



Advances and challenges for obtaining human milk oligosaccharides: Extraction from natural sources and synthesis by intentional design

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ABSTRACT

Background: Human milk oligosaccharides (HMOs) are a group of structurally diverse unconjugated carbohydrates that are bioactive and functional beyond their important function as the reference for prebiotics. As the health benefits of HMOs are becoming more apparent, both academia and industry have shown increasing interest in accessing HMOs.

Scope and approach: This review describes the structure and endogenous biosynthesis of HMOs and provides an overview of current approaches to obtain HMOs, which are classified based on source materials and production technologies. Strategies of each approach are highlighted. Challenges and future needs for HMO production are discussed.

Key findings and conclusions: HMOs can be extracted from natural sources, such as human milk as the original source for a wide spectrum of HMOs and domestic animal milk for certain oligosaccharides. Oligosaccharides that are chemically and structurally identical to their naturally occurring counterpart present in human milk can be synthesized through various approaches, including chemical synthesis, enzymatic synthesis, chemoenzymatic synthesis, microbial fermentation, and mammary cell cultivation. Systematically considering the strategies and challenges of these approaches for obtaining HMOs may lead to new solutions and accelerate the advances in this field. The advances in HMO generation and production will accelerate their application in infant formulas, medical foods, and dietary supplements to improve the health of infants, mothers, those with special healthcare needs, and the general population.

1. Introduction

Human milk oligosaccharides (HMOs) are a group of structurally diverse unconjugated carbohydrates with three or more monosaccharide units that are essential components of human milk. Resistant to enzymatic hydrolysis in the upper gastrointestinal tract, HMOs are non-nutritive, but bioactive with various functions (Engfer, Stahl, Finke, Sawatzki, & Daniel, 2000; Li et al., 2021). HMOs can reach the distal intestine and serve as prebiotics that shape the gut microbiome, supporting beneficial bacteria such as infant-type bifidobacteria and reducing pathogen infections in infants, indirectly via reduction of intestinal pH and production of specific metabolites such as SCFAs and

more directly by acting as decoy receptors or bacteriostatic agents (Moore, Xu, & Townsend, 2021; Zivkovic, German, Lebrilla, & Mills, 2011). In addition, HMOs have the capacity to modulate immune responses and gut barrier function by mediating cytokine production (He, Lawlor, & Newburg, 2016; Xiao et al., 2018; Šuligoj et al., 2020), delivering protection against intestinal and immunological disorders (e.g., necrotizing enterocolitis) (Jantscher-Krenn et al., 2012; Masi et al., 2021; Wu et al., 2019). Recent interventional clinical trials further provide evidence for the safety, tolerability, and health effects of specific HMOs in infants as well as adults (Berger et al., 2020; Palsson et al., 2020; Parschat, Melsaether, Jäpelt, & Jennewein, 2021; Schönknecht, Moreno Tovar, Jensen, & Parschat, 2023; Vandenplas et al., 2020, 2022).

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Abbreviations for oligosaccharides

2'-FL	2'-fucosyllactose;
3-FL	3-fucosyllactose;
3'-GL	3'-galactosyllactose;
3'-SL	3'-sialyllactose;
4'-GL	4'-galactosyllactose;
6'-GL	6'-galactosyllactose;
6'-SL	6'-sialyllactose;
6'-SLN	6'-sialyl- <i>N</i> -acetyllactosamine;
DFL	difucosyllactose;
DFpLNnH	difucosyl- <i>para</i> -lacto- <i>N</i> -neohexaose;
DSL	disialyl lactose;
DSLNT	disialyllacto- <i>N</i> -neotetraose
DSLNT	disialyllacto- <i>N</i> -tetraose;

LDFH I	lacto- <i>N</i> -difucohexaose I;
LNB	lactose- <i>N</i> -biose I.
LNDFH I	lacto- <i>N</i> -difucohexaose I;
LNDFH II	lacto- <i>N</i> -difucohexaose II;
LNFP I	lacto- <i>N</i> -fucopentaose I;
LNFP II	lacto- <i>N</i> -fucopentaose II;
LNFP III	lacto- <i>N</i> -fucopentaose III;
LNFP VI	lacto- <i>N</i> -fucopentaose VI;
LNH	lacto- <i>N</i> -tetraose;
LNnDFH II	lacto- <i>N</i> -neodifucohexaose II;
LNnH	lacto- <i>N</i> -neotetraose;
LNnT	lacto- <i>N</i> -neotetraose;
LNT	lacto- <i>N</i> -tetraose;
LNT II	lacto- <i>N</i> -triose II;
TFpLNnH	trifucosyl- <i>para</i> -lacto- <i>N</i> -neohexaose;

As the health benefits of HMOs are being revealed, developing HMOs as dietary ingredients has received increasing attention. Due to past difficulties producing HMOs on a large scale, alternative oligosaccharides have been applied in the food industry. Milk oligosaccharides from domestic animals and other derived oligosaccharides, such as short chain galacto-oligosaccharides (scGOS) and long chain fructo-oligosaccharides (lcFOS), have been utilized in a ratio of 9:1 at 8 g/L ready to feed, to mimic the prebiotic effects of HMOs (Salminen, Stahl, Vinderola, & Szajewska, 2020). Over the past decade, technological advances in synthesizing oligosaccharides chemically and structurally identical to HMOs and regulatory approvals for such products have made it possible to bring HMOs to specialized nutrition market. The first regulatory approvals were obtained for 2'-FL and LNnT chemically synthesized by Glycom A/S and 2'-FL biotechnologically synthesized by Jennewein Biotechnologie GmbH through the Generally Recognized as Safe (GRAS) registration in the United States in 2015 (U.S. Food and Drug Administration, 2015a; 2015b; 2015c). In the European Union, 2'-FL and LNnT produced by chemical synthesis and microbial fermentation were registered as novel foods in 2016 (Bode et al., 2016; Bych et al., 2019). Currently, HMOs including 2'-FL, 3-FL, DFL, LNnT, LNT, 3'-SL, and 6'-SL have gone through or are undergoing safety evaluation by different authorities, provoking worldwide commercialization of these ingredients and their applications in infant formulas, medical foods, as well as dietary supplements.

Obtaining HMOs has drawn increasing attention in both academia and industry. HMOs can be isolated from human milk or oligosaccharides with identical structures can be obtained by other means. Bode et al. discussed HMO generation by isolating it from human donor milk or dairy streams, chemoenzymatic synthesis, and microbial metabolic engineering (Bode et al., 2016). Walsh et al. described HMO production through chemical synthesis, chemoenzymatic synthesis, and microbial metabolic engineering (Walsh, Lane, van Sinderen, & Hickey, 2020). Enzymatic synthesis and microbial production of HMOs have been described in several reviews (Bych et al., 2019; Fajjes, Castejón-Vilatersana, Val-Cid, & Planas, 2019; Zhou, Jiang, Wang, Liang, & Mao, 2021). The synthesis and isolation of HMOs have advanced rapidly in recent years. An overview of the ways to get HMOs may inspire new solutions. From a systematic point of view, HMOs can be extracted from natural sources and synthesized by intentional human design; the approaches can be further classified based on source materials and production technologies. In this article, we will briefly describe the structure and endogenous biosynthesis of HMOs, systematically update the approaches to obtain HMOs, and discuss current challenges as well as future needs.

2. Structure and endogenous biosynthesis of HMOs

HMOs are composed of five monosaccharide residues: glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid, with *N*-acetylneuraminic acid (Neu5Ac) as the predominant if not the only form of sialic acid. These residues are linked by various glycosidic bonds, generating linear and branched structures (Bode, 2012; Chen, 2015). All known HMOs have a lactose (Gal β -1,4-Glc) core at their reducing end. The lactose core can be extended with β -1,3- or β -1,6-linked *N*-acetyllactosamine (Gal β -1,4-GlcNAc-, type 2) or lacto-*N*-biose (Gal β -1,3-GlcNAc-, type 1) (Fig. 1A). While *N*-acetyllactosamine can be further extended by the addition of one of the two disaccharides, lacto-*N*-biose terminates the chain and only serves as the non-reducing-end terminal disaccharide. Lactose or the extended core structures can be fucosylated in α -1,2, α -1,3 or α -1,4 linkages and/or sialylated in α -2,3 or α -2,6 linkages. Besides the major structures known for HMOs, galactosyllactoses (GLs) with Gal linked to lactose with β -1,3, β -1,4 or β -1,6 linkages have been reported, leading to 3'-GL, 4'-GL, or 6'-GL, respectively (Salminen et al., 2020; Urashima, Hirabayashi, Sato, & Kobata, 2018). Recently, a tentative class of high galactose-HMO based on 6'-GL rather than lactose and containing *N*-acetylglucosamine branches extended by oligogalactoses has been proposed (Hanisch & Kunz, 2021). With the improvement of analytical techniques, HMOs with low abundance and more complex structures may be revealed. Determination of HMO structures is essential to decipher their functions and biosynthetic pathways.

HMO biosynthesis starts with the lactose core since all known HMOs carry lactose at their reducing end. The lactose synthesis occurs in the Golgi of mammary gland epithelial cells, which is catalyzed by a lactose synthase complex containing β -1,4-galactosyltransferase and α -lactalbumin (Bode, 2012; Ramakrishnan, Boeggeman, & Qasba, 2002; Urashima, Sato, Nio-Kobayashi, & Hirabayashi, 2021). It is believed that the lactose is extended, branched, fucosylated and sialylated sequentially by various glycosyltransferases in the mammary gland to form different structures of HMOs. Currently, most of the specific glycosyltransferases for the endogenous biosynthesis of HMOs have not been identified.

Based on enzymatic reactions in other glycan synthesis, Kobata and Urashima et al. proposed that core structures of HMOs (without fucosylation and sialylation) are potentially synthesized by four glycosyltransferases: β -1,3-*N*-acetylglucosaminyltransferase (iGnT) for linear chain extension, β -1,6-*N*-acetylglucosaminyltransferase (iGnT) for branching, β -1,3-galactosyltransferase (β 3GalT) for type 1 termination, and β -1,4-galactosyltransferase (β 4GalT) for type 2 formation (Kobata, 2017; Urashima et al., 2018, 2022). Hypothetical biosynthetic pathway has been mapped for 19 core structures excepting lacto-*N*-novopentaose I (Urashima et al., 2022; Urashima, Katayama, Fukuda, & Hirabayashi, 2021). Indeed, human milk contains galactosyllactoses including 3'-GL

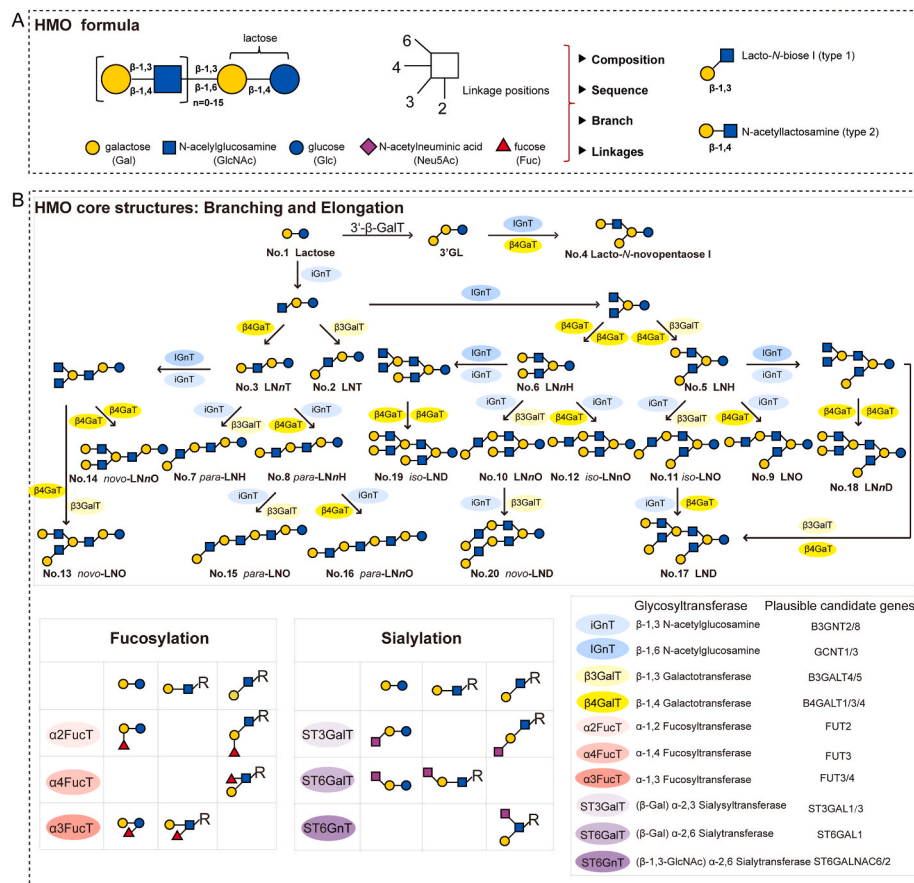


Fig. 1. Structure and endogenous biosynthesis of human milk oligosaccharides (HMOs). (A) Typical structure of HMOs. The five monosaccharides glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac) are building blocks for HMOs. These monosaccharide residues are linked by various glycosidic linkages in linear and branched sequences. (B) Hypothetical endogenous biosynthetic pathway of HMOs. Ten elementary enzymatic reactions are involved in the elongation, branching, fucosylation and sialylation for HMO biosynthesis. The glycosyltransferase responsible for each reaction is illustrated with a shaded background. The reaction of the glycosyltransferase and the candidate gene(s) of specific isozymes are described in the lower right. Abbreviations of HMO core structures (without fucosylation and sialylation): LNT, lacto-*N*-tetraose; LNnT, lacto-*N*-neotetraose; 3'-GL, 3'-galactosyllactose; LNH, lacto-*N*-hexaose; LNnH, lacto-*N*-neohexaose; *para*-LNH, *para*-lacto-*N*-hexaose; *para*-LNnH, *para*-lacto-*N*-neohexaose; LNO, lacto-*N*-octose; LNnO, lacto-*N*-neooctose; *iso*-LNO, *iso*-lacto-*N*-octose; *iso*-LNnO, *iso*-lacto-*N*-neooctose; *nov*-LNO, *nov*-lacto-*N*-octose; *nov*-LNnO, *nov*-lacto-*N*-neooctose; *para*-LNO, *para*-lacto-*N*-octose; *para*-LNnO, *para*-lacto-*N*-neooctose; LND, lacto-*N*-decaose; LNnD, lacto-*N*-neodecaose; *iso*-LND, *iso*-lacto-*N*-decaose; *nov*-LND, *nov*-lacto-*N*-decaose.

(Salminen et al., 2020; Urashima et al., 2018), which might be derived from lactose by β -1,3-galactotransferase, and lacto-*N*-novopentaose I is possibly derived from 3'-GL by IGnT and β 4GalT (Fig. 1B). The IGnT that acts on 3'-GL is expected to have different specificity and activity than the IGnT that acts on LNT II (GlcNAc β -1,3-Gal β -1,4-Glc) (McDonald, Mariethoz, Davey, & Lisacek, 2022). HMO fucosylation is highly dependent on the Secretor and Lewis status of the lactating mother. Fucosyltransferase 2 (FUT2) encoded by the Secretor gene (*Se*) is responsible for α -1,2-fucosylation of terminal Gal; fucosyltransferase 3 (FUT3) encoded by Lewis gene (*Le*) facilitates α -1,4-fucosylation of subterminal GlcNAc on type 1 chains; FUT3 or other FUTs independent of *Se* and *Le* may involve in the α -1,3-fucosylation of Glc and subterminal GlcNAc. Several sialyltransferases such as ST3Gal, ST6Gal, and ST6GnT may be involved in HMO sialylation, adding one or more Neu5Ac to terminal Gal or subterminal GlcNAc (Bode, 2012; Jin et al., 2022).

Indeed, each enzymatic reaction in HMO synthesis can be performed by multiple isozymes with the same activity. Specific enzymes that are involved in the *in vivo* reactions remain unclear. Recently, network-based approaches have been applied to decipher the biosynthesis of HMOs (Bao et al., 2021; Kellman et al., 2022; McDonald et al., 2022). Enzymatic reactions for HMO elongation, branching, fucosylation, and sialylation were simultaneously considered for model construction (Kellman et al., 2022; McDonald et al., 2022). Furthermore, by integrating HMO quantification and gene expression data, Kellman et al.

predicted candidate genes for these reactions (as shown in Fig. 1B) (Kellman et al., 2022). Further insights integrating more datasets from different experimental approaches would be valuable to elucidate the biosynthetic network of HMOs. A better understanding of the endogenous biosynthesis may inspire new strategies facilitating the production and utilization of HMOs as ingredients, supplements, or potential therapeutics.

3. Extraction of HMOs from milk sources

Endogenously synthesized and secreted by the mammary glands, HMOs naturally exist in human milk; therefore, human milk is the original source for various HMOs. Similarly, a few oligosaccharides with identical structures can be found in milk from other species; thus, certain oligosaccharides can be extracted from the milk of dairy animals.

3.1. Isolation from human milk

Human milk contains a wide spectrum of HMOs. HMOs are the major solid component of human milk after lactose and fat. Mean concentrations of macronutrients in mature term milk were reported as 67–78 g/L for lactose, 32–36 g/L for fat, and 9–12 g/L for proteins (Ballard & Morrow, 2013). The mean concentration of total HMOs in human milk ranges from 11 to 18 g/L, in which the variations are attributable to

various factors such as gestation length, secretor status of the mother, lactation period and analytical methods (Soyyilmaz et al., 2021; Thurl, Munzert, Boehm, Matthews, & Stahl, 2017). Over 200 unique HMO species have been found, and the structures of over 100 HMOs have been fully elucidated (Chen, 2015; Remoroza, Mak, De Leoz, Mirokhin, & Stein, 2018; Totten et al., 2014; Urashima, Katayama, et al., 2021).

Isolation of HMOs from human milk is mostly for research purposes, such as structural characterization, *in vitro* discovery studies, and analytical standard preparation. Human milk is a complex matrix, containing carbohydrates, fat, proteins, cells, metabolites, and other biological molecules; extracting the oligosaccharide fraction out of this complex matrix is a common procedure for HMO analysis. Fat is removed by centrifugation or solvent extraction. Proteins are often precipitated using organic solvents, such as ethanol, chloroform/methanol, acetone or acetonitrile, or removed by membrane filtration (Auer, Jarvas, & Guttman, 2021). In some earlier studies, gel permeation chromatography (GPC), ion-exchange chromatography (IEC), and affinity chromatography (AC) were employed to isolate HMOs in small amounts (Grönberg et al., 1989; Kitagawa et al., 1989; Kobata, Ginsburg, & Tsuda, 1969; Thurl, Offermanns, Müller-Werner, & Sawatzki, 1991). Recently, preparative high-performance liquid chromatography (HPLC) provides an effective means to obtain large quantities of compounds from natural products (Cao et al., 2021; Liang et al., 2015). In human milk, the content of HMOs is less abundant compared to lactose. In addition, HMOs are highly complex with diverse neutral and sialic oligosaccharides, and multiple isomeric structures exist. Therefore, single chromatography is difficult to isolate individual HMOs (Yan, Ding, & Liang, 2017). In our lab, a three-dimensional preparative chromatography method based on hydrophilic interaction liquid chromatography (HILIC) and porous graphitized carbon chromatography (PGC) separation was developed to purify natural HMOs from human milk (Li et al., 2022). Briefly, sample preparation started with the removal of lipids and proteins from 20 L of human milk by centrifugation and membrane filtration. To enrich neutral and sialylated HMOs, the first dimensional (1D) separation was performed on a Click TE-GSH column, which is a zwitterionic stationary phase with mixed-mode action of hydrophilic interaction and cation-exchange. In ethanol/water system with pH > 2, both sialylated HMOs and Click TE-GSH materials are negatively charged, so that sialylated HMOs are eluted quickly due to electrostatic repulsion. With hydrophilic interaction, lactose and neutral HMOs are eluted subsequently from the column. This process effectively removes large amounts of lactose and separates sialylated oligosaccharides from neutral ones (Yan et al., 2018). Then, sialylated and neutral HMO fractions were applied to HILIC phase XAmide with superior hydrophilicity as the second dimensional (2D) chromatography for polarity-based separation. HMOs can gradually flow through the column with increased degree of polymerization. For HMO isomers with similar polarity coeluted from the 2D column, PGC stationary phase with molecular shape-based selectivity was employed as the third dimensional (3D) chromatography for isomer isolation. Subtle differences in monosaccharide composition, linkages or branches can be resolved. Using this strategy, many abundant HMOs and some complex HMOs with high degree of polymerization or novel core structures in low abundance were obtained in the gram or milligram scale. This purification strategy not only provides an effective way for the acquisition of novel natural oligosaccharides with complex structures, but also lays foundation for the study of the structure-activity relationship between individual HMOs and glycan binding proteins.

In addition to research purposes, HMOs isolated from human milk has the potential for human consumption, especially for infants in need. WHO and UNICEF call for use of donor human milk for infants who cannot be fed their mother's own milk or who need to be supplemented (World Health Organization, 2018). Although the main focus of donor milk processing nowadays is assuring the safety and preserving the nutritional and biological properties (Moro et al., 2019), further processing to enrich certain components of human milk has been applied for

specific health benefits. For example, Prolacta Bioscience has produced human milk derived cream supplement, which enhances the energy density of feeds, enabling very low birth weight preterm infants to be discharged earlier (Hair et al., 2016). Recent studies revealed that preterm infants receiving less DSLNT in mother's milk are prone to abnormal microbiome development and necrotising enterocolitis (NEC), suggesting that DSLNT supplementation may prevent the disease (Autran et al., 2018; Masi et al., 2021). It is possible to produce human milk derived HMO products, which can be a specific HMO or a mixture of certain oligosaccharides, or even containing the original spectrum of HMOs. This approach can acquire a variety of HMOs that cannot be obtained from any other sources. However, HMO isolation starts with a large amount of donor human milk with intrinsic variability, which is expensive and with ethical concerns. These are key constraints to use human milk as a source of ingredients. Therefore, alternative sources are needed to produce HMOs as dietary or pharmaceutical ingredients for human consumption.

3.2. Enrichment from dairy streams

Due to the limited supply and ethical constraints, the quantity of HMOs obtained from human milk is difficult to meet the need for interventional clinical trials, let alone commercialization as ingredients. Bovine milk oligosaccharides (BMOs) and caprine milk oligosaccharides (CMOs) that bear structural similarities to HMOs may exert similar biological functions. For example, sialylated oligosaccharides from human milk and bovine milk are both associated with growth promotion in malnourished infants. Analysis of milk from Malawian mothers revealed that sialylated HMOs are less abundant in those with undernourished infants; meanwhile, sialylated BMO supplementation has shown to promote growth in gnotobiotic mice and piglets colonized with a stunted infant's gut microbiota (Charbonneau et al., 2016; Cowardin et al., 2019).

Bovine milk is the most used raw material of dairy products. So far, at least 37 BMO structures have been identified (Albrecht et al., 2014; Mariño et al., 2011; Remoroza et al., 2020). The concentration of BMOs is much lower than HMOs, about 1 g/L in colostrum and 0.03–0.06 g/L in mature milk. Unlike oligosaccharides in human milk, fucosylated neutral oligosaccharides are less common while sialylated oligosaccharides account for most of total oligosaccharides in bovine milk (Hickey & Urashima, 2022). Most of the BMOs consist of 3'-SL, 6'-SL, 6'-SLN and DSL, with 3'-SL constituting 70% of the total oligosaccharide content. There are two types of sialic acids in BMOs: N-glycolylneuraminic acid (Neu5Gc) and Neu5Ac. Neu5Ac is the main sialic acid in sialylated oligosaccharides in bovine colostrum. Fig. 2A shows the typical BMOs, the core units of most BMOs are lactose or N-acetyllactosamine, unlike human milk oligosaccharides with longer core units including LNT, LNnT, LNH and LNnH, etc. It is interesting that some phosphorylated oligosaccharides have been detected in bovine colostrum (Cumar, Ferchmin, & Caputto, 1965; Parkkinen & Finne, 1985). So far, phosphorylated oligosaccharides have not been found in human milk.

CMOs have attracted attention in recent years since goat milk is another important raw material for dairy industry as an approved protein source for infant formula and with higher levels and diversity of oligosaccharides compared to bovine milk (Barnett, Roy, McNabb, & Cookson, 2016; van Leeuwen et al., 2020). The concentrations of CMOs range from 0.2 to 0.65 g/L in colostrum and from 0.06 to 0.35 g/L in mature milk. Up till now, 78 compositions of CMOs were found and 40 structures among them were well characterized. Very similar to BMOs, more than 80% of CMOs are sialylated oligosaccharides and sialic acids contain both Neu5Gc and Neu5Ac. However, the ratio of Neu5Gc/Neu5Ac in CMOs is significantly higher than that in BMOs (Martín-Ortiz et al., 2017; Stahl, Zens, & Boehm, 2007; Yan et al., 2018). Neu5Gc is a non-human sialic acid, and its impact on immune responses in infants remains to be investigated (Bode, 2012).

Considering the wide availability of dairy processing byproducts

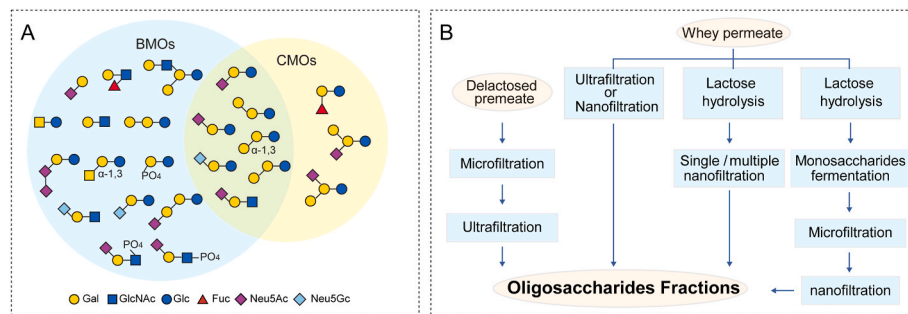


Fig. 2. Typical structures of oligosaccharides in dairy streams and their enriching process. (A) Typical structure of bovine milk oligosaccharides (BMOs) and caprine milk oligosaccharides (CMOs). Glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acids *N*-acetylneuraminic acid (Neu5Ac) as well as *N*-glycolylneuraminic acid (Neu5Gc) monosaccharide residues are building blocks for BMOs and CMOs. Besides, phosphorylated BMOs have been detected. (B) Enrichment of oligosaccharides from dairy streams by membrane-based technology.

from which oligosaccharides can be isolated, researchers are committed to enriching BMOs and CMOs by membrane-based technology (Cohen, Barile, Liu, & de Moura Bell, 2017; de Moura Bell et al., 2018; Mehra et al., 2014). The main challenge of the enrichment process is to isolate trace amounts of BMOs or CMOs from a large amount of lactose. Recently, Wang and Yu reviewed the membrane separation process for enrichment of BMOs and CMOs from dairy byproducts, such as whey, permeate, and delactosed permeate (Wang & Yu, 2021). Enrichment

approaches including single-membrane approaches, the combination of lactose hydrolysis and nanofiltration, and the integrated approach were described (Fig. 2B). Though some studies showed recovery yield and purity of oligosaccharides have been improved, challenges still exist regarding reducing the membrane fouling, improving the recovery yield and purity, and overcoming the change of milk oligosaccharides composition. In addition to these challenges, another important issue that has not been addressed is the cost of production. Due to the low

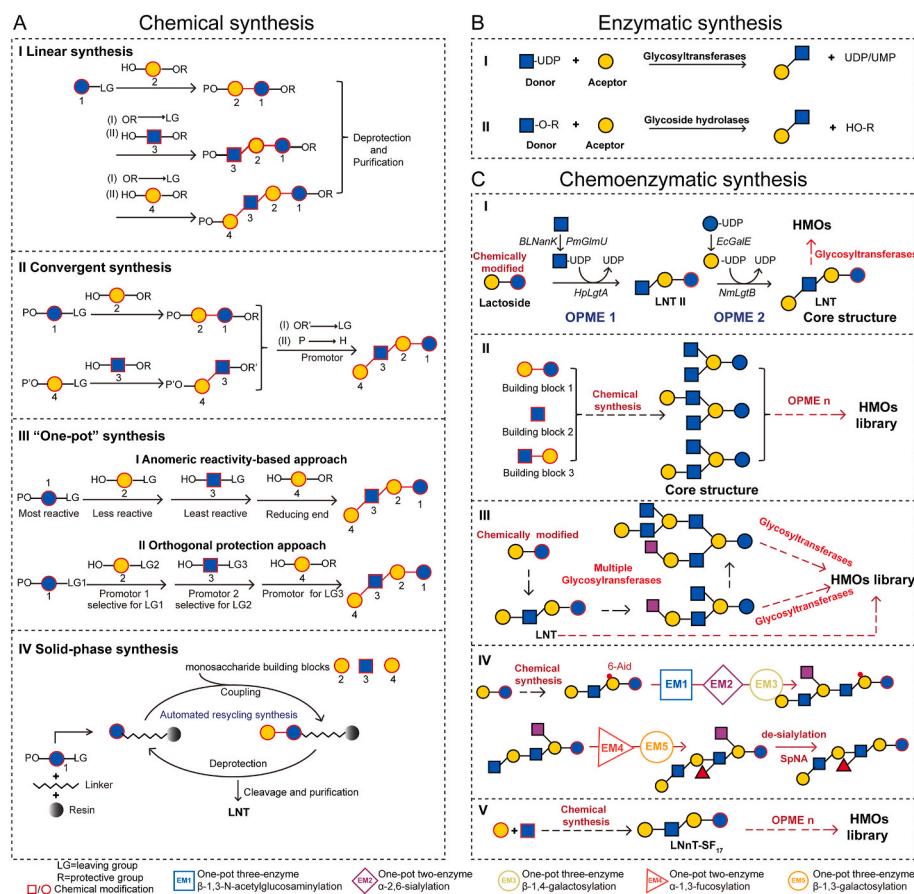


Fig. 3. Chemical and/or enzymatic synthesis strategies for HMOs. (A) Major strategies for chemical synthesis of HMOs include linear synthesis (I), convergent synthesis (II), one-pot synthesis (III), and solid-phase synthesis (IV). (B) Glycosyltransferases (I) and glycoside hydrolases (II) are key enzymes for enzymatic synthesis of HMOs. (C) Some strategies for chemo-enzymatic synthesis of HMOs include sequential one-pot multienzyme (OPME) glycosylation of chemically modified lactoside for linear structure synthesis (I), convergent chemically synthesizing core structures followed by enzymatic extension for branched structure generation (II), chemically modification of lactose with a multifunctional anomeric linker followed by sequential enzymatic actions utilizing substrate specificities for complex asymmetrical multibranched structure synthesis (III), and redox-controlled strategy and sulfo-fluorous tagging strategy for site-selective fucosylation/sialylation (IV and V). Chemically modified residues are depicted with red outline (e.g., ● is regular galactose, while ● is chemically modified galactose). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

abundance and compositional difference of oligosaccharides in dairy streams versus in human milk, it takes up considerable resources obtaining BMOs and CMOs apart from lactose, not to mention purifying specific oligosaccharides that share the same structures of certain HMOs. A more economical way to utilize the BMOs and CMOs is to produce oligosaccharide-enriched products like BMO-enriched whey. Cost-benefit analysis is needed to determine the appropriate manufacturing processes and industrial applications.

4. Synthesis of HMOs by intentional design

Oligosaccharides chemically and structurally identical to their naturally occurring counterparts present in human milk can be synthesized by intentional human design with various techniques. Here, we categorized the HMO synthesis by design into the following five approaches based on production technologies: chemical synthesis, enzymatic synthesis, chemoenzymatic synthesis, microbial fermentation, and mammary cell cultivation.

4.1. Chemical synthesis

The key reaction in oligosaccharide synthesis is glycosylation, which involves the formation of a glycosidic bond between two monosaccharides. Chemical synthesis of HMOs requires extensive protection and deprotection manipulations to control the position of the glycosidic bond (Pérez-Escalante et al., 2022; Wen et al., 2018). First, hydroxyl groups that are not involved in the desired reaction need to be selectively protected; then, the glycosyl donor is activated into an electrophilic intermediate that reacts with a free hydroxyl group from the acceptor to form a glycosidic linkage; next, protecting groups are removed for next round reactions or to obtain the final desired molecule. Major strategies for chemical synthesis of HMOs include linear synthesis, convergent synthesis, one-pot synthesis, and solid-phase synthesis (Fig. 3A).

Linear synthesis is a conventional sequential extension strategy involving the stepwise assembly of the oligosaccharide chain from one end to the other. The convergent synthesis involves the preparation of a set of properly protected modular disaccharide blocks and the assembly of these blocks into the desired oligosaccharides (Arungundram et al., 2009). Each glycosidic bond formation and deprotection process requires purification steps, which are labor-intensive and time-consuming. Thus, one-pot and solid-phase synthetic strategies were employed to avoid excessive purification steps.

One-pot synthesis is achieved in a single reaction chamber, where glycosyl donors are sequentially coupled with the previous product. The availability of well-designed donors and acceptors are required in these consecutive reactions. In the anomeric reactivity-based approach, oligosaccharide synthesis is mainly based on distinct anomeric reactivities of differentially protected glycosyl donors; with proper planning, an acceptor for the first glycosylation can be a donor for the second glycosylation, and sequential construction of glycosidic linkages can be achieved (Lee, Greenberg, & Wong, 2006). In the orthogonal protection approach, orthogonal leaving groups and a pair of suitable activators are adopted for sequential activation (Kaeothip & Demchenko, 2011).

Automated solid-phase synthesis has been developed by Seeberger's group (Lin et al., 2023; Plante, Palmacci, & Seeberger, 2001). Through appropriate linker arms, glycosyl acceptor is immobilized onto the resin, which is extended by alternating cycles of selective deprotection and coupling reactions. Ultimately, the oligosaccharide is cut off from the resin and the desired target oligosaccharide could be obtained after removal of protective groups.

Using these chemical synthetic strategies, a series of HMOs including 2'-FL, 3-FL, DFL, LNT, LNnT, LNH, LNnH, LNFP I, and LNFP III and sialylated HMOs have been acquired, detailed in previous review (Pérez-Escalante et al., 2022). With laborious and multi-step purification processes, the yield of HMOs produced by chemical synthesis is still

low (ranging from 20% to 55%), leading to high production cost. In addition, toxic reagents employed in deprotection steps also limit the application of chemical synthesis in HMO manufacturing. Although kilogram scale chemical synthesis of 2'-FL has been reported (Agoston, Hederos, Bajza, & Dekany, 2019), the chemical synthesis is not preferred for large scale industrial production. Thus, chemical synthetic strategies are mainly employed to produce structurally well-defined HMOs for function and structure-activity relationship studies. In recent years, chemical synthesis has been used in producing certain precursors for chemo-enzymatic synthesis.

4.2. Enzymatic synthesis

As an alternative to chemical synthesis, enzymatic synthesis confers regioselectivity and stereoselectivity without tedious operations for protection or deprotection of hydroxyl groups. Enzymatic synthesis of HMOs has been reviewed recently (Fajjes et al., 2019; Pérez-Escalante et al., 2022; Zheng, Xu, Fang, & Zhang, 2022). Two classes of enzymes have been used to synthesize oligosaccharides: glycosyltransferases (GTs) and glycoside hydrolases (GHs) (Fig. 3B).

GTs catalyze the formation of glycosidic bonds via the transfer of an activated nucleotide sugar donor to a glycosyl acceptor. Disaccharide units like Gal β -1,4-Glc, Gal β -1,3-GlcNAc and Gal β -1,4-GlcNAc are common acceptors in the enzymatic synthesis, while nucleotide sugars including UDP-Glc, UDP-GlcNAc, UDP-Gal, GDP-Fuc and CMP-Neu5Ac are the most common donors. The activity of GTs is usually affected by the chemical nature of different substrates, making the identification of suitable GTs a laborious process. In addition, the limited stability and availability of GTs as well as the high cost of nucleotide donors are limiting factors for large-scale production. Synthesizing nucleotide sugars with multi-enzyme cascades has been explored by Lothar Elling's group; the Elling group has utilized high-throughput screening, repetitive-batch-mode synthesis, and the immobilization of enzyme cascades to reduce the cost of nucleotide sugar production (Frohnmeier & Elling, 2023; Rexer et al., 2021).

Glycoside hydrolases (GHs), also known as glycosidases, catalyze the hydrolysis of glycosidic bonds of glycosides. GHs can also be utilized in glycoside synthesis by forcing transglycosylation against hydrolysis (Bissaro, Monsan, Fauré, & O'Donohue, 2015; Franceus, Lormans, & Desmet, 2022). To improve the regioselectivity, substrate specificity and product yield, engineered glycoside hydrolases with suppressed hydrolysis activity have been developed to efficiently catalyze transglycosylation and produce HMOs (Franceus et al., 2022; Nekvasilová et al., 2022; Teze et al., 2021; Zeuner, Teze, Muschiol, & Meyer, 2019). Compared to the expensive activated nucleotide sugars required in GTs-catalyzed reaction, the donors involved in GHs-catalyzed transglycosylation are cheaper and can be acquired in large quantities (Zeuner et al., 2019).

To date, over twenty HMOs have been provided enzymatically, including 2'-FL, 3-FL, LNT, LNT II, DFL, LNnT, DFpLNnH, TFpLNnH, LNFP I, LNFP II, LNFP III, LNFP VI, LDFH I, LNDFH I, LNDFH II, LNnDFH II, 3'-SL, 6'-SL, LNB and DSLNnT (Nishimoto & Kitaoka, 2007; Pérez-Escalante et al., 2022; Zheng et al., 2022). This method effectively produces more structurally complex HMOs than chemical method and promotes the biological function research of HMOs in lab-scale experiments. Despite the relative high efficiency of enzymatic synthesis, the diversity of HMO structures acquired by enzymatic methods is limited by the substrate specificity and the availability of the glycosidases. Engineering stable enzymes with superior specificities and activities and reducing the production cost are areas with focus.

4.3. Chemoenzymatic synthesis

Chemoenzymatic approach, which integrates the flexibility of chemical synthesis and the high selectivity of enzymatic synthesis, is efficient to obtain complex oligosaccharides. Chemoenzymatic synthesis

of HMOs has been described in recent reviews (Bode et al., 2016; Faijes et al., 2019; Pérez-Escalante et al., 2022; Walsh et al., 2020; Xu & Townsend, 2021; Zhao et al., 2017; Zheng et al., 2022). Generally, chemical synthesis of the desired substrate followed by enzymatic extension and modification is applied in this approach to produce HMOs with complex structures (Fig. 3C).

Using a sequential one-pot multienzyme (OPME) glycosylation process, Xi Chen's group (Chen et al., 2015; Huang, Yu, & Chen, 2011) synthesized LNT, LNnT and their sialyl and fucosyl derivatives from chemically generated 3-azidopropyl lactosides (Fig. 3C I). The OPME process avoids tedious purification of intermediates, which is an effective strategy and often adopted in the production of more complex HMOs. In order to obtain branched HMOs, Peng George Wang's group (Xiao et al., 2016) firstly synthesized 3 branched core structures by convergent chemical methods, and then enzymatically extended these core structures by 4 glycosyltransferases to generate a total of 31 branched HMOs, where OPME were used for β -1,4-galactosyltransferase and α -2,6-sialyltransferase catalyzed steps (Fig. 3C II). To obtain complex asymmetrical multibranched HMOs, Geert-Jan Boons' group (Prudden et al., 2017) exploited inherent substrate specificities of a limited number of glycosyltransferases and generated a library of 60 linear, biantennary, and triantennary HMOs. Lactose was chemically modified with a multifunctional anomeric linker, and sequential enzymatic actions for extension, branching, and selective fucosylation or sialylation were performed based on substrate specificities of the added glycosyltransferases (Fig. 3C III). Recently, the asymmetrically branched LNH has been synthesized by using selective enzymatic glycan extension on a branched scaffold (Ooi, Zhang, Kuo, Liu, & Yu, 2022).

More strategies for site-selective fucosylation and sialylation have been developed. For example, Hongzhi Cao's group developed a redox-controlled site-specific α -2,6-sialylation strategy which masks unwanted sialylation sites by site-specific enzymatic oxidation of galactose units (Lu et al., 2019). Furthermore, Cao's group also designed a strategy for site-specific fucosylation by introducing α -2,6-sialylation to specific sites to block unwanted fucosylation, and de-sialylation is performed after defined fucosylation to yield final desired structures (Ye et al., 2019) (Fig. 3C IV). In addition, Ching-Ching Yu's group (Huang et al., 2021) developed site-selective fucosylation/sialylation of HMOs by using removable sulfo-fluorous tag at the reducing end of lactose to alter the substrate specificity of readily available bacterial glycosyltransferases. Combining this strategy with sequential one-pot enzymatic reactions (Fig. 3C V), over 30 linear HMOs with varying degrees of fucosylation and sialylation were obtained in milligram scale.

Ever-increasing chemoenzymatic methods have improved the diversity, yield, and purity of HMOs produced in labs. HMOs including 3-FL, LNT, LNT II, bi-antennary and asymmetry multi-antennary HMOs with or without fucosylation and/or sialylation have been obtained in milligram scale. Although scaling up of chemoenzymatic synthesis of HMOs for cost effective commercial applications is challenging, this approach enables the development of elaborate libraries of diverse HMOs for further investigations of their structure-function relationships.

4.4. Microbial fermentation

With the advances in metabolic engineering and synthetic biology, metabolically engineered microbial strains have been developed to produce HMOs (Bych et al., 2019; Endo, Koizumi, Tabata, & Ozaki, 2000; Faijes et al., 2019; Koizumi, 2003; Zhu, Cao, Wang, & Mu, 2022). The overall strategy is to restructure metabolic networks and construct biosynthetic pathways favorable for target molecules in host microbial strains using genetic modification techniques, so that the strains can utilize simple sugars as energy sources and other substrates to generate oligosaccharides during fermentation, and afterwards target HMOs will be obtained by isolation and purification. The more complex a target HMO structure is, the more challenging the metabolic engineering becomes. Over 40 HMO structures including building blocks have been

generated by microbial metabolic engineering, while they are restricted to small oligosaccharides (Faijes et al., 2019). Since 2015, HMOs produced by fermentation with genetically modified strains that have been approved in the U.S. and EU include 2'-FL, LNnT, LNT, 2'-FL/DFL, 3'-SL, 6'-SL, and 3-FL (EFSA Panel on Nutrition et al., 2022; U.S. Food and Drug Administration). These HMOs were developed and commercialized at first since they are the most abundant oligosaccharides in human milk and have relatively simple structures with only 3 or 4 monosaccharide units. Currently, microbial fermentation is the main approach for commercial production of HMOs due to its cost-effectiveness, scalability, and large-scale manufacturing capacity.

Developing a microbial strain suitable for HMO generation is the first critical step. Frequently used for molecular cloning, *Escherichia coli* has become the most common host organism for HMO production. Host strain is modified to develop the production strain by introducing genes required for the HMO synthesis and removing genes interfering the metabolic pathway. Taking 2'-FL as an example, basic requirements for its biosynthesis include internalization of lactose, synthesis of GDP-L-fucose, and glycosylation of lactose with GDP-L-fucose. Thus, *lacY* is often overexpressed to encode lactose permease to actively take up lactose, while *lacZ* is inactivated to prevent the hydrolysis of lactose by lactose cleaving β -galactosidase; GDP-L-fucose can be synthesized via the *de novo* pathway by introducing enzyme activities including mannose-6-phosphate isomerase (*manA*), phosphomannomutase (*manB*), mannose-1-phosphate guanylyltransferase (*manC*), GDP-mannose 4,6-dehydratase (*gmd*), and GDP-fucose synthase (*wcaG*), while *wcaJ* knockout is performed to prevent the consumption of GDP-L-fucose; and then fucosyltransferase encoded by the introduced genes such as *fucT2*, *wbgL* or *futC* synthesizes the 2'-FL (Bode et al., 2016; Bych et al., 2019; U.S. Food and Drug Administration, 2015c, 2016, 2019). Besides *E. coli*, other microbes, like *Corynebacterium glutamicum* and *Saccharomyces cerevisiae*, have been used as production microorganisms (U.S. Food and Drug Administration, 2021b; Xu et al., 2023).

Developing microbial strains that can produce more complex HMOs has attracted interests from both industry and academia. Identifying enzymes to construct appropriate metabolic pathways is the key, and knowledge gained in enzymatic or chemoenzymatic synthesis may help with enzyme selection and engineering in microbial fermentation. Even if capacity for HMO generation has developed in the strains, many of them are not suitable or competitive for industrial productions. Challenges like genetic instability of engineered strains are involved at scale-up. During large-scale fermentation, high-performing production strains may lose the production capacity due to metabolic burden and product toxicity, turning into low-producing or nonproducing variants (Rugbjerg & Sommer, 2019). To mitigate the loss of desired phenotypes in production, measures can be taken in the phase of strain development, such as chromosomal integration rather than plasmid-mediated engineering, knockout of an entire gene rather than single-point mutations, or design of plasmid addition systems (Lee & Kim, 2015; Rugbjerg & Sommer, 2019; Snoeck et al., 2019). Besides, cost saving is a lasting theme in the industry. Current fermentation processes require lactose to be added as a substrate, which is an expensive starting material. *E. coli* strain for *de novo* biosynthesis of 2'-FL using inexpensive sucrose as the sole carbon source has been developed to reduce the costs, and the final production strain produced over 60 g/L of 2'-FL after fermentation for 84 h (Parschat, Schreiber, Wartenberg, Engels, & Jennewein, 2020). More strategies for developing industrial microbial strains have been discussed in previous reviews (Choi et al., 2019; Lee & Kim, 2015; Rugbjerg & Sommer, 2019). Overall, to be industrially competitive, strain development needs to be integrated with process optimization.

The manufacturing process can be broadly divided into upstream processing and downstream processing. Upstream processing is often referred to fermentation stage, at which the production strain will grow and express the target HMO. Upstream processing can be further divided into media preparation, seed cultivation, and product fermentation (U.S. Food and Drug Administration, 2016, 2019). Major HMO manufacturers

have fermenter volumes over 200 m³ (Bych et al., 2019). Fermentation is performed in a chemically defined medium mainly consisting of simple sugars like glucose and/or sucrose as well as glycerol as carbon source, mostly lactose as substrate for HMO synthesis, inorganic salts as nutrients, and certain trace elements as inducer, pH control agent, defoamer, complexation aid or additional processing aids (U.S. Food and Drug Administration, 2015c, 2016, 2019). Process conditions such as pH, temperature, pressure, and oxygen availability are tightly controlled. The fermentation process continues until a certain level of target HMO is achieved. Key performance measurements of the process include productivity (rate), product concentration (titer), and yield. Ideally, the HMO product is excreted into the fermentation medium, and concentrations and yields of the HMO are high. Otherwise, undesirable fermentation performance will create burdens on downstream processing and overall cost.

Downstream processing is the post-fermentation stage, often referred to isolation and purification (U.S. Food and Drug Administration, 2018a, 2019). After upstream processing, the fermentation broth contains cell biomass, residual fermentation media, various carbohydrates, and other byproducts that need to be separated from the target HMO. Thus, a series of isolation and purification steps are applied to generate the final high-purity product. Alternative purification technologies or unit operations are adopted to accommodate production at different manufacturing sites, and the sequence of operations can vary if the targeted product purity is achieved. In general, for HMOs excreted into the medium where cell lysis is unnecessary, downstream processing usually starts with the separation of microbial biomass (fermentation strains and cell debris) from fermentation medium either by centrifugation or by filtration. Large biomolecules (e.g., proteins, nucleic acids, and lipopolysaccharides) are often removed by ultrafiltration, and small molecules (e.g., small carbohydrates, organic acids, amino acids, minerals, and salts) are removed by nanofiltration. Charged compounds (e.g., organic acids, inorganic salts, trace metals, proteins, and DNA) are often removed by ion-exchange resins, and residual charged components can be further removed via electrodialysis. Color and off-odor compounds are removed from solution by treatment with an adsorbent of charcoal, activated carbon or polymeric resin. Between different steps, concentration of the solution can be achieved by evaporation, nanofiltration or reverse osmosis. In some cases, the target HMO is purified by crystallization from the concentrated solution, and then solid crystallized product is isolated and washed followed by drying and packaging. In other cases, the concentrated HMO solution is directly packaged or dried into powder. For 2'-FL produced by microbial fermentation approved in the U.S., the minimum contents of 2'-FL documented in product specifications range from 82% to 96%, and most of them are above 90% (U.S. Food and Drug Administration, 2015c, 2016; 2018a; 2018b, 2019, 2020; 2021a; 2021b; 2022a; 2022b).

4.5. Mammary cell cultivation

Regarding biotechnologically synthesized HMOs, enzymatic synthesis and microbial fermentation are the approaches that often mentioned nowadays, while cell cultivation technology is overlooked. Indeed, using mammary cell culture to produce milk product has received growing interest along with the rising attention to cell-cultured meat. Despite technical and regulatory uncertainties, biotech startups have been founded and raised funds to accelerate the research and production of cell-cultured milk (Cohen & Cassidy, 2022). Up to date, it is difficult to produce the whole milk with cellular technology; thus, startup companies often start with culturing mammary cells to secrete high-value milk components such as bioactive proteins and oligosaccharides (Gabison, 2020; Strickland, 2022; Yu, 2021).

Mammary cells are proliferated and differentiated under hormonal regulation during pregnancy; aqueous solutes of milk including major proteins and oligosaccharides are secreted by polarized mammary epithelial cells into the lumen through an exocytotic pathway *in vivo*

(McManaman & Neville, 2003). Producing oligosaccharides from cultured cells is mimicking such process outside of the body. Obtaining functional mammary cells or mammary-like cells is the fundamental step (Fig. 4). There are two common approaches to generate functional mammary cells *in vitro* or *ex vivo*. One approach is isolating milk producing cells from mammary tissues, which is adopted by several startups such as USA-based Biomilk and Israel-based Wilk (formerly BioMilk). The other approach is using stem cell technology to generate mammary like cells. TurtleTree Labs, a Singapore-based startup, separates mesenchymal stem cells from milk or other mammalian samples (e.g., adipose tissue, mammary tissue, umbilical cord and cord blood), and then differentiates the stem cells into engineered mammary cells (e.g., mammary epithelial and mammary luminal cells) that are capable of secreting milk components (Rye, Lin, Toor, Yong, & Shahid, 2021). Next, these mammary cells need to grow and proliferate under suitable culturing conditions.

Bioreactor processing is critical for milk component production. Biotech startups are filing patents on bioreactor systems for culturing cells to produce milk products (Fima, 2022; Goh, Wu, & Yong, 2022; Strickland, 2021). Unlike microbial strains in fermentation, mammary cells are polarized with a basal surface for nutrient absorption and an apical surface for milk secretion. Thus, bioreactors are designed to let mammary cells attach to structural scaffolds, which support the compartmentalization and ensure nutrient supply and product secretion in a correct direction. Culturing media and atmosphere are controlled for mammary cell cultivation. Then, the secretion of milk components can be stimulated by certain environmental conditions, such as hormones, stimulants as well as temperature alteration. Secreted milk components are collected, and products are further isolated.

5. Summary and outlook

Nowadays, various approaches have been developed to obtain HMOs. Each approach has its characteristics and challenges, determining its suitable applications. Systematically considering the strategies in these approaches and integrating the knowledge may create new solutions to address the challenges and accelerate the advancement of this field.

Isolation of oligosaccharides from human milk is the original way to obtain HMOs, which paved the way for a detailed exploration of this group of bioactive compounds. Although this approach is not suitable to produce oligosaccharides as dietary ingredients due to the availability and ethical issues, it is irreplaceable in certain research regarding HMOs, including elucidating their endogenous biosynthetic pathways and mapping their variations in different populations or individuals. Also, over 200 HMO species have been found, but most of them are hard to be obtained via other approaches. Human milk is a valuable source of oligosaccharides for the discovery of unrevealed structures and biological functions.

Dairy streams are sources of milk oligosaccharides suitable for industrial processing. Although BMOs and CMOs are less abundant than HMOs in milk, they are concentrated in dairy byproducts and sufficiently available for enrichment by large-scale membrane separation (Wang & Yu, 2021). Thorough removal of lactose to isolate highly purified milk oligosaccharides is technically and economically challenging, so that producing oligosaccharide-enriched products rather than isolating pure oligosaccharides is more feasible in the dairy industry. BMOs have simpler and less fucosylated structures than HMOs. Using enriched BMOs as precursors for chemical and/or enzymatic synthesis can bridge the gap and produce more structures identical to HMOs (Bode et al., 2016; Weinborn et al., 2020).

Strategies for chemical and/or enzymatic synthesis of oligosaccharides have been investigated independently and in combination. Chemical, enzymatic, and chemoenzymatic synthesis can assemble a wide range of HMOs with defined structures. However, the isolation of product oligosaccharides from the reaction mixture is usually labor

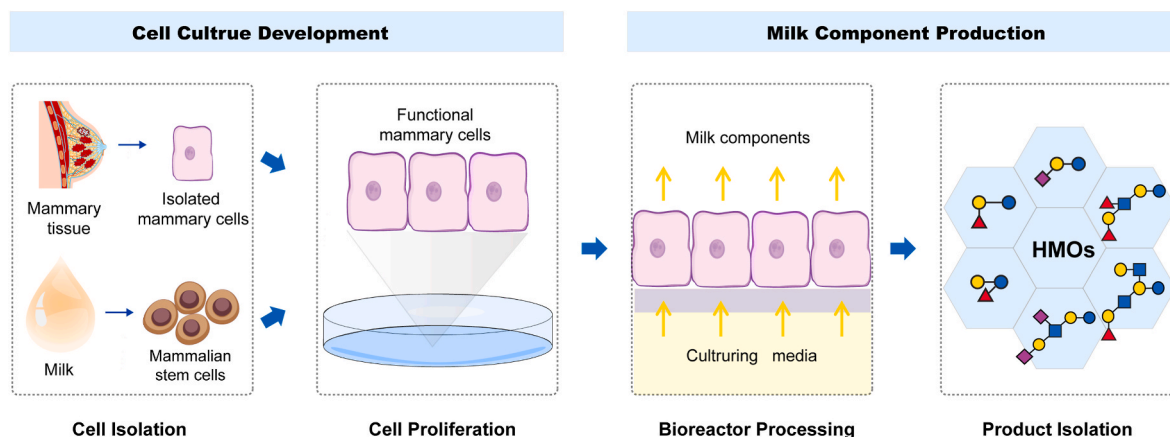


Fig. 4. Strategies to produce milk components including HMOs by mammary cell cultivation. Functional mammary cells are generated by isolating milk producing cells from mammary tissues or by differentiating mammalian stem cells (isolated from milk or other mammalian samples) into engineered mammary cells. Bioreactors are designed to let mammary cells attach to structural scaffolds, which support the compartmentalization and ensure nutrient supply and product secretion in a correct direction; then, secreted milk components are collected, and products are further isolated.

intensive, time-consuming, and typically with low yields of desired products. Consolidation of automated reaction platforms may help to streamline the synthetic process (Li et al., 2019; Lin et al., 2023). Nevertheless, considering the cost and scale, chemical, enzymatic, and chemoenzymatic synthesis are more suitable to produce HMOs as research materials rather than dietary ingredients at this stage.

With technological advances in metabolic engineering and the cost-effectiveness for large-scale production, microbial fermentation is promising and has become the prevailing approach for industrial manufacturing of targeted small HMOs as dietary ingredients. Currently, microbial fermentation of large HMOs remains challenging (Zhu et al., 2022). To address this challenge, identifying appropriate enzymes to construct suitable metabolic pathway is critical. Other approaches can offer knowledge that could assist in the metabolic engineering for fermentation approach. For example, enzymatic and chemoenzymatic synthesis approaches improve our understanding of selectivity and specificity of various glycosyltransferases. Meanwhile, further investigations on oligosaccharides obtained from human milk and other animal milk may provide insights into key enzymes and strategies involved in the endogenous synthesis and transport of these oligosaccharides as well as their precursors. Recently, Thomès et al. leveraged evolutionary relationships and network methodology to analyze a comprehensive dataset of milk oligosaccharides from >100 mammals without relying on known enzymatic activities, providing a better understanding of glycan biosynthetic pathways despite missing information (Thomès, Karlsson, Lundström, & Bojar, 2023). Taken together, all the information may help with pathway construction in metabolic engineering. Regarding manufacturing processes for microbial fermentation, quality improvement and cost saving are major trends in the industry. Currently, product testing is necessary to ensure the final product fulfils the product specification. Indeed, quality control measures can be applied throughout the whole process, for example, pre-screening of the attributes of added materials and in-process evaluation of the efficiency for microorganism removal. Process Analytical Technology, a risk-based framework that has been promoted in the pharmaceutical industry for manufacturing and quality assurance through timely measurements, can also be adopted in the HMO production to refine manufacturing processes and ensure product quality. In addition to product quality, reducing the processing cost is another critical consideration for large-scale HMO production. Cost reduction can be achieved by increasing productivity and enhancing efficiencies. Potential measures include using cost-effective raw materials as well as high-capacity membranes or resins and optimizing fermentation conditions and purification parameters. Moreover, advanced methods or

techniques can be integrated into the manufacturing with sophisticated designs, for example, incorporating selective fermentation for removal of mono- and disaccharides from oligosaccharides solutions to lower downstream processing costs and using simulated moving bed technology for continuous purification of large quantities of HMOs with high purity (Kruschitz & Nidetzky, 2020).

In recent years, biotech startups have demonstrated promising proof-of-concept methods of producing milk components, including oligosaccharides, with cellular technology. However, producing cell cultured milk oligosaccharides is still at an early stage. Key technical challenges include sustainably sourcing functional mammary cells, optimizing culture media and extracellular matrix, regulating cellular synthesis and HMO secretion, organizing cells in a three-dimensional space, and scaling up the bioreactor production. Besides, regulatory challenges and consumer perspectives need to be tackled before bringing cultured milk products to market. Knowledge from endogenous and exogenous synthetic strategies of HMOs can be integrated to address certain technical issues. Regulations on microbial fermentation or cell-cultured meat may shed light on the risk and safety assessment for cellular production of milk components. Singapore Food Agency was the first to approve the sale of cell-based meat in December 2020, the U.S. Food and Drug Administration completed its first pre-market consultation for a human food made from cultured animal cells in November 2022, and the European Food Safety Authority organized a scientific colloquium on cell culture-derived foods and food ingredients in May 2023. The landscape for cell-cultured milk is expected to be expanded with the development of cell culture technologies and regulatory frameworks.

6. Conclusions

Due to the discovery of bioactivities and health benefits of human milk oligosaccharides, accessing HMOs has been received increasing interest in academia and in industry. In this review, we provide an update on the approaches to obtain HMOs, which are: isolation from human milk, enrichment from dairy streams, chemical synthesis, enzymatic synthesis, chemoenzymatic synthesis, microbial fermentation, and mammary cell cultivation. Strategies of endogenous HMO biosynthesis and all other approaches to obtain HMOs can be systematically considered to inspire new solutions addressing current challenges. The advances in HMO generation and production will accelerate their applications in infant formulas, medical foods, and dietary supplements to improve the health of infants, mothers, those with special healthcare needs, and the general population.

Data availability

No data was used for the research described in the article.

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