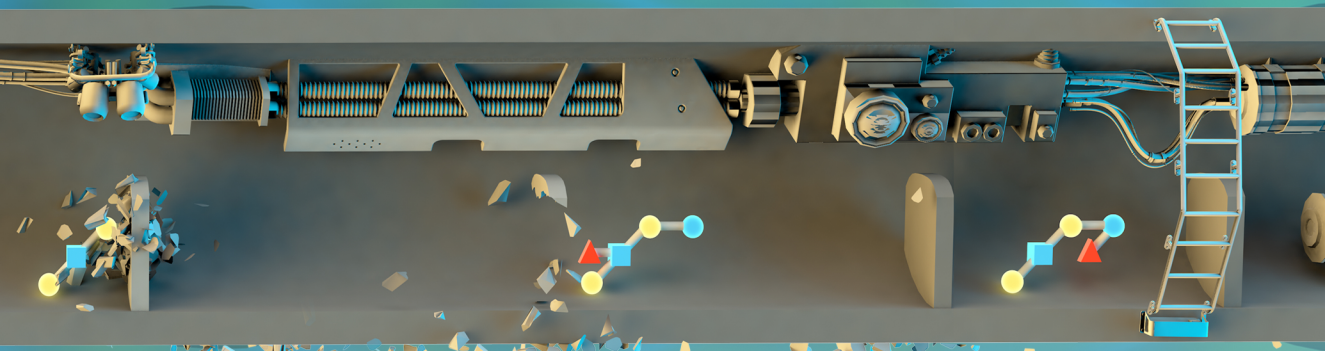
Variation in gastrointestinal metabolization of prebiotics

learnings from human milk oligosaccharides and modified starch



Fangjie Gu

Propositions

1. The dynamic interaction between gut microbiome and prebiotic fermentation in the colon is like a chicken and egg situation.
   (this thesis)

2. It is unrealistic to aim for an infant formula that would meet the needs of every baby.
   (this thesis)

3. Biofilms have potential to become environment-friendly and sustainable lubricants due to their favorable physicochemical properties (Hou et al., *Appl Environ Microbial* **2018,** *84* (1), e01516-17).

4. "Rectal seeding" to stimulate beneficial gut colonization of caesarean section born babies would be more helpful than vaginal seeding (Mitchell et al., *bioRxiv* preprint **2020)**.

5. Reduced feelings of guilt brought by the environment-friendly claims of products will eventually lead to production of more waste.

6. Decluttering is not only about throwing away what you don't need, but more importantly, to figure out what you really need.

Propositions belonging to the thesis, entitled:
Variation in gastrointestinal metabolization of prebiotics - learnings from human milk oligosaccharides and modified starch

Fangjie Gu
Wageningen, 26 January 2021

Variation in gastrointestinal metabolization of prebiotics – learnings from human milk oligosaccharides and modified starch

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Variation in gastrointestinal metabolization of prebiotics – learnings from human milk oligosaccharides and modified starch

Fangjie Gu

Thesis

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ABSTRACT

Prebiotics and its close interactions with human gut microbiota is gaining attention in recent years, as it exerts multiple impacts on human health. Studying the metabolization of metabolic fates of indigestible oligo- and poly- saccharides in the gastro-intestinal tract is vital for understanding the mechanism of how the carbohydrates influence gut environment and immune system. The current thesis describes the metabolization of indigestible carbohydrates, human milk oligosaccharides (HMOs) and isomalto/malto-polysaccharides (IMMPs) by infant or adult gut microbiota using *in vivo* or *in vitro* study approaches.

HMOs are considered as the first natural prebiotic for life, providing essential benefits to support infant health. Concentrations of 19 major HMOs present in human milk and infant fecal samples were obtained by combining high performance anion exchange chromatography - pulsed amperometric detection (HPAEC-PAD) and porous graphitized carbon - liquid chromatography mass spectrometry (PGC-LC-MS). Relative levels of fucosylated and sialylated HMO structural elements was obtained by one-dimensional ^{1}H nuclear magnetic resonance (1D ^{1}H NMR). Mother milk and paired infant fecal samples at one-month postpartum from the KOALA cohort study and at two-, six-, and 12- weeks postpartum from the BINGO cohort study were analyzed for HMOs present. Fecal microbiota composition was characterized using Illumina HiSeq amplicon 16S rRNA sequencing. The results showed inter-individual variations regarding HMO synthesis by mothers and metabolism by infant gut microbiota during the first three months of life. HMOs with different structural elements were differentially consumed when passing through infant gut, and their metabolic fates depended on both linkage types and decoration of fucose/sialic acid moieties. Three distinct consumption patterns of HMOs were found among infants under three months of age, which correlated to three microbial community clusters found in the infant fecal samples. Caesarean section and early exposure to hospital/clinic associated surroundings were found to delay the gradual progression of infant gut development stages. Several phylotypes (OTUs) within specific genera were suggested to be the key taxa responding for HMO metabolization.

Prebiotic fermentation behaviors of IMMPs were studied using a batchwise *in vitro* fermentation model with human fecal inoculum. Structural degradation on molecular levels, metabolites production, enzyme expression and microbiota composition changes during *in vitro* fermentation were linked together, pointed out a slow-fermenting behavior and structure-specific prebiotic effects of IMMPs.

Overall, these findings provide valuable evidence for researchers and industry to identify functional structures of prebiotics for humans at different developmental stages.

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Chapter 1

General introduction

The project

In recent years, both researchers and industries have shown increased interest in bio-active oligo- and polysaccharides, especially those that contribute to a healthy gut and immune system. The microbiota in the gut is crucial for human life in maintaining homeostasis of the human immune system and metabolism. Many indigestible carbohydrates such as human milk oligosaccharides (HMOs) naturally present in maternal milk and lactose-based galacto-oligosaccharides (GOS) have been shown to have a strong effect on the microbiota composition, thereby supporting immunological and metabolic homeostasis. However, the number of healthy carbohydrates that reach the market is still limited, partly due to a lack of targeted approaches to identify specific effects of prebiotics.

The current research was part of a Carbohydrate Competence Centre (CCC) programme named CarboHealth, with the theme "Carbohydrates for improving health". The CarboHealth project aimed at developing a multidisciplinary technology platform to rapidly identify and validate the health promoting properties of indigestible carbohydrates in a highthrough put manner. The current thesis focuses on structural analysis of indigestible oligo- and polysaccharides, as well as investigating the metabolism of bacteria utilizing these carbohydrates in the gut using different study approaches, identifying intermediates and end products that may accumulate during fermentation, and recognizing synbiotic effects between prebiotics and microbiome. HMOs and isomalto/malto-polysaccharides (IMMPs) were used as representative oligosaccharides and polysaccharides, respectively. In the current thesis, we focused on metabolic fates of carbohydrates in gastrointestinal tract, while the gut microbiota analysis was addressed through collaboration with a partner of CCC CarboHealth.

Prebiotics

A prebiotic was previously defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health".[1] In 2017, the definition of a prebiotic was updated to "a substrate that is selectively utilized by host microorganisms conferring a health benefit".[2] A large and dynamic bacterial community has evolved and adapted to reside in the human intestine, and this microbiota has significant influence on human health.[3] A series of studies suggested that the human colon microbiota has important and specific metabolic, trophic and protective functions, e.g. the production of short chain fatty acids and the interactions between gut bacteria and host immunity. [4-7] Besides, it has been demonstrated that changes in the microbiota are associated with diseases such as Crohn's disease, ulcerative colitis and obesity.[8] A conceivable approach towards manipulation of the microbiota composition with a highly industrial

relevance is by administration of prebiotic carbohydrates. A few carbohydrates such as inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) have been well documented for their beneficial effects on gut microbiota and on immunological and metabolic homeostasis.[9-14] Health benefits of prebiotics can be proved by different study approaches, including human trials, animal experiments, *in vitro*, and computational research.[3]

HMOs as the first prebiotic for infants

Human milk oligosaccharides (HMOs) belong to the most important bioactive oligosaccharides present in nature. Research on HMOs started in the last century, and gained more and more attention in recent years.[15] When searching in the literature database of PubMed.gov, the results showed a clear increase from around 2011 onwards (Figure 1.1). HMOs were firstly known as "bifidus factor", which played a role in the differences in gut microbiota composition between breast-fed and bottle-fed infants.[16] Later on, more studies showed the benefits of HMOs in pathogen inhibition, immune-modulation and brain development for infants.[16] The HMO profiles of human milk are unique in their high concentrations and complexity compared to milk of other mammals.[17] As shown in Figure 1.2, bovine milk contains only trace amounts of oligosaccharides.[16, 18] In contrast, HMOs are present as the third largest fraction in human milk, after lactose and lipids. Despite large variations in reported values of HMO level in mature human milk, a commonly accepted range of HMOs concentration is 5-20 g/L.[16]

According to literature, more than 240 HMOs have been discovered, from which more than 160 structures have been characterized.[19-22] A blueprint of HMOs structures is given in Figure 1.2. Although present in high complexity, all HMOs structures are built from five monosaccharides, including glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc) and *N*-acetylneuraminic acid (Neu5Ac). The core structures are formed by adding lacto-*N*-biose (Galβ1-3GlcNAc-) or *N*-acetyllactosamine (Galβ1-4GlcNAc-) to the lactose, and are named type 1 and type 2 chain, respectively.[16] Fucosylated HMOs are formed by attaching fucose to core structures via (α1-2)-, (α1-3)-, or (α1-4)-linkages. HMOs can also be sialylated as a result of attaching sialic acid (*N*-acetylneuraminic acid; Neu5Ac) via (α2-3)- or (α2-6)- linkages.[16] Furthermore, fucose and Neu5Ac can decorate the lactose backbone unit directly, e.g. 2′-fucosyllactose (2′FL) and 6′-sialyllactose (6′SL).

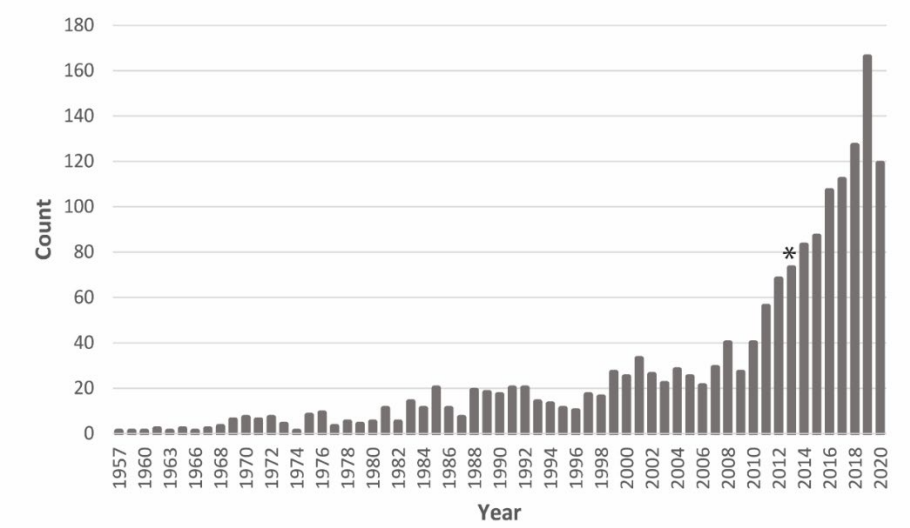


Figure 1.1. Counts of search papers per year using the keywords “human milk oligosaccharide” in the database of PubMed.gov (https://pubmed.ncbi.nlm.nih.gov/). The asterisk indicates the year when the current PhD research started.

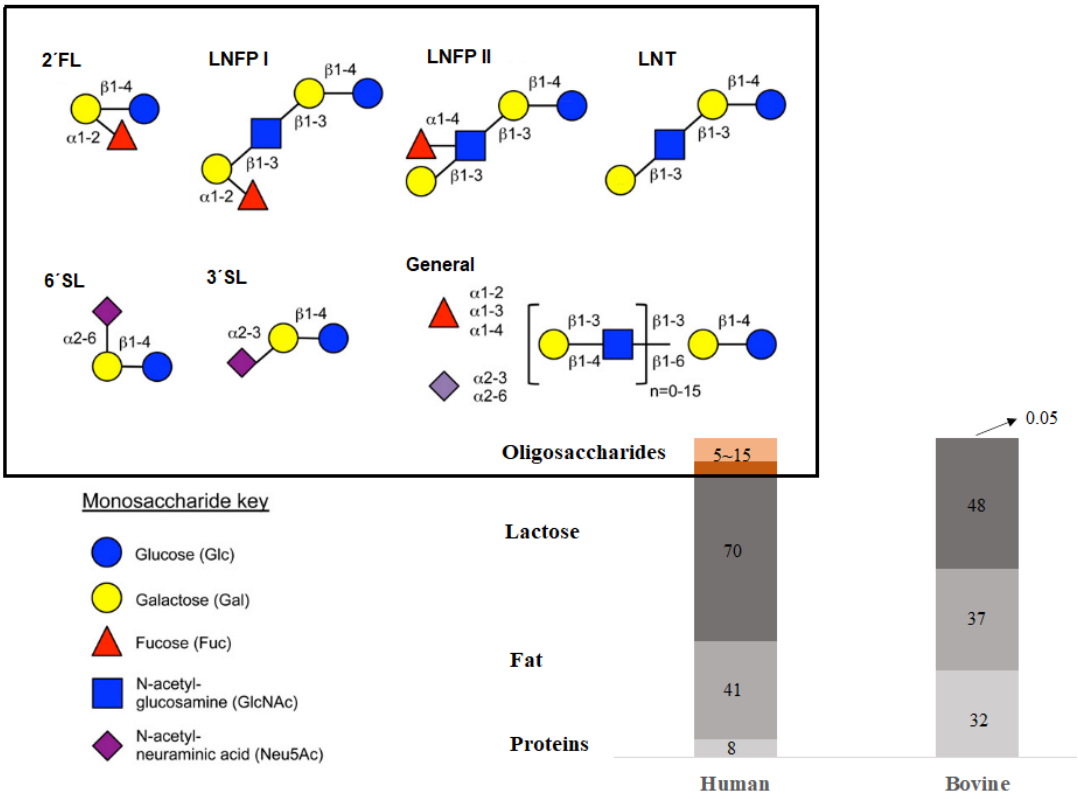
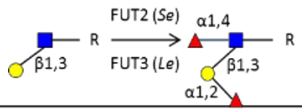
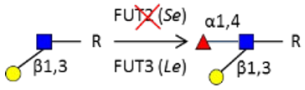
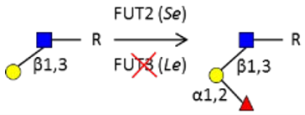
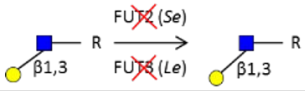


Figure 1.2. Approximate values of macronutrients and oligosaccharides in mature human and bovine milk (g/L) are given in the stacked column.[16] General structural blueprint of human milk oligosaccharides (HMOs) and several major HMOs are shown in the squared area, with the monosaccharide key given at the bottom left. When n = 0, either Fuc or Neu5Ac will be linked onto the lactose backbone. Adapted from Bode et al.[23]

Variation in HMO profiles among lactating women

Large variations exist in HMO compositions among lactating women, regarding amount as well as structures present in the ensemble. Lewis blood types and Secretor status of mothers are two of the genetic factors that decide the inter-individual variation of HMO synthesis (Table 1.1).[24] There are four milk groups reported, as a result of expression of the *Le* gene and the *Se* gene, which encodes the enzyme α1,-3/4-fucosyltransferase (FUT3) and α1,-2-fucosyltransferase (FUT2), respectively.[25] In the milk group of Lewis-positive Secretor (Le+Se+), FUT2 and FUT3 are both expressed for producing (α1-2)-, (α1-3)- and (α1-4)-linked fucosylated HMOs. Mothers from milk group of Lewis-negative Secretors (Le-Se+) are not able to express FUT3, thus their milks lack (α1-4)-linked fucosylated HMOs. The milk group of Lewis-positive non-Secretors (Le+Se-) lacks (α1-2)-linked structures, due to absence of FUT2 expression. Lewis-negative non-Secretors (Le-Se-) milk group contains merely (α1-3)-linked structures, which are produced with FUTs independent from *Le* gene and *Se* gene.[25] It has been shown that Lewis blood types and Secretor status of mothers could be determined according to the presence of specific fucosylated HMOs, such as 2′FL, lacto-*N*-fucopentaose I (LNFP I), lacto-*N*-fucopentaose II (LNFP II), and lacto-*N*-difucohexaose I (LNDFH I), in their milks instead of the traditional serological examination.[25-28]

Table 1.1. Milk groups as a result of Secretor status and Lewis blood types of mothers and the distribution in Caucasian population.[16, 29-30] HMO epitopes are designed as: yellow circle - galactose; blue square - *N*-acetylglucosamine; red triangle – fucose.

Milk Groups	Fucosylation	Caucasian population %
Group 1 : (Se+Le+) Secretor - Lewis-positive		69%
Group 2 : (Se-Le+) Non-Secretor - Lewis-positive		20%
Group 3 : (Se+Le-) Secretor - Lewis-negative		5-10%
Group 4 : (Se-Le-) Non-Secretor - Lewis-negative		1%

The frequency of these four milk groups varied geographically. As given in Table 1.1, milk group Se+Le+ is the most predominant (69%) in the Caucasian population, followed by Se-Le+ (20%) and Se+Le-

(5-10%), and Se-Le- is quite rare (1%).[29-30] In contrast, Lewis negative and non-Secretor phenotypes are found in much higher frequency in West Africa (32-38%), as well as in Central Asia, Pacific and Far East.[27, 31] Nissan et al. even reported 50% of Bangladeshi mothers to be Non-Secretors in a birth cohort study.[32] In the study of Erney et al.,[33] 2´FL, a specific structure of Secretor milks, was detected in only 46% of milk samples from the Philippines, but was in 100% of milk samples from Mexican donors. These findings showed the influence of geographical origin on distribution of *Se* gene among population, although the biological implications out of this requires further investigation.

Lactational stage is another well-known factor that causes the temporal changes of HMO synthesis of mothers. In contrast to lactose concentrations which increased from colostrum to day 120,[34] HMO concentrations decreased from ~20 g/L at day 4 to ~16 g/L at day 30,[35] and further decreased to ~13g/L at day 120,[34] and less than 10 g/L after 6 months postpartum.[36] The longitudinal change pattern of HMOs was found to be structure-specific. Absolute concentrations of major sialylated HMOs decreased over the course of lactation.[37] Regarding fucosylated HMOs produced by Le+Se+ mothers, the ratio of structures containing (α1-2)-linked fucose to those not containing (α1-2)-linked fucose changed from 5:1 in colostrum to 1:1 in the end of the first year of life.[36]

It is unclear whether gestational age would influence the HMO profiles. De Leoz et al. reported a higher abundance of lacto-*N*-tetraose (LNT) present in preterm milk compared to term milk.[38] The same study also found higher inter-individual variations of fucosylated HMO production within mothers who delivered preterm babies compared to those who delivered term babies, indicating an immature HMO regulation of the former.[38] However, some other studies did not find significant differences in HMO patterns between preterm and term milk.[39-40] The body mass index (BMI) of mothers, as an indicator of their nutritional status, was reported to be correlated with HMO concentrations, where mothers with lower BMI (14 – 18) synthesized significantly less HMOs than those with higher BMI (24 – 28).[41]

Besides the possible reasons such as genetic profiles of mothers and lactation duration, Urashima et al. proposed that methodological differences in HMO analysis also played an important role in obtaining a wide range of concentrations.[31] Table 1.2 shows concentrations of major HMOs as quantitated by different techniques in literature.[31] Large variations existed on the values among different studies on both neutral and sialylated HMOs. For example, the average concentration of 2´FL was reported by Kunz et al. to be 0.45 g/L in milks collected 2 – 4 weeks postpartum,[42] in contrast to the values 3.93 – 1.84 g/L from day 4 to day 60 postpartum as found by Coppa et al.,[35] although both studies used high performance anion exchange chromatography - pulsed amperometric detection (HPAEC-PAD) for analysis. Chaturvedi et al. and Asakuma et al. analyzed derivatized HMOs by reverse-phase (RP) HPLC, and found the average concentrations of 2´FL to be 2.43 g/L over the first year of lactation,[36] or 2.01 g/L at day 2 postpartum,[43]

respectively. Therefore, even with the same technique, inter-laboratory variations also existed regarding the quantitation of HMOs in human milk samples. Almost all the studies presented in Table 1.2 found higher levels of fucosylated HMOs and LNT compared to sialylated HMOs, with 2′FL, LNT, LNFP I, LNFP II being the most abundant structures.

Table 1.2. Concentrations of major HMOs determined by different analytical methods from literature. Adapted from Urashima et al.[31] Days means specific day postpartum. Some values are shown as averages ± standard deviation.

Neutral HMOs (g/L)	**Kunz et al.[42]**	**Chaturvedi et al.[36]**	**Thurl et al.[44]**	**Coppa et al.[35] (d 4)**	**Coppa et al.[35] (d 60)**	**Asakuma et al.[43] (d 2)**
Analytical method	HPAEC-PAD	RP-HPLC (derivatization)	HPAEC-PAD	HPAEC-PAD	HPAEC-PAD	RP-HPLC (derivatization)
2′FL	0.45 ± 0.43	2.43 ± 0.26	1.84	3.93 ± 1.11	1.84 ± 0.39	2.01 ± 1.07
3FL	0.07 ± 0.08	0.86 ± 0.10	0.46	0.34 ± 0.06	0.71 ± 0.07	0.28 ± 0.26
DFL		0.43 ± 0.04	0.17			0.28 ± 0.30
LNT	1.09 ± 0.47	0.55 ± 0.08	0.86	0.84 ± 0.29	1.56 ± 0.57	1.44 ± 0.70
LNnT	Trace	0.17 ± 0.03	0.11	2.04 ± 0.55	0.95 ± 0.83	0.54 ± 0.14
LNFP I	1.26 ± 1.11	1.14 ± 0.18	0.67	1.36 ± 0.18	0.97 ± 0.61	2.08 ± 1.67
LNFP II			0.20	0.29 ± 0.22	0.29 ± 0.16	
LNFP III			0.28			
LNDFH I		0.50 ± 0.06	0.58	0.79 ± 0.25	1.18 ± 0.22	1.87 ± 1.55
LNDFH II	0.16 ± 0.11	0.09 ± 0.01	0.25			0.02 ± 0.03
LNH			0.13	0.07 ± 0.07	0.09 ± 0.02	
Sialylated HMOs (g/L)	**Kunz et al.[42]**	**Martin-Sosa et al.[37]**	**Bao et al.[45] (d 3-5)**	**Bao et al.[45] (d 9-21)**	**Asakuma et al.[46] (d 1-3)**	
Analytical method	HPAEC-PAD	HPLC	Capillary electrophoresis	Capillary electrophoresis	RP-HPLC (derivatization)	
3′SL	0.30-0.50	0.10-0.30	0.097 ± 0.038	0.076 ± 0.014	0.297 ± 0.096	
6′SL	0.10-0.30	0.20-0.30	0.335 ± 0.033	0.396 ± 0.054	0.370 ± 0.108	
LST a	0.03-0.20	1.70-3.80	0.026 ± 0.011		0.141 ± 0.107	
LST b			0.131 ± 0.064	0.074 ± 0.026	0.065 ± 0.025	
LST c	0.10-0.60	1.40-3.00	0.232 ± 0.058	0.148 ± 0.060	0.686 ± 0.264	
DSLNT	0.20-0.60	0.70-1.50	1.274 ± 0.503	0.795 ± 0.234	0.462 ± 0.128	

Analysis of individual HMOs in milk, feces and other biological fluids

Investigation of both HMO profiles of human milk and their metabolic fates in the gastrointestinal tract of infants require analytical methods that enable us to identify HMO structures, as well as to quantitate their concentrations present in milk, infant feces and other biological fluids. Separating HMOs from other major components, like fat, protein, lactose, monosaccharides and salt, etc., in human milk is an essential step of sample preparation to avoid interference with subsequent HMO analysis. Centrifugation at lower

temperature is a common practice for fat removal or reduction.[47] Proteins could be separated through organic solvent precipitation or ultrafiltration.[47] Furthermore, porous graphitized carbon (PGC) material could also trap proteins during a solid phase extraction (SPE) step.[47] Although the mechanism of PGC is not fully understood yet, PGC-SPE is used in different studies to remove salt, monosaccharides and lactose by washing with water, acetonitrile/water mixtures with/without fluoroacetic acid.[24, 48-49] Alternative ways to isolate HMOs from lactose include gel-permeation chromatography, and enzymatic hydrolysis with lactase followed by membrane-based separations.[50] As to fecal samples, dilution, centrifugation and filtration are usually applied before loading to PGC-SPE.[51]

Structures of HMOs are quite complex and their hydrophilic nature and the presence of numerous isomers makes the separation of HMOs a challenge.[52] Besides, lack of intrinsic chromophore in HMO structures is adding up to the difficulty of detection for their quantitation.[52] HPAEC-PAD has been often used in early studies to analyze underivatized HMOs.[35, 42, 44] Other liquid chromatography (LC) methods used for separation of HMOs include RP-HPLC following derivatization with chromophoric active labels (1-phenyl-3methyl-5-pyrazolone, 2-aminopyridine, and 2-aminobenzoic acid), as well as hydrophilic interaction chromatography (HILIC) with preceded labeling with e.g. 2- aminobenzamide.[53-54] However, identification of HMO structures are merely based on retention time compared to standards in these methods. To improve detection and identification of HMOs, application of mass spectrometry (MS) following the chromatographic separation has shown to be quite helpful.[54] Capillary electrophoresis with laser induced fluorescence detection (CE-LIF) coupled to mass spectrometry (MS) was developed by Albrecht et al. to analyze HMOs extracted from human milk and infant feces,[48] where HMO structures were derivatized with 8-aminopyrene-1,3,6-trisulfonic acid for separation and fluorescence detection. However, structural isomers often eluted at about the same time in CE-LIF method, which lead to compromised separation and identification if not coupled to MS. The most employed method in the recent years is PGC-LC-MS, which allows good separation of HMO isomers as well as unambiguous structure identification with MS.[20-21, 54] HMOs usually are reduced before PGC-LC-MS analysis, in order to avoid split peaks of α- and β- anomers, and also for higher response factors.[52]

Furthermore, analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) could generate compositional fingerprinting of HMOs in a rapid and high-throughput manner.[54] Neutral HMOs could be analyzed as monosodium adducts in positive mode, and sialylated HMOs in both positive and negative mode, but required careful selection of matrices due to inferior stability of the sialylated structures.[55] However, isomeric structures are hardly distinguishable in MALDI-TOF-MS.[54, 56] Nuclear magnetic resonance (NMR) spectroscopy is another method that could provide comprehensive information on the structures of pure HMOs or HMOs within a mixture.[54] With the structural-reporter-

group concept, NMR spectrum could also indicate specific structural elements in a relative and quantitative way.[25, 47] However, NMR is found sensitive to interfering components present in biological fluids, making identification and quantitation of HMOs still difficult.[47]

Metabolization of HMOs by infant gut microbiota

After ingestion, most components in human milk are digested and absorbed by infants.[57] In contrast, only < 5% of HMOs are degraded during enzymatic digestion throughout the transit in the upper small intestine, with the majority of them reaching the large intestine, as shown by both lactulose breath hydrogen test,[58] as well as in vitro digestion models using pancreas and intestinal brush border membranes.[59-60] A very small part (0.5% - 2%) of the HMOs are absorbed intact into the circulation system and then excreted into the urine,[15, 57, 61] which explained their presence in the plasma and urine of infants.[61-62] The rest of the ingested HMOs would be fermentation substrates for the infant gut microbiota, with the undegraded ones and metabolites leaving the body in feces.[57, 63-64]

The gut intestinal microbiota of adults constitutes over 1000 species with around 7000 strains, with the number of genes exceeding the number of genes of the human genome by > 150 times.[65] The microbes reside along the gastrointestinal tract, with the highest density and diversity found to be located at the distal gut.[65] It is reported that the gut microbiota plays an essential role in maintaining both short- and long-term health of the host, regarding immunomodulation, physiology and metabolism.[65]

The initiation of bacterial colonization starts when infants are delivered or already *in utero*.[66-67] The first strains are mostly facultative anaerobic bacteria that originate from mother's vaginal and fecal microbiota for vaginally delivered infants, or skin microbiota from mothers as well as hospital environment for caesarian-section (C-section) delivered infants.[68] Therefore, mode of delivery is often considered as an important determinant of early colonization, and infants born by C-section tend to show a delayed acquisition of *Bifidobacterium* and *Bacteroides*.[69] The development of gut microbiota follows a step-wise manner, until it reaches an adult-like composition after 2-3 years of life.[65] The characteristics of gut microbiota at different stages are listed in Table 1.3. Following the first colonization by mainly facultative anaerobic bacteria in the first several weeks postpartum, the gut is gradually resided by more anaerobes such as bifidobacteria as a result of diminished oxygen in the gut lumen, as well as by selective stimulation by HMOs from breastmilk.[68-69] Maternal milk is also an important source of bacteria that influence the establishment of infantile gut microbiota.[69] It is estimated that nursing infants would intake approximately 1×10^5 - 1×10^7 bacteria from human milk per day, which are predominantly Staphylococci, Streptococci, lactic acid bacteria and bifidobacteria.[70] Therefore, the lack of HMOs and bacteria in bovine milk derived formula might exert different effects on the succession of microbiota for formula-fed infants, if not

supplemented with other prebiotics or probiotics. With introduction of solid food and weaning, the gut flora would experience a major change, and eventually resemble that of adults, which is predominated by anaerobes, and has high microbial richness and diversity.[71] Healthy adult gut microbiota is dominated by two major phyla, *Firmicutes* and *Bacteroidetes*, followed by *Proteobacteria* and *Actinobacteria*[72-73] , although high inter-individual variations in species and strains level phylotypes exist. Several factors that would influence human gut microbial community have been identified to be obesity, antibiotic treatment, genetics, diet and bowel disease.[72, 74] Aging is also found to be related to alterations of human gut microbiota composition. In the population of over 55 – 65 years of age, a significant decline of bifidobacterial level in the colon was found,[75] although the reason behind this remained a question.

Table 1.3. Comparison of developmental stages of infant gut microbiota and oligosaccharide excreted in infant feces.[64]

	Microbiota composition[69, 76-77]	**Fecal oligosaccharide composition**[51, 78]
1. First weeks (stage 1)	Mixed microbiota; large contribution of facultative anaerobe species	Neutral HMOs/ acidic HMOs/ (degradation-, metabolization products)
2. Pre-weaning (stage 2)	Anaerobic, "simple" microbiota: *Bifidobacterium/ Bacteroides*	Blood group characteristic oligosaccharides; no HMOs
3. Weaning (stage 3)	Mixed microbiota: anaerobic/facultative anaerobic	Oligosaccharides from solid food; no HMOs
4. Post-weaning	Installation of "adult" microbiota; anaerobic; predominantly *Bacteroides*	No experimental data available; no oligosaccharides expected

The microbial consumption of HMOs was reported in several studies applying *in vitro* fermentation systems, either with mixtures of HMOs extracted from human milk samples,[79-82] or with individual HMO structures.[82-86] Some studies focused on the impact of HMOs on the entire population of gut microbiota by involving infant fecal materials,[81-82, 85] while other studies tested the growth of single strains with HMOs as the sole carbon source.[79-80, 87] In general, *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) was reported as the most efficient utilizer of HMOs, followed by *Bifidobacterium longum* subspecies *longum* (*B. longum*), *Bifidobacterium breve* (*B. breve*), and several strains from *Bacteroides fragilis* and *Bacteroides vulgatus*.[79-80, 87] HMO mixtures were also shown to selectively promote the abundance of *Bifidobacterium* and *Lactobacillus*, meanwhile significantly reducing the presence of *E. coli* and *Clostridium perfringens*.[82] Furthermore, specific strains showed preferences to degrade certain structures of HMOs. One study reported that the major fucosylated HMOs were strongly digested by specific

Bifidobacterium and *Bacteroides*.[84] Ruiz-Moyano et al. found LNT and LNnT were most preferred by *B. breve*, which also consumed LNnH at a higher level compared to its structural isomers.[87] When fermenting with infant fecal inoculum, 2′FL and LNnT were more rapidly degraded compared to 6′SL.[85] Partly due to the limited production of HMOs, animal studies on their metabolization are rare at the time this thesis started. One study by Jantscher-Krenn et al. fed several HMO ingredients to neonatal rats and followed the compositional pattern along the intestine and in serum and urine.[88] Only 3′SL, which was specific in rat milk, was found to be absorbed into the blood and then excreted into the urine.[88] The change of HMO composition along the intestine showed a structure-specific pattern, where 2′FL and LST b increased while LNFP I and LST c reduced.[88]

As to human studies on HMO metabolization in the gastrointestinal tract, most researchers examined the HMO profile as excreted in infant fecal samples, and compared to that from the respective milk samples. Sabharwal et al. identified blood group specific oligosaccharides in feces of term and preterm infants, suggesting biosynthesis of these compounds in the intestine.[89-92] Differences in HMO metabolization between preterm and term infants were noticed by Sabharwal et al.[91] A study involving 16 mother-infant dyads found comparable oligosaccharide patterns among human milk, infant feces and infant urine.[57] Coppa et al. reported the same observations based on six infants at one month postpartum, besides the complete absence or significant decrease of LST a, disialyllacto-*N*-neotetraose and LNT in feces of some study subjects.[93] These metabolization studies reported varied observations, based on limited study subjects and random sampling time points.

Then, a stage-wise pattern of fecal oligosaccharide profiles during the first six months of life was proposed by Albrecht et al., by analyzing 11 mother-infant pairs with a CE-LIF-MS method.[78] This study also compared the fecal oligosaccharide composition to the development of gut microbiota of infants at different stages as reported in literature, as adapted in Table 1.3.[64] At the first stage, usually the first few weeks after delivery, infant gut was mainly occupied by facultative anaerobic bacteria, which has lower degradation capability of HMOs, leading to presence of intact neutral and acidic HMOs in feces. With predominance of *Bifidobacterium* and *Bacteroides* at stage 2, which are major HMO-utilizers in the infant gut, original HMOs were degraded, meanwhile blood group specific oligosaccharides were detected. With introduction of solid food at weaning, only dietary oligosaccharides were recovered, meanwhile the gut microbiota composition started to adapt and approach towards an adult-like composition. However, structural isomers of HMOs were hardly differentiated due to the limitation of their analytical CE-LIF method. Information on the infant gut microbiota was merely proposed from literature, but not measured in the same infant fecal samples in the study of Albrecht et al.[78] Therefore, studies using updated analytical

methodology, with involvement of more subjects sampled longitudinally, as well as paring HMO metabolization with fecal microbiota analysis in the same sample set, are warranted.

Structure-specific benefits of HMOs to infants

As one of the major components in human milk, HMOs have been reported on their beneficial effects brought to the newborns, with a summary given in Table 1.4. As shown, specific HMO structures are responsible for different functions and protective effects for infants. HMOs are indigestible carbohydrates and could reach the large intestine of infants, serving as fermentation substrates for beneficial microbes present, like bifidobacteria.[83-85] In an *in vitro* fermentation study, the major fucosylated HMOs (2′FL, 3FL and DFL) showed a strong influence on growth of specific *Bifidobacterium* spp. and *Bacteroides* spp., while 3′SL and 6′SL moderately stimulated growth of those bacteria.[83] Similarly, Vester Boler et al. also found a higher *in vitro* fermentation speed of infant fecal microbiota on 2′FL and LNnT compared to 6′SL.[85] Furthermore, fucosylated HMOs, especially (α1-2)-linked structures could protect gastrointestinal epithelial cells from attachment of pathogens, *Campylobacter jejuni,*[94-95] calicivirus,[95] stable toxin of enterotoxigenic *Escherichia coli,*[96] enteropathogenic *Escherichia coli* and *Pseudomonas aeruginosa*,[97] and thereby reducing the occurrence of infant diarrhea. Fucosylated HMOs, but not non-fucosylated structures such as 3′SL, 6′SL nor LNnT, could diminish colon motor contractions, which might help to prevent disorders of gut motility.[98]

However, in other cases, fucosylation of core structures could lead to loss of pathogen preventative capabilities. One of the major neutral core HMOs, Lacto-*N*-tetraose (LNT) was found to prevent infections of intestinal epithelial cells from parasite *Entamoeba histolytica* and cytotoxicity, whereas e.g. lacto-*N*-fucopentaose I (LNFP I) showed no such effect.[99] Another major core structure, lacto-*N*-neotetraose (LNnT), showed protective effects against postnatal transmission of HIV,[**100**] as well as preventive effects against rotavirus infection.[101]

One of the major sialylated HMOs, 3′SL, showed ability in inhibiting the ulcer pathogen *Helicobacter pylori* from binding to epithelial cells of human stomach.[102] Bode et al. described anti-inflammatory effects of acidic HMO fraction while such effects are absent in case of neutral HMOs. The authors proposed acidic HMOs to be associated with lower incidence of necrotizing enterocolitis (NEC) with breastfeeding.[103] Later on, the key protective structure against NEC was evidenced to be disialyllacto-*N*-tetraose (DSLNT) using a neonatal rat model.[104] Another well-known benefit of sialylated HMOs is their contribution to brain development and cognition, provided that sialic acid is a key component in building neural cells.[105] This

functionality has been demonstrated in a piglet model where the group being supplemented with sialic acid in the feed had an increased memory and learning ability.[106]

Table 1.4. Health benefits of HMOs reported in literature and the corresponding HMO structures playing the role.

Reported health benefits	Corresponding HMO structures	References
Inhibit gut infection caused by *Campylobacter jejuni*	Fucosylated HMOs, especially (α1-2)-linked ones,	Ruiz-Palacios et al.[94] Morrow et al.[95]
Protect from diarrhea caused by calicivirus	(α1-2)-linked fucosylated HMOs	Morrow et al.[95]
Inhibit binding of stable toxin of enterotoxigenic *Escherichia coli*	(α1-2)-linked fucosylated HMOs	Morrow et al.[96]
Reduce *Entamoeba histolytica* attachment and cytotoxicity	LNT	Jantscher-Krenn et al.[99]
Diminish colon motor contractions	3FL, 2′FL	Bienenstock et al.[98]
Inhibit adhesion of enteropathogenic *Escherichia coli*, *Pseudomonas aeruginosa* to human intestinal and respiratory cell lines	3FL, 2′FL	Weichert et al.[97]
Inhibit adhesion of *Salmonella fyris* to human intestinal cell lines	2′FL	Weichert et al.[97]
Stimulate growth of key species of mutualist symbionts, e.g. *Bifidobacterium, Bacteroides, Lactobacillus*	2′FL, 3FL, DFL > 3′SL and 6′SL	Yu et al.[83]
Anti-inflammatory by inhibiting adhesion of monocyte, lymphocyte and neutrophil to endothelial cells	3′SL, 3′-sialyl-3-fucosyl-lactose	Bode et al.[107]
Inhibit rotavirus infection	3′SL, 6′SL, LNnT	Hester et al.[101]
Inhibit binding of *Helicobacter pylori* to gastrointestinal epithelial cells	3′SL	Simon et al.[102]
Support brain development and cognition	Sialic acid	Wang et al.[105]
Protect against postnatal HIV transmission	Non-3′SL HMOs, especially LNnT	Bode et al.[100]
Protect from necrotizing enterocolitis (NEC)	Disialyllacto-*N*-tetraose (DSLNT)	Jantscher-Krenn et al.[104]

Dietary fibre with prebiotic potential

Human milk is considered as the golden standard for infant food; however, breastfeeding is not always available or practical. For infants who are not able to consume enough mother milk, infant formula which is often based on bovine milk becomes an alternative option. Oligosaccharides present in bovine milk are both much lower in concentrations as well as in structure diversity. [16-17] Formulas supplemented with HMOs (2´FL and/or LNnT) have only been launched to American and European market in the year 2016.[108] Traditionally, non-digestible carbohydrates were supplemented to infant formula to mimic the role of HMOs, with the most popular representatives being galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS).[109] GOS is produced from lactose with the fungal or bacterial enzyme β-galactosidase, resulting in varying chain length composing 2 to 10 units of galactose and a terminal glucose.[110-112] Galactose moieties can be linked to each other or to glucose moieties in (β1-2), (β1-3), (β1-4) or (β1-6) linkages.[111] FOS and another well-documented prebiotic inulin belong to fructans with a wide range of degree of polymerization and a glucose at the terminal end.[113] Inulin can be extracted from fruits and vegetables such as chicory roots, containing exclusively (β1-2) linked fructan backbones with degree of polymerization from 2 to 60.[13] FOS was generated from partial hydrolyzing inulin with degree of polymerization (DP) of 3 – 8.[13] These substrates have been reported to selectively promote the growth and activity of bifidobacteria and lactic acid bacteria in the gut.[2] Supplementing infant formula with GOS and/or FOS also improved stool characteristics of formula-fed infants,[114] as well as manipulating their gut microbiota towards a comparable pattern of that of breast-fed infants.[115] Besides the application of GOS and FOS in infant formula, they are also widely used for adult nutrition because of their prebiotic benefits. Nevertheless, the search for other novel prebiotics is on-going, which requires development of experimental tools for identifying and validating the prebiotic potential of novel indigestible carbohydrates.[109]

Isomalto/malto-polysaccharides (IMMPs), are novel α-glucans which could possess potential prebiotic effects (Figure 1.3). A 4,6-α-glucanotransferase (GTFB) from *Lactobacillus reuteri* 121 could remove a glucose moiety from the non-reducing end of (α1-4) glucan segments of starch or maltodextrins, and then link it to the terminal glucose residue of another chain by (α1-6) linkages.[116] Native (α1-4,6) linked branching points of starch were found to limit GTFB activity.[117] Consequently, depending on starch origin and application of debranching enzymes, this stepwise translocation of glucose results in the synthesis of IMMPs with varying levels and lengths of linear (α1-6) glucan segments.[118] Nothing was known about the consequences of decreased levels of easily digestible (α1-4) linkages from starch, and increased levels of linear (α1-6) linkages present in IMMPs; however, other glucans or gluco-oligosaccharides with predominantly (α1-6) linkages were shown to be bifidogenic in the colon, such as isomalto-oligosaccharides (IMOs) and dextran.[119-121] IMOs are oligosaccharides with DP 2 – 10, and dextran is polysaccharides

synthesized from sucrose by bacterial enzyme dextransucrase, with 50 – 97% (α1-6) linkages and branched at (α1-3) linkages.[122] Considering a similar structure compared to IMOs and dextran and their bifidogenic effects, it is worthwhile to further explore the fermentation behaviour of IMMPs and its influence on gut microbiota.

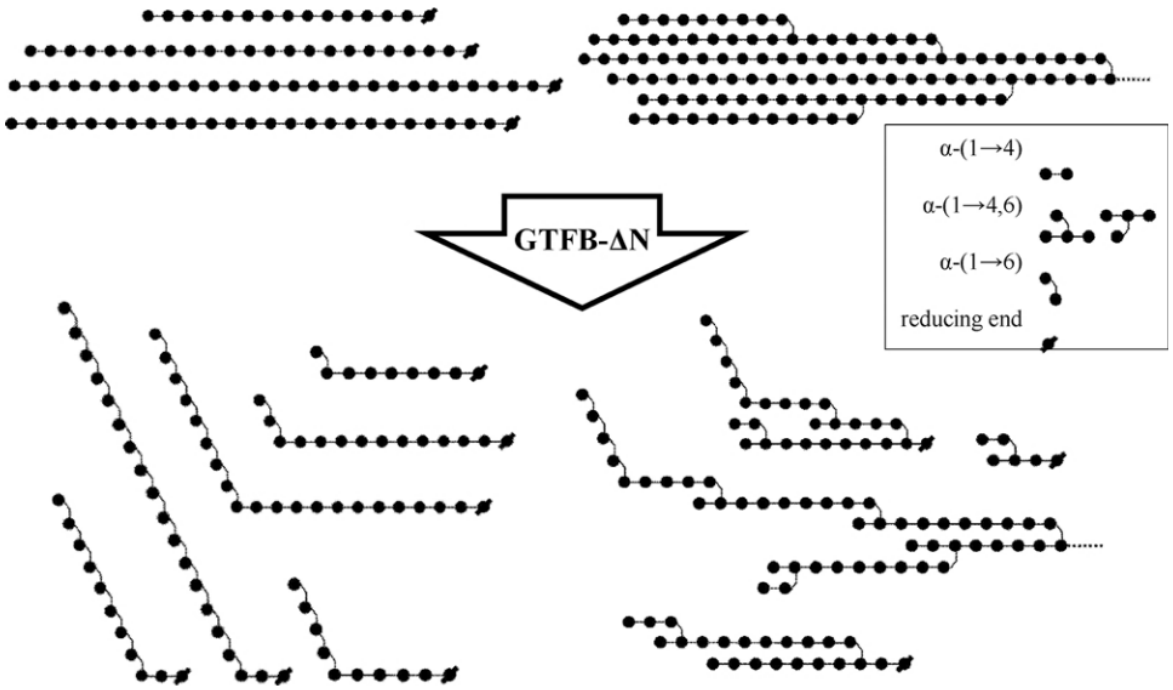


Figure 1.3. Schematical overview of an IMMP derived from an amylose-rich starch. Figure from van der Zaal et al.[117]

Approaches for investigating prebiotic potential

To investigate the fermentation behaviour of indigestible oligo- and polysaccharides in the human gut as well as their associations with the microbiota, *in vitro* fermentation models and *in vivo* animal models are often used as alternative study approaches besides human studies, due to practical and ethical issues as well as time and budget considerations.[123]

In vitro fermentation model provides a screening platform which allows studying fermentation of larger number of substrate candidates in a simulated gut environment. One type of the *in vitro* fermentation model is a static system using sealed serum bottles with buffer and inoculum. The inoculum could be single strain, mixed strains or fecal material.[124] Figure 1.4 shows the schematic diagram of a batch fermentation model as previously described by Rosch et al.[125] Selected fibres are fermented anaerobically with a standardized fecal inoculum which was pooled from several human beings, in a pH- and temperature-

controlled serum bottle filled with standard ileal efflux medium (SIEM). Digesta are sampled at certain time intervals to be analysed for molecular degradation, short chain fatty acid production, as well as temporal changes of bacterial composition and enzyme expression, in order to explain the mechanism behind the microbial consumption. The batch fermentation model is inexpensive, and enables high-throughput screening of large number of substrates.[123] However, pH is not well controlled due to accumulation of metabolites and end products. A more sophisticated design expands the fermentation vessel to multiple stages, that represent different parts of the human gut, e.g. the TNO in vitro model of the colon (TIM-2),[126] and the Simulator of the Human Intestinal Microbial Ecosystem model (SHIME).[127] The continuous *in vitro* fermentation models have better control of fermentation conditions, and allow the researchers to monitor the fermentation behaviour of substrates at different parts of the GI tract. However, large amount of substrates, and much longer equilibration times are required per fermentation experiment. Furthermore, both batchwise and continuous *in vitro* fermentation models usually lack immune or neuroendocrine system, as well as epithelial and other colonic cells.[123] This has been improved by incorporating mucosal microbiota in the SHIME model.[127]

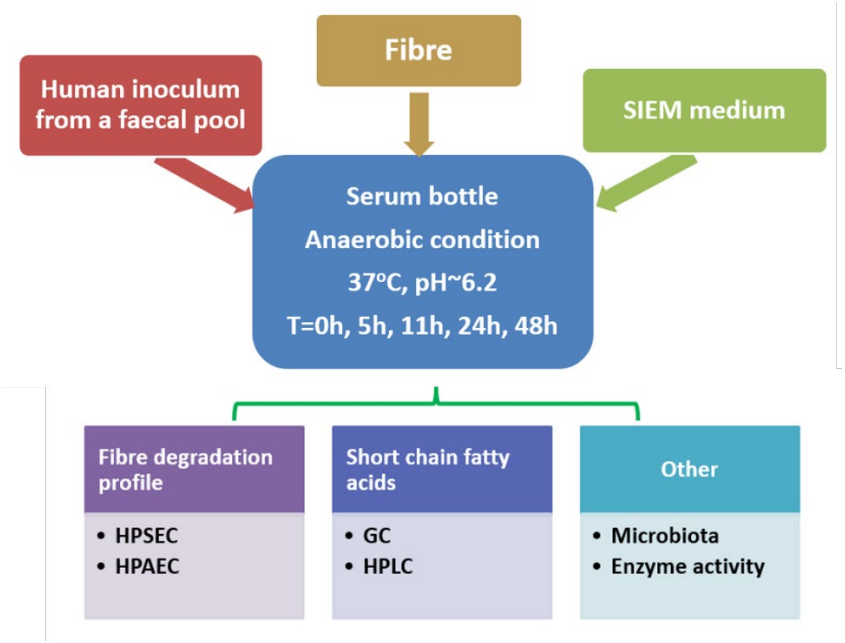


Figure 1.4. Schematic diagram of batch *in vitro* fermentation model and subsequent analysis.

Results from *in vitro* models need to be validated in *in vivo* animal models, and eventually human beings. Rodents and pigs are commonly used animal models for fibre fermentation and gut microbiota studies. Although requiring more budget and facilities compared to *in vitro* models, animal models are more comparable to human models. Animal models are more convenient than human studies, as digesta from

different regions of the gut, tissues and organs can be sampled for analysis.[123] Rodent models could be conventional rodents, germ-free rodents, or germ-free rodents colonized with human fecal microbiota.[124] For examples, fermentation patterns of different types of pectins as well as influence on microbiota composition and short chain fatty acid production could be compared using a rat model.[128] Piglets were also shown to be a suitable model to study the metabolic fate of GOS, or other dietary fibre in gastrointestinal tract of infants.[129-130] However, the animal models were sometimes questioned about the translatability of the results to humans, considering the differences in microbial distributions and compositions between animals and humans.[88]

Thesis outline

Studying the metabolization of indigestible oligo- and polysaccharides in the gastro-intestinal tract is vital for understanding the mechanism of how the carbohydrates influence gut environment and immune system. The aim of the thesis is to investigate degradation of representative oligo- and polysaccharides during fermentation and their interactions with gut microbiota composition.

Human milk oligosaccharides (HMOs) are considered as the first natural prebiotic for life, providing essential benefits to support infant health. In **Chapter 2**, we describe the optimization of PGC-LC-MS and HPAEC-PAD methods to quantitate 17 major HMOs present in human milk and infant fecal samples. The developed methods were applied to a pilot sample set consisting of human milk and paired infant feces collected at 1 month postpartum. Results were combined with 1D ^{1}H NMR analysis of the same samples, in order to understand metabolization of HMOs at 1 month old. In **Chapter 3**, we expanded the sampling time duration in another cohort study to week 2, week 6 and week 12 postpartum, as well as a larger sample size, to study temporal changes of HMO composition in mother milk, and their metabolization in infant gut during the first few months of life. HMO groups having different structure properties were compared regarding their metabolic fates. Furthermore, potential infant and maternal characteristics that seem to influence HMO metabolization are discussed.

The associations between HMO compositions in maternal milk and infant gut microbiota compositions at 1 month postpartum and during the first 3 months of life are described for the two different cohort studies in **Chapter 4** and **Chapter 5**, respectively. Other factors that would contribute to infant gut microbiota establishment are discussed. Furthermore, degradation patterns of HMOs ingested by infants were correlated to fecal microbiota composition. Specific bacterial taxa in infant gut that played key roles in degrading major HMOs were identified by microbial network analysis.

Chapter 6 focuses on isomalto/malto-polysaccharides (IMMPs), which is a novel type of indigestible polysaccharides. We demonstrated the application of a batchwise *in vitro* fermentation model with human adult inoculum, on the evaluation of the prebiotic potential of several types of IMMPs. Structural degradation and short chain fatty acid production were monitored during fermentation, and were associated to microbiota composition.

Finally, **Chapter 7** summarises highlights from this research, and provides insights on intestinal metabolization of indigestible carbohydrates and their interactions with gut microbiota community.

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Chapter 2

Combining HPAEC-PAD, PGC-LC-MS and 1D ^{1}H NMR to investigate metabolic fates of human milk oligosaccharides in one-month old infants: a pilot study

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ABSTRACT

Human milk oligosaccharides (HMOs) are important nutrients present in high concentrations in human milk, providing myriad health benefits for infants. In the current study we optimized the solid phase extraction procedure to extract 3-fucosyllactose (3FL) and other HMOs from human milk samples separately, followed by absolute quantitation of 3FL and 16 other HMOs using high performance anion exchange chromatography - pulsed amperometric detection (HPAEC-PAD), and porous graphitized carbon - liquid chromatography mass spectrometry (PGC-LC-MS), respectively. The approach developed was applied on a pilot sample set of 20 human milk samples from the KOALA Birth Cohort Study, and was also used to quantitate HMOs excreted in feces collected from the corresponding exclusively breastfed infants at around 1 month postpartum. Furthermore, one-dimensional ^{1}H nuclear magnetic resonance (1D ^{1}H NMR) was employed on the same set of samples to determine relative levels of fucosylated-epitopes and sialylated (Neu5Ac) structural elements. Using results of either individual fucosylated HMO concentrations, or relative levels of fucosylated structural elements, Lewis/Secretor phenotypes of the mothers were identified unambiguously. Based on different HMO consumption patterns in the gastrointestinal tract, the one-month-old infants were assigned to three clusters: complete consumption; specific consumption of non-fucosylated HMOs; and thirdly, considerable levels of HMOs still present with consumption showing no specific preference. Consumption of HMOs by infant microbiota at one month postpartum also showed structure-specificity, with HMO core structures and Neu5Ac(α2-3) decorated HMOs being the most prone to degradation. Degree and position of fucosylation of HMOs also impacted their metabolization differently. Matching the HMO profiles of maternal milk to paired infant feces as obtained from HPAEC-PAD combined with PGC-LC-MS led to consistent findings with that from 1D ^{1}H NMR. The developed analytical approach is now available for studies in the future on the temporal metabolic fates of HMOs within longitudinal cohort studies with larger number of participants.

INTRODUCTION

Exclusive breastfeeding is recommended by the World Health Organization for infants under six months of age, making human milk the preferred sole source of nutrition in the first months of life.[1] As the third most abundant component in human milk, human milk oligosaccharides (HMOs) have been examined in increasing number of studies on the myriad health benefits it brought to infants. After ingestion by infants, a majority of HMOs in human milk escape the digestion of stomach and upper small intestine, reaching the large intestine of infants,[2-3] where they can be assimilated by beneficial microbes, mostly bifidobacteria.[4-5] HMOs are also known to play a role in the immune system of infants,[6] as well as to prevent infectious diseases among infants by inhibiting the adherence of enteric pathogens.[7-10] Specific HMO structures, including 2´-fucosyllactose (2'FL) and 3/6´-sialyllactose (3'SL; 6'SL), are considered beneficial on cognitive development of individuals by e.g. improving learning and memory.[11-14] HMOs consist of five monosaccharides: glucose, galactose, *N*-acetylglucosamine, fucose and *N*-acetylneuraminic acid (also called, sialic acid). With a lactose at the reducing end, a number of lacto-*N*-biose or *N*-acetyllactosamine repeats can be added via (β1-3) or (β1-6) linkages to form so-called core structures.[15] Core structures can be further decorated by variable amount of fucoses via (α1-2)-, (α1-3)-, or (α1-4)- linkages, and/or sialic acids via (α2-3)- or (α2-6)- linkages, contributing to the complexity of HMO structures.[15] Heretofore, over 240 HMOs have been recognized, with approximately 160 structures fully characterized.[16-19] It should be noted that the HMO composition in human milk (both amount and presence of specific structures) differ between mothers, depending on their genetic profiles. One key factor that determines the fucosylated HMO profiles is the Secretor status and Lewis blood types of mothers.[20] The Lewis positive mothers have the *Le* gene which encodes the enzyme α1,-3/4-fucosyltransferase (FUT3), while the Secretor mothers have the *Se* gene that corresponds to α1,-2-fucosyltransferase (FUT2). Depending on different combinations of FUT2 and FUT3 activities, different ensembles of HMOs are produced in four groups of human milk.[21] Lewis-positive Secretors produce (α1-2)-, (α1-3)- and (α1-4)-linked fucosylated HMOs, which contain Le^a/ Le^b/ (pseudo-) Le^x /(pseudo-) Le^y/H epitopes. Lewis-negative Secretors lack (α1-4)-linked fucosylated HMOs, only contained (pseudo-) Le^x /(pseudo-) Le^y/H epitopes. Lewis-positive non-Secretors produce only Le^a/(pseudo-) Le^x and lack (α1-2)-linked structures. Only (α1-3)-linked structures are observed in Lewis-negative non-Secretors, corresponding to (pseudo-) Le^x epitopes.[21] In a Vietnamese cohort study individuals have been observed that are Lewis-negative and are unable to produce (pseudo-)Le^x epitopes,[22] a feature also observed in an early Japanese study.[23]

Besides Lewis Secretor status, there are many other factors reported to influence HMO composition of human milk, e.g. lactation stages,[24-25] geographic origins,[26-27] and gestational ages.[20, 28] A systematic review by Thurl *et al.* summarized concentration ranges and mean values of 33 HMOs present in human

milk from 21 eligible studies.[29] Large interlaboratory variations in the HMO concentrations were summarized in the review, partly due to numerous analytical methods employed, aside of other determinants.[29] The major techniques for HMO analysis include high performance anion exchange chromatography - pulsed amperometric detection (HPAEC-PAD),[30-31] capillary electrophoresis with laser induced fluorescence detection (CE-LIF),[32-33] matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)[34-35], nuclear magnetic resonance (NMR) spectroscopy,[21] liquid chromatography (LC) either by derivatization with a fluorescent label at the reducing end,[36] or by reduction to alditols prior to porous graphitized carbon (PGC) separation combined to mass spectrometry (MS).[17-18, 37] A review by Van Leeuwen critically evaluated the pros and cons of these techniques, as well as the sample preparation steps.[38] One important step of sample preparation is extracting HMOs from other interfering fractions in human milk, which is often achieved by solid phase extraction (SPE). PGC material has commonly been used in SPE to remove salt, monosaccharides and lactose.[20, 32, 39] However, van Leeuwen,[38] as well as Xu et al.,[40] pointed out the loss of 3-fucosyllactose (3FL) during the clean-up step of PGC-SPE, which was attributed to a lower affinity of 3FL to the column material. Xu et al. skipped the SPE step and directly injected the samples containing an overload of lactose directly into the UPLC to prevent loss of 3FL.[40] However, mature human milk contains ±70 g/L of lactose,[15] therefore, lactose removal by SPE is usually done to avoid interference like co-elution or ion-suppression of HMOs.[38] In this context, a new clean-up method should be developed to include 3FL in the HMO analysis in order to avoid under-estimation. Beyond human milk, HMO analysis has also been performed in other biological fluids ranging from infant feces,[32, 41] to urine,[42-43] and from plasma to amniotic fluid,[44-46] in order to understand the fate of HMOs after ingestion. Regarding the metabolization of HMOs by infant gut microbiota, most studies either took only HMO profiles of infant feces into consideration without matching to the corresponding human milk,[47] or were using only small sample numbers.[48-49] Therefore, the full picture of the metabolic fate of HMOs during gut transit, as well as inter-individual differences, is not yet clear.

The aim of the present study is to develop a new approach to quantitate HMOs including 3FL from human milk, and to combine three analytical methods, HPAEC-PAD, PGC-LC-MS and one-dimensional ^{1}H NMR, to profile the major HMOs present in human milk and infant feces. This approach was then applied on a pilot sample set of 20 maternal milk and 20 paired infant fecal samples, collected one month postpartum, to determine Lewis Secretor phenotype of the mothers, and to investigate the consumption patterns of HMOs by the gut microbiota of one-month-old infants. Preferences to different HMO structures (groups) by the infant's microbiota has been examined by comparing the HMO profiles of mother-infant dyads.

MATERIALS & METHODS

Materials

Milk oligosaccharide standards, 3-fucosyllactose (3FL), lacto-*N*-neotetraose (LNnT), lacto-*N*-hexaose (LNH), lacto-*N*-neohexaose (LNnH), lacto-*N*-fucopentaose I (LNFP I), lacto-*N*-fucopentaose II (LNFP II), lacto-*N*-fucopentaose III (LNFP III), lacto-*N*-difucohexaose I (LNDFH I), sialyl-lacto-*N*-tetraose a (LST a), sialyl-lacto-*N*-tetraose b (LST b), and sialyl-lacto-*N*-tetraose c (LST c) were purchased from Dextra Laboratories (Reading, UK). As to the other milk oligosaccharide standards: 2′-fucosyllactose (2′FL) and lacto-*N*-tetraose (LNT) were purchased from Carbosynth Ltd (Compton, UK); difucosyllactose (DFL) was purchased from ELICITYL (Crolles, France); lacto-*N*-fucopentaose V (LNFP V), 3′-sialyllactose (3′SL) and 6′-sialyllactose (6′SL) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Twenty human milk and infant fecal samples were analyzed in the current study, from 20 mother-infant dyads that were included in the KOALA (acronym in Dutch for Child, Parent and health: Lifestyle and Genetic constitution) Birth Cohort Study (N = 2834). The KOALA study was conducted in the south of the Netherlands, and approved by the medical ethics committee of the Maastricht University/University Hospital of Maastricht, with informed consent signed by all participants.[50] Design of the KOALA study, including baseline characteristic registration of mother-infant dyads, and collection procedures of human milk and infant feces, have been published previously. [37, 50-51] The 20 infants included in the current study were born from November 2002 to August 2003, at home or hospital by vaginal delivery or Caesarean section. The human milk and infant feces samples were collected at around 1 month postpartum on the same day. All the infants were exclusively breastfed till the time of sampling, without antibiotic administration. The milk and feces were collected in sterile tubes and sent to the lab within 1 day. As to collection of feces, a diaper and a spoon were used, with a sanitary napkin to prevent absorption. Before storage, fecal samples were 10-fold diluted in peptone glycerol solution, and milk samples were centrifuged at 4 °C to remove lipid fraction. The processed samples were stored at -80 °C before further analysis.

Sample preparation

Milk or fecal samples were thawed at 4 °C overnight. A volume of 0.6 mL of each sample was taken and diluted with an equal volume of Milli-Q^{TM} water, followed by centrifugation at 21,000 × g (4 °C, 15 min.). Exactly 1.0 mL of the clear liquid fraction of milk or fecal solution was taken, and applied on a Carbograph solid phase extraction (SPE) cartridge (Grace, Breda, the Netherlands, 300 mg bed volume for milk samples and 150 mg bed volume for feces). The SPE procedure was based on Albrecht et al.,[32] with

modifications. The cartridges were activated with 3 × 1.5 mL of 80% (v/v) acetonitrile (ACN) containing 0.1% (v/v) trifluoracetic acid (TFA), thereafter rinsed with 3 x 1.5 mL of Milli- Q™ water. After sample loading, the cartridges were first washed with 3 x 1.5 mL Milli-Q™ water to remove salts and monomers. Subsequently, the fraction of 3FL and lactose was collected by applying 3 × 1.5 mL of 3% ACN in water, and then the fraction of other milk oligosaccharides was collected with 3 × 1.5 mL of 40% ACN containing 0.05% TFA into scintillation vials. Both fractions were evaporated under nitrogen (N_2) stream to remove acetonitrile and eventually lyophilised. The step of washing with 3% ACN was omitted for fecal samples.

HPAEC-PAD quantitation

The 3% ACN fraction of human milk samples were further analyzed by high performance anion exchange chromatography - pulsed amperometric detection (HPAEC-PAD). The lyophilized human milk was rehydrated and diluted with Milli-Q™ water, reaching the final dilution factors of 100. Ten µL of each sample was injected to an ICS 5000 system (Dionex, Sunnyvale, CA) equipped with a CarboPac PA-1 column (250 mm x 2 mm ID), which is preceded by a CarboPac PA guard column (25 mm x 2 mm ID). The column temperature was 20 °C, and the flow rate was 0.3 mL/min. Mobile phase A and B were 0.1 M NaOH and 1 M NaOAc in 0.1 M NaOH, respectively, with a gradient elution of B from 0% to 10% in the first 10 min. Following the gradient elution, 100% B was used to wash the column for 5 min, and 15 min 0% B to equilibrate to starting condition. A pulsed amperometric detector (Dionex ICS-5000 ED) monitored the oligosaccharide signals, which were subsequently processed by using Chromeleon™ 7.1 (Dionex). A 3FL calibration curve (0.4 – 20 µg/mL Milli-Q™ water) was used for quantification.

HMO reduction and PGC-LC-MS quantitation

HMOs present in the 40% ACN SPE fraction of human milk samples and infant feces were further chemically reduced at their reducing end sugar residue before being analyzed by porous graphitized carbon - liquid chromatography mass spectrometry (PGC-LC-MS). The reduction of HMOs was performed to avoid split peaks of α- and β- anomers, caused by the strong separation ability of PGC column, as well as to achieve higher response factors in MS.[52] The lyophilized samples were rehydrated with Milli-Q™ water to make a 10-fold dilution of human milk, and two-fold dilution of infant feces. A volume of 200 µl of the rehydrated samples was mixed with 200 µl of freshly prepared 0.5 M sodium borohydride ($NaBH_4$), subsequently left overnight at room temperature, to reduce the HMOs into alditols. A stock solution of HMO standards containing 40 µg/mL of 3FL, 2´FL, LNT, LNnT, LNH, LNnH, LNFP I, LNFP II, LNFP III, LNDFH I, 3´SL, 6´SL, LST a, LST b and LST c, respectively, as well as 20 µg/mL of DFL, was given the same treatment. The reduced samples and standards were purified on Carbograph SPE cartridges: firstly

1.5 ml of 80:20 (v/v) ACN/water containing 0.1% (v/v) TFA followed by 1.5 ml Milli-Q^{TM} water to activate the cartridges; then the 400 µl reduced samples were loaded and washed with 4 x 1.5 mL Milli-Q^{TM} water to remove salts; the HMO fraction was eluted with 1.5 ml of 40% ACN containing 0.05% TFA. The eluted HMO fractions were dried under N_2 stream and lyophilized.

The lyophilized human milk and infant feces were rehydrated and diluted with Milli-Q^{TM} water to reach the final dilution factors of 20 and 4, respectively. HMO standard solutions of different concentrations ranging from around 0.40 to 20 µg/mL (0.20 to 10 µg/mL for DFL) were prepared from serial dilutions of the reduced stock solution. A volume of 5 µl of reduced samples was injected onto an Accela ultra-high-pressure liquid chromatography system (Thermo Scientific, Waltham, MA, USA), which was equipped with a Thermo Hypercarb column (100 x 2.1 mm, 3 µm particle size) preceded by a Hypercarb guard column (10 x 2.1 mm, 3 µm particle size). The elution gradient, using 1% (v/v) ACN in water containing 0.1% (v/v) formic acid as eluent A, and ACN containing 0.1% (v/v) formic acid as eluent B, was set as published previously:[37] isocratic, 3% B in the first 5 min; 5-22 min, 3 to 20% B; 22-32 min, 20 to 40% B; and washing step with 100% B for 10 min and an equilibration step with 3% B for 21 min. The column oven was set at 25 °C, and sample tray at 10 °C. The flow rate was 200 µl/min, except for the washing step and the first 10 min of equilibration, where the flow rate was 300 µl/min. The Velos Pro mass spectrometer (Thermo Scientific) with an electrospray ionization probe was operated in negative-ion mode over a mass-to-charge ratios (*m/z*) range of 300-2000. Samples composed of a mixture of HMO standards were included both in the beginning and the end of sample sequences to verify the stability of MS signal during the analysis. HMOs were identified by the same retention times with the specific standards, as well as checking the *m/z* values and confirming with literature.[17-18] Xcalibur 4.3 (Thermo Scientific) was used for data processing. Quantitation of each identified HMOs was based on integrated peak area from MS signals, and corresponding calibration curves. The limit of quantitation (LOQ) of a given HMO structure was determined as the lowest concentration at which the signal/noise ratio ≥ 6. If needed, individual samples were 10 x further diluted to have the concentrations of certain HMOs within the corresponding linear ranges.

NMR spectroscopy

The 40% ACN fraction of human milk samples as well as infant feces were further analyzed by one-dimensional ^{1}H nuclear magnetic resonance (1D ^{1}H NMR). Based on published methods,[21-22] the lyophilized samples were exchanged with 99.9%$_{atom}$ D_2O (Cambridge Isotope Laboratories, Andover, MA, USA), followed by another lyophilization and exchange with D_2O. Subsequently, 650 µL D_2O containing acetone as internal standard (δ_H 2.225) was used to dissolve the sample, before analysis on a Varian Inova 600 spectrometer (Groningen Biomolecular Sciences and Biotechnology Institute, NMR center, University

of Groningen, The Netherlands). With probe temperatures set at 300 K, a spectral width of 4800 Hz, as well as zero filled to 32k, 1D ^{1}H NMR spectra were recorded at 16k complex points. Suppression of the HOD signal was by applying a pre-saturation Wet1D pulse. MestReNova 10.2 (Mestrelabs Research SL, Santiago de Compostela, Spain) was used for spectra processing, employing a Whittaker smoother baseline correction with a median filter 20 and smooth factor 100. Interpretation of structural-reporter-group signals, and integration of specific spectral regions were described in previous studies.[21-22]

Data analysis

Hierarchical cluster analysis (HCA) was performed for a subset of samples that fulfilled two criteria that human milk belonged to Le+Se+ group, and total HMO concentrations in fecal samples exceeded 100 μg/mL as diluted solution, which equals to 1 μg/mg fresh feces. HCA was conducted in R (Version 3.4.0) using factoextra package, based on the relative compositional changes of the 17 human milk oligosaccharides milk from milk to feces. Firstly, initial relative compositional changes were calculated by subtracting relative abundance (%) of individual HMO in milk from the corresponding relative abundance (%) in paired feces, then divided by the former value. Then, all the positive values were normalized to 0 – 100%, by defining the initial maximum positive values in each sample as 100%. The relative compositional changes were visualized as heatmap, using color scales function in Microsoft Excel 2019. When total HMO concentrations in a fecal sample was below 1 μg/mg, it was considered as complete degradation. Other statistical analyses, including Kruskal-Wallis test and Pearson's chi-squared test for evaluating baseline data of study subjects among different groups, as well as Wilcoxon signed ranks test for comparing proportions of different HMO structure groups between human milk and infant feces, were conducted in SPSS Statistics Version 26 (IBM Corp., Armonk, NY, USA).

RESULTS

HMO analysis of human milk samples

Huge variations exist among studies in terms of HMO concentrations present in human milk, and one major HMO, 3FL, has been often undetected or underestimated according to previous reports.[38, 40] Therefore, in the current study, we developed a separate extraction and quantitation method of 3FL from the starting HMO pool. The PGC-SPE method was adapted from a previous report,[32] to collect 3FL in the 3% ACN fraction along with the major fraction of lactose before eluting the total HMO fraction. Subsequently, the 3% ACN fraction was analyzed by HPAEC-PAD as shown in Figure 2.1, with the 3FL peak being identified by the same retention time as a commercial standard run concurrently. No interference with the huge

amount of lactose was observed. Quantitation of 3FL was performed by using a calibration curve (y = 1.6929x - 0.0823) generated on a serial dilution of 3FL standards, with linear range covering 0.40 – 19.94 µg/mL, and linear correlation (R^2) being 0.999.

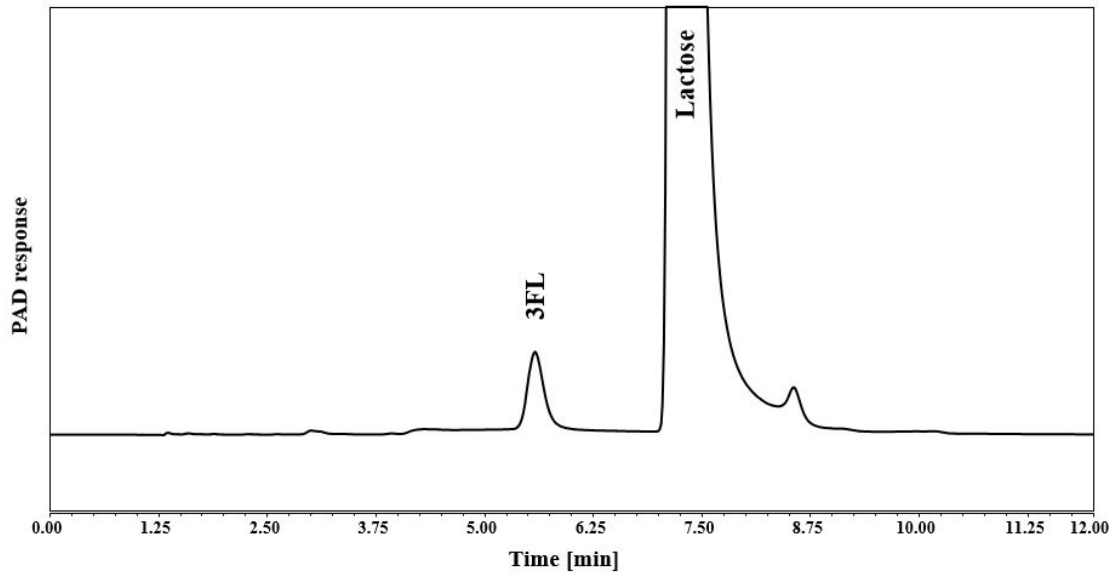


Figure 2.1. HPAEC-PAD elution of 3FL in the 3% acetonitrile fraction of PGC-SPE of a human milk sample.

The 40% ACN fraction of human milk eluted from SPE, which contained all other HMOs, was analyzed by PGC-LC-MS. Elution pattern of a representative human milk sample is given in Figure 2.2 (A), with names, structures and retention times of the annotated peaks listed in Table 2.1. Most of the neutral HMOs were detected in both $[M-H]^-$ and $[M+FA-H]^-$, with $[M+FA-H]^-$ being predominantly; the sialylated HMOs were detected only as $[M-H]^-$. As shown in Figure 2.2 (A), the extracted ion spectra of the HMOs listed in Table 2.1 constituted over 90% of the full mass spectrum, with a few minor peaks excluded in the present study. A small amount of lactose could still be detected, indicating its incomplete removal; nevertheless, its minor presence did not interfere with HMO analysis. The peak of 3FL was absent in the LC chromatograms of the 40% ACN elution fraction, confirming its full elution into the 3% ACN fraction. Other identified HMOs eluted during 13-31 min, with neutral HMO peaks being eluted first within this window, followed by sialylated HMO peaks (23-28 min), except for 6´SL that was eluted at around 18.1 min.

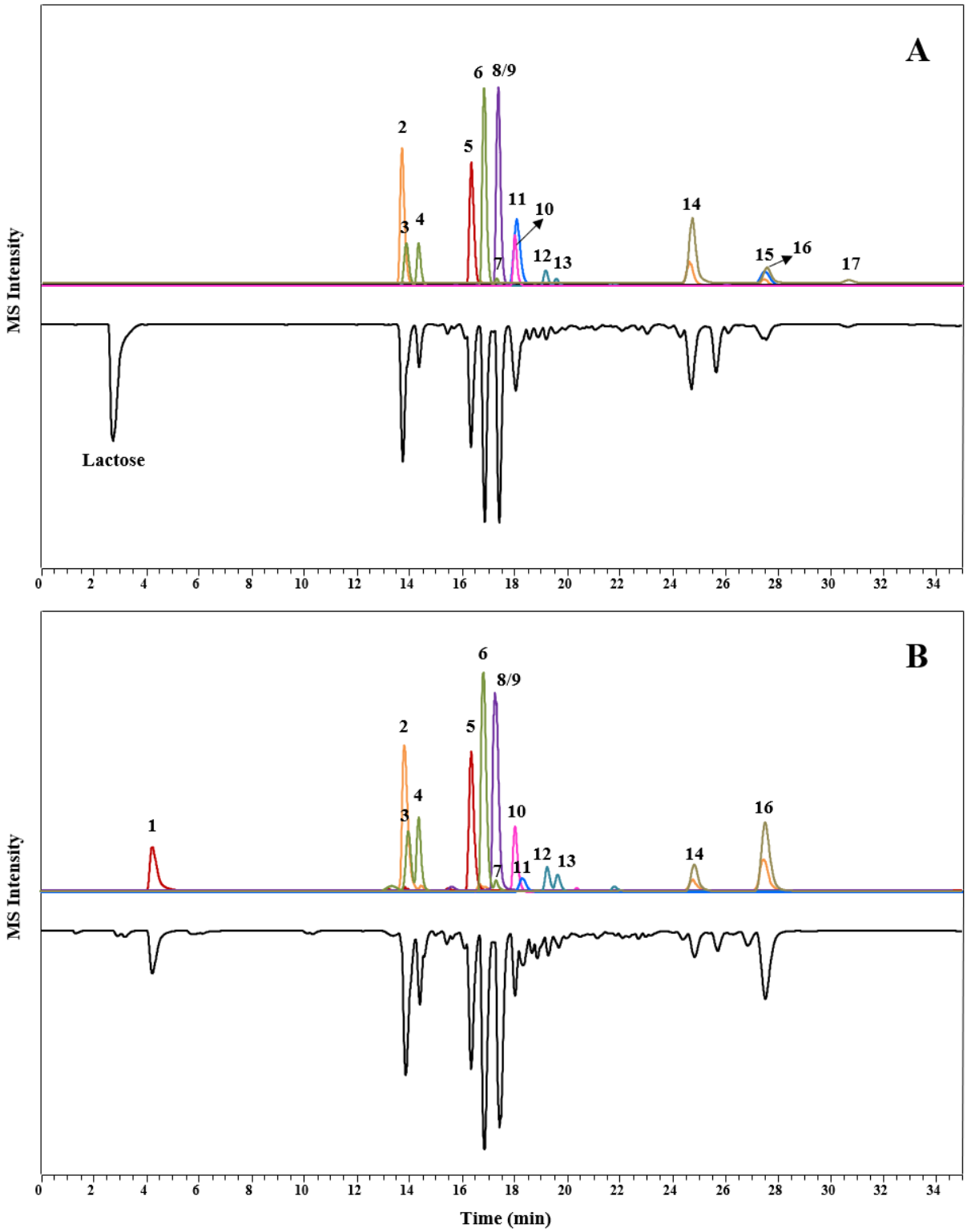
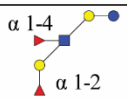
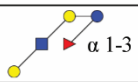
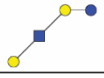
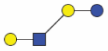
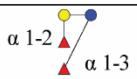
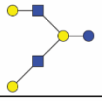
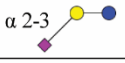
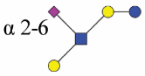
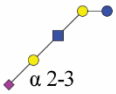


Figure 2.2. PGC-LC-MS elution patterns of a representative mother-infant pair samples (A) human milk and (B) infant feces, in extracted ion spectrum (colored lines, overlaid) and full spectrum (black lines, mirrored view). Names and structures of the identified HMO peaks 1-17 are listed in Table 2.1.

Table 2.1. Overview of the HMOs quantitated in the current method by PGC-LC-MS. HMO structures were designed by using GlycoWorkbench,[53] and kept consistent with previous studies:[17-18] blue circle - glucose; yellow circle - galactose; blue square - N-acetylglucosamine; red triangle – fucose; purple diamond - N-acetylneuraminic acid. The linkages, if not stated in the table, are β linkages indicated following the rule of 

Peak No.	RT (min)	*m/z*	Name	Abbreviation	Structure	Se/Le[#]
1	4.24	489.4, 535.4	3-Fucosyllactose	3FL		Ps-Le^{x}
2	13.8	1000.5, 1046.5	Lacto-*N*-difucohexaose I	LNDFH I		Le^{b}
3	13.9	854.4, 900.4	Lacto-*N*-fucopentaose III	LNFP III		Le^{x}
4	14.4	854.4, 900.3	Lacto-*N*-fucopentaose II	LNFP II		Le^{a}
5	16.3	489.3, 535.2	2′-Fucosyllactose	2′FL		Ps-H
6	16.9	854.4, 900.4	Lacto-*N*-fucopentaose I	LNFP I		H
7	17.4	854.4, 900.4	Lacto-*N*-fucopentaose V	LNFP V		Ps-Le^{x}
8	17.4	708.4, 754.3	Lacto-*N*-tetraose	LNT		NA
9	17.4	708.4, 754.3	Lacto-*N*-neotetraose	LNnT		NA
10	18.0	681.3	Difucosyllactose	DFL		Ps- Le^{y}
11	18.1	634.3	6′-Sialyllactose	6′SL		NA
12	19.2	1119.5, 1073.5	Lacto-*N*-hexaose	LNH		NA
13	19.6	1119.4, 1073.4	Lacto-*N*-neohexaose	LNnH		NA
14	24.7	999.5	Sialyl-lacto-*N*-tetraose c	LST c		NA
15	27.4	634.3	3′-Sialyllactose	3′SL		NA
16	27.6	999.5	Sialyl-lacto-*N*-tetraose b	LST b		NA
17	30.7	999.5	Sialyl-lacto-*N*-tetraose a	LST a		NA

Secretor and Lewis based histo-blood groups are assigned according to van Leeuwen et al.[22] NA means not applicable.

Integrated peak areas from MS signals were used to quantitate each identified HMO, based on calibration curves which were generated on serial dilutions of corresponding standards. Calibration curves of the 17 HMOs quantitated in the current study are given in supplementary Table 2.1, with linear ranges, limit of quantitation (LOQ) and R^2 of each curve evaluated. All of the 17 calibration curves showed good linearity with $R^2 > 0.99$, covering concentrations mostly from 0.39 to 20 µg/mL (except for DFL, 0.078 – 10 µg/mL; LNnH, LST c, 3´SL and LST a, 0.078 – 20 µg/mL; LNH and LST b, 0.156 – 20 µg/mL). Due to the fact that LNT eluted at the same time as its isomer LNnT, which has a rather similar MS spectrum, it is hardly possible to differentiate between the two components, consistent with a previous report.[54] Considering the similar response factors of their calibration curves, LNT and LNnT in human milk samples were integrated as one peak and quantitated using the calibration curve of LNT. Despite co-elution of LNT and LNnT, we are able to separate and quantitate the absolute concentrations of almost all major HMOs present in human milk samples with the PGC-LC-MS method.

For comparison, a previously developed system of a 1D ^{1}H NMR structural-reporter-group signals was applied on the same set of samples for the annotation of Fuc- and Neu5Ac- containing HMOs.[21-22] Based on this system, structure-dependent regions were determined for the α-anomeric region (δ 5.00-5.50), NAc CH_3 region (δ 1.80-2.20), Fuc CH_3 region (δ 1.00-1.40), and Neu5Ac CH_3 region (δ2.50-2.90), as shown in Figure 2.3 (B). In the Fuc CH_3 region (δ1.00-1.40), three structure regions are observed. The bracket δ1.24-1.30 contains CH_3 signals for Fuc(α1-2), Fuc(α1-3) and Fuc(α1-4) in case of two neighboring residues are fucosylated, i.e. in Le^b or Le^y epitopes. The δ1.19-1.24 bracket contains CH_3 signals belonging to Fuc-(α1-2) residues in an H-antigen epitope and the δ1.14-1.19 bracket contains the CH_3 signals for Fuc(α1-4) and Fuc(α1-3) in Le^a and Le^x epitopes.[21] Beside these brackets the α-anomeric region shows a fingerprint of the Fuc-epitopes in 8 brackets **a** – **h**. Using these fingerprint brackets, together with the Fuc CH_3 brackets it is possible to distinguish Le^b from Le^y, and Le^a from Le^x.[21] Also, a separate peak is observed for Fuc-(α1-2)- in 2´FL (bracket **b**), allowing a distinction of this HMO structure from other H-antigen structures (e.g. LNFP I). An estimation of the relative levels of Le^a, Le^b, Le^x and Le^y can be made based on the peak intensity of these regions, as well as a differentiation between 2´FL derived H-antigen and other H-antigen epitopes.

KOALA Birth Cohort Study pilot samples

The three analytical methods of HMOs, HPAEC-PAD, PGC-LC-MS and 1D ^{1}H NMR, were applied on a subset of participants who enrolled in the KOALA Birth Cohort Study. A total of 20 human milk samples from 20 mothers were analyzed in the current study, with the overall baseline data of these study subjects summarized in Table 2.2. Sample collection was conducted at 32 days postpartum on average (SD

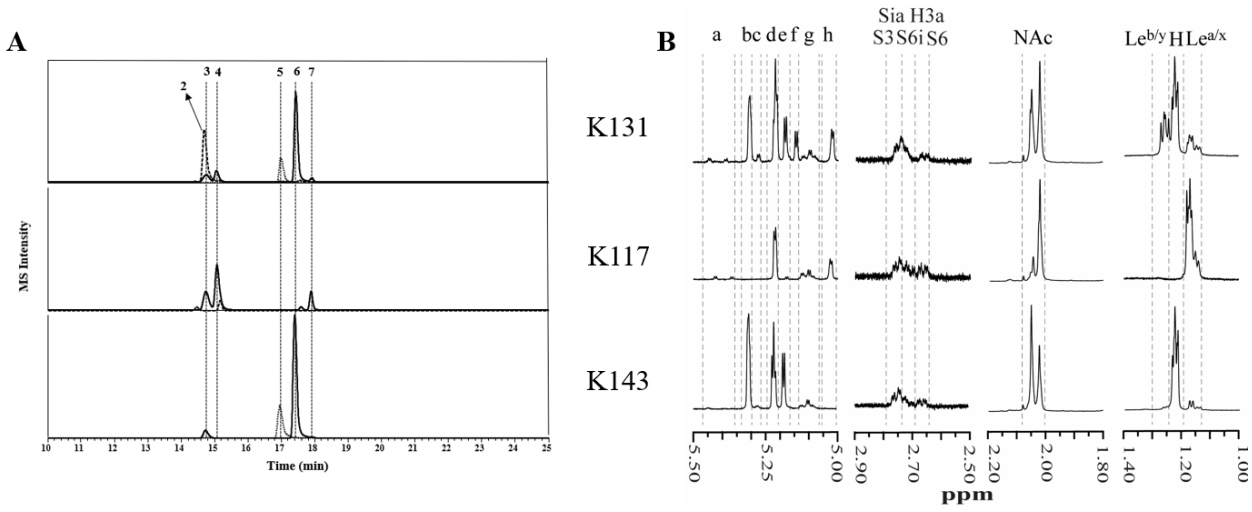
= 4), with all infants born in full term and of normal birthweight, as well as exclusively breastfed till samples were collected. Out of the 20 mothers, only one mother delivered via Caesarean section, 75% of the mothers delivered at home. Concentrations of 3FL and the other 16 HMOs in the 20 human milk samples were quantitated by HPAEC-PAD and PGC-LC-MS, respectively, with the results of each individual sample given in supplementary Table 2.2. Relative levels of Fuc-epitopes, namely Le^{a}, Le^{b}, Le^{x}, Le^{y}, 2′FL and non-2′FL H-antigen, derived from 1D ^{1}H NMR for the 20 milk samples are presented in supplementary Table 2.4. Results of selected samples will be described in the following sections.

Table 2.2. Baseline data of the study subjects, overall and from different designated consumption pattern clusters (only Lewis-positive Secretor milk group). The values of each variable were evaluated among different clusters using Kruskal-Wallis Test, or Pearson's chi-squared test, yet no significant differences were found (data not shown).

Variables	Overall n = 20	Cluster A n = 3	Cluster B n = 9	Cluster C n = 5
Age postpartum, day, mean (SD)	32 (4)	32 (6)	32 (3)	32 (4)
Gestational age, week, mean (SD)	40 (1)	39 (0)	40 (1)	40 (1)
Birthweight, g, mean (SD)	3657 (395)	3780 (505)	3643 (376)	3611 (394)
Gender, male, n (%)	15 (75)	2 (67)	7 (78)	3 (60)
Delivery mode, vaginal, n (%)	19 (95)	3 (100)	8 (89)	5 (100)
Delivery place, home, n (%)	15 (75)	1 (33)	6 (67)	5 (100)

Lewis/Secretor milk group identification

In the present study, Secretor status and Lewis blood groups of the 20 mothers were identified by examining HMO concentrations as quantitated by PGC-UPLC-MS. Results of Fuc-epitope levels as integrated from 1D ^{1}H NMR spectroscopy led to exactly the same grouping of mothers. Out of 20 human milk samples, 18 samples fit the profile of Le+Se+ (90%), one sample (K117) fits with the pattern of Le+Se- (5%), and one (K143) fits Le-Se+ (5%). LC-MS chromatograms and 1D ^{1}H NMR spectra of one Le+Se+ milk K131 are given in Figure 2.3 (A) and (B), respectively, together with that of K117 and K143. Extracted ion spectra of FL, LNFP and LNDFH, showing their absolute intensities, are presented in Figure 2.3 (A), as 2′FL, LNFP I (H-antigen), LNFP II (Le^{a}) and LNDFH I (Le^{b}) are major and typical components for Lewis and Secretor determination. Presence of the other two annotated peaks, LNFP III (Le^{x}) and LNFP V (pseudo-Le^{x}), are also important fucosylated structures, although independent from Lewis and Secretor status of mothers. Concentrations of each annotated peak of the three milk samples, as well as the relative levels of Fuc-epitopes, are listed in Figure 2.3 (C).



C

	Concentrations by PGC-LC-MS (µg/mL)						Relative levels of Fuc-epitopes by 1D ^{1}H NMR					
	LNDFH I (2)	LNFP III (3)	LNFP II (4)	2´FL (5)	LNFP I (6)	LNFP V (7)	Le^a	Le^b	Le^x	Le^y	2´FL	H
K131	473.7	88.2	201.9	460.4	650.0	30.2	0.01	0.17	0.14	0.06	0.35	0.26
K117	0	216.5	831.5	0	26.2	118.5	0.49	0.00	0.51	0.00	0.00	0.00
K143	7.4	87.3	0	698.1	981.5	0	0	0	0.03	0.06	0.60	0.31

Figure 2.3. (A) Extracted and overlaid ion spectra of PGC-LC-MS chromatograms of three human milk samples: K131 (Le+ Se+), K117 (Le+ Se-), K143 (Le- Se+). Ion spectra extracted with different *m/z* are shown in lines of different formats: solid (LNFP), square dot (LNDFL), round dot (FL). (B) Relevant parts (δ 5.00-5.50, δ2.50-2.90, δ 1.80-2.20, and δ 1.10-1.30 ppm) of 1D ^{1}H NMR spectra of the same human milk samples as in (A). Bracket identities of anomeric region δ5.00-5.50: **a**. Fuc-(α1-3)- H-1 in pseudo-Le^x (3FL) and pseudo-Le^y (DFL) epitopes, **b**. Fuc-(α1-2)- H-1 in 2´FL, **c**. Fuc-(α1-2)- H-1 in Le^y epitopes, **d**. α-D-Glc*p* H-1, **e**. Fuc-(α1-2)- H-1 in H-antigen epitopes and α-D-Glc*p* H-1 in 3FL and DFL, **f**. Fuc-(α1-2)- H-1 in Le^b epitopes, **g**. Fuc-(α1-3)- H-1 in Le^x and Le^y epitopes **h**. Fuc-(α1-4)- in Le^a and Le^b epitopes. Neu5Ac region δ2.50-2.90: **S3.** Neu5Ac-(α2-3)- H-3e, **S6.** Neu5Ac-(α2-6)-Gal H-3e, and **S6i.** Neu5Ac-(α2-6)-GlcNAc H-3e. Fuc CH_3 region δ 1.00-1.40: **$Le^{b/y}$.** CH_3 signals of Fuc residues in Le^b and Le^y epitopes, **H**. CH_3 signals of Fuc residues in H-antigen epitopes, **$Le^{a/x}$**. CH_3 signals of Fuc residues in Le^a and Le^x epitopes. Note: the Fuc anomeric region is not scaled in the same proportion as the Neu5Ac H-3e, NAc and Fuc CH_3 regions. (C) Names and concentrations of each annotated peaks of (A) in the three human milk samples, as well as their relative levels of Fuc-epitopes derived from 1D ^{1}H NMR integration of anomeric region sections a – h in (B).

Similar to K131 milk sample, 18 milk samples fitting Le+Se+ profile contained significant levels of $Le^{b/y}$, H-antigen and $Le^{a/x}$ CH_3 peaks (supplementary Table 2.4), in consistency with the high abundances of 2´FL, LNFP I, LNFP II and LNDFH I present in the milk samples (supplementary Table 2.2). The relative levels of four Le-epitopes and H-antigen showed different balances among these Le+Se+ milks; likewise, large variations existed in concentrations of 2´FL, LNFP I, LNFP II and LNDFH I presented in these samples, as measured by PGC-LC-MS. As shown in Figure 2.3, the milk sample from Le-Se+ mother K143 contained trace amount of LNDFH I (7.4 µg/mL); however, the value is less than 2% of that in K131 milk. Moreover, K143 showed a strong H-antigen peak (e.g. 2´FL, LNFP I), despite the hardly detected $Le^{b/y}$ epitopes (e.g. LNDFH I), which was most likely hidden in the background noise. With a minor peak in the

$Le^{a/x}$ region belonging only to Le^{x} epitopes (e.g. LNFP III), as evidenced by the absence of a Fuc(α1-4) anomeric signal (e.g. LNFP II), K143 was confirmed as Le-Se+ milk group. As to milk sample K117, no peaks in $Le^{b/y}$ nor H-antigen Fuc CH_3 regions were detected, while anomeric peaks fitting Le^{a} as well as Le^{x} structures are observed, implying the mother's phenotype being Le+Se-. This is confirmed by high abundances of LNFP II, LNFP III and LNFP V in LC-MS, whereas LNDFH I was hardly detected, and LNFP I was found only in trace amounts.

HMO analysis of infant fecal samples

Together with human milk samples, 20 infant feces were collected around the same day postpartum from the 20 mother-infant dyads. The collected infant feces were processed to extract HMOs and then analyzed by PGC-LC-MS and 1D ^{1}H NMR. Unlike human milk, lactose content significantly decreases during the transit through infants' gastro-intestinal tract, ending up in almost complete absence.[31] Therefore, the step of washing with 3% ACN was omitted for feces during SPE, which led to elution of 3FL in the 40% ACN fraction of fecal samples, as well as analysis of 3FL by PGC-LC-MS together with other HMOs. Figure 2.2 (B) presents chromatograms of the infant fecal sample corresponding to the human milk shown in Figure 2.2 (A). 3FL eluted after 4.2 min (Figure 2.2 (B); Table 2.1), much earlier than the other HMOs, but close to the retention time of lactose (2.6 min) as seen in Figure 2.2 (A), which was consistent with the loss of 3FL in the lactose removal step during PGC-SPE. Similar to the other 16 HMOs, the concentration of 3FL in fecal samples was quantitated based on its corresponding calibration curve as shown in supplementary Table 2.1. Three infant fecal samples (K109, K117, K143) were only analyzed by 1D ^{1}H NMR, and PGC-LC-MS results were not available. Quantitation results of the 17 HMOs (only sum of LNT and LNnT were given) detected in each of the other 17 infant fecal samples are given in supplementary Table 2.3. Relative levels of Le^{a}, Le^{b}, Le^{x}, Le^{y}, 2′FL and non-2′FL H-antigen in the 20 infant feces, based on 1D ^{1}H NMR, are listed in supplementary Table 2.4.

HMO concentrations of Le+Se+ group in both milk and feces

To further study the 18 mothers whose milk samples were assigned to the Le+Se+ milk group, the means and standard deviations of each quantitated HMO in the milk and paired infant feces samples, as determined by HPAEC-PAD and PGC-LC-MS, are summarized in Table 2.3. In the human milk samples, the sum of the 17 HMOs ranged from 2.1 – 5.6 mg/mL, with an average of 3.8 mg/mL. Fucosylated HMOs accounted for the largest proportion (60%), followed by non-fucosylated core structures (26%), and sialylated structures (14%). The HMOs having the highest average concentrations are LNT and LNnT, which were quantitated together. Other abundantly present HMOs include 2′FL, LNFP I and LNDFH I,

while LST b is the most abundant sialylated HMO on average. As to the fecal samples, variation of single HMO concentrations between individuals is quite large and hardly any solid generic conclusions on individual HMO concentrations could be made. Average proportions of the total fucosylated, total non-fucosylated core, and total sialylated HMOs in fecal samples were compared to those in human milk, with significant increase found in relative amounts of fucosylated structures, and highly significant reduction in relative amounts of non-fucosylated core structures. This added new observations concerning the consumption preference by infant colonic microbiota to specific HMO structural elements, and allowed us to look in more detail to the metabolization of HMOs ingested.

Table 2.3. Concentrations of 17 human milk oligosaccharides (μg/mL; only sum of LNT and LNnT was given) and proportions of different HMO structure groups, in human milks (n=18) and in paired infant feces in 10-fold diluted solutions (n=17) from Lewis-positive Secretor milk group by using HPAEC-PAD or PGC-LC-MS. The results are shown as means (standard deviations). Significant differences were evaluated on proportions of different HMO structure groups between human milk and infant feces by Wilcoxon signed ranks test, with results noted by * $p < 0.05$, ** $p < 0.001$.

Compound	Human milk n = 18	Infant feces n = 17
Fucosylated*	*61.0% (5.19%)*	*75.3% (15.7%)*
3FL	146 (54.7)	74.4 (76.7)
2′FL	561 (117)	91.3 (94.6)
LNFP I	536 (165)	132 (139)
LNFP II	290 (158)	108 (108)
LNFP III	134 (46.3)	40.1 (37.3)
LNFP V	40.6 (26.1)	1.70 (4.84)
DFL	84.8 (89.8)	116 (122)
LNDFH I	505 (142)	189 (115)
Non-fucosylated core*	*25.5% (5.23%)*	*8.60% (6.30%)*
LNT + LNnT	869 (367)	45.7 (52.4)
LNH	66.7 41.5)	0.694 (1.54)
LNnH	71.6 (69.1)	1.21 (2.27)
Sialylated, %	*13.6% (2.12%)*	*16.0% (13.4%)*
3′SL	94.3 (24.0)	1.01 (1.64)
LST a	20.9 (7.18)	1.52 (3.73)
6′SL	74.3 (30.0)	16.1 (27.6)
LST c	134 (58.9)	37.2 (64.0)
LST b	194 (100)	131 (281)
Total	3822 (853)	986 (880)

Metabolization of HMO in infants

To further investigate different metabolic fates of HMOs by infant colonic microbiota, relative compositional changes of the 17 HMOs between milk and paired feces were calculated for 14 selected mother-infant pairs. The 14 samples fulfilled two criteria that human milk belonged to Le+Se+ group, and total HMO concentrations in fecal samples exceeded 100 µg/mL as diluted solution, which equals to 1 µg/mg fresh feces. Concentrations of individual HMOs of fecal sample K109 was unavailable. Feces K026, K028 and K090 were excluded because of the limited presence of HMOs in the infant feces. These three fecal samples were designated as Cluster A of consumption pattern in the present study, which indicated (almost) complete consumption of HMOs by infant colonic microbiota.

The relative changes of HMO composition between milk and feces of the 14 included mother-infant pairs were visualized with a heatmap, with hierarchical clustering performed to show similarities between these subjects (Figure 2.4; calculated values in supplementary Table 2.5). There are two main clusters, with 9 subjects in the one cluster (designated as Cluster B) and 5 in the other (designated as Cluster C). Clear differences could be observed between the two clusters: subjects in Cluster B showed increased relative levels of most fucosylated HMOs, while Cluster C showed considerable reductions of those. As to core structures and sialylated structures, both clusters showed comparable reductions of relative levels in feces. This observation implies a specific consumption of non-fucosylated structures by Cluster B infants; conversely, Cluster C infants utilized HMOs from all three structural groups at considerable levels without obvious preferences. It is also noted that all infants from Cluster B excreted higher concentrations of total HMOs (839.9 – 3065.3 µg/mL) compared to those from Cluster C (120.9 – 641.7 µg/mL). To explore any maternal or infant factors associated with these consumption patterns, baseline variables of subjects from the three clusters were evaluated and summarized in Table 2.2; however, no significant differences were found among the clusters. When comparing the relative compositional changes of individual HMO structures in both Cluster B and Cluster C, it is noted that LNT, LNnT, LNH and LNnH, which are core structures, as well as 3´SL and LST a, which are Neu5Ac(α2-3) structures, were the heavily degraded HMOs in most cases. Compared to Neu5Ac(α2-3) structures, Neu5Ac(α2-6) structures, including 6´SL, LST b and LST c, were degraded to a lesser degree, as well as in fewer cases. This shows general preferences of colonic microbiota for different isomeric HMO structures. As to Fuc-containing structures, it is noted that DFL and LNDFH I were in general excreted in higher amounts in infant feces than structures with only one fucose residue, implying that a higher degree of fucosylation results in a lower degree of enzymatic degradation by colonic bacteria. The three mother-infant pairs grouped in Cluster A showed almost no HMO peaks in the LC-MS chromatograms of their fecal samples (results not shown), indicating high consumption activities of gut microbiota which fully utilized the ingested HMOs.

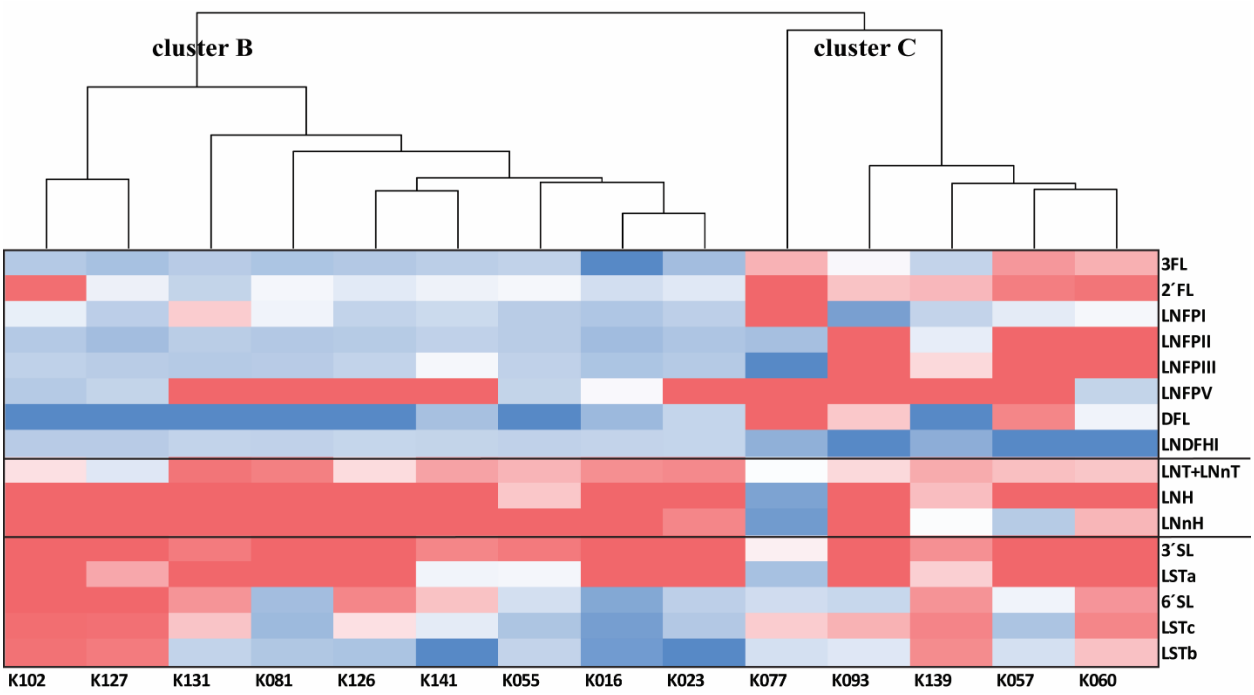


Figure 2.4. Dendrogram and heatmap of relative compositional changes of human milk oligosaccharides between human milk samples and the corresponding infant feces. The colors range from red (relative reduction in feces) to blue (relative increment in feces). Hierarchical clustering results of the 14 mother-infant pairs, whose human milk samples were classified into the Lewis-positive Secretor milk group, and were not completely degraded in the colon of the corresponding infants. Cluster A consisting of 3 mother-infant pairs is not shown here because of full utilization of HMOs.

To compare with the observations from the heatmap and hierarchical clustering, 1D ^{1}H NMR spectra of one representative mother-infant pair from each of the three clusters were shown in Figure 2.5. All three milk samples showed patterns with occurrence of all Fuc-containing epitopes and H-antigen structural elements, as well as presence of Neu5Ac(α2-3) and Neu5Ac(α2-6) structures (Figure 2.5 Cluster A – C). As to infant feces, K090 from Cluster A showed no presence of HMO-like structures, confirming the proposed full consumption of HMOs from the milk with no structural bias. K023 from Cluster B showed reduction in 2´FL, combined with minor increase in Le^{a}, Le^{b} and Le^{x}. Here the Neu5Ac(α2-3) was consumed, while Neu5Ac(α2-6) showed a relative reduction in levels. Referring to supplementary Table 2.5, which provides concentrations of individual HMOs as quantitated by LC-MS, 2´FL relatively decreased by 28%, where LNFP II and LNFP III relatively increased by 20% and 13%, respectively. Although LNDFH I remained relatively unchanged, the relative composition of 3FL showed 31% of increment. Full consumption of 3´SL and LST a is consistent with NMR spectra. However, levels of 6´SL, LST b and LST c relatively increased in feces compared to milk, which was different from observation of NMR spectra. Therefore, examining relative changes of individual HMOs could indicate slightly different

utilization levels compared with what was expected from the NMR structural groups. The difference was mainly caused by presence of other HMOs with the same fucosylated structural elements in the same sample, which also contributed to the overall signal of NMR spectra. It is important for researchers to be aware of the limitations of each technique applied in their study, in order to draw objective conclusions. As to K077 from Cluster C, a nearly complete disappearance of H-antigen structures and Le^{y} specific anomeric peaks is observed, with relatively reduced Le^{b} epitopes, whereas Le^{x} epitopes are still relatively abundant. Likewise, 2´FL and DFL were completely absent in K077 feces, while LNFP III increased the most in relative levels compared to LNDFH I, as quantitated by LC-MS (supplementary Table 2.5). Nevertheless, it is noted that even a relative increase of an HMOs-structural element in feces when compared to the paired milk could still mean that consumption by gut microbiota has taken place.

Furthermore, NMR spectra of the corresponding infant fecal samples of Le-Se+ (K143) and Le+Se- (K117) milk groups were also examined (Figure 2.5). The LC-MS results of fecal samples of these two pairs were not available for comparison. K143 showed almost no carbohydrate related peaks, with minor levels of H-antigen still observed. Absence of 2´FL peaks at δ 5.31 indicates only H-antigen in LNFP I-like structures remain. K117 showed only trace amounts of carbohydrate peaks, and a preferred consumption of Le^{a} for this sample set according to the change in relative levels of Le^{a} and Le^{x} from milk (0.49, 0.51) to feces (0.13, 0.88) as given in supplementary Table 2.4. Nevertheless, no solid conclusions could be made on these two milk groups, due to limited sample numbers.

DISCUSSION

Three commonly used HMO analytical techniques, HPAEC-PAD, PGC-LC-MS and 1D ^{1}H NMR, were combined to determine HMO profiles of a set of human milk and infant fecal samples from 20 mother-infant dyads, who were enrolled in the KOALA Birth Cohort Study. Absolute concentrations of 3FL in human milk were determined by using HPAEC-PAD, while its concentration in infant fecal samples was quantitated by PGC-LC-MS. The other 16 major HMO structures in both milk and feces were quantitated by PGC-LC-MS. Loss of 3FL in the SPE clean-up step has been occasionally mentioned previously. Such losses could explain a lower content or even absence of 3FL in human milk samples as reported in some studies compared to others.[38] Some analytical techniques could hardly differentiate structural isomers due to co-elution, e.g. CE-LIF or MALDI-TOF-MS, which were only able to report combined intensity of 2´FL and 3FL. Despite the fact that 2´FL, LNnT and sialylated HMOs were the most studied structures regarding health benefits, limited studies allude to certain health potentials brought by 3FL.[55-56] The current study adapted the collection of 3FL in a separate fraction and then analyzed 3FL by HPAEC-PAD, which opens the window of including 3FL in efficacy studies in the future. Although the interaction mechanism between

HMOs and PGC material is not fully clear,[38] it is observed that presence, number and linkage-type of fucosylation and sialylation have distinct influences on the retention times in PGC-LC-MS method, which is also true for HPAEC-PAD.[57] However, identification and quantitation of multiple isomeric structures by HPAEC-PAD was not optimal, as only retention time was used without further confirmation by MS.[52, 58]

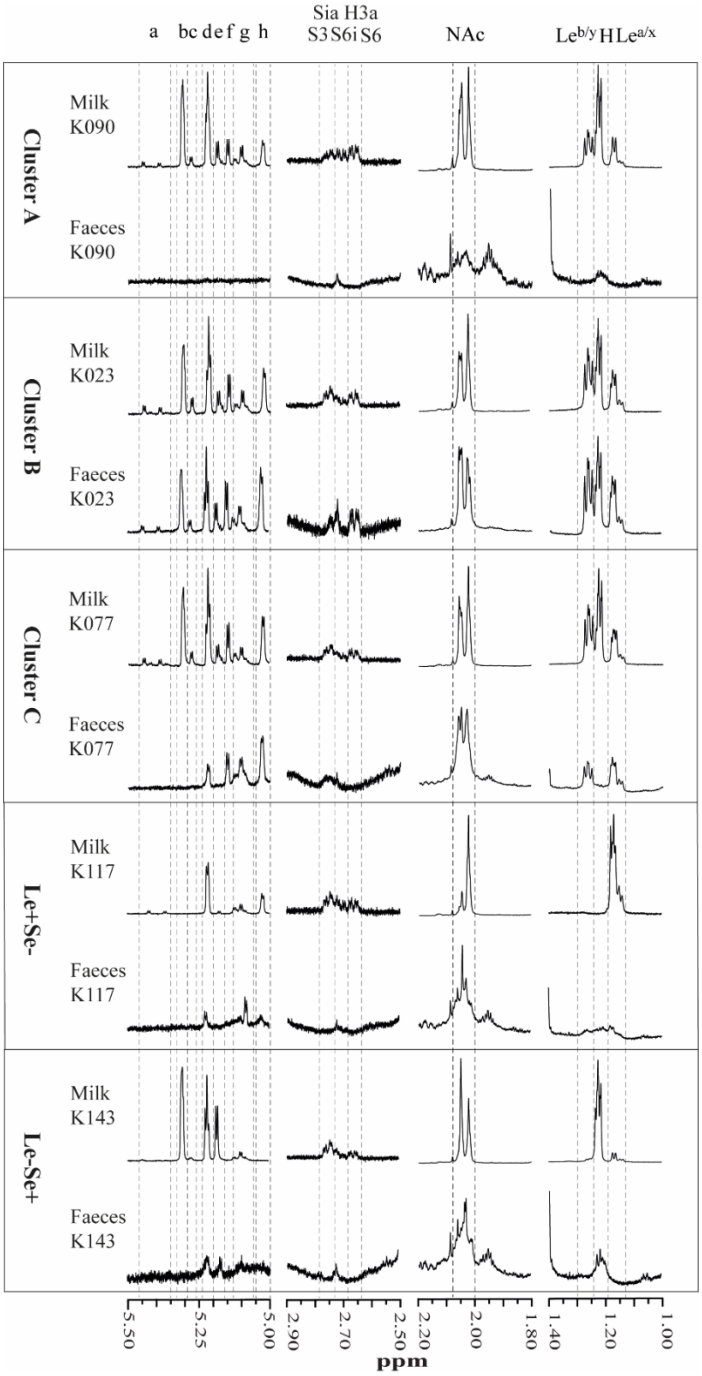


Figure 2.5. Relevant parts (δ 5.00-5.50, δ2.50-2.90, δ 1.80-2.20, and δ 1.10-1.30 ppm) of 1D ^{1}H NMR spectra of representative human milk samples and their corresponding infant feces. The structure-dependent regions are annotated in Figure 2.3.

It is noted that the total HMO concentrations present in human milk samples included in the current study (2.1 – 5.6 g/L) were lower than the reported value 5-20 g/L in literature.[15] The systematic review by Thurl *et al*. concluded the average total concentration of neutral HMOs to be 14.78 g/L in mature milk of Secretor mothers and acidic HMOs to be 2.13 g/L.[29] The values given in the review also included some larger, multi-fucosylated structures such as trifucosyllacto-*N*-hexaose,[29] which was reported to be around 2.6 – 3.1 g/L in only a few studies,[24, 59-60] resulting in increased relative HMO levels. Despite the fact that less HMO structures were included in the calculation in the current study, concentrations of individual HMOs were all lower than corresponding values from the review.[29] The lower concentrations might be attributed to possible degradation of HMOs during the long storage time, considering that the samples were collected over ten years ago in the period 2002-2003. Concentrations of relatively simple structures, like 2´FL and 6´SL, were much lower compared to literature than more complicated structures like LNDFH I and LST,[29] indicating the simple HMOs were more prone to degradation during long-term storage. Furthermore, the concentrations of HMOs presented were based on the watery fraction of the maternal milk samples after lipid removal, which also contributed to the lower concentrations. Nevertheless, there are other studies that also reported lower total HMO concentrations (5-8 g/L),[61-62] which were closer to the current study. Besides other determinants that would influence HMO results, large variations between different laboratories were already noticed in the previous review, in which a proposal of performing interlaboratory tests in parallel on the same standardized human milk samples was made.[29] One limitation of the current method is the exclusion of disialyllacto-*N*-tetraose (DSLNT) from quantitation. Previous studies showed relatively high abundance of DSLNT in human milk (0.37-0.71 g/L in mothers delivering at full term),[29] and indicated its preventive effects on necrotizing enterocolitis in preterm infants.[63-64] However, the presence of two sialic acid moieties on DSLNT required a much longer retention time than the HMOs quantitated in the current LC-MS method. We decided to continue with the current high throughput LC-MS method which will be applied to larger size of sample sets, and leave DSLNT for future method development studies. Furthermore, a few new peaks emerged in the LC-MS chromatograms of infant feces compared to the paired milk (Figure 2.2), which represented presence of metabolites that originated from HMO degradation or endogenous sources such as intestinal glycoconjugates.[49, 65] Metabolite investigation in infant feces could be another potential direction for future research.

Several previous studies have presented four milk groups with representative HMO profiles corresponding to mothers who belong to Lewis-positive Secretor (Le+Se+), Lewis-negative Secretor (Le-Se+), Lewis-positive non-Secretor (Le+Se-) and Lewis-negative non-Secretor (Le-Se-).[21, 25, 66-67] In the current study, we successfully assigned the mothers to different Lewis Secretor milk groups using the HPAEC-PAD combined with LC-MS method, and with results confirmed by NMR spectroscopy. The frequencies of milk groups in the pilot study was found to be 90% of Le+Se+, 5% of Le+Se- and 5% of Le-

Se+, which numbers slightly deviated from previously reported values (~70%: 20%: 10%) for European mothers.[21, 25, 66, 68] This distribution is within the expected range, considering the rather small sample size (n = 20) in the current pilot study. All three aforementioned analytical methods led to consistent results for milk group assignment, with the difference being that HPAEC-PAD and LC-MS examined concentrations of individual HMOs, whereas NMR compared the relative levels of Le-epitopes and H-antigen structural elements. With either HPAEC-PAD combined with PGC-LC-MS, or NMR method alone, it should be sufficient to correctly identify milk groups based on HMO profiles in future studies.

It is noted that when using PGC-LC-MS alone, it is quite demanding to quantitate all HMOs in biological samples (17 HMOs in our methods), considering time and labor required. Instead, NMR mainly provides information on specific structural elements (Le-epitopes, H-antigen, and Neu5Ac structural groups) without any preceded separation of individual HMOs. In this study, NMR provided a helicopter view of all HMO structural elements present in the samples, but lacking information on individual HMOs. Therefore, which techniques to use should depend on the research question.

With the developed methods, we identified three characteristic HMO consumption patterns among the infants in the present study (Figure 2.4), with Cluster A (n =3) showing complete consumption, Cluster B (n = 9) showing specific consumption of non-fucosylated structures, and Cluster C infants (n = 5) utilizing HMOs without preference to specific structures but considerable level of HMOs could still be detected in feces. These patterns were also recognized by a previous study of a Vietnamese cohort at 1 month postpartum using NMR spectroscopy,[22] where three profile types were proposed, namely full consumption, presence of all structural elements, and significant consumption of specific structural elements, especially 2´FL and H-antigens. However, not all studies observed the same varying consumption patterns, e.g. Chaturvedi *et al.* found generally similar HMO profiles between all the infant feces and paired human milk being analyzed, using reverse-phase HPLC-MS.[65] Albrecht *et al.* reported dominance of either neutral or acidic HMOs in infant feces in the first weeks to months, with empty fecal HMO profiles mostly occurring after 6 month postpartum.[49] Besides the non-specific consumption patterns shown by infants from Cluster C, their fecal HMO concentrations were found lower than those from Cluster B. Albrecht et al. has found a gradual decrease of HMO excretion in infant feces with age as a result of ripening of gut microbiota.[49] It is likely that infants from Cluster C had developed the gut microbiota composition more quickly towards a HMO-consuming microbial community compared to those from Cluster B. However, it is noted that the comparisons were made based on wet weight of infant feces, which were affected by the water content in the samples. The amount of milk consumed by the infants, and the amount of feces excreted by the infants were not available. Therefore, the observations here on infant fecal HMO quantitation were used for indications, but no solid conclusions could be made yet. Despite differences in HMO composition secreted

by mothers, the large variations observed in infant fecal HMO patterns were mainly caused by different microbiota compositions of infant's colon.[47] Considering some studies reporting the influence of ingested HMOs on maturity of infant gut microbiota,[69-70] the exact mechanism of how these two factors interact with each other is much more complicated and not fully understood heretofore. Isomer-specific consumption was recognized previously in a proof-of-concept study, which involved two infants, showing that certain HMOs were consumed at much higher degree compared to their structural isomers.[48] Our results showed that non-fucosylated core structures were more likely to be degraded compared to those decorated by fucose and/or sialic acid; while fucosylated structures were more resistant to degradation and remained mostly intact till their excretion in feces. As the most abundant HMOs in human milk, LNT and LNnT were in most cases significantly reduced after transit through infant's GI tract, this implies that these structures are more prone to degradation by infant colon microbiota compared to other structures. This observation was supported by a few studies where infant feces were found to be devoid of LNT and/or LNnT,[32, 41, 71] albeit one study presented the opposite conclusion.[47] HMO core structures decorated with fucose at different levels and linkage types were found to exert different influences on their metabolic fates in infant colon. Di-fucosylated HMO structures, despite their lower levels in human milk than mono-fucosylated ones, had relatively higher survival in the infant gut, which fits with the higher likelihood of anti-adhesive properties because of their multivalent interaction with glycan binding proteins from enteric pathogens.[72] As to sialylated HMOs, it is found that Neu5Ac(α2-3) linked HMOs were more readily consumed compared to Neu5Ac(α2-6) linked ones. Furthermore, correlation between maternal HMO synthesis and infant fecal microbiota composition, as well as the key microbial phylotypes responding for HMO degradation, was published elsewhere,[37] with samples from KOALA cohort study including the current pilot sample set. The current study is limited by only one time point of sample collection, lacking information of how fecal HMO excretion patterns would progress with maturity of infant gut microbiota composition, as shown in literature.[47, 49]

CONCLUSIONS

Human milk oligosaccharides are valuable nutrients provided by mothers to their nursing infants, which makes it important to develop analytical methods for HMO profiling in human milk as well as infant feces, in order to find out the optimal HMO composition for infant's health, as well as how different HMO structures were metabolized by infants. Extraction and quantitation of 3FL separately from the major HMOs pool provided more accurate information on this relevant HMO. With the combination of HPAEC-PAD and PGC-LC-MS, concentrations of individual HMO structures were quantitated; meanwhile, 1D ^{1}H NMR

provided a clear picture showing relative levels of different structural elements. Depending on the future research questions, the most suitable method should be chosen to obtain the correct information.

In the current pilot study, inter-individual variation was shown not only in mother's milk, but also in consumption of HMOs by the infant gut microbiota. Three distinct HMO consumption patterns were recognized at one-month postpartum. Level of degradation of HMOs was different depending on the specific structures and linkage types. Due to limited sample size and only one-time sample collection, future studies involving more mother-infant pairs as well as multiple timepoints are warranted to define the maternal or infant factors that determined these variations we observed. Answers to all these questions would help with making decisions regarding infant formula development with personalized HMO combinations for individual babies.

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SUPPORTING INFORMATION

Table S2.1. Linear range, linear regression equation, linear correlation and limit of quantitation (LOQ) of the HMOs quantitated by using the current PGC-LC-MS method.

Compound	Linear range (µg/mL)	Linear regression equation	Linear correlation	LOQ (µg/mL)
3FL	0.039 -20	y = 1E+06x - 117516	0.999	0.039
LNDFH I	0.039 -20	y = 1E+06x - 72426	1	0.039
LNFP III	0.039 -20	y = 1E+06x - 104818	0.999	0.039
LNFP II	0.039 -20	y = 1E+06x - 272696	0.999	0.039
2´FL	0.039 -20	y = 2E+06x + 57627	0.998	0.039
LNFP I	0.039 -20	y = 2E+06x - 364643	0.999	0.039
LNFP V	0.039 -20	y = 1E+06x - 195263	0.999	0.039
LNT	0.039 -20	y = 2E+06x - 234160	0.999	0.039
LNnT	0.039 -20	y = 2E+06x - 368694	0.998	0.039
6´SL	0.039 -20	y = 4E+06x - 854590	0.998	0.039
DFL	0.078 - 10	y = 1E+06x + 86115	0.999	0.078
LNH	0.156 -20	y = 675039x - 116719	0.999	0.156
LNnH	0.078 - 20	y = 878156x - 86410	0.999	0.078
LST c	0.078 - 20	y = 2E+06x - 393948	0.999	0.078
3´SL	0.078 - 20	y = 3E+06x - 753917	0.998	0.078
LST b	0.156 -20	y = 1E+06x - 186087	0.999	0.156
LST a	0.078 - 20	y = 3E+06x - 590263	0.999	0.078

Table S2.2. Concentrations of human milk oligosaccharides in the individual human milk samples (µg/mL), analyzed by HPAEC-PAD (3FL) and PGC-LC-MS (other HMOs). Mother K117 was assigned to the group Lewis-positive non-Secretor. Mother K143 was assigned to the group Lewis-negative Secretor. All other mothers were assigned to the group Lewis-positive Secretor.

Subject No.	3FL	2'FL	LNT& LNnT	LNFP III	LNFP II	LNFP I	LNFP V	LNH	LNnH	LNDFH I	DFL	6'SL	3'SL	LST c	LST b	LST a	Sum
K016	216.3	389.4	734.0	131.3	410.6	333.3	47.2	17.9	24.3	540.8	63.1	63.6	83.9	126.8	196.9	11.4	3390.6
K023	164.5	436.0	644.3	116.7	299.4	294.6	73.2	99.9	40.9	446.3	438.9	74.8	96.0	119.2	145.4	19.7	3509.7
K026	129.8	523.4	1158.9	133.0	402.7	656.9	48.8	88.0	110.6	646.1	46.6	61.9	97.7	112.7	323.6	29.6	4570.3
K028	123.0	497.6	926.8	192.3	216.8	659.0	42.8	17.2	15.7	469.9	53.9	82.0	137.4	134.2	319.0	21.1	3908.5
K055	179.6	687.5	214.2	69.4	70.0	222.6	0.0	24.9	55.7	268.0	67.5	60.0	125.6	30.4	53.2	13.2	2141.9
K057	140.9	460.0	1363.3	137.4	457.3	684.3	59.3	59.4	40.3	693.4	47.2	154.8	69.7	293.9	392.3	14.6	5067.9
K060	59.7	852.8	408.9	50.7	51.1	661.4	0.0	68.1	78.2	313.9	53.2	78.6	80.2	178.0	69.1	16.6	3020.5
K077	294.8	580.1	722.7	168.6	421.0	338.8	41.7	44.5	56.5	558.9	70.9	35.3	111.4	110.1	162.1	17.7	3735.2
K081	147.0	629.0	907.4	101.7	287.0	509.1	33.6	199.5	201.0	464.1	56.6	87.5	94.7	194.5	153.8	26.7	4093.2
K090	181.8	699.7	484.2	133.1	160.3	411.3	24.5	29.1	22.6	389.9	58.9	113.8	77.3	131.1	107.6	13.2	3038.2
K093	152.7	483.1	1583.7	233.0	562.1	713.9	72.3	47.8	51.1	706.9	63.6	42.8	41.4	214.2	114.2	23.9	5106.8
K102	185.9	508.3	1053.8	148.8	319.1	535.7	42.3	81.8	69.2	684.4	65.7	76.1	116.3	188.5	217.1	24.6	4317.7
K109	146.0	596.5	1510.7	206.8	614.2	759.0	81.9	75.2	50.5	752.0	74.8	93.4	108.7	116.2	357.5	17.0	5560.2
K117	213.6	0.0	1223.5	216.5	831.5	26.2	118.5	80.4	55.4	0	0.3	77.5	87.5	124.0	244.4	19.3	3352.2
K126	158.8	574.2	597.9	98.2	231.5	384.7	27.7	55.8	33.5	543.3	83.3	116.5	99.8	114.4	115.2	26.4	3261.1
K127	65.6	729.7	628.8	103.9	107.8	653.3	0.0	99.3	299.1	365.9	39.8	45.3	128.1	89.4	121.4	15.1	3492.6
K131	95.7	460.4	920.7	88.2	201.9	650.0	30.2	50.8	26.7	473.7	44.3	62.0	93.2	69.9	232.2	30.6	3530.3
K139	98.8	526.2	640.1	116.7	143.6	464.1	19.4	47.2	76.4	328.5	37.6	42.9	63.4	98.8	120.9	15.3	2839.9
K141	93.9	459.2	1141.6	174.2	263.8	720.3	85.5	93.9	36.4	446.0	160.6	45.8	73.3	92.9	289.5	38.6	4215.6
K143	16.7	698.1	707.8	87.3	0.0	981.5	0.0	46.6	42.0	7.4	15.0	76.3	103.4	68.1	142.4	26.9	3019.7

Table S2.3. Concentrations of human milk oligosaccharides in the diluted infant fecal solutions (μg/mL), analyzed by HPAEC-PAD (3FL) and PGC-LC-MS (other HMOs). Mother K117 was assigned to the group Lewis-positive non-Secretor. Mother K143 was assigned to the group Lewis-negative Secretor. All other mothers were assigned to the group Lewis-positive Secretor.

Subject No.	3FL	2'FL	LNT& LNnT	LNFP III	LNFP II	LNFP I	LNFP V	LNH	LNnH	LNDFH I	DFL	6'SL	3'SL	LST c	LST b	LST a	Sum
K016	232.4	166.4	43.3	81.3	279.7	201.8	10.6	0.0	0.0	273.6	44.7	53.3	0.0	116.1	185.5	0.0	1688.7
K023	207.5	194.9	42.0	104.5	308.5	221.1	0.0	0.0	2.4	276.2	270.9	55.2	0.0	112.4	391.0	0.0	2186.4
K026	1.4	1.9	7.0	1.1	2.9	6.2	0.0	0.0	0.0	10.4	0.0	1.2	1.5	1.3	3.2	1.0	39.2
K028	0.1	0.0	2.4	0.6	1.1	1.9	0.0	0.0	0.0	0.9	0.0	1.0	0.0	0.9	2.0	0.0	11.0
K055	146.3	240.3	34.1	58.5	74.0	233.2	0.0	4.9	0.0	225.6	304.5	34.7	5.2	43.8	38.8	4.8	1448.7
K057	1.0	1.6	17.2	0.0	0.0	21.8	0.0	0.0	2.7	150.6	0.2	4.2	0.0	24.7	15.5	0.0	239.4
K060	1.2	3.4	10.6	0.0	0.0	30.6	0.0	0.0	1.7	216.6	2.7	1.0	0.0	1.5	1.7	0.0	271.0
K077	2.2	0.0	10.9	21.0	24.3	0.0	0.0	4.1	5.8	43.5	0.0	1.0	1.5	1.1	4.4	1.0	120.9
K081	135.2	125.6	25.7	65.7	222.0	110.5	0.0	0.0	0.0	227.5	165.2	100.5	0.0	248.5	124.9	0.0	1551.3
K090	0.0	0.8	1.8	0.7	1.1	1.3	0.0	0.0	0.0	1.5	0.0	1.1	0.0	0.9	1.2	1.0	11.4
K093	1.8	3.6	14.6	0.0	0.0	49.1	0.0	0.0	0.0	61.3	0.5	1.1	0.0	1.3	2.2	0.0	135.4
K102	84.7	1.9	75.9	39.3	146.1	67.1	18.3	0.0	0.0	268.9	135.4	0.0	0.0	0.7	1.5	0.0	839.9
K109	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
K117	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
K126	159.7	212.8	113.6	59.4	206.6	233.8	0.0	0.0	0.0	283.7	299.3	5.9	0.0	22.5	139.1	0.0	1736.5
K127	73.1	177.6	184.1	69.6	131.3	389.6	0.0	0.0	0.0	252.4	124.2	0.0	0.0	1.0	3.1	1.2	1407.1
K131	101.4	194.7	15.5	104.7	185.8	77.6	0.0	0.0	0.0	259.1	295.1	3.3	2.3	7.7	123.3	0.0	1370.6
K139	24.7	29.2	29.7	9.2	21.2	112.6	0.0	2.8	7.9	330.4	64.7	1.3	1.8	2.0	3.0	1.1	641.7
K141	91.8	198.2	149.3	65.3	225.0	483.1	0.0	0.0	0.0	329.9	264.7	9.2	4.8	45.8	1182.	15.7	3065.3
K143	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table S2.4. Relative levels of Fuc-epitopes derived from 1D ^{1}H NMR integration of anomeric region sections a – h (**Error! Reference source not found.**)[21] of HMOs isolated from milk and the corresponding fecal samples. Mother K117 was assigned to the group Lewis-positive non-Secretor. Mother K143 was assigned to the group Lewis-negative Secretor. All other mothers were assigned to the group Lewis-positive Secretor.

Subject No.	Human milk						Infant feces					
	Le^a	Le^b	Le^x	Le^y	2′FL	H	Le^a	Le^b	Le^x	Le^y	2′FL	H
K016	0.08	0.20	0.16	0.07	0.34	0.14	0.10	0.24	0.16	0.08	0.29	0.13
K023	0.09	0.17	0.20	0.07	0.36	0.11	0.12	0.20	0.22	0.05	0.29	0.12
K026	0.08	0.19	0.14	0.04	0.36	0.20	0.00	0.00	0.00	0.00	0.00	0.00
K028	0.04	0.16	0.18	0.05	0.36	0.21	0.00	0.00	0.00	0.00	0.00	0.00
K055	0.00	0.10	0.06	0.08	0.65	0.11	0.00	0.12	0.09	0.05	0.61	0.14
K057	0.08	0.22	0.18	0.04	0.26	0.22	0.05	0.50	0.00	0.00	0.00	0.45
K060	0.02	0.06	0.00	0.06	0.67	0.19	0.03	0.62	0.00	0.00	0.00	0.35
K077	0.09	0.18	0.17	0.06	0.39	0.11	0.22	0.28	0.49	0.00	0.00	0.01
K081	0.05	0.16	0.13	0.07	0.41	0.19	0.11	0.23	0.26	0.04	0.16	0.20
K090	0.02	0.14	0.18	0.05	0.47	0.14	0.00	0.00	0.00	0.00	0.00	0.00
K093	0.10	0.20	0.22	0.04	0.25	0.18	0.00	0.14	0.48	0.00	0.00	0.38
K102	0.25	0.15	0.04	0.06	0.39	0.11	0.11	0.44	0.33	0.05	0.00	0.07
K109	0.09	0.19	0.18	0.05	0.31	0.19	0.08	0.37	0.00	0.00	0.06	0.49
K117	0.49	0.00	0.51	0.00	0.00	0.00	0.13	0.00	0.88	0.00	0.00	0.00
K126	0.04	0.17	0.12	0.08	0.44	0.15	0.08	0.19	0.14	0.05	0.41	0.13
K127	0.00	0.10	0.07	0.06	0.57	0.20	0.00	0.16	0.09	0.04	0.33	0.38
K131	0.01	0.17	0.14	0.06	0.35	0.26	0.04	0.17	0.24	0.06	0.31	0.18
K139	0.01	0.13	0.09	0.06	0.51	0.20	0.10	0.69	0.00	0.00	0.06	0.15
K141	0.04	0.15	0.19	0.03	0.34	0.24	0.07	0.15	0.18	0.02	0.17	0.41
K143	0	0	0.03	0.06	0.60	0.31	0	0	0	0	0	1

Table S2.5. Relative compositional changes of the 17 quantitated HMOs between human milk samples and paired infant fecal samples.

Subject No.	3FL	2´FL	LNFPI	LNFPII	LNFPIII	LNFPV	DFL	LNDFHI	LNT+LNnT	LNH	LNnH	3´SL	LSTa	6´SL	LSTc	LSTb
K102	14%	-98%	-36%	14%	4%	13%	100%	11%	-63%	-100%	-100%	-100%	-100%	-100%	-98%	-96%
K127	26%	-40%	7%	30%	10%	0%	100%	11%	-27%	-100%	-100%	-100%	-80%	-100%	-97%	-94%
K131	11%	1%	-69%	8%	13%	-100%	100%	3%	-96%	-100%	-100%	-94%	-100%	-86%	-72%	2%
K081	21%	-47%	-43%	16%	11%	-100%	100%	4%	-93%	-100%	-100%	-100%	-100%	30%	35%	17%
K126	15%	-30%	2%	12%	2%	-100%	100%	-2%	-64%	-100%	-100%	-100%	-100%	-90%	-63%	22%
K141	7%	-41%	-8%	4%	-48%	-100%	27%	0%	-82%	-100%	-100%	-91%	-44%	-72%	-32%	100%
K055	4%	-48%	10%	10%	4%	0%	100%	4%	-76%	-71%	-100%	-94%	-46%	-14%	20%	1%
K016	100%	-14%	19%	32%	21%	-55%	36%	1%	-88%	-100%	-100%	-100%	-100%	59%	72%	77%
K023	31%	-28%	6%	20%	13%	-100%	-1%	-1%	-90%	-100%	-91%	-100%	-100%	6%	15%	100%
K077	-77%	-100%	-100%	28%	100%	-100%	-100%	49%	-53%	65%	76%	-58%	26%	-12%	-69%	-16%
K093	-56%	-72%	70%	-100%	-100%	-100%	-70%	100%	-65%	-100%	-100%	-100%	-100%	-3%	-77%	-27%
K139	2%	-75%	1%	-35%	-65%	-100%	100%	52%	-79%	-74%	-54%	-87%	-68%	-87%	-91%	-89%
K057	-85%	-93%	-33%	-100%	-100%	-100%	-91%	100%	-73%	-100%	12%	-100%	-100%	-43%	22%	-16%
K060	-78%	-96%	-48%	-100%	-100%	0%	-43%	100%	-71%	-100%	-76%	-100%	-100%	-86%	-91%	-73%

Chapter 3

Structure-specific and individual-dependent metabolization of human milk oligosaccharides in infants: a longitudinal birth cohort study

Manuscript submitted for publication:

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ABSTRACT

Human milk contains a unique high level of complex oligosaccharides, named human milk oligosaccharides (HMOs), which are of great importance for growth and health of infants. To follow the dynamics of HMO profiles in human milk, as well as their metabolization by the infant gut microbiome over the course of lactation, mother milk and paired infant feces from 71 mother-infant dyads were collected at week two, week six and week 12 after delivery, and 18 major HMOs were quantitated in each sample. Levels of fucosylated and neutral core HMOs in milk were dependent on mothers' Lewis/Secretor status, whereas sialylated HMO levels were independent, except for sialyl-lacto-*N*-tetraose c. Infant fecal excretion of HMOs gradually declined when babies aged, especially of non-substituted core structures like lacto-*N*-tetraose, lacto-*N*-neotetraose, lacto-*N*-hexaose, lacto-*N*-neohexaose. Although decreasing in absolute concentrations in milk during lactation, the relative abundance of the sum of all fucosylated HMOs increased in both milk and fecal samples. There seemed no differences regarding microbial degradation of (α1-2)-fucosylated HMOs compared to (α1-3/4)-fucosylated HMOs; whereas mono-fucosylated HMOs were more consumed than those decorated with two fucose moieties. The group of (α2-6)-sialylated HMOs accounted for around 90% of sialylated structures in the milk at early lactation, while higher proportion of (α2-3)-sialylated HMOs were present at later lactation stages. Infant gut microbiota showed stronger assimilation abilities on (α2-3)-sialylated HMOs at all three time points. Speed of transitions of HMO metabolization by infant gut microbiota from either a non-specific or a HMO structure-specific consumption stage to the final complete consumption stage was individual-dependent. Where two infants reached the final stage already at two weeks postpartum, almost half of the infants still excreted significant levels of HMOs at 12 weeks of life. Variation was found to be associated with mode and place of delivery, where caesarean section or exposure to hospital/clinic environment at birth seemed to delay the transition to complete HMO consumption.

INTRODUCTION

Human milk oligosaccharides (HMOs) have gained increasing research interest due to their high abundance in human milk (5-15 g/L),[1] as well as the health benefits for infants' immune system,[2] gut microbiota establishment,[3] and cognitive development.[4-5] After ingestion by infants, HMOs are minimally digested in the upper gastrointestinal (GI) tract (< 5%),[6-7] or absorbed through the brush border into the circulation system and excreted into urine (0.5% - 2%).[8] Instead, HMOs are mainly utilized by intestinal bacteria as fermentation substrate, with the remaining HMOs, if any, excreted into the feces.[9-10] Colonization of the infant's intestine by microorganisms initiates at delivery or already *in utero*,[11-12] and follows a stage-specific development.[13] *Bifidobacterium* species are known to be predominant in breastfed infants' colon, partially attributed to their HMO assimilation abilities.[14-15] Despite inter-individual differences, gut microbiota composition progresses towards adult-like patterns in the first three years of life with microbial richness and diversity being highly increased.[13] A healthy gut microbiota in early life is vital for maintaining normal functions of both metabolic- and immune systems, both having a persisting long-term influence.[16] Several factors have been reported as driving force to shape the gut microbiota of infants and these include mode of delivery, antibiotic exposure, premature birth, and feeding method.[17-18] Infants who are exclusively breastfed, formula-fed, or mixed-fed showed different transitions across developmental stages regarding their gut microbiota.[12] HMOs are a group of complex glycans comprising over 240 reported structures, all built from the five monosaccharides glucose, galactose, *N*-acetylglucosamine, fucose and *N*-acetylneuraminic acid.[19-22] The core structures of HMOs start with lactose, extended at the non-reducing end with one or more specific disaccharides, i.e. lacto-*N*-biose or *N*-acetyllactosamine, in a linear or a branched manner.[1] Fucosylated HMOs or sialylated HMOs are formed when fucose (Fuc) or *N*-acetylneuraminic acid (Neu5Ac; also referred to as sialic acid) residues are covalently bound to the core structures, respectively. HMOs can be further grouped depending on number and linkage types of these fucose/sialic acid moieties, e.g., Fuc-(α1-2) or (α1-3) or (α1-4) substituted HMO groups, mono- versus di-fucosylated HMOs, or Neu5Ac(α2-3) versus (α2-6) substituted HMOs. Composition and amounts of HMOs secreted into the milk by women are influenced by maternal genetics and lactation duration. Considerable amount of Fuc-(α1-2) and Fuc-(α1-3/4) structures are secreted by mothers with an active Secretor (Se)- and Lewis (Le)- gene, respectively.[23] When mothers are non-Secretors, or Lewis-negative, Fuc-(α1-2) and Fuc-(α1-3/4) structures are nearly absent in their milks, respectively. HMOs with different structural elements showed distinct temporal changes regarding concentrations in human milk with lactation duration.[24]

Previous studies showed that specific HMO structures undergo different metabolic fates in the GI tract.[25-26] Coincident with the temporal development of infant gut microbiota, metabolization of HMOs follow a three-stage pattern, as proposed by Albrecht et al. based on fecal HMO profiles at several time

points.[27] Infant feces in the first weeks postpartum still contained either neutral HMOs or sialylated ones. This was followed by a transition to HMO-derived metabolites, and finally to the absence of any HMO-related structure with the introduction of solid foods over six months of age.[27] However, contradictory findings or exceptional cases have been reported. Where Chaturvedi et al. found that HMO patterns in infant feces highly resembled that in mother milk[28], Dotz et al. reported on an infant showing higher abundances of fecal neutral HMOs at 7 month postpartum compared to that at 2 months.[29] Furthermore, we recognized three distinct HMO consumption patterns already at 1 month postpartum in another birth cohort which were 1) complete consumption, 2) considerable consumption on specific structures, and 3) low consumption.[26] However, these studies have in common that they were limited by either small sample sizes, single time points, or did not analytically differentiate between structural isomers.

To further investigate HMO metabolization by infant gut microbiota in a longitudinal manner, human milk and paired infant fecal samples from a longitudinal birth cohort study collected at 2-, 6-, and 12-weeks postpartum were analyzed, with 18 major HMOs quantitated. Temporal changes of HMO profiles in human milk were examined, as well as metabolization of different structural HMO groups during the first 12 weeks of life. Progress of HMO consumption patterns were compared among infants, in order to find out possible factors that influence these different transitional stages.

MATERIALS & METHODS

Birth cohort and sample collection

The study design of the BINGO study has been published before,[30-31] where BINGO stands (in Dutch) for *Biological Influences on Baby's Health and Development*. The study recruited participants in the Nijmegen-Arnhem region in the Netherlands with the aim to identify prenatal and early postnatal factors that predict infant health and development. The ethical committee of the Faculty of Social Sciences of the Radboud University approved the study [ECSW2014-1003-189], and informed consent were signed by participants. In general, healthy mothers and infants who were delivered > 37 weeks of gestational age were eligible participants, with more detailed initial and postnatal inclusion criteria described elsewhere.[30-31] The infants included in the current study were born from December 2014 to November 2016. Human milk and infant fecal samples were collected when each infant reached 2, 6 and 12-week of age. Collection of milk samples from the breast into sterile cups was performed by mothers with hand expression before the first morning feeding. Infant feces were collected within 48 hours, being transferred from diapers into sterile stool vials. These samples were temporarily stored in the participants' home freezers until the samples from all three time points had been collected. At the end of this collection period (i.e. after 12 weeks postpartum),

the samples were transferred to a -80 °C laboratory freezer until further HMO analysis. More details about sample collection procedures can be found in a previous article.[32]

In total, 201 human milk samples and 202 infant fecal samples were received from 80 mother-infant dyads. Thirteen fecal samples corresponding to five mother-infant pairs were excluded from further analysis, because the infants were formula-fed at all three time points, without any breast milk samples collected. Six milk samples and 4 fecal samples from 2 pairs were excluded, because the gestational age at birth was not provided. Eighteen fecal samples from 9 infants were excluded due to either unknown feeding type, or mislabeling. The remaining 195 milk samples and 167 fecal samples were analyzed for HMO concentrations.

HMO quantitation

HMO concentrations of the milk and fecal samples were quantitated based on the method as described by Gu et al. with some adaptations.[26] Briefly, milk samples were diluted two-fold, followed by centrifugation at 4 °C (21,000 × g, 20 min) to remove the fat layer. Subsequently, a solid phase extraction (SPE) procedure was applied to extract oligosaccharides, using Supelclean™ ENVI-Carb™ 250 mg/3 mL cartridges (Sigma-Aldrich; St. Louis, MO, USA). Two fractions were lyophilized and rehydrated for further analysis: the fraction containing 3-fucosyllactose (3FL) was analyzed by high performance anion exchange chromatography - pulsed amperometric detection (HPAEC-PAD), whereas the other one containing all other HMOs was analyzed by porous graphitized carbon - liquid chromatography mass spectrometry (PGC-LC-MS). Reduction of oligosaccharides with sodium borohydride overnight, and a following purification step with SPE was performed before loading samples to PGC-LC-MS. Thawed fecal samples were weighed and diluted to 100 mg/mL with Milli-Q™ water, followed by centrifugation. Then an aliquot of 100 µL supernatant was taken directly to reduction and purification steps, and was analysed by PGC-LC-MS.

An ICS 5000 system (Dionex, Sunnyvale, CA) equipped with a CarboPac PA-1 column (250 mm x 2 mm ID), and a CarboPac PA guard column (25 mm x 2 mm ID) was employed for 3FL analysis. The column temperature was maintained at 20 °C, and the flow rate was set as 0.3 mL/min. Injection volume was 10 µL. Mobile phase A was 0.1 M NaOH, and mobile phase B was 1 M NaOAc in 0.1 M NaOH. The elution profile was as follows: 0 – 10 min, 0 – 10% B; 10 – 10.1 min, 10 – 100% B; 10.1 – 15 min, 100% B; 15 – 15.1 min, 100 – 0% B; 15.1 – 30 min, 0% B. The eluted oligosaccharides were monitored by a pulsed amperometric detector (Dionex).

An Accela ultra-high-pressure liquid chromatography system (Thermo Scientific, Waltham, MA, USA) equipped with a Thermo Hypercarb column (100 x 2.1 mm, 3 µm particle size) preceded by a Hypercarb

guard column (10 x 2.1 mm, 3 μm particle size), was used to analyze HMOs present in the reduced samples. Eluent A was 1% (v/v) ACN in water containing 0.1% (v/v) formic acid, eluent B was ACN containing 0.1% (v/v) formic acid. The elution profile was as follows: 0 – 5 min, 3% B; 5 – 22 min, 3 – 20% B; 22 – 32 min, 20 – 40% B; followed by washing with 100% B for 10 min and equilibrating with 3% B for 21 min. Temperature of the column oven and sample tray were set at 25 °C or 10 °C, respectively. Injection volume was 5 μL. The flow rate of the washing step and the first 10 min of equilibration was 300 μl/min, and that of the rest of elution was 200 μl/min. The Velos Pro mass spectrometer (Thermo Scientific) with an electrospray ionization probe was operated in negative-ion mode over a mass-to-charge ratios (*m/z*) range of 300-2000 Da. Chromeleon™ 7.1 (Dionex) and XCalibur 4.3 (Thermo Scientific) were used for processing data obtained from HPAEC-PAD or LC-MS, respectively.

A number of HMOs was identified by comparing retention times and mass-to-charge ratios (*m/z*) to commercial standards, including 3FL, 2′-fucosyllactose (2′FL), lacto-*N*-fucopentaose I (LNFP I), lacto-*N*-fucopentaose II (LNFP II), lacto-*N*-fucopentaose III (LNFP III), lacto-*N*-fucopentaose V (LNFP V), lacto-N-difucohexaose I (LNDFH I), difucosyllactose (DFL), lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), lacto-*N*-hexaose (LNH), lacto-*N*-neohexaose (LNnH), 3′-sialyllactose (3′SL), 6′-sialyllactose (6′SL), sialyl-lacto-*N*-tetraose a (LST a), sialyl-lacto-*N*-tetraose b (LST b), and sialyl-lacto-*N*-tetraose c (LST c). Calibration curves were constructed for each HMO for quantitation, based on integrated peak area from PAD or MS signals. Two structural isomers, LNT and LNnT, were quantitated together because of co-elution. Beyond the abovementioned HMOs, an adjacent peak following LNDFH I with the same *m/z* value was identified as lacto-*N*-difucohexaose II (LNDFH II), consistent with literature.[21] Quantitation of LNDFH II was based on the calibration curve of LNDFH I, by assuming a similar response factor.

Data and statistical analysis

Five infants were delivered under 37 weeks of gestation, so they were considered as preterm infants. Results from these five pairs were exploratorily compared separately from the rest of infants who were delivered full-term. Presence or (near) absence of 2′FL and LNFP I in human milk were used as indicators of a mother's Secretor status, whereas LNFP II acted as indicator of Lewis blood groups.[26] The mothers were assigned to four milk groups based on their milk oligosaccharide profiles: milk group 1, Lewis-positive Secretor (Le+Se+), milk group 2, Lewis-positive non-Secretor (Le+Se-); milk group 3, Lewis-negative Secretor (Le-Se+); milk group 4, Lewis-negative non-Secretor (Le-Se-). In the current study, the total HMOs was defined as the sum of the 18 annotated HMOs. The sum of 2′FL, LNFP I/II/III/V, DFL, LNDFH I/II, and 3FL was defined as total fucosylated HMOs; the sum of LNT, LNnT, LNH, and LNnH was defined as total neutral core HMOs; the sum of 3′SL, 6′SL, LST a/b/c was defined as total sialylated

HMOs. Furthermore, the group of Fuc-(α1-2) structural HMOs included 2´FL, LNFP I, DFL and LNDFH I; Fuc-(α1-3/4) group included 3FL, LNFP II/III/V, and LNDFH II; mono-Fuc included 2´FL, LNFP I/II/III/V, and 3FL; di-Fuc included DFL, and LNDFH I/II. The sialylated Neu5Ac-(α2-3) HMOs contained 3´SL and LST a, whereas Neu5Ac-(α2-6) group included 6´SL and LST b/c. Relative abundances of total fucosylated, neutral core, and total sialylated HMOs were calculated as the concentrations of each group divided by the sum of all individual HMO concentrations. Relative abundances of Fuc-(α1-2), Fuc-(α1-3/4), mono-Fuc, and di-Fuc structures were calculated as the concentration of each group divided by the total of fucosylated HMOs. Relative abundances of Neu5Ac-(α2-3) and Neu5Ac-(α2-6) groups were calculated as the concentration of each group divided by the total of sialylated HMOs.

To study longitudinal changes of HMO profiles in human milk, all milk samples from full-term mother-infant pairs were included for statistical analysis (n = 180). With respect to longitudinal fecal HMO profiles, only fecal samples collected from full-term infants who were breast-fed at that specific time point were included (n = 115). To compare the relative abundances of different structural HMO groups between human milk and infant fecal samples, mother-infant pairs were excluded if total fecal HMO concentrations were less than 1 μg/mg, considering full utilization. Continuous variables, including age postpartum, gestational age, birthweight, HMO absolute concentrations as well as relative abundances in milk and feces, were compared between milk group 1 and milk group 2 with Mann-Whitney tests, or among the three time points within each milk group with Kruskal-Wallis with *post hoc* stepwise multiple comparisons. Pearson's chi-squared test was used to compare categorical variables, i.e. gender, delivery mode, and delivery place, between the milk groups. The calculation of averages, standard deviations, percentages, and abovementioned comparisons were performed with SPSS Statistics Version 26 (IBM Corp., Armonk, NY, USA). The Spearman rank-order correlation coefficients with associated *p*-values between individual HMOs in human milk samples were calculated by using scipy.stats.spearmanr, then visualized with heatmap using matplotlib.pyplolt in Python (Version 3.7.7). Hierarchical cluster analysis (HCA) was performed for milk group 1 and 2 separately, based on relative compositional changes of each quantitated HMO between milk and paired fecal samples at all the three time points, with only full-term mother-infant pairs that had full sets of samples included. Initial relative compositional changes were calculated by subtracting relative abundance of individual HMO in milk from corresponding relative abundance in paired feces, then divided by the former value. All the positive values were normalized to 0 – 100%, by defining the initial maximum positive values in each sample as 100%. When total HMO concentrations in a fecal sample was below 1 μg/mg, it was considered as complete degradation, and the relative composition changes of all individual HMO structures were designated as -100%. The relative compositional changes were visualized as heatmaps, using color scales function in Microsoft Excel 2019. Influence of maternal-infant variables on relative HMO compositional changes at each time point and overall were evaluated by

Multiple Response Permutation Procedures (MRPP). HCA and MRPP were conducted in R (Version 3.4.0), using factoextra and vegan packages, respectively. Results were considered as statistically significant when *p*-values were less than 0.05, or highly significant when less than 0.001.

RESULTS

Maternal and infant characteristics

Among the 68 full-term pairs, human milk samples from 51 mothers (75%) contained considerable concentration of 2´FL, LNFP I and LNFP II, indicating active Lewis (Le)- and Secretor (Se)- genes, therefore assigned to the milk group 1 (Le+Se+). The peaks of 2´FL and LNFP I in milks of the remaining 17 full-term pairs (25%) were all found to be (nearly) absent, while LNFP II was present in considerable levels, based on which these pairs were assigned to milk group 2 (Le+Se-). Mothers with genotype Se+Le- or Se-Le- were absent among the full-term pairs. As to the preterm pairs, human milk samples of three pairs were assigned to milk group 1, that of one pair to milk group 2, and one pair to milk group 3, with the latter two pairs excluded from further analysis due to limited sample sizes. Maternal and infant characteristics of the full-term pairs and milk group 1 from preterm pairs were summarized and compared in Table 3.1. There are no significant differences between the two milk groups within full-term pairs, regarding sample collection timepoints, gestational age at birth, birthweight, gender, and delivery mode. However, significantly fewer mothers from milk group 2 (6%) delivered at home instead of clinic/hospital, when compared to that from milk group 1 (33%). The difference in delivery place should be taken into consideration when comparing HMO profiles between the two milk groups, as the exposure to hospital/clinic surroundings could influence the initial acquisition of infant gut microbiota.[11] The three preterm infants are all girls who were vaginally delivered in hospital, with normal birthweight (3212 g on average), though a bit lower than the full-term ones from the same milk group (3591 g on average).

Longitudinal changes of HMO in human milk

A total of 18 major HMOs were quantitated in 180 full-term human milk samples, results of which are summarized in Table 3.2. Based on their averages, the most abundant HMOs in milk group 1 were 2´FL and LN(n)T, followed by LNFP I and LNDFH I at week 2; the concentration of 3FL increased to be the second most abundant after 2´FL at week 12, while the other abovementioned HMOs dropped in concentrations. In milk group 2 samples, LN(n)T, 3FL and LNFP II were the most predominant HMOs at all three time points, but HMOs containing (α1-2)-linked fucose residues were hardly detected. All fucosylated HMOs, except for LNFP III at week 12, showed highly significant differences in concentrations

between milk samples from group 1 and group 2 at all three time points. In general, (α1-2)-linked fucosylated HMOs with or without other fucose residues were present in higher concentrations in milk group 1, whereas HMOs containing only (α1-3)- and/or (α1-4)-linked fucose residues were higher in milk group 2. As to the neutral core structures, milk group 1 contained less LN(n)T at week 2 and week 12, but more LNH at week 2, and more LNnH along the first 12 weeks compared to milk group 2. No differences were found in sialylated HMOs between the two milk groups except for LST c, which was naturally more abundant in milk group 1 at all the time points. Despite lacking (α1-2)-linked structures, total HMO concentrations secreted by milk group 2 mothers was found to be lower than milk group 1 only at week 2 (5.9 g/L versus 6.7 g/L, respectively), while becoming comparable at later time points, though both groups showed decreases with longer lactation duration. This longitudinal reduction in concentrations was found for most individual HMOs, with a few exceptions: DFL remained stable over lactation in milk group 1; LNFP V, LNDFH II, and 3´SL were almost unchanged over time in both milk groups; 3FL levels increased over time in both milk groups yet the increase in milk group 2 was not significant (p = 0.065); LNFP II reduced significantly in milk group 2 samples, but remained constant in group 1, while LNFP III showed exactly the opposite results. It should be noted that these observations over time were based on concentrations in human milk, instead of total volume ingested by infants at that time, which would most likely lead to different conclusions on total intake, considering the increasing milk consumption when babies grew up. Borewicz et al. suggested a rather stable amount of HMO intake during the first 12 weeks postpartum, with an estimated changing daily intake of human milk over time.[32]

Table 3.1. Characteristics of the full-term study subjects from milk group 1 (Le+Se+) and milk group 2 (Le+Se-), and preterm study subjects from milk group 1. Data are presented in means (standard deviations, SD), or number of subjects (percentages).

Variables	**Full-term**			**Preterm**
	Milk group 1 (n = 51)	Milk group 2 (n = 17)	p-value*	Milk group 1 (n = 3)
Age postpartum, day, mean (SD)				
Week 2	15 (1) §	15 (2) ‡	0.169	11 (4)
Week 6	43 (1) §	43 (2) #	0.557	41 (1)
Week 12	85 (5) §	85 (3) §	0.716	84 (1)
Gestational age, week, mean (SD)	40.1 (1)	40.0 (1) #	0.542	36.6 (0.2)
Birthweight, g, mean (SD)	3591 (400)	3580 (456) #	0.652	3212 (99)
Gender, male, n (%)	26 (53) #	10 (59)	0.651	0 (0)
Delivery mode, vaginal, n (%)	46 (90)	15 (88)	0.818	3 (0)
Delivery place, home, n (%) †	17 (33)	1 (6) §	**0.024**	0 (0)

* Difference between milk group 1 and milk group 2 full-term subjects.
§ One case with missing value.
# Two cases with missing values.
‡ Three cases with missing values.
† Other places, including hospital and Clinic, are considered as *Hospital/Clinic*.

Table 3.2. Concentrations of 18 human milk oligosaccharides (g/L) in full-term human milk at three lactation time points. The results are shown as means (standard deviations). [#]

Compound	WEEK 2			WEEK 6			WEEK 12			*p*-value‡	
	Group 1 (n = 48)	Group 2 (n = 16)	*p*-value†	Group 1 (n = 48)	Group 2 (n = 13)	*p*-value†	Group 1 (n = 42)	Group 2 (n = 13)	*p*-value†	Group 1	Group 2
Fucosylated											
2′FL*	1.22 [b] (0.36)	0 (0)	**< 0.001**	1.03 [a] (0.30)	0 (0)	**< 0.001**	0.91 [a] (0.26)	0 (0)	**< 0.001**	**< 0.001**	
LNFP I*	0.99 [c] (0.42)	0.01 (0.01)	**< 0.001**	0.59 [b] (0.29)	0.01 (0.01)	**< 0.001**	0.36 [a] (0.23)	0.01 (0.01)	**< 0.001**	**< 0.001**	
DFL*	0.10 (0.06)	0 (0)	**< 0.001**	0.09 (0.05)	0 (0)	**< 0.001**	0.11 (0.05)	0 (0)	**< 0.001**	0.135	
LNDFH I*	0.93 [c] (0.27)	0.05 (0.02)	**< 0.001**	0.78 [b] (0.24)	0.05 (0.01)	**< 0.001**	0.65 [a] (0.21)	0.06 (0.01)	**< 0.001**	**< 0.001**	
3FL	0.33 [a] (0.21)	1.14 (0.40)	**< 0.001**	0.55 [b] (0.30)	1.39 (0.52)	**< 0.001**	0.88 [c] (0.50)	1.59 (0.50)	**< 0.001**	**< 0.001**	0.065
LNFP II	0.30 (0.24)	1.12 [b] (0.37)	**< 0.001**	0.29 (0.17)	0.93 [a, b] (0.31)	**< 0.001**	0.27 (0.15)	0.77 [a] (0.22)	**< 0.001**	0.939	**0.023**
LNFP III	0.31 [a] (0.10)	0.46 (0.09)	**< 0.001**	0.36 [b] (0.10)	0.47 (0.09)	**0.002**	0.43 [b] (0.16)	0.46 (0.09)	0.215	**< 0.001**	0.998
LNFP V	0.03 (0.02)	0.13 (0.04)	**< 0.001**	0.03 (0.02)	0.12 (0.04)	**< 0.001**	0.03 (0.02)	0.11 (0.03)	**< 0.001**	0.446	0.124
LNDFH II	0.03 (0.07)	0.22 (0.11)	**< 0.001**	0.03 (0.02)	0.20 (0.12)	**< 0.001**	0.03 (0.02)	0.15 (0.07)	**< 0.001**	0.095	0.316
Neutral core											
LNT + LNnT	1.22 [c] (0.50)	1.64 [b] (0.55)	**0.009**	0.99 [b] (0.39)	1.24 [b] (0.45)	0.054	0.74 [a] (0.32)	0.93 [a] (0.31)	**0.023**	**< 0.001**	**0.002**
LNH	0.08 (0.04)	0.06 [b] (0.04)	**0.091**	0.05 (0.05)	0.04 [b] (0.03)	0.221	0.02 (0.03)	0.02 [a] (0.02)	0.213	**< 0.001**	**< 0.001**
LNnH	0.06 [b] (0.04)	0.03 (0.02)	**< 0.001**	0.06 [b] (0.01)	0.02 (0.02)	**< 0.001**	0.04 [a] (0.01)	0.01 (0.01)	**0.001**	**0.01**	0.144
Sialylated											
3′SL	0.18 (0.04)	0.18 (0.39)	0.379	0.17 (0.04)	0.18 (0.05)	0.658	0.16 (0.05)	0.17 (0.04)	0.735	0.511	0.564
LST a	0.04 [c] (0.02)	0.04 [b] (0.02)	0.752	0.02 [b] (0.01)	0.02 [a] (0.01)	0.601	0.01 [a] (0.01)	0.01 [a] (0.01)	0.066	**< 0.001**	**< 0.001**
6′SL	0.36 [c] (0.10)	0.37 [c] (0.17)	0.313	0.17 [b] (0.06)	0.18 [b] (0.12)	0.345	0.07 [a] (0.04)	0.07 [a] (0.03)	0.548	**< 0.001**	**< 0.001**
LST c	0.31 [c] (0.14)	0.19 [c] (0.10)	**0.001**	0.10 [b] (0.04)	0.07 [b] (0.03)	**0.007**	0.05 [a] (0.03)	0.02 [a] (0.01)	**< 0.001**	**< 0.001**	**< 0.001**
LST b	0.21 [b] (0.08)	0.26 (0.12)	0.113	0.20 [b] (0.12)	0.24 (0.12)	0.192	0.15 [a] (0.07)	0.17 (0.07)	0.122	**< 0.001**	0.110
Total	6.70 [c] (0.97)	5.90 [b] (1.12)	**0.003**	5.50 [b] (1.01)	5.15 [a, b] (0.96)	0.271	4.91 [a] (0.98)	4.56 [a] (0.67)	0.294	**< 0.001**	**0.003**

Values with different superscript letters were significantly different from each other in the same milk group over time, with their averages ranked from lower to higher following the order a to c.
† Difference between milk group 1 and milk group 2 at the same time point.
‡ Difference among the three time points within the same milk group.
*Comparison for the milk group 2 was not performed due to (near) absence in the samples.

To further investigate the relations between individual HMOs, correlations were calculated between each structure, involving all the 180 milk samples, with significant results visualized as a heatmap in Figure 3.1. The heatmap clearly divided fucosylated HMOs into two groups, one group containing (α1-2)-linked fucose (2´FL, LNFP I, DFL and LNDFH I), the other devoid of (α1-2)-linked fucose (3FL, LNFP II/III/V and LNDFH II). HMOs were positively correlated with each other within the same group (r = 0.42 – 0.93), whereas negatively correlated with those from the other group (r = -0.17 – -0.86). LN(n)T showed weak negative correlation with (α1-2)-fucosylated group (r = -0.30), and positive correlation with (α1-3/4)-fucosylated group (r = 0.29 – 0.54); LNH and LNnH showed the opposite correlations with the two fucosylated groups (r = 0.23-0.61, or -0.17 – -0.64, respectively), although also belonging to the same neutral core group as LN(n)T. These observations were consistent with their different concentrations detected in milk samples from milk group 1 and milk group 2. Hardly any strong correlations appeared for sialylated HMOs and fucosylated HMOs, except for LSTc, which was positively correlated with LNFP I (r = 0.57) and negatively correlated with 3FL (r = -0.55), partly due to its higher abundance in milk group 2 milks. Except 3´SL, which was found to be independent from all other HMOs, other sialylated structures were in general positively correlated with core HMOs and other sialylated HMOs, with the strongest correlations seen among 6´SL, LST a and LST c (r = 0.70 – 0.88), and between LST b and LN(n)T (r = 0.74).

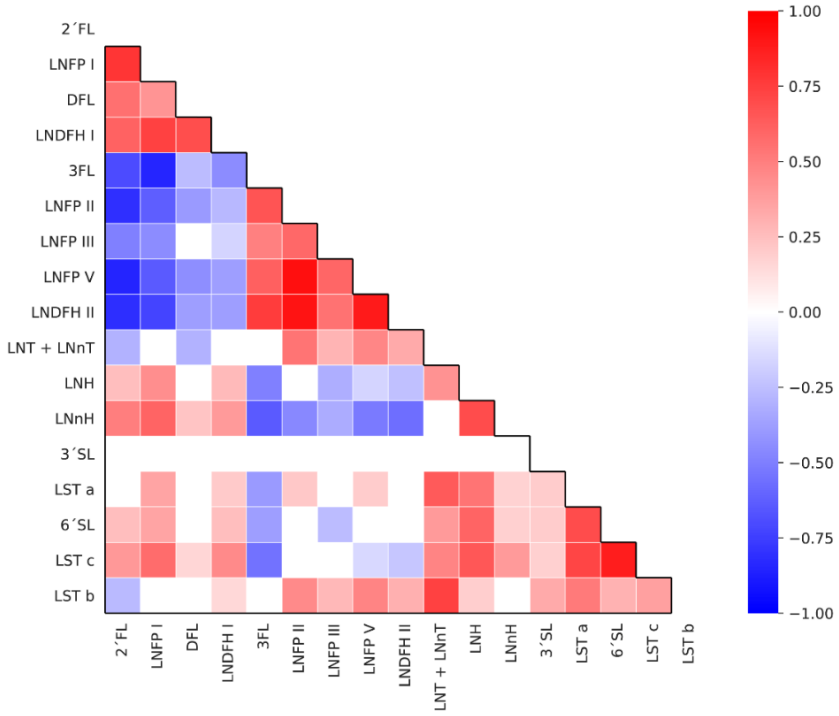

Figure 3.1. Spearman correlation heatmap between individual HMOs in milk samples from mothers of full-term delivered infants during the first 12 weeks, including both milk groups at all time points. Color represents correlation from -1 (blue) to +1 (red) for cells with significant levels ($p < 0.05$), or white if not passing the significance threshold.

The 18 annotated HMOs were further grouped depending on their common structural elements, i.e. fucosylated group, neutral core group, sialylated group, (α1-2)-fucosylated group, (α1-3/4)-fucosylated group, mono-fucosylated group, di-fucosylated group, (α2-3)-sialylated group, and (α2-6)-sialylated group, with the sum of each structural group compared between the two milk groups at three time points (Figure 3.2). Milk group 1 contained higher total fucosylated HMOs at week 2 and week 12 than milk group 2, while milk group 2 contained higher total neutral core structures at week 2 than milk group 1, but comparable at other time points. Total concentrations of sialylated HMOs were not influenced by Secretor status, despite the higher abundance of LST c in milk group 1 (Table 3.2). Total core and total sialylated HMOs decreased with lactation time for both milk groups. Meanwhile, total fucosylated HMOs only reduced during week 2-6 for milk group 1, and remained unchanged from week 6 to week 12, or kept constant at all time points for milk group 2. Furthermore, the sums of (α1-3/4)-fucosylated HMOs, mono-fucosylated HMOs, and di-fucosylated HMOs for milk group 2 all remained comparable over the course of lactation. Unlike milk group 2, (α1-2)-fucosylated HMOs decreased while (α1-3/4)-fucosylated HMOs increased from week 2 to week 12; mono-and di-fucosylated HMOs both dropped from week 2 to week 6, but remained unchanged at the later time point. The two milk groups showed quite different distribution of fucosyl linkage types; nevertheless, no differences were found in their total mono-fucosylated HMO levels. As to di-fucosylated HMOs, a higher level was found in milk group 1 compared to the other milk group. Concentrations of (α2-3)- and (α2-6)-sialylated HMOs were comparable between milk groups, indicating their independence from secretor status of mothers. Albeit present in higher concentrations, (α2-6)-sialylated HMOs decreased from week 2 to week 12 in both milk groups, whereas (α2-3)-sialylated HMO levels remained stable in milk group 2, and dropped only from week 2 to week 6 in milk group 1.

Longitudinal changes of HMO in infant feces

HMO quantitation results of 115 infant fecal samples, which were collected from full-term infants at breast-fed time points, were included to investigate longitudinal changes of fecal HMO excretion, with results summarized in Table 3.3. It should be noted that concentrations values given here were based on wet weight of feces, which were influenced by varied water contents of these feces. Only one infant (B119) was reported to have diarrhea problem at 6 weeks postpartum, while the rest of fecal samples were collected under healthy conditions. Among infants who were fed with Secretor milk (milk group 1), the most abundant HMOs in milk, 2´FL, LN(n)T, LNFP I and LNDFH I, also accounted for the major part of HMOs present in their feces at week 2, with LNDFH I present in much higher concentration than the others. At later time points, fecal concentrations of 3FL, LNFP II/III and LST b were relatively higher, whereas LN(n)T and LNFP I levels dropped most significantly, meanwhile LNDFH I level remained to be

predominant. Infants who received non-Secretor milk (milk group 2) showed high concentrations of 3FL in their feces at all three time points, followed by LNFP II. These two HMOs were also most abundant in the corresponding milks. Another high-level HMO in the non-Secretor milk, LN(n)T, was present as the third richest fecal HMO at week 2 (6.48 µg/mg); however, its average concentration strongly reduced to 0.84 µg/mg at week 6, and to trace amounts (0.06 µg/mg) at week 12. Three other HMOs, LNFP II, LNFP III, LNDFH II and LST b, became the most concentrated ones at week 6 and week 12. In general, infant fecal HMO concentrations displayed larger inter-individual variations compared to that of human milk samples. A regular pattern was observed that included a gradual decrease in HMO concentrations excreted into the feces, especially of neutral core structures (LNT, LNnT, LNH, LNnH), despite their high concentrations in milk. The lower fecal HMO excretion reflects the development of infant gut microbiota with an increased colonization of HMO-consuming bacteria.[32] It is noted that the relative level of 3FL in milk group 1 feces increased slightly, although not significantly, from week 2 to week 12. This even may indicate some microbial degradation of 3FL, since one should also consider the rising amount of milk 3FL ingested by the nursing infants.

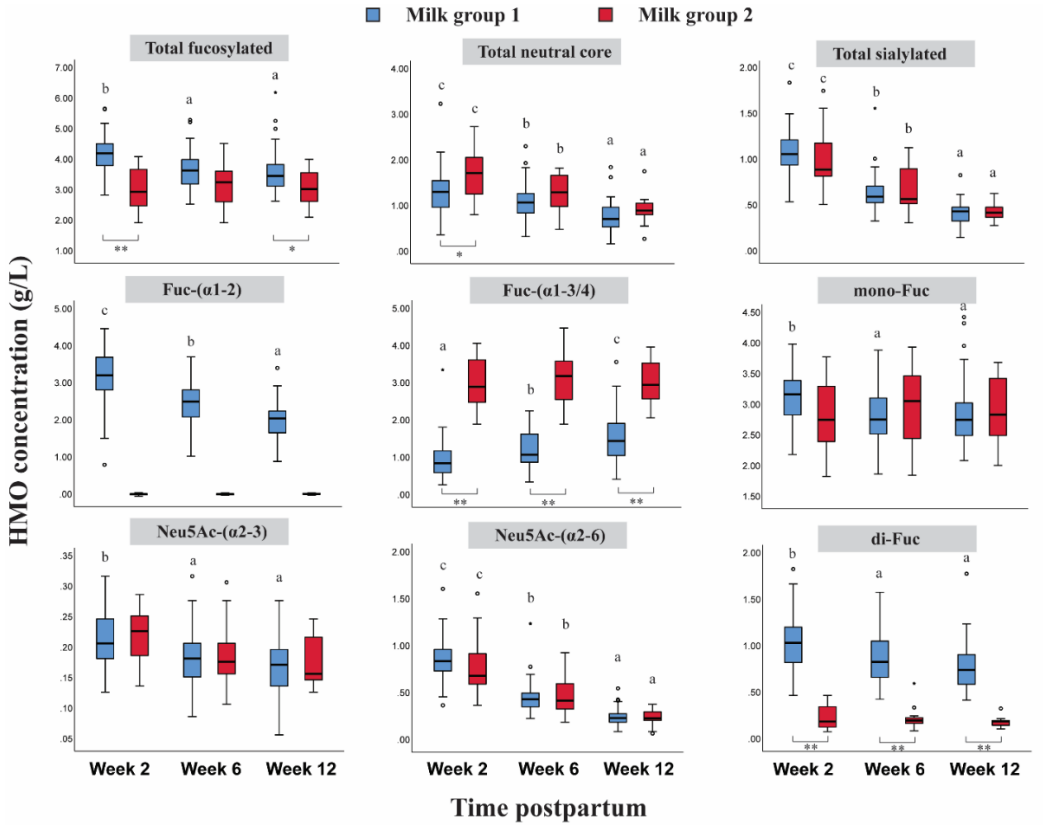

Figure 3.2. Concentrations of different HMO structural groups in milk samples from mothers of full-term delivered infants at three time points from milk group 1 (Le+Se+, blue) or milk group 2 (Le+Se-, red). Box-whisker plots represent minimum, first quartile, median, third quartile, maximum and outlier. Asterisks below the bars indicate significant differences (* $p < 0.05$, ** $p < 0.001$) between the milk groups at single time point. Different lower-case letters above the bars indicate significant differences ($p < 0.05$) from each other time point within the same milk group.

Table 3.1. Concentrations of 18 human milk oligosaccharides (µg/mg) in fecal samples from breastfed and full-term delivered infants. The results are shown as means (standard deviations). [#]

Compound	WEEK 2			WEEK 6			WEEK 12			*p*-value‡	
	Group 1 (n = 33)	**Group 2 (n = 8)**	***p*-value†**	**Group 1 (n = 31)**	**Group 2 (n = 7)**	***p*-value†**	**Group 1 (n = 29)**	**Group 2 (n = 7)**	***p*-value†**	**Group 1**	**Group 2**
Fucosylated											
2′FL*	3.09 (3.33)	0.01 (0.01)	**0.002**	2.65 (4.08)	0 (0)	**0.010**	2.14 (3.64)	0 (0)	**0.026**	0.146	
LNFP I*	3.73 [c] (3.47)	0.05 (0.04)	**0.002**	2.12 [b] (2.64)	0.01 (0.01)	**0.002**	0.69 [a] (1.22)	0.01 (0.01)	0.151	**< 0.001**	
DFL*	0.51 (0.43)	0 (0.01)	**< 0.001**	0.45 (0.66)	0 (0)	**< 0.001**	0.44 (0.77)	0 (0)	**0.001**	0.115	
LNDFH I*	9.77 [c] (5.68)	0.70 (0.44)	**< 0.001**	8.28 [b] (7.63)	0.57 (0.37)	**0.009**	4.05 [a] (6.16)	0.48 (0.83)	0.239	**0.001**	0.192
3FL	1.49 (1.42)	7.49 (3.86)	**< 0.001**	2.62 (3.07)	9.18 (7.53)	**0.019**	3.08 (4.65)	6.08 (10.0)	0.427	0.470	0.332
LNFP II	1.60 [b] (1.74)	7.35 (4.38)	**0.001**	1.91 [b] (2.66)	6.23 (4.25)	**0.010**	1.08 [a] (1.81)	2.25 (2.83)	0.650	**0.014**	0.059
LNFP III	1.48 [b] (1.24)	3.99 (2.80)	**0.007**	1.34 [a, b] (1.63)	2.98 (2.07)	0.061	1.26 [a] (2.19)	1.27 (2.11)	0.914	**0.047**	0.067
LNFP V	0.07 (0.11)	0.49 [b] (0.45)	**0.002**	0.07 (0.13)	0.20 [a, b] (0.25)	**0.028**	0.02 (0.05)	0.06 [a] (0.13)	0.520	0.150	**0.012**
LNDFH II	0.15 (0.12)	2.16 (1.80)	**< 0.001**	0.20 (0.27)	2.09 (2.43)	**0.002**	0.13 (0.20)	1.27 (1.68)	0.442	0.274	0.509
Neutral core											
LNT + LNnT	2.90 [b] (4.48)	6.48 [b] (6.60)	0.063	1.87 [b] (3.03)	0.84 [b] (0.83)	0.610	0.25 [a] (0.57)	0.06 [a] (0.09)	0.897	**< 0.001**	**0.002**
LNH	0.26 [b] (0.79)	0.34 [b] (0.51)	0.123	0.07 [b] (0.13)	0.06 [b] (0.07)	0.493	0.01 [a] (0.01)	0 [a] (0.01)	0.685	**0.001**	**0.020**
LNnH	0.11 [b] (0.21)	0.10 [b] (0.21)	0.725	0.09 [a, b] (0.17)	0 [a, b] (0.01)	0.145	0.01 [a] (0.03)	0 [a] (0)	0.196	**0.001**	**0.018**
Sialylated											
3′SL	0.69 [b] (1.03)	0.23 [b] (0.43)	0.304	0.40 [a, b] (0.69)	0 [a] (0)	**0.033**	0.26 [a] (0.64)	0 [a] (0)	0.358	**0.020**	**0.015**
LST a	0.14 [b] (0.17)	0.08 [b] (0.11)	0.440	0.05 [a] (0.08)	0 [a] (0)	**0.033**	0.03 [a] (0.05)	0 [a] (0.01)	0.371	**0.001**	**0.013**
6′SL	2.15 [c] (2.39)	1.17 (1.53)	0.161	1.09 [b] (1.54)	0.01 (0.01)	0.057	0.34 [a] (0.82)	0.26 (0.43)	0.873	**< 0.001**	0.256
LST c	1.60 [c] (1.34)	1.00 [b] (1.18)	0.211	0.75 [b] (1.13)	0 [a] (0)	**0.005**	0.29 [a] (0.58)	0.14 [a, b] (0.29)	0.981	**< 0.001**	**0.014**
LST b	1.97 [b] (2.80)	2.38 (2.13)	0.308	2.07 [a, b] (4.88)	0.59 (0.94)	0.676	0.67 [a] (1.14)	0.53 (0.85)	0.917	**0.005**	0.083
Total	31.7 [b] (16.1)	34.0 [b] (14.0)	0.818	26.0 [b] (23.3)	22.7 [a, b] (14.4)	0.985	14.7 [a] (20.0)	12.4 [a] (17.4)	0.484	**0.003**	**0.041**

Values with different superscript letters are significantly different from each other in the same milk group over time, with their averages ranked from lower to higher following the order a to c.

† Difference between milk group 1 and milk group 2 at the same time point.

‡ Difference among the three time points within the same milk group.

*Comparison for the milk group 2 was not performed due to (near) absence in the milk.

Fecal concentrations of different HMO structural groups are shown in Supplementary Figure S3.1. Despite high variations, total fucosylated HMOs were relatively more enriched, and present in almost double the concentration of neutral core and sialylated HMOs. Furthermore, the longitudinal reduction in neutral core and sialylated HMOs was much more than that found for total fucosylated HMOs.

Longitudinal changes of HMO metabolization

To investigate metabolic fates of HMOs within the infant gut, we first compared the relative abundance of each structural group between human milk and infant feces at different time points (Figure 3.3). Inclusion criteria of data in Figure 3.3 were: full-term delivery, milk group 1, exclusive breastfeeding, total HMO concentrations higher than 1 μg/mg in infant feces. Although the total level of fucosylated HMOs in milk decreased in absolute concentrations from week 2 to week 6, its relative abundance slightly rose up over lactation (from 65% to 76%). Both the absolute concentrations and relative abundances of the total neutral core HMOs (from 19% to 16%) and total sialylated HMOs (from 16% to 9%) in human milk decreased from week 2 to week 12. Similar trends in their relative abundances were observed in infant feces, where the total fucosylated structures accounted for the largest proportion at all three time points, and increased from 71% at week 2 to 86% at week 12. Also, total sialylated HMOs and total neutral core HMOs dropped from 20% to 11%, and from 9% to 3%, respectively, over lactation. Although the core structures accounted for a higher proportion in milk than sialylated HMOs, their relative abundances were the opposite in feces. When comparing the distribution of the abovementioned three structural groups, fecal HMO profiles showed relatively more fucosylated structures, less neutral core, and comparable level of sialylated HMOs than milk at all the time points. This difference implies a general utilization preference by infant gut microbiota, with neutral core HMOs being the most quickly consumed, followed by sialylated HMOs, and fucosylated HMOs being the least consumed during the first 12 weeks of life. Figure 3.3 (B) shows a rather comparable distribution for (α1-2)- and (α1-3/4)-fucosylated HMOs between milk and feces for all time points, which indicates no preference in microbial consumption of these two structures in infants fed with Secretor milk. However, the proportion of (α1-2)-fucosylated HMOs was much higher than that of (α1-3/4)-fucosylated HMOs at week 2 (~80% vs. 20%); although the abundance of these two groups became similar at week 12. The longitudinal distribution of mono-fucosylated and di-fucosylated HMOs remained relatively stable in both milk samples (75-79% vs. 25-21%) and fecal samples (48-65% vs. 52-35%), as can be seen in Figure 3.3 (C); however, the lower abundance of mono-fucosylated HMOs in feces compared to milk indicates that this structural group was more prone to microbial degradation than di-fucosylated structures. Within the sialylated HMOs (Figure 3.3 D), (α2-3)-sialylated structures accounted for only 21% of all sialylated HMOs in milk at week 2, while their proportion increased with prolonged lactation to 39%

at week 12. Interestingly, their distribution in fecal samples remained unchanged during all 12 weeks, with around 90% being (α2-6)-sialylated group, which might be attributed to a growing assimilating ability of gut microbes towards (α2-3)-sialylated HMOs with longer duration of breastfeeding.

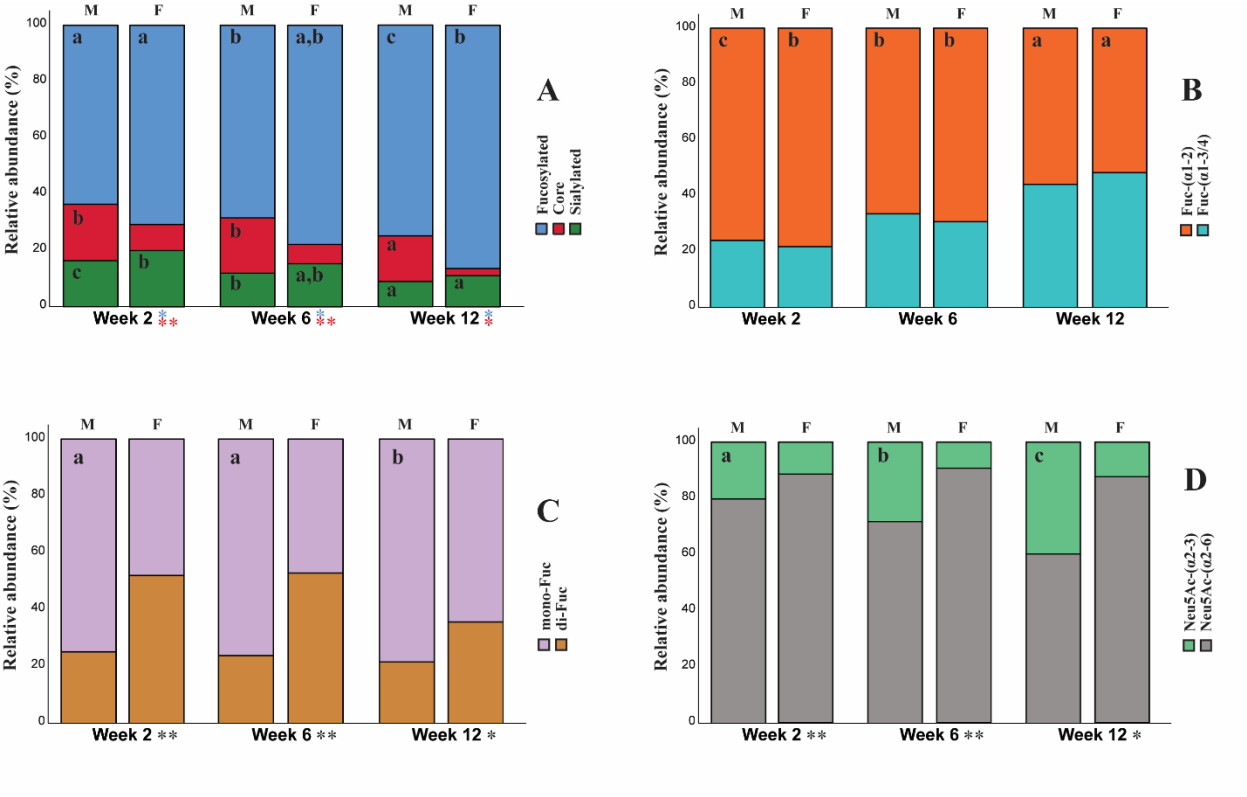

Figure 3.3. Relative abundances of different HMO structural groups (A, total fucosylated, total core, and total sialylated HMO structures; B, (α1-2)-fucosylated, and (α1-3/4)-fucosylated HMO structures; C, mono-fucosylated, and di-fucosylated HMO structures; D, (α2-3)-sialylated, and (α2-6)-sialylated HMO structures in human milk (M) and in paired breastfed infant feces (F), in which fecal HMOs were not completely utilized at week 2 (n = 30), week 6 (n = 26), week 12 (n = 13). All subjects belong to full-term and milk group 1. Colored asterisks in (A) below the bars indicate significant differences (* $p < 0.05$, ** $p < 0.001$) for the structural groups of the same color, between milk and feces at single time point; while the black asterisks indicate significant differences for both structural groups in (B), (C) and (D). Different lower-case letters in the bars indicate significant differences ($p < 0.05$) time points within the same type of samples (milk, or feces), with each structural group annotated separately in (A), but both groups annotated by one letter in (B), (C) and (D).

Besides the general trend in metabolization of different HMO groups, we also investigated the temporal HMO consumption pattern of individual infants, with results visualized in Figure 3.4. Only mother-infant pairs that provided milk and fecal samples from all the three time points were included in the evaluation, with milk group 1 and milk group 2 clustered separately. The milk group 1 was divided over two main clusters (Figure 3.4 A), with cluster A1 not reaching the complete HMO consumption stage in

the first 12 weeks of life, while cluster A2 showed complete consumption at week 12 the latest. The cluster A1 was further divided into three sub-clusters. Cluster A1-1 (B071, B022, B031, B157 and B122) showed growing consumption ability towards sialylated HMOs from week 2 to week 12, while the fucosylated HMOs remained the most abundant in feces. Cluster A1-2 (B020, B015, B021 and B018) showed especially utilization of the neutral core structures, the degree of which enhanced with time, meanwhile fucosylated and sialylated HMOs were predominantly excreted in feces. Sub-cluster A1-3 (B140 and B101) showed a preference for the consumption of fucosylated HMOs at week 2, but switched to utilization of neutral core structures at later time points. Conversely, B039 from the same sub-cluster A1-3 showed mainly consumption of sialylated HMOs at week 6, and changed to a fucosylated-HMO consumption pattern at week 12. The other major cluster A2 also contained three sub-clusters. In general, sub-cluster A2-1 (B117, B115, B119, B118) showed utilization of neutral core structures, 2′FL, LNFP I, 3FL, and LNFP V at week 2, with 3′SL and LST a started to be consumed as well from week 6, finally all HMOs were consumed at week 12. In contrast, sub-cluster A2-2 (B045, B013, B113, B146, B048) showed a stronger ability to degrade sialylated HMOs than fucosylated HMOs, especially those containing (α1-3/4)-fucose moieties and at an early lactation stage. Both sub-clusters reached complete consumption stage later than six weeks postpartum. Two subjects from sub-cluster A2-3 (B104, B08, B091, B007, B130, B069) showed empty fecal HMO profiles already at two weeks postpartum, whereas the other four subjects from the same sub-cluster reached the final stage between week 2 and week 6. The relative HMO compositional changes of subjects from milk group 2 and preterm group are shown in Figure 3.4 (B) and (C), respectively. Inter-individual differences were also observed in these two groups. One subject from milk group 2 and one from preterm group reached the complete consumption stage between week 2 and week 6; B067 from milk group 2 could utilize all HMOs at week 12. All other samples from these two groups were not at the final stage of complete consumption at 12 weeks. All subjects from milk group 2 and the preterm group were delivered vaginally at hospital/clinic, except one that was delivered via caesarean section. There were still HMOs left in the B050 fecal samples at week 12, especially sialylated structures, 6′SL, LST c and LST b. Due to limited subject numbers, no clear differences were observed between mother-infant pairs from milk group 1 and milk group 2, or between term and preterm infants.

When considering the compositional changes of individual HMOs, LNT, LNnT, LNH and LNnH decreased the most at all the three time points, followed by 2′FL, LNFP I, LNFP V, 3′SL and LST a. The HMOs that showed the strongest increase in relative compositional levels were LST b and LNDFH I and LNDFH II. This observation was consistent with our deduction on preferred degradation of different structural groups, that neutral core, mono-fucosylated, and (α2-3)-sialylated HMOs were more prone to microbial consumption.

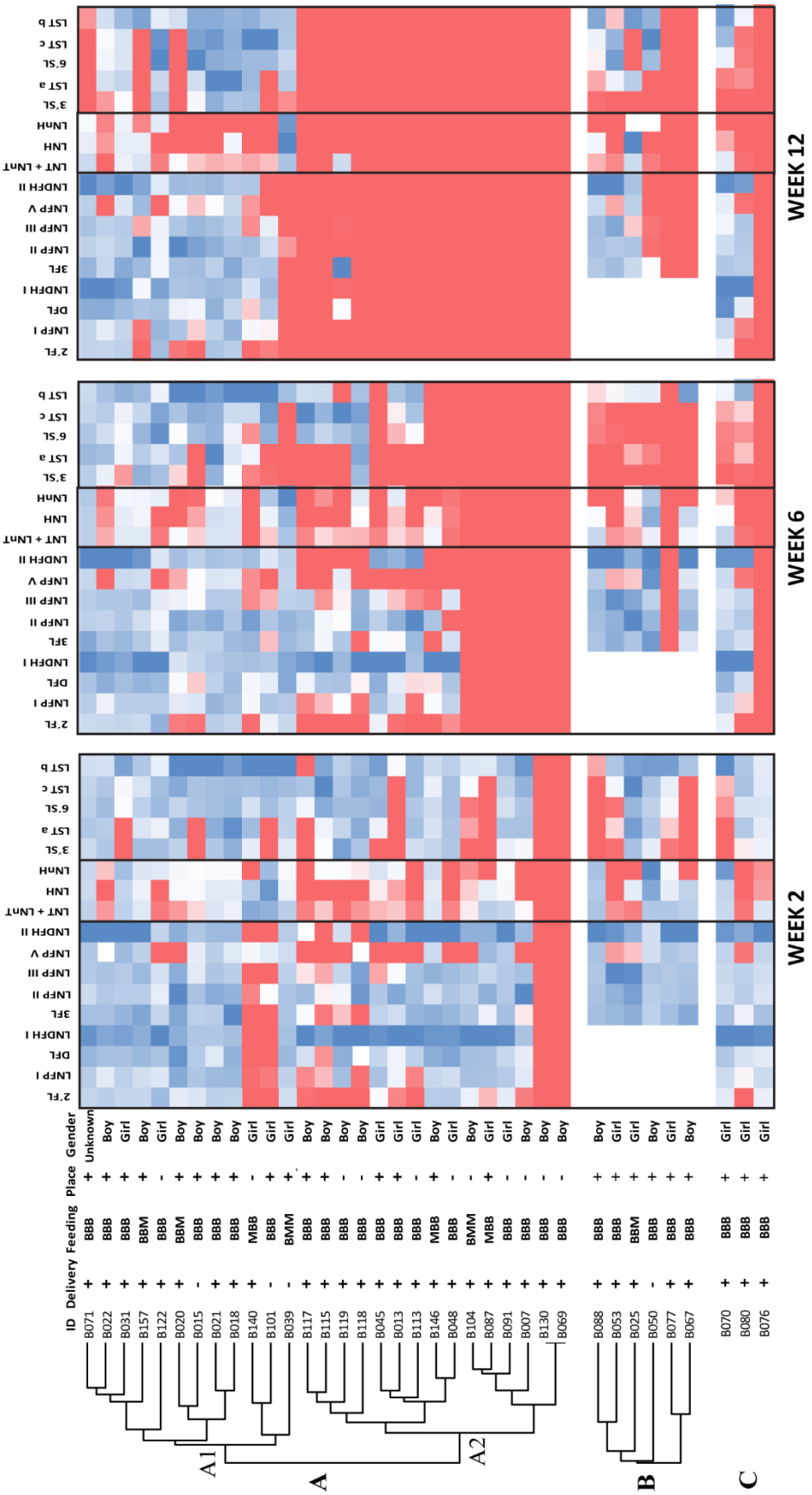

Figure 3.4. Heatmap of relative HMO compositional changes between human milk samples and paired infant feces. The colors range from dark red – white – dark blue, representing -100% (reduction) to 100% (increment) in feces compared to milk after normalization. Hierarchical clustering results of the mother-infant pairs are presented in a dendrogram for milk group 1 (A) and milk group 2 (B) separately. Three preterm pairs from milk group 1 are listed in (C) without hierarchical clustering. Delivery: +, vaginal delivery; -, Caesarean section. Feeding: B/M, breastfeeding/mixed-feeding, with the three successive letters represent feeding type at week 2-6-12, respectively. Place: +, hospital/clinic; -, home.

As discussed, quite diverse patterns of HMO metabolization among these subjects were observed, even within Cluster A, where infants were all delivered full-term and fed with Secretor milk. This indicates the influence of other determinants on HMO metabolization. It is noted that all the caesarean-section born infants belonged to the main cluster A1, and the infants in A2 were exclusively vaginally-delivered. Furthermore, occurrence of infants who were delivered at home were higher in cluster A2 compared to A1, whereas those delivered at hospital or clinic more likely clustered into A1. Therefore, multiple response permutation procedures (MRPP) were performed to identify any possible differences in HMO consumption clustering that were caused by maternal-infant variables (Table 3.4). Both delivery mode and delivery place had marginally significant influences on the overall consumption pattern across the 12 weeks. When considering single time points, the influence of delivery mode was marginally significant at week 2, and became significant at week 12. Likewise, delivery place only exerted significant influence starting from week 6. Although both delivery mode and place had an effect on the initial acquisition of gut microbes, their influences on HMO-consuming abilities were more obvious with the babies' increasing age. No significant differences were found between boys and girls, or breast-fed and mixed-fed infants in the current subset of samples. Due to limited samples size, we did not perform MRPP on milk group 2 or preterm group.

DISCUSSION

Longitudinal changes of HMO in human milk

The longitudinal set-up of the BINGO birth cohort study enabled us to follow the changes in HMO profiles of human milk and infant fecal samples over the first 12 weeks of age in healthy Dutch mother-infant pairs. A total of 18 major HMOs were quantitated in the current study, despite the presence of over 100 structures. However, this is not regarded as a major problem, as it is generally accepted that more than 90% of total HMO concentrations in human milk was accounted for by 20 most abundant HMOs.[33-34] In the current cohort, 75% of mothers were assigned to milk group 1 (Le+Se+) and 25% to milk group 2 (Le+Se-), which was comparable with the range of distribution as given in literature (70% Le+Se+ versus 20% Le+Se-).[34-37] The average of total HMO concentrations were found to be 6.7 g/L and 5.9 g/L in full-term milk from milk group 1 and 2 at two weeks postpartum, respectively, both declined over lactation to 4.9 g/L and 4.6 g/L at 12 weeks. The concentrations were in the lower range of that given by widely cited reviews (5-15 g/L),[1, 38] although similar concentrations have been reported (5-8 g/L in mature milk).[39-40] Based on our results, the between-milk group differences were significant at early lactation stage, and disappeared at week 6. It should be noted that there were no Lewis-negative subjects included in our study,

which might partly explain the slight difference with a previous finding,[40] stating that the total HMO amount were still higher in Secretor's milk than non-Secretor at around 5 weeks postpartum.

Table 3.4. Multiple Response Permutation Procedures (MRPP) results to evaluate any differences in HMO consumption clustering of the study subjects, as caused by the listed maternal-infant variables, at each time point and overall. Only milk group 1 (Le+Se+) samples were included for calculation. Significant *p*-values (< 0.05) are bold, and marginally significant *p*-values (< 0.1) are underlined.

Time	Variables	*A* [#]	*p*-value
WEEK 2	*Delivery mode* [a]	0.01831	0.08
	Delivery place [b]	0.01032	0.172
	Infant gender [c]	-0.01302	0.748
	Feeding type [d]	-0.01345	0.851
WEEK 6	*Delivery mode*	0.01758	0.164
	Delivery place	0.05845	**0.016**
	Infant gender	0.00766	0.293
	Feeding type	-0.01969	0.879
WEEK 12	***Delivery mode***	0.04958	**0.042**
	Delivery place	0.05358	**0.045**
	Infant gender	0.01589	0.349
	Feeding type	0.00120	0.351
OVERALL	*Delivery mode*	0.01992	0.074
	Delivery place	0.01904	0.076
	Infant gender	-0.00107	0.497
	Feeding pattern [e]	-0.00080	0.465

[#] Chance corrected within-group agreement *A* values.
[a] Vaginal delivery vs. Caesarean section
[b] Home/clinic vs. home
[c] Boy vs. girl
[d] Breastfeeding vs. mixed-feeding
[e] Breastfeeding at all time points vs. involvement of mixed-feeding at any time point

Although one study pointed out that human milk samples were predominated by the non-fucosylated HMOs,[9] our samples and another longitudinal study showed the clear abundance of fucosylated HMOs in milk,[41] with relative levels continuing to increase over lactation. Synthesis of fucosylated HMOs in the mammary gland required supply of both donor substrate nucleotide sugars as well as acceptor core structures, like LN(n)T, LN(n)H as well as lactose. Due to competition for substrates, the (near) absence of (α1-2)-fucosylated HMOs in milk group 2 was compensated by higher amount of LN(n)T and (α1-3/4)-fucosylated HMOs, which was also observed by other studies.[41-42] However, mothers from milk group 1 seemed to produce higher amounts of LNH and LNnH in the milk compared to those from milk group 2, although both structures are only present in the milk in low concentrations. Both Azad et al. and Tonon et

al. also reported higher levels of LNH and/or LNnH in Le+Se+ milks than those in Le+Se- milks.[33, 43] The difference in correlations between LN(n)T or LN(n)H with milk group 1 and milk group 2 implied that the *Se* gene is only one of the multiple factors influencing the actual concentrations of these neutral core HMOs in mother milk. The negative correlations between (α1-2)- and (α1-3/4)-fucosylated HMOs in milk group 1 samples were in line with donor- and acceptor- substrate competition between FUT2 and FUT3 enzymes, as suggested by Samuel et al.[41] It can be seen from our results that FUT2 had higher activity in early lactation, and FUT3 caught up at a later stage, which was reflected by the declining concentrations of 2´FL and LNFP I, while 3FL and LNFP III increased, confirming earlier literature.[24, 41, 44] The concentration of LNFP II in milk group 1 remained constant over lactation, which could be a balance between a stronger FUT3 activity and a general diluting effect of HMO synthesis. Two HMOs that contain (α1-3)-linked fucose moieties at the reducing end glucose of base structure LNT, namely LNFP V and LNDFH II, kept constant during lactation in both milk groups, consolidating previous suggestions that their fucosylation was independent from FUT3 activity.[41] Competition for core structures might existed between sialylation and fucosylation, but the levels of 3´SL, 6´SL, LST and LST b were not different between milk groups, except for a higher concentration of LST c in Secretor milk. This was contradictory to other findings that sialylation of LNnT was less influenced by FUT activities than LNT.[41] Nevertheless, the independency of lactose-derived sialylated structures (3´SL and 6´SL) from Secretor status was confirmed, as reported in a previous study.[45] As to the temporal changes of 3´SL concentrations in milk, our study showed stable concentrations in the first 3 months of life, in line with Sprenger et al.[45] Note, however, that Ma et al. found a trend for an increase of 3´SL in Chinese and Malaysian populations from 2 months to one year of age.[44] Furthermore, concentrations of 6´SL, LST c and LST b, which belonged to the (α2-6)-sialylated HMO group, all declined, albeit their predominance over (α2-3)-sialylation at 2 weeks postpartum, in agreement with Austin et al.[24]

Longitudinal changes of HMO metabolization

HMOs excreted intact in infant feces were those that escaped from gut microbiota consumption or, to a smaller extent, that escaped from being absorbed; therefore, by studying the longitudinal changes of fecal HMO profiles, we could deduce the metabolic fates of different HMOs in the GI tract, as well as the development of the gut microbiota of the infants. Previously, we showed that neutral core structures had the highest degree of degradation, while fucosylated HMOs the lowest, at around one-month postpartum.[26] The same microbial preferences were confirmed in the current study and, thanks to the longitudinal design, even found to reach up to 12 weeks postpartum. Despite the general higher frequency of 2´FL and LNFP I showing lower relative abundances in infant feces compared to those in the paired human milk (Figure 3.4), no differences were found between degradation of (α1-2)- or (α1-3/4)-fucosylated HMOs at any time point.

Di-fucosylated HMOs were more difficult to be degraded than mono-fucosylated ones, resulting in higher fecal levels of the former. HMOs containing (α2-6)-sialic acid were more abundant than those containing (α2-3)-sialic acid in milk samples, especially at early stages; however, gut microbes seemed to favor the latter structural group as fermentation substrates, with their assimilation abilities even stronger with time. Ingested HMOs are reported to be catabolized by *Bifidobacterium*, the most predominant bacteria in early life, with varying abilities to utilize HMOs among species and strains.[14] Already in *in vitro* experiments, consumption of HMOs by *Bifidobacterium* and *Bacteroides* were found to be structure-specific.[29] The same observations have been reported by *in vivo* studies. LNT, for instance, was one of the most consumed HMOs as indicated by its lower level in feces,[24] similarly to the (α1-2)-fucosylated HMO.[25] De Leoz et al. also found a lower microbial degradation level of LNFP II, compared to LNFP I/III/V, in the first 13 weeks of age.[25]

Next to a general trend of consumption of HMO structural groups, each infant displayed variation in their development trajectory of consumption patterns from two weeks to 12 weeks. We recognized three consumption patterns among one-month old babies in a previous study,[26] including complete consumption, specific assimilation of core and sialylated HMOs, or no specific degradation with all HMOs still present at considerable levels. When taking the three time points of the present study into consideration, almost all infants showed growing abilities to degrade HMOs with increased duration of breastfeeding, with several infants having already developed the gut microbiota to completely utilize all ingested HMOs at two or six weeks postpartum. This transition reflected the development of infant gut microbiota from a non-HMO-consuming population, acquired from the mothers or surrounding environment after delivery, towards a population dominated by HMO-consuming microbes.[25] The infant fecal microbiota compositions of the same set of BINGO samples were analyzed by Borewicz et al.,[32] and a colonization pattern towards *Bifidobacterium* dominated microbial communities in these infant fecal samples was found. By examining the HMO metabolization in the current study, we found that the speed of this abovementioned transition across developmental stages was individual-dependent, since almost half of the infants had not reached the final stage yet at the last time point, 12 weeks postpartum. This inter-individual variation in developmental trajectories was also noticed in other studies.[13, 27, 29] The current study found delivery mode to be one factor that influenced the transition speed, with infants delivered by caesarean section displaying a delayed colonization and enrichment of HMO-consuming microbes compared to those vaginally delivered, in consistence with previous literature.[11, 18] Besides delivery mode, infants who were delivered at home tended to show a faster transition to reach the final stage, compared to those exposed to hospital or clinic environment at birth. The influence of delivery mode on infant gut microbiota was also proved by Borewicz et al. based on fecal microbiota of these same 'BINGO' infants; however, no significant difference between different delivery places was found there.[32] Nevertheless, all these findings point at the importance of initial

colonization of infant gut for the metabolization of HMOs. Our results did not find any obvious differences in HMO metabolization transition between infants fed with Secretor or non-Secretor milk, although some studies indicated a negative impact of feeding non-Secretor milk on the gut development,[15] especially those delivered by caesarean section.[42] Furthermore, our results did not find significant differences in HMO metabolization between breast-fed and mixed-fed infants, although feeding infants with infant formula to replace human milk is believed to significantly alter infant gut microbiota.[17, 46-47] This could be partly due to the exclusion of entirely formula-fed subjects, and the limited number of mixed-fed subjects. Prebiotics, such as galacto- and fructo-oligosaccharides, supplemented to infant formula nowadays, might partially alleviate the negative influence on gut microbial maturation due to lack of HMOs.[12]

A limitation of the current study is the lack of Lewis negative mothers and their babies, which could have provided more insights of enzymatic regulation of HMO synthesis as influenced by FUT3 activity. More subjects from milk group 2 with more variations regarding delivery mode and delivery place should be included in future studies, in order to draw solid conclusions about how these variables possibly affect infant gut microbiota and fecal HMO profiles. Furthermore, data about infants' body growth and development, as well as health status, should be followed, to find out any biological significance of these differences in HMO consumption development.

CONCLUSIONS

With more participants and samples from multiple time points, we were able to follow the temporal changes of HMO synthesis in mother milk, as well as HMO metabolization by paired infant gut microbiota, during the first three months of life. Lactation duration had significant influence on concentrations of individual HMOs in mother milk, as well as mothers' Lewis/Secretor status, especially on the fucosylated and neutral core HMOs, and LST c. The diverse HMO metabolization patterns by the infant gut microbiota found in the current study further confirmed the observations of our previous pilot study and indicated the different stages of gut microbiota development. The transition of HMO metabolization from a low/non-specific pattern towards a complete utilization pattern is individual-dependent, and can be partly attributed to differences in mode and place of delivery. Although it remains unknown how the gut microbiota developmental pattern and HMO metabolization stages would link to long-term health outcomes, more attention is suggested to be paid on personalized nutrition, in order to provide infants with the most appropriate HMOs at specific developmental stage. Furthermore, HMOs with different structures were utilized with different preferences by infant gut microbiota, which could lead researchers and product developers to identify specific compounds of interest to be applied into infant nutrition, with right dosing at a given stage of growth.

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SUPPORTING INFORMATION

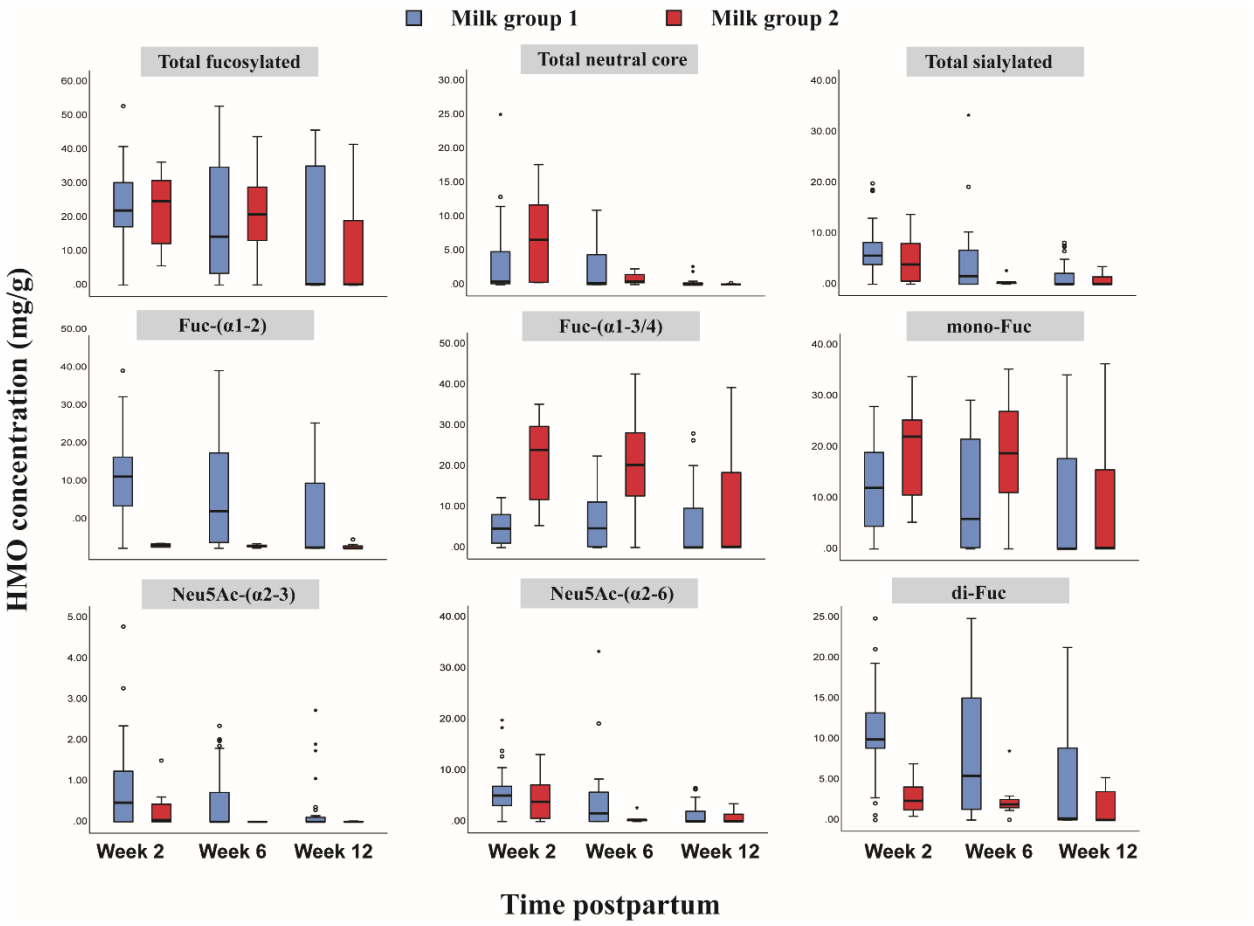

Figure S3.1. Concentrations of different HMO structural groups in full-term infant fecal samples, from milk group 1 (blue) or milk group 2 (red), at three time points. Box-whisker plots represent minimum, first quartile, median, third quartile, maximum and outliers. Significant differences are not indicated in the figure.

Chapter 4

Correlating infant fecal microbiota composition and human milk oligosaccharide consumption by microbiota of one-month old breastfed infants

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ABSTRACT

Understanding biological functions of different free human milk oligosaccharides (HMOs) in shaping gastrointestinal tract microbiota during infancy is of great interest. We examined a link between HMOs in maternal milk and infant fecal microbiota composition and investigated the role of microbiota in degrading HMOs within the GI tract of healthy, breastfed, one-month old infants. Maternal breast milk and corresponding infant feces originated from the KOALA Birth Cohort. HMOs were quantified in milk and infant fecal samples using PGC-LC-MS and HPAEC-PAD. Fecal microbiota composition was characterised using Illumina HiSeq amplicon 16S rRNA sequencing. The composition associated with gender, mode of delivery, and milk HMOs: Lacto-*N*-fucopentaose I and 2′-Fucosyllactose. Overall, *Bifidobacterium, Bacteroides, Escherichia-Shigella* and *Parabacteroides* were predominating genera. We detected three different patterns in the infant fecal microbiota structure. Gastrointestinal degradation of HMOs was strongly associated with fecal microbiota composition, and there was a link between utilisation of specific HMOs and abundance of various phylotypes (OTUs). HMOs in maternal milk are among important factors shaping GI tract microbiota composition in one-month old breastfed infants. Infant's ability to metabolise different HMOs strongly correlate with fecal microbiota composition, and with phylotypes within genera *Bifidobacterium, Bacteroides* and *Lactobacillus*.

INTRODUCTION

During and after birth, microorganisms from the mother and other environmental sources colonize an infant. Genetic and various environmental factors and life events further shape the microbial communities, making them specific to each body site and to each individual. These microbial ecosystems acquired and developed in early life play an important role in well-being and health, both during infancy and beyond.[1] One of the body sites that undergoes a rapid microbial colonisation in early life is the gastrointestinal (GI) tract.[1] The anaerobic conditions in the lower GI tract favour the establishment of bacteria, such as *Bifidobacterium*, *Bacteroides* and *Clostridium*.[1] Besides the absence of oxygen, diet is another key factor that has a strong influence on shaping the GI microbial ecosystem.

In breastfed infants, breast milk is the sole source of nourishment during the first few months of life. Breast milk is a complex biofluid that contains high concentrations of lactose, lipids, proteins, and milk glycans, the latter being present as either glycoproteins or free human milk oligosaccharides (HMOs).[2] HMOs play an important role in intestinal cell proliferation and maturation, maintaining epithelial barrier function, and protecting the GI tract against bacterial and viral pathogens and toxins.[2-5] Despite being the third most abundant component of human milk, HMOs are not affected by infant digestive enzymes.[2] As a result, milk HMOs reach the infant colon, where they are degraded by bacteria. Since not all bacteria have the necessary enzymes to utilize HMOs, these milk glycans facilitate the establishment of a highly specialized microbial ecosystem dominated by bifidobacteria and *Bacteroides* amongst others, while indirectly limiting growth of other bacteria. This prebiotic effect has been demonstrated for selected bacterial species, both *in vitro*,[3, 6] and *in vivo*,[7] and it has been recognised as one of the key drivers for bacterial species succession in the infant GI tract [5]

Maternal genotype (e.g. mother's Secretor status) determines the HMO composition in breast milk, and the concentrations of different HMOs vary between individuals and across lactation stages.[8-10] This variability might target the distinct and changing needs of a growing infant and orchestrate the stepwise development of infant GI tract microbiota. Recent developments in glycomics led to the recognition of over 200 different HMOs in human milk.[2] The core structures of HMOs include galactose, glucose and *N*-acetyl-glucosamine, which are further decorated with fucose and/or sialic acid. The fucosylation of HMOs depends on the presence of specific glycosyltransferases, including the α1-2-fucosyltransferase FUT2 and the α1-3/4-fucosyltransferase FUT3, in lactating women. Milk of Secretor women having active *Se* gene locus that encodes the FUT2 has high amounts of α1-2-fucosylated HMOs, while milk of Lewis positive women with active *Le* gene locus that encodes the FUT3, is abundant in α1-4-fucosylated HMO. In contrast, milk of non-Secretor or Lewis negative women lack α1-2- or α1-4-fucosylated HMO, respectively.[11] Based on the presence or absence of sialic acid, HMOs can be classified into two categories: the neutral and the acidic HMOs. In this study we measured 17 highly abundant HMOs, including 12 neutral and five acidic HMOs (Table 2.1). The concentrations of these

major HMO structures account for around 86% of the total HMOs in breast milk of secretors 35 days postpartum.[12]

In the light of growing evidence supporting the role of the early colonization of GI tract microbial ecosystem in health, understanding the biological function of the different HMOs is of a great interest. Previous studies focused mainly on *in vitro* fermentation of HMOs by fecal bacterial inoculum, or by fecal isolates.[3] A proof-of concept study showed correlation between HMO degradation and gut microbiota development in early life by analysing samples from two infants.[12] However, the HMO degradation within an infant GI tract is still not fully understood. Here, we analysed 121 mother-infant pairs to investigate the association between selected maternal HMOs and the infant fecal microbiota composition. Our two main research questions were: i) whether there was an association between the composition of HMOs in breast milk and the composition of fecal microbiota in healthy, breastfed, one-month old infants, and; ii) if the degradation of these HMOs could be linked to infant fecal microbial communities, and to specific bacterial taxa found in the infant's GI tract.

MATERIALS & METHODS

Milk and fecal sample collection

The milk and fecal samples used in this study originated from the KOALA Birth Cohort (Dutch acronym for: Child, Parents and Health: Lifestyle and Genetic Constitution). The design, selection criteria and feces collection procedure have been described elsewhere and the study was approved by the Ethics Committee of the University Hospital of Maastricht.[13-15] Briefly, the KOALA study included healthy pregnant women living in the south of the Netherlands (N=2834), and the exclusion criteria included prematurity (birth before 37 weeks of gestation), twins, congenital abnormalities related to growth, and administration of antimicrobial agents before feces collection. Only infants who were exclusively breastfed, and for whom both the fecal and the corresponding maternal breast milk samples were available were included in the analyses (n=121). All infants were born in the years 2002-2003, healthy, full term, at home or hospital via either vaginal delivery or C-Section. Three infants were reported by the parents as sick on the sample collection day, but none of the infants received antibiotics during the first month of life (Table S4.1). Infant fecal samples were collected at approximately one month postpartum from infants' diapers, refrigerated, and sent to the lab by post within one day after collection. Fecal samples were stored in peptone glycerol solution (10 g/L peptone water in 20 v/v% glycerol) at 1g of feces in 9 mL of solution. Breast milk samples were collected into sterile tubes (Cellstar PP-test tubes, Kremsmünster, Austria), in the morning on the same day as the fecal samples, refrigerated (±4 °C), transported on ice and processed in the lab on the same day. Breast milk samples were centrifuged (400 × g, 12 min, no brake, 4 °C) and the lipid and aqueous fraction were separated

and stored in plastic vials (Sarstedt, Nümbrecht, Germany) at -80 °C in the European Biobank, Maastricht. The remaining debris was not used to avoid contamination with cell fragments.

Dosage information/ Dosage regime

The total amount of HMOs ingested daily by each infant depended on the HMO concentration in breast milk and the total amount of breast milk consumed per day. We estimated the daily intake based on the literature data on infants of similar age (approximately one month postpartum) who were breastfed without feeding problems and consumed amounts as regulated according to their needs.[16]

DNA extraction

Total DNA was extracted from the stool samples as previously described [17], using the double bead-beating procedure followed by the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The resulting DNA templates (5-20 ng) were used for subsequent PCR amplification and Illumina HiSeq sequencing of the V4 region of 16S ribosomal RNA (rRNA) genes.[18]

HMO Analysis

HMOs were isolated and purified from milk and infant feces using solid phase extraction (SPE) [19]. Then HMOs were reduced to alditols using sodium borohydride,[20-21] and were analysed by an Accela Ultra High Pressure Liquid Chromatography (UHPLC) system (Thermo Scientific, Waltham, MA, USA) which was coupled to a Velos Pro mass spectrometer (Thermo Scientific) with an electrospray ionisation probe. A volume of 5 µl of reduced sample or HMO commercial standard was injected onto a Thermo Hypercarb™ Porous Graphitic Carbon LC Columns column (100 x 2.1 mm, 3 µm particle size) with a Hypercarb guard column (10 x 2.1 mm, 3 µm particle size). The separation was performed using two eluents (A) 1% (v/v) ACN in water with 0.1% (v/v) formic acid and (B) ACN with 0.1% (v/v) formic acid. The elution profile was applied as follows: 0-5 min, 3% B; 5-22 min, 3-20% B; 22-32 min, 20-40% B; 32-33 min, 40-100% B; 33-43 min, 100% B; 43-44 min, 100-3% B; 44-65 min, 3% B. In total, 12 neutral HMOs (2´FL, LNT and LNnT, 3FL, DFL, LNDFHI, LNFPI, LNFPII, LNFPIII, LNFPV, LNH, LNnH) and five acidic HMOs (3´SL, 6´SL, LSTa, LSTb, LSTc) were measured. Data processing was done by using XCalibur™ software (Thermo Scientific) and peak area as extracted from MS signal was used for quantitation. Quantitation of 3FL was by High Performance Anion Exchange Chromatography- Pulsed Amperometric Detection (HPAEC-PAD). The analysis was applied on a ICS 5000 system (Dionex, Sunnyvale, CA), equipped with a CarboPac PA-1 column (250 mm x 2 mm ID) and a CarboPac PA guard column (25 mm x 2 mm ID) and with a column temperature of 20°C. The two mobile phases were (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH. With a flow rate of 0.3

mL/min, the gradient elution profile was as follows: 0-10 min, 0-10% B; 10-10.1 min, 10-100% B; 10.1-15 min, 100% B; 15-15.1 min, 100-0% B; 15.1-30 min, 0% B. The elution was monitored by a pulsed amperometric detector (Dionex ICS-5000 ED). Data processing from HPAEC was done by using ChromeleonTM 7.1 software (Dionex). The HMOs concentrations were measured in µg per mL of milk or µg per gram of feces.

Data analysis

The 16S rRNA gene sequencing data was analysed using the NG-Tax analysis pipeline using default settings.[22] In brief, libraries were filtered to contain only read pairs with perfectly matching barcodes that were subsequently used to separate reads by sample. Operational taxonomic units (OTUs) were assigned using an open reference approach and SILVA_111_SSU 16S rRNA gene reference database (https://www.arb-silva.de/).[23] Microbial composition data was expressed as a relative abundance of each OTU obtained with NG-Tax.

Infants were classified into three distinct microbial cluster types based on genus level microbial abundance data using Dirichlet Multinomial Mixture (DMM) modelling.[24] Briefly, the number of Dirichlet components was selected by inspection of the fit of the model to the count data for varying number of components (1 to 7). Goodness of fit was assessed using the Laplace and the Akaike information criteria. Finally, each sample was assigned to the component for which it had the largest fitted value using the DirichletMultinomial R package in R (version 3.3.1).[25] Microbial composition of each DMM cluster is shown in supplementary Figure S4.1.

Redundancy analysis (RDA) was done in Canoco5 using the log transformed OTU level relative abundance data with significance assessed using a permutation test.[26] Explanatory variables included concentrations of milk HMOs: 2´FL, LNT and LNnT, LNFPIII, LNFPII, LNFPI, LNFPV, LNH, LNnH, LNDFHI, DFL, 6´SL, 3´SL, LSTc, LSTb, LSTa, 3FL, delivery mode (normal vaginal, assisted vaginal and C-section), delivery place (home, hospital), gender, gestational age, mother antibiotic use, infant signs of sickness (more specifically, the signs of gastroenteritis including vomiting, fever and diarrhoea) at the time of sample collection, infant age in days, and birth weight. The association between fecal microbiota composition, the assignment of each infant to a specific microbial cluster and the HMO concentrations in corresponding breast milk samples of the infant's mother were investigated with Partial Least Squares regression (PLS) using MatlabR2107a, and resulting p values were corrected for multiple comparisons using FDR. The Chi-square test was used to assess the significance of the association between infant gender and infant microbial cluster type, and between mother's secretor status (positive, negative) and infant microbial cluster type.

HMO degradation (consumption) in the infant GI tract was estimated based on profiles in breast milk and corresponding infant feces. Based on the utilisation of the 17 individual HMOs, infants were

assigned to consumption categories: “Complete”, “Non-specific” and “Specific” (acidic, neutral or other). The Chi-square test was used to assess the significance of the association between the assigned consumption category for each HMO and the microbial DMM cluster type of each infant.

Based on the extent to which each individual HMO was consumed (calculated as a ratio of the HMO concentration in infants’ feces and the concentration of the same HMO measured in mothers’ milk) infants were divided into tertiles (“low”, “medium”, or “high”) for consumption levels of each individual HMO. If a given HMO was not detected in milk, the consumption score was not included in the analysis, and if the amount in feces exceeded the amount detected in milk, the infant was assigned to the “low” category for that HMO. Associations between fecal microbiota composition and the assignment of each infant to a “low”, “medium”, or “high” consumption category for each HMO were investigated with RDA analysis in Canoco5, with significance assessed using a permutation test.[26] Kruskal-Wallis analysis was performed in QIIME,[27-28] to identify bacterial OTUs that differed significantly between infants who were classified as “high”, “medium” and “low” consumers for each individual HMO.

Nucleotide sequences

KOALA data sets cannot be made publicly available due to data confidentiality and the potential to identify individual study participants from the data. Data are available to the research community through the Dataverse repository (https://dataverse.nl/; 10411/CEGPGR) upon request to Prof. C. Thijs of the KOALA Study Management Committee at: Maastricht University, Department of Epidemiology, PO Box 616, 6200 MD Maastricht, The Netherlands, e-mail: c.thijs@maastrichtuniversity.nl, tel: +31(0)43 3882374.

RESULTS

HMO Analyses

HMOs in maternal milk and in infant feces were quantified. The minimum, maximum, median, average and standard deviation of the concentrations of each HMO, the HMO type (neutral, fucosylated, and sialylated), and the total amounts are summarised in Table 4.1. Total concentrations of the measured HMOs in milk ranged from 2.0 to 6.5 mg/mL, and were lower than those reported in literature.[11, 29-30] We observed large individual variation in the HMO concentrations in both the maternal milk samples, and in infant feces. In most samples the percentage of neutral HMOs was higher than that of acidic HMOs (Table 4.1). The fucosylated HMOs accounted for the vast majority of neutral HMOs. The composition of fucosylated HMOs in milk depends on the Lewis and Secretor status of a mother, and in some samples we did not detect fucosylated HMOs at all. Structures like 2’FL and LNFP I were present in high concentrations in milk of Secretors, while they were absent in Non-secretors. Other structures,

like LNFP II, were present as main HMOs in the Lewis positive mothers, while they were absent in the Lewis negative mothers. We could not detect LNDFH I and DFL in breast milk samples containing α1-2- and α1-4-fucoses from mothers who were Lewis or Secretor negative. Some neutral HMOs were detected in all milk samples, i.e. 3'FL, LNFP III, LNT and LNnT. Only one mother lacked LNH and LNnH in her milk. The acidic HMOs were detected in all milk samples. These observations were close to those published before.[19, 29] Similar to the HMOs in breast milk samples, there was also a large variation in HMOs concentrations in the infant feces. However, the median values of milk HMO concentration matched well with the average values, while this was not the case for the HMO levels in feces, indicating a right-skewed distribution for the fecal profiles that was different from the bell-shaped distribution observed for the milk HMOs profiles (Table 4.1).

Table 4.1. Average, minimum, maximum, and median concentrations of individual HMOs, classes and total measured HMOs and corresponding standard deviations (SD),* in breast milk and in infants' fecal solution (1 g faeces/ 9 mL of peptone glycerol medium). For abbreviations and structures of HMOs, please refer to Table 2.1.

	Concentrations of HMO, or HMO category									
	Human milk (μg/mL)					**infant fecal solution (μg/mL)**				
HMO	**Min**	**Max**	**Median**	**Average**	**SD**	**Min**	**Max**	**Median**	**Average**	**SD**
3FL	5.0	1098.0	182.0	248.0	222.0	NA	NA	NA	NA	NA
2'FL	0.0	852.8	460.0	372.7	242.3	0.0	240.3	0.5	29.6	61.4
LNT and LNnT	214.2	1806.7	948.0	976.2	319.0	0.0	372.7	15.8	48.6	75.0
LNFPIII	50.7	758.0	243.9	270.1	140.7	0.0	726.7	0.0	41.0	98.0
LNFPII	0.0	1341.5	236.3	339.0	294.3	0.0	549.0	3.1	81.9	125.5
LNFPI	0.0	1493.7	517.2	467.3	367.5	0.0	505.7	0.0	41.8	91.3
LNFPV	0.0	191.4	27.7	41.8	50.1	0.0	75.0	0.0	2.5	8.7
LNH	0.0	313.0	89.5	105.0	64.1	0.0	161.2	0.0	4.4	17.3
LNnH	0.0	299.1	56.1	72.2	56.3	0.0	563.4	0.4	12.1	57.0
LNDFHI	0.0	1856.2	548.3	475.5	388.3	0.0	889.8	28.0	204.3	258.2
DFL	0.0	125.9	42.1	40.4	32.1	0.0	68.4	0.6	9.7	17.0
6´SL	16.6	385.7	97.3	110.8	63.5	0.0	298.5	0.1	18.4	46.7
3´SL	16.8	194.8	91.5	90.7	38.8	0.0	100.0	0.0	4.2	14.9
LSTc	14.7	334.1	98.8	116.2	68.8	0.0	248.5	0.8	28.9	56.8
LSTb	53.2	804.4	244.4	256.2	118.7	0.0	499.9	1.8	52.2	104.5
LSTa	6.3	83.4	24.6	28.2	15.4	0.0	31.7	0.0	2.1	6.1
Sum Neutral	1542.0	5717.3	3064.8	3160.2	824.2	0.0	1671.6	237.9	475.9	523.1
Sum Fucosylated	266.5	4489.4	2042.5	2006.8	737.6	0.0	1591.7	186.1	410.8	463.4
Sum Sialylated	174.1	1273.2	564.7	602.1	210.6	0.0	956.3	3.3	105.8	197.1
Sum Total	1917.4	6545.2	3635.9	3762.3	939.1	0.0	2169.9	267.3	581.7	648.0

* NA- not determined.

Fecal microbiota composition and microbial clusters

Illumina HiSeq sequencing of the V4 region of bacterial 16S rRNA genes yielded 14,474,685 high quality reads that passed the quality check and could be assigned to 531 OTUs from 113 genera. In case an OTU could not be classified to a given genus level, it was assigned to the next available taxonomic rank. The predominating genera in this infant cohort were *Bifidobacterium, Bacteroides, Escherichia-Shigella* and *Parabacteroides*, with a mean relative abundance of 32% (0-91.5%), 21.3% (0-76.7%), 11.8% (0-57.8%) and 6.7% (0-64%), respectively. Genera with the highest number of contributing OTUs included *Bacteroides* (64 OTUs), *Bifidobacterium* (38 OTUs), *Parabacteroides* (31 OTUs), *Lactobacillus* (24 OTUs), and *Streptococcus* (20 OTUs). Eighty-two OTUs of the total 531 OTUs were found in at least six or more infants of the 121 infants studied (Table S4.2). The remaining low prevalence OTUs found in five or fewer infants and were summarised as "Other" (Table S4.2). Overall, the fecal microbiota composition of infants in this cohort was highly variable, yet we could distinguish presence of three universal microbial patterns based on DMM cluster analysis (Figure S4.1). These clusters were characterised by microbial communities with a mixed structure (Cluster A), or by communities with either a high relative abundance of *Bifidobacterium* (Cluster C), or a high relative abundance of both *Bifidobacterium* and *Bacteroides* (Cluster B).

The effect of HMOs in breast milk and other factors on fecal microbiota composition

We used RDA to identify factors affecting fecal microbiota composition of infants in the study (Figure 4.1). Together the explanatory variables explained 21.7% of the variation in the OTU data. However, only mode of delivery and gender had a significant effect on microbiota composition ($p<0.05$), and milk 2′FL concentration was borderline significant ($p=0.06$). PLS regression also showed a significant association between 2′FL (and LNFPI) concentrations in milk with infant microbiota (FDR<0.05, Table S4.3). When samples were color-coded by infant's DMM cluster type, we also noted that, based on the RDA vector distribution, high levels of 2′FL and LNFPI in milk, as well as C-section, were all associated with microbial cluster A, which is characterised by a mixed microbial profile (Figure 4.1). The association between breast milk HMO composition and the assignment of each infant to a specific microbial cluster type was further investigated using RDA *(data not shown)*. The results indicated an association between overall HMO composition in milk and the DMM cluster type ($p<0.05$, 3.1% explained). We used Chi-square analysis to test if the mother's secretor status (yes/no), which is known to affect the ability to synthesize (α1-2)-linked fucose, and thus, the amount of some major neutral HMOs in milk (e.g. 2′FL and LNFPI), had an effect on infant microbiota profiles, as characterised by different DMM cluster types, but the association was not significant ($p=0.08$) (Table S4.4). Also, infant gender was not significantly associated with any of the DMM cluster types.

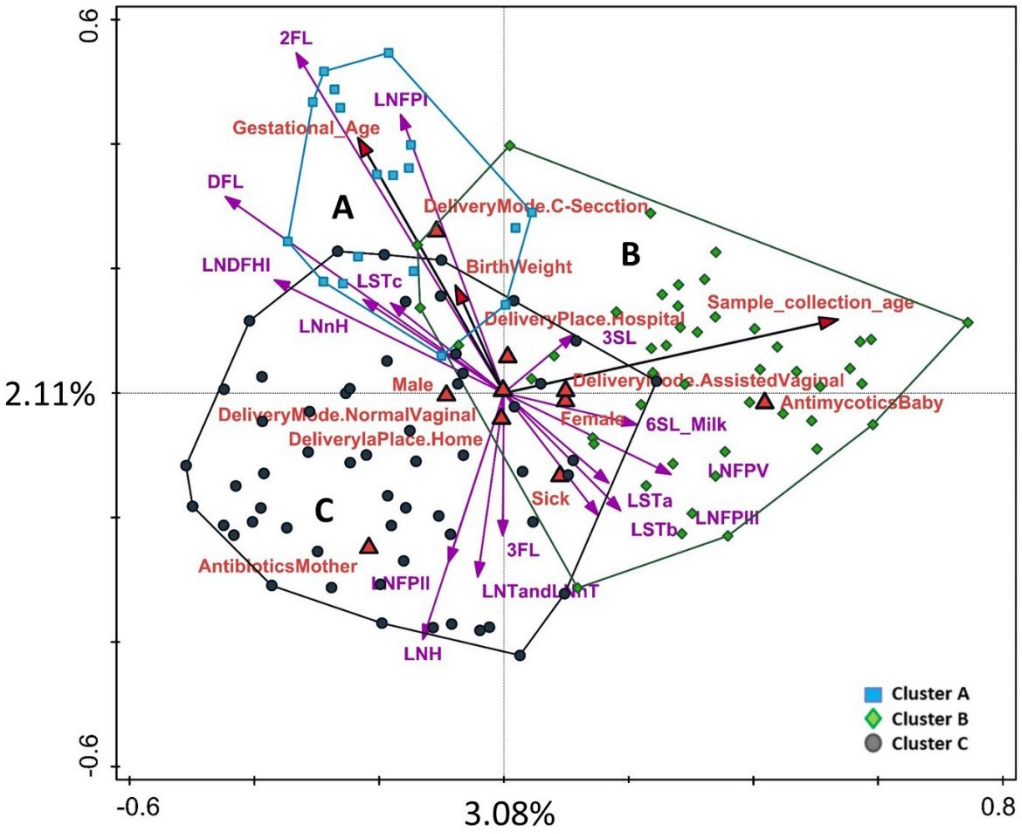

Figure 4.1. Constrained Analysis (RDA) of different factors and milk HMO levels and their association with the fecal OTU profile of infants. Samples are labelled and enveloped based on the infant assignment to microbial cluster type A, B or C.

The association between infant fecal microbiota composition and HMO degradation

By comparing the HMO profiles in breast milk and in corresponding infant feces we detected presence of five patterns in the HMO consumption (Figure 4.2). The first pattern ("Complete") was characterised by low or undetectable amounts of any of the HMOs in infant feces, suggesting a complete consumption of all HMOs from the breast milk. The second pattern ("Non-specific") showed a fecal HMOs profile that was comparable to that of breast milk and contained high concentrations, thus implying a non-specific (or broad) and incomplete (or slow) consumption of HMOs by the infant GI tract microbiota. The third pattern ("Specific") indicated selective consumption of specific HMOs, and was further divided into three types. "Specific acidic" showed a high level of neutral HMOs in feces, meaning that the acidic HMOs (3´SL, 6´SL, LSTa, LSTb, LSTc) were predominantly utilized. "Specific neutral" was characterised by the acidic HMO profile of the feces, meaning that neutral HMOs were predominantly utilized by the infant GI tract microbiota. The third type was "Specific other", which could not be categorized as neither acidic nor neutral HMOs *(data not shown)*.

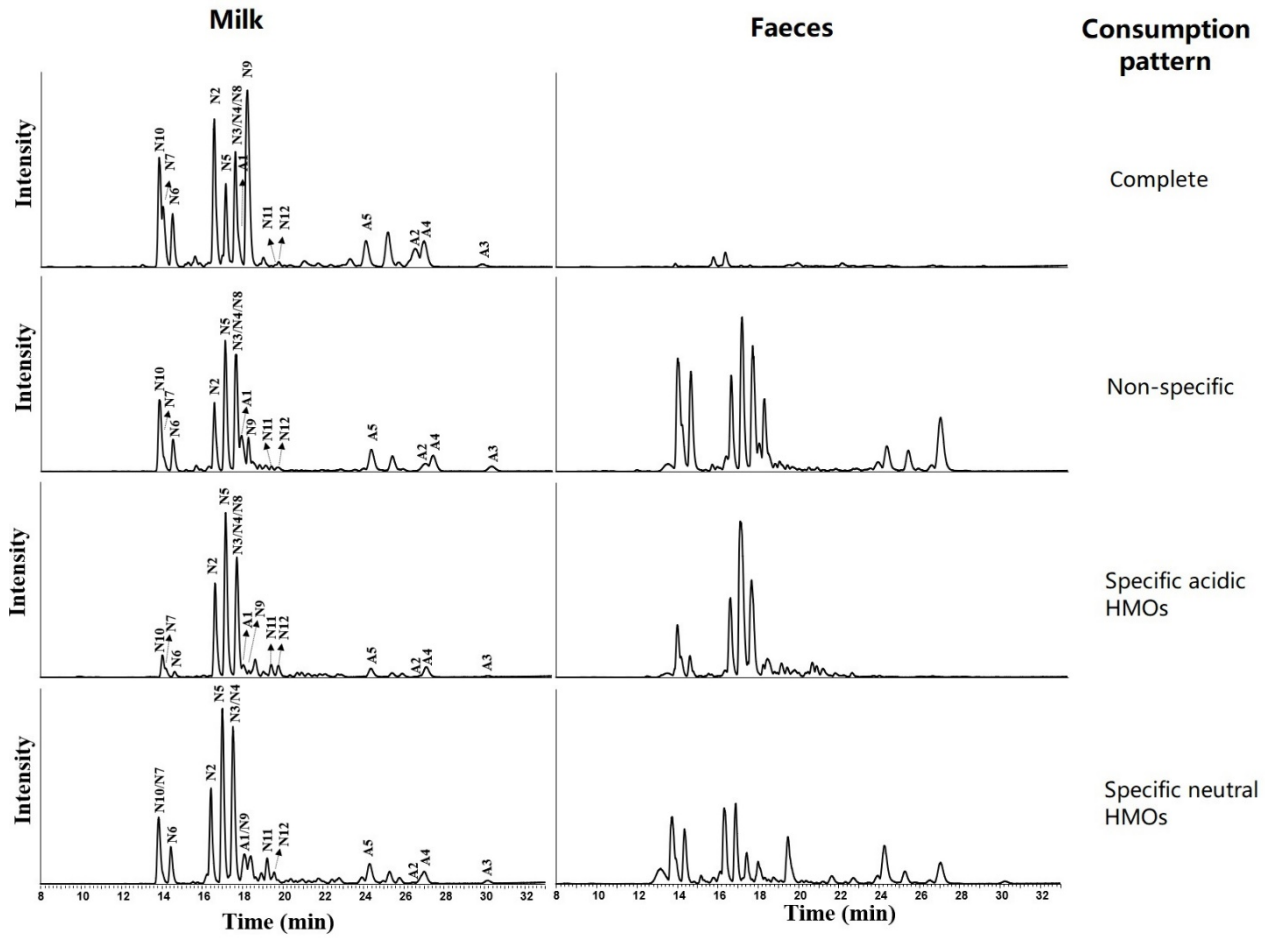

Figure 4.2. Different utilization patterns of infants defined based on the comparison of HMO profiles in breastmilk and infant feces: complete consumption, non-specific, specific consumption of acidic HMOs and specific consumption of neutral HMOs. Peak assignments are as follows: N2-2′FL, N3-LNT, N4-LNnT, N5-LNFP I, N6-LNFP II, N7-LNFP III, N8-LNFP V, N9-DFL, N10-LNDFH I, N11-LNH, N12-LNnH; A1-6′SL, A2-3′SL, A3-LSTa, A4-LSTb, A5-LSTc.

We used RDA analysis to investigate the association between microbiota composition and different HMO consumption patterns. We noted that “Complete”, “Non-specific” and “Specific acidic” consumptions were significantly associated with infant microbiota composition (FDR<0.05), while the association of “Specific neutral” and “Specific other” was not significant. In addition, “Complete” consumption correlated with high relative abundance of bifidobacteria, including the two highly abundant *Bifidobacterium* OTUs 614 and 418 (Figure 4.3a). Furthermore, the Chi-Square test showed a strong and significant association (χ^2 = 32.28; p<0.00001) between the frequency of different consumption patterns and each DMM microbial cluster. Forty percent of infants who were classified in the mixed microbial cluster A also showed a non-specific HMO consumption pattern (Figure 4.3b).

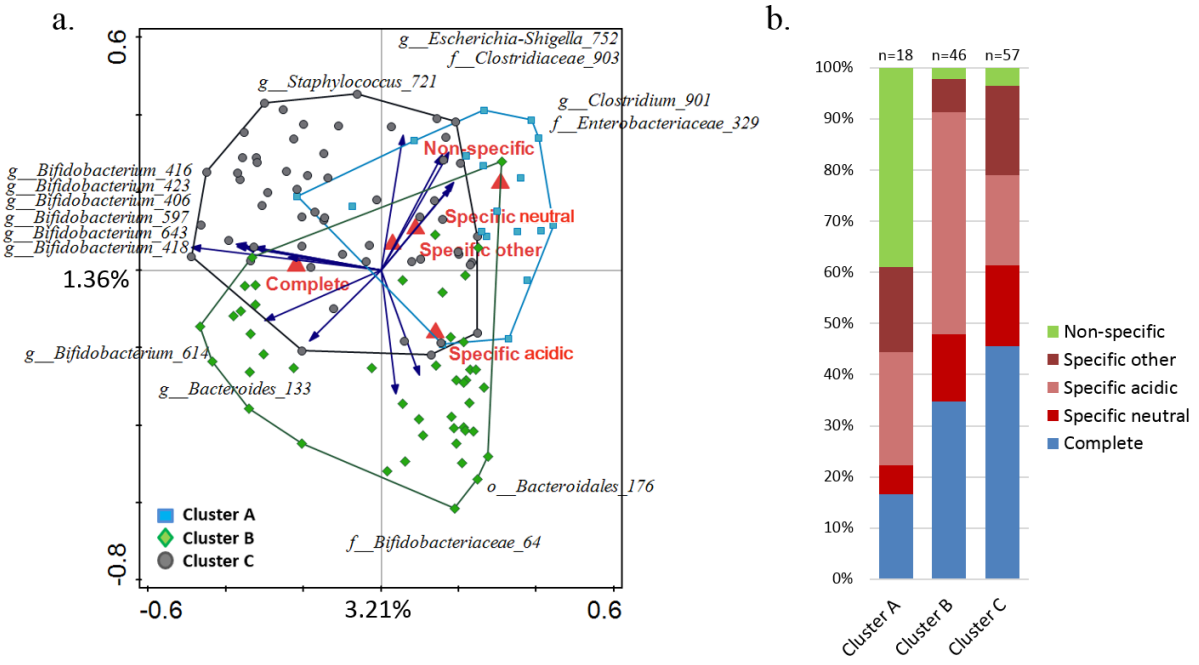

Figure 4.3. Association of general HMO consumption patterns with microbial cluster types A, B, C; a. RDA showing the association between HMO consumption patterns and microbial OTUs. 15 best fitting OTUs are displayed and samples are color-coded based on their cluster type assignment; b. Segregation of infants based on their HMO consumption pattern in relation to their microbial cluster type classification.

In order to investigate the association between microbiota composition and consumption of specific HMOs in more depth, we classified infants as “low”, “medium” or “high” consumers for each of the measured HMOs. We then used this classification in the multivariate RDA analysis and showed that the HMO consumption explained 61.5% of variation in microbiota. Infant’s degradation ability of 2′FL, LNT and LNnT, DFL, 6′SL, LNH, LNFPII and LNFPIII (FDR<0.05), and LSTb (FDR = 0.06), LSTc and 3′SL (FDR=0.07) was associated with differences in the fecal microbiota composition (Figure 4.4). For all HMO types, there was a general trend relating consumption efficiency and infant fecal microbiota cluster class. RDA showed that microbial DMM cluster type alone could explain 8.4% of variation in the consumption category and that the cluster effect was statistically significant (FDR<0.05). The lowest efficiency of consumption was linked to microbial cluster type A, with 40.6% at “high” level, 10% at “medium” level, and 49.4% of all HMOs consumed at “low” level. Infants classified in microbial cluster B showed high HMO consumption levels, with 47.3% of all HMO types consumed at “high” level, 21.3% consumed at “medium” level and 31.4% at “low” level. Infants classified in microbial cluster C, showed “high” consumption for 49.8% of all HMOs, “medium” consumption for 24.4% and “low” consumption for 25.8%. The microbial cluster type consumption efficiency pattern varied for different HMO types (Figure 4.5). Chi-square analysis was used to test the correlation between the proportion of infants in “high”, “medium”, and “low” consumption categories for each HMO and the infant microbial cluster groups. Significant ($p<0.05$) differences were detected between clusters with respect to consumption of 2′FL, LNFPIII, LNFPII, DFL and 6′SL. For the aforementioned HMOs, the highest

proportion of infants with the lowest ability to break down these HMOs was found in cluster A (Figure 4.5).

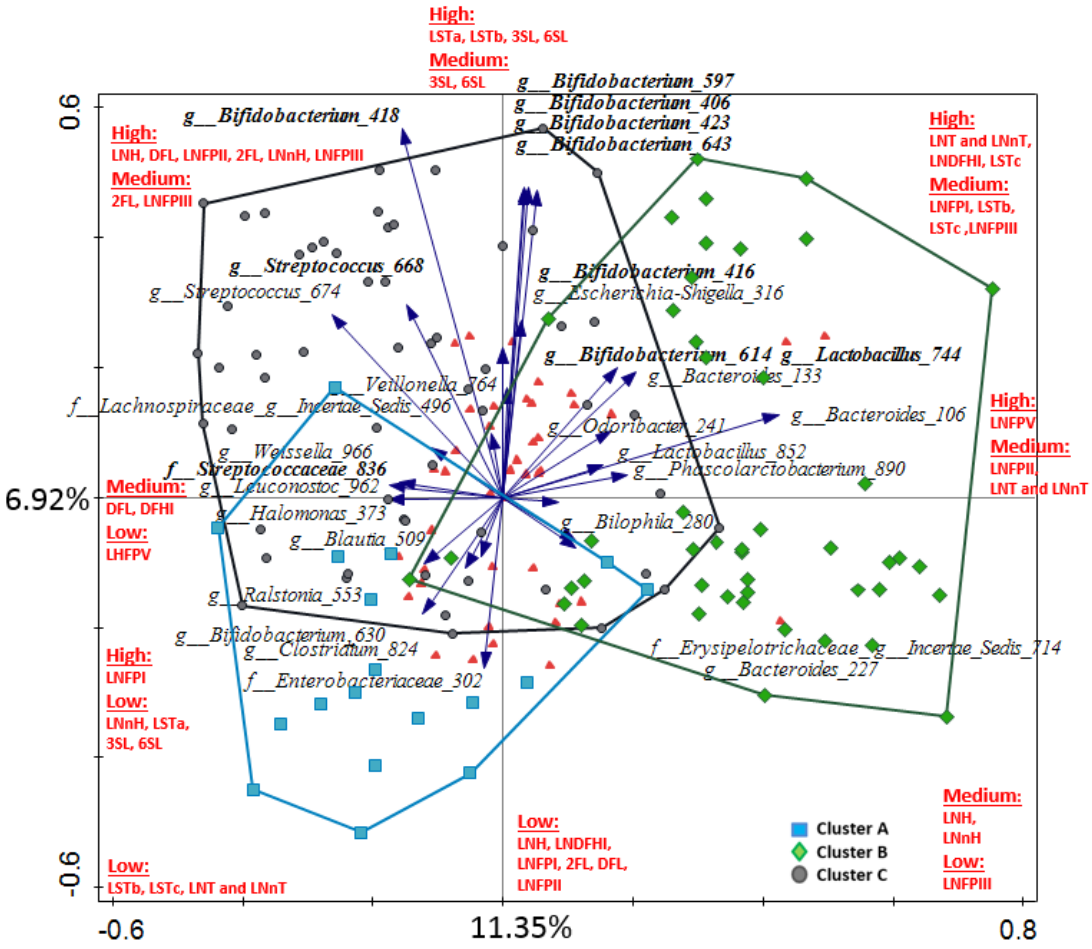

Figure 4.4. RDA showing the association between the degree of degradation of individual HMOs and microbial OTUs. OTUs which were significantly ($p<0.05$) increased in high-degrading infants for at least one of the HMOs are displayed. Taxa with FDR<0.05 are highlighted in bold. For more information on average relative abundance of the displayed OTUs in the study population and the detailed results of Kruskal-Wallis analyses, see Tables S4.3 and S4.5. Samples are color-coded based on microbiota cluster type assignment. Red triangles indicate consumption of each HMO, as summarized in red text.

Kruskal-Wallis analysis was used to compare microbiota composition at the OTU level between infants who were classified as either "high" or "low" consumers for each HMO measured in this study (Figure 4.6, Table S4.5). Infants who showed "high" consumption of 2′FL and DFL had significantly higher relative abundance of OTUs *Bifidobacterium* 418 and *Lactobacillus* 744 (FDR<0.05). In addition, "high" DFL consumption was associated with significantly higher relative abundance of *Bifidobacterium* OTUs 406, 643, 423, and 597. Similarly, infants who showed "high" consumption of LNT and LNnT, LNFPIII, LNFPII, LNH had a significantly higher relative abundance of *Bifidobacterium* OTUs 418, 406, 643, 423, and 597. Relative abundance of *Bifidobacterium* 418 was higher in infants who were efficient degraders of LNnH and LNDFHI, and *Bifidobacterium* 416 was associated with degradation of LNFPII, and *Bifidobacterium* 614 with LNFPII and LNH. We could not

detect statistically significant differences (with FDR<0.05) in the relative abundance of taxa between infants characterised as "high" and "low" degraders of LNFPI, LNFPV, 3´SL, LSTa, LSTb and LSTc.

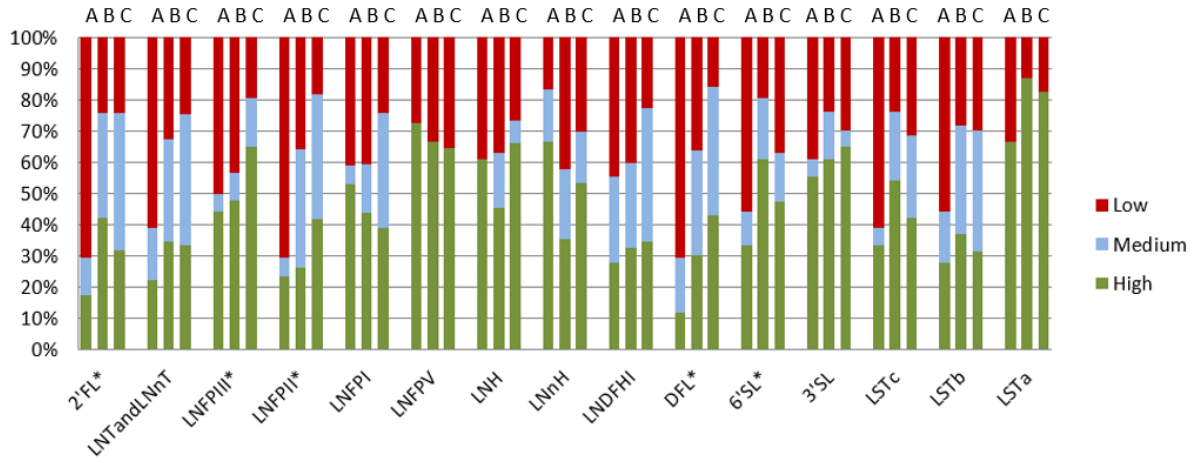

Figure 4.5. Proportion of infants showing either "high", "medium" or "low" HMO consumption levels, within each microbial cluster class: A, B, C. Significant differences in distribution as determined by Chi-square analysis are indicated with an asterisk.

Kruskal-Wallis test comparing infants who were classified as "high" and "medium" consumers for different HMOs showed no statistically significant differences in distribution of any of the OTUs *(data not shown)*. Infants classified as "low" and "medium" consumers showed significant differences (FDR<0.05) in relative abundance of *Bifidobacterium* 418 for 2´FL and LNFPII; *Bifidobacterium* OTUs 418, 643, 406 and 423 for DFL; *Bifidobacterium* 418 and *Bifidobacterium* 416 for LNFPIII; *Bifidobacterium* OTUs 418, 643, 406, 597 and 423 for LNT and LNnT *(data not shown)*. The observed patterns in the abundance of these taxa between the "low" and "medium" consumption classes mimicked those seen between "low" and "high" consumers for these HMOs (Table S4.5).

DISCUSSION

Fecal microbiota composition of healthy, one-month old breastfed infants was characterised in this study using 16S rRNA gene sequencing and revealed high inter-individual variability in the fecal microbiota composition. Microbial patterns could be observed in the sequencing data and the DMM analysis revealed that all infants could in fact be classified into three categories based on their fecal microbial profiles. Even though the high variability in infant fecal microbiota composition had been reported,[31] the occurrence of similar microbial clusters had been described previously in only one study.[32] The origin of these microbial patterns seen during infancy and their health implications are still unknown.

Several factors in early life may affect the dynamics of the developing infant GI microbiota. Our results showed that at about four weeks of age, mode of delivery and gender could explain the observed

variation in the microbiota composition. The effect of mode of delivery has been indicated in infants of similar age in another study.[33] Also, the effect of gender in infants of that age had been implied previously using qPCR/RTqPCR analyses for specific species/strain detection in feces.[34] Our study shows that gender associated differences can also be detected at the microbial community level in healthy breastfed infants.

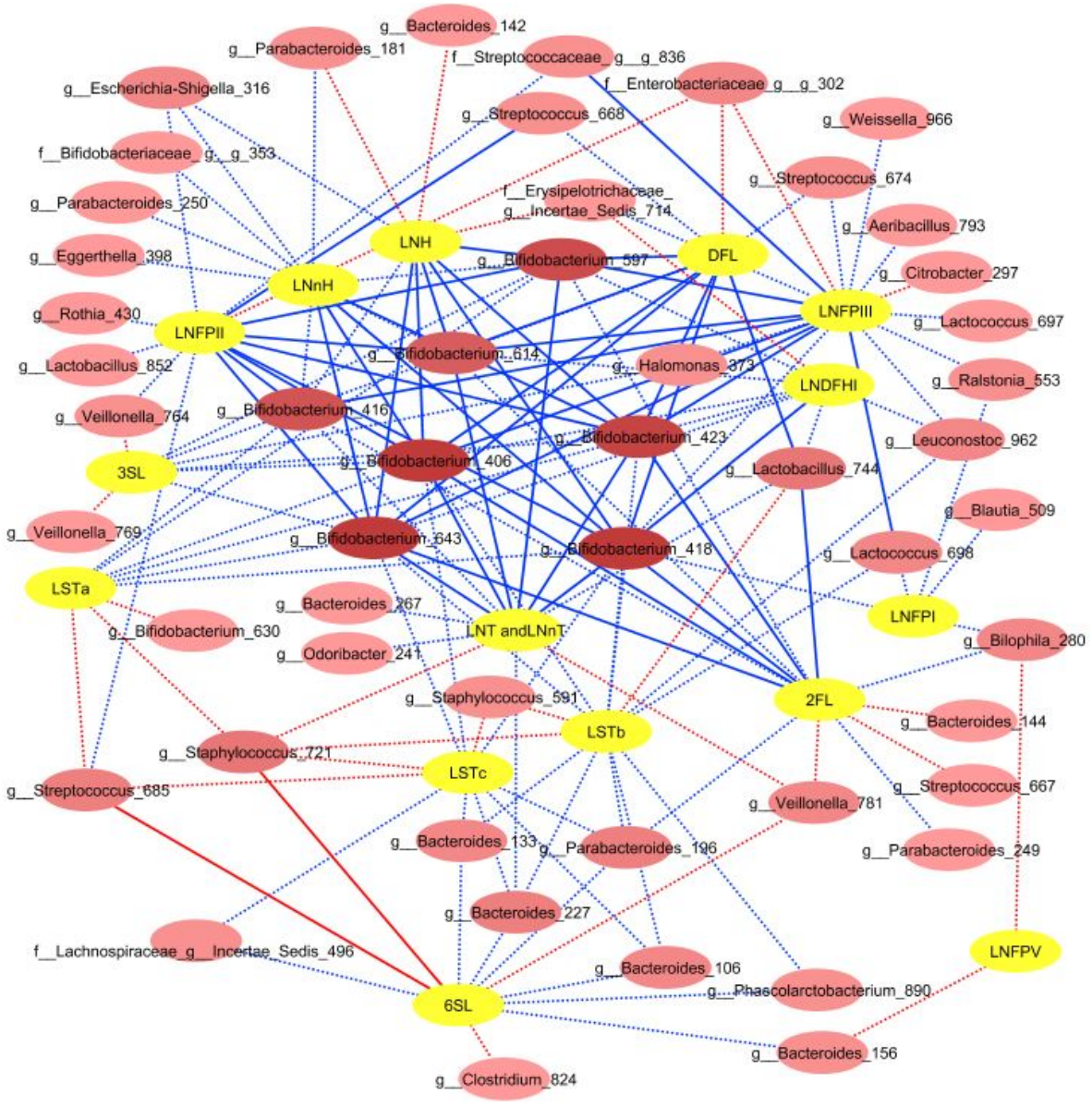

Figure 4.6. OTUs significantly associated with HMO consumption based on Kruskal-Wallis test including infants classified as “high” and “low” consumers for each HMO. Red lines indicate higher OTU relative abundance in relation to “low” HMO consumption, blue lines indicate higher relative abundance in relation to “high” consumption. OTU nodes which are connected with the highest number of HMOs are indicated by darker shades of pink. Dotted lines indicate associations with $p<0.05$, solid lines indicate associations with FDR<0.05.

Breast milk HMO levels have a limited effect on fecal microbiota composition in one-month old infants

One of the key factors shaping infant microbiota in early life is infant diet. Exclusively breastfed infants show high inter-individual variability, leading to the question whether these differences can be linked to the breast milk properties, including the unique HMO composition of the milk. It has been estimated that in healthy breastfed infants most of the ingested HMOs can reach the colon undigested.[35-37] These HMOs serve as an abundant and diverse carbon source available for bacterial fermentation.[5] Using PLS modelling we could detect statistically significant associations between infant fecal microbiota composition and milk LNFPI and 2´FL levels (Table S4.3). Both, LNFPI and 2´FL are neutral, fucosylated, unbranched HMOs with an α1,2 linkage joining fucose and galactose, and they were on average the third and fourth most abundant HMO measured in our dataset. The same association was found in another study of three-month-old breastfed infants, albeit using a much smaller cohort (n=16), which further indicated that LNFPI was positively associated with *Bacteroides* and *Bifidobacterium,* and 2´FL with *Bacteroides*.[7] Based on our RDA analysis (Figure 4.1), these two HMOs were associated with mixed microbiota cluster type A, which is characterised by low relative abundance of *Bacteroides* and *Bifidobacterium*. In addition, none of the other HMOs showed significant association with microbiota composition, but based on the vector positions (Figure 4.1), one could argue that higher concentrations of a number of different HMOs in maternal milk could be driving the infant microbiota away from cluster A, but not specifically towards clusters B or C. Some of the possible explanations could be that a combined effect of a number of HMO structures may be necessary to guide microbiota development in early life, or that stronger associations develop over a longer period, and that at one month of age the microbial profile of infants in our study was still largely in its transitional phase.[38-39] In addition, other HMO structures that were not measured in our study might also play a role, as well as the undigested residual lactose from breast milk, which in the lower intestine could also be readily fermented by the resident microbiota.

Other breast milk components, such as secretory IgA, lactoferrin, lysozyme, as well as the breast milk microbiota itself, are all likely to contribute to shaping the structure of microbial communities within infants' GI tract,[2, 40-43] and possibly concealing the effect of the individual HMOs. Over 200 different microbial genera had been identified in human milk to date, of which *Streptococcus* and *Staphylococcus* tend to be the most predominant taxa.[44-46] Vertical transfer between mother's breast milk and infant GI tract of viable populations of different microbial groups, including few species and strains of *Lactobacillus* and *Bifidobacterium* had also been confirmed and implied as one of the key factors in establishing infant's gut microbiota.[46] Breast milk also contains large amounts of lysozyme, up to 400µg/mL, which acts selectively on different microbial species, for example both *B. bifidum* and *B. longum* had been shown to be resistant to lysozyme, whereas clostridia, and many other Gram-positive and Gram-negative bacteria were highly susceptible.[43] Thus, the modulatory function of breastmilk

likely comes from the synergetic effect of all of its components, and HMOs alone might not be the driving factor in shaping the microbiota in early life.

HMO consumption patterns are associated with specific microbial groups

The matched analysis of HMO profiles from breast milk and fecal samples allowed us to estimate the *in vivo* HMO degradation levels within each infant, and to study in much greater detail the relationship between infant GI microbiota composition and HMO utilisation. Based on this novel approach we were able to classify infants into five HMO consumption groups. A previous pilot study reported only two types of HMO fecal profiles in infants, namely the neutral and the acidic profiles.[47] However, links between these utilization patterns and the infant fecal microbiota composition have remained largely unknown. Our data showed a strong significant association between "Non-specific" (or low) consumption and the microbial DMM cluster A, whereas the "Complete" consumption pattern was related with cluster C and "Specific-acidic" with cluster B (Figure 4.3). Thus, even though the GI tract microbial ecosystem is still in its early establishment phase at one month of age, we showed that the degradation of different types of HMOs was carried out by specific bacterial assemblages, which evolved mechanisms for efficient consumption of these abundant food components in milk.

Bifidobacteria are the main group of microorganisms in the lower GI tract of healthy infants, and also the main consumers of HMOs.[48-50] Our data supports this, as the most abundant and prevalent OTUs detected were of bifidobacterial origin (Table S4.2). Earlier *in vitro* studies showed highly specific metabolic behaviour of different bifidobacterial species and strains with respect to their ability to utilize different HMOs.[6] The most prevalent OTU in our set was *Bifidobacterium* 614*,* with an average relative abundance of 23.3 % and prevalence of 92%. NCBI blast analysis revealed that the OTU sequence (Table S4.6) matched several different species and strains of *Bifidobacterium,* including various strains of *B. longum (infantis)*, commonly found in the infant GI tract. Our analysis showed that *Bifidobacterium* 614 was associated with high *in vivo* consumption of various HMOs, specifically 2´FL, DFL, LNDFHI, LNFPII, LNFPIII, LNH, and LNT and LNnT (Table S4.5). The second most abundant OTU was *Bifidobacterium* 418*,* found in 45% of infants, with an average relative abundance of 6.6%. The NCBI blast analysis of OTU 418 returned a 100% match to several different strains of *Bifidobacterium bifidum* (DSM 20456 = ATCC 29521 = JCM 1255**,** NBRC100015, KCTC3202). The presence of *Bifidobacterium* 418 correlated strongly (FDR<0.05) with high consumption levels of 2´FL, LNT and LNnT, LNFPIII, LNFPII, LNH and its isomer LNnH, LNDFHI, and DFL, and with LNFPI, LSTa, LSTb and LSTc ($p<0.05$). *B. bifidum* has been shown to be an efficient HMO degrader in *in vitro* fermentation studies able to secrete glycosidases to degrade HMOs extracellularly, also making it possible for other species/subspecies to access the HMO degradation by-products and metabolites during cross-feeding.[6] *In vitro* studies showed that *B. bifidum* DSM 20456 could

efficiently degrade LNT, 2´FL, LNnT, LNFPI, LNFPII, LNFPIII, and LNDFHI, though the rate at which it was degrading these HMOs varied.[6]

Streptococcus and *Staphylococcus* OTUs showed an increase in relative abundance specifically in relation to high consumption of the fucosylated HMOs - DFL, LNFPII and LNFPIII. Although, *in vitro* studies showed that *Streptococcus* and *Staphylococcus* cannot effectively metabolize HMOs,[51] it has been shown that presence of HMOs may enhance growth of breast milk associated *Staphylococcus* by activating growth-promoting signalling and without being actively metabolised by this strain.[51] *Streptococcus* and *Staphylococcus* cross-feeding on HMO metabolites might also play a role though to date there are no studies documenting it.

Bacteroides and *Parabacteroides* (formerly also *Bacteroides*) are among the first dominant bacterial groups, next to bifidobacteria, established in the infant GI tract.[52] In general, members of the genus *Bacteroides* can degrade a broad range of simple and complex sugars, oligosaccharides, and polysaccharides, including HMOs, mucus glycans, and plant derived polysaccharides.[3] Like bifidobacteria, *Bacteroides* spp. can grow on milk glycans as a sole carbon source, however bifidobacteria might be better adapted to utilize a wider range of HMO structures, including simple HMO structures, as it has been shown for *B. infantis* and LNnT.[52] The trophic niche overlap might explain why infants with high levels of *Bifidobacterium,* such as those classified in the DMM cluster C tend to have lower levels of *Bacteroides*. On the other hand, *Bacteroides* has been shown to efficiently degrade mucus glycans, and because of the similarity of HMO structures and mucus glycans, some *Bacteroides* species could also effectively degrade specific HMOs by activating the mucus degrading pathway.[52-53] These species might be better at competing with bifidobacterial groups, especially those species of *Bifidobacterium* which might be less adapted for HMO utilisation. Our analysis indicated that infants who were efficient degraders of the sialylated (acidic) HMOs (3´SL, 6´SL, LSTa, LSTb, LSTc) and classified into "Specific acidic" consumption category were also often assigned to the *Bacteroides* dominated DMM cluster B (Figure 4.2). This was in agreement with another study which showed that among others, the HMOs 3´SL and 6´SL could be used as sole carbon source to support growth of *Bacteroides fragilis, Bacteroides vulgatus* and *Bacteroides thetaiotaomicron*.[54] Furthermore, these species, as well as few other species, including certain strains of *B. longum* were also shown to metabolise sialic acid.[55]

Finally, a biologically important microbial group commonly detected in infant feces are the lactobacilli. Our results show that high levels of degradation of 2´FL, DFL, LNDFHI, LNT and LNnT and LNFPII were significantly correlated with higher relative abundance of this group. Remarkably, the opposite effect was noted for LSTb (Figure 4.6). Unfortunately, the two interesting lactobacilli OTUs which were identified in our data, namely *Lactobacillus* 744 and *Lactobacillus* 852, had sequence reads which returned a hundred percent match to more than a dozen species and strains of lactobacilli in the

NCBI blast analysis, making it impossible to unequivocally identify these populations to the species level. Several *Lactobacillus* spp. have been frequently isolated from neonate feces, including: *L. fermentum, L. casei, L. paracasei, L. delbrueckii, L. gasseri, L. rhamnosus* and *L. plantarum*.[56] These lactobacilli were shown to be unable to efficiently ferment HMOs *in vitro*,[57-58] however, they have been shown to grow well on HMO metabolites *in vitro*.[58] Thus, via the cross-feeding with other bacteria, for example bifidobacteria, it is possible that HMO degradation can be linked with higher relative abundance of lactobacilli, and other community members in the microbial ecosystem within GI tract.

The roles of different HMOs in the development of infant GI tract microbiota, the occurrence of microbial clusters, and the nutritional and health consequences relating to the existence of different trophic networks that are built upon the degradation of specific HMOs are still mostly unknown. Our results confirmed the central role of bifidobacteria in the HMO breakdown and provided an insight into different microbial assemblages in healthy, one-month old infants. Furthermore, carrying out the analyses at the OTU level allowed us to uncover a higher level of detail showing that, for example, bifidobacteria were associated with both clusters B and C (17% and 41%, respectively), but the distribution of specific bifidobacterial OTUs within these two clusters was not identical *(data not shown)*. Until now, few *in vitro* studies demonstrated that closely related species or strains might exhibit different metabolic activities and be involved in a range of complementary trophic interactions. Future studies should strive to identify the species or strains that are present in the infant gut and to build understanding on the interactions between these species. In the future, a better understanding on how the bacterial assemblages form *in vivo* and the identification of the key species and their roles in driving the colonisation, as well as their effects on the host could be translated into practical applications within infant nutrition and health.

CONCLUSIONS

GI tract microbiota composition in one-month old breastfed infants is shaped by multiple factors, including HMOs. We observed a direct link between 2′FL and LNFP I in breast milk and microbial community composition in this cohort, but it is likely that the infant microbiota is shaped through the combined effect of all HMOs and other bioactive components in breast milk. We showed that breast milk HMO degradation patterns differed among infants belonging to different microbial cluster types. Degradation of specific HMOs could be correlated with an increase in relative abundance of various phylotypes (OTUs) within the genus *Bifidobacterium*, and to lesser extent within the genera *Bacteroides* and *Lactobacillus*.

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SUPPORTING INFORMATION

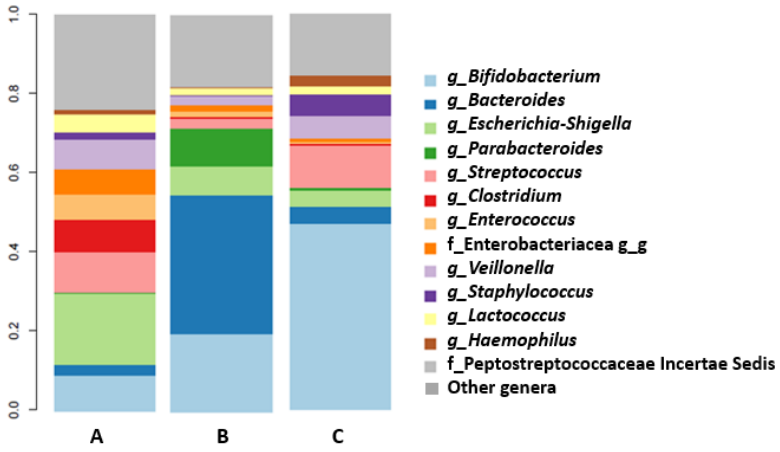

Figure S4.1. Microbial cluster composition based on DMM modelling of KOALA samples

Table S4.1. Infant – mother pair demographics

Infant/Mother pairs (n=121)		**n**
Delivery mode	Normal Vaginal	100
	Assisted Vaginal	11
	C-Section	10
Delivery Place	Home	72
	Hospital	49
Gender	Female	59
	Male	62
Gestation (weeks)	Mean ± SEM	40.24 ±1.16
	Minimum	37.14
	Maximum	42.86
Birth Weight (g)	Mean ± SEM	3651 ±43.48
	Minimum	2140
	Maximum	4780
Age at collection (days)	Mean ± SEM	32.56 ±0.5
	Minimum	24
	Maximum	56
Health at collection	Sick	3
	Not Sick	118
Medication Use at time of collection	Antibiotics (Baby)	0
	Antimycotics (Baby)	2
	Antibiotics (Mother)	2

Table S4.2. Average relative abundance and prevalence of the OTUs shared by at least 5% of infants in this study.

OTU	Average RA (%)	Prevalence (%)
f_Bifidobacteriaceae_64_g_g	0.03	11
f_Bifidobacteriaceae_353_g_g	0.01	7
g_Bifidobacterium_614	23.32	92
g_Bifidobacterium_418	6.64	45
g_Bifidobacterium_622	1.33	50
g_Bifidobacterium_643	0.13	29
g_Bifidobacterium_406	0.13	29
g_Bifidobacterium_423	0.12	27
g_Bifidobacterium_597	0.11	26
g_Bifidobacterium_416	0.03	16
g_Bifidobacterium_630	0.02	9
g_Bifidobacterium_356	0.02	7
Other *g_Bifidobacterium* (n=26)	0.12	26
g_Bacteroides_106	8.61	59
g_Bacteroides_149	4.58	22
g_Bacteroides_144	2.04	24
g_Bacteroides_159	1.94	23
g_Bacteroides_157	0.78	25
g_Bacteroides_125	0.60	26
g_Bacteroides_133	0.49	20
g_Bacteroides_156	0.33	12
g_Bacteroides_126	0.30	20
g_Bacteroides_142	0.20	6
g_Bacteroides_139	0.08	5
g_Bacteroides_88	0.08	5
g_Bacteroides_267	0.05	5
g_Bacteroides_227	0.02	5
Other *g_Bacteroides* (n=50)	1.26	27
g_Parabacteroides_181	3.29	37
g_Parabacteroides_179	2.00	18
g_Parabacteroides_249	0.53	19
g_Parabacteroides_250	0.05	5
g_Parabacteroides_196	0.02	6
Other *g_Parabacteroides* (n=25)	0.79	22
g_Lactobacillus_852	1.03	26
g_Lactobacillus_744	0.78	36
Other g_Lactobacillus (n=22)	0.48	12
f_Streptococcaceae_836_g_g	0.06	18
g_Streptococcus_685	2.46	74
g_Streptococcus_668	0.18	19
g_Streptococcus_674	0.16	36
g_Streptococcus_684	0.15	6
g_Streptococcus_667	0.13	12
Other *g_Streptococcus* (n=14)	0.67	13
g_Lactococcus_698	0.35	50
g_Lactococcus_697	0.03	10
g_Lactococcus_696	0.01	6
f_Enterobacteriaceae_302_g_g	3.83	31
g_Escherichia-Shigella_328	11.70	72
g_Escherichia-Shigella_316	0.04	17
g_Staphylococcus_721	2.08	58
g_Staphylococcus_591	0.02	7
g_Veillonella_781	0.83	48
g_Veillonella_769	0.50	20
g_Veillonella_764	0.27	22
g_Veillonella_776	0.09	10
g_Haemophilus_368	0.88	49
g_Clostridium_824	0.85	7
g_Clostridium_885	0.48	9
g_Blautia_509	0.47	7
g_Blautia_471	0.04	5
g_Phascolarctobacterium_890	0.21	7
g_Phascolarctobacterium_892	0.12	5
f_Lachnospiraceae *Incertae_Sedis*_487	0.77	9
f_Lachnospiraceae *Incertae_Sedis*_496	0.05	5
f_Lachnospiraceae *Incertae_Sedis*_941	0.19	7
g_Rothia_430	0.16	22
g_Sutterella_565	0.79	21
g_Leuconostoc_962	0.06	19
g_Enterococcus_842	0.20	18
g_Halomonas_373	0.06	18
g_Haemophilus_371	0.08	17
g_Bilophila_280	0.12	13
g_Flavonifractor_737	0.33	11
g_Aeribacillus_793	0.04	10
g_Collinsella_528	0.10	10
g_Eggerthella_398	0.02	7
g_Ralstonia_553	0.02	7
g_Weissella_966	0.01	7
g_Odoribacter_241	0.10	6
g_Citrobacter_297	0.02	5
g_Negativicoccus_759	0.02	5
g_Varibaculum_438	0.02	5
f_Erysipelotrichaceae *Incertae_Sedis*_714	0.69	7
f_Ruminococcaceae_6_g_g	0.31	6
f_Coriobacteriaceae_929	0.03	8
f_Peptostreptococcaceae *Incertae_Sedis*_394	0.02	9
Remaining Other OTUs (n=312)	6.81	87
Total (n=531)	100.0	100

Table S4.3. PLS analysis results showing the association of milk and faecal HMOs with microbiota of 121 infants. p<0.05 highlighted in bold.

	MILK		FAECES	
Compound	R2	p-value	R2	p-value
2′FL	0.449014	**0.01**	0.474618	**0.008**
LNT and LNnT	0.365672	0.321	0.486463	**0.005**
LNFPIII	0.411369	0.1	0.334021	0.489
LNFPII	0.428876	0.059	0.538998	**0.001**
LNFPI	0.453009	**0.006**	0.352368	0.395
LNFPV	0.361059	0.29	0.411458	0.268
LNH	0.296281	0.711	0.419855	0.311
LNnH	0.372147	0.267	0.407214	0.372
LNDFHI	0.348777	0.378	0.425054	**0.02**
DFL	0.375545	0.193	0.484093	**0.001**
6′SL	0.308867	0.679	0.307907	0.651
3′SL	0.330597	0.538	0.335586	0.548
LSTc	0.40498	0.101	0.284052	0.797
LSTb	0.3488	0.387	0.366729	0.303
LSTa	0.37228	0.267	0.303566	0.667

Table S4.4. Chi-square analysis results. There is no association between mother secretor status and infant microbial DMM cluster type (χ^2 (2) = 5.13; p=0.08)

	Secretor +			Secretor -			Totals
Cluster	Observed	Expected	χ^2	Observed	Expected	χ^2	
A	31	33.45	0.18	15	12.55	0.48	46
B	17	13.09	0.17	1	4.91	3.11	18
C	40	41.45	0.05	17	15.55	0.14	57
Totals	88			33			121

Table S4.5. OTUs significantly different (p<0.05) in relative abundance between infants classified as high or low consumers of specific HMOs. Differences in relative abundance with FDR<0.05 are indicated in bold.

Taxonomy	OTU	HMO	p	FDR	High	Low
g_Bacteroides	106	6′SL	0.008	0.137	0.1021	0.0355
	106	LSTb	0.010	0.192	0.0745	0.0279
	106	LSTc	0.006	0.129	0.1047	0.0288
	133	6′SL	0.005	0.136	0.0079	0.0002
	133	LSTb	0.005	0.131	0.0078	0.0003
	133	LSTc	0.002	0.129	0.0087	0.0002
	142	LNH	0.034	0.276	0.0001	0.0056
	144	2′FL	0.017	0.168	0.0269	0.0271
	156	6′SL	0.021	0.263	0.0020	0.0010
	156	LNFPV	0.040	0.848	0.0005	0.0047
	227	6′SL	0.040	0.296	0.0004	0
	227	LNTandLNnT	0.022	0.176	0.0004	0
	227	LSTb	0.022	0.227	0.0006	0
	227	LSTc	0.030	0.273	0.0004	0
	267	LNTandLNnT	0.041	0.280	0.0011	0
g_Bifidobacterium	**406**	**2′FL**	**0.001**	**0.018**	**0.0015**	**0.0002**
	406	3′SL	0.033	0.410	0.0014	0.0005
	406	**DFL**	**0.000**	**0.001**	**0.0015**	**0**
	406	LNDFHI	0.002	0.059	0.0015	0.0004
	406	**LNFPII**	**0.000**	**0.000**	**0.0024**	**0**
	406	**LNFPIII**	**0.000**	**0.003**	**0.0015**	**0.0001**
	406	**LNH**	**0.000**	**0.000**	**0.0017**	**0.0001**
	406	**LNTandLNnT**	**0.000**	**0.000**	**0.0017**	**0.0001**
	406	LSTa	0.005	0.147	0.0013	0.0001
	406	LSTb	0.022	0.227	0.0012	0.0004
	406	LSTc	0.038	0.279	0.0011	0.0004
	416	2′FL	0.029	0.194	0.0004	0.0001
	416	3′SL	0.048	0.441	0.0003	0.0001
	416	DFL	0.011	0.108	0.0003	0
	416	**LNFPII**	**0.000**	**0.002**	**0.0006**	**0**
	416	**LNFPIII**	**0.003**	**0.030**	**0.0003**	**0**
	416	LNH	0.006	0.072	0.0004	0.0000
	416	LNTandLNnT	0.008	0.091	0.0004	0.0000
	416	LSTa	0.026	0.294	0.0003	0
	418	**2′FL**	**0.000**	**0.004**	**0.0668**	**0.0211**
	418	**DFL**	**0.000**	**0.000**	**0.0742**	**0.0022**
	418	**LNDFHI**	**0.000**	**0.005**	**0.0704**	**0.0265**
	418	LNFPI	0.036	0.588	0.0737	0.0160
	418	**LNFPII**	**0.000**	**0.000**	**0.0967**	**0**
	418	**LNFPIII**	**0.000**	**0.000**	**0.0757**	**0.0031**
	418	**LNH**	**0.000**	**0.000**	**0.0884**	**0.0108**
	418	**LNTandLNnT**	**0.000**	**0.000**	**0.0930**	**0.0105**
	418	LSTa	0.002	0.147	0.0654	0.0254
	418	LSTb	0.001	0.070	0.0717	0.0217
	418	LSTc	0.006	0.129	0.0699	0.0299

	423	**2′FL**	**0.001**	**0.022**	**0.0014**	**0.0001**
	423	3′SL	0.027	0.410	0.0012	0.0005
	423	**DFL**	**0.000**	**0.002**	**0.0012**	**0**
	423	LNDFHI	0.007	0.110	0.0014	0.0004
	423	**LNFPII**	**0.000**	**0.000**	**0.0022**	**0**
	423	**LNFPIII**	**0.000**	**0.005**	**0.0013**	**0.0001**
	423	**LNH**	**0.000**	**0.000**	**0.0016**	**0.0001**
	423	**LNTandLNnT**	**0.000**	**0.000**	**0.0016**	**0.0001**
	423	LSTa	0.008	0.169	0.0012	0.0001
	423	LSTb	0.030	0.254	0.0011	0.0003
	597	2′FL	0.005	0.071	0.0013	0.0001
	597	3′SL	0.016	0.410	0.0012	0.0004
	597	**DFL**	**0.000**	**0.002**	**0.0012**	**0**
	597	LNDFHI	0.019	0.255	0.0013	0.0003
	597	**LNFPII**	**0.000**	**0.000**	**0.0021**	**0**
	597	**LNFPIII**	**0.001**	**0.010**	**0.0013**	**0.0001**
	597	**LNH**	**0.000**	**0.001**	**0.0015**	**0.0001**
	597	**LNTandLNnT**	**0.000**	**0.002**	**0.0015**	**0.0001**
	597	LSTa	0.012	0.190	0.0011	0.0001
	614	2′FL	0.016	0.168	0.2053	0.1097
	614	**DFL**	**0.001**	**0.017**	**0.2532**	**0.1215**
	614	LNDFHI	0.039	0.377	0.2223	0.1482
	614	**LNFPII**	**0.000**	**0.002**	**0.2718**	**0.1180**
	614	LNFPIII	0.008	0.065	0.2290	0.1357
	614	**LNH**	**0.000**	**0.006**	**0.2418**	**0.1266**
	614	**LNTandLNnT**	**0.002**	**0.024**	**0.2272**	**0.1167**
	630	LSTa	0.017	0.231	0.0001	0.0004
	643	**2′FL**	**0.001**	**0.018**	**0.0015**	**0.0002**
	643	3′SL	0.035	0.410	0.0014	0.0005
	643	**DFL**	**0.000**	**0.001**	**0.0014**	**0**
	643	LNDFHI	0.002	0.059	0.0015	0.0004
	643	**LNFPII**	**0.000**	**0.000**	**0.0023**	**0**
	643	**LNFPIII**	**0.000**	**0.003**	**0.0015**	**0.0001**
	643	**LNH**	**0.000**	**0.000**	**0.0017**	**0.0001**
	643	**LNTandLNnT**	**0.000**	**0.000**	**0.0017**	**0.0001**
	643	LSTa	0.005	0.147	0.0013	0.0001
	643	LSTb	0.019	0.227	0.0012	0.0004
	643	LSTc	0.036	0.279	0.0012	0.0005
g_Bilophila	280	2′FL	0.047	0.270	0.0019	0.0002
	280	LNFPI	0.024	0.588	0.0017	0
	280	LNFPV	0.047	0.848	0.0005	0.0016
g_Blautia	509	LNFPI	0.024	0.588	0.0122	0
g_Citrobacter	297	LNFPIII	0.018	0.103	0.0000	0.0004
g_Clostridium	824	6′SL	0.038	0.296	0.0022	0.0193
g_Escherichia-Shigella	316	LNFPII	0.029	0.210	0.0005	0.0002
	316	LNH	0.044	0.327	0.0004	0.0002
g_Halomonas	373	3′SL	0.017	0.410	0.0003	0.0012
	373	LNFPIII	0.008	0.065	0.0009	0.0000

g_Lactobacillus	**744**	**2′FL**	**0.000**	**0.004**	**0.0080**	**0.0013**
	744	**DFL**	**0.000**	**0.001**	**0.0086**	**0.0002**
	744	LNDFHI	0.006	0.110	0.0057	0.0016
	744	LNTandLNnT	0.035	0.255	0.0064	0.0048
	744	LSTb	0.031	0.254	0.0036	0.0049
	852	LNFPII	0.013	0.102	0.0207	0.0019
g_Lactococcus	697	LNFPIII	0.015	0.095	0.0004	0
	698	LNFPI	0.007	0.588	0.0045	0.0010
	698	**LNFPIII**	**0.001**	**0.019**	**0.0044**	**0.0008**
	698	LSTb	0.047	0.273	0.0045	0.0020
g_Leuconostoc	962	LNDFHI	0.042	0.377	0.0005	0.0003
	962	LNFPIII	0.011	0.074	0.0007	0.0001
	962	LSTb	0.040	0.269	0.0008	0.0003
g_Odoribacter	241	LNTandLNnT	0.022	0.176	0.0018	0
g_Parabacteroides	181	LNH	0.012	0.125	0.0130	0.0437
	196	2′FL	0.040	0.251	0.0005	0
	196	6′SL	0.026	0.263	0.0004	0
	196	LSTb	0.042	0.269	0.0003	0
	196	LSTc	0.019	0.244	0.0004	0
	249	2′FL	0.029	0.194	0.0111	0.0002
g_Phascolarctobacterium	890	6′SL	0.026	0.263	0.0036	0
	890	LSTb	0.043	0.269	0.0052	0.0001
g_Ralstonia	553	LNFPI	0.041	0.588	0.0004	0
	553	LNFPIII	0.034	0.162	0.0003	0
g_Rothia	430	LNFPII	0.036	0.226	0.0025	0.0003
g_Staphylococcus	591	LSTb	0.022	0.227	0	0.0003
	591	LSTc	0.018	0.244	0	0.0002
	721	**6′SL**	**0.001**	**0.048**	**0.0087**	**0.0334**
	721	LNTandLNnT	0.047	0.295	0.0099	0.0181
	721	LSTa	0.030	0.294	0.0155	0.0297
	721	LSTb	0.002	0.080	0.0092	0.0239
	721	LSTc	0.005	0.129	0.0078	0.0231
g_Streptococcus	667	2′FL	0.021	0.168	0	0.0005
	668	DFL	0.026	0.194	0.0034	0.0001
	668	**LNFPII**	**0.002**	**0.024**	**0.0036**	**0.0001**
	674	DFL	0.016	0.144	0.0019	0.0003
	674	LNFPIII	0.042	0.181	0.0018	0.0006
	685	**6′SL**	**0.001**	**0.048**	**0.0138**	**0.0289**
	685	LNFPII	0.031	0.210	0.0194	0.0168
	685	LSTa	0.033	0.294	0.0165	0.0360
	685	LSTc	0.021	0.244	0.0150	0.0253
g_Veillonella	764	3′SL	0.035	0.410	0.0018	0.0030
	764	LNFPII	0.043	0.246	0.0038	0.0011
	769	3′SL	0.015	0.410	0.0011	0.0117
	781	2′FL	0.019	0.168	0.0018	0.0097
	781	6′SL	0.008	0.137	0.0022	0.0121
	781	LNTandLNnT	0.010	0.100	0.0023	0.0118
g_Weissella	966	LNFPIII	0.034	0.162	0.0002	0

g_Aeribacillus	793	LNFPIII	0.010	0.074	0.0005	0
f_Enterobacteriaceae_g_g	302	DFL	0.024	0.192	0.0106	0.0782
f_Enterobacteriaceae_g_g	302	LNFPII	0.007	0.067	0.0014	0.0717
f_Enterobacteriaceae_g_g	302	LNFPIII	0.042	0.181	0.0182	0.0565
f_Erysipelotrichaceae	714	LNDFHI	0.041	0.377	0.0001	0.0227
f_Erysipelotrichaceae	714	LNFPIII	0.023	0.123	0.0121	0
f_Erysipelotrichaceae	714	LNH	0.034	0.276	0.0001	0.0200
f_Lachnospiraceae	496	6′SL	0.040	0.296	0.0009	0
f_Lachnospiraceae	496	LSTc	0.030	0.273	0.0010	0
f_Streptococcaceae_g_g	**836**	**LNFPIII**	**0.002**	**0.019**	**0.0008**	**0.0000**
f_Streptococcaceae_g_g	836	LNFPII	0.046	0.249	0.0009	0.0002

Table S4.6. DNA sequences of OTUs that differ in relative abundance between high and low consumer groups.

Taxonomy	**OTU**	**Sequences (5'-3')**
g_Aeribacillus	793	TACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGGCCAGAGAGCCGCCTTCGCCACTGGTG
g_Bacteroides	106	TACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGATGGATGTTTAAGTCAGTTGCCTGTTTGATACCCACACTTTCGAGCCTCAATGTCAGTTGCAGCTTAGCAGGCTGCCTTCGCAATCGGAG
	133	TACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGGCGGATTGTTAAGTCAGTTGCCTGTTTGATACCCACACTTTCGAGCATCAGCGTCAGTTACACTCCAGTGAGCTGCCTTCGCAATCGGAG
	142	TACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGGCGGGTTGTTAAGTCAGTTGCCTGTTTGATACCCACACTTTCGAGCATCAGCGTCAGTTACAATCCAGTAAGCTGCCTTCGCAATCGGAG
	144	TACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGGTGGACAGTTAAGTCAGTTGCCTGTTTGATACCCACACTTTCGAGCATCAGTGTCAGTTGCAGTCCAGTGAGCTGCCTTCGCAATCGGAG
	156	TACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGGTGGATTGTTAAGTCAGTTGCCTGTTTGATACCCACACTTTCGAGCATCAGTGTCAGTAACAGTCTAGTGAGCTGCCTTCGCAATCGGAG
	227	TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGATGGATGTTTAAGTCAGTTGCCTGTTTGATACCCACACTTTCGAGCCTCAATGTCAGTTGCAGCTTAGCAGGCTGCCTTCGCAATCGGAG
	267	TACGGAGGATTCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGATGGATGTTTAAGTCAGTTGCCTGTTTGATACCCACACTTTCGAGCCTCAATGTCAGTTGCAGCTTAGCAGGCTGCCTTCGCAATCGGAG
g_Bifidobacterium	406	TACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTAACGGCCCAGAGACCTGCCTTCGCCATTGGTG
	416	TACGTAGGGCGCAAGCGTTATCCGGATTTATTGGGCGTAAAGGGCTCGTAGGCGGCTCGTCGCGTCCGGTGCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTAACGGCCCAGAGACCTGCCTTCGCCATCGGTG
	418	TACGTAGGGCGCAAGCGTTATCCGGATTTATTGGGCGTAAAGGGCTCGTAGGCGGCTCGTCGCGTCCGGTGCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTGACGGCCCAGAGACCTGCCTTCGCCATCGGTG
	423	TACGTAGGGCGCAAGCGTTATCCGGATTTATTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTAACGGCCCAGAGACCTGCCTTCGCCATTGGTG
	597	TACGTAGGGTGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGCTCGTCGCGTCCGGTGCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTGACGGCCCAGAGACCTGCCTTCGCCATCGGTG

	614	TACGTAGGGTGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGTT CGTCGCGTCCGGTGCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTAACGGC CCAGAGACCTGCCTTCGCCATTGGTG
	630	TACGTAGGGTGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGTT CGTCGCGTCCGGTGCCTGTTTGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTAACGGC CCAGAGACCTGCCTTCGCCATTGGTG
	643	TACGTAGGGTGCAAGCGTTATCCGGATTTATTGGGCGTAAAGGGCTCGTAGGCGGCT CGTCGCGTCCGGTGCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTGACGGC CCAGAGACCTGCCTTCGCCATCGGTG
g_Bilophila	280	TACGGAGGGTGCAAGCGTTAATCGGAATCACTGGGCGTAAAGCGCACGTAGGCGGC TTGGTAAGTCAGGGGCCTGTTTGCTACCCACGCTTTCGCACCTCAGCGTCAGTTACCG TCCAGGTGGCCGCCTTCGCCACCGGTG
g_Blautia	509	TACGTAGGGGGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGT GTGGCAAGTCTGATGCCTGTTTGCTCCCCACGCTTTCGAGCCTCAACGTCAGTTACCG TCCAGTAAGCCGCCTTCGCCACTGGTG
g_Citrobacter	297	TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT CTGTCAAGTCGGATGCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCG TCCAGGGGGCCGCCTTCGCCACCGGTA
g_Clostridium	824	TACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGTAGGCGGA TTTTTAAGTGGGATGCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAG TCCAGAAAGTCGCCTTCGCCACTGGTG
g_Escherichia-Shigella	316	TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT TTGTTAAGTCAGATGCCTGTTCGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCG TCCAGGGGGCCGCCTTCGCCACCGGTA
g_Halomonas	373	TACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGT CTGATAAGCCGGTTGCCTGTTTGCTACCCACGCTTTCGCACCTCAGCGTCAGTGTCAG TCCAGAAGGCCGCCTTCGCCACTGGTA
g_Lactobacillus	744	TACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTT TTTTAAGTCTGATGCCTGTTCGCTACCCATGCTTTCGAGCCTCAGCGTCAGTTACAGA CCAGACAGCCGCCTTCGCCACTGGTG
	852	TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGTGCAGGCGGTT CAATAAGTCTGATGCCTGTTCGCTACCCATGCTTTCGAGCCTCAGCGTCAGTTGCAGA CCAGAGAGCCGCCTTCGCCACTGGTG
g_Lactococcus	697	TACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTT TAATAAGTCTGATGCCTGTTTGCTACCCACGCTTTCGAGCCTCAGTGTCAGTTACAGT CCAGAGAGCCGCTTTCGCCACCGGTG
	698	TACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTT TATTAAGTCTGGTGCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGTGTCAGTTACAGG CCAGAGAGCCGCTTTCGCCACCGGTG
g_Leuconostoc	962	TACGTATGTCCCGAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACGGTT GATTAAGTCTGATGCCTGTTTGCTACCCACACTTTCGAGCCTCAACGTCAGTTGTTGT CCAGTAAGCCGCCTTCGCCACTGGTG
g_Odoribacter	241	TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGTT TATTAAGTTAGTGGCCTGTTCGCTACCCACGCTCTCGTGCATCAGCGTCAGTTACAGT CTGGTAAGCTGCCTTCGCTATCGGAG
g_Parabacteroides	181	TACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGCC TTTTAAGTCAGCGGCCTGTTTGATCCCCACGCTTTCGTGCATCAGCGTCAGTCATGGC TTGGCAGGCTGCCTTCGCAATCGGGG
	196	TACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGTGGTG ATTTAAGTCAGCGGCCTGTTTGATCCCCACGCTTTCGTGCTTCAGTGTCAGTTATGGT TTAGTAAGCTGCCTTCGCAATCGGAG
	249	TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGTGGTG ATTTAAGTCAGCGGCCTGTTTGATCCCCACGCTTTCGTGCTTCAGTGTCAGTTATGGT TTAGTAAGCTGCCTTCGCAATCGGAG
g_Phascolarctobacterium	890	TACGTAGGTGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCATGTAGGCGGCT TAATAAGTCGAGCGCCCGTTCGCTACCCTGGCTTTCGCATCTCAGCGTCAGACACAG TCCAGAAAGGCGCCTTCGCCACTGGTG
g_Ralstonia	553	TACGTAGGGTCCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTT GTGCAAGACCGATGCCTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAGTGTTATC CCAGGGGGCTGCCTTCGCCATCGGTA
g_Rothia	430	TACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTT TGTCGCGTCTGCTGCCTGTTCGCTCCCCATGCTTTCGCTTCTCAGCGTCAGTTACAGC CCAGAGACCTGCCTTCGCCATCGGTG

g_Staphylococcus	591	TACGTAGGGTGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTT TTTTAAGTCTGATGCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAGA CCAGAAAGTCGCCTTCGCCACTGGTG
	721	TACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTT TTTTAAGTCTGATGCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAGA CCAGAAAGTCGCCTTCGCCACTGGTG
g_Streptococcus	667	TACGTAGGTCCCGAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTT AGATAAGTCTGAAGCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAA GCCAGAGAGCCGCTTTCGCCACCGGTG
	668	TACGTAGGTCCCGAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTT AGATAAGTCTGAAGCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAG ACCAGAGAGCCGCTTTCGCCACCGGTG
	674	TACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTT AGATAAGTCTGAAGCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAA GCCAGAGAGCCGCTTTCGCCACCGGTG
	685	TACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTT TGATAAGTCTGAAGCCTGTTCGCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAG ACCAGAGAGCCGCTTTCGCCACCGGTG
g_Veillonella	764	TACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGA TAGGTCAGTCTGTCTCCCGTTCGCTCCCCTGGCTTTCGCGCCTCAGCGTCAGTTTTCG TCCAGAAAGTCGCCTTCGCCACTGGTG
	769	TACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGA TCAGTCAGTCTGTCTCCCGTTCGCTCCCCTGGCTTTCGCGCCTCAGCGTCAGTTTTCGT CCAGAAAGTCGCCTTCGCCACTGGTG
	781	TACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGA TTGGTCAGTCTGTCTCCCGTTCGCTCCCCTGGCTTTCGCGCCTCAGCGTCAGTTTTCGT CCAGAAAGTCGCCTTCGCCACTGGTG
g_Weissella	966	TACGTATGTTCCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACGGTT ATTTAAGTCTGAAGCCTGTTTGCTACCCACACTTTCGAGCCTCAACGTCAGTTACAGT CCAGAAAGCCGCCTTCGCCACTGGTG
f_Enterobacteriaceae	302	TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT CTGTCAAGTCGGATGCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTG TCCAGGGGGCCGCCTTCGCCACCGGTA
f_Erysipelotrichaceae *Incertae_Sedis*	714	TACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGGGAGCAGGCGGC AGCAAGGGTCTGTGGCCTATTTGCTCCCCACGCTTTCGGGACTGAGCGTCAGTTGCA GGCCAGATCGTCGCCTTCGCCACTGGTG
f_Lachnospiraceae *Incertae_Sedis*	496	TACGTAGGGGGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGC GAAGCAAGTCTGAAGCCTGTTTGCTCCCCACGCTTTCGAGCCTCAACGTCAGTTATC GTCCAGTAAGCCGCCTTCGCCACTGGTG
f_Streptococcaceae	836	TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTT CATTAAGTCTGATGCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAGT CCAGAGAGCCGCTTTCGCCTCCGGTG

Chapter 5

The association between breastmilk oligosaccharides and fecal microbiota in healthy breastfed infants at two, six, and twelve weeks of age

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ABSTRACT

Several factors affect gut microbiota development in early life, among which breastfeeding plays a key role. We followed 24 mother-infant pairs to investigate the associations between concentrations of selected human milk oligosaccharides (HMOs) in breastmilk, infant feces, and the fecal microbiota composition in healthy, breastfed infants at two, six and 12 weeks of age. Lactation duration had a significant effect on breastmilk HMO content which decreased with time, except for 3-fucosyllactose (3FL) and Lacto-*N*-fucopentaose III (LNFP III). We confirmed that microbiota composition was strongly influenced by infant age and was associated with mode of delivery and breastmilk LNFP III concentration at two weeks, with gender, delivery mode, and concentrations of 3'sialyllactose (3´SL) in milk at six weeks, and gender and Lacto-*N*-hexaose (LNH) in milk at 12 weeks of age. Correlations between levels of individual breastmilk HMOs and relative abundance of OTUs found in infant feces, including the most predominant *Bifidobacterium* OTUs, were weak and varied with age. However, the level of degradation of HMOs in the infant gut increased with age and was strongly and positively correlated with relative abundance of OTUs within genera *Bifidobacterium, Parabacteroides*, *Escherichia-Shigella, Bacteroides, Actinomyces, Veillonella,* Lachnospiraceae *Incertae Sedis,* and Erysipelotrichaceae *Incertae Sedis,* indicating importance of these taxa for HMO metabolism *in vivo*.

INTRODUCTION

Microbial colonisation of the infant gastrointestinal (GI) tract begins before or at birth, and in healthy, breastfed infants it progresses towards a microbial community that is dominated by bifidobacteria and is metabolically adapted to thrive on human milk.[1-2] Many host specific and environmental factors have been identified to play a role in the development of human GI tract microbiota,[3] and understanding of the impact of these factors and their associated health outcomes has been a growing area of research during recent years.[4-5] Breastfeeding is essential for optimal colonization and maturation of the infant GI microbiota; breastmilk not only provides an important medium for the transfer of microbes between the mother and her infant, but it also contains high concentrations of prebiotic human milk oligosaccharides (HMOs) which further facilitate microbial colonisation.[6] High concentrations of HMOs in breastmilk are believed to be the main force shaping the bifidobacteria dominated GI ecosystem in breastfed infants, although up to date only few *in vivo* studies have been able to demonstrate this.[2, 7] The type and amount of the secreted HMOs in breastmilk are genetically predetermined and are highly variable among mothers and across lactation stages.[8-11] Up to date over 200 different HMO structures had been identified,[12] and this variability in the chemical and structural conformations of HMOs might be biologically relevant. Colonisation and growth of highly specialised taxa and consequently the progression of the microbial succession within the infant gut might be supported by specific HMO types. The specific utilisation of individual HMOs by infant's colon microbiota measured in the feces during the first month after birth has been reported by Albrecht et al.[13-14] Microbiota composition during infancy plays an important role in processes that have life-long health consequences, such as educating the immune system, metabolic programming, and facilitating nutrient utilisation.[6] Thus, it is important to gain better understanding of the biological function of the different HMOs in shaping microbiota development and function. Until now, there have been no reports on longitudinal studies investigating the establishment of infant GI microbiota in relation to changes in breastmilk HMO composition, and previous studies focused mainly on *in vitro* fermentation of HMOs by fecal bacterial inoculum, or by fecal isolates.[15] Little is known about how the GI microbial community development affects an infant's ability to digest different HMOs, and how this ability changes during early infancy. In order to fill these knowledge gaps, we followed a cohort of 24 full term, healthy, breastfed Dutch infants and analysed maternal breastmilk and infant fecal samples collected at two, six and 12 weeks post-delivery. We measured 18 highly abundant HMOs, which account for approximately 86 % of the total HMOs in breastmilk,[16] including 13 neutral and five acidic HMOs (Table 7.2).[17] We measured the association between the concentrations of these breastmilk HMOs and infant fecal microbial composition through the first three months of life. We also investigated how the microbiota composition correlated with the degradation of HMOs *in vivo*.

MATERIALS & METHODS

Sample collection

The infants included in this study were born at term (after 37 weeks of gestation) from single pregnancies, were healthy and had no congenital abnormalities related to growth, did not receive oral or systemic antibiotic treatments, were exclusively breastfed during the study period, and had the maternal breastmilk and infant fecal samples available from each study time point. The samples originated from the BINGO (Dutch acronym for Biological Influences on Baby's Health and Development) cohort, which is an ongoing longitudinal study investigating prenatal predictors of infant health and development. This study and all its experimental protocols were approved by and carried out in accordance with the ethical committee of the Faculty of Social Sciences of the Radboud University [ECSW2014-1003-189]. Parents and/or legal guardians of all participating infants were asked to sign an informed consent form and were free to withdraw from the study at any point. The study design and infant recruitment criteria can be found at http://www.bingo-onderzoek.nl/deelname/. Both, the infant fecal samples and the maternal breastmilk samples were collected between years 2015-2016, within a 48-hour period, by the mothers at home, at two, six and 12 weeks post-partum. Breastmilk samples (approximately 20ml) were collected in the morning before feeding the infant, into clean, sterile collection cups as previously described.[49] Mothers were asked to wash hands, breasts and nipples before collecting the sample by hand, or in case of mechanical collection, to first sterilize the breast pump compartments by boiling. Stool samples were collected from infants' diapers using sterile stool collection vials (80×16.5mm; cat#:80.623.022, Sarstedt; Nümbrecht, Germany). Mothers were asked to save all fecal sample up to one-third of the vial. Milk and fecal samples were stored by the participants in their home freezers until collected by the experimenter within a week after the last collection time point. Subsequently all milk and fecal samples were stored at -80 °C until further processing and analysis. Samples were analysed for breastmilk HMOs, corresponding fecal HMOs, and microbiota composition.

HMO analysis in breastmilk and feces

Eighteen different HMO structures were analysed in milk and infant feces, including 13 neutral HMOs (2′FL, 3FL, DFL, LNDFH I, LNDFH II, LNFP I, LNFP II, LNFP III, LNFP V, LNH, LNnH, pLNH, LNT and LNnT) and five acidic HMOs (3´SL, 6´SL, LST a, LST b, LST c) (Table 7.2). The HMOs were extracted, purified by solid phase extraction (SPE), and quantified by using porous graphitized carbon- liquid chromatography - mass spectrometry (PGC-LC-MS),[50-52] or by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).[53]

DNA extraction, amplification of 16S rRNA genes and sequencing

Total bacterial DNA was extracted from 0.1-0.15g of feces using the double bead-beating procedure and the Maxwell® 16 Total RNA system (Promega; Wisconsin, USA) customized with Stool Transport and Recovery Buffer (STAR; Roche Diagnostics Corporation, Indianapolis, IN) for the use with the DNA samples, as previously described.[54] The resulting DNA templates (20 ng) were used for subsequent PCR amplification of the V4 region of 16S ribosomal RNA (rRNA) genes using barcoded primers 515F-n (5'-GTGCCAGCMGCCGCGGTAA-) and 806R-n (5'-GGACTACHVGGGTWTCTAAT) following conditions described previously.[54] Seventy unique barcode tags were used in each library.[55] Negative control blanks were included during DNA extraction and PCR to check for any possible contamination. PCR products were purified, 100 ng of each barcoded sample was added to an amplicon pool and the pools were adjusted to 100 ng/µL final concentration. The libraries were sent for adapter ligation and Illumina HiSeq2000 sequencing at GATC-Biotech, Konstanz, Germany.[54]

Data analysis

The 16S rRNA sequencing data analysis was carried out using the NG-Tax analysis pipeline with standard parameters.[55] Filtered libraries contained only read pairs with perfectly matching barcodes that were subsequently used to separate reads by sample. OTUs were assigned using an open reference approach and the SILVA_111_SSU 16S rRNA gene reference database (https://www.arb-silva.de/).[56] Microbial composition data was expressed as a relative abundance of each OTU obtained with NG-Tax.

Statistical analyses

Milk and fecal HMOs concentrations were measured in µg per mL of milk or µg per gram of feces. Readout values were normalised for each time point separately around mean using the Probabilistic Quotient Normalization (PQN) method in R (version 3.3.2) to correct for sample to sample variability due to naturally occurring differences in milk dilutions. Changes in HMO concentrations between study time points were assessed using one-way repeated measures ANOVA for HMOs for normally distributed data sets as assessed with Shapiro-Wilk's normality test. Differences between not-normally distributed HMO sets were tested using nonparametric Kruskal-Wallis test. We estimated average daily volumes of ingested breastmilk based on literature data to be 480 g at week two, 580 g at week six, and 630 g at week 12 of life.[18] These values were then used to calculate the daily amount of each HMO consumed by infants in our study at each time point.

Microbial composition data was expressed as relative abundance (RA) of each OTU obtained in the NG-Tax pipeline. OTUs which had a prevalence of less than 5% across all samples were removed and their values were summarized as "Other OTUs". Alpha diversity index determinations (Shannon,

Chao1, and PD Whole Tree) were carried out in QIIME on rarefied data with OTU cut-off of 3380 reads per sample.[57] Spearman correlations were calculated using R to evaluate associations between OTU members of the fecal microbial community, and between the fecal OTUs and milk or fecal HMO concentrations. We only reported associations which passed the correlation threshold of ±0.3 and significance of $p<0.05$. Unconstrained (PCA) and constrained (RDA) multivariate analyses were carried out in Canoco5 on log transformed HMO concentrations data and on microbial OTU level relative abundance data with the significance assessed using the Monte Carlo permutation test at 499 random permutations.[58] For better image clarity, only the top twenty best fitting taxa were displayed on the PCA and RDA plots. These taxa were those for which the highest percentage of variation in the relative abundance data was explained by the ordination axes. The vectors corresponding to these taxa point towards the ordination plane where samples containing higher relative abundance of these taxa are located and the lengths of these vectors equal to R- squared calculated by dividing the taxa scores by their SD.[58] The explanatory variables used in the RDA included infant age at the time of collection, estimated amounts of the 18 milk HMO ingested during a 24 h period (mg/24 h) or fecal HMOs (μg/g of feces), gender, place and mode of delivery, maternal secretor status, and if an infant was sick at the time of sample collection, as recalled by the mother. Degradation of each breastmilk HMO was calculated as a ratio of HMO concentration in infants' feces and the concentration of the same HMO measured in mothers' milk. If a given HMO was not detected in milk, the consumption score was not included in the analysis, and if the concentration in feces exceeded the amount detected in milk, the infant was assigned to the "low" category for that HMO. Resulting values (ratios) and the natural breaks in the data were then used for separating and assign infants to either a "low", "medium", or a "high" consumption category for each HMO at each time point. The association between fecal microbiota composition and the assignment of each infant to a "low", "medium", or "high" consumption category for each HMO were investigated with RDA in Canoco5, with significance assessed using a permutation test.[58] Differentially abundant OTUs were then identified by comparing the microbial abundance data of infants assigned into "high" and "low" consumption groups and for each individual HMO using Kruskal-Wallis analysis in QIIME with a significance cut-off set at $FDR<0.05$.[57]

Nucleotide sequences

BINGO data sets cannot be made publicly available due to the data being part of an ongoing longitudinal study. Parts of the data are available to the research community for scientific collaborations upon request to Prof. dr. C. de Weerth at: Department of Cognitive Neuroscience, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, 6525 HR Nijmegen, the Netherlands, e-mail: Carolina.deWeerth@radboudumc.nl.

RESULTS

The infants included in this study were delivered vaginally (n=22) or via C-Section (n=2). For one of the infants, data on gender, weight and place of delivery were not reported. The remaining infants included 13 boys and ten girls, and their average body weight at birth was 3,536g and ranged from 3,024g to 4,140g. Five infants were born at a clinic, seven at home, nine at a hospital, and for two infants birth started at home but was completed at a hospital. Six infants had a common cold and one had diarrhoea during the study. The actual ages in days ranged within each collection time point; at two weeks' time point infant age ranged from seven to 20 d (*M* = 14.28, *SD* = 2.06), at six weeks it was 41 d to 44 d (*M* = 41.12, *SD* = 1.42), and at 12 weeks it was 80 d to 93 d (*M* =84.42, *SD* =2.48).

The Illumina HiSeq sequencing resulted in a total number of 8,550,719 (range: 3,383-421,482 per sample, *M* = 118,760, *SD* = 86,261, *SE* = 10,166) sequencing reads that passed the quality filtering (Figure S5.1). A total number of 411 Operational Taxonomic Units (OTUs) were identified of which 83 OTUs were found in more than 5%, or at least four samples (Table S5.1).

The development of infant fecal microbiota

Alpha diversity analyses showed no statistically significant differences in richness and diversity in fecal microbiota of infants at different time points when tested with Kruskal-Wallis and Wilcoxon tests (Figure S5.2). The Principle Component Analysis (PCA) showed that composition of fecal microbiota of individual infants was diverse, but despite the high inter-individual variation, the observed changes in microbial profiles were directional and progressed with time towards bifidobacteria dominated communities (Figure 5.1). Redundancy analysis (RDA) was performed on the OTU level fecal microbiota data, and together all explanatory variables could explain 56.58% of variation in infant microbiota, with the statistically significant effect (False Discovery Rate (FDR)<0.05) of infant age (4.3% variation explained), gender (6.1%), place (8.2%) and mode of delivery (3.5%), secretor status (1.7%), and the breastmilk HMO concentrations of 6'-sialyllactose (6´SL) (5.1%), 3'-sialyllactose (3´SL) (3.2%), 2'-fucosyllactose (2′FL) (2.9%), Lacto-*N*-difucohexaose II (LNDFH II) (2.6%), and 3FL (2%). RDA was repeated separately at each time point, and showed that infant fecal microbiota composition was associated (FDR>0.05; p<0.05) at two weeks of age with mode of delivery (6.7% variation explained) and breastmilk LNFP III levels (8.1%), at six weeks with gender (6.9%), mode of delivery (6.3%) and breastmilk 3´SL levels (7.3%), and at 12 weeks with gender (7.2%) and breastmilk LNH levels (6.6%).

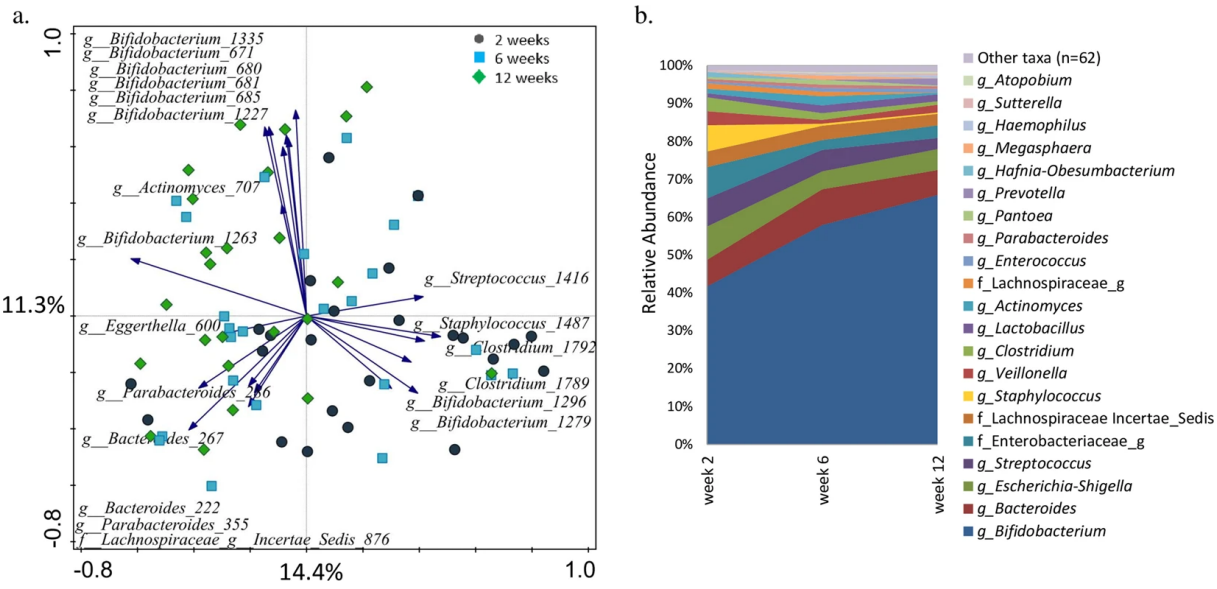

Figure 5.1. Infant fecal microbiota composition. a) PCA showing spatial distribution of samples from 24 infants based on their fecal microbiota composition at two, six and 12 weeks of age and the twenty best fitting OTUs; b) Average relative abundance of genus level taxa in the 24 infants at different time points. In case the taxonomic assignment could not be made at genus level, the lowest classifiable taxonomy assignment is used, and the unidentified genus is indicated with "_g".

Milk HMO content and changes during lactation

HMO profiles of each breastmilk sample were analysed (Table S5.2a), and samples from six mothers revealed consistent lack of 2´FL and low levels of Lacto-N-fucopentaose I (LNFP I), Lacto-N-difucohexaose I (LNDFH I) and Difucosyllactose (DFL) (non-secretors). PCA of milk HMOs showed a clear separation of samples in relation to collection time point postpartum and secretor status (Figure 5.2a). This finding was confirmed with RDA which showed that collection time point and secretor status had a significant effect on the types and levels of the HMOs present, explaining respectively 16.7% and 2.5% of variation in the data. When analysed separately for each time point, neither the mode of delivery nor maternal stress measured via saliva cortisol, were significantly associated with the HMO levels in mothers' milk (data not shown). The average concentration (µg/ml) of the HMOs measured in the milk decreased with time of lactation, except for the concentrations of 3FL and LNFP III, which were secreted at higher amounts at later time points (Figure 5.2b, Table S5.2a). After adjusting the HMO concentrations for the literature-based averages of the amounts of milk consumed (480 g, 580 g and 630 g at weeks two, six and 12, respectively),[18] the estimated average HMO intake remained stable in the first three months of life (Figure 5.2c).

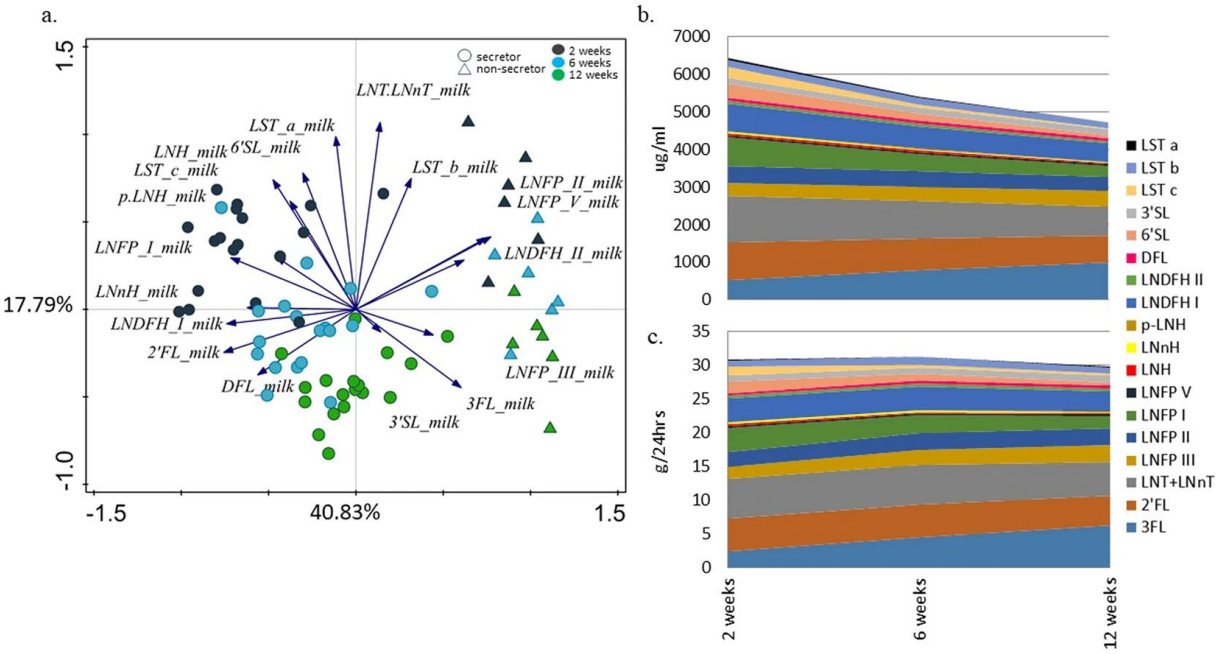

Figure 5.2. Breastmilk HMO profiling. a) PCA showing separate clustering of breastmilk samples of 24 mothers at two, six and 12 weeks of lactation. The 3FL, LNFP III, and 3SL (3SL between week six and 12 only) are positively associated with duration of lactation; b) Average concentration of different HMOs found in the breastmilk samples from the 24 mothers included in the study at two, six and 12 weeks of lactation; c) Estimated average daily intake of each HMO in the 24 infants at two, six and 12 weeks of age.

Correlation between milk HMOs and infant fecal microbiota

The estimated daily intake (g/day) of each HMO in each infant, and the relative abundance of OTUs for the corresponding fecal samples were correlated to examine the potential link between the levels of ingested HMOs and the abundance of different microbial taxa in infant feces (Figure 5.3). The strongest positive correlations were detected between few of the *Staphylococcus* OTUs and milk levels of 6´SL, Sialyl-lacto-N-tetraose c (LST c), Sialyl-lacto-N-tetraose a (LST a), LNH, Lacto-N-neohexaose (LNnH), and LNFP I. *Streptococcus* OTUs were positively correlated with 3FL, LNFP III, and Para-lacto-N-hexaose (pLNH). Positive correlations were also found between OTUs within the genus *Actinomyces* and 3´SL, and *Enterococcus* OTUs and 3FL, Lacto-N-fucopentaose II (LNFP II), LNFP III, Lacto-N-fucopentaose V (LNFP V) and LNDFH II. No significant correlations were found between the most predominant *Bifidobacterium* OTU 1263 and any of the milk HMOs. Spearman correlation analysis at each separate time point (Figure S5.3) showed that at two weeks of age three bifidobacterial OTUs were positively associated with LNH, pLNH and LNFP I, and the main *Bifidobacterium* OTU 1263 was negatively associated with LNFP II and LNFP III (Figure S5.3a). At six weeks three different low abundance bifidobacterial OTUs were positively associated with LNFP V, LNnH, 6´SL, Sialyl-lacto-N-tetraose b (LST b) and LST c (Figure S5.3b), and at 12 weeks LNnH, LNFP II, and LNDFH II showed a low positive correlation with two low abundance bifidobacterial OTUs. Overall, the

associations did not exceed ± 0.6, there was no consistency in the type (positive or negative), or strength (passing threshold of ± 0.3, $p<0.05$) of associations between specific OTU-HMO pairs when data from all time points was combined, or when individual time points were analysed separately.

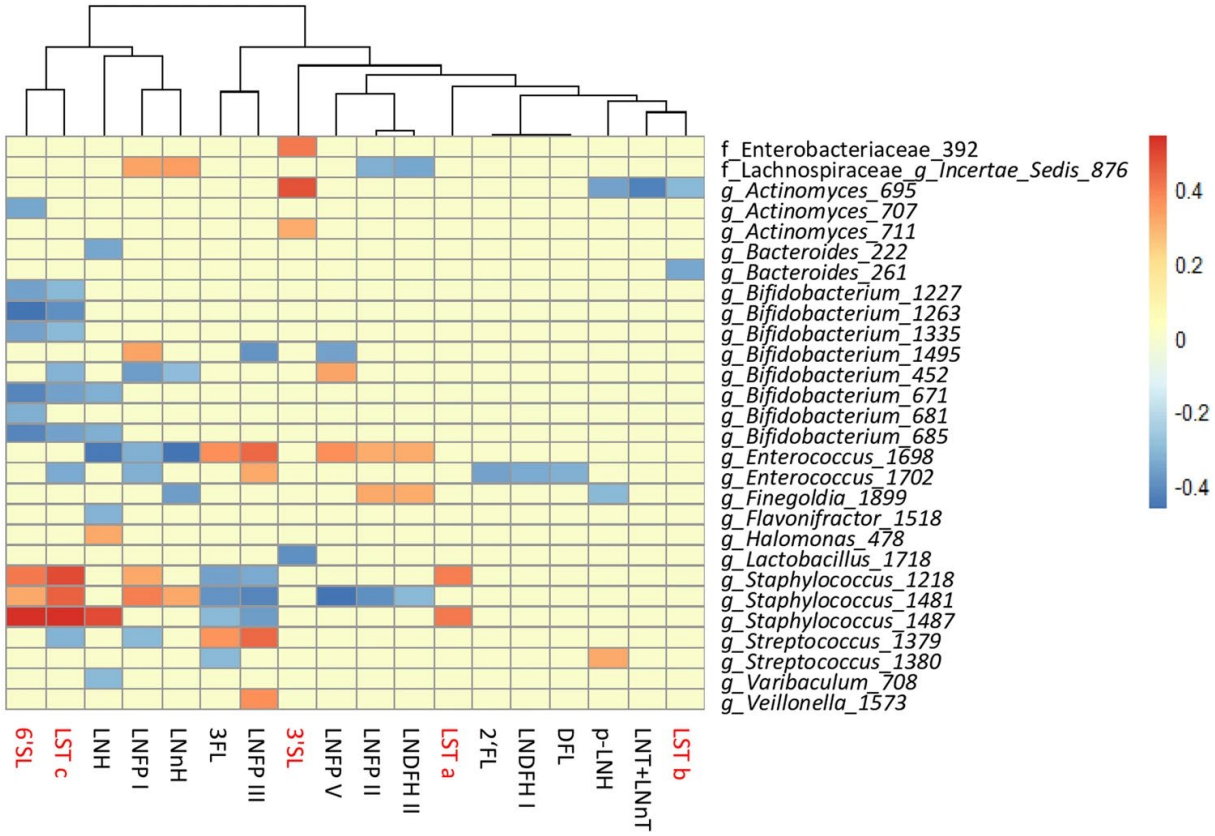

Figure 5.3. Statistically significant ($p<0.05$) Spearman correlations (correlation threshold ±0.3) between estimated daily intake of different milk HMOs and fecal microbiota composition at OTU level of 24 infants across the study duration. Positive associations are indicated in red, negative in blue, and correlations that did not pass significance or correlation threshold are marked in yellow. The names of acidic HMOs are highlighted in red.

Correlation between infant fecal microbiota and HMOs excreted in infant feces

Undigested milk HMOs were secreted in infant feces and their concentrations varied between infants and across study time points (Table S5.2b). Our hypothesis was that aside of being directly dependent on the amounts of the HMOs ingested with the milk, the fecal HMO concentrations could serve as an indicator of the utilisation of different HMOs by infant GI tract microbiota. PCA (Figure 5.4a) showed that samples clustered by infant age, and to lesser extent also by the maternal secretor status. RDA confirmed these findings showing a significant (FDR<0.05) effect of infant age (7.5% explained variation) and maternal secretor status (7.3%) on the HMO concentrations in infant feces (Figure not shown). The concentrations of all HMOs in feces decreased with infant age, with the exception of 3FL (Figure 5.4b). Spearman correlation between the fecal HMO concentrations and fecal microbiota showed that the majority of statistically significant negative associations were between bifidobacterial OTUs and thirteen different HMOs (Figure 5.5). The main *Bifidobacterium* OTU 1263

was negatively correlated with nine different HMOs, of which LNH, LNFP V, and the LNT / LNnT group showed strongest correlations, suggesting an important role of *Bifidobacterium* OTU 1263 in consumption of these HMOs in the infant GI tract. Positive associations were also observed between various OTUs of *Streptococcus, Staphylococcus, Clostridium* and *Escherichia-Shigella* and thirteen different HMOs. Spearman correlation analysis at each separate time point showed that at two weeks of age the strongest negative correlations were found between various HMOs and highly abundant *Bifidobacterium* OTU 1263 and 681 (Figure S5.4a). At week six, an additional six bifidobacterial OTUs showed negative correlations with pLNH, LNH, LNFP II and III. At both time points *Bifidobacterium* OTU 1263 was the only one correlating negatively with 3FL, possibly highlighting the unique link between this HMO and the major *Bifidobacterium* OTU during the initial stages of the development of GI tract microbiota (Figure S5.4b). At 12 weeks of age, the HMO concentrations in feces were very low or no longer within the detectable range, leading to overall less clear correlation patterns between bifidobacteria and fecal HMOs. Still, at 12 weeks of age *Bifidobacterium* OTU 1263 correlated strongly with 2′FL, LNDFH II and LNFP V (Figure S5.4c).

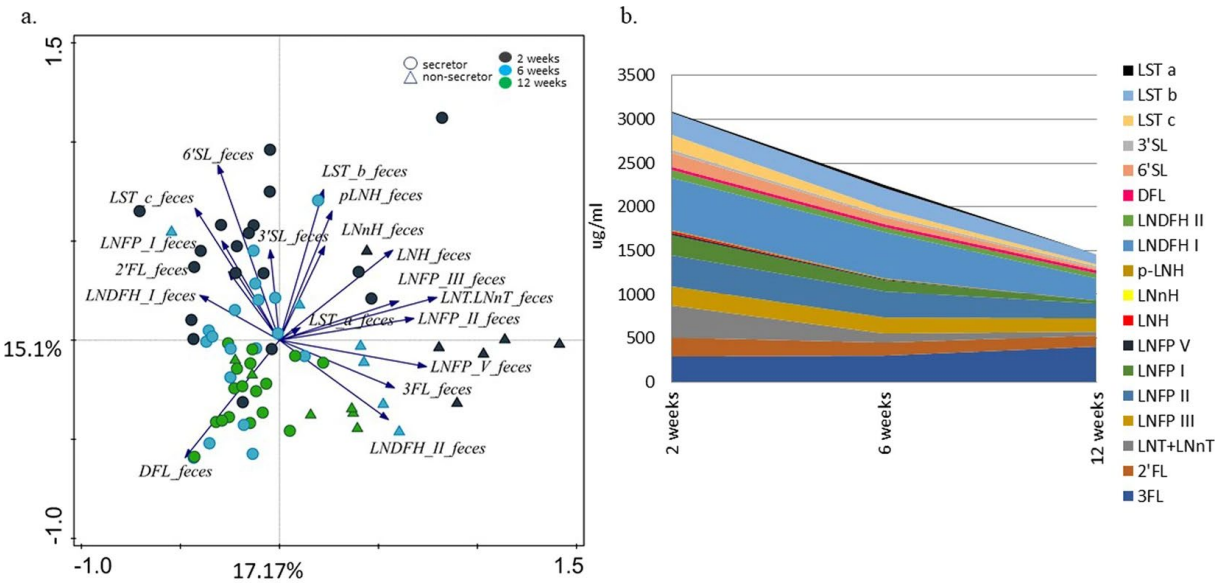

Figure 5.4. Fecal HMO profiling. a) PCA showing spatial distribution of fecal samples of 24 infants at two, six and 12 weeks of age based on the concentrations of residual HMOs detected in infant feces, and indicating mother's secretor status; b) Average concentration of different HMOs (µg/ml of feces) found in infant feces decreases with infant age, except for 3FL, which for most infants increased with age.

In order to account for the initial availability of the different HMOs in the milk, we calculated ratios between each fecal and milk HMO, for each mother-infant pair at each time point. The resulting ratios were then used to estimate the consumption level for each HMO as either "high", "medium" or "low" based on tertiles. RDA showed that high consumption was associated with older infant age and higher levels of bifidobacteria. Low consumption of pLNH, 2′FL, LNH, LNnH, LNTandLNnT, LNFP I and V, 3´SL and LNDFH I, medium consumption of LNDFH II, LNFP II and 6´SL, and high consumption of

pLNH, LNH, LNnH, LNFP I and V, LST a, LNTandLNnT, and 3´SL were significantly associated with the microbial composition (FDR<0.05; Figure 5.6). Overall, high consumption was detected in association with various bifidobacterial OTUs including the most predominant *Bifidobacterium* OTU 1263, and several OTUs within genera *Parabacteroides*, *Escherichia-Shigella, Bacteroides, Actinomyces, Veillonella,* and Erysipelotrichaceae *Incertae Sedis* (Figures 5.5 and 5.6). The result was confirmed when relative abundance of OTUs between infants assigned into a low and high consumption groups for each HMO were compared (Table S5.3) showing significantly higher relative abundance of various bifidobacterial OTUs, and in most cases the most predominant OTU *Bifidobacterium* 1263 in the high consumption group (Figure 5.7). Interestingly, high consumption of 6´SL, DFL, LST a and LST c was not significantly linked with the presence of bifidobacteria, but instead was linked with other bacterial groups (p<0.05; Table S5.3).

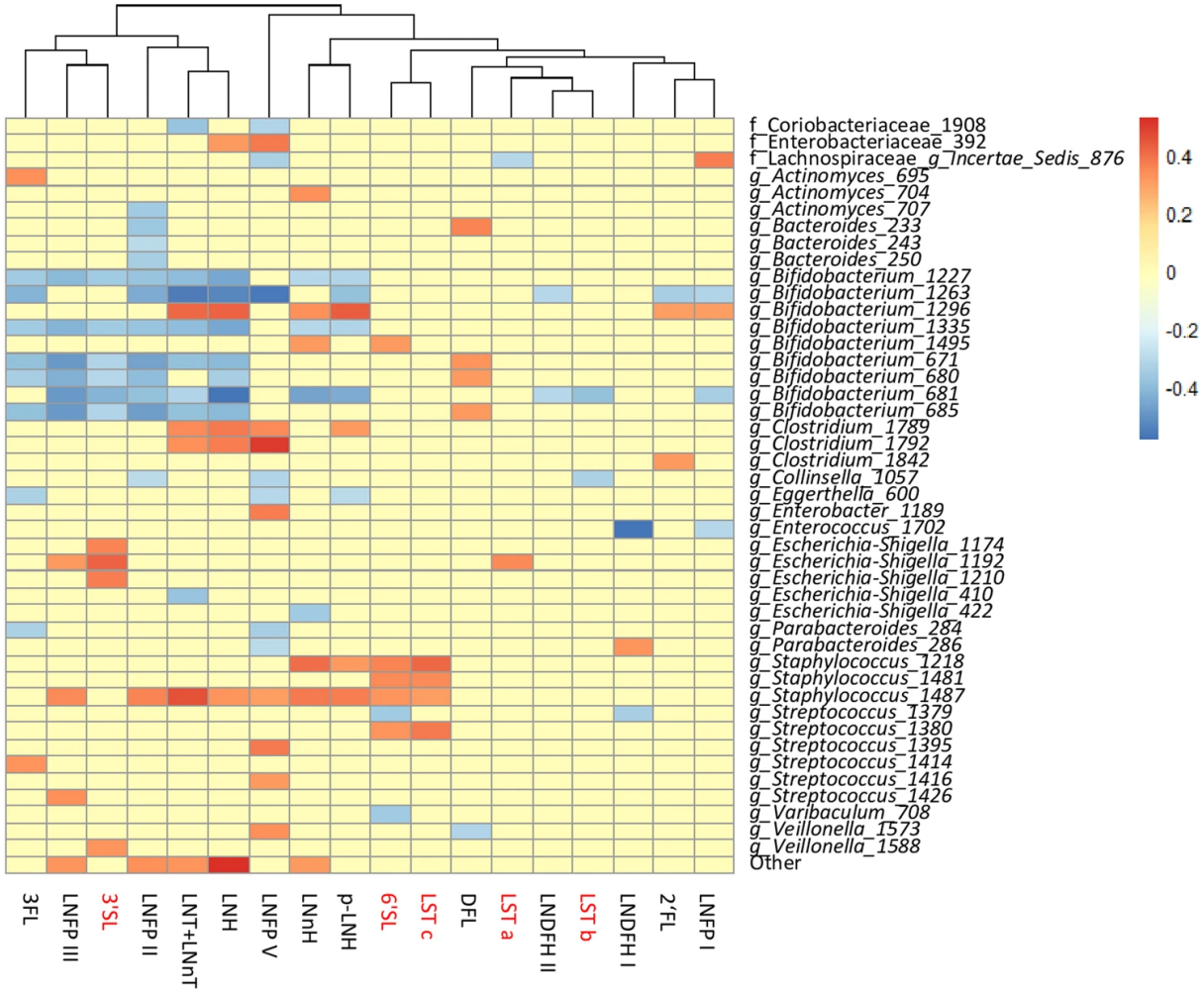

Figure 5.5. Statistically significant (p<0.05) Spearman correlations (correlation threshold ±0.3) between HMOs detected in infant feces and fecal microbiota composition at OTU level of 24 infants across the study duration. Positive associations are indicated in red, negative in blue, and correlations that did not pass significance or correlation threshold are marked in yellow. The names of acidic HMOs are highlighted in red.

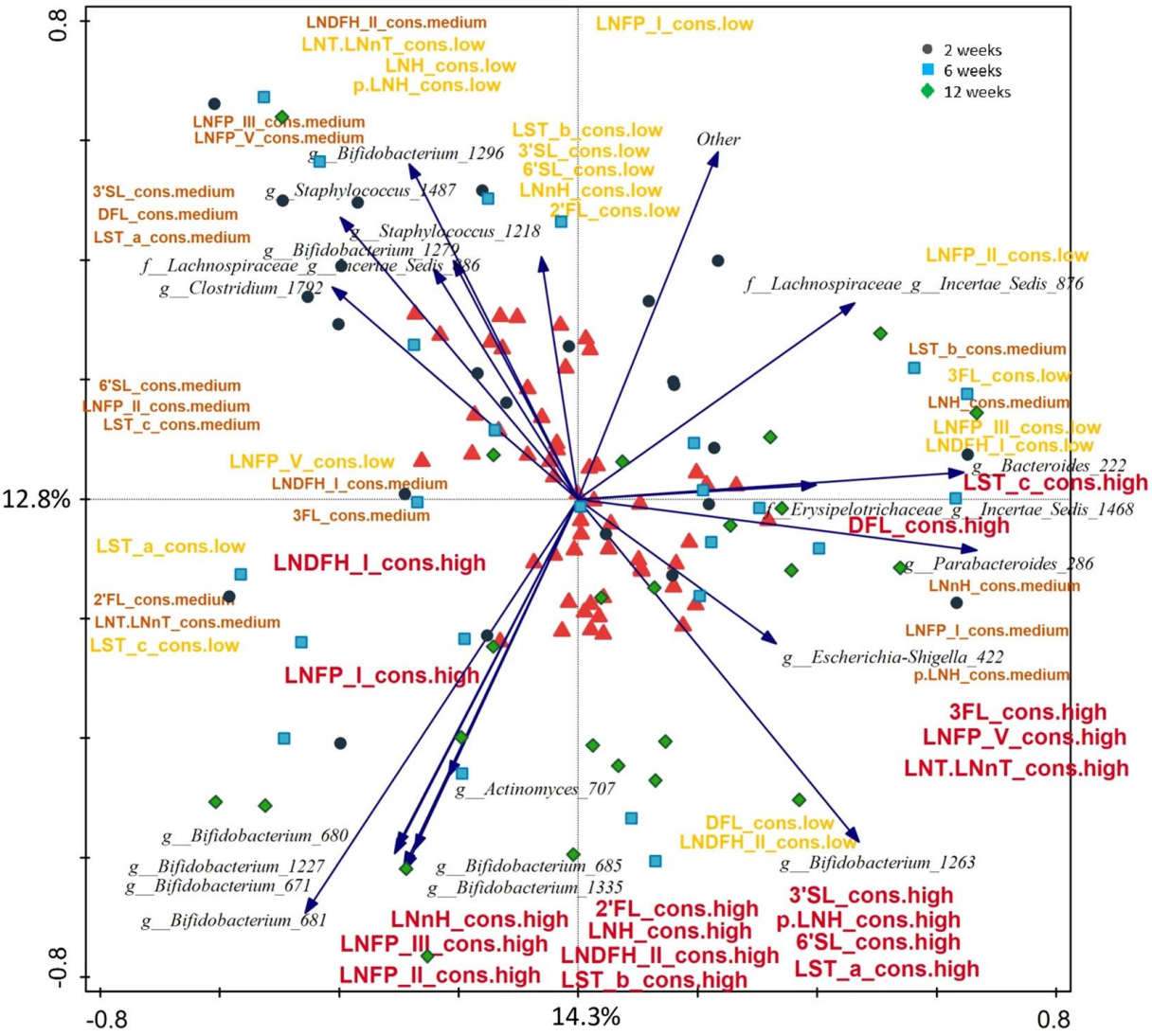

Figure 5.6. RDA showing spatial distribution of fecal samples of 24 infants at two, six and 12 weeks of age based on their OTU composition and using the estimated level of consumption (low, medium and high) for each HMO as explanatory variables. The red triangles represent centroids of each consumption group (high, medium and low) for each of the HMOs. For better image clarity only top twenty best fitted microbial taxa are displayed.

DISCUSSION

In the cohort described in this study we observed directional changes in both HMO concentrations in breast milk and in infant faces, and in the composition of infant fecal microbiota. HMO concentrations differed between individuals and depended on lactation time and infant age. Earlier studies showed that fecal microbiota structure is highly dynamic during infancy and is associated with multiple factors, including diet.[19-22] The variation in the fecal microbiota of infants in our study could be explained by infant age, gender, place and mode of delivery and certain milk HMOs, namely 6´SL, 2′FL, 3FL, 3´SL, LNDFH II ($p<0.05$). The effect of factors varied with age. Mode of delivery and breastmilk LNFP III concentrations showed a significant association with fecal microbiota structure at two weeks of age. The

important role of the mode of delivery in the initial seeding of the GI tract has been previously reported, [23-24] and has been linked to various health outcomes, both in infancy and beyond.[25-26] At six weeks the significant effect of the mode of delivery could still be detected in our data, and also gender and milk 3´SL seemed to play a role. A recent study using animal models showed that gender specific, microbiota-independent differences in immunity may lead to the selection of a gender-specific GI microbiota in adult germ free mice.[27] Gender-related differences in fecal microbiota have been also reported in adults,[28] in pre-term,[29] and term infants,[30] yet the timing and the possible mechanisms that might underlie the gender differentiation of GI microbiota in healthy full term infants remain largely unknown. As male infants tend to have a higher daily milk intake, it is possible that they receive higher doses of microbial and HMO components from the mother's milk.[31] At 12 weeks of age, both gender and LNH were significantly associated with microbiota, however, the significant association between microbiota composition and mode of delivery was no longer present. With the accumulating evidence linking mode of delivery with various health effects later in life,[25-26, 32] it is likely that the microbiota related programming of the host happens soon after birth, or during specific "windows of opportunity", thus, even when microbiota recovers to its normal state, the long term health effects of such disturbance might persist throughout life.[32]

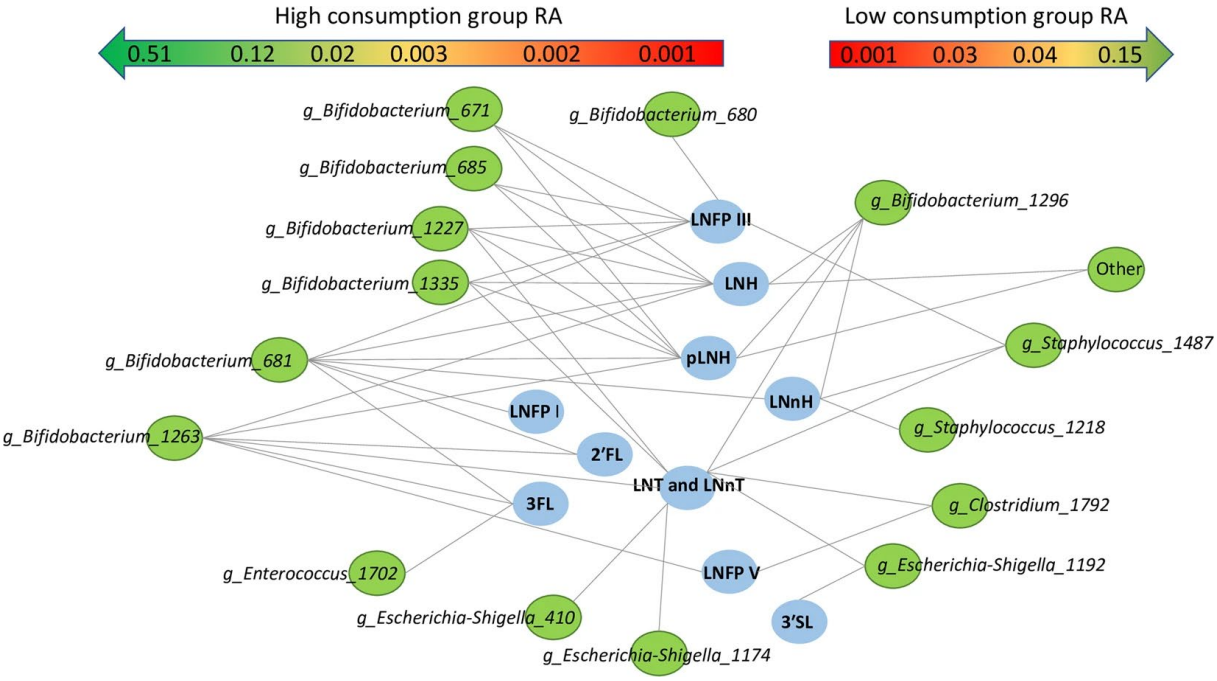

Figure 5.7. Differentially abundant OTUs in fecal samples of infants classified into high or low HMO consumption categories for each HMO type (Kruskal-Wallis; FDR<0.05). OTUs enriched in infants classified as high consumers are displayed on the left half of the figure, and OTUs enriched in infants classified as low consumers are displayed on the right half of the figure. The approximate RA of the OTUs is indicated by the gradient arrows on top of the figure.

One of the crucial factors shaping the development of GI microbial community during infancy is the type of feeding that an infant receives, with formula- and breastfed infants showing very different

microbial profiles.[33-37] Human milk is a source of prebiotic HMOs which support microbial colonisation in the infant GI tract.[7, 15, 38] The HMO content is variable and highest in colostrum, and the concentrations of HMOs decrease in mature milk.[8-10] Our data agrees with these findings showing that concentrations of the HMOs measured per mL of milk varied between mothers, and decreased between two and 12 weeks of lactation, except for 3FL and LNFP III, which increased in concentration as lactation progressed. Earlier studies showed that the daily intake of milk increases in the first months of life likely compensating for the decreased HMO concentrations.[18] Considering the daily milk intake, we could conclude that in our study the amounts of ingested HMOs remained relatively stable during the first 12 weeks of life. The exception was in the intake of 3FL and LNFP III which gradually increased in time. Interestingly, this increase in the 3FL and LNFP III intake corresponded with the increase in the fecal concentrations of these two HMOs, suggesting that, on average, their supply likely exceeded the ability of the infant GI tract microbiota to utilise these two HMOs. Furthermore, the role of these structures might go beyond their prebiotic effect, as both 3FL and LNFP III can act as decoys against pathogen binding and infection.[39-41]

One of the signature bacterial groups found in feces of breastfed infants is the genus *Bifidobacterium*.[2] *In vitro* studies showed that *Bifidobacterium bifidum* JCM1254,[38] *Bifidobacterium longum* subsp. *infantis*, *Bacteroides fragilis* and *Bacteroides vulgatus,*[15] grow well on HMOs as sole carbon source. Thus, we expected to find positive correlations between certain breastmilk HMOs and the bifidobacterial OTUs. We did not measure the absolute abundance of bacteria in infant feces and are aware of the limitation of using relative abundance data. However, culture-based studies showed that the total fecal bacterial load, as well as *Bifidobacterium* counts tend to increase in the first month of life.[34] Thus, the observed increase in the number of sequencing reads and the relative abundance of bifidobacteria in our data likely reflect the actual increase in the abundance of this group (Figure S5.1). We hypothesized that quantity of HMOs might selectively promote growth of either the primary or secondary HMO degraders, leading to increase in their abundance within the microbial community. However, when using data from the three time points combined, we saw an opposite trend - as the predicted daily intake of most HMOs was stable in time, the relative abundance of bifidobacteria was increasing. The analyses repeated for individual time points showed few positive correlations and the results varied between time points (Figure S5.3 a, b, c). This could be due to the fact that, in addition to individual HMOs promoting growth of selected microbes capable to utilise this carbon source, there may be other mechanisms controlling the microbial community structure. For example, presence of other breastmilk components, such as lysozyme, secretory IgA and other endogenous factors can suppress growth of certain members of the community and thus, indirectly allow other bacterial species to dominate the infant GI tract ecosystem.[34] Furthermore, the possible effect of HMOs on microbiota may already start in the breastmilk itself. A recent study on human milk investigated the associations between the HMO content and microbiota composition in colostrum and reported strong positive

correlations between different HMOs and various microbial groups, including streptococci, staphylococci, enterococci and bifidobacteria, in particular *Bifidobacterium breve* and LNFP III.[42] In the present study, RDA showed a significant association of milk LNFP III with infant fecal microbiota in all time points combined. There was a strong positive association of milk LNFP III with OTUs belonging to the genera *Veillonella*, *Enterococcus* and *Streptococcus* (Figure S5.3). Positive associations at all time points for breastmilk 3´SL and unidentified OTUs within family Enterobacteriaceae and *Actinomyces* 695 were also observed. 6´SL was positively associated with clostridia – *Clostridium* 1789 at week two and *Clostridium* 1639 at week 12. Finally, LNFP III was positively associated with *Enterococcus* 1698 at six and 12 weeks. At two and at six weeks Lachnospiraceae *Incertae Sedis* 876 was negatively associated with LNDFH II, *Lactobacillus* 1718 was negatively associated with 3´SL, and *Bifidobacterium* 1295 with LNFP I. Studies on mature breastmilk microbiota and the microbial transfer of microbiota from mother to infant show that breastmilk contains a distinct microbial community and that breastfed infants receive on average nearly 30% of the bacteria from breastmilk and 10% from areolar skin in the first 30 days of life.[43] The study also concluded that the association was lower in older infants, and it was proportional to the frequency of breastfeeding that an infant received.[43] Thus, it is then likely that some of the correlations observed here were due to a combined effect of the HMOs modulating the microbiota of the mother's milk and the infant GI tract, as well as due to the transfer of bacteria during breastfeeding.

Infant GI microbiota plays an important role in energy metabolism via utilising otherwise indigestible HMOs. Our data showed that the average concentrations of fecal HMOs decreased with age, suggesting that microbiota of older infants was more adapted and efficient in degrading these compounds (Figure 5.5b). Furthermore, we noted that the increase in efficiency was correlated with the increase in the relative abundance of several bifidobacterial OTUs, but also *Parabacteroides*, *Escherichia-Shigella, Bacteroides, Actinomyces, Veillonella,* and Erysipelotrichaceae *Incertae Sedis* (Figure 5.6). High ability to degrade a wide range of HMOs was associated with higher relative abundance of one or more *Bifidobacterium* OTUs confirming the important role of this bacterial group in the HMO metabolism (Figure 5.6). Thirteen fecal HMOs negatively correlated with nine different *Bifidobacterium* OTUs, and the highly abundant *Bifidobacterium* OTU 1263 was negatively correlated with nine different HMOs in feces, especially LNH, LNT and LNnT and LNFP V. Aside of bifidobacteria, members of the genus *Bacteroides* were significantly more abundant in infants who were good degraders of 2′FL, LNFP I, II, V, and pLNH, and *Parabacteroides* in the high degraders of 3FL, LNFP V, LNH, LNT and LNnT, indicating that these microbial groups might have a mutualistic or symbiotic relationship degrading those compounds. In addition, *Halomonas*, *Enterococcus*, *Lactobacillus,* St*aphylococcus, Suterella*, *Varibaculum*, *Veillonella*, *Streptococcus, Actinomyces*, and Lachnospiraceae *Incertae Sedis* were also associated with degradation of the same HMOs as bifidobacteria. Interestingly, high levels of degradation of 6´SL, DFL, LST a and LST c were not

associated with high levels of any of the bifidobacterial OTUs, but with various OTUs belonging to *Bacteroides*, *Streptococcus*, *Varibacullum* (6′SL), *Actinomyces*, *Clostridium*, *Collinsella* and *Streptococcus* (LST c), and *Haemophilus*, *Veillonella* (DFL), and Lachnospiraceae *Incertae Sedis*, and *Halomonas* (LST a).

Negative associations were also observed for LNFP II and *Bacteroides,* and for LNFP V and *Parabacteroides* suggesting the role of these bacteria in the HMO degradation. The fact that *Bacteroides* and *Parabacteroides* (formerly also *Bacteroides*) were identified in our analysis is in line with earlier studies showing growth of *Bacteroides* spp. on selected milk glycans by activating the mucus degradation pathway.[44] Finally, LNDFH I in both milk and feces was negatively associated with *Enterococcus* OTU 1702, but the association was stronger in feces. Even though *in vitro* studies showed that in a monoculture *Enterococcus* was not able to grow on milk HMOs,[15] another study showed that this group was found in breastmilk,[42] that its abundance in infant feces could be predicted from the maternal HMO profile and that it was positively correlated with the abundance of *Bifidobacterium, Streptococcus* and *Veillonella*.[7] One of the suggested explanations was that *Enterococcus* can cross feed on HMO fermentation products or HMO breakdown by-products that are released in the ecosystem by HMO degrading bifidobacteria or *Bacteroides* spp.[7]

The correlation analysis of infant fecal HMOs and infant fecal OTUs for all time points combined also detected numerous significant positive associations between various HMOs and *Streptococcus, Staphylococcus, Escherichia-Shigella*, and *Clostridium* OTUs (Figure 5.5). In both, milk and feces LST c, 6′SL and LNnH showed significant correlation with staphylococci, while LNFP III and 3FL were positively correlated with streptococci. Earlier studies showed that neither *Streptococcus, Staphylococcus, Escherichia-Shigella*,[7, 15] nor *Clostridium*,[15] could effectively utilise and grow on milk HMOs. However, all these bacterial groups are members of the microbiota of breastmilk and areolar skin,[43, 45-46] and the positive link might be due to breastfeeding practises, for example with more frequent feedings resulting in higher ingested doses of the bacteria and the HMOs. If the HMOs are not well digested, the positive associations may still persist in the feces.

Our study has few important limitations. The number of participating mother-infant pairs was relatively small and future studies should be done on larger sample sizes to verify our findings. Larger sample size would also allow for stratification of data based on maternal secretor status. Another limitation of this study is the lack of measurements of the actual volumes of the breast milk ingested and the mass of the feces produced by each infant in a 24-hour sampling period. Therefore, our calculations had to be based on estimates from other studies that measured the daily milk intake in infants of similar ages. Actual intakes, although likely increasing over time, can vary greatly between infants, and thus, our calculated values are only an estimate and should therefore be considered as such. Measurements of volumes or weights of breastmilk samples and fecal samples should be included in

future studies. Finally, with our methods we could not identify species or strains of the bacteria, nor could we quantify their actual numbers which could further support our findings.

In this study we found only weak associations between selected breastmilk HMOs and fecal microbiota community structure in breastfed infants. However, we found a strong link between degradation levels of various HMOs and specific microbial groups, in particular different species of *Bifidobacterium*. Earlier studies showed that different bifidobacterial species vary in their ability to break down HMOs, and some species can degrade HMOs without experiencing a detectable population growth.[47-48] Thus, including metatranscriptome or metaproteome analyses in this set would have been very helpful in understanding the community dynamics in regard of HMO metabolism in the infant GI tract. Our findings could provide the basis for assembling simple synthetic communities to study microbial interactions and community structure changes which are centred around degradation of different HMO structures. *In vitro* fermentation studies incorporating purified compounds would also help to eliminate confounders, such as presence of milk's own microbiota and presence of milk components, which have a regulatory effect on microbiota in both, milk and in the infant GI tract.

CONCLUSIONS

Fecal microbiota of breastfed infants during the first 12 weeks of life is highly diverse, dynamic and influenced by age and other factors. The effect of mode of delivery disappeared after six weeks of age, whereas the effect of gender became detectable over time. Overall, microbiota development in this cohort followed a normal colonization pattern resulting in fecal microbial communities dominated by *Bifidobacterium,* in particular the most predominant *Bifidobacterium* OTU 1263. Breastmilk HMO analyses showed that the composition of the 18 HMOs that were measured varied between mothers and throughout the duration of lactation. In our analysis we did not observe strong and consistent positive correlations between the HMOs in maternal breastmilk and specific microbial OTUs including bifidobacteria in infants' feces. Thus, we believe that HMO composition is only one of many factors regulating colonization and structure of the infant GI microbial community. However, the findings of this study supported the key role of bifidobacteria in the infants' ability to degrade most of the measured HMOs, in addition to indicating the role of other microbial taxa in the degradation or metabolism of specific HMOs.

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SUPPORTING INFORMATION

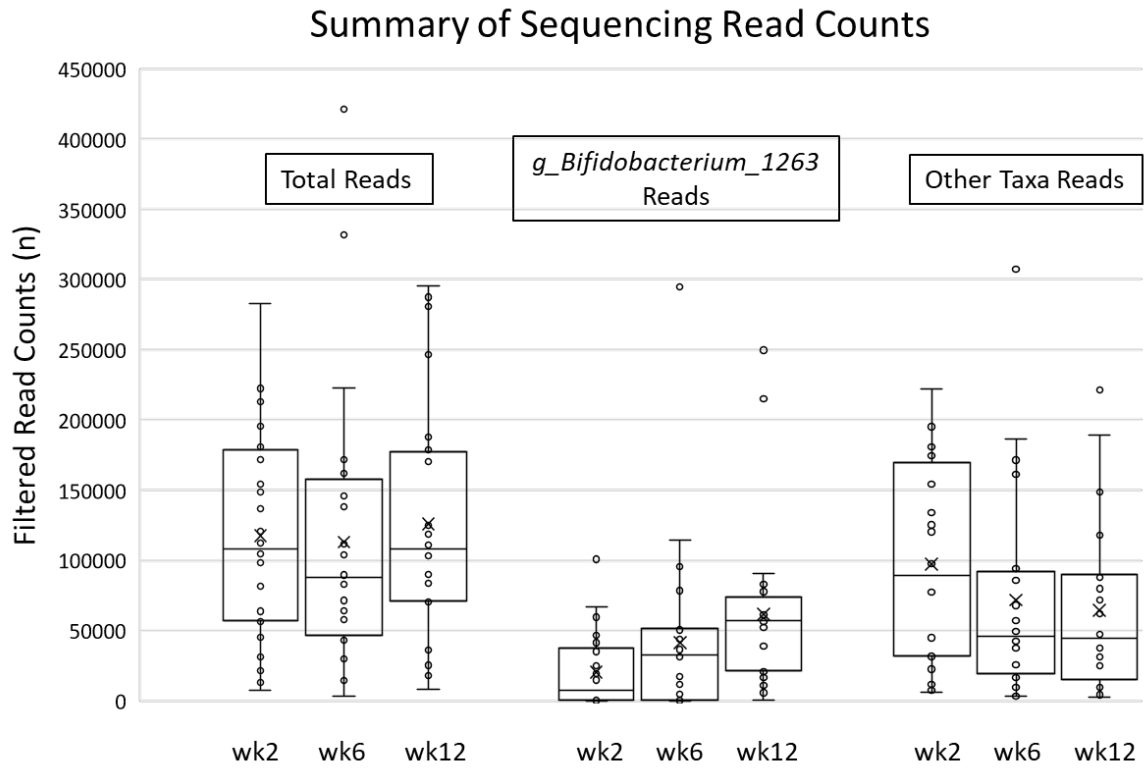


Figure S5.1. Box and whisker plots showing the minimum, first quartile, median, third quartile, and maximum number of the filtered sequencing reads from infants' fecal samples at two, six, and 12-week collection timepoints. Total number of filtered reads, a subset of reads that were assigned to *g_Bifidobacterium_1263*, and a sum of reads from all other taxa are displayed.

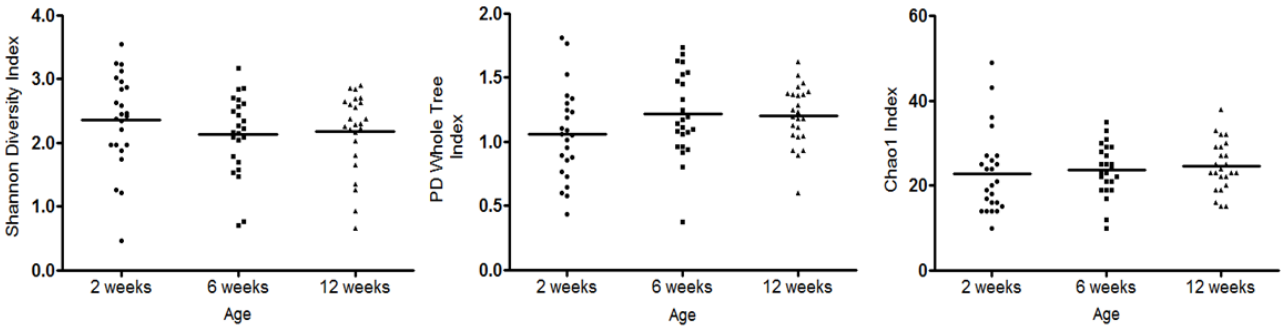
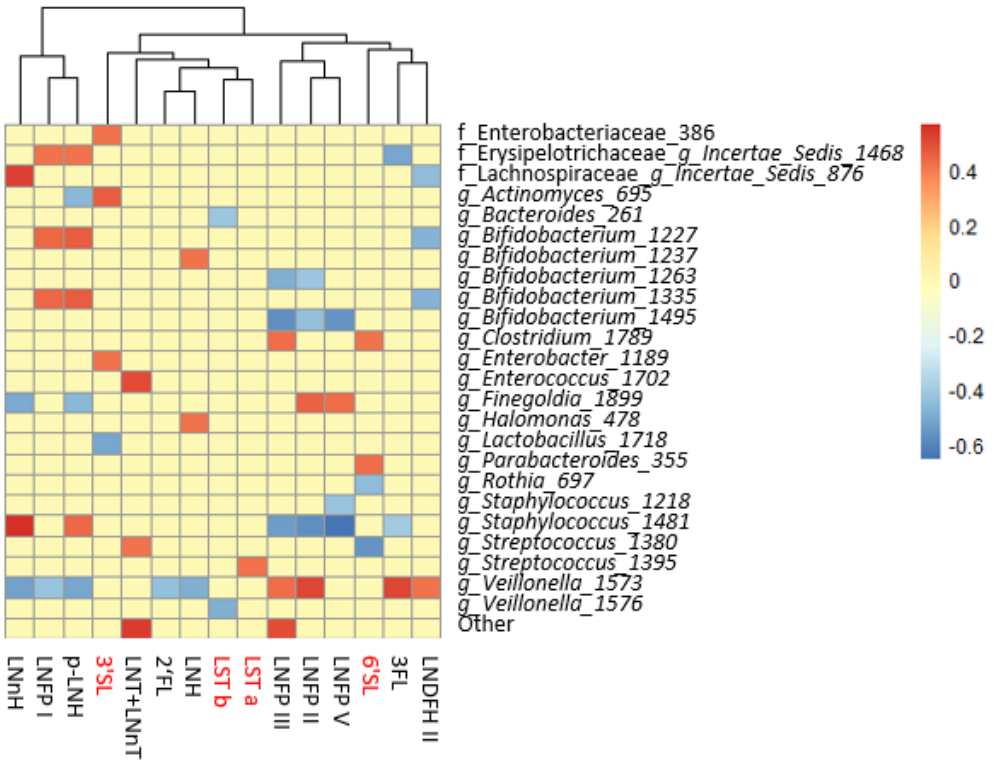


Figure S5.2. Alpha diversity indices at different time points. Diversity was estimated using Shannon diversity index, which evaluates both the number of species and the evenness of their distribution. Richness, which estimates the number of different species present in each sample, was measured with the Chao1 Index and with PD Whole Tree, the latter of which takes into account phylogenetic differences between species (OTUs). No significant differences were found between any of the age groups.

a.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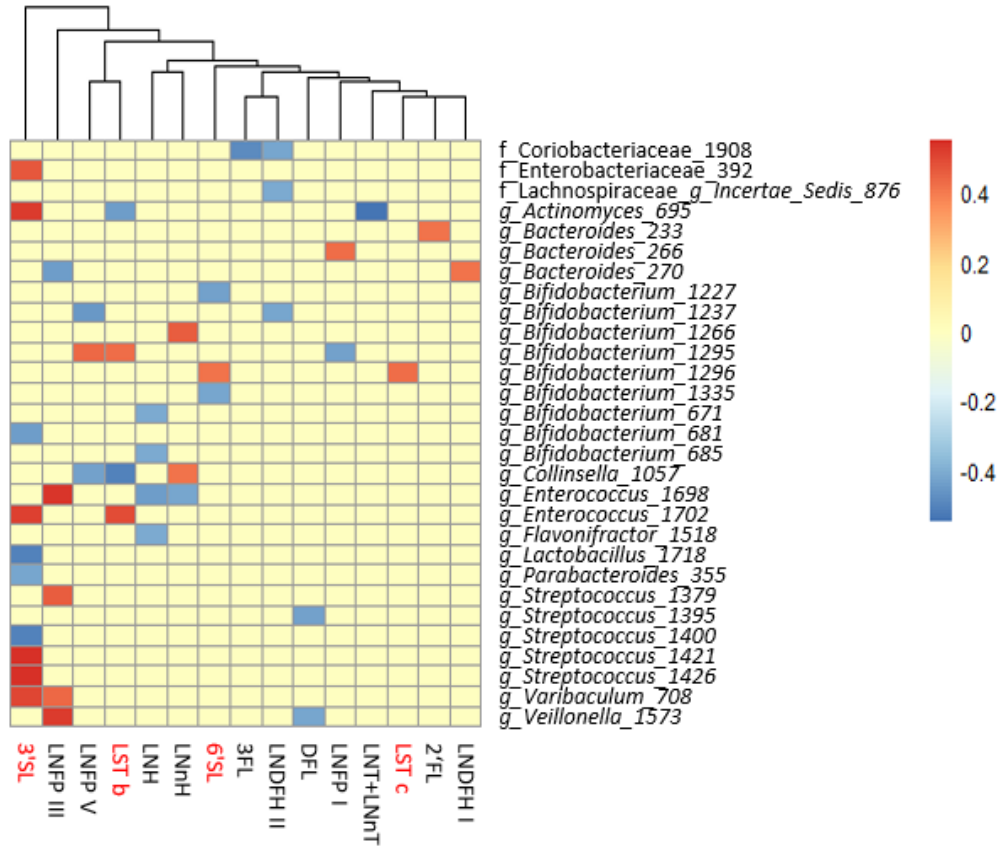
g_Actinomyces_695
g_Bacteroides_261
g_Bifidobacterium_1227
g_Bifidobacterium_1237
g_Bifidobacterium_1263
g_Bifidobacterium_1335
g_Bifidobacterium_1495
g_Clostridium_1789
g_Enterobacter_1189
g_Enterococcus_1702
g_Finegoldia_1899
g_Halomonas_478
g_Lactobacillus_1718
g_Parabacteroides_355
g_Rothia_697
g_Staphylococcus_1218
g_Staphylococcus_1481
g_Streptococcus_1380
g_Streptococcus_1395
g_Veillonella_1573
g_Veillonella_1576
Other
0.4
0.2
0
-0.2
-0.4
-0.6
LNnH
LNFP I
p-LNH
3'SL
LNT+LNnT
2'FL
LNH
LST b
LST a
LNFP III
LNFP II
LNFP V
6'SL
3FL
LNDFH II

b.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g_Bacteroides_270
g_Bifidobacterium_1227
g_Bifidobacterium_1237
g_Bifidobacterium_1266
g_Bifidobacterium_1295
g_Bifidobacterium_1296
g_Bifidobacterium_1335
g_Bifidobacterium_671
g_Bifidobacterium_681
g_Bifidobacterium_685
g_Collinsella_1057
g_Enterococcus_1698
g_Enterococcus_1702
g_Flavonifractor_1518
g_Lactobacillus_1718
g_Parabacteroides_355
g_Streptococcus_1379
g_Streptococcus_1395
g_Streptococcus_1400
g_Streptococcus_1421
g_Streptococcus_1426
g_Varibaculum_708
g_Veillonella_1573
0.4
0.2
0
-0.2
-0.4
3'SL
LNFP III
LNFP V
LST b
LNH
LNnH
6'SL
3FL
LNDFH II
DFL
LNFP I
LNT+LNnT
LST c
2'FL
LNDFH I

c.

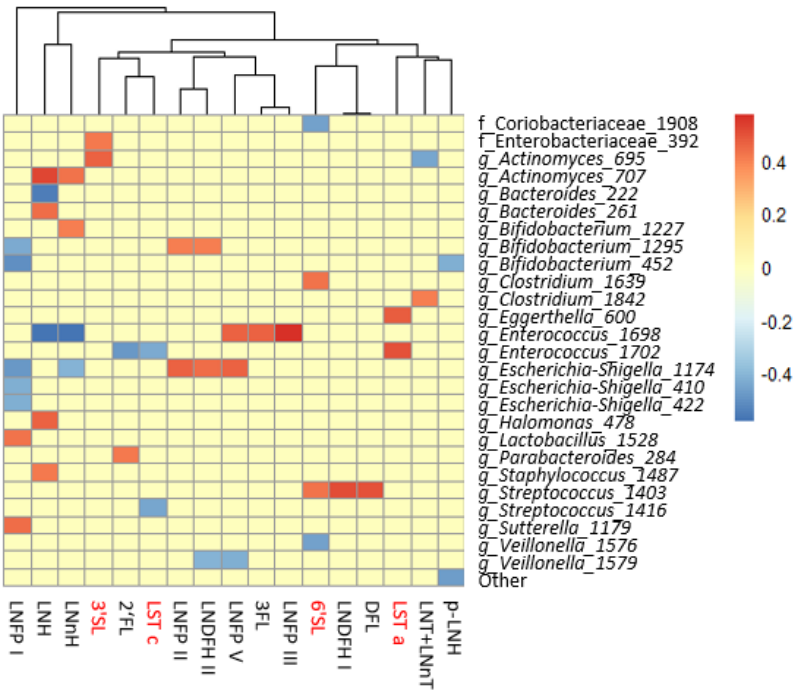
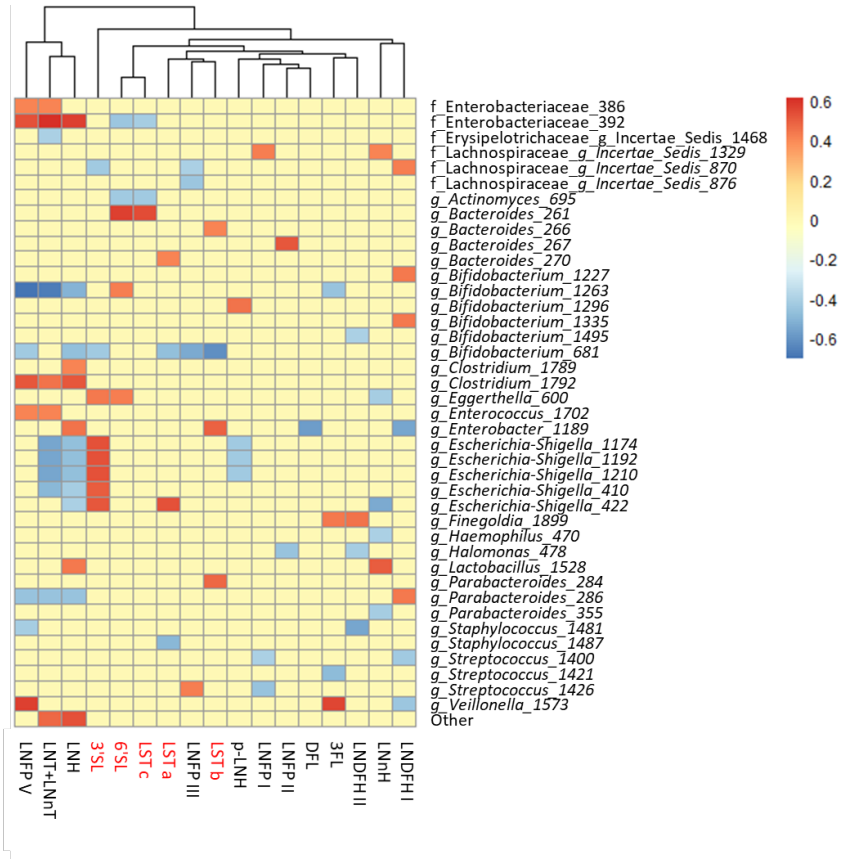
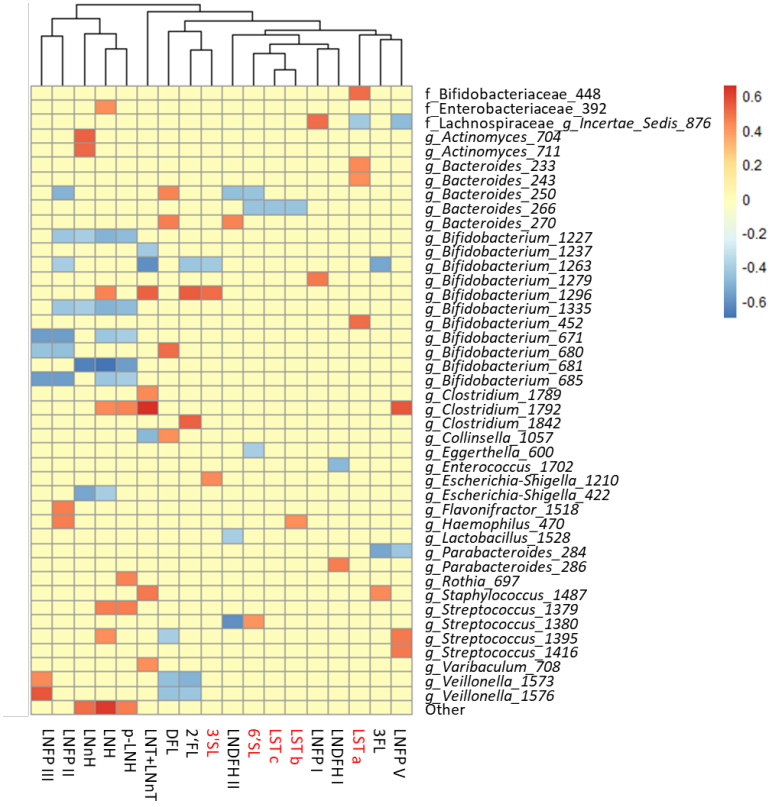


Figure S5.3. Statistically significant ($p<0.05$) Spearman correlations (correlation threshold ±0.3) between an estimated daily intake of different breastmilk HMOs and faecal microbiota composition at OTU level of 24 infants at two (a), six (b) and 12 (c) weeks after birth. Positive associations are indicated in red, negative in blue, yellow denotes correlations that did not pass the significance or the correlation thresholds. The names of acidic HMOs are highlighted in red.

a.



b.



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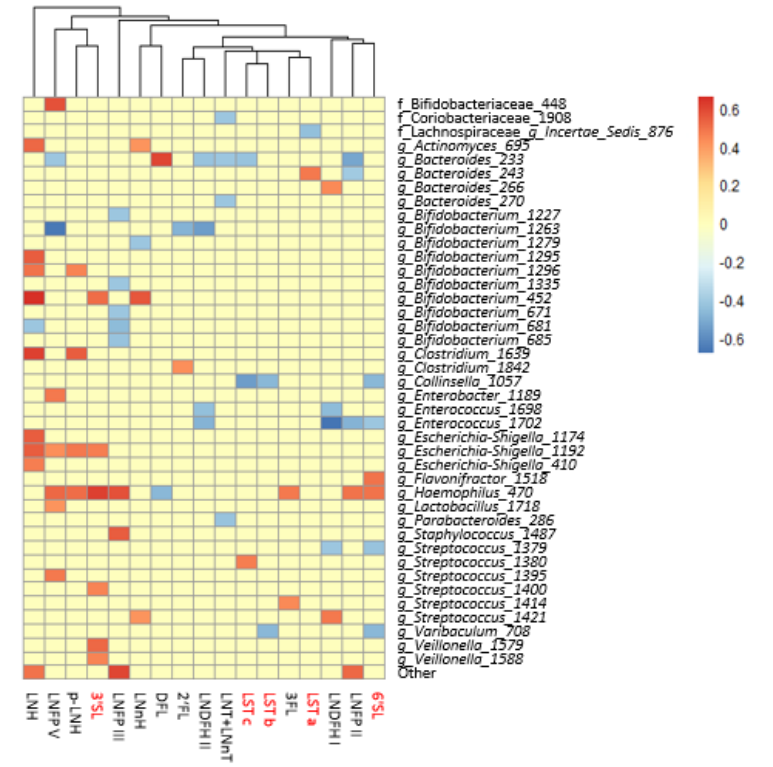

Figure S5.4. Statistically significant ($p<0.05$) Spearman correlations (correlation threshold ±0.3) between different HMOs detected in infant feces and fecal microbiota composition at OTU level of 24 infants at two (a), six (b) and 12 (c) weeks after birth. Positive associations are indicated in red, negative in blue, and correlations that did not pass the significance or the correlation thresholds are marked in yellow. The names of acidic HMOs are highlighted in red.

Table S5.1. Average relative abundance (Avg RA), standard error of abundance means (SEM), and OTU prevalence of OTUs (n=411) at two, six and 12 weeks of age. Only OTUs which were present in at least 5% of all samples are shown

Genus	OTU#	Week two (n=24)			Week six (n=24)			Week 12 (n=24)			Total (n=72)		
		Avg RA (%)	± SEM	Prevalance (%)	Avg RA (%)	± SEM	Prevalance (%)	Avg RA (%)	± SEM	Prevalance (%)	Avg RA (%)	± SEM	Prevalance (%)
Actinomyces	695	0.89	0.88	8.33	2.17	2.16	12.5	0.17	0.16	8.33	1.08	0.77	9.72
	704	0.3	0.28	12.5	0.06	0.04	12.5	0.01	0.01	4.17	0.12	0.09	9.72
	707	0	0	0	0.01	0.01	4.17	0.07	0.03	20.83	0.03	0.01	8.33
	711	0	0	0	0.07	0.05	8.33	0.12	0.08	16.67	0.06	0.03	8.33
Aeribacillus	1603	0.01	0.01	4.17	0.02	0.01	12.5	0.01	0.01	4.17	0.01	0	6.94
Bacteroides	222	2.67	1.17	33.33	7.97	3.24	50	5.48	2.16	50	5.37	1.36	44.44
	233	0.09	0.08	8.33	0.04	0.02	16.67	0.1	0.04	25	0.07	0.03	16.67
	243	0.03	0.03	8.33	0.07	0.05	8.33	0.39	0.2	20.83	0.16	0.07	12.5
	250	0.05	0.04	8.33	0.07	0.05	12.5	0.23	0.12	16.67	0.12	0.04	12.5
	261	1.31	1.29	12.5	0.07	0.07	4.17	0.04	0.02	12.5	0.47	0.43	9.72
	266	0.36	0.28	8.33	0.08	0.05	12.5	0.02	0.01	8.33	0.15	0.1	9.72
	267	0.5	0.4	12.5	0.27	0.19	16.67	0.1	0.08	12.5	0.29	0.15	13.89
	270	0.32	0.29	12.5	0.08	0.04	16.67	0.07	0.04	12.5	0.16	0.1	13.89
Bifidobacterium	1227	0.11	0.08	8.33	0.14	0.07	20.83	0.28	0.1	37.5	0.17	0.05	22.22
	1237	0.13	0.09	8.33	0.17	0.1	16.67	0.21	0.14	25	0.17	0.06	16.67
	1263	22.23	5.39	83.33	35.1	6.23	91.67	51.26	4.96	100	36.2	3.46	91.67
	1265	0.04	0.02	12.5	0.01	0.01	4.17	0.01	0.01	4.17	0.02	0.01	6.94
	1266	0.01	0.01	4.17	0.02	0.01	8.33	0.03	0.02	8.33	0.02	0.01	6.94
	1279	11.92	3.18	58.33	11.93	3.82	79.17	6.01	2.86	79.17	9.95	1.91	72.22
	1295	0.05	0.03	16.67	0.06	0.03	20.83	0.07	0.03	20.83	0.06	0.02	19.44
	1296	0.07	0.04	16.67	0.05	0.03	12.5	0.02	0.02	4.17	0.05	0.02	11.11
	1335	0.09	0.06	8.33	0.12	0.06	20.83	0.24	0.08	37.5	0.15	0.04	22.22
	1495	0.06	0.03	16.67	0	0	0	0	0	0	0.02	0.01	5.56
	452	0.02	0.02	4.17	0.02	0.01	8.33	0.05	0.02	20.83	0.03	0.01	11.11
	671	0.03	0.03	4.17	0.08	0.04	16.67	0.24	0.08	37.5	0.12	0.03	19.44
	680	0.01	0.01	4.17	0.02	0.01	12.5	0.09	0.04	25	0.04	0.01	13.89
	681	5.92	3.51	16.67	9.46	4.41	41.67	6.48	2.38	58.33	7.29	2.02	38.89
	685	0.03	0.03	4.17	0.08	0.04	16.67	0.28	0.1	37.5	0.13	0.04	19.44
Clostridium	1629	0.02	0.02	4.17	0.52	0.52	4.17	0.18	0.18	4.17	0.24	0.18	4.17
	1639	0.03	0.03	4.17	0.04	0.04	4.17	0.28	0.2	8.33	0.12	0.07	5.56
	1789	0.06	0.03	12.5	0.02	0.01	8.33	0	0	0	0.03	0.01	6.94
	1792	1.87	0.84	20.83	0.79	0.37	45.83	0.32	0.15	25	0.99	0.31	30.56
	1842	0	0	0	0.08	0.07	12.5	0.08	0.07	8.33	0.06	0.03	6.94
Collinsella	1057	0.05	0.05	4.17	0.09	0.06	12.5	0.12	0.07	16.67	0.09	0.03	11.11
Eggerthella	600	0.09	0.05	12.5	0.14	0.08	20.83	0.09	0.06	12.5	0.1	0.04	15.28
Enterobacter	1189	0.02	0.02	8.33	0.01	0.01	4.17	0.02	0.01	8.33	0.02	0.01	6.94
Enterococcus	1698	0.01	0.01	4.17	0.15	0.1	12.5	0.18	0.1	20.83	0.11	0.05	12.5
	1702	0.48	0.25	25	0.77	0.35	37.5	0.84	0.3	54.17	0.7	0.17	38.89
Escherichia-Shigella	1174	0.04	0.02	16.67	0.02	0.01	12.5	0.06	0.03	20.83	0.04	0.01	16.67
	1192	0.04	0.02	16.67	0.01	0.01	4.17	0.02	0.02	8.33	0.02	0.01	9.72
	1210	0.04	0.02	16.67	0.03	0.02	16.67	0.05	0.02	16.67	0.04	0.01	16.67
	410	0.12	0.05	29.17	0.08	0.03	29.17	0.17	0.05	50	0.12	0.02	36.11

	422	8.45	3.68	66.67	4.42	1.58	58.33	5.09	1.46	75	5.99	1.42	66.67
Finegoldia	1899	0.06	0.05	12.5	0.06	0.06	4.17	0.01	0.01	4.17	0.04	0.03	6.94
Flavonifractor	1518	0	0	0	0.02	0.01	8.33	0.04	0.03	8.33	0.02	0.01	5.56
Gemella	1606	0.01	0.01	4.17	0.01	0.01	4.17	0.02	0.01	8.33	0.01	0.01	5.56
Haemophilus	470	0.18	0.08	25	0.32	0.15	37.5	0.7	0.41	41.67	0.4	0.15	34.72
Halomonas	478	0.01	0.01	8.33	0.03	0.02	12.5	0.01	0.01	8.33	0.02	0.01	9.72
Lactobacillus	1205	0	0	0	0.01	0.01	4.17	0.04	0.02	16.67	0.01	0.01	6.94
	1334	0.01	0.01	4.17	0.01	0.01	4.17	0.03	0.02	12.5	0.02	0.01	6.94
	1528	0.55	0.31	20.83	1.28	0.41	41.67	1.7	0.51	50	1.18	0.25	37.5
	1718	0.46	0.32	12.5	0.71	0.34	25	0.04	0.03	8.33	0.4	0.16	15.28
Parabacteroides	284	0.19	0.14	12.5	0.12	0.09	16.67	0.04	0.03	8.33	0.12	0.06	12.5
	286	0.34	0.17	29.17	0.72	0.31	29.17	0.47	0.28	29.17	0.51	0.15	29.17
	355	0.07	0.05	12.5	0.08	0.06	8.33	0.14	0.08	12.5	0.1	0.04	11.11
Rothia	697	0.02	0.01	8.33	0.04	0.02	20.83	0.06	0.03	20.83	0.04	0.01	16.67
Staphylococcus	1218	0.28	0.08	45.83	0.01	0.01	4.17	0	0	0	0.1	0.03	16.67
	1481	0.09	0.03	33.33	0	0	0	0	0	0	0.03	0.01	11.11
	1487	6.45	1.08	91.67	0.54	0.13	70.83	0.32	0.13	54.17	2.44	0.49	72.22
Streptococcus	1379	0.05	0.05	4.17	0.02	0.02	8.33	0.22	0.09	25	0.1	0.04	12.5
	1380	0.33	0.1	50	0.13	0.05	33.33	0.25	0.11	37.5	0.24	0.05	40.28
	1395	1.18	1.13	8.33	0.7	0.57	8.33	0.03	0.02	8.33	0.64	0.42	8.33
	1400	0.11	0.04	33.33	0.17	0.06	45.83	0.35	0.08	62.5	0.21	0.04	47.22
	1403	0.22	0.17	12.5	0.05	0.05	4.17	0.02	0.01	12.5	0.1	0.06	9.72
	1414	0.12	0.12	4.17	0.24	0.18	8.33	0.2	0.17	8.33	0.19	0.09	6.94
	1416	5.22	1.4	87.5	4.18	1.65	83.33	1.82	0.61	62.5	3.74	0.76	77.78
	1421	0.03	0.01	16.67	0.03	0.01	12.5	0.01	0.01	8.33	0.02	0.01	12.5
	1426	0.07	0.02	25	0.03	0.02	12.5	0.01	0.01	4.17	0.03	0.01	13.89
Sutterella	1179	0.12	0.12	4.17	0.08	0.05	12.5	0.41	0.35	8.33	0.2	0.12	8.33
Varibaculum	708	0	0	0	0.18	0.13	16.67	0.12	0.06	16.67	0.1	0.05	11.11
Veillonella	1573	0.61	0.44	25	0.18	0.12	20.83	0.81	0.35	45.83	0.53	0.19	30.56
	1576	1.04	0.61	37.5	0.12	0.06	16.67	0.22	0.11	29.17	0.46	0.21	27.78
	1578	0.11	0.1	16.67	0.01	0.01	4.17	0	0	0	0.04	0.03	6.94
	1579	0.04	0.04	4.17	0	0	4.17	0.03	0.01	16.67	0.03	0.02	8.33
	1588	1.74	0.7	54.17	0.65	0.36	54.17	0.95	0.48	41.67	1.11	0.31	50
f_Bifidobacteriaceae	448	0.01	0.01	4.17	0.02	0.01	8.33	0.02	0.01	12.5	0.02	0.01	8.33
f_Coriobacteriaceae	1908	0.02	0.02	4.17	0.08	0.04	12.5	0.13	0.07	16.67	0.08	0.03	11.11
f_Enterobacteriaceae	386	0.03	0.02	8.33	0.04	0.03	8.33	0.09	0.04	29.17	0.05	0.02	15.28
	392	6.02	3.97	29.17	2.45	1.6	37.5	3.07	1.59	37.5	3.85	1.51	34.72
f_Erysipelotrichaceae *Incertae_Sedis*	1468	0.13	0.1	12.5	0.08	0.08	4.17	0.06	0.04	8.33	0.09	0.05	8.33
f_Lachnospiraceae *Incertae_Sedis*	1329	0.05	0.03	12.5	0.03	0.02	12.5	0.02	0.02	8.33	0.03	0.01	11.11
	870	0.15	0.08	16.67	0.07	0.04	12.5	0.03	0.02	12.5	0.08	0.03	13.89
	876	3.21	1.33	33.33	2.02	0.9	25	1.25	0.64	20.83	2.16	0.58	26.39
Other OTUs (n=328)		12.07	3.94	95.83	9.09	2.44	95.83	6.71	2.18	95.83	9.29	1.7	95.83

Table S2. Average concentrations of HMOs in a) maternal breast milk; b) infant faeces, indicating significant differences in concentrations between study timepoints ($p<0.05$)

a.

	Concentrations of HMO, or HMO category in breast milk(µg/mL)												
	2 weeks				6 weeks				12 weeks				
HMO	**Min**	**Max**	**Average**	**SD**	**Min**	**Max**	**Average**	**SD**	**Min**	**Max**	**Average**	**SD**	**p-value**
3FL	79.4	1466.1	521.7	388.1	120.6	1959.0	779.7	482.3	115.3	2381.4	1002.7	567.8	$p<0.05$
2'FL	0.0	1839.0	1006.6	663.1	0.0	1704.5	839.9	567.6	0.0	1394.4	702.1	482.0	$p=0.058$
LNT+LNnT	477.9	2330.6	1228.2	466.9	353.0	2228.1	1006.4	417.1	227.5	1820.8	781.3	333.8	$p<0.001$
LNFP III	149.7	559.0	357.0	120.0	230.7	555.1	379.0	88.4	228.4	642.7	406.8	112.8	$p<0.05$
LNFP II	69.2	1550.2	448.3	425.9	53.0	1514.8	431.0	377.2	68.4	1110.9	382.2	278.1	$p=0.95$
LNFP I	0.0	1909.6	760.6	580.6	0.0	1335.5	446.5	366.6	0.0	1264.1	295.8	289.7	$p<0.05$
LNFP V	0.0	186.2	49.6	58.0	0.0	203.8	53.7	54.5	0.0	136.3	48.7	40.9	$p=0.60$
LNH	26.4	143.6	63.8	33.5	8.7	92.9	42.5	25.4	3.4	38.2	16.8	8.9	$p<0.001$
LNnH	8.8	85.3	41.6	25.6	4.9	84.8	37.9	26.0	1.6	90.9	24.0	21.7	$p=0.20$
p-LNH	0.0	50.6	11.6	11.1	0.0	44.1	8.6	9.9	0.0	47.3	8.2	11.9	$p<0.05$
LNDFH I	31.7	1650.5	735.8	513.8	39.4	1311.1	603.1	409.9	29.5	1047.0	488.8	315.4	$p=0.19$
LNDFH II	0.0	431.8	81.5	127.0	0.0	570.2	75.9	128.0	0.0	303.6	64.4	79.6	$p=0.63$
DFL	0.0	262.7	80.6	73.8	0.0	201.2	66.1	53.5	0.0	155.1	71.5	54.6	$p=0.87$
6'SL	183.2	632.6	362.1	96.5	70.1	450.5	178.6	83.7	15.9	193.5	73.9	40.7	$p<0.001$
3'SL	99.5	233.2	174.8	34.3	87.5	209.2	162.9	29.0	94.9	233.7	164.2	35.6	$p<0.05$
LST c	98.6	708.2	274.6	167.3	28.0	199.4	88.1	46.5	5.7	81.1	34.4	20.9	$p<0.001$
LST b	53.9	435.1	193.5	84.2	71.7	489.4	185.4	97.4	33.0	306.6	147.0	66.7	$p=0.09$
LST a	4.5	107.2	40.8	22.7	3.0	37.8	15.8	8.1	1.7	58.9	11.0	11.0	$p<0.001$
Total	4412.8	8601.4	6432.7	1249.6	3588.1	7085.8	5401.0	1060.4	3206.5	6159.6	4723.7	840.3	$p<0.001$

b.

	Concentrations of HMO, or HMO category in infant faecal solution (µg/mL)												
	2 weeks				6 weeks				12 weeks				
HMO	**Min**	**Max**	**Average**	**SD**	**Min**	**Max**	**Average**	**SD**	**Min**	**Max**	**Average**	**SD**	**p-value**
3FL	0.0	1554.2	286.4	383.0	0.0	1795.2	305.1	476.9	0.0	2497.7	404.7	672.0	p=0.34
2'FL	0.0	953.1	217.2	316.3	0.0	1121.8	147.1	316.2	0.0	894.8	128.5	304.1	p=0.41
LNT+LNnT	0.2	2015.3	369.4	536.1	0.0	909.1	106.0	215.5	0.0	606.9	37.8	123.3	p<0.001
LNFP III	0.0	950.2	221.5	224.3	0.0	1245.7	177.2	278.1	0.0	785.9	152.1	250.0	p=0.06
LNFP II	0.2	1468.4	347.1	436.0	0.0	1937.6	295.5	496.5	0.0	1271.4	169.1	309.8	p<0.05
LNFP I	0.0	987.9	233.6	314.5	0.0	709.2	130.6	224.9	0.0	352.7	36.5	90.1	p<0.05
LNFP V	0.0	127.3	17.7	33.6	0.0	36.8	5.6	11.1	0.0	35.3	4.4	10.4	p=0.28
LNH	0.0	419.3	25.0	84.8	0.0	25.6	4.1	7.4	0.0	5.5	0.7	1.7	p<0.05
LNnH	0.0	70.6	5.8	14.6	0.0	38.7	2.9	8.3	0.0	14.6	0.6	3.0	p<0.05
p-LNH	0.0	149.8	9.1	30.4	0.0	121.4	6.6	24.6	0.0	4.3	0.4	1.0	p=0.07
LNDFH I	0.0	1731.1	599.8	533.3	0.0	2263.1	529.2	688.4	0.0	1782.7	244.9	454.5	p<0.05
LNDFH II	0.0	580.1	90.5	171.3	0.0	738.0	57.8	154.6	0.0	552.0	60.6	142.5	p=0.25
DFL	0.0	113.5	31.4	40.1	0.0	269.0	30.2	65.4	0.0	303.8	29.6	71.4	p=0.63
6'SL	0.0	410.1	161.9	148.7	0.0	426.8	82.9	129.3	0.0	190.8	31.2	57.4	p<0.05
3'SL	0.0	204.2	38.6	55.9	0.0	179.8	19.7	50.6	0.0	258.8	19.2	60.5	p=0.16
LST c	0.0	475.9	160.6	151.2	0.0	484.9	78.7	127.8	0.0	122.8	20.9	36.9	p<0.05
LST b	0.0	1355.6	258.5	340.9	0.0	2536.1	235.0	552.8	0.0	1404.4	112.0	292.6	p<0.05
LST a	0.0	66.8	14.4	18.8	0.0	769.6	35.2	156.6	0.0	15.5	1.7	4.2	p=0.09
Total	2.6	5889.6	3088.4	1780.1	1.7	6728.3	2249.2	2188.5	0.3	4922.3	1455.1	2033.2	p<0.05

Table S5.3. Differentially abundant OTUs in fecal samples of 24 infants at two, six and 12 weeks of age associated with high and low level of HMO consumption (Kruskal-Wallis; p<0.05, FDR<0.05 where indicated with*). The higher values of the average relative abundances (RA) are marked in bold.

HMO	OTU	Test-Statistic	p	FDR	High consumption group RA	Low consumption group RA
2′FL	*g_Bacteroides_261*	5.60	0.018	0.223	**0.002**	0
	*g_Bifidobacterium_1263**	11.16	0.001	0.036	**0.549**	0.198
	*g_Bifidobacterium_681**	16.59	0.000	0.004	**0.094**	0
	g_Bifidobacterium_1227	5.60	0.018	0.223	**0.001**	0
	g_Bifidobacterium_1335	5.60	0.018	0.223	**0.001**	0
	g_Bifidobacterium_671	4.36	0.037	0.247	**0.001**	0
	g_Bifidobacterium_685	4.36	0.037	0.247	**0.001**	0
	g_Bifidobacterium_1296	5.60	0.018	0.223	0	**0.001**
	g_Clostridium_1842	4.36	0.037	0.247	0	**0.001**
	g_Escherichia-Shigella_1210	4.63	0.031	0.247	0	**0.001**
	g_Escherichia-Shigella_410	4.06	0.044	0.255	0.001	**0.002**
	g_Halomonas_478	5.60	0.018	0.223	**0**	0
	g_Streptococcus_1416	4.09	0.043	0.255	0.016	**0.047**
	g_Veillonella_1588	4.84	0.028	0.247	0.006	**0.022**
	Other	4.63	0.031	0.247	0.045	**0.094**
3FL	*g_Bifidobacterium_1263**	12.04	0.001	0.023	**0.529**	0.235
	g_Bifidobacterium_685	7.81	0.005	0.124	**0.002**	0
	g_Bifidobacterium_1227	4.94	0.026	0.208	**0.002**	0.001
	g_Bifidobacterium_671	7.65	0.006	0.124	**0.002**	0.000
	g_Bifidobacterium_1335	5.07	0.024	0.208	**0.002**	0.001
	g_Bifidobacterium_680	6.41	0.011	0.165	**0.001**	0
	g_Bifidobacterium_452	4.09	0.043	0.215	**0.001**	0
	g_Bifidobacterium_1296	4.45	0.035	0.215	0	**0.001**
	g_Enterococcus_1698	5.23	0.022	0.208	**0.002**	0
	*g_Enterococcus_1702**	12.10	0.001	0.023	**0.017**	0.002
	g_Finegoldia_1899	4.45	0.035	0.215	0	**0.001**
	g_Lactobacillus_1528	5.45	0.020	0.208	**0.018**	0.006
	g_Parabacteroides_284	4.11	0.043	0.215	**0.003**	0
	g_Staphylococcus_1218	4.04	0.044	0.215	0	**0.001**
	g_Staphylococcus_1487	6.41	0.011	0.165	0.010	**0.034**
	g_Sutterella_1179	4.09	0.043	0.215	**0.006**	0
	g_Varibaculum_708	4.09	0.043	0.215	**0.001**	0
	g_Veillonella_1588	5.60	0.018	0.208	0.003	**0.016**
3′SL	f_Lachnospiraceae *Incertae_Sedis_1329*	4.85	0.028	0.151	**0.001**	0
	g_Bifidobacterium_1263	4.93	0.026	0.151	**0.418**	0.267
	*g_Bifidobacterium_681**	10.87	0.001	0.043	**0.104**	0.028
	g_Bifidobacterium_1227	7.87	0.005	0.055	**0.003**	0
	g_Bifidobacterium_1335	7.87	0.005	0.055	**0.002**	0
	g_Bifidobacterium_685	6.33	0.012	0.084	**0.002**	0
	g_Bifidobacterium_671	6.24	0.013	0.084	**0.002**	0

	g_Bifidobacterium_680	6.25	0.012	0.084	**0.001**	0
	g_Bifidobacterium_452	4.28	0.039	0.198	0	**0.001**
	g_Escherichia-Shigella_422	8.67	0.003	0.055	0.021	**0.129**
	g_Escherichia-Shigella_410	6.98	0.008	0.080	0.001	**0.002**
	g_Escherichia-Shigella_1174	9.13	0.003	0.055	0	**0.001**
	g_Escherichia-Shigella_1210	8.19	0.004	0.055	0	**0.001**
	*g_Escherichia-Shigella_1192**	11.85	0.001	0.043	0	**0.001**
	g_Escherichia-Shigella_382	5.67	0.017	0.107	0	**0**
	g_Staphylococcus_1487	3.95	0.047	0.214	**0.026**	0.025
	g_Varibaculum_708	4.18	0.041	0.198	**0.001**	0
	g_Veillonella_1579	6.47	0.011	0.084	0	**0.001**
	g_Veillonella_1588	8.10	0.004	0.055	0.004	**0.022**
6′SL	*g_Bacteroides_250*	4.20	0.040	0.440	**0.002**	0
	g_Bifidobacterium_1495	4.26	0.039	0.440	0	**0.001**
	g_Collinsella_1057	3.79	0.051	0.497	**0.002**	0.001
	g_Haemophilus_470	4.81	0.028	0.410	0.001	**0.007**
	g_Staphylococcus_1218	5.45	0.020	0.410	0	**0.002**
	g_Staphylococcus_1481	5.16	0.023	0.410	0	**0.001**
	g_Streptococcus_1379	5.01	0.025	0.410	**0.002**	0
	g_Streptococcus_1380	7.72	0.005	0.410	0.001	**0.003**
	g_Varibaculum_708	6.68	0.010	0.410	**0.002**	0
DFL	f_Coriobacteriaceae*_1908*	7.14	0.008	0.425	0	**0.002**
	g_Bacteroides_233	3.85	0.050	0.425	0	**0.001**
	g_Bacteroides_250	3.96	0.047	0.425	0.001	**0.002**
	g_Bacteroides_266	4.12	0.042	0.425	0.001	**0.004**
	g_Collinsella_1057	4.96	0.026	0.425	0	**0.003**
	g_Eggerthella_600	4.45	0.035	0.425	0	**0.002**
	g_Haemophilus_470	4.22	0.040	0.425	**0.008**	0.001
	g_Varibaculum_708	5.80	0.016	0.425	0	**0.001**
	g_Veillonella_1573	4.45	0.035	0.425	**0.012**	0.001
LNDFH I	f_Enterobacteriaceae*_386*	4.26	0.039	0.339	**0.001**	0
	g_Actinomyces_707	4.26	0.039	0.339	**0.001**	0
	g_Bifidobacterium_671	4.77	0.029	0.339	**0.002**	0.001
	g_Bifidobacterium_685	4.89	0.027	0.339	**0.002**	0.001
	g_Enterococcus_1702	10.79	0.001	0.089	**0.015**	0.003
	g_Flavonifractor_1518	4.26	0.039	0.339	0	**0.001**
	g_Parabacteroides_286	6.41	0.011	0.328	0.001	**0.011**
	g_Streptococcus_1416	5.76	0.016	0.339	0.012	**0.049**
	g_Streptococcus_1421	6.68	0.010	0.328	0	**0.000**
	g_Streptococcus_1426	5.44	0.020	0.339	0	**0.001**
LNDFH II	*g_Actinomyces_695*	4.27	0.039	0.476	0	**0.009**
	g_Bifidobacterium_1263	6.33	0.012	0.258	**0.516**	0.318
	g_Enterococcus_1698	6.71	0.010	0.258	**0.003**	0
	g_Enterococcus_1702	4.40	0.036	0.476	**0.016**	0.003
	g_Halomonas_478	3.81	0.051	0.476	**0**	0

	g_Lactobacillus_1528	9.53	0.002	0.088	**0.019**	0.005
	g_Streptococcus_1400	9.66	0.002	0.088	**0.003**	0.001
LNFP I	*g_Actinomyces_707*	4.27	0.039	0.259	**0.001**	0
	g_Bacteroides_233	4.75	0.029	0.232	**0.001**	0.001
	g_Bifidobacterium_1263	7.02	0.008	0.117	**0.450**	0.221
	*g_Bifidobacterium_681**	21.93	0.000	0.000	**0.162**	0.004
	g_Bifidobacterium_1227	9.06	0.003	0.064	**0.003**	0
	g_Bifidobacterium_1335	9.06	0.003	0.064	**0.003**	0
	g_Bifidobacterium_685	6.20	0.013	0.124	**0.002**	0
	g_Bifidobacterium_671	6.20	0.013	0.124	**0.002**	0
	g_Bifidobacterium_1296	6.71	0.010	0.119	0	**0.001**
	g_Clostridium_1792	4.28	0.039	0.259	0.002	**0.027**
	g_Streptococcus_1400	8.84	0.003	0.064	**0.003**	0.001
	g_Sutterella_1179	5.46	0.019	0.169	**0.006**	0
	Other	7.60	0.006	0.101	0.073	**0.120**
LNFP II	f_Enterobacteriaceae_*386*	3.93	0.048	0.180	**0.001**	0.000
	f_Lachnospiraceae *Incertae_Sedis_870*	4.12	0.042	0.174	0	**0.002**
	g_Actinomyces_707	6.68	0.010	0.083	**0.001**	0
	g_Bacteroides_233	5.01	0.025	0.146	**0.001**	0
	g_Bacteroides_243	6.68	0.010	0.083	**0.003**	0
	g_Bacteroides_250	6.68	0.010	0.083	**0.002**	0
	g_Bifidobacterium_1227	5.16	0.023	0.143	**0.003**	0.002
	g_Bifidobacterium_1263	9.57	0.002	0.033	**0.546**	0.274
	g_Bifidobacterium_1335	5.16	0.023	0.143	**0.002**	0.001
	g_Bifidobacterium_671	11.03	0.001	0.026	**0.003**	0
	g_Bifidobacterium_680	9.30	0.002	0.033	**0.001**	0
	g_Bifidobacterium_681	6.55	0.011	0.083	0.084	**0.085**
	g_Bifidobacterium_685	11.21	0.001	0.026	**0.003**	0
	g_Collinsella_1057	4.26	0.039	0.169	**0.001**	0
	g_Enterococcus_1698	4.48	0.034	0.169	**0.003**	0
	g_Enterococcus_1702	4.28	0.039	0.169	**0.013**	0.005
	g_Haemophilus_470	7.18	0.007	0.083	0	**0.008**
	g_Parabacteroides_284	4.06	0.044	0.174	0	**0.002**
	g_Staphylococcus_1218	9.30	0.002	0.033	0	**0.002**
	g_Staphylococcus_1481	5.44	0.020	0.142	0	**0.001**
	g_Staphylococcus_1487	11.51	0.001	0.026	0.011	**0.032**
	g_Varibaculum_708	4.48	0.034	0.169	**0.002**	0
	Other	4.34	0.037	0.169	0.034	**0.093**
LST c	*g_Actinomyces_695*	6.68	0.010	0.170	**0.032**	0
	g_Clostridium_1792	6.79	0.009	0.170	**0.011**	0.001
	g_Collinsella_1057	6.68	0.010	0.170	**0.002**	0
	g_Staphylococcus_1218	6.68	0.010	0.170	0	**0.002**
	g_Streptococcus_1380	11.90	0.001	0.049	0	**0.003**
	g_Streptococcus_1395	4.26	0.039	0.445	**0.008**	0
	g_Streptococcus_1403	4.26	0.039	0.445	0	**0.001**

LNFP III	*g_Actinomyces_707*	5.44	0.020	0.124	**0.001**	0
	g_Bifidobacterium_1263	4.97	0.026	0.150	**0.504**	0.313
	*g_Bifidobacterium_681**	10.72	0.001	0.023	**0.120**	0.034
	*g_Bifidobacterium_685**	12.84	0.000	0.016	**0.003**	0
	*g_Bifidobacterium_1227**	9.18	0.002	0.030	**0.003**	0.001
	*g_Bifidobacterium_671**	12.65	0.000	0.016	**0.003**	0
	*g_Bifidobacterium_1335**	9.34	0.002	0.030	**0.003**	0.001
	*g_Bifidobacterium_680**	10.69	0.001	0.023	**0.001**	0
	g_Enterococcus_1702	5.41	0.020	0.124	**0.015**	0.005
	g_Escherichia-Shigella_1192	5.44	0.020	0.124	0	**0.001**
	g_Escherichia-Shigella_422	6.04	0.014	0.124	0.022	**0.116**
	g_Halomonas_478	4.26	0.039	0.212	**0**	0
	g_Parabacteroides_355	5.60	0.018	0.124	0	**0.003**
	*g_Staphylococcus_1487**	10.07	0.002	0.026	0.015	**0.028**
	g_Streptococcus_1426	5.75	0.016	0.124	0	**0.001**
	g_Varibaculum_708	5.44	0.020	0.124	**0.001**	0
LNFP V	f_Enterobacteriaceae_392	4.43	0.035	0.171	0.008	**0.101**
	f_Lachnospiraceae *Incertae_Sedis_876*	7.31	0.007	0.090	**0.034**	0.006
	g_Bacteroides_222	8.57	0.003	0.074	**0.054**	0.007
	g_Bacteroides_261	4.53	0.033	0.170	**0.011**	0
	g_Bacteroides_267	4.14	0.042	0.183	**0.006**	0
	*g_Bifidobacterium_1263**	18.02	0.000	0.001	**0.475**	0.156
	g_Bifidobacterium_1296	5.17	0.023	0.143	0	**0.001**
	g_Clostridium_1639	4.53	0.033	0.170	0	**0.002**
	g_Clostridium_1789	7.84	0.005	0.089	0	**0.001**
	*g_Clostridium_1792**	18.33	0.000	0.001	0.001	**0.028**
	g_Collinsella_1057	5.39	0.020	0.135	**0.002**	0
	g_Eggerthella_600	4.93	0.026	0.153	**0.002**	0
	g_Enterobacter_1189	6.16	0.013	0.104	0	**0.001**
	g_Parabacteroides_284	7.21	0.007	0.090	**0.003**	0
	g_Parabacteroides_286	6.61	0.010	0.098	**0.009**	0.001
	g_Staphylococcus_1487	9.09	0.003	0.074	0.013	**0.032**
	g_Streptococcus_1395	6.16	0.013	0.104	0	**0.002**
	g_Streptococcus_1416	6.63	0.010	0.098	0.031	**0.055**
	g_Veillonella_1573	4.30	0.038	0.175	0.002	**0.014**
	g_Veillonella_1588	5.71	0.017	0.122	0.004	**0.024**
LNH	f_Coriobacteriaceae *1908*	3.90	0.048	0.175	**0.001**	0
	f_Enterobacteriaceae *392*	4.67	0.031	0.145	0.025	**0.069**
	g_Actinomyces_695	6.99	0.008	0.061	0	**0.032**
	g_Actinomyces_704	5.89	0.015	0.095	0	**0.003**
	g_Actinomyces_707	3.90	0.048	0.175	**0**	0
	g_Bacteroides_222	3.90	0.048	0.175	**0.055**	0.044
	g_Bacteroides_261	4.62	0.032	0.145	**0.009**	0
	*g_Bifidobacterium_1263**	20.45	0.000	0.000	**0.480**	0.161

	*g_Bifidobacterium_681**	21.88	0.000	0.000	**0.116**	0
	*g_Bifidobacterium_1227**	12.26	0.000	0.007	**0.003**	0
	*g_Bifidobacterium_1335**	12.26	0.000	0.007	**0.003**	0
	*g_Bifidobacterium_685**	10.37	0.001	0.014	**0.002**	0
	*g_Bifidobacterium_671**	10.37	0.001	0.014	**0.002**	0
	g_Bifidobacterium_680	6.93	0.008	0.061	**0.001**	0
	*g_Bifidobacterium_1296**	12.84	0.000	0.007	0	**0.001**
	g_Clostridium_1639	5.16	0.023	0.134	0	**0.002**
	g_Clostridium_1789	8.88	0.003	0.025	0	**0.001**
	g_Clostridium_1792	9.23	0.002	0.023	0.002	**0.027**
	g_Collinsella_1057	3.90	0.048	0.175	**0.001**	0
	g_Parabacteroides_355	4.62	0.032	0.145	**0.002**	0
	g_Staphylococcus_1487	4.79	0.029	0.145	0.021	**0.037**
	g_Streptococcus_1395	6.02	0.014	0.094	0.000	**0.019**
	g_Veillonella_1588	3.94	0.047	0.175	0.006	**0.022**
	Other*	16.51	0.000	0.001	0.042	**0.165**
LNnH	*g_Actinomyces_704*	8.75	0.003	0.054	0	**0.004**
	g_Bifidobacterium_1263	6.51	0.011	0.085	**0.417**	0.241
	*g_Bifidobacterium_681**	15.41	0.000	0.008	**0.114**	0.005
	g_Bifidobacterium_1227	6.97	0.008	0.080	**0.003**	0
	g_Bifidobacterium_1335	7.06	0.008	0.080	**0.002**	0
	g_Bifidobacterium_685	5.43	0.020	0.115	**0.002**	0
	g_Bifidobacterium_671	5.52	0.019	0.115	**0.002**	0
	*g_Bifidobacterium_1296**	10.65	0.001	0.026	0	**0.001**
	g_Bifidobacterium_1495	7.84	0.005	0.069	0	**0.001**
	g_Clostridium_1629	5.79	0.016	0.115	0	**0.007**
	g_Clostridium_1789	5.03	0.025	0.136	0	**0.001**
	g_Escherichia-Shigella_410	4.75	0.029	0.150	**0.002**	0.001
	g_Escherichia-Shigella_422	7.69	0.006	0.069	**0.070**	0.039
	*g_Staphylococcus_1218**	13.02	0.000	0.013	0	**0.003**
	g_Staphylococcus_1481	6.67	0.010	0.085	0	**0.001**
	*g_Staphylococcus_1487**	10.50	0.001	0.026	0.013	**0.048**
	g_Streptococcus_1400	3.89	0.049	0.235	**0.003**	0.001
	Other	5.67	0.017	0.115	0.057	**0.146**
LST a	f_Bifidobacteriaceae_*448*	6.65	0.010	0.189	0	**0.000**
	f_Lachnospiraceae *Incertae_Sedis_1329*	4.88	0.027	0.215	**0.001**	0
	f_Lachnospiraceae *Incertae_Sedis_870*	6.49	0.011	0.189	**0.001**	0
	f_Lachnospiraceae *Incertae_Sedis_876*	6.69	0.010	0.189	**0.037**	0.001
	g_Bacteroides_243	4.00	0.046	0.331	0	**0.002**
	g_Escherichia-Shigella_1174	5.46	0.020	0.215	0	**0.001**
	g_Escherichia-Shigella_1192	8.45	0.004	0.185	0	**0.001**
	g_Escherichia-Shigella_1210	5.25	0.022	0.215	0	**0.001**
	g_Escherichia-Shigella_382	4.91	0.027	0.215	0	**0**
	g_Escherichia-Shigella_422	5.42	0.020	0.215	0.022	**0.119**
	g_Halomonas_478	4.88	0.027	0.215	**0**	0

	g_Veillonella_1588	8.17	0.004	0.185	0.004	**0.018**
LST b	*g_Actinomyces_707*	4.26	0.039	0.622	**0.001**	0
	g_Bifidobacterium_1296	5.44	0.020	0.427	0	**0.001**
	g_Bifidobacterium_681	5.84	0.016	0.427	**0.149**	0.045
	g_Collinsella_1057	6.68	0.010	0.427	**0.002**	0
	g_Staphylococcus_1218	6.47	0.011	0.427	0	**0.002**
pLNH	*g_Bacteroides_261*	4.49	0.034	0.198	0.009	0
	*g_Bifidobacterium_1263**	15.91	0.000	0.002	**0.457**	0.173
	*g_Bifidobacterium_681**	16.21	0.000	0.002	**0.118**	0.028
	*g_Bifidobacterium_1227**	10.16	0.001	0.022	**0.003**	0
	*g_Bifidobacterium_1335**	10.16	0.001	0.022	**0.003**	0
	*g_Bifidobacterium_685**	8.40	0.004	0.041	**0.002**	0
	*g_Bifidobacterium_671**	8.40	0.004	0.041	**0.002**	0
	g_Bifidobacterium_680	5.98	0.015	0.105	**0.001**	0
	*g_Bifidobacterium_1296**	15.57	0.000	0.002	0	**0.002**
	g_Clostridium_1639	7.27	0.007	0.068	0	**0.004**
	g_Clostridium_1789	5.36	0.021	0.128	0	**0.001**
	g_Clostridium_1792	6.68	0.010	0.077	0.003	**0.022**
	g_Eggerthella_600	6.76	0.009	0.077	**0.002**	0
	g_Rothia_697	5.64	0.018	0.117	0	**0.001**
	g_Staphylococcus_1487	3.98	0.046	0.225	0.020	**0.032**
	g_Veillonella_1588	3.97	0.046	0.225	0.006	**0.022**
	Other*	10.08	0.001	0.022	0.063	**0.136**
LNT and LNnT	f_Bifidobacteriaceae_*448*	4.26	0.039	0.154	**0**	0
	f_Coriobacteriaceae_*1908*	6.68	0.010	0.065	**0.002**	0
	*g_Bifidobacterium_1263**	18.97	0.000	0.001	**0.561**	0.162
	g_Bifidobacterium_681	5.82	0.016	0.081	0.054	**0.060**
	*g_Bifidobacterium_1296**	7.96	0.005	0.046	0	**0.001**
	*g_Bifidobacterium_1227**	10.69	0.001	0.016	**0.002**	0
	*g_Bifidobacterium_1335**	10.69	0.001	0.016	**0.002**	0
	g_Bifidobacterium_452	6.68	0.010	0.065	**0.001**	0
	*g_Bifidobacterium_671**	10.69	0.001	0.016	**0.002**	0
	g_Bifidobacterium_680	6.68	0.010	0.065	**0.001**	0
	*g_Bifidobacterium_685**	10.69	0.001	0.016	**0.002**	0
	g_Clostridium_1789	5.44	0.020	0.081	0	**0.001**
	*g_Clostridium_1792**	8.03	0.005	0.046	0.002	**0.027**
	g_Eggerthella_600	6.02	0.014	0.081	**0.002**	0
	*g_Escherichia-Shigella_1174**	9.30	0.002	0.029	**0.001**	0
	g_Escherichia-Shigella_1192	5.44	0.020	0.081	**0.001**	0
	g_Escherichia-Shigella_1210	5.75	0.016	0.081	**0.001**	0
	*g_Escherichia-Shigella_410**	12.93	0.000	0.014	**0.002**	0
	g_Escherichia-Shigella_422	5.77	0.016	0.081	0.068	**0.072**
	g_Parabacteroides_284	5.44	0.020	0.081	**0.002**	0
	*g_Staphylococcus_1487**	7.64	0.006	0.050	0.010	**0.035**

	Other	5.72	0.017	0.081	0.053	**0.153**
*FDR<0.05						

Chapter 6

In vitro fermentation behaviour of isomalto/malto-polysaccharides using human faecal inoculum indicates prebiotic potential

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ABSTRACT

This study characterized intestinal fermentation of isomalto/malto-polysaccharides (IMMPs), by monitoring degradation of IMMPs, production of short chain fatty acids (SCFAs), lactic acid and succinic acid as well as enzyme activity and microbiota composition in time. IMMP-94 (94% α-(1→6) glycosidic linkages), IMMP-96, IMMP-27 and IMMP-dig27 (after removal of digestible starch segments from IMMP-27) were fermented in a batchwise *in vitro* fermentation model using human faecal inoculum. Fermentation digesta samples were taken for analysis in time up till 48 h. The fermentation of α-(1→6) glycosidic linkages in IMMP-94, IMMP-96 and IMMP-dig27 started after 12 h and finished within 48 h. IMMP-27 fermentation started directly after inoculation utilising α-(1→4) linked glycosidic linkages, however, the utilization of α-(1→6) linked glycosidic linkages was delayed and started only after depletion of α-(1→4) glycosidic linkages. SCFAs were produced in high amounts with acetic acid and succinic acid being the major products next to propionic acid and butyric acid. The polysaccharide fraction was degraded into isomalto-oligosaccharides (IMOs) mainly by extracellular enzymes. The smaller IMOs were further degraded mainly by cell-associated enzymes. Overall microbial diversity, as well as the relative abundance of *Bifidobacterium* and *Lactobacillus*, significantly increased during fermentation of IMMPs. In conclusion, α-(1→6) linked segments of IMMPs are slowly-fermentable fibres with prebiotic potential.

INTRODUCTION

Prebiotics and their health benefits are of growing research interest nowadays. A dietary prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit".[1] Well-documented prebiotics include lactulose, inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS). These substrates have been shown to selectively stimulate the growth and activity of bifidobacteria, lactic acid bacteria and other health beneficial bacteria.[2,3] Fermentation of prebiotics in the colon by these and other bacterial groups leads to production of short chain fatty acids (SCFAs) that are beneficial for gut health.[4] The present study focuses on a novel type of undigestible α-glucans, the isomalto/malto-polysaccharides (IMMPs).

IMMPs are produced from starch with the use of a 4,6-α-glucanotransferase (GTFB) enzyme from *Lactobacillus reuteri* 121.[5,6] The GTFB enzyme transfers a glucose moiety from the non-reducing end of α-(1→4) linked glycosidic chains, as present in starch and starch-derived maltodextrins, to the non-reducing end of other glycosidic chains generating α-(1→6) linked glycosidic linkages in a stepwise manner, which results in the formation of IMMPs containing linear α-(1→6) linked glycosidic chains.[6] The conversion rate to α-1,6-linkages is positively correlated with the amylose content of the substrates, and negatively correlated with the original level of α-(1→4,6) linked branches present in amylopectin.[6] For this reason, the joint action of GTFB and debranching enzymes, e.g. isoamylase or pullulanase, leads to higher conversion rates from α-(1→4) to α-(1→6) glycosidic linkages.[6] The percentage of α-(1→6) glycosidic linkages can reach more than 90%, depending on the origin of the starch used as the substrate and the involvement of debranching enzymes.[6]

IMMPs have been suggested to have potential health-beneficial effects because the α-(1→6) rich segments can escape digestion in the upper gastrointestinal tract, and can be utilized as carbon source by microbiota in the large intestine.[6] This has been reported for compounds such as isomalto-oligosaccharides (IMOs) and dextran, which have similarities in structure when compared to IMMPs. IMOs are gluco-oligosaccharides consisting of predominantly α-(1→6) linkages, with the degree of polymerization (DP) ranging from 2 to 10.[7] IMOs have been shown to promote growth of lactobacilli and bifidobacteria in both *in vitro* fermentation and *in vivo* rat models.[8–10] Dextran, another well-known glucose homopolysaccharide with consecutive α-(1→6) linkages, has been reported to stimulate bifidobacteria and lactobacilli during *in vitro* fermentation with human faecal microbiota, and to lead to increased production of butyrate.[11] Therefore, based on the structural similarity between IMMPs, IMOs and dextran, we expected that IMMPs would bear prebiotic potential as well.

The starch origin and involvement of debranching enzymes during synthesis of IMMPs lead to structural differences, which in turn, may influence the IMMPs' fate during fermentation in the colon. The difference can be in the proportion of α-(1→6) and α-(1→4) linked glycosidic linkages. It has been shown by NMR spectroscopy that the relative amount of α-(1→6) linkages can be very different, ranging

from 7% to over 90%,[6] with the remaining linkages being α-(1→4). Although α-(1→4) linkages are in general readily digested by human digestive enzymes, introduction of α-(1→6) linkages may help neighbouring α-(1→4) linked units to escape digestion and to enter the colon. Such starches that have been chemically or enzymatically modified to resist digestion, are considered to be Resistant Starch type IV [12]. It remains unclear to what extent the α-(1→4) linked segments of IMMPs would end up in the colon and have an influence on fermentation of α-(1→6) linkages. Based on earlier studies on the fate of retrograded tapioca starch, it can be speculated that the presence of resistant starch could influence the fermentation of other fibres.[13] IMMPs with similar percentages of α-(1→6) linkages could differ in the distribution of molecular chain length, depending on the side-chain length distribution of the parental starch. It remains unknown whether such differences in molecular chain length would influence the fermentation behaviour of IMMPs.

Leemhuis et al. showed preliminary results of *in vitro* fermentation of IMMPs,[6] including an increase in microbial biomass, as monitored by optical density, and an increase in concentrations of acetic acid and propionic acid. However, the influence of additional factors on IMMPs fermentation, including molecular weight and the presence of α-1,4-linkages, still needs to be determined. Furthermore, previous research showed that the production of enzymes by faecal microbiota varies depending on substrate properties, including sugar composition, linkage type and chain length.[13–15] The prebiotic potential of IMMPs is still unknown since detailed effects on microbiota composition have yet to be established.[6]

Therefore, to evaluate the prebiotic potential of IMMPs, a comprehensive *in vitro* batch fermentation of selected types of IMMPs with a standardized human faecal inoculum was performed in the present study. The fermentation behaviour of IMMPs at a molecular level and production of individual organic acids were studied, and a link to microbiota composition was made. In addition, bacterial enzyme activities involved in the IMMP degradation were studied in order to help explaining the mechanism of bacterial utilization of IMMPs.

MATERIALS & METHODS

Materials

Three different types of isomalto/-malto-polysaccharides (IMMPs) were used in this study. In order to facilitate the comparison of results, the IMMPs were named after their percentages of total α-(1→6) content in this study. The total α-(1→6) content, consisting of both α-(1→6) and α-(1→4,6) linked glycosidic linkages, was determined by hydrogen-1 nuclear magnetic resonance (^{1}H NMR) spectroscopy, with the methodology and results already published previously.[16] IMMP-94 (94% α-(1→6) linkages) originate from potato starch (AVEBE, Veendam, the Netherlands) modified with *Lactobacillus reuteri* 121 GTFB 4,6-α-glucanotransferase,[6] and pullulanase (Promozyme D2) (Novozymes, Bagsvaerd,

Denmark) and was kindly provided by Dr. Hans Leemhuis (AVEBE). IMMP-27 (27%) and IMMP-96 (96%) were synthesized from potato starch and Etenia 457 starch (AVEBE) respectively, as published by van der Zaal et al. and described below:[16]

IMMP-27: Potato Starch was suspended at 2.5% (w/v) in 20 mM sodium acetate buffer, pH=4.9, containing 5 mM $CaCl_2$. The suspension was autoclaved at 121°C for 15 min and cooled to 37°C. IMMP synthesis was carried out by adding 0.3 mg GTFB-ΔN /g substrate and incubating the reaction mixture at 37°C for 24 h. GTFB-ΔN is GTFB with N-terminal truncation,[17] and the synthesis of GTFB-ΔN is described elsewhere.[16] GTFB-ΔN was inactivated in a water bath at 95°C for 15 min. The solution was cooled to 50°C, Amberlite® MB-20 resin (Dow, Midland, MI, USA) was added to remove salts and then incubated at 50°C for 2 h. The resin was sieved out and the IMMP solution was freeze-dried.

IMMP-96: Amylomaltase treated potato starch (Etenia 457) was used as substrate, and treated similarly as described for the synthesis of IMMP-27, with some modifications. Besides GTFB-ΔN, pullulanase (Promozyme D2) was also added at an amount of 2 μL/g substrate, and the incubation time was extended to 41 h.

Experimental set-up and removal of digestible starch segments

A schematic overview of the present study is shown in Figure 6.1. Three types of IMMPs, IMMP-27, IMMP-94 and IMMP-96 were used, representing extremes with respect to the percentage of total α-(1→6) linked glycosidic linkage content. Each of the three IMMPs were split and parts were either left untreated or treated with pancreatic α-amylase and amyloglucosidase (Resistant Starch assay kit, Megazyme, Bray, Ireland) to remove α-(1→4) linked glycosidic linkages in order to obtain the resistant fibre. Hereto, IMMP-27, IMMP-94 and IMMP-96 were treated with two starch digesting enzymes. The concentrations of both enzymes and the incubation conditions were according to Megazyme protocols. After inactivating the enzymes at 100ºC for 5 min, IMMPs were recovered by ethanol precipitation with a final ethanol concentration of 70%. The supernatant containing glucose and small maltodextrins was removed by decanting after centrifugation at 10,000 g for 15 min at room temperature. The ethanol precipitation step was repeated twice. Afterwards, the pellet was washed once with pure ethanol and air-dried at 30ºC. The sample obtained after the removal of digestible α-1,4-linked glucose segments from IMMP-27 was named IMMP-dig27.

In order to determine the level of removal of α-(1→4) linked glycosidic linkages in IMMPs after enzymatic digestion, the sugar content of the supernatant collected from the ethanol precipitation step was measured colorimetrically by a phenol-sulphuric acid assay, using D-glucose as standard for calibration[18,19] Since less than 2% of glucose moieties were removed from both IMMP-94 and IMMP-96, only the parental IMMP-94 and IMMP-96 were included in the following *in vitro* fermentation, whereas both untreated IMMP-27 and IMMP-dig27 were used.

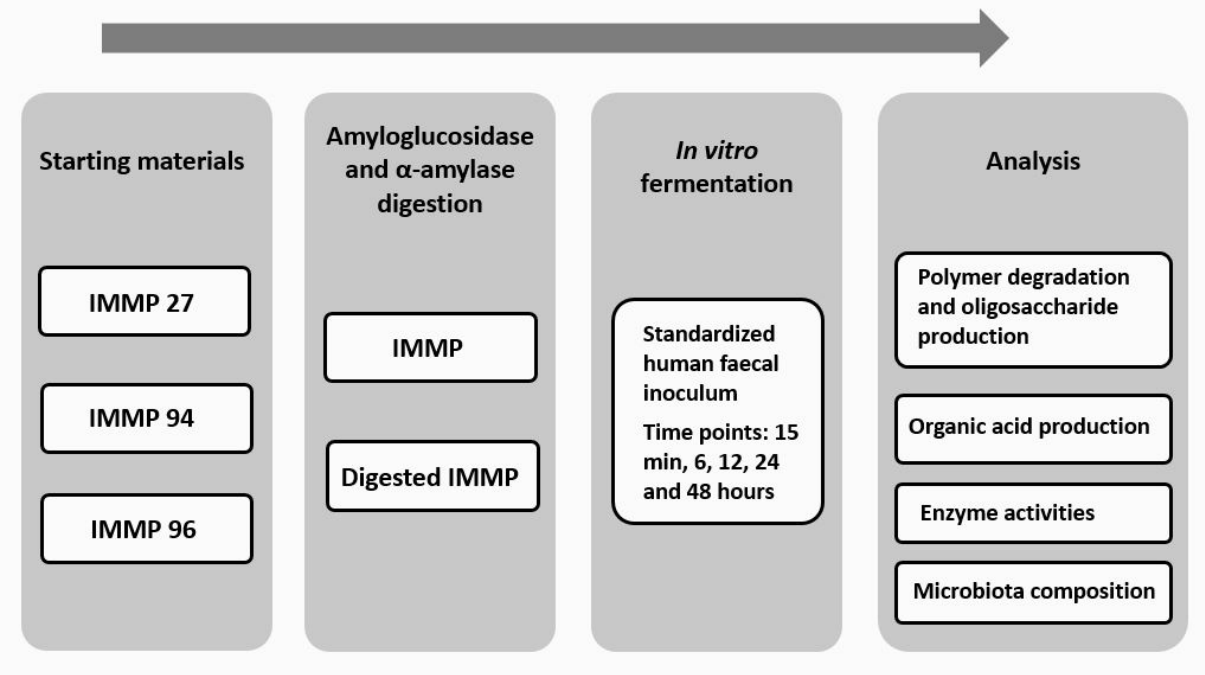

Figure 6.1. Experimental set-up of the *in vitro* fermentation of IMMPs

In vitro fermentation

An *in vitro* fermentation was performed to simulate the fermentation of IMMPs in the human colon according to the procedure described by Rösch et al.,[14] with the modification that carbohydrates (pectin, xylan, arabinogalactan, amylopectin and starch) and Tween 80 were left out of the standard ileal efflux medium (SIEM), in order to reduce as much as possible background fermentation from the medium components. The modified SIEM medium contained 40% (v/v) BCO medium, 1.6% (v/v) salt solution, 0.8% (v/v) $MgSO_4$ (50 g/L), 0.4% (v/v) cysteine hydrochloride (40 g/L), 0.08% (v/v) vitamin solution and 10% (v/v) MES buffer (1 M, pH 6.0) in water. The BCO medium contained (g/L): Bacto Peptone, 60.0; casein, 60.0; Ox bile, 1. The salt solution contained (g/L): $K_2HPO_4 \cdot 3H_2O$, 156.25; NaCl, 281.25; $CaCl_2 \cdot 2H_2O$, 28.13; $FeSO_4 \cdot 7H_2O$, 0.31; hemin, 0.63. The vitamin solution contained (mg/L): menadion, 1.0; biotine, 2.0; vitamin B12, 0.5; pantothenate, 10.0; nicotinamide, 5.0; *p*-aminobenzoic acid, 5.0; thiamine, 4.0. The ingredients used to make SIEM were purchased from Tritium Microbiologie (Veldhoven, the Netherlands).

A standard human faecal inoculum was prepared by TNO (Zeist, the Netherlands), and was kindly provided by Prof. Dr. K. Venema. The faecal inoculum was pooled from seven healthy volunteers (male: n=3, average age=46.3 y (range: 26-57), BMI= 24.1 ± 2.42 kg/m2; female: n=4, average age=37.7 y (27-52), BMI= 24.2 ± 1.91 kg/m2). The pooling procedure was described and validated previously.[20,21]

Each *in vitro* fermentation took place in a 20 mL serum bottle sealed with a butyl rubber stopper and with the final volume of the fermentation liquid being 10 mL. The final concentration of IMMP was

10 mg/mL and faecal inoculum was added to a final concentration of 1% (v/v). The fermentation was performed in duplicate. All handling procedures were performed in an anaerobic cabinet (gas phase: 81% N_2, 15% CO_2 and 4% H_2). Negative control incubations were included and did not receive any faecal inoculum (inoculum blanks) or IMMP substrate (IMMP blanks). A baseline sample (defined as 0 h) was taken within the first 15 min after addition of the inoculum, after which bottles were incubated at 37ºC and shaking at 140 rpm. For sampling, sterile syringes and needles were used to take aliquots (2 – 3.5 mL) at time points 15 min, 6, 12, 24 and 48 h.

Analytical methods

Assessment of IMMP degradation by HPSEC-RI

Part of the fermentation digest was heated at 100ºC for 5 min and then centrifuged at 18,600 g for 10 min at room temperature. The supernatant was diluted four times with water to be used for high performance size exclusion chromatography (HPSEC). A set of four TSK-Gel® SuperAW columns (Tosoh Bioscience, Tokyo, Japan) were used on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA) system: a guard column (SuperAW-L, 3.5 cm x 4.6 mm ID) and three analytical columns (SuperAW 4000, 3000 and 2500; 15 cm x 6.0 mm ID). Ten µL of sample was injected and eluted at 0.6 mL/min 0.2 M $NaNO_3$ isocratically. The column temperature was 55ºC. Eluted components were monitored by an RI detector (Shodex RI-101, Showa Denko K.K., Kawasaki, Japan). Molecular weights of IMMPs were estimated using a pullulan (Polymer Laboratories, Palo Alto, CA, USA) calibration curve. Chromeleon™ 7.1 software (Dionex) was used to process data from HPSEC.

Analysis of oligosaccharide production by HPAEC-PAD

The supernatant obtained after centrifugation of the fermentation digest was tenfold diluted before analysis using high performance anion exchange chromatography in combination with pulsed amperometric detection (HPAEC-PAD). The oligosaccharide peaks were annotated using dextranase-treated IMMP-94 as a standard. The dextranase-treated IMMP-94 was prepared as follows: 0.25 unit of dextranase from *Chaetomium erraticum* (Sigma-Aldrich, St. Louis, MO, USA) was added to 5 mg IMMP-94 in 1 mL of 0.1 M sodium maleate buffer containing 5 mM $CaCl_2$ at pH 6, and incubated at 37ºC for 30 min. The reaction was stopped by heating at 99ºC for 5 min, and the supernatant was diluted five times for HPAEC analysis after centrifuging at 18,600 g for 10 min at room temperature.

Ten µL of sample was injected to a Dionex ICS 5000 system (Dionex) with a CarboPac PA-1 column (250 mm x 2 mm ID) and a CarboPac PA guard column (25 mm x 2 mm ID). The column temperature was 20ºC. The flow rate of the two mobile phases (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH was set to 0.3 mL/min. The gradient elution was applied as follows: 0 – 40 min, 0 – 40% B; 40 – 40.1 min, 40 – 100% B; 40.1 – 45 min, 100% B; 45 – 45.1 min, 100 – 0% B; 45.1 – 60 min, 0%

B. The elution was monitored by a PAD (Dionex ISC-5000 ED). Chromeleon™ 7.1 software (Dionex) was used to process data from HPAEC.

Extraction and activity of bacterial enzymes

Part of the fermentation digest (0.4 mL) was snap frozen in liquid nitrogen and stored at -80ºC before enzyme extraction. The following fermentation digests were selected according to HPAEC results (see section Results and Discussion for further details): IMMP blank, IMMP-27 at 12 and 48 h, IMMP-dig27 at 12 and 24 h, and IMMP-94 at 12 and 24 h. Protein extraction was performed as described elsewhere with some modifications.[14] To obtain the fraction of extracellular enzymes (EE), the fermentation digest was first centrifuged (21,000 g, 4ºC, 10 min), and the supernatant was applied on a 10kDa centrifugal filter (VWR®, Amsterdam, the Netherlands) at 4ºC and 18,600 g to remove any mono- and oligosaccharides produced during fermentation. A volume of 0.4 mL 25 mM MES buffer pH 5.8 containing 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol was used to reconstitute the retentate (EE). The pellet from the first centrifugation step was washed once with 1.5 mL buffer, centrifuged again and then suspended in 0.4 mL of the same MES buffer. The suspension was sonicated at 30% amplitude for 30 s and repeated three times with 40 s break in between.[13] The supernatant after centrifugation was used as cell-associated enzymes (CE).

The enzyme activity of EE and CE towards PNP-glucose substrates and starch was determined using a colour reaction, as described previously with some modifications.[14] In the glycosidase assay, only PNP-α-D-glucopyranoside and PNP-β-D-glucopyranoside were included as substrates. In the polysaccharide assay, soluble potato starch (Sigma-Aldrich) and IMMP-94 were used as substrates. Potato starch was incubated at 99ºC until solubilized. The substrate (3.125 mg/mL) was mixed with enzyme extracts in a 4:1 ratio, yielding a final substrate concentration of 2.5 mg/mL. The amount of reducing sugar released after 1 h incubation was determined by 4-hydroxybenzoic acid hydrazide (PAHBAH) assay using glucose as a standard.[13] Enzyme activities were expressed in mU (nmol-reduced-end-formed* mL-digest^{-1} min^{-1}).

Analysis of SCFAs and other organic acids by GC-FID and HPLC-RI

Determination of SCFAs (acetic acid, propionic acid and butyric acid) by gas chromatography (GC) and of lactic acid and succinic acid by high performance liquid chromatography (HPLC) was done as described previously with some modifications.[22] For GC, 70 µL of twofold diluted supernatant of the fermentation digest was mixed with 70 µL 0.15 M oxalic acid and allowed to stand at room temperature for 30 min. Then 199 µL water and 1 µL of 5 mg/mL 2-ethylbutyric acid was added. The temperature profile during GC analysis was as follows: from 100ºC to 165ºC at 5ºC min^{-1}, then held at 165ºC for 1 min. Chromeleon™ 7.1 software (Dionex) was used to process data from HPLC. Xcalibur TM software (Thermo Scientific, Breda, the Netherlands) was used to process data from GC.

DNA extraction, 16S ribosomal RNA gene sequencing and microbial composition analysis

The pellets obtained from centrifugation of fermentation digest were snap frozen in liquid nitrogen, stored at -80ºC and used for microbial composition analysis. Total bacterial DNA was extracted using the Maxwell® 16 Total RNA system (Promega, Wisconsin, USA) with Stool Transport and Recovery Buffer (STAR; Roche Diagnostics Corporation, Indianapolis, IN). Briefly, bacterial pellets were homogenized with 0.25 g of sterilized 0.1 mm zirconia beads and three glass beads (2.5 mm) in 300 µL STAR buffer for 3 × 1 min at 5.5 m/s using a bead beater (Precellys 24, Bertin Technologies), with cooling on ice for 1 min in between. Samples were incubated with shaking at 100 rpm for 15 min at 95ºC and pelleted by 5 min centrifugation at 4ºC and 14,000 g. Supernatant was removed and the pellets were processed again using 200 µL fresh STAR buffer. Samples were incubated at 95ºC and centrifuged as before. Supernatant was removed, pooled with the first supernatant and 250 µL was used for purification with Maxwell® 16 Tissue LEV Total RNA Purification Kit (AS1220) customized for DNA extraction in combination with the STAR buffer. DNA was eluted with 50 µL of DNAse and RNAse free water (Qiagen, Hilden, Germany). DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA) and adjusted to 20 ng/µL with DNAse and RNAse free water. The V4 region of 16S ribosomal RNA (rRNA) genes was amplified. PCR reactions were done in duplicates, each in a total volume of 50 µL and containing 20 ng of template DNA. Each sample was amplified with a unique barcoded primer pair 515F-n (5'-GTGCCAGCMGCCGCGGTAA-) and 806R-n (5'-RGGATTAGATACCC) (10 µM each/reaction [23]), 1x HF buffer (Finnzymes, Vantaa, Finland), 1 µL dNTP Mix (10 mM each, Roche Diagnostics GmbH, Mannheim, Germany), 1 U Phusion® Hot Start II High Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) and 36.5 µL of DNAse and RNAse free water. The amplification program included 30 s initial denaturation step at 98ºC, following by 25 cycles of denaturation at 98ºC for 10 s, annealing at 56ºC for 10 s and elongation at 72ºC for 10 s, and a final extension at 72ºC for 7 min. The PCR product presence and size (~290 bp) was confirmed with gel electrophoresis using the Lonza FlashGel® System (Lonza, Cologne, Germany). Seventy unique barcode tags were used in each library and artificial control (Mock) communities representative of human intestinal microbiota were included.[23] PCR products were purified with HighPrep® PCR kit (MagBio Genomics, Alphen aan den Rijn, the Netherlands), and DNA concentrations were measured with Qubit® dsDNA BR Assay Kit (Life Technologies, Leusden, the Netherlands). 100 ng of each barcoded sample was added to an amplicon pool that was subsequently concentrated with HighPrep® PCR kit to 20 µL volume. The concentration was measured with Qubit® dsDNA BR Assay Kit and adjusted to 100 ng/µL final concentration. The libraries were sent for adapter ligation and HiSeq sequencing (GATC-Biotech, Konstanz, Germany). Data processing and analysis was carried out using NG-Tax.[23] Diversity analyses were carried out in QIIME.[24,25] Relative abundance at genus level was used for calculating pairwise Pearson correlation scores between biological replicates, and the values for the different taxa were averaged for each replicate pair.

RESULTS & DISCUSSION

Fermentation reproducibility

The *in vitro* fermentation experiments were run in two separate batches, the first one using IMMP-27 and IMMP-94, and the second batch with IMMP-dig27 and IMMP-96. Between batch similarity was estimated based on Pearson correlation scores of genus-level microbiota composition data for the IMMP blank samples from different batches, at time 0, 24 and 48 h and were 0.98, 0.94 and 0.88 respectively. A high reproducibility for the results between the two batches was found, validating the between batch comparisons to be carried out when necessary. Pearson correlation scores also showed high levels of similarity between the biological duplicates at genus level (average Pearson score of 0.97, SD ± 0.03 for IMMP treatment groups and 0.90, SD ± 0.22 for IMMP blank groups).

Physicochemical characterization of IMMP-94 and IMMP-96

IMMP-94 and IMMP-96 contained high percentages of α-1,6-linkages, as a result of including the debranching enzyme pullulanase during the synthesis by GTFB. The two IMMPs were derived from different starches, namely normal potato starch and amylomaltase treated potato starch. Amylomaltase treatment results in a disappearance of the amylose fraction and a broader chain length distribution of the amylopectin fraction, due to the disproportionating effect of the amylomaltase enzyme.[26] In order to verify potential differences in the molecular weight distribution, both IMMPs were compared by HPSEC using samples prior to fermentation (Figure 6.2, Aa and Ba). IMMP-94 showed a broad molecular weight distribution with populations being eluted between 10 – 12.9 min (1.7 – 65 kDa). IMMP-96 showed a slightly clearer bimodal distribution, with higher RI response towards both ends of the same elution window (10 – 12.9 min), indicating that IMMP-96 contained both shorter and longer chains and fewer medium length chains, as compared to IMMP-94.

Influence of IMMP molecular weight distribution on its in vitro fermentation

Polymer degradation and oligosaccharide formation upon in vitro fermentation of IMMP-94 and IMMP-96

The degradation of IMMPs during *in vitro* fermentation was monitored by HPSEC up to 48 h (Figure 6.2). The fermentation behaviour of IMMP-94 and IMMP-96 was similar, and the same types of oligomeric dextran fragments were formed and utilized in time. For both IMMPs, the HPSEC elution patterns remained the same during the first 12 h, followed by a shift in molecular size from larger to smaller molecules from 12 h to 24 h. No further difference was noted between 24 h and 48 h of incubation suggesting that the degradation of the polysaccharides fraction of IMMP-94 and IMMP-96

mainly took place between 12 and 24 h of fermentation. To have a better overview of smaller size molecules being formed during fermentation, HPAEC was performed (Figure 6.3). For both IMMPs, a broad peak being eluted between 20 and 25 min was seen during the first 12 h of fermentation. This peak included a wide range of not well-separated IMMP molecules, which partly corresponded to the 10 – 65 kDa population in the HPSEC chromatograms (Figure 6.2, A&B, lines a, b, c). At 24 h of fermentation, these polymers had disappeared, and a series of well-separated oligosaccharide peaks which eluted between 11 and 20 min could be observed. The oligosaccharide peaks were annotated according to the HPAEC elution pattern of IMMP-94 treated with a pure dextranase from *Chaetomium erraticum* (results not shown). The oligosaccharide fraction of the fermentation digest comprised α-(1→6) linked IMOs with a DP of 7 to over 20. IMOs with DP < 7 were absent at 24 h, which could be due to instant consumption of smaller oligosaccharides by the microbiota during fermentation, indicating a preference of the microbiota for the utilization of small molecules. At 48 h of incubation, the oligosaccharide fraction had disappeared, and no carbohydrate peaks were present in the chromatogram (Figure 6.3). Overall, the HPAEC results of IMMP-94 and IMMP-96 were in accordance with HPSEC results.

Despite similarities in the fermentation behaviour of IMMP-94 and IMMP-96, the overall rate of fermentation of IMMP-96 was slower (Figure 6.3), as indicated by the presence of polymeric material being eluted between 18 and 20 min after 24 h of incubation. This difference in fermentation rate could be due to the difference in chain length distributions of the two IMMPs. This finding agreed with a previous research which reported that IMOs of different chain length led to different utilization and fermentation rate when using human faecal microbiota.[27]

pH and production of organic acids upon in vitro fermentation of IMMP-94 and IMMP-96

Analysis of the pH of fermentation digesta and organic acid production at different time points confirmed that the fermentation of IMMP-94 and IMMP-96 started after 12 h of incubation (Figure 6.4). For both IMMP-94 and IMMP-96, the pH remained stable at around pH 6.2 during the first 12 h, followed by a decrease to around pH 5.2 at 24 h, and a slight further decrease at 48 h (Figure 6.4). It is noteworthy that the drop of the pH to 5.0 at 48 h was larger as compared to a drop of pH to 6.0 at 48 h previously observed for resistant gluco-dextrin fermentation in a comparable set-up.[14] The pH decreased as a result of organic acid production. In line with the change of pH, the largest increase in the concentration of SCFAs was observed from 12 h to 24 h, followed by a further increase from 24 h to 48 h (Figure 6.4). Acetic acid, propionic acid and butyric acid are in general the three main SCFAs produced during *in vitro* fermentation of carbohydrates. Lactic acid and succinic acid should also be taken into consideration, since they are intermediates in SCFA production during fermentation.[28]

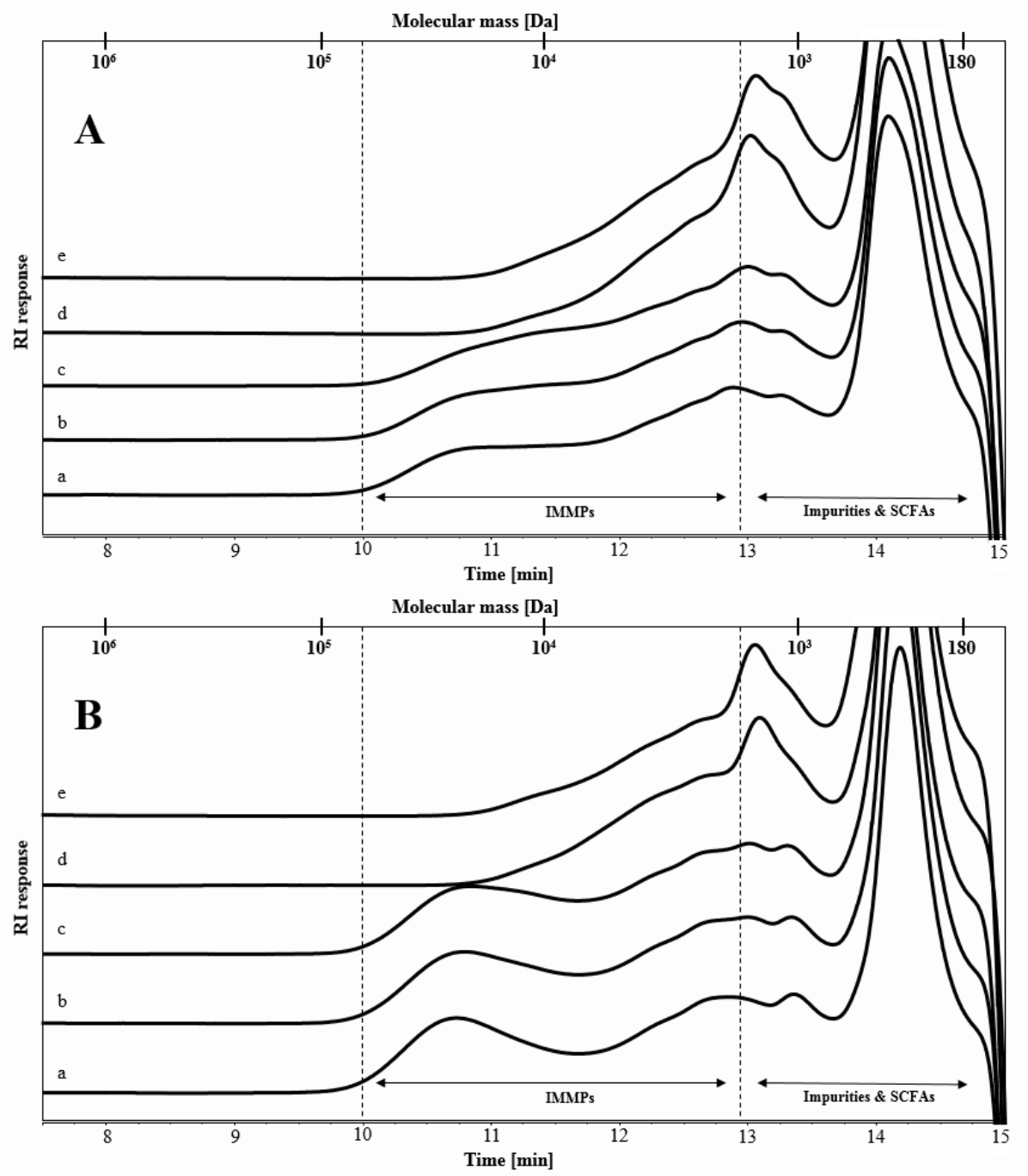

Figure 6.2. High performance size exclusion chromatography (HPSEC) elution patterns of (A) IMMP-94 originating from potato starch, and (B) IMMP-96 originating from Etenia 457, before (a) and after *in vitro* fermentation for 6 h (b), 12 h (c), 24 h (d) and 48 h (e). Calibration of the system with pullulan standards is indicated.

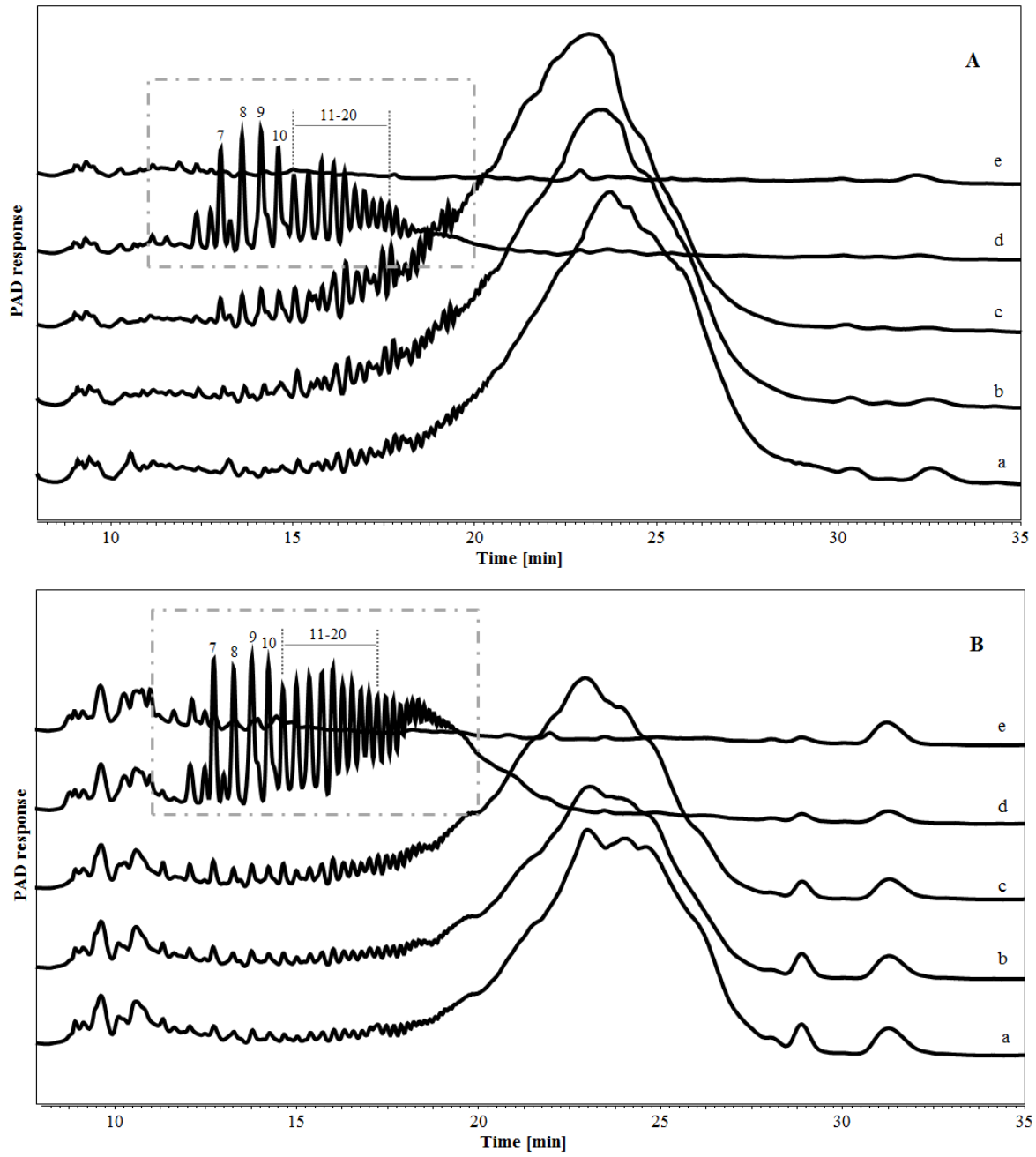

Figure 6.3. High performance anion exchange chromatography (HPAEC) elution patterns of (A) IMMP-94 originating from potato starch, and (B) IMMP-96 originating from Etenia 457, before (a) and after *in vitro* fermentation for 6 h (b), 12 h (c), 24 h (d) and 48 h (e). Isomalto-oligosaccharides are annotated in a box, with the number indicating their degree of polymerization (DP).

For both IMMPs, the most predominant SCFA produced was acetic acid, with minor amounts of propionic acid and butyric acid (Figure 6.4). Unexpectedly, the second most produced organic acid for both IMMPs was succinic acid. Succinic acid is an intermediate of intestinal SCFA production, and is utilized by members of the phylum Bacteroidetes and the family Veillonellaceae to form propionic acid.[29–31] In the current study, however, succinic acid accumulated during the incubation without further

conversion. This accumulation of succinic acid can also explain the lower pH at the end of fermentation, since succinic acid has a pK_{a1} of 4.2,[32] which is lower than that of other SCFAs (approximately 4.8,[4]).

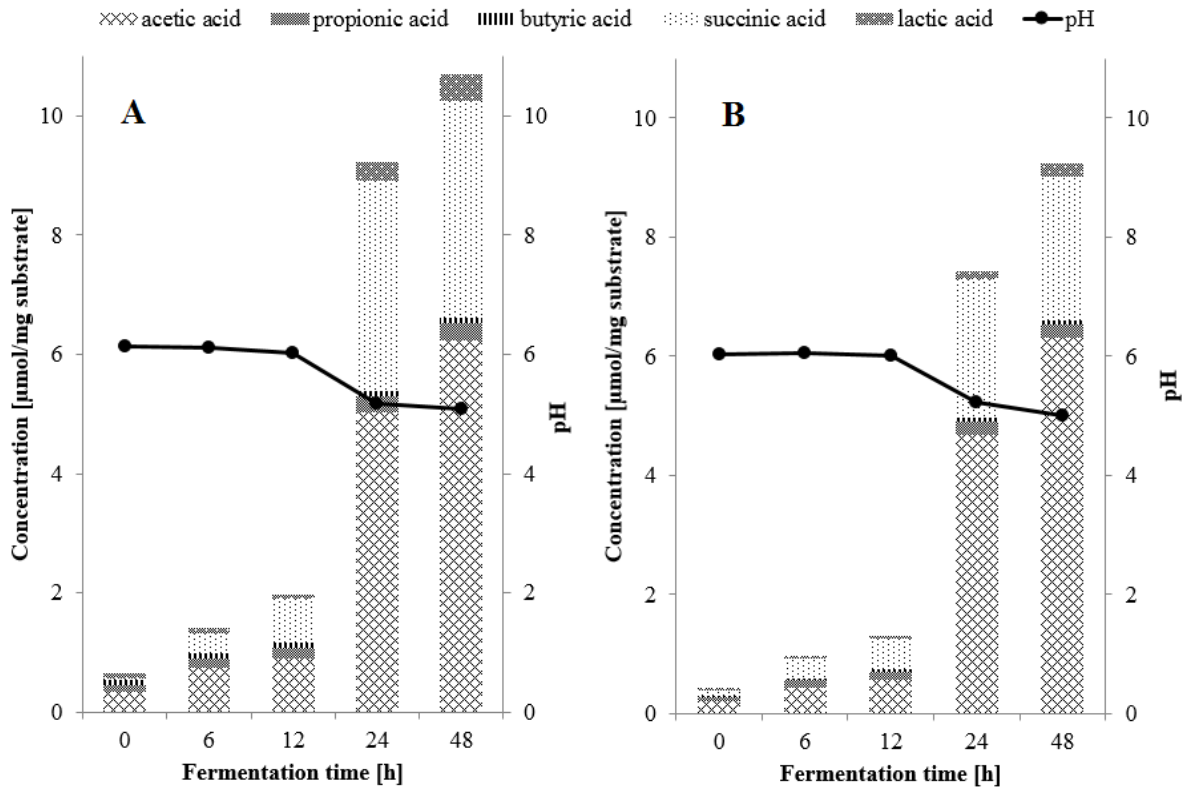

Figure 6.4. Concentrations of short chain fatty acids, lactic acid and succinic acid and the pH (-●-) during *in vitro* fermentation with human faecal inoculum of (A) IMMP-94 and (B) IMMP-96.

The drop of pH and the predominant production of acetic acid are in line with a previous report on *in vitro* fermentation of IMMPs.[6] Formation of succinic acid was not reported in that study, however, it should be noted that only acetic acid and propionic acid were measured. Information about succinic acid was also not presented for studies where IMOs or dextrans were fermented,[8,10,11] but was reported for fermentation studies where other prebiotics were used as substrate, e.g. lactulose and inulin.[33–35] It has been reported previously that *Bacteroides fragilis* produced acetate and succinate mainly in the presence of sufficient carbon source, whereas it converted succinate to propionate when carbon sources were limited.[28] An *in vivo* study was performed in collaboration with the University Medical Centre Groningen (the Netherlands), where IMMPs were fed to mice (unpublished results). Also, in the mice faeces, significant amounts of succinic acid were found, providing additional evidence that the production and accumulation of succinic acid during IMMPs fermentation was not an artefact of the *in vitro* fermentation set up.

The degradation of IMMPs started later and continued over a longer time than that of other commonly studied prebiotics. In the comparable *in vitro* fermentation set-up, utilization of FOS started

at around 2 h after faecal inoculation and was completed within 9 h.[36] Therefore, IMMPs can be considered to be a slowly fermentable fibre, although a direct comparison between the substrates might be necessary to unequivocally confirm observations described here. Slowly fermentable fibres are of great interest, because most colonic diseases occur distally, where proteolytic fermentation may take place when carbohydrates are lacking.[2,37] The slow fermentability of IMMPs makes them beneficial to gut health by increasing the delivery of SCFAs to the distal colon. Besides, given the fact that IMOs of DP <10 were shown to be bifidogenic,[9,38,39] results presented here indicate that IMMPs are a good fibre source to make these IMOs available for the fermentation by the colonic microbiota.

Physicochemical characterization of IMMP-27 and IMMP-dig27

Starch, due to its high content of α-(1→4) linked glycosidic linkages, is mostly digested in the human small intestine. In contrast, when mixed with α-(1→6) linked glycosidic linkages such as in IMMPs, it is possible that part of the α-(1→4) linked glycosidic linkages could escape digestion and enter the large intestine. To investigate the influence of α-(1→4) linked glycosidic linkages on the fermentation of α-(1→6) linked glycosidic linkages by colonic microbiota, the *in vitro* fermentation of IMMP-27 and IMMP-dig27 were compared. IMMP-27 contains 27% α-(1→6) linked glycosidic linkages, whereas IMMP-dig27 is the α-(1→6) enriched fraction of IMMP-27, after being treated with an excess of α-amylase and amyloglucosidase that removed >70% of glucose moieties.

The molecular size distribution of IMMP-27 and IMMP-dig27 was determined by HPSEC (lines a, Figure 6.5A, B). The overall molecular size of IMMP-27 was larger than that of IMMP-dig27. Molecules that eluted at 8 – 10 min (65 – 850 kDa) in IMMP-27 were not observed in IMMP-dig27, indicating that this fraction of molecules was digested to smaller fragments due to the removal of α-(1→4) linked glucose moieties by α-amylase and amyloglucosidase.

Influence of α-1,4-linkages on bacterial utilization of α-1,6-linked glucose during in vitro fermentation of IMMPs

Polymer degradation and oligosaccharide formation upon fermentation of IMMP-27 and IMMP-dig27

The change in molecular size distribution of IMMP-27 and IMMP-dig27 during *in vitro* fermentation was monitored using HPSEC (Figure 6.5). For IMMP-27, HPSEC chromatograms showed differences between 0 h and 6 h, with molecules ranging in size between 65 – 850 kDa being degraded within 6 h of fermentation. After 6 h, the chromatograms of IMMP-27 did not show any further increase in the proportion of the smaller molecules which eluted at 8 – 10 min (65 – 850 kDa). In contrast, the chromatograms of IMMP-dig27 remained the same in the first 12 h, and there was a shift in molecular

size distribution to smaller molecules between 12 h and 24 h. No changes in the elution patterns were observed from 24 h to 48 h, indicating that the degradation of IMMP polymers was completed. The oligomer profiles of IMMP-27 and IMMP-dig27 during fermentation obtained by HPAEC showed that for IMMP-27, α-1,4-linked maltodextrin peaks were already present at 15 min, and were still present at 6 h (Figure 6.6). At 12 h, these maltodextrin peaks were hardly present, whereas new peaks, probably representing oligosaccharides consisting of both α-(1→4) and α-(1→6) linkages, became more apparent (Figure 6.6). At 24 h, a series of well-separated α-1-6-linked IMO peaks which eluted between 11 and 20 min appeared, and a broad fraction eluting between 20 and 24 min representing unseparated dextran oligomers of higher DPs was clearly seen. The peaks of IMOs (11 – 20 min) were still present at 48 h of fermentation, whereas the unseparated fraction (20 – 24 min) disappeared. For IMMP-dig27, with hardly any α-1,4-linkages present in the substrate, the IMMP molecules remained intact during the first 12 h of fermentation. However, no carbohydrates were detected at 24 h of fermentation, indicating that a very quick and complete fermentation took place between 12 h and 24 h.

The different degradation patterns of IMMP-27 and IMMP-dig27 suggest that in the *in vitro* fermentation model, human faecal microbiota could utilize the α-(1→4) linkages directly, whereas α-(1→6) linkages were utilized only after the α-(1→4) linkages were depleted. Different enzymes are required to digest α-(1→4,6) linkages, and bacteria present in the faecal inoculum could be induced to produce corresponding hydrolytic enzymes by the presence of specific substrates in the colon.[40] However, when mixtures of compounds are present, the availability of one substrate could delay the fermentation of another, possibly less favourable substrate.

Our results suggest that the presence of α-(1→4) linked glycosidic linkages could postpone the utilization of α-(1→6) linked glycosidic linkages *in vitro* and that fermentation of IMMPs with high levels of α-(1→6) linkages may require colonic microbiota to undergo an adaptation period. Furthermore, this adaptation period might relate to the molecular size of the α-(1→6) linked glycosidic linkage chains. The fermentation behaviour of IMMP-dig27 resembled that of IMMP-94 and IMMP-96, in line with the facts that all three substrates are rich in α-(1→6) linked- and depleted in α-(1→4) linked glucose residues.

The complete degradation of IMMP-dig27, however, was faster than that of the other two IMMPs. This could be explained by the smaller molecular sizes of IMMP-dig27 ‘dextran’-segments as compared to IMMP-94 and IMMP-96, indicating that fermentation of α-(1→6) linkages is quicker for smaller IMMP molecules. Therefore, the fermentation of IMMPs depends not only on the presence of α-(1→4) linkages, but also on molecular length distribution of IMMPs, although it would be necessary to further investigate whether α-(1→4) linked glycosidic linkages that mixed with IMMPs would escape digestion and to enter the colon *in vivo*.

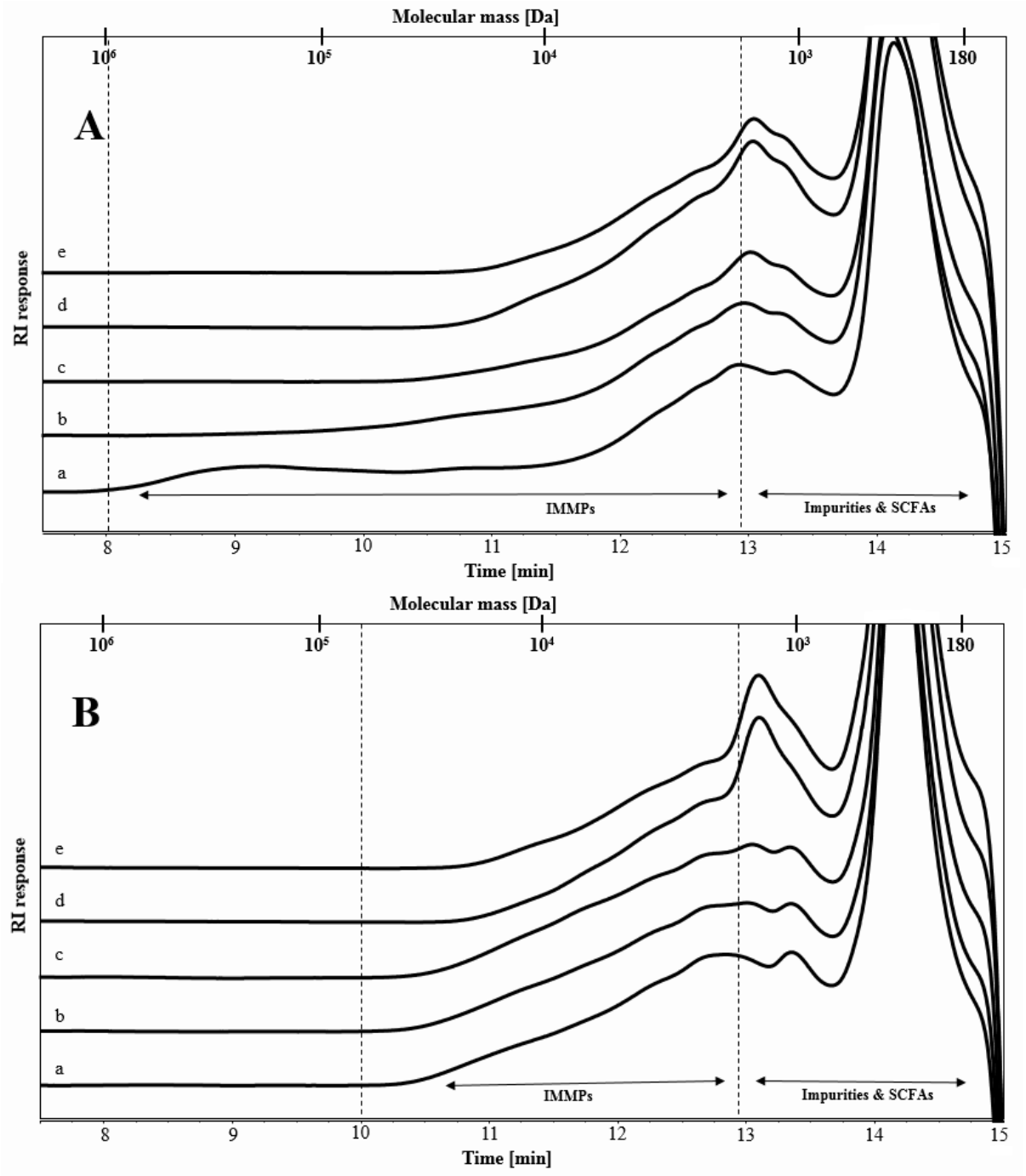

Figure 6.5. High performance size exclusion chromatography (HPSEC) elution patterns of (A) IMMP-27 and (B) IMMP-dig27, before (a) and after *in vitro* fermentation for 6 h (b), 12 h (c), 24 h (d) and 48 h (e). Calibration of the system with pullulan standards is indicated.

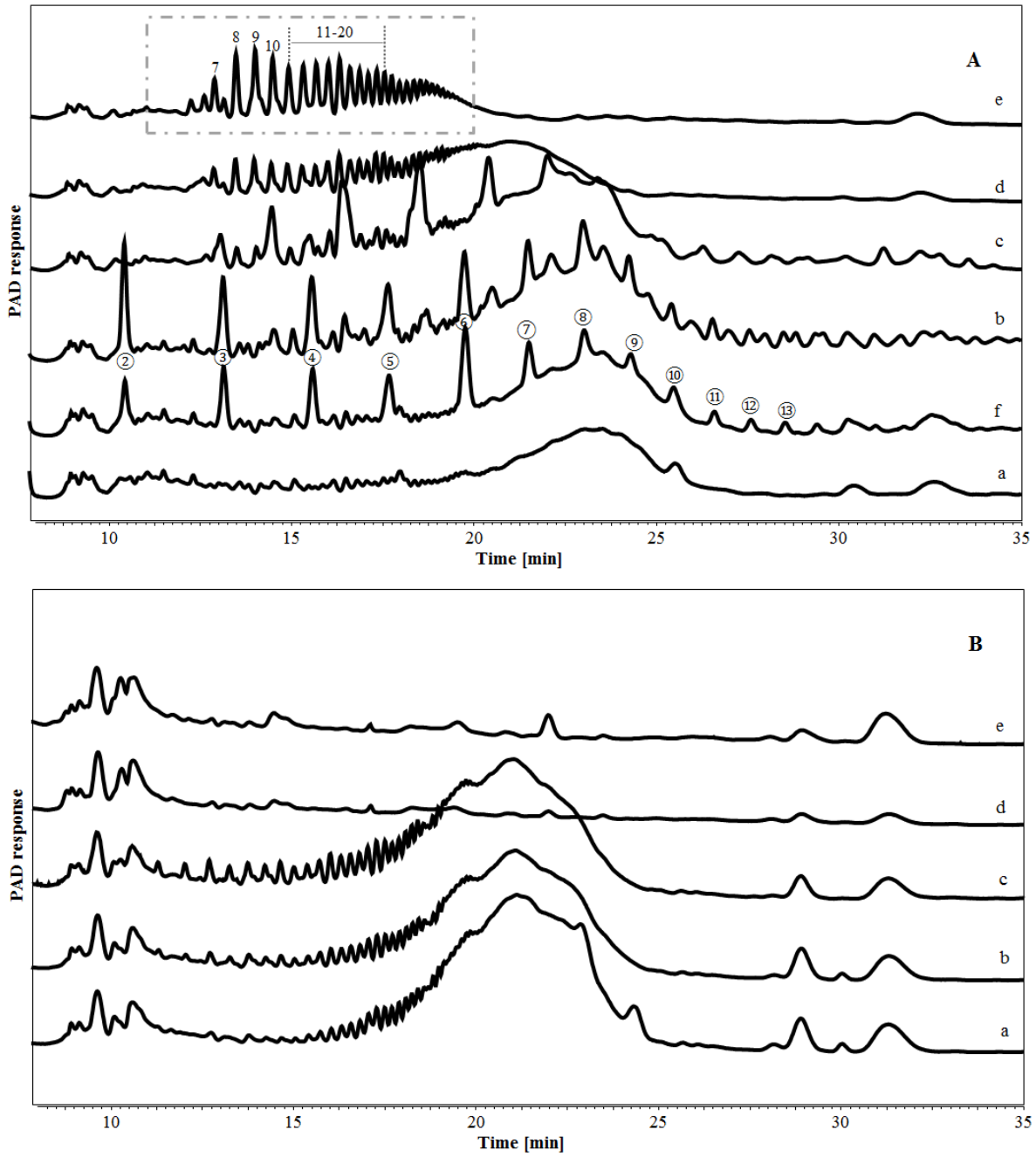

Figure 6.6. High performance anion exchange chromatography (HPAEC) elution patterns of (A) IMMP-27 and (B) IMMP-dig27, before (a) and after *in vitro* fermentation for 15 min (f), 6 h (b), 12 h (c), 24 h (d) and 48 h (e). Isomalto-oligosaccharide peaks (7-20) in a box and maltodextrin peaks (②-⑬) are annotated, with the number indicating the DP.

pH and production of organic acids upon fermentation of IMMP-27 and IMMP-dig27

For IMMP-27, the pH dropped continuously from the beginning of the fermentation until 24 h, which agrees with the steadily increasing level of SCFAs, lactic acid and succinic acid produced during the first 24 h (Figure 6.7). From 24 h to 48 h, the pH remained stable and the concentrations of lactic

acid and succinic acid decreased. During the fermentation of IMMP-dig27, the pH dropped between 12 and 24 h, concomitant with the most pronounced increase in the level of total organic acids, resembling the results for IMMP-94 and IMMP-96. The pH profiles and SCFAs production of IMMP-27 and IMMP-dig27 fermentation further confirmed that the human faecal microbiota used here readily utilized the α-(1→4) linked glycosidic chains, whereas the utilization of the α-(1→6) linked glycosidic chains was delayed. A slight increase of pH (from 5.5 to 5.7) was observed between 24 and 48 h when fermenting IMMP-dig27. This could be an indication of proteolytic fermentation, of which one of the end-products is ammonia (not measured in this study).[41] The onset of proteolytic fermentation was possibly a result of carbohydrate depletion of IMMP-dig27 after 24 h fermentation.

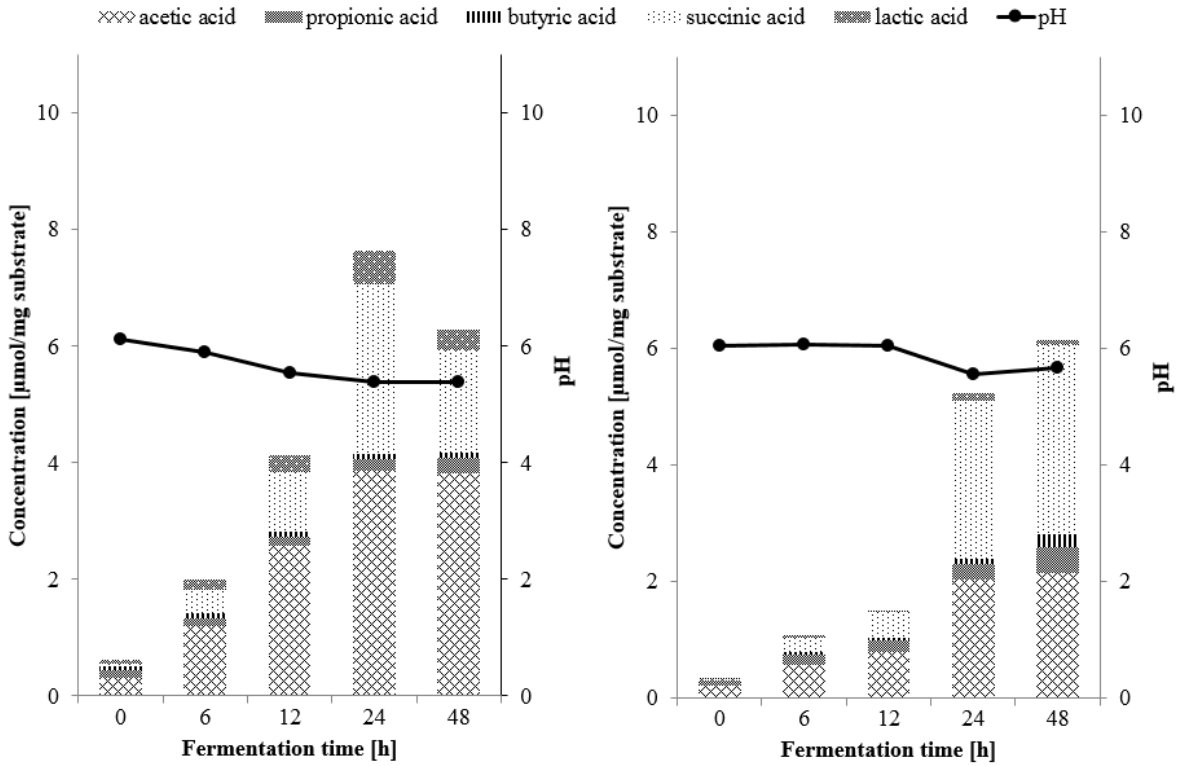

Figure 6.7. Concentrations of short chain fatty acids, lactic acid and succinic acid present and the pH (-●-) during *in vitro* fermentation with human faecal inoculum of (A) IMMP-27 and (B) IMMP-dig27.

Furthermore, acetic acid and succinic acid were the two major products for IMMP-27 and IMMP-dig27, as reported above for IMMP-94 and IMMP-96. Overall, the production of SCFAs with IMMP-dig27 resembled that with IMMP-94 and IMMP-96, except that the production of acetic acid was much lower in final concentration for IMMP-dig27 between 24 and 48 h (Figure 6.4, Figure 6.7). The lower production of acetic acid explained the slightly higher pH at 48 h in fermentation of IMMP-dig27 (5.7) compared to that of IMMP-94 (5.0) and IMMP-96 (5.0). Furthermore, within the first 12 h of fermentation of IMMP-27, where mainly the α-(1→4) glucose was utilized by faecal bacteria, succinic

acid was already produced in large quantity (Figure 6.7). Therefore, the production of succinic acid was not specific to fermentation of the α-(1→6) linked glycosidic linkages of IMMPs.

Enzyme activity upon fermentation of IMMPs

During *in vitro* fermentation, IMMP molecules with higher degree of polymerization (DP) were degraded into molecules with lower DP by enzymes that were produced by faecal microbiota, followed by further degradation into glucose, which was then utilised by bacteria present. To investigate which enzymes were produced during IMMP fermentation, proteins were extracted from fermentation digests at selected time points, chosen based on the HPAEC patterns: IMMP-94 (12 h & 24 h), IMMP-27 (12 h & 48 h) and IMMP-dig27 (12 h & 24 h). These time points indicated the time before the α-(1→6) linked glycosidic chains started to be degraded (all three IMMPs), the time when IMOs of DP 7 – 20 were predominantly present (IMMP-27 and IMMP-94) or even fully utilized (IMMP-dig27). IMMP-94 was used to represent IMMPs that were rich in α-(1→6) linkages. Besides, the IMMP blank which contained inoculum with no IMMPs at time 0 h was included as the baseline of enzyme activity. From all time points, two types of enzyme extracts were obtained: extracellular enzyme extract (EE) and cell-associated enzyme extract (CE). Four substrates, PNP-α-D-glucopyranoside and PNP-β-D-glucopyranoside, potato starch and IMMP-94, were tested to determine the presence and activity of α- and β-1,4-glucosidases, starch-degrading enzymes and dextran-degrading enzymes (Table 6.1).

Table 6.1. Enzyme activity in mU (nmol mL-digest^{-1} min^{-1}) of enzyme extracts (EE: extracellular enzymes; CE: cell-associated enzymes) from *in vitro* fermentation samples.

In vitro fermentation substrates	Fermentation time [h]	Enzyme extract	Enzyme assay substrates			
			PNP-α-D-glucopyranoside	PNP-β-D-glucopyranoside	Soluble potato starch	IMMP 94
Inoculum blank	0	EE	31	27	-	-
		CE	0.5 [I]	0.2	-	-
IMMP 94	12	EE	32	47	43	46
		CE	71 [II]	2.5	1	0.4
	24	EE	7 [II]	2	20	69
		CE	448	46	6	29
IMMP 27	12	EE	45	25	14	21
		CE	127 [II]	10 [I]	1	-
	48	EE	61	4	60	20
		CE	101 [I]	13	28	8
IMMP dig27	12	EE	64 [I]	N.A.	36	31
		CE	1 [I]	0.03	1	7
	24	EE	25	6	17	32
		CE	76	7	4	74

[I] Results given by single test; [II] results given by duplicates; all other results given by triplicates.
-Not detectable.
N.A. Not analysed.

At baseline (IMMP blank-0 h), all enzyme activities measured were neglectable, especially the starch/dextran-degrading enzymes, as neither CE nor EE showed detectable activity towards soluble potato starch or IMMP-94 (Table 6.1). When IMMPs were present during the fermentation, the enzyme activities towards PNP-α-D-glucopyranoside increased at 12 h, especially in CE of IMMP-27-12 h (127 mU compared to 0.5 mU in IMMP blank). For both IMMP-94 and IMMP-dig27, the enzyme activities of CE towards PNP-α-D-glucopyranoside at 24 h were much higher than those at 12 h. In general, the enzyme activity towards PNP-α-D-glucopyranoside was much higher than the activity towards PNP-β-D-glucopyranoside for all enzyme extracts, suggesting that the microbiota was induced to produce enzymes to degrade the α-glucans used in this study. Enzyme activities towards soluble potato starch and IMMP-94 were also higher when IMMPs were present as substrates in the fermentation, and the enzyme activities of EE were much higher than that of CE. The EE enzyme extracts of IMMP-94 showed an increasing activity towards IMMP-94 from 12 h (46 mU) to 24 h (69 mU), whereas a declining activity towards soluble potato starch from 12 h (43 mU) to 24 h (20 mU) was observed. This confirms that the production of α-(1→6) linkage hydrolytic enzymes was induced by the presence of α-(1→6) linkage-rich substrates after the disappearance of α-(1→4) linkages. The decrease in activity of α-(1→4) glucose endo-acting enzyme was most probably due to the absence of starch, and the α-(1→4) linked glucose hydrolytic enzyme that was found active at the beginning was no longer produced during later stages of the fermentation.

The overall distribution of the four enzyme activities in CE and EE followed a certain tendency: activities towards soluble potato starch and IMMP-94, i.e. α-amylase and dextranase, were higher in EE than in CE, whereas activities towards PNP-α/β-D-glucopyranoside were higher in CE than in EE. This suggests that α-(1→4) and α-(1→6) linked glucose polysaccharide-degrading enzymes, which comprise mainly of endo-acting enzymes,[14] were excreted by microbes to cleave IMMP polysaccharides into smaller oligosaccharides. These smaller oligosaccharides could then be taken up by microbial cells to be further degraded by glucosidases, which are exo-acting enzymes. This agrees with previous findings that exo-acting enzymes were mostly cell-bound whereas endo-acting enzymes were mostly extracellular.[14] Also the absence of IMOs of DP lower than 7 in the well-separated IMO fraction in the HPAEC chromatograms (Figure 6.3 and Figure 6.6) seems to match this theory, because bacterial cells, together with the smaller oligosaccharides that had already passed the cell membrane, were removed from fermentation digest by centrifugation before HPAEC analysis.

According to HPAEC (Figure 6.6), degradation of IMMP-27 was mainly targeting α-(1→4) linkages in the first 12 h, and switched to α-(1→6) linked glycosidic linkages afterwards. Furthermore, at 48 h, IMOs of DP 7-20, which were products of degradation of IMMP polysaccharides by endo-acting enzymes, were present. This means that the α-(1→4) linkage degrading enzymes were active during the first 12 h of fermentation, whereas afterwards, glycanase activity was taken over by the α-(1→6) linkage degrading enzymes. However, this did not agree with the enzyme activities measured during

fermentation of IMMP-27: the combined CE and EE enzyme activities towards soluble potato starch were higher at 48 h (88 mU) than at 12 h (15 mU). In addition, the enzyme extracts of IMMP-27-48 h showed higher combined CE and EE enzyme activities towards soluble potato starch (88 mU) than towards IMMP-94 (28 mU). This suggests that production of α-1,4-linkage degrading enzymes was not suppressed after the substrates were depleted.

As to IMMP-dig27, the combined CE and EE enzyme activity towards soluble potato starch declined from 12 h (37 mU) to 24 h (21 mU), whereas the activity towards IMMP-94 increased from 12 h (38 mU) to 24 h (106 mU). This observation suggested that the microbial enzyme production of IMMP-dig27 fermentation resembled that of IMMP-94 fermentation. This agreed with the results of molecular degradation patterns and SCFA production, as discussed above.

Microbiota composition during fermentation of IMMPs

The microbiota composition during fermentation of IMMPs was analysed to evaluate the prebiotic potential of IMMPs, and to make a link with the structural changes of IMMPs and production of SCFAs, lactic acid and succinic acid. Multivariate analysis of bacterial community dynamics over time in the different *in vitro* fermentations, using weighted Unifrac distances as a measure for differences in microbial composition, showed a directional shift in community composition in relation to incubation time and the type of IMMPs used. A strong segregation of samples with IMMPs present after 24/48 h of incubation can be seen in Figure 6.8. This indicates that both the duration of incubation and the presence of different IMMP substrates played an important role in shaping the microbial communities *in vitro*. A similar segregation of samples was also found with unweighted analyses that only take presence and absence of microbial groups into account (data not shown). The microbial alpha diversity, as determined based on Shannon's diversity index, changed as the fermentation progressed and decreased in the blank, but increased in digesta with the IMMPs present (Figure 6.9). Shannon's diversity index accounts for both abundance and evenness of the species present. There was a high predominance of *Escherichia-Shigella* group at the beginning of the fermentation, possibly due to the presence of residual amounts of oxygen during initial inoculum activation. As fermentation progressed, the presence of IMMPs and depletion of oxygen enabled growth of other bacterial groups leading to an increase in the evenness of the community. Although the microbiota composition at the start of fermentation was different from that normally found in faeces of healthy adults, it is interesting to note that such a dysbiotic community was "normalized" by IMMPs towards a more typical colonic microbiota. It is tempting to speculate that this "normalization" effect might also occur *in vivo* and could facilitate ecosystem recovery following situations of dysbiosis (e.g. after diarrhea). In the IMMP blank sample, the ecosystem was starved, thus the growth of other bacteria groups was much slower. Phylogenetically weighted species richness, as measured by the PD (Phylogenetic Diversity) Whole Tree index, decreased in all treatment groups in the first hours of incubation, whereas it remained

relatively stable after 24 h. Despite high structural similarities between IMMP-94 and IMMP-96, Pearson correlation scores at genus level were 0.77, 0.89 and 0.34 at times 0, 24 and 48 h, respectively, suggesting different microbial response patterns towards these two substrates. In line with this observation, the average relative abundance of different phyla changed with time, and was influenced by the type of IMMP being fermented (Figure 6.10 A). Levels of Proteobacteria decreased in all groups until 24 h of incubation and remained stable or slightly increased at 48 h. This was accompanied by a gradual increase in Bacteroidetes up to 24 h, followed by decrease at 48 h. Firmicutes showed a rapid decrease in abundance at 6 h and gradual increase at later time points, except for IMMP blank where their relative abundance continued to decline. The levels of Actinobacteria were very low, and decreased to 4.7% in the IMMP blank at 48 h. Their relative abundance was higher in the IMMP digesta as compared to the IMMP blank.

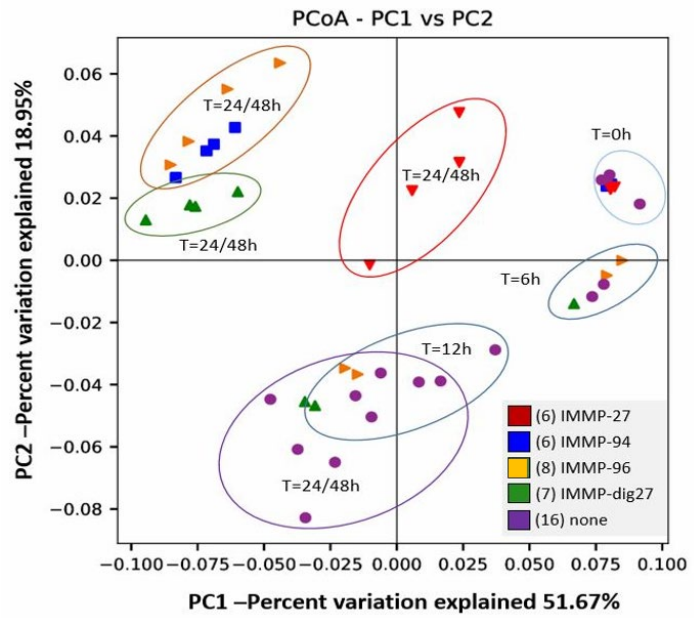

Figure 6.8. PCoA based on weighted (relative abundance) Unifrac distances between observed microbial communities for *in vitro* fermentation of IMMPs with human faecal inoculum at different time points.

At the genus level, four bacterial taxa, namely *Escherichia-Shigella, Bacteroides, Bifidobacterium and Lactobacillus* were predominant in all IMMP digesta, with their combined relative abundance ranging from 41% to 97% of all detected reads (Figure 6.10 B). The detailed relative abundance of taxa during IMMPs fermentation at genus level is given in Supporting Table S6.1. The duration of *in vitro* fermentation was positively correlated with the increase of *Bacteroides* and a corresponding decrease in *Escherichia-Shigella*. The presence of IMMPs correlated with high (up to 50%) relative abundance of genera *Bifidobacterium* and *Lactobacillus*, as compared to the IMMP blank group, in which less than 5% of all reads belonged to these taxa. This prebiotic effect was especially strong in IMMP-27, IMMP-94 and IMMP-96 after 24 h of incubation, at which time the fermentation of α-(1→6) linked glycosidic linkages was predominant. The increase in relative abundance of *Bifidobacterium* and *Lactobacillus* was

specific to the presence of IMMPs and was not observed in the IMMP blank, indicating that fermentation of IMMPs promoted the growth of *Bifidobacterium* and *Lactobacillus* (Table 6.2).

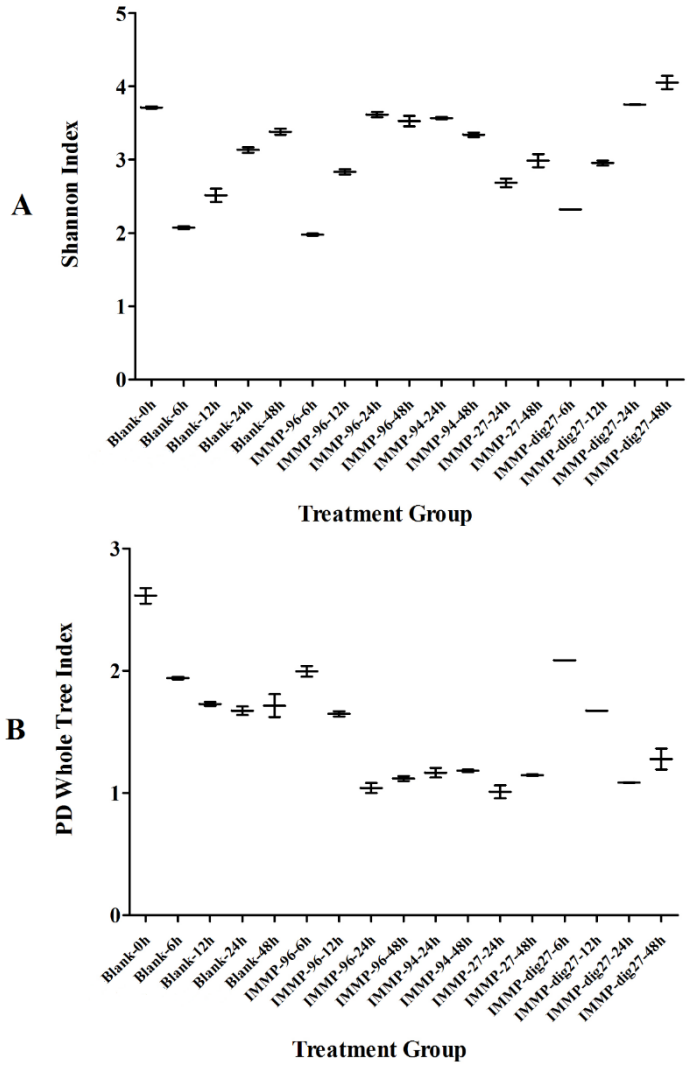

Figure 6.9. Microbial alpha-diversity estimates, including (A) Shannon diversity index, (B) Phylogenetic Diversity Whole Tree for *in vitro* fermentation of IMMPs with human faecal inoculum at different time points.

For IMMP-96 and IMMP-dig27, the relative abundance of *Bifidobacterium* remained very low in the first 12 h (2 – 5%), then increased rapidly to a high level at 24 h (27% for IMMP-96; 9% for IMMP-dig27). From 24 h to 48 h, *Bifidobacterium* relative abundance continued to largely increase for IMMP-96 (44%), whereas it only slightly increased for IMMP-dig27 (11%). The growth pattern of bifidobacteria was in line with the degradation pattern of IMMPs which consisted mostly of α-1,6-linkages, as both started only after 12 h of fermentation. The highest increase in relative abundance of bifidobacteria occurred from 12 h to 24 h, where IMMP polysaccharides were degraded into α-1,6-linked IMOs with DP of 7 to over 20. The growth pattern of bifidobacteria also agreed with the formation of SCFAs, as shown previously. Formation of SCFAs during fermentation contributes to acidification

of the colonic lumen.[4] A lower pH in the colon is favourable for bifidobacteria and lactic acid bacteria, while impeding the overgrowth of more pH-sensitive pathogenic bacteria.[2,42] For IMMP-94 and IMMP-27, microbiota composition at 6 h and 12 h was not analysed, due to a scarcity of the fermentation digest. Both IMMPs showed high levels of *Bifidobacterium* at 24 h (29% for IMMP-94; 25% for IMMP-27), and at 48 h the relative abundance of this genus remained almost the same for IMMP-94 (30%) but slightly decreased for IMMP-27 (21%).

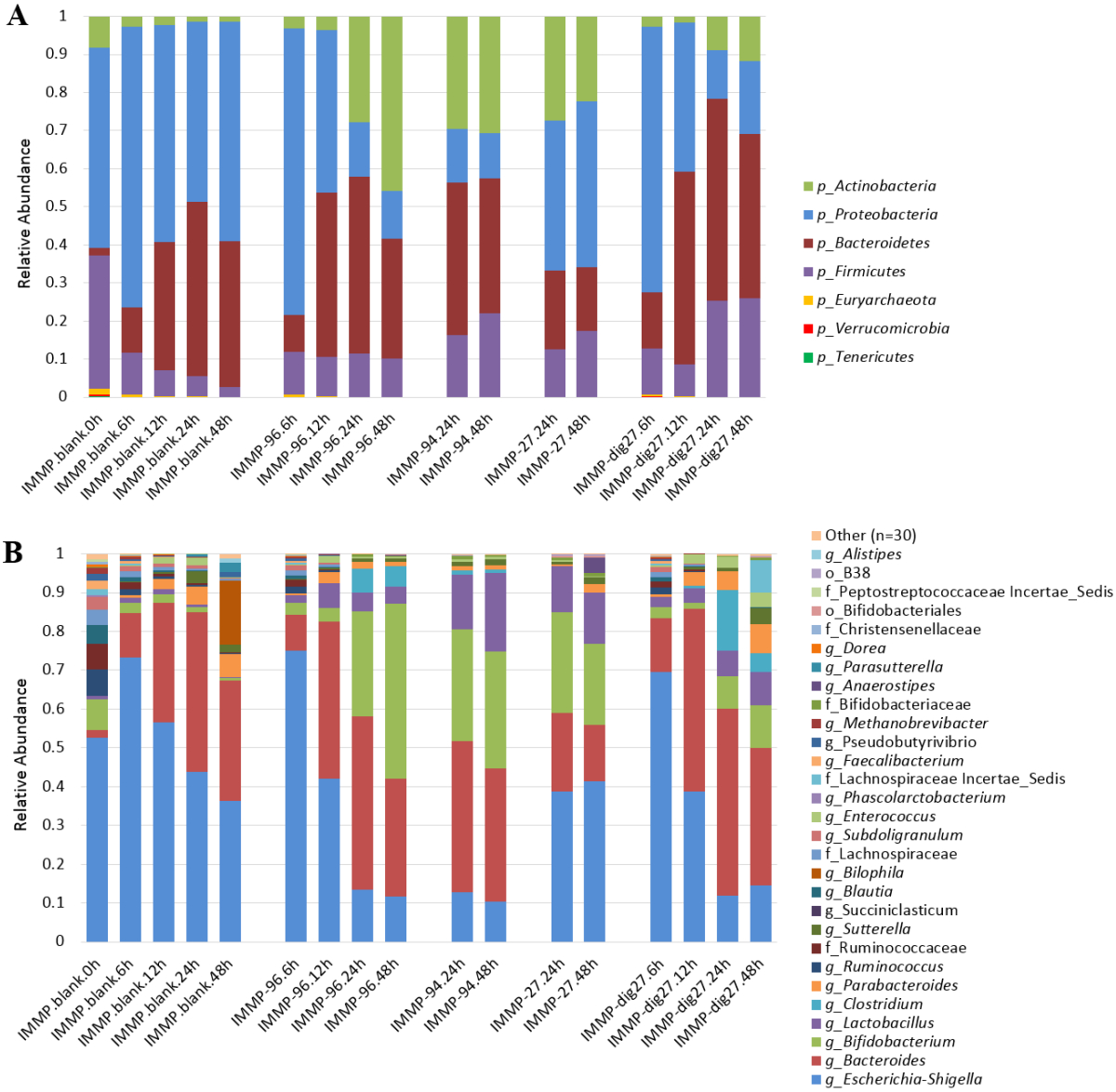

Figure 6.10. Relative abundance of taxa detected during *in vitro* fermentation of IMMPs with human faecal inoculum at different time points, considering phylum (A) and genus (B) levels.

The observed changes in relative abundance of *Lactobacillus* differed among different IMMPs, with the strongest increase observed for IMMP-94 and IMMP-27 at 24 h and 48 h, whereas the increase in relative abundance was weaker with IMMP-96 and IMMP-dig27. In the presence of IMMP-96 there was a rapid increase in the relative abundance at 6 h, followed by a gradual decline at later time points,

whereas with IMMP-dig27 the relative abundance of this genus showed a steady increase with time. The more pronounced increase in relative abundance of *Lactobacillus* during the fermentation of IMMP-94 and IMMP-27 as compared to the other two substrates was in line with the higher level of lactic acid produced during fermentation of IMMP-94 and IMMP-27 (Figure 6.4 and Figure 6.7). In addition, there was an increase in the relative abundance of genera *Enterococcus* and *Parabacteroides* during the fermentation of IMMP-dig27 and with the IMMP blank, but not for the other IMMPs (Figure 6.10 B), a result which we cannot explain in a straightforward way. However, there is a growing evidence suggesting that metabolic webs and complex polysaccharide utilization networks exist between different members of intestinal microbiota, with different species specializing to utilize different polysaccharides, expanding the number and types of glycoside hydrolase produced in the presence of a competitor, or acting as producers or recipients of the polysaccharide breakdown products.[43,44]

Table 6.2. Genus level taxa with significantly different relative abundance in combined IMMP groups at 24 h and 48 h of incubation as compared to IMMP blank groups at 24 h and 48 h using Kruskal-Wallis analysis.

Taxon	P	FDR_P	Bonferroni_P	Average RA	
				IMMP	Blank
g__Bifidobacterium	0.00001	0.00006	0.00029	0.263	0.008
g__Escherichia-Shigella	0.01045	0.02438	0.43878	0.184	0.319
g__Lactobacillus	0.00001	0.00006	0.00029	0.096	0.005
g__Parabacteroides	0.00922	0.02278	0.38733	0.023	0.042
g__Sutterella	0.00023	0.00109	0.00981	0.014	0.030
f__Bifidobacteriaceae_g__g	0.00000	0.00006	0.00017	0.006	0.000
o__Bifidobacteriales_g__g	0.00479	0.01341	0.20118	0.002	0.000
g__Parasutterella	0.00000	0.00005	0.00005	0.000	0.013
g__Bilophila	0.00011	0.00060	0.00476	0.000	0.078
g__Pseudobutyrivibrio	0.00164	0.00492	0.06895	0.000	0.005
g__Alistipes	0.00164	0.00492	0.06895	0.000	0.005
g__Eggerthella	0.00045	0.00171	0.01879	0.000	0.003
f__Lachnospiraceae_g__g	0.00001	0.00006	0.00024	0.000	0.003
g__Ruminococcus	0.00003	0.00016	0.00111	0.000	0.003
g__Subdoligranulum	0.00003	0.00016	0.00111	0.000	0.002
g__Butyricimonas	0.00045	0.00171	0.01879	0.000	0.002
f__Ruminococcaceae_g__g	0.00164	0.00492	0.06895	0.000	0.001
g__Methanobrevibacter	0.00565	0.01484	0.23749	0.000	0.001

We observed a high accumulation of succinate during the *in vitro* fermentation of all IMMPs. This might be due to activity of *Bacteroides* which in the gut can use CO_2 to reduce formate to succinate to

generate ATP in a primitive electron transport chain.[43] Succinate is then excreted as an end product and can be utilised by secondary fermenters, or it can be further converted by *Bacteroides* to propionate if the CO_2 is limiting. In fact, the ability to convert succinate to propionate has been described for both Bacteroidetes and Veillonellaceae.[31] In auxotrophic *Bacteroides* spp. this conversion of succinate to propionate is modulated by the availability of vitamin B12, which in the gut is produced by certain members of Firmicutes and Actinobacteria.[45] Thus, the accumulation of succinic acid in our experiment could be, among others, a result of high CO_2 levels, vitamin B12 limitation, or might be linked to the absence of members of the family Veillonellaceae, which were not detected in our microbiota composition analysis (Figure 6.10 B).

CONCLUSIONS

IMMPs showed delayed and slow-fermenting behaviour compared to other prebiotics during their *in vitro* fermentation by a human faecal inoculum. Measurable production of enzymes targeting α-1,6-linked glucose was detected after 12 h of incubation. The presence of α-(1→4) linked glycosidic linkages in the IMMPs further postponed the bacterial utilization of α-(1→6) linked glycosidic linkages, suggesting that when available, the α-(1→4) linked glycosidic linkages are preferentially used by the faecal microbiota. We also found that α-(1→6) linked glucose oligomers with lower DP were preferentially used, as compared to those with higher DP. Organic acids were produced at high total amounts during IMMPs fermentation, with acetic acid and succinic acid being the predominant metabolites in all incubations. The HPAEC chromatograms and enzyme production analysis showed that the polysaccharide fraction of IMMPs was degraded mainly by extracellular enzymes into α-(1→6) linked IMOs, among which the IMOs with DP lower than 7 might be transported into microbial cells and further degraded by cytoplasmic enzymes. Fermentation of IMMPs led to the increase in diversity and evenness of bacterial communities, and promoted the increase in relative abundance especially of genera *Bifidobacterium* and *Lactobacillus*, lending a strong support for the prebiotic potential of these fibres.

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SUPPORTING INFORMATION

Table S6.1. Relative abundance of taxa detected during *in vitro* fermentation of IMMPs with human faecal inoculum at different time points at genus level. Where no classification was available at genus level, higher taxonomic level was used and the unclassified genus is denoted as g_g.

	All	IMMP27		IMMP-94		IMMP-96				IMMP-dig27				BLANK B1		BLANK B2			
Taxon	t0	t24	t48	t24	t48	t6	t12	t24	t48	t6	t24	t24	t48	t24	t48	t6	t12	t24	t48
g_Escherichia-Shigella	52.618	38.682	41.411	12.699	10.416	75.068	42.092	13.361	11.631	69.45	38.67	11.84	14.61	26.784	12.781	73.33	56.50	43.73	36.41
g_Bifidobacterium	7.793	26.145	20.966	28.706	30.168	3.057	3.562	27.120	45.132	2.722	1.576	8.396	10.98	0.866	0.317	2.78	2.18	1.30	0.80
g_Ruminococcus	6.780	0	0	0	0	1.730	0.482	0	0	1.635	0.426	0	0	0.131	0	1.676	0.706	0.579	0.3187
f_Ruminococcaceae_g_g	6.557	0	0	0	0	1.683	0.352	0	0	1.67	0.441	0	0	0	0	1.639	0.624	0.398	0.0757
g_Blautia	4.892	0	0	0	0	0.996	0.176	0	0	1.149	0.152	0	0.176	0	0.357	1.199	0.445	0.186	0
f_Lachnospiraceae_g_g	3.868	0	0	0	0	1.337	0.569	0	0	1.355	0.522	0	0	0.197	0	1.496	0.880	0.535	0.3278
g_Subdoligranulum	3.364	0	0	0	0	1.266	0.440	0	0	1.236	0.278	0	0	0.082	0	1.333	0.670	0.680	0.2201
g_Faecalibacterium	2.261	0	0	0	0	0.506	0.086	0	0	0.509	0	0	0	0	0	0.559	0.089	0	0
g_Bacteroides	2.046	20.211	14.532	39.051	34.342	9.282	40.414	44.688	30.485	14.05	47.19	48.2	35.32	44.166	27.561	11.33	30.94	41.23	30.91
g_Pseudobutyrivibrio	1.819	0	0	0	0	0.513	0.063	0	0	0.46	0	0	0	0	0	0.503	0.192	0	1.256
g_Incertae_Sedis	1.525	0	0.059	0	0	0.364	0	0	0	0.443	0	0	8.392	0	0	0.422	0.151	0	0
g_Methanobrevibacter	1.411	0	0	0	0	0.624	0.229	0	0	0.43	0.145	0	0	0	0	0.642	0.267	0.273	0.0314
g_Lactobacillus	0.983	11.893	13.007	14.092	20.128	1.917	6.409	4.875	4.411	2.692	3.706	6.721	8.609	1.362	0	1.253	1.318	0.722	0.100
f_Christensenellaceae_g_g	0.660	0	0	0	0	0.049	0	0	0	0.237	0	0	0	0	0	0.21	0	0	0
g_Dorea	0.647	0	0	0	0	0.191	0	0	0	0.254	0	0	0	0	0	0.212	0.130	0.065	0
f_Peptostreptococcaceae_g_Incertae_Sedis	0.533	0	0	0	0	0.220	0	0	0	0.136	0	0	0	0	0	0.214	0.075	0.067	0
g_Phascolarctobacterium	0.415	0	0	0	0	0	0	0	0	0	0	0	0	0	15.203	0	0	0	0
g_Akkermansia	0.405	0	0	0	0	0.063	0	0	0	0.162	0	0	0	0	0	0	0	0	0
o_RF9_g_g	0.305	0	0	0	0	0.032	0	0	0	0	0	0	0	0	0	0	0	0	0
f_Bifidobacteriaceae_g_g	0.188	0.715	0.783	0.708	0.425	0	0	0.595	0.419	0	0	0.386	0.614	0	0	0	0.1	0	0
g_Dialister	0.181	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
f_Erysipelotrichaceae_g_Incertae_Sedis	0.137	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
f_Coriobacteriaceae_g_g	0.125	0	0	0	0	0	0	0	0	0	0	0	0	0	1.949	0	0	0	0
f_Ruminococcaceae_g_Incertae_Sedis	0.112	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
g_Coprococcus	0.106	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
g_Anaerostipes	0.077	0	3.89	0	0	0	0	0	0.165	0	0	0	0	0	0	0	0	0	0
f_Erysipelotrichaceae_g_g	0.058	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
o_Bifidobacteriales_g_g	0.052	0.471	0.490	0.175	0	0	0	0.063	0.216	0	0	0	0.182	0	0	0	0	0	0
g_Clostridium	0.030	0	0	1.202	0.990	0	0	6.096	5.260	0	0.541	15.55	4.854	19.354	1.130	0	0	0	0
g_Parabacteroides	0.023	0.458	2.260	1.015	1.022	0.520	2.734	1.711	1.090	0.697	3.509	4.747	7.463	2.299	1.975	0.576	2.554	4.502	5.941
g_Marvinbryantia	0.016	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
f_S24-7_g_g	0.012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
g_Succiniclasticum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25.584	0	0	0	0
g_Bilophila	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.590	0	0	0	16.539
g_Eggerthella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.746	0	0	0	0.489
g_Parasutterella	0	0	0	0	0	0	0	0	0.035	0	0	0	0.099	0.232	0.266	0	0	0.62	2.465
g_Butyricimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.189	0	0	0	0.429
f_Enterobacteriaceae_g_g	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.164	0	0	0	0
f_Veillonellaceae_g_g	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.141	0	0	0	0
k_NA_g_g	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.095	0	0	0	0
g_Alistipes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.146
g_Pantoea	0	0	0	0	0	0	0	0	0	0.245	0	0	0	0	0	0.134	0	0	0
f_Clostridiaceae_g_g	0	0	0	0	0	0	0	0	0	0	0	0	0	0.329	0	0	0	0	0
o_Bacteroidales	0	0	0	0	0	0	0	0	0	0	0	0	0.168	0.061	0	0	0	0	0
o_Desulfovibrionales	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.204
f_Bacteroidaceae_g_g	0	0	0	0	0	0	0	0	0	0	0	0	0.056	0.101	0	0	0	0	0
o_Chromatiales_g_g	0	0	0	0	0	0	0	0	0	0	0	0	0	0.138	0	0	0	0	0
f_Enterococcaceae_g_g	0	0	0	0	0	0	0	0	0	0	0	0	0.076	0	0	0	0	0	0
f_Lactobacillaceae_g_g	0	0.146	0.106	0.081	0.139	0	0	0	0	0	0	0	0	0	0	0	0	0	0
o_Lactobacillales_g_g	0	0	0	0.215	0.171	0	0	0	0	0	0	0.072	0.184	0	0	0	0	0	0
g_Halomonas	0	0	0	0	0	0.027	0	0	0	0	0	0	0	0	0	0	0	0	0
g_Aeribacillus	0	0	0	0	0	0.043	0	0	0	0	0	0	0	0	0	0	0	0	0
o_B38_g_g	0	0.341	0.440	0.111	0	0	0.155	0	0	0	0	0	0.168	0	0	0	0	0	0
o_Clostridiales_g_g	0	0	0	0	0	0	0	0.038	0	0	0	0.334	0.126	0.098	0.221	0	0	0	0
g_Enterococcus	0	0.478	0.359	0.758	0.596	0.311	1.767	0.543	0.272	0.473	2.293	2.736	3.634	0.558	0.280	0.301	1.594	2.007	0.286
g_Sutterella	0	0.459	1.700	1.187	1.604	0.202	0.469	0.911	0.885	0	0.546	1.021	4.29	3.241	4.450	0.199	0.580	3.108	2.052

Chapter 7

General discussion

Research aim and approach

Prebiotics, with the definition "a substrate that is selectively utilized by host microorganisms conferring a health benefit",[1] is a popular topic in both academic and industrial area. To help with the development of a technology platform which could be applied for identification and validation of prebiotic potential of indigestible carbohydrates, Carbohydrate Competence Centre (CCC) initiated a "Carbohydrates for improving health" programme with the acronym 'CarboHealth'. As part of CarboHealth, this PhD research investigated the metabolic fates of indigestible oligo- and poly-saccharides at molecular level under fermentation by human gut microbiota, from infancy to adulthood.

We started with human milk oligosaccharides (HMOs), which is a natural prebiotic synthesized in human milk and fed to nursing infants. We firstly adapted the analytical methodologies to be able to quantify accurately the major HMOs present in human milk and infant fecal samples without the loss of specific, relevant HMO structures. With samples collected from two cohort studies, we followed the HMO dynamics during lactation period, and inter-individual differences among Dutch mothers. By comparing compositional changes from mother milk to paired infant feces, different utilization patterns and developmental trajectories of HMOs by infant gut microbes were found, associated with several infant-maternal characteristics. Additionally, metabolization of HMOs varied depending on their specific structures. By correlating HMOs in maternal milk to infant fecal microbiota composition, influence of HMOs on shaping infant gut microbiota was discussed. Association between HMO metabolization and infant fecal microbiota composition was investigated, and several phylotypes within specific genera were suggested to be the key taxa responding for HMO metabolization.

As an attempt to search for novel prebiotics for adulthood, we demonstrated the effectiveness of a batchwise *in vitro* fermentation model for investigating fermentation behaviour of isomalto/malto-polysaccharides (IMMPs). Structural degradation on molecular level, metabolite production, enzyme expression and microbiota composition changes during *in vitro* fermentation were linked together, pointing out the structure-specific prebiotic effects of IMMPs.

The findings on prebiotic fermentation behaviour of the PhD study are connected in this chapter, from oligosaccharides to polysaccharides, from infancy to adulthood, from *in vivo* to *in vitro* model. Furthermore, implications of these findings to future research and product development related to infant nutrition and prebiotic supplement will be discussed.

The KOALA and BINGO Birth Cohort Studies

There are two birth cohort studies involved in the current thesis research, the KOALA Birth Cohort Study (Chapter 2 and Chapter 4) and the BINGO study (Chapter 3 and Chapter 5). Both cohorts were conducted in the Netherlands, with infants from KOALA being born in the years 2002 – 2003, and those from BINGO born in the years 2014 – 2016.[2-6] Table 7.1 lists relevant infant and maternal characteristics of all subjects from the two cohorts. Milk and feces samples from KOALA were collected at a single time point (~ one-month postpartum), while those from BINGO were collected at three time points (~ 2, 6 and 12-weeks postpartum). A pilot sample set (n = 20) from KOALA cohort was used for method development in Chapter 2, and the whole sample set (n =121) was described in terms of associations between HMO metabolization and infant fecal microbiota in Chapter 4. Samples from all the mother-infant pairs from BINGO (n = 68) were analysed and discussed in terms of longitudinal variations of HMO synthesis and metabolization in Chapter 3. Finally, in Chapter 5, only subjects with both human milk and infant feces available at all three time points (n = 24) were included to correlate HMO metabolization to infant fecal microbiota.

Table 7.1. Characteristics of the full-term study subjects from KOALA cohort study (Chapter 4) and BINGO cohort study (Chapter 3 and Chapter 5). Data are presented in means (standard deviations, SD), or counts (percentages).

Variables	KOALA	BINGO
	n = 121	**n = 68**
Age postpartum, day, mean (SD)		
Week 2	NA	15 (1)
Week 4	33 (5)	NA
Week 6	NA	43 (2)
Week 12	NA	85 (6)
Gestational age, week, mean (SD)	40.2 (1)	40 (1)
Birthweight, g, mean (SD)	3651 (476)	3589 (410)
Gender, male, n (%)	62 (51)	36 (53)
Delivery mode, vaginal, n (%)	111 (92) *	61 (90)
Delivery place, home, n (%) †	72 (60)	18 (26)
Maternal Lewis Secretor phenotypes, n (%)		
Le+Se+	81 (67)	51 (75)
Le+Se-	29 (24)	17 (25)
Le-Se+	7 (6)	0
Le-Se-	4 (3)	0

* Include both normal and assisted vaginal delivery

† Other places, including hospital and clinic, are considered as *Hospital/Clinic*.

Subjects from KOALA and BINGO were comparable in terms of average gestational age (~40 weeks), average infant birthweight (~3.6 kg), infant gender (~50% boys), and frequency of vaginal delivery (~90%). There is a big difference in delivery place between the two cohorts, where only 26% of infants from BINGO were delivered at home, much lower than that from KOALA (60%) which was conducted over 10 years earlier. Despite any sampling bias that we could see, the difference indicated a trend of delivering more often at hospital or clinic in the Netherlands in the past decade, which was also mentioned in literature.[7] The trend could possibly lead to changes in health and growth of newborns, as delivery place played a role in shaping infant gut microbiota in the first three months of life (Chapter 3).

The Lewis/Secretor phenotypes of the mothers from KOALA and BINGO were determined based on the HMO profiles of their milk samples, as explained in Chapter 2. In literature, the frequencies of the four milk groups in Caucasian population were 69% Le+Se+, 20% Le+Se-, 5 – 10% Le-Se+ and 1% Le-Se-.[8-9] In Chapter 2, with the 20 milk samples from KOALA, we found 90% of mothers to be Le+Se+, and 5% Le+Se- and 5% Le-Se+. When considering the full sample set from KOALA (n = 121), the frequencies were found to be 81%, 29%, 7% and 4% for the four milk groups, respectively (Table 7.1), which becomes quite comparable to literature. Among the 68 mothers from BINGO, 75% of them were assigned to Le+Se+ group and 25% Le+Se-, with no one belonging to Le-Se+ or Le-Se-. Therefore, temporal changes of HMO synthesis were only compared between these two milk groups in Chapter 3.

Development of analytical techniques used in HMO study

Comprehensive HMOs profiling by combining HPAEC-PAD, PGC-LC-MS and 1D ^{1}H NMR

In Chapter 2, three different analyzing techniques were applied to profile HMOs contained in pilot samples from KOALA cohort (n = 20). High performance anion exchange chromatography - pulsed amperometric detection (HPAEC-PAD) was used to quantitate 3FL present in human milk; porous graphitized carbon - liquid chromatography mass spectrometry (PGC-LC-MS) to quantitate 3FL in infant feces and other 16 major HMO structures in both milk and feces; one-dimensional ^{1}H NMR was used to obtain relative levels of fucosylated-epitopes and sialylated (*N*-acetylneuraminic acid; Neu5Ac) structural elements. The combination of HPAEC-PAD and PGC-LC-MS generated absolute concentration of individual HMO structures, whereas NMR provided a helicopter view of structural elements, but missing details of individual HMOs, especially neutral core structures. Results from the three techniques kept consistency on identifying the mother's Lewis/Secretor phenotypes. In order to focus on longitudinal variations of individual HMOs synthesized by lactating mothers, as well as their specific metabolization through infants' gastrointestinal tract, we decided to use HPAEC-PAD and PGC-LC-MS for HMO

quantitation in the following research in order to be able to distinguish between different members from one HMO substituent group.

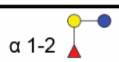
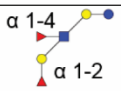
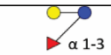
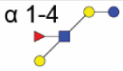
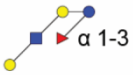
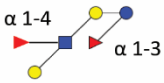
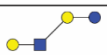
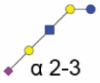
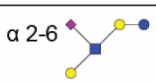
With the combination of HPAEC-PAD and PGC-LC-MS methods employed in the current thesis, we analyzed in total 121 human milk samples from 121 mothers registered in KOALA cohort (Chapter 4) which were collected 1 month postpartum, and 180 human milk samples from 68 mothers registered in BINGO cohort (Chapter 3; week 2, n=64; week 6, n=61; week 12, n=55). The average total HMO concentrations of KOALA was 3.8 g/L (Chapter 4), and that of BINGO was 6.4 g/L, 5.4 g/L and 4.8 g/L for week 2, 6 and 12, respectively (Chapter 3). There is no solid consensus yet on the actual HMO concentration in human milk, with some studies reported it to be 5 – 15 g/L,[10-11] and others 5 – 8 g/L in mature milk.[12-13] Besides maternal factors that would influence HMO level in mother milk, which will be discussed in following section, interlaboratory variations caused by different techniques that were employed has been noticed.[11]

In total 18 HMOs were quantitated in the current thesis, as listed in Table 7.2. Lacto-N-difucohexaose II (LNDFH II) was only included in the BINGO cohort (Chapter 3 and Chapter 5). These HMOs were grouped based on structures (Chapter 3), namely total fucosylated HMOs, total neutral core HMOs, total sialylated HMOs, (α1-2)-fucosylated HMOs with abbreviation Fuc-(α1-2), (α1-3/4)-fucosylated HMOs with abbreviation Fuc-(α1-3/4), mono-fucosylated HMOs with abbreviation mono-Fuc, di-fucosylated HMOs with abbreviation di-Fuc, (α2-3)-sialylated HMOs with abbreviation Neu5Ac-(α2-3), and (α2-6)-sialylated HMOs with abbreviation Neu5Ac-(α2-6). The HMO structural groups (one distinct structure might be covered by multiple groups) were compared in terms of their dynamic concentrations in human milk, and their metabolic fates by the infant gut microbiota.

3FL concentrations in mother milk revisited

Sample preparation is an essential step before loading human milk or infant fecal samples in analytical equipment, such as liquid chromatography. By applying solid phase extraction (SPE) using porous graphitized carbon (PGC) cartridges, lactose being present as the major solid fraction in human milk, could be largely separated from HMOs, thus eliminating its interferences during further analysis. We started with the SPE procedure which was described by Albrecht et al.;[14] however, loss of 3-fucosyllactose (3FL) in the lactose clean-up fraction was noticed during analysis. The same problem with PGC-SPE was also reported by van Leeuwen,[15] and Xu et al.[16] Therefore, the previous PGC-SPE procedure was adapted to extract 3FL in a separate fraction, followed by quantitative analysis by HPAEC-PAD, as described in Chapter 2. The other most dominant HMO structures present in the milk were quantitated by PGC-LC-MS, which allows good separation of isomeric structures and unambiguous identification with MS (Chapter 2).

Table 7.2. Overview of the HMOs quantitated in the current thesis. HMO structures: blue circle - glucose; yellow circle - galactose; blue square - N-acetylglucosamine; red triangle – fucose; purple diamond - N-acetylneuraminic acids.

No.	Name	Abbreviation	Structure	Structural group
1	2´-Fucosyllactose	2´FL		Fucosylated/ mono-Fuc/ Fuc-(α1-2)
2	Lacto-*N*-fucopentaose I	LNFP I		Fucosylated/ mono-Fuc/ Fuc-(α1-2)
3	Difucosyllactose	DFL		Fucosylated/ di-Fuc/ Fuc-(α1-2)
4	Lacto-*N*-difucohexaose I	LNDFH I		Fucosylated/ di-Fuc/ Fuc-(α1-2)
5	3-Fucosyllactose	3FL		Fucosylated/ mono-Fuc/ Fuc-(α1-3/4)
6	Lacto-*N*-fucopentaose II	LNFP II		Fucosylated/ mono-Fuc/ Fuc-(α1-3/4)
7	Lacto-*N*-fucopentaose III	LNFP III		Fucosylated/ mono-Fuc/ Fuc-(α1-3/4)
8	Lacto-*N*-fucopentaose V	LNFP V		Fucosylated/mono-Fuc/ Fuc-(α1-3/4)
9	Lacto-*N*-difucohexaose II	LNDFH II		Fucosylated/ di-Fuc/ Fuc-(α1-3/4)
10	Lacto-*N*-tetraose	LNT		Neutral core
11	Lacto-*N*-neotetraose	LNnT		Neutral core
12	Lacto-*N*-hexaose	LNH		Neutral core
13	Lacto-*N*-neohexaose	LNnH		Neutral core
14	3´-Sialyllactose	3´SL		Sialylated/ Neu5Ac-(α2-3)
15	Sialyl-lacto-*N*-tetraose a	LST a		Sialylated/ Neu5Ac-(α2-3)
16	6´-Sialyllactose	6´SL		Sialylated/ Neu5Ac-(α2-6)
17	Sialyl-lacto-*N*-tetraose c	LST c		Sialylated/ Neu5Ac-(α2-6)
18	Sialyl-lacto-*N*-tetraose b	LST b		Sialylated/ Neu5Ac-(α2-6)

We made a comparison between the 3FL levels in mother milk as measured by our method and concentrations provided in literature (Table 7.3). In human milk samples within the KOALA cohort (Chapter 2 and Chapter 4), an average concentration of 0.19 g 3FL/L was found in milk samples from Lewis positive Secretor positive (Le+Se+) mothers, and 0.48 g/L from Lewis positive non-Secretor (Le+Se-) mothers. The values from Le-Se+ and Le-Se- milk groups were lower, 0.04 g/L and 0.11 g/L respectively. Compared to these milk samples from KOALA cohort, human milk samples provided by the BINGO cohort (Chapter 3 and Chapter 5) contained higher concentrations of 3FL, 0.33 g/L and 1.14 g/L for Le+Se+ and Le+Se- mothers at 2 weeks postpartum, respectively. The 3FL concentrations also increased from 2 weeks to 12 weeks postpartum for both milk groups. The 3FL results from BINGO samples is almost three times higher than that from KOALA samples for the same milk group, which is unexpected and will be discussed further in the following section. No mothers with phenotypes Le-Se+ or Le-Se- were identified in the BINGO cohort. It is noted that mothers with Le+Se- phenotype generally synthesized higher levels of 3FL, although its production is independent from *Le* and *Se* genes. This observation could be explained by fucose donor availability during the various fucosyltransferase reactions during HMO biosynthesis, which has been explained in Chapter 3 with BINGO samples. Two studies of De Leoz et al.[17-18] could not detect any 3FL in over half of the human milk samples, with the rest of samples reported to contain no more than 0.71% of total HMO abundance. If we assume the total HMO concentration in human milk samples to be 10 g/L on average, 0.71% equals to 0.071 g/L of 3FL. Two studies of Kunz et al. gave totally different 3FL levels where one of the studies reported on co-elution of another HMO with 3FL.[19-20] Totten et al. analyzed 69 human milk samples collected during the first 6 months postpartum, but only found 0.05% - 0.11% of total oligosaccharide intensity to be 3FL.[21] It seems that some published studies coincidently underestimated 3FL levels in human milk. Therefore, with our adapted extraction and quantitation methods of 3FL, which is explained in Chapter 2, a more accurate result could be obtained. This is important for monitoring 3FL synthesis during lactation, learning about metabolization of 3FL by the infant gut microbiota, as well as for the quality control of 3FL supplemented infant formula that might be commercialized in the future.

Variations in HMO synthesis by lactating mothers

Comparison between the KOALA and BINGO cohort studies

Although analyzed with the same technique (described in Chapter 2), HMO concentrations of human milk samples from KOALA and BINGO showed variations, as shown in Figure 7.1. It is known that most HMOs and their total concentrations generally decrease with lactation during the first three months of life (Chapter 3), we would expect the values from KOALA to be in between those of week 2 and week 6 from BINGO. However, the average concentrations of total fucosylated HMOs and total HMOs present in

KOALA milk samples were much lower than those in BINGO ones (Figure 7.1). The values of total neutral core and total sialylated HMOs were generally comparable between the two cohorts. Nevertheless, fucosylated HMOs accounted for the most abundant fraction in milk samples from both cohorts. When comparing the relative abundances of individual HMO structures, samples from both cohorts contained 2′FL, LNFP I, LNDFH I, 3FL, LNFP II, LNFP III, LN(n)T as the major HMOs. It is noted that the relative abundances of both 2′FL and 3FL in milk samples from KOALA were much less than those from BINGO, even after taking consideration of temporal changes. In contrast, LN(n)T and LST b were more abundant in KOALA milk samples compared to BINGO milk samples, while the other HMOs remained comparable between the two cohorts. As mentioned in Chapter 2, we suspected a continuous degradation of HMO structures during sample storage. Samples from KOALA were collected over 10 years before those from BINGO, so the lower concentrations of the fucosylated HMOs, especially those of lower molecular weight, of the former sample set confirmed our assumption. This also implies that infant formula industry might need to pay attention on 3FL, 2′FL and other small HMOs in the supplemented products during shelf life.

Table 7.3. Reported levels of 3-fucosyllactose (3FL) in human milk samples in literature and in the current thesis.

References/Chapter	Milk group	Sample size	Postpartum age	3FL level
KOALA cohort study (Chapter 2&4)	Le+Se+	81	1 month	0.19 (0.15) g/L[#]
	Le+Se-	29	1 month	0.48 (0.25) g/L[#]
	Le-Se+	7	1 month	0.04 (0.06) g/L[#]
	Le-Se-	4	1 month	0.11 (0.06) g/L[#]
BINGO cohort study (Chapter 3&5)	Le+Se+	48	2 weeks	0.33 (0.21) g/L[#]
		48	6 weeks	0.55 (0.30) g/L[#]
		42	12 weeks	0.88 (0.50) g/L[#]
	Le+Se-	16	2 weeks	1.14 (0.40) g/L[#]
		13	6 weeks	1.39 (0.52) g/L[#]
		13	12 weeks	1.59 (0.50) g/L[#]
De Leoz et al.[17]	All included	41	1 – 16 weeks	Not detected in 22 samples, the rest with max. 0.71% of total HMO abundance
De Leoz et al.[18]	Not mentioned	1	Not mentioned	Not detected
Kunz et al.[19]	All included	10	2 – 4 weeks	0.07 (0.08) g/L
Kunz et al.[20]	Secretor	21	1 – 30 days	Day 1-7, 1.03 g/L; day 8-15, 1.28 g/L; day 16-30, 1.11 g/L[†]
	Nonsecretor	11	1 – 30 days	Not detected
Totten et al.[21]	Secretor	44	0 – 6 months	0.05% [‡]
	Nonsecretor	16	0 – 6 months	0.11% [‡]

[#] Values are shown as averages (standard deviation).
[†] Values are shown as sum of 3FL and lacto-*N*-difucohexaose I, since not well separated during analysis. The values given here are medians.
[‡] Values are shown as average percentage of total oligosaccharide intensity

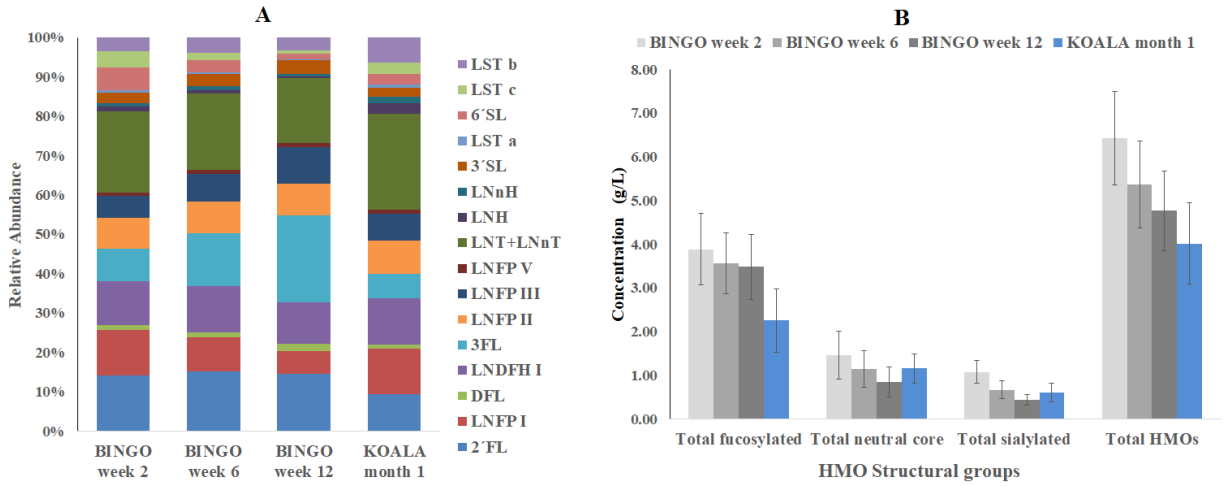


Figure 7.1. Comparison of HMO levels in human milk samples between KOALA study cohort (1-month postpartum, n=121) and BINGO study cohort (week 2, n=64; week 6, n=61; week 12, n=55). A) relative abundances of each quantitated HMO; B) average concentrations and standard deviations of total and different HMO structural groups.

Inter-individual and longitudinal variations of HMO synthesis

Two main determinants of HMO synthesis in human milk are Lewis/Secretor phenotypes of mothers, and lactation duration, which has been discussed in Chapter 3. Not only the presence of Fuc-(α1-2) and Fuc-(α1-3/4) HMO structures were influenced by the Secretor- and Lewis- genes of mothers, but also levels of neutral core HMO structures (LNT, LNnT, LNH and LNnH) were significantly different between Le+Se+ and Le+Se- milk (Chapter 3). For the BINGO cohort, synthesis of sialylated HMOs was generally found to be independent from milk groups, except for level of LST c. Contradictory findings were reported in literature regarding the distribution of different HMO structural groups, where one study stated non-fucosylated HMOs being predominant in human milk,[18] whereas another study found fucosylated HMOs to be the most abundant fraction.[22] Our results from BINGO samples confirmed the findings of the latter study,[22] and we also observed that the relative fraction of the fucosylated HMO group continuously increased during lactation. The total HMO concentrations of Le+Se+ milk were generally higher than that of Le+Se- milk at all the three time points, but the between-group differences were only statistically significant at week 2 postpartum and became more similar at later lactation stages. Over the course of lactation, concentrations of total and most individual HMO structures in human milk showed a trend in getting lower over time, with a few exceptions such as increasing level of 3FL. The temporal reduction in HMO concentrations was also found to be milk group dependent. When taking the consumption volume by infants into calculation, the absolute amounts of most individual as well as total HMOs ingested by babies remained comparable in the first three months of life, as a higher amount of milk intake compensated for lower concentrations at later lactation stages (Chapter 5).[23]

One limitation of the BINGO cohort is the absence of Le-Se+ and Le-Se- milk samples, therefore no comparison was made between Secretor and non-Secretor milk within the Lewis negative mothers. Among the samples from the KOALA cohort, there are 7 milk samples from Le-Se+ mothers, and 4 from Le-Se- mothers. Although the sample size is limited, a preliminary comparison was made among the four milk groups from KOALA cohort in Table 7.4. The total HMO concentrations of Le+Se+ milk was the highest, followed by comparable values of Le+Se- and Le-Se+ milk, and the lowest values were from Le-Se- milk. The fraction of fucosylated HMOs was the largest in all the milk groups except for Le-Se- group, which contained mostly neutral core HMOs. Le-Se- milk also contained higher level of 6′SL compared to other milk groups. Within the BINGO cohort, we found that Secretor mothers produced higher amount of LST c compared to non-Secretors (Chapter 3). As shown in Table 7.4, also the KOALA cohort milk samples at week 4 postpartum from both Le+Se+ and Le-Se+ groups contained almost double amount of LST c than that from Le+Se- and Le-Se- groups, confirming the influence of Secretor status on LST c synthesis. Our findings challenge a previous report which stated that sialylation of LNnT was less influenced by fucosyltransferase activity compared to LNT.[22] It is speculated that the differences of HMO profiles between milk groups might lead to different influences on long-term health and growth of infants of mothers with different Lewis/Secretor phenotypes.

Variations in HMO metabolization in infant GI tract

Developmental trajectories of individual infants

With a pilot sample set from KOALA cohort (n= 20), three differential HMO consumption patterns by infant gut microbiota at approximately one month of age were found (Chapter 2). Both relative compositional changes of HMO profiles between human milk and paired infant feces, and total HMO concentrations excreted into infant's feces, were different for each consumption pattern. Infants whose fecal samples contained less than 1 μg/mg of total HMO structures were considered as 'complete degradation' group. The second group of infants showed considerable consumption level of specific HMO structures, with intermediate amounts of ingested HMOs excreted in feces. In Chapter 2, the total HMO concentrations in infant feces of the 'specific consumption' group was between 1.2 – 6.4 μg/mg. The third group of infants contained the highest total concentrations of HMOs in their feces (8.4 – 30.7 μg/mg) compared to the former two groups. Additionally, the HMO profiles between mother milk and paired infant feces from this third group were relatively comparable with no obvious specificity to certain structural groups. The varying HMO metabolization patterns reflected variation in infant gut microbiota composition. In Chapter 4 and Chapter 5,[4, 23] microbiota composition of the infant fecal samples from KOALA and BINGO studies were described. Interestingly, despite the high inter-individual variations of infant fecal microbiota composition,

three distinct microbial patterns (Cluster A, B and C) at genus level were recognized in the whole sample set from KOALA (Chapter 4), as shown in Figure 7.2a. Infant fecal microbiota from Cluster A showed a mixed structure, with *Bifidobacterium* accounting for a lower proportion and *Streptococcus* and others more predominant (Figure S4.1). Cluster B is characterized by high abundance of both *Bacteroides* and *Bifidobacterium*, while Cluster C is dominated by *Bifidobacterium*. Also, in Chapter 4, the full sample set of KOALA samples was assigned to the different HMO metabolization patterns: "Complete", "Non-specific", and "Specific". The "Specific" pattern was further divided into "specific acidic" (sialylated HMOs were predominantly utilized), "specific neutral" (fucosylated and neutral core HMOs were predominantly utilized), and "specific other" (samples that did not belong to the other two groups). Strong correlations were found between microbial Cluster A and low/non-specific HMO consumption group, as well as between microbial Cluster B and specific acidic consumption group, while the microbial Cluster C was associated with the 'complete consumption' group (Chapter 4). Similar enterotypes of infant gut microbiota have also been recognized in other studies.[24-25] Furthermore, these three different microbial community patterns were also found in the sample set from BINGO, with only slight fluctuations of relative abundances of specific taxa.[26]

The distribution of infants belonging to the three microbial community clusters is given in Figure 7.2b, at different time points postpartum, from both BINGO and KOALA cohorts. Any direct comparison between the two cohorts should be cautious, since the two sample sets were collected over 10 years apart. Within BINGO samples, from week 2 to week 12 postpartum, a clear directional transition of the cluster distribution was observed for the infants. Number of infants belonging to Cluster A gradually decreased from 50% at week 2 to less than 20% at week 12; meanwhile, those of Cluster C steadily increased from 25% at week 2 to almost 50% at week 12. The clustering results of KOALA samples were more comparable to that of BINGO week 12. This gradual transition of clustering indicated that the infant gut microbiota developed towards a *Bifidobacterium* dominated community during the first three months of life.[26]

Table 7.4. Concentrations of the 17 human milk oligosaccharides (g/L) in full-term human milk collected around 1 month postpartum, from KOALA cohort study (n=121). The results were shown as means (standard deviations) per milk group depending on mother's Lewis Secretor phenotypes.

Compound	Le+Se+ (n = 81)	Le+Se- (n = 29)	Le-Se+ (n = 7)	Le-Se- (n = 4)
Fucosylated				
2′FL	0.49 (0.10)	0.02 (0.09)	0.66 (0.06)	0 (0)
LNFP I	0.61 (0.22)	0 (0.01)	1.05 (0.44)	0 (0)
DFL	0.06 (0.02)	0 (0.01)	0.02 (0.02)	0 (0)
LNDFH I	0.70 (0.27)	0.02 (0.02)	0.04 (0.10)	0 (0)
3FL	0.19 (0.15)	0.48 (0.25)	0.04 (0.06)	0.11 (0.06)
LNFP II	0.24 (0.15)	0.73 (0.29)	0.01 (0.03)	0 (0)
LNFP III	0.24 (0.12)	0.40 (0.14)	0.12 (0.05)	0.30 (0.06)
LNFP V	0.03 (0.03)	0.09 (0.07)	0 (0)	0.08 (0.05)
Total fucosylated	2.55 (0.54)	1.74 (0.64)	1.95 (0.28)	0.49 (0.16)
Neutral core				
LNT + LNnT	0.93 (0.32)	1.11 (0.27)	0.79 (0.39)	1.20 (0.21)
LNH	0.11 (0.06)	0.09 (0.06)	0.10 (0.09)	0.18 (0.09)
LNnH	0.09 (0.06)	0.03 (0.02)	0.09 (0.06)	0.02 (0.03)
Total neutral core	1.13 (0.34)	1.23 (0.28)	0.97 (0.49)	1.41 (0.23)
Sialylated				
3′SL	0.09 (0.04)	0.10 (0.04)	0.10 (0.05)	0.11 (0.06)
LST a	0.03 (0.01)	0.03 (0.02)	0.03 (0.02)	0.05 (0.03)
6′SL	0.11 (0.05)	0.11 (0.07)	0.11 (0.06)	0.22 (0.13)
LST c	0.13 (0.07)	0.08 (0.06)	0.14 (0.10)	0.07 (0.05)
LST b	0.25 (0.12)	0.30 (0.10)	0.22 (0.12)	0.23 (0.09)
Total sialylated	0.59 (0.20)	0.62 (0.22)	0.59 (0.31)	0.68 (0.20)
Total	4.27 (0.85)	3.60 (0.81)	3.52 (1.02)	2.58 (0.40)

The directional transition of infant gut microbiota was also reflected on the HMO metabolization, based on relative compositional changes between fecal and milk HMO profiles (Chapter 3). Almost all infants from the BINGO cohort displayed an increased degradation of ingested HMOs from week 2 to week 12, though the succession from low/non-specific to intermediate/specific consumption, and eventually complete consumption stage was highly variable among individuals. Two infants already reached the complete consumption pattern at week 2; however, considerable levels of HMOs were still excreted by almost half of the BINGO infants in their feces. There were few studies in literature on *in vivo* degradation of ingested HMOs by infants with large sample size, with diverse results reported.[27-30] Chaturvedi et al. found fecal HMO profiles highly resembling that of paired mother milk after analysing 16 pairs of milk-feces samples collected from subjects between 2 – 19 weeks of age.[27] Similarly, the study of Coppa et al. included 6 mother-infant pairs at 1 month postpartum, and reported quite comparable HMO profiles

between milk and feces, except for decreases of three HMO structures (LST a, disialyllacto-*N*-neotetraose and LNT) in fecal excretion.[28] With a higher number of study subjects (n = 76) included, van Leeuwen et al. recognized the same three HMO consumption patterns in infants at 1 month of age as we found in Chapter 2.[30] The abovementioned studies were based on single time point of sample collection, and no indication on temporal changes of the consumption pattern was provided. A stage-wise development model of HMO metabolization was first proposed by Albrecht et al., with evidences from 11 mother-infant pairs followed from 2 weeks to 7 months of life.[29] However, infant fecal microbiota composition was not analyzed for the same sample set that Albrecht used in her study, so the development of gut microbiota part of the proposed model was only based on other studies.[29] Combining results of both HMO metabolization and infant fecal microbiota composition found for the same sample sets of both the BINGO and KOALA cohort, we would like to update the development model originally proposed by Albrecht et al (shown in Chapter 1).[29]

- In the original model, the first stage covers the first few weeks of life, when the infant gut was occupied by mixed microbiota dominated by facultative anaerobes with low ability to degrade HMOs, and resulted in either acidic- or neutral HMO dominated fecal profile. Based on evidence of low/non-specific consumption pattern and its strong association with microbial Cluster A recognized in BINGO and KOALA samples, the mixed microbiota of the **first stage** is validated in the current model, and the facultative anaerobes were mainly *Streptococcus* and others (Figure S4.1).[26] The fecal HMO profile at the first stage in the new model was characterized by low and non-specific consumption, which led to high similarity with the profile in the paired mother milk.

- In the new model, when infant microbiota composition progressed to the **second stage**, which was dominated by both *Bacteroides* and *Bifidobacterium*, intermediate level of ingested HMOs would be degraded, and fecal HMO profile displayed a pattern of specific structures (Figure 4.2).

- At the **third stage** of this model, we proposed that infant gut microbiota was dominated by *Bifidobacterium* which degraded almost all HMOs consumed from mother milk, leading to (almost) empty fecal HMO profiles.

When matching the HMO consumption patterns to the infant fecal microbiota clustering, the key role of *Bifidobacterium* in degrading HMOs in the infant gastrointestinal tract was confirmed, followed by *Bacteroides*, which was also reported to be able to utilize mucus glycans.[31-32] Although Albrecht et al. indicated approximate time phase of succession across developmental stages,[29] we found a high variation regarding the transition speed across different stages of individual infants. There were infants who already reached the third stage at 2 weeks postpartum, whereas half of the breastfed infants from BINGO were still at the first or the second stage at 3 months postpartum.

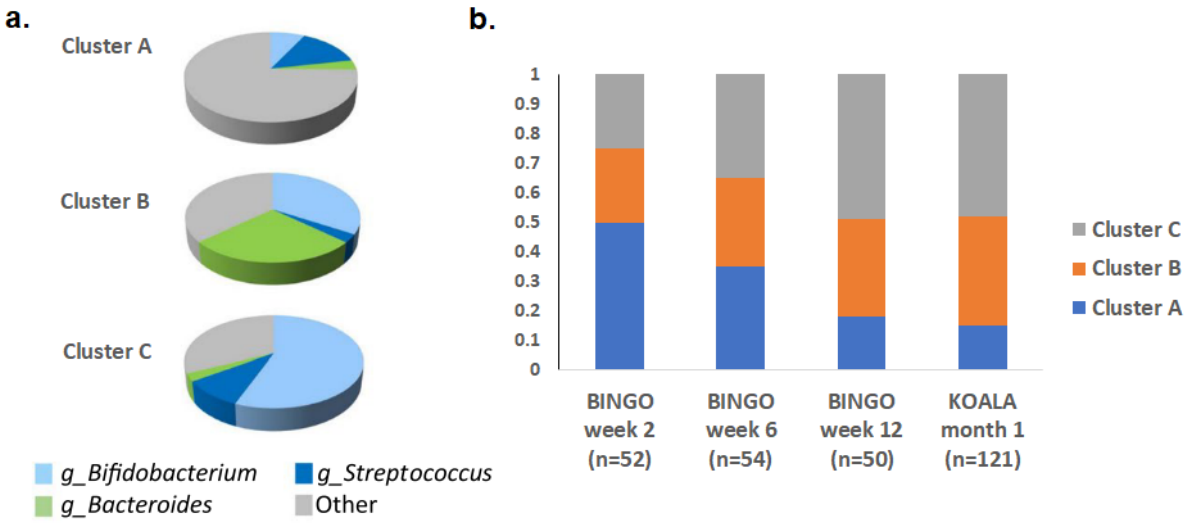


Figure 7.2. Dirichlet Multinomial Mixture (DMM) clustering of samples on the basis of fecal microbiota composition at genus level; a) average relative abundance of microbial groups characteristic to individual cluster A, B and C, KOALA full sample set, 1-month postpartum; b) fraction of samples from infants within each cluster category. Figure adapted from Borewicz et al.[26]

Structure-specific metabolization of HMOs by the infant gut microbiota

Quite a few *in vitro* fermentation studies found the strong HMO-degrading abilities of especially *Bifidobacterium (e.g. Bifidobacterium longum* subspecies *infantis, Bifidobacterium longum* subspecies *longum*, *Bifidobacterium breve*), followed by some members from *Bacteroides (e.g. Bacteroides fragilis* and *Bacteroides vulgatus)*.[33-37] The growth of specific species and strains from *Bifidobacterium* and *Bacteroides* was dependent on the specific HMO structures present in the medium.[35, 38-40] One *in vivo* study had addressed the isomer-specific consumption of infant gut microbiota with samples from two infants analyzed.[41] No systematic comparison was made in terms of different metabolic fates of HMO structures through the transit of infant gastrointestinal tract.

As described in Chapter 2, by comparing relative concentrations of infant fecal HMOs to the paired milk from a pilot sample set of KOALA study, it was found that certain HMO structural groups were more prone to microbial degradation compared to others. Subsequently, with the whole sample set of BINGO collected at three time points from week 2 to week 12 postpartum, the structure-specific consumption of ingested HMOs by the infant gut microbiota was further confirmed by the variation in relative abundances of different HMO structural groups (Figure 3.3). To compare what we had found with BINGO samples, HMO levels present in 69 milk and fecal samples from KOALA were shown in Figure 7.3. Only samples that contained over 1 μg/mg of total HMO structures in infant feces were selected in the calculation. Total

fucosylated HMOs increased from 53% in milk to 71% in feces, while neutral core HMOs decreased from 31% in milk to 11% in feces, and total sialylated HMOs remained around 16% - 18% (Figure 7.3A). This trend indicated a general higher consumption level of neutral core HMOs, followed by sialylated HMOs, and the fucosylated HMOs were the least consumed. No obvious difference was observed between degradation of (α1-2)-fucosylated HMOs compared to that of (α1-3/4)-fucosylated HMOs, as their relative abundances were quite comparable between milk and feces (Figure 7.3B). Mono-fucosylated HMOs accounted for 74% in milk, but reduced to 48% in feces, while di-fucosylated HMOs increased from 26% to 52%, indicating lower degradation ability of gut microbes for HMOs with more fucose moieties (Figure 7.3C). Within the sialylated HMO group where (α2-6)-sialylated HMOs were predominant in both milk and feces, the reduction in relative abundance of (α2-3)-sialylated HMOs showed a stronger microbial degradation ability of the latter (Figure 7.3D).

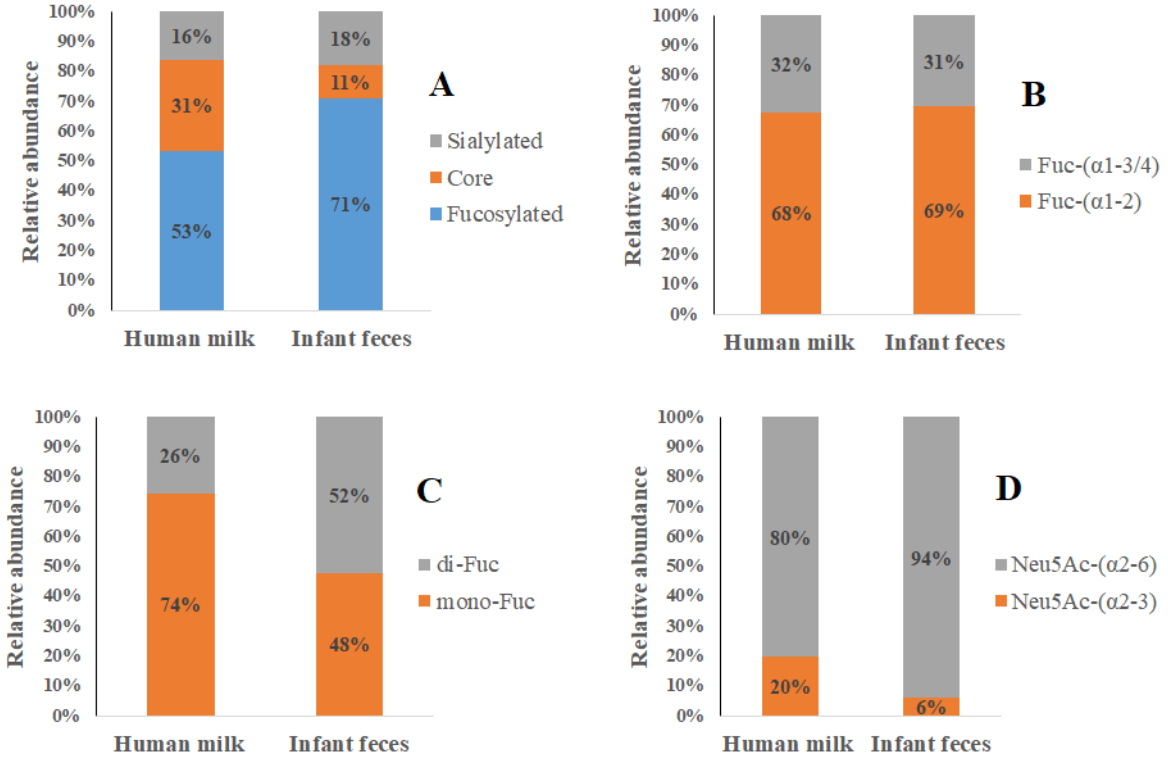
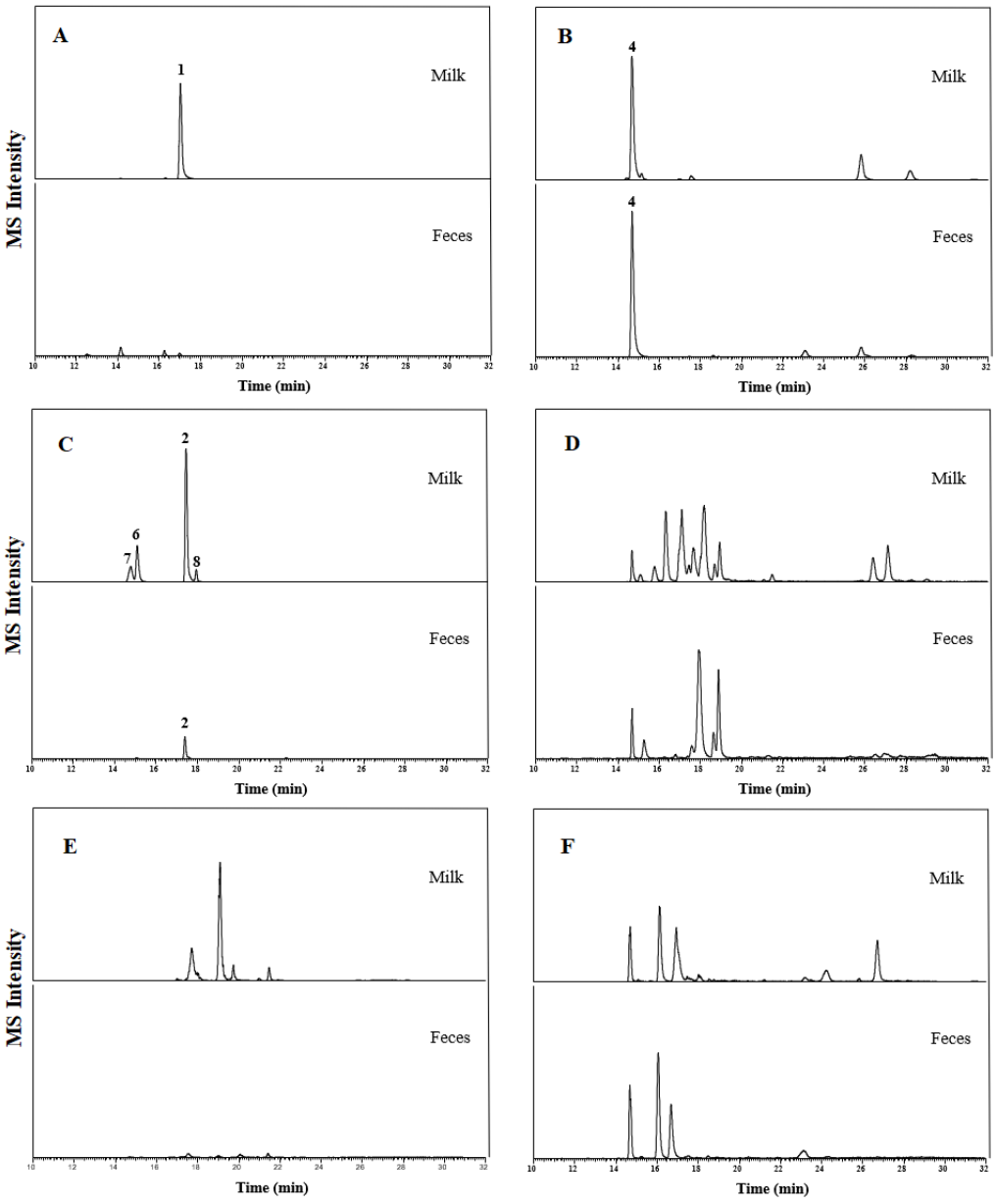


Figure 7.3. Relative abundances of different HMO structural groups (A, total fucosylated, total core, and total sialylated HMO structures; B, (α1-2)-fucosylated, and (α1-3/4)-fucosylated HMO structures; C, mono-fucosylated, and di-fucosylated HMO structures; D, (α2-3)-sialylated, and (α2-6)-sialylated HMO structures) in human milk and in paired breastfed infant feces, in which total fecal HMOs > 1 µg/mg. All subjects belong to KOALA cohort study (n=69).

The preferred consumption pattern also reflected on HMO profiles of individual sample subjects. The MS peak intensities of HMOs with different numbers of fucose moieties in milk and fecal sample of one mother-infant pair from KOALA study are given in Figure 7.4, where all the different isomers are shown

for a given oligomer. It is found that FL (Figure 7.4A), LNFP (Figure 7.4C) and monofucosyllacto-*N*-hexaose (MFLNH, Figure 7.4E) were (almost) fully utilized by the infant, whereas LNDFH (Figure 7.4B), difucosyllacto-*N*-hexaose (DFLNH, Figure 7.4D) and trifucosyllacto-*N*-hexaose (TFLNH, Figure 7.4F) still largely remained in the feces, compared to their presences in the mother milk. In Figure 7.4C and 7.4E, even all the different isomers of LNFP and MFLNH behaved similar. But in Figure 7.4D and 7.4F, several isomers (not identified here) of DFLNH and TFLNH, respectively, behaved differently. Overall, quite some of the di- and tri- fucosylated HMOs remained in the feces, with the levels strongly depending on the isomers. As given in Figure 7.4G, the ratios of total peak area between feces and milk of each extracted ion spectrum was 0.11- 0.18 for HMOs with one fucose moiety, which was much lower compared to that for HMOs with multiple fucose moieties (0.69 – 1.06). Therefore, more fucose decoration onto HMO structures reduced the assimilation ability of infant gut microbes. Linkage types also influenced the metabolization efficiency of sialylated HMOs, as can be seen in another mother-infant pair shown in Figure 7.5. Peak intensity of 3´SL (peak 14) relatively reduced to a larger extent compared to 6´SL (peak 16) from milk to feces (Figure 7.5A), and LST b (peak 18) reduced the least, compared to LST c (peak 17) and LST a (peak 15; (Figure 7.5B). Both 6´SL and LST b are (α2-6)-sialylated HMOs, which were less degraded than 3´SL and LST a that belong to (α2-3)-sialylated HMOs. It is noted that LST c with (α2-6)-sialyl linkages was considerably degraded in this sample, which implies the core structure of sialylated HMOs also exert influence on microbial degradation. However, the gut microbiota of other infants from KOALA and BINGO studies showed varying preferences to LST c and LST b, without consensus.

In both KOALA and BINGO samples, the most predominating genera in infant fecal microbiota were found to be *Bifidobacterium*, *Bacteroides*, *Escherichia-Shigella* and *Parabacteroides* (Chapter 4 and Chapter 5). When correlating relative abundances of phylotypes from infant fecal microbiota with degradation levels of specific HMO structures, consumption of most HMOs was associated with several Operational Taxonomic Units (OTUs) from *Bifidobacterium*, especially the most abundant *Bifidobacterium* OTU 1263 (Chapter 5). Strongest associations with *Bifidobacterium* OTU 1263 were observed for LNH, LNT and LNnT belonging to the neutral core HMO group, and LNFP V. Several *Bacteroides* OTUs were present in higher abundance in infants who showed higher consumption abilities of 2′FL and LNFP I/II/V, while *Parabacteroides* OTUs were more abundant in those degrading more 3FL, LNFP V, LNH, LNT and LNnT (Chapter 5). Furthermore, infants that showed efficient consumption on sialylated HMOs were more often assigned to microbial Cluster B which was dominated by *Bacteroides* (Chapter 4). The methods for analyzing infant fecal microbiota in the current study provided information on relative abundance of bacterial taxa, instead of absolute abundances. Furthermore, species or strains of the infant fecal microbes were not identified. Future studies regarding strain or species level microbiota composition analysis are warranted, in order to confirm our findings with previous *in vitro* fermentation studies.



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	Sum of Peak Area		Feces/Milk
	Milk	Feces	
FL	24763304	4573365	0.18
LNFP	1.14E+08	12992910	0.11
MFLNH	19814251	3061662	0.15
LNDFH	79118340	80464183	1.02
DFLNH	12849465	8905586	0.69
TFLNH	6088500	6465123	1.06

Figure 7.4. (A – F) Extracted ion spectra of PGC-LC-MS chromatograms of one human milk sample and its paired infant fecal sample from KOALA cohort: A) FL, fucosyllactose; B) LNDFH, lacto-*N*-difucohexaose; C) LNFP, lacto-*N*-fucopentaose; D) DFLNH, difucosyllacto-*N*-hexaose; E) MFLNH, monofucosyllacto-*N*-hexaose; F) TFLNH, trifucosyllacto-*N*-hexaose. Names and structures of the identified HMO peaks 1-8 in (A – C) are listed in Table 7.2. (G) total peak area of extracted ion spectra in (A – F) and ratios between human milk and paired infant feces.

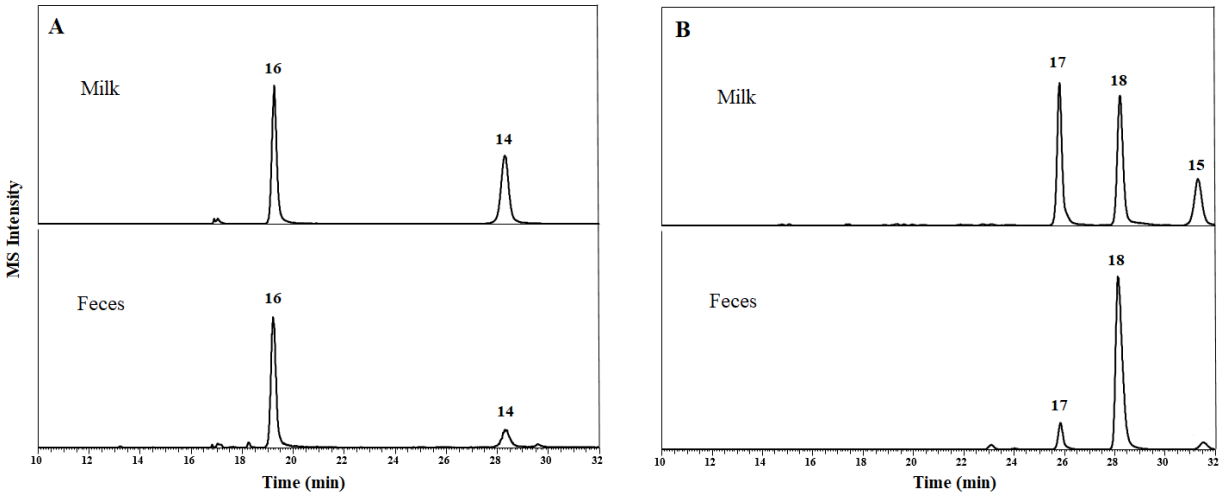

Figure 7.5. Extracted ion spectra of PGC-LC-MS chromatograms of one human milk sample and its paired infant fecal sample from KOALA cohort: A) SL, sialyllactose; B) LST, sialyl-lacto-*N*-tetraose. Names and structures of the identified HMO peaks 14 - 18 are listed in Table 7.2.

Key determinants for the HMO metabolization in infants

Aside from infant age, other factors, including delivery mode, delivery place, infant gender, specific HMOs in mother milk, and feeding mode, that would influence infant gut microbiota and HMO metabolization are discussed in Chapter 3, Chapter 4 and Chapter 5. Delivery mode and delivery place were found to be among the factors leading to the differences of infant gut microbiota composition, especially at week 2 postpartum. The influence of delivery mode and place on gut microbiota disappeared at 12 weeks of age (Chapter 5). However, when examining the HMO consumption patterns of infant microbiota, differences associated with delivery mode and delivery place only became significant at 12 weeks postpartum (Chapter 3). Caesarean section delivery, or exposure to hospital/clinic environment at delivery, tended to delay the transition of HMO consumption pattern towards the final stage of complete utilization, compared to vaginal delivery, or home delivery (Chapter 3). Both factors would influence the initial seeding of infant gut at birth, which has been reported in previous studies.[42-45] With the effects on infant fecal microbiota composition already detected at early age, the indirect effects on HMO metabolization were observed at later time points.

Infant gender was another factor that showed strong association with infant fecal microbiota composition, especially at 12 weeks of age (Chapter 5). It has been reported previously that gender was related to fecal microbiota in adults as well as infants.[46-48] However, no statistically significant differences were found between boys' and girls' HMO consumption patterns (Chapter 3). The mechanism of the gender-related differences of fecal microbiota remained further investigation.

Feeding non-Secretor milk to infants was found to negatively affect infant gut development by a previous study.[49] Interestingly, in Chapter 4, positive associations were found between 2´FL, LNFP I with microbial Cluster A (mixed microbiota). Both 2´FL and LNFP I are major components of Secretor milk, but absent in non-Secretor milk. Therefore, the associations aforementioned implied an effect exerted by mother's phenotypes on the infant gut microbiota development. To confirm the assumption with KOALA samples, we calculated the distribution of infants that showed complete, intermediate and low consumption levels of ingested HMOs within the four milk groups (Figure 7.6). Among one-month old infants, already 68 – 75% of those fed with non-Secretor mother milk (Le+Se- and Le-Se-) could utilize all the ingested HMOs, whereas only 17 – 33% of those fed with Secretor milk (Le+Se+ and Le-Se+) reached the complete utilization stage. However, the sample size of Le-Se+ and Le-Se- group were too small to draw strong conclusions. We are also aware that serving as prebiotic and carbon source during colonic fermentation is only one out of the myriad health benefits of HMOs, other functions such as preventing adherence of enteric pathogens might lead to fecal excretion.[50-53]

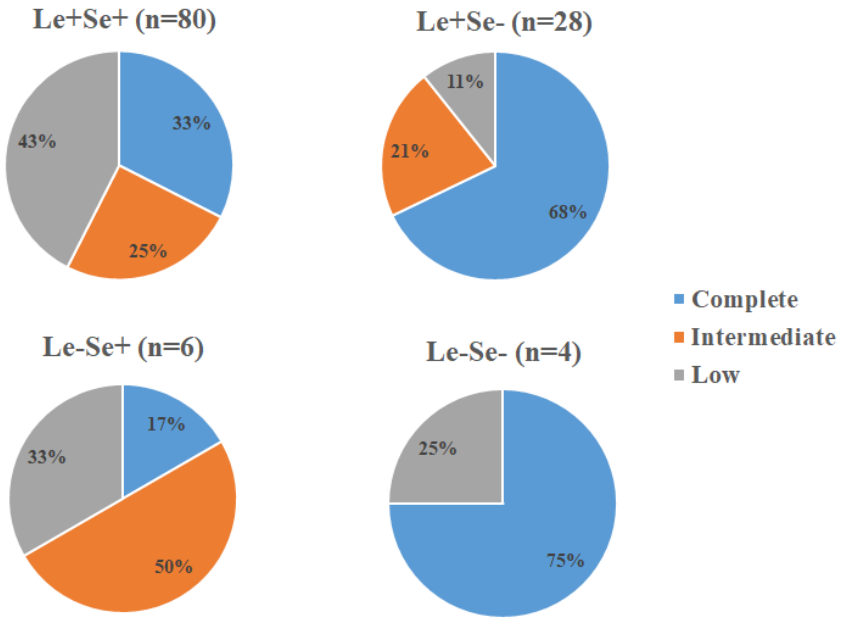

Figure 7.6. Distribution of complete, intermediate and low consumption levels of HMOs by infant gut microbes fed with human milk from mothers with different Lewis Secretor phenotypes, from KOALA cohort (1 month postpartum). Consumption levels based on total HMO concentrations present in infant fecal samples: complete, < 1 μg/mg; intermediate, 1-8 μg/mg; low, 8-22 μg/mg.

No formula-fed infants were included in the current thesis, and only a limited number (n = 7) of mixed-fed infants were compared to breast-fed ones in Chapter 3, with no significant differences found in terms of HMO metabolization pattern. However, in another study involving formula-fed and more mixed-fed

infants from KOALA and BINGO,[26] clear influence of feeding mode was observed on infant gut microbiota composition. In KOALA samples collected in the period 2002-2003, when prebiotic enriched infant formula was limited on the market, more than 90% of formula-fed and mixed-fed infants were assigned to microbial Cluster A and Cluster B at 1 month postpartum.[26] With development of infant formula and application of prebiotics such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), formula-feeding assigned more infants to microbial Cluster C (*Bifidobacterium* dominated) at 6 -12 weeks of age than breastfed and mixed-fed in BINGO samples that were collected 2014 – 2016.[26] Besides, possible effects of HMOs present in breastmilk, milk microbiota and other bioactive components in milk, as well as many environmental factors could lead to the differences in infant gut development.[26]

Evaluating prebiotic potential of IMMPs by *in vitro* and *in vivo* fermentation models

Aside from the well-documented prebiotics in the market, researchers and industry are spending effort on searching for novel prebiotics of varying bifunctionalities. Both *in vitro* and *in vivo* methods are commonly used study approaches in prebiotic research. In Chapter 6, with a batch *in vitro* fermentation model inoculated with standardized human fecal microbiota, we investigated fermentation behavior of a type of enzymatically modified starch isomalto/malto-polysaccharides (IMMPs). Three types of IMMPs with different proportions of α-(1→6) and α-(1→4) linked glycosidic linkages were compared in terms of polymer degradation, oligosaccharide formation, short chain fatty acid (SCFA) production, enzyme expression and microbiota composition changes during *in vitro* fermentation performed separately. Fermentation behavior of IMMPs was found to be structure-dependent. IMMPs rich in α-(1→6) linked glycosidic linkages showed slow-fermenting manner compared to other prebiotics such as FOS,[54] and only started to be utilized by microbiota after an adaption period (12 h *in vitro* fermentation). When a considerable level of α-(1→4) linked glycosidic linkages was present, fermentation of these structures started readily but had a delayed microbial degradation of α-(1→6) linked glycosidic linkages later on. Furthermore, molecular length distribution of α-(1→6) rich IMMPs also influenced the speed of *in vitro* fermentation, with smaller IMMP molecules fermenting faster, which is consistent with the reported good fermentability of isomalto-oligosaccharides (IMOs).[55] SCFAs were produced at high levels during IMMP fermentation, especially acetic acid. Besides, accumulation of succinic acid in the digesta was observed, which was unexpected, and required further investigation. Increased relative abundances of *Bifidobacterium* and *Lactobacillus* were detected, growth patterns of which were consistent with timing of degradation of α-(1→6) linkages.

In a study published elsewhere,[56] a mouse model was used to validate prebiotic potentials of IMMPs found with *in vitro* fermentation as discussed in Chapter 6. Briefly, C57BL/6 wild-type mice (nine weeks

old) were fed *ad libitum* with either control or IMMP supplemented diet. The IMMP diet contained 10% w/w IMMP 94 (94% α-(1→6) linked glycosidic linkages) to replace starch in the control diet. Fecal samples collected from day 0 – 21 were analyzed for SCFA contents and microbiota composition. Digesta samples from different parts of the gastrointestinal tract at day 21 were analyzed for oligosaccharide profiles, with results from one mouse per diet shown in Figure 7.7 A and B. Maltodextrin peaks resulting from starch digestion by murine digestive enzymes were observed in stomach and small intestine of both mice. Oligosaccharide products of IMMP fermentation were observed mainly in the cecum and large intestine of IMMP-fed mice (Figure 7.7B), while the control mice contained hardly any carbohydrate oligomers in cecum and large intestine (Figure 7.7A). The well-separated α-(1→6) linked oligosaccharide peaks in cecum and large intestine of IMMP-fed mice were similar to those observed during *in vitro* fermentation of IMMPs (Chapter 6). The broad peak eluted at 17-24 min representing undegraded IMMP polymer fraction, which was still present in significant amounts in the large intestine, confirming the slow-fermenting properties of IMMPs.

In the mousestudy,[56] the fecal excretion of SCFAs, succinic acid and lactic acid from day 0 to day 21 did not show any clear trend in response to IMMP supplementation, despite some minor variations between the control and IMMP-fed group. Increased production of succinic acid was observed at day 2 and day 3 for both groups.[56] However, SCFAs content in feces does not fully present the actual amount of production during microbial fermentation, due to instant uptake of these metabolites by colonocytes.[56-58] Additionally, the results of gut microbiota composition of mice were not fully in line with what we found with *in vitro* fermentation model using human/adult fecal inoculum. Relative abundance of *Lactobacillus* in the digesta from cecum and large intestine of IMMP-fed mice were higher compared to that of control mice; *Bacteroides* increased in large intestinal digesta in IMMP fed mice.[56] Different from the bifidogenic effect of IMMPs observed in *in vitro* fermentation (Chapter 6), *Bifidobacterium* was reduced in relative abundance in both cecum and large intestinal digesta when IMMP was supplemented in diet.[56] Nevertheless, IMMP feeding positively associated with relative abundance of *Lachnospiraceae*, *Roseburia* and *Odoribacter*, which are known as important microbes responsible for butyrate production.[56] Butyrate is known to possess many health benefits to the host such as helping with maintaining the colonic homeostasis.[7]

In vitro fermentation models and animal models are usually applied for screening potential prebiotics, due to practical and ethical reasons that limit human trials. Both models provided detailed information on structural metabolization of indigestible carbohydrates through gastrointestinal tract at molecular level, and allowed monitoring at different time points. However, the batchwise *in vitro* fermentation model is a closed environment, where the pH and temperature are often less maintained; in the meantime nutrients deplete and metabolites accumulate, resulting in less reliable observations with elongated fermentation time.[59] The

rodents model has been questioned regarding differences in gut microbiota composition and metabolization between rodents and humans.[60] We are aware of the limitations of these models, and further validation using piglet models or human trials are required before future application in food supplement.

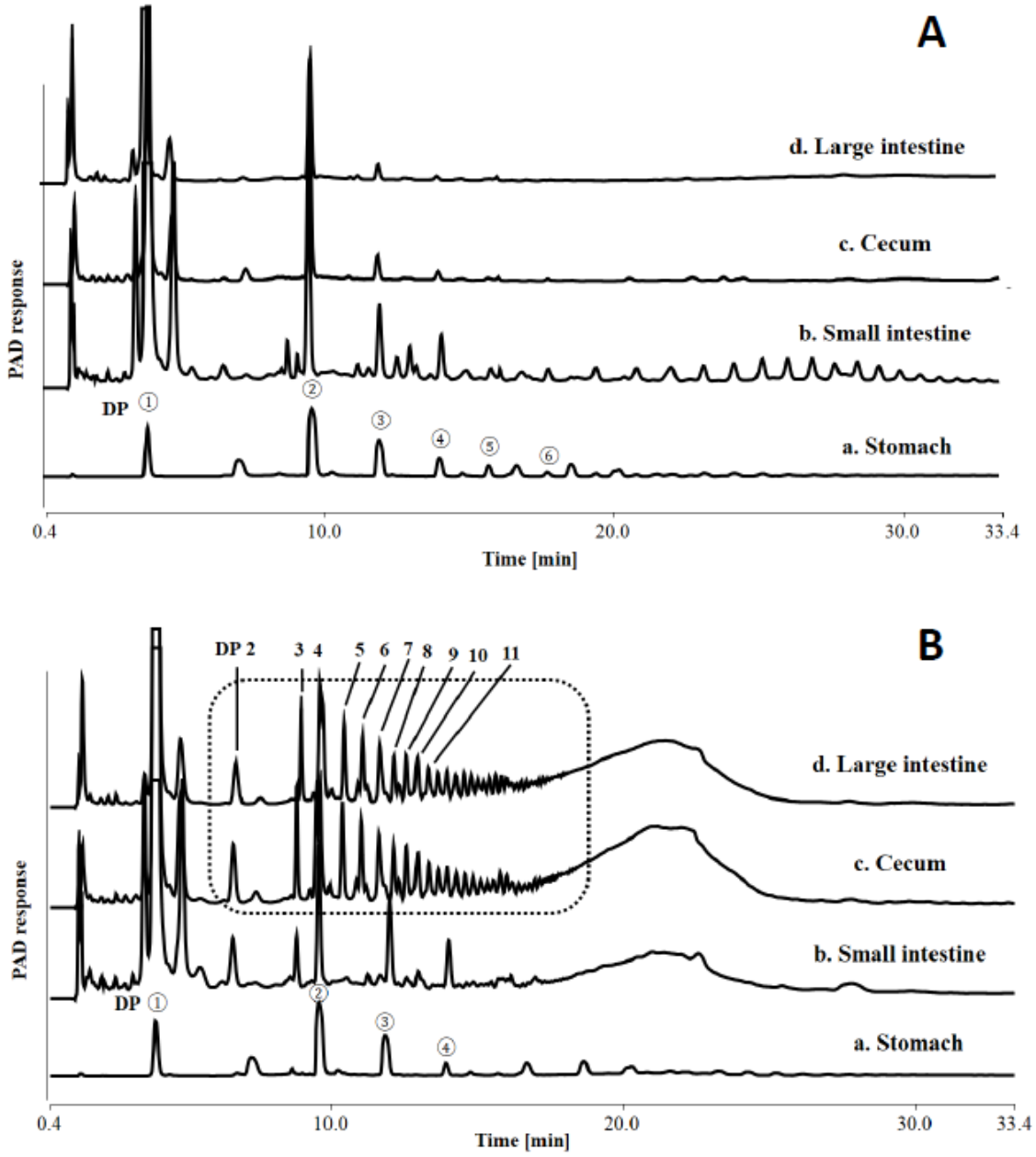

Figure 7.7. High performance anion exchange chromatography (HPAEC) elution patterns of digesta from one mouse fed with A) control diet or B) IMMP supplemented diet. The digesta are taken from different parts of the gastrointestinal tract: a) stomach, b) small intestine, c) cecum, and d) large intestine. IMMP peaks (2-11) in a box and maltodextrin peaks (①-⑥) are annotated, with the number indicating the degree of polymerization (DP). Figure from Mistry et al.[56]

Conclusions and future perspectives

The currently described research examined metabolization of indigestible carbohydrates, human milk oligosaccharides (HMOs) and isomalto/malto-polysaccharides (IMMPs), by infant or adult gut microbiota, using *in vivo* or *in vitro* study approaches. The optimized methods of HMO quantitation can also be applied for quality control of HMO supplemented formula or other products in the future market. The infant gut development model proposed in literature was updated based on evidence we found in KOALA and BINGO sample sets. The findings provide valuable evidence to guide researchers and industry to identify HMO structures and ratios to be applied for infant formula, as well as to make adjustments to suit requirements of infants at different developmental stages. The transition duration of infant gut microbiota and HMO metabolization pattern is highly variable among infants. As a preliminary observation, Secretor milk seemed to negatively influence the transition speed from reaching the complete utilization stage, where the infant gut microbiota composition is predominated by *Bifidobacterium*. However, the relation between colonization speed during infancy and gut microbiota growth later in adulthood, as well as other health outcomes later in life, remains a question. Due to low frequencies of Le-Se+ and Le-Se- mothers among population, we only collected limited numbers of samples from mothers of these two phenotypes. Future studies with involvement of more participants from Lewis-negative milk group are warranted to validate our current findings.

Lewis/Secretor status of the infants involved in the current study were unknown, so we were unable to make any comparison regarding possible influence of Lewis/Secretor milk groups on nursing infants with different Lewis/Secretor phenotypes. Furthermore, it remains a question from which age onwards the genuine Lewis phenotypes of infants could be establish, with one study proposing this to be at 5 months to 9 months after birth for the different Lewis phenotypes.[61] Results of a previous study for both *in vitro* and *ex vivo* experiments showed that binding of *Campylobacter jejuni*, which often causes bacterial diarrhea, requires (α1-2)-fucosyl epitope as present in either Secretor specific HMOs or as H(O) antigen in intestinal mucosa.[62] Therefore, it was hypothesized that feeding Secretor breastmilk to Secretor infants is essential in inhibiting campylobacter adherence in gastrointestinal tract, but not necessarily for non-Secretor infants.[62] Future research focusing on infants with different Lewis/Secretor phenotypes needs more attention to be paid on study design and participant recruitment.

Last but not least, we demonstrated the batch *in vitro* fermentation model for screening prebiotic potential of IMMPs, and for predicting its fermentation behavior that would happen *in vivo*. A standardized human fecal inoculum was applied in the fermentation model, which indicated a general trend within larger

population. The effect of IMMPs on different human individuals might vary and is worthy further investigations.

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Summary

Prebiotics and their close interactions with human gut microbiota has gained attention in recent years, as they exert multiple impacts on human health. Studies described in the current thesis focused on metabolism of prebiotics or indigestible carbohydrates at molecular level, as well as their associations with gut microbiota composition. In **Chapter 1**, we start with introducing human milk oligosaccharides (HMOs), which is the natural prebiotic during infancy. HMO structures are quite complex, and their concentrations in human milk vary depending on mothers' genetic profiles such as Lewis/Secretor genotypes, as well as lactation duration. The metabolization of HMOs in infant gastrointestinal tract has been investigated in some studies, however only with limited numbers of participants or time points, insufficiently linked to microbiota composition and activity. We also introduced an enzymatically modified starch, isomalto/malto-polysaccharides (IMMPs), having potential as prebiotic based on preliminary results from previous studies. Commonly applied approaches for prebiotic study, including *in vitro* fermentation model, *in vivo* animal and human model, are described.

In **Chapter 2**, the optimization of analytical methods to quantify HMOs in maternal milk and infant fecal samples is described, including revisited protocol for 3-fucosyllactose (3FL) analysis. Absolute concentrations of 18 major HMOs present in human milk and infant fecal samples were obtained by combining high performance anion exchange chromatography - pulsed amperometric detection (HPAEC-PAD) and porous graphitized carbon - liquid chromatography mass spectrometry (PGC-LC-MS). Relative levels of fucosylated and sialylated HMO structural elements were obtained by one-dimensional ^{1}H nuclear magnetic resonance (1D ^{1}H NMR). Application of these three techniques was compared regarding efficiencies and accuracy for the identification of mothers' Lewis/Secretor phenotypes, as well as for monitoring HMO metabolization in the infant gut. Using a pilot sample set from KOALA cohort study, three HMO consumption patterns by infant gut microbiota at 1 month of age were identified. Degree of HMO consumption was also found to be varied depending on specific structural elements.

To validate the findings from KOALA pilot samples, human milk and infant fecal samples from 71 mother-infant pairs registered in the BINGO cohort study, and collected at two, six, and 12 weeks postpartum were analyzed for HMO presence and level as described in **Chapter 3**. Mothers' Lewis/Secretor phenotypes influenced the concentrations of fucosylated and neutral core HMOs and sialyl-lacto-*N*-tetraose c (LST c) in the milk. During the first three months of life, gut microbiota favored utilization of neutral core HMOs the most, followed by sialylated HMOs, and utilized fucosylated HMOs the least. Di-fucosylation of HMOs lead to less microbial degradation compared to mono-fucosylation. Comparable consumption levels of (α1-2)-fucosylated HMOs and (α1-3/4)-fucosylated HMOs were observed. The majority of sialylated HMOs include the (α2-6) linked substituent; however, degradation levels of their (α2-3) isomeric structures were higher at all time points. High variation existed among infants regarding the

time required for gradual transition from low/non-specific HMO consumption pattern, to intermediate/specific pattern, and finally reaching the complete consumption stage. Caesarean section, or early exposure to hospital/clinic associated surroundings were found to delay the gut developmental transition.

Chapter 4 describes the association between HMOs in mother milk and infant fecal microbiota composition at one month postpartum with 121 breastfed infants from KOALA study. Levels of two (α1-2)-fucosylated HMOs, 2′-fucosyllactose (2'FL) and lacto-*N*-fucopentaose I (LNFP I) were associated with infant fecal microbiota composition, besides delivery mode and infant gender. Infant fecal microbial community showed three different microbial clusters, one showed mixed community structure, one being predominated by *Bacteroides* and *Bifidobacterium*, and the third cluster being predominated by *Bifidobacterium*. Furthermore, it was found that several *Bifidobacterium* OTUs played a major role in degrading specific HMOs, followed by the genera *Bacteroides* and *Lactobacillus*.

In **Chapter 5**, maternal HMO profiles, infant fecal HMO profiles and infant fecal microbiota composition from 24 mother-infant pairs from BINGO study at two, six, and 12 weeks postpartum are described. Only weak correlations were found between maternal HMO levels and microbial OTUs in infant fecal samples. Other factors, including mode of delivery, delivery place and infant gender showed stronger influence on shaping infant gut microbiota. In general, the gut microbiota development of the infants in the study showed a gradual progression towards a *Bifidobacterium* dominated pattern; meanwhile, microbial degradation of HMOs increased with age. OTUs within genera *Bifidobacterium, Parabacteroides*, *Escherichia-Shigella, Bacteroides, Actinomyces, Veillonella,* Lachnospiraceae *Incertae Sedis,* and Erysipelotrichaceae *Incertae Sedis* were found to be important taxa of HMO metabolization.

Applying a batchwise *in vitro* fermentation model with human fecal inoculum to study prebiotic fermentation behaviors of isomalto/malto-polysaccharides (IMMPs) is described in **Chapter 6**. Overall, IMMPs with over 90% of α-(1→6) linked glycosyl residues showed a delayed and slow-fermenting behavior. IMMPs were degraded into α-(1→6) linked isomalto-oligosaccharides by extracellular enzymes, followed by absorption of these smaller molecules into the microbial cells and further degradation by cell-associated enzymes. When considerable amounts of α-(1→4) linked glycosidic linkages were present within the IMMPs, fecal microbiota instantly utilized these structures from the start of fermentation. Switching from α-(1→4) linked glucose consumption to α-(1→6) linked glucosyl residues required quite an adaption period of the microbes, which further postponed the α-(1→6) linked glucose associated fermentation. Acetic acid and succinic acid were the main metabolites during IMMP fermentation, besides production of propionic acid, butyric acid and lactic acid. Microbiota composition analysis pointed to an increase in relative abundance of genera *Bifidobacterium* and *Lactobacillus* in the IMMPs fermentation

digesta, especially during degradation of α-(1→6) glycosidic linkages of IMMPs. All observations pointed towards the prebiotic potential of IMMPs, rich in α-(1→6) glycosidic linkages.

In **Chapter 7**, findings from both KOALA and BINGO cohort samples in the current thesis are summarized regarding HMO synthesis during lactation, as well as structure-specific metabolization by infant gut microbiota. The methodological improvement in HMO quantitation, including 3FL, was discussed and outcomes on a first pilot of paired milk – fecal samples were related to data from literature. The infant gut development model incorporating microbiota composition and HMO metabolization pattern was updated from previous studies. Several factors that could influence the infant gut microbiota development and hence HMO metabolization at different developmental stages were described. Furthermore, the *in vitro* fermentation behavior of IMMPs are compared to findings from *in vivo* mice study using the same substrates. Limitations of the current thesis are discussed, and suggestions for future research are provided.

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I'm now standing at the end of this journey, looking back, with mixed feelings in my heart.

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About the author

Curriculum Vitae

Fangjie Gu was born on October 4th, 1989, in Heze, Shandong province, China. In 2007, she received a full scholarship from the Chinese University of Hong Kong to pursue a bachelor's degree there, majoring in Food and Nutritional Sciences. In 2008, she participated in a two-month exchange program to study at the University of California, Berkeley, USA. In 2011, she finalized her bachelor study with a thesis that investigated the influence of calorie intake on breast cancer development using nude mice. In the same year, she moved to the Netherlands, and continued with a master program in Food Technology, specialized in Ingredient Functionality, at Wageningen University. Her master thesis was entitled "characterization of novel pectin acetyl esterase from *Bacillus licheniformis*", under supervision of Dr. Connie Remoroza and Prof. Henk Schols, at the Laboratory of Food Chemistry, Wageningen University. As part of her master program, she did a five-month internship at Dairy Application department of DuPont Nutrition and Health, Brabrand, Denmark. She investigated the correlation between alcohol test results and real-time stability for neutral pH dairy beverages. Right after her graduation in 2013, she started her PhD research at the Laboratory of Food Chemistry, Wageningen University, under supervision of Prof. Henk Schols. The results of her PhD research are presented in this thesis. In 2018 – 2020, next to finalizing her PhD research, she worked as an assistant scientist of Maternal and Infant Nutrition of Yili, based at Yili Innovation Center Europe, Wageningen. In 2020, she moved to Copenhagen, Denmark, and is now employed as a postdoctoral researcher at CP Kelco, Denmark.

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List of Publications

Gu, F.[#]; Borewicz, K.[#]; Richter, B.; van der Zaal, P. H.; Smidt, H.; Buwalda, P. L.; Schols, H. A., *In Vitro* Fermentation Behavior of Isomalto/Malto-Polysaccharides Using Human Fecal Inoculum Indicates Prebiotic Potential. *Mol Nutr Food Res* **2018**, *62* (12), e1800232.

Gu, F.; Ten Kate, G. A.; Arts, I.; Penders, J.; Thijs, C.; Lindner, C.; Nauta, A.; van Leusen, E.; van Leeuwen, S. S.; Schols, H. A., Combining HPAEC-PAD, PGC-LC-MS and 1D ^{1}H NMR to investigate metabolic fates of human milk oligosaccharides in one-month old infants: a pilot study. *(Submitted)*

Gu, F.; Wang, S.; Beijers, R.; de Weerth, C.; Schols. H. A., Structure-specific and individual-dependent metabolization of human milk oligosaccharides in infants: a longitudinal birth cohort study. *(Submitted)*

Borewicz, K. [#]; **Gu, F.** [#]; Saccenti, E.; Arts, I. C. W.; Penders, J.; Thijs, C.; van Leeuwen, S. S.; Lindner, C.; Nauta, A.; van Leusen, E.; Schols, H. A.; Smidt, H., Correlating Infant Faecal Microbiota Composition and Human Milk Oligosaccharide Consumption by Microbiota of One-Month Old Breastfed Infants. *Mol Nutr Food Res* **2019**, *63* (13), e1801214.

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[#] *Shared first authorship*

Overview of completed training activities

Discipline specific activities	
Fibres in food and feed	VLAG, Wageningen, 2013
Symposium Developments in carbohydrate analysis	Eurofins, Amsterdam, 2013
Summer course glycosciences [a]	VLAG, Wageningen, 2014
The intestinal microbiome and diet in human and animal health	VLAG, Wageningen, 2014
Design of experiments	WIAS, Wageningen, 2014
The future of pre and probiotics	WUR, Wageningen, 2015
Advanced food analysis [a]	VLAG, Wageningen, 2015
Origins and benefits of biologically active components in human milk	FASEB, Big Sky, Montana, USA, 2015
CCC Seminar [a,b]	CCC, Groningen, 2014-2017
General courses	
Information literacy including endnote introduction	WGS, Wageningen, 2013
Project and time management	WGS, Wageningen, 2014
Techniques for writing and presenting a scientific paper	WGS, Wageningen, 2014
Scientific writing	WGS, Wageningen, 2014
Presentation skills	WGS, Wageningen, 2014
Optional	
Research proposal preparation	FCH, Wageningen, 2014
PhD study trip 2014 [a,b]	FCH, Germany, Denmark, Sweden and Finland, 2014
PhD study trip 2016 [a,b]	FCH, Japan, 2016
MSc thesis student supervisions, presentations and colloquia	FCH, Wageningen, 2013-2017
PhD presentations	FCH, Wageningen, 2013-2017
Project meetings	CCC, 2013-2017

[a] Poster presentation. [b] oral presentation.
Abbreviations: CCC, Carbohydrate Competence Centre. FASEB, Federation of American Societies for Experimental Biology. FCH, Laboratory of Food Chemistry. VLAG, Graduate School for Advanced Studies in Food Technology, Agrobiotechnology, Nutrition and Health Science. WGS, Wageningen Graduate School. WIAS, Wageningen Institute of Animal Sciences. WUR, Wageningen University and Research.

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