Advanced Characterization of Biofunctional Human Milk Oligosaccharides by Mass Spectrometry and Complementary Methods

Marko Mank

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Geavanceerde Karakterisering van
Biofunctionele Moedermelk Oligosacchariden
met behulp van Massaspectrometrie en Complementaire Methoden

(met een samenvatting in het Nederlands)

Marko Mank

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CHAPTER I

Introduction and Thesis Outline

THE MOLECULAR COMPOSITION OF HUMAN MILK

Human milk (HM) is considered to be the optimal nutrition for the neonate. Therefore, the world health organization (WHO) recommends exclusive breastfeeding at least for the first 6 month post-partum (pp) and continued BF with adequate complementary foods for up to two years and beyond [1]. Next to approximately 87% of water [2] to ensure proper hydration, the necessary building blocks for healthy somatic growth of the nursling are delivered by the macro and micronutrient fractions of human milk which are complex in composition. Apart from the molecular composition of HM, also somatic cells are contained in HM [3]. The caloric density of mature human milk is about 68-70 kcal/dL, [4]. Human milk ingredients originate from three different sources: 1. Synthesis in the lactocyte, 2. actual maternal nutrition/nutritional metabolites and 3. maternal storages. More recently, also the transmission of whole bacteria from the gut into breastmilk has been suggested [5] which explains in part why human milk also contains microorganisms and is not sterile. The macronutrient fraction of mature, term milk mainly consists of lactose (63-78 g/L [6, 7]), lipids (~32-36 g/L) [6], oligosaccharides (~ 5-20 g/L [8]) , and proteins (~9-12 g/L [6]). Furthermore, also endogenous and partly HM-protein derived peptides are present in HM[9, 10]. They may exert important (e.g. anti-microbial) functions [11, 12] but are detected in only low abundances (~ 0,1% of protein concentration)[13].

If breastfeeding is not possible, breast milk substitutes (BMS) [2] become necessary to ensure healthy growth of the infant. Frequently, modern commercially available BMS are based on cow's milk. Therefore, a short comparison of human and bovine milk ingredients (including non-digestible oligosaccharides) is depicted in Table 1. More information about human and bovine milk micronutrients can be found in a publication by Atkinson et al. 1995 [14].

Table 1 | Macronutrients in mature human and bovine milk

Milk Macronutrient Fraction	Human	Bovine
Lactose	~63-78 g/L [6, 7]	~48 [15]
Lipids	~32-36 g/L) [6]	~37 [15]
Proteins	~9-12 g/L [6]	~32 [15]
Oligosaccharides	~5-20 g/L* [8]	~0.05 [16]
Number identified oligosaccharides	> 150[a,b]	~40 [17, 18]
Percentage of fucosylated OS	50-80%[a,b,c]	~1% [17, 18]
Percentage of sialylated OS	10-20%[a,b]	~70% [17, 18]

* for women with positive genetic secretor status even >20g/L

[a] Compilation of data from the following references: Coppa et al., 1999; Kunz et al., 1999; Newburg et al.,2000; Chaturvedi, Warren, Altaye, et al., 2001; Davidson et al., 2004; Bao et al., 2007; Gabrielli et al., 2011, Thurl et al. 2017.

[b] Compilation of data from the following references: Ninonuevo et al., 2006; and Wu et al., 2010; 2011; and reviewed in Kobata, 2010.

[c] Depending on maternal Se/Le genotype

Furthermore, metabolites contained in human milk have been compared on a worldwide basis by Gay et al. 2018 [19]. Other animal milks could also serve as future alternative macronutrient sources for infant milk formula production. A recent side by side comparison of macronutrients has been compiled for human, goat, cow and sheep milk by van Leeuwen et al. in 2020 [20] with a specific focus on goat milk oligosaccharides.

The composition of human milk is relatively conserved across populations but not static. The dynamic adaptation of human milk composition between parturition and the following course of lactation (which may last up to 2 years pp [21]) is believed to be tailored to the changing needs of the infants in this period [22]. To better address and categorize distinct milk qualities resulting from different stages of lactation, colostral milk ~1-5 days post partum (pp)), transitional milk (~ 6-15 days pp) and mature milk (> 15days pp) are generally distinguished.

HUMAN MILK OLIGOSACCHARIDES AND THEIR FUNCTIONAL RELEVANCE

Beyond well-known nutritional properties of HM macronutrients, many individual human milk compounds may exert additional biological functions which may be crucial for the healthy development of the infant [6]. In this context, and although present in much lower levels, peptides [23, 24] and glycoconjugates (e.g. glycoproteins and glycolipids) [25] gain more and more scientific interest [26]. Even more, human milk oligosaccharides (HMOs) seem to be a promising class of human milk constituents as they may confer many intriguing functional properties of which some are likely to facilitate or influence healthy development of neonates [27-31]. The increasing commercial availability of authentic HMO-structures via novel biotechnological production processes [32] facilitated the addition of individual HMOs like 2'-Fucosyllactose (2'-FL) [33, 34] and Lacto-N-neotetraose (LNnT) [35] to infant milk formula (IF). The safety of the former and additional HMOs like e.g. 3'-Sialyllactose (3'-SL) and 6'-Sialyllactose (6'-SL), which are all considered as "novel food" ingredients with respect to infant formulae (IF), has been investigated in several studies [37-39] and approved by various food safety authorities such as the European Food Safety Authority (EFSA) [39-44] or the United States Food and Drug Administration (FDA).

In literature, distinct structure-function relationships have already been attributed to several HMOs and HM glycans, but for the vast majority of HMOs early life functions still need to be explored. The already known beneficial properties of HMOs, which are non-digestible in the upper gastrointestinal tract [45], range from direct anti-infective [46] and immunomodulatory effects to indirect influences on the hosts

health by modulation of the developing infant's microbiome [47-49]. Of note, the HM concentrations of individual HMO-structures may strongly differ (e.g. between 2,74 g/L (2'-FL) [50] and 0,02 g/L (3'-GL [51]), but these particular abundances of specific HMOs might not necessarily predict the magnitude of their functional effect in early life (especially with respect to immunomodulation). Recently, also first evidence for in utero exposure of the developing embryo to HMOs was found [52]. This may further emphasize the probable importance and influence of HMOs for healthy development starting already in utero.

Today, human milk research is increasingly enriched by a constantly growing body of published evidence for the multiple functional properties and potential benefits of HMOs and their associations with early life infant development. A short overview about some of these interesting HMOs functional effects have been compiled in Table 2 below. Furthermore, a complementary extended description of HMOs functional implications for early life was recently summarized by Hundshammer et al. in 2020 [53].

Table 2 | Selected functional effects of HMOs or dietary glycans with possible relevance for healthy early life development

HMO Effect / Underlying Mechanism	Active HMO or Functionally Analogous Glycan(s)	Ref.
Anthropometric effects		
Microbiome mediated stunting by lack of sialylated and fucosylated HMOs	α1-2 fucosylated 2'-FL & LNFP I, and a2-6 sialylated LST b	[54]
Concentration of (LNnT) inversely associated and 2'-FL directly associated with child height and weight z scores;	Type II core LNnT, α1-2 fucosylated 2'-FL	[55]
Anti-infective effects		
HMO as decoy receptors for bacterial and viral pathogens (e.g. *C. jejunii*, *V. colera*, *E.coli* (heat stable enterotoxin*), H. pylori*, *H. influenza*, *S. pneumonia*, Norwalk virus, avian influenza viruses etc.)	Various neutral and acidic HMOs	[56-58]
Increased protection of infants against diarrhoea (in vivo)	α1-2 fucosylated HMOs	[59]
Higher HMO-levels associated with protection against respiratory and gastrointestinal infections at 6 and 12 weeks pp but not 24 weeks pp	α1-4 fucosylated LNFP II as surrogate marker for total HMOs levels	[60]
Inhibition of norovirus-like-particle binding to histo blood group antigen surrogates (*in vitro)*	2'-FL and 3-FL	[61]
Concentration dependent influence on risk for HIV transmission mother to child	3-'SL	[62-63]
Reduction of HIV envelope glycoprotein gp120 binding to DC-SIGN receptors on dendritic cells	Total HMOs and (multivalent) Le^x structures	[64]
Prevention / attenuation of necrotizing enterocolitis (NEC) in rodents	2'-FL, sialylated galacto-oligosaccharides (siaGOS), DSLNT	[65], [66]
Reduction of *entamoeba histolytica* attachment and cytotoxicity (*in vitro*)	LNT and galactooligosaccharides (GOS)	[67]
In vitro protection of epithelial bladder cells against uropathogenic *E. Coli* invasion by total HMOs or GOS, cytotoxic and proinflammatory effects by total (sialylated) HMOs	(Sialylated) total HMOs and GOS	[68]
HMOs as prebiotic adjuvants mitigate Group B *Streptococcus* antibiotic resistance via perturbation of bacterial membrane metabolic pathways	Lactose and monosaccharide depleted total HMO fraction	[69]
Effects on cognition & brain		
Improved memory / learning (rats *in vivo*)	2'-FL	[70]
Infant cognitive development at 1 month influenced by increased 2'-FL exposure	2'-FL	[71]
Increased brain ganglioside concentrations and modulation of microbiota (*piglets in vivo*)	3'-SL, 6'-SL and other sialylated HMOs	[72], [73]
Adjustment of cognitive development by short-term upregulation of genes modulating neuronal patterning in the prefrontal cortex (mice in vivo)	6'-SL	[74]

Table 2 | Continued.

HMO Effect / Underlying Mechanism	Active HMO or Functionally Analogous Glycan(s)	Ref.
Immunomodulatory effects		
Association of LNFP III levels with reduced risk to develop cow's milk allergy in at risk population	LNPF III	[75]
Differential modulation of Toll-Like Receptor-mediated Inflammation by various HMOs and HM compounds	HMOs and HM compounds including proinflammatory (multivalent?) LNFP III, 3'-SL and anti-inflammatory 2'-FL, 3'-GL and Lactoferrin	[49]
Down-regulation of Th-1 Tcells by induction of B220+ cell interleukin 10 production via schistosome-infection (mice *in vivo*)	Multivalent LNFP III	[76]
Alternative activation of murine macrophages (possibly via C-type lectins) promotes Th2-response (mice *in vivo*)	Multivalent LNFP III-dextran conjugate	[77]
Various HMO as DC-SIGN receptor ligands and possible immunomodulatory effects in developing infants	α-Fucosylated HMOs (e.g. 2'-FL, 3-FL, LNFPs etc.)	[78]
Reduction of selectin-mediated human peripheral blood cell interactions and possible anti-inflammatory effect	Acidic fraction of total HMOs	[79]
Attenuation of mucosal responses to inflammatory stimuli and probable support of intestinal mucosal immune system maturation (*ex vivo*)	Colostral HMOs (cHMOs) including Galactosyllactose 3'-GL	[80]
Selective binding of recombinant human galectins ((hGal)-1, -3, -4, -7, -8 and -9) to individual human milk glycans (HMG) suggests possible role of HMGs in infant immunity. Exception: hGal2 (no binding to HMGs to human blood group A Type 2 determinants on other microarrays)	Diverse HMG-motifs; hGal-7 prefers terminal Type 1 residues (Galβ1-3 GlcNAc)	[81]
HMOs as regulators of host physiology and immune responses: Human DC-SIGN and Siglecs bind specific human milk oligo-saccharides with α-Linked fucose residue	2'-FL, 3-FL and higher order mono- and polyvalent α-Fucosylated HMOs (DC-SIGN); 3'-SL, 6'-SL and other motifs (Siglecs)	[82]
In vitro modulation of Toll-like receptor (TLR) signaling (activation or inhibition)	Distinct individual or synergistic effects of 2'-FL, 3-FL, 6'-SL and LNT2 on individual TLRs 2, 5 and 7. Potent effects on all investigated TLRs by LNT2	[83]
Modulation of rotavirus-associated dysbiosis and TLR gene expression in neonatal rats	2'-FL, scGOS/lcFOS 9:1 and combination of both	[84]

Table 2 | Continued.

HMO Effect / Underlying Mechanism	Active HMO or Functionally Analogous Glycan(s)	Ref.
Non digestible oligosaccharides are direct ligands for TLR4 (*in vitro* assessment with intestinal epithelial cell assays (IEC18, HT29, and Caco-2 cells))	Fructooligosaccharides (FOS), Galactooligosaccharides (GOS), goat milk oligosaccharides	[85]
In vitro promotion of immune regulation / tolerance via interactions of HMOs with human dendritic cells (DCs). "Regulatory effects of HMOs seemed to be mediated by interactions with receptors including but not limited to TLR4 and DC-SIGN on human moDCs."	Lactose depleted total HMOs fraction	[86]
In vitro maturation of cord blood mononuclear cells (CBMC) by modulation of cytokine production; reduction of peanut allergen-specific Th-2 type response and direction of neonatal Tcell phenotype towards balanced Th1 / Th2 profile	Effective: Total acidic HMO fraction; Ineffective: Total acidic bovine milk oligosaccharide (BMO) fraction, scGOS/lcFOS, pectin derived acidic oligosaccharides (pAOS)	[87]
Prebiotic & gastro-intestinal effects (incl. modulation of gut maturation & microbiome)		
Selective and preferred utilization of (HM)Os by microorganisms conferring health benefits to the host	HMOs (total fraction + individual structures) & dietary OS (e.g. short chain galactooligo-saccharides/long chain fructooligo-saccharides scGOS/lcFOS))	[88-95]
Lacto-N-biosidase (of *B. bifidum*) enables degradation / utilization of (specific) HMOs structures	Type I HMOs	[96]
HMO specific utilization mechanisms of bifidobacterial commensals	Various HMOs	[97, 98]
Predominance of Type I HMOs in human breast milk versus other mammalian milks and their importance for beneficial bifidobacteria	Type I HMOs	[99]
Modification of intestinal epithelial cell glycosylation (in vitro)	3'-SL	[100]
Differential impact of 3'-SL vs. 6'-SL (or vs. mixture of 3'-SL + 6'-SL) on early life intestinal bacterial colonization and susceptibility to DSS induced colitis in adulthood (mice *in vivo*)	3'-SL and 6'-SL	[101]
Reduction of colon motor contractions in mouse distal colon segments (*ex vivo*)	3-FL and 2'-FL (but not 3'-SL, 6'-SL nor GOS or LNT)	[102]
Modulation of infants' fecal microbiome by distinct HMO-profiles and association of HMOs with specific bacterial genera	Distinct HMOs including e.g. 2'-FL, DFL, LNFP I and DSLNT	[103]

Table 2 | Continued.

HMO Effect / Underlying Mechanism	Active HMO or Functionally Analogous Glycan(s)	Ref.
Impact of human milk groups on human microbiota diversity; higher frequency of *B. dolescentis* and absence of *B. catenulatum* in HM group IV	Abundances of Human milk group- / Se-/ Le-dependent HMOs	[104]
Differential impact of diverse HMOs on microbiome of Chinese infants; levels of fucosylated HMOs & milk N-glycans highly correlated with growth of *Bifidobacterium spp*. and *Lactobacillus spp.*	Se-gene dependent HMOs and N-glycans; changes of HMOs abundances during different stages of lactation	[105]
Maternal Secretor (Se) status affects gut *bifidobacterial* communities of breastfed infants: Better growth of Se^+ human milk fed fecal isolates on 2'-FL	Se-genotype dependent HMOs, 2'-FL	[106]
Human salivary MUC5B glycoprotein o-glycosylation reflects maternal Se- & ABO status	O-glycans structurally similar to Se-genotype dependent HMOs	[107]

OBJECTIVES IN THE PRESENTED WORK AND OUTLINE OF THIS THESIS

HMOs represent a promising class of HM compounds with many beneficial, biofunctional features and the potential to support healthy early life development of infants. Nevertheless, HMOs are still relatively understudied e.g. with respect to establishment of more clear structure-effect relationships and more detailed mechanistic explanations of their functional benefits or features.

This is partly due to the limitations in existing analytical approaches. The analytical challenge which did prevent a more detailed qualitative and quantitative characterization of HMOs so far, can partly be attributed to the huge structural complexity and variety exhibited by these HM glycans. HMOs quantities and patterns may vary for multiple reasons between individual donors and over time during the different stages of lactation. As explained in more detail in the following chapters, HMOs variations can be influenced by e.g. the maternal genetic predisposition but also environmental, nutritional or other early life factors. Furthermore, neither the individual contributions of particular HMOs structures nor the combined effects of the total HMOs fraction on the developing immunity of the breast fed infant, its microbiome or other important developmental aspects impacting infant's health are completely deciphered.

For the reasons described above, this thesis was designed to increase the knowledge about the complexity of the HMOs fraction in HM including its qualitative and quantitative variation under changing early life (EL) conditions.

Consequently, (semi) quantitative changes of individual HMO-structures (up to hexaoses) over defined stages of lactation up to 12 month post-partum and between different existing human milk groups should be determined.

Here, the in-depth characterisation of individual HMOs in various individual HM specimens with novel analytical approaches should be a key element to elicit the desired insights. It was further intended to characterize also low abundant HMOs and also closely related isomeric HMOs like e.g. Lacto-N-fucopentaose II (LNFP II) and Lacto-N-fucopentaose III (LNFP III) or Lacto-N-Tetraose (LNT) and Lacto-N-neotetraose (LNnT). However, the latter intension posed an analytical challenge as most contemporary analytical methods reported so far could distinguish such highly similar HMOs structures only incompletely.

Hence, a focus of this work was the development of powerful, sensitive, and highly selective analytical methods building on liquid chromatography coupled to mass spectrometry (LC-MS) and on capillary gel electrophoresis coupled to laser induced

fluorescence detection (xCGE-LIF). xCGE-LIF should also open access to a high throughput screening capability. The improved analytical method portfolio outlined above should finally allow for correct identification and quantitation of even isobaric HMO-isomers in HM specimens and in vitro models addressing HMO functional aspects. To ensure profound and statistically sound biological conclusions, the developed LC-MS and xCGE-LIF methods should only be applied to HM specimens derived from well controlled clinical studies or (large) birth cohort studies with dedicated HM sampling.

Another goal of this thesis was to review the current status in knowledge about impact of HMOs on early life immunological development as well as resilience against EL threats. To extend the mechanistic understanding about prebiotic HMO-effects, the desired novel LC-MS approach should finally be employed to monitor differential in vitro utilization of individual HMOs in bacterial mono or co-cultures. These in vitro fermentation experiments should also serve as early life model-systems to explore cross feeding between *Bifidobacterium infantis*, a prototypic HMOs consuming strain of the EL microbiome, and the butyrate producer *Anaerostipes caccae*.

The scientific objectives outlined above were addressed by the individual chapters of this thesis as listed below:

- In **CHAPTER II** recent in vitro and in vivo findings about the impact of individual HMOs or the total HMO-fraction on early life immune development and susceptibility to neonatal and childhood infections were reviewed. This chapter was entitled "Diversity of Human Milk Oligosaccharides and Effects on Early Life Immune Development".

- **CHAPTER III** describes the development of a novel and advanced analytical approach for "Label-Free Targeted LC-ESI-MS^2 Analysis of Human Milk Oligosaccharides (HMOs) and Related Human Milk Groups with Enhanced Structural Selectivity". This new analytical tool was designed to enable characterization of relevant (isomeric) HMOs up to hexaoses in *e.g.* real-life HM study samples or microbial mono and co-cultures as demonstrated in CHAPTERS 4-6.

- **CHAPTER IV** entails how application of the advanced LC-ESI-MS^2 methodology described in CHAPTER III reveals differences of HMO-patterns between milk groups I-IV and between different stages of lactation. Here, human milk samples from the INFAT clinical study were investigated. The title of this chapter is "Targeted LC-ESI-MS^2-Characterization of Human Milk Oligosaccharide Diversity at 6 to 16 Weeks post-partum Reveals Clear Staging Effects and Distinctive Milk Groups".

- The same LC-ESI-MS^2 method was also employed in **CHAPTER V** for differential HMO-analyses in probiotic bacterial mono and co-cultures. The goal of this

study was to evaluate the prebiotic potential of the total HMOs fraction and specific individual HMOs thereof in in vitro model systems for cross feeding of the developing gut microbiome. The headline of the publication which resembles this chapter was "Cross-Feeding Between *Bifidobacterium infantis* and *Anaerostipes caccae* on Lactose and Human Milk Oligosaccharides".

- "Human milk oligosaccharide profiles over 12 months of lactation: The Ulm SPATZ Health Study" is the title of **CHAPTER VI**. This chapter highlights the first application of an even further improved, fully validated targeted isomer specific LC-ESI-MS^2 method for absolute quantitation of HMOs. It was deployed to more than 1200 HM study samples. These HM samples were collected in Germany at 6 weeks, 6 months and 12 months post-partum, respectively. By taking advantage of the described hyphenated analytical approach and the size of the chosen HM cohort, we attempted to reveal new insights about variety and longitudinal concentration changes of HMOs between different stages of lactation and human milk groups.

- **CHAPTER VII** delineates a novel high throughput (HTP) methodology for efficient semi-quantitative HMOs characterization. The related publication was entitled "Development of a High-Throughput Glycoanalysis Method for the Characterization of Oligosaccharides in Human Milk Utilizing Multiplexed Capillary Gel Electrophoresis with Laser-Induced Fluorescence Detection (xCGE-LIF)". The purpose behind the development of this new methodology was to facilitate efficient HMO-analyses for modern HM cohorts with high sample numbers (see e.g. CHAPTER VIII) in a sensibly (short) time frame.

- The HTP characterization of more than 700 individual HM specimens yielded from 371 donors enrolled in the PreventCD study by xCGE-LIF could be exemplified in **CHAPTER VIII**. In contrast to other HM studies, the Prevent CD study featured HM specimens collected in monthly frequency between 1-12 month post-partum. This study design together with the high sensitivity of xCGE-LIF should allow precise monitoring of monthly HMO-level-changes including high abundant HMOs like 2'-FL but also low abundant structures like beta 3'-Galactosyllactose.

- **CHAPTER IX** recapitulates the main findings from **CHAPPTERS II-VIII** and draws overall conclusions

Finally, **CHAPTER X** summarizes the main content of the previous **CHAPTERS II-IX**. This summary was translated into Dutch in **CHAPTER XI** ("Nederlandse Samenvatting") and into German in **CHAPTER XII**.

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CHAPTER II

Diversity of Human Milk Oligosaccharides and Effects on Early Life Immune Development

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ABSTRACT

One of the well-known features of human milk, is the capacity to protect against the risk and impact of neonatal infections, as well as to influence the onset of allergic and metabolic disease manifestations. The major objective of this review is to provide a detailed overview regarding the role of human milk, more specifically the diversity in human milk oligosaccharides (HMOs), on early life immune development. Novel insights in immune modulatory effects of HMOs obtained by *in vitro* as well as *in vivo* studies, adds to the understanding on how early life nutrition may impact immune development. Extensive description and analysis of single HMOs contributing to the diversity within the composition provided during breastfeeding will be discussed with specific emphasis on immune development and the susceptibility to neonatal and childhood infections.

Keywords

Human milk oligosaccharides, mucosal immunity, tolerogenic dendritic cells, infections, early life nutrition

THE PROTECTIVE EFFECT OF BREASTFEEDING AGAINST INFECTIONS

It has been long noted that breastfeeding protects newborns against infections. Infant formula has been developed over many decades into adequate nutrition for those infants who cannot receive human milk. However, even modern infant formulas lack many components tailor made by each mother for the immune imprinting of her baby, such as specific antibodies (based on the immunologic history of the mother) and human milk oligosaccharides (HMOs) (based on the mother's specific genetic makeup regarding e.g., Lewis (Le) blood group and secretor (Se) status). Exclusive breastfeeding until the age of 4 months followed by partial breastfeeding is associated with a reduction in respiratory and gastrointestinal infectious diseases (1). For example, infants admitted to the hospital with Respiratory Syncytial Virus infection (RSV) are less likely to have been breastfed (2, 3). Similarly, infants who are not exclusively breastfed at 6–8 weeks of age, have a higher risk of hospitalization in early life in relation to a wide range of common infections (2). On the contrary, specific prebiotic oligosaccharides [like short chain galacto- and long chain fructo-oligosaccharides (scGOS/lcFOS)] are already added in a 9:1 ratio to plain infant formula and have been shown to reduce the development of atopic eczema and allergies as well as reduce the impact of pediatric infections (4, 5). Therefore, further optimization of infant nutrition when breastfeeding is unavailable is a principal factor required to further support immune development in early life. The protective effect of human milk is postulated to be achieved by several mechanisms including the provision of pathogen specific maternal antibodies. This will provide the infant with pathogen specific protection during the first months of life, in which infant's own B cell development has not reached its full potential. Specifically, within the first years of life, the B cell repertoire matures upon encounter of pathogens, eventually providing a full range of protection against the recurrent pathogens. The immunoglobulins in human milk possess a broad range of pathogen specificity, which mirrors the maternal antigenic state. In addition, the concentration of soluble IgA (sIgA) are remarkably high and variable, and correlate to levels of IgG and IgM detected within different regions (6). At birth (for example via vaginal delivery as well as during breastfeeding) the neonate encounters a large variety of microorganisms which are determinant in the establishment of the microbiome in adult-life (7, 8). The initial colonization follows successive steps and is altered through the first year of life by diverse factors including genetic as well as environmental factors such as, introduction of oral feeding. Human milk was shown to stimulate healthy intestinal microbial diversity which includes colonization of several Bifidobacteria and Lactobaccillus species, which in turn, will result in the development of a balanced metabolic response (8, 9). In addition, human milk provides direct support to further development of the immune system in the neonate (10, 11).

The immune system of the neonates needs to adapt and respond to diverse stimuli encountered in early life. Immune homeostasis is determined by the cross-talk

between exposure to the mucosal surfaces encompassing cross talk between epithelial cells and underlying immune cells (12). Human milk contains diverse factors like HMOs, milk epidermal growth factor or vitamin A, which contribute to the development of the neonatal mucosa and thus, to the promotion of the neonatal immune system by counterbalancing the deficiencies in early life. These variations are designated as poor IgA production, defective antimicrobial peptide secretion, lack of epithelial chemokine secretion as well as increased permeability among others (6, 10, 13).

Beyond functional components in human milk also the intestinal microbiota can help to further develop these aspects of the mucosal immune system (2). This emphasizes the relevance of a healthy intestinal microbiome diversity for adequate immune development in the first years of life (9). Next to the various classes of pathogen specific immunoglobulins in human milk, the presence of antimicrobial molecules, including specific non-digestible free carbohydrate structures and other molecules like glycoconjugates in breast milk have been shown to bind to pathogens (14). The nutrient source in early life, in particular the non-digestible human milk oligosaccharides (HMOs) in case of breastfeeding are of importance for healthy neonatal microbial colonization (7, 8, 12), immune development (15–19), as well as B cell development (20). This review aims to reveal the current state of knowledge regarding the immunomodulatory properties of HMOs and its unique complexity with differences in short chain as well as long chain structures. Recently some of these structures have become available via manufacturing procedures, and it might be considered to apply these in future generations of infant milk formula (21).

DIVERSITY OF HUMAN MILK OLIGOSACCHARIDE COMPOSITION

HMOs are the third most abundant class of biomolecules found in mature human milk (21,22) after lactose and lipids, reaching between 5 and 20 g/L in mature human milk (22). Up to 1% of HMOs are absorbed in the gastrointestinal tract and found available in systemic circulation (23). This diversity and abundance is unique in humans and not seen in other mammals (24). The concentration of total HMOs is subjected to variations dependent on lactational stage (25), maternal nutrition (26), genetic predisposition (27) or even geographic localization and socioeconomic environment of milk donors (28). Although HMOs are composed out of only 5 different monosaccharides, the structural complexity of HMOs encountered in human milk is unique (21). The monosaccharides which are used as building blocks for HMOs are glucose (Glc), galactose (Gal), N-Acetyl-Glucosamine (GlcNAc), fucose (Fuc), and sialic acid (Neu5Ac). These single monosaccharides are conjugated via several linkage types (i.e., glycosidic bonds). With only a few exceptions, HMOs structures do follow a strict building plan (**Figure 1**).

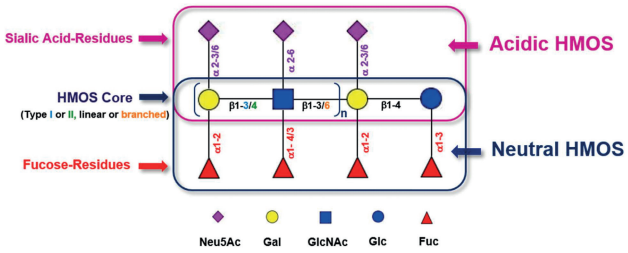

Figure 1 | Generic building scheme of HMOs. Lactose and Type I (Gal (1-3)GlcNAc-R) or Type II (Gal (1-4)GlcNAc-R) HMOs core structures can be further extended linearly by adding additional Gal-GlcNAc building blocks to terminal Galactoses via 1-3 glycosidic linkages or via 1-6 glycosidic linkages. In the latter case, branching of the HMOs structure occurs. The (elongated) HMOs core structures can be further decorated with Fucoses (Fuc) and/or Sialic Acid (Neu5Ac) residues following distinct rules. Symbolic representation of monosaccharides according to CFG guidelines (29).

Each HMOs structure starts with a lactose unit "Gal (1-4) Glc" which results from formation of a 1-4 glycosidic linkage between galactose and glucose catalyzed by the lactose synthase protein complex (30). Several tri-saccharides can be synthesized by appending either galactose or fucose to the reducing or non-reducing end of the lactose residue, which is performed through galactosyl- or fucosyl-transferase activity. Resulting components are e.g., 3'-galactosyllactose (Gal(1-3)Gal(1-4)Glc), 4'-galactosyllactose (Gal(1-4)Gal(1-4)Glc), 6'-galactosyllactose (Gal(1-6)Gal(1-4)Glc), 2'-fucosyllactose (2'-FL, (Fuc(1-2)Gal(1-4)Glc), and 3-fucosyllactose (3-FL) (Gal(1-4)[Fuc1-3]Glc). If sialic acids are connected to the non-reducing end of lactose via Sialyltransferases, 3'-sialyllactose (3'-SL; Neu5Ac (2-3)Gal(1-4)Glc) and 6'-sialyllactose (6'-SL) (Neu5Ac(2-6)Gal(1-4)Glc) are formed. Further elongation of lactose via the free 3-OH group of galactose can occur by addition of Gal (1-x)GlcNAc units of either type I (Gal (1-3)GlcNAc, Lacto-N-biose) or type II (Gal (1-4)GlcNAc, N-Acetyllactosamine). Up to now, 19 different human milk oligosaccharide core structures have been described. These core structures may be linear or branched and can be further decorated with fucoses or sialic acid residues. This indicates that a myriad of different HMOs structures may be produced in the human mammary gland. The cellular localization of HMO-synthesis in the mammary gland epithelium is believed to be the Golgi apparatus. Among other early life factors, the individual maternal genetic disposition has a huge influence on the - profile of human milk. More specifically, the individual expression pattern of Lewis (Le) and Secretor (Se) gene alleles codes for different fucosyl-transferases (FUTs), as shown in **Table 1**. The activity of these FUTs can lead to fucosylation of lactose and various other human milk core structures as indicated.

An active Se gen codes for FUT2 which transfers fucose via an α 1-2 glycosidic linkage. Prominent HMOs resulting from FUT2 activity are e.g., 2'-FL and Lacto-N-fucopentaose I (LNFP I). Glycans like LNFP I which are carrying the reducing terminus Fuc (α1-2)Gal (1-3)GlcNAc belong to the group of Le^d or H type 1 antigens. H type

antigens link the Le/Se system with the blood group ABH system (31). In contrast, an active Le gene codes for FUT3 which in turn enables fucosylation via either α1-3 or α1-4 glycosidic linkage. FUT3 related structures are e.g., Lacto-N-fucopentaose II (LNFP II), and Lacto-N-fucopentaose III (LNFP III), LNFP III is also an example for an Lewis^{x} (Le^{x}) structural motif, whereas LNFP II represents a Lewis^{a} (Le^{a}) epitope. Le^{a} epitopes are characterized by the carbohydrate sequence Gal (1-3)[Fuc (1-4)] GlcNAc-R. Le^{x}-antigens contain type II structures with the following residue: Gal (1-4)[Fuc (1-3)] GlcNAc-R. If both, Se and Le genes are active, fucosylated HMO-structures bearing either one, two or all the possible types of fucosylation (i.e., via 1-2, 1-3, and 1-4 glycosidic linkages) can occur. Lacto-N-difucohexaose I (LNDFH I) which also resembles a Lewis^{b} epitope with the monosaccharide motif Fuc (α1-2)Gal (1-3)[Fuc (1-4)]GlcNAc-R, is a known metabolite of joined FUT2 and FUT3 activity. It is noteworthy to mention that also other, Le/Se-system independent fucosyl-transferases may contribute to formation of 1-3-fucosylated HMOs such as 3'-FL or Lacto-N-Fucopentaose V (LNFP V).

The complexity and relative abundance of different HMOs contained in human milk can for instance be characterized by size exclusion chromatography (SEC) and coupled refractive index detection (RI). A resulting SEC-RI trace is shown in **Figure 2**. Even more detailed information about complexity and individual monosaccharide compositions of HMOs could be derived by a subsequent MALDI-MS (33) analysis of individual SEC HMO-fractions. The acidic sub-fraction adds a further dimension to the overall variety of HMOs. The total number of neutral and acidic HMO-structures based on the MALDI-MS analyses of total human milk carbohydrate SEC-fractions is estimated to exceed the number of 1,000 different structures (24).

Based on the Le/Se status of the mother and specifically the related fucosylated HMO-structures found in the respective human milks, milk group systems of 4 different milk types have been defined (27). Therefore, it is possible to determine individual human milk types by probing presence of specific fucosylated HMOs like 2'-FL, DFL, LNFP I, LNFP II, LNDFH I, and LNDFH II with suited analytical means. An overview of the relationship between maternal Le and Se genotype and some major HMO-structures present in the respective milk types is given in **Table 1**. A recent review has summarized most of the qualitative and quantitative approaches to characterize the diversity of HMO-structures present within human milk (21).

BIOLOGICAL FUNCTIONS OF THE DIFFERENT HMOS

The presence of the unique diversity of HMOs, suggests different biological functions and mechanisms by which they may influence the infant's microbiome and immune maturation and their susceptibility to infections as summarized below and shown

in **Figure 3**. The topics exemplified in **Figure 3** are further substantiated point by point in the following section.

Table 1 | Relationship between maternal genotype and exemplified Le- or Se- related major HMOs expected to be present in milks of respective milk types.

Maternal genotype		Frequency in France / Europe (31)	Prominent HMOs expected in milk group	Milk group
Secretor	Lewis			
Se/–	Le/–	69	2'-FL, 3-FL, DFL, LNT, LNnT, LNFP I, LNFP II, LNFP III LNDFH I, LNDFHII, 3'-SL, 6'-SL	Type I
se/se	Le/–	20	3-FL, LNT, LNnT, LNFP II, LNFP III, LNDFH II, 3'-SL, 6'-SL	Type II
Se/–	le/le	9	2'-FL, 3-FL, DFL, LNT, LNnT, LNFP I, LNFP III, 3'-SL, 6'-SL	Type III
se/se	le/le	1	3-FL, LNT, LNnT, LNFP III, 3'-SL, 6'-SL	Type IV

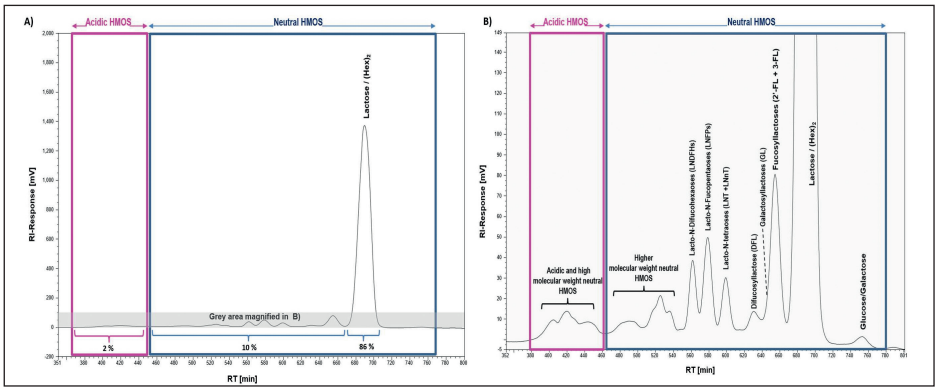

Figure 2 | SEC-RI profiles of the total HMOs and mineral fraction from pooled human milk; (A) Full SEC-RI Profile, 86% of compounds detected by RI consist of Lactose/(Hex)2, 10% of other neutral HMOs and 2% of acidic HMOs; (B) Magnified section of (A) zooming into acidic and neutral HMOs; HM sampling, pooling, isolation of the total HMOs fraction and SEC-RI analysis have been performed as described earlier (32).

ANTIMICROBIAL AND ANTIVIRAL EFFECTS OF HMOS

HMOs play a role in the prevention of infections in breastfed infants by direct blockage of viral and bacterial cellular pathogens and toxins infection by mimicking cell entry receptors (34– 36). The first mechanisms by which HMOs may exert their anti-infective properties are through the inhibition of virus binding to the host cells by mimicking viral receptors and/or by blocking virus entry into the cell, as well as

intracellularly, by blocking viral replication. The anti-infective potential of HMOs has been demonstrated for both neutral as well as acidic HMOs, for example different strains of Norovirus have affinity for specific HMO-structures (35, 37). In addition, both sialylated and fucosylated milk oligosaccharides reduced the infectivity of rotavirus (38). Interestingly, HMOs with multiple Le^x epitopes were shown to inhibit HIV-1 transfer to CD4+ T lymphocytes more efficiently than other HMO-structures (39).

HMOs may also block microbial pathogen entry, since HMOs from pooled human milk were shown to significantly reduce *Escherichia coli* attachment to cultured epithelial cells (40). Likewise, it has been shown *in vitro* that LNT, or its fucosylated derivative LNFPI, both can inhibit the growth of Group B Streptococci (41). Moreover, the presence of 3'-FL within the complex mixture of HMO-structures has been inversely correlated with Group-B Streptococci abundancy in infants (42). In addition, α(1-2)-fucosylated HMOs like 2'-FL, or LNDFH I may reduce of early life diarrhea incidence and severity, via their ability to block specific diarrhea inducing pathogens (43).

PREBIOTIC EFFECT OF HMOS

Development of selective bacterial strains is subjected to their capacity to metabolize HMOs (44). The role of microbial modulation i.e., the prebiotic capacity of specific HMO-structures have in addition been subject of extensive studies. More specifically, secretor positivity of mothers, hence expressing FUT2 and therefore able to produce α(1-2)-glycosidic-fucosylated HMOs, have been shown to affect the gut bifidobacterial communities of breastfed infants (45). Bifidobacteria and Bacteroides species are known to metabolize HMOs with high efficiency in contrast to other bacterial species such as *E. coli, Clostridia, Eubacteria, Enterococci* (44). This appears strain specific and selective for specific HMO-structure (44, 46, 47). For example, *Bifidobacteria* exhibited strong growth stimulation while expansion of *Clostridium perfringens* and *E. coli* were suppressed within cultures using specific HMOs (like 2'-FL, 3-FL, and LDFT), whereas Enterobacteria could not grow on 2'-FL or 6'-SL cultures (48). In addition, utilization of fucosylated type human milk oligosaccharides by isolated human gut microbes was shown (49). These data indicate selective and specific prebiotic capacities of different functional HMO-structures, showing growth of commensal bacteria such as Bifidobacteria at the expense of pathogens, as shown in **Figure 3**. Hence beyond directly blocking viral and bacterial entrance to the host also these prebiotic capacities of HMOs may help to reduce the susceptibility to infection of the host.

MUCOSAL BARRIER MATURATION BY HMOS

HMOs interact with glycans present in the surface of intestinal epithelial cells (IEC) or with dendritic cells (DC) which protrude to the gut lumen from lamina propria. This results in direct support of epithelial barrier maturation or an indirect effect on barrier integrity via modulation of the microbiota and consequent short chain fatty acid (SCFA) production (50). In this regard, beyond blocking pathogen invasion, HMOs may also promote mucosal barrier maturation by increasing the differentiation of IECs. Indeed, synthetic HMOs or HMOs isolated from human milk were shown to promote differentiation and reduce proliferation of various IEC cultures (HT-29 and Caco-2). Similarly, expression of mucosal maturation factors was promoted in fetal intestine cultures after exposure to HMOs isolated from colostrum. These findings suggest that some specific HMOs may be able to promote gut maturation and contribute to epithelial barrier integrity in the gastrointestinal tract of neonates (18, 50, 51).

MODULATION OF PATHOGEN RECOGNITION BY HMOS

Receptors involved in the recognition of microbes such as toll-like receptors (TLR) are suggested to be modulated by HMOs. Subsequently the response of the host cell to pathogens is altered (17, 37). *In vitro* studies to elucidate the receptors involved in HMO-effects have been performed mostly in cells isolated from adult individuals which might not translate directly to the neonatal situation. Specific HMO-structures have been postulated to modulate bacterial and viral signaling on epithelial cells and/or DC (19). For instance, 2'-FL modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation *in vitro* (17). On the contrary, HMOs such as sialyllactoses, human galactosyllactoses and/or LNFP III may be ligands for toll like receptors (TLR). For example, TLR-3 signaling seems specifically inhibited by human milk 3'-galactosylactose (52). Moreover, it has been shown that the addition of human milk as well as HMOs interacts directly with DCs, through DC-SIGN, Siglecs and related glycan-binding proteins which are also essential in immune regulation (53–55). DCs are key in directing the adaptive immune response toward effective immunity identification and clearance pathogens. Alpha-fucosylated HMOs (2'-FL and 3-FL) showed specific binding to DC-SIGN (54). Effects of scGOS/lcFOS were suggested to be mediated by TLR-4 (56).

Similarly, TLR-4 as well as TLR-3 have also been related to modulate the effects of HMOs. 3'-GL was able to modulate TLR-3 and elicit an anti-inflammatory effect, while exposure to 2'-FL inhibited inflammation through TLR-4 (52). More specifically it has clearly been shown that the addition of scGOS/lcFOS ameliorates the microbial composition reducing the presence of clinically relevant pathogens (57). Selectins were also suggested as possible receptors for binding of HMOs due to their ability to block P-selectin (58). Several receptors are hypothesized to be involved in the

recognition of HMOs. The diversity of HMO-structures present in human milk might determine HMO-glycan receptor binding. HMOs target TLRs and C-type lectins which are vital in pathogen recognition, immune modulation and essential during development of the immune system in early life. Therefore, HMOs may contribute to the development of a balanced and effective immune response, hereby providing protection toward infections.

Diversity in structures and function of HMOs

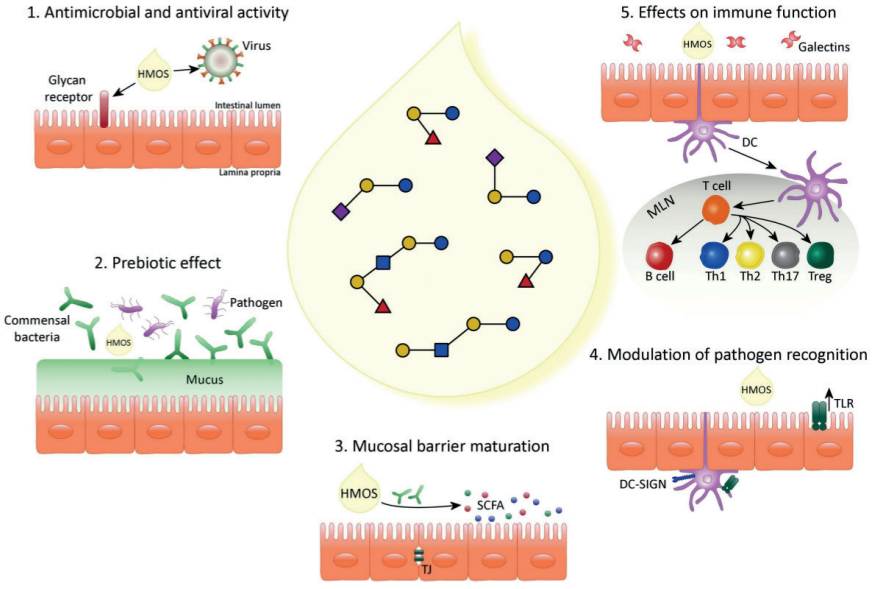

Figure 3 | Schematic overview indicating the diversity in structure and function of HMOs. HMOs are composed of a complex mixture of oligosaccharides. This diversity of structures results in various roles in the epithelial cell layer, surrounding mucosa and immune system composing the digestive tract of breastfed infants. (1) HMOs have shown antimicrobial and antiviral effects by binding to virus bacteria, toxins and/or eukaryotes reaching the mucosal surfaces as well as by direct binding to epithelial surface receptors and blocking the access of pathogens. Thus, avoiding their replication and subsequent infection. (2) Commensal bacteria, illustrated as Bifidobacteria, metabolize HMOs and thus, their growth is promoted while pathogens less able to metabolize HMOs will experience growth suppression. (3) HMOs and Short Chain Fatty Acid (SCFA), metabolites of HMOs, were shown to influence intestinal epithelial cell (IEC) maturation by promoting differentiation while suppressing proliferation as well as tight junction development, required for proper intestinal barrier function. (4) Expression of receptors involved in pathogen recognition, such as TLR as well as their signal transduction was increased after HMOs exposure which in turn impacts the immune homeostasis. (5) DC in close proximity to the intestinal epithelial barrier are involved in the immunomodulatory effects described for HMOs. DCs exposed to HMOs play a role in the DC/T cell interaction leading to T cell differentiation and/or T cell/B cell interaction which may occur in secondary lymphoid organs, depicted as mesenteric lymph nodes (MLN), subsequently promoting immune homeostasis.

EFFECT ON IMMUNE SYSTEM DEVELOPMENT BY HMOS

Specific HMOs, such as 2'-FL, 3'-SL, 6'-SL, and LNT have been detected within the intestine as well as in systemic circulation of breastfed infants (23, 59, 60). Increasing evidence collected during the past two decades suggesting a role of HMOs directly on immune cells. Despite all efforts, the effects described remain rather incomplete (19). Nevertheless, it is suggested that HMOs play a role in supporting the developing mucosal and systemic immune system (13, 16). HMOs derived from human colostrum can modulate intrinsic expression of inflammatory markers associated with cell trafficking and modulate signaling pathways related to maturation of lymphoid tissue and influence cytokine and chemokine networks that regulate Th1/Th2 lymphocyte balance. The anti-inflammatory effect of for instance 2'-FL is known. 2'-FL from pooled human milk showed the ability to dampen pro-inflammatory mediator IL-8 release from T84 IEC line after type 1 pili *E. coli* infection (17). Similarly, reduced IL-8 expression was measured in fetal intestinal human tissue when exposed to 3'-, 4- and 6'-galactosyllactoses from human milk colostrum (17, 52). 2'-FL was shown to inhibit the inflammatory mediators secreted after TNFα induced *in vitro*, possibly through the inhibition of NF-KB activation (61). Furthermore, *in vitro* data demonstrate 2'-FL and LNFP I to be able to reduce monocyte activation and to modulate the release of IFNγ, IL-12, and IL-10 (62).

In addition, specific prebiotic oligosaccharides have been demonstrated to be immune modulatory (63–65). Immunomodulatory effects have been demonstrated for 2'-FL, suggesting an additional function of specific oligosaccharides (66–68). However, if these effects also relate to improved infection susceptibility in infants remains to be established. From the *in vitro* based human milk immune cell interaction studies some specific anti-inflammatory effects have been identified.

Galectins are another class of lectins involved in the regulation of immune and inflammatory processes (55). Interestingly, HMOs are reported to bind to various recombinant human galectins like hGal-1, -3, -4, -7, -8, and -9 in a very structure dependent and selective way. Human milk glycans with terminal type I sequences (Galβ1-3 GlcNAc) preferentially bind to hGal-7, whereas hGal-2 did not bind to human milk glyco-types but to a human blood group A Type 2 determinant (55). Beyond serving as a glycan receptor, galectins can also be secreted as soluble mediators and affect immune function. In this regard, IEC derived galectin-9 was increased after exposure of IEC to a mixture of scGOS/lcFOS in combination with a TLR-9 ligand in an *in vitro* co-culture model of IEC and activated immune cells (69). Galectin-9 played a key role in enhancing IFNγ and IL-10 production by immune cells underlying the IEC in this model (55). Further research will reveal the specific role of galectins in immunomodulation after exposure to HMOs, as well as their similarities with the immunomodulatory properties seen by scGOS/lcFOS. However, it is important

to realize that an efficient immune response remains to be mounted against the intruding pathogen. Providing efficient protection, in most cases, will go hand in hand with the induction of inflammation. If an anti-inflammatory response is beneficial in relation to the protection against pathogens, will be pathogen and host specific, and can only be elucidated *in vivo.*

HUMAN MILK OLIGOSACCHARIDES' IMPACT IN VIVO

It is the unique complexity of human milk oligosaccharides which leads to the speculation that these abundantly available structures in human milk play a key role in providing protection against infections in neonates. From the limited *in vivo* studies, we know that specific HMO-structures can reduce the interaction of specific pathogens like *Salmonella*, *Shigella*, *Vibrio cholerae*, *E. coli*, Polioviruses, Rotavirus and Respiratory Syncytial virus (RSV) with the host (11, 70). Within some studies, levels of the α 1-2-linked fucosyloligosaccharides 2'-FL and Lacto-N-difucohexaose I (LNDFH I) and ratios between 1-2-linked and 1-3-/1-4-linked oligosaccharides were associated with the presence of specific pathogens like *E. coli*, *Campylobacter* and *Norovirus* (35, 43). Interestingly, the provision of secretory type related complex mixtures of HMOs, have been associated with a direct protection against specific infections (71). Fucosyltransferase 2 non-secretor and low secretor status seems to associate with severe outcomes in premature infants. Meaning that within this study a low secretor phenotype was associated with the onset of NEC, and non-secretor genotype was associated with gram negative sepsis (71). In addition, it has been suggested that FUT2, the regulator of Lewis and ABO(H) antigens in the intestinal mucosa, could be a host genotypic feature affecting susceptibility to ETEC infection (72). Several intervention studies have reported the functional benefit of adding prebiotic oligosaccharides to infant formula. More specifically, specific prebiotic oligosaccharides have been shown to ameliorate the development of allergies as well as reduce the impact of pediatric infections (4, 5, 73–75). In this regard, the immune modulating effect that seems to decrease the risk on developing atopy and allergy, also may lower the infection risk in neonates, which is suggestive for basic immune modulation early in life. The clinical consequences of specific individual HMO-structures however, remain to be further elucidated (76). The first clinical safety studies are now reported on the use of specific HMOs combinations i.e., 2'-FL and scGOS (60) or the combination of two single oligosaccharides 2'-FL and LNnT (68, 77). Although growth and 2'-FL uptake were similar between formula receiving infants and as seen in breastfed infants, the possible functional benefits regarding immune development and/or infection susceptibility related to a single oligosaccharide are however not extractable from these studies. Therefore, the identification and understanding of protective elements in human breast milk decreasing infant's susceptibility to infection remains limited (75).

In conclusion, components of breast milk (including HMOs) play a key role in the development of the neonatal immune system by preventing pathogen replication, promoting healthy microbial diversity, inducing maturation of intestinal mucosa and by modulation of immune cells as well as pathogen recognition receptors. Currently, there is little understanding about the role of the diverse HMO-structures in optimally inducing microbiome and immune development and consequently how they may provide protection against infections. Therefore, we postulate that HMOs are involved in regulation of mucosal immune and barrier function in multiple ways, although the specific mechanisms remain poorly understood and may be a compilation of the biological functions of individual structures and their interactions. Further investigation into the components of breast milk and their roles in providing protection to infants is required, irrespective of the mechanism by which specific HMO-structures can provide protection toward certain pathogens.

AUTHOR CONTRIBUTIONS

VA-M, AvS, and MM have written the review. LW, JG, and BvL supervised the program. BS made specific contributions to the program with regard to human milk and in particular functional oligosaccharides. All authors listed have approved it for publication.

CONFLICT OF INTEREST STATEMENT

JG is head of the Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science at the Utrecht University and partly employed by Nutricia Research. BvL is leading a strategic alliance between University Medical Centre Utrecht/Wilhelmina Children's Hospital and Nutricia Research. BS, MM, and BvL are employed by Nutricia Research.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER III

Label free targeted LC-ESI-MS^2 analysis of human milk oligosaccharides (HMOs) and related human milk groups with enhanced structural selectivity

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ABSTRACT

Human milk (HM) supports the healthy development of neonates and exerts many of its beneficial effects via contained free human milk oligosaccharides (HMOs). These HMOs exhibit a complexity and structural diversity that poses a significant analytical challenge. A detailed characterization of HMOs is essential as every individual structure may have a different function/activity. Certain HMOs isomers may even fundamentally differ in their biological function, and especially their characterization by LC or LC-MS is often impaired by co-elution phenomena. Thus, more efficient analytical methodologies with enhanced structural selectivity are required.

Therefore, we developed a negative ion mode LC-ESI-MS^2 approach featuring straightforward sample preparation, environmentally friendly EtOH-gradient elution and enhanced, semi-quantitative characterization of distinct native HMOs by multiple reaction monitoring (MRM). Our MRM-LC-MS setup takes advantage of highly selective, glycan-configuration dependent CID-fragments to identify individual neutral and acidic HMOs. Notably, many human milk oligosaccharide isomers could be distinguished in a retention time independent manner. This contrasts with other contemporary MRM-approaches relying on rather unspecific MRM-transitions.

Using our method, the most abundant human milk Tri-, Tetra-, Penta- and Hexaoses could be determined semi-quantitatively in a single LC-MS assay. Detected HMO structures included Fucosyllactoses (e.g., 2'-FL), Lacto-N-Dicfuotetraose (LDFT), Lacto-N-Tetraoses (LNTs), Lacto-N-fucopentaoses (e.g., LNFPI, LNFP II & III), Lacto-N-Difcuohexaoses (LNDFHs) as well as Sialylactoses (SLs) and tentatively assigned blood group A and B tetrasaccharides from which also correct human milk type assignment could be demonstrated.

Correctness of milk typing was validated for milk groups I-IV by HPAEC-PAD.

Keywords

Human milk oligosaccharides (HMOs), structural identification, milktyping, targeted LC-MS^2, MRM, label free relative quantitation

INTRODUCTION

With concentrations between 3-18 g/L [1, 2, 3, 4, 5, 6] and next to lactose and lipids [7], human milk oligosaccharides (HMOs) represent the 3rd most abundant class of biomolecules in human milk (HM). Distinct structure-function relationships have been attributed to various HMOs which may be a crucial factor in the healthy development of infants. The beneficial early life functions of HMOs which are non-digestible in the upper gastrointestinal tract[8] range from direct anti-infective[9] and immunomodulatory effects to modulation of the developing infant's microbiome [10-12] e.g., via prebiotic activity. Many of these beneficial effects are also highly dependent on the primary fine structures of these glycans. Furthermore, the genetic predisposition of the mother with regard to polymorphisms of Se/Le genes [13] and in addition to other early life factors, considerably influences individual HMO glycoprofiles. Se- and Le-genes code for Fucosyltransfrase 2 (FUT 2, active Se gene) and Fucosyltransferase 3 (active Le gene). FUT 2 is described to primarily catalyze fucosylation of Galactoses (Gal) terminating glycans such as lactose or other HMO core-structures (e.g., Lacto-N-tetraose (LNT)) via alpha 1-2 glycosidic linkages [14]. The resulting fucosylated HMOs are e.g., 2'-Fucosyllactose (2'-FL), Difucosyllactoes (DFL), or Lacto-N-fucopentaose I (LNFP I). In contrast, FUT 3 activity forms alpha 1-3- or alpha 1-4-linkages between Fucose and type I or type II oligosaccharide-backbone-structures. In this case for example Lacto-N-fucopentaose II (LNFP II) or Lacto-N-Difucohexaose II (LNDFH II) may result. To date, due to the donor specific, individual FUT 2 and FUT3 expression patterns and the related HMO profiles detectable in human milk, 4 different human milk groups or types have been defined: Milk group I, II, III and IV [15]. Independent from milk group related glycans, a plethora of other compositional and structural HMO variants (> 1000) may be inferred from matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) data of high molecular weight human milk oligosaccharide fractions obtained by gel permeation chromatography (GPC) [2].

Research in the field of early life nutrition continuously strives to better understand biological functions of different human milk types, in particular, HMOs and other dietary glycans by deciphering the active molecular structures. Here, one prerequisite is the analytical ability to resolve, identify and quantify as many individual HMO structures as possible including configurational isomers. In the optimal case, multiple target molecules should be detectable reliably and in one quick analytical run. This would facilitate the analysis of larger sample numbers from cohorts or clinical studies and thereby help to describe biological benefits of HMOs with improved statistical quality. (Semi-) quantitative assays may be preferred over mere qualitative ones as some biological HMOs-effects may be of concentration dependent nature [16].

To address these analytical needs, many sensitive and accurate methods have already been developed to characterize native and derivatized human milk oligosaccharides, dairy prebiotics and other glycans. These methods include preparative and analytical size exclusion chromatography (SEC) combined with refractive index (RI) detection [17, 18] or offline MALDI-MS detection [19-23], high pressure anion exchange chromatography (HPAEC) coupled to pulsed amperometric detection (PAD) [15, 24-27] and high pressure liquid chromatography (HPLC) offering high sensitivity [28, 29]. Furthermore, electrophoretic attempts like polyacrylamide gel electrophoresis [30] and capillary electrophoresis (CE) [31, 32] have been employed. More advanced techniques such as fast atom bombardment mass spectrometry (FAB-MS) [33, 34], CE with laser induced fluorescence and MS detection (CE-LIF-MS) [35-37], and capillary gel electrophoresis (CGE)-LIF [38, 39] were applied, too. Furthermore, nuclear magnetic resonance spectroscopy (NMR) [40-44], MALDI-MS [19, 45-47], untargeted or targeted liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) [48] , multiple fragmentation stage ESI-MS (ESI-MS^n) [49, 50] or isotope ratio mass spectrometry (IRMS) [51] proved to be suited for compositional or structural identification and quantitation of known or even unknown glycans.

Most of these carefully designed methods perform quite well, but especially LC or CE based analytical attempts often depend on the availability of pure oligosaccharide (OS)- standards to calibrate and assign correct retention times (RTs) to specific OS-structures. Moreover, the identification of compounds based on specific RTs also requires very stable chromatographic systems. Even if provided, still co-elution of difficult to resolve isomers can impair detection and quantitation of target molecules. In addition, standards of e.g., rare HMOs like LNFP V or more complex glycans may be not commercially available or very expensive.

To mitigate those analytical challenges, novel automated, targeted and sensitive nano- or micro-LC-MS applications like capillary-HPAEC-ESI-MS [52] have been developed. By introducing the option to determine molecular weights of eluting compounds via MS, individual HMO structures can be analyzed with more specificity and selectivity compared to traditional HPLC/HPAEC methods with photometric-, fluorescence- or amperometric- detection. Thereby, identification of target glycans is achieved by combining structure specific retention time information with intact molecular mass and MS^2-fragment ion monitoring. In consequence, additional information about the monosaccharide composition, linkage and sequence can be obtained.

Further improvement of HMOs quantitation was published by Bao et al in 2013. Here, a simple, efficient and robust quantitative LC-ESI-MS method was described. It operates in negative ion mode and can resolve 11 neutral HMOs (Tri- to Hexaoses,

incl. LNFPI, II, III, V, and VI) after reduction into alditols [53]. Identification is based on retention time and precursor mass information. Wu et al. presented highly sensitive chipLC-nESI-TOF-MS approaches for analysis of 45 neutral [54], and 30 acidic [55] HMO structures after reduction with sodium borohydrite. A combination of structure specific retention times together with selected precursor and fragment ions served to identify glycan structures and construction of a HMOs database. Another approach by Tao in 2011 employed chipLC-nESI-TOF-MS in positive ion mode for identification and relative quantification of milk OS from 7 different primate species including human milk. Based on retention time and accurate mass detection, approximately 50-100 different milk OS per species were claimed to be found [56]. Furthermore, Fong et al. demonstrated the use of hydrophilic interaction HPLC-high-resolution-MS with selected reaction monitoring (SRM) for measuring six different oligosaccharides in bovine milk, bovine colostrum, and infant milk formulae [57]. This method is able to detect e.g., native 3'-SL, 6'-SL and Disialylactose (DSL)) in negative ion mode by using distinct pairs of precursor- and selected fragment-ions. Further targeted, label free MRM UHPLC-MS methods have been introduced: They can monitor native monosaccharides (Fucose, Sialic acid, Glucose), Lactose, 2'-FL, 3'-SL, 6'-SL and LNneoT in rat serum or detect HMOs-alditols after reduction with $NaBH_4$ in negative [58] or in positive ion mode [6]. These approaches utilize specific retention time information combined with either specific or in the case of HMO alditols rather unspecific MRM transitions for absolute quantitation of individual compounds.

Until today, no efficient analytical strategy for HMOs has been developed to overcome both, the dependence on availability of pure standards for compound identification and the frequent impairment of compound quantitation by co-elution effects. A key element to progress from here would be to define and to exploit structure specific diagnostic MS^2- or MS^n -fragments for direct mass spectrometric identification and quantification of important HMOs. This basic idea has already been suggested a decade ago by e.g., Pfenninger et al. [49, 50] who characterized several isolated HMO structures in static nESI-MS^n experiments with high sensitivity on a classical quadrupole ion trap (QIT) instrument. Thereby, fundamental fragmentation rules could be established which can aid in characterization of composition, sequence but also partly in determination of glycosidic linkages in known or novel HMOs. Due to the nature of static nESI which requires manual change of static nESI which required manual exchange of spraying needles in the nESI-ion-source for every new experiment, the sample throughput was quite limited then.

To further enhance throughput and parallel characterization of relevant isomeric HMO structures in human milk up to pentaoses, we developed a novel targeted LC-ESI-MS^2 method operating in negative ion MRM mode. This approach builds on the aforementioned diagnostic ion strategy for identification and relative quantitation of particular HMOs. The novelty of this approach is to exploit highly specific, HMOs

configuration dependent, diagnostic MS^2-CID fragments for identification and relative quantification of distinct oligosaccharide structures rather than using the more unspecific MRM-transitions published so far. Thereby our method circumvents challenges in HMO quantitation posed by co-elution phenomena, the need to use rare HMO standards for compound identification and also tedious derivatization of HMOs prior to analysis.

EXPERIMENTAL SECTION

Chemicals & reagents

1,5-α-L-Arabinopentaose (> 95%) was purchased from Megazyme International, Ireland. Ammonium acetate (p.a.), Acetonitrile (LC-MS grade), and Ethanol (gradient grade for HPLC) were from Merck, Darmstadt, Germany. LC-MS grade H_2O (HiPerSolv Chromanorm) came from Prolabo, VWR International, Darmstadt, Germany. Alternatively, Ultrapure lab water, 18 kW, <5ppm TOC was produced using a MilliQ Advantage Ultrapure Water System from Merck Millipore, Darmstadt, Germany.

Isolation and characterization of defined molecular weight HMO fractions by gel permeation chromatography (GPC) and sub-fractionation into isomerically pure Lacto-N-tetraoses (LNTs), Lacto-N-fucopentaoses (LNFPs), and Lacto-N-difucohexaoses (LNDFHs) by high performance liquid chromatography (HPLC)

Availability of defined molecular weight HMO fractions and isomerically pure LNT, LNnT, LNFP I, LNFP II, LNFP III, LNFP V, LNDFH I, and LNDFH II were mandatory for subsequent method development i.e. assignment of isomer specific, diagnostic CID MS^2-fragments, further definition of specific MRM transitions and future quantitation purposes e.g., construction of calibration curves. Defined molecular weight HMO fractions (ranging from human milk derived trioses to hexaoses) and individual isomers were isolated from pooled human milk as described in more detail by Stahl et al. 1994 [19]. Briefly, the protocol included milk skimming by centrifugation (3000rpm, 4°C, 30min), protein precipitation (2 volumes of cold EtOH added to 1 volume of skimmed milk), and chromatographic fractionation of the resulting carbohydrate (CH) fraction by preparative (GPC) into *defined molecular weight HMO fractions (trioses, tetraoses, pentaoses and hexaoses). Defined GPC fractions were sub-fractionated using* C18rp-HPLC (see [19]) to yield pure HMO isomers. In modification of the original procedure, no anion exchange chromatographic (AEC) separation of neutral and acidic HMOs was employed. Instead, the CH fraction was lactose depleted prior to GPC and C18rp-HPLC fractionation by simulated moving bed chromatography as outlined by Geisser et al. 2005 [59]. Further technical *details about GPC and c18rp-HPLC fractionation including pretreatment and storage of yielded HMO fractions can be found in the supplements of this article.* Purity of HMO fractions was assessed by MALDI-MS [19], HPAEC [24] and xCGE-LIF [38]. In case of pure LNT

and LNFP isomers, only purity grades exceeding 95% were accepted to be used for preparation of calibration solutions and determination of HMO structure specific tandem MS fragments.

Oligosaccharide-solutions for determination of HMO structure specific diagnostic tandem MS fragments and a-arabinopentaose (a-AP) internal standard solution

HMO GPC fractions or pure OS isomers also served for tandem MS experiments via flow injection analyses (FIA) or LC-MS^2 (see below). Solutions of defined molecular weight HMO GPC-fractions and isomerically pure HMO fractions (see above) were prepared by using ultrapure lab water (18 kW, <5ppm TOC). The final concentrations after 0.2-micron filtration through a PES syringe filter (Minisart, Sartorius, Goettingen, Germany) were ~ 0.01 µg/µl. a-Arabinopentaose internal standard (ITS) was introduced to continuously monitor instrumental performance. Furthermore, we intended to (at least partly) compensate for possible deviations in ionisation efficiency due to expected biological inter sample variations of the human milk matrix. Hence, 5 mg a-arabinopentaose (a-AP, Megazyme International, Ireland) were dissolved in 1 ml of ultrapure water to yield a to 5mg/ml stock solution. Subsequently, 33.9 µl of this stock solution were pipetted into a glass vial and filled up to 5.0 ml with water (equal to 0.05 mmol/l or 0.0339 mg/ml). This solution was stored at -20°C until further use.

Eluent-solutions for MRM LC-ESI-MS

LC-MS eluent solutions A and B have been prepared as follows:

Preparation of 5 mM Amonium Acetate solution ("Eluent A")

385,4 mg ammonium acetate were dissolved in one liter of water (LC-MS-Grade) and the solution was filtered through a 0,22 µm filter device (bottle top filter, Sartolab, 0,22 µm PES, 500ml , Sartorius, Göttingen, Germany) directly into a 1 L Duran glass bottle (Schott, Mainz, Germany) by applying appropriate vacuum. Final pH of eluent solution was approximately 6,0. The bottle was connected to the 1200 series binary HPLC system.

Preparation of 5 mM Amonium Acetate solution in 80 % EtOH ("Eluent B")

385.4 mg ammonium acetate were dissolved in 200 ml ultrapure lab water and 800 ml gradient grade EtOH (Merck, Darmstadt, Germany) were added. The solution was filtered through a 0.22 µm filter device into a 1 L duran glass bottle (Schott, Mainz, Germany) as described for "Eluent A" and connected to the 1200 series binary HPLC system.

4 Human milk samples of known milk group type

In order to verify if our MRM LC-ESI-MS approach can distinguish among the 4 commonly known human milk groups by deciphering the sample specific LNFP

patterns, we utilized a subset of 4 donor milks with known milk group type from previous studies conducted by Thurl et al. 1997 and 2010 [15, 60]. These human milks had been provided by healthy 20-35 year old Caucasian volunteers from the regions of Frankfurt/Main or Dresden in Germany who had given birth to healthy infants. Already in this study, each human milk sample had been assigned to one of the 4 different milk groups via HPAEC by virtue of its specific HMO pattern. Also, donor Lewis blood group status had been confirmed by hemagglutination blood testing in the same study. Samples were anonymized before re-analyzes by HPAEC and LC-MS.

Fast sample clean-up by ultrafiltration and addition of internal standard a-Arabinopentaose prior to MRM LC-ESI-MS

The purity of the target compounds (oligosaccharides) is crucial to protect the analytical LC-MS system against contamination, clogging and to enhance the sensitivity for lead molecules by depleting competing matrix compounds. Ultrafiltration (UF) is a fast means to facilitate sample clean up by separating smaller molecules like HMOs from most of proteins and other compounds of the sample matrix exceeding the UF cut off. In this study, clean-up of all samples (i.e. human milks and isolated HMO standards) was performed by ultrafiltration with 3 kDa membrane centrifugal device at room temperature before LC-MS as follows:

500 µl Amicon Ultra centrifugal filter devices (Millipore, Billerica, MA, USA) with 3kDa nominal cut off were first cleaned by adding 450 µl of H_2O (LC-MS grade) into each device and centrifuging at 14000 g for 20 minutes at room temperature. This step was introduced to remove possible residual surfactants from the UF membranes. Filtrate was discarded afterwards. Then, human milk samples were thawed at room temperature and vortexed well until an optically homogeneous milk solution was achieved. Afterwards, 135 µl of milk were mixed with with 15 µl of a-Arabinopentaose internal standard (ITS) solution (0.05 mmol/l, for preparation see above). This combined sample-ITS-solution was further diluted 1:11 (v/v) in 2 mL-Eppendorf-test tubes by adding 1350 µl H_2O (LC-MS grade). 450 µl of the diluted mixture were transferred into the previously washed filter device. After centrifugation at 14000 g for 1 hour at room temperature, the complete filtrate containing HMOs was vortexed again for 5 seconds and pipetted into a clean 300 µl glass LC-MS-vial with screw top (Thermo Fisher Scientific, Waltham, MA, USA). Samples were either directly subjected to MRM LC-ESI-MS or stored at -20°C until further use.

HPAEC analysis of 4 human milk samples with known donor Le-/Se gene status

Classification of 4 human milk samples with known donor Le-/Se gen status (see above) into one of 4 human milk groups was reconfirmed by HPAEC following the approach published by Coppa et al. 2011 [61]. In deviation from the method of Coppa et al., we applied a Carbopac PA-200 column (Dionex, Idstein Germany) instead of PA-1 column. Unlike in the method formerly described by Thurl 1997[15], Lactose was not removed

prior to HPAEC in our approach. Nevertheless, the resulting milk group assignments were identical with both approaches: The four selected samples comprised milk group I, II, III and IV. Aliquots of these 4 human milk samples had been prepared for HPAEC and MRM LC-ESI-MS analysis by 3kDa ultrafiltration as described before.

Qualitative and semi-quantitative analysis of human milk oligosaccharides by negative ion MRM LC-ESI-MS

General MRM LC-ESI-MS setup: The employed LC-ESI-MS configuration consisted of a 3200 Qtrap triple quadrupole linear ion trap mass spectrometer (MS) equipped with a Turbo V-ion source (all ABSciex, Framingham, MA, USA) connected to a 1200 series HPLC-stack (Agilent, Santa Clara, CA, USA). All MS experiments were carried out in negative ion mode. The modules of the HPLC-stack included a binary pump, a degasser, a temperature controlled autosampler kept at 22°C, a column compartment kept at 45°C, and a DAD detector monitoring possible peptide or protein contaminations at 215 & 280 nm wavelength. A Hypercarb 2.1x30mm porous graphitized carbon (PGC) column with 5µm particle diameter and a 2.1x10 mm Hypercarb pre-column (Thermo Fisher Scientific, Waltham, MA USA) were used as stationary LC phase. A ~18 minutes gradient elution at 400ml/min flow rate was used to separate HMOs by LC before online infusion into the mass spectrometer. The gradient was formed by varying the ratio of eluents A (5 mM aqueous NH_4CH_3COO) and B (80 % EtOH (v/v) with 5 mM aqueous NH_4CH_3COO) over time as described in Table S 1 (see supplementary information). For preparation of eluents A and B see section above. The sample injection volume per analytical run was 5µl. After each injection, the autosampler needle was flushed externally for 5 seconds with 20% aqueous isopropanol to minimize sample carryover. The inbuilt Valco-diverter valve of the 3200 Qtrap was directing the PGC-column effluent either to the ion source or to waste. It was switched towards the MS-ion source 0.2 min after sample injection and switched back to waste after 17 minutes. Effluent switching was introduced to protect the ion source from excess effluent between runs or during automated column cleaning procedures. In addition to the MS detection also UV-profiles of each sample were recorded at 215 and 280 nm wavelength and 360nm reference wavelength. An Agilent 1200 series diode array detector and an analytical flow cell were used to sample UV signals with a frequency > 20Hz. At the start of each run the UV signal was set to zero automatically. Analyst 1.4 software (ABSciex, Framingham, USA) was used to control the complete LC-MS configuration and for further evaluation of LC-MS data.

Optimization of ion source and compound specific MS-parameters for HMOs

To achieve maximum sensitivity for EPI-ESI-MS and the final MRM LC-ESI-MS assay, ion source and target compound related parameters of the 3200 Qtrap were optimized. Flow injection analysis (FIA) MS of a GPC derived Lacto-N-Fucopentaose-mixture (for purification see above) was employed to infer the optimized settings as shown by Table S 2 in the supplements.

Determination of HMO structure-specific diagnostic tandem MS fragments for construction of MRM transitions.

In order to determine/confirm isomer specific diagnostic fragment masses, MS^2 experiments were performed by direct syringe- or HPLC infused flow-injection MS/MS analysis or LC-MS analysis in negative ion mode. HMO GPC fractions or pure OS isomers were analyzed using the general linear ion trap triple quadrupole LC-MS setup and optimized ion source parameters already described above in enhanced product ion (EPI) mode. A quadrupole 3D ion trap (QIT) instrument (LCQ, Thermo Fisher) equipped with a syringe pump was used alternatively. All experiments were performed at room temperature with or without PGC column. HPLC- or syringe flow rates were 400ml/min using an isocratic eluent consisting of 25 % EtOH (v/v) and 5 mM aqueous NH_4CH_3COO. Both MS instruments were operated with standard µ-spray ion sources in negative ion mode using default ion source and MS^2 conditions if not further specified below. Isolation widths for precursor ions of interest were set to *m/z* +/- 3 in case of QIT-MS and to unit resolution in case of the linear ion trap MS instrument. Collision energies were adjusted to yield precursor ion intensities of ~ 10-30% relative to the most intense fragment ion in resulting tandem MS spectra. Fragment ions were considered to be diagnostic if they were uniquely detected in MS/MS spectra of individual HMO structures but not in others and exceeded at least 1% of the signal intensity of the most prominent peak in the MS^2-spectrum. The identified diagnostic molecular fragment masses were further employed in MRM transitions of the final MRM LC-ESI-MS method after compound specific ESI-MRM-optimization on the 3200 Qtrap MS instrument.

Application of final negative ion MRM LC-ESI-MS method for analysis of HMOs in human milk samples.

Using the optimized final MRM LC-ESI-MS setup, human milk samples were analyzed in randomized manner. Each sample was followed by two blanks to minimize possible carryover. First blank was 80 % isopropyl alcohol and second blank ultrapure water.

Software settings for identification of HMOs, peak integration and relative quantitation after negative ion mode MRM LC-ESI-MS analysis.

Identification of most individual HMOs primarily relied on the specific MRM transitions (see results). As the employed Analyst software also stipulates to assign specific LC-retention times for compound recognition, a window of 6 min retention time (RT) tolerance was allowed to ensure correct peak assignment to HMO structures for most runs. Larger, rather erratic RT shifts were occasionally recognized especially for Hex_2, 3-FL, 2'-FL and DFL in some exceptional cases. Then the RT tolerance window was manually extended to up to 15 minutes. To also compensate for different LC-peak-shapes, bunching factors were adapted accordingly between 5 and 30 to achieve complete integration of peak areas.

The complete software settings to ensure proper glycan peak assignment and quantitation are summarized in Table S 3 (see supplementary).

Manual assignment of human milk groups by individual inspection of LC-MS and HPAEC data

For further processing, generated LC-MS data sets were exported from Analyst 1.4 software to Microsoft Excel version 14.0.7145.5000 (32 bit) or ABSciex Markerview version 1.2.1..

To facilitate relative quantitation, dimensionless responses were calculated for each individual glycan in Excel or Analyst software according to Equation 1. If needed, dimensionless responses yielded for individual HMO structures after analyses of different HM samples were compared to each other and used to calculate e.g., changes in abundance between samples in percent.

Equation 1

$$R = \frac{\mathrm{A_S}}{A_{\mathrm{its}}}$$

R	Response
A_S	peak area specific OS
A_{its}	peak area a-Arabinopentaose (internal standard (its))

Assignment of human milk samples to one of the four known milk groups was based on the absence, presence or concomitance of LNFP I and LNFP II [15] as determined by MRM LC-ESI-MS. Peaks were considered for evaluation if the detected peak responses R exceeded the value of 0.2. In case of the 4 human milk samples with known donor Le-/Se gen status, additional HPAEC analysis (see above) was performed prior to MRM LC-ESI-MS to cross validate milk group assignment. Determination of milk groups by HPAEC was facilitated by detection of milk group specific marker HMOs like LNFP I, LNFP II, 2'-FL, DFL, LNDH I and LNDHII as proposed in literature (see Thurl et al. 1997 [15]). HMO peaks were inspected manually in each LC-MS or HPAEC plot.

Estimation of HMO quantitative inter run variations in MRM LC-ESI-MS

To estimate the inter run variation of detected HMOs quantities, a human milk sample was injected 10 times after sample pretreatment (see above). Between every injection an 80% isopropanol blank and an ultrapure water blank were run. Responses R (see Equation 1) for all detected HMOs were averaged over the 10 runs and relative standard deviations were calculated in percent.

RESULTS

Determination of characteristic, isomer specific HMO CID fragments and derived MRM transitions

A crucial step in MRM method optimization was to define suitable negative ion mode MRM transitions being specific enough to distinguish between (partly isomeric) HMOs of interest such as 2'-FL, 3-FL, 3'-SL, 6'-SL, DFL, LNT, LNnT, LNFP I, LNFP II, and LNFP III. The necessary $[M-H]^-$-precursor masses for definition of MRM precursor-fragment ion pairs could be readily deduced from chemical sum formulae of the respective HMO compounds. Then, the corresponding MRM fragment ion masses were derived from literature [49, 50, 62, 63] as described for negative ion mode CID induced fragmentation pathways of linear or branched HMOs (ranging from tetraoses up to hexaoses). Afterwards literature information was confirmed and completed by own negative ion mode ESI-MS^2 CID-experiments with purified HMO standards. A selection of resulting spectra for defined HMO structures by negative ion mode enhanced product ion ESI-MS^2 (EPI-ESI-MS^2) are exemplified in **Figures 1**, **2**, and **3**. The depicted EPI-ESI-MS^2 spectra were yielded for pure LNFP I, II, and III (see **Figure 1a**), a mixture of LNFP I, II, III and V (**Figure S1a** in supplements), pure LNT and LNnT (**Figure 1b**), a mixture of LNT and LNnT (see **Figure S1b** in supplements), as well as isomerically pure 2'-FL (**Figure 2a**), 3-FL (**Figure 2b**), 3'-SL (**Figure 3a**) and 6'SL (**Figure 3b**).

Diagnostic C, C/Z or A fragment ions of LNFP I, II, III and V at *m/z* 325, 348, 364 and 544 and of LNT and LNnT at *m/z* 202 and 263 have already been described in literature by Pfenninger et al in 2002 [49, 50]. These authors used classical quadrupole 3D ion trap instruments and nESI in negative ion mode for structural MS^n-elucidation. Low energy resonant collision induced dissociation (CID) was applied.

Interestingly, the same HMO-isomer specific diagnostic fragments could also be detected under non-resonant low energy CID conditions by us with a triple quadrupole-linear ion trap MS (see **Figure 1)** but also by Chai et al. [62]using a quadrupole-quadrupole-TOF-MS. This might be surprising as different ion dissociation processes take place during resonant and non-resonant CID. Encouraged by these findings, we integrated also other structure specific C-, C/Z- or A-fragments into our MRM setup which had been formerly described e.g., for LNFP V, LNDFH I, or LNDFH II by Chai et al.. Moreover, additional diagnostic fragment ions specific for 6'-SL at *m/z* 470, 3'-SL at *m/z* 408, 2'-FL at *m/z* 325 and 3-FL at *m/z* 179 (see **Figures 2 + 3**) could be revealed or re-confirmed by own EPI-ESI-MS^2 experiments on the triple quadrupole linear ion trap MS platform. Noteworthy, we did not find any indication for gas phase rearrangement of fucosylated human milk oligosaccharides and prompt fucose migration as described by Wuhrer et al 2011 [64] and Aldredge et al. 2003 [65]. These authors conducted positive ion mode MS/

MS analysis of protonated OS precursor ions. Gas phase rearrangement can lead to misinterpretation of MS/MS data if structural elucidation of oligosaccharides is attempted. Obviously, this drawback can not only be circumvented by analyzing permethylated milk oligosaccharides as outlined by Dong et al. 2016 [66] and Oursel et al. 2017 [67], but also by utilizing negative ion mode CID of underivatized HMOs as demonstrated by us and others.

According to literature, Kelly et al. [68] already successfully attempted to distinguish and to quantify native 3-'SL and 6'-SL in bovine milk by negative ion mode 3D-ion-trap-FIA-MS. Unlike in our results, the *m/z* 470 fragment wasn't exclusively found after CID fragmentation of 6'-SL, but also appeared in low abundance in 3'-SL standards. It is not clear if this divergence in results might stem from 3'-SL-traces contained in the commercial 6'-SL standards they used or has other causes.

At least no statement about the purity of the used standards was made in this article. Intriguingly, the 3'-SL specific fragment which we detected at *m/z* 408 was not at all described in the 3D-ion-trap 3'-SL spectra by Kelly and colleagues. In consequence, they used ratios of the fragment ions *m/z* 572 and *m/z* 290 rather than diagnostic fragment ions to determine proportions of 3'-SL and 6'-SL being present in milk samples. Regarding ease of use in method development or application, our and other FIA or LC-MS approaches might be considered as an improvement in structural HMO analysis compared to static nESI tandem MS experiments such as those conducted by Pfenninger et al. [49,50]. This is because these static nESI approaches were much more tedious to conduct due to the need for manual loading of the nano spray needle, containing the next sample, into the ion source.

On the other hand, carry over is theoretically much less probable in such static nESI applications and MS^n for elucidation of monosaccharide sequences and glycosidic linkages can be conveniently performed with sub µl sample volumes. By analyzing further HMO standards, we succeeded to define unambiguous MRM transitions for 11 HM isomers employing one unique diagnostic fragment ion per structure. These structures represent different configurational isomers of the following HMOs: Fucosyllactoses (FLs), Difucosyllactose (DFL), Lacto-N-tetraoses (LNTs), Lacto-N-Fucopentaoses (LNFPs), and Silalylactoses (SLs).

It's noteworthy to mention that the diagnostic fragment found for LNFP I at *m/z* 325 presents an exception, as it would not be specific enough to distinguish between LNFP I and its type II core isomer LNFP IV [64]. This LNFPI C_2-fragment (see Table 1) indicates the position of Fucose at the glycan's nonreducing end which doesn't differ between LNFP I and IV. Fortunately, the existence of LNFP IV has not been reported for human milk but for seal milk so far [69]. Therefore, it still appears to be reasonable to employ the C_2 fragment at *m/z* 325 as marker for LNFP I in human

milk analysis. On the contrary, if LNFP IV would be present in significant abundance in HM, the appearance of an additional LC-MS peak or at least peak broadening might be expected.

This was not the case in the subsequent MRM LC-ESI-MS analyses we conducted with 4 HM samples. Also for the MRM transition which we established for LNFP V, in theory another structure called LNFP VI could be picked up. LNFP V is known to have a Fucose residue at the reducing end of a type I core LNT backbone. Its type II core counterpart LNFP VI could theoretically produce the same C_3-fragment at *m/z* 544. LNFP VI has recently been detected by Bao et al. [53] in 4 different human milks collected between 3 and 29 days pp. Here, contained HMOs were converted into alditols prior to LC-MS. With and without reduction of HMOs into alditols, LNFP V and LNFP VI were discernable by gradient elution on a 100x2.1 mm PGC column via different retention times. In contrast to Bao et al. we used a 2.1 mm ID PGC stationary phase but with a shorter column length of 30mm. However, in our HM sample set we couldn't detect a second peak in addition to LNFP V for the transition *m/z* 882 to 544 which might have been an indication for presence of LNFP VI. With our setup, only one peak with two shoulders was detected, most probably reflecting the two anomeric isomers of LNFP V. Assignment to LNFP V was done as this compound is a well-known and confirmed part of the HMO composition in HM [70]. It will be interesting to investigate if this outcome will persist if more milks e.g., from a HM cohort might be analyzed in future. Biological differences between the characterized sample sets regarding gestational ages of donors as well as a better sensitivity of Bao's method for LNFP VI and co-elution of LNFP V and VI in our case may account for the diverging observations, too.

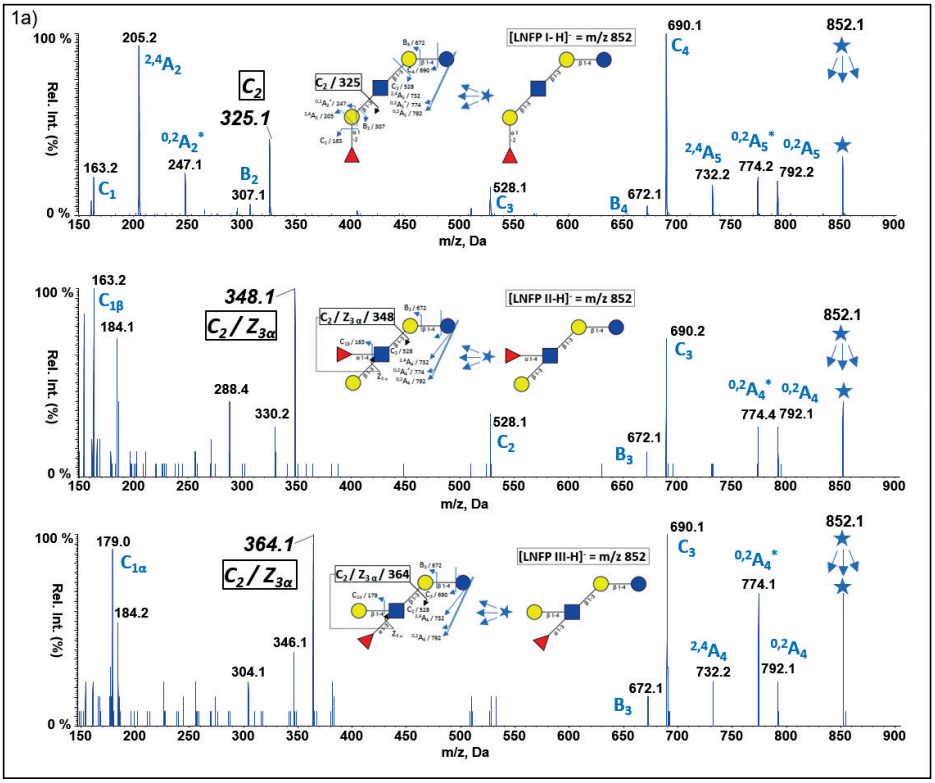


Figure 1a | Negative ion mode EPI-ESI-MS² spectra of pure LNPF I, II, III. [M-H]⁻-precursor ions selected for MS² in EPI mode were at *m/z* 852. Isomer specific diagnostic MS²-fragments are highlighted by boxes and in bold Italic letters for LNFP I/IV (*m/z* 325), LNFP II (*m/z* 348), LNFP III (*m/z* 364), LNFP V/VI (*m/z* 544). Detected LNFP-isomer specific diagnostic CID fragments comply with published findings described for direct static nESI-QIT- and qTOF-MS/MS experiments [49, 50, 62]. All MS²-spectra were acquired at -29eV collision energy.

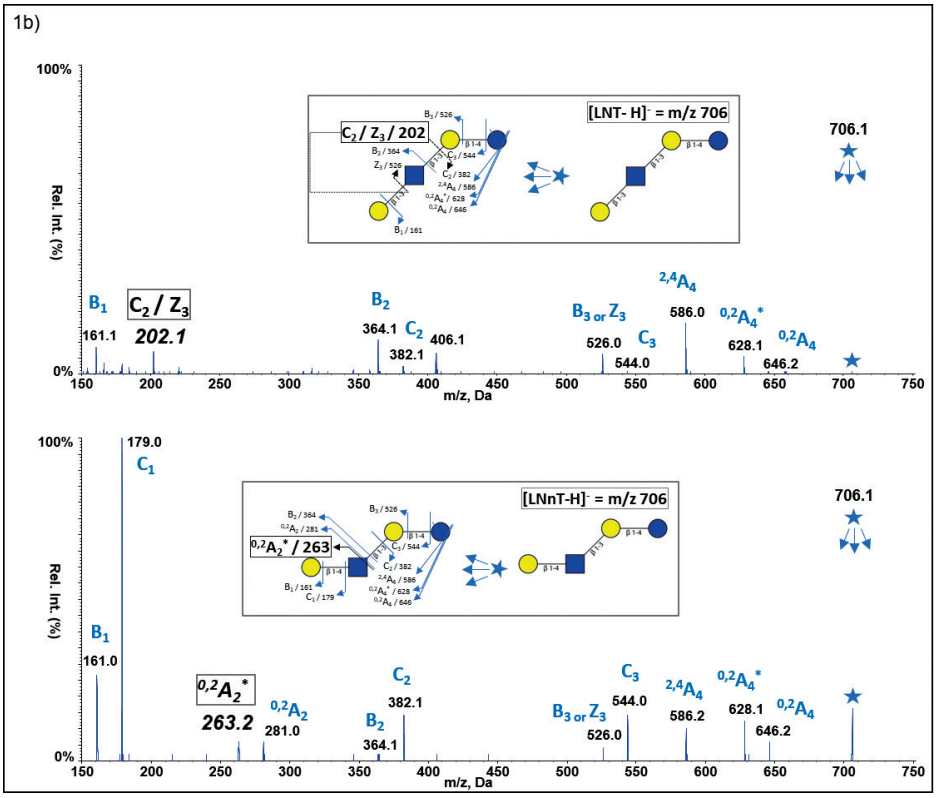


Figure 1b | Negative ion mode EPI-ESI-MS² spectra of pure LNT and LNnT. [M-H]⁻-precursor ions selected for MS² in EPI mode were at *m/z* 706. Isomer specific diagnostic MS²-fragments are highlighted by boxes and in bold italic letters for LNT (*m/z* 202) and LNnT (*m/z* 263). Detected LNT-isomer specific diagnostic CID fragments comply with published findings described for direct static nESI-QIT- and qTOF-MS/MS experiments [49, 50, 62]. All MS²-spectra were acquired at -29eV collision energy.

Implementation of HMO isomer specific MRM transitions into the new negative ion mode MRM LC-ESI-MS method for identification and relative quantitation of individual HMO structures

Based on translation of all diagnostic HMO fragments described in the paragraphs above, we attempted to create a highly HMO structure selective negative ion mode MRM LC-ESI-MS approach.

An overview about the characteristic isomer specific MRM-transitions consisting of respective pseudomolecular precursor-ions and diagnostic fragment-ions is given in Table 1 for 10 HMO structures. The sketches used to visualize intact HMO structures and hypothetical fragment ion structures as shown in Table 1 and Table 2 comply with the nomenclature first introduced by Domon and Costello 1988 [73] and continued by Pfenninger et al. 2002 [49], Chai et al. 2001 [62], and Harvey et

al. 2005 [74, 75]. Although considering published suggestions by literature [49, 62, 75], the shown structural representations of these fragment ions are only likely variants which may actually still differ from the real fragment-ion-configuration. Analyzing the exact nature of such fragment ions is out of the scope of this study. An extended set of additional 13 MRM-transitions conveying less configurational but at least compositional specificity was included into the final MRM method, too (see Table 2 below).

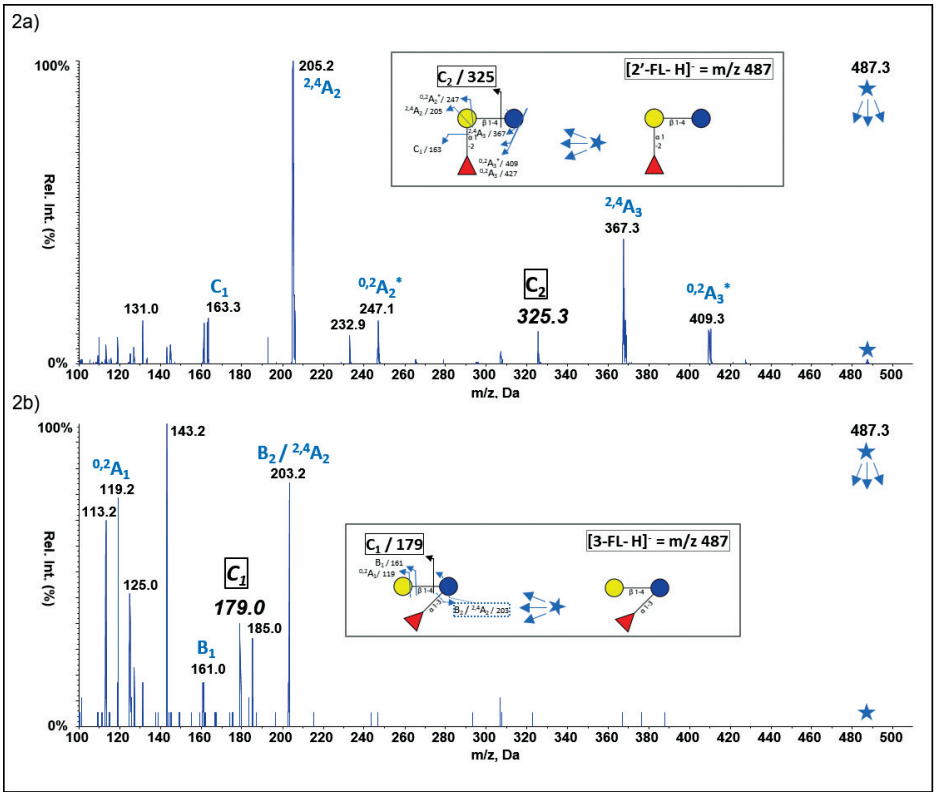


Figure 2 | Negative ion mode EPI-ESI-MS² spectra of a) pure 2'-FL and b) pure 3-FL. Isomer specific diagnostic MS²-fragments were highlighted by boxes and bold italic letters and are found for 2'FL at *m/z* 325 and for 3-FL at *m/z* 179. The [M-H]⁻- precursor ion selected for MS² of both isomers was at *m/z* 487. MS²-spectra were acquired at -29eV collision energy. Monosaccharide-symbols and structural representations of oligosaccharides were drawn with Glycoworkbench [71] according to CFG proposals [72]: Glucose (Glc)●, Galactose (Gal)●, Fucose (Fuc)▲. Annotation of fragments follows the nomenclature introduced by Domon and Costello [73]

Among other OS, this set contains MRM transition for LNFP V, LNDFH I, LNDFH II or their respective type II core isomers, tentative histo blood group antigen A [76] and B like tetrasaccharides, and a-Arabinopentaose which served as internal standard.

After implementing the entire set of compound specific MRM transitions into the new MRM LC-ESI-MS-method and subsequent optimization of source and detector settings, analyses of GPC- and C18rp-HPLC-purified HMO structures were performed to assign individual retention times to the respective HMOs.

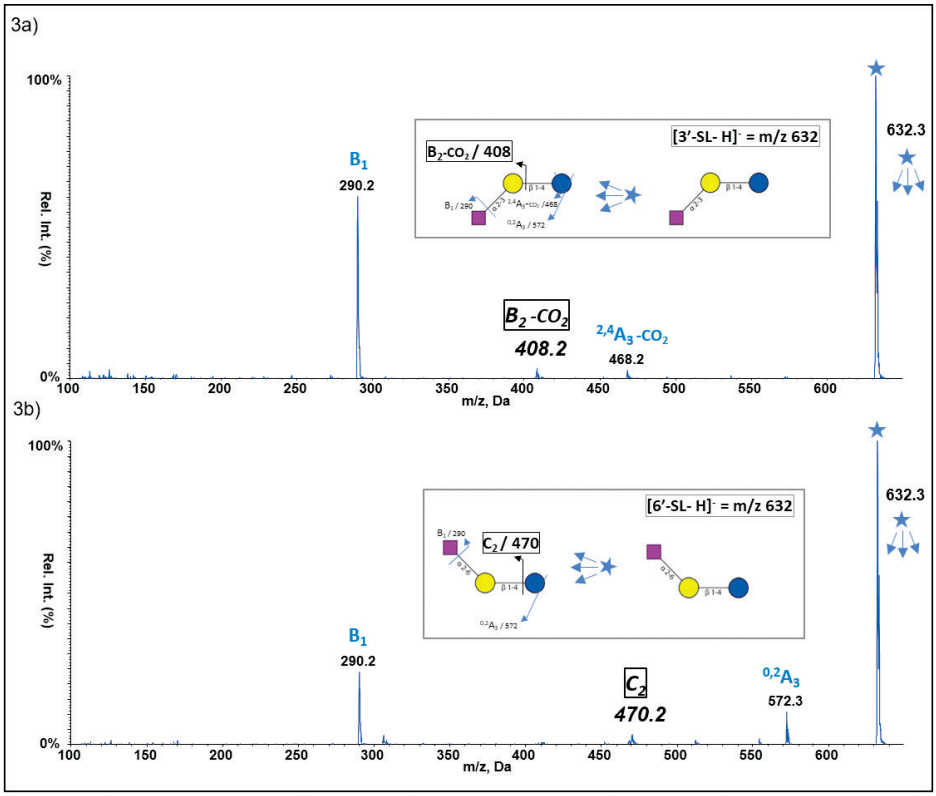


Figure 3 | Negative ion mode EPI-ESI-MS² spectra of a) pure 3'- and b) pure 6'-Sialylactose standards. Isomer specific diagnostic MS²-fragments are found for 3'-SL at *m/z* 408 and for 6'-SL at *m/z* 470 and are highlighted by boxes and bold italic letters. [M-H]⁻- precursor ion selected for MS² in EPI mode was at *m/z* 632 for both isomers. All MS²-spectra were acquired at -29eV collision energy. Monosaccharide-symbols and structural representations of oligosaccharides were drawn with Glycoworkbench [71] according to CFG proposals [72]: Glucose (Glc)●, Galactose (Gal)●, N-Acetylneuraminic acid (Neu5Ac)◆. Annotation of fragments follows the nomenclature introduced by Domon and Costello [73]

Then, by analyzing HMOs in a complete human milk sample, we tested the specificity of the selected MRM transitions together with the resolving power of the employed multistep H_2O-EtOH gradient on porous graphitized carbon (PGC) columns designed to resolve especially the different LNFP isomers. Both, a good chromatographic resolution and the clear distinction between LNFP isomers I and II by the chosen

MRM transitions would be valuable advantages for the desired determination of different human milk groups.

Table 1 | Characteristic, isomer specific MRM-transitions (monoisotopic *m/z* values) for HMOs including precursor ions [M-H]$^-$ and diagnostic fragment ions as employed in final negative ion mode MRM LC-ESI-MS method. Monosaccharide symbols and structural representations of oligosaccharides were drawn with Glycoworkbench [71] according to CFG proposals [72]: Glucose (Glc)●, Galactose (Gal)●, N-Acetylglucosmanine (GlcNAc)■, Fucose (Fuc)▲, N-Acetylneuraminic acid (Neu5Ac)◆. Annotation of fragments follows the nomenclature introduced by Domon and Costello [73]

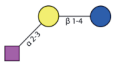
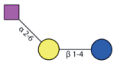
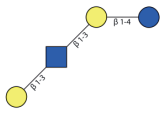
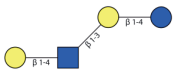
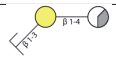
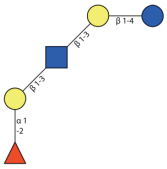
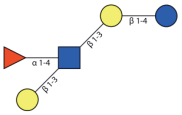
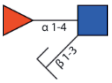
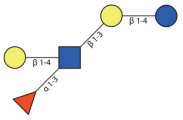
			MRM-Transitions		
Nr.	**Oligo-saccharide**	**Structure**	**Precursor [*m/z*]**	**Fragment [*m/z*]**	**Possible Fragment ion types** [49, 62, 73, 75]
1	2´-FL		487.1668	325	 C_2
2	3-FL		487.1668	179	 C_1
3	3´-SL		632.2044	408	 $B_2{}^{1,3}X_3$ (double cleavage)
4	6´-SL		632.2044	470	 C_2
5	DFL		633.2248	325	 C_2
6	LNT		706.2411	202	 C_2Z_3 (double cleavage)
7	LNnT		706.2411	263	 $^{0,2}A_2\ Z_3$ (double cleavage)
8	LNFP I		852.2990	325	 C_2

Table 1 | Continued.

Nr.	Oligosaccharide	Structure	MRM-Transitions Precursor [*m/z*]	Fragment [*m/z*]	Possible Fragment ion types [49, 62, 73, 75]
9	LNFP II		852.2990	348	 C_2Z_{3a} (double cleavage)
10	LNFP III		852.2990	364	 C_2Z_{3b} (double cleavage)

As published by Thurl et al 1997 [15] and others, human milk groups can be determined by monitoring absence, presence or concomitance of certain Secretor and Lewis gen dependent oligosaccharides like LNFP I and LNFP II.

Table 2 | Characteristic, composition specific MRM-transitions (nominal *m/z* values) for oligosaccharides, tentative blood group related haptens and internal standard a-Arabinopentaose including pseudomolecular precursor ions $[M-H]^-$ and fragment-ions as used in final negative ion mode MRM LC-ESI-MS method. Structural representations of oligosaccharides were drawn with Glycoworkbench [71] according to CFG proposals [72]: Hexose (Hex) ○, N-Acetylhexosamine (HexNAc) □, a-Arabinose (Ara), Glucose (Glc) ●, Galactose (Gal) ●, N-Acetylglucosmanine (GlcNAc) ■, Fucose (Fuc) ▲, N-Acetylneuraminic acid (Neu5Ac) ◆. Annotation of fragments follows the nomenclature introduced by Domon and Costello [73]

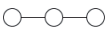
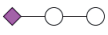
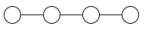
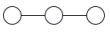
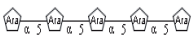
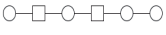
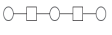
Nr.	Oligosaccharide	Oligosaccharide Structure	MRM-Transitions Precursor [*m/z*]	Fragment [*m/z*]	Possible Fragment ion types
11	$(Hex)_2$ / Lactose		341.1089	179	C_1
12	$(Hex)_3$ / Galactosyllactose		503.1618	341	 C_2
13	SL / $(Hex)_2(NeuAc)_1$		632.2044	290	 B_1
14	$(Hex)_4$		665.2146	503	 C_3
15	a-Arabinopentaose (internal standard)		677.2148	587	 $^{0,2}X_5$
16	LST / $(Hex)_3(HexNAc)_1(Neu5Ac)_1$		997.3365	290	 B_1
17	LNH / $(Hex)_4(HexNAc)_2$		1071.3733	909	 C_5

Table 2 | Continued.

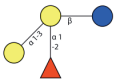
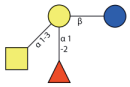
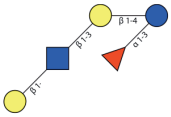
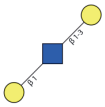
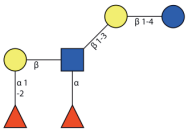
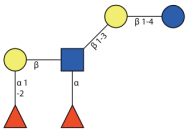
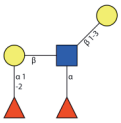
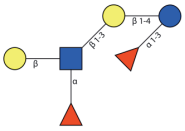
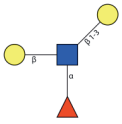
Nr.	Oligosaccharide	Oligosaccharide Structure	MRM-Transitions Precursor [*m/z*]	MRM-Transitions Fragment [*m/z*]	Possible Fragment ion types
18	Tentative Blood Group B-Tetrasaccharide / $(Hex)_3(Fuc)_1$		649.2197	179	 C_1
19	Tentative Blood Group A-Tetrasaccharide / $(Hex)_2(HexNAc)_1(Fuc)_1$		690.2462	220	 C_1
20	LNFP V or LNFP VI		852.2990	544	 C_3
21	LNDFH I or LnNDFH I		998.3569	325	 C_2
22	LNDFH I or LNnDFH I		998.3569	836	 C_4
23	LNDFH II or LNnDFH II		998.3569	690	 C_3

Figure 4 shows the resulting UV trace at 215nm wavelength, the total ion chromatogram (TIC), and the MRM traces of multiple HMO structures. By tracking the UV absorbance of the LC effluent at 215 and 280 nm wavelength we intended to monitor the presence of possible additional non HMO compounds not targeted by MRM. For example, peptides or other small molecules might also pas through the 3kDa ultrafiltration devices used for sample clean up. Fortunately, no indication for abundant elution of such compounds was found among samples subjected to analysis. The 3kDA ultrafiltration obviously retained the majority of other compounds like e.g., HM proteins or peptides. The peaks visible in the blue shaded ranges of the UV trace in **Figure 4a**) do only represent absorbance variations triggered by changes in the used LC-gradient rather than additional HM compounds or small molecules co-eluting with HMOs. Those eluent dependent peaks reproducibly occurred throughout all sample runs but also in the blank runs.

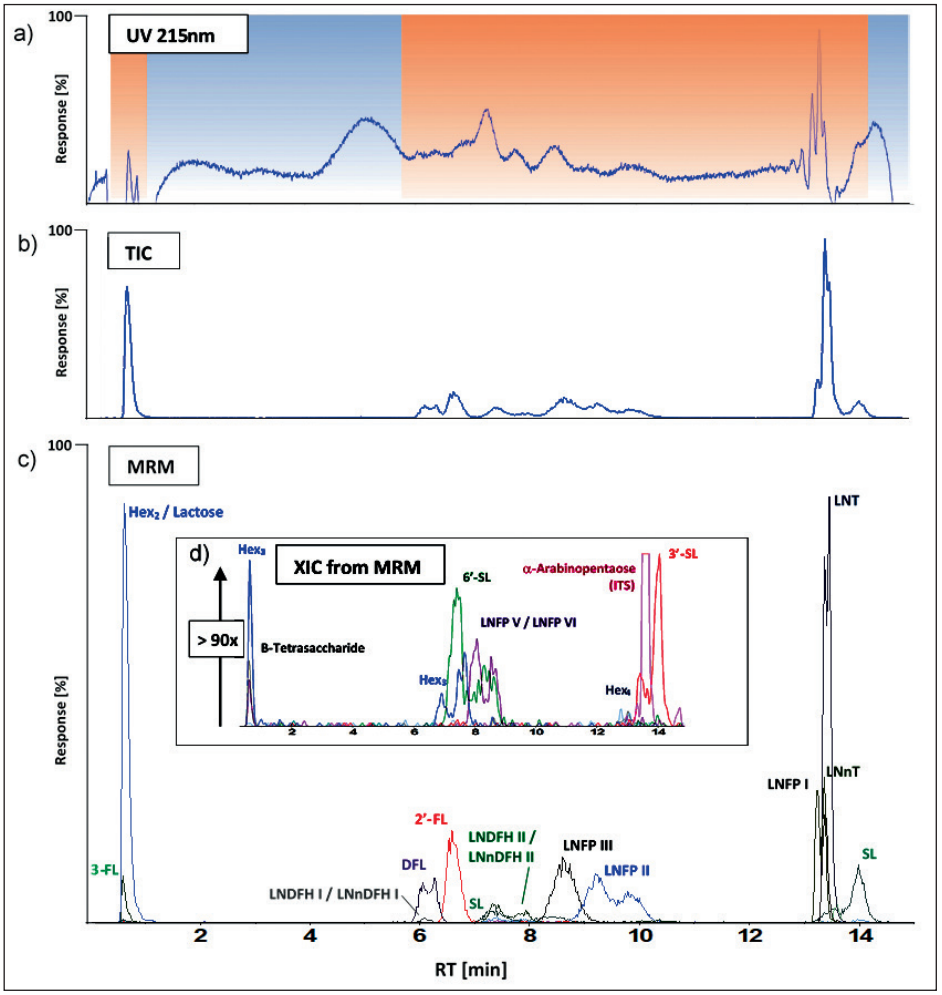


Figure 4 | MRM LC-ESI-MS analysis of OS in human milk; a) UV trace at 215nm wavelength; HMO range shaded in red; b) Total ion chromatogram (TIC); c) MRM traces of individual HMOs (for compound specific MRM-transitions and related HMO structures see Table 1 + Table 2); HMOs in order of elution: Hex_2/Lactose (blue); 3-FL (green); LNDFH II/LNnDFH II (grey); DFL (magenta); 2'-FL (red); LNDFH I/LNnDFH I (green); SL (blue green); LNFP III (black); LNFP II (blue); LNDFH I/LNnDFH I (green); LNFP I (dark yellow); LNnT (brown); LNT (dark blue); SL (blue green); insert d): Magnified section of extracted ion chromatograms (XIC) derived from c) and normalized to 3'-SL, showing low abundant glycans: Hex_3 (blue), Tentative Blood Group B-Tetrasaccharide (grey); Hex_3 (blue); LNFP V (magenta); 6'-SL (green); Hex_4 (light blue); a-Arabinopentaose (internal standard, pink), 3'-SL (red)

If UV and TIC-traces are compared to the MRM-profiles (**Figure 4 a)-d)**), the advantage of increased specificity for particular HMO structures which is inherent in the applied MRM methodology becomes evident. The UV or TIC signals alone do not provide any structural or compositional information to confirm peak identity without prior

calibration by appropriate and often rarely available standards. In contrast, the applied MRM LC-MS technology does: By using specific MRM transitions, multiple and partly isomeric HMO structures could be identified in parallel within only one run. Even HMO isomers with the same molecular weight as LNFP I, II, III or V could be clearly distinguished. Moreover, the applied H_2O-EtOH gradient also facilitated full separation of LNFP I from the other human milk LNFP-isomers. LNFP II, III and V were at least partly resolved as well. Compared with published LC-MS approaches of Wu et al. 2010 [54], the LC separation of LNFPI, LNFP III and DFL was clearly improved with our adapted EtOH-gradient. These authors used PGC as stationary phase in form of a HPLC chip and converted HMOs into their respective alditols to avoid appearance of anomeric peaks. Nevertheless, LNFP III and DFL did still elute at identical retention times while these HMOs were well separated with our gradient although we injected native HMOs. Also the LNFP I and LNFP III retention differed only by 0.14 min in the 45 min gradient applied in the method of Wu et al.. In contrast, the difference in retention time was approximately 7 min in our approach which enabled secure distinction within a total LC run time of only 17 min.

However, despite optimization, some major HMOs like the Lacto-N-tetraose-isomers LNT and LNnT were still not sufficiently resolved and co-eluted (see **Figure 4 c**). The same finding applied to $(Hex)_2$/ Lactose, 3-FL, some low abundant HM glycans (like the tentative Blood Group B-Tetrasaccharide / $(Hex)_3(Fuc)_1$), but also to one of the different detected $(Hex)_3$-peaks. Usage of the applied compound specific MRM-transitions clearly obliterated the dependence on (baseline) resolution for both, identification and (relative) quantitation. Although, at the current state of development, our approach could monitor fewer HMO compositions compared to the impressive MRM- or chip TOF LC-MS methods published e.g., by Hong [6] et al. or De Leoz et al. [77], we benefited from higher throughput by shorter analysis times. Furthermore and in contrast to the MRM approaches published before [6], higher HMO compound specificity is achieved here by replacement of rather unspecific oxonium ions in MRM-transitions by more diagnostic fragment ions (see Table 1 & Table 2). Usage of such diagnostic fragment ions allows the analyst to discern the following major structural features of HMOs: Positon of fucose (Fuc) residues and neuraminic acid (Neu5Ac) residues relative to the HMO backbone and also type 1 or 2 backbone structures. Thus, inherent structural chemical features of glycans can now directly be monitored through targeted MS/MS provided by MRM. In theory, this should be possible even without prior LC-separation.

Quantitative variation in the detection of individual HMOs was estimated by 10 repetitive injections of the same HM sample and subsequent determination of relative standard deviations (RSD) per HMO. The resulting RSDs ranged from < 12 % for LNFP I, LNFP II, LNFP III, LNT, LNnT over up to 20% for total SL, $(Hex)_3$, 3'-SL, 6'-SL, $(Hex)_2$ / Lac to ≤ 38 % for all other HMOs detectable in this sample (see

Figure S2 in the supplements). One reason for HMO structure related differences in precision of quantitation could be that the properties of the internal standard α-Arabinopentaose better resemble LNFP I, LNFP II, LNFP III, LNT, LNnT and the Sialyllactoses. Therefore, additional complementary internal standards like α-Arabinoheptaose or α-Arabinotriose might be used in future to achieve better results also for HMOs now detected with less precision.

In sum, with negative ion mode MRM LC-ESI-MS we achieved parallel identification and relative quantification for more than 20 human milk glycan structures in one efficient approach (see Table 1, Table 2, **Figure 4** and **Figure 5**). Applicability of this method could be proven for purified, defined HMO fractions but also for whole human milk samples. Identified glycan structures included $(Hex)_2$ (=Lactose by specification via retention time), neutral non fucosylated or partly fucosylated HMOs such as trioses (Hex_3 (e.g., galactosylactoses), 2'-FL or 3-FL), tetraoses (DFL, LNT, LNnT), pentaoses (LNFPI–V), and Hexaoses (e.g., LNDFH I-II or LNnDFH I-II). Also, acidic HMOs (3'-SL, 6'-SL) and tentative blood group A and B like determinants in form of free human milk tetraoses could be traced. Moreover, the introduction of an affordable internal standard (α-Arabinopentaose) facilitated normalization and relative quantitation of MS raw data. Given the fact that ethanol, a less harmful replacement of more commonly used acetonitrile, was employed for gradient formation, this LC-MS method might be considered as an environmentally friendlier analytical alternative for HMO characterization.

Suitability of negative ion mode MRM LC-ESI-MS for determination of human milk groups in comparison with HPAEC

In a next step, four human milk samples representing the four currently known human milk groups were compared with the final MRM LC-ESI-MS method. The milk group status of each individual sample had been thoroughly characterized by HPAEC before. Thereby it was possible to benchmark the milk typing performance of our label free MRM LC-ESI-MS method against the well-established HPAEC methodology (see **Figures 5** & **6**) for oligosaccharide analysis. Compared to HPAEC or to other commonly used analytical methods like untargeted LC-MS or CE, targeted MRM LC-ESI-MS offered the advantage of relatively short run times and better selectivity. For example, it took only ~17 min to perform one MRM LC-ESI-MS run. In contrast, 40-70 minutes are consumed on average to finish OS analyses by HPAEC [60, 78], by nanoESI-LC chipTOF-MS [79] or by CE [38, 80]. As an exception, Xu et al described an impressive label free 10 minute MRM LC-ESI-MS approach similar to ours [81]. This ultra-high pressure LC-MS (UPLC-MS) approach relies on positive ion mode. Although being very fast and able to monitor at least 31 different HMOs - monosaccharide-compositions, it lacks the ability to separate between isomers like type I and type II tetrasaccharides LNT and LNnT. Also 3'-SL, 6'-SL, or Lacto-N-Fucopenatose isomers different from LNFP I can't be distinguished. One of those LNFP isomer is LNPF II

another Le-/Se-gene related compound which can be used for determination of human milk groups in conjunction with the also Le-/Se-gene dependent LNFP I. As outlined before, our negative ion MRM LC-ESI-MS setup on the other hand provided clear distinction between such (isomeric) HMOs as shown in **Figure 4**. Thus, this methodological advantage helped to perform human milk group analysis based on detection of marker HMOs like LNFP I and LNFP II. The extracted ion chromatograms displayed in **Figure 5** visualize that group I milk contains e.g., LNFPI and LNFP II, group II milk lacks LNFP I but contains LNFP II, group III milk only contains LNFP I and group IV milk finally doesn't show any of these Lacto-N-fucopentaose-markers. The same findings had been elicited by prior HPAEC analysis of these samples. Consequently, MRM LC-ESI-MS was correctly confirming HPAEC results in line with the biological expectations dictated by the known Se-/Le-status of tested HM specimens [82]. For comparison with LC-MS (**Figure 5**), **Figure 6** exemplifies one HPAEC HMO profile yielded for the group I human milk sample. Furthermore, HPAEC and MRM LC-ESI-MS did not only correspond in Lacto-N-fucopentaose or Lacto-N-tetraose-profiles but also in all other HMO structures accessible by both methods (data not shown). This suggests comparable suitability of HPAEC and MRM LC-ESI-MS for human milk typing and glycorofiling with the advantage of shorter analysis times using MRM LC-ESI-MS.

DISCUSSION

In this study, several diagnostic, HMO configuration-dependent fragment ions could be described and translated into a novel MRM LC-ESI-MS method. Thereby, multiplexed and targeted MRM LC-ESI-MS analysis of relevant HMOs up to DP 6 (neutral and acidic) became possible utilizing more than 20 MRM-transitions in parallel in negative ion mode. Identification as well as relative quantitation of such HMOs and tentative blood group A and B determinants could be accomplished with efficient LC-MS run times of ~17 min.

Compared to similar contemporary LC-MS approaches [6], a higher selectivity for particular HMO structures was obtained by exploiting specific MS/MS signatures and their translation into MRM-transitions. Especially the position of fucose residues relative to the HMO backbone and, moreover, the HMO backbone type itself (type I or type II) could be determined thereby. The analytical LC-MS setup was further facilitated by an environmentally benign ethanol-gradient. Overall our MRM LC-ESI-MS approach offered increased efficiency and ease of use also with respect to a more effortless sample pretreatment (only aqueous dilution of HM, addition of internal standard and 3kDa UF).

Application of this MRM LC-ESI-MS concept to real world human milk study samples proved proper distinction of partly isomeric HMOs and correct assignment of individual HM samples to one of the 4 currently known human milk groups.

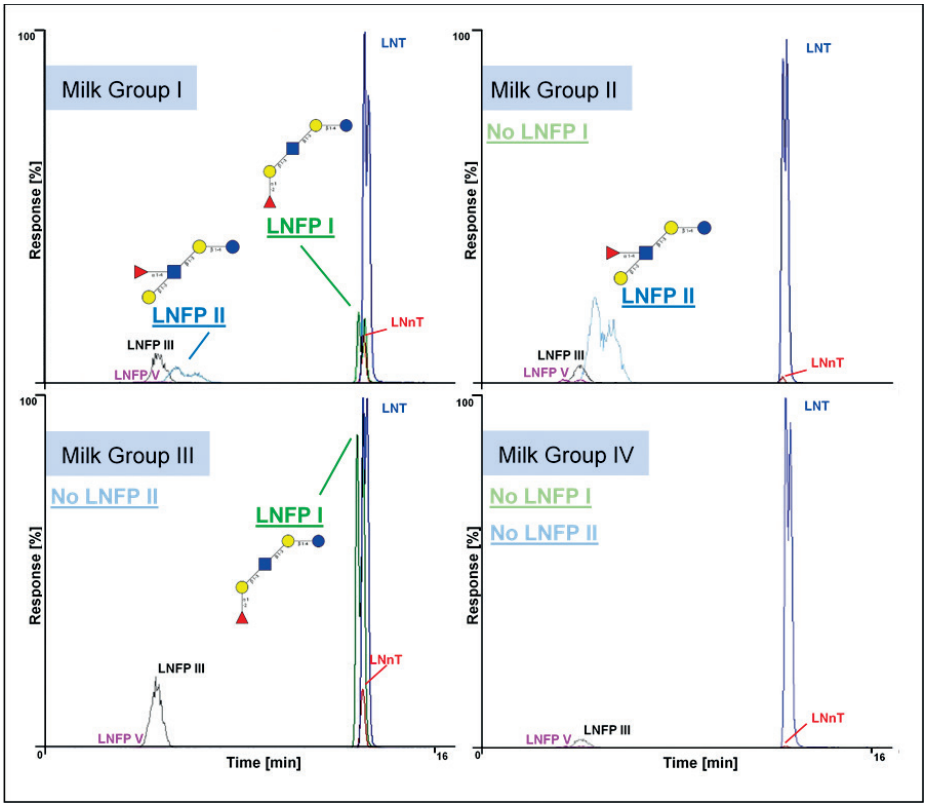


Figure 5 | Extracted ion chromatograms (XIC) of specific LNT- & LNFP-isomer after MRM LC-ESI-MS analyses of human milk samples belonging to either human milk group I, II, III, or IV. Multi-compound detection of HMOs including LNFP- and LNT-isomers was achieved. The 4 different milk groups are discernible on the basis of the absence or presence of LNFP I, LNFP II or other fucosylated HMOs which are products of fucosyltransferases FUT2 and FUT3. FUT 2 and FUT 3 are controlled by Le- and Se-gene activity. Milk group I is characterized by e.g., presence of LNPF I and LNFP II. Milk group II contains LNFP II but no LNPF I. Milk group III has LNFP I but not LNFP II. Finally, milk group IV neither contains LNFP I nor LNFP II. All milk groups display FUT 2 and FUT3 unrelated HMOs like LNT or LNnT. Unlike with HPAEC (see Figure 6), all present LNT and LNFP isomers including the low abundant LNFP V could be clearly distinguished from each other due to selectivity of MRM LC-ESI-MS. Structural representations of oligosaccharides were drawn with Glycoworkbench[71] according to CFG proposals [72]: Glucose (Glc)●, Galactose (Gal)○, N-Acetylglucosmanine (GlcNAc)■, Fucose (Fuc)▲.

The high specificity of this MRM LC-ESI-MS concept for particular HMO structures bears potential for further methodological improvement like column free MRM ESI-MS milk group typing.

This might allow for even faster direct detection and identification of several prominent fucosylated or sialylated human milk oligosaccharides like 2'-FL, 3-FL, 3'-SL, 6'-SL, DFL, LNT, LNnT, LNFP I, LNFP II, LNFP III, LNFP V (see Table 1, results and conclusions section). Of course, possible adverse influences of LC free MRM ESI-MS due to co-elution of major compounds like Lactose and less abundant HMOs should be carefully investigated before. They could compromise ionization efficiency and sensitivity for low concentrated HMOs.

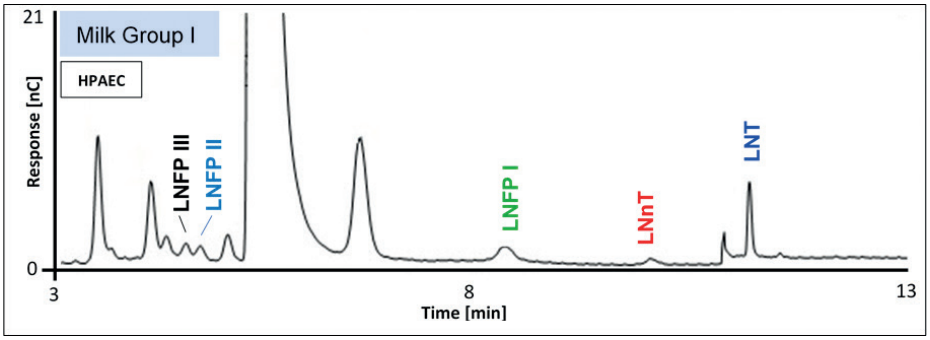


Figure 6 | Section of a HMO HPAEC-PAD-profile recorded for a milk group I sample. For easier comparison with extracted ion chromatograms (XICs) shown in Figure 5, only LNT and LNFP isomers have been labeled. With HPAEC and in contrast to MRM LC-ESI-MS (see Figure 5), LNFP V could not be unambiguously assigned as it was either below detection limit or co-eluted with other HMOs. HPAEC requires more extensive sample preparation and longer elution times to accommodate sufficient resolution and proper identification of HMOs compared to MRM LC-ESI-MS

Although our current approach is limited to HMOs up to DP 6, theoretically the identification and relative quantitation of higher molecular weight glycans with more than two fucose- or one sialic acid residue might be possible. In this case, information derived from multiple specific or unspecific MRM-transitions needs to be combined to achieve a reliable determination. The limitation is that we couldn't reveal any single diagnostic MS/MS-fragment by low energy CID which enabled unambiguous identification of (multiply) fucosylated or sialyated HMOs exceeding DP 6.

Due to the advantages of relatively short run times and ease of use, we hope that our new method will facilitate HMO analyses of larger sample sets and complete human milk cohort [83-85]). The expected expanded and thus statistically more meaningful HMO data sets could be useful to decipher the influence of certain early life factors (e.g., maternal genetic background and nutrition) on HMOs patterns. This

might in turn help to better understand the impact of human milk oligosaccharides on offspring's longitudinal health trajectories.

Finally, also absolute quantitation of recently introduced next generation infant milk ingredients like 2'-FL, LNnT or other HMOs could be imagined. At least, necessary pure 2'-FL and LNnT standards needed for validation of absolute HMOs quantitation are becoming commercially available at lower costs now. Apart from mere scientific application of MRM LC-ESI-MS and after slight adaption, quality control (QC) of HMOs, mammalian milk OS or dietary prebiotic glycans like galacto-/or fructooligosaccharides (GOS/FOS) may be an interesting additional option.

COMPLIANCE WITH ETHICAL STANDARDS

The usage and analysis of the described human milk samples has been performed in accordance with ethical standards and guidelines as laid down in the Declaration of Helsinki. Ethical approval and written consents of donors were given as stated in the publications of Thurl et al. 1993, 1997 and 2010 [15, 60, 86]. The same samples were already subjected to HMOs analysis in these prior studies. They were now used for optimization of a new LC-MS method but the purpose of usage (HMO analysis) was the same as stated in the former publications of Thurl et al. [60, 86] for which ethical approval was received.

CONFLICT OF INTEREST

Marko Mank and Bernd Stahl are employees of Nutricia Research B.V.. Philipp Welsch was enrolled as PhD student at Technical University of Dresden during this study and performed the practical part of his PhD thesis at Danone Nutricia Research in Utrecht. Albert J.R. Heck is Professor at Utrecht University. The present research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors. None of the authors have further conflicts of interest with regard to the content of this manuscript.

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SUPPLEMENTS

Isolation and characterization of defined molecular weight human milk oligosaccharide (HMO) fractions by gel permeation chromatography (GPC)

GPC fractionation was performed on a 2 x 110 cm low pressure LC-system using 5 cm inner diameter glass columns (Kronlab, Germany) filled with Toyopearl HW 40S stationary phase (Tosoh Bioscience). Approximately 1g of a carbohydrate (CH) mineral fraction dissolved in 5ml ultrapure H_2O was injected per LC run. Isocratic elution of HMOs was done with 2% isopropanol for 2100 minutes at a flow rate of 1,65ml / min at 55 °C. The effluent was monitored by refractive index (RI) detection. HMOs typically eluted between 700 and 1700 minutes. In the latter time range, fractions were collected in 7 min intervals by means of a Superfrac fraction collector (Pharmacia LKB). Resulting HMO fractions of defined molecular weights were concentrated by vacuum evaporation to dryness (40°C, 20mbar), re-dissolved in ultrapure lab water (MilliQ, 18 kW, <5ppm TOC), filtrated (0.2-micron polyethersulfone (PES) filters), freeze dried (-5°C, 20mbar) and stored at -20°C until further use.

Sub-fractionation of HMO GPC fractions into isomerically pure Lacto-N-tetraoses (LNTs), Lacto-N-fucopentaoses (LNFPs), and Lacto-N-difucohexaoses (LNDFHs) by high performance liquid chromatography (HPLC)

The reversed phase sub fractionation of HMO GPC fractions of defined molecular weights to isolate pure HMO isomers was accomplished as follows: A 10 mg / ml HMO-solution was prepared from freeze dried GPC fractions by re-dissolution in ultrapure H_2O. Per run, 50 ml of this HMO solution was injected into a Varian Pro Star HPLC system and separation was enabled by a series of two 250 mm Nucleosil C18-rp columns and a 10mm pre-column (Machery & Nagel, Germany). Nucleosil particle size was 5mm. The HPLC columns had 8 mm inner diameter. Isocratic elution of HMOs was achieved with ultrapure water at a flow rate of 1,2 ml / min at 25 °C. Eluting HMO fractions were monitored via UV detection at 215 nm and collected in 15 second intervals. Resulting HMO isomer fractions were concentrated to dryness by vacuum evaporation (40°C, 20 mbar), re-dissolved in ultrapure lab water (MilliQ, 18 kW, < 5ppm TOC), filtrated (0.2-micron polyethersulfone (PES) filters), freeze dried (-5°C, 20mbar) and stored at -20°C until further use.

Table S1 | LC-Gradient of the ESI-LC-MS/MS method

Time [min]	A (%)	B (%)
0.00	98.0	2.0
1.00	98.0	2.0
1.54	98.0	2.0
2.64	91.5	8.5
4.18	91.0	9.0
8.00	91.0	9.0
9.50	89.0	11.0
9.82	86.0	14.0
10.70	75.0	25.0
11.80	70.0	30.0
12.90	0.0	100.0
14.22	0.0	100.0
14.66	98.0	2.0
17.74	98.0	2.0

Table S2 | Optimized ion source and HMOs compound related parameters for final negative ion mode ESI-LC-MS/MS method

Source voltage	-4500 V
Temperature	400 °C
Curtain gas (N_2)	11.0 psi
Ion source gas 1 (zero air)	50 psi
Ion source gas 2 (zero air	36 psi
Declustering potential	-44.0 V
CAD gas (N_2)	pressure = high
Entrance potential	-4.6 V
Collision energy	-29.0 eV
Collision cell exit potential	- 3.0 V

Table S3 | LC-MS peak detection and integration settings

Parameter	**Value**
Bunching factor	From 5 (narrow peaks) up to 30 (wide peaks)
Smoothing factor	6 (9 for LNFPV)
Separation width	0.2
Noise threshold	50 cps
Area threshold	40 cps
Retention time	Compound dependent
Retention time tolerance	6 minutes (manual extension up to 15min if required)

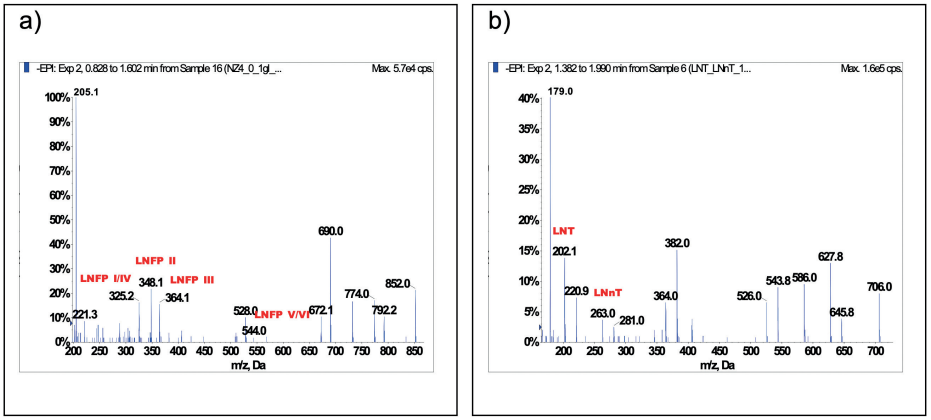

Figure S1 | Negative ion mode EPI LC-ESI-MS² spectra of a) a Lacto-N-Fucopentaose standard containing 4 isomers (LNPF I, II, III, and V) and b) a Lacto-N-tetraose-standard consisting of 2 isomers (LNT and LNnT). [M-H]⁻-precursor ions selected for MS² in EPI mode were at *m/z* 852 in case of LNFPs and at *m/z* 706 for LNTs. Isomer specific diagnostic MS²-fragments are assigned in red letters as LNFP I/IV (*m/z* 325), LNFP II (*m/z* 348), LNFP III (*m/z* 364), LNFP V/VI (*m/z* 544), LNT (*m/z* 202) and LNnT (*m/z* 263). Detected LNFP- or LNT-isomer specific diagnostic CID fragments comply with published findings achieved with direct static nESI-QIT-MS/MS experiments [1, 2]. All MS²-spectra were acquired at -29eV collision energy.

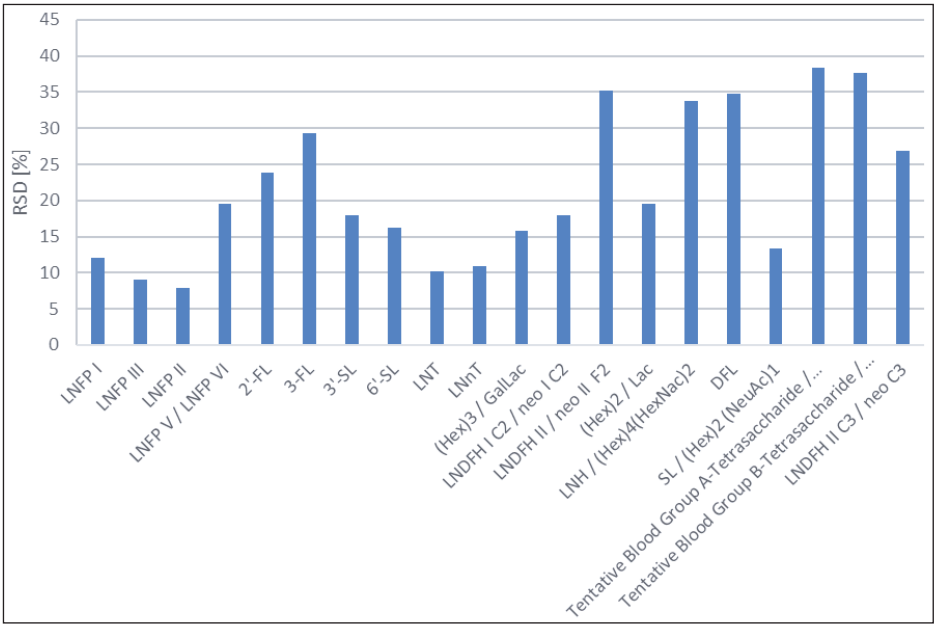

Figure S2 | Relative standard deviations (RSD) in percent for particular HMOs after 10 repetitive MRM LC-ESI-MS analyses of a human milk sample

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CHAPTER IV

Targeted LC-ESI-MS^2-characterization of human milk oligosaccharide diversity at 6 to 16 weeks post partum reveals clear staging effects and distinctive milk groups

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ABSTRACT

Many molecular components in human milk (HM), such as human milk oligosaccharides (HMOs), assist in the healthy development of infants. It has been hypothesized that the functional benefits of HM may be highly dependent on the abundance and individual fine structures of contained HMOs and that distinctive HM groups can be defined by their HMO-profiles.

However, the structural diversity and abundances of individual HMOs may also vary between milk donors and at different stages of lactations. Improvements in efficiency and selectivity of quantitative HMO-analysis are essential to further expand our understanding about the impact of HMOs variations on healthy early life development.

Hence, we applied here a targeted, highly selective and semi-quantitative LC-ESI-MS^2 approach by analyzing 2 x 30 mature human milk samples collected at 6- and 16-weeks post-partum. The analytical approach covered the most abundant HMOs up to hexasaccharides and, for the first time, also assigned blood group A and B tetrasaccharides.

Principal component analysis (PCA) was employed and allowed for automatic grouping and assignment of human milk samples to four human milk groups which are related to the maternal Secretor (Se) and Lewis (Le) genotypes.

We found that HMO-diversity varied significantly between these four HM groups. Variations were driven by HMOs being either dependent or independent of maternal genetic Se- and Le-status. We found preliminary evidence for an additional HM subgroup within the Se and Le positive HM group I. Furthermore, the abundances of 6 distinct HMOs structures (including 6'-SL and 3-FL) changed significantly with progression of lactation.

Keywords

Human milk oligosaccharides (HMOs), stages of lactation, human milk groups / milktyping, targeted LC-MS^2, principal compound analysis (PCA), secretor (Se) and lewis (Le) gene dependent or independent HMOs, 3-FL, 6'-SL

INTRODUCTION

Human milk (HM) is largely defined by the abundance of 5 major constituents comprising lactose, lipids, oligosaccharides, proteins, and water. Published values for lactose, lipid and protein concentrations in mature milk 1-2 month post-partum (pp) are 67-78 g/L, 32-36 g/L, and 9-12 g/L [1] respectively. Even broader ranges in concentration of HM components apply if also colostral and transitional milks (5-14 days pp) are considered [2]. The reported concentrations for total HMOs in mature milk range between ~3-18 g/L [3-8]. Until now, more than 150 HMO-structures have been revealed and confirmed by various analytical approaches including HPLC, mass spectrometry and NMR to name but a few [9-13]. Abundant individual HMOs like 2'-Fucosyllactose (2'-FL), Lacto-N-tetraose (LNT), Lacto-N-neotetraose (LNnT), Lacto-N-fucopentaose I (LNFP I), Difucosyl-lacto-N-hexaose II (DF-LNH II), Trifucosyl-lacto-N-hexaose (TF-LNH), 6'-Sialyllactose (6'-SL), and Disialyl-lacto-N-tetraose(DSLNT) may be present in the low gram per liter range [3]. This set of known HMO-structures represents only a few of more than 1000 compositional or structural variants as anticipated from MALDI-MS data of high molecular weight HMO-fractions with molecular masses > 8 kDa. The latter mass range translates into a degree of polymerization (DP) for particular HMOs > 43 [4].

Additional variations in human milk oligosaccharide profiles can be introduced by preterm delivery (lower lactose concentrations compared to term birth [14]) or are observed at different stages of lactation. In fact, literature reports an overall decrease of HMOs [15-17] over time during stages of lactation and the highest concentration of approximately 20 g/L total HMOs for term delivered infants is described to appear on around day 4 postpartum [18]. Staging effects are also observed for other mammalian milks like bovine or caprine milk [19-22]. Finally, also regional differences may have an influence on HMO-variations exemplified by 100 % presence of 2'-FL in milks of 156 Mexican women versus only 46% presence in milks of 26 Philippine donors [23]. Beyond their presence in human milk, HMOs have also been found in blood samples of both, mothers (gestational week 10-35) and breastfed offspring [24-28] and are even found in the amniotic fluid [29].

Bovine milk which is the basis for many infant milk formulations contains on average less lactose (approximately 40-50 g/L lactose[30]) and only minute amounts of bovine milk oligosaccharides (BMOs). BMOs comprise about 40 structures [31]. Among these BMOs, 3'- and 6'-Sialylactose are prevalent, but also Galactosyllactoses (Gal-Lacs) may be of interest. As reported by Nakamura et al. in 2003 [20], Sialyllactose-concentrations in bovine milk samples from 3 days post-partum range between 0.1 g/L for 3'-SL and < 0.05g/L for 6'-SL. Mature bovine milk contains approximately 0.03-0.12 g/L 3'-SL and 0.03-0.09 g/L 6´-SL [32] which is below the mean concentrations found in term human milk for 3'-SL (0.16 g/L) and 6'-SL (0.6 g/ L) [3].

Biosynthesis of HMOs is governed by a limited set of glycosyltransferases present in the mammary gland and human milk itself [12]. Therefore, with only a few exceptions, the structural arrangement of most human milk oligosaccharides can be described by the following general scheme (see also Fig. 1): The sequence of each HMO-molecule starts with a Lactose building block (Galβ1–4Glc) introducing a reducing end which is a common feature of all known HMOs. This lactose building block can be extended with a disaccharide residue consisting of galactose and N-Actetylglucosamine (Gal-GlcNAc). As suggested by Kobata et al. 2010 [33], the transfer of Gal-GlcNAc to the terminal Gal of Lactose may be accomplished by consecutive action of either β-1,3-N-acetylglucosaminyltransferase (iGnT) [34] and β1,4-galactosyltransferases (β4GalTs) [35] or iGnT and β1,3-galactosyltransferases (β3GalTs)[36]. Thereby, two basic types of HMO-core-structures can be created: A type I core (Galβ1–3GlcNAcβ1–3Galβ1–4Glc, LNT) or a type II core molecule (Galβ1–4GlcNAcβ1–3Galβ1–4Glc, LNnT). In HM, type I core HMOs are predominant over type II structures [37]. This feature distinguishes HM from other species including apes and monkeys. According to Urashima et al. [37], one possible explanation for this phenomenon is the role of type I oligosaccharides as important substrates for beneficial bifidobacteria. Moreover, the two types of neutral core entities (i.e. LNT or LNnT) may be further extended by successive addition of more Gal-GlcNAc blocks. If Gal-GlcNAc residues are appended to the core structures via β 1,3 glycosidic linkage, no further elongation will occur after this Gal-GlcNAc block. If, in contrast, Gal-GlcNAc dimers are added via action of β6N-acetylglucosaminyltransferase (IGNT) [38] through β1,6-glycosidic linkages, branching will occur and further elongation of the β1,6-glycosidically linked branches is possible. Thereby, up to 19 complex HMO-core structures can be created [39]. Finally, these core structures or lactose itself may be furthermore decorated by additional fucoses (Fuc), and sialic acid (Neu5Ac) moieties through human fucosyl-[40] or Sialyl-transferases[41]. However, it remains to be explored which of the known human Sialyl- and Fucosyltranferases are actually actively involved in the mammary gland biosynthesis of HMOs. More certainty already exists with respect to HMO-fucosylation exerted by the Se-enzyme [42]and Fucosyltransferase 3 [43]as explained in the next paragraph. Finally, exceptions from the general HMO building scheme outlined above may exist and are e.g. exemplified by the presence of Galactosyllactoses (GL) in human milk. Here, only a single Gal is added to Lactose. Literature describes several Galactosyllactose-isomers such as 6'-GL, 3'-GL, and 4-'GL. To date, information about GL concentrations in HM is still rare, but first published levels are in the milligram per liter range [44].

Inherited maternal genetic predispositions may induce remarkable inter-individual differences in oligosaccharide synthesis and resulting individual HMO-profiles. For instance, Secretor (Se) and Lewis (Le) genes on chromosome 19 encode for two different fucosyltranferases: The Se-enzyme and Fucosyltransferase 3 (Fuc-TIII) [40]. The Se-enzyme is expressed in the case of an active Se gene (FUT 2) and Fuc-TIII in

the case of an active Le gene (FUT 3). The Se-enzyme specifically appends Fucose (Fuc) via a 1,2-glycosidic linkage to type 1 HMOs (e.g. Lacto-N-tetraose (LNT)) or to type 2 HMOs (e.g. Lacto-N neotetraose (LNnT)), whereas in contrast Fuc-TIII catalyzes the formation of a 1,3/4-fucosylated HMOs. Primarily catalyzed by the Se-enzyme and Fuc-TIII, 4 different ways of HMO-fucosylation at distinct monosaccharides and distinct sites within type I or II core HMO-structures can occur [12]: a1,2-linkage to non-reducing end Gal, a1,3- or a1,4-linkage to N-Acetylglucosamine (GlcNAc), or a1,3-linkage to reducing end Glc. Neutral or sialylated acidic HMOs with core structures smaller or larger than tetraoses can be decorated in the same way and lead to a multitude of known or hypothetically possible fucosylated HMO-structures.

Figure 1 schematically exemplifies fucosylation of LNT (resembling a Lec-antigen[45]) and LNnT (Le I-antigen) by activity of Se-enzyme, FucTIII or further a1,3-Fucosyltransferases [46]. Resulting HMOs are e.g. Lacto-N-Fucopentaoses (LNFPs) such as LNFP I, II, III, and V. Such fucosylated HMOs can resemble ABO- or Se/Le-system related antigens like the Le^d-antigen in case of LNFP I, the Le^a-antigen in case of LNFP II, or the Le^x-antigen in case of LNFP III [45, 47]. Additionally, LNFP VI (the type II core variant of LNFP V) may be formed, too. It was recently detected in human milk by Bao et al. [48] using negative ion mode LC-MS after conversion of HMOs into alditols. Other products of the Se-enzyme, Fuc-TIII (or other a1,3-Fucosyltransferases) are human milk oligosaccharides like Fucosyllactoses (2'FL or 3-FL), Difucosyllactose (DFL), the Lacto-N-difucohexaoses LNDFH I (Le^b-antigen), LNnDFHI (Le^Y-antigen), LNDFH II and LNnDFH II or higher molecular weight neutral and acidic HMOs with either linear or branched core structures.

Based on the presence, absence or concomitance of a 1,2- and a 1,3/4-fucosylated HMOs like LNFP I and LNFP II in human milk, 4 different human milk groups (group I, II, III and IV) are currently distinguished [49, 50]. However, just recently, group I milks from Chinese donor were further sub-divided into 2 new subgroups based on significantly different levels in e.g. 2'-FL, DFL, LNT, LNFP I or F-LNO [51]. Assignment of human milk samples to one of the 4 main human milk groups I-IV can e.g. be performed by ESI-LC-MS or HPAEC HMO analysis and subsequent evaluation e.g. of the respective LNFP I and LNFP II patterns. Moreover, corresponding Le- and Se gene-product dependent glycan epitopes may also be found in maternal blood i.e. on erythrocytes or glycoproteins like mucins [52]. As mentioned before, other α 1,3-Fucosyltransferases such as plasma α-1,3-fucosyltransferase (E.C. 2.4.1.152), different from Se-enzyme or Fuc-TIII and independent from the Le blood group system, may be active in human milk donors as well [45, 53, 54].

Table 1 summarizes the interdependence of maternal Lewis and Secretor genotypes, encoded fucosyltransferases, Lewis blood group phenotypes, related carbohydrate

epitopes, resulting milk groups and some prevalent HMOs structures found in specific milk groups.

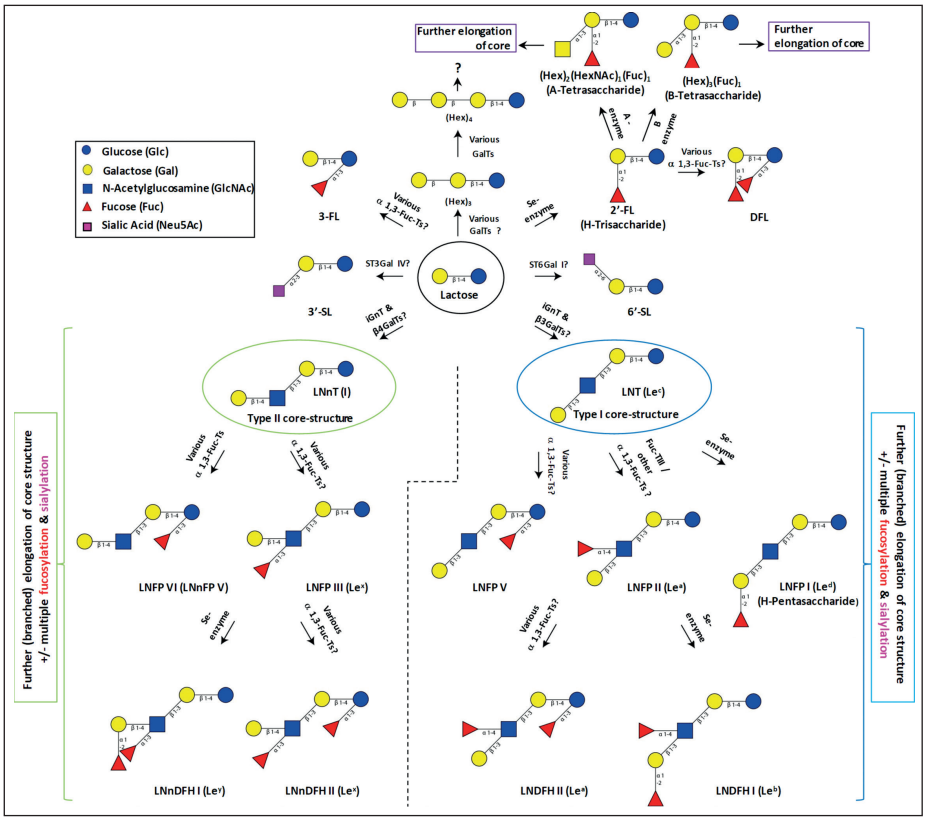


Figure 1 | Major HM HMO-structures and related glycosyltransferases [9, 40-42, 55] involved in their mammary gland biosynthesis starting from Lactose as precursor. Certain HMOs resemble ABH-[47] or Se/Le-system related antigens [39, 45] 3-Fucosyllactose (3'-FL), 2'-Fucosyllactose (2'-FL), 3'-Sialyllactose (3'-SL), 6'-Sialyllactose (6'-SL), Difucosyllactose (DFL), Lacto-N-Tetraose (LNT); Lacto-N-neoTetraose (LNnT); Tentative Blood Group B tetrasaccharide $(Hex)_3(Fuc)_1$; Tentative Blood Group A tetrasaccharide $(Hex)_2(HexNAc)_1(Fuc)_1$; 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-Fucopentose VI (LNFP VI = LNnFP V), Lacto-N-neoDifucohexaose I (LNnDFH I), Lacto-N-neoDifucohexaose II (LNnDFH II), Lacto-N-Difucohexaose I (LNDFH I), Lacto-N-Difucohexaose II (LNDFH II); Monosaccharide-symbols and structural representations of oligosaccharides were drawn with Glycoworkbench [56] according to CFG proposals [57].

Table 1 | Interdependence of maternal Lewis and Secretor genotypes, encoded fucosyltransferases (Fuc-Ts), Lewis blood groups, resulting carbohydrate epitopes, HMO-structures and milk groups. 2'-Fucosyllactose (2'-FL); Lacto-Di-Fucotetraose (LDFT); 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); Lacto-N-Difucohexaose I (LNDFH II). Adapted from: Oriol et al. 1986, Thurl et al. 1997, Blank et al. 2011

Genotypes		**Encoded Fucosyl-transferases / Gene Loci**	**Resulting Phenotypes**			**Human milk group**	**Prominent neutral HMO structures** (up to Hexaoses[a])
Secretor	Lewis		Erythrocytes		Human Milk		
			Lewis	Prevalence in France [45]	Fucosylated Carbohy-drate Epitopes		
Se/-	Le/-	Se-enzyme / FUT2, Fuc-TIII / FUT3	a-b+c-d-	69	Fuc (α1-2)R, Fuc (α1-4)R, Fuc (α1-3)[c]R	I	**2'-FL,** 3'-FL, **DFL,** LNT, LNnT, **LNFP I, LNFP II,** LNFPIII, **LNDFH I, LNDFH II**
se/se	Le/-	Fuc-T III / FUT3	a+b-c-d-	20	Fuc (α1-4)R, Fuc (α1-3)[c]R	II	3-FL, LNT, LNnT, **LNFP II**, LNFPIII, **LNDFH II;** *(2-'FL, DFL, LNFP I, LNDFH I)*
Se/-	le/le	Se-enzyme / FUT2	a-b-c-d+	9	Fuc (α1-2)R Fuc (α1-3)[c]R	III	**2'-FL**, 3'-FL, **DFL**, LNT, LNnT, **LNFP I**, LNFPIII; *(LNFP II, LNDFH I, LNDFH II)*
se/se	le/le	Le- and Se- indepen-dent Fuc-Ts (e.g. Fuc-TVI / FUT6 [54]	a-b-c+[b]d-	1	Fuc (α1-3)[c]R	IV	3-FL, LNT, LNnT, LNFPIII; *(2'-FL, DFL, LNFP I, LNFP II, LNDFH I, LNDFH II)*

[a]bold black letters indicate major HMO-structures present in human milks due to active Le or Se genes; italic letters in parentheses indicate HMO-structures lacking or being present in very low concentrations in human milk due to fully or partly inactive Le or Se genes; regular black letters indicate prominent HMOs occurring in HM independent from maternal Le/Se activity.

[b]According to Hanfland et al. 1986 [58], there may be more than one Le[c] antigenic determinant: Le[c] epitopes may comprise non-fucosylated Type I or Type II glycan residues or extended unbranched or branched glycan residues with α1-3-fucosylation at the penultimate GlcNAc positioned before the non-reducing end of the glycan chain.

[c] Fuc (α1-3-)R Epitope can also be catalyzed by Le/Se-independent FUT like e.g. FUT 6 [54]

HMOs confer many intriguing functional properties of which some are likely to facilitate or influence healthy development of neonates [59-63]. Many of the published prebiotic, anti-infective or immunomodulatory properties are highly structure specific [64-70]. They may vary between HMOs of the same monosaccharide composition, but different core types or fucosylation sites like LNFP I, LNFP II, or LNFP III. Also blood group A and B carbohydrate determinants can be present as free carbohydrates in HM [71]. Together with Galactosyllactoses, they deviate from the HMOs building scheme described above. Nevertheless, they may bear anti-infective potential relevant for early life and beyond e.g. by blocking the cholera toxin (CT) subunit B (CTB) of *V. cholerae* from adhesion to mucins and intestinal epithelial cells of the small intestine [72].

As outlined above, different structure-effect relationships could be attributed to HMOs which are isomers but share the same monosaccharide composition. Thus, development and application of hyphenated analytical methods like LC-MS which can distinguish such subtle structural HMOs features may be key to decipher still unknown (beneficial) biological functions of HMOs and other glycans. To accelerate the analysis of larger sample numbers available from e.g. human milk cohorts or clinical studies, analytical approaches with short run times and avoidance of tedious sample pre-treatment would be of advantage. In turn this will help to improve statistical association of distinct HMO-patterns with related health benefits for infants. In addition, (semi-) quantitative assays should be preferred over mere qualitative ones to allow for elucidation of concentration dependent biological HMO-effects.

Therefore in this work, we applied a recently developed targeted semi-quantitative LC-ESI-MS^2 approach with high structural selectivity [73], easy sample preparation and comparatively low run times of ~18min per sample to 2x30 human milk specimens. These specimens were derived from a control group of the INFAT study which was not subjected to nutritional intervention. The used sample set comprised human milks collected at 6 and 16 weeks post-partum to elicit possible changes in HMO-profiles between those 2 different stages of lactation.

Principal component analysis (PCA) was employed as convenient and efficient strategy for mining of structural HMO information inherent in the acquired MRM LC-ESI-MS^2 data sets.

EXPERIMENTAL SECTION

Human milk samples from INFAT study with unknown milk group and HMO-status

A subset of 60 human milk samples derived from 30 healthy women resident in southern Germany and enrolled in a dietary intervention study called the INFAT

study [74, 75] was subjected to MRM LC-ESI-MS^2 analysis. Every woman donated 2 milk samples at 6 and 16 weeks post-partum. Written consent from the donors was provided prior to analysis and approval was given by the ethical committee of the Faculty of Medicine of the Technical University of Munich, Germany (project number 1479/06 on 14th of September 2012). The human milk samples used for this LC-MS investigation were derived from a control group not subjected to dietary intervention. Samples were obtained as described elsewhere [74]. Briefly, maternal breast milk samples were collected in the study centre in the morning after an overnight fast in a standardized manner using a breast pump, aliquoted, and immediately stored at −86 °C until analysis. Samples were thawed once to take aliquots for different kinds of analysis and stored again at -20°C until further usage, such as for negative ion mode multiple reaction monitoring (MRM) LC-MS^2 analysis.

Chemicals, reagents & disposables

Ammonium acetate (p.a.), Acetonitrile (LC-MS grade), and Ethanol (gradient grade for HPLC) were from Merck, Darmstadt, Germany. LC-MS grade H_2O (HiPerSolv Chromanorm) was from Prolabo, VWR International, Darmstadt, Germany. Alternatively, Ultrapure lab water, 18 kW, <5ppm TOC produced by a MilliQ Advantage Ultrapure Water System from Merck Millipore (Darmstadt, Germany) was used. Clear glass 300 µl micro LC-MS vials were from Thermo Fisher Scientific (Waltham, MA, USA , #60180-507). Micro vial screw caps with pre-slit PTF/silicone membrane were from Thermo Scientific (Langerwehe, Germany). Two milliliter polypropylene LoBind® test tubes were from Eppendorf AG (Hamburg, Germany, #022431102). 500 µl volume Amicon Ultra centrifugal filter devices with 3kDa cut off were from Millipore (Billerica, MA, USA).

Preparation of human milk samples and MRM LC-ESI-MS2 analyses of HMOs

Pre-treatment of human milk samples, targeted semi-quantitative negative ion MRM LC-ESI-MS^2 analyses of HMOs, software settings, evaluation of data and manual assignment of human milk groups were essentially conducted as recently published in more detail by Mank et al. [73]. Briefly, preparation of HM milk samples was accomplished by adding 15 µl of α-L-Arabinopentaose internal standard (ITS) solution (0.05 mmol/l) to 135 µl of human milk. Frozen human milk samples were thawed at room temperature before analysis and were vortexed well. The combined sample-ITS-solution was further diluted 1:11 (v/v) in 2 mL-Eppendorf-test tubes by adding 1350 µl H_2O (LC-MS grade). 450 µl of the diluted mixture were transferred into a previously washed 500 µl Amicon Ultra centrifugal filter device with 3kDa cutoff. Ultrafiltration (UF) was performed at 14000 x g for 1 hour. The UF permeate was transferred into a 300 µl glass LC-MS- vial with screw top (Thermo Fisher Scientific, Waltham, MA, USA) and either stored at -20°C until further use or directly subjected to LC-MS analysis. Sample preparation steps were conducted at room temperature.

For negative ion mode multipe reaction monitoring (MRM) LC-ESI-MS^2 analyses, a 1100 series HPLC stack (Agilent, Santa Clara, CA, USA) with solvent rack, degasser, binary solvent pump, high performance autosampler, column oven, and DAD detector was coupled with a 3200 Qtrap triple quadruple linear ion trap MS instrument (Sciex, Framingham, MA, USA). A Hypercarb 2.1 x 30 mm porous graphitized carbon (PGC) column (5µm particle diameter) following a 2.1 x 10 mm Hypercarb pre-column (both Thermo Fisher Scientific, Waltham, MA USA) served as stationary phase. Column temperature was kept at 45°C, and sample temperature in the autosampler was 22°C. The DAD detector was used to monitor possible peptide or protein contaminations at 215 & 280 nm wavelength. 5µl sample solutions prepared as described above were injected into the LC-MS system. Carryover was minimized by flushing the sample needle externally for 5 seconds with 20% aqueous isopropanol after each injection and by running a water and isopropanol blank between the HM samples. Elution of HMOs was achieved with an ~18 min H_2O-EtoH gradient at a flow rate of 400 ml/min (see Table S1 in supplements). The solvents used to form the gradient were A (5 mM aqueous NH_4CH_3COO) and B (80 % EtOH (v/v) with 5 mM aqueous NH_4CH_3COO). Analyst 1.4 software (Sciex, Framingham, USA) was used to control the complete LC-MS configuration and for further evaluation of LC-MS data.

Using this LC-MS platform, a first set of 10 distinct, specific and partly isomeric HMO structures could be integrated into the targeted MRM LC-MS^2 method. HMO-specific MRM transition were based on monoisotopic molecular masses of deprotonated precursor ions and respective diagnostic fragment ions. Unit resolution was chosen to conduct MRM analyses. MRM transitions were continuously monitored in negative ion mode during the entire MRM LC-MS^2 gradient elution (see [73]). One LC-MS run took approximately 18 minutes. HMO-structures which were determined by this first set of (isomer-) specific MRM transitions included 2´-FL, 3-FL, 3´-SL, 6´-SL, DFL, LNT, LNnT, LNFP I, LNFP II, and LNFP III (see Table S2 in supplements). The correct identification of the latter HMOs structures via their respective MRM transitions was proven by analysis of pure HMO-standards (concentrations ~ 0,03-0,05 mg/ml) which were run together with the INFAT human milk study samples. The used pure HMOs standards had been isolated from pooled human milk and were identical to those already described in more detail by Mank et al 2019 [73].

Furthermore, a second set of 11 additional MRM transitions representing further OS structures could be included into the same LC-MS^2 approach as mentioned before (see Table S3 in supplements). Compared to the first MRM set, HMO monosaccharide compositions rather than exact structural features could be directly deduced from the fragment ions used in these MRM transitions (see [73]). Therefore, either one HMO-composition or up to two different structures were assigned per MRM transition in set 2. Compounds which could be characterized by this 2^{nd} set of

MRM transitions were: $(Hex)_2$ or Lactose, $(Hex)_3$ or Glactosyllactose, Sialyllactose or $(Hex)_2$ $(NeuAc)_1$, $(Hex)_4$, α-Arabinopentaose (internal standard), Tentative Blood Group B-Tetrasaccharide or $(Hex)_3(Fuc)_1$, Tentative Blood Group A-Tetrasaccharide or $(Hex)_2(HexNAc)_1(Fuc)_1$, LNFP V or LNFP VI, LNDFH I or LnNDFH I, LNDFH II or LNnDFH II. Despite the more putative assignment of the latter HMOs, at least LN(n)DFH I could also be distinguished from LN(n) DFH II due to a retention time delta of approximately 2 minutes as shown in Mank et al 2019 [73] All 60 human milk samples derived from the INFAT study were analysed by the MRM LC-ESI-MS^2 approach described above in a randomized and anonymized manner. For exemplary MRM LC-ESI-MS^2 traces of some pure HMOs standards (LNFPI, II, III, LNT, and LNnT) and of abundant HMOs in a HM study sample please see Figure S1 a)-f) in the supplements.

If not otherwise mentioned, the settings for identification of HMOs, peak integration, relative quantitation of HMOs using negative ion mode MRM LC-ESI-MS have been applied as described by Mank et al. [73]. Also, run to run variations for individual HMOs were determined as described in this reference by 10 repetitive injections of the same HM sample. Results were expressed as individual relative standard deviations (RSDs) for distinct HMOs: The resulting RSDs were less than 12% for LNFP I, LNFP II, LNFP III, LNT, and LNnT, up to 20% for total SL, $(Hex)_3$, 3'-SL, 6'-SL, $(Hex)_2$/Lac, and maximally 38% for all other targeted HMOs. After integration of peak areas, dimensionless responses R were calculated for each HMO peak in a MRM trace by dividing its area under the curve by the area under the curve yielded for the internal standard α-Arabinopentaose. The limit of detection (LOD) for individual HMOs was defined by a signal to noise (S/N) ratio of approximately 3:1 [76]. Peaks below LOD were not considered and reported as zero values.

For further processing and evaluation, original LC-MS raw data sets were exported from Analyst 1.4 software to Microsoft Excel version 14.0.7145.5000 (32 bit), Sciex Markerview version 1.2.1., or IBM SPSS version 19. Basic statistics (t-test, Welsch's t-test) were carried out within Markerview. Further statistical tests (see below) were accomplished with SPSS.

Manual assignment of HM samples to HM groups

Manual assignment of human milk INFAT study samples to one of the four commonly known human milk groups was based on the absence, presence or concomitance of LNFP I and LNFP II. Biosynthesis of these two HMOs and other HMOs is governed by the maternal Se/Le status (see Table 1). If both, LNFP I and LNFP II were detected in a sample, human milk group I was assigned. If LNFP I was absent but LNFP II was present, milk group II was assigned. If only LFNP I was detected but not LNFP II, group III was assigned and if neither LNFP I nor LNFP II could be detected then milk group IV was assigned. Peaks were considered for evaluation if the detected

responses R exceeded the value of 0.2. The distribution of HM samples across human milk groups I, II, II and IV was expressed in percentages and graphically visualized using SPSS software.

Automated HM group determination by principal component analyses (PCA) and statistical evaluation of lactation and HM group specific HMO features

To relieve future milk grouping attempts from tedious, time consuming manual inspection of analytical LC-MS raw data, principal component analysis (PCA) was used as a means to automatically recognize different milk groups. Dedicated software (Markerview version 1.2.1. (Sciex)) facilitated PCA analysis of 60 MRM LC-ESI-MS^2 HMO data sets derived from analyses of 2x30 HM samples (30 samples from 6 weeks and 30 samples from 16 weeks pp). The following parameters were used by the Markerview software to extract peak areas from MRM LC-ESI-MS^2 raw data: Smoothing half width: 9 points; noise percentage: 50.0 %; baseline subtraction window: 2.0 minutes; peak splitting factor: 2 points; retention time tolerance: 15.00 min; minimum peak intensity: 100 cps; minimum peak width: 4 points; minimum signal/noise ratio was set at 3:1. Prior to performing PCA, MRM LC-MS^2 peak areas of specific HMOs were first normalized against corresponding peak areas of the internal standard α-Arabinopentaose. Then normalized peak areas were converted into their natural logarithms and finally scaled by mean centering. Subsequently, PCA was performed. To investigate if abundances of individual HMO-structures differed significantly between human milk groups as identified by PCA, non-parametric Kruskal-Wallis H testing [77] was performed with SPSS statistics software version 19.0.0 (IBM, Armonk, USA) . Non-parametric Kruskal-Wallis H testing was applied as prior exploration of data sets by the Shapiro Wilk test [78] indicated non normal distribution of HMOs. The null hypothesis for non-parametric Kruskal-Wallis H testing was that medians of detected responses R for particular HMOs were the same between the milk groups. The considered significance level α was 0.05 and the confidence interval was 95%. The same LC-ESI-MS^2 HMO raw data sets as for PCA analysis were used, but quality of integration was carefully inspected and manually re-adjusted if peaks were not fully integrated using automatic integration. Welch's t-test [79] was applied to investigate if significant differences between HMO peak areas (normalized against the internal α-Arabinopentaose) were existing between major HM groups and possible sub-groups. Significance was reported if calculated P values were < 0.05 (α level of 0.05).

Moreover, variations in HMO abundances between HM samples from either 6 or 16 weeks pp were statistically elucidated by using student's t-test [80, 81] and Welch's t-test. Prior to t-testing, all data were interrogated by Levene's test for homoscedasticity [82]. Peak areas normalized against the internal α-Arabinopentaose were used as input for calculations. Significance was stated if calculated P values were < 0.05 (α level of 0.05). Only HMO- structures were considered which exhibited fold changes above 0.1 between the decadic logarithms

of arithmetric mean at 6 compared to 16 weeks pp. For HMOs showing significant variations under these conditions, the differences between arithmetic mean values between 6 and 16 weeks pp of individual HMOs were expressed in percentages relative to the 6 weeks values. Results were displayed as bar charts created with Microsoft Excel for Office 365.

RESULTS AND DISCUSSION

Our MRM LC-ESI-MS^2 approach with enhanced specificity for fucose positional HMO-isomers was applied to 60 unknown human milk samples. These milks originated from 30 donors resident in southern Germany which were enrolled in the INFAT study [74]. Specimens were randomly selected from a larger control group of women not subjected to dietary intervention during the study. Per donor, two human milk samples from two different stages of lactation (6 and 16 weeks post-partum) were analysed.

Figure 2 and **Figure 3** are summarizing resulting MRM LC-ESI-MS2 responses R (see experimental section) for HMO-structures as detected across the 60 studied HM samples. These major HMO-structures cover approximately > 50% of all HMOs contained in HM by quantity. It's noteworthy to mention that the applied semi-quantitative MRM LC-ESI-MS2 approach does not yield equimolar responses for all investigated HMO-structures. This is due to many factors including variations in ionization efficiency and fragmentation yields between specific HMOs. Nevertheless, relative differences in HMO concentration between samples or different stages of lactation can be accurately followed for individual HMO structures. Moreover, for some HMO-isomers like LNFP I, II, and III the MRM LC-ESI-MS^2 responses are similar enough to discriminate between biological abundances of these HMOs. This allows e.g. for reliable milk group assignment [73]. Also other HMOs like 3'SL and 6'-SL show quite similar MRM LC-ESI-MS^2 responses which enable reasonable direct comparisons of relative abundances. The same is true for LNT and LNnT.

Human milk groups shown in **Figure 2** and **Figure 3** were assigned following the "manual approach" (see experimental section) based on absence or presence of LNFP I and LNFP II. Hence, 77% of all HM samples (= 23 donors) could be binned into HM group I, 13% (= 4 donors) into HM group III, 7% (= 2 donors) into HM group II and 3% (=1 donor) into HM group IV. These proportions didn't change when the PCA based milk-grouping approach was applied alternatively (see below).

Principal Component Analyses (PCA) of HMO MRM LC-ESI-MS^2 data

As outlined in the experimental section, yielded LC-MS data were also subjected to unsupervised multivariate statistics using principal component analysis (PCA). This was done in an attempt to investigate three aspects:

1. The distinction of HM samples into (sub-)groups not discovered by the manual approach
2. The effect of progressing lactation on human milk group proportions between 6 to 16 weeks pp
3. The variations of Le/Se-status related or independent HMOs between HM groups

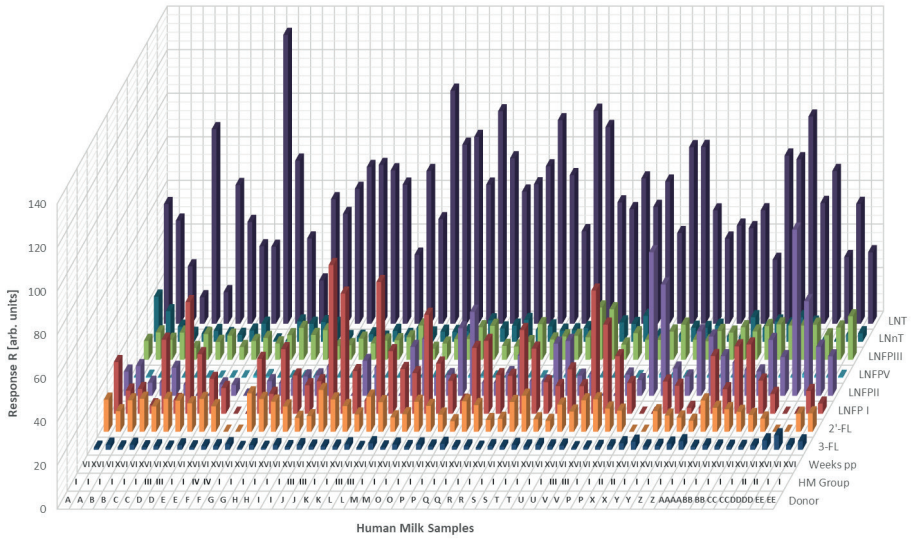


Figure 2 | HMOs detected across 60 HM samples with LC-ESI-MS2 (N=30 donors, 2 stages of lactation (6 w and 16w) per donor). Responses R are not strictly equimolar and ranged between 0 and 132. HMO-responses below LOD are reported with a zero value. Individual milk donors are indicated by different capital letters A-EE, HM groups by Roman numbers I-IV, and stages of lactation by Roman numbers (VI = 6 weeks pp, XVI=16 weeks pp).

PCA based grouping of HM milk samples

Fig. 4 shows the resulting PCA score plot which could explain approximately 77% of variances in the MRM LC-ESI-MS^2 data set (PC 1: ~45%, PC 2: ~32%). Notably, four separate sample clusters distinguished from each other (blue, red, cyan and orange dots). After post hoc inspection of LC-MS data sets for Se/Le-gene dependent milk group marker HMOs like 2'-FL, LNFP I, LNFP II, DFL, LNDH I and LNDH II, each cluster could be unambiguously assigned to one of the 4 commonly known human milk groups (HM). Blue and dark blue circles in Fig. 4 represent samples belonging to HM group I, red circles indicate HM group II, cyan circles milk group II, and orange circles milk group IV. Filled circles are HM samples collected at 6 weeks after delivery whereas open circles represent HM samples from 16 weeks pp.

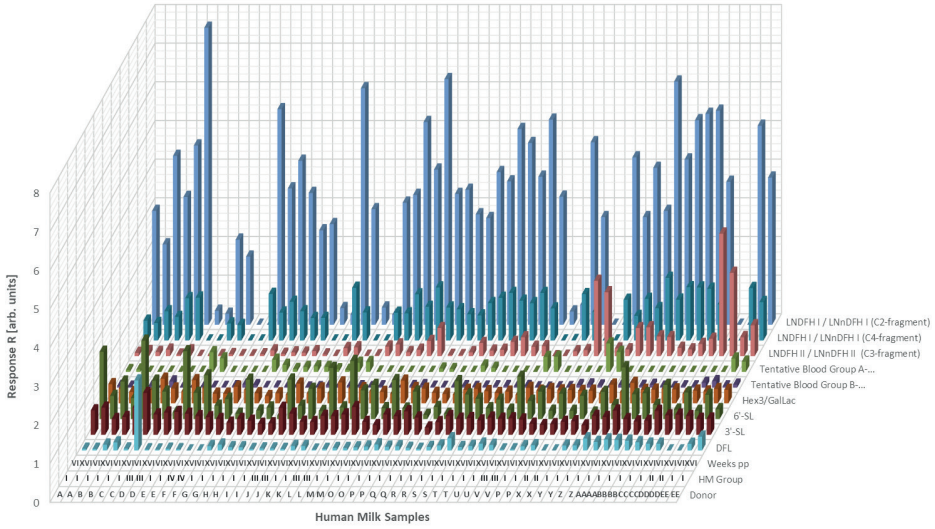


Figure 3 | HMOs detected across 60 HM samples (N=30 donors, 2 stages of lactation (6 w and 16w) per donor) with MRM LC-ESI-MS². Responses R are not strictly equimolar and ranged between 0 and 7.7. HM O-responses below LOD are reported with a zero value. Individual milk donors are indicated by different capital letters A-EE, HM groups by Roman numbers I-IV, and stages of lactation by Roman numbers (VI = 6 weeks pp, XVI=16 weeks pp).

Unexpectedly, within cluster I (blue circles, HM group I) a tendency towards formation of a possible subgroup (full and open dark blue circles in **Figure 4**) became visible. We named this subgroup "HM group Ia". To investigate if responses of certain HMOs might differ significantly between HM group I and HM group Ia, we performed t-testing as described in the experimental section. Thereby, the following findings were obtained: At 6 weeks post-partum, LNFP V/VI, LNFP II, LNDFH II or LNnDFH II (C3-Fragment), LNFP III, 2'-FL varied significantly (all P< 0,001), also 3'-SL (P=0,03) and DFL (P=0,04) were significantly different in abundance. At 16 weeks pp, LNFP V/VI, LNFP II, 3-FL, LNDFH II or LNnDFH II (C3-Fragment) exhibited significant abundance changes (all P< 0,001). Also levels of Tentative Blood Group B-Tetrasaccharide / (Hex)3(Fuc)1 (P=0,002), and 2'-FL (P=0,003) were found to be significantly different. In general, median values for Le+/FUT 3 related HMOs like LNFP II and LNDFH II were always lower in HM group Ia compared to HM group I. On the other hand, Se+ dependent HMOs like 2'-FL appeared in much higher levels in HM group Ia compared to HM group I.

The observed HM group Ia subset is concordant with similar findings for Dutch and Chinese human breast milks [51]. In 2018, Elwakiel et al. characterized HMO patterns derived from 30 HM from different Chinese donors via CE-LIF. Milk from each donor was sampled between the first and 20th week after birth. For comparison, also 28

different Dutch breast milks which had been donated approximately at 4 weeks pp to a milk bank were analysed. Hierarchical clustering of Chinese milk HMO-patterns revealed a division of HM group I into two subgroups. These subgroups differed significantly in concentrations of 2'-FL , DFL, LNFP I and LNFO. However, separation of Dutch Se+Le+ breast milks into subdivisions was driven by 2'-FL, LNT and LNFO. Unlike in our study, 3-FL could not be evaluated as it was removed during sample pre-treatment. Also, no information was provided about the impact of LNFP II, LNFP III, LNFP V or LNDFH II. In sum, the "manual " approach for assignment of HM groups and the unsupervised PCA assignment resulted in the same 4 major milk groups with the same relative proportions of samples and also identical individual samples assigned to these major groups.

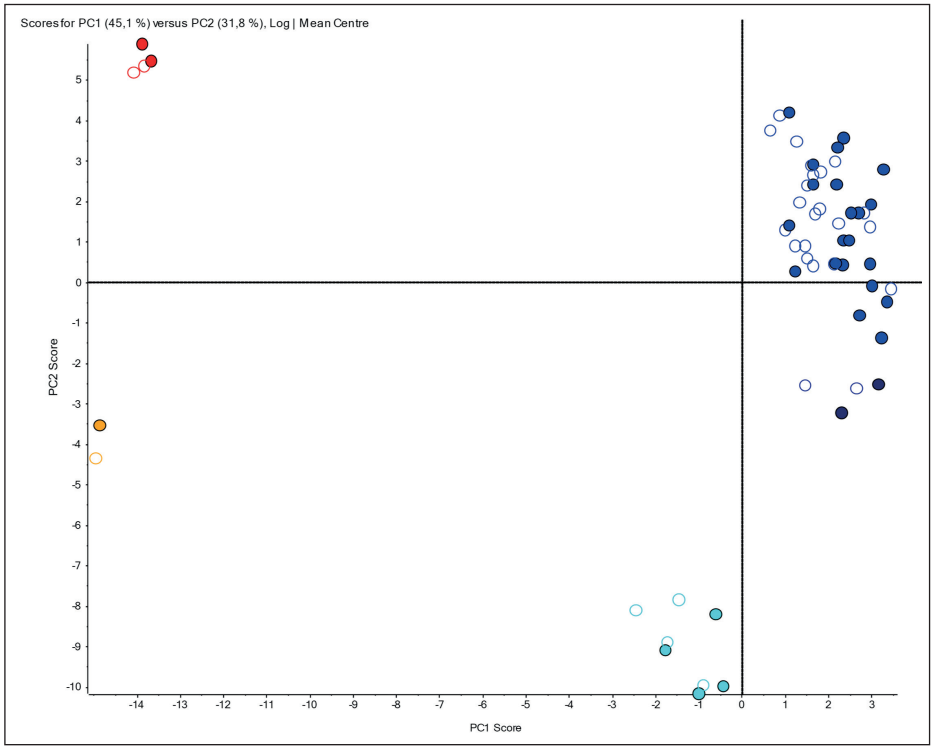


Figure 4 | PCA evaluation of MRM LC-ESI-MS2 data from 2 x 30 human milk samples (collected at 6 and 16 weeks pp). Every HM sample is represented by a dot in the PCA scoring plot depicted below. Blue and dark blue circles: cluster 1 / group I human milks; red circles: cluster 2 / group II human milks; cyan blue circles: cluster 3/group III human milks; yellow circles: cluster 4 / group IV human milks. Filled circles represent HM samples collected at 6 weeks pp. Open circles represent HM samples collected at 16 weeks pp.

Figure 5 summarizes the resulting proportions of the 4 milk groups in percentages after characterization of HM samples by MRM LC-ESI-MS^2. HM group Ia (contributing 5% to the total HM group I +Ia) was not considered separately but accounted for in one HM group labelled as HM group I + Ia. This was done to be consistent with the number of milk groups published earlier [49] and to acknowledge the preliminary nature of HM group Ia identification: In our approach, this HM sub-group consisted of only 4 HM samples from 2 donors. Thus, future confirmation by analysis of many HM samples is warranted. The final PCA based proportions of human milk groups (Fig. 5) were 77% (HM group I + Ia), 7% (HM group II) , 13% (HM group III), and 3% (HM group IV). This result was identical to the proportions yielded by "manual" assignment relying on presence of absence of LNFP I and LNFP II (see above). A comparable frequency of ~76% for human milk group I was reported by Tonon et al. in 2019 [83] for mature Brazilian human milks from 78 women. Noteworthily, the proportions for group II and group III of ~ 12% each differed from our results especially with regard to group II. This may be explained by regional differences or varying ethnicities between Germany and Brazil. Furthermore in 2019, Samuel et al. [84] reported new findings for HM group distributions for milks collected during the first 4 month of lactation from 290 subjects across Europe (not including Germany). In their publication 72% of donors could be assigned to HM group I, whereas ~18 % of donors belonged to HM group II, ~7% to HM group 3, and ~ 4% to HM group IV. Again, differences between our findings and these numbers may be attributed to regional, ethnic or yet unknown genetic or exogenous factors.

Similar proportions for human milk groups as found by us were also received by analyses of maternal Lewis blood group determinants directly in plasma of French (Oriol et al. 1986 [45]) or German donors (Thurl et al. in 2010 [16]). Oriol reported values of 70% for Le (a-b+c-d-) phenotypes, 9% for Le (a-b-c-d+), and 1% for Le (a-b-c+d-). Thurl et al tested blood specimens from 30 subjects but only for Le a and Le b epitopes and found the following distribution: 73% Le (a- b+), 17% Le (a+ b-), and 10% Le (a- b-). In general, the outcome of our study for HMOs and the results of Oriol et al. and Thurl et al. re-confirm the relationship of the Le-blood-group-system and the maternal Le/Se-status dependent HM group system (see also Table 1). In this context, HM group II seems to be underrepresented in our data as only 7% of samples were assigned to this group. The corresponding Lewis blood group proportions published by Oriol et al. and Thurl et al. were 20 % (Le a+b-c-d-) and 17% Le (a+b-), respectively. This difference may be attributed to the different geographies from which HM samples had been derived (Germany vs France) or to different proportions of ethnicities in the probed sample sets. Also, variations introduced by the targeted biomolecules themselves (human milk oligosaccharides (this study) vs erythrocytes) might be reasons for deviating findings. The set of fucosyltransferases (FUTs) affecting formation of Le-epitopes on erythrocytes is related but not identical to the FUTs active in HM. Moreover, the relatively low number of 30 donors in our

study might have contributed to the observed variations from results found by Oriol et al. in 1986, too. Therefore, in future the parallel analysis of statistically meaningful numbers of blood and HM specimens from donors of the same geographies and ethnicities may be helpful to mount further evidence.

PCA based investigation of effects related to progression of lactation

In the PCA score plot depicted in **Figure 4**, only minor shifts in ordination of HM samples could be observed between sampling at either 6 (filled circles) or 16 weeks (open circles) after birth. On the contrary, the pronounced distances between HM group clusters I-IV did persist despite changes of lactational stages. Also the assignment of individual HM samples to HM group I-IV stayed the same at 6 and 16 weeks pp. Consequently, no percentual change in milk group ratios could be attributed to progression of lactation. Thus, we conclude that at least between 6 and 16 weeks pp, progression of lactation has no influence on milk group distribution and milk group related diversity (but not abundance) of particular HMOs.

Variation of Le-/Se-status related or independent HMOs between milk groups

The PCA loading plot displayed in **Figure 6** helped to reveal specific HMO compounds which were driving the separation into 4 major milk group clusters most: The highest impact on separation of milk groups was exerted by a set of MRM LC-MS^2 transitions specific for LNFP I, 2'-FL, and DFL (light blue rectangle). These HMOs are linked with a secretor-positive (Se+) genetic maternal status. To a lower degree, also LNDFH I /LNnDFH I transitions (light red and blue rectangle) which are concatenated with a positive secretor- and lewis-gene status (Se+/Le+), had a strong impact on HM grouping. HM group separation was less but still considerably influenced by MRM-transitions of Le-positive status (Le+) related HMOs like LNFP II and LNDFH II/LNnDFH II (light red rectangle). Intriguingly, another set of Se- and Le-gene independent HMOs like LNFP V/VI, and LNnT, but also the tentative blood group A and B tetrasaccharides seemed to contribute to clustering as well, but to an even lesser extent than the Le/Se gene linked glycans. Other compounds like LNFP III, Lactose/ $(Hex)_2$, $(Hex)_3$, and 6'-SL have almost no effect on group separation. This means that the latter structures are quite stable in presence and abundance across all probed HM samples. Variability of Le- and Se-gene regulated HMOs is much higher.

The observations explained above were further substantiated by nonparametric statistics. The null hypothesis that distribution and abundance of particular HMOs is the same across the 4 human milk groups as distinguished by PCA was rejected for all measured Le/Se-dependent but also some independent HMOs by nonparametric statistics (Kruskal Wallis method, see experimental section) as well. As a result, actually 12 HMO structures differed significantly in abundance between human milk group I-IV (see **Table 2**). Moreover, some HMOs could only be detected in specific milk groups (see column 3 in **Table 2** or **Figure 2** and **Figure 3**). If HMOs

were detectable in more than one milk group, the group with the highest median was highlighted by red bold letters in Table 2. For example, 2'-FL and LNFP I could both be detected in HM groups I and III but medians were higher in HM group III. Thus the roman number III is displayed in bold red for both structures.

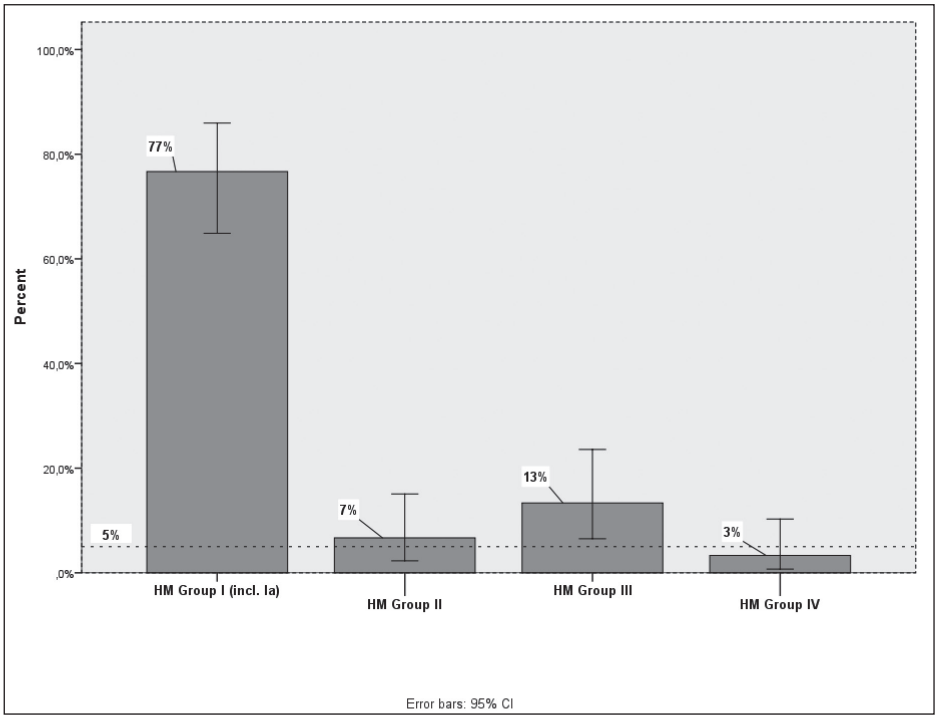


Figure 5 | HM-group-distribution in percentages based on MRM ESI-LCMS² analysis of 60 HM samples. Error bars represent 95% confidence intervals.

Noteworthily, HMOs related to a positive maternal genetic Se-status (Se+), positive Le-status (Le+) or positive Se- and Le- status (Se+Le+) such as 2'-FL, DFL, LNFPI, LNFP II, LN(n)DFH I, and LN(n)DFH II exhibited significantly different levels between milk groups (significance level α = 0.05, see Table 2). The latter findings approximately corroborate data published e.g. by Thurl et al. in 2010 [16] for milk group dependent abundances of distinct neutral, fucosylated HMOs.

This outcome complies with the well accepted biological expectation that distribution of specific HMO-structures should differ in HM samples due to known differences in maternal Le/Se status which can occur between individual milk donors: If the maternal Secretor and/or Lewis gene is active, it will encode expression of fucosyltransferases FUT 2 and / or FUT 3 and subsequent synthesis of either α1,2- and / or α1,4-fucosylated HMOs in the mammary gland. As a result, the presence or

absence of α1,2- and / or α1,4-fucosylated human milk OS should lead to formation of 4 different milk groups defined by presence (or absence) of α1,2- and / or α1,4-fucosylated HMOs. As mentioned before, not only the expected appearance of these different milk groups, but also significant variations in α1,2- and α1,4-fucosylated oligosaccharides between the 4 HM groups could be confirmed after interrogation of the LC-MS results through PCA and nonparametric statistics (see **Table 2**). More surprisingly and in addition, PCA and non-parametric statistical testing suggested that some HMOs such as α1,3-fucosylated 3-FL and LN(n)FP V or e.g. non-fucosylated LNT which are all independent from maternal Le/Se status did also significantly vary between HM groups. A further examination of milk groups for specific HMOs structures revealed another unexpected finding: The appearance of the MRM signal for LNDFH I based on its C_2 fragment (see **Table 3**) in milk group III samples (with lower abundance than in milk group I). This finding is in contradiction with the published knowledge describing a lack or strongly reduced activity of FUT 3 for Le-negative HM group III donors. FUT III would be needed in biosynthesis to append a fucose in α 1,4 glycosidic linkage to the GlcNAc in thy type I LNT backbone of LNDFH I. An explanation for this paradoxon may be provided by the fact that the used LNDFH I C_2-fragment based MRM transition obviously picks up both, the backbone-type I and- type II isomer of LNDFH I. Therefore, the LNDFH I variant determined in the group III milks is most probably LNneoDFH I, also named LNDFH III. LNDFH III consists of a type II LNnT backbone and the α 1,4 linked Fucose present in LNDFH I is replaced by a α 1,3 linked Fucose. The latter structure is FUT 3 independent and could therefore be present also in HM group III samples. In contrast, LNDFH I (with type I backbone) is detected only in group I milks as expected if the MRM transition of the LNDFH C_4-fragment is monitored. Thus, the latter transition is obviously more specific and suitable for correct identification of LNDFH I.

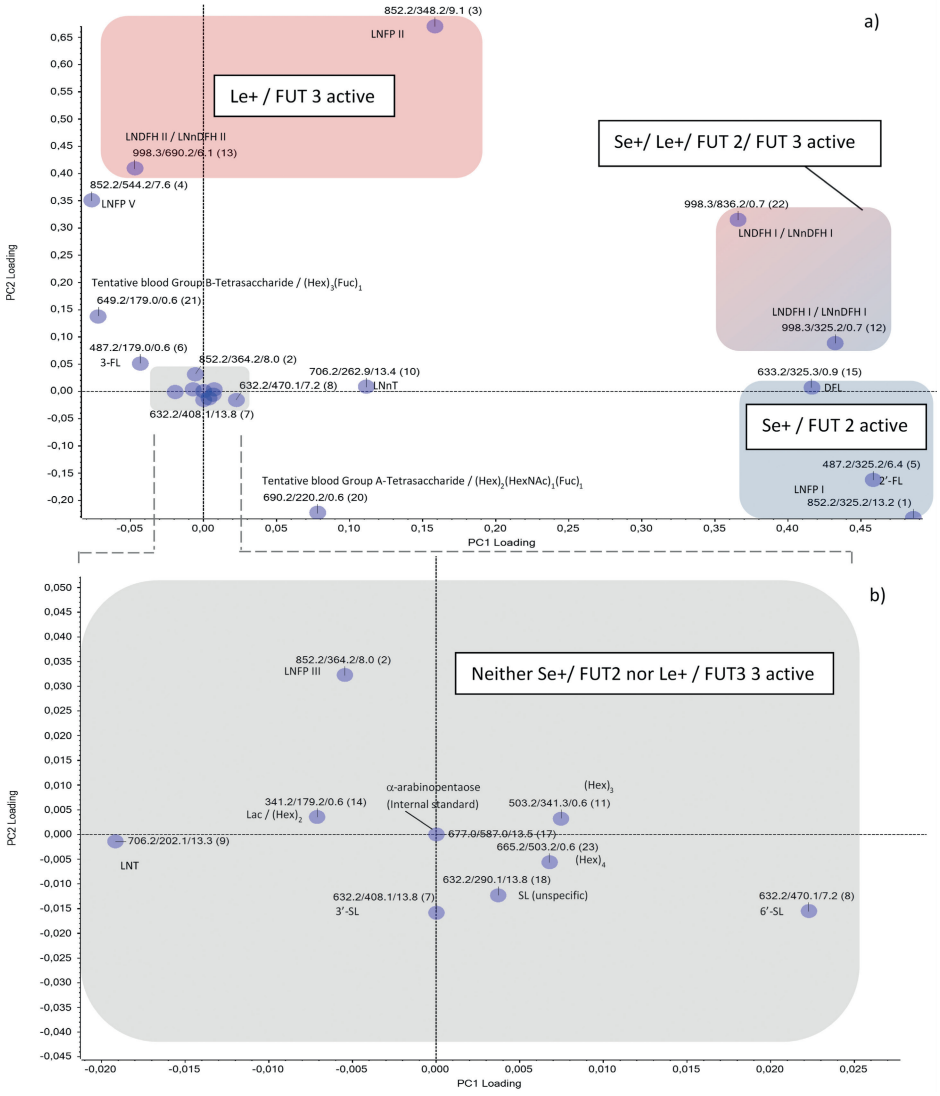
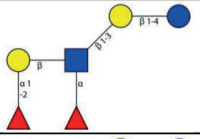
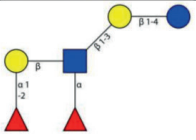
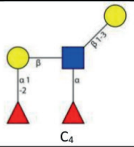
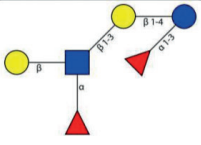
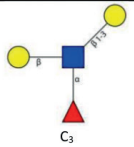


Figure 6a | PCA loading plot based on LC-ESI-MS² responses R for 21 MRM-transitions of individual, partly isomeric HMOs as detected in 2 x 30 human milk samples collected at 6 and 16 weeks pp. HMOs specific MRM transitions are represented by individual dots including information about precursor masses, fragment masses and retention times. The order number of transition in MRM scan is reported in parentheses. b) Magnification of grey shaded section in a) depicting MRM transitions of HMOs with no or low contribution to clustering of HM.

Table 2 | Milk group specific presence of individual HMOs. P-values < α indicate statistically significant differences between milk groups I, II, III and IV found for median values of 12 particular HMOs (Nr.1-12, grey shaded). α = 0.05, non-parametric Kruskal Wallis test, N = 2x30 samples (from 6 and 16 weeks pp)). Bold red letters in column 3 indicate elevated median values of specific HMOs in this particular milk group relative to other milk groups.

Nr.	HMOs	Presence in Milk Group	P-Value
1	LNDFH I / LNnDFH I, (MRM transition C_2-Fragment)	**I**+III	**0,000**
2	LNFP II	I+**II**	**0.000**
3	LNDFH I / LNnDFH I, (MRM transition C_4-Fragment)	I	**0,000**
4	LNDFH II / LNnDFH II, (MRM transition C_3-Fragment)	I+**II**	**0,000**
5	LNFP V / LNnFP V	I, **II**, III, IV	**0,000**
6	DFL	**I**+III	**0,000**
7	LNFP I	I+**III**	**0,000**
8	2'-FL	I+**III**	**0,000**
9	Tentative Blood Group B-Tetrasaccharide / $(Hex)_3(Fuc)_1$	I, **II**, III, IV	**0,001**
10	3-FL	I, **II**, III, IV	**0,004**
11	LNnT	I, II, III, IV	**0,014**
12	Tentative Blood Group A-Tetrasaccharide / $(Hex)_2(HexNAc)_1(Fuc)_1$	I, **III**	**0,032**
13	$(Hex)_3$ / e.g. Galactosyllactose	I, II, III, IV	0,158
14	$(Hex)_2$ / Lac	I, **II**, III, IV	0,191
15	LNFP III	I, **II**, III, IV	0,213
16	LNT	I, II, III, **IV**	0,256
17	3'-SL	I, II, III, IV	0,284
18	Total SL	I, II, III, IV	0,542
19	6'-SL	I, II, III, IV	0,668
20	$(Hex)_4$	I, II, III, IV	0,899
21	α-Arabinopentaose (internal standard)	I, II, III, IV	1,000

Table 3 | Isomeric LNDFH structures, possible tandem MS fragment ion types, and related MRM-Transitions as utilized in the applied HMO LC-ESI-MS^2 method

Nr.	Oligosaccharide	Oligosaccharide Structure	MRM-Transitions		Possible Assignments Fragment ion types
			Precursor [m/z]	Fragment [m/z]	
1	LNDFH I or LnNDFH I		998.3569	325	 C_2
2	LNDFH I or LNnDFH I		998.3569	836	 C_4
3	LNDFH II or LNnDFH II		998.3569	690	 C_3

Relative HMO-abundance changes between 2 stages of lactation (6 and 16 weeks post-partum)

Another important aspect which may contribute to functional adaptation of human milk during staging of lactation is the change of concentrations observed for particular HMOs. To further investigate such staging effects, we compared the human milks which were either collected at 6 or 16 weeks after parturition. This was done with no distinction between milk groups.

As shown in **Figure 7 a** and **b**, only six HMO-structures were changing significantly in abundance between the lactational stages at 6 and 16 weeks post-partum (αlevel of 0.05; fold change threshold of +/- 0.1). This finding could be confirmed for 6'-SL, 3-FL, LNFP I, and $(Hex)_3$ also with Wilcoxon signed rank test for non-normally distributed, interdependent samples (data not shown). Among these significantly changing HMOs, only one glycan (LNFP I) was related to maternal Se+ status. LNFP I decreased in its relative abundance. Additional HMO compounds including 6'- SL, total SL, $(Hex)_3$, and LNT showed the same tendency and decreased. In opposition to this trend, 3-FL exhibited an increase in relative abundance between 6 and 16 weeks pp. Except for LNFPI, all other 5 HMOs were independent from maternal Le- or Se-status.

Figure 8 visualizes quantitative abundance changes for the six selected HMO-structures visible in **Figure 7 b**. Level changes were expressed in percentages relative to the original abundances measured at 6 weeks pp. Here, 6'-SL showed the strongest quantitative variation among all investigated HMOs with a decrease of 73%. On the contrary, 3'-FL increased by approximately 57% during the same time span. Interestingly, in 2016 Seppo et al. [85] published similar data with highly significant shifts of 6'-SL (decrease) and 3-FL (increase) between 0 and 6 month pp. They also showed a negative correlation between LNFP I concentration and increasing duration of breastfeeding. In contrast to our analytical LC-MS based approach, these authors relied on HPLC analysis of HMOs after labelling with 2-aminobenzamide. Also other reports state a steady increase for 3'-FL levels either between approximately 0 and 8 month pp for HM collected from Chinese women [86] or (at least) between 0 and 4 month pp for HM from Pan-European populations [84]. Furthermore, Austin et al. could show that 2'-FL, 3'-SL, 6'-SL, LNT, LNnT, LNFP-I and LNFP-V decreased by trend over lactational stages. Although we analysed human milks from a different geography (Gemany), these trends were reflected in our data, too. Another publication by Coppa et al. 1999 [18] was in line with our results as well. In the latter study 3'-SL decreased between 0 and 3 mon pp in HM, but the differences in 3'-SL were not significant. Also the 6'-SL concentration declined over the same time range, but in this case and similar to our findings, the concentration-changes were significant.

CONCLUSIONS

In this study, application of a novel LC-MRM-MS concept to 2 x 30 mature human milk samples collected at 6- and 16-weeks post-partum was demonstrated. This approach covered (partly isomeric) HMOs up to hexasaccharides and as a novelty also the (tentatively assigned) blood group A and B tetrasaccharides. More than approximately 50% of the HMOs quantity present in HM could be characterized .

Principal component analysis (PCA) was successfully utilized for automated interrogation of LC-MS data. The chosen PCA approach allowed for unattended, effortless and quick assignment of HM samples to one of the 4 currently known human milk groups. The additional advantage of PCA on top of advanced data processing speed and convenience was the immediate visualisation and indication for possible HM subgroups. Taking these findings into account, unsupervised PCA seems to be well suited for convenient HM group recognition if applied as described in the experimental section.

Intriguingly, mining of HMOs LC-MS data via PCA and comparison with complementary non parametric statistics revealed first evidence for possible distinction of HM group I into a further HM subgroup termed HM group Ia. Compared to other HM group I samples, HM group Ia specimens had significantly elevated levels of Se+ related HMOs (e.g. 2'-FL) and decreased levels of Le+ structures like LNFP II and LNDFH.

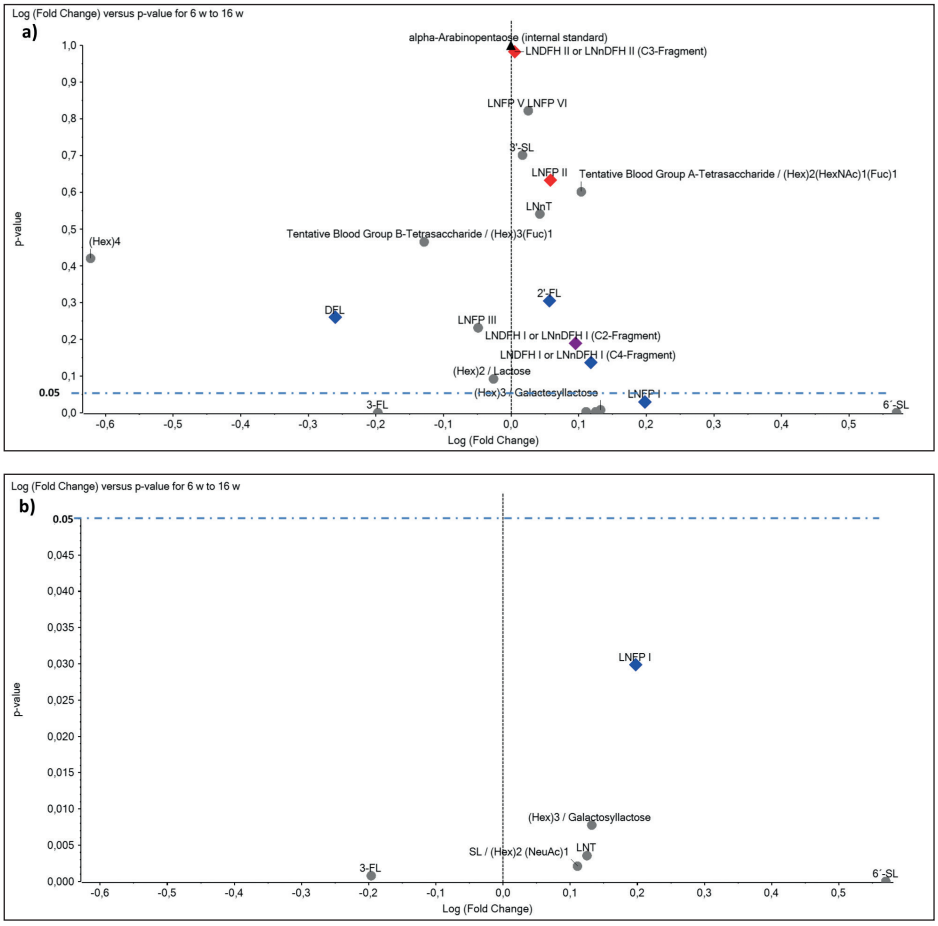


Figure 7 | Variations of individual HMOs levels between stages of lactation. a) P- values for variations in HMO-mean values between 6 and 16 weeks pp were plotted over respective fold changes. Negative values for fold changes indicate an increase of HMOs levels over time, positive values a decrease. b) Section of a) displaying only significantly changing HMOs ($p < \alpha$). Red diamonds: Le+ dependent HMOs, blue diamonds: Se+ dependent HMOs, purple diamonds: Se+ & Le+ depended HMOs, grey dots: Le/Se–independent HMOs, black triangle: internal standard alpha-Arabinopentaose.

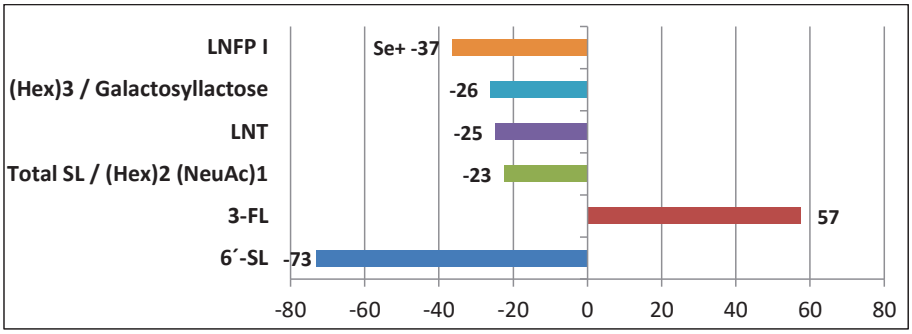


Figure 8 | Quantitative differences of HMO-mean values with significant level changes (t-test, p < 0.05) between 6 and 16 weeks pp. Level changes are expressed in percentages relative to the mean value at 6 weeks pp. Highest increase over time is found for 3-FL (57%), highest decrease for 6'-SL (-73%) , followed by LNFP I (-37%).

Also Le/Se independent HMOs such as LNFP V/VI or putatively assigned Blood Group B-Tetrasaccharide / $(Hex)_3(Fuc)_1$ were found to differ.

12 HMO-structures exhibited significantly different abundances between the major 4 HM groups. As expected and in line with contemporary literature, most of those 12 HMOs were related to either a positive maternal genetic Se-status, a positive Le-status, or a positive Se- and Le- status. However, also a second set of Le/Se-status- independent α1,3-fucosylated compounds like 3-FL and LN(n)FP V or non fucosylated LNT did vary significantly.

During progression of lactation between 6 and 16 weeks pp, staging effects could be confirmed for 6 distinct HMOs structures. Most prominent level changes were revealed for 6'-SL (decrease by 73 %) and 3-FL (increase by 57%).

It is challenging to find a rationale why there is a second set of Le/Se-gene independent HMOs, seemingly associated with milk grouping. Neither milk group relevant active FUT2 or FUT3 genes are required for biosynthetic formation of those human milk glycans. Finally, and in accordance with first interpretation of PCA outcomes, Kruskal Wallis ANOVA testing could confirm the existence of a third set of human milk oligosaccharides which were assessed to be permanently present across all samples without significant difference between the 4 human milk groups (see Nr. 13-21 in Table 2).

Whether milk group independent, ubiquitously present glycans like Lactose/ $(Hex)_2$, $(Hex)_3$, SL, LNT or LNFP III can therefore be considered as more "essential" with regard to healthy infant development rather than the Le-/Se milk group dependent HMOs is yet still open. At least growing published evidence states their association with relevant early life health benefits like bifidogenic effect, anti-infective efficacy

(e.g. directed against parasitic protozoans), and modulation of the developing immune-system including less incidence of cows' milk allergy [68, 85, 87-91]. On the other hand, it may be hypothesized that the synthesis and secretion of the Le-/Se-dependent HMOs like 2'-FL, DFL, LNFPI [92-96], or LNPF II [97] could also be interpreted as a complementary additional response of evolution to optimize protection of the infant against specific threats like pathogenic bacteria or viruses.

Diversity and abundance of these pathogens however probably did or does still vary by geography due to socioeconomic and environmental circumstances. Therefore, several tailor-made but geographically variable sets of HMOs (i.e. the Le/Se-gene regulated HMOs) may be required to optimally protect the infant with respect to geographically variable, specific early life threats. The profile of a HMOs set fitting optimally to the infants needs may also depend on the infants' own Le/Se status. The latter aspect is important as the Le/Se status also co-defines glyco-epitopes expressed on gut epithelial linings or other inner surfaces [52]. Pathogens can adhere to these epitopes via glycan specific receptors and subsequently cause infections e.g. of the gastrointestinal tract. An optimal free human milk OS pattern would therefore resemble all glycan epitopes possibly displayed by the host (the infant) to bacteria or viruses. Thereby, effective blocking of glycan receptors of these pathogens could be achieved and adhesion and subsequent infection could be avoided.

However, if pathogenic threats throughout human evolution exerted a selective pressure on HMO-profiles, the evolutional adaption should have manifested itself in geographically different Le/Se-gene dependent HMO- patterns. These patterns may still be traceable today in diverse geographic regions or ethnicities. In this sense, some publications propose a possible link between history of human FUT 2 polymorphisms [98] or blood group variations with partly pathogen driven selective pressures [99]. Moreover, there is at least a growing body of evidence published by e.g. Erney et al. [23] and other authors [100-103] that prevalence of the Se-gene and related HMOs like 2'-FL differs in a geographically specific manner.

Whether the idea of "essential" HMOs which are always present across all human milk groups and other milk group dependent "specialized" e.g. anti-infective HMOs can be substantiated by further studies, remains to be elusive and an exciting question. The same applies for the influence of HMO-abundances on infants' healthy development which are significantly changing during lactational staging. In this publication, we investigated only the segment of human milk lactation between 6 and 16 weeks pp. An extended milk sampling including specimens from the first week until 6 or even 12 month pp and a much higher number of samples could reveal more insights in future.

Other biofluids than HM such as faecal slurries or plasma could probably also become amenable to sensitive MRM-LC-MS analyses by slight adaptions of the current sample pre-treatment procedure. For example, additional microfiltration steps or SPE could be exploited in addition or as an alternative to 3kDA ultrafiltration. Thus in future, more holistic insights may be elicited by improved analyses with regard to systemic effects of HMOs on healthy infants' development during the relevant first 1000 days of life.

Finally, we hope that the described multiple reaction monitoring (MRM) LC-ESI-MS^2 methodology might help to study HMO-profiles of even larger human milk sample sets e.g. provided by prospective human milk cohorts. If combined with PCA analysis, this in turn may contribute to reveal new associations between variations in HM glycan composition and their impact on healthy infant development and early life immuno-modulation.

COMPLIANCE WITH ETHICAL STANDARDS

The usage and analysis of the described human milk samples have been performed in accordance with ethical standards and guidelines as laid down in the Declaration of Helsinki. Ethical approval and written consents of donors were given as stated above for HM samples from the INFAT study.

CONFLICT OF INTEREST

Marko Mank and Bernd Stahl are employees of Nutricia Research B.V., Utrecht, The Netherlands. Albert J.R. Heck is Professor at Utrecht University, Utrecht, The Netherlands. Hans Hauner is holding a professorship at the Technical University of Munich, Munich, Germany. The present research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. None of the authors have further conflicts of interest with regard to the content of this manuscript.

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SUPPLEMENTS

Table S1 | LC-Gradient of the applied MRM LC-ESI-MS² method.

Time [min]	A (%)	B (%)
0.00	98.0	2.0
1.00	98.0	2.0
1.54	98.0	2.0
2.64	91.5	8.5
4.18	91.0	9.0
8.00	91.0	9.0
9.50	89.0	11.0
9.82	86.0	14.0
10.70	75.0	25.0
11.80	70.0	30.0
12.90	0.0	100.0
14.22	0.0	100.0
14.66	98.0	2.0
17.74	98.0	2.0

Table S2 | Characteristic, isomer-specific MRM transitions for HMOs as applied in our negative ion mode MRM LC-ESI-MS² method. Precursor ions are given as monoisotopic *m/z* values, fragment ions are given as nominal *m/z* values.

Nr.	Oligosaccharide	MRM-Transitions	
		Precursor [*m/z*]	Fragment [*m/z*]
1	2´-FL	487.1668	325
2	3-FL	487.1668	179
3	3´-SL	632.2044	408
4	6´-SL	632.2044	470
5	DFL	633.2248	325
6	LNT	706.2411	202
7	LNnT	706.2411	263
8	LNFP I	852.2990	325
9	LNFP II	852.2990	348
10	LNFP III	852.2990	364

Table S3 | Characteristic, composition-specific MRM transitions for oligosaccharides, tentative blood group-related haptens, and the internal standard α-Arabinopentaose as applied in our negative ion mode MRM LC-ESI-MS² method. Precursor ions are given as monoisotopic *m/z* values, fragment ions are given as nominal *m/z* values.

Nr.	Oligosaccharide	MRM-Transitions	
		Precursor [*m/z*]	Fragment [*m/z*]
11	$(Hex)_2$ / Lactose	341.1089	179
12	$(Hex)_3$ / Galactosyllactose	503.1618	341
13	SL / $(Hex)_2(NeuAc)_1$	632.2044	290
14	$(Hex)_4$	665.2146	503
15	a-Arabinopentaose (internal standard)	677.2148	587
16	Tentative Blood Group B-Tetrasaccharide / $(Hex)_3(Fuc)_1$	649.2197	179
17	Tentative Blood Group A-Tetrasaccharide / $(Hex)_2(HexNAc)_1(Fuc)_1$	690.2462	220
18	LNFP V or LNFP VI	852.2990	544
19	LNDFH I or LnNDFH I	998.3569	325
20	LNDFH I or LNnDFH I	998.3569	836
21	LNDFH II or LNnDFH II	998.3569	690

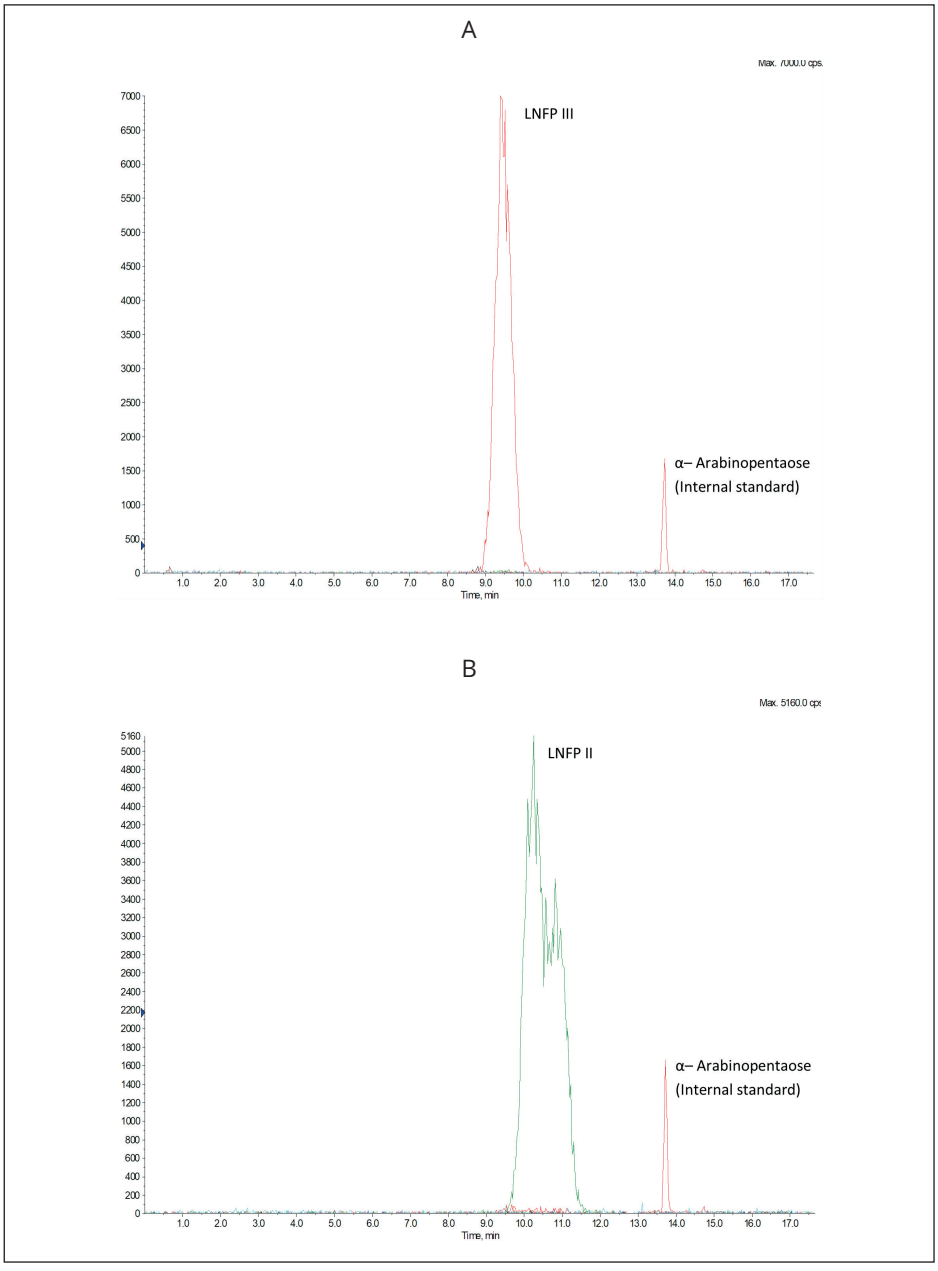

Figure S1 | Exemplary extracted ion chromatograms yielded after MRM LC-ESI-MS^2 analyses of pure HMOs standards (a) LNFP III, b) LNFP II, c) LNFP I, d) LNT, e) LNnT) and e) HMOs in a group I HM specimens selected from the INFAT study sample set. Traces and assignments of most abundant HMOs: RT window 0-5min: Lactose/Hex2 (brown), 3-FL (pink); RT window 5-8min: DFL (blue), 2'-FL (light blue), 6'-SL (green); RT window 8-12 min: LNFP III (red), LNFP II (green); RT window 12-15 min: LNFP I (black), LNT (turquoise), LNnT (blue), 3'SL (green), internal standard α-Arabinopentose (red).

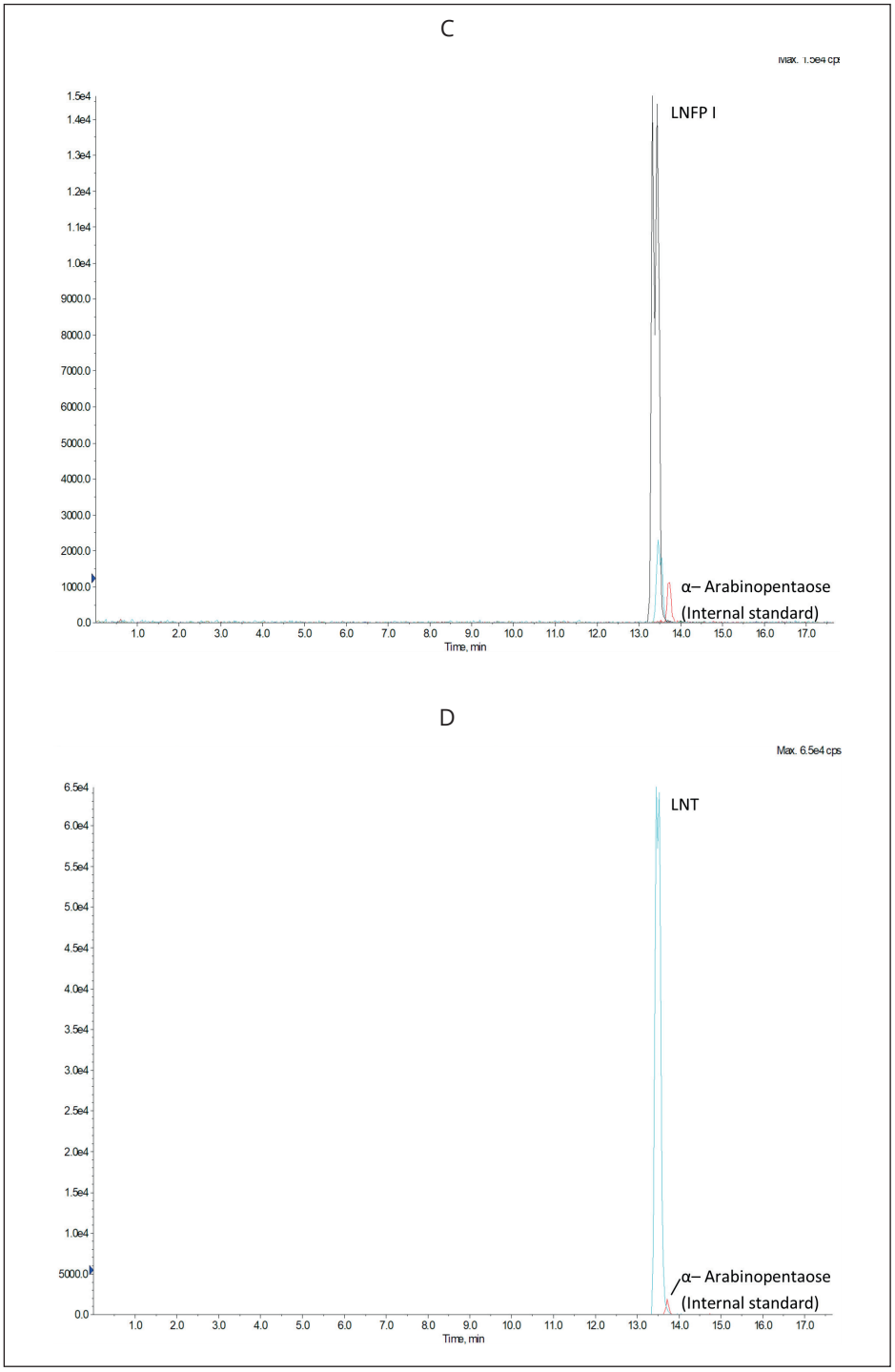

Figure S1 | Continued.

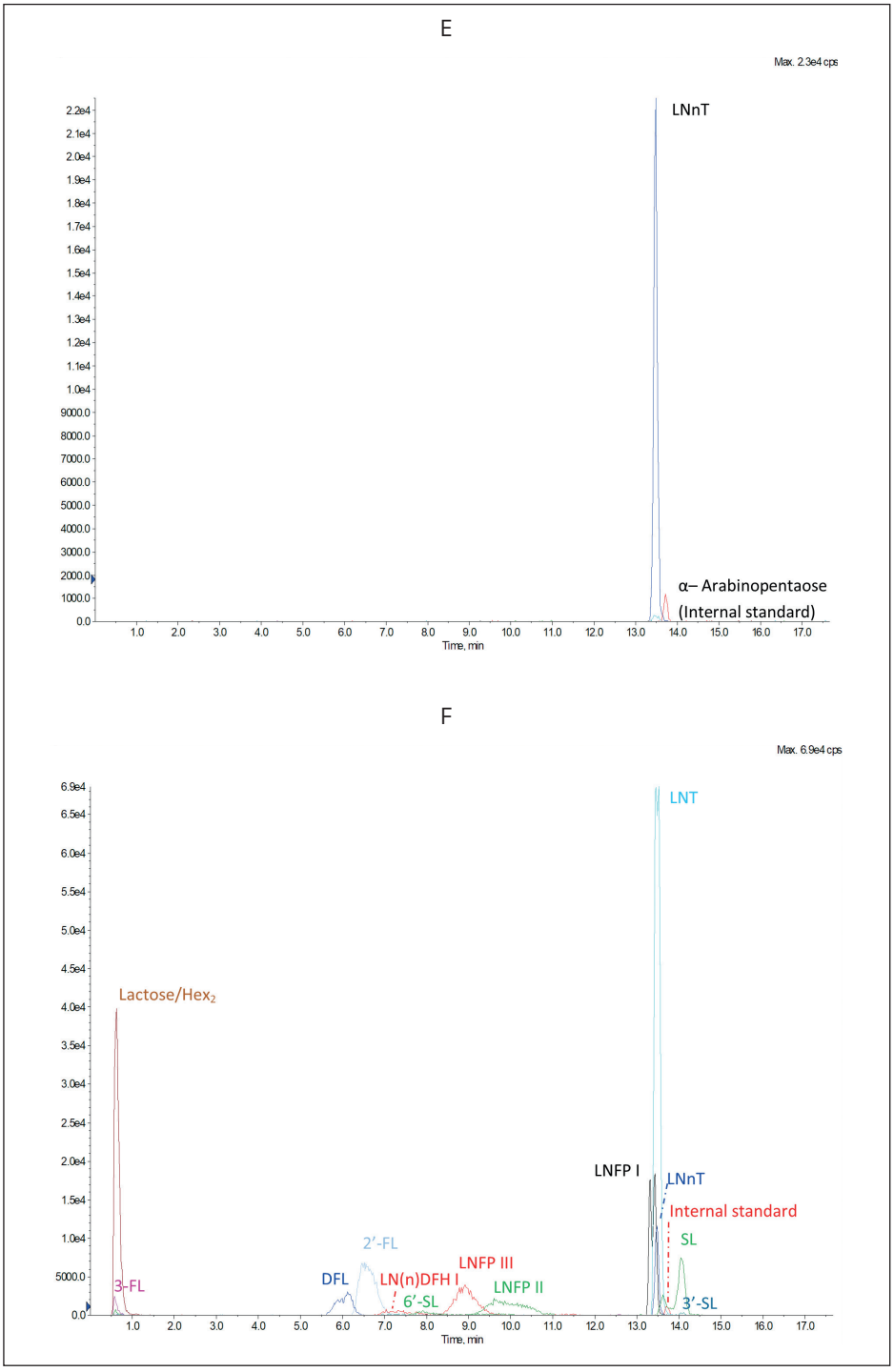

Figure S1 | Continued.

CHAPTER V

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

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ABSTRACT

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

Keywords

Bifidobacteria, butyrate, *Lachnospiraceae*, microbiome, pH

INTRODUCTION

The succession of microbial species in the infant gut microbiota is a profound process in early life (Backhed et al., 2015; Koenig et al., 2011), which coincides with the important development of the immune, metabolic and neurological systems (Arrieta et al., 2014; Sherman et al., 2015; Thompson, 2012). At this developmental stage, human milk is recognised as the best nourishment for infants (Neville et al., 2012). Human milk contains a range of microbial active components and all human milk oligosaccharides (HMOs) play an important role in the development of the infant gut microbiota (Zivkovic et al., 2011). HMOs are complex carbohydrates composed of a lactose core, which may be elongated by N-acetylglucosamine (GlcNAc), galactose and/or decorated with fucose and/or sialic acid (Smilowitz et al., 2014). The composition of HMOs in human milk is highly individual, driven by maternal genetic factors (Kunz et al., 2017; McGuire et al., 2017) and varies with the phases of lactation. The concentration of HMOs ranges from 23 g/L in colostrum to 7 g/L in matured human milk (Coppa et al., 1993; Gabrielli et al., 2011).

The majority of the HMOs escapes digestion by the host's enzymes in the upper gastrointestinal tract (Engfer et al., 2000). HMOs confer important physiological traits by acting both as a decoy for the binding of pathogenic bacteria and viruses, and as a prebiotic to stimulate the growth and activity of specific microbes in the infant gut (Bode, 2012). These complex carbohydrates exert a selective nutrient pressure to promote the HMO-utilising microbes, especially *bifidobacteria* belonging to the *Actinobacteria* phylum (Marcobal et al., 2010). Specifically, infant-associated *bifidobacteria* are well adapted to utilise HMOs by employing an extensive range of glycosyl hydrolases and transporters, which lead to their dominance in the infant gut ecosystem (Sela and Mills, 2010). Upon weaning, the relative abundance of *bifidobacteria* decreases with the increase of *Firmicutes* and *Bacteroidetes* phyla whilst the gut microbial diversity increases (Laursen et al., 2017).

The early dominance of *bifidobacteria* could be important for the maturation of the overall microbial community. In healthy children, the relative abundance of *bifidobacteria* is positively associated with the butyrate-producing *Firmicutes* from the family of *Lachnospiraceae* (also known as *Clostridium* cluster XIVa) and *Ruminococcaceae* (also known as *Clostridium* cluster IV) (Cheng *et al.*, 2015). This butyrogenic community is often present at much lower relative abundance in the gut of new-borns (Jost et al., 2012). The subdominant butyrogenic species could however quickly become more dominant upon weaning as a result of the cessation of breast-feeding and the introduction of solid food (Backhed et al., 2015; Laursen et al., 2016). The expansion of the strict anaerobic, butyrate-producing bacteria could be a critical step for the gut and immune maturation (Arrieta et al., 2015; Wopereis et al., 2017). The interactions between lactate-producing bacteria (such as *bifidobacteria*)

and lactate-utilising bacteria (such as *Ruminococcaceae* and *Lachnospiraceae*) are suggested to be associated with colicky symptoms and atopy disease in infants (Arrieta et al., 2015; De Weerth et al., 2013; Pham et al., 2017; Wopereis et al., 2017). To date, cross-feeding between glycan-degrading *bifidobacteria* and butyrate-producers using complex dietary carbohydrates (including starch, inulin, fructo-oligosaccharides, and arabinoxylan oligosaccharides) has been demonstrated in *in vitro* co-culturing experiments (Belenguer et al., 2006; De Vuyst and Leroy, 2011; Falony et al., 2006; Rios-Covian et al., 2015; Riviere et al., 2015). However, limited studies have shown the cross-feeding between these groups of bacteria on host-secreted glycans, such as HMOs (Schwab et al., 2017) and mucins (Bunesova et al., 2017).

In this study, we elucidated the trophic interaction between a versatile HMO-utiliser, *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) (Sela et al., 2008) and a butyrogenic non-degrader. To this end the butyrate-producer *Anaerostipes caccae* was selected as the representative species for the *Lachnospiraceae* family as it is detected in the early life gut microbiota (Backhed et al., 2015; Yatsunenko et al., 2012) with demonstrated *in vitro* cross-feeding interaction with adult-associated *Bifidobacterium adolescentis* (Belenguer et al., 2006). *A. caccae* is also recently identified as a key bacterium critical for regulating food allergy in early life (Feehley et al., 2019). We showed that *B. infantis* metabolises HMOs into monosaccharides and metabolites including lactate and acetate, to support the growth and concomitant butyrate production by *A. caccae.* This butyrogenic cross-feeding postulates the role of bifidobacteria in driving both the maturation of microbial ecology and physiology of infant gut.

MATERIALS AND METHODS

Screening of 16S rRNA gene amplicon libraries

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

Bacterial strains and growth conditions

Bacterial pre-cultures were grown in anaerobic serum bottles filled with gas phase of N_2/CO_2 (80/20 ratio) at 1.5 atm. Pre-cultures were prepared by overnight 37 °C incubation in basal minimal medium (Plugge, 2005) containing 0.5% (w/v) tryptone (Oxoid, Basingstoke, UK), supplemented with 30mM lactose (Oxoid) for *Bifidobacterium longum* subsp. *infantis* ATCC15697; and 30 mM glucose (Sigma-Aldrich, St. Louis, MO, USA) for *Anaerostipes caccae* L1-92 (DSM 14662) (Schwiertz et al., 2002). Growth was measured by a spectrophotometer at an optical density of 600 nm (OD600) (OD600 DiluPhotometerTM, IMPLEN, München, Germany).

Carbohydrate substrates

Lactose (Oxoid) and total human milk (HM) carbohydrates were tested as the carbohydrate substrates for bacterial growth. The usage and analysis of pooled HM samples described in this study were performed in accordance with ethical standards and guidelines as laid down in the Declaration of Helsinki. Ethical approval and written consents of donors were given as stated in the publications (Thurl et al., 1993). For preparation of total HM carbohydrates, a total carbohydrate mineral fraction was derived from pooled human milk after protein depletion by ethanol precipitation and removal of lipids by centrifugation as described by Stahl *et al.* (1994). Deviant from this workflow, no anion exchange chromatography was used to further separate neutral from acidic oligosaccharides present in the resulting total carbohydrate mineral fraction. The total HM carbohydrates contained approximately 90% of lactose, 10% of both acidic and neutral HMOs as well as traces of monosaccharides, as estimated by gel permeation chromatography (GPC) described below (Supplementary Figure S1).

Anaerobic bioreactor

Fermentations were conducted in eight parallel minispinner bioreactors (DASGIP, Jülich, Germany) with 100 ml filling volume at 37 °C and a stirring rate of 150 rpm. Culturing experiments were performed in autoclaved basal minimal media (Plugge, 2005) containing 0.5% (w/v) tryptone (Oxoid), supplemented with 8 g/L of 0.2 µM filter-sterilised lactose or total HM carbohydrates. Anaerobic condition was achieved by overnight purging of anaerobic gas mixture containing 5% CO_2, 5% H_2, and 90% N_2. Overnight pre-cultures were inoculated at starting OD600 of 0.05 for each bacterial strain. Online signals of pH values and oxygen levels were monitored by the DASGIP control software. Cultures were maintained at pH 6.5 by the addition of 2 M NaOH.

Gel permeation chromatography (GPC)

Total HM carbohydrates were analysed using GPC. Glycans were separated by the GPC stationary phase and eluted according to size and charge. Neutral mono-, di-, and oligosaccharides, and acidic oligosaccharides with different degree of

polymerisation (DP) could be detected. HM carbohydrate solution was prepared by dissolving 0.2 g/ml of total HM carbohydrates in ultrapure water (Sartorius Arium Pro, Goettingen, Germany) containing 2% (v/v) 2-propanol at 37 °C. 5 ml of 0.2 µM filter-sterilised HM carbohydrate solution was injected for each GPC run. The sample loop was cleaned by ultrapure water prior to analysis. Two connected Kronlab ECO50 columns (5×110 cm; YMC Europe, Dinslaken, Germany) packed with Toyopearl HW 40 (TOSOH Bioscience, Tokyo, Japan) were used. Milli-Q water was maintained at 50 °C using heating bath (Lauda, RE 206) for columns equilibration. Milli-Q (Millipore; Merck Millipore, Burlington, MA, USA) water containing 2% (v/v) of 2-propanol was used as the eluent. The flow rate of the eluent was set at 1.65 ml/min. Eluting glycans were monitored by refractive index detection (Shodex RI-101; Showa Denko America, Inc., New York, NY, USA). The resulting chromatograms were analysed by using the Chromeleon® software (v 6.80; ThermoFisher Scientific, Waltham, MA, USA).

High-performance liquid chromatography

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

Human milk oligosaccharide extraction

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

Targeted liquid chromatography electrospray ionisation tandem mass spectrometry analysis

The identification and relative quantitation of HMOs were determined by liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS_2) as described in Mank *et al.* (2019). This method allowed the study of distinct HMOs structures differing in monosaccharide sequence, glycosidic linkage or the molecular conformation. Thereby even the HMOs isobaric isomers such as lacto-N-fucopentaose (LNFP) I, II, III and V could be distinguished. LC-ESI-MS^2 analysis was performed on a 1200/1260 series HPLC stack (Agilent, Waldbronn, Germany) consisting of solvent tray, degasser, binary pump, autosampler and DAD detectorcoupled to a 3200 Qtrap mass spectrometer (ABSciex, Framingham, MA, USA). After HMOs extraction (see above) 5 µl of HMOs extract was injected into the LC-MS system. Oligosaccharides were separated by means of a 2.1×30 mm Hypercarb porous graphitized carbon (PGC) column with 2.1×10 mm PGC pre-column (ThermoFisher Scientific) using a 19 min water-ethanol (Merck, Darmstadt, Germany) gradient following 2 min pre-equilibrium according to Mank et al. (2019), with an additional 1 min for stabilisation at the end. Eluent flow was 400 µl/min and the columns were kept at 45 °C. The LC-effluent was infused online into the mass spectrometer and individual HMOs structures were analysed qualitatively and quantitatively by multiple reaction monitoring (MRM) in negative ion mode. Specific MRM transitions for neutral HMOs up to pentaoses and acidic HMOs up to trioses were included. The spray voltage was -4,500 V, declustering potential was at 44 V, and collision energy was set to 29 eV. Each MRM-transition was performed for 50 ms (except for LNT and lactose for 25 and 10 ms, respectively). The instrument was calibrated with polypropylene glycol according the instructions of the manufacturer. Unit resolution setting was used for precursor selection whereas low resolution setting was used to monitor fragment ions of the MRM transitions.

Quantitative real-time PCR

The abundance of *B. infantis* and *A. caccae* in mono- and co-culture were determined by quantitative real-time PCR. Bacterial cultures were harvested at 16,100×g for 10 min. DNA extractions were performed using MasterPureTM Gram Positive DNA Purification Kit (Lucigen, Middleton, WI, USA). The DNA concentrations were determined fluorometrically (Qubit dsDNA HS assay; Invitrogen, Carlsbad, CA, USA) and adjusted to 1 ng/µl prior to use as the template in qPCR. Primers targeting the 16S rRNA gene of Bifidobacterium spp. (F-bifido 5'-CGCGTCYGGTGTGAAAG-3'; R-bifido 5'-CCCCACATCCAGCATCCA-3'; 244 bp product (Delroisse et al., 2008)) and A. caccae (OFF2555 5'-GCGTAGGTGGCATGGTAAGT-3'; OFF2556 5'-CTGCACTCCAGCATGACAGT-3'; 83 bp product (Veiga et al., 2010)) were used for quantification. Standard template DNA was prepared by amplifying genomic DNA of each bacterium using primer pairs of 35F (5'-CCTGGCTCAGGATGAACG-3' (Hayashi et al., 2004)) and 1492R (5'-GGTTACCTTGTTACGACTT-3') for *B. infantis*; and

27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R for *A. caccae*. Standard curves were prepared with nine standard concentrations of 100 to 108 gene copies/ µl. PCRs were performed in triplicate with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a total volume of 10 µl with primers at 500 nM in 384-well plates sealed with optical sealing tape. Amplification was performed with an iCycler (Bio-Rad) with the following protocol: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 30 s; 95 °C for 1 min and 60 °C for 1 min followed by a stepwise temperature increase from 60 to 95 °C (at 0.5 °C per 5 s) to obtain the melt curve data. Data was analysed using the Bio-Rad CFX Manager 3.0.

Fluorescent in situ hybridisation

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

Carbohydrate-active enzymes prediction

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

RESULTS

Occurrence of Bifidobacterium infantis and Anaerostipes caccae across the life span

To understand the occurrence of *B. infantis* and *A. caccae* in the gut microbiota across life stages, a published dataset (Yatsunenko et al., 2012) was mined. The two infant-associated bacteria demonstrated opposite trajectories in early life. *Bifidobacterium* genus showed high abundance at the first year followed by a sharp decline, with a negative correlation between age and relative abundance (Spearman ρ=-0.38, P<0.05) (**Figure 1**), a similar finding as presented by Schwab et al. (2017). In contrast, *Anaerostipes* genus (Spearman ρ=0.56, P<0.05) and *Lachnospiraceae* family (Spearman ρ=0.37, P<0.05) were present at low abundance early in life and increased in relative abundance during the aging process (**Figure 1**).

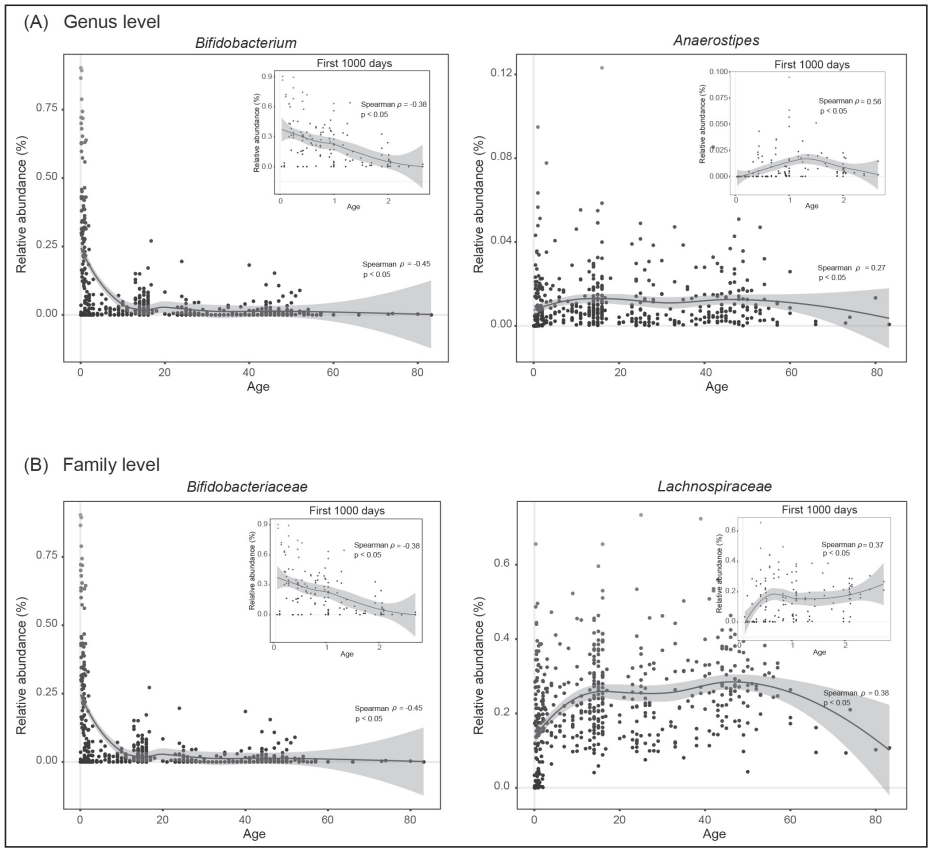

Figure 1 | Occurrence of (A) *Bifidobacterium* and (B) *Anaerostipes* genus, as well as (C) *Bifidobacteriaceae* and (D) *Lachnospiraceae* family in the gut microbiota across age. The plot was generated from a published dataset (Yatsunenko et al., 2012) using R package ggplot2 version 2.2.1. The trend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

Model for Bifidobacterium infantis and Anaerostipes caccae co-occurrence

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

Bacterial strains were cultured in anaerobic bioreactors controlled at pH 6.5 supplemented with either lactose or total human milk (HM) carbohydrates. *B. infantis* monoculture reached maximal cell density around 12 h at growth rate around 0.33 h-1 (ODmax = 1.40±0.38 on lactose and ODmax = 1.37±0.25 on total HM carbohydrates) **(Figure 2**). For *A. caccae* monoculture, no growth or substrate breakdown was detected in identical media (ODmax = 0.02±0.01 on lactose and ODmax = 0.03±0.02 on total HM carbohydrates) (Supplementary Table S1).

The co-culture of *B. infantis* with *A. caccae* reached a growth rate of 0.70 h^{-1} on lactose and 0.72 h^{-1} on total HM carbohydrates, with maximal optical density at 11 h on lactose (ODmax = 3.63±0.61) and at 9 h on total HM carbohydrates (ODmax = 3.54±0.60). The cell density of the co-cultures was higher than the monocultures initially but significantly decreased after about 12 h of growth due to cell lysis. qPCR results showed that the number of *B. infantis* in the monocultures was about 2-fold higher than in the co-cultures from 9 h onwards. The community dynamic in the co-cultures was monitored over time by qPCR. An equal number of *B. infantis* and *A. caccae* (around 10^6 copy number/ml) was inoculated at the start of the fermentation. During the first 7 h, *B. infantis* and *caccae* increased 100-fold, after which growth halted. FISH analysis of samples harvested at 11 h showed a *infantis* to *A. caccae* ratio of 1:6 (Supplementary **Figure S2** and Supplementary **Table S1**). These results accounted for both conditions either on lactose and total HM carbohydrates supplemented cultures.

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

Substrate consumption and metabolite production were monitored over time (**Figure 3**). A similar profile was observed between the fermentation of lactose and total HM carbohydrates, probably because total HM carbohydrates was shown to consist of approximately 10% HMOs and 90% lactose (Supplementary **Figure S1**). On both substrates, the monoculture of *B. infantis* degraded the lactose present into glucose and galactose resulting in the accumulation of monomeric sugars in the supernatant (**Figure 3**). Lactose was completely degraded at 9 h. At the same time point, 17.49±1.83 mM of glucose and 15.24±2.06 mM of galactose were detected in the media supplemented with lactose, whereas 14.77±1.59 mM of glucose and 10.91±1.77 mM of galactose were detected in the media supplemented with total HM carbohydrates.

The monomeric sugars were fully consumed after 31 h. *B. infantis* produced acetate (56.96±4.48 mM on lactose and 50.76±3.23 mM on total HM carbohydrates), lactate (22.73±3.02 mM on lactose and 17.69±1.21 mM on total HM carbohydrates) and

formate (6.56±0.09 mM on lactose and 8.04±0.21 mM on total HM carbohydrates) as the major end metabolites. The final acetate to lactate ratio for *B. infantis* on lactose was 2.4:1 and 2.6:1 on total HM carbohydrates.

The co-culture of *B. infantis* with *A. caccae* also degraded lactose completely within 9 h. However, the co-cultures depleted glucose and galactose faster compared to the monocultures of *B. infantis*. The concentration of monomeric sugars peaked around 7 h in media supplemented with lactose, with 4.62±1.21 mM glucose and 7.10±0.97 mM galactose. In media supplemented with the total HM carbohydrates, the maximum concentration for glucose (4.20±2.10 mM) and galactose (7.39±4.45 mM) was detected after 5 h. Only traces of monomeric sugars were still detectable after 9 h. The major end products of fermentation in the co-cultures were butyrate (31.39±2.15 mM on lactose and 25.80±2.45 mM on total HM carbohydrates), acetate (5.44±0.30 mM on lactose and 9.05±0.71 mM on total HM carbohydrates) and formate (2.53±0.16 mM on lactose and 4.78±1.16 mM on total HM carbohydrates). Butyrate, the signature metabolic end product of A. caccae reached levels of 31.39±1.36 mM with lactose and 27.68±1.38 mM with total HM carbohydrates at 24 h. In contrast to the *B. infantis* monocultures, no lactate was detected after 11 h in the co-cultures.

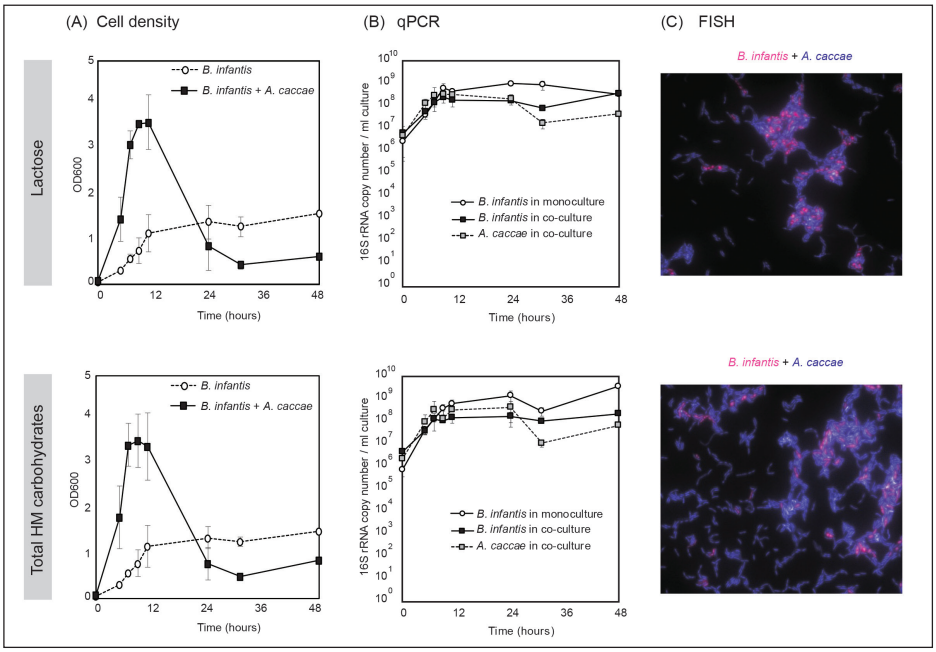

Figure 2 | *Bifidobacterium infantis* supported the growth of *Anaerostipes caccae* in human milk carbohydrates. (A) The optical density (OD600) indicating bacterial growth and (B) qPCR results showing the microbial composition in the mono- and co-cultures over time with lactose or with total human milk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

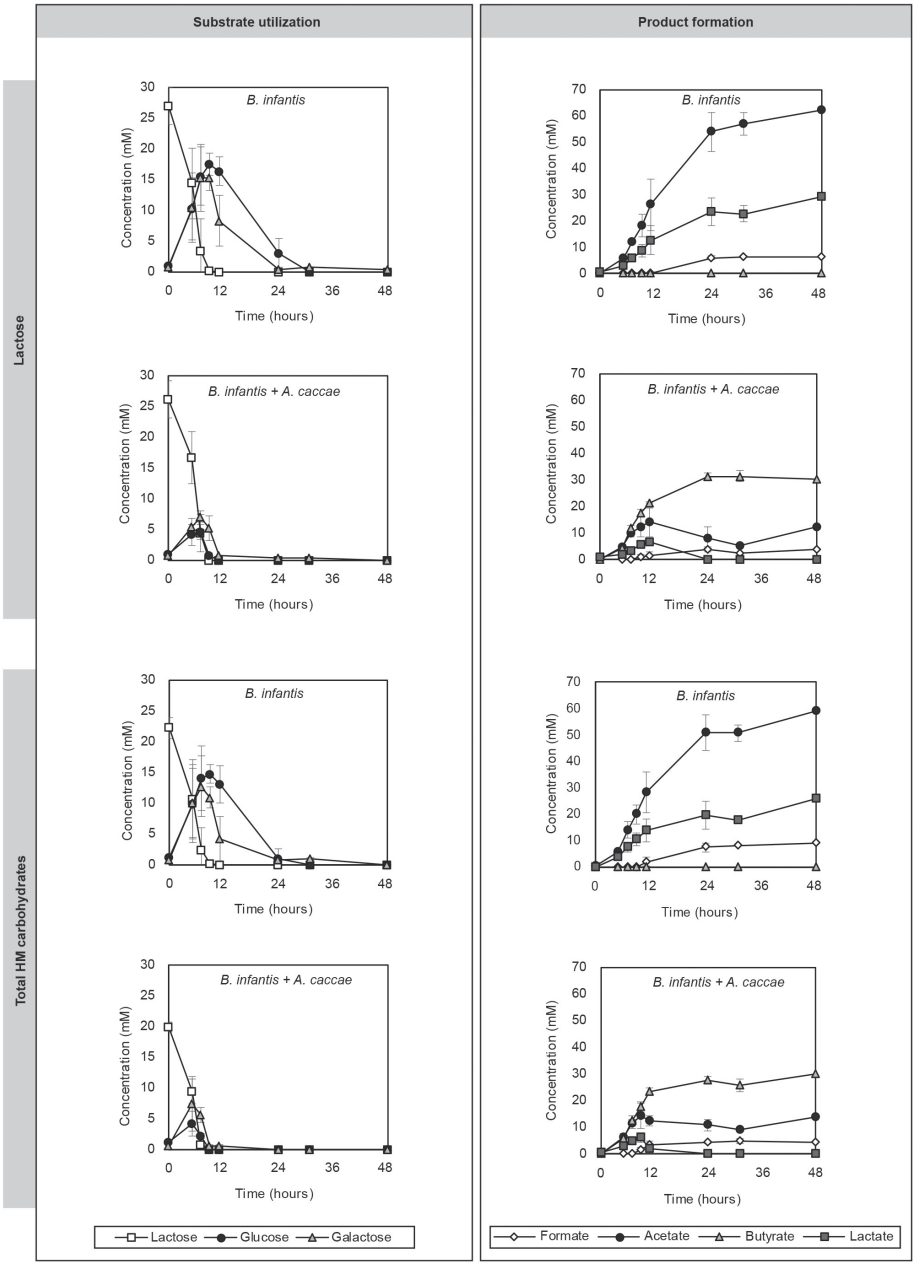

Figure 3 | *Bifidobacterium infantis* supported butyrate production of *Anaerostipes caccae*. The substrate utilisation and product formation of co-cultures on lactose or total HM carbohydrates. Error bars represent the standard deviation for biological triplicates, except for time point 31 h (n=2) and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

The low molecular weight HMOs structures in the total HM carbohydrates were determined by LC-ESI-MS2 for 0 and 24 h cultures in order to understand the specific glycan utilisation by these bacteria (**Figure 4**). The monoculture of *B. infantis* completely degraded the full range of neutral trioses (including 2'-fucosyllactose [2'-FL] and 3-fucosyllactose [3-FL]), tetraoses (including difucosyllactose [DFL], lacto-N-tetraose [LNT], lacto-N-neotetraose [LNnT]), pentaoses (lacto-N-fucopentaose I [LNFP I], lacto-N-fucopentaose II [LNFP II], lacto-N-fucopentaose III [LNFP III], lacto-N-fucopentaose V [LNFP V]), and acidic trioses (including 3'-sialyllactose [3'-SL] and 6'-sialyllactose [6'-SL]). No degradation of HMOs was observed in the *A. caccae* monoculture. On the other hand, the glycan utilisation pattern in the co-culture was identical to the profile of *B. infantis* monoculture indicative of the degrader role of B. infantis in the co-cultures. In addition, substrate quantification using HPLC showed that specific HMO-derived sugars, such as GlcNAc and fucose, were not detected, likely because these stay below the detection limit (0.5 mM) or due to overlap with other HPLC peaks.

Microbial cross-feeding results in a shift of acids pool

The cultures were maintained at pH 6.5 with the addition of 2 M NaOH. *B. infantis* monocultures required a higher amount of base addition compared to the co-culture with *A. caccae* (**Figure 5A**). The acidification of the cultures was reflected in the composition of acids. The total amount of acids at 31 h were higher in the monocultures (86.76±7.78 mM on lactose and 76.75±3.86 mM on total HM carbohydrates) in comparison to the co-cultures (39.36±1.68 mM on lactose and 39.88±3.97 mM on total HM carbohydrates). Furthermore, as a result of microbial cross-feeding in the co-cultures, lactate (pKa=3.86) produced by *B. infantis* monocultures was converted to butyrate (pKa=4.82).

The pKa value indicates the quantitative measurement of the strength of an acid in the solution with lower values for stronger acid. As the pKa values are expressed in log scale, the decrease by one numerical value in lactate compared to butyrate may result in a 10-fold higher concentration of soluble protons.

To investigate the dynamic of pH in early life, the data from Wopereis et al. (2017) was employed. We observed that the faecal pH for infants (n=138) increased from pH 5.7 at 1 week to pH 6.0 at 6 months of life (**Figure 5B**).

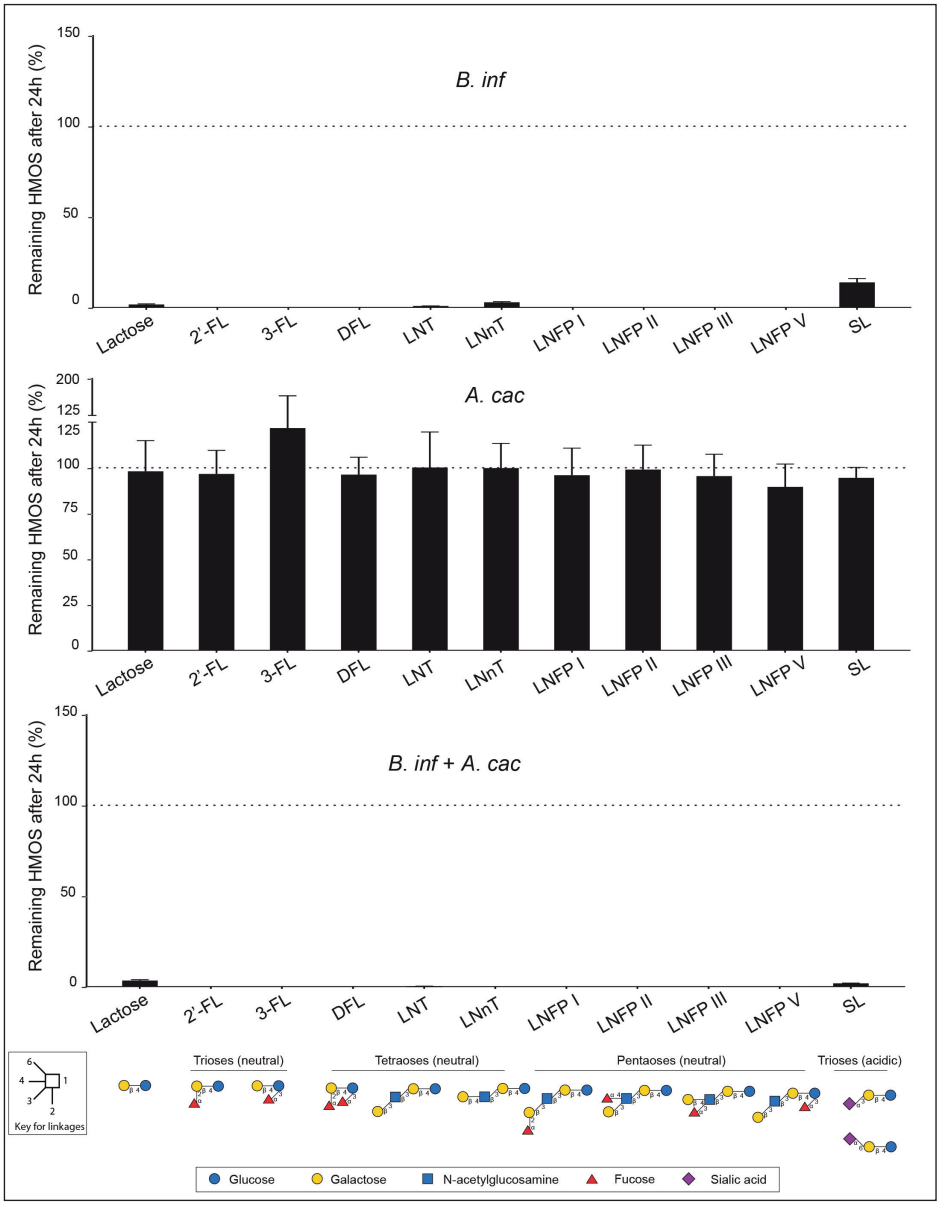

Figure 4 | *Bifidobacterium infantis* monoculture and co-culture with *Anaerostipes caccae* utilised the full range of low molecular weight HMOs. Error bars represent the error propagation for mean of three (for A. caccae) or four (for *B. infantis* and *B. infantis* + *A. caccae*) biological replicates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

DISCUSSION

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

The dominance of *bifidobacteria* is often observed in the infant gut microbiota (Tannock et al., 2016). Infant-associated *bifidobacteria* have evolved to be competitive in utilising human milk as substrate by employing a large arsenal of enzymes to metabolise HMOs (O'Callaghan and Van Sinderen, 2016). We showed that *B. infantis* effectively degrades the full range of the low molecular weight HMOs structures including neutral trioses, tetraoses, and pentaoses as well as acidic trioses. This is consistent with the unique HMOs utilisation capability of *B. infantis* by encoding a 43 kb gene cluster that carries the genes for different oligosaccharides transport proteins and glycosyl hydrolases (Underwood et al., 2014). However, we did not detect the production of 1,2-propanediol from fucosylated HMOs, as demonstrated by Bunesova et al. (2016). No signal peptide or transmembrane domain was predicted for *B. infantis* enzymes involved in the cleavage of the monitored HMOs structures (Supplementary **Table S2**), indicating intracellular degradation of these substrates (Sela *et al.*, 2008). The monosaccharides including glucose and galactose were detected in the growth media of mono- and co-cultures (**Figure 3**) potentially released upon cell lysis. Furthermore, the distinct 'bifid shunt pathway' centred around the enzyme fructose-6-phosphate phosphoketolase (F6PPK) could also account for the competitiveness of *bifidobacteria* (O'Callaghan and Van Sinderen, 2016). The fermentation of sugars via F6PPK-dependent bifid shunt pathway yields more energy compared to the usual glycolysis or Emden-Meyerhof Parnas pathway which could give *bifidobacteria* an additional advantage compared to other gut bacteria (Palframan et al., 2003).

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

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

In the first months of life butyrate levels in the faeces are generally low (Oozeer et al., 2013; Pham et al., 2016) and the major adult-type butyrate-producing population (*Roseburia* and *Faecalibacterium* spp.) remained undetectable up to 30 days postnatal (Jost et al., 2012). We observed an increase of relative abundance for *Lachnospiraceae* family and *Anaerostipes* genus in the first year of life (Yatsunenko et al., 2012). The majority of butyrate-producing bacteria from the *Lachnospiraceae* and *Ruminococcaceae* are not capable of utilising HMOs (Sheridan et al., 2016). For *caccae*, no growth or metabolism was detected in the media containing lactose and HMOs. These subdominant butyrogenic bacteria in the infant gut could depend on cross-feeding with species like *bifidobacteria*. Our results indicate that *A. caccae* could utilise the monomeric sugars and end products like acetate and lactate derived from *infantis* for metabolic activity and growth (Duncan et al., 2004). *A. caccae* is known to convert 1 mol of acetate and 2 mol of lactate to yield 1.5 mol of butyrate (Duncan et al., 2004). Our previous study (Belzer et al., 2017) demonstrated that *A. caccae* in glucose has a specific growth rate around 0.2 h^{-1}. Glucose supplemented with 10 mM of acetate enhanced the growth rate of *A. caccae* to around 0.6 h^{-1}. In the monocultures of *B. infantis* (**Figure 3**), we showed that monosaccharides (glucose and galactose) were detected in the supernatant at the intermediary stage of fermentation, with acetate and lactate as end metabolites. We deduced that the growth rate for *A. caccae* in co-culture with *B. infantis* could be on par of the previously tested condition with glucose and acetate, due to the presence of key substrate to support the growth of *A. caccae*. This is supported by the observation that the total carbon in the end metabolites of the co-cultures was about 1.5-fold lower compared to the *B. infantis* mono-culture, indicating carbon utilisation for the growth of *A. caccae* to synthesise cell components. This metabolic interaction

could also benefit the microbial community by reducing the metabolic burden (Seth and Taga, 2014), shown by the formation of a relatively weaker acid pool. The infant faecal pH showed an increasing trend with age (**Figure 5**) (Wopereis et al., 2017), potentially due to the shift of colonic acids pool driven by changes in diet, microbiota composition and function, and physiological development. Faecal pH provides an indication on the acids pool although other factor such as the production of alkaline molecules, i.e. ammonia could affect the acidity level. Osuka *et al.* (2012) showed that the total organic acid was increased in acidic faeces (pH<6.0) and decreased in alkaline faeces (pH>7.2). Specifically, lactate, succinate, and formate were the main contributors to acidity in acidic faeces. Acetate and lactate as well as a small amount of propionate and butyrate can be detected in the faeces of infants (Pham et al., 2016; Wopereis et al., 2017). Whereas, the typical short chain fatty acids (SCFA) ratio in adult faeces is around 3:1:1 for acetate, propionate and butyrate respectively (Schwiertz et al., 2010; Scott et al., 2011). The shift of the SCFA pool goes hand in hand with the transition of the gut microbiota, likely induced by dietary changes. Upon weaning, the diversification of indigestible fibres due to the introduction of solid foods results in conditions leading to the decrease of the relative numbers of bifidobacteria and the relative increase of *Lachnospiraceae*, *Ruminococcaceae*, and *Bacteroides* spp. (Laursen et al., 2017).

Although the contributing factors to the progression from bifidobacteria dominant community to *Firmicutes* and *Bacteroides* dominant community are not well understood, the well-orchestrated transition is important for health.

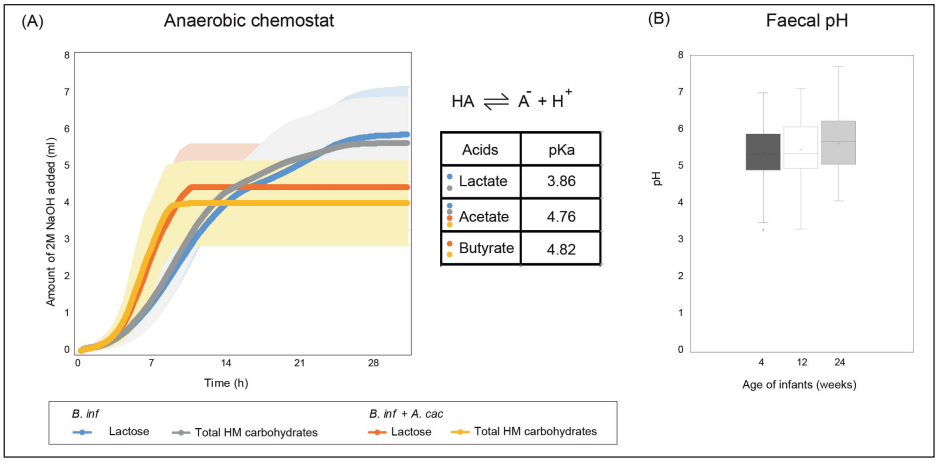

Figure 5 | The acidification of cultures and faecal pH. (A) Base (2 M NaOH) added to maintain the anaerobic chemostat at pH 6.5. The shaded error bars indicate standard deviation for biological triplicates. (B) The faecal pH for infants (n=138). Data adapted from Wopereis et al. (2017).

An aberrant microbial composition and/or SCFA production are associated with colicky symptoms and atopy diseases in infants (Arrieta et al., 2015; De Weerth et al., 2013; Pham et al., 2017; Stokholm et al., 2018; Wopereis et al., 2017). We demonstrated the role of *B. infantis* in driving the butyrogenic trophic chain by metabolising human milk carbohydrates.This microbial cross-feeding is indicative of the key ecological role of bifidobacteria as substrates provider for subdominant butyrate-producing bacteria. The compromised health outcomes as a result of the delayed transition from bifidobacteria-dominant to butyrogenic microbial community highlight the importance of proper developmental stages in the infant gut.

CONFLICT OF INTEREST

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SUPPLEMENTS

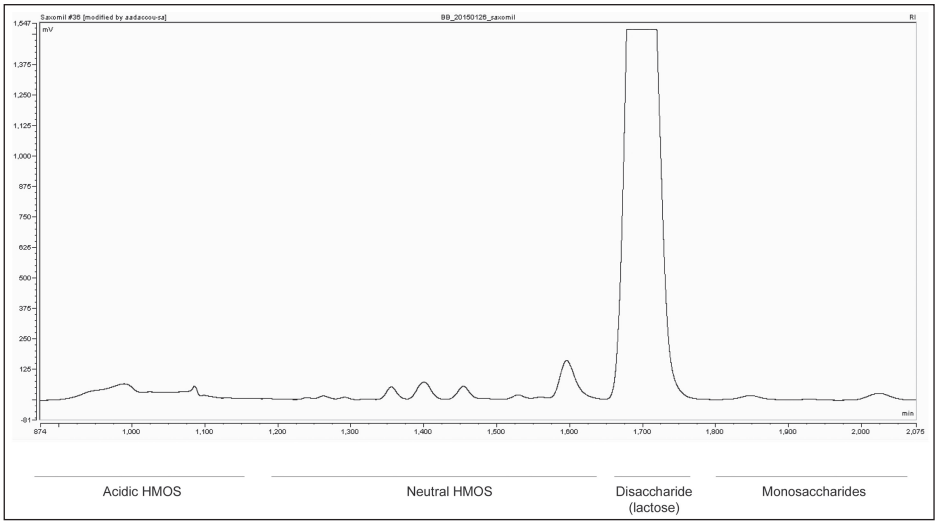

Figure S1 | The GPC-RI chromatogram for total human milk carbohydrates showing a composition of approximately 90% of lactose, 10% of both acidic and neutral HMOs as well as traces of monosaccharides.

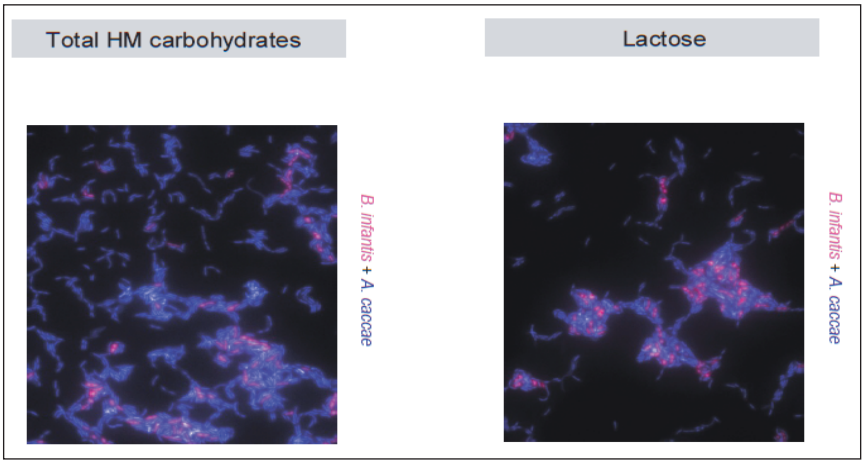

Figure S2 | *Bifidobacterium infantis* supported the growth of *Anaerostipes caccae* in human milk carbohydrates: fluorescent in situ hybridisation of co-cultures at 11 h (*B. infantis* in pink and *A. caccae* in purple). No growth or substrate utilisation was detected for *A. caccae* monocultures in identical media (see Supplementary Table S1).

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

	OD (600nm)	Lactose (mM)	Glucose (mM)	Galactose (mM)	GlucNAc (mM)	GalNAc (mM)	Fucose (mM)	Malate (mM)	Fumarate (mM)	Succinate (mM)	Citrate (mM)	Formate (mM)	Acetate (mM)	Butyrate (mM)	Isobutyrate (mM)	Lactate (mM)	1,2 propanediol (mM)	Propionate (mM)
Basal media	0.009± 0.016	-	0.013± 0.022	-	-	-	0.042 ± 0.073	0.121 ± 0.106	-	-	-	-	-	-	-	-	-	0.369 ± 0.640
Basal media + lactose	0.024 ± 0.005	24.732 ± 2.810	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Basal media + total HM carbohydrates	0.022 ± 0.001	16.107 ± 0.858	0.076 ± 0.107	-	-	0.200 ± 0.003	0.032 ± 0.045	0.091 ± 0.128	-	-	-	-	-	-	-	-	-	0.862 ± 1.205
Basal media + lactose + A.cac	0.016 ± 0.012	27.417 ± 3.359	0.107 ± 0.219	-	-	-	0.022 ± 0.061	0.138 ± 0.152	-	-	-	-	-	-	-	0.342 ± 0.969	-	0.360 ± 0.668
Basal media + total HM carbohydrates + A.cac	0.029 ± 0.016	22.105 ± 5.273	0.616 ± 1.582	-	-	0.186 ± 0.163	0.018 ± 0.051	0.106 ± 0.162	-	-	-	-	-	-	-	0.461 ± 1.305	-	0.147 ± 0.417

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

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

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

None of the proteins in the list is predicted for signal peptide and transmembrane domain.
Abbreviations: 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; DFL, difucosyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose I; LNFP II, lacto-N-fucopentaose II; LNFP III, lacto-N-fucopentaose III; LNFP V, lacto-N-fucopentaose V; SL, sialyllactose.
Supplementary material can also be found online at https://doi.org/10.3920/BM2020.0005.

CHAPTER VI

Human milk oligosaccharide profiles over 12 months of lactation: The Ulm SPATZ Health Study

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L.P.S. and J.Ge. conceived the study question. L.P.S. conducted the statistical analyses. L.P.S. and J.Ge. interpreted the data and wrote the manuscript. M.M, J.Go. and BB. designed the LC-MS^2 method for HMOs analysis. J.Go. and B.B. validated the method for HMOs analysis and analysed the HMOs in the lab. J.Ge. and D.R. conceived and designed the Ulm SPATZ Health Study. J.Ge., D.R., M.M., and B.S. conceptualized the AMICA project for advanced compositional human milk analysis of SPATZ human milk samples. J.Ge. and M.M. manage the AMICA project. All authors (including B.S., M.M., J.Go. and B.B.) critically reviewed the manuscript and approved its final version.

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ABSTRACT

Background: Human milk oligosaccharides (HMOs) have specific dose-dependent effects on child health outcomes. The HMO profile differs across mothers and is largely dependent on gene expression of specific transferase enzymes in the lactocytes.

Objective: This study investigated the trajectories of absolute HMO concentrations at three time points during lactation, using a more accurate, robust and extensively validated method for HMO quantification.

Design: We analysed human milk sampled at 6 weeks (*n*=682), 6 months (*n*=448) and 12 months (*n*=73) of lactation in a birth cohort study conducted in south Germany, using label-free targeted liquid chromatography mass spectrometry (LC-MS^2). We assessed trajectories of HMO concentrations over time, and used linear mixed models to explore the effect of secretor status and milk group on these trajectories. Generalized linear model-based analysis was used to examine associations between HMOs measured at 6 weeks of lactation and maternal characteristics.

Results: Overall, 74%, 18%, 7% and1 % of human milk samples were attributed to milk groups I, II, III and IV, respectively. Most HMO concentrations declined over lactation, but some increased. Cross-sectionally, HMOs presented high variations within milk group and secretor groups. The trajectories of HMO concentrations during lactation were largely attributed to the milk group and secretor status. None of the other maternal characteristics were associated with the HMO concentrations.

Conclusions: The observed changes in the HMO concentrations at different time points over lactation and variations of HMOs between milk groups warrant further investigation of their potential impact on child health outcomes. These results will aid in the evaluation and determination of adequate nutrient intakes, as well as urge to further investigate(or future) investigation of the dose-dependent impact of these biological components on infant and child health outcomes.

Keywords

Human milk oligosaccharides (HMOs), absolute quantitation, most abundant HMOs (trioses to hexaoses), targeted LC-MS/MS, stages of lactation; human milk groups; maternal secretor and lewis (Le/Se) status

INTRODUCTION

Human milk is considered the most suitable source of nutrition for infants during infancy. In addition to other biological components, human milk oligosaccharides (HMOs) constitute the third largest fraction of biomolecules in human milk and comprise approximately 20% of its carbohydrate content (1). More than 100 HMOs have been identified, but, less than 20 typically account for more than 90% of the total HMO content in human milk (1). Mature human milk typically contains between 3-18 g/L of HMOs which are potentially indigestible for the infant [2,3]. Lower HMO concentrations have been reported in preterm milk (~15 g/L) compared to term milk (~ 17 g/L) and higher concentrations of HMOs have been reported in secretor milks (~ 18 g/L) (4). However, evidence suggests that HMOs function as prebiotics for the gut microbiota (5), possess antimicrobial activity (6), prevent pathogen binding and infections (7), modulate the immune system (8–10) and may also be necessary for brain (11,12) and cognitive development (13).

A wide variety of HMOs are synthesized in the mammary gland by the glycosyltransferase enzymes which sequentially add N-acetyl glucosamine, galactose, fucose, and N-acetylneuraminic acid to the basic acceptor molecule, lactose (4). Thus, the pattern and abundance of HMOs are genetically determined and largely dependent on the expression of these enzymes and other maternal factors (14). On one hand, the secretor gene encodes for the α1,2-fucosyltransferase Se-enzyme, which is responsible for elongating the HMO chain by linking fucose in a α1-2 linkage. On the other hand, Lewis blood group gene encodes for the Fuc-TIII (α1-3 fucosyltransferase) enzyme which catalyses the reaction with fucose in a α1-3/4 linkage, creating further fucosylated oligosaccharides (15,16). As such, not every lactating woman synthesises the same set of HMOs due to genetic and other maternal factors.

In this regard, there are four different human milk groups that have been characterised based on the expression of Se- and Fuc-TIII enzymes. Most studies (4) report HMO concentrations of secretor and non-secretor milks. In which case, secretors produce milk attributed to milk groups I and III, while non-secretors produce milk which belongs to milk groups II and IV. It is only until recently that a few studies have compared the HMO concentrations between these four milk groups (17–22). Conversely, concentrations of HMOs that have been reported in literature vary widely, and these variations are attributable to general biological variability, lactation stage at sampling, geographic region (23,24), influences of other maternal factors as well as differences in analytical methods (4).

Although data on human milk are often used as a standard for establishing infant feeding regimens, not many studies have reported HMOs as absolute concentrations in large sample sizes in real world observational cohorts. In addition,

accurate, reliable and robust methods for identifying and quantifying exact HMO concentrations are needed to identify specific concentrations of HMOs (4) as well as understand the exact concentrations at which they could impact infant health (25). To our knowledge only a few other studies (26–29) have investigated a variety of absolute individual HMO concentrations using current and recent liquid chromatography mass spectrometry (LC-MS) technologies. However, the methodology used in the current study has been validated recently (30) and has overcome limitations from these previous studies which include its sensitivity to measure more HMOs in a larger sample size.

Moreover, most of the studies investigating HMO concentrations and their trajectories over time have focused on the first few weeks of lactation. Some have explored the composition of HMOs beyond one or two months (17,21,23,31–35), but none up to 12 months of lactation. Granted that individual HMOs have been shown to have specific effects on infant health and disease, there is a need for comprehensive research that will aid in understanding the origin and implications of changes in HMO composition during lactation.

In light of this, we measured absolute HMO concentrations in large and well-defined human milk sample sets in the Ulm SPATZ Health Study collected at 6 weeks, 6 months and 12 months of lactation using a more accurate, robust, and extensively validated method, developed for HMO quantification. We further assessed the trajectories of HMOs over time, taking into account the four milk groups that have previously been identified. Our study therefore aims to provide novel information regarding longitudinal changes of HMOs during 12 months of lactation thereby adding to the already existing literature on HMO variations during lactation.

MATERIAL AND METHODS

Study design and population

Data were obtained from the Ulm SPATZ Health Study, an ongoing birth cohort study, in which a total of 970 mothers and their 1006 new born infants were recruited from the general population, soon after their delivery, during their hospital stay at the University Medical Centre Ulm, southern Germany, between April 2012 and May 2013 (36). Mothers were excluded if they had inadequate German language skills, outpatient childbirth, maternal age <18 years, postpartum transfer of mother or child to intensive care unit, or still birth. Participation in the study was entirely voluntary and written informed consent form was obtained from all study participants. Ethical approval was obtained from the Ethics board of Ulm University (No. 311/11).

Data collection and measurement

Self–administered questionnaires were used to collect demographic, lifestyle and birth-related data including child sex, delivery mode, birth season, birthweight, maternal age, education, parity, pre-pregnancy body mass index (BMI calculated as [mass(kg)/height(m)^2]) and smoking status in the 12 months prior to and during pregnancy. Electronic hospital charts and routine screening examinations were further used to collect additional information. Human milk samples were collected at approximately 6 weeks [Mean (SD), 5.9 (0.7) weeks], 6 months [25.8 (0.8) weeks] and 12 months [53.9 (4.3) weeks] post-delivery from those lactating women who were still breastfeeding at the time of sample collection and who were willing to provide a sample. Lactating mothers were instructed to manually express or pump breast milk between 9am and 12pm, after breakfast and before lunch, but at least one hour after the last feeding. In some cases where necessary, trained study nurses helped mothers with expression. Mothers stored the milk in the fridge until study nurses collected it from their homes on the same day if milk was expressed between 9am and 12pm (76.9%), or on the next day if milk was expressed in the evening [after 12pm, 9%; and before 9am, 13.8%)] and delivered it refrigerated to the study center. Additional data was collected at 6 weeks, 6 months and 12 months post-delivery by telephone interview or self-administered questionnaires sent by post.

Analysis of HMOs

Human milk samples were stored at -80 °C until analysis of HMOs in 2019. Samples were transported to and received at Danone Nutricia Research on dry ice. The samples were defrosted overnight in a refrigerator (4-8 °C) and homogenized on a flatbed shaker for 30 min at room temperature. The samples were gently inverted 10 times prior to aliquoting for subsequent analysis and stored at -80 °C. The identification and quantitation of individual native HMOs was accomplished by targeted liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS^2) in negative ion mode. This method allowed studying distinct HMO structures differing in monosaccharide composition, sequence, glycosidic linkages or the molecular conformation. Thereby even isobaric HMO-isomers such as Lacto-N-fucopentaose (LNFP) I, II, III and V could be distinguished. Our LC-ESI-MS^2 approach is based on the method as described by Mank et al., 2019 (30) utilizing the concept of pre-defined precursor ions and collision induced (CID) diagnostic fragment ions for unambiguous identification and quantitation of lactose and 16 various (isomeric) HMOs. For this study, the latter approach was adapted and improved in major aspects like choice of stationary phase and LC-gradient as described subsequently.

Clean up of human milk samples was achieved by delipidation and subsequent 3kDa ultrafiltration (UF) in 96 well format. Aliquoted human milk samples (40 µl) were thawed for 15 minutes at room temperature and 200 µl of 80 mM ammonium

formate (purity ≥ 99.0%, Sigma Aldrich, St. Louis, Missouri United States of America (USA)) containing 0.12 g/L internal standard alpha-Arabinopentaose (purity ~90%, Megazyme, Bray, Ireland) was added (30). The samples were briefly vortexed and the lipids were separated by centrifugation at (20,000xg for 10 min at 4°C). 160 µl of the clear HMOs fraction was aspired carefully piercing through the lipid layer and by transferring the aspirate to a 350 µL 3kDa UF 96 well plate (Omega membrane filter plate, Pall, New York, USA) which was preflushed by centrifuging 200 µl of 67 mM ammonium formate in each well for one hour at 25 °C and 1500xg. A HMO filtrate was yielded by centrifugation at 1500xg, for 2 h at 25°C.

The filtrate was stored at -20°C until further usage. Prior to LC-ESI-MS^2 analysis, the filtrate was thawed at RT, vortexed for one minute, 20 µl diluted 1:1 (v/v) with acetonitrile and subjected to LC-ESI-MS^2 analysis by injection of 5µL sample. LC-ESI-MS^2 analysis was performed on a 1200 series HPLC stack (Agilent, Waldbronn, Germany) consisting of solvent tray, degasser, binary pump and auto sampler coupled to a 3200 Qtrap mass spectrometer (ABSciex, USA). Oligosaccharides were separated by means of an Acquity UPLC BEH Amide Column (1.7µm, 2.1x100mm) (Waters, USA) with a precolumn (1.7µm 2.1x5mm) (Waters, USA). HMOs were separated using a 25 min water-acetonitrile gradient following a 4 min pre-equilibration step. Solvent A consisted of 10mM ammonium formate and 0.05% ammonia in water and solvent B of 10mM ammonium formate and 0.05% ammonia (Merck, Burlington, Massachusetts, USA) in 90% acetonitrile (VWR, Radnor, Pennsylvania, USA). A 4 min pre-equilibration step was performed with a flowrate of 500 µl/min using 90% solvent B. The following gradient elution of HMOs was performed with a flowrate of 400 µl/min, starting with 90% solvent B for 0.5 min. Then solvent B was decreased to 70% in 16 minutes. In the next step, solvent B was further decreased to 50% in 0.5 min and was kept at 50% for 5 min. Then B was increased to 90% in 0.1 min and was kept at 90% for 2.9 min. The column was constantly kept at 65°C. HMO structures eluting from the column were infused into the mass spectrometer and analysed qualitatively and quantitatively by multiple reaction monitoring (MRM) in negative ion mode. Per HMO, two specific MRM transitions for neutral HMOs up to hexaoses and acidic HMOs up to trioses were monitored (Supplementary **Table S1**). The spray voltage and source temperature were -4500 V and 500 °C, respectively. The declustering potential, entrance potential, exit potential and collision energy were optimized for the individual compounds measured. For optimizing the individual compounds 0.1 g/L in water was infused at 10 µl/min together with eluent (20% A+ 80%B) at a flow rate of 200 µl/min and the $[M-H]^-$ ion was selected. The optimal declustering MRM potential per compound was assessed by injecting a mixed standard and varying the declustering potential. Scheduled MRM was used with a target scan time of 2 sec and a 150 sec detection window. The instrument was calibrated with polypropylene glycol according the instructions of the manufacturer. Absolute quantitation was

achieved by an external calibration curve using standards obtained from Isosep (Tullinge, Sweden), Prozym (Ballerup, Denmark), Carbosynth (Berkshire, UK), Sigma Aldrich (Missouri, USA) and Elicityl (Crolles, France) of minimally 90 % purity. Quantification of absolute HMO concentrations was done for lactose and 16 of the most abundant HMOs comprising: 2'-fucosyllactose (2'-FL); 3-fucosyllactose (3'-FL); 3'-sialyllactose (3'-SL); 4'-Galactooligosaccharide (4'-GL); 6'-Galactooligosaccharide (6'-GL); 3,2'-difucosyllactose (DFL); 6'-sialyllactose (6'-SL); lacto-N-tetraose (LNT); lacto-N-neotetraose (LNnT); 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-difusohexaose I (LNDFH I); and the sum of Lacto-N-difusohexaose II and Lacto-N-neodifucohexaose II (LNDFH II + LNnDFH II, standard containing both).

Standards for quantitation (n=8) were prepared in 20 mM ammonium formate, diluted 1:1 with acetonitrile, and injected together with the prepared samples in a batch. The calibration standards were run at the beginning, after 48 samples and at the end of the batch. Two MRM transitions were monitored per compound, one served as a quantifier and one as a qualifier. The area ratio of the two transitions in the human milk samples had to be within 20% compared to the area ratio of the standards. This threshold was set to verify that no interference from other matrix compounds would affect the correct determination of HMO concentrations. The quality control (QC) sample was a colostrum milk sample attributed to milk group I and a written informed consent form was obtained. A human milk sample batch consisted of 94 samples. The human milk-QC was prepared in duplicate together with the human milk study samples.

Method validation

The above described method was validated for repeatability, reproducibility, bias, batch stability and linearity. The repeatability, reproducibility, and bias were determined in water on 3 different days at 4 different concentrations in triplicate to assess how calibration graphs are affected. The repeatability and bias in human milk were determined in three individual donors per milk group, fortified with HMOs, and performed in duplicate (n=24). The reproducibility in human milk was calculated using the human milk-QC samples analysed with the different batches. The linearity was investigated using prepared solutions (n=8) with different known concentrations. The fit (linear or quadratic) and weighting (none, 1/x or 1/x2) were assessed for the best back-calculated concentration. To obtain a satisfying fit and weighting, a high and a low concentration range were determined for the oligosaccharides. If the concentration of the compound in the sample was below the high concentration range the concentration of the compound was calculated on the calibration graph for the low concentrations. To assess the stability of the LC-MS^2 when analyzing a full batch of samples, the QC sample was prepared 96 times, pooled and 40 µl was distributed in each of the 96 wells. The batch was

injected and the RSD of the concentration per compound was calculated. The RSD was between 4-8% except for 3'-SL and 6'-SL which were 15% and 13% respectively. To continuously monitor performance of the LC-ESI-MS^2 analyses, a human milk-QC sample was introduced at the beginning and end of each human milk sample batch. Results of the validation are shown in the supplementary **Table S1**.

Secretor phenotype and Milk typing

Milk typing was based on the presence of α1,2- and α1,4-fucosylated HMOs, following previous (15,37) and validated approaches (30). Milk attributed to group I contains α1,2- and α1,4-fucosylated HMOs such as 2'-FL and LNDFH I; milk which belongs to group II contains α1,4- fucosylated HMOs, such as LNDFH II, but does not contain α1,2-fucosylated HMOs, such as 2'-FL, LNFP I and LNDFH I; group III milk contains α1,2 fucosylated HMOs, such as 2'-FL and LNFP I, but does not contain α1,-4 fucosylated HMOs, such as LNDFH I and II and; group IV, the least common phenotype, contains neither α1,2- nor α1,4- but only α1,3-fucosylated HMOs, such as 3'-FL, which are present in all the four milk groups since their synthesis apparently is not influenced by the secretor and Lewis genes (15,17). Briefly, in this study, lactating mothers whose milk presented both α1,2 and α1,4 fucosylated HMOs were attributed to group I. Women whose milk samples did not present LNFP I and LNDFH I [below the lower limit of quantification (<LLOQ)] were classified as group II. Women whose milk samples did not present LNFP II and LNDFH I (<LLOQ) were classified as group III. Those whose human milk sample did not present LNFP I, LNDFH I and LNFP II (<LLOQ) were classified as type IV. For the categorization according to secretor phenotypes, milk groups I and III were grouped as secretors, while milk groups II and IV were grouped as non-secretors. HMO concentrations were presented as absolute values (g/L). The relative proportion of HMOs as percentages of total HMOs was then calculated from absolute values. The total HMO concentration was calculated as the total sum of the specific oligosaccharides detected.

Statistical Analysis

Data were checked for normal distribution using the Kolmogorov–Smirnov test and visual inspection of histogram plots. HMO profiles were summarised using descriptive statistics and values presented as mean (SD) as well as median [min, max]. Data for 4'-GL were excluded because >90% of values were found to be <LLOQ. For the remaining HMOs, all values <LLOQ were replaced by LLOQ / √ (2) and the values higher than the upper limit of quantification (>ULOQ) were extrapolated for 3-FL, 3'-SL, DFL, 6'-GL, 6'-SL, LNT, LNFP I, LNFP II, LNDFH I, LNDFH II + LNnDFH II. The Blom's normal ranks transformation (38) was applied to individual HMO concentrations. General linear univariate and multivariate models were used to investigate associations between individual HMOs with maternal pre-pregnancy BMI, parity, delivery mode, gestational age and maternal BMI at 6 weeks, infant sex and exclusive breastfeeding. Generalized linear model based-repeated measure

analysis was used to evaluate changes in lactose and total HMO concentrations over lactation. Linear mixed models were used to assess milk group and secretor status differences in the HMO changes over lactation. Bonferroni-adjusted level of statistical significance (α=0.05/14=0.0036) was applied to control for multiple testing. All statistical analyses were performed with R (version 3.5.1; R Foundation for Statistical Computing) and SAS version 9.4 (The SAS Institute, Cary, NC, USA).

RESULTS

Human milk samples were available for HMO analysis from 682, 448 and 73 lactating women at 6 weeks, 6 months and 12 months, respectively (**Table 1**).

A total of 66 lactating mothers had HMO data available from samples collected at all three time points (6 weeks, 6 months and 12 months). All women were aged 32.7 ± 4.7 years. At 6 weeks more than 70% of the lactating women were attributed to milk group I and 1% were attributed to milk group IV. In rare cases, i.e. 7 lactating women, were initially attributed to milk group IV at 6 and 12 months. LNFPI was not present or <LLOQ in some cases, while other FUT2 related HMOs (2'-FL, DFL, LNDFH I) were found in partly quite high abundance. We then confirmed the milk groups by recognising additional surrogate markers for active FUT2 and FUT3 (i.e. LNDFH I). As a result, they were re-assigned to milk group I.

Table 1 | Characteristics of lactating mothers whose human milk samples were available for human milk oligosaccharide (HMO) analysis in the Ulmer SPATZ Health Study

Sums (n) may not always add up to total because of missings for certain items. Percentages exclude missings. Milk groups were based on quantification and presence of certain human milk oligosaccharides.

Characteristics	All 6 weeks samples (*n* = 682)		All 6 months samples (*n* = 448)		All 12 months samples (*n* = 73)	
	n	% or mean	n	% or mean	n	% or mean
Mother						
Age	681	33.1	447	33.5	72	34.3
Maternal body weight (6 weeks) [kg]	587	70.5	385	69.9	58	70.8
Maternal BMI (6 weeks) [kg]	662	24.7	438	24.8	72	25.1
Parity						
0 births	368	54	243	54	42	57.5
≥1 births	314	46	205	46	31	42.5
Milk group						
I	502	74	330	74	55	75
II	122	18	80	18	13	18
III	49	7	32	7	5	7
IV	9	1	6	1	0	0
Infant						
Female	339	50	216	48	29	40
Male	343	50	232	52	44	60
Gestation weeks	682	38.7	448	38.8	73	38.6
Birth weight [g]	682	3272	448	3282	73	3292
Delivery mode						
Vaginal spontaneous	432	63	294	65	48	66
Elective caesarean	80	12	52	11	8	11
Emergency caesarean	118	17	66	14	13	18
Vaginal assisted	52	8	36	8	4	5

Individual HMO concentrations over lactation irrespective of milk group

We assessed the trajectory of individual HMO concentrations during lactation amongst lactating women who had HMO data available at all three time points (*n*=66). Following Bonferroni correction, most HMO concentrations were significantly lower at 12 months of lactation (α threshold = 0.0036) than at earlier time points with the exception of 3-FL, DFL, 3'-SL and LNDFH II +LNnDFH II, which were significantly higher (**Table 2**). The trajectories of HMO concentrations over lactation up to 6 months were the same when the sample size was restricted to mothers with human milk samples available at both 6 weeks and 6 months (*n*=422; Supplementary **Table S2-S4**).

Trajectory of total HMOs and lactose concentrations during lactation

Lactose concentrations increased at 6 months ($\beta = 0.6922$, $p < 0.0001$) and decreased significantly at 12 months ($\beta = -0.5602$, $p = 0.0002$) compared to 6 weeks of lactation (**Figure 1A**). These changes remained significant even after excluding the 6 mothers whose lactose concentrations were extremely low in comparison to the other values. Overall, total HMOs (all three time points combined) ranged from 1.66 to 12.65 g/L (mean 5.7 ± 1.35). Total HMO concentrations decreased significantly at 6 months ($\beta = -1.3487$, $p < 0.0001$) and 12 months ($\beta = -1.0606$, $p < 0.0001$) compared to 6 weeks of lactation (**Figure 1B**), regardless of secretor status or milk group. These changes in total HMO concentrations and lactose were similar when the available data for 6 weeks and 6 months (*n*=422) were considered (Supplementary **Figure S1**).

Relative proportions (%) of HMOs according to milk group and secretor status

As expected, individual and total HMO concentrations varied significantly between the four milk groups. Overall, total combined HMOs (sum of 14 detected structures) in all human milk samples (Mean ± SD; Median [Min, Max]) were significantly lower in group IV milk (3.02 ± 0.65; 2.88 [1.84, 4.19]) compared to group I (5.76 ± 1.22; 5.63 [2.49, 10.44]), group II (4.30 ± 1.07; 4.13 [1.66, 7.42]), and group III (6.44 ± 1.57; 6.52 [2.62, 10.67]). At both 6 weeks and 6 months, total HMO concentrations were different between all four milk groups before and after adjustments ($p < 0.0001$) and at 6 months total HMOs did not differ significantly between groups II and IV ($p = 0.0545$). The relative proportion of 3-FL was more abundant in group II milk and LNT in group V milk (**Figure 2**, and Supplementary **Tables S5-S7**). Most of the individual HMOs differed significantly between the four milk groups, except for 6'-SL, LNFP III and LNFP II. When lactating mothers were stratified according to secretor phenotype, non-secretor mothers produced significantly lower concentrations of HMOs compared with their secretor counterparts (**Figure 3**).

This difference was primarily due to the absence of 2'-FL which accounted for more than 40% of total detected HMOs in secretor milk. Within the secretor groups, total HMOs irrespective of time point were variable between lactating mothers in terms of HMO content (range among secretors, 2.62 – 12.60 g/L; non-secretors 1.66 – 8.64 g/L) as well as in relative composition (e.g. 2.81- 77.40% 2'-FL among secretors; 0.99 - 65.80% LNT among non-secretors; Supplementary **Tables S8-S9**). Most of the other HMOs also differed significantly by secretor status, except 6'-GL, 6'-SL and LNFP III.

Table 2 | Absolute concentrations of human milk oligosaccharides (HMOs) measured in g/L at 6 weeks, 6 months and 12 months of lactation in the Ulm SPATZ Health Study

HMOs	6 weeks (n=66)	6 months (n=66)	12 months (n=66)	p[1]	p[2]	p[3]
2'-FL						
Mean (SD)	2.45 (1.32)	1.65 (1.08)	1.43 (0.85)	<0.001	0.305	<0.001
Median [Min, Max]	2.60 [0.13, 6.00]	1.65 [0.13, 4.30]	1.45 [0.13, 3.30]			
3'-FL						
Mean (SD)	0.64 (0.57)	1.24 (0.74)	1.52 (1.26)	<0.001	0.446	<0.001
Median [Min, Max]	0.44 [0.04, 2.40]	1.05 [0.08, 3.10]	1.05 [0.12, 6.90]			
3'-SL						
Mean (SD)	0.12 (0.03)	0.14 (0.04)	0.25 (0.12)	0.470	<0.001	<0.001
Median [Min, Max]	0.13 [0.08, 0.24]	0.14 [0.07, 0.34]	0.22 [0.09, 0.58]			
6'-GL						
Mean (SD)	0.02 (0.01)	0.01 (0.00)	0.01 (0.01)	<0.001	0.188	<0.001
Median [Min, Max]	0.01 [0.01, 0.04]	0.01 [0.00, 0.02]	0.01 [0.00, 0.03]			
DFL						
Mean (SD)	0.18 (0.14)	0.20 (0.14)	0.40 (0.41)	0.198	0.010	<0.001
Median [Min, Max]	0.15 [0.01, 0.56]	0.20 [0.01, 0.51]	0.27 [0.01, 2.20]			
6'-SL						
Mean (SD)	0.24 (0.09)	0.04 (0.02)	0.01 (0.00)	<0.001	<0.001	<0.001
Median [Min, Max]	0.22 [0.08, 0.53]	0.03 [0.01, 0.12]	0.01 [0.01, 0.03]			
LNT						
Mean (SD)	0.86 (0.44)	0.44 (0.28)	0.36 (0.24)	<0.001	0.038	<0.001
Median [Min, Max]	0.75 [0.15, 1.90]	0.41 [0.10, 1.50]	0.32 [0.05, 1.20]			
LNnT						
Mean (SD)	0.08 (0.05)	0.05 (0.05)	0.02 (0.02)	<0.001	<0.001	<0.001
Median [Min, Max]	0.07 [0.01, 0.23]	0.03 [0.01, 0.23]	0.02 [0.01, 0.13]			
LNFP I						
Mean (SD)	0.43 (0.37)	0.19 (0.21)	0.20 (0.21)	<0.001	0.696	<0.001
Median [Min, Max]	0.31 [0.04, 1.60]	0.09 [0.04, 1.10]	0.12 [0.04, 1.00]			

Table 2 | Continued.

HMOs	6 weeks (n=66)	6 months (n=66)	12 months (n=66)	p[1]	p[2]	p[3]
LNFP V						
Mean (SD)	0.03 (0.03)	0.02 (0.02)	0.02 (0.02)	0.641	0.996	0.598
Median [Min, Max]	0.02 [0.01, 0.14]	0.02 [0.01, 0.10]	0.02 [0.01, 0.08]			
LNFP III						
Mean (SD)	0.17 (0.07)	0.19 (0.07)	0.14 (0.05)	0.089	<0.001	0.002
Median [Min, Max]	0.18 [0.05, 0.31]	0.18 [0.07, 0.36]	0.13 [0.04, 0.30]			
LNFP II						
Mean (SD)	0.30 (0.40)	0.24 (0.24)	0.24 (0.24)	0.567	0.800	0.376
Median [Min, Max]	0.13 [0.04, 1.70]	0.15 [0.04, 1.40]	0.17 [0.04, 1.20]			
LNDFH I						
Mean (SD)	0.50 (0.34)	0.27 (0.19)	0.36 (0.26)	<0.001	0.033	0.012
Median [Min, Max]	0.54 [0.02, 1.30]	0.27 [0.02, 0.70]	0.35 [0.02, 0.90]			
LNDFH II + LNnDFH II						
Mean (SD)	0.06 (0.12)	0.06 (0.08)	0.08 (0.11)	0.002	0.441	<0.001
Median [Min, Max]	0.01 [0.01, 0.70]	0.03 [0.01, 0.30]	0.03 [0.01, 0.46]			
Total HMOs						
Mean (SD)	6.09 (1.05)	4.74 (0.84)	5.03 (1.05)	<0.001	0.093	<0.001
Median [Min, Max]	5.91 [3.86, 8.32]	4.84 [3.01, 6.59]	5.07 [1.66, 8.64]			

P values in **Table 2** are derived from Wilcoxon signed-rank test comparing HMOs concentrations between [1]6 weeks and 6 months, [2]6 months and 12 months, [3]6 weeks and 12 months. The Bonferroni-adjusted level of statistical significance is α=0.05/14=0.0036. HMOs, human milk oligosaccharides. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactosyllactose; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

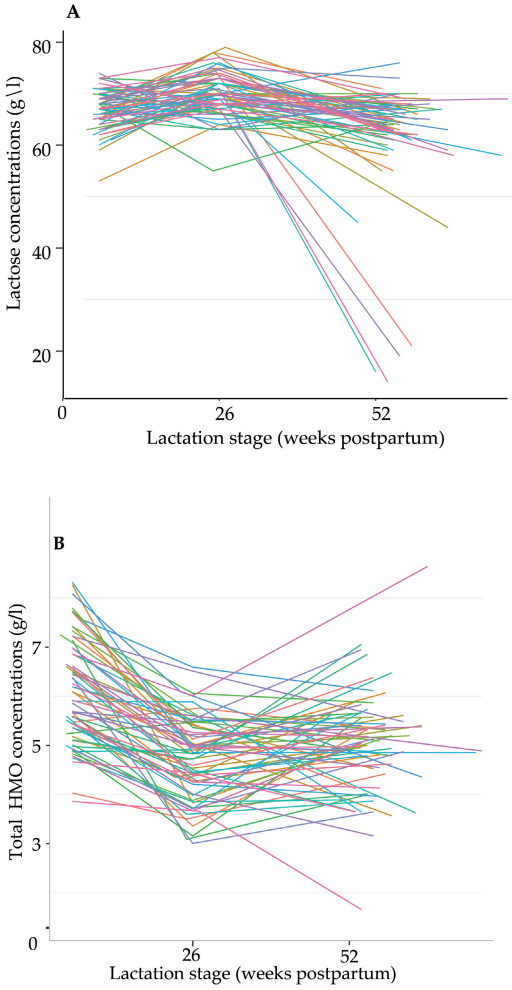

Figure 1 | Multiple line plot of absolute (A) lactose and (B) total human milk oligosaccharide (HMO) concentrations (g/L) during lactation. Each line represents 1 sample among the lactating mothers with human milk samples collected at all three time points, 6 weeks, 6 months and 12 months (n = 66). Lactation stage was determined by the real age of the infant at the stage of lactation at which their respective mothers provided a human milk sample.

Factors associated with HMO concentrations at 6 weeks

Milk group was strongly associated with most individual HMO concentrations ($p < 0.0001$), except 6'-GL ($p = 0.0953$) and 6'-SL ($p = 0.0353$, Bonferroni correction α threshold = 0.0036). Also as expected, secretor status was strongly associated with most individual HMOs except 6'-SL ($p = 0.4537$), 6'-GL ($p = 0.0523$) and 3'-SL ($p = 0.0478$).

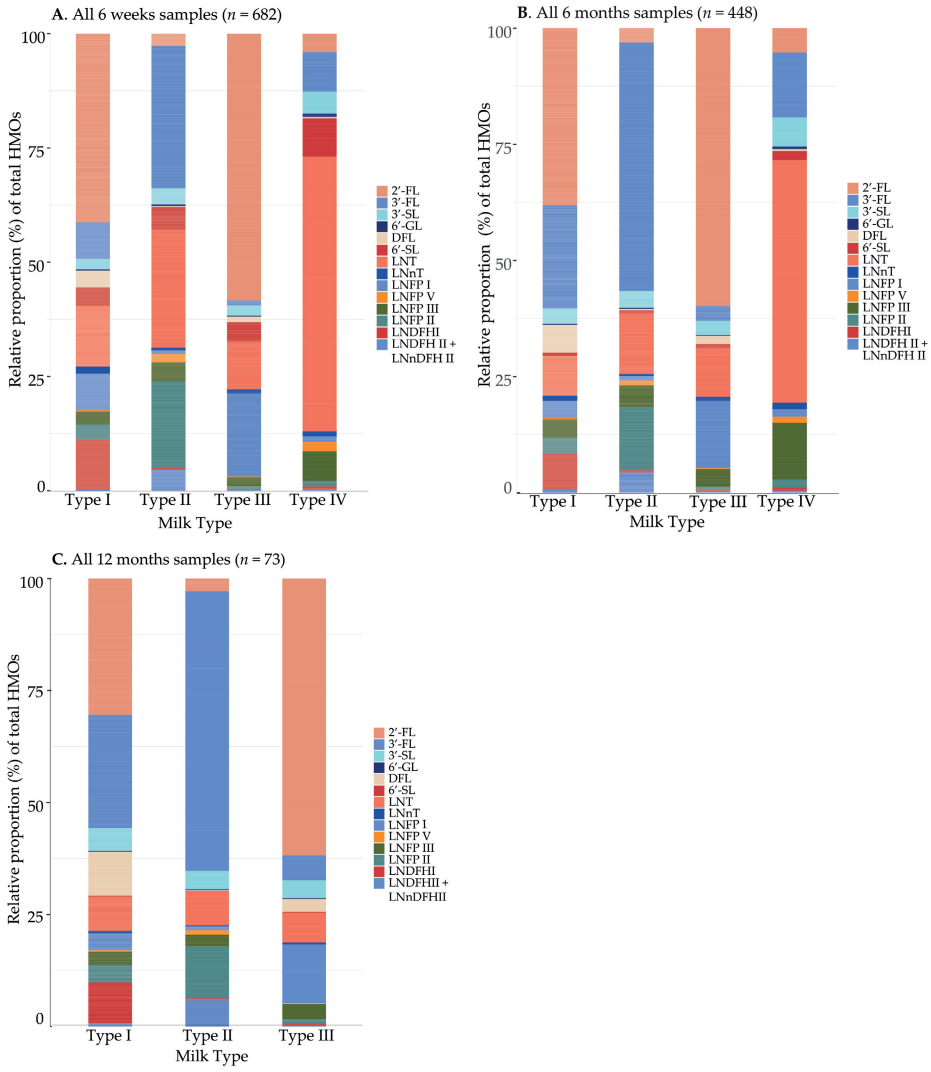

Figure 2 | Relative proportion [%] of total human milk oligosaccharide (HMO) concentrations according to human milk group, in samples collected at 6 weeks, 6 months and 12 months of lactation. Percentages of total HMOs were calculated for all women at A) 6weeks; B) 6 months; and C) 12 months according to the four different milk groups. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactosyllactose; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

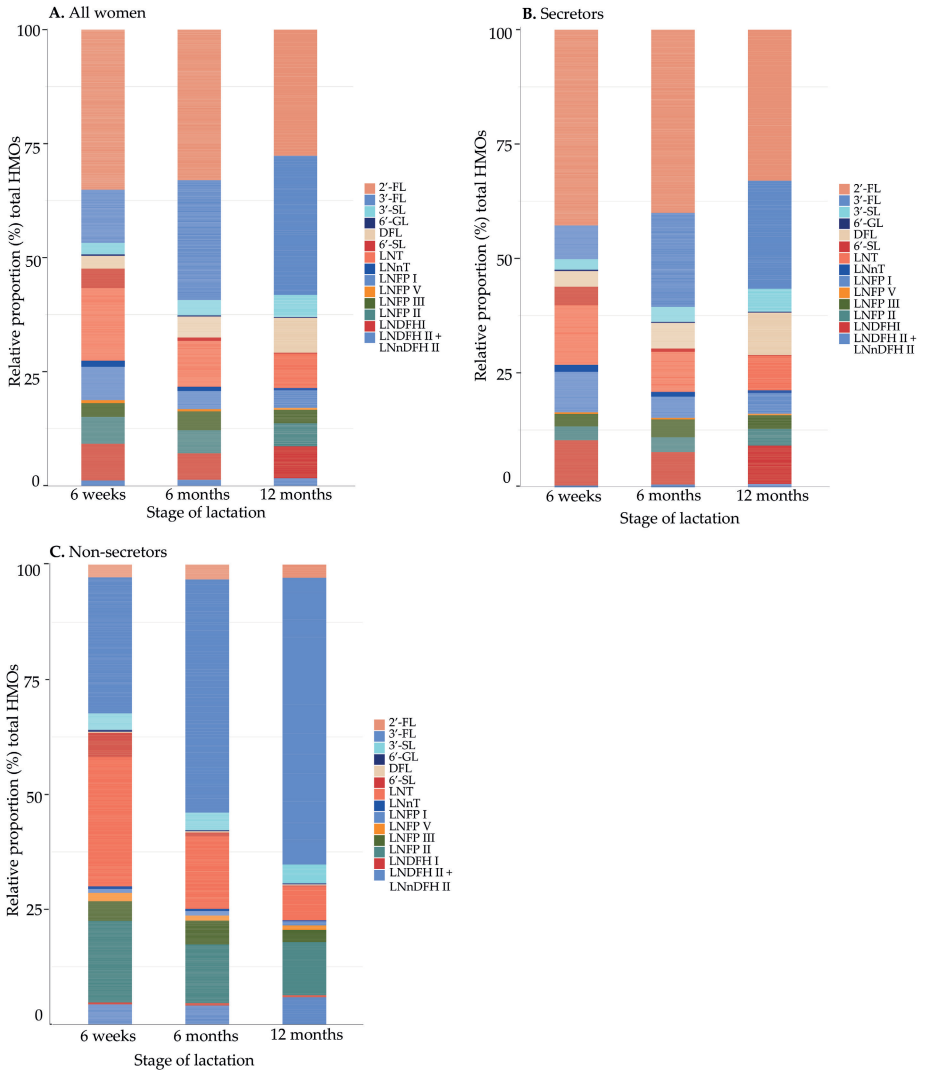

Figure 3 | Relative proportion [%] of human milk oligosaccharides (HMOs) in human milks samples collected at 6 weeks, 6 months and 12 months. Percentages of total HMOs were calculated for A) all women regardless of milk group or secretor status; B) secretors milks attributed to by milk groups I and III; and C) non-secretor milks attributed to by milk groups II and IV. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactosyllactose; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

Other maternal factors associated with HMOs were pre-pregnancy BMI [lower 3'-SL (β = -0.1669, p = 0.0358) in overweight women]; maternal BMI at 6 weeks [higher 6'-SL (β = 0.1556, p = 0.0028) and lower 3'-SL (β = -0.2141, p = 0.0068) in overweight women; parity[higher 6'-GL(β = 0.1193, p = 0.0476), LNFP I (β = 0.0314, p = 0.0256) and LNFP III (β = 0.1858, p = 0.0189) in multiparous women; and gestation period (lower 6'-GL (β = -0.1527, p = 0.0453) in the milk of lactating mothers with a gestation period of ≥ 41 weeks). Lactose (β = 0.2788, p < 0.0001) and LNDFH I (β = 0.1355, p = 0.0259) were higher in exclusively breastfed infants.

Although infant sex was not associated with individual HMO concentrations, total HMO concentrations were higher in the milk for girls than that for boys (β = 0.2332, p = 0.0179). However, all these associations except for lactose lost significance after adjustments and following correction for multiple testing (α threshold = 0.0036).

Factors associated with HMO variations and longitudinal changes within milk groups I and II

We investigated associations of individual HMOs with maternal characteristics within milk groups I and II because the small number of mothers whose milk were attributed to milk groups III and IV would limit conclusions. Amongst the mothers whose milk was attributed to milk group I, parity was associated with LNDFH I and 6'-GL (higher in multiparous women; β = 0.1350, p = 0.0239 and β = 0.1963, p = 0.0055, respectively), delivery mode with LNnT (higher in mothers who delivered by elective or emergency caesarean; β = 0.1843 p = 0.0055), exclusive breastfeeding with DFL and lactose (higher DFL; β = 0.1514, p = 0.0356 and lower lactose; β = -0.2824, p = 0.0007 in exclusively breastfed infants), pre-pregnancy BMI with 6'-SL and DFL (higher in overweight mothers; β = 0.1547, p = 0.0096 and β = 0.1503, p = 0.0207, respectively), maternal BMI at 6 weeks with 6'-SL and 3'-SL (higher β = 0.1560, p = 0.0099 and lower β = -0.2157, p = 0.0168, in overweight mothers, respectively).

Among the mothers whose milk was attributed to milk group II, LNFP V (β = -0.3383, p = 0.0.0281) and lactose (β = -0.5290, p = 0.0.0448) were lower in the milk of mothers with a gestation period of < 37weeks, lactose (β = -0.5250, p = 0.0448) was lower in non-exclusively breastfed infants, 6'-GL (β = 0.2356, p = 0.0236) and the sum of LNDFH II + LNnDFH II (β = 0.4113, p = 0.0050) were higher in overweight women (pre-pregnancy BMI). However, all associations observed amongst mothers whose milks were attributed to types I and II with the exception of lactose lost significance following adjustments and Bonferroni correction.

In both unadjusted and adjusted mixed models, we observed milk group differences in the HMO changes up to 12 months of lactation (n=66) for all HMOs excluding 3'-SL (p = 0.5693). This was also the case when the sample size was restricted to the *n* = 422 mothers for changes between 6 weeks and 6 months. Significant secretor

and non-secretor differences in the HMO changes were also observed for all HMOs with the exception of 6'-GL ($p = 0.4068$) and DFL ($p = 0.2520$), in both sub-groups (*n*=66 and *n*=422). We then investigated the effect of parity, delivery mode, gestation period and maternal BMI on the changes in HMO concentrations at 6 months, and up to 12 months of lactation within milk group I and II. Parity, gestation and BMI were associated with the trajectories of the individual HMOs amongst mothers whose milk was attributed to milk group I or II ($p < 0.050$, Supplementary **Table S10-S11**). However, these lost significance following adjustments for maternal age, pre-pregnancy BMI, delivery mode, infant sex and correction for multiple testing.

DISCUSSION

In the present study including lactating women from a birth cohort in Germany, we evaluated absolute HMO concentrations and their trajectories in human milk samples collected over 12 months of lactation, using a considerably more precise and comprehensively validated method developed for HMO quantification. Our results show that HMO concentrations are highly variable between mothers and change considerably over the course of lactation. The variations observed between mothers were strongly associated with milk group and secretor status. Of the factors we examined, parity, gestation period, pre-pregnancy BMI, and exclusive breastfeeding were associated with at least one HMO at 6 weeks. In addition, pre-pregnancy BMI, gestation, maternal BMI and parity seem to likely influence some HMO trajectories within milk groups I and II over lactation. However, these associations disappeared after further adjustments and correction for multiple testing.

The novelty of this study includes the quantification method (negative ion mode targeted MRM LC-ESI-MS^2) used to determine absolute HMO concentrations in breast milk sampled repeatedly across lactation in a large number of mothers. In comparison to other HMO-analytical approaches which only allow relative quantification, the method used in this study offered a higher efficiency of detecting HMOs. The enhanced selectivity of our method for highly similar HMO structures based on the concept of diagnostic tandem MS fragment ions allowed a precise identification and quantitation of even partly isomeric HMOs. This also facilitated assignment of individual human milk samples to one of the known four milk groups (30). We measured lactose and 16 individual HMOs including the group isomers of LNDFH II and LNnDFH II which could not be separated but were measured together. The method employed in this study also allowed the separation of LNT and LNnT which were previously quantified together in another study (20) that also reported absolute concentrations quantified using LC-MS technology. Thus, the quantification method used in this study in particular eliminates the problem of co-elution of HMOs (of large concentrations) during quantification, the need to use uncommon

HMO standards for identifying compounds during HMO quantification as well as tedious derivatization of HMOs prior to analysis (30).

In addition, milk group I was the predominant milk group, and only 1% of the samples were attributed to milk group IV, which is known to be a rare milk group (15,20,22,39). Although very rare (33,40), we identified seven lactating women in our study with peculiar HMO profiles. In principle, these lactating women were attributed to group IV milk at 6 and 12 months of lactation because of the complete absence or undetectable levels of LNFP I (< LLOQ). However, given the presence of 2'-FL in these human milk samples, we considered other FUT2 related HMOs such as DFL and LNDFH I which were present in partly quite high abundances to reliably confirm their milk group. The finding that LNFP I was absent or below LLOQ in a subgroup of group I human milk samples while other α1,2-fucosylated HMO-structures like 2'-FL, DFL and LNDFH I were present is quite unusual.

A plausible explanation for this finding could be that a so far unknown α1-2 fucosyltransferase being active in parallel to the FUT2 encoded secretor enzyme produces LNFP I which in turn is less active in the milks of these lactating women. Our findings may therefore indicate changes in the maternal genome with respect to activation of FUT2 and possibly other FUTs which impact the synthesis of fucosylated HMOs in the mammary gland as lactation progresses. Although the reason for this phenomenon is unknown, our findings further confirm that milk group or secretor status may not be predicted based on a single HMO profiling-scheme within a human milk sample collected at one single time point of lactation (41). Further insight into the genetic background of these lactating mothers or those with unusual HMO profiles could further elucidate and help researchers understand how HMO synthesis is regulated.

Consistent with other studies (17,21–23,31,32,34,35), our results show a considerable decline of the total content and most individual HMOs over lactation, irrespective of milk group or secretor status. However, 3-FL, DFL and 3'-SL concentrations were consistently higher at 6 months and 12 months of lactation. On the other hand, the increase of DFL concentrations over lactation has only been reported in one recent study (Plows et al. 2021). It is plausible that the discrepancies in these results are attributable to several factors which include but are not limited to genetic variants, geographic regions, ethnicity and other maternal factors.

Nonetheless, 3'-SL is among some of the specific HMO structures that have been detected within the intestine as well as systematic circulation of breastfed infants (42–44). Despite the efforts towards providing evidence suggesting an important role of HMOs on immune cells, these previously proposed effects remain understudied. Also, infants whose mother's milk presented with low

concentrations of α1,2-fucosylated HMOs were more likely to have *Campylobacter*, calicivirus and moderate-to-severe diarrhoea compared to their counterparts whose mother's milk contained higher concentrations of α1,2 fucosylated HMOs (45). Therefore, the changes that we observed in HMO concentrations over the course of lactation suggest that there is a need for these individual HMO structures during early infancy and perhaps later in childhood. Thus, an adequate intake should be ensured during this period of critical growth and development.

Furthermore, we primarily used the concentrations of 2'-FL, LNFP I, LNFP II and LNDFH I as proxies for FUT2 and FUT3 activities. In this study, HMO concentrations were highly variable between lactating mothers. The presence and absence of the functional FUT2 and FUT3 encoded enzymes is known to greatly influence the HMO profile of lactating mothers (17,18,20–22). Our results are in line with other studies (17,18,20–22) that also reported the presence and absence of some individual fucosylated HMOs in the different milk groups. However, Kunz et al. (22) reported LNT as a major component in milk group II and Gabrielli et al. (18) a higher concentrations of LNnT than LNT in milk group I. This was not the case in our study. Nonetheless, the highest concentrations of these HMOs were observed at 6 weeks followed by a gradual decrease of most individual structures while others increased by 12 months of lactation. We also investigated the effect of other maternal factors on the trajectories of HMOs within milk groups I and II only. We observed significant effects of parity, gestation and maternal BMI on the trajectories of some individual HMO structures in the milk of mothers attributed to milk group I and II. However, statistical significance was lost following consecutive adjustments for other maternal characteristics and correction for multiple testing. Thus, in this study maternal characteristics did not contribute largely as milk group does to the variability of HMOs and their trajectories over lactation.

Moreover, classifying the samples according to secretor and non-secretor status, we observed large differences in the total amount of HMOs and the trajectories of individual HMO concentrations. Other studies (18,35,46,47) have also shown higher 3'-FL in non-secretor milks compared to their secretor counterparts. Similar differences were also observed in this study, confirming that the relative levels of the fucosylated oligosaccharides are a result of the competition between the enzymes for a limited substrate (21). The differences between the groups were largely attributed to the presence and absence of α1,2-fucosylated HMOs such as 2'-FL, LNFP I and LNDFH I in secretor and non-secretor milks. The considerable effect of milk group within secretor and variations within secretor and non-secretor mothers also confirm that there are indeed other factors that play a role in overall HMO concentrations in human milk (35,48). Nonetheless, certain HMO structures in secretor and non-secretor milks have been associated with health outcomes in infancy. Higher incidence of diarrhoea was observed among infants whose mother's

milks had low concentrations of 2'-FL (non-secretors milk) compared to those who received human milk with higher amounts of 2'-FL (secretor milk) (45). Also, higher concentrations of FUT2 dependent HMOs such as 2'-FL were associated with lower risk of allergy at age 2 and 5 in infants with high hereditary risk of allergy (34). Therefore, early exposure of the infant to FUT2 dependent HMOs such as 2'-FL could be an important temporal window of opportunity to improve child health and possibly growth outcomes.

Although the evidence is conflicting, several maternal factors like parity, pre-pregnancy BMI, mode of delivery, exclusive breastfeeding and gestation period have been associated with some individual HMOs (17,18,20–22,24,39,46). In contrast to these studies, we found marginal associations with parity, pre-pregnancy BMI, exclusive breastfeeding, gestation period and infant sex. The effect sizes of these associations were small, and all associations lost significance following adjustment for respective maternal characteristics and correction for multiple testing. However, these observations could be an indication of the subtle changes that are associated with early post-partum physiology, which in turn for some HMOs, results in additional variations in the glycosylation pathways attributable to pre-pregnancy BMI, parity and mode of delivery (21).

The strengths of this study include the presentation of absolute HMO concentrations across lactation and use of a more accurate and extensively validated method with high specificity for HMO quantification. Also, milk groups and grouping milks into secretor and non-secretor milks explains a large part of inter-individual variability in HMO concentrations. Thus, the confirmation of the four milk groups and assignment of lactating mothers to these milk groups based on a very high number of HM samples adds new information to the limited existing literature. However, we do not cover 100% of the abundance of HMOs but quantifying 16 individual HMOs.

Despite the reduced number of human milk samples available at all three time points, not many studies have assessed the variations and changes in HMO concentrations up to 12 months of lactation. Therefore, this study provides new information regarding changes in HMO concentrations during a long-term period of lactation which could be further used to look at associations with child health outcomes. Also, the study protocol for the collection of human milk samples was standardised in order to reduce variability of HMOs and potential between feed variations of human milk components. However, due to the lack of dietary information, we could not investigate its impact on HMOs, if any. Future studies should investigate the potential impact of HMO trajectories over lactation on child health and growth outcomes. Further exploration of the synergistic mechanisms by which other human milk components potentially influence HMO concentrations may also contribute additional information in the relevance of research in human milk composition.

CONCLUSIONS

In conclusion, HMO concentrations vary considerably between mothers and may change over the course of lactation. These changes could be an indication of the temporal relevance of HMOs and their unique role later in infancy and childhood. Our findings warrant further exploration of their physiological relevance for the breastfed infant. Findings from this study also showed that the variations in HMO profiles that exist between mothers are largely attributed to milk group and secretor status, which are in turn dependent on the maternal genome and expression of FUT2 and FUT3 encoded fucosyltransferases in the lactocytes. However, in some rare instances milk typing based on a specific scheme of HMOs needs confirmation by recognizing additional surrogate markers for FUT2 and FUT3 activity as outlined above. This phenomenon warrants further research into the genetic background of and HMO regulation by these mothers who exhibit such HMO profiles.

DISCLAIMER:

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SUPPLEMENTS

Table S1 | Human milk oligosaccaharide analysis method validation

Compound Retention time (min)	Quantifier Q1/Q2 (*m/z*) (DP) (CE)	Qualifier Q1/Q2 (*m/z*) (CE)	Low calibration (g/L)[a] High calibration (g/L)[a] Fit and weighting	Concentration added to water (g/L) (n=9)	Bias (%) Repeatability (%) Reproducibility (%)	Concentration added to milk (g/L) (n=24)	Bias (%) Repeatability (%)	Reproducibility QC sample (%) (n=26)	Bias +/- combined deviation (%)
Lactose 4.95	341.1/179 (-40) (-10)	341.1/161 (-10)	2.5-25 25-100 Quadratic, none	- - - 80	-/-/- -/-/- -/-/- -2/2/3	-	-/-	4	x ± 4
2'-Fucosyllactose 6.60	487.1/325 (-45) (-12)	487.1/205 (-24)	- 0.19-7.5 Quadratic, none	0.19 0.56 2.49 6.0	-15/5/5 -4/3/3 -3/3/4 -5/4/4	1.25	-4/7	6	-4 ± 9
3-Fucosyllactose 7.25	487.1/179 (-40) (-14)	487.1/89 (-34)	- 0.038-1.5 Quadratic, 1/x	0.038 0.11 0.50 1.2	-16/5/4 10/3/5 0/2/2 -3/3/4	0.25	-13/4	17	-13 ± 17
3'-Sialyllactose 8.50	632.2/290 (-60) (-35)	632.2/142 (-46)	0.013-0.062 0.030-0.50 Quadratic, 1/x	0.013 0.038 0.17 0.40	17/8/7 -1/5/7 -2/5/5 3/4/4	0.083	-40/11	12	-40 ± 16
4'-Galactooligosaccharide 8.20	503.1/101 (-30) (-35)	503.1/161 (-17)	0.0013-0.0062 0.0030-0.050 Quadratic, 1/x	0.0013 0.0038 0.017 0.040	7/9/15 -8/10/14 4/7/7 8/4/4	0.0083	-31/12	-	-31 ± 16[b]
6'-Galactooligosaccharide 8.65	503.1/179 (-40) (-22)	503.1/221 (-24)	0.0013-0.0062 0.0030-0.050 Quadratic, 1/x	0.0013 0.0038 0.017 0.040	4/7/8 -14/9/13 8/6/15 18/7/4	0.0083	-38/15	-	-38 ± 19[b]

Table S1 | Continued.

Compound Retention time (min)	Quantifier Q1/Q2 (*m/z*) (DP) (CE)	Qualifier Q1/Q2 (*m/z*) (CE)	Low calibration (g/L)[a] High calibration (g/L)[a] Fit and weighting	Concentration added to water (g/L) (n=9)	Bias (%) Repeatability (%) Reproducibility (%)	Concentration added to milk (g/L) (n=24)	Bias (%) Repeatability (%)	Reproducibility QC sample (%) (n=26)	Bias +/- combined deviation (%)
3,2'-Difucosyllactose 9.15	633.2/325 (-45) (-27)	633.2/205 (-17)	0.019-0.094 0.045-0.75 Quadratic, 1/x	0.019 0.056 0.49 0.60	9/5/5 13/8/9 1/2/2 -4/3/2	0.13	-17/7	6	-17 ± 9
6'-Sialyllactose 9.50	632.2/290 (-60) (-34)	632.2/572 (-32)	0.013-0.062 0.030-0.50 Quadratic, 1/x	0.013 0.038 0.17 0.40	18/4/4 -3/10/10 1/8/9 3/4/6	0.083	-41/20	8	-41 ± 22
Compound Retention time (min)	Quantifier Q1/Q2 (*m/z*) (DP) (CE)	Qualifier Q1/Q2 (*m/z*) (CE)	Low calibration (g/L)[a] High calibration (g/L)[a] Fit and weighting	Concentration added to water (g/L) (n=9)	Bias (%) Repeatability (%) Reproducibility (%)	Concentration added to milk (g/L) (n=24)	Bias (%) Repeatability (%)	Reproducibility QC sample (%) (n=26)	Bias +/- combined deviation (%)
Lacto-N-tetraose 10.80	706.2/202 (-35) (-28)	706.2/142 (-34)	0.068-0.062 0.17-2.7 Quadratic, 1/x	0.068 0.20 0.90 2.2	11/3/2 6/5/4 13/2/2 15/3/3	0.45	-20/18	17	-20 ± 25
Lacto-N-neotetraose 11.00	706.2/263 (-35) (-24)	706.2/281 (-22)	0.013-0.062 0.031-0.50 Quadratic, 1/x	0.013 0.038 0.17 0.40	8/3/5 -12/7/6 8/3/4 -1/2/3	0.083	-35/16	19	-35 ± 25
Lacto-N-fucopentaose I 12.85	852.5/325 (-45) (-30)	852.5/205 (-45)	0.050-0.25 0.13-2.0	0.05 0.15 0.67 1.6	4/2/3 -8/7/8 4/2/3 4/4/3	0.33	-56/15	8	-56 ± 17

Table S1 | Continued.

Compound Retention time (min)	Quantifier Q1/Q2 (*m/z*) (DP) (CE)	Qualifier Q1/Q2 (*m/z*) (CE)	Low calibration (g/L)[a] High calibration (g/L)[a] Fit and weighting	Concentration added to water (g/L) (n=9)	Bias (%) Repeatability (%) Reproducibility (%)	Concentration added to milk (g/L) (n=24)	Bias (%) Repeatability (%)	Reproducibility QC sample (%) (n=26)	Bias +/- combined deviation (%)
Lacto-N-fucopentaose V 13.20	852.5/544 (-40) (-20)	852.5/382 (-28)	- 0.013-0.25 Quadratic, 1/x	0.013 0.038 0.17 -	8/7/7 14/4/7 4/2/6 -/-	0.083	-45/9	20	-45 ± 22
Lacto-N-fucopentaose III 13.55	852.5/364 (-40) (-26)	852.5/179 (-34)	0.025-0.13 0.060-1.0 Quadratic, 1/x	0.025 0.075 0.33	11/5/7 -7/6/7 2/3/4 -12/2/2	0.17	-34/7	13	-34 ± 15
Lacto-N-fucopentaose II 13.65	852.5/348 (-35) (-26)	852.5/288 (-34)	0.050-0.25 0.13-2.0 Quadratic, 1/x	0.05 0.15 0.67	4/6/7 -7/7/6 0/2/4 2/3/3	0.33	-51/10	21	-51 ± 23
Lacto-N-difucohexaose I 15.25	998.4/205 (-50) (-52)	998.4/836 (-24)	0.025-0.13 0.062-1.0 Quadratic, 1/x	0.025 0.075 0.33	3/4/6 -3/9/9 12/4/4 10/5/5	0.17	-68/5	11	-68 ± 12
Lacto-N-difucohexaose II + Lacto-N-neodifucohexaose II 15.25	998.4/690 (-50) (-24)	998.4/528 (-30) 998.4/364 (-30)	0.013-0.062 0.031-0.50 Quadratic, 1/x	0.013 0.038 0.17 0.40	-/13/11 -/6/6 -/7/7 -/5/6	0.083	-56/6	12	-56 ± 13

DP: declustering potential
CE: collision energy
Combined deviation $\sqrt{repeatability\ milk^2 + reproducibility\ QC^2}$
a: expressed as concentration in milk, absolute concentration 6 time lower
b: Concentration 4-GL <LLOQ and 6-GL >ULOQ in QC sample, used averaged reproducibility in water

Table S2 | Absolute concentrations (g/L) of individual human milk oligosaccharides in all available human milk samples at 6 weeks, 6 months and 12 months of lactation in the Ulm SPATZ Health Study

HMOs	6 weeks (n=682)	6 months (n=448)	12 months (n=73)
2'-FL			
Mean (SD)	2.31 (1.39)	1.68 (1.08)	1.36 (0.84)
Median [Min, Max]	2.50 [0.13, 6.60]	1.70 [0.13, 4.80]	1.40 [0.13, 3.30
3'-FL			
Mean (SD)	0.65 (0.54)	1.21 (0.71)	1.57 (1.24)
Median [Min, Max]	0.47 [0.03, 3.00]	1.00 [0.05, 3.90]	1.10 [0.12, 6.9]
3'-SL			
Mean (SD)	0.15 (0.05)	0.16 (0.05)	0.24 (0.12)
Median [Min, Max]	0.15 [0.05, 0.54]	0.15 [0.05, 0.57]	0.21 [0.09, 0.58
6'-GL			
Mean (SD)	0.02 (0.01)	0.01 (0.01)	0.01 (0.01)
Median [Min, Max]	0.02 [0.00, 0.15]	0.01 [0.01, 0.04]	0.01 [0.00, 0.03
DFL			
Mean (SD)	0.19 (0.17)	0.24 (0.23)	0.38 (0.40)
Median [Min, Max]	0.17 [0.01, 1.80]	0.22 [0.01, 2.90]	0.26 [0.01, 2.20
6'-SL			
Mean (SD)	0.26 (0.10)	0.04 (0.02)	0.02 (0.00)
Median [Min, Max]	0.24 [0.05, 0.730]	0.03 [0.01, 0.29]	0.01 [0.01, 0.03
LNT			
Mean (SD)	0.94 (0.50)	0.47 (0.29)	0.36 (0.24)
Median [Min, Max]	0.84 [0.09, 3.10]	0.41 [0.05, 1.80]	0.32 [0.05, 1.20
LNnT			
Mean (SD)	0.09 (0.06)	0.05 (0.0)	0.02 (0.02)
Median [Min, Max]	0.07 [0.01, 0.38]	0.03 [0.01, 0.24]	0.02 [0.01, 0.13
LNFP I			
Mean (SD)	0.51 (0.50)	0.21 (0.25)	0.19 (0.20)
Median [Min, Max]	0.39 [0.04, 4.10]	0.12 [0.04, 1.90]	0.12 [0.04, 1.00
LNFP V			
Mean (SD)	0.04 (0.04)	0.02(0.02)	0.022 (0.02)
Median [Min, Max]	0.02 [0.01, 0.24]	0.02 [0.01, 0.12]	0.02 [0.01, 0.08
LNFP III			
Mean (SD)	0.18 (0.08)	0.20 (0.08)	0.14 (0.06)
Median [Min, Max]	0.18 [0.03, 0.56]	0.19 [0.03, 0.45]	0.14 [0.04, 0.30
LNFP II			
Mean (SD)	0.33 (0.38)	0.24 (0.23)	0.25 (0.23)
Median [Min, Max]	0.17 [0.04, 2.50]	0.15 [0.04, 1.40]	0.18 [0.04, 1.20
LNDFHI			
Mean (SD)	0.53 (0.39)	0.30 (0.22)	0.35 (0.25)
Median [Min, Max]	0.57 [0.02, 1.90]	0.31 [0.02, 0.99]	0.35 [0.02, 0.90
LNDFHII + LNnDFHII			
Mean (SD)	0.06 (0.11)	0.06 (0.07)	0.08 (0.11)
Median [Min, Max]	0.02 [0.01, 0.73]	0.03 [0.01, 0.44]	0.03 [0.01, 0.46
Total HMOs			
Mean (SD)	6.24 (1.33)	4.87 (0.89)	5.00 (1.01)
Median [Min, Max]	6.18 [2.41, 12.6]	4.85 [1.84, 8.35]	5.02 [1.66, 8.64

HMOs, human milk oligosaccharides. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactooligosaccharide; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetrose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

Table S3 | Relative proportion (%) of human milk oligosaccharides (HMOs) all available samples at each time point in the Ulm SPATZ Health study

HMOs	6 weeks (n=682)	6 months (n=449)	12 months (n=73)
2'-FL			
Mean (SD)	35.1 (19.1)	33.0 (18.7)	27.7 (16.7)
Median [Min, Max]	39.0 [1.55, 74.2]	35.8 [2.16, 77.3]	29.8 [1.50, 71.4]
3'-FL			
Mean (SD)	11.6 (11.0)	26.3 (17.0)	30.5 (19.6)
Median [Min, Max]	7.68 [0.31, 53.5]	21.6 [0.85, 75.5]	23.9 [3.29, 81.8]
3'-SL			
Mean (SD)	2.56 (0.94)	3.39 (1.10)	4.85 (1.98)
Median [Min, Max]	2.36 [0.84, 8.32]	3.22 [0.91, 9.38]	4.13 [2.00, 10.2]
6'-GL			
Mean (SD)	0.29 (0.12)	0.20 (0.10)	0.20 (0.09)
Median [Min, Max]	0.26 [0.07, 2.45]	0.18 [0.03, 1.03]	0.18 [0.09, 0.57]
DFL			
Mean (SD)	2.84 (2.26)	4.61 (3.61)	7.63 (8.59)
Median [Min, Max]	2.70 [0.12, 18.3]	4.72 [0.17, 34.7]	5.76 [0.12, 56.5]
6'-SL			
Mean (SD)	4.27 (1.84)	0.73 (0.49)	0.23 (0.09)
Median [Min, Max]	3.90 [0.78, 16.1]	0.61 [0.15, 4.58]	0.22 [0.14, 0.60]
LNT			
Mean (SD)	15.9 (10.0)	10.1 (7.49)	7.48 (4.87)
Median [Min, Max]	13.7 [1.33, 65.2]	8.58 [0.81, 61.8]	7.12 [0.73, 25.1]
LNnT			
Mean (SD)	1.34 (0.94)	0.93 (0.82)	0.50 (0.46)
Median [Min, Max]	1.15 [0.12, 7.72]	0.65 [0.15, 5.17]	0.37 [0.12, 2.71]
LNFP I			
Mean (SD)	7.33 (5.93)	3.97 (4.16)	3.88 (4.00)
Median [Min, Max]	6.37 [0.48, 32.4]	2.47 [0.62, 26.6]	2.39 [0.46, 18.6]
LNFP V			
Mean (SD)	0.62 (0.68)	0.47 (0.38)	0.45 (0.36)
Median [Min, Max]	0.33 [0.09, 5.23]	0.31 [0.13, 2.16]	0.32 [0.14, 1.69]
LNFP III			
Mean (SD)	3.03 (1.43)	4.14 (1.79)	2.93 (1.30)
Median [Min, Max]	2.87 [0.34, 9.18]	3.88 [0.51, 20.2]	2.80 [0.54, 6.31]
LNFP II			
Mean (SD)	5.85 (6.83)	5.07 (4.78)	5.05 (4.36)
Median [Min, Max]	2.73 [0.32, 29.8]	3.20 [0.52, 23.3]	3.60 [0.73, 19.8]
LNDFHI			
Mean (SD)	8.10 (5.36)	5.82 (3.80)	6.99 (4.55)
Median [Min, Max]	9.24 [0.16, 22.5]	6.54 [0.26, 15.4]	7.97 [0.23, 15.3]
LNDFHII + LNnDFHII			
Mean (SD)	1.09 (1.84)	1.26 (1.58)	1.62 (2.21)
Median [Min, Max]	0.26 [0.08, 9.95]	0.59 [0.13, 7.75]	0.62 [0.14, 10.3]

HMOs, human milk oligosaccharides. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactooligosaccharide; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetrose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

Table S4 | Absolute concentrations of individual human milk oligosaccharides restricted to mothers with samples collected at both 6 weeks and 6 months of lactation in the Ulm SPATZ Health Study

HMOs	6 weeks (n=422)	6 months (n=422)	p value
Lactose			
Mean (SD)	66.7 (3.66)	70.5 (4.24)	< 0.001
Median [Min, Max]	67.0 [52.0, 77.0]	71.0 [46.0, 85.0]	
2'-FL			
Mean (SD)	2.35 (1.41)	1.69 (1.08)	< 0.001
Median [Min, Max]	2.50 [0.13, 6.60]	1.70 [0.13, 4.80]	
3'-FL			
Mean (SD)	0.62 (0.51)	1.20 (0.70)	< 0.001
Median [Min, Max]	0.45 [0.03, 2.60]	1.00 [0.05, 3.60]	
3'-SL			
Mean (SD)	0.15 (0.04)	0.16 (0.05)	0.003
Median [Min, Max]	0.15 [0.05, 0.37]	0.15 [0.05, 0.57]	
6'-GL			
Mean (SD)	0.02 (0.01)	0.01 (0.01)	< 0.001
Median [Min, Max]	0.02 [0.01, 0.15]	0.01 [0.00, 0.04]	
DFL			
Mean (SD)	0.18 (0.16)	0.24 (0.23)	< 0.001
Median [Min, Max]	0.16 [0.01, 1.80]	0.22 [0.01, 2.90]	
6'-SL			
Mean (SD)	0.26 (0.10)	0.04 (0.02)	< 0.001
Median [Min, Max]	0.24 [0.05, 0.73]	0.03 [0.01, 0.12]	
LNT			
Mean (SD)	0.93 (0.51)	0.47 (0.29)	< 0.001
Median [Min, Max]	0.85 [0.09, 2.90]	0.41 [0.05, 1.80]	
LNnT			
Mean (SD)	0.086 (0.06)	0.05 (0.04)	< 0.001
Median [Min, Max]	0.0750 [0.0100, 0.340]	0.03 [0.01, 0.24]	
LNFP I			
Mean (SD)	0.50 (0.46)	0.21 (0.25)	< 0.001
Median [Min, Max]	0.38 [0.04, 3.00]	0.12 [0.04, 1.90]	
LNFP V			
Mean (SD)	0.03 (0.03)	0.02 (0.02)	< 0.001
Median [Min, Max]	0.02 [0.01, 0.17]	0.02 [0.01, 0.12]	
LNFP III			
Mean (SD)	0.17 (0.07)	0.19 (0.07)	< 0.001
Median [Min, Max]	0.17 [0.03, 0.48]	0.20 [0.03, 0.43]	
LNFP II			
Mean (SD)	0.32 (0.38)	0.23 (0.23)	0.189
Median [Min, Max]	0.16 [0.04, 2.00]	0.1503 [0.04, 1.40]	
LNDFHI			
Mean (SD)	0.52 (0.36)	0.30 (0.22)	< 0.001
Median [Min, Max]	0.57 [0.02, 1.90]	0.31 [0.02, 0.99]	
LNDFHII + LNnDFHII			
Mean (SD)	0.06 (0.10)	0.06 (0.07)	< 0.001
Median [Min, Max]	0.01 [0.01, 0.70]	0.03 [0.01, 0.44]	
Total HMOs			
Mean (SD)	6.18 (1.26)	4.86 (0.90)	< 0.001
Median [Min, Max]	6.17 [2.41, 11.6]	4.85 [1.84, 8.35]	

P values derived from Wilcoxon signed-rank test comparing HMOs concentrations between 6 weeks and 6 months. Bonferroni-adjusted level of statistical significance is α=0.05/14=0.0036. HMO, human milk oligosaccharides. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactooligosaccharide; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetrose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

Table S5 | Absolute concentrations of human milk oligosaccharides according to milk types at 6 weeks

HMOs	Type I (n=502)	Type II (n=122)	Type III (n=49)	Type IV (n=9)
Lactose				
Mean (SD)	66.3 (3.73)	67.2 (3.78)	66.4 (4.38)	66.9 (4.01)
Median [Min, Max]	67.0 [52.0, 77.0]	67.0 [56.0, 79.0]	67.0 [56.0, 74.0]	68.0 [57.0, 70.0]
2'-FL				
Mean (SD)	2.68 (0.88)	0.13 (0)	4.27 (1.04)	0.13 (0)
Median [Min, Max]	2.60 [0.13, 6.30]	0.13 [0.13, 0.13]	4.30 [1.00, 6.60]	0.13 [0.13, 0.13]
3'-FL				
Mean (SD)	0.48 (0.26)	1.57 (0.48)	0.08 (0.05)	0.30 (0.15)
Median [Min, Max]	0.425 [0.0900, 2.40]	1.60 [0.63, 3.00]	0.06 [0.03, 0.21]	0.25 [0.08, 0.58]
3'-SL				
Mean (SD)	0.15 (0.04)	0.18 (0.05)	0.16 (0.04)	0.16 (0.04)
Median [Min, Max]	0.14 [0.05, 0.54]	0.16 [0.07, 0.32]	0.15 [0.09, 0.34]	0.16 [0.08, 0.22]
6'-GL				
Mean (SD)	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)
Median [Min, Max]	0.02 [0.00, 0.09]	0.02 [0.01, 0.15]	0.02 [0.01, 0.04]	0.02 [0.01, 0.04]
DFL				
Mean (SD)	0.24 (0.17)	0.01 (0)	0.10 (0.12)	0.01 (0)
Median [Min, Max]	0.21 [0.02, 1.80]	0.01 [0.01, 0.01]	0.08 [0.01, 0.75]	0.01 [0.01, 0.01]
6'-SL				
Mean (SD)	0.26 (0.10)	0.25 (0.10)	0.30 (0.12)	0.27 (0.05)
Median [Min, Max]	0.24 [0.05, 0.70]	0.23 [0.07, 0.55]	0.28 [0.08, 0.73]	0.28 [0.19, 0.38]
LNT				
Mean (SD)	0.85 (0.41)	1.32 (0.593)	0.75 (0.45)	2.00 (0.40)
Median [Min, Max]	0.79 [0.09, 2.90]	1.20 [0.24, 3.10]	0.66 [0.20, 2.00]	1.90 [1.50, 2.70]
LNnT				
Mean (SD)	0.10 (0.06)	0.03 (0.02)	0.07 (0.04)	0.03 (0.03)
Median [Min, Max]	0.09 [0.01, 0.38]	0.02 [0.01, 0.16]	0.06 [0.02, 0.20]	0.02 [0.01, 0.08]
LNFP I				
Mean (SD)	0.55 (0.38)	0.04 (0)	1.40 (0.72)	0.04 (0)
Median [Min, Max]	0.45 [0.04, 2.30]	0.04 [0.04, 0.04]	1.30 [0.37, 4.10]	0.04 [0.04, 0.04]
LNFP V				
Mean (SD)	0.02 (0.01)	0.09 (0.04)	0.01 (0.01)	0.07 (0.06)
Median [Min, Max]	0.02 [0.01, 0.09]	0.09 [0.03, 0.24]	0.01 [0.01, 0.05]	0.06 [0.01, 0.21]
LNFP III				
Mean (SD)	0.18 (0.07)	0.21 (0.08)	0.15 (0.09)	0.22 (0.07)
Median [Min, Max]	0.17 [0.04, 0.48]	0.20 [0.08, 0.56]	0.12 [0.03, 0.47]	0.19 [0.11, 0.30]
LNFP II				
Mean (SD)	0.20 (0.16)	1.00 (0.40)	0.04 (0)	0.04 (0)
Median [Min, Max]	0.15 [0.04, 1.20]	0.95 [0.24, 2.50]	0.04 [0.04, 0.04]	0.04 [0.04, 0.04]
LNDFHI				
Mean (SD)	0.71 (0.28)	0.02 (0)	0.02 (0)	0.02 (0)
Median [Min, Max]	0.65 [0.07, 1.90]	0.02 [0.02, 0.02]	0.02 [0.02, 0.02]	0.02 [0.02, 0.02]
LNDFHII + LNnDFHII				
Mean (SD)	0.02 (0.02)	0.25 (0.14)	0.01 (0)	0.01 (0.00)
Median [Min, Max]	0.01 [0.01, 0.24]	0.23 [0.04, 0.73]	0.01 [0.01, 0.01]	0.01 [0.01, 0.02]
Sum of HMOs				
Mean (SD)	6.46 (1.11)	5.12 (1.06)	7.37 (1.59)	3.32 (0.60)
Median [Min, Max]	6.37 [3.58, 11.1]	4.99 [2.76, 8.40]	7.08 [4.43, 12.6]	3.18 [2.41, 4.14]

HMO, human milk oligosaccharides. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactooligosaccharide; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetrose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

Table S6 | Absolute concentrations of human milk oligosaccharides according to milk types at 6 months

HMOs	Type I (n=330)	Type II (n=80)	Type III (n=32)	Type IV (n=6)
Lactose				
Mean (SD)	70.2 (4.15)	70.7 (4.87)	71.0 (3.37)	73.5 (4.18)
Median [Min, Max]	70.0 [51.0, 81.0]	71.0 [46.0, 85.0]	70.5 [63.0, 76.0]	72.5 [69.0, 81.0]
2'-FL				
Mean (SD)	1.93 (0.74)	0.130 (0)	3.34 (0.92)	0.13 (0)
Median [Min, Max]	1.90 [0.13, 3.90]	0.13 [0.13, 0.13]	3.35 [0.54, 4.8]	0.13 [0.13, 0.13]
3'-FL				
Mean (SD)	1.06 (0.45)	2.30 (0.51)	0.17 (0.08)	0.37 (0.12)
Median [Min, Max]	0.98 [0.33, 3.20]	2.30 [1.10, 3.90]	0.17 [0.05, 0.41]	0.38 [0.16, 0.59]
3'-SL				
Mean (SD)	0.16 (0.06)	0.16 (0.04)	0.16 (0.05)	0.16 (0.03)
Median [Min, Max]	0.15 [0.05, 0.57]	0.15 [0.09, 0.23]	0.15 [0.08, 0.38]	0.15 [0.12, 0.21]
6'-GL				
Mean (SD)	0.01 (0.00)	0.01 (0.01)	0.01 (0.00)	0.01 (0.04)
Median [Min, Max]	0.01 [0.00, 0.04]	0.01 [0.00, 0.04]	0.01 [0.00, 0.02]	0.01 [0.01, 0.02]
DFL				
Mean (SD)	0.31 (0.22)	0.01 (0)	0.10 (0.13)	0.01 (0)
Median [Min, Max]	0.26 [0.02, 2.90]	0.01 [0.01, 0.01]	0.07 [0.01, 0.73]	0.01 [0.01, 0.01]
6'-SL				
Mean (SD)	0.03 (0.02)	0.03 (0.02)	0.04 (0.03)	0.05(0.02)
Median [Min, Max]	0.03 [0.01, 0.29]	0.03 [0.010, 0.11]	0.03 [0.01, 0.12]	0.04 [0.03, 0.08]
LNT				
Mean (SD)	0.42 (0.26)	0.56 (0.29)	0.50 (0.32)	1.35 (0.35)
Median [Min, Max]	0.38 [0.05, 1.60]	0.51 [0.12, 1.50]	0.46 [0.17, 1.40]	1.40 [0.71, 1.80]
LNnT				
Mean (SD)	0.05 (0.05)	0.016 (0.01)	0.05 (0.03)	0.03 (0.03)
Median [Min, Max]	0.04 [0.01, 0.24]	0.01 [0.01, 0.08]	0.03 [0.01, 0.12]	0.020 [0.01, 0.08]
LNFP I				
Mean (SD)	0.18 (0.18)	0.04 (0)	0.81 (0.35)	0.04 (0)
Median [Min, Max]	0.14 [0.04, 1.00]	0.04 [0.04, 0.04]	0.77 [0.21, 1.90]	0.04 [0.04, 0.04]
LNFP V				
Mean (SD)	0.02 (0.00)	0.05 (0.02)	0.01 (0.00)	0.04 (0.02)
Median [Min, Max]	0.01 [0.01, 0.06]	0.05 [0.01, 0.12]	0.01 [0.01, 0.02]	0.03 [0.01, 0.06]
LNFP III				
Mean (SD)	0.19 (0.07)	0.20 (0.06)	0.21 (0.08)	0.30 (0.11)
Median [Min, Max]	0.18 [0.06, 0.45]	0.19 [0.08, 0.34]	0.20 [0.03, 0.34]	0.32 [0.14, 0.43]
LNFP II				
Mean (SD)	0.17 (0.11)	0.61 (0.24)	0.04 (0)	0.04 (0)
Median [Min, Max]	0.14 [0.04, 0.73]	0.59 [0.19, 1.40]	0.04 [0.04, 0.04]	0.04 [0.04, 0.04]
LNDFHI				
Mean (SD)	0.40 (0.16)	0.02 (0)	0.02 (0)	0.02 (0)
Median [Min, Max]	0.38 [0.03, 0.99]	0.02 [0.02, 0.02]	0.02 [0.02, 0.02]	0.02 [0.02, 0.02]
LNDFHII + LNnDFHII				
Mean (SD)	0.03 (0.02)	0.19 (0.08)	0.01 (0.00)	0.01 (0.00)
Median [Min, Max]	0.03 [0.01, 0.19]	0.18 [0.06, 0.44]	0.01 [0.01, 0.01]	0.01 [0.01, 0.01]
Sum of HMOs				
Mean (SD)	4.98 (0.81)	4.32 (0.68)	5.51 (1.02)	2.55 (0.40)
Median [Min, Max]	4.94 [3.01, 8.35]	4.29 [2.91, 6.01]	5.46 [2.62, 7.74]	2.67 [1.84, 2.91]

HMO, human milk oligosaccharides. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactooligosaccharide; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetrose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

Table S7 | Absolute concentrations of human milk oligosaccharides according to milk types at 12 months

HMOs	Type I (n=55)	Type II (n=13)	Type III (n=5)
Lactose			
Mean (SD)	62.6 (10.2)	56.4 (18.6)	64.4 (2.19)
Median [Min, Max]	65.0 [16.0, 76.0]	65.0 [14.0, 73.0]	63.0 [63.0, 68.0]
2'-FL			
Mean (SD)	1.54 (0.63)	0.13 (0)	2.64 (0.29)
Median [Min, Max]	1.50 [0.13, 3.30]	0.13 [0.13, 0.13]	2.60 [2.40, 3.10]
3'-FL			
Mean (SD)	1.25 (0.643)	3.43 (1.57)	0.245 (0.12)
Median [Min, Max]	1.00 [0.52, 3.70]	3.10 [0.63, 6.90]	0.27 [0.12, 0.40]
3'-SL			
Mean (SD)	0.26 (0.12)	0.22 (0.12)	0.17 (0.06)
Median [Min, Max]	0.22 [0.10, 0.59]	0.18 [0.10, 0.53]	0.17 [0.09, 0.23]
6'-GL			
Mean (SD)	0.01 (0.01)	0.02 (0.01)	0.01 (0.00)
Median [Min, Max]	0.01 [0.00, 0.03]	0.01 [0.00, 0.03]	0.01 [0.01, 0.01]
DFL			
Mean (SD)	0.49 (0.40)	0.01 (0)	0.12 (0.07)
Median [Min, Max]	0.33 [0.10, 2.20]	0.01 [0.01, 0.01]	0.12 [0.05, 0.22]
6'-SL			
Mean (SD)	0.01 (0.00)	0.01 (0.03)	0.01 (0.00)
Median [Min, Max]	0.01 [0.01, 0.03]	0.01 [0.01, 0.02]	0.01 [0.01, 0.02]
LNT			
Mean (SD)	0.37 (0.24)	0.35 (0.27)	0.30 (0.16)
Median [Min, Max]	0.32 [0.05, 1.20]	0.34 [0.05, 0.87]	0.22 [0.13, 0.48]
LNnT			
Mean (SD)	0.03 (0.03)	0.01 (0.01)	0.02 (0.01)
Median [Min, Max]	0.02[0.01, 0.13]	0.01 [0.01, 0.01]	0.02 [0.01, 0.02]
LNFP I			
Mean (SD)	0.19 (0.17)	0.04 (0)	0.59 (0.31)
Median [Min, Max]	0.12 [0.04, 0.62]	0.04 [0.04, 0.04]	0.42 [0.28, 1.00]
LNFP V			
Mean (SD)	0.02 (0.01)	0.05 (0.02)	0.01 (0)
Median [Min, Max]	0.02 [0.01, 0.03]	0.05 [0.02, 0.08]	0.01 [0.01, 0.01]
LNFP III			
Mean (SD)	0.15 (0.06)	0.12 (0.04)	0.15 (0.04)
Median [Min, Max]	0.14 [0.04, 0.30]	0.12 [0.05, 0.19]	0.16 [0.09, 0.18]
LNFP II			
Mean (SD)	0.19 (0.11)	0.58 (0.32)	0.04 (0)
Median [Min, Max]	0.16 [0.04, 0.43]	0.51 [0.20, 1.20]	0.04 [0.04, 0.04]
LNDFHI			
Mean (SD)	0.46 (0.19)	0.02 (0)	0.02 (0)
Median [Min, Max]	0.420 [0.0620, 0.900]	0.02 [0.02, 0.02]	0.02 [0.02, 0.02]
LNDFHII + LNnDFHII			
Mean (SD)	0.04 (0.03)	0.29 (0.09)	0.01 (0)
Median [Min, Max]	0.03 [0.01, 0.21]	0.26 [0.17, 0.46]	0.01 [0.01, 0.01]
Sum of HMOs			
Mean (SD)	5.00 (0.83)	5.27 (1.61)	4.33 (0.76)
Median [Min, Max]	5.04 [3.16, 6.95]	5.13 [1.66, 8.64]	4.36 [3.64, 5.48]

HMO, human milk oligosaccharides. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactooligosaccharide; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetrose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

Table S8 | Absolute concentrations of human milk oligosaccharides (HMOs) in human milk samples from the Ulm SPATZ Health Study by secretor status

HMOs	6 weeks		p value	6 months		p value	12 months		p value
	Non-secretor (n=131)	Secretor (n=551)		Non-secretor (n=86)	Secretor (n=362)		Non-secretor (n=13)	Secretor (n=60)	
Lactose									
Mean (SD)	67.2 (3.78)	66.3 (3.79)	0.022	70.9 (4.86)	70.3 (4.09)	0.145	56.4 (18.6)	62.8 (9.77)	0.474
Median [Min, Max]	67.0 [56.0, 79.0]	67.0 [52.0, 77.0]		71.0 [46.0, 85.0]	70.0 [51.0, 81.0]		65.0 [14.0, 73.0]	65.0 [16.0, 76.0]	
2'-FL									
Mean (SD)	0.130 (0)	2.83 (1.00)	<0.001	0.130 (0)	2.05 (0.86)	<0.001	0.13 (0)	1.63 (0.68)	<0.001
Median [Min, Max]	0.13 [0.13, 0.13]	2.70 [0.13, 6.60]		0.13 [0.13, 0.13]	2.00 [0.13, 4.80]		0.13 [0.13, 0.13]	1.50 [0.13, 3.30]	
3'-FL									
Mean (SD)	1.48 (0.57)	0.45 (0.27)	<0.001	2.17 (0.70)	0.98 (0.50)	<0.001	3.43 (1.57)	1.17 (0.68)	<0.001
Median [Min, Max]	1.50 [0.08, 3.00]	0.40 [0.03, 2.40]		2.30 [0.16, 3.90]	0.94 [0.05, 3.20]		3.10 [0.63, 6.90]	0.10 [0.12, 3.70]	
3'-SL									
Mean (SD)	0.17 (0.05)	0.15 (0.04)	<0.001	0.16 (0.03)	0.16 (0.06)	0.661	0.22 (0.12)	0.25 (0.12)	0.225
Median [Min, Max]	0.16 [0.07, 0.32]	0.14 [0.05, 0.54]		0.15 [0.09, 0.23]	0.15 [0.05, 0.57]		0.18 [0.10, 0.53]	0.22 [0.09, 0.58]	
6'-GL									
Mean (SD)	0.02 (0.02)	0.02 (0.01)	0.058	0.01 (0.01)	0.01 (0.00)	0.041	0.02 (0.01)	0.01 (0.01)	0.116
Median [Min, Max]	0.02 [0.01, 0.15]	0.02 [0.00, 0.09]		0.01 [0.00, 0.04]	0.01 [0.00, 0.04]		0.01 [0.00, 0.03]	0.01 [0.00, 0.03]	
DFL									
Mean (SD)	0.01 (0)	0.23 (0.17)	<0.001	0.01 (0)	0.29 (0.22)	<0.001	0.01 (0)	0.46 (0.39)	<0.001
Median [Min, Max]	0.01 [0.01, 0.01]	0.20 [0.01, 1.80]		0.01 [0.01, 0.01]	0.25 [0.01, 2.90]		0.01 [0.01, 0.01]	0.32 [0.05, 2.20]	
6'-SL									
Mean (SD)	0.25 (0.10)	0.26 (0.10)	0.267	0.03 (0.02)	0.04 (0.02)	0.645	0.01 (0.00)	0.01 (0.00)	0.758
Median [Min, Max]	0.24 [0.07, 0.55]	0.25 [0.05, 0.73]		0.03 [0.01, 0.11]	0.030 [0.01, 0.29]		0.01 [0.01, 0.02]	0.01 [0.01, 0.03]	
LNT									
Mean (SD)	1.37 (0.61)	0.84 (0.42)	<0.001	0.62 (0.35)	0.43 (0.26)	<0.001	0.35 (0.30)	0.36 (0.23)	0.746
Median [Min, Max]	1.30 [0.24, 3.10]	0.76 [0.09, 2.90]		0.53 [0.12, 1.80]	0.38 [0.05, 1.60]		0.34 [0.05, 0.87]	0.32 [0.05, 1.20]	
LNnT									

Table S8 | Continued.

HMOs	6 weeks		p value	6 months		p value	12 months		p value
	Non-secretor (n=131)	Secretor (n=551)		Non-secretor (n=86)	Secretor (n=362)		Non-secretor (n=13)	Secretor (n=60)	
Mean (SD) Median [Min, Max]	0.03 (0.02) 0.02 [0.01, 0.16]	0.01 (0.06) 0.09 [0.01, 0.39]	<0.001	0.02 (0.01) 0.01 [0.01, 0.08]	0.05 (0.05) 0.04 [0.01, 0.24]	<0.001	0.01 (0.01) 0.01 [0.01, 0.01]	0.03 (0.02) 0.02 [0.01, 0.13]	<0.001
LNFP I									
Mean (SD) Median [Min, Max]	0.04 (0) 0.04 [0.04, 0.04]	0.62 (0.49) 0.51 [0.04, 4.10]	<0.001	0.04 (0) 0.04 [0.04, 0.04]	0.25 (0.26) 0.15 [0.04, 1.90]	<0.001	0.04 (0) 0.04 [0.04, 0.04]	0.22 (0.21) 0.14 [0.04, 1.00]	<0.001
LNFP V									
Mean (SD) Median [Min, Max]	0.10 (0.04) 0.09 [0.01, 0.24]	0.02 (0.01) 0.02 [0.01, 0.09]	<0.001	0.05 (0.02) 0.05 [0.01, 0.12]	0.02 (0.01) 0.013 [0.01, 0.06]	<0.001	0.05 (0.02) 0.05 [0.02, 0.08]	0.02 (0.01) 0.01 [0.01, 0.03]	<0.001
LNFP III									
Mean (SD) Median [Min, Max]	0.21 (0.08) 0.20 [0.08, 0.56]	0.17 (0.08) 0.17 [0.03, 0.48]	<0.001	0.20 (0.07) 0.19 [0.08, 0.43]	0.19 (0.07) 0.19 [0.03, 0.45]	0.284	0.12 (0.05) 0.10 [0.05, 0.19]	0.12 (0.06) 0.14 [0.04, 0.30]	0.206
LNFP II									
Mean (SD) Median [Min, Max]	0.94 (0.46) 0.92 [0.04, 2.50]	0.19 (0.16) 0.13 [0.04, 1.20]	<0.001	0.57 (0.27) 0.55 [0.04, 1.40]	0.18 (0.11) 0.13 [0.04, 0.73]	<0.001	0.58 (0.32) 0.51 [0.20, 1.20]	0.18 (0.11) 0.15 [0.04, 0.43]	<0.001
LNDFHI									
Mean (SD) Median [Min, Max]	0.02 (0) 0.02 [0.02, 0.02]	0.65 (0.33) 0.63 [0.02, 1.90]	<0.001	0.02 (0) 0.02 [0.02, 0.02]	0.36 (0.18) 0.36 [0.02, 0.99]	<0.001	0.02 (0) 0.02 [0.02, 0.02]	0.43 (0.22) 0.41 [0.02, 0.90]	<0.001
LNDFHII + LNnDFHII									
Mean (SD) Median [Min, Max]	0.23 (0.15) 0.21 [0.01, 0.73]	0.020 (0.02) 0.01 [0.01, 0.24]	<0.001	0.18 (0.09) 0.18 [0.01, 0.44]	0.03 (0.02) 0.02 [0.01, 0.19]	<0.001	0.29 (0.09) 0.26 [0.17, 0.46]	0.03 (0.03) 0.03 [0.01, 0.210]	<0.001
Total HMOs									
Mean (SD) Median [Min, Max]	4.99 (1.13) 4.91 [2.41, 8.40]	6.54 (1.19) 6.44 [3.58, 12.6]	<0.001	4.20 (0.81) 4.25 [1.84, 6.01]	5.02 (0.84) 4.96 [2.62, 8.35]	<0.001	5.27 (1.61) 5.13 [1.66, 8.64]	4.95 (0.84) 5.01 [3.16, 6.95]	0.32

P values derived from Wilcoxon signed-rank test comparing Secretor vs. Non-Secretor human milk. Bonferroni-adjusted level of statistical significance is α=0.05/14=0.0036. HMO, human milk oligosaccharides. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactooligosaccharide; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetrose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

Table S9 | Relative proportion (%) of human milk oligosaccharides (HMOs) in human milk samples from the Ulm SPATZ Health study by secretor status

HMOs	6 weeks		6 months		12 months	
	Non-secretor (n=131)	Secretor (n=551)	Non-secretor (n=86)	Secretor (n=363)	Non-secretor (n=13)	Secretor (n=60)
2'-FL						
Mean (SD)	2.75 (0.671)	42.8 (11.9)	3.24 (0.77)	40.0 (13.2)	2.84 (1.56)	33.0 (13.3)
Median [Min, Max]	2.65 [1.55, 5.40]	43.1 [2.71, 74.2]	3.06 [2.16, 7.08]	40.2 [2.85, 77.3]	2.53 [1.50, 7.84]	33.0 [2.35, 71.4]
3'-FL						
Mean (SD)	29.6 (10.3)	7.38 (5.30)	50.7 (13.6)	20.5 (11.7)	62.4 (13.7)	23.6 (12.6)
Median [Min, Max]	29.4 [3.45, 53.5]	6.39 [0.31, 45.4]	53.3 [8.72, 75.5]	18.8 [0.85, 73.3]	60.6 [38.0, 81.8]	22.0 [3.29, 67.0]
3'-SL						
Mean (SD)	3.61 (1.14)	2.31 (0.68)	3.83 (1.15)	3.29 (1.06)	4.08 (1.34)	5.01 (2.07)
Median [Min, Max]	3.45 [1.48, 8.32]	2.23 [0.84, 7.02]	3.60 [1.66, 8.72]	3.10 [0.91, 9.38]	3.70 [2.63, 7.51]	4.54 [2.00, 10.2]
6'-GL						
Mean (SD)	0.40 (0.28)	0.27 (0.12)	0.26 (0.16)	0.19 (0.08)	0.23 (0.11)	0.19 (0.078)
Median [Min, Max]	0.34 [0.12, 2.45]	0.25 [0.08, 0.91]	0.23 [0.07, 1.03]	0.17 [0.03, 0.67]	0.20 [0.13, 0.57]	0.18 [0.09, 0.42]
DFL						
Mean (SD)	0.21 (0.05)	3.46 (2.07)	0.25 (0.06)	5.64 (3.25)	0.22 (0.12)	9.23 (8.68)
Median [Min, Max]	0.20 [0.12, 0.42]	3.10 [0.12, 18.3]	0.24 [0.17, 0.55]	5.27 [0.38, 34.7]	0.20 [0.12, 0.60]	6.00 [1.29, 56.5]
6'-SL						
Mean (SD)	5.26 (2.38)	4.04 (1.60)	0.82 (0.57)	0.71 (0.47)	0.24 (0.13)	0.25 (0.09)
Median [Min, Max]	4.79 [1.35, 15.8]	3.78 [0.78, 16.1]	0.71 [0.19, 4.09]	0.59 [0.15, 4.58]	0.21 [0.14, 0.60]	0.22 [0.14, 0.52]
LNT						
Mean (SD)	28.1 (13.1)	13.0 (6.27)	15.8 (11.8)	8.74 (5.21)	7.35 (5.13)	7.51 (4.86)
Median [Min, Max]	25.1 [4.43, 65.2]	12.0 [1.33, 41.8]	12.4 [2.43, 61.8]	7.92 [0.81, 45.8]	8.86 [0.89, 14.3]	7.08 [0.729, 25.1]
LNnT						
Mean (SD)	0.57 (0.55)	1.52 (0.92)	0.45 (0.55)	1.05 (0.83)	0.22 (0.122)	0.56 (0.48)
Median [Min, Max]	0.39 [0.12, 3.24]	1.33 [0.16, 7.72]	0.29 [0.17, 4.47]	0.80 [0.15, 5.17]	0.20 [0.12, 0.60]	0.43 [0.14, 2.7]
LNFP I						
Mean (SD)	0.85 (0.21)	8.88 (5.58)	0.10 (0.24)	4.68 (4.33)	0.87 (0.48)	4.53 (4.13)
Median [Min, Max]	0.81 [0.48, 1.66]	8.08 [0.69, 32.4]	0.94 [0.67, 2.18]	3.12 [0.62, 26.6]	0.78 [0.46, 2.41]	2.78 [0.58, 18.6]
LNFP V						
Mean (SD)	1.82 (0.65)	0.34 (0.24)	1.11 (0.36)	0.31 (0.16)	0.10 (0.51)	0.34 (0.15)

Table S9 | Continued.

HMOs	6 weeks		6 months		12 months	
	Non-secretor (n=131)	Secretor (n=551)	Non-secretor (n=86)	Secretor (n=363)	Non-secretor (n=13)	Secretor (n=60)
Median [Min, Max]	1.72 [0.54, 5.23]	0.27 [0.09, 1.58]	1.07 [0.28, 2.16]	0.27 [0.13, 1.33]	1.15 [0.28, 1.69]	0.30 [0.14, 0.82]
LNFP III						
Mean (SD)	4.30 (1.57)	2.73 (1.22)	5.15 (2.77)	3.90 (1.36)	2.61 (1.41)	2.99 (1.28)
Median [Min, Max]	4.04 [1.71, 9.18]	2.56 [0.34, 7.63]	4.57 [2.02, 20.2]	3.77 [0.51, 8.99]	2.67 [0.58, 5.79]	2.96 [0.54, 6.31]
HMOs	Non-secretor (n=131)	Secretor (n=551)	Non-secretor (n=86)	Secretor n=363)	Non-secretor (n=13)	Secretor (n=60)
LNFP II						
Mean (SD)	17.7 (6.09)	3.03 (2.73)	12.8 (4.67)	3.24 (2.37)	11.6 (5.80)	3.63 (2.22)
Median [Min, Max]	18.6 [0.97, 29.8]	2.14 [0.32, 20.6]	13.3 [1.37, 23.3]	2.68 [0.52, 16.6]	13.1 [3.56, 19.8]	3.11 [0.73, 9.97]
LNDFHI						
Mean (SD)	0.423 (0.103)	9.93 (4.26)	0.498 (0.119)	7.08 (3.09)	0.437 (0.240)	8.41 (3.70)
Median [Min, Max]	0.407 [0.238, 0.831]	10.4 [0.158, 22.5]	0.471 [0.333, 1.09]	7.52 [0.258, 15.4]	0.390 [0.231, 1.21]	8.59 [0.37, 15.3]
LNDFHII + LNnDFHII						
Mean (SD)	4.32 (2.02)	0.321 (0.389)	4.07 (1.51)	0.589 (0.485)	5.88 (1.90)	0.69 (0.57)
Median [Min, Max]	4.51 [0.241, 9.95]	0.192 [0.0791, 4.30]	4.24 [0.344, 7.75]	0.477 [0.129, 4.16]	5.61 [3.26, 10.3]	0.54 [0.14, 3.80]

HMO, human milk oligosaccharides. 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactooligosaccharide; DFL, 3,2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetrose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II.

Table S10 | Unadjusted[1] and adjusted[2] effects of maternal characteristics on the trajectories of human milk oligosaccharides in milk type I and II over 6 months of lactation in the Ulm SPATZ Health Study

Characteristic	6 months			
	β^1	p value	β^2	p value
TYPE I MILK (*n* = 306)				
Pre-pregnancy BMI				
3'-SL	0.3186	0.0129	0.2951	0.0606
Parity				
LNDFH II + LNnDFHII	-0.1762	0.0125	-0.1698	0.0597
3'-FL	-0.1571	0.0043	-0.1521	0.0272
2'-FL	0.1406	0.0357	0.0918	0.2785
Gestation				
Total HMOs	-0.3565	0.0233	-0.3786	0.0514
Maternal BMI at 6 weeks				
LNFP I	0.2027	0.0096	0.214	0.0222
3'-SL	0.4137	0.0009	0.3334	0.0237
3'-FL	-0.1314	0.0226	-0.1044	0.1298
Maternal BMI at 6 months				
LNFP I	0.2452	0.004	0.213	0.0394
3'-SL	0.322	0.0152	0.2385	0.1372
3'-FL	-0.1552	0.0152	-0.1424	0.0618
TYPE II MILK (*n* = 72)				
Pre-pregnancy BMI				
6'-GL	0.3563	0.0147	0.289	0.0927
Parity				
6'-SL	0.3305	0.0233	0.1005	0.5671

HMO, human milk oligosaccharides; BMI, Body Mass Index; 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-GL, 6'-Galactooligosaccharide; DFL, 3, 2'-difucosyllactose; 6'-SL; 6'-sialyllactose; LNT, lacto-N-tetrose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II. Adjustments made for maternal age, gestation, infant age, infant sex, pre-pregnancy BMI. Bonferroni-adjusted level of statistical significance is α=0.05/14=0.0036.

Table S11 | Unadjusted[1] and adjusted[2] influence of maternal characteristics on the trajectories of human milk oligosaccharides in milk type I and II over 12 months of lactation in the Ulm SPATZ Health Study

Characteristic	6 months				12 months			
	β[1]	p value	β[2]	p value	β[1]	p value	β[2]	p value
TYPE I MILK (*n* = 44)								
Parity								
LNFP III	-0.8078	0.0053	-0.2164	0.4571	-0.8385	0.0037	-0.3607	0.2166
LNFP V	-0.7269	0.0132	-0.3487	0.2848	-0.7109	0.0147	-0.5768	0.0787
LNFP I	-0.3299	0.0548	-0.2715	0.1642	-0.5581	0.0013	-0.5581	0.0076
LNnT	-0.4715	0.0192	-0.4086	0.072	-0.5617	0.0053	-0.531	0.0203
LNT	-0.396	0.065	-0.3747	0.1339	-0.6105	0.0047	-0.6983	0.0069
Gestation								
LNDFH I	0.7196	0.0874	0.5774	0.1647	1.0422	0.0214	0.8879	0.0468
DFL	0.4572	0.4258	0.2924	0.3281	1.91941	0.0113	1.252	0.0085
2′-FL	0.3002	0.3178	0.2684	0.3446	0.7457	0.0224	0.7502	0.0291
TYPE II MILK (*n* = 11)								
Parity								
LNnT	0.8824	0.0576	1.4203	0.0859	1.0066	0.0337	1.4236	0.0853
Delivery mode								
6′-SL	-0.05109	0.8875	0.4712	0.4129	-0.0754	0.0481	0.0926	0.8676
Gestation								
6′-SL	-0.5633	0.2354	-0.2026	0.4612	-1.2867	0.0115	-0.1352	0.7861
6′-GL	-0.2562	0.7911	0.3356	0.9949	-2.7575	0.0095	-1.2541	0.4769
3′-SL	0.4577	0.5797	3280	0.703	-1.8837	0.0318	-0.6219	0.1102
Maternal BMI at 12 months								
6′-GL	0.4497	0.406	-0.5731	0.4426	-1.4358	0.0142	0.6956	0.3582

BMI, Body Mass Index; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-GL, 6′-Galactooligosaccharide; DFL, 3,2′-difucosyllactose; 6′-SL; 6′-sialyllactose; LNT, lacto-N-tetrose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNnDFH II, Lacto-N-neodifucohexaose II; Respective adjustments made for maternal age, gestation, infant age, infant sex, pre-pregnancy BMI. Bonferroni-adjusted level of statistical significance is α=0.05/14=0.0036.

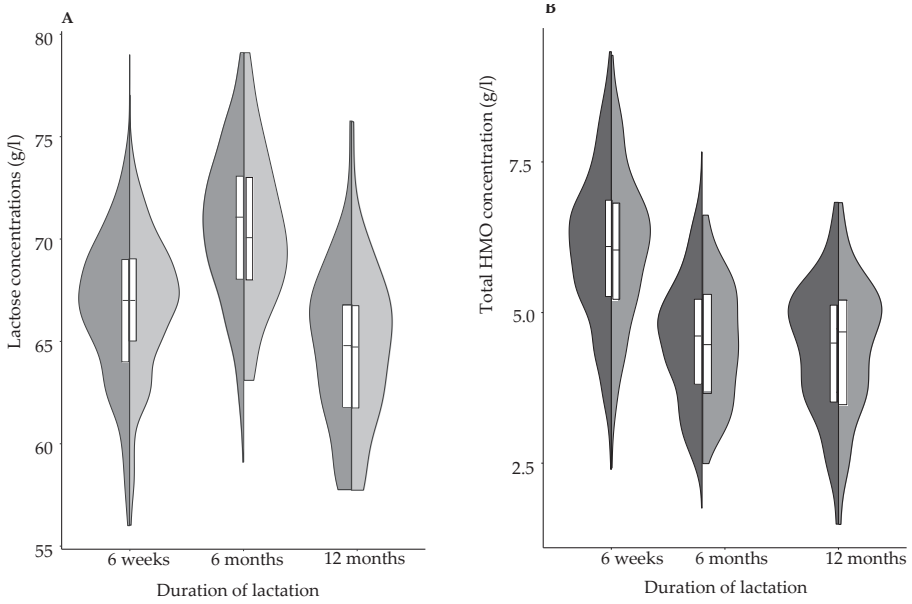

Figure S1 | Split violin plots of the distribution of A) lactose and B) total human milk oligosaccharide concentrations (g/L) during the first 12 months of lactation regardless of milk type and secretor status. Shaded areas show the split violin plots of the density function with inserted boxed indicating the first and third quartile and the median. Dark grey ( ): Total sample size [6 weeks (*n* = 682), 6 months (*n* = 448), 12 months (*n* = 73)]; light grey ( ): Sub-sample size [6 weeks samples restricted to those who provided samples at 6 months (*n* = 422), 6 months samples restricted to those who provided samples at 12 months (*n* = 68), 12 months samples restricted to those who provided samples at 12 all three time point (*n* = 66)].

CHAPTER VII

Development of a high-throughput glycoanalysis method for the characterization of oligosaccharides in human milk utilizing multiplexed capillary gel electrophoresis with with laser induced florescence detection (xCGE-LIF)

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ABSTRACT

During the last decade, enormous progress regarding knowledge about composition and properties of human milk (HM) has been made. Besides nutrition, the three macro-nutrients: proteins, lipids, and carbohydrates combine a large variety of properties and functions. Especially, complex oligosaccharides emerge as important dietary factors during early life with multiple functions. The characterization of these HM oligosaccha-rides (HMOs) within the total carbohydrate fraction is prerequisite to understand the relationship between milk composition and biological effects. Therefore, extended studies of large donor cohorts and thus, new high-throughput glycoanalytical methods are needed. The developed method comprises sample preparation, as well as analysis of HMOs by multiplexed CGE with LIF detection (xCGE-LIF). Via a respective database the generated "fingerprints" (normalized electropherograms) could be used for structural elucidation of HMOs. The method was tested on HM samples from five different donors, partly sampled as a series of lactation time points. HMOs could be easily identified and quantified. Consequently, secretor and Lewis status of the donors could be determined, milk typing could be performed and quantitative changes could be monitored along lactation time course. The developed xCGE-LIF based "real" high-throughput HMOs analysis method enables qualitative and quantitative high-performance profiling of the total carbohydrate fraction composition of large sets of samples.

Keywords

xCGE-LIF; fingerprint; human milk; oligosaccharides

INTRODUCTION

From various points of view, like evolutionary, nutritional, and economical, human milk (HM) is ideal for infant nutrition for the first months of life, and thus it has been recommended by the WHO [1]. Despite the steps that already have been taken regarding knowledge about composition and properties, the functionality of HM in its complexity is still not fully understood [2–6].Besides water, the three macronutrients of HM are proteins, lipids, and carbohydrates [7–9]. They combine a large variety of properties and functions, which were closely surveyed in several studies. It could be shown that during breastfeeding, antibodies pass from mother to child [10, 11], and that HM contains several antiinfective factors such as lactoferrin [4, 6] and immunoglobulin A, protecting against pathogenic microorganisms [12–14]. All this supports the development of the newborns immune system [15–17], e.g. by lowering the risk of infections like otitis media [18] and urinary tract infections [19]. Besides these important factors within the protein fraction ofHM, also the total carbohydrate fraction (TCF) is known to contain compounds bearing a variety of complex and important functions beyond simple energy delivery to the infants [14, 20, 21]. The composition of the HM oligosaccharide (HMOs) pool, i.e. the HM-TCF differs, comparing milks from different mothers or from different lactation time points (LTP). In the past, most studies concerning HM and breastfeeding were looking at HM on its whole and the studies, focusing on the HM-TCF, were analyzing just small donor cohorts (e.g. [2, 6, 21–41]) that can only provide limited information. The HMOs pool mainly consists of lactose (70–85% of the HM-TCF [31]) and a large variety of neutral and acidic oligosac-charides (OS). Until now, more than 1000 of these HMOs have been detected/identified via different low- and medium-throughput techniques like chromatography (e.g. paper chromatography [41–45], HPLC [25, 46–48], high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [26, 49–54] or hydrophilic interaction chromatography [55], mass spectrometry (MS) [56] (e.g., using infrared laser desorption MS [57], fast atom bombardment ionization combined with MS/MS, [58] MALDI-MS/MS [23, 24, 59–65], (nano) ESI multistage MS (LC-MSn), nuclear magnetic resonance (NMR) spectroscopy [80,81] or capillary electrophoresis (CE) with laser-induced fluorescence detection (LIF) [82,83], sometimes additionally coupled online with MS [84–86]. But, as the characterization of HMOs within the HM-TCF is prerequisite to understand the complex relationship between their structures and their biological effects [28], there is a need for high-performance (HP) analytical methods that combine high sensitivity, high selectivity, and high resolution with robustness, reliability, and reproducibility in a high-throughput (HT) manner [83].Consequently, our work is focusing on the development and establishment of an HP glycoanalytical technique, based on multiplexed CGE with LIF detection (xCGE-LIF) that allows in-depth characterization of the HM-TCF composition in large numbers of individual (human) milk samples. The presented method comprises

sample preparation, as well as xCGE-LIF analysis of HMOs, both ready for "real" HT. First neat HMOs were isolated out of the HM-TCF. These neat compounds (HMOs standards) were then used for method development and reproducibility testing. Following on that, we initiated a database (containing said HMOs standards and their normalized migration times ($t_{mig'}$), for a later on fast and easy identification of HMOs beyond peaks of "fingerprints" (normalized electropherograms) generated from real HM samples. To approve the methods capability for monitoring TCF compositions of larger cohorts of individ-ual HM samples in future, we tested the developed method with the HM-TCF of four different donors (representing the four known HM types, according to Thurl et al. [87]), as well as a series of eight HM samples of one donor from different time-points along the lactation time course. It turned out that this xCGE-LIF based "real" HT technique represents a valuable alternative to existing glycoanalysis tools that are more time, lab space, and manpower consuming. This technique will enable unequivocal qualitative and quantitative HP profiling of the HM-TCF (i.e. the HMOs pool) composition of large cohorts.

MATERIALS AND METHODS

Samples

All HM samples (**Table 1**) were provided from healthy volunteers; Caucasian women living in the regions of Dresden (Germany) and Frankfurt/Main (Germany) (previously used by Thurl et al. [27,87], see also for further sample details). The HM samples were immediately frozen after delivery and stored at -20°C until analysis. Pooled sample HM I was used for the isolation of neat HMOs. Samples HM II–V were used for HM type determination of their donors. The series of eight HM samples of one donor from different time-points (HM VI) was taken to demonstrate the methods capability for monitoring HM-TCF composition along the lactation time course.

Table 1 | Samples and lactation time points

LPT	1	2	3	4	5	6	7	8	Pool
(days postpartum)	(1-3)	(4)	(8)	(15)	(22)	(30)	(60)	(90)	
HM I									Yes
HM II									Yes
HM III									Yes
HM IV									Yes
HM V									Yes
HM VI	x	x	x	x	x	x	x	x	No

Sample preparation and analysis workflow for HMOs profiling via xCGE-LIF

Figure 1 shows the HMOs sample preparation and analysis workflow. Briefly, all samples were heated for 30 min at 70°C according to [88] (pasteurization) to inactivate pathogens and to prevent degradation of HM. In the following, proteins and lipids were separated from carbohydrates either via ultrafiltration (UF) (**Figure. 1**, left branch) or via a two-step purification, with first skimming via centrifugation and second deproteinization via UF (**Figure 1**, right branch), resulting in the isolated HM-TCF. Subsequently, the HM-TCF could be analyzed directly or optionally further fractionated by chromatographic steps (**Figure 1**, right branch) to generate HMOs fractions and neat HMOs thereof. To unravel and detect the extracted HMOs using xCGE-LIF, they were fluorescently labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS). Sulfonated aromatic amines, like 8-aminonaphthalene-1,3,-disulfonic acid, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), and APTS are common fluorescent derivatives widely used in the field of electrokinetic separation techniques [89–91]. Due to the utilization of an argon-ion laser inside the xCGE-LIF system with an excitation wavelength of 488 nm and a band filter at 524 nm, APTS (with a X_{exc_max} of 455 nm and a X_{em_max} of 512 nm) was chosen as fluorescent tag, perfectly fitting the requirements.

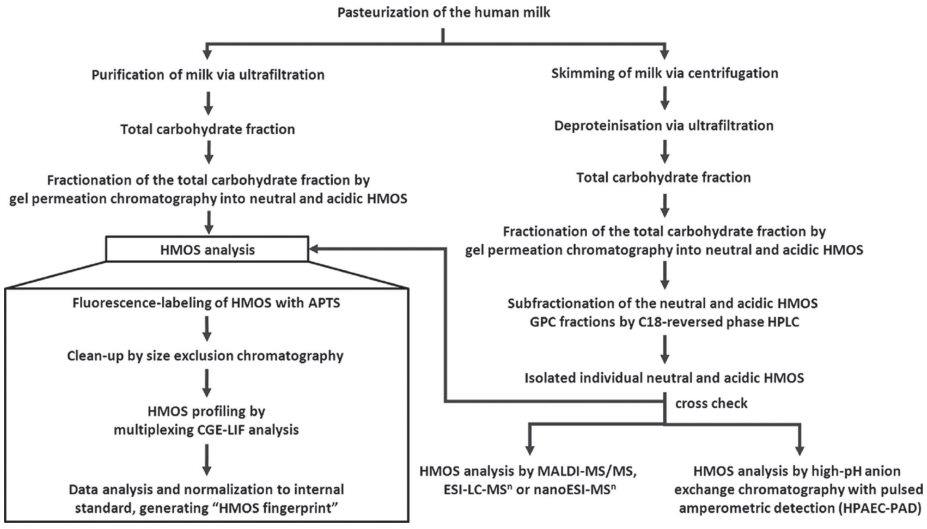


Figure 1 | Workflow of xCGE-LIF based OS analysis from HM (left branch) and isolation / verification of the HMOs standards (right branch)

For postlabeling purification, the mixture of excess APTS and APTS-labeled HMOs was fractionated via SEC according to Schwarzer et al. [92]. Following this postlabeling and clean-up step, the HMOs containing fractions were identified, pooled, and analyzed using xCGE-LIF according to [92–94].

Sample preparation of the pooled HM samples HM II-V
Lipid and protein contents were reduced by centrifugation and UF at 2000 ´ g and 6°C using the Centrifree micropartition system (centrifugal filter devices, 30 MWCO, regenerated cellulose, 1 mL, 4104, Amicon, Germany) using an Avanti J-26 XP centrifuge (with JS-7.5 rotor, Beckman Coulter, USA) according to [88]. By gel permeation chromatography (GPC), the remaining aqueous UF-permeate (crude TCF) was separated into monosaccharides, lactose, the total acidic HMOs fraction (aHMOs) and the total neu-tral HMOs fraction (nHMOs), as described in detail by Thurl et al. [88]. Briefly, the separations were performed on a semipreparative LC system (pump P-500 (GE, Netherlands); 3-channel-degasser (ERC, Germany); two serially connected, thermo jacket (4.0 ´ 110 cm) glass columns (Kro-nlab, Germany), packed with Toyopearl HW 40 (S) (14681, Tosoh Bioscience, Japan); column temperature: 60°C, via column heating bath RK 8 KS (Lauda, Germany) connected to thermo jacket of the glass columns; refractive index (RI) detector LKB Bromma 2142 differential refractometer (GE); fractionator (SuperFrac, GE); recorder SE 120 (BBC Goerz Metrawatt, Germany), and software AI–450 (Version 3.1, Dionex, USA)). The eluent was MilliQTM water + 2% v/v 2-propanol (278 475, Sigma Aldrich, Germany); packing flow rate was 1.7 mL/min; separation flow rate was 1.3 mL/min; injected sample weight/volume was 1 g/5 mL. The aHMOs and nHMOs fractions were dried by lyophilization using the Alpha I-12 lyophilizer (Christ, Germany) with RC-5 pump (VacuuBrand, Germany) (temperature: -20°C and pressure: 20 mbar (isp 3 mbar)) and then redissolved in 1 mL or 0.5 mL of MilliQTM water, respectively.

Sample preparation of HM samples HM VI (lactation time course series)
Here the starting point was 1 mL of HM already mixed with 0.1 mL of an aqueous solution containing stachyose (400 mg/100 mL) and galacturonic acid (80 mg/100 mL), which were added as internal standards in previous studies [26,27]. Again first, lipid and protein contents were reduced, as described above. The remaining crude HM-TCF was fractionated by GPC, dried, and dissolved, as described above.

Sample preparation for generation of HMOs standards from pooled HM sample HM I

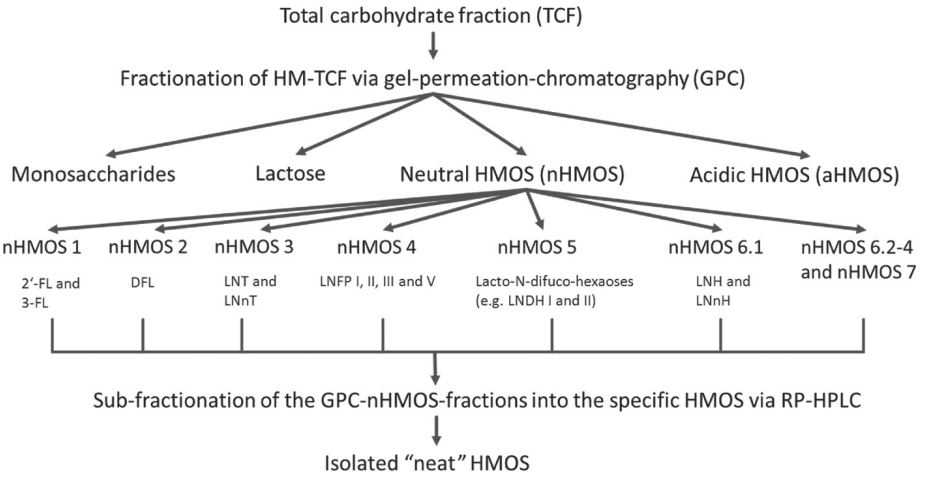


Figure 2 | Scheme for fractionation of HM-TCF into monosaccharides, lactose, the neutral and the acidic HMOs fractions, followed by further subfractionation into neat HMOs (HMOs standards for xCGE-LIF database).

To generate HMOs standards and to build-up a HMOs library and a database, neat HMOs were isolated from HM I (pooled HM). Again first, the lipid and protein contents were reduced. Therefore, a two-step method was used: First, the HM was skimmed using an Avanti J-26 XP centrifuge (15 min, 5000 rpm and 4°C, with JS-7.5 rotor, Beckman Coulter). The remaining aqueous solution was filtered through glass wool (18 421, Sigma Aldrich) to totally skim the cream. In a second step, the filtered aqueous solution was deproteinized via UF (reservoirs: 150 L; feed flow rate: 2.4 m^3/h with max. 8.5 bar; circulation flow rate: 20 m^3/h). For this purpose a 5 m^2 polyethersulfone/polysulfone plate module membrane with a molecular cut off of 30 kDa (Lenntech, Netherlands) was used. Afterwards, the remaining filtrate was spray dried. The spray dried sample was dissolved (1 g/5 mL) in MilliQ™ water. By GPC (see Fig. 1, right branch) and similar as described above, this crude HM-TCF solution again was separated into monosaccha-rides, lactose, aHMOs, and nHMOs fractions. Briefly, the separations were performed on a semipreparative LC system (pump LKB 2150 (GE); double jacketed (1.6 ´ 90 cm) glass column (Kronlab) packed with TSK HW-40(S) (Merck, Germany); column temperature: 60°C, via column heating bath RK 8 KS (Lauda); refractive index-detector LKB Bromma 2142 differential refractometer (GE); frac-tionator LKB Frac100 (GE)). The eluent was MilliQ™ water + 0.02% v/w sodium azide (71 289, Sigma Aldrich); packing flow rate was 1.5 mL/min; separation flow rate was 1.0 mL/min; injected sample volume was 100 pL. But, this time the GPC nHMOs and aHMOs fractions were collected as subfractions (nHMOs 1-7 and aHMOs 1-6) (Fig. 2). Subsequently, the individual subfractions were vacuum dried using a vacuum centrifuge RVC 2–25 with cooling trap CT 02–50 at

-50°C (Christ) and vacuum pump MZ25 (VacuuBrand) and were stored at -20°C until further processing.

According to Stahl et al. [60], for the purpose of HMOs isolation, each of these nHMOs and aHMOs subfractions were resolved in MilliQ™ water, at a concentration of 50 g/L and individually reinjected and further separated and fractionated (see also Fig. 2) on a semipreparative RP-HPLC system, (two pumps: model 215 with 5 mL stainless-steel pump heads (Varian, Germany); two serially connected (8.0 ´ 250 mm) columns with a 30 mm precolumn, packed with NUCLEOSIL® 100–5 $_{C18/5}$ pm/100 $^{A^*}$ (MACHEREY-NAGEL, Germany); UV-detector UV1 (Dynamax, USA) (195 nm); fractionator model 704 (Varian), fractionation intervals: 10–30 s). Eluent: MilliQ™ water; flow rate of 0.8– 1.2 mL/min (at room temperature): injected sample volume was 25 pL; ethanol (99.8%, 34 852, Sigma Aldrich) was used for column regeneration. To isolate the compounds, the RP-HPLC flow rates and fractionation intervals had to be optimized for each GPC fraction. If required, the RP chromatographic step was repeated for the collected subfractions until just one single HMOs structure was identified within them (data not shown). HMOs identification and purity checking was done either via MALDI-MS/MS, according to [60, 61], nanoESI-MS^n (where MS^n is multistage MS) according to Pfenniger et al. [66,69], ESI-LC-MS^n, according to Thomsson et al. [74], and/or HPAEC-PAD, according to Thurl et al. [26].

Differing from [60,61] a MALDI-TOF/TOF 4800 instrument (ABSciex, USA) was used and operated in positive reflector ion-mode utilizing dried droplet preparation and DHBS-MALDI-Matrix (20 g/L in 5% ACN (34 967, Sigma Aldrich) with 0.1% v/v TFA (>99%, 40967, Sigma Aldrich)) as matrix, according to Karas et al. [95]. In contrast to Thoms-son et al. [74] for ESI-LC-MS^n analysis of native HMOs, we employed an LTQ Velos linear ion trap instrument (Thermo Scientific, USA) online coupled to an Ultimate 3000 HPLC system (both: ThermoScientific) operated with a porous graphitized carbon (PGC) column (1.0 ´ 100 mm, packing: Hypercarb, 3 pm particle size, ThermoScientific) at room temperature and a flow rate of 20 pL/min. Furthermore, a 170 min MilliQ™ water-ethanol gradient was used instead of the original H2O-ACN gradient. Eluents were MilliQ™ water (= eluent A) and ethanol (= eluent B) (99.8%, 34 852, Sigma Aldrich), both contained 0.1% formic acid v/v (98%, 94 318, Sigma Aldrich). Gradient profile was as follows: 0–11 min 100% A, 11–75 min 100–70% A, 75–85 min 70– 0% A, 85–125 min 0% A, 125–130 min 0–100% A, 130– 170 min 100% A. IEC-based HMOs analyses were performed according to [26] on an HPAEC-PAD system (DX-300 BIO-LC-system (Dionex); AS3500 autosampler (Spectra Physics, USA); Carbo-Pac PA–100 (4.0 ´ 250 mm) column with CarboPac PA–100 (4.0 ´ 50 mm) precolumn (both: Dionex); pulsed electrochemical detector PED-2 (Dionex)). Eluents were MilliQ™ water and aqueous solutions of sodium hydroxide (NaOH, 306 576, Sigma Aldrich) and sodium acetate (NaOAc, S3272, Sigma Aldrich). They were used to generate the following gradient: 0–20 min, 30 mM NaOH; 20–34 min,

30–100 mM NaOH; 34–48 min 100 mM NaOH/ 0–28 mM NaOAc; 48–55 min, 100 mM NaOH/28–200 mM NaOAc; 55–60 min 100 mM NaOH/200 mM NaOAc; flow rate was 1.0 mL/min (at room temperature).

With this procedure, at least 1 mg of each of the 17 neat HMOs compounds was sampled pooling the respective fractions from several semipreparative RP-chromatographic runs.

Fluorescent labeling, postlabeling clean-up, and xCGE-LIF based HMOs analysis
After protein and lipid content of HM were reduced via UF, the TCF was analyzed as shown in Fig. 1 (left branch of the scheme). Therefore, the HMOs were fluorescently labeled according to the method of Callewaert et al. [96] and modified by Schwarzer et al. [92] with APTS (>96.0%, A7222, Sigma-Aldrich) by reductive amination. Briefly, 2 pL 20 mM APTS and 2 pL 1M sodium cyanoborohydride (NaCNBH3) (reagent grade, 156 159, Sigma Aldrich) both in 15% v/v acetic acid (99.7%, A3701, Applichem, Germany) in MilliQ™ water were added to 2 pL of the TCF. Derivatization was allowed for 18 hat 37°C. Afterwards, the excess of APTS (not reacted) and salt were removed using SEC. Therefore, MultiScreen Deep Well Solvinert Filter Plates (MDRL N04, low-binding hydrophilic PTFE, Millipore, USA) were packed with Toy-opearl HW-40(F) (19 808, Tosoh Bioscience, Japan). Before packing, the slurry was washed six times with MilliQ™ water in 50 mL centrifuge tubes. Then, deep well filter plates were packed with 2 mL washed slurry and washed again with three column volumes MilliQ™ water. After sample application, HMOs were eluted with MilliQ™ water by centrifugation ((50 x g/step)). Up to 25 fractions were collected per well (each in one 96-well plate (V-bottom Polystyrene, 9292.1, Roth, Germany)) and screened for HMOs content by analyzing the fractions with a 4-capillary DNA-sequencer "ABI PRISM 3100-Avant Genetic Analyzer" (Applied Biosys-tems, USA). Afterwards, HMOs containing fractions were pooled. HMOs "fingerprints" were obtained by analyzing the HMOs pools via xCGE-LIF according to Schwarzer et al. [92]. Each of the four parallel capillaries had an effective capillary length of 50 cm. Undiluted POP-6™ polymer (4 316 357, Applied Biosystems) was used as separation matrix. Samples were diluted 1:10 in HiDi™-Formamide (4 311 320, Applied Biosystems) and injected for 5 sat 15 kV. Separation was performed for 130 min at 15 kV and 30°C. According to Schwarzer et al. [92] internal standard was added to the samples after diluting with HiDi™-Formamide. Based on this standard, a migration time normalization was performed using the Matlab R based program (glyXtoolTM) [97], that enabled automated data processing and analysis. Doing so, the migration time t_{mig} in (data points) was automatically normalized to $t_{mig'}$ with normalized migration time units (MTU').

Reproducibility testing and HMOs database built-up
For reproducibility testing and to initiate a respective HMOs database, normalized xCGE-LIF data resulting from repeated analysis of the 17 isolated and identified neutral HMOs standards (see above) were compiled. Over a timespan of six months, these standards were measured, at least ten times each. Based on these repeated measurements, the mean normalized migration times and the resulting RSDs were calculated (**Table 2**).

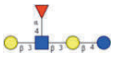
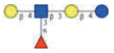
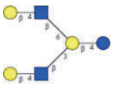
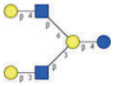
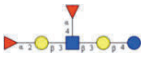
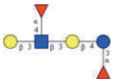
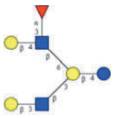
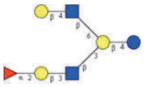
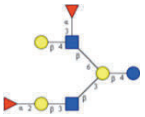
Peak identification via migration time matching with HMOs database entries
Based on the results ofthe reproducibility testing (RSD < 1%, for all isolated and tested HMOs standards), the new HMOs database (correlating HMOs structures with their respective $t_{mig'}$ (Table 2)) was used to assign HMOs to xCGE-LIF peaks. Peaks with the same $t_{mig'}$ most likely represent the same HMOs. For selected peaks/HMOs, this was cross-checked via spiking experiments (as described below/data not shown).

Peak identification via spiking experiments
For spiking experiments, the isolated and labeled HMOs standards were used. The respective HMOs standard was added to the sample, the HiDi™-Formamide volume to be added was accordingly reduced. Thus, end-volume and concentration of the sample were the same and the run was conducted as described above (data not shown).

Quantitative comparison of HMOs fingerprints at different lactation time points via the relative peak height proportions (PHP)
HMOs concentration changes during lactation were analyzed by calculating relative PHP (as published previously for *N*-glycosylation patterns [92, 98–103]). Briefly, the same set of 18 peaks was picked in all HMOs fingerprints of the samples from the LTP series (peak picking criteria: peaks > 200 relative fluorescence units (RFU) or already known peaks). To determine the relative PHP of the peaks, the peak heights (intensities in RFU) of all picked peaks were summed up to the total peak height.

Then the relative PHP of each picked peak could be determined in percent of the total peak height, i.e. the peak height of each picked peak was normalized to the sum of the peak heights of all picked peaks. Despite the fact, that peaks with the same $t_{mig'}$ most likely represent the same HMOs and thus, easily could be assigned via $t_{mig'}$ matching with database entries again, all the respective peaks were verified via cross-checking with spiking experiments (data not shown).

Table 2 | Initiated HMOs/xCGE-LIF $t_{mig'}$ database.

Name	HMOS		Peak number (PN)
	Structure	Migration time in MTU′ (%RSD)	
Lactose		22.07 (3.1)	1
3-FL		46.50 (0.2)	2
2′-FL		47.92 (0.8)	3
DFL		64.87 (0.3)	4
LNnT		87.62 (0.9)	5
LNT		90.40 (0.7)	6
LNFP II		109.88 (0.5)	7
LNFP V		111.67 (0.4)	8
LNFP I		113.03 (0.6)	9
LNFP III		115.09 (0.3)	10
LNnH		121.25 (0.6)	11
LNH		121.73 (0.6)	12
LNDH I		126.63 (0.2)	13
LNDH II		128.68 (0.2)	14
3-F-LNH		140.47 (0.6)	15
2′-F-LNH		141.72 (0.6)	16
2,3-DF-LNH		161.24 (0.5)	17

Mean and RSD values of $t_{mig'}$ were calculated from >10 repeated measurements over 6 months. Schematic HMOs structures were generated with GlycoWorkbench (EuroCarbDB) [132], according to CFG nomenclature; 2′–FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 2′–F–LNH: 2′–fucosyl-lacto-N-hexaose; 3-F-LNH: 3-fucosyl-Lacto-N-hexaose; DFL: Difucosyllactose; LNDH I–II, Lacto-N-difuco-hexaose I–II; 2,3–DF–LNH: 2,3–difucosyl-Lacto-N-hexaose; LNFP I–III and V, Lacto-N-fuco-pentaose I–III and V; LNH: Lacto-N-hexaose; LNnH: Lacto-N-neo-hexaose; LNT: Lacto-N-tetraose; LNnT: Lacto-N-neo-tetraose

Table 3 | Classification of HM types based on the presence and absence of specific HMOs fucosylation

Milk type	Specific fucosylation		Relative distribution (%)[a)]
	α1-2 (*Se*)	α1-4 (*Le*)	
I	+	+	69
II	—	+	20
IIII	+	—	10
IV	—	—	1

Se–secretor gene encodes the fucosyltransferase II that catalyzes OS epitopes with an a1-2 fucosylation at end terminal galactose; *Le*–Lewis gene encodes the fucosyltransferase III that catalyzes OS epitopes with an a1-4 fucosylation at *N*-acetylglucosamine a)[87]

RESULTS

Reproducibility testing and initialization of an xCGE-LIF based HMOs database and library

In order to build up an HMOs-database, first, the reproducibility of the normalized migration times of the respective HMOs standards had to be demonstrated. Therefore, a reproducibility study was performed, periodically (N > 10) measuring these HMOs standards over a timespan of six months. As described in Section 2 (**Figure 1**), the standards were isolated from HM via RP-HPLC after GPC-fractionation of total HM-TFC, identified and labeled. In addition, the purity of the standards has been cross-checked via HPAEC-PAD, MALDI-MS/MS, nanoESI-MS^n, and/or ESI-LC-MS^n – and their structures were elucidated by these orthogonal methods (described in Section 2 – data not shown). Evaluating the repeated measurements of the isolated APTS-labeled HMOs standards (data not shown), the needed high reproducibility of t_{mig}, was approved (long-term RSD < 1%). Doing so, an initial HMOs database could be built up, including a first set of 17 HMOs (**Table 2**).

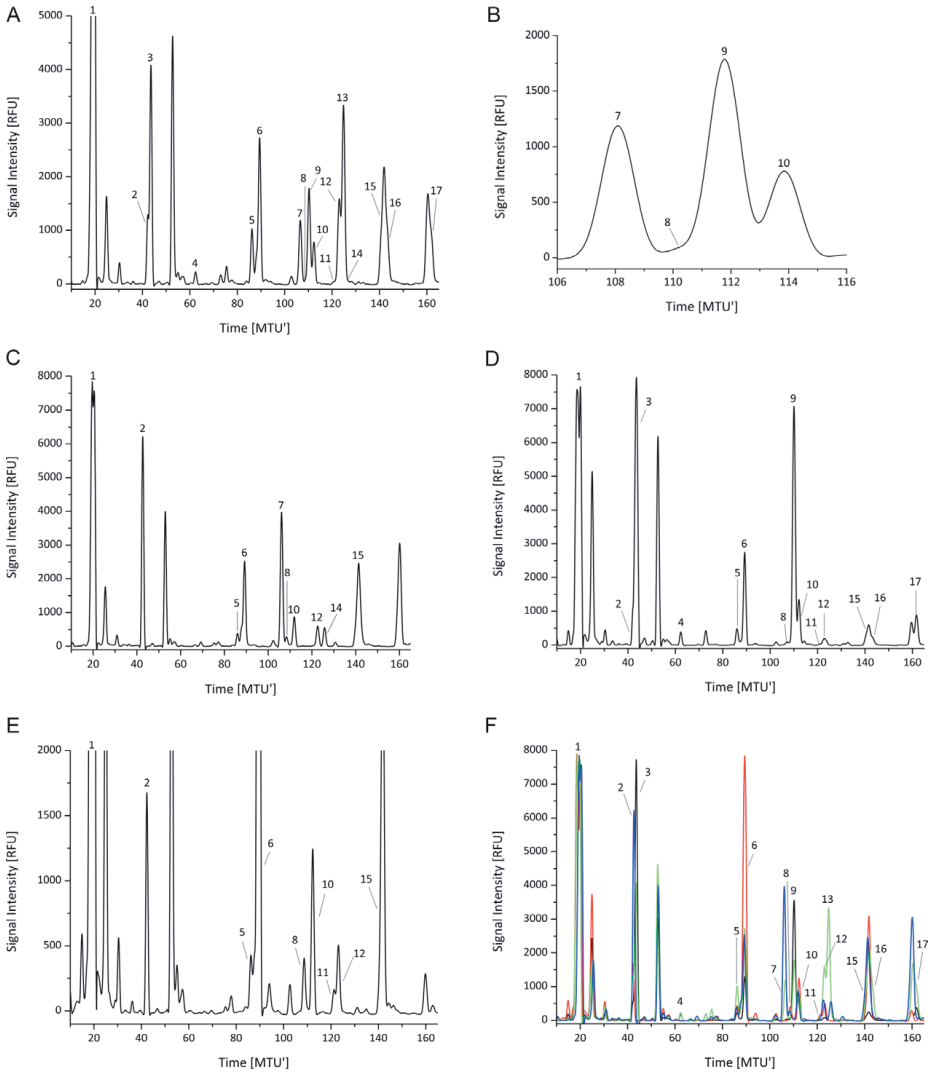


Figure 3 | HMOs fingerprints (normalized electropherograms) representing the four HM types. Fingerprints (relative fluorescence units [RFU] plotted over normalized migration time units [MTU']) of the HM-TCF generated via xCGE-LIF. Peak numbers (PN 1–17) according to Table 2. Fingerprints of HM samples (Table 1): (A) HM II; (B) zoom-in (106–116 MTU') of (A); (C) HM III; (D) HM IV; and (E) HM V— representing HM type I, II, III, and IV, respectively (according to Table 3) and (F) Overlay of the HMOs fingerprints (green: HM type I (HM II); blue: HM type II (HM III); black: HM type III (HM IV) and red: HM type IV (HM V).

Approval of xCGE-LIF based method regarding qualitative and quantitative analysis of real HMOs samples

Method validation and feasibility testing was done via two series of experiments. First, exemplary HMOs fingerprints of four different donors were recorded via xCGE-LIF with respect to HM typing (**Table 3**) and structural elucidation of HMOs in complex mixtures. The generated individual fingerprints are shown in **Figure. 3**, i.e. the qualitative HMOs composition of different HM was investigated. Second, HMOs fingerprints of different LTP of one donor along the lactation time course were measured in three technical replicates to demonstrate methods feasibility regarding relative quantitation. The series of generated individual fingerprints representing the lactation time course is shown in **Figure. 4A**. According to Thurl et al. [87], to determine the four different HM types (Table 3), represented by the samples HM II-V, the presence or absence of distinct HMOs had to be investigated. For doing so, the peaks within the HMOs fingerprints (**Figure. 3A** and **C–E** and peak numbers (PN) 1–17) were assigned via normalized migration time matching with the database entries of **Table 2**. In addition, this HMOs determination was confirmed by consecutive reconducting the fingerprints, after spiking each of the 17 isolated and labeled HMOs standards of **Table 2** to each of the four individual HM samples (i.e. 17 ´ 4 additional runs; data not shown). **Figure 3A** represents the HMOs fingerprint of HM II, in which all peaks (PN 1–17 (Table 2)) appear. **Figure 3B** shows an additional zoom into this HMOs fingerprint to reveal the ability of the xCGE-LIF based HMOs analysis method to resolve/separate the four isomeric Lacto-N-fuco-pentaoses (LNFP I, II, III, and V (PN 7–10)). As a result (according to **Table 3** and Thurl et al. [87]), HM II clearly represents HM type I, which corresponds to secretor gene (Se) as well as Lewis gene (Le) positive status of the donor. In **Figure. 3 C**, the peaks PN 3, 4, 9, 13, 16, and 17 do not appear in the HMOs fingerprint of HM III. Thus, according to Table 3 and [87], by the lack of these peaks HM type II is indicated for this donor. In **Figure. 3D**, LNFP II (PN 7) and additionally, the hexaoses LNDH I (PN 13) and LNDH II (PN 14) are absent, i.e. according to Table 3 and [87], the HMOs fingerprint of HM IV corresponds to the TCF composition of an HM type III. **Figure 3E** illustrates the HMOs fingerprint of HM V. According to Table 3 and [87], this donor could be identified to be HM type IV, as the peaks PN 3, 4, 7, 9, 13, 14, and 16 do not appear in this HMOs fingerprint. Compared to the HM types I–III (**Fig. 3A**, **C**, and **D**) here the lowest number of peaks was found. In addition, the large proportion of Lacto-N-tetraose (PN 6) is striking in **Fig. 3E**, making Lacto-N-tetraose (beside PN 1 (lactose)) to the by far most abundant HMOs within this sample. During the examination of the HMOs fingerprints (**Figure 3A** and **C–E**), significant differences could be identified and based on these differences HM typing was easily possible. For better illustration of the differences between the four HM types, all four fingerprints are plotted as an overlay in **Figure. 3 F**. Via overlay, the excellent separation performance is demonstrated and the striking reproducibility of the developed xCGE-LIF based HMOs analysis method is shown by the coincidence of the peaks within the overlayed fingerprints.

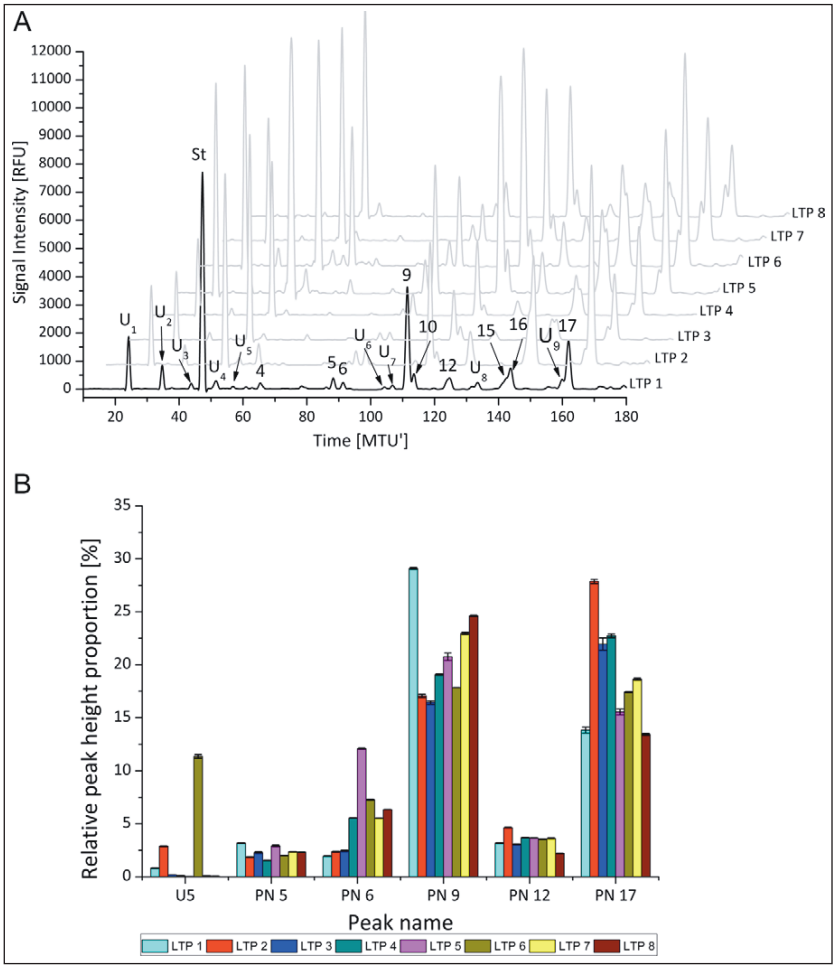


Figure 4 | HMOs fingerprints from samples taken along lactation time course and bar plot of the relative PHP of selected peaks. A) Shifted waterfall overlay of HMOs fingerprints (normalized electropherograms) plotted over [MTU'] of the nHMOs fraction (HM VI/HM type III) from eight LTP (day 1– 3, 4, 8, 15, 22, 30, 60, and 90 post-partum) of one donor, conducted via xCGE-LIF. For each LTP, the same 18 peaks were picked. (B) Bar plot of the relative PHP (see Table 4) of selected HMOs peaks (U5, PN 5, 6, 9, 12, and 17) along the lactation time course. The chosen peaks represent relatively constant HMOs (PN 5and 12), respectively, HMOs significantly changing their concentration along the lactation time course (A PHP >> 10% of the actual PHP: U5, PN 6, 9, and 17).
Peak nomenclature: St: stachyose peak, covering the fuco-syllactoses; PN 4–17 (according to Table 2) and U1–U9 (unknown peaks). Stachyose was added to each sample during the sample preparation (HM-TCF fractionation via GPC) for subsequent quantification of the HMOs components via HPAEC-PAD analysis (according to Thurl et al. [26]). During the CGE-LIF based OS analysis, it became clear that stachyose interferes with two HMOs peaks. The migration time of stachyose is nearly the same as for the two fucosylactoses (PN 2 and 3). In Figure 4, this sum peak is marked with St and is later on not considered in detail, since there is no possibility to distinguish between stachyose, 3-FL, and 2'-FL.

Table 4 | Summary of the relative PHP (%) of selected peaks along lactation time course of HM VI (see Table 1)

Peak PN	LTP 1	LTP 2	LTP 3	LTP 4	LTP 5	LTP 6	LTP 7	LTP 8
U1	14.95	11.03	22.71	19.64	20.08	16.43	18.53	25.90
U2	6.82	2.72	2.40	1.76	1.65	1.43	0.70	0.65
U3	1.64	0.31	0.36	0.20	0.08	0.13	0.10	0.03
U4	2.42	0.94	2.06	1.37	2.07	1.25	1.43	2.41
U5	0.82	2.89	0.18	0.09	0.03	11.37	0.09	0.05
4	1.74	0.19	1.71	0.65	1.04	0.58	0.76	0.52
5	3.17	1.87	2.30	1.55	2.92	2.01	2.36	2.32
6	1.94	2.37	2.45	5.54	12.11	7.27	5.52	6.35
U6	0.59	0.49	0.78	0.59	0.16	0.34	0.32	0.24
U7	1.11	0.56	0.29	0.09	0.03	0.05	0.06	0.08
9	29.10	17.06	16.42	19.07	20.76	17.84	22.97	24.62
10	4.35	1.78	4.45	2.60	3.96	2.95	3.96	4.97
12	3.17	4.65	3.06	3.69	3.65	3.56	3.63	2.22
U8	1.94	0.26	0.69	0.21	0.18	0.31	0.33	0.26
15	3.61	7.68	6.95	10.31	7.79	8.23	8.69	6.40
16	6.02	15.17	6.81	6.76	5.93	5.94	6.01	3.65
U9	2.77	2.19	4.42	3.14	2.01	2.90	5.91	5.88
17	13.82	27.87	21.96	22.73	15.54	17.42	18.63	13.43

PN 4–17 (according to Table 2) and U_1–U_9 (unknown peaks). Color code of the comparison of the relative PHP (always against predecessor), black: constant (change of PHP below 10% = RSDmax), red: decrease of PHP > 10%; green: increase of PHP > 10%.

Second, as mentioned above, samples HM VI of one donor (**Table 1**) from eight consecutive LTP were analyzed in three technical replicates to prove the feasibility of the method and to demonstrate the feature of relative quan-titation for the purpose of monitoring lactation time courses of larger cohorts in the future. For this series of experiments, first, the HM-TCF of each LTP was separated into the nHMOs fraction and the aHMOs fraction (see Section 2). In the following, only the nHMOs fractions were analyzed via xCGE-LIF, shown in Fig. 4. To visualize the (quantitative) changes along the lactation time course, the HMOs fingerprints of these HM samples are plotted as shifted waterfall overlay (Fig. 4A). Based on the absence of the peaks corresponding for LNFP II (PN 7) and the LNDH I and II (PN 13 and 14) inside the HMOs fingerprint, easily HM type III could be determined for this HM. The concrete numbers of quantitative changes of the nHMOs fraction composition, corresponding to relative changes of single peaks along the lactation time course, are given in **Table 4** as relative PHP. The HMOs fingerprints of the nHMOs fraction of all eight LTP (LTP 1 (day 1–3) in black and LTP 2–8 in gray) are shown for the normalized migration time range of 10 to 180 MTU' in **Figure. 4 A**. This timespan was chosen to be shown, because all high abundant HMOs compounds as well as the already identified HMOs migrate within this window. Within all eight examined HMOs fingerprints of the samples taken along the lactation time course, the same 18 peaks were selected for peak assignment and relative quantitation. The database matching resulted in

the following findings: unfortunately 3-FL and 2'-fucosyllactose (2'-FL) were covered by the stachyose-peak (St) (Fig. 4A), PN 4–17 could be assigned (according to **Table 2**) and the peaks U1–U9 had to be annotated as unknown. See Table 4, which also summarizes the relative PHP of all 18 picked peaks at all LTP. For the three technical replicates of the samples HM VI from eight LTP, an RSD <10% was found for the relative PHP values. As a result, it was found that it is easily possible to monitor quantitatively changes in HM-TCF composition during lactation time course using the developed xCGE-LIF based HMOs analysis method. Interestingly, the qualitative composition of the nHMOs fraction remains constant along the lactation time course (all peaks could be identified at all time points (**Figure. 4** and **Table 4**)), while its relative quantitative composition changes greatly. Exemplarily, peak U5 shows large changes within its relative PHP: there is a threefold change from nearly 1% at the LTP 1 to almost 3% at LTP 2 and a drop-down to about 1/15 of this value afterwards (**Table 4** and **Figure. 4 B**). In the further lactation time course, the relative PHP stays on this "very" low level, except for the LTP 6 (day 30 postpartum). Here the relative PHP of U5 reaches by far the biggest value (>11%), and U5 is the fourth abundant peak within the HMOs fingerprint. Another example showing major changes is PN 9 (LNFP I). During the lactation time course, its relative PHP decreases from 29% at LTP 1 to a minimum of 16.4% at LTP 3 (**Table 4** and **Figure 4B**). In contrast to these strongly changing peaks, there are also peaks like PN 5 or 12 that show a quite stable relative PHP during lactation period (**Table 4** and Figure. 4B).

DISCUSSION

The aim of the present study was the establishment of a fast, robust, reliable, and reproducible (i.e. high-throughput), but also sensitive, selective, and highly resolving (i.e. high-performance) HMOs analysis method. It should be shown that the developed xCGE-LIF based method allows generation of HMOs fingerprints to be used for HM typing and can be used for in-depth analysis of HMOs, i.e. structural elucidation of the single compounds and their relative quantification. Therefore first, neat HMOs compounds were isolated out of the HM-TCF (see Section 2, **Figure. 2**) and were used for method development as well as robustness/reproducibility testing. Second, these neat HMOs were used to initiate a respective database (HMOs structures with their associated $t_{mig'}$) for fast and easy identification of HMOs beyond peaks of generated HMOs fingerprints from real samples via migration time matching. Finally, for method establishment, the HM-TCF of four different donors, representing the four known different HM types (see **Table 1** and Thurl et al. [87]), as well as a set of eight HM samples of one donor collected at different time-points along the lactation time course, were analyzed repeatedly. Thus, the method could be approved to be a valuable tool for qualitative and quantitative monitoring HMOs pool composition of large HM donor cohorts in future.

Method development—reproducibility testing and initialization of an xCGE-LIF based HMOs database and library

The successful reproducibility study was prerequisite for the unambiguous assignment of HMOs structures to normalized xCGE-LIF migration times. Reproducibility is the basis for the establishment of an HMOs database that combines structural and $t_{mig'}$ information for each HMOs compound entry. For the future, this database has to be increased to facilitate fast and efficient analysis of the HM-TCF, but also of other OS samples, e.g. animal milk [104,105] or plants as sources of dietetic OS. As already demonstrated for the human blood serum glycome [93], a respective database will also allow the HT analysis of biotechnological HMOs [106] or for HT glycoprofiling of bacterial HMOs consumption [107].

The decisive reproducibility test (technical replicates and repeated measurements along a timespan of 6 months, with a total *N* 10 per HMOs) was passed successfully. The achieved mean $t_{mig'}$ and RSD of all 17 repeatedly measured neutral HMOs can be found in **Table 2**. During this long-term study, two aspects became apparent and should be noted: older capillary arrays lose sensitivity and overaged polymer hampers the overall stability of the system. To avoid these phenomena, both the capillary array and the polymer were changed regularly. As the temperature has an effect on the viscosity of the polymer, the observed slight fluctuations in $t_{mig'}$ could be further reduced by air conditioning.

Having a closer look on the four isomeric fucopentaoses (LNFP I, II, III, and V), it is obvious that their structure has a significant influence on $t_{mig'}$. Their fucose-position (a1-2, a1-3, or a1-4) combined with their core structure has a significant effect on their migration behavior (Fig. 3B and Table 2). Despite identical molecular weights, a $t_{mig'}$ difference of about 5 MTU' can be observed between LNFP II and LNFP III. This can be explained by the two orthogonal separation mechanisms working together in CGE: separation by *m/z* and separation by molecular shape, i.e. all LNFP isomers have the same *m/z* (three negative charges from APTS and identical molecular weights) but show different glycosidic linkages between their constituent monosaccharides and therefore different molecular shapes. At present, only broad rules with respect to the migration order can be applied (e.g. (i) larger (HM)OS show longer $t_{mig';}$ (ii) negative charges (like additional sialic acid) reduce $t_{mig';}$ (iii) nonfucosylated (HM)OS with Galβ1–3 N-acetylglucosamine (GlcNAc) core structure (type 1 core) migrate slower than corresponding (HM)OS with Galβ1–4GlcNAc neo-core-structure (type 2 core); and (iv) fucosylated (HM)OS with a Galβ1–3GlcNAc core structure migrate faster than corresponding (HM)OS with Galβ1– 4GlcNAc (neo)-core structure). According to Rohrer [108], who already summarized regularities for the HPAEC-PAD-based (HM)OS analysis, in future the increasing number of HMOs database entries and the increasing knowledge regarding structure-specific CGE separation characteristics will allow further preclassification or maybe even prediction of unknown (HM)OS structures within those fingerprints.

As a result of the performed long-term reproducibility testing, it can be stated that the $t_{mig'}$ are quite stable. Over the entire reproducibility study, the RSD of $t_{mig'}$ was <1%, for all 17 isolated and tested HMOs. This result indicates that the developed HT and HP xCGE-LIF based HMOs analysis method is appropriate to initialize a respective HMOs database, as its migration time variance is significantly below its typical peak width. The database could now be tested regarding its feasibility to be used for structural elucidation of peaks in real samples.

Method application—approval of xCGE-LIF based method regarding qualitative and quantitative analysis of real HMOs samples

After successful approval of the methods reproducibility and the initialization of a respective HMOs database and library, HMOs fingerprints of HM-TCF of different donors were conducted via xCGE-LIF to demonstrate its performance and applicability. This was successfully demonstrated for HM from donors representing the four known different HM types, as described in Thurl et al. [87] (**Figure. 3**) and also for HM samples of one donor from eight different LTP along the lactation time course, as described in [27] (**Figure. 4**).

Figure 3 A and **C–E** show the HMOs fingerprints of the four HM donors with HMOs structures assigned to the peaks. Despite the large concentration differences, structures could be easily assigned via database matching (**Table 2**), e.g. all four isomeric LNFP structures (see zoom-in, Fig. 3B). The assignment of all entries was additionally confirmed by spiking experiments with the corresponding isolated neat HMOs compounds (data not shown). The fingerprints of HM-TCF in **Figure. 3** reveal that xCGE-LIF based HMOs analysis nicely resolves very complex HMOs mixtures. This complexity is due to the possibility of different glycosidic linkages that enable a low number of different monosaccharides to form out a broad variety of HMOs structures [5,28,109–111]. At this point, it should be noted that some of the various HMOs structures that exist may elute closely together or even may co-elute (e.g. the hexoses LNH (PN 12) and LNnH (PN 11) which are only 0.5 MTU' apart, or the stachyose peak which is co-eluting with 3-FL (PN 2) and 2'-FL (PN 3) (**Figure 4**). But the majority of these HMOs (including isomeric structures like LNFP (**Figure 3 B**)) can be clearly separated from each other. Due to the high resolution of the xCGE-LIF based HMOs analysis, all four HM types could easily be determined, according to Thurl et al. [87]. The (HM)OS that contain antigenic determinants of the Le and/or Se [37, 41, 112–114] are synthesized by fucosyltransferases that are not distributed uniformly throughout the human population (**Table 3**) [28, 115–120]. The two mentioned antigenic determinants (Se and Le) are responsible for the presence of specific fucosyltransferases (FucT-II and III). A secretor (Se +) bears FucT-II and will build up (HM)OS-epitopes, such as 2'-FL, which carry a fucosylation in α1-2 position on the terminal galactose and a nonsecre-tors will not assemble such (HM)OS-epitopes (Table 3). Le encodes FucT-III, which catalyzes the addition of

fucose in α3 and α1-4 position on the subterminal GlcNAc. Experiments showed that other enzymes can compensate the α1-3 fucosylation at GlcNAc [27], but apparently only FucT-III is able to fucosylate GlcNAc in an α1-4 position. Le-dependent OS are normally found as components of glycosphingolipids and glycoproteins in red blood cells and other tissues and biological fluids such as saliva and plasma [121]. However, HM contains these OS in a free form. Based on the presence or absence of these specific HMOs, it is possible to classify HM into specific groups. **Table 3** lists and classifies the possible glycosidic linkages (fucosylation) based on Se and Le. Donors classified with HM type I are Se and Le positive and thus hold the genetic requirements for a "complete" HMOs equipment. Donors classified with HM type IV are Seas well as Le negative. Consequently, they do not have FucT-II and III and do not build up the respective fucosylated HMOs structures, i.e. HMOs with α1-2 fucosysated terminal galactose or a α1-4 fucosylated GlcNAc cannot be synthesized by donors with HM type IV. Accordingly, HMOs structures like 2'-FL (PN 3), LNFP II (PN 7), LNFP I (PN 9), LNDH I (PN 13), LNDH II (PN 14), 2'-fucosyl-Lacto-N-hexaose (PN 16), and 2,3-difucosyl-Lacto-N-hexaose (PN 17) are missing for HM type IV (**Table 2** and **Figure. 3 E**). Absence of the respective fucosylated HMOs structures could also be revealed within the HMOs fingerprints of HM type II (**Figure. 3 C**) and III (**Figure. 3 D**), while all expected fucosylated HMOs were found within the HM type I fingerprint (**Figure. 3 A**).

Furthermore, the high concentration of lactose (PN 1) in all four HMOs fingerprints (that can be explained by the extremely high lactose concentration from 53–61 g/L within the HM-TCF [122]) should be noted: by far this signal exceeds the linear dynamic range (above 9000 RFU) of the detector and consequently the lactose peak tip appears split-ted as can be seen in **Figure 3 C** and D (PN 1). But, in contrast to chromatographic methods, for electrophoretic techniques there is no effect whether lactose is within the sample at high concentrations or not. Almost no peak broadening in xCGE-LIF can be observed for the lactose peak compared to what has to be expected for chromatographic techniques. When using LC techniques for HMOs profiling, the lactose content of each sample has to be reduced [26, 27, 87, 108], to avoid column overloading leading to peak broadening and disturbed chromatograms. This superior loading capacity of electrophoretic techniques, like xCGE-LIF, is due to the fact, that electrophoresis is a volume-based separation technique, while adsorption/dispersion chromatography is surface based and thus provides only limited area for the adsorption/dispersion process. Another striking advantage of xCGE-LIF based HMOs analysis is the possibility to simultaneously analyze acidic and neutral HMOs, which can be allocated to its superior separation performance.

The comparative investigation of the TCF of different HM types showed, that not only the qualitative HMOs pool composition differs, but also the relative abundance

of specific HMOs can do so. The possibility to measure such quantitative changes within the HM-TCF composition is shown to be indispensable with respect to monitoring along the lactation time course [9, 28, 32]. Therefore as the final part of this initial work, HM samples from eight different LTP of one donor (HM VI, see **Table 1**) were analyzed to demonstrate the feasibility of this method to monitor changes within the HMOs composition during the lactation time course. The analyzed time points are summarized in **Table 1**. To confirm the results of the xCGE-LIF based HMOs analysis, the samples were additionally analyzed using HPAEC-PAD according to [27, 87]. HPAEC-PAD was used to validate the xCGE-LIF results, as this orthogonal method is the most accepted reference technique in the field of (HM)OS analysis (data not shown). As HPAEC-PAD is also routinely applied to quantify HMOs, a defined amount of stachyose had been already added to these samples before we started sample preparation (see Section 2). Stachyose is a tetraose and within an HPAEC-PAD chromatogram and there, shows no impact to HMOs peaks (data not shown). However, for the xCGE-LIF based HMOs analysis of these eight samples, it became evident that stachyose and the fucosyllactoses (PN 2 and 3) show almost identical migration times (about 47 MTU'). Thus, the sum peak of stachyose, 3-FL, and 2'-FL as shown in **Figure 4 A** (marked with St) was not further considered. As depicted in **Figure 4** and listed in **Table 4**, the individual changes within the HMOs pool composition along the lactation time course could easily be monitored. The relative PHP of 18 specific peaks over the lactation period are summarized in **Table 4**. Looking at this table, it is obvious that some of the HMOs change quite strongly in their relative PHP. Large quantitative changes can be seen, e.g. for PN 6, 9, 17 and in particular, for U5 that showed by far the biggest changes in its relative PHP (**Figure 4 B**). But there are also peaks remaining relatively constant (e.g. PN 5 and 12 - **Table 4** and **Figure 4B**), over the lactation period. At this time point, an explanation of these changes has to wait for further investigations, as up to now only few attempts were made to understand the quantitative changes of HMOs within (human) milk throughout the lactation time course and also regarding the elucidation of their functions. Despite the known importance of complex carbohydrates (like N-glycans, O-glycans, and free OS) in (human) milk [2,20,77,83,123–129], only a few publications are concerning this topic and were analyzing larger donor cohorts [22,27–32,39] therefore. One reason is probable, that up to now, there was no "real" HT analysis method available, that allows the measurement of thousands of samples within a couple of days [83,130].

Next task will be to enhance the initiated HMOs database (starting with structural elucidation of the unknown peaks (U1–U9)), to reach a sufficient number of entries. In combination the presented sample preparation, the HMOs database, the software glyXtoolTM [97], and the possibility to use instruments with up to 96 capillaries in parallel will empower the developed xCGE-LIF based method for HMOs analysis to conduct parallel measurements with an effective runtime <1 min for each sample

[131]. This HP and "real" HT glycoanalytical technique will enable large-scale studies, which will allow further insights into functional glycomics of free HMOs, digging deeper into the relation of mothers genotype, HMOs pool composition, and HM properties/effects as lately requested from Neville et al. [2].

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CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

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7

CHAPTER VIII

Presence and levels of galactosyllactoses and other oligosaccharides in human milk and their variation during lactation and according to human milk group

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ABSTRACT

Among the human milk oligosaccharides (HMOs), the galactosyllactoses (GLs) are only limitedly studied. This study aims to describe the presence and relative levels of HMOs, including GLs, in human milk (HM) according to HM group and lactation stage. Relative levels of 19 HMOs were measured in 715 HM samples collected in the first 4 months postpartum from 371 donors participating in the PreventCD study. From a subset of 24 Dutch women (171 HM samples), samples were collected monthly up to 12 months postpartum and were additionally analyzed for relative and absolute levels of β6'-GL, β3'-GL and α3'-GL. Most HMOs, including β6'- and β3'-GL, were present in the vast majority (≥75%) of HM samples, whereas others (e.g. LNDFH II, 2'-F-LNH and α3'-GL) only occurred in a low number (<25%) of samples. Clear differences were observed between the presence and relative levels of the HMOs according to HM group and lactation stage. Absolute concentrations of β6'-GL and β3'-GL were higher in HM group IV samples compared to samples of the other three HM groups. β3'-GL was also higher in HM group II samples compared to HM group I samples. β3'-GL and β6'-GL were stable over lactation stages. In conclusion, presence and levels of HMOs vary according to HM group and lactation stage. Not all HMOs behave similarly: some HMOs depend strongly on maternal genetics and/or lactation stage, whereas others do not. β3'-GL and β6'-GL were present in low concentrations in over 75% of the analyzed HM samples and showed differences between HM groups, but not between lactation stages.

Keywords

Human milk; oligosaccharides; HMOs; galactosyllactoses; lactation; human milk groups

INTRODUCTION

Human milk oligosaccharides (HMOs) are the third most abundant solid component in human milk (HM) after lactose and lipids, reaching between 5 and 20 g/L in mature HM [1]. The concentrations of oligosaccharides in HM vary within and between women and are influenced by various factors, including stage of lactation, parity, maternal diet, body mass index, ethnicity, socioeconomic status and genetic predisposition [2-10]. Maternal genetic predisposition, particularly the individual expression pattern of Secretor *(Se)* and Lewis *(Le)* blood group genes [2, 11-14], has a huge influence on the oligosaccharides profile of HM. The α1-2-fucosyltransferase gene (FUT2) is responsible for producing the *Se*-enzyme, which is necessary for generating α1-2 fucosylated HMOs, whereas the *Le* α1-3/4-fucosyltransferase gene (FUT3) is responsible for encoding the enzyme fucosyltransferase III (FucT-III), which is necessary for generating α1-4 fucosylated HMOs [10]. Depending on the activity of the *Se*- and *Le*-gene products (i.e. *Se*- and FucT-III-enzymes), four distinct HM types with specific and individual HMOs patterns, usually referred to as HM groups I-IV, can be characterized [4, 11, 15].

HMOs have been suggested to play an important role in healthy infants' development, ranging from reported effects on growth [16] and early life immune function [7] to effects on the gut microbiota [17] and intestinal functions [18]. HM is proposed to comprise more than 1000 individual and unique HMOs of which only approximately 200 are fully structurally characterized [19-24]. Yet, most research in the field of breastfeeding and HM has focused on about 20 HMOs, including the most abundant ones, like 2'-fucosyllactose (2'-FL), lacto-N-fucopentaose (LNFP) I, lacto-N-difucohexaose (LNDFH) I, and lacto-N-tetraose (LNT) [1]. Recently, the galactosyllactoses (GLs), a group of small oligosaccharides, have been identified in HM [25, 26]. GLs occur in form of several structurally distinct isomers, such as β3'- and β6'-galactosyllactose (β3'- and β6'-GL, respectively) which only differ in the glycosidic linkage of the terminal galactose. Although the GLs appear in low concentrations in HM, β3'- and β6'-GL have recently been reported to modulate major immunologic pathways of immature human intestinal cells [27] and to attenuate inflammation [25]. The anti-inflammatory effects of the GLs may be mediated via inhibition of toll like receptor 3 (TLR3) signaling [28]. Insights into the presences of the GLs in HM is still rare [29]. Moreover, little is known about the role of maternal expression pattern of *Se* and *Le* blood group genes and lactation stage on the levels of GLs.

This study aims to describe the presence and relative levels of HMOs in a large number of HM samples (n=715) collected in the first 4 months postpartum from 371 donors participating in the PreventCD study (www.preventCD.com). In order to substantiate evidence about the presence of the low-abundant GLs in HM,

relative and absolute levels of these HMOs were determined in a sample selection comprising 171 HM samples collected from 24 Dutch donors in the first year of infants' life (Dutch subset) using an adapted analytical approach. To better assess the impact of maternal genetics and lactation stage on the presence and levels of specific HMOs, the results are presented separately according to HM group and the stage of lactation.

MATERIALS AND METHODS

Human milk samples

The HM samples were collected from 371 mothers participating in the PreventCD study. The PreventCD study is a multicenter study aimed to assess the role of infant nutrition and also of genetic, immunologic and environmental factors, on the risk of developing coeliac disease (CD) and related autoimmune phenomena [30,31] (www.preventCD.com). Participating mothers were resident in Germany, Hungary, Italy, the Netherlands and Spain. Ethical approval for the study was obtained from the Leiden University Medical Centre (LUMC) on the 1st December 2006 (P06.177), as well as from the medical ethics committees of each participating center. An independent ethical advisor, Professor Moshe Berant (Helsinki Committee, Rambam Health Care Campus, Haifa, Israel), was appointed to provide guidance on ethical issues and decisions throughout the research period. An ethical sub-committee, chaired by the ethical advisor, monitored the project's management of documentation and reporting, and its safety issues. The study complied with Good Clinical Practice (ICH-GCP) guidelines and all participants signed an informed consent form. The full details of the study have been reported previously in Hogen Esch et al. [31] and Vriezinga et al. [30]. In brief, term-born infants at risk of developing CD were recruited up to the age of 3 months. Included infants had at least one first-degree family member with CD and were genotyped to be HLA-DQ2 or HLA-DQ8 positive. The infants were randomized to receive either daily 200 mg wheat gluten or placebo (200 mg/day lactose) between 4 and 6 months of age [31]. HM samples (10–30 mL) were collected on an explorative basis. Mothers were asked to express HM manually or by pump once a month, around the same day of each month [32]. For most women (n=347), samples were collected between 1 and 4 months postpartum. For a subset comprised of 24 Dutch women, HM samples were collected monthly up to 12 months postpartum. The collected HM samples were immediately frozen and then stored overnight at -20ºC in home freezers. Afterwards, the samples were transferred to the hospital on ice, where they were defrosted, homogenized and subsequently aliquoted in 1-2 mL portions and finally stored at -80ºC until analysis. For the HMOs analysis reported here, samples were transported on dry ice to the glycoanalytical laboratory (glyXera GmbH, Magdeburg, Germany). The PreventCD study was approved by the medical ethics committee at each participating center and complied with Good Clinical Practices (ICH-GCP) guidelines [30].

Analysis of human milk oligosaccharides

Relative peak areas (rPA) of HMOs were determined using highly sensitive multiplexed capillary-gel-electrophoresis with laser-induced-florescence detection (xCGE-LIF) [33].

In brief and in accordance with the glyXera GmbH kit protocol (KIT-glyX-Quant-DPV, glyXera GmbH, Magdeburg, Germany), the pure HM samples were diluted 1:100 and then spiked with an internal standard (IS) (oligosaccharide quantification standard solution, OS-A5-N-1mL-01, glyXera GmbH, Magdeburg, Germany). For this step, 2 µL of an IS solution and 3 µL of ultrapure water [34] were added to 20 µL of each diluted HM sample. Subsequently, the IS spiked samples were treated with denaturation solution (C-DeNatSol-100uL-01, glyXera GmbH, Magdeburg, Germany). Next, the free oligosaccharides within the denaturized HM/IS samples, comprising the free HMOs and IS, were labeled with APTS dye (KIT-glyX-OSP-A-96-01, glyXera GmbH, Magdeburg, Germany). Afterwards, the excess of APTS (not reacted) and salt were removed using hydrophilic interaction chromatography solid phase extraction (HILIC-SPE, in accordance with the glyXera GmbH kit protocol KIT-glyX-OS.H-APTS-96-01, glyXera GmbH, Magdeburg, Germany). In the next step, the purified APTS-labeled oligosaccharides (HILIC-SPE eluates) were analyzed via xCGE-LIF in accordance with the glyXera GmbH measurement kit protocol (KIT-glyX-OS.M-96-01, glyXera GmbH, Magdeburg, Germany). Finally, data processing, analysis, and evaluation of the HMOs Fingerprints (normalized electropherograms) were performed via glyXtool™ software (version 5.3.1, glyXera GmbH, Magdeburg, Germany), and data analysis and evaluation of the relative quantitative HMOs Fingerprints was performed via glyXtoolGUI™ software (Beta v0.4 [r166+], glyXera GmbH, Magdeburg, Germany).

The Limit of Quantification (LOQ) was determined using the signal-to-noise ratio (S/N) of each HMOs *Fingerprint* that was calculated according to Ullsten et al. [35]. The LOQ was defined at an S/N of 10. The respective noise within each sample was determined after the migration time alignment of the unsmoothed data in the late migration time range (approximation range = degree of polymerization (DP) 18<DP<20). Consequently, the LOQ is specific with regard to the sample and measurement. Presence and IS-normalized peak area of the HMOs were calculated if the detected signals were ≥LOQ.

For the low-abundant GLs (i.e. β6'-, β4'-, β3'-, and α3'-GL), which were of particular interest in this study, the relative and absolute levels were determined down to the Limit of Detection (LOD), defined at an S/N of 3. Absolute concentrations (*c*) were determined according to the following formula: *c* = IS-normalized peak height of the analyte × concentration of IS × dilution factor × response factor (KIT-glyX-Quant-DPV, glyXera GmbH, Magdeburg, Germany).

Selection of human milk oligosaccharides

A total of 200 valid peaks, synonymous with just as many HMOs, were detected in at least one of the 715 samples by xCGE-LIF. Following their abundance and/or suggested prominent role in infants' health [1, 25, 27], we focused on the following subset of HMOs: 2'-FL, 3-fucosyllactose (3-FL), difucosyllactose (DFL), LNT, lacto-N-neotetraose (LNnT), LNFP I, LNFP II, LNFP III, LNFP V, LNDFH I, LNDFH II, 3' and 6'-sialyllactose (3'- and 6'-SL), disialyllacto-N-tetraose (DSLNT), sialyllacto-N-tetraose (LST)a, LSTb, and LSTc, 3'-fucosyllacto-N-hexaose (3'-F-LNH), 2'-fucosyllacto-N-hexaose (2'-F-LNH), β1-3'-galactosyllactose (β3'-GL), β1-4'-galactosyllactose (β4'-GL), β1-6'-galactosyllactose (β6'-GL) and α1-3'-galactosyllactose (α3'-GL).

Yet, the HMOs *Fingerprints* demonstrated that β4'-GL was not discernible due to co-migration with 2'-FL and was, for this reason, excluded from the analysis. Additionally, co-migration was observed for LNFP I and LNFP V, and therefore these HMOs were analysed as the sum of both compounds, i.e. LNFP I+V. For the HM groups that contain LNFP I (i.e. HM groups I and III, Table 1), LNFP I+V is predominantly composed of LNFP I with a small contribution of LNFP V. For the HM groups that do not contain LNFP I (i.e. HM groups II and IV, Table 1), LNFP I+V is only composed of LNFP V. This selection ultimately led to a subset of 22 HMOs. Over all HM samples, the sum of the IS-normalized peak areas of this subset of HMOs, further referred to as 'selected HMOs', comprised a median (Q1-Q3) of 85.5% (82.6-87.8) of the IS-normalized total peak area of all valid HMOs peaks.

Table 1 | Identification of human milk (HM) groups I to IV based on the presence or absence of specific fucosylated HMOs (adapted from Oriol et al., 1986 [14])

HM group (Lewis Epitope)	Specific fucosylation		Prominent α1-2, α1-3 and α1-4 fucosylated HMOs
	α1-2 *(Se)*	α1-4 *(Le)*	
I (a-b+c-d-)[1]	+	+	2'-FL, 3-FL, DFL, LNFP I, LNFP II, LNFP III, LNDFH I and LNDFH II
II (a+b-c-d-)[1]	-	+	3-FL, LNFP II, LNFP III and LNDFH II
III (a-b-c-d+)[1]	+	-	2'-FL, 3-FL, DFL, LNFP I and LNFP III
IV (a-b-c+d-)[1]	-	-	3-FL and LNFP III

[1] According to Blank et al. [36]; α1-2 *(Se)*: The gene is responsible for encoding the *Se*-enzyme, which is necessary for generating α1-2 fucosylated HMOs; α1-4 *(Le)*: The gene is responsible for encoding the enzyme FucT-III, which is necessary for generating α1-4 fucosylated HMOs

Maternal Secretor- and Lewis type (HM group)

The HM samples were assigned to a maternal Secretor- and Lewis *(SeLe)* type (HM group) dependent on the presence or absence of specifically fucosylated HMOs [11, 33]. In HM group I *(Se+/Le+)*, HMOs with α1-2 fucosylation at the terminal

galactose (e.g. 2'-FL and LNFP I), as well as HMOs with α1-4 fucosylation at the N-acetylglucosamine (e.g. LNFP II and LNDFH II) are present. HM group II *(Se-/Le+)* contains HMOs with α1-4 fucosylation at N-acetylglucosamine, but HMOs with α1-2 fucosylation are missing, whereas HM group III *(Se+/Le-)* contains HMOs with α1-2 fucosylation at end terminal galactose but does not contain HMOs with α1-4 fucosylation at N-acetylglucosamine. In HM group IV *(Se-/Le-)*, all HMOs with α1-2 fucosylation and α1-4 fucosylation are lacking (**Table 1**).

Data presentation and statistical analysis

Data were checked for normality using the Shapiro-Wilk test and visual inspection of histograms. As data for nearly all HMOs were non-normally distributed, data are expressed as median (Q1-Q3) relative peak areas (rPA), representing the relative proportion that each HMOs contributed to the subset of selected HMOs (summed peak area of selected HMOs divided by the peak area of the IS), in which the selected HMOs (see section 2.3.) summed up to 100% (relative total peak area (rTPA)). Similarly, IS-normalized total peak area (nTPA) of selected HMOs is presented as median (Q1-Q3) and calculated as the sum of the selected IS-normalized HMOs signals (see section 2.3.). Data are presented for the subset of HM samples collected in the first 4 months postpartum (371 donors; in total 635 HM samples), and separately for the Dutch subset comprising longitudinally collected HM samples over the full first year postpartum (24 donors; in total 171 HM samples). The Mann-Whitney U-test was used to assess differences in medians between the different HM groups. Bonferroni-adjusted level of statistical significance was applied to control for multiple testing. A p-value <0.05 was considered statistically significant. All statistical analyses were performed with IBM® SPSS® Statistics version 26 (IBM Corp. Int., Armonk, New York, USA).

RESULTS

Human milk samples

Table 2 summarizes the number of HM samples at each donation time point for the 371 women who donated HM samples in the first 4 months postpartum, as well as for the subset of 24 Dutch women who donated HM samples up to 12 months postpartum. From the original set of samples, a total of 19 HM samples derived from six donors were excluded because of incongruent HM group typing (i.e. different HM groups assignment dependent on lactation stage) and an additional three samples derived from three donors were excluded because of missing data for lactation stage.

Human milk samples collected over the first 4 months of lactation

The next sections summarize the findings related to the subset of 635 HM samples collected in the first 4 months postpartum from 371 donors. In this dataset, the GLs were not determined resulting in a total of 19 selected HMOs.

Maternal Secretor- and Lewis type (HM group)

A total of 99.5% of the HM samples could be categorized into one of the four HM groups. The HM typing showed that 66.1% of the 635 HM samples corresponded to HM group I, 21.6% to HM group II, 8.3% to HM group III and 3.5% to HM group IV. The nTPA showed that the total abundance of HMOs was highest in HM samples assigned to HM group I, closely followed by those of HM group III and II. HM samples assigned to HM group IV had about 60% of the HMOs abundance of HM group I (**Table 3**).

Table 2 | Overview of the number of human milk (HM) samples collected at different time-points over the first 4 months (middle column) and for the subset of 24 Dutch women over the first 12 months (right column) of lactation.

Lactation month	First 4 months Number of HM samples	First 12 months (Dutch subset) Number of HM samples
1 month postpartum	240	21
2 months postpartum	92	24
3 months postpartum	54	23
4 months postpartum	249	23
5 months postpartum	-	23
6 months postpartum	-	21
7 months postpartum	-	12
8 months postpartum	-	9
9 months postpartum	-	7
10 months postpartum	-	4
11 months postpartum	-	2
12 months postpartum	-	2
Total	635 (371 donors)	171 (24 donors)

Presence of HMOs according to maternal Secretor- and Lewis type and lactation stage

Levels of 15 out of the 19 HMOs, i.e. 2′-FL, 3-FL, DFL, LNT, LNnT, LNFP I+V, LNFP II, LNFP III, 3′-SL, 6′-SL, DSLNT, LSTb, LSTc and 3′-F-LNH, were ≥LOQ in more than 75% of analyzed HM samples. Levels of LNT, LNFP I+V, LNFP III, 3′-SL, 6′-SL, DSLNT, LSTb and 3′-F-LNH were ≥LOQ in nearly all (>98%) samples (Table 4). In contrast, some HMOs occurred only in a low number of HM samples, i.e. LNDFH II and 2′-F-LNH were ≥LOQ in only 25.2% and 12.9% of samples respectively.

Following the assignment of the HM groups according to the presence or absence of specific fucosylated HMOs, clear differences were observed in the HMOs patterns occurring in the different HM groups (Table 4). Levels of 2′-FL and DFL were ≥LOQ

only in the vast majority of samples assigned to HM groups I and III, LNFP II level was ≥LOQ only in the vast majority of samples assigned to HM groups I and II, LNDFH I level was ≥LOQ only in the vast majority of samples assigned to HM group I and LNDFH II level was ≥LOQ only in the vast majority of samples assigned to HM group II (**Table 4**).

There were no clear trends in presence of HMOs according to lactation stage, with the exception of LSTa which showed a significant decrease in presence in the HM samples from 1 month postpartum (≥LOQ in 86% of samples) to 4 months postpartum (≥LOQ in 37% of samples).

Relative levels of HMOs according to maternal Secretor- and Lewis type

Figure 1 and **Table 3** present the median rPA of the selected HMOs in the HM samples according to HM group. As expected, clear differences in HMOs were observed between the four known different HM groups [4, 11, 15]. HM group I is particularly high in 2'-FL (median rPA: 23.2%) and LNDFH I (17.3%); HM group II is particularly high in 3-FL (28.4%), LNFP II (20.6%) and LNT (19.1%); HM group III is particularly high in 2'-FL (40.9%) and LNFP I+V (24.9%); and HM group IV is particularly high in LNT (51.8%) and LNFP III (14.3%) (**Figure 1** and **Table 3**).

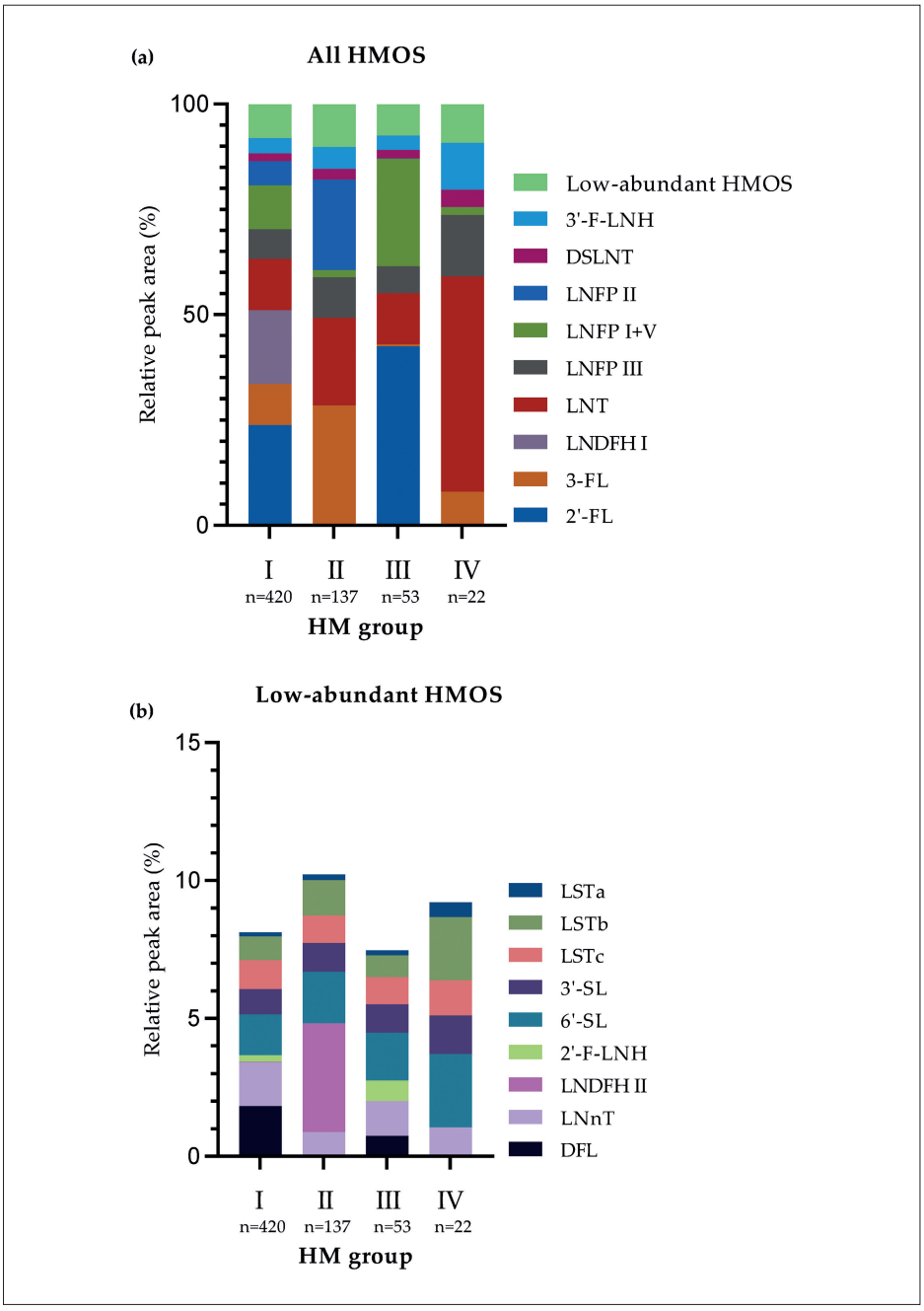

Figure 1 | Median relative peak areas (rPA) of all selected human milk oligosaccharides (HMOs) a) and of the subset of low-abundant HMOs b) in the human milk (HM) samples (n=635) collected in the first 4 months of lactation according to the HM groups [4, 11, 15]. Three samples could not be assigned to a HM group and are for this reason not depicted in these figures.

Table 3 | Total peak area of the selected human milk oligosaccharides (HMOs) (nTPA) and relative peak area (rPA, %) of the HMOs (median (Q1-Q3)) according to maternal human milk (HM) group [4, 11, 15] in HM samples (n=635) collected in the first 4 months postpartum from 371 donors. Three samples could not be assigned to a HM group and are therefore not depicted in this table.

	HM group I (n=420 samples, 241 donors) *median (Q1-Q3)*	HM group II (n=137 samples, 83 donors) *median (Q1-Q3)*	HM group III (n=53 samples, 32 donors) *median (Q1-Q3)*	HM group IV (n=22 samples, 14 donors) *median (Q1-Q3)*
nTPA[1]	12.3 (10.0-17.6)[a]	10.7 (8.33-14.1)[a,b]	10.9 (8.73-14.7)[a,b]	8.18 (5.75-10.5)[b]
rPA (%)				
2'-FL	23.2 (18.4-29.0)[a]	0.00 (0.00-0.00)[2 b]	40.9 (34.2-48.9)[c]	0.00 (0.00-0.00)[2 b]
3-FL	7.33 (3.70-13.8)[a]	28.4 (17.2-35.8)[b]	0.00 (0.00-0.00)[2 c]	6.25 (4.03-11.3)[a]
DFL	1.61 (1.13-2.20)[a]	0.00 (0.00-0.00)[2 b]	0.65 (0.33-1.08)[c]	0.00 (0.00-0.00)[2 b]
LNT	11.9 (9.18-14.6)[a]	19.1 (14.5-26.5)[b]	11.8 (9.10-13.8)[a]	51.8 (46.4-56.7)[c]
LNnT	1.44 (1.02-2.04)[a]	0.68 (0.45-1.16)[b]	1.10 (0.82-1.50)[c]	0.91 (0.45-1.44)[a,b,c]
LNFP I+V	9.69 (6.00-14.0)[a]	1.65 (1.39-1.97)[b]	24.9 (19.3-31.5)[c]	1.60 (1.32-2.36)[b]
LNFP II	5.13 (3.57-7.34)[a]	20.6 (17.6-25.4)[b]	0.00 (0.00-0.00)[2 c]	0.00 (0.00-0.00)[2 c]
LNFP III	6.81 (4.62-8.83)[a]	9.25 (7.30-11.3)[b]	6.44 (3.85-8.49)[a]	14.3 (9.58-18.0)[c]
LNDFH I	17.3 (14.9-20.2)[a]	0.00 (0.00-0.00)[2 b]	0.00 (0.00-0.00)[2 b]	0.00 (0.00-0.00)[2 b]
LNDFH II	0.00 (0.00-0.00)[2 a]	3.77 (2.84-4.88)[b]	0.00 (0.00-0.00)[2 a]	0.00 (0.00-0.00)[2 a]
3'-SL	0.89 (0.70-1.07)[a]	1.01 (0.82-1.25)[b]	0.94 (0.76-1.22)[a,b]	1.45 (1.09-1.63)[c]
6'-SL	1.22 (0.76-2.22)[a]	1.58 (0.92-2.67)[a,b]	1.31 (0.88-2.51)[a]	2.42 (1.80-3.45)[b]
DSLNT	1.71 (1.32-2.27)[a]	2.37 (1.69-3.13)[b]	1.94 (1.31-2.48)[a,b]	4.09 (3.22-5.05)[c]
LSTa	0.17 (0.00-0.26)[a]	0.21 (0.00-0.31)[a]	0.00 (0.00-0.32)[a]	0.47 (0.32-0.75)[b]
LSTb	0.80 (0.60-1.04)[a]	1.21 (1.03-1.49)[b]	0.75 (0.57-0.89)[a]	2.21 (1.89-2.75)[c]
LSTc	0.75 (0.42-1.37)[a]	0.75 (0.39-1.32)[a]	0.60 (0.45-1.42)[a]	1.01 (0.74-1.79)[a]
3'-F-LNH	3.42 (2.35-4.59)[a]	4.60 (2.71-7.18)[b]	2.65 (1.99-4.72)[a]	10.5 (8.48-11.3)[c]
2'-F-LNH	0.00 (0.00-0.00)[2 a]	0.00 (0.00-0.00)[2 a]	0.00 (0.00-1.20)[a]	0.00 (0.00-0.00)[2 a]

[1] Displayed as the internal standard (IS)-normalized total peak area of selected HMOs; [2] '0.00' does not necessarily mean that the respective HMOs is not present at any level, but it means that any amount of HMOs present is below the Limit of Quantification (LOQ); [a,b,c and d] Different superscript letters indicate statistical significance ($p<0.05$). Significance values have been adjusted by the Bonferroni correction for multiple tests.
Abbreviations used: 2'-FL: 2'-fucosyllactose; 3-FL: 3-fucosyllactose; DFL: difucosyllactose; LNT: lacto-N-tetraose; LNnT: lacto-N-neotetraose; LNFP I: lacto-N-fucopentaose I; LNFP II: lacto-N-fucopentaose II; LNFP III: lacto-N-fucopentaose III; LNFP V: lacto-N-fucopentaose V; LNDFH I: lacto-N-difucohexaose I; LNDFH II: lacto-N-difucohexaose II; 3'-SL: 3-sialyllactose; 6'-SL: 6-sialyllactose; DSLNT: disialyllacto-N-tetraose; LSTa: Sialyl-lacto-N-tetraose a; LSTb: Sialyl-lacto-N-tetraose b; LSTc: Sialyl-lacto-N-tetraose c; 2'-F-LNH: 2'- fucosyllacto-N-hexaose; 3'-F-LNH: 3'-fucosyllacto-N-hexaose

Table 4 | Percentage of human milk (HM) samples (n=635) in which the specific human milk oligosaccharide (HMOs) was detected (Presence, % of total) and relative peak area (rPA, %) of the selected HMOs (median (Q1-Q3)) according to maternal HM group [4, 11, 15]. HM samples were collected in the first 4 months postpartum from 371 donors. Three samples could not be assigned to a HM group and are therefore not depicted in this table.

	HM group I (n=420 samples, 241 donors)		HM group II (n=137 samples, 83 donors)		HM group III (n=53 samples, 32 donors)		HM group IV (n=22 samples, 14 donors)	
	Presence *% of total*[1]	rPA (%) *median (Q1-Q3)*	Presence *% of total*[1]	rPA (%) *median (Q1-Q3)*	Presence *% of total*[1]	rPA (%) *median (Q1-Q3)*	Presence *% of total*[1]	rPA (%) *median (Q1-Q3)*
2'-FL	100	23.2 (18.4-29.0)[a]	0	-	100	40.9 (34.2-48.9)[b]	0	-
3-FL	95	7.76 (4.17-14.5)[a]	100	28.4 (17.2-35.8)[b]	15	2.86 (2.47-4.18)[c]	100	6.25 (4.03-11.3)[a]
DFL	100	1.61 (1.13-2.20)[a]	4.4	0.44 (0.25-0.65)[b]	89	0.68 (0.54-1.15)[c]	4.5	1.29 (1.29-1.29)[b]
LNT	100	11.9 (9.20-14.6)[a]	99	19.3 (14.6-26.5)[b]	100	11.8 (9.10-13.8)[a]	100	51.8 (46.4-56.7)[c]
LNnT	99	1.45 (1.03-2.06)[a]	85	0.78 (0.54-1.26)[b]	98	1.10 (0.83-1.54)[c]	95	1.04 (0.45-1.50)[a,b,c]
LNFP II	100	5.14 (3.58-7.34)[a]	100	20.6 (17.6-25.4)[b]	3.8	0.18 (0.13- .)[2 c]	0	-
LNFP III	100	6.81 (4.62-8.83)[a]	100	9.25 (7.30-11.3)[b]	100	6.44 (3.85-8.49)[a]	100	14.3 (9.58-18.0)[c]
LNFP I+V	100	9.69 (6.00-14.0)[a]	100	1.65 (1.39-1.97)[b]	100	24.9 (19.3-31.5)[c]	100	1.60 (1.32-2.36)[b]
LNDFH I	100	17.4 (14.9-20.2)[a]	1.5	1.44 (0.043- .)[2 b]	3.8	0.59 (0.46- .)[2 b]	0	-
LNDFH II	2.1	1.25 (0.99-1.59)[a]	100	3.77 (2.84-4.88)[b]	0	-	0	-
3'-SL	100	0.89 (0.70-1.07)[a]	100	1.00 (0.82-1.25)[b]	98	0.95 (0.77-1.22)[a,b]	100	1.45 (1.09-1.63)[c]
6'-SL	100	1.22 (0.76-2.23)[a]	100	1.58 (0.92-2.67)[a,b]	98	1.35 (0.89-2.52)[a]	100	2.42 (1.80-3.45)[b]
DSLNT	100	1.71 (1.33-2.27)[a]	99	2.37 (1.72-3.14)[b]	98	1.94 (1.33-2.48)[a,b]	100	4.09 (3.22-5.05)[c]
LSTa	60	0.24 (0.18-0.30)[a]	58	0.29 (0.23-0.44)[a]	45	0.33 (0.27-0.45)[a]	95	0.48 (0.34-0.78)[b]
LSTb	99	0.80 (0.61-1.04)[a]	99	1.21 (1.04-1.50)[b]	92	0.76 (0.62-0.93)[a]	100	2.21 (1.89-2.75)[c]
LSTc	96	0.79 (0.46-1.42)[a]	93	0.84 (0.42-1.33)[a]	92	0.71 (0.47-1.71)[a]	95	1.02 (0.76-1.82)[a]
3'-F-LNH	98	3.46 (2.40-4.61)[a]	99	4.63 (2.77-7.18)[b]	98	2.71 (2.05-4.76)[a]	100	10.5 (8.48-11.3)[c]
2'-F-LNH	12	1.72 (1.17-2.45)[a]	0	-	36	1.69 (1.04-3.19)[a]	0	-

[1] Percentage of HM samples in which the specific HMOs was detected (≥Limit of Quantification (LOQ)); [2] In only two samples, the respective HMOs was present and hence Q3 could not be calculated; [a,b,c] and [d] superscript letters indicate statistical significance ($p<0.05$). Significance values have been adjusted by the Bonferroni correction for multiple tests.Abbreviations used: see Table 3

Relative levels of HMOs according to lactation stage
The nTPA showed that the total levels of the HMOs decreased to less than half during the first 4 months of lactation.

Figure 2 presents the median rPA of the selected HMOs in the HM samples according to lactation stage. The results show that the rPA of 3-FL, DFL, LNFP III and 3'-SL increased over lactation, whereas the rPA of LNT, LNFP I+V, 6'-SL, DSLNT, LSTa, LSTc, 3'-F-LNH and 2'-F-LNH decreased. Other HMOs like 2'-FL, LNnT, LNFP II, LNDFH I, LNDFH II and LSTb were stable (i.e. no consistent in- or decreasing trends and/or <10% in- or decrease compared to previous lactation month(s)) during the first 4 months of lactation. Similar findings were observed when samples were distinguished according to HM group (**Figure 3**).

Human milk samples collected over the first 12 months of lactation (Dutch subset)
The next sections summarize the findings related to the subset of HM samples (n=171) collected longitudinally during the first year postpartum from 24 Dutch donors (Dutch subset; **Table 2**). In this subset, also relative and absolute levels of the low-abundant GLs, β6'-GL, β3'-GL and α3'-GL, were determined.

Presence of HMOs according to maternal Secretor- and Lewis type and lactation stage
HMOs presences (≥LOQ) according to HM group in the Dutch subset of HM samples collected longitudinally during the first year postpartum were very similar to the samples collected in the first 4 months postpartum. For the GLs, results show that β6'-GL, β3'-GL and α3'-GL were detected (≥LOD) in 100%, 82% and only 1% of HM samples, respectively. β3'-GL was detected in nearly all (≥95%) samples of HM groups II and IV, compared to presences in 83% and 73% of samples assigned to HM group I and III, respectively (**Supplemental Tables 1 and 2**).

Interestingly, although no clear in- or decreasing trends in the presence (≥LOQ) of LSTc was found during the first 4 months, after these first months LSTc presence decreased from about 90% to 20% at month 8.

Relative levels of HMOs according to maternal Secretor- and Lewis type and lactation stage
Median rPA of the selected HMOs according to HM group in the Dutch subset of HM samples collected during the first year postpartum were very similar to the samples collected in the first 4 months of lactation.

β3'-GL and β6'-GL appeared in low rPA, with significantly higher levels in samples assigned to HM group IV (β3'-GL (median rPA): 0.42%; β6'-GL: 1.07%) compared to HM group I (β3'-GL: 0.074%; β6'-GL: 0.36%), group II (β3'-GL: 0.10%; β6'-GL: 0.52%), and group III (β3'-GL: 0.11%; β6'-GL: 0.42%). rPA of β6'-GL was also significantly higher in samples assigned to HM group II compared to HM group I (**Supplemental Table 1**).

Only 2 out of 171 HM samples (1.2%) derived from one donor at lactation month 8 and 9 showed levels of α3'-GL ≥LOD.

Figure 4 presents the median rPA of the selected HMOs in the HM samples per month during the first year postpartum. The increasing trends in rPA observed for 3-FL, DFL, LNFP III and 3'-SL, and as well as the decreasing trends observed for LNT, LNFP I+V, 6'-SL, LSTa, LSTc, 3'-F-LNH and 2'-F-LNH in the first 4 months of lactation, continued in subsequent postpartum months (**Figure 4**). In contrast, after the decline in rPA observed in the first 4 months, DSLNT slightly increased after this period. rPA of other HMOs, including β3'-GL and β6'-GL, remained stable over lactation.

nTPA showed that, following the initial decrease in the total amount of selected HMOs to about half in the first 4 months of lactation, HMOs amounts remained largely stable until the end of sample collection (month 12). It should be noted that the results from postpartum months 8 to 12 are based on samples from <10 participants.

Absolute concentrations of GLs according to maternal Secretor- and Lewis type and lactation stage

Median absolute concentrations of the GLs, analyzed in the subset of 171 HM samples, were 2.42 mg/L and 8.04 mg/L for β3'-GL and β6'-GL, respectively.

Absolute concentrations of β3'-GL were higher in HM samples assigned to HM group IV (5.48 mg/L) compared to samples assigned to HM group I (2.12 mg/L), HM group II (3.16 mg/L) and HM group III (2.21 mg/L) ($p<0.05$ for all). Additionally, samples of HM group II had higher absolute concentrations of β3'-GL compared to samples of HM group I ($p<0.001$).

The absolute concentration of β6'-GL was also higher in samples assigned to HM group IV (14.7 mg/L) compared to HM groups I (7.62 mg/L) and II (8.30 mg/L) ($p<0.05$ for all) (**Figure 5**). Absolute levels of β3'-GL and β6'-GL remained stable over lactation stages (**Figure 6**).

Only 2 out of 171 HM samples (1.2%) derived from one donor at month 8 and 9 showed absolute levels of α3'-GL ≥LOD. The α3'-GL concentrations in those samples were 3.35 mg/L and 7.74 mg/L, respectively.

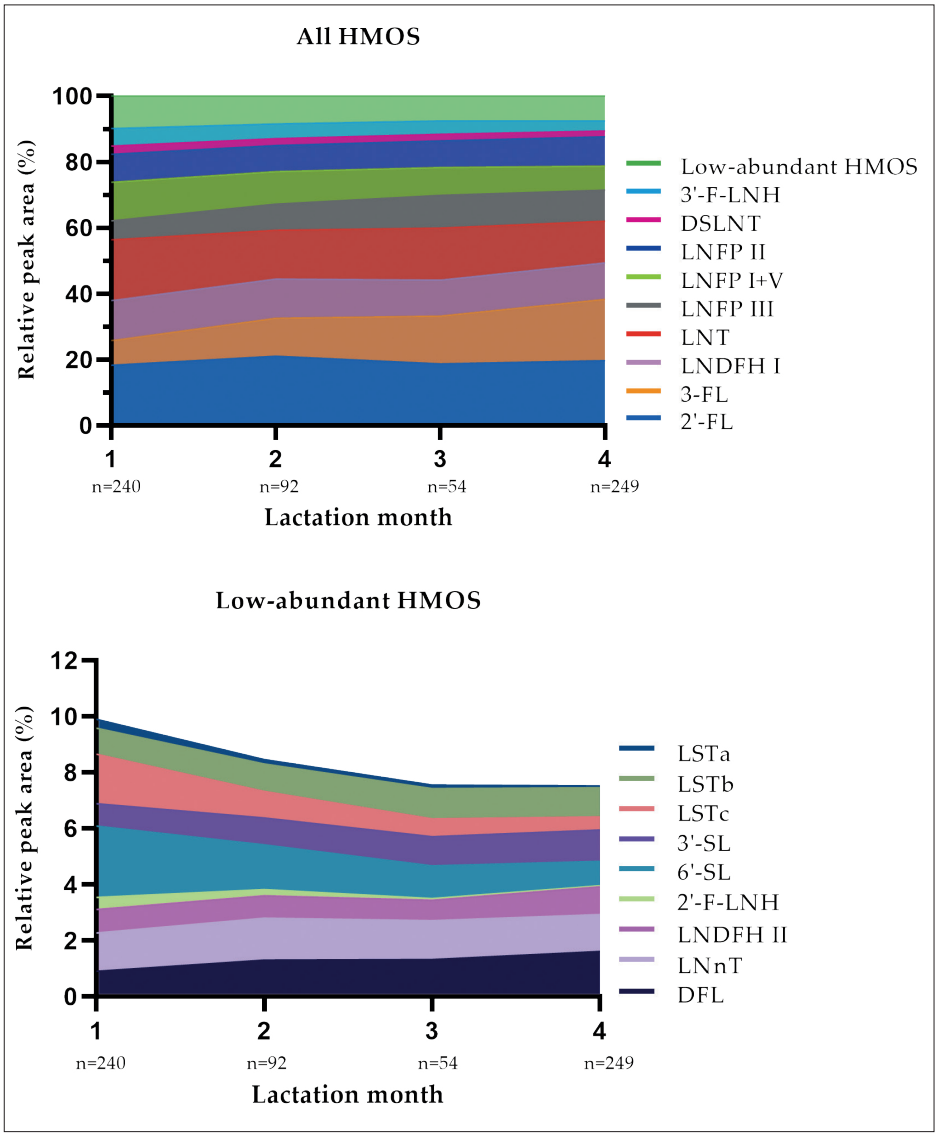

Figure 2 | Median relative peak areas (rPA) of all selected human milk oligosaccharides (HMOs) a) and of the subset of low-abundant HMOs b) in the human milk samples (n=635) according to lactation stage.

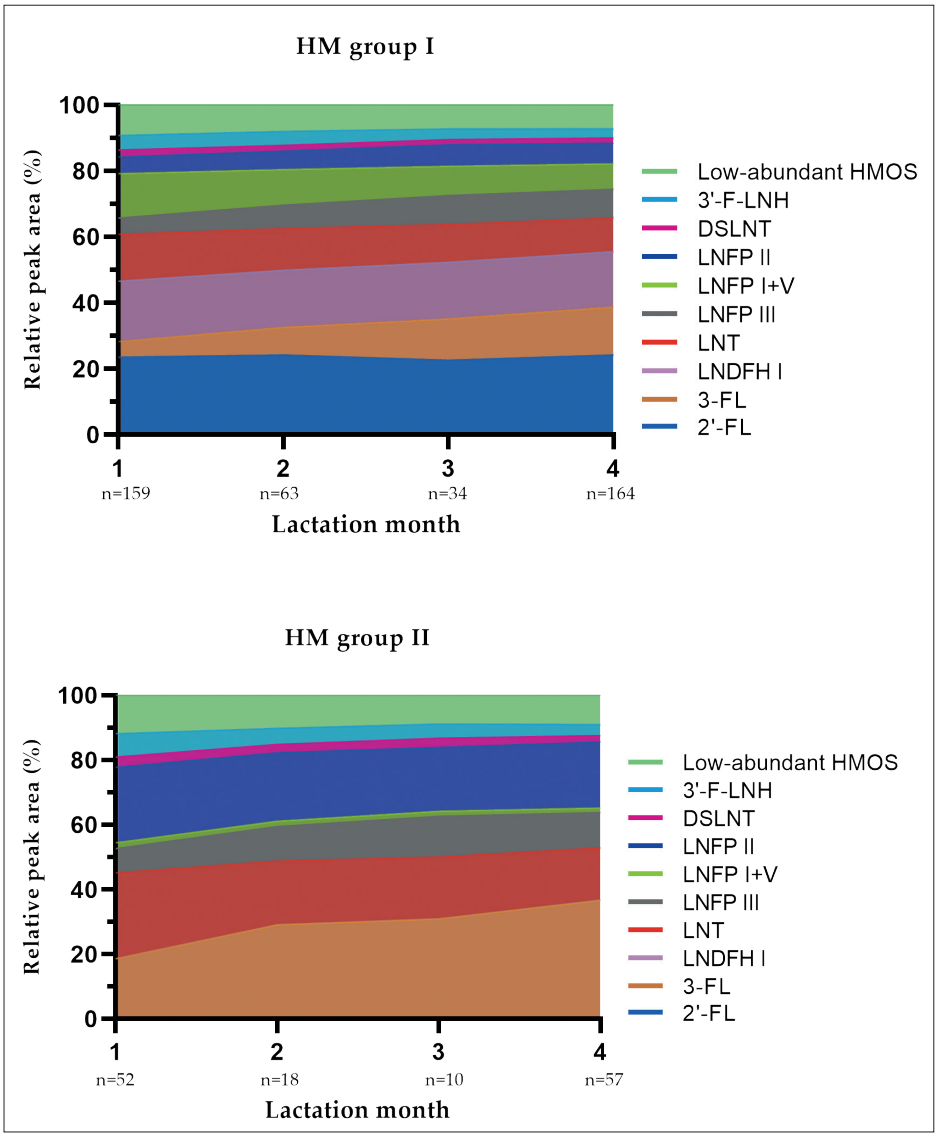

Figure 3 | Median relative peak areas (rPA) of all selected human milk oligosaccharides (HMOs) in the human milk (HM) samples (n=635) collected in the first 4 months of lactation according to HM group [4, 11, 15] and lactation stage. a) HM group I; b): HM group II; c): HM group III and d): HM group IV. Three samples could not be assigned to a HM group and are for this reason not depicted in these figures.

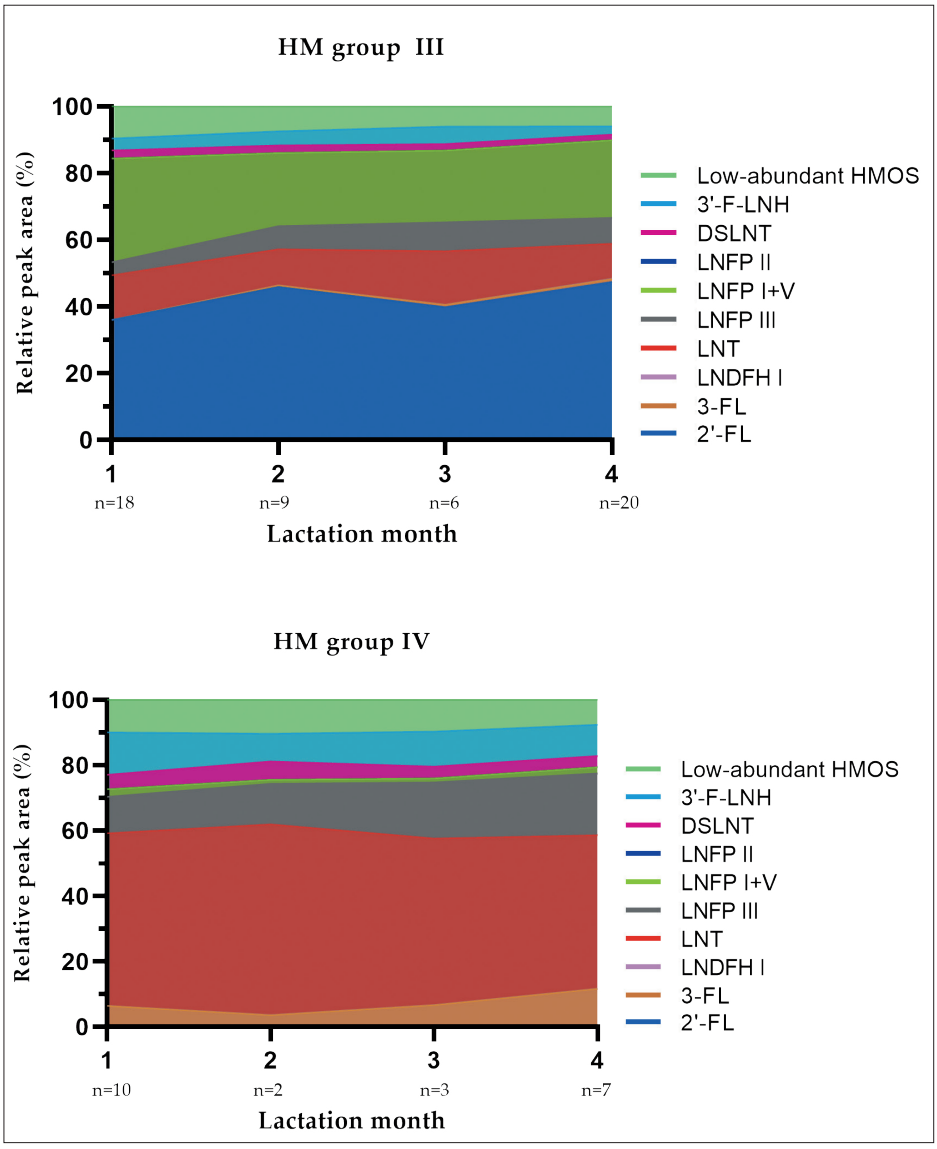

Figure 3 | Continued.

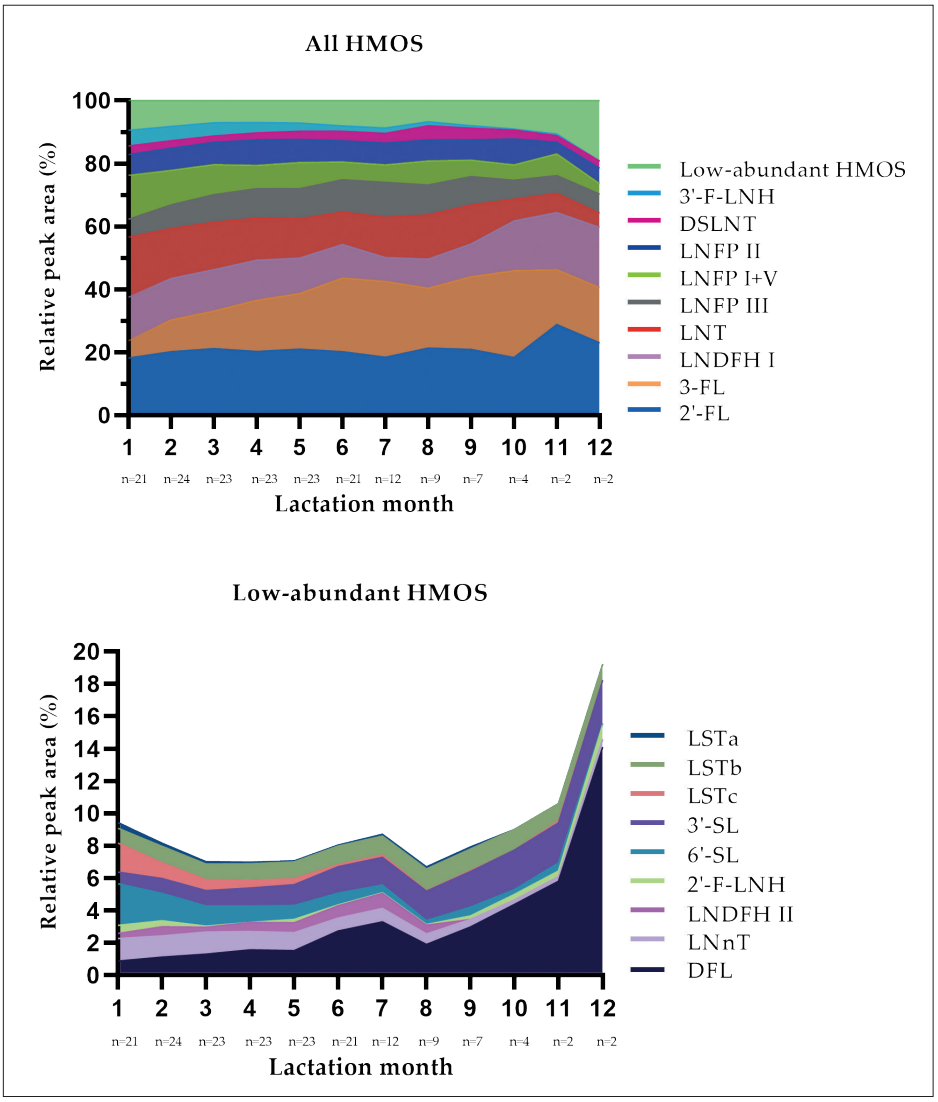

Figure 4 | Median relative peak areas (rPA) of all selected human milk oligosaccharides (HMOs) (a) and of the subset of low-abundant HMOs (b) in the human milk (HM) samples (n=171, Dutch subset) collected in the first year postpartum according to lactation month. Symbols used: ⬚, results from postpartum months 8 to 12 are based on samples from <10 participants and should be interpreted with caution

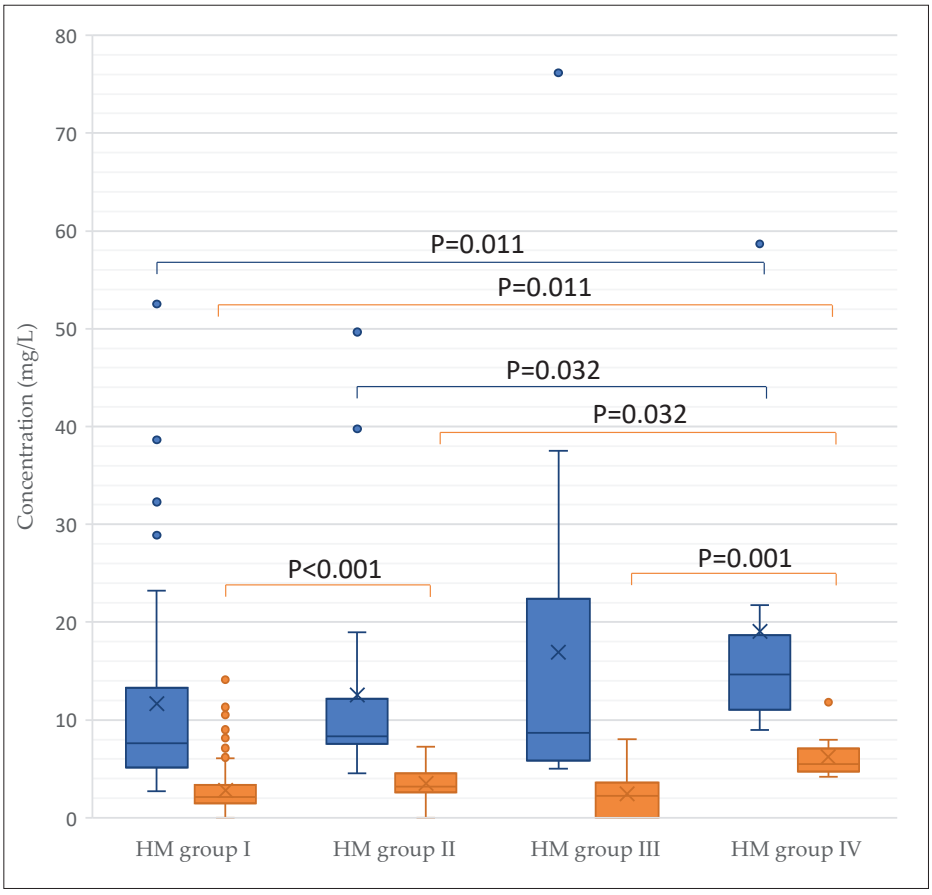

Figure 5 | Absolute concentrations of β1-6'-galactosyllactose (β6'-GL) (■) and β1-3'-galactosyllactose (β3'-GL) (■) in the human milk (HM) samples in the first year of lactation according to HM group [4, 11, 15]. One sample assigned to HM group I with a β6'-GL concentration of 210.1 mg/L was not included in the figure for display purposes but was included in the statistical analysis. Significance values have been adjusted by the Bonferroni correction for multiple tests. A p-value <0.05 was considered statistically significant.

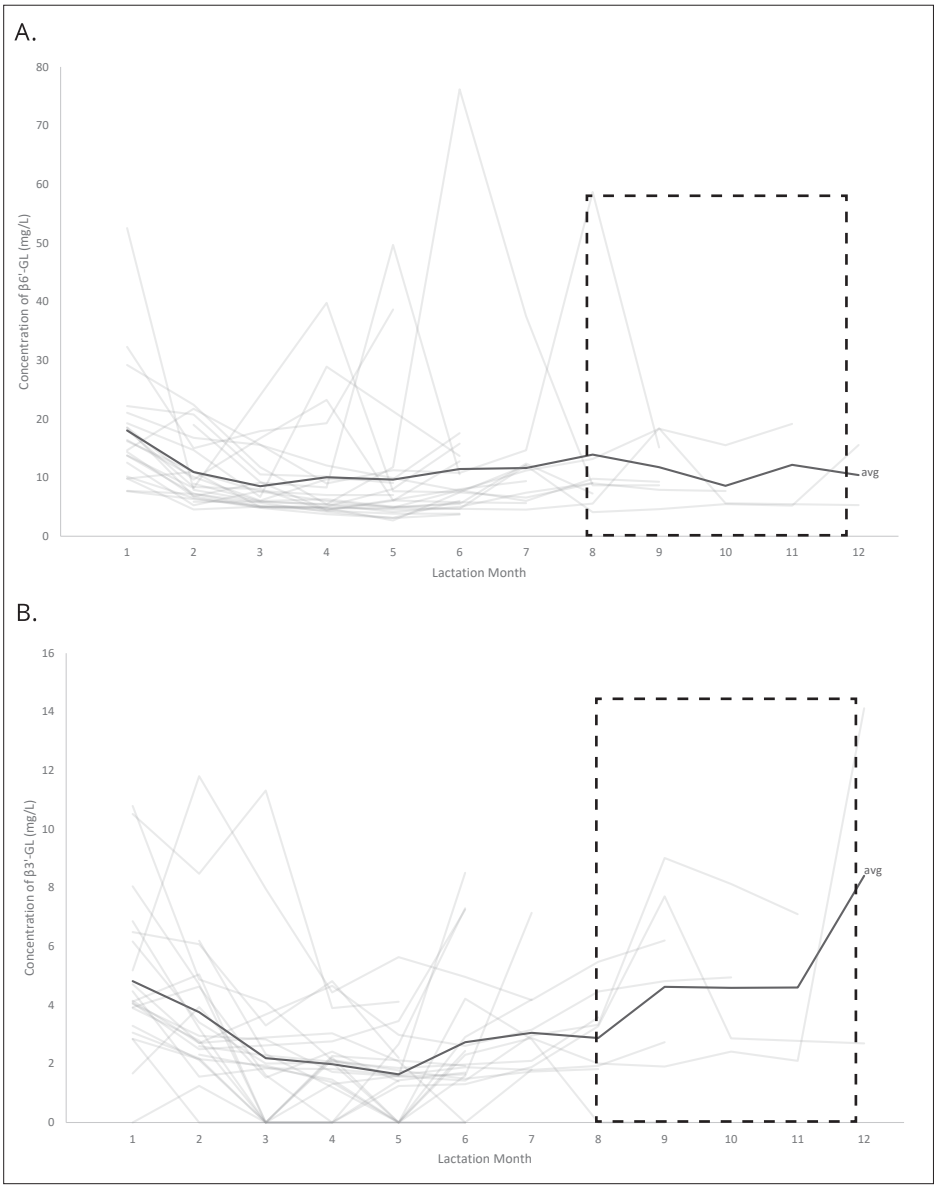

Figure 6 | Absolute concentrations of β1-6'-galactosyllactose (β6'-GL) (a) and β1-3'-Galactosyllactose (β3'-GL) (b) in the human milk (HM) samples throughout the first year of lactation according to lactation month.
One HM group I sample collected at lactation month 5 with a determined β6'-GL concentration of 210.1 mg/L was not included in the figure for display purposes. Abbreviation/symbols used: avg, average; ⊏ ⊐, results from postpartum months 8 to 12 are based on samples from <10 participants and should be interpreted with caution.

DISCUSSION

The World Health Organization (WHO) recommends HM as the sole source of nutrition for infants during their first 6 months of life, and continued breastfeeding up to two years and beyond [37]. The composition of HM adapts to the changing needs of the infant to provide the newborn with appropriate nutrients and bioactive components for each stage of growth [38]. HMOs are one of the key components of HM, contributing to a healthy growth and development of the infant, including its immune system and gut microbiota [7, 16, 17].

In this work, we report the presence and relative levels of HMOs in a large set of HM samples (n=715) collected from 371 mothers with a term-born infant at risk of developing CD residing in Germany, Hungary, Italy, the Netherlands or Spain over the first 4 months postpartum and a subset of 24 Dutch women over the first year postpartum (**Table 2**). To the best of our knowledge, our study is one of the largest studies to date describing relative levels of a high number of HMOs and collecting HM samples on a monthly basis. Our results show that the composition of HM with regards to HMOs is highly variable according to maternal *SeLe* type, i.e. the four HM groups. Yet not all HMOs behave similarly. 2'-FL, DFL, LNFP I, LNFP II and LNDFH I and II are examples of HMOs which presence in HM is heavily dependent on maternal genetics, whereas the sialyllactoses (3'- and 6'-SL) and the Sialyl-lacto-N-tetraoses (LSTa, LSTb, and LSTc) are very similar across the different HM groups. This finding is in line with previous studies [2-4, 10, 15].

A total of 99.5% of the HM samples could be categorized into one of the four HM groups. Most HM samples were categorized as HM group I (66.1%), while 21.6% were assigned as HM group II, 8.3% as HM group III and 3.5% as HM group IV. This is nicely in line with the distribution of *Se* and *Le* polymorphisms as reported by Oriol et al. (HM group I: 69%; HM group II: 20%; HM group III: 9% and HM group IV: 1%) [14]. Three HM samples (0.5% of all samples) could not be grouped into one of the four HM groups. These HM samples had in common the absence or very low relative levels of 2'-FL, DFL and LNFP I(+V), whereas – in contrast to expectations – LNDFH I was abundantly present. LNDFH I is an α1-2-fucosylated HMOs and its presence indicates a functional FUT2 allele. The finding that other α1-2-fucosylated HMOs like 2'-FL, DFL and LNFP I(+V) were absent despite FUT2 activity is unusual. A plausible explanation of this phenomenon could be the presence and activity of another α1-2-fucosyltransferase different from the well know *Se*-enzyme. The *Se*-enzyme may catalyse synthesis of 2'-FL from lactose, DFL from 3'-FL and LNFP I from LNT [29]. A hypothetic additional novel α1-2-fucosyltransferase which could be the product of a currently unknown FUT2 polymorphism may catalyse the synthesis of LNDFH I from LNFP II. This synthesis may occur in competition with or independent from the *Se*-enzyme. Hence, even if the *Se*-enzyme is inactive or only expressed in low

levels, the novel α1-2-fucosyltransferase might still catalyse formation of LNDFH I from LNFP II, whereas 2'-FL, DFL and LNFP I might not be present or only found in low levels. As the detection of LNDFH I in the discussed three HM samples still indicates presence of α1-2-fucosylated HMOs (but in an unusual pattern compared to regular HM group I specimens), but on the other hand also FucT-III-enzyme dependent HMOs (e.g. 3-FL, LNFP II, LNDFH II) are found, this finding could indicate the existence of a possible subgroup of HM group I. This new hypothetical HM group might be different from the hypothetical HM group Ia as recently postulated by Mank et al. [29] and therefore could be named HM group Ib. The existence of this FUT2 polymorphism and its influence on the HMOs patterns and HM groups needs further confirmation by analyses of oligosaccharides in HM from cohorts with high numbers of subjects and maybe different between geographies and ethnicities.

In contrast to earlier findings showing that LNDFH II is present in both HM groups I and II [10], we found that only 2.1% of the analyzed HM samples in group I showed LNDFH II (≥LOQ) with a median rPA of 1.25% (Table 4). So far, not many studies have reported on LNDFH II presence (≥LOD) and/or levels (≥LOQ), and therefore we cannot speculate as to whether it is a spurious result caused by a methodological reason, or a true finding. Yet, the fact that in the first 4 months postpartum, 100% of the samples of HM group II contain LNDFH II advocates for the latter.

Besides maternal genetic predisposition linked to the expression pattern of *Se* and *Le* blood group genes, total amounts of HMOs also varied according to lactation stage. In line with others (e.g. [3-5, 39, 40]), we showed that the nTPA of selected HMOs dropped sharply to about half in the first 4 months, and thereafter remained largely stable until the end of the first year postpartum. Our group recently showed similar results in a large number of samples (n=1203) from a birth cohort study conducted in South Germany [40]. In that study, we found that - in line with the decrease in total HMOs amounts - absolute concentrations of most individual HMOs decreased from 6 weeks to 6 and 12 months postpartum. The added value of expressing the changes in individual HMOs as relative values is that these data are not obscured by the changes in the total amount of oligosaccharides present in HM. For example, it has been shown previously that absolute concentrations of 2'-FL and LNnT are continuously decreasing over the first months postpartum [4, 16, 40], whereas we here show that relative levels of these HMOs are very stable during this period (Figures 1-3). In line with the increasing trends in absolute levels of 3-FL, 3'-SL and DFL over the first 6 to 12 months postpartum observed by Siziba et al. [40], we found increased relative levels of these HMOs (**Figures 1-3**).

In addition to the relative quantification of the 22 HMOs, we describe also absolute concentrations of the GLs for a subset of 171 HM samples from 24 Dutch donors. The median concentrations of β3'-GL and β6'-GL were 2.42 mg/L and 8.04 mg/L

respectively, i.e. in agreement with previous findings [26]. Absolute concentrations of β6'-GL and β3'-GL were higher in HM group IV samples compared to samples of the other three HM groups. β3'-GL was also higher in HM group II samples compared to HM group I samples. Only 2 out of 171 HM samples (1.2%) derived from one donor at month 8 and 9 showed levels of α3'-GL ≥LOD. Although the GLs have been suggested to have key roles in inflammation and immune functions, the median concentrations of the GLs are much lower than those of many other HMOs, including 2'-FL, 3-FL, LNT, LNnT, and LNFP I, II and III. Yet, as for other bioactive substances, the nutritional value and bioactivity of HMOs may not depend on the dose. Indeed, studies by He et al. [27] and Newberg et al. [25] have pointed towards the involvement of GLs in major immunologic pathways and inflammation attenuation. The relevance of GLs for infant growth and development remains the subject of further research.

Strengths of this study include the large number of HM samples collected from various centers in five countries (Germany, Hungary, Italy, the Netherlands and Spain) and the longitudinal assessment of HMOs covering the full first year postpartum. Moreover, our high-sensitive and high-throughput method of analysis (xCGE-LIF) allowed for the relative quantification of more than 200 HMOs, including the up to now little-studied GLs, as well as a large number of HMOs that have not yet been fully structurally characterized.

To the best of our knowledge, our method is unique in its ability to separate over 200 different HMOs on the basis of their size, charge and shape, with only one single and simple sample preparation and one measurement. The present work focused on 22 abundant and/or prominent HMOs, comprising about 85% of the total amount of oligosaccharides in HM over all HM groups. Yet, future efforts should focus on the full characterization of the remaining HMOs.

Despite the extremely high peak resolution and peak capacity of the xCGE-LIF-based analysis, it was still not possible to baseline separate all HMOs. Some specific HMOs (e.g. β4'-GL and LNFP I and LNFP V in some *Se* positive donors) were not automatically discernible due to partially co-migrating or overlapping peaks. In the future, a further developed automated data analysis will enable the *in-silico* separation of these peaks. Other limitations of our study are the low number of samples collected from 8 months postpartum and onwards resulting from mother's decision to stop breastfeeding. Moreover, although our study included a large number of HM samples, only a small fraction (3.5%) of samples was assigned to HM group IV due to the low frequency of this particular HM group in the general population, contributing to a lower accuracy of the data in this group. Additionally, the unequal sample sizes could have affected statistical power and Type I error rates [41]. Further, for the majority of HMOs no absolute concentrations were measured, limiting the ability to directly compare our findings to previously published data.

Moreover, by definition, quantitative results for the HMOs (with the exception of the GLs) were provided for HM samples with HMOs intensities at or above the LOQ. As a consequence, a (low) number of additional HM samples may contain specific HMOs (≥LOD, but <LOQ) which have not been considered because HMOs levels in these samples were <LOQ, and therefore their rPA was set to 0.00.

Important areas for future research include investigations of the impact of maternal diet, ethnicity and country of origin, and other maternal or infant characteristics, including the presence of the HLA-DQ2 and/or HLA-DQ8 genotype, on HMOs abundance, regional variations in HMOs abundance and further exploration of the impact of HMOs on infants' growth and development. Also potential differences in HMOs concentrations driven by maternal phenotype *within* the HM groups as recently described by Elwakiel et al. [3] need further investigation.

CONCLUSIONS

In conclusion, this study displays the presence and relative levels of HMOs in a large number of HM samples collected in five countries (Germany, Hungary, Italy, the Netherlands and Spain) over the first year postpartum and highlights the large intra- and inter-individual variability according to maternal *SeLe* type and lactation stage. Not all HMOs behave similarly: some HMOs are heavily dependent on maternal genetics (e.g. 2'-FL, LNFP I(+V) and LNDFH I) or lactation stage (e.g. 3-FL, 6'-SL and DFL), whereas other HMOs were very similar across the different HM groups (e.g. 3'- and 6'-SL or LST a/b/c) or lactation stages (e.g. 2'-FL, LNnT and LNDFH I). β3'-GL and β6'-GL were – in low concentrations – present in more than 75% of analyzed HM samples and showed differences between HM groups dependent on maternal genetics, but not between lactation stages.

Further research on the impact and correlation of the presence and levels of individual HMOs on the healthy development of infants is needed.

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CONFLICTS OF INTEREST

S.R.B.M.E., M.M. and B.S. are employees of Danone Nutricia Research, Utrecht, The Netherlands. R.K., X.K.H., A.B. and E.R. are employees of glyXera GmbH, Magdeburg, Germany. M.L.M. and B.K. declare no conflict of interest with respect to the contents of this manuscript.

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SUPPLEMENTS

Table S1 | Total peak area of the selected human milk oligosaccharides (HMOs) (nTPA) and relative peak area (rPA, %) of the HMOs (median (Q1-Q3)) according to maternal human milk (HM) group [4, 11, 15] in HM samples (n=171) collected longitudinally over the first year postpartum from 24 Dutch mothers (Dutch subset)

	HM group I (n=126 samples, 18 donors) *median (Q1-Q3)*	HM group II (n=21 samples, 3 donors) *median (Q1-Q3)*	HM group III (n=15 samples, 2 donors) *median (Q1-Q3)*	HM group IV (n=9 samples, 1 donor) *median (Q1-Q3)*
nTPA[1]	11.4 (9.33-13.4)[a]	9.30 (7.53-10.8)[b]	9.95 (8.51-10.7)[a,b]	6.22 (5.27-9.83)[b]
rPA (%)				
2'-FL	22.2 (15.0-29.5)[a]	0.00 (0.00-0.00)[2 b]	42.0 (35.5-53.0)[c]	0.00 (0.00-0.00)[2 b]
3-FL	11.7 (5.53-21.4)[a]	35.9 (26.4-45.3)[b]	0.00 (0.00-3.38)[c]	6.78 (3.04-7.31)[d]
DFL	1.86 (1.29-2.50)[a]	0.00 (0.00-0.00)[2 b]	1.01 (0.62-1.48)[a]	0.00 (0.00-0.00)[2 b]
LNT	10.6 (7.87-13.1)[a]	15.3 (11.3-20.6)[b]	9.77 (6.50-13.8)[a]	51.8 (47.8-59.3)[c]
LNnT	0.98 (0.67-1.48)[a]	0.66 (0.00-0.91)[b]	1.21 (0.83-1.78)[a]	1.35 (0.94-1.89)[a,b]
LNFP I+V	7.56 (3.29-12.5)[a]	1.51 (1.36-1.63)[2 b]	18.8 (15.6-21.5)[c]	1.29 (1.16-1.55)[2 b]
LNFP II	5.79 (3.31-9.01)[a]	19.7 (17.1-21.2)[b]	0.00 (0.00-0.00)[2 c]	0.00 (0.00-0.00)[2 c]
LNFP III	7.52 (5.66-9.12)[a]	9.28 (8.06-10.6)[b]	8.94 (6.99-12.3)[a,b]	15.6 (10.1-18.4)[b]
LNDFH I	16.3 (13.5-19.1)[a]	0.00 (0.00-0.00)[2 b]	0.00 (0.00-0.00)[2 b]	0.00 (0.00-0.00)[2 b]
LNDFH II	0.00 (0.00-0.00)[2 a]	3.50 (2.87-4.16)[b]	0.00 (0.00-0.00)[2 a]	0.00 (0.00-0.00)[2 a]
3'-SL	1.08 (0.78-1.43)[a]	1.37 (1.04-1.91)[b]	1.18 (1.04-2.15)[a,b]	1.76 (1.05-2.21)[c]
6'-SL	0.83 (0.47-1.64)[a]	0.70 (0.50-1.64)[a]	0.91 (0.37-1.62)[a]	1.74 (0.66-2.71)[a]
DSLNT	1.97 (1.40-2.47)[a]	3.20 (2.86-3.71)[b]	2.95 (1.94-4.10)[a,b]	5.53 (5.00-8.83)[c]
LSTa	0.00 (0.00-0.20)[a]	0.00 (0.00-0.21)[a]	0.00 (0.00-0.21)[a]	0.78 (0.75-0.93)[b]
LSTb	0.92 (0.68-1.08)[a]	1.43 (1.29-1.89)[b]	0.64 (0.51-0.98)[a]	2.36 (2.18-2.72)[c]

Table S1 | Continued.

	HM group I (n=126 samples, 18 donors) *median (Q1-Q3)*	HM group II (n=21 samples, 3 donors) *median (Q1-Q3)*	HM group III (n=15 samples, 2 donors) *median (Q1-Q3)*	HM group IV (n=9 samples, 1 donor) *median (Q1-Q3)*
LSTc	0.34 (0.00-0.84)[a]	0.28 (0.00-0.77)[a]	0.26 (0.00-0.57)[a]	0.99 (0.27-1.60)[a]
3'-F-LNH	2.38 (1.42-3.85)[a]	3.65 (0.76-6.23)[ab]	2.51 (1.44-3.94)[a]	7.46 (4.94-8.41)[b]
2'-F-LNH	0.00 (0.00-0.00)[2 a]	0.00 (0.00-0.00)[2 a]	0.00 (0.00-0.00)[2 a]	0.00 (0.00-0.00)[2 a]
β3'-GL[3]	0.074 (0.049-0.12)[a]	0.10 (0.080-0.13)[a]	0.11 (0.00-0.13)[a]	0.42 (0.38-0.51)[b]
β6'-GL[3]	0.36 (0.25-0.53)[a]	0.52 (0.44-0.61)[b]	0.42 (0.38-1.01)[ab]	1.07 (0.87-1.36)[c]
α3'-GL[3]	0.00 (0.00-0.00)[2 a]	0.00 (0.00-0.00)[2 a]	0.00 (0.00-0.00)[2 a]	0.00 (0.00-0.00)[2 a]

[1] Displayed as the internal standard (IS)-normalized total peak area of selected HMOs; [2] '0.00' does not necessarily mean that the respective HMOs is not present at any level, but it means that any amount of HMOs present is below the Limit of Quantification (LOQ); [3] For β6'-GL, β3'-GL and α3'-GL, the Limit of Detection was defined at a signal-to-noise ratio (S/N) of 3. For all other HMOs, the Limit of Quantification was defined at a S/N of 10; [a,b,c and d] Different superscript letters indicate statistical significance ($p<0.05$). Significance values have been adjusted by the Bonferroni correction for multiple tests.

Abbreviations used: 2'-FL: 2'-fucosyllactose; 3-FL: 3-fucosyllactose; DFL: difucosyllactose; LNT: lacto-N-tetraose; LNnT: lacto-N-neotetraose; LNFP I: lacto-N-fucopentaose I; LNFP II: lacto-N-fucopentaose II; LNFP III: lacto-N-fucopentaose III; LNFP V: lacto-N-fucopentaose V; LNDFH I: lacto-N-difucohexaose I; LNDFH II: lacto-N-difucohexaose II; 3'-SL: 3-sialyllactose; 6'-SL: 6-sialyllactose; DSLNT: disialyllacto-N-tetraose; LSTa: Sialyl-lacto-N-tetraose a; LSTb: Sialyl-lacto-N-tetraose b; LSTc: Sialyl-lacto-N-tetraose c; 2'-F-LNH: 2'-fucosyllacto-N-hexaose; 3'-F-LNH: 3'-fucosyllacto-N-hexaose; β3'-GL: β3'-galactosyllactose; β6'-GL: β6'-galactosyllactose; α3'-GL: α3'-galactosyllactose.

Table S2 | Percentage of human milk (HM) samples (n=171) in which the specific human milk oligosaccharide (HMOs) was detected (Presence, % of total) and relative peak area (rPA, %) of the selected HMOs (median (Q1-Q3)) according to maternal HM group [4, 11, 15]. HM samples were collected longitudinally over the first year postpartum from 24 Dutch mothers

	HM group I (n=126 samples, 18 donors)		HM group II (n=21 samples, 3 donors)		HM group III (n=15 samples, 2 donors)		HM group IV (n=9 samples, 1 donor)	
	Presence *% of total*[1]	rPA (%) *median (Q1-Q3)*	Presence *% of total*[1]	rPA (%) *median (Q1-Q3)*	Presence *% of total*[1]	rPA (%) *median (Q1-Q3)*	Presence *% of total*[1]	rPA (%) *median (Q1-Q3)*
2'-FL	100	22.3 (15.0-29.9)[a]	0	-	100	42.0 (35.5-53.0)[b]	0	-
3-FL	96	12.2 (6.29-21.7)[a]	100	35.9 (26.4-45.3)[b]	33	3.61 (3.04-4.40)[c]	100	6.78 (3.04-7.31)[d]
DFL	100	1.86 (1.29-2.50)[a]	0	-	100	1.01 (0.62-1.48)[a]	0	-
LNT	100	10.6 (7.87-13.1)[a]	100	15.3 (11.3-20.6)[b]	100	9.77 (6.50-13.8)[a]	100	51.8 (47.8-59.3)[c]
LNnT	94	1.03 (0.73-1.51)[a]	71	0.73 (0.55-1.13)[b]	93	1.22 (0.96-1.80)[a]	100	1.35 (0.94-1.89)[a,b]
LNFP I+V	100	7.56 (3.29-12.5)[a]	100	1.51 (1.36-1.63)	100	18.8 (15.6-24.5)[b]	100	1.29 (1.16-1.55)
LNFP II	100	5.79 (3.31-9.01)[a]	100	19.7 (17.1-21.2)[b]	0	-	0	-
LNFP III	100	7.52 (5.66-9.12)[a]	100	9.28 (8.06-10.6)[b]	100	8.94 (6.99-12.3)[a,b]	100	15.6 (10.1-18.4)[b]
LNDFH I	100	16.3 (13.5-19.1)[a]	0	-	0	-	0	-
LNDFH II	6.3	1.20 (1.04-1.85)[a]	90	3.91 (3.09-4.19)[b]	0	-	0	-
3'-SL	100	1.08 (0.78-1.43)[a]	100	1.37 (1.04-1.91)[a]	100	1.18 (1.04-2.15)[a]	100	1.76 (1.05-2.21)[a]
6'-SL	95	0.88 (0.52-1.66)[a]	95	0.74 (0.51-1.71)[a]	100	0.91 (0.37-1.62)[a]	100	1.74 (0.66-2.71)[a]
DSLNT	100	1.97 (1.40-2.47)[a]	95	3.30 (2.91-3.75)[b]	100	2.95 (1.94-4.10)[a,b]	100	5.53 (5.00-8.83)[c]
LSTa	46	0.20 (0.17-0.28)[a]	33	0.26 (0.20-0.31)[a]	27	0.23 (0.21-0.42)[a]	100	0.78 (0.75-0.93)[b]
LSTb	99	0.93 (0.69-1.08)[a]	100	1.43 (1.29-1.89)[b]	100	0.64 (0.51-0.98)[a]	100	2.36 (2.18-2.72)[c]
LSTc	73	0.57 (0.27-1.08)[a]	57	0.70 (0.32-1.20)[a]	60	0.53 (0.29-0.77)[a]	89	1.17 (0.51-1.64)[a]
3'-F-LNH	93	2.49 (1.59-3.90)[a]	86	4.06 (1.23-6.82)[a,b]	87	2.76 (1.75-4.37)[a]	100	7.46 (4.94-8.41)[b]
2'-F-LNH	16	1.00 (0.61-2.14)[a]	0	-	20	0.90 (0.32- .)[3 a]	0	-
β3'-GL[2]	83	0.087 (0.062-0.15)[a]	95	0.11 (0.081-0.14)[a]	73	0.12 (0.11-0.13)[a]	100	0.42 (0.38-0.51)[b]
β6'-GL[2]	100	0.36 (0.25-0.53)[a]	100	0.52 (0.44-0.61)[b]	100	0.42 (0.38-1.01)[a,b]	100	1.07 (0.87-1.36)[c]
α3'-GL[2]	1.6	0.18 (0.13- .)[3 a]	0	-	0	-	0	-

[1] Percentage of HM samples in which the specific HMOs was detected (≥Limit of Quantification (LOQ)); [2] For β6'-GL, β3'-GL and α3'-GL, the Limit of Detection was defined at a signal-to-noise ratio (S/N) of 3. For all other HMOs, the Limit of Quantification was defined at an S/N of 10; [3] In only two samples, the respective HMOs was present and hence Q3 could not be calculated; [a, b, c and d] Different superscript letters indicate statistical significance ($p<0.05$). Significance values have been adjusted by the Bonferroni correction for multiple tests.
Abbreviations used: see Table S1

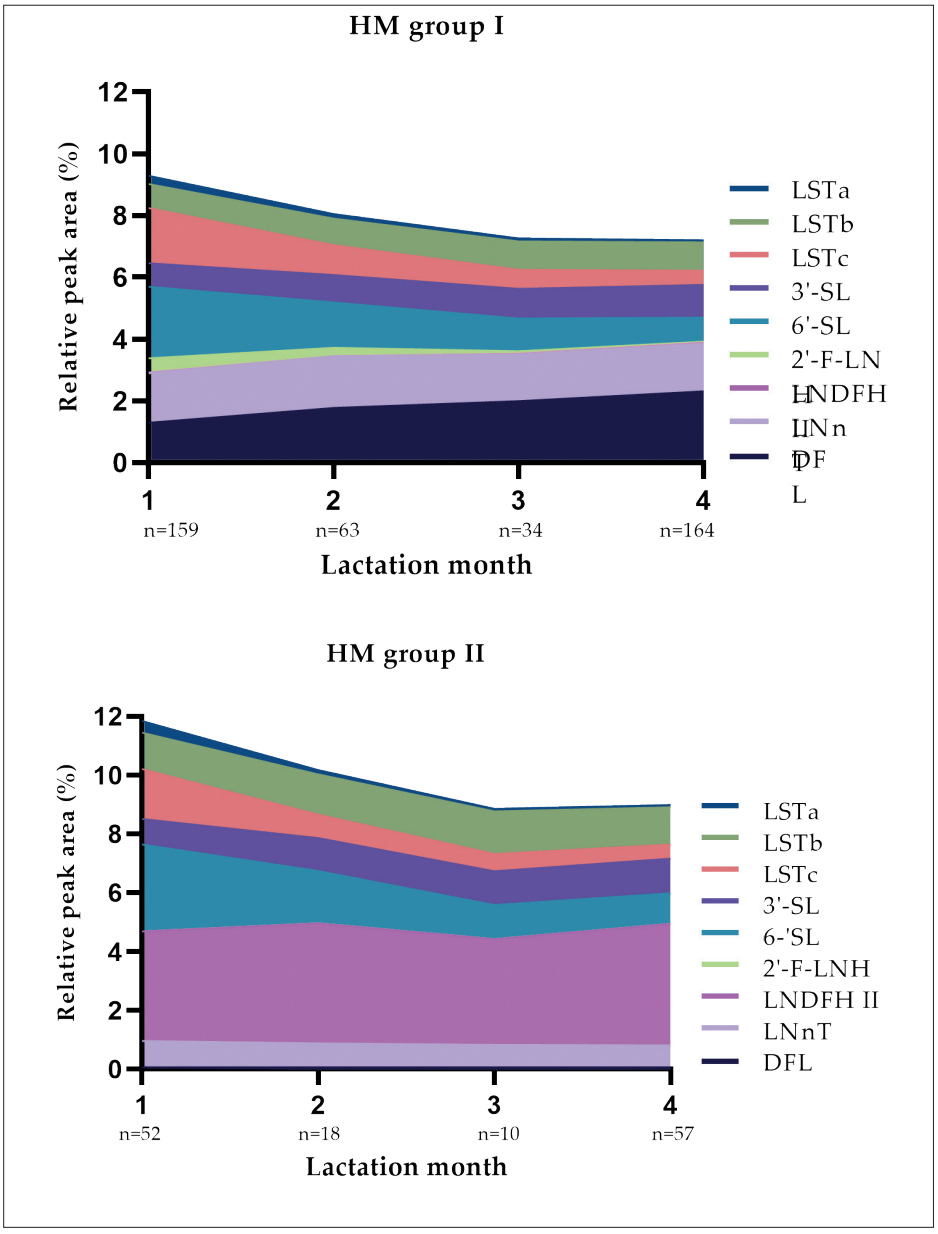

Figure S1 | Median relative peak areas (rPA) of low-abundant human milk oligosaccharides (HMOs) in the human milk (HM) samples (n=635) collected in the first 4 months of lactation according to HM group [4, 11, 15] and lactation stage. a) HM group I; b): HM group II; c): HM group III and d): HM group IV. Three samples could not be assigned to a HM group and are for this reason not depicted in these figures.

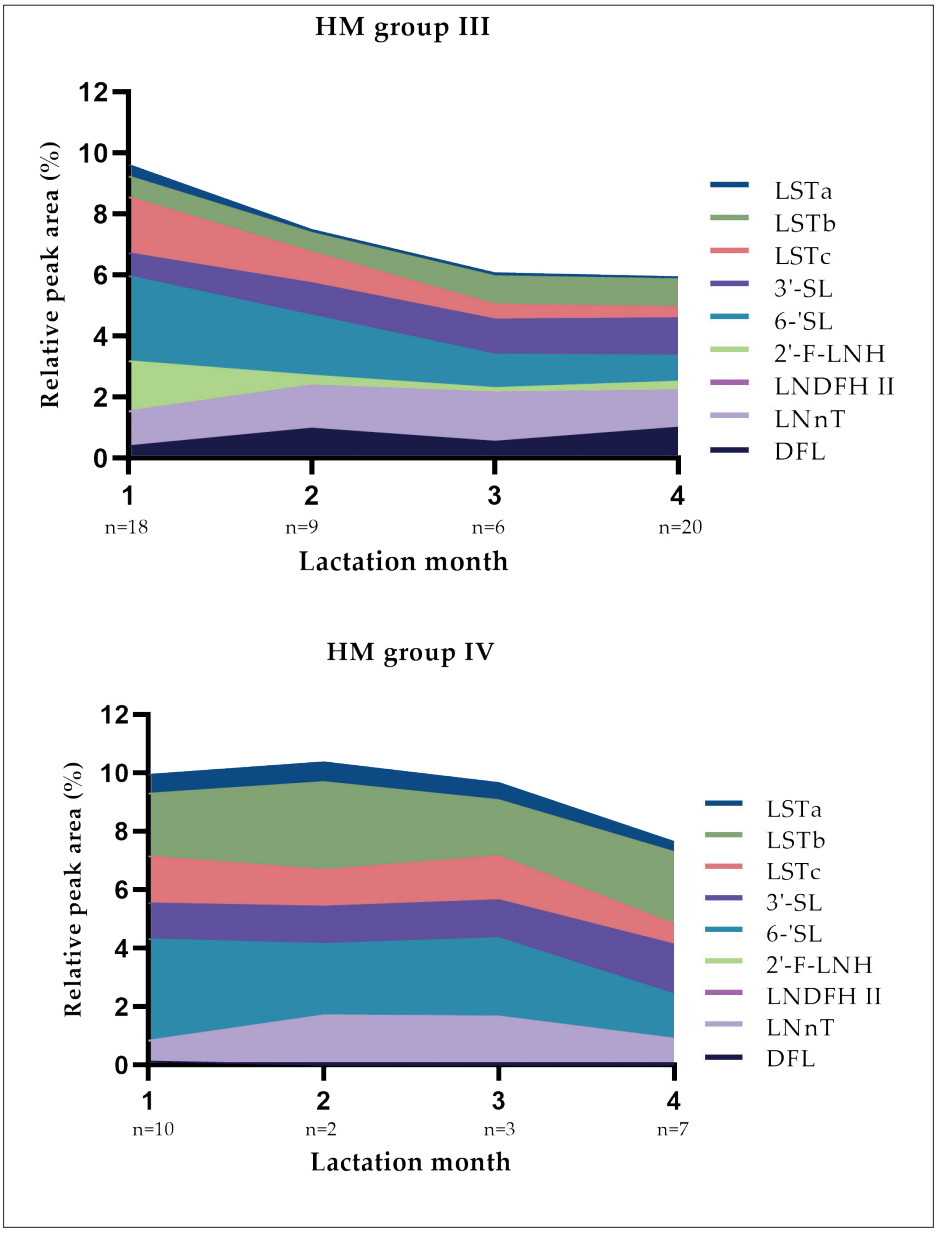

Figure S1 | Continued.

CHAPTER IX

Overall Conclusions and Future Perspectives

In the following paragraphs, the major aspects and findings from **CHAPTERS II-VII** will be recapitulated and brought into context with each other to draw overall conclusions. Also future perspectives for improved analytical approaches and extended research in the field of HMOs will be discussed.

DEVELOPMENT OF HYPHENATED TARGETED METHODS FOR HMO-ANALYSIS AND FUTURE PERSPECTIVES FOR ADVANCED CHARACTERIZATION OF HMOS OR GLYCOCONJUGATES

Two new analytical approaches fully dedicated to detailed characterization of HMOs have been established and outlined in this thesis. The new approaches comprised a specific and highly selective LC-MS^2 method (i.e. label-free targeted LC-ESI-MS^2 (see **CHAPTER III**)) and an electrophoretic method (i.e. multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (xCGE-LIF)). For direct identification and quantitation of relevant HMOs, the label-free targeted LC-ESI-MS^2 approach is taking advantage of "diagnostic" tandem MS fragments. The latter are resulting from so called collision induced dissociation (CID) in the 2^{nd} quadruple of triple quadrupole mass spectrometer. As indicated in **CHAPTER VII,** xCGE-LIF was designed to facilitate future high throughput analysis of HMOs complementary to mass spectrometry (MS)**.** The most important features of these novel methods will be summarized below together with possible further improvement or applications. Also emerging analytical approaches using e.g. alternative MS^2-fragmentation principles will be discussed. These alternative analytical technologies might help to ameliorate expected limitations in the structural characterization of higher molecular weight (complex) HMOs or glycoconjugates.

Label free targeted LC-ESI-MS^2 of HMOs building on the diagnostic ion approach

The novel LC-ESI-MS^2 method builds on multiple reaction monitoring (MRM). MRM LC-ESI-MS^2 relies on analysis of preselected HMOs structures by detection of their intact mass and identification of individual HMOs by diagnostic MS^2-fragment ions after LC separation. One of the most remarking new features of this MRM LC-ESI-MS^2 approach is its ability to directly identify (and quantify) the 10 most abundant, partly (regio-)isomeric HMOs structures in HM. This is accomplished in a retention time independent manner. Moreover, HMOs can be directly analysed in native form without the need for derivatization prior to analyses and without loss of specificity. Solely MS-based characterization of multiple important HMOs became possible by shifting their structural identification from the LC or MS-only level to the MS^2-level. A further crucial element for direct distinction of the 10 most abundant HMOs found in HM was to employ glycan-structure-specific diagnostic MS/MS fragments. Finally, this advantage allowed to perform LC-MS^2 analysis of abundant isomeric HMO-structures ranging from trioses (e.g. 2'-FL, 3-FL, 3'-SL, and

6'-SL) over tetraoses (LNT and LNnT) to pentaoses (LNFP I, II , III or V (see **Table 1** of **CHAPTER III**). Remarkably, these HMOs could be analysed devoid of the future need for pure HMO-standards which are difficult to isolate or not commercially available. In contrast, most contemporary LC-MS approaches still identify (HMO-) molecules based on retention time (RT) or unspecific fragments. In these cases, the option to execute analyses is limited by availability of said rare standards. The new targeted LC-ESI-MS^2 approach was then applied to identify and quantify ~ 20 HMOs in HM study samples (**CHAPTER IV**). Also, relative changes of HMO levels during in vitro fermentation with probiotic bacteria (**CHAPTER V**) could be differentiated. With regard to stability of HMO-retention times and signal intensity, the MRM LC-ESI-MS^2 setup was stable enough to characterize a batch of approximately 60 HM as delineated in the **CHAPTERs III-V**. However, for reliable analysis of > 1000 HM samples and in order to yield absolute quantitation of HMOs as planned for **CHAPTER VI**, the analytical approach had to be adapted and improved in major aspects. These improvements included for example the choice of another stationary LC phase (BEH Amide column instead of porous graphitized carbon (PGC) column) and optimization of the LC-gradient for compliance with the new stationary phase. In addition, the original sample pre-treatment process was extended by a de-lipidation step prior to 3kDA ultrafiltration. Efficiency of the ultrafiltration step could also be enhanced by exchanging single ultrafiltration devices by a 96 well format. To afford absolute quantitation, also the MS-specific parameters of the method were adjusted. Per HMO, two instead of only one specific MRM transitions were monitored. The precursor ions in those two transitions were the same but the fragment ions differed. One (mostly diagnostic) fragment ion served to identify the respective HMO, the second ion served as quantifier. As more transitions (33 instead of 20) needed to be scanned compared to the original method, this was counterbalanced by using a "nested" MRM approach. "Nested" MRM means to scan transitions of a specific HMO only within the expected retention time (RT) window and not during the complete MS run. Thereby an effective MS duty cycle with a sufficient number of data points assembled for each MS-peak was guaranteed. LC-resolution of HMO-isomers was slightly worsened compared to usage of the PGC-column. Nevertheless, this could be settled by extension of the column length to 100 mm and usage of a longer gradient. Consequently, the only disadvantage of this hydrophilic interaction chromatography (HILIC) based LC-MS^2 method was a prolongation of the run time per sample to 29 min. On the other hand, this fully optimized and validated targeted HILIC MRM LC-MS^2 approach facilitated stable runs and absolute quantitation of neutral and acidic HMOs up to hexaoses for more than >1000 individual HM samples. To date, this might be one of the first validated and stable MRM LC-MS^2 methods suitable to analyse such a high number of HM specimens. Moreover, the method covered a wide validated HMO-concentration range reaching from 7,5 g/L 2'-FL to 1,3 mg/L beta 6'-GL and resolved all relevant HMO isomers up to hexaoses.

In general, the here established HMO-diagnostic ion principle bears further potential to develop even more improved MS^2 applications. As demonstrated in **CHAPTER III**, the unambiguous identification of HMOs by automated on line LC-MS^2 using only one diagnostic ion per structure might be limited to HMOs up to Lacto-N-fucopentaoses. Nevertheless, this means that at least for this molecular weight (MW) range often difficult to obtain pure HMOs standards might be spared and throughput might be tremendously increased in future by creation of direct flow injection analysis (FIA) MS applications. Usage of more than one diagnostic ion would probably allow to identify even larger HMO structures carrying e.g. more than one Lewis-epitope just based on MS^2. Promising first steps to identify such complex HMOs have already been demonstrated by using microflow ESI-CID-MS^2 [1, 2]. However, until now these methods have not been translated into LC-MS^2 applications able to screen higher numbers of HM samples in a convenient and efficient way.

The existence of high molecular weight (HMW) HMOs exceeding 6 kDa and a degree of polymerization (DP) of 28 has been reported [3]. HMW HMOs are by far beyond the mass range studied in this thesis by application of xCGE-LIF or label free targeted LC-ESI-MS^2. It is conceivable that physical limitations of CID fragmentation as applied in the here established LC-ESI-MS^2 approach could hamper the in-depth structural elucidation of such large and structurally complex HMOs. One physical effect among others which might complicate the successful complete fragmentation of such large molecules by CID is the increase of inner degrees of freedom. The degrees of freedom which a molecule can exploit to absorb energy (e.g imparted by collision with other molecules during CID) are increasing with molecular weight. The more degrees of freedom are available to distribute the collision energy deposited on the molecule, the higher is the needed collision energy to achieve complete fragmentation. This may eventually result in insufficient fragment-ion yields especially for those fragment ion species presumably created by multistep scission processes like the C/Z fragments. Alternatively, C/Z-ions are sometimes assigned as D-type ions [4]). The latter proved to be very useful to discern HMO fuco-regioisomers (e.g. LNFP I, LNFP II, LNFP III resembling different Lewis (Le) and secretor (Se) determinants (see **Figure 1 CHAPTER III** and **Figure 1 CHAPTER IV**)). In contrast to the HMOs which have been investigated in this thesis and which mostly exhibited one Le-epitope, higher molecular weight HMOs may even carry multiple Le-determinants. This adds to their structural complexity on top of possible (multiple) branching and additional (multiple) sialylation. Multiple Le-epitopes or other biologically relevant epitopes could increase the avidity of such polyvalent HMOs for carbohydrate receptors like C-type lectins, Siglecs, toll like receptors (TLR) or Galectins (see e.g. also **CHAPTER II Figure 3**). Those receptors are widely expressed on various cells of the inherited and adaptive immune system (T-cells, B-cells, dendritic cells, etc.). Thus, polyvalent HMOs may profoundly modulate immune homeostasis of the developing infant by binding or even crosslinking

these immune cells with higher avidity than monovalent HMOs or other ligands. Consequently, further in depth characterisation of polyvalent (HMW) HMOs might be important to better understand their impact on healthy and balanced EL immune development. Due to the aforementioned possible limitations of the CID approach especially for polyvalent HMOs of higher MW, alternative fragmentation methods for MS^2 or MS^n are warranted. Here, infrared multiphoton dissociation (IRMPD) [5], ultraviolet photodissociation (UVPD) [6], or charge transfer dissociation CTD [7] may be superior to CID in producing enough (diagnostic) MS^2-fragment ions for extensive structural characterization. Next to improvement of compound identification by more versatile fragmentation methods, also the addition of a further dimension for improved separation of OS compounds prior to MS^2 on top of LC could be helpful. Here the integration of the ion mobility (IM) principle into LC-MS^2 approaches for HMOs might be a promising option [8-10].

Nevertheless, negative ion mode diagnostic MS^2-fragments generated by CID, UVPD, or IRMPD might be exploited even beyond structural analysis of free native glycans or HMOs. In future, they could also facilitate better direct characterization of the glycan-fine-structures exposed as residues of glycoconjugates like glycoproteins, glycopeptides and glycolipids. Currently, detailed information about branching and glycosidic linkages of glycan residues can only partly be delivered by mass spectrometric analysis. Modern proteomic approaches are e.g. suitable to localize the attachment sites of glycan residues relative to the amino acid sequence of glycoproteins or glycopeptides [11]. Furthermore, ESI-MS^2-based proteomic characterization of *N*-and *O*-glycopeptides exemplified that fine tuning of MS^2 CID-energies can also yield both, fragment ion series of peptide glycan residues and peptide backbone [12]. Taken together, contemporary advanced glycoproteomics approaches can routinely identify the monosaccharide composition of glycan residues and also assign further structural features of *N*- and *O*-glycans like the right monosaccharide sequence or N-glycan class [13, 14]. However, the obtained structural information is often insufficient to also distinguish between glycan epitopes such as Le^x or Le^a as mentioned above before. These Lewis-determinants are typical examples for glycosylation patterns with the same monosaccharide composition but with subtle, yet functionally important differences in their fine structure. Here, the development of diagnostic ion empowered LC-MS^2 applications which could also improve identification of N- and O-glycan microheterogeneities in glycoproteins like lactoferrin, kappa-casein, sIgA, osteopontin, or butyrophillin would be a crucial step ahead. A more detailed characterization of these post translational modifications (PTMs) would allow to significantly extend our knowledge about the functional effects of many protein bound glyco-epitopes in early life which are still not fully understood. The obvious potential of CID-MS^2-based approaches for in depth elucidation of glycan residue fine structures has been demonstrated decades ago. In 1988, Domon and Costello already described the fragmentation patterns obtained from intact glycosphingolipids,

glycopeptides and other biomolecules by fast atom bombardment (FAB)-CID MS^2 analyses [15]. They generated for example fragment ions from carbohydrate moieties of asialo-gangliosid GM1 in negative ion mode which included a series of A-, B-, C- and Y-ions for which they also introduced the nomenclature. Ions of the same type, namely A- and C-type, were also useful to distinguish between the two HMO linkage isomers LNT and LNnT (see **CHAPTERS III-VI** above). Besides this, c-type ions were aiding in successful distinction of the regioisomers of LNFP I, II, III and IV/V.

More recently, Ko et al. [16] published an article in which they employed negative ion mode ultraviolet photodissociation (UVPD) ESI-MS^2. This approach afforded highly diagnostic information about both, glycan structure and peptide sequence of *O*-glycopeptides from fetuin and kappa-casein. Interestingly, it was stated in this manuscript that negative ion mode UVPD tandem MS (but not CID-MS^2) of doubly deprotonated kappa casein *O*-peptides resulted in fragment ion masses at *m/z* 290 and *m/z* 468. Those fragment ions originated from $(HexNAc)_1(Hex)_1(Neu5Ac)_1$ residues. The same fragment masses were found after CID-MS^2 of the free acidic HMO 3'-SL as depicted in **Figure 3** of **CHAPTER III**. Following the nomenclature of Domon and Costello [15], the 3'-SL fragment at *m/z* 290 was assigned as B_1 which represents a glycosidic linkage fragment. The *m/z* value of 468 could be assigned as $^{2,4}A_3$-CO_2 which denotes a ring fragment of glucose. The observation of identical fragment masses originating from MS^2-spectra of a free HMO (3'-SL) and an O-glycosylated peptide which resembled the monosaccharide composition and sequence of the HMO in its glycoresidue is intriguing. This could be a first indication that negative ion mode ESI-UVPD-MS^2 of glycopeptides could be a strategy to deliver diagnostic ion information for more advanced structural characterization of glycoepitopes comparable to ESI-CID-MS^2 of free native HMOs.

Next to using electrospray ionisation (ESI) [17] for charging of biomolecules like HMOs prior to their subsequent mass spectrometric (MS) analysis, also matrix-assisted laser desorption/ionization (MALDI [18]) can be employed for the same purpose. Both ionisation technologies, ESI and MALDI, suffer from a certain sensitivity loss if deployed in negative ion mode as. On the other hand, negative ion MS^2 provides the valuable A-, C-, and Z-type (diagnostic) ions useful for direct structural characterization and identification of different HMOs isomers as outlined in this thesis. A new promising opportunity to overcome the negative ion mode sensitivity loss for HMOs could probably be provided by an improved MALDI ionisation process: This new MALDI-PI or MALDI-2 ionisation principle exploits so called laser post ionization (PI) [19] and already increased negative ion mode sensitivity to attomole detection levels.

Finally, an elegant hybrid approach combining tandem mass spectrometry and 3 µm infrared (IR) molecular spectroscopy (i.e. IRMPD) was introduced by

Schindler et al in 2017 [20]. This new "top down" metric allows for monosaccharide sequence elucidation by multistage positive ion mode MS (MS^n) and subsequent IR characterization of resulting C-type MS-fragments. Complete IR resolution of resulting monosaccharide isomers, regio-isomers and stereo-isomers including epimers (e.g. GlcNAc and GalNAc) could be demonstrated. This method is suitable for unambiguous and complete de novo structural elucidation of oligosaccharides. Currently, analysis time per monosaccharide residue is approximately 10 min.

High Throughput Multiplexed Capillary Gel Electrophoresis of HMOs with Laser-Induced Fluorescence Detection (xCGE-LIF)

To also consider the need for higher sample throughput as additional feature on top of e.g. enhanced selectivity and other improvements provided by LC-MS^2, xCGE-LIF was established as alternative tool. This electrophoretic method primarily separates by capillary gel electrophoresis (CGE) according to their hydrodynamic volume. Also, many important HMO-isomers like 2'-FL and 3-FL or LNFP I-IV can be resolved by xCGE-LIF. However, visual inspection of xCGE-LIF electropherograms (**Figure 3** B CHAPTER VII) and MRM-LC-ESI-MS^2 traces (**Figure 6**, **CHAPTER III**), shows that xCGE-LIF resolution of isomeric HMOs like LNFP I-IV is slightly inferior to targeted LC-ESI-MS^2. Extracted ion chromatograms (IEC) of the latter reach near baseline resolution for these compounds. In addition, co-elution of HMO structures with similar molecular mass can occur in xCGE-LIF as observed for beta 4'-GL and 2'-FL (see **CHAPTER VIII**). Comparing xCGE-LIF with LC-ESI-MS^2, the higher selectivity of the new MRM LC-ESI-MS^2 method will recommend it over xCGE-LIF if maximum distinction of HMO isomers in milks with high structural HMO-diversity (e.g. HM group I milks) is the analytical goal. On the other hand, xCGE-LIF will be superior in other aspects and its capacity for high throughput HMO screening as explained further below. Through introduction of one fluorophore (i.e. APTS[21]) per HMO molecule via reductive amination (see **CHAPTER VII)** a uniform structure independent response factor during laser induced fluorescence detection (LIF) is expected. This is an advantage over LC-MS^2 (different response factors for different HMO structures) if individual HMOs or the totality of HMOs need to be quantified. Finally, relative retention times are extremely stable in xCGE-LIF as demonstrated in **CHAPTER VII**. Thereby, reliable CE-migration time-based identification of Lactose and 17 HMOs was routinely attained for HM samples. The identifiable HMO-structures are ranging from simple trioses (e.g. 2'-FL, 3-FL) to a complex, branched, and difucosylated octaose structure (2,3-DF-LNH) as listed in Table 2 of **CHAPTER VII**. With 2,3 DF-LNH a HMO comprising two Le-epitopes became accessible by xCGE-LIF which was not yet included in the LC-ESI-MS^2 approach. 2,3 DF-LNH entails Le^x and Le^d [22]. So, the main advantages of this new xCGE-LIF method are uniform response-factors (glycan structure independent) for effortless relative quantitation of Lactose and HMOs, enhanced sensitivity and high robustness. Eventually, the development of this method might have paved the road to a real high throughput

workflow: In our approach, multiplexing of 4 analytical runs on one instrument could be achieved while taking advantage of a widely available and affordable DNA sequencer technology. Although one xCGE-LIF run takes 130 min, the multiplexing option of the used DNA analyser platform allows to shorten the effective run time per sample. Hence, the approximate effective run time is approximately 30 min (130 min divided by 4 runs in parallel). As there are also 96 capillary CGE-instruments available, the effective run time per sample could be reduced to only approximately 1 min / sample in future. This would allow for currently unmatched high throughput screening of HMOs.

The established xCGE-LIF approach is generally applicable to various types of oligosaccharides which can be derivatized via reductive amination with the fluorophore 8-aminopyrene-1,3,6-trisulfonic acid (APTS). Therefore, another important future application could also be the quality control of prebiotic trans-galactooligosaccharides (TOS or GOS) in infant milk formulations (IF). Just recently, suitability of xCGE-LIF for in process control of biotechnologically produced HMOs (2'-FL, 3-FL, LNT2, 3'-SL, 6'-SL, DFL, LNT, LNnT, p-LNH) was indeed established. [23]. Using a borate buffered electrophoresis gel, extremely short runtimes < 6 min were sufficient to resolve said HMOs. Although, separation of isomers such as 3'-SL and 6'-SL or LNT and LNnT appeared to be inferior to our xCGE-LIF method described in **CHAPTER VII**.

The identification of oligosaccharide structures by xCGE-LIF still depends on the availability of pure standards for definition of compound dependent retention times. This is a limiting factor for the addition of new complex oligosaccharide-structures to the xCGE-LIF portfolio due to their frequent commercial unavailability. Fortunately, new advances in the combined chemo enzymatic synthesis of complex HMO [24] might be able to mitigate this issue in future.

The advantages of our hyphenated xCGE-LIF and targeted MRM LC-ESI-MS^2 methodologies were fully exploited to identify and quantify the most abundant HMOs in a multitude of HM samples. These HM samples were received from diverse HM cohorts, clinical studies and in vitro assays as summarized in the following paragraphs.

EARLY LIFE HMO *IN VIVO* AND *IN VITRO* VARIANCES IN SPECIMENS FROM HM-COHORTS, CLINICAL HM STUDIES AND MICROBIAL FERMENTATION ASSAYS AS REVEALED BY LC-ESI-MS^2 OR xCGE-LIF ANALYSES

The targeted LC-ESI-MS^2 method for HMOs explicated in **CHAPTER III** was applied to 60 real life HM study samples (healthy controls of "INFAT study") and after further improvement also to more than 1000 HM specimens from a prospective HM cohort

("Ulm SPATZ health cohort"), see **CHAPTERS IV + VI**). Moreover, specific utilization of individual HMOs serving as carbohydrate source in microbial mono and co-cultures could be monitored by this MRM LC-ESI-MS^2 approach (**CHAPTER V**). In these applications, relevant neutral and acidic HMOs up to hexaoses (e.g. LNDFH I) could be accurately characterized building on the improved selectivity of targeted LC-ESI-MS^2 for isomeric HMOs. Various isomeric structures like 3-FL, 2'-FL, 3'-SL, 6'-SL, LNT, LNnT, LNFP I, LNPF II, LNFP III, LNFP V or tentative blood group A and B haptens were quantified in absolute or relative manner. By taking advantage of the xCGE-LIF technology outlined in **CHAPTER VII**, relative quantitation of HMOs up to Heptaoses (e.g. 2'-FLNH) was accomplished for more than 700 individual HM yielded from 371 donors of the "Prevent CD study". After further validation and extension of the original xCGE-LIF method, further acidic HMOs like LSTb, LSTc and DSLNT could be integrated into the new approach. Even absolute quantitation of the low abundant human milk Galactosyllactose-isomers β3'-GL, β4'-GL, β6'-GL and α3'-GL was obtained. Taken together, more than 1900 individual European HM samples could be investigated by application of LC-ESI-MS^2 or xCGE-LIF to decipher HMO diversity and quantitative changes.

Maternal Le- and Se-genotype dependent and independent HMOs vary between HM groups I-IV and tentative new HM group I subtypes

HM specimens from the European HM cohorts and studies mentioned before were subjected to LC-ESI-MS^2 or xCGE-LIF. This was done to confirm known or elicit new HMO-patterns or typical individual HMOs driving the separation of HM into characteristic human milk groups. Furthermore, level changes of individual HMOs over the course of HM lactation up to 12-month pp should be monitored.

Literature frequently refers to 4 distinct lactotypes or human milk groups (HM groups I, II, III and IV) [25, 26]. There is also a growing body of evidence for the existence of HM group I related sub-groups [27]. So far it is believed that formation of HM groups is mainly directed by expression of specific maternal genes (FUT 2 or Se-gene and FUT 3 or Le-gene on Chromosome 19) which translate into respective fucosyltransferases (Se-enzyme and FucT III) and finally into characteristic products of these fucosyltansferases which are α1,2- or α1,4/1,3-fucosylated HMOs. HM groups can be determined by measuring the presence and levels of Se-gene dependent α1,2-fucosylated HMOs like 2'-FL, DFL, LNFPI I, together with Le-gene dependent α1,4/1,3-fucosylated HMOs like LNFP II, and Se- plus Le-dependent α1,2- plus α1,4-fucosylated HMOs like LNDFH I.

60 healthy control HM samples of the INFAT study were scrutinized by MRM LC-ESI-MS^2 for more than 20 neutral- and acidic HMOs up to DP 6 including tentatively assigned blood group A and B haptens (**CHAPTER IV**). Principal component analysis (PCA) of the obtained HMO data sets resulted in formation of four HM clusters in

the PCA score plot. These clusters could be assigned to the known HM groups I-IV based on presence of Se- and Le-gene dependent marker HMOs which were defining the separation into these 4 HM groups (see **CHAPTER IV Figure 4** and **Figure 6**). In general, and next to effortless interrogation of large data sets, PCA therefore seems to offer the potential for identification of new HM groups and the HMOs which drive the separation into new or existing milk groups.

Notably, the HMO diversity especially regarding the Se-/Le-gene dependent HMOs was significantly different between the 4 HM groups/clusters. This was expected and in line with previous literature [26]. At least between 6 weeks and 16 weeks pp, the changes in HMO diversity were dominated by influence of maternal genetic predisposition rather than by HM lactational stage. These results could be further confirmed after HMO analysis of > 1000 HM samples from the ULM SPATZ HM cohort (see **CHAPTER VI**) and > 700 HM samples from the Prevent CD study (see **CHAPTER VIII**).

In more detail, the highest impact on separation of the HM groups received by PCA was conveyed by HMO like 2'-FL, DFL, LNFP I (resembling the H 1 type or Le^d epitope) which are linked to an active FUT 2 gene, by LNDFH I (Le^b) resulting from combined FUT 2 and FUT 3 activity, and by LNFP II and LNDFH II (both Le^a) linked to FUT 3 activity. Unexpectedly, also other HMOs which are independent from FUT 2 or FUT 3 (i.e. from maternal Le-/Se-status) showed significant differences between HM groups I-IV (see e.g. **CHAPTER IV Table 2**). This finding applied for the following HMOs: 3-FL, LNnT, tentative blood group B-tetrasaccharide/$(Hex)_3(Fuc)_1$, and tentative blood group A-tetrasaccharide/$(Hex)_2(HexNAc)_1(Fuc)_1$.

All other detected non Se/Le-status dependent HMOs did not significantly vary between HM groups. This "core" set of HMOs included $(Hex)_3$ /Galactosyllactoses, $(Hex)_2$/Lactose, LNFP III, LNT, 3'-SL, Total SL, 6'-SL, and $(Hex)_4$.

Intriguingly, ordination of HMO data hinted towards the existence of a novel HM subgroup within HM group I. Some Se- and Le-positive HM visually separated from the HM group I cluster in the PCA score plot. This subgroup was termed "HM group Ia". t-testing demonstrated that HM group Ia significantly distinguished from HM group I by higher abundances of specific Se+ HMOs like 2'-FL. In turn, abundances of Le+ related HMOs like LNFP II and LNDFH II were significantly lower in HM group Ia compared to HM group I. These findings corroborated similar results published by Elwakiel et al in 2018 [27]. They also identified a subgroup in HM group I (i.e. Se+ and Le+) in breast milks from Europe (Netherlands) and Asia (China).

Closer inspection of HMO data derived from the Ulm SPATZ HM cohort and from the Prevent CD HM study (see **CHAPTERS VI + VIII**) revealed a further subtype within the

HM group I which was termed “HM group Ib”. A new interesting feature of HM group Ib was the clear presence of the HMO LNDFH I while other Se+ (i.e. FUT 2 dependent) HMOs like 2’-FL, and LNFP I were absent or detected at extremely low levels. Presence of LNDFH I (resembling a Le^b epitope) is thought to be dependent on expression of the Se- and the FucT-III fucosyltransferases in the mammary gland. LNDFH I may result from Se enzyme catalyzed alpha 1,2-fucosylation of the terminal galactose on LNT and addition of a α1,4-linked Fucose to the subterminal GlcNAc moiety of LNT. The Se enzyme may also catalyze the α1,2 fucosylation of Lactose leading to 2’-FL, and the transformation of LNT into LNFP I by attachment of an α1,2 linked fucose. As the Se-enzyme would be required for formation of all these α1,2-fucosylated HMOs, it is left unclear how LNDFH I can be found in HM while other FUT II activity linked HMOs are absent. Due to the novelty of this phenomenon, no proven functional explanation regarding e.g. altered pathways in mammary gland HMO biosynthesis is currently available. However, one could speculate that a so far unknown α1,2-fucosyltransferase (FucT-x2) with increased preference for e.g. LNPF II as substrate could be active here. This enzyme may be the product of a novel FUT 2 polymorphism and may catalyse the synthesis of LNDFH I from LNFP II. This synthesis could occur in competition with or independent from the Se enzyme. Thus, even if the Se enzyme is inactive or only expressed in low levels, the novel α1-2-fucosyltransferase might still catalyse formation of LNDFH I from LNFP II, whereas 2’-FL and LNFP I might not be present or only found in low levels. This would exactly lead to the observed HMO patterns found within the “HM group Ib” which was quite rare and comprised approximately less than 0,1% of all studied HM samples. Of course, the latter hypothesis for Se-enzyme independent formation of LNDFH I warrants further confirmation. FucTx2 was preliminary chosen as designation for the postulated new α1-2-fucosyltransferase to distinguish it from FucTx. FucTx was assigned by van Leeuwen et al. in 2018 to another new hypothetical α1-3-fucosyltransferase which may produce Le_X and Le_Y HMO in FucT III negative subjects [28].

In this thesis, we studied neutral HMO compounds up to a degree of polymerization (DP) of 7 (e.g. 2’-FLNH) and acidic HMOs up to DP 6 (e.g. DSLNT). By abundance this covers approximately between 50% (MRM LC-ESI-MS^2 approaches of CHAPTERs III + IV) and 80% (extended xCGE-LIF approach CHAPTER VIII) of all HMOs contained in HM. However, the analytical coverage in terms of individual HMO structures which are probably concealed in HM might be much lower. MALDI-MS based investigations of GPC subfractions yielded from pooled HM disclosed the existence of HMOs up to DP28 (i.e. a molecular mass > 6000 Da) [3]. This could translate into a plethora of compositional and structural HMO variants exceeding the number of 1000 [29]. Only 162 HMO have been completely structurally elucidated so far [30-35]. Consequently, it seems to be sensible to assume that much more individual Se/Le-status independent (i.e. HM group independent) glycans are attributable to the afore defined core set of HMOs. In addition, also the number of distinct but unknown

HMOs > DP7 which are not part of the HMO-core-set and which will substantially vary between the HM groups might be very high as well.

If the results from all tested European human milks collected up to 6 month pp are taken together, the following distributions of HM groups and Se-status (positive (+) or negative (-) were obtained (see **Table 1**):

Table 1 | Distributions of distinct HM groups and maternal Se-status as derived after HMO analyses in milks from 3 European HM-cohorts or studies

Cohort/study	HM group I [%]	HM group II [%]	HM group III [%]	HM group IV [%]	Se (+) HM [%]	Se (-) HM [%]
Infat [36]	77	7	13	3	90	10
Ulm SPATZ [37]	74	18	7	1	81	19
Prevent CD [38]	66	22	8	4	74	26

Compared to the publication of McGuire et al in 2017 [39], our findings for proportions of secretor positive HM visible from **Table 1** are approximating those described for other European countries like Spain (76%) and Sweden (79%), or the United States (68-95%) However, HM collected in other geographies like Peru (98%) in South America, or rural Ethiopia (65%) and Gambia (65%) in Africa differ more in the found percentages for secretor positive HM. Furthermore and in contrast to our results, 50-70% of Iranian or Iraqi-Arabic HM were found to be from non-secretors. The rate of non-secretors in Cambodian HMs was even > 70% [40].

Disclosure of diametrically opposed variations versus relative stability of particular HMOs during different stages of lactation within 6weeks-12 month pp

The concentrations of the total HMOs fraction in mature milks ranges between 3-18 g/L [29, 41, 42, 43, 44, 45]. Colostrum i.e. human milk sequestered during the first week after birth may contain up to 20 g/L [46] total HMOs. By applying the improved targeted LC-ESI-MS^2 approach described in **CHAPTER VI** to > 1000 HM samples from the ULM SPATZ cohort, absolute concentrations of the 14 most abundant HMOs structures could be obtained. This set of HMO structures resembled more than approximately 50% of the HMOs quantity expected in HM. To investigate the absolute changes of HMOs concentrations during progressing lactation, a LC-ESI-MS^2 data subset of HM from 66 different donors participating in the Ulm Spatz cohort was interrogated. Each of the 66 donors provided a complete series of HM after 6 weeks, 6 month and 12month post-partum (see **Figure 1**). The found levels for total HMO concentrations based on the 15 most abundant HMOs declined from 6.1 g/L at 6 weeks after birth to 4.7 g/L at 6 month and stabilized at 5 g/L until 12-month pp. If not stratified according to HM milk groups, 2'-FL was the most abundant individual HMOs. Its concentrations ranged between 2.45 g/L in HM collected at 6

weeks after birth to 1.4 g/L in HM from 12-month pp. The second highest abundant HMO-structure was 3-FL (0.6 g/L at 6 w, 1.5 g/L at 12 mon), followed by LNT (0.9 g/L at 6 w, 0.4 g/L at 12 mon), LNDFH I (0.5 g/L at 6w, 0.4 g/L at 12 mon), LNFP I (0.4 g/L at 6w, 0.3 g/L at 12 mon), LNFP II (0.3 g/L at 6w, 0.2 g/L at 12 mon), DFL (0.2 g/L at 6w, 0.4 g/L at 12 mon), LNFP III (0.2 g/L at 6w, 0.1 g/L at 12 mon), 3'SL (0.1 g/L at 6w, 0.3 g/L at 12 mon) and 6'-SL (0.2 g/L at 6w, <0.1 g/L at 12 mon). All other measured HMOs were below 0.1 g/L at any stage between 6weeks and 12 months pp. Following the assumption that approximately 50% of the estimated total HMO quantity were covered by the applied LC-ESI-MS^2 method, the found results are well in line with the range of 3-18 g/L published before for the complete HMOs fraction in HM (see e.g. Thurl et al. 2017 [41] and further references above).

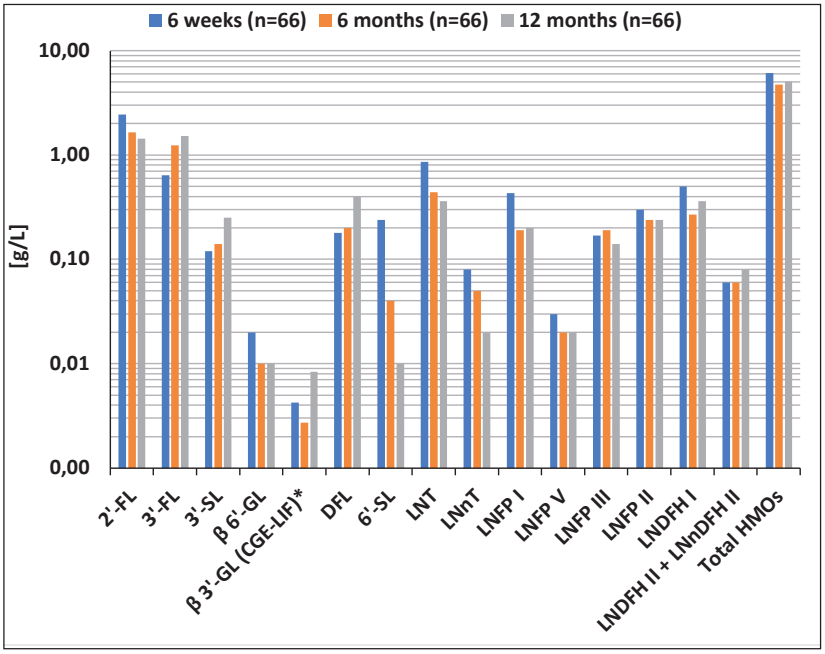

Figure 1 | Average concentrations of individual and total HMOs as detected by MRM LC-ESI-MS^2 in HM from 66 European donors collected over 3 stages of lactation (see CHAPTER VI))

*β 3'-GL data were accumulated by xCGE-LIF analysis (see CHAPTER VII); n=22 HM at 1-6 mon, n=2 HM at 12 mon

Stratification of HMO variations to the lactation period from 6 weeks to 6 month post partum

The overall decrease in total HMOs concentration between 6 weeks and 6 months after birth could be confirmed by the findings yielded from the INFAT study (**CHAPTER IV**) and the Prevent CD study (**CHAPTER VIII)**. This trend was true irrespective of the fact

if HM were collected as a series from one donor or different donors (see e.g. **Figure S1.B**, **CHAPTER VI**). To depict the directionality of variations in HMO abundances, decadic logarithms of the HMO ratios 6 month/6 weeks and 12 month/6 month were calculated (see **Figure 4** - **Figure 7**). A positive value indicates and increase in HMO concentration over the course of lactation, a negative value a decrease. Significances of level changes had been determined as explained in **CHAPTERS IV** and **VI**. β 3'-GL levels as determined in **CHAPTER VIII** were excluded because the number of HM donors providing complete HM series from 6 weeks to 12-month pp with β 3'-GL levels > LLOQ was not matching to the numbers obtained for the other HMOs.

Some individual HMOs deviated from the overall trend of total HMOs by either decreasing much stronger in concentrations or by increasing in concentrations during lactation. This group of particular glycans with significant variations was not homogenous if the lactation periods from 6 weeks to 6-months pp and from 6 month to 12-months pp were compared. Nevertheless, some structures were found to have stable trends in both studied periods of lactation. Interestingly, the concentrations of some isomeric HMOs developed into diametrically opposite directions during lactation.

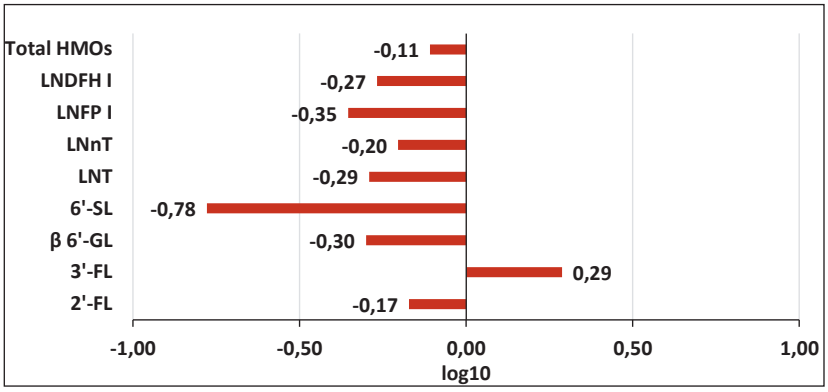

Figure 4 | Variations of HMO-abundances between 6 weeks and 6-months pp (only glycans with significant changes shown, p < 0.0036, n=66))

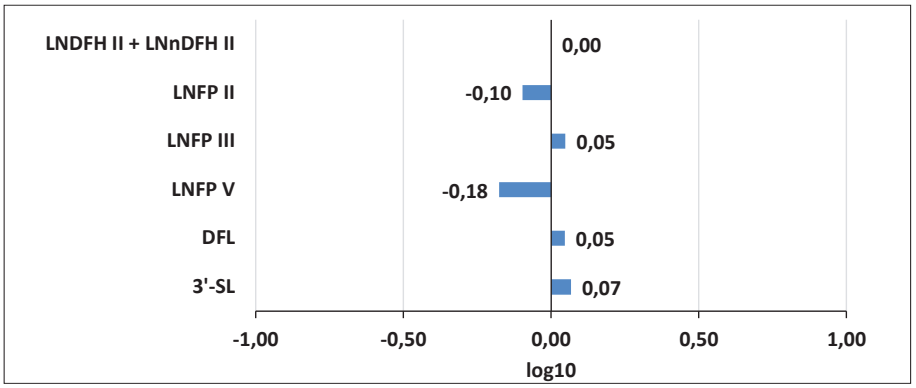

Figure 5 | Variations of HMO abundances between 6 weeks and 6 months pp (only glycans with insignificant changes shown, p < 0.0036, n=66))

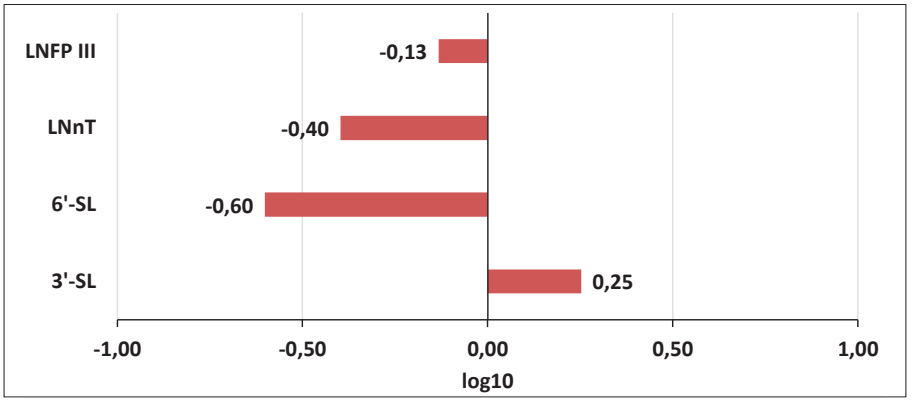

Figure 6 | Variations of HMO abundances between 6 month and 12 months pp (only glycans with significant changes shown, p < 0.0036, n=66))

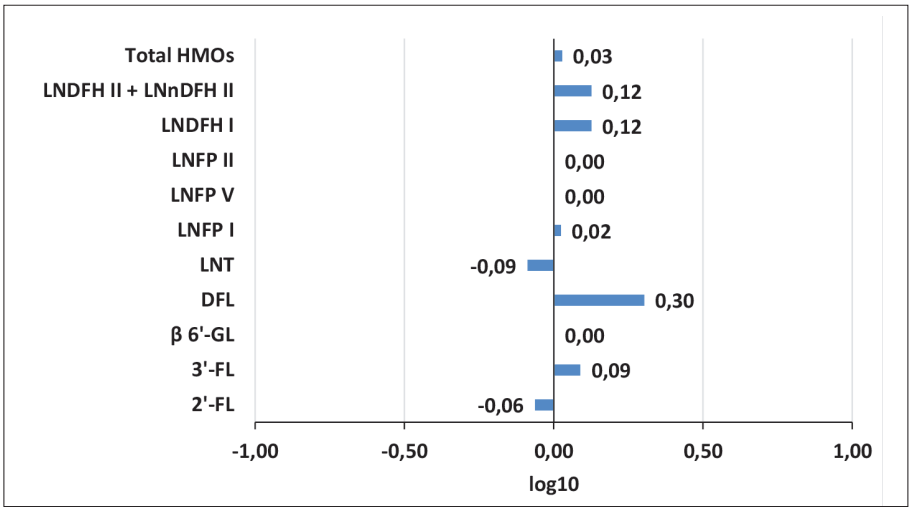

Figure 7 | Variations of HMO abundances between 6 months and 12 months pp (only glycans with insignificant changes shown, p < 0.0036, n=66))

For example, **Figure 4** shows a decrease of 2'-FL between 6 weeks and 6 months after birth by a log10 value of -0.17 (equal to a decrease of 32%) while 3-FL strongly increased by a log 10 value of +0.29 (equal to an increase of 95%). Similar trends for temporal changes of 2'-FL and 3-FL over the course of lactation were observed by Austin et al. in 2016. They analyzed 90 Chinese breast milks collected between 1 week and 4-months pp [47]. As also visible from **Figure 4**, 3-FL was in fact the only compound among the 15 tested most abundant HMOs which significantly increased in the period between 6w and 6 months post-partum. All other HMOs with significant variations in this period of lactation did decrease in concentrations. By sorting HMO structures according to the magnitude of their concentration decrease, the following order appeared: 6'-SL >> LNFP I > β 6'-GL > LNT > LNDFH I > LNnT > 2'-FL > total HMOs. If the proportions of type I and type II HMO-core structures in this group are considered, a clear preponderance of type I-core HMOs (LNDFH I, LNFP I, LNT) can be recognized. LNnT was the only type II HMO.

In contrast, the set of HMOs which appeared to be stable in this important period for early life development until the approximate start of weaning included the sum of LNDFH II and LNnDFH II, LNFP II, LNFP III, LNFP V, DFL and 3'-SL (**Figure 5**).

Apparently, LNFP III, LNFP V, and 3'-SL were also among the HMOs which did not change significantly in concentrations between the 4 HM groups. These HMOs are independent from maternal Se-and Le-genotype. Their exceptional low variation across HM groups and during progressing lactation until the approximate start of weaning (at 4-6 months pp) could indicate a prominent functional role of

these glycans for healthy infant development. The latter might also be true for LNnDFH II which is also not dependent on expression of maternal Se- or Le-genes. Unfortunately, the levels of this structure could not be determined independently but as a sum including the isomer with opposite HMO-core type (i.e. LNDFH II). By restricting the criterion of concentration stability across milk types and over staging until 6 months to either the Se(+) HM groups I and III, or the Se(-)Le(+) HM group II, more HMOs would match this condition. In this case, DFL contained in HM groups I and III and LNFP II from HM group II would be eligible and would share the feature of high concentration-stability up to 6 months pp.

Stratification of HMO variations to the lactation period from 6 month to 12 month post partum

Intriguingly, the group of HMOs which significantly declined in concentrations in HM from 6 month to 12 months was reduced to only 3 structures (see **Figure 6**). Here, 6'-SL and LNnT abated while also LNFP III started to drop significantly. Moreover, 3'-SL was the sole HMO which significantly increased by concentration in this period of HM lactation. Thereby, 3'-SL and 6'-SL manifest as the second pair of HMO-isomers with opposing concentration changes in this period of late lactation next to 2'-FL and 3-FL in early lactation. Regarding type I and type II HMO core-structures, now only type II-glycans (LNFP III and LNnT) were part of the group with significant concentration changes in addition to the Lactose core structures. In the time range from 6 month to 12 months pp, all type I core structures didn't vary significantly in concentration anymore and were very stable (see **Figure 7**).

Stratification of HMO variations to the lactation period from 6 weeks to 12 month post partum

If the view is expanded to the lactation range from 6 weeks up to 12 months pp, the number of HMO structures exhibiting stable concentrations across this period and across all HM groups is further reduced to LNFP V and LNFP III. Considering also the stable HMOs which were belonging to only one of the individual HM groups, LNDFH I needs to be regarded with respect to HM group I and LNFP II needs to be added for HM group II.

Of note, 3 HMOs with Lactose-core (6'-SL, 3'-SL and 3-FL) exposed the most extreme abundance-changes over various HM lactational stages (see **Figure 1**, **Figure 5**, and **Figure 6**). 6'-SL showed the most continuous and strongest decline, 3'-SL and 3-FL had the strongest increases (at different periods of staging). Surprisingly, they shared also another communality: They belonged to the HM-group-independent "core set" of compounds (i.e. $(Hex)_3$ /Galactosyllactoses, $(Hex)_2$/Lactose, LNFP III, LNT, 3'-SL, 6'-SL, and $(Hex)_4$) as defined in **CHAPTER IV**.

In sum, the observed trends for variations of individual HMOs and total HMOs were again very similar in the other studied HM sets comprising the Infat study (**CHAPTER IV**) or the Prevent CD study (**CHAPTER VIII**). Noteworthily, the unveiled HMO-variations did apply for two different categories of HM donors: One category consisted of predominantly healthy subjects resident in Germany (INFAT and Ulm SPATZ study/cohort) and the other consisted of milks received from mothers with the offspring at risk to develop celiac disease (Prevent CD study). The latter HM donors were resident across Europe (Germany, Hungary, Italy, the Netherlands and Spain). Obviously, the partly present genetic predisposition to inherit celiac disease had at least no major influence on the staging trends received for abundant HMOs up to Hexaoses.

IN VITRO UTILIZATION OF HMOS IN A MODEL SYSTEM FOR EARLY LIFE BACTERIAL CROSSFEEDING BETWEEN *BIFIDOBACTERIUM INFANTIS* AND BUTYRATE PRODUCING *ANAEROSTIPES CACCAE*

Prebiotic effects of HMOs but also dietary fibers like scGOS/lcFOS foster the early life colonization and the growth of anaerobic bifidobacterial species and other beneficial microorganisms. *Bifidobacerium* and *Bacteroides* are so called keystone taxa of the early life microbiome [48]. A bifidobacteria-dominated early life microbiome contributes to gut health by lowering the pH in the colon, production of short chain fatty acids (SCFA)[49], and reduction of clinically relevant pathogens. Among the released SCFA, acetate is dominating over other SCFA. Wopereis et al. [50] indicated that the gradual shift of the early life gut microbiome towards a more adult microbiome occurs approximately between 4 to 6 months pp but this shift is further continued up to 3 y and beyond. This timing corresponds with the initiation of complementary feeding by solid food (weaning). In healthy subjects, a shift in microbial diversity during this period is also characterized by an increase of butyrate sequestering species like e.g. *Bacteroides.* Nevertheless, levels of bifidobacterial species remain to be high. A well-tailored phasing and balanced transition of the early life gut microbial diversity towards a more adult like microbiome may also prevent development of allergic diseases. *Bifidobacterium* and *Bacteroides* may modulate balanced immune development by increasing the number of interleukin 10 (IL 10) producing regulatory T cells (Treg) e.g. through Toll like receptor 2 (TLR 2) signaling. An increased level of the enteric SCFA butyrate is and indicator for gut microbiome maturation. Importantly, short chain fatty acids (SCFA) like acetate, propionate, or butyrate serve as substrates for enteric epithelial cells. Beyond this SCFA may benefit the integrity of the gut barrier [51]. The gut barrier can be comprehended as an integral part of the innate immune system forming a first line of defense against pathogens or “foreign” molecules. SCFA may also function as

epigenetic regulators by modulation of e.g. histone acetylation [52], [53]. Thereby, they may contribute to "the link between early nutrition, microbiome, and long-term health development" as outlined by Indrio et al in 2017 [54]. Here, butyrate and propionate may be more effective than acetate [55]. Finally, an increased butyrate production may also be useful to ameliorate inflammatory processes of the intestine like inflammatory bowel disease (IBD) [56] or even severity of COVID-19 by promotion of Treg cells [57, 58].

Owing to the various possible benefits of butyrate producing bacteria, the role of HMOs in an early life in vitro model system for crossfeeding between *Bifidobacteria* and typical butyrate producing microorganisms was investigated. By employing bacterial mono- and co-cultures of *Anaerostipes caccae* and *Bifidobacterium infantis*, it could be demonstrated that *B. infantis* utilizes the complete pattern of lactose and HMOs from a type I HM (see **Figure 4**, **CHAPTER V**). These insights were obtained via differential HMO analysis at the start and the end of bacterial fermentation by MRM LC-ESI-MS^2. The set of completely consumed HMOs included 2'-FL, 3-FL, DFL, LNT, LNnT, LNFP I, LNFP II, LNFP III, LNFP V, 3'-SL and 6'-SL. This result is corroborating well with massive, published evidence for the ability of *Bifidobacterium infantis* and other strains from this genus to grow well on various types of HMOs (acidic, neutral, type I and type II)[59-61]. It again underlines the strong bifidogenic/prebiotic effect exerted by HMOs. *Anaerostipes caccae* alone could not grow on HMOs but did thrive and produce butyrate in co-culture with *B. infantis*. Hence, trophic interaction between the two species and a key ecological role of *Bifidobacteria* could be confirmed: At least in vitro, *B. infantis* crossfeeds butyrate producing species like *A. caccae* with metabolites such as liberated HMO-monosaccharides and acetate or lactate. Thereby, supply with butyrate may be ensured which may support EL health by its benefits as discussed above. In a similar in vitro set up, Chia et al revealed in 2020 [62] that crossfeeding also takes place between *Bacteroides thetaiotaomicron* and *Anaerostipes caccae.* Changes of HMO levels in the bacterial mono- and co-cultures were characterized by the same MRM LC-ESI-MS^2 approach as described in **CHAPTER III**. Again, A. caccae alone could not consume HMOs. Compared to the results obtained for the monoculture of *B. infantis* in **CHAPTER V**, *B. thetaiotaomicron* did consume HMOs, but not as quickly and completely. Surprisingly, *B. thetaiotaomicron* followed a specific differential kinetic of HMO consumption. After 24h, still residual amounts of lactose and all HMOs up to pentaoses, which were detectable by the applied MRM LC-ESI-MS^2 method, were present in different abundances. However, the HMOs 2'-FL, 3-FL, 3'-SL and 6'-SL were utilized with preference and consumed to more than to 50%. In the co-culture, consumption of all HMOs was much more pronounced compared to the monoculture. After 48h in co-culture, lactose, 2'-FL, 3-FL, DFL, LNnT, LNFP II, LNFP III, LNFP V and 3'-SL were not detectable any more. Only trace amounts of 6'-SL, but approximately > 6% of the initial LNT- and LNFP I-concentrations were still left in the supernatants derived from the co-culture.

These results show that also *B. thetaiotaomicron* can utilize HMOs with various structural features such as fucosylation, sialylation, and type I or type II glycan backbones. While thriving on HMOs, *B. thetaiotaomicron* also provides crossfeeding to butyrate producers like *A. caccae.*

In sum, the insights discussed in this chapter further complement the hypothesis that *Bifidobacterium infantis* and further species from the genus *Bifidobacterium* have an advantage in early colonization. Supported by our in vitro experiments, this advantage of *Bifidobacterium infantis* builds on its ability to completely utilize the full range of (investigated short chain) HMO-structures as carbohydrate substrate. *B. thetaiotaomicron* also thrives on HMOs, however, follows a slower kinetics compared to the prototypical HMO-degrader *B. infantis.* This might be caused by the slightly higher preference of this bacterium for longer chain HMOs according to Asakuma et al. 2011 [61].

The described results further fit to the well-established notion that prebiotic and bifidogenic HMOs (see e.g. **CHAPTER II**, **Figure 3**), support early colonization of Bifidobacterial species in the developing gut (see **Figure 1A** and **1B**, **CHAPTER V**). Bifidobacterial colonization is one step in the gradual conversion of the EL microbiome and occurs after establishment of anaerobic conditions by facultative anaerobes like e.g. *Enterobacterium and Lactobacillus genera* [48, 63, 64]. After appropriate establishment, *Bifidobacteria* together with e.g. species from the genus *Bacteriodes* may help to cross feed butyrate producers which start to thrive in a later phase of gut microbiome maturation after approximately 3 month of age (see **Figure 1A** and **B**, **CHAPTER V**). These observations may also raise the question if (immune-) regulatory effects of butyrate need to be compensated by other functional molecules in the earlier phases of microbiome maturation before 3-4 months pp. The overall level of HMOs is highest in the first weeks after parturition and declines until weaning (see **Figure 4**). This happens just before numerical increase of butyrate producers like Lachnospiraceae, Ruminococcaceae and some Bacteroidetes [65] is starting. It is therefore tempting to speculate if other HM ingredients including particular HMOs or even free butyrate in HM [66] could be involved in filling the functional butyrate gap prior to weaning.

POSSIBLE IMPLICATIONS OF INVESTIGATED HMOS AND THEIR VARIANCES ACROSS HM GROUPS AND THE STAGES OF HM LACTATION ON A BALANCED EARLY LIFE IMMUNE DEVELOPMENT

The nutritional value of HM is defined by its macronutrients largely represented by Lactose, lipids, and proteins. Nevertheless, this macronutrient composition of HM shows a high variability e.g. between different subjects and is influenced by many factors such as HM staging, maternal age and body composition [67-69]. It has been

proposed that HM composition might adapt dynamically to the nutritional needs of the growing infant. Beyond mere supply with energy and molecular building blocks for growth, HM ingredients like HMOs may convey other important functions (see **CHAPTER II**). They may e.g. support the balanced development of innate and adaptive immunity in early life (EL) by direct or indirect effects. Direct influence of HMOs on the neonate's immune systems is for example mediated via (immune-cell-) receptors like C-type lectin receptors (CLR)[70], Toll-like receptors (TLR) [71], sialic acid–binding immunoglobulin-like lectins (Siglecs) [72], selectins [73], or galectins [74, 75] which do recognize (multimeric to polymeric) carbohydrates. Indirect influence on immunity and healthy development is e.g. exerted by beneficial microorganisms like bifidobacteria [76-78] [79, 80] or lactobacilli [81] which can utilize otherwise non digestible oligosaccharides (NOD) like HMOs [82] as substrates. Here, the microorganisms themselves or their metabolites [83] may be the active immune modulating factors. The observable changes in levels and proportions of individual HMOs over various stages of HM lactation up to 12 months pp might reflect a purposeful necessity: Beyond adaptation to the changing nutritional needs of the growing infant, the variations of HMO might also be exactly tailored to support the different developmental stages of infant's' immunity in an optimal way. The beneficial impact of HMOs, conveyed to the offspring e.g. via HM, might last even beyond cessation of breastfeeding.

In the following paragraphs, it will be attempted to interpret the detected variations of HMOs regarding possible implications on the developing early life immunity and other aspects of early life.

Possible effects of characterized HMOs and HMO-variations on innate an adaptive immunity

Literature suggests stimulation of neonatal immune maturation by HMOs [84]. Regarding the evolving adaptive immunity of the neonate, HMO-epitopes like Le^x do for example modulate differentiation of T helper cells (Th) into Th2-polarized T cells via DC SIGN signalling [85]. In general, Th cells can differentiate into various subsets including Th1, Th2, Th 17, Tfh (follicular T helper cells) and regulatory T cells (Treg) [86]. Selected HMO may, together with further EL-effector molecules, help to instruct the developing immune system in mounting a higher Th2-capacity while avoiding Th2 linked overreactions like allergic responses (e.g. cow's milk allergy, peanut allergy etc.). A strengthened Th2 immune response favours the protection against threats by certain parasites, viruses or bacteria. In parallel, another subfraction of HMO might continuously increase Th1-capacity in concert with other EL factors to prevent intracellular bacterial or viral infections more effectively.

There is at least first in-vitro evidence for the Th1 promoting effects of the acidic HMOs subfraction directing the neonatal Th2 T-cell phenotype towards a more balanced Th1/Th2 profile [87]. Furthermore, administration of 2'-FL to the feed of

mice led to a Th1-skewing and increase in Treg markers [88]. These effects were independent from possible microbial interaction and dose dependent. In this respect, it is intriguing that 2'-FL showed the highest concentration of all HMO in HM of Se(+) donors (**Figure 1**) and that this HMO significantly decreased in concentration from 6 weeks pp to 6 months pp. Because neonates are born with a Th2 skewed phenotype, the high 2'-FL levels and its Th1 promoting effects may be a contribution of HM to gradually develop Th1/Th2 homeostasis in the infant. 2'-FL might employ these effects via TLR [89] and / or DC-SIGN [90] signalling. It has not yet been investigated in detail if other α 1,2-fucosylated HMOs like LNFP I or LNDFH I which show the same staging trends can retain or even amplify the effect of 2'-FL. Moreover, it might be interesting to verify if also HMOs like LNT or 3'-FL which are present in both, Se(+) and Se(-) HM can have a similar function. At least 3'-FL also binds to DC-SIGN)[90] in addition to its other beneficial effects [90-92].

Remarkably, a low Th1/Th2 immune status seems to be an unfavorable situation. It was hypothesized earlier that neonates with allergies are born with a weaker Th1 response, but apparently, they are presented with an overall low Th1/Th2 response [93]. Just recently and apart from EL development, Gutiérrez-Bautista et al [94] reported that negative clinical evolution in COVID 19 patients was frequently accompanied with an increased proportion of undifferentiated Th cells and a strong underrepresentation of the Th1-subset.

More generally, it will be crucial to better understand which specific glyco-moieties of HMOs or HM glycoconjugates (e.g. glycoproteins [95] and glycosphingolipids) are essentially contributing to early life immune maturation. Furthermore, the interaction with other HM ingredients [96] and exogenous [97] or endogenous [98] EL-factors need to be considered here. Consequently, the improved analytical distinction of HMO-isomers carrying immuno-functional residues like Le-epitopes could be a further step to achieve this goal. A Th1/Th2 based immune homeostasis, well-tailored to the developmental stages of the infant, may be particularly facilitated by distinct variations of HMO-levels across HM groups and during HM staging (see **CHAPTERs IV, VI,** and **VIII)**. In future, it may also be important to unveil in more detail how individual HMOs or distinct functional HMO-groups interact with the innate and the adaptive immune system. This may allow to extend hypothesizing about (beneficial) implications of HMOs on immune homeostasis in early or adult life. Consequently, these aspects will be explicated in the following paragraphs.

Anti-infective effects of HMOs contributing to innate immunity

Activation of innate immune pathways occurs if pathogens or other "threats" (i.e. pathogens, "non self" molecules) managed to overcome a first line of defence assembled by innate immunity. This first line of defence are chemical, physical or biological barriers like epithelial surfaces and mucus (e.g. the "gut barrier") or a

protective microbiota preventing colonization of pathogens. Meanwhile, HMOs are part of this initial innate defence mechanism [99]. As reported by Natividad et al. in 2020, a mix of short chain HMOs containing 2'-FL, DFL, LNT, LNnT, 3'-SL and 6'-SL improved integrity of a Caco-2: HT29- methotrexate (MTX) cell line model for the gut barrier after a challenge with TNF-α and IFN-γ [100]. 2'-FL proved to be most effective among the tested HMOs. In a Caco-2 cell intestinal model, DP2 and DP3 from a mixture of dietary galactooligosaccharides (GOS) restored the epithelial barrier function most effectively after exposure to the mycotoxin deoxynivalenol (DON) [101, 102]. Some of the structures contained in these DP2 and DP3 fractions are also HM ingredients like lactose (DP2) or the HMOs β 4'-GL and β 3'-GL (DP3) [103, 104].

HMOs do also convey a first line protection against EL-viral or bacterial infections which can infest the gastrointestinal, urogenital or respiratory tract. HMOs block specific receptors of these pathogens which otherwise would bind to glycan residues on inner surfaces like the intestinal epithelial glycocalyx [99, 105]. Thus, acting as "decoy" ligands for harmful microorganisms, HMOs prevent the initial step of infection which is adhesion. The early life threat caused by infectious diseases and the variety of causative pathogens is substantially high. Worldwide, more than half (51.8 %) of the deaths of children who succumb to infections occur within the first two years of life. [106]. Examples for such detrimental early life pathogens are for example *Respiratory syncytial virus* (RSV), *Measles morbillivirus*, *Influenza, Mycobacterium tuberculosis*, *Enterotoxic E. coli* (ETEC), *Enteropathogenic E. coli* (EPEC), *Shigella*, *Vibrio cholerae, Rotavirus*, *Norovirus*, and Plasmodium spp. e.g., falciparum (malaria) [106]. A subset of these early life pathogens which utilize glyco-epitopes to adhere to inner surfaces (i.e. the first step of infection) include e.g. *Norovirus*, *Rotavirus, ETEC, Uropathogenic E. coli, Influenza, HIV.* In such cases, infections could be prevented or ameliorated by ingested HMOs locally in the GI-tract but also in other body compartments: Approximately 1-2% ingested HMOs also become systemically available[107, 108]. This reasoning is primarily supported by *in vitro* experiments and considerations about the known receptor specificities for glycans resembled by distinct HMOs [109-119]. In 2017, Morozov et al. [120] reviewed e.g. the antiviral capacity of HMOs. Individual HMOs were described to very effectively block noroviruses, rotaviruses, various strains of influenzaviruses, or HIV by virtue of their pathogen receptor specificities. In more detail, 2´-FL, 3-FL, LNFP I, LNFPIII, LNDFH I, and DFLNHa were effective for / interacting with (different) norovirus strains. LNDFH I, LNFP I, LNT, LNnT were recognized by various rotavirus strains whereas 6´-SL and HMOs with Neu5Acα2,6-Gal-β1,4-GlcNAc-residues interacted with influenza strains. In addition, multimeric Le^x-glycans inhibited DC-SIGN-mediated transfer of HIV-1 to CD4+ T lymphocytes. Most interestingly, this could not be achieved with the monovalent Le^x-glycans [121]. More examples for HMO- or glycan-residues interacting with bacteria or other microorganisms are tabulated in **Table 2** of **CHAPTER I**. Further examples for distinct anti-infective HMO effects *in vivo* have

been demonstrated in several studies. It was concluded that very specific HMO-structures were able to reduce the interaction of specific pathogens like Salmonella, Shigella, *Vibrio cholerae, E. coli*, Polioviruses, and *Respiratory syncytial virus* (RSV) with the host [122, 123]. Additional studies revealed that HM levels of 2'-FL, Lacto-N-difucohexaose I (LNDFH I) and other α 1,2-linked fucosyloligosaccharide as well as the ratios between alpha 1,2-linked to α 1,3/4-linked fucooligosaccharides in HM were negatively associated with presence of specific pathogens like *E. coli, Campylobacter* and *Norovirus* and incidences of gastrointestinal symptoms [124, 125]. A further publication stated that levels of LNFP II (containing one α 1,4-linked fucose-residue) measured at 2 weeks pp were associated with decreased risks to develop respiratory problems at 6 and 12 weeks after parturition [126].

Strikingly, many HMOs for which literature most frequently states essential anti-infective properties (see above) are among the HMO-compounds with the highest detected concentrations as confirmed by this thesis (see e.g. **CHAPTER VI**). If averaged over the complete range of investigated stages of lactation (i.e. across 6 weeks, 6 months and 12 months pp) the order by concentration for this high abundant group of HMOs as visualized in **Figure 1** is: 2'-FL >> 3-FL > LNT > LNDFH I > LNFP I > LNFP II > DFL > LNFP III > 3'SL > 6'-SL.

Taking the previous paragraphs together, it becomes evident that distinct strains of the same viral or bacterial genus do prefer various, structurally different HMOs as ligands to adhere to. Hence, effective adhesion-blocking of all possibly existing or evolving pathogens on strain-level would need a plethora of HMO-glyco-epitopes to create a "future proof" pool. This might, at least in part, explain why "nature" created the astonishingly high structural diversity of HMOs which comprises also high molecular weight "long chain" and complex HMO [127].

Furthermore, the formation of the 4 HM groups could be a result of evolutionary pressures imposed by regional epidemic outbreaks of infections. The geographically diverse and distinct distribution of maternal predisposition to express secretor-positive or -negative HMs [39] may be one indication to substantiate this idea. Unfortunately, complete structural characterization has only be obtained for the most prominent out of more than 1000 probably existing HMO-structures [29]. A better structural characterization of also high molecular weight "long chain" HMOs by modern analytical means as proposed in the paragraphs above may reduce this gap of knowledge in future. Once identified, these new HMOs could be synthesized and subsequently tested in vitro for binding to relevant pathogens [24, 120]. Thereby, novel decoy ligands for pathogen receptors could be defined. Successful HMO candidates would then extend the portfolio of desperately needed new anti-infective agents which may e.g. be effective against antibiotic resistant pathogens. Surprisingly, also monovalent "short" chain HMOs have just recently been reported

to inhibit the growth of group B *streptococcus* (GBS). This pathogen is a "leading cause of invasive bacterial infections of newborns" [128]. Neutral type I core HMOs like LNT and LNFP I exerted host independent direct bacteriocidic effects and did also synergistically boost efficiency of established antibiotics. Noteworthily, LNFP I and LNT are among those HMOs with the highest average concentrations across lactation up to 12 months pp as stated in **CHATPER VI** or **Figure 1**. In addition, LNT is part of the maternal genetic Se-/Le predisposition independent group of "core HMOs" and present in virtually all HMs (see above and **CHAPTER IV**). LNFP I is at least present in all Se(+) HM i.e. in approximately 80 % of all milks from Europe, China, or North America. The set of HMOs active against GBS is complemented by 3-FL and LNDFH I. Both structures were negatively correlated with colonisation of GBS in breast fed Gambian infants [129].

Especially in early life, glycoproteins of intestinal epithelial cells are sialylated. Pathogens can bind to these sialylated sites. In vitro, 3'-SL and 6'-SL can competitively inhibit pathogen binding and thereby potentially reduce infections with *Salmonella*, S-fimbriated *E. coli* strains, *Vibrio cholera*, *Campylobacter jejunii*, *Helicobacter pylori*, and rotavirus[130]. Sialylactoses were unfortunately ineffective against *H. pylori* in *in vivo* studies. In 2018, Pandey et al. confirmed a broad neutralization capacity of both SL-isomers against avian influenza viruses [131]. This included the subtypes AI H1N1, H1N2, H3N2, H5N1, H5N8 and H9N2. Those revelations might add to the idea that HMOs could be used as promising antiviral agents for emerging threats. By coincidence, the first transmission of the H5N8 subtype to humans was reported in Russia in 2021. The infection inflicted mild symptoms.

If HMOs with multiple functional epitopes (e.g. difucosylated LNDFH I) have stronger anti-infective properties (e.g. mediated through higher receptor avidity) compared to HMOs with only one functional epitope (e.g. monofucosylated LNFP I or 2'-FL) remains to be investigated. Nevertheless, in vitro results do give first hints: For example, an up to 1-million-fold higher avidity for binding to *Norovirus* P-domain by polyvalent fucosylation compared to an univalent ligand was revealed by Krammer et al. in 2018 [113]. The identification of rare HM groups like HM group Ib (see **Chapter VIII**) which e.g. contain LNDFH I (divalent) but no or low levels of monovalent LNFP I and 2'-FL compared to HM groups I or Ia could be very helpful here. For example, in vivo data about severity, duration and frequency for episodes of gastrointestinal infection available from HM cohorts could be compared for milks from HM group Ib and I+Ia. If no significant statistical difference would e.g. be found between these groups, one could assume that the divalent HMOs would exert the major protection.

Hypothetical roles of HMOs in linking homeostasis of innate and adaptive immunity
The innate immune system recognizes pathogens or "foreign" substances and eliminates them via an inflammatory response, the complement system or

lymphocytes. Early life threats are detected by specific pattern recognition receptors (PRR). PRR include membrane bound toll like receptors (TLRS) and C-type lections, or cytoplasmic NOD like receptors (NLRs). These receptors sense conserved pathogen-associated-molecular-patterns (PAMPS) or danger-associated-molecular-patterns (DAMPS). Among other molecules like nucleic acids, glycans and glycoconjugates exposed by the outer cell membranes of gram-negative or gram-positive bacteria (e.g. lipopolysaccharides (LPS) or lipoteichoic acids, respectively), do act as PAMPS. LPS are for example sensed by TLR 4 (expressed by macrophages, B-cells, dendritic cells (DC), or mast cells), whereas lipoteichoic acids or peptidoglycans are detected by TLR2 (expressed e.g. on macrophages, DCs, mast cells).

Furthermore, the innate immune system also activates the adaptive immune system by antigen presentation via antigen presenting cells (APCs) such as macrophages or dendritic cells. Antigen presentation by APCs to undifferentiated T-cells occurs via MHC class I or II molecules and the T cell receptor (TCR) [132]. If T-cell CD28 co-stimulation is provided by e.g. CD80 or CD86 on APCs, T-cells differentiate into Th1, Th2, or Th17 T-cell types. DC toll like receptor (TLR) signalling, e.g. induced by glycan motifs of microorganisms [133] or HMOs, can instruct expression of the necessary co-stimulatory CD 80 or CD86 patterns by NF-kB activation and promotes maturation of DCs. In this context, it needs to be mentioned that 2'-FL did increase the in vitro expression of CD86 and CD40 on DCs [88].

Without co-stimulation and the absence of danger signalling, C-type lectin receptor (CLR) mediated uptake of antigens by DCs and presentation to T cells leads to a switch into regulatory T cells (Treg). Treg-cells dampen both, Th1 and Th2 responses and may ameliorate Th2 or Th1 related clinical phenotypes like allergy [134] or Type I diabetes [135]. CLRs comprise for example DC-SIGN or dectins. Of note, it has been proposed that the extent of CLR receptor crosslinking on APCs (DCs) by multimeric glycan epitopes might influence the strength and endurance of signalling cascades. Moreover, the nature of glycan epitopes recognized by DC receptors (e.g. GlcNAC vs Le-epitopes) could result in disparate stimulation of intracellular immunological pathways. Interestingly, Lewis Y determinants expressed by *H. pylori* during a specific phase variation of this pathogen triggered development of a Th2 phenotype [136]. The free soluble form of the Lewis Y determinant is for example resembled by the HMOs LNnDHF I (see **Figure 1 CHAPTER IV**). Besides this, a GlcNAc (β 1-3)-Gal (β 1-4)-Glc-residue on a LgtB mutant form of *Neisseria* meningitidis caused a Th1 profile via DC-SIGN recognition [137]. Accessible univalent GlcNAc (β 1-3)-Gal (β 1-4)-Glc structures can be created from type I HMOs like LNT by acid hydrolysis in the stomach and low concentrations have also been detected directly in HM. Acid hydrolysis of more complex HMO-core structures [75, 138] may result in multi- or polyvalent GlcNAc (β 1-3)-Gal- epitopes inducing Th1 polarization via DC-SIGN recognition. Intriguingly, in 2019 Cheng et al. [139] reported a dose dependent

induction of macrophage-TLR signalling by univalent LNT2, 2'-FL, 3-FL, 6'-SL, and LNnT. LNT2 had the most potent effects and activated a broad range of TLRs from TLR 2 to TLR9. Among the other tested HMOs, 3-FL activated TRL2 and TLR1/6, but inhibited TLR 5, 7, and TLR 8. 2'-FL, 6'-SL, and LNnT inhibited TLR 5 and TLR7. An interesting example indicating that the same glycan residues can provoke opposite immunological reactions if the monovalent form is compared to its multivalent counterparts is the Le^x epitope. Helminths expose multivalent Le^x e.g. on the surface of their eggs and induce a Th2-shift as well as IL-10 secretion mediated by DCs [140]. This leads to a kind of immunological anergy and allows these parasites to escape from immunological clearance. Surprisingly, the state of anergy can be reverted by monovalent form of Le^x which is represented by the HMO LNFP III.

Sialic-acid-binding immunoglobulin-like lectins (Siglecs) are a group of transmembrane-receptors which are found on many immune cells like for instance macrophages, B-Cells or also DCs [141]. Their function can be interpreted as immunosuppressive [142]. Through their cytosolic immune receptor tyrosine-based inhibitory motifs (ITIMs) they can suppress activation signals that emanate from receptors associated with immunoreceptor tyrosine-based activation motifs (ITAMs) [143]. ITAMs or ITAM like motifs are also expressed by DC CLRs like Dectin-1 or DC-SIGN. Especially multivalent glycan patterns (on surfaces of microorganisms or host cells) can crosslink APC receptors and thereby enhance signalling as mentioned above. Receptor crosslinking could probably also be accomplished by multivalent HMOs with similar structural motifs. Thereby these HMOs may link and guide innate and adaptive immune responses. If more specific (polyvalent) HMO-residues which may help to repolarize disparate immune responses could be identified in future, new remedies for many early and adult life impairments may be inspired. A comprehensive review about acidic oligosaccharids in human and other mammalian milks with implications for brain and gut health has been published by Hobbs et al. in 2021 [144]. Some of the described benefits of sialylated HMOs and animal oligosaccharides might be mediated by their function as Siglec ligands.

Different Siglecs are characterized by their unique specificity for sialylated ligands: For example, Siglec-2 (CD22, (with ITIM)) on B-cells prefers a Neu5Ac α2-6 Gal β 1-4 GlcNAc-residue with 6-sulfation of GlcNAc. CD 22 is associated with lymphoma, leukemia, SLE, and rheumatoid arthritis. Siglec-3 (CD 33) which is displayed e.g. by macrophages, microglia, granulocytes or monocytes and signals via ITIM binds to a Neu5Ac α2-6 Gal β 1-4 GlcNAc-residue. The latter epitope is exhibited partly by the acidic HMO 6'-SL and fully by type II core LSTc or F-LSTc. In F-LSTc, the reducing end is also carrying a by a α1-3 linked Fucose. CD33 is linked to acute myeloid leukemia (AML) and Alzheimers disease. Human Siglec-10 (ITIM, B-cells, Monocytes, Eosinophils) has a relevance for lymphoma, leukemia, eosinophilia, and allergy and recognizes Neu5Ac α2-6 Gal β 1-4 GlcNAc - but also Neu5Gc α2-6 Gal β 1-4

GlcNAc-motifs. Neu5Gc α2-6 Gal β 1-4 GlcNAc-motifs are absent from human milk but present among oligosaccharides found in other mammalian milks such as goat milk [145].

Siglecs on APCs (DCs, macrophages) may also modulate TLR cytokine responses by ligation with host glycans e.g. sialylated residues on colonic epithelial cells [142]. Interestingly, Sialyl 6-sulfo Lewis^x and Disialyl Lewis^a which are expressed by healthy colonic epithelial cells have for example been identified as ligands for Siglec-7 and -9 on resident macrophages. These Siglec ligands maintain immunological homeostasis in colonic mucosal membranes. On the contrary, loss of immunosuppressive glycans and replacement by carcinogenesis associated Sialyl-Lewis^a and Sialyl-Lewis^x [146] enhances macrophage related inflammatory mediator production [142]. The Disialyl Lewis^a epitope is also present in HM in form of the free HMO FDS-LNT. In addition the free HMO-forms of Sialyl-Lewis^a and Sialyl-Lewis^x have been identified in HM as F-LSTa and LSTd, respectively [147]. The elucidation of the unknown staging behaviour of these rare HMOs might shed more light on their possible role in early life healthy development. Following structural similarity of residues, also α2-3 sialylated HMOs with 6-sulfo-GlcNAc might partly be able to suppress proinflammatory immune signalling by activation of CLRs and/or TLRs e.g. carried by DCs. Moreover, the comparably high HM levels of 6'-SL might probably help to protect the just colonizing and developing microbiome by (low affinity) binding to e.g. Siglec 3. Astonishingly, the decline of 6'-SL concentration over the course of lactation (see **Figure 4**) happens in parallel to the transformation of a predominantly (α2,6?-) sialylated gut lining to a fucosylated one [148, 149]. This transformation has been observed in mice and is assumed to pertain for other mammalian species. On the other hand, 3'-SL which increases during staging could counterbalance 6'-SL to prevent a to high level of immunotolerance. This could occur in synergistic action with (monovalent) LNFP III through their TLR4 mediated proinflammatory effects [89]. Indeed, it could be proven that anti-inflammatory impact of 6'-SL alone or in combination with 3'-SL could ameliorate induced colitis in a mouse model. However, mice fed with 3'-SL only developed even aggravated colitis [150]. The TLR 4 mediated proinflammatory role of 3'-SL in an intestinal IL-10 knockout mouse model could also be demonstrated by Kurakevich et al [151]. They stated that "3'-SL directly stimulated mesenteric lymph node CD11c+ dendritic cells and induced production of cytokines required for expansion of Th1 and Th17". If 6'-SL is directly involved via Siglec binding or if it is co-inducing the early life dominance of gut epithelial sialylation may remain to be clarified.

To shed more light onto the question which structural features of HMOs are most relevant for a healthy development of infants and their immune system, also more complex "long chain" HMOs should be considered in future. The reasoning behind is that with increasing molecular weight also the chance to bear multivalent functional epitopes increases. The latter might be more potent in e.g. crosslinking several

immunoreceptors as discussed in the paragraphs above. To meet this goal in an efficient way, it may be promising to define new HMO-subgroups based on the 4 known HM groups. Those groups could e.g. be subdivided by features like HMO chain length, branching, multivalency or ratios of Le-epitopes etc. This proposal follows the idea that HM groups sharing very similar structural features may trigger comparable biological effects. These effects might differ in magnitude dependent on further HMO-structural features next to those of the functional glycan epitopes. The latter aspect might be neglectable in the first instance if the primary research goals is to define new hypotheses about functional HMO epitopes located at the non-reducing HMO terminus. Then, just a selection of HMOs, the representatives for defined HM subgroups, would need to be analysed in specimens e.g. derived from HM studies or cohorts. Such an approach would enormously reduce the necessary number of short and long chain HMOs to be characterized from the possible range of > 1000 individual HMO-structures. Nevertheless, most of the probably relevant new long chain but also short chain HMO-structures would be covered. Once sufficiently characterized in HM by hyphenated analytical approaches (see paragraphs above), differences in levels or proportions of marker HMOs between such predefined HM groups could be statistically tested and associated with health trajectories of infants. Also selected HMOs with promising structural features could be preclinically tested relative to each other. This might accelerate the prediction of EL health benefits associated with the structural microheterogeneity of yet unknown HMOs. It would also support timely translation of possible new insights for example into patents or improved infant milk formula recipes. Functional preclinical testing of HMO with promising structural features could also be extended to glycoconjugates with epitopes resembling those of selected HMOs. Thereby, a more holistic approach would be created which is more representative of HM compounds other than HMOs.

Taken together, HMOs may foster the gradual establishment of a more balanced Th1/Th2 phenotype in the developing infant in synergy with other exogenous and endogenous EL factors [152, 153]. This may in part be achieved by distinct interactions with various immunoreceptors including CLRs, TLRs, siglecs, galectins and selectins. In general, many HMOs (e.g. 2'-FL, 3'-GL, DSLNT) but also various other HM components like the glycoproteins lactoferrin and lactadherin, peptides like β-defensin 2 [154-157], or soluble TLR inhibitors (sTLR2, sCD4, sTLR4, sTLR5) do exert a strong anti-inflammatory effect [89]. Moreover, the beneficial prebiotic, bifidogenic and immunological influences of HMOs may not be restricted to local compartments of the human physiology (e.g. mucosal immunity), but may also apply systemically [84]. It might be this synergistic impact which does not only co-drive the maturation of a favourable early life (gut) microbiome, but which benefits the healthy development of infants in general. Such beneficial effects of HMOs maybe closely tailored to the developmental stages of infants by purposeful variation of HMOs over the stages of HM lactation [158, 159].

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CHAPTER X

English Summary

This thesis is focused around the topic of **"Advanced Characterization of Biofunctional Human Milk Oligosaccharides by Mass Spectrometry and Complementary Methods**". After a general introduction into the compositional features of human milk (HM) and comparison to cow's milk (the most widely used natural basis for infant milk formula production), the structure and objectives of this work are outlined in **CHAPTER I**.

Human milk oligosaccharides (HMOs) contribute substantially to the composition of human milk (HM) and represent its third most abundant molecular fraction. HMOs may exert various beneficial effects favoring the healthy development of the neonate. The relationship of structural and quantitative HMOs variations and their consequences for healthy early life (EL) development of breast feed infants is of high interest. Moreover, immunomodulatory and EL-protective ramifications of HMOs are a promising fields of human milk research. In this context, the specific relevance of HMOs for the early life immune development and susceptibility to neonatal and childhood infections are reviewed in **CHAPTER II.** This chapter summarizes how HMOs exert intriguing anti-infective, prebiotic, and immune homeostatic functions e.g. via distinct dendritic cell (DC) receptors or galectins. In addition, modulation of pathogen recognition, and strengthening of the mucosal barrier by HMOs are discussed. As summarized in **CHAPTER II** and **Table 2** in **CHAPTER I**, most contemporary *in vitro* or *in vivo* investigations of prebiotic, immunological or other HMO functions are limited to a few characteristic HMO-fractions: The total HMO-fraction isolated from HM, the total neutral or acidic HMO-subfractions thereof. This is complemented by results about (mixtures of) a few commercially available low molecular weight HMOs like 2'-Fucosyllactose (2'-FL), 3-Fucosyllactose (3-FL), 3'-Sialyllactose (3'-SL), 6'-Sialyllactose (6'-SL), Lacto-N-tetraose (LNT) or Lacto-N-neotetraose (LNnT). The HMOs within this MW range include mono or maximally bivalent (immuno-) functional epitopes. Consequently, multi- or polyvalent HMOs appear to be understudied with regard to their influence on early life immunity and warrant more attention in future.

As described in **CHAPTER I**, a major challenge in the accurate analytical characterization of HMOs is their broad structural diversity, which is further complicated by the presence of various highly similar (regio-) isomeric HMO-compounds. The latter may e.g. only differ in one glycosidic linkage or the position of a monosaccharide residue relative to one of the multiple HMO-backbone structures [1]. It has been proposed that the composition of human milk (HM) ingredients adapts dynamically to the nutritional needs of the growing infant [2]. Thus, a main goal of this thesis was to create a deeper understanding of the dynamic variations of the approximately 20 most abundant individual HMO-structures contained in HM. Furthermore, associations of HMOs (and their variations in HM) with probable benefits for healthy EL development should be elicited if possible.

In this regard, the effect of two main EL-factors impacting HMO variations were studied in more detail:

- The maternal genetic predisposition with respect to Secretor (Se)- and Lewis (Le)-status
- The stages of HM-lactation up to 12 month post-partum

A further ambition of this thesis, to study quantitative changes of abundant isomeric HMOs up to Hexaoses, was apparently dependent on the availability of accurate analytical approaches. In general, appropriate analytical approaches should allow for the identification, distinction and quantitation of the molecules of interest. However, a summary of advanced HMO-methods in the introduction of **CHAPTER III** indicated the need to improve resolution and selectivity for several isomeric HMOs like e.g. LNFP II and LNFP III. Therefore, the development, improvement and application of such advanced analytical approaches was a focus of this work. The major analytical challenges exemplified above could be successfully addressed. As detailed in **CHAPTERS III** and **VII,** this became possible by establishment of two novel hyphenated analytical approaches namely "Label-Free Targeted LC-ESI-MS^2" and "High Throughput Multiplexed Capillary Gel Electrophoresis with Laser-Induced Fluorescence Detection (xCGE-LIF)".

The novel targeted LC-ESI-MS^2 method employs the diagnostic ion principle in negative ion mode as outlined in **CHAPTER III**. This principle facilitated direct identification and relative quantitation of the 10 most abundant acidic and neutral HMOs in their native form including isomeric variants of Fucosyllactoses, Sialyllactoses, Lacto-N-tetraoses and Lacto-N-fucopentaoses. Notably, identification of these partly regioisomeric HMO-structures could be achieved independent from recognition of structure specific retention times (RT) in liquid chromatography. Here, identification of targeted HMO structures was exclusively obtained by tandem MS. In more detail, predefined specific pairs of intact molecular masses and distinct diagnostic ions were screened by a triple quadrupole MS in negative ion mode. In this respect and according to the fragment ion nomenclature introduced by Domon and Costello[3], mainly C-, C/Z-, A/Z- and B/X- type MS^2 fragments ions were appropriate to serve as diagnostic ions. Ten more HMO structures ranging from Lactose over Galactosylactsoes (e.g. 3'-GL) up to Hexaoses (e.g. Lacto-N-Difcucohexaoses) and tentatively assigned soluble blood group A and B tetraoses could be added by using this approach. In this case, a combination of analytical information based on diagnostic or non-diagnostic MS- and MS^2 -ion pairs as well as specific RTs was sufficient to identify these further HMOs.

To afford analysis of high HM sample numbers (see **CHAPTER VI**) and absolute HMO quantitation, the targeted LC-ESI-MS^2 approach had to be further improved. Improvements involved e.g. optimization of sample pre-treatment protocols

(additional delipidation), adaptation of stationary LC phases (HILIC amide instead of porous graphitized carbon columns) and the introduction of quantifier fragment ions next to (diagnostic) identifier fragment ions (see **CHAPTER VI**).

The second analytical approach introduced in this work to analyse HMOs was xCGE-LIF. This method combined sensitivity and high robustness together with the future option to further multiplex analytical runs. Moreover, this technology was built on a widely available 4 capillary DNA analyzer platform (see **CHAPTER VII**). If the full capacity of nowadays available 96 capillary DNA analyzers would be exploited, run times per sample around only 1 minute would result. This recommends this method for future high throughput screening of HMOs, complementary to LC-ESI-MS^2. The HMO-structures accessible by xCGE-LIF were ranging from simple trioses (e.g. 2'-FL, 3-FL, 3'-GL) to a complex, branched, and difucosylated octaose structure (2,3-DF-LNH).

Based on the higher analytical specificity, resolution and accuracy of the established new HMO-methods, the resulting HMOs data sets were expected to be more informative compared to similar contemporary approaches.

Consequently, targeted LC-ESI-MS^2 was e.g. used to elucidate the *in vitro* utilization of HMOs up to LNFPs in an early life model for bacterial cross feeding (**CHAPTER V**). As a result, it could be proven that the total range of detectable HMOs served as preferred carbohydrate substrate for the health promoting microorganism *Bifidobacterium infantis*. *B. infantis* was also able to cross feed with butyrate producer *Anaerostipes caccae* while thriving on various HMO structures (neutral, acidic, with and without fucosylation).

Furthermore, more detailed insights about variations of distinct (isomeric) HMOs, so far still concealed in the compositional complexity of HM, were hoped to be revealed by the optimized analytical approaches described in this work.

Therefore, the new targeted LC-ESI-MS^2 and xCGE-LIF methods were also employed to quantify the ~20 most abundant HMOs in a multitude of diverse HM specimens. As demonstrated in the **CHAPTERS IV, VI,** and **VIII,** together more than 1900 HM specimens derived from European HM studies and cohorts could be analyzed. The provided high numbers of HM samples ensured solid statistical confirmation of HMO variations over main stages of lactation from the first weeks to 12 months post-partum. Moreover, also HMO-variations between the 4 known HM milk groups (lactotypes) I-IV could be confirmed. Cumulatively, this large dataset corroborated not only published knowledge, but also revealed novel unexpected insights.

The mining of combined HMO data from **CHAPTERS IV, VI,** and **VIII** and stratification according to HM groups I-IV, revealed specific HMO patterns characterizing these

individual HM groups. As expected, many HMO-compounds like e.g. 2'-FL, DFL, LNFPI, LNFP II, LNDFH I, which were driving the separation of HM into the 4 different milk groups were dependent on the maternal genetic Se- and Le-status. This is in line with literature [4, 5]. Remarkably, also some Se-/Le-independent glycans were contributing to milk group formation (for instance 3-FL, LNnT, tentative blood group B-tetrasaccharide/$(Hex)_3(Fuc)_1$, and tentative blood group A-tetrasaccharide/$(Hex)_2(HexNAc)_1(Fuc)_1$. Besides, a group of "core HMOs" was found to be present in every milk group. This "core" HMO-set included particular compounds like e.g. $(Hex)_3$/Galactosyllactoses, $(Hex)_2$/Lactose, LNFP III, LNT, 3'-SL, 6'-SL, and $(Hex)_4$.

Of note, new evidence was eventually generated regarding the existence of additional novel HM subgroups within HM group I. They were tentatively named HM group Ia and Ib (see **CHAPTERs IV, VI, VIII** and **IX**). The rare pattern of α1,2-fucosylated HMOs (LNDFH I present, but 2'-FL and LNFP I absent) typical for HM group Ib might be explained by the existence of a so far unknown α 1,2-fucosyltransferase. This potentially novel fucosyltransferase was tentatively assigned as FucT-x2. Up to now, only 2 other publications mentioned similar observations [6, 7]. Overall, the measured proportions of 74-90% Secretor positive (Se+) HM relative to the totality of all investigated human milk samples resembled published values for diverse European countries [8].

Without stratification to HM groups, the order of abundances for the 10 most prominent individual HMO-structures at 2 defined stages of lactation is depicted in **Table 1**. Values are based on mean concentrations obtained via targeted LC-ESI-MS^2 (see **CHAPTER IX Figure 1**).

Table 1. Order of abundances for individual HMOs in HM of N= 66 representative healthy European donors. Mean concentrations as detected by MRM LC-ESI-MS^2

Order	HMOs	Concentrations [g/L] (6 weeks pp)	Concentrations [g/L] (12 mon pp)
1.	2'-FL	2.45	1.4
2.	3-FL	0.6	1.5
3.	LNT	0.9	0.4
4.	LNDFH I	0.5	0.4
5.	LNFP I	0.4	0.3
6.	LNFP II	0.3	0.2
7.	DFL	0.2	0.4
8.	LNFP III	0.2	0.1
9.	3'-SL	0.1	0.3
10.	6'-SL	0.2	<0.1

Here, 2'-FL was by far the most abundant HMO, followed by 3-FL, LNT, LNDFH I and 6 more HMOs as displayed in **Table 1**. The concentration of all other detected HMOs including e.g. LNFP V, LNnT, or LNDFH II which are not displayed in **Table 1** was below 0.1g/L at any stage of lactation.

Considering the staging characteristics of HMOs up to 12 months post-partum, further interesting aspects evolved after evaluation of the combined HMOs data sets from Chapters **IV, VI, VIII** and **IX.** Next to the well-known overall decline of the total HMO-fraction [9], some distinct HMOs didn't follow this trend (see **CHAPTER IX**). Moreover, there were even isomeric HMO dyads which exposed diametrically opposed staging trends. The most striking examples belonged to compounds with Lactose-core-structures and comprised 3'-SL (increase), 6'-SL (decrease) as well as 3-FL (increase) and 2'-FL (decrease). In contrast, the most stable HMOs over various stages of lactation were LNFP V and LNFP III. Considering also HMOs with low to no variations, which belong to only one individual HM group, LNDFH I needs to be mentioned with respect to HM group I and LNFP II needs to be added for HM group II.

Finally, **CHAPTER IX** addressed how the observed individual HMO structures, their variations between HM groups and over progression of lactation (see **CHAPTERS IV & VIII**) might fit into the context of healthy infant development. This chapter also provided an overall conclusion regarding the major aspects and insights from the previous chapters. In addition, future perspectives with regard to development and application of sophisticated analytical methods suitable for complex HMOs and glycoconjugates were discussed in **CHAPTER IX**.

Finally, the probable implications of HMOs on a balanced immune development of the neonate were extensively discussed in **CHAPTER IX**, too**.** This was accomplished with special consideration of possible HMO-interactions with various immune receptors ranging from sialic acid-binding immunoglobulin-type lectins (Siglecs) over c-type lectin receptors (CLRs) to toll-like receptors (TLRs). The hypothetical link HMOs make between the innate and adaptive immune system was also addressed. An example of an aspect highlighted in **CHAPTER IX**, was the striking coincidence of declining 6'-SL concentrations from 6 weeks to 6 months post-partum with the decrease of gut epithelial cell sialylation in the same time frame [10].

Future mining of the hyphenated HMO data sets discussed above may pave the road to disclose further associations of HMOs with (longitudinal) health trajectories of breast-fed infants. Based on the new insights as described in **CHAPTERS II-IX**, novel functional hypothesis about the role of HMOs for healthy infant development may be postulated. Ultimately, such revelations could inspire future innovation and personalization of infant milk formula (IF) design bringing it even closer to the natural blueprint for infant nutrition, human milk.

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CHAPTER XI

Nederlandse Samenvatting

Na een algemene inleiding over de samenstelling van moedermelk (MM) en de vergelijking met koemelk (de meest gebruikte natuurlijke basis voor de productie van zuigelingenvoeding), worden de structuur en doelstellingen van dit proefschrift uiteengezet in **HOOFDSTUK I**.

Oligosacchariden in moedermelk, ook wel Human Milk Oligosaccharides (HMOs) genoemd, dragen in een belangrijke mate bij aan de samenstelling van MM en vertegenwoordigen de op twee na meest voorkomende moleculaire fractie. Naast een nutrionele bijdrage, leveren HMOs verschillende gunstige effecten op de ontwikkeling van de zuigeling. De samenhang tussen de hoeveelheden verschillende HMOs en hun positieve effecten op de ontwikkeling in het vroege leven van borstgevoede zuigelingen is van groot belang. Tevens zijn de immuunmodulerende en de beschermende effecten van HMOs in het vroege leven van de baby een veelbelovend onderzoeksveld op het gebied van MM. **HOOFDSTUK II** omvat een review over de specifieke bijdragen van HMOs aan de ontwikkeling van het immuunsysteem en weerstand tegen infecties in het vroege leven. Dit hoofdstuk vat samen hoe HMOs intrigerende anti-infectieuze, prebiotische en immuun-regulerende functies uitoefenen, bijvoorbeeld via verschillende dendritische cel receptoren of galectines. Bovendien worden de modulerende effecten van HMOs op pathogeenherkenning en de versterking van de slijmvliesbarrière besproken. Zoals samengevat in **HOOFDSTUK II** en Tabel 2 in **HOOFDSTUK I**, richten de meeste huidige *in vitro* en *in vivo* onderzoeken naar de prebiotische, immuunmodulerende of andere effecten van HMOs zich slechts op een paar karakteristieke HMO-fracties: de totale hoeveelheid HMOs in MM, de totale hoeveelheid neutrale HMOs in MM en de total hoeveelheid zure HMOs in MM. Dit wordt aangevuld met resultaten over (mengsels van) een beperkt aantal commercieel verkrijgbare HMOs met een laag molecuulgewicht zoals 2'-Fucosyllactose (2'-FL), 3-Fucosyllactose (3-FL), 3'-Sialyllactose (3'-SL), 6'-Sialyllactose (6'-SL), Lacto-N-tetraose (LNT) en Lacto-N-neotetraose (LNnT). De HMOs binnen dit bereik van molecuulgewichten omvatten mono- of ten hoogste bivalente (immuno-) functionele epitopen. Als gevolg hiervan worden de immuunmodulerende effecten van polyvalente HMOs in het vroege leven op dit moment onvoldoende belicht. Dit verdient in de toekomst meer aandacht.

Zoals vermeldt in **HOOFDSTUK I** is de brede structurele diversiteit van HMOs een grote uitdaging in hun analytische karakterisering v. De analytische karakterisering wordt verder bemoeilijkt door de aanwezigheid van verschillende zeer vergelijkbare isomere HMO-verbindingen. Dit laatste kan bijvoorbeeld leiden tot verschillen in slechts één glycosidische koppeling of de positie van een monosaccharide-residu ten opzichte van een van de meervoudige HMO-backbone-structuren [1]. Deze kleine structurele verschillen zijn de oorzaak van het bestaan van meerdere isobarische HMO-regioisomeren [1]. Men suggereert dat de samenstelling van de ingrediënten van MM zich dynamisch aanpast aan de voedingsbehoeften van de groeiende

zuigeling [2]. Een hoofddoelstelling van dit proefschrift was om meer inzicht te krijgen in de dynamische variaties van de ~20 meest voorkomende individuele HMO-structuren in MM. Tevens werden, indien mogelijk, associaties tussen HMOs (en hun variaties in MM) en mogelijke effecten op de gezonde ontwikkeling in het vroege leven opgehelderd. In deze context werd de invloed van twee belangrijke factoren in het vroege leven die van invloed zijn op de variaties in de HMOs nader onderzocht:

- De maternale genetische aanleg met betrekking tot Secretor (Se)- en Lewis (Le)-status
- De fases van MM-lactatie tot 12 maanden post-partum

Een belangrijke doelstelling van dit proefschrift, d.w.z. het bestuderen van de veranderingen in de hoeveelheden van de meest voorkomende isomere HMOs tot de Hexaoses, hangt af van de beschikbaarheid van accurate analytische methodes. Geschikte analytische methodes moeten de identificatie, de onderscheiding en de kwantificering van de belangrijkste moleculen mogelijk maken. Een samenvatting van geavanceerde methoden om HMOs te analyseren in de introductie van **HOOFDSTUK III** wijst echter op de noodzaak om de resolutie en selectiviteit voor isomeer-HMOs, zoals bijvoorbeeld LNFP II en LNFP III, te verbeteren. Daarom was de ontwikkeling, verbetering en toepassing van dergelijke analytische benaderingen een verdere focus van dit proefschift. De hierboven beschreven belangrijkste analytische uitdagingen werden met de nieuwe analytische strategie met succes aangepakt, zoals benadrukt in de titel van dit proefschift. Zoals beschreven in de **HOOFDSTUKKEN III** en **VII,** werd dit mogelijk gemaakt door het opzetten van twee nieuwe analytische methodes, namelijk "Label-Free Targeted LC-ESI-MS^2" en "High Throughput Multiplexed Capillary Gel Electrophoresis with Laser-Induced Fluorescence Detection (xCGE-LIF)".

De nieuwe gerichte LC-ESI-MS^2-methode maakt gebruik van het diagnostische ionenprincipe in negatieve ionenmodus zoals beschreven in **HOOFDSTUK III**. Dit principe maakt de directe identificatie en relatieve kwantificering van de 10 meest voorkomende zure en neutrale HMOs in natieve vorm mogelijk, met inbegrip van isomeervarianten van de Fucosyllactoses, Sialyllactoses, Lacto-N-tetraoses en Lacto-N-fucopentaoses. De identificatie van deze deels regioisomerische HMO-structuren zou onafhankelijk van de herkenning van specifieke retentietijden (RT) in vloeistofchromatografie kunnen worden bereikt. Bij deze methode werd de identificatie van HMO-structuren uitsluitend verkregen door tandem-MS-detectie. Meer specifiek werden vooraf gedefinieerde specifieke paren van intacte moleculaire massa's en van verschillende diagnostische ionen gescreend door een drievoudige quadrupool massaspectrometer (MS) in negatieve ionen stand. In dit verband en in lijn met de fragment-ionen-nomenclatuur die door Domon en Costello [3], waren voornamelijk C-, C/Z-, A/Z- en B/X-type MS^2 fragmenten ionen geschikt

om als diagnostische ionen te dienen. Tien verdere HMO-structuren, variërend van Lactose tot de Galactosylactoses (bijv. 3'-GL) en de Hexaoses (bijv. Lacto-N-Difcucohexaoses) en mogelijke bloedgroep A- en B-tetraoses, kunnen ook aan deze aanpak worden toegevoegd. Een combinatie van analytische informatie op basis van de diagnostische of niet-diagnostische MS- en MS^2-ionenparen, evenals specifieke RTs, waren in dit geval voldoende om deze verdere HMOs te identificeren.

Om de analyse van zeer hoge aantallen MM-monsters (zie **HOOFDSTUK VI**) en absolute HMO-kwantificering mogelijk te maken, werd de gerichte LC-ESI-MS^2 analyse verder verbeterd. De verbeteringen omvatten bijvoorbeeld een optimalisatie van de voorbehandelingsprotocollen (aanvullende delipidatie) van de MM-monsters, aanpassing van de stationaire LC-fases (HILIC-amide in plaats van poreuze gegrafitisierte koolstofkolommen) en de introductie van kwantificator fragment ionen naast de (diagnostische) identificatie fragment ionen (zie **HOOFDSTUK VI**).

De tweede analytische methode die in dit proefschrift werd geïntroduceerd om HMOs te analyseren is xCGE-LIF. Deze methode combineert een hoge robuustheid en gevoeligheid met de toekomstige optie om multiplex analytische runs uit te voeren. Bovendien is deze technologie gebaseerd op een breed beschikbaar 4 capillair DNA analysator platform (zie **HOOFDSTUK VII**). Als de volledige capaciteit van de huidige beschikbare 96 capillaire DNA-analysatoren zou worden benut, zou de looptijd per monster ongeveer 1 minuut bedragen. Dit geeft de potentie van deze methode aan voor de toekomstige high-throughput screening van HMOs, complementair aan LC-ESI-MS^2. De HMO-structuren die toegankelijk bleken te zijn voor xCGE-LIF varieerden van eenvoudige trioses (bijv. 2'-FL, 3-FL en 3'-GL) tot een complexe, vertakte en difucosyleerde octaosestructuur (2,3-DF-LNH).

Op basis van de hogere analytische specificiteit, resolutie en nauwkeurigheid van de opgezette nieuwe HMO-methoden werd verwacht dat de resulterende HMO bepalingen informatiever zouden zijn in vergelijking met vergelijkbare hedendaagse methodes.

Daaropvolgend werd LC-ESI-MS^2 bijvoorbeeld gebruikt om het *in vitro* gebruik van HMOs tot en met de LNFPs op te helderen in een vroeg leven model voor bacteriële crossfeeding (**HOOFDSTUK V**). Er kon bewezen worden dat het totale scala aan detecteerbare HMOs dienden als voorkeur koolhydraat-substraten voor de gezondheid bevorderende micro-organismen *Bifidobacterium infantis*. *Bifidobacterium infantis* gedijde goed op verschillende HMO-structuren (neutraal, zuur, met en zonder fucosylatie), en kon als voeding dienen voor butyraatproducerende *Anaerostipes caccae*, zoals in **HOOFDSTUK V** nader wordt uitgelegd.

Tevens was de verwachting dat betere en meer gedetailleerdere inzichten verkregen konden worden over de variaties van de afzonderlijke (isomeer) HMOs door de geoptimaliseerde analytische methodes die in dit werk zijn ontwikkeld, welke tot nog niet opgehelderd konden worden vanwege de complexe samenstelling van MM.

Daarom werden ook de nieuw opgezette LC-ESI-MS^2 en xCGE-LIF-methoden gebruikt om de ~ 20 meest voorkomende HMOs in een groot aantal verschillende MM-monsters te kwantificeren. Zoals aangetoond in de **HOOFDSTUKKEN IV, VI** en **VIII,** konden opgeteld meer dan 1900 MM-monsters, verkregen van verschillende Europese studies en cohorten, worden geanalyseerd. De hoge aantallen MM-monsters zorgden voor een solide statistische bevestiging van variaties in HMOs in de belangrijkste fases van lactatie van de eerste weken tot 12 maanden post-partum. Bovendien konden ook HMO-variaties tussen de vier bekende moedermelkgroepen I-IV worden bevestigd. Deze bevindingen bevestigen gepubliceerde kennis, terwijl tevens enkele onverwachte nieuwe inzichten werden onthuld.

De gecombineerde HMO resultaten van **HOOFDSTUKKEN IV, VI** en **VIII** en de stratificatie volgens de moedermelkgroepen I-IV, onthulde zeer specifieke HMO-patronen die deze individuele MM-groepen kenmerken. Zoals verwacht waren alle HMO-verbindingen die de verdeling van MM over de vier verschillende melkgroepen bepaalden afhankelijk van de maternale genetische Se- en Le-status (bijv. 2'-FL, DFL, LNFPI, LNFP II, LNDFH I). Dit is in overeenstemming met de literatuur [4, 5]. Verrassend genoeg waren er ook enkele Se-/Le-onafhankelijke glycanen die bijdroegen aan de vorming van de melkgroepen (bijvoorbeeld 3-FL, LNnT, mogelijke bloedgroep B-tetrasaccharide/$(Hex)_3(Fuc)_1$, en mogelijke bloedgroep A-tetrasaccharide/$(Hex)_2(HexNAc)_1(Fuc)_1$). Bovendien bleek in elke melkgroep een groep "kern-HMOs" aanwezig te zijn. Deze "kern HMO"-set omvatte bijzondere verbindingen zoals $(Hex)_3$/Galactosyllactoses, $(Hex)_2$/Lactose, LNFP III, LNT, 3'-SL, 6'-SL en $(Hex)_4$.

Belangrijk te vermelden is dat er uiteindelijk nieuw bewijs is gegenereerd met betrekking tot het bestaan van aanvullende nieuwe moedermelk-subgroepen binnen moedermelk-groep I. Ze werden moedermelk-groep Ia en Ib genoemd (zie **HOOFDSTUKKEN IV, VI, VIII** en **IX**). Het zeldzame patroon van α1,2-fucosyleerde HMOs (LNDFH I aanwezig, maar 2'-FL en LNFP I afwezig) typisch voor moedermelk-groep Ib zou verklaard kunnen worden door het bestaan van een tot nu toe onbekende α1,2-fucosyltransferase. Deze potentieel nieuwe fucosyltransferase werd voorlopig toegewezen als FucT-x2. Tot nu toe vermeldden slechts twee andere publicaties soortgelijke waarnemingen [6, 7]. Over het geheel genomen lijken de gemeten verhoudingen van 74-90% Secretor positieve (Se+) MM-monsters ten opzichte van het geheel van alle onderzochte monsters op de gepubliceerde waarden voor diverse Europese landen[8].

De volgorde van concentraties voor de 10 meest prominente individuele HMO-structuren (zie **HOOFDSTUK IX Figuur 1**) word weergegeven in **Tabel 1**. De waarden zijn gebaseerd op gemiddelde HMO-concentraties voor de lactatiefasen 6 weken en 12 maanden verkregen via gerichte LC-ESI-MS.

Tabel 1. Volgorde van concentraties voor individuele HMOs in representatieve HM van N=66 gezonde Europese donoren. Gemiddelde concentraties zoals gedetecteerd d.m.v. MRM LC-ESI-MS²

Order	HMOs	Concentraties [g/L] (6 weken pp)	Concentraties [g/L] (12 maanden pp)
1.	2'-FL	2.45	1.4
2.	3-FL	0.6	1.5
3.	LNT	0.9	0.4
4.	LNDFH I	0.5	0.4
5.	LNFP I	0.4	0.3
6.	LNFP II	0.3	0.2
7.	DFL	0.2	0.4
8.	LNFP III	0.2	0.1
9.	3'-SL	0.1	0.3
10.	6'-SL	0.2	<0.1

2'-FL was verreweg de meest abundante HMO, gevolgd door 3-FL, LNT, LNDFH I en nog 6 verdere HMOs zoals weergegeven in **Tabel 1**. De concentratie van alle andere gedetecteerde HMOs inclusief b.v. LNFP V, LNnT of LNDFH II die niet in **Tabel 1** worden weergegeven, was in elke fase van de lactatie lager dan 0,1 g/L.

Gezien de afhankelijkheid van de HMOs van de lactatieperiode tot 12 maanden post-partum, zijn - na evaluatie van de gecombineerde HMOs resultaten uit de **HOOFDSTUKKEN IV, VI, VIII** en **IX** - verdere interessante aspecten tevoorschijn gekomen**.** Naast de bekende algemene daling van de totale HMO-concentratie [9] volgden bepaalde afzonderlijke HMOs deze trend niet (zie **HOOFDSTUK IX**). Bovendien waren er zelfs isomeer HMO-paren dietegenstrijdige trends per lactatieperiode blootlegden. De meest opvallende voorbeelden daarvan behoorden tot de verbindingen met Lactose-kern-structuren en bestonden uit 3'-SL (toename) en 6'-SL (afname), evenals 3-FL (toename) en 2'-FL (afname). Daarentegen waren LNFP V en LNFP III de meest stabiele HMOs gedurende verschillende fases van lactatie. Met inachtneming van HMOs met weinig tot geen variatie in concentratie die tot slechts één individuele moedermelk-groep behoren, moet LNDFH I worden toegevoegd aan moedermelk-groep I en LNFP II aan moedermelk-groep II.

Tot slot werd in **HOOFDSTUK IX** ingegaan op de vraag hoe de waargenomen individuele HMO-structuren, hun variaties tussen de MM-groepen en over de lactatieperiodes (zie **HOOFDSTUKKEN IV** en **VIII**) in de context van een gezonde ontwikkeling van zuigelingen geplaatst zouden kunnen worden. In dit hoofdstuk wordt ook een algemene conclusie over de belangrijkste aspecten en

inzichten uit de vorige hoofdstukken gegeven. Daarnaast worden toekomstige perspectieven met betrekking tot de ontwikkeling en toepassing van geavanceerde analysemethoden besproken die geschikt zijn voor de bestudering van complexe HMOs en glycoconjugaten. Ten slotte worden de mogelijke implicaties van HMOs voor een evenwichtige immuunontwikkeling van de zuigeling uitvoerig besproken in **HOOFDSTUK IX.** Hierbij word speciale aandacht gegeven aan mogelijke interacties tussen HMOs en verschillende immuunreceptoren, variërend van zogenaamde "sialic acid-binding immunoglobulin-type lectins" (Siglecs) over "c-type lectin receptors" (CLRs) tot "toll-like-receptors" (TLRs). De hypothetische link van het aangeboren en adaptieve immuunsysteem door HMOs wordt ook besproken. Een voorbeeld hiervan dat in **HOOFDSTUK IX** wordt benadrukt, is het opvallende samengaan van een afnemende 6'-SL concentratie tussen 6 weken en 6 maanden post-partum en het dalen van darmepitheel celsialylation in hetzelfde tijdsbestek[10].

De verdere evaluatie van de hierboven besproken HMO-datasets kan de weg vrijmaken om verdere associaties van HMOs met (longitudinale) gezondheidstrajecten van borstgevoede zuigelingen te openbaren. Op basis van de nieuwe inzichten zoals beschreven in de **HOOFDSTUKKEN II-IX**, kan een nieuwe functionele hypothese over de rol van HMOs voor een gezonde ontwikkeling van zuigelingen worden gevormd. Uiteindelijk zouden dergelijke onthullingen toekomstige innovaties in de samenstelling van zuigelingenvoeding kunnen inspireren, dichter bij de natuurlijke blauwdruk voor zuigelingenvoeding, moedermelk.

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CHAPTER XII

Zusammenfassung in deutscher Sprache

In **KAPITEL I** wird zunächst eine allgemeine Einführung bezüglich der Zusammensetzung von Muttermilch (MM) im Vergleich zur Kuhmilch geben. Letztere wird am häufigsten als Basis zur Herstellung von Säuglingsmilchnahrung genutzt. Danach werden Struktur und Ziele dieser Arbeit erläutert.

Die in der Muttermilch vorkommenden Oligosaccharide (HMOs) tragen wesentlich zur Zusammensetzung dieser Koerperfluessigkeit bei. HMOs stellen dabei die Fraktion mit dem dritthöchsten Anteil gemessen an den insgesamt in der Muttermilch vorkommenden Substanzen dar. Jenseits des ernährungsphysiologischen Beitrags von HMOs können diese Mehrfachzucker durch weitere funktionelle Effekte die gesunde Entwicklung des Neugeborenen fördern. Die Zusammensetzung von MM und der darin enthaltenen HMOs ist nicht statisch und kann z.B. zwischen verschiedenen Müttern oder abhängig vom Laktationszeitpunkt quantitativ variieren. Die weitere Erforschung der Ursachen und Auswirkungen dieser HMO-Variationen auf die gesunde frühkindliche Entwicklung ist von hohem Interesse geprägt. Darüber hinaus sind mögliche immunmodulatorische und protektive Eigenschaften von HMOs ein weiteres vielversprechendes Feld der Humanmilchforschung. In diesem Zusammenhang wurde der aktuelle Kenntnisstand bezüglich des Einflusses von HMOs auf die Entwicklung des Immunsystems von Neugeborenen und deren Anfälligkeit für neonatale und frühkindlich Infektionen in **KAPITEL II** zusammengefasst**.** Dieses Kapitel erklärt unter anderem die faszinierenden anti-infektiösen, präbiotischen und immun-homöostatischen Wirkmechanismen der HMOs. Diese werden z. B. über bestimmte Rezeptoren wie den Toll-Like-Rezeptoren (TLRS), Galektinen oder anderen Rezeptoren, die auch auf Immunzellen vorkommen, vermittelt. Darüber hinaus wurden die Modulation der Pathogenerkennung und die Stärkung der Schleimhautbarriere durch HMOs diskutiert.

Wie in **KAPITEL II** und **Tabelle 2** in **KAPITEL I** zusammengefasst, waren die meisten *in-vitro* und *in-vivo* Untersuchungen zu präbiotischen, immunologischen oder auch anderen HMO-Funktionen bisher auf nur wenige charakteristische HMOs oder HMO-Fraktionen beschränkt. Dazu zählen die aus MM isolierte gesamte HMO-Fraktion bestehend aus neutralen und sauren Komponenten, sowie die Subfraktionen der sauren und neutralen HMOs. Weitere Forschungsergebnisse gibt es zu Wirkungen von wenigen, kommerziell erhältlichen niedermolekularen HMOs. Dazu zählen z.B 2'-Fucosyllactose (2'-FL), 3-Fucosyllactose (3-FL), 3'-Sialyllactose (3'-SL), 6'-Sialyllactose (6'-SL), Lacto-N-tetraose (LNT) oder auch Lacto-N-neotetraose (LNnT). Diese niedermolekularen HMOs tragen mono- oder maximal bivalente (immun-)funktionelle Epitope. Daher sollte in Zukunft multi- oder polyvalenten HMOs mit Hinblick auf ihr (immunologisches) Funktionspotenzial mehr Aufmerksamkeit geschenkt werden.

Wie in **KAPITEL I** erwähnt, stellt die genaue analytische Charakterisierung von HMOs aufgrund ihrer strukturellen Vielfalt eine große Herausforderung dar. Diese wird durch

das Vorhandensein verschiedener sehr ähnlicher HMO-Isomere noch erschwert. Letztere unterscheiden sich z.B zum Teil nur in einer glykosidischen Bindung oder der Position eines Monosaccharidrestes relativ zu einer der vielen möglichen HMO-Grundstrukturen [1]. Schon diese leichten strukturellen Unterschiede führen zum Vorhandensein von multiplen HMO-Regioisomeren[1]. Weiterhin besteht zum Teil die Auffassung, dass sich Muttermilch in ihrer Zusammensetzung dynamisch an die Ernährungsbedürfnisse des heranwachsenden Säuglings anpasst [2]. Ein Hauptziel dieser Arbeit war es daher auch, mögliche dynamische Konzentrationsschwankungen der rund 20 am häufigsten in der Muttermilch vorkommenden individuellen HMO-Strukturen zu erforschen. Weiterhin sollten mögliche Assoziationen von HMOs und deren Konzentrationsveränderungen mit Merkmalen der gesunden frühkindlichen Entwicklung untersucht werden. In diesem Zusammenhang wurde überdies der Einfluss von zwei Faktoren, welche wiederum die Komposition von HMOs in MM beeinflussen können, genauer betrachtet:

- Die mütterliche genetische Veranlagung in Bezug auf Sekretor (Se)- und Lewis (Le)-Status
- Die Stadien der HM-Laktation bis zu 12 Monaten nach der Geburt

Die Möglichkeit der genauen Untersuchung quantitativer HMO-Veränderungen in Muttermilch, speziell von isomeren HMOs, ist jedoch abhängig von der Verfügbarkeit geeigneter analytischer Methoden. Prinzipiell sollten zielführende analytische Ansätze die Identifizierung, Unterscheidung und Quantifizierung der Zielmoleküle zulassen. Eine Zusammenfassung leistungsfähiger Methoden der instrumentellen HMO-Analytik wurde daher in die Einleitung von **KAPITEL III** aufgenommen. Diese Zusammenfassung verdeutlichte jedoch auch die Notwendigkeit, dass die analytische Auflösung und Selektivität insbesondere für isomere HMOs wie z.B. LNFP II und LNFP III noch stark verbessert werden musste. Ein weiterer Schwerpunkt dieser Arbeit war daher die Entwicklung und Verbesserung leistungsfähiger analytischer Methoden für HMOs. Durch die Entwicklung neuer analytischer Strategien konnten die oben beschriebenen analytischen Herausforderungen erfolgreich gelöst werden. Wie in den **KAPITELN III** und **VII** beschrieben, wurde die exakte Analyse isomerer HMOs letztendlich durch zwei ganz auf HMO abgestimmte Techniken ermöglicht: „Label-Free Targeted LC-ESI-MS^2" und „HighThroughput Multiplexed Capillary Gel Electrophoresis with Laser-Induced Fluorescence Detection (xCGE-LIF)". Die Etablierung und der erfolgreich Einsatz dieser Methoden wurde auch noch einmal durch den Titel dieser Arbeit **„Advanced Characterization of Biofunctional Human Milk Oligosaccharides by Mass Spectrometry and Complementary Methods**" verdeutlicht.

Die neue LC-ESI-MS^2-Methode für HMOs basiert auf dem Prinzip der diagnostischen Fragment-Ionen, welches im **KAPTIEL III** beschrieben wird. Dieses Prinzip

erleichtert die direkte Identifizierung und relative Quantifizierung der 10 am häufigsten vorkommenden sauren und neutralen HMOs in nativer Form. Dies schließt auch ausdrücklich isomere Varianten von Fucosyllactosen, Sialyllactosen, Lacto-N-Tetraosen und Lacto-N-fucopentaosen ein. Insbesondere konnte die korrekte Identifizierung dieser teilweise regioisomeren HMO-Strukturen unabhängig von deren LC -Retentionszeiten (RT) verwirklicht werden. Vielmehr wurde die Identifizierung relevanter HMOs ausschließlich durch Tandem-MS basierte Strukturerkennung erreicht. Genauer gesagt beruht dieses neu entwickelte massenspektrometrische Analysenprinzip auf der kontinuierlichen Messung von speziellen vordefinierten Ionen-Paaren durch ein Triplequadrupol-Massenspektrometer im negativen Ionenmodus. Diese Ionen-Paare repräsentieren einerseits die intakte Molekularmasse des Zielmoleküls und anderseits die schon erwähnten strukturspezifischen diagnostischen HMO- Fragmentionen. Der Typus dieser diagnostischen Fragmentionen umfasst der von Domon und Costello [3] eingeführten Nomenklatur folgend C-, C/Z-, A/Z- und B/X-MS^2-Fragmente. Zusätzlich konnte der Anwendungsbereich dieser neuen selektiven LC-ESI-MS^2-Methode durch weitere 10 Glykane erweitert werden. Diese reichen von Lactose über Galactosyllactosen (z.B. β 3'-GL) bis hin zu Hexaosen (z.B. Lacto-N-Difukohexaosen) oder (vorläufig zugeordneten) Blutgruppen A- und B-Tetraosen. Zur Bestimmung dieser weiteren 10 Komponenten müssen jedoch weiterhin analytische Informationen auf Grundlage diagnostischer oder nichtdiagnostischer MS/MS-Ionenpaare sowie spezifische RT-Daten herangezogen werden.

Um die Analyse sehr hoher HM-Probenzahlen (siehe **KAPITEL VI**) bei absoluter Quantifizierung von HMOs zu ermöglichen, wurde der zuvor beschriebene LC-ESI-MS^2-Ansatz noch weiter verbessert. Die Verbesserungen beinhalteten z.B. die Optimierung von Probenvorbehandlungsprotokollen (zusätzliche Delipidation), die Anpassung der stationären LC-Phase (HILIC-Amid statt poröser graphitisierter Karbonsäulen) und die Einführung von zusätzlichen Fragmentionen zur Quantifizierung der Zielkomponenten (siehe **KAPITEL VI**).

Der zweite analytische Ansatz, der in diese Arbeit zur Analyse von HMOs eingeführt wurde, war xCGE-LIF. Diese Methode kombiniert Empfindlichkeit und hohe Robustheit mit der zukünftigen Option des weiteren Multiplexens von Analysenlaeufen. Darüber hinaus wurde diese Technologie mittels einer weit verbreiteten 4 Kapillar-DNA-Analysatoren-Plattform etabliert (siehe **KAPITEL VII**). Würde die volle Kapazität der heute verfügbaren 96 Kapillar-DNA-Analysatoren genutzt, würden die Laufzeiten pro Probe nur 1 Minute betragen. Dies empfiehlt diese Methode für zukünftige Hochdurchsatz-Screenings von HMOs ergänzend zu LC-ESI-MS^2. Die durch xCGE-LIF erfassbaren HMO-Strukturen reichen von einfachen Triosen (z.B. 2'-FL, 3-FL, 3'-GL) bis hin zu einer komplexen, verzweigten und difukosylierten Oktaosestruktur (2,3-DF-LNH).

Basierend auf der höheren analytischen Spezifität, Auflösung und Genauigkeit der neu etablierten Methoden für die HMO Analyse, sollten auch die resultierenden HMOs-Datensätze im Vergleich zu bisher genutzten analytischen Anwendungen informativer sein.

Folglich wurde z.B. die LC-ESI-MS^2 Methode angewendet, um *in-vitro* die Rolle von HMOs als Substrat beim Phänomen des bakteriellen „Crossfeedings" (**KAPITEL V**) aufzuklären. Als Ergebnis konnte gezeigt werden, dass die gesamte Bandbreite der gemessenen HMOs als bevorzugtes Kohlenhydratsubstrat für das gesundheitsfördernde Bakterium *Bifidobacterium infantis* dient. Während *B. infantis* auf verschiedenen HMO-Strukturen (neutral, sauer, mit und ohne Fucosylierung) gedieh, konnten spezielle *B. infantis-Metabolite* von dem Butyrat-produzierenden Bakterium *Anaerostipes caccae* utilisiert und damit für dessen eigenes Wachstum nutzbar gemacht werden. Außerdem wurde versucht, durch die Anwendung der optimierten analytischen Verfahren weitere Einblicke bezüglich bestimmter milchtypus- oder laktationszeitabhängiger HMO-Variationen zu erlangen.

Daher wurden sowohl LC-ESI-MS^2 als auch xCGE-LIF eingesetzt, um die 20 am häufigsten vorkommenden HMOs in einer Vielzahl unterschiedlicher HM-Proben zu quantifizieren. Wie in den **KAPITELN IV, VI** und **VIII** beschrieben, konnten dabei mehr als 1900 HM-Proben aus verschiedenen europäischen HM-Studien oder Kohorten analysiert werden. Die hohe Anzahl analysierter HM-Proben sorgte für eine statistisch valide Erkennung spezifischer quantitativer und qualitativer Schwankungen der HMO in MM. So konnten signifikante Schwankungen über den gesamten Laktationsverlauf bis zu einem Kindesalter von 12 Monaten verfolgt werden. Darüber hinaus konnten auch signifikante Unterschiede zwischen den HMO-Mustern der Milchgruppen I-IV gefunden werden. Diese Ergebnisse bestätigten bereits veröffentlichtes Wissen, legten aber im Detail auch einige neue unerwartete Erkenntnisse offen.

Wie zu erwarten, konnten die 4 verschiedenen bekannten Milchgruppen durch Unterschiede im Muster von HMOs wie 2'-FL, DFL, LNFPI, LNFP II, LNDFH I differenziert werden. Dieses Ergebnis steht im Einklang mit bisher veröffentlichter Literatur [4, 5]. Das Vorhandensein der erwähnten HMO-Strukturen wird jedoch auch vom genetischen Se- und Le-Status der Mutter gesteuert. Erstaunlicherweise trugen aber auch einige Se-/Le-Status unabhängige Glykane zur Separierung der 4 Gruppen bei. Zu diesen HMO gehörten z.B. 3-FL, LNnT, ein mögliches Blutgruppen B-Tetrasaccharid/$(Hex)_3(Fuc)_1$, und ein mögliches Blutgruppen A-tetrasaccharid/$(Hex)_2(HexNAc)_1(Fuc)_1$. Außerdem wurde festgestellt, dass eine spezifische Gruppe von bestimmten HMOs in jeder Milchgruppe vorhanden war. Diese Auswahl von "Kern-HMOs" umfasste Strukturen wie z.B. $(Hex)_3$/Galactosyllactosen, $(Hex)_2$/Lactose, LNFP III, LNT, 3'-SL, 6'-SL und $(Hex)_4$."

Bemerkenswerterweise konnten auch neue Beweise für die Existenz zusaetzlicher und neuartiger HM-Untergruppen innerhalb der HM-Gruppe I generiert werden. Diese Untergruppen wurden vorläufig HM-Gruppe Ia und Ib genannt (siehe **KAPITEL IV, VI, VIII** und **IX**). Ein seltenes Muster von α1,2-fucosylierten HMOs war prototypisch für die HM-Gruppe Ib. Dabei war jeweils LNDFH I detektierbar während 2'-FL und LNFP I nicht nachweisbar waren. Dieses außergewöhnliche HMO Muster könnte durch die Existenz einer bisher unbekannten α1,2-Fucosyltransferase erklärt werden. Diese potentiell neuartige Fucosyltransferase wurde vorläufig als FucT-x2 bezeichnet. Bisher erwähnen nur 2 weitere Publikationen ähnliche Beobachtungen[6, 7].

Addiert man die Anzahlen der Milchproben aus den **KAPITELN IV, VI** und **VIII,** die den Milchgruppen I und III zugeordnet werden konnten und damit von Sekretor positiven (Se+) Müttern stammen, ergibt sich ein Anteil von a. 74-90% relativ zur Gesamtanzahl von 1900 untersuchten Muttermilchen. Dieser Anteil korreliert sehr gut mit publizierten Werten für Humane Milchen aus unterschiedlichen anderen europäischen Ländern[8].

Nach Höhe der durchschnittlichen HMO-Konzentrationen geordnet, ergibt sich dabei die in **Tabelle 1** dargestellte generelle Rangfolge von HMO-Strukturen in MM. Den Werten in **Tabelle 1** liegen LC-ESI-MS^2 Analysen von repräsentativen MM (N=66 gesunde Donoren) zugrunde, die zu den Laktationzeitpunkten 6 Wochen bzw. 12 Monaten post-partum gesammelt wurden.

Tabelle 1 | Rangfolge von individuellen HMO-Strukturen in Muttermilch basierend auf mittleren Konzentrationen, wie durch MRM LC-ESI-MS^2 nachgewiesen (N = 66 repräsentative gesunde Europäische Donoren)

Order	HMOs	Konzentration [g/L] (6 Wochen pp)	Konzentration [g/L] (12 Monate pp)
1.	2'-FL	2.45	1.4
2.	3-FL	0.6	1.5
3.	LNT	0.9	0.4
4.	LNDFH I	0.5	0.4
5.	LNFP I	0.4	0.3
6.	LNFP II	0.3	0.2
7.	DFL	0.2	0.4
8.	LNFP III	0.2	0.1
9.	3'-SL	0.1	0.3
10.	6'-SL	0.2	<0.1

Wie aus **Tabelle 1** ersichtlich, war 2'-FL dabei das bei weitem abundanteste HMO, gefolgt von 3-FL, LNT, LNDFH I und 6 weiteren HMOs. Die Konzentration aller anderen nachgewiesenen HMOs einschließlich LNFP V, LNnT oder LNDFH II, die in Tabelle 1 nicht aufgeführt sind, lagen in jedem Laktationsstadium unter 0,1 g/L.

Durch Auswertung der kombinierten HMOs-Datensätze aus den **Kapiteln IV, VI, VIII** und **IX** hinsichtlich des „Stagingverhaltens" von bis zu 12 Monaten nach der

Geburt, ergaben sich weitere interessante Aspekte. Schon aus der Literatur ist für die HMO-Gesamtfraktion eine kontinuierliche Konzentationsabnahme über diesen nachgeburtlichen Laktationszeitraum bekannt[9]. Jedoch folgten bestimmte individuelle HMOs diesem Trend nicht (siehe **KAPITEL IX**). Vielmehr gab es sogar isomere HMO-Paare, deren Konzentrationsverlauf diametral entgegengesetzt war. Zu den auffälligsten Beispielen hierfür gehörten z.B. Verbindungen mit Lactose-Kernstrukturen wie 3'-SL (Erhöhung), 6'-SL (Abnahme) sowie 3-FL (Erhöhung) und 2'-FL (Abnahme). Im Gegensatz dazu waren die HMOs mit der höchsten Konzentrationsstabilität während der verschiedenen Stadien der Laktation LNFP V und LNFP III. Betrachtet man zusaetzlich das Stagingverhalten von HMOs in spezifischen HM-Gruppen, so zeigen LNDFH I in HM-Gruppe I und LNFP II in HM-Gruppe II ebenfalls keine oder nur niedrige Konzentrationsschwankungen.

In **Kapitel IX** wurde auch ausgeführt, wie die beobachteten Variationen einzelner HMO-Strukturen z.B. zwischen den HM-Gruppen oder über den fortschreitenden Laktationsverlauf (siehe **KAPITEL IV & VIII**) eine gesunde Säuglingsentwicklung fördern könnten. Dieses Kapitel fasste darüber hinaus auch noch einmal die wichtigsten Aspekte und Erkenntnisse aus den vorhergehenden Kapiteln zusammen. Zusätzlich wurden in **KAPITEL IX** Zukunftsperspektiven in Bezug auf die Entwicklung und Anwendung anspruchsvoller neuer Analysemethoden diskutiert, die für komplexe HMOs und Glykokonjugate geeignet sein könnten.

Abschließend wurden in **KAPITEL IX** noch ausführlich die möglichen Einflüsse von spezifischen HMOs auf eine ausgewogene Immunentwicklung des Neugeborenen betrachtet. Dies wurde unter besonderer Berücksichtigung möglicher HMOWechselwirkungen mit verschiedenen Immunrezeptoren, angefangen von Siglecs über CLRs bis hin zu TLRs, vorgenommen. Die hypothetische Möglichkeit einer gezielten Verbindung des angeborenen und adaptiven Immunsystems durch HMOs wurde ebenfalls angesprochen. Ein Beispiel für Aspekte, die in **KAPITEL IX** hervorgehoben wurden, war die auffällige Koinzidenz der Konzentrationsabnahme von 6'-SL zwischen 6 Wochen und 6 Monaten post-partum mit der Abnahme der Darmepithelzell-Sialisierung im gleichen Zeitraum[10].

Eine tiefergehende weitere zukünftige Auswertung der oben erörterten umfangreichen HMO-Datensätze könnte den Weg ebnen, um weitere Assoziationen von HMOs und günstigen Einflüssen auf die Gesundheit von gestillten Säuglingen offenzulegen. Basierend auf den schon erzielten neuen Erkenntnissen, die in den **KAPITELN II-IX** beschrieben wurden, könnten neue funktionelle Hypothesen zur Rolle von HMOs für eine gesunde Säuglingsentwicklung postuliert werden. Diese könnten möglicherweise mit zur Entwicklung von innovativen Formel-Nahrungen für Kleinkinder beitragen, die noch besser and das natürliche Vorbild Muttermilch angepasst sind.

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CHAPTER XIII

Curriculum Vitae, List of Publications & Acknowledgements

CURRICULUM VITAE

Date & Location of Birth	**02.12.1970 Giessen, Germany**
Address	Bachlaan 66, 3706 BD, Zeist, The Netherlands
University Entrance Diploma	1990: Abitur Liebigschule Gießen
Military Service	1990 -1991: Panzergrenadier, 2./ Panzerbataillon 61, 34466 Wolfhagen, Germany
Academic Education	1991- 1996: University of Applied Sciences Gießen-Friedberg / Technische Hochschule Mittelhessen /THM), Germany; degree program: Biotechnology 1996: Graduation to Diplom-Ingenieur (FH), Biotechnologie (Graduate Engineer Biotechnology); Thesis-title: "Monoclonal antibodies against a specific fungal elongation factor-Isolation and characterisation of antibodies, immuno-affinitychromatographic purification of the antigen" Supervisors: Prof. D. Kitzrow (THM) , Drs. Schönfeld & Ziegelbauer (Bayer AG)
Internships	1994: Institut für Hygiene und Umwelt, Lollar, Germany for 3 weeks: Microbiological, physicochemical and instrumental (GC, AAS, WWWAOX) analysis of drinking water according to „Trinkwasser-verordnung", sampling & sample pretreatment 1995-1996: Bayer AG Wuppertal, Germany: 4 month at Central Technical- / Process-Technical Research: Laboratory Dr. Henzler: Up and downstream process technology (micro- & ultrafiltration, determination of energy -input, mixing times & kla-values, enzymatic turnover in continuously stirred tank reactors) 5 month at Pharma-Research-Center: Laboratory Drs. Schönfeld / Ziegelbauer: Project activity in the development of an antimycotic agent using biochemical and immunological methods (immunoassays, immunography, immunoblotting, SDS-PAGE, HPLC, immunaffinity-chromatography etc.), LC-based protein isolation strategies, and in vitro translation-assay to test protein activity
Employment History	1997: Abbott GmbH Wiesbaden, Germany: GMP-production of in vitro diagnostica
Employment History	1997-2010: Laboratory-Engineer at Milupa GmbH Friedrichsdorf / Royal Numico N.V, Germany, Carbohydrate-research: Carbohydrate-/protein- and lipid-research, analytical method development (mass spectrometry and general instrumental analytics & bio analytics), isolation / purification of glycans (human milk oligosaccharides, prebiotics), glycopeptides/-proteins, -lipids, lipids & oligonucleotides; Preparation of presentations, publications, patent files, scientific information acquisition

	2010: Scientist / manager at Danone Research Centre For Specialised Nutrition, Germany: Scientific + technical projects as well as supervision and mentoring of students and lab-staff 2012: Scientist / Line Manager at Danone Research Centre For Specialised Nutrition, Germany: Scientific and technical projects, supervision of students and 1 employee 2012: Team Leader "Advanced Analytical Discovery" at Danone Research Utrecht: Interdisciplinary analytical and technical research projects relating to Human Milk Research, immunity, gut microbiology, medical nutrition; supervision and mentoring of students, line management of 4 employees
Selected Industrial (PPP-) Research Projects 1997-2020	"Neue Antiinfektiva": Proteomics of pathogens via 1D- or 2D gel based MALDI-MS-Peptide-Mass Fingerprinting & shotgun approaches (cooperation with university of Würzburg, Germany (Prof. Göbel)) Large scale separation of lactose from oligosaccharides by preparative scale ultra-& nanofiltration (cooperation with Biotechnologie-Institut-Mittelhessen, Germany (BIM, Prof. Czermak) Characterization of human milk oligosaccharides up to *m/z* 6000 by MALDI and direct nanoESI-MSN in cooperation with University of Frankfurt (Prof. Karas) Characterisation of novel CD 22 isoforms in different species by accurate mass nanoESI-FTICR-MS "composition based sequencing" (CBS) in cooperation with University of Giessen, Germany (Prof. Spengler) Proteomics of iTRAQ-labelled human milks from different stages of lactation "MikrOligo": Technical projectleader; effects exerted on microorganisms by oligo-and polysaccharides from plants or bacterial production "ATHENA" Danone Nutricia: Local project leader: Relocation of complete research facility from Germany to the Netherlands; also "Building Project", "IT/IS" & „WoW". Establishment of the "Advanced Analytical Discovery" team (AAD) at Nutricia Research Center in Utrecht: Substantiation of human milk / life science research & functional innovations; focus mass spectrometry and (bio-) analytical methods "Advanced milk cohort analysis" (AMICA) DNR project manager in cooperation with Profs. Genuneit & Rothenbacher, Universities of Ulm & Leipzig:

Promotion	2021: Utrecht University, Utrecht Institute for Pharmaceutical Sciences (UIPS) Thesis-title: "Advanced Characterization of Biofunctional Human Milk Oligosaccharides by Mass Spectrometry and Complementary Methods" Promotor: Prof. Dr. Albert J. R. Heck Co-promotor: Dr. Bernd Stahl
Further training & development (selection)	Danone Nutrica Research Trainings: Danone Leadership College (DLC), CODE-Developer (management & coaching), "Getting things done"; "Presenting convincingly", "Leading as a coach"; "Situational leadership", "1st 1000 days ambassador", "Intuitive Biostatistics", "Development conversation for line managers", "Finance for non-financial managers", '"Finance Project management tools", "Advanced biostatistics", "Immunology", "WISE leadership", "Compliance training", "OPOH, "Insights", "Boost R&D Impact", etc;
Scientific conferences	Multiple MS-vendor user meetings (Sciex, Dionex, Thermo Scientific, etc.); Desorption (2000), Bioanalytical Quantum Steps (2003), Bioperspectives (2004 + 2005), Prokagen (2005), HUPO (2005), Glycan Forum (2011, 2012 + 2013), Bioinspired Therapies (2016), GlycoBioTec Berlin (2017 + 2019), Eurocarb (2019), Annual meeting DGMS (several), Regular meetings IMSC (several), Deutscher Lebensmittelchemiker Tag (several), Nutricia Forum Muttermilchforschug/Geburtshilfe Dialog Mannheim (several), Forum medicine in Pregnancy Leipzig, EACCI (several), , Annual conference Espghan, Annual conference ISRHML, Annual conference ASMS (several), etc.
Publications & Patents:	Co-author- and authorship: papers peer-reviewed (>15), posters (>20), several lectures, reviews (2); patents (3)
Languages:	German, English, Latin (basic skills), Dutch (basic skills)
Hobbies:	Ski alpine, Jogging, Dancing (standard)

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"The reason why we can look so far is because we are sitting on the shoulders of giants" - (John B. Fenn, Nobel laureate, anniversary symposium Justus von Liebig "Bioanalytical Quantum Steps" in 2003)

Now that this thesis is almost finalized, it is a real pleasure to express my appreciation to all the people who made its completion possible.

Dear Albert, thanks a lot for accepting me as your PhD student and your openness to start the adventure of a new research topic which was "human milk" and the contained human milk oligosaccharides (HMOs). I was honoured to create this thesis under your supervision. You did not only guide me scientifically e.g. regarding high end mass spectrometric analysis of "HMOs" and beyond, but also helped to set the right milestones during my PhD journey. You did this with a lot of patience and the right kind of input at the right time. I am honestly grateful for all your support and hopefully there will be new opportunities for joined research in future!

Dear Bernd, this PhD thesis is another landmark in our joined history of human milk research which is now lasting for more than 20 years! You were my co-promotor but also my mentor in biomolecular mass spectrometry, human milk research and many topics apart from science. I still remember the moment in August 1997 when you told me that you are looking forward to collaborate with me. Shortly after this meeting I began to dive into the field of human milk research as a young lab-engineer in your carbohydrate research group at Milupa Research in Friedrichsdorf, Germany. Back then and in the following years, it was a great experience to gather "hands on" knowledge about (MALDI-) mass spectrometry, oligosaccharides, and the secrets of human milk. This occurred in an environment which offered a lot of scientific and experimental freedom motivating everyone in the former Friedrichsdof R&D teams. The prebiotics "scGOS/lcFOS" which are nowadays improving many infant milk formulae are only one achievement resulting from these times. After restructuring of our research department which became part of Royal NUMICO and later on Danone Nutricia Research (DNR) in Utrecht, our ways split for a while. Today, I am happy to further evolve analytical sciences tailored to human milk and infant milk formula together with my team and as integral part of your centre of excellence within DNR. Thanks a lot for this amazing journey! I am really looking forward to contribute to new improved nutritional solutions for infants (and beyond) inspired by human milk.

Dear Johan, did you know that you actually made this PhD thesis possible? After the end of our very first meeting with Albert and Bernd at a time when the building

of the DNR research site in Utrecht was not even finalized you said: “Shall I ask Albert if he would like to be your PhD promotor?”. I was happy about this idea and so you directly went back to Albert who indeed could imagine to be my supervisor. Incredible! Most interestingly, the topics we discussed back then are still of scientific interest today: oligosaccharides, peptides, proteins and their influence on healthy (immune-) development of neonates. Hopefully, it will be possible in the next years to further deepen analytical insights about human milk composition and its immunological role together with you and your team!

Nana, also you need to be mentioned of course. Not only, because of your continued support as team leader in Friedrichsdorf and thereafter which enabled people (including myself) to grow in many respects, but also for your continued help beyond professional engagement. You once gave me the good advice to think about doing a PhD in the Netherlands instead of Germany. This was one of the best advices I ever got!

Dear Alma, thanks a lot for providing such a great working atmosphere within your Human and Essential Milks team at DNR. I admire how you manage and link both, in depth scientific work and the needs of daily business duties. I am also grateful that you could spent the time to read relevant chapters of my thesis and that you, together with Bernd, granted me the time to finalize my thesis. I am looking forward to new research insights and innovation inspired by human milk!

For sure, I will also not forget the support of you Hans, the first R&I and site director of Nutricia Research in Utrecht. Dear Hans, thanks for believing in “advanced analytical discovery (AAD)” and for helping me to start up the team and for supporting the start of this thesis!

Talking about early days of the AAD team in Utrecht: Without you Philipp, Joost and Amra it would not have been possible to do anything. Philipp, you deserve many of the credits for re-animating all the instrumentation we saved from Friedrichsdorf and for development of our first MRM-LC-MS methods for HMOs. It was also inspiring to supervise your Diploma thesis and to outline your PhD thesis about HM protein glycation. Joost, without having you in the small team of the early days we wouldn’t have been able to develop the next level of proteomics or to establish a working lab (IT-) infrastructure. Thanks a lot for all your contributions until today. Amra, you are one of the most loyal and warm hearted colleagues I had the pleasure to become acquainted with. I wanted to thank you for the good times we had back in the early days of AAD and continue to have at multiple occasions still today.

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inspiring and something I wouldn't like to miss. I am grateful that you welcomed me during my time in the GBM team and for the nice projects we could conduct.

Dear Simone! It appears to me that we are colleagues in the HMR-AS team since many years although you just joined not too long ago. Thanks a lot, not only for editing my "Dutch PhD summary" but for the good and inspiring teamwork in HMR-AS!

LooWee, special thanks also to you! Your perseverance in pursuing the publication of our joined research in the field of HMOs and probiotics was great and added a whole chapter to this thesis!

Ingrid, it is clear that your ability to anticipate the future was always an incentive for me to turn your prophecy ("Hello Dr. Mank") one day into reality. It's a real asset to have great colleagues like you around!

The same is of course true for you Joris, Saskia, Selma, Bea, Leon and the whole team of the CoE Immunology at DNR! I admire your expertise as well as your elegant ideas to reveal the functional benefits of e.g. HMOs and other HM ingredients by various in vitro and vivo approaches. Chapter II of this thesis was therefore dedicated to these aspects. I was happy to contribute to the published review about the diversity of HMOs and their immunological early life implications together with Arthur, Veronica and Bea.

Bernadet, John, Aliye and Sibel! You are the heart of our advanced analytical discovery team! I feel privileged to be in lead of a team with such a dedication, expertise and enthusiasm! You are the reason why we are able to develop and apply unique front edge analytical approaches suited to unravel still unknown secrets and functional benefits of human milk and beyond. I gratefully appreciate your continuous and crucial contributions to analytical sciences, human milk research and functional innovations of infant milks! The perspective to continue our journey through this fascinating field of life sciences, now and in the coming years, is very rewarding and a great motivation.

Gerrit, Eline, and Martin! We have been working together for so long now, even before Nutricia Research in Utrecht was created. Your expertise and accomplishments with regard to almost any field of analytical chemistry are indispensable for our joined early life research. Thanks a lot for this!

Jing and Kelly, you were the drivers of advanced proteomics and peptidomics applied to human milk via your own PhD theses in the last 3 years. Without you and Albert, we wouldn't have revealed the incredible diversity of the human milk proteome,

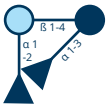
peptidome and glycoproteome which becomes more and more important today. The functional implications of these insights as well as the analytical advances and publications which resulted from our collaboration are fabulous. I was privileged to facilitate our regular meetings which were linking your research with DNR and to discuss all the new findings together with you Bernd and Albert. For sure, we are not at the end but just at the beginning of “cracking” the human milk “omics” composition. It will be fascinating to assign early life functions to the newly revealed human milk compounds!

Dear Robert and Erdmann, I owe you gratefulness for the long lasting research relationship we developed since the days when Robert accomplished his diploma research in Friedrichsdorf. It is both, exciting and encouraging to steadily drive the development and application of advanced analytical methods like xCGE-LIF together with you. Our joined efforts are paving the road to expand the knowledge about human milk oligosaccharides and their relevance for early life development, now and in future!

Dear Jon, Linda, Katerina, Frazer, Joris and Selma! It is also time to thank you sincerely for the very fruitful and open collaboration in the AMICA project. It was always a pleasure to exchange and learn from you e.g. during our project meetings. The HM cohort studies were and hopefully will again be one of the biggest opportunities to find new associations between the most relevant human milk ingredients and their impact on a health early life.

Liebe Eltern. Natürlich kann ich Euch und meine ganze Familie (in Mittelhessen und im Münsterland) hier nicht unerwähnt lassen. Ihr habt mich immer unterstützt und tut es noch heute. Dafür bin ich Euch mehr als dankbar. Wie Du siehst liebe Anneliese, war das tägliche Abfragen von Englisch-Vokabeln im 5. und 6. Schuljahr nicht nur mit viel Spass verbunden, sondern hat sich letztendlich doch auch anderweitig ausgezahlt 😉!

Den größten Dank aber schulde ich Euch, Britta und Matteo. Meine Lieben, ihr seid mir nicht nur in das „Abenteuer Niederlande“ gefolgt, sondern wart trotz vieler Herausforderungen immer an meiner Seite. Dafür und für vieles mehr liebe ich Euch von ganzem Herzen.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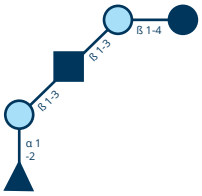
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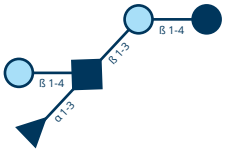
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