glycan degradation by the microbiota members could support the growth and activity of butyrogens. A reductionist approach using anaerobic culturing in minimal environment was used to facilitate the understanding of the complex metabolic interactions in the gut ecosystem. It is crucial to understand the molecular link between host-secreted glycans and key degrader species as well as the subsequent microbial interactions in order to better understand the drivers of gut microbial ecology, which can help in the design of effective nutrition strategies targeting the gut microbiota.

Chapter 2 investigates the trophic interaction between a HMOS-degrader, *Bifidobacterium infantis* (*Actinobacteria*) and a butyrogenic non-HMOS-degrader, *Anaerostipes caccae* (a member of the *Lachnospiraceae* from the *Firmicutes* phylum) in human milk carbohydrates. *Anaerostipes caccae* was not able to metabolise lactose and HMOS but the presence of *Bifidobacterium infantis* supported its growth and butyrate production. This points towards the key ecological role of bifidobacteria in providing substrates for other important species in the infant gut. The gradual shift of the microbiota composition in the ecosystem contributing to the slow induction of butyrate could be important for gut maturation.

Chapter 3 investigates the microbial network formation in the infant gut driven by another glycan-degrader, namely *Bacteroides thetaiotaomicron*. *Bacteroides thetaiotaomicron* could also drive the butyrogenic trophic chain by metabolising lactose and HMOS to support the growth of *Anaerostipes caccae*. This indicates that *Bacteroides thetaiotaomicron* could drive the establishment of the microbial network in the infant gut, leading to the sequential establishment of adult-like functional groups

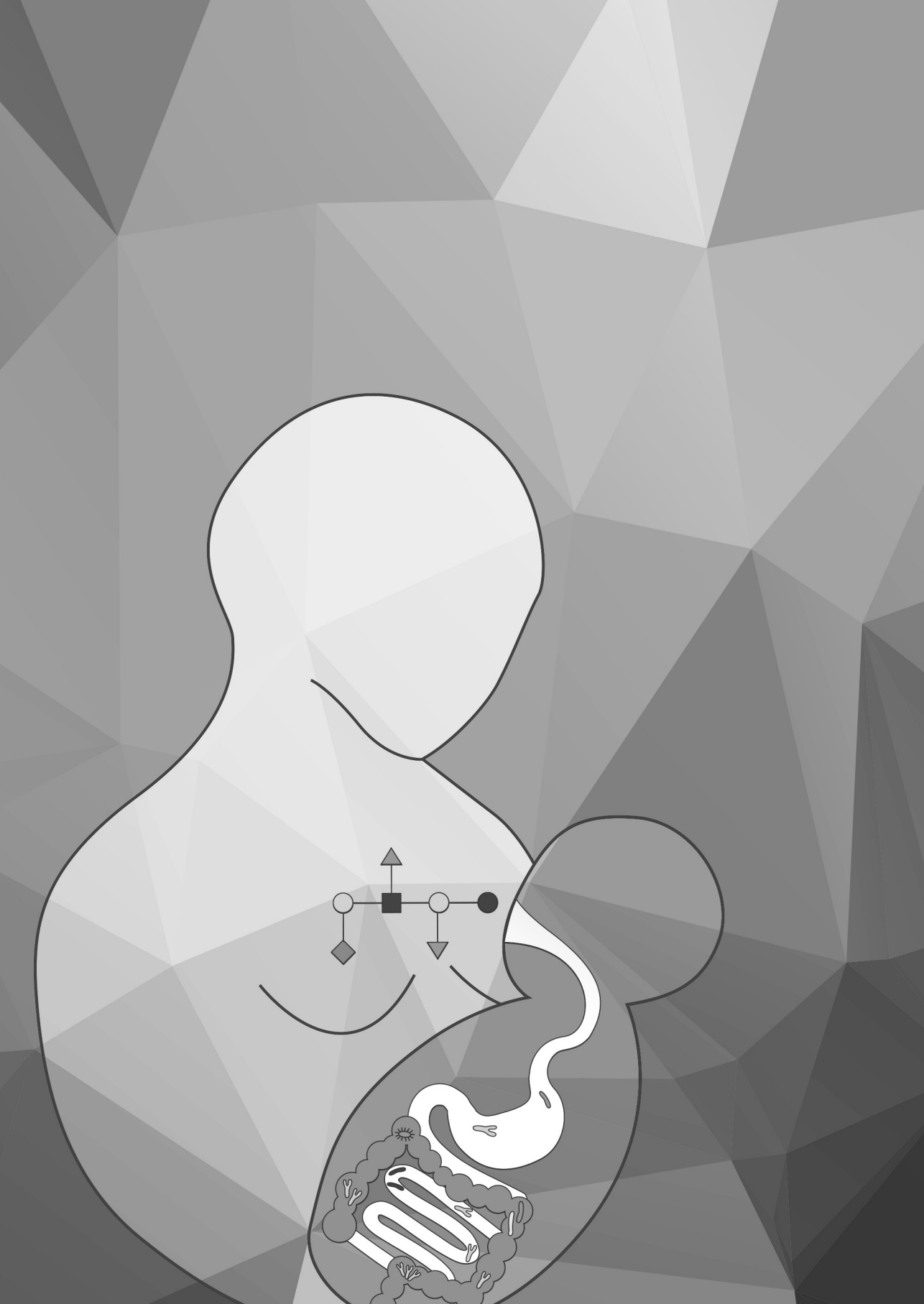
such as lactate-utilising and butyrate-producing bacteria. Furthermore, we observed stereospecific lactate isomer production in which *Bacteroides* spp. and *Bifidobacterium* spp. produced predominantly D- and L-lactate, respectively. The distinct lactate isomer production by these major glycan-degrading genera might affect the gut microbiota compositions by differential cross-feeding interaction with the specific D- and L-lactate-utilisers.

Chapter 4 studies the microbial metabolic network at the mucosal layer. The complex mucin glycans presents as a selective substrate to stimulate the dominance of *Akkermansia muciniphila*. The subsequent metabolite exchange promotes the growth and butyrate production of the butyrogens including *Anaerostipes caccae*, *Eubacterium hallii*, and *Faecalibacterium prausnitzii*. Interestingly, a bidirectional cross-feeding was observed between *Akkermansia muciniphila* and *Eubacterium hallii.* Pseudo-vitamin B12 produced by *Eubacterium hallii* facilitated propionate production by *Akkermansia muciniphila* via the methylmalonyl-CoA pathway. Propionate could be beneficial to the human host by regulating satiety and lipid biosynthesis in the liver, indicative of a mutualistic host-microbial interaction driven by mucin glycans. The beneficial microbial network at the mucosal layer could confer colonisation resistance to the host against pathogens.

Chapter 5 further investigates the mechanism of trophic interactions between *Akkermansia muciniphila* and *Anaerostipes caccae* by using a metatranscriptomic approach. Metatranscriptomics is employed to understand the transcriptional changes of a bacterium in response to environmental stimuli. We observed that *Akkermansia muciniphila* behaves differently when it is grown in monoculture compared to growth in co-culture with *Anaerostipes caccae*. In particularly, *Akkermansia muciniphila* increased the expression of the extracellular mucin-degrading enzymes in the co-culture. This inferred that *Akkermansia muciniphila* increased its mucolytic activity to support the growth and activity of the mucosal bacteria.

Chapter 6 provides a general discussion by summarising the insights generated within this thesis with regards to the butyrogenic microbial network and putting the knowledge into a broader ecological context. The understanding of the molecular mechanisms may contribute to the design of novel nutritional approaches to modulate the gut microbiota. As such, the concept of next generation gut modulator encompassed of

novel probiotic strains (key species including *Akkermansia muciniphila*, *Bacteroides* spp. and butyrate-producing *Clostridium*), prebiotics (HMOS in early nutrition), and nutrients (iron and vitamin B12) is put forward.



Chapter 2

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

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Submitted

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 promote the growth of gut microbes particularly the group of *Actinobacteria* (bifidobacteria). This is shifted to the colonisation of *Firmicutes* and *Bacteroidetes*, which generally dominate the human gut throughout adulthood. The well-orchestrated transition is important for health as an aberrant microbial composition and/or SCFA production for example are associated with colicky symptoms and atopic diseases in infants. Here, we study the trophic interactions between a HMOS-degrader, *Bifidobacterium infantis* and the butyrogenic *Anaerostipes caccae* using carbohydrate substrates that are relevant in this early life period i.e. 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. *Anaerostipes caccae* was unable to grow on these substrates except when grown in co-culture with *Bifidobacterium infantis*, leading to growth and concomitant butyrate production. Two levels of cross-feeding were observed, in which *Anaerostipes caccae* utilised the liberated monosaccharides as well as lactate and acetate produced by *Bifidobacterium 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 gradual shift of the microbiota composition in the ecosystem contributing to the gradual production of butyrate could be important for host-microbial cross talk and gut maturation.

Keywords

Bifidobacteria, butyrate, human milk oligosaccharides, lactose, *Lachnospiraceae*, microbiome, pH

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 among 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 HMOS-utilising microbes, especially bifidobacteria belonging to the *Actinobacteria* phylum (Marcobal *et al* 2010). Bifidobacteria are specifically adapted to utilise HMOS by employing an extensive range of glycosyl hydrolases and transporters, which lead to their dominance in the infant gut (Sela and Mills 2010b). 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 often presents 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 colonisation by 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 atopic 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 HMOS-degrader, *Bifidobacterium infantis* and a butyrogenic non-degrader. To this end the butyrate-producer *Anaerostipes caccae* was used as the representative species for the *Lachnospiraceae* family as it is detected in the early life gut microbiota (Backhed *et al* 2015, Yatsunenko *et al* 2012) and is one of the prevalent members of the gut microbiota in human adults (Arumugam *et al* 2011). *Bifidobacterium infantis* supports the development of the microbial ecosystem by metabolising complex carbohydrates into monosaccharides and short chain fatty acid (SCFA) including lactate and acetate, to support the growth and concomitant butyrate production by *Anaerostipes caccae.* This butyrogenic cross-feeding demonstrates the importance of bifidobacteria in the establishment of a healthy microbial ecosystem in early life.

Materials and Methods

16S rRNA gene amplicon libraries screen. 16S rRNA gene amplicon sequencing datasets published by Yatsunenko *et al.* (Yatsunenko *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 (RDP) (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_2/CO_2 (80/20 ratio) at 1.5 atm. Pre-cultures were prepared by overnight 37ºC incubation in basal minimal medium (Plugge 2005) containing 0.5% (w/v) tryptone (Oxoid, Basingstoke, UK), supplemented with 30mM lactose (Oxoid, Basingstoke, UK) for *Bifidobacterium longum* subsp. *infantis* ATCC15697; and 30 mM glucose (Sigma-Aldrich, St. Louis, 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™, IMPLEN, Germany).

Carbohydrate substrates. Lactose (Oxoid, Basingstoke, UK) and total human milk (HM) carbohydrates were tested as the carbohydrate substrates for bacterial growth. 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.* (Stahl *et al* 1994) . Deviant from this workflow, no anion exchange chromatography (AEC) 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 (Fig. S1).

Anaerobic bioreactor. Fermentations were conducted in eight parallel minispinner bioreactors (DASGIP, 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, Basingstoke, UK), supplemented with 0.2 µM filter-sterilized lactose or total HM carbohydrates. Anaerobic condition was achieved by overnight purging of anaerobic gas mixture containing 5% CO_2, 5% H_2, and 90% N_2. Overnight pre-cultures were inoculated at starting OD600 of 0.05 for each bacterial strain. Online signals of pH values and oxygen levels were monitored by the DASGIP control software (DASGIP, Germany). Cultures were maintained at pH 6.5 by the addition of 2 M NaOH.

Gel permeation chromatography (GPC). 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) containing 2% (v/v) 2-propanol at 37^0C. 5 ml of 0.2 µM filter-sterilized 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) packed with Toyopearl HW 40 (TOSOH BIOSCIENCE) were used. Milli-Q water was maintained at 50°C using heating bath (Lauda, RE 206) for columns equilibration. Milli-Q 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). The resulting chromatograms were analysed by using the Chromeleon® software (ThermoScientific 6.80).

High-performance liquid chromatography (HPLC). For metabolites analysis, 1 ml of bacterial culture was centrifuged and the supernatant was stored at -20°C until 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 conversion and product formation 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, Breda, the Netherlands).

HMOS extraction. HMOS were recovered from 1 ml aliquots of bacterial cultures. Internal standard 1,5-α-L-arabinopentaose (Megazyme) was added, at the volume of 10 µl per sample to minimize 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 Milipore) at 14,000 g for 1 h at room temperature. Finally, the filtrate was vortexed and stored at -20°C until further electrospray ionisation liquid chromatography mass spectrometry (ESI-LC-MS) analysis.

Electrospray ionisation liquid chromatography mass spectrometry (ESI-LC-MS) analysis. The identification and relative quantitation of HMOS were determined with ESI-LC-MS. This method allowed the study of distinct HMOS structures differed 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. Micro ESI-LC-MS analysis was performed on a 1200 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, 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 2.1x30 mm Hypercarb porous graphitized carbon (PGC) column with 2.1x10 mm PGC pre-column (Thermo Scientific, USA) using water-acetonitrile gradient for 18 min protocol. The gradient started with a ratio of 98% (v/v) water and 2% (v/v) acetonitrile in 5 mM ammonium acetate at 0 min and ended with a ratio of 20% (v/v) water and 80% (v/v) acetonitrile in 5 mM ammonium acetate at 15 min. Re-equilibration

was established between 15 and 18 min with 98% (v/v) water and 2% (v/v) acetonitrile in 5 mM ammonium acetate. Eluent flow was 400 µl/min and the columns were kept at 45^0C. 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 -4500 V, declustering potential was at 44 V, and collision energy was set to 29 eV. Each MRM-transition was performed for 50 ms. The instrument was calibrated with polypropylene glycol (PPG) 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 (q-PCR). The abundance of *Bifidobacterium infantis* and *Anaerostipes 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. The DNA concentrations were determined fluorometrically (Qubit dsDNA HS assay; Invitrogen) 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 *Anaerostipes 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 *Bifidobacterium infantis*; and 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R for *Anaerostipes caccae*. Standard curves were prepared with nine standard concentrations of 10^0 to 10^8 gene copies/µl. PCRs were performed in triplicate with iQ SYBR Green Supermix (Bio-Rad) 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* hybridization (FISH): 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 5 min of 4°C centrifugation at 8,000 g, 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. Hybridization was performed using rRNA-targeted oligonucleotide probes specific for *Bifidobacterium* genus (Bif164m 5'-CATCCGGYATTACCACCC -3' [5']Cy3) (Dinoto *et al* 2006). 10 µl of hybridization mixture containing 1 volume of 10 µM probe and 9 volumes of hybridization buffer (20 mM Tris–HCl, 0.9 M NaCl, 0.1% SDS, pH 7.2 – pH 7.4) was applied on each well. The slides were hybridized 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 – pH 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 and a coverslip. The slides were enumerated using an Olympus MT ARC/HG epifluorescence microscope. 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 (CAZymes) prediction. 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).

Results

The occurrence of *Bifidobacterium infantis* and *Anaerostipes caccae* across the life span

A published dataset (Yatsunenko *et al* 2012) was mined for the occurrence of *Bifidobacterium infantis* and *Anaerostipes caccae* in the microbiota across life stages. 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 ρ = -0.38, $p < 0.05$) (Fig. 1). On the contrary, *Anaerostipes* genus (Spearman ρ = 0.56, $p < 0.05$) and *Lachnospiraceae* family (Spearman ρ = 0.37, $p < 0.05$) were present at low abundance early in life and increased in relative abundance during the aging process (Fig. 1).

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

Bacteria strains were cultured in anaerobic bioreactors controlled at pH 6.5 supplemented with either lactose or total human milk (HM) carbohydrates. *Bifidobacterium infantis* monoculture reached maximal cell density around 12 h (OD_{max} = 1.40 ± 0.38 in lactose and OD_{max} = 1.37 ± 0.25 in total HM carbohydrates) (Fig. 2). For *Anaerostipes caccae* monoculture, no growth or substrate breakdown was detected in identical media (OD_{max} = 0.02 ± 0.01 in lactose and OD_{max} = 0.03 ± 0.02 in total HM carbohydrates) (Table S1). The co-culture of *Bifidobacterium infantis* with *Anaerostipes caccae* grew rapidly reaching maximal optical density at 11 h in lactose (OD_{max} = 3.63 ± 0.61) and at 9 h in total HM carbohydrates (OD_{max} = 3.54 ± 0.60). The community dynamics in the co-cultures was monitored over time by qPCR. An equal amount of *Bifidobacterium infantis* and *Anaerostipes caccae* (around 10^6 copy number/ml) was inoculated at the start of the fermentation. During the first 7 h, *Bifidobacterium infantis* and *Anaerostipes caccae* increased 100-fold based on the increase of 16S rRNA gene copy number, after which growth slowed down. FISH analysis of samples harvested at 11 h showed *Bifidobacterium infantis* to *Anaerostipes caccae* ratio of 1:6. These results accounted for both conditions either in lactose or total HM carbohydrates supplemented cultures.

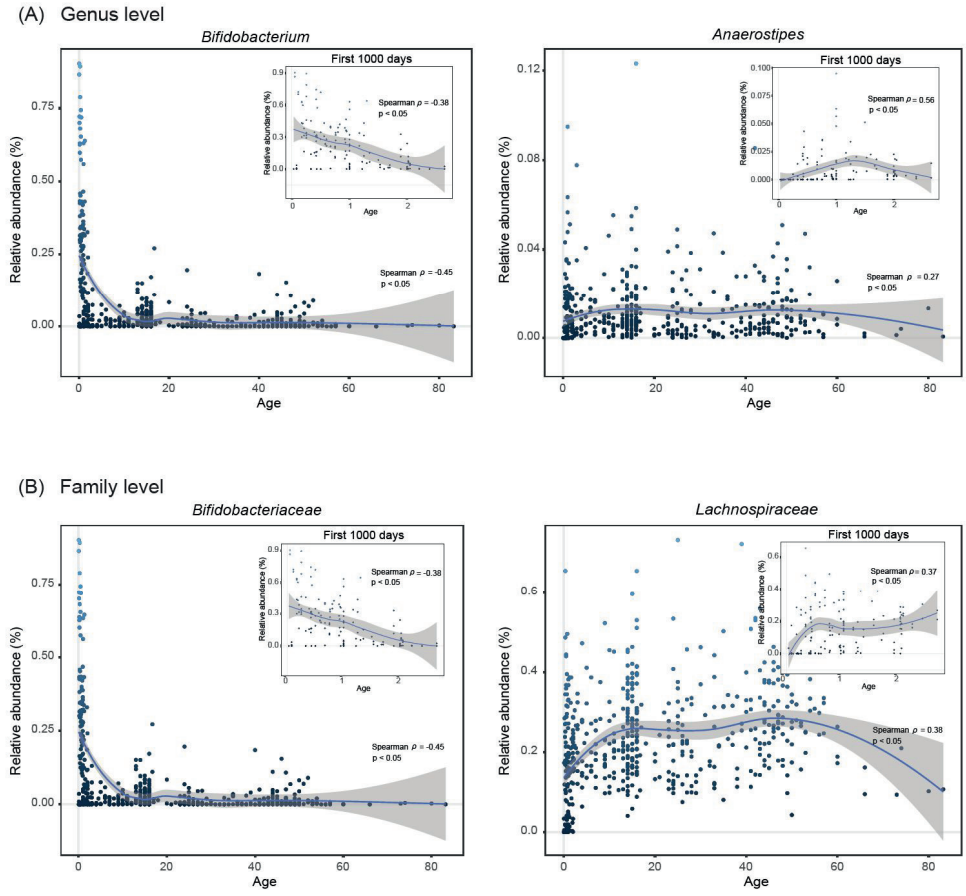


Figure 1. **The occurrence of (A) *Bifidobacterium* and *Anaerostipes* genus (B) *Bifidobacteriaceae* and *Lachnospiraceae* family in the gut microbiota across age.** The plot was generated from a published dataset (Yatsunenko *et al* 2012) using R package ggplot2 version 2.2.1. The trend lines represent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

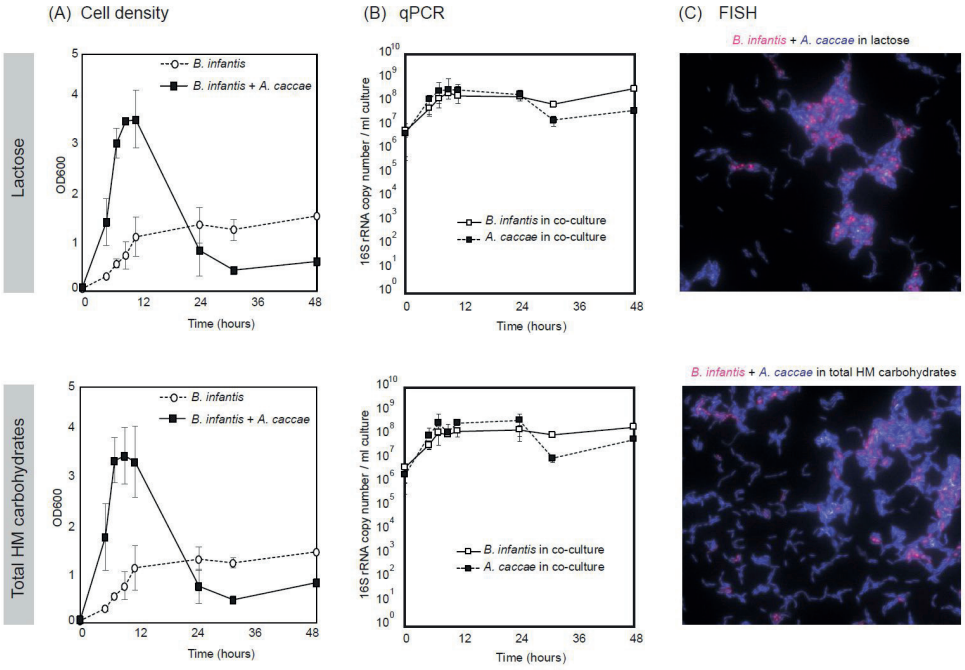


Figure 2. ***Bifidobacterium infantis*** **supported the growth of** ***Anaerostipes caccae*** **in human milk carbohydrates.** (A) The optical density (OD600) indicating bacterial growth and (B) qPCR results showing the microbial composition in the co-cultures over time with lactose or with total HM carbohydrates. Error bars represent the standard deviation for biological triplicates, except for time point 31 h (n=2) and 48 h (n=1). (C) Fluorescent *in situ* hybridisation (FISH) of co-cultures at 11h (*Bifidobacterium infantis* in pink and *Anaerostipes caccae* in purple). No growth or substrate utilisation was detected for *Anaerostipes caccae* monocultures in identical media (Table S1).

Bifidobacterium infantis supported the growth and metabolism of *Anaerostipes caccae* in lactose and HMOS

The substrates consumption and SCFA production were monitored over time (Fig. 3). A similar profile was observed between the fermentation of lactose and total HM carbohydrates, probably because total HM carbohydrates is consisted of approximately 10% HMOS and 90% lactose (Fig. S1). On both substrates, the monoculture of *Bifidobacterium infantis* degraded the lactose present into glucose and galactose resulting in the accumulation of monomeric sugars in the supernatant (Fig. 3). Lactose was completely degraded at 9 h. At the same time point, 17.49 ± 1.83 mM of glucose and 15.24 ± 2.06 mM of galactose were detected in the media supplemented with lactose, whereas 14.77 ± 1.59 mM of glucose and 10.91 ± 1.77 mM of galactose were detected in the media supplemented with total HM carbohydrates. The monomeric sugars were fully consumed after 31 h. *Bifidobacterium infantis* produced acetate (56.96 ± 4.48 mM in lactose and 50.76 ± 3.23 mM in total HM carbohydrates), lactate (22.73 ± 3.02 mM in lactose and 17.69 ± 1.21 mM in total HM carbohydrates) and formate (6.56 ± 0.09 mM in lactose and 8.04 ± 0.21 mM in total HM carbohydrates) as the major end metabolites. The final acetate to lactate ratio for *Bifidobacterium infantis* in lactose was 2.4:1 and 2.6:1 in total HM carbohydrates.

The co-culture of *Bifidobacterium infantis* with *Anaerostipes caccae* also degraded lactose completely within 9 h. However, the co-cultures depleted glucose and galactose faster compared to the monocultures of *Bifidobacterium infantis*. The concentration of monomeric sugars peaked around 7 h in media supplemented with lactose, with 4.62 ± 1.21 mM glucose and 7.10 ± 0.97 mM galactose. In media supplemented with the total HMOS carbohydrates, the maximum concentration for glucose (4.20 ± 2.10 mM) and galactose (7.39 ± 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 ± 2.15 mM in lactose and 25.80 ± 2.45 mM in total HM carbohydrates), acetate (5.44 ± 0.30 mM in lactose and 9.05 ± 0.71 mM in total HM carbohydrates) and formate (2.53 ± 0.16 mM in lactose and 4.78 ± 1.16 mM in total HM carbohydrates). In contrast to the *Bifidobacterium 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 ESI-LC-MS for 0 h and 24 h cultures in order to understand the specific glycan utilisation by these bacteria (Fig. 4). The monoculture of *Bifidobacterium 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 *Anaerostipes caccae* monoculture. On the other hand, the glycan utilisation pattern in the co-culture was identical to the profile of *Bifidobacterium infantis* monoculture indicative of the degrader role of *Bifidobacterium infantis* in the co-cultures. 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.

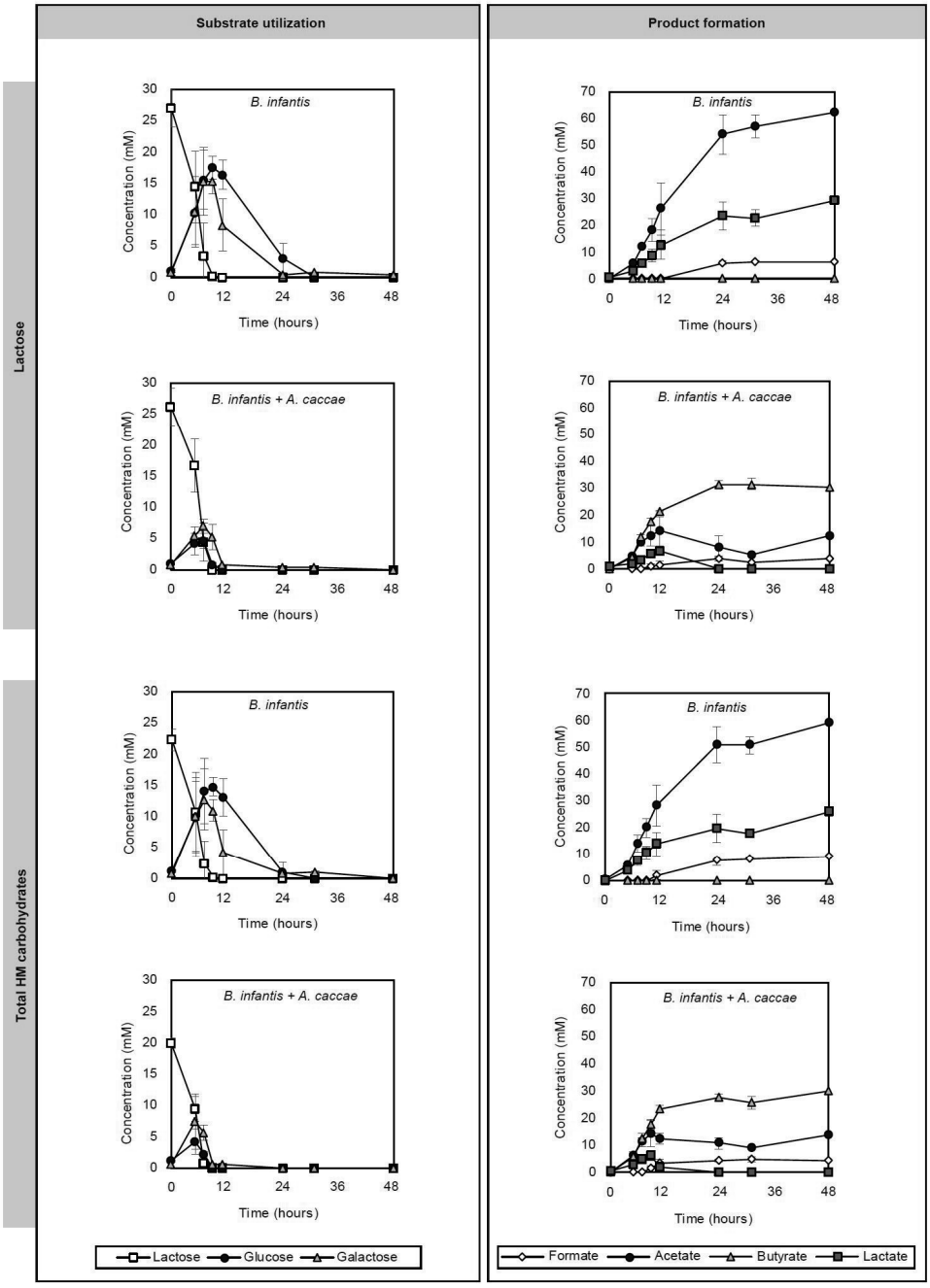


Figure 3. ***Bifidobacterium infantis* supported butyrate production of *Anaerostipes caccae*.** The substrate utilisation and SCFA formation of co-cultures in lactose or total HM carbohydrates. Error bars represent the standard deviation for biological triplicates, except for time point 31 h (n=2) and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

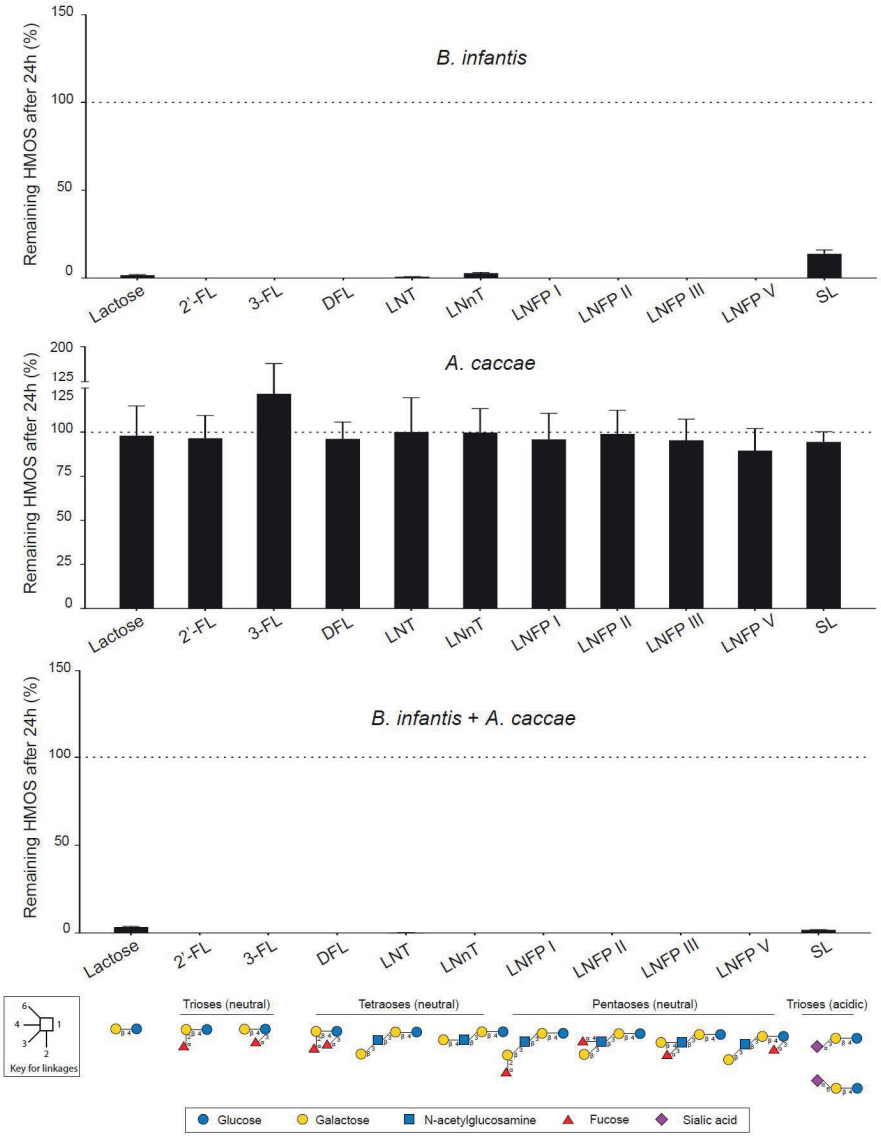


Figure 4. ***Bifidobacterium infantis*** **monoculture and co-culture with** ***Anaerostipes caccae*** **utilised the full range of low molecular weight HMOS.** Error bars represent the error propagation for mean of three (for *Anaerostipes caccae*) or four (for *Bifidobacterium infantis* and *Bifidobacterium infantis* + *Anaerostipes caccae*) biological replicates measured in technical triplicates. The HMOS structures and glycosidic linkages are depicted according to Varki *et al.* (Varki *et al* 2015). *Abbreviations*: 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.

Microbial cross-feeding results in a shift of SCFA pool

The cultures were maintained at pH 6.5 with the addition of 2 M NaOH. *Bifidobacterium infantis* monocultures required a higher amount of base addition compared to the co-culture with *Anaerostipes caccae* (Fig. 5a). The acidification of the cultures was reflected in the composition of SCFAs. The total amount of SCFAs at 31 h were higher in the monocultures (86.76 ± 7.78 mM in lactose and 76.75 ± 3.86 mM in total HM carbohydrates) in comparison to the co-cultures (39.36 ± 1.68 mM in lactose and 39.88 ± 3.97 mM in total HM carbohydrates). Furthermore, as a result of microbial cross-feeding in the co-cultures, lactate (pKa = 3.86) produced by *Bifidobacterium infantis* monocultures was converted to butyrate (pKa = 4.82). The pKa value indicates the quantitative measurement of the strength of an acid in the solution with lower values for stronger acid. As the pKa 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.* (Wopereis *et al* 2017) was employed. We observed that the faecal pH for infants (n=138) increased from pH 5.7 at 4 weeks to pH 6.0 at 6 months of life (Fig. 5b).

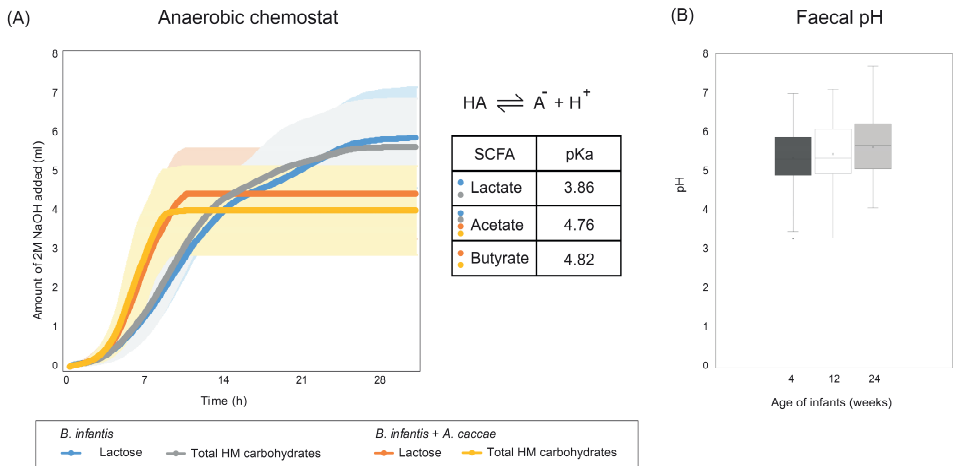


Figure 5. **The acidification of cultures and faecal pH.** (A) Base (2M NaOH) added to maintain the anaerobic chemostat at pH 6.5. The shaded error bars indicate standard deviation for biological triplicates. (B) The faecal pH for infants (n=138). Data adapted from Wopereis *et al.* (Wopereis *et al*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

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 *Bifidobacterium infantis* could support the metabolism and growth of another important species in early life, the butyrate-producing *Anaerostipes caccae* via cross-feeding. This microbial cross-feeding resulted in the shift of the SCFA 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 (GPR) and by inhibiting histone deacetylase (HDAC) (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). 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 *Bifidobacterium infantis* effectively degraded 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 *Bifidobacterium infantis* by encoding a 43kb gene cluster that carries the genes for different oligosaccharides transport proteins and glycosyl hydrolases (Underwood *et al* 2014). No signal peptide or transmembrane domain was predicted for *Bifidobacterium infantis* enzymes involved in the cleavage of the monitored HMOS structures (Table S2), indicating intracellular degradation of these substrates. Furthermore, the distinct “bifid shunt pathway” centred 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 (EMP)

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. 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 slightly acidic 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 *Klebseilla 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). Data mining of a published dataset showed 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 *Anaerostipes 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 indicated that *Anaerostipes caccae* could utilise the monomeric sugars and end products like acetate and lactate derived from *Bifidobacterium infantis* for metabolic activity and growth (Duncan *et al* 2004). *Anaerostipes 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). 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 (Wopereis *et al* 2017). 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 SCFA ratio in adult faeces is around 3:1:1 for acetate, propionate and butyrate respectively (Schwiertz *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 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 atopic 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 *Bifidobacterium 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 substrate 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.

Acknowledgements

We thank Heleen de Weerd for 16S rRNA amplicon sequencing analysis.

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

Supplementary materials

Table S1. The optical density, substrates and end products (mean ± standard deviation) detected in *Anaerostipes caccae* monoculture in lactose and total HM carbohydrates and media blanks at the end of fermentation.

	OD (600nm)	Lactose (mM)	Glucose (mM)	Galactose (mM)	GlucNAc (mM)	GalNAc (mM)	Fucose (mM)	Malate (mM)	Fumarate (mM)	Succinate (mM)	Citrate (mM)	Formate (mM)	Acetate (mM)	Butyrate (mM)	Isobutyrate (mM)	Lactate (mM)	1,2 propanediol (mM)	Propionate (mM)
Basal media	0.009 ± 0.016	-	0.013 ± 0.022	-	-	-	0.042 ± 0.073	0.121 ± 0.106	-	-	-	-	-	-	-	-	-	0.369 ± 0.640
Basal media + lactose	0.024 ± 0.005	24.732 ± 2.810	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Basal media + total HM carbohydrates	0.022 ± 0.001	16.107 ± 0.858	0.076 ± 0.107	-	-	0.200 ± 0.003	0.032 ± 0.045	0.091 ± 0.128	-	-	-	-	-	-	-	-	-	0.852 ± 1.205
Basal media + lactose + *A. caccae*	0.016 ± 0.012	27.417 ± 3.359	0.107 ± 0.219	-	-	-	0.022 ± 0.061	0.138 ± 0.152	-	-	-	-	-	-	-	0.342 ± 0.969	-	0.360 ± 0.668
Basal media + total HM carbohydrates + *A. caccae*	0.029 ± 0.016	22.105 ± 5.273	0.616 ± 1.582	-	-	0.186 ± 0.163	0.018 ± 0.051	0.106 ± 0.162	-	-	-	-	-	-	-	0.461 ± 1.305	-	0.147 ± 0.417

Table S2. **HMOS structures and prediction of degradation by *Bifidobacterium infantis* ATCC15697**. The HMOS structures and glycosidic linkages are depicted according to Varki *et al.* (Varki *et al* 2015). Arrows indicate glycosidic linkages cleavable by the bacterial glycosidases.

Cleavage site	Lactose	2'-FL	3-FL	DFL	LNT	LNnT	LNFP I	LNFP II	LNFP III	LNFP V	3'-SL	6'-SL
Cleavage site 1 Enzyme CAZymes Protein Accession	β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	α1-2 fucosidase GH 95 BAJ69989.1	α1-3/4 fucosidase GH 29 BAJ67848.1 BAJ68029.1 BAJ69990.1	α1-3/4 fucosidase GH 29 BAJ67848.1 BAJ68029.1 BAJ69990.1	Lacto-N-biose phosphorylase GH 112 BAJ69828.1	Lacto-N-biose phosphorylase GH 112 BAJ69828.1	α1-2 fucosidase GH 95 BAJ69989.1	α1-3/4 fucosidase GH 29 BAJ67848.1 BAJ68029.1 BAJ69990.1	α1-3/4 fucosidase GH 29 BAJ67848.1 BAJ68029.1 BAJ69990.1	α1-3/4 fucosidase GH 29 BAJ67848.1 BAJ68029.1 BAJ69990.1	α2-3 sialidase GH 33 BAJ68244.1 BAJ69999.1	α2-6 sialidase GH 33 BAJ68244.1 BAJ69999.1
Cleavage site 2 Enzyme CAZymes Protein Accession		β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	α1-2 fucosidase GH 95 BAJ69989.1	β-hexosaminidase GH 2/20/42 BAJ67866.1 BAJ68060.1 BAJ69539.1 BAJ68336.1 BAJ69988.1 BAJ70005.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β-hexosaminidase GH 2/20/42 BAJ67866.1 BAJ68060.1 BAJ69539.1 BAJ68336.1 BAJ69988.1 BAJ70005.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	Lacto-N-biose phosphorylase GH 112 BAJ69828.1	Lacto-N-biose phosphorylase GH 112 BAJ69828.1	Lacto-N-biose phosphorylase GH 112 BAJ69828.1	Lacto-N-biose phosphorylase GH 112 BAJ69828.1	β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1
Cleavage site 3 Enzyme CAZymes Protein Accession				β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β-hexosaminidase GH 2/20/42 BAJ67866.1 BAJ68060.1 BAJ69539.1 BAJ68336.1 BAJ69988.1 BAJ70005.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β-hexosaminidase GH 2/20/42 BAJ67866.1 BAJ68060.1 BAJ69539.1 BAJ68336.1 BAJ69988.1 BAJ70005.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β-hexosaminidase GH 2/20/42 BAJ67866.1 BAJ68060.1 BAJ69539.1 BAJ68336.1 BAJ69988.1 BAJ70005.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β-hexosaminidase GH 2/20/42 BAJ67866.1 BAJ68060.1 BAJ69539.1 BAJ68336.1 BAJ69988.1 BAJ70005.1 BAJ69670.1 BAJ69777.1 BAJ70064.1		
Cleavage site 4 Enzyme CAZymes Protein Accession							β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1	β1-4 galactosidase GH 1/2/35/42 BAJ69551.1 BAJ67866.1 BAJ69539.1 BAJ69988.1 BAJ69670.1 BAJ69777.1 BAJ70064.1		

Key
- Glucose
- Galactose
- N-acetylglucosamine
- Fucose
- Sialic acid

Non of the proteins in the list is predicted for signal peptide and transmembrane domain.

Abbreviations: 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.

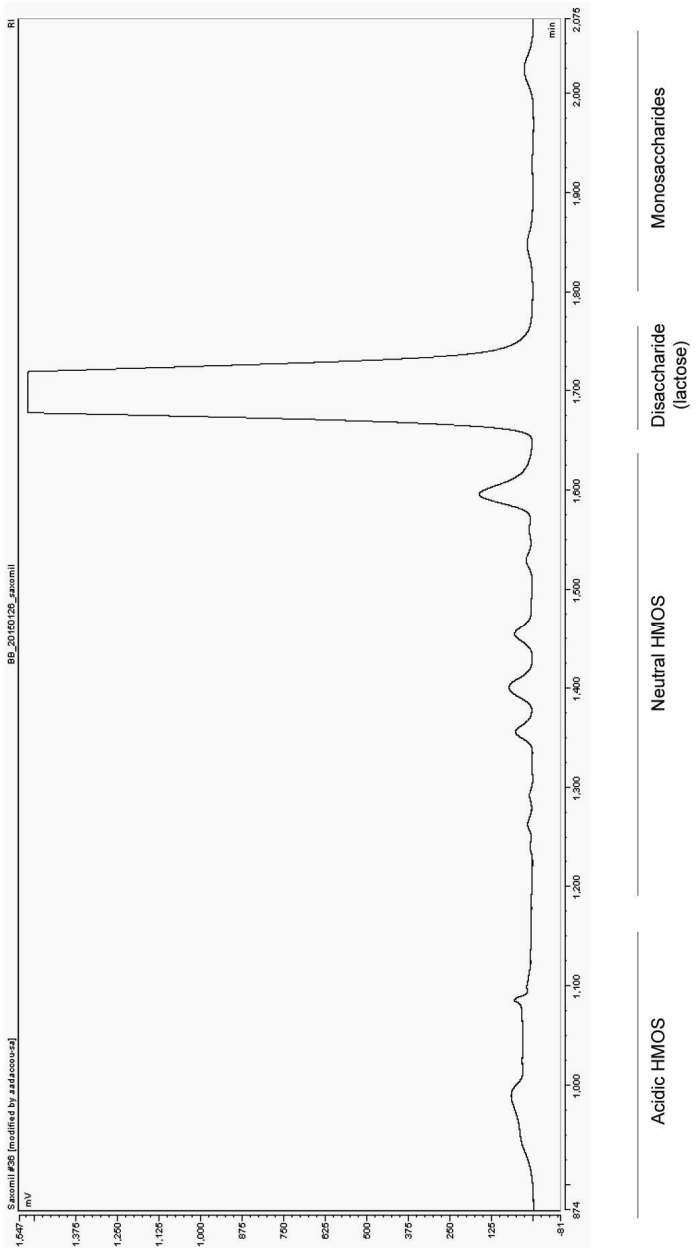
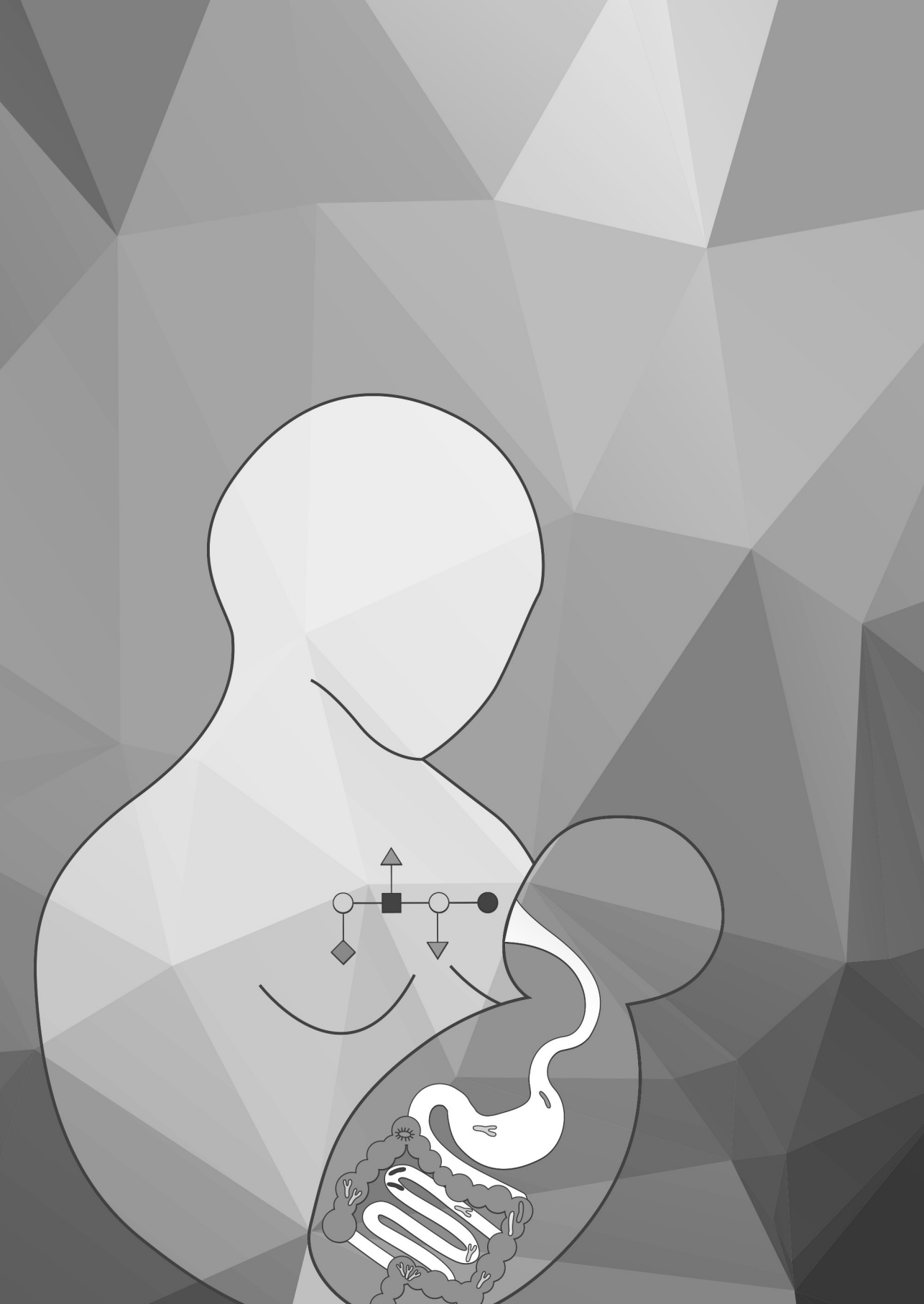


Figure S1. The GPC-RI chromatogram for total human milk (HM) carbohydrates showing a composition of approximately 90% of lactose, 10% of both acidic and neutral HMOS as well as traces of monosaccharides.



Chapter 3

Bacteroides thetaiotaomicron stimulates the growth of butyrate-producing *Anaerostipes caccae* in the presence of lactose and human milk oligosaccharides

Loo Wee Chia, Marko Mank, Bernadet Blijenberg, Steven Aalvink, Roger S. Bongers, Bernd Stahl, Jan Knol, Clara Belzer

In preparation

Abstract

The development of the infant gut microbiota is strongly influenced by nutritional and environmental factors. Human milk oligosaccharides (HMOS) in breast milk selectively promote the growth of glycan-degrading microbes particularly members of the genera *Bifidobacterium* and *Bacteroides*. As such, these genera are dominant in the early life microbiota and they form the basis of the microbial network formation. In this study, we investigated the trophic interactions between the HMOS-degrading *Bacteroides thetaiotaomicron* and the butyrate-producing *Anaerostipes caccae* in the presence of human milk carbohydrates. *Anaerostipes caccae* was not able to utilise lactose and HMOS but its signature metabolite, butyrate was detected in the co-culture with *Bacteroides thetaiotaomicron. Bacteroides thetaiotaomicron* displayed glycan catabolic capability, and it showed preference in utilising specific HMOS structures. As such, *Anaerostipes caccae* cross-fed on *Bacteroides thetaiotaomicron*-derived monosaccharides, acetate and D-lactate for growth and concomitant butyrate production. Furthermore, we investigated the lactate isomer production by *Bacteroides* spp. and *Bifidobacterium* spp. We observed differential isomer production by these major lactate-producing degraders in the infant gut, in which *Bacteroides* spp. and *Bifidobacterium* spp. produced D- and L-lactate respectively. The distinct lactate isomer production by these major glycan-degrading genera might affect the composition of the gut microbiota by the sequential cross-feeding interaction with specific D- and L- lactate-utilisers.

Keywords

Bifidobacteria, cross-feeding, D-lactate, infant, L-lactate, microbiota

Introduction

The establishment of the infant gut microbiota is a dynamic process manifested by the successive colonisation of functionally distinct microbial groups (Backhed *et al* 2015, Laursen *et al* 2017). The infant gut microbiota displays high temporal and inter-individual variation that is influenced by factors including genetic background, the mode of the delivery, hospitalisation, use of antibiotics and early life nutrition (Scholtens *et al* 2012). Even though it has not been well-defined what a healthy infant gut microbiota is comprised of, it has been associated with desirable criteria such as infants delivered at full term via natural birth mode as well as received breast-feeding and no administration of antibiotics. Breast-feeding selectively promotes the growth of bacteria capable of utilising human milk oligosaccharides (HMOS). The HMOS-degrading members from the *Bifidobacterium* and *Bacteroides* genera are dominant taxonomic groups in the gut of healthy infants (Backhed *et al* 2015).

Infants delivered by caesarean section are often deprived of *Bacteroidetes* in the first days of life, likely due to the absence of exposure to members of this genus which are present in the birth canal (Backhed *et al* 2015, Martin *et al* 2016). The common *Bacteroides* spp. found in the infant gut are *Bacteroides thetaiotaomicron, Bacteroides fragilis* and *Bacteroides vulgatus* (Backhed *et al* 2015, Martin *et al* 2016). *Bacteroides thetaiotaomicron* is generally recognised as a symbiont that contributes to the postnatal gut development and host physiology (Wexler 2007). *Bacteroides thetaiotaomicron* is able to forage on host-secreted glycans including HMOS and mucins (Marcobal *et al* 2011). It is described that the distinct capability to utilise glycans using the archetypal starch utilisation system (*Sus*), and the ability to sense and respond to the environmental cues, lead to the colonisation of *Bacteroides thetaiotaomicron* in human gut (Reeves *et al* 1997, Xu *et al* 2003). The relative abundance of *Bacteroides* spp. increases with age, in which a stable adult gut microbiota is predominantly comprised of *Bacteroidetes* and *Firmicutes* (Laursen *et al* 2017).

The primary HMOS-degraders could drive the establishment of the microbial community in the infant gut via cross-feeding. As such, the intermediate breakdown products by one bacterial species could serve as the substrate to support growth of other microbes in the environment, often resulting in indirect benefits for all species

involved (Belenguer *et al* 2006, Duncan *et al* 2004, Freilich *et al* 2011). For instance, *Bifidobacterium* spp. degrade host-produced 2'-fucosyllactose (2'-FL, an abundant HMOS) and mucin glycans that in turn supports the growth of butyrate-producing bacteria (Bunesova *et al* 2017, Schwab *et al* 2017). Furthermore, the *in vivo* interaction between *Bacteroides thetaiotaomicron* and butyrate-producing bacteria in gnotobiotic mice lead to the increase of intestinal butyrate (Mahowald *et al* 2009, Wrzosek *et al* 2013). However, to date, no study has addressed the interaction between *Bacteroides* spp. and butyrogen on early life substrates. We hypothesise that *Bacteroides* spp. could also drive the butyrate metabolic network in the infant gut.

This study investigates the trophic interaction between the early life colonisers *Bacteroides thetaiotaomicron* and butyrate-producing *Anaerostipes caccae* from the *Lachnospiraceae* family. Anaerobic bioreactor culturing of the bacteria on human milk carbohydrates including lactose and total human milk (HM) carbohydrates were conducted. We demonstrated that *Bacteroides* spp. could support the butyrogenic trophic chain using lactose or HMOS. *Anaerostipes caccae* cross-fed on monosaccharides, acetate and D-lactate released by *Bacteroides thetaiotaomicron* from lactose and HMOS metabolism. Moreover, we observed that *Bacteroides* spp. showed differential lactate isomer production in comparison to the early life colonising *Bifidobacterium* spp. that produced L-lactate predominantly.

Materials and Methods

Bacterial strains and growth conditions. Bacterial pre-cultures were prepared by overnight growth in anaerobic serum bottles sealed with butyl-rubber stoppers at 37ºC with gas phase of N_2/CO_2 (80/20 ratio) at 1.5atm. Basal medium (Plugge 2005) containing 0.5% (w/v) tryptone (Oxoid, Basingstoke, UK) was used for culturing. For *Bacteroides thetaiotaomicron* DSM 2079 (VPI 5428), 30mM of lactose (Oxoid, Basingstoke, UK) and 5 mg/L of hemin (Sigma-Aldrich, St. Louis, USA) were supplemented; whereas for *Anaerostipes caccae* DSM 14662 (L1-92) (Schwiertz *et al* 2002), 30mM of glucose (Sigma-Aldrich, St. Louis, USA) was supplemented. Growth was measured by a spectrophotometer as optical density at 600nm (OD600) (OD600 DiluPhotometer™, IMPLEN, Germany).

Growth substrates. Lactose (Oxoid, Basingstoke, UK) and total human milk (HM) carbohydrate fraction were tested as the growth substrates. For preparation of total HM carbohydrate fraction, 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.* (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 carbohydrate fraction contained approximately 10% HMOS and 90% of lactose as estimated by gel permeation chromatography (GPC) (Fig. S1).

Anaerobic bioreactor. Fermentations were conducted in eight parallel minispinner bioreactors (DASGIP, Germany) with 100 ml filling volume at 37°C and a stirring rate of 150 rpm. Culturing experiments were performed in autoclaved basal media (Plugge 2005) containing 0.5% (w/v) tryptone (Oxoid, Basingstoke, UK), supplemented with 8 g/L of 0.2 µM filter-sterilized lactose or total HM carbohydrate fraction. Experiments were performed with 1% (v/v) supplementation of 0.2 µM filter-sterilized 0.5 g/L hemin stock solution and the B vitamins stock solution. The B vitamins stock solution contains 11 g/L $CaCl_2$, 20 mg/L biotin, 200 mg/L nicotinamide, 100 mg/L *p*-aminobenzoic acid, 200 mg/L thiamine, 100 mg/L pantothenic acid, 500 mg/L pyridoxamine, 100 mg/L cyanocobalamin, and 100 mg/L riboflavin. Anaerobic condition was achieved by overnight purging of anaerobic gas mixture that contained 5% CO_2, 5% H_2, and 90% N_2. Overnight pre-cultures were inoculated at starting OD600 of 0.05 for each bacterial

strain. Online signals of pH values and oxygen levels were monitored by the DASGIP Control software (DASGIP, Germany). Cultures were maintained at pH 6.5 by dosing 2 M sodium hydroxide (NaOH).

Gel permeation chromatography (GPC). 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 degrees 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) containing 2% (v/v) 2-propanol at 37^0C. 5 ml of 0.2 µM filter-sterilized 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) packed with Toyopearl HW 40 (TOSOH BIOSCIENCE) were used. Milli-Q water was maintained at 50°C using a heating bath (Lauda, RE 206) for columns equilibration. Milli-Q 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). The resulting chromatograms were analysed by using the Chromeleon® software (ThermoScientific 6.80).

High-performance liquid chromatography (HPLC). For metabolites analysis, 1 ml of bacterial culture was centrifuged and the supernatant was stored at -20°C until HPLC analysis. Crotonate was used as the internal standard, and the external standards tested were lactose, glucose, galactose, N-acetylglucosamine (GlcNAc), fucose, malate, fumarate, succinate, citrate, formate, acetate, butyrate, isobutyrate, lactate, 1,2-propanediol, and propionate. Substrates conversion and products formation 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 organic acids and carbohydrates. 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, Breda, the Netherlands).

HMOS extraction. HMOS were recovered from 1 ml aliquots of bacterial cultures. The internal standard 1,5-α-L-arabinopentaose (Megazyme) was added, at the volume of 10 µl per sample to minimize 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 a 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 Milipore) at 14,000 g for 1 h at room temperature. Finally, the filtrate was vortexed and stored at -20°C until further electrospray ionisation liquid chromatography mass spectrometry (ESI-LC-MS) analysis.

Electrospray ionisation liquid chromatography mass spectrometry (ESI-LC-MS) analysis. The identification and relative quantitation of HMOS were determined with ESI-LC-MS. This method allowed the study of distinct HMOS structures differed 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. Micro ESI-LC-MS analysis was performed on a 1200 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, 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 2.1x30 mm Hypercarb porous graphitized carbon (PGC) column with 2.1x10 mm PGC pre-column (Thermo Scientific, USA) using water-acetonitrile gradient for 18 min protocol. The gradient started with a ratio of 98% (v/v) water and 2% (v/v) acetonitrile in 5 mM ammonium acetate at 0 min and ended with a ratio of 20% (v/v) water and 80% (v/v) acetonitrile in 5 mM ammonium acetate at 15 min. Re-equilibration was established between 15 and 18 min with 98% (v/v) water and 2% (v/v) acetonitrile in 5 mM ammonium acetate. Eluent flow was 400 µl/min and the columns were kept at 45^0C. 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 -4500 V, declustering potential was at 44 V, and collision energy was set to 29 eV. Each MRM-transition was performed for 50 ms. The instrument was calibrated with polypropylene glycol (PPG) according to 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 (q-PCR). The abundances of *Bacteroides thetaiotaomicron* and *Anaerostipes caccae* in 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. The DNA concentrations were determined fluorometrically (Qubit dsDNA HS assay; Invitrogen) and adjusted to 1 ng/µl prior to use as the template in qPCR. Primers targeting 16S rRNA gene of *Bacteroides thetaiotaomicron* (g-Bfra-F 5'-ATAGCCTTTCGAAAGRAAGAT-3'; g-Bfra-R 5'-CCAGTATCAACTGCAATTTTA-3'; 501 bp product (Matsuki *et al* 2002)) and *Anaerostipes 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 from the 16S rRNA gene of each bacterium by amplification with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Standard curves were prepared with nine standard concentrations of 10^0 to 10^8 gene copies/µl. PCRs were performed in triplicate with iQ SYBR Green Supermix (Bio-Rad) 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: one cycle of 95°C for 10 min; 40 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 30 s each; one cycle of 95°C for 1 min, one cycle of 60°C for 1 min, and a stepwise increase of the temperature from 60 to 95°C (at 0.5°C per 5 s) to obtain melt curve data. Data were analysed using the Bio-Rad CFX Manager 3.0.

Anaerobic microtiter plate culturing. Anaerobic microtiter plate culturing was performed to test the production of lactate isomers on different growth substrates. Bacteria strains used were *Bifidobacterium longum* subsp. *infantis* DSM 20088, *Bifidobacterium breve* DSM 20213, *Bifidobacterium bifidum* DSM 20456, *Bacteroides fragilis* DSM 2151, and *Bacteroides vulgatus* DSM 1447. Growth substrates tested were lactose, glucose, mixture of short-chain galacto-oligosaccharides (Friesland Campina, the Netherlands) and long-chain fructo-oligosaccharides (Orafti Beneo, Belgium) at 9:1 ratio (GOS/FOS), 2'-fucosyllactose (2'-FL) and 3'-sialyllactose (3'-SL). All the procedures were performed under anaerobic condition (Backtron anaerobic

chambers), with incubation at 37°C. Basal medium (Plugge 2005) containing 0.5% (w/v) tryptone (Oxoid, Basingstoke, UK), supplemented with 8 g/L of growth substrates were used as the test media. Overnight pre-cultures, which were grown in basal medium (Plugge 2005) containing 0.5% (w/v) tryptone (Oxoid, Basingstoke, UK) and 8 g/L of lactose (Oxoid, Basingstoke, UK), were added to 200 µl of test media at a starting OD600 of 0.1. Anaerobic fermentation was performed in 96-wells plate using a microtiter plate reader (Synergy HTX Multi-Mode Reader) at 37°C for 48 h. OD600 readings were taken at 30 min interval with 3 s agitation prior to measurement. Gen5™ software was used data collection and analysis. The experiment was repeated 2 times with technical triplicates. Bacterial cultures at 48 h were pooled for the determination of lactate isomers.

Determination of D- and L-lactate. Lactate isomers were measured enzymatically by using D- and L- lactate dehydrogenase according to manufacturer's instructions (Boehringer Mannheim, Darmstadt, Germany).

Results

Bacteroides thetaiotaomicron supported the growth of Anaerostipes caccae in the presence of human milk carbohydrates

Bacteroides thetaiotaomicron in mono- and co-culture with *Anaerostipes caccae* were cultured in anaerobic bioreactor regulated at pH 6.5 to simulate the condition in early life gut. Lactose or total human milk (HM) carbohydrates were tested as the carbohydrate sources. The monoculture of *Bacteroides thetaiotaomicron* grew in both lactose and total HM with the continuous increase of cell density up to 72 h (OD_{max} = 4.07 ± 0.10 in lactose and OD_{max} = 3.58 ± 4.31 in total HM carbohydrates) (Fig. 1). No growth was observed for *Anaerostipes caccae* (OD_{max} = 0.04 ± 0.01 in lactose and OD_{max} = 0.05 ± 0.01 in total HM carbohydrates) (Table S1). The co-culture of *Bacteroides thetaiotaomicron* with *Anaerostipes caccae* resulted maximum cell density at 24 h (OD_{max} = 5.47 ± 0.43 in lactose and OD_{max} = 4.84 ± 0.26 in total HM carbohydrates). The growth was also reflected by the acidification of the cultures (Fig. 1). qPCR was performed to monitor the growth of each bacterium in the co-culture. Around 6 log of cells were inoculated for both *Bacteroides thetaiotaomicron* and *Anaerostipes caccae.* Both strains decreased 10-fold in abundance at the first 5 h. Hereafter, *Bacteroides thetaiotaomicron* grew exponentially to 1.24 x 10^9 copy number/ ml in lactose and 1.09 x 10^9 copy number/ ml in total HM carbohydrates at 11 h, after which growth slow down (Fig. 1). *Anaerostipes caccae* showed similar trend with an increase of abundance to 8.56 x 10^6 copy number/ ml in lactose and 1.93 x 10^7 copy number/ ml in total HM carbohydrates at 11 h. In both substrates, *Bacteroides thetaiotaomicron* outnumbered *Anaerostipes caccae* by 100-fold.

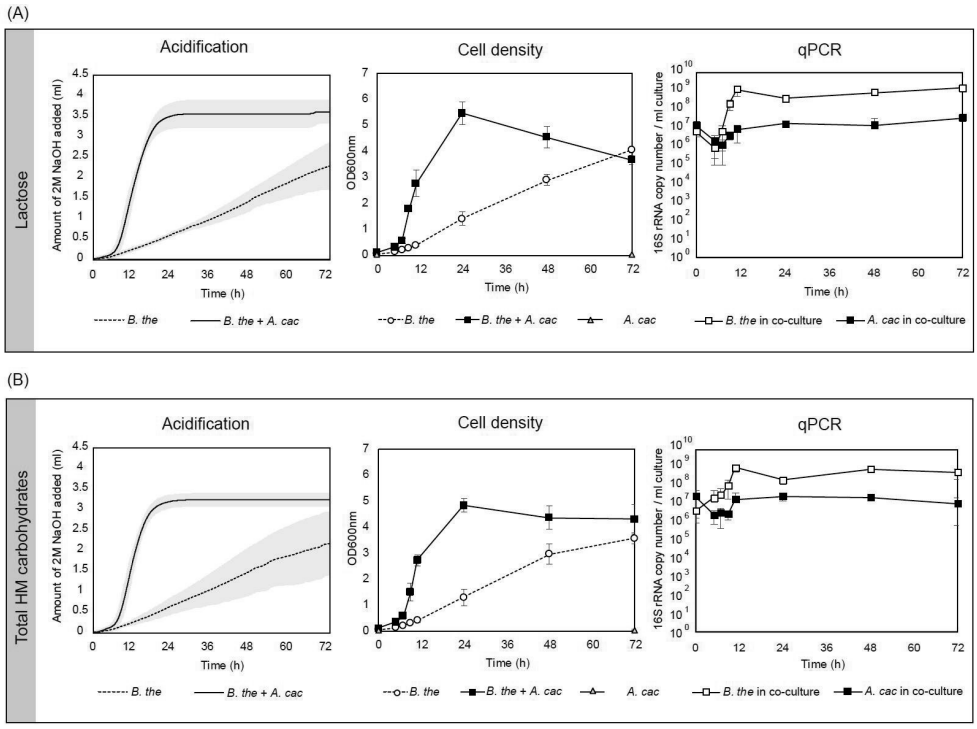


Figure 1. ***Bacteroides thetaiotaomicron* supported the growth of *Anaerostipes caccae* in the human milk carbohydrates.** The acidification, cell density (OD600nm), and microbial composition (qPCR) of *Bacteroides thetaiotaomicron* monocultures and co-cultures with *Anaerostipes caccae* in (A) lactose and (B) total HM carbohydrates. Fermentation was performed in anaerobic bioreactor regulated at pH 6.5. Error bars represent the standard deviation for biological triplicates. No growth was observed for *Anaerostipes caccae* in the identical medium cultured in anaerobic tubes (Table S1).

Cross-feeding between Bacteroides thetaiotaomicron and Anaerostipes caccae led to butyrate production

The sugar consumption and short chain fatty acid (SCFA) production were monitored over time (Fig. 2). Similar changes in the composition of metabolites were observed for the fermentation of lactose and total HM carbohydrates probably because total HM carbohydrates are consisted of approximately 90% of lactose (Fig. S1). *Bacteroides thetaiotaomicron* monoculture showed lower substrate catabolism in monoculture as compared to co-cultures. Lactose was not completely consumed and still detected after 72 h (4.24 ± 0.73 mM in lactose and 1.15 ± 0.83 mM in total HM carbohydrates). Low

amounts (around 1 mM) of the monosaccharides glucose and galactose were also detected in the supernatant throughout the course of fermentation (Fig. 2). Furthermore, *Bacteroides thetaiotaomicron* produced acetate, propionate, succinate, and lactate as well as a low amount of malate from lactose and total HM carbohydrate fermentation. The co-culture of *Bacteroides thetaiotaomicron* with *Anaerostipes caccae* showed faster consumption of lactose compared to mono-culture, with complete depletion within 24 h. Glucose and galactose (around 3 mM) were only detected in the supernatant of co-culture in the first 12 h of fermentation when lactose was still detected. The major metabolites in the co-cultures were propionate, succinate, acetate, butyrate, and formate. Butyrate, the signature product of *Anaerostipes caccae* was produced at 11.85 ± 0.32 mM in lactose and 12.23 ± 1.80 mM in total HM carbohydrates. In contrast to monoculture, several additional changes were observed in the metabolite composition of the co-culture, including the absence of lactate, decrease of acetate and malate after 24h and the production of formate (5.70 ± 0.64 mM in lactose and 4.32 ± 0.65 mM in total HM carbohydrates).

To investigate the glycan degradation capability of the bacteria, HMOS-specific sugars and low molecular weight HMOS structures were measured. Both of the monitored HMOS-specific sugars i.e. N-acetylglucosamine (GlcNAc) and fucose were below detection limit of 0.5 mM throughout the fermentation. The glycoprofiling analysis (Fig. 3) at 0 h and 24 h showed no HMOS degradation for both monoculture of *Bacteroides thetaiotaomicron* and *Anaerostipes caccae*. Interestingly, the majority of the HMOS structures were degraded in the co-culture, with complete degradation of 2'-fucosyllactose (2'-FL), lacto-N-fucopentaose III (LNFP III), lacto-N-fucopentaose V (LNFP V), lacto-N-neotetraose (LNnT), and sialyllactose (SL) as well as limited degradation of 3-fucosyllactose (3-FL), difucosyllactose (DFL), lacto-N-tetraose (LNT), lacto-N-fucopentaose I (LNFP I), and lacto-N-fucopentaose II (LNFP II).

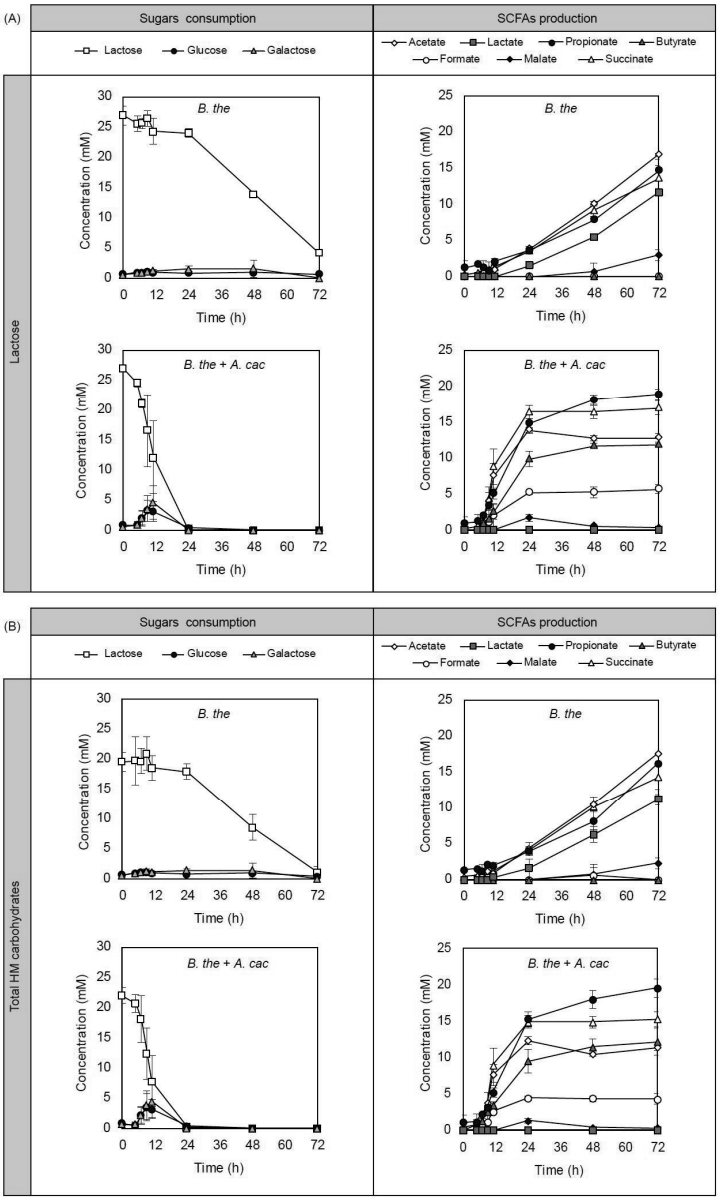


Figure 2. ***Bacteroides thetaiotaomicron* supported butyrate production of *Anaerostipes caccae*.** The sugar and SCFA profile of *Bacteroides thetaiotaomicron* monoculture and co-culture with *Anaerostipes caccae* in basal medium containing (A) lactose or (B) total HM carbohydrates. Error bars represent the standard deviation for biological triplicates. No metabolites production was detected for *Anaerostipes caccae* mono-culture in the identical media (Table S1).

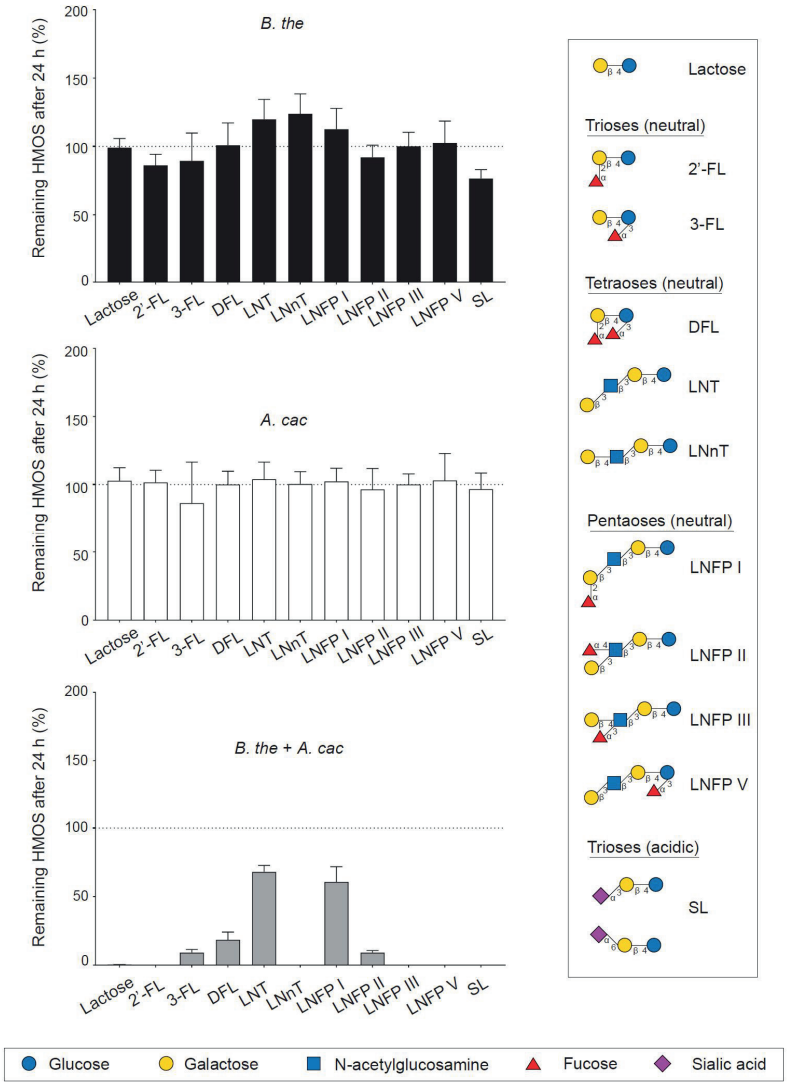


Figure 3. **Utilisation of HMOS structures by *Bacteroides thetaiotaomicron* and *Anaerostipes caccae* co-culture**. No HMOS degradation was detected for the monoculture of *Bacteroides thetaiotaomicron* and *Anaerostipes caccae* after 24 h of fermentation. The HMOS structures and glycosidic linkages are depicted according to Varki *et al.* (Varki *et al* 2015). Error bars represent the error propagation for mean of two biological replicates measured in technical triplicates. Abbreviations: 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.

Differential D- and L-lactate metabolism by gut commensals

The lactate isomers in *Bacteroides thetaiotaomicron* mono- and co-cultures at 24 h were quantified using enzymatic assay (Fig. 4a). Interestingly, only D-lactate was detected in the *Bacteroides thetaiotaomicron* monocultures grew in both lactose (1.05 ± 0.10 mM) and total HM carbohydrates (1.09 ± 0.30 mM). For *Bacteroides thetaiotaomicron* in co-culture, a very low amount of D-lactate (0.29 ± 0.27 mM in lactose and 0.43 ± 0.30 mM in total HM carbohydrates) and L-lactate (nil detection in lactose and 0.25 ± 0.27 mM in total HM carbohydrates) were detected, consistent with the HPLC finding of no lactate detection (Fig. 2). We further quantified the lactate production of *Anaerostipes caccae* after growth in glucose. *Anaerostipes caccae* produced predominantly L-lactate (10.87 ± 2.63 mM) but also low amounts of D-lactate (0.57 ± 0.12 mM) (Fig. 4a).

The stereospecific lactate production by *Bacteroides thetaiotaomicron* roused the query if this trait is specific and consistent for the major lactate-producers in the infant gut. Hence, further analysis of lactate production was performed by culturing common infant gut residents, including *Bacteroides* spp. (*Bacteroides fragilis* and *Bacteroides vulgatus*) and *Bifidobacterium* spp. (*Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium breve*, and *Bifidobacterium bifidum*) (Fig. 4b). Bacterial strains were cultured using anaerobic microtiter plate in media supplemented with different early life substrates including lactose, mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (GOS/FOS), 2'-FL and 3'-SL. *Bacteroides fragilis* and *Bacteroides vulgatus* grew in all of the tested carbohydrates and produced only D-lactate. In contrast, *Bifidobacterium* spp. produced L-lactate in all of the carbohydrate substrates tested.

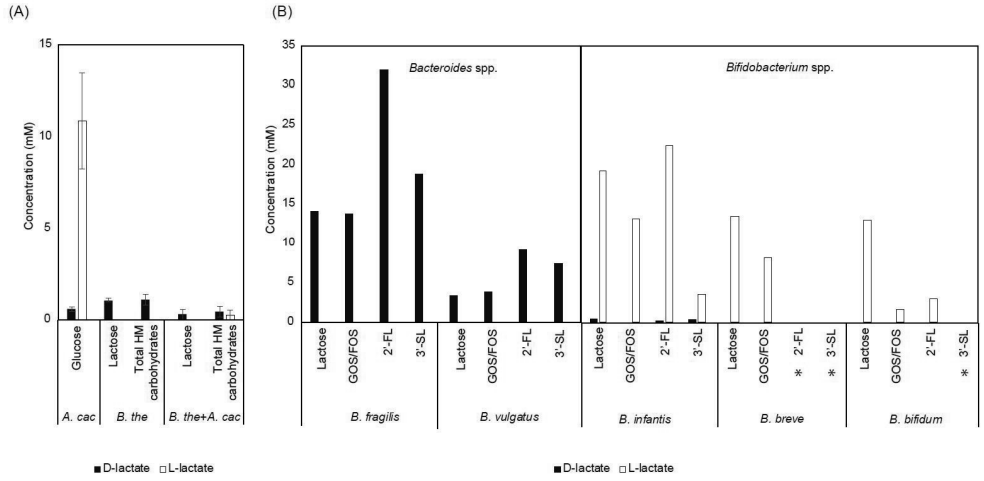


Figure 4. **Differential lactate isomers produced by gut commensals**. (A) The concentration of D- and L-lactate for *Bacteroides thetaiotaomicron* in mono- or co-culture with *Anaerostipes caccae* at 24 h. The experiments were performed in anaerobic bioreactor with error bars represent standard deviation for biological triplicate. *Anaerostipes caccae* monoculture was grown in 30mM glucose harvested at 24 h. (B) The concentration of D- and L-lactate in the monoculture of *Bifidobacterium* spp. and *Bacteroides* spp. in lactose, GOS/FOS, 2'-FL, or 3'-SL. The fermentation experiment was performed using anaerobic microtiter plate at biological duplicates with technical triplicates. Bacterial cultures at 48 h were pooled for the determination of lactate isomers. (*) No growth was observed for *Bifidobacterium breve* in 2'-FL and 3'-SL as well as *Bifidobacterium bifidum* in 3'-SL.

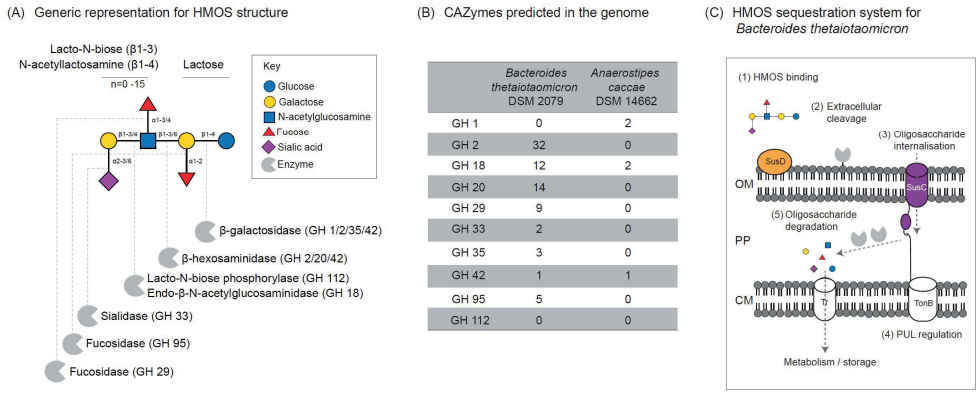
Discussion

Bacterial cross-feeding on non-digestible dietary components drives the microbial network formation in the infant gut. The intricate relationship among key functional groups is vital for the maintenance of a healthy state. Dysbiosis of the infant gut microbiota could result in short-term consequences such as intestinal discomfort and colics (de Weerth *et al* 2013, Pham *et al* 2017) as well as atopic and metabolic syndrome that compromise life-long health (Arrieta *et al* 2015, Dogra *et al* 2015, Scheepers *et al* 2014, Wopereis *et al* 2017). In this study, we investigated the role of human milk carbohydrates i.e. lactose and HMOS in driving a butyrogenic microbial interaction.

The major microbial-derived SCFAs detected in infant faeces are acetate and lactate as well as a small amount of propionate and butyrate (Pham *et al* 2016), in contrast to the adult gut with the faecal SCFA composition ratio of 3:1:1 for acetate, propionate and butyrate (Schwiertz *et al* 2010). The distinction in faecal metabolites across age could be partially explained by the compositional difference in the gut microbiota. The gut of breast-fed infant is primarily colonised by HMOS-utilising *Bifidobacterium* spp. and to a lesser extent *Bacteroides* spp. (Backhed *et al* 2015). As complementary feeding progresses, the abundance of the butyrate-producing bacteria from the family of *Lachnospiraceae* and *Ruminococcaceae* gradually increase with age (Laursen *et al* 2017). Metabolic dependency has been observed between *Bifidobacterium* spp. and butyrate-producing bacteria in utilising 2'-FL (Schwab *et al* 2017). Here, we demonstrated that *Bacteroides* spp. could also fuel the butyrogenic trophic chain in the presence human milk carbohydrates. *Anaerostipes caccae* was not able to metabolise either lactose or HMOS, but was dependent on the intermediates produced by *Bacteroides thetaiotaomicron* for growth. *Anaerostipes caccae* could scavenge on free monosaccharides i.e. glucose and galactose liberated by *Bacteroides thetaiotaomicron* from carbohydrates catabolism.

Bacteroides thetaiotaomicron possesses a range of carbohydrate-active enzymes (CAZymes) predicted to degrade HMOS including fucosidases, sialidases, β-galactosidases, and β-hexosaminidases (Fig. 5). In contrast, *Anaerostipes caccae* has a limited catabolic capability. *Bacteroides thetaiotaomicron* encodes for *Sus* system consisted of several membrane-bound proteins and lipoproteins involved in

substrate binding, degradation, and internalisation into the periplasm (Martens *et al* 2014, Reeves *et al* 1997). The fucosidases and sialidases required to initiate HMOS degradation are often organised in a modular manner adjacent to transcriptional regulator such as hybrid two-component systems (HTCS) and extracytoplasmic function (ECF) σ-factors (Fig. S2). The *Sus* system thus appeared to be an efficient and well-coordinated machinery to sequester HMOS by employing the mucus-utilisation machinery (Marcobal *et al* 2011). In the co-culture with *Anaerostipes caccae*, the rapid depletion of lactose was coupled with the degradation of HMOS. However, no HMOS degradation was observed in the monoculture of *Bacteroides thetaiotaomicron* at 24 h when lactose was hardly consumed. This could infer slow metabolism of *Bacteroides thetaiotaomicron* monoculture or the bacterium repressed the degradation of HMOS in the presence of lactose as alternative substrate. Pudlo *et al.* showed that *Bacteroides thetaiotaomicron* deprioritizes mucin glycans metabolism in the presence of competing complex carbohydrates and monosaccharides (Pudlo *et al* 2015). Hence, glycoprofiling analysis of 48 h cultures will be performed for further mechanistic insight. This metabolic plasticity of *Bacteroides thetaiotaomicron* has also been demonstrated by the alteration of CAZymes gene expression to switch its metabolism from milk to plant carbohydrates after weaning in mice (Bjursell *et al* 2006). We found that *Bacteroides thetaiotaomicron* effectively utilised most of the low molecular weight HMOS as previously reported (Yu *et al* 2013), with differential preference for specific HMOS structures. The glycan-foraging capability could enhance the bacterial fitness and colonisation in the infant gut (Martens *et al* 2008) and the fine-structure prioritisation at species level has been accounted for the collective fitness of *Bacteroides* spp. (Tuncil *et al* 2017). As such, several HMOS structures including 2'-FL, LNFP I, and LDFT were found to correlate positively with the abundance of *Bacteroides* spp. in breast-fed infants (Wang *et al* 2015). Intriguingly, *Bacteroides thetaiotaomicron* could reciprocally affect the gut glycosylation by regulating the production of fucosylated glycans for its competitive advantage (Hooper *et al* 1999).



	Bacteroides thetaiotaomicron DSM 2079	*Anaerostipes caccae* DSM 14662
GH 1	0	2
GH 2	32	0
GH 18	12	2
GH 20	14	0
GH 29	9	0
GH 33	2	0
GH 35	3	0
GH 42	1	1
GH 95	5	0
GH 112	0	0

Figure 5. ***Bacteroides thetaiotaomicron* degraded lactose and HMOS in the co-culture**. (A) The schematic representation for HMOS structure and prediction of bacterial glycosyl hydrolase (GH) required to cleave the specific linkage. (B) Genome prediction for HMOS-degrading carbohydrate-active enzymes (CAZymes) for *Bacteroides thetaiotaomicron* and *Anaerostipes caccae*. (C) The proposed mechanism for HMOS sequestration by *Bacteroides thetaiotaomicron* using polysaccharide utilisation loci (PULs). Abbreviation: HMOS, human milk oligosaccharides; OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane; Tr, transporter; *Sus*C, *Sus*C-like TonB-dependent transporter; *Sus*D, *Sus*D-like outer membrane-binding protein.

We revealed that the major milk glycan-degraders i.e. *Bifidobacterium* spp. and *Bacteroides* spp. contributed differently to the lactate isomer pool in the gut. A consistent trait was observed in which *Bacteroides* spp. produced specifically D-lactate whereas *Bifidobacterium* spp. produced only L-lactate when grown in the human milk carbohydrates. Bacterial lactate production from pyruvate involves the catalysis of stereospecific lactate dehydrogenase. We found that the genome of *Bacteroides thetaiotaomicron* encodes only for a D-lactate dehydrogenase (BT_1575) leading to specific D-lactate production. The lactate-utilising butyrate-producing bacteria (LUB) could subsequently convert acetate and lactate into butyrate. Our model organism, *Anaerostipes caccae* was reported to metabolise both D- and L- lactate (Duncan *et al* 2004). Despite the phenotypic observation, two L-lactate dehydrogenases (ANACAC_01148 and ANACAC_03769) and no D-lactate dehydrogenase or lactate racemace was found in the genome of *Anaerostipes caccae*. The homologous protein check against D-lactate dehydrogenase from *Lactobacillus helveticus* (UniProt identifier P30901) showed no match in *Anaerostipes caccae* genome except the general NAD-binding domain. Besides, no domain matching the lactate racemase from

Lactobacillus plantarum (UniProt identifier F9USS9) was found in *Anaerostipes caccae* genome. Nevertheless, as D-lactate conversion was required for the accumulation of butyrate up to 12mM in the co-cultures, novel gene / genes could be involved. Other LUB such as *Eubacterium hallii* is reported to metabolise both D- and L- lactate whereas *Roseburia intestinalis*, *Eubacterium rectale* and *Faecalibacterium prausnitzii* can only metabolise the D-form (Duncan *et al* 2004). Ecologically, the lactate isomer pool produced by the degrader community could directly affect the substrate availability for LUB. Furthermore, this could potentially incur physiological effect to the host. As the tolerance for D-lactate is lower compared to L-lactate due to the lack of D-lactate dehydrogenase in human genome, the accumulation of D-lactate is associated with a higher susceptibility to acidosis (Uribarri *et al* 1998). The balance of D- and L- lactate was also linked to the risk for D-encephalopathy in patients with short bowel syndrome (Mayeur *et al* 2013).

The degrader community could drive the establishment of microbial network in the infant gut. This forms the basis for the sequential colonisation of adult gut-like functional groups including the LUB, followed by hydrogen-utilising community (i.e. sulphur-reducing bacteria, reductive acetogens and methanogens). We showed for the first time that *Bacteroides* spp. enabled the formation of butyrogenic trophic chain in the presence of human milk carbohydrates. Besides, we highlighted the distinct lactate isomer production by *Bifidobacterium* spp. and *Bacteroides* spp. suggesting that the balance of D- and L- lactate could impact gut bacterial structure via cross-feeding.

Acknowledgement

We thank Yan Qin and Eline Voogd for D- and L- lactate analysis, Edoardo Saccenti for advice on statistical analysis as well as Bastian V.H. Hornung for homologous protein check.

Supplementary materials

Table S1. The optical density, substrates and end products (mean ± standard deviation) detected in *Anaerostipes caccae* monoculture in lactose and total HM carbohydrates and media blanks at the end of fermentation.

	OD (600nm)	Lactose	Glucose	Galactose	GlucNAc	GalNAc	Fucose	Malate	Fumarate	Succinate	Citrate	Formate	Acetate	Butyrate	Isobutyrate	Lactate	1.2 propanediol	Propionate
Basal media	0.009 ± 0.016	-	0.013 ± 0.022	-	-	-	0.042 ± 0.073	0.121 ± 0.106	-	-	-	-	-	-	-	-	-	0.369 ± 0.640
Basal media with hemin and B vitamins + lactose + *A. caccae*	0.039 ± 0.008	26.484 ± 1.797	-	-	-	-	-	0.055 ± 0.110	-	-	-	-	-	-	-	-	-	0.793 ± 0.935
Basal media with hemin and B vitamins + total HM carbohydrates + *A. caccae*	0.049 ± 0.011	21.832 ± 1.863	1.402 ± 2.138	-	-	0.210 ± 0.145	0.036 ± 0.072	0.103 ± 0.119	-	-	-	-	-	-	-	-	-	0.757 ± 0.917

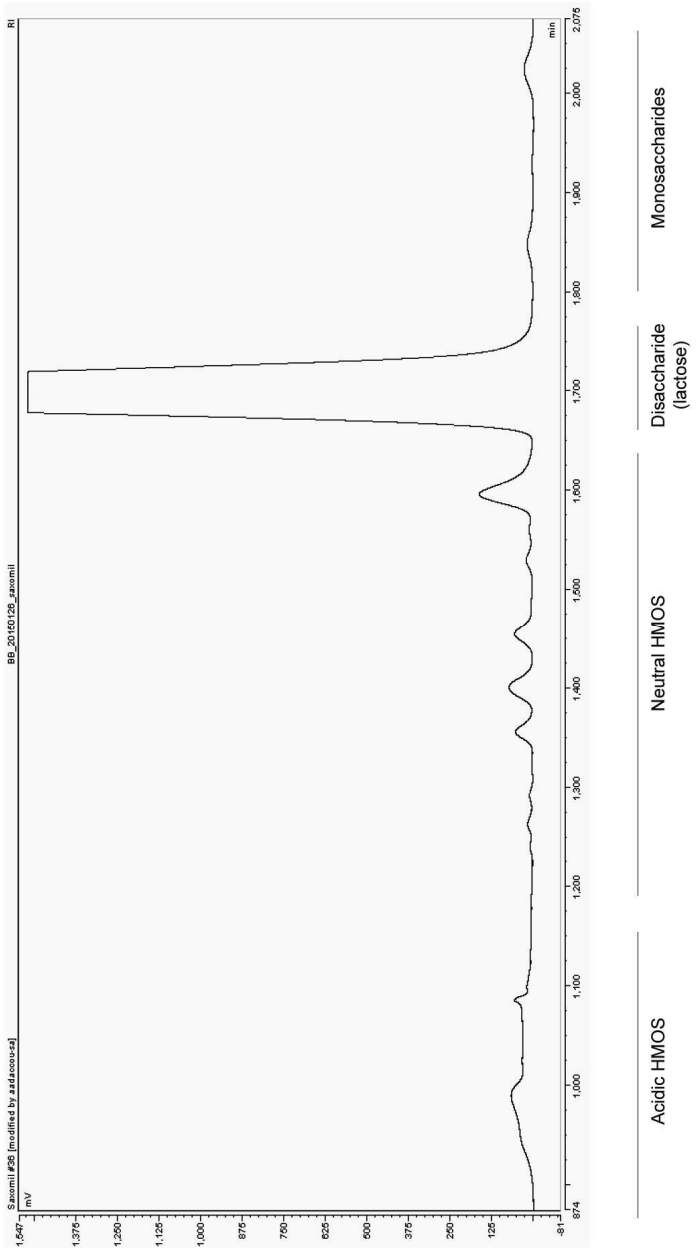


Figure S1. The GPC-RI chromatogram for total human milk (HM) carbohydrates showing a composition of approximately 90% of lactose, 10% of both acidic and neutral HMOS as well as traces of monosaccharides.

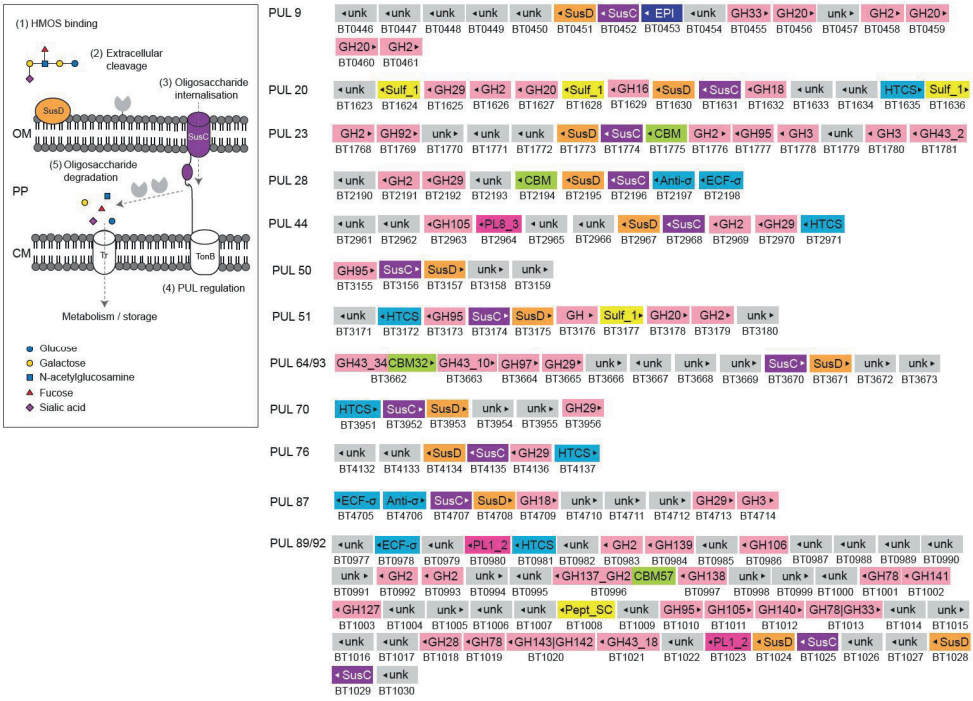
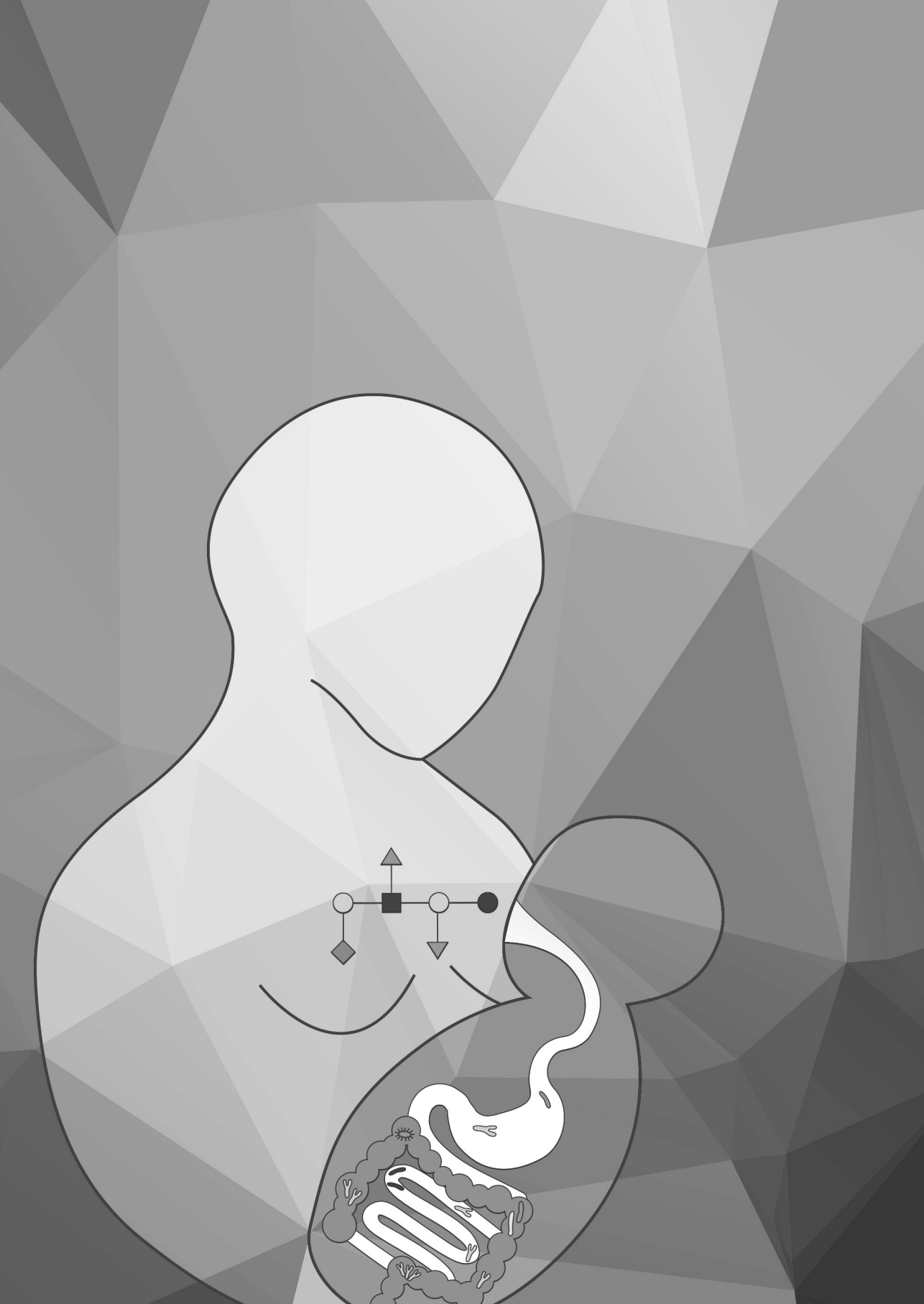


Figure S2. **Polysaccharide utilisation loci (PULs) for *Bacteroides thetaiotaomicron* DSM2079 predicted from PULDB (Terrapon *et al* 2017) with fucosidase (GH29/GH95) and sialidase (GH33) activity.** Abbreviation: HMOS, human milk oligosaccharides; OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane; Tr, transporter; ECF-σ, extracytoplasmic function sigma factor; Anti-σ, extracytoplamic function anti sigma factor; CBM, carbohydrate-binding module; EPI, epimerase; GH, glycoside hydrolase; HTCS, hybrid two-component system; PL, polysaccharide lyase; Pept_SC, peptidase + 2-letter MEROPS clan; *Sus*C, *Sus*C like TonB-dependent transporter; *Sus*D, *Sus*D-like outer membrane-binding protein; Sulf, sulfatase; unk, unknown.



Chapter 4

Microbial Metabolic Networks at the Mucus Layer Lead to Diet-Independent Butyrate and Vitamin B12 Production by Intestinal Symbionts

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This chapter has been published as Belzer C, **Chia LW**, Aalvink S, Chamlagain B, Piironen V, Knol J, de Vos WM. 2017. Microbial metabolic networks at the mucus layer lead to diet-independent butyrate and vitamin B12 production by intestinal symbionts. mBio 8:e00770-17.

Abstract

Akkermansia muciniphila has evolved to specialize in the degradation and utilisation of host mucus, which it may use as the sole source of carbon and nitrogen. Mucus degradation and fermentation by *Akkermansia muciniphila* are known to result in the liberation of oligosaccharides and subsequent production of acetate, which becomes directly available to microorganisms in the vicinity of the intestinal mucosa. Co-culturing experiments of *Akkermansia muciniphila* with non-mucus-degrading butyrate-producing bacteria *Anaerostipes caccae*, *Eubacterium hallii*, and *Faecalibacterium prausnitzii* resulted in syntrophic growth and production of butyrate. In addition, we demonstrate that the production of pseudo-vitamin B12 by *Eubacterium hallii* results in production of propionate by *Akkermansia muciniphila*, which suggests that this syntrophy is indeed bidirectional. These data are proof of concept for syntrophic and other symbiotic microbe-microbe interactions at the intestinal mucosal interface. The observed metabolic interactions between *Akkermansia muciniphila* and butyrogenic bacterial taxa support the existence of colonic vitamin and butyrate production pathways that are dependent on host-secreted glycan production and independent of dietary carbohydrates. We infer that the intestinal symbiont *Akkermansia muciniphila* can indirectly stimulate intestinal butyrate levels in the vicinity of the intestinal epithelial cells with potential health benefits to the host.

Importance

The intestinal microbiota is said to be a stable ecosystem where many networks between microorganisms are formed. Here we present a proof of principle study of microbial interaction at the intestinal mucus layer. We show that indigestible oligosaccharide chains within mucus become available for a broad range of intestinal microbes after degradation and liberation of sugars by the species *Akkermansia muciniphila*. This leads to the microbial synthesis of vitamin B12, 1,2-propanediol, propionate, and butyrate, which are beneficial to the microbial ecosystem and host epithelial cells.

Keywords

Akkermansia muciniphila, anaerobes, butyrate, cross-feeding, intestine, microbiome, mucus, syntrophy

Introduction

The mammalian intestinal tract harbours complex microbial ecosystems that have been forged by millennia of co-evolution between microbes and hosts. It is suggested that the evolution of metabolic interdependencies has led to strong deterministic processes that shape the composition of the microbiota during development (Ley *et al* 2008). The diversity and richness of the gut microbiota within individuals, as well as the similarity in composition between individuals, are governed by several selective pressures within host habitats, such as diet (Bokulich *et al* 2016, Zoetendal and de Vos 2014). Recent extreme interventions have illustrated the importance of dietary carbohydrates on the intestinal microbial community succession (David *et al* 2014, O'Keefe *et al* 2015). While dietary fibres affect substrate availability for the colonic microbiota, the mucus lining that covers the epithelial cells forms a consistent factor along its internal surface and is proposed to function as an endogenous prebiotic (Johansson *et al* 2008, Ouwehand *et al* 2005, Pacheco *et al* 2015, Tailford *et al* 2015). The mucosal layer of the intestine is characterised by specific microbiota communities enriched with taxa affiliated with the family *Lachnospiraceae* (also known as *Clostridium* cluster *XIVa*) and the phylum *Verrucomicrobia* (Arrieta *et al* 2014, Chen *et al* 2014, Hong *et al* 2011, Jakobsson *et al* 2015, Koropatkin *et al* 2012, Ouwerkerk *et al* 2013).

Akkermansia muciniphila is a mucus-colonising member of the gut microbiota that has evolved to specialize in the degradation and utilisation of host mucus, which it may use as the sole source of carbon and nitrogen (Belzer and de Vos 2012, Derrien *et al* 2004). Its mucin degradation activity leads to the production of 1,2-propanediol, propionate, and acetate (Derrien *et al* 2004). In addition, its mucus foraging results in the availability of sugars liberated from mucus glycans and subsequent acetate production can stimulate co-existence of butyrogenic bacteria within the same mucosal niche (Belzer and de Vos 2012). Microbe-produced short-chain fatty acids are described as major health-promoting compounds (Flint *et al* 2012b, Smith *et al* 2013). Because of its location close to the host cells, a symbiotic mucobiome could therefore be particularly important in fostering health in terms of nutrient exchange, communication with the host, regulation of the immune system, and resistance against invading pathogens.

Dietary intervention studies (Hong *et al* 2011), *in vitro* mucosal model studies (Van den Abbeele *et al* 2013), and microbiota comparisons of gut lumen and epithelial biopsy specimens (Chen *et al* 2014) have revealed strong co-occurrence of specific mucolytic bacteria (*Akkermansia muciniphila*, *Bacteroides* spp., and *Ruminococcus* spp.) and second-line butyrate producers (*Anaerostipes caccae*, *Eubacterium* spp., *Faecalibacterium prausnitzii*, and *Roseburia intestinalis*). This co-occurrence may be indicative of shared metabolic networks among the different microbial groups. *In vitro* isotope labelling has identified lactate and acetate as important precursors of butyrate production in human faecal samples (Morrison *et al* 2006). On top of this, kinetic modelling showed the likelihood for the dominant butyrate producers, such as *Anaerostipes coli* and *Eubacterium hallii*, to use short-chain fatty acids for butyrate production by utilising lactate and acetate via the butyryl coenzyme A (CoA):acetate CoA transferase route, the main metabolic pathway for butyrate synthesis in the human colon (Munoz-Tamayo *et al* 2011).

In this study, we test the hypothesis that *Akkermansia muciniphila* can serve as the keystone species supporting a syntrophic network in a mucosal environment. Therefore, we studied the metabolic interactions between *Akkermansia muciniphila* and representative intestinal butyrate-producing bacteria; *Faecalibacterium prausnitzii* (representative of the family *Ruminococcaceae* also known as *Clostridium* cluster *IV*) and *Anaerostipes caccae* and *Eubacterium hallii* (representatives of *Lachnospiraceae* also known as *Clostridium* cluster *XIVa*). The results indicate the existence of trophic chains on mucus between *Akkermansia muciniphila* and the butyrate-producing *Faecalibacterium prausnitzii* and *Anaerostipes caccae*, while true bidirectional metabolic cross-feeding dependent on vitamin B12 was observed between *Akkermansia muciniphila* and *Eubacterium hallii*, indicative of a mutualistic symbiosis.

Materials & Methods

Bacterial growth conditions. *Akkermansia muciniphila* Muc^T (ATTC BAA-835) was grown as described previously (Derrien *et al* 2004, Duncan *et al* 2002b). Purified mucin was prepared as follows. Ten grams of hog gastric mucins (type III; Sigma-Aldrich) was dissolved in 500 ml of 0.1 M sodium chloride (NaCl) (pH 7.8) containing 0.02 M phosphate buffer (0.02 M NaH_2PO_4 and Na_2HPO_4) (pH 7.8), stirring for 24 h at 4°C. After 1 h, the pH was adjusted to pH 7.2 using 1 M sodium hydroxide (NaOH). After centrifugation, the supernatant was cooled on ice and precipitated with 60% (v/v) pre-chilled ethanol. After centrifugation, the pellet was dissolved in 0.1 M NaCl. These last two steps were repeated twice. After the last centrifugation step, the pellet was washed once with 100% ethanol, dissolved in 100 ml Milli-Q, and dialyzed using Spectra/Por 6 8,000-Da MWCO (molecular weight cut-off) protein dialysis with four changes. Last, the dialyzed mucins were freeze dried and dissolved in Milli-Q at a concentration of 5% (w/v). Mucins were added to the medium after autoclaving. The resulting purified mucins were tested for the absence of oligosaccharides. Incubations were performed in serum bottles sealed with butyl rubber stoppers at 37°C under anaerobic conditions provided by a gas phase of 182 kPa (1.5 atm) N2/CO2 (80/20 ratio). Growth was measured by a spectrophotometer as the optical density at 600 nm (OD600).

Faecalibacterium prausnitzii A2-165 was grown anaerobically at 37°C in YCFA medium supplemented with 33 mM acetate and 25 mM glucose (Duncan *et al* 2002b). *Anaerostipes caccae* L1-92 (Schwiertz *et al* 2002) was grown anaerobically at 37°C in either PYG medium (DSMZ) or minimal medium (Plugge 2005) containing 25 mM glucose. *Eubacterium hallii* L2-7 was grown anaerobically at 37°C in YCFA medium without the addition of fatty acids (propionate, isovaleric acid, valeric acid, isobutyrate, and butyrate). Mucin sugar utilisation was performed in minimal medium with or without the addition of 10 mM acetate. In some cases, the experiments were performed with mucin-derived single sugars i.e. mannose (Sigma-Aldrich), fucose (Sigma-Aldrich), galactose (Biochemika), N-acetylgalactosamine (GalNAc; Sigma-Aldrich), or N-acetylglucosamine (GlcNAc; Sigma-Aldrich); these were used at a concentration of 25 mM. Growth was monitored for 24 h, and samples were collected regularly for OD600 and high-performance liquid chromatography (HPLC) analysis.

Co-culture experiments were performed in minimal medium supplemented with mucus (Derrien *et al* 2004), and the medium was buffered to reduce pH changes due to fermentation products. Optimal co-culture conditions were established as follows. *Akkermansia muciniphila* was added to media containing mucins, and the media containing bacteria were incubated for 8 h to reach measurable concentrations of acetate and liberate sugars. Subsequently, 10^8 cells of *Anaerostipes caccae*, *Eubacterium hallii*, or *Faecalibacterium prausnitzii* were added to the *Akkermansia muciniphila* containing cultures. All cells had been washed twice with phosphate-buffered saline (PBS) before being added to the co-culture to prevent carryover of products from the pre-culture. During the co-culture, 0.15% mucins was added to the medium every 48 h to maintain sufficient substrate availability for *Akkermansia muciniphila*. All growth experiments were repeated a minimum of three times in duplicate.

High-performance liquid chromatography (HPLC). For fermentation product analysis, 1 ml of bacterial culture was centrifuged, and the supernatant was stored at -20°C for HPLC analysis. Substrate conversion and product formation were measured with a Thermo Scientific Spectrasystem HPLC system equipped with a Varian Metacarb 67H column (300 by 6.5 mm) kept at 45°C and with 0.005 mM sulphuric acid as the eluent. The eluent had a flow rate of 0.8 ml/min, and metabolites were detected by determining the refractive index. Carbon balances were calculated by the amount of carbon of the products/amount of carbon of the substrate x 100%, using sugars and short-chain fatty acid (SCFA) as measured by HPLC with biological triplicate samples and technical duplicate samples. We used theoretical CO_2 calculations: 6 mol glucose yields 8 mol CO_2, and 1 mol lactate yields 1 mol CO_2.

Ultrahigh performance liquid chromatography-mass spectrometry (UHPLC-MS). For vitamin B12 analysis, *Eubacterium hallii* cells (0.2 g) were mixed with 10 ml of extraction buffer (8.3 mM NaOH and 20.7 mM acetic acid [pH 4. 5]) containing 100 µl of 1% sodium cyanide. The vitamin was extracted in its cyano form by subjecting the mixture to a boiling water bath for 30 min. After cooling, the extract was recovered by centrifugation (6,900 x *g* for 10 min; Hermle, Wehingen, Germany) and finally purified by immunoaffinity column chromatography (Easy-Extract; R-Biopharma, Glasgow, Scotland). The reconstituted extract was analysed for vitamin content using an HSS T3 C_{18} column (2.1 by 100 mm; 1.8 µm) on a Waters Acquity UPLC (ultraperformance

liquid chromatography) system (Milford, MA) equipped with a photodiode array detector (PDA) (210 to 600 nm) and interfaced to a high-resolution quadrupole time of flight mass spectrometer (QTOF; Synapt G2-Si, Waters). The eluent was a gradient flow (0.32 ml/min) of water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid: 0 to 0.5 min (95 parts solvent A to 5 parts of solvent B [95:5]), 0.5 to 5 min (60:40), 5 to 6 min (60:40), and 6 to 10 min (95:5). The column was maintained at 30°C, and the UV detection was recorded at 361 nm. The MS analysis was done in positive ion mode with electrospray ionization, using a scanning range set for m/z of 50 to 1,500. The parent ions corresponding to the vitamin peak were further fragmented (tandem mass spectrometry [MS/MS]) and analysed.

Fluorescent *in situ* hybridization (FISH). The following rRNA-targeted oligonucleotide probes were used: (i) Cy3-labeled universal EUB338 (5'-GCTGCCTCCCGTAGGAGT-3'), which is complementary to a conserved region of the bacterial 16S rRNA molecule specific to most eubacteria except phyla of *Plantomycetales* and *Verrucomicrobia* (Derrien *et al* 2004); and (ii) Cy5-labeled EUB338 III (5'-GCTGCCACCCGTAGGTGT-3'), the supplementary probes for eubacteria to target *Verrucomicrobia* (Daims *et al* 1999).

Cell fixation, *in situ* hybridization, DAPI staining, and microscopy. Bacterial cultures (0.5 ml) were fixed overnight with 1.5 ml of 4% paraformaldehyde (PFA) at 4°C. Working stocks were prepared by harvesting bacterial cells by 5 min centrifugation at 8,000 x *g*, followed by resuspension in ice-cold phosphate-buffered saline (PBS) and 96% ethanol at a 1:1 (v/v) ratio. Three microliters of the PBS-ethanol working stocks were spotted into 18 wells (round wells with a 6-mm diameter) on gelatine-coated microscope slides. The slides were hybridized with the DNA probes by applying 10 µl of hybridization mixture per well, which contained 1 volume of probe mixture (probe concentration of 20 µM) and 9 volumes of hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.1% SDS [pH 7.2]). The slides were hybridized for at least 3 h in a moist chamber at 50°C; this was followed by 30 min incubation in washing buffer (20 mM Tris-HCl, 0.9 M NaCl [pH 7. 2]) at 50°C for washing. The slides were rinsed briefly with Milli-Q and air dried. The slides were stained with a 4,6-diamine-2-phenylindole dihydrochloride (DAPI) mixture containing 200 µl PBS and 1 µl DAPI dye (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 and a coverslip. The bacteria

on the slides were enumerated using an Olympus MT ARC/HG epifluorescence microscope. A total of 25 positions per well were automatically analysed in three-color channels (DAPI, Cy3, and Cy5) using a quadruple band filter.

Quantitative real-time PCR. The abundances of *Akkermansia muciniphila* and butyrate producers in co-culture were determined by quantitative real-time PCR. Bacterial cultures were harvested at 16,100 x *g* for 10 min. DNA extractions were performed using MasterPure Gram-positive DNA purification kit. The DNA concentrations were determined fluorometrically (Qubit dsDNA HS [double-stranded DNA highsensitivity] assay; Invitrogen) and adjusted to 1 ng/µl prior to use as the template in quantitative PCR (q-PCR). Primers targeting *Akkermansia muciniphila*, *Anaerostipes caccae*, and *Eubacterium hallii* based on specific variable regions of the 16S rRNA gene (Table 1) were used for quantification. Standard template DNA was prepared from the 16S rRNA gene of each bacterium by amplification with primers 27F (F stands for forward) and 1492R (R stands for reverse). Standard curves were prepared with nine standard concentrations of 10^0 to 10^8 gene copies/µl. PCRs were performed in triplicate with iQ SYBR green supermix (Bio-Rad) in a total volume of 10 µl with primers at 500 nM in the wells on 384-well plates with the wells sealed with optical sealing tape. Amplification was performed with an iCycler (Bio-Rad) and the following protocol: one cycle of 95°C for 10 min; 35 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s each; one cycle of 95°C for 1 min; one cycle of 60°C for 1 min; and a stepwise increase of the temperature from 60 to 95°C (at 0.5°C per 5 s) to obtain melt curve data. Data were analysed using the Bio-Rad CFX Manager 3.0.

Statistics. Statistics were performed using *t* test and corrected for multiple testing using false discovery rate (FDR) correction for multiple comparisons. P values of < 0.05 were considered significant.

Table 1. PCR primers used in this study and their amplification products.

Bacterium	Primer	Primer Sequence	Product size (bp)	Reference
Akkermansia muciniphila	AM1 AM2	5'-CAGCACGTGAAGGTGGGGAC-3' 5'-CCTTGCGGTTGGCTTCAGAT-3'	327	(Collado *et al* 2007)
Anaerostipes caccae subgroup	OFF2555 OFF2556	5'-GCGTAGGTGGCATGGTAAGT-3' 5'-CTGCACTCCAGCATGACAGT-3'	83	(Veiga *et al* 2010)
Eubacterium hallii L2-7	EhalF EhalR	5'-GCGTAGGTGGCAGTGCAA-3' 5'-GCACCGRAGCCTATACGG-3'	278	(Ramirez-Farias *et al* 2009)
Faecalibacterium prausnitzii	FPR2F Fprau645R	5'-GGAGGAAGAAGGTCTTCGG-3' 5'-AATTCCGCCTACCTCTGCACT-3'	248	(Ramirez-Farias *et al* 2009)

Results

Growth and metabolism of intestinal butyrate producers on mucus or mucus-derived sugars

In order to test whether *Akkermansia muciniphila* can serve as a keystone species in an environment where mucus is the main nutrient source, we first tested the ability of butyrate-producing mucosal colonisers to grow on mucus and mucus-derived sugars in the absence of *Akkermansia muciniphila*. When incubated in culture media with mucus as the sole carbon and nitrogen source, none of the butyrate-producing strains tested, *Anaerostipes caccae*, *Eubacterium hallii*, and *Faecalibacterium prausnitzii*, were able to grow or produce metabolites (Table S2A).

The mucin sugars D-galactose, D-mannose, GlcNAc, GalNAc, and L-fucose and the non-mucin sugar glucose were subsequently tested as possible carbon sources for each butyrate-producing species. Minimal media used for the bacteria differed as a result of different minimal requirements for protein and spore elements (see Materials and Methods for details on the composition of the media). *Faecalibacterium prausnitzii* is known to be able to grow on GlcNAc and galactose (Munoz-Tamayo *et al* 2011). In addition, we tested the growth of *Faecalibacterium prausnitzii* on mannose and GalNAc, but no growth was observed (Table S2B). *Anaerostipes caccae* was observed to use glucose, D-mannose, D-galactose, and GlcNAc for growth, and the main fermentation products were acetate, butyrate, and lactate (Fig. 1). The highest *Anaerostipes caccae* cell numbers and acetate production were reached with GlcNAc, possibly due to the fact that fermentation of this amino sugar can replace the need for acetate in the medium (Fig. 1). *Eubacterium hallii* showed the same preference for sugars as *Anaerostipes caccae* did, resulting in growth on glucose, D-mannose, D-galactose, and GlcNAc (Fig. 2). The main fermentation products of *Eubacterium hallii* were observed to be acetate, butyrate, and formate. Again, GlcNAc resulted in the highest production of acetate and butyrate compared to the other sugars, but this was not accompanied with increased cell numbers of *Eubacterium hallii* (Fig. 2).

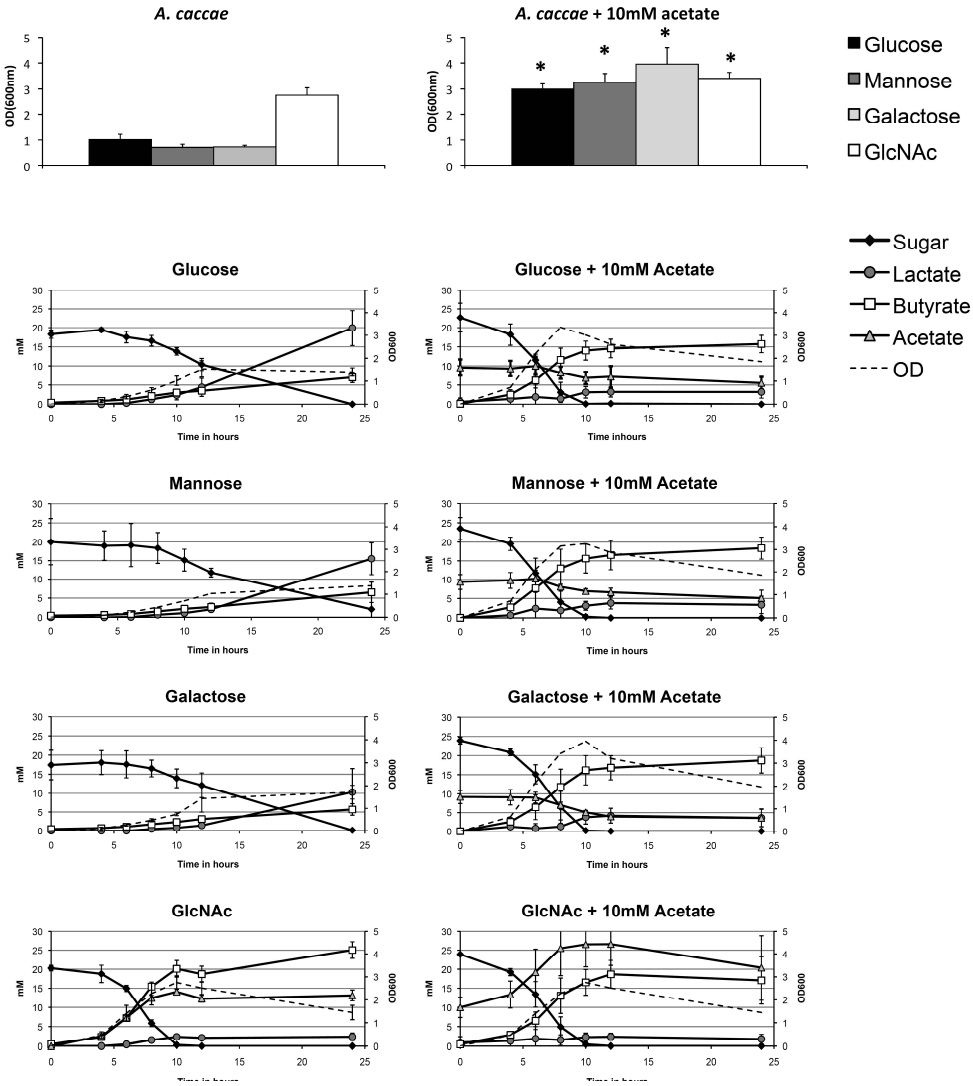


Figure 1. **Metabolic activity of *Anaerostipes caccae* on mucin-derived sugars.** *Anaerostipes caccae* was grown on monosaccharide present in the glycan chain of mucins. The OD600 values and HPLC profiles are shown for the sugars that resulted in positive growth. The sugars that gave positive test results were also used to perform experiments with the addition of 10 mM acetate. The graphs show the mean values for the experiments performed a minimum of three times in duplicate. Values that are significantly different ($P < 0.05$) in the presence of 10 mM acetate or absence of acetate are indicated by an asterisk.

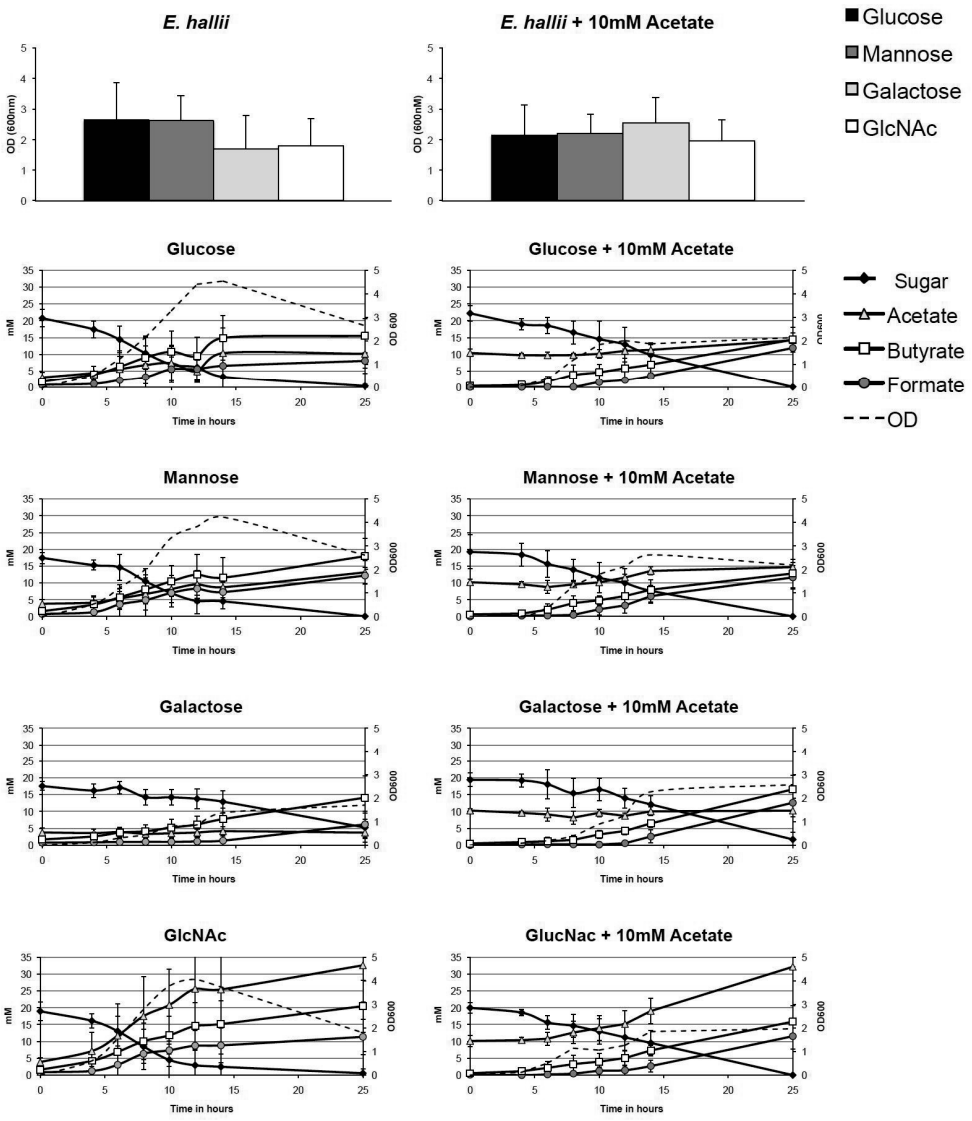


Figure 2. **Metabolic activity of *Eubacterium hallii* on mucin-derived sugars.** *Eubacterium hallii* was grown on monosaccharide present in the glycan chain of mucins. The OD600 value and HPLC profiles are shown for sugars that resulted in positive growth. The sugars that gave positive test results were also used to perform experiments with the addition of 10 mM acetate. The graphs show the mean values for the experiments performed a minimum of three times in duplicate.

Acetate enhances growth of Anaerostipes caccae but not Eubacterium hallii on mucin-derived sugars

The average production of 10 mM acetate by *Akkermansia muciniphila* grown in medium containing mucins could serve as the substrate for growth of butyrogens. Therefore, we added 10 mM acetate to cultures growing on glucose, D-mannose, D-galactose, and GlcNAc. In the case of *Anaerostipes caccae*, this did indeed lead to the production of butyrate, acetate, lactate, and formate as measured in a minimal medium. Furthermore, these butyrate production levels were significantly higher than the observed butyrate production without added acetate (Fig. 1). Weak growth of *Anaerostipes caccae* on L-fucose was observed after the addition of acetate but without detected metabolite production. Acetate alone did not support growth (Table S2C). The addition of acetate to the growth media of *Eubacterium hallii* did not result in differences in growth or metabolite profile, possibly due to its own production of acetate (Fig. 2). The overall fermentation efficiency was determined by calculating the carbon balance at each monosaccharide condition. The recovery of carbon atoms varied in between 70 and 100%, depending on the biomass produced that explains the loss (Tables 2 and 3).

Table 2. Carbon balance of *Anaerostipes caccae* on mucin-derived sugars with or without acetate

Sugar	No. of carbons (mM)							Carbon recovery (%)	
	Substrates		Products						
	Sugar	Acetate	Lactate	Acetate	Butyrate	Formate	CO_2	Avg	SD
Glucose	110		60		26		24	101	13
Glucose + 10 mM acetate	136	8	8		62	2	82	71	0
Mannose	121		55		27		27	85	12
Mannose + 10 mM acetate	140	8	10		73	2	76	78	8
Galactose	99		38		26		22	88	10
Galactose + 10 mM acetate	144	11	11		75	2	59	77	12
GlcNAc	162		7	26	98		27	98	2
GlcNAc + 10mM acetate	192		5	31	84	3	34	81	11

Table 3. Carbon balance of *Eubacterium hallii* on mucin-derived sugars with or without acetate

Sugar	No. of carbons (mM)							Carbon recovery (%)	
	Substrates		Products						
	Sugar	Acetate	Lactate	Acetate	Butyrate	Formate	CO_2	Avg	SD
Glucose	122			14	55	7	27	87	30
Glucose + 10 mM acetate	133			8	56	12	29	79	18
Mannose	106			19	66	12	24	117	24
Mannose + 10 mM acetate	115			9	49	12	26	85	21
Galactose	74	0.1			50	5	16	96	29
Galactose + 10 mM acetate	106	0.1			64	13	24	93	14
GlcNAc	147			57	76	11	25	116	40
GlcNAc + 10mM acetate	160			44	61	11	27	90	19

Mucus-induced trophic chains of Akkermansia muciniphila and butyrate producers Anaerostipes caccae, Eubacterium hallii, and Faecalibacterium prausnitzii results in butyrate production

After the monoculture experiments, a series of co-cultures of approximately equal amounts of *Akkermansia muciniphila* and butyrate producers were set up to test whether sugars and acetate produced as a result of mucin degradation by *Akkermansia muciniphila* would enable butyrate production of the chosen isolates. Remarkably, this co-culturing on mucin-containing media supported growth and butyrate production for all three tested species (Fig. 3). *Anaerostipes caccae* produced butyrate in levels comparable to those found in the monoculture conditions that were supplemented with acetate. Similarly, *Faecalibacterium prausnitzii* also produced butyrate in co-culture with *Akkermansia muciniphila* and also produced 5 mM formate indicative of acetate consumption. Butyrate levels produced by *Eubacterium hallii* were in the range of what was seen in the monocultures growing on single sugars. The pH was monitored in all experiments and stayed around pH 6.5 throughout the experiments. Determination by quantitative PCR (q-PCR) and qualitative presence (fluorescent *in situ* hybridization [FISH]) of the butyrate-producing species within the co-cultures indicated a difference in abundance of the butyrate producers of several

log units compared to the abundance of *Akkermansia muciniphila* (Fig. 3 and Table S1). The abundance of *Anaerostipes caccae* increased 100-fold over the first 8 days of incubation based on the increase in its 16S rRNA gene copy number. Maximum butyrate levels were reached after 11 days of incubation. In contrast to the results for cultures, no lactate was measured during the cross-feeding experiments with *Anaerostipes caccae.* Both q-PCR and FISH results indicated a ratio of *Akkermansia muciniphila* to *Anaerostipes caccae* of approximately 100:1.

In the *Faecalibacterium prausnitzii-Akkermansia muciniphila* co-cultures, *Faecalibacterium prausnitzii* 16S rRNA gene copy numbers decreased, and a small amount of butyrate appeared after 8 days of incubation. FISH staining revealed the presence of *Faecalibacterium prausnitzii* cells within the co-cultures but confirmed its slow growth. Finally, within the *Eubacterium hallii-Akkermansia muciniphila* co-cultures, low levels of butyrate started to build up after 8 days. This was associated with an increase in 16S rRNA gene copy numbers of *Eubacterium hallii* on day 8. Q-PCR and FISH staining showed an *Akkermansia muciniphila*-to-*Eubacterium hallii* ratio of 100:1 after 8 to 24 days (Fig. 3 and Table S1).

Vitamin B12-dependent syntrophy between Eubacterium hallii and Akkermansia muciniphila

Analyses of the metabolites produced in co-cultures showed that in the *Akkermansia muciniphila-Eubacterium hallii* co-culture, the proportion of succinate to propionate had shifted compared to the proportion in monocultures of *Akkermansia muciniphila* (Fig. 3). This was not observed in the other co-cultures. Notably, 1,2-propanediol, found as a result of fucose degradation by *Akkermansia muciniphila* in monocultures, was not detected in the co-culture with *Eubacterium hallii*.

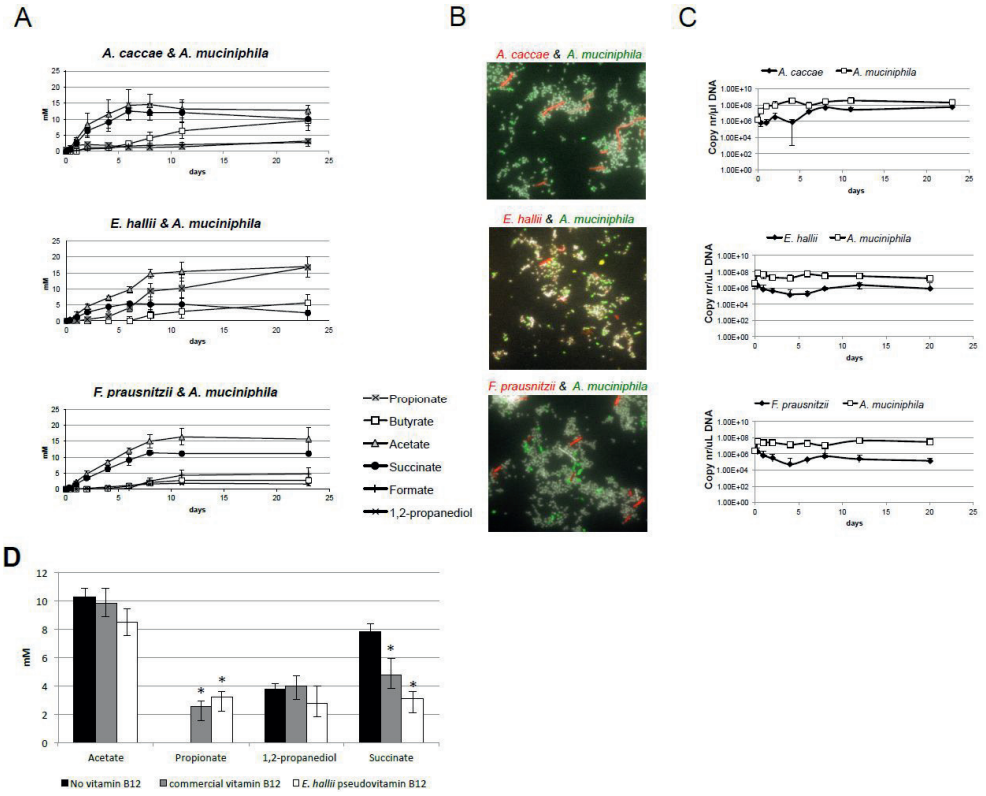


Figure 3. ***Akkermansia muciniphila* degradation and fermentation of mucus enables cross-feeding by the butyrate-producing gut isolates.** (A to C) Co-cultures of *Akkermansia muciniphila* with butyrate-producing isolates were performed and measurements of product formation and consumption (A), FISH staining (B), and q-PCR (C) were performed. (D) Measurement of *Akkermansia muciniphila* metabolites on mucus-containing media without the addition of vitamin B12 or with vitamin B12 from *Eubacterium hallii* or pseudo-vitamin B12 from *Eubacterium hallii*. The graph shows the mean values for the experiment performed a minimum of three times in duplicate. Asterisks indicate a significant difference ($P < 0.05$) compared to the condition without vitamin B12 added.

Conversion of propionate to succinate involves vitamin B12-dependent methylmalonyl-CoA mutase

Detailed mass spectroscopy analysis confirmed that Eubacterium hallii is capable of synthesizing a B12 vitamer in monocultures as described previously (Engels *et al* 2016). Our analyses show that the structure of this vitamer (Fig. 4) is pseudo-vitamin B12, as the lower ligand contained adenine instead of 5,6-dimethyl benzimidazole

(DMBI). No effect of DMBI addition was observed on the structure of the produced B12 vitamer.

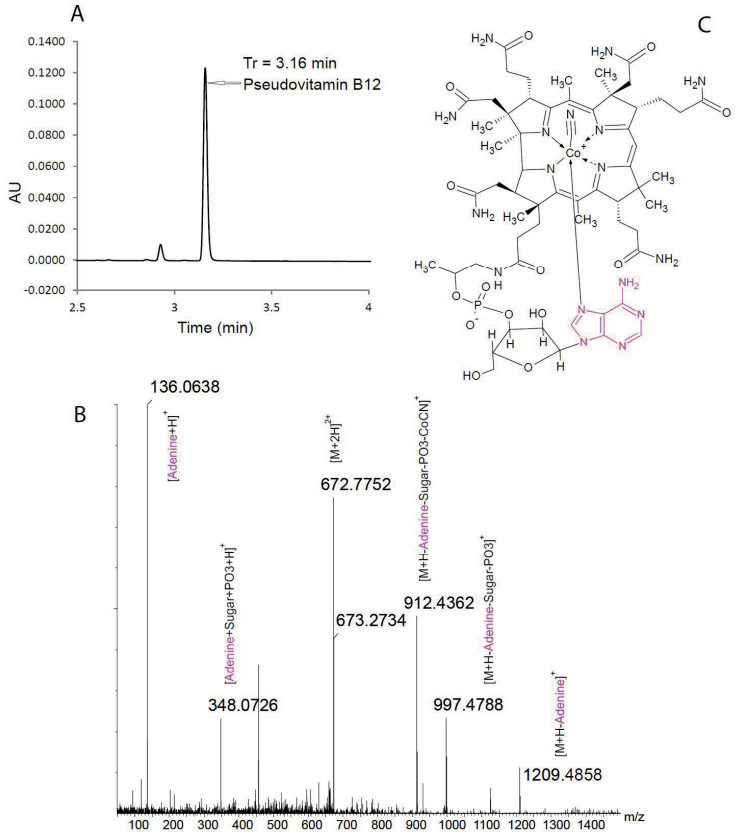


Figure 4. UHPLC-UV chromatogram of *Eubacterium hallii* vitamin B12. (A) Immunoaffinity-purified cell extract of *Eubacterium hallii* (in arbitrary units [AU]) is shown on the y axis, and time (in minutes) is shown on the x axis. Tr, retention time. (B) LC-MS/MS identified a peak at 3.16 min. (C) Chemical structure of pseudo-vitamin B12 from *Eubacterium hallii*.

To test the hypothesis that *Akkermansia muciniphila* can use the pseudo-vitamin B12 produced by *Eubacterium hallii* for the conversion of succinate to propionate, the effects of both purified *Eubacterium hallii* and commercially available vitamin B12 on *Akkermansia muciniphila* growth were tested. Indeed, the addition of pseudo-vitamin B12 and vitamin B12 resulted in significant lower succinate levels and significant higher propionate production. The addition of either vitamin B12 resulted in a profile identical to the profile observed for *Akkermansia muciniphila*- *Eubacterium*

hallii co-culture (Fig. 3). These observations provide evidence for bidirectional metabolic cross-feeding between *Akkermansia muciniphila* and *Eubacterium hallii*. *Akkermansia muciniphila* liberates sugars from mucus and produces 1,2-propanediol for growth support of *Eubacterium hallii*. In return, *Akkermansia muciniphila* is provided with a vitamin B12 analogue used as a cofactor for the conversion of succinate to propionate via methylmalonyl-CoA synthase (Fig. 5). Apparently both vitamin B12 and pseudo-vitamin B12 can be used as a cofactor by *Akkermansia muciniphila* to activate the methylmalonyl-CoA synthase. Hence, the B12 vitamer produced by *Eubacterium hallii* is in the pseudo-vitamin B12 form and can be used by other intestinal microorganisms, but it has lower affinity than vitamin B12 for the human intrinsic factor (Stupperich and Nexo 1991).

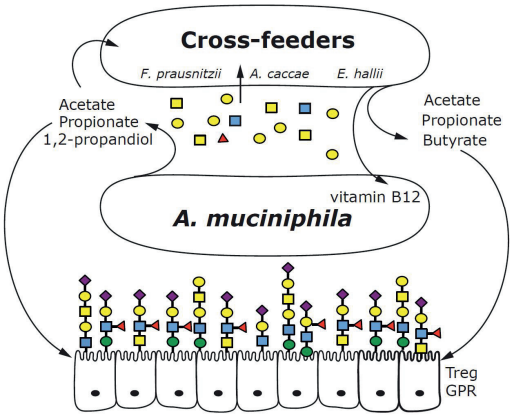


Figure 5. **Schematic overview of mucus-dependent cross-feeding network.** Keystone mucolytic bacteria, such as *Akkermansia muciniphila*, degrade mucin glycans resulting in oligosaccharides (mainly galactose, fucose, mannose, and GlcNAc) and SCFAs (acetate, propionate, and 1,2-propanediol) that can be used for growth, as well as for propionate, butyrate, and vitamin B12 production by cross-feeding partners. Treg GPR, regulatory T cell G-protein-coupled protein receptor.

Discussion

In spite of the great interest in metabolic conversions in the human gut, there is limited information on actual product sharing mechanisms and trophic dependencies of individual members of the intestinal microbiota. One such syntrophic relationship has been described for the species *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii* (Wrzosek *et al* 2013). *Faecalibacterium prausnitzii* can metabolise acetate produced by Bacteroides thetaiotaomicron to produce butyrate. This butyrate is then utilised by host epithelial cells and regulates host immunity via epithelial cell signalling, colonic T regulatory cells, and macrophages (Chang *et al* 2014, Smith *et al* 2013). In addition, a few studies demonstrated the use of lactate and acetate produced by *Bifidobacterium* spp. by colonic butyrate producers (Belenguer *et al* 2006, Falony *et al* 2006, Rios-Covian *et al* 2015). Specifically, this form of cross-feeding has been described for *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii* (Rios-Covian *et al* 2015).

Moreover, co-cultivation of amylolytic bacteria from the human colon, such as *Eubacterium rectale*, *Bacteroides thetaiotaomicron*, or *Bifidobacterium adolescentis*, with *Ruminococcus bromii* L2-63 can lead to increased starch utilisation (Ze *et al* 2012). In addition, co-culturing of the non-starch-degrading species *Anaerostipes hadrus* with *Ruminococcus bromii* has been shown to result in the removal of the reducing sugars that accumulate in *Ruminococcus bromii* monocultures (Ze *et al* 2013). Similarly, by stable isotope probing with ^{13}C-labeled resistant starch has revealed a butyrogenic trophic chain between *Ruminococcus bromii* and *Eubacterium rectale* in an *in vitro* human colon model (Kovatcheva-Datchary *et al* 2009, Walker *et al* 2011).

Various studies have coupled co-occurrence networks of bacteria to their genome content to model possible metabolic cross-feeding (Levy and Borenstein 2013, Munoz-Tamayo *et al* 2011). It should be noted that the studies discussed above all focus on cross-feeding that relies on diet-derived colonic sugars. However, mucin-derived sugars are the main source of energy for a group of microbiota members that can directly impact host cross talk at the mucosa (Wrzosek *et al* 2013). Mucus-dependent microbial networks at the mucosal layer would yield butyrate and other components with health benefits to the host (Wrzosek *et al* 2013). Our study supports the hypothesis that cross-feeding between microbiota members can take place when

mucus is the only carbon source to support growth. Such mucosal trophic networks could determine host microbial cross talk in immune and metabolic regulation.

The mucosa-colonising bacterium *Akkermansia muciniphila* is strongly correlated with a lean phenotype and increased barrier function (Everard *et al* 2013, Mack *et al* 2016, Reunanen *et al* 2015). The correlation between *Akkermansia muciniphila* and host might depend on an additional microbial player. Indeed, we have shown that the mucus-degrading capacity of *Akkermansia muciniphila* may provide substrates to butyrate producers tested. Two distinct types of trophic chains between *Akkermansia muciniphila* and butyrate-producing species were observed in this study. In the case of *Anaerostipes caccae*, liberated sugars from mucus could sustain growth but *Akkermansia muciniphila*-derived acetate increased growth and metabolic production of butyrate even further, indicative of metabolic syntrophic interactions. In the case of *Eubacterium hallii*, a specific metabolic and cofactor syntrophic interaction was observed; pseudo-vitamin B12 affected the carbon flux within *Akkermansia muciniphila*, resulting in propionate production.

It is known from human studies that propionate delivered to the colon has various beneficial effects, including the regulation of satiety (Chambers *et al* 2015). Remarkably, *Eubacterium hallii* was able to utilise mucus sugars, in agreement with an earlier report (Duncan *et al* 2004). However, *Eubacterium hallii* had no clear advantage when acetate was present, possibly due to its own production of acetate when grown on mucus-derived sugars that already reached levels comparable to that of *Akkermansia muciniphila* monoculture.

Recently, it was reported that *Eubacterium hallii* is also able to use 1,2-propanediol for the production of propionate. Our data show the lack of 1,2-propanediol in the *Akkermansia muciniphila- Eubacterium hallii* co-culture and supports the previous suggested syntrophic possibilities between intestinal microbes (Engels *et al* 2016). 1,2-propanediol is produced by *Akkermansia muciniphila* from fucose. As such, the presence or absence of fucose in the intestinal mucosa (FUT2 polymorphism) may help explain microbial networks at the mucosal layer (Tanaka *et al* 2009). Furthermore, in co-culture experiments with *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, low levels of butyrate were measured accompanied by the presence of cells and 16S rRNA copies of this butyrate producer as opposed to monocultures of the

organism on the same medium (Table S2A). These results further indicate that the association of butyrate *Clostridium* cluster *XIVa* and *IV* species could indeed yield the production of butyrate as a result of a microbial metabolic network in the mucosal layer, which is poor in usable carbon sources.

The fact that a changed metabolic profile for *Akkermansia muciniphila* in the presence of *Eubacterium hallii* was found is further evidence supporting a mutualistic syntrophic interaction. The availability of pseudo-vitamin B12 *in vivo* can be of importance for the microbial ecosystem as well as the host. Microorganisms are the only natural sources of the pseudo-vitamin B12 derivatives, and several intestinal microbes have been reported to contribute to the pseudo-vitamin B12 levels in the intestine (Krautler 2005). The approximate concentration of the cobalamin analogue adenine (as produced by *Eubacterium hallii*) is 164 ng/g (wet weight) of faeces (Allen and Stabler 2008), and this is also in the range of what we found to be needed for *Akkermansia muciniphila* propionate induction (100 ng/ml). It is not clear whether pseudo-vitamin B12 can be used by intestinal cells. While the affinity of human intrinsic factor for pseudo-vitamin B12 is lower than that for vitamin B12, it is equally bound by transcobalamin and haptocorrin human intrinsic factors (Stupperich and Nexo 1991) and is not antagonistic to vitamin B12 (Watanabe *et al* 1999), and it may be transported without intrinsic factor (Doets *et al* 2013). Moreover, it has been shown that pseudo-vitamin B12 produced by *Lactobacillus reuteri*, also an abundant mouse intestinal bacterium, can alleviate vitamin B12 deficiency in mice (Molina *et al* 2009, Santos *et al* 2007).

In summary, the present data indicate that pseudo-vitamin B12 is biologically active in *Akkermansia muciniphila* propionate metabolism that involves methylmalonyl-CoA mutase (van Passel *et al* 2011). Hence, the syntrophic partners together produce a higher propionate-to-succinate ratio, and this in turn is beneficial for host cell metabolism. It also implies that stimulating or diminishing a keystone species, such as *Akkermansia muciniphila*, from the microbiota can have drastic effect on a complete microbial network and associated host-microbe homeostasis. In this case, stimulating or administrating *Akkermansia muciniphila* within the intestine might benefit from addition of another organism or solely pseudo-vitamin B12 to stimulate the organism's production of propionate and a healthy mucosal environment (Fig. 5).

Many gastrointestinal disorders have been associated with mucosal damage and lower gut barrier function. The fact that intestinal bacteria may have an impact on both these factors, either directly or via specific immune and metabolic stimulation, further emphasizes the importance of having the right bacteria at the right place. Loss of mucosal integrity and the associated mucobiome could be indicative of disease states and its development. *Akkermansia muciniphila* has been positively associated with a lean phenotype and beneficial metabolic gene regulation in human cell types (Everard *et al* 2013, Lukovac *et al* 2014). Its presence might be essential for a mucosal adherent network of beneficial microorganisms that together prompt these effects of the host. As a matter of fact, weight loss studies usually report increased abundance of *Verrucomicrobia* (mainly *Akkermansia muciniphila*) as well as several other microbial species (Liou *et al* 2013, Remely *et al* 2015, Ward *et al* 2014). Taken together, these results further indicate the possible importance of mucosa-associated microbial networks and their metabolic cross-feeding for regulation of host health-related parameters and prevention of disease.

Acknowledgements

We thank Harm Wopereis, Nam Bui, Caroline Plugge, and Fons Stams for their constructive comments.

This work was supported by Advanced research grant 250172 (MicrobesInside) of the European Research Council to W.M.D.V., the Gravity 024.002.002 (SIAM) and Spinoza grants of the Netherlands Organization for Scientific Research (NWO) to W.M.D.V.

Jan Knol and Loo Wee Chia are financially supported by Nutricia Research, but there was no involvement of the company in the content of this work.

Supplementary material

Table S1. The count of mucin-degrading and butyrate-producing bacteria in the co-cultures assessed by FISH.

Co-culture	Time (day)	Cy5-labelled Mucin degrader		Cy3-labelled Butyrate producer		Ratio of Mucin degrader / Butyrate producer
		Avg	Stdev	Avg	Stdev	
A. muciniphila + A. caccae						
t=0h inoculation	1	8.73E+07	2.32E+07	4.90E+06	5.92E+06	17.82
	2	8.52E+07	1.37E+07	3.96E+06	1.08E+06	21.49
	4	3.25E+07	3.95E+06	2.33E+07	1.49E+07	1.40
	6	2.40E+07	2.54E+06	1.99E+07	1.97E+07	1.20
t=8h inoculation	1	1.31E+08	7.20E+06	2.59E+06	1.95E+06	50.57
	2	7.38E+07	2.73E+07	1.20E+07	1.27E+07	6.13
	4	3.04E+07	7.10E+05	1.04E+07	1.09E+07	2.92
	6	1.59E+07	1.11E+06	1.72E+07	1.63E+07	0.92
t=0h with	1	2.52E+08	1.25E+07	6.80E+05	7.46E+05	370.37
washed	2	2.20E+08	1.48E+08	2.25E+07	2.54E+07	9.79
inoculum	4	5.62E+07	3.34E+07	1.38E+07	8.90E+05	4.07
	6	2.68E+07	7.01E+06	3.76E+06	1.60E+06	7.13
t=8h with	1	2.24E+08	2.68E+07	5.46E+06	6.93E+06	40.98
washed	2	1.92E+08	4.81E+07	6.33E+06	6.26E+06	30.26
inoculum	4	3.55E+07	1.83E+07	3.77E+07	4.12E+07	0.94
	6	2.28E+07	-	2.38E+06	-	9.56
A. muciniphila + E. hallii						
	1	1.79E+07	1.06E+07	8.83E+06	4.31E+06	2.03
	2	5.08E+07	2.22E+07	3.61E+06	2.32E+06	14.10
	4	4.22E+07	2.50E+07	6.46E+06	3.06E+06	6.53
	6	1.91E+07	4.03E+06	5.11E+06	7.79E+05	3.74
A. muciniphila + F. prausnitzii						
	1	1.69E+07	1.03E+07	1.60E+07	8.56E+06	1.06
	2	7.48E+07	3.24E+07	8.04E+06	4.22E+06	9.31
	4	2.59E+07	5.63E+06	3.14E+06	8.81E+05	8.26
	6	2.34E+07	1.18E+07	6.84E+06	2.40E+06	3.42

Table S2. The optical density, substrates and end products detected in the positive and negative controls at the end of fermentation.

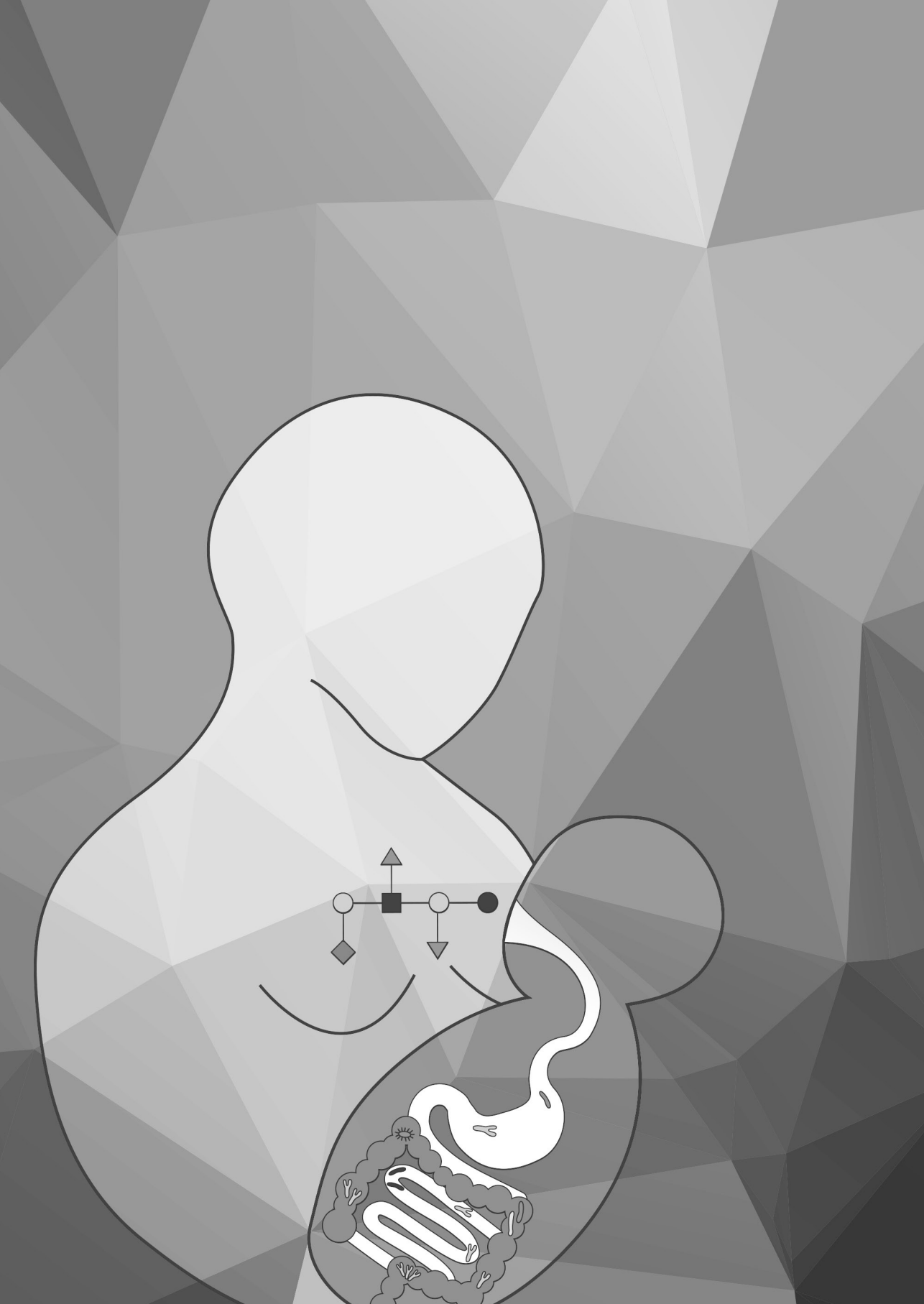
	OD600 Max		GlucNac		GalN		Galactose		Mannose		L-Fucose		1.2 propanediol		Acetate		Galactose		Glucose		Propionate		Succinate	
growth on mucin media	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
Unicoculated mucin media	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
A. muciniphila	1,24	0,23	-	-	0,03	0,06	0,12	0,12	-	-	0,03	0,05	1,63	0,39	16,10	3,53	-	-	-	-	0,47	0,69	13,79	2,72
A. caccae	0,10	0,19	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
E. hallii	0,015	0,08	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
F. prausnitzii	-0,055	0,02	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-

N.D. = Not detected

	OD600 Max		GalNac		Mannose		Acetate		Butyrate		Formate		Glucose		Lactate		Propionate	
F. prausnitzii	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
YCFA + glucose	2,17	0,12	N.D.		N.D.		2,013092	4,09044	12,165432	2,6320785	20,57033	4,673598	-14,2957	5,439799	0,780973	0,423085	1,48768	1,264443
YCFA + mannose	0,07	0,01	N.D.		-3,77	2,31	-3,11	1,90	0,52	0,48	N.D.		N.D.		N.D.		-0,75	0,84
YCFA + GalNac	0,03	0,01	-1,90	0,96	N.D.		-3,61	4,02	0,48	0,37	N.D.		N.D.		N.D.		-1,33	0,80

	OD600 at 24h		L-Fucose		Acetate		Butyrate		Glucose		Isobutyrate		Lactate		Propionate		Succinate	
A. caccae	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
CP + Glucose	1,40	0,18	-	-	0,17	0,41	6,98	1,32	-	-	-	-	19,93	4,35	0,46	0,71	-	-
CP + Acetate	0,03	0,03	-	-	-	-	8,61	1,21	-	-	0,08	0,11	-	-	-	-	0,27	0,40
CP + Acetate & Lactate	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
CP + L-Fucose	0,01	0,02	22,38	1,04	-	-	0,82	0,13	-	-	-	-	0,06	0,16	0,93	1,45	-	-
CP + Acetate & L-Fucose	0,06	0,01	24,67	0,91	9,13	1,36	0,90	0,38	0,05	0,11	0,31	0,75	0,35	0,86	0,43	0,84	-	-

Chapter 4



Chapter 5

Deciphering trophic interaction between *Akkermansia muciniphila* and the butyrogenic gut commensal *Anaerostipes caccae* using a metatranscriptomic approach

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This chapter has been published as **Chia LW**, Hornung BVH, Aalvink S, Schaap PJ, de Vos WM, Knol J, Belzer C. Deciphering trophic interaction between *Akkermansia muciniphila* and the butyrogenic gut commensal *Anaerostipes caccae* by metatranscriptomics. Antonie van Leeuwenhoek. 10.1007/s10482-018-1040-x.

Abstract

Host-secreted glycans are paramount in regulating the symbiotic relationship between humans and their gut bacteria. The constant flux of host-secreted mucins at the mucosal layer creates a steady niche for bacterial colonisation. Mucin degradation by keystone species subsequently shapes the microbial community. This study investigated the transcriptional response of mucin-driven trophic interaction between the specialised mucin-degrader *Akkermansia muciniphila* and a butyrogenic gut commensal *Anaerostipes caccae*. *Akkermansia muciniphila* monocultures and co-cultures with non-mucolytic *Anaerostipes caccae* from the *Lachnospiraceae* family were grown anaerobically in minimal media supplemented with mucins. We analysed for growth, metabolites (HPLC analysis), microbial composition (quantitative reverse transcription PCR), and transcriptional response (RNA-seq). Mucin degradation by *Akkermansia muciniphila* supported the growth of *Anaerostipes caccae* and concomitant butyrate production predominantly via acetyl-CoA pathway. Differential expression analysis (DESeq2) showed the presence of *Anaerostipes caccae* induced changes on *Akkermansia muciniphila* transcriptional response with increased expression of mucin degradation genes and reduced expression of ribosomal genes. Two putative operons that encode for uncharacterised proteins and an efflux system, and several two-component systems were also differentially regulated. This indicated *Akkermansia muciniphila* changed its transcriptional regulation in response to *Anaerostipes caccae*. This study provides insight to understand the mucin-driven microbial ecology using metatranscriptomics. Our findings show that the expression of mucolytic enzymes by *Akkermansia muciniphila* increases upon the presence of a community member. This could indicate its role as a keystone species that supports the microbial community in the mucosal environment by increasing the availability of mucin sugars.

Keywords

Butyrate; cross-feeding; keystone species; microbiome; mucin; transcriptional regulation; *Verrucomicrobia*

Introduction

The bacterial assembly at the mucosal layer of the human gastrointestinal tract is associated with gut health and disease (Ouwerkerk *et al* 2013, Tailford *et al* 2015). Although the microbial composition of the healthy mucosa has not been properly defined, it has been observed that strong deviations in the mucosal microbiota are associated with inflammatory bowel disease (IBD) (Kostic *et al* 2014) and irritable bowel syndrome (IBS) (Lopez-Siles *et al* 2014). At this mucosal site, host-produced mucin glycans and bioactive compounds collectively exert a selective pressure that enriches for a sub-population of mucosa-associated bacteria (Koropatkin *et al* 2012, Ouwerkerk *et al* 2013, Schluter and Foster 2012). Mucins are large and complex glycoproteins consisting of a protein core that is rich in proline, threonine and serine moieties, to which oligosaccharides are attached (Tailford *et al* 2015). Mucins can function as an indigenous prebiotic in which only specialised members of the intestinal microbiota are able to utilise it as the substrate for growth (Marcobal *et al* 2013, Ouwehand *et al* 2005, Tailford *et al* 2015).

The intestinal symbiont, *Akkermansia muciniphila* is the sole human intestinal representative of the phylum *Verrucomicrobia* (de Vos 2017). *Akkermansia muciniphila* has adapted to mucosal environment in the gut (Derrien *et al* 2008). The genome of *Akkermansia muciniphila* is equipped with an arsenal of mucin-degrading enzymes including proteases, glycosyl hydrolases (GH), and sulfatases (Derrien *et al* 2016, van Passel *et al* 2011). The mucin-degrading capacity and oxygen tolerance of *Akkermansia muciniphila* render it a key species in the mucosal niche (Ouwerkerk *et al* 2016). This specialised mucin-degrading bacterium is detected at high prevalence (over 96%) in healthy Western adults (Collado *et al* 2007, Derrien *et al* 2008, Shetty *et al* 2016). The abundance of *Akkermansia muciniphila* in the gut microbiota is inversely correlated with syndromes such as IBDs (both Crohn's disease and ulcerative colitis) (Png *et al* 2010), appendicitis (Swidsinski *et al* 2011) and obesity (Everard *et al* 2013). Furthermore, the potential therapeutic role of *Akkermansia muciniphila* has been demonstrated in mice by remedying symptoms of obesity and diabetes (Plovier *et al* 2017) as well as alcoholic liver disease (Grander *et al* 2017).

In addition to the health-promoting role of *Akkermansia muciniphila* via immune modulation, the extracellular mucin degradation by this bacterium could provide growth

benefits to community members via trophic interactions (Belzer and de Vos 2012, Belzer *et al* 2017, Derrien *et al* 2016). Several *in vitro* studies have demonstrated the butyrogenic effect of complex carbohydrates via cross-feeding between glycan-degrading bifidobacteria and butyrogenic bacteria (Belenguer *et al* 2006, De Vuyst and Leroy 2011, Falony *et al* 2006, Rios-Covian *et al* 2015, Riviere *et al* 2015, Schwab *et al* 2017). In the mucosal environment, mucolytic bacteria such as *Akkermansia muciniphila*, *Bacteroides spp.* and *Ruminococcus spp.* as well as butyrogenic members of the family *Lachnospiraceae* (also known as *Clostridium* cluster *XIVa*) and *Ruminococcaceae* (also known as *Clostridium* cluster *IV*) are enriched (Nava *et al* 2011, Van den Abbeele *et al* 2013). However, no mucolytic capacities of these butyrogenic bacteria are known, which suggested potential metabolic cross-feeding between the microbial groups. Butyrate production in the vicinity of epithelial cells is suggested to be important in maintaining gut health (Koh *et al* 2016, Louis and Flint 2017).

In a previous study (Belzer *et al* 2017), we showed that mucin degradation by *Akkermansia muciniphila* yields short chain fatty acid (SCFA) and mucin-derived monosaccharides that support the growth and concomitant butyrate production of non-mucolytic butyrogens. In this paper, we used metatranscriptomics (RNA-seq) to study the molecular response of mucin-directed trophic interaction between *Akkermansia muciniphila* and a butyrogenic bacterium from the family *Lachnospiraceae* (*Anaerostipes caccae*) which possesses metabolic capacity to convert acetate and lactate into butyrate (Duncan *et al* 2004) and shows frequent occurrence at the mucosal niche (Nava *et al* 2011, Van den Abbeele *et al* 2013). We demonstrated the use of metatranscriptomics as an explorative approach to study the expressional changes of *Akkermansia muciniphila* in response to community member. Notably, we showed that *Akkermansia muciniphila* increased its mucolytic activity to sustain the community.

Materials and Methods

Bacterial strains and growth conditions. All bacteria were grown in anaerobic serum bottles sealed with butyl-rubber stoppers at 37ºC with N_2:CO_2 (80:20 ratio) in the headspace at 1.5 atm. Bacterial pre-cultures were prepared by overnight growth in: minimal media supplemented with type III hog gastric mucins (Sigma-Aldrich, St. Louis, USA) for *Akkermansia muciniphila* Muc^T (ATCC BAA-835) (Derrien *et al* 2004), and peptone yeast glucose (PYG) medium for *Anaerostipes caccae* L1-92 (DSM 14662) (Schwiertz *et al* 2002). Growth was measured by spectrophotometer as optical density at 600 nm (OD_{600}) (OD600 DiluPhotometer^{TM}, IMPLEN, Germany).

Co-culture experiment. Co-culture experiments were performed in minimal media (Plugge 2005) supplemented with purified hog gastric mucins (Miller and Hoskins 1981). Culture conditions were established as previously described (Belzer *et al* 2017). *Akkermansia muciniphila* was inoculated at $1x10^6$ cells to mucin media followed by 8 h of incubation to allow accumulation of metabolites. Subsequently, $1x10^6$ cells of *Anaerostipes caccae* (*A.muc-A.cac* co-cultures) were added to the *Akkermansia muciniphila* cultures. Cells were washed twice with phosphate-buffered saline (PBS) before addition to the co-cultures to prevent carryover of metabolites from the pre-cultures. Purified mucins (1.25 g/L) were added to the media every 48 h. A schematic setup of the experiment is depicted in Fig. 1a. Cultures were sampled at 0, 1, 2, 4, 6, 8, 11, and 23 days for metabolites analysis. For transcriptomic analysis at day 8, bacteria pellets were preserved in Trizol® reagent (Invitrogen, Carlsbad, CA, USA) at -20^0C storage till further RNA purification.

High-performance liquid chromatography (HPLC). For metabolites analysis, 1 ml of bacterial culture was centrifuged and the supernatant was stored at -20°C until HPLC analysis. Crotonate was used as the internal standard, and the external standards were lactate, formate, acetate, propionate, isobutyrate, butyrate, citrate, malate, succinate, fumarate, 1,2-propanediol, methanol, ethanol, 2-propanol, lactose, N-acetylgalactosamine (GalNAc), N-acetylgalactosamine (GlcNAc), glucose, and galactose. Substrates conversion and products formation 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 organic acids and carbohydrates. 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, Breda, the Netherlands).

RNA purification. Total RNA was isolated by a method combining the Trizol® reagent and the RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany) as described previously (Chomczynski 1993, Zoetendal *et al* 2006). Four microliters of *p*-mercaptoethanol and 0.4 ml of buffer RLT were added to 1 ml of Trizol® reagent containing the bacterial pellet. The mixture was transferred to a tube containing 0.8 g of glass beads (diameter 0.1 mm), followed by three times of bead beating for 1 min at 5.5 m/s with ice cooling steps in between. Subsequently, 0.2 ml of ice-cold chloroform was added. The solution was mixed gently followed by centrifugation at 12,000 g for 15 min at 4°C. The RNA isolation was continued with the RNA clean-up according to the manufacturer's instructions for the RNeasy Mini kit. Genomic DNA was removed by an on-column DNase digestion step during RNA purification (DNase I recombinant, RNase-free, Roche Diagnostics GmbH, Mannheim, Germany). Yield and RNA quality was assessed using the Experion™ RNA StdSens Analysis Kit in combination with the Experion™ System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Quantitative reverse transcription PCR (RT-qPCR). cDNA was synthesised using the ScriptSeq v2 RNA-Seq library preparation kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions followed by purification using CleanPCR (CleanNA, the Netherlands). The cDNA was analysed by quantitative real-time PCR. Primers targeting 16S rRNA gene of *Akkermansia muciniphila* (AM1 5'-CAGCACGTGAAGGTGGGGAC-3' and AM2 5'-CCTTGCGGTTGGCTTCAGAT-3') (Collado *et al* 2007), and *Anaerostipes caccae* (OFF2555 5'-GCGTAGGTGGCATGGTAAGT-3' and OFF2556 5'-CTGCACTCCAGCATGACAGT -3') (Veiga *et al* 2010) were used for quantification. Standard template DNA was prepared by 16S rRNA gene amplification of each bacterium with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Standard curves were prepared with nine standard concentrations from 10^0 to 10^8 gene copies/µl. qPCR was performed in technical triplicate with iQ SYBR Green Supermix (Bio-Rad) 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: one cycle of 95°C for 10 min, 35 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s each, one cycle of 95°C for 1 min, one cycle of 60°C for 1 min, and a stepwise increase of the temperature from 60 to 95°C (at 0.5°C per 5 s) to obtain melt curve data. Data were analysed using the Bio-Rad CFX Manager 3.0.

Transcriptome sequencing (RNA-seq). Total RNA samples were further processed by Baseclear for RNA-seq (Leiden, the Netherlands). Depletion of ribosomal RNA was performed using the Ribo-Zero™ Kit for bacteria (Epicentre, Madison, WI, USA) followed by quality monitoring using the Agilent 2100 BioAnalyser system. Library construction for whole transcriptome sequencing was done using the TruSeq Stranded mRNA Library Prep Kit (Illumina, USA). The barcoded cDNA libraries were analysed using BioAnalyser and were subsequently pooled and sequenced. Single read 50 bp sequencing was performed on two lanes using the Illumina HiSeq2500 platform.

Transcriptome analysis. The RNA-seq data was pre-processed for quality control. Ribosomal RNA was removed with SortMeRNA v2.0 (Kopylova *et al* 2012) followed by all TruSeq adapters removal with Cutadapt v1.1.a (Martin 2011). Next, quality trimming was performed using Sickle v1.33 (Joshi and Fass 2011) with a score of 30 for threshold indicating a base calling confidence of 99.9%. Reads trimmed to a length <50 bp were removed. Reads were subsequently mapped to the relevant bacterial genomes with Bowtie2 v0.6 (Langmead and Salzberg 2012) using default settings. HTSeq v0.6.1p1 was used to determine the read count for each protein coding region (Anders *et al* 2015). All these steps were performed within a local Galaxy environment (Afgan *et al* 2016). More detailed information about the data analysis can be found in Table S1. Non-mapping reads of the two samples with the lowest mapping rate (both of the *Akkermansia muciniphila* monocultures) were collapsed to unique reads with the fastx toolkit version 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/). A blast search (with standard parameters, except for an e-value of 0.0001) of these unique reads was performed against the NCBI NT database (download 22.01.2014), against the human microbiome (download 08.05.2014), the NCBI bacterial draft genomes (download 23.01.2014), and the human genome (download 30.12.2013, release 08.08.2013, NCBI *Homo sapiens* annotation release 105). Taxonomy was estimated with a custom version of the LCA algorithm as implemented in MEGAN (Huson *et al* 2011). Default parameters were used with the customization that only hits exceeding a bitscore of 50 and a length of more than 25 nucleotides were considered. 98% of the non-mapping

reads were not classified, with *Akkermansia* accounting for 1.15% of the classified reads (Table S2). Differential gene expression was assessed using DESeq2 (Love *et al* 2015). Raw RNA-seq sequence files can be accessed at the European Nucleotide Archive under accession numbers ERR1907419, ERR1907420, ERR1907423, and ERR1907424.

Carbohydrate-active enzymes (CAZymes) prediction. 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).

Results

Metabolite profile of Akkermansia muciniphila monocultures and co-cultures with Anaerostipes caccae

Co-culturing of *Akkermansia muciniphila* and *Anaerostipes caccae* was performed followed by RT-qPCR, HPLC and metatranscriptomic analysis. The metabolites detected in the cultures were comparable with previous findings (Belzer *et al* 2017). *Akkermansia muciniphila* grown as monoculture produced acetate, succinate and 1,2-propanediol as the major metabolites from pure mucin degradation (Fig. 1c). On day 8 the *A.*muc-*A.cac* co-cultures yielded around 2 mM butyrate and a low amount of propionate was detected (Fig. 1c). The mucin sugars (galactose, GalNAc, and GlcNAc) were below the detection limit of 0.5 mM.

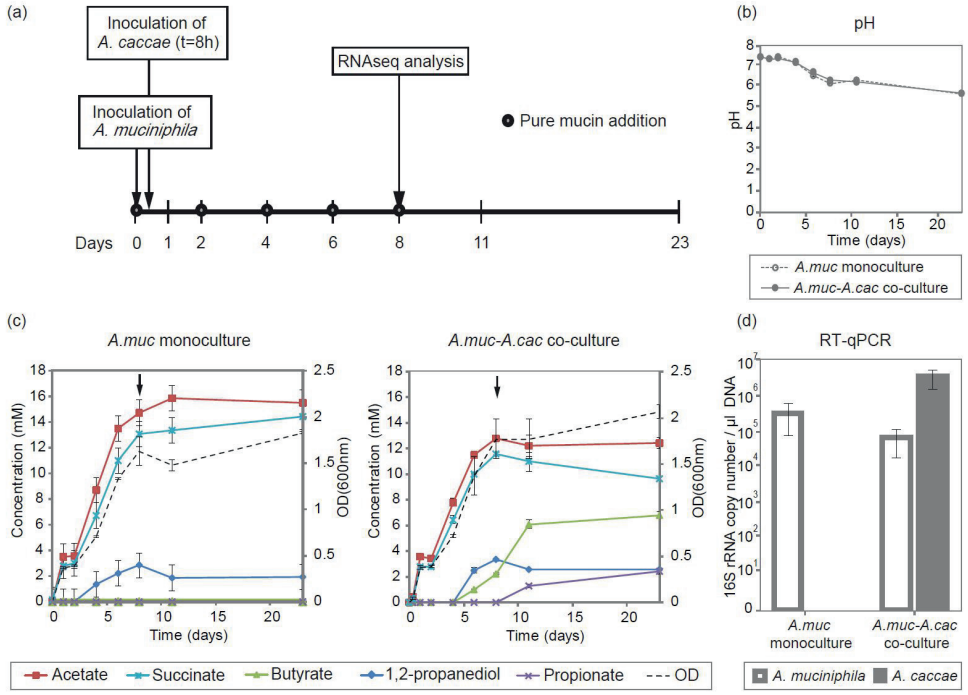


Figure 1. (a) Schematic overview of the interval-fed batch culture setup. *Akkermansia muciniphila* was inoculated at t=0 followed by *Anaerostipes caccae* at t=8h to ensure substrate availability for butyrogen via extracellular mucin degradation by *Akkermansia muciniphila*. Limited amounts of pure mucins, 0.15% (v/v) were supplemented at two days intervals to maintain the abundance of *Akkermansia muciniphila* and to support the emergence of *Anaerostipes caccae*. A sample for RNA-seq analysis was collected on day 8. (b) The pH and (c) metabolite profile of monocultures and co-cultures of the interval-fed batch culture, with arrow showing day 8. (d) Quantification of microbial composition on day 8 by RT-qPCR targeting 16S rRNA on total RNA. Error bars indicate the standard deviation of biological duplicates.

The transcriptomes of Akkermansia muciniphila monocultures and co-cultures with Anaerostipes caccae

Transcriptomic samples were analysed on day 8 of the interval-fed batch cultures, when the major metabolites were accumulated (Fig. 1c) and a stable bacterial composition was established (Belzer *et al* 2017). On average 27 million reads were generated per sample, which is above the recommended sequence depth of 5-10 million reads for a single bacterial transcriptome (Haas *et al* 2012). The detailed

information about the data analysis can be found in Table S1. The RT-qPCR targeting 16S rRNA on total RNA showed *Akkermansia muciniphila* to *Anaerostipes caccae* ratio of 1:50 (Fig. 1d). On the other hand, the ratio of sequenced transcripts mapped to the genome of *Akkermansia muciniphila* versus *Anaerostipes caccae* was 1:1 (Table S1).

Differential expression between Akkermansia muciniphila in monocultures and co-cultures with Anaerostipes caccae

The genome of *Akkermansia muciniphila* possesses a total of 2,176 predicted protein-coding sequences (CDSs) (van Passel *et al* 2011) of which 2,137 (98%) were found to be expressed in this study (Table S3). Differential expression analysis (DESeq2) was performed to compare the gene expression of *Akkermansia muciniphila* in mono- and co-culture conditions. The overall transcriptional response differentiated between the mono- and co-cultures (Pearson's correlation = 0.88 ± 0.02) (Fig. 2).

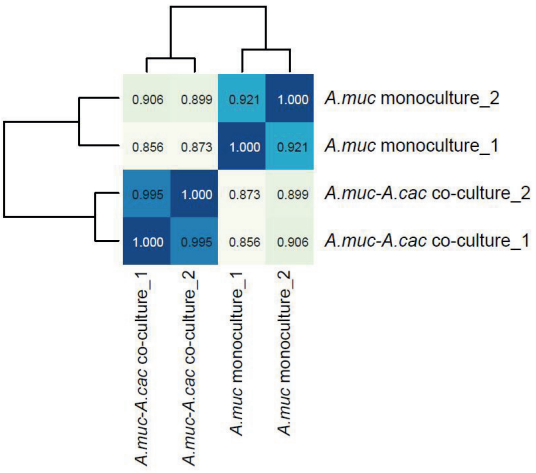


Figure 2. Hierarchical clustering showing the Pearson's correlation of the transcriptome samples as calculated from *Akkermansia muciniphila* CDS count performed with Python 2.7.12 and SciPy version 0.17.1 (van der Walt *et al* 2011).

We used cut-offs of q<0.05 and fold change>2 for significantly regulated genes (Schurch *et al* 2016). A total of 12% *Akkermansia muciniphila* genes were differentially

regulated between mono- and co-cultures, with 148 upregulated genes and 132 downregulated genes (Table S3). Interestingly, two groups of contiguous genes were differentially regulated at high fold change (Fig. 3a). In the co-cultures, the upregulation of the annotated response regulator Amuc_1010 was coupled with the upregulation of a putative operon containing the genes Amuc_1011, Amuc_1012, Amuc_1013, and Amuc_1014 (Fig. 3b). Whereas, the putative operon consisting of Amuc_2041, Amuc_2042 and Amuc_2043 was downregulated in the co-cultures (Fig. 3c). Furthermore, several putative two-component systems were differentially expressed (Table 1).

Gene ontology analysis (Table 2) showed overall increase expression of hydrolase activity, DNA recombination enzymes, and sulphuric ester hydrolase activity in the co-cultures whereas ribosome, structural constituent of ribosome and translation were downregulated. The list of *Akkermansia muciniphila* CAZymes is summarised in Table S4. The overall expression of glycosyl hydrolases was upregulated in the co-cultures. Signal peptides and transmembrane domains prediction showed putative extracellular activity for glycosyl hydrolases required for the degradation of mucin *O*-glycan chains including GH2, GH20, GH29, GH33, GH84, GH89, and GH98.

Table 1. The differential expression of putative two-component systems in *Akkermansia muciniphila*. Negative values indicate upregulation in monocultures and positive values indicate upregulation in co-cultures.

Locus tag	q value	Fold change	Function
Amuc_0311	<0.05	1.96	Signal transduction histidine kinase, nitrogen specific, NtrB
Amuc_0312	<0.05	2.19	Two-component, sigma54 specific, transcriptional regulator, Fis family
Amuc_0827	<0.05	1.44	Osmo-sensitive K^+ channel signal transduction histidine kinase
Amuc_0828	<0.05	1.74	Two-component transcriptional regulator, winged helix family
Amuc_1109	<0.05	-1.89	Histidine kinase
Amuc_1110	0.53	-1.07	Two-component transcriptional regulator, winged helix family
Amuc_1727	0.63	1.06	Integral membrane sensor signal transduction histidine kinase
Amuc_1728	0.25	1.13	Two-component transcriptional regulator, winged helix family
Amuc_1010	<0.05	5.28	Response regulator receiver protein

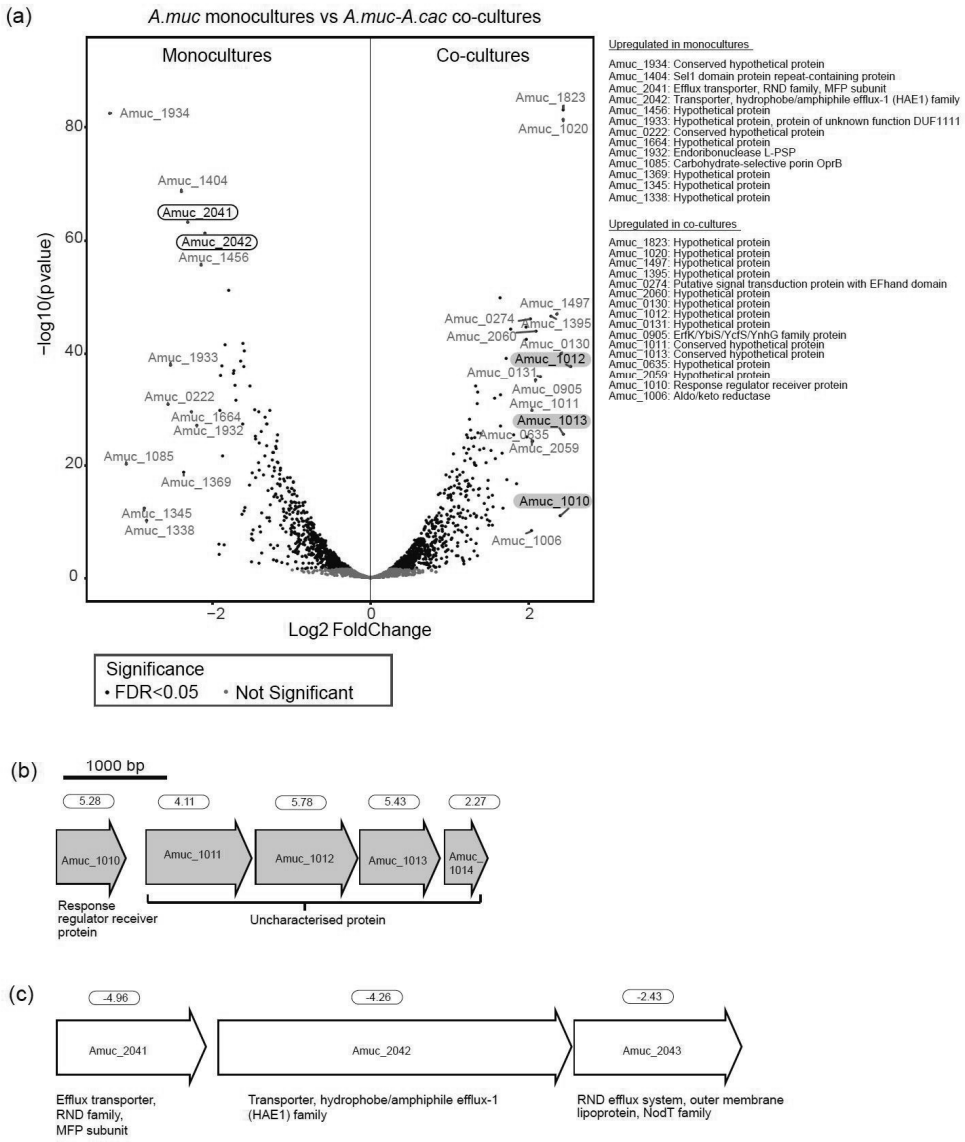


Figure 3. (a) Volcano plots showing p-values correlated to fold changes in gene expression of *Akkermansia muciniphila* observed in monocultures versus co-cultures with *Anaerostipes caccae*. Positive fold changes indicate upregulation in co-cultures, and negative fold changes indicate upregulation in monocultures. Locus tags for genes with Log2 fold change >2 (or fold change >4) are labelled. (b) Response regulator and putative operon upregulated in the co-cultures (c) Putative operon upregulated in the monocultures. Fold changes are listed above the respective genes.

Table 2. Gene ontology (GO) analysis of the differentially regulated *Akkermansia muciniphila* genes (q<0.05) in co-cultures. The list contains GO with total count in genome higher than 10 and absolute percentage difference higher than average value.

GO term	Total count in *A.muc* genome	Percentage upregulated	Percentage downregulated
Overall expression upregulated			
GO:hydrolase activity, hydrolysing O-glycosyl compounds	30	0.60	0.03
GO:DNA recombination	17	0.53	0.06
GO:sulphuric ester hydrolase activity	12	0.50	0.17
Overall expression downregulated			
GO:transporter activity	27	0.22	0.52
GO:magnesium ion binding	16	0.19	0.44
GO:tRNA processing	11	0.18	0.55
GO:cytoplasm	66	0.17	0.48
GO:pyridoxal phosphate binding	20	0.15	0.45
GO:RNA binding	37	0.14	0.46
GO:GTP binding	20	0.10	0.55
GO:transferase activity	21	0.10	0.43
GO:tRNA aminoacylation for protein translation	24	0.08	0.71
GO:cellular amino acid metabolic process	12	0.08	0.50
GO:aminoacyl-tRNA ligase activity	25	0.08	0.72
GO:nucleotide binding	40	0.08	0.58
GO:intracellular	42	0.07	0.79
GO:NAD binding	15	0.07	0.33
GO:ribosome	50	0.02	0.88
GO:structural constituent of ribosome	55	0.02	0.89
GO:translation	57	0.02	0.88

Genes expression in relation to the metabolites production

We examined the transcripts of the co-cultures to reconcile the metabolite findings. The transcripts for *Anaerostipes caccae* showed median of relative abundance around 0.005% and maximum value of 2.07%. The list of *Anaerostipes caccae* genes is displayed in Table S5. It is described that *Anaerostipes caccae* metabolises acetate to butyrate by employing the most prevalent butyrate production pathway via acetyl-

coenzyme A (CoA) (Vital *et al* 2014). The relative abundances of all transcripts involved in the metabolism pathways are summarised in Table 3.

Table 3. The relative abundance (%) of *Anaerostipes caccae* transcripts for genes involved in butyrate synthesis pathway.

Enzyme	Locus tag	Dup1	Dup2
Interconversion of pyruvate to acetyl-CoA			
Pyruvate dehydrogenase complex	ANACAC_01488	<0.00	<0.00
	ANACAC_01489	<0.00	<0.00
	ANACAC_01490	<0.00	<0.00
	ANACAC_01491	<0.00	<0.00
	ANACAC_01492	<0.00	<0.00
Formate C-acetyltransferase	ANACAC_01621	<0.00	<0.00
	ANACAC_00664	<0.00	<0.00
Pyruvate synthase	ANACAC_00834	1.83	1.85
Interconversion of pyruvate to lactate			
L-lactate dehydrogenase	ANACAC_01148	0.01	0.01
	ANACAC_03769	0.02	0.02
Acetyl-CoA pathway			
Acetyl-CoA C-acetyltransferase	ANACAC_00256	0.34	0.37
Acetoacetyl-CoA reductase	ANACAC_00254	0.35	0.39
3-hydroxybutyryl-CoA dehydratase	ANACAC_03496	0.01	0.02
	ANACAC_00255	0.21	0.23
Butyryl-CoA dehydrogenase	ANACAC_00252	0.50	0.50
	ANACAC_00253	0.54	0.56
	ANACAC_03492	0.00	0.00
Phosphate acetyltransferase	ANACAC_00344	0.13	0.15
Acetate kinase	ANACAC_00343	0.17	0.18
Butyryl-CoA: acetate CoA-transferase	ANACAC_01149	0.16	0.17
4-aminobutyrate / succinate pathway			
Hydroxybutyrate dehydrogenase	ANACAC_00166	<0.00	<0.00
4-hydroxybutyrate coenzyme A transferase	ANACAC_00165	<0.00	<0.00
4-hydroxybutanoyl-CoA dehydratase	ANACAC_00167	<0.00	<0.00
	ANACAC_02698	<0.00	<0.00

Our data indicated that the majority of enzymes involved in the acetyl-CoA pathway were expressed at a relative abundance higher than 0.1%, with over 2% of total transcripts accounted for butyrate production. In addition, *Anaerostipes caccae* possesses genomic capacity to synthesis butyrate by using 4-aminobutyrate or succinate as the precursor. However, the expression of this pathway was low, with the relative abundance of transcripts lower than 0.01%, indicating that acetyl-CoA was the dominant pathway.

Nutrients interdependency between Akkermansia muciniphila and Anaerostipes caccae

The genomes of *Akkermansia muciniphila* and *Anaerostipes caccae* were inspected for B vitamins and amino acids auxotrophy to investigate potential nutrient interdependency. *Akkermansia muciniphila* lacked the upstream genes required for vitamin B12 biosynthesis including CbiL, CobG, CbiGF, CobF, CbiECA and CobAT. Complementarily, *Anaerostipes caccae* was predicted to possess complete vitamin B12 biosynthesis pathway (Table 4). However, no vitamin B12 transporter was found in *Anaerostipes caccae* genome. We found indications for aspartate auxotrophy of *Anaerostipes caccae* (Table S6) however the bacterium was reported to grow in minimal defined media supplemented with glucose without additional nitrogen source (Belzer *et al* 2017). Furthermore, *Anaerostipes caccae* lacked the genes to synthesise the cofactor lipoate required for dehydrolipoate dehydrogenase, EC 1.8.1.4. The different enzyme complexes containing this enzyme were involved in citrate cycle, glycine, serine, and threonine metabolism, and valine, leucine, and isoleucine degradation. Nevertheless, *Anaerostipes caccae* could acquire lipoate via salvage pathway and we observed the upregulation of lipoate biosynthesis by *Akkermansia muciniphila* in co-cultures.

Table 4. Genomic prediction of B vitamins biosynthesis (presence = 1 and absence = 0) based on the combination of essential functional roles by Magnusdottir *et al*. (Magnusdottir *et al* 2015).

	B1 Thiamin	B2 Riboflavin	B3 Niacin	B5 Pantothenate	B6 Pyridoxin	B7 Biotin	B9 Folate	B12 Cobalamin
Akkermansia muciniphila Muc^T	1	1	1	1	1	1	1	0
Anaerostipes caccae L1-92	1	1	1	1	1	0	1	1

Discussion

In this study, we demonstrated the use of metatranscriptomics as an explorative approach to decipher bacterial interaction in the mucosal environment. Two representative mucosa-associated species, namely *Akkermansia muciniphila* and *Anaerostipes caccae*, were used to show the ecological dependency between a mucin-degrader and a butyrate producer. Importantly, this study revealed changes in the expression of genes involved in host-glycan catabolism and trophic interactions between the gut commensals. This interplay leads to the formation of butyrate at the mucosal layer that is proposed to be beneficial to the host (Koh *et al* 2016, Louis and Flint 2017).

In the presence of *Anaerostipes caccae*, *Akkermansia muciniphila* upregulated mucin-degrading genes involved in hydrolase and sulphuric ester hydrolase activity. The majority of these mucin-degrading enzymes were predicted to function in the extracellular compartment (Ottman *et al* 2016), which could lead to the degradation of oligosaccharide chains consisting of GalNAc, GlcNAc, mannose, galactose, fucose and sialic acid (Moran *et al* 2011). Previous work demonstrated that *Anaerostipes caccae* as well as *Eubacterium hallii* and *Faecalibacterium prausnitzii* could utilise the mucin-derived sugars including galactose, mannose and GlcNAc for growth (Belzer *et al* 2017, Lopez-Siles *et al* 2012). The fermentation of these monosaccharides results in butyrate production. Since both *Akkermansia muciniphila* and the butyrate-producer rely on the uptake of mucin-derived sugars for growth in our model, a higher

extracellular concentration of *Akkermansia muciniphila*-derived mucolytic enzymes could contribute to available substrate in the community. Concurrently, *Akkermansia muciniphila* showed downregulation of ribosomal genes in the co-cultures, which implied a lower growth rate of *Akkermansia muciniphila*. The qPCR results of genomic 16S rRNA gene ratio from a previous publication on extracted DNA showed a *Akkermansia muciniphila* to *Anaerostipes caccae* ratio of 100:1 (Belzer *et al* 2017). In this study, the ratio of 16S rRNA in total RNA samples quantified by RT-qPCR showed a *Akkermansia muciniphila* to *Anaerostipes caccae* ratio of 1:50, whereas, the sequenced transcripts ratio was 1:1. The discrepancy could be the result of differential expression between ribosomal and messenger RNA. Note that total RNA could contain 95-99% of ribosomal RNA (Zoetendal *et al* 2006) and that the number of ribosomes per cell correlates with the growth rate (Fegatella *et al* 1998). In addition, *Akkermansia muciniphila* and *Anaerostipes caccae* contain 3 and 12 copies of the rRNA operon, respectively. Taken together, these results indicate that *Akkermansia muciniphila* dominated in terms of cells number but *Anaerostipes caccae* showed proportionally higher growth rate and transcriptional activity.

The co-culturing of two representative mucosa-associated bacteria has demonstrated the major pathways for intestinal SCFA biosynthesis. The overview of this mucin-directed trophic interaction is shown in Fig. 4. *Anaerostipes caccae* cross-fed on a part of the mucin sugars liberated by *Akkermansia muciniphila* for central metabolism. In addition, *Anaerostipes caccae* can incorporate *Akkermansia muciniphila*-derived acetate for butyrate production via butyryl-CoA: acetate CoA-transferase enzyme (Duncan *et al* 2004, Louis and Flint 2009, Louis and Flint 2017). Moreover, *Akkermansia muciniphila* could benefit from the corrinoids released by *Anaerostipes caccae* (Degnan *et al* 2014). Pseudo-vitamin B12 from *Eubacterium hallii* could activate the propionate production by *Akkermansia muciniphila* via the succinate pathway (Belzer *et al* 2017). A low level of propionate was detected after day 8 in *A.muc-A.cac* co-cultures (Belzer *et al* 2017). Propionate is likely produced by *Akkermansia muciniphila* because *Anaerostipes caccae* is not known to produce propionate and it does not possess the genes involved in the known propionate biosynthesis pathways i.e. the succinate, acrylate, and propanediol pathways (Louis and Flint 2017). Nevertheless, *Anaerostipes caccae* is predicted to synthesise vitamin B12 but lacked a vitamin B12 transporter.

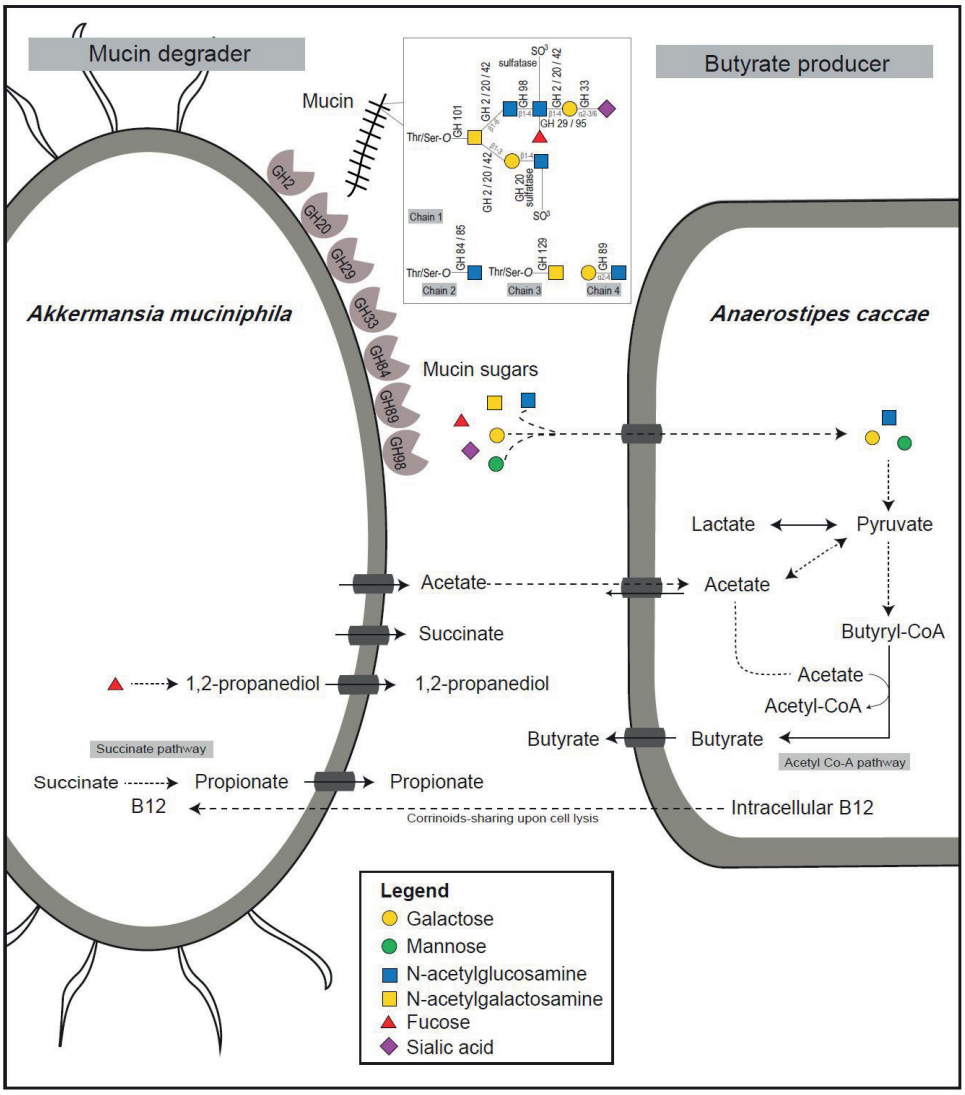


Figure 4. Schematic representation of mucin-driven trophic interaction between *Akkermansia muciniphila* and *Anaerostipes caccae*. *Akkermansia muciniphila* degrades oligosaccharides chain of mucins by extracellular glycosyl hydrolases. The structure for *O*-linked glycan chains and CAZymes action sites are adapted from Tailford *et al.* (Tailford *et al* 2015). Chain 1 is a hypothetical mucin glycan chain, chain 2 is *O*-GlcNAc often found on other glycoproteins, chain 3 (Tn antigen) and chain 4 are found in gastro-duodenal mucins. In addition, mannose could be released from degradation of *N*-linked glycan chains. *Anaerostipes caccae* utilises some of the mucin-derived sugars (galactose, mannose and GlcNAc) and acetate released by *Akkermansia muciniphila* for growth and concomitant butyrate production.

Upon cell lysis, the release of cellular vitamin B12 by *Anaerostipes caccae* could facilitate methylmalonyl-CoA mutase enzymes (Amuc_1983 and Amuc_1984) of *Akkermansia muciniphila* to produce propionate (Degnan *et al* 2014). The upregulation of cobalamin-dependent methylmalonyl-CoA mutase genes in monocultures indicated an attempt by the organism to activate the propionate production pathway in the absence of the essential cofactor (Fig. S1), as the conversion of methylmalonyl-CoA to propionyl-CoA is thermodynamically favourable (Dimroth and Schink 1998). The exergonic decarboxylation of methylmalonyl-CoA could be coupled to sodium ion export to extracellular space for the establishment of a proton gradient via a sodium-proton antiporter to generate ATP (Ottman *et al* 2017a).

Interestingly, two putative operons and several two-component systems were differentially regulated, indicating the mode of transcriptional regulation by *Akkermansia muciniphila* in response to *Anaerostipes caccae*. A previous study has demonstrated that the presence of one organism is often associated with transcriptional changes in the other (Plichta *et al* 2016). In the co-culture with *Anaerostipes caccae*, *Akkermansia muciniphila* downregulated a putative operon consisting of Amuc_2041 (efflux transporter, RND family, MFP subunit), Amuc_2042 (transporter, hydrophobe/amphiphile efflux-1 (HAE1) family) and Amuc_2043 (RND efflux system, outer membrane lipoprotein, NodT family). The membrane fusion protein (MFP) is described as a component of drug resistance, nodulation, and the cell division (RND) involved in the transportation of drug molecules (Anes *et al* 2015). HAE1 is involved in toxin production and resistance processes (Anes *et al* 2015). The outer membrane lipoproteins from the NodT are predicted to primarily export small molecules rather than proteins. This efflux system was reported to play a role in multidrug resistance of Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* (Nikaido and Takatsuka 2009). A similar resistance mechanism could be employed by the Gram-negative *Akkermansia muciniphila*, and this study suggested the down-tuning of the efflux pump expression in the presence of a community member.

The annotated response regulator Amuc_1010 and the adjacent predicted operon consisting of Amuc_1011, Amuc_1012, Amuc_1013, and Amuc_1014, were upregulated in the co-cultures. Amuc_1010 is likely not a two-component system as it encoded only for the LytTR DNA-binding domain without the CheY-like receiver

domain. Above all, Amuc_1010 could be autoregulatory as *cis*-acting regulatory elements were predicted at its upstream region using MEME (Bailey *et al* 2009) (data not shown). Amuc_1011, Amuc_1012, Amuc_1013, and Amuc_1014 were annotated as uncharacterised proteins, and Amuc_1011 was predicted as an outer membrane protein (Ottman *et al* 2016). Further research is needed to investigate this interesting gene cluster with unidirectional arrangement and a short intercistronic region that could likely be co-transcribed. The upregulation of the outer membrane protein could be associated with host colonisation, persistence and immunomodulation (Galdiero *et al* 2012). A recent study showed that an immune-stimulatory outer membrane protein of *Akkermansia muciniphila* (Amuc_1100) (Ottman *et al* 2017b) is able to ameliorate the metabolic symptoms of obese and diabetic mice (Plovier *et al* 2017). However, Amuc_1100 was not found to be differentially regulated in this study.

In addition, *Akkermansia muciniphila* upregulated several two-component systems in the co-cultures. Two-component systems consist of a membrane bound sensor histidine kinase and a cytoplasmic response regulator, which are often encoded by adjacent genes, enable bacteria to response to changing environment by altering gene expression (Monedero *et al* 2017). However, the roles of two-component systems in *Akkermansia muciniphila* grown in the co-cultures were not yet identified. Studies showed that they could be involved in the regulation of physiological processes in commensal bacteria, such as stress responses, regulation of metabolism, and resistance to antimicrobial peptides (Monedero *et al* 2017). The gastrointestinal pathogen, enterohemorrhagic *Escherichia coli* (EHEC), was reported to encode the two-component system FusKR. This system provides a growth advantage and modulates the expression of virulence genes upon sensing of fucose liberated by *Bacteroides thetaiotaomicron* during growth in media containing mucins (Pacheco *et al* 2012). The metabolism of mucin-derived fucose by *Akkermansia muciniphila* yielded 1,2-propanediol (Ottman *et al* 2017a). As such, fucose metabolism by *Akkermansia muciniphila* could confer colonisation resistance against the fucose-dependent enteric pathogens (Pickard and Chervonsky 2015).

In conclusion, we demonstrated the use of metatranscriptomics to provide in-depth mechanistic understanding of bacterial interaction. The trophic interaction between mucosal keystone species *Akkermansia muciniphila* and *Anaerostipes caccae* could result in beneficial butyrate production at close proximity to host

epithelium. We revealed the expressional changes of *Akkermansia muciniphila* in response to *Anaerostipes caccae* and demonstrated the provider role of *Akkermansia muciniphila* by upregulating the mucolytic activity to sustain the community at the mucosa niche.

Acknowledgements

We thank Thi Phuong Nam Bui, Kees C.H. van der Ark, Maria Suarez-Diez, Teunke van Rossum and Fons Stams for their constructive comments. Furthermore, we would like to thank Bart Nijsse for technical support. This work was partly supported by a special grant from Nutricia Research, the SIAM Gravitation Grant 024.002.002 of the Netherlands Organization for Scientific Research, and the IP/OP program Systems Biology (project KB-17-003.02-023).

JK is the employee of Nutricia Research. LWC and CB are financially supported by Nutricia Research. There was no involvement of the company in the content of this work.

Supplementary materials

Table S1. The general features of RNA-seq data analysis.

	A.muc monoculture		*A.muc-A.cac* co-culture	
	Dup 1	Dup 2	Dup 1	Dup 2
Total no. of reads	30812972	27356734	26799549	22755873
Average quality scores (Phred)	37.72	37.72	38.73	38.72
rRNA removed (%)	6.4	4.1	3.9	4.4
Adapters removed (%)	4.3	4.7	5.4	5.4
No. of reads after Cutadapt	28826013	26236614	25748139	21756579
Trimmed reads by Sickle (%)	24.90	25.24	13.23	13.63
No. of reads after Sickle (quality threshold=30; length threshold=50)	21647836	19613896	22342387	18790981
Total no. of reads mapped to the concatenated genome of *A.muciniphila* & butyrogens	NA	NA	21433553	17850364
Total no. of reads mapped to the genome of *A.muciniphila*	17531380	15516414	10135288	8630425
Total no. of reads mapped to the genome of butyrogens	NA	NA	11298279	9219949
Total no. of reads mapped to the concatenated genome of *A.muciniphila* & butyrogens (%)	NA	NA	95.93	94.99
Total no. of reads mapped to the genome of *A.muciniphila* (%)	80.98	79.11	45.36	45.93
Total no. of reads mapped to the genome of butyrogens (%)	NA	NA	50.57	49.07
Sum of % of reads mapped to the genomes	80.98	79.11	95.93	95.00
Total no. of reads mapped to the concatenated protein coding regions of *A.muciniphila* & butyrogens	NA	NA	10286240	8436520
Total no. of reads mapped to the protein coding regions of *A.muciniphila*	8368120	8505786	3991352	3189066
Total no. of reads mapped to the protein coding regions of butyrogens	NA	NA	6294888	5247454
Total no. of reads mapped to the protein coding regions	8368120	8505786	10286240	8436520

NA denotes not available

Table S2. The blast result for the unique non-mapping reads from both of the *Akkermansia muciniphila* monocultures. (Available on request)

Table S3. Summary of the differentially expressed *Akkermansia muciniphila* CDS in the co-cultures with *Anaerostipes caccae*.

	A.muc-A.cac co-culture
Total number of CDS	2137
Number of CDS with q>0.05	1003
Number of CDS with q<0.05	
Upregulated	
Fold change < 2	416
2 < Fold change < 4	132
Fold change > 4	16
Downregulated	
Fold change < 2	438
2 < Fold change < 4	119
Fold change > 4	13

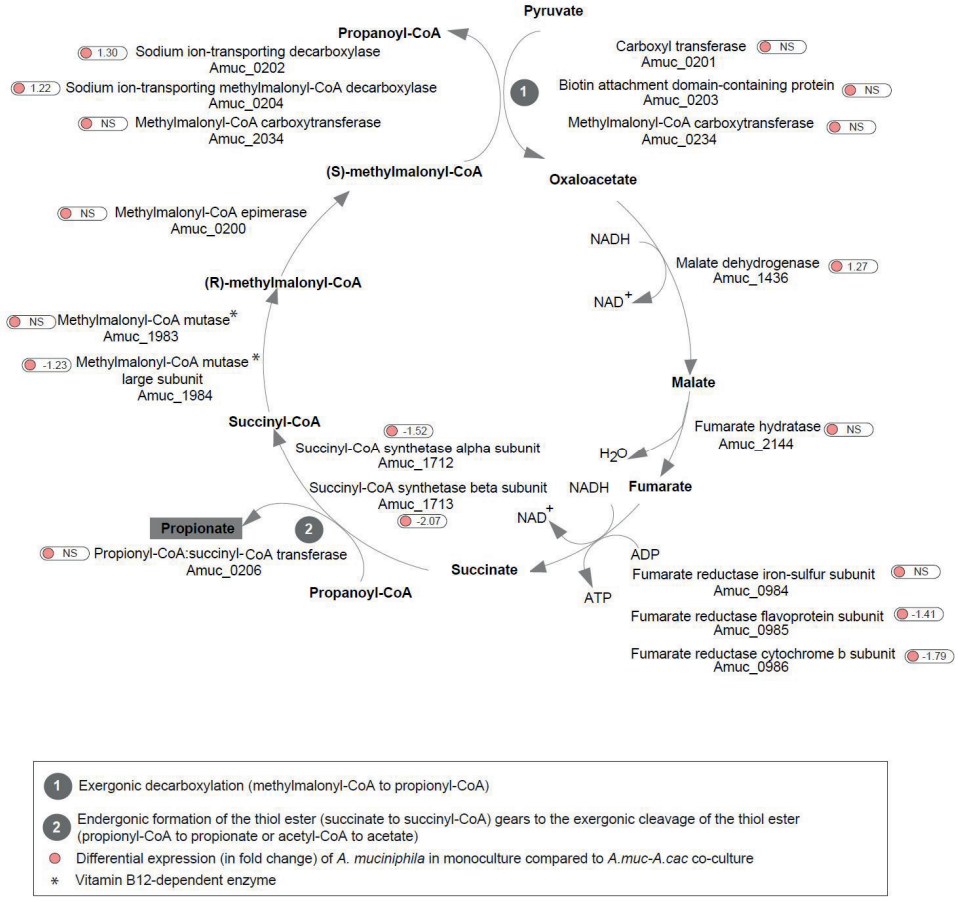
Table S4. Differentially regulated CAZymes of *Akkermansia muciniphila* in *A.muc-A.cac* co-cultures (q<0.05). Signal peptide and transmembrane protein were predicted using SignalP and TMHMM respectively. (Available on request)

Table S5. The relative abundance and putative function of transcripts for *Anaerostipes caccae*. (Available on request)

Table S6. The genomic prediction of amino acids biosynthesis.

Amino acids	*Akkermansia muciniphila* Muc^T	*Anaerostipes caccae* L1-92
Alanine (Ala)	Complete	Complete
Arginine (Arg)	Complete	Complete
Asparagine (Asn)	Complete	Complete
Aspartic acid (Asp)	Complete	NA
Cysteine (Cys)	Complete	Complete
Glutamic acid (Glu)	Complete	Complete
Glutamine (Gln)	Complete	Complete
Glycine (Gly)	Complete	Complete
Histidine (His)	Complete	Complete
Isoleucine (Ile)	Complete	Complete
Leucine (Leu)	Complete	Complete
Lysine (Lys)	Complete	Complete
Methionine (Met)	Complete	Complete
Phenylalanine (Phe)	NA	NA
Proline (Pro)	Complete	Complete
Serine (Ser)	Complete	Incomplete
Threonine (Thr)	NA	Complete
Tryptophan (Trp)	Complete	Complete
Tyrosine (Tyr)	Incomplete	Incomplete
Valine (Val)	Complete	Complete
Selenocysteine (Sec)	Incomplete	Complete
Pyrrolysine (Pyl)	Incomplete	Incomplete
Homocysteine	Complete	Incomplete
Homoserine	Complete	Complete
Ornithine	Complete	Complete

NA denotes not available



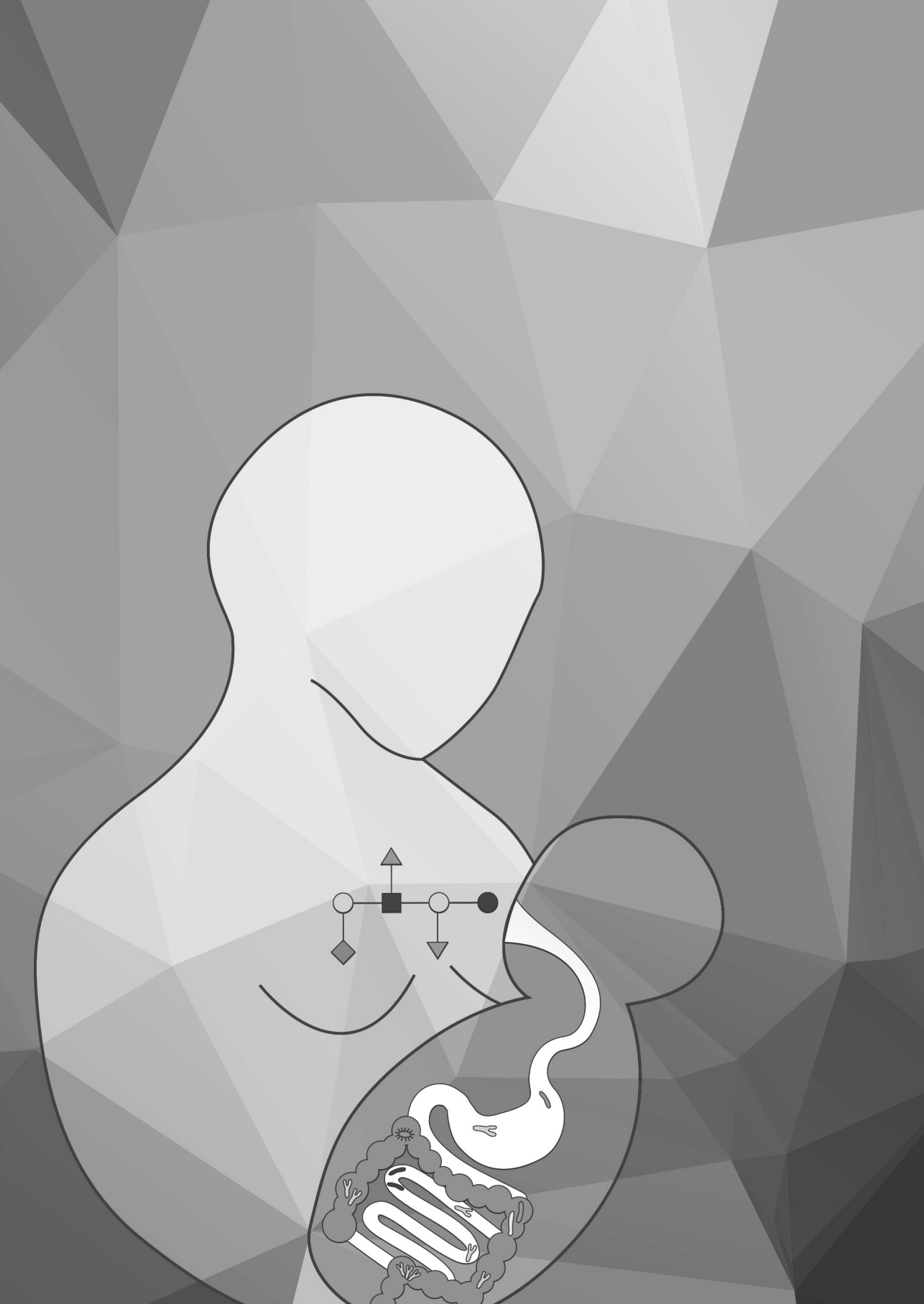
1 Exergonic decarboxylation (methylmalonyl-CoA to propionyl-CoA)

2 Endergonic formation of the thiol ester (succinate to succinyl-CoA) gears to the exergonic cleavage of the thiol ester (propionyl-CoA to propionate or acetyl-CoA to acetate)

Differential expression (in fold change) of *A. muciniphila* in monoculture compared to *A.muc-A.cac* co-culture

* Vitamin B12-dependent enzyme

Figure S1. *Akkermansia muciniphila* pathway for pyruvate fermentation to propionate (succinate pathway). Fold changes for differentially expressed genes (q <0.05) comparing *Akkermansia muciniphila* in monoculture and co-cultures with *Anaerostipes caccae* are listed next to the respective genes. Negative values indicate upregulation in monocultures, positive values indicate upregulation in co-cultures and NS indicates non-significant regulation with q >0.05. Figure is adapted from MetaCyc (Caspi *et al* 2014) with curation.



Chapter 6

General discussion

General discussion

Contemplating the complexity of the gut microbiota and host-secreted glycans

The human gut microbiota is essential for health by supporting the metabolic, immune and neurological functions of the host (El Kaoutari *et al* 2013, Honda and Littman 2016, Rogers *et al* 2016). The ecological landscape of the human gut microbiota is highly complex, and consists of trillions of microorganisms including bacteria, archaea, microeukaryotes and viruses (Sender *et al* 2016). Bacteria are the most studied microorganisms in the human gut. The bacterial diversity is rather limited at the phylum level in comparison to other environments, and consists predominantly of *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (Falony *et al* 2016). However, high diversity is displayed at the level of operational taxonomic units (OTU) that is commonly used to recapitulate microbial phylogeny from sequencing data (Ursell *et al* 2012). Over 2000 species-level phylotypes of which approximately 1000 cultured isolates have been detected in the human gut (Rajilic-Stojanovic and de Vos 2014, Ritari *et al* 2015, Zoetendal *et al* 2008). The human gut epithelium in essence, separates host cells from the resident microbes and incoming dietary components. The gut epithelial lining, covered with host-secreted mucin glycans, is an important interaction site between host and commensal as well as pathogenic bacteria (Tailford *et al* 2015). Also, the vertical transfer of glycans and bioactive compounds via human milk from mothers to their offsprings is thought to be an important evolutionary force of mammalian hosts to select for symbiotic bacteria in the infant gut (Ballard and Morrow 2013). Host-secreted glycans are consisted of oligosaccharides synthesised in a non-template-driven fashion. Hence, they are often branched and show complex regio- and stereo-chemistry resulting in an enormous number of sugar structures in human body (Hofmann and Pagel 2017).

The complexity of the gut microbiota and host-secreted glycans poses an interesting challenge to understand the link between both parties. The bacterial glycan-utilisation can be predicted from genomic data by *in silico* assignment of the biological and biochemical role to proteins (Gabaldon and Huynen 2004). Based on the amino acid sequence similarities, the catalytic capability of an ecosystem or a bacterium can be predicted from metagenome or genome respectively (Lombard *et al* 2014). Yet,

despite the vast amount of sequencing data generated in the last decade, our understanding of the metabolic functions of the gut microbiota is progressing rather slow (El Kaoutari *et al* 2013). The analysis of microbiome data using genomic-based predictions is limited to answer "what could the bacteria do?" instead of "what are the bacteria actually doing?". Therefore, it is critical to generate more physiological and biochemical data by culturing (both monoculture and co-culturing with other microbes) in order to gain mechanistic insights, which is the main topic of this thesis.

This thesis focuses on unravelling the interaction of gut symbionts in the presence of human milk oligosaccharides (HMOS) or host-derived mucins. More specifically, we studied the interactions between two groups of bacteria that are predicted to cross-feed i.e. milk and mucin glycan-degraders (including *Bifidobacterium infantis* – **Chapter 2**, *Bacteroides thetaiotaomicron* – **Chapter 3**, and *Akkermansia muciniphila* – **Chapter 4** and **5)** and butyrate producers (including *Anaerostipes caccae* – **Chapter 2** to **5**, *Eubacterium hallii* – **Chapter 4**, and *Faecalibacterium prausnitzii* – **Chapter 4**). A reductionist approach using anaerobic culturing in minimal environment was applied to investigate the physiology and interaction between the aforementioned gut symbionts. The ability of the glycan-degraders to support the growth and activity of butyrogens is clearly demonstrated. Cross-feeding between these two bacterial groups leads to the formation of a microbial network with a specific metabolic output that could have a potential health impact. In the following paragraphs, the insights generated within this thesis with regards to the butyrogenic microbial networks will be summarised and put into a broader ecological context. The understanding of the microbe-microbe interactions and the underlying molecular mechanisms might contribute to the design of interventions to modulate the gut microbiota in order to sustain human health. As such, the concept of next generation gut microbiota modulators composed of novel probiotic strains, prebiotics and microbiota-targeted nutrients will be discussed.

Host-secreted glycans support butyrogenesis in the gut microbiota

Microbial activity on the host- and/or diet-derived fermentative substrates leads to the formation of metabolites in the gut (Flint *et al* 2014). The profile of short chain fatty acids (SCFAs) is distinct between the infant and adult gut (Fig. 1). One of the most

apparent differences between these two niches is the availability of specific microbial substrates. The gut of a breast-fed infants is provided with HMOS from human milk, whereas the gut of adults is provided through the diet with components from plant and animal origin. At the same time, host-secreted mucins present as a stable source of endogenous substrate in both infants and adults. The genes of CAZymes to degrade milk and mucin glycans are predicted in the genomes of gut microbes but seem to be concentrated in the specialised bacteria as illustrated at Figure 2 in **Chapter 1**. The difference in glycan-degrading capabilities among bacteria results in the dominance of degrader species in the glycan-rich niches. The milk and mucin glycan-degraders can subsequently support the growth of sub-ordinate cross-feeders in the infant gut or the mucosal layer.

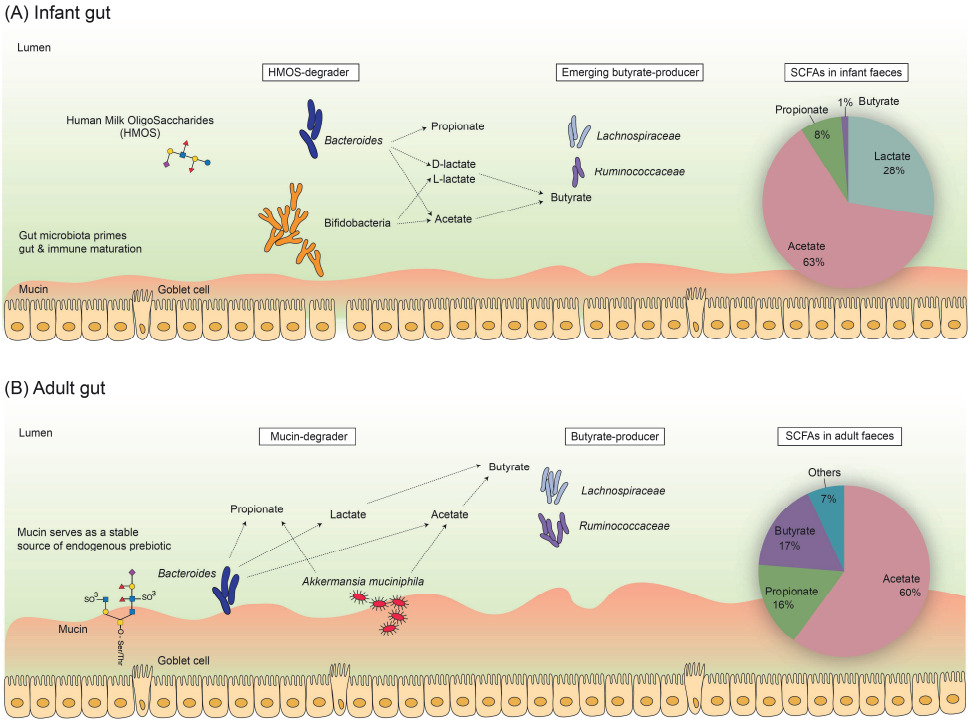


Figure 1. **Host-secreted glycans drive butyrogenic microbial networks in the infant and adult gut.** The microbial trophic chain for SCFA formation (A) in the infant gut and (B) at the mucosal niche of the adult gut. The SCFA composition for 3 months old infant and adult faeces is derived from Pham *et al.* and Schwiertz *et al*. (Pham *et al* 2016, Schwiertz *et al* 2010). Others SCFAs in adult faeces include iso-butyrate, iso-valerate, and valerate, which are mainly the products of protein fermentation.

The role of HMOS in guiding the establishment of microbial networks is well recognised and appreciated (Bode 2012, Smilowitz *et al* 2014). The results described in this thesis show that HMOS degradation by *Bifidobacterium* spp. (**Chapter 2**) and *Bacteroides* spp. (**Chapter 3**) supports butyrogenic cross-feeding. From an ecological point of view, our results support the hypothesis that, HMOS-degraders in the infant gut could enable the sequential colonisation of other functional bacterial groups and support the ecosystem towards a more stable and mature situation. The infant gut microbiota undergoes drastic changes upon the introduction of solid food and the cessation of breast-feeding (Backhed *et al* 2015, Laursen *et al* 2016), after which, a mature gut composition resembling an adult gut microbiota is gradually established (Yatsunenko *et al* 2012). Results from **Chapter 2** suggest that the initial HMOS-degraders could provide substrates and create the environment for other important species that will colonise the infant gut after weaning. Microbial fermentation of non-digestible carbohydrates in the gut of exclusively breast-fed infants leads to a relatively high concentration of acetate and lactate (a relative strong acid with pKa= 3.86) (Pham *et al* 2016, Wopereis *et al* 2017). Hence, infants have a lower faecal pH than that of adults (Henrick *et al* 2018, Oozeer *et al* 2013). The pH of the gut lumen 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). The change of the intestinal microbiota composition is reflected by the shift in faecal SCFA ratios, characterised by the relative decrease of lactate and increase of butyrate and propionate (Fig. 1). HMOS-degraders like *Bifidobacterium* (**Chapter 2**), *Bacteroides* (**Chapter 3**), *Lactobacillus*, *Streptococcus*, and *Enterococcus* spp. produce lactate as a major metabolite (Chassard *et al* 2014, Jost *et al* 2012). Upon weaning, the relative abundance of lactate-producing bacteria decreases in contrast to the increase of butyrate- and propionate-producing bacteria (Laursen *et al* 2017). *Firmicutes* from the *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae* families are known for their butyrate-producing capacity, whereas propionate-producing bacteria mainly include *Bacteroidetes* members from the *Bacteroidaceae*, *Prevotellaceae*, and

Rikenellaceae families, and *Firmicutes* members from the *Lachnospiraceae, Veillonellaceae*, and *Acidaminococcaceae* families as well as *Verrucomicrobia* (*Akkermansia muciniphila* as the only cultured representative in human gut) (Louis and Flint 2017).

Physiologically, the conversion of lactate in the gut by microbiota members could prevent excessive lactate accumulation, which could potentially lead to detrimental health consequences such as acidosis, neurotoxicity, and cardiac arrhythmia (Ewaschuk *et al* 2005, Hove *et al* 1994). Further metabolic conversion of lactate by butyrate-producing bacteria such as *Anaerostipes* spp. (**Chapter 2** and **3**), *Eubacterium hallii*, and *Eubacterium* spp. or sulphate-reducing bacteria (SRB) such as *Desulfovibrio piger* could lead either to the accumulation of hydrogen gas or hydrogen sulphide respectively (Chassard and Bernalier-Donadille 2006, Pham *et al* 2016, Robert *et al* 2001). The balance between these activities is essential for gut health as the accumulation of hydrogen can result in discomfort such as bloating and cramping (Pham *et al* 2017), whereas the accumulation of hydrogen sulphide is toxic for epithelial cells and can inhibit butyrate oxidation leading to autophagy (Donohoe *et al* 2011, Kim *et al* 2013). Subsequently, the accumulation of hydrogen gas generated from bacterial sugar fermentation as a mechanism to recover NAD^+, poses a metabolic burden on the microbial community (Gibson *et al* 1990). Hence, further establishment of hydrogen gas-consuming community including SRB, reductive acetogens (such as *Blautia hydrogenotrophica* via Wood-Ljungdahl pathway) and methanogens (archaea such as *Methanobrevibacter smithii*) could increase the overall metabolic efficiency of the gut microbiota (Chassard and Bernalier-Donadille 2006, Koropatkin *et al* 2012). No significant methane production has been reported for children below three years of age, suggesting that a fully functional adult microbial ecosystem has not been established yet at this period of life (Peled *et al* 1985, Rutili *et al* 1996). To date, a healthy developmental trajectory or stages of the infant gut microbiota remains elusive, despite the physiological importance that could impact lifelong health. Longitudinal clinical study and attention on individual development could start to fill this gap in our knowledge.

In adulthood, the gut microbiota is more diverse and stable compared to that of infants (Mehta *et al* 2018, Yatsunenko *et al* 2012). The gut microbial assemblage could fluctuate with the intake of specific dietary components (Turnbaugh *et al* 2009) and is

shown to be strongly affected by extreme dietary changes or the use of antibiotics (Modi *et al* 2014, O'Keefe *et al* 2015). Despite the environmental variations due to dietary habits, the host mucin glycans secreted by the goblet cells at the epithelial lining forms a consistent nutrient source for bacterial colonisation. The bacterial community at the mucosal surface could incur disproportionate health impact because of the close proximity to host cells (Koropatkin *et al* 2012). On one side, pathogenic bacteria could degrade mucins to facilitate them to penetrate the mucosal layer for host invasion or thrive on mucus-derived nutrients liberated via microbial-dependent mechanisms (Sicard *et al* 2017). On the other side, *Akkermansia muciniphila*, an intestinal symbiont specialised in degrading mucins, can support the formation of a beneficial microbial network leading to the production of important compounds like butyrate, propionate and vitamin B12 (**Chapter 4**). Through its mucolytic activity, *Akkermansia muciniphila* plays a key role as nutrients provider to sustain the community at the mucosal layer (**Chapter 5**). As such, the commensalistic community at the host-microbe interface could protect the host against intestinal pathogens via competitive exclusion and contribute to the restoration of the microbial ecosystem after perturbations (Kamada *et al* 2013, Reid *et al* 2011).

The importance of butyrate production by the gut microbiota for host health

Butyrate is a product of bacterial fermentation that is closely associated with host health. It is used as the preferred energy source for colonic epithelial cells (Donohoe *et al* 2011). Physiologically, butyrate is associated with the enhancement of colonic barrier function, increase satiety, pain relief, anti-inflammation, and protection against colorectal cancer (Banasiewicz *et al* 2013, Bolognini *et al* 2016, Donohoe *et al* 2011, Furusawa *et al* 2013, Geirnaert *et al* 2017, Goncalves and Martel 2013). Also, it can regulate host immune and metabolic states by signalling through G-protein-coupled receptors (GPR) and by inhibiting histone deacetylase (HDAC) (Bolognini *et al* 2016, Fellows *et al* 2018, Koh *et al* 2016). Most of the mechanistic evidence for the effect of butyrate is generated through studies with adults. Therefore, further mechanistic experiments are required to understand the physiological relevance of butyrate in the infant gut.

Early in life, butyrate is normally observed at a low level (Fig. 1), with around 2 mM of faecal butyrate detected in 3 months old infants, compared to 14 mM in healthy adults (Pham *et al* 2016, Schwiertz *et al* 2010). Butyrate is important to support gut maturation by fostering the development of the gut barrier function (Ploger *et al* 2012, Yan and Ajuwon 2017). Yet, the immature gut epithelium of infants may not be able to cope with the high levels of butyrate, which could potentially lead to apoptosis (Hague *et al* 1996, Ryu *et al* 2016). In principle, the direct infusion of butyrate should be able to confer the beneficial effects similar to that induced by the butyrate-producing bacteria. The supplementation of butyrate salts is found to exert a wide range of beneficial effects to pre- and post-weaning calves and piglets, including the improvement of the gut barrier function, immune system, digestibility, and growth rate (Kato *et al* 2011, Kotunia *et al* 2004). This has been performed in human adults via oral administration of butyrate tablets (Roda *et al* 2007, Vernia *et al* 2000), butyrate-producing precursors such as tributyrin (Conley *et al* 1998, Vanhoutvin *et al* 2009) or rectal enemas (Scheppach *et al* 1992). However, limited efficacy has been reported with direct administration of butyrate as several modes of action such as the effective concentration range and the site of action remain elusive. Hence, the pre-, pro- or synbiotic approach to stimulate butyrate-producing bacteria could be best to mimic the natural way to locally deliver butyrate.

Furthermore, butyrate could play a role in the immune maturation of infants. An aberrant SCFA profile and/or microbial composition are/is shown to be associated with colicky symptoms and allergy in infants (Arrieta *et al* 2015, de Weerth *et al* 2013, Pham *et al* 2017, Stokholm *et al* 2018, Wopereis *et al* 2017). A significant decrease of *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* genera, as well as reduced levels of faecal acetate at 3 months old have been observed in children at risk of asthma (Arrieta *et al* 2015). Similar findings have been reported in 6 months old children diagnosed with eczema, with increased levels of lactate, and decreased levels of butyrate and propionate in the faeces. These eco-physiological changes are correlated to the lower abundance of lactate-utilising butyrate-producing bacteria (LUB) *Eubacterium* spp. and *Anaerostipes* spp. from the *Lachnospiraceae* family (Wopereis *et al* 2017). Butyrate-producing bacteria are suggested to stimulate host immune system directly by posing as antigens or indirectly via their metabolites (Ivanov and Honda 2012). The lack of certain stimulants during infancy could lead the

manifestations of allergy later in life, due to the failure to provide necessary challenge to train the immune system during the window of plasticity (the hygiene hypothesis) (Azad *et al* 2013b).

Next generation microbiota modulators

In light of the importance for health, continuous efforts are being taken to improve the physiological state of a person by modulating his/her gut microbiota. Already in the early 20th century, Ilya Mechnikov proposed that the consumption of microbe-containing food improves health by displacing health-threatening bacteria in the large intestine (McGuire and McGuire 2015). In this thesis, novel avenues to modulate gut ecology are explored. As such, novel probiotics that are not traditionally used such as *Akkermansia muciniphila*, *Bacteroides* spp. and butyrate-producing *Clostridium* (referring to the butyrate-producing members from the *Lachnospiraceae* and *Ruminococcaceae* families) are studied. Besides, the potential of supplementing infant nutrition with HMOS is highlighted, and an update based on recent (clinical) findings is provided. Furthermore, the prospect of administrating microbiota-targeted nutrients including iron and vitamin B12 is discussed.

Novel probiotic strains

This thesis explores the physiology of a few potential probiotic strains. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill *et al* 2014). The conventional probiotics often originate from a narrow taxonomic range of bacteria mainly consisting of *Bifidobacterium* spp. and *Lactobacillus* spp. (O'Toole *et al* 2017). *Bifidobacterium* spp. (**Chapter 2**) are commonly administered as probiotic with well demonstrated health benefits, safety, and history of use (FAO/WHO 2002). Generally, novel probiotic strains are first tested *in vitro* for resistance to gastric acidity, bile acid and host digestive enzymes, anti-microbial activity, and safety aspects (including antibiotic resistance, metabolic activities i.e. D-lactate production, bile salt deconjugation, toxin production and haemolytic activity), followed by *in vivo* studies to substantiate health effects (FAO/WHO 2002, Huys *et al* 2013). The scope for probiotics has recently been revised

to include new commensals and consortia comprising defined strains from human samples (Hill *et al* 2014). Alternatively, the current legislative framework diverts the development of these beneficial microbes as live biotherapeutic product (LBP) (Sun *et al* 2016). LBP is defined by the US Food and Drug Administration (FDA) as a biological product that contains live organisms, such as bacteria; that are applicable for prevention, treatment, or cure of a disease or condition of human beings; and is not a vaccine (Hill *et al* 2014).

Akkermansia muciniphila is the first and only cultured representative of the *Verrucomicrobia* phylum from human gut (Derrien *et al* 2004). The mucin-utilisation lifestyle of this bacterium allows it to colonise the mucosal niche in the distal ileum, ascending colon and rectum (Wang *et al* 2005). The high relative abundance of *Akkermansia muciniphila* is often associated with a healthy state and negatively correlated to acute appendicitis (Swidsinski *et al* 2011), metabolic syndrome including obesity, dyslipidaemia and type 2 diabetes (Derrien *et al* 2016), irritable bowel syndromes including Crohn's disease and ulcerative colitis (Png *et al* 2010) and autism (Wang *et al* 2011). The administration of *Akkermansia muciniphila* can ameliorate metabolic symptoms of obesity, diabetes, and alcoholic liver disease in mice (Everard *et al* 2013, Grander *et al* 2017). The beneficial effect of this bacterium could also be conferred by administrating pasteurised bacteria and an immuno-stimulatory outer membrane protein (Amuc_1100) in mice (Cani and de Vos 2017, Ottman *et al* 2017b, Plovier *et al* 2017). The mode of health-promoting mechanisms include the increase of mucus thickness and gut barrier function (Everard *et al* 2013), direct host immune stimulation (Ottman *et al* 2017b), and stimulation of a beneficial mucosal community (**Chapter 4** and **5**) (Belzer *et al* 2017, Chia *et al* 2018). Nevertheless, the relative abundance of *Akkermansia muciniphila* has also been associated with negative health consequences such as Parkinson's disease (Heintz-Buschart *et al* 2018), Alzheimer's disease (Vogt *et al* 2017), multiple sclerosis (Cekanaviciute *et al* 2017) and colorectal cancer (Wang *et al* 2017). These health associations are mainly demonstrated in mouse models, and often the design of the above-mentioned studies are not as robust as those that demonstrate beneficial effect. However, it cannot be excluded that under certain conditions, *Akkermansia muciniphila* could modulate the host response differently, as shown by its exacerbation of gut inflammation in the *Salmonella* Typhimurium-infected gnotobiotic mice (Ganesh *et al* 2013). Given the above,

Akkermansia muciniphila appears promising, especially in modulating metabolic syndrome, and its application as a probiotic has already passed the safety evaluation in the first human trial (Plovier *et al* 2017).

Bacteroides spp. are dominant members in the gut microbiota of human adults. Some potential health-promoting *Bacteroides* spp. have been evaluated for probiotic functionality and include *Bacteroides xylanisolvens* DSM23694 (Ulsemer *et al* 2016), *Bacteroides ovatus* D-6 (Ulsemer *et al* 2013), *Bacteroides ovatus* V975 (Hamady *et al* 2010, Hamady *et al* 2011), *Bactroides dorei* D8 (Gerard *et al* 2007), *Bacteroides fragilis* ZY-312 (Deng *et al* 2016), *Bacteroides acidifaciens* JCM10566 (Yanagibashi *et al* 2013). However, the lipopolysaccharides (LPS) of *Bacteroides* spp. are also described to inhibit the innate immune signalling and endotoxin tolerance, leading to immune-silencing and manifestations of allergy in children in Finland and Estonia (Vatanen *et al* 2016). **Chapter 3** points out a potential metabolic concern with the specific D-lactate production by *Bacteroides* spp. Nevertheless, the relative abundance of *Bacteroidetes* family in human gut is highly subjected to environmental fluctuation (Goodrich *et al* 2014). For example, caesarean birth leads to the defective colonisation of *Bacteroides* spp. in new-borns due to disruptive vertical transfer via the natural birth route (Azad *et al* 2013a, Backhed *et al* 2015, Martin *et al* 2016). Therefore, it could be plausible to restore this lost taxon for C-section delivered infants by introducing *Bacteroides* spp. as a probiotic. *Bacteroides thetaiotaomicron* is generally recognised as a symbiont that contributes to the postnatal gut development and host physiology (Wexler 2007). Furthermore, **Chapter 3** shows that in the presence of human milk carbohydrates, *Bacteroides thetaiotaomicron* could confer a benefit to the host by supporting the growth of *Anaerostipes caccae*, leading to butyrate production.

Butyrate-producing bacteria from the family of *Lachnospiraceae* and *Ruminococcaceae* (collectively termed butyrate-producing *Clostridium*) have also been investigated for potential probiotic function. The majority of the bacteria from this group are not equipped to degrade complex carbohydrates. This thesis shows close metabolic interactions of several butyrate-producing bacteria including *Anaerostipes caccae* (**Chapter 2** to **5**), *Eubacterium hallii* (**Chapter 4**), and *Faecalibacterium prausnitzii* (**Chapter 4**) with the glycan-degrading species. *Faecalibacterium prausnitzii* is an abundant species found in the gut of healthy adults and has been demonstrated to mitigate inflammatory bowel diseases and atopic diseases (Miquel *et*

al 2013, Rossi *et al* 2016, Sokol *et al* 2008, Song *et al* 2016). Whilst, *Eubacterium hallii* is shown to improve insulin sensitivity in obese and diabetic mice as well as detoxify dietary carcinogen (Fekry *et al* 2016, Udayappan *et al* 2016). Other promising butyrate-producing *Clostridium* include *Roseburia* spp. (Louis *et al* 2007), *Clostridium butyricum* CBM 588 (Seo *et al* 2013) and *Intestinimonas butyriciproducens* AF211 (Bui *et al* 2015). The administration of butyrate-producing bacteria in an *in vitro* system inoculated with the gut microbiota of Crohn's disease patients is shown to increase the butyrate production and enhance barrier function of Caco-2 cells (Geirnaert *et al* 2017). Furthermore, the abundance of butyrate-producing bacteria could also be elevated by the supplementation of specific carbohydrates such as inulin, which are shown to increase the abundance of *Bifidobacterium* and *Anaerostipes* spp. (Vandeputte *et al* 2017). Judging from the metabolic interdependencies of butyrogens and the glycan-degrading bacterial species, a cocktail of probiotic strains instead for a strain could be administered to stimulate the beneficial butyrate production.

Novel prebiotics

A prebiotic is defined as a substrate that is selectively utilised by microorganisms to confer a health benefit (Gibson *et al* 2017). This includes carbohydrates or non-carbohydrate substrates, which could not be digested by the host but selectively promotes the metabolism of health-promoting microorganisms, either the indigenous gut residents or probiotic strains. At present, prebiotics are mostly plant- or dairy-derived. Galactans i.e. galacto-oligosaccharides (GOS) synthesised from lactose, and fructans i.e. fructo-oligosaccharides (FOS) and inulin derived from chicory root, have a demonstrated long history of use as prebiotics (Oozeer *et al* 2013, Roberfroid *et al* 1998). The linkages in GOS and FOS can be readily degraded by β-galactosidase and β-fructanosidase enzymes respectively, which are prevalent in *Lactobacillus* and *Bifidobacterium* spp. (Fig. 2) (Roberfroid *et al* 1998). A specific mixture of short chain GOS and long chain FOS at 9:1 ratio (scGOS+lcFOS), is widely applied in infant nutrition to mimic the molecular size distribution of HMOS (Boehm *et al* 2003, Boehm *et al* 2005). scGOS+lcFOS is able to lower the faecal pH, enhance the growth of bifidobacteria and reduce the load of pathogenic bacteria (Boehm *et al* 2004, Knol *et al* 2005a, Knol *et al* 2005b). This specific mixture has been extensively studied and is

shown to confer potential health benefits that are similar to those of human milk (Oozeer *et al* 2013).

Recent technological advances provide the opportunity for better nutrition. The supplementation of some HMOS structures to food could close one of the compositional gaps between human milk and infant formula. The application of HMOS in infant formula faces several challenges. First of all, it is technically challenging to obtain large quantities of HMOS for commercial use (Barile and Rastall 2013). The current supply of purified HMOS are isolated from bovine milk or synthesised by chemical or microbial means (Donovan and Comstock 2016). Secondly, it involves a lengthy legislative process towards the application of novel ingredients in infant formula (O'Toole *et al* 2017). At present, researchers are engaged in safety and tolerance studies to evaluate the suitability of HMOS as novel additives (Table 1). To date, 2'-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT) are supplemented in some infant formulas (Fig. 2). However, not much is known for the use of selected individual HMOS structure, on contrary to an assortment of over 100 structurally distinct HMOS present in human milk (Milani *et al* 2017). The application of HMOS in modulating the infant gut microbiota is an emerging field that warrants more research on the administration of fucosylated oligosaccharides (Milani *et al* 2017).

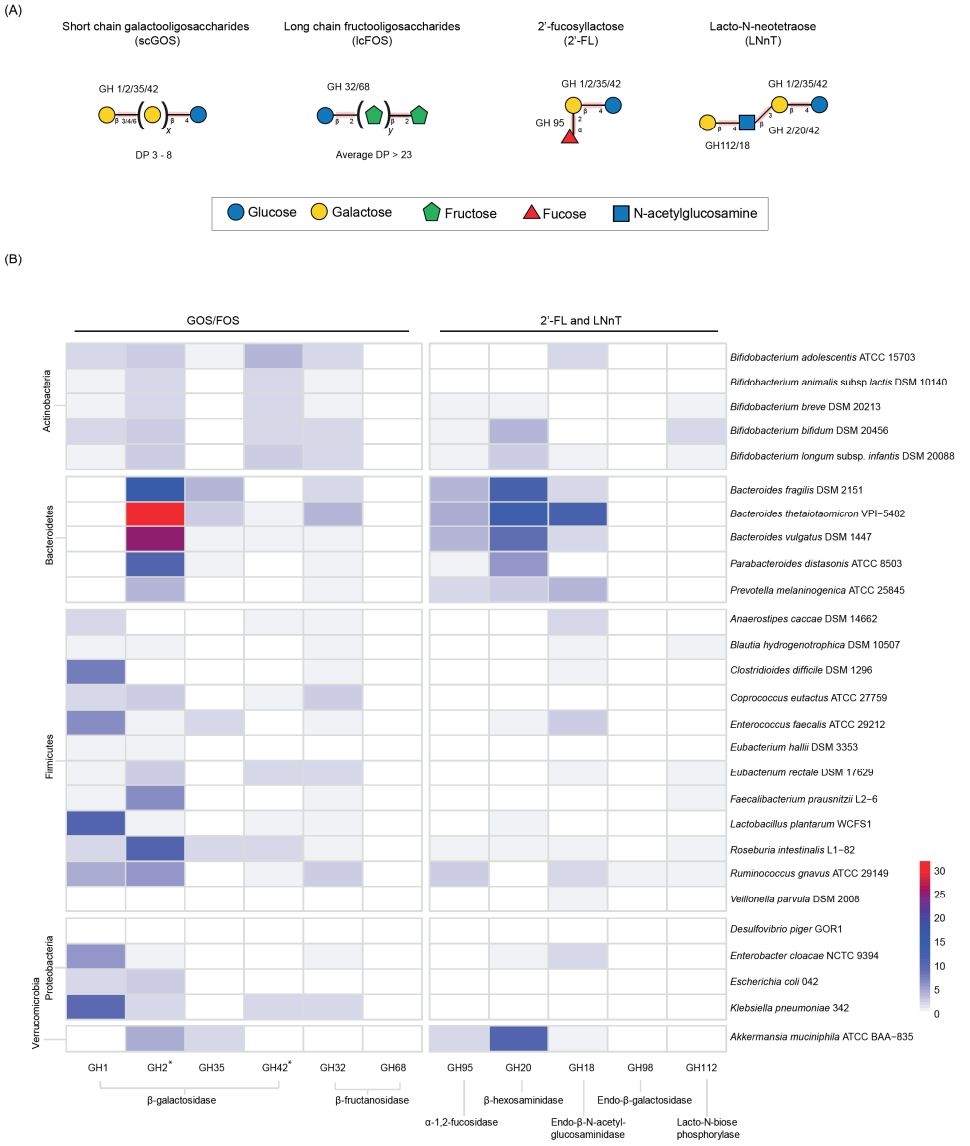


Figure 2. **Prebiotics in infant nutrition**. (A) The combination of scGOS and lcFOS at 9:1 ratio has been widely supplemented in infant formula whereas HMOS including 2'-FL and LNnT are currently evaluated in clinical study. (B) Prediction for bacterial carbohydrate-active enzymes (CAZymes) involved in the catabolism of prebiotics for the representative species in the human gut. Colour key indicating the number of CAZymes predicted in the genome. *Abbreviations*: DP, degree of polymerization; GH, glycosyl hydrolase. (*) GH 2 and GH 42 are also predicted for β-hexosaminidase function.

Table 1. The summary of clinical studies that investigated the application of HMOS in infant nutrition.

HMOS structure	Key finding	Type of study	Reference
2'-FL (2.4 g/L of total oligosaccharides with GOS + 0.2 or 1 g/L 2'-FL)	**Immune outcome**: 2'-FL supplementation induced similar inflammatory cytokines profile as in the breast-fed infants.	Randomised, double-blind controlled growth and tolerance study on healthy infants (5 days to 3 months old)	(Goehring *et al* 2016)
2'-FL (2.4 g/L of total oligosaccharides with GOS + 0.2 or 1 g/L 2'-FL)	**Growth and tolerance outcome**: Comparable growth (weight, length, and head circumference) and tolerance (stool consistency, number of stool per day and occurrence of vomit) as in breast-fed infants.	Prospective, randomised, controlled, growth and tolerance study on healthy infants (5 days to 3 months old)	(Marriage *et al* 2015)
2'-FL and LNnT (1 g/L of 2'-FL and 0.5 g/L of LNnT)	**Growth and tolerance outcome**: Similar growth and tolerance compared to control formula without HMOS supplementation. HMOS supplementation reduced parent-reported morbidity (particularly bronchitis) and medication use including antipyretics and antibiotics.	Parallel, double-blind, randomised, growth and tolerance study on healthy infants (HMOS supplementation from 0 to 6 months old, followed by standard follow-up formula without HMOS from 6 to 12 months)	(Puccio *et al* 2017)
2'-FL and LNnT (5, 10, or 20g of 2'-FL, LNnT, or 2:1 mix of 2'-FL: LNnT)	**Tolerance outcome**: Daily doses up to 20g is well tolerated accessed by the gastrointestinal symptoms rating scale. **Microbiology outcome**: HMOS supplementation increased the relative abundance of Actinobacteria and *Bifidobacterium* community. No significant changes detected in the composition of faecal SCFAs.	Parallel, double-blind, randomised, placebo-controlled study on healthy adults (2 weeks intervention)	(Elison *et al* 2016)

Abbreviation: GOS, galacto-oligosaccharides; 2'-FL, 2'-fucosyllactose; LNnT, lacto-N-neotetraose.

Microbiota-targeted nutrients

Nutrients such as trace elements and vitamins could also be used to modulate the gut microbiota. For example, iron could be a potential modulator, as excess iron in the diet could end up in the colon to stimulate the microbial community (Kortman *et al* 2014). Iron is essential for the electron transport in anaerobic bacteria (Cronin *et al* 2012). Several mechanisms are used by bacteria to acquire iron either by the secretion of chelating agents i.e. siderophores or via direct membrane protein binding (Raymond *et al* 2015). Members of *Enterobacteriaceae* family e.g. *Salmonella* Typhimurium and enteropathogenic *Escherichia coli* (EHEC) are highly competent in sequestering iron leading to their competitiveness and pathogenicity in the gut (Kortman *et al* 2012, Zimmermann *et al* 2010). On the other hand, the gut symbiont, *Bacteroides thetaiotaomicron* (**Chapter 3**) requires hemin, an iron-containing porphyrin for growth. The membrane-bound iron transport systems are found to be present in *Bacteroides* spp., with *Bacteroides fragilis* expresses the ferrous iron transport system (FeoAB) and the iron storage protein ferritin (FtnA) to acquire and store iron (Rocha and Smith 2013). Furthermore, *Bifidobacterium* spp. are also known to secrete siderophores for iron acquisition. *Bifidobacterium* spp. isolated from anaemic children display high-iron sequestration property (Vazquez-Gutierrez *et al* 2015), and are demonstrated to inhibit the growth of enteropathogens and the adhesion of the enteropathogens to the epithelial cells (Vazquez-Gutierrez *et al* 2016). Moreover, iron could modulate the child gut microbiota, in which decreased butyrate production and lower relative abundance of the members of *Lachnospiraceae* and *Bacteroides* families are observed in low iron conditions (Dostal *et al* 2013, Dostal *et al* 2015). Considering the above, the level of iron in the gut can be manipulated to prevent the outgrowth of pathogens. For instance, the co-supplementation of iron and GOS to anaemic Kenyan infants has been shown to increase iron absorption, thereby mitigates the adverse effects of iron on the gut microbiota (Paganini *et al* 2017b). The abundances of *Bifidobacterium* and *Lactobacillus* spp. are increased and the virulence and toxin genes of pathogens are reduced as a result of the co-supplementation (Paganini *et al* 2017a).

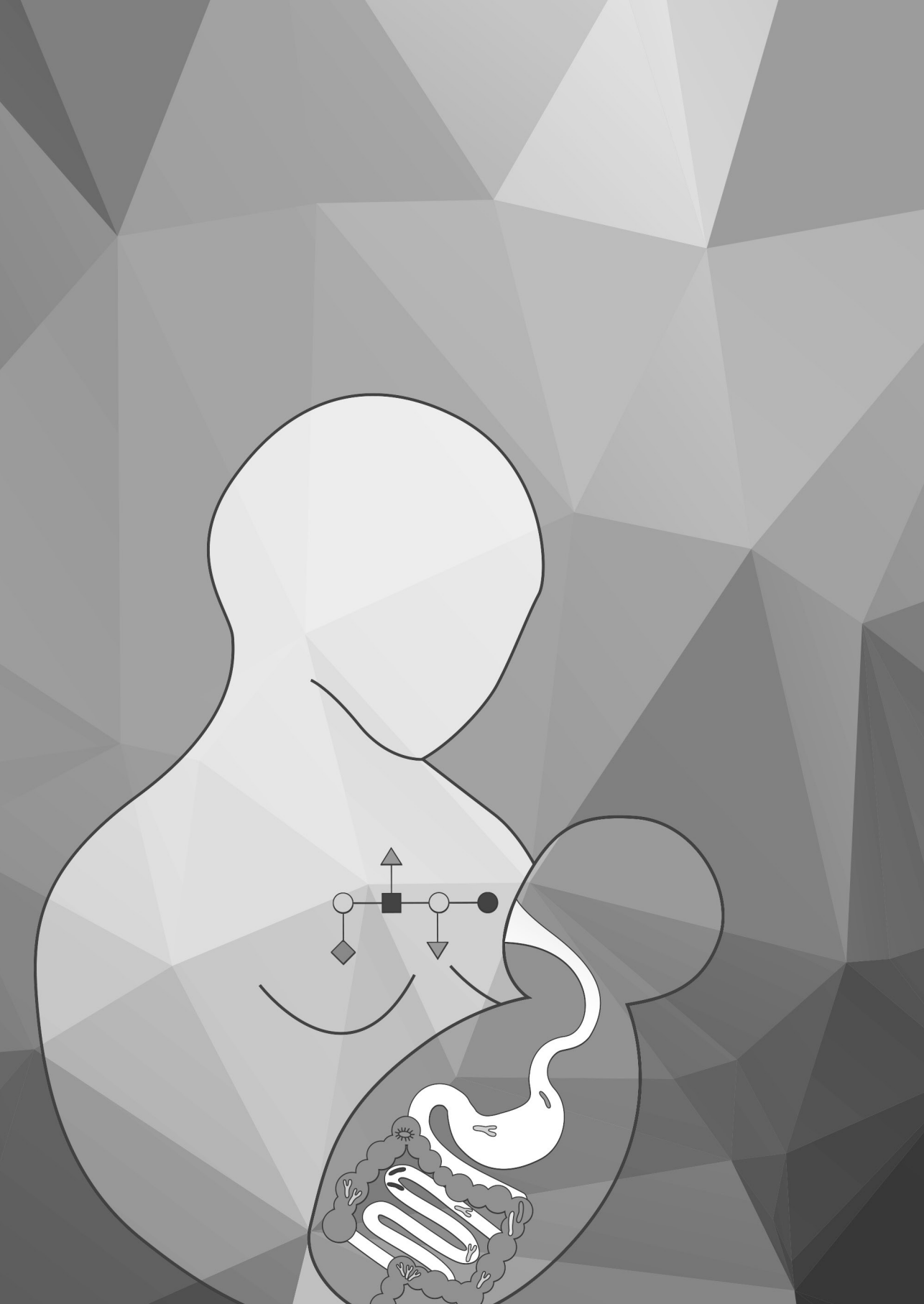
Also, vitamin B12 is known to modulate the gut microbial ecology (Degnan *et al* 2014). The gut microbiota exhibits close cooperation in B-vitamins biosynthesis (Magnusdottir *et al* 2015). Vitamin B12 is an essential co-factor for the synthesis of propionate (Takahashi-Iniguez *et al* 2012) and amino acids including folate, ubiquinone,

and methionine (Romine *et al* 2017). **Chapter 4** demonstrates that pseudo-vitamin B12 synthesised by *Eubacterium hallii* shifted the metabolism of *Akkermansia muciniphila* towards propionate production. Whilst, an accidental tri-culture of *Akkermansia muciniphila* with *Eubacterium hallii* and *Anaerostipes caccae* lead to the discovery of pseudo-vitamin B12 transportation in *Eubacterium hallii* (data not shown). The expression data suggests that *Eubacterium hallii* exported pseudo-vitamin B12 actively using an ECF transporter complex CbrTUV via ATP-dependent toppling mechanism (Slotboom 2014). Furthermore, bacterial-derived vitamin B12 can be taken up by the host (Krautler 2005). Nevertheless, the pseudo-vitamin B12 produced by *Eubacterium hallii* exhibits a lower affinity for the host intrinsic factor involving in vitamin B12 absorption (Stupperich and Nexo 1991). As such, pseudo-vitamin B12 is likely to be more beneficial for the bacterial community than for the host. The supplementation of vitamin B12 can be devised to modulate the gut microbiota towards the production of host-beneficial compounds such as propionate.

Main conclusion and future outlook

The complex nature of the human gut microbiota and host-produced glycans such as HMOS and mucins demands special scrutiny in order to comprehend the intricate host-microbial interactions. This thesis demonstrates the key functional role of glycan-degrading symbionts in fostering intestinal butyrate production via cross-feeding. As such, this confers ecological importance to the respective niche, as HMOS-degrading microbes drive the establishment of a healthy infant gut microbiota, and mucin-degrading microbes drive a beneficial mucosal community contributing to colonisation resistance and the production of host-beneficial compounds. These understandings could facilitate a rational design for intervention studies to modulate the gut microbiota leading to potential health benefits. To this end, innovative avenues using novel probiotic strains, prebiotics and microbiota-targeted nutrients seem promising. The key species investigated in this thesis, which include *Akkermansia muciniphila*, *Bacteroides* spp. and butyrate-producing *Clostridium*, could serve as novel probiotic candidates to restore the aberrant gut microbiota. Besides, the supplementation of HMOS in early nutrition could close the nutritional gap between human milk and infant formula. Furthermore, microbiota-targeted nutrients including iron and vitamin B12 could be used to modulate the microbial composition and/or activity community. Future research should focus on improving the synergistic effect of different microbiota modulators by using a holistic approach, with the combination of probiotics, prebiotics and microbiota-targeted nutrients.

Chapter 6



Chapter 7

Thesis summary &

Nederlandse samenvatting

Thesis summary

The co-evolution of the human host and the gut microbiota has led to bacterial adaption to forage on host-produced glycans such as human milk oligosaccharides (HMOS) and mucins. In early life, the HMOS that are present in mother milk contribute to the establishment of a healthy gut microbiota. Furthermore, mucins that cover the intestinal lining create a stable niche for bacterial colonization throughout a person's life. Due to the highly complex nature of these host-secreted glycans, bacteria equipped with specific glycan-degrading enzymes can exploit them as substrate for growth. Subsequently, the glycan-degrading bacteria can drive microbial networks via cross-feeding. The glycan-foraging microbial population exerts a large influence on the host physiology, by influencing the immune, metabolic, and neurological development early in life, and by conferring colonisation resistance throughout life. The work described in this thesis aims to improve our understanding of the metabolic dependencies between the milk- and mucin-degrading microbes (*Akkermansia muciniphila*, *Bifidobacterium* spp. and *Bacteroides* spp.) and butyrate-producing bacteria (*Anaerostipes caccae*, *Eubacterium hallii*, and *Faecalibacterium prausnitzii*). Bacteria-derived butyrate is the preferred energy source for host epithelial cells and is associated with a range of beneficial effects including enhancement of colonic barrier function, increased satiety, and protection against inflammation, and cancer. In the introductory chapter, the role of human milk and mucin glycans in fostering the gut microbial network is discussed. As such, the molecular mechanisms of glycan-foraging by key microbial species and the potential butyrate-inducing interaction among gut symbionts is presented.

In Chapter 2 and 3, the role of HMOS as selective substrates for microbial growth that drive the establishment of the infant gut microbiota was investigated. At this developmental stage, HMOS promote the dominance of bifidobacteria from the *Actinobacteria* phylum. Upon weaning, the gut microbiota shifts towards an adult gut microbiota composition that is predominantly comprised of bacteria from the *Firmicutes* and *Bacteroidetes* phyla. In chapter 2, the interaction between a HMOS-degrader, *Bifidobacterium infantis* (*Actinobacteria* phylum) and a butyrogenic non-HMOS-degrader, *Anaerostipes caccae* (a member of the *Lachnospiraceae* from the *Firmicutes* phylum) was studied. *Anaerostipes caccae* in monoculture was not able to metabolise

lactose or HMOS but its growth and concomitant butyrate production were detected in co-cultures with *Bifidobacterium infantis*. *Anaerostipes caccae* was sustained by cross-feeding on the monosaccharides, lactate and acetate derived from *Bifidobacterium infantis*. *Bifidobacterium infantis* fully degraded lactose and the complete range of low molecular weight HMOS, pointing towards the key ecological role of bifidobacteria in providing substrates for other important emerging species in the infant gut. The gradual shift of the microbiota composition in the ecosystem contributing to the slow induction of butyrate could also be important for gut maturation.

In chapter 3, the microbial network formation in the infant gut driven by another HMOS-degrading species, namely *Bacteroides thetaiotaomicron* was studied. We showed that *Bacteroides thetaiotaomicron* could drive the butyrogenic trophic chain with *Anaerostipes caccae*. *Bacteroides thetaiotaomicron* could metabolise lactose and HMOS. The bacterium showed different preference for specific HMOS structures when grown in co-culture. Subsequently, *Anaerostipes caccae* cross-fed on *Bacteroides thetaiotaomicron*-derived monosaccharides, lactate and acetate for growth and butyrate production. *Bacteroides thetaiotaomicron* might drive the establishment of the microbial network in the infant gut, leading to the sequential establishment of adult-like functional groups such as lactate-utilising and butyrate-producing bacteria. Furthermore, we observed stereospecific lactate isomer production in which *Bacteroides* spp. and *Bifidobacterium* spp. produced predominantly D- and L-lactate, respectively. The distinct lactate isomer production by these major glycans-degrading genera might affect the gut microbiota compositions by differential cross-feeding interaction with the lactate-utilisers.

Chapter 4 and 5 studied the role of mucins in creating a micro-environment that leads to the formation of a microbial network at the intestinal mucosal layer. In chapter 4, we demonstrated that *Akkermansia muciniphila*, a gut symbiont specialised in mucin degradation, could support the growth of the butyrate-producing cross-feeders *Anaerostipes caccae*, *Eubacterium hallii*, and *Faecalibacterium prausnitzii*. *Akkermansia muciniphila* metabolised the complex mucin glycans into short chain fatty acids including acetate, propionate and 1,2-propanediol as well as the mucin-derived sugars. Subsequently, acetate and the liberated sugars could be used by the butyrate-producers for growth and concomitant butyrate production. Interestingly, a bidirectional cross-feeding was observed between *Akkermansia muciniphila* and *Eubacterium hallii.*

Pseudo-vitamin B12 produced by *Eubacterium hallii* facilitated propionate production by *Akkermansia muciniphila* via the methylmalonyl-CoA pathway. Propionate could be beneficial to the human host by regulating satiety and lipid biosynthesis in the liver, indicative of a mutualistic host-microbial interaction driven by mucin glycans.

In chapter 5, we studied the molecular mechanism of cross-feeding between *Akkermansia muciniphila* and *Anaerostipes caccae* by metatranscriptomics. We observed a differential transcriptional response of *Akkermansia muciniphila* grown in monoculture as compared to a co-culture together with *Anaerostipes caccae*. In particular, the expression of the extracellular mucin-degrading enzymes by *Akkermansia muciniphila* was heightened in co-cultures. As a result, the monosaccharides liberated from the breakdown of mucin oligosaccharides chain could support the central metabolism of both *Akkermansia muciniphila* and the butyrate-producer. This suggested that *Akkermansia muciniphila* plays a key role in supporting the microbial community at the mucosal environment of the intestine by increasing the availability of substrates.

In summary, this thesis demonstrated the key functional role of milk- and mucin-degrading symbionts in fostering a butyrogenic microbial network via cross-feeding. Ecologically, HMOS-degraders are critical to drive the establishment of a healthy infant gut microbiota, whilst mucin-degraders are vital to maintain a beneficial mucosal community. A better understanding of the complex nature of both the microbial network and the host-secreted glycans could aid in the design of nutritional intervention for health improvement. To this end, innovative avenues using novel probiotic strains (key species including *Akkermansia muciniphila*, *Bacteroides* spp. and butyrate-producing *Clostridium*), prebiotics (HMOS in early nutrition) and microbiota-targeted nutrients (iron and vitamin B12) deem promising.

Nederlandse samenvatting

De co-evolutie van de menselijke gastheer en de darmbacteriën hebben tot gevolg dat de bacteriën zich hebben aangepast op het verbruik van door de gastheer geproduceerde glycanen zoals humane melk oligosachariden (HMOS) en mucinen. Tijdens de babytijd leveren de in de moedermelk aanwezige HMOS een bijdrage aan de totstandkoming van een gezonde darm-microbiota. Daarnaast , creëren de mucinen, die het slijmvlies van de darm bedekken, een mensenleven lang, een stabiele niche voor de kolonisatie van bacteriën. Door de zeer complexe aard van deze door de gastheer geproduceerde glycanen kunnen enkel bacteriën die zijn uitgerust met specifieke afbraak enzymen, ze benutten als substraat voor de groei. Vervolgens kunnen de bacteriën die glycanen afbreken het microbiële netwerk vormgeven via cross-feeding. De door glycanen vormgegeven microbiële populatie heeft grote invloed op de gesteldheid van de gastheer en is onder andere betrokken bij de ontwikkeling van het immuunsysteem, het zenuwstelsel en een gezonde spijsvertering. Tevens beschermt de microbiële populatie tegen de kolonisatie van pathogenen. Het werk beschreven in dit proefschrift heeft als doel het inzicht te verbeteren in de metabolische afhankelijkheid tussen melk- en mucine-afbrekende bacteriën (*Akkermansia muciniphila*, *Bifidobacterium* spp. en *Bacteriodes* spp.) en butyraat producerende bacteriën (*Anaerostipes caccae*, *Eubacterium hallii* en *Faecalibacterium prausnitzii*). Het door bacteriën uitgescheiden butyraat is de geprefereerde energiebron voor de epitheelcellen van de gastheer en is betrokken bij een scala aan gunstige invloeden, met inbegrip van de verbetering van de fysieke slijmvlies barrière, het opwekken van het verzadigingsgevoel en bescherming tegen ontstekingen en kanker. In het inleidende hoofdstuk werd de rol van HMOS en mucinen met betrekking tot de totstandkoming van het microbiële netwerk in de darmen besproken. In dat licht werden voor zogenaamde bacteriële sleutelsoorten de moleculaire afbraakmechanismen van glycanen besproken alsook hoe de interactie tussen darm symbionten leidt tot butyraat productie.

In de hoofdstukken 2 en 3 was de rol van HMOS als selectief substraat voor de microbiële groei die de totstandkoming stuurt van de kinder darm-microbiotica onderzocht. In deze ontwikkelingsfase bevorderen HMOS de dominantie van de

Bifidobacteria van het *Actinobacteria* phylum. Tijdens het spenen evolueert de darm-microbiota naar een samenstelling zoals die gevonden wordt bij volwassenen en welke voornamelijk bestaat uit bacteriën van de *Firmicutes* en *Bacteriodetes* phyla. In hoofdstuk 2 was de interactie tussen de HMOS-verbruiker *Bifidobacterium infantis* (*Actinobacteria* phylum) en de butyrogene *Anaerostipes caccae* (een lid van de *Lachnospiraceae* van het *Firmicutes* phylum) die geen HMOS kan afbreken bestudeerd. *Anaerostipes caccae* in monocultuur was niet in staat lactose of HMOS te metaboliseren, maar zijn groei en gelijktijdige butyraat productie werden waargenomen in co-culturen met *Bifidobacterium infantis*. In de co-cultuur voedde *Bifidobacterium infantis* zich met de monosacchariden, lactaat en acetaat die *Anaerostipes caccae* beschikbaar maakte (cross-feeding). *Bifidobacterium infantis* brak lactose en het complete scala HMOS met een laag moleculair gewicht volledig af, wat wijst op een ecologische sleutelrol van bifidobacteria in het voorzien van andere belangrijke opkomende soorten met substraten in de darm-microbiota van kinderen. De geleidelijke verschuiving van de samenstelling van darm-microbiotica, de gelijktijdig toenemende butyraat productie zouden ook belangrijk kunnen zijn voor de maturatie van de darmen.

In hoofdstuk 3 werd ook de formatie van de darm-microbiota in kinderen onder sturing van een HMOS-afbrekende bacteriesoort bestudeerd, alleen in dit hoofdstuk draaide het om *Bacteroides thetaiotaomicron*. We toonden aan dat *Bacteroides thetaiotaomicron* butyraat-productie door *Anaerostipes caccae* kon aansturen en lieten zien dat *Bacteroides thetaiotaomicron* lactose en HMOS kon metaboliseren. Het bleek dat in co-cultuur *Bacteroides thetaiotaomicron* een voorkeur had voor andere HMOS. *Anaerostipes caccae* groeide op monosacchariden, lactaat en acetaat die werden geproduceerd door *Bacteroides thetaiotaomicron* en produceerde zelf butyraat. Het is mogelijk dat via cross-feeding *Bacteroides thetaiotaomicron* de formatie van de darm-microbiota aanstuurt in kinderen, wat uiteindelijk leidt tot kolonisatie van lactaat-afbrekende en butyraat-producerende bacteriën die worden gevonden in volwassenen. We vonden dat *Bacteroides* spp. voornamelijk D-lactaat produceerden terwijl *Bifidobacterium* spp. voornamelijk L-lactaat produceerden. De gedifferentieerde productie van lactaat isomeren van deze belangrijke glycanen-afbrekende geslachten hebben mogelijk invloed op de samenstelling van de darm-microbiota door gedifferentieerde cross-feeding interactie met de lactaat-gebruikers.

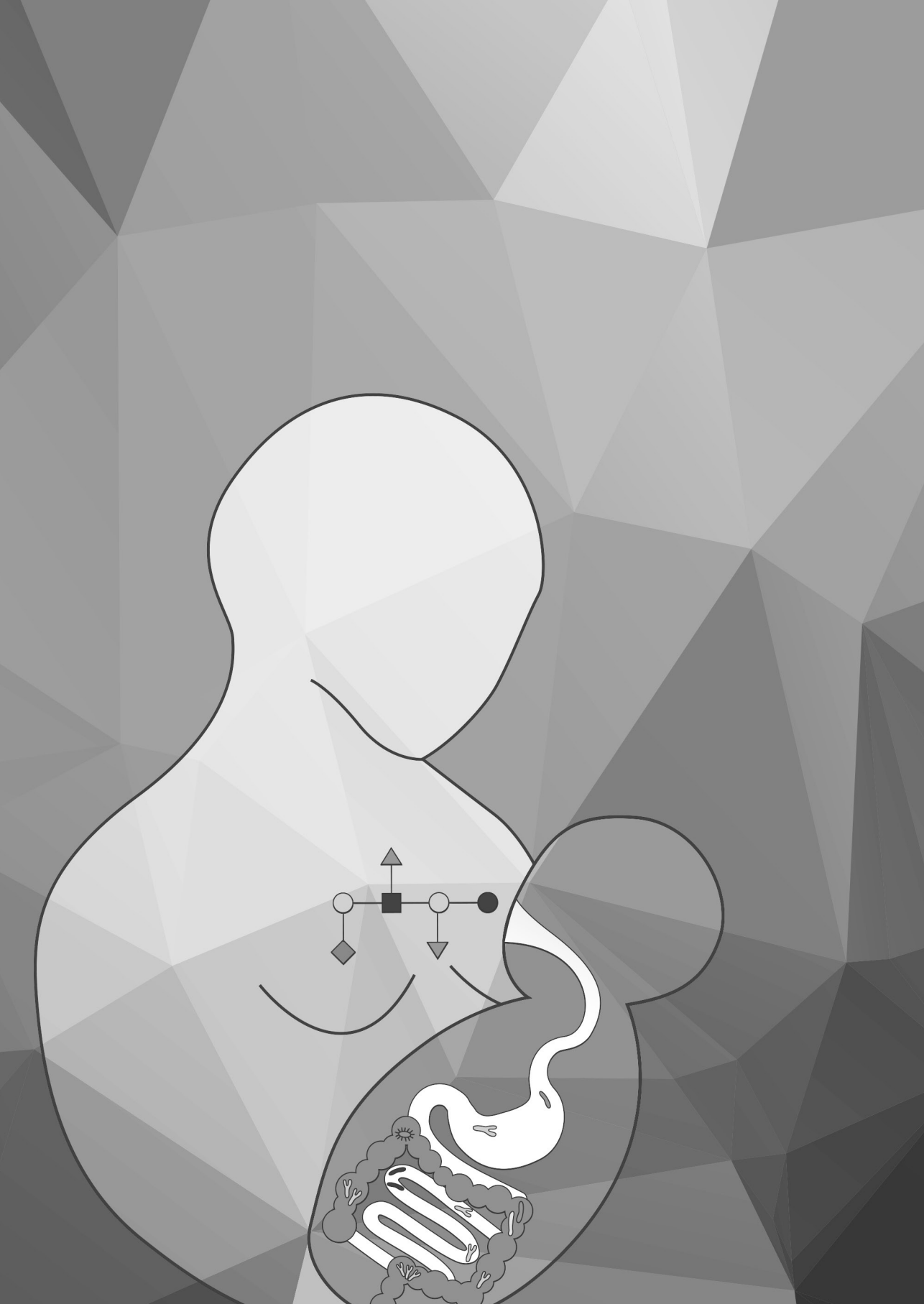
De hoofdstukken 4 en 5 bestudeerden de rol van mucinen in het creëren van een micro-niche die leidt tot de formatie van een microbieel netwerk bij het darmslijmvlies. In hoofdstuk 4 lieten we zien dat *Akkermansia muciniphila* (een darm symbiont die gespecialiseerd is in de afbraak van mucinen) de groei van de butyraat-producerende cross-feeders *Anaerostipes caccae*, *Eubacterium hallii*, en *Faecalibacterium prausnitzii* kon ondersteunen. *Akkermansia muciniphila* metaboliseert de complexe mucinen in (1) korte keten vetzuren, met inbegrip van acetaat, propionaat en propaan-1,2-diol , en (2) van mucinen afgeleide suikers. De acetaat en het vrijgekomen suiker kunnen vervolgens worden gebruikt door butyraat-produceerders voor groei en de daarmee samengaande butyraat productie. Het was belangwekkend dat er een bi directionele cross-feeding werd waargenomen tussen *Akkermansia muciniphila* en *Eubacterium hallii*. De door *Eubacterium hallii* geproduceerde pseudo-vitamine B12 bevorderde de productie van propionaat door *Akkermansia muciniphila* via de methylmalonyl-CoA route. Propionaat zou nuttig kunnen zijn voor de menselijke darm-microbiota middels het regelen van het verzadigingsgevoel en de vetsynthese in de lever, wijzend op een interactie tussen de gastheer en zijn darmbacteriën die wordt aangedreven door mucinen.

In hoofdstuk 5 bestudeerden we het moleculaire mechanisme van cross-feeding tussen *Akkermansia muciniphila* en *Anaerostipes caccae* met behulp van metatranscriptomics. We vonden dat de transcriptionele reactie van *Akkermansia muciniphila* anders was in monocultuur dan wanneer de bacterie werd gegroeid in co-cultuur met *Anaerostipes caccae*. Met name de expressie van de extracellulaire mucine-afbrekende enzymen door *Akkermansia muciniphila* was toegenomen in de co-cultuur. Het gevolg was dat de vanuit de mucinen bevrijdde suikers het centrale metabolisme konden ondersteunen van zowel *Akkermansia muciniphila* als de butyraat produceerder. Dit suggereert dat *Akkermansia muciniphila* een sleutelrol speelt in het ondersteunen van de microbiële gemeenschap in het milieu van het darmslijmvlies door het verhogen van de beschikbaarheid van substraten.

Samenvattend, het werk besloten in dit proefschrift toont aan dat melk- en mucinen-afbrekende symbionten in de darmen het butyrogene netwerk bevorderen via cross-feeding. Ecologisch gesproken zijn HMOS-afbrekers cruciaal voor het vormgeven van een gezonde darm-microbiotica bij jonge kinderen terwijl mucine-afbrekers van cruciaal belang zijn voor het behouden van een gezonde mucosale

gemeenschap de rest van het leven. Een beter begrip van de complexe aard van zowel het microbiële netwerk als de door de gastheer uitgescheiden glycanen, kan een bijdrage leveren aan de volksgezondheid, als het opzetten van voedingsinterventies. Met het oog daarop worden innovatieve remedies die gebruik maken van probiotica (sleutelsoorten, met inbegrip van *Akkermansia muciniphila*, *Bacteroides* spp. en butyraat-producerende *Clostridium*), prebiotica (HMOS, bij voeding op jonge leeftijd) en op darm- microbiotica gerichte nutriënten (ijzer en vitamine B12) als veelbelovend beschouwd.

Chapter 7



Appendices

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Acknowledgements

~ When you want something, all the universe conspires in helping you to achieve it. ~

Paulo Coelho

I would like to acknowledge many people who have shone on my universe throughout this PhD journey.

First of all, I would like to thank Jan and Clara for giving me the opportunity to start my PhD at the Laboratory of Microbiology. Your supervision and help during the past years have been essential for the quality work presented in this thesis. Jan, I am particularly thankful for your trust and support as well as providing me with the opportunity to work at the interface between academia and industry, which I enjoyed very much. Clara, your curiosity in science and creativity are my greatest learning points.

Next, I would like to thank all my collaborators whom I have pleasant and fruitful cooperation with. Willem, thanks for your insightful and critical feedback on the manuscript. Steven, thank you for your help, especially for your guidance in the lab during the first year of my PhD. Bastian, it has been a pleasure to work together with you in analysing the metatranscriptomic data. I would also like to extend my gratitude to others in Systems and Synthetic Biology including Bart, Jasper K., Benoit, Maria, Edoardo, and Peter for your help in solving bioinformatics and statistical problems. Nam, Fons, and Caroline, thanks for answering my endless physiology related questions. Roger, Kees, and Sebastian, thanks for co-supervising the students and help in setting up the co-culturing experiment. Harm and Rob, thanks for your help for FISH analysis. Bernadet, Marko, and Bernd, your expertise in human milk research has been the key for several chapters in this thesis.

I am very proud to be a part of the Laboratory of Microbiology and would like to express my gratitude to Willem for creating a stimulating learning environment. Furthermore, I am grateful to the support team (Wim, Anja, Carolien, Sjon, Phillippe, and Mirelle) who makes sure that everything is running smooth and neat. To all the

technicians (Tom vdW, Tom S., Ineke, Steven, Wilma, Monika, Ton, Hans, Jorn, and Merlijn), thank you for your patience and technical support during these years. I would also like to thank all members of the Molecular Ecology group for nice scientific meetings and cake gathering every Thursday. And, to all the colleagues from Bacterial Genetic, Microbial Physiology, and Systems and Synthetic Biology, who made these 4.5 years an enjoyable and memorable epoch!

Danone Nutricia Research has been the second home of my PhD. I am grateful for the help I received and cooperation I enjoyed over the past years with the Gut Biology and Microbiology team (Roger, Kees, Desiree, Rob, Amra, Heleen, Tiemen, Harm, Sebastian, Jolanda, Mona, Guus, Raish, and Kaouther) and the Advance Analytical Discovery team (Bernadet, Eline, Marko, and Bernd).

I am pleased to be part of the Early Life Microbiology team, a nice platform to share and discuss laboratory trivial and science. Lennart, Romy, and Harm, I am glad to have your companion since the beginning and I have learnt a lot from you. I am also glad to get to know the energetic newbies in our team, Emmy, Giannis K., Charmaine, Jannie, and Patrick.

I would also like to acknowledge the work from my students that were involved in my research both at the Laboratory of Microbiology and at Danone Nutricia Research. So, Joost, Rick, Yan (x2), Sharon, and Fitriari (Pipit), thanks for your help.

Next, I would like to thank my paranymphs, Johanna and Emmy for your support. Johanna, this PhD journey would be bland without you, and I am glad that you have been there since the beginning. Emmy, I am also glad to share a lot of commonality and fought the uphill battle together with you.

Also, to my office mates, Tom vdB, Kees, Johanna, Hanne, Sudarshan, Jie, Dat, Grant, Menia, and Leyre, thanks for the nice chat and discussion. One thing in common among us, that we are not good in taking care of plants (to be fair, except Johanna). Worth mentioning that at the moment of writing this acknowledgements, a new leaf has miraculously grown out from the dried banana plant (an office insider news).

The PhD trip to California was another memorable moment. Thanks to the 2015 PhD trip committee members for the almost flawless planning. Yue, Anna, Lara, Alex

U., Alex K, Johanna, Ying, Ioannis M., Nico, Klaudyna, and Michiel, thanks for the amazing trip all the way from San Diego to Yellowstone!

The Veluweloop team run has been something that I look forward to every year. Thanks Tom vdW for organising it and all the runners throughout the years (Fons, Alex K., Jorrit, Jasper, Nico, Monika, Elleke, YiFan, Noora, Romy, Daan S., Thomas, Melvin, CaiFang, Janneke, Rob, Yuan, Emma, Wen, Thomas, Mark, and Daan vV).

Furthermore, I am glad to be part of 'the Chinese food group' with YiFan, Ying, Yue, Yuan, CaiFang, Jie, Ran, TaoJun, Peng, ZhiFei, and occasionally Charmaine. Nothing can be better than having good food with good companions.

I am also grateful to my housemates, the Pomonians (Sacha, Kim, Marlies, Marcus, Marcel, Simon, SiewLing[6.5], Mariana, Anna, Anita, Marta, Helene, Lieke, Chihiro, Diana, Arely, Megan, Jane, and Ivette) with whom I have shared daily trivial, cooking experiments, meals, gossips and laughter with.

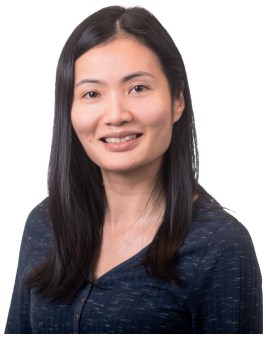
Thank you Dennis and family (Agnes, Teus, Astrid, Stephan and Sophieje) for the nice company and support throughout the years. A special thanks to Agnes for helping me to translate the Nederlandse samenvatting of this thesis.

Finally, a big thank you to _____, IPMen, all friends and family for your companion and support! 我要感谢家人们一直以来，远距离的支持。妈，你对生活的坚持，是我最好的榜样。

Loo Wee

May 2018, Wageningen

About the author



Picture by Sven Menschel

Loo Wee Chia was born on the 27th of October 1986 in Kajang, Malaysia. After completing her secondary and pre-university education at SMJK Yu Hua, Kajang in 2006, she continued her study with BSc in Food Technology and Bioprocess at Universiti Malaysia Sabah, Sabah. Her BSc thesis investigated the effect of fructooligosaccharides and inulin on the fermentation kinetics of probiotics in cultured milk. She fulfilled her BSc with internship at Nestlé Manufacturing (Malaysia) Bhd., Negeri Sembilan (Malaysia) working with product and process optimisation for chocolate. In 2010, She furthered her education with MSc in Food Technology Specialised in European Master in Food Studies, a multinational food technology program at four European universities including Wageningen University & Research (the Netherlands), University Cork College (Ireland), AgroParisTech (France), and Lund University (Sweden). She concluded her MSc study with internships at Danone Research Centre for Specialised Nutrition, Wageningen (the Netherlands) working on the optimisation of mutagenesis assays in *Lactobacilli*. After obtaining her MSc degree in 2012, she started working as Junior Product Developer at Dutch Lady Milk Industries Bhd., a subsidiary of Royal Friesland Campina, Selangor (Malaysia). There, she was responsible for the new product development of long shelf life dairy-based beverages. She went back to the academia and started her PhD in 2013 at the Laboratory of Microbiology in collaboration with Danone Nutricia Research under the supervision of Prof. Dr Jan Knol and Dr Clara Belzer. Her PhD research, which is described in this thesis, investigated the beneficial cross-feeding interactions of gut symbionts driven by human milk oligosaccharides and mucins as well as the ecophysiology of (novel) probiotic strains.

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List of publications

Ng SY, **Chia LW**, Padam BS, Chye FY (2014). Effect of selected oligosaccharides on the viability and fermentation kinetics of *Lactobacillus acidophilus* and *Lactobacillus casei* in cultured milk. Journal of Pharmacy and Nutrition Sciences **4** (2): 92-99.

Belzer C, **Chia LW**, Aalvink S, Chamlagain B, Piironen V, Knol J, de Vos WM (2017). Microbial metabolic networks at the mucus layer lead to diet-independent butyrate and vitamin B12 production by intestinal symbionts. mBio **8**:e00770-17.

Chia LW, Hornung BVH, Aalvink S, Schaap PJ, de Vos WM, Knol J, Belzer C (2018). Deciphering trophic interaction between *Akkermansia muciniphila* and the butyrogenic gut commensal *Anaerostipes caccae* by metatranscriptomics. Antonie van Leeuwenhoek. 10.1007/s10482-018-1040-x.

Ceapa C, Bongers RS, **Chia LW**, Lambert JM, Knol J, Kleerebezem M. L-fucose metabolization in *Lactobacillus rhamnosus*: identification and analysis of the operon. Submitted.

Bäumel S., Tytgat HLP, Nemec B, Schmidt R, **Chia LW**, Smidt H. Fifty percent human – how art brings us in touch with our microbial co-habitants. Submitted.

Chia LW, Mank M, Blijenberg B, Bongers RS, Aalvink S, van Limpt K, Wopereis H, Tims S, Stahl B, Belzer C, Knol J. Cross-feeding between *Bifidobacterium infantis* and *Anaerostipes caccae* on lactose and human milk oligosaccharides. Submitted.

Chia LW, Mank M, Blijenberg B, Aalvink S, Bongers RS, Stahl B, Knol J, Belzer C. *Bacteroides thetaiotaomicron* stimulates the growth of butyrate-producing *Anaerostipes caccae* in the presence of lactose and human milk oligosaccharides. In preparation.

Overview of completed training activities

Discipline specific activities

Courses

Laboratory Animal Science, Wageningen, NL	2014
Epigenesis and epigenetics – Physiological consequences of perinatal nutritional programming, Wageningen, NL	2014
The intestinal microbiota and diet in human and animal health, Wageningen, NL	2014
R course, Detroit, US	2015

Meetings

Novel Anaerobes symposium, Wageningen, NL – poster presentation	2014
ENGIHR conference: The gut microbiota throughout life, Karlsruhe, DE – poster presentation	2014
KNVM fall meeting, Amsterdam, NL	2014
Gut day symposium, Amsterdam, NL – poster presentation	2014
The future of pre- and probiotics symposium, Wageningen, NL – poster presentation	2015
KNVM spring meeting, Arnhem, NL – oral presentation	2015
Gut day symposium, Rotterdam, NL – poster presentation	2015
KNVM spring meeting, Arnhem, NL – oral presentation	2016
INRA-ROWETT conference: Gut microbiology, Clermont-Ferrand, FR – poster presentation	2016
KNVM spring meeting, Arnhem, NL – poster presentation	2017
Microbiology centennial conference, Wageningen, NL – oral pitch presentation	2017
Nutricia symposium, Utrecht, NL – oral presentation	2017

General courses

VLAG PhD week, Baarlo, NL	2014
Techniques for Writing and Presenting a Scientific Paper, Wageningen, NL	2015
Scientific Writing, Wageningen, NL	2016
Competence assessment, Wageningen, NL	2016
Career Orientation, Wageningen, NL	2017

Optionals

Preparing project proposal, Wageningen, NL	2014
Laboratory of Microbiology PhD trip, California, US	2015
Nutricia LEAN microbiota meetings, Utrecht, NL	2014-2015
Journal club, Wageningen, NL	2014-2017
Laboratory of Microbiology PhD meetings, Wageningen, NL	2014-2017
Molecular Ecology group meetings, Wageningen, NL	2014-2017

The research described in this thesis was financially supported by Danone Nutricia Research.

Printing of this thesis was financially supported the Netherlands Society of Medical Microbiology (NVMM) and the Royal Netherlands Society for Microbiology (KNVM), Danone Nutricia Research, and the Laboratory of Microbiology, Wageningen University & Research.

Colophon

Cover design: Stefanie van den Herik and Loo Wee Chia

Layout design: Loo Wee Chia

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