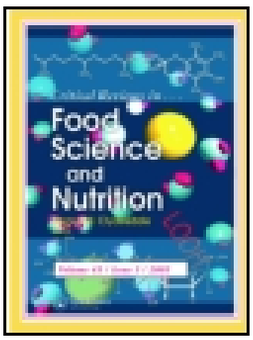

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REVIEW

Human milk oligosaccharides as bioactive compounds in infant formula: recent advances and trends in synthetic methods

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ABSTRACT

Human milk oligosaccharides (HMO) have attracted great interest in recent years due to their role in boosting infants and adults health. According to several *in vitro*, *in vivo* and clinical studies, gastrointestinal and immune physiological systems benefit the most from HMO intake. Other organ systems, such as the respiratory, central nervous, circulatory, locomotor, and urinary systems have also been found to be affected by the HMO consumption in the recent decade. Due to their positive impact on human health, the incorporation of HMO into the infant formula or other functional foods has become highly desirable. Currently, their large-scale production is limited to 2′-fucosyllactose (2′FL) and lacto-*N*-neotetraose (LNnT) that are obtained through fermentation and added to the infant formula as fortifiers. Fewer advances have been made for other HMO to reach the industrial scale synthesis. The present paper summarizes the latest research on HMO in terms of their health benefits and synthetic methodologies, with the overall aim to establish the current status and trends in both fields.

KEYWORDS

Bioactive oligosaccharides; functional foods; health benefits; human milk; infant formula; synthetic production

Introduction

Oligosaccharides are molecules formed by monosaccharide units which degree of polymerization can vary between 3–10 and 2–20 monomers depending on the scientific or food agency, such as the International Union of Biochemistry and Molecular Biology (IUBMB) or the U.S. Food and Drug Administration (FDA), respectively. (Meyer et al. 2015; Mussatto and Mancilha 2007; Nobre et al. 2015).

Importantly, the degree of polymerization and the type of monosaccharide units that comprise any given oligosaccharide have been directly related not only to its physicochemical properties, for example, solubility, viscosity, and digestibility, but also with different health benefits for human (Meyer et al. 2015; Nobre et al. 2015). Indeed, based on the *in vitro*, *in vivo* and clinical studies, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), xylo-oligosaccharides (XOS) and arabin-oligosaccharides (AOS) have been all recognized as functional biomolecules due to their prebiotic and immunomodulatory potential (Arzamasov, Van Sinderen, and Rodionov 2018; D. Chen et al. 2017; Ding et al. 2018; Le Bourgot et al. 2014; S. H. Lin et al. 2016; F. Liu et al. 2017; Manosroi, Khositsuntiwong, and Manosroi 2014; Neyrinck et al. 2018; Perdijk et al. 2019; Tian et al. 2018).

As it could be expected, food and pharmaceutical industries have manifested a strong interest in incorporation of those oligosaccharides into functional or medicinal foods (S. Kim et al. 2017; Nobre et al. 2015; Sharma et al. 2016).

Another important family of oligosaccharides that has attracted a remarkable attention in recent decade constitutes human milk oligosaccharides (Ballard and Morrow 2013; Bych et al. 2019; McGuire, McGuire, and Bode 2016; Olivares et al. 2015; Sprenger, De Castro, et al. 2017). They have been shown to have a tight relation with the prevention and treatment of diseases in infants, in particular gastrointestinal, respiratory, immune, nervous and blood system disorders (Ackerman et al. 2017; Korpela et al. 2018; Sprenger, Odenwald, et al. 2017; Weichert et al. 2013; Weiss and Hennet 2012; Wickramasinghe et al. 2015). Various studies have established that HMO act as prebiotics, can modulate the intestinal microbiota, support the cognitive development, as well as exhibit antimicrobial, and immunomodulatory properties (X. Chen 2015; Fanos et al. 2018; McGuire, McGuire, and Bode 2016). In response to these

CONTACT Luis Guillermo González-Olivares lgonzales@uaeh.edu.mx Universidad Autónoma del Estado de Hidalgo. Área Académica de Química. Ciudad del Conocimiento. Carretera Pachuca-Tulancingo km 4.5, Colonia Carboneras. CP. 42184. Mineral de la Reforma, Hidalgo, México; Sergio Alatorre-Santamaría salatorre@xanum.uam.mx Universidad Autónoma Metropolitana, Unidad Iztapalapa. División de Ciencias Biológicas y de la Salud. Departamento de Biotecnología, Colonia Vicentina AP 09340, Ciudad de México, México.

findings, the food industry has incorporated HMO into its products, with the main market formed by infant formula (Baker et al. 2016; Gallier et al. 2015; Rollins et al. 2016).

Previously, infant formula was fortified with FOS and GOS as main sources of oligosaccharides (Bode et al. 2016; R. Kent et al. 2015) until they were found to have lesser effect on the intestinal microbiota modulation in comparison to HMO (Alliet et al. 2016; Cristofalo et al. 2013; Lönnerdal 2014). The resulting reformulation of the formula market portfolio toward the HMO - fortified products has been intended to reflect the nutritional and bioactive functions of human milk as closely as possible (Alliet et al. 2016; Gallier et al. 2015; G. Kent 2015).

As for the HMO production, four different approaches have been proposed: chemical synthesis, whole cell biotransformation (fermentation), enzymatic and chemo-enzymatic routes (Craft and Townsend 2017; Fang et al. 2018; Prudden et al. 2017). At present, biocatalytic methods are considered the most efficient in terms of HMO production yields (Ammann 2017; Fang et al. 2018; Hollands et al. 2019; H. Yu et al. 2017). Additionally, new functions of HMO were discovered, and some toxicological studies were carried out to ensure their safe incorporation into the infant formula or other functional foods (Monaco, Gurung, and Donovan 2019; Phipps, Baldwin, Lynch, Flaxmer, et al. 2018; Phipps, Baldwin, Lynch, Stannard, et al. 2018).

Notwithstanding the advances in synthetic methods, the HMO addition to the commercial foods have been limited. So far, only 2'FL and LNnT have been added to the special lines of infant formulas (Bych et al. 2019; Vandenplas et al. 2018). The main reasons that account for this situation have been identified as the lack of methodologies to access oligosaccharides with a high degree of polymerization or asymmetry, as well as the poor yields obtained for the up-scaled processes or the multi-steps followed to achieve the desired structure. For these reasons, the present review is focused on the recent improvements in HMO synthesis and summarizes the most up-to-date studies related to the health benefits provided by these biomolecules.

Human milk: Composition and bioactive compounds

Human milk is considered the golden standard for infant nutrition since it contains macro and micronutrients, which promote the best physical and intellectual development of a newborn (Andreas, Kampmann, and Le-Doare 2015; Ballard and Morrow 2013).

In addition, human milk has been proven to be a complex biofluid with highly variable composition affected by both intrinsic (country of origin, genetics, endogenous synthesis enzymes, and special diseases) and extrinsic factors (feed, day/night lactation, lactation stage, storage, and heat treatments). Moreover, depending on the analytical methods applied for the HMO determination, further discrepancies can be found among the studied populations (Andreas, Kampmann, and Le-Doare 2015; Ballard and Morrow 2013; Kunz et al. 2017; Olivares et al. 2015; Sprenger, De Castro, et al. 2017; van Leeuwen 2019).

Despite these differences in milk composition, lactose and lipids are its major components to supply energy to the infants. Lactose is the main source of carbohydrates at the average concentration of $70\,g{\cdot}L^{-1}$, while the lipid fraction ($30\text{-}60\,g{\cdot}L^{-1}$) accounts for 40-55% of total energy. Other macromolecules that add to the energy balance are proteins with an average concentration of $12\,g{\cdot}L^{-1}$. The caseins account for 13% (wt) of total protein content, which is considered the optimal proportion for the infant growth (Andreas, Kampmann, and Le-Doare 2015; Ballard and Morrow 2013; Urashima et al. 2013).

In addition to nutritional elements, human milk contains other molecules known as bioactive compounds such as medium chain monoglycerides, lactoferrin, lysozyme, immunoglobulins, cytokines and oligosaccharides (Andreas, Kampmann, and Le-Doare 2015; Ballard and Morrow 2013; Fanos et al. 2018). The latter possess a number of functions, among which the most important is protection against pathogenic bacteria or viruses, that provoke gastrointestinal or respiratory diseases (Duska-McEwen et al. 2014; El-Hawiet, Kitova, and Klassen 2015; Hester et al. 2013; Patel et al. 2013; Weichert et al. 2013). Also, their presence has been linked to the preventive activity against bowel inflammation and positive modulation of the immune system response through diverse signaling pathways (He, Lawlor, and Newburg 2016; McGuire and McGuire 2015; Walker 2013). Around 200 types of HMO have been described so far, with some structures showing interesting and beneficial effects on the general health (Kunz, Kuntz, and Rudloff 2014; McGuire, McGuire, and Bode 2016).

Human milk oligosaccharides: Structure, endogenous synthesis and metabolism

HMO chemical structure

HMO are composed of five structural units: three monosaccharides, which are D-glucose (Glc), D-galactose (Gal), and L-fucose (Fuc); one amino sugar, namely *N*-acetylglucosamine (GlcNAc), and one acid monosaccharide which is known as *N*-acetylneuraminic acid, or sialic acid (Sia). The combination of these five units forms different oligosaccharides present in human milk, all having in common to contain a lactose moiety (Galβ-1,4Glc) on their structure at the reducing end (X. Chen 2015; McGuire, McGuire, and Bode 2016).

Furthermore, HMO can be classified into three groups: fucosylated oligosaccharides (FucOS), sialylated oligosaccharides (SiaOS), or so-called core oligosaccharides. In FucOS, Fuc can be attached to lactose, lacto-*N*-biose (Galβ1-3GlcNAc) or *N*-acetyllactosamine (Galβ1-4GlcNAc) residues by alpha bonds (Figure 1). Likewise, Sia that belongs to SiaOS can be bound to the same residues as Fuc in FucOS. Finally, core oligosaccharides are formed through lactose elongation, where it links to lacto-*N*-biose (LNB) *via* β1-3 bonds or to *N*-acetyllactosamine (LacNAc) *via* β1-3 or β1-6 linkages. (Bode 2012; X. Chen 2015; McGuire, McGuire, and Bode 2016).

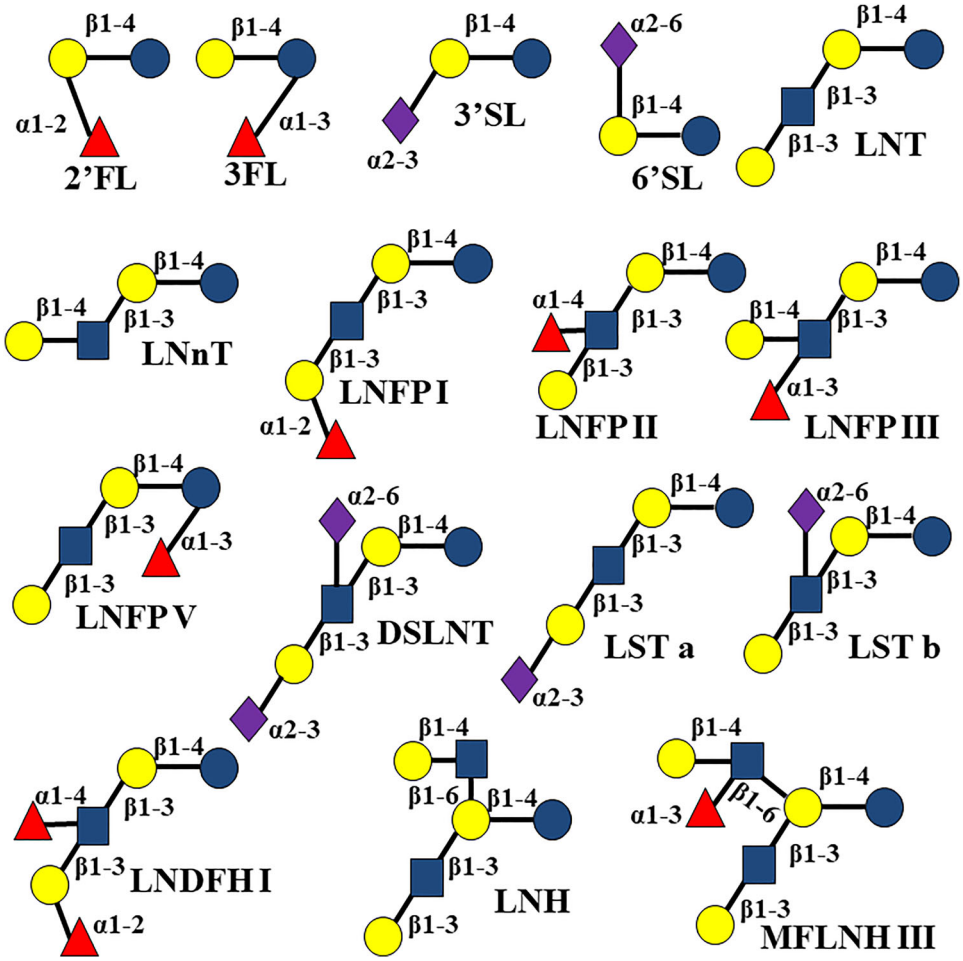

Figure 1. Structure of the main HMO produced and studied. Glc, Gal, Fuc, GlcNAc, Sia.

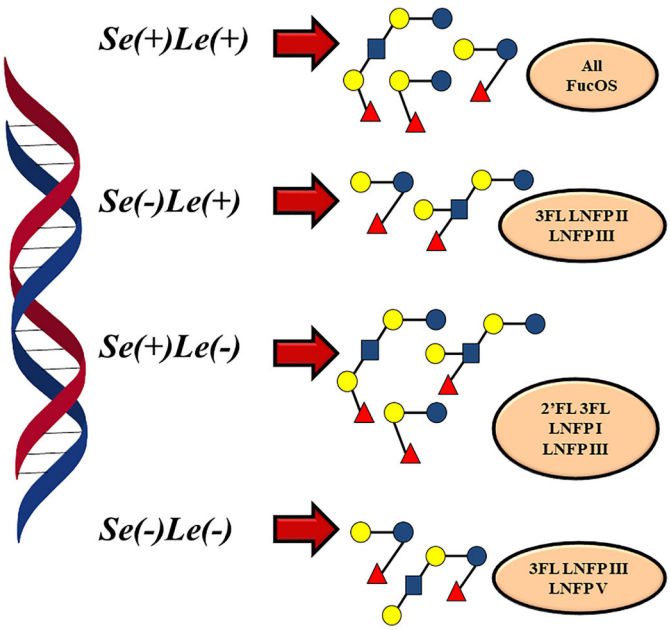

Figure 2. Phenotypes to production of FucOS and main FucOS synthesized. Image based on Vandenplas et al. (2018).

The general classification of a vast number of different oligosaccharide structures is based on their size. There can be distinguished short molecules of fucosylated and sialylated oligosaccharides, such as 2′-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 3′-sialyllactose (3'SL), or 6′-sialyllactose (6'SL) (Figure 1), and large structures comprised of core oligosaccharides with the attached Fuc or Sia moieties, like lacto-*N*-fucopentaose I (LNFP I), lacto-*N*-fucopentaose II (LNFP II), lacto-*N*-fucopentaose III (LNFP III), lacto-*N*-difuco-hexaose I (LNDFH I), or disialyllacto-*N*-tetraose (DSLNT) (Bych et al. 2019; Kunz, Kuntz, and Rudloff 2014).

Endogenous synthesis of HMO

Several different genes are implicated in the endogenous synthesis of HMO. In the case of fucosylated oligosaccharides, two genes, namely *Secretor* (*Se*) and *Lewis* (*Le*), are considered crucial for their production. The first gene encodes α1,2-fucosyltransferase (Fut2), while the second one allows the expression of fucosyltransferase Fut3. The latter catalyzes the Fuc linkage in α1,3 and α1,4 positions. Based on the genetic profile, mothers can be classified as either positive (+) or negative (-) for both genes (Bode 2012; Bode 2018; X. Chen 2015; McGuire, McGuire, and Bode 2016).

Furthermore, considering the expression of both *Se* and *Le* genes, four main phenotypes of fucosyl oligosaccharides can be identified: *Se*(+)*Le*(+), *Se*(-)*Le*(+), *Se*(+)*Le*(-), and *Se*(-)*Le*(-), as shown in Figure 2 (Austin et al. 2016; X. Chen 2015; Kunz, Kuntz, and Rudloff 2014; Kunz et al. 2017;

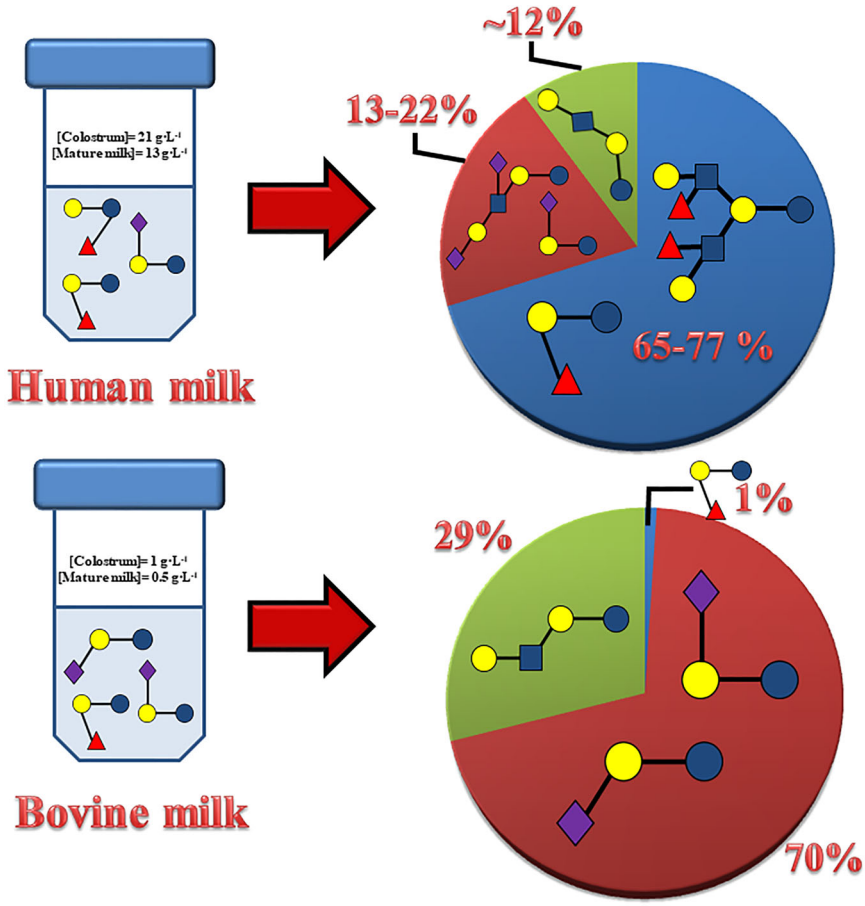

Figure 3. Differences in oligosaccharide composition between human and bovine milk. Image based on Urashima et al. (2013) and Thurl et al. (2017).

McGuire, McGuire, and Bode 2016; Vandenplas et al. 2018). Nonetheless, in some instances FucOS synthesis cannot be fully explained by the phenotypic group representation. The most recent studies have suggested either the existence of the alternative synthetic routes independent from fucosyl-transferases, or participation of a third and unidentified fucosyl-transferase. This is especially the case for *Le* gene (Austin et al. 2019; Ayechu-Muruzabal et al. 2018; Elwakiel et al. 2018; Tonon et al. 2019; van Leeuwen et al. 2018; Yan et al. 2019).

Thus, mothers with the *Se*(+)*Le*(+) phenotype are capable of synthesizing all types of FucOS, while those identified as *Se*(-)*Le*(+) can produce FucOS with α1,3 and α1,4 bonds, such as 3FL, LNFP II, and LNFP III, but not with α1,2 linkages, for example 2'FL or LNFP I. For women who belong to the *Se*(+)*Le*(-) phenotype, 2'FL or LNFP I are the main oligosaccharides found in their milk. They express Fut2 enzyme which only catalyzes α1,2 bond formation. Interestingly, this phenotype has been also found to synthesize 3FL or LNFP III. Finally, the *Se*(-)*Le*(-) females produce milk containing FucOS with α1,3 linkages such as 3FL, LNFP III, and lacto-*N*-fucopentaose V (LNFP V). To sum up, *Le* gene has been associated with higher variability of FucOS structures defined for each phenotype. Although the reasons remain unknown, it has been speculated the involvement of subgroups or epitopes from *Le* gene, mutations, co-regulation in enzyme activity by GDP-fucose or conditions from preterm birth (Austin et al. 2016; Austin et al. 2019; Elwakiel et al. 2018; Galeotti et al. 2012; Kunz et al. 2017; Totten et al. 2012; Tonon et al. 2019; Yan et al. 2019).

According to statistics, approximately 15-20% of women worldwide do not express *Se* gene and they are consideraded as non-secretor mothers, while the rest named as secretor mothers can synthesize α1,2 FucOS. Moreover, nearly 70% of female population is *Le* positive, while 5-10% is *Le* negative. The remaining 20% of women express Fut3 enzyme that binds Fuc to GlcNAc residue of LNB only through α1,4 bonds (Kunz, Kuntz, and Rudloff 2014; Sprenger, De Castro, et al. 2017).

Similarly, the biosynthesis of sialylated oligosaccharides is encoded by two genes, known as the *ABH* and *Lewis* blood groups. Mothers with the *ABH*(+)*Le*(+) phenotype can produce high levels of sialylated oligosaccharides, while those representing the *ABH*(-)*Le*(-) phenotype can express sialylated oligosaccharides at low levels (ten Bruggencate et al. 2014). Apart from 6'SL, which is the main oligosaccharide comprising SiaOS group, 3'SL, DSLNT, and LS-tetrasaccharide (LSTa) are also found among its members (Bode 2018; Goehring et al. 2014; Leo et al. 2010).

As for biosynthesis of core oligosaccharides, four glycosyltransferases are implicated in this process: two are involved in the Gal transfer, and the other two take part in the GlcNAc relocation. Particularly, galactosyltransferases (β3 or β4) and β1,3-*N*-acetylglucosaminyltransferase (iGnT) participate in a linear formation of core oligosaccharides, while β1,6-*N*-acetylglucosaminyltransferase (IGnT) and both

β3 and β4-galactosyltransferases, catalyze formation of the branched structures, which can be linearly elongated by iGnT (Bode 2012; Kobata 2010).

Despite the high variability in HMO abundance in breast milk, their average concentration stands at 21 $g{\cdot}L^{-1}$ at fourth day postpartum (colostrum). During the next 120 days counted from the partum, the HMO concentration decreases gradually to a value of 13 $g{\cdot}L^{-1}$ maintaining this level up to one year, and it is known as mature milk (Andreas, Kampmann, and Le-Doare 2015; Ballard and Morrow 2013; Urashima et al. 2013).

Analysis of the HMO structural profile from the secretor mothers of term and preterm infants revealed that FucOS account for 65-77% (wt) of total oligosaccharides, among which 2'FL and difucosyllacto-*N*-hexaose II (DF-LNH II) are the predominant structures, both found at ~17% (wt) of total HMO content. The second most abundant group consists of SiaOS, which represent between 13 and 22% (wt) of total oligosaccharides found in human milk. The SiaOS main structures were identified as 6'SL and DSLNT at ~ 4%, followed by 3'SL at ~1.5% (wt) of total HMO content. Finally, core oligosaccharides constitute around 12% (wt) of total oligosaccharides with lacto-*N*-tetraose (LNT) and its isomer lacto-*N*-neotetraose (LNnT) as the main components at ~ 6 and ~ 4%, respectively, and lacto-*N*-neohexaose (LNnH) at ~ 0.7% (wt) (Bych et al. 2019; Thurl et al. 2017). In contrast, for non-secretor mothers the amount of core oligosaccharides and α1,4 FucOS, like LNT and LNFP II are considerably different, as these two oligosaccharides represent 53 and 21% (wt) of HMO total content, respectively (Elwakiel et al. 2018; Kunz et al. 2017).

Importantly, remarkable differences have been found in the oligosaccharide profile when comparing human milk with other mammal sources, such as bovine milk. As shown in Figure 3, not only is human milk richer in oligosaccharides than bovine milk (up to 22-26 times higher), but also the structural distribution in bovine milk is very different from human milk. For example, SiaOS are the predominant structures in cow milk with 70% (wt) of total content, while FucOS only represent 1% (wt). In contrast, FucOS are major oligosaccharides in human milk (Andreas, Kampmann, and Le-Doare 2015; Thurl et al. 2017; Urashima et al. 2013).

Metabolism of HMO

Results of the clinical studies confirmed several in vitro experiments that HMO could pass unchanged through the gastrointestinal tract (Dotz et al. 2015; Gnoth et al. 2000; Goehring et al. 2014; Underwood et al. 2015). In general, they were observed to reach the proximal intestine without structural changes, and be later fermented by intestinal microbiota, such as Proteobacteria and Firmicutes phyla, and in particular by *Bifidobacterium* spp. and *Bacteroides* spp., in the distal intestine (De Leoz et al. 2014; Z. Liu et al. 2020; Underwood et al. 2015; M. Wang, Li, et al. 2015). According to the experimental results, depending on the type of HMO consumed, certain microbiota predominated in the gut. Thanks to the gene clusters present in bacteria, the HMO catabolism was possible (Bidart et al. 2018; De Leoz et al. 2014; Hirvonen et al. 2019; James et al. 2016; James et al. 2018; Lawson et al. 2020; Özcan and Sela 2018; Underwood et al. 2015; Underwood et al. 2017; Zabel et al. 2019).

Microbiota of infants fed with milk from non-secretor mothers exhibited higher abundance of Proteobacteria than Firmicutes phyla, whereas milk with higher content of LNFP I, LNFP III, LNFP V, DSLNT, monofucosyllacto-*N*-hexaose (MFLNH III) and sialyllacto-*N*-tetraose b (LSTb) favored the growth of *Bifidobacterium* spp. In addition, the predominant presence of bacteria from *Bacteroides* spp. in human intestine has been related to 2'FL, lacto-*N*-hexaose (LNH) and two of its isomers found in human milk (De Leoz et al. 2014; Underwood et al. 2015; M. Wang, Li, et al. 2015).

According to Dotz et al. (2015) and Goehring et al. (2014), nearly 99% (wt) of total HMO ingested are processed into the intestine, with renal and fecal excretion between 1-4% and 40-50% (wt), respectively. Around 45% (wt) of total HMO consumed by infants are used by intestinal microbiota and the remaining 1% (wt) is absorbed at concentrations of 0.10-0.20 $g{\cdot}L^{-1}$ and then transported to the bloodstream, reaching concentrations of 0.01-0.10 $mg{\cdot}L^{-1}$, sufficient to have an impact on infants health (Andreas, Kampmann, and Le-Doare 2015; Kulinich and Liu 2016; Vazquez et al. 2017).

Noteworthy, the recent studies have shown differences in the HMO absorption and excretion patterns among infants. For example, Goehring et al. (2014) investigated the HMO levels in urine and plasma from breastfed infants but only considering those metabolites with known chemical structure. 2'FL and 6'SL were identified as those HMO that were mostly excreted to urine and only 0.1% (wt) of total HMO ingested were found in plasma with a prolonged circulation. In other work, Dotz et al. (2015) followed the metabolic fate of neutral HMO, as well as identified novel metabolites. The analysis of infants urine and feces showed the presence of LNnT, monofucosylated LNnT, acetylated HMO, and some elongated HMO not associated with common HMO, all from microbial metabolism. In conclusion, the available data suggest that HMO absorption and pharmacokinetics in breastfed infants is directly linked to the mother secretory status that in turn determines the infant gut microbiota.

Health benefits related to the consumption of HMO

The most beneficial health effect of HMO has been demonstrated for the gastrointestinal system, although other physiological systems such as respiratory, nervous, immune, blood, urinary and locomotor systems benefit from HMO intake. Selected examples of these advantages are summarized in Table 1.

Effect of HMO on the gastrointestinal system

In relation to the gastrointestinal system, HMO have been effective at limiting infections caused by different pathogens including bacteria and viruses. (El-Hawiet, Kitova, and

Table 1. Summary of the most recent studies about health benefits provide by HMO.

HMO tested	Benefited system	Main benefits	Type of study	Reference
HMO pooled	Gastrointestinal	Decrease the invasion of *Candida albicans* on intestinal epithelial cells. Reduce the hypha length and the yeast cell production.	*In vitro*	Gonia et al. (2015)
HMO pooled	Gastrointestinal	Attenuate inflammation in H4 cells through reduction in expression of IL-8, MIP-3α, and MCP-1. Mitigate NFκB signaling pathway.	*In vitro*	Newburg et al. (2016)
HMO pooled	Gastrointestinal	Diminish the IL-8 secretion in IPEC-J2 cells. Stimulate gut gene-expression of pro- and anti-inflammatory factors such as: IL-10, IL-12, TGFβ, and TLR4.	*In vitro* and *In vivo*	Rasmussen et al. (2017)
HMO pooled	Gastrointestinal	Inhibit adhesion of *Clostridium butyricum* on Caco-2 and HT29-MTX lines cells.	*In vitro*	Musilova et al. (2017)
HMO pooled	Gastrointestinal	Provide a protective effect on NEC by reduction of IL-8 concentrations, downregulation of protein expression from TLR4, inhibition of NFκB signaling pathway, restoration of enterocytes proliferative ability, and improvement in the maturation organoid cells.	*In vivo*	C. Wang et al. (2019)
HMO pooled	Gastrointestinal, respiratory and blood	Protect against infection and sepsis development produced by *Staphylococcus aureus*, *Streptococcus* group B, *Enterococcus* spp., *E. coli* spp., *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp. and *Serratia* spp.	Clinical	Patel et al. (2013)
HMO pooled	Gastrointestinal and immune	Reduce symptoms of rotavirus infection through decrease diarrhea, modulating intestinal microbiota and improve immune response.	*In vivo*	Li et al. (2014)
HMO pooled	Gastrointestinal and immune	Provide higher adhesion on Caco-2 cells by *Bifidobacterium infantis* and *Bifidobacterium breve*. Regulate cytokine activity. Downregulate pro-inflammatory markers involved in NEC.	*In vitro*	Wickramasinghe et al. (2015)
HMO pooled and 2′FL	Gastrointestinal	Supply a prebiotic effect on *Bifidobacteria longum* ATCC15697 and *Lactobacillus acidophilus* NRRL B-4495. Inhibit the growth of *Campylobacter jejuni* S107 and *Escherichia coli* K12 through fermentation products from *Bifidobacteria longum*.	*In vitro*	J. Wang et al. (2017)
2′FL	Gastrointestinal	Provides spread antiviral spectrum on norovirus genotypes.	*In vitro*	Koromyslova et al. (2017)
2′FL	Gastrointestinal	Provides a prebiotic effect. Increases butyrate concentrations in lumen. Promotes the growth of *Bifidobacterium adolescentis* and the other bacteria with capacity to produce butyrate.	*In vitro*	Van den Abbeele et al. (2019)
2′FL	Gastrointestinal and immune	Protects against NEC through downregulation of pro-inflammatory biomarkers. Maintains the health architecture of mucus small intestine by expression of eNOS.	*In vivo*	Good et al. (2016)
2′FL	Gastrointestinal and immune	Modulates the intestinal microbiota. Improves the growth of *Bifidobacterium* spp. and diminishes the *Enterobacter* spp. growth, which generates inflammatory effects. Alleviates negative effects on intestinal microbiota produce by cesarean birth.	Clinical	Korpela et al. (2018)
2′FL and 3FL	Gastrointestinal	Decrease colon motor contractions and possess an antinociceptive effect, while 3′SL, 6′SL and LNnT not show these effects.	*In vivo*	Bienenstock et al. (2013)
2′FL and 3FL	Gastrointestinal and respiratory	Inhibit the adhesion of enteropathogenic *E.coli*, *Salmonella fyris* and *Pseudomonas aueruginosa* on epithelial cell lines.	*In vitro*	Weichert et al. (2013)
2′FL and 3FL	Gastrointestinal	Block the linkage of norovirus to HBGA, inhibiting gastroenteritis development.	*In vitro*	Weichert et al. (2016)
2′FL, LNT, LNFP I, LNFP II and LNFP III	Gastrointestinal	Link to Shiga toxin 1 and 2 from *E.coli*. Link to homopentameric B subunit of choleric toxin from *Vibrio cholerae*.	*In vitro*	El-Hawiet, Kitova, and Klassen (2015)
2′FL, 3FL, LNT and LNnT	Gastrointestinal	Modulate the intestinal microbiota. Promotes the growth of *Bifidobacterium longum* subsp.*infantis*.	*In vitro*	Garrido et al. (2015)
2′FL and LNnT	Gastrointestinal	Modulate the intestinal microbiota. Increase *Bifidobacterium* spp. concentration. Boost the propionate production. Increase the intestinal moving.	Clinical	Elison et al. (2016)
2′FL, LNT, LNFP I and LNFP II	Gastrointestinal and immune	Modulate neonatal rotavirus infection. Enhance infectivity of rotavirus commercial vaccine and could improve vaccine effectivity during immunization.	*In vitro*	Ramani et al. (2018)

2′FL, 3FL, LDFT, 3′SL and 6′SL	Gastrointestinal	Produce lactate and short fatty acid through *Bifidobacterium* spp. and *Bacteroides* spp. growth.	*In vitro*	Z. T. Yu, Chen, and Newburg 2013
2′FL, LNnT and 6′SL	Gastrointestinal	Inhibit growth of isolated *Enterobacteriaceae* spp.	*In vitro*	Hoeflinger et al. 2015
2′FL, 3FL, LNnT, 3′SL and 6′SL	Gastrointestinal	Provide a prebiotic effect on microbiota intestinal strains and commercial probiotics strains.	*In vitro*	Thongaram et al. 2017
2′FL, LNnT, 3′SL and 6′SL	Gastrointestinal	Diminish the infection produced by different rotavirus serotypes.	*In vivo*	Comstock et al. 2017
2′FL, 3′SL and 6′SL	Gastrointestinal	Reduce infectivity of human rotavirus G1P[8] and G2P[4].	*In vitro*	Laucirica et al. 2017
2′FL and 3′SL	Gastrointestinal	Protect against NEC by inhibition of TLR4, which reduce apoptosis, inflammation, weight lost and histologic damage.	*In vivo*	Sodhi et al. 2020
2′FL and 6′SL	Gastrointestinal	Provide an antiadhesive effect on *E.coli* 0119 in Caco-2 cellular lines.	*In vitro*	Facinelli et al. 2019
3′SL	Gastrointestinal	Diminishes *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium virulence through the metabolites from *Bifidobacterium mongoliense* fermentation.	*In vitro*	Bondue et al. 2020
3′SL, LNnT and 6′SL	Gastrointestinal	Inhibit cellular binding of rotavirus OSU and its replication.	*In vitro* and *in situ*	Hester et al. 2013
6′SL	Gastrointestinal	Diminishes susceptibility to development colitis in adult stage.	*In vivo*	Weiss and Hennet 2012
DSLNnT	Gastrointestinal	Protects against NEC and improves the health architecture of ileum.	*In vivo*	H. Yu et al. 2014
DSLNT	Gastrointestinal	Prevents NEC in rats and humans.	*In vitro* and clinical	Autran et al. 2018
LNB	Gastrointestinal	Modulates the intestinal microbiota. Increases the acetic acid concentrations.	*In vitro*	Satoh et al. 2013
HMO pooled	Immune	Diminish the HIV infection risk in infants with mothers with this virus.	Clinical	Bode et al. 2012
HMO pooled	Immune	Delay and suppress the diabetes type 1. Reduce the development of pancreatic insulitis. Modulate the intestinal microbiota and produce anti-inflammatory metabolites.	*In vivo*	L. Xiao, Van't Land, et al. 2018
HMO pooled	Immune	Supply immune homeostasis through immunomodulation of NO, PGE_2, ROS, TNF-α, IL-1β, IL-2, IL-6, IL-10, and NFκB and MAPK signaling pathways.	*In vitro*	Zhang et al. 2019
2′FL	Immune	Diminishes the risk of acquire allergic diseases especially atopic dermatitis.	Clinical	Sprenger, Odenwald, et al. 2017
2′FL	Immune	Increases thymus index. Improves T-lymphocytes proliferation, natural killer cell activity and degree of delayed-type hypersensitivity reactivity.	*In vivo*	Mao et al. 2019
2′FL	Immune and respiratory	Enhances the influenza vaccine-specific delayed-type hypersensitivity responses by increasing in IgG1, IgG2a, splenic B-cells, CD8+ and CD4+ T-cells proliferation, and γ-interferon. 2′FL blended with GOS and FOS can increase the responses to influenza immunization compared to 2′FL alone.	*In vivo*	L. Xiao, Leusink-Muis, et al. 2018 L. Xiao et al. 2019
2′FL y 3FL	Immune	Inhibit HIV through the linkage to dendritic cells.	Clinical	Kuhn et al. 2015
2′FL, 3FL, LNnT and 6′SL	Immune	Protect against the development of cystic fibrosis, systemic lupus erythematosus and lung disease by TLR 5, 7 and 8 inhibition.	*In vitro*	Cheng et al. 2019
2′FL and 6′SL	Immune	Diminish the symptoms of ovalbumin allergy as diarrhea and hypothermia.	*In vivo*	Castillo-Courtade et al. 2015
2′FL and 6′SL	Immune	Decrease allergy symptoms through attenuation in releasing of chemokines IL-8 and CCL20.	*In vitro*	Zehra et al. 2018
3′SL	Immune	Downregulates the pro-inflammatory factors as IL-8, IL-12 and TNF-α.	*In vitro*	Zenhom et al. 2011
LNFP III	Immune	Prevents the cow milk allergy development.	Clinical	Seppo et al. 2017
LNnT, LNFP I, LNFP II, sialyllacto-*N*-tetraose c (LST c) and fucodisialyllacto-*N*-hexaose (FDSLNH)	Immune	Reduce the risk of food sensitization.	Clinical	Miliku et al. 2018
2′FL, LNnT, 3′SL and 6′SL	Respiratory	2′FL and 3′SL decrease viral charge of respiratory syncytial virus, while LNnT and 6′SL decrease viral charge of Influenza type A virus.	*In vitro*	Duska-McEwen et al. 2014
HMO pooled	Blood	Promote a less colonization of *Streptococcus* group B especially with LNDFH I.	Clinical	Andreas et al. 2016
HMO pooled	Blood	Provide a bacteriostatic effect on *Streptococcus* from B group. Modulate the growth and biofilm formation of *Streptococcus* group B.	*In vitro*	Ackerman et al. 2017
HMO pooled	Blood, respiratory and nervous	Reduce the growth of *Streptococcus* group B by bacteriostatic effect. Promote a synergistic effect with antibiotics for the treatment of *Streptococcus* group B infections.	*In vitro*	A.E. Lin et al. 2017
2′FL			*In vitro*	Craft and Townsend 2019

(*continued*)

Table 1. Continued.

HMO tested	Benefited system	Main benefits	Type of study	Reference
	Blood, respiratory and nervous	Reduces the growth of *Streptococcus* group B. 2′FL amination improves anti-biofilm activity.		
2′FL	Blood and nervous	Provides a neural protection and repairment in stroked brain by attenuation of calcium flux and glutamic excitoxicity.	*In vitro* and *In vivo*	K. J. Wu et al. 2020
LDFT	Blood	The unique HMO that inhibits the platelet adhesion.	*In vitro*	Newburg, Tanritanir, and Chakrabarti 2016
3′SL	Blood	Works as anti-leukemia agent through the linkage to CD33 receptor from leukemia cells.	*In vitro*	Ha et al. 2020
2′FL	Nervous central	Improves the learning. Increases the molecules related with recently acquired knowledge.	*In vivo*	Vázquez et al. 2015
HMO pooled	Urinary	Inhibit the adhesion of uropathogenic *E. coli* on cell lines. Protect against invasion and cytotoxicity produced by uropathogenic *E. coli*.	*In vitro*	A. E. Lin et al. 2013
Bovine sialylated oligosaccharides from whey with similar structure	Locomotor	Promote bone growth. Increase corporal mass.	*In vivo*	Charbonneau et al. 2016
3′SL	Locomotor	Protects against the osteoarthritic development by restoration synthesis of Col2a1 and accumulation of sulfated proteoglycan, crucial factors in cartilage regeneration, and also through the blocking of Mmp3, Mmp13 and Cox-2, which promote cartilage degradation.	*In vitro* and *ex vivo*	Jeon et al. 2018
3′SL	Locomotor	Provides a therapeutic effect on the progression of rheumatoid arthritis through reduction of synovitis and pannus formation, and also suppression of cartilage destruction.	*In vitro* and *In vivo*	Kang et al. 2018
HMO pooled	————————	Prevent the infant obesity. Increase the corporal mass fat free.	Clinical	Alderete et al. 2015 Gridneva et al. 2019
2′FL	————————	Maintains a positive association between its consumption and body mass index.	Clinical	Lagström et al. 2020
LNFP II	————————	Protects against the fast gain of weight and diminishes the risk of obesity development.	Clinical	Berger et al. 2019

Klassen 2015; Hester et al. 2013; Li et al. 2014; Weichert et al. 2013; Weichert et al. 2016). It is important to highlight this particular function since diseases involving diarrhea represent the second place in deaths among children under five years old, amounting to around 1.4 million deaths of infants per year in developing countries (ten Bruggencate et al. 2014).

Moreover, HMO have been successfully applied in the treatment of two important viral gastrointestinal infections caused by rotavirus and norovirus (Hester et al. 2013; Koromyslova et al. 2017; Laucirica et al. 2017; Li et al. 2014; Ramani et al. 2018; Weichert et al. 2016). It was estimated that an average intake of $4\,g{\cdot}L^{-1}$ of a pooled HMO could lessen the diarrhea episodes caused by rotavirus. Mechanistic studies revealed that HMO played a role in preventing the virus from attaching to epithelial cells (Hester et al. 2013; Li et al. 2014). Another important function of oligosaccharides is to stimulate the immune response through γ-interferon and IL-10 expression, thus decreasing rotavirus virulence (Comstock et al. 2017; Li et al. 2014).

In particular, 2'FL, 3'SL and 6'SL were among the HMO structures that showed a notable antiviral activity against human rotavirus strains such as G1P[8] and G2P[4], which preferentially bound to the sequences characteristic to these oligosaccharides (Laucirica et al. 2017; Morozov et al. 2018). Based on these findings, HMO have been considered promising molecules to prevent and to assist treatment of this viral infection (Comstock et al. 2017; Hester et al. 2013; Laucirica et al. 2017; Li et al. 2014). Nonetheless, not all rotavirus infections in infants can be treated with HMO as it was the case of neonatal rotavirus G10P[11]. In this study carried out by Ramani et al. (2018), LNT, LNnT, and HMO pooled samples were investigated as active agents. Unfortunately, the opposite effect of enhancing viral infectivity was observed for all oligosaccharides, which led to the conclusion that a complex linkage between HMO, milk microbiome, and infant gut microbiome modulation took place for this specific instance. Similarly, 2'FL did not show any antiviral activity against rotavirus OSU in contrast to 3'SL, LNnT or 6'SL (Hester et al. 2013).

In the case of the antiviral potential of HMO against norovirus, 2'FL and 3FL were found to be the most efficient agents since L-fucose and D-glucose residues of these oligosaccharides maintained the hydrophilic and hydrophobic interactions with certain amino acids present at GI.1, GII.17 and GII.10 noroviruses. As a result, the binding of the virus to human histo-blood group antigens (HBGA) was inhibited, which is a crucial step in the infection development (Koromyslova et al. 2017; Weichert et al. 2016).

Other advantage that offers HMO ingestion for the gastrointestinal system is their antimicrobial activity against certain pathogenic bacteria, such as enteropathogenic *E.coli*, *Salmonella fyri*s and *Campylobacter jejuni*. These molecules were found to play a role in impeding the adhesion of the bacteria to the intestine (Morrow et al. 2004a; Morrow et al. 2004b; Morrow et al. 2005; Newburg et al. 2004; Newburg, Ruiz-Palacios, and Morrow et al. 2005; Patel et al. 2013; Ruiz-Palacios et al. 2003).

In particular, the *in vitro* studies showed that 2'FL decreased *E.coli*, *S. fyri*s and *C. jejuni* adhesion by 18-31, 12 and 26% respectively, while 3FL or 6'SL lowered *E.coli* adhesion by 26-29% (Facinelli et al. 2019; Weichert et al. 2013; Z. T. Yu, Nanthakumar, and Newburg 2016). Higher antipathogenic activity was observed for 2'FL, LNT, LNFP I, LNFP II and LNFP III since they can bind to six different exotoxins produced by *E.coli* and *Vibrio cholerae*, while 3'SL, 3′-sialyl-3-fucosyllactose (3'S3FL) or DSLNT exhibited affinity to three endotoxins (El-Hawiet, Kitova, and Klassen 2015). Likewise, *Enterobacteriaceae* spp. has been totally inhibited by 2'FL and 6'SL but not by LNnT, which only partially limited their growth. Also, 6'SL presented a bacteriostatic effect extending the lag phase of *E.coli* O1:K1:H7 (Hoeflinger et al. 2015).

Moreover, pooled HMO decreased by 46% the adhesion to epithelial cells of *Clostridium butyricum*, which has been considered as a new emerging pathogen (Cassir, Benamar, and La Scola 2016; Musilova et al. 2017). In addition, in a study carried out by Gonia et al. (2015) using pooled HMO at $15\,g{\cdot}L^{-1}$ was observed a 52% decline in the invasive potential of *Candida albicans*, a fungal pathogen with high prevalence in infant intestine, especially in premature ones.

Another beneficial property of HMO intake is the ability to modulate intestinal microbiota in children by stimulating the growth of specific probiotic bacteria, such as *Bifidobacterium* spp. and *Bacteroides* spp. These bacteria produce short-chain fatty acids that provide a hostile ambient for pathogens by lowering the intestinal pH (Elison et al. 2016; Garrido et al. 2015; James et al. 2019; Lawson et al. 2020; Z. Liu et al. 2020; Satoh et al. 2013; Thongaram et al. 2017; Van den Abbeele et al. 2019; J. Wang et al. 2017; Wickramasinghe et al. 2015; Z. T. Yu, Chen, and Newburg 2013).

Likewise, 2'FL and LNnT have been successfully tolerated by adults (Elison et al. 2016). No adverse effects were reported when the subjects were supplemented with 20 g per day of each tested HMO. Patients showed an increase in *Actinobacterium* spp. and *Bifidobacterium* spp. populations, with *Bifidobacterium adolescentis* as the most abundant strain in comparison to *Bifidobacterium longum* subsp. *infantis* that predominates in infants.

HMO have been also involved in the prevention of necrotizing enterocolitis (NEC), an important gastrointestinal disease in premature infants (Autran et al. 2018; Maffei and Schanler 2017; Rollins et al. 2016; Underwood et al. 2015; Walker 2013). Administration of the pooled HMO at physiological concentrations was shown to reduce NEC incidence by 83% (Maffei and Schanler 2017; Wickramasinghe et al. 2015). Furthermore, 2'FL at a concentration of $5\,g{\cdot}L^{-1}$ (Good et al. 2016), DSLNT and disialyllacto-*N*-neotetraose (DSLNnT) at concentrations between $0.20\text{-}0.40\,g{\cdot}L^{-1}$ (Autran et al. 2018; H. Yu et al. 2014), pooled HMO at $20\,g{\cdot}L^{-1}$ (C. Wang et al. 2019), as well as a mixture of 2'FL and 6'SL at $5\,g{\cdot}L^{-1}$ each (Sodhi et al. 2020) helped to rebuild the architecture of the damaged intestine through downregulation of the pro-inflammatory biomarkers related with this disease, such as iNOS, IL-6, IL-1β and TLR4.

Table 2. Summary of HMO toxicological evaluation.

HMO tested	Type of study	Tests realized	NOAEL ($g \cdot kg^{-1} \cdot day^{-1}$)	Reference
2′FL	*In vitro* and *In vivo*	Genotoxicity. 14, 90-day dose toxicity in rats.	5.00	Coulet et al. 2014
2′FL	*In vivo*	21-day oral toxicity study in farm neonatal piglets.	0.29	Hanlon and Thorsrud 2014
2′FL	*In vitro* and *In vivo*	Genotoxicity. 90-day dose toxicity in rats.	>7.25	Van Berlo et al. 2018
2′FL and LDFT blend	*In vitro* and *In vivo*	Genotoxicity. 90-day dose toxicity in neonatal rats.	5.00	Phipps, Baldwin, Lynch, Flaxmer, et al. 2018
2′FL, 3FL, LNT, 3′SL and 6′SL blend	*In vitro* and *In vivo*	Genotoxicity. 7-day oral tolerance study in rats. 90-day oral toxicity study in rats.	5.67	Parschat et al. 2020
3FL	*In vitro* and *In vivo*	Acute oral toxicity. Genotoxicity. 90-day subchronic rodent feeding study.	5.00	Pitt et al. 2019
LNT	*In vitro* and *In vivo*	Genotoxicity. 90-day dose toxicity in neonatal rats.	4.00	Phipps, Baldwin, Lynch, Stannard, et al. 2018
LNnT	*In vitro* and *In vivo*	Genetic toxicity. 14, 28, 90-day dose toxicity in rats.	5.00	Coulet et al. 2013
3′SL	*In vitro* and *In vivo*	Acute oral toxicity. Genotoxicity. Oral dose toxicity study in Beagle dogs. 28, 90-day oral toxicity study in rats.	>2.00	D. Kim et al. 2018
3′SL	*In vivo*	21-day oral toxicity study in neonatal piglets.	0.17	Monaco, Gurung, and Donovan 2019
6′SL	*In vitro* and *In vivo*	Acute oral toxicity. Genotoxicity. 90-day oral toxicity study in rats.	5.00	Gurung et al. 2018

The most recent studies have shown different protective mechanisms against NEC. For example, R. Y. Wu et al. (2019) demonstrated the HMO mediated the increase of mucin expression which affected cell wall permeability and thus prevented the adhesion of pathogens to the bowel epithelial cells. Secondly, it was observed that HMO induced the production of disulfide isomerase known as a chaperon protein related to NEC. In another work, C. Wang et al. (2019) linked the presence of these biomolecules with the enhanced proliferation of crypt cells.

However, it must be noted that the contradictory results were reported regarding the HMO activity against NEC. For instance, Rasmussen et al. (2017) found similar lesions in the HMO treated group to those shown by a control. HMO mixture used in this experiment comprised 25 different oligosaccharides. When a mixture was reduced to four components, namely 2′FL, LNT, LNnT and 6′SL (final concentration $5\,g \cdot L^{-1}$), no effect on NEC was observed. Authors suggested that either LNT or LNnT might show antagonistic effects but this hypothesis needs further investigation.

Effect of HMO on the immune system

The immune system is another physiological system that is positively affected by the HMO consumption. The gastrointestinal and immune systems are closely related because an adequate modulating of intestinal microbiota can positively influence the immunomodulatory mechanisms (Barile and Rastall 2013; Mao et al. 2019; McGuire and McGuire 2015; Walker 2013). In the most cases of gastrointestinal diseases that were treated or prevented with HMO, it was observed an immune response that consisted of the anti-inflammatory cytokines production, or by downregulating the expression of pro-inflammatory molecules (Morrow, Newburg, and Ruiz-Palacios 2018; Morrow, Newburg, and Ruiz-Palacios 2019). For example, such mechanisms were reported for the rotavirus and NEC treatment, respectively (Good et al. 2016; Li et al. 2014; Newburg, Tanritanir, et al. 2016; Rasmussen et al. 2017).

Among a variety of the oligosaccharide structures tested, 3′SL has been reported to downregulate the expression of IL-8, IL-12 and TNF-α in *in vitro* models (Zenhom et al. 2011). Similarly, 2′FL and LNFP I have shown a remarkable potential to alter the anti-inflammatory response through the IL-10 induction and suppress the expression of pro-inflammatory factors, such as IL-8, IL-12, or overexpression of CD4 and IFN-γ (Kulinich and Liu 2016). Likewise, it has been demonstrated that administration of pooled HMO at 1% (wt) could prevent the development of autoimmune diabetes type 1 in non-obese diabetic mice. The effect was transmitted by modulating the microbiome that could induce anti-inflammatory and anti-diabetogenic cytokines (L. Xiao, Van't Land, et al. 2018). Additionally, HMO intake at concentrations of $1.87\,g \cdot L^{-1}$ have been correlated with lower possibility of HIV transmission in infants *via* breastfeeding, which is linked to the LNT presence (Bode et al. 2012). Similarly, 2′FL, 3FL, LNFP I, LNFP II and LNFP III have all been demonstrated to have a protective function in infants from Zambia exposed to HIV, but only during breastfeeding (Kuhn et al. 2015). Both activities have been linked to the rise in CD4 cell counts.

Since HMO can modulate the anti- and pro-inflammatory responses, it was suggested their role as protective agents against allergic diseases (Castillo-Courtade et al. 2015; Miliku et al. 2018; Seppo et al. 2017; L. Xiao, Leusink-Muis,

et al. 2018; L. Xiao et al. 2019; Zehra et al. 2018). The ingestion of 2'FL or other FucOS with α1,2 bonds has been linked to the decline in the allergy cases associated with IgE, such as the atopic dermatitis (Sprenger, Odenwald, et al. 2017).

Effect of HMO on other physiological systems

Besides the gastrointestinal and immune systems, the beneficial properties of the HMO intake expand to other physiological systems including the respiratory, blood, nervous central, locomotor and urinary systems (Table 1).

In the case of respiratory diseases and sepsis, HMO have been successfully applied as bacteriostatic agent against *Streptococcus* bacteria from Group B, which is a strain directly related to the highly invasive infections in infants and adults (Ackerman et al. 2017; Craft, Thomas, and Townsend 2018; Craft and Townsend 2019; A. E. Lin et al. 2017; Patel et al. 2013). Pooled HMO at concentrations between 1-2 $mg{\cdot}L^{-1}$ have been also reported to delay the growth of *Streptococcus* bacteria Group B by 96-98%, with LNT and LNDFH I showing the highest inhibition potential. These oligosaccharides can work synergistically with certain antibiotics, such as vancomycin or ciprofloxacin, to enhance the overall outcome of the treatment (Ackerman et al. 2017; Andreas et al. 2016; A. E. Lin et al. 2017).

Moreover, the location and degree of fucosylation play a key role in the antimicrobial activity of HMO (Craft, Thomas, and Townsend 2018). It was shown that 2'FL at 5 $g{\cdot}L^{-1}$ could reduce the growth of Group B *Streptococcus* by 15% but did not affect the biofilm formation. The antibiofilm activity was improved through the 2'FL amination at the anomeric carbon resulting in the decrease of biofilm production by 37-46% (Craft and Townsend 2019).

It was also reported that the nervous central system could benefit from the HMO consumption. The study involved administration of 2'FL at doses of 350 $mg{\cdot}kg^{-1}$ per day for the duration of 5- and 12-weeks *in vivo* models (rodents). Analysis of the biological response to this fucooligosaccharide showed the increase in production of molecules related to memory, like postsynaptic density protein 95, calcium calmodulin kinase II, and brain neurotrophic factor (Vázquez et al. 2015).

Finally, HMO intake can support the health of the urinary system when applied at the dose of 15 $g{\cdot}L^{-1}$. They prevent uropathogenic *E. coli* strain from attaching to epithelial cells, and in this way delaying the MAPK and NF-κB signaling pathways associated with infections (A. E. Lin et al. 2013).

Incorporation of HMO to infant formula

Owing to the numerous benefits that the HMO ingestion provides to infant health, the World Health Organization (WHO) highly recommends the exclusive breastfeeding during the first six months of a newborn infant life (Gallier et al. 2015). However, diverse factors such as personal decision, socio-cultural or economic reasons, and health problems of the mother disfavor this practice. Only 38% of the worldwide infant population has been exclusively breastfed (Martin, Ling, and Blackburn 2016). The infant formula market observed a sharp rise in sales up to 40.8% in the period between 2008 and 2013, especially in the emerging economies (Baker et al. 2016; G. Kent 2015; Rollins et al. 2016).

Currently, milk formulations for newborns have turned away from the traditionally fortifying oligosaccharides, such as FOS and GOS, prebiotics with higher availability in the past years (Alliet et al. 2016; Elison et al. 2016; Marriage et al. 2015; Puccio et al. 2017). Thus, a new concept of infant formula has emerged, where industry try to offer the closest formulation to human milk, through the incorporation of fat, proteins and HMO (Alliet et al. 2016; Bode et al. 2016; Gallier et al. 2015; Lönnerdal 2014).

The incorporation of HMO in milk formulations for children is especially recommended when the concentrations or type of HMO produced by mothers are not sufficient for a good growth of an infant, or with the objective to give additional protection against different diseases related to the child early years (R. Kent et al. 2015; Olivares et al. 2015; Wejryd et al. 2018). For example, an infant formula composed by cow proteins, 2'FL at 1 $g{\cdot}L^{-1}$ and LNnT at 0.5 $g{\cdot}L^{-1}$, promoted a similar microbiome like that obtained from breastfed healthy infants (Alliet et al. 2016).

Other studies have shown a good assimilation and tolerance by infants when they were fed with 2'FL and LNnT incorporated into the milk formulation (Marriage et al. 2015; Puccio et al. 2017). In the case of the addition of 2'FL at concentrations between 0.2-1.0 $g{\cdot}L^{-1}$, the formula was well tolerated with the absorption rate between 0.05-0.07% (wt), however, no significant differences in anthropometric measurements were found among infants when compared to those fed with the formulas fortified with GOS (Marriage et al. 2015). On the other hand, Puccio et al. (2017) reported lower incidence of respiratory infections and reduced usage of antipyretic and antibiotics in infants fed with 2'FL and LNnT at concentrations of 1.0 $g{\cdot}L^{-1}$ and 0.5 $g{\cdot}L^{-1}$, respectively.

To conclude, the above studies confirm the good tolerance and safety of 2'FL and LNnT as additives in infant milk formulations. More evidence on the toxicological properties of the HMO mentioned above and other types have been evaluated mainly through pre-clinical studies.

In this context, No Adverse Effect Level (NOAEL) is the parameter developed to ensure the toxicological safety of HMO in infant formula or in other functional foods. As shown in Table 2, the NOAEL oscillates between 4 and 5 $g{\cdot}kg^{-1}{\cdot}day^{-1}$ for most HMO tested. Some discrepancies were found only for 2'FL, which NOAEL was evaluated $>$ 7.5 $g{\cdot}kg^{-1}{\cdot}day^{-1}$ and it could be associated with the purity grade (Van Berlo et al. 2018). On the contrary, Hanlon and Thorsrud (2014) estimated a NOAEL value for 2'FL at 0.29 $g{\cdot}kg^{-1}{\cdot}day^{-1}$ since they carried out experiments using that as the highest concentration possible. Their results were consistent with those obtained by Monaco, Gurung, and Donovan (2019) for 3'SL. In conclusion, the HMO risk

assessment studies have confirmed the safety status of HMO when used as food additives.

Presently, HMO can be found in infant formulas produced by Nestlé® and Abbott® that have incorporated mainly 2'FL and LNnT (Bych et al. 2019). The addition of more complex HMO has been hampered by inefficient synthetic processes and production costs (Bode et al. 2016). In fact, these are the main obstacles that have affected the growth of HMO market, valued at 14.6 billion dollars in 2017, with an expected annual increase of 21.9% (GVR (Grand View Research) 2018).

Synthetic methods for HMO production

Since scientific evidence has clearly shown the involvement of HMO in a variety of beneficial physiological effects in infants, the research efforts have been focused on gaining access to these valuable biomolecules *via* synthetic routes. Indeed, the HMO demand in the next years could grow either by reaching out to new consumers, or by the HMO incorporation in other products. So far, four different synthetic routes have been proposed for the HMO production: chemical synthesis, the whole cell biotransformation, enzymatic and chemo-enzymatic pathways.

Chemical synthesis of HMO

Chemical synthesis has been widely applied to access oligosaccharides for the last twenty years (Chernyak, Oscarson, and Turek 2000; Hsu et al. 2010; Love and Seeberger 2005; Plante, Palmacci, and Seeberger 2001). However, its usefulness in HMO production has been limited (Table 3). Among main obstacles that cause difficulties to readily afford HMO through chemical process are the multi-step synthesis to obtain the final HMO structure, and usage of the reactants banned in the manufacture of food additives (Bode et al. 2016; X. Chen 2015; Hanson et al. 2004).

The chemical route usually consists of sequential steps for selective protection of the hydroxyl groups from the monosaccharides. It is an indispensable procedure to guide the position of the glycosidic bond. In general, the main protector groups used in the HMO chemical synthesis are benzoyl, benzyl, and acetic esters (Codee et al. 2011; Lipták, Borbás, and Bajza 2007; Pétursson 1997). Following the protection step, the glycosidic bond formation is carried out using chemical catalysts, such as trimethylsilyl trifluoromethanesulfonate (TMS-OTf), triethylsilyl trifluoromethanesulfonate (TESOTf), silver trifluoromethanesulfonate (AgOTf), triflic acid (TfOH), *N*-iodosuccinimide (NIS), and tetra-n-butylammonium bromide (TBABr). Finally, the protected HMO structure must be deprotected to obtain the desired molecule.

The reaction conditions and solvents used to form the glycosidic bond are other limiting factors that hamper advances in synthetic HMO production (Arboe Jennum et al. 2014; Craft and Townsend 2017; Pereira and McDonald 2012; Schmidt and Thiem 2010). For example, application of low temperatures for certain reactions adds to the production costs, while usage of toxic solvents implies extensive purification processes that in some instances cannot be avoided.

Nonetheless, chemical synthesis of HMO has become a tool to produce the backbone structures that are later used in enzymatic or chemo-enzymatic processes to afford HMO with higher degree of complexity (Saumonneau et al. 2016; Schmidt and Thiem 2010; Pereira and McDonald 2012). Thus, the use of one-pot or sequential reactions allows the excessive purification stages to be avoided, which significantly increases the global yields.

LNT has been successfully synthesized through three building blocks: 1) carbamate derivative from glucosamine, 2) acetylated galactose and 3) lactose selectively protected with acetic and benzyl esters. The latter compound acts as acceptor of a protected LNB derivative, which is synthesized from carbamate glucosamine and acetylated galactose (Craft and Townsend 2017). The complete synthesis of LNT requires sixteen steps, of which eight are protection of lactose hydroxyl residues, other two steps are required to form LNB derivative, and the remaining six steps correspond to LNT formation, including glycosidic bond formation and deprotection stages, with a global yield close to 31% (mol).

Likewise, 2'FL and 3FL can be used for the construction of other FucOS, such as lactodifucotetraose (LDFT), LNFP II and LNFP III (Saumonneau et al. 2016). Pereira and McDonald (2012) reported the synthesis of 2'FL and 3FL, using allyl lactoside as an acceptor of fucose trichloroacetimidate, which in turn acts as a fucose donor. Fucose trichloroacetimidate was obtained through L-fucose protection with benzyl and acetic esters and subsequent activation of anomeric carbon with 1-*O*-trichloroacetimidate to obtain a global yield of 25% (mol) after five steps. The fucose acceptor was synthesized at overall yield of 40% (mol) in a four-step process, starting from lactose octaacetate and allyl alcohol, and maintaining the hydroxyl group at the C-2 free for glycosidic bond formation. Then, the α1,2 bond between acceptor and fucose donor was formed using TMS-OTf at 0 °C in CH_2Cl_2 with 60% (mol). Deprotection phase was achieved in seven steps at 32% (mol). Although 3FL was synthesized following the same protocol, the yield of fucose acceptor was lower than that obtained for 2'FL, affording only 3.4% (mol). The α1,3 bond formation was carried out under the same conditions that were used for 2'FL, with glycosylation and deprotection yields at 82% and 34% (mol), respectively.

Another approach to the HMO precursors was proposed by Gangwar, Sahu, and Deepak (2018), who developed "reverse building blocks of milk oligosaccharides", with fucose and glucose moieties at the reducing end instead of lactose. The synthetic route is similar to that reported by Pereira and McDonald (2012), although in this case lactose trichloroacetimidate acted as a lactose donor while methyl-3,4-isopropylidene-α-L-fucopyranoside and methyl-2,3,4-tri-*O*-acetyl-D-glucopyranoside were used as acceptors. Likewise, glycosylation was carried out with TMS-OTf as a catalyst to afford reverse trisaccharides, which are considered the 2'FL and GOS analogues with similar properties yet

Table 3. Summary of the most recent advances in HMO synthetic methods.

HMO synthesized	Type of synthesis	Donor	Acceptor	Summary of synthesis	Global Yield	Reference
2′FL	Chemical	1-*S*-phenyl-2,3,4-*O*-benzyl-fucose	Lactose acetonide	Sequential and one-pot synthesis to obtain fucosyl donor and lactose acceptor. 4 steps to the donor synthesis and 2 steps to acceptor synthesis. 2′FL obtained in three steps starting from donor and acceptor compounds by glycosylation in dry CH_2Cl_2 at 5-20 °C for 48 h using TBABr as catalyst.	~27[a]	Agoston et al. 2019
2′FL and 3FL	Chemical	Fucose trichloroacetimidate	1-*O*-alyllactosyl derivative	Sequential synthesis with derivatives of fucose and lactose. Fucose acceptor synthesized in 4 steps. Fucose donor synthesized in 5 steps. Glycosylation in CH_2Cl_2 at 0 °C with TMS-OTf as catalyst with 8 steps.	2′FL: ~20[a] 3FL: ~34[a]	Pereira and McDonald 2012
LNFP I	Chemical	1-*S*-phenyl-2,3,4-*O*-benzyl-fucose	*O*-benzyl-LNT	Sequential and one-pot synthesis to obtain donor and acceptor compounds. 4 steps to the acceptor synthesis. Product obtained in 2 steps under glycosylation in CH_2Cl_2 at −20 °C with NIS/TESOTf as catalysts.	~50[a]	Arboe Jennum et al. 2014
SLNT	Chemical	3-sialyllactosamine derivative	Methyl-β-lactoside	Sequential synthesis with 5 steps. Selective protection and deprotection. Glycosylation in toluene at −40 and −10 °C with NIS and TfOH as catalysts.	~49[a]	Schmidt and Thiem 2010
LNT	Chemical	LNB derivative	Lactose protected selectively	Sequential synthesis with derivatives of glucosamine, galactose and lactose as building blocks. 16 steps for final formation. Glycosylation in CH_2Cl_2 at −10 °C with TfOH as catalyst. Deprotection through deacetylation and hydrogenolysis.	~31[a]	Craft and Townsend 2017
LNT	Chemical	LNB derivative	Lactose protected selectively	Lineal synthetic strategy with 7 steps to obtain the final product. Glycosylation in CH_2Cl_2 at −30 °C for 15 min with AgOTf as catalyst.	~48[a]	Bandara, Stine, and Demchenko 2019b
LNnT	Chemical	LacNAc derivative	Lactose protected selectively	Lineal synthetic strategy with 7 steps to obtain the final product. Glycosylation in CH_2Cl_2 at −30 °C for 15 min with AgOTf as catalyst.	~42[a]	Bandara, Stine, and Demchenko 2019c
LNH	Chemical	Lactosamine thioglycoside	Diol-tetrasaccharide similar to LNT	Sequential synthesis with 8 steps. Glycosylation in CH_2Cl_2 from −40 °C to room temperature for 60 min with NIS/AgOTf as catalysts.	~25[a]	Bandara, Stine, and Demchenko 2019a
LNnH	Chemical	Lactosamine thioglycoside	Diol-tetrasaccharide similar to LNnT	Sequential synthesis with 7 steps. Glycosylation in CH_2Cl_2 from −40 °C to −18 °C for 60 min with NIS/AgOTf as catalysts.	~36[a]	Bandara, Stine, and Demchenko 2020
Reverse building blocks of milk oligosaccharides	Chemical	Lactose trichloroacetimidate	Glucose and fucose derivatives	Sequential synthesis with derivatives of glucose, fucose and lactose as building blocks. Multi-step synthesis with use of protector groups. Glycosylation in CH_2Cl_2 at 0 °C with TMS-OTf as catalyst for 60 min.	55[a]	Gangwar, Sahu, and Deepak 2018
2′FL	Fermentation	GDP-fucose	Lactose	Synthesis through *E. coli* JM109(DE3) with α1,2-fucosyltransferase.	1.23[b]	Lee et al. 2012

(*continued*)

Table 3. Continued.

HMO synthesized	Type of synthesis	Donor	Acceptor	Summary of synthesis	Global Yield	Reference
				Batch Fermentation. LB medium, 25 °C, pH= 6.8, 250 rpm, 96 h. [Lactose] = 14.54 g·L^{-1}		
2′FL	Fermentation	GDP-fucose	Lactose	Synthesis through recombinant *E. coli* JM109 with α1,2-fucosyltransferase. Batch Fermentation 13 L. Mineral salt medium with glycerol as carbon source, pH = 7 at 37 °C, 35.5 h.	20.28[b]	Baumgärtner et al. 2013
2′FL	Fermentation	GDP-fucose	Lactose	Batch fermentation with recombinant *E.coli* BL21star(DE3) in LB medium at 25 °C, pH= 6.8, 250 rpm, 20 g·L^{-1} of glycerol as carbon source and 54 h. [Lactose]: 20 g·L^{-1}	6.40[b]	Chin et al. 2015
2′FL	Fermentation	GDP-fucose	Lactose	Synthesis through recombinant *E. coli* BL21star(DE3). LB medium with 20 g·L^{-1} of glycerol as carbon source at pH= 6.8, 25 °C and 59 h. Bioreactor with volume of 2.5 L. [Donor and acceptor]: 10 g·L^{-1}	23.10[b]	Chin et al. 2016
2′FL	Fermentation	GDP-fucose	Lactose	Batch fermentation in two steps with recombinant *E.coli* BL21(DE3). Fermentation conditions: Proposed medium by authors at pH= 8, 25 °C and 22 h.	10.75[b]	Qin et al. 2016
2′FL	Fermentation	GDP-fucose	Lactose	Batch fermentation with recombinant *E.coli* in medium proposed by author at pH= 6.8, 25 °C, 250 rpm with 20 g·L^{-1} of lactose and 20 g·L^{-1} of glycerol as carbon source. Fermentation for 29 h.	15.40[b]	Chin et al. 2017
2′FL	Fermentation	GDP-fucose	Lactose	Batch fermentation with recombinant *E.coli* JM109 with new approach of fermentation adding α-ketoglutarate as energy source and modifying the Krebs cycle. Fermentation medium: PBS buffer at pH= 7.7, 250 rpm, 37 °C with 5 g·L^{-1}of fucose and 5 g·L^{-1} of lactose for 24 h.	3.30[b]	Guan et al. 2018
2′FL	Fermentation	GDP-fucose	Lactose	Batch fermentation with recombinant *Saccharomyces cerevisae* in YP medium with 40 g·L^{-1} of glucose as carbon source, 3 g·L^{-1} of lactose at 30 °C, 250 rpm for 96 h.	0.56[b]	J. J. Liu et al. 2018
2′FL	Fermentation	GDP-fucose	Lactose	Batch fermentation with recombinant *Sacharomyces cerevisae* in Verduyn medium with 20 g·L^{-1} of glucose as carbon source, 2 g·L^{-1} of lactose, 2 g·L^{-1} of fucose at pH= 5.5, 30 °C, 250 rpm for 120 h.	0.50[b]	S. Yu et al. 2018
2′FL	Fermentation	GDP-fucose	Lactose	Batch fermentation with recombinant *Saccharomyces cerevisae* and *Yarrowia lipolytica*. SC medium with glucose as carbon source, 0.5% (wt) of lactose at 30 °C, 220 rpm, pH= 6.3 and 6.5 for *S. cerevisae* and *Y. lipolytica* respectively, for 24 h.	15-24[b]	Hollands et al. 2019
2′FL	Fermentation	GDP-fucose	Lactose	Batch fermentation with recombinant *Bacillus subtilis*. 3 L bioreactor. Medium proposed by authors with glycerol at 800 g·L^{-1} as carbon source, 20 g·L^{-1} of	5.01[b]	Deng et al. 2019

				$MgSO_4 \cdot 7H_2O$, 20 g·L^{-1} of lactose, 5 g·L^{-1} of fucose at pH= 6.0, 30-50% dissolved oxygen for 50 h.		
2′FL	Fermentation	GDP-fucose	Lactose	Biosynthesis using the recombinant *E.coli* ΔL M15 strain. Fermentation in 50 mL of medium proposed by authors with phosphates, citrates, metallic salts, 20 g·L^{-1} of glycerol as carbon source, 50 mg·L^{-1} of ampicillin/kanamycin and 5 g·L^{-1} of lactose at 25-37 °C, pH= 6.8, 250 rpm, aerobic conditions for 72 h.	0.49[b]	Seydametova et al. 2019
2′FL and 3FL	Fermentation	GDP-fucose	Lactose	Batch fermentation with recombinant *E.coli* BL21(DE3) in M9 minimal medium with 12 g·L^{-1} of glucose as carbon source, 10 g·L^{-1} of lactose at pH = 7.2 and ∼12 h.	2′FL: 9.5[b] 3FL: 13[b]	Huang et al. 2017
3FL	Fermentation	GDP-fucose	Lactose	Batch fermentation with recombinant *E.coli* BL21(DE3), which contain an genetic modified α1,3-fucosyltransferase. Fermentation in LB medium at pH = 6.8 25-37 °C, 200 rpm and 72 h. [Acceptor]: 5 g·L^{-1}	0.58[b]	J. Yu et al. 2018
3FL	Fermentation	GDP-fucose	Lactose	Biosynthesis using the recombinant *E.coli* BL21(DE3) strain. Flask fermentation in 250 mL of minimum media with phosphates, citrates, metallic salts, thiamin, EDTA, 5 g·L^{-1} of glycerol as carbon source and feeding at 1.5 g·L^{-1}, 50 mg·L^{-1} of kanamycin/streptomycin, 10 g·L^{-1} of yeast extract, 1.64 g·L^{-1} of fucose and 6.84-13.68 g·L^{-1} of lactose at 30-37 °C, pH= 7.0-7.5, 250 rpm for 60.5 h.	4.60[b]	Choi et al. 2019
LNFP I	Fermentation	GDP-fucose	LNT	Batch fermentation with *in situ* formation of LNT through a recombinant *E.coli* strain. Minimal medium at 30 °C, 90 rpm, and 26 h for LNT synthesis and 30 °C, 90 rpm, and 65 h for LNFP I production. [Lactose]: 2 g·L^{-1} [Fucose]: 2 g·L^{-1}	0.27[b]	Baumgärtner et al. 2015
LNT	Fermentation	UDP-GlcNAc and UDP-Gal	Lactose	Synthesis through recombinant *E. coli* K-12 LJ110. Minimal medium with 10 g·L^{-1} of glucose as carbon source, 0.2% (wt) of lactose, at 30 °C for 24 h.	0.22[b]	Baumgärtner et al. 2014
LNnT	Fermentation	UDP-GlcNAc and UDP-Gal	Lactose	Batch fermentation with recombinant *Bacillus subtilis*. 3 L bioreactor. Medium proposed by authors with phosphates, metallic salts, 30 g·L^{-1} of glucose, 1.8 g·L^{-1} of lactose, 20 g·L^{-1} of yeast extract, 20 g·L^{-1} of triptone and 8 g·L^{-1} of urea. Feeding solution with 500 g·L^{-1} of glucose and 30 g·L^{-1} of lactose. Fermentation at pH = 7, 37 °C, 800 rpm, 1 vvm aeration for 72 h.	4.52[b]	Dong et al. 2019
2′FL	Enzymatic	GDP-fucose	Lactose	One-pot multi-enzymatic synthesis with *in situ* production of GDP-fucose. Fucosylation reaction catalyst by α1,2-fucosyltransferase from *wbgL* gene of *E. coli* O126.	100[a]	Engels and Elling 2013

(*continued*)

Table 3. Continued.

HMO synthesized	Type of synthesis	Donor	Acceptor	Summary of synthesis	Global Yield	Reference
				[Donor]: 4.3 mM [Acceptor]: 8 mM pH= 7.6, 22 °C and 22 h.		
2′FL	Enzymatic	pNP-fucose	Lactose	Use of an α-L-fucosidase isolated from soil metagenome and α-L-fucosidase from *Thermotoga maritima* at optimum pH for each enzyme, 30 °C and 75 min. [Donor]: 25 mM [Acceptor]: 100 mM	0.6-6.4[a]	Lezyk et al. 2016
2′FL	Enzymatic	pNP-fucose	Lactose	Use of α-L-fucosidase from *Thermotoga maritima* at pH= 5, 60 °C and 3 h. [Donor]: 3.5 mM [Acceptor]: 584 mM	25.2[a]	Guzmán-Rodríguez et al. 2018a
2′FL	Enzymatic	Citric Xyloglucan	Lactose	Use of α-L-fucosidase from *Fusarium graminearum* at pH= 4.6, 40 °C and 24 h. [Donor]: 2 mM [Acceptor]: 100 mM	14[a]	Zeuner, Muschiol, et al. 2018
2′FL	Enzymatic	pNP-fucose	Lactose	Use of α-L-fucosidase from *Lactobacillus rhamnosus* GG at pH= 7, 37 °C and 12 h. [Donor]: 1 g·L^{-1} [Acceptor]: 200 g·L^{-1}	21[a]	Escamilla-Lozano et al. 2019
2′FL and 3FL	Enzymatic	pNP-fucose	Lactose	Use of recombinant α-L-fucosidase from *Pedobacter* sp. CAU209 at pH= 8.5, 25-40 °C 0.5 U·mL^{-1} of enzyme and 3 h. [Donor]: 2.85 g·L^{-1} [Acceptor]: 239 g·L^{-1}	2′FL: 14.5[a] 3FL: 70.5[a]	Shi et al. 2020
3FL	Enzymatic	GDP-fucose	Lactose	Use of mutant α1,3-fucosyltransferase from *H. pylori* 26695. Reaction conditions: pH = 7.6, 37 °C, 5 mM of $MgCl_2$ and 1 h. [Donor]: 5 mM [Acceptor]: 15 mM	>96[a]	Choi et al. 2016
3FL LNFP III LNDFH II LNnDFH II	Enzymatic	GDP-fucose	Lactose LNnT LNT Azidopropyl-LNnT	Multi-enzymatic synthesis with *in situ* production of GDP-fucose through bifunctional enzyme from *Bacteroides fragilis* and inorganic pyrophosphatase from *Pasteurella multocida*. Fucosylation with fucosyltransferase from *H. pylori* UA948 with ability to catalyst α1,3 and α1,4 bonds. Enzyme with great fucosyltransferase activity at pH = 6-9 and 30 °C.	90[a] 88[a] 98[a] 99[a]	H. Yu et al. 2017
3FL LNFP III LNFP VI LNnDFH II DF-*para*-LNnH TF-*para*-LNnH	Enzymatic	GDP-fucose GDP-fucose and UDP-Gal GDP-fucose	Lactose LNnT LNT II LNnT *para*-LNnH *para*-LNnH	Multi-enzymatic synthesis approach using the truncated version of the α1,3-fucosyltransferase from *Helicobacter pylori* for fucosylation process at pH= 8 and 37 °C. LNT II attached to fucose was then elongated with the NmLgtB glycosyltransferase to obtain the product. [Acceptors]: 10 mM [GDP-fucose]: 12-40 mM [UDP-Gal]: 12 mM	88[a] 80[a] 80[a] 68[a] 80[a] 94[a]	Bai et al. 2019
LNFP I	Enzymatic	GDP-fucose	LNT	Transfucosylation reaction with α1,2-fucosyltransferase from *Thermosynechococcus*	95[a]	Zhao et al. 2016

				elongatus BP-1. One-pot synthesis with three different enzymes: L-fucokinase, fucosyltransferase and inorganic pyrophosphatase. Fucosylation with 10 mM of acceptor and 25 mM of donor at pH= 7.4 and 25 °C.		
LNFP II	Enzymatic	3FL	LNT	Use of mutated α-L-fucosidase from *Clostridium perfringens* and *Bifidobacterium bifidum* at pH= 7.0 or 5.5, 40 °C and 3 h. [Donor]: 10 mM [Acceptor]: 100 mM	39-50[a]	Zeuner, Vuillemin, et al. 2018
LDFT LNFP II LNFP III LDFH I	Enzymatic	3FL	2′FL LNT LNnT LNFP I	Transfucosylation with a mutant α1,3/4-fucosidase from *Bifidobacterium longum* subsp. *infantis*. Reaction conditions: pH= 6, 37 °C [Donor]: 20 mM [Acceptor]: 20 mM	17[a] 17[a] 21[a] 11[a]	Saumonneau et al. 2016
LNFP V LNFP VI LNDFH II LNnDFH II	Enzymatic	UDP-GlcNAc, UDP-Gal and GDP-fucose	Azidohexyl-lactose	One-pot multi-enzymatic synthesis with *in situ* production of GDP-fucose by bifunctional enzyme from *Bacteroides fragilis*, transfucosylation with α1,3/4 fucosyl-transferase from *H. pylori* DSM6709, and β1,3-*N*-acetylglucosaminyltransferase from *H. pylori*, β1,3-galactosyltransferase from *E. coli* O55 and/or β1,4-galactosyltransferase from *Neisseria meningitides* to elongate acceptor. Fucosylation for LNFP V obtaining at pH = 6.5, 37 °C for 18 h. Fucosylation for LNFP VI obtaining at pH= 6.5, 37 °C for 16 h. Fucosylation for LNDFH II obtaining at pH= 6.5, 37 °C for 26.5 h. Fucosylation for LNnDFH II obtaining at pH= 6.5, 37 °C for 20.5 h. [Acceptor]: 10 mM [GDP-fucose]: 12-25 mM	∼61[a] ∼70[a] ∼53[a] ∼88[a]	Tsai et al. 2019
LNT II LNT LNFP II LNFP V LNDFH I	Enzymatic	UDP-GlcNAc UDP-Gal GDP-fucose GDP-fucose GDP-fucose	Lactose LNT II LNT LNT II LNT	One-pot multi-enzymatic systems with *in situ* production of UDP-GlcNAc, UDP-Gal and GDP-fucose. General reaction conditions: 2.92 mmol of lactose, 3.51 mmol of GlcNAc, 3.8 mmol of ATP and 3.8 mmol of UTP at pH= 8, 30 °C and 72 h for LNT II. 0.18 mmol of LNT II, 0.24 mmol of galactose, ATP, and UTP at pH= 8, 37 °C and 30 h for LNT. 0.71 mmol of LNT and 0.78 mmol of fucose at pH= 7.5, 30 °C and 2 days for LNFP II. 0.29 mmol of fucosyl-LNT II, 0.43 mmol of galactose at pH= 8, 30 °C and 48 h for LNFP V. 0.12 mmol of LNFP I, 0.23 mmol of fucose at pH= 7.5, 30 °C and 2 days for LNDFH I.	97[a] 96[a] 81[a] 93[a] 80[a]	McArthur, Yu, and Chen 2019
LNT II	Enzymatic	*N,N′*-diacetylchitobiose	Lactose	Synthesis of precursor for production of LNT and LNnT. Use of two different β-*N*-acetylhexosaminidases. Reaction at pH= 6 or 8, 25 °C and 1-2 h. [Donor]: 100 mM [Acceptor]: 500 mM	2-8[a]	Nyffenegger et al. 2015
LNB	Enzymatic	*O*-nitrophenyl-β-D-galactopyranoside	GlcNAc	Transgalactosylation reaction with phospho-β-galactosidase from *Lactobacillus casei* BL23	∼69[a]	Bidart et al. 2017

(*continued*)

Table 3. Continued.

HMO synthesized	Type of synthesis	Donor	Acceptor	Summary of synthesis	Global Yield	Reference
				[Donor]: 40 mM [Acceptor]: 200 mM pH= 7.5, 42 °C, 3 h.		
LNT	Enzymatic	UDP-Gal and UDP-GlcNAc	6-azidohexyllactoside	One-pot multi-enzymatic synthesis with *in situ* production of donors. LNT production through donor transglycosylation with β1,3-*N*-acetylglucosaminyltransferase from *H. pylori* and β1,3-galactosyltransferase from *E. coli* O55:H7. Working range of temperatures: 40-65 °C.	85[a]	Fang et al. 2018
LNnT	Enzymatic	UDP-Gal and UDP-GlcNAc	6-azidohexyllactoside	One-pot multi-enzymatic synthesis with *in situ* production of donors. LNnT production through donor transglycosylation with β1,3-*N*-acetylglucosaminyltransferase from *H. pylori* and β1,4-galactosyltransferase from *H. pylori*. Incubation at 37 °C for 30 min.	94[a]	Fang et al. 2018
LNnT	Enzymatic	Lactose	LNT II	Synthesis with three different thermostable β-galactosidases from *Thermus thermophiles* HB27, *Pyrococcus furiosus* and truncated enzyme of *Bacillus circulans*. Transgalactosylation at pH= 6, optimum temperatures for each enzyme and times between 10-30 min. [Donor]: 20 mM [Acceptor]: 100 mM	1.0-7.1[a]	Zeuner, Nyffenegger, et al. 2016
1-propylazide-LNnT	Enzymatic	UDP-Gal	Tri-saccharide formed by lactosyl-propyl-azide and UDP-GlcNAc	Multi-enzymatic synthesis one-pot. Tri-saccharide formation through trienzymatic one-pot system at pH= 8, 37 °C and 18 h with a yield of 88% (mol). Tri-saccharide galactosylation with β1,4-galctosyltransferase from *Neisseria meningitidis* at pH= 7.5, 37 °C, and 12 h with yield of 93% (mol).	~82[a]	C. Chen et al. 2015
3′SL	Enzymatic	cGMP	Lactose	Use of trans-sialidase from *Trypanosoma cruzi* at pH= 5.8 and 30 °C with 38.3 g·L^{-1} of cGMP and 14.1 g·L^{-1} of lactose for 22 h in reactor with final volume of 5.8 L.	64[a]	Holck et al. 2014
3′SL	Enzymatic	cGMP	Lactose	Use of mutated sialidase Tr6 from *Trypanosoma rangeli*. Reaction conditions: pH= 5.5, 25 °C, 20 min and volume production of 5 L. [Donor]: 26 g·L^{-1} [Acceptor]: 40 g·L^{-1}	50[a]	Michalak et al. 2014
3′SL	Enzymatic	cGMP	Lactose	Use of 25% (v/v) of *t*-butanol as co-solvent in reaction catalyst by mutant enzyme Tr6 from *Trypanosoma rangeli*. Reaction conditions: pH= 5.5, 25 °C and 60 min [Donor]: 4.6 mM equivalent to sialic acid [Acceptor]: 117 mM	32[a]	Zeuner et al. 2014
3′SL and 6′SL	Enzymatic	cGMP	Lactose	Trans-sialylation with sialyltransferase from recombinant *Pasteurella multocida*. Optimum conditions for 3′SL: pH= 6.4, 40 °C, 5% (wt) of cGMP, 100 mM of lactose and 6 h.	3′SL: 30.6[a] 6′SL: 37[a]	Y. Guo et al. 2014

				Optimum conditions for 6′SL: pH= 5.4, 40 °C, 5 % (wt) of cGMP, 10 mM of lactose and 8 h.		
3′SL and 6′SL	Enzymatic	CMP-Neu5Ac	Lactose	Use of native and mutant sialyltransferase from *Pasteurella dagmatis*. Reaction conditions: pH= 8 with 1 mM of donor and acceptor. Mutation allow a higher production of 6′SL	72-75[a]	Schmölzer et al. 2015
6′SL	Enzymatic	cGMP	Lactose	Trans-sialylation with mutant sialyltransferase from *Pasteurella multocida*. Reaction conditions: pH= 6.4, 40 °C and 20 h [Donor]: 5% (wt) [Acceptor]: 100 mM	15-22[a]	Y. Guo et al. 2015
6′SL	Enzymatic	Sialic acid dimer and oligomer with α2,8-linkages	Lactose	Use of trans-sialidase from *Bacteroides fragilis* NCTC 9343 at pH= 6.5, 50 °C and 10 min. [Dimer]: 40 mM [Oligomer]: 40 $g \cdot L^{-1}$ [Acceptor]: 1 M	20-26[a]	L. Guo et al. 2018
DSLNnT	Enzymatic	CMP-Neu5Ac	LNnT	One-pot multi-enzyme synthesis with *in situ* formation of donor and acceptor from other precursors. Trans-sialylation with α2,6-sialyltransferase from *Photobacterium damsalae* using a ratio Sialic acid: LNnT of 2.4:1	99[a]	H. Yu et al. 2014
3′SL	Chemo-enzymatic	CMP-Neu5Ac	Lactose	Synthesis of lactose from protected thioglucose and thiogalactose bound to a photo cleavable resin, acting as an acceptor of sialic acid. Sialylation with α2,3-sialyltransferase from *Pasteurella multocida* with 1.1 equivalents of donor and alkaline phosphatase at pH= 8 and 37 °C.	∼30[a]	Fair, Hahm, and Seeberger 2015
LNT II	Chemo-enzymatic	GlcNAc Oxazoline	Lactose	Chemical synthesis of GlcNAc Oxazoline under the following conditions: GlcNAc in trimethylamine at 0-4 °C and using 2-chloro-1,3-dimethylimidazolinium chloride as catalyst for 15-120 min. Enzymatic reaction with hexosaminidase at pH= 8, 25 °C and 24 h. [Donor]: 100 mM [Acceptor]: 500 mM	25[a]	Muschiol and Meyer 2019
LNT II	Chemo-enzymatic	GlcNAc Oxazoline	Lactose	Continuous flow reaction on the immobilized *β-N*-acetylhexosaminidase deficient in hydrolytic activity from *Bifidobacterium bifidum*. General conditions: Laminar flow at 0.25 or 0.5 $mL \cdot min^{-1}$, 37 °C and pH= 7.5 with residence time of 2 min. [Donor]: 145 mM [Acceptor]: 580 mM	>95[a]	Ruzic, Bolivar, and Nidetzky 2020
LNT	Chemo-enzymatic	Lacto-*N*-biose-1,2-oxazoline	Lactose	Chemo-enzymatic synthesis of a donor through lacto-*N*-biose phosphorylase and 2-chloro-1,3-dimethyl-1H-benzimi-dazol-3-ium chloride. LNT production catalyzed by *β-N*-hexosaminidase from *Bifidobacterium bifidum* for < 5 min. [Donor]: 12 mM and [Acceptor]: 600 mM	∼60[a]	Schmölzer et al. 2019
Bi-antennary HMO	Chemo-enzymatic	UDP-Galactose CMP-Neu5Ac GDP-fucose	Tetra-saccharide and pentasaccharide	Construction of two pentasaccharides and one tetrasaccharide as building blocks by chemical synthesis in CH_2Cl_2 at temperatures between	∼70-90[a]	Z. Xiao et al. 2016

(*continued*)

Table 3. Continued.

HMO synthesized	Type of synthesis	Donor	Acceptor	Summary of synthesis	Global Yield	Reference
			with GlcNAc in antennary form	−78 °C and −20 °C using TfOH and TMSOTf as catalysts. Use of robust glycosyltransferases to extend building blocks overnight.		
Asymmetry multi-antennary HMO	Chemo-enzymatic	UDP-GlcNAc UDP-Galactose CMP-Neu5Ac GDP-fucose	*N*-methyl-hidroxy-amine-lactose	Sequential synthesis through production of LNnT as core structure with use of β1,3-*N*-acetylglucosaminyltransferase II and β1,4-galactosyltransferase. Later elongation, fucosylation or sialylation with β1,6-*N*-acetylglucosaminyltransferase, α1,3/4 fucosyltransferase or α2,6-sialyltransferase. General reaction conditions: pH between 4.2 and 7.3 at 37 °C overnight.	78-90[a]	Prudden et al. 2017

Global yields are reported in: [a]mol% for chemical, enzymatic, and chemo-enzymatic routes, and [b]$g \cdot L^{-1}$ for fermentation procedures.

to be studied, with a global yield without deprotection stage of 55% (mol).

In the case of SiaOS, an example of their synthesis *via* a chemical process is that described by Schmidt and Thiem (2010), gaining access to the sialyl-lacto-*N*-tetraose (SLNT), also named LSTa. It was synthesized from methyl ester of peracetylated 3-sialyl-lactosamine that acted as a donor, while methyl-lactoside was used as an acceptor. NIS and TfOH were used to catalyze the glycosylation reaction with 61% (mol) while partial deprotection was carried out at 81% (mol).

Finally, more recent advances in chemical routes have been described by the Bandara research group (Bandara, Stine, and Demchenko 2019a; Bandara, Stine, and Demchenko 2019b; Bandara, Stine, and Demchenko 2019c; Bandara, Stine, and Demchenko 2020) and by Agoston et al. (2019). In the first case, a similar synthetic approach was used for the production of some core oligosaccharides like LNT, LNnT, LNH, and LNnH at ∼48, ∼42, ∼25 and ∼36% (mol), respectively. Such strategies reduced the number of steps and improved LNT yield previously described by Craft and Townsend (2017). Interestingly, Agoston et al. (2019) proposed a novel route to afford one kilogram of 2'FL, using ∼10 kg of 1-*S*-phenyl-2,3,4-*O*-benzyl-fucose and ∼1 kg of lactose acetonide. However, this procedure has not been awarded the GRAS certification by FDA for its use in infant formula as in the case of the microbial fermentation procedure. Therefore, valuable progress obtained by Agoston et al. (2019) can only be used for analytical or bioactivity test purposes.

Synthesis of HMO by whole cell and enzyme biotransformations

The whole cell biotransformations, commonly known as fermentation, together with the isolated-enzyme processes have acquired great importance and have undergone substantial development in the last decade (Table 3). The first methodology is centered on the utilization of genetically modified microorganisms, which are able to express the genes that encode enzymes involved in the biosynthesis of these oligosaccharides (Baumgärtner et al. 2013; Baumgärtner et al. 2014; Baumgärtner et al. 2015; Hollands et al. 2019; Huang et al. 2017; Lee et al. 2012; S. Yu, Shin, et al. 2018). Then, the extraction and purification of HMO from the cell culture must be carried out. When HMO are not secreted to the medium, the lysis of the microorganisms is performed, usually by sonication (Bych et al. 2019; Hollands et al. 2019; J. J. Liu et al. 2018; J. Yu, Shin, et al. 2018; S. Yu, Shin, et al. 2018).

The second approach consists of the usage of enzymes from different microorganisms, which are isolated, purified and genetically engineered and exhibit a desired enzymatic activity (Y. Guo et al. 2015; Michalak et al. 2014; Saumonneau et al. 2016; Schmölzer et al. 2015; Zeuner, Vuillemin, et al. 2018). In order to obtain HMO, enzyme preparations of glycosyltransferases and glycosidases among others, are added into the systems at optimal for a given enzyme reaction conditions.

HMO production by fermentation

Fermentation has been an important tool of the HMO production and most probably, it is a strategy with the best advances in recent years. Generally, strains of *E. coli* are modified by genetic engineering to express the necessary enzymes for the biosynthesis of the target oligosaccharides. For example, 2'FL has been one of the most synthesized HMO and *E. coli* is utilized as a vector for the expression of necessary enzymes implicated in the endogenous synthesis of GDP-fucose, a fucose donor (Baumgärtner et al. 2013; Chin et al. 2015; Chin et al. 2016; Chin et al. 2017; Guan et al. 2018; Lee et al. 2012; Qin et al. 2016; Seydametova et al. 2019). The bond formation between the donor molecule and lactose is catalyzed by an intracellular α1,2-fucosyltransferase, affording 2'FL as a final product. In addition, the deletion of some genes involved in the lactose assimilation avoids its depletion and allow the efficient lactose transfucosylation (Chin et al. 2017; Huang et al. 2017; Qin et al. 2016).

A classical example of the whole cell biotransformation is a work by Lee et al. (2012) who produced 2'FL through batch fermentation in Lysogeny broth (LB) where the *E. coli* JM109(DE3) DNA was modified with a *H. pylori* gene to express FucT2, and its slow lactose assimilation was also exploited. Under these metabolic conditions, 2'FL could be found at the maximum intracellular concentration of 1.23 $g \cdot L^{-1}$ and a yield of 0.09 g per 1 g of lactose after 96 h. Similarly, in the most recent study by Seydametova et al. (2019), *E.coli* ΔL M15 strain encoding the α1,2-fucosyltransferase from *Thermosynechococcus elongatus* was employed to obtain 2'FL at 0.49 $g \cdot L^{-1}$ and with 94% of this concentration in an extracellular way.

In another work carried out by Baumgärtner et al. (2013), the improved production yield of 2'FL in a shorter time (20.28 $g \cdot L^{-1}$ in 35.5 h) was reported for a recombinant strain of *E. coli* JM109 in batch fermentation. Additional copies of *fkp* gene from *Bacteroides fragilis* and *futC* gene were inserted into the vector DNA that resulted in a rise of the GDP-fucose concentration and acceleration of the transfucosylation reaction. Moreover, the addition of glycerol as a carbon source enhanced the biomass production directly linked to the improved 2'FL concentration.

Among other advances of the 2'FL production *via* fermentation, it was reported the lactose operon modulation in *E. coli* BL21star(DE3) that led to the increased production of 2'FL up to 23.10 $g \cdot L^{-1}$ after 59 h. The changes included *lacZ, fucI* and *fucK* genes deletion while *lacY* gene was retained (Chin et al. 2016). In the previous work published by the same authors, the insertion of operon *lacZΔM15* and FucT2 mutation allowed the 2'FL production at a concentration of 6.40 $g \cdot L^{-1}$ after 59 h and a yield of 0.225 g per 1 g of lactose (Chin et al. 2015).

Other strategies for the improved 2'FL biosynthesis also included the deletion of *lacZ* gene and substitution of *fucT2* gene with *wcfB* from *Bacteroides fragilis* to generate a

recombinant *E.coli* strain, that afforded 2'FL at a concentration of 15.40 g·L^{-1} after 29 h (Chin et al. 2017). While, certain modifications in *E.coli* BL21(DE3) metabolism such as lactose and NADPH availability, as well as improve in GDP-fucose production through the insertion of *rcsA* and *zwf* genes, resulted in the production of 2'FL at 9.5 g·L^{-1} with a relative intra and extracellular distribution of 50:50 (Huang et al. 2017). In addition, genetic modifications to the *E.coli* BL21(DE3) vector to enhance GDP-fucose production through *de novo* pathway, and the division of fermentation process into two steps, produced 2'FL at 10.75 g·L^{-1} after 22 h (Qin et al. 2016).

Recently, new approaches to produce 2'FL have been proposed (Guan et al. 2018, Hollands et al. 2019; J. J. Liu et al. 2018; S. Yu, Shin, et al. 2018). Guan et al. (2018) presented the modification in the Krebs cycle of *E.coli* JM109 to provide additional cellular energy for oligosaccharide synthesis and to avoid the formation of sub-products from glycolysis. This strategy involved the addition of α-ketoglutarate, citrate or succinate, and the obtained 2'FL concentrations were at 3.3, 1.5 and 0.5 g·L^{-1}, respectively, after 24 h. Thanks to the modifications of this methodology, it was possible to avoid pH control and to achieve the improved fermentation time since the glycerol metabolism is too slow, one of the main limitations in traditional fermentation to obtain 2'FL. Likewise, Yun et al. (2019) biosynthesized 2′-fucosylgalactose, which can be considered a 2'FL precursor, at a concentration of 17.74 g·L^{-1}. For that purpose, *E.coli* BL21(DE3) vector was used with a number of genes inserted, such as *manB*, *manC*, *gmd*, *wcaG* y *fucT2*.

In the light of significant advances and improved methodologies developed for the 2'FL biosynthesis, its scaled-up production is of utmost importance for application in milk formulas. It has been achieved through fermentation with *E. coli* K12 cells, which are genetically modified to use D-sucrose as carbon source and lactose as final acceptor. Some modifications include the insertion of the genes for GDP-fucose production *via de novo*, *lacZ* knockout to avoid lactose consumption, and other gene deletions such as *lacA*, *melA*, and *mdoH* to improve yields and purification processes. Following this methodology, 2'FL is produced with the highest fermentation yield known to date of 180 g·L^{-1} (Ammann 2017; FDA (U.S. Food and Drug Administration) 2016a; FDA (U.S. Food and Drug Administration) 2017).

Nevertheless, the scale-up production of 2'FL with *E.coli* implies some risks, like the presence of endotoxins and the mandatory usage of antibiotics during fermentation (FDA (U.S. Food and Drug Administration) 2016a; FDA (U.S. Food and Drug Administration) 2017; J. J. Liu et al. 2018; S. Yu, Shin, et al. 2018). Given this, the alternative up-to-date procedures are based on the application of GRAS microorganisms, such as some industrial type of yeasts or bacilli (Deng et al. 2019; Hollands et al. 2019; J. J. Liu et al. 2018; S. Yu, Shin, et al. 2018). For this purpose, *Saccharomyces cerevisiae* was genetically modified to produce GDP-fucose through the *salvage* or *de novo* pathway, overexpressing *fkp* and *gmd* genes. Additionally, the insertion of *LAC12* and *fucT2* genes promoted the lactose permeability and transfucosylation, respectively, which contributed to the higher production of 2'FL. Such modified *S. cerevisiae* could synthesize 2'FL at concentrations of 0.56 and 0.42 g·L^{-1} intra and extracellularly, respectively, in the YP medium after 96 h (J. J. Liu et al. 2018), and up to 0.50 g·L^{-1} in the Verduyn medium after 120 h (S. Yu, Shin, et al. 2018), using glycerol and even ethanol produced during fermentation, as an extra carbon source.

Furthermore, Hollands et al. (2019) have synthesized 2'FL using *S. cerevisiae* and *Yarrowia lipolytica* that were genetically modified to consume lactose, produce GDP-fucose and carry out the transfucosylation reaction through insertion of *LAC12*, *gmd/wcaG* and *futC* genes. Thus, production of 2'FL could reach concentrations of 15 and 24 g·L^{-1} after 24 h by *S. cerevisiae* and *Y. lipolytica*, respectively. Moreover, the authors demonstrated the improvement in secretion of 2'FL to medium, which remains the main challenge in the HMO production by fermentation, achieved by the insertion of *cdt2* conveyor gene. Likewise, Deng et al. (2019) could access 2'FL through GRAS bacilli, a recombinant strain of *Bacillus subtilis* inserted with *fkp*, *futC*, *lacY*, and *LAC12* genes. It was also characterized by the enhanced fucose transportation and lactose consumption, and 2'FL concentration was at 5.01 g·L^{-1}.

Other HMO that have been successfully obtained by fermentation are 3FL, LNT, LNnT, and LNFP I. For example, 3FL was synthesized by Huang et al. (2017) using a recombinant strain of *E.coli* BL21(DE3), which was inserted with *lacY*, *rcsA*, *zwf* and *futB* genes to enhance its production, reaching a concentration of 13 g·L^{-1}. J. Yu et al. (2018) and Choi et al. (2019) could also produce 3FL through the growth of another recombinant *E.coli* BL21(DE3) strain in LB or minimal medium. In the case of J. Yu et al. (2018), the strain was upgraded by insertion of a modified α1,3-fucosyltransferase from *H. pylori* that improved the solubility and affinity to cellular membrane, with a production yield of 0.58 g·L^{-1}. Finally, Choi et al. (2019) reached a 3FL concentration of 4.6 g·L^{-1} through the overexpression of L-fucokinase/GDP-fucose pyrophosphorylase, *lacZ* modification, and mutated FutA expression.

For the LNT synthesis, it has been reached through construction of an *E.coli* K-12 LJ110 recombinant strain (Baumgärtner et al. 2014), inserted with *LgtA* and *wbgO* genes to express *β*1,3-*N*-acetylglucosaminyltransferase and *β*1,3-galactosyltransferase, respectively. Moreover, *lacZ* gene was removed from the vector DNA and a product concentration of 0.22 g·L^{-1} was reached after 24 h. By analogy, LNFP I has been produced through fermentation (Baumgärtner et al. 2015) by means of a recombinant *E.coli* that was able to synthesize LNT that in the next step was fucosylated through α1,2-fucosyltransferase expressed by *futC* gene that was also inserted to the vector. Under these conditions, LNFP I reached a concentration of 0.27 g·L^{-1}, while applied to the synthesis of lacto-*N*-difuco-hexaose II (LNDFH II), it was achieved a concentration of 0.55 g·L^{-1} using the same recombinant strain but with the additional *fucT14* gene inserted to express α1,4-fucosyltransferase.

Lastly, the large scale biosynthesis of LNnT utilizing *E.coli* K-12 vector was achieved through genetic modifications that involved the deletion of *lacZ* and *lacA* genes, as well as the insertion of genes from *Neisseria meningitides* and *H. pylori* for the expression of *β*1,3-*N*-acetylglucosaminyltransferase and *β*1,4-galactosyltransferase, respectively (FDA (U.S. Food and Drug Administration) 2016b). Furthermore, Dong et al. (2019) have proposed its biosynthesis in GRAS bacilli, using a recombinant *Bacillus subtilis* strain with *lacY* and other genes necessary to express transferases identical to those described in the previous example. In this way, LNnT was afforded at a concentration of 4.52 $g \cdot L^{-1}$.

HMO production by enzymatic processes

Like the whole cell processes, enzymatic reactions that use transferases or hydrolases have become of great importance to access HMO during the last years (Table 3). In the case glycosyltransferases, the enzymatic reaction requires the presence of nucleotide sugars, so that they could be transferred to an acceptor molecule to form the glycosidic bond. For example, GDP-fucose is transferred to lactose through an α1,2-fucosyltransferase to form 2'FL (Engels and Elling 2013). Another alternative to the HMO biosynthesis is the application of glycosidases whose catalytic activity is modified in such a way that transglycosylation is favored over hydrolysis. It is achieved through the reduction of water activity (a_w), the use of co-solvents, or through protein engineering (Champion et al. 2019; Guzmán-Rodríguez et al. 2018a; Guzmán-Rodríguez et al. 2018b; Holck et al. 2014; Lezyk et al. 2016; Michalak et al. 2014; Nyffenegger et al. 2015; Saumonneau et al. 2016; Wada et al. 2008; Zeuner et al. 2014; Zeuner, Nyffenegger, et al. 2016; Zeuner, Vuillemin, et al. 2018).

Enzymatic production of FucOS

Not only the fermentation processes, but also enzyme-assisted synthetic routes have been focused on the production of FucOS. Currently, 2'FL is one of the most frequently synthesized oligosaccharides. For example, Engels and Elling (2013) successfully obtained 2'FL at the quantitative yield in a one-pot multi-enzymatic synthesis using α1,2-fucosyltransferase expressed from *wbgL* gene of *E. coli* O126. GDP-fucose was obtained after 3 h incubation, followed by the preparative step in which the remaining nucleotides were eliminated (4 h). Then the transfucosylation reaction was carried out for 22 h to reach the total synthesis of 2'FL after 29 h.

2'FL has been also produced using α-L-fucosidases as catalysts from diverse sources. It can be illustrated by a study conducted by Zeuner, Muschiol, et al. (2018) who applied α-L-fucosidase from *Fusarium graminearum* and a xyloglucan from citrus peel as a fucose donor to obtain this oligosaccharide at 14% (mol). Another work was carried out by Lezyk et al. (2016) who achieved to synthesize 2'FL at 0.6% (mol) using an enzyme with transglycosylation activity identified through screening of the soil-derived metagenome library and expressed in *E. coli*., while *para*-Nitrophenyl-L-fucose (pNP-Fuc) and lactose were utilized as a fucose donor and acceptor, respectively. Similarly, Escamilla-Lozano et al. (2019) reported 21% (mol) for 2'FL synthesis using α-L-fucosidase from *Lactobacillus rhamnosus* GG and the same donor and acceptor compounds as described in the previous example.

In addition, Guzmán-Rodríguez et al. (2018a) reported a yield of 25.2% (mol) for 2'FL synthesis. The reaction system consisted of α-L-fucosidase from *Thermotoga maritima* acting as a biocatalyst, pNP-Fuc and lactose as a fucose donor and acceptor, respectively, at a donor/acceptor ratio of 0.006. Further modification of this ratio to 0.001 resulted in the yield increase to 32.5% (mol). In another work of the same authors (Guzmán-Rodríguez et al. 2018b), the transfucosylation conditions for 2'FL formation were altered in such a way that a_w was decreased by the addition of 1.1 M $CaCl_2$ and a donor/acceptor ratio was 0.024. However, the obtained yield was 12.48% (mol). In comparison, Lezyk et al. (2016) reported only 6.4% (mol) for 2'FL biosynthesis when α-L-fucosidase from *T. maritima* was used and a donor/acceptor ratio was 0.25.

To synthetically obtain 3FL, fucosyltransferases has been employed as biocatalysts in the presence of GDP-fucose acting as a donor. For instance, H. Yu et al. (2017) achieved 90% (mol) in a one-pot reaction system composed by the enzyme from *H. pylori* UA948 and a donor produced *in situ*, followed by the addition of Fuc and a fucokinase from *Bacteroides fragilis*. When a mutant of α1,3-fucosyltransferase (Choi et al. 2016) was used, the obtained yield was nearly quantitative which was explained by the improved solubility, catalytic activity, and substrate specificity. In the most recent study, 3FL has been obtained as a main product in a process catalyzed by α-L-fucosidase from *Pedobacter* sp. CAU209 (Shi et al. 2020) that also allowed to obtain 2'FL in the same reaction conditions.

Likewise, more complex FucOS have been produced *via* enzymatic synthesis, for example LNFP I, LNFP II, LNFP III, LNFP V, lacto-*N*-fucopentaose VI (LNFP VI), LNDFH II, lacto-*N*-neodifucohexaose II (LNnDFH II), difuco-*para*-lacto-*N*-neohexaose (DF-*para*-LNnH), and trifuco-*para*-lacto-*N*-neohexaose (TF-*para*-LNnH) (Bai et al. 2019; Champion et al. 2019; McArthur, Yu, and Chen 2019; Saumonneau et al. 2016; Tsai et al. 2019; H. Yu et al. 2017; Zeuner, Vuillemin, et al. 2018; Zhao et al. 2016). In the case of LNFP I, it was successfully synthesized by Zhao et al. (2016) in a one-pot multi-enzymatic reaction by employing α1,2-fucosyltransferase from *Thermosynechococcus elongatus* BP-1 to generate GDP-fucose *in situ* and LNT as a fucose acceptor. The afforded yields were 94-95% (mol). In another study, Fang et al. (2018) also obtained LNFP I with α1,2-fucosyltransferases from *H. pylori* NCTC 11639 and *E. coli* O128:B12 at 92-96% (mol).

In contrast, LNFP II and LNFP III have been synthesized using α-L-fucosidases from different sources. In a study carried out by Saumonneau et al. (2016), LNFP II was obtained by application of a mutated enzyme from *Bifidobacterium longum* subsp. *infantis* at 28% (mol). Similarly, Zeuner,

Vuillemin, et al. (2018) also applied protein engineering to enhance the transfucosylation activity of α-L-fucosidases from *Bifidobacterium bifidum* and *Clostridium perfringens,* reaching the maximum yields of 39 and 50% (mol), respectively. Additionally, in both studies LNFP III was biosynthesized at the yield range between 11 and 21% (mol). The conversion was improved up to 60% (mol) by Champion et al. (2019) through another mutation in α-L-fucosidase from *Bifidobacterium longum* subsp. *infantis*. High reaction yields, in the range between 80 and 88% (mol), were reported by H. Yu et al. (2017) and Bai et al. (2019) who obtained LNFP III using different α-fucosyltransferases as biocatalysts from *H. pylori* strains.

Other fucosyl-saccharides that need to be described, despite not having the same stereochemistry as HMO, are disaccharides obtained by Black et al. (2012), Alatorre-Santamaría et al. (2018), and Oh et al. (2019). These authors achieved the production of *β*-D-galactosyl-fucosides using cellular extract of *Lactobacillus bulgaricus* (Black et al. 2012), a *β*-galactosidase from *Aspergillus oryzae* (Alatorre-Santamaría et al. 2018), and a recombinant bifidobacterial *β*-galactosidase (Oh et al. 2019). The synthetic routes described in these studies add to the advances in the fucosyl backbone formation of FucOS analogues, which could exhibit similar health benefits like HMO. It was shown that these galactosyl-fucosides acted as prebiotics modulating bifidobacterial microbiota, especially *Bifidobacterium infantis* (Oh et al. 2019). Other compounds of interest that represent HMO analogues with biological activity are those that mimic FucOS from bovine milk and show antiadhesive properties to protect against *E.coli* O157:H7 (Weinborn et al. 2020).

Enzymatic production of SiaOS

The enzyme-catalyzed reactions have been mainly employed to produce 3'SL and 6'SL that comprise SiaOS. In the studies carried out by Holck et al. (2014) and Michalak et al. (2014), the 3'SL synthesis was accomplished utilizing a trans-sialidase from *Trypanosoma cruzi* and a mutated sialidase from *Trypanosoma rangeli* together with a casein glycomacropeptide (cGMP) acting as a source of sialic acid and lactose as a sialic acid acceptor. After optimization of the both processes, 3'SL was afforded at 64 and 50% (mol) as reported by Holck et al. (2014) and Michalak et al. (2014), respectively.

The second SiaOS structure, namely 6'SL, was synthesized by L. Guo et al. (2018) by means of sialidase from *Bacteroides fragilis* NCTC 9343 that showed an excellent transglycosylation activity in the reaction system consisting of lactose and sialic acid oligomers with α2,8-linkages, yielding this HMO at 20-26% (mol) after 10 min. In the previous work of W. Wang et al. (2011), a 6'SL analogue was produced with the improved yield of 35% (mol) thanks to the application of a pH-responsive, water-soluble polymer support for the purification step. The authors worked with α2,6-sialyltransferase and cytidine-monophosphate sialic acid (CMP-Neu5Ac).

Several research groups have focused their efforts on enzymes that exhibit dual activity and can form both 3'SL and 6'SL with the aim of obtaining only one major product. Y. Guo et al. (2014) reported the production of 3'SL as the main product after 30 min when the reaction was catalyzed by a sialyltransferase from the recombinant *Pasteurella multocida* acting as trans-sialidase due to its special dual activity, making it possible to use cGMP as a donor. The same reaction resulted in the 6'SL formation after 2 h. Although the reaction conditions such as pH, temperature, and donor/acceptor ratio, were optimized, 3'SL and 6'SL were obtained at 30.6 and 37% (mol), respectively. In another work, the 3'SL yield was improved to 40% (mol) in the presence of co-solvents such as *tert*-butanol at 25% (v/v). In the experiments was employed a mutant of *Trypanosoma rangeli* sialidase showing trans-sialylation activity (Zeuner et al. 2014).

Likewise, protein engineering has been used to increase enzyme activity and specificity to produce 3'SL and 6'SL. For example, a double mutation in the glucose binding site and a simple mutation in the CMP-Neu5Ac catalytic site of a sialyltransferase from *Pasteurella multocida* were carried out to improve enzymatic activity toward 3'SL formation. In addition, higher conversion yields for the 6'SL production were achieved through a double mutation in the CMP-Neu5Ac recognition site of sialyltransferase from *Photobacterium damselae* (Choi et al. 2013).

In the same way, Y. Guo et al. (2015) increased the regioselectivity of *Pasteurella multocida* sialyltransferase to afford 3'SL or 6'SL as a main product. The mutation at P34H resulted in the improved 6'SL yields, although they were 15 and 22% (mol) after 8 and 20 h, respectively. Another research group reported that a double mutation in the active site of *Pasteurella dagmatis* sialyltransferase enhanced its stereoselectivity toward 6'SL production with the maximum yield of 72% (mol), while 3'SL was observed at a yield less than 1% (mol) (Schmölzer et al. 2015).

SiaOS have been also mimicked through GOS sialylation (Y. Wang, Li, et al. 2015; Wilbrink et al. 2015; Zeuner, Holck, et al. 2016). In the work carried out by Y. Wang, Li, et al. (2015), a one-pot reaction was used to obtain in situ CMP-Neu5Ac followed by the action of sialyltransferases from *Pasteurella multocida* and *Photobacterium damselae* ATCC 33539 to afford α2,3-Sia-GOS and α2,6-Sia-GOS, respectively. The estimated yield was ∼86% (mol) for these oligosaccharides, which were fermented by *Bifidobacterium longum* subsp. *infantis* due to the structural resemblance to SiaOS. In the same way, Wilbrink et al. (2015) and Zeuner, Holck, et al. (2016) reported the α2,3-Sia-GOS production for which a trans-sialidase from *Trypanosoma cruzi* and an engineered sialidase from *Trypanosoma rangeli,* respectively, were used, both in combination with cGMP as a source of Sia.

Enzymatic production of core oligosaccharides

Among the core oligosaccharides that have been successfully produced through the enzyme-catalyzed synthesis are LNT and LNnT. The LNT structure was achieved in a sequential enzymatic process, where UDP-Gal and UDP-GlcNAc acting as donors, were synthesized *in situ*. Then, these

intermediates were linked to lactose using β1,3-*N*-acetylglucosaminyltransferase and β1,3-galactosyltransferase, which formed LNT at 85% (mol). Similar approach was followed to obtain LNnT with a yield of 94% (mol) (Fang et al. 2018).

Another synthetic pathway to access LNnT was proposed by C. Chen et al. (2015) who synthesized this HMO as 1-propyl-azide-LNnT in a one-pot multi-enzymatic reaction. In this work, UDP-GlcNAc was bound to propyl-azide-lactoside using a β1,3-*N*-acetylglucosaminyltransferase from *H. pylori* as a catalyst. Once a trisaccharide acceptor was formed, it underwent β1,4-galactosylation catalyzed by β1,4-galactosyltransferase from *Neisseria meningitides*, reaching an overall yield of 82% (mol).

Similarly, Zhu et al. (2017) achieved synthesizing of LNnT by the application of β1,3-*N*-acetylglucosaminyltransferase and β1,4-galactosyltransferase but in the solid phase. First, lactosyl-propyl-amine was linked to a cationic resin (Dowex 50wX2) and then lacto-*N*-triose (LNT II) was formed in the enzymatically catalyzed transfer of UDP-GlcNAc to lactoside. After this intermediate product was removed from the resin by washing with ammonium bicarbonate, the second enzyme was applied to bind Gal and produce LNnT.

In another attempt, LNnT has been produced with thermostable β-galactosidases from *Thermus thermophilus* HB27, *Pyrococcus furiosus* and a truncated enzyme from *Bacillus circulans* (Zeuner, Nyffenegger, et al. 2016). For this purpose, lactose and LNT II were used as a donor and acceptor, respectively, and the afforded yields were between 1 and 7.1% (mol). Moreover, these thermostable β-galactosidases can also assist the production of LacNAc, which is considered a precursor of core oligosaccharides, with yields between 5.4 and 32% (mol). Noteworthy, the best results for LNT II and LacNAc production were achieved using the enzyme from *Bacillus circulans*.

Indeed, production of core oligosaccharides building blocks for a synthesis of various HMO, such as LacNAc, LNT II and LNB, has gained considerable attention in recent years. For example, LNT II was obtained by Nyffenegger et al. (2015) using two new β-*N*-acetylhexosaminidases that could hydrolyze *N*,*N*'-diacetylchitobiose and generated an oxazoline intermediate. The latter compound was later used by the same enzyme in a transglycosylation reaction to lactose, reaching yields between 2 and 8% (mol). In another study, Bidart et al. (2017) synthesized LNB through transgalactosylation of GlcNAc, where a phospho-β-galactosidase from *Lactobacillus casei* BL23 used a *O*-nitrophenyl-β-D-galactopyranoside to form LNB with a yield of 69% (mol) and at a concentration of 10.7 g·L^{-1} at semi-preparative scale.

Chemo-enzymatic synthesis of HMO

Chemo-enzymatic synthetic methods have become an emerging and powerful tool for the HMO production according to the most recent results from the last 5 years.(Muschiol and Meyer 2019; Prudden et al. 2017; Ruzic, Bolivar, and Nidetzky 2020; Schmölzer et al. 2019; Z. Xiao et al. 2016).

Muschiol and Meyer (2019) succeeded in synthesizing LNT II through lactose transglycosylation using a mutated β-*N*-acetylhexosaminidase and GlcNAc oxazoline, obtained by chemical synthesis, as a GlcNAc donor. In this synthetic route, the HMO precursor was produced at 25% (mol) after 6.5 h. In another work, Ruzic, Bolivar, and Nidetzky (2020) improved the LNT II yields up to 95% (mol) by application of a flow process, where a mutated β-*N*-acetylhexosaminidase was immobilized on a Cu^{2+} - agarose support.

In addition, Schmölzer et al. (2019) synthesized LNT at 60% (mol) using a wild type of β-*N*-hexosaminidase from *Bifidobacterium bifidum* as a catalyst and lacto-*N*-biose-1,2-oxazoline, obtained *via* a chemo-enzymatic approach, as a donor. In earlier work, Fair, Hahm, and Seeberger (2015) produced 3'SL through the automated solid phase chemical synthesis of a selectively protected lactose on a photo-cleavable resin. Then, lactose was sialylated using α2,3-sialyltransferase from *Pasteurella multocida* obtaining a yield close to 30% (mol).

Finally, the major progress in chemo-enzymatic synthesis has been marked by the production of libraries for the asymmetrical multi-antennary HMO by Z. Xiao et al. (2016) and Prudden et al. (2017). In the first step, both authors synthesized the antennary core structures composed of lactose linked to GlcNAc in the β1,3 or β1,6 positions with respect to Gal from lactose. Then, these core structures were expanded by action of β1,3-*N*-acetylglucosaminyltransferase, β1,6-*N*-acetylglucosaminyltransferase and β1,4 galactosyltransferase. As a result, the elongated HMO were also fucosylated and sialylated by glycosyltransferases, giving a total of 11 and 60 new structures reported by Z. Xiao et al. (2016) and Prudden et al. (2017), respectively, with a similar yield rage between 70 and 90% (mol).

Although the synthetic strategies to access HMO presented in this review have seen remarkable advances, it is important to highlight the fact that the reported results refer mainly to the lab-scale experiments. Nevertheless, it is an important first step of any process before the scale-up production of a particular saccharide can be possible, like in the case of 2'FL and LNnT.

Conclusions and remarks

Endogenous synthesis of HMO is a genetically controlled process that can explain observed differences in the oligosaccharide profiles among mothers. This diversity in HMO production directly affects the infant metabolism and intestinal microbiota, which can impact immunological response, as well as the prevention and treatment of different infant diseases. Given this, the HMO-fortified infant formula has been considered an excellent option to provide specific oligosaccharides in those cases where a link between a disease and HMO deficiency exists. Moreover, such modified infant formula offers an improved source of nutritional and bioactive compounds to infants that are not breastfed.

Currently, the HMO synthesis has been successfully achieved through the whole-cell and isolated enzyme biotransformations that outdo chemical and chemo-enzymatic processes. For example, 2'FL and LNnT have been produced *via* fermentation in the efficient scaled-up synthesis, which allows their incorporation in new infant formulas. Nevertheless, further application of GRAS microorganisms at the industrial scale, would require improvements of fermentation times, purification steps and yields. As for enzymatic processes, remarkable advances have been made to access HMO with higher degree of polymerization. To make this approach feasible at the large scale, reaction conditions at batch scale need to be optimized to afford higher yields.

Another approach to HMO synthesis involves the application of chemo-enzymatic methods that have been showed to be a powerful tool to access branched oligosaccharides. Since limited investigation have been carried out in this field, which other methods do not cover, more research is needed to explore the potential and feasibility of this mixed synthetic route.

Finally, application of chemical synthesis for a total HMO production on the industrial scale is not a viable strategy since it implies the usage of toxic solvents and temperatures difficult to apply. However, it could be used to provide some common precursors for the chemo-enzymatic methodologies.

In conclusion, HMO have shown excellent properties that could be used in prevention of certain diseases by boosting infant and adult health, and to support their treatment. Hence, the research focus should be placed on the improvement of synthetic and purification methods, so that the access to these compounds could be economically feasible.

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