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Publication Date

2024-06-01

DOI

10.1016/j.cofs.2024.101154

Peer reviewed

HHS Public Access

Author manuscript

Curr Opin Food Sci. Author manuscript; available in PMC 2024 October 11.

Published in final edited form as:

Curr Opin Food Sci. 2024 June ; 57: . doi:10.1016/j.cofs.2024.101154.

Recent Advances in the Microbial Production of Human Milk Oligosaccharides

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Abstract

Human milk oligosaccharides (HMOs) are naturally occurring, non-digestible sugars found in human milk. They have recently become a popular target for industrial synthesis due to their positive effects on the developing gut microbiome and immune system of infants. Microbial synthesis has shown great promise in driving down the cost of these sugars and making them more available for consumers and researchers. The application of common metabolic engineering techniques such as gene knockouts, gene overexpression, and expression of exogenous genes has enabled the rational design of whole-cell biocatalysts which can produce increasingly complex HMOs. Herein, we discuss how these strategies have been applied to produce a variety of sugars from sialylated to complex fucosylated HMOs. With increased availability of HMOs, more research can be done to understand their beneficial effects.

Introduction

Human milk oligosaccharides (HMOs) are a structurally diverse class of mostly non-digestible sugars that are the third most abundant component of milk solids, after lactose and lipids [1]. To date, over 200 species of HMOs have been detected in samples of human milk with sizes ranging from 3 to 18 monosaccharide units [2,3]. All HMOs share a lactose or N-acetyllactosamine moiety at the reducing end that is decorated with one or multiple of the monosaccharides N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), fucose, or N-acetylneuraminic acid (Neu5Ac). Different numbers, combinations, and branching patterns of these decorating monosaccharides gives rise to the diversity of HMOs.

A variety of studies have provided evidence for HMOs being important for multiple aspects of infant health. These non-digestible sugars help to promote the growth of beneficial gut microbiota such as *Bifidobacteria* which reduce gut inflammation and exert an immunomodulatory effect by fermenting HMOs into short chain fatty acids [4–6]. The presence of HMOs in the infant gut has also been shown to protect the mucosal lining of against pathogenic microbes and viruses by serving as ‘decoy receptors’ that prevent their adhesion [7,8]. A clinical trial conducted in 693 infants revealed that an infant formula

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supplemented with a blend of 5 HMOs ultimately improved infants' gut barrier health and intestinal immune systems and resulted in a gut microbiome more like that of breastfed infants [9]. The WHO and USDA recommend exclusive breastfeeding for the first 6 months of life, citing the benefits of bioactive substances present in human milk. However, in 2020 only about 44% of infants globally and 25% of infants in the United States were exclusively breastfed for their first 6 months, with the rest receiving some form of dietary supplementation with infant formula (USDA; URL: https://www.dietaryguidelines.gov/resources/2020-2025-dietary-guidelines-online-materials). Moreover, 17% of infants in the US were never breastfed. Due to the many valid reasons for not breastfeeding, it is therefore important to close the gap in nutrition between infant formula and human milk. Because HMOs have been shown to be effective in this regard, commercial manufacturers have begun to include one or more HMOs in formula and there is a growing market for them.

While several HMOs have been detected in cow's and goat's milk, they are not abundant enough to justify the cost of extraction and purification from these sources [2]. It is possible to chemically synthesize HMOs, however these syntheses involve the use of many expensive reagents and extensive protection-deprotection strategies which dramatically reduces overall atom economy [10]. Enzymatic syntheses avoid the use of protecting groups and have been used to synthesize an array of HMOs on small scale, however the use of purified enzymes and cofactors limits the scalability of this strategy [11]. A biosynthetic approach avoids the use of expensive reagents while still retaining the high level of specificity afforded by enzyme catalysts, making it a much more scalable alternative to *in vitro* synthesis of HMOs. Given the high genetic plasticity of production hosts such as *Escherichia coli* and extensive knowledge on their previous use for biosynthetic applications, the use of these organisms for the biosynthesis of HMOs has been heavily investigated. Herein, the recent developments in the use of this strategy to economically synthesize increasingly complex HMOs have been discussed. This review emphasizes important details of the most recent studies of the bioproduction of complex HMOs as well as their bioactivities in infants and adults.

2'-Fucosyllactose (2'-FL)

2'-FL is trisaccharide made from three monosaccharides: D-glucose, D-galactose, and L-fucose. Among the HMOs present in milk, 2'-FL is the most abundant, although its total value can differ significantly from mother to mother [29]. Infants born to mothers with an active fucosyltransferase2 gene, responsible for 2'-FL synthesis, tend to establish bifidobacterial populations faster and more frequently than those born to mothers with an inactive allele [30]. 2'-FL also has antiadhesive properties towards a variety of pathogens [31]. Additionally, 2'-FL can improve the cognitive ability of rats, indicating that it has the potential to promote brain development in humans [32]. Due to its beneficial properties, 2'-FL has gained interest as an additive in infant formula milk. While 2'-FL can be synthesized via chemical synthesis or chemoenzymatically, there are many challenges in establishing an inexpensive and efficient manufacturing process [33,34]. An alternative to these production methods is the microbial production of 2'-FL [35]. Importantly, 2'-FL produced by engineered *E. coli* received its GRAS rating by the FDA, and it is being used as a supplement in infant formula in several countries.

To generate 2'-FL in a microbial host, the sugar donor GDP-fucose is consumed by an (α1,2)-fucosyltransferase to add fucose to the galactose moiety on lactose. There are two main pathways to produce GDP-fucose, the *de novo* pathway and the salvage pathway (Fig. 1) [36]. The *de novo* pathway consists of 5 enzymes which converts fructose-6-phosphate into GDP-fucose. The salvage pathway uses a single bifunctional fucokinase/fucose-1-phosphate guanylyl transferase enzyme (Fkp) to convert exogenously supplied fucose into FDP-fucose. The last step of the 2'-FL pathway requires the use of an (α1,2)-fucosyltransferase. While many (α1,2)-fucosyltransferases have been successfully used to produce 2'-FL, these enzymes are commonly rate limiting and many produce unwanted byproducts [37]. Recently, an (α1,2)-fucosyltransferase from a *Helicobacter* species was shown to improve 2'-FL production and remove by-product formation [37].

Early work showed that low levels of 2'-FL could be produced by overexpressing an (α1,2)-fucosyltransferase and *rcsA*, a transcriptional activator for capsular polysaccharide biosynthesis in a *lacZ* deficient strain of *E. coli* [38]. This strain was fed with glucose and lactose, making use of the natural *de novo* pathway. More recently, robust 2'-FL production was shown using sucrose as a sole carbon source, producing ~60 g/L 2'-FL after 84 hours [15]. In this strain, the *de novo* pathway was directly overexpressed and several gene knockouts were employed to prevent substrate loss from the production pathway. Additionally, the simultaneous overexpression of *de novo* and salvage pathways was shown to elevate the intracellular GDP-fucose concentration above those achieved by expressing either pathway alone [39]. Adaptive laboratory evolution (ALE) is a strategy that is commonly used to identify beneficial mutations for a given set of growth conditions. ALE was used to improve the performance of the 2'-FL producing strain by subjecting it to UV mutagenesis followed by growth on endpoint fermentation broth containing 2'-FL [40]. This selection process revealed a mutation in the gene that encodes the β subunit of RNA polymerase (*rpoC*) and showed that it could boost 2'-FL titer from 46 g/L to 61 g/L. Lastly, *E. coli* was engineered to produce over 100 g/L of 2'-FL in a fed-batch fermentation using glycerol and glucose [17]. This strain used a combination of the strategies described above, including direct and RcsAB-mediated overexpression of the *de novo* pathway, knocking out competing pathways, and overexpressing the (α1,2)-fucosyltransferase. In addition to overexpression, a solubility tag was added to the (α1,2)-fucosyltransferase to improve the kinetics of this bottleneck step. In addition to *E. coli*, similar strategies have been employed to produce 2'-FL in other industrially relevant GRAS strains such as *Saccharomyces cerevisiae* (15 g/L), *Bacillus subtilis* (5 g/L), and *Yarrowia lipolytica* (24 g/L) (Table 1) [14,16].

3-Fucosyllactose (3-FL)

Although it is comparatively less concentrated in human milk, 3-FL shares many of the same prebiotic and anti-pathogenic effects of 2'-FL and many other HMOs as well as FDA-granted GRAS status [41]. 3-FL is structurally unique among HMOs for bearing a modification to the reducing glucose of lactose. Therefore, an α−1,3-fucosyltransferase is used in this position. Aside from the choice of glycosyltransferase, the strategy to produce 3-FL is largely the same as that of 2'-FL since both pathways use GDP-fucose as a sugar donor to modify lactose. Two recent studies highlight the use of the *de novo* GDP-fucose

pathway to power efficient syntheses of 3-FL from lactose and glycerol [18,19]. Both studies overexpressed the *de novo* pathway enzymes on a plasmid to enhance GDP-fucose supply, but the two studies used different gene knockouts to direct carbon flux. Among these differences, Li et. al. found that knocking out *pfkA* to decrease carbon leakage to lower glycolysis was advantageous [18], while Ni et. al. found the same knockout to be detrimental to cell growth and 3-FL production, hypothesizing that this may have been due to a restricted supply of ATP [19]. Both studies used α−1,3-glycosyltransferases which had been engineered to enhance solubility and activity, which was important given the native enzyme's low activity in *E. coli* [18,19]. Li et. al. further optimized α−1,3-glycosyltransferase activity by screening various ribosomal binding sites and gene copy numbers, which allowed them to improve 3-FL titer almost 2-fold in shake flasks [18]. Each study was able to improve 3-FL titers upon scaling up their best-performing strain to a fed-batch bioreactor. Li et. al. and Ni et. al. achieved 40.7 g/L and 35.7 g/L, respectively. (Table 1).

Lactodifucotetraose (LDFT)

LDFT is the fourth most abundant HMO present in the milk of individuals with the secretor genotype (S+) [42]. Fucosylated HMOs have been identified as key molecules in giving rise to the beneficial immunomodulatory effects attributed to HMOs [43]. LDFT was produced from lactose, fucose, and glycerol in *E. coli* [24]. The base strain was first modified to prevent side product formation by deleting the *lacZ* and *fucU* genes which enable the catabolism of lactose and fucose, respectively. Three enzymes necessary for LDFT synthesis were then introduced via a two-plasmid expression system (Fig. 2).

Fkp, an enzyme from *Bacteroides fragilis*, catalyzes the two steps in the salvage pathway which generates GDP-L-fucose from the supplied L-fucose. WbgL, an α1–2-fucosyltransferase from *E. coli* O126, consumes GDP-L-fucose to fucosylate lactose and generate 2'-FL. Hp3/4FT, an α1–3/4-fucosyltransferase from *Heliobacter pylori* consumes another equivalent of GDP-L-fucose to further fucosylate 2'-FL to LDFT. The LDFT is then secreted via the transporter SetA. To ensure adequate intracellular availability of lactose and fucose, the respective membrane transporters LacY and FucP were additionally expressed. The additional expression of FucP proved to be important to LDFT production, increasing LDFT titer ~9 fold.

Sialylated HMOs

Sialic acids are a family of amino sugars, with the main form being N-acetylneuraminic acid (Neu5Ac). They play key roles in biology, regarded as the method by which viruses infect their host [44]. Sialic acids are found bound to glycoproteins and glycolipids, commonly localized on nerve and brain cells [44]. Sialylated HMOs account for approximately 13% of the total HMO composition in human milk [45]. They provide a range of benefits to their neonatal host, including being an antiadhesive antimicrobial, being antiviral, modulate intestinal epithelial cell response, and can improve brain development and cognition [46]. The sialylated HMOs 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL) have been designated GRAS by the FDA.

E. coli has been engineered to produce 3'-SL [20]. The production of 3'-SL involves the production of CMP-Neu5Ac, followed by the formation of an α−2,3 linkage between Neu5Ac and lactose (Fig. 3). Many bacterial (α2,3)-sialyltransferases have been identified and characterized, and this library of enzymes was compared for 3'-SL production. Nst from *Neisseria meningitidis* has long been established for 3'-SL production, but the (α2,3)-sialyltransferase Vs16 from *Vibrio* sp. JT-FAJ-16 improved 3'-SL production 1.14-fold over Nst [47].

Balancing precursor pools of UDP-GlcNAc and CMP-Neu5Ac intermediates is also important for 3'-SL biosynthesis. Further overexpressing GlmM, GlmU, and GlmS to increase production of UDP-GlcNAc decreased 3'-SL production. However, these efforts in conjunction with increased expression of the downstream enzymes responsible for CMP-Neu5Ac production, lead to increased titers of 3'-SL. This work highlights the importance of balancing UDP-GlcNAc and CMP-Neu5Ac fluxes toward 3'-SL. The final strain was grown on continuously fed glycerol and lactose in a 5 L bioreactor and produced 31.4 g/L of 3'-SL after 38.5 hours [20].

B. subtilis has also been used as a host for 3'-SL production [21]. CMP-Neu5Ac is synthesized by an exogenous pathway modified from a similar pathway established in *E. coli* [47]. An (α2,3)-sialyltransferase gene from *Neisseria meningitidis* was introduced to convert CMP-Neu5Ac to 3′-sialyllactose in the presence of lactose, which serves as the donor substrate for the glycosyltransferase enzyme. Protein-based scaffolds have been used to keep enzymes of the same pathway spatially close to one another to improve the efficiency of a cascade of enzymatic reactions [21]. When supplemented with lactose and glucose, a final titer of 1.25 g/L of 3'-SL was produced from a 50 mL flask culture after 12 hours.

Like the 3'-SL biosynthetic strategy, synthesis of 6'-sialyllactose (6'-SL) uses the same exogenous pathway to produce CMP-Neu5Ac, which is instead transferred to a lactose with an α2,6-sialyltransferase [22]. Genes for competing pathways (*nanA, nagB, lacZ, pfkA*) were deleted to direct carbon flux towards CMP-Neu5Ac. 6'-SL titer was improved by modulating the expressions of the pathway genes. This allowed for CMP-Neu5Ac to accumulate, then transfer of the Neu5Ac onto the lactose base could occur efficiently.

Efforts to biosynthesize 6'-SL are also hindered by the relatively low expression and activity of (α2,6)-sialyltransferases. Pst6–244 is a (α2,6)-sialyltransferase from *Photobacterium* sp. JT-ISH-224 has been previously established for 6'-SL biosynthesis [23], meanwhile multiple (α2,6)-sialyltransferases have been identified and engineered, and this library of enzymes was compared for 6'-SL production [48–51]. A truncated (α2,6)-sialyltransferase from *P. leiognathi* JT-SHIZ-119 (Plst6) improved 6'-SL production over the previously established Pst6–244 [22]. The final strain was grown on continuously fed glycerol and lactose in a 3 L bioreactor and produced 22.9 g/L of 6'-SL after 75 hours [22]. While this did not surpass the 23 g/L of 6'SL previously produced in a 2 L reactor using the Pst6–244 (α2,6)-sialyltransferase, a direct comparison cannot be made due to the differences in base strains (*E. coli* DH1 and *E. coli* BL21 (DE3), respectively) and their gene knockouts (Δ*lacZ* Δ*nanA* Δ*nanK* and Δ*lacZ* Δ*nanA* Δ*nagB* Δ*pfkA*, respectively) [23].

Lacto-*N*-tetraose (LNT) and Lacto-*N*-neotetraose (LNnT)

LNT is a linear tetrasaccharide composed of lactose decorated with GlcNAc and galactose, sequentially. LNT and its structural isomer LNnT missing a terminal galactose are very abundant in human milk, making up 20–30% of total HMOs in samples [42]. Lacto-*N*-triose II (LNT II) is the immediate precursor to LNT and LNnT. LNT and LNnT serve as core structures which are further decorated to yield many of the more complex HMOs [42]. Therefore, developing robust syntheses of LNT and LNnT will help make these complex HMOs more available for further study.

E. coli was engineered to produce LNT from lactose and glycerol [25]. Two glycosyltransferases LgtA (a β−1,3-*N*-acetylglucosaminyltransferase) and WbgO (a β−1,3-galactosyltransferase) were expressed. Similarly to other HMO-producing strains, the *lacZ* gene was deleted to prevent the catabolism of lactose. The LNT production was improved with the overexpression of *galETK*, encoding for enzymes in the Leloir pathway that generates UDP-Gal. Given the apparent importance of UDP-Gal abundance, the *ugd* gene was deleted to eliminate UDP-glucose-6-phosphate dehydrogenase that consumes UDP-Gal. This final strain produced 31.6 g/L LNT and 5.7 g/L LNT II after 56 hours in a 3-L bioreactor.

The common strategy of enzyme library screening was used to optimize the production of LNT and LNnT from glycerol and lactose in *E. coli* [26]. The main production pathway included the enzymes LgtA and one of either HpgalT or SewbdO, galatcosyltransferases were used to convert LNT II to LNT or LNnT, respectively. These glycosyltransferases were chosen as the result of a plasmid screening experiment in which many combinations of enzymes and expression levels were tested in the context of *lacZ* deletion. The *galETKM* operon was also overexpressed on the chromosome of this strain to strengthen the supply of UDP-Gal. Next, a series of knockouts was performed to further improve sugar nucleotide availability. The *ushA* deletion removed a UDP-sugar hydrolase and was the most impactful single knockout tested, although the effect was still marginal. Five additional knockouts preventing leakage from the UDP-Gal production module further optimized production and yielded the LNT and LNnT-producing strains. In a fed-batch 5 L bioreactor, these strains produced 48.4 g/L LNT and 22.1 g/L LNnT, respectively after 54 hours.

Lacto-N-Fucopentaoses (LNFP)

LNFP I, LNFP II, and LNFP III together represent the second and third most abundant class of HMOs in non-secretors and secretors, respectively [42]. *E. coli* was engineered to produce LNFP I [27]. The main pathway consisted of glycosyltransferases LgtA and WbgO to generate LNT from lactose, and an additional transferase α−1,2-fucT which selectively yields LNFP I (Fig. 5).

The starting strain for LNFP I production contained Δ*lacZ* Δ*wecB* Δ*nagB* Δ*ugD* to prevent carbon leakage from the production pathway, and *galE* was overexpressed on the chromosome to enhance the supply of UDP-Galactose. To improve the stability of gene expression in this relatively long pathway, a multi-copy chromosomal integration of *lgtA*

under a constitutive promoter was utilized. This was accomplished using CRISPR-associated transposase-based multicopy chromosomal integration which yielded 1 through 5-copy insertion at IS186 loci [52]. Variable insertion of *lgtA* revealed that a 4-copy insertion of *lgtA* was optimal. The next glycosyltransferase in the pathway, WbgO, was expressed from a plasmid (Fig. 5). Finally, the α1–2-fucosylation of LNT involved two main challenges: first, the intracellular supply of GDP-Fuc needed to be increased and second, an LNT-specific α1,2-fucosyltransferase needed to be found. To strengthen the GDP-Fuc pathway, five genes were overexpressed on a plasmid system: *manBC, gmd*, and *wcaG*. When this plasmid was tested with the commonly used α1,2-fucosyltransferase FucT2, a modest amount of LNFP I was produced but 2'-FL was also produced, indicating promiscuity of FucT2 towards lactose. To remedy this, 6 analogous enzymes from various species were tested. Among these, WbsJ from *E. coli* O128 displayed the highest substrate specificity towards LNT and this strain had minimal 2'-FL production and residual LNT and LNT II left over. This 3-plasmid strain was carried forward for testing in a 5-L bioreactor, where it was batch-fed with glycerol and lactose for 48 hours, yielding 30.5 g/L LNFP I, 5.9 g/L LNT, 5.4 g/L LNT II, and no detectable 2'-FL.

E. coli was engineered to produce LNFP III [28]. The starting strain for LNFP III production could produce the precursor LNnT using the heterologous enzymes NpLgtA and HpGalT in the context of gene deletions of *lacZ* and *wcaJ* and overexpression of the lactose transporter gene *lacY* and the transcriptional activator gene of the GDP-Fucose pathway *rcsA* (Fig. 6).

The main challenge that LNFP III faces is the promiscuity of known α1,3-fucosyltransferases. While the necessary final step of LNFP III production involves the α1,3-fucosylation of LNnT on the GlcNAc moiety, many α1,3-fucosyltransferases are known to be promiscuous towards lactose-like as well as LacNAc-like structures, meaning that they will also catalyze the α1,3-fucosylation of the reducing glucose of LNnT or lactose [53,54]. To prevent these unwanted reactions, α1,3-fucosyltransferases were screened by their ability to fucosylate added lactose or LacNAc. Eight α1,3-fucosyltransferases were tested. One candidate, PgsFucT from *Parabacteroides goldsteinii*, showed substrate specificity for LacNAc over lactose *in vivo*, with no detectable activity toward lactose. With this enzyme, the final strain produced 3.8 g/L LNFP III in a 3 L fermenter after 48 hours and full cytolysis, although preliminary shake-flask fermentations showed that the vast majority of the produced LNFP III remained intracellular.

Conclusions

The past few years have seen many advances in the understanding of how to employ common metabolic engineering strategies such as pathway overexpression, gene knockouts, enzyme screening, and fine-tuning enzyme expression to efficiently biosynthesize many HMOs. Such efforts will certainly help to inform future industrial-scale biosynthesis of HMOs for diet and research. However, there is still room for improvement in the scope and scale of bioproduction of complex HMOs. As seen, glycosyltransferase specificity is key to achieving efficient production in the presence of many intermediates. Future endeavors into discovery and rational modification of glycosyltransferases will surely prove crucial to future HMO production. Also, upon analysis of studies reporting complex HMO

biosynthesis, it becomes apparent that cellular export of these products may be limiting to production. Reports of complex HMO products that are significantly more concentrated in the cytosol compared to the fermentation broth could be an indication of export issues which would prematurely stall production [28]. Therefore, as the field advances toward the synthesis of more complex HMOs, it would be advantageous to better understand the transport properties of more membrane proteins. Increased availability of these complex HMOs will help support future research on their bioactivities. This knowledge, coupled with scalability and efficiency of microbial production platforms, will pave the way for a new generation of food products and nutraceuticals that are accessible and effective.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (GM145842 to SA).

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fucosyllactose. Bioresour Technol 2023, 372:128667. [PubMed: 36702325] The export and intracellular tolerance of HMOs are important areas to consider improving product titers. In this study, ALE revealed a single gene mutation that improved the host's tolerance of 2'-FL. Furthermore, 2'-FL export into the production media was facilitated by overexpressing the membrane transporter SetA. Although this study was done in the context of producing a relatively simple HMO, similar strategies will surely aid in the production of complex HMOs where sugar export and tolerance starkly limit production.

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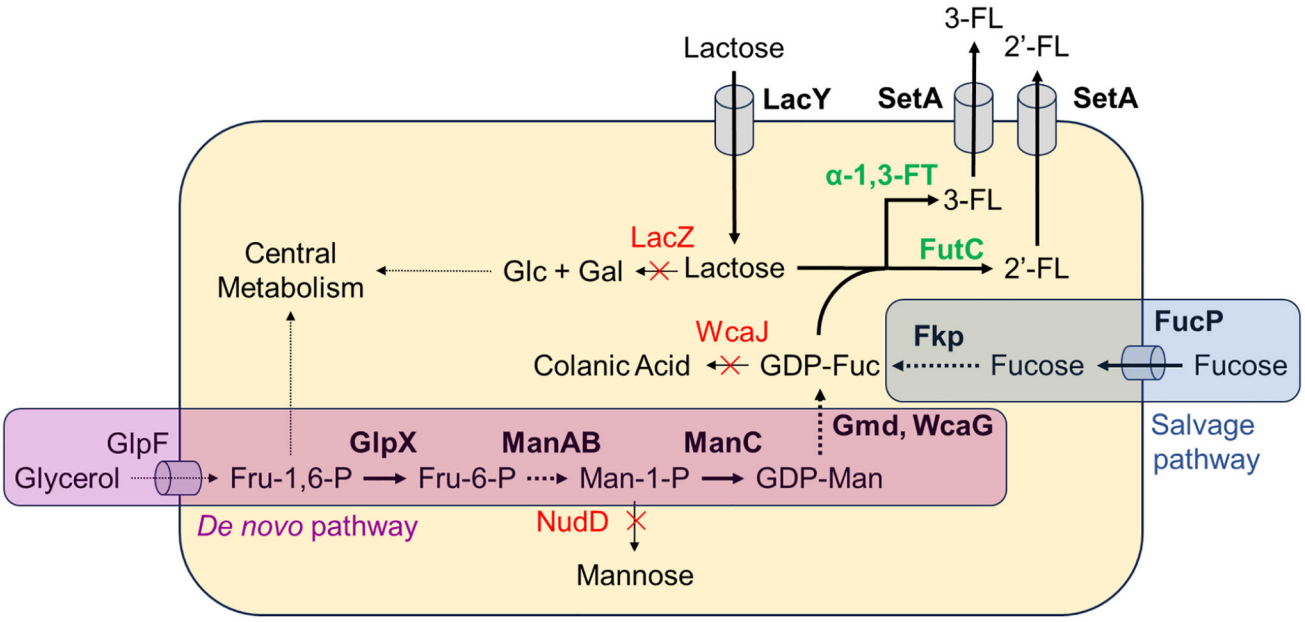


Figure 1. Biosynthetic pathways for 2'-FL and 3-FL.

Green indicates reaction steps with exogenous enzymes. Red indicates deletion of the reaction steps. Bold indicates overexpression of the corresponding gene. **Metabolite abbreviations:** Glc, D-glucose; Gal, D-galactose; Fru-1,6-P, D-fructose-1,6-bisphosphate; Fru-6-P, D-fructose-6-phosphate; Man-6-P, D-mannose-6-phosphate; Man-1-P, D-mannose-1-phosphate; GDP-Man, Guanosine diphosphate mannose; GDP-Fuc, Guanosine diphosphate fucose; 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose. **Enzyme abbreviations:** LacY, lactose permease; SetA, Sugar efflux transporter; lacZ, β-galactosidase; FutC, α−1,2-fucosyltransferase; α−1,3-FT; α−1,3-fucosyltransferase; WcaJ, Undecaprenyl-phosphate glucose phosphotransferase; WcaG, GDP-4-keto-6-deoxy-D-mannose epimerase/reductase; Gmd, GDP-mannose 4,6-hydro-lyase; ManC, Mannose-1-phosphate guanylyltransferase; NudD, GDP-mannose mannosyl hydrolase; ManB, Phosphomannomutase; ManA, Mannose-6-phosphate isomerase; GlpX, fructose-1,6,-bisphosphatase 2; GlpF, Glycerol facilitator; Fkp, Fucokinase/fucose-1-phosphate guanylyl transferase; FucP, L-fucose:H^+ symporter.

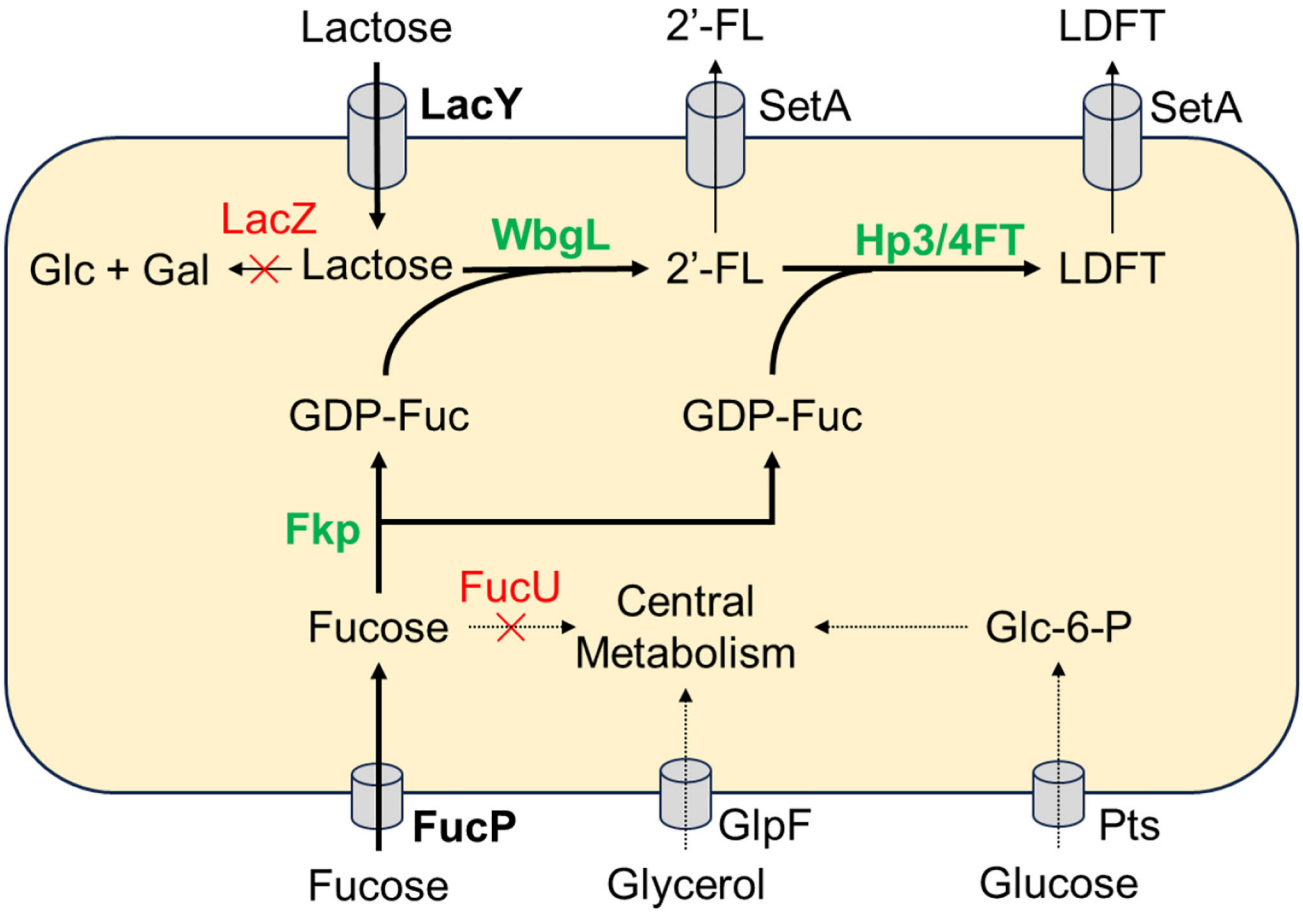


Figure 2. Biosynthetic pathway for LDFT.

Green indicates reaction steps with exogenous enzymes. Red indicates deletion of the reaction steps. Bold indicates overexpression of the corresponding gene. **Metabolite abbreviations:** Glc, D-glucose; Gal, D-galactose; GDP-Fuc, Guanidine-5'-diphosphate-fucose; Glc-6-P, Glucose-6-phosphate; 2'-FL, 2'-Fucosyllactose; LDFT, Lactodifucotetraose. **Enzyme abbreviations:** LacY, Lactose permease; GlpF, Glycerol facilitator; SetA, Sugar efflux transporter; Pts, Phosphoenolpyruvate-carbohydrate phosphotransferase system; lacZ, β-galactosidase; FucU, L-fucose mutarotase; Fkp, Fucokinase/fucose-1-phosphate guanylyl transferase; WbgL, α1–2-fucosyltransferase; Hp3/4FT, α1–3/4-fucosyltransferase. [24].

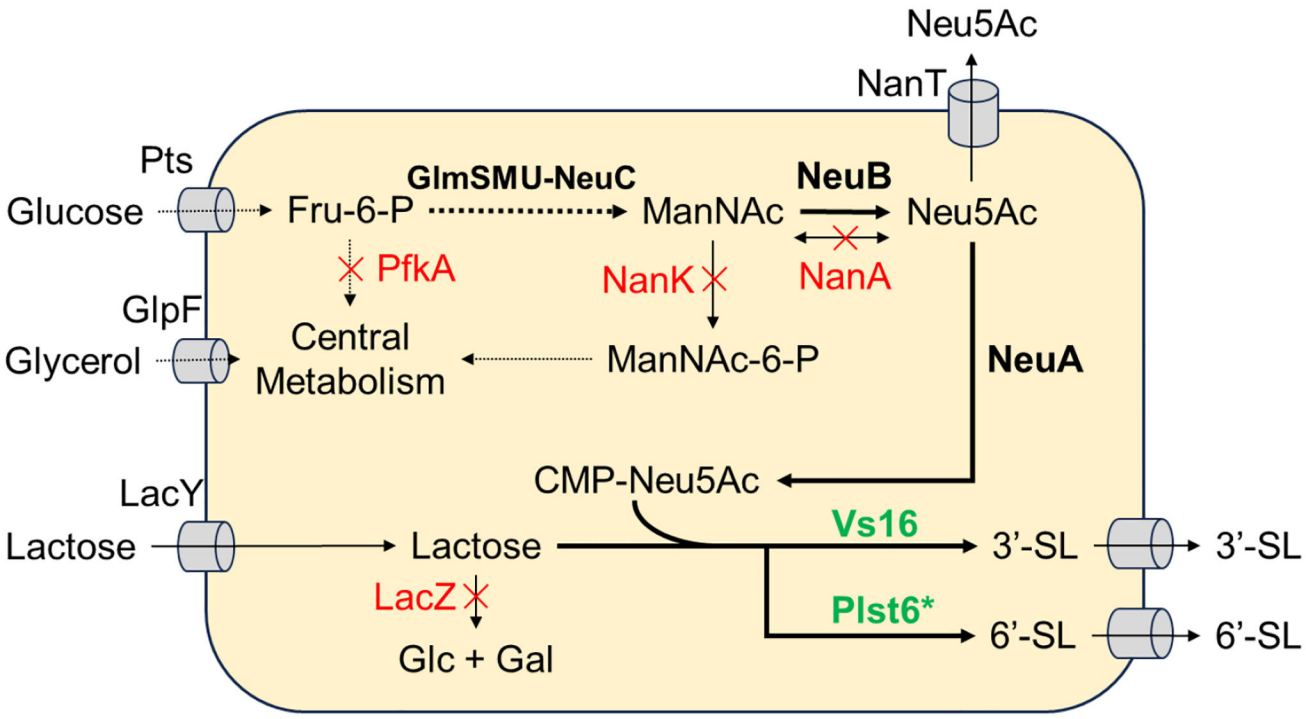


Figure 3. Biosynthetic pathways for 3'-SL and 6'-SL.

Green indicates reaction steps with exogenous enzymes. Red indicates deletion of the reaction steps. Bold indicates overexpression of the corresponding gene. **Metabolite abbreviations:** Glc, D-glucose; Gal, D-galactose; Fru-6-P, D-fructose-6-phosphate; ManNAc, *N*-acetylmannosamine; ManNAc-6-P, *N*-acetylmannosamine-6-phosphate; Neu5Ac, *N*-acetylneuraminic acid; CMP-Neu5Ac, Cytidine-5'-monophospho-*N*-acetylneuraminic acid; 3'-SL, 3'-Sialyllactose; 6'-SL, 6'-Sialyllactose**. Enzyme abbreviations:** LacY, lactose permease; GlpF, glycerol facilitator; NanT, *N*-acetylneuraminate:H^+ symporter; Pts, Phosphoenolpyruvate-carbohydrate phosphotransferase system; lacZ, β-galactosidase; Vs16, 3'-Sialyltransferase; Plst6*, 6'-Sialyltransferase; NeuA, CMP-Neu5Ac synthase; NeuB, *N*-acetylneuraminic acid synthase; NanA, *N*-acetylneuraminate lyase; NanK, *N*-acetylmannosamine kinase; NeuC, UDP-*N*-acetylglucosamine-2-epimerase; GlmS, glutamine-fructose-6-phosphate aminotransferase; GlmM, phosphoglucosamine mutase; GlmU, uridyltransferase/glucosamine-1-phosphate acetyltransferase; PfkA, 6-phosphofructokinase.

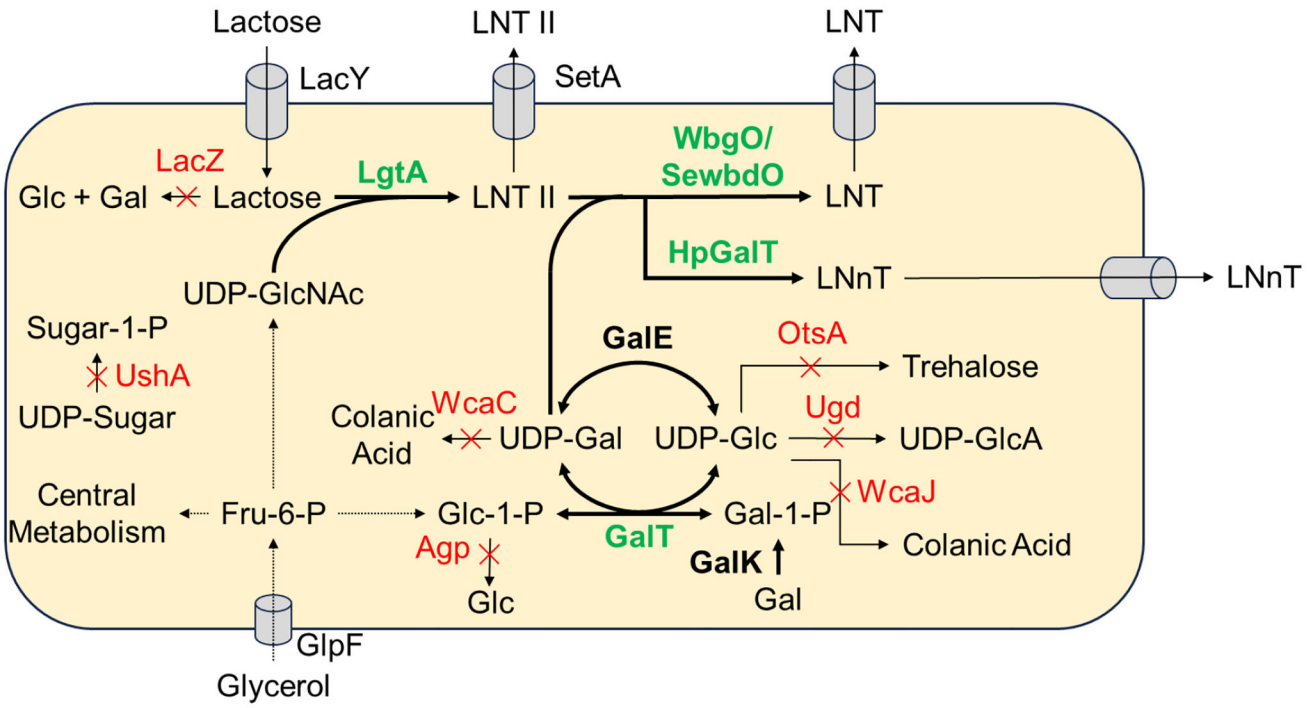


Figure 4. Biosynthetic pathways for LNT and LNnT.

Green indicates reaction steps with exogenous enzymes. Red indicates deletion of the reaction steps. Bold indicates overexpression of the corresponding gene. **Metabolite abbreviations:** Glc, D-glucose; Gal, D-galactose; Fru-6-P, D-fructose-6-phosphate; UDP-GlcNAc, Uridine diphosphate *N*-acetylglucosamine; LNT II, Lacto-*N*-triose II; LNT, Lacto-*N*-tetraose; LNnT, Lacto-*N*-neotetraose; UDP-Gal, Uridine diphosphate galactose; UDP-Glc, Uridine diphosphate glucose; UDP-GlcA, Uridine diphosphate glucuronate; Gal-1-P, D-galactose-1-phosphate; Glc-1-P, D-glucose-1-phosphate. **Enzyme abbreviations:** LacY, Lactose permease; GlpF, glycerol facilitator; SetA, Sugar efflux transporter; lacZ, β-galactosidase; LgtA, β−1,3-*N*-acetylglucosaminyltransferase; WbgO/SewbdO, β−1,3-galactosyltransferase; GalE, UDP-galactose-4-epimerase; GalT, Galactose-1-phosphate uridylyltransferase; GalK, Galactokinase; OtsA, Trehalose-6-phosphate synthase; Ugd, UDP-glucose-6-dehydrogenase; WcaJ, Undecaprenyl-phosphate glucose phosphotransferase; WcaC, Colanic acid biosynthesis galactotransferase; Agp, Glucose-1-phosphatase; UshA, UDP-sugar hydrolase..

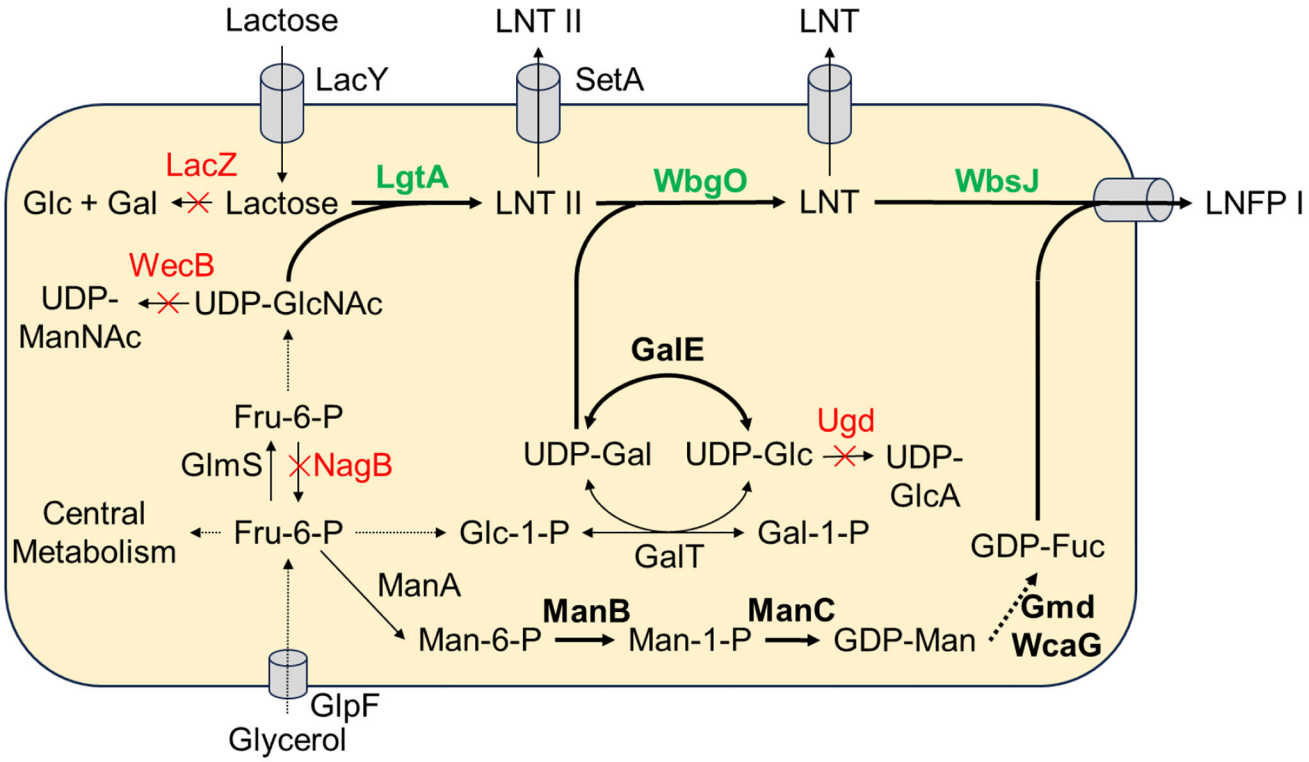


Figure 5. Biosynthetic pathway for LNFP I.

Green indicates reaction steps with exogenous enzymes. Red indicates deletion of the reaction steps. Bold indicates overexpression of the corresponding gene. **Metabolite abbreviations:** Glc, D-glucose; Gal, D-galactose; Fru-6-P, D-fructose-6-phosphate; Fru-1-P, D-fructose-1-phosphate; UDP-GlcNAc, Uridine diphosphate *N*-acetylglucosamine; LNT II, Lacto-*N*-triose II; LNT, Lacto-*N*-tetraose; LNFP I, Lacto-*N*-fucopentaose I; UDP-Gal, Uridine diphosphate galactose; UDP-Glc, Uridine diphosphate glucose; UDP-GlcA, Uridine diphosphate glucuronate; Gal-1-P, D-galactose-1-phosphate; Glc-1-P, D-glucose-1-phosphate; GDP-Fuc, Guanidine-5'-diphosphate-fucose; GDP-Man, Guanidine-5'-diphosphate-mannose; Man-1-P, Mannose-1-phosphate; Man-6-P, Mannose-6-phosphate. **Enzyme abbreviations:** LacY, Lactose permease; GlpF, glycerol facilitator; SetA, Sugar efflux transporter; WbsJ, α1,2-fucosyltransferase; lacZ, β-galactosidase; LgtA, β−1,3-*N*-acetylglucosaminyltransferase; WbgO, β−1,3-galactosyltransferase; GalE, UDP-galactose-4-epimerase; GalT, Galactose-1-phosphate uridylyltransferase; Ugd, UDP-glucose-6-dehydrogenase; WcaG, GDP-4-keto-6-deoxy-D-mannose epimerase/reductase; Gmd, GDP-mannose 4,6-hydro-lyase; ManC, Mannose-1-phosphate guanylyltransferase; ManB, Phosphomannomutase; ManA, Mannose-6-phosphate isomerase; GlmS; L-glutamine-D-frutcose-6-phosphate aminotransferase; NagB, Glucosamine-6-phosphate deaminase.

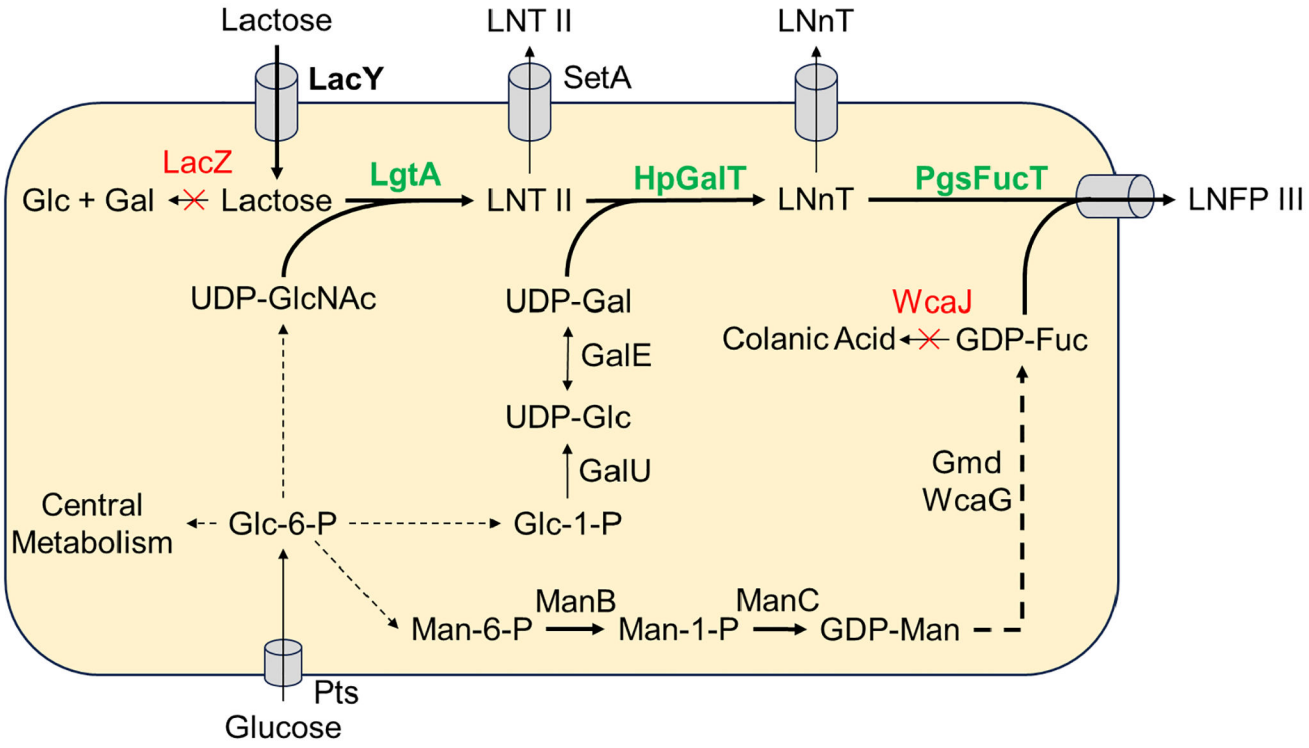
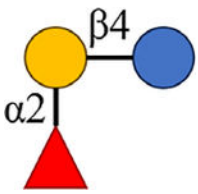
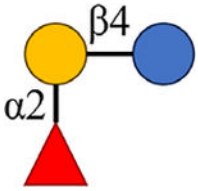
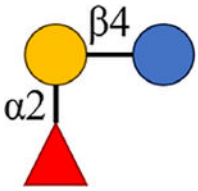
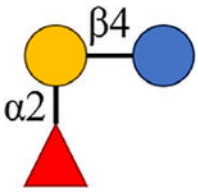
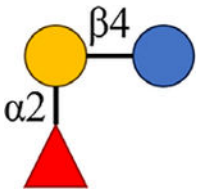
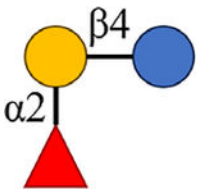


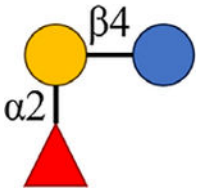
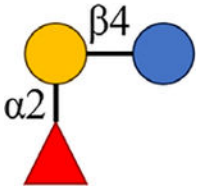
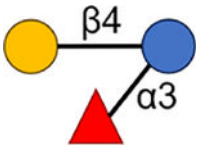
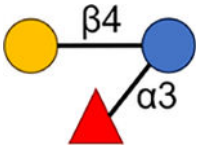
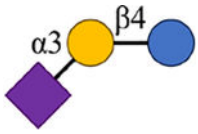
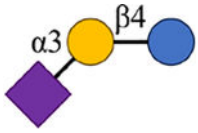
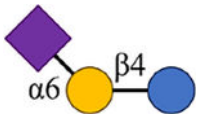
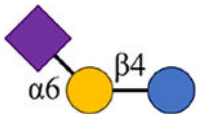
Figure 6. Biosynthetic pathway for LNFP III.

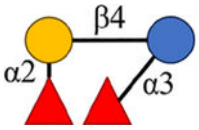
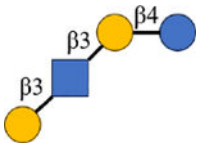
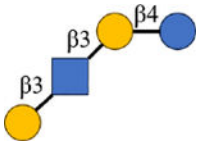
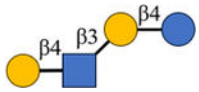
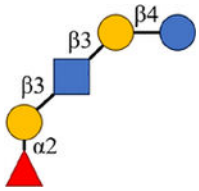
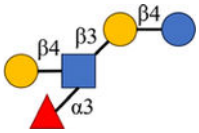
Green indicates reaction steps with exogenous enzymes. Red indicates deletion of the reaction steps. Bold indicates overexpression of the corresponding gene. **Metabolite abbreviations:** Glc, D-glucose; Gal, D-galactose; UDP-GlcNAc, Uridine diphosphate *N*-acetylglucosamine; LNT II, Lacto-*N*-triose II; LNnT, Lacto-*N*-neotetraose; LNFP III, Lacto-*N*-fucopentaose III; UDP-Gal, Uridine diphosphate galactose; UDP-Glc, Uridine diphosphate glucose; Glc-1-P, D-glucose-1-phosphate; Glc-6-P, D-glucose-6-phosphate; GDP-Fuc, Guanidine-5'-diphosphate-fucose; GDP-Man, Guanidine-5'-diphosphate-mannose; Man-1-P, Mannose-1-phosphate; Man-6-P, Mannose-6-phosphate. **Enzyme abbreviations:** LacY, Lactose permease; SetA, Sugar efflux transporter; Pts, Phosphoenolpyruvate-carbohydrate phosphotransferase system; WbsJ, α1,2-fucosyltransferase; lacZ, β-galactosidase; LgtA, β−1,3-*N*-acetylglucosaminyltransferase; HpGalT, β−1,4-galactosyltransferase; PgsFucT, α−1,3-fucosyltransferase; GalE, UDP-galactose-4-epimerase; GalU, UTP-glucose-1-phosphate uridylyltransferase; WcaJ, UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase; WcaG, GDP-4-keto-6-deoxy-D-mannose epimerase/reductase; Gmd, GDP-mannose 4,6-hydro-lyase; ManC, Mannose-1-phosphate guanylyltransferase; ManB, Phosphomannomutase.

Table 1

Summary of HMO Bioproduction

Product	Structure	Host Organism	Feedstocks	Fermentation Type and Duration	Highest Product Titer	Reference
2'-FL		*E. coli* MG1655	Lactose, Glycerol	5L fed-batch bioreactor, 38.5h	61.06 g/L	[12]
2'-FL		*E. coli* BL21	Lactose, Glycerol	3L fed-batch bioreactor, 38.5h	22.3 g/L	[13]
2'-FL		*S. cerevisiae* HS07	Lactose, Glucose	2L fed-batch bioreactor, 68h	15 g/L	[14]
2'-FL		*Y. lipolytica* HY28	Lactose, Glucose	2L fed-batch bioreactor, 68h	24 g/L	[14]
2'-FL		*E. coli* BL21 (DE3)	Sucrose	1L fed-batch bioreactor, 84h	64 g/L	[15]
2'-FL		*B. subtilis*	Lactose, Glucose, Fucose	3L fed-batch bioreactor, 48h	5.01 g/L	[16]

Product	Structure	Host Organism	Feedstocks	Fermentation Type and Duration	Highest Product Titer	Reference
2'-FL		*E. coli* BL21 (DE3)	Lactose, Glycerol	3L fed-batch bioreactor, 84h	121.9 g/L	[17]
2'-FL		*E. coli* BL21 (DE3)	Lactose, Glucose	3L fed-batch bioreactor, 84h	111.6 g/L	[17]
3-FL		*E. coli* BL21 (DE3)	Lactose, Glycerol	3L fed-batch bioreactor, 100h	40.68 g/L	[18]
3-FL		*E. coli* C41 (DE3)	Lactose, Glycerol	5L fed-batch bioreactor, 78h	35.72 g/L	[19]
3'-SL		*E. coli* BL21 (DE3)	Lactose, Glucose, Glycerol	5L fed-batch bioreactor, 38.5h	31.4 g/L	[20]
3'-SL		*B. subtilis* 168	Lactose, Glucose	50mL shake flask, 12h	1.25 g/L	[21]
6'-SL		*E. coli* BL21 (DE3)	Lactose, Glycerol	3L fed-batch bioreactor, 75h	22.85 g/L	[22]
6'-SL		*E. coli* DH1	Lactose, Glycerol	2L fed-batch bioreactor, 75h	23 g/L	[23]

Product	Structure	Host Organism	Feedstocks	Fermentation Type and Duration	Highest Product Titer	Reference
LDFT		*E. coli* MG1655 Z1	Glucose, Glycerol, Lactose, L-Fucose	3 mL shake-flask, 24h	5.0 g/L	[24]
LNT		*E. coli* BL21 (DE3)	Lactose, Glycerol	3L fed-batch bioreactor, 56h	31.56 g/L	[25]
LNT		*E. coli* BL21 star (DE3)	Lactose, Glycerol	2L fed-batch bioreactor, 66h	48.41 g/L	[26]
LNnT		*E. coli* BL21 star (DE3)	Lactose, Glycerol	2L fed-batch bioreactor, 66h	22.07 g/L	[26]
LNFP I		*E. coli* BL21 (DE3)	Lactose, Glycerol	5L fed-batch bioreactor, 48h	30.47 g/L	[27]
LNFP III		*E. coli* K-12 W3110	Lactose, Glucose	3L fed-batch bioreactor, 48h	3.84 g/L, after cell lysis	[28]

Abbreviations: *E. coli, Escherichia coli*; *S. cerevisiae, Saccharomyces cerevisiae*; *Y. lipolytica, Yarrowia lipolytica*; *B. subtilis, Bacillus subtilis*; 2'FL, 2'-Fucosyllactose; 3-FL, 3-Fucosyllactose 3'SL, 3'-Sialyllactose; 6'SL, 6'-Sialyllactose; LDFT, Lactodifucotetraose; LNT, Lacto-*N*-tetraose; LNnT, Lacto-*N*-neotetraose; LNFP I, Lacto-*N*-fucopentaose I; LNFP III, Lacto-*N*-fucopentaose III; *E. coli, Escherichia coli*. Structural abbreviations: blue circle, D-glucose; yellow circle, D-galactose; blue square, N-acetylglucosamine; red triangle, L-fucose; purple diamond, N-acetylneuraminic acid (Neu5Ac).