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# From lab bench to formulated ingredient: Characterization, production, and commercialization of human milk oligosaccharides

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ABSTRACT

Human milk oligosaccharides (HMOs) are known to positively influence infant health. Extensive variation exists in the levels, diversity, and complexity of oligosaccharides in the milk of a lactating mother. Until recently, limited availability of HMOs hampered their use in clinical applications. Most HMOs are unique to human milk, and have proven difficult and expensive to isolate and synthesize. Added to that, analysis of these complex glycans in milk samples requires state-of-the-art analytical instruments and associated technologies. The current review provides a critical overview of methods used in HMO analysis, and highlights the importance of understanding the factors which influence their composition and structural diversity. We also discuss recently employed strategies to overcome the availability of HMOs at industrial scale including microbial metabolic engineering and chemoenzymatic techniques. Finally, we examine how these recent advancements have opened up new avenues for future research and nutraceutical applications.

## 1. Introduction

Human breast milk is recognised as the gold standard in infant nutrition (Walker, 2010), with the World Health Organization (WHO) and the United Nations Children's Fund recommending exclusive breastfeeding up to at least 6 months of age (WHO, 2001). According to a systematic review of over 400 studies, breastfeeding was found to reduce the likelihood of atopic dermatitis, asthma, respiratory tract infections, gastrointestinal infections, type 1 and 2 diabetes, obesity, and necrotizing enterocolitis in preterm infants (Ip et al., 2007). The properties and chemical composition of human breast milk have been researched since the beginning of the 20th century, in an attempt to understand how specific structures in breast milk contribute to its diverse functionalities (Elgood, 1910; Gerstenberger, Ruh, Brickman, Leslie, & Ochsner, 1919). The composition of maternal milk changes constantly throughout lactation and adapts in order to fulfil the nutritional needs of the neonate for healthy growth and development (Andreas, Kampmann, & Mehring Le-Doare, 2015). The human infant enters the world with a functionally naïve immune system, which leaves the newborn at risk of infection. Human milk affords protection to the infant during this initial period by providing bioactive compounds which facilitate immune tolerance (Turfkruyer & Verhasselt, 2015), prevent pathogenic infection (Newburg, Ruiz-Palacios, & Morrow, 2005), reduce intestinal permeability (Taylor, Basile, Ebeling, & Wagner, 2009), and act as highly specific prebiotics for particular commensal micro-organisms (Zivkovic, German, Lebrilla, & Mills,

*Abbreviations:* HMO, human milk oligosaccharide; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Neu5Ac, *N*-acetylneuraminic acid; GOS, galactooligosaccharides; FOS, fructooligosaccharides; LNB, lacto-*N*-biose; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; DFL, difucosyllactose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-*N*-tetraose; LNnT, lacto-*N*-neotetraose; LNH, lacto-*N*-hexaose; LNnH, lacto-*N*-neohexaose; LNFP, lacto-*N*-fucopentaose; FLNH, fucosyl-lacto-*N*-hexaose; LNDFH, lacto-*N*-difucohexaose; LDFT, lactodifucotetraose; LST, sialyllacto-*N*-tetraose; DSLNT, disialyllacto-*N*-tetraose; FSLNnH, fucosyllacto-*N*-neohexaose; EFSA, European Food Safety Authority; WHO, World Health Organisation; FDA, Food and Drug Administration; GRAS, Generally Regarded as Safe; QPS, Qualified Presumption of Safety; HPLC, high performance liquid chromotography; RP-LC, reversed phase liquid chromotography; NP-LC, normal phase liquid chromtography; HPAEC, high-pH anion-exchange chromatography; SEC, size exclusion chromatography; SPE, solid phase extraction; NMR, nuclear magnetic resonance; CE, capillary electrophoresis; MS, mass spectrometry; MS/MS, tandem mass spectrometry; QqQ, triple quadruple; TOF, time of flight; ESI, electrospray ionization; LIF, laser-induced fluorescence; PGC, porous graphitic carbon; IMS, ion mobility spectrometry; GTs, glycosyltransferases; GHs, glycosylhydrolases; PMP, 1-phenyl-3-methyl-5-pyrazolone; APTS, 8-aminopyrene, 3, 6-trisulfonic acid; FD, field desorption; MALDI, matrix-assisted laser desorption/ionization; UV, ultraviolet; CV, coefficient of variation; BMI, body mass index; OPME, one-pot multi-enzyme; BF, breast fed; FF, formula fed

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2011). However, in cases where breast-feeding is not possible, or when the nutrient demands of the infant surpass what is provided by human milk, fortified infant formulas which aim to mimic the functions of breast-milk have been developed. Targeting the prebiotic components in human milk which guide progression of infant's microbiota represents an important strategy aimed at replicating the beneficial effects of breast-feeding (van den Elsen, Garssen, Burcelin, & Verhasselt, 2019). One of the most important components imparting this prebiotic function is believed to be represented by the human milk oligosaccharide (HMO) fraction. In addition to guiding the progression of the infant intestinal microbiota, HMOs also demonstrate antimicrobial properties against Group B *Streptococcus* and other pathogens (Ackerman et al., 2017, 2018; Lin et al., 2017). HMOs have even recently been shown to act as adjuvants to potentiate the function of antibiotics (Chambers et al., 2020; Craft, Caddy, & Townsend, 2018). These molecules also act as soluble decoy receptors reducing colonisation of and infection by pathogenic bacteria (Laucirica, Triantis, Schoemaker, Estes, & Ramani, 2017). Studies have also demonstrated that HMOs support immune development (Donovan & Comstock, 2016), moderate intestinal permeability (Chichlowski, De Lartigue, German, Raybould, & Mills, 2012), influence intestinal cell responses (Kong et al., 2019), and reduce occurrences of necrotizing enterocolitis (Autran et al., 2018; Good et al., 2016). Substantiation of a cause-effect relationship between HMOs and infant health has encouraged the design of more functional formulas, such as through the inclusion of complex oligosaccharide structures (Smilowitz, Lebrilla, Mills, German, & Freeman, 2014). Recent studies by Steenhout et al. and Puccio et al. have shown that consumption of a formula supplemented with two HMOs (2′-fucosyllactose [2′-FL] and lacto-*N*-neotetraose [LNnT]) is safe and well-tolerated, but most importantly results in gut microbiota progression similar to that witnessed in breastfed infants (Puccio et al., 2017; Steenhout et al., 2016). However, it is unlikely that the introduction of one or two HMOs will be sufficient in fully mimicking all the beneficial effects associated with the total HMO pool. It is anticipated that incorporation of more and more distinct HMOs, as they become available at commercial levels, will revolutionize the landscape of paediatric nutrition, and will offer opportunities to improve infant health worldwide.

Such a scenario cannot be achieved without accurate measurements of individual HMO structures and examination of how these HMO components are degraded/metabolized in the gastrointestinal tract. While the powerful tool sets of metagenomics, metatranscriptomics, and metabolomics have been employed routinely over the past decades, glycomics still has its technological challenges and may thus be considered a critical technology/knowledge gap in dairy, nutrition and clinical research. The concentrations of HMOs reported in the literature vary significantly (Thurl, Munzert, Boehm, Matthews, & Stahl, 2017) which to a considerable degree can be explained by the influence of lactation stage, genetic/ethnicity factors (such as secretor status/lewis factor), and environment on the expression of HMOs (Erney et al., 2000; Xu et al., 2016). However, one major contributor to discrepancies in literature is the wide range of non-standardized analytical methods used across various laboratories. A recent systematic review by Thurl et al. emphasised this lack of standardization, stating that "nearly every study used a unique methodology with regard to the preparation, derivatization, and separation of samples, and the detection and quantification of HMOs" (Thurl et al., 2017). Advancements and standardisation of analytical methods, which allow for HMO quantification that is rapid enough for routine and high-throughput applications, will be crucial to understanding how the mother-infant dyad functions to protect the vulnerable and developing neonate. Considering the multitude of functions assigned to HMOs, and often to specific structures, it is very important to have robust methods to accurately determine the levels of these structures in human milk. The aims of this review are to give a critical overview of the methods used in the analysis of human milk oligosaccharides, while also highlighting the importance of understanding the factors which influence the composition and structural diversity of HMOs. The review also aims to provide a summary of recent strategies which have been employed to overcome the lack of availability of HMOs and the regulatory framework involved to allow such artificial HMOs to be incorporated in commercial milk formulas. Finally, we examine the importance of placebo-controlled clinical intervention trials in supporting and furthering our understanding of HMO biology and the significance of these compounds in infant nutrition. As a semi-quantitative review, PubMed databases were searched (last search on 04 May 2020) according to a predetermined search strategy using the following keywords: Human milk oligosaccharides, synthesis, secretor, lactation. Original studies and review articles published in English involving HMOs were eligible for inclusion.

## 2. HMO characterization

Analytical characterization of HMOs has proven problematic, in particular due to the complex stereochemistry of these compounds, as well as their branched-chain structures (Grabarics, Csernák, Balogh, & Béni, 2017). Early analyses on the glycome of human milk were directed towards identifying and measuring a limited number of individual sugars, and the technology platforms applied only captured an incomplete snapshot of the whole picture (Mizoguchi, Mizuochi, & Kobata, 1982). The past decade has witnessed major advancements in glycomics technology platforms, which allow for in-depth rapid, sensitive, and reproducible characterization of structural diversity in complex biofluids such as milk (Yan, Ding, & Liang, 2017). Such technologies are capable of resolving many individual HMO structures in a single analytical run, with (semi) quantitative assays preferred over basic qualitative analysis (Mantovani, Galeotti, Maccari, & Volpi, 2016). Documenting and understanding the inter-individual and intra-individual variations of HMO concentrations is of utmost importance not only in basic biological research, but also for the development of infant nutritional products.

The majority of published HMO analytical methods incorporate similar isolation and purification processes in order to generate a highly pure HMO fraction from human milk. Initial centrifugation at low temperatures removes lipids, while precipitation and C-18 solid phase extraction (SPE) eliminates proteins and peptides from the sample. This is generally followed by porous graphitized carbon SPE, which removes lactose, monosaccharides, and salts from the sample. Once extracted, the HMOs must be separated in order to perform quantification analysis. Gel Permeation was one of the earliest separation techniques to be employed for the separation of HMOs (Thurl, Offermanns, Müller-Werner, & Sawatzki, 1991). Gel matrices such as Sephadex G-25 (Obermeier, Rudloff, Pohlentz, Lentze, & Kunz, 1999), Bio-Gel P-4 (Donald & Feeney, 1988) and TSK HW40 (Thurl, Henker, Taut, Tovar, & Sawatzki, 1993) separated HMOs on the basis of acidity and size. Although gel permeation is still used in purification of enriched HMO fractions, it is no longer employed for analysis due to its limited resolution.

Rapid developments in chromatography have taken place in recent years and are summarised in a recent review by O'Sullivan, Salcedo, and Rubert (2018). These methods are accurate and sensitive, and are capable of resolving many HMO structures including configurational isomers. Traditionally used methods for this purpose are reverse phase (RP) or normal phase (NP) high performance liquid chromatography (HPLC) coupled to UV detectors (Chaturvedi, Warren, Ruiz-Palacios, Pickering, & Newburg, 1997; Sumiyoshi et al., 2003). Oligosaccharides in their native form have poor UV absorption and therefore derivatization of HMOs is required which not only facilitates their separation and retention but also increases sensitivity when using UV detectors (Sumiyoshi et al., 2003). Derivatization methods employed for chromatographic analysis of HMO are summarised in Table 1, which include reduction of HMOs with sodium borohydride to their alditol form (Bao, Chen, & Newburg, 2013). However, these approaches have

**Table 1**
Derivitization, Separation, and Detection Techniques Employed for Analysis of HMOs.

| Separation | Detection | Derivitization | HMO Analytes | Location | Gestation | Secretor status | N | Reference |
|---|---|---|---|---|---|---|---|---|
| HPAEC | PAD | No derivitization | Neutral/Acidic | France | Term | NS | 15 | Viverge et al., 1990 |
| HPAEC | PAD | No derivitization | Neutral/Acidic | Germany | Term | NS | 2 | Kunz et al., 1996 |
| HPAEC | PAD | No derivitization | Neutral/Acidic | NS | NS | NS | NS | Thurl et al., 1996 |
| RP-LC | UV | Perbenzoylation | Neutral | Mexico | Term | NS | 50 | Chaturvedi et al., 1997 |
| HPAEC | PAD | No derivitization | Neutral/Acidic | Italy | Term | NS | 18 | Coppa et al., 1999 |
| NP-LC/RP-LC | MALDI-TOF MS/MS | 2-Aminoacridone | Neutral/Acidic | UK | Term | +/− | 3 | Charlwood, Tolson, Dwek, & Camilleri, 1999 |
| HPAEC | PAD | No derivitization | Neutral | Global (10 countries) | Term | + | 435 | Erney et al., 2000 |
| CE | LIF | 2-Aminoacridone | Neutral/Acidic | China | Term | NS | 25 | Song, Weng, Wu, & Xia, 2002 |
| RP-LC | UV | Pyridylamination | Neutral | Japan | Term | NS | 16 | Sumiyoshi et al., 2003 |
| RP-LC | UV | No derivitization | Acidic | Spain | Term | NS | 12 | Martín-Sosa, Martín, García-Pardo, & Hueso, 2003 |
| PGC-Nano-LC | TOF-MS | Reduction with borohydride | Neutral/Acidic | USA | Term | | 5 | Ninonuevo et al., 2008 |
| CE | UV | No derivitization | Acidic | USA | Term | NS | 13 | Bao, Zhu, & Newburg, 2007 |
| RP-LC | UV | PMP | Acidic | Japan | Term | NS | 20 | Asakuma et al., 2007 |
| RP-LC | UV | PMP | Neutral | Japan | Term | + | 12 | Asakuma et al., 2008 |
| RP-LC | UV | 2-Anthranilic Acid | Neutral | Samoa | Term | NS | 12 | Leo et al., 2009 |
| PGC- HPAEC | PAD | No derivitization | Neutral/Acidic | Germany | Term | +/− | 30 | Thurl et al., 2010 |
| CE | LIF-MS | APTS | Neutral | Netherlands | 32 week | NS | 5 | Albrecht et al., 2010 |
| NP-LC | FD-EI-MS | 2-Aminoacridone | Neutral/Acidic | Italy | Term | +/− | NS | Galeotti et al., 2012 |
| PGC-LC | MS | Reduction with borohydride | Neutral | USA | Term | + | 20 | Bao et al., 2013 |
| NMR | | No dervitization | Neutral | USA | Term | +/− | 52 | Smilowitz et al., 2013 |
| LC and GC | MS and MS/MS | No derivitization | Neutral/Acidic | USA | Term | +/− | 17 | Goehring, Kennedy, Prieto, & Buck, 2014 |
| NP-LC | ESI-MS | Reduction with borohydride | Neutral/Acidic | USA | Term | +/− | 20 | Hong et al., 2014 |
| GC | ESI-MS | *O*-trimethylsilyl-oxime | 2′-FL, 3-FL | Hungary | Term | + | 2 | Balogh, Szarka, & Béni, 2015 |
| CE | LIF | APTS | Neutral | Netherlands | Term | +/− | 12 | Olivares et al., 2015 |
| NMR | | No dervitization | Neutral | USA | Term/Preterm | NS | 28 | Spevacek et al., 2015 |
| RP-LC | MS/MS | Reduction with borohydride & permethylation | Neutral | NS | Term | NS | NS | Oursel, Cholet, Junot, & Fenaille, 2017 |
| PGC-LC | MS/MS | Reduction with borohydride | Neutral/Acidic | | | | | |
| UPLC | QqQ-MS | No derivitization | Neutral/Acidic | US/Malawi | Term | +/− | 45/88 | Xu et al., 2017 |
| PGC-LC | ESI-MS | Reduction with borohydride | Neutral/Acidic | Brazil | Term | NS | 41 | Tonon et al., 2019 |

NS = not specified.

disadvantages; namely, sample clean-up is required which is time-consuming and considerable disparity has been observed between analyses. As an alternative to derivatization and UV detection, high-pH anion-exchange chromatography (HPAEC) is more commonly used for quantification of HMO fractions (Kunz, Rudloff, Hintelmann, Pohlentz, & Egge, 1996; Viverge, Grimmonprez, Cassanas, Bardet, & Solere, 1990). One of the earliest applications of HPAEC described in literature was in 1996 when Thurl et al. employed SEC for pre-fractionation, and pulsed amperometric detection (PAD) for quantification of neutral and acidic structures (Thurl, Müller-Werner, & Sawatzki, 1996). Although RP/NP HPLC and HPAEC are regarded historically as important chromatographic techniques, researchers have also recently developed methods outside of standard liquid chromatography. These include Nuclear Magnetic Resonance (NMR) methods (Smilowitz et al., 2013) and electrophoresis techniques such as capillary electrophoresis (CE) (Olivares et al., 2015).

Mass Spectrometry (MS) determines molecular weights of eluting compounds and, is therefore, capable of analysing individual HMO structures. This allows for measurement of HMOs with more specificity versus detection using UV or amperometric techniques. In recent years, MS has begun to play an increasingly important role in HMO characterization (Mirgorodskaya, Karlsson, Sihlbom, Larson, & Nilsson, 2018; Remoroza, Mak, De Leoz, Mirokhin, & Stein, 2018; Tonon, Miranda et al., 2019). Low resolution tandem mass spectrometric analysers such as triple quadruple (QqQ) have been employed for quantification of HMOs (Xu et al., 2017). Analysis of HMOs using LC-QqQ methods is robust, sensitive, cost-effective and accurate. However, detection requires the use of analytical standards, and doesn't allow for the detection of unknown compounds. Technological improvements in high-resolution mass spectrometric analysers have opened new possibilities for analysis of glycans. The resolving power of instruments such as time of flight (TOF) and Orbitrap analysers is up to 10 times higher than that of QqQ and low resolution instruments (O'Sullivan et al., 2018). One of the major advantages of high-resolution mass spectrometers is the identification of unknown compounds. The development of novel, combinational techniques using MS has allowed for the analysis of multiple HMO structures in large sample numbers. These methods include NanoLC-Chip/Q-TOF MS methods (Totten et al., 2014), capillary electrophoresis with laser-induced fluorescence and MS (CE-LIF-MS) (Albrecht, Schols, van den Heuvel, Voragen, & Gruppen, 2010), and liquid chromatography electrospray ionization MS (LC-ESI-MS) (Tonon, de Morais et al., 2019). Ion mobility spectrometry (IMS) techniques have been developed in recent years and offer improved separation and identification of structurally similar HMO structures (Hofmann et al., 2017). IMS coupled to MS instruments has also been employed recently for analysis of HMOs and other glycan structures (Ben Faleh, Warnke, & Rizzo, 2019; Khanal, Masellis, Kamrath, Clemmer, & Rizzo, 2018)

During the last ten years the glycomics field has gone through an extraordinary expansion in terms of technology developments, which have allowed detailed and a more comprehensive understanding of the structure of individual HMOs as well their concentrations in human breast milk at different lactation stages and across geographically distinct areas. Accurate measurement of the physiological intakes of HMOs across various population groups has furthered our understanding of the factors that contribute to variations in HMO composition.

## 3. Variations in HMO structure and concentration in human milk

Inter-individual variation of HMO concentrations is pronounced, and is strongly dependent on maternal genetics and allelic variation in the Secretor (*Se*) and Lewis (*Le*) genes (Thurl et al., 2010). Besides the influence of *Se* and *Le,* substantial differences between HMO profiles occur in the milk produced by women with the same genetic background. Therefore, non-genetic factors must also influence HMO composition (Samuel et al., 2019). Although studies are limited, it is thought that maternal factors such as parity and body mass index (BMI) (Azad et al., 2018; Jantscher-Krenn et al., 2019), as well as environmental factors such as geographical location, influence the composition of HMOs (Erney et al., 2000; van Leeuwen et al., 2018). An observational study of breastfeeding mothers in Brazil has also described apparent associations between maternal allergic disease and infant's sex on the one hand and HMO concentrations on the other (Tonon, Miranda et al., 2019). Moreover, results from a recent intervention study have demonstrated that HMO composition can be influenced by maternal probiotic supplementation during the late stages of pregnancy. The study, conducted by Seppo et al. in Finland, analysed data from 81 women who were fed either a placebo or a twice-daily dose of a combination of *B. breve* Bb99, *L. rhamnosus* GG, *L. rhamnosus* LC705 and *P. freudenreichii* subsp. *shermanii* JS. Significantly higher concentrations of 3-Fucosyllactose (3-FL) and 3′-Sialyllactose (3′-SL) were found in the colostrum of the probiotic group when compared to the control group (Seppo et al., 2019). While it has long been established that HMOs shape microbial communities, the latter study suggests that microbes play a role in modifying HMO composition and thus revises the hypothesis that interactions occurring between HMOs and the human microbiome is a one-way street.

Additional to inter-individual variations, intra-individual variations are pronounced with HMO composition altering constantly throughout the nursing period (Sprenger, Lee, De Castro, Steenhout, & Thakkar, 2017). Concentrations of HMOs alter throughout breast-feeding according to lactation stage (decreasing from 20 to 25 g/L in colostrum to 5–20 g/L in mature milk), time of day, and mother's diet while continuously adapting to the requirements of the infant (Austin et al., 2016; Thurl et al., 1993; Xu et al., 2017). A number of studies assessing the HMO concentrations in the milk of mother's who had delivered preterm infants have highlighted that significant differences in HMO composition exist when compared to term milk. (De Leoz et al., 2012; Gabrielli et al., 2011; Nakhla, Fu, Zopf, Brodsky, & Hurt, 2007). Most notably, a recent study analysed HMO concentrations in 500 samples from 28 mothers nursing term infants, and 25 mothers who had delivered very preterm neonates. The study revealed elevated concentrations of sialylated HMOs were present in preterm milk, particularly 3′-SL (Austin et al., 2019). These observations align with the theory that the composition of milk adapts to satisfy the nutritional requirements of the neonate, though the underlying mechanistic process that drives these compositional changes is as yet obscure.

Oligosaccharides in human milk are characterised by remarkable structural diversity (Ayechu-Muruzabal et al., 2018). This diversity is brought about by 12 glycosidic linkage options between the monosaccharide building blocks of Glucose (Glc), Galactose (Gal), *N*-acetylglucosamine (GlcNAc), Fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac). The chemical structure of monosaccharide building blocks are illustrated in Supplementary Fig. 1. HMO synthesis occurs in the lactating mammary gland and is under the control of a series of glycosyltransferases, including fucosyltransferases, sialyltransferases, galactotransferases, and acetylglucosaminyltransferases (Bode, 2006; Urashima, Fukuda, & Messer, 2011). The pathways for HMO synthesis are summarised in Fig. 1. All HMOs follow a basic blueprint and contain a lactose molecule (Galβ1-4Glc) at their reducing end (Bode, 2012). Linear chains form when the monosaccharide GlcNAc attaches to the galactose moiety through a β1-3 linkage, while chain branching is introduced via β1-6 glycosidic linkages. Linear structures are described as "para-HMO", while those with chain branching are termed "iso-HMO". Human milk is particularly rich in type 1 oligosaccharides, with lacto-*N*-biose (LNB; Galβ1-3GlcNAc-) as the building unit, while the less prevalent type 2 oligosaccharides contain *N*-acetyllactosamine as the building block (Galβ1-4GlcNAc) (Fig. 1 & Supplementary Fig. 1) (Smilowitz et al., 2014). Lactose, linear, and branched oligosaccharides can be sialylated by α2-3 or α2-6 linkages and/or fucosylated by α1-2, α1-3, or α1-4 linkages (Fig. 1). The smallest oligosaccharides are formed when the sialic acid, NeuAc, attaches to the lactose core to form

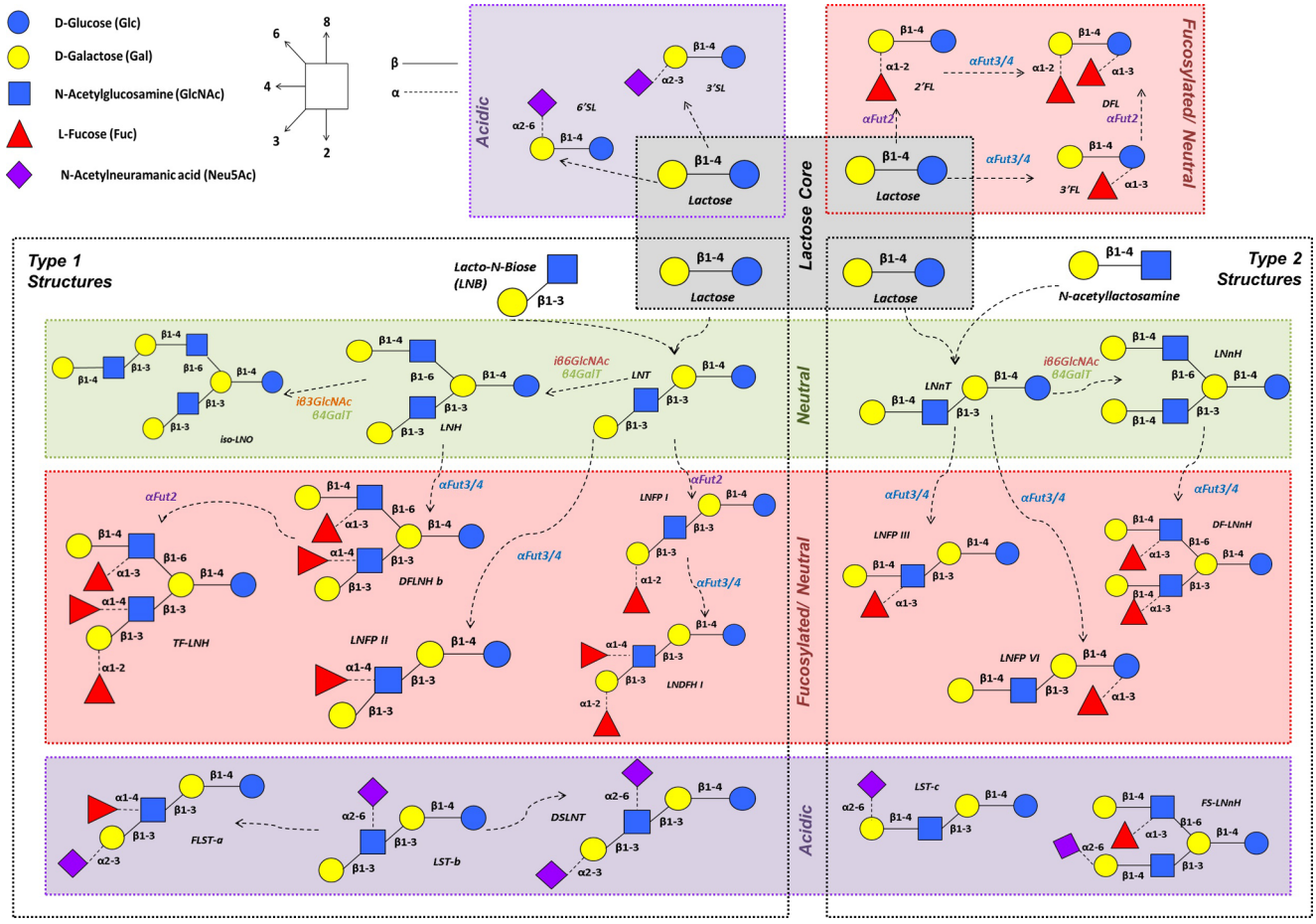


**Fig. 1.** Biosynthetic pathways for assembly of complex human milk oligosaccharide (HMO) structures. Diversity is brought about by conjugation of 5 monosaccharide building blocks via 12 potential glycosidic linkages. Synthesis occurs in the mammary gland and is directed by a series of glycosyltransferases including galactotransferases (β4-GalT), acetylglucosaminyltranferases (iβ3- and iβ6-GlcNAc), fucosyltransferases (α-Fut2 and α-Fut3/4) and sialyltransferases. All HMOs contain lactose at their core. Type 1 HMO chains incorporate lacto-*N*-biose (LNB) disaccharide units, while type 2 HMO chains incorporate *N*-acetyllactosamine disaccharide units. The simplest sugars are formed through the addition of fucose to the lactose core to form the trisaccharides 2′- and 3- fucosyllactose (2′-FL and 3-FL) or addition of the sialic acid, Neu5Ac, to lactose to form the trisaccharides 3′- and 6′- siallylactose (3′-SL and 6′-SL).

the siallylactoses, 3'-SL and 6'-SL (Supplementary Fig. 1), or when fucose is added to lactose to generate the fucosyllactoses, 2′-FL and 3-FL (Supplementary Fig. 1) (Bode, 2012). The presence of sialic acid means that HMOs are often separated into two groups on the basis of their chemical charge: Neutral oligosaccharides which contain no charged monosaccharides (Glu, Gal, *N*-acetylglucosamine, Fucose) and acidic oligosaccharides which contain negatively charged residues of NeuAc. Acidic HMOs make up 12–14% of the total HMO, while 35–50% are fucosylated structures with the remaining 42–55% structures being non-fucosylated (Thurl et al., 2017). Decoration of HMOs with fucose is dependent on the mother's Secretor and Lewis blood group status (Fig. 2). Polymorphisms of Lewis and Secretor genes have one of the greatest impacts on the phenotypic profile of HMOs present in human milk (Blank, Dotz, Geyer, & Kunz, 2012).

### 3.1. Secretor status

While the biological variation (denoted as coefficient of variation or CV) for total HMOs is as high as 28% (Xu et al., 2017), even greater disparity is observed for certain HMO structures such as 2′-FL, 3-FL, difucosyllactose (DFL), lacto-*N*-fucopentaose (LNFP) I, and LNFP II. The CV for these structures in mature breast milk ranges from 42% to 84.3% (Smilowitz et al., 2014). Variations in these fucosylated HMO structures can be largely accounted for by allelic variations in the expression of specific glycosyltransferases. HMO composition mirrors maternal blood group characteristics (Newburg, Warren, Chaturvedi, & Ruiz-Palacios, 1999), whereby expression of Secretor and Lewis genes in the mammary gland determines the structure and abundance of HMOs (Blank et al., 2012; Thurl, Henker, Siegel, Tovar, & Sawatzki, 1997). The Secretor gene (*Se*) encodes the α1-2 fucosyltransferase (FUT2) enzyme, which attaches fucose residues via an α1-2 linkage to acceptor HMO chains, while the Lewis gene (*Le*) encodes for fucosyltransferase FUT3, which attaches fucose residues via α1-3 and α1-4 linkages to subterminal GlcNAc of type 1 HMO chains (Kunz et al., 2017). Four milk groups can be assigned based on the *Se/Le* blood group system, with each resulting in a different phenotypic HMO pattern as summarised in Table 2.

Mothers with a functional *FUT2* allele produce milk which is abundant in α1-2 fucosylated HMOs and are described as "Secretors". Secretor milk contains significant amounts of 2′-FL, lacto-*N*-difucohexaose (LNDFH I), and lacto-*N*-fucopentaose I (LNFP I) (Fig. 2). In contrast, mothers who are classified as non-secretors are homozygous for a non-active secretor gene, and their milk has low or undetectable levels of α1-2 fucosylated oligosaccharides. The milk of non-secretor mothers also contains significantly lower amounts of total oligosaccharides, but higher abundances of lacto-*N*-tetraose (LNT), LNFP II, LNFP III, and LNDFH II (Fig. 2). Globally, a substantial number of allelic variants of the secretor phenotype have been detected. In a study by Ferrer-Admetlla et al. the coding region of the *FUT2* gene was re-sequenced in 732 individuals from 39 human populations (Ferrer-

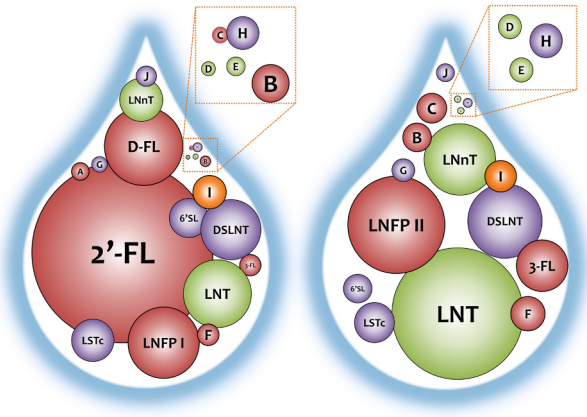


**Fig. 2.** Schematic representation of human milk oligosaccharide (HMO) profile in the milk of secretor mothers (left) and non-secretor mothers (right). Secretor milk contains large amounts of α1-2 fucosylated oligosaccharides, while non-secretor milk has undetectable levels of α1-2 fucosylated oligosaccharides. Non-secretor milk also contains lower concentrations of total oligosaccharides but has higher concentrations of LNT, LNFP II, and LNFP III. Diameters of each circle depict the concentration of quantified HMO. Concentrations adapted from (Thurl et al., 2017). Neutral non-fucosylated HMO = green. Neutral, fucosylated HMO = red. Acidic, non-fucosylated HMO = purple. Acidic, fucosylated HMO = orange. **A** = LNFP II, **B** = LNFP III, **C** = LNDFHII, **D** = LNH, **E** = LNnH, **F** = FLNH II, **G** = LST a, **H** = LST b, **I** = FSLNnH I, **J** = 3′-SL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Admetlla et al., 2009). The researchers identified 55 single-nucleotide polymorphisms (SNPs) in the *FUT2* gene. Non-secretor phenotypes were found to be present in all populations, but inter-population differences in secretors and non-secretors were notable (Ferrer-Admetlla et al., 2009).

Cross-sectional, observational studies have been conducted aimed at exploring the relationship between maternal genetics and HMO concentrations across various geographical locations. Perhaps the first comprehensive study of this kind was conducted by Erney et al. where HMO concentrations were measured in the milk of 435 lactating mothers from ten different countries. Significant differences in the concentrations of 2′-FL were observed, which were quantifiable in all milk samples from Mexican and Swedish mothers, but detected in only 46% of the milk samples collected in the Philippines (Erney et al., 2000). Expanding on this work, McGuire et al. analysed the HMO composition in the milk of 410 breastfeeding women from 11 distinct cohorts (McGuire et al., 2017). More advanced analytical methods and sampling approaches were used when compared to those used in the earlier study, but similar distribution trends were observed. The number of secretor mothers ranged from 98% in the Peruvian cohort to 65% in the Rural Gambian and Rural Ethiopian cohort. The percentage of secretors in Sweden, Spain, Urban Ethiopia, Kenya, and Urban Gambia was similar ranging from 76% to 85%. The Hispanic cohort in the USA was similar to that of Peru (95% secretors), while the other cohorts in the USA (of unspecified ethnicity) contained just 68% secretors. The authors speculated that the high percentage of secretors in Peruvian and US-Hispanic cohorts was due to historical evolutionary pressures that conferred α1-2 fuc related health benefits to these populations (McGuire et al., 2017). Perhaps the most interesting finding from this study was the divergence in secretors between rural and urban populations in both Ethiopia and Ghana. It would be expected that the populations would be genetically related, implying that environmental influences might also hold significance.

The precise reason for the geographical disparities is unknown, but scientists have speculated that the phenomenon could be the result of evolutionary forces acting on different continental groups (Ferrer-Admetlla et al., 2009). Since pathogens are powerful selective agents, it is probable that different pathogenic environments have acted on the *FUT2* locus throughout human history, driving the varying halotypes observed across populations. Results from population-based observational studies in infants verify that a possible relationship exists between *FUT2* gene expression and exposure to infectious diseases. Morrow et al. was one of the first groups to observe that α1-2 fucosylated glycans in breast milk confer protection against pathogens (Morrow, Ruiz-Palacios, Jiang, & Newburg, 2005). Consumption of secretor milk was shown to correlate with a lower incidence of infant diarrhoea caused by *Campylobacter*, calciviruses, and the heat stable enterotoxin of enterotoxigenic (ETEC) *E. coli* (Newburg et al., 2003). Conversely, it has been reported that non-secretor milk confers protection against Norwalk-like virus (Jiang et al., 2004), while a non-functional *FUT2* allele is also linked with slower development of HIV-1 infection (Kindberg et al., 2006). It is clear, therefore, that specific HMO structures act as anti-adhesion agents either directly, by competing with epithelial cell-receptor binding (Morrow et al., 2005; Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003) or indirectly, by promoting the colonization of beneficial bacteria in the gut (Garrido et al., 2015; James et al., 2019; Kavanaugh et al., 2013)

Strong associations have been observed between maternal genetics, HMOs and the type of commensal communities found in the infant gut (van den Elsen et al., 2019). Specifically, secretor positivity of mothers is associated with higher abundances of *Bifidobacterium* in the infant gut microbiome (Lewis et al., 2015; Smith-Brown, Morrison, Krause, & Davies, 2016). Conversely, studies have shown delayed colonisation of these beneficial microbes in the gut of infants fed non-secretor milk. Higher numbers of *Clostridium* and *Enterobacteria* were also observed in the fecal microbiome of infants fed non-secretor milk (Mottram, Wiklund, Larson, Qadri, & Svennerholm, 2017). It is therefore apparent that differences in HMO profiles between secretor and non-secretor mothers results in differences in the microbial communities of their infants. This highlights a huge level of stereospecific activity, with the different HMO structures and conformations accounting for their various biological functions.

## 4. Oligosaccharide production

Until recently manufacturing of HMOs in large quantities was a tedious and costly process which resulted in insufficient yields for clinical applications. For over two decades, paediatric nutritional products were fortified with alternative, non-human sources of complex oligosaccharides such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS) in an attempt to close the gap between breastmilk and formula (Vandenplas, De Greef, & Veereman, 2014). Chains of GOS consist of 3–10 galactose molecules, frequently with a glucose unit at

**Table 2**
Milk groups according to Lewis and Secretor Status.

| Milk Group | Classification | *Se* (FUT2) | *Le* (FUT3) | Fucose linkages | HMOs Secreted |
|---|---|---|---|---|---|
| 1 | Lewis Positive, Secretors | + | + | α1-2, α1-3, α1-4 | All HMOs |
| 2 | Lewis Positive, Non-Secretors | – | + | α1-3, α1-4 | 3-FL, LNFP-II, LNFP-III |
| 3 | Lewis Negative, Secretors | + | – | α1-2, α1-3 | 2′-FL, 3-FL, LNFP-I, LNFP-III |
| 4 | Lewis Negative, Non-Secretors | – | – | α1-3 | 3-FL, LNFP-III and LNFP-V |

the reducing end, and are synthesised through the enzymatic action of β-galactosidase, while FOS can be isolated in large quantities from chicory roots (Davani-Davari et al., 2019). Although a vast body of pre-clinical and clinical studies exist assessing the properties of GOS and FOS, the beneficial effects are indeterminate. The EFSA Panel on Dietetic Products, Nutrition and Allergies has subsequently stated that there is no necessity to add GOS and FOS to infant formulae (Bode et al., 2016; EFSA, 2014).

When compared to human milk, the oligosaccharides found in the milk of domestic farm animals are present in significantly lower concentrations and have less structural diversity when compared to HMOs (Urashima, Saito, Nakamura, & Messer, 2001). Despite these facts, milk from farm animals such as cows and goats, represent attractive alternatives for obtaining large quantities of oligosaccharides (reviewed by Quinn, Joshi, & Hickey, 2020). Milk oligosaccharides are found in whey permeate, which is a lactose-rich effluent remaining after protein extraction from milk-derived cheese whey, an abundant dairy waste. A number of studies have highlighted the potential use of whey permeate as an alternative source of oligosaccharides. (Bode et al., 2016; Mehra et al., 2014). Bode et al. calculated a theoretical yield of 220,000 kg of bovine milk oligosaccharides annually from US dairy industry waste by-products (Bode et al., 2016). However, bovine milk contains only trace amounts of fucosylated oligosaccharides, while HMOs are predominantly fucosylated. The differences in oligosaccharides profiles could potentially be resolved through the use of chemoenzymatic synthesis to construct fucosylated and elongated glycans. Bovine milk oligosaccharides therefore offer themselves as precursors for chemoenzymatic processes (Yu & Chen, 2019).

Considering the difficulties associated with large-scale synthesis of HMOs, characterisation and lab scale research of HMOs has traditionally been conducted using fractions isolated from human milk. The advantage of this was that HMO structures were obtained in their natural form. The challenge was that they could not be isolated in high enough quantities sufficient for clinical trials due to limited access to human donor milk. Recently, development of new synthetic methods have begun to emerge that are able to solve this scale issue (Faijes, Castejón-Vilatersana, Val-Cid, & Planas, 2019). These methods, which include chemoenzymatic techniques and microbial metabolic engineering processes, have resulted in the production of unprecedented quantities of certain HMO structures, and have furthered our understanding of the biological role of specific HMOs in clinical settings. Moreover, progress in HMO manufacturing allows for design of HMO-containing products for pharmaceutical and nutraceutical applications (Sprenger, Baumgärtner, & Albermann, 2017).

### 4.1. Chemical synthesis

The first HMOs (2′-FL and LNnT) registered as “novel foods” in Europe were produced by Glycom A/S through the use of *in vitro* chemical synthesis. In comparison to other biopolymers, significant challenges are encountered during chemical synthesis of carbohydrate molecules. Due to the structure–function relationships of HMOs and other oligosaccharides, correct branching and stereospecific linkages are required for biological function to be preserved (Smilowitz et al., 2014). Therefore, issues such as regioselective protection of hydroxyl groups on acceptor monosaccharides and stereospecific construction of glycosidic linkages must be overcome (Kulkarni et al., 2018). Despite these challenges, stepwise solid-phase methods have been applied for the synthesis of a significant number of complex HMO structures such as 2′-FL, 3-FL, DFL, LNT, LNnT, LNFP I, and LNFP III (Arboe Jennum, Hauch Fenger, Bruun, & Madsen, 2014; Craft & Townsend, 2017). Methods for chemical synthesis of sialylated molecules have also been described (Fair, Hahm, & Seeberger, 2015; Lai, Hahm, Liang, & Seeberger, 2015). Most recently, Bandara et al. have developed methods for the chemical synthesis of LNnH using a convergent synthetic strategy (Bandara, Stine, & Demchenko, 2020). New synthetic protocols continue to be examined and refined and allow for HMOs to be obtained in a homogenous fashion (Bandara, Stine, & Demchenko, 2019). Thus, chemical synthesis can serve to enhance our understanding of the role of HMOs and guide larger scale production. However, it is often a laborious and inefficient process, resulting in low yields of the targeted HMO. To address these problems, methods are being developed whereby automated chemical synthesis is used in combination with enzymatic glycan synthesis (Wen et al., 2018).

### 4.2. Chemoenzymatic synthesis

The enzymes used for chemoenzymatic synthesis of HMOs are largely glycosyltransferases (GTs) from pathogenic bacteria such as *Helicobacter*, *Neisseria*, or *Pasteurella* (Sprenger et al., 2017). To date, researchers have exploited fucosyltransferases, sialyltransferases, galactosyltransferases, and *N*-acetyl-glucosaminyltransferases for enzymatic synthesis of HMOs. Commonly employed chemoenzymatic pathways have been described in detail in many recent reviews (Zeuner, Teze, Muschiol, & Meyer, 2019). Pioneering works in this field revealed that recombinant β1,3-*N*-acetyl-glucosaminyltransferase (LgtA) and β1,4-galactosyltransferase (LgtB) from pathogenic *Neisseria* species can be employed to synthesize LNnT with product yield of more than 85% (Johnson, 1999). More recent work by Zeuner and colleagues demonstrated synthesis of LNnT using glycosidases from natural sources. Here, the enzymatic action of β-galactosidase cloned from *Bacillus circulans* was exploited to add lactose moieties to lacto-*N*-triose acceptor molecules (Zeuner, Nyffenegger, Mikkelsen, & Meyer, 2016). Enzymatic synthesis of the core HMO structure, lacto-*N*-biose (LNB) has also been described. Nishomoto and Kitoaka used the concurrent actions of four enzymes, a sucrose phosphorylase ), a UDP-glucose-hexose-1-phospate uridylyltransferase, a UDP-glucose-4-epimerase (all from *Bifidobacterium longum*), and a LNB phosphorylase (from *Bifidobacterium bifidum*) to produce LNB in kilogram quantities (Nishimoto & Kitaoka, 2007). Other methods to construct neutral HMO structures include the use of LNB phosphorylase from *Bifidobacterium longum* subsp. *infantis*, resulting in the synthesis of β1,3-linked molecules including LNB and LNT (Yao, Yan, Chen, Wang, & Cao, 2015; Yu et al., 2010).

Fucosylation of HMO structures can also be carried out using GTs cloned from microbial sources. Early work from Albermann et al. demonstrated that 2′-FL could be produced with a 65% yield through a stepwise synthetic pathway (Albermann, Piepersberg, & Wehmeier, 2001). This involved conversion of GDP-Fucose from mannose using recombinant *E. coli* enzymes, before transfer of GDP-Fucose to a lactose acceptor. This final enzymatic step was catalysed by recombinant α1,2-FucT cloned from *Helicobacter pylori* (Albermann et al., 2001). FutT glycosyltransferases from *Helicobacter* strains are commonly used for synthesis of Fuc-borne HMO. However, the enzymes demonstrate higher affinity for LNB and LacNAc than lactose which hampers their use in the production of 2′-FL, 3-FL, and DFL. Choi et al. overcame these challenges by introducing mutations in the C-terminal domain of a 1,3-FucT, thereby significantly increasing yields of 3-FL (Choi, Kim, Park, & Kim, 2016). Mutagenesis techniques have also been applied in the engineering of α1,2-fucosynthase from a glycosidase. Mutations in a 1,2-α-fucosidase from *B. bifidum* abolishes the enzyme’s hydrolase activity, while retaining its transferase activity. The use of this mutagenesis method has been described for the synthesis of 2′-FL (lactose acceptor), 3-FL (lactose acceptor), and DFL (2′-FL acceptor) using β-L-fucopyranosylfluoride as the donor molecule (Sugiyama et al., 2016; Wada et al., 2008).

Chen et al. employed a one-pot multi-enzymatic (OPME) approach to produce 148 mg of LNnT with a 81% yield (Chen et al., 2015). Further enzymatic steps resulted in the decoration of this LNnT receptor with fucose or sialic acid. In this study, the enzymatic action of α1,3-FucT from *H. pylori* resulted in the production of LNFP III, while the sialyllacto-*N*-tetraoses LST-a and LST-c were synthesised by addition of

sialic acid residues using an α2,3-sialyltranferase (from *Pasteurella multocida*) and α2,6-sialyltranferase (from *Photobacterium damselae*), respectively (Chen et al., 2015). Sialyltransferases from *P. multocida* have also been employed in the production of the trisaccharides 3′-SL and 6′-SL (Guo et al., 2015).

A more recent study from Chen's group at UC Davis employed similar one pot multieznyme (OPME) glycosylation processes to produce milligram quantities of LNT (McArthur, Yu, & Chen, 2019). Here, McArthur et al. identified a β3GalT from *Chromobacterium violaceum* (CVβ3GalT) which was homologous to a previously assessed β3GalT from *E. coli* but demonstrated much higher efficiency in catalysing the formation of LNT from lacto-*N*-triose II. Yields of 99.3% LNT from lactose were achieved after two OPME processes in sequence (McArthur et al., 2019). The high titres achieved using this CVβ3GalT facilitated the subsequent fucosylation and/or sialylation of LNT to produce HMO structures such as LNFP II, LNDFH, and LNFP V (McArthur et al., 2019).

Despite an ever-increasing number of studies in this area, these approaches can still not satisfy the HMO demands associated with commercial applications. Therefore, use of HMO produced through chemoenzymatic pathways is more suitable for development of elaborate libraries of structurally diverse HMOs. Milligram quantities of HMO produced during chemoenzymatic synthesis can be used for small-scale analysis of structure–activity relationships, and may ultimately guide the development of HMO production in larger scale biotechnological processes (Bode et al., 2016).

### 4.3. Microbial metabolic engineering

The potential use of microbial host organisms for large-scale HMO production was first demonstrated nearly two decades ago and since then, microbial fermentation processes have been employed routinely resulting in the production of commercially viable yields of 2′-FL and LNnT, with production of several other HMO structures imminent (Bych et al., 2019). Indeed, 42 HMO structures (including building blocks) have been produced using the cell factory approach to date (Faijes et al., 2019). To the best of our knowledge, no microbial organism naturally produces HMO. Therefore, metabolic engineering must be applied, and the appropriate glycosyltransferases expressed in microbes in order to generate HMOs. Owing to their extensive use as a research and cloning tool, non-pathogenic *E. coli* strains have become the preferred host organism for HMO synthesis (Sprenger et al., 2017). Despite this, there is no evidence that *E. coli* is the optimal microbial host, and efforts to develop alternative hosts, such as *Saccharomyces cerevisiae* (Yu et al., 2018), *Bacillus* species, and *Corynebacterium glutamicum* (Seo, Young Wook, & Hae Yong, 2018) are on-going. The production of HMOs via enzymatic and cell factory approaches has been reviewed in detail recently (Bych et al., 2019; Sprenger et al., 2017).

Beneficial properties associated with host organisms include efficient uptake of precursors (such as lactose), proficient export of HMO end-products, and suitable pathways for production of nucleotide-activated sugar donors (such as GDP-Fuc). In addition, preferred host organisms have a generally regarded as safe (GRAS) or qualified presumption of safety (QPS) status, have high growth rates, and possess features which reduce down-stream processing costs and eliminant ingredient quality issues. Challenges in HMO production now lie with the development of efficient, cost-effective downstream processing lines to produce high quality HMO. Low levels of contaminants (such as endotoxins, DNA and protein), low biomass production, and low levels of viscosity are preferable for down-stream processes. *E. coli* satisfies some of these requirements, but certainly not all (Bych et al., 2019). Until now, research has mostly focused on the large-scale production of 2′-FL since it is the most abundant HMO in human breast milk (in secretor women), and one of the simplest structures. Regardless of the chosen host strain, three elements are required for biosynthesis of 2′-FL: (a) intracellular transport of lactose into the cell, (b) overproduction of nucleotide-activated L-fucose (GDP-Fuc), and (c) a fucosyltransferase enzyme capable of fucosylating lactose via α1-2 linkages.

Although wild-type *E. coli* is able to take up lactose via lactose permease (LacY), it also has metabolic pathways dedicated to the degradation of lactose. Therefore, for the purpose of 2′-FL synthesis, research groups have engineered *E. coli* strains with altered or deleted β-galactosidase (LacZ) activity (Chin, Seo, Kim, & Seo, 2016; Dumon et al., 2001). Currently, the preferred method is to feed lactose produced during whey processing directly into the fermentation system (Bych et al., 2019). However, low solubility of lactose is an issue, as is the isomerization of lactose to lactulose. Small amounts of lactulose present in the fermentation system results in the production of 2′-fucosyllactulose. Therefore, researchers have proposed *in vivo* synthesis of lactose as a potential alternative (Bode et al., 2016).

GDP-L-fucose is produced naturally in *E. coli* strains as a precursor to colonic acid via the *de novo* pathway (Stevenson, Andrianopoulos, Hobbs, & Reeves, 1996), but is required at much higher concentrations for 2′-FL production. Overproduction of enzymes along the *de novo* pathway has been investigated including ManB and ManC to increase flux towards GDP-D-mannose production, as well as increased expression of GDP-D-mannose-4,6-dehydratase (Gmd) and GDP-L-fucose synthetase (WcaG) to increase conversion of GDP-D-mannose to GDP-L-fucose (Wang et al., 2019). In addition, genes downstream of GDP-L-fucose along the *de novo* pathway can be switched off to prevent conversion to colonic acid. A second pathway, the salvage pathway, present in mammalian and some *Bacteroides* species can be introduced to *E. coli* strains to increase the concentration of GDP-L-fucose. Introduction of the *fkp* gene encoding fucokinase/GDP-L-fucose pyrophosphorylase (Fkp) from *Bacteroides fragilis* results in conversion of extracellular fucose to GDP-Fucose (Chin et al., 2016; Chin, Kim, Lee, & Seo, 2015). Despite the high cost of supplying fucose to the system, the salvage pathway is still a viable option for overproduction of GDP-fucose.

The final element required for production of 2′-FL using cell factories is the introduction of an exogenous fucosyltransferase to catalyse addition of fucose to the lactose acceptor. Fucosyltransferases such as FutC from *H. pylori* are commonly employed, although a more favourable by-product profile is observed using the α1,2-fucosyltransferase, WcfB, from *B. fragilis* (Chin, Kim, Kim, Jung, & Seo, 2017), which results in reduced production of DFL (Engels & Elling, 2013). Through the employment of the engineering strategies described above, whole-cell microbial synthesis of substantial quantities of 2′-FL has been achieved. In 2012, Lee et al demonstrated production of 2′-FL from lactose through engineering of the *E. coli* strain JM109. The wild-type JM109 is incapable of consuming lactose and, through overexpression of the *de novo* pathway genes *manB, manC, gmd,* and *wcaG*, 1.2 g/L of 2′-FL was produced after consumption of 14.5 g/L of lactose (Lee et al., 2012). Baumgartner et al. introduced salvage pathway genes to the strain in addition to the *de novo* genes, and produced significantly higher yields of 2′-FL when compared with overexpression of the *de novo* pathway genes alone (Baumgärtner, Seitz, Sprenger, & Albermann, 2013). Knockout of the fucose degradation genes *fucI* and *fucK* by Chin et al. resulted in production of 20.6 g/L of 2′-FL from the consumption of 27 g/L of L-fucose and 53.6 g/L lactose (Chin et al., 2016). To date, the highest yield reported for the production of 2′-FL is 180 g/L according to http://jennewein-biotech.de/cms/assets/uploads/2017/11/EDMbuntNov17.pdf.

In 2014, Baumgartner et al. described the use of engineered strains for production of LNT. The protein products of two genes, *lgtA,* which encodes β1,3-*N*-acetylglucosaminyltransferase, and *wgbO,* which specifies a β1,3-galactosyltransferase, were overexpressed in *E. coli.* Lactose and glucose were used as substrates, and 0.219 g/L of LNT was produced. This was increased to 0.81 g/L when glucose was replaced with galactose as an energy source (Baumgärtner, Conrad, Sprenger, & Albermann, 2014). Up-scaling to fed-batch fermentation resulted in formation of 12.7 g/L of LNT. Similar methods were applied by Glycom A/S for microbial production of LNnT. These methods included introduction of *lgtA* from *Neisseria meningitidis* and a *galT* from *H. pylori* into *E. coli* strains and utilisation of lactose, glycerol and D-glucose as

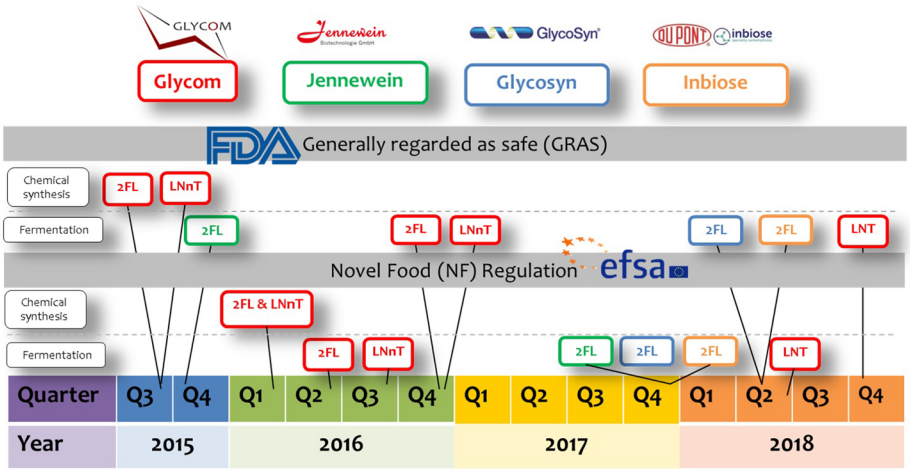


**Fig. 3.** Summary of the regulatory landscape for HMOs adapted from Bych et al. (2019). Timeline includes overview of the major HMO manufacturers, the technology utilized to synthesise HMO structures, the regulatory bodies in the US (FDA which grants GRAS status) and EU (EFSA which grants NF regulation), and highlights the progress which has been made over past number of years in achieving regulatory approval for synthetically manufactured HMO structures.

**Table 3**
Outcomes from clinical intervention studies in infants.

| Population | Formula(e) | Outcomes | Reference |
|---|---|---|---|
| FF (n = 189)<br>BF (n = 65) | 1. GOS (2.4 g/L)<br>2. GOS (2.2 g/L) + 2′-FL (0.2 g/L)<br>3. GOS (1.4 g/L + 2′-FL (1 g/L) | Consumption of 2′-FL is safe and well-tolerated. Growth of FF infants similar to those who are BF | Marriage, Buck, Goehring, Oliver, & Williams, 2015 |
| FF (n = 317)<br>BF (n = 107) | 1. GOS (2.4 g/L)<br>2. GOS (2.2 g/L) + 2′-FL (0.2 g/L)<br>3. GOS (1.4 g/L) + 2′-FL (1 g/L) | Compared to GOS alone, infants who consumed formula with 2′-FL had lower levels of immune biomarkers comparable to BF infants | Goehring et al., 2016 |
| FF (n = 88)<br>BF (n = 43) | 1. Control (no oligosaccharide)<br>2. FOS (1.8 g/L) + 2′-FL (0.2 g/L) | Formula with 2′-FL safe for consumption. Stool consistency of infants fed 2′-FL appears more similar to BF infants. | Kajzer, Oliver, & Marriage, 2016 |
| FF (n = 175)<br>BF (n = 38) | 1. Control (no oligo)<br>2. 2′-FL(1 g/L) + LNnT (0.5 g/L) | Formula with 2′-FL and LNnT shifts stool microbiota and metabolic profile of infants closer to BF infants | Steenhout et al., 2016 |
| FF (n = 175) | 1. Control (no oligo)<br>2. 2′-FL(1 g/L) + LNnT (0.5 g/L) | Formula with 2′-FL and LNnT supports age appropriate-growth, and results in lower incidences of infection and medication use vs control group | Puccio et al., 2017 |

substrates (Bajza et al., 2015). LNnT produced by Glycom through these recombinant technologies received regulatory approval in the US and EU in 2016 (Fig. 3). Despite decisive advancements in metabolic engineering practices for production of specific HMOs, significant limitations still exist. Harnessing the benefits of HMOs for inclusion in infant food products will require further developments in the large-scale synthesis of multiple HMO structures. For more complex HMOs, fermentation yields are low or the products are not exported to the external environment rendering purification difficult. Of particular significance is the identification of an appropriate β1,6-*N*-acetylglucosaminyltransferase for manufacture of branched chain HMOs.

## 5. Regulatory framework and nutraceutical applications

HMOs manufactured through the synthetic processes described above display exact structural identity to those found in human breast milk. Irrespective of this fact, introduction of these ingredients into European, American and Asian commercial arenas requires regulatory approval (summarised in Fig. 3). The regulatory framework in the EU involves designation of Novel Food status by the European Food Safety Authority (EFSA) following mandatory safety assessment, while in the US, HMOs must receive GRAS notification in order to be used as food ingredients. GRAS approval is granted by the Food and Drug Administration (FDA) (Salminen, 2017) and strong competition has ensued between manufacturers since the first regulatory approval of HMO in 2015. While Glycom A/S were the first to receive GRAS notification for their chemically synthesised 2′-FL and LNnT, Jennewein were the first company to receive regulatory approval for 2′-FL produced by microbial fermentation in November 2015 (Fig. 3). In 2016, Glycom A/S received Novel Food status in the EU and GRAS notification in the US for their microbially fermented 2′-FL and LNnT. More recently, Glycosyn/Friesland Campina Domo and Inbiose/DuPont have entered into HMO production with both companies receiving GRAS notification for 2′-FL. Genechem have recently received a positive response from the FDA for GRAS notification of 3′-SL produced by chemoenzymatic synthesis. Most recently, Novel Food status has been granted to Glycom A/S for their combination of 2′-FL and DFL in a ratio close to the composition naturally found in breast milk (Bych et al., 2019).

## 6. Clinical intervention trials

After decades of research in the field, HMOs are now available in quantities and at prices accessible for a broad range of applications. 2′-FL and/or LNnT are currently found in infant products in more than 30 countries and non-infant products are beginning to emerge (Zeuner et al., 2019). A limited number of intervention studies in infants have been performed to assess the safety and efficacy of HMO-fortified infant formulae (Vandenplas et al., 2018). These studies are summarised in Table 3, and show that formula milk containing 2′-FL and LNnT is safe and well-tolerated while beneficial effects on the microbiota have also been reported.

The clinical data available to date indicates potential health benefits for the inclusion of 2′-FL and LNnT in formula, but it remains to be seen whether a limited number of HMO structures will close the compositional and physiological gap between breast milk and formula (Vandenplas et al., 2018). Many more prospective, intervention trials in infants are required to establish benefits for addition of HMO to formula. According to https://clinicaltrials.gov/, and as of the end of 2019, eight intervention studies assessing the impact of HMO on infants are active and/or recruiting with one further study planned which

targets adult populations. Companies such as Nestle and Jennewein are both undertaking trials to assess the safety of new HMO formulations which contain a blend of five HMO structures, while others attempt to expand on previous studies involving 2′-FL and LNT/LNnT. Targeted outcome measures in these studies include colonisation of *Bifidobacterium*, iron absorption, and frequency of illness. It is anticipated that findings from these studies will expand our view of HMO as bioactive compounds.

## 7. Conclusions

Recent expansion of technologies for the characterization, purification, and production of HMOs promises successful translation of fundamental discoveries into commercial and therapeutic applications. Although there have been substantial breakthroughs in our understanding of HMOs, numerous uncertainties still remain. Owing to the fact that the beneficial effects of HMOs are highly structure-specific, it is pertinent that levels of these compounds in human milk are quantified with utmost precision. Furthermore, the maternal, genetic, and environmental factors that drive the inter-individual variations in HMO composition need to be fully defined. As researchers in the field look to verify the beneficial effects of HMOs in human populations and expand their use in nutraceutical applications, it is important to be specific about which structures are responsible for each function and to avoid assigning all of the observed benefits of HMOs to one or two structures. Future research should examine whether dietary supplementation of HMOs not present in the milk of some mothers may be counterproductive or even have potentially negative effects on their infants. Further well-designed clinical studies, both observational and placebo-controlled interventions, are necessary to further substantiate and grow our understanding of HMO biology and its significance for the advancement of infant nutrition. Overall, we are still a long way from artificially replicating the specified complexity of HMOs and many questions about their clinical implications remain unanswered. Findings from HMO research should therefore serve, above all else, as yet another compelling motive to encourage and support breastfeeding as the “gold standard” in infant nutrition.

## Ethical statements

This is a review article. It has not involved any human subjects and animal experiments.

## CRediT authorship contribution statement

**Clodagh Walsh:** Writing - review & editing. **Jonathan A. Lane:** Writing - review & editing. **Douwe van Sinderen:** Writing - review & editing, Supervision. **Rita M. Hickey:** Writing - review & editing, Supervision.

## Declaration of Competing Interest

The work was financially supported by H & H Group, Ireland. Jonathan A. Lane is employee of H & H, Ireland.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.104052.

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