Food Science of Animal Products

Review Article

# Insight into the key bridge for infant's nutrition and health: how to explore personalized utilization pathways from diverse human milk oligosaccharides

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**Abstract:** Breast milk is the preferred gold standard food for infants. Human milk oligosaccharides (HMOs) are the third most natural component in breast milk. But breast milk is often insufficient, so they rely solely on breast milk substitutes. HMOs provide nutrients to beneficial gut microbiota such as *Lactobacilli* and *Bifidobacteria*, helping to establish and maintain a balance of microbial communities in the infant gut. HMOs mimic the receptors of pathogens, preventing them from attaching to the baby's intestinal cells, thereby preventing pathogen infection. This function is particularly crucial for newborns and infants. How to individually use HMOs is important. We focused on classification and characteristics of HMOs, their impact, intake, development/utilization mechanism on infant health, aiming to provide HMOs data support for the development. HMOs are quite different (more than 200 kinds), so it is necessary to make targeted selection, and those associated with intestinal microorganisms (*Bifidobacterium*), which can utilize HMOs, have the greatest application potential. Oligosaccharide-binding protein (OBPs) are an important medium for ATP-binding cassette transporter channel of intestinal HMOs transport; the influence of key OBPs of *Bifidobacterium* on HMOs recognition in infants from various countries has been explored, which is helpful to accelerate the establishment of precise and personalized milk powder in the future. The more important significance of the results of this review is to help consumers better choose HMOs, thereby promoting the long-term health of infants, especially the early development of their immune system.

**Keywords:** *Bifidobacterium*; human milk oligosaccharides; intestinal microorganisms

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## 1 Introduction

Breast milk is the most natural, safe and complete food for infants. Therefore, breast milk is widely considered as the best food necessary for infants' growth and development[1–3]. It contains all the nutrients and antibodies needed for infant growth, which cannot be replaced by any exogenous foods for special energy supply, nutrient absorption, and brain and body development[4–6]. Among them, various nutrients in which breast milk is rich can also be metabolized by intestinal microorganisms and then effectively regulate the intestinal immune response in early life, which is crucial for early intestinal immune system development in infants[7]. Specifically, the immune system is the first line of defence against exposure to allergens, bacteria and viruses in the body[8–10]. In this golden age of immune system development, the immune system is still immature. As a special life substance, breast milk not only provides nutritional support for infants but also helps them quickly build immune defences[11], including stimulating the development of the gastrointestinal mucosa, affecting the composition and development of intestinal microbiota[12]. Therefore, the World Health Organization and relevant regulations and standards emphasize the recommendation that infants should be exclusively breast-fed for 6 months at least[13]. Human milk oligosaccharides (HMOs) are carbohydrates other than lactose found in breast milk[14–16]. More than 200 kinds of oligosaccharides have been found. The concentrations of HMOs in mature milk and colostrum are 7–14 and 20–24 g/L, respectively, making HMOs the third most abundant solid component after lactose and lipids. As antibacterial agents, HMOs can resist pathogens, and reduce the risk of parasitic viral, bacterial and protozoan infections[17–22]. It can be seen that HMOs, using breast milk as a carrier, play an extremely important role in the healthy growth of infants in early life (Figure 1).

Breast milk is the best nutrition for infants' growth and development, and it is also rich in antibodies, providing the best source of adaptive immunity for newborns' intestines. Especially for premature or low birth weight newborns, breast milk is the first choice[23–25]. However, physiological status, social pressure and other reasons often lead to insufficient breast milk, and some infants cannot be fed with breast milk at all. For 6 months old, the prolonged breastfeeding was only 34.8%[26]. Therefore, these infants rely solely on breast milk substitutes, of which the largest demand is

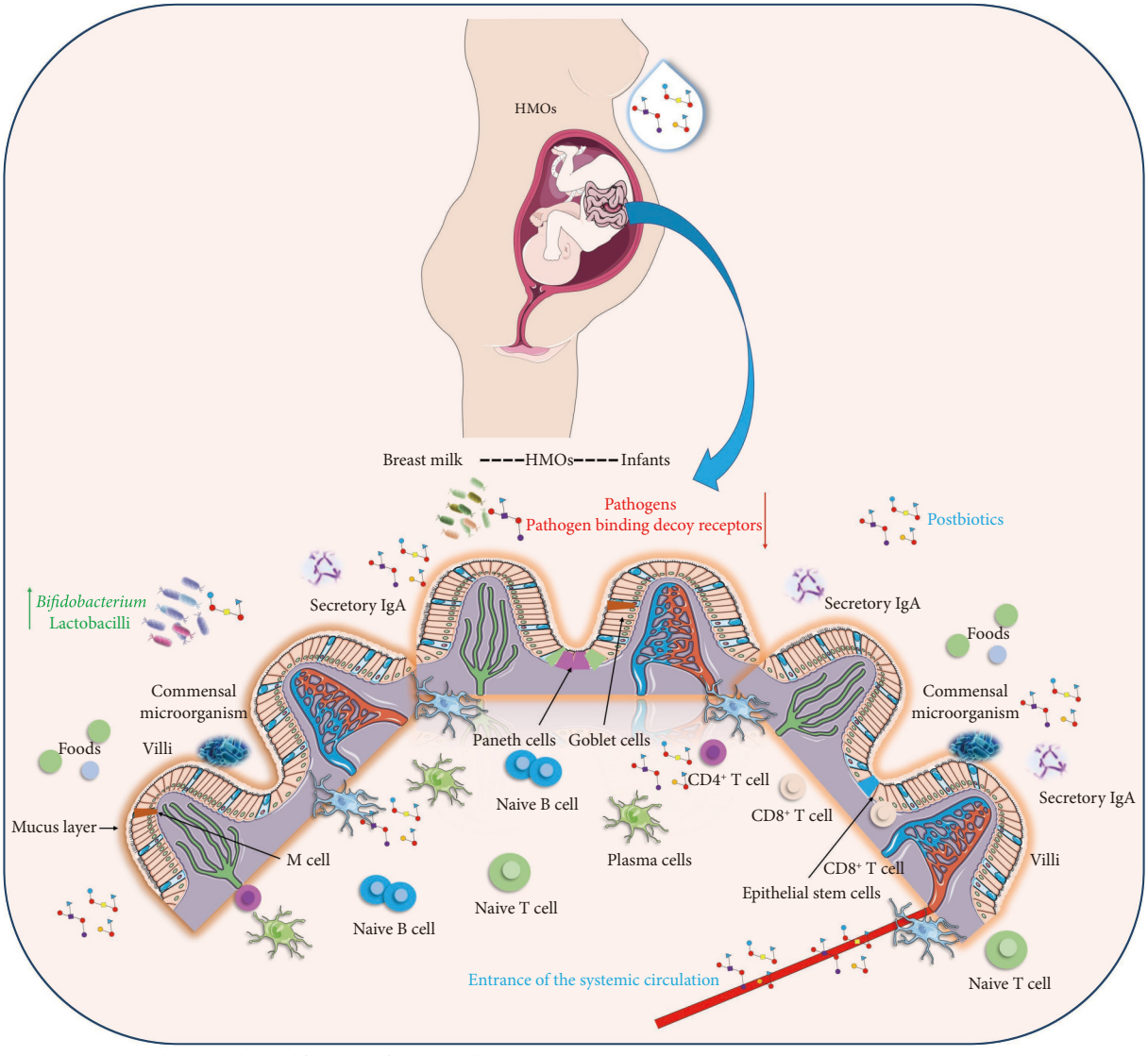


**Figure 1** Intestinal immune barrier and related functions of human milk oligosaccharides (HMOs).

for infant formula milk powder. Infant formula milk powder is generally prepared with cow milk as the base material and strengthened with various nutrients contained in breast milk. As the third largest natural component of breast milk, HMOs can affect the growth and metabolism of intestinal microorganisms and thus affect the intestinal immune system[27]. How to accurately and individually use HMOs and develop corresponding products for infants around the world has become an important problem in the industry.

In this review, we have focused on the classification and characteristics of HMOs, their impact on infant health, their intake mechanism and their development and utilization, aiming to provide data support and a theoretical reference for the development of more diversified baby formula milk powder and other breast milk substitutes produced from dairy products.

## 2 Classification and characteristics of HMOs

### 2.1 HMOs have a wide variety and rich structures

HMOs are the third largest solid component in breast milk after lactose and fat, they have a short-chain polymer structure of monosaccharides connected by 3–10 covalent bonds. Up to now, more than 200 kinds of HMOs have been confirmed[18,28], they are mainly composed of 5 basic monosaccharides: glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (SA)[29], but they have various structures and changes. However, almost all HMOs have lactose as the reducing end; under enzymatic action, the core structure is expanded by a $\beta$-1,3 or $\beta$-1,6-glycosidic bond connecting lacto-*N*-diose and *N*-acetyllactamine, an $\alpha$-1,2/$\alpha$-1,3/$\alpha$-1,4 glycosidic bond connecting Fuc residues, or $\alpha$-2,3 or $\alpha$-2,6-glycosidic linkage to SA residues[30–31] (Figure 2).

In addition, HMOs can be classified by their chemical charge into 2 categories, neutral HMOs and acidic HMOs. Neutral HMOs, mainly composed of charge-free monosaccharides (Glu, Gal, GlcNAc, and Fuc), can be further divided into fucosylated HMOs and non-fucosylated HMOs, accounting for 35%–50% and 42%–55%, respectively[32]. Acidic HMOs mainly refer to those carrying negatively charged residues of *N*-acetylneuraminic acid (Neu5Ac) and sialylated HMOs accounting for 12%–14%, and the most abundant of which are 6'-sialyllactose (6'-SL), 3'-SL, disialolacto-*N*-tetraose (DSLNT) and lactosyl-*N*-tetraose (LnT)[33].

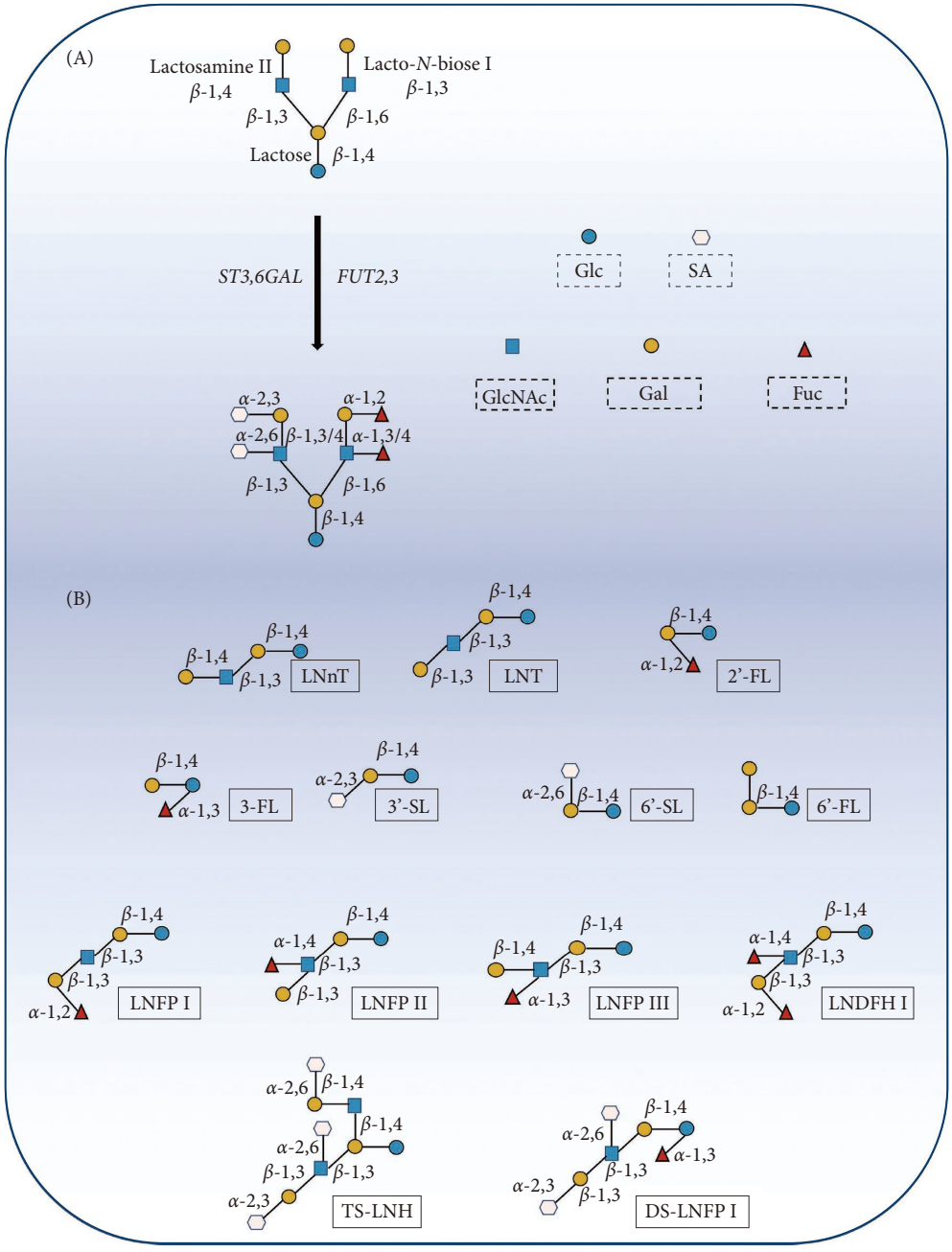


**Figure 2** (A) Diagrammatic sketch of the human milk oligosaccharides (HMOs) biosynthesis and (B) examples of HMOs structures.

## 2.2 HMOs in breast milk differ in type and content

The HMOs composition in breast milk is not constant, and the type and content of HMOs can be influenced by various factors such as regional differences, genetic differences and changes in the stage of lactation[34]. First, the concentration of HMOs also varies throughout breastfeeding depending on the stage of lactation, being 20–24 g/L in colostrum and 12–14 g/L in mature milk. Second, the composition of HMOs in breast milk is influenced by fucosyltransferase 2 (*FUT2*, *Se* gene) and fucosyltransferase 3 (*FUT3*, *Le* gene), which are strongly associated with the Lewis blood group[35]. Based on the expression of *FUT2* and *FUT3*, HMOs distribution can be divided into 4 groups: Lewis-positive secretory ($Se^+Le^+$), Lewis-positive non-secretory ($Se^-Le^+$), Lewis-negative secretory ($Se^+Le^-$) and Lewis-negative non-secretory ($Se^-Le^-$)[36]. The most abundant HMOs in secretory breast milk are fucosyllactose (FL) and lacto-*N*-fucopentaose (LNFP I), which are not present in non-secretory breast milk where *FUT2* is not expressed[37]. Further, as an example of the species distribution of HMOs, 78% of Chinese mothers' breast milk contains 2'-FL, while only 46% of Philippine breast milk does. The concentration of FL in the breast milk of Swedish mothers is 0.4 times higher than that in the breast milk of rural Gambian mothers; DSLNT in the breast milk of Swedish mother's ranges from 216 to 614 nmol/mL, while in rural Gambian mothers it varies from 668 to 870 nmol/mL[38]. Therefore, the selection of HMOs and their ratio to the base material in breast milk substitutes developed for infants from different countries should also be precise and individualized according to their distribution characteristics.

### 2.3 The relationship of structure and physiological function of HMOs

HMOs have rich structures, and their functions vary with changes in structure. For monomeric structured HMOs, as early as 1954, a research team defined HMOs containing GlcNAc as "bifidogenic factors", suggesting that they could promote the growth of *Bifidobacterium* and *Bacteroidetes*[39]. Newborn infants and young children, due to their immature immune system, have much lower immunity to pathogens than adults. Researchers have found that higher concentrations of non-3'-SL are associated with preventing the transmission of human immunodeficiency virus (HIV) after birth[40]. Oliveros et al.[41] found that supplementing 6'-SL could improve memory in rats. For complex structures, rotavirus is the main cause of severe dehydrated gastroenteritis in children under 5 years old. Laucirica et al.[42] found that specific 3 types of HMOs (2'-FL, 3'-SL, and 6'-SL) have anti-infective ability against 2 human rotavirus strains (G1P and G2P), and 2'-FL has a stronger inhibitory effect on G1P, while 3'-SL and 6'-SL have better inhibitory effects on G2P. Moreover, the mixture of 3'-SL and 6'-SL has stronger anti-infective ability compared to 3'-SL or 6'-SL alone. Facinelli et al.[43] used 2'-FL and 6'-SL as potential inhibitors and found that both HMOs significantly reduced the adhesion of *Escherichia coli*. In addition, specific combinations of HMOs (sialylate *N*-tetrasaccharides and 2'-FL) also have the potential to reduce the risk of necrotizing enterocolitis[44].

### 2.4 Separation and quantification of HMOs

#### *2.4.1 High performance liquid chromatography*

High performance liquid chromatography is a separation method that uses liquid as the mobile phase. It uses the difference in distribution coefficients between different components in the mobile phase and the stationary phase to achieve the separation of target components. Breast milk is a highly complex biological fluid, and there is significant interference in the analysis of HMOs in the breast milk matrix. At present, the main methods for separating HMOs include high performance anion exchange chromatography[45], porous graphitized carbon chromatography[46], reversed phase high performance liquid chromatography, and normal phase high performance liquid chromatography, supplemented by pulsed amperometric detector, ultraviolet (UV) detector, and fluorescence detector for detection.

#### *2.4.2 Mass spectrometry*

In recent years, the application of mass spectrometry platforms has made tremendous progress, and the combination of liquid phase and mass spectrometry has greatly facilitated the composition analysis and quantification of HMOs. Among them, time of flight-mass spectrometry and Fourier transform particle cyclotron resonance mass spectrometry are 2 commonly used high-resolution mass spectrometry platforms. Nijman et al.[45] used Nano-LC-chip/Q-TOF to detect 133 different structures of HMOs in breast milk samples.

#### *2.4.3 Capillary electrophoresis (CE)*

CE is a new type of liquid phase separation technology that uses capillaries as separation channels and high-voltage direct current electric fields as driving forces. CE has advantages such as low sample size requirements, fast and convenient operation, and high resolution, and is often used for the detection of sialylated HMOs[47]. Galeotti et al.[47] performed 2-aminoacridone fluorescence labeling on breast milk samples and detected derived HMOs using CE-UV. Although 2'-FL and 3'-FL were well separated in this study, some isomers still exhibited co-elution, such as LNFP II and LNFP III, as well as 3'-SL and 6'-SL.

#### *2.4.4 Nuclear magnetic resonance (NMR) method*

NMR could provide richer compound structural information (such as connection methods), and is mainly used for the structural analysis of HMOs. van Leeuwen[48] used one-dimensional $^{1}$H NRM to analyze the structure of HMOs purified from breast milk, which can quickly identify the Fuc connection mode in the sample, such as $\alpha$-1,2/3/4 was used to identify different genotypes (i.e. *Se* and *Le*) groups, and significant differences were found in the fucosylated epitopes within different genotypes.

## 3 Effect of HMOs on the healthy development of infants

As prebiotics, HMOs cannot be directly digested in the intestine, they need to reach the intestine smoothly and then be used by intestinal microorganisms, playing an irreplaceable important role in the healthy development of infants[49–51].

### 3.1 HMOs promote rapid brain development

The rapid development of the infant's brain benefits from nutrients such as HMOs and lipids, which are especially important for the neurodevelopment of the brain. The main source of SA in breast milk is sialylated HMOs, which are involved in processes such as production of brain gangliosides and modification of nerve cells. SA is an important component of gangliosides and neural cell adhesion molecules (NCAMs). Gangliosides can promote axonal elongation and regeneration, maintain axonal stability, and affect the interaction between axons and myelin sheaths[52]. The polymeric SA fragments of NCAMs can affect cell migration and neurite growth, enhance synaptic connectivity, promote neural growth and memory formation[53]. The SA in breast milk mainly exists in the form of SA functionalized oligosaccharides, and studies have shown that breastfeeding can increase the distribution of SA content in the brain of newborn pigs by about 10%[54]. In general, gangliosides in the brain increase rapidly in late pregnancy, while premature infants have relatively less storage of gangliosides in the brain, which is not conducive to neural development. In theory, breastfeeding can increase the storage of SA, gangliosides, and NCAMs in the brain of premature infants. SA-based gangliosides and polysialic acid-based glycoproteins are important components of brain tissue and play a major role in the mechanism of brain development[55]. Oliveros et al.[56] suggested that the intake of 2'-FL during lactation can affect neurodevelopment and cognitive performance. Studies on oral 2'-FL have confirmed that the cognitive abilities of infants and adults are increased during lactation. Therefore, it is important to conduct studies on the

cognitive development of infants ingesting 2'-FL in breast milk during lactation as soon as possible to accelerate the development of breast milk substitutes containing 2'-FL.

### 3.2 HMOs reduce virus transmission in infants

The antiviral mechanisms of HMOs are also diverse. Research has suggested that HMOs can enhance the T cell response and produce more balanced Th1/Th2 cytokines by promoting the maturation of the immune system[57]. Meanwhile, HMOs stimulate the immune response and maturation of epithelial cells to protect the host from viral infection. In addition, they can also affect the diversity and concentration of microorganisms and stimulate the growth of symbiotic bacteria. Based on their biological structure being similar to that of various cell surface carbohydrates, HMOs will mimic the surface glycans of epithelial cells and capture viruses that do not stick to cells. An HMOs-blocked viral lectin receptor cannot participate in the recognition of host cell surface glycoprotein, thus preventing its adhesion and colonization. Weichert et al.[58] and Etzold et al.[59] found that a mixture of sialylated HMOs can reduce selectin-mediated neutrophil rolling and adhesion, platelet-neutrophil complex formation and neutrophil activation *in vitro*. Monosialylated HMOs (3'-SL and 6'-SL) have been shown to reduce influenza infection in cell culture experiments. Bode et al.[40] and Kuhn et al.[60] found that breast feeding infants may reduce the transmission of HIV from mother to child. Rotaviruses (RVs) cause life-threatening diarrhoea in infants and children worldwide. Recent biochemical and epidemiological studies have underscored the importance of histo-blood group antigens (HBGAs) as both cell attachment and susceptibility factors for the globally dominant P[4], P[6] and P[8] genotypes of human RVs. Hu et al.[61] determined the crystal structures of P[4] and a neonate-specific P[6] VP8*s alone and in complex with H-type I HBGA, revealing a unique glycan binding site that is conserved in the globally dominant genotypes and allows for the binding of ABH HBGAs, consistent with their prevalence. Remarkably, the VP8* of P[6] RVs isolated from neonates displays subtle structural changes in this binding site that may restrict its ability to bind branched glycans. This provides a structural basis for the age-restricted tropism of some P[6] RVs as developmentally regulated unbranched glycans are more abundant in the neonatal gut (Figures 3A–D).

Morozov et al.[30] believed that the current discovery of anti-adhesion may be the mechanism of HMOs' anti-virus action. Simulating the structure of viral receptors could block adhesion to target cells and prevent infection. For example, HMOs structures are similar to those of carbohydrates on the surface of various cells and act as a soluble receptor trap for viruses (direct-acting antiviral drugs (DAAs)). As a host-acting antiviral drug, HMOs can stimulate the immune response and epithelial cell maturation (Figure 3E).

*In vitro* studies have shown that HMOs can resist Norwalk virus, RVs, influenza virus and HIV. Due to the diversity of HMOs structures, the complexity of their reaction with viruses and the barriers of separation and structural characterization technology, they have not yet been developed into antiviral drugs. However, in view of the clinical effect of HMOs added to infant formula, it is expected that their antiviral effect will be applied in clinical practice.

### 3.3 Prevention of necrotizing enterocolitis with HMOs

In neonates with necrotizing enterocolitis, there are usually 3 factors in the small intestine: persistent intestinal ischaemic damage, bacterial colonization and substrate in the intestinal cavity (such as in enteral feeding). Some studies have suggested that bacterial colonization and excessive neutrophil activity are 2 of the key characteristics of the pathogenesis of necrotizing enterocolitis. Through clinical research, researchers have found that from the perspective of the incidence rate of necrotizing enterocolitis in infants, the risk ratio of formula-fed infants is 6–10 times higher than that of breast-fed infants[62]. Autran et al.[44] then analysed individual studies of mothers and infants, and found that the content of bisial-*N*-tetraose in the breast milk of children with necrotizing enterocolitis was lower than that in control groups. Henrick et al.[63] believed that there is an orderly pattern of immune spectrum changes after birth, such as the expansion of mucosal-specific T cells in response to microbial colonization. A lack of bifidobacteria and HMOs utilization genes is related to systemic inflammation and immune imbalance in early life. Supplementation with *Bifidobacterium infantis* EVC001 (expressing all HMOs utilization genes) can reduce intestinal inflammation and intestinal Th2 and Th17 cytokines and upregulate interferon (IFN)-β in breast-fed infants. A faecal suspension from these infants can make T cells polarize to Th1 and Treg cells, and the metabolite indole-3-lactic acid, abundant in faeces, inhibits the activation of Th2 and Th17 by upregulating the expression of galactin 1, which is closely related to enteritis (Figure 4A). Zhao et al.[64] found that oral pretreatment of mice with 2'-FL can inhibit the weight loss caused by intraperitoneal injection of 5-fluorouracil (5-FU) and reduce the intestinal inflammation score, proinflammatory cytokine production, villus shortening, epithelial cell apoptosis, goblet cell loss and destruction of tight junction complex. However, simultaneous administration of 2'-FL and 5-FU improve intestinal mucositis relatively little (Figure 4B).

### 3.4 HMOs affect immune development

The immune system of neonates is different from that of adults, and it is not yet mature. During the first 6 months of life, whether a baby is breast-fed or not will affect the development of their subsequent immune system. There is evidence that exclusive breastfeeding can reduce the incidence rate of many diseases, such as asthma, allergy, inflammatory bowel disease, type I diabetes, celiac disease and leukemia[65]. HMOs can regulate the production of lymphocyte cytokines, which may mediate a more balanced Th1/Th2 response. In addition, HMOs can also reduce selectin-mediated intercellular interaction in the immune system, reduce the rolling of leukocytes on activated endothelial cells, which may lead to the reduction of mucosal leukocyte infiltration and activation, thus playing an immune role[66]. Goehring et al.[67] conducted a prospective, randomized, controlled growth and tolerance study in 210 healthy full-term infants. At the age of 6 weeks, blood samples of the subjects were collected to detect 10 kinds of cytokines. The results showed that the blood concentrations of the interleukins (IL-1Ra, IL-1α, IL-1β, IL-6) and tumor necrosis factor α (TNF-α) in the control group were significantly higher than those in the breast milk and 2'-FL formula groups. There was no significant difference in the 10 inflammatory factors between the 2'-FL formula group and breast-fed infants.

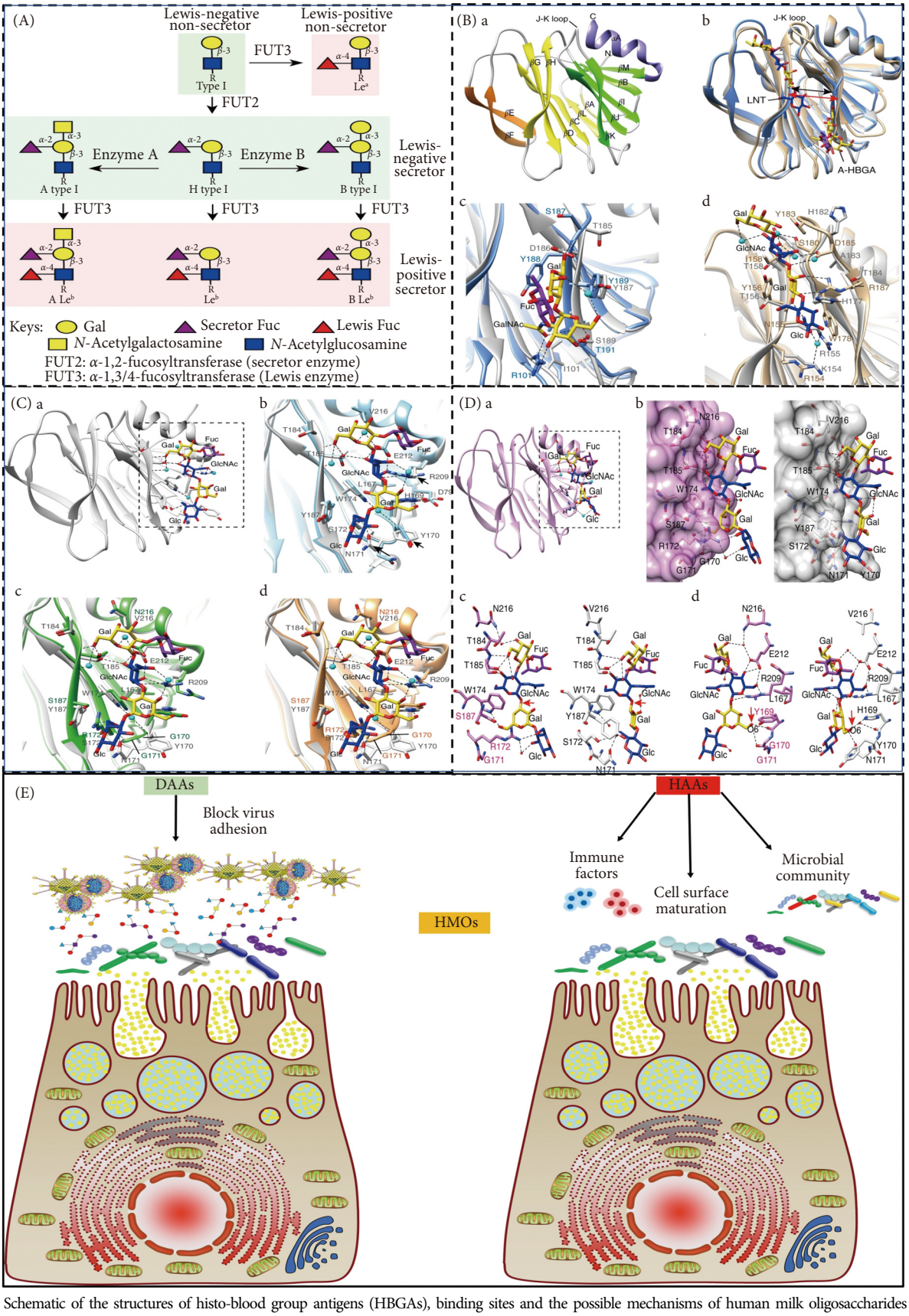


**Figure 3** Schematic of the structures of histo-blood group antigens (HBGAs), binding sites and the possible mechanisms of human milk oligosaccharides (HMOs) antiviral protection. (A) The structures of HBGAs[61]; (B) VP8* structure of a neonatal human rotavirus strain P[6] RV3 and structural comparison with VP8*s of other HRVs in complex with HBGAs[61]; (C) Structure of P[6] RV3 VP8* in complex with H type I pentasaccharide[61]; (D) Structure of P[4] Indian VP8* in complex with H type I pentasaccharide[61]; (E) Prediction of antiviral mechanism of HMOs; DAAs, direct-acting antiviral drugs; HAAs, host acting antivirals.

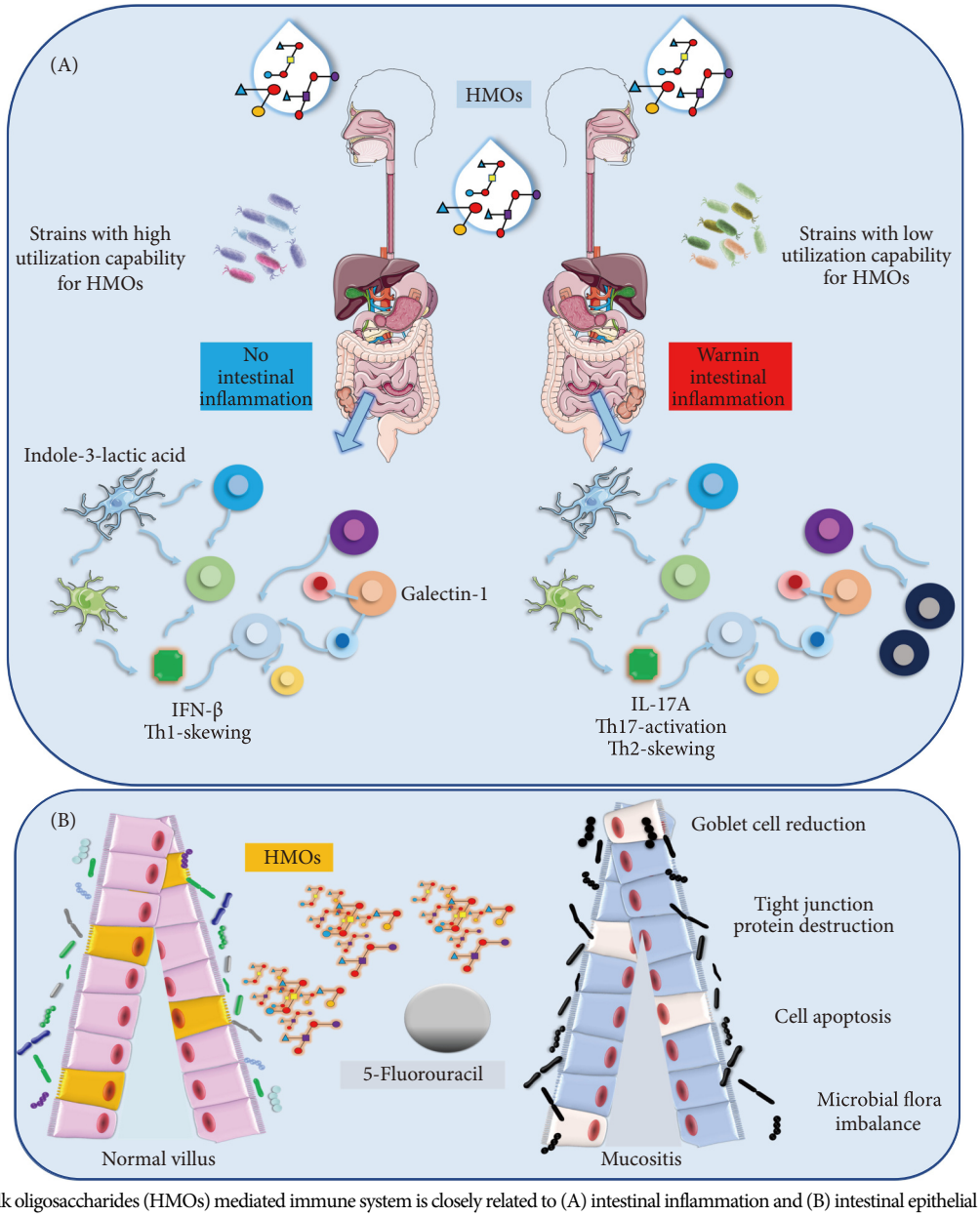


**Figure 4** Human milk oligosaccharides (HMOs) mediated immune system is closely related to (A) intestinal inflammation and (B) intestinal epithelial cells.

## 4 Utilization and uptake mechanism of HMOs

Several studies have reported that different HMOs are explored and mined in different living organisms[19,68]. Targeted studies in humans, animals, cells, pathogens and other aspects are shown in Table 1. More and more evidence supports the role of early colonization of the gastrointestinal microbial ecosystem in health, especially its interaction mechanism with different HMOs.

### 4.1 HMOs enhance the metabolism of short-chain fatty acids (SCFAs) and improve intestinal microorganism structure

SCFAs are an important energy source of intestinal cells and a key molecule in maintaining intestinal health[69]. In the intestine, HMOs can indirectly increase the production of SCFAs, and these increased levels are mediated by bifidobacteria, the main product of which is acetic acid which can reduce the pH value of the intestine, have a bacteriostatic effect and inhibit the growth of pathogenic bacteria. In addition to acetic acid, fermentation products also include butyric acid and propionic acid. In their latest research, Button et al.[70] explored the implantation of live biological therapy products into the human intestinal microbiota in a predictable and sustainable way as a promising method to regulate the human intestinal microbiota. The matching of infant intestinal microbiota *Bifidobacterium longum* subspecies and HMOs improved butyric acid levels in both *in vivo* and *in vitro* models. Pichler et al.[71] believed that HMOs utilization sites are diverse and ubiquitous in *Roseburia*. In addition, HMOs catabolism is upregulated during co-culture with *Akkermansia muciniphila* bacteria that degrade mucin, indicating that this pathway plays an additional role in promoting cross-feeding and obtaining mucin *O*-glycans. This proves that the catabolism of HMOs by butyrate-producing *Clostridium* bacteria may help to improve the competitive advantage of this flora in the process of microbial maturation induced by weaning (Figure 5A).

Borewicz et al.[72] believed that the ability of infants to metabolize different HMOs is closely related to the composition of the faecal

**Table 1** Studies on human milk oligosaccharides (HMOs) in different living organisms.

| Classification of living organisms | Living organisms | HMOs | Key findings | References |
|---|---|---|---|---|
| Human beings | Human | LNnT | Promoted the growth of beneficial microorganisms | [96] |
| Human beings | Human | HMOs | Affected gut microbiota | [97] |
| Human beings | Human | 2'-FL/LNnT | Positive regulation of intestinal microbiota profiles | [98] |
| Human beings | Human | HMOs | Associated with protection against postnatal HIV transmission | [99] |
| Human beings | Human | 2'-FL | Changes of inflammatory factors in infants | [67] |
| Human beings | Human | HMOs | Related to infants necrotising enterocolitis (NEC) | [44] |
| Human beings | Human | 2'-FL | Related to infants' cognitive ability | [100] |
| Animals | Pig | HMOs | Related to systemic and gastrointestinal immunity | [101] |
| Animals | Pig | 2'-FL | HMOs not affect NEC incidence | [102] |
| Animals | Pig | Sialic acid | Related to learning and memory levels | [103] |
| Animals | Pig | 3'-SL/6'-SL | Related to brain' ganglioside Sia | [54] |
| Animals | Pig/mouse | 2'-FL/6'-SL | Affected NEC in part via TLR4 pathway | [104] |
| Animals | Mouse | LNnT | The relationship of LNnT and *Bifidobacteria* | [105] |
| Animals | Mouse | 2'-FL | Inhibited colonization and adherence ability of *Campylobacter jejuni* | [106] |
| Animals | Mouse | HMOs | Related to immune responses of Th1/Th17 | [107] |
| Animals | Mouse | 2'-FL | Promoted immune cell differentiation level | [108] |
| Animals | Mouse | 2'-FL/6'-SL | Regulated immunity level of IL-10$^+$ T regulatory/mast cells | [109] |
| Animals | Mouse | 2'-FL | Protected against NEC via eNOS pathway | [110] |
| Animals | Mouse | HMOs | Prevented NEC via goblet cell and mucin level | [111] |
| Animals | Rat | Pooled HMOs | Related to survival of NEC | [112] |
| Animals | Rat | 2'-FL | Related to NEC pathology | [113] |
| Animals | Rat | 2'-FL | Related to cognitive and memory ability | [56] |
| Animals | Rat | 2'-FL | Related to cognitive and memory ability via gut-brain axis | [114] |
| Animals | Rat | 2'-FL/3'-SL | Promoted the growth of the strain *Bifidobacterium* spp. by the 2'-FL and 3'-SL | [115] |
| Cells | HT-29 cells | 3'-SL/6'-SL | Promoted adhesion of *Bifidobacterium* | [116] |
| Cells | Caco-2 cells | 2'-FL | Promoted adhesion of *B.* bifidum | [117] |
| Cells | Cells | 3'-SL/6'-SL, 2'-FL | Affected proliferation/epithelial differentiation | [118] |
| Cells | Cells | 2'-FL/3-FL | Affected glycocalyx development | [119] |
| Cells | Cells | 2'-FL/3-FL, LNT2 | Affected goblet cells gene expression level | [120] |
| Cells | Cells | Acidic HMOs | Acidic HMOs can stimulate an Affected the Th1/Th2 type cytokine level | [121] |
| Cells | Cells | 2'-FL | Promoted the level of regulatory Th1 response | [122] |
| Pathogen | Norwalk virus | HMOs | Related to the Norwalk virus | [123] |
| Pathogen | *Campylobacter jejuni* | 2'-FL | Related to the *Campylobacter jejuni* | [124] |
| Pathogen | *Vibrio cholera* | HMOs | Related to the *Vibrio cholera* | [125] |
| Pathogen | *Streptococcus pneumoniae* | Sialyllactose | Related to the Norwalk *Streptococcus pneumoniae* | [126] |
| Pathogen | *Helicobacter pylori* | Sialyllactose | Related to the *Helicobacter pylori* | [127] |
| Pathogen | HIV-1 | HMOs | Related to the HIV-1 | [128] |
| Pathogen | Norovirus | 2'-FL/LNFP I | Related to the Norovirus | [129–130] |
| Pathogen | Rotavirus | LNT/LNnT | Related to the Rotavirus | [131–132] |
| Pathogen | Influenza H1N1 | Sialyllactose | Related to the influenza H1N1 | [133] |

microbiota, especially to the phylogenetic types of *Bifidobacterium*, *Bacteroides* and *Lactobacillus*. Bidart et al.[73] found that the lactose operon from *Lactobacillus casei* is involved in the transport and metabolism of the HMOs core-2 *N*-acetyllactosamine. Yamada et al.[74] believed that breast-fed infants usually have a flora rich in bifidobacteria, the growth of which is selectively promoted by HMOs. Meanwhile, they determined the crystal structure of the catalytic domain of the enzyme (LnbX) possessed by *B. longum* and found that LnbX is essential for *B. longum* to grow by using lacto-*N*-tetrasaccharide, which is also a key genetic factor for the persistence of *B. longum* in the intestinal tract of breast-fed infants (Figure 5B). Asakuma et al.[75] cultured *B. longum* subsp. *infantis* JCM1222 with HMOs as the only carbohydrate source; they found that the number of this strain increased significantly and it could completely consume HMOs, including their degraded monosaccharide and disaccharide products. However, cultured *B. bifidum* JCM1255 grew slowly and could not fully utilize the monosaccharide degradation products of HMOs. In contrast, *B. longum* subsp. *longum* JCM1217 and *B. breve* JCM1192 were difficult to grow. These 2 strains can only metabolize LNT and cannot metabolize

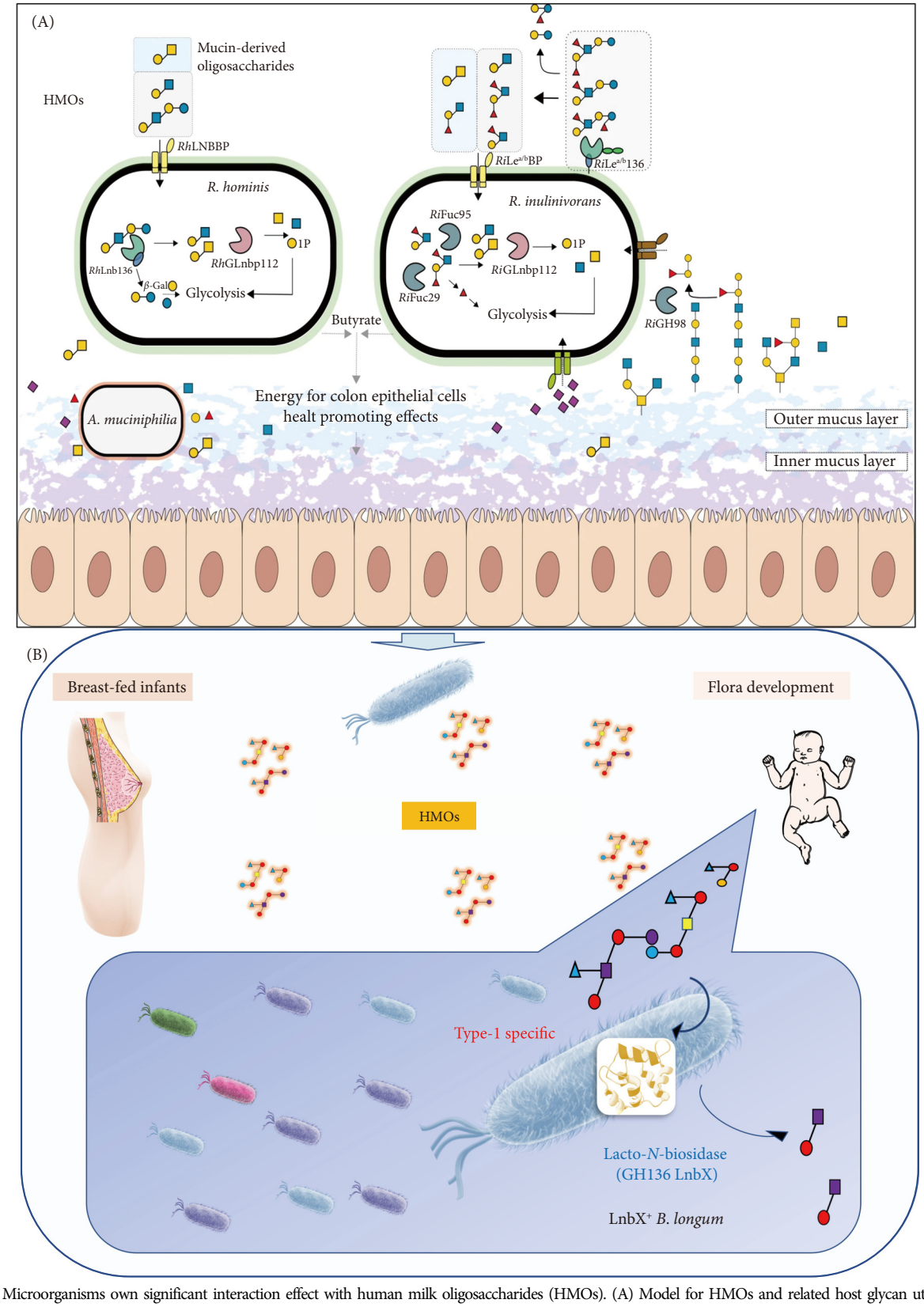


**Figure 5** Microorganisms own significant interaction effect with human milk oligosaccharides (HMOs). (A) Model for HMOs and related host glycan utilization by *Roseburia* and other Lachnospiraceae[71]; (B) LnbX is the key molecule in the coevolution of breast-fed infants and *Bifidobacteria*.

LNnT. Wang et al.[76] found that the composition of the intestinal flora of infants was different in groups fed breast milk and infant powder. Although *Bifidobacterium* spp. were the main bacteria, the proportion of *Bacteroides* in the intestinal flora of infant powder-fed group was high, while the proportions of *Clostridium* XVIII cluster, *Trichospirillum*, *Streptococcus* and *Enterococcus* were low.

de Leoz et al.[77] found that the intestinal flora of breast-fed infants gradually changes from not consuming HMOs to consuming HMOs within a few weeks of birth. During this period, the proportion of *Bacteroides* and *Bifidobacterium* increases, and the proportion of Enterobacteriaceae and *Staphylococcus* decreases. It can be seen that the mechanism of the interaction between HMOs and intestinal flora needs to be further explored.

### 4.2 HMOs enhance the amino acid metabolism of intestinal microorganisms

For the growth and development of infants, there is also an urgent need for all kinds of amino acids, including essential amino acids such as phenylalanine and tryptophan, and non-essential amino acids such as tyrosine. As the best protein source, breast milk can effectively meet the needs of infant growth. Amino acids are closely related to histogenesis, organogenesis and the development and function of the immune system. Among them, intestinal immune function is an indispensable part of intestinal mucosal barrier function. Amino acids can be used as the energy and raw material sources of intestinal mucosal metabolism, effectively maintaining the integrity of intestinal structure and normal development of the immune system[78]. Among these amino acids, it is particularly important that aromatic amino acids (phenylalanine, tryptophan and tyrosine) can be metabolized and transformed by intestinal microorganisms such as bifidobacteria to form aromatic lactic acids such as indole lactic acid, phenyllactic acid and 4-hydroxyphenyllactic acid, which play a role in regulating the intestinal immune response. In addition, the metabolites of tryptophan formed by the indoleamine 2,3-dioxygenase pathway and tetrahydrobiopterin-dependent tryptophan hydroxylase decomposition pathway can affect the expression of T cell surface receptors and the growth of B cells and natural killer cells[79]. It can be seen that various nutrients including aromatic amino acids in breast milk play an extremely important role in the growth and development of infants. The multiple metabolism mechanisms involving intestinal microorganisms have been found to have an important impact on the regulation of mechanisms including the development of the intestinal immune system, especially those involving aromatic amino acid metabolites. For example, indole-3-propionic acid can bind to the pregnane X receptor and play a role in strengthening the intestinal barrier, while indole-3-formaldehyde, indole-3-acetic acid, indole-3-propionic acid and indole-3-acetaldehyde, ligands of aromatic hydrocarbon receptors, can effectively participate in regulation of the early intestinal immune response[80]. However, the relevant metabolic studies mainly focus on Bacteroides and Firmicutes, and the complete aromatic amino acid metabolic pathways and products have only been determined for a small number of intestinal microorganisms. For example, *L*-tryptophan generates indole-3-lactic acid under the action of *Lactobacillus*, *Bifidobacterium* and *Bacteroides*, and *L*-phenylalanine and *L*-tyrosine generate phenyllactic acid and 4-hydroxyphenyllactic acid under the action of *Lactobacillus*[81]. The latest research has found that in the feces of breast-fed infants, the aromatic lactic acid content is positively correlated with the abundance of bifidobacteria that can utilize HMOs[82], and bifidobacteria can efficiently convert aromatic amino acids into aromatic lactic acids (such as indole lactic acid) by using aromatic lactate dehydrogenase (ALDH), while indole lactic acid has antifungal, anti-*E. coli* and anti-*Bacillus cereus* functions[83]. More importantly, indoleacetic acid is closely related to the activation of aryl hydrocarbon receptors that control the intestinal immune response, and regulates the *in vitro* immune response of $CD4^+$ T cells and monocytes in a dose-dependent manner, which plays an important role in the development of the infant intestinal immune system[82]. In a previous study, our research team found that a strain of *Bifidobacterium* F1-7 can metabolize tryptophan and synthesize related metabolites, proving that it has good potential to metabolize aromatic amino acids[84]. However, ALDH is a new aromatic amino acid-metabolizing enzyme that has never been recognized or reported. Whether and how HMOs regulates the expression of this enzyme in infant intestinal microorganisms in various countries is still unknown. The important promoting effect of HMOs on the above metabolic processes of intestinal microorganisms such as *Bifidobacterium* should also be the focus of subsequent research.

### 4.3 Conventional mechanism of HMOs ingestion by intestinal microorganisms

With the development of microbial genome research in recent years, it has become possible to systematically elucidate the physiological and metabolic mechanisms of intestinal microorganisms at the molecular level. Two ways of utilizing HMOs (intracellular and extracellular digestion) have evolved for infant intestinal-associated *Bifidobacterium*. *B. bifidum* and LnbX (an important HMOs-degrading enzyme)-positive *B. longum* secrete an extracellular glycosidase to degrade extracellular HMOs, and then the released monosaccharides or disaccharides are introduced into cells for further degradation[85–86]. *Bifidobacterium* has another way to dominate the ecosystem, that is, species/strains can use HMOs cooperatively[85]. Tannock et al.[87] studied the relationship between the presence and dominance of *Bifidobacterium* spp. in infants' intestines. They found that when *B. bifidum* accounted for more than 10% of bifidobacteria, the abundance of *Bifidobacterium* spp. in the corresponding intestinal microbiota was higher. This result showed that there was cross-feeding of HMOs degradation products in the *Bifidobacterium* group, and HMOs degradation products produced by bifidobacteria can be shared among different kinds of bifidobacteria, thus promoting the growth of other *Bifidobacterium* species. Recent studies have confirmed that *Bacteroides* may also dominate the intestinal microorganisms of some infants. As the main fermentation bacteria in the microbiota, *Bacteroides* spp. have the ability to use a variety of HMOs from the intestinal environment[88]. This special glycolytic ability can be attributed to the special mechanism of polysaccharide utilization site coding in the *Bacteroides* genome, each one of which seems to be responsible for sensing and acquiring different kinds of oligosaccharides or polysaccharides[89].

### 4.4 Special transport channel mechanism of HMOs ingestion by intestinal microorganisms

Studies have continued to confirm that there are special transport channels used in the intake of HMOs (Figure 6).

Among them, the ATP-binding cassette (ABC) transporter channel has become the current research focus. The ABC transporter system is a large class of transmembrane proteins which have an ATP-binding domain. It relies on the hydrolysis of ATP to provide energy for the transport of specific substrates and carries out the transmembrane transport of multiple substrates by active transport. The genome of intestinal microorganisms encodes a

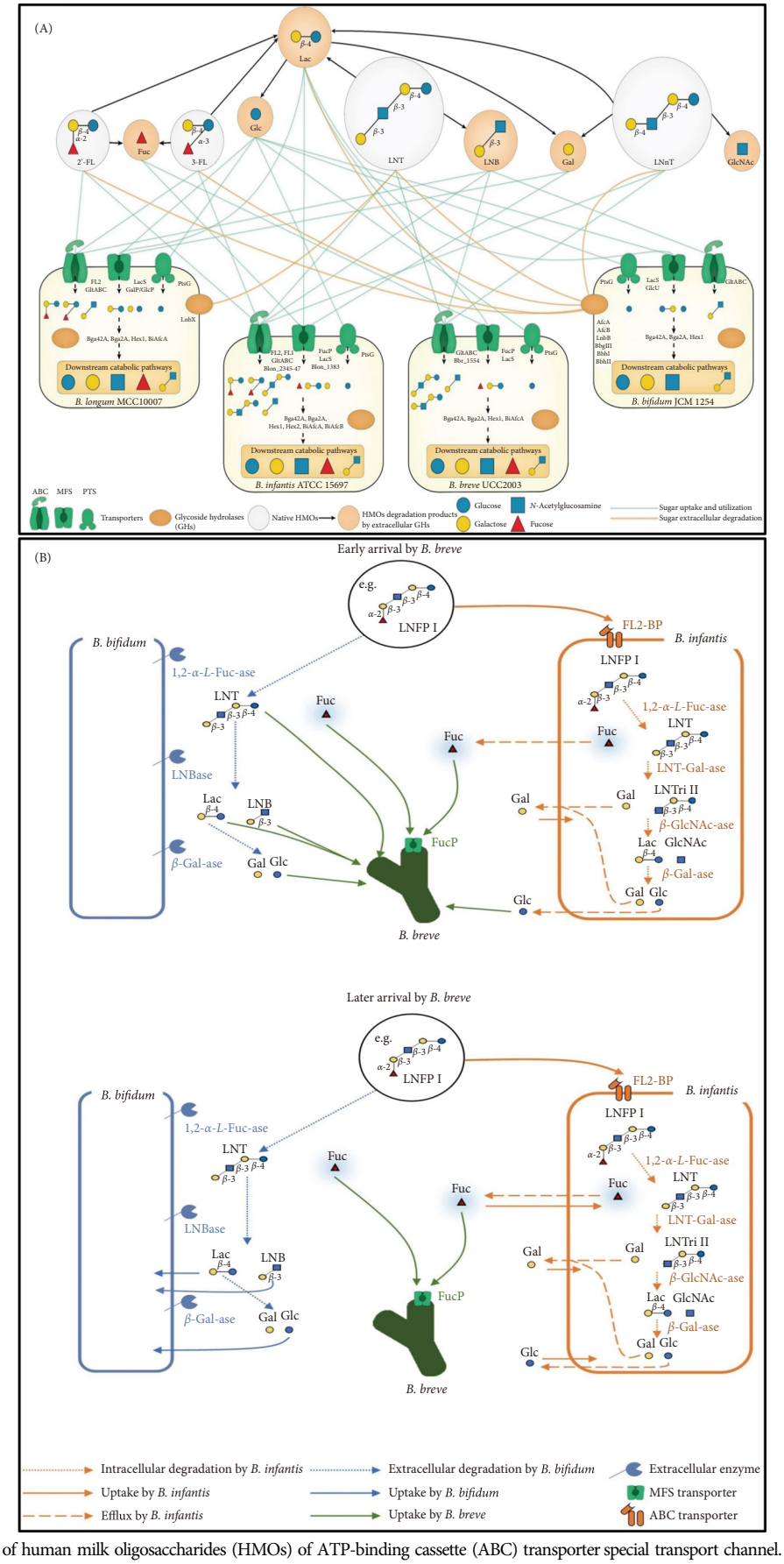


**Figure 6** Utilization mode of human milk oligosaccharides (HMOs) of ATP-binding cassette (ABC) transporter special transport channel. (A) Schematic summary of predicted HMOs-utilization phenotypes of each bifidobacterial strain[92]; (B) Predicted HMOs-utilization pathways that enable *B. breve* to benefit from priority effects[92].

series of ABC channels that can recognize and transport oligosaccharides. One type of ABC channel plays an important role in the intake of oligosaccharides by intestinal microorganisms: oligosaccharide substrates are recognized by substrate-binding proteins (SBPs) and then transferred into cells[90]. As a kind of SBPs (including Bl6GBP, BlMnBP and BlAXBP), oligosaccharide-binding proteins (OBPs) have important structural commonness: they are composed of 2 structural domains, $\alpha$ and $\beta$. When it is not combined with its substrate, an OBP will display more flexible features, meaning $\alpha$ and $\beta$ domains can rotate freely near the hinge region, so that its conformation exists in an 'open' state. When binding with the substrate, the protein conformation exists in the 'off' state. At this time, the oligosaccharide is recognized as the substrate and utilized by the contact surface. This process is called the 'fly trap' mechanism[91]. Ojima et al.[92] revealed the assembly characteristics of *B. infantis* under the condition of HMOs as substrate through genome prediction and an *in vitro* culture test. Among them, the preferential effect contributes greatly to the assembly of the community. *B. bifidum* and *B. infantis* benefit strongly from the presence of HMOs. *B. brevis* can monopolize fucose (the downstream product of HMOs) for proliferation. Faecal metagenomic data from breast-fed infants also showed that the abundance of *B. brevis* is high. Therefore, it is particularly important to explore the key OBPs involved in recognition and utilization of HMOs by intestinal microorganisms to reveal the mechanism by which HMOs regulate the growth of intestinal microorganisms such as *Bifidobacterium* and aromatic amino acid metabolism.

## 5 Development and prospects of HMOs

The known HMOs that can be industrialized include 2'-FL, lacto-*N*-neotetraose, lacto-*N*-tetraose, 3'-FL, 3'-SL, 6'-SL, etc. In 2017, 2'-FL and lacto-*N*-neotetraose were approved by the European Union for use in infant formula. Dairy companies such as Nestlé and Abbott have launched infant formulas and follow-on formulas with milk oligosaccharides added. At present, products containing HMOs have been marketed in more than 45 countries and regions. According to the composition of oligosaccharides in European breast milk, the Experts Committee of the European Food Safety Authority stipulated that the concentration of 2'-FL added should not be higher than 1.2 g/L. *Food Standards Australia New Zealand* stipulated that the concentration of 2'-FL added shall not exceed 2.4 g/L. Because of the uniqueness of HMOs, it is impossible to extract a large amount from breast milk for research and application, so it is important to develop more extraction methods. At present, the options for synthesizing HMOs include chemical, enzymatic, chemical enzymatic, genetically engineered bacteria-coupled fermentation and whole-cell synthesis methods[93–94]. HMOs are abundant carbohydrates fundamental to infant health and development. Although these oligosaccharides were discovered more than half a century ago, their biosynthesis in the mammary gland remains largely uncharacterized. Kellman et al.[95] used a systems biology framework that integrates glycan and RNA expression data to construct a biosynthetic HMOs network and predict the glycosyltransferases involved. The US Food and Drug Administration, the European Commission, the Australian and New Zealand Food Standards Agency have allowed 2'-FL and lactose-*N*-neotetraose to be used in food categories such as infant formula. As a food nutritional enhancer, 2'-FL can be used in children's formula milk powder, infant formula food, and special medical infant formula food, with a usage amount of 0.7–2.4 g/L (calculated as pure product). The usage range of lactose-*N*-neotetraose is the same, with a usage amount of 0.2–0.6 g/L (calculated as pure product). In 2023, 2 kinds of HMOs (active nutrient breast milk oligosaccharides) raw materials, namely 2'-FL and LNnT, were officially approved for use in domestically produced milk powder products in China. Within the foreseeable future, the addition of synthetic HMOs to infant formula will become a hot field of development.

In brief, there are various types of HMOs and they have various functions. They have different specificity for different diseases and strains, but those associated with *Bifidobacterium* have the greatest application potential. With the development of synthetic HMOs technology, the research and development and popularization of infant formula milk powder containing HMOs has become the major trend of infant formula research and design. Influenced by many factors, the distribution and content of HMOs are quite different in different mothers, so it is necessary to make targeted selection. It is of great significance to accelerate the establishment of an HMOs database suitable for infants in various countries, create precise and personalized milk nutritional formulas for infants in various countries and accelerate the development of independent infant milk substitutes.

## Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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