CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
2022, VOL. 62, NO. 8, 2083–2092
https://doi.org/10.1080/10408398.2020.1850413

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REVIEW

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Recent advances on 2′-fucosyllactose: physiological properties, applications, and production approaches

Yingying Zhu[a], Li Wan[a], Wen Li[a], Dawei Ni[a], Wenli Zhang[a], Xin Yan[b], and Wanmeng Mu[a,c]

[a]State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, China; [b]Department of Microbiology, College of Life Sciences, Key Laboratory for Microbiological Engineering of Agricultural Environment of Ministry of Agriculture, Nanjing Agricultural University, Nanjing, China; [c]International Joint Laboratory on Food Safety, Jiangnan University, Wuxi, China

ABSTRACT

The trisaccharide, 2′-fucosyllactose (Fucα1-2Galβ1-4Glc; 2′-FL), is the most abundant oligosaccharide in human milk. It has numerous significant biological properties including prebiotics, antibacterial, antiviral, and immunomodulating effects, and has been approved as "generally recognized as safe" (GRAS) by the Food and Drug Administration (FDA) and as a novel food (NF) by the European Food Safety Authority (EFSA). 2′-FL not only serves as a food ingredient added in infant formula, but also as a dietary supplement and medical food material in food bioprocesses. There is considerable commercial interest in 2′-FL for its irreplaceable nutritional applications. This review aims at systematically elaborating key functional properties of 2′-FL as well as its applications. In addition, several approaches for 2′-FL production are described in this review, including chemical, chemo-enzymatical, and cell factory approaches, and the pivotal research results also have been summarized. With the rapid development of metabolic engineering and synthetic biology strategies, using the engineered cell factory for 2′-FL large-scale production might be a promising approach. From an economic and safety point of view, microbial selection for cell factory engineering in 2′-FL bioprocess also should be taken into consideration.

KEYWORDS

Biological production; 2′-fucosyllactose; human milk oligosaccharide; metabolic engineering; physiological properties

Introduction

Human milk is generally considered as the most pivotal source of nutrition for neonates. It has a specific composition providing unique benefits beyond the common nutrition found in infant formula milk. In the last few decades, thorough studies of human milk have been driven by pediatricians, microbiologists, chemists, and other scientists with different perspectives and interests. An accumulating body of evidence suggests that human milk oligosaccharides (HMOs) probably play a key role in the excellent effects of breast milk (Bode 2012). Originally, HMOs were discovered as a prebiotic that promotes the growth of desired bacteria and modulates intestinal microbiota composition, with health benefits for the breastfed newborn. Later on, comprehensive data stemming from in vitro and in vivo experiments confirmed that HMOs directly reduce microbial infections by modifying the host's epithelial cell-surface glycome and indirectly affect the infant's immune system by modulating the immune responses. In addition, HMOs have been proven to be an essential nutrient for brain development, especially during the first months of life of neonates (Bode 2009, 2012, 2015; Walker and Iyengar 2015; Yu et al. 2013).

Shown in Figure 1, HMOs are the third most abundant component of mature human milk (5-15 g/L) after lactose (70 g/L) and lipids (40 g/L) (Bode 2009; Zivkovic et al. 2011). HMOs comprise a broad variety of oligosaccharide structures and more than 200 different isomers, and more than 100 HMOs structures have been well-documented (German et al. 2008; Kobata 2010; Wu et al. 2011; Wu et al. 2010). Among them, approximately over half of HMOs are fucosylated (Smilowitz et al. 2014). Fucosylated HMOs, mainly including 2′-fucosyllactose (2′-FL), 3-fucosyllactose (3-FL), lacto-*N*-fucopentaose (LNFP), lacto-*N*-difucohexaose (LNDFH), have been reported to have many important biological activities for breastfed newborns (Chen 2015). 2′-FL, a kind of trisaccharide formed with lactose and fucose via α-(1,2)-linkage, is the most abundant fucosyloligosaccharide with more than 30% of total HMOs. To date, 2′-FL, with irreplaceable physiological effects, is one of the most promising oligosaccharides in HMOs and has gathered great interest not only in large-scale production but also in commercial applications.

Because of its important physiological functionalities, there is great interest in introducing 2′-FL into infant formula milk. In recent years, the 2′-FL-containing products have appeared in different infant formula milks produced by various producers worldwide. However, the high cost has restricted the availability of commercialized 2′-FL (Bych et al. 2019). In turn, economic considerations have stimulated the development of biosynthesis of 2′-FL. Although several approaches have been established for large-scale 2′-FL production, including chemical, chemo-enzymatical, and

CONTACT Wanmeng Mu wmmu@jiangnan.edu.cn State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China.

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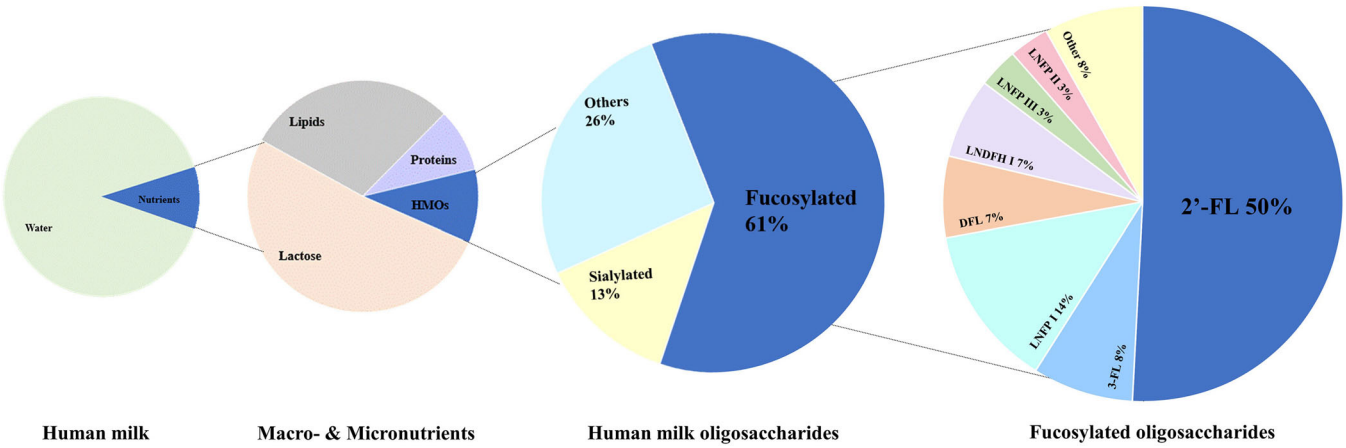

Figure 1. The general composition and proportion of human milk (based on Thurl et al. 2017).

cell factory methods, it is obvious that chemical synthesis is not suitable to meet the 2′-FL production demands because of the complicated multiple protection and deprotection procedures along with low yield (Roussel, Takhi, and Schmidt 2001; Schmidt and Thiem 2010). Thus, biological synthesis methods are more advantageous for large-scale production of 2′-FL. This review mainly introduces recent researches on the chemical and advanced biotechnological synthesis methods for 2′-FL production. Furthermore, to the best of our knowledge, this is the first systematic review of the physiological properties of 2′-FL. And its applications in the infant formula milk field also have been described.

Brief introduction to HMOs

Structure

HMOs contain diverse and complex sugars. In the last few decades, the structural studies of HMOs have been performed by analysts and chemists. There are several analytical methods used for identifying the composition and structure of oligosaccharides in human milk, including capillary electrophoresis, mass spectrometry (MS), high-performance liquid chromatography (HPLC), high-performance anion-exchange chromatography (HPAEC), and nuclear magnetic resonance (NMR) (Amano et al. 2009; Coppa et al. 1999; Kobata 2010; Pfenninger et al. 2008). Particularly, the frontier HPLC-Chip/MS analytical means provides a new strategy to profile a series of oligosaccharides (Ninonuevo et al. 2006, 2007). More than 200 separated HMOs have been found with different polymers, in which five monosaccharides have been confirmed to form the backbone of HMOs, including D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac) (Han et al. 2012). At least 13 core oligosaccharides have been identified, which are mainly responsible for elongation and branching of the complex sugars. More than 100 oligosaccharides are formed by adding fucosylated residues and sialylated residues to these core oligosaccharides (Kobata 2010; Wu et al. 2010, 2011). The most abundant oligosaccharides are fucosylated ones, with the proportion of higher than 70%, and the remaining are sialylated oligosaccharides (Ninonuevo et al. 2006).

Physiological properties

It is well-known that human milk contains all the essential nutrients for the infant to meet growth demands, and there is ample evidence that most of these functional nutrient components are HMOs (Bode 2009). HMOs are oligosaccharides with a degree of polymerization of 3-20 in human milk acting as prebiotics, which are preferably consumed by beneficial microorganisms such as *Bifidobacterium bifidum*, *Bifidobacterium longum*, and *Bifidobacterium infantis*. Bifidobacteria produce special metabolites (lactic acid and short-chain fatty acids) in the gut, which provide undesirable conditions for pathogen growth. Bifidobacteria also occupy the surface binding sites and inhibit the adhesion of harmful bacterial. In addition, human milk glycan fixed on the surface of the gut serves as soluble decoy receptors for pathogenic microorganisms, thus lowering the risk for infection (Chichlowski et al. 2012; Gibson and Wang 1994; Locascio et al. 2007; Newburg, Ruiz-Palacios, and Morrow 2005; Zivkovic et al. 2011). HMOs are believed to have significant effects on lowering the incidence of diarrhea in breastfed infants (Morrow et al. 2004). Furthermore, several studies have been reported on other effects such as modulating the immune system and promoting brain development for the newborn (Smilowitz et al. 2014; Wang 2012; Yatsunenko et al. 2012).

Existing sources

HMOs are mainly found in mother's milk during the lactation period and are very rare in the mature milk of domestic animals (Bode 2012). The oligosaccharide amount is very different between mothers and varies throughout the course of lactation, ranging from 5-25 g/L (Chaturved et al. 2001; Gabrielli et al. 2011). The oligosaccharide content of goat's milk is 0.25-0.30 g/L, which is higher than that in bovine and ovine milk, with 0.03-0.06 and 0.02-0.04 g/L, respectively, but still far below the concentration existing in human milk (Lane et al. 2010). In human milk, many

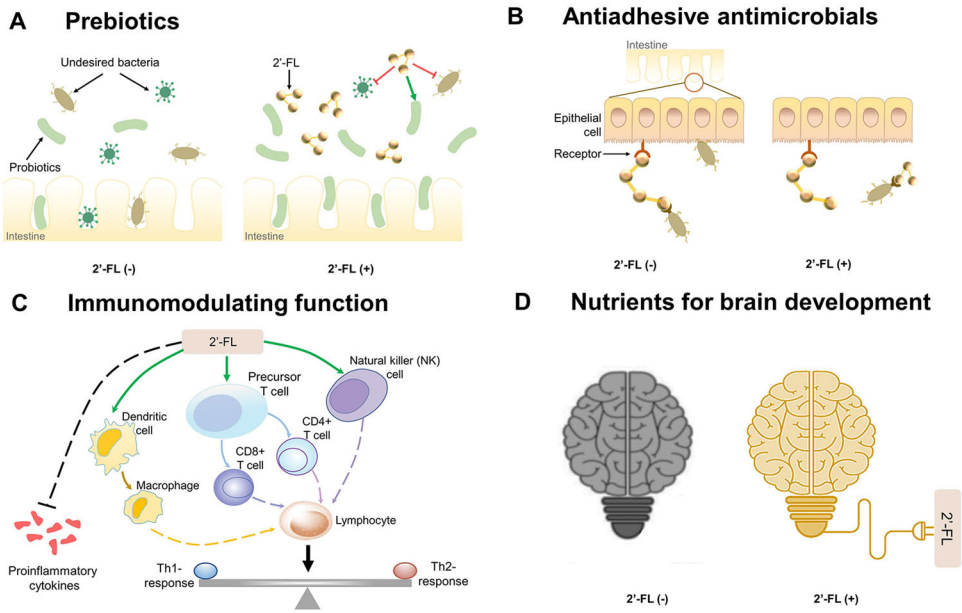

Figure 2. The main physiological properties of 2′-FL, including prebiotics (A), antiadhesive antimicrobials (B), immunomodulators (C), and nutrient providers for brain (D).

oligosaccharide structures are unique, and the fucosylated oligosaccharides are one of the most important components in human milk. Bovine milk does not have these components, and caprine milk has a very low content just as identified level (Oliveira et al. 2015). These unique fucosylated oligosaccharides in human milk might play a crucial role in providing special benefits to the breastfed newborn. Among fucosylated oligosaccharides, 2′-FL is the most well-documented oligosaccharide to have these unique physiological effects.

Physiological functionalities of 2′-FL

Prebiotics effect

A prebiotic was first defined as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson et al. 1995, 2004). 2′-FL is one of the simplest fucosylated oligosaccharides, which meets all criteria and acts as prebiotics to selectively stimulate the growth of beneficial bacteria (Figure 2A). 2′-FL has been tested as a supplement in the in vitro cultivation of four separation representative strains of infant gut microbes. *Bifidobacterium longum* JCM7007 and ATCC15697 efficiently utilize 2′-FL accompanied with short-chain fatty acids and lactate production, and the generated low-pH condition inhibits the growth of *Escherichia coli* K12 and *Clostridium perfringens*, exhibiting the key feature of a prebiotic (Yu et al. 2013). In addition, the small-mass fucosylated oligosaccharides could be preferentially consumed by *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (Locascio et al. 2007, 2009). It has been reported that 2′-linked fucosyloligosaccharides could significantly lower the incidence of diarrhea in breastfed infants (Newburg et al. 2004). The primary reason could be that 2′-FL or the similar 2′-linked fucose inhibits the toxin stably produced by *E. coli*, preventing the risk of toxin-induced secretory diarrhea (Newburg et al. 1990). Additionally, infants breastfed with milk containing 2′-FL show higher Bifidobacterial populations than infants fed with milk lacking 2′-FL (Lewis et al. 2015).

Antiadhesive antimicrobials

Many documents have reported the antiadhesive antimicrobials effect of fucosylated oligosaccharides (Figure 2B). α-1,2-Fucosylated oligosaccharides are shown to inhibit binding of Std fimbriae with UEA-I to Caco-2 cells. The *std* operon encodes an adhesin that binds to an α-1,2-fucosylated oligosaccharides receptor in the cecal mucosa (Chessa et al. 2009). In addition, fucosylated oligosaccharides also inhibit the adhesion of *Campylobacter jejuni* to the H(O) blood group epitope and decrease the risk of infection (Ruiz-Palacios et al. 2003). The amount of 2′-FL consumed by breastfed infants is related to the incidence of *Campylobacter diarrhea* (Morrow et al. 2004). 2′-FL also acts as an antifungal modulator, and it inhibits the adhesion of *Candida albicans* on human buccal epithelial cells (Cravioto et al. 1991). It was recently investigated whether HMOs composition influences the survival of HIV-infected and HIV-exposed-uninfected (HEU) infants. The results revealed that higher concentrations of α-1-2 fucosylated oligosaccharides result in lower mortality of breastfed infants (Kuhn et al. 2015).

Immunomodulating function

It is known that HMOs change the intestinal microbial environment of the infant, and the latest in vitro results indicated that HMOs directly modulate immune responses (Bode 2012). Two groups of experimental mice were selected to identify the effect of 2′-FL on immunity study, and 2′-FL

was effective in reducing colonization by adherent-invasive *E. coli* (AIEC), modulating AIEC-induced CD14 expression, and inhibiting proinflammatory induced signaling (He et al. 2016). Additionally, an experiment was performed on over 400 healthy singleton infants to investigate the effects of feeding formulas with added 2′-FL on immune activity, and the results suggested that 2′-FL fortification promotes the ratio of activated CD8+ T cells and lowers the inflammatory cytokine production and dietary of single 2′-FL increases immune development in infants (Goehring et al. 2016) (Figure 2C).

Nutrients for brain development

2′-FL is generally considered to have functional effects related to immunity and intestinal health during the newborn stage, and there is less information about its effect on cognitive capabilities. In the 1990s, there was a report that 2′-FL increases the hippocampal long-term potentiation (LTP) (Matthies, Staak, and Krug 1996), but more experiments are needed to prove that 2′-FL is mainly responsible for improving cognitive abilities in the breastfed newborn. For the first time, the Vázquez group conducted a series of in vitro experiments, on orally administered 2′-FL on learning and memory capabilities in rodents. In summary, animals that consumed 2′-FL had obviously better behavior than the controls, showing that 2′-FL affects cognitive domains and promotes learning and memory abilities (Vazquez et al. 2015). The next year, another oral supplementation experiment was conducted in rats to test the effect of 2′-FL on brain development. It was indicated that the addition of 2′-FL could enhance brain development, showing an effect in both childhood and adulthood (Figure 2D) (Oliveros et al. 2016).

Commercialization and application of 2′-FL

Because of the outstanding physiological effects mentioned above, the abundant HMO (2′-FL) has potential application in the pharmaceutical and food industries, especially in infant formula. New breakthroughs are being explored in the development of fortified formula milk that more closely resembles human milk, because it is universally known that breast milk plays a vital role in the development of neonates (Faijes et al. 2019). In recent years, 2′-FL has been approved as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) in the USA (FDA, GRN 650), and as a novel food (NF) by the European Food Safety Authority (EFSA) (EU, 2017/2470). It is permitted to not only serve as the food ingredient added to infant formulas, but also as dietary supplements and medical foods (Bych et al. 2019). The first regulatory permission was acquired in the US in September of 2015 for two HMOs 2′-FL and LNnT, produced by chemical synthesis by Glycom A/S. In 2016, 2′-FL manufactured by microbial fermentation by Glycom A/S was approved. Currently, there are over ten 2′-FL-related approvals by the FDA and/or EFSA from five companies including Glycom A/S (Esbjerg, Denmark), BASF Corporation (Ludwigshafen, Germany), DuPont (Wilmington, USA), Glycosyn (Gracefield, New Zealand), and Jennewein Biotechnologie GmbH (Rheinbreitbach, Germany). These entrepreneurs and scientists have made great efforts toward the development of 2′-FL commercialization, and have fueled 2′-FL-related product research and novel product development. The first infant formula product containing 2′-FL started to appear on the market in 2016, and then non-infant products containing 2′-FL also started appearing. Abbott and Nestle are the pioneers for introducing HMOs (especially 2′-FL) into infant formula. Obviously, 2′-FL has promising market potential in the infant formula industry and now it is the best time to develop novel applications. Although 2′-FL has been produced industrially and successfully introduced into formula milk products, 2′-FL remains difficult to obtain at a reasonable price. From an economic point of view, the manufacturing cost of 2′-FL might be decreased by improving the various synthesis methods.

Chemical production of 2′-FL

So far, three approaches have been applied to produce 2′-FL, including chemical, chemo-enzymatical, and cell factory methods. In the early 1980s, there was the first report about 2′-FL production by a chemical synthesis method (Abbas, Barlow, and Matta 1981), and 2′-FL was synthesized by benzylation substrate such as partially benzylated lactose derivatives (Fernandez-Mayoralas and Martin-Lomas 1986). However, these methods require more expensive and toxic reagents for the glycosylation reaction. Compared to previous chemical synthesis, Pereira and McDonald (2012) utilized milder reaction conditions and fewer toxic reagents to replace the toxic stoichiometric metal reagents for chemical synthesis of 2′-FL (Pereira and McDonald 2012). Recently, Agoston et al. from Glycom A/S company established a chemical synthesis method to produce kilogram-scale 2′-FL, which only needs a single chromatographic purification procedure and meets the industrial environment requirements. More specifically, the fucosyl donor (1-*S*-phenyl donor) was prepared from commercial L-fucose by a four-step reaction and the lactose acceptor was obtained by a routine two-step reaction. After final glycosylation and deprotection reactions, the resulting 2′-FL could be obtained at a high purity and large quantities scale (Agoston et al. 2019).

Biological production of 2′-FL

Enzymatic production

Generally, the enzymatic synthesis of 2′-FL requires performing glycosylation reactions with two different types of enzymes, fucosyltransferases and α-L-fucosidases, with glycosynthase activity (Faijes et al. 2019). Fucosyltransferases can transfer a nucleotide donor substrate (GDP-fucose) to lactose to complete the fucosylation reaction. A crucial work on the in vitro synthesis of 2′-FL was reported by two-step enzymatic catalysis, wherein GDP-fucose was first produced from GDP-mannose and NADPH using recombinant GDP-mannose 4,6-dehydratase (Gmd) and GDP-fucose synthetase

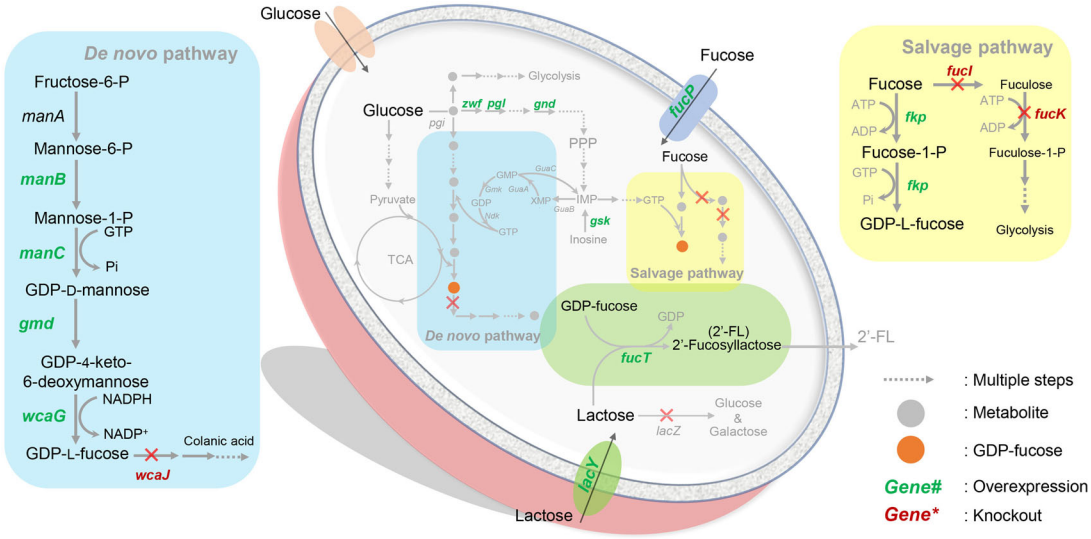

Figure 3. Metabolic pathways for the whole cell biosynthesis of 2′-FL in *E. coli* via the *de novo* pathway and salvage pathway of GDP-fucose.

(WcaG) with a yield of 78% and was further converted to 2′-FL by α-1,2-fucosyltransferase (FucT2) with a yield of 65% on a milligram scale (Albermann, Piepersberg, and Wehmeier 2001).

α-L-Fucosidases are classified into retaining glycosyl hydrolase family 29 (GH29) and inverting α-L-fucosidases (GH95) (Lombard et al. 2014). In recent years, researchers have paid much attention to improve the efficiency of fucosylation reaction by α-L-fucosidases (Sakurama et al. 2012; Saumonneau et al. 2016; Sugiyama et al. 2016; Wada et al. 2008). Seven novel GH29 α-L-fucosidase-encoding genes were screened from metagenome, and expressed and identified in *E. coli*, among them, Mfuc5 were able to produce FL with a yield of 3.6% using 25 mM pNP-Fuc as donor and 100 mM lactose as acceptor. While the *Thermotoga maritima* α-L-fucosidase capable of producing 2′-FL with a maximum yield of 6.4% at the acceptor: donor ratio of 4 (Lezyk et al. 2016). An α-1,2-L-fucosidase derived from an inverting glycosidase could synthesize 2′-FL from *β*-L-fucosyl fluoride triacetate and lactose. Several mutations have been designed and tested, and the D766G had the most synthetic efficiency for 2′-FL (Wada et al. 2008). However, the reaction efficiency was still low for the large-scale synthesis of 2′-FL. Eight years later, Sugiyama and coworkers found that N423H and N423D/D766N mutants showed the highest activity at pH 5.5 and 5.0, respectively, and the synthase activity of both mutants producing 2′-FL was significantly higher than that of the previously reported mutant D766G. Both the N423H and N423D/D766N mutants could produce approximately 8.5 mM 2′-FL, whereas mutant D766G produced less than 1 mM 2′-FL in the presence of 10 mM *β*-fucosyl fluoride and 10 mM lactose (Sugiyama et al. 2016).

Cell factory synthesis

Nowadays, metabolic engineering has been playing increasingly pivotal roles in the development of microbial cell factories for the production of valuable chemicals from cheap materials (Chae et al. 2017). It is a promising strategy with a wide use for HMOs production. Various advanced tools have been explored to maximize the yield of target products, such as synthetic biology, systems biology tools, and modular metabolic pathway assembly (Faijes et al. 2019). Biosynthesis of 2′-FL by cell factory strategy is the best case for the production of HMOs. In general, biosynthesis of 2′-FL requires a glycosylation reaction in the presence of fucosyltransferases. Using lactose as the acceptor of the fucosyltransferases, the common strategies to more efficiently utilize lactose in the cell factory include the overexpression of the lactose permease (LacY) to improve internalized lactose and the inhibition of the galactosidase (LacZ) activity to decrease lactose catabolism. GDP-fucose, the donor of the fucosyltransferases, could be synthesized by two types of pathways, the *de novo* and salvage pathway (Figure 3). Generally, for the *de novo* pathway, the first step is a dehydration reaction catalyzed by GDP-mannose-4,6-dehydratase (GMD) leading to the formation of intermediate GDP-4-keto-6-deoxy-mannose. GDP-fucose is synthesized by a bifunctional enzyme GDP-4-keto-6-deoxy-mannose-3,5-epimerase/4-reductase (WcaG) in the last step. The salvage pathway starts from supplemented fucose, which is first phosphorylated by fucokinase to fucose-1-P and then combined with guanosine triphosphate (GTP) by GDP-fucose pyrophosphorylase to produce GDP-fucose (Mattila et al. 2000).

Comparison of 2′-FL biosynthesis using cell factory strategy by various engineered host strains was shown in Table 1. *E coli* is the most common host for the production of GDP-fucose for 2′-FL production, because it contains the endogenous GDP-fucose synthesis gene cluster of the *de novo* pathway. The Samain group engineered *E. coli* with to produce H-Antigen related oligosaccharides (Drouillard, Driguez, and Samain 2006; Dumon et al. 2006). As for 2′-FL production, the α-1,2-fucosyltransferase was expressed in *lacZ*-inactivated host *E. coli* JM107. To optimize the GDP-fucose flux, they overexpressed the *rcsA* gene, a positive regulator for the colanic acid operator, and eliminated the *wcaJ* gene encoding for the enzyme catalyzing the conversion from GDP-fucose to colanic acid. The final

Table 1. Comparison of 2′-FL biosynthesis using cell factory strategy by various engineered host strains.

Strain	GDP-fucose synthesis pathway	Overexpressed genes	Cultivation	Titer (g/L)	Yield	Reference
E. coli JM107 (Δ*wcaJ*)	*De novo*	*futC*, *rcsA*	Glc and Lac; Fed-batch	3 (intracellularly); 11 (extracellularly)	0.56 mol/mol Lac	Drouillard, Driguez, and Samain 2006
E. coli JM109	*De novo*	*manB*, *manC*, *gmd*, *wcaG*, *fucT2*	Lac; Batch	1.23 (extracellularly)	0.09 g/g Lac	Lee et al. 2012b
E. coli JM109 (Δ*fucI*, Δ*fucK manB*$^+$, *manC*$^+$, *gmd*$^+$, *wcaG*$^+$, *fkp*$^+$, *futC*$^+$)	*De novo* and salvage	NR	Gly, Lac, and Fuc; Fed-batch	10.22 (extracellularly); 10.18 (intracellularly)	309.16 mg/g $_{CDW}$	Baumgärtner et al. 2013
E. coli BL21 (DE3) (*lacZ*Δ*M15*)	*De novo*	*manB*, *manC*, *gmd*, *wcaG*, *fucT2*	Gly and Lac; Fed-batch	6.4 (extracellularly)	0.225 g/g Lac	Chin et al. 2015
E. coli BL21 (DE3) (Δ*lacZ*, Δ*fucI*, Δ*fucK*)	Salvage	*fucT2*, *fkp*	Gly, Fuc, and Lac; Fed-batch	23.1 (extracellularly)	0.37 mol/mol Lac; 0.36 mol/mol Fuc	Chin et al. 2016
E. coli BL21 (DE3) (Δ*lacZ*)	*De novo*	*manB*, *manC*, *gmd*, *wcaG*, *wcfB*	Gly and Lac; Fed-batch	15.4 (extracellularly)	0.858 g/g Lac	Chin et al. 2017
E. coli BL21(DE3) (Δ*lacZ*, Δ*lon*, Δ*wcaJ*)	*De novo*	*manA*, *manB*, *manC*, *gmd*, *wcaG*, *futC*, *zwf*, *pntAB*, *lacY*, *rcsA*	Glc and Lac; Batch	9.1	NR[a]	Huang et al. 2017
S. cerevisiae	Salvage	*fkp*, *fucT2*, *LAC12*	Glc, Fuc, and Lac; Fed-batch	0.503	0.3 mol/mol Lac; 0.63 mol/mol Fuc	Yu et al. 2018
S. cerevisiae and *Y. lipolytica*	*De novo*	*GMD*, *GMER*, *futC*, *CDT2*, *LAC12*	Glc and Lac; Fed-batch	15-24 (extracellularly)	NR	Hollands et al. 2019
E. coli BL21 (DE3) (*lacZ*Δ*M15*)	*De novo*	*manB*, *manC*, *gmd*, *wcaG*, *Te2FT*	Gly and Lac; Fed-batch	0.486	NR	Seydametova et al. 2019
E. coli BL21 (DE3) (Δ*lacZ*, Δ*fucI*, Δ*fucK*, Δ*araA*, Δ*rhaA*)	Salvage	*fkp*, *futC*	Gly, Fuc, and Lac; Fed-batch	47.0	0.52 mol /mol Fuc	Jung et al. 2019
B. subtilis (Δ*yesZ*, Δ*ykfN*, Δ*guaC*)	Salvage	*glcP*, *LAC12*, *ndk*, *gmk*, *futC*	Gly, Fuc, and Lac; Fed-batch	5.01	0.27 mol/mol Lac; 0.85 mol /mol Fuc	Deng et al. 2019a
B. subtilis	Salvage	*fkp*, *futC*	Gly, Fuc, and Lac; Shake-flask	0.674	0.187 g/g Lac	Deng et al. 2019b

[a]NR, Not reported.

Abbreviations: Wcaj: UDP-glucose lipid carrier transferase; *futC*: α-1,2-fucosyltransferase from *H. pylori*; *rcsA*: transcriptional regulatory protein; *lacY*: lactose permease; *fucI*: fucose isomerase; *fucK*: fucokinase; *fkp*: L-fucokinase/L-fucose 1-phosphate guanylyltransferase; *manB*: phosphomannomutase; *manC*: α-D-mannose 1-phosphate guanylyltransferase; *gmd*: GDP-mannose-6-dehydrogenase; *wcaG*: GDP-fucose synthase; *fucT2*: α-1,2-fucosyltransferase from *H. pylori*; *wcfB*: α-1,2-fucosyltransferase from *Bacteroides fragilis*; *lacZ*: β-galactosidase; *lon*: lon protease; *zwf*: glucose 6-phosphate 1-dehydrogenase; *pntAB*: NAD(P) transhydrogenase subunit α part 2; *LAC12*: lactose permease from *K. lactis*; *GMD*: GDP-mannose dehydratase from *Mortierella alpine*; *GMER*: GDP-β-L-fucose: NADP$^+$ 4-oxidoreductase (3,5-epimerizing) from *M. alpine*; *CDT2*: cellodextrin transporter from *N. crassa*; Te2FT: α-1,2-fucosyltransferase from *Thermosynechococcus elongatus*; *araA*: arabinose isomerase; *RhaA*: rhamnose isomerase; *yesZ*: β-galactosidase; *ykfN*, *guaC*, *ndk*, and *gmk*: proteins involved in GTP metabolic pathway in *B. subtilis*.

concentration of 2′-FL was on the gram scale (Drouillard, Driguez, and Samain 2006). To make GDP-fucose available for the synthesis of 2′-FL, Seo and coworkers modulated the guanosine nucleotides biosynthetic pathways and GDP-mannose metabolism pathways and introduced the cofactor NADPH recycle pathway to enhance GDP-fucose production (Byun et al. 2007; Lee et al. 2009, 2011, 2012a, 2013). *E. coli* BL21 was engineered to produce 2′-FL from glycerol and lactose via the *de novo* pathway of GDP-fucose biosynthesis (Lee et al. 2012b). To improve 2′-FL production, the endogenous lac operon was replaced with the modified lac operon to reduce β-galactosidase activity, resulting in 2′-FL titer of 6.4 g/L and yield of 0.225 g/g based on lactose (Chin et al. 2015). In another work, the genes *manA*, *manB*, *manC*, *gmd*, and *wcaG* involved in the *de novo* pathway and the selected genes *futC*, *lacY*, *zwf*, *pntAB*, and *rcsA* encoding for *Helicobacter pylori* α-1,2-fucosyltransferase (FutC), lactose permease (LacY), glucose-6-phosphate dehydrogenase (G6PDH), membrane-bound transhydrogenase (PntAB), and regulator for colanic acid production, respectively, were overexpressed in *E. coli* BL21(DE3). Furthermore, the *wcaJ*, *lacZ*, and *lon* genes were removed, and the engineered strains were capable of producing 9.12 g/L 2′-FL (Huang et al. 2017). Baumgärtner et al. developed an effective combined production strategy through chromosomally integrating related genes involved in the *de novo* and salvage pathways in *E. coli* JM109 strains. In this approach, cells with the stable expression of the *de novo* and salvage pathway genes along with *futC*, produced approximately twice as much 2′-FL compared with cells with only *de novo* pathway for GDP-fucose synthesis (380 vs. 200 mg/g $_{CDW}$). A further improvement of 2′-FL made in the engineered *E. coli* host by additionally expressing *futC* gene with a titer of 20.28 g/L in a 13.5-L fed-batch fermentation (Baumgärtner et al. 2013).

Chin et al. (2016) introduced the genes involved in salvage pathway, *fkp* gene encoding fucokinase/GDP-fucose pyrophosphorylase (Fkp) from *Bacteroides fragilis* and the *fucT2* gene encoding α-1,2-fucosyltransferase from *Helicobacter pylori*, to allow the engineered *E. coli* to produce 2′-FL. In addition, the gene cluster *fucI-fucK* encoding fucose isomerase (FucI) and fuculose kinase (FucK) and gene *lacZ* were deleted to further enhance the 2′-FL production, resulting in 2′-FL formation with a titer of 23.1 g/L in fed-batch fermentation cultivation (Chin et al. 2016). In addition to overexpressing *fkp* gene from *B. fragilis* and *fucT2* gene and deleting *lacZ*, *fucI*, and *fucK* genes, Jung et al. (2019) additionally eliminated *araA* gene encoding L-arabinose isomerase and *rhaA* gene encoding L-rhamnose isomerase, led to 2′-FL production with a titer of 47.0 g/L (Jung et al. 2019). Except for *E. coli*, several GRAS status

microorganims have been selected as the hosts to produce 2′-FL. A new attempt was implemented to produce 2′-FL in engineered *Saccharomyces cerevisiae* by overexpressing the salvage pathway related gene (*fkp*). The genes *LAC12* from *Kluyveromyces lactis* and *fucT2* were also selected to be expressed in *S. cerevisiae* to biosynthesize 2′-FL. Although the fermentation result was not satisfactory, this was the first report on 2′-FL production using food-grade yeast *S. cerevisiae* (Yu et al. 2018). In recent years, Hollands et al. demonstrated that both *S. cerevisiae* and *Yarrowia lipolytica* could be engineered to produce 2′-FL via the *de novo* pathway from GDP-mannose to GDP-fucose. Overexpressing *LAC12* gene from *K. lactis* to import acceptor lactose and genes encoding α-1,2-fucosyltransferases from various organisms were used to complete biosynthesis of 2′-FL. Furthermore, several different sources of transporters were identified to export 2′-FL from yeast. And the *Neurospora crassa* cellobiose transporter (CDT2) was selected to be the best option used for 2′-FL efflux. The final engineered *S. cerevisiae* and *Yarrowia lipolytica* capable of producing 2′-FL with titers of 15 and 24 g/L, respectively (Hollands et al. 2019). As a typical GRAS strain, *Bacillus subtilis* as a host also have been developed for an effective production of 2′-FL. The authors constructed the engineered *B. subtilis* by introducing the salvage pathway genes for GDP-fucose production, overexpressing sugar transporter-encoding *glcP* and lactose permease-encoding gene *LAC12*, knocking out *guaC*, *yfkN*, and *yesZ* genes involved in bypass pathway, and engineering guanosine 5′-triphosphate (GTP) cofactor regeneration systems. The final engineered *B. subtilis* could synthesize 5.01 g/L 2′-FL in a 3-L fed-batch bioreactor (Deng et al. 2019a). Another strategy was performed by the same author which developed an aptamer-based regulatory mechanism for dynamic metabolic engineering to improve 2′-FL production (Deng et al. 2019b).

Conclusions and future outlook

2′-FL is a versatile ingredient that can be employed by food developers not only as a food ingredient added to infant formulas, but also as dietary supplements and medical foods. Since the irreplaceable biological functions of human milk that convey numerous benefits to newborns, developing infant formula that more closely mimics human milk is becoming increasingly urgent and important. Notably, the extensive demand of 2′-FL has accelerated 2′-FL production. Currently, three methods are applied to produce 2′-FL, including chemical, chemo-enzymatic, and cell factory methods. The chemical method for 2′-FL production on the industrial scale is not very practical mostly due to low stereoselectivity and the usage of toxic solvents. While the chemo-enzymatic approach might be mainly limited by the required enzymes and costly substrates. Owing to the development of metabolic engineering and synthetic biology strategies, the cell factory approach for 2′-FL production presents a more promising alternative. And many successful cases have demonstrated that it is feasible to produce scaled-up through engineered strains.

Until now, *E. coli* is most widely used as the engineered host to produce 2′-FL. And a number of desirable results have been obtained by cell factory approach using *E. coli*. Nonetheless, more efforts should be paid to improve 2′-FL production from multidimensional strategies. GDP-fucose is a crucial and costly precursor. The salvage pathway with the supplemented fucose might increase the cost of 2′-FL synthesis, while the *de novo* pathway with inexpensive substrates generally have low yields. The balanced consideration between production cost and efficiency is an important issue. FucT is recognized as a rate-limiting enzyme, which hampers the efficient production of 2′-FL. Advanced protein engineering might be a good strategy to improve the catalytic efficiency of FucT. In addition, the effective supply of cofactors using cofactor engineering strategies and relieving feedback inhibition of intermediate metabolites using specific feedback-resistant genes are of great importance for the efficient synthesis of 2′-FL. Recently, the food-grade *B. subtilis* and yeasts *S. cerevisiae* and *Y. lipolytica* were successfully engineered to synthesize 2′-FL. They all have an advantage over toxin-producing bacteria in terms of food safety and downstream isolation and purification. Although these engineered strains are capable of biosynthesizing 2′-FL, the yields are still unfavorable for practicable application. These reported food-grade strains generally lack of effective endogenous lactose permeases for the utilization of lactose substrate. And thus, an applicable strategy to increase 2′-FL yield is the directed evolution of lactose permease for improving the lactose utilization.

Funding

This work was supported by the National Natural Science Foundation of China (No. 31922073), the Key-Area Research and Development Program of Guangdong Province (No. 2020B020226007), and Zhangjiagang international cooperation project (ZKH1905).

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