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HUMAN MILK OLIGOSACCHARIDES (HMOS): STRUCTURE, FUNCTION, AND ENZYME-CATALYZED SYNTHESIS

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Abstract

The important roles of human milk oligosaccharides (HMOS), the third major component of human milk, in the health of breast-fed infants have been increasingly recognized. Structures of more than 100 different HMOS have now been elucidated. Despite the recognition of the various functions of HMOS as prebiotics, antiadhesive antimicrobials, and immunomodulators, the roles and the applications of individual HMOS species are less clear. This is mainly due to the limited accessibility to large amounts of individual HMOS in their pure forms. Current advance on the development of enzymatic, chemoenzymatic, whole cell, and living cell systems allows for the production of a growing numbers of HMOS in increasing amounts. This effort will greatly facilitate the elucidation of the important roles and exploring the applications of HMOS as individual compounds and as a mixture of defined structures with desired functions. The structures, functions, and enzyme-catalyzed synthesis of HMOS are briefly surveyed to provide a general picture about the current progress on these aspects. Future efforts should be devoted to elucidating the structures of more complex HMOS, synthesizing more complex HMOS including those with branched structures, and to develop HMOS-based or HMOS-inspired prebiotics, additives, and therapeutics.

Keywords

human milk oligosaccharides (HMOS); prebiotic; antiadhesive; antimicrobial; immunomodulator; structure; function; synthesis

1. Introduction

Carbohydrates in human milk are presented in diverse forms including monosaccharides such as glucose and galactose, lactose (a disaccharide), oligosaccharides, glycoproteins, glycopeptides, and glycolipids.¹ Human milk oligosaccharides (HMOS) containing a diverse array of oligosaccharides with three or more monosaccharide units are the subject of investigation here.

HMOS are the third major component of human milk after lactose (55–70 g/L) and lipids (16–39 g/L).^{2–4} Historically, purified HMOS were used to synthesize glycan antigens to obtain antibodies^{5, 6} which was later used as important bioreagents to identify novel glycans

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and detect glycoconjugates.⁷ The amounts of HMOS vary in lactation stages with 12–14 g/L in mature milk and 20–24 g/L in colostrum.^{8–10} HMOS were found to be presented in higher concentrations in preterm human milk than those in term human milk.¹¹ The presence and the quantity of HMOS also vary among individuals and are related to the secretor status and the Lewis group type of the nursing mothers.^{10–13} Four human milk groups have been classified based on the HMOS profiles controlled by the Secretor (*Se*) status and the Lewis (*Le*) blood type of the nursing mother.¹¹ Individuals (*Se*⁺/*Le*⁺) with both α 1–2-fucosyltransferase FUT2 encoded by the Secretor (*Se*) gene and α 1–3/4-fucosyltransferase FUT3 encoded by the Lewis (*Le*) gene represent about 70% of the European population and contain all types of fucosylated HMOS with α 1–2/3/4-fucosyl linkages. Those (*Se*⁻/*Le*⁺) with no FUT2 but with FUT3 represent 20% of the population and do not have α 1–2-fucosylated HMOS. Those (*Se*⁺/*Le*⁻) with FUT2 but no FUT3 represent 9% of the general population and do not have α 1–4-fucosyl oligosaccharides. Finally, those (*Se*⁻/*Le*⁻) without FUT2 nor FUT3 represent 1% of the general population contains α 1–3-fucosylated HMOS but not other fucosylated HMOS due to the expression of a Lewis-independent α 1–3-fucosyltransferase.^{11, 14}

Due to its structure complexity and the lack of efficient analytic methods, the presence and the functions of HMOS were unaware of in early time. For example, lactose was first isolated from milk in 1633.¹⁵ In comparison, three centuries years later in the early 1930, Polonowski and Lespagnol found and named nitrogen-containing “gynolactose”^{16, 17} which was confirmed two decades later to be a mixture of more than ten oligosaccharides by two-dimensional paper chromatography separation.¹⁸ In 1954, György, Kuhn, and *et al.* reported β -linked *N*-acetylglucosamine (GlcNAc)-containing oligosaccharides and polysaccharides in human milk as “bifidus factors”¹⁹ that promote the growth of *Lactobacillus bifidus var. Penn* (now *Bifidobacterium bifidum*).^{20–23} This ignited the efforts on elucidating the structures of HMOS. Several papers published in 1956 reported the structures of lacto-*N*-tetraose (LNT), 2'-fucosyllactose (2'FL), lacto-*N*-fucopentaose I (LNFP I), and 3-fucosyllactose (3FL).^{24–27} By 1965, 14 HMOS structures have been reported, mainly by the groups of Kuhn and Montreuil.²⁸ Additional structures were soon elucidated by the efforts of Ginsburg, Kobata, and others. The introduction of mass spectrometry to the identification of HMOS²⁹ further speeds up the progress. Modern advance on the separation and analysis method development allows fast profiling of HMOS and the structure identification of additional HMOS. More than 200 HMOS species have now been observed^{30, 31} and more than 100 HMOS structures have been elucidated.^{28, 32–34}

Unlike lactose, the primary component and the principle carbohydrate of human milk which is digestible by infants and provides them nutritional needs,³⁵ HMOS are not digestible by the infant.^{1, 36, 37} Therefore, the direct physiological roles of HMOS are not clear. Accumulating evidence has shown that HMOS can survive the obstacles encountered upon suckling and reach the infant gut where they regulate the microbiota population which in turn can affect the health of breastfed infants.^{36–39} HMOS are believed to contribute significantly to the health of breast-fed infants in lowering their risk of diarrheal disease, respiratory infections, allergy, and other infectious diseases including otitis media.^{15, 40–42} The prebiotic (stimulating the growth and colonization of beneficial bacteria, mainly bifidobacteria, in the gut), antiadhesive antimicrobial (acting as decoys to inhibit specific

pathogenic bacteria, viruses, or parasites binding to epithelial surface and translocation), immunomodulating, and brain development nutritional functions of HMOS have also been reported.^{1, 15, 38, 43–46} The enrichment of bifidobacteria in the gut also leads to the increased production of lactate and short-chain fatty acids thus the decrease of pH, worsening the environment for the growth and colonization of some pathogens.¹ Additional mechanism of pathogen inhibition may include the release of other anti-microbial substances by bifidobacteria.⁴⁷

The functions of individual structures, however, are less clear. Only a handful of HMOS have known specific roles, and only a limited number of HMOS have been synthesized. The current knowledge about the structures, functions, and production of HMOS by enzyme-catalyzed processes is presented here.

2. Structures Of HMOS

2.1 HMOS monosaccharide building blocks, core structures, and glycosidic linkages.

Human milk is unique in containing a large number of oligosaccharides compared to the milk of other mammals.⁴⁸ Five monosaccharides have been found to be major building blocks for HMOS which include D-glucose (Glc), D-galactose (Gal), *N*-acetyl-D-glucosamine (GlcNAc), L-fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac). These monosaccharide building blocks in HMOS are presented in the six-membered ring pyranose (for Glc) or pyranoside (for Gal, GlcNAc, Fuc, and Neu5Ac) structures. HMOS are extended from lactose (Gal β 1–4Glc with Glc at the reducing end) by *N*-acetylglucosaminylation and/or galactosylation with or without fucosylation and/or sialylation. Among the five major HMOS monosaccharide building blocks (TABLE I), the glucose (Glc) is at the reducing end with a mix of α - and β - configuration at the anomeric carbon. While Gal and GlcNAc are always presented with β -glycosidic linkages, Fuc and Neu5Ac are always presented with α -glycosidic linkages.

Other than lactose (which itself is not considered an HMOS), at least fifteen neutral oligosaccharides (TABLE II), including linear and branched structures, have been identified to be able to serve as the core structures of other HMOS.^{32–34} It is interesting to observe that these structures rarely have GlcNAc as the terminal unit at the non-reducing end, indicating the high efficiency of galactosyltransferases (either β 1–3- or β 1–4-galactosyltransferase) in capping the GlcNAc residues in these HMOS. In addition, unlike *N*-acetylglucosamine (LacNAc, Gal β 1–4GlcNAc) which can serve as both internal and non-reducing-end terminal disaccharide units, lacto-*N*-biose (LNB, Gal β 1–3GlcNAc) can only serve as the non-reducing-end terminal disaccharide. Linear structures only contain β 1–3-linked GlcNAc residue, while any β 1–6-linked GlcNAc generates branching.³⁸

Other than the exceptions mentioned above, twelve glycosidic linkages constitute the structures of HMOS and the list is shown in TABLE III which include three types of galactosidic, two types of *N*-acetyl-glucosaminidic, four types of fucosidic, and three types of sialidic linkages.

2.2 HMOS structures

Over 200 individual HMOS molecular species have been found^{30, 31} and the structures of more than 100 HMOS have been successfully elucidated (see TABLE IV).^{28, 32–34} These were achieved using chromatography separation, tritium labeling, derivatization with a chromophore or fluorophore, methylation analysis, glycosidase digestion, ¹H and ¹³C nuclear magnetic resonance spectroscopy (NMR) characterization, high performance liquid chromatography (HPLC), high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), capillary electrophoresis, and various mass spectrometry (MS) techniques.^{8, 11, 30, 58, 60, 61} Diversity of HMOS comes from five different monosaccharide building blocks (TABLE I), the length, the size, the sequence (TABLE II), and twelve glycosidic linkages (TABLE III) of the glycans.¹⁵ TABLE IV lists 144 structures (lactose is not counted) in the **I–XVI** categories based on the differences of core structures and 12 structures in the **XVII** category of deviant structures.

Several exceptions have been found to the general structure featured described above for HMOS. For example, a few HMOS containing a terminal *N*-acetylgalactosamine (GalNAc) such as A antigen-tetrasaccharide, pentasaccharide, hexasaccharide,⁶² and heptasaccharide⁶³ were isolated from urine or feces of blood group A breast-fed infants. In addition, several HMOS containing 6-*O*-sulfated monosaccharides have been identified.^{64, 65} Several HMOS missing the glucose⁵⁹ or lactose^{34, 59, 66} at the reducing end have been identified. In addition, an unusually Galβ1–3Gal,^{59, 67} Galβ1–4Gal,⁶⁸ or Galβ1–6Gal⁶⁹ component has been found in several HMOS (see **XVII** Deviant structures in TABLE IV).

The presence of some HMOS is related to the secretor status and the Lewis blood type of the mother.⁷⁰ The milk produced by Le^{a+b+} secretors, presenting in 70% of the general population, has the highest diversity of HMOS.¹⁴ Fuca1–2Gal-containing HMOS such as 2'-fucosyllactose (2'FL),^{71, 72} lactodifucotetraose (LDFT), lacto-*N*-fucopentaose I (LNFP I), and lacto-*N*-difuco-hexoase I (LNDFH I) are missing in the milk of Le^{a+b-} non-secretors.⁷³ Fuca1–4GlcNAc-containing HMOS including LNFP II, LNDFH I, and LNDFH II are missing in the milk of Lewis negative (Le^{a-b-}) individuals⁷⁴ (TABLE IV). It has been shown that in the absence of blood samples, the ratios of 2'FL versus 3'FL; LNFP I, LDFT, and LNDFH I versus LNT; and 6'SL versus 3'SL in human milk can be used as specific and sensitive markers for determining the secretor status of individuals.⁷⁵

High molecular weight HMOS (Mr 2242–8000),¹¹² complex neutral HMOS with up to 10 fucose residues on a core structure containing 7 LacNAc units,⁶⁰ HMOS with up to 32 monosaccharide units,¹¹³ neutral HMOS with up to 35 monosaccharides and HMOS with more than 50 monosaccharide units¹¹⁴ have been observed. Nevertheless, the identified of HMOS containing more than 14 monosaccharide units have not been elucidated and are not presented in TABLE IV.

Overall, about 70% of HMOS in pooled milk are fucosylated and about 20% are sialylated.³⁰ The major components of HMOS are lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), as well as monofucosylated, monosialylated, difucosylated, and disialylated lactose, LNT, and LNnT (TABLE IV).⁵⁷ Oligosaccharides with both sialic acid and fucose are also presented in HMOS. The top 10 most abundant HMOS species are

responsible for about 46% of the HMOS mass. The top 50 most abundant HMOS species in the pooled human milk sample account for 83% of the total intensity and the least abundant half of the total constitute only 8% of the entire intensities.³⁰ Among HMOS, 20–25 of them are considered to be the major components.¹¹ Most of them contain 3–9 monosaccharide units.⁸ 2'FL, LNFP I, LNDFH I, and LNT were shown to be the most abundant HMOS in the colostrum of Japanese Women (85% are secretors) and in mature human milk.^{10, 61, 115–117} The most abundant acidic HMOS were LSTc, DSLNT, 6'SL, 3'SL and LSTa.^{61, 115}

Compared to the milk oligosaccharides (MOS) characterized for some domestic animals^{118–120} and other primates,^{48, 121} HMOS are higher in quantities and complexity with more diversity and longer structures. In general, HMOS are high in fucosylation which is rare in the MOS of cows and pigs. In comparison, sialylation is more abundant in the MOS of cows and pigs.^{118–120} Furthermore, *N*-glycolylneuraminic acid (Neu5Gc)-containing MOS found in the milk of cows, pigs, and primates^{48, 118–121} and 4-*O*-acetyl-*N*-acetylneuraminic acid (Neu4,5Ac₂)-containing MOS found in the milk of monotremes including echidna^{122, 123} and platypus^{124–126} have not been observed in human milk. Except for 3'-galactosyllactose (3'GL), other oligo- β 1–3-galactoside structures abundant in the milk of metatheria (marsupials)^{127–129} such as common brushtail possum, tammar wallaby, red kangaroo, and koala, have not been found in HMOS.

3. Biosynthesis Of HMOS

Our understanding on the biosynthesis of HMOS is limited. Most, if not all, HMOS are extended from lactose at the non-reducing ends and are believed to be catalyzed by glycosyltransferases in the mammary gland.^{130–133} Glucose and galactose can be *de novo* synthesized in the mammary gland by a process named hexoseogenesis although plasma glucose is the major carbon source of milk lactose.^{134, 135} Lactose and other MOS are most likely accumulated in the secretory vesicle and secreted by exocytotic fusion with the apical plasma membrane.¹³⁶ Lactose itself is produced in the mammary gland by β 1–4-galactosyltransferase 1 (β 1–4GalT1) bound to α -lactalbumin in a lactose synthase complex.^{137–139} However, most of the specific glycosyltransferases that are responsible for the formation of HMOS structures with specific glycosidic linkages have not been identified. The best understood examples are human α 1–2-fucosyltransferase FUT2 encoded by the secretor (*Se*) gene^{71, 140} and α 1–3/4-fucosyltransferase FUT3 encoded by the Lewis (*Le*) gene⁷⁴ that are responsible for the formation of α 1–2- and α 1–3/4-linked fucosides, respectively, in human mammary glands.^{130, 141} Lewis (*Le*) gene-independent α 1–3-fucosyltransferase presented in all women has also been described.^{11, 14} Transgenic introduction of a human α 1–2-fucosyltransferase gene to mice was shown to allow the mice to express large quantities of 2'-fucosyllactose¹⁴² which is a good indication of the ability of the mammary gland in producing corresponding oligosaccharides in the presence of suitable glycosyltransferases. Similar success was achieved for the transgenic manipulation of mice, but not rabbit, using other glycosyltransferases including a homologous galactosyltransferase and different fucosyltransferases.¹⁴³

The presence of several glycosyltransferases in human milk has been confirmed. For example, β 1–4GalT1,^{144, 145} α 1–3- and α 1–4-fucosyltransferases,¹⁴⁶ as well as an

α 1–3/4-fucosyltransferase^{147–149} have been purified from human milk. The activity of β 1–3-*N*-acetylglucosaminyltransferase was identified in human colostrums but not in bovine (Holstein and Jersey cow) colostrums studied.¹⁵⁰ In addition to the presence of fucosyltransferase activity in human milk, α -fucosidase activity has also been identified.¹⁵¹

Enzymes purified from human milk have been used for the synthesis. For example, partially purified α 1–3/4-fucosyltransferase from human milk was used for synthesizing sialyl Lewis a,¹⁵² Lewis a and Lewis x¹⁵³ including their deoxy analogs,¹⁵⁴ sulfated Lewis x,¹⁵⁵ and multivalent tyrosinamide-tagged Lewis x structures.^{156, 157} Purified human milk fucosyltransferase preparation was also used for the synthesis of tumor-associated trimeric Lewis x¹⁵⁸ and its sialylated structures,¹⁵⁹ sialyl Lewis a and sialyl Lewis x tetrasaccharide structures modified at the C-2 position of the glucose residue at the reducing end.¹⁶⁰ Fucosylation of lacto-*N*-neohexaose (LNnH) by a partially purified human milk α 1–3-fucosyltransferase was found to add fucose at the LacNAc units of LNnH in the non-reducing end.^{161, 162} Human milk α 1–3FucTs were also shown to fucosylate chitin oligosaccharides containing 2–4 GlcNAc units.¹⁶³ Purified human milk β 1–4GalT was used together with a partially purified rat liver β 1–3GalT, a recombinant core 2 β 1–6GlcNAcT, and a recombinant human α 1–3FucT in synthesizing a sialyl Lewis x hexasaccharide.¹⁶⁴ Carbon 13-labeled linear *N*-acetylpolylactosamines (LacNAc)_n were enzymatically synthesized at 10–100 μ mol scale using the partially purified and immobilized bovine milk β 1–4GalT and human serum β 1–3GlcNAcT.¹⁶⁵ *N*-Acetylglucosaminyltransferase I (GnT-I) purified from human milk was shown to be able to catalyze the transfer of deoxy derivatives of GlcNAc.^{166, 167} These synthetic applications of human milk enzymes provide important information about their properties. Nevertheless, the syntheses were limited to small scales and were mostly used for HMOS derivatives instead of natural HMOS structures with a free reducing end.

4. Functions Of HMOS

The functional studies of HMOS were usually carried out using mixtures of HMOS that were isolated from human milk pools. The benefits of breast-feeding was observed as early as the end of the 19th century.³⁸ Increasing evidence has now shown that HMOS contribute significantly to the health of breast-fed infants via several mechanisms by serving as listed in the following:^{1, 2, 15, 35, 38, 44, 45, 57, 133, 168–177}

1. **Prebiotics:** HMOS are carbon and energy sources preferably used by beneficial bacteria such as probiotic bifidobacteria, thus promoting their growth, which in turn produce lactic acid and short chain fatty acid to decrease the pH of the gut, making it less desirable for the growth of pathogens. The predominant growth and colonization of bifidobacteria allow them to compete well pathogens for the limited nutrient available in the gut. Bifidobacteria also occupy the epithelia binding sites and make them less available for the binding of pathogens. Some antimicrobial substances released by bifidobacteria also generate an unfavorable environment for pathogens.⁴⁷
2. **Antiadhesive antimicrobials:** HMOS mimic the glycan structures presented on the surface of gut epithelium and serve as soluble decoy receptors to pathogenic

bacteria to decrease their binding to infant gut surface for colonization, thus lowering the risk for viral, bacterial and protozoan parasite infections. HMOS can also serve as inhibitors for toxins released by pathogenic bacteria.

3. Immunomodulators: Evidence has shown that HMOS can modulate epithelial and immune cell responses. Some HMOS can directly influence the gut epithelium functions,¹⁷⁸ reduce excessive mucosal leukocyte infiltration and activation which can lower the risk for necrotizing enterocolitis (NEC), one of the most common and fatal intestinal disorders in preterm infants. *Bifidobacterium infantis* grown on HMOS can also change the functions of intestinal cells.⁴³
4. Nutrient providers for brain: Some HMOS, mainly sialylated ones, may also be providers of sialic acid for the synthesis of sialic acid-containing glycolipids (gangliosides) and glycoproteins important for the development of brain and cognition of infants.

These functions have been discussed quite thoroughly in several excellent reviews published recently.^{1, 35, 38, 44, 45, 61, 133, 168–172} The functional roles of individual HMOS species, however, are less clear. This is mainly due to the unavailability of sufficient amounts of pure HMOS for detailed functional studies. Only a handful examples have been shown. These are discussed briefly in the sections below as three categories.

4.1 Neutral non-fucosylated HMOS

Neutral non-fucosylated HMOS constitute the core structures or the backbones of all HMOS. Despite earlier studies on identifying β -GlcNAc-containing oligosaccharides and polysaccharides in human milk as “bifidus factors”,^{19, 179} their identities have not been elucidated conclusively. The discovery of a novel galactose operon responsible for the assembly of GNB/LNB pathway in *Bifidobacterium longum* JCM1217 for galacto-*N*-biose (GNB) and lacto-*N*-biose (LNB) consumption 10 years ago pointed to lacto-*N*-biose (LNB, Gal β 1–3GlcNAc) presented at the non-reducing end of many neutral non-fucosylated HMOS as a potential “bifidus factor”.⁹ This was further supported by the property of LNB in selective stimulating the growth of bifidobacteria, but not *Clostridia*, *Enterococci*, and *Lactobacillus*.^{180–182} The extracellular lacto-*N*-biosidase, α 1–2-fucosidase, α 1–3/4-fucosidase, and sialidase of *B. bifidum*^{183–185} can de-cap fucosylated and/or sialylated HMOS to release their core structures which can then be used by its extracellular lacto-*N*-biosidase to produce LNB.¹⁸⁶ LNB can be transported into the bacterium by the GNB/LNB transporter in the GNB/LNB pathway and be metabolized by other enzymes involved in the GNB/LNB pathway.¹⁸⁷ On the other hand, LacNAc-terminated core HMOS can be broken down by extracellular β -galactosidase and β -*N*-acetylhexosaminidase of *B. bifidum*.¹⁸⁸

Among bifidobacteria species commonly found in breast-fed infant such as *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. bifidum*, and *B. breve*¹⁷⁵ *B. longum* subsp. *infantis* (e.g. JCM1222) and *B. bifidum* (e.g. JCM1254) were both found to consume both type I and type II HMOS core structures equally well. The other two species tested had preference towards LNT, but not LNnT. The *B. longum* subsp. *infantis* strain tested also consume mono- and di-fucosylated LNT/LNnT, disaccharides and monosaccharides

monitored in the experiment quite well.¹⁸² LNnT was further confirmed to provide advantages for *B. infantis* versus *B. thetaiotaomicron* in both *in vitro* growth studies and germ-free mice studies.¹⁸⁹

Different from *B. bifidum* which express extracellular glycosidases, *B. longum* subsp. *infantis* express internal glycosidases^{190–192} and rely on their glycan ABC transporters¹⁹³ for internalization of the corresponding HMOS.¹⁷⁵ The extracellular glycosidases on some bacteria such as *B. bifidum* could be used as a mechanism to release components from HMOS which can be readily transported into *B. longum* subsp. *infantis* or other bacteria for consumption. The symbiotic sharing of HMOS and components could be one of the mechanisms used to shape the gut microbiota. The enrichment of bifidobacteria in infant gut could be the result of coevolution of the bacteria and milk ingredients including HMOS.^{175, 182}

LNT is a carbon source that can be used by most bifidobacteria.¹⁸² LNT in HMOS, and may be other HMOS with Gal at the non-reducing end, was shown to reduce *Entamoeba histolytica* (a protozoan parasite infecting ~ 50 million people and causing ~100,000 deaths per year¹⁹⁴) attachment and its cytotoxicity towards human intestinal epithelia HT-29 cells in a dose-dependent manner.¹⁹⁴ Further *in vivo* studies are needed to show the prebiotic and antimicrobial potentials of LNT.

In comparison, LNnT was shown to be a selective carbon source for certain bifidobacteria such as *B. longum* subsp. *infantis* and *B. bifidum*. LNnT was also shown to have immunosuppressive functions¹⁹⁵ and can inhibit the binding of *Streptococcus pneumoniae* to ciliated chinchilla tracheal epithelium.¹⁹⁶ Higher concentrations of LNnT in the milk of HIV-infected women was found to be associated with reduced postnatal transmission via breastfeeding.¹⁹⁷ Therefore, LNnT is a potential candidate for developing prebiotics and therapeutics against infectious disease.¹⁹⁸

4.2 Fucosylated HMOS

Fucosylated HMOS are the most abundant HMOS species.³³ Their prebiotic, antiadhesive antimicrobial, and immunomodulation activities have been shown.

2'FL, 3FL, and LDFT were shown to selectively promotes the growth of bifidobacteria.¹⁹⁹ Fucosylated HMOS including 2'FL, 3FL, LDFT, LNFP I/II/III, LNDFH I, and LNDFHII showed preferred consumption by *B. longum* subsp. *infantis* and *B. bifidum* compared to *B. longum* subsp. *longum* or *B. breve*.¹⁸² In fact, five fucosidases have been identified from *B. longum* subsp. *infantis* strain ATCC 15697 and characterized. Their ability in using fucosylated HMOS was confirmed.¹⁹⁰

Several examples have been shown for the antiadhesive antimicrobial functions of fucosylated HMOS including antibacterial, antiyeast, and antiviral activities. Fucosylated HMOS were also found to bind norovirus²⁰⁰ and inhibit the adhesion of an enteropathogenic *Escherichia coli* (EPEC) to HEp-2 cells.²⁰¹ A minor neutral fucosylated HMOS component was shown to protect suckling mice from the diarrheagenic effects caused by heat-stable enterotoxin of *E. coli*.²⁰² α 1–2-Fucosylated HMOS were shown to inhibit the adherence

of Std fimbriated *Salmonella enterica* serotype *Typhimurium* to Caco-2 cells.²⁰³ They also inhibit the binding of *Campylobacter jejuni* to intestinal H(O) antigen and lower the chance of infection²⁰⁴ and potentially protect infants against diarrhea caused by Campylobacter or calicivirus.²⁰⁵ More specifically, high levels of 2'FL in mother's milk corresponded to lower occurrences of Campylobacter diarrhea of the infants. LDFH I was also shown to correlated to lower incidences of calicivirus diarrhea.²⁰⁵ In addition, α 1-2-fucosylated HMOS, but not those of Lewis blood group-type, were found to inhibit the binding of *Candida albicans* yeasts to human buccal epithelia cells.²⁰⁶ On the other hand, Lewis blood group antigen-containing HMOS bind well to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), competing against human immunodeficiency virus (HIV) surface glycoprotein gp120 binding to DC-SIGN *in vitro*.²⁰⁷ Indeed, breastfeeding with human milk with high concentrations of α 1-2-fucosylated HMOS and α 1-3-fucosylated was found to be protective against mortality for HIV-exposed uninfected (HEU) children during breastfeeding.²⁰⁸ Lewis b (Le^b) antigens including Le^b-terminated LNDFH I that was synthesized enzymatically were shown to bind to *Helicobacter pylori*.^{209, 210}

The immunomodulating function of fucosylated HMOS was represented by Lewis x-type LNFP III which was shown to have immunosuppressive functions.¹⁹⁵ It was able to activate macrophages *in vitro* which can further activate natural killer (NK) cells.²¹¹ HMOS containing Lewis blood group antigens were also shown to reduce selectin-mediated cell-cell interactions.^{176, 212} 2'FL and 3FL were shown to decrease colon motor contractions in a dose-dependent fashion with a better activity observed for 3FL than for 2'FL.¹⁷⁸

The understanding of the important roles of α 1-2- and α 1-3/4-fucosylated HMOS for infant health is greatly facilitated by the presence of nursing mothers with differences on the secretor status (determined by α 1-2-fucosyltransferase FUT2) and Lewis blood type (determined by α 1-3/4-fucosyltransferase FUT3). Bifidobacteria were shown to be established earlier and more often in infants fed by secretor mothers.²¹³ Mother's milk with a higher ratio of α 1-2-fucosylated versus non- α 1-2-fucosylated HMOS was shown to provide protection of breast-fed infants against diarrhea.²¹⁴

The secretor status of premature infants was also shown to be a predictor for the outcome of infants on their survival or susceptibility to diseases. Low or non-secretor status was associated with a higher death rate, higher incident of necrotizing enterocolitis (NEC) and Gram-negative sepsis.²¹⁵

4.3 Sialylated HMOS

Sialylated HMOS are charged species and represent about 20% of HMOS.³⁰ Their prebiotic, antiadhesive antimicrobial, and immunomodulating activities as well as their nutritional value for infant brain development have been shown.

A sialylated HMOS fraction was shown to inhibit the adhesion of *Escherichia coli* serotype O119, *Vibrio cholerae*, and *Salmonella fytis* to differentiated Caco-2 cells.²¹⁶ As hemagglutinins on the surface of influenza viruses bind to sialylated glycans on host cell surface, it is not a surprise that sialylated HMOS bind to influenza virus or inhibit the viral hemagglutinin binding to its ligand.²¹⁷

Sialylated HMOS have been shown to influence lymphocyte maturation²¹⁸ and have anti-infective and immunomodulating effects.³⁸ Sialylated HMOS, but not non-sialylated HMOS, reduce leukocyte rolling and adhesion in a dose-dependent manner.¹⁷⁶ In fact, sialylated HMOS fraction in a physiological range (12.5–125 µg/mL) was shown to be even better than soluble sialyl Lewis x in inhibiting leukocyte rolling and adhesion. 3'SL and 3'S-3FL were further identified to be the key ingredients and were suggested to contribute to the lower incidence of inflammatory diseases in breast-fed infants.¹⁷⁶ Similarly, sialylated HMOS reduce platelet-neutrophil complex formation and subsequent neutrophil activation in an ex vivo model with whole human blood.²¹²

Sialylated HMOS may also be used as source of sialic acid for the synthesis of sialic acid-containing glycolipids (gangliosides) and glycoproteins important for the development of brain and cognition of infants.⁴⁵

The simplest and the most well studied sialylated HMOS are sialyllactose including 6'SL and 3'SL. Sialyllactose inhibited cholera toxin induced fluid accumulation in a rabbit intestinal loop model. These effects are believed to be responsible for the activity of human milk and its low molecular weight fraction in inhibiting cholera toxin B subunit binding to monosialoganglioside (GM1).²¹⁹ Sialyllactose was also shown to inhibit the binding of *Aspergillus fumigatus* conidia to laminin extracted from mouse sarcoma tumor²²⁰ and the binding of *Pseudomonas aeruginosa* 8830 to immobilized asialo GM1 in a microtiter plate assay²²¹ although the mechanism for the latter is unknown. Sialyllactoses were also shown to induce differentiation in transformed human intestinal cells HT-29 and human intestinal epithelial cells HIEC.²²² 6'SL alone or with 3'SL, but not 3'SL alone or oligofructose alone, was shown to enhance the adhesion of *B. longum* subsp. *infantis* strain ATCC15697 to HT-29 human intestinal cells.²²³ 3'SL was shown to bind to polyomavirus.²²⁴ It inhibited the binding of S fimbriated *E. coli* to endothelial and epithelial.^{225, 226} It also inhibited the adhesion of *Helicobacter pylori* binding to human epithelial cells *in vitro* and was shown to decrease *Helicobacter pylori* colonization in a rhesus monkey antiadhesive therapy model.²²⁷ 3'SL was shown to inhibit the binding of some sialyl oligosaccharides to *Helicobacter pylori*,²²⁸ *E. coli S-fimbriate*,²²⁹ and influenza viruses.¹⁷³

The 3'SL level in human milk, however, can also be an indicator of HIV infection. Higher relative abundances of 3'SL were shown in the milk of HIV-infected mothers²³⁰ and in the milk of mothers who transmit HIV to their babies via breastfeeding.¹⁹⁷

Another exiting example about the potential use of sialylated HMOS is their application in treating necrotizing enterocolitis, one of the most common and fatal intestinal disorders in preterm infants^{231, 232} that does not currently have an ideal therapeutic outcome.^{233–235} A single sialylated HMOS, disialyllacto-*N*-tetraose (DSLNT), but not its non-sialylated or mono-sialylated analog, was identified as a specific HMOS component that is effective for preventing necrotizing enterocolitis (NEC) in a neonatal rat model.²³⁶ Low concentrations of DSLNT in mother's milk are corresponding to an increased risk of NEC in the preterm very-low-birth weight infants.²³⁰

5. Production Of HMOS By Enzyme-Catalyzed Processes

Chemical synthesis of more than 15 different HMOS and derivative (2'FL,²³⁷ 3FL,²³⁷ LDFT,²³⁷ LNT,^{238–240} LNnT,^{238, 241} LNFP I,^{239, 242} LNFP III^{242–245} and its protected form,²⁴⁶ LSTa and LSTd (Neu5Ac α 2–3LNnT, not found in human milk) with an aglycon,^{247, 248} LNDFH I with a β -linked aglycone,²⁴⁹ pLNnH,^{241, 250} LNnH²⁵¹ and its protected forms,^{252, 253} lLNO,²⁵⁴ pLNnO²⁴¹ and its protected form,²⁵⁵ trifucosylated pLNnO in its protected form,²⁵⁵ DF-LNH II,^{256, 257} and DF-LNnH^{256, 257}) with 3–11 monosaccharide units have been reported including recent successes in the synthesis of LNFP I and its α 1–2-fucosylated LNnT analog using one-pot glycosylation approaches.²⁵⁸ These chemical synthetic efforts are out of the scope of this review. The focus of this section will be a survey on enzyme-catalyzed processes for the production of HMOS.

The production of only a handful of HMOS has been reported using enzyme-catalyzed processes²⁵⁹ and the synthesized HMOS are limited to those with relatively simple structures. Despite the success on the characterization of mammalian enzymes and purification of several glycosyltransferases from human milk, their application in synthesis has been limited due to the difficulties in obtaining them in large amounts and in an economically efficient manner. On the other hand, bacteria express a wide array of glycosyltransferases which are responsible for the construction of diverse lipopolysaccharides (LPS) and capsular polysaccharide structures. Some of these glycan structures mimic those found on human cell surfaces and those in HMOS.^{260, 261} Therefore, bacteria are a rich source of glycosyltransferases that can be used for the synthesis of HMOS as well as the glycans and glycoconjugates presented on human surface.^{262–265} Recombinant bacterial glycosyltransferases have been increasingly used for the synthesis of several HMOS structures in enzymatic, chemoenzymatic, whole cell, and living cell approaches.

Early enzymatic methods used expensive sugar nucleotides as donor substrates for glycosyltransferases for the production of HMOS. Glycosyltransferase-catalyzed reactions with in situ donor regeneration cycles that applied for preparative-scale synthesis of oligosaccharides^{266–268} can also be used for the synthesis of HMOS. Recently, highly efficient one-pot multienzyme (OPME) systems have been established for the synthesis of HMOS.^{264, 269–271} These systems use inexpensive, free monosaccharides as starting materials, which are enzymatically converted to sugar nucleotides with or without the formation of sugar-1-phosphate intermediates. The activated sugars in the forms of sugar nucleotides are supplied to the corresponding glycosyltransferases in one-pot for the formation of the corresponding oligosaccharides. Multiple OPME systems can be used in sequential to build up more complex oligosaccharides.^{269, 272} The high efficiency of the systems is facilitated by the elucidation of novel salvage pathways of sugar nucleotide biosynthesis as well as the identification and characterization of new bacterial glycosyltransferases and mutants with high expression levels in *E. coli*, good solubility and stability, and high activity.

Much progress has been made recently in identifying glycosyltransferase mutants with improved functions and many of these successes are based on protein crystal structure-

based rational design and some are from directed evolution coupled with high-throughput screening methods.^{262, 273} These are effective approaches for obtaining additional or better catalysts that are not readily available from nature.

If not all glycosyltransferases that are responsible for formation of desired HMOS are available, enzymatically synthesized oligosaccharide derivatives can be as building blocks (or synthons) for chemical synthesis of more complex HMOS and derivatives. Such chemoenzymatic methods have been explored for the synthesis of sialyl galactosides,^{274, 275} sialyl Lewis x tetrasaccharides,^{276, 277} protected sialyllacto-*N*-tetraose a (LSTa, Neu5Ac α 2–3LNT) and LSTd (Neu5Ac α 2–3LNnT,^{126, 263} has not been found in human milk),²⁷⁸ and an LNT derivative²⁷⁹. The obtained LNT derivative was further used as a glycosyltransferase acceptor for the production of LSTa derivatives by OPME enzymatic sialylation.²⁷⁹

A limited number of HMOS have also been synthesized by whole cell synthesis and engineered *E. coli* living cell fermentation approaches. Both approaches take a good use of microorganisms' own metabolic machinery for the production of some components (such as nucleotides, monosaccharides, and/or or sugar nucleotides) from less expensive materials (simple carbon and energy source such as glycerol or glucose). One of the limitations of the living cell system is the restriction of the oligosaccharide transporter systems for transfer acceptors from external sources into the cells for the product formation.²⁶²

Alternative enzymatic synthetic strategies using glycosidases, trans-glycosidases, and glycosidase mutants designed for synthesizing carbohydrates (e.g. glycosynthase²⁸⁰) have also been developed for obtaining HMOS. These methods require the use of glycosylated donors which may not be readily available. The synthetic donors used have to be chemically synthesized and may not be stable.^{281, 282} Low yields and poor regioselectivity are also common problems for glycosidase-catalyzed reactions. Strategies to improve the trans-glycosylation reactions of glycosidases including controlling acceptor/donor ratio and reaction time, removing product continuously, enzyme immobilization and recycling, using cosolvents, and enzyme engineering have been reviewed recently.²⁸³

Examples of HMOS that have been synthesized as their natural oligosaccharide forms with a free reducing end using enzymatic, whole cell, and living cell approaches are shown in the following sections.

5.1 2'FL

Enzymatic production of 2'FL (18 mg, 65% yield) from lactose was achieved using a reaction catalyzed by *Helicobacter pylori* NCTC 364 α 1–2-fucosyltransferase (glutathione S-transferase or GST fusion was shown to improve the expression of soluble protein)²⁸⁴ using GDP-L-fucose (78 mg, 78% yield) produced from GDP-D-mannose by enzymatic reactions catalyzed by *E. coli* K-12 GDP-D-mannose 4,6-dehydratase and GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase-4-reductase.²⁸⁵

Living cell biosynthesis of 2'FL (1.23 g/L, 20% yield) from lactose (14.5 g/L) in batch fermentation was achieved using *E. coli* JM109(DE3) cells engineered to overexpress *Helicobacter pylori* 26695 strain (ATCC 700392) α 1–2-fucosyltransferase²⁸⁶

and overproduce GDP-fucose.²⁸⁷ The production of 2'FL (6.4 g/L) was further improved using an engineered *Helicobacter pylori* α 1–2-fucosyltransferase by adding three aspartate residues at its N-terminus in an alternative expression host obtained by engineering *E. coli* BL21star(DE) strain to delete its endogenous lactose operon and to introduce a *lacZ* *M15*-containing modified lactose operon from *E. coli* K-12.²⁸⁸

An improved large-scale production of 2'FL (20 g/L) from lactose and glycerol was achieved using an antibiotic-free fed batch fermentation (13 L) of engineered *E. coli* JM109 (*lacY*⁺, *lacZ*⁻) cells. The cells were engineered by chromosome incorporation of genes involved in the *de novo* GDP-L-fucose biosynthetic pathway, two copies of *Helicobacter pylori* α 1–2-fucosyltransferase *futC* gene, and *Bacteroides fragilis* bifunctional fucokinase and GDP-fucose pyrophosphorylase *fkp* gene involved in the salvage pathway of GDP-fucose formation to the chromosome.²⁸⁹

2'FL production was also achieved in the milk of transgenic mice by introducing to mice a fusion gene containing a human α 1–2-fucosyltransferase gene downstream of a murine whey acidic protein promoter and upstream of a polyadenylation signal.¹⁴² The same transgenic manipulation on rabbits seemed to interfere with their lactation process.²⁹⁰ The presence of glycoconjugates containing Fuca1–2Gal epitope reduces the rate and duration of pathogen colonization in pups inoculated with pathogenic strains of *Campylobacter jejuni*.²⁰⁴

Several α 1–2-fucosynthases were obtained from *Bifidobacterium bifidum* α 1–2-fucosidase (AfcA), an inverting glycosidase, by mutating the amino acid residues involved in catalysis (N421G, N423G, or D766G).^{183, 291} The D766G mutant was found to be the most effective enzyme in catalyzing the synthesis of 2'FL from β -L-fucosyl fluoride (10 mM) and lactose (30 mM). A 6% yield was obtained based on the β -L-fucosyl fluoride donor substrate used.²⁹²

5.2 3'SL and 3'SLN

Neu5Ac α 2–3Lac (3'SL) and Neu5Ac α 2–3LacNAc (3'SLN) were synthesized using a one-pot three-enzyme (OP3E) system containing an *E. coli* sialic acid aldolase (EcNanA),^{262, 293} *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS),²⁹³ and a multifunctional *Pasteurella multocida* α 2–3-sialyltransferase 1 (PmST1).²⁹⁴ The amount of the enzyme used and the reaction time needed to be controlled to allow the optimal production of the product due to the multi-functionality of PmST1. The synthesis can be improved by replacing the wild-type PmST1 with a PmST1 E271F/R313Y double mutant which has retained α 2–3-sialyltransferase activity while with >6000-fold decreased α 2–3-sialidase activity.²⁹⁵ PmST1 M144D mutant with decreased donor hydrolysis and lowered α 2–3-sialidase activities²⁹⁶ can also be used for high efficient synthesis of 3'SL and 3'SLN. The sialosides can also be synthesized from Neu5Ac and a suitable acceptor using a one-pot two-enzyme system containing NmCSS and a sialyltransferase.

Production of 3'SL and 3'SLN has also been reported from CMP-Neu5Ac and lactose by catalyzed a *Pasteurella multocida* α 2–3-sialyltransferase²⁹⁷ or *Pasteurella dagmatis* α 2–3-sialyltransferase.^{298, 299}

The α 2–3-trans-sialidase activity of *Pasteurella multocida* α 2–3-sialyltransferase (GenBank accession number [AAK02272](#)) (PmST) which differs from PmST1 protein sequence by three amino acid residues (N105D, Q135R, and E295G) and has α 2–3- and α 2–6- dual trans-sialidase activities was used for the synthesis of 3'SL from lactose and casein glycomacropeptide (whey protein). The product 3'SL was accumulated up to 2.75 mM from lactose (100 mM) and 5% (w/v) casein glycomacropeptide (containing 9 mM bound sialic acid) under an optimal condition at pH 6.4 and 40 °C for 6 hours.³⁰⁰

The trans-sialidase activities of *Bacteroides fragilis* sialidase,³⁰¹ *Arthrobacter ureafaciens* or *Bifidobacterium infantis* sialidase,³⁰² and *Trypanosoma cruzi* α 2–3-trans-sialidase have also been explored for the synthesis of 3'SL.^{303, 304} Low or moderate yields were achieved.

A fusion protein of NmCSS and *Neisseria meningitidis* α 2–3-sialyltransferase (Nm α 2–3ST) was used in a sugar nucleotide regeneration reaction for the synthesis of 3'SL (68 g in a partial purified solid form, 68% yield) at the 100 gram scale from lactose, Neu5Ac, phosphoenolpyruvate, and catalytic amounts of ATP and CMP.³⁰⁵

Large-scale production of 3'SL was also achieved using a whole cell approach.³⁰⁶ In this process, *Corynebacterium ammoniagenes* DN510 cells (for the production of UTP from inexpensive orotic acid and converting CMP to CDP) and three recombinant *E. coli* strains (containing *E. coli* K12 CTP synthetase, *E. coli* K1 CMP-Neu5Ac synthetase, and *Neisseria gonorrhoeae* α 2–3-sialyltransferase respectively) were permeabilized by treating cell pellets with polyoxyethylene octadecylamine (Nymeen S-215) and dimethylbenzenes (xylene). Multiple grams of 3'SL (0.99 g, 36% yield and 72 g, 44% yield) were synthesized from lactose, Neu5Ac, and orotic acid at 32 °C for 11 h.³⁰⁶

3'SL (2.6 g/L, 49% yield) has also been produced from Neu5Ac and lactose fed to living *E. coli* (*lacY*⁺, *lacZ*⁻, *nanT*⁺, *nanA*⁻) cells engineered to express *N. meningitidis* CMP-Neu5Ac synthetase (NmCSS) and an *N. meningitidis* L3 strain MC58 α 2–3-sialyltransferase (Nm2–3ST). The knockout of *lacZ*⁻ and *nanA*⁻ genes was to ensure that the lactose and Neu5Ac fed to the cells were not broken down by the β -galactosidase and sialic acid aldolase, respectively. Neu5Ac was transported into the cells by Neu5Ac permease NanT and β -galactoside permease LacY endogenous to the *E. coli* host cells were responsible for transporting exogenous Neu5Ac and lactose, respectively, into *E. coli* cells for the production of 3'SL.³⁰⁷

To decrease the cost for 3'SL production, the engineered 3'SL biosynthetic *E. coli* K12 cells were modified further by deleting ManNAc kinase *nanK* gene and incorporating plasmids for the expression of *Campylobacter jejuni* strain ATCC43438 *neuABC* genes encoding GlcNAc-6-phosphate 2-epimerase, sialic acid synthase, and CMP-Neu5Ac synthetase to produce CMP-Neu5Ac from endogenous UDP-GlcNAc and avoid the need of exogenous Neu5Ac. Using this improved engineered bacterial strain, a higher concentration (25 g/L) of 3'SL was obtained.³⁰⁸

5.3 6'SL and 6'SLN

Neu5Ac α 2–6LacNAc (6'SLN) was synthesized using a similarly OP3E sialylation system as described above for the synthesis of 3'SL and 3'SLN except for replacing the PmST1 by *Photobacterium damselae* α 2–6-sialyltransferase (Pd2,6ST).³⁰⁹ Neu5Ac α 2–6Lac (6'SL) can also be synthesized similarly using the same OP3E system as shown for the synthesis of 6'SL derivatives.

Both 6'SL and 6'SLN have been synthesized from CMP-Neu5Ac and lactose using *Pasteurella dagmatis* α 2–3-sialyltransferase P7H/M117A double mutant which was completely switched to an α 2–6-sialyltransferase.²⁹⁸

More recently, 6'SL (3.33 mM) was synthesized from lactose (100 mM) and casein glycomacropeptide (containing 9 mM bound sialic acid) at pH 5.4 and 40 °C for 8 hours using the α 2–6-trans-sialidase activity of PmST (GenBank accession number [AAK02272](#)) which has the dual α 2–3- and α 2–6-trans-sialidase activities.³⁰⁰ PmST1 P34H mutant with α 2–6-trans-sialidase activity was used to further improve the regio-selective production of 6'SL versus 3'SL.

6'SL was also produced together with its disialylated derivative, 6,6'-disialyllactose, using a living cell system engineered to overexpress *Photobacterium sp.* JT-ISH-224 α 2–6-sialyltransferase (Psp2,6ST)^{304, 310} with *Campylobacter jejuni* strain ATCC43438 *neuABC* genes encoding GlcNAc-6-phosphate 2-epimerase, sialic acid synthase, and CMP-Neu5Ac synthetase.³¹¹ A 6'SL derivative Kdo α 2–6Lac was also able to be produced using a similar system with Psp2,6ST gene under the control of a strong P_{trc} promoter and *neuABC* genes under the control of a weaker P_{lac} promoter.³¹¹

5.4 LNT2, LNnT, LNnH, LNnO, LNnD, LSTd, and disialyl oligosaccharides

Recently, two β -*N*-acetylhexosaminidases HEX1 and HEX2 identified from soil-derived metagenomic library screening were found to be able to catalyze trans-glycosylation reactions using chitin oligosaccharides as donor substrates and lactose as the acceptor for the formation of lacto-*N*-triose II (LNT2, GlcNAc β 1–3Lac),³¹² the precursor for the synthesis of LNT and LNnT. Although the yields are low (2% and 8% respectively), they have the potential for improvement by mutagenesis.

LNT2 (106.3 mg) was also synthesized from lactose and UDP-GlcNAc catalyzed by bovine serum β 1–3-*N*-acetylglucosaminyltransferase. LNnT (12 mg) was subsequently produced from LNT2 and *ortho*-nitrophenyl β -galactoside by a commercially available *Bacillus circulans* β -D-galactosidase.³¹³

Large-scale production of LNT2 trisaccharide and LNnT in several hundred grams in a 100 L reactor has been reported. LNT2 trisaccharide (250 grams) was synthesized from lactose and UDP-GlcNAc using *E. coli* cells expressing β 1–3-*N*-acetylglucosaminyltransferase (LgtA). LNnT (300 grams, >85% yield) was subsequently synthesized from LNT2 and UDP-galactose using *E. coli* cells expressing β 1–4GalT (LgtB). Sialyllacto-*N*-tetraose d (LSTd, Neu5Ac α 2–3LNnT, has not been identified in human milk) was further produced

in 50 grams with a 90% yield from LNNt and Neu5Ac α 2–3Lac using a recombinant *Trypanosoma cruzi* α 2–3-trans-sialidase expressed in *E. coli*.²⁶³

LNT2 and LNNt were reported to be produced in kilograms in a fermentation-based system to allow the conduct of clinical trials.^{290, 314} At the tested concentration, LNNt was proven to be stable and safe to use as a component of infant formula although it did not reduce oropharyngeal colonization of *Streptococcus pneumoniae* in children of 6 months or older.³¹⁵

LNNt was also synthesized from 1-thio- β -LNT2 conjugated to a polyethylene glycol (PEG)-based dendrimeric support and UDP-Glc using reactions catalyzed by UDP-Gal 4-epimerase and bovine milk β 1–4GalT. In this system, the UDP-Gal 4-epimerase was responsible for the formation of UDP-Gal from less expensive UDP-Glc, thus providing donor substrate for the bovine milk β 1–4GalT for the formation of LNNt. The thio-linked PEG-support was readily cleaved off using mercuric (II) trifluoroacetate (CF₃CO₂)₂Hg (2 equivalents) in acetic acid (0.05 M) at room temperature to release free LNNt (18 mg).³¹⁶

Large-scale production of LNT2 (6 g/L, 73% yield) and LNNt (> 5 g/L), and lower level formation of lacto-*N*-neohexaose (LNNH), lacto-*N*-neooctaose (LNNO), and even lacto-*N*-neodecaose (LNNd) were reported using living *E. coli* JM109 cells (*lacY+* *lacZ*⁻) engineered to overexpress *Neisseria meningitidis* β 1–3-*N*-acetylglucosaminyltransferase (NmLgtA) and *Neisseria meningitidis* β 1–4GalT (NmLgtB).³⁰⁷

Enzymatic synthesis of LNT2 (1.36 g, 95% yield), LNNt (1.19 g, 92% yield) and disialyl glycans was successfully achieved using sequential one-pot multienzyme (OPME) systems as shown in FIG. 1 and FIG. 2.²⁶⁹ In these systems, free monosaccharides were added one-by-one at each one-pot systems containing multiple enzymes responsible for catalyzing monosaccharide activating followed by transfer processes. Multiple OPME systems were used sequentially for building up complex HMOS structures. The combination of several OPME systems were used for the synthesis of disialyl oligosaccharides milk including DSLNNt (236 mg, 99% yield), GD3 tetraose (239 mg, 82% yield), DSLac (112 mg, 93% yield), and DS'LNT (268 mg, 98% yield) which are analogs of disialyl lacto-*N*-tetraose (DSLNT), a hexaose commonly found in human. A monosialylpentaose LSTd (or 3''-sLNNt) (138 mg, 98% yield) was synthesized similarly using sequential OPME systems.²⁶⁹ Similar to DSLNT and HMOS pool,²³⁶ both synthetic DSLNNt and DS'LNT were shown to protect neonatal rats from necrotizing enterocolitis.²⁶⁹

5.5 Fuca1–2LNNt

Fuca1–2LNNt, a monofucosylated pentaose that has not been identified from human milk, was produced together with 2'FL using *E. coli* living cells engineered to overproduce GDP-fucose³²⁵ and LNNt³⁰⁷ with an additional introduction of a modified *H. pylori* strain 26695 α 1–2-fucosyltransferase.³²⁶

5.6 LNFP III, LNnFP V, and LNnDFH

Lacto-*N*-neofucopentaose (LNnFP V), lacto-*N*-neodifucohexaose (LNnDFH), and a lacto-*N*-neodifucooctaose [Gal β 1–4GlcNAc β 1–3Gal β 1–4(Fuca1–3)GlcNAc β 1–3Gal β 1–

4(Fuca1–3)Glc] have been synthesized from lactose using living *E. coli* cells engineered to inactivate genomic *wcaJ* gene involved in colanic acid synthesis and to express NmLgtA, NmLgtB, *Helicobacter pylori* strain 26695 α 1–3-fucosyltransferase FutA (encoded by *HP0379* gene), and RcsA (a positive regulator of the colanic acid operon). Glucose was used as a carbon source.³²⁵ The construct was further modified to improve the yield for the synthesis of LNnDFH (1.7 g/L). In addition, the living cell system containing another *Helicobacter pylori* strain 26695 α 1–3-fucosyltransferase *futB* gene (*HP0651*) was shown to produce both lacto-*N*-neofucopentaose III (LNFP III) (260 mg/L) and LNnFP V (280 mg/L).³²⁷

5.7 LNT

LNT was enzymatically synthesized from LNT2 and *ortho*-nitrophenyl β -D-galactoside using a *Bacillus circulans* ATCC31382 β -galactosidase-catalyzed transglycosylation reaction. Alternatively, LNT (7.1 mg) was able to be synthesized from lactose and Gal β 1–3GlcNAc β pNP using *Aureobacterium sp.* L-101 lacto-*N*-biosidase-catalyzed transglycosylation reaction.³¹³ Inherent low yields (19–26%) were observed for typical glycosidase-catalyzed trans-glycosylation reactions.

An LNT benzyl glycoside was efficiently produced from LNT2 benzyl glycoside (synthesized by NmLgtA-catalyzed glycosylation reaction from lactose benzyl glycoside and UDP-GlcNAc) and UDP-Gal using a GST-tagged *Escherichia coli* O55:H7 β 1–3-*N*-acetylglucosaminyltransferase WbgO fusion protein.³¹³

Large-scale production of LNT was not achieved until recently using *E. coli* strain LJ110 (with intact LacY but with *lacZ* knockout) chromosomally integrated with *Neisseria meningitidis* β 1–3-*N*-acetylglucosaminyltransferase *IgtA* and *Escherichia coli* O55:H7 β 1–3-*N*-acetylglucosaminyltransferase *wbgO* genes.³²⁸ Nevertheless, when glucose was used as the carbon source, LNT2 was the major product and only about 5% of the lactose was converted to LNT (219 mg/L).³²⁸ By substituting the glucose with galactose, the yield of LNT production (811 mg/L) was improved by 3.6-fold. Fed-batch cultivation with galactose further improved the efficiency and produced LNT in 173 grams (12.72 g/L).³²⁹

5.8 3FL, LDFT, LNFP II, Le^a tetrasaccharide, and Le^x tetrasaccharide

Several α 1–3/4-fucosyltransferases were obtained from *Bifidobacterium bifidum* α 1–3/4-fucosyltransferase (BbAfcB), a retaining glycosidase, by mutating the amino acid residue that was predicted to serve as a nucleophile (D703). Among the D703A, D703C, D703G, and D703S four mutants, D703S mutant was found to be the best α 1–3/4-fucosyltransferase and was used for the production of several fucosylated HMOS and derivatives using β -L-fucosyl fluoride (40 mM) and a suitable acceptor (100 mM) such as Lac, 2'FL, LNT, LNB, and LacNAc. HMOS and derivatives 3FL, LDFT, LNFP II, Le^a tetrasaccharide, and Le^x tetrasaccharide were obtained in 13%, 5.5%, 41%, 47%, and 55% yields, respectively, based on the β -L-fucosyl fluoride donor substrate used. Increasing the LNB concentration to 200 mM was able to improve the yield to 56%.³³⁰

5.9 LNFP I and LNDFH I

LNFP I (7% yield) and LNDFH I (6% yield) was synthesized from lactose using several glycosyltransferase-catalyzed reactions with the corresponding sugar nucleotides and a galactosidase-catalyzed reaction with a corresponding synthetic donor. LNT2 (44% yield) was initially synthesized from lactose and UDP-GlcNAc catalyzed by a β 1–3GlcNAcT that was partially purified from bovine blood. LNT (22% yield) was then produced from LNT2 and *ortho*-nitrophenyl- β -galactoside (Gal β oNP) using a recombinant *Bacillus circulans* β 1–3-galactosidase. The production of LNFP I (71% yield) was achieved from LNT and GDP-Fuc using a recombinant human α 1–2-fucosyltransferase 1 (FUT1) expressed in a baculovirus system. Finally, LNDFH I (85% yield) was produced from LNFP I and GDP-Fuc by a FUT3-catalyzed reaction using a commercial enzyme.²¹⁰

5.10 Other oligosaccharides

Gram-scale production of globotriose (Gb₃) and globotetraose (Gb₄) oligosaccharides was achieved using bacterial glycosyltransferases and sugar-nucleotides. Gb₃ trisaccharide (5 grams, 75% yield) was synthesized from lactose and UDP-galactose using *Neisseria gonorrhoeae* α 1–4-galactosyltransferase (NgLgtC). Gb₄ tetrasaccharide (1.5 grams, 60% yield) was synthesized from Gb₃ trisaccharide and UDP-GalNAc using *Neisseria gonorrhoeae* β 1–3-*N*-acetylgalactosaminyltransferase (NgLgtD).²⁶³

UDP-galactose (44 g/L) and globotriose Gal α 1–4Lac (188 g/L) were also produced using permeabilized *Corynebacterium ammoniagenes* cells (for the production of UTP from orotic acid) and *E. coli* cells engineered to overexpress UDP-Gal biosynthetic genes with or without *Neisseria gonorrhoeae* α 1–4-galactosyltransferase.³³¹ A whole cell approach using permeabilized *Corynebacterium ammoniagenes* cells (for the production of GTP from GMP) and *E. coli* cells engineered to overexpress *de novo* GDP-Fuc biosynthetic enzymes, phosphoglucomutase, phosphofructokinase, and *Helicobacter pylori* α 1–3-fucosyltransferase was also developed for the production of Lewis x trisaccharide (21 g/L in a 30 mL scale, purification yield 32%) from GMP, mannose, and *N*-acetyllactosamine (LacNAc).³³²

The engineered *E. coli* living cell strategy can be used for the synthesis of other HMOS by introducing plasmids for the expression of necessary glycosyltransferases and sugar nucleotide biosynthetic enzymes. For example, gram-scale synthesis of Lewis x tetrasaccharides Gal β 1–4(Fuc α 1–3)GlcNAc β 1–4GlcNAc (0.62 g) and Gal β 1–4(Fuc α 1–3)GlcNAc β 1–3Gal (1.84 g) was achieved by placing four *de novo* GDP-Fuc biosynthetic gene under *Plac* promoter without knocking out *wcaJ* involved in colanic acid synthesis or introducing RcsA (a positive regulator of the colanic acid operon).³³³ The engineered living cells strategy was also used to produce the oligosaccharide components of gangliosides GM2 and GM1, GalNAc β 1–4(Neu5Ac α 2–3)Lac and Gal β 1–3GalNAc β 1–4(Neu5Ac α 2–3)Lac,³³⁴ has been achieved.

6. Perspectives

Future efforts on HMOS analysis should be focused on elucidating the structures of more complex and longer chain HMOS including those with more than 14 monosaccharide residues that are not currently presented in TABLE IV. These more complex structures are unlikely the byproducts of biosynthesis of HMOS. Although less abundant, they could have significant biological functions. Profiling HMOS in a high-throughput format will also help to find correlation of disease states and the roles of certain populations of HMOS. Various enzyme-catalyzed synthetic methods that have been successfully used in production of relatively simple HMOS include glycosyltransferase-catalyzed reactions with or without co-factor recycling, sialidase and trans-glycosidase-catalyzed reactions, one-pot multienzyme (OPME) and sequential OPME systems, whole cell approaches, and living cell strategies. Such efforts, however, have not been applied for the production of more complex structures, especially the ones with branches. With the limited access to all enzymes needed, especially essential glycosyltransferases, the combination of chemical and enzymatic methods can be used. Such methods have been developed but have not been broadly used for the synthesis of HMOS. The strategy of chemoenzymatic synthesis of asymmetrically branched N-glycan structures^{335, 336} can be readily applicable for the synthesis of branched HMOS structures. Efficient purification methods for large scale production of HMOS either by chemical, enzymatic, or cell-based systems are also in a great demand. The availability of structurally defined more complex individual HMOS species will greatly facilitate their function studies and to explore their prebiotic and therapeutic potentials. Other than functional studies using pooled HMOS and individual pure synthetic HMOS, the synergistic effect of a mixture of two or more structurally defined HMOS should also be investigated as, most likely, a single compound will not provide the desired protection against multiple pathogens.¹⁵

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Abbreviations

ATP	adenine 5'-triphosphate
<i>B. breve</i>	<i>Bifidobacterium breve</i>
<i>B. bifidum</i>	<i>Bifidobacterium bifidum</i>
<i>B. infantis</i>	<i>Bifidobacterium longum</i> subsp. <i>infantis</i>
BiNahK	<i>B. infantis</i> N-acetylhexosamine-1-kinase
<i>B. longum</i>	<i>Bifidobacterium longum</i> subsp. <i>longum</i>
BLUSP	<i>B. longum</i> UDP-sugar synthase
CDP	cytidine 5'-diphosphate

CMP	cytidine 5'-monophosphate
CMP-Neu5Ac	cytidine 5'-monophosphate- <i>N</i> -acetylneuraminic acid
CstII	<i>Campylobacter jejuni</i> α 2-8-sialyltransferase II
CTP	cytidine 5'-triphosphate
DSLNT	disialyllacto- <i>N</i> -tetraose
EcGalK	<i>E. coli</i> K-12 galactose kinase
EcNanA	<i>E. coli</i> sialic acid aldolase
NmCSS	<i>Neisseria meningitidis</i> CMP-sialic acid synthetase
PmGlmU	<i>P. multocida</i> <i>N</i> -acetylglucosamine 1-phosphate uridylyltransferase
PmPpA	<i>P. multocida</i> inorganic pyrophosphatase
PmST	<i>Pasteurella multocida</i> α 2-3-sialyltransferase (GenBank accession number AAK02272)
PmST1	multifunctional <i>Pasteurella multocida</i> α 2-3- sialyltransferase 1
2'FL	2'-fucosyllactose
3FL	3-fucosyllactose
Fuc	fucose
FucT	fucosyltransferase
FUT2	human α 1-2-fucosyltransferase encoded by the Secretor (<i>Se</i>) gene
FUT3	human α 1-3/4-fucosyltransferase encoded by the Lewis (<i>Le</i>) gene
FutA	<i>Helicobacter pylori</i> strain 26695 α 1-3-fucosyltransferase
Gal	galactose
GalT	galactosyltransferase
GalNAc	<i>N</i> -acetylgalactosamine
GDP	guanidine 5'-diphosphate
GMP	guanidine 5'-monophosphate
GTP	guanidine 5'-triphosphate
Glc	glucose

GlcNAc	<i>N</i> -acetylglucosamine
GlcNAcT	<i>N</i> -acetylglucosaminyltransferase
GNB	galacto- <i>N</i> -biose
GnT-I	<i>N</i> -acetylglucosaminyltransferase I
GST	glutathione S-transferase
<i>E. coli</i>	<i>Escherichia coli</i>
HMOS	human milk oligosaccharides
HPAEC-PAD	high-pH anion-exchange chromatography with pulsed amperometric detection
HPLC	high performance liquid chromatography
Lac	lactose
LacNAc	<i>N</i> -acetylglucosamine
LacY	β -galactoside permease
LDFT	lactodifucotetraose
Le	Lewis
LNB	lacto- <i>N</i> -biose
LNDFH	lacto- <i>N</i> -difuco-hexoase
LNFP	lacto- <i>N</i> -fucopentaose
LNnD	lacto- <i>N</i> -neodecaose
LNnDFH	lacto- <i>N</i> -neodifucohexaose
LNnFP	lacto- <i>N</i> -neofucopentaose
LNnH	lacto- <i>N</i> -neohexaose
LNnT	lacto- <i>N</i> -neotetraose
LNT	lacto- <i>N</i> -tetraose
LNT2	lacto- <i>N</i> -triose II
LPS	lipopolysaccharides
LST	sialyllacto- <i>N</i> -tetraose
MOS	milk oligosaccharides
MS	mass spectrometry

NanT	Neu5Ac permease
NEC	necrotizing enterocolitis
Neu5Ac	<i>N</i> -acetylneuraminic acid, the most abundant sialic acid form in nature
Neu4,5Ac₂	4- <i>O</i> -acetyl- <i>N</i> -acetylneuraminic acid
NK	natural killer
Nma2-3ST	<i>Neisseria meningitidis</i> α 2-3-sialyltransferase
NmLgtA	<i>Neisseria meningitidis</i> β 1-3- <i>N</i> -acetylglucosaminyltransferase
NmLgtB	<i>Neisseria meningitidis</i> β 1-4-galactosyltransferase
NMR	nuclear magnetic resonance spectroscopy
OPME	one-pot multienzyme
Pd2,6ST	<i>Photobacterium damsela</i> α 2-6-sialyltransferase
Psp2,6ST	<i>Photobacterium sp.</i> JT-ISH-224 α 2-6-sialyltransferase
UTP	uridine 5'-triphosphate
Se	Secretor
3'S-3FL	3'-sialyl-3-fucosyllactose
SiaT	sialyltransferase
3'SL	3'-sialyllactose
6'SL	6'-sialyllactose
3'SLN	3'-sialyl- <i>N</i> -acetyllactosamine
6'SLN	6'-sialyl- <i>N</i> -acetyllactosamine

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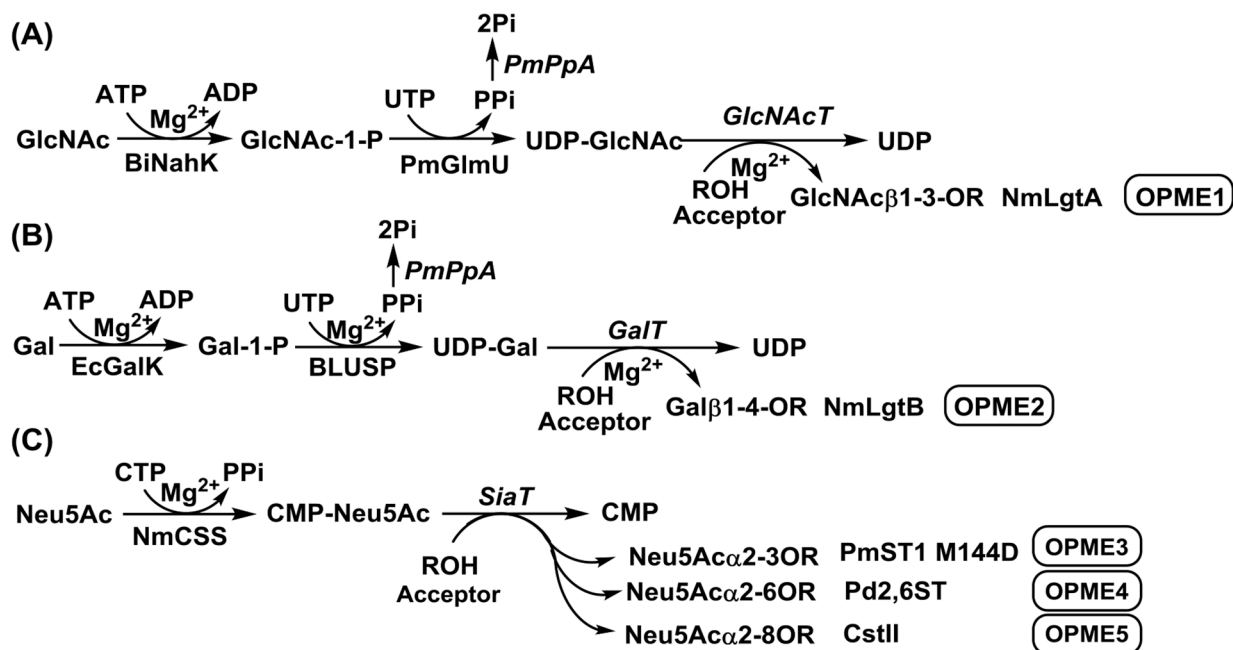
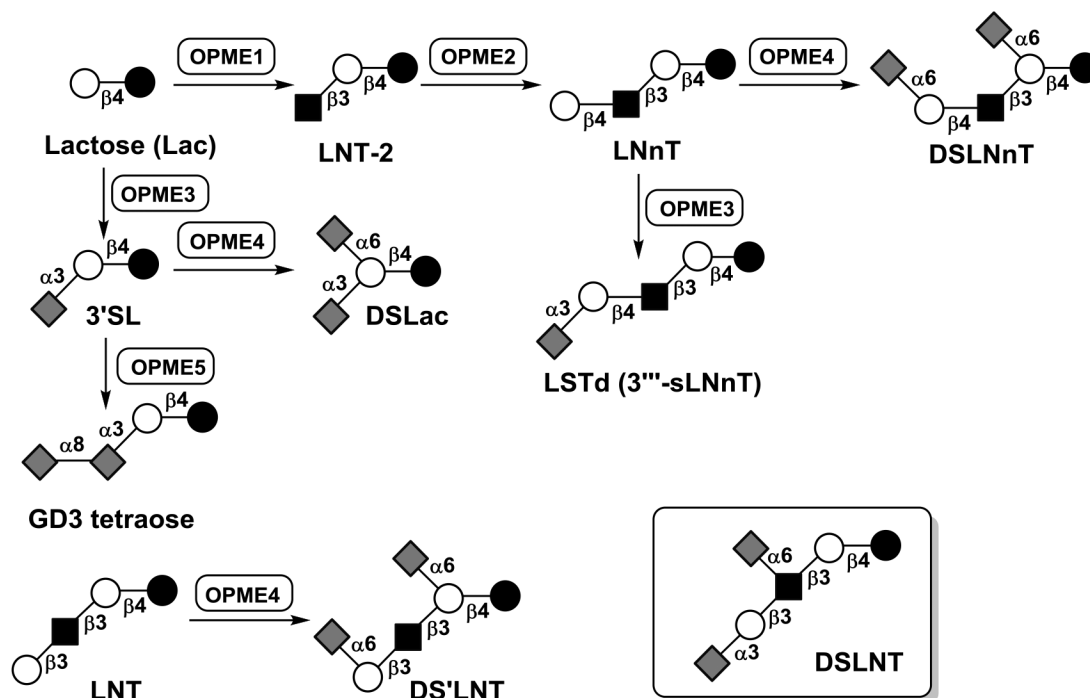


FIG. 1.

One-pot multienzyme (OPME) GlcNAc (A), Gal (B), and Neu5Ac (C) activation and transfer systems.^{269, 270} Enzyme abbreviations: BiNahK, *B. infantis* *N*-acetylhexosamine-1-kinase;³¹⁷ PmGlmU, *P. multocida* *N*-acetylglucosamine 1-phosphate uridylyltransferase;³¹⁸ PmPpA, *P. multocida* inorganic pyrophosphatase;³¹⁸ GlcNAcT, *N*-acetylglucosaminyltransferase; NmLgtA, *Neisseria meningitidis* β1–3-*N*-acetylglucosaminyltransferase;³¹⁹ EcGalK, *E. coli* K-12 galactose kinase;³²⁰ BLUSP: *B. longum* UDP-sugar synthase;³²⁰ GalT, galactosyltransferase; NmLgtB, *Neisseria meningitidis* β1–4-galactosyltransferase;³²¹ NmCSS, *Neisseria meningitidis* CMP-sialic acid synthetase;²⁹³ SiaT, sialyltransferases; PmST1 M144D, *Pasteurella multocida* α2–3-sialyltransferase 1 M144D mutant;²⁹⁶ Pd2,6ST, *Photobacterium damsela* α2–6-sialyltransferase;^{309, 322} CstII, *Campylobacter jejuni* α2–8-sialyltransferase II.^{323, 324}

**FIG. 2.**

OPME and sequential OPME systems for the synthesis of disialyl oligosaccharides including DSLNnT, GD3 tetraose, DSLac, DS'LNT, and a monosialylpentaose LSTd (3'''-sLNnT).²⁶⁹ The structure of DSLNT found in human milk is shown for comparison purpose.

TABLE I.

Major monosaccharide building blocks for HMOS.


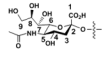

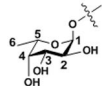

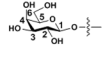

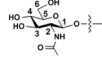

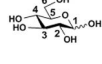
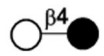
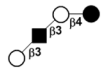
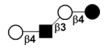
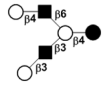

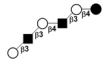
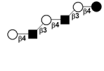


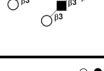
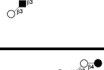

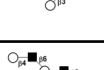
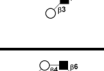
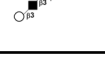

HMOS monosaccharide building blocks	Abbreviations	Symbols	Structures	Glycosidic Linkages in HMOS
<i>N</i> -Acetylneuraminic acid	Neu5Ac			α -linkages
Fucose	Fuc			α -linkages
Galactose	Gal			β -linkages
<i>N</i> -Acetylglucosamine	GlcNAc			β -linkages
Glucose	Glc			None (at the reducing end, a mix of α and β configurations at the anomeric carbon)

TABLE II.

Lactose and neutral non-fucosylated HMOS that can serve as the core structures for other HMOS.^{28, 32–34}

Core #	Lactose and HMOS core structures	Abbreviations	Symbols	Ref.
I	Lactose (not considered an HMOS itself)	Lac		49
II	Lacto- <i>N</i> -tetraose	LNT		24
III	Lacto- <i>N</i> -neotetraose	LNTn		50
IV	Lacto- <i>N</i> -hexaose	LNH		51
V	Lacto- <i>N</i> -neohexaose	LNTnH		52
VI	<i>para</i> -Lacto- <i>N</i> -hexaose	<i>p</i> LNH		53
VII	<i>para</i> -Lacto- <i>N</i> -neohexaose	<i>p</i> LNTnH		53
VIII	Lacto- <i>N</i> -octaose	LNO		54
IX	Lacto- <i>N</i> -neooctaose	LNTnO		54
X	<i>iso</i> -Lacto- <i>N</i> -octaose	<i>i</i> LNO		55
XI	<i>para</i> -Lacto- <i>N</i> -octaose	<i>p</i> LNO		56
XII	<i>para</i> -Lacto- <i>N</i> -neooctaose	<i>p</i> LNTnO		57
XIII	Lacto- <i>N</i> -decaose	LND		58
XIV	Lacto- <i>N</i> -neodecaose	LNTnD		58
XV				33

Core #	Lactose and HMOS core structures	Abbreviations	Symbols	Ref.
XVI				59

Symbols and abbreviations: ○ galactose (Gal), ■ *N*-acetylglucosamine (GlcNAc), ● glucose (Glc).

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










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



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TABLE III.

Twelve glycosidic linkages that constitute diverse HMOS.^{33, 34}

Glycosidic linkages	Abbreviations	Symbol
Galactosidic bonds	Gal β 1-4Glc	
	Gal β 1-3GlcNAc	
	Gal β 1-4GlcNAc	
N-acetyl-glucosaminidic bond	GlcNAc β 1-3Gal	
	GlcNAc β 1-6Gal	
Fucosidic bond	Fuca.1-2Gal	
	Fuca.1-3Glc	
	Fuca.1-3GlcNAc	
	Fuca.1-4GlcNAc	
Sialidic bond	Neu5Aca.2-3Gal	
	Neu5Aca.2-6Gal	

Glycosidic linkages	Abbreviations	Symbol
	Neu5Ac α 2-6GlcNAc	

Symbols and abbreviations:  *N*-acetylneuraminic acid (Neu5Ac),  fucose (Fuc),  galactose (Gal),  *N*-acetylglucosamine (GlcNAc),  glucose (Glc).

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





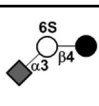

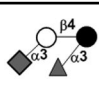

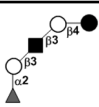
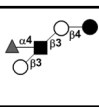

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








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TABLE IV.

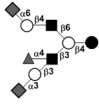









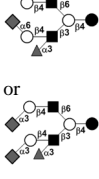



Structures of HMOS grouped by their core structures.^{28, 32–34, 61}

Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
I	Lactose (not considered as HMOS itself)	Lac		49
		LNTri II		76
	^a ₂ -Fucosyllactose	2'FL		25
	3-Fucosyllactose	3FL		27
	^a Lactodifucotetraose	LDFT		77
	3'-Sialyllactose	3'SL		78
	6'-Sulfo-3'-sialyllactose	6'-Sulfo-3'SL		64
	6'-Sialyllactose	6'SL		79
	3'-Sialyl-3-fucosyllactose	3'S3FL		80
II	Lacto- <i>N</i> -tetraose	LNT		24
	^a Lacto- <i>N</i> -fucopentaose I	LNFP I		26
	^b Lacto- <i>N</i> -fucopentaose II	LNFP II		81
	Lacto- <i>N</i> -fucopentaose V	LNFP V		82















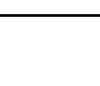
Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
	^{a, b} Lacto- <i>N</i> -difuco-hexaose I	LNDFH I		83
	^b Lacto- <i>N</i> -difuco-hexaose II	LNDFH II		84
	Sialyllacto- <i>N</i> -tetraose a	LSTa		66
	Sialyllacto- <i>N</i> -tetraose b	LSTb		66
	Disialyllacto- <i>N</i> -tetraose	DSLNT		85
	Sialylfucosyllacto- <i>N</i> -tetraose	S-LNF II or F-LSTa		86
	Fucosylsialyllacto- <i>N</i> -tetraose	S-LNF I or F-LSTb		86
	Fucosyldisialyllacto- <i>N</i> -tetraose	DS-LNF II or FDS-LNT I		87
	Disialylfucosyllacto- <i>N</i> -tetraose	DS-LNF V or FDS-LNT II		87
III	Lacto- <i>N</i> -neotetraose	LNnT		50
	Lacto- <i>N</i> -fucopentaose III	LNFP III		88
	Lacto- <i>N</i> -neofucopentaose V	LNnFP V		89
	Lacto- <i>N</i> -neodifucohexaose II	LNnDFH II (LNDFH III)		67
	Sialyllacto- <i>N</i> -neotetraose c	LSTc		90
	Fucosylsialyllacto- <i>N</i> -neotetraose	F-LSTc		91
















Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
IV	Lacto- <i>N</i> -hexaose	LNH		51
	Fucosyllacto- <i>N</i> -hexaose I	FLNH I		92
	Fucosyllacto- <i>N</i> -hexaose II	FLNH II		93
	^c 4120a			33
	Difucosyllacto- <i>N</i> -hexaose a	DF-LNH a		92
	Difucosyllacto- <i>N</i> -hexaose b	DF-LNH b		51
	Difucosyllacto- <i>N</i> -hexaose c	DF-LNH c		33
	Trifucosyllacto- <i>N</i> -hexaose	TF-LNH		55
	Sialyllacto- <i>N</i> -hexaose	S-LNH		51
	^c 4021a			34
	Disialyllacto- <i>N</i> -hexaose I	DS-LNH I		94

Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
	Disialyllacto- <i>N</i> -hexaose II	DS-LNH II		94
	Trisialyllacto- <i>N</i> -hexaose	TS-LNH		95
	Fucosylsialyllacto- <i>N</i> -hexaose	FS-LNH		92
	Fucosylsialyllacto- <i>N</i> -hexaose I	FS-LNH I		96
	Fucosylsialyllacto- <i>N</i> -hexaose II	FS-LNH II		96
	Fucosylsialyllacto- <i>N</i> -hexaose III	FS-LNH III		96
	Fucosylsialyllacto- <i>N</i> -hexaose IV	FS-LNH IV		97
	Difucosylsialyllacto- <i>N</i> -hexaose I	DFS-LNH I		96
	Difucosylsialyllacto- <i>N</i> -hexaose II	DFS-LNH II		97
	Fucosyldisialyllacto- <i>N</i> -hexaose I	FDS-LNH I		98
	Fucosyldisialyllacto- <i>N</i> -hexaose II	FDS-LNH II		98

Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
	Fucosyl disialyllacto- <i>N</i> -hexaose III	FDS-LNH III		97
V	Lacto- <i>N</i> -neohexaose	LNnH		52
	Fucosyllacto- <i>N</i> -neohexaose	F-LNnH		52
	Difucosyllacto- <i>N</i> -neohexaose	DF-LNnH		56
	Sialyllacto- <i>N</i> -neohexaose I	S-LNnH I		51
	Sialyllacto- <i>N</i> -neohexaose II	S-LNnH II		80
	Disialyllacto- <i>N</i> -neohexaose	DS-LNnH		87
	Fucosylsialyllacto- <i>N</i> -neohexaose I	FS-LNnH I		80
	Fucosylsialyllacto- <i>N</i> -neohexaose II	FS-LNnH II		52
	Difucosylsialyllacto- <i>N</i> -neohexaose	DFS-LNnH		80
	Fucosyl disialyllacto- <i>N</i> -neohexaose	FDS-LNnH	 Or 	98
VI	<i>para</i> -Lacto- <i>N</i> -hexaose	<i>p</i> LNH		53
	Fucosyl- <i>para</i> -lacto- <i>N</i> -hexaose I	F- <i>p</i> LNH I		99

Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
	Fucosyl- <i>para</i> -lacto- <i>N</i> -hexaose II	F- <i>p</i> LNH II		100
	Fucosyl- <i>para</i> -lacto- <i>N</i> -hexaose IV	F- <i>p</i> LNH IV		100
	Difucosyl- <i>para</i> -lacto- <i>N</i> -hexaose	DF- <i>p</i> LNH		53
	Trifucosyl- <i>para</i> -lacto- <i>N</i> -hexaose I	TF- <i>p</i> LNH I		101
	Trifucosyl- <i>para</i> -lacto- <i>N</i> -hexaose II	TF- <i>p</i> LNH II		100
	Difucosyl- <i>para</i> -lacto- <i>N</i> -hexaose sulfate I	DF- <i>p</i> LNH sulfate I		65
	Difucosyl- <i>para</i> -lacto- <i>N</i> -hexaose sulfate II	DF- <i>p</i> LNH sulfate II		65
	Difucosyl- <i>para</i> -lacto- <i>N</i> -hexaose sulfate III	DF- <i>p</i> LNH sulfate III		65
VII	<i>para</i> -Lacto- <i>N</i> -neohexaose	<i>p</i> LNnH		53
	Fucosyl- <i>para</i> -lacto- <i>N</i> -neo-hexaose	F- <i>p</i> LNnH or IFLNH III		99
	Difucosyl- <i>para</i> -lacto- <i>N</i> -neo-hexaose	DF- <i>p</i> LNnH		53
	Trifucosyl- <i>para</i> -lacto- <i>N</i> -neo-hexaose	TF- <i>p</i> LNnH		100
	^c 4021b			34
	^c 4121a			34
	^c 4121b			34
VIII	Lacto- <i>N</i> -octaose	LNO		54


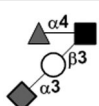
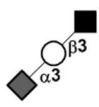





Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
	^c 5130c			33
	^c 5130b			33
	Fucosyllacto- <i>N</i> -octaose	F-LNO		102
	Difucosyllacto- <i>N</i> -octaose I	DF-LNO I		54
	Difucosyllacto- <i>N</i> -octaose II	DF-LNO II		54
	Trifucosyllacto- <i>N</i> -octaose	TF-LNO		54
	^c 5031a			34
	Fucosylsialyllacto- <i>N</i> -octaose	FS-LNO		103
	^c 5131a			34
	^c 5231a			34
	^c 5231b			34
	Difucosylsialyllacto- <i>N</i> -octaose	DFS-LNO		104
	^c 5331a			34
IX	Lacto- <i>N</i> -neooctaose	LNnO		54
	Fucosyllacto- <i>N</i> -neooctaose	F-LNnO		54

Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
	Difucosyllacto- <i>N</i> -neo-octaose I	DF-LNnO I		54
	Difucosyllacto- <i>N</i> -neo-octaose II	DF-LNnO II		54
	Trifucosyllacto- <i>N</i> -neo-octaose I	TF-LNnO I		54, 55
	Trifucosyllacto- <i>N</i> -neo-octaose II	TF-LNnO II		55
X	<i>iso</i> -Lacto- <i>N</i> -octaose	λ LNO		55
	^c _{5130a} Fucosyl- <i>iso</i> -Lacto- <i>N</i> -octaose	F- λ LNO		33, 105
	Difucosyl- <i>iso</i> -lacto- <i>N</i> -octaose I	DF- λ LNO I		106
	Difucosyl- <i>iso</i> -lacto- <i>N</i> -octaose II	DF- λ LNO II		106
	^c _{5230a}			33
	Trifucosyl- <i>iso</i> -lacto- <i>N</i> -octaose I	TF- λ LNO I		55
	Trifucosyl- <i>iso</i> -lacto- <i>N</i> -octaose II	TF- λ LNO II		56, 105
	^c _{5330a}			33
	Tetra-fucosyl- <i>iso</i> -lacto- <i>N</i> -octaose	TetraF- λ LNO		56
	penta-fucosyl- <i>iso</i> -lacto- <i>N</i> -octaose	PentaF- λ LNO		56
	Fucosylsialyl- <i>iso</i> -lacto- <i>N</i> -octaose	FS- λ LNO		104

Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
	Difucosylsialyl- <i>iso</i> -lacto- <i>N</i> -octaose I	DFS- <i>l</i> LNO I		104
	Difucosylsialyl- <i>iso</i> -lacto- <i>N</i> -octaose II	DFS- <i>l</i> LNO II		104
	Trifucosylsialyl- <i>iso</i> -lacto- <i>N</i> -octaose	TFS- <i>l</i> LNO		94
XI	<i>para</i> -Lacto- <i>N</i> -octaose	<i>p</i> LNO		56
	Tetrafucosyl- <i>para</i> -lacto- <i>N</i> -octaose	TetraF- <i>p</i> LNO		56
XII	<i>para</i> -Lacto- <i>N</i> -neo-octaose	<i>p</i> LNO		57
XIII	Lacto- <i>N</i> -decaose	LND		58, 107
	Fucosyl-lacto- <i>N</i> -decaose I	F-LND I		107
	Difucosyl-lacto- <i>N</i> -decaose I	DF-LND I		58
	Difucosyl-lacto- <i>N</i> -decaose II	DF-LND II		58
	Difucosyl-lacto- <i>N</i> -decaose III	DF-LND III		58
	Difucosyl-lacto- <i>N</i> -decaose IV	DF-LND IV		58
	Difucosyl-lacto- <i>N</i> -decaose V	DF-LND V		58
	Difucosyl-lacto- <i>N</i> -decaose VI	DF-LND VI		58

Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
				58
				58
	Trifucosyl-lacto- <i>N</i> -decaose I	TriF-LND I		58
	Trifucosyl-lacto- <i>N</i> -decaose II	TriF-LND II		58
	Trifucosyl-lacto- <i>N</i> -decaose III	TriF-LND III		58
	Trifucosyl-lacto- <i>N</i> -decaose IV	TriF-LND IV		58
				58
				58
	^c 6340a			33
	Tetrafucosyl-lacto- <i>N</i> -decaose I	TetraF-LND I		58
	Tetrafucosyl-lacto- <i>N</i> -decaose II	TetraF-LND II		58
	Tetrafucosyl-lacto- <i>N</i> -decaose III	TetraF-LND III		58
				58

Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
XIV	Lacto- <i>N</i> -neodecaose	LNdD		58
	Fucosyllacto- <i>N</i> -neodecaose I	F-LNdD I		58
	Fucosyllacto- <i>N</i> -neodecaose II	F-LNdD II		58
	Difucosyllacto- <i>N</i> -neodecaose	DF-LNdD		58
	^c 6041a			34
XV				33
XVI				59
				59
				59
XVII Deviant structures	A antigen-tetrasaccharide	A-Tri		62, 108
	A antigen-pentasaccharide	A-Penta		62, 108-110
	A antigen-hexasaccharide	A-Hexa		62
	A antigen-heptasaccharide	A-Hepta		62, 108, 109

Core #	Lactose and HMOS	Abbreviations	Symbols	Ref.
				111
	3'-Sialyl Lewis a	3'SLe ^a		111
	3'-Sialyl-N-acetylglucosamine	3'SLN		34
	6'-Sialyl-N-acetylglucosamine	6'SLN		66
	Fucosylsialyl-novo-lacto-N-pentaose I	FS-novo-LNP I		96
	3'-Galactosyllactose	β 3'GL		67
	4'-Galactosyllactose	β 4'GL		68
	6'-Galactosyllactose	β 6'GL		69

Symbols and abbreviations: \blacklozenge *N*-acetylneuraminic acid (Neu5Ac), \blacktriangle fucose (Fuc), \circ galactose (Gal), \blacksquare *N*-acetylglucosamine (GlcNAc), \bullet glucose (Glc), \square *N*-acetylgalactosamine (GalNAc).

^aMissing in the milk of Le^{a+b-} non-secretors.⁷³

^bMissing in the milk of Lewis negative (Le^{a-b-}) individuals.⁷⁴

^cIndicate the number of Hexose, Fucose, HexNAc, and Neu5Ac in the oligosaccharide.^{33, 34}