Special Article

Overcoming the limited availability of human milk oligosaccharides: challenges and opportunities for research and application

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Human milk oligosaccharides (HMOs) are complex sugars highly abundant in human milk but currently not present in infant formula. Rapidly accumulating evidence from in vitro and in vivo studies, combined with epidemiological associations and correlations, suggests that HMOs benefit infants through multiple mechanisms and in a variety of clinical contexts. Until recently, however, research on HMOs has been limited by an insufficient availability of HMOs. Most HMOs are found uniquely in human milk, and thus far it has been prohibitively tedious and expensive to isolate and synthesize them. This article reviews new strategies to overcome this lack of availability by generating HMOs through chemoenzymatic synthesis, microbial metabolic engineering, and isolation from human donor milk or dairy streams. Each approach has its advantages and comes with its own challenges, but combining the different methods and acknowledging their limitations creates new opportunities for research and application with the goal of improving maternal and infant health.

INTRODUCTION

The existence of complex soluble oligosaccharides in human milk was first reported in the 19th century during investigations to determine why breastfed infants had significantly higher survival rates than nonbreastfed infants at that time.[1–3] Human milk oligosaccharides (HMOs) consist of 5 monosaccharide building blocks: glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid (Figure 1A). Human milk oligosaccharides carry lactose (Galβ1–4Glc) at the reducing end, which can be elongated by the addition of β1–3- or β1–6-linked lacto-*N*-biose (Galβ1–3GlcNAc-, type 1 chain) or *N*-acetyllactosamine (Galβ1–4GlcNAc-, type 2 chain). Lactose or the elongated oligosaccharide chain can be fucosylated in α1–2, α1–3, or α1–4 linkage and/or sialylated in α2–3 or α2–6 linkage. More than 150 different HMOs have been identified thus far.

Colostrum, the thick, yellowish fluid secreted by the mammary gland a few days before and after parturition, contains HMOs in concentrations as high as 20 to

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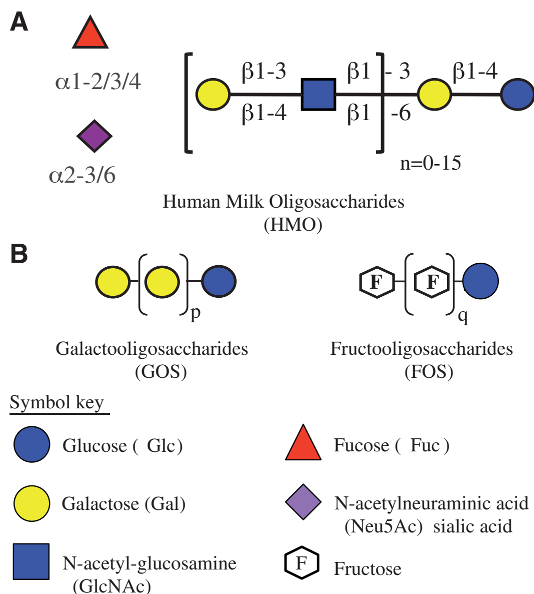

Figure 1 **Symbol representation of (A) human milk oligosaccharides (HMOs) and (B) nonhuman milk oligosaccharides currently added to infant formula.** (A) HMOs contain lactose at the reducing end and can be elongated by up to 15 disaccharide units of either lacto-*N*-biose (Gal *β*1–3-linked to GlcNAc) or *N*-acetyllactosamine (Gal *β*1–4-linked to GlcNAc). In addition, HMO can be fucosylated in α1–2-, α1–3-, or α1–4-linkage or/and sialylated in α2–3- or α2–6-linkage. (B) Galactooligosaccharides (GOS), which are not present in human milk, are oligomers of galactose that often carry glucose at the reducing end. Fructooligosaccharides (FOS), which are also not present in human milk, are oligomers of fructose that also carry glucose at the reducing end. Fructose itself is not a building block of the oligosaccharides naturally found in human milk. GOS and FOS are currently added to some infant formulas but are structurally very different from human milk oligosaccharides.

25 g/L.[4,5] As milk production matures, HMO concentrations decline to 5 to 20 g/L,[4–8] which often exceeds the concentration of total milk protein.

To date, research on HMOs has demonstrated a wide range of possible benefits.[2] In vitro and in vivo work continues to show that HMOs serve as prebiotics that help shape microbiota composition, act as soluble decoy receptors to block pathogens from attaching to host cells and causing disease, and have direct antimicrobial effects to halt pathogen growth and survival. In addition, HMOs may modulate epithelial and immune cell responses and contribute to the maturation of the immune system.

Epidemiological studies have correlated low levels of a specific HMO called 2′-fucosyllactose (2′-FL) in mother's milk with increased susceptibility to *Campylobacter jejuni* infection in a cohort of Mexican infants.[9] High levels of 2'-FL and other oligosaccharides in the mother's milk correlate with decreased infant mortality.[10] In addition, HMO composition was associated with body composition during the first 6 months of life in the United States[11] and with growth and body composition in undernourished infants in Malawi.[12]

The vast body of in vitro and in vivo studies, combined with epidemiological associations and correlations, supports the need for further preclinical studies and randomized controlled trials to fully elucidate the potential health benefits of HMOs. Until recently, however, research on HMOs has been limited by the insufficient availability of HMOs. To fill that void, alternative oligosaccharides not present in human milk have been used in many infant formulas. Technological advances in oligosaccharide isolation and purification procedures, chemoenzymatic synthesis, and microbial bioengineering now offer several different opportunities to produce HMOs for research and commercial application.

The challenge: limited availability of HMOs for study of potential benefits

Most HMOs are found only in human milk. Oligosaccharide concentrations in the milk of most other mammals are much lower, and the structures are less complex and less diverse.[13,14] Even the milk of nonhuman primates, the closest relatives of humans, differs widely with respect to oligosaccharide amount and structural diversity.[15] Why oligosaccharide amount and composition are so unique to human milk remains unknown, but the uniqueness creates major challenges to investigate the biological relevance of HMOs.

First, results obtained from preclinical models often do not translate to humans because animals assessed in these models do not produce HMOs, are usually not exposed to HMOs, and are likely to respond to HMOs very differently than humans. For example, HMOs are partially absorbed in the intestine and appear in the systemic circulation as well as the urine of breastfed infants.[16–18] In contrast, most orally administered HMOs given to neonatal rats and mice, often used in preclinical models, are not absorbed,[19] likely because these animals are usually not exposed to HMOs and therefore have no need to develop intestinal absorption mechanisms. Similarly, absorption, distribution, metabolism, and excretion data are not available for other preclinical models, which makes it very difficult to study HMO effects that are potentially occurring in tissues and organs other then the gut.

Second, the unique presence of HMOs in human milk has resulted in a major bottleneck in the availability of these oligosaccharides for research purposes, not to mention a lack of large-scale availability for clinical intervention studies and commercial products. Not too

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long ago, small amounts of HMOs had to be isolated from donated human milk, which was extremely tedious and labor intensive. Small amounts of individual human milk tri- or tetrasaccharides were generated chemically, but synthesis took months and was very expensive. On one hand, the limited availability of HMOs made it difficult to study HMO effects, even in small-volume tissue culture assays and certainly in preclinical models with long-term HMO exposure. Randomized intervention studies to assess safety and efficacy in humans were not feasible at all. On the other hand, the lack of robust research data on beneficial HMO effects made it very difficult to justify the investment of time and resources in HMO isolation or synthesis. For years, this vicious cycle placed a heavy burden on HMO research.

Temporary alternative: use of nonhuman oligosaccharides

The inability to produce genuine HMOs on a sufficient scale led to the use of alternative complex oligosaccharides such as fructans and galactooligosaccharides, which were available from more abundant and easily accessible sources (Figure 1B). In particular, the availability of these alternative oligosaccharides in large amounts at reasonable prices led to their inclusion in most infant formulas from the 1990s onward. Galactooligosaccharides have a chain length of 3 to 8 galactose units from a terminal glucose and are synthesized from lactose by enzymatic transgalactosylation using β-galactosidase.[20] Fructans, such as inulin and its partial hydrolysates, known as fructooligosaccharides,[21] can be isolated in large quantities from chicory roots. Neither fructooligosaccharides nor galactooligosaccharides are structurally similar to HMOs; indeed, they are not found in the milk of any domestic mammal. Given that the health benefits of these substitutes are unclear, the European Food Safety Authority (EFSA) recently classified them as unnecessary ingredients in infant formula.

The solution(s): technological advances that increase the availability of HMOs

Recent advances in technologies for isolating or synthesizing HMOs created a breakthrough in HMO research. The amount and diversity of available HMOs increased and facilitated in vitro and in vivo studies that shed light on potential HMO benefits. The data generated by these studies spurred new academic and commercial interest, encouraged the investment of additional resources, and further accelerated the development of technologies to isolate and synthesize HMOs. The different approaches currently used to generate HMOs for research and commercial application are outlined in the sections below. Advantages and challenges for each of the different approaches are summarized in Table 1.

Purification of oligosaccharides from human donor milk and dairy streams. While human milk would be the ideal source of HMOs for isolation and purification, the lack of access to large volumes of human milk is currently a limitation. Oligosaccharides that are similar to – but simpler than – those in human milk have been described in bovine milk, and more than 50 different structures have been identified to date.[14,22–24] Although the characterization of bovine milk oligosaccharides has been a focus of research for several years, the analysis of these oligosaccharides remains challenging because of their overall complexity and their low concentration in bovine milk. Recent advances in analytical instrumentation allow the detailed annotation of oligosaccharides not previously identified in bovine milk. Understanding the extent and types of oligosaccharides present in bovine milk is an important step toward determining the feasibility of developing commercial sources of these oligosaccharides. Bovine milk oligosaccharides contain the key monosaccharides found in HMOs; however, the concentration of bovine milk oligosaccharides is much lower than that of HMOs: 0.7 to 1 g/L in bovine colostrum, and just trace amounts in mature bovine milk.

Colostrum is especially rich in sialylated oligosaccharides and is potentially suitable for isolating individual oligosaccharides for addition to infant formulas, but its availability is so limited that it is impossible to obtain the amounts required for industrial use. Because mature bovine milk contains lower amounts of sialylated oligosaccharides, there is a need to identify suitable methods for concentrating these valuable dietary components.

Whey is the thin, watery part of milk that separates from the solid part (curds) of milk following acid or enzymatic coagulation. Whey has traditionally been considered a byproduct of cheese and casein manufacture. Whey production has grown enormously in recent decades and, along with cheese production, will continue to grow. Whey permeate is the byproduct obtained when cheese whey is passed through an ultrafiltration membrane to concentrate whey protein. Whey proteins are retained by the membrane, whereas smaller molecules such as lactose and salts pass through the membrane, forming the permeate. Although the protein concentrate can be used in food products, the permeate has thus far been of little commercial value. Worldwide, the whey industry is facing the problem of disposal and utilization of surplus whey permeate: global production of whey protein amounted to 240 million metric tons in

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Table 1 **Advantages and challenges of different methods to generate human milk oligosaccharides (HMOs)**

Method	Advantages	Challenges
Isolation from human milk	Contains "authentic" HMOs	Scale-up somewhat limited because of limited availability of human donor milk
Isolation from dairy streams	Several oligosaccharides are identical to those in human milk. Scale-up possible because of large volumes available from dairy streams. Bovine milk oligosaccharides can be used as precursors for chemoenzymatic synthesis	Oligosaccharide concentrations much lower in bovine milk than in human milk. Not all HMOs are present in bovine milk (eg, only trace amounts of fucosylated oligosaccharides present in bovine milk). Bovine milk contains several nonhuman oligosaccharides (safety?)
Chemoenzymatic synthesis	Allows synthesis of clearly defined structures and HMO libraries, which are especially valuable for investigating structure–function relationships	Scale-up challenging
Bioengineering of microorganisms	Scale-up possible and almost unlimited	Produces a limited set of HMOs at a time. Use of genetically engineered organisms

2014, and production is expected to increase 3.5% annually.[25]

The recent discovery that whey permeate contains oligosaccharides presents a promising alternative for industrial production of oligosaccharides analogous to those in human milk. It has been shown that milk oligosaccharides pass through the ultrafiltration membranes commonly used by the dairy industry, ending up in the whey permeate.[26] Utilizing specific whey fractions is an attractive idea based on the wide availability and low cost of whey permeate as compared with liquid bovine milk. This approach is attractive to the dairy industry because it is easily scalable: it takes the byproduct of the protein ultrafiltration process and repurposes it using the same equipment, requiring only a change in the membrane pore size to isolate the oligosaccharide fraction.

Considering that the US dairy industry produces 1.1 billion liters of whey per year, and assuming an average bovine milk oligosaccharide concentration of approximately 200 mg/L, theoretically 220 000 kg of bovine milk oligosaccharides could be extracted every year. The biggest challenge associated with the development of large-scale or industrial processes to recover oligosaccharides from mammalian milks is to achieve a high degree of purity. More specifically, the challenge is to remove simple sugars – such as lactose, glucose, and galactose – that lack prebiotic activities and might have adverse effects, while simultaneously maximizing product recovery. Recent work by Thum et al.[27] demonstrates the use of lactose hydrolysis combined with membrane filtration for the recovery of oligosaccharides from goat milk. Similar methods could be adapted to the extraction of bovine milk oligosaccharides from dairy streams.

The main difference between HMOs and bovine milk oligosaccharides is that human milk contains predominantly neutral (fucosylated) oligosaccharides, whereas bovine milk contains primarily sialylated oligosaccharides. Solutions to overcome these structural differences include the utilization of bovine milk oligosaccharides from dairy streams as precursors for chemical and/or enzymatic synthesis to generate extended and fucosylated oligosaccharides that structurally and functionally more closely resemble HMOs.

Strategies to access HMOs via chemoenzymatic synthesis. Recent advances in the development of new synthetic methods (including automated oligosaccharide production) and in the production of key enzymes in active form promise to increase the availability of structurally well-defined HMOs in the coming years.[28–32] Whereas automated synthesis methods promise the facile synthesis of libraries of natural HMOs and their analogs just as automated peptide synthesis has done for the study of peptides, synthetic approaches specifically to HMOs to date have focused largely on chemoenzymatic processes. Several key enzymes involved in the synthesis of linkages associated with HMO structures have been successfully produced in recombinant form and employed in the synthesis of milligram quantities of certain oligosaccharide structures, as outlined below. These enzymes have also been shown to be active in extending the sugar chains of chemically synthesized substrates, thereby expanding the potential range of targets that are accessible.

Chemoenzymatic approaches begin with a chemically created target compound that can be further modified by one or more glyco-related enzymes. If an acceptor is initially synthesized, its design allows specific saccharide units to be recognized for further enzymatic derivatization.

To date, the literature contains only a few examples of HMO synthesis accomplished by means of a

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chemoenzymatic approach. In 2003, Furuike et al.[33] chemically synthesized a branched tetrasaccharide containing an unnatural 1,6-anhydro-β-*N*-acetyl lactosamine-reducing terminus with Gal extended by a β1–3 and β1–6 GlcNAc. The two GlcNAc units were recognized by the bovine β1–4 galactosyl transferase, which resulted in the installation of two Gal monosaccharides to provide a pseudo lacto-*N*-neohexaose. Further elongation with α2–3-sialyl transferase produced the bis-sialylated branched octasaccharide target.

In a similar fashion, Fair et al.[34] constructed lacto-*N*-neotetraose and para-lacto-*N*-neohexaose using an automated solid phase strategy. The final step in the synthesis required the installation of an α2–3 sialic acid cap, and this proved inefficient using standard chemical glycosylation strategies (<20% yield). To circumvent this setback, a sialyl transferase from *Pasteurella multocida*[35] was used to install sialic acid, resulting in yields in excess of 75%, with absolute regioselectivity and stereospecificity.

Recently, Yao et al.[36] took advantage of D-galactosyl-β1-3-D-hexosamine phosphorylase (BiGalHexNAc-P), an enzyme involved in the degradation of lacto-*N*-biose, which is derived from *Bifidobacterium infantis* (Figure 2).[37] Because of the reversible nature of this enzyme, α-Gal-1-phosphate could be glycosylated to an ethyl-β-D-2-deoxy-trifluoroacetamido-1-thio-glucoside acceptor. The protected lacto-*N*-biose disaccharide was then coupled to a lactose acceptor bearing a free 3′-hydroxyl under standard glycosylation conditions. Base treatment of the resulting tetrasaccharide provided lacto-*N*-tetraose in high yield. Subsequent α2–3-sialylation produced sialyllacto-*N*-tetraose (LST)-a in excess of 20 mg.

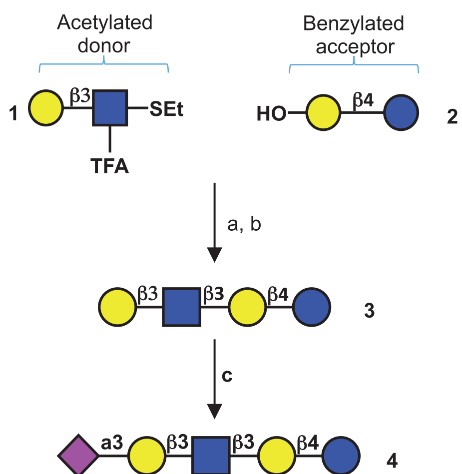

Figure 2 **Chemoenzymatic synthesis of sialyllacto-*N*-tetraose (LST-a).** (A) Chemical glycosylation. (B) Removal of all ester- and acetamido-protecting groups. (C) α2–3-sialyltransferase.

Chen et al.[38] describe a robust one-pot multienzyme approach that starts with a chemically created lactoside bearing an azido alkyl linker. Using a collection of kinases, epimerases, uridylyltransferases, and glycosyl transferases, 148 mg of lacto-*N*-neotetraose (LNnT) could be synthesized in 81% yield over two steps. Further enzymatic extension of LNnT with α2–3-sialyltransferase from *Pasteurella multocida*,[35] α2–6-sialyltransferase from *Photobacterium damselae*,[39] or α1–3-fucosyltransferase from *Helicobacter pylori* produced LST-a, LST-c, and lacto-*N*-fucopentaose III, respectively.

Prudden et al.[40] synthetically created a lactose acceptor bearing a cleavable anomeric linker. This approach, unlike reductive amination, preserved the reducing terminus and provided a handle for rapid purification of reaction intermediates via reverse-phase chromatography (Figure 3). Sequential treatment of lactose with a β1–3-*N*-acetylglucosamine transferase from *H. pylori* and a β1–4-galactosyl transferase from bovine milk produced LNnT, which could be differentially fucosylated or sialylated to produce lacto-*N*-neo-difucohexaose II (LNnD-II), LST-a, and α2–3-sialyl LNnD-II.

Chemoenzymatic synthesis is currently most suitable for developing comprehensive libraries of HMOs populated with clearly defined structures of different lengths, branching patterns, and fucosylation/sialylation arrangements. Using a chemoenzymatic strategy, a carefully protected acceptor could be synthesized in such a way as to allow enzymatic extension at select positions, thereby affording a unique collection of targets. An elaborate library of compounds, in milligram quantities, would allow preliminary analysis detailing how certain classes of HMOs interact with various pathogenic adhesion proteins and gut microbiota or modulate immune responses. Results from such studies would begin to describe how HMO structure correlates with biological function. It is expected that these data would act as a guide for the development of interesting targets to be produced on a larger scale for more elaborate biological investigation.

Microbial metabolic engineering for large-scale production of HMOs. Microbial fermentation has served as a crucial manufacturing process of food and pharmaceutical ingredients for decades. For instance, amino acids, antibiotics, and vitamins are currently manufactured by microbial fermentation using natural or engineered microorganisms. As there is no microorganism capable of producing HMOs naturally, metabolic engineering approaches are necessary to generate HMOs from microbes.

2′-Fucosyllactose is one of the most abundant HMOs in human milk, and it has a relatively simple

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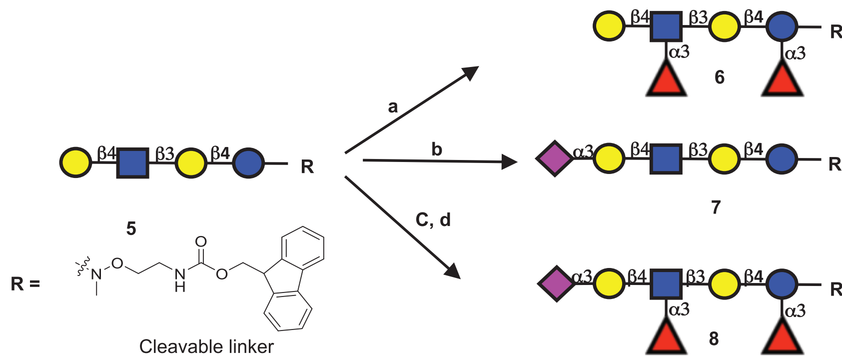

Figure 3 **LNnT diversification.** (A) *Helicobacter pylori* α1–3-fucosyltransferase; (B) ST3Gal4; (C) ST3Gal, *H. pylori* α1–4-fucosyltransferase. *Abbreviations*: HO, hydroxyl; SEt, thioethyl; TFA, trifluoroacetimide.

structure as compared with other HMOs. For example, *Escherichia coli*, which is a preferred host for metabolic engineering, has been engineered to produce 2′-FL (Figure 4).[41–43] Other host strains, such as *Saccharomyces cerevisiae*, lactic acid bacteria, and *Bacillus* species, which are generally recognized as safe (GRAS), might be engineered to produce 2′-FL as well. Regardless of the host strain, the biosynthesis of 2′-FL in engineered host strains requires 3 elements, outlined below, which are also necessary for the biosynthesis of HMOs other than 2′-FL.

First, lactose, which covers the terminal end of all HMOs, needs to be transported into the cytosol. *E. coli* can assimilate lactose through the enzymes lactose permease (lacY) and β-galactosidase (lacZ), but intracellular hydrolysis of lactose into glucose and galactose by β-galactosidase needs to be minimized for the efficient synthesis of 2′-FL and other HMOs. Therefore, *E. coli* strains with attenuated or eliminated β-galactosidase activities have been used to produce 2′-FL. Instead of using extracellular lactose, lactose can be synthesized intracellularly from glucose and uridine diphosphate galactose. The intracellular synthesis of lactose can be more advantageous for producing pure 2′-FL than external lactose feeding. Currently, lactose is produced mainly from whey processing, but thermal processing during lactose production can accelerate the isomerization of lactose into lactulose. Therefore, small amounts of lactulose are always contaminated in lactose, resulting in the production of 2′-fucosyllactulose as well as 2′-FL during microbial fermentation.

Second, the fucosyl donor guanosine diphosphate fucose (GDP-$_L$-fucose) needs to be overproduced in the cytosol. There are two biosynthetic pathways that yield GDP-$_L$-fucose in microorganisms. First, GDP-$_L$-fucose can be synthesized intracellularly from guanosine diphosphate mannose (GDP-$_D$-mannose) via the *de novo* pathway. The *de novo* pathway consists of two metabolic reactions: GDP-$_D$-mannnose-4,5-dehydratase (gmd) and GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (wcaG).[44] *E. coli* has the *de novo* pathway and can synthesize exopolysaccharide colanic acid and GDP-$_L$-fucose for biosynthesis of the cell wall component.[45] However, it is necessary to increase intracellular GDP-$_L$-fucose concentrations for the enhanced production of 2′-FL. Overexpression of the enzymes involved in GDP-$_L$-fucose synthesis was effective in increasing the intracellular GDP-$_L$-fucose concentration. In particular, overexpression of manB and manC to increase metabolic fluxes toward GDP-$_D$-mannose and overexpression of gmd and wcaG to convert GDP-$_D$-mannose to GDP-$_L$-fucose led to increased availability of GDP-$_L$-fucose in the cytosol. Second, the salvage pathway, consisting of $_L$-fucose permease (fucP) and bifunctional $_L$-fucose kinase/$_L$-fucose-1-phosphate guanylyltransferase (fkp), can convert extracellularly provided $_L$-fucose into GDP-$_L$-fucose.[46] While $_L$-fucose is a very expensive sugar, it is feasible to overproduce GDP-$_L$-fucose using the salvage pathway.

Third, an α 1-2-fucosyltransferase capable of fucosylating into the C-2 position of the galactosyl residue of lactose needs to be overexpressed. The α1,2-fucosyltransferase (FucT2) from *H. pylori* has been used to synthesize 2′-FL in *E. coli*, but the activity of FucT2 after overexpression was limited because of inclusion body formation in *E. coli*. Lowering the temperature of the culture or adding an amino acid tag to the *N*-terminus of FucT2[42] increases the soluble expression of FucT2. In addition to FucT2, *wbgL* from *E. coli* O126[47] and *wbsJ* from *E. coli* O128,[48] which code for endogenous fucosyltransferases, have been identified as α1,2-fucosyltransferases. Since they are endogenous enzymes, they are good targets of overexpression for the production of HMOs.

By combining the 3 elements described above, various engineered *E. coli* strains capable of producing 2′-FL

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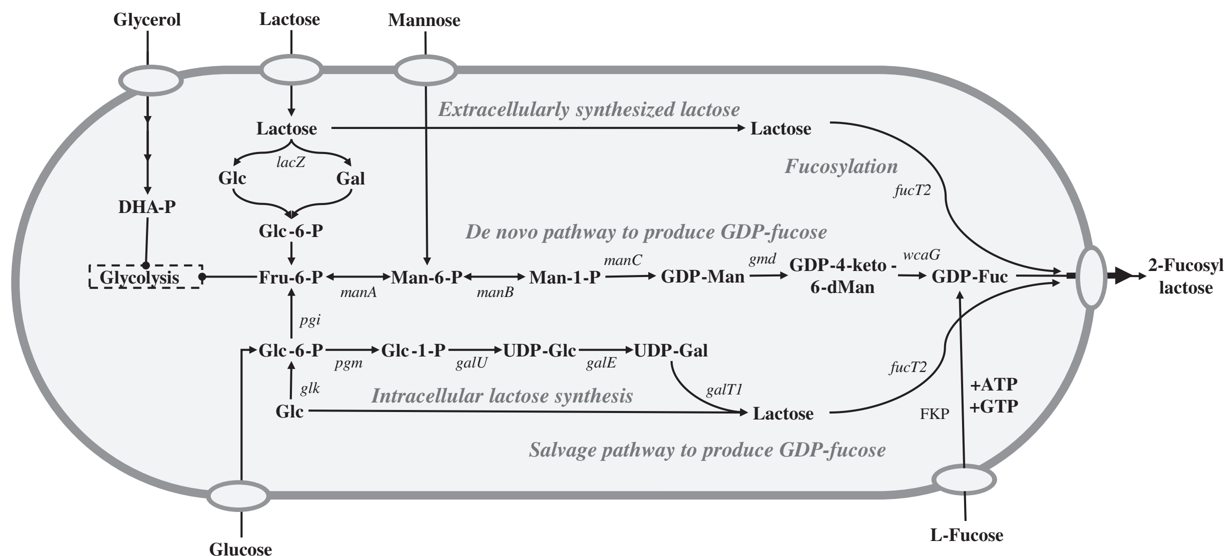

Figure 4 **Gene targets of metabolic engineering to produce 2′-fucosyllactose (2′-FL) in *E. coli*.** Three elements for producing 2′-FL in *E. coli* are illustrated. Either extracellularly fed or intracellularly synthesized lactose can be used as a backbone. *De novo* and/or salvage pathways for producing GDP-L-fucose can be used. Fucosyltransferases from various sources can be used for fucosylation of lactose.
Abbreviations: ATP, adenosine triphosphate; DHA-P, dihydroxyacetone phosphate; FKP, L-fucokinase/guanosine 5′-diphosphate-L-fucose pyrophosphorylase; Fru-6-P, fructose-6-phosphate; *fucT2*, α1–2-fucosyltransferase; Gal, galactose; *galE*, UDP-glucose 4 epimerase; *galT1*, galactose-1-phosphate uridylyltransferase; *galU*, UTP-glucose-1-phosphate uridylyltransferase; GDP-Fuc, guanosine diphosphate fucose; GDP-Man, guanosine diphosphate mannose; Glc, glucose; *gmd*, GDP-mannose 4,6-dehydratase; GDP-4-keto-6-dMan, GDP-4-keto-6-deoxy-D-mannose epimerase/reductase; Glc-6-P, glucose-6-phosphate; GTP, guanosine triphosphate; *lacZ*, β-galactosidase; *manA*, mannose-6-phosphate isomerase; *manB*, phosphomannomutase, *manC*, mannose-1-phosphate guanylyltransferase; Man-6-P, mannose-6-phosphate; *pgi*, glucose-6-phosphate isomerase; *pgm*, phosphoglucomutase; *wcaG*, GDP-4-keto-6-deoxy-d-mannose-3,5- epimerase/4-reductase.

have been constructed. Lee et al.[43] demonstrated the feasibility of whole-cell biosynthesis of 2′-FL from lactose using engineered *E. coli*. Using a host strain incapable of consuming lactose, such as JM109, was critical for producing 2′-FL from lactose. The resulting engineered *E. coli* strain JM109(DE3), which overexpressed *manB*, *manC*, *gmd*, and *wcaG*, was able to produce 2′-FL at a concentration of 1.2 g/L after consuming lactose at 14.5 g/L.[43]

Baumgärtner et al.[41] reported two advances in 2′-FL production by engineered *E. coli*. They introduced both *de novo* and salvage pathways for producing GDP-L-fucose into *E. coli*. In addition, all necessary genetic perturbations were made through chromosomal integration, ie, without using plasmids. Therefore, it was not necessary to add antibiotics to maintain plasmids in the engineered *E. coli*. With the advanced strain and supplementation of L-fucose, a much higher 2′-FL yield of 20.3 g/L was obtained in an antibiotic-free fed-batch fermentation.[41] While detailed performances are not known, there are patent applications claiming production of 2′-FL with higher yields. In addition, production of 6′-sialyllactose (6′-SL) by engineered *E. coli* has been demonstrated.[49]

In order to fully exploit the beneficial effects of HMOs, the development of additional metabolic engineering strategies to produce HMOs other than 2′-FL or 3′/6′-SL is needed. Baumgärtner et al.[50] constructed an engineered *E. coli* strain capable of producing lacto-*N*-tetraose through overexpression of *lgtA* and *wbgO* coding for β 1-3-*N*-acetylglucosaminyltransferase and β 1-3-galactosyltransferase. The resulting strain produced lacto-*N*-tetraose at a concentration of 0.219 g/L from a mixture of glucose and lactose, and the concentration further increased up to 0.810 g/L when a mixture of lactose and galactose was used.[50,51] Using fed-batch fermentation with galactose as the sole carbon source, lacto-*N*-tetraose at a concentration of 12.7 g/L was produced by the engineered *E. coli*.[51] These results suggest that metabolic engineering can also be used to make longer oligosaccharide backbones. Identification of precise transferases capable of adding fucose or sialic acid into the desired locations of the backbones will enable the production of complex HMOs by engineered strains. Finally, the discovery of sugar transporters capable of secreting HMOs is crucial for expanding the biosynthesis of HMOs beyond 2′-FL.

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The most outstanding feature of microbial fermentation is the feasibility of scale-up for economic and large-scale production, although purification of HMOs from large volumes of fermentation broth might be challenging if HMO concentrations are low. Therefore, higher HMO yields and the use of minimal medium will be desirable for large-scale production.

The unique feature of microbial production of HMOs is that it might allow the production of HMOs in the consumer's gut or in food if probiotic or food-fermenting microorganisms such as yeast or lactic acid bacteria are engineered to produce HMOs. For instance, a 2′-FL–containing kefir or yogurt can be produced using engineered yeast or lactic acid bacteria, as milk contains substantial amounts of lactose. While there are concerns about genetically modified organisms in food, many of the organisms are already deemed safe. In addition, rapid advances in genome engineering[52] have enabled a more targeted gene-editing approach with limited off-target effects, thus providing an effective method for obtaining safe, genetically modified strains in the near future.

From the lab into the world

More recent developments in the area of metabolic engineering have addressed the economic drawbacks of total chemical synthesis and chemoenzymatic/biocatalytic processes. Fermentation is less expensive per se but is also much more scalable, making the large-scale production of HMOs for infant formula more feasible. However, removing the bottleneck in upstream production has merely shifted it downstream, and now the main challenge is to increase the scalability of HMO purification, which is also a major hurdle for isolating oligosaccharides from human donor milk or dairy streams. Most importantly, the commercial production of HMOs by fermentation requires the rigorous removal of any traces of the production strain, which is classified as a genetically modified organism. The advent of economical large-scale purification processes based on nanofiltration, electrodialysis, and simulated moving bed chromatography has made the commercial production and purification of HMOs feasible at last, and the first food-grade HMOs are now available at prices appropriate for the infant formula market.[53,54]

Despite high production costs, two HMOs produced by chemical synthesis are approaching market approval.[55,56] Glycom A/S is registering both LNnT and 2'-FL for use as novel ingredients for infant and toddler formula, among other uses in Europe (Novel Food applications 157 and 166) and the United States (Generally Recognized as Safe [GRAS] registration). In contrast, Jennewein Biotechnologie GmbH has developed a large-scale production process based on bacterial fermentation and is currently registering 2′-FL in Europe (Novel Food applications 168 and 174) and the United States (GRAS Notice Inventory, Government Reference Number [GRN] 571) as well as in other markets.

Although HMOs are natural carbohydrates that have been recognized as safe for many years, the use of chemical- or biotechnology-based production processes means that HMO products must undergo a rigorous registration process assessing the safety of the novel process through which the compound is derived. In the United States, all 3 precommercial HMOs (chemically synthesized 2′-FL and LNnT, and fermentation-derived 2′-FL) are under review by the US Food and Drug Administration, and GRN numbers have been assigned. In the European Union (EU), the same products are undergoing the application process for registration as novel foods. Both regulatory processes examine the safety of the premarket food product; however, whereas the GRAS application process is driven by the applicant company, which must take an active role in the assembling an appropriate expert panel (self-determined GRAS), the novel food registration process in Europe is controlled by the authorities representing each EU member state. In principle, a novel food application can be submitted through any member state, but most of the 179 applications submitted thus far have been filed in the Republic of Ireland, the Netherlands, and the United Kingdom, with a smaller number filed in Germany and France. The competent authorities from the member state should provide a safety evaluation within a certain specified time limit, but if this is not possible the case is referred to the EFSA. Such a referral was recently made in the Glycom novel food applications for 2′-FL and LNnT, with the EFSA ultimately issuing positive statements.

Once novel food status in the EU and/or GRAS status in the United States is obtained, infant food manufacturers can then register their infant food products (eg, by filing an infant food petition in the United States) that contain the novel ingredient. For the registration of the final formula containing the novel ingredient, a clinical examination of the formula becomes necessary. The first clinical trial is a so-called growth and tolerance study to confirm the appropriate growth and tolerance of infants consuming the premarket formula. The first growth and tolerance study for 2′-FL was recently published,[57] and further clinical trials for 2′-FL and LNnT are expected to follow soon.

The increasing popularity of organic infant formula has led to suggestions that HMOs produced by chemical synthesis or bacterial fermentation would not qualify. However, according to European Regulation

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No. 834/2007, up to 5% nonorganic material can be included in products labeled as organic. Similarly, under the US Department of Agriculture's National Organic Program (7 CFR Part 205), the inclusion of natural concentrations of HMOs, such as those for 2′-FL and LNnT in organic infant formula, would not be restricted.

Many of the regulatory and economic hurdles preventing the inclusion of HMOs in infant food products for human consumption have now been overcome. However, the commercial development of the first HMO products (2′-FL and LNnT) took 8 to 10 years, which is in the same league as the timeline for an innovative pharmaceutical product. The development of the first HMOs as infant food ingredients has covered new territory in terms of the production technology and regulatory oversight, paving the way for additional HMOs as innovative food products in the near future.

CONCLUSION

Technological advances in oligosaccharide production, isolation, and purification have provided an exciting opportunity to study the many potential benefits of HMOs. Human milk oligosaccharide mixtures and individual HMOs have become available in unprecedented quantities that allow the assessment of their metabolic fate, their efficacy, and their structure–function relationships using the entire spectrum from in vitro and in vivo models to human intervention studies.

This article outlined several different approaches to synthesize or isolate HMOs, each one accompanied by a set of advantages and challenges. Chemoenzymatic synthesis can create structurally defined HMOs and HMO libraries to facilitate initial research studies for identification of targets. Isolation of HMOs from human milk generates the entire spectrum of "authentic" HMOs, but the limited availability of human donor milk might create a ceiling for large-scale production. Isolation of milk oligosaccharides from bovine milk can generate some HMOs that are also present in bovine milk, with the advantage of a seemingly unlimited supply. Bovine milk oligosaccharides that are structurally different from those found in human milk could serve as precursors for chemoenzymatic production and help create "authentic" HMOs. Bioengineered microorganisms also have the advantage of nearly ceilingless scale-up opportunities. A better understanding of how HMOs are produced in the human mammary gland would guide chemoenzymatic synthesis and inform the bioengineering process.

With improved technologies for synthesizing or isolating the oligosaccharide structures in human milk comes the exciting possibilities of creating infant formulas with a composition closer to that of human milk, designing personalized formulas as supplements to human milk, and, potentially, utilizing synthesized oligosaccharides as ingredients in dietary supplements for use in adult populations. Therefore, it will become increasingly important to determine both the efficacy and the safety of HMOs.

Each unique process to generate HMOs may introduce different components into the final product. Whether additional safety studies are needed must, therefore, be given careful consideration. In addition to the typical safety considerations, such as tolerance and toxicology, there may be some considerations unique to HMOs. For example, certain types of gut bacteria are "cross-feeders" that feed and grow on the metabolic byproducts of other bacteria within the consortium.[58] Thus, it may be necessary to demonstrate not only the direct prebiotic benefit of HMOs but also the indirect impact of HMOs on bacteria that may utilize the metabolic byproducts of HMO consumption.

As the field moves to confirm the benefits and safety of HMOs in human populations and to expand the production of commercial supplies of HMOs, it is important to be specific about which structures (or groups of structures) are responsible for which benefits and to avoid attributing all of the observed benefits of HMOs to a single structure. The advancement of technologies for the identification, purification, and scaled-up production of HMOs promises to bring a "bench-to-bedside" translation of the many potential benefits of HMOs.

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