Gastrointestinal-active oligosaccharides from human milk and functional foods

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Thesis

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Abstract

Oligosaccharides, as present in human milk or supplemented to food, are renowned for their biological activity in the gastrointestinal tract. So far, little is known about the implication of oligosaccharide structures on their gastrointestinal fate. The influence of diet-related oligosaccharides on the postnatal gastrointestinal development and on the establishment of a balanced microflora is of special interest. Therefore, the present research aimed at an advanced understanding of the gastrointestinal metabolization of diet-related oligosaccharides, focusing on infant nutrition.

Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was introduced as a sensitive, qualitative and quantitative method for the analysis of individual galactooligosaccharides (GOS) from complex food matrices. The method also showed to be useful for the monitoring and characterization of complex konjac glucomannan (KGM) oligosaccharides, resulting from enzymatic digestion of the KGM polysaccharide and *in vitro* fermentation with human gut flora. The analysis and identification of human milk oligosaccharides (HMOs) in breast milk and the characterization of oligosaccharides as present in the feces of breast-, formula- and mixed-fed babies was performed by CE-LIF coupled to a mass spectrometer (CE-LIF-MS^n). The type of feeding determines the presence of diet-related oligosaccharides in baby feces. For breast-fed babies a gradual change in fecal oligosaccharide profile was found during the first six months postpartum. Three continuous stages of fecal oligosaccharide profiles were defined, comprising the presence of the genetically determined HMO-profile of the breast milk consumed (stage 1), the presence of HMO-units conjugated to blood group determinants from gastrointestinal mucins (stage 2) and predominantly oligosaccharides characteristic for follow-up feeding when solid food is introduced (stage 3). In total, sixteen fecal oligosaccharides, which pointed to the degradation and gastrointestinal metabolization of diet-related oligosaccharides and which were not present in human milk or infant formula, were identified in this research.

Monitoring the gastrointestinal fate of diet-related oligosaccharides pointed to an individual-dependent gastrointestinal adaptation to enteral food during the postnatal period.

Keywords: human milk oligosaccharides (HMOs), galacto-oligosaccharides (GOS), konjac glucomannan (KGM), breast milk, baby feces, gastrointestinal metabolization, blood-group specific conjugates, CE-LIF-MS^n

List of abbreviations

AHC	agglomerative hierarchical clustering
APTS	9-aminopyrene-1,4,6-trisulfonate
BG	blood group
CE-LIF	capillary electrophoresis with laser-induced fluorescence detection
DA	degree of acetylation
DF-L	difucosyllactose
DF-LNH	difucosyllacto-N-hexaose
DF-LnNH	difucosyllacto-N-neo-hexaose
DF-LNO	difucosyllacto-N-octaose
DS-LNH	disialyllacto-N-hexaose
DS-LnNH	disialyllacto-N-neo-hexaose
DS-LNT	disialyllacto-N-tetraose
EG	endo-glucanase
EM	endo-mannanase
FL	fucosyllactose
F-LND	fucosyllacto-N-decaose
F-LNH	fucosyllacto-N-hexaose
F-LnNH	fucosyllacto-N-neo-hexaose
F-LNO	fucosyllacto-N-octaose
FOS	fructooligosaccharides
FS-LNH	fucosyl-sialyllacto-N-hexaose
FS-LNT	fucosyl-sialyllacto-N-tetraose
Fuc	fucose
Gal	galactose
GalNAc	N-acetylgalactosamine
Glc	glucose
GlcNAc	N-acetylglucosamine
GOS	galactooligosaccharides
HPAEC	high performance anion exchange chromatography
Hex	hexose
HexNAc	N-acetyl-hexosamine
HMO	human milk oligosaccharides
ISTD	internal standard
KGM	Konjac glucomannan
L	Lactose
LND	lacto-N-decaose
LNDFH	lacto-N-difucosylhexaose
LNFP	lacto-N-fucopentaose
LNH	lacto-N-hexaose
LNT	lacto-N-tetraose
LnNT	lacto-N-neo-tetraose
MALDI-TOF MS	matrix-assisted laser desorption/ ionisation time-of-flight mass spectrometry
MS^n	mass $spectrometry^n$
Man	mannose
Neu5Ac	sialic acid
Neu5Gc	glycolylic acid
PAD	pulsed amperometric detection
SL	sialyllactose
S-LNH	sialyllacto-N-hexaose
S-LnNH	sialyllacto-N-neo-hexaose
S-LNT	sialyllacto-N-tetraose
SPE	solid phase extraction
TFA	trifluoroacetic acid
TF-LNH	trifucosyllacto-N-hexaose
TF-LNO	trifucosyllacto-N-octaose
TS-LNH	trisialyllacto-N-hexaose

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Chapter 1

General introduction

Project outline

Health-promoting oligosaccharides are of broad public interest, but little is known about their structure-function relationship. In order to understand the gastrointestinal fate of these oligosaccharides, it is necessary to:

1) Have methods available to isolate, identify and quantify the oligosaccharides in complex food and body matrices.
2) Monitor their gastrointestinal degradation and metabolization *in vitro* and *in vivo*.

These requirements formed the basis of this research project. The research presented in this PhD thesis focuses on the characterization and gastrointestinal metabolization of human milk oligosaccharides and functional oligosaccharides supplemented to infant nutrition. For this purpose, valuable sample material, e.g. resulting from a hospital study with preterm born babies, was provided by the project partners.

Functional oligosaccharides play an important role in infant nutrition. They influence the development and bacterial colonization and the gastrointestinal functioning of neonates, which are connected to short- and long-term infant health. An advanced understanding of the gastrointestinal fate of functional oligosaccharides in baby foods is thus of nutritional, medical, scientific as well as economical relevance.

Gastrointestinal development and functioning

The maturation of the gastrointestinal tract during the post-conceptional period is a gradual process (**Figure 1**). At term birth (> 36 weeks gestation), the lipid- and protein-digestion is still immature,[1,2] but is expected to quickly reach its mature activity with the introduction of enteral food.[3] The carbohydrases maltase and sucrase are present in mature levels at term birth, showing a gradual development *in utero*.[1] On the other hand, lactase activity only increases markedly between the 24^{th} and 40^{th} post-conceptional week of gestation and its full activity is considered as a marker for gastrointestinal maturity.[1,4] Rapid maturational changes in terms of digestive functioning, nutrient absorption, endocrinology, microbiology and immunology occur with the introduction of enteral food, and, later on, during weaning, which also implies a marked change in dietary habits.[3] Special attention has to be paid to the development of the gastrointestinal microbiota. At birth, the gut presents a sterile environment, which is gradually colonized, depending on the feeding provided. The composition of the colonic microbiota is closely connected to the host's well-being.[5]

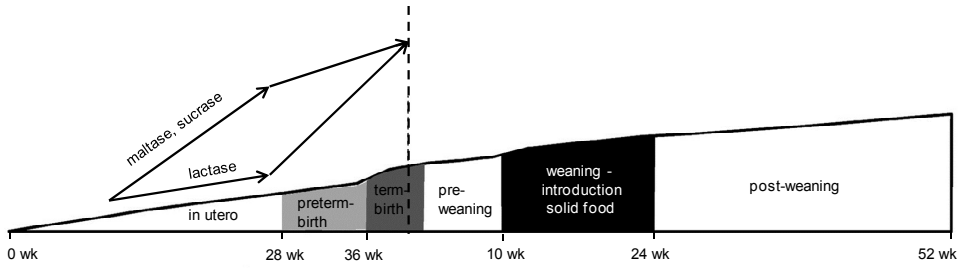


Figure 1. Phases of intestinal maturation *in utero* and during first year postpartum, as adapted from Sangild (copyright © 2006 by the Society for Experimental Biology and Medicine).[3] The development of the carbohydrases maltase, sucrase and lactase is schematized.

Development of the gastrointestinal microbiota

Approximately 10^{12} bacteria per gram gut content inhabit the adult colon and these bacteria are distributed within the gastrointestinal lumen or are attached to the mucosal surface layer.[5] The intestinal flora comprises health-beneficial bacteria (e.g. Bifidobacteria) and potentially harmful bacteria (e.g. Clostridia), which commensally live together in a balanced proportion in healthy individuals.[5] The gastrointestinal microbiota gradually develops during the first year of life.[6] An overview on the postnatal development of the gastrointestinal microbiota is given in **Figure 2**, which summarizes the results from various studies on the neonatal development of the gastrointestinal microbiota.[6-10] Oxygen is present in the colon at birth and is made responsible for the pronounced presence of facultative anaerobe bacteria (e.g. *E.coli* or other *Enterobacteriaceae* spp) during the first weeks. The development of this initial flora is feeding-dependent. During the first weeks of life the colonization pattern changes gradually to a stabilized flora, with a relatively "simple" flora in case of breast feeding. Bifidobacteria form a considerable part of this "simple" flora, as present during the pre-weaning phase.[6,9] *Bifidobacterium longum*, *Bifidobacterium bifidum* and *Bifidobacterium breve* are characteristic representatives, with *Bifidobacterium longum* ssp *infantis* being the hallmark of the microbiota of breast-fed babies.[6,11] Formula-fed babies show similar Bifidobacteria counts, but the contribution of other anaerobic bacteria (e.g. Bacteroides) and facultative anaerobic bacteria (e.g. *E.coli*) to the total microbiota is higher.[6,9]

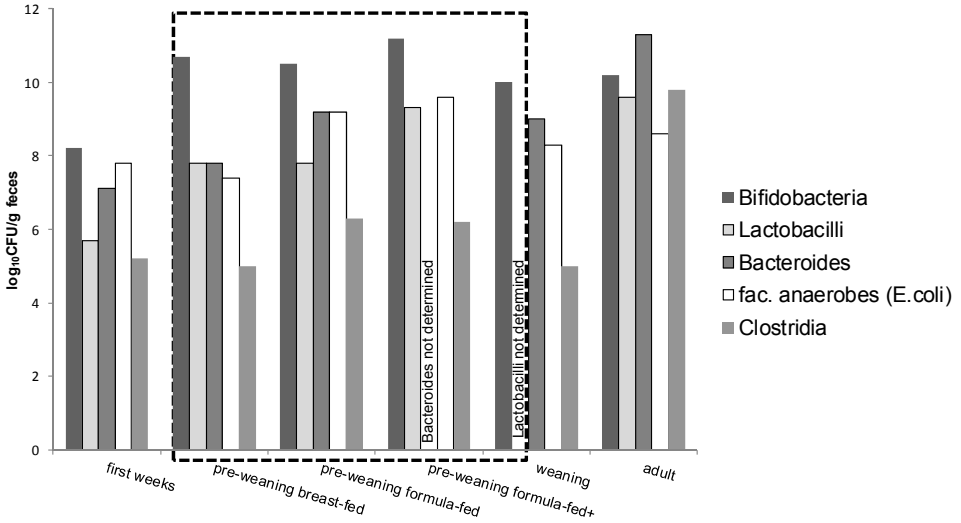


Figure 2. Succession of gastrointestinal colonization in the postnatal period, as influenced by different ways of feeding. Dashed box summarizes different possibilities of infant nutrition during pre-weaning phase. Formula-fed+ = formula supplemented with prebiotic oligosaccharides. Mean values are only indicative, as they were derived from different studies.[6-10]

The supplementation of prebiotic oligosaccharides to infant formula leads to increased counts of Bifidobacteria and Lactobacilli, while similar counts of facultative anaerobic bacteria is similar to what is observed for babies fed with non-supplemented formula.[8]

A mixed flora is characteristic for the weaning cluster when solid food is introduced. The change in flora composition during weaning is less pronounced for formula-fed babies, but presents a major disturbance in the colon of breast-fed babies. During the first years of life, the microbiota gradually develops to its adult-like form, which is dominated by Bacteroides, followed by Bifidobacteria and Firmicutes (e.g. Clostridia and Ruminococci).[6,9] Variations of the colonization pattern are mainly observed in case of caesarean delivery and preterm birth, which implicate a longer time period for the installation of the commensal gut flora.[8,12]

Gastrointestinal oligosaccharide metabolization in relation to health

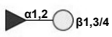
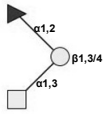
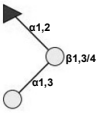
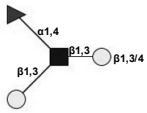
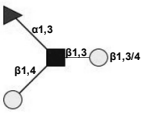
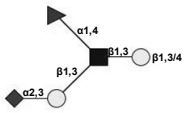
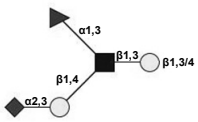
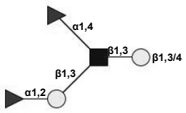
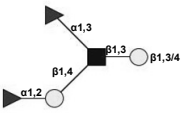
Gastrointestinal bacteria have different nutritional requirements and can be sub-divided into proteolytic bacteria, gas-utilizers and saccharolytic bacteria, with the latter group being most abundant. Besides endogenous carbohydrates, non-digestible, feeding-related carbohydrates are the main substrate available for colonic fermentation.[5,13,14] Feeding can influence the gastrointestinal flora composition to a certain extent and non-digestible oligosaccharides specifically stimulating the growth of health-beneficial bacteria in the large intestine are called prebiotics.[5] Due to their indigestibility in the upper gastrointestinal tract, HMOs from breast milk and oligosaccharides added to breast milk replacers are substrates for colonic fermentation and are of special interest in view of the postnatal establishment of the microbiota and infant health.

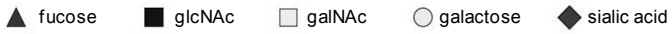
Endogenous gastrointestinal carbohydrates

Endogenous gastrointestinal carbohydrates are represented by glycoproteins (e.g. mucins) and other polysaccharide derivatives (e.g. glycosaminoglycans).[5] The bioactive functionality of mucins, which are part of the gastrointestinal mucus layer are of special scientific interest.[15-17] The gut surface is covered by a mucus gel layer bound to the epithelial cell surface and mucus is secreted into the gut lumen by gastrointestinal goblet cells.[18,19] The production and erosion of mucus is a balanced system, with the latter one occurring by mechanical shear and enzymatic digestion by luminal proteases.[20] These mucus gel layers are, besides mainly water and electrolytes, composed of mucins, which are polypeptides heavily glycosylated with mucin-type O-glycans.[19] Colonic mucins have a core structure of Gal-(β1,3)-GalNAc (core 1), GlcNAc-(β1,6)-[Gal-(β1,3)]-GalNAc (core 2) or GlcNAc-(β1,3)-GalNAc (core 3), which are α-linked to a serine or threonine in the polypeptide backbone. The core structures can be decorated with sialic acid or elongated by linear or branched Gal-(β1,4)-GlcNAc-units, which can be further modified by sulfate esters. The terminal determinants of mucins are A, B, H(0)-blood group antigens, Lewis-antigens and sialyl-Lewis-antigens.[18] The main antigens of mucins are summarized in **Table 1**. Their expression depends on the host's Lewis (Le/le) and Secretor (Se/se) gene system. The Lewis-blood group system is genetically independent from the ABH(0) system, but the expression of the Lewis phenotypes is dependent of the ABH(0) secretor-status, which determines the presence of (α1,2)-fucosylated oligosaccharides.[21] In addition, ABH(0)-non-secretors show Le^a-character [Le(a+b-)] and ABH(0)-secretors show Le^b-character [Le(a-b+)]. [Le(a-b-)]-secretors neither show Le^a- nor Le^b –character.[21]

Besides being lubricants, important other biological functions are ascribed to mucins. They participate in the control of the immune system and, dependent on their structural composition, they present receptors for specific bacteria and inhibit the adhesion of others.[18] The mucosal glycoproteins can serve as nutrients for bacterial growth, e.g. for *Bifidobacterium* and *Ruminococcus* spp.[15,22,23] Due to the consequent decrease of the protective mucus barrier, an advanced bacterial mucin-degradation is not desired.[24]

Table 1. Antigenic determinant structures as found at the non-reducing terminus of mucin-type O-glycans. Their presence depends on the Secretor-/ Lewis type [Le(a-b+), Le(a+b-), Le(a-b-)], as indicated.

		Le(a-b+)	*Le(a+b-)*	*Le(a-b-)*
H(0)		+	-	+
A		+	-	+
B		+	-	+
Le^a		+	+	-
Le^x		+	+	+
Sialyl-Le^a		+	+	-
Sialyl-Le^x		+	+	+
Le^b		+	-	-
Le^y		+	-	+



Human milk oligosaccharides (HMOs)

Lactose and HMOs are the main components of the carbohydrate fraction in human milk.[25] Colostrum milk (1-4 days postpartum) contains 21-24 g/L HMOs, whereas for mature milk (as established approx. one month postpartum) the concentration ranges between 12-13 g/L and then decreases with proceeding time postpartum.[25-29] The reducing end of HMOs is composed of a lactose unit, to which lacto-N-biose units (Gal-(β1,3)-GlcNAc) or lactosamine units (Gal-(β1,4)-GlcNAc) are attached.[30] This results in a set of basic structures, either based on type 1 [Gal-(β1,3)-GlcNAc-(β1,3)-Gal-(β1,4)-Glc] or type 2 [Gal-(β1,4)-GlcNAc-(β1,3)-Gal-(β1,4)-Glc] (**Figure 3**). High-Mw HMOs can be either linear or branched.

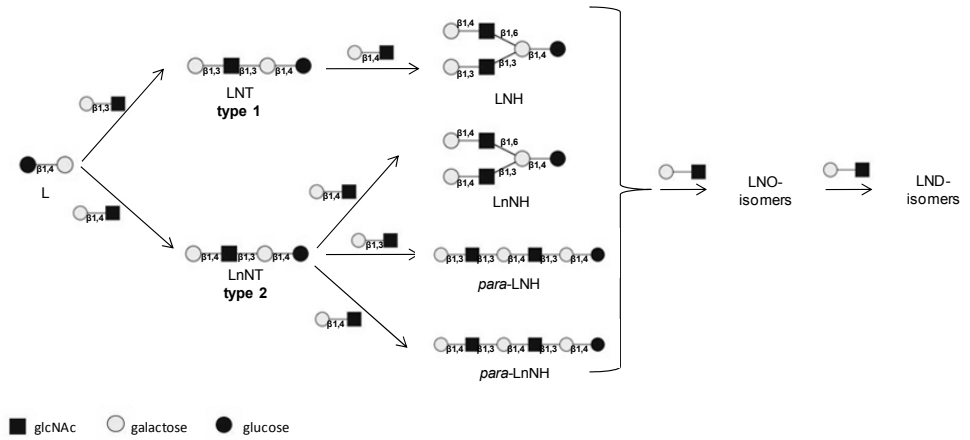
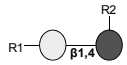
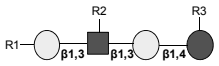
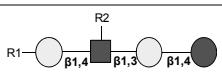
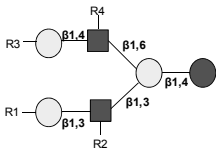
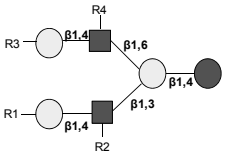
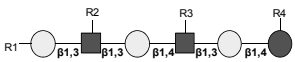
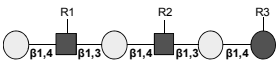


Figure 3. Structural composition of core oligosaccharides of HMOs. L: lactose; LNT: lacto-N-tetraose; LnNT: lacto-N-neo-tetraose; LNH: lacto-N-hexaose: LnNH: lacto-N-neo-hexaose. The elongation of the core chains to lacto-N-octaose- and lacto-N-decaose-isomers is indicated.

HMOs are synthesized in the mammary glands and can be further decorated by glycosyltransferases with fucose and sialic acid.[30] Oligosaccharides in human milk can thus be divided into a neutral and an acidic fraction, with acidic oligosaccharides being present in a tenfold lower concentration than neutral oligosaccharides.[31] The structural composition of HMOs has been extensively studied and results obtained by Kuhn et al,[32,33] Kobata et al,[34,35] Montreuil,[36] Strecker et al[37,38] and many others contributed to a well-defined picture on the glycobiology of oligosaccharides in human milk. **Table 2** gives an overview on the structural composition of fucosylated and sialylated HMOs up to a core structure of six sugar units, as reviewed by Urashima et al.[30]

Table 2. Fucosylation and sialylation of HMO-core structures[30] and their concentration in Le(a-b+)-, Le(a+b-)- and Le(a-b-)-breast milk.[39] [lactose (L), lacto-N-tetraose (LNT), lacto-N-neo-tetraose (LnNT), [*para*]-lacto-N-hexaose ([*para*-]LNH) and [*para*-]lacto-N-neo-hexaose ([*para*-]LnNH)].
Fuc: fucose; Neu5Ac: sialic acid. nd: not determined, low concentration is expected.

core-HMO structures (Figure 3)	Fuc-(α1,2)-	Fuc-(α1,3)-	Fuc-(α1,4)-	Neu5Ac-(α2,3)-	Neu5Ac-(α2,6)-	name	g/L Le(a-b+)	g/L Le(a+b-)	g/L Le(a-b-)
 **L**	-	-	-	-	-	**L**	57	58	55
	R1	-	-	-	-	**2'FL**	3.13	-	4.57
	-	R2	-	-	-	**3'FL**	0.42	1.79	0.15
	R1	R2	-	-	-	**DF-L**	0.41	-	0.17
	-	-	-	R1	-	**3'SL**	0.27	0.24	0.31
	-	-	-	-	R1	**6'SL**	1.22	1.14	1.31
 **LNT**	-	-	-	-	-	**LNT**	0.88	1.19	0.85
	R1	-	-	-	-	**LNFP I**	1.58	-	3.18
	-	-	R2	-	-	**LNFP II**	0.22	1.25	-
	-	R3	-	-	-	**LNFP V**	nd	nd	nd
	R1	-	R2	-	-	**LNDFH I**	1.28	-	-
	-	R3	R2	-	-	**LNDFH II**	0.20	0.45	-
	-	-	-	R1	-	**S-LNTa**	0.04	0.04	0.06
	-	-	-	-	R2	**S-LNTb**	0.08	0.11	0.08
	-	-	-	R1	R2	**DS-LNT**	0.34	0.42	0.41
 **LnNT**	-	-	-	-	-	**LnNT**	0.23	0.08	0.23
	-	R2	-	-	-	**LNFP III**	0.38	0.38	0.31
	-	-	-	-	R1	**S-LNTc**	0.29	0.21	0.31
 **LNH**	-	-	-	-	-	**LNH**	0.12	0.09	0.10
	R1	-	-	-	-	**F-LNH I**	0.21	-	0.26
	-	R4	-	-	-	**F-LNH II**	0.16	0.35	0.13
	R1	R4	-	-	-	**DF-LNH I**	0.33	-	0.58
	-	R4	R2	-	-	**DF-LNH II**	nd	nd	nd
	R1	R4	R2	-	-	**TF-LNH**	nd	nd	nd
	-	-	-	-	R3	**S-LNH**	nd	nd	nd
	-	-	-	R1	R3	**DS-LNH I**	nd	nd	nd
	-	-	-	R1	R2	**DS-LNH II**	nd	nd	nd
	-	-	-	R1	R2,R3	**TS-LNH**	nd	nd	nd
 **LnNH**	-	-	-	-	-	**LnNH**	nd	nd	nd
	R1/R3	-	-	-	-	**F-LnNH**	nd	nd	nd
	-	R2, R4	-	-	-	**DF-LnNH**	nd	nd	nd
	-	-	-	-	R3	**S-LnNH I**	nd	nd	nd
	-	-	-	-	R1	**S-LnNH II**	nd	nd	nd
	-	-	-	-	R1,R3	**DS-LnNH**	nd	nd	nd
 ***para*-LNH**	-	R3	-	-	-	**F-*para*-LNH I**	nd	nd	nd
	-	-	R2	-	-	**F-*para*-LNH II**	nd	nd	nd
	-	R3	R2	-	-	**DF-*para*-LNH**	nd	nd	nd
	R1	R3	R2	-	-	**TF-*para*-LNH I**	nd	nd	nd
	-	R3,R4	R2	-	-	**TF-*para*-LNH-II**	nd	nd	nd
 ***para*-LnNH**	-	R1,R2	-	-	-	**DF-*para*-LnNH**	nd	nd	nd
	-	R1,R2,R3	-	-	-	**TF-*para*-LnNH**	nd	nd	nd

The larger the molecules are, the lower is their concentration in breast milk. Oligosaccharides based on type 1 (LNT) are dominant in human milk.[30]
Although not presented in **Table 2**, HMOs can as well be simultaneously sialylated and fucosylated, which increases the structural complexity of the molecules. So far, approximately 200 different structures have been detected in human milk[40] whereof approximately 100 have been identified.[30]

Genetic fingerprint of HMOs

The presence and abundancy of HMOs in breast milk is genetically determined.[41] The (α1,2)-fucosyltransferase (FUT2) and (α1,3/4)-fucosyltransferases (FUT3, 4, 5, 6, 7, 9), which are responsible for the fucosylation of the HMOs in the mammary glands, correspond to the fucosyltransferases responsible for fucosylation of other body glycoproteins (e.g. on erythrocytes and mucins).[42] The expression of FUT2 and FUT3 depends on the maternal Secretor- and Lewis type, respectively.[42] Accordingly, human milk can be classified into four groups:[41]

1) Human milk from Le(a-b+)-secretors contains (α1,2)-, (α1,3)- and (α1,4)-fucosylated oligosaccharides, which accounts for 70% - 80% of the European population.[41,43,44]
2) Human milk from Le(a+b-)-non-secretors contains (α1,3)- and (α1,4)-fucosylated oligosaccharides, but lacks (α1,2)-fucosylated oligosaccharides. This is found for approximately 20% of the European population.[41,43,44]
3) Le(a-b-)-secretor-milk contains (α1,2)- and (α1,3)-fucosylated oligosaccharides and does not contain (α1,4)-fucosylated oligosaccharides. The occurrence of the Le(a-b-)-secretor status is only approximately 10%, as was determined for the French population. [41,43]
4) Le(a-b-)-non-secretor-milk is composed of (α1,3)-fucosylated oligosaccharides, but lacks (α1,2)- as well as (α1,4)-fucosylated oligosaccharides. The occurrence of this milk type was estimated as rather low (1%, as was determined for the French population).[41,43]

The concentration of HMOs in breast milk of Le(a-b+)- Le(a+b-)- and Le(a-b-)-mothers is indicated in **Table 2**.

HMOs and health

Breast-feeding is connected to a lowered risk of inflammatory bowel diseases and gastrointestinal, respiratory and urinary infections in the postnatal period.[45-48] The health-beneficial characteristics of breast milk are mainly connected to the complex HMO-structures, which were also proposed to be involved in the development of the immune system.[49] Even an influence on brain development has been proposed.[50] Direct pathogen inhibition and bifidogenicity are well-studied examples of health-related structure-function relationships of HMOs.

Direct pathogen inhibition

HMOs can act as receptor analogs for preventing the adhesion of pathogenic bacteria to the mucosal surface. This ligand-receptor mechanism is based on the structural characteristics of HMOs, which are complementary to the structures of the carbohydrate epitopes in the mucosa.[30] *In vitro* experiments showed that sialylated HMOs prevent the adhesion of S-fimbriated enteropathogenic *E.coli* and Influenza A virus.[51] Special attention has to be paid to (α1,2)-fucosylated HMOs, which showed inhibition of campylobacter, norovirus and toxin-producing *E-coli in vitro* and a lower incidence of diarrhea in breast-fed infants *in vivo*.[52-55] Babies who consumed milk rich in (α1,2)-fucosylated HMOs showed less incidence for diarrhea than babies, who received milk with low (α1,2)-fucosylated HMO–levels.[54] There is still insufficient knowledge in order to judge different types of breast milks on their functional value.

Bifidogenicity

The microbiota of breast-fed infants is described as “simple”, with a considerable contribution of Bifidobacteria.[9] Bifidobacteria are known to inhibit the growth of pathogens by short chain fatty acid production and to stimulate the immune response, cholesterol assimilation and the synthesis of vitamins.[56-58] Infant-specific *Bifidobacterium* species exhibit cellular transporter systems for intact HMOs[59] or exhibit extracellular enzymes necessary to degrade HMOs, such as sialidase, (α1,2)-/(α1,3/4)-fucosidase and lacto-N-biosidase-phosphorylase.[11,60-62] Lacto-N-biose, the building block of type-I HMOs (**Figure 3**), has been described as essential bifidogenic factor of breast milk.[63]

Non-digestible oligosaccharides in infant food

Prebiotic oligosaccharide-supplementation to infant formula aims at simulating the health-promoting effect of HMOs. Due to the complexity of HMOs, their synthesis or extraction cannot be performed on a production scale to date. In view of the high galactose content in human milk and the lactose-based structural composition of HMOs, galactooligosaccharides (GOS) are frequently used for the supplementation of infant formula. In addition, long-chain fructooligosaccharides (FOS) are nowadays added to infant formula in order to simulate the higher molecular weight oligosaccharide fraction of human milk.[64,65]

Preparation of GOS

GOS are produced by transgalactosylation from lactose using fungal, yeast-derived or bacterial β-galactosidases (EC 3.2.1.23). The structural composition of the resulting GOS-mixture is highly dependent on the origin of the enzyme and the incubation parameters, such as pH of the medium, temperature and substrate-concentration.[66] The size of the oligosaccharides in the mixture ranges from dimers to pentamers, with β-(1,2)- and/or β-(1,3)- and/or β-(1,4)- and/or β-(1,6)-linkages between galactose-galactose or galactose-glucose.[67-69] Nonreducing galactooligosaccharides, containing α-(1,1)-linkages between glucose and galactose can be produced as well.[70]

Preparation of FOS

FOS can be obtained by extraction and hydrolysis of inulin from plants, which is a storage polysaccharide in e.g. chicory root and artichoke.[71] Most inulin-type fructans are composed of (β-D-Fru*f*-(1,2))$_n$-linkages with a non-reducing β-D-Fru*f*-(2,1)-α-D-Glc*p* terminus. The chain length of the fructans is dependent on the plant (usually DP4 - DP < 200).[71,72] Commercial inulin-type short chain and long chain FOS may consist of both, (β-D-Fru*f*-(1,2))$_n$-D-Fru*f*–series (F_n) and (β-D-Fru*f*-(1,2))$_n$-β-D-Fru*f*-(2,1)-α-D-Glc*p*-series (GF_n).

FOS can also be prepared by transfructosylation of sucrose with β-fructofuranosidases (EC 3.2.1.26) or β-fructosyltransferases (EC 2.4.1.9).[73-75] The result is a homologous GF_n-oligosaccharide-series of DP 2-5.[75]

Health functions of GOS and FOS

In vitro- and *in vivo* studies with prebiotic GOS and FOS demonstrated a specific stimulation of health-beneficial gut bacteria, although structurally different oligosaccharide mixtures were used in the respective studies.[76-79] Correspondingly, the presence and expression of saccharidases (β-galactosidases and β-fructofuranosidases) from health-beneficial bacteria and their mode of action has been described.[80-82] Similar to HMOs, anti-adhesive functions towards an enteropathogenic *E.coli*-strain were found for GOS *in vitro*.[83]

For the application in infant formula, a mixture of GOS:FOS (9:1) has been used.[84] This proportioning emulates the molecular size distribution of HMOs in breast milk. Furthermore, a synergistic effect of GOS and FOS on the stimulation of health-beneficial colonic bacteria was proposed.[84] The FOS added to the infant formula have a size of DP > 10, as short-chain FOS can cause increased flatulence.[71] Feeding infant formula supplemented with a combination of GOS and FOS resulted in the stimulation of bifidobacterial growth and was accompanied by increased stool frequency, soft stools and decreased fecal pH.[85] The health-promoting effects observed were dose-dependent, with 0.8 g/dL supplementation being considered as optimal and approved as safe by the EFSA authority.[85,86]

Other potential oligosaccharides for functional food supplementation

The supplementation of infant formula with GOS and/or FOS neglects the presence of acidic oligosaccharides in human milk. These may contribute to the gastrointestinal defense of pathogenic bacteria and to the systemic effects proposed for HMOs. So far, synthetically produced, sialylated oligosaccharides are not suitable for the application in food.[87] Instead, acidic oligosaccharides from pectin (AOS) are being considered for their potential use in infant formula.[88] Pectin, which is a plant cell wall polysaccharide contains acidic polygalacturonan-chains. AOS are obtained by enzymatic hydrolysis and are renowned for their curative functioning for diarrhea, which may be related to the anti-adhesive effects found for these oligosaccharides towards *E. Coli*.[89]

GOS and FOS are structurally less complex than HMOs. The “simple” prebiotic oligosaccharide structures are, therefore, readily fermented in the proximal colon, although at different rates.[90] Many colonic diseases, such as ulcerative colitis and Crohn’s disease occur in the distal colonic part and were suggested to result from a reduced immune

response to the commensal gut flora.[91] Nature is rich in dietary fibres, which are defined to reach the colon intact.[92] The more complex these substrates are, the higher the potential to survive the whole gastrointestinal tract passage and to reach the distal part of the colon. Complex storage polysaccharides can be found in various plants, such as acetylated glucomannan in the tubers of *Amorphophallus*-species and highly branched fructans in the stems of *Agave*-species.[93,94] Industrial by-products, such as cereal bran, fruit pomace and sugar beet pulp contain large amounts of complex plant cell wall polysaccharides, such as pectic arabinogalactan, arabinan, rhamnogalactorunan, arabinoxylan and glucurono-arabinoxylan, which can be used for the enzymatic production of complex oligosaccharide mixtures.[95] In fact, a slower fermentability of acetylated xylo-oligosaccharides and xylo-oligosaccharides decorated with methylglucuronic acid groups was found *in vitro*,[96] making them potential candidates for novel prebiotics.

Analysis of oligosaccharides from human milk and functional foods

The analysis of oligosaccharides from human milk and functional foods is challenging, due to the wide structural variety and the presence of numerous isomeric forms in these mixtures. HMOs and functional oligosaccharides are usually embedded in low concentrations in food (< 10 % (w/w))[97] and body liquids. These complex matrices may also contain other components, which disturb the analysis, due to their presence in larger quantities than the components of interest, e.g. proteins, fat, monosaccharides, lactose, sucrose and starch-derived oligosaccharides. Extraction, fractionation and enrichment may, therefore, be required prior to analysis. High-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) is the most frequently used method for analyzing HMOs and functional oligosaccharides.[31,41,98-100] Although good resolutions are obtained by HPAEC-PAD, several disadvantages in view of the analysis of complex oligosaccharide mixtures are faced. These include the susceptibility of alkali-instable substituents to the eluent and the difficulty of a direct MS-coupling.[101-103] For further mass information of the structural components, a supplementary method, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), has to be applied.[100,104] Accurate quantification of HMOs and functional oligosaccharides with techniques such as HPAEC is limited due to the fact that few standard substrates are commercially available or may even have to be self-produced by tedious extraction and purification. Usually approx. 10-20 HMOs are taken as reference.[31,41,98-100] The use of different standard-sets combined with the use of different analytical instrumentation and

methods complicates the comparison of literature data. As a consequence of these limitations, the quantitative determination of functional oligosaccharides added to food matrices is usually not performed on an individual oligosaccharide level. Their quantification is mostly based on the monosaccharide concentration, which is determined after enzymatic hydrolysis of the extracted oligosaccharides as defined in official methods (AOAC 997.08 for FOS and AOAC 2001.02 for GOS).[97,105] The lack of knowledge and quantifiability of individual oligosaccharides is, however, disadvantageous for studying their structure-function relationship and quality control. Furthermore, it delays the legal approval of functional food ingredients, which requires advanced scientific proof of functionalities and quantities.[106]

The coupling of separation techniques with on-line mass spectrometry is of increasing interest for the application to complex oligosaccharide mixtures. HPLC-Chip/MS on porous graphitized carbon material has been introduced for the analysis of HMOs and enabled the annotation of approximately 200 oligosaccharide structures.[40] In other research fields dealing with complex oligosaccharides (e.g. enzymatic digests of plant polysaccharides), capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) and CE-MS-hyphenation showed to be promising analytical tools of high sensitivity.[103,107,108]

Oligosaccharide analysis in feces and urine

Analysis of oligosaccharides in baby feces and urine provide information on the gastrointestinal metabolization. Their analysis does not solely focus on one or several structural components or bacteria as is the case for *in vitro* studies.[11] Several studies have been performed on the recovery of HMOs and their metabolization products or of prebiotic oligosaccharides from infant formula in baby feces and urine. They are summarized in **Table 3**.[28,64,109-113] The studies performed mainly focus on HMOs and only few studies deal with the gastrointestinal fate of prebiotic oligosaccharides. Most studies on the fecal recovery of HMOs and their metabolization products were performed in the 1980's and early 1990's, when analytical oligosaccharide screening was not yet established as a standard laboratory method. The sample throughput was limited, as preparative chromatography, which is time-consuming and requests large sample amounts, preceded analysis (usually ^{1}H NMR or FAB-MS).[110-112] The results obtained were diverse. In some studies exclusively HMOs were reported to be present in feces or urine,[28,109,110] whereas in other studies blood group characteristic oligosaccharides were detected.[111-113] Several factors, such as time of sampling, time of birth and genetic characteristics may influence the presence of oligosaccharides in feces and urine (**Table 3**). Unfortunately, the

oligosaccharide sets, as initially present in the respective diet, were mostly not studied. In case of breast milk, the HMO-composition was assumed, according to serological tests performed.[110-113] Although indications were made on the gastrointestinal metabolization of diet-related oligosaccharides, no general conclusions could be drawn.

Table 3. Overview on studies performed on the recovery of HMOs and diet-related oligosaccharides in baby feces and urine. n: number of participants. BG: blood group. Neu5Ac: sialic acid

matrix	*n*	*BG*	*birth*	*age sampling*	*feeding*	*recovery*	*year*	*ref.*
feces	1	A	term	1 month	breast milk (secretor)	BGA-oligosaccharides	1984	[111]
feces	1	B	term	3.5 months	breast milk (non-secretor)	non-secretor HMOs	1988	[110]
feces	2	A	preterm	1 week	breast milk (pooled)	non-secretor HMOs	1988	[112]
	1			2 months		BGA- oligosaccharides		
feces	4	A/0	preterm	1-5 weeks	breast milk (pooled)	rate of free to bound Neu5Ac increased in time	1991	[113]
	5	A/0	term	4-11 weeks				
	1	A	term	2/4/6/8/10/12 months	breast milk (pooled)	HMO- /BGA-content decreased in time		
feces	6	n.d.	term	1 month	breast milk	HMOs	2001	[28]
	1	n.d.	n.d.	n.d.	formula	few unknown oligosaccharides		
feces	22	n.d.	term	1 month	formula (GOS/FOS 9:1 0.8 g/dL)	GOS/FOS	2005	[64]
urine	9	n.d.	preterm	approx. 24days	breast milk	HMOs	1996	[109]
	9				formula (based on bovine milk)	sialylated oligosaccharides (from bovine milk)		

Thesis outline

As stated above, little is known on the gastrointestinal metabolization of diet-related oligosaccharides. A reason for that is the diversity of study approaches and analytical methods used. The aim of this PhD research was to provide advanced knowledge on the characteristics and gastrointestinal metabolization of functional oligosaccharides with (potential) relevance for infant nutrition, by firstly introducing novel analytical techniques for the analysis of these oligosaccharides and secondly applying them for a selection of samples originating from *in vivo* and *in vitro* studies.

In **chapter 2**, CE-LIF is introduced as a suitable analytical tool for the qualitative and quantitative analysis of GOS, as extracted from complex food matrices. The on-line coupling of CE-LIF to a mass spectrometer is applied for the analysis of HMOs in human milk and baby feces in **chapter 3** and two novel HMO-degradation products are identified in the feces by means of MS^n.

Next, fecal oligosaccharide profiles of preterm born, two months old breast- and formula-fed babies are studied in **chapter 4** and diet- and genetically dependent trends are pointed out. In **chapter 5**, the fecal oligosaccharide profiles from breast-fed babies and the HMO-profiles of the corresponding breast milks are followed during the first six months postpartum. Three distinct stages of fecal oligosaccharide profiles are highlighted and blood group characteristic HMO metabolization products are identified by CE-LIF(-MS^n). The impact of the gastrointestinal development on the metabolization of diet-related oligosaccharides is discussed.

Chapters 6 and **chapter 7** deal with the complex konjac glucomannan polysaccharide and oligosaccharide sets obtained after enzymatic digestion of the polysaccharide with endo-β-(1,4)-mannanase and endo-β-(1,4)-glucanase, which are potential prebiotic substrates. In **chapter 6**, the *in vitro* fermentation of the konjac glucomannan polysaccharide and oligosaccharides is described and in **chapter 7**, the structural composition of the oligosaccharides, which emerged during *in vitro* fermentation, as well as the enzymatically produced oligosaccharide sets are studied.

Finally, the results obtained in this research are discussed in **chapter 8** and their impact on further research towards the gastrointestinal metabolization of feeding-related oligosaccharides is reflected.

References

1. Antonowicz, I.; Lebenthal, E. Developmental pattern of small intestinal enterokinase and disaccharidase activities in human fetus. *Gastroenterology*, **1977**, *72*, 1299-1303.
2. Manson, W.G.; Coward, W.A.; Harding, M.; Weaver, L.T. Development of fat digestion in infancy. *Arch. Dis. Child.*, **1999**, *80*, F183-F187.
3. Sangild, P.T. Gut responses to enteral nutrition in preterm infants and animals. *Exp. Biol. Med.*, **2006**, *231*, 1695-1711.
4. Shulman, R.J.; Schanler, R.J.; Lau, C.; Heitkemper, M.; Ou, C.-N.; Smith, E.O.B. Early feeding, feeding tolerance, and lactase activity in preterm infants. *J. Pediatr.*, **1998**, *133*, 645-649.
5. Gibson, G.R.; Roberfroid, M.B. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *J. Nutr.*, **1995**, *125*, 1401-1412.
6. Adlerberth, I.; Wold, A.E. Establishment of the gut microbiota in western infants. *Acta Pædiatr.*, **2009**, *98*, 229-238.
7. Benno, Y.; Sawada, K.; Mitsuoka, T. The intestinal microflora of infants - composition of fecal flora in breast-fed and bottle-fed infants. *Microbiol. Immunol.*, **1984**, *28*, 975-986.
8. Penders, J.; Thijs, C.; Vink, C.; Stelma, F.F.; Snijders, B.; Kummeling, I.; van den Brandt, P.A.; Stobberingh, E.E. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics*, **2006**, *118*, 511-521.
9. Stark, P.L.; Lee, A. The microbial ecology of the large bowel of breastfed and formula-fed infants during the first year of life. *J. Med. Microbiol.*, **1982**, *15*, 189-203.
10. Yoshioka, H.; Iseki, K.; Fujita, K. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics*, **1983**, *72*, 317-321.
11. Lo Cascio, R.G.; Ninonuevo, M.R.; Freeman, S.L.; Sela, D.A.; Grimm, R.; Lebrilla, C.B.; Mills, D.A.; German, J.B. Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. *J. Agric. Food Chem.*, **2007**, *55*, 8914-8919.
12. Schwiertz, A.; Gruhl, B.; Lobnitz, M.; Michel, P.; Radke, M.; Blaut, M. Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. *Pediatr. Res.*, **2003**, *54*, 393-399.
13. Egert, M.; de Graaf, A.A.; Smidt, H.; de Vos, W.M.; Venema, K. Beyond diversity: Functional microbiomics of the human colon. *Trends Microbiol.*, **2006**, *14*, 86-91.
14. Macfarlane, G.T.; Gibson, G.R. Metabolic interactions between colonic bacteria and the host. In *Human health: The contribution of microorganisms*; Gibson, S.A.W., Ed; Springer: London, UK, **1994**; pp 17-52.
15. Hoskins, L.C.; Agustines, M.; McKee, W.B.; Boulding, E.T.; Kriaris, M.; Miedermeyer, G. Mucin degradation in human colon ecosystems; isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. *J. Clin. Invest.*, **1985**, *75*, 944-953.
16. Hoskins, L.C.; Boulding, E.T. Degradation of blood group antigens in human colon ecosystems;
part 1: In-vitro production of ABH blood group degrading enzymes by enteric bacteria. *J. Clin. Invest.*, **1976**, *57*, 63-73.
17. Herrmann, A.; Davies, J.R.; Lindell, G.; Martensson, S.; Packer, N.H.; Swallow, D.M.; Carlstedt, I. Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. *J. Biol. Chem.*, **1999**, *274*, 15828-15836.
18. Brockhausen, L. Biosynthesis of mucin-type o-glycans. In *Comprehensive glycoscience. From chemistry to system biology; vol.3*, 1st ed; Kamerling, J.P., Boons, G.J., Lee, Y.C., Suzuki, A., Taniguchi, N.,Voragen, A.G.J., Eds; Elsevier: Oxford, **2007**; pp 33-59.
19. Neu, J. Gastrointestinal maturation and implications for infant feeding. *Early Hum. Dev.*, **2007**, *83*, 767-775.
20. Allen, A. Gastrointestinal mucus. In *Handbook of physiology. The gastrointestinal system. Salivary, gastric, pancreatic, and hepatobiliary secretion.*; Forte, J.G.,Schultz, S.G., Eds; Am. Physiol. Soc: Bethesda, MD, USA, **1989**; pp 359-382.
21. Schenkel-Brunner, H. Blood group antigens. In *Comprehensive glycoscience. From chemistry to system biology; vol.3*; Kamerling, J.P., Boons, G.J., Lee, Y.C., Suzuki, A., Taniguchi, N.,Voragen, A.G.J., Eds; Elsevier: Oxford, UK, **2007**; pp 343-372.
22. Fujita, K.; Oura, F.; Nagamine, N.; Katayama, T.; Hiratake, J.; Sakata, K.; Kumagai, H.; Yamamoto, K. Identification and molecular cloning of a novel glycoside hydrolase family of core 1 type O-glycan-specific endo-α-N-acetylgalactosaminidase from *Bifidobacterium longum*. *J. Biol. Chem.*, **2005**, *280*, 37415-37422.

23. Ruas-Madiedo, P.; Gueimonde, M.; Fernandez-Garcia, M.; de los Reyes-Gavilan, C.G.; Margolles, A. Mucin degradation by Bifidobacterium strains isolated from the human intestinal microbiota. *Appl. Environ. Microbiol.*, **2008**, AEM.02509-02507.
24. Zhou, J.S.; Gopal, P.K.; Gill, H.S. Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and Bifidobacterium lactis (HN019) do not degrade gastric mucin *in vitro*. *Int. J. Food Microbiol.*, **2001**, *63*, 81-90.
25. Newburg, D.S.; Neubauer, S.H. Carbohydrates in milks: Analysis, quantities and significance. In *Handbook of milk composition*; Jensen, R.G., Ed; Academic Press: San Diego, **1995**; pp 273-249.
26. Asakuma, S.; Urashima, T.; Akahori, M.; Obayashi, H.; Nakamura, T.; Kimura, K.; Watanabe, Y.; Arai, I.; Sanai, Y. Variation of major neutral oligosaccharides levels in human colostrum. *Eur. J. Clin. Nutr.*, **2007**, *62*, 488-494.
27. Chaturvedi, P.; Warren, C.D.; Altaye, M.; Morrow, A.L.; Ruiz-Palacios, G.; Pickering, L.K.; Newburg, D.S. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology*, **2001**, *11*, 365-372.
28. Coppa, G.V.; Pierani, P.; Zampini, L.; Bruni, S.; Carloni, I.; Gabrielli, O. Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. In *Bioactive components of human milk*; Newburg, D.S., Ed; Kluwer Academic/Plenum Publishers: New York, **2001**; pp 307-314.
29. Martin-Sosa, S.; Martin, M.J.; Garcia-Pardo, L.A.; Hueso, P. Sialyloligosaccharides in human and bovine milk and in infant formulas: Variations with the progression of lactation. *J. Dairy Sci.*, **2003**, *86*, 52-59.
30. Urashima, T.; Asakuma, S.; Messer, M. Milk oligosaccharides. In *Comprehensive glycoscience. From chemistry to system biology; vol.4*; Kamerling, J.P., Boons, G.J., Lee, Y.C., Suzuki, A., Taniguchi, N.,Voragen, A.G.J., Eds; Elsevier: Oxford, UK, **2007**; pp 695-722.
31. Coppa, G.V.; Pierani, P.; Zampini, L.; Carloni, I.; Carlucci, A.; Gabrielli, O. Oligosaccharides in human milk during different phases of lactation. *Acta Paediatr.*, **1999**, *88*, 89-94.
32. Kuhn, R.; Baer, H.H.; Gauhe, A. Die Konstitution der lacto-N-fucopentaose ii - ein Beitrag zur Spezifität der Blutgruppensubstanz LeA. *Chem. Ber.-Recl.*, **1958**, *91*, 364-374.
33. Kuhn, R.; Gauhe, A. Die konstitution der lacto-N-neotetraose. *Chem. Ber.-Recl.*, **1962**, *95*, 518-522.
34. Kobata, A. Structures and functions of the sugar chains of glycoproteins. *Eur. J. Biochem.*, **1992**, *209*, 483-501.
35. Kobata, A.; Ginsburg, V. Oligosaccharides of human milk .3. Isolation and characterization of a new hexasaccharide, lacto-N-hexaose. *J. Biol. Chem.*, **1972**, *247*, 1525-&.
36. Montreuil, J. Structure de deux triholosides isoles du lait de femme. *Cr. Hebd. Acad. Sci.*, **1956**, *242*, 192-193.
37. Strecker, G.; Fievre, S.; Wieruszeski, J.M.; Michalski, J.C.; Montreuil, J. Primary structure of 4 human-milk octa-saccharides, nona-saccharides, and undeca-saccharides established by H-1-nuclear and C-13-nuclear magnetic-resonance spectroscopy. *Carbohydr. Res.*, **1992**, *226*, 1-14.
38. Strecker, G.; Hondiassah, T.; Fournet, B.; Spik, G.; Montreuil, J.; Maroteaux, P.; Durand, P.; Farriaux, J.P. Structure of 3 major sialyl-oligosaccharides excreted in urine of 5 patients with 3 distinct inborn diseases - I cell disease and 2 new types of mucolipidosis. *Biochim. Biophys. Acta*, **1976**, *444*, 349-358.
39. Thurl, S.; Munzert, M.; Henker, J.; Boehm, G.; Muller-Werner, B.; Jelinek, J.; Stahl, B. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Brit. J. Nutr.*, **2010**, *104*, 1261-1271.
40. Ninonuevo, M.R.; Park, Y.; Yin, H.; Zhang, J.; Ward, R.E.; Clowers, B.H.; German, J.B.; Freeman, S.L.; Killeen, K.; Grimm, R.; Lebrilla, C.B. A strategy for annotating the human milk glycome. *J. Agric. Food Chem.*, **2006**, *54*, 7471-7480.
41. Thurl, S.; Henker, J.; Siegel, M.; Tovar, K.; Sawatzki, G. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconjugate J.*, **1997**, *14*, 795-799.
42. Oriol, R.; Mollicone, R.; Cailleau, A.; Balanzino, L.; Breton, C. Divergent evolution of fucosyltransferase genes from vertebrates, invertebrates, and bacteria. *Glycobiology*, **1999**, *9*, 323-334.
43. Oriol, R.; Le Pendu, J.; Mollicone, R. Genetics of ABO, H, Lewis, X and related antigens. *Vox Sang.*, **1986**, *51*, 161-171.
44. Erney, R.M.; Malone, W.T.; Skelding, M.B.; Marcon, A.A.; Kleman-Leyer, K.M.; O'Ryan, M.L.; Ruiz-Palacios, G.; Hilty, M.D.; Pickering, L.K.; Prieto, P.A. Variability of human milk neutral oligosaccharides in a diverse population. *J. Pediatr. Gastr. Nutr.*, **2000**, *30*, 181-192.
45. Lucas, A.; Cole, T.J. Breast-milk and neonatal necrotizing enterocolitis. *Lancet*, **1990**, *336*, 1519-1523.
46. Pisacane, A.; Graziano, L.; Mazzarella, G.; Scarpellino, B.; Zona, G. Breast-feeding and urinary-tract infection. *J. Pediatr.*, **1992**, *120*, 87-89.

47. Pisacane, A.; Graziano, L.; Zona, G.; Granata, G.; Dolezalova, H.; Cafiero, H.; Coppola, A.; Scarpellino, B.; Ummarino, M.; Mazzarella, G. Breast-feeding and acute lower respiratory-infection. *Acta Paediatr.*, **1994**, *83*, 714-718.
48. Popkin, B.M.; Adair, L.; Akin, J.S.; Black, R.; Briscoe, J.; Flieger, W. Breast-feeding and diarrheal morbidity. *Pediatrics*, **1990**, *86*, 874-882.
49. Klein, N.; Schwertmann, A.; Peters, M.; Kunz, C.; Strobel, S. Immunomodulatory effects of breast milk oligosaccharides. In *Short and long term effects of breast feeding on child health*; Koletzko, B., Michaelsen, K.F.,Hernell, O., Eds; Kluwer Academic / Plenum Publ: New York, **2000**; pp 251-259.
50. Wang, B.; McVeagh, P.; Petocz, P.; Brand-Miller, J. Brain ganglioside and glycoprotein sialic acid in breastfed compared with formula-fed infants. *Am. J. Clin. Nutr.*, **2003**, *78*, 1024-1029.
51. Zopf, D.; Roth, S. Oligosaccharide anti-infective agents. *Lancet*, **1996**, *347*, 1017-1021.
52. Huang, P.W.; Farkas, T.; Marionneau, S.; Zhong, W.M.; Ruvoen-Clouet, N.; Morrow, A.L.; Altaye, M.; Pickering, L.K.; Newburg, D.S.; LePendu, J.; Jiang, X. Noroviruses bind to human ABO, Lewis, and secretor histo-blood group antigens: Identification of 4 distinct strain-specific patterns. *J. Infect. Dis.*, **2003**, *188*, 19-31.
53. Newburg, D.S.; Pickering, L.K.; McCluer, R.H.; Cleary, T.G. Fucosylated oligosaccharides of human milk protect suckling mice from heat-stabile enterotoxin of *Escherichia coli*. *J. Infect. Dis.*, **1990**, *162*, 1075-1080.
54. Newburg, D.S.; Ruiz-Palacios, G.M.; Altaye, M.; Chaturvedi, P.; Meinzen-Derr, J.; Guerrero, M.d.L.; Morrow, A.L. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology*, **2004**, *14*, 253-263.
55. Ruiz-Palacios, G.M.; Cervantes, L.E.; Ramos, P.; Chavez-Munguia, B.; Newburg, D.S. Campylobacter jejuni binds intestinal H(0) antigen (Fucα1,2Galβ1,4GlcNac), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J. Biol. Chem.*, **2003**, *278*, 14112-14120.
56. Kirjavainen, P.V.; Arvola, T.; Salminen, S.J.; Isolauri, E. Aberrant composition of gut microbiota of allergic infants: A target of bifidobacterial therapy at weaning? *Gut*, **2002**, *51*, 51-55.
57. Deguchi, Y.; Morishita, T.; Mutai, M. Comparative studies on synthesis of water-soluble vitamins among human species of bifidobacteria. *Agr. Biol. Chem. Tokyo*, **1985**, *49*, 13-19.
58. Biavati, B., M. Vescovo, S. Torriani, and V. Bottazzi Bifidobacteria: History, ecology, physiology and applications. *Ann. Microbiol.*, **2000**, *50*, 117-131.
59. Ventura, M.; O'Flaherty, S.; Claesson, M.J.; Turroni, F.; Klaenhammer, T.R.; van Sinderen, D.; O'Toole, P.W. Genome-scale analyses of health-promoting bacteria: Probiogenomics. *Nat. Rev. Microbiol.*, **2009**, *7*, 61-U77.
60. Katayama, T.; Sakuma, A.; Kimura, T.; Makimura, Y.; Hiratake, J.; Sakata, K.; Yamanoi, T.; Kumagai, H.; Yamamoto, K. Molecular cloning and characterization of *Bifidobacterium bifidum* 1,2-alpha-L-fucosidase (AfcA), a novel inverting glycosidase (glycoside hydrolase family 95). *J. Bacteriol.*, **2004**, *186*, 4885-4893.
61. Ashida, H.; Miyake, A.; Kiyohara, M.; Wada, J.; Yoshida, E.; Kumagai, H.; Katayama, T.; Yamamoto, K. Two distinct alpha-l-fucosidases from *Bifidobacterium bifidum* are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates. *Glycobiology*, **2009**, *19*, 1010-1017.
62. Wada, J.; Ando, T.; Kiyohara, M.; Ashida, H.; Kitaoka, M.; Yamaguchi, M.; Kumagai, H.; Katayama, T.; Yamamoto, K. *Bifidobacterium bifidum* lacto-N-biosidase, a critical enzyme for the degradation of human milk oligosaccharides with a type 1 structure. *Appl. Environ. Microbiol.*, **2008**, *74*, 3996-4004.
63. Kitaoka, M.; Tian, J.S.; Nishimoto, M. Novel putative galactose operon involving lacto-N-biose phosphorylase in *Bifidobacterium longum*. *Appl. Environ. Microbiol.*, **2005**, *71*, 3158-3162.
64. Moro, G.E.; Stahl, B.; Fanaro, S.; Jelinek, J.; Boehm, G.; Coppa, G.V. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants. *Acta Paediatr.*, **2005**, *94*, 27-30.
65. Boehm, G.; Fanaro, S.; Jelinek, J.; Stahl, B.; Marini, A. Prebiotic concept for infant nutrition. *Acta Paediatr.*, **2003**, *92*, 64-67.
66. Zarate, S.; Lopezleiva, M.H. Oligosaccharide formation during enzymatic lactose hydrolysis - a literature-review. *J. Food Prot.*, **1990**, *53*, 262-268.
67. Onishi, N.; Yamashiro, A.; Yokozeki, K. Production of galacto-oligosaccharide from lactose by Sterigmatomyces Elviae cbs8119. *Appl. Environ. Microbiol.*, **1995**, *61*, 4022-4025.
68. Toba, T.; Yokota, A.; Adachi, S. Oligosaccharide structures formed during the hydrolysis of lactose by *Aspergillus oryzae* beta-galactosidase. *Food Chem.*, **1985**, *16*, 147-162.
69. Usui, T.; Morimoto, S.; Hayakawa, Y.; Kawaguchi, M.; Murata, T.; Matahira, Y.; Nishida, Y. Regioselectivity of beta-D-galactosyl-disaccharide formation using the beta-D-galactosidase from *Bacillus circulans*. *Carbohydr. Res.*, **1996**, *285*, 29-39.
70. Fransen, C.T.M.; Van Laere, K.M.J.; van Wijk, A.A.C.; Brüll, L.P.; Dignum, M.; Thomas-Oates, J.E.; Haverkamp, J.; Schols, H.A.; Voragen, A.G.J.; Kamerling, J.P.; Vliegenthart, J.P.G. α-D-glc*p*-(1↔1)-β-D-gal*p*-

containing oligosaccharides, novel products from lactose by the action of β-galactosidase. *Carbohydr. Res.*, **1998**, *314*, 101-114.
71. Roberfroid, M.B.; Van Loo, J.A.E.; Gibson, G.R. The bifidogenic nature of chicory inulin and its hydrolysis products. *J. Nutr.*, **1998**, *128*, 11-19.
72. Kiss, A.; Forgo, P. Investigations on inulin-type oligosaccharides with regard to HPLC analysis and prospective food applicability. *Mon. Chem.*, **2011**, *142*, 547-553.
73. Sangeetha, P.T.; Ramesh, M.N.; Prapulla, S.G. Fructooligosaccharide production using fructosyl transferase obtained from recycling culture of Aspergillus oryzae cfr 202. *Process Biochem.*, **2005**, *40*, 1085-1088.
74. Fernandez, R.C.; Maresma, B.G.; Juarez, A.; Martinez, J. Production of fructooligosaccharides by beta-fructofuranosidase from *Aspergillus* sp 27H. *J. Chem. Technol. Biotechnol.*, **2004**, *79*, 268-272.
75. Grizard, D.; Barthomeuf, C. Enzymatic synthesis and structure determination of neo-FOS. *Food Biotechnol.*, **1999**, *13*, 93-105.
76. Choi, N.; Shin, H.S. Effect of oligosaccharides and inulin on the growth and viability of Bifidobacteria in skim milk. *Food Sci. Biotechnol.*, **2006**, *15*, 543-548.
77. Knol, J.; Scholtens, P.; Kafka, C.; Steenbakkers, J.; Gross, S.; Helm, K.; Klarczyk, M.; Schopfer, H.; Bockler, H.M.; Wells, J. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: More like breast-fed infants. *J. Pediatr. Gastroenterol. Nutr.*, **2005**, *40*, 36-42.
78. Rycroft, C.E.; Jones, M.R.; Gibson, G.R.; Rastall, R.A. A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. *J. Appl. Microbiol.*, **2001**, *91*, 878-887.
79. Tzortzis, G.; Goulas, A.K.; Gee, J.M.; Gibson, G.R. A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous *in vitro* fermentation system and in the proximal colonic contents of pigs in vivo. *J. Nutr.*, **2005**, *135*, 1726-1731.
80. Dumortier, V.; Brassart, C.; Bouquelet, S. Purification and properties of a beta-D-galactosidase from *Bifidobacterium bifidum* exhibiting a transgalactosylation reaction. *Biotechnol. Appl. Biochem.*, **1994**, *19*, 341-354.
81. Moller, P.L.; Jorgensen, F.; Hansen, O.C.; Madsen, S.M.; Stougaard, P. Intra- and extracellular beta-galactosidases from *Bifidobacterium bifidum* and *B. Infantis*: Molecular cloning, heterologous expression, and comparative characterization. *Appl. Environ. Microbiol.*, **2001**, *67*, 2276-2283.
82. Warchol, M.; Perrin, S.; Grill, J.P.; Schneider, F. Characterization of a purified beta-fructofuranosidase from *Bifidobacterium infantis* ATCC 15697. *Lett. Appl. Microbiol.*, **2002**, *35*, 462-467.
83. Shoaf, K.; Mulvey, G.L.; Armstrong, G.D.; Hutkins, R.W. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. *Infect. Immun.*, **2006**, *74*, 6920-6928.
84. Boehm, G.; Lidestri, M.; Casetta, P.; Jelinek, J.; Negretti, F.; Stahl, B.; Marini, A. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. *Arch. Dis. Child.*, **2002**, *86*, 178-181.
85. Moro, G.; Minoli, I.; Mosca, M.; Fanaro, S.; Jelinek, J.; Stahl, B.; Boehm, G. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. *J. Pediatr. Gastroenterol. Nutr.*, **2002**, *34*, 291-295.
86. Report of the committee on food on the revision of essential requirement of infant formulae and follow-on formulae; SCF/CS/NUT/IF/65; **2003**: Scientific committee on food; European Commission, Brussels.
87. Lee, K.B.; Lee, Y.C. Transfer of modified sialic acids by *Trypanosoma cruzi* trans-sialidase for attachment of functional-groups to oligosaccharide. *Anal. Biochem.*, **1994**, *216*, 358-364.
88. Fanaro, S.; Jelinek, T.; Stahl, T.; Boehm, T.; Kock, R.; Vigi, V. Acidic oligosaccharides from pectin hydrolysate as new component for infant formulae: Effect on intestinal flora, stool characteristics, and pH. *J. Pediatr. Gastroenterol. Nutr.*, **2005**, *41*, 186-190.
89. Guggenbichler, J.P.; de Bettignies-Dutz, A.; Meissner, P.; Schellmoser, S.; Jurentsch, J. Acidic oligosaccharides from natural sources block adherence of *Escherichia coli* on uroepithelial cells. *Pharm. Pharmacol. Lett.*, **1997**, *7*, 35-38.
90. Macfarlane, G.T.; Gibson, G.R.; Cummings, J.H. Comparison of fermentation reactions in different regions of the human colon. *J. Appl. Bacteriol.*, **1992**, *72*, 57-64.
91. Cummings, J.H.; Macfarlane, G.T.; Macfarlane, S. Intestinal bacteria and ulcerative colitis. *Curr. Iss. Int. Microb.*, **2003**, *4*, 9-20.
92. AACC Dietary Fiber Technical Committee. Report of the dietary fiber definition committee to the board of directors of AACC international. *Cereal Food World*, **2001**, *46*, 112-126.
93. Cescutti, P.; Campa, C.; Delben, F.; Rizzo, R. Structure of the oligomers obtained by enzymatic hydrolysis of the glucomannan produced by the plant *Amorphophallus konjac*. *Carbohydr. Res.*, **2002**, *337*, 2505-2511.

94. Lopez, M.G.; Mancilla-Margalli, N.A.; Mendoza-Diaz, G. Molecular structures of fructans from *Agave tequilana weber* var. Azul. *J. Agric. Food Chem.*, **2003**, *51*, 7835-7840.
95. Van Laere, K.M.J.; Hartemink, R.; Bosveld, M.; Schols, H.A.; Voragen, A.G.J. Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. *J. Agric. Food Chem.*, **2000**, *48*, 1644-1652.
96. Kabel, M.A.; Kortenoeven, L.; Schols, H.A.; Voragen, A.G.J. *In vitro* fermentability of differently substituted xylo-oligosaccharides. *J. Agric. Food Chem.*, **2002**, *50*, 6205-6210.
97. de Slegte, J. Determination of *trans*-galactooligosaccharides in selected food products by ion-exchange chromatography: Collaborative study. *J. AOAC Int.*, **2002**, *85*, 417-423.
98. Nakhla, T.; Fu, D.; Zopf, D.; Brodsky, N.L.; Hurt, H. Neutral oligosaccharide content of preterm human milk. *Brit. J. Nutr.*, **2007**, *82*, 361-367.
99. Thurl, S.; Müller-Werner, B.; Sawatzki, G. Quantification of individual oligosaccharide compounds from human milk using high-pH anion-exchange chromatography. *Anal. Biochem.*, **1996**, *235*, 202-206.
100. Finke, B.; Stahl, B.; Pfenninger, A.; Karas, M.; Daniel, H.; Sawatzki, G. Analysis of high-molecular-weight oligosaccharides from human milk by liquid chromatography and MALDI-MS. *Anal. Chem.*, **1999**, *71*, 3755-3762.
101. Lee, Y.C. Carbohydrate analyses with high-performance anion-exchange chromatography. *J. Chromatogr. A*, **1996**, *720*, 137-149.
102. Schols, H.A.; Kabel, M.; Bakx, E.; Daas, P.; van Alebeek, G.-J.; Voragen, A.G.J. HPLC of oligosaccharides: New developments in detection and peak identification. *AVH Association - 7th Symposium*, **2000**, 39-45.
103. Hilz, H.; de Jong, L.E.; Kabel, M.A.; Schols, H.A.; Voragen, A.G.J. A comparison of liquid chromatography, capillary electrophoresis, and mass spectrometry methods to determine xyloglucan structures in black currants. *J. Chromatogr. A*, **2006**, *1133*, 275-286.
104. Stahl, B.; Thurl, S.; Zeng, J.R.; Karas, M.; Hillenkamp, F.; Steup, M.; Sawatzki, G. Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem.*, **1994**, *223*, 218-226.
105. Prosky, L.; Hoebregs, H. Methods to determine food inulin and oligofructose. *J. Nutr.*, **1999**, *129*, 1418-1423.
106. Corrigendum to regulation (EC) No.1924/2006 of the European Parliament and of the council of 20 december 2006 on nutrition and health claims made on foods; L12/3; **2007**, European Union Publishers' Forum; Office for Official Publications of the European Communities ed.: Official Journal of the European Union, Brussels.
107. Coenen, G.J.; Kabel, M.A.; Schols, H.A.; Voragen, A.G.J. CE-MS^n of complex pectin-derived oligomers. *Electrophoresis*, **2008**, *29*, 2101-2111.
108. Kabel, M.A.; Heijnis, W.H.; Bakx, E.J.; Kuijpers, R.; Voragen, A.G.J.; Schols, H.A. Capillary electrophoresis fingerprinting, quantification and mass-identification of various 9-aminopyrene-1,4,6-trisulfonate-derivatized oligomers derived from plant polysaccharides. *J. Chromatogr. A*, **2006**, *1137*, 119-126.
109. Rudloff, S.; Pohlentz, G.; Diekmann, L.; Egge, H.; Kunz, C. Urinary excretion of lactose and oligosaccharides in preterm infants fed human milk or infant formula. *Acta Paediatr.*, **1996**, *85*, 598-603.
110. Sabharwal, H.; Nilsson, B.; Chester, M.A.; Lindh, F.; Grönberg, G.; Sjöblad, S.; Lundblad, A. Oligosaccharides from faeces of a blood-group B, breast-fed infant. *Carbohydr. Res.*, **1988**, *178*, 145-154.
111. Sabharwal, H.; Nilsson, B.; Chester, M.A.; Sjöblad, S.; Lundblad, A. Blood group specific oligosaccharides from faeces of a blood group A breast-fed infant. *Mol. Immunol.*, **1984**, *21*, 1105-1112.
112. Sabharwal, H.; Nilsson, B.; Grönberg, G.; Chester, M.A.; Dakour, J.; Sjöblad, S.; Lundblad, A. Oligosaccharides from feces of preterm infants fed on breast milk. *Arch. Biochem. Biophys.*, **1988**, *265*, 390-406.
113. Sabharwal, H.; Sjöblad, S.; Lundblad, A. Sialylated oligosaccharides in human milk and feces of preterm, full-term, and weaning infants. *J. Pediatr. Gastr. Nutr.*, **1991**, *12*, 480-484.

Chapter 2

Introducing capillary electrophoresis with laser-induced fluorescence (CE-LIF) as a potential analysis and quantification tool for galactooligosaccharides extracted from complex food matrices

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Abstract

The analysis and quantification of (galacto)oligosaccharides from food matrices demands both a reproducible extraction method as well as a sensitive and accurate analytical method. Three typical matrices, namely, infant formula, fruit juice, and a maltodextrin-rich preparation, to which a commercial galactooligosaccharide mixture was added in a product concentration range from 1.25 to 30%, served as model substrates. Solid-phase extraction on graphitized carbon material upon enzymatic amyloglucosidase pretreatment enabled a good recovery and a selective purification of the different galactooligosaccharide structures from the exceeding amounts of particularly lactose and maltodextrins. With the implementation of capillary electrophoresis in combination with laser-induced fluorescence (CE-LIF) detection, a new possibility facilitating a sensitive qualitative and quantitative determination of the galactooligosaccharide contents in the different food matrices is outlined. Simultaneous monitoring and quantifying prebiotic oligosaccharides embedded in food matrices presents a promising and important step toward an efficient monitoring of individual oligosaccharides and is of interest for research areas dealing with small quantities of oligosaccharides embedded in complex matrices, e.g., body liquids.

Introduction

Prebiotic carbohydrates, mainly nondigestible oligosaccharides (NDOs), play a significant role in the fortification of food products with beneficial gut flora components. The amount of prebiotics added to a food matrix is usually low, because studies have revealed already beneficial effects by the ingestion of 2-10 g of NDOs/day.[1-3] Evenmore, a supplementation with doses ≥ 15 g of NDOs/day may lead to abdominal pain, flatulence, and even diarrhea.[4,5] Maltodextrins, lactose, and monomeric sugars are the common predominating carbohydrates in these food matrices, for example, in powdered milk-based products.[6] Therefore, quality control of functional food products is a demanding field regarding the sensitivity and accuracy of analytical methods. The presence of such methods is indispensable, and this need has even been reinforced by the recently established European Union (EU) Health Claim Regulation (EG 1924/2006), which demands scientific substantiation of the functional ingredients, concerning both their gastrointestinal functionality and quantity.[7] Galactooligosaccharides are prominent representatives of prebiotic carbohydrates, implying their fermentability by beneficial intestinal bacteria and selective stimulation of the latter as well as the resistance to acid hydrolysis, enzymatic digestion, and absorption in the upper gastrointestinal tract.[8] The frequent use of galactooligosaccharides in all kind of processed foods, such as acidic beverages, fermented milk products, confectionery, or baby food, can be related to their low caloric value, bulking capacity, low sweetness, noncariosity, excellent solubility, and heat and storage stability, even in an acidic environment.[9-11] Galactooligosaccharides are usually produced from lactose. The incubation of lactose with fungal, yeast-derived, or bacterial β-galactosidases enables transglycosylation reactions and results in a complex mixture of reducing as well as, in minor amounts, non-reducing galactooligosaccharides or also called trans-galactooligosaccharides (DP2-DP ≥ 4). In these mixtures, lactose, glucose, and galactose can be present as well, because of the fact that galactooligosaccharides are kinetic intermediates and also substrates for hydrolysis during production.[9,12] The DP and linkages formed are strongly dependent upon the reaction conditions and enzymes used.[11] The existing Association of Official Analytical Chemists (AOAC) method for the determination of trans-galactooligosaccharides in food products [AOAC 2001.02][6] is based on high performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) analysis of galactose after β-galactosidase incubation of the galactooligosaccharide-containing matrix, yielding the total amount of galactose present in the mixture. According to this AOAC method, the term galactooligosaccharides is defined

to include dimers (excluding lactose) and larger oligosaccharides. Although being a robust method, it does not provide any information on the individual oligosaccharides present. Such information would be highly desirable in view of the vast variability in structural features characteristic for galactooligosaccharide mixtures because the composition is used as a quality and identification parameter. Another disadvantage of the AOAC method is that high lactose contents present in the food matrix tend to influence the measurement of galactose.[6] Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) has been shown to be a powerful and sensitive technique in the separation of 9-aminopyrene-1,4,6-trisulfonate (APTS)-tagged oligosaccharides derived from plant polysaccharides and other natural sources.[13-15] Therefore, this method could also be of great value for the analysis of prebiotic oligosaccharides in complex matrices. Labeling carbohydrates with the fluorescent dye APTS is based on a reductive amination reaction.[16] APTS carries three negative charges, which provides the neutral carbohydrates with charge, a prerequisite for electrophoretic separation.[17] Each carbohydrate molecule is attached to one fluorescent molecule. When an internal standard is included, CE-LIF is, therefore, a potential technique for quantitative analysis. Because APTS is only attached to reducing oligosaccharides, non-reducing oligosaccharides are not included in this derivatization method. Galactooligosaccharide mixtures are usually composed of predominantly reducing oligosaccharides. They are often applied in combination with fructooligosaccharides (mainly non-reducing oligosaccharides), e.g., in infant formula.[18] A labeling of exclusively reducing oligosaccharides thus guarantees a selective detection of galactooligosaccharides in these mixtures. However, the small amount of non-reducing galactooligosaccharides is not included in the analysis and has to be taken into account differently (e.g., by means of a calibration curve). Besides a preliminary quantitative screening in the beginning of the 1990s, in which APTS was introduced as the fluorescent dye of choice for CE-LIF analysis of carbohydrates,[16] the quantitative use of CE-LIF has only been studied in detail for monosaccharides and not for oligosaccharides.[14,15] Characterization and quantification of galactooligosaccharides from food matrices in the first instance demands a reproducible extraction and purification from the predominating and disturbing components present, mainly monomers, lactose, and maltodextrins. To evaluate the suitability of CE-LIF as an analysis tool for commercially available galactooligosaccharides, the first part of this research is aimed at a reproducible extraction of galactooligosaccharides from complex food matrices. It focuses on the fine-tuning of a solid-phase extraction (SPE) method for oligosaccharides, on the basis of the use of graphitized carbon cartridges,[19] in combination with an enzymatic amyloglucosidase pretreatment to remove maltodextrins.[20] Next, CE-LIF

is tested for a reproducible quantification of oligosaccharides in general and, more specifically, galactooligosaccharides extracted from food matrices.

Materials and Methods

Vivinal GOS, Vivinal GOS-containing samples, enzymes and chemicals

Vivinal GOS syrup, Vivinal GOS/maltodextrin preparation, and infant formula were provided by FrieslandCampina DOMO (Zwolle, The Netherlands). The Vivinal GOS syrup (reference material) was prepared by a β-galactosidase from Bacillus circulans and specified by the supplier with a dry matter of 75% (w/w), of which 59% (w/w) is galactooligosaccharides, 21% (w/w) is lactose, 19% (w/w) is glucose, and 1% (w/w) is galactose, as determined according to AOAC 2001.02.[6] The galactooligosaccharides present in the mixture are predominantly reducing oligosaccharides, with $[\beta\text{-D-Gal-}(1,4)\text{-}\beta\text{-D-Gal-}(1,4)]_n\text{-Glc}$ being the most abundant structural element for oligosaccharides of DP $\geq$ 3, as determined by nuclear magnetic resonance (NMR) analysis.[21] Non-reducing oligosaccharides are present predominantly in the form of dimers; β-D-Gal-(1,1)-β-D-Glc and β-D-Gal-(1,1)-α-D-Glc make up about 3% of the oligosaccharide mixture.[21] The Vivinal GOS/maltodextrin preparation was specified by the supplier to be composed of 48.5% (w/w) maltodextrins, 28.5% (w/w) Vivinal galactooligosaccharides, 10.1% (w/w) lactose, and 9.7% (w/w) mono- and other disaccharides. For infant formula, an approximate content of 3% (w/w) Vivinal galactooligosaccharides was given. No further sample specification was provided. Lactose (40%, w/w) and maltodextrin (10%, w/w) were found to be present in this matrix by analysis in our lab, using HPAEC-PAD. The fruit juice is a commercial product and was obtained from a local shop. It is specified to contain 12.3% (w/v) sugars (glucose, fructose, and saccharose) and 1.25% (w/v) Vivinal galactooligosaccharides.

Amyloglucosidase (source: *Aspergillus niger*) was obtained from Boehringer (B208469, Boehringer, Mannheim, Germany). Monosaccharides and oligosaccharides used as standards were D-xylose (Sigma-Aldrich, St. Louis, MO), α-L-1,5-arabinooligosaccharides DP3-DP5 (Megazyme, Bray, Ireland), maltodextrin-oligosaccharides (α-D-(1,4)-glucooligosaccharides) DP3-DP5 (Sigma-Aldrich, St. Louis, MO), and cellodextrin-oligosaccharides (β-D-(1,4)-glucooligosaccharides) DP3-DP5 (Sigma-Aldrich, St. Louis, MO). All other chemicals used were of analytical grade.

Fractionation of Vivinal GOS

Vivinal GOS was fractionated according to its DP by gel filtration on a BioGel P2 column (26 mm x 900 mm, 200-400 mesh; Bio-Rad, Hercules, CA). A total of 300 mg of VivinalGOS syrup dissolved in 10 mL of water was applied to the column and was eluted with millipore water (1.5 mL/min) at 60 ºC using an Akta Explorer system (GE Amersham, Uppsala, Sweden). The column efflux was first led through a refractive index (RI) detector (Shodex RI-72, Showa Denko K.K., Tokyo, Japan) before it was collected in fractions of 5 mL. The fractions were pooled according to the RI profile and freeze-dried.

Oligosaccharide extraction, enzymatic pretreatment and purification

For extraction of galactooligosaccharides, 100 mg of Vivinal GOS-containing matrix, respectively, infant formula, fruit juice, or the Vivinal GOS/maltodextrin preparation, was suspended in 50 mL of boiling water and kept at 80 ºC for 30 min [AOAC 2001.02].[6] Fruit juice was adjusted to pH 7 prior to extraction. In the case of dominant levels of maltodextrins being present, specific enzymatic degradation of these maltodextrins using 148 µL of a 1% (w/v) amyloglucosidase solution per 1 mg/mL substrate solution was performed in 0.2 M sodium acetate (NaOAc) buffer (pH 4.5, 55 ºC, 24 h). The enzyme dosage was chosen according to the best result in the oligosaccharide profile and maltodextrin degradability obtained after testing different substrate/enzyme ratios. The enzyme was inactivated (10 min, 100 ºC), and the sample was centrifuged (5 min, 10000g, room temperature). High levels of mono- and dimers present in the food matrix or resulting from enzymatic degradation of maltodextrins were removed by SPE on nonporous graphitized carbon cartridges (bed weight, 150 mg; tube size, 4 mL; Alltech, Deerfield, IL). The cartridges were washed with 1.5 mL of 80:20 (v/v) acetonitrile (ACN)/water containing 0.1% (v/v) trifluoroacetic acid (TFA) followed by 1.5 mL of millipore water. A total of 1 mL of the GOS-containing sample was loaded onto the cartridge, which was eluted with 1.5 mL of water. Subsequently, mono- and dimers were eluted with 1.5 mL of 2-5% (v/v) aqueous ACN, depending upon the sample matrix. Sample flowthrough, wash water, and mono- and dimers were trapped as one fraction. The oligosaccharides were eluted with 1.5 mL of 25:75 (v/v) ACN/water containing 0.05% (v/v) TFA and trapped as a separate fraction. The fractions were dried under a stream of air. The fraction containing mono- and dimers was rehydrated with 1mL of millipore water, and the oligosaccharide-containing fraction was rehydrated with 500 µL of millipore water. The sample matrices (infant formula, fruit juice, and Vivinal GOS/maltodextrin preparation) were extracted in duplicate.

Analytical methods

MALDI-TOF MS

For matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of oligosaccharides, an Ultraflex workstation (Bruker Daltronics, Bremen, Germany) equipped with a nitrogen laser of 337 nm was used. The measurement was performed in the positive mode. After a delayed extraction time of 200 ns, the ions were accelerated with a 25 kV voltage. The data were collected from averaging 100 laser shots, with the lowest laser energy necessary to obtain sufficient spectra intensity. The mass spectrometer was calibrated with a mixture of maltodextrins (AVEBE, Veendam, The Netherlands; mass range of 527-2309 Da) as sodium adducts. Samples (1mg/mL) were desalted with AG 50W-X8 resin (H^+ form; Bio-Rad, Hercules, CA). The desalted samples (1 µL) were mixed directly on a MS target plate with 1 µL of matrix solution consisting of an aqueous solution of 2,5-dihydroxybenzoic acid (10 mg/mL; Bruker Daltonics, Bremen, Germany). The mix was dried under a stream of warm air.

HPAEC

HPAEC of lactose was performed on a Dionex ISC 3000 system (Dionex, Sunnyvale, CA), equipped with a Dionex Carbopac PA-1 column (2 mm x 250 mm) in combination with a Carbopac PA-1 guard column (2 mm x 50 mm). Samples (20 µL) were injected by means of a Dionex ISC 3000 autosampler. Elution was performed at 0.3 mL/min at room temperature using a gradient of 0-150 mM sodium acetate in 100 mM sodium hydroxide (NaOH) for 15 min. Each elution was followed by a washing step (10 min, 1 M NaOAc in 100 mM NaOH) and an equilibration step (15 min, 100 mM NaOH). Detection was performed using a Dionex ED40 detector in the PAD mode.

CE-LIF

Separation of APTS-derivatized carbohydrates was performed using a ProteomeLab PA 800 characterization system (Beckman Coulter, Fullerton, CA) equipped with a polyvinyl alcohol-coated capillary (50 µm x 50.2 cm). Detection was performed with a LIF detector (Beckman Coulter, Fullerton, CA) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm after 40 cm capillary length. The capillary was pressure-rinsed with water (3 min, 30 psi) and separation buffer (2 min, 30 psi), composed of 25 mM acetate (pH 4.75) containing 0.4% (v/v) polyethylene oxide. The vials of the rinsing solutions were changed every 12 samples. Supplementary to the pressure rinsing, the capillary was rinsed

with water by applying current (20 min, 30 kV) to prevent sample carryover. Samples were loaded hydrodynamically (4 s at 0.5 psi, representing approx. 14 nL of sample solution) on the capillary, which was kept at 25 ºC. Separation was performed in the reversed polarity mode (20 min, 30 kV) in separation buffer.

Sample preparation for CE-LIF analysis

Samples for CE-LIF were prepared using the ProteomeLab Carbohydrate Labeling and Analysis Kit (Beckman Coulter, Fullerton, CA). A total of 2 nmol of xylose was added as an internal standard and mobility marker to the samples (0.02-0.05 mg) instead of the 5 nmol of maltose internal standard proposed by the guidelines of the kit. This mixture was dried using a SpeedVac Concentrator Savant ISS110 (Thermo Electron Corporation, Waltham, MA). The mono- and oligosaccharides present in the dried sample were derivatized with 2 µL of APTS and 2 µL of sodium cyanoborohydride and, afterward, filled up to 50 µL by millipore water. For CE-LIF, the samples were diluted 10-40 times.

Results and Discussion

Composition of Vivinal GOS

Vivinal GOS syrup (75% DM) was used as a reference in this study. For characterizing the reference material, pools of Vivinal GOS were prepared by fractionation on BioGel P2 size-exclusion material (**Figure 1A**). The pools each consisted of saccharides of one DP, with minor contamination of the preceding and following DP, as determined by MALDI-TOF MS. Evaluating the proportion (%) of the respective pool areas (RI response) in relation to the total area (representing the total carbohydrate content of Vivinal GOS) resulted in 23% pool 1 (P1; DP1), 39% pool 2 (P2; DP2), 23% pool 3 (P3; DP3), 10% pool 4 (P4; DP4), 4% pool 5 (P5; DP5), and 1% pool 6 (P6; not considered for analysis). HPAEC analysis of Vivinal GOS resulted in a lactose content of 20%, which allowed us to define pool 2 as a mixture consisting of 20% lactose and 19% galactooligosaccharide dimers. The addition of components results in a galactooligosaccharide content of 57%, which is close to what was specified by the supplier (59%). In view of the subsequent extraction of Vivinal galactooligosaccharides from complex matrices and analysis and quantification by CE-LIF, it is of prime importance to analyze this reference galactooligosaccharide mixture itself by CE-LIF. The electropherograms of the DP1-DP5 pools, derivatized with the fluorescent dye APTS, showed distinct migration times (**Figure 1B**).

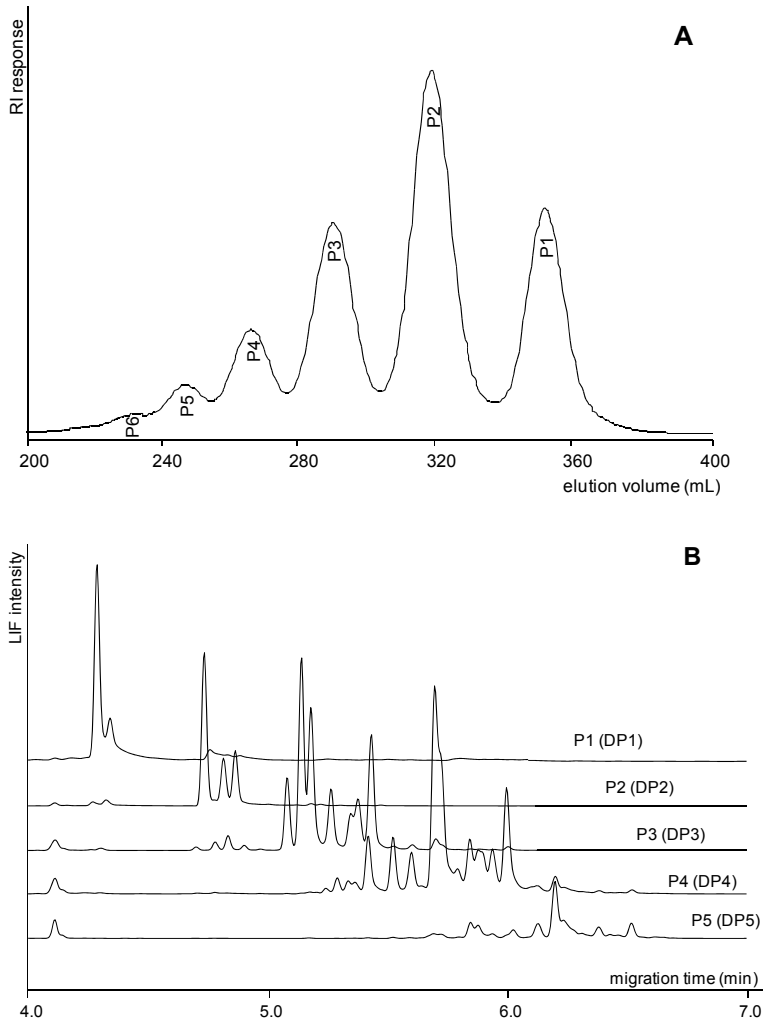

Figure 1. (A) Size-exclusion chromatography profile (RI response) obtained by BioGel P2 column separation of Vivinal GOS. P1-P6 = pool 1-6. (B) CE-LIF electropherograms of BioGel P2 pools. Pools representing DP1 and DP2 were diluted ten times before CE-LIF analysis.

Although a separation according to the Mw of the molecules is known for CE-LIF,[14] one has to accept a narrow time window of peak overlap in the beginning and end of the respective DP pools, as is visible for the last abundant peak of the DP3 pool and the first abundant peak of the DP4 pool. This may also slightly influence the subsequent

quantification. In the present case, the overlapping peak was accounted for DP3 because the DP3 peak is more abundant than the respective DP4 peak. With increasing DP, increased numbers of peaks were found as was expected for complex transgalactosylation products, indicating the gradual extension of oligosaccharides formed by the β-galactosidase used. A good peak resolution was obtained for the respective DPs. The presence of at least seven oligosaccharides could be found for the pool representing DP3, with at least ten for DP4 (pool 4) and at least eight for DP5 (pool 5) (**Figure 1B**). Our findings are comparable to those by Coulier et al.[21] These authors annotated seven reducing DP3 oligosaccharides by NMR spectroscopy after isolation using size-exclusion chromatography (SEC) and hydrophilic interaction liquid chromatography (HILIC), as well as methylation analysis. In addition, four non-reducing minor oligosaccharides were tentatively assigned.[21] The standard APTS-labeling conditions advised by the manufacturer (60 ºC, 1.5 h) were applied in this study. Testing different labeling conditions (change of derivatization time and temperature and APTS concentration) from those indicated, showed that the conditions set by the manufacturer were most optimal.

Extraction of galactooligosaccharides from complex matrices

Three different matrices (infant formula, fruit juice, and Vivinal GOS/maltodextrin preparation) were chosen to study the extractability and purification of Vivinal galactooligosaccharides added during the production process. The amount of galactooligosaccharides specified for these matrices was determined by the supplier according to the total galactose content after enzymatic hydrolysis of the galactooligosaccharides with β-galactosidase [AOAC 2001.02].[6] The crude aqueous sample extracts obtained after heat treatment (80 ºC, 30 min) yielded electropherograms showing a number of dominating peaks representing monomers (t = 4-4.5 min) and/or lactose (t = 4.8 min), as exemplified for infant formula (curve I in **Figure 2A**). The large peaks caused an unstable baseline, and peaks resulting from oligosaccharides (t = 5-6.5 min) were hardly visible. For the precise analysis of the galactooligosaccharides, the aqueous sample extracts were loaded on cartridges filled with nonporous graphitized carbon material, for which the capability to separate carbohydrates stepwise after elution by an organic solvent is known.[19] The concentration of the organic solvent applied (ACN) had to be adjusted for the different types of samples [2% (v/v) ACN for the Vivinal GOS/maltodextrin preparation and 5% (v/v) ACN for infant formula and fruit juice] to remove selectively the high amounts of mono- and/or dimers and not to affect the galactooligosaccharides ≥ DP3, which were used later on for quantification. CE-LIF analysis of the APTS-derivatized

galactooligosaccharide fractions showed an improved resolution of oligomeric peaks compared to the electropherograms, resulting from the initial aqueous extracts. However, maltodextrin-containing samples showed clearly the comigration of peaks originating from maltodextrin and galactooligosaccharides, as shown for infant formula (curve II in **Figure 2A**). Maltodextrins in food matrices can be removed by enzymatic incubation with amyloglucosidase.[20] Applying the enzymatic pretreatment for the removal of maltodextrins prior to mono- and dimer removal in sample matrices containing Vivinal galactooligosaccharides was successful. The peak profile of oligomeric structures was comparable to the Vivinal GOS reference, as shown for infant formula in curve III in **Figure 2A** and curve I **Figure 2B**. As expected, the incubation of the galactooligosaccharide pools with amyloglucosidase did not result in any breakdown of galactooligosaccharides. Similar to the removal of maltodextrins, an appropriate enzymatic incubation would be necessary for matrices containing other disturbing reducing oligosaccharides. The quantitative recovery of galactooligosaccharides was investigated by standard addition. To the crude aqueous sample extract of infant formula, a known amount of Vivinal GOS was added. The standard addition yielded a recovery of 90% in the CE-LIF electropherograms, which was considered good because of the fact that Vivinal GOS is a multiple component analyte. The fingerprints of the galactooligosaccharides extracted from all three sample matrices are shown in **Figure 2C**. From the matrices tested, galactooligosaccharides could successfully be extracted on the basis of the comparison to the reference substrate. For only the fruit juice, some small irregularities were found in the trimeric (▲ in **Figure 2C**) and tetrameric (■ in **Figure 2C**) structures, which may point to a different batch of Vivinal galactooligosaccharides added to this food matrix.

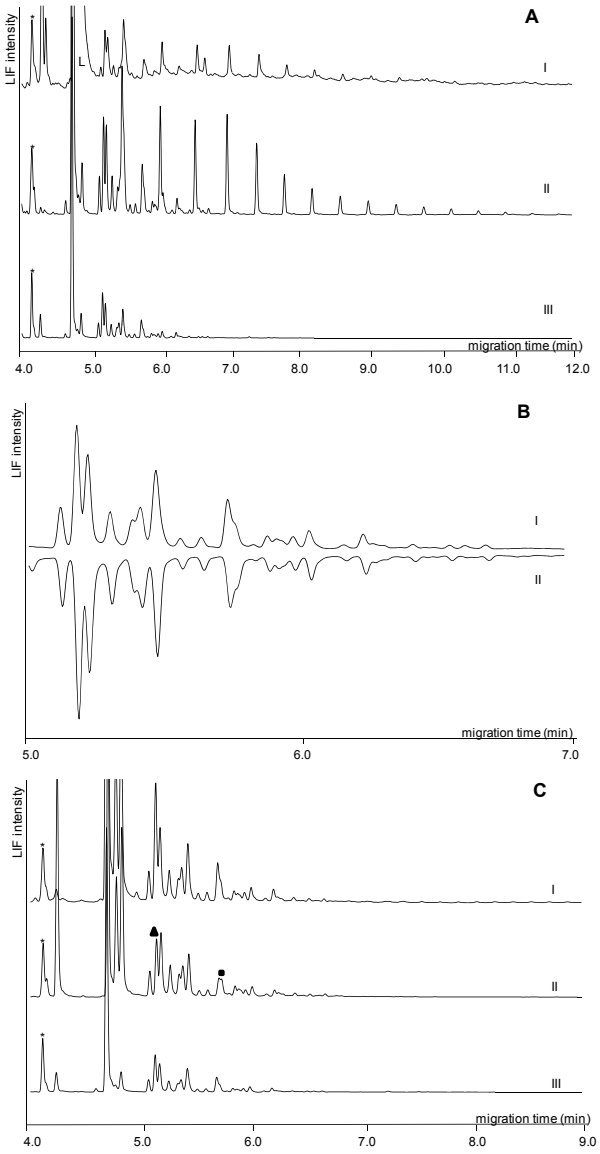

Figure 2. CE-LIF electropherograms of galactooligosaccharides. (A) (I) Aqueous extract infant formula, (II) infant formula after SPE pretreatment, and (III) infant formula after amyloglucosidase and SPE pretreatment. Curve I in panel B and curve III in panel C = curve III in panel A. (B) (II) reference Vivinal GOS (mirrored view), after amyloglucosidase and SPE pretreatment. (C) (I) Vivinal GOS/maltodextrin preparation and (II) fruit juice, both after amyloglucosidase and SPE pretreatment.
L = lactose. (*) = internal standard xylose. (▲/■) = deviating peak ratio in trimeric/tetrameric structures.

The mole-based fluorescent derivatization allows for the evaluation of the DP ratios of the galactooligosaccharides extracted. The ratio of DP3/DP4/DP5 was 1:0.6:0.1, 1:0.5:0.1, and 1:0.5:0.1 for galactooligosaccharides extracted from infant formula, fruit juice, and Vivinal GOS/maltodextrin preparation, respectively. The abundancy ratio for the Vivinal GOS reference CE-LIF profile was 1:0.5:0.2 and similar to that found by others.[21] Summarizing, the reproducible extraction of Vivinal galactooligosaccharides from complex matrices is nicely demonstrated.

Quantification of oligosaccharides by CE-LIF

The use of an internal standard carbohydrate and the fact that one APTS molecule is linked to the reducing end of each molecule theoretically enable the quantification of the components present in the oligosaccharide mixtures. Kabel et al successfully proved the quantifiability of monomers ranging in a concentration from 0.5 to 50 nmol by CE-LIF, dealing with an error of about 10%.[14] Evangelista et al started the relative quantification of oligosaccharides by analyzing different amounts of APTS-derivatized gluco-heptaose and gluco-tetraose, which resulted in a linear correlation between peak area and molar amount of sugar.[16] However, focusing on oligosaccharides, their quantification accuracy on CE-LIF should be validated in more detail with an extended range of different oligosaccharides. Commercially available standards of single DPs (DP3, DP4, and DP5) of maltodextrin-, cellodextrin-, and arabinooligosaccharides were chosen, representing α-D-(1,4)- and β-D-(1,4)-linked hexose-oligosaccharides and α-L-(1,5)-linked pentose-oligosaccharides, respectively. The CE-LIF response was investigated for stepwise, twofold increasing amounts of oligosaccharides, relative to their accurate respective starting concentration at 1 nmol. A good correlation was found between the relative amount of labeled oligosaccharides and the LIF signal obtained for concentrations up to approximately 30 nmol (**Figure 3**). The single measuring points ranged within an average deviation of ≤ 10% to the regression line ($R^2 = 0.98$). Increasing the amounts of labeled carbohydrates above a concentration of 30 nmol showed partly increased deviation values (10-25%), which emphasizes the necessity of operating in low nanomole ranges for accurate quantification purposes.

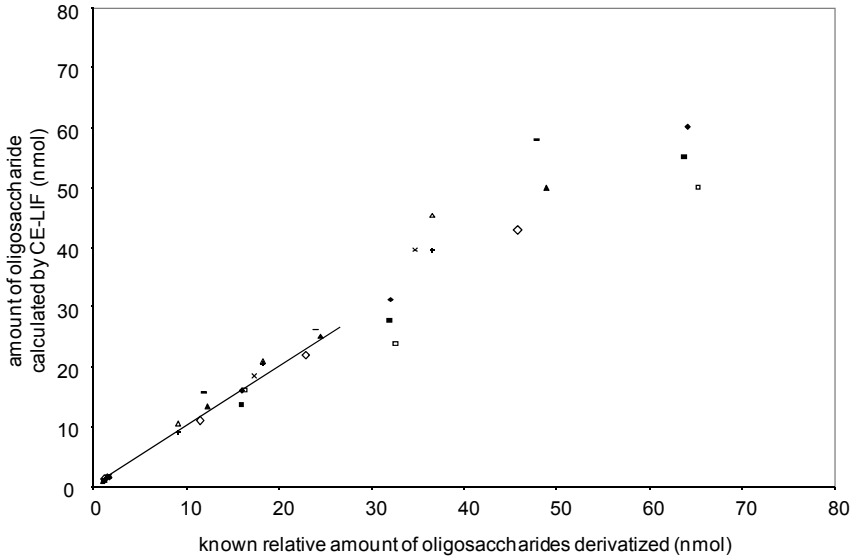

Figure 3. Relative quantification of oligosaccharides by CE-LIF, regression line ($R^2 = 0.98$). (⧫) ara 3, (□) ara 4, and (■) ara 5 = arabinotri-, tetra-, and penta-ose. (◊) m 3, (Δ) m 4, and (▲) m 5 = maltotri-, tetra-, and penta-ose. (+) c 3, (x) c 4, and (-) c 5 = cellotri-, tetra-, and penta-ose.

Quantification of Vivinal galactooligosaccharides extracted from complex food matrices

Having established an appropriate extraction method and having proven the quantifiability of oligosaccharrides by CE-LIF allows the quantitative determination of the galactooligosaccharides extracted from food matrices. To simulate extraction and purification conditions, the amyloglucosidase- and nonamyloglucosidase-incubated Vivinal GOS reference mixtures were subjected to graphitized carbon material for setting up a calibration curve (**Figure 4A**). The mono- and dimeric sugars were partly removed by elution with 2% (v/v) ACN. Solutions containing 30, 50, 90, 120, and 240 µg of galactooligosaccharides [as defined by AOAC][6] were derivatized with APTS and analyzed by CE-LIF. From the electropherograms obtained, the amount (nmol) of galactooligosaccharides representing DP3-DP5 was calculated on the basis of the internal standard xylose added. Similar to the quantification trial with standard oligosaccharides, the calibration curve exhibits a good linear range for low nanomole amounts (approximately 3-10 nmol) of derivatized oligosaccharides ($R^2 = 0.98$). One has to take into account that the effective total amount of carbohydrates labeled in these oligosaccharide mixtures was

higher, but only oligosaccharides of DP3-DP5 were taken into consideration for quantification because of a partial and sample-dependent removal of the mono- and dimers. For 120 µg of reference galactooligosaccharides [according to AOAC][6] approximately 11 nmol of DP3-DP5 was calculated (left scale y axes of **Figure 4A**), whereas the total amount of carbohydrates labeled (including residual mono- and dimers) was roughly calculated to be approximately 30 nmol (right scale y axes of **Figure 4A**). It highlights again that quantification of oligosaccharides should not be performed with amounts of total carbohydrates exceeding 30 nmol. It is the reason for the exclusion of the data points for 240 µg of reference galactooligosaccharides (approximately 55 nmol of total carbohydrate content) for setting up the calibration curve (**Figure 4A**). Consecutively, the amount of galactooligosaccharides (nanomoles of DP3-DP5) was calculated from the electropherograms of the respective food extracts. The value was then translated to the galactooligosaccharide content (micrograms) in the product using the calibration curve. Galactooligosaccharides extracted from fruit juice were also quantified using the reference calibration curve because the small structural irregularities were only of intra-DP importance but did not affect the inter-DP ratio of DP3/DP4/DP5 oligosaccharides, as shown (Vivinal GOS reference, 1:0.5:0.2; fruit juice extract, 1:0.5:0.1). Samples were extracted in duplicate, and mean values were determined (**Table 1**).

Table 1. Galactooligosaccharide contents in selected food products quantified by CE-LIF and according to product specifications

	galactooligosaccharides calculated by CE-LIF	***product specifications***
Infant Formula	2.59 ± 0.11 g/100g	±3 g/100g
Fruit juice	1.20 ± 0.01 g/100mL	1.25 g/100mL
Vivinal GOS/ maltodextrin preparation	27.87 ± 1.84 g/100g	28.5 g/100g

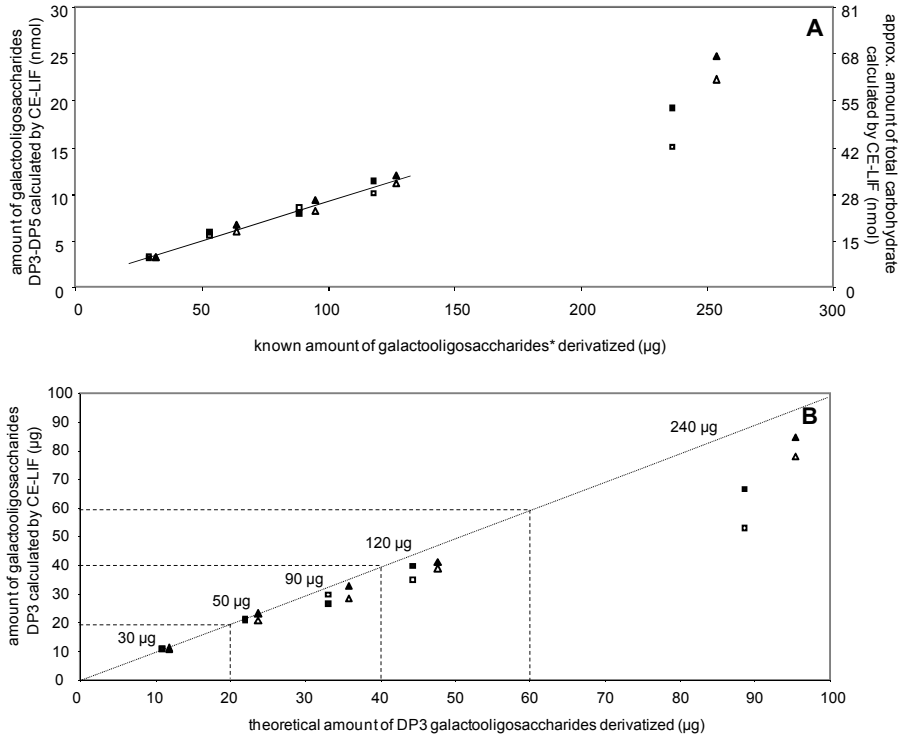

Figure 4. Quantification of Vivinal galactooligosaccharides using CE-LIF. (A) calibration curve (R^2 = 0.98). (B) Recovery of Vivinal galactooligosaccharides DP3 based on content specifications of reference Vivinal GOS. Dotted lines = 100% recovery. Reference values = 30, 50, 90, 120, and 240 µg. (■) AGOS 1 and (□) AGOS 2 = amyloglucosidase-pretreated Vivinal GOS (duplicate measurement). (▲) GOS 1 and (Δ) GOS 2 = nonamyloglucosidase-pretreated Vivinal GOS (duplicate measurement).
(*) Amount (micrograms) of galactooligosaccharides as defined by AOAC 2001.02.[6]

The results (infant formula, 2.59 ± 0.11 g/ 100 g, approximately 3 g/100 g specified; fruit juice, 1.20 ± 0.01 g/ 100 mL, 1.25 g/100 mL specified; and Vivinal GOS/maltodextrin preparation, 27.87 ± 1.84 g/100 mL, 28.5 g/100 g specified) showed that, irrespective of the matrix, the introduced method is a promising way for quantifying galactooligosaccharides with a low intersample deviation. To validate this new method, extensive collaborative studies as described by de Slegte would be necessary.[6] This was not performed in the scope of this research.

Vivinal GOS, which was available as a reference, was used as a prebiotic in all food matrices used in this study. However, there may be cases in which quantification should be

performed while the prebiotic reference material is not available. This issue can also be of interest for the determination of prebiotic oligosaccharides in complex body liquids, such as gastrointestinal contents and feces, to monitor the fate of prebiotic oligosaccharides in the human gastrointestinal tract. After unknown oligosaccharides were extracted and purified from the complex matrix, the peak migration times in the electropherogram should be assigned with the respective DP of the oligosaccharide in the first instance. This can be performed by MALDI-TOF MS after fractionation on size-exclusion material or by CE-LIF-MS coupling. The concentration of the respective peaks can subsequently be calculated on the basis of their Mw and CE-LIF area, related to the area of the internal standard used. As an example, the concentration of the DP3-DP5 structures present in the Vivinal GOS reference was determined, without using the calibration curve. The results were compared to the theoretical concentrations, which were determined on the basis of the BioGel P2 proportions (%) of DP3 (23% on DM), DP4 (10% on DM), and DP5 (4% on DM). The galactooligosaccharide reference solutions were investigated in their amyloglucosidase- and non-amyloglucosidase-pretreated forms, as exemplified for DP3 in **Figure 4B**. The theoretical amount (micrograms) of DP3 galactooligosaccharides calculated is represented by the x axis, whereas the DP3 galactooligosaccharides calculated (micrograms) from the CE-LIF response is indicated by the y axis in **Figure 4B**. The calculations were based on the internal standard xylose. The dotted line in **Figure 4B** represents the theoretical 100% recovery. DP4 and DP5 were quantified in the same way (data not shown). Remarkably, the data points belonging to the successively increasing concentrations of galactooligosaccharides were deviating to lower values with respect to the theoretical recovery to a small extent (**Figure 4B**). This results from the fact that one should stay in low nanomol ranges (≤ 30 nmol) for quantification. It may also result from an underestimation of peaks belonging to the respective DPs, because of the small overlap in the beginning and end of the DP pools, as was previously described. Quantifying complex oligosaccharides from scratch is possible, provided an appropriate sample cleanup and dilution is at hand. Recent studies on the lactose content of Vivinal GOS resulted in a concentration of only 10% lactose, because of the application of an improved gradient for HPAEC analysis,[21] which allowed for the separation of lactose from other DP2 components. Because dimers were not included for quantification in our study and sample specifications were based on AOAC 2001.02,[6] these new data have no effect on our setup. However, the method should be reconsidered if DP2 oligosaccharides are of special interest. The same counts if non-reducing oligosaccharides are of importance, because they are not labeled by the fluorescent APTS.

With the method proposed, galactooligosaccharides could be selectively extracted, despite being embedded in complex food matrices in low amounts. The implementation of CE-LIF showed a promising possibility for the quantification of reducing oligosaccharides. The application of CE-LIF may be of high value for the investigation on residual prebiotic (galacto)oligosaccharides in complex body liquids, providing insight into the fate of these carbohydrates during their fermentation in the human gastrointestinal tract.

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References

1. Ben, X.-M.; Li, J.; Feng, Z.-T.; Shi, S.-Y.; Lu, Y.-D.; Chen, R.; Zhou, X.-Y. Low level of galacto-oligosaccharide in infant formula stimulates growth of intestinal *Bifidobacteria* and *Lactobacilli*. *World J. Gastroenterol.*, **2008**, *14*, 6564-6568.
2. Bouhnik, Y.; Flourié, B.; D'Agay-Abensour, L.; Pochart, P.; Gramet, G.; Durand, M.; Rambaud, J.-C. Administration of transgalacto-oligosaccharides increases fecal Bifidobacteria and modifies colonic fermentation metabolism in healthy humans. *J. Nutr.*, **1997**, *127*, 444-448.
3. Ito, M.; Deguchi, Y.; Matsumoto, K.; Kimura, M.; Onodera, N.; Yajima, T. Influence of galactooligosaccharides on the human fecal flora. *J. Nutr. Sci. Vitaminol.*, **1993**, *39*, 635-640.
4. Pedersen, A.; Sandström, B.; van Amelsvoort, J.M.M. The effect of ingestion of inulin on blood lipids and gastrointestinal symptoms in healthy females. *Br. J. Nutr.*, **1997**, *78*, 215-222.
5. Teuri, U.; Korpela, R.; Saxelin, M.; Montonen, L.; Salminen, S. Increased fecal frequency and gastrointestinal symptoms following ingestion of galactooligosaccharides containing yoghurt. *J. Nutr. Sci. Vitaminol.*, **1998**, *44*, 465-471.
6. de Slegte, J. Determination of *trans*-galactooligosaccharides in selected food products by ion-exchange chromatography: Collaborative study. *J. AOAC Int.*, **2002**, *85*, 417-423.
7. Corrigendum to regulation (EC) No.1924/2006 of the European Parliament and of the council of 20 december 2006 on nutrition and health claims made on foods; L12/3; **2007**, European Union Publishers' Forum; Office for Official Publications of the European Communities ed.: Official Journal of the European Union, Brussels.
8. Gibson, G.R.; Probert, H.M.; van Loo, J.; Rastall, R.A.; Roberfroid, M.B. Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutr. Res. Rev.*, **2004**, *17*, 259-275.
9. Angus, F.; Smart, S.; Shortt, C. Prebiotic ingredients with emphasis on galacto-oligosaccharides and fructo-oligosaccharides. In *Probiotic dairy products*; Tamine, A., Ed; Blackwell Publ.: Oxford, **2005**; pp 120-137.
10. Hartemink, R.; Van Laere, K.M.J.; Peeters, C.C.K.M.; Nout, M.J.R.; Rombouts, F.M. *In vitro* cariogenicity of trans-galactosyl-oligosaccharides. *Lett. Appl. Microbiol.*, **1997**, *25*, 38-42.
11. Macfarlane, G.T.; Steed, H.; Macfarlane, S. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J. Appl. Microbiol.*, **2008**, *104*, 305-344.
12. Fransen, C.T.M.; Van Laere, K.M.J.; van Wijk, A.A.C.; Brüll, L.P.; Dignum, M.; Thomas-Oates, J.E.; Haverkamp, J.; Schols, H.A.; Voragen, A.G.J.; Kamerling, J.P.; Vliegenthart, J.P.G. α-D-glc*p*-(1↔1)-β-D-gal*p*-containing oligosaccharides, novel products from lactose by the action of β-galactosidase. *Carbohydr. Res.*, **1998**, *314*, 101-114.
13. Albrecht, S.; van Muiswinkel, G.C.J.; Schols, H.A.; Voragen, A.G.J.; Gruppen, H. Introducing capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) for the characterization of konjac glucomannan oligosaccharides and their *in vitro* fermentation behavior. *J. Agric. Food Chem.*, **2009**, *57*, 3867-3876.
14. Kabel, M.A.; Heijnis, W.H.; Bakx, E.J.; Kuijpers, R.; Voragen, A.G.J.; Schols, H.A. Capillary electrophoresis fingerprinting, quantification and mass-identification of various 9-aminopyrene-1,4,6-trisulfonate-derivatized oligomers derived from plant polysaccharides. *J. Chromatogr. A*, **2006**, *1137*, 119-126.
15. Shaheen, R.; Senn, J.P. Quantification of polysaccharides in water using capillary electrophoresis. *Int. J. Environ. Anal. Chem.*, **2005**, *85*, 177 - 198.
16. Evangelista, R.A.; Liu, M.-S.; Chen, F.-T.A. Characterization of 9-aminopyrene-1,4,6-trisulfonate derivatized sugars by capillary electrophoresis with laser-induced fluorescence detection. *Anal. Chem.*, **1995**, *67*, 2239-2245.
17. O'Shea, M.G.; Samuel, M.S.; Konik, C.M.; Morell, M.K. Fluorophore-assisted carbohydrate electrophoresis (FACE) of oligosaccharides: Efficiency of labelling and high-resolution separation. *Carbohydr. Res.*, **1998**, *307*, 1-12.
18. Moro, G.E.; Stahl, B.; Fanaro, S.; Jelinek, J.; Boehm, G.; Coppa, G.V. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants. *Acta Paediatr.*, **2005**, *94*, 27-30.
19. Redmond, J.W.; Packer, N.H. The use of solid-phase extraction with graphitised carbon for the fractionation and purification of sugars. *Carbohydr. Res.*, **1999**, *319*, 74-79.
20. Prosky, L.; Hoebregs, H. Methods to determine food inulin and oligofructose. *J. Nutr.*, **1999**, *129*, 1418-1423.
21. Coulier, L.; Timmermans, J.; Bas, R.; van den Dool, R.; Haaksman, I.; Klarenbeek, B.; Slaghek, T.; van Dongen, W. *In-depth* characterization of prebiotic galacto-oligosaccharides by a combination of analytical techniques. *J. Agric. Food Chem.*, **2009**, *57*, 8488-8495.

Chapter 3

CE-LIF-MS^n profiling of oligosaccharides in human milk and feces of breast-fed babies

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Abstract

Mixtures of the complex human milk oligosaccharides (HMOs) are difficult to analyse and gastrointestinal bioconversion products of HMOs may complicate analysis even more. Their analysis, therefore, requires the combination of a sensitive and high-resolution separation technique with a mass identification tool. This study introduces for the first time the hyphenation of capillary electrophoresis (CE) with an electrospray mass spectrometer, capable to perform multiple MS analysis (ESI-MS^n) for the separation and characterization of HMOs in breast milk and feces of breast-fed babies. Laser-induced fluorescence (LIF) was used for on- and off-line detections. From the overall 47 peaks detected in off-line CE-LIF electropherograms, 21 peaks could be assigned and 11 peaks could be tentatively assigned. The detailed structural characterization of a novel lacto-N-neo-tetraose isomer and a novel lacto-N-fucopentaose isomer was established in baby feces and pointed to gastrointestinal hydrolysis of higher-Mw HMOs. CE-LIF-ESI-MS^n presents, therefore, a useful tool which contributes to an advanced understanding on the fate of individual HMOs during their gastrointestinal passage.

Introduction

Still today, in times of rapid innovations in food technology with respect to nutritional needs, human breast milk is unambiguously the generally recommended form of nutrition for neonate infants.[1-3] Human milk contains a broad range of bioactive components[4] among them a complex mixture of oligosaccharides (3–19 g/L).[5,6] Research on human milk oligosaccharides (HMOs) is challenging due to their enormous structural complexity, which is substantiated by the presence of a large number of isomers. HMOs contain a lactose (L) unit at their reducing end, elongated by N-acetylglucosamine (GlcNAc) ((β1,3)-linked to the galactose (Gal) residue) as well as by consecutive linear or branched extension consisting of alternating Gals and GlcNAcs. The sugar units can be fucosylated (either (α1,2)- (α1,3)- or (α1,4)-linked) or sialylated (either (α2,3)- or (α2,6)-linked).[7] So far, more than 100 structures have been unraveled in human milk.[5,8,9] The structural composition of HMOs is closely related to their biological function (e.g. bifidobacterial stimulation, anti-inflammatory properties).[5,7,10-13] However, the exact relationship is not yet known. Referring to this, the gastrointestinal tract plays a key role as the location of possible bioconversion and brush border absorption of HMOs. Feces of breast-fed babies are rich in oligosaccharides[14,15] and their analysis can give essential information on the fate HMOs undergo throughout the gastrointestinal tract passage. So far, only few publications have reported on the analysis of HMOs extracted from the feces of breast-fed babies.[14-17] In the past, normal- and reversed-phase HPLC, high performance anion exchange chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis were the methods most frequently used for HMO analysis.[18-21] Nowadays, the hyphenation of effective separation techniques with an MS device plays an important role in the structural analysis of complex analyte mixtures.[22] HPLC-Chip/TOF-MS has been recently introduced as a potential analytical tool for the analysis of HMOs in breast milk.[9,23] Capillary electrophoresis (CE) and CE-MS coupling are other state-of-the-art methods, which recently show a tremendous development. They were introduced to a broad range of research fields (e.g. biomedical analysis) and are outstanding in view of separation efficiency and selectivity.[22] Regarding the analysis of oligosaccharide mixtures, CE is well known for its high resolving power and sensitivity.[24-27] Some commercially available HMO isomeric standards have even been successfully separated by CE, e.g. 2'fucosyllactose (2'FL)/3'FL or lacto-N-fucopentaose I/II (LNFP I/II) .[28-31] Laser-induced fluorescence (LIF) detection presents one of the most favorable and most frequently used ways of routine detection for carbohydrates separated by CE.[32] The nonchromophoric

carbohydrates are commonly derivatized with a fluorophore, e.g. 9-aminopyrene-1,4,6-trisulfonate (APTS) or 8-aminonaphtalene- 1,3,6-trisulfonate via reductive amination[33,34] prior to CE-LIF analysis. This results in a low-picomole sensitivity, mole-based fluorescent detection and the provision of charged groups, which is a prerequisite for electrophoretic separation.[35] CE-LIF presents an attractive technique for the analysis of complex biological samples, but the identification of the migrating analytes depends on the analysis of reference standards, which are often expensive, difficult to obtain or even not available. Furthermore, co-migration cannot be excluded solely based on migration times of standards. CE-MS hyphenation presents an attractive way of overcoming these limitations. Some of the parameters applied for routine CE-LIF analysis have to be modified due to technical and compatibility limitations between CE and MS device, e.g. the extension of capillary length and the composition of the separation buffer.[26,32,36-38] In contrast to LC-(UV/DAD)-MS, CE-LIF analysis implies a proceeding separation in the CE capillary, even after the LIF-detector window, due to the voltage set between cathode and anode at the MS interface. A solution for interlinking off-line CE-LIF analysis (no MS detector included) and CE-MS is the inclusion of an on-line LIF detector in front of the MS inlet to assure comparison between off-line CE-LIF electropherograms with MS base peak chromatograms. The first studies on the use of on-line CE-LIF-MS for the analysis of N-linked glycans showed promising results.[37] This study introduces for the first time the hyphenation of ESI-MS with CE for the characterization of HMOs in breast milk and feces of breast-fed babies, simultaneously making use of an on-line LIF detector. Special attention was paid to the elucidation of a lacto-N-neo-tetraose (LnNT) and LNFP isomer not present in breast milk, but present in the feces of breast-fed babies.

Materials and Methods

Sample material, HMO reference material and chemicals

Breast milk samples of five human individuals and fecal slurries (1:10 (w/v) in phosphate-buffered saline) of three individual breast-fed preterm babies were provided by FrieslandCampina (FrieslandCampina, Deventer, The Netherlands). Preterm birth took place at 32 weeks of pregnancy. Feces were collected 22–24 days after birth. 3'FL, lacto-N-tetraose (LNT), LNFP I-III, lacto-N-difucosylhexaose (LNDFH), fucosyllacto-N-hexaose II and a mixture composed of 3'sialyllactose, 6'sialyllactose and 6'sialyl-N-acetyllactosamine were purchased from Dextra Laboratories (Reading, UK). LNFP V was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of analytical grade.

HMO extraction from breast milk and fecal extracts

The extraction of carbohydrates from breast milk was accomplished as performed by Stahl et al[20] and included a mild temperature treatment (30 min, 70 ºC), defatting at 4 ºC as well as a protein precipitation by adding the double amount (v/v) of cold ethanol. Diluted fecal slurries were kept overnight at 4 ºC and were filtered through a 0.22 µm membrane according to Moro et al,[39] after a short enzyme inactivation (5 min, 100 ºC). The carbohydrates extracted from breast milk and fecal extracts were purified by SPE on graphitized carbon column cartridges (150 mg bed weight, 4 mL tube size; Alltech, Deerfield, IL).[9] The cartridges were washed with 1.5 mL of 80/20 (v/v) ACN/water containing 0.1% (v/v) TFA followed by 1.5 mL of millipore water. One milliliter of the sample extract was loaded onto a cartridge, which was subsequently eluted with 1.5 mL millipore water to remove salts. Next, monomers and L were largely removed by eluting with 1.5 mL of an aqueous 2% (v/v) ACN solution. This fraction was not further considered for analysis. The remaining carbohydrates (HMOs) on the cartridge were eluted with 1.5 mL of 40/60 (v/v) ACN/water containing 0.05% (v/v) TFA. The solution was dried under a stream of air and the dried sample was then rehydrated with 500 mL of millipore water.

Off-line CE-LIF

For CE analysis, SPE-extracted carbohydrates present were derivatized with the fluorescent APTS overnight at room temperature as reported elsewhere.[24] Briefly, 1 nmol xylose was added to each sample volume (100–200 µL) as internal standard and mobility marker. The solutions containing the fluorescent derivatives were diluted twenty times before CE-LIF analysis. Off-line CE-LIF was performed on a ProteomeLab PA 800 characterization system (Beckman Coulter, Fullerton, CA), equipped with a LIF detector (excitation: 488 nm, emission: 520 nm) (Beckman Coulter) and a polyvinyl alcohol-coated capillary (50 µm x 50.2 cm (Beckman Coulter), detector after 40 cm), kept at 25 ºC. The samples were loaded hydrodynamically (4 s at 0.5 psi, representing approx. 14 nL sample solution) on the capillary. Separation was performed in the reversed polarity mode (30 kV, 20 min) in the 25 mM acetate buffer containing 0.4% polyethylene oxide provided in the derivatization kit. Due to the low pK_a of the sialic acid residues (pK_a 2.6) and in order to provide the same pH as for CE-MS, the separation buffer was adjusted to pH 2.4 by adding 1.2% (v/v) formic acid.[36] Peaks were integrated manually using Chromeleon software 6.8 (Dionex, Sunnyvale, CA).

On-line CE-LIF-ESI-MS^n

On-line CE-LIF-ESI-MS^n experiments were performed on a P/ACE^{TM} System MDQ (Beckman Coulter). For the fluorescent detection, a Picometrics ZetaLIF discovery system was used (Picometrics, Toulouse, France) (excitation: 488 nm, emission: 520 nm). The LIF detection cell was placed approx. 25 cm from the ESI-MS source by an adjustable arm. Separation was performed on a fused silica capillary (50 µm x 85 cm (Beckman Coulter), capillary window fitted with an ellipsoid for LIF detection after 60 cm) in reversed polarity mode. LIF signals were sent to Beckmann 32Karat software via an Agilent SSXL4002 converter (Agilent Technologies, Santa Clara, CA). Best separation results were obtained at 20 and 15 kV in 0.3% (v/v) formic acid (pH 2.4) and a capillary temperature of 15 ºC, after sample injection at 10 psi for 2 s. The sample solutions containing the fluorescent derivatives were diluted four times or ten times before CE-LIF-MS analysis. The ESI-MS (LTQ ion trap, Thermo Fisher Scientific, Waltham, MA) was operated in the negative mode using a spray voltage of 1.9 kV and an MS-capillary temperature of 190 ºC. The end of the CE capillary was installed in front of the ESI source by leading it through a T-part designed in our laboratory[38] and provided the coaxial addition of a sheath liquid (50/50 isopropanol/water) at 2 mL/min. Mass spectra were acquired from m/z 300 to 2000. MS^n was performed in the data-dependent mode using a window of m/z 1 and collision energy of 35%. For increasing the S/N, ions of m/z 311, 314 and 329 were excluded from detection in MS^n experiments. MS^n data were interpreted using Xcalibur software 2.0.7 (Thermo). Base peak chromatograms are shown in their smoothed forms.

Results and Discussion

Introducing on-line CE-LIF-MS for the analysis of breast milk and fecal samples

Assuring the compatibility of CE separation and ESI-MS analysis requires the adaptation of several parameters commonly applied for off-line CE-LIF assays. In our system, it included the change of capillary material from neutral surface to fused silica[36] and the introduction of a volatile separation buffer (formic acid 0.3% v/v, pH 2.4).[32,38] Of main importance is the capillary length, which had to be extended considerably to a length of 85 cm in order to reach the MS inlet, leading to an on-going separation and longer migration times. In off-line CE-LIF analysis, capillaries with a detection window after 40 cm are generally used.[24,36-38] Placing an on-line LIF detector just in front of the MS inlet presents, therefore, an attractive means for transferring mass information to the electropherograms recorded by the off-line CE-LIF detector.[37] In our system, this was accomplished by a Picometrics LIF

detection cell located on an adjustable arm, which was placed after 60 cm capillary length. An overview of the off-line and on-line CE-LIF electropherograms as well as the respective MS base peak chromatograms is shown in **Figure 1A–E** for a randomly chosen breast milk extract and a fecal extract of a breast-fed preterm baby.

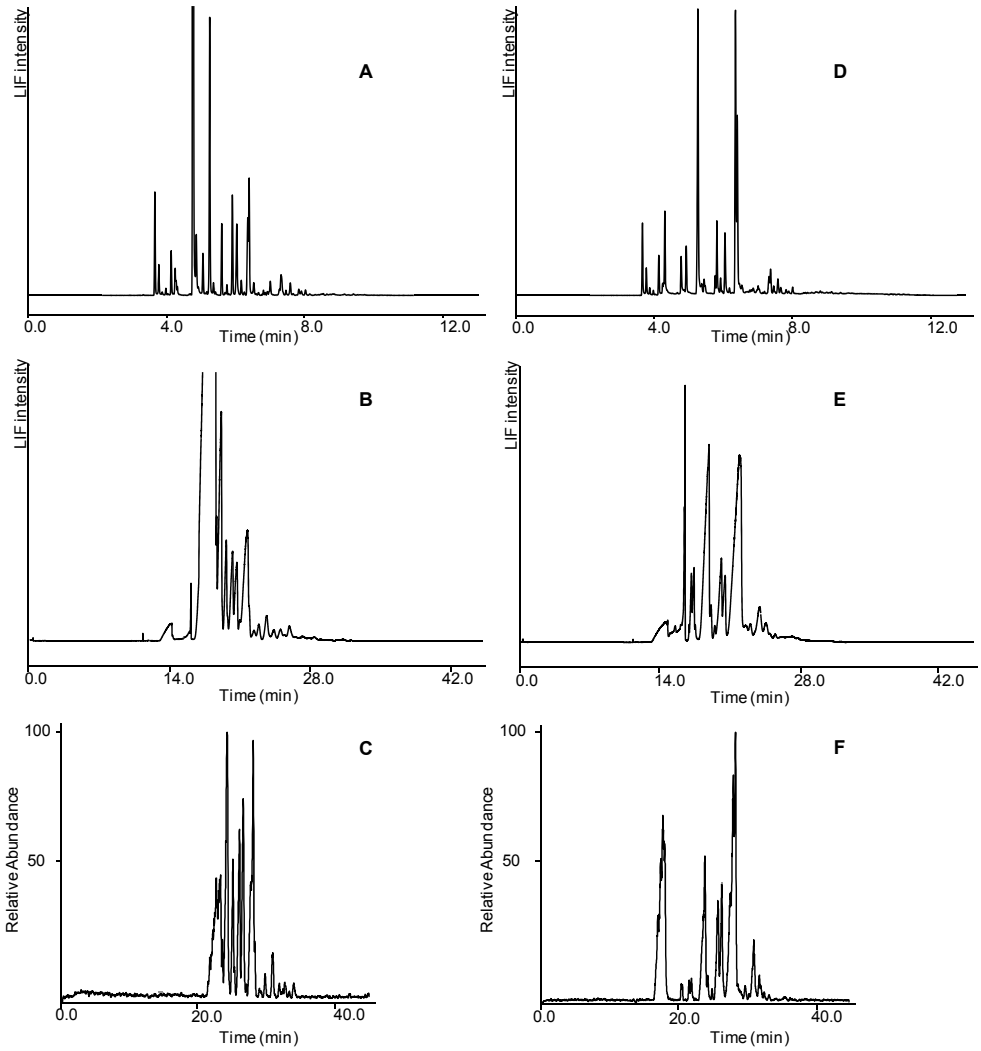

Figure 1. APTS-derivatized HMOs extracted from breast milk (A–C) and feces of a breast-fed baby (D–F). (A, D) Off-line CE-LIF electropherograms. (B, E) On-line CE-LIF electropherograms. (C, F) MS base peak chromatograms.

Off-line CE-LIF resulted in the most detailed separation profile of the samples and could be well compared with the MS base-peak chromatograms. On-line LIF electropherograms showed some peak overlap, which was not the case for the MS base peak chromatograms, due to ongoing electrophoretic separation between LIF and MS detector. The time difference between on-line LIF peak detection and MS detection was 5 min, which is small compared with ≥ 10 min reported in earlier studies.[26,36]

A sheath-flow interface was used for transferring liquid from the CE capillary outlet to the MS inlet. This results, however, in a dilution of the analyte and a reduced detection sensitivity.[32] Appropriate MS detection requires thus a sample injection of increased pressure and length (2 psi/10 s in place of 0.5 psi/7 s) and less dilution of the solutions containing the APTS-derivatized carbohydrates compared with off-line CE-LIF (four times instead of twenty times). The higher injection volume resulted in overloaded on-line LIF electropherograms. Lowering the separation voltage from 20 to 15 kV and injecting a ten times instead of a four times diluted sample resulted in the most optimal on-line LIF separation profile, as shown for the electropherogram of breast milk (**Figure 2**).

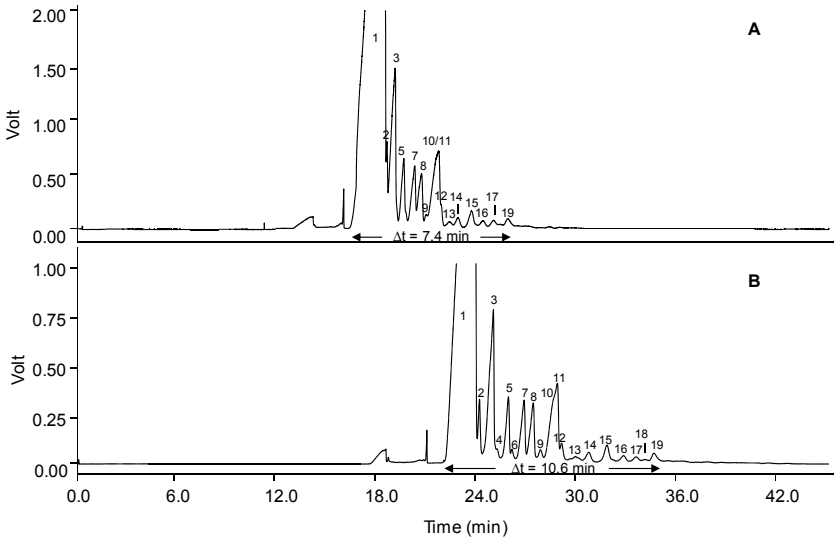

Figure 2. On-line CE-LIF electropherograms of APTS-derivatized HMOs extracted from breast milk. Separation conditions: FA buffer (0.3%, pH 2.4); separation-T: 15 ºC; injection: 2 s, 10 psi; (A) separation voltage: 20 kV, sample dilution: four times. (B) separation voltage: 15 kV, sample dilution: ten times. 1– 19: peaks detected; partly identified by assigning with MS data (**Table 1**). Δt indicates the total sample migration range.

The overall oligosaccharide migration window was extended from 7.4 min (20 kV) to 10.6 min (15 kV) (Δt = 3.2min). As a consequence, baseline separation was improved (e.g. peaks 9 and 10 or 11 and 12 (**Figure 2**)). Because of peak widening, which accompanies the separation at lowered voltage, now only the most abundant HMOs (**Figure 2**, peaks 1–3 and 5, 7, 8, 10/11) resulted in peaks in the base peak chromatograms (data not shown) and thus implies less mass information. For breast milk, 19 peaks were detected in the on-line CE-LIF electropherograms, whereas 37 peaks were detected with off-line CE-LIF analysis. Off-line CE-LIF detection showed superior sensitivity, however, no mass information is obtained during off-line CE-LIF measurement. On-line LIF detection was, therefore, considered a valuable tool for properly interlinking the simultaneously obtained information on the m/z values of peaks to the corresponding off-line CE-LIF electropherogram.

Characterization of breast milk and fecal extracts by CE-LIF-MS

General overview

The off-line CE-LIF electropherograms of a breast milk extract and of a fecal sample of a breast-fed baby are shown in **Figure 3**. The samples chosen represent the oligosaccharide patterns of individuals and the breast milk depicted does not correspond to the breast milk the preterm baby was fed with. However, the oligosaccharide pattern is representative for the patterns commonly observed in all samples extracted, taking into account individual variation. For both extracts, a detailed profile of major and minor peaks was obtained within a separation window of 4.5 min. Peaks observed between 3.5 and 4 min were defined as degradation products of the fluorescent APTS, as defined in an earlier study.[38] The peak marked with an asterisk (*) represents the internal standard and mobility marker xylose, which was added during sample preparation. For a better overview, peaks were classified as ‘‘low-Mw HMOs’’ (t_{migr} = 4.8–6.4 min), ‘‘intermediate-Mw HMOs’’ (t_{migr} = 6.5–8 min) and ‘‘high-Mw HMOs’’ (t_{migr} > 8 min) in this study. The number of peaks detected were 37 and 36 for breast milk and fecal sample, respectively. Summing up, there were 47 different peaks present in the two electropherograms (peaks present in both electropherograms were counted once only). In total, 22 of these 47 peaks were assigned and 11 were tentatively assigned (**Table 1**), mainly by means of MS data obtained from on-line CE-LIF-MS analysis and supported by MS^2 fragmentation and the analysis of standard substrates. The mole-based labeling with APTS allowed to calculate the relative amounts of HMOs present per sample, based on the area of internal standard added (xylose, 1 nmol)

(**Table 1**). Monomers and lactose (L) were not considered as the sample pretreatment on graphitized carbon material and the ethanolic extraction of breast milk is connected with a loss of monomers and L.[40,41] Components migrating t > 8 min could not be identified due to their low abundance. As the electrophoretic mobility is generally decreasing with the Mw of the oligosaccharides,[24,38] these are most probably representing high-Mw HMOs. MS data gave first indications hereof (**Table 1**).

Identification of oligosaccharides in breast milk and fecal extracts

Obviously, HMOs are present in breast milk in adequate proportions for surviving the gastrointestinal tract passage and the overall CE pattern can be found back in the feces of breast-fed babies (**Figure 3**). Though, considering individual peaks of the electropherograms, marked differences were observed concerning their presence and relative abundance in breast milk and feces. The fecal samples originated from preterm babies. Preterm birth might imply differing gastrointestinal bioconversion compared with term babies. However, this was not studied within the scope of this research.

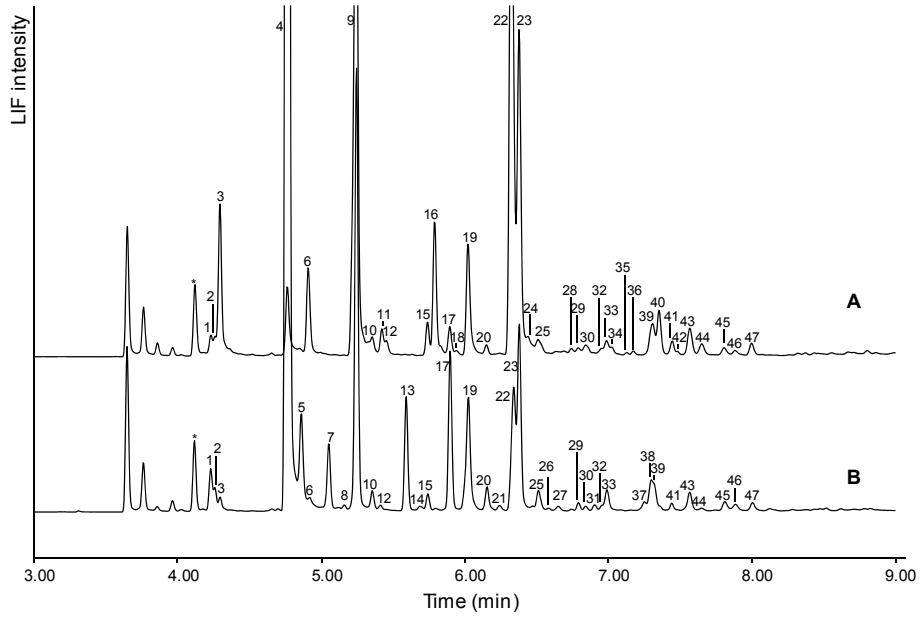

Figure 3. Off-line CE-LIF electropherograms of APTS-derivatized HMOs extracted from (A) feces of a breast-fed baby and (B) breast milk. 1– 47: peaks detected; partly identified by assigning with MS data (**Table 1**). *internal standard xylose.

Table 1. Structural assignment, composition and relative abundancy of peaks detected in representative off-/ on-line CE-LIF electropherograms of APTS-derivatized HMOs extracted from breast milk and feces of a breast-fed baby. APTS-derivatized carbohydrates detected in threefold negatively charged state by ESI-MS analysis (APTS = three protoneable sulfonate groups).

Peak off-line CE-LIF (**Figure 3**)	Peak on-line CE-LIF (breast milk) (**Figure 2**)	$t_{migr.}$ (min) off-line CE-LIF (breast milk) (**Figure 3**)	$t_{migr.}$ (min) off-line CE-LIF (fecal extract) (**Fig.ure 3**)	m/z (APTS 3-)	Hexose	GlcNAc	Fucose	Neu5Ac	name (abbreviation)	Peak area rel. to ISTD (**Fig.ure 3**, breast milk)	Peak area rel. to ISTD (**Figure 3**, fecal extract)
*	-	4.12	4.13	196	-	-	-	-	Xylose ISTD	1	1
1	-	4.23	4.24	206	1	0	0	0	Glucose	n.d.	n.d.
2	-	4.26	4.27	206	1	0	0	0	Galactose	n.d.	n.d.
3	-	4.30	4.30	201	0	0	1	0	Fucose	n.d.	n.d.
4	1	4.75	4.77	260	2	0	0	0	Lactose	n.d.	n.d.
6	-	4.92	4.92	255	1	0	1	0	F-Hex	0.2	1.5
7	2	5.06	-	357	2	0	0	1	SL	1.0	-
9	3	5.25	5.25	309	2	0	1	0	FL	6.9	9.6
11	-	-	5.43	382	3	1	0	0	LnNT Y	-	0.4
13	5	5.60	-	358	2	0	2	0	DF-L	1.8	-
15	6	5.75	5.75	382	3	1	0	0	LnNT	0.2	0.5
16	-	-	5.80	431	3	1	1	0	LNFP Y	-	2.2
17	7	5.90	5.91	382	3	1	0	0	LNT	2.5	0.5
19	8	6.03	6.03	431	3	1	1	0	LNFP II	2.4	2.0
				576	3	1	0	2	DS-LNT		
20	9	6.16	6.16	479	3	1	0	1	S-LNT	0.4	0.2
22	10	6.34	6.34	431	3	1	1	0	LNFP I/III	2.5	9.2
23	11	6.38	6.39	479	3	1	2	0	LNDFH	3.2	5.5
25	12	6.52	6.52	479	3	1	3	0	LNDFH	0.4	0.4
				479	3	1	0	1	S-LNT/		
26-32	13	6.59-6.95		479	3	1	2	0	LNDFH	0.3	0.1
				504	4	2	0	0	LNH		
				528	3	1	1	1	FS-LNT		
33	14	6.99	7.00	552	4	2	1	0	F-LNH II	0.4	0.3
38	15	7.30	-							0.6	-
39	15	7.32	7.32	601	4	2	2	0	DF-LNH	0.4	0.7
40	15	-	7.37							-	0.8
43	16	7.57	7.58	650	4	2	3	0	TF-LNH	0.4	0.5
				601	4	2	2	0	DF-LNH/		
				601	4	2	0	1	S-LNH		
44-47	17-19	7.65-8.01		649	4	2	1	1	FS-LNH	0.6	0.7
				650	4	2	3	0	TF-LNH		
				674	5	3	1	0	F-LNO		
				723	5	3	2	0	DF-LNO		
				771	5	3	3	0	TF-LNO		
-	-	> 8.01		820	5	3	4	0	tetra-F-LNO	-	-
									sialylated HMOs		

Low-Mw, neutral HMOs

L was clearly the most abundant peak found in breast milk samples (**Figure 3B**, peak 4). Although *in vivo* studies reported immature lactase activity with preterm babies,[42] the low L peak present in fecal extracts (**Figure 3A**, peak 4) indicated increased lactase activity at 22–24 days after birth or a different gastrointestinal utilization, e.g. membrane transport by intestinal bacteria. It was not possible to define peak 5 (**Figure 3B**) present in breast milk, but not in the fecal extract, in spite of its abundance. MS base peak chromatograms of breast milk did not give any information here over. In fecal samples, a peak composed of a hexose and fucose unit (F-Hex) migrated at t = 4.92 min (**Figure 3A**, peak 6), which was present in much smaller quantities in breast milk. F-Hex was, therefore, considered as a fragment resulting from gastrointestinal degradation of HMOs. Within both, fecal extracts and breast milk, FL was the most abundant HMO (**Figure 3**, peak 9). Unfortunately, it was not possible in this study to distinguish between 3'FL (Gal-(β1,4)-[Fuc-(α1,3)]-Glc) and 2'FL (Fuc-(α1,2)-Gal-(β1,4)-Glc), which is the more prominent isomer present in breast milk.[7,31] Having a closer look on the HMOs up to an m/z 500 in breast milk, LNDFH (**Figure 3B**, peak 23), LNT (**Figure 3B**, peak 17), LNFP I/III (**Figure 3B**, peak 22), LNFP II (**Figure 3B**, peak 19) and difucosyllactose (DF-L) (**Figure 3B**, peak 13) were found to be prevailing (ratio 1:0.8:0.8:0.7:0.5). Within the fecal extract, these peaks were found to be present in a completely different ratio (ratio 1:0.1:1.7:0.4:0), indicating an active gastrointestinal bioconversion. The absence of DF-L within the fecal extract indicated gastrointestinal fucosidase activity (**Figure 3A**).[11] On the contary, the remaining high abundance of FL and LNFP isomers in fecal extracts (**Figure 3A**, peaks 9 and 16, 19, 22), which probably results from the enzymatic degradation of higher-Mw HMOs and DF-L, indicates a certain limitation of gastrointestinal fucosidase activity toward HMO structures. LNT, which is a nondecorated tetramer, seems to be a highly susceptible substrate for enzymatic attack, as it was present in only low amounts in the fecal extract (**Figure 3A**, peak 17). The peak ratios found were highly individual dependent, but always showed the same trend.

Acidic HMOs and intermediate-Mw HMOs

Besides neutral HMOs, acidic HMOs decorated with one or more sialic acid groups form an important part of HMOs, although present in much lower amounts.[5] Sialyllactose (SL), disialyllacto-N-tetraose (DS-LNT) and sialyllacto-N-tetraose (S-LNT) (**Figure 3B**, peaks 7 and 19, 20) were identified in breast milk. Fecal extracts did not contain any SL or DS-LNT and showed a reduced abundance of S-LNT (**Figure 3A**, peak 20). These observations support results of *in vivo* studies on the gastrointestinal sialidase activity.[11,15] The assignment of intermediate-Mw sialylated HMOs is complicated by the fact that their m/z values partly coincide with the m/z values of neutral HMOs. Therefore, peaks 26–32 and 44–47 present in breast milk and fecal extracts (**Figure 3**) were assigned tentatively only. More detailed MS^n analysis of these intermediate-Mw HMOs is necessary. In general, intermediate-Mw HMOs (**Figure 3**, peaks 24–47) showed a large complexity. A definite assignment was not possible so far due to frequent co-migration caused by the increasing number of possible isomers for these structures.

Unknown HMOs

The individual variation of HMOs in breast milk and their recovery in feces has been reported previously.[7,17,23,31] However, in most cases the identification of HMOs was limited to the purified HMOs commercially available or connected with tedious preparative fractionation. CE-LIF-MS presents an efficient way for a sensitive and mole-based separation of the complex HMOs combined with a simultaneous availability of mass information. This is of special importance for the identification of peaks in the fecal extract, which were not present in breast milk. MS base peak chromatograms indicated the presence of a new LNFP structure (**Figure 3A**, peak 16) and a new LNT structure (**Figure 3A**, peak 11). Their characterization, based on MS fragmentations of commercially available HMO isomers, was of special interest in order to understand the gastrointestinal breakdown of HMO structures. It is, therefore, addressed separately.

Structural characterization of LNFP Y and LnNT Y in fecal extracts

LNFP I, II, III and V

Compared with breast milk, additional peaks were detected in the feces of breast-fed babies. In order to be able to characterize the unknown LNFP structure in fecal extracts of breast-fed babies (LNFP Y, m/z 431, **Figure 3A**, peak 16), CE-LIF-MS^2 experiments on commercially available LNFP isomers were performed. Four isomeric LNFP structures are known to be present in breast milk,[43,44] namely LNFP I, II, III and V (**Figure 4A**). The migration time of the unknown LNFP isomer did not coincide with any of the LNFP standards. MS^2 fragmentation of underivatized LNFP isomers has been studied earlier.[29,45] Compared hereto, deviating MS^2 fragmentation patterns were obtained in this study due to the APTS derivatization of the isomers LNFP I, II, III and V (**Figure 4A**). The fragments of interest were exclusively Y fragments.[46] Due to the derivatization at the reducing end, no cross-ring cleavages were expected.[29,44] LNFP I has a linear structure and m/z 328 (corresponding to the loss of the terminal Fuc and Gal unit) is characteristic for this linear structure.[29] For the branched LNFP II, III and V, m/z 328 demands the cleavage of two glycosidic linkages and explains the lower abundance of this fragment. Another main characteristic feature is the ratio of the fragments m/z 377 (loss of the terminal Gal; not possible for LNFP I) and 382 (loss of the Fuc residue). LNFP II and V, which both possess a (β3,1)-linked terminal Gal, show an abundance of m/z 377 < m/z 382, whereas for LNFP III, which possesses a (β4,1)-linked terminal Gal, the abundancy is m/z 377 > m/z 382. LNFP V showed a predominating fragmentation ion of m/z 464 [Gal-[Fuc]-Glc-APTS]. It is characteristic for a Fuc residue being attached to the reducing glucose (Glc) moiety and, therefore, such fragmentation is not possible for any of the other LNFP isomers.

LNFP Y

The fragmentation pattern of LNFP Y (**Figure 3A**, peak 16) did not coincide with any of the known LNFP isomers. Based on the assumption that LNFP Y results from gastrointestinal degradation, the structural characteristics of higher-Mw HMOs were studied. The unit –Gal-(β1,6)- frequently occurs at the branching points of these higher-Mw HMOs (exemplified for fucosyllacto-N-hexaose II (F-LNH II), m/z 553, **Figure 4B**).

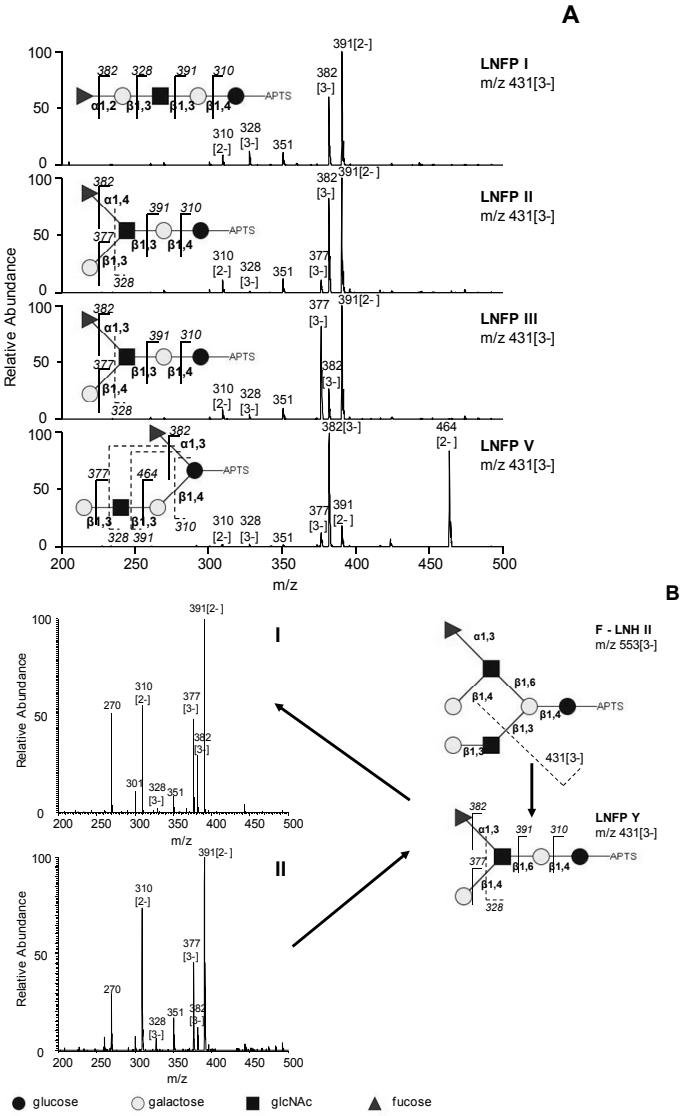

Figure 4. (A) MS^2 fragmentation patterns of APTS-derivatized standards LNFP I, II, III and V (m/z 431). (B) Identification of LNFP Y in feces of breast-fed babies. (B I) MS^2 fragmentation pattern of m/z 431 (precursor ion: APTS-derivatized standard F-LNH II, m/z 553). (B II) MS^2 fragmentation pattern of LNFP Y. [2-] [3-]: charge state of APTS-molecule.

F-LNH II (m/z 553) was chosen for a MS-fragmentation study. The MS^2 spectrum of F-LNH II contained the fragment m/z 431 (LNFP), which corresponds to the loss of a Gal and a GlcNAc unit at the branching point of F-LNH II. This m/z 431 fragment was of special interest as it contained the –Gal-(β1,6)- unit and was, therefore, subjected to a subsequent MS fragmentation. The fragmentation pattern showed m/z 377 > m/z 382, which is representative for its (β1,4)-linked terminal Gal (see LNFP III), and an increased signal for m/z 310 (Hex-APTS), deduced from its –Gal-(β1,6)- unit. The m/z–value 310 was not prominently present in any other LNFP isomer fragmentation pattern (**Figure 4B**). LNFP Y in fecal extracts showed the same MS-fragmentation pattern and was, therefore, characterized as a breakdown product of higher-Mw HMOs, possessing a –Gal-(β1,6)- unit and a (β4,1)-linked terminal Gal (**Figure 4B**). LNFP isomers are present in considerable quantities in fecal extracts. The hydrolysis of the –GlcNAc-(β1,3)-Gal linkage at branching points of higher-Mw HMOs (**Figure 4B**) results in the formation of LNFP structures or larger structures further hydrolysable to LNFP. The –GlcNAc-(β1,3)-Gal- linkage was therefore assumed to be a preferred site of enzymatic attack during the gastrointestinal passage. Compared hereto, defucosylation was considered to be of minor importance.

LNT, LnNT and LnNT Y

Further characterization was as well needed for the unknown LnNT structure found in fecal extracts of breast-fed babies (LnNT Y, m/z 382, **Figure 3A**, peak 11). Two LNT isomers were detected in breast milk (**Figure 3B**, peaks 15 and 17). For breast milk, the presence of two LNT isomers is known, namely LNT (Gal-(β1,3)-GlcNAc-(β1,3)-Gal-(β1,4)-Glc) and LnNT (Gal-(β1,4)-GlcNAc-(β1,3)-Gal-(β1,4)-Glc), representing nonfucosylated LNFP I, II, V and LNFP III, respectively.[7,9,47] The migration time and MS^2 pattern of the LNT standard was the same as peak 17 (**Figure 3**). Peak 15 (**Figure 3**) was, therefore, assigned as LnNT and showed a very similar MS^2 pattern to LNT, as expected. In the fecal extract, three isomeric LNT structures were detected (**Figure 3A**, peak 11 [LnNT Y], 15 [LnNT] and 17 [LNT]). The MS^2 pattern of LnNT Y differed from the ones of LnNT and LNT, but showed the same pattern as LNFP Y, including an abundantly present fragment m/z 310, typical for a Gal-(β1,6)-linkage. LnNT Y was therefore characterized as a defucosylated LNFP Y structure (Gal-(β1,4)-GlcNAc-(β1,6)-Gal-(β1,4)-Glc) and was present in lower abundance compared with LNFP Y (1:0.2, **Table 1**).

Concluding remarks

The hyphenation of on-line CE-LIF with ESI-MS showed to be a useful tool for the annotation of off-line CE-LIF separation profiles of APTS-derivatized HMOs from breast milk. Analyzing fecal extracts of breast-fed babies gave a valuable insight in the fate of HMOs during the gastrointestinal passage. The identification of LnNT Y and LNFP Y, two new isomers not initially present in breast milk allowed to draw conclusions on the gastrointestinal degradation of higher-Mw HMOs.

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References

1. Leung, A.K.C.; Sauve, R.S. Breast is best for babies. *J. Natl. Med. Assoc.*, **2005**, *97*, 1010-1019.
2. Mc Guire, W.; Henderson, G.; Fowlie, P.W. ABC of preterm birth - feeding the preterm infant. *Br. Med. J.*, **2004**, *329*, 1227-1230.
3. Work Group on Breastfeeding. Breastfeeding and the use of human milk. *Pediatrics*, **1997**, *100*, 1035-1039.
4. Neu, J. Gastrointestinal maturation and implications for infant feeding. *Early Hum. Dev.*, **2007**, *83*, 767-775.
5. Kunz, C.; Rudloff, S.; Baier, W.; Klein, N.; Strobel, S. Oligosaccharides in human milk: Structural, functional, and metabolic aspects. *Ann. Rev. Nutr.*, **2000**, *20*, 699-722.
6. Viverge, D.; Grimmonprez, L.; Cassanas, G.; Bardet, L.; Bonnet, H.; Solère, M. Variations of lactose and oligosaccharides in milk from women of blood types secretor A or H, secretor Lewis, and secretor H/nonsecretor Lewis during the course of lactation. *Ann. Nutr. Metab.*, **1985**, *29*, 1-11.
7. Kunz, C.; Rudloff, S. Biological functions of oligosaccharides in human milk. *Acta Paediatr.*, **1993**, *82*, 903-912.
8. Bao,Y.; Newburg, D.S. Capillary electrophoresis of acidic oligosaccharides from human milk. *Electrophoresis*, **2008**, *29*, 2508-2515.
9. Ninonuevo, M.R.; Park, Y.; Yin, H.; Zhang, J.; Ward, R.E.; Clowers, B.H.; German, J.B.; Freeman, S.L.; Killeen, K.; Grimm, R.; Lebrilla, C.B. A strategy for annotating the human milk glycome. *J. Agric. Food Chem.*, **2006**, *54*, 7471-7480.
10. Coppa, G.V.; Gabrielli, O.; Giorgi, P.; Catassi, C.; Montanari, M.P.; Varaldo, P.E.; Nichols, B.L. Preliminary study of breastfeeding and bacterial adhesion to uroepithelial cells. *Lancet*, **1990**, *335*, 569-571.
11. Lo Cascio, R.G.; Ninonuevo, M.R.; Freeman, S.L.; Sela, D.A.; Grimm, R.; Lebrilla, C.B.; Mills, D.A.; German, J.B. Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. *J. Agric. Food Chem.*, **2007**, *55*, 8914-8919.
12. Newburg, D.S.; Ruiz-Palacios, G.M.; Altaye, M.; Chaturvedi, P.; Meinzen-Derr, J.; Guerrero, M.d.L.; Morrow, A.L. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology*, **2004**, *14*, 253-263.
13. Yoshioka, H.; Iseki, K.-I.; Fujita, K. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics*, **1983**, *72*, 317-321.
14. Sabharwal, H.; Nilsson, B.; Grönberg, G.; Chester, M.A.; Dakour, J.; Sjöblad, S.; Lundblad, A. Oligosaccharides from feces of preterm infants fed on breast milk. *Arch. Biochem. Biophys.*, **1988**, *265*, 390-406.
15. Sabharwal, H.; Sjöblad, S.; Lundblad, A. Sialylated oligosaccharides in human milk and feces of preterm, full-term, and weaning infants. *J. Pediatr. Gastroenterol. Nutr.*, **1991**, *12*, 480-484.
16. Chaturvedi, P.; Warren, C.D.; Buescher, C.R.; Pickering, L.K.; Newburg, D.S. Survival of human milk oligosaccharides in the intestine of infants. In *Bioactive components of human milk*; Kluwer Academic/Plenum Publ: New York, **2001**; pp 315-323.
17. Coppa, G.V.; Pierani, P.; Zampini, L.; Bruni, S.; Carloni, I.; Gabrielli, O. Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. In *Bioactive components of human milk*; Newburg, D.S., Ed; Kluwer Academic/Plenum Publishers: New York, **2001**; pp 307-314.
18. Charlwood, J.; Tolson, D.; Dwek, M.; Camilleri, P. A detailed analysis of neutral and acidic carbohydrates in human milk. *Anal. Biochem.*, **1999**, *273*, 261-277.
19. Finke, B.; Stahl, B.; Pfenninger, A.; Karas, M.; Daniel, H.; Sawatzki, G. Analysis of high-molecular-weight oligosaccharides from human milk by liquid chromatography and MALDI-MS. *Anal. Chem.*, **1999**, *71*, 3755-3762.
20. Stahl, B.; Thurl, S.; Zeng, J.R.; Karas, M.; Hillenkamp, F.; Steup, M.; Sawatzki, G. Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem.*, **1994**, *223*, 218-226.
21. Thurl, S.; Henker, J.; Siegel, M.; Tovar, K.; Sawatzki, G. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconjugate J.*, **1997**, *14*, 795-799.
22. Klampfl, C.W. CE with MS detection: A rapidly developing hyphenated technique. *Electrophoresis*, **2009**, *30*, S83-S91.
23. Ninonuevo, M.R.; Perkins, P.D.; Francis, J.; Lamotte, L.M.; LoCascio, R.G.; Freeman, S.L.; Mills, D.A.; German, J.B.; Grimm, R.; Lebrilla, C.B. Daily variations in oligosaccharides of human milk determined by microfluidic chips and mass spectrometry. *J. Agric. Food Chem.*, **2008**, *56*, 618-626.

24. Albrecht, S.; van Muiswinkel, G.C.J.; Schols, H.A.; Voragen, A.G.J.; Gruppen, H. Introducing capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) for the characterization of konjac glucomannan oligosaccharides and their *in vitro* fermentation behavior. *J. Agric. Food Chem.*, **2009**, *57*, 3867-3876.
25. El Rassi, Z. Recent developments in capillary electrophoresis and capillary electrochromatography of carbohydrate species. *Electrophoresis*, **1999**, *20*, 3134-3144.
26. Kabel, M.A.; Heijnis, W.H.; Bakx, E.J.; Kuijpers, R.; Voragen, A.G.J.; Schols, H.A. Capillary electrophoresis fingerprinting, quantification and mass-identification of various 9-aminopyrene-1,4,6-trisulfonate-derivatized oligomers derived from plant polysaccharides. *J. Chromatogr. A*, **2006**, *1137*, 119-126.
27. Sei, K.; Nakano, M.; Kinoshita, M.; Masuko, T.; Kakehi, K. Collection of α_1-acid glycoprotein molecular species by capillary electrophoresis and the analysis of their molecular masses and carbohydrate chains: Basic studies on the analysis of glycoprotein glycoforms. *J. Chromatogr. A*, **2002**, *958*, 273-281.
28. An, H.J.; Franz, A.H.; Lebrilla, C.B. Improved capillary electrophoretic separation and mass spectrometric detection of oligosaccharides. *J. Chromatogr. A*, **2003**, *1004*, 121-129.
29. Que, A.H.; Novotny, M.V. Structural characterization of neutral oligosaccharide mixtures through a combination of capillary electrochromatography and ion trap tandem mass spectrometry. *Anal. Bioanal. Chem.*, **2003**, *375*, 599-608.
30. Shen, Z.; Warren, C.D.; Newburg, D.S. High-performance capillary electrophoresis of sialylated oligosaccharides of human milk. *Anal. Biochem.*, **2000**, *279*, 37-45.
31. Song, J.-F.; Weng, M.-Q.; Wu, S.-M.; Xia, Q.-C. Analysis of neutral saccharides in human milk derivatized with 2-aminoacridone by capillary electrophoresis with laser-induced fluorescence detection. *Anal. Biochem.*, **2002**, *304*, 126-129.
32. Gennaro, L.A.; Salas-Solano, O.; Ma, S. Capillary electrophoresis-mass spectrometry as a characterization tool for therapeutic proteins. *Anal. Biochem.*, **2006**, *355*, 249-258.
33. Evangelista, R.A.; Liu, M.-S.; Chen, F.-T.A. Characterization of 9-aminopyrene-1,4,6-trisulfonate derivatized sugars by capillary electrophoresis with laser-induced fluorescence detection. *Anal. Chem.*, **1995**, *67*, 2239-2245.
34. Larsson, M.; Sundberg, R.; Folestad, S. On-line capillary electrophoresis with mass spectrometry detection for the analysis of carbohydrates after derivatization with 8-aminonaphthalene-1,3,6-trisulfonic acid. *J. Chromatogr. A*, **2001**, *934*, 75-85.
35. O'Shea, M.G.; Samuel, M.S.; Konik, C.M.; Morell, M.K. Fluorophore-assisted carbohydrate electrophoresis (FACE) of oligosaccharides: Efficiency of labelling and high-resolution separation. *Carbohydr. Res.*, **1998**, *307*, 1-12.
36. Coenen, G.J.; Kabel, M.A.; Schols, H.A.; Voragen, A.G.J. CE-MS^n of complex pectin-derived oligomers. *Electrophoresis*, **2008**, *29*, 2101-2111.
37. Gennaro, L.A.; Salas-Solano, O. On-line CE-LIF-MS technology for the direct characterization of N-linked glycans from therapeutic antibodies. *Anal. Chem.*, **2008**, *80*, 3838-3845.
38. Hilz, H.; de Jong, L.E.; Kabel, M.A.; Schols, H.A.; Voragen, A.G.J. A comparison of liquid chromatography, capillary electrophoresis, and mass spectrometry methods to determine xyloglucan structures in black currants. *J. Chromatogr. A*, **2006**, *1133*, 275-286.
39. Moro, G.E.; Stahl, B.; Fanaro, S.; Jelinek, J.; Boehm, G.; Coppa, G.V. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants. *Acta Paediatr.*, **2005**, *94*, 27-30.
40. Albrecht, S.; Schols, H.A.; Klarenbeek, B.; Voragen, A.G.J.; Gruppen, H. CE-LIF as an analysis and quantification tool for galactooligosaccharides extracted from complex food matrices. *J. Agric. Food Chem.*, **2010**, *58*, 2787-2794.
41. Machado, J.J.B.; Coutinho, J.A.; Macedo, E.A. Solid-liquid equilibrium of α-lactose in ethanol/water. *Fluid Phase Equilib.*, **2000**, *173*, 121-134.
42. Shulman, R.J.; Feste, A.; Ou, C. Absorption of lactose, glucose polymers, or combination in premature infants. *J. Pediatr.*, **1995**, *127*, 626-631.
43. Ginsburg, V.; Zopf, D.A.; Yamashita, K.; Kobata, A. Oligosaccharides of human milk : Isolation of a new pentasaccharide, lacto-N-fucopentaose V. *Arch. Biochem. Biophys.*, **1976**, *175*, 565-568.
44. Spengler, B.; Dolce, J.W.; Cotter, R.J. Infrared laser desorption mass spectrometry of oligosaccharides: Fragmentation mechanisms and isomer analysis. *Anal. Chem.*, **2002**, *62*, 1731-1737.
45. Pfenninger, A.; Karas, M.; Finke, B.; Stahl, B. Structural analysis of underivatized neutral human milk oligosaccharides in the negative ion mode by nano-electrospray MS^n (part 1: Methodology). *J. Am. Soc. Mass Spectrom.*, **2002**, *13*, 1331-1340.

46. Domon, B.; Costello, C.E. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. *Glycoconjugate J.*, **1988**, *5*, 397-409.
47. Urashima, T.; Asakuma, S.; Messer, M. Milk oligosaccharides. In *Comprehensive glycoscience. From chemistry to system biology; vol.4*; Kamerling, J.P., Boons, G.J., Lee, Y.C., Suzuki, A., Taniguchi, N.,Voragen, A.G.J., Eds; Elsevier: Oxford, UK, **2007**; pp 695-722.

Chapter 4

Oligosaccharides in feces of breast- and formula-fed babies

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Abstract

So far, little is known on the fate of oligosaccharides in the colon of breast- and formula-fed babies. Using capillary electrophoresis with laser induced fluorescence detector coupled to a mass spectrometer (CE-LIF-MS^n), we studied the fecal oligosaccharide profiles of twenty-seven two months old breast-, formula- and mixed-fed preterm babies. The interpretation of the complex oligosaccharide profiles was facilitated by beforehand clustering the CE-LIF data points by agglomerative hierarchical clustering (AHC). In the feces of breast-fed babies, characteristic human milk oligosaccharide (HMO) profiles, showing genetic fingerprints known for human milk of secretors and non-secretors, were recognized. Alternatively, advanced degradation and bioconversion of HMOs, resulting in an accumulation of acidic HMOs or HMO bioconversion products was observed. Independent of the prebiotic supplementation of the formula with galactooligosaccharides (GOS) at the level used, similar oligosaccharide profiles of low peak abundance were obtained for formula-fed babies. Feeding influences the presence of diet-related oligosaccharides in baby feces and gastrointestinal adaptation plays an important role herein. Four fecal oligosaccharides, characterized as HexNAc-Hex-Hex, Hex-[Fuc]-HexNAc-Hex, HexNAc-[Fuc]-Hex-Hex and HexNAc-[Fuc]-Hex-HexNAc-Hex-Hex, highlighted an active gastrointestinal metabolization of the feeding-related oligosaccharides. Their presence was linked to the gastrointestinal mucus layer and the blood-group determinant oligosaccharides therein, which are characteristic for the host's genotype.

Introduction

Dietary prebiotic carbohydrates, such as human milk oligosaccharides (HMOs) and galactooligosaccharides (GOS), have a considerable impact on the neonatal gastrointestinal development. They influence the intestinal microbial colonization, which is responsible for the absorption and metabolisation of food ingredients, the establishment of the immune system, act as receptor analogs for pathogenic bacteria and prevent constipation.[1-6] HMOs are a mixture of structurally complex, lactose-based oligosaccharides, composed of galactose (Gal), glucose (Glc), N-acetyl-glucosamine (GlcNAc) and frequently decorated by fucose (Fuc) and sialic acid (Neu5Ac).[7] HMOs are present in human milk to an extent of 3-19 g/L.[7,8] The qualitative and quantitative composition of HMOs in breast milk is individual-dependent. Human milk can be classified according to the presence and absence of (α1,3/4)- and (α1,2)-fucosylated oligosaccharides, which indicate the activity of the Lewis (Le)- and Secretor (Se)-gene of the mother, with the latter being responsible for the expression of (α1,2)-fucosyltransferase (FUT2).[5,8,9] Due to the complexity and individual-dependent profile of HMOs it has not been possible to reproduce HMOs for the use as prebiotic supplements of breast milk replacers so far. Prebiotic GOS are structurally similar to HMOs, however, less complex. They are high in galactose content and carry a lactose unit at the reducing end. GOS are frequently added to infant formulas for term and preterm babies.[10] Especially preterm infants (born after a gestational age of 24-36 weeks), are vulnerable for infections due to their impaired immunity.[11] This results in frequent antibiotic use and this is one of the factors, which can distort the intestinal flora. Although human milk is the preferred feeding to these infants, human milk is not always available and substitution with preterm formula is important,[12] e.g. in order to establish a balanced microflora.

Several studies on the intestinal flora composition of term and preterm born babies have been performed in order to evaluate the value of HMOs in breast milk and prebiotic oligosaccharides in infant formula.[13,14] However, little is known on the gastrointestinal fate of the individual oligosaccharide structures. Diet-related oligosaccharides can be traced back in baby feces, but they have rarely been studied in detail.[14-16] A more profound knowledge of the gastrointestinal fate of oligosaccharides would be helpful for the understanding of the structure-function relation of feeding-related oligosaccharides.

We recently introduced the hyphenation of capillary electrophoresis with laser-induced fluorescence detection to a mass spectrometer capable to perform multiple MS analysis

(CE-LIF-MS^n) for the separation and characterization of HMOs in breast milk and feces of breast-fed babies.[17]

In the present study we applied CE-LIF-MS^n for a broad-range screening of oligosaccharides present in feces of breast-, formula- and mixed-fed preterm babies. The formula was either fortified with prebiotic GOS (0.4 g/dL) or did not contain any prebiotic oligosaccharides. Agglomerative hierarchical clustering (AHC) was performed on the CE-LIF data points in order to observe general trends. Subsequently, CE-LIF-MS^n oligosaccharide profiles were described in order to study the individual-dependent and genetically determined gastrointestinal degradation of feeding-related carbohydrates. MS^n analysis was applied for the identification of unknown oligosaccharides.

Materials and Methods

Set-up study and sample collection

Twenty-seven fecal samples of approximately two months old preterm infants (born after 27-35 weeks of gestation and a birth weight between 770 g and 2285 g) were selected from a clinical study on the investigation of the effect of the supplementation of preterm infant formula with prebiotic GOS or lactoferrin on the performance of preterm babies, which was set up and performed prior to our research intention. The study was performed at the level III neonatal intensive care unit of the Isala clinics, Zwolle, The Netherlands. Enteral feeding of the babies was started after birth as soon as possible and full enteral feeding was established 5-19 days after birth. Fecal samples were taken 6 weeks after full enteral feeding was established and samples were frozen at -20°C until analysis. The babies received either exclusively expressed human milk from their own mother (n = 5) or preterm infant formula (n = 6) [supplemented with 0.4 g/dL GOS (Frisolac prematuur, FrieslandCampina DOMO, Zwolle, The Netherlands), or without GOS (Frisolac prematuur, GOS replaced by maltodextrin), or supplemented with 0.1 g/dL lactoferrin]. Sixteen babies got mixed feeding (breast milk and formula). Breast milk meals contributed to > 80%, > 60% or < 55% of the total number of feeding, which the babys got during the study period (**Table 1**). The babies were randomly assigned to one of the three formula groups. The study was performed double-blind and was approved by the medical ethical review board of the hospital. Written informed consent was obtained from all the parents. Except for baby A2, the blood group of the babies was known (**Table 1**). Among the twenty-seven babies studied were two twin pairs and one triplet pair, but they were not further specified in this study. Lactoferrin-supplementation was not of interest for the present study on the

analysis of fecal oligosaccharides and no differences were observed in the fecal oligosaccharide profiles of formula-fed babies with and without lactoferrin supplementation. Babies who got preterm infant formula supplemented with lactoferrin (A7, B4, B7, B8, B14) were thus assigned to the control group and were not further specified.

Table 1. Feeding groups, blood groups of babies and sample codes for fecal samples used in this study and examined by AHC and CE-LIF-MS. *Formula supplemented with 0.4 g/dL GOS. n.d.: not defined.

Composition of feeding ***(% of feeding to total feeding)***	**n (babies)**	**AHC codes (Figure 1)** ***(blood group indicated in brackets)***
Breast milk (100%)	5	A1*(0)*, A4*(A)*, A9*(A)*, A11*(A)*, A13*(B)*
Breast milk (> 80%) and formula	6	A2*(n.d.)*, A3*(0)*, A5*(0)*, A8*(0)*, A10*(A)*, A12*(0)*
Breast milk (> 60%) and formula	2	A6*(A)*, A7*(A)*
Formula (> 45%) and breast milk	8	B5*(A)*, B6*(A)*, B7*(A)*, B8*(A)*, B10*(A)*, B12*(B)*, B13*(A)*, B14*(AB)*
Formula (100%)	6	B1*(A)*, B2*(0)*, B3*(B)*, B4*(0)*, B9*(A)*, B11*(0)*

HMO reference material and chemicals

2'Fucosyllactose, lacto-N-tetraose, lacto-N-fucopentaose I-III, lacto-N-difucohexaose, fucosyllacto-N-hexaose II and a mixture composed of 3'sialyllactose, 6'sialyllactose and 6'sialyl-N-acetyllactosamine were purchased from Dextra Laboratories (Reading, UK). All other chemicals used were of analytical grade.

Oligosaccharide extraction from feces

Oligosaccharides from feces were extracted according to Moro et al.[14] Watery fecal slurries (50 mg/mL) were kept overnight at 4 °C and were centrifuged (15 min, 3500g) and filtered through a 0.22 μm membrane. Fecal enzymes were inactivated by heat (5 min, 100 °C).

The carbohydrates extracted from fecal extracts were purified by solid phase extraction on graphitized carbon column cartridges (150 mg bed weight, 4 mL tube size; Alltech, Deerfield, Il) as described previously.[17,18] In short, the cartridges were washed with 80/20 (v/v) acetonitrile (ACN)/water containing 0.1% (v/v) trifluoroacetic acid (TFA) followed

by millipore water. After loading the sample extract onto the cartridge, monomers and lactose were largely removed by eluting with an aqueous 2% (v/v) ACN solution. The remaining carbohydrates on the cartridge were eluted with 40/60 (v/v) ACN/water containing 0.05% (v/v) TFA. The solution was dried under a stream of air and the dried sample was then rehydrated with millipore water.

Monosaccharide composition

The crude fecal extracts (not purified on graphitized carbon columns) were hydrolyzed with 2 M TFA for 1 h at 121 °C followed by evaporation and repeated washing with methanol. The hydrolysates were re-dissolved in millipore water and analyzed with HPAEC-PAD for their monosaccharide composition.[19] For the determination of sialic acid, mild sample hydrolysis with 0.1 M TFA for 40 min at 80 °C was applied[20] before HPAEC analysis[19].

CE-LIF

For CE-LIF analysis, the carbohydrates present in the fecal extracts were derivatized with the fluorescent 9-aminopyrene-1,4,6-trisulfonate (APTS) overnight at room temperature as reported elsewhere.[21,22] One nanomole xylose was added as internal standard and mobility marker. CE-LIF was performed on a ProteomeLab PA 800 characterization system (Beckman Coulter, Fullerton, CA), equipped with a laser induced fluorescence detector (LIF) (excitation: 488 nm, emission: 520 nm) (Beckman Coulter) and a polyvinyl alcohol-coated capillary (50 µm x 50.2 cm (Beckman Coulter), detector after 40 cm), kept at 25 °C. Samples were loaded hydrodynamically (4 s at 0.5 psi, representing approx. 14 nL sample solution) on the capillary. Separation was performed in the reversed polarity mode (30 kV, 20 min) in a 25 mM acetate buffer containing 0.4% polyethylene oxide provided in the ProteomeLab Carbohydrate Labeling and Analysis Kit (Beckman Coulter). Due to the low pK_a of the sialic acid residues (pK_a 2.6) and in order to provide the same buffer-pH as for CE-MS analysis, the separation buffer was adjusted to pH 2.4 by adding 1.2% (v/v) formic acid.[17] Peaks were integrated manually using Chromeleon software 6.8 (Dionex, Sunnyvale, CA).

CE-LIF-ESI-MS^n

CE-LIF-ESI-MS^n experiments were performed on a P/ACE™ System MDQ (Beckman Coulter) according to Albrecht et al.[17] For the fluorescent detection (excitation: 488 nm, emission: 520 nm), a Picometrics ZetaLIF discovery system was used (Picometrics, Toulouse, France). Separation in 0.3% (v/v) formic acid (pH 2.4) was performed on a fused

silica capillary (50 µm x 85 cm (Beckman Coulter), capillary window fitted with an ellipsoid for LIF detection after 60 cm) in reversed polarity mode (20 kV, 15 °C, 40 min). Samples were injected at 10 psi for 2 s. LIF signals were sent to Beckmann 32Karat software via a SSXL4002 converter (Agilent Technologies, Santa Clara, CA). The ESI-MS (LTQ ion trap, Thermo Fisher Scientific Inc., Waltham, MA) was operated in the negative mode using a spray voltage of 1.9 kV and an MS-capillary temperature of 190 °C. The end of the CE capillary was installed in front of the ESI source by leading it through a T-part designed in our laboratory[23] and provided the coaxial addition of a sheath liquid (50/50 isopropanol/water) at 2 µL/min. Mass spectra were acquired from m/z 300 to 2000. MS^n was performed in the data dependent mode using a window of 1 m/z and collision energy of 35%. For increasing the S/N ratio, ions of m/z 311, 314 and 329 were excluded from detection in MS^n experiments (mass exclusion list). MS^n data were interpreted using Xcalibur software 2.0.7 (Thermo).

Statistical analysis

The areas of the peaks in the CE-LIF electropherograms were normalized on the internal standard xylose. Agglomerative hierarchical clustering (AHC) was performed on the CE-LIF data points of all the fecal extracts using XLSTAT 2010.2.03 (Addinsoft, New York, NY). Data points starting from the migration time of the internal standard xylose (t = 4.1 min) were included in the statistical analysis. 3334 data points were subjected to AHC per sample electropherogram. The similarities for AHC were determined by the Pearson correlation coefficient and agglomerated using the unweighted pair group method. The results are presented as dendrogram, showing clustering patterns and the similarities among the clusters.

Results

Twenty-seven fecal samples from preterm babies were analysed. Thirteen babies received a feeding exclusively or predominantly composed of breast milk, with breast milk contributing to > 60% of the total feeding, which the baby received during the study period. The other fourteen babies received a feeding exclusively or predominantly composed of preterm infant formula (Frisolac prematuur), supplemented with 0.4 g/dL GOS or the same formula without GOS supplementation. The infant formula contributed to > 45% to the total feeding (**Table 1**).

Monosaccharide composition of the fecal extracts

For a first screening, the HMO-constituting sugars [fucose (Fuc), galactose (Gal), glucose (Glc), N-acetylglucosamine (GlcNAc), sialic acid (Neu5Ac)] and N-acetylgalactosamine (GalNAc) and mannose (Man), which are known to be supplementary present in the glycoconjugates of the human gastrointestinal mucosa,[7,20] were determined by high performance anion exchange chromatography (HPAEC) after acid hydrolysis of the fecal samples. The molar sugar proportions, presented relative to Gal = 1 in **Table 2A**, are grouped according to the main feeding the babies received. Similar molar proportions were obtained for samples analyzed in duplicate but highly diverse proportions were found for the different samples. Nevertheless, mean values were calculated. The high standard deviations (stdev) indicated variations in the fecal sugar compositions despite of the similar feeding received. Overall, the sugar proportions found for the predominantly breast-fed babies (group A) can be described as Fuc, GlcNAc > Glc >> GalNAc, Man, Neu5Ac, whereas for predominantly formula-fed babies (group B) it was GlcNAc > Fuc > Glc, GalNAc > Man, Neu5Ac. The mean proportions of GlcNAc, GalNAc, Man and Neu5Ac for formula-fed babies were higher than for breast-fed babies. Some sub-groups could be observed within the feeding groups. The predominantly breast-fed babies A4-A10 showed similar fecal relative proportions of Fuc, Glc and GlcNAc, but sub-divisions of the sample sub-groups were indicated by the different GalNAc-, Man- and Neu5Ac-proportions, which were low or even zero for samples A4, A9 and A10. Samples A1-A3 and A11-A13 showed highly diverse sugar proportions. The proportions of GalNAc, found for these specific breast-fed babies, were more comparable to what was found for formula-fed babies. The predominantly formula-fed babies B1-B2, who got GOS-supplemented formula, and B13-B14 could be distinguished within the formula-fed group due to the lowered proportions of all or several of the sugars Fuc, Glc, GlcNAc, GalNAc and Neu5Ac and may indicate an increased relative galactose content.

Table 2A. Sugar composition of fecal samples, presented relative to Gal = 1 and ordered according to the feeding groups of babies. Dashed lines indicate sample groups for which similarities in sugar composition were observed. Mean values and their standard deviations (mean±stdev) are presented for the feeding groups.

	Fuc	Gal	Glc	GlcNAc	GalNAc	Man	Neu5Ac
Exclusively or predominantly breast-fed babies (A)							
A1	0.09	1.00	0.34	0.78	0.19	0.09	0.21
A2	1.07	1.00	0.32	0.84	0.23	0.00	0.07
A3	0.56	1.00	0.51	0.50	0.04	0.08	0.08
A4	0.97	1.00	0.69	0.69	0.00	0.00	0.01
A5	0.82	1.00	0.51	0.58	0.05	0.04	0.01
A6	0.81	1.00	0.43	0.63	0.13	0.05	0.03
A7	0.85	1.00	0.42	0.57	0.09	0.05	0.03
A8	0.72	1.00	0.38	0.70	0.10	0.05	0.10
A9	0.90	1.00	0.55	0.53	0.03	0.00	0.01
A10	0.86	1.00	0.51	0.51	0.05	0.00	0.05
A11	0.55	1.00	0.65	0.85	0.58	0.19	0.00
A12	0.63	1.00	0.32	0.83	0.36	0.23	0.18
A13	0.44	1.00	0.42	1.21	0.20	0.08	0.07
mean±stdev	*0.71±0.25*	*1.00*	*0.46±0.11*	*0.71±0.19*	*0.16±0.15*	*0.07±0.07*	*0.06±0.06*
Exclusively or predominantly formula-fed babies (B)							
B1	0.41	1.00	0.34	0.60	0.19	0.48	0.08
B2	0.43	1.00	0.71	0.80	0.15	0.13	0.14
B3	0.50	1.00	0.57	0.73	0.28	0.17	0.10
B4	0.43	1.00	0.71	1.44	0.35	0.37	0.26
B5	0.66	1.00	0.11	1.05	0.20	0.13	0.07
B6	0.57	1.00	0.12	0.97	0.35	0.19	0.09
B7	0.75	1.00	0.23	0.96	0.50	0.06	0.16
B8	0.56	1.00	0.26	0.97	0.23	0.05	0.12
B9	0.75	1.00	0.27	1.11	0.31	0.08	0.10
B10	0.58	1.00	0.20	1.09	0.22	0.07	0.08
B11	0.48	1.00	0.84	1.16	0.28	0.18	0.25
B12	0.67	1.00	0.30	1.02	0.30	0.11	0.14
B13	0.29	1.00	0.30	0.46	0.21	0.14	0.16
B14	0.33	1.00	0.24	0.85	0.32	0.25	0.00
mean±stdev	*0.53±0.14*	*1.00*	*0.37±0.23*	*0.94±0.24*	*0.28±0.09*	*0.17±0.12*	*0.12±0.06*

Agglomerative hierarchical clustering (AHC) of CE-LIF oligosaccharide profiles

Sugar composition analysis indicated variations within feeding groups and the possible presence of sub-groups with high similarities. Lower standard deviations were obtained by averaging the sugar proportions obtained for A5-A7, A9-A10 and B3-B12 (**Table 2B**), compared to what was obtained for the total feeding group. These sample-groups thus represent sub-groups.

Table 2B. Mean±stdev of sugar composition of fecal sample-sub-groups A5-A7, A9-A10 and B3-B12, presented relative to Gal = 1.

	Fuc	**Gal**	**Glc**	**GlcNAc**	**GalNAc**	**Man**	**Neu5Ac**
A5-A7							
mean±stdev	*0.83±0.01*	*1.00*	*0.45±0.04*	*0.59±0.02*	*0.09±0.03*	*0.05±0.01*	*0.02±0.01*
A9-A10							
mean±stdev	*0.88±0.02*	*1.00*	*0.53±0.02*	*0.52±0.01*	*0.04±0.01*	*0.00±0.00*	*0.03±0.02*
B3-B12							
mean±stdev	*0.58±0.11*	*1.00*	*0.37±0.23*	*1.07±0.17*	*0.29±0.08*	*0.14±0.09*	*0.13±0.06*

The sugar building units can represent numerous, structurally different oligosaccharides. The fecal extracts were, therefore, analyzed for their oligosaccharide profiles by CE-LIF. AHC was then performed on the data points for all CE-LIF electropherograms in order to investigate the similarity between the samples. Two main clusters and one sample (A1), which did not match with any of the two clusters, are obtained (**Figure 1**). The first cluster was composed of samples from exclusively and predominantly breast-fed babies (A2-A10). Samples A2 and A3 showed low similarity to samples A4-A10, which could be further divided into the sub-clusters A4/A5-A7 and A8/A9-A10.

The second cluster was mainly composed of samples from exclusively and predominantly formula-fed babies. Three breast-fed babies (samples A11-A13) were also located in the second cluster. Several sub-clusters were observed for feces from the formula-fed group, with the most homogenous cluster being composed of samples B4-B11. In line with the exceptional sugar proportions observed for babies A1-A3, A11-A13, B1-B2 and B13-B14, low similarities to the other samples of the same feeding group were found with AHC for these samples as based on the CE-LIF profiles.

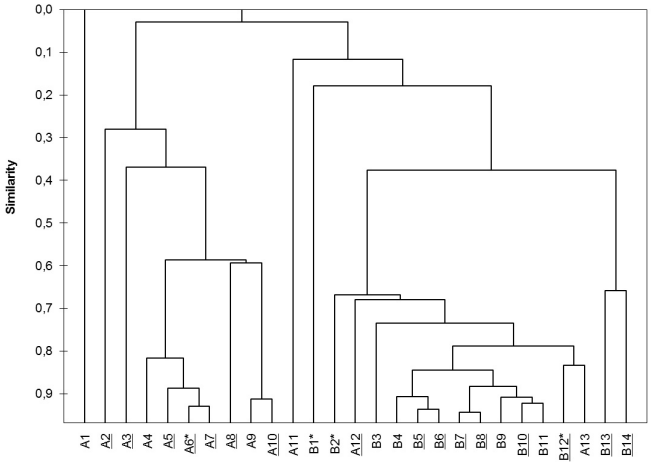

Figure 1. AHC dendrogram of CE-LIF data sets from the 27 fecal samples analyzed. A: exclusively breast-fed babies. A: predominantly breast-fed babies. B: exclusively formula-fed babies. B: predominantly formula-fed babies. *: supplementation of formula with 0.4 g/dL GOS. See **Table 1** for the assignment of the sample codes to the respective feeding group.

Characterization of the CE-LIF oligosaccharide profiles

Breast-fed babies showing fecal oligosaccharide profiles similar to breast milk

Fucosyllactose (FL), difucosyllactose (DF-L), lacto-N-neo-tetraose(LnNT)-isomers, lacto-N-tetraose(LNT), lacto-N-fucopentaose(LNFP)-isomers, lacto-N-difuco-hexaose(LNDFH)-isomers, fucosyllacto-N-hexaose(F-LNH)-isomers, difucosyllacto-N-hexaose(DF-LNH)-isomers and trifucosyllacto-N-hexaose(TF-LNH)-isomers are HMOs and their degradation products, which were found to be present in individual-dependent proportions in the feces of two exclusively and five predominantely breast-fed babies (A4-A10), corresponding to what was previously reported.[17] Representative electropherograms for these sub-groups are shown in **Figure 2**. Peaks, which have been annotated by CE-LIF-MS^n and/or using standards are listed in **Table 3**. The oligosaccharide profiles could be discriminated according to their respective secretor-(A4-A8) or non-secretor-(A9, A10) fingerprint. The (α1,2)-fucosylated HMOs, such as DF-L (Fuc-(α1,2)-Gal-(β1,4)-[Fuc-(α1,3)]-Glc; peak 7), LNFP-I (Fuc-(α1,2)-Gal-(β1,3)-GlcNAc-(β1,3)-Gal-(β1,4)-Glc; peak 18) or LNDFH-I (Fuc-(α1,2)-Gal-(β1,3)-[Fuc-(α1,4)]-GlcNAc-(β1,3)-Gal-(β1,4)-Glc; peak 20) are characteristic for Le(a-b+)[9]-secretor-milk and were present in the fecal oligosaccharide profiles A4-A8. Two representative examples (A4, A7) are shown in **Figure 2**. Besides the

well-known HMO-peaks and their degradation products[17] (**Figure 2**-A4) also an unknown structure of m/z 328 (**Figure 2**-A7, peak 6) and two unknown structures of m/z 377 (**Figure 2**-A7, peaks 12 and 13) could be present in the secretor-profiles of breast–fed babies, as exemplified by sample A7 in **Figure 2**. Strikingly, m/z 328 and m/z 377-I were never present in the HMO-profiles from exclusively breast-fed babies, but in some cases present in the profiles of mixed-fed babies.

Secretor-milk-specific HMOs containing (α1,2)-fucose residues were absent in samples A9 and A10. This phenomenon is typical for non-secretor milk Le(a+b-).[5,9] CE-MS analysis indicated isomeric structures for F-LNH and DF-LNH in secretor-profiles, visible as shoulders (**Figure 2**, peaks 24 and 25) which thus may contain (α1,2)-fucose linkages. Only single-shaped peaks were observed for F-LNH and DF-LNH in these non-secretor profiles, as exemplified by sample A10 in **Figure 2**. On the other hand, LNFP II and two not further specified LNDFH isomers contributed with a higher proportion to the total oligosaccharide content in non-secretor profiles (**Figure 2**-A10, peaks 15 and 21,22).

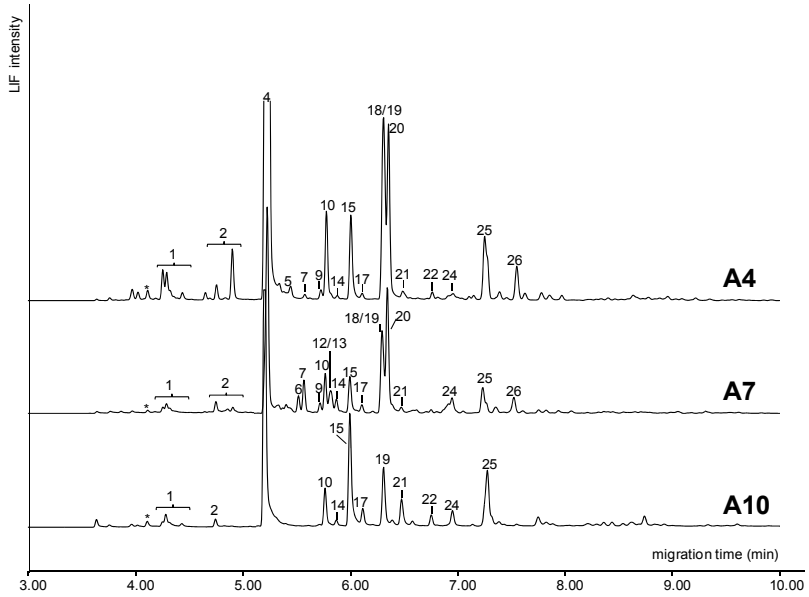

Figure 2. CE-LIF electropherograms of APTS-derivatized oligosaccharides extracted from baby feces. (A4) HMO secretor profile of exclusively breast-fed baby A4. (A7) HMO secretor profile including unknown oligosaccharides of mixed-fed baby A7. (A10) HMO non-secretor profile of mixed-fed baby A10. See **Table 3** for peak annotation.*internal standard xylose.

Table 3. Structural composition of oligosaccharides detected in CE-LIF electropherograms (**Figures 2-4**). The oligosaccharides are attached to an APTS-molecule and are present in their threefold negative charge in the ESI-MS profiles.

Peaks (Figures 2-4)	m/z (APTS 3-)	hexose	GlcNAc	fucose	Neu5Ac	name (abbreviation)	Peaks (Figures 2-4)	m/z (APTS 3-)	hexose	GlcNAc	fucose	Neu5Ac	name (abbreviation)
*						xylose ISTD	15	431	3	1	1	0	LNFP II
1						monomers	16	576	3	1	0	2	DS-LNT
2						dimers	17	479	3	1	0	1	S-LNT*
3	357	2	0	0	1	SL*	18	431	3	1	1	0	LNFP I
4	309	2	0	1	0	FL*	19	431	3	1	1	0	LNFP III
5	382	3	1	0	0	LnNT Y	20	479	3	1	2	0	LNDFH I
6	328	2	1‘	0	0	m/z 328	21	479	3	1	2	0	LNDFH*
7	358	2	0	2	0	DF-L	22	479	3	1	2	0	LNDFH*
8	479	3	1	0	1	S-LNT*	23	498	3	2	1‘	0	m/z 498
9	382	3	1	0	0	LnNT	24	552	4	2	1	0	F-LNH*
10	431	3	1	1	0	LNFP Y	25	601	4	2	2	0	DF-LNH*
11	479	3	1	0	1	S-LNT*	26	650	4	2	3	0	TF-LNH*
12	377	2	1‘	1	0	m/z 377-I	III[a-d]	314	3	0	0	0	Hexo-trioses*
13	377	2	1‘	1	0	m/z 377-II	IV[a-c]	368	4	0	0	0	Hexo-tetraoses*
14	382	3	1	0	0	LNT	**: isomers not further specified; ‘: GlcNAc=HexNAc*						

Breast-fed babies showing fecal oligosaccharide profiles not similar to breast milk

For three exclusively and three partially breast-fed babies (samples A1-A3, A11-A13), which stood out in the clustering profile due to their low similarity, fecal oligosaccharide profiles were found, that did not depict a characteristic HMO pattern with CE-LIF (**Figure 3**). Exclusively acidic HMOs predominated in sample A1 (**Figure 3**-A1). In human milk, acidic HMOs are present in a tenfold lower concentration compared to neutral oligosaccharides.[7] SL, three sialyllacto-N-tetraose (S-LNT)-isomers (**Figure 3**-A1, peaks 8 and 11,17) and one disialyllacto-N-tetraose (DS-LNT)-isomer (**Figure 3**-A1, peak 16) were found in this oligosaccharide profile by CE-LIF-MS analysis. Their origin from breast milk

was confirmed by their presence in the acidic fraction obtained by anion exchange chromatography of HMOs (results not shown). Remarkably, in sample A12 solely SL was found (results not shown).

Only a few, but dominant HMOs were found for A2 and A3, which were LNFP II and LNDFH I as shown for A2 (**Figure 3**-A2, peaks15 and 20). An unknown structure of m/z 377 was present as well (**Figure 3**-A2, peak 12), which corresponded to peak 12 in **Figure 2**-A7, assigned as 377-I. No oligosaccharides were found to be present in the profile of sample A13 (results not shown).

Interestingly, an oligosaccharide, previously assigned as 377-II in the fecal profiles of breast-fed babies showing secretor-HMO-profiles (**Figure 2**-A7), was found to prevail in the profile of the breast-fed baby A11 (**Figure 3**-A11, peak 13), together with a structure of m/z 498 (**Figure 3**, A11, peak 23). Besides the presence of m/z 377-II and m/z 498, the oligosaccharide level found for sample A11 was quite low.

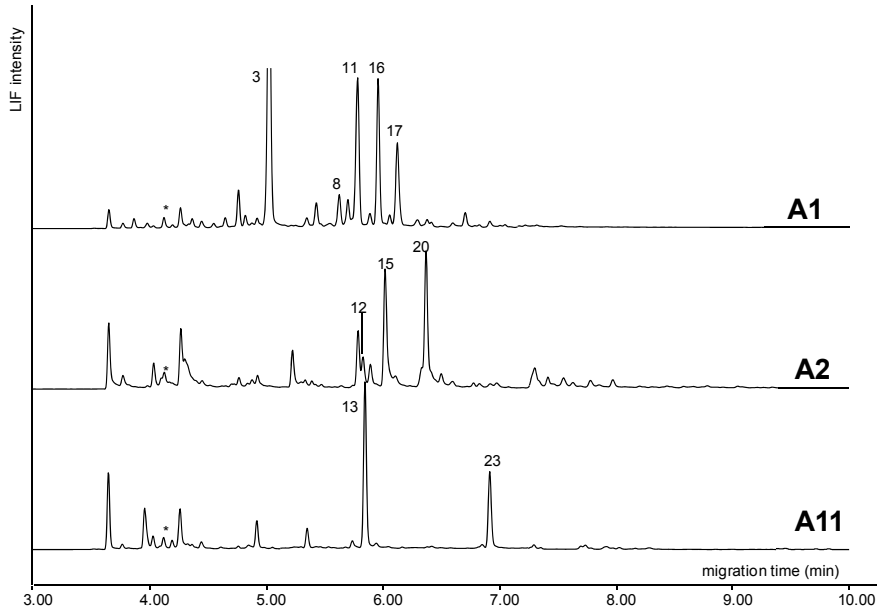

Figure 3. CE-LIF electropherograms of APTS-derivatized oligosaccharides extracted from baby feces. (A1) Exclusively acidic HMOs of exclusively breast-fed baby A1. (A2) Accumulation of LNFP II/LNDFH I of mixed-fed baby A2. (A11) Accumulation of structures with m/z 377-II/498 of exclusively breast-fed baby A11. See **Table 3** for peak annotation.*internal standard xylose.

Fecal oligosaccharide profiles of formula-fed babies

Similar fecal oligosaccharide profiles were obtained for ten formula-fed or predominantly formula-fed babies (B2, B4-B12), irrespective of having received formula-feeding supplemented with 0.4 g/dL GOS. Representative electropherograms are shown in **Figure 4** for GOS-fed baby B12 and formula-fed baby B5. By means of CE-MS, the two main oligosaccharide peak-clusters were identified as hexose-trimers and –tetramers (**Figure 4**, peaks III^{a-d} and IV^{a-c}, respectively). The oligosaccharide profiles of samples B1, B3, B13 and B14 could be distinguished from the other peak profiles of formula-fed babies due to the abundant presence of an unknown structure of m/z 328, as represented by baby B3, (**Figure 4**-B3, peak 6), which was as well present as a minor component in the oligosaccharide profiles of predominantly breast-fed babies showing a secretor-profile (**Figure 2**-A7, peak 6).

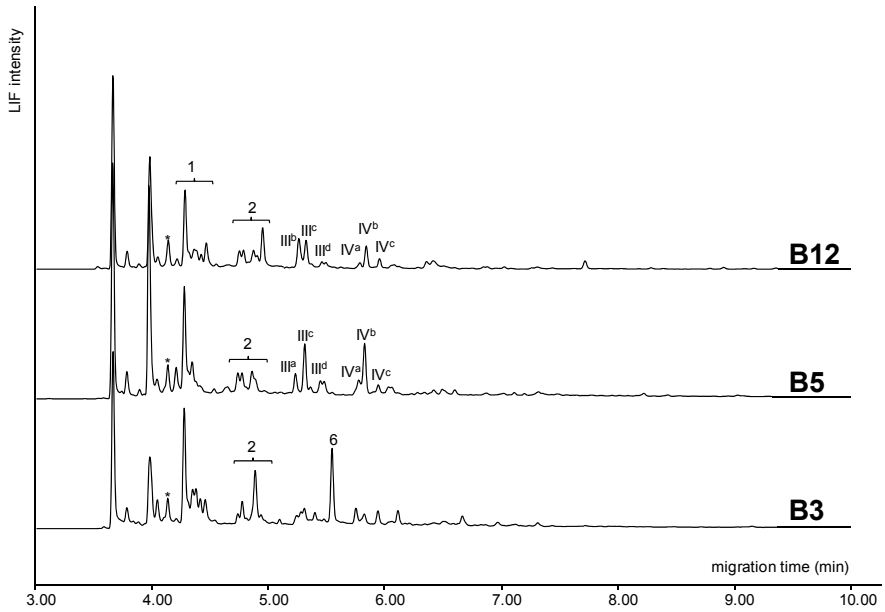

Figure 4. CE-LIF electropherograms of APTS-derivatized oligosaccharides extracted from baby feces. (B12) mixed-fed baby B12 (GOS-supplemented formula). (B5) mixed-fed baby B5 (control formula). (B3) exclusively formula-fed baby B3 (control) showing abundant oligosaccharide of m/z 328.
See **Table 3** for peak annotation.*internal standard xylose.

Annotation of the unknown peaks m/z 377-I/-II, 328 and 498

As described above, four unknown compounds of the masses m/z 328, m/z 377 (isomers I and II) and m/z 498 were observed in several fecal extracts. Although having the same m/z values and migrating closely together in CE-LIF, the MS fragmentation patterns of m/z 377-I (found for several partially breast-fed babies (**Figure 2** and **Figure 3**, peak 12)) and m/z 377-II (found for several exclusively and partially breast-fed babies (**Figure 2** and **Figure 3**, peak 13)) showed marked differences (**Figure 5A**).

The compound m/z 377-I was annotated as Hex-[Fuc]-HexNAc-Hex-APTS (**Figure 5A**), as supported by its mass fragments Hex-APTS, HexNAc-Hex-APTS, Fuc-HexNAc-Hex-APTS and Hex-HexNAc-Hex-APTS (**Table 4**).

For the compound 377-II, the presence of the fragments Hex-APTS, Hex-Hex-APTS, Fuc-Hex-Hex-APTS and HexNAc-Hex-Hex-APTS, as summarized in **Table 4**, led to an annotation of HexNAc-[Fuc]-Hex-Hex-APTS (**Figure 5A**).

The fragmentation pattern of m/z 328 (found as minor component for several partially breast-fed babies and as dominant peak for several formula-fed babies (**Figure 2** and **Figure 4**, peak 6)) lacked the fragment Hex-APTS, although showing an abundant Hex-Hex-APTS fragment (**Figure 5B** and **Table 4**). This characteristic fragmentation was also found for a mixture of the single-substituted, Hex-Hex-based structures 3'SL (Neu5Ac-(α2,3)-Gal-(β1,4)-Glc) and 6'SL (Neu5Ac-(α2,6)-Gal-(β1,4)-Glc) (data not shown). HexNAc-Hex-Hex-APTS was thus annotated for m/z 328.

The MS fragments, which resulted for m/z 498 (**Figure 5B**) (breast-fed baby A11 (**Figure 3**-A11, peak 23)) are summarized in **Table 4**. The presence of fragments Fuc-Hex-HexNAc-Hex-Hex-APTS and HexNAc-Hex-HexNAc-Hex-Hex-APTS provide complementary information on the substitution of the terminal hexose unit with Fuc as well as HexNAc. This led to the annotation of HexNAc-[Fuc]-Hex-HexNAc-Hex-Hex-APTS for m/z 498. Unfortunately, the definitive identification of the new structures by NMR was not possible due to the low sample amounts available.

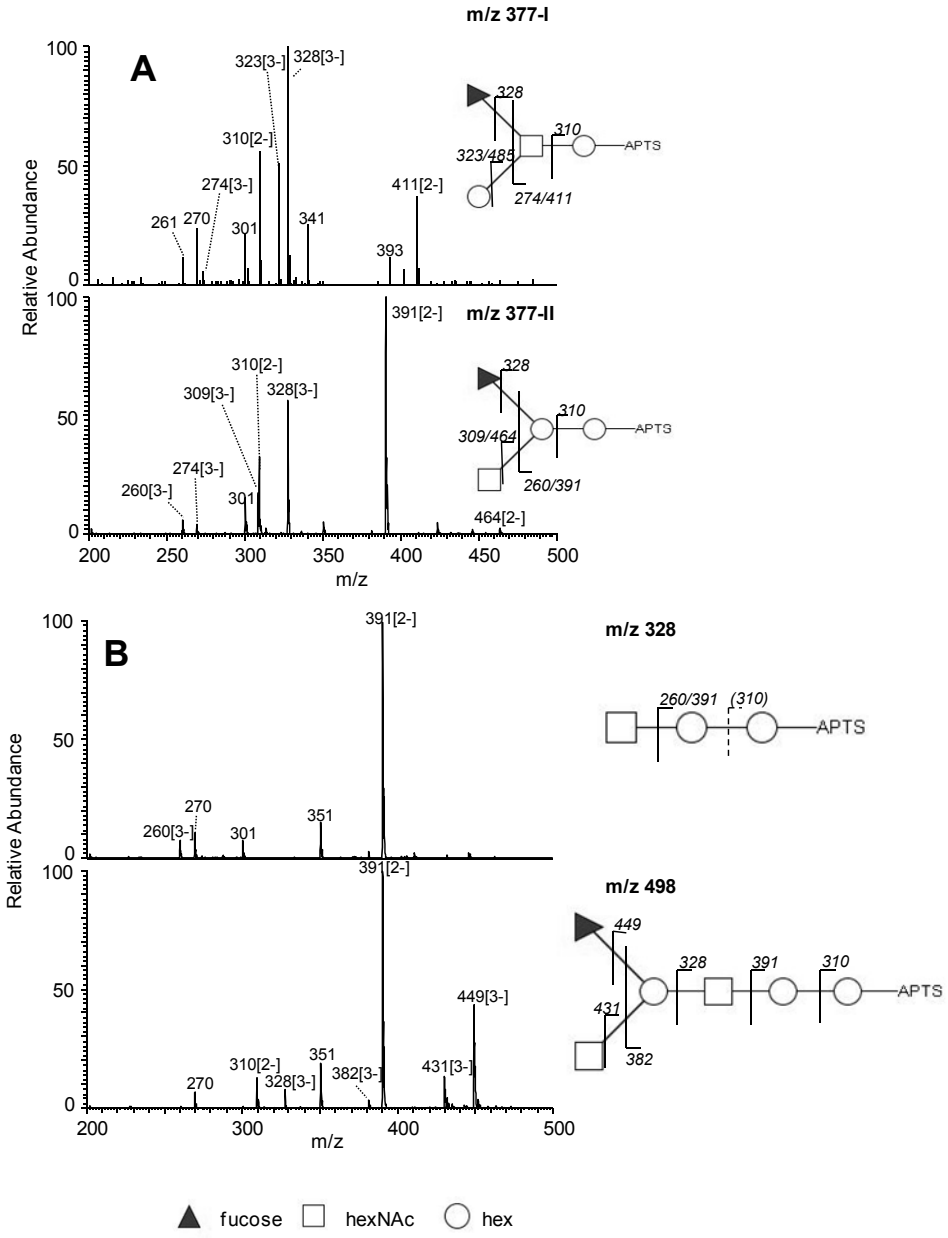

Figure 5. MS^2 fragmentation patterns and structural composition of (A) m/z 377-I/-II. (B) m/z 328 and m/z 498. [2-] [3-]: charge state of APTS-molecule.

Table 4. Presence and annotation of MS^2 fragments of m/z 328 (**Table 3**, peak 6), 377-I.-II (**Table 3**, peaks 12 and 13) and 498 (**Table 3**, peak 23).

		m/z 328	m/z 377-I	m/z 377-II	m/z 498
m/z fragment pair	**structural composition**				
m/z 207[3-]/310[2-]	Hex-APTS	-	+	+	+
m/z 255[3-]/383[2-]	Fuc-Hex-APTS	-	-	-	-
m/z 260[3-]/391[2-]	Hex-Hex-APTS	+	-	+	+
m/z 274[3-]/411[2-]	HexNAc-Hex-APTS	-	+	-	-
m/z 309[3-]/464[2-]	Fuc-Hex-Hex-APTS	-	-	+	-
m/z 323[3-]/485[2-]	Fuc-HexNAc-Hex-APTS	-	+	-	-
m/z 328[3-]/492[2-]	HexNAc-Hex-Hex-APTS/ Hex-HexNAc-Hex-APTS	-	+	+	+
m/z 382[3-]/574[2-]	Hex-HexNAc-Hex-Hex-APTS	-	-	-	+
m/z 431[3-]/647[2-]	Fuc-Hex-HexNAc-Hex-Hex-APTS	-	-	-	+
m/z 449[3-]/674[2-]	HexNAc-Hex-HexNAc-Hex-Hex-APTS	-	-	-	+

Discussion

Breast-fed babies

Three out of the five exclusively breast-fed babies showed the absence of a characteristic HMO profile after approximately two months of life. On the other hand, for the majority of predominantly, but not exclusively, breast-fed babies (five out of eight), fecal HMO-profiles were obtained. The individual-dependent, genetically determined HMO-profiles of secretor- [Le(a-b+)]- and non-secretor- [Le(a+b-)] breast milk[5] were mirrored in these fecal HMO-profiles and resulted in different degrees of similarities among the samples.

Mixed feeding can thus delay an advanced HMO-degradation and metabolization at two months after birth. A lower adaptation to the feeding and a microbiota not exclusively specified in the degradation and metabolization of HMOs for these babies was indicated.

The presence of exclusively acidic oligosaccharides, such as SL, S-LNT, DS-LNT-isomers, as observed in the feces of the breast-fed baby A1 (**Figure 3**-A1), the accumulation of specific HMOs (**Figure 3**-A2) or unknown structures (**Figure 3**-A11) and the absence of specific oligosaccharides (sample A13) can thus be interpreted as biomarkers for an advanced gastrointestinal and gastromicrobial development and explain the diversity found for these samples with CE-LIF, expressed by their low similarities in AHC. The increased

presence of GlcNAc and the non-HMO sugars GalNAc and Man, which are known to be present in the glycoconjugates of the human gastrointestinal mucosa,[20] may indicates an involvement of mucin oligosaccharides in the gastrointestinal re-design of diet-related HMOs. To understand the gastrointestinal bioconversion of HMOs, more study is needed.

Blood group A-specific metabolization products in the feces of breast-fed babies

Breast-fed babies, for whom HexNAc-[Fuc]-Hex-Hex-APTS (m/z 377-II) and HexNAc-[Fuc]-Hex-HexNAc-Hex-Hex-APTS (m/z 498) were annotated in their fecal oligosaccharide profiles, all had blood group A (**Table 1**), as was exemplified by babies A7 and A11 in **Figure 2** and **Figure 3**. GalNAc-(α1,3)-[Fuc-(α1,2)]-Gal is the antigenic determinant structure of blood group A,[24] which corresponds to the non-reducing HexNAc-[Fuc]-Hex-group found for m/z 377-II and m/z 498 and is further supported by the increased presence of the non-HMO-sugar GalNAc in the respective samples. The oligosaccharides GalNAc-(α1,3)-[Fuc-(α1,2)]-Gal-(β1,4)-Glc (corresponding to m/z 377-II) and GalNAc-(α1,3)-[Fuc-(α1,2)]-Gal-(β1,3)-GlcNAc-(β1,3)-Gal-(β1,4)-Glc (corresponding to m/z 498), which are unknown for human milk, were previously identified in the feces of a single, eight weeks old, prematurely born breast-fed blood group A-secretor baby.[15,16] Blood group characteristic oligosaccharides are not expected to occur in human milk.[15,25] However, the occurrence of trace amounts has previously been suggested but the structural composition of these oligosaccharides has not been proven.[26] The gastrointestinal mucosa is known to carry a broad range of cell-surface glycoconjugates, which are built up from Gal, Glc, GalNAc, GlcNAc, Man, Fuc and Neu5Ac.[20] The antigenic determinant structures of these glycoconjugates are, among other factors, determined by the host's ABO histo-blood group type.[5] A sole microbial degradation of these structures from mucus epitopes[27,28] was not expected. Our results indicate a gastrointestinal bioconversion of the HMOs, with an involvement of these blood group specific mucus epitopes. This is similar to what has been hypothesized in previous studies in view of the high yield of these fecal oligosaccharides detected in the respective sample.[15,16] Our assumption of bioconversion is further supported by the fact that neither m/z 377-II nor m/z 498 was detected in the feces of any of the formula-fed babies.

Blood-group specific oligosaccharides can be absent in the feces of breast-fed blood group A secretor babies (**Figure 2**-A4), they can be present additionally to HMOs (**Figure 2**-A7, peak 13) or they can be present as exclusive components (**Figure 3**-A11, peaks 13 and 23). Blood group A non-secretor babies (A9 and A10; **Figure 2**-A10) lacked the presence of theses oligosaccharides, due to the presence of a terminal (α1,2)-Fuc-

linkage for these structures.[15,29] The extent to which blood group specific oligosaccharides are present in feces depends on the secretor status of the baby and may depend on the presence of secretor-breast milk and on the state of development and adaptation of the gastrointestinal microbiota to the HMOs provided.

Presence of unknown oligosaccharide m/z 377-I in the feces of breast-fed babies

Hex-[Fuc]-HexNAc-Hex-APTS (m/z 377-I), which was found for several predominantly but not exclusively, breast-fed babies (**Figure 2**-A7 and **Figure 3**-A2) seems to be dependent on the gastrointestinal presence of HMOs, but does not depend on the respective blood group (**Table 1**). The compound m/z 377-I may result from the gastrointestinal degradation of the *para*-isomers of higher-Mw HMOs, which carry a terminal Gal-(β1,3)-GlcNAc-(β1,3)-Gal-(β1,4)-unit (e.g. F-*para*-LNH I, Gal-(β1,3)-GlcNAc-(β1,3)-Gal-(β1,4)-[Fuc-(α1,3)]-GlcNAc-(β1,3)-Gal-(β1,4)-Glc).

Another possibility is the origin from gastrointestinal mucin-type-O-glycans, for which repeats of Gal-GlcNAc-units were described.[30] The presence of intestinal bacteria-/enzyme-sets able to degrade these structures for these HMO-fed babies has to be assumed in that case.

Formula-fed babies

Ten out of fourteen fecal extracts from exclusively and predominantly formula-fed babies showed similar fecal CE-LIF patterns, independent of feeding a preterm formula without GOS or a preterm formula supplemented with 0.4 g/dL GOS, as was represented in **Figure 4** by samples B12 and B5. The hexo-trioses and hexo-tetraoses, which are present in a low abundance in these fecal profiles, as well as the numerous unidentified peaks were, therefore, assumed to origin from the gastrointestinal mucus layer and not from the supplemented GOS, as also indicated by the high GlcNAc-, GalNAc- and Man-proportions found for these samples (**Table 2A** and **Table 2B**). A higher contribution of monomeric Gal was expected for the exclusively GOS-fed babies (B1, B2), due to the low proportions of the other sugars determined in these two samples relative to Gal (**Table 2A**). However, no conclusions could be drawn from the CE-LIF profiles due to the fact that monomeric sugars were partly removed during sample-preparation for CE-LIF-MS. For fecal samples originating from 24 days old preterm born babies who received formula supplemented with 0.5 g/dL, we found hexose-oligosaccharides related to the supplemented GOS (unpublished results). In addition, GOS and fructooligosaccharides (FOS) were recovered in the feces of one months old babies, who received a formula supplemented with a mixture of 0.8 g/dL

GOS/FOS (9:1) by Moro et al.[14] In the present study, the fecal samples were taken at the age of two months and the fecal flora may be more developed and adapted to GOS than in the study of Moro et al[14] and able to completely degrade GOS. The fecal recovery of supplemented oligosaccharides to infant formula may thus depend on their concentration and on the stage of gastrointestinal development of the baby.

Presence of unknown oligosaccharide m/z 328 in the feces of breast- and formula-fed babies

HexNAc-Hex-Hex-APTS (m/z 328) was found in minor proportions for several mixed-fed babies showing secretor-HMO profiles and as major component for the four formula-fed babies B1, B3, B13, B14 (exemplified by **Figure 2**-A7 and **Figure 4**-B3). The presence of HexNAc-Hex-Hex is not dependent on the gastrointestinal presence of HMOs or the type of infant formula. Furthermore, this unknown structure was not present in any of the fecal profiles of exclusively breast-fed babies and babies showing non-secretor HMO-profiles. A mucosal origin is assumed for HexNAc-Hex-Hex and the pronounced expression of HexNAc-Hex-Hex for formula-fed babies may indicate an enhanced mucus-degradation. This may point to a lack of intestinal feeding and thus to the need of the microbiota to degrade sugar units from the gastrointestinal mucus layer. The observed dependency of HexNAc-Hex-Hex on the presence of secretor-breast milk in case of breast feeding may implicate the involvement of (α1,2)-fucosylation prior to the gastrointestinal release of this compound.

Concluding remarks

Overall, the fecal recovery of oligosaccharides from human milk and formula seems to be dependent on the adaptation of the gastrointestinal microbiota to the respective feeding and the concentration of oligosaccharides herein. The host's genotype plays also an important role in the gastrointestinal oligosaccharide metabolisation and was identified as a determinant factor responsible for the presence of the blood group characteristic oligosaccharides GalNAc-[Fuc]-Gal-Glc, GalNAc-[Fuc]-Gal-GlcNAc-Gal-Glc, and may be involved in the formation of the structures annotated as Hex-[Fuc]-HexNAc-Hex and HexNAc-Hex-Hex in the feces.

Fecal oligosaccharide profiles contain valuable information. To understand the gastrointestinal bioconversion of HMOs, more study is needed.

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References

1. Cummings, J.H.; Macfarlane, G.T. Role of intestinal bacteria in nutrient metabolism. *Clin. Nutr.*, **1997**, *16*, 3-11.
2. Lo Cascio, R.G.; Ninonuevo, M.R.; Freeman, S.L.; Sela, D.A.; Grimm, R.; Lebrilla, C.B.; Mills, D.A.; German, J.B. Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. *J. Agric. Food Chem.*, **2007**, *55*, 8914-8919.
3. Lu, L.; Walker, W.A. Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. *Am. J. Clin. Nutr.*, **2001**, *73*, 1124S-1130S.
4. Moro, G.; Minoli, I.; Mosca, M.; Fanaro, S.; Jelinek, J.; Stahl, B.; Boehm, G. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. *J. Pediatr. Gastroenterol. Nutr.*, **2002**, *34*, 291-295.
5. Newburg, D.S.; Ruiz-Palacios, G.M.; Altaye, M.; Chaturvedi, P.; Meinzen-Derr, J.; Guerrero, M.d.L.; Morrow, A.L. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology*, **2004**, *14*, 253-263.
6. Vanderhoof, J.A.; Young, R.J. Role of probiotics in the management of patients with food allergy. *Ann. Allergy Asthma Immunol.* , **2003**, *90*, 99-103.
7. Kunz, C.; Rudloff, S.; Baier, W.; Klein, N.; Strobel, S. Oligosaccharides in human milk: Structural, functional, and metabolic aspects. *Ann. Rev. Nutr.*, **2000**, *20*, 699-722.
8. Viverge, D.; Grimmonprez, L.; Cassanas, G.; Bardet, L.; Bonnet, H.; Solère, M. Variations of lactose and oligosaccharides in milk from women of blood types secretor A or H, secretor Lewis, and secretor H/nonsecretor Lewis during the course of lactation. *Ann. Nutr. Metab.*, **1985**, *29*, 1-11.
9. Thurl, S.; Henker, J.; Siegel, M.; Tovar, K.; Sawatzki, G. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconjugate J.*, **1997**, *14*, 795-799.
10. Fanaro, S.; Boehm, G.; Garssen, J.; Knol, J.; Mosca, F.; Stahl, B.; Vigi, V. Galacto-oligosaccharides and long-chain fructo-oligosaccharides as prebiotics in infant formulas: A review. *Acta Paediatr.*, **2005**, *94*, 22-26.
11. Sangild, P.T. Gut responses to enteral nutrition in preterm infants and animals. *Exp. Biol. Med.*, **2006**, *231*, 1695-1711.
12. Neu, J. Gastrointestinal maturation and implications for infant feeding. *Early Hum. Dev.*, **2007**, *83*, 767-775.
13. Boehm, G.; Lidestri, M.; Casetta, P.; Jelinek, J.; Negretti, F.; Stahl, B.; Marini, A. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal Bifidobacteria in preterm infants. *Arch. Dis. Child.*, **2002**, *86*, 178-181.
14. Moro, G.E.; Stahl, B.; Fanaro, S.; Jelinek, J.; Boehm, G.; Coppa, G.V. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants. *Acta Paediatr.*, **2005**, *94*, 27-30.
15. Sabharwal, H.; Nilsson, B.; Chester, M.A.; Sjöblad, S.; Lundblad, A. Blood group specific oligosaccharides from faeces of a blood group A breast-fed infant. *Mol. Immunol.*, **1984**, *21*, 1105-1112.
16. Sabharwal, H.; Nilsson, B.; Grönberg, G.; Chester, M.A.; Dakour, J.; Sjöblad, S.; Lundblad, A. Oligosaccharides from feces of preterm infants fed on breast milk. *Arch. Biochem. Biophys.*, **1988**, *265*, 390-406.
17. Albrecht, S.; Schols, H.A.; van den Heuvel, E.G.H.M.; Voragen, A.G.J.; Gruppen, H. CE-LIF-MS^n profiling of oligosaccharides in human milk and feces of breast-fed babies. *Electrophoresis*, **2010**, *31*, 1264-1273.

18. Ninonuevo, M.R.; Park, Y.; Yin, H.; Zhang, J.; Ward, R.E.; Clowers, B.H.; German, J.B.; Freeman, S.L.; Killeen, K.; Grimm, R.; Lebrilla, C.B. A strategy for annotating the human milk glycome. *J. Agric. Food Chem.*, **2006**, *54*, 7471-7480.
19. Sengkhamparn, N.; Verhoef, R.; Schols, H.A.; Sajjaanantakul, T.; Voragen, A.G.J. Characterisation of cell wall polysaccharides from okra (*Abelmoschus esculentus* (L.) Moench). *Carbohydr. Res.*, **2009**, *344*, 1824-1832.
20. Herrmann, A.; Davies, J.R.; Lindell, G.; Martensson, S.; Packer, N.H.; Swallow, D.M.; Carlstedt, I. Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. *J. Biol. Chem.*, **1999**, *274*, 15828-15836.
21. Albrecht, S.; van Muiswinkel, G.C.J.; Schols, H.A.; Voragen, A.G.J.; Gruppen, H. Introducing capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) for the characterization of konjac glucomannan oligosaccharides and their *in vitro* fermentation behavior. *J. Agric. Food Chem.*, **2009**, *57*, 3867-3876.
22. Evangelista, R.A.; Liu, M.-S.; Chen, F.-T.A. Characterization of 9-aminopyrene-1,4,6-trisulfonate derivatized sugars by capillary electrophoresis with laser-induced fluorescence detection. *Anal. Chem.*, **1995**, *67*, 2239-2245.
23. Hilz, H.; de Jong, L.E.; Kabel, M.A.; Schols, H.A.; Voragen, A.G.J. A comparison of liquid chromatography, capillary electrophoresis, and mass spectrometry methods to determine xyloglucan structures in black currants. *J. Chromatogr. A.*, **2006**, *1133*, 275-286.
24. Schenkel-Brunner, H. Blood group antigens. In *Comprehensive glycoscience. From chemistry to system biology; vol.3*; Kamerling, J.P., Boons, G.J., Lee, Y.C., Suzuki, A., Taniguchi, N.,Voragen, A.G.J., Eds; Elsevier: Oxford, UK, **2007**; pp 343-372.
25. Urashima, T.; Asakuma, S.; Messer, M. Milk oligosaccharides. In *Comprehensive glycoscience. From chemistry to system biology; vol.4*; Kamerling, J.P., Boons, G.J., Lee, Y.C., Suzuki, A., Taniguchi, N.,Voragen, A.G.J., Eds; Elsevier: Oxford, **2007**; pp 695-722.
26. Kobata, A.; Ginsburg, V. Uridine diphosphate-N-acetyl-d-galactosamine:D-galactose α-3-N-acetyl-d-galactosaminyltransferase, a product of the gene that determines blood type A in man. *J. Biol. Chem.*, **1970**, *245*, 1484-1490.
27. Hoskins, L.C.; Agustines, M.; McKee, W.B.; Boulding, E.T.; Kriaris, M.; Miedermeyer, G. Mucin degradation in human colon ecosystems; isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. *J. Clin. Invest.*, **1985**, *75*, 944-953.
28. Hoskins, L.C.; Boulding, E.T. Degradation of blood group antigens in human colon ecosystems; part 1: *In-vitro* production of ABH blood group degrading enzymes by enteric bacteria. *J. Clin. Invest.*, **1976**, *57*, 63-73.
29. Kobata, A. Structures and functions of the sugar chains of glycoproteins. *Eur. J. Biochem.*, **1992**, *209*, 483-501.
30. Brockhausen, L. Biosynthesis of mucin-type o-glycans. In *Comprehensive glycoscience. From chemistry to system biology; vol.3*; Kamerling, J.P., Boons, G.J., Lee, Y.C., Suzuki, A., Taniguchi, N.,Voragen, A.G.J., Eds; Elsevier: Oxford, **2007**; pp 33-59.

Chapter 5

Occurrence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk

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Abstract

The characterization of oligosaccharides in the feces of breast-fed babies is a valuable tool for monitoring the gastrointestinal fate of human milk oligosaccharides (HMOs). In the present study we monitored fecal oligosaccharide profiles together with the HMO-profiles of the respective breast milks up to six months postpartum, by means of capillary electrophoresis with laser induced fluorescence detection and mass spectrometry. Eleven mother/child pairs were included. Mother's Secretor- and Lewis-type included all combinations [Le(a-b+), Le(a+b-), Le(a-b-)]. The fecal HMO-profiles in the first few months of life are either predominantly composed of neutral or acidic HMOs and are possibly effected by the HMO-fingerprint in the respective breast milk. Independent of the initial presence of acidic or neutral fecal HMOs, a gradual change to blood group specific oligosaccharides was observed. Their presence pointed to a gastrointestinal degradation of the feeding-related HMOs, followed by conjugation with blood group specific antigenic determinants present in the gastrointestinal mucus layer. Eleven of these "hybride"-oligosaccharides were annotated in this study. When solid food was introduced, no HMOs and their degradation- and metabolization products were recovered in the fecal samples.

Introduction

Exclusive breast feeding during the first six months after birth is officially recommended by the WHO[1] and efforts are made to support and promote breast feeding among mothers worldwide.[2,3] Important components of breast milk are human milk oligosaccharides (HMOs), present to 3-19 g/L.[4,5] They are composed of a core of galactose, glucose, N-acetyl-glucosamine and decorated with fucose and sialic acid to different extents.[4] Numerous studies have pointed out the biological importance of HMOs, e.g. their role in inhibiting the adhesion of pathogenic bacteria to the epithelial surface or the establishment of gut microbiota.[6,7] The HMO-composition of breast milk cannot be generalized though, as it is genetically determined.[7] The mother's Secretor- and Lewis type determine the fucosylation pattern and thus the set of HMOs present in breast milk.[8] Breast milks from Le(a-b+)-secretors, Le(a+b-)-non-secretors and Le(a-b-)-secretors/-non-secretors can be distinguished and have been thoroughly studied in view of their structural composition as well as their development during different stages of lactation.[8,9] Differences in HMO-profiles may have an influence on the biological functioning of breast milk.[7] The gastrointestinal fate of the complex HMOs remains vague, though. In the 1980s and early 1990s, several studies have been performed in order to investigate fecal oligosaccharides from breast-fed babies.[10-13] Blood group A active oligosaccharides were found in the feces of a single blood group A breast-fed baby and a gastrointestinal metabolization of the feeding-related HMOs was supposed.[10] On the other hand, no HMO metabolization products were found for a single blood group B breast-fed baby.[12] In a third study, no indication for gastrointestinal HMO-metabolization was found as HMO-profiles similar to the respective breast milks were observed.[14] In other studies HMOs and blood group characteristic oligosaccharides were detected in urine of breast-fed babies and lactating women,[15,16] pointing out their gastrointestinal absorbance and importance on a systemic level. Clearly, further research is needed in order to understand the gastrointestinal fate of complex HMOs and their possible conjugation with blood group antigenic structures. The application of novel analytical methods, which provide low detection limits and require limited preparative sample work in combination with a short analysis time, may open new possibilities in this context. It may help to establish an advanced scientific underpinning of the feeding-guidelines set up for neonates. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF), combined with mass spectrometry, has been shown to be a suitable tool for the analysis of HMOs in breast milk and baby feces.[17]

In the present study we, therefore, followed the HMO-profiles in breast milk and feces over time by CE-LIF/MS. Eleven mothers, representing three different Secretor- and Lewis groups [Le(a-b+), Le(a-b-), Le(a+b-)], and their breast-fed babies were included in this study.

Materials and Methods

Set-up study and sample collection

The eleven mothers, who were recruited in this study, each gave birth to a healthy child after 36-39 weeks of gestation. Ten of these respective babies, who were exclusively breast-fed during several months postpartum were as well recruited. Breast milk (5 mL) and fecal samples (±200 mg) were collected at 14-19 days (assigned as 14d), 1 month (assigned as 1m), 2 months (assigned as 2m), 3 months (assigned as 3m), 4 months (assigned as 4m), 5 months (assigned as 5m) and 6-7 months (assigned as 6m) postpartum. Not for all mother/child-pairs, samples were obtained at each time point. An overview of the samples collected is given in **Table 1**. The blood groups of the mothers were obtained by A,B,H(0) secretor testing or oral communication with the mothers. Except for baby I (blood group H(0)), the blood groups of the babies were not known.

Table 1. Overview on breast milk/fecal sample pairs A-K, classified according to the respective secretor-status of the mothers, as determined by CE-LIF/MS. º: secretor-status confirmed by saliva-testing. (14d/1m/2m/3m/4m/5m/6m): time points, for which samples were collected. [BG(x)]: [blood group(mother)]. #: baby and mother had same blood group. *: only breast milk samples obtained. n.d.: not determined

Secretor [Le (a-b+)]	Aº (14d/1m/2m/3m/4m) *[BG(A)]*	Eº (14d/1m/2m/6m) *[BG(H(0))]*
	Bº (2m/3m/4m/5m/6m) *[BG(H(0))]*	F (14d/1m/2m) *[BG(n.d.)]*
	Cº (14d/1m/2m/3m) *[BG(H(0))]*	G (2m/3m) *[BG(B)]*
	Dº (1m/2m/3m/6m) *[BG(B)]*	Hº (6m) *[BG(A)]*
Secretor [Le (a-b-)]	Iº (14d/1m/2m/3m/4m/5m/6m) *[BG(H(0))]*#	
non-secretor [Le(a+b-)]	Jº (14d/1m/2m)* [*BG(H(0))]*	K (14d/1m) *[BG(n.d.)]*

It was not possible to study the development of fecal oligosaccharide profiles for babies from non-secretor mothers, due to limited numbers of samples available.
For two babies (E, H) solid food was already introduced when the fecal sample was collected at 6m postpartum. All babies showed an average growth during their first six months of life. Written informed consent was obtained from all the mothers. Samples were stored at -80°C until further analysis.

HMO reference material and chemicals

2'Fucosyllactose, lacto-N-tetraose, lacto-N-fucopentaose I-III, lacto-N-difucohexaose I, fucosyllacto-N-hexaose II and a mixture composed of 3'sialyllactose, 6'sialyllactose and 6'sialyl-N-acetyllactosamine were purchased from Dextra Laboratories (Reading, UK). All other chemicals used were of analytical grade.

A,B,H(0)-secretor-testing

Saliva (5 mL) was collected from eight of the participating mothers. The A,B,H(0)-secretor-status was tested by the Dutch blood bank (Sanquin Diagnostiek, Amsterdam, The Netherlands), using an immunohematology method as established by the American Red Cross.[18] The expression of secretor-gene-specific antigens may not yet be fully developed with newborns during the first months postpartum.[19] Therefore, A,B,H-secretor-typing was not performed for the respective babies.

Oligosaccharide extraction from breast milk and feces

The extraction of carbohydrates from breast milk was accomplished as performed by Stahl et al.[20] It included pasteurization (30 min, 70 °C), defatting at 4 °C as well as a protein precipitation by adding the double amount (v/v) of cold ethanol. Oligosaccharides from feces were extracted according to Moro et al.[21] Aqueous fecal slurries (50–100 mg/mL) were kept overnight at 4 °C, centrifuged (15 min, 3500g) and filtered through a 0.22 μm membrane. Fecal enzymes were inactivated by heat (5 min, 100 °C). The carbohydrates extracted from milk and fecal extracts were purified by solid phase extraction on graphitized carbon column cartridges (150 mg bed weight, 4 mL tube size; Alltech, Deerfield, Il) as described previously.[17,22] In short, the cartridges were washed with 80/20 (v/v) acetonitrile (ACN)/water containing 0.1% (v/v) trifluoroacetic acid (TFA) followed by millipore water. After loading the sample extract onto the cartridge, monomers and lactose were largely removed by eluting with an aqueous 2% (v/v) ACN solution. The remaining carbohydrates on the cartridge were eluted with 40/60 (v/v) ACN/water

containing 0.05% (v/v) TFA. The solution was dried under a stream of air and the dried sample was then rehydrated with millipore water.

Monosaccharide composition

The crude fecal extracts (not purified on graphitized carbon columns) were hydrolyzed with 2 M TFA for 1 h at 121 °C followed by evaporation and repeated washing with methanol. The hydrolysates were re-dissolved in millipore water and analyzed with HPAEC-PAD for their monosaccharide composition.[23] For the determination of sialic acid, mild sample hydrolysis with 0.1 M TFA for 40 min at 80 °C was applied[24] before HPAEC analysis.[23]

CE-LIF

For CE-LIF analysis, the carbohydrates present in the fecal extracts were derivatized with the fluorescent 9-aminopyrene-1,4,6-trisulfonate (APTS) overnight at room temperature as reported elsewhere.[25] One nanomole xylose was added as internal standard and mobility marker. CE-LIF was performed on a ProteomeLab PA 800 characterization system (Beckman Coulter, Fullerton, CA), equipped with a laser induced fluorescence detector (LIF) (excitation: 488 nm, emission: 520 nm) (Beckman Coulter) and a polyvinyl alcohol-coated capillary (50 µm x 50.2 cm (Beckman Coulter), detector after 40 cm), kept at 25 °C. Samples were loaded hydrodynamically (4 s at 0.5 psi, representing approx. 14 nL sample solution) into the capillary. Separation was performed in the reversed polarity mode (30 kV, 20 min) in the 25 mM acetate buffer containing 0.4% polyethylene oxide provided in the ProteomeLab Carbohydrate Labeling and Analysis Kit (Beckman Coulter). Due to the low pK_a of the sialic acid residues (pK_a 2.6) and in order to provide the same buffer-pH as for CE-MS analysis, the separation buffer was adjusted to pH 2.4 by adding 1.2% (v/v) formic acid.[17] Peaks were integrated manually using Chromeleon software 6.8 (Dionex, Sunnyvale, CA).

CE-MS^n

CE-MS^n experiments were performed on a PA800+ System (Beckman Coulter) according to Albrecht et al.[17] Samples were injected at 10 psi for 2 s. Separation in 0.3% (v/v) formic acid (pH 2.4) was performed on a fused silica capillary (50 µm x 85 cm (Beckman Coulter) in reversed polarity mode (20 kV, 15 °C). The end of the CE capillary was installed in front of the ESI source (LTQ Velos, Thermo Fisher Scientific Inc., Waltham, MA) by leading it through a T-part designed in our laboratory[26] and provided the coaxial addition of a sheath liquid (50/50 isopropanol/water) at 3 µL/min. The ESI-MS was operated in the negative

mode using a spray voltage of 1.9 kV and an MS-capillary temperature of 190 °C. Mass spectra were acquired from m/z 300 to 2000. Due to the known migration behavior of HMOs and related structures, no supplementary detector was used between CE instrument and MS, as described before,[17] providing a more robust analysis set-up. MS^n was performed in the data dependent mode using a window of 1 m/z and collision energy of 35%. For increasing the S/N ratio, ions of m/z 311, 314 and 329 were excluded from detection in MS^n experiments. MS^n data were interpreted using Xcalibur software 2.0.7 (Thermo).
Based on the m/z-value as determined by CE-MS, the nanomole-concentration of oligosaccharides was translated to g/L.

Results and Discussion

Oligosaccharides in breast milk

Characterization of breast milk oligosaccharides (HMOs)

In order to study the fecal oligosaccharide profile of breast-fed babies during their first months of life, it is first of all important to characterize the breast milk, which served as feeding at the respective time points. **Table 1** presents an overview of the samples obtained from the eleven mother/child pairs A-K. Three representative HMO-fingerprints, as found for breast milk samples 14 days postpartum, are shown in **Figure 1**.
The HMO-profiles were recorded by CE-LIF and the structural compositions of the peaks were characterized by $CE\text{-}MS^n$ (**Table 2**). Secretor-characteristic (α1,2)-, (α1,3)- and (α1,4)-fucosylated HMOs[8] were found in the milk of eight out of the eleven mothers. They were, therefore, assigned as Le(a-b+)-secretors (**Figure 1-I** and **Table 1**). This is in accordance with the predominance of Le(a-b+)-secretors as found among the European population.[27,28] The breast milks of Le(a-b+)-secretors could be sub-classified into two groups according to their peak proportions of LNFP I/III (peak 25) / LNDFH I (peak 26). For the first group, a rather equal ratio was observed, with LNFP I/III / LNDFH I < 1.5:1 on average, as exemplified in **Figure 1-I**. The second group had a LNFP I/III / LNDFH I -ratio of ≥ 2:1. An example hereof is shown in **Figure 2**. From the seven Le(a-b+)-breast-milk samples available during the first two months, four (B, C, F, G) could be sub-classified in group 1 and three (A, D, E) in group 2.

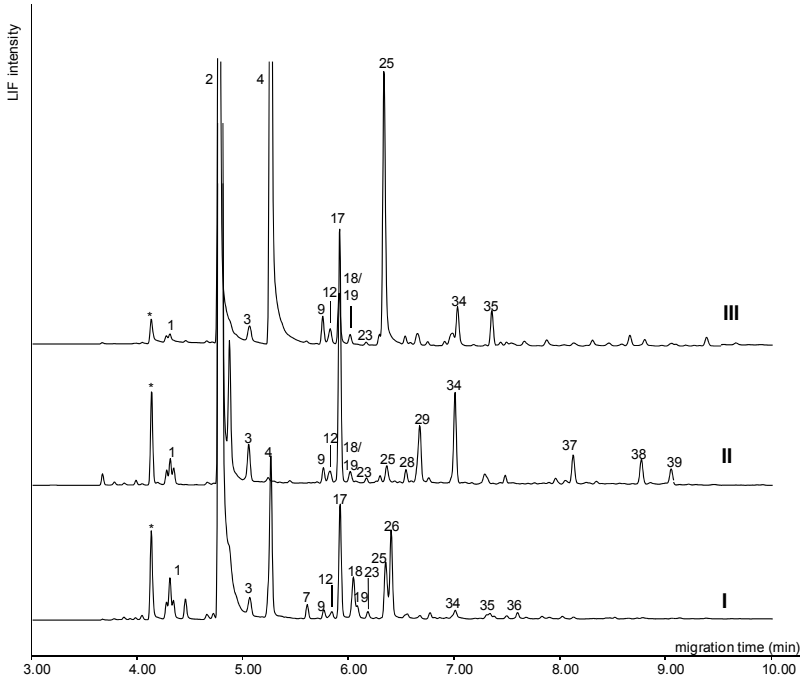


Figure 1. CE-LIF electropherograms of APTS-derivatized HMOs extracted from (I) breast milk of Le(a-b+)-secretor-mother C. (II) Le(a+b-)-non-secretor-mother K. (III) Le(a-b-)-secretor-mother I.
See **Table 2** for peak annotation.*internal standard xylose.

Due to the inactivity of the secretor-gene, non-secretors [Le(a+b-)] are not able to synthesize (α1,2)-fucosylated HMOs, such as 2'fucosyllactose (2'FL; peak 4, co-migrating with 3'FL), lacto-N-fucopentaose I (LNFP I; peak 25, co-migrating with LNFP III) and lacto-N-difucohexaose I (LNDFH I; peak 26). The respective structures were not present in the electropherograms of mothers J and K. They were, therefore, assigned as non-secretors. A non-secretor-milk pattern is shown in **Figure 1-II** and represents a characteristic oligosaccharide profile observed for this group. Higher-Mw HMOs, such as fucosyl-/difucosyl-/trifucosyl- and lacto-N-hexaose-isomers (F-LNH, DF-LNH, TF-LNH and LNH), represented a higher percentage of the total oligosaccharide-content in the non-secretor profile (24±8%) compared to the Le(a-b+)-secretor group (7±2%). Even lacto-N-octaose- and lacto-N-decaose isomers were detectable. With regard to the low-Mw HMOs, lacto-N-tetraose was the main HMO in the non-secretor profiles,

constituting 33±2% of the total oligosaccharide content, whereas for Le(a-b+)-secretors this value was 9±5%.

Table 2. Structural composition of oligosaccharides detected in CE-LIF electropherograms (**Figures 1-5**). APTS-derivatized oligosaccharides were present in their threefold negative charge in the ESI-MS profiles.

Peaks (Figures 1-5)	m/z (APTS 3-)	hexose	hexNAc	fucose	neu5Ac	name (abbreviation)	Peaks (Figures 1-5)	m/z (APTS 3-)	hexose	hexNAc	fucose	Neu5Ac	name (abbreviation)
*						xylose ISTD	20	601	4	2	0	1	S-LNH*
1						monomers	21	412	3	0	2	0	m/z 412[#]
2	260	2	0	0	0	lactose	22	425	2	1	2	0	m/z 425[#]
3	357	2	0	0	1	SL*	23	479	3	1	0	1	S-LNT*
4	309	2	0	1	0	FL*	24	601	4	2	0	1	S-LNH*
5	309	2	0	1	0	m/z 309[#]	25	431	3	1	1	0	LNFP I/III
6	382	3	1	0	0	LnNT Y	26	479	3	1	2	0	LNDFH-I
7	358	2	0	2	0	DF-L	27	479	3	1	2	0	m/z 479[#]
8	479	3	1	0	1	S-LNT*	28	650	4	2	1	1	FS-LNH*
9	382	3	1	0	0	LnNT	29	504	4	2	0	0	LNH
10	528	3	1	1	1	FS-LNT*	30	485	4	1	1	0	m/z 485[#]
11	358	2	0	2	0	m/z 358[#]	31	533	4	1	2	0	m/z 533[#]
12	479	3	1	0	1	S-LNT*	32	547	3	2	2	0	m/z 547[#]
13	363	3	0	1	0	m/z 363[#]	33	498	3	2	1‘	0	m/z 498[#]
14	377	2	1	1	0	m/z 377[#]	34	552	4	2	1	0	F-LNH*
15	431	3	1	1	0	LNFP Y	35	601	4	2	2	0	DF-LNH*
16	358	2	0	2	0	m/z 358[#]	36	650	4	2	3	0	TF-LNH*
17	382	3	1	0	0	LNT	37	674	5	3	1	0	F-LNO*
18	431	3	1	1	0	LNFP II	38	747	6	4	0	0	LND*
19	576	3	1	0	2	DS-LNT*	39	795	6	4	1	0	F-LND*

: isomers not further specified; [#]see Figure 4B for structural characterization

(Alpha1,4)-fucosylated HMOs, such as LNFP II (peak 18) and LNDFH I (peak 26), are absent in the milk of Lewis-negative secretors Le(a-b-).[8] These oligosaccharides were not present in the breast milk of mother “I”, who was, therefore, assigned as Le(a-b-)-secretor (**Figure 1-III**). Instead, (α1,2)- and (α1,3)-fucosylated oligosaccharides dominated, as visualized by the FL- and LNFP I/III -peaks (**Figure 1-III**, peaks 4 and 25). They made up 76% of the total oligosaccharide content of the Le(a-b-)-profile. The FL-peak encompasses 2’FL (Fuc-(α1,2)-Gal-(β1,4)-Glc) and 3’FL (Gal-(β1,4)-[Fuc-(α1,3)]-Glc). The LNFP I/III -peak includes LNFP I [Fuc-(α1,2)-Gal-(β1,3)-GlcNAc-(β1,3)-Gal-(β1,4)-Glc] and

LNFP III [Gal-(β1,4)-[Fuc-(α1,2)]-GlcNAc-(β1,3)-Gal-(β1,4)-Glc]. These structures co-migrated in CE-LIF.

Sialyllactose(SL; peak 3)-, two sialyllacto-N-tetraose(S-LNT; peaks 12 and 23)-, disialyllacto-N-tetraose(DS-LNT; peak 19)- and fucosyl-sialyllacto-N-hexaose(FS-LNH; peak 28)-isomers were the most abundant acidic HMOs found in the breast milks, as determined by CE-MS. These acidic oligosaccharides constituted 9±2% of the total oligosaccharide content in the milk of Le(a-b+)- and Le(a+b-)-mothers and 3% in the milk of the Le(a-b-)-mother. However, the actual number may be higher as high-Mw acidic HMOs were not taken into account and the abundant DS-LNT co-migrated with LNDFH II, influencing the quantification of this compound.

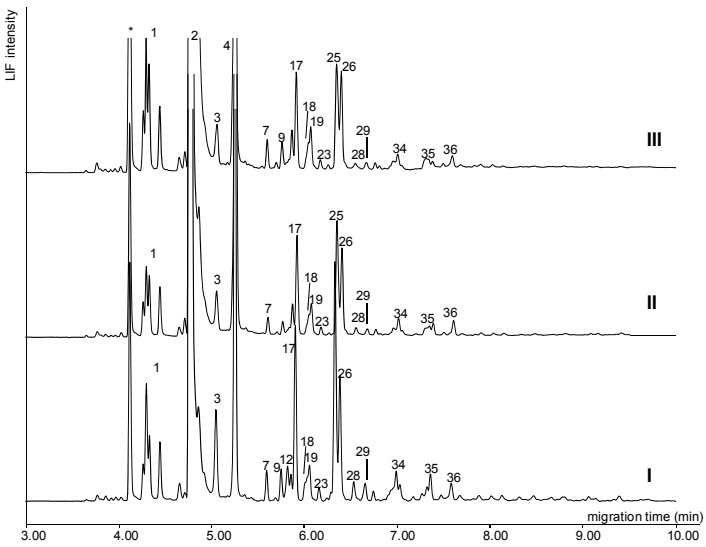
By means of saliva analysis, the secretor-status of eight out of the eleven mothers was determined and the prediction on the secretor-status on basis of the CE-LIF patterns was confirmed (**Table 1**).

Development of the HMO-profile of breast milk in time

In order to include non-identified oligosaccharides for quantification, which is possible with CE-LIF due to the mole-based detection of the APTS-derivatized oligosaccharides,[29] concentrations are expressed in nmol in this study. The total HMO-concentration was translated to 14±5 g/L for the 14d-breast milk samples (*see materials and methods*). This is a common concentration range found for breast milk early after birth.[9] The concentration of HMOs as present in breast milk 14d postpartum decreased by ± 40% on average during the first three months and is comparable to what has been previously reported.[9] Overall, the proportions of the individual HMOs changed during the first months after birth. A representative example is given for breast milk A at the time points 14d, 2m and 4m (**Figure 2**). For comparison, peak ratios are presented relative to LNDFH I in the table of **Figure 2**. A characteristic ratio of LNFP I/III / LNDFH I ≥ 2:1 was observed 14d after birth for breast milk A (*vide supra*). This ratio decreased during the course of lactation, which was also found for the other individuals of this group. On the contrary, such a change was not observed for individuals who initially showed a ratio of LNFP I/III / LNDFH I < 1.5:1.

Within most profiles, but most noticeably for the Le(a-b-)-secretor-profile, an increased contribution of FL (**Figure 1**, peak 4, composed of 2'FL and 3'FL) to the oligosaccharide profile after advanced lactation time was observed. This increase resulted in a plateau value of the reduced oligosaccharide concentration as present after three months or even resulted in a subsequent increase of on average 14% between three and six months. Previous studies related this increase to the increase of (α1,3)-fucosylated HMOs in time.[9] As (α1,2)- and

(α1,3)-fucosylated oligosaccharides co-migrate with CE-LIF, this explanation could only be assumed for the present study. As milk samples from non-secretors were only available for a limited time-range (**Table 1**), a decrease in total oligosaccharide concentration during the first two months could only be indicated for this group in our study. A decrease in total oligosaccharide content in the milk of non-secretors during the first three months postpartum has been previously stated by Thurl et al.[9]



	SL (3)	FL (4)	LNT (17)	LNFP I/III (25)	LNDFH I (26)
I - 2 weeks	0.8	5.6	1.3	2.0	1.0
II - 2 months	0.5	6.1	1.2	1.5	1.0
III - 4 months	0.5	6.2	0.9	1.3	1.0

Figure 2. CE-LIF electropherograms of APTS-derivatized HMOs extracted from breast milk of Le(a-b+)-secretor-mother A at 14 days (I), 2 months (II), 4 months (III) postpartum.
Table indicates peak ratios relative to LNDFH I = 1. See **Table 2** for peak annotation.*internal standard xylose.

Fecal oligosaccharides of breast-fed babies

One or more fecal samples were obtained from ten breast-fed babies at the time points indicated in **Table 1**. Contrary to the corresponding breast milks, which only changed in HMO-proportions and not in their overall HMO-fingerprint during the first six months postpartum, two major changes in the fecal oligosaccharide profiles were observed. Although the time-period inbetween these major changes was individual-dependent, three

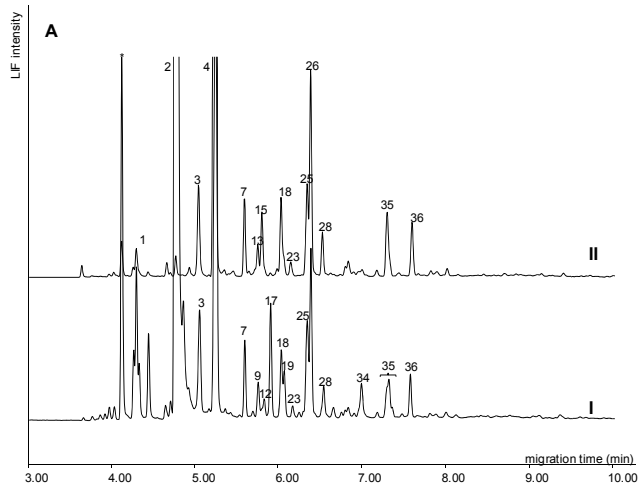
successive stages of fecal oligosaccharide profiles in time could be defined. The first stage comprises the first weeks or months of life, when the gastrointestine is not yet adapted to the enteral food. The second stage represents a stabilized gastrointestinal ecosystem, which is then disturbed by the introduction of solid food in the third stage. The findings will be discussed below per stage, followed by a general discussion.

***Stage 1*: Neutral or acidic HMO-profiles in feces**

Fecal oligosaccharide profiles obtained just after birth and up to two months postpartum could be sub-divided according to their respective fingerprints, which were dominated by either neutral or acidic oligosaccharides.

Neutral HMOs

Fecal oligosaccharide profiles, which were overall corresponding to the HMO-profile of the respective breast milks, were found for four babies (E, F, G, I). As a representative, the HMO-profile of the breast milk G and the corresponding fecal oligosaccharide profile, both taken two months postpartum, are shown in **Figure 3A** and the peak ratios relative to LNDFH = 1 are indicated. Changes in peak presence and abundance in the fecal extract can be linked to the activity of gastrointestinal enzymes: Lactose is absent in the fecal extract, indicating lactase activity.[30] The lowered proportion of the FL- and LNFP I/III -peak in the fecal extract (**Figure 3A**, peaks 4 and 25) points to fucosidase activity.[31] The decreased proportion of SL (**Figure 3A**, peak 3) and the reduction in DS-LNT (**Figure 3A**, peak 19) can be connected to intestinal sialidase activity. LNT [Gal-(β1,3)-GlcNAc-(β1,3)-Gal-(β1,4)-Glc] (**Figure 3A**, peak 17) is absent in the fecal sample, as was previously reported.[14,17] LNT, which contains the bifidogenic factor lacto-N-biose [Gal-(β1,3)-GlcNAc]^6 is thus indeed a desired substrate for the developing gastrointestinal microbiota characteristic for breast-fed babies in an early stage postpartum. Even more, due to the presence of the degradation product LNFP Y [Gal-(β1,4)-[Fuc-(α1,3)]-GlcNAc-(β1,6)-Gal-(β1,4)-Glc] (**Figure 3A**, peak 15) and the absence of F-LNH-isomers (**Figure 3A**, peak 34), it can be assumed, that F-LNH-isomers, which carry a non-substituted lacto-N-biose unit at their branching point (e.g. F-LNH II: Gal-(β1,4)-[Fuc-(α1,3)]-GlcNAc-(β1,6)-[Gal-(β1,3)-GlcNAc-(β1,3)]-Gal-(β1,4)-Glc), are important oligosaccharides for gastrointestinal bacteria during the first months postpartum. A non-HMO-component of m/z 363 (**Figure 3A**, peak 13) was present in the feces of baby G in *stage 1*, but only contributed to 2% of the total oligosaccharide content. As this is a dominant oligosaccharide of *stage 2*, the component will be discussed there.



	SL (3)	FL (4)	DF-L (7)	m/z 363 (13)	LNFP Y (15)	LNT (17)	LNFP-II/ DS-LNT (18/19)	LNFP-I/-III (25)	LNDFH-I (26)	FS-LNH (28)	F-LNH (34)	DF-LNH (35)	TF-LNH (36)
I - milk	0.7	5.7	0.4	0.0	0.0	0.7	0.7	0.7	1.0	0.2	0.3	0.5	0.3
II - feces	0.5	3.9	0.3	0.2	0.3	0.0	0.5	0.5	1.0	0.2	0.0	0.5	0.3

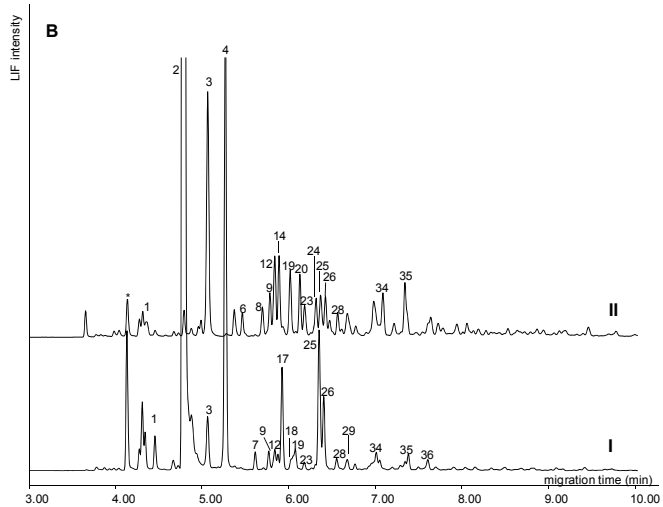


Figure 3. CE-LIF electropherograms showing (A) HMOs of Le(a-b+)-secretor-milk from mother G (A-I) and neutral fecal HMO-profile of baby G (A-II) at 2 months postpartum. (B) HMOs of Le(a-b+)-secretor-milk from mother A (B-I) and acidic fecal HMO-profile of baby A (B-II) at 14 days postpartum. Table indicates peak ratios relative to LNDFH I = 1 for **Figure 3A**.

See **Table 2** for peak annotation.*internal standard xylose.

Acidic HMOs

Remarkably, for three babies (A, D, K), a fecal oligosaccharide profile composed of 39±13% acidic HMOs was found. As a comparison, for babies F, G and I, who showed a mainly neutral fecal HMO-profile, the percentage of the acidic oligosaccharides was only 7±1%. An exception was baby E, for which, besides a characteristic neutral HMO-profile, acidic oligosaccharides contributed to 15%. The babies showing predominantly acidic fecal oligosaccharides had Le(a+b-)-non-secretor-(K)- or Le(a-b+)-secretor-mothers (A, D). In the HMO-profiles of the respective breast milks no elevated contribution of acidic HMOs was found. As a representative, the HMO-profile of the breast milk A and the corresponding fecal oligosaccharide profile, both taken 14d postpartum, are shown in **Figure 3B**. Noticeably, babies A, D and E, who showed increased proportions of acidic fecal HMOs, consumed milk with a LNFP I/III / LNDFH I -proportion of ≥ 2:1 (exemplified by **Figure 2** and **Figure 3B**). Instead, for breast milk of the mothers F and G, whose babies showed neutral HMO-profiles, the proportion of LNFP I/III / LNDFH I was < 1.5:1 (exemplified by **Figure 3A**).

The HMO-profiles of breast milk from Le(a-b+)-secretors may thus have an influence on the gastrointestinal fate of these oligosaccharides in the first few months postpartum. The HMO-profiles of Le(a-b-)-secretor- and Le(a+b-)-non-secretor-milk may influence the fecal oligosaccharide profiles in a different way. On the other hand, also other factors than the composition of the respective breast milk may determine the fecal oligosaccharide profile, e.g. a reduced gastrointestinal sialidase activity, which may inhibit a further enzymatic degradation of the acidic oligosaccharides.[32]

For the fecal samples, which were taken up to two months postpartum from baby C, no oligosaccharide profile comparable to human milk was found and the prevailing peaks could not be identified by CE-MS. The values (m/z) in the MS fragmentation pattern were unusual for carbohydrates and may point to the presence of glycoprotein-components in this single case. For baby B, fecal samples were collected only from month two onwards and the oligosaccharide profiles observed were already indicative for *stage 2*, as will be discussed next.

Stage 2: Predominance of metabolization products

Development of the fecal oligosaccharide profiles in time

The absence of HMOs and the prevalence of a few, individual-dependent, dominant peaks, which were subsequently characterized as gastrointestinal HMO-metabolization products (*see below*) is characteristic for fecal oligosaccharide profiles of *stage 2*. The time-point, at which the neutral or acidic fecal HMO-profile of *stage 1* had completely disappeared was baby-dependent. For all babies, oligosaccharides characteristic for *stage 2* were at three months postpartum present to a larger proportion than oligosaccharides characteristic for *stage 1*. This development of the fecal oligosaccharide profile is exemplified for baby A in **Figure 4**. Mainly acidic oligosaccharides are still observed after two months and exclusively metabolization products are observed after four months. The inlay in **Figure 4** shows the total fecal oligosaccharide content, which was set to 100% for day 14. Similar to what was found for other babies, an increase in fecal oligosaccharide concentration could be observed during the first few months, which may be correlated to an increased intake of breast milk. Good feeding practice estimates an approximate intake of breast milk equivalent to one fifth of the baby's weight per day. The average decrease of approximately 40% of the initial HMO-concentration in breast milk during the first three months, as shown for the mothers in this study, may therefore be compensated by an increased milk intake. The considerable drop in fecal total oligosaccharide concentration after three months is remarkable and could be the consequence of an adapted bacterial flora, which has colonized under the influence of breast milk during the first few months postpartum.[33] The inlay in **Figure 4** also indicates the contribution of the novel oligosaccharides to the total oligosaccharide content, related to the total oligosaccharide content of 100%, as set for day 14. After four months, the predominant metabolization products represent more than 80% of the total oligosaccharide peaks present at that time point for baby A. This dominant presence of gastrointestinal metabolization products corresponds to what was found for all the other babies. The corresponding breast milk for baby A is shown in **Figure 2** and does not indicate major changes in oligosaccharide profile and only indicated changes in the total oligosaccharide concentration at the respective time points. Although having a secretor-mother of the type Le(a-b-), baby I showed the same trend in fecal oligosaccharide development as found for babies from Le(a-b+)-mothers, albeit with a slower switch from *stage 1* to *stage 2*. A HMO-profile corresponding to the respective breast milk was still present after two months, not showing any indication for metabolization (which was already clearly visible for baby G at two months, **Figure 3A**). Although no conclusions

could be drawn, this may be connected to the preterm delivery of baby I and points at a delayed gastrointestinal development.[34]

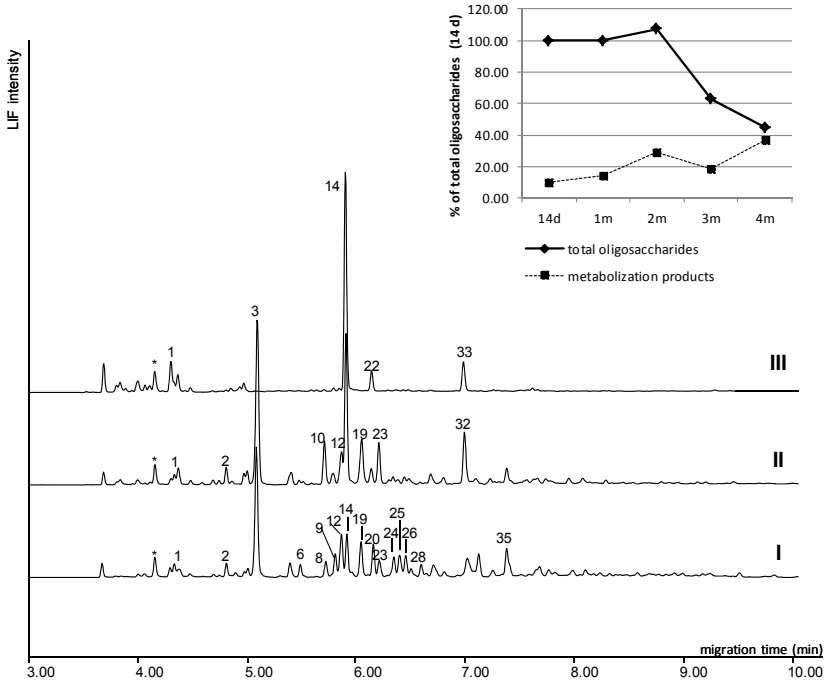


Figure 4. CE-LIF electropherograms showing fecal oligosaccharide profiles for baby A at 14 days (I), 2 months (II) and 4 months (III) postpartum. Inlay depicts development of total oligosaccharide concentration and contribution of metabolization products to total oligosaccharide content in time.
See **Table 2** for peak annotation.*internal standard xylose.

Characterization of metabolization products

Four different oligosaccharide sets were found for the eight babies from whom samples showing metabolization products (*stage 2*) were available. Representative electropherograms are shown in **Figure 5A**. The structures characteristic of the oligosaccharide profiles were studied by MS-fragmentation after separation with CE. A summary of the annotated MS-fragments is given in **Table 3**. The fragments could be affiliated to oligosaccharides characteristic for either blood group A, B, or H(0). In addition, the fragments indicated the presence of similar structural backbones, which may result from HMO-building blocks (**Figure 5B**). Blood groups were not determined for the

babies participating in the present study, but for the respective mothers, the blood group was mostly known (**Table 1**). A correlation to the baby's blood group may thus be possible in some cases. Due to the low amounts of sample, the annotation of the emerging structures could not be confirmed by means of NMR.

Blood group A-characteristic oligosaccharides

For two babies (A, F), oligosaccharide profiles corresponding to **Figure 5A-I** were observed. The three predominating structures present in **Figure 5A-I** had m/z-values of 377 (peak 14), 425 (peak 22) and 498 (peak 33). Their structural compositions [HexNAc-[Fuc]-Hex-Hex (m/z 377), HexNAc-[Fuc]-Hex-[Fuc]-Hex, (m/z 425) and HexNAc-[Fuc]-Hex-HexNAc-Hex-Hex (m/z 498)] were annotated according to their respective MS^n fragmentation patterns (**Table 3**). All had a terminal HexNAc-[Fuc]-Hex-unit (**Figure 5B**). Supplementary, a fourth structure with a terminal HexNAc-[Fuc]-Hex-unit, assigned as HexNAc-[Fuc]-Hex-[Fuc]-HexNAc-Hex-Hex (m/z 547), was found in the fecal sample taken after two and three months from baby A (**Figure 4**, peak 32). It was only present in trace amounts and co-migrated with structure m/z 498 in the four-month-sample (**Figure 4** and **Figure 5A-I**, peak 33). The m/z 498 represents the monofucosylated analogon of the difucosylated m/z 547 (**Figure 5B**). The increase of m/z 498 at the expense of m/z 547 may thus indicate enzymatic defucosylation in time. HexNAc-[Fuc]-Hex is the antigenic determinant for blood group A and composed of GalNAc-(α1,3)-[Fuc-(α1,2)]-Gal.[35] In a previous study we detected m/z 377 and m/z 498 in the feces of a breast-fed two months old preterm baby of blood group A.[36] Previously, the same blood group A characteristic oligosaccharides were detected in the feces of an eight weeks old preterm baby of blood group A.[10] Hence, the presence of m/z 377 (peak 14), m/z 425 (peak 22), m/z 547 (peak 32) and m/z 498 (peak 33) in the present study was associated with a blood group A-affiliation of the babies A and F, with baby A having a mother belonging to blood group A.

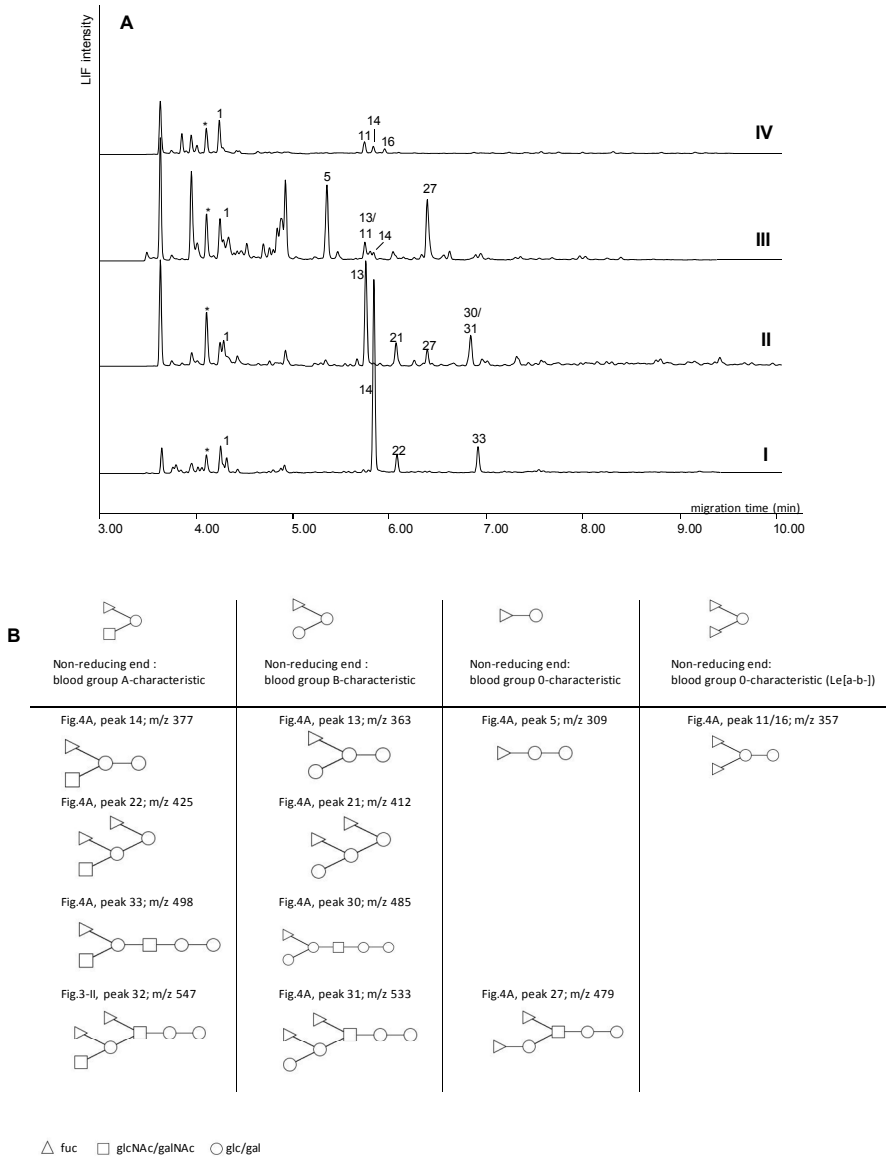


Figure 5. (A) CE-LIF electropherograms showing (A-I) blood group A-characteristic oligosaccharides from baby A after 4m. (A-II) blood group B-characteristic oligosaccharides from baby G after 3 months. (A-III/-IV) Blood group H(0)-characteristic oligosaccharides from baby D after 6 months and baby I after 3 months. (B) Summary of oligosaccharides resulting from gastrointestinal metabolization of HMOs, as present in **Figures 4** and **Figure 5A** and characterized by CE-MS.*internal standard xylose.

Table 3. Mass fragments and structural composition of unknown, APTS-derivatized oligosaccharides of the CE-LIF electropherograms (**Figure 5**). The APTS-derivatized oligosaccharides were present in their twofold [2-] and threefold [3-] negative charge in the fragmentation profiles.

		m/z 377	**m/z 425**	**m/z 498**	**m/z 363**	**m/z 412**	**m/z 533**	**m/z 309**	**m/z 479**	**m/z 358**
m/z fragment pair	structural composition									
207[3-]/ 310[2-]	Hex-APTS	+	±	+	+	±	+	+	+	+
255[3-]/ 383[2-]	Fuc-Hex-APTS	-	±	-	-	+	-	-	-	-
260[3-]/ 391[2-]	Hex-Hex-APTS	+	+	+	+	±	+	+	+	+
309[3-]/ 464[2-]	Fuc-Hex-Hex-APTS	+	+	-	+	+	+	-		+
314[3-]/ 472[2-]	Hex-Hex-Hex-APTS	-	-	-	+	±	-	-		-
328[3-]/ 492[2-]	HexNAc-Hex-Hex-APTS	+	±	+	-	-	+	-		-
358[3-]/ 537[2-]	Fuc-Hex-[Fuc]-Hex-APTS	-	+	-	-	+	-	-		-
363[3-]/ 545[2-]	Hex-Hex-[Fuc]-Hex-APTS Hex-[Fuc]-Hex-Hex-APTS	-	-	-	-	+	-	-		-
377[3-]/ 566[2-]	Fuc-HexNAc-Hex-Hex-APTS HexNAc-[Fuc]-Hex-Hex-APTS HexNAc-Hex-[Fuc]-Hex-APTS	-	+	-	-	-	+	-	+	-
382[3-]/ 574[2-]	Hex-HexNAc-Hex-Hex-APTS	-	-	+	-	-	+	-	+	-
431[3-]/ 647[2-]	Fuc-Hex-HexNAc-Hex-Hex-APTS	-	-	+	-	-	+	-	+	-
449[3-]/ 674[2-]	HexNAc-Hex-HexNAc-Hex-Hex-APTS	-	-	+	-	-	-	-	-	-
479[3-]/ 719[2-]	Fuc-Hex-[Fuc]-HexNAc-Hex-Hex-APTS	-	-	-	-	-	+	-	-	-
485[3-]/ 723[2-]	Hex-[Fuc]-Hex-HexNAc-Hex-Hex-APTS Hex-Hex-[Fuc]-HexNAc-Hex-Hex-APTS	-	-	-	-	-	+	-	-	-

Blood group B-characteristic oligosaccharides

Baby G showed an oligosaccharide profile as shown in **Figure 5A-II** during *stage 2*. The three characteristic structures found in the fecal oligosaccharide profile have the values m/z 363 (peak 13), m/z 412 (peak 21) and m/z 533 (peak 31). Their structural compositions [Hex-[Fuc]-Hex-Hex (m/z 363), Hex-[Fuc]-Hex-[Fuc]-Hex, (m/z 412) and Hex-[Fuc]-Hex-[Fuc]-HexNAc-Hex-Hex (m/z 533)] were determined according to their

respective MS^n fragmentation patterns (**Table 3**). All carry a terminal Hex-[Fuc]-Hex-unit (**Figure 5B**). Hex-[Fuc]-Hex is the antigenic determinant for blood group B and is composed of Gal(α1,3)-[Fuc(α1,2)]-Gal.[35] The mother of baby G belongs to blood group B. This is the first time that blood group B-characteristic oligosaccharides have been detected in the feces of a breast-fed baby. A fourth structure of m/z 485 (peak 30) was found in minor amounts and co-migrated with m/z 533. The m/z 485-value represents the monofucosylated analogon of m/z 533 (**Figure 5B**), similar to the monofucosylated m/z 498, which replaced the difucosylated analogon m/z 547 for blood group A after four month. Due to its low abundance an MS-fragmentation was not possible for m/z 485.

Oligosaccharides indicating affiliation to blood group H(0)

Three babies (B, D, E) showed fecal oligosaccharide profiles corresponding to what is shown in **Figure 5A-III**. The two dominating structures in **Figure 5A-III** had the m/z-values 309 (peak 5) and 479 (peak 27) and were assigned as Fuc-Hex-Hex (m/z 309) and [Fuc]-Hex-[Fuc]-HexNAc-Hex-Hex (m/z 479) according to their fragmentation patterns (**Table 3**). The fragmentation patterns and m/z values corresponded to the HMO-structures 2'FL and LNDFH I, but showed different migration times. Thus, the fecal oligosaccharides may represent structural isomers of these HMOs as proposed in **Figure 5B**. Alternatively, the reducing end sugar may be galactose instead of the glucose. The unknown structure m/z 479 may correspond to a LNDFH-isomer, not known for breast milk (lacto-N-neo-difucohexaose: Fuc-(α1,2)-Gal-(β1,4)-[Fuc-(α1,3)]-GlcNAc-(β1,3)-Gal-(β1,4)-Glc), which was previously identified in the urine of lactating women.[15] The antigenic determinant for blood group H(0) is a pre-cursor of the antigenic determinant of blood groups A and B and is composed of Fuc-(α1,2)-Gal.[35]

The components m/z 377 (peak 14) (blood group A specific) and m/z 363 (peak 13) (blood group B-specific) were present only in minor proportions in the profiles of babies B, D and E, as exemplified by baby D in **Figure 5A-III**. On the other hand, m/z 479, which is abundantly present in the profiles of babies B, D and E, was found in low proportions only in the blood group B characteristic profile (**Figure 5A-II**). Hence, an affiliation to blood group H(0) was proposed for babies B, D and E. Indeed, two of the respective mothers were known to have blood group H(0).

Baby I, who had a Le(a-b-)-secretor-mother, had blood group H(0). A different fecal peak profile for *stage 2* was observed (**Figure 5A-IV**) compared to what was described for the three babies B, D, E. For these babies, blood group H(0) was only expected, due to the

oligosaccharide profiles observed (**Figure 5A-III**). Their mothers were Le(a-b+)-secretor-mothers.

Three peaks of exceptionally low abundance were detected from three months onwards for the baby of the Le(a-b-)-secretor. Two of the peaks (**Figure 5A-IV**, peaks 11 and 16; m/z 358) were assigned as Fuc-[Fuc]-Hex-Hex according to their mass fragmentation pattern (**Table 3**). They are structurally related to Fuc-Hex-Hex (m/z 309), as present in the feces of the babies for whom blood group H(0) was expected. A double-fucosylated non-reducing end was found for m/z 358 (**Figure 5B**). Component m/z 358 was as well present in the profiles of the babies, for whom blood group H(0) was expected (**Figure 5A-III**), but was dominated by other structures in that case. Similar to the babies for whom blood group H(0) was expected, traces of the blood group A-characteristic structure m/z 377 were detected in the fecal profile of baby I from the Le(a-b-)-secretor-mother (**Figure 5A-IV**, peak 14). The genetically determined HMO-fingerprint in breast milk may influence the gastrointestinal formation of metabolization products in *stage 2*.

***Stage 3*: Oligosaccharides characteristic for follow-up feeding**

For two babies (E, H), fecal samples were collected at t ≥ six months, when solid food was part of the diet next to breast milk. No fecal HMOs, and also no oligosaccharides characteristic for *stage 2* were found. Instead, a homologous series of hexose-oligosaccharides was observed. These oligosacchrides co-migrated with a maltodextrin-standard and probably result from diet-related (resistant) starch. The glucose/galactose proportion was exceptionally high for samples of *stage 3* (3.8:1) as resulted from monosaccharide composition analysis of these samples. As a comparison, the glucose/galactose proportion was 0.7:1 for *stage 1* and 0.4:1 for *stage 2* on average. The presence of a diverse gastrointestinal microbiota by the time solid food is introduced may be reflected in the peak profile.[33] The microflora present thus largely degrades HMOs and does not stimulate the bioconversion to blood group characteristic structures. Yet, it is not adapted to fully degrade the carbohydrate structures newly introduced by the feeding, e.g. starch. Indeed, microbiological studies have shown a significant change in gastrointestinal flora composition with the introduction of solid food for breast-fed babies.[33]

Overview of HMO-metabolization in the gastrointestine of neonates

Following the fecal oligosaccharide profiles of breast-fed babies and comparing them to the HMO-profiles of the respective breast milks during the first months postpartum enabled us to get a detailed picture on the gastrointestinal fate of HMOs. The ongoing gastrointestinal development and the adaptation towards the feeding during that time was depicted, which finally led to an almost complete disappearance of the feeding-related HMOs at the time when solid food was introduced. A schematic overview on the suggested gastrointestinal bioconversion of HMOs is given in **Figure 6**. Although some general trends were observed, the timing of the fecal oligosaccharide development remained baby-specific. In the first few months (*stage 1*), HMOs are degraded to monomers and dimers, serving as energy source for the developing gastrointestinal microbiota, with among them lacto-N-biose, which can be used by the health-promoting Bifidobacteria.[6] Degradation products (such as LNFP Y) are detected in the feces of these babies in combination with a HMO-profile depicting the fingerprint of their mother's milk. Alternatively, a selection of acidic HMOs is detected, which may be a result of the set of HMOs provided with the respective breast milk. Independent of the initial presence of neutral or acidic oligosaccharides (*stage 1*), a gradual change to an oligosaccharide profile dominated by parts of HMOs decorated with blood group specific epitopes was observed with proceeding time (*stage 2*). HMOs are degraded in the gastrointestinal tract during *stage 2*, resulting in a uniform set of basic units, which are subsequently conjugated to blood group characteristic oligosaccharides. The involvement of blood group characteristic epitopes, which are located in the gastrointestinal mucus layer,[35] are proposed to play an important role for this enzymatic re-structuration of the HMOs. This results in a personalized fecal oligosaccharide profile during *stage 2*. The exact mechanism and biological significance of this gastrointestinal re-design remains to be investigated. Finally, the absence of HMOs and their blood group-characteristic derivatives in *stage 3*, points to the installation of an adult-like flora at the time of weaning.[33] We thus propose, that fecal oligosaccharide profiles can be used as mirrors for monitoring the individual-dependent gastrointestinal adaptation to enteral food during the first months postpartum.

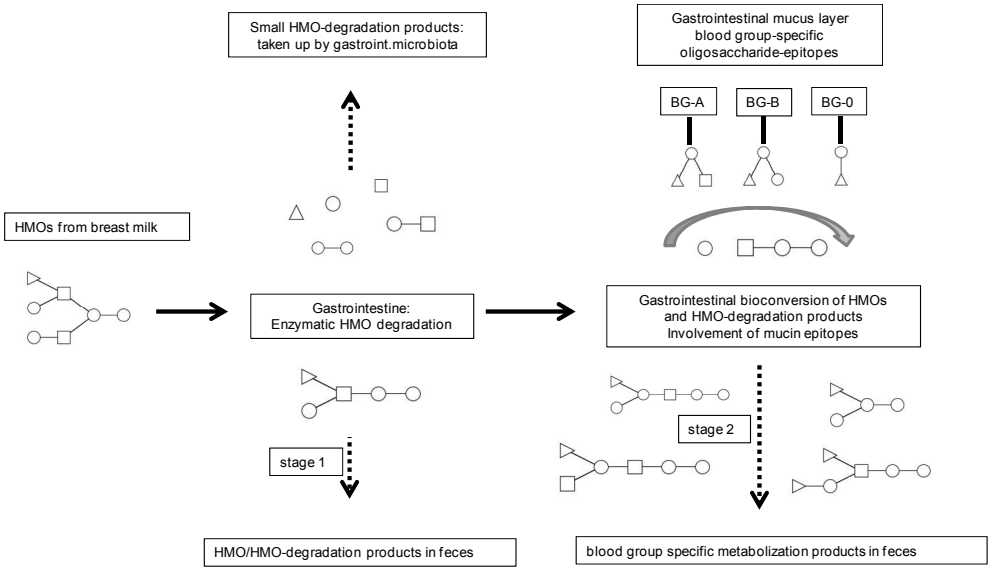


Figure 6. Schematic overview on the proposed gastrointestinal degradation and metabolization of HMOs as observed for *stage 1* and *stage 2*.

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References

1. Kramer, M.S.; Kakuma, R. The optimal duration of exclusive breastfeeding: A systematic review. *WHO/NHD/01.08, WHO/FCH/CAH/01.23*, **2002**.
2. American Academy of Pediatrics. Breastfeeding and the use of human milk. *Pediatrics*, **1997**, *100*, 1035-1039.
3. Leung, A.K.C.; Sauve, R.S. Breast is best for babies. *J. Natl. Med. Assoc.*, **2005**, *97*, 1010-1019.
4. Kunz, C.; Rudloff, S.; Baier, W.; Klein, N.; Strobel, S. Oligosaccharides in human milk: Structural, functional, and metabolic aspects. *Ann. Rev. Nutr.*, **2000**, *20*, 699-722.
5. Viverge, D.; Grimmonprez, L.; Cassanas, G.; Bardet, L.; Bonnet, H.; Solère, M. Variations of lactose and oligosaccharides in milk from women of blood types secretor a or H, secretor Lewis, and secretor H/nonsecretor Lewis during the course of lactation. *Ann. Nutr. Metab.*, **1985**, *29*, 1-11.
6. Kitaoka, M.; Tian, J.S.; Nishimoto, M. Novel putative galactose operon involving lacto-N-biose phosphorylase in *Bifidobacterium longum*. *Appl. Environ. Microbiol.*, **2005**, *71*, 3158-3162.
7. Newburg, D.S.; Ruiz-Palacios, G.M.; Altaye, M.; Chaturvedi, P.; Meinzen-Derr, J.; Guerrero, M.d.L.; Morrow, A.L. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology*, **2004**, *14*, 253-263.
8. Thurl, S.; Henker, J.; Siegel, M.; Tovar, K.; Sawatzki, G. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconjugate J.*, **1997**, *14*, 795-799.
9. Thurl, S.; Munzert, M.; Henker, J.; Boehm, G.; Muller-Werner, B.; Jelinek, J.; Stahl, B. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Brit. J. Nutr.*, **2010**, *104*, 1261-1271.
10. Sabharwal, H.; Nilsson, B.; Chester, M.A.; Sjöblad, S.; Lundblad, A. Blood group specific oligosaccharides from faeces of a blood group A breast-fed infant. *Mol. Immunol.*, **1984**, *21*, 1105-1112.
11. Sabharwal, H.; Nilsson, B.; Grönberg, G.; Chester, M.A.; Dakour, J.; Sjöblad, S.; Lundblad, A. Oligosaccharides from feces of preterm infants fed on breast milk. *Arch. Biochem. Biophys.*, **1988**, *265*, 390-406.
12. Sabharwal, H.; Nilsson, B.; Chester, M.A.; Lindh, F.; Grönberg, G.; Sjöblad, S.; Lundblad, A. Oligosaccharides from faeces of a blood-group B, breast-fed infant. *Carbohydr. Res.*, **1988**, *178*, 145-154.
13. Sabharwal, H.; Sjöblad, S.; Lundblad, A. Sialylated oligosaccharides in human milk and feces of preterm, full-term, and weaning infants. *J. Pediatr. Gastr. Nutr.*, **1991**, *12*, 480-484.
14. Coppa, G.V.; Pierani, P.; Zampini, L.; Bruni, S.; Carloni, I.; Gabrielli, O. Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. In *Bioactive components of human milk*; Newburg, D.S., Ed; Kluwer Academic/Plenum Publishers: New York, **2001**; pp 307-314.
15. Hallgren, P.; Lindberg, B.S.; Lundblad, A. Quantitation of some urinary oligosaccharides during pregnancy and lactation. *J. Biol. Chem.*, **1977**, *252*, 1034-1040.
16. Obermeier, S.; Rudloff, S.; Pohlentz, G.; Lentze, M.J.; Kunz, C. Secretion of C-13-labelled oligosaccharides into human milk and infant's urine after an oral C-13 galactose load. *Isot. Environ. Health Stud.*, **1999**, *35*, 119-125.
17. Albrecht, S.; Schols, H.A.; van den Heuvel, E.G.H.M.; Voragen, A.G.J.; Gruppen, H. CE-LIF-MS^n profiling of oligosaccharides in human milk and feces of breast-fed babies. *Electrophoresis*, **2010**, *31*, 1264-1273.
18. American Red Cross. Method 31. In *Immunohematology methods and procedures*: ISBN 0-86536-208-4, **1993**.
19. Ameno, S.; Kimura, H.; Ameno, K.; Zhang, X.; Kinoshita, H.; Kubota, T.; Ijiri, I. Lewis and secretor gene effects on Lewis antigen and postnatal development of Lewis blood type. *Biol. Neonate*, **2001**, *79*, 91-96.
20. Stahl, B.; Thurl, S.; Zeng, J.R.; Karas, M.; Hillenkamp, F.; Steup, M.; Sawatzki, G. Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem.*, **1994**, *223*, 218-226.
21. Moro, G.E.; Stahl, B.; Fanaro, S.; Jelinek, J.; Boehm, G.; Coppa, G.V. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants. *Acta Paediatr.*, **2005**, *94*, 27-30.
22. Ninonuevo, M.R.; Park, Y.; Yin, H.; Zhang, J.; Ward, R.E.; Clowers, B.H.; German, J.B.; Freeman, S.L.; Killeen, K.; Grimm, R.; Lebrilla, C.B. A strategy for annotating the human milk glycome. *J. Agric. Food Chem.*, **2006**, *54*, 7471-7480.
23. Sengkhamparn, N.; Verhoef, R.; Schols, H.A.; Sajjaanantakul, T.; Voragen, A.G.J. Characterisation of cell wall polysaccharides from okra (*Abelmoschus esculentus* (L.) Moench). *Carbohydr. Res.*, **2009**, *344*, 1824-1832.
24. Herrmann, A.; Davies, J.R.; Lindell, G.; Martensson, S.; Packer, N.H.; Swallow, D.M.; Carlstedt, I. Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. *J. Biol. Chem.*, **1999**, *274*, 15828-15836.

25. Albrecht, S.; van Muiswinkel, G.C.J.; Schols, H.A.; Voragen, A.G.J.; Gruppen, H. Introducing capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) for the characterization of konjac glucomannan oligosaccharides and their in vitro fermentation behavior. *J. Agric. Food Chem.*, **2009**, *57*, 3867-3876.
26. Hilz, H.; de Jong, L.E.; Kabel, M.A.; Schols, H.A.; Voragen, A.G.J. A comparison of liquid chromatography, capillary electrophoresis, and mass spectrometry methods to determine xyloglucan structures in black currants. *J. Chromatogr. A.*, **2006**, *1133*, 275-286.
27. Erney, R.M.; Malone, W.T.; Skelding, M.B.; Marcon, A.A.; Kleman-Leyer, K.M.; O'Ryan, M.L.; Ruiz-Palacios, G.; Hilty, M.D.; Pickering, L.K.; Prieto, P.A. Variability of human milk neutral oligosaccharides in a diverse population. *J. Pediatr. Gastr. Nutr.*, **2000**, *30*, 181-192.
28. Oriol, R.; Le Pendu, J.; Mollicone, R. Genetics of ABO, H, Lewis, X and related antigens. *Vox Sang.*, **1986**, *51*, 161-171.
29. Albrecht, S.; Schols, H.A.; Klarenbeek, B.; Voragen, A.G.J.; Gruppen, H. CE-LIF as an analysis and quantification tool for galactooligosaccharides extracted from complex food matrices. *J. Agric. Food Chem.*, **2010**, *58*, 2787-2794.
30. Antonowicz, I.; Lebenthal, E. Developmental pattern of small intestinal enterokinase and disaccharidase activities in human fetus. *Gastroenterology*, **1977**, *72*, 1299-1303.
31. Katayama, T.; Sakuma, A.; Kimura, T.; Makimura, Y.; Hiratake, J.; Sakata, K.; Yamanoi, T.; Kumagai, H.; Yamamoto, K. Molecular cloning and characterization of *Bifidobacterium bifidum* 1,2-alpha-L-fucosidase (AfcA), a novel inverting glycosidase (glycoside hydrolase family 95). *J. Bacteriol.*, **2004**, *186*, 4885-4893.
32. Strecker, G.; Hondiassah, T.; Fournet, B.; Spik, G.; Montreuil, J.; Maroteaux, P.; Durand, P.; Farriaux, J.P. Structure of 3 major sialyl-oligosaccharides excreted in urine of 5 patients with 3 distinct inborn diseases - I cell disease and 2 new types of mucolipidosis. *Biochim. Biophys. Acta*, **1976**, *444*, 349-358.
33. Stark, P.L.; Lee, A. The microbial ecology of the large bowel of breastfed and formula-fed infants during the first year of life. *J Med Microbiol*, **1982**, *15*, 189-203.
34. Sangild, P.T. Gut responses to enteral nutrition in preterm infants and animals. *Exp. Biol. Med.*, **2006**, *231*, 1695-1711.
35. Schenkel-Brunner, H. Blood group antigens. In *Comprehensive glycoscience. From chemistry to system biology; vol.3*; Kamerling, J.P., Boons, G.J., Lee, Y.C., Suzuki, A., Taniguchi, N.,Voragen, A.G.J., Eds; Elsevier: Oxford, UK, **2007**; pp 343-372.
36. Albrecht, S.; Schols, H.A.; van Zoeren, D.; van Lingen, R.A.; Groot Jebbink, L.J.M.; van den Heuvel, E.G.H.M.; Voragen, A.G.J.; Gruppen, H. Oligosaccharides in feces of breast- and formula-fed babies. *Carbohydr. Res.*, **doi: 10.1016/j.carres.2011.06034**.

Chapter 6

Introducing capillary electrophoresis with laser-induced fluorescence (CE-LIF) for the characterization of konjac glucomannan oligosaccharides and their *in vitro* fermentation behavior

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Abstract

The application of capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) as a tool for the characterization of complex carbohydrate structures was investigated for konjac glucomannan (KGM) oligosaccharide mixtures and the monitoring of their structural changes during 72 h of *in vitro* fermentation with human gut flora. Different types of KGM oligosaccharide mixtures were produced from a KGM polysaccharide using endo-β-(1,4)-mannanase and endo-β-(1,4)-glucanase. Distinction of structures emerging from different enzymatic KGM digests and detection of acetylated oligosaccharides were possible by both CE-LIF and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Using CE-LIF it could be shown that the endo-β-(1,4)-glucanase digest exhibited a large degradability of the DP2, DP3, DP5, and DP6 components during *in vitro* fermentation, whereas the endo-β-(1,4)-mannanase digest was digested only slightly, thereby highlighting the influence of structural characteristics on the fermentability by human gut flora.

Introduction

The profiling of complex oligosaccharides is an analytical challenge, complicated by the presence of numerous isomeric structures. However, the monitoring of structural features of complex carbohydrate substrates during *in vitro* and *in vivo* fermentation trials with human gut flora is a crucial step for judging their fate in the human colon and thus their prebiotic potential. Prebiotics are defined to be nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or more desired bacteria species in the colon and thus improve host health.[1] Prebiotics are associated with nondigestible oligosaccharides (NDOs), meaning they are transiting the human small intestine inert[2] and, being oligosaccharides, they are defined to consist of 3-10 monosaccharide units according to IUB-IUPAC. However, there is no rational or physiological limit for setting the latter limits of DP10.[3] Frequently used prebiotic substrates are lactose-based galactooligosaccharides (GOS) and fructooligosaccharides (FOS)/inulin, and large numbers of studies have been performed that investigated the prebiotic potential of GOS and FOS,[4,5] confirming their selective stimulation of beneficial gut bacteria. A drawback of these commonly used prebiotics might be their fast fermentation in the proximal part of the colon.[3,6] Many colonic diseases, such as ulcerative colitis and tumors, occur in the distal part of the gastrointestinal tract. Targeting the more distal colonic regions is therefore an important issue in the fields of prebiotic research and design.[3,7] Due to their possible resistance to bacterial metabolization in the proximal colon, complex carbohydrate structures are of increasing interest and need to be tested *in vitro* and *in vivo* for their prebiotic suitability. Earlier studies on the *in vitro* fermentability of differently substituted xylooligosaccharides (XOS) showed that nonsubstituted XOS were fermented more easily than the more complex structures of acetylated XOS, although the effect of adaptation to the substrate has not been described.[8]

Hence, having a combination of acetylation of the sugars and a backbone of two different sugars, konjac glucomannan (KGM) and oligosaccharides derived thereof seem to be promising prebiotic substrates. KGM is a neutral polysaccharide derived from the tuber of *Amorphophallus konjac* C. Koch and native to Southeast Asia.[9] It is composed of β-(1,4)-linked D-mannose and D-glucose residues in a molar ratio of 1.6:1.[10] Five to ten percent of the hexose residues are acetylated.[11] However, it has not yet been established if the acetyl groups are attached to the glucose or mannose residues, and our knowledge concerning the sequence of the mannose and glucose residues over the polysaccharidic chain is still limited. Although efforts have been made to investigate the monosaccharide sequence, still

blockwise[12] as well as random distributions[10] are being considered. KGM is estimated to be a slightly branched polysaccharide with branches consisting of 11-16 hexose residues (mannose, glucose, and galactose).[13] Besides its potential prebiotic characteristics, KGM is also known to have beneficial effects on human health and has been consumed in Asia for centuries. Being a soluble dietary fiber, it can positively influence hyperglycemia and hypercholesterolemia and may result in a delayed stomach emptying, hence providing a feeling of satiety and possibly facilitating weight loss.[14-16] Studies of the effect of KGM on the fecal microflora in Balb/c mice revealed a dose-dependent bifidogenic effect, especially for hydrolyzed KGM.[17] However, the influence of fermentation on KGM structures has not yet been studied in detail, mainly due to limited analytical capabilities. Recently, capillary electrophoresis with laser induced fluorescence detection (CE-LIF) has been shown to be a suitable technique for the study of complex oligosaccharide mixtures, which are laborious and difficult to analyze using reversedphase high-performance liquid chromatography (RP-HPLC) or high-performance anion exchange chromatography (HPAEC) and mass spectrometry coupling (HPAEC-MS).[18-21] Therefore, in this study CE-LIF and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were used to elucidate the structural characteristics and the *in vitro* fermentation by human gut flora of structurally different KGM oligosaccharide mixtures.

Materials and Methods

Materials and enzymes

The konjac glucomannan polysaccharide (KGM native) was obtained from Kalys (Kalys, St-Ismier, France). The standards used for HPAEC and CE-LIF were cellodextrin [β-(1,4)-linked glucose oligosaccharides] and D-(+)-mannose, both from Sigma-Aldrich (St. Louis, MO), as well as manno-β-(1,4)-biose, manno-β-(1,4)-triose, manno-β-(1,4)-tetraose, and manno-β-(1,4)-pentaose, all obtained from Megazyme (Bray, Ireland). The crude cellulase preparation used was produced by *Trichoderma viride* (Maxazyme, DSM Food Specialties, Delft, The Netherlands). Endo-β-(1,4)-glucanase VI [EC 3.2.1.4, GH family 7, specific activity = 11 mU/mg of protein on CM-cellulose (type Akucell AF 0305, Akzo, Arnhem, The Netherlands), 20 mM sodium citrate buffer, pH 3.5, 30 ºC] was purified from this commercial cellulase preparation according to the method of Beldman et al.[22] Endo-β-(1,4)-mannanase [EC 3.2.1.78, GH family 5, specific activity = 33 U/mg of protein on palm kernel mannan (extracted and prepared by Düsterhöft et al,[23] 0.05 M sodium acetate buffer, pH 5, 30 ºC] was purified from a

commercial *Aspergillus niger* enzyme mixture (Gamanase III, Novozymes, Bagsvaerd, Denmark) according to the method of Düsterhöft et al.[24]

Preparation of oligosaccharides from konjac glucomannan polysaccharide

Digestions with the crude cellulase preparation and with the pure endo-β-(1,4)-mannanase were performed in 0.05 M sodium acetate buffer at pH 5. KGM results in highly viscous solutions and tends to form clumps. Therefore, it had to be prepared by adding the KGM powder slowly to vigorously stirred water. The KGM polysaccharide solution (10 mg/mL) was incubated with a 0.1% (v/v) cellulase concentration. The enzyme level applied for endo-β-(1,4)-mannanase was 70 µL of enzyme solution (3.3 U/mL) per milliliter of substrate solution (5 mg/mL). The incubation with endo-β-(1,4)-glucanase VI was performed in 20 mM sodium citrate buffer at pH 3.5, and 363.6 µL of enzyme solution (0.8 U/mL) was added per milliliter of substrate solution (5 mg/mL). The enzyme dosages were chosen according to the best results in oligosaccharide profile obtained after testing different substrate to enzyme ratios. The incubation parameters were 30 ºC and 24 h at shaking conditions for all enzymes. After hydrolysis, the enzymes were inactivated (10 min, 100 ºC), and the hydrolysate was centrifuged (5 min, 10000g, room temperature). Saponification was performed for 16 h at 4 ºC, shaking conditions (25 mL of 0.2 M sodium hydroxide (NaOH) per 200 mg of substrate), with parts of the KGM digests to obtain deacetylated material. The solutions were desalted by adding an excess of anion-exchange resin (AG 50W-X8 resin; Bio-Rad, Hercules, CA) and freeze-dried.

For a more detailed structural characterization, the oligosaccharides in the digests were fractionated according to their DP. Fractionation was performed on a Spectra System HPLC (Thermo Finnigan, Waltham, MA) equipped with a BioGel P2 column (26 mm x 900 mm, 200-400 mesh, Bio-Rad) thermostated at 60 ºC and eluted with Milli-Q water at 0.3 mL/min. Samples (3-13 mg) were either injected by means of an autosampler or directly applied onto the column. The column efflux was first led through a refractive index detector (Shodex RI72, Showa Denko K.K., Tokyo, Japan) before it was collected in fractions of 10 min by a fraction collector (Superfrac, GE Amersham, Uppsala, Sweden). Appropriate fractions were pooled and freeze-dried for further analysis.

Prior to the fermentation experiment, monomers were removed from the initial KGM digests. The cellulase and endo-β-(1,4)-mannanase digest were subjected to gel filtration on a Superdex 30 prep grade column (600 mm x 120 mm, 24-49 µm bead size; GE Amersham, Uppsala, Sweden). Samples of up to 2 g were applied on the column and were eluted with distilled water (25 mL/min) at room temperature using an Akta Explorer system

(GE Amersham). A Shodex RI-72 detector (Showa Denko K.K.) was used to monitor the refractive index. Fractions were collected each 2 min with a Superfrac fraction collector (GE Amersham) and examined for monomers using HPAEC-PAD. Monomer-free fractions were freeze-dried.

Fermentation

The fermentation broth used was prepared according to the method of Kabel et al.[8] A solution of each konjac glucomannan substrate in water [1% (w/v)] was prepared (S1); for the blank S1 consisted of pure water. The substrates used were fermented in duplicate. For solution 2 (S2) bacto yeast nitrogen base (Difico, Detroit, MI) was dissolved in water (6.7 g/L). Solution 3 (S3) was composed of 40% (v/v) of a watery salt solution [magnesium sulfate (0.2 g/L), calcium chloride (0.2 g/L), potassium hydrogen phosphate (1 g/L), potassium dihydrogen phosphate (1 g/L), sodium hydrogen carbonate (10 g/L), and sodium chloride (2 g/L)], 5% (w/v) casein enzymatic hydrolysate (N-Z Amine A, Sigma-Aldrich), and 0.5% (w/v) sodium thioglycolate]. S2 and S3 were adjusted to pH 6-7 by 1 M NaOH. The three solutions S1, S2, and S3 were combined in a ratio of (v/v) 17:1:2 in serum flasks, which were filled at 50% and closed by a butyl rubber stopper. Taking into account Maillard reactions, filter-sterilized S2 was added only after the flasks had been autoclaved (15 min, 121 ºC).

To create anaerobic conditions, the air in the serum flasks was exchanged for a nitrogen atmosphere (0.7 bar underpressure) by using the Anaerobic Lap system (8 cycles) (GR Instruments B.V., Wijk bij Duurstede, The Netherlands).

Fecal samples were obtained from three healthy human volunteers in sterile plastic cups and were processed shortly after defecation to avoid aerobic conditions. The processing of the samples was performed according to methods of Kabel et al. and Hartemink.[8,25] A 10% (w/v) mixed fecal slurry was prepared by adding one spoon (~5 g) of each fecal sample to 150 mL of buffered peptone water containing L-cysteine hydrochloride (0.5 g/L, pH 6.7). The fecal slurry was mixed, and after an equilibration time of 30 min, the 10-fold diluted feces were diluted 100 times further by adding them to serum flasks containing a sterilized solution (pH 6.7) of neutralized bacterial peptone (1 g/L), sodium chloride (8 g/L), and L-cysteine hydrochloride (0.5 g/L) and a nitrogen atmosphere. Serum flasks containing fermentation medium were inoculated with 20% (v/v) of the volume of S1, S2, and S3 present in the flask using the 1000-fold fecal dilutions. The serum flasks, representing one KGM substrate each, were incubated at 37 ºC.

Samples were removed eight times between 0 and 72 h. Enzymes were inactivated by heating (5 min, 100 ºC, 1100 rpm). The samples were centrifuged, and the supernatants were stored at -80 ºC for further analysis. For a screening of the bacterial growth, the pH of the supernatants was measured, and the optical density of the bacterial residue was determined at 600 nm (GeneQuant pro spectrophotometer, Biochrom, Cambridge, U.K.).

Analytical methods

Monosaccharide composition

The neutral sugar content and composition were determined by gas-liquid chromatography according to the method of Englyst et al using inositol as an internal standard.[26] Samples were pretreated with aqueous 72% sulfuric acid (H_2SO_4) (w/w) (1 h, 30 ºC), followed by hydrolysis with 1M H_2SO_4 (3 h, 100 ºC) before they were analyzed as volatile alditol acetates on a Carbo Erba Mega 5160 GC (Thermo Finnigan).

Degree of acetylation (DA)

The degree of acetylation was determined according to the method of Voragen et al.[27] The ester bonds were saponified by 0.4 N NaOH in a water-isopropanol mixture, and the released acetic acid was determined on a Spectra System HPLC (Thermo Finnigan) equipped with an Aminex HPX 87H column (Bio-Rad) and a Shodex RI72 detector (Showa Denko K.K.). The eluent used was 5.0 mM H_2SO_4 (0.6 mL/min, 40 ºC).

HPSEC

The molecular weight distribution of large oligosaccharide and polysaccharide fractions was investigated by high performance size exclusion chromatography (HPSEC) using a Spectra System HPLC (ThermoFinnigan) equipped with a refractive index detector Shodex RI72 (Showa Denko K.K.). Twenty microliter sample solutions (5 mg/mL) were injected automatically, using a Thermo AS3000 autosampler. Separation was performed on three TosoH Biosep-TSK-Gel G columns in series (4000PW_{XL}-3000PW_{XL}-2500PW_{XL}, each 7.5 mm x 300 mm; TosoH, Japan) in combination with a PW_{XL} guard column (6 mm x 40 mm; TosoH). For elution, 0.2 M sodium nitrate was used at a flow rate of 0.8 mL/min at 30 ºC. The system was calibrated with pullulan standards (Sigma-Aldrich; mass range from 180 Da to 790 kDa).

MALDI-TOF MS

For MALDI-TOF MS of oligosaccharides, an Ultraflex workstation (Bruker Daltronics, Bremen, Germany) equipped with a nitrogen laser of 337 nm was used. The measurement was performed in the positive mode. After a delayed extraction time of 200 ns, the ions were accelerated with a 25 kV voltage. The data were collected from averaging 100 laser shots, with the lowest laser energy necessary to obtain sufficient spectra intensity. The mass spectrometer was calibrated with a mixture of maltodextrins (AVEBE, Veendam, The Netherlands; mass range of 527-2309 Da as sodium adducts). For sample preparation, samples (1 mg/mL) were desalted with anion-exchange material (AG 50W-X8 resin; Bio-Rad). The desalted samples (1 µL) were mixed directly on a MS target plate with 2 µL of matrix solution consisting of a watery solution of 2,5-dihydroxybenzoic acid (10 mg/mL; Bruker Daltonics). The mix was dried under a stream of warm air.

HPAEC

The oligosaccharide profile was monitored by HPAEC (Dionex ISC 3000; Dionex, Sunnyvale, CA), equipped with a Dionex Carbopac PA-1 column (2 mm x 250 mm) in combination with a Carbopac PA-1 guard column (2 mm x 50 mm). Samples (20 µL) of a concentration of 0.1 mg/mL were injected by means of a Dionex ISC3000 autosampler. The oligomers were eluted (0.3 mL/min) by using a gradient of 0-400 mM NaOAc in 100 mM NaOH during 40 min. Each elution was followed by a washing step (5 min, 1 M NaOAc in 100 mM NaOH) and an equilibration step (20 min, 100 mM NaOH). Detection was performed using a Dionex ED40 detector in the pulsed amperometric detection mode.

CE-LIF

Samples for CE-LIF were prepared using the ProteomeLab Carbohydrate Labeling and Analysis Kit (Beckman Coulter, Fullerton, CA). To samples containing approx. 0.1 mg of carbohydrate was added 5 nmol of maltose as an internal standard and mobility marker. This mixture was dried using the SpeedVac Concentrator Savant ISS110 (Thermo Electron Corp., Waltham, MA). The mono- and oligosaccharides present in the dried sample were labeled with 9-aminopyrene-1,4,6-trisulfonate (APTS). Separation of the derivatized carbohydrates was performed using a ProteomeLab PA 800 characterization system (Beckman Coulter) equipped with a polyvinyl alcohol-coated capillary (50 µm x 50.2 cm). Detection was done with a laser-induced fluorescence detector (LIF) (Beckman Coulter) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm after 40 cm

capillary length. The capillary was rinsed with water (5 min, 30 psi) and then with separation gel buffer (2 min, 30 psi); the rinsing buffer was exchanged every 20 samples. Subsequently, samples were loaded hydrodynamically (4 s at 0.5 psi, representing approx. 14 nL of sample solution) on the capillary, which was kept at 25 ºC. Separation was performed in the reversed polarity mode (30 kV, 20 min) in a 25 mM acetate buffer (pH 4.75) containing 0.4% polyethylene oxide.

Results

Production and characterization of KGM material

Knowledge of the characteristics of KGM material is essential for understanding the changes the material undergoes upon fermentation with human gut flora. All KGM materials produced were derived from the native polymeric KGM. The sugar composition of the native KGM showed a mannose:glucose molar ratio of 1.5:1 and galactose contributing to the total sugar content with a mole percentage of 1%. The DA was determined to be 7.9%. For a structural characterization, incubation of the polysaccharide was performed with endo-β-(1,4)-mannanase and endo-β-(1,4)-glucanase. MALDI-TOF MS was used for a first screening of the KGM oligosaccharides obtained after the enzymatic digestion of the polymer. The mass spectra obtained showed the sodium (Na) and potassium (K) adducts of hexose oligomers. Besides, masses were found that indicate the presence of one to three acetyl groups bound to hexose oligomers. Applying a mass window ranging from m/z 500 to 2500 made it possible to detect oligosaccharides of DP3-DP14. Monomers, dimers, and oligosaccharides with DP > 14 were not detected with this method. The use of a purified endo-β-(1,4)-glucanase resulted in the appearance of oligosaccharides covering the whole range of DP3-DP14 (**Figure 1A**).

Instead, incubation with endo-β-(1,4)-mannanase resulted in oligosaccharides showing a smaller DP range of DP3-DP9 (**Figure 1B**). Comparison of the MALDI-TOF mass spectra of the endo-β-(1,4)-mannanase digest and the endo-β-(1,4)-glucanase digest revealed a different distribution of acetyl groups on the oligosaccharides with different DPs. The insets in **Figure 1** show the degree of acetyl substitution of the oligosaccharides, based on the signal heights of the MALDI-TOF mass spectra. Within one DP the total height of all Na and K adduct signals was set to 100% and the relative amount of DP having x, y, z, or k acetyl groups was indicated. This is merely an indication, because it was only assumed that all oligomers would have the same response. The higher the DP, the higher seems to be the amount of single-acetylated and multiple-acetylated oligosaccharides for both substrates.

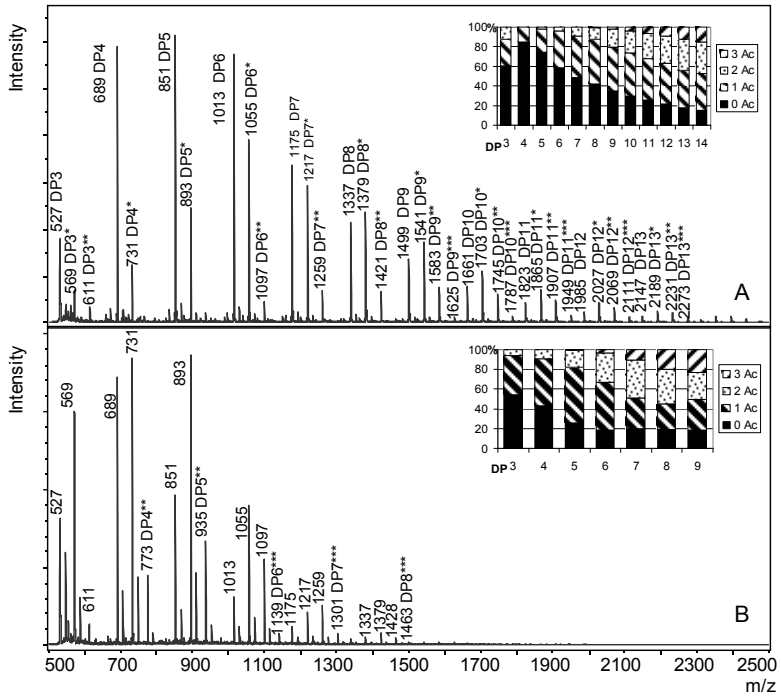

Figure 1. MALDI-TOF mass spectra of (A) KGM endo-β-(1,4)-glucanase digest and (B) KGM endo-β-(1,4)-mannanase digest. Masses indicated represent sodium adducts. *, Single-acetylated DP; **, double-acetylated DP; ***, triple-acetylated DP. Insets represent the degree of acetylation per DP. (Ac = acetyl group.)

The DA of smaller oligomers is higher for the endo-β-(1,4)-mannanase digest.

Because MALDI-TOF MS is only a screening of the oligomers present, another method was needed for a more detailed characterization. HPAEC-PAD is commonly used for characterizing oligosaccharidic material. Comparison of the HPAEC-PAD separation of the series mannose, mannobiose, and mannooligosaccharides with the series glucose, cellobiose, and cellooligosaccharides (**Figure 2A**) revealed a complex elution profile in which elution was certainly not based on size alone. Cellobiose is eluting almost contemporaneously with mannopentaose. The mixed glycosidic linkages of glucose and mannose present in KGM make the chromatograms of KGM oligomers even more complex and an assignment of the DPs impossible (data not shown). Therefore, the recently established CE-LIF method was tested for its capacity to separate complex oligomer mixtures resulting from KGM digests.

Electrophoretic separation demands charged molecules. Therefore, the neutral oligosaccharides were labeled with the threefold negatively charged fluorescent dye APTS.

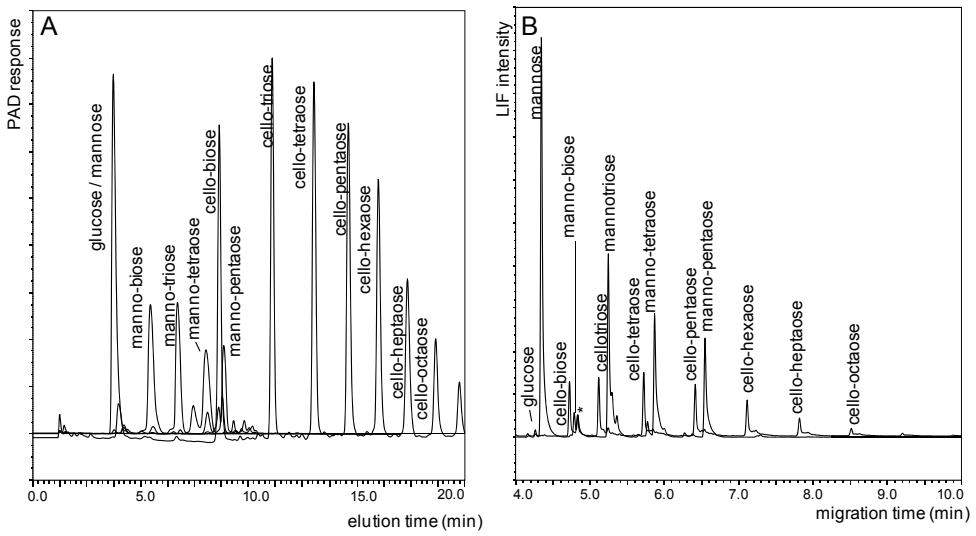

Figure 2. (A) HPAEC PAD chromatogram of cello-oligomers and manno-oligomers; (B) CE-LIF electropherogram of cello-oligomers and manno-oligomers. *, Maltose (internal standard).

For the standard mixtures a groupwise migration according to the DP was obtained with CE-LIF (**Figure 2B**), which presents a crucial advantage compared to the separation obtained by HPAEC-PAD. Analyzing the endo-β-(1,4)-mannanase and endo-β-(1,4)-glucanase digests with CE-LIF resulted in electropherograms showing distinct peak clusters with a repeating pattern, especially for the endo-β-(1,4)- glucanase digest (**Figure 3**). Free APTS as well as mono- and oligosaccharidic components of the endo-β-(1,4)-mannanase digest migrated completely between the migration times 3 and 7.5 min, resulting in a separation window of < 4.5 min. The peak clusters of the endo-β-(1,4)-glucanase digest were distributed over the whole separation time of 20 min (electropherograms shown were all zoomed in to a maximum of 15 min). For an identification of the DPs in these complex mixtures of nonacetylated and acetylated oligosaccharides, BioGel P2 fractions of the endo-β-(1,4)-mannanase and endo-β-(1,4)-glucanase digests were analyzed with CE-LIF. The DPs of those fractions were estimated by MALDI-TOF MS. Subsequently, it was possible to assign each of these distinct peak clusters appearing in **Figure 3** with their respective DP.

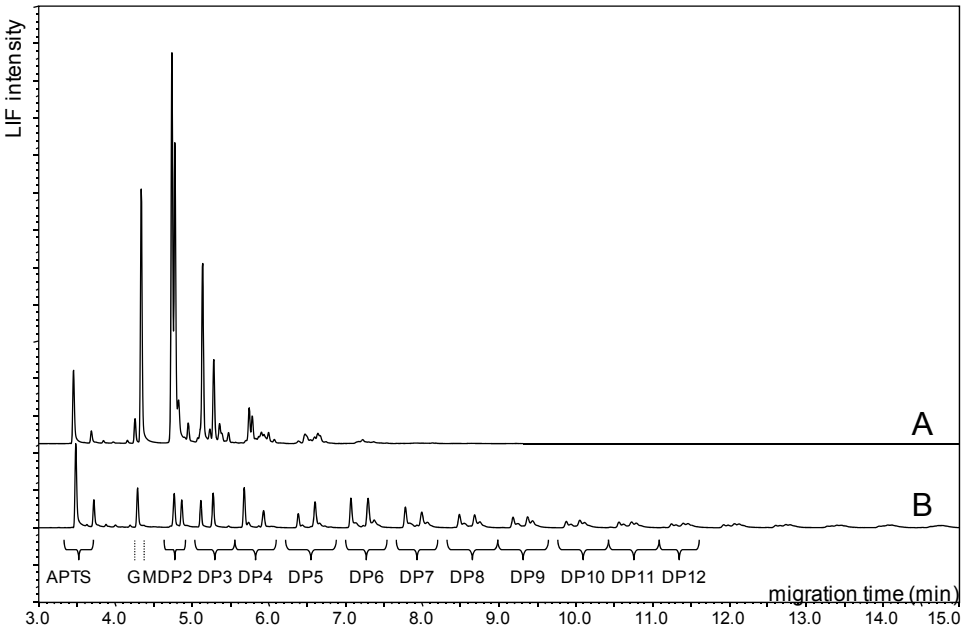

Figure 3. CE-LIF electropherogram of (A) KGM endo-β-(1,4)-mannanase digest and (B) KGM endo-β-(1,4)-glucanase digest.

The endo-β-(1,4)-mannanase and endo-β-(1,4)-glucanase digest showed complementary peak patterns. Clearly, CE is able to separate both acetylated and nonacetylated oligosaccharides in a single run, whereas the alkaline HPAEC elution does not give any information on the presence of acetylated oligosaccharides. Saponified substrates were subjected to CE-LIF analysis to investigate if all peaks originally present in electropherograms of nonsaponified substrates were still present after saponification. Peaks that were not present any more were assigned as peaks representing acetylated oligomers. For the endo-β-(1,4)-mannanase digest, disappearance of peaks mainly at the end of each DP cluster was observed (**Figure 4A**), whereas for endo-β-(1,4)-glucanase, the disappearence of peaks was not restricted to the end of the DP clusters (**Figure 4B**). Acetylation is more abundant for higher DPs in the endo-β-(1,4)-glucanase digest, whereas saponification effects already clear changes with DP3 and DP4 in the endo-β-(1,4)-mannanase digest.

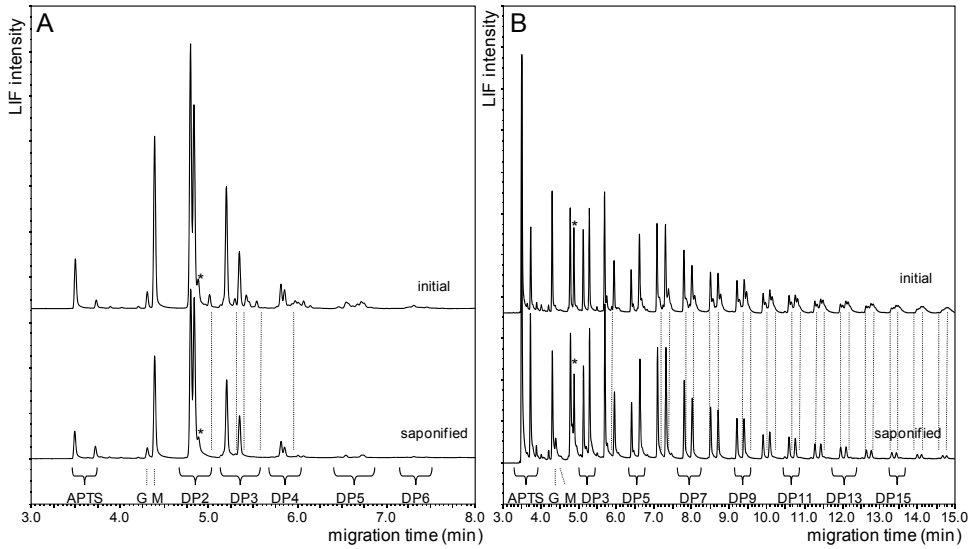

Figure 4. CE-LIF electropherograms of (A) initial and saponified KGM endo-β-(1,4)-mannanase digest and (B) initial and saponified KGM endo-β-(1,4)-glucanase digest. *, Maltose (internal standard). Vertical dotted lines indicate peaks that are no longer present after the saponification experiment.

In vitro fermentation of KGM material

Because methods were now available for a structural characterization, they were applied to the monitoring of the *in vitro* fermentation of KGM substrates. Therefore, oligosaccharides were prepared by enzymatic digestion of the KGM polymer with endo-β-(1,4)-mannanase and a crude cellulase mixture. Four different KGM substrates and a fermentation blank (without any carbohydrate source added) were fermented in duplicate by a fecal slurry consisting of a mix of feces from three human volunteers. In summary, the substrates used were a cellulase and an endo-β-(1,4)-mannanase digest of KGM. The enzymatic digests were used for the fermentation experiments in their monomer-free forms. To test the influence of the presence of monomers, the cellulase digest was as well fermented in its monomer-containing form. The behaviour of the KGM oligosaccharides was compared to the fermentation behavior of the parental native polysaccharide KGM. The substrates were autoclaved to exclude bacterial contamination. MALDI-TOF mass spectra of autoclaved enzymatically treated KGM material showed largely reduced signal intensity or even a lack of signals for acetylated oligosaccharides. All oligosaccharidic KGM mixtures could, therefore, be considered as substrates low in or without acetyl groups. For estimating bacterial growth and activity, OD and pH in the samples taken during the fermentation

experiment (up to 72 h) were measured. HPSEC, MALDI-TOF MS, and CE-LIF analysis were used to monitor the *in vitro* fermentation and utilization of the polymer or the individual oligosaccharidic carbohydrate components. Results from the duplicate experiment were comparable and are not further considered. All KGM samples showed an increase in optical density and a decrease of pH from an average pH 6.5 to a common end point of pH 4.7-4.8 during 72 h of *in vitro* fermentation. With the monomer-containing cellulase digest, the pH reached the end point after only 24 h, whereas for the polysaccharide a more gradual decrease to the end point pH after 72 h was found. The result for the monomer-free cellulase digests was situated between these two extrema mentioned. In contrast, the monomer-free endo-β-(1,4)-mannanase digest behaved similarly to the monomer-containing cellulase digests. The fermentation blank showed neither an increase of OD nor a decrease in pH upon 72 h of fermentation time. Studying the degradation pattern of the monomer-containing cellulase digest by CE-LIF revealed the following characteristics: Monomeric material was fermented during the first 24 h, and di- and trimers were largely fermented especially between 24 and 72 h. For the larger oligomers, no changes were observed during the *in vitro* fermentation (**Figure 5A**). The fermentation of the monomer-free cellulase digest resulted in a different pattern compared to its monomer containing analogon (**Figure 5B**). During the first 24 h, no changes within the oligosaccharidic material were observed. Between 24 and 72 h, DP6 was completely and DP5 largely degraded, whereas DP4 was more resistant. The areas of the first peak, identified as a dimer (migration time of 4.7 min), and the peaks belonging to DP3 (migration time between 5.05 and 5.3 min) decreased also over time. The decrease in areas over time for the latter peaks described was 50% higher in the monomer-containing analogue, compared to the monomer-free preparation. The CE-LIF electropherograms of the cellulase digest at t = 0 h (**Figure 5**) did not completely match with the electropherogram obtained for the endo-β-(1,4)-glucanase digest (**Figure 3B**), as the latter one showed additional side peaks aside from the common main peaks found for both electropherograms. The CE-LIF electropherograms of the monomer-free endo-β-(1,4)-mannanase digest did not show a distinct degradation pattern. The increase of oligosaccharides with the size of DP3 indicated, nevertheless, an ongoing carbohydrate fermentation upon 72 h of fermentation. MALDI-TOF MS results of the KGM mixtures confirmed the degradation of oligosaccharidic material during the 72 h *in vitro* fermentation and did not provide any further information.

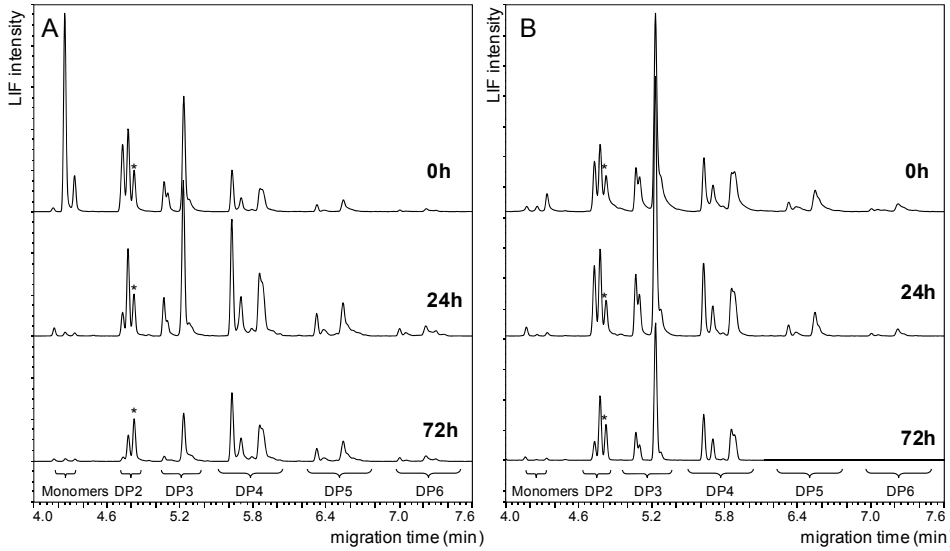

Figure 5. CE-LIF electropherograms obtained after 0, 24, and 72 h of fermentation of (A) KGM cellulase digest containing monomers and (B) monomer-free KGM cellulase digest. *, Maltose (internal standard).

Analysis of CE-LIF electropherograms, MALDI-TOF mass spectra, and HPSEC chromatograms resulted in complementary information concerning the fermentation of the KGM polymer. As observed by the shift in molecular weight in **Figure 6A**, the polymer, which had originally a Mw of ≥ 500 000 (≥ DP 3000), was gradually degraded to oligomeric material during 72 h of fermentation. By integrating the areas under the curves it could be stated that the oligomers formed were also utilized to some extent. The peak areas representing oligomeric material increased to only 35% of the decreased area belonging to polymeric material in the HPSEC chromatograms. In **Figure 6B**, the emergence of oligomers during the fermentation of the polymer is shown by CE-LIF electropherograms. The intensity of the oligomers increased, with DP5 being the most abundant DP after 72 h. MALDI-TOF mass spectra showed, in addition to the emerging series of hexoses, the appearances of series of masses representing single-acetylated hexoses.

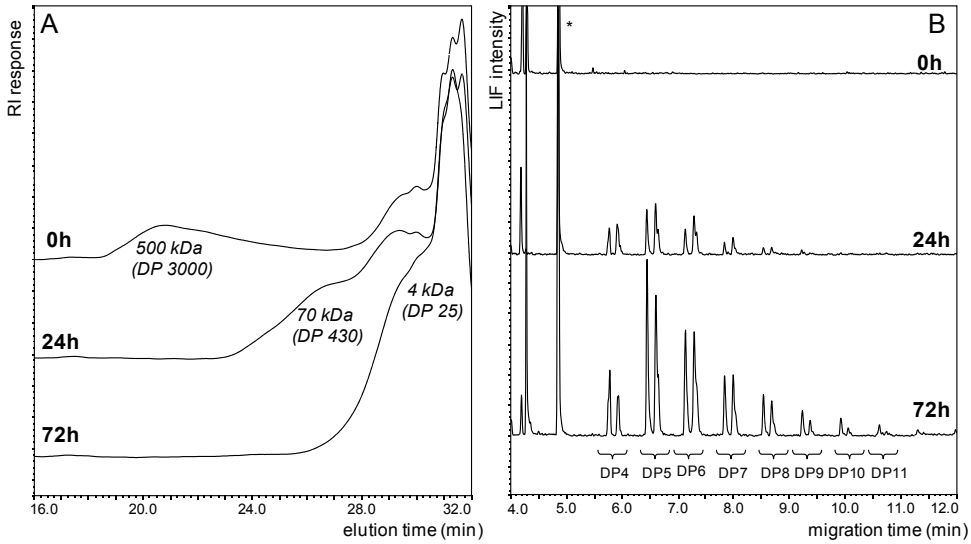

Figure 6. (A) HPSEC chromatograms obtained after 0, 24, and 72 h of fermentation of the KGM polysaccharide; (B) CE-LIF electropherograms obtained after 0, 24, and 72 h of fermentation of the KGM polysaccharide. *, Maltose (internal standard).

Discussion

Characterization of KGM material

To study the influence of the chemical structure and the KGM mixture composition on the fermentability, the KGM material used for fermentation was characterized in the first part of this study. Combination of MALDI-TOF MS with results obtained by applying the recently established CE-LIF method contributed to a structural approach on endo-β-(1,4)-mannanase and endo-β-(1,4)-glucanase digests of KGM. For KGM with a M:G ratio of 1.5:1, the KGM chain provided more cleaving sites for an enzymatic digest with endo-β-(1,4)-mannanase, which is expected to cleave the glycosidic linkage between two mannose residues (DP3-DP9 in MALDI-TOF mass spectra) than for an enzymatic digest with endo-β-(1,4)-glucanase, which is expected to cleave the glycosidic linkage between two glucose residues (DP3-DP14 in MALDI-TOF mass spectra). Not only the excess of mannose residues in the KGM polysaccharide but also the fact that endo-β-(1,4)-mannanases cleave M-G bonds in the konjac glucomannan polysaccharidic chain[28] explains the good degradability of KGM with endo-β-(1,4)-mannanase as observed with MALDI-TOF MS. The enhanced degradation with endo-β-(1,4)-mannanase was confirmed with

CE-LIF, which provided oligosaccharide separation that showed advantages over the commonly performed separation with HPAEC-PAD and can be considered as the method of choice for the structural analysis of complex carbohydrate oligosaccharides. Besides the fast separation (20 min per run), the mole-based detection, and the detection of acetylated oligosaccharides, a distinct separation in peak clusters representing oligosaccharides of the same DP was obtained with CE-LIF. A complementary structural composition was found for the endo-β-(1,4)-mannanase and the endo-β-(1,4)-glucanase digests of KGM. Saponification experiments resulted in the detection of acetylated oligosaccharides that differed in position in the electropherograms of the endo-β-(1,4)-mannanase and endo-β-(1,4)-glucanase digest. The results obtained for the saponification experiment with CE-LIF analysis (**Figure 4**) have to be compared with the results found for the acetylation pattern with MALDI-TOF MS (**Figure 1**). Both digests showed an increased intensity for acetylated oligosaccharides with increasing DP in their mass spectra as well as in their electropherograms. However, the endo-β-(1,4)-glucanase digest showed a lower release of acetylated oligosaccharides for DP2-DP4. The endo-β-(1,4)-glucanase is therefore assumed to be less tolerant toward acetylation in the glucomannan backbone.

Hypothesizing the chemical structure of KGM

By combining results, it was possible to form a hypothesis on the chemical structure. Peak assignment was done by transferring information given by Cescutti et al,[10] who examined enzymatic digests of KGM produced by endo-β-(1,4)-mannanase (issuing from *Aspergillus niger*) and cellulase (issuing from *Penicillum funiculosum*) with CE and ^{1}H NMR spectroscopy. Additionally, the migration profile of oligosaccharide standards consisting of β-linked glucose or mannose moieties was considered. The action pattern of an enzyme is highly dependent on its origin as well as the sugar composition of the substrate.[29] Threfore, the action pattern of the endo-β-(1,4)-glucanase and endo-β-(1,4)-mannanase used can be the easiest verified by evaluating the dimers formed by these two enzymes (**Figure 7A**). The endo-β-(1,4)-glucanase digest resulted in one dimer only, which did not migrate at the same time as either the cellobiose standard (GG; migration time of standard GG is indicated as ** in **Figure 7A**; G stands for glucose) or the mannobiose standard (MM; migration time of standard MM is indicated as *** in **Figure 7A**; M stands for mannose). The peak was identified as MG, as endo-β-(1,4)-glucanases are not supposed to cleave glycosidic linkage after mannose residues, but it showed clearly that the endo-β-(1,4)-glucanase used is capable of cleaving the linkage between glucose and mannose residues.

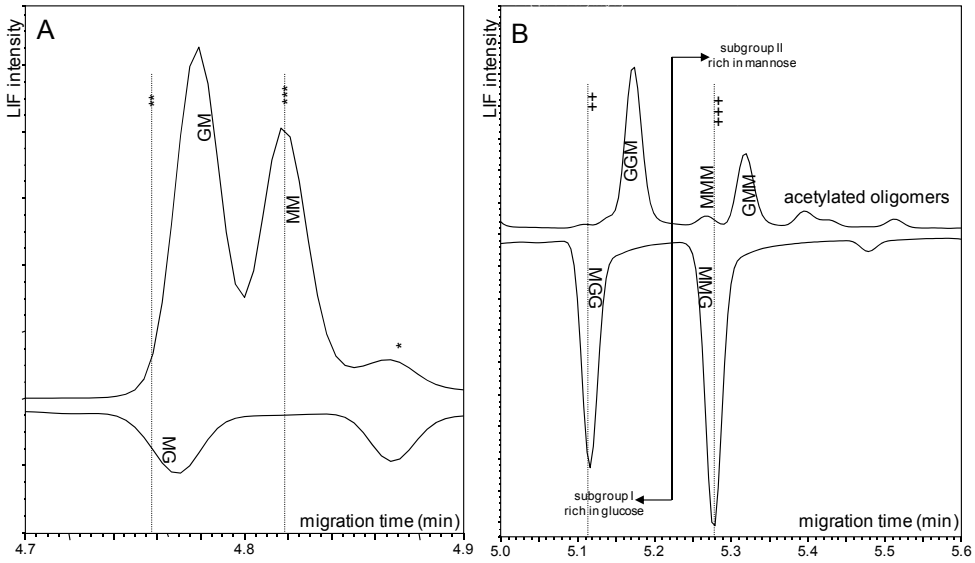

Figure 7. Hypothesis on the structure of KGM; CE-LIF electropherograms of mirrored zoom-ins of **Figure 3** for the KGMendo-β-(1,4)-mannanase digest (normal view) and endo-β-(1,4)-glucanase digest (mirrored view) for (A) DP2 and (B) DP3. *, Maltose (internal standard); ++/**, migration time of GG/GGG standard, respectively; +++/***, migration time of MM/MMM standard, respectively.

For the endo-β-(1,4)-mannanase digest, two dimers were identified, of which one could be identified as MM. The earlier-migrating dimer was assigned as GM, having a slightly later migration time than the dimer assigned as MG in the endo-β-(1,4)-glucanase digest, and explains the capability of the endo-β-(1,4)-mannanas used to cleave linkages between mannose and glucose. Within each DP, standards composed of glucose units migrated earlier than standards composed of mannose units (**Figure 2B**). On the basis of the latter fact, each peak cluster representing one DP in the KGM digests was divided into two subgroups, as demonstrated for DP3 in **Figure 7B**. According to the migration profile of the references, the subgroup migrating earlier (subgroup I) was assigned to be the one richer in glucose (migration time of standard GGG is indicated as ++ in **Figure 7B**), whereas the subgroup migrating later (subgroup II) was assigned to be the one richer in mannose (migration time of standard MMM is indicated as +++ in **Figure 7B**). A hypothesis on the structures was formed on the basis of the relative concentrations of trimeric structures as identified by Cescutti et al.[10] For the endo-β-(1,4)-mannanase digest GGM and GMM were reported to occur in a ratio of 1.00:0.52. Therefore, the peak in

subgroup I was assigned as GGM and the most abundant peak in subgroup II as GMM. Also, MMM was identified in subgroup II by its respective standard and was, as well as MGM, reported to be a minor component of the trimeric fraction.[10] However, no peak was assigned to MGM in the electropherogram. Comigration was assumed. Due to the saponification trials performed, peaks migrating later than GMM were assigned to acetylated trimers. Having used a cellulase preparation, the information given by Cescutti et al (MMG:MMM:MGG 1.83:1.00:0.2)[10] was not completely adaptable to the digestion with endo-β-(1,4)-glucanase performed in the present study. Neglecting the formation of MMM, which is, considering the formation of dimers, not probable to occur by using this pure endo-β-(1,4)-glucanase, the two trimers were assigned for MGG in subgroup I and MMG in subgroup II. This structural characterization confirmed the action expected for the enzymes applied and justified the assumption of a clustered migration according to the predominance of glucose or mannose in the oligomers. Oligomers found in the endo-β-(1,4)-glucanase digest were assigned to carry exclusively glucose residues at the reducing end, revealing that endo-β-(1,4)-glucanases are not able to cleave the frequently occurring mannotri, -tetra, and -pentaose segments present in the KGM polymer chain.[30] On the other hand, oligomers found in the endo-β-(1,4)-mannanase digest were assigned to carry exclusively mannose residues at the reducing end but predominantly glucose residues at the non-reducing end. Similar observations were made by Shimahara et al.[28,31] The repeating peak pattern obtained in the electropherograms [especially for the endo-β-(1,4)-glucanase digest] supports the block model for the KGM polymer instead of a random distribution of glucose and mannose residues. However, the higher the DP, the more peaks remain unknown, due to the increasing complexity of the KGM oligosaccharides and possible comigrations. From the literature, it is not known on which sugar residue acetyl groups are preferably situated in KGM. However, as acetylation intensity is increasing for the higher-MW oligosaccharides in the endo-β-(1,4)-glucanase digest, which are assumed to be especially rich in mannose, it can be assumed that the acetyl groups are preferably attached to the mannose residues. Furthermore, saponification of endo-β-(1,4)-mannanase digests resulted in a preferred disappearance of peaks belonging to subgroup II, representing oligosaccharides richer in mannose. The investigations made for the acetylation of a glucomannan from *Lupinus varius* seed showed as well acetylation of the mannose residues in the glucomannan chain.[32]

***In vitro* fermentation of KGM material**

In the second part of this study, native KGM and oligosaccharide mixtures representing cellulase and endo-β-(1,4)-mannanase digests of KGM were successfully fermented by mixed fecal slurries. Autoclaving the substrates before fermentation significantly decreased the level of acetylation. Autoclaving should be replaced by another sterilization method (e.g., based on filtration) in subsequent fermentation studies. However, choosing a sterilization method based on filtration may be difficult to apply for viscous polysaccharides such as KGM. During the fermentation course of 72 h, different degradation patterns for the respective KGM substrates were observed. Due to the use of a cellulase preparation instead of a pure endo-β-(1,4)-glucanase, the initial peak pattern on CE-LIF showed additional side peaks for the cellulase digest, which can be explained by the presence of endo-β-(1,4)-glucanases as well as exo-β-(1,4)-glucosidases in the cellulase preparation used.[22] With the monomer-containing cellulase digest, a complete utilization of the abundant amount of monomers occurred during the first 24 h. Dimers and trimers were largely degraded, and oligosaccharides of DP4-DP6 did not change upon 72 h of fermentation. Monomers effect the inhibition of enzymes responsible for degrading oligosaccharides of higher Mw and promote the prevalence of certain species. The promotion of lactic acid bacteria (esp. *Streptococcus* species) by monomeric material and the simultaneous suppression of *Bacteroides* and *Bifidobacteria* species have been reported by Olano-Martin et al[33] and explain the rapid drop in pH during the first 24 h of fermentation. For the monomer-free cellulase digest, differences in the degradation pattern were observed when compared to the monomer-containing analogue. This emphasizes the necessity of removing monomers before fermentation experiments, as they are also absorbed in the upper gastrointestinal tract and do not reach the colon. Besides the degradation of DP2 and DP3 a degradation of oligomers ≥ DP5 was observed after an adaptation time of 24 h. DP4 was more resistant to fermentation. Comparable to that, Kabel et al found a preferential fermentation of low substituted acetylated xylooligosaccharides with a DP of 3 and a DP > 7.[8] The lack of easily accessible material in the monomer-free cellulase digest stimulated the development of an environment having other bacterial enzymes and/or even bacterial species present, which are responsible for the degradation of higher-Mw oligomers. The contribution of a broader range of bacteria may explain the less pronounced decrease in pH during the first 24 h (pH ≥ 5). Nevertheless, a final pH of 4.6 was reached after 24 h, which is suitable for a suppression of pathogenic species.[1] According to the mass spectra and electropherograms, there was hardly any change in the carbohydrate profile during the 72 h fermentation course of the monomer-free

KGM endo-β-(1,4)-mannanase digest. The large pH drop and increase in OD during the first 24 h pointed nevertheless to an ongoing degradation, most probably consisting of an utilization of low-Mw material hidden by a meanwhile degradation of high-Mw oligomers out of the range of detection by MALDI-TOF MS and CE-LIF. The structural composition has obviously a big influence on the fermentation behavior: studying the structure of the endo-β-(1,4)-mannanase digest led to the conclusion that glucose or cellobiose is most frequently situated at the non-reducing end. For the cellulase digests it was assumed to be mainly mannose. Consequently, by the activity of bacterial exoenzymes, another set of mono- and dimers is provided for bacterial metabolization. High amounts of released glucose and therefore a predominance of lactic acid bacteria are as well indicated by the rapid drop in pH during the first 24 h of the fermentation of the endo-β-(1,4)-mannanase digest, which is similar to the behavior observed for the monomer containing cellulase digest.

The fermentation of the parental polysaccharide was monitored by HPSEC, which showed the gradual degradation of the polymer (~500 kDa) to an oligosaccharide mixture (≤ 4 kDa) after 72 h. Emerging oligomers were characterized by MALDI-TOF MS and CE-LIF. The peak profile in the electropherograms, of which each DP cluster could be subdivided into two subgroups again, did not match exclusively with the profile of one of the enzymatic digests but was a combination of both, indicating the action of a broad range of endo- and exoenzymes expressed in the human colonic flora in order to break down a complex polysaccharide. The pH decreased very gradually and was still ≥ 5 after 24 h. As was described by Van Laere et al, the degradation of a polymer is a synergistic action, resulting in the expression of a broad variety of enzymes and bacteria.[34]

Our study showed that the availability of advanced analytical methods is crucial for monitoring and understanding the behavior of complex carbohydrate structures during their *in vitro* fermentation. Structural features, composition, and method of preparation of the KGM mixtures had a large influence on the respective fermentation with human fecal flora. Combining results obtained from fermentation studies is necessary for a good understanding of bacterial action. Therefore, the inclusion of the on-line CE-LIF-MS coupling, allowing the assignment of peaks present in CE-LIF electropherograms with their respective masses, is aimed for. Furthermore, biomolecular methods such as Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE), a method used for investigating complex bacterial communities, and quantitative PCR, with which it is possible to establish a prebiotic index for potential prebiotic substrates, are currently applied for *in vitro* fermented konjac glucomannan substrates in our laboratory.

The determination of the bacterial metabolization products (organic acids) with GC and GC-MS will be our further approach to completely understand the fate of prebiotic substrates subjected to human gut flora. Although *in vitro* fermentation experiments do not allow a precise prediction of the fermentation *in vivo* (lack of buffered gut environment, strict anaerobicity and influence of other food components, adaptation of the flora subjected to prolonged exposure to specific prebiotic, strong influence of chosen concentrations of substrate to inoculum in *in vitro* experiments), they show clearly that the structural modification of oligosaccharides presents an effective way to influence the microbial community in the gut.

References

1. Gibson, G.R.; Roberfroid, M.B. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *J. Nutr.*, **1995**, *125*, 1401-1412.
2. Asp, N.-G. Dietary carbohydrates: Classification by chemistry and physiology. *Food Chem.*, **1996**, *57*, 9-14.
3. Voragen, A.G.J. Technological aspects of functional food-related carbohydrates. *Trends Food Sci. Technol.*, **1998**, *9*, 328.
4. Ito, M.; Deguchi, Y.; Matsumoto, K.; Kimura, M.; Onodera, N.; Yajima, T. Influence of galactooligosaccharides on the human fecal microflora. *J. Nutr. Sci. Vitaminol.*, **1993**, *39*, 635-640.
5. Wang, X.; Gibson, G.R. Effects of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *J. Appl. Bacteriol.*, **1993**, *75*, 373-380.
6. Macfarlane, G.T.; Gibson, G.R.; Cummings, J.H. Comparison of fermentation reactions in different regions of the human colon. *J. Appl. Bacteriol.*, **1992**, *72*, 57-64.
7. Gibson, G.R.; Probert, H.M.; van Loo, J.; Rastall, R.A.; Roberfroid, M.B. Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutr. Res. Rev.*, **2004**, *17*, 259-275.
8. Kabel, M.A.; Kortenoeven, L.; Schols, H.A.; A.G.J., V. *In vitro* fermentability of differently substituted xylo-oligosaccharides. *J. Agric. Food Chem.*, **2002**, *50*, 6205-6210.
9. Takigami, S. Konjac mannan. In *Handbook of hydrocolloids*; Phillips, G.O.,Williams, P.A., Eds; Woodhead Publishing Ltd: Cambridge, UK, **2000**; pp 413-423.
10. Cescutti, P.P.; Campa, C.C.; Delben, F.F.; Rizzo, R.R. Structure of the oligomers obtained by enzymatic hydrolysis of the glucomannan produced by the plant *Amorphophallus konjac*. *Carbohydr. Res.*, **2002**, *337*, 2505.
11. Maekaji, K. Determination of acidic component of konjac mannan *Agr. Biol. Chem. Tokyo*, **1978**, *42*, 177-178.
12. Katsuraya, K.; Okuyama, K.; Hatanaka, K.; Oshima, R.; Sato, T.; Matsuzaki, K. Constitution of konjac glucomannan: Chemical analysis and 13C NMR spectroscopy. *Carbohydr. Polymers*, **2003**, *53*, 183.
13. Stephen, A.M. Other plant polysaccharides; volume 2. In *The polysaccharides*; Aspinall, G.O., Ed; Academic Press: New York, **1983**; pp 97-192.
14. Vuksan, V.; Jenkins, D.J.; Spadafora, P.; Sievenpiper, J.L.; Owen, R.; Vidgen, E.; Brighenti, F.; Josse, R.; Leiter, L.A.; Bruce-Thompson, C. Konjac-mannan (glucomannan) improves glycemia and other associated risk factors for coronary heart disease in type 2 diabetes. A randomized controlled metabolic trial. *Diabetes Care*, **1999**, *22*, 913-919.
15. Wu, J.; Peng, S. Comparison of hypolipidemic effect of refined konjac meal with several common dietary fibers and their mechanisms of action. *Biomed. Environ. Sci*, **1997**, *10*, 27-37.
16. Walsh, D.E.; Yaghoubian, V.; Behforooz, A. Effect of glucomannan on obese patients: A clinical study. *Int. J. Obes.*, **1984**, *8*, 289-293.
17. Chen, H.H.; Fan, Y.Y.; Chen, M.M.; Chan, Y.Y. Unhydrolyzed and hydrolyzed konjac glucomannans modulated cecal and fecal microflora in balb. *Nutrition*, **2005**, *21*, 1059.
18. Hilz, H.; de Jong, L.E.; Kabel, M.A.; Schols, H.A.; Voragen, A.G.J. A comparison of liquid chromatography, capillary electrophoresis, and mass spectrometry methods to determine xyloglucan structures in black currants. *J. Chromatogr. A*, **2006**, *1133*, 275-286.
19. Kabel, M.A.; Heijnis, W.H.; Bakx, E.J.; Kuijpers, R.; Voragen, A.G.J.; Schols, H.A. Capillary electrophoresis fingerprinting, quantification and mass-identification of various 9-aminopyrene-1,4,6-trisulfonate-derivatized oligomers derived from plant polysaccharides. *J. Chromatogr. A*, **2006**, *1137*, 119.
20. Lee, Y.C. Carbohydrate analyses with high-performance anion-exchange chromatography. *J. Chromatogr. A.*, **1996**, *720*, 137-149.
21. Schols, H.A.; Kabel, M.A.; Bakx, E.J.; Daas, P.J.H.; Alebeek, G.-J.; Voragen, A.G.J. HPLC of oligosaccharides: New developments in detection and peak identification. *AVH Association - 7th symposium*, **2000**, 39-45.
22. Beldman, G.; Searle van Leeuwen, M.F.; Rombouts, F.M.; Voragen, A.G.J. The cellulase of *trichoderma viride* - purification, characterization and comparison of all detectable endoglucanases, exoglucanases and β-glucosidases. *Eur. J. Biochem.*, **1985**, *146*, 301-308.
23. Düsterhöft, E.-M.; Posthumus, M.A.; Voragen, A.G.J. Non-starch polysaccharides from sunflower (*helianthus annuus*) meal and palm-kernel (*elaeis guineensis*) meal - investigation of the structure of major polysaccharides. *J. Sci. Food Agric.*, **1992**, *59*, 151-160.
24. Düsterhöft, E.-M.; Bonte, A.W.; Voragen, A.G.J. Solubilisation of non-starch polysaccharides from oil-seed meals by polysaccharide-degrading enzymes. *J. Sci. Food Agric.*, **1993**, *63*, 211-220.

25. Hartemink, R. Prebiotic effects of non-digestible oligo- and poysaccharides. PhD thesis, Food Microbiology, Wageningen University, the Netherlands,**1999**.
26. Englyst, H.; Wiggins, H.S.; Cummings, J.H. Determination of the non-starch polysaccharides in plant foods by gas-liquid-chromatography of constituent sugars as alditol acetates. *Analyst*, **1982**, *107*, 307-318.
27. Voragen, A.G.J.; Schols, H.A.; Pilnik, W. Determination of the degree of methylation and acetylation of pectins of H. P. L. C. *Food Hydrocolloid.*, **1986**, *1*, 65-70.
28. Shimahara, H.; Suzuki, H.; Sugiyama, N.; Nishizawa, K. Isolation and characterization of oligosaccharides from an enzymatic hydrolyzate of konjac mannan. *Agr. Biol. Chem. Tokyo*, **1975**, *39*, 293-299.
29. Tenkanen, M.; Makkonen, M.; Perttula, M.; Viikari, L.; Teleman, A. Action of *trichoderma reesei* mannanase on galactoglucomannan in pine kraft pulp. *J. Biotechnol.*, **1997**, *57*, 191-204.
30. Biely, P.; Tenkanen, M. Enzymology of hemicellulose degradation. In *Trichoderma and gliocladium*; Kubicek, C.P., Harman, G.-E.,Ondik, K.-L., Eds; Taylor & Francis Ltd: London, **1998**; pp 25-47.
31. Shimahara, H.; Suzuki, H.; Sugiyama, N.; Nisizawa, K. Partial purification of β-mannanases from the konjac tubers and their substrate specifity in the relation to the structure of konjac glucomannan. *Agr. Biol. Chem. Tokyo*, **1975**, *39*, 301-312.
32. Ishurd, O.; Kermagi, A.; Elghazoun, M.; Kennedy, J.F. Structural of a glucomannan from *lupinus varius* seed. *Carbohydr. Polymers*, **2006**, *65*, 410-413.
33. Olano Martin, E.; Gibson, G.R.; Rastall, R.A. Comparison of the *in vitro* bifidogenic properties of pectins and pectic-oligosaccharides. *J. Appl. Microbiol.*, **2002**, *93*, 505-511.
34. Van Laere, K.M.J.; Hartemink, R.; Bosveld, M.; Schols, H.A.; Voragen, A.G.J. Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. *J. Agric. Food Chem.*, **2000**, *48*, 1644-1652.

Chapter 7

Enzymatic production and characterization of konjac glucomannan oligosaccharides

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Abstract

Enzymes from a balanced human gut flora are promising tools to design prebiotic oligosaccharides. Konjac glucomannan (KGM) and oligosaccharides thereof are potentially prebiotic substrates, which may even reach the distal part of the gut due to their structural complexity. In this study, the structural composition of oligosaccharides produced from KGM by enzymes of fecal bacteria was compared to two KGM digests produced by fungal endo-β-(1,4)-glucanase (EG) or endo-β-(1,4)-mannanase (EM). The oligosaccharide mixtures were separated according to their size and pools were subsequently analysed for their sugar composition. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF) was used for the characterization of the oligosaccharide pools as such and after digestion by the exo-acting enzymes exo-β-(1,4)-glucanase and β-(1,4)-mannosidase. The KGM-oligosaccharides produced by the fecal enzyme mixture resulted in a series of homologous oligosaccharides with a constant mannose/glucose ratio of 1.5:1, representing the mannose/glucose ratio of the initial polysaccharide. A homologous series composed of mannose-rich oligosaccharides, which must be partly branched was observed for the EG-digest, whereas a short series of oligosaccharides with an equal ratio of mannose and glucose was observed for the EM-digest. A backbone composed of short mannose- and glucose sequences, to which branches rich in mannose are attached was indicated for the KGM polysaccharide. Finally, the *in vitro* fermentability of the structurally different oligosaccharides is evaluated and their use as prebiotic substrates is discussed.

Introduction

Oligosaccharides, intended to specifically stimulate gastrointestinal bacteria beneficial to the host's health[1,2] are frequently added to food products. Commonly used oligosaccharides are galactooligosaccharides (GOS) and fructooligosaccharides (FOS), which have been extensively studied for their prebiotic potential.[3,4] These short-chain oligosaccharides are rapidly fermented in the colon. In order to prevent colonic diseases, which mainly take place in the distal part of the colon, more complex prebiotic oligosaccharides are needed, able to survive the entire gastrointestinal transit.[5,6] The production of tailor-made oligosaccharides for this purpose is of great interest.[2] Complex polysaccharides can be used for the enzymatic production of a variety of oligosaccharide mixtures.

Glucomannan from *Amorphophallus konjac* C. Koch (KGM) represents an interesting polysaccharide for the production of structurally different oligosaccharides. KGM, which is composed of β–(1,4)-linked mannose- and glucose-units in a molar ratio of 1.5:1 enables the enzymatic hydrolysis by endo-β-(1,4)-glucanase (EG) as well as by endo-β-(1,4)-mannanase (EM).[7] The monosaccharide units are acetylated to an extent of 5-10%.[8] Branches are estimated to occur once upon every 10-11 hexose units in the main chain.[9] Little is known about the precise structure of KGM, such as the sequence of glucose and mannose within the backbone, precise length and distribution of the side chains and location of acetyl groups, although several studies have been performed.[10-12] The complex structural composition make the KGM polysaccharide and the oligosaccharides derived hereof potential substrates for reaching the distal colon, which is the desired target site for bioactive carbohydrates.[13] In a previous study, we tested KGM as well as two different KGM oligosaccharide sets, produced by EM and EG, for their *in vitro* fermentability by human gut flora.[7] The structural composition of the oligosaccharides showed to influence the fermentability by human fecal bacteria. For the *in vitro* fermentation of the polysaccharide, the formation of an unknown oligosaccharide series, different from the mixtures obtained by the glucomannan hydrolysis with fungal EG or EM, was described.[7]

No conclusions on the structural composition of the oligosaccharides, which were formed during *in vitro* fermentation could be drawn.[7] The structural characteristics of oligosaccharides produced during the *in vitro* fermentation of polysaccharides provide information on the action of the enzymatic machinery of the colonic flora and has been recognized as valuable tool for the design of target-specific prebiotic oligosaccharides.[14,15]

In the present study, the structural composition of oligosaccharides produced from KGM by enzymes of fecal bacteria is determined. For this purpose, it is compared to two KGM

digests produced by fungal EG and EM. The KGM-digests were fractionated according to their size and subsequently studied for their monosaccharide composition. Their degradability by exo-β-(1,4)-glucanase and β-(1,4)-mannosidase was followed quantitatively by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF).[16] Besides enabling the evaluation of the different enzymatic degradation mechanisms on KGM, the combination of the results allowed to draw some conclusions on the molecular structure of the KGM polysaccharide.

Materials and Methods

Materials and Enzymes

The KGM polysaccharide was obtained from Kalys (St-Ismier, France). The KGM of the species *Amorphophallus konjac* had a molecular mass of approx. 500 kDa, a mannose/glucose molar ratio of 1.5:1 and a degree of acetylation (DA) of 7.9%.[7] All chemicals used were of analytical grade.

Endo-β-(1,4)-glucanase VI (EC 3.2.1.4) and exo-β-(1,4)-glucanase III (EC 3.2.1.91) were purified from a crude cellulase preparation from *Trichoderma viride* (Maxazyme, DSM Food Specialities, Delft, The Netherlands) as described by Beldman et al.[17] Endo-β-(1,4)-mannanase (EC 3.2.1.78) was purified from a commercial *Aspergillus niger* enzyme mixture (Gamanase III, Novozymes, Bagsvaerd, Denmark) according to Düsterhöft et al.[18] Beta-(1,4)-mannosidase from *Helix pomatia* (E.C.3.2.1.25)[19] was purchased from Sigma-Aldrich (St Louis, MO).

The enzymes from human fecal bacteria originated from a supernatant, which was obtained from an *in vitro* fermentation of KGM after 24 h fermentation time. The *in vitro* fermentation was performed with a mix of three human fecal samples, according to Albrecht et al.[7] The composition of the medium was adapted according to Sunvold et al.[20] The concentration of KGM at the start of the fermentation was 3 mg/mL and the fermentation bottle was incubated with 200-fold diluted fecal slurry. The incubation volume was 20% (v/v) of the volume of substrate and medium present in the fermentation bottle. In order to prevent the loss of acetyl groups from KGM, the fermentation flasks were not autoclaved, but pasteurized prior to fermentation (70 ºC, 30 min). The fermentation liquid taken after 24h incubation was centrifuged (5 min, 2300g, room temperature), the bacterial cell material was discarded and the supernatant containing the soluble enzymes from human fecal bacteria was stored at -40 ºC.

Enzymatic hydrolysis and sample preparation

The enzymatic digests of KGM using endo-β-(1,4)-glucanase VI and endo-β-(1,4)-mannanase were prepared according to Albrecht et al.[7] The enzymatic digests of KGM with enzymes from human fecal bacteria were prepared by adding 2 mL of the supernatant to 10 mL of a 1.5 mg/mL KGM solution in water prior to incubation for 24 h at 37 ºC. Samples were mixed continuously during incubation (head-over-tail). Enzymes were inactivated (10 min, 100 ºC) and the samples were centrifuged (5 min, 10 000g, room temperature).

The KGM digests were fractionated into oligosaccharide pools on a BioGel P2 column (26 mm x 900 mm, 200-400 mesh; Bio-Rad, Hercules, CA). Five mL of the supernatants were applied onto the column and were eluted with millipore water (1.5 mL/min) at 60 °C using an Akta Explorer system (GE Amersham, Uppsala, Sweden). The eluate was monitored by a refractive index detector (Shodex RI-72, Showa Denko K.K., Tokyo, Japan) before fractions (5 mL) were collected. The fractions were pooled according to the RI profile and freeze-dried.

For their characterization, 100 µL of the oligosaccharide pools (approx. 0.1 mg/mL) were incubated with 1.35 mU exo-β-(1,4)-glucanase III or 19.7 mU β-(1,4)-mannosidase. The incubation (24 h) with exo-β-(1,4)-glucanase III was performed in 20 mM sodium citrate buffer pH 3.5 at 30 ºC and the incubation with β-(1,4)-mannosidase (24 h) was performed in 10 mM sodium acetate buffer at pH 4.5 at 45 ºC, shaking conditions. The enzymes were inactivated by heating the samples for 10 min at 100 ºC and the hydrolysate was centrifuged (5 min, 10 000g, room temperature) prior to analysis.

Analytical methods

Monosaccharide composition

The enzymatic digests and the oligosaccharide pools derived thereof were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 121 °C. Subsequently, the acid was evaporated and the residue was repeatedly washed with methanol. The hydrolysates were re-dissolved in millipore water and analyzed for their monosaccharide composition by HPAEC-PAD on a Dionex ISC 3000 system (Dionex, Sunnyvale, CA). The system was equipped with an analytical Carbopac PA–1 column (2 mm x 250 mm; Dionex) and a guard column (2 mm x 50 mm; Dionex). Separation was performed by elution with water, and 0.5 M sodium hydroxide was added post-column to the eluent for detection by PAD.[21]

Pools of DP2 were supplementary reduced prior to acid hydrolysis in order to determine the monosaccharide units located at the reducing end. The pools were, therefore, treated with sodium borohydride in ammonium hydroxide as described elsewhere.[22]

Degree of acetylation (DA)

Acetyl groups released from KGM during incubation with the enzyme mixture from fecal bacteria were determined by the analysis of acetic acid on a Dionex Ultimate 3000 HPLC system (Dionex). The system was equipped with an Aminex HPX 87H column (Bio-Rad) and a Shodex RI72 detector (Showa Denko K.K., Tokyo, Japan). The eluent used was 5 mM sulphuric acid (0.6 mL/min, 40 °C).

HPSEC

The molecular weight distribution of the polysaccharides and the enzymatic digests was determined by High Performance Size Exclusion Chromatography **(**HPSEC) using an Ultimate 3000 HPLC system (Dionex), equipped with an RI72 refractive index detector (Showa Denko K.K., Tokyo, Japan). 20 µL sample solutions (1 mg/mL) were injected and separation was performed on three Tosoh TSK gel superAW columns in series (AW4000-AW3000-AW2500, each 6 mm x 150 mm; Tosoh Bioscience, Tokyo, Japan) in combination with a guard column (3.5 mm x 46 mm; Tosoh). For elution, 0.2 M sodium nitrate was used at a flow rate of 0.6 mL/min at 55 °C. The system was calibrated with pullulan standards (Sigma Aldrich; mass range 180 Da – 790 kDa).

MALDI-TOF MS

For matrix–assisted laser desorption/ionisation time-of-flight mass spectrometry **(**MALDI-TOF MS) of oligosaccharides, an UltraflexTreme TOF/TOF (Bruker, Bremen, Germany) equipped with a nitrogen laser of 337 nm was used. The measurement was performed in the positive mode. After a delayed extraction time of 200 ns, the ions were accelerated with a 25 kV voltage. Data from averaging 100 laser shots were collected, with lowest laser energy applied in order to obtain sufficient spectra intensity.

The mass spectrometer was calibrated with maltodextrin. Samples were prepared as described previously.[7]

CE-LIF

The sample preparation for CE-LIF, which includes the derivatization of the carbohydrates with the fluorescent dye APTS (9-aminopyrene-1,4,6-trisulfonate) was done using the ProteomeLab Carbohydrate Characterization kit (Beckman Coulter, Fullerton) as described previously.[7] Separation of the derivatized carbohydrates was performed using a ProteomeLab PA 800 CE system (Beckman Coulter) equipped with a polyvinyl alcohol-coated capillary (50 µm x 50.2 cm). Detection was done with a laser induced fluorescence detector (LIF) (Beckman Coulter) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm after 40 cm capillary length.

Samples were loaded hydrodynamically (7 s at 0.5 psi, representing approx. 14 nL sample solution) on the capillary, which was kept at 25 °C. Separation was performed in the reversed polarity mode (30 kV, 20 min) in a 25 mM acetate buffer (pH 4.75) containing 0.4 % polyethylene oxide. Between sample runs, the capillary was rinsed with water (5 min, 30 psi) and with separation buffer (2 min, 30 psi).

Results and Discussion

Production of oligosaccharides from the KGM polysaccharide

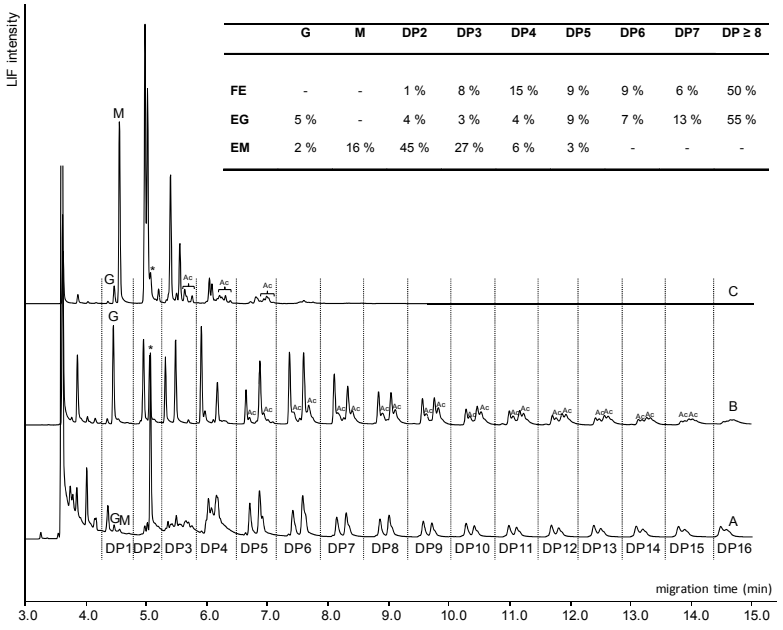
A digest of KGM with a mixture of soluble enzymes from an *in vitro*-fermentation of KGM with human inoculum (fecal enzymes; FE) was produced in this study. In order to compare the hydrolysis of the KGM chain by fecal enzymes with the hydrolysis of KGM by fungal enzymes, an endo-β-(1,4)-glucanase (EG)- and an endo-β-(1,4)-mannanase (EM)-digest of KGM were included, which were available from our previous research.[7] The oligosaccharides resulting from the different KGM digests could be distinguished by CE-LIF and the proportions of the DPs to the total digests were calculated (**Figure 1** and **Table** in **Figure 1**).

A repeating pattern, composed of two peaks per DP was observed for the FE-digest, with oligosaccharides DP ≥ 8 contributing to 50% to the total digest (**Figure 1A**). Monomers and dimers were present in trace amounts only in the FE-digest. Although only soluble enzymes were present in the enzyme mixture from fecal bacteria, the degradation profile of KGM (**Figure 1A**) was comparable to the oligosaccharide profile, which was previously obtained during *in vitro* fermentation.[7] During *in vitro* fermentation also cell-associated bacterial enzymes may have been involved. Acetylated oligosaccharides were present in trace amounts only in the FE-digest, as investigated by MALDI-TOF MS (results not

shown). The lack of acetylated oligosaccharides in the FE-digest points to the activity of acetyl-esterases present in the FE-mixture.

The oligosaccharides resulting from the EG-digest comprise a large DP-range (DP > 15; **Figure 1B**), with oligosaccharides of DP ≥ 8 contributing for 55% of the total digest. Acetylated oligosaccharides, as previously[7] determined by MALDI-TOF MS before and after saponification of the oligosaccharides, are indicated in **Figure 1B**. Although showing an oligosaccharide pattern similar to the FE-digest, most peaks of the EG-digest showed different migration times compared to the FE-digest.

Mannose (16%), dimers (45%) and trimers (27%) are the main degradation products in the EM-digest (**Figure 1C**). Tetramers and pentamers are minor enzymatic hydrolysis products (6% and 3%, respectively). This can be explained by the mannose/glucose ratio of the polymer (mannose/glucose 1.5:1) and the ability of EM from *A.niger* to cleave mannose-mannose and mannose-glucose-linkages.[23] Acetylated oligosaccharides, as previously[7] determined by MALDI-TOF MS and deacetylation experiments are indicated in **Figure 1C**.



	G	M	DP2	DP3	DP4	DP5	DP6	DP7	DP ≥ 8
FE	-	-	1 %	8 %	15 %	9 %	9 %	6 %	50 %
EG	5 %	-	4 %	3 %	4 %	9 %	7 %	13 %	55 %
EM	2 %	16 %	45 %	27 %	6 %	3 %	-	-	-

Figure 1. CE-LIF electropherograms of konjac glucomannan hydrolyzed by (A) enzymes from human fecal bacteria (FE) (B) endo-β-(1,4)-glucanase (EG) and (C) endo-β-(1,4)-mannanase (EM). *: maltose (internal standard). Ac: acetylated oligosaccharides. Inlay represents relative proportions of oligosaccharides per DPs within the digest.

Characterization of enzymatic hydrolysis products from KGM

Size exclusion chromatography (SEC) of the enzymatically digested polysaccharide resulted in pools of distinct DPs for DP2-DP7, with minor contamination of the preceding and following DP (data not shown). CE-LIF electropherograms of the FE-, EG- and EM-digest, which were used for further investigation, are shown in **Figure 2**. Tri- and tetra-saccharides present in the respective pools of the FE-digest (**Figure 2A**) were separated more clearly compared to the total digest (**Figure 1A**), most probably due to impurities present in the total digest.

Monosaccharide composition of KGM pools

For an overview, monosaccharide composition analysis of the oligosaccharide pools was performed. The molar ratio of mannose/glucose for the oligosaccharide pools is presented in **Table 1**. For each digest, a characteristic trend in mannose/glucose ratio was observed.

The oligosaccharide pools of the FE-digest showed a constant mannose/glucose ratio (1.5:1, mannose/glucose) up to DP7. The initial mannose/glucose ratio of 1.5:1 in the KGM polysaccharide chain is thus maintained. It can, therefore, be concluded, that the hydrolysis of KGM by fecal enzymes is not sugar-dependent.

Although the EG-digest showed a homologous series of peaks, which is also found for the FE-digest, the trends in mannose/glucose ratio were not similar. The molar ratio for the EG-digest was 1.4 mol mannose per mol glucose for DP3 and DP4. The mannose/glucose ratio significantly increased to 2.4:1 for DP7.

Table 1. Molar ratios of mannose (M) and glucose (G) in pools of enzymatic konjac glucomannan digests. FE = digest with enzymes from human fecal bacteria. EG = endo-β-(1,4)-glucanase-digest. EM = endo-β-(1,4)-mannanase-digest. For dimers, the ratios after sample reduction are indicated.

	FE-digest ***M/G***	***EG-digest*** ***M/G***	***EM-digest*** ***M/G***
DP1	-	0 : 1	8.8 : 1
DP2	-	0.9 : 1	2.3 : 1
DP2 after reduction*	-	*6.4 : 1*	*0.7 : 1*
DP3	1.3 : 1	1.4 : 1	0.9 : 1
DP4	1.5 : 1	1.4 : 1	1.0 : 1
DP5	1.5 : 1	1.9 : 1	1.1 : 1
DP6	1.5 : 1	2.3 : 1	-
DP7	1.5 : 1	2.4 : 1	-

*: only the sugar located at non-reducing end is detected.

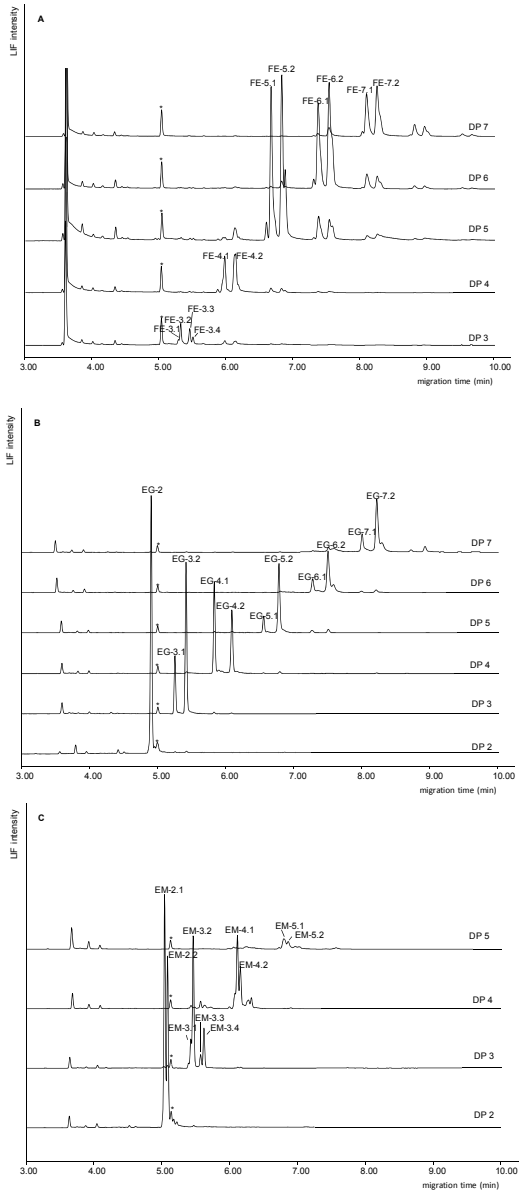


Figure 2. CE-LIF electropherograms of BioGel P2-pools from konjac glucomannan digest with (A) enzymes from human fecal bacteria (FE), (B) endo-β-(1,4)-glucanase (EG) and (C) endo-β-(1,4)-mannanase (EM). *: maltose (internal standard). The peaks within the respective pools are indicated.

Increased mannose ratios were expected for the higher-Mw pools of the EG-digest since mannose is present to a larger extent than glucose in the KGM polysaccharide, and EG is only able to cleave the glycosidic linkage between glucose-glucose and glucose-mannose, but not between mannose-mannose and mannose-glucose.[24]

The mannose ratio in the DP3-DP5-oligosaccharide pools of the EM-digest was constantly low and equal to glucose (0.9-1.1 mol mannose per mol glucose). DP5 is the highest-Mw oligosaccharide pool produced by the hydrolysis of KGM by EM. With a mannose/glucose ratio of 1.1:1 for DP5, sequences of > 2 glucose units are indeed not expected to occur in the KGM polysaccharide.[23] Taking into account monomeric mannose, which was present in considerable amounts in the total digest (16% of the total peak area; **Table** in **Figure 1**) these results highlight the good accessibility of the enzyme to the glucomannan chain, as was indicated before.[23]

Summarizing, for both, EG- and EM-digest, a more sugar-dependent enzymatic degradation of the KGM than for the fecal enzymes was indicated by the monosaccharide compositions of the oligosaccharide pools.

Structural composition of dimers from endo-β-(1,4)-glucanase-(EG) and endo-β-(1,4)-mannanase-(EM) digests

Before characterizing higher-Mw oligosaccharides from the FE-, EG- and EM-digests, we firstly confirmed the composition of the dimers as present in the EG- and EM-digest. For these dimers a theoretical assignment has previously been established,[7] as discussed below.

The sugar compositions of the dimer-pools were also determined after reduction with sodium borohydride in order to determine the sugar unit located at the reducing end (**Table 1**). Combining this information with the number and proportion of compounds present in the respective EG- and EM-pool (**Figure 2B** and **Figure 2C**) enabled the assignment of the dimers. MG was assigned as the component present in the dimer-pool of the EG-digest (**Figure 2B**, peak EG-2), which is in agreement with the structure hypothesized in our previous study.[7] Supplementary, a small proportion of GG can be assumed to be present, due to the ratio of 0.9 mannose per mol glucose.

GM (EM-2.1) and MM (EM-2.2) were assigned for the EM-digest (**Figure 2C**) and the assignment is in agreement with previous data.[7] The ratios of 2.3 mannose per mol glucose before reduction and 0.7 mol mannose per mol glucose after reduction in the EM-digest match with the CE-LIF peak proportions of EM-2.1 (GM; 43%) and EM-2.2 (MM; 57%). The structural assignments as made for the dimer-pools is summarized in **Table 2**.

Table 2. Composition of dimer-pools from endo-β-(1,4)-glucanase (EG)- and endo-β-(1,4)-mannanase-(EM)-digest of konjac glucomannan, as determined by monosaccharide composition analysis. The relative proportion of the respective structure in the pool is indicated. Peak numbers are according to **Figure 2**.

	peak	structure	% of pool
EG-digest	2	MG	~ 100%
	-	GG	traces
EM-digest	2.1.	GM	43%
	2.2.	MM	57%

Characterization of KGM oligosaccharide pools with exo-enzymes

Knowledge of the monosaccharide composition is not sufficient for the assignment of oligosaccharides of DP > 2, due to the increase in structural possibilities. The hydrolysis of the oligosaccharides by exo-enzymes, which results in a sequential release of mono- and disaccharide units located at the non-reducing end will provide additional structural information. As KGM is composed of glucose and mannose, a cellobiase (exo-β-(1,4)-glucanase III (exo-glucanase) from *Trichoderma viride*)[17] and a β-(1,4)-mannosidase (β-mannosidas; from *Helix pomatia*)[19] were used. In order to firstly understand the action of these enzymes on KGM, the trimer-pools of the EG- and EM-digests were used. A theoretical assignment has previously been established for these oligosaccharides and will be discussed below. Subsequently, the information obtained was used for the assignment of the oligosaccharide structures as present in the FE-digest.

Mode of action of exo-β-(1,4)-glucanase III (exo-glucanase) and β-(1,4)-mannosidase (β-mannosidase) towards KGM trisaccharides

The dimers and monomers, which resulted from the incubation of the trimer-pools with the exo-enzymes, are listed in **Table 3**. The dimers MG, GM and GG can hardly be distinguished by CE-LIF if present together. Therefore, their combined presence is annotated with H2 in **Table 3**.

The trimer-pool of the EG-digest is composed of two structures (**Figure 2B**), previously theoretically assigned as MGG (EG-3.1) and MMG (EG-3.2).[7] EG-3.1 was only slightly degraded by exo-glucanase to H2 and glucose (G), whereas EG-3.2 was not at all degraded. For the EM-digest GGM, MMM and GMM were previously assigned to the compounds EM-3.2, EM-3.3 and EM-3.4, respectively (**Figure 2C**).[7] Only GGM (EM-3.2.) was degraded by exo-glucanase.

Table 3. Mode of action of exo-(1,4)-glucanase (exo-glucanase) and β-(1,4)-mannosidase (β-mannosidase) on trisaccharides from konjac glucomannan hydrolyzed by endo-β-(1,4)-glucanase (EG), endo-β-(1,4)-mannanase (EM) and fecal enzyme mixture (FE). Peak numbers are according to **Figure 2**. The M/G ratio was calculated based on the relative proportion of the respective structure in the pool. H2: GM/MG/GG.

	peak	exo-glucanase	β-mannosidase	structure	% of pool	M/G
EG digest	3.1.	(G, H2)	M, G	MGG	26 %	1.4 : 1
	3.2.	-	GG	MMG	74 %	
EM digest	3.1.	-	M, H2*	MGM	12 %	0.9 : 1
	3.2.	M, GG	-	GGM	64 %	
	3.3.	-	M, H2*	MMM	6 %	
	3.4.	-	-	GMM	18 %	
FE digest	3.1.	-	-	MGG	8%	1.5 : 1
	3.2.	-	M, G	MGM	42%	
	3.3.	-	H2	MMG	36%	
	3.4.	-	-	GMM	14%	

( ): no complete degradation. *: hydrolysis products result from EM-3.1 and EM-3.3

Thus, exo-glucanase can cleave off the terminal disaccharide unit GG attached to a reducing mannose-unit. A release of the terminal MG-unit, attached to a reducing glucose-unit is limited. GM linked to a reducing mannose-unit cannot be hydrolysed by exo-glucanase.

Except for GGM (EM-3.2) and GMM (EG-3.4), all other structures were degraded by β-mannosidase. Thus, β-mannosidase showed to be able to remove terminal mannose-units, independent of being β-linked to a mannose- or glucose-unit. Beta-mannosidase completely hydrolyses repeating mannose-units (e.g. MMG to 2M and G or MMM to 3M), which was concluded from the quantitative amounts of mannose released in the digested pools (data not shown).

Although not detected by us before,[7] a fourth structure (MGM, **Figure 2C**, peak EM-3.1) was assigned in the DP3 pool of the EM-digest, due to its resistance to exo-glucanase digestion and its degradation to M and H2 by β-mannosidase. The presence of MGM in a KGM digest produced by EM from *A.niger* has been previously described.[11]

Information on the mode of action of the exo-enzymes was thus obtained by hydrolysing the trisaccharide-pools. Simultaneously, the mono- and disaccharides resulting from the hydrolysis of the trisaccharide-pools confirm our previous theoretical assignments of the oligosaccharide structures.[7] The structural assignments of the trisaccharides is in agreement with the monosaccharide composition as mentioned in **Table 1**, considering the relative proportions of the individual oligosaccharides within the pools (**Table 3**).

The trisaccharides FE-3.1, FE-3.2, FE-3.3 and FE-3.4 of the FE-digest were assigned as MGG, MGM, MMG and GMM, respectively (**Figure 3** and **Table 3**). The presence of both, glucose and mannose as reducing end units in the fecal enzyme digest indicates a multiple-enzyme-system and a different mode of action of the bacterial enzymes on the KGM polysaccharide compared to the fungal enzymes.

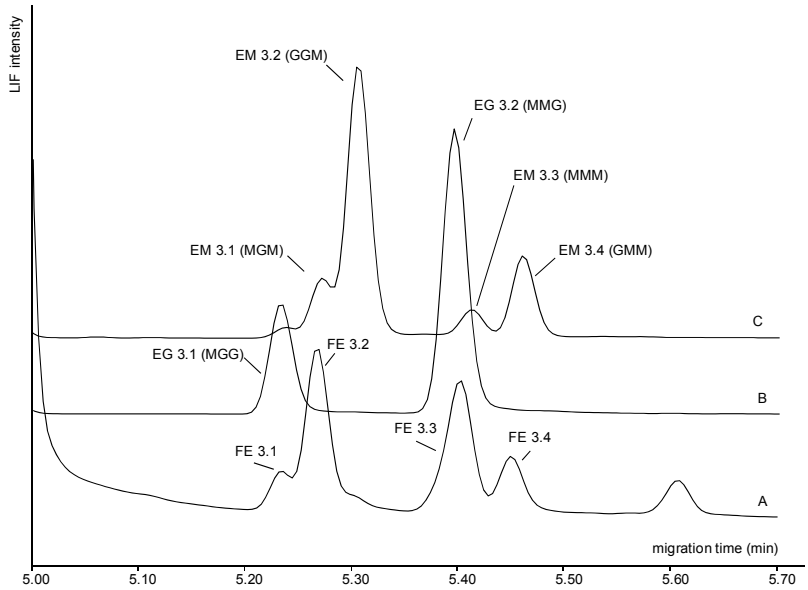


Figure 3. CE-LIF electropherograms of DP3-pools of konjac glucomannan hydrolyzed by (A) enzymes from human fecal bacteria (FE), (B) endo-β-(1,4)-glucanase (EG) and (C) endo-β-(1,4)-mannanase (EM). The peaks within the respective pools are indicated.

Assignment of higher-Mw KGM-oligosaccharides based on their degradation with exo-enzymes

Knowing the structure of the various DP2- and DP3-peaks and the mode of action of the enzymes allowed the use of the exo-enzymes for the structural investigation of higher-Mw KGM oligosaccharides. In order to subsequently draw some conclusions on the structural composition of the KGM polysaccharide, attention was paid not only to the investigation of oligosaccharides from the FE-digest, but also to the oligosaccharides in the EG- and EM-digest. The KGM-pools investigated were degraded to different extents by the exo-

enzymes. **Table 4** summarizes the decrease in total initial peak area of the oligosaccharides in the pools upon incubation.

FE-digest

For the FE-digest, the susceptibility to hydrolysis by exo-glucanase increased with increasing size of the oligosaccharides (**Table 4**). Oligosaccharides of the DP5-pool showed the lowest degradability by exo-glucanase as well as by β-mannosidase. This low degradability may be deduced to a complicated structural composition and may thus explain the predominant presence of DP5 oligosaccharides during the *in vitro* fermentation.[7]

Table 4. Degradability by exo-β-(1,4)-glucanase and β-(1,4)-mannosidase of oligosaccharide-pools from konjac glucomannan hydrolyzed by fecal enzymes (FE), endo-β-(1,4)-glucanase (EG) and endo-β-(1,4)-mannanase (EM).

	FE-digest		***EG-digest***		***EM-digest***	
	exo-glucanase	β-mannosidase	exo-glucanase	β-mannosidase	exo-glucanase	β-mannosidase
DP3	0 %	86 %	7 %	97 %	64 %	31 %
DP4	23 %	43 %	59 %	86 %	95 %	72 %
DP5	22 %	34 %	n.d.	n.d.	49 %	50 %
DP6	85 %	38 %	81 %	83 %	-	-
DP7	96 %	54 %	n.d.	n.d.	-	-

Interestingly, all FE-pools degraded by exo-glucanase showed the presence of GGM, MGM, MMG and GMM, as exemplified by the hydrolysis of the DP4-pool in **Figure 4A**. These tri-saccharides are the components of the DP3-pool from the FE-digest (**Table 3**). The homologous peak series observed for the FE-digest can thus be explained by the presence of a number of core-structures, which are extended either linear or branched. Clearly, a variety of structures is present.

Beta-mannosidase-digestion of the FE-pools of a given DPx resulted in the emergence of mainly oligosaccharides of DPx-1 and mannose, as exemplified for the hydrolysis of DP4 in **Figure 4A**. The action of β-mannosidase is thus hindered by the presence of a subsequent glucose unit and indicates the frequent occurrence of mannose-glucose repeats in the oligosaccharides of the FE-pool.

Considering the ratio of 1.5 mannose per mol glucose, which represents the distribution as present for the intact KGM polysaccharide, the FE-mixture seems to act less sugar-dependant on the KGM-chain compared to the fungal enzymes.

EG-digest

For the EG-pools the degradability of oligosaccharides with exo-glucanase and β-mannosidase significantly increased with increasing DP (**Table 4**). The degradability by exo-glucanase was surprising, since high mannose ratios were found for the higher-Mw EG-pools (**Table 1**). A simple structural extension by mannose-units at the non-reducing end of the oligosaccharides present in the preceding pool is thus not the case. The profiles of the tetramer-pool, composed of EG-4.1 and EG-4.2, after digestion with exo-glucanase and β-mannosidase are shown in **Figure 4B**.

The presence of a branched oligosaccharide was assumed for EG-4.1, as exo-glucanase resulted in the appearance of glucose, dimers and a trimer at the migration time of MMG, whereas β-mannosidase resulted in a complete degradation to monomers and dimers. Exo-glucanase may act differently on branched structures than on linear structures as observed for the DP3-pools. Nevertheless, the good degradability by both enzymes indicates the presence of mannose at the non-reducing ends and a core unit which contains glucose for DP-4.1, e.g. M[M]GG (**Table 5**). For DP-4.2 a single or combined presence of MMGG or/and MMMG was assumed (**Table 5**), as concluded by its resistance to exo-glucanase digestion and degradation by β-mannosidase (**Figure 4B**).

An increased presence of branched oligosaccharides rich in mannose may explain the presence of the homologous series up to DP > 15, as observed for the EG-digest.

Table 5. Structural propositions for tetrasaccharides from endo-β-(1,4)-glucanase-(EG) and endo-β-(1,4)-mannanase-(EM)-digest of konjac glucomannan. Peak numbers are according to **Figure 2**.

	peak	***structural proposition***
EG-digest	4.1.	M[M]GG
	4.2.	MMGG/MMMG
EM-digest	4.1.	[M]GGM
	4.2.	GMGM.

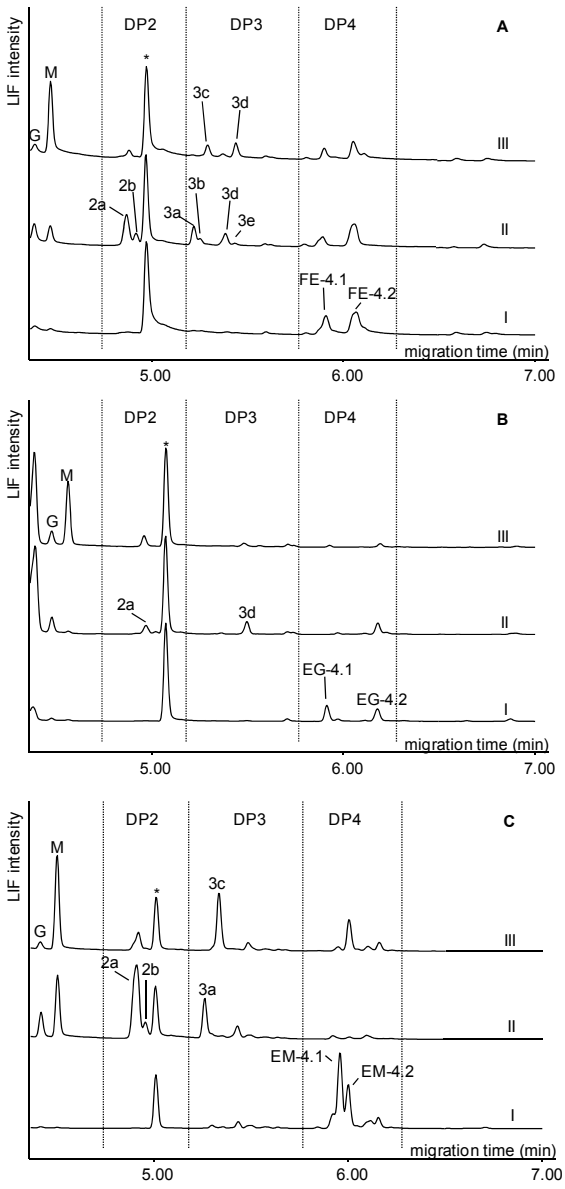


Figure 4. Action of exo- β-(1,4)-glucanase (exo-glucanase) (A/B/C-II) and β-(1,4)-mannosidase (β-man) (A/B/C-III) on tetrasaccharides resulting from konjac glucomannan digest with (A-I) enzymes from human fecal bacteria (FE), (B-I) endo-β-(1,4)-glucanase (EG) and (C-I) endo-β-(1,4)-mannanase (EM). FE-4.1/-4.2, EG-4.1/-4.2, EM-4.1/-4.2 are according to **Figure 2**. 2a: GG/GM/MG. 2b: MM. 3a: MGG°. 3b: MGM°. 3c: GGM°. 3d: MMG°. 3e: GMM°. °:structures are linear or branched. *:maltose internal standard.

EM-digest

Although DP4- and DP5-pools were present in the EM-digest, their contribution to the total digest is minor, as shown in **Figure 1**. These DP-pools are thus less important. Equal proportions of glucose and mannose were found for the EM-tetramer-pool (**Table 1**). The oligosaccharides in the DP4-pool (EM-4.1 and EM-4.2) are well-degraded by exo-glucanase (**Figure 4C**). A branched structure was assumed for EM-4.1 ([M]GGM, **Table 5**), due to the presence of GGM in the β-mannosidase-digest. Additionally, a compound at the migration time corresponding to MGG was present in the exo-glucanase digest, which may correspond to the branched [M]GG. The dominant presence of dimers (H2) in the exo-glucanase digest was assumed to result from EM-4.2. EM-4.2 was not degraded by β-mannosidase and was thus assigned as GMGM (**Table 5**).

No conclusions could be drawn on the structural composition of oligosaccharides present in the DP5-pool.

Characterization of the KGM polysaccharide

Different opinions on the structure of the KGM polysaccharide exist. A blockwise distribution of mannose units within the KGM polysaccharide chain was claimed by Shimahara et al,[10] whereas a random distribution was proposed by Cescutti et al.[11] The significantly increased ratio of mannose within oligosaccharides DP > 4 in the EG-digest as observed in our research supports the existence of a blockwise distribution of mannose units within the KGM polysaccharide chain. Ramification of the KGM-backbone with side chains rich in mannose would explain the homologous peak series of DP > 15 as obtained for the EG-digest. It would also justify the good degradability of the polysaccharide by EM to mainly monomers, dimers and trimers. The small hydrolysis products and the equal mannose/glucose ratio in the DP-pools for EM confirm the absence of more than two sequential glucose units in the KGM chain as suggested by Shimahara et al.[10] Repeating glucose/mannose-containing core structures in the KGM main chain, to which mannose-rich branches are attached, were assumed by us, as indicated by the structural assignments M[M]GG (EG-4.1) and [M]GGM (EM-4.1). Our research supports the attachment of the side chains to glucose-units in the backbone, as was proposed by Katsuraya et al,[12] but disagrees with the findings of Maeda et al[9] and Smith et al[25] who propose branching at both glucose and mannose units in the polysaccharide backbone.

KGM is an acetylated polysaccharide (DA 5-10%).[8] The distribution of the acetyl groups has not yet been determined. An attachment of the acetyl group to the mannose unit can be assumed due to the presence of higher-Mw acetylated oligosaccharides in the EG-digest

(**Figure 1**), which are rich in mannose. A hypothetical structure of the KGM polysaccharide as revealed by our study is shown in **Figure 5**.

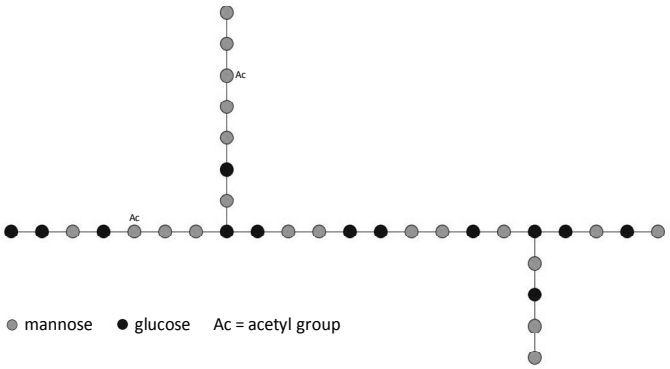


Figure 5. Excerpt of the konjac glucomannan polysaccharide as revealed by this study. Acetyl groups are placed arbitrarily on the mannose units.

Evaluation and implication of the hydrolysis of KGM by fecal enzymes

For the degradation of KGM by human gut bacteria, an enzyme machinery is activated, which results in the presence of a broad range of non-acetylated oligosaccharides. The FE-mixture seems to act in a less sugar-dependent way on the KGM-chain compared to the fungal enzymes tested. This can be concluded from the ratio of 1.5 mannose per mol glucose for the oligosaccharides produced, which represents the sugar distribution of the intact KGM polysaccharide. A multi-enzyme-system expressed by the fecal bacteria is indicated by the presence of KGM hydrolysis products of consecutive mannose-glucose-units. Gut bacteria adapt their enzyme expression to the substrates provided, as has been previously concluded from an *in vitro* fermentation of arabinoxylan.[26]

Surprisingly, in the FE-digest mono- and dimers were absent. With the absence of mono- and dimers and the presence of a large range of oligosaccharides obtained for the FE-digest and the *in vitro* fermentation,[7] endo-acting enzymes, which have different modes of action on KGM compared to the fungal enzymes used in this study, may therefore be prevailing. The oligosaccharides observed may represent suitable intermediate degradation products, which serve as nutrients for the survival of other gut bacteria. Bacterial cross-feeding has been recognized as gastrointestinal utilization mechanism for polysaccharides.[27]

Monitoring and characterizing intermediately formed oligosaccharides during *in vitro* fermentation provides an insight into the degradation mechanism of complex polysaccharides in the gastrointestinal tract. The structural composition of oligosaccharide mixtures influences their utilization by fecal bacteria, as was previously observed.[7] Oligosaccharides produced by EM, which are of small size carrying a glucose unit at their non-reducing end, resisted more to *in vitro* fermentation than oligosaccharides produced by cellulase.[7] The endo-enzymes of the fecal microbiota show a different mode of action on KGM and only the determination of the prebiotic index[28] (PI; defined as PI = [(Bifidobacteria/total bacteria) – (Bacteroides/total bacteria) + (Lactobacilli/total bacteria) – (Clostridia/total bacteria)]) would give information on the prebiotic value of the different oligosaccharide mixtures. Prebiotic oligosaccharides produced by fecal enzymes would require the characterization and identification of these enzymes for their subsequent large-scale use for the production of oligosaccharides. The production of functional oligosaccharides by the enzymes from health beneficial bacteria or the gut microbiota has been already recognized for the production of galactooligosaccharides,[14,15] although not yet commercially applied.

References

1. Gibson, G.R.; Roberfroid, M.B. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *J. Nutr.*, **1995**, *125*, 1401-1412.
2. van den Broek, L.A.M.; Hinz, S.W.A.; Beldman, G.; Vincken, J.-P.; Voragen, A.G.J. Bifidobacterium carbohydrases-their role in breakdown and synthesis of (potential) prebiotics. *Mol. Nutr. Food Res.*, **2008**, *52*, 146-163.
3. Ito, M.; Deguchi, Y.; Matsumoto, K.; Kimura, M.; Onodera, N.; Yajima, T. Influence of galactooligosaccharides on the human fecal microflora. *J. Nutr. Sci. Vitaminol.*, **1993**, *39*, 635-640.
4. Wang, X.; Gibson, G.R. Effects of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *J. Appl. Bacteriol.*, **1993**, *75*, 373-380.
5. Gibson, G.R.; Probert, H.M.; van Loo, J.; Rastall, R.A.; Roberfroid, M.B. Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutr. Res. Rev.*, **2004**, *17*, 259-275.
6. Kabel, M.A.; Kortenoeven, L.; Schols, H.A.; Voragen, A.G.J. *In vitro* fermentability of differently substituted xylo-oligosaccharides. *J. Agric. Food Chem.*, **2002**, *50*, 6205-6210.
7. Albrecht, S.; van Muiswinkel, G.C.J.; Schols, H.A.; Voragen, A.G.J.; Gruppen, H. Introducing capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) for the characterization of konjac glucomannan oligosaccharides and their in vitro fermentation behavior. *J. Agric. Food Chem.*, **2009**, *57*, 3867-3876.
8. Maekaji, K. Determination of acidic component of konjac mannan *Agr. Biol. Chem. Tokyo*, **1978**, *42*, 177-178.
9. Maeda, M.; Shimahara, H.; Sugiyama, N. Detailled examination of the branched structure of konjac glucomannan. *Agr. Biol. Chem. Tokyo*, **1980**, *44*, 245-252.
10. Shimahara, H.; Suzuki, H.; Sugiyama, N.; Nisizawa, K. Partial purification of β-mannanases from the konjac tubers and their substrate specifity in the relation to the structure of konjac glucomannan. *Agr. Biol. Chem. Tokyo*, **1975**, *39*, 301-312.
11. Cescutti, P.; Campa, C.; Delben, F.; Rizzo, R. Structure of the oligomers obtained by enzymatic hydrolysis of the glucomannan produced by the plant *Amorphophallus konjac*. *Carbohydr. Res.*, **2002**, *337*, 2505.
12. Katsuraya, K.; Okuyama, K.; Hatanaka, K.; Oshima, R.; Sato, T.; Matsuzaki, K. Constitution of konjac glucomannan: Chemical analysis and ^{13}C NMR spectroscopy. *Carbohydr. Polymers*, **2003**, *53*, 183.
13. Voragen, A.G.J. Technological aspects of functional food-related carbohydrates. *Trends Food Sci. Technol.*, **1998**, *9*, 328.
14. Splechtna, B.; Nguyen, T.H.; Steinboeck, M.; Kulbe, K.; Lorenz, W.; Haltrich, D. Production of prebiotic galacto-oligosaccharides from lactose using beta-galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.*, **2006**, *54*, 4999-5006.
15. Tzortzis, G.; Goulas, A.K.; Gee, J.M.; Gibson, G.R. A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous *in vitro* fermentation system and in the proximal colonic contents of pigs *in vivo*. *J. Nutr.*, **2005**, *135*, 1726-1731.
16. Albrecht, S.; Schols, H.A.; Klarenbeek, B.; Voragen, A.G.J.; Gruppen, H. CE-LIF as an analysis and quantification tool for galactooligosaccharides extracted from complex food matrices. *J. Agric. Food Chem.*, **2010**, *58*, 2787-2794.
17. Beldman, G.; Searle van Leeuwen, M.F.; Rombouts, F.M.; Voragen, A.G.J. The cellulase of *Trichoderma viride* - purification, characterization and comparison of all detectable endoglucanases, exoglucanases and β-glucosidases. *Eur.J.Biochem.*, **1985**, *146*, 301-308.
18. Düsterhöft, E.-M.; Bonte, A.W.; Voragen, A.G.J. Solubilisation of non-starch polysaccharides from oil-seed meals by polysaccharide-degrading enzymes. *J.Sci.Food Agric.*, **1993**, *63*, 211-220.
19. McCleary, B.V. β-D-mannosidase from *Helix pomatia*. *Carbohydr. Res.*, **1983**, *111*, 297-310.
20. Sunvold, G.D.; Hussein, H.S.; Fahey, G.C., Jr; Merchen, N.R.; Reinhart, G.A. *In vitro* fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and ruminal fluid from cattle. *J. Anim Sci.*, **1995**, *73*, 3639-3648.
21. Sengkhamparn, N.; Verhoef, R.; Schols, H.A.; Sajjaanantakul, T.; Voragen, A.G.J. Characterisation of cell wall polysaccharides from okra (*Abelmoschus esculentus* (L.) Moench). *Carbohydr. Res.*, **2009**, *344*, 1824-1832.
22. Kühnel, S.; Westphal, Y.; Hinz, S.W.A.; Schols, H.A.; Gruppen, H. Mode of action of Chrysosporium lucknowense C1 α-L-arabinohydrolases. *Bioresource Technol.*, **2011**, *102*, 1636-1643.
23. Shimahara, H.; Suzuki, H.; Sugiyama, N.; Nishizawa, K. Isolation and characterization of oligosaccharides from an enzymatic hydrolyzate of konjac mannan. *Agr. Biol. Chem. Tokyo*, **1975**, *39*, 293-299.

24. Tenkanen, M.; Niku-Paavola, M.; Linder, M.; Viikari, L. Cellulase in food processing. In *Handbook of enzymology*; Whitaker, J.R., Voragen, A.G.J., Ed; Marcel-Dekker: New York, **2003**; pp 771-790.
25. Smith, F.; Srivastava, H.C. Constitution studies on the glucomannan of konjac flower. *J. Am. Chem. Soc.*, **1959**, *81*, 1715-1718.
26. Vardakou, M.; Palop, C.N.; Christakopoulos, P.; Faulds, C.B.; Gasson, M.A.; Narbad, A. Evaluation of the prebiotic properties of wheat arabinoxylan fractions and induction of hydrolase activity in gut microflora. *Int. J. Food Microbiol.*, **2008**, *123*, 166-170.
27. Rossi, M.; Corradini, C.; Amaretti, A.; Nicolini, M.; Pompei, A.; Zanoni, S.; Matteuzzi, D. Fermentation of fructooligosaccharides and inulin by Bifidobacteria: A comparative study of pure and fecal cultures. *Appl. Environ. Microbiol.*, **2005**, *71*, 6150-6158.
28. Palframan, R.; Gibson, G.R.; Rastall, R.A. Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides. *Lett. Appl. Microbiol.*, **2003**, *37*, 281-284.

Chapter 8

General discussion

Research aim and approach

Knowledge on the gastrointestinal fate of diet-related oligosaccharides is limited. Nevertheless it is necessary in view of the structure-function relationship of these oligosaccharides. The aim of this PhD research was, firstly, to provide a suitable method for the isolation, identification and quantification of oligosaccharides in complex food and body matrices, and, secondly, make use of the method for an advanced understanding of the gastrointestinal fate of diet-related oligosaccharides, with focus on infant nutrition.
Human milk oligosaccharides (HMOs), which represent the most optimal functional oligosaccharides in baby nutrition, galactooligosaccharides (GOS), which are representatives of a prebiotic with market position, and konjac glucomannan (KGM) and oligosaccharides derived hereof, representing a structurally complex and potentially prebiotic substrate, were used in this PhD research. Their gastrointestinal fate was monitored *in vitro* or *in vivo*, by making use of the sensitive analytical tool CE-LIF/MS. Screening the gastrointestinal metabolization of oligosaccharides showed to provide a mirror for monitoring the gastrointestinal adaptation towards different kinds of diet-related oligosaccharides. The findings of this PhD research project and their possible nutritional, scientific and application-related implications are discussed in this chapter.

Qualitative and quantitative analysis of complex oligosaccharides by CE-LIF/MS

The increasing scientific, public and industrial interest in the bioactive mechanism of functional oligosaccharide mixtures demands their advanced qualitative and quantitative analysis from complex matrices, such as food and body liquids. Furthermore, high requirements for the assessment of gastrointestinal functionality and quantity in the product are imposed for the approval of these oligosaccharides as functional ingredients by European legislation (EG 1924/2006).[1] At present, oligosaccharide contents in food are usually determined by the total monosaccharide content after enzymatic incubation of the samples with β-galactosidase (AOAC 2001.02 for GOS)[2] or inulinase (AOAC 997.08 for inulin and FOS).[3] Unfortunately, high concentrations of dimers and monomers (lactose in case of milk-based products; glucose/fructose/sucrose in case of fruit-based products) can influence the enzymatic determination of the low amounts of oligosaccharides present in the matrix. In order to fulfil the high demands of oligosaccharide analysis at present, an analysis on an individual oligosaccharide level is preferable.

CE-LIF was introduced as a sensitive qualitative and quantitative tool for the analysis of individual oligosaccharides from food matrices in chapter 2. Preceding the quantification step, low amounts of GOS were reproducibly extracted from matrices rich in monosaccharides, sucrose, lactose and maltodextrin. A selective purification of the oligosaccharides was achieved by the combination of the enzymatic degradation of maltodextrins by amyloglucosidase pretreatment and solid phase extraction on graphitized carbon material.[3,4] Due to the mole-based derivatization of oligosaccharides with the fluorescent APTS,[5] the reducing oligosaccharides can be quantified, even if individual oligosaccharide standards are not available. Furthermore, the migration of neutral oligosaccharides of low Mw by CE-LIF is predictable, as it is influenced by the size of the molecules. With other advanced techniques based on liquid chromatography (HPAEC and separation based on porous graphitized carbon), a prediction of the migration time of unknown compounds is not possible.[6,7] In our research, CE-LIF analysis showed to be a valuable method for the analysis of complex oligosaccharide mixtures, namely GOS (chapter 2), HMOs (chapter 3-5) and KGM oligosaccharides (chapter 6 and chapter 7). In previous research, CE-LIF was successfully used for the separation of oligosaccharides from pectins and hemicelluloses.[8-10] Supplementary, the possibility of on-line coupling to mass spectrometry makes CE-LIF an attractive tool for studying oligosaccharides before and after gastrointestinal metabolization, where numerous unknown structures can be formed.
CE-LIF can thus be considered as a candidate for the establishment of official, validated methods for the qualitative and quantitative analysis of a broad range of reducing oligosaccharides on an individual level. Nevertheless, validation including ring-tests should be done for a broad range of matrices.

Some analytical challenges remain. CE-LIF analysis requires the derivatization of the carbohydrates with a charged fluorescent. In this research APTS is used, which reacts with the reducing carbohydrates via reductive amination. Non-reducing carbohydrates can be important components in functional oligosaccharide mixtures (e.g. fructooligosaccharides or α-(1,1)-linked GOS), but are not labeled by APTS-derivatization. Alternative methods (e.g. liquid chromatography based methods on porous graphitized carbon (PGC) or hydrophilic interaction liquid chromatography (HILIC)) have, therefore, to be considered, if non-reducing oligosaccharides are of interest. Or else, harsher labeling techniques than reductive amination with APTS should be applied for the non-reducing oligosaccharides, e.g. techniques based on hydrazone-bond formation.[11] Peak overlap is a general problem for

the analysis of complex oligosaccharides with any analytical technique. It is of special concern for higher-Mw oligosaccharides, for which the number of isomers is usually high and the concentration of the respective oligosaccharides is low. The use of complementary techniques may give a broader insight into the oligosaccharide composition.
The recent developments on CE-MS hyphenation by sheathless interface[12] and the possibility to use polyvinylalcohol-coated capillaries not only for off-line CE-LIF analysis, but also for CE-MS coupling[13] may make CE even more powerful for the analysis of low amounts of complex oligosaccharides in near future.

HMOs from breast milk

The composition of the HMOs in breast milk depends on the mother's Secretor- and Lewis-type and has extensively been studied.[14-16] In chapter 5, the breast milks of eleven mothers were monitored during six months of lactation. For eight mothers, Le(a-b+)-secretor-characteristic HMO-profiles composed of non-substituted and (α1,2)-/(α1,3)-/(α1,4)-fucosylated and sialylated HMOs were obtained. For two mothers, Le(a+b-)-non-secretors-characteristic HMO-profiles composed of non-substituted and (α1,3)-/(α1,4)-fucosylated and sialylated HMOs were obtained. Only one mother showed Le(a-b-)-secretor-characteristic HMO-profiles composed of non-substituted and (α1,2)-/(α1,3)-fucosylated and sialylated HMOs. The Le-/Se- gene expression of the European population, which is approximately 70% Le(a-b+), 20% Le(a+b-) and 10% Le(a-b-),[17,18] is represented by this study group. The geographical distribution of the Le-/Se- gene expression has to be taken into account when performing research on the HMO-composition in breast milk. Studies on the breast milk composition among Mexican mothers resulted in a prevalence of > 99 % secretors, whereas a study among Philippine mothers resulted in a percentage of only 46% secretors.[18] Secretor-characteristic (α1,2)-fucosylated oligosaccharides were recognized as receptor analogs for pathogenic bacteria and the quantity of these oligosaccharides in breast milk is even discussed as a quality parameter of human milk.[19] The increased proportion of secretors among certain populations was proposed to be a genetic response to the increased presence of certain pathogenic bacteria in the regions of these populations.[19]
Markedly during the first few months of lactation differences in the expression of fucosylated oligosaccharides within the Le(a-b+)-secretor-group were observed in the present study, as was shown in chapter 5 by the LNFP I/III / LNDFH I ratio. This ratio was either $< 1.5:1$ or $\geq 2:1$. With CE-LIF, it is not possible to distinguish between (α1,2)- and

(α1,3)-fucosylated oligosaccharides, which is a disadvantage in view of the biological significance of (α1,2)-fucosylated oligosaccharides.
The HMO-content decreases with proceeding lactation time.[16,20-22] In the present study, a decrease of 40% of the total, initially present HMO-concentration was found after three months lactation. This corresponds well with previous studies, where a decrease of approximately 20% and 50% was found after two months and three months, respectively.[16,21] Overall, the HMO-profile of breast milk does not significantly change during the lactation course. (Alpha1,3)-fucosylated oligosaccharides present an exception, as their concentration increases with time.[16,20,22] Correspondingly, after 3 months, a plateau in HMO-concentration was stated in the present research (chapter 5) and could be deduced to an increase in (α1,2)-/(α1,3)-FL-concentration. The change in abundance from (α1,2)- to (α1,3)-fucosylated oligosaccharides was proposed to be a consequence of a decreased activity of the secretor gene in time[20] or might be an adjustment of breast milk to different biological needs in time.

Gastrointestinal metabolization of diet-related oligosaccharides

In the present research, 16 oligosaccharides in baby feces, which could not directly be linked to the oligosaccharide structures in the respective diet, were annotated by means of CE-MS^n (**Table 1**). Their presence was associated with the gastrointestinal metabolization of diet-related oligosaccharides, as discussed below.

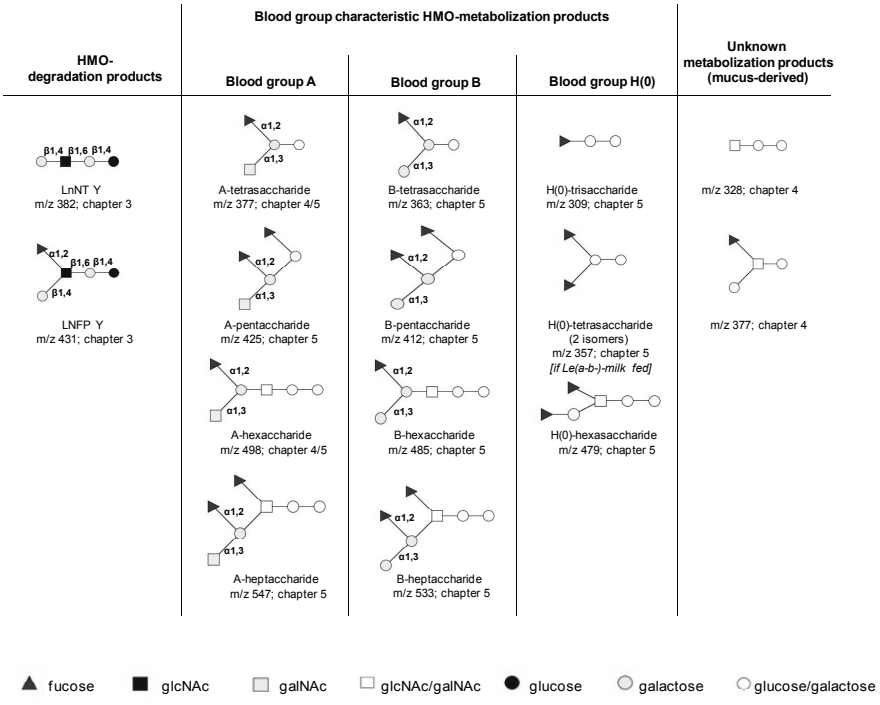
Breast-fed babies

Three-stage model of gastrointestinal development
By studying feces of breast-fed babies and the corresponding breast milks during the first six months postpartum (chapter 5), the gastrointestinal metabolization of HMOs could be schematized and was sub-divided into three continuous stages. Shortly after birth (*stage 1*), fecal oligosaccharide profiles of exclusively breast-fed babies are composed of either neutral or acidic HMOs. HMO-degradation products (LnNT Y, LNFP Y) can be present. Their origin from higher-Mw HMOs, which contain the bifidogenic lacto-N-biose unit (LNB),[23] indicates the easy utilization of this unit by the characteristic breast-fed microbiota. The fact that no HMO-degradation products were detected in previous studies can be deduced to the peak identification, which was then limited to HMO-standards.[24-26] The screening of the individual oligosaccharides was not performed.[27-30]

With advancing time postpartum, we monitored the gradual development to a fecal oligosaccharide profile composed exclusively of blood group characteristic oligosaccharides (*stage 2*). These oligosaccharides were assigned as conjugates of HMO-units and blood group-specific antigenic determinants (either, A, B or H(0)) from the gastrointestinal mucus (chapter 5). Comparable results were previously found for fecal samples of a preterm- and aterm-baby both belonging to blood group A.[28,29] As sample amounts were limited, no results could previously be drawn for other blood group types.[27] Even more, the fecal presence of neutral HMOs in a previous study, in which fecal samples were exclusively collected shortly after birth thus led to the conclusion, that there occurs no gastrointestinal metabolization of HMOs in the neonatal gut.[26]

At an advanced time postpartum, when solid food has been introduced, these personalized oligosaccharide profiles disappear (*stage 3*). Oligosaccharides, which were newly introduced with the diet are now present (starch-derived structures). No information on fecal oligosaccharide profiles during weaning (*stage 3*) has been available so far.

Table 1. Overview on oligosaccharides resulting from gastrointestinal metabolization, as assigned in the present study.



Genetic influence

During this research we observed a considerable influence of the oligosaccharide composition of the breast milk on the oligosaccharide profiles of the feces of the baby.

The breast milk profiles, as found for the mothers, were present in their initial Secretor- and Lewis-specifc form in the baby feces during *stage 1* (chapter 5). For none of the babies studied, a catabolism or re-synthesis of Secretor-/Lewis-specific HMOs was observed in the feces as was suggested in previous research.[27,29] Furthermore, the ratio of LNFP I/III : LNDFH I in breast milk seems to have an influence on the presence of either an acidic or neutral HMO-profile during *stage 1* (chapter 5). Whereas consumption of breast milk having a LNFP I/III / LNDFH I -ratio of ≥ 2:1 favoured the presence of a fecal oligosaccharide profile composed of acidic HMOs, predominantly neutral HMOs were found to be present in the feces if the LNFP I/III / LNDFH I -ratio in the breast milk was < 1.5:1.

The ABH(0)-blood group system does not influence the presence of HMOs in human milk.[31] In the feces of breast-fed babies, ABH(0)-specific metabolization products are abundantly present during *stage 2* (chapter 4 and chapter 5), resulting in personalized oligosaccharide profiles. For formula-fed babies, blood group specific oligosaccharides are absent (chapter 5). Thus, their production clearly depends on the gastrointestinal presence of HMOs. Our study also indicated a dependence of the formation of these blood group characteristic oligosaccharides on the HMO-composition in breast milk. A baby with blood group H(0), having a Le(a-b-)-mother, showed a different fecal oligosaccharide profile during *stage 2* than three assumed blood group H(0)-babies having Le(a-b+)-mothers (chapter 5). We, therefore, assume that the presence of blood group specific oligosaccharides in *stage 2* largely depends on the composition of the breast milk, which is dependent on the mother's Secretor- and Lewis-type. Secondly, the secretor-status of the child has to be considered, as the presence of ABH(0)-oligosaccharides at the non-reducing terminus of mucin glycoproteins in the colon is dependent on the host's ability to express (α1,2)-fucosidase.[31]. The ability of expressing (α1,2)-fucosylated oligosaccharides may change during the first years of life.[32]

Among the 27 fecal samples of breast-fed, formula-fed and mixed-fed two months old preterm babies (chapter 5) were the samples of two twin-pairs and one triplet-pair. One twin-pair was predominantly breast-fed (A6/A7, chapter 5), and the other twin pair (B7/B8, chapter 5) and the triplet pair (B5/B6/B10, chapter 5) were predominantly formula-fed. High similarities in fecal oligosaccharide profiles, resulting in high similarity by statistical means (AHC), were observed for the twin pairs and two siblings of the triplet pair.

The oligosaccharide profiles as were found for the breast-fed twin-pair A6/A7 are shown in **Figure 1**.
The highly similar fecal oligosaccharide profiles highlight the influence of breast milk and a similar gastrointestinal metabolisation in the gastrointestines of the siblings.
These results confirm the influence of the host's genotype on the gastrointestinal microflora, which was concluded previously, after studying the gastrointestinal microflora of genetic relatives.[33]

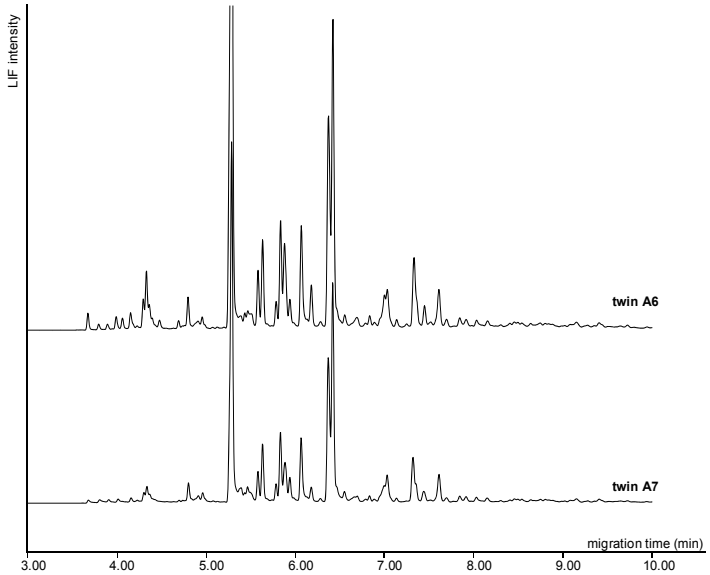


Figure 1. Fecal oligosaccharide profiles (CE-LIF) of predominantly breast-fed twin pair A6/A7 (chapter 5) at the age of two months.

Possible gastrointestinal function of blood-group characteristic metabolization products
Blood group specific oligosaccharides were assumed to be conjugation products of HMO-units and antigen-specific, non-reducing termini of intestinal mucin glycoproteins (chapter 5). The production of blood group active oligosaccharides in the intestine of breast-fed babies results in soluble analogs of the antigenic structures, which were initially fixed to the intestinal mucus layer. Having soluble receptor analogs available may be useful for a better distribution of these immuno-determinant groups within the colon, and may enable their transport to distanced body tissues subsequent to their absorption.

This may result in an enhanced protection against pathogenic bacteria, which can be bound to the respective soluble receptor analog before attaching to a similar epitope within the gastrointestinal mucus layer. The formation of personalized, soluble receptors may thus contribute to the overall health effects, which are attributed to the consumption of breast milk and the stimulation of the immune system.

Breast- and formula-fed babies

Influence of gestational age, dosage and kind of oligosaccharides

Overall, after two months, the fecal oligosaccharide profiles obtained from preterm born breast-fed babies (chapter 4 and chapter 5, baby "I") indicated a less advanced gastrointestinal metabolization and degradation than the profiles of term born breast-fed babies after the same time postpartum (chapter 5). From a medical point of view, preterm birth is characterized with immature digestive functions[34-36] and a slower microbial colonization pattern.[37] Additionally, the intestine of preterm born babies is still short.[38] The under-developed digestive functioning in a short intestine may result in a supply of residual small carbohydrates (e.g. lactose) to the colon and serve as nutrient for the microflora, which thus does not have to utilize the more complex carbohydrate structures.

GOS was absent in the feces of formula-fed, preterm babies (0.4 g/dL GOS) after two months (chapter 4). Blood group characteristic oligosaccharides were not detected. Instead, some babies showed an abundance of a structure assigned as HexNAc-Hex-Hex in their fecal extracts, which was assumed to originate from gastrointestinal mucins. The functional role of this oligosaccharide is not known.

In a parallel study, results indicated a possible dependency of the fecal recovery of GOS on the gestational age and concentration of GOS in the formula. For babies, who got formula supplemented with 0.5 g/dL GOS, we observed fecal oligosaccharide profiles of hexose-oligosaccharides of mainly DP4, when the samples were collected at an age of < one month and the baby was born at a relatively early gastrointestinal age (**Figure 2**).

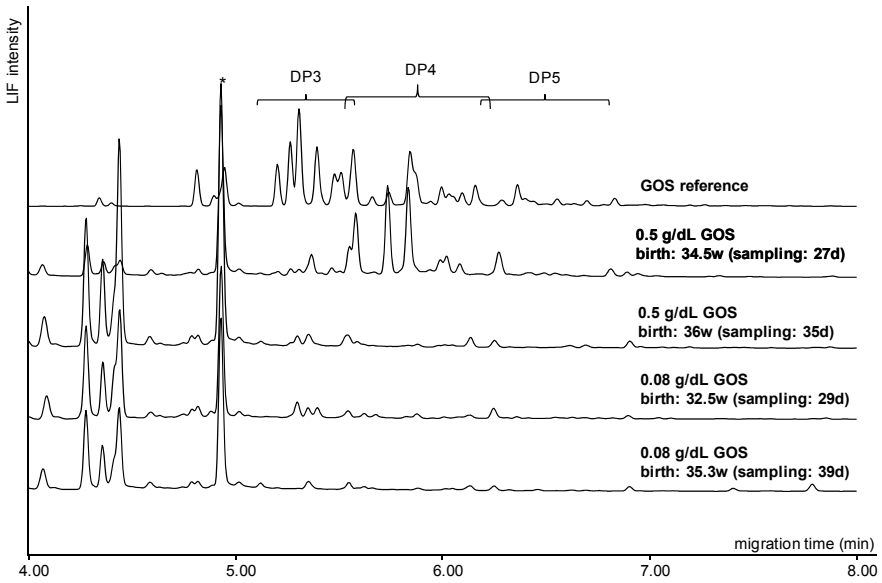


Figure 2. Fecal oligosaccharide profiles (CE-LIF) of preterm born babies fed with infant formula supplemented with 0.08 g/dL GOS or 0.5 g/dL GOS and GOS reference. Gestational age and sampling time are indicated (w: weeks; d: days). *: internal standard maltose.

As the fecal oligosaccharide profiles showed only limited similarity with the GOS-standard, gastrointestinal degradation and metabolization of GOS is already ongoing at that time point. No fecal oligosaccharide profiles showing a pronounced presence of hexose-oligosaccharides were observed for preterm babies, who got a formula supplemented with a low dosis of GOS (0.08 g/dl), independent of the time of gestation and sampling after birth (**Figure 2**).

In an earlier microbiological study on the microflora of formula-fed babies, a dosage-dependent bifidogenic effect of GOS/FOS (9:1)-supplementation was found.[39] Higher Bifidobacteria-counts were observed for the babies who got formula supplemented with 0.8 g/dL GOS/FOS (9:1) compared with the babies who got formula supplemented with 0.4 g/dL GOS/FOS (9:1).[39] For the babies who got formula supplemented with 0.8 g/dL GOS/FOS (9:1), fecal oligosaccharide profiles showing high-Mw FOS and low-Mw oligosaccharides were recovered after one month postpartum (**Figure 3**).[24] As the oligosaccharide profile of the initial GOS-mixture was not shown by the authors and the fecal oligosaccharide profile was not confirmed by MS analysis,[24] a partial metabolization of GOS at that time point cannot be ruled out.

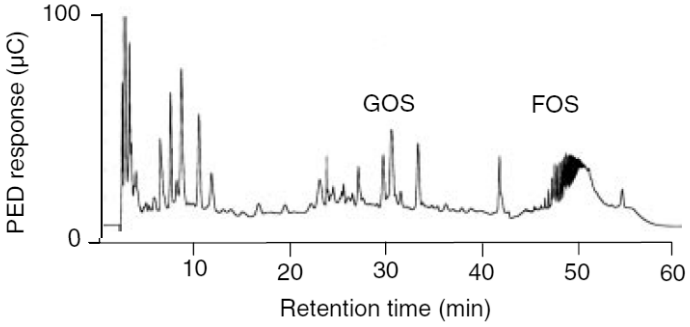


Figure 3. Fecal oligosaccharide profile (HPAEC-PAD) of a term born baby fed with infant formula supplemented with 0.8 g/dL GOS/FOS (9:1), at 28 days postpartum. Reprinted from Thurl et al, with permission from WILEY publishers.[24]

Remarkably, the higher–Mw FOS (DP > 10)[40] seem to survive the gastrointestinal passage, although supplied in a ten times lower dose than GOS. Thus, the presence of GOS may have influenced the degradability of the higher-Mw FOS. Correspondingly, GOS may delay the gastrointestinal metabolization of HMOs and may stimulate the appearance of unknown structures (Hex-[Fuc]-HexNAc-Hex and HexNAc-Hex-Hex) in the feces, as was observed for mixed-fed babies and mixed- and formula-fed babies, respectively (chapter 4).

Influence of gastrointestinal microflora development on oligosaccharide metabolization

The gastrointestinal postnatal microbiota succession of breast- and formula-fed babies is different. Results from several studies were summarized in chapter 1.[41-45] **Table 2** gives a literature overview on the colonic microflora development. It incorporates the observations made for the oligosaccharide profiles at the respective postnatal stages in our study. For all stages, relations between bacterial composition and oligosaccharide profiles are observed.
The mixed, facultative anaerobe flora, as present in the first few weeks of life, is primarily not capable to completely degrade or metabolize the complex HMOs. The presence of HMO-building blocks, such as lacto-N-biose (LNB), stimulates the gradual development to the "simple" flora of *stage 2*, of which Bifidobacteria contribute to a considerable level.[44]

Table 2. Gastrointestinal colonization and fecal oligosaccharide composition in the postnatal period. Microflora composition is merely indicative, as information was derived from different studies.[41-45]

			***microflora composition*[41-45]**	***fecal oligosaccharide composition***
1.	first weeks (*stage 1*)	breast-fed/ formula-fed	mixed flora; large contribution of facultative anaerobe species	*breast feeding:* neutral HMOs/acidic HMOs/ (degradation-, metabolization products) *formula feeding:* supplemented oligosaccharides/ degradation-, metabolization products
2.	pre-weaning (*stage 2*)	breast-fed	anaerobic, "simple" flora: Bifidobacteria/ Bacteroides	*breast feeding:* blood group characteristic oligosaccharides; no HMOs
		formula-fed	maintenance of mixed flora: anaerobic/ facultative anaerobic *with prebiotic supplementation:* mixed flora with increased counts of Bifidobacteria/Lactobacilli	*formula feeding:* absence of diet-related oligosaccharides/ predominant "mucus"-derived structure possible
3.	weaning (*stage 3*)	breast-fed/ formula-fed	mixed flora: anaerobic/ facultative anaerobic	*breast feeding:* oligosaccharides from solid food; no HMOs *formula feeding:* no experimental data available; no oligosaccharides expected
4.	post-weaning	breast-fed/ formula-fed	installation of "adult" flora; anaerobic; predominantly Bacteroides	*breast-/formula feeding:* no experimental data available; no oligosaccharides expected

On the contrary, the flora of formula-fed babies is not specifically stimulated with factors such as LNB during *stage 1*. The flora composition remains thus diverse and facultative anaerobes are still present in *stage 2*.

If functional oligosaccharides are supplemented to infant formula, beneficial colon bacteria are stimulated.[39] The species stimulated are probably different to the species stimulated during breast feeding and the overall flora composition was found to be more diverse.[43]

Overall, less dramatic changes in flora development were thus observed for formula-fed babies compared to breast-fed babies in the postnatal period.[44] The residing microbiota of (exclusively) formula-fed babies should, therefore, adapt to the degradation of the diet-related oligosaccharides earlier than the microbiota of (exclusively) breast-fed babies. This has indeed been observed in the present research (chapter 4).

The specific flora of breast-fed babies is able to conjugate HMO-units to blood group specific carbohydrate residues from the gastrointestinal mucosa during *stage 2*. The less specific flora of formula-fed babies is able to degrade a broad range of carbohydrates. Such carbohydrates include mucins from the colon.[46] The presence of mucin-related oligosaccharides (HexNAc-Hex-Hex; Hex-[Fuc]-HexNAc-Hex) in the feces of formula-fed and mixed-fed babies may be a mirror for a diverse microbiota. The gastrointestinal mucus layer is a barrier, which hinders the systemic invasion of toxins and pathogenic bacteria. Its use as main nutritional source for the gastrointestinal microbiota is judged as not favorable.[47] Enhanced mucin-degradation in case of a too low enteral supply of nutrients was previously proposed.[48] Alternatively, increased mucin secretion may be the response to the presence of luminal factors, such as organic acids.[49]

The change to *stage 3* (weaning) has been described as less pronounced for formula-fed babies than for breast-fed babies as the flora-composition does not change significantly.[44] A rapid adaptation to the new food and thus no, or minor, fecal recovery of diet-related oligosaccharides is expected. In contrast, weaning has been described as a critical period for breast-fed babies, with a possibility of increased susceptibility to gastrointestinal disorders.[44] The flora changes significantly and the recovery of diet-related oligosaccharides may be a witness of a not yet stabilized flora, similar to what was observed for *stage 1*. During the development of an "adult" flora in the post-weaning period, an adaptation of the microflora to the diet-related oligosaccharides is expected in time also for breast-fed babies, leading to the disappearance of diet-related oligosaccharides in the feces.

Microflora adaptation to carbohydrate substrates

Our study showed that the enzyme machinery produced by the colonic microflora adapts gradually to the respective carbohydrate substrates, as introduced at the beginning of each stage. The composition of this respective microflora may be different, depending on the substrate, to which the flora has to adapt to. Several studies proved the adaptation to carbohydrate substrates *in vitro*, e.g. to arabinoxylan and arabinogalactan,[50,51] which was

indicated by elevated levels of enzyme activity necessary for the degradation of the respective substrate.

In this research, a broad range of carbohydrate substrates was incubated with an enzyme cocktail from fecal bacteria in order to test its degradation potential. An overview is given in **Table 3**. The enzyme cocktail, resulted from fecal bacteria, which had been adapted *in vitro* to the degradation of konjac glucomannan (chapter 6 and chapter 7). Remarkably, the cocktail of fecal enzymes showed only pronounced activity towards substrates with backbones similar to konjac glucomannan, except for its activity towards maltodextrin. Even high degrees of substitution, as is the case for locust bean gum galactomannan, did not hinder its activity, reflecting the complexity of the initial glucomannan substrate the fecal bacteria were initially grown on.

Enzymes, which are not necessary to degrade the carbon source provided are thus not expressed by the microflora. Correspondingly, the change to another carbohydrate substrate in the colon of breast-fed babies during weaning requires an adaptation of the microflora and its enzyme machinery. The fecal presence of diet-related oligosaccharides at the beginning of *stage 3* (chapter 5) represents this adaptation period.

Interestingly, the incubation of maltodextrin, cellodextrin and galactomannan polymers (**Table 3**) did not result in oligosaccharide profiles reflecting a straightforward degradation to smaller oligosaccharides and monomers, which was the case when using fungal enzymes (e.g. endo-β-(1,4)-mannanase) to digest these substrates. Instead, a vast variety of oligosaccharide structures was obtained, indicating transglycosylation. This is exemplified by the incubation of tara galactomannan with either fungal endo-β-(1,4)-mannanase or the mixture of fecal enzymes in **Figure 4**.

The transglycosylation activity of beneficial gut bacteria has already been recognized as a potential source for producing target-specific functional oligosaccharides.[52,53] The gene-sequencing of beneficial gut bacteria, which exhibit transglycosylation activity may be a helpful tool for this purpose.[54]

Table 3. Incubation of carbohydrate structures with enzyme cocktail from *in vitro* fermentation of KGM by human inoculum. M: mannose; G: galactose

carbohydrate substrate	***Result from incubation with cocktail of fecal enzymes, pre-grown on konjac glucomannan***
pectin	decrease in Mw; no oligosaccharide formation
dextran	no reaction
arabinogalactan	no reaction
inulin	no reaction
GOS	no reaction
maltodextrin	transglycosylation
cellodextrin	transglycosylation
tara galactomannan *M/G 1.5:1*	degradation/transglycosylation
guar galactomannan *M/G 2.9:1*	degradation/transglycosylation
locust bean gum galactomannan *M/G 3.4:1*	degradation/transglycosylation
konjac glucomannan	de-acetylation/degradation

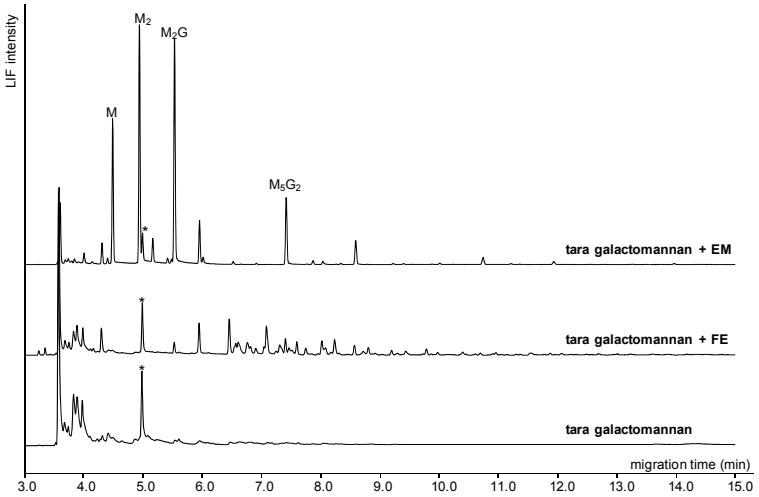


Figure 4. CE-LIF oligosaccharide profiles of tara galactomannan degraded by endo-β-(1,4)-mannanase (EM), fecal enzyme mixture (FE) and tara galactomannan blank. Assignment according to Daas et al.[55] M: mannose; G: galactose; *: internal standard maltose.

Future perspectives

Feeding practice

A general protocol for postnatal feeding does not exist as gastrointestinal maturation and, thus, the response to enteral feeding is individual-dependent. Nevertheless, some guidelines have been implemented in current practice. A six-months period exclusive breast feeding is recommended by the WHO[56] and the introduction of complementary food between four and six months was considered as safe by the EFSA.[57] There are, however, cases where breast feeding is not possible or not advisable, e.g. for HIV-infected mothers or in case of preterm birth.[58,59] Since the neurological and gastrointestinal functioning of preterm born babies may still be immature, parenteral nutrition may be necessary after birth. Furthermore, excessive amounts of enteral food may lead to inflammatory bowel diseases in the underdeveloped intestine of preterm born babies.[60] On the other hand, enteral feeding stimulates gastrointestinal maturation and an increase in mucosal mass.[61] Minimal enteral nutrition, which combines parenteral nutrition with small amounts of enteral nutrition has, therefore, been recognized as method of choice for preterm nutrition.[62]

Individual needs may have to be considered when choosing appropriate infant food and appropriate timing. Fecal oligosaccharide profiles mirror the gastrointestinal response to enteral feeding and may thus contribute to design an individual postnatal feeding protocol.

Supplementation of infant formula

Although infant formula have been on the market for many years, research is still on-going in order to improve the present formulations towards more optimal breast milk replacers.

Breast milk has a unique composition and cannot simply be replaced by animal milk. **Figure 5** exemplifies the proportions of the major components from bovine milk and human milk.

Bovine milk has a higher protein content compared to human milk, which can, besides of evoking allergies, stimulate a too rapid growth and thus increase the risk of overweight when feeding it to neonates.[63] The composition of the protein-fraction is casein-based in bovine milk. In human milk, the protein fraction is whey-based and contains the bioactive lysozym and lactoferrin.[63] These differences have to be considered in the production of infant formula. Furthermore, the supplementation of infant formula with breast milk characteristic long-chain polyunsaturated fatty acids (e.g. arachidonic acid and docosahexaenoic acid) are currently common practice.[64]

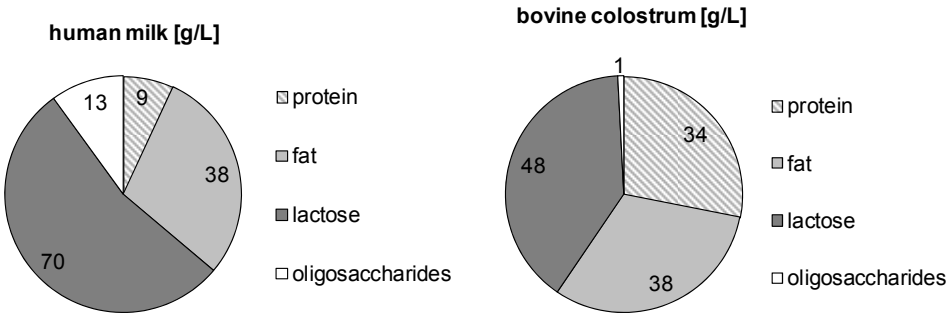


Figure 5. Composition of human milk and bovine colostrum.[63,65]

The emulation of the bioactive HMOs in infant formula is of special importance, but it is also very challenging due to the complexity of the HMO-structures. GOS and FOS are currently used as prebiotic supplements in infant formula. Although bifidogenicity has been proven for these oligosaccharides,[39,66-68] also other gastrointestinal species may be stimulated and the fermentation speed may be relatively quick, especially for the short-chain GOS. More complex substrates may further penetrate the colon, but may stimulate a rather diverse flora.[69] A better understanding on the gastrointestinal metabolization and successive flora development in breast- and formula-fed babies provides knowledge for an advanced supplementation of infant formula in the direction of breast milk. Results of *in vitro* fermentation studies of HMOs with specific bacterial strains and the present research highlight the importance of lacto-N-biose-containing structures for the establishment of a breast-fed-like microflora.[23] The decoration of these oligosaccharides with e.g. fucose (especially (α1,2)-linked) is necessary in view of their direct anti-adhesive functions.[19] The role of secretor-specific fucosylated oligosaccharides in the defence mechanism and their possible impact on the gastrointestinal metabolization has to be considered. An advanced knowledge on the bioactivity of these structures may even result in a selective supplementation of secretor-specific oligosaccharides to the feeding of babies who receive non-secretor-milk. To date, the enzymatic synthesis of HMOs or their production with recombinant bacteria is not feasible in terms of yield and costs.[70] The extraction of oligosaccharides from animal milk may offer an alternative. The oligosaccharide content of domestic animals is, however, low, e.g. 1-2 g/L for bovine colostrum, and even less for mature bovine milk or milk from goat or sheep, compared to

approximately 12-13 g/L in human milk.[65,71,72] In addition, the composition of these oligosaccharides is different compared to HMOs. Only few oligosaccharides of bovine milk are similar to the ones occurring in human milk. About 40 different oligosaccharides are known to be present in bovine milk.[73] These are mainly acidic oligosaccharides.[73,74]
Bovine milk oligosaccharides, for which a structural composition has been established are listed in **Table 4**. Besides sialylated oligosaccharides, bovine milk contains acidic oligosaccharides, which are decorated with glycolylic acid (Neu5Gc). Concentrations of neutral oligosaccharides have not yet been determined and may thus be quite low. Few phosphorylated oligosaccharides are present in bovine milk.

Table 4. Summary of oligosaccharides from bovine colostrum, and their corresponding presence in human milk. As investigated by Parkkinen et al,[74] Thurl et al[16] and as reviewed by Urashima et al.[65] n.d.: not determined. Neu5Ac: sialic acid. Neu5Gc: glycolylic acid. PO_4: phosphate group

structures	***bovine colostrum (mg/L)[65,74]***	***human milk (mg/L)[16,65]***
neutral oligosaccharides		
Gal-(β1,4)-GlcNAc	conc. n.d.	-
GalNAc-(β1,4)-Glc	conc. n.d.	-
Gal-(β1,3)-Gal-(β1,4)-Glc	conc. n.d.	traces
Gal-(β1,4)-Gal-(β1,4)-Glc	conc. n.d.	traces
Gal-(β1,6)-Gal-(β1,4)-Glc	conc. n.d.	traces
Gal-(α1,3)-Gal-(β1,4)-Glc	conc. n.d.	-
Gal-(β1,4)-[Fuc-(α1,3)]-GlcNAc	conc. n.d.	-
GalNAc-(α1,3)-Gal-(β1,4)-Glc	conc. n.d.	-
Gal-(β1,4)-GlcNAc-(β1,6)-[Gal-(β1,3)]-Gal-(β1,4)-Glc	conc. n.d.	-
acidic oligosaccharides		
Neu5Ac-(α2,3)-Gal		
Neu5Ac-(α2,3)-Gal-(β1,4)-Glc (3'SL)	95	240-310
Neu5Ac-(α2,6)-Gal-(β1,4)-Glc (6'SL)	19	1140-1310
Neu5Gc-(α2,6)-Gal-(β1,4)-Glc	conc. n.d.	-
Neu5Gc-(α2,3)-Gal-(β1,4)-Glc	1	-
Neu5Ac-(α2,6)-Gal-(β1,4)-GlcNAc	47	-
Neu5Gc-(α2,6)-Gal-(β1,4)-GlcNAc	conc. n.d.	-
Neu5Ac-(α2,3)-Gal-(β1,3)-Gal-(β1,4)-Glc	2	-
Neu5Ac-(α2,8)-Neu5Ac-(α2,3)-Gal-(β1,4)-Glc	28	-
phosphorylated oligosaccharides		
Gal-(β1,4)-Glc-3-PO_4	conc. n.d.	-
Neu5Ac-(α2,6)-Gal-(β1,4)-GlcNAc-1-PO_4	2	-
Neu5Ac-(α2,6)-Gal-(β1,4)-GlcNAc-6-PO_4	0.7	-

Milks from other animal species have to be taken into consideration when looking for a potential source of HMO-analogs. A remarkably, high concentration of fucosylated and sialylated oligosaccharides, structurally corresponding to HMOs, was found in the milk of elephants (20 g/L).[75] The oligosaccharide structures of elephant milk are, however, mainly based on LnNT instead of LNT.[75] On the opposite, high levels of LNT and considerable amounts of sialylated and fucosylated oligosaccharides were found in the milk from chimpanzee.[76] ABH(0)-specific oligosaccharides showed to be predominant components of feces from breast-fed babies in *stage 2*. Milk of polar bears contains ABH(0)-blood group specific oligosaccharides[77] and may thus be of scientific interest to find out more on the potential health-beneficial functionalities of these oligosaccharides.

Concluding remarks

The results obtained during this study broadened the knowledge on the gastrointestinal fate of diet-related oligosaccharides, mainly with reference to infant nutrition. Screening the gastrointestinal metabolization of oligosaccharides points to the gastrointestinal adaptation to enteral food. Future studies have to focus on the correlation of analytical information on individual oligosaccharides, information on the respective gastrointestinal microflora composition, and medical information. With the emergence of sophisticated analytical techniques for oligosaccharide analysis and advanced approaches on the identification of the gastrointestinal microflora composition by means of molecular techniques, e.g. phylogenetic microarrays,[78] scientists have potential tools at hand for an advanced understanding of the structure-function relationship of diet-related oligosaccharides.

Genetic factors, such as ABH(0) blood groups, Lewis- and Secretor type, influence the gastrointestinal fate of diet-related oligosaccharides in the gastrointestine of neonates. A large and diverse subject group has, therefore, to be involved in the design of *in vivo* studies in future. The high-throughput and short analysis times of the present analytical techniques do not limit the large sample numbers necessary for these studies.

References

1. Corrigendum to regulation (EC) No.1924/2006 of the European Parliament and of the council of 20 december 2006 on nutrition and health claims made on foods; L12/3; **2007**, European Union Publishers' Forum; Office for Official Publications of the European Communities ed.: Official Journal of the European Union, Brussels.
2. de Slegte, J. Determination of *trans*-galactooligosaccharides in selected food products by ion-exchange chromatography: Collaborative study. *J. AOAC Int.*, **2002**, *85*, 417-423.
3. Prosky, L.; Hoebregs, H. Methods to determine food inulin and oligofructose. *J. Nutr.*, **1999**, *129*, 1418-1423.
4. Redmond, J.W.; Packer, N.H. The use of solid-phase extraction with graphitised carbon for the fractionation and purification of sugars. *Carbohydr. Res.*, **1999**, *319*, 74-79.
5. Evangelista, R.A.; Liu, M.-S.; Chen, F.-T.A. Characterization of 9-aminopyrene-1,4,6-trisulfonate derivatized sugars by capillary electrophoresis with laser-induced fluorescence detection. *Anal. Chem.*, **1995**, *67*, 2239-2245.
6. Lee, Y.C. Carbohydrate analyses with high-performance anion-exchange chromatography. *J. Chromatogr. A*, **1996**, *720*, 137-149.
7. Westphal, Y.; Schols, H.A.; Voragen, A.G.J.; Gruppen, H. Introducing porous graphitized carbon liquid chromatography with evaporative light scattering and mass spectrometry detection into cell wall oligosaccharide analysis. *J. Chromatogr. A*, **2010**, *1217*, 689-695.
8. Westphal, Y.; Kuhnel, S.; Schols, H.A.; Voragen, A.G.J.; Gruppen, H. LC/CE-MS tools for the analysis of complex arabino-oligosaccharides. *Carbohydr. Res.*, **2010**, *345*, 2239-2251.
9. Kabel, M.A.; Heijnis, W.H.; Bakx, E.J.; Kuijpers, R.; Voragen, A.G.J.; Schols, H.A. Capillary electrophoresis fingerprinting, quantification and mass-identification of various 9-aminopyrene-1,4,6-trisulfonate-derivatized oligomers derived from plant polysaccharides. *J. Chromatogr. A*, **2006**, *1137*, 119-126.
10. Coenen, G.J.; Kabel, M.A.; Schols, H.A.; Voragen, A.G.J. CE-MS^n of complex pectin-derived oligomers. *Electrophoresis*, **2008**, *29*, 2101-2111.
11. Hermanson, G.T. Bioconjugate chemistry. In *Bioconjugate techniques*, 2nd ed; Academic Press: London, UK, **2008**; pp 3-169.
12. Haselberg, R.; Ratnayake, C.K.; de Jong, G.J.; Somsen, G.W. Performance of a sheathless porous tip sprayer for capillary electrophoresis-electrospray ionization-mass spectrometry of intact proteins. *J. Chromatogr. A*, **2010**, *1217*, 7605-7611.
13. Gennaro, L.A.; Salas-Solano, O. On-line CE-LIF-MS technology for the direct characterization of N-linked glycans from therapeutic antibodies. *Anal. Chem.*, **2008**, *80*, 3838-3845.
14. Newburg, D.S.; Pickering, L.K.; McCluer, R.H.; Cleary, T.G. Fucosylated oligosaccharides of human milk protect suckling mice from heat-stabile enterotoxin of Escherichia Coli. *J. Infect. Dis.*, **1990**, *162*, 1075-1080.
15. Thurl, S.; Henker, J.; Siegel, M.; Tovar, K.; Sawatzki, G. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconjugate J.*, **1997**, *14*, 795-799.
16. Thurl, S.; Munzert, M.; Henker, J.; Boehm, G.; Muller-Werner, B.; Jelinek, J.; Stahl, B. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Brit. J. Nutr.*, **2010**, *104*, 1261-1271.
17. Oriol, R.; Le Pendu, J.; Mollicone, R. Genetics of ABO, H, Lewis, X and related antigens. *Vox Sang.*, **1986**, *51*, 161-171.
18. Erney, R.M.; Malone, W.T.; Skelding, M.B.; Marcon, A.A.; Kleman-Leyer, K.M.; O'Ryan, M.L.; Ruiz-Palacios, G.; Hilty, M.D.; Pickering, L.K.; Prieto, P.A. Variability of human milk neutral oligosaccharides in a diverse population. *J. Pediatr. Gastr. Nutr.*, **2000**, *30*, 181-192.
19. Newburg, D.S.; Ruiz-Palacios, G.M.; Altaye, M.; Chaturvedi, P.; Meinzen-Derr, J.; Guerrero, M.d.L.; Morrow, A.L. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology*, **2004**, *14*, 253-263.
20. Chaturvedi, P.; Warren, C.D.; Altaye, M.; Morrow, A.L.; Ruiz-Palacios, G.; Pickering, L.K.; Newburg, D.S. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology*, **2001**, *11*, 365-372.
21. Coppa, G.V.; Pierani, P.; Zampini, L.; Carloni, I.; Carlucci, A.; Gabrielli, O. Oligosaccharides in human milk during different phases of lactation. *Acta Paediatr.*, **1999**, *88*, 89-94.
22. Sumiyoshi, W.; Urashima, T.; Nakamura, T.; Arai, I.; Saito, T.; Tsumura, N.; Wang, B.; Brand-Miller, J.; Watanabe, Y.; Kimura, K. Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. *Brit. J. Nutr.*, **2003**, *89*, 61-69.
23. Kitaoka, M.; Tian, J.S.; Nishimoto, M. Novel putative galactose operon involving lacto-N-biose phosphorylase in *Bifidobacterium longum*. *Appl. Environ. Microbiol.*, **2005**, *71*, 3158-3162.

24. Moro, G.E.; Stahl, B.; Fanaro, S.; Jelinek, J.; Boehm, G.; Coppa, G.V. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants. *Acta Paediatr.*, **2005**, *94*, 27-30.
25. Rudloff, S.; Pohlentz, G.; Diekmann, L.; Egge, H.; Kunz, C. Urinary excretion of lactose and oligosaccharides in preterm infants fed human milk or infant formula. *Acta Paediatr.*, **1996**, *85*, 598-603.
26. Coppa, G.V.; Pierani, P.; Zampini, L.; Bruni, S.; Carloni, I.; Gabrielli, O. Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. In *Bioactive components of human milk*; Newburg, D.S., Ed; Kluwer Academic/Plenum Publishers: New York, **2001**; pp 307-314.
27. Sabharwal, H.; Nilsson, B.; Chester, M.A.; Lindh, F.; Grönberg, G.; Sjöblad, S.; Lundblad, A. Oligosaccharides from faeces of a blood-group B, breast-fed infant. *Carbohydr. Res.*, **1988**, *178*, 145-154.
28. Sabharwal, H.; Nilsson, B.; Chester, M.A.; Sjöblad, S.; Lundblad, A. Blood group specific oligosaccharides from faeces of a blood group A breast-fed infant. *Mol. Immunol.*, **1984**, *21*, 1105-1112.
29. Sabharwal, H.; Nilsson, B.; Grönberg, G.; Chester, M.A.; Dakour, J.; Sjöblad, S.; Lundblad, A. Oligosaccharides from feces of preterm infants fed on breast milk. *Arch. Biochem. Biophys.*, **1988**, *265*, 390-406.
30. Sabharwal, H.; Sjöblad, S.; Lundblad, A. Sialylated oligosaccharides in human milk and feces of preterm, full-term, and weaning infants. *J. Pediatr. Gastr. Nutr.*, **1991**, *12*, 480-484.
31. Schenkel-Brunner, H. Blood group antigens. In *Comprehensive glycoscience. From chemistry to system biology; vol.3*; Kamerling, J.P., Boons, G.J., Lee, Y.C., Suzuki, A., Taniguchi, N.,Voragen, A.G.J., Eds; Elsevier: Oxford, UK, **2007**; pp 343-372.
32. Ameno, S.; Kimura, H.; Ameno, K.; Zhang, X.; Kinoshita, H.; Kubota, T.; Ijiri, I. Lewis and secretor gene effects on Lewis antigen and postnatal development of Lewis blood type. *Biol. Neonate*, **2001**, *79*, 91-96.
33. Zoetendal, E.G.; Akkermans, A.D.L.; Akkermans-van Vliet, W.M.; de Visser, A.G.M.; de Vos, W.M. The host genotype affects the bacterial community in the human gastrointestinal tract. *Microbial Ecol. Health Dis.*, **2001**, *13*, 129-134.
34. Antonowicz, I.; Lebenthal, E. Developmental pattern of small intestinal enterokinase and disaccharidase activities in human fetus. *Gastroenterology*, **1977**, *72*, 1299-1303.
35. Manson, W.G.; Coward, W.A.; Harding, M.; Weaver, L.T. Development of fat digestion in infancy. *Arch. Dis. Child.*, **1999**, *80*, F183-F187.
36. Shulman, R.J.; Schanler, R.J.; Lau, C.; Heitkemper, M.; Ou, C.-N.; Smith, E.O.B. Early feeding, feeding tolerance, and lactase activity in preterm infants. *J. Pediatr.*, **1998**, *133*, 645-649.
37. Schwiertz, A.; Gruhl, B.; Lobnitz, M.; Michel, P.; Radke, M.; Blaut, M. Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. *Pediatr. Res.*, **2003**, *54*, 393-399.
38. Shanklin, D.R.; Cooke, R.J. Effects of intrauterine growth on intestinal length in the human fetus. *Biol. Neonate*, **1993**, *64*, 76-81.
39. Moro, G.; Minoli, I.; Mosca, M.; Fanaro, S.; Jelinek, J.; Stahl, B.; Boehm, G. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. *J. Pediatr. Gastroenterol. Nutr.*, **2002**, *34*, 291-295.
40. Boehm, G.; Fanaro, S.; Jelinek, J.; Stahl, B.; Marini, A. Prebiotic concept for infant nutrition. *Acta Paediatr.*, **2003**, *92*, 64-67.
41. Adlerberth, I.; Wold, A.E. Establishment of the gut microbiota in western infants. *Acta Pædiatr.*, **2009**, *98*, 229-238.
42. Benno, Y.; Sawada, K.; Mitsuoka, T. The intestinal microflora of infants - composition of fecal flora in breast-fed and bottle-fed infants. *Microbiol. Immunol.*, **1984**, *28*, 975-986.
43. Penders, J.; Thijs, C.; Vink, C.; Stelma, F.F.; Snijders, B.; Kummeling, I.; van den Brandt, P.A.; Stobberingh, E.E. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics*, **2006**, *118*, 511-521.
44. Stark, P.L.; Lee, A. The microbial ecology of the large bowel of breastfed and formula-fed infants during the first year of life. *J. Med. Microbiol.*, **1982**, *15*, 189-203.
45. Yoshioka, H.; Iseki, K.; Fujita, K. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics*, **1983**, *72*, 317-321.
46. Fujita, K.; Oura, F.; Nagamine, N.; Katayama, T.; Hiratake, J.; Sakata, K.; Kumagai, H.; Yamamoto, K. Identification and molecular cloning of a novel glycoside hydrolase family of core 1 type O-glycan-specific endo-α-N-acetylgalactosaminidase from *Bifidobacterium longum*. *J. Biol. Chem.*, **2005**, *280*, 37415-37422.

47. Zhou, J.S.; Gopal, P.K.; Gill, H.S. Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin *in vitro*. *Int. J. Food Microbiol.*, **2001**, *63*, 81-90.
48. Hoskins, L.C.; Agustines, M.; McKee, W.B.; Boulding, E.T.; Kriaris, M.; Miedermeyer, G. Mucin degradation in human colon ecosystems; isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. *J. Clin. Invest.*, **1985**, *75*, 944-953.
49. Barcelo, A.; Claustre, J.; Moro, F.; Chayvialle, J.-A.; Cuber, J.-C.; Plaisancié, P. Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. *Gut*, **2000**, *46*, 218-224.
50. Vardakou, M.; Palop, C.N.; Christakopoulos, P.; Faulds, C.B.; Gasson, M.A.; Narbad, A. Evaluation of the prebiotic properties of wheat arabinoxylan fractions and induction of hydrolase activity in gut microflora. *Int. J. Food Microbiol.*, **2008**, *123*, 166-170.
51. Degnan, B.A.; Macfarlane, G.T. Arabinogalactan utilization in continuous cultures of *Bifidobacterium longum*: Effect of co-culture with Bacteroides thetaiotaomicron. *Anaerobe*, **1995**, *1*, 103-112.
52. Splechtna, B.; Nguyen, T.H.; Steinboeck, M.; Kulbe, K.D.; Lorenz, W.; Haltrich, D. Production of prebiotic galacto-oligosaccharides from lactose using beta-galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.*, **2006**, *54*, 4999-5006.
53. Tzortzis, G.; Goulas, A.K.; Gee, J.M.; Gibson, G.R. A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous *in vitro* fermentation system and in the proximal colonic contents of pigs in vivo. *J. Nutr.*, **2005**, *135*, 1726-1731.
54. van den Broek, L.A.M.; Hinz, S.W.A.; Beldman, G.; Vincken, J.-P.; Voragen, A.G.J. Bifidobacterium carbohydrases-their role in breakdown and synthesis of (potential) prebiotics. *Mol. Nutr. Food Res.*, **2008**, *52*, 146-163.
55. Daas, P.J.H.; Schols, H.A.; de Jongh, H.H.J. On the galactosyl distribution of commercial galactomannans. *Carbohyd. Res.*, **2000**, *329*, 609-619.
56. Kramer, M.S.; Kakuma, R. The optimal duration of exclusive breastfeeding: A systematic review. *WHO/NHD/01.08, WHO/FCH/CAH/01.23*, **2002**.
57. European Food Safety Authority (EFSA) - Panel on Dietetic Products, Nutrition and Allergies(NDA). Scientific opinion on the appropriate age for introduction of complementary feeding of infants. *EFSA Journal*, **2009**, *7:1423*.
58. Leung, A.K.C.; Sauve, R.S. Breast is best for babies. *J. Natl. Med. Assoc.*, **2005**, *97*, 1010-1019.
59. Neu, J. Gastrointestinal maturation and implications for infant feeding. *Early Hum. Dev.*, **2007**, *83*, 767-775.
60. Wasif, G.S.; Yelly, O.S. The systematic review of necrotizing enterocolitis; risk, prevention and outcomes. *HealthMED*, **2010**, *4*, 297-309.
61. Sangild, P.T. Gut responses to enteral nutrition in preterm infants and animals. *Exp. Biol. Med.*, **2006**, *231*, 1695-1711.
62. Mishra, S.; Agarwal, R.; Jeevasankar, M.; Deorari, A.K.; Paul, V.K. Minimal enteral nutrition. *Indian J. Pediatr.*, **2008**, *75*, 267-269.
63. Hernell, O. Human milk vs. Cow's milk and the evolution of infant formulas. In *Milk and milk products in human nutrition*; Clemens, R.A., Hernell, O.,Michaelsen, K.M., Eds; Karger: Basel, Switzerland, **2011**; pp 17-28.
64. Simmer, K.; Patole, S.K.; Rao, S.C. Longchain polyunsaturated fatty acid supplementation in infants born at term. *Cochrane Database Syst Rev.*, **2008**.
65. Urashima, T.; Saito, T.; Nakamura, T.; Messer, M. Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconjugate J.*, **2001**, *18*, 357-371.
66. Choi, N.; Shin, H.S. Effect of oligosaccharides and inulin on the growth and viability of bifidobacteria in skim milk. *Food Sci. Biotechnol.*, **2006**, *15*, 543-548.
67. Knol, J.; Scholtens, P.; Kafka, C.; Steenbakkers, J.; Gross, S.; Helm, K.; Klarczyk, M.; Schopfer, H.; Bockler, H.M.; Wells, J. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: More like breast-fed infants. *J. Pediatr. Gastroenterol. Nutr.*, **2005**, *40*, 36-42.
68. Rycroft, C.E.; Jones, M.R.; Gibson, G.R.; Rastall, R.A. A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. *J. Appl. Microbiol.*, **2001**, *91*, 878-887.
69. Van Laere, K.M.J.; Hartemink, R.; Bosveld, M.; Schols, H.A.; Voragen, A.G.J. Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. *J. Agric. Food Chem.*, **2000**, *48*, 1644-1652.
70. Urashima, T.; Asakuma, S.; Messer, M. Milk oligosaccharides. In *Comprehensive glycoscience. From chemistry to system biology; vol.4*; Kamerling, J.P., Boons, G.J., Lee, Y.C., Suzuki, A., Taniguchi, N.,Voragen, A.G.J., Eds; Elsevier: Oxford, UK, **2007**; pp 695-722.

71. Martinez-Ferez, A.; Rudloff, S.; Guadix, A.; Henkel, C.A.; Pohlentz, G.; Boza, J.J.; Guadix, E.M.; Kunz, C. Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology. *Int. Dairy J.*, **2006**, *16*, 173-181.
72. Newburg, D.S.; Neubauer, S.H. Carbohydrates in milks: Analysis, quantities and significance. In *Handbook of milk composition*; Jensen, R.G., Ed; Academic Press: San Diego, **1995**; pp 273-249.
73. Tao, N.; Depeters, E.J.; Freeman, S.; German, J.B.; Grimm, R.; Lebrilla, C.B. Bovine milk glycome. *J. Dairy Sci.*, **2008**, *91*, 3768-3778.
74. Parkkinen, J.; Finne, J. Isolation of sialyl oligosaccharides and sialyl oligosaccharide phosphates from bovine colostrum and human-urine. *Method Enzymol.*, **1987**, *138*, 289-300.
75. Kunz, C.; Rudloff, S.; Schad, W.; Braun, D. Lactose-derived oligosaccharides in the milk of elephants: Comparison with human milk. *Brit. J. Nutr.*, **1999**, *82*, 391-399.
76. Tao, N.A.; Wu, S.A.; Kim, J.; An, H.J.; Hinde, K.; Power, M.L.; Gagneux, P.; German, J.B.; Lebrilla, C.B. Evolutionary glycomics: Characterization of milk oligosaccharides in primates. *J. Proteome Res.*, **2011**, *10*, 1548-1557.
77. Urashima, T.; Yamashita, T.; Nakamura, T.; Arai, I.; Saito, T.; Derocher, A.E.; Wiig, O. Chemical characterization of milk oligosaccharides of the polar bear, *ursus maritimus*. *Biochim. Biophys. Acta-Gen. Subj.*, **2000**, *1475*, 395-408.
78. Rajilic-Stojanovic, M.; Heilig, H.; Molenaar, D.; Kajander, K.; Surakka, A.; Smidt, H.; de Vos, W.M. Development and application of the human intestinal tract chip, a phylogenetic microarray: Analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environ. Microbiol.*, **2009**, *11*, 1736-1751.

Summary

Samenvatting

Zusammenfassung

Summary

The consumption of functional oligosaccharides can positively influence host's health, as these oligosaccharides show bioactivity during their gastrointestinal passage. Functional oligosaccharides are of special interest for infant nutrition. During the postnatal period, the gastrointestinal tract undergoes a profound development. The changes in morphology and functionality, including the establishment of a balanced microbiota, are influenced by enteral food. Non-digestible oligosaccharides play an important role. Nevertheless, little is known on the gastrointestinal fate of these oligosaccharides, which would help to understand their biological functioning. A general introduction on the neonatal gastrointestinal development and functional oligosaccharides, including their potential gastrointestinal metabolization, is given in **chapter 1**.

The research project firstly aimed at providing a suitable analytical method for the analysis of diet-related oligosaccharides in complex matrices. This method was used in order to gain advanced knowledge on the gastrointestinal metabolization of these oligosaccharides. A variety of *in vitro* and *in vivo* sample materials was studied, focusing on human milk oligosaccharides (HMOs) and galactooligosaccharides (GOS). These oligosaccharides frequently occur in infant nutrition. Supplementary, the potentially prebiotic konjac glucomannan polysaccharide (KGM) and oligosaccharides derived thereof were included in the study.

In **chapter 2**, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) is introduced for the sensitive qualitative and quantitative determination of individual GOS extracted from food matrices. The matrices investigated were infant formula, fruit juice, and a maltodextrin-rich preparation, to which a commercial GOS-mixture was added in a product concentration range from 1.25 to 30%. It was possible to reproducibly separate GOS from the high amounts of particularly lactose and maltodextrins as present in the matrices by means of solid-phase extraction on graphitized carbon material following enzymatic amyloglucosidase pretreatment. Additionally, the quantification of a range of structurally different oligosaccharides with a size of DP3-DP5 by CE-LIF was evaluated. CE-LIF showed to be a valuable tool for the analysis of oligosaccharids on nanomole-scale.

Next, the hyphenation of CE-LIF with an electrospray mass spectrometer, capable to perform multiple MS analysis (ESI-MS^n), was introduced for the profiling of oligosaccharides in human milk and feces of breast-fed babies (**chapter 3**). HMOs and gastrointestinal HMO-degradation products could be assigned. In total, 21 peaks could be assigned and 11 peaks could be tentatively assigned. The gastrointestinal metabolization of

HMOs was indicated (e.g. de-fucosylation and de-sialylation). The detailed structural characterization of a so far unknown lacto-N-neo-tetraose isomer and lacto-N-fucopentaose isomer was established in baby feces and pointed to gastrointestinal hydrolysis of higher-Mw HMOs, releasing the bifidogenic unit lacto-N-biose.

Having introduced CE-LIF(-MS^n) as a suitable analytical tool for the analysis of oligosaccharides from complex matrices, this method was subsequently applied for the study of the fecal oligosaccharide profiles of twenty-seven two months old breast-, formula- and mixed-fed preterm babies (**chapter 4**). Feeding was found out to determine the presence of diet-related oligosaccharides in baby feces. In the feces of breast-fed babies, Secretor- and Lewis-characteristic HMO-profiles or profiles indicating an advanced degradation and bioconversion of HMOs, were observed. GOS supplemented to the formula was not recovered in baby feces. The involvement of gastrointestinal mucins in the bioconversion of feeding-related oligosaccharides was indicated by the fecal presence of four unknown structures. Two of these structures were assigned as blood group A characteristic tetra-saccharide and hexa-saccharide.

In **chapter 5**, the occurrence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk was studied. The study comprised eleven mother/child pairs, with mother's Secretor- and Lewis-type including all combinations [Le(a-b+), Le(a+b-), Le(a-b-)]. Whereas no major changes in HMO-profiles of breast milk were observed over time, three continuous stages of fecal oligosaccharide profiles were determined. During *stage 1* (up to three months postpartum) either predominantly neutral or acidic HMOs were detected in the feces, which is most probably effected by the HMO-composition of the respective breast milk. Next, a gradual change to blood group specific oligosaccharides was observed. Their presence pointed to a gastrointestinal degradation of the HMOs, followed by conjugation with blood group specific antigenic determinants present in the gastrointestinal mucus layer. Eleven of these "hybride"-oligosaccharides present during *stage 2* were annotated in this study. Finally, predominantly oligosaccharides characteristic for follow-up feeding were present in the fecal samples at the time solid food was introduced (*stage 3*).

The complex konjac glucomannan polysaccharide (KGM) and oligosaccharide-mixtures, obtained after endo-β-(1,4)-mannanase (EM) and endo-β-(1,4)-glucanase (EG) digestion of the polysaccharide, were fermented *in vitro* by human fecal inoculum (**chapter 6**). The structural characteristics of the oligosaccharide mixtures and the *in vitro* fermentation of the polysaccharide and oligosaccharides were studied by CE-LIF and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Different

fermenatation characteristics were observed, which were related to the structural composition of the oligosaccharide mixtures. A hypothesis on the oligosaccharide structure of the EM- and EG-digest was established. Subsequently, an in-depth study of the structural composition of oligosaccharides from KGM produced by EM and EG and a mix of enzymes from human fecal bacteria is described in **chapter 7**. EM and EG showed a structured degradation mechanism of KGM. EM resulted in a short series of oligosaccharides with equal proportions of mannose and glucose. EG resulted in a homologous series composed of probably branched, mannose-rich oligosaccharides with a glucose- and mannose-containing core-structure. Contrarily, degradation with the enzymes from human fecal bacteria resulted in a large variety of structurally different oligosaccharides, showing characteristics of both, EM- and EG-digest. The results obtained were also used to discuss the structural composition of the KGM polysaccharide.

Finally, in **chapter 8**, the results obtained in this research and their possible nutritional, scientific and application-related implications are discussed. The gastrointestinal metabolization of diet-related oligosaccharides, as observed in the postnatal period, is compared to studies published on the neonatal development of the intestinal microflora.

Samenvatting

De inname van functionele oligosacchariden is gezondheidsbevorderend, aangezien deze oligosacchariden tijdens hun passage door de darm bioactieve effecten vertonen. Functionele oligosacchariden zijn van bijzonder belang voor babyvoeding. Het maagdarmkanaal ondergaat tijdens de postnatale periode ingrijpende ontwikkelingen. Enterale voeding beïnvloed morfologische en functionele veranderingen en het instellen van een evenwichtige microbiota. Onverteerbare oligosacchariden spelen hierin een belangrijke rol. Kennis over de gastrointestinale lot van deze oligosacchariden is essentieel om hun biologische werking te verklaren maar hierover is echter tot nu toe nog weinig bekend. In **hoofdstuk 1** wordt een algemene inleiding gegeven over de neonatale gastrointestinale ontwikkeling, de functionele oligosacchariden en hun gastrointestinale omzetting.

De eerste doelstelling van dit onderzoeksproject was het introduceren van een geschikte methode voor het analyseren van voeding-gerelateerde oligosacchariden in complexe matrices. Vervolgens werd deze methode toegepast om meer inzicht te krijgen in de gastrointestinale omzetting van deze oligosacchariden. Hiervoor werd een groot aantal *in vitro* en *in vivo* monsters geanalyseerd. Deze studies richtten zich voornamelijk op moedermelk oligosacchariden (human milk oligosaccharides; HMOs) en galactooligosacchariden (GOS) omdat deze oligosacchariden vaak voorkomen in babyvoeding. Daarnaast werden konjac glucomannan polysaccharide en daarvan afgeleide oligosacchariden bestudeerd vanwege hun potentieel prebiotische werking.

In **hoofdstuk 2** wordt capillaire elektroforese met laser-geïnduceerde fluorescentie-detectie (capillary electrophoresis with laser-induced fluorescence detection; CE-LIF) ingevoerd voor de gevoelige kwalitatieve en kwantitatieve bepaling van individuele GOS uit voedingsmatrices. Hiervoor werd babyvoeding, vruchtensap en een met maltodextrine verrijkt preparaat bestudeerd, waaraan een commercieel GOS-mengsel was toegevoegd in een productconcentratie van 1.25 tot 30%. Door middel van vaste fase extractie (solid phase extraction) op gegrafitiseerde koolstof, na enzymatische amyloglucosidase voorbehandeling, was het mogelijk om GOS reproduceerbaar te scheiden van met name de lactose en maltodextrinen, die in grote hoeveelheden in de matrices aanwezig zijn. Daarnaast werd de kwantificatie van een aantal structureel verschillende oligosacchariden met een grootte van DP3-DP5 door CE-LIF geëvalueerd. CE-LIF bleek een waardevolle techniek voor het analyseren van oligosacchariden op nanomol-niveau te zijn.

Vervolgens wordt het koppelen van CE-LIF met een electrospray-massaspectrometer (ESI-MS^n, met de mogelijkheid om meerdere MS-fragmentaties achter elkaar uit te voeren) toegepast voor het analyseren van oligosacchariden in moedermelk en faeces van borstgevoede baby's (**hoofdstuk 3**). Eenentwintig pieken werden geannoteerd als HMOs of HMO-afbraakproducten en 11 pieken werden tentatief geannoteerd. De resultaten toonden de gastrointestinale metabolisatie van HMOs aan (bijv. de eliminatie van fucose en siaalzuur). De struktuur van een tot dusver onbekende lacto-N-neo-tetraose isomeer en lacto-N-fucopentaose isomeer in baby feces werd in detail gekarakteriseerd. Hierbij werd de gastrointestinale hydrolyse van HMOs met een hoger molecuulgewicht en het vrijkomen van de bifidogene eenheid lacto-N-biose aangetoond.

Nadat CE-LIF(-MS^n) was ingevoerd als geschikte techniek voor de analyse van oligosacchariden uit complexe matrices werd de techniek gebruikt voor het bestuderen van feces van zevenentwintig, twee maanden oude borst-, formule- en gemengd gevoed premature baby's (**hoofdstuk 4**). De aanwezigheid van voeding-gerelateerde oligosacchariden in baby feces bleek afhankelijk te zijn van de voeding. In de feces van borstgevoede baby's werden Secretor- en Lewis- karakteristieke HMO-profielen gevonden of profielen die een geavanceerde afbraak en bioconversie van HMOs lieten zien. GOS, die oorspronkelijk aan de formule waren toegevoegd, werden niet teruggevonden in de feces. De aanwezigheid van vier onbekende structuren in de feces toonde aan dat gastrointestinale mucines bij de bioconversie van voeding-gerelateerde oligosacchariden betrokken zijn. Twee van deze structuren werden herkend als een bloedsgroep A karakteristieke tetra- en hexa-saccharide.

In **hoofdstuk 5** werden de oligosacchariden in de feces van borstgevoede baby's en de bijbehorende moedermelk in de eerste zes maanden na geboorte bestudeert. De studie omvatte elf moeder/kind paren. De deelnemende moeders vertegenwoordigden alle combinaties aan Secretor- en Lewis-types [Le(a-b+), Le(a+b-), Le(a-b-)]. Hoewel tijdens de eerste zes maanden geen grote veranderingen in de HMO-profielen van moedermelk werden waargenomen, werden er drie achtereenvolgende stadia van fecale oligosaccharide-profielen herkend. Tijdens *stadium 1* (tot drie maanden na de geboorte) werden er of voornamelijk neutrale of zure oligosacchariden in de feces gevonden. De aanwezigheid van deze oligosacchariden hangt waarschijnlijk af van de HMO-samenstelling van de desbetreffende moedermelk. Vervolgens werd er een geleidelijke verandering van het profiel naar bloedgroep-specifieke oligosacchariden waargenomen. Hun aanwezigheid wijst op een gastrointestinale afbraak van HMOs, gevolgd door een conjugatie van de afbraakprodukten met bloedgroep-specifieke antigenen, die in de gastrointestinale mucus

aanwezig zijn. Elf van deze "hybride"-oligosacchariden die tijdens *stadium 2* aanwezig zijn werden in deze studie geannoteerd. Ten slotte, op het tijdstip van invoeren van vast voedsel waren voornamelijk oligosacchariden in de feces aanwezig (*stadium 3*), die karakteristiek zijn voor deze opvolg-voeding.

De complexe polysaccharide konjac glucomannan (KGM) en oligosaccharide-mengsels hiervan, die gemaakt werden door enzymatische afbraak van de polysaccharide met endo-β-(1,4)-mannanase (EM) en endo-β-(1,4)-glucanase (EG), werden door incubatie met menselijke feces *in vitro* gefermenteerd (**hoofdstuk 6**).

De structurele kenmerken van de oligosaccharide-mengsels en het *in vitro* fermentatie-gedrag van de polysaccharide en de oligosacchariden werd bestudeerd met behulp van CE-LIF en matrix-assisted laser desorption/ionization time-of-flight massaspektrometrie (MALDI-TOF MS). Vastgestelde verschillen in fermentatiegedrag konden gerelateerd worden aan de structurele opbouw van de oligosacchariden in de mengsels. Vervolgens werd er een hypothese over de structurele opbouw van de oligosacchariden in de mengsels, geproduceerd door EM of EG, opgesteld. **Hoofdstuk 7** beschrijft een diepgaande studie over de structurele samenstelling van de oligosacchariden aanwezig na afbraak van KGM met EM, EG of een mix van enzymen afkomstig van bacteriën aanwezig in menselijke feces. EM en EG vertoonden een gestructureerde afbraak van KGM. EM resulteerde in een series van korte oligosacchariden, die gelijke hoeveelheden aan mannose en glucose bevatten. EG resulteerde in een homologe series van waarschijnlijk vertakte oligosacchariden rijk aan mannose. Deze oligosacchariden zijn waarschijnlijk gebaseerd op een structuur-eenheid, die mannose en glucose bevat. In tegenstelling hiermee resulteerde de afbraak met enzymen afkomstig van bacteriën uit menselijke feces in een groot aantal structureel verschillende oligosacchariden. Deze oligosacchariden vertonen kenmerken van zowel EM- als EG-afbraak. Aan de hand van de behaalde resultaten werd ook de structurele samenstelling van de KGM polysaccharide besproken.

Tenslotte worden in **hoofdstuk 8** de resultaten van dit onderzoek en de mogelijke voeding-gerelateerde, wetenschappelijke en applicatie-gerelateerde implicaties besproken. De postnatale gastrointestinale omzetting van voeding-gerelateerde oligosacchariden, zoals aangetoond in deze studie, wordt vergeleken met gepubliceerde studies over de neonatale ontwikkeling van de darmflora.

Zusammenfassung

Die Einnahme funktioneller Oligosaccharide kann den Gesundheitszustand positiv beeinflussen, da diese Oligosaccharide während ihrer Passage durch den Gastrointestinaltrakt bioaktive Wirkung aufweisen. Funktionelle Oligosaccharide sind für Babynahrung von besonderer Bedeutung. Nach der Geburt durchläuft der Gastrointestinaltrakt tiefgreifende Entwicklungen. Enteral zugeführte Nahrung beeinflusst morphologische und funktionelle Veränderungen und den Aufbau einer ausgeglichenen Darmflora. Unverdauliche Oligosaccharide spielen hierbei eine wichtige Rolle. Über das Schicksal dieser Oligosaccharide im Darm ist jedoch wenig bekannt. Erweiterte Kenntnis hierüber wäre nützlich um die biologische Funktion dieser Oligosaccharide zu verstehen.

Kapitel 1 gibt eine allgemeine Einführung in die gastrointestinale Entwicklung und die funktionellen Oligosaccharide, einschließlich ihrer möglichen gastrointestinalen Umsetzung. Das Ziel dieses Forschungsprojektes war es, zuerst eine geeignete Methode für die Analyse von Oligosacchariden aus der Nahrung, welche in komplexe Grundmassen enthalten sind, bereitzustellen. Diese Methode wurde dann benutzt um unser Wissen über die gastrointestinale Umsetzung dieser Oligosaccharide zu erweitern. Hierfür wurde eine Auswahl an Proben von *in vitro* und *in vivo* Studien analysiert, wobei der Schwerpunkt auf Muttermilch-Oligosacchariden (human milk oligosaccharides; HMOs) und Galaktooligosacchariden (GOS) lag. Diese Oligosaccharide kommen oft in Babynahrung vor. Zusätzlich waren das möglicherweise präbiotische Konjac Glucomannan (KGM) Polysaccharid und hiervon abgeleitete Oligosaccharide Teil dieser Studie.

In **Kapitel 2** wird Kapillarelektrophorese mit Laser-induzierter Fluoreszenz-Detektion (capillary electrophoresis with laser-induced fluorescence detection; CE-LIF) für die qualitative und quantitative Bestimmung von individuellen GOS, welche aus Lebensmittelmatrices extrahiert wurden, eingeführt. Die untersuchten Lebensmittelmatrices (Babynahrung, Fruchtsaft und eine maltodextrinhaltige Mischung), enthielten ein handelsübliches GOS-Präparat in einer Produktkonzentration von 1.25 bis 30%. Amyloglukosidase-Vorbehandlung und anschließende Festphasenextraktion (solid phase extraction) auf Graphitkohlematerial ermöglichte die reproduzierbare Trennung von GOS von den hohen Konzentrationen an vor allem Laktose und Maltodextrin, welche in den Lebensmittelmatrices vorhanden sind. Zusätzlich wurde das Quantifizieren mit CE-LIF anhand einer Serie strukturell unterschiedlicher Oligosaccharide mit einer Größe von DP3 – DP5 bewertet. CE-LIF erwies sich als eine nützliche Methode zur Analyse von Oligosacchariden die in nanomol-Konzentrationen vorliegen. Als nächster Schritt wurde

die Kopplung von CE-LIF an einen Electrospray-Massenspektrometer (ESI-MS^n), welcher mehrere MS-Analysen hintereinander ausführen kann, für das Screening von menschlicher Muttermilch und Fäzes von mit Muttermilch gefütterter Babys eingeführt (**Kapitel 3**). Sowohl HMOs als auch gastrointestinale Abbau-produkte von HMOs konnten identifiziert weden. Insgesamt wurden 21 Peaks benannt. Für 11 weitere Peaks war eine provisorische Benennung möglich. Die Abspaltung von z.B. Fucose- und Sialsäure-Einheiten deutete auf eine gastrointestinale Umwandlung der HMOs hin. Die Strukturen eines bisher unbekannten Lacto-N-neo-tetraose- und eines Lacto-N-fucopentaose-isomeres, die in Babyfäzes vorkommen, wurden im Detail charakterisiert. Die gastrointestinale Hydrolyse von HMOs mit höheren Molekulargewichten, bei welcher die bifidogene Einheit Lacto-N-biose freigesetzt wird, wurde aufgezeigt.

Nach dem Einführen von CE-LIF(-MS^n) als geeignete Methode zur Analyse von Oligosacchariden aus komplexen Grundmassen wurde diese Methode für die Untersuchung von Oligosaccharid-Profilen in den Fäzes von 27 zu früh geborenen Babys angewendet (**Kapitel 4**). Diese Babys wurden mit Muttermilch, Muttermilchersatz oder beidem gefüttert. Es konnte gezeigt werden, dass die Art der Nahrung die Anwesenheit von nahrungsmittelbezogenen Oligosacchariden in Babyfäzes beeinflusst.

In den Fäzes mit Muttermilch gefütterter Babys wurden entweder HMO-Profile gefunden, welche charakteristisch für die jeweilige Sekretor- und Lewistyp-Zugehörigkeit sind, oder es wurden Profile gefunden, welche einen fortgeschrittenen Abbau und Umwandlung der HMOs aufwiesen. GOS, die ursprünglich in der Muttermilch-Ersatznahrung enthalten waren, wurden in den Babyfäzes nicht zurückgefunden. Die Anwesenheit vier unbekannter Strukturen in den Fäzes deutete auf eine Beteiligung des gastrointestinalen Mucins an der Umwandlung der aus der Nahrung stammenden Oligosaccharide hin. Eine dieser Strukturen wurde als Blutgruppe A-spezifisches Tetrasaccharid und eine andere als Blutgruppe A-spezifisches Hexasaccharid identifiziert.

In **Kapitel 5** wurde das Vorkommen von Oligosacchariden in den Fäzes mit Muttermilch gefütterter Babys während ihrer ersten sechs Lebensmonate sowie die dazugehörige Muttermilch untersucht. Die Studie umfasste elf Mutter/Kind-Paare. Alle Kombinationen an Lewis- und Secretor-Typen [Le(a-b+), Le(a+b-), Le(a-b-)] waren durch die Mütter vertreten. In den Fäzes wurden in den ersten Lebensmonaten drei aufeinanderfolgende Stadien an Oligosaccharid-Profilen gefunden, wohingegen Muttermilch keine signifikanten Veränderungen aufwies. Während *Stadium 1* (bis zu 3 Monate nach Geburt) wurden entweder vor allem neutrale oder saure Oligosaccharide in den Fäzes gefunden, was höchstwahrscheinlich von der Zusammenstellung der HMOs in der jeweiligen Muttermilch

abhängt. Daraufhin wurde ein Wechsel zu blutgruppenspezifischen Oligosacchariden festgestellt. Die Anwesenheit dieser Oligosaccharide deutete auf einen gastrointestinalen Abbau der HMOs hin, gefolgt von einer Konjugation der Abbauprodukte mit blutgruppen-spezifischen Antigen-Strukturen, welche in der gastrointestinalen Mukus-Schicht vorkommen. Elf dieser Hybrid-Oligosaccharide, welche während Stadium 2 vorkommen, konnten in dieser Studie benannt werden. Ab der Einführung von fester Nahrung wurden vor allem Oligosaccharide, welche typisch für Folgenahrung sind, in den Fäzes gefunden (*Stadium 3*).

Das komplexe Polysaccharid Konjac Glucomannan (KGM) und Oligosaccharid-Mischungen, welche nach Inkubation des Polysaccharides mit Endo-β-(1,4)-mannanase (EM) and Endo-β-(1,4)-glucanase (EG) erhalten wurden, wurden *in vitro* fermentiert. Hierfür wurde ein Inokulum aus menschlichen Fäzes verwendet (**Kapitel 6**). Die strukturellen Eigenschaften der Oligosaccharid-Mischungen und die *in vitro* Fermentation des Polysaccharides und der Oligosaccharide wurden mittels CE-LIF und matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) untersucht. Verschiedene Fermentations-Charakteristika wurden beobachtet, welche an die strukturelle Zusammenstellung der Oligosaccharid-Mischungen zurückgeführt wurden.

Für die Oligosaccharid-Strukturen des EM- und EG-Digestes wurde eine Strukturhypothese aufgestellt. Anschließend wird in **Kapitel 7** eine detaillierte Strukturstudie der Oligosaccharide, welche mittels EM, EG oder der Enzymmischung von menschlichen Fäkalbakterien produziert wurden, beschrieben. KGM wird durch EM und EG mittels eines selektiven Mechanismus abgebaut. Für EM wurde eine kurze Oligosaccharid-Serie mit gleichen Anteilen an Mannose und Glukose gefunden. Für EG wurde eine homologe Serie gefunden. Diese besteht wahrscheinlich aus verzweigten, mannosereichen Oligosacchariden, welche auf einer Grundstruktur basieren, die aus Glukose und Mannose aufgebaut ist. Für den Digest mit Enzymen aus Fäkalbakterien wurde hingegen eine große Anzahl strukturell verschiedener Oligosaccharide, welche sowohl charakteristische Eigenschaften des EM- als auch EG-Digestes aufwiesen, gefunden. Die Ergebnisse wurden auch für die Diskussion des strukturellen Aufbaus des KGM Polysaccharides benutzt. Die Diskussion der in dieser Studie erlangten Ergebnisse und die möglichen nahrungsmittelbezogenen, wissenschaftlichen und anwendungsbezogenen Konsequenzen sind in **Kapitel 8** beschrieben. Die gastrointestinale Umwandlung der nahrungsmittel-bezogenen Oligosaccharide, welche in dieser Studie beobachtet wurden, wurden mit Literaturdaten über die frühkindliche gastrointestinale Mikroflora-Entwicklung verglichen.

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Mama, Papa und Andreas: obwohl ihr weit weg seid, steht ihr mir sehr nahe und ich kann jederzeit und in allen Lebenslagen mit eurer Unterstützung rechnen. Ich freue mich auf unser erstes "Familientreffen" in den Niederlanden. Andreas – einen Bruder so wie du zu haben ist Gold wert. Mit niemand anders hätte ich lieber den Umschlages dieser Arbeit entworfen!

Simone

About the author

Curriculum Vitae

Simone Albrecht was born on the 27th of October 1982 in Blaubeuren, Baden Württemberg, Germany and grew up in Allmendingen, Baden Württemberg, Germany. In July 2002 she finished secondary school (Gymnasium Ehingen, Baden Württemberg, Germany) and started studying Food Chemistry in the same year. The basic study (October 2002- July 2004) was carried out at the University of Stuttgart (Baden Württemberg, Germany) and the main study (October 2004 – December 2006) was carried out at the University of Stuttgart-Hohenheim, Institute of Food Chemistry, which is chaired by Prof. Dr. W. Schwack. In January 2007 she came to Wageningen University, The Netherlands, as an ERASMUS student. During her six months stay she did her diploma thesis on the characterization and *in vitro* fermentation of konjac glucomannan oligosaccharides at the Laboratory of Food Chemistry. She finished her study with the 1st state examination for food chemists and received her diploma in Food Chemistry in July 2007. From August 2007 until June 2011 she conducted her PhD research on the gastro-intestinal activity of oligosaccharides from human milk and functional foods as described in this thesis at the Laboratory of Food Chemistry. She now temporarily works at the same department within the same project.

List of publications

Albrecht, S.; van Muiswinkel, G.C.J.; Schols, H.A.; Voragen, A.G.J.; Gruppen, H. Introducing capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) for the characterization of konjac glucomannan oligosaccharides and their *in vitro* fermentation behaviour. *J. Agric. Food Chem*., **2009**, *57*, 3867-3876.

Albrecht, S.; Schols, H.A.; Klarenbeek, B.; Voragen, A.G.J.; Gruppen, H. Introducing capillary electrophoresis with laser-induced fluorescence (CE-LIF) as a potential analysis and quantification tool for galactooligosaccharides extracted from complex food matrices. *J.Agric.Food Chem*., **2010**, *58*, 2787-2794.

Albrecht, S.; Schols, H.A.; van den Heuvel, E.G.H.M.; Voragen, A.G.J.; Gruppen, H. CE-LIF-MS^n profiling of oligosaccharides in human milk and feces of breast-fed babies. *Electrophoresis*, **2010**, *31*, 1264-1273.

Albrecht, S.; Schols, H.A.; van Zoeren, D.; van Lingen, R.A.; Groot Jebbink, L.J.M.; van den Heuvel, E.G.H.M.; Voragen, A.G.J.; Gruppen, H. Oligosaccharides in feces of breast- and formula-fed babies. *Carbohydr. Res*., doi: 10.1016/j.carres.2011.06.034, *in press*

Albrecht, S.;Schols, H.A.;van den Heuvel, E.G.H.M.;Voragen, A.G.J.;Gruppen, H. Occurence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk. *Carbohydr. Res., accepted*

Albrecht, S.; van Muiswinkel, G.C.J.; Schols, H.A.; Voragen, A.G.J.; Gruppen, H. Enyymatic production and characterization of konjac glucomannan oligosaccharides. *J. Agric. Food Chem*., *submitted*

Xu, C.L.; Pranovich, A.; Hemming, J.; Holmbom, B.; **Albrecht, S.**; Schols, H.A.; Willfor, S. Hydrolytic stability of water-soluble spruce O-acetyl galactoglucomannans. *Holzforschung*, **2009**, *63*, 61-68.

Martinez, R.C.R.; Aynaou, A.-E.; **Albrecht, S.**; Schols, H.A.; De Martinis E.C.P.; Zoetendal, E.; Venema, K.; Saad, S.M.I.; Smidt, H.; *In vitro* evaluation of gastrointestinal survival of *Lactobacillus amylovorus* DSM 16698 alone and combined with galactooligosaccharides, milk and/or *Bifidobacterium animalis subsp. lactis* Bb-12. *Int. J. Microbiol*., **2011**, *149*, 152-158.

Overview of completed training activities

Discipline specific activities

Courses

Food Enzymology, VLAG, Wageningen, The Netherlands (2008)
Summer School Glycoscience, VLAG, Wageningen, The Netherlands (2008)
Ecophysiology of the gastrointestinal tract, ABS/VLAG, Helsinki, Finland (2009)
Advanced Food Analysis, VLAG, Wageningen, The Netherlands (2010)

Conferences and meetings

Deutscher Lebensmittelchemikertag, GDCH, Kaiserslasutern, Germany (2008)
Deutscher Lebensmittelchemikertag, GDCH, Berlin, Germany (2009)
Nederlandse Vereniging voor Massaspektrometrie (NVMS)-spring-meeting, Vlaardingen, The Netherlands (2009)
Deutscher Lebensmittelchemikertag, Stuttgart, Germany (2010)
Capillary electrophoresis - user day, Leiden, The Netherlands (2011)
CCC meetings, Wageningen/Groningen, The Netherlands (2007-2010)

General courses

PhD week, VLAG, Bilthoven, The Netherlands (2008)
Interpersonal Communication, WGS, Wageningen, The Netherlands (2008)
Food Chemistry Seminars, Wageningen, The Netherlands (2007-2011)
Food Chemistry Colloquia, Wageningen, The Netherlands (2007-2011)

Additional activities

Preparation PhD research proposal, Wageningen, The Netherlands (2007)
Food Chemistry Study trip, University Gent, Belgium (2009)
PhD Study trip, China (2008)
PhD Study trip, Switzerland, Italy (2010)
Member of the Organization Committee for the PhD study trip to Switzerland/Italy (2010)

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