I Production and Bioactivity of Oligosaccharides

1.1 Naturally Occurring Oligosaccharides

1 Bioactivity of Human Milk Oligosaccharides

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1.1 Introduction

Since the discovery of human milk oligosaccharides (HMO) in the mid-twentieth century, research has faced major challenges including (i) the development of methods to identify and characterize these components, (ii) the need to use HMO fractions for functional studies since single HMO were not available, (iii) the uncertainty of the purity of HMO fractions, which were often "contaminated" with remainders of lactose, proteins or glycoconjugates as well as lipopolysaccharides, and (iv) the low availability of large quantities of single HMO for animal and human studies (Table 1.1). Since the early 2000s there has been tremendous progress in all these areas, particularly in the development of methods for detailed structural analysis in extremely low milk volumes. At the same time large amounts of single HMO have been produced by chemical and biotechnical means, which will allow human studies to be conducted in the future.

New data from cell culture experiments, animal studies, and metabolic studies in humans strongly support the unique properties of HMO. Some of these recent observations will be presented including interactions with gut microbiota and direct effects on human intestinal cells (Figure 1.1). In addition, the potential for anti-inflammatory and anti-infective effects will be discussed.

With regard to biological functions, an intriguing aspect is the susceptibility of infants to diseases depending on the amount and type of oligosaccharides they receive via their mother's milk. Depending on the mother's Lewis blood group and secretor status, the oligosaccharide pattern and the total amount of HMO an infant receives per day vary significantly (Egge 1993; Kunz *et al*. 1996; Coppa *et al*. 1999; Kobata 2000; Le Pendu 2004; Asakuma *et al*. 2008; Urashima *et al*. 2011; Ruhaak and Lebrilla 2012; Thurl *et al*. 2010; Gabrielli *et al*. 2011; Prieto 2012). Therefore, the question to be addressed is whether this difference has an effect on the infant's health – i.e. are some infants more prone to certain diseases such as infections or inflammation due to a lower intake of specific oligosaccharides (Kunz *et al*. 2003)?

1.2 Structural uniqueness of human milk oligosaccharides

1.2.1 Lewis blood group and secretor-specific components in milk

Table 1.2 shows basic HMO structures that are present in human milk. Their composition has been described in several recent reviews (Blank *et al.* 2011; Urashima *et al.* 2011; Bode and Jantscher-Krenn 2012). Of particular importance is the influence of the mothers' Lewis blood group and secretor status. The presence of different neutral oligosaccharides in human milk depends on the activity of specific fucosyltransferases (FucT) in the lactating mammary gland

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Table 1.1 Milestones in milk carbohydrate and HMO research.

Around 1900	Observation: different fecal composition of breast-fed and bottle-fed infants First indications that difference is linked to milk composition First description of micro-organisms and their importance for health
Since 1930	Characterization of the first individual HMO
1950 to 1980s	Further identification of HMO and functional studies Biochemical interest due to similar epitopes on blood and tumor cells Intensive work on growth factors for micro-organisms and antiadhesive and anti-inflammatory properties
1990 to 2012	Enormous progress in developing analytical tools to characterize glycoconjugates in biological fluids Increasing number of *in vitro* functional studies with milk carbohydrate or HMO fractions (antiadhesion, anti-inflammation, direct effects on epithelial cells) First animal studies using single HMO to investigate their effects on microbiota, inflammation, infections and others First observational studies in humans relating a specific HMO pattern to diseases First metabolic studies in term and preterm infants

Notes: See also: *Advances in Nutrition*, **3**, 379S-488S, 2012. This supplement contains up to date information and comprehensive reviews of the plenary presentations at the First International Conference on the Glycobiology of Human Milk Oligosaccharides, organized by Dr. Sharon Donovan (USA) and Dr. Clemens Kunz (Germany).

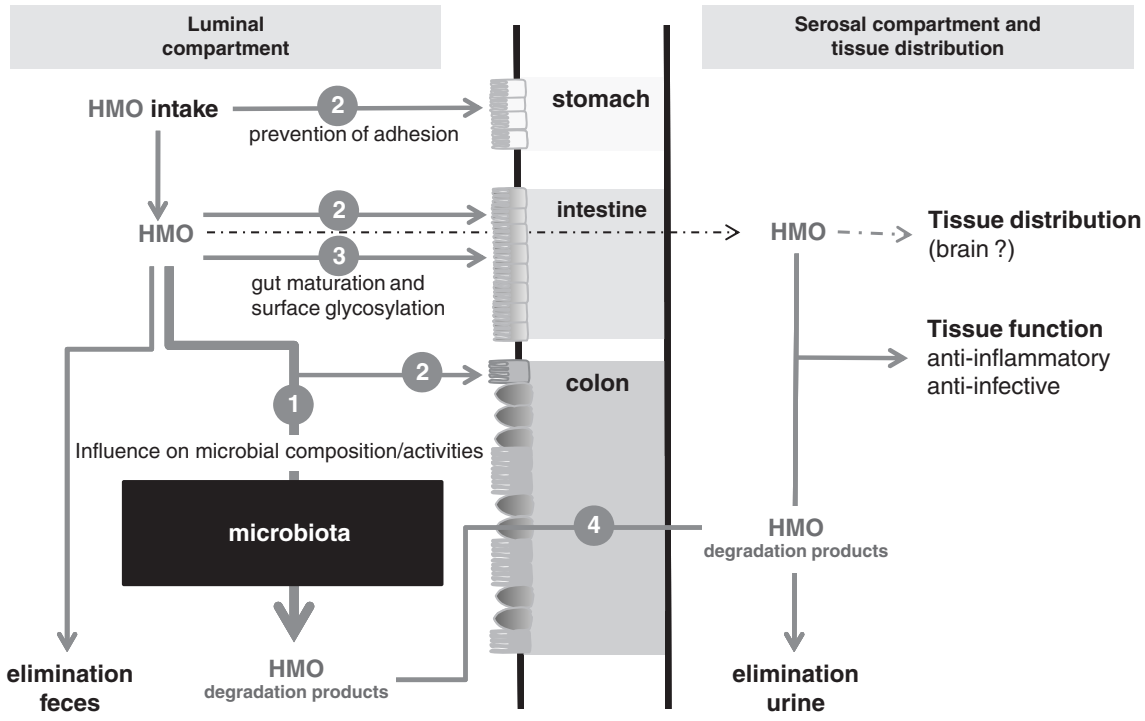


Figure 1.1 Overview of HMO metabolism and potential functions in human milk-fed infants. The numbers 1 to 4 indicate specific functions, i.e. (1) influence on the microbiota composition and/or activity, (2) prevention of pathogen adhesion, (3) direct effects on epithelial cells and (4) systemic effects.

Table 1.2 Basic chemical structures and their common abbreviations.

HMO	Abbreviation	Structure
2′-FL	2′-fucosyllactose	Fuc-α-(1→2)-Gal-β-(1→4)-Glc
3-FL	3-fucosyllactose	Gal-β-(1→4)-[Fuc-α-(1→3)]-Glc
LNT	lacto-*N*-tetraose	Gal-β-(1→3)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
LNnT	lacto-*N*-neotetraose	Gal-β-(1→4)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
LNFP I	lacto-*N*-fucopentaose I	Fuc-α-(1→2)-Gal-β-(1→3)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
LNFP II	lacto-*N*-fucopentaose II	Gal-β-(1→3)-[Fuc-α-(1→4)]-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
LNFP III	lacto-*N*-fucopentaose III	Gal-β-(1→4)-[Fuc-α-(1→3)]-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
LNDFH I	lacto-*N*-difucohexaose I	Fuc-α-(1→2)-Gal-β-(1→3)-[Fuc-α-(1→4)]-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
LNDFH II	lacto-*N*-difucohexaose II	Gal-β-(1→3)-[Fuc-α-(1→4)]-GlcNAc-β-(1→3)-Gal-β-(1→4)-[Fuc-α-(1→3)]-Glc
3′-SL	3′-sialyllactose	Neu5Ac-α-(2→3)-Gal-β-(1→4)-Glc
6′-SL	6′-sialyllactose	Neu5Ac-α-(2→6)-Gal-β-(1→4)-Glc
LST a	sialyl lacto-*N*-tetraose a	Neu5Ac-α-(2→3)-Gal-β-(1→3)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
LST b	sialyl lacto-*N*-tetraose b	Gal-β-(1→3)-[Neu5Ac-α-(2→6)]-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
LST c	sialyl lacto-*N*-tetraose c	Neu5Ac-α-(2→6)-Gal-β-(1→4)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
DS-LNT	disialyl lacto-*N*-tetraose	Neu5Ac-α-(2→3)-Gal-β-(1→3)-[Neu5Ac-α-(2→6)]-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Type I chain		Gal-β-(1→3)-GlcNAc
Type II chain		Gal-β-(1→4)-GlcNAc

(Egge *et al.* 1993; Kobata 2000; Kunz *et al.* 2003; Prieto 2012). Milk of so-called "secretors" is characterized by the activity of FucT2 forming Fuc-α-(1→2)-Gal units (compound 1, Table 1.3) like 2′-Fucosyl-Lactose (compound 2, Table 1.3) or Lacto-*N*-fucopentaose I (compound 3, Table 1.3).

In Lewis (a+b-) individuals, constituing about 20% of the population, FucT3 attaches Fuc residues in α-(1→4) linkages to a subterminal GlcNAc residue of type 1 chains. Therefore, in milk from Lewis (a+b-) nonsecretors the major fucosylated oligosaccharide is lacto-N-fucopentaose II (Gal-ß-(1→3)-[Fuc-α-(1→4)]-GlcNAc-ß-(1→3)-Gal-ß-(1→4)-Glc; compound 5, Table 1.3).

In Lewis (a-b+) donors who represent about 70% of the population, both, FucT2 and FucT3, the secretor gene and the Lewis gene dependent form, are expressed. Here, one of the major milk oligosaccharides is lacto-N-difucohexaose I (Fuc-α-(1→2)-Gal-ß-(1→3)-[Fuc-α-(1→4)]-Glc*N*Ac-ß-(1→3)-Gal-ß-(1→4)-Glc; compound 7, Table 1.3).

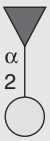
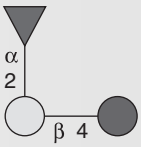
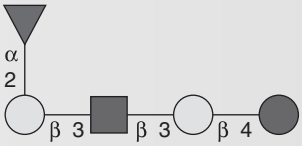
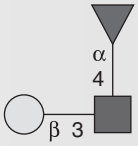
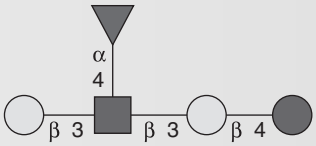
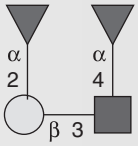
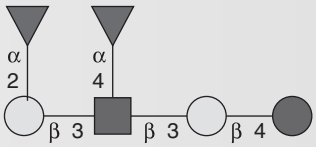
In about 5–10% of the population belonging to blood group Lewis (a-b-), FucT2 but not FucT3 is active, instead. The major oligosaccharide in their milk is lacto-*N*-fucopentaose I (Fuc-α-(1→2)-Gal-ß-(1→3)-Glc*N*Ac-ß-(1→3)-Gal-ß-(1→4)-Glc; compound 3, Table 1.3).

1.2.2 Total human milk oligosaccharides content and concentrations of single components

With regard to total HMO concentrations, published data vary for various reasons. There is no routine method available for the quantification of HMO and, despite of very sophisticated advances in mass spectrometry, this technique is still not the first choice. Therefore, components are often quantified by high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Kunz *et al.* 1996; Coppa *et al.* 1999; Thurl *et al.* 2010; Rudloff *et al.* 2012) or after fluorescence labeling and HPLC separation (Asakuma *et al.* 2008, Urashima *et al.* 2012a). According to our own data using HPAEC-PAD the concentration of oligosaccharides in human milk is estimated to be about 10 to 15 g/L with large variations mostly due to the dependency of the Lewis and secretor status.

Table 1.4 shows some of the major HMO and their quantity in milk according to various publications summarized by Urashima *et al.* (2012a).

Table 1.3 HMO – structural elements and Lewis blood group and secretor-specific components. (*For color details please see color plate section.*)

Compound	Abbreviation	Name	Epitopes	Characteristics
1				Secretor epitope
2	2′-Fuc-Lac	2′-Fucosyllactose		Secretor
3	LNFP I	Lacto-*N* fucopentaose I		Secretor
4				Lewis a epitope
5	LNFP II	Lacto-*N*-fucopentaose II		Lewis (a+b-)
6				Lewis b epitope
7	LNDFH I	Lacto-*N*-difucohexaose I		Lewis (a-b+)

Notes: Glycan structures are depicted according to the recommendations of the Consortium of Functional Glycomics using the GlycoWorkbench software tool (Ceroni *et al.* 2008); ○ galactose; ● glucose; ■ *N*-acetylglucosamine; ▲ fucose.

1.3 Human milk oligosaccharides and their functions in the gastrointestinal tract

Based upon a variety of *in vitro* experiments, animal studies and a few association studies in humans, many functions of HMO have been proposed. Examples are given in Table 1.5 (for reviews see Kunz *et al.* 2000; Newburg *et al.* 2005;

Table 1.4 (a) Concentrations of neutral HMO from different studies (according to Urashima *et al.* 2012a, with modifications).

	Concentration (g/L)					
HMO	Chaturvedi *et al.* 2001	Thurl *et al.* 2010	Coppa *et al.* 1999 (d 4)	Coppa *et al.* 1999 (d 60)	Asakuma *et al.* 2008 (d 2)	Kunz *et al.* 1999
2′-FL	2.43 ± 0.26	1.84	3.93 ± 1.11	1.84 ± 0.39	2.01 ± 1.07	0.45 ± 0.43
3-FL	0.86 ± 0.10	0.46	0.34 ± 0.06	0.71 ± 0.07	0.28 ± 0.26	0.07 ± 0.08
LNT	0.55 ± 0.08	0.86	0.84 ± 0.29	1.56 ± 0.57	1.44 ± 0.70	1.09 ± 0.47
LNnT	0.17 ± 0.03	0.11	2.04 ± 0.55	0.95 ± 0.83	0.54 ± 0.14	Trace
LNFP I	1.14 ± 0.18	0.67	1.36 ± 0.18	0.97 ± 0.61	2.08 ± 1.67	1.26 ± 1.11
LNFP II		0.20	0.29 ± 0.22	0.29 ± 0.16		
LNDFH I	0.50 ± 0.06	0.58	0.79 ± 0.25	1.18 ± 0.22	1.87 ± 1.55	
LNDFH II	0.09 ± 0.01	0.25			0.020 ± 0.025	0.16 ± 0.11

Table 1.4 (b) Concentrations of acidic HMO from different studies (according to Urashima *et al.* 2012a, with modifications)

	Concentration (g/L)				
HMO	Kunz *et al.* 1999	Martin-Sosa *et al.* 2003	Bao *et al.* 2007 (d 3–5)	Bao *et al.* 2007 (d 9–21)	Asakuma *et al.* 2008 (d 1–3)
3′-SL	0.30 – 0.50	0.10 – 0.30	0.097 ± 0.038	0.076 ± 0.014	0.297 ± 0.096
6′-SL	0.10 – 0.30	0.20 – 0.30	0.335 ± 0.033	0.396 ± 0.054	0.370 ± 0.108
LST a	0.03 – 0.20	1.70 – 3.80	0.026 ± 0.011		0.141 ± 0.107
LST b			0.131 ± 0.064	0.074 ± 0.026	0.065 ± 0.025
LST c	0.10 – 0.60	1.40 – 3.00	0.232 ± 0.058	0.148 ± 0.060	0.686 ± 0.264
DS-LNT	0.20 – 0.60	0.70 – 1.50	1.274 ± 0.503	0.795 ± 0.234	0.462 ± 0.128

Espinosa *et al.* 2007; Donovan 2011; Bode 2012; Kunz 2012). In the following we focus on the potential of HMO to influence (i) the microbial composition in the gastrointestinal tract, (ii) the adhesion of micro-organisms to the epithelium, (iii) gut maturation and cell surface glycosylation, (iv) systemic effects after intestinal absorption, and (v) association studies in humans.

1.3.1 Human milk oligosaccharides and gut microbiota

Intestinal colonization with balanced microbiota is of major importance for the appropriate development of the immune system, and there is an enormous scientific and commercial interest in modifying the microbiota for health promotion (Walker *et al.* 2010). As the gut is sterile at birth, it is an organ sensitive to environmental influences. Furthermore, there is an intensive crosstalk between gut microbes and the intestinal epithelium throughout life (Kau *et al.* 2011; Lozupone *et al.* 2012; Maynard *et al.* 2012). The mechanisms by which the intestinal mucosa perceives and responds to microbes, both pathogenic and commensal, are not completely known yet. Here, it is intriguing to investigate the role of HMO or specific single components and their effects on the selective growth of micro-organisms in the gut.

Since the pioneering work of György and coworkers in the middle of the twentieth century demonstrating the effect of *N*-acetylglucosamine containing oligosaccharides on the growth of *Bifidobacteria bifidum* subsp. *Pensylvanicum*, a strain

György isolated from infant feces, this topic is still of great scientific interest today (György *et al.* 1954; Sela *et al.* 2008; Sela and Mills 2010; Donovan *et al.* 2012; Kitaoka 2012) (Table 1.5).

Recently, Marcobal *et al.* (2011) showed that *B. longum* subsp. *infantis* can use HMO as sole carbon source, whereas most of the other intestinal bacteria they studied were unable to assimilate HMO. The genome of *B. longum* subsp. *infantis* (ATCC15697) contains a large gene cluster that comprises several glycosidases and specific transporters suggested to be involved in the metabolism of HMO (Sela *et al.* 2008). A comparative genomic survey showed that the occurrence of the cluster correlates with the survival of this subspecies on HMO (LoCascio *et al.* 2010). Therefore, David Mills, one of the leading experts in this field, and co-workers suggested that an HMO-consuming phenotype (HMO^+) of the subspecies can be attributed to the presence of this cluster (designated as the HMO cluster-1) (LoCascio *et al.* 2010). At that time, not all of the enzymes involved in the metabolism of HMO by certain Bifidobacteria had been determined.

Using a different methodological approach, Asakuma *et al.* (2011) reported the occurrence and localization of HMO-degrading enzymes in different *bifidobacterial* strains (Table 1.6). The presence of external enzymes such as specific fucosidases and lacto-*N*-biosidases, which are secreted by bacterial cells and specific transporters for lacto-*N*-biose (LNB; Gal-β-(1→3)-Glc*N*Ac) and galacto-*N*-biose (GNB; Gal-β-(1→3)-Gal*N*Ac), explains why specific HMO can be used differently by bifidobacteria. Kitaoka and co-workers have recently elucidated that *B. bifidum*, which is another consumer of HMO, has a special pathway for degrading type-1 HMO (Kitaoka *et al.* 2005; Wada *et al.* 2008). According to the authors, *B. bifidum* uses a secretory lacto-*N*-biosidase to hydrolyze LNT to LNB and lactose. The liberated LNB is then incorporated into the cells by an ABC transporter specific for LNB and GNB (Suzuki *et al.* 2008). LNB and GNB are converted to Gal and GlcNAc-1-phosphate or GalNAc-1-phosphate by the action of GLB/LNB phosphorylase (Kitaoka *et al.* 2005).

Table 1.5 (a) Examples for functional studies with HMO *in vitro*.

Effect	Factor	Investigated in/with	Reference
Prebiotic effects; influence on different *Bifdobacteria*; description of special HMO using pathways	Neutral HMO	*B. longum* ssp. *Infantis*; *B. bifidum*, *B. breve*; *B. longum* subsp. *longum*	Sela *et al.* (2008); Asakuma *et al.* (2011); Garrido *et al.* (2012); Kitaoka (2012); Yoshida *et al.* (2012)
Inhibition of adhesion of *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris* to Caco-2 cells	HMO	Caco-2 cells	Coppa *et al.* (2006)
Reduction of Entamoeba attachment and cytotoxicity	HMO with terminal Gal (e.g. LNT)	HT-29 cells	Jantscher-Krenn *et al.* (2012b)
Increase in INFγ-, IL-13-producing T cells	Sialylated HMO	Cord blood T cells	Eiwegger *et al.* (2004)
Influence on rolling and adhesion of human leukocytes	Sialylated and fucosylated HMO	High umbilical vein endothelial cells	Bode *et al.* (2004a)
Reduction of platelet neutrophil complex formation and neutrophile activation	Sialylated and fucosylated HMO	*Ex vivo* model with fresh human blood	Bode *et al.* (2004b)
Changes in cell surface glycosylation EPEC adhesion reduced	3′-SL	Human intestinal cell lines, gene expression	Angeloni *et al.* (2005)
Effects depend on cell lines; Inhibition/reduction of proliferation; alteration of cell dynamics; induction of differentiation and/or influence on apoptosis	Neutral/sialylated HMO fraction or single HMO	Transformed and nontransformed intestinal cell lines(HT 29, Caco 2, HIEC)	Kuntz *et al.* (2008, 2009)

Table 1.5 (b) Examples for animal studies with single HMO and HMO fractions.

Effect	Factor	Investigated in	Reference
Influence on brain sialic acid content	Sialic acid, sialyllactose	Rats	Witt *et al.* (1979); Carlson and House (1986); Wang *et al.* (2009)
Reduction of *S. pneumoniae* and *H. influenzae* adhesion	LNnT and sialylated LNnT	Rat pubs	Idäänpään-Heikkil *et al.* (1997)
Activation of NK cells by LNFP III stimulated macrophages	LNFP III/Lewis x	SCID and BALB/c mice	Atochina and Harn (2005)
Cure of *H. pylori* infections (50%)	3′SL	Rhesus monkeys	Mysore *et al.* (1999)
Microbial composition influenced, DSS-induced colitis reduced	3′SL	Sialyltransferase-deficient mice	Fuhrer *et al.* (2010); Weiss and Hennet (2012)
Necrotizing enterocolitis reduced	DS-LNT	rats	Jantscher-Krenn *et al.* (2012a)
HMO consumption by *Bacteroides* via mucus utilizing pathways	HMO, LNnT,	Gnotobiotic mice	Marcobal *et al.* (2011)
Effects on SCFA and microbial modulation	HMO, LNnT	Pigs	Li *et al.* (2012)

Table 1.5 (c) Examples for effects of single HMO or specific fucosylated oligosaccharides in humans.

Effect	Factor	Investigated in	Reference
No reduction of colonization of throat and nasopharynx with *S. pneumonia* or *H. pylori*; tendency of reduced "abnormal" ears	LNnT supplemented formula	Infants	Prieto (2005)
No effects in acute otitis media	LNnT	Infants (placebo-controlled trial)	Ukkonen *et al.* (2000)
Reduction of diarrhea (e.g. *Campylobacter* diarrhea)	Association with total fucosylated oligosaccharides and 2′-FL in milk	Infants	Ruiz-Palacios *et al.* (2003); Morrow *et al.* (2004)
Association with Crohn's disease	Nonsecretor status	Genotyping in young and adult individuals	McGovern *et al.* (2010)
Association with mortality, gram negative sepsis and necrotizing enterocolitis	Low or nonsecretor status	Preterm infants; secretor genotyping/phenotyping	Morrow *et al.* (2011)

This pathway, the GNB/LNB pathway, which involves LNB as a key intermediate, occurs in *B. bifidum* and some strains of *B. longum* subsp. *longum* (Wada *et al.* 2008), but apparently does not work in *B. longum* subsp. *infantis* because all of the genomes having been determined so far do not contain lacto-*N*-biosidase homologs (LoCascio *et al.* 2010). *B. longum* subsp. *infantis* possesses a GNB/LNB phosphorylase, but this enzyme exclusively acts on the disaccharide and not on LNT (Kitaoka *et al.* 2005; Hidaka *et al.* 2009).

In a study with *B. longum* subsp. *Infantis*, Yoshida *et al.* (2012) demonstrated that this strain directly incorporates LNT and hydrolyzes it inside the cell by a specific ß-galactosidase (the authors suggest the name: LNT ß1,3-galactosidase). This β-galactosidase seems to have the substrate specificity for the type-1 chain (Gal-β-(1→3)-GlcNAc), but this strain also has another β-galactosidase that is specific for lactose and type-2 chain (Gal-β-(1→4)-GlcNAc) (Urashima *et al.*

Table 1.6 HMO metabolism by *Bifidobacteria* – involved enzymes and their location.

Activities	*B. bifidum* JCM 1254	*B. longum* subsp. *infantis* JCM1222	*B. longum* subsp. *longum* JCM1217	*B. breve* JCM1192
1,2-α-L-Fucosidase	Cell bound	Cytosol	Does not exist	Gene not active[a]
1,3-1,4-α-L-Fucosidase	Cell bound	Cytosol	Does not exist	Does not exist
1,4-β-Galactosidase	Cell bound	Cytosol	Cytosol	Cytosol
β-*N*-Acetylhexosaminidase	Cell bound	Cytosol	Cytosol	Cytosol
Lacto-*N*-biosidase	Cell bound	Does not exist	Cell bound[b]	Does not exist
GLBP (GNB/LNB transporter)	+	+	+	+
Transporters in HMO cluster	does not exist	Transmembrane	Does not exist	Does not exist
LNT β-galactosidase	Cytosol	Cytosol	Cytosol	Cytosol

Note: According to Asakuma *et al.* (2011), Yoshida *et al.* (2012) and Kitaoka (2012): [a] 1,2-α-L-Fucosidase activity not detected; this strain does not utilize 2′fucosyllactose; [b] no gene encoding GH20 LNBase. (M. Kitaoka and T. Katayama, personal communication.)

2012a; Yoshida *et al.* 2012). This new data indicates that the organism uses two different ß-galactosidases to degrade type-1 and type-2 HMO selectively. It also supports the view that the HMO^+ phenotype of this subspecies should not be attributed solely to the presence of the HMO cluster-1.

So far, investigations of growth-promoting factors have primarily been focused on neutral HMO although a few studies on the effects of sialylated oligosaccharides have been published and involved sialidases have been found in *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium bifidum* (Kiyohara *et al.* 2011; Sela *et al.* 2011).

In recent animal studies, however, interesting observations regarding gut colonization have been reported by Fuhrer *et al.* (2010) using α2,3- and α2,6-sialyltransferase-deficient mice and examining the effect of the milk oligosaccharides α2,3- and α2,6-sialyllactose on mucosal immunity (Table 1.5 (b)). This study proves the influence of sialyllactose on the colonization of intestinal microbiota in mice and thus on the susceptibility to DSS-induced colitis. For the first time it was observed that one single component, namely 3′-SL, influenced the microbial composition *in vivo*. An influence on regulatory functions and on the mucosal immune system of the animals has not been detected.

1.3.2 Human milk oligosaccharides and antiadhesion effects

Since the early 1990s, numerous studies have been conducted on HMO in cell culture systems (for reviews see Newburg *et al.* 2005; Bode 2012). Some examples are given in Table 1.5 (a). The conclusion from these studies is that various HMO are potential decoy receptors for bacterial or viral pathogens relevant to infections of the gastrointestinal, urogenital or respiratory tract. Although such *in vitro* systems are pivotal for studies investigating potential underlying mechanisms which often cannot be investigated in human studies, the data obtained need careful interpretation with regard to the situation *in vivo*. It has to be shown whether effects of HMO can also be shown in animals and/or in humans as the mucus layer and the glycocalyx covering the epithelial cell surface are major barriers which aggravate a direct contact of luminal components (e.g. micro-organisms) with epithelial cells (Figure 1.2).

The mucus layer as defense system The mucosal surface of the gastrointestinal tract is a complex ecosystem, which is composed of micro-organisms, immune cells and the epithelial layer (McGuckin *et al.* 2011). The latter is covered by a mucus layer which represents the largest surface in man (200–300 m^2). This mucus layer, which can be found in the whole GIT, is in permanent contact with the environment. Two layers are to be distinguished, one of which being very loose and the other adhering tightly to the mucosal surface (Figure 1.2). Thickness ranges from 300 µm in the stomach to 700 µm in the intestine. This layer represents the very first defense system in human tissue.

The protective physico-chemical characteristics of the mucus can be traced back to the high carbohydrate content of the mucins, which at the same time interact with microbial lectins and glycans. On the other hand, those glycans can also be influenced by glycosidases and other enzymatic activities induced by the microbiota, and can thus directly affect

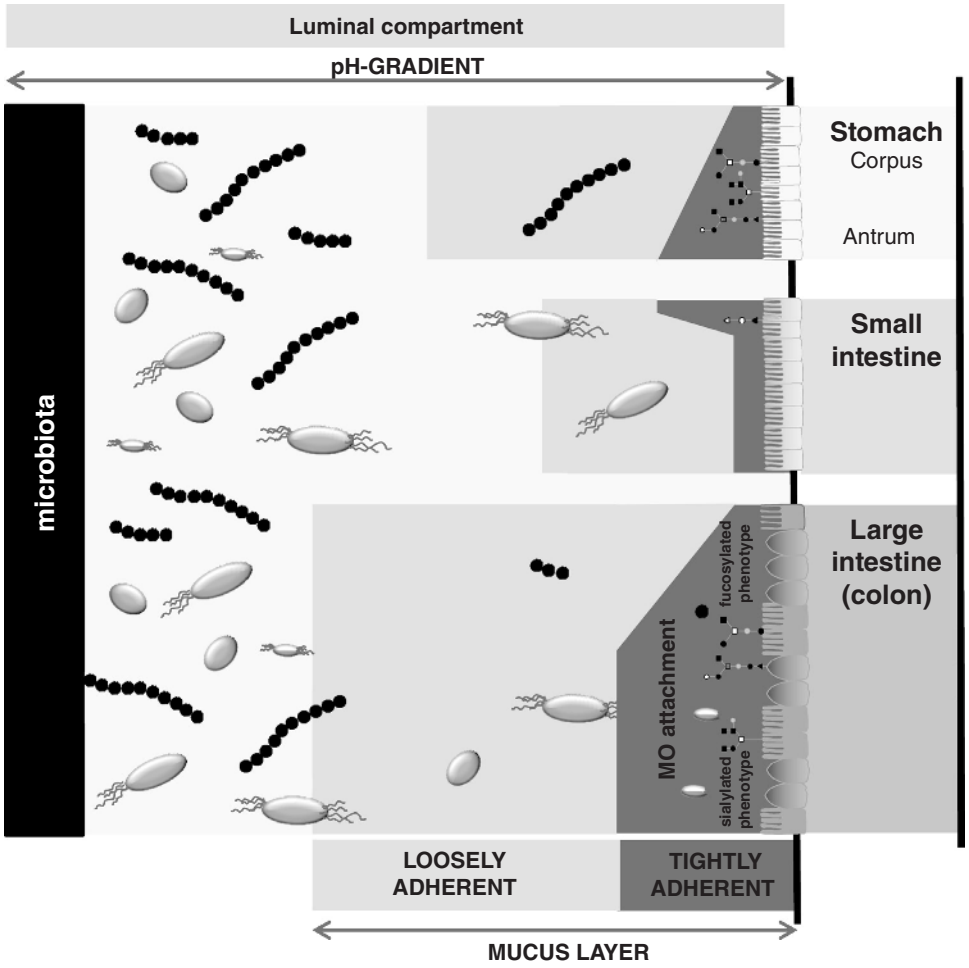


Figure 1.2 Scheme of different layers along the gastrointestinal tract and the presence of micro-organisms. If an infant receives HMO via breast milk, most of these oligosaccharides are transported to the colon. During the transit they can get immediately in intimate contact with potential luminal pathogens preventing their adhesion to the epithelial cell surface. Some of the HMO might be taken up by special epithelial cells such as M cells which are not covered by a dense layer of mucus. In general, the glycocalyx and the tight and loose mucus layer above the epithelial cell surface are very effective barriers to prevent the penetration of pathogens (see text).

these interactions. This strategy is an important mechanism to form binding ligands and to supply glucose for bacterial metabolism. A bacterial interaction with the supramucosal layer may lead to a chronic colonization of the mucus – on one hand the mucosal microbiota is able to protect cells from the invasion of pathogenic microbes and on the other hand pathogenic microbes exert strategies to adhere to the epithelial cell in order to permeate this layer.

The glycocalyx as defense system The second defense system is the epithelial glycocalyx that is located underneath the mucus layer (Figure 1.2). This glycocalyx is composed of numerous glycoproteins and glycolipids being expressed on the epithelial membrane. Depending on the tissue, the glycocalyx is ranging from 100 and 500 nm thickness in intestinal microvillus tips and only between 30 and 60 nm in the lateral microvilli. The glycosylation of mucosal epithelial cells does not only vary in dependence of the cell type but is also strongly influenced by the sub- and supramucosal environment,

for example by the hormonal status, inflammation or microbial colonization. Like the mucus layer, the glycocalyx is continually being renewed. It interacts with the overlying mucosal layer, the bile juice, and the resident microbiota to prevent or reduce the colonization of pathogenic microbes. It will be intriguing to gain more information on the effect of nutritional factors such as HMO on these very complex specific and unspecific defense systems in the human gut.

1.3.3 Human milk oligosaccharides and effects on epithelial cells and immune modulation

Recent studies demonstrated effects of HMO on the glycosylation pattern of epithelial cells, on cell proliferation, differentiation, and apoptosis as well as on cell signaling pathways (Table 1.5 (a)). In an *in vitro* study with human intestinal epithelial cell lines, Angeloni *et al.* (2005) were able to induce a differential expression of glycosylation-related genes and cell surface glycome changes with 3′-SL in HT-29 cells, which then led to a reduced adhesion of enteropathogenic *E. coli* (EPEC). This suggests that it may be possible to influence cell surface glycosylation and thereby reduce the susceptibility for pathogenic bacteria by HMO given orally (Figure 1.3).

Using a variety of neutral and sialylated HMO we have shown a reduced proliferation of intestinal epithelial cell lines (HT-29, Caco-2 cells) and nontransformed small intestinal epithelial crypt cells of fetal origin (HIEC) without having cytotoxic effects on any of the cell lines tested. Effects on proliferation, differentiation, apoptosis or cell dynamics depended on the cell lines used (Kuntz *et al.* 2008). Subsequent studies showed effects of pooled HMO on cell cycle regulation, potentially by signaling effects through EGF receptor and Ras/Raf/ERK pathway (Kuntz *et al.* 2009).

Human milk oligosaccharides may also have an influence on the immune system for the following reasons: the involvement of intestinal epithelial cells in inflammatory processes of the gastrointestinal tract is increasingly being recognized (Subramanian *et al.* 2006; Green-Johnson 2012). *In vivo*, that is, in an experimentally induced colitis, intestinal epithelial cells release cytokines such as IL-8, IL-6, TGF-ß and IL-1ß (Chang *et al.* 2012). Production of proinflammatory cytokines such as the potent chemoattractant IL-8 from epithelial cells can be expected to have a major impact on neighboring intraepithelial and lamina propria macrophages and neutrophils. Furthermore, changes in the cytokine

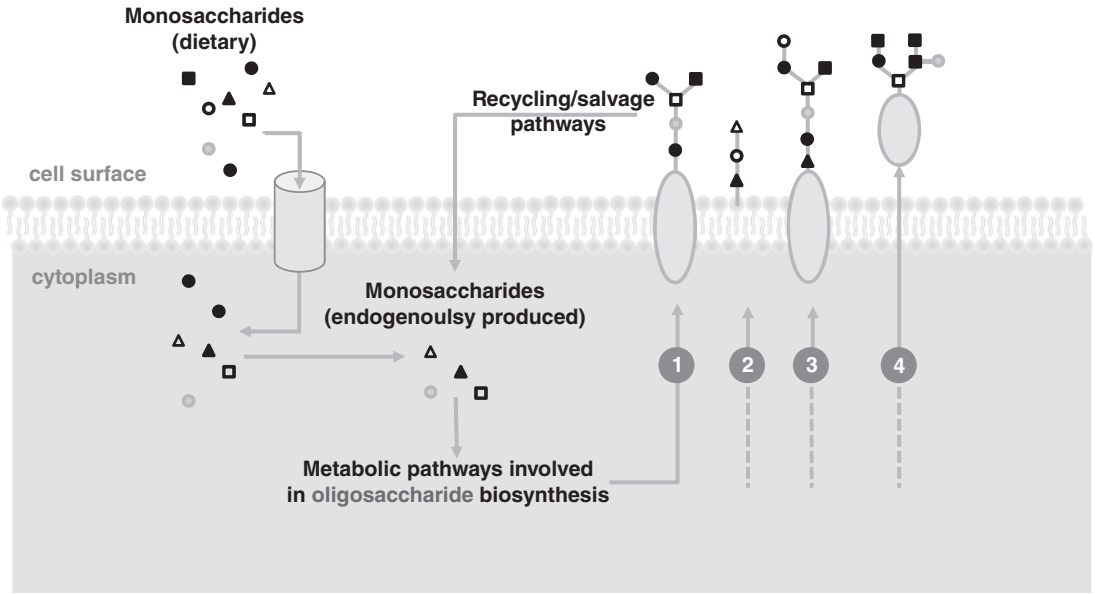


Figure 1.3 Hypothetical model indicating that dietary monosaccharides might be taken up by the intestinal cell and used for the synthesis of cell surface glycoconjugates. Numbers 1 to 4 represent the intracellular machinery for glycan synthesis.

balance may stimulate macrophages leading to a further production of proinflammatory cytokines which then influence T-lymphocyte response (Funakoshi *et al.* 2012). Secretion of proinflammatory cytokines in response to bacterial lipopolysaccharides or dietary components is a well known concept in ongoing intestinal inflammation and its progression (Subramanian *et al.* 2006). Therefore, it is of interest to know to what extent HMO affects the initial step of an inflammatory process in intestinal cells by inhibiting the secretion of proinflammatory cytokines.

1.4 Human milk oligosaccharides and systemic effects

Metabolic studies in lactating mothers and their infants showed that intact HMO and degradation products can be detected in the urine of term and preterm infants (Rudloff *et al.* 2012; Rudloff and Kunz 2012). Therefore, HMO may also exert systemic effects like influencing the adhesion of leukocytes to endothelial cells or the interaction of platelets with neutrophils. Hence, besides local functions of HMO within the gastrointestinal tract an influence on systemic infectious, inflammatory and immune processes seems likely (Figure 1.1).

Eiwegger *et al.* (2004) demonstrated that if cord blood T-cells were exposed to sialylated HMO, the number of INFγ-producing CD3+CD4+ and CD3+CD8+ lymphocytes as well as IL-13-producing CD3+CD8+ lymphocytes would increase. The authors speculated that sialylated HMO influence lymphocyte maturation and promote a shift in T-cell response towards a more balanced Th1/Th2-cytokine production.

For the neutral HMO fraction, LNFP III and LNnT have been shown to influence peritoneal macrophages capable of suppressing naïve CD4+ Tcell responses (Table 1.5 (b)) (Atochina *et al.* 2001). LNFP III also stimulated macrophage activity *in vitro* and increases secretion of prostaglandin E2, IL-10, and TNFα (Atochina and Harn 2005).

In previous experiments we have shown that both sialylated and fucosylated HMO influence leukocyte infiltration and activation in an *in vitro* flow model with TNFα-activated human umbilical vein endothelial cells and isolated human leukocytes (Bode *et al.* 2004a). In an *ex vivo* model with fresh human blood we observed a reduced platelet neutrophil complex formation and neutrophil activation in the presence of sialylated and fucosylated HMO (Bode *et al.* 2004b).

Besides these effects, an impact of HMO on brain glycoconjugate composition has also been discussed (Wang 2009, 2012). In 1986, Carlson and House compared an intraperitoneal administration to an intragastric application of sialic acid on rat brain composition and found that both oral and systemic application routes resulted in significantly more cerebral and cerebellar glycolipid and glycoprotein sialic acid than glucose injections did (Carlson and House 1986). Compared to free sialic acid, orally given sialyllactose (SL), the major acidic oligosaccharide in human milk, affected brain composition even more. These data supported an earlier observation by Witt *et al.* (1979) comparing radiolabeled free sialic acid and SL who showed a preferential incorporation of ^{14}C-SL in rat brain gangliosides. The importance of individual monosaccharides for humans, either as precursor for the production of HMO or as components having a direct effect on specific processes is currently being investigated (Sprenger and Duncan 2012). For example, Duncan *et al.* (2009) speculate that during the neonatal suckling period, *de novo* sialic acid production may not be sufficient to meet the needs of all tissues in the rapidly developing newborn and that sialic acid could serve as a conditionally essential nutrient for the suckling neonate.

1.5 Human milk oligosaccharides and studies in animals and humans

Due to the recent progress in producing certain HMO there is an increasing amount of data from animal studies supporting the high potential of HMO for various health effects. *Campylobacter jejuni* is one of most common causes of diarrheal morbidity and mortality in infants. Fucosylated oligosaccharides are considered to be very effective in preventing such infections although the definite proof is still missing. In *in vitro* and *ex vivo* studies, Newburg and co-workers have shown that α1,2-fucosylated carbohydrate moieties containing the H2 blood group epitope (Fuc-α-(1→2)-Gal-ß-(1→4)-Glc*N*Ac-…) were able to inhibit the adherence of *C. jejuni* to epithelial cells *in vitro* (Table 1.5 (c)) (Ruiz-Palacios *et al.* 2003). In concomitant experiments Campylobacter colonization of nursing mouse pups were inhibited when their

dams had been transfected with a human α1,2-fucosyltransferase gene that caused overexpression of H-antigen (Fuc-α−(1→2)-Gal-ß-(1→4)-Glc*N*Ac-…) in Chinese hamster ovary cells. The authors concluded that fucosylated HMO contributed to the protection of infants against *C. jejuni* and other enteric pathogens. However, it needs to be kept in mind that the definite proof that it is 2′fucosyl-lactose that is responsible for preventing infections has still not been shown yet. The effects demonstrated in the experiments have been found for fucosylated components with the minimal epitope fucosyl-lactosamin (Fuc-α−(1→2)-Gal-ß-(1→4)-Glc*N*Ac-…).

In another study using *a* neonatal rat model of induced necrotizing enterocolitis, it could be shown that disialylated LNT (DSLNT) increased survival rates and improved pathology scores (Jantscher-Krenn *et al.* 2012a). The effect was structure specific as the removal of one or both sialic acid residues led to a loss of function. It was the first study showing these effects in a specific disease. However, as the authors themselves stated, the general question is whether data obtained from rats can be translated to human preterm infants.

So far, only a few human studies addressed questions related to the potential effects of HMO on certain diseases (Table 1.5 (c)). Morrow *et al.* (2004) reported an association between HMO and protection against diarrhea in breastfed infants. They found a strong negative association between the amount of total fucosylated oligosaccharides and the degree of moderate to severe diarrhea of all causes, e.g., Campylobacter diarrhea was low when 2′-FL in milk was high and the occurrence of calcivirus diarrhea seemed less when Lacto-N-Difucohexaose I was high in milk (Morrow *et al.* 2004). As 2′-FL is now available in larger quantities it will be interesting to see whether the observed effects can be supported by placebo controlled clinical studies in the future.

These data raised the question again of whether the Lewis blood group and secretor status, which are known to have an influence on the pattern of fucosylated HMO, have an impact on the infant's health (Kobata 2000; Kunz *et al.* 2003; Le Pendu 2004; Prieto 2012).

In a recent study, Morrow *et al.* (2011) examined whether polymorphisms in the secretor gene (FUT2) and in the secretor phenotype affected the outcome of premature infants. The study comprised 410 infants with a gestational age of less than 32 weeks of whom 26 died, 30 developed necrotizing enterocolitis and 95 sepsis. The authors distinguished between a low-secretor and a nonsecretor phenotype depending on the amount of H2-antigen (Fuc-α−(1→2)-Gal-ß-(1→4)-Glc*N*Ac-…) determined in the infants' saliva. A low secretor phenotype was associated with necrotizing enterocolitis and a nonsecretor genotype with a gram-negative sepsis but not an overall sepsis. Thus, secretor genotype and phenotype may potentially be used as prognostic biomarkers for the outcomes in premature infants (Morrow *et al.* 2011).

1.6 Conclusion and perspective

In recent decades, research has progressed fast with regard to the characterization of individual HMO structures and patterns in milk. It is known that human milk contains a broad variety of complex oligosaccharides in concentrations ranging from 10 to 20 g/l. However, the quantity of these components does not only depend on the lactational stage but is also affected by the expression of specific glycosyltransferases in the mammary gland. The large amount of *N*-acetyl-glucosamine containing oligosaccharides in milk, which may favor the growth of specific micro-organisms, is still a matter of discussion (Garrido *et al.* 2012; Kitaoka 2012). The analysis of the genome of some strains of *Bifidobacteria* indicates their evolutionary adaptation to use specific milk components preferentially, particularly HMO as substrates. But even today, the bifidogenic effect of HMO and their direct impact on the intestinal microbiota are difficult to demonstrate in humans. The same applies to other specific *in vitro* functions of HMO such as their potential to influence inflammatory and infectious processes via inhibition of the attachment of pathogens to epithelial cells, to influence leukocyte endothelial and neutrophil platelet interactions or to affect cell recognition and cell signaling, cell adhesion or neurodevelopment.

Recent animal studies support HMO functions shown *in vitro* (Table 1.5). Concomitantly with these observations, progress in biotechnology today allows the production of at least some of the major milk oligosaccharides to be potentially added to infant formula. However, to be able to decide which compound should be used in which concentrations or combinations, studies are needed regarding absorption, metabolism and physiological functions in infants.

Previous human studies indicated that the infants' intake of HMO ranges within several hundred milligrams per suckling and that some of these components are excreted as intact molecules or as metabolites in the infants' urine (Rudloff *et al.* 2006; 2012) as well as in feces (Albrecht *et al.* 2011; Rudloff and Kunz 2012). Therefore, HMO have the potential to benefit the infants by preventing gastrointestinal or inflammatory diseases. Recent observations indicate that the genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome; and here, HMO might be of particular importance (Sela *et al.* 2008; Sela and Mills 2010). It is striking, however, that oligosaccharides in human milk are mainly characterized by type 1 structures (Gal-β-(1→3)-GlcNAc-linkages) (Urashima *et al.* 2012a). Milk of other species, including apes and monkeys, either contain only type 2 oligosaccharides (Gal-β−(1→4)-GlcNAc-linkages) or type 2 predominate over type 1. It seems likely that type 1 HMO may have, for example, importance for beneficial *bifidobacteria* in breast-fed infants (Urashima *et al*. 2012b). This interesting hypothesis needs further studies, both in animals and humans regarding structure-function relations and specific metabolic aspects.

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