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Unlocking the mysteries of milk oligosaccharides: Structure, metabolism, and function

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ABSTRACT

Milk oligosaccharides (MOs), complex carbohydrates prevalent in human breast milk, play a vital role in infant nutrition. Serving as prebiotics, they inhibit pathogen adherence, modulate the immune system, and support newborn brain development. Notably, MOs demonstrate significant variations in concentration and composition, both across different species and within the same species. These characteristics of MOs lead to several compelling questions: (i) What distinct beneficial functions do MOs offer and how do the functions vary along with their structural differences? (ii) In what ways do MOs in human milk differ from those in other mammals, and what factors drive these unique profiles? (iii) What are the emerging applications of MOs, particularly in the context of their incorporation into infant formula? This review delves into the structural characteristics, quantification methods, and species-specific concentration differences of MOs. It highlights the critical role of human MOs in infant growth and their potential applications, providing substantial evidence to enhance infant health and development.

1. MOs structure and composition

1.1. The finding of MOs

The discovery of milk oligosaccharides (MOs) traces back to early 20th-century investigations by microbiologists and chemists into the health benefits of human milk. In 1900, Moro independently noted different bacterial compositions in the feces of breastfed infants compared to those fed formula. This observation was complemented in 1926 by Schönfeld et al., who identified a key growth factor for *Bifidobacterium bifidum* in human milk, a factor scarcely present in cow's milk. Meanwhile, chemists Polonowski and Lespagnol discovered a mysterious carbohydrate fraction in human milk, termed "gynolactose," in 1929. It wasn't until 1954 that these compounds were isolated into individual MOs by Polonowski and Montreuil. The functional significance of these oligosaccharides, however, remained unclear until Görgy et al. (1954) linked them to the earlier bacterial studies by Moro (1900). They demonstrated that MOs were vital for the growth of *Lactobacillus bifidus*, influencing the bacterial composition in infants' feces.

As research progressed, it became evident that MOs constitute a diverse array of carbohydrates, ranking as the third most crucial nutritional component in human breast milk. Furthermore, they confer numerous beneficial effects on children, including promoting brain development, facilitating immune system maturation, and modulating

Abbreviations: 2′-FL, 2′-fucosyllactose; 2-AA, 2-aminobenzoic acid; 2-AMAC, 2-aminoacridone; 3′-FL, 3′-fucosyllactose; 3′-SL, 3′-sialyllactose; 3-GSL, 3′-galactosyllactose; 6′-SL, 6′-sialyllactose; CE, capillary electrophoresis; ESI, electrospray ionization; FOS, fructooligosaccharide; Fuc, fucose; FUT, fucosyltransferase; Gal, galactos; GCB, graphitized carbon column; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GOS, galactooligosaccharide; HILIC, hydrophilic interaction chromatography; HMOs, human milk oligosaccharides; HPAEC, high pH anion-exchange chromatography; HPLC, high performance liquid chromatography; LC, liquid chromatography; Le, Lewis gene; LNB, lacto-N-biose; LNDFH I, lacto-N-difuco-hexaose I; LNDFH II, lacto-N-difuco-hexaose II; LNFP I, lacto-N-fucopentaose I; LNFP II, lacto-N-fucopentaose II; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; LPS, lipopolysaccharide; MALDI, matrix assisted laser desorption/ionization; MOs, milk oligosaccharides; MRM, multi-reaction monitoring; MS, mass spectrometry; MUC2, mucin 2; Neu5Ac, *N*-acetylneuraminic acid; NeuAc, sialic acid; NMR, nuclear magnetic resonance; PAD, photodiode array detector; PGC, porous graphitized carbon; RPLC, reversed-phase LC; Se, secretor gene; SEC, size exclusion chromatography; TLRs, toll-like receptors; UDP-Gal, uridine diphosphate-Gal; UV, ultraviolet.

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gut microbiota composition. These effects will be succinctly elucidated in the ensuing sections.

1.2. The structures of MOs

In conjunction with lactose, the carbohydrate constituents or elements present in milk encompass glycoconjugates and independent MOs.

Regarding glycoconjugates, protein glycans are categorized into three fundamental groups based on their localization and function: N-linked, O-linked, and proteoglycans (Lyons et al., 2015; Schnaar, 2015). Specifically, a subset of asparagine (ASP) residues in the Asn-X-Ser/Thr motif is associated with N-glycans, while certain serine and threonine residues are linked to O-glycans (Yan & Lennarz, 2005). Proteoglycans are also linked to serine or threonine residues, but through long disaccharide repeats (Esko & Selleck, 2002). In glycolipids, glycans are attached to a ceramide lipid moiety (Schnaar, 2015). Collectively, these glycans form diverse cell surface structures with varying functions. Glycoconjugates are known for their vital role in regulating immunological responses and in diseases (Freeze et al., 2014), although this topic is not explored further here.

As for MOs, they are composed of a distinctive combination of monosaccharide building blocks, including glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid (NeuAc) (Chaturvedi et al., 2001). Broadly speaking, lactose, composed of Gal and Glc, serves as the fundamental unit for MOs. This lactose unit is then elongated in either a linear or branched manner through the addition of varying numbers of Galβ1–3GlcNAc (lacto-N-biose, type I chain) or Galβ1–4GlcNAc units (*N*-acetyllactosamine, type II chain) (Bode, 2012). The addition of lacto-N-biose terminates further extension of the chain, whereas *N*-acetyllactosamine permits continued elongation (Fig. 1). The incorporation of disaccharides into the chain through β1–3 or β1–6 bonds results in the formation of linear or branched chains. These chains are further modified with Fuc and NeuAc residues. This variety of linkages, branching patterns, and decorative residues contribute to the diverse structural landscape of MOs. To date, over 200 structures of human milk oligosaccharides (HMOs) have been identified (Leo et al., 2009; Ruhaak & Lebrilla, 2012).

1.3. The classification of MOs

The structural diversity of MOs arises from their various linkages—sialylation in a2-3 or a2-6 and fucosylation in α1-2, α1-3, or α1-4—leading to distinct MOs groups, namely sialylated, fucosylated, and non-fucosylated neutral MOs (Totten et al., 2012).

Focusing on the fucosylated MOs subset, these structures carry at least one fucose (Fuc) residue, with 2′-fucosyllactose (2′-FL) and 3′-fucosyllactose (3′-FL) being prominent representatives (Fig. 2). Notably, approximately 70 %–80 % of MOs in human milk are fucosylated, characterized by the presence of at least one *N*-acetylneuraminic acid (NeuAc) residue (Ninonuevo et al., 2006). Among the sialylated MOs, 3′-sialyllactose (3′-SL) and 6′-sialyllactose (6′-SL) are the most abundant (Fig. 2). Conversely, animal milk predominantly features sialylated MOs. Additionally, there are non-fucosylated neutral MOs, such as lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT), which lack Fuc or NeuAc residues. The diverse chemical properties of these MO types suggest potentially distinct functional roles, which will be comprehensively outlined in the following section.

2. Beneficial effects of MOs

MOs exhibit resistance to digestion. In breastfed infants, over 95 % of MOs remain intact as they pass through the gastrointestinal tract, eventually reaching the intestine where they play a crucial role in modulating the microbiota population (Goehring et al., 2014). Approximately 1 % of digested MOs are absorbed into the bloodstream, enabling them to extend their beneficial effects to various target organs (Bode, 2015). Presently, a growing body of evidence highlights the multifaceted mechanisms through which these benefits are conferred.

1. MOs influence the composition of gut microbiota by selectively promoting the proliferation of beneficial microorganisms while inhibiting the growth of pathogens.
2. Direct interactions between MOs and glycan-binding proteins contribute to gut maturation and strengthen the intestinal barrier.
3. MOs play a role in shaping the responses and development of the immune system, thereby enhancing overall immune functionality.
4. MOs provide essential components that contribute to brain development.

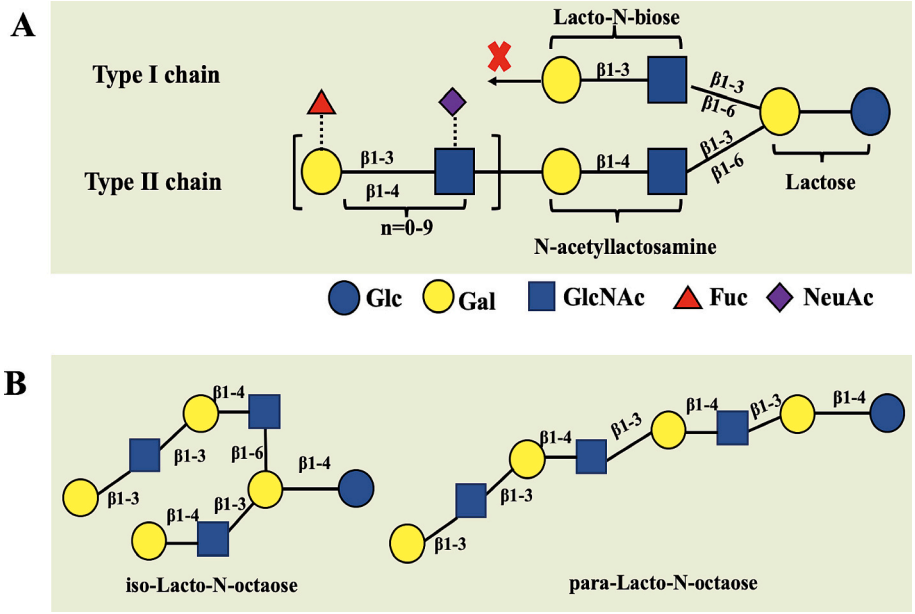

Fig. 1. Basic MO units and structure. A, The structural blueprint of MOs; B, an example of structural isomers generated by different linkages.

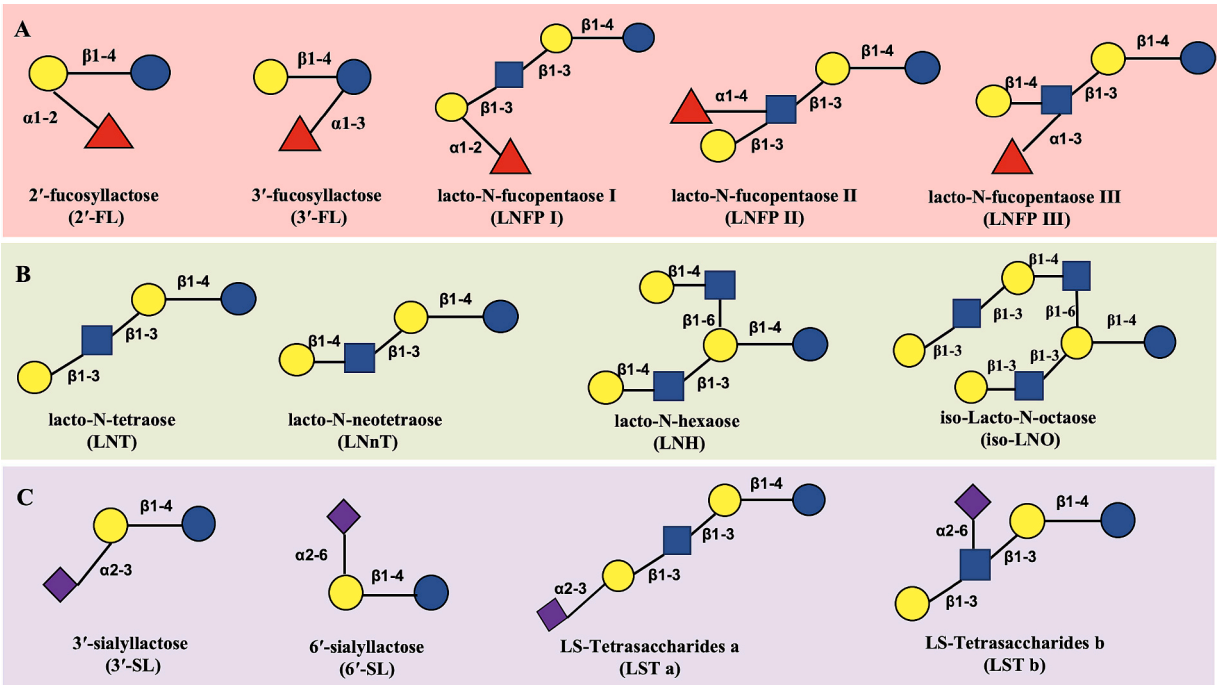

Fig. 2. Three classifications of MOs. A, Representative fucosylated neutral MOs; B, representative non-fucosylated neutral MOs; C, representative sialylated MOs.

An overview of the collective beneficial functions of MOs present in breast milk is summarized in Fig. 3, with more detailed descriptions to be provided in the following sections.

2.1. Regulate the composition of gut microbiota

2.1.1. Fermented by specific bacteria

In breastfed infants, *Bifidobacteria*, including *Bifidobacterium longum* subsp. *longum* (*B. longum*), *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), *Bifidobacterium breve*, and *Bifidobacterium bifidum*, predominantly populate the gut microbiota. Distinct strategies for utilizing MOs, particularly the fucosylated ones, have evolved among various *Bifidobacteria* species. For instance, *B. bifidum* strains initiate extracellular degradation of MOs using 1,2-α-fucosidase (AfcA) and a 1-3/4-α-fucosidase (AfcB) to release fucose (Katayama et al., 2004). Then, lacto-N-biosidase from *B. bifidum* or *B. longum* releases lacto-N-biose (LNB) from MO chains that lack fucose or sialic acid residues (Wada et al., 2008). These liberated LNB molecules are transported into cells via ABC transporters, providing a substrate for *B. bifidum* (Fig. 4A, Suzuki et al., 2008). In contrast, *B. infantis* employs a distinct approach to MO metabolism. This subspecies possesses a comprehensive set of essential enzymes, such as galactosidases, fucosidases, sialidases, and hexosaminidases, as well as transporters including ABC transporters (LoCascio et al., 2007). This enables *B. infantis* to utilize a broad range of MOs, importing smaller MOs into cells via specialized ABC or solute-binding protein (SBP) transporters. These MOs are then further metabolized into monosaccharides through glycosidases (Fig. 4B, Zivkovic et al., 2011). Meanwhile, *B. longum* and *B. breve* exhibit the ability to consume non-fucosylated neutral MOs, such as LNnT, with the latter also capable of fermenting several other monosaccharides (Ward et al., 2007).

Beyond *Bifidobacteria*, several other bacterial strains also exhibit the capability to metabolize MOs. In a study by Salli et al. (2021), the utilization of 2′-FL, 3′-FL, and difucosyllactose (DFL) as sole carbon sources were compared among 57 bacterial strains encompassing *Lactobacillaceae*, *Bacteroides*, *Bifidobacterium*, and potentially pathogenic bacteria. Apart from *Bifidobacterium*, *Bacteroides fragilis*, *Bacteroides vulgatus*, and *Bacteroides thetaiotaomicron* were identified as consumers of 2′-FL, 3′-FL, and DFL. However, none of the *Lactobacillaceae* strains or pathogenic bacteria showed growth on these MOs (Salli et al., 2021). Similar findings were reported by Yu et al. (2013), where *Lactobacillus delbrueckii* ATCC7830, *Enterococcus faecalis* ATCC19433, and *Streptococcus thermophilus* ATCC19258 exhibited limited growth on 2′-FL or 3′-FL, but not LDFT. *Bifidobacterium longum*, *Bacteroides vulgatus*, and *B. thetaiotaomicron* displayed growth promotion in the presence of 3′-SL or 6′-SL. Conversely, *Clostridium* spp., *Enterobacter* spp., *Staphylococcus* spp., and *Escherichia coli* K12 did not exhibit growth in MOs-enriched medium. While the supportive role of other MOs for gut bacteria is likely, comprehensive exploration in this area remains relatively limited.

2.1.2. Prevent pathogen adhesion

The ability of MOs to prevent pathogen adhesion is attributed to the structural similarities between the mucosal cell surface of pathogens and MOs. Pathogens bind to MOs and pass through the intestinal tract without adhering to epithelial cells. To date, fucosylated and acidic MOs have been reported to prevent the adhesion of pathogens, including *Campylobacter jejuni* and *Escherichia coli* (Newburg et al., 2005). These pathogens are major causes of bacterial infections and pose significant health challenges to young children. The mechanism by which MOs inhibit infection by *Campylobacter jejuni* has been extensively studied. *Campylobacter jejuni* attaches to and colonizes the host through interactions between host cell surface glycans and protein-assembling adhesins on the pathogen's surface (Morrow et al., 2005). Among the various glycans, the fucosylated ones are crucial for fucose binding of *Campylobacter jejuni*. The pathogenesis of *Campylobacter jejuni* can indeed be inhibited by α 1-2-linked fucosyl MOs, such as 2′-FL (Morrow et al., 2004).

In another study, researchers found an additional mechanism by which MOs inhibit pathogen growth. Here, the antibacterial properties of MOs against various harmful bacteria were compared, and MOs showed antimicrobial activity against *Acinetobacter baumannii* and Group B *Streptococcus* by increasing the membrane permeability of bacterial cells. However, they had no effects against *Staphylococcus aureus*. Moreover, the activity of MOs was found to be strain-dependent, meaning different bacteria showed varied susceptibilities to MOs (Craft

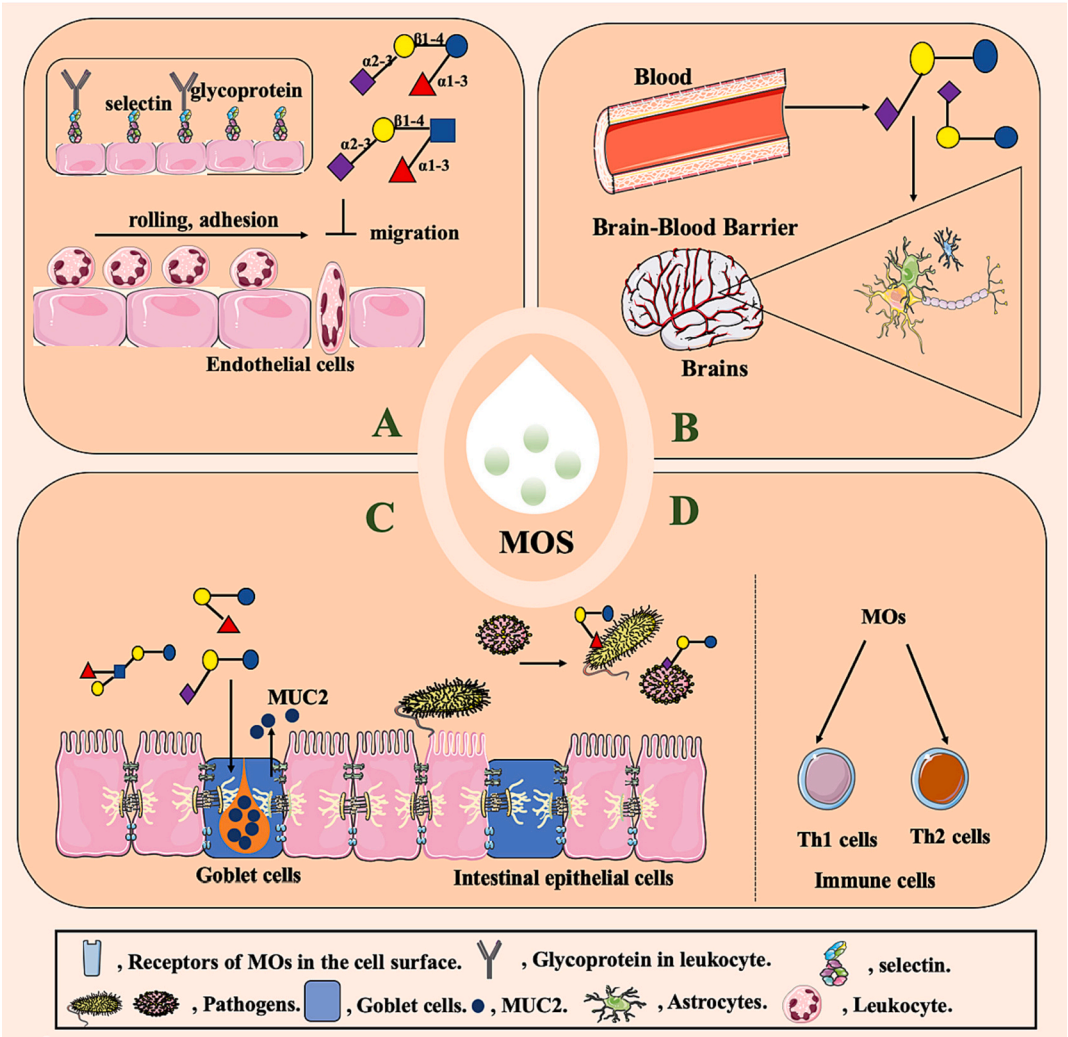

Fig. 3. Overview of beneficial functions of MOs in breast milk for the body. A, MOs prevent leukocyte extravasation. Sialyl-Lewis × (3-sialyl-3-fucosyllactosamine) and also 3-sialyl-3-fucosyllactose, having both fucose and sialic acid, could interact with selectins and then decrease leukocyte rolling and adhesion which finally lead to the decline of leukocyte extravasation. B, MOs promote brain development. The sialic acid can go through the blood-brain barrier and concentrate in the brain area and then is utilized as a neurotransmitter. C, MOs enhance intestinal barrier functions. Pathogens attach to and continue to colonize through interaction between host cell surface glycans and protein-assembling adhesins of pathogens' surface. MOs could bind to pathogens and then prevent them from colonizing the intestine. D, MOs promote the systemic immune response. MOs could affect immune responses by shifting neonatal Th-2 type T-cell phenotype toward a more balanced Th-1/Th-2 ratio.

& Townsend, 2019).

2.2. Enhance intestinal barrier function

MOs exert influence not only on the modulation of gut microbiota populations but also on the regulation of intestinal barrier functionality, particularly the physical and chemical barriers. This includes various intestinal epithelial cells and the mucin layer, which collectively act as a defense against bacterial transmission to the lamina propria. Direct effects of MOs on intestinal epithelial cells have been documented. Kuntz et al. (2008) reported that under homeostatic conditions, MOs inhibit the growth of intestinal cells (HT-29 and HIEC) by impeding cell cycle progression, either through induction of differentiation or apoptosis regulation. This effect is notably attributed to the nature of MOs; for instance, only acidic MOs induce differentiation, while neutral MOs induce pronounced apoptosis. Angeloni et al. (2005) demonstrated that distinct glycoprotein types contribute to the alteration in pathogen binding to cell surfaces. In their study, treatment with 3′-SL led to reduced expression of sialyltransferases ST3Gal1, ST3Gal2, and ST3Gal4 in Caco2 cells. Consequently, this decreased a2-3- and a2-6-sialylation glycans on the cell surface and substantially reduced *Escherichia coli* adherence.

Apart from these direct influences, MOs contribute to the enhancement of the intestinal barrier through their degradation products or via the secretion of MO-utilizing bacteria. Notably, *Bifidobacteria* fermentation of MOs generates acetate, which safeguards the host against *E. coli* O157:H7 infection (Fukuda et al., 2011). Furthermore, cell-free spent media containing secretions from *Bifidobacterium infantis* (*B. infantis*), as demonstrated by Guo et al. (2017) and Lewis et al. (2017), have a protective impact on intestinal epithelial barrier function. Specifically, *B. infantis* conditioned media (BCM) protects Caco2 cells against IL-1β stimulation and promotes claudin-1 and occluding protein expression, thereby bolstering intestinal barrier integrity.

Mucins in the colon are high molecular weight glycoproteins characterized by extensive glycosylation at serine and threonine residues with oligosaccharides composed of *N*-acetylgalactosamine (GalNAc), GlcNAc, Fuc, Gal, and NeuAc (Bansil & Turner, 2006). These mucins are classified into membrane-bound (e.g., MUC1 and MUC4) and secreted (e.g., MUC2, MUC5AC, MUC5B, MUC6, MUC7) categories, with MUC2 being the predominant secreted mucin in humans (Hansson, 2020). An

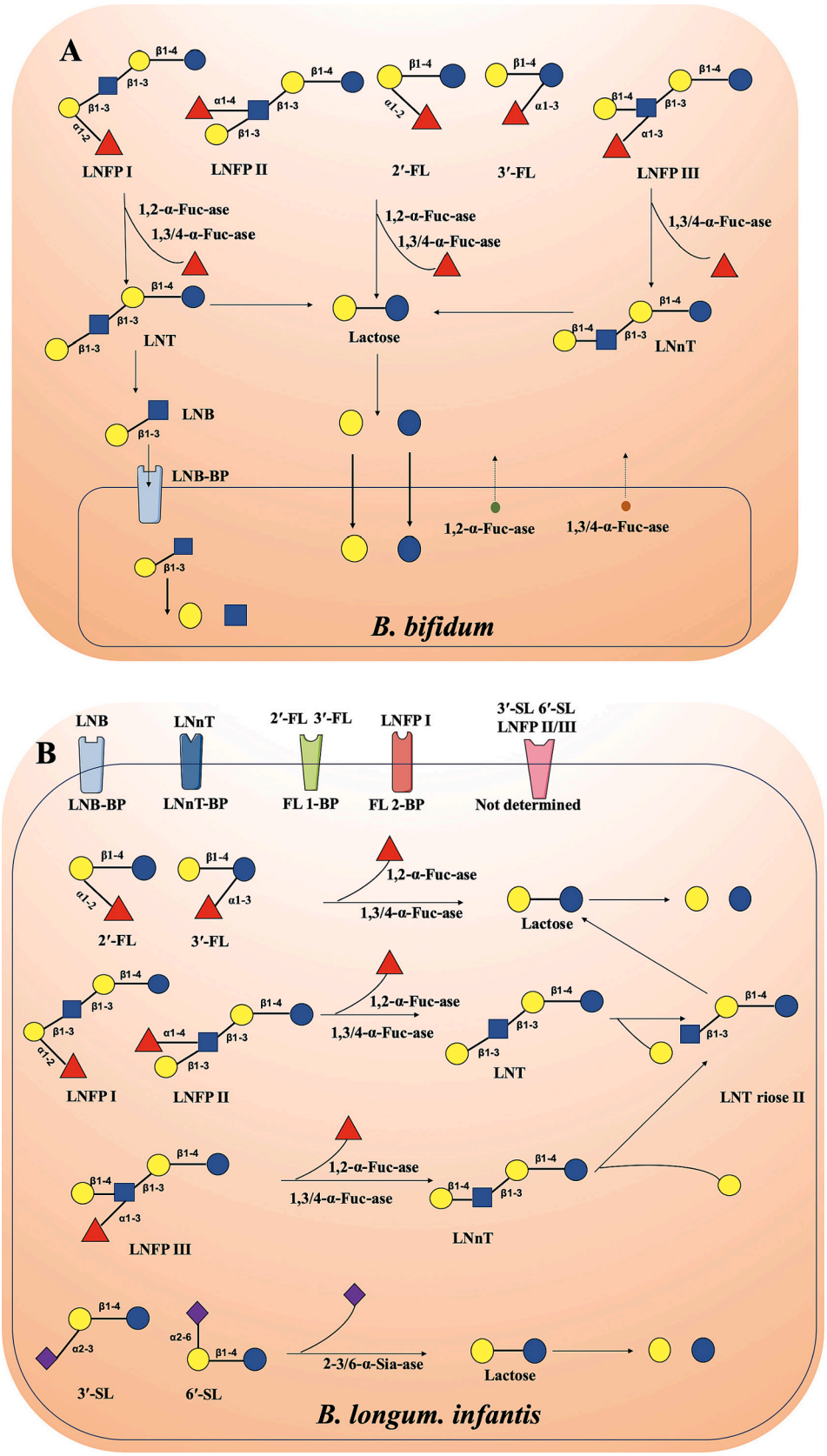

Fig. 4. The specific strategies of *Bifidobacterium bifidum* and *Bifidobacterium longum. infantis* to utilize MOs. A. *B. bifidum* strains initiate MOs degradation extracellularly by 1,2-α-fucosidase and a 1-3/4-α-fucosidase to release fucose. Next, lacto-N-biosidase liberates LNB, which then is transported into cells. B, the process of *B. longum* subsp. *infantis* ferments MOs. As possessing almost all essential sialidase, fucosidase, galactosidase, and hexosaminidase, a wide range of MOs are metabolized to monosaccharide intracellularly.

increasing body of evidence supports the role of MOs in promoting mucin secretion and sustaining gut health. Table 1 provides a compilation of studies investigating the impact of MOs on intestinal functions using diverse models, including in vivo animal models, in vitro cell models, and ex vivo intestinal organoids. The mixture of HMOs was observed to enhance goblet cell counts in neonatal mice with necrotizing enterocolitis, elevate MUC2 expression in cell models (Wu et al., 2019), and reduce diarrheal duration while enhancing intestinal functions in newborn piglets (Li et al., 2014; Rasmussen et al., 2017). Beyond infant models, the beneficial effects of HMOs extend to adult models as well. Daddaoua et al. (2006) demonstrated that goat MOs reduced anorexia, body weight loss, IL-1β expression, and mucin 3 levels, while enhancing TFF3 production in adult colitic rats.

Currently, research primarily focuses on exploring the influence of MOs on MUC2 production, while their effects on other mucin proteins remain less understood. Most studies used the whole HMOs mixture, it is unclear the crucial individual MOs to exert the main function. Although progress has been made in comprehending the impact of MOs on mucin secretion, translating these findings into tangible clinical applications, particularly therapeutic interventions for gastrointestinal disorders, presents a significant challenge.

2.3. Immunomodulatory effects

A multitude of immunological receptors, prominently expressed on immune cell surfaces, are designed to recognize different types of MO types (Walsh et al., 2020). These receptors can discern glycoprotein ligands of MOs, thereby enabling alterations in cell-to-cell communication. This, in turn, may modify the length, quality, or affinity of cell surface receptors and their ligands (Rabinovich et al., 2012; Rana & Haltiwanger, 2011). A notable example of this is the role of MOs in mediating leukocyte extravasation. Selectins, a class of cell-surface proteins, facilitate the movement of leukocytes from the bloodstream to inflammatory tissue in the cellular immune response (Ley, 2003). Given that MOs possess binding ligands for selectins, they have the potential to interfere with leukocyte recruitment and potentially reduce extravasation. Schumacher et al. (2006) demonstrated that sialyl-Lewis × (3-sialyl-3-fucosyllactosamine) and 3-sialyl-3-fucosyllactose interacted with selectins, resulting in diminished leukocyte enrollment and adhesion, ultimately leading to reduced leukocyte extravasation. However, the efficacy of this function depends on the structure, as non-fucosylated MOs like 3′-SL and 6′-SL do not exhibit this effect. Moreover, Sodhi et al. (2021) revealed that 2′-FL and 6′-SL could directly bind to the LPS binding pocket of TLR4, subsequently mitigating necrotizing enterocolitis in mice and piglets. Additionally, 2′-FL suppressed LPS-mediated inflammation by reducing CD14 expression—a factor mediating LPS-TLR4 interaction—thereby inhibiting inflammation (He et al., 2016). However, the universality of this function across all Fuc-borne MOs remains uncertain.

Furthermore, MOs contribute to the systemic immune response by influencing the expression of immune cell populations. An imbalance between Th1 and Th2 phenotypes is observed in T-helper cell populations among newborns, often leaning toward a Th2 phenotype (Adkins, 2000). Th1 cells primarily release cytokines such as interferon (IFN)-γ and IL-2, crucial in defending against viruses and intracellular bacterial infections. Conversely, Th2 cells secrete cytokines like IL-4 and IL-5, critical for humoral immunity and immune responses to bacterial infections and hypersensitivity reactions (Akbari et al., 2003). An imbalanced Th1/Th2 ratio can contribute to specific clinical conditions, with excessive Th2 activity associated with allergic diseases and Th1 predominance linked to inflammatory bowel disease (Nakayama et al., 2017). Eiwegger et al. (2004) were pioneers in demonstrating that sialylated MOs increased both Th-1 type cytokines (IFN-γ) and Th2-cytokines, alongside Treg cells. Subsequently, they revealed that sialylated MOs could mitigate postnatal allergen-specific immune responses by shifting neonatal Th-2 type T-cell phenotype toward a more balanced Th-1/Th-2 ratio (Eiwegger et al., 2010). Additionally, MOs have been shown to directly modulate immunological signaling in juvenile intestinal mucosa, suppressing the production of acute phase inflammatory cytokines like IL-6 and IL-1β, and curbing the overexpression of Th17-polarizing cytokines such as TNF-β, TGF-β, IL-4, and IL-17 (He et al., 2014).

2.4. Improve brain development

Facilitating brain development stands as a distinct function attributed to sialylated MOs, largely due to the pivotal role of Sia, a critical component of sialylated glycoconjugates including polySia and gangliosides. Sia plays an essential role in synaptic transmission, impacting learning, memory, and cognition (Becker et al., 1996). A study by Wang et al. (2007) demonstrated that newborn piglets supplemented with sialylated MOs exhibited enhanced performance in a learning and memory task involving an 8-arm radial maze, coupled with elevated expression of two learning-associated genes, ST8SIA4 and GNE,

Table 1
The present studies on MOs promoting intestinal functions using various models.

MOs	Model/subject	Age/period	Dose and duration	Outcomes	Reference
HMOs mixture	Neonatal mice	Newborn	Daily intake HMOs of 2–3 mg/g body weight	HMO-fed pups displayed intact ileal morphology; ↑ Muc2+ cells (goblet cell) in HMOs-fed group	Wu et al., 2019
HMOs mixture	Intestinal organoid Caco-2Bbe1 cells; LS174T cells	/	20 mg/mL HMOs treat for 24 h	↑Upregulated the expression of Muc2 ↓Decreased bacterial attachment and dextran permeability	Wu et al., 2019
2′-FL, LNT, their mix	SHIME® Caco2 cells	26-year-old healthy female feces /	10 g 2′-FL, 2′-FL + LNnT (4:1), and LNnT for 3 weeks 24 h fermented -HMO stimulation	↑Increase of bifidobacteria and SCFAs; ↓Reduction in paracellular permeability and IL-6 expression in 2-FL and mix groups	Šuligoj et al., 2020
HMOs mixture	Piglets	Newborn	4 g/L HMO	↓Reduced the duration of diarrhea ↑Increased pH and lowered colonic DM ↑Enhanced the abundance of *Lachnospiraceae*	Li et al., 2014
HMOs mixture	Preterm pig	Newborn	0.7 g/kg/day 4-HMO for 5 d 0.84 g/kg/day 25-HMO for 5 d	↓Both HMOs decreased IL-8 content ↑4-HMO increased IL-10, IL-12, TGF-β, TLR4 Not change Bacterial adherence and diversity	Rasmussen et al., 2017
Caprine MOs	Co-culture of HT29-MTX cells and Caco-2 cells	/	0.4, 1.0, 2.0 and 4.0 mg/mL of caprine MOs for 24 h	↑4.0 mg/mL caprine MOs enhanced TEER, mucin gene expression and mucin protein	Barnett et al., 2016
Goat MOs	Colitic rats	Female Wistar rats (175–225 g)	500 mg/(kg·d) of the goat MOs, starting 2 d before the colitis induction until d 6	↓Decreased anorexia and body weight loss; ↓Downregulated colonic expression of IL-1β and mucin 3; ↑Increased TFF3	Daddaoua et al., 2006

compared to the control group. Furthermore, in another investigation, pre-term piglets supplemented with sialylated MOs displayed augmented expression of genes linked to Sia metabolism, myelination, and ganglioside production in the hippocampus (Obelitz-Ryom et al., 2019). Interestingly, the extent of this ability varies among individual MOs; for instance, rats consuming 6′-SL, but not 3′-SL, exhibited improved cognitive ability alongside enhanced expression of polysialic acids-neural cell adhesion (PolySia-NCAM) (Oliveros et al., 2018).

However, the intricate metabolic pathway of dietary sialylated MOs within the context of in vivo settings, including how they traverse to the brain to exert their functions, remains less elucidated. Researchers have endeavored to address this inquiry through the utilization of models involving rats, mice, and pigs. Wang, Downing, et al. (2007) sought to investigate whether sialic acid (*N*-acetylneuraminic acid 6 ^{14}C) could traverse the blood-brain barrier and whether brain regions associated with cognition were more prone to uptake. Their findings revealed that 80 % of the injected activity was cleared from piglet blood within 2 min. By 120 min, only 8 % of the activity remained in the blood, while a substantially higher amount was detected in the brain. In a mouse model, orally administered sialic acids were effectively absorbed, yielding a concentration of 3 %–4 % within the brain within 6 h after ingestion (Nohle & Schauer, 1981), suggesting that sialic acid can traverse the blood-brain barrier in a small proportion.

3. MOs quantification and structural analysis

The analysis and quantification of MOs are challenging due to their numerous coexisting isomeric structures and multiple connectivity sites. Several methods have been developed to quantify MOs, which generally include three main steps: MOs preparation, separation of individual MOs, and quantification of individual MOs.

3.1. MOs preparation

Milk is a complex matrix containing various nutrients that can interfere with the MOs analysis. Therefore, before identifying MOs, lipids and proteins need to be eliminated using centrifugation and organic solvents, such as ethanol, chloroform/methanol and/or acetonitrile (Porfirio et al., 2020; Tonon et al., 2019). However, crystallization of lactose and co-crystallization of MOs with lactose caused by organic solvents can hinder MOs detection (Tonon et al., 2019). After removing protein and lipid, crude MOs are further purified using gel permeation chromatography and solid phase extraction (SPE) with porous graphitized carbon (PGC) to eliminate these organic solvents and some other small molecules. Depending on the detection tools, MOs are correspondingly labeled with fluorescent or hydrophobic tags or converted to a permethylated form to enhance the sensitivity and specificity of the analytical techniques (Oursel et al., 2017; Ruhaak & Lebrilla, 2012; Tonon et al., 2019).

3.2. MOs separation and quantification

Once pure MOs is isolated, the next step involves their separation and quantification. Currently, high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are the two prevailing techniques for MOa separation.

Over the recent years, an array of HPLC methods have been established. Reversed-phase liquid chromatography (RPLC) is the primary technique for non-polar carbohydrate separation, operating on the principle of the solute's hydrophobicity relative to the stationary phase (C18 column) (Porfirio et al., 2020). The greater the carbohydrate's hydrophobicity, the stronger its binding to the stationary phase, necessitating a higher concentration of organic solvent for elution, thereby influencing its retention time. This method mandates the derivatization of MOs with a hydrophobic label, such as a methylated tag, to retain their hydrophobic properties (Kurz et al., 2021). Hydrophilic interaction chromatography (HILIC) is commonly used for separating polar carbohydrates with fluorescent tag labeling. The effectiveness of MO separation in HILIC hinges on the number of polar groups present (Pabst & Altmann, 2011). However, HILIC's efficacy is limited for closely related small carbohydrates, like trisaccharides (Balogh et al., 2015). High pH anion-exchange chromatography (HPAEC) is another well-established technique for MO separation, eliminating the requirement for labeling. MOs exhibit distinct ion exchange capabilities with the stationary phase, enabling their separate elution through tailored buffer solutions (Cataldi et al., 2000). Neutral MOs (with no charge) lack compatibility with the stationary phase's ion exchange, allowing for their elution with water. Conversely, negatively charged acidic MOs bind to the stationary phase and require individual elution via saline solutions of appropriate concentrations (Kunz et al., 1999).

In addition to chromatographic-based techniques, CE is another effective technique for the separation of MOs. In CE, charged MOs (sialylated MOs) can be separated based on their size-to-charge ratio. Neutral MOs often require derivatization to impart a charge or fluorescent labels to enhance sensitivity and allow for fluorescence detection (Mantovani et al., 2018). The choice of buffer in CE. It affects the pH and the ionic strength of the medium, which in turn influences the charge and the migration of MOs. For MOs, buffers are often chosen to optimize the separation of specific types of oligosaccharides, like sialylated ones (Toppazzini et al., 2016).

For quantitative characterization of MOs, appropriate detection techniques are crucial. For LC and CE methods, each individual MO can be quantified combined with photodiode array (PAD), UV detection, and mass spectrum (MS). Yan et al. (2018) successful identified sialylated MOs in human breast milk and seven other animal milks (cow, yak, buffalo, camel, donkey, swine, and sheep) through online solid-phase extraction (SPE)-HILIC-MS. Li et al. (2021) employed the SPE-HILIC-MS approach to segregate acidic MOs in rat and mouse samples, unveiling 15 acidic MOs in rat milk and 14 in mouse milk. To date, the total number of quantified individual MOs in human milk is up to 40 (Grabarics et al., 2017). Among them, about 16 main MOs are commonly quantified in various mammal species, which are detailed described in Section 4. The major barrier to quantifying more MOs is the shortage of corresponding MOs standards, which is expected to be addressed by producing MOs through artificial methods (see Section 6.1).

3.3. Structural analysis of MOs

As mentioned above, only 40 human MOs, mostly abundant and low molecular weight, have been quantified. Although the 10 most abundant MOs represent about 75 % of the total MO concentration in human milk (Grabarics et al., 2017), the greatest structural diversity of MOs resides in the remaining 25 % of high molecular weight ones. To address these challenges, the scientific community has turned toward advanced analytical techniques. High-resolution mass spectrometry methods, such as MALDI-MS, nLC-MS/MS and NMR, have become indispensable tools, offering detailed insights into the molecular structure of MOs (Table 2). To date, over 200 MO structures in human milk have been found, of which approximately 162 have been subject to structural characterization (Urashima et al., 2018).

In MALDI ionization, the MOs sample is mixed with a matrix substance in a MALDI plate. The matrix, a small organic molecule, absorbs laser energy and assists in the ionization of the analyte (MOs). Then the ionized molecules are introduced into the TOF-MS or FTICR-MS analyzer, where they are determined based on their mass-to-charge ratio (m/z) (Calvano et al., 2018). FTICR-MS provides fine branching patterns and linkage positions of MOs compared to TOF-MS. Yang et al. (2011) showcased the utility of MALDI-FTICR-MS in identifying MOs patterns, particularly in distinguishing among α(1→4)-, α(1→6)-, β(1→4)-, and β(1→3)-linked isobaric structures.

In nano-LC-MS/MS, ESI or NSI are commonly used to change

Table 2
Examples of main MOs found in human milk and colostrum.

MOs	Abb.	Mean (mg/L)	Minimum (mg/L)	Maximum (mg/L)
Fucosylated neutral MOs				
Fuc(α1-2)Gal(β1-4)Glc	2′-FL	1990 ± 998	410	4130
Fuc(α1-3)Gal(β1-4)Glc	3′-FL	766 ± 487	147	2350
Gal(β1-3)GlcNAc-(β1-3)Gal (β1-4)GlcFuc(α1-2)	LNFP I	904 ± 654	158	2250
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcFuc(α1-3)	LNFP III	364 ± 331	60	1869
Fuc(α1-3)Gal(β1-3)Fuc(α1-4) GlcNAc(β1-3)Gal(β1-4)Glc	LNDFH I	859 ± 404	156	1550
Gal(β1-3)Fuc(α1-4)GlcNAc (β1-3)Gal(β1-4)GlcFuc(α1-3)	LNDFH II	326 ± 319	64	960
Non-fucosylated neutral MOs				
Gal(β1-3)GlcNAc(β1-6)Gal(β1-4)GlcGal(β1-3)GlcNac(β1-3)	LNH	95 ± 50	17	170
Gal(β1-4)GlcNAc(β1-6)Gal(β1-4)GlcGal(β1-4)GlcNAc(β1-3)	LNnH	63 ± 52	18	160
Gal(β1-3)/Gal(β1-4)GlcNAc (β1-3)Gal(β1-4)Glc	LNT + LNnT	1143 ± 553	380	2972
Sialylated MOs				
Neu5Ac(α2-3)Gal (β1-4)Glc	3′-SL	218 ± 189	97	1125
Neu5Ac(α2-6)Gal(β1-4)Glc	6′-SL	451 ± 368	73	1420
Neu5Ac(α2-3)Gal(β1-3) GlcNAc(β1-3)Gal(β1-4)Glc	LSTa	48 ± 37	10	141
Gal(β1-3)Neu5Ac(α2-6) GlcNAc(β1-3)Gal(β1-4)Glc	LSTb	100 ± 56	33	193
Neu5Ac(α2-6)Gal(β1-4) GlcNAc(β1-3)Gal(β1-4)Glc	LSTc	247 ± 227	29	940
Neu5Ac(α2-3)Gal(β1-3) GlcNAc(β1-3) Neu5Ac(α2-6) Gal(β1-4)Glc	DSLNT	358 ± 174	121	795
Total		7935	1773	21,025

analytes from the solution phase into numerous charged ions (Zhong et al., 2014). Once ionized, MS/MS analyzes these ions. The first MS stage identifies the mass-to-charge ratio of the ions, providing molecular weight information. Then the ions are further fragmented and analyzed in the second MS stage. This step is crucial for determining the sequence of monosaccharide units and the type of glycosidic linkages in the MOs. Porfirio et al. (2020) identified 80 permethylated MOs (including sialylated and/or fucosylated MOs) in human milk samples by nLC-NSI-MS/MS method, with the highest permethylated masses >5000 Da. Remoroza et al. (2018) developed a searchable MO reference library from 100 human breast milk samples by HILIC-ESI-MS/MS, which contains over 469 positive and negative ion spectra. This reference standard mixture provides a promising option when standards are not available.

NMR, both One-Dimensional (1D) NMR and 2D NMR techniques, focuses on the analysis of specific combinations within MOs (Smilowitz et al., 2013). Proton NMR (^{1}H NMR) and Carbon NMR (^{13}C NMR) are frequently used for MOs as they provide information about the hydrogen atoms or carbon skeleton within the molecule (Hamagami et al., 2020). The most outstanding advantage of this method is its ability to effectively detect different MOs with different Lewis epitopes (van Leeuwen et al., 2018), with minimal or no clean-up steps. However, the similar structures among MOs result in overlapping signals, making differentiation difficult. To address, 2D NMR, such as COSY, TOCSY and HSQC, are developed to determine the connectivity between atoms and to resolve overlapping signals. They can further provide information about the sequence of sugar units and the type of α-linkages/β-linkages (Gunjan Kumar & Deepak, 2018).

The processes of MOs quantification and structural analysis is shown in Fig. 5. Despite the establishment of advanced methods expected to provide more information for the quantification and structural sequences of MOs, there are several areas for improvement. First, sample preparation is complicated and time-consuming, with the risk of losing MOs at low concentrations. More importantly, when MOs are derivatized, the difference in labeling efficiency should be considered (Austin & Bénet, 2008). For high-throughput analysis, simpler sample preparation is required to facilitate method development. Second, the comparison of MOs concentrations in different research is challenging because there are large differences in the techniques adopted to analyze MOs.

4. MOs concentration in various species

4.1. General comparison of MOs between human milk and animal milk

The concentrations and patterns of Milk Oligosaccharides (MOs) significantly vary across different species. Human milk, in particular, exhibits a unique composition of MOs, standing out with the highest counts of detected (247), structurally analyzed (162), and quantified MOs (40) compared to other mammalian milk. Notably, the quantity of MOs in human milk is substantially higher, ranging from 10 to 100 times more than that found in animal milk (Wang et al., 2020). Furthermore, MOs in human milk are characterized by their increased complexity and diversity. Among these, fucosylated neutral MOs are predominant, making up about 80 % of the total. This is in sharp contrast to their limited presence, ranging from 2 % to 17 %, in domestic animal milk, with the exception of donkey milk (Fig. 6).

Current research predominantly focuses on the structural diversity and concentration of MOs. However, there is a notable gap in studies specifically addressing the structure of MOs in animal milk, and even less so regarding their concentration. In this context, our study provides a comparative overview of the MOs profile across various mammalian species based on the previous research. We analyze the number of detected, structurally analyzed, and quantified MOs, explore the distribution ratios among different types of MOs, and highlight the top five individual MOs, as detailed in Fig. 6.

4.2. MOs concentration and composition in human milk

Although about 247 structures of MOs have been identified, only a few MOs can be quantified. Table 2 shows the concentrations of 16 MOs in breast milk samples throughout lactation (Asakuma et al., 2007; Bao et al., 2013, 2007; Borewicz et al., 2019; Leo et al., 2009, 2010; Ma et al., 2018; Samuel et al., 2019; Thurl et al., 2010; Zhang et al., 2019; Siziba et al., 2021; Alderete et al., 2015). The mean total MOs (16 MOs) is 7935 mg/L, ranging from 1773 to 21,025 mg/L. Among them, 2′-FL is most abundant, with an average concentration of 1990 mg/L, followed by 3′-FL and LNFP I. LNT and LNnT are the dominant non-fucosylated neutral MOs (1143 mg/L in average), widely ranging from 380 to 2872 mg/L. Human milk contains relatively few sialylated MOs, 3′-SL, 6′-SL and DSLNT being the most prevalent. Additionally, the ratio of fucosylated MOs: non-fucosylated MOs: sialylated MOs in human milk is about 66 %:16 %:18 %, calculated by data in Table 2.

The nutrients concentrations vary during the whole lactation, as do MOs. To explore the influence of lactation time on MOs concentrations, the postpartum period is classified into four periods: 0–14 days (colostrum), 14–30 days, 30–90 days and >90 days. The data in Table 2 are reanalyzed according to the lactation periods, as shown in Fig. 7. The contents of majority of analyzed MOs decline with prolongation of lactation. Compared to the first period, the concentrations of 2′-FL, LNFP I, LNT + LNnT, 6′-SL and DSLNT decreased by 1.5, 3.7, 1.8, 1.7, and 2.2 folds in the period, respectively. However, the 3′-FL sees a 2.0-fold rise during the fourth period. The ratio of 2′-FL to 3′-FL in 0–14 days, 14–30 days, 30–90 days, and >90 days is 1: 4.6, 1:4.4, 1:2.4, and 1:1.5,

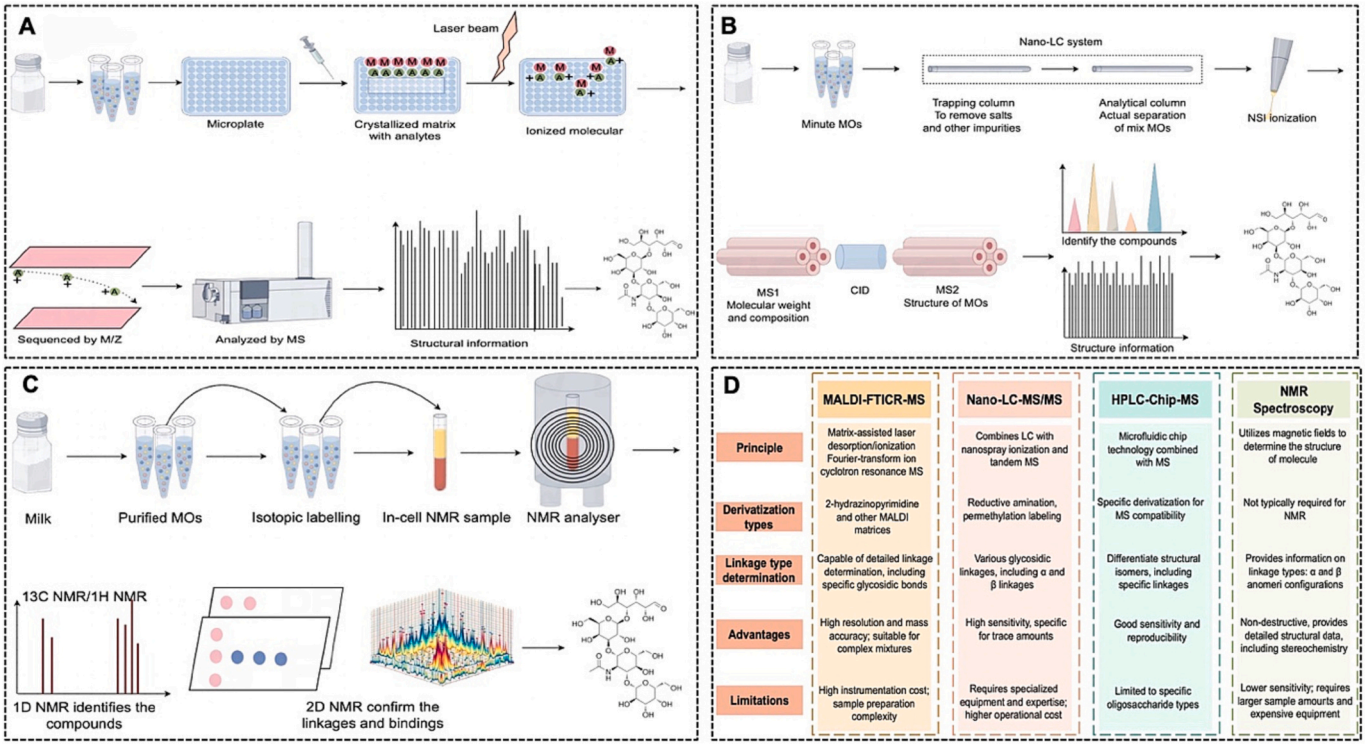

Fig. 5. Workflow of current technologies for MOs structural analysis. A, MALDI-based technologies; B, nano-LC-MS/MS; C, NMR spectroscopy; D, a comparison among the structural analysis methods, including MALDI-FTICR-MS, Nano-LC-MS/MS, HPLC-chip-MS/MS and NMR spectroscopy.

respectively. The concentrations of LNH, 3′-SL and LSTb seem to be stable during the whole lactation.

However, data obtained from different research teams vary considerably. For example, the concentration of 3′-SL reported by Asakuma et al. (2007) was threefold higher than that obtained by Bao et al. (2007). Due to this, the MOs concentration and composition described in this work may differ somewhat from reality. Also, the variation in MOs concentration is attributed to biological parameters like gestational age, secretor status, lactation period, or general biological variability.

Summarized from references of MOs quantification, including Asakuma et al. (2007), Bao et al. (2007, 2013), Borewicz et al. (2019), Leo et al. (2009, 2010), Ma et al. (2018), Samuel et al. (2019), Thurl et al. (2010), Zhang et al. (2019), Siziba et al. (2021), Alderete et al. (2015).

4.3. MOs concentration and composition in animal milk

Besides breast milk, MOs are also present in milk from domestic animals. Albrecht et al. (2014) identified 48, 40, 40, 38 and 35 MO structures in camel, porcine, equine, ovine, and bovine milk, respectively, which is much lower than the number identified in human milk. The MOs in other animals exhibit similar structural elements to those in human milk; however, they are less complicated and abundant. Table 3 shows the concentrations of the main quantified MOs in domestic animal milk (Albrecht et al., 2014; Chatziioannou et al., 2021; Difilippo et al., 2016, 2015; Kunz et al., 1999; Licitra et al., 2019; Liu et al., 2014; McJarrow & van Amelsfort-Schoonbeek, 2004; Shi et al., 2021; Wang et al., 2022, 2020; Yan et al., 2019, 2018). Most studies on MOs quantification from animal milk samples focus on dairy cows, sheep, and horse (colostrum), with their MOs concentrations around 414–1720, 562–1349 and 2420–13,850 mg/L, respectively. Due to the lack of adequate data, it is hard to summarize MOs concentration in camels, monkeys, yaks, elephants and pigs.

Different from human milk, the sialylated MOs are predominant in concentration (80 %–90 % of the total MOs) in domestic animals, with 3′-SL, 6′-SL detected in almost all samples (Albrecht et al., 2014). However, the situation is slightly different in goat milk. Proportions ranging from 15.6 % to 18.2 % were quantitatively found for fucosylated neutral MOs, from 7.3 % to 18.9 % for non- fucosylated neutral MOs, and 68.2 % to 74.1 % for sialylated MOs (Chatziioannou et al., 2021; Shi et al., 2021; Wang et al., 2020).

It is important to acknowledge that the quantification of MOs in animal milk exhibits variability, introducing complexities when comparing research outcomes across various species. On one hand, differences could stem from the utilization of diverse analytical methods. On the other hand, the composition of MOs is influenced by genetic diversity and the progression of lactation. Thus, acquiring supplementary data is imperative to establish a robust and comprehensive understanding of the MOs composition in animal milk.

5. Influencing factors of MOs

5.1. Species

As described earlier, MOs exhibit remarkable structural diversity, leading to substantial variations in their concentration and composition among different species, particularly between human and other animal milk sources. Compared to other mammals, human milk is unique, containing 10–100 times more total MOs and featuring a more intricate MOs profile. Notably, fucosylated neutral MOs dominate human milk, constituting 50 %–80 % of the total MOs, whereas sialylated MOs prevail in domestic animal milk (Albrecht et al., 2014). The genetic basis is believed to underlie the presence of MOs (Erney et al., 2001), yet elucidating why MOs in human milk differ from those in other species remains challenging. A plausible explanation stems from evolutionary adaptations and species-specific requirements (Urashima et al., 2022). As proposed by Urashima et al. (2022), evolutionary forces have shaped the MOs composition in each species to cater to the unique needs of their offspring. For instance, MOs have conferred evolutionary advantages in other animals as a significant energy source for nursing infants. In contrast, human infants possess an underdeveloped immune system and

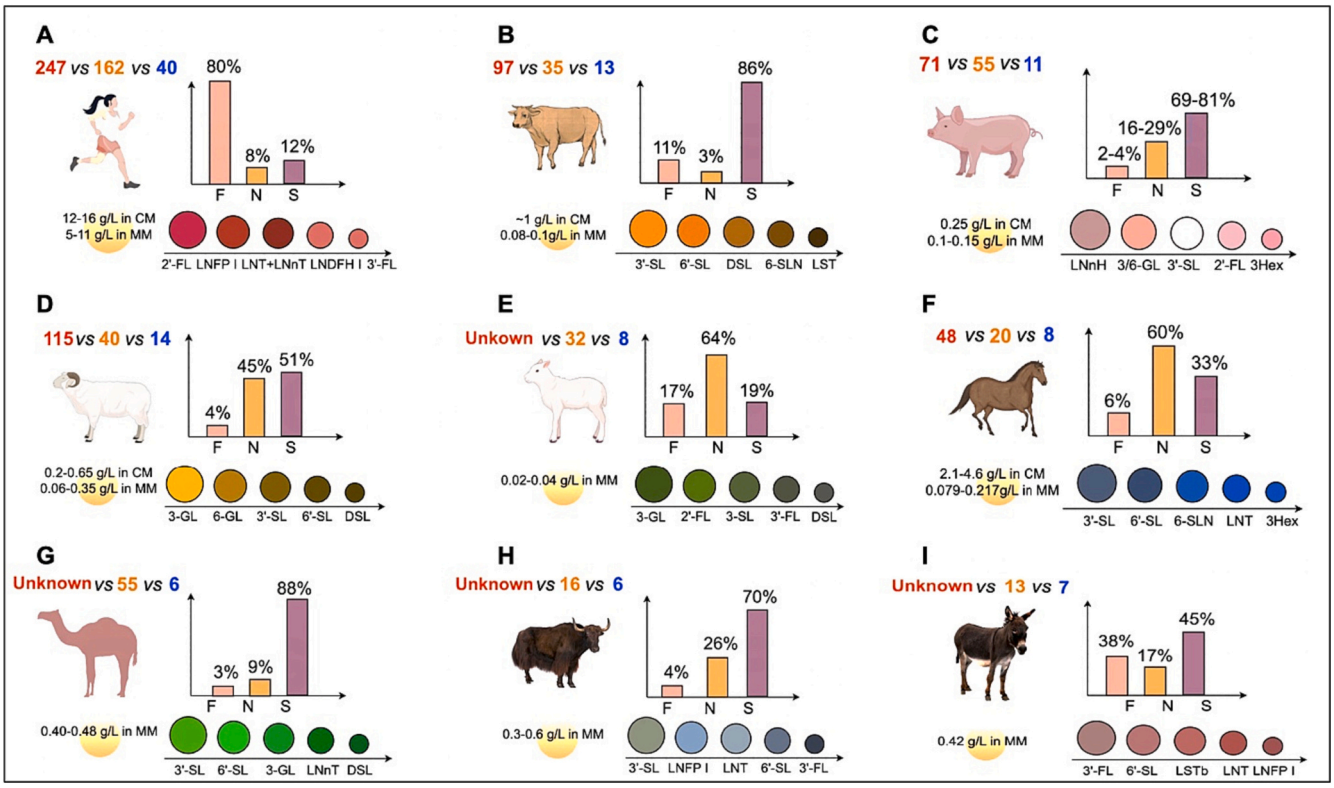

Fig. 6. Comparative overview of MOs across mammalian species. The panels of A–I are humans, dairy cows, sows, goats, sheep, mares, camels, yaks, and donkeys, respectively. The number in red, reddish-orange, and blue, represents the counts of detected, structurally analyzed, and quantified MOs, respectively. F, Fucosylated neutral MOs; N, non-fucosylated neutral MOs; S, sialylated MOs. The bar graph shows the distribution ratios among different MO types, according to their absolute concentration in the previous research. CM, colostrum; MM, mature milk; The Top five MOs individuals are shown by the round shape in different circles. Summarized from references of MOs quantification, including Samuel et al. (2019), Borewicz et al. (2019), Zhang et al. (2019), Albrecht et al. (2014), Durham et al. (2023), Fischer-Tlustos et al. (2020), Karav et al. (2018), Licitra et al. (2019), Mudd et al. (2016), Shi et al. (2021), van Leeuwen et al. (2020), Wang et al. (2020), Wei et al. (2018), Zhang et al. (2023), and Zhong et al. (2024).

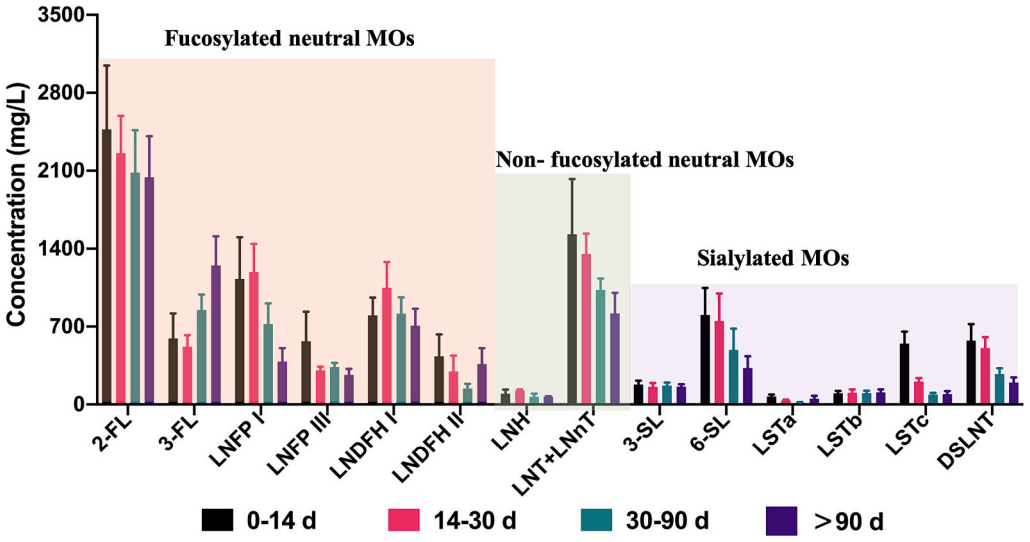

Fig. 7. Concentration changes of representative neutral and acidic MOs in human milk during lactation. Summarized from references of MOs quantification, including Asakuma et al. (2007), Bao et al. (2007, 2013), Borewicz et al. (2019), Leo et al. (2009, 2010), Ma et al. (2018), Samuel et al. (2019), Thurl et al. (2010), Zhang et al. (2019), Siziba et al. (2021), Alderete et al. (2015).

a rapidly growing brain. Consequently, MOs play pivotal roles in bolstering the immune system, enhancing brain development, and establishing symbiosis with colonic bacteria. Researchers have examined whether the co-evolution of MOs with colonic bacteria extends to other species (Akazawa et al., 2021; Li et al., 2021). *Enterococcus gallinarum* isolated from the feces of neonatal rats demonstrated the capability to hydrolyze 3′-SL to Neu5Ac and lactose (Akazawa et al., 2021). However, considering that *Bifidobacteria* are not dominant in infant rats

Table 3
Examples of main neutral and acidic oligosaccharides found in milk and colostrum from domestic animals.

MOs	Abb.	Concentration in milk samples (mg/L)								References
		Bovine	Goat	Camel	Monkey	Yak	Horse (colostrum)	Elephant	Swine	
Fucosylated neutral MOs										
Fuc(α1-2)Gal(β1-4)Glc	2′-FL	nd	3.11–41.41	0.19	nd	nd	nd	nd	nd	c, d, e, h, i, j
Fuc(α1-3)Gal(β1-4)Glc	3′-FL	0.36–86.55	0.44–71.21	3.02	123.05	63.3	nd	160–310	nd	c, e, h, i
Gal(β1-3)GlcNAc-(β1-3)Gal (β1-4)GlcFuc(α1-2)	LNFP I	71.17	98.59	nd	34.84	99.89	30–110	370–126	nd	e, f, h, i, k
Gal(β1-3)GlcNAc(β1-3)Gal (β1-4)GlcFuc(α1-4)	LNFP II	nd	nd	–	nd	–	nd	–	nd	i
Gal(β1-4)GlcNAc(β1-3)Gal (β1-4)GlcFuc(α1-3)	LNFP III	nd	nd	nd	nd	–	nd	–	–	i
Fuc(α1-3)Gal(β1-3)Fuc(α1-4) GlcNAc(β1-3)Gal(β1-4)Glc	LNDFH I	nd	nd	–	–	–	–	–	–	i
Gal(β1-3)Fuc(α1-4)GlcNAc (β1-3)Gal(β1-4)GlcFuc(α1-3)	LNDFH II	nd	nd	–	–	–	–	–	–	i
Non-fucosylated neutral MOs										
Gal(β1-4)Gal(β1-4)Glc	3′-GSL	4.18	31.69–147.94	1.15	nd	–	40–230	1201–4000	190–1980	c, d, f, h, k
Gal(β1-6)Gal(β1-4)Glc	6′-GSL	nd	8.05–65.67	–	nd	–	550–3150	–	640–1230	d, f, k
Gal(β1-3)GlcNAc(β1-6)Gal (β1-4)GlcGal(β1-3)GlcNac (β1-3)	LNH	–	–	–	nd	–	10–70	–	–	f
Gal(β1-4)GlcNAc(β1-6)Gal (β1-4)GlcGal(β1-4)GlcNAc (β1-3)	LNnH	–	1–5	nd	nd	–	nd	–	100–430	h, I, k
Gal(β1-3)/Gal(β1-4)GlcNAc (β1-3)Gal(β1-4)Glc	LNT + LNnT	0.41–37.66	0–1.40	1.57	42.17	10.74	20–390	1160–2490	–	c, e, f, g, h
Sialylated MOs										
Neu5Ac(α2-3)Gal (β1-4)Glc	3′-SL	116–867	43.63–198.92	–	7.32–100.17	121.82	290–1730	860–2790	1.86–20.98	d, e, f, g, h, k
Neu5Ac(α2-6)Gal(β1-4)Glc	6′-SL	6–136	41.47–284.41	–	16.35–36.16	34.98	20–230	130–340	nd	a, b, d, e, f, g, h
Neu5Ac(α2-8)Neu5Ac(α2-3) Gal(β1-4)Glc	DSL	201–283	1.9	–	nd	–	20–400	–	–	b, c, f, j
Neu5Ac(α2-3)Gal(β1-3) GlcNAc(β1-3)Gal(β1-4)Glc	LSTa	nd	nd	–	nd	nd	20–830	50–520	100–250	e, f, j
Gal(β1-3)Neu5Ac(α2-6) GlcNAc(β1-3)Gal(β1-4)Glc	LSTb	15.39	30.69	–	49.16	nd	–	nd	–	e, i
Neu5Ac(α2-6)Gal(β1-4) GlcNAc(β1-3)Gal(β1-4)Glc	LSTc	nd	9.92	–	0.30–0.77	nd	–	360–1090	nd	e, g, h, j
Neu5Ac(α2-3)Gal(β1-3) GlcNAc (β1-3) Neu5Ac(α2-6)Gal(β1-4)Glc	DSLNT	nd	284.80	–	nd	nd	–		–	e
Neu5Ac(α2-6)Gal(β1-4) GlcNAc	6-SLN	0.27–220	7.36–107.2	nd	nd	nd	1420–6710	nd	nd	b, d, f, j

References: a, Liu et al., 2014; b, McJarrow & van Amelsfort-Schoonbeek, 2004; c, Shi et al., 2021; d, Chatziioannou et al., 2021; e, Wang et al., 2020; f, Difilippo et al., 2015, g, Licitra et al., 2019, h, Kunz et al., 1999; i, Albrecht et al., 2014; j, Yan et al., 2018; k, Difilippo et al., 2016.
nd, the concentration of the oligosaccharides were not determined but the oligosaccharides were structurally characterized.
–, not reported; NED, no enough data.

(Korgan et al., 2022), the applicability of *Bifidobacteria*-type microbiota to rats might differ from other species.

Regarding the synthesis of MOs, distinctions also emerge between human milk and other animal milk sources. The synthetic pathways of MOs in humans exhibit significantly higher complexity compared to animals like bovines (Urashima et al., 2022). Kellman et al. (2022) used glycan and RNA expression data to construct an HMO biosynthetic network, revealing the involvement of multiple fucosyltransferases and sialyltransferases in biosynthesis, including FUT1, FUT2, FUT3, FUT4, FUT5, FUT9, FUT11, ST6GAL1, ST6GAL2, and ST3GAL3. In contrast, MOs found in bovine milk showcase unique attributes, such as the presence of β1-4*N*-acetylgalactosaminyl transferase (β4GalNAcT), which is absent in humans.

5.2. Lewis blood group of mothers

The composition of MOs in human milk displays substantial individual variation. While the fundamental structure and types of MOs remain consistent, the specific combinations and quantities of individual MOs can differ significantly. Notably, even a minute genetic variation of 1 base pair (bp) out of the genome's approximately 3 billion base pairs can dramatically alter the human MOs composition, leading to the classification of secretor and non-secretor lactotypes (Bode, 2020). Within lactocytes, two distinct fucosyltransferases have been identified: (1) α1-2-fucosyltransferase (FUT2), encoded by the Secretor (Se) gene, which catalyzes the attachment of Fucose to terminal Gal through α1-2 bonds, and (2) α1-3/4-fucosyltransferase (FUT3), encoded by the Lewis (Le) gene, responsible for linking Fucose to GlcNAc via α1-4 bonds (Bode, 2015). Consequently, "secretor mothers," possessing the *Se* gene, can produce MOs such as 2′-FL (Fucα1-2Galβ1-4Glc) and LNFP I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc). The absence of functional FUT2 activity influences the concentration of almost all other MOs, extending beyond the scope of solely 1-2-fucosylated ones, thereby impacting the entire HMO biosynthesis process. Conversely, non-secretors exhibit an inactive or non-functional FUT2 gene, resulting in reduced or absent production of fucosylated MOs in their milk.

Similarly, Single Nucleotide Polymorphisms (SNPs) in the FUT3 gene, responsible for the Lewis blood group antigen, also contribute to this genetic diversity (Bode, 2020). Those that carry *Le* gene can produce α1-4-fucosylated MOs, such as LNFP II (Fucα1-4Galβ1-3GlcNAcβ1-3Glβ1-4Glc) (Xu et al., 1996). The presence of Le b antigens in milk relies on the expression of both FUT2 and FUT3. In cases where only FUT3 is active, the milk includes MO with Le a antigens. Conversely, if FUT3 expression is absent, the milk lacks Le a or b antigens. Accordingly, there are four different groups of mothers: Se + Le + (Le a-b+), Se + Le- (Le a-b−), Se-Le + (Le a + b−) and Se-Le- (Le a-b−) (Stahl et al., 2001), which is summarized in Table 4. Notably, the milk of mothers expressing neither FUT2 nor FUT3 contains fucosylated HMOs such as 3′-FL or LNFP III, indicating the potential involvement of other Se- and Le-independent FUTs (FUT4, 5, 6, 7, or 9) in fucosylated MO synthesis (Newburg et al., 2005).

5.3. Maternal diets

Diet plays a substantial role in determining the composition of MOs, especially those in human milk. Evidence suggests that the maternal diet can predictably and significantly affect specific MO concentrations in human milk (Azad et al., 2018; Seferovic et al., 2020). The types and amounts of carbohydrates, fibers, and fats consumed by the mother have a direct impact on MO composition. For example, increased glucose intake corresponds with a reduction in overall fucosylated MOs compared to a galactose-based diet. However, no statistically significant differences in individual non-fucosylated MOs were observed between these dietary approaches (Seferovic et al., 2020). Similarly, the concentration of total sialylated MOs decreased in response to a high-fat diet compared to a carbohydrate-rich diet during an 8-day intervention initiated at 10 weeks postpartum. This decrease coincided with elevated energy expenditure and output, without affecting gluconeogenesis (Mohammad et al., 2009; Seferovic et al., 2020). Conversely, a lipid-based nutrient supplement did not significantly impact MOs composition in another study (Jorgensen et al., 2017). Consistent with these findings, Azad et al. (2018) reported that the total concentration of MOs was not directly correlated with specific maternal diets, although certain dietary elements displayed associations with individual MOs. For instance, LSTb showed an inverse correlation with total protein and empty calories, while fucosyllacto-N-hexaose exhibited a positive correlation with whole grains. Moreover, positive correlations were established between LNT and difucosyllacto-N-hexaose and overall energy intake (Azad et al., 2018).

It is pertinent to recognize that the extent of dietary influence on MOs composition can vary among individuals. Genetic factors, maternal health, and other physiological processes also contribute to shaping the final MOs composition in breast milk. Ongoing research is exploring the complex relationship between diet and MOs composition, necessitating further investigations to fully understand the intricate mechanisms and effects of diverse dietary components on MO synthesis.

Table 4
Fucosyltransferases determining the diverse structure of fucosylated MOs.

Groups	Mother			
	Secretors		Non-secretors	
	Se + Le+	Se + Le−	Se-Le+	Se-Le−
Fucosyltransferase	FUT2, FUT3	FUT2	FUT3	–
Linkage	α1–2; α1–3/4	α1–2	α1–3/4	–
Representative MOs	All fucosylated MOs	2′-FL, LNFP I	3′-FL, LNFP II/III,	–
Le blood antigen	Le^b (Le a-b+)	Le a-b−	Le^a (Le a + b−)	Le a-b−
Proportion (%) of European country*	70 %	9 %	20 %	1 %

* Data from Gabrielli et al. (2011).

6. Potential applications of MOs

6.1. Artificial synthesis of MOs

In humans, MOs are synthesized within the epithelial cells of the mammary gland, orchestrated by specific enzymes that facilitate the gradual construction of these intricate MO structures. The synthesis journey begins with lactose, formed through the action of active UDP-Gal and Glc (Ramakrishnan et al., 2002). A sequence of enzymes including glycosyltransferases such as fucosyltransferases (FUT2, FUT3, FUT4, etc.), sialyltransferases (including six distinct ST6GalNAc enzymes), and *N*-acetylglucosaminyltransferases (β3GlcNAcT for type I and β6GlcNAcT for type II), catalyze glycosylation reactions that intricately link specific monosaccharide units to the evolving MO chain in a precise sequence and linkage pattern (Stahl et al., 2001). Fucosyltransferases and sialyltransferases play pivotal roles in appending fucose and sialic acid residues, contributing to the diversity observed in fucosylated and sialylated MOs (Fig. 8A). Once synthesized, these MOs are enclosed within vesicles and transported through the Golgi apparatus to the apical side of mammary epithelial cells, where they are ultimately released into milk via exocytosis.

Given the relatively low concentration of MOs in human milk, several industrial methodologies have been developed for MOs production, including chemical synthesis, enzymatic synthesis, chemoenzymatic synthesis, and microbial fermentation (Pérez-Escalante et al., 2022). Chemical synthesis, involving step-by-step assembly of the MO chain, is limited by its multistep nature and the use of various reactants (Bode et al., 2016). Enzymatic synthesis uses glycosyltransferases under mild reaction conditions but is constrained by enzyme ability (Schmölzer et al., 2015; Zeuner et al., 2018). Chemoenzymatic synthesis combines chemical and enzymatic techniques but often yield MOs in low quantities, at the milligram scale (Muschiol & Meyer, 2019). Microbial fermentation has emerged as a highly effective approach, utilizing microbial hosts include *E. coli*, *Corynebacterium glutamicum*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*, engineered to produce MOs (Faijes et al., 2019). *E. coli*, known for its rapid growth and efficient DNA manipulation, has been predominantly employed in MO development, achieving significant progress in producing of 2′-FL, 3′-FL, LNT, LNnT, 3′-SL, and 6′-SL at a gram scale (Fig. 8B). The synthesis of complex fucosylated MOs often relies on LNT and LNnT as core structures, followed by the addition of fucose via α-glycosidic linkages, as evidenced by the production of LNFP I/II/II and LNDFH I/II (Dumon et al., 2001, 2004). However, due to its endotoxin-bearing nature, *E. coli* is not regarded as the optimal host strain. Other candidates, such as *S. cerevisiae*, *B. subtilis*, and *C. glutamicum*, are seen as safer alternatives, although their utilization in MO production is still relatively limited (Lu et al., 2021).

6.2. The applications

MOs, particularly those derived from human milk, are poised to play a transformative role in various health and nutrition sectors in the future. They have been identified as key contributors to neonatal health, offering protection against certain pathologies, aiding in brain development, and shaping the infant's immune system (Corona et al., 2021; Urashima et al., 2018). Beyond infant nutrition, the scope of MOs extends into the realm of functional food ingredients for adult (Schönknecht et al., 2023; Šuligoj et al., 2020), offering benefits such as non-carcinogenicity, low calorific value, and the promotion of beneficial gut bacteria (Fig. 9). The burgeoning field of biotechnological production of these MOs opens new avenues for their incorporation into food and pharmaceutical products.

Among these applications, incorporating MOs into infant formula stands out as a primary use, aiming to replicate the protective benefits of human milk to provide a more comprehensive nutritional profile for

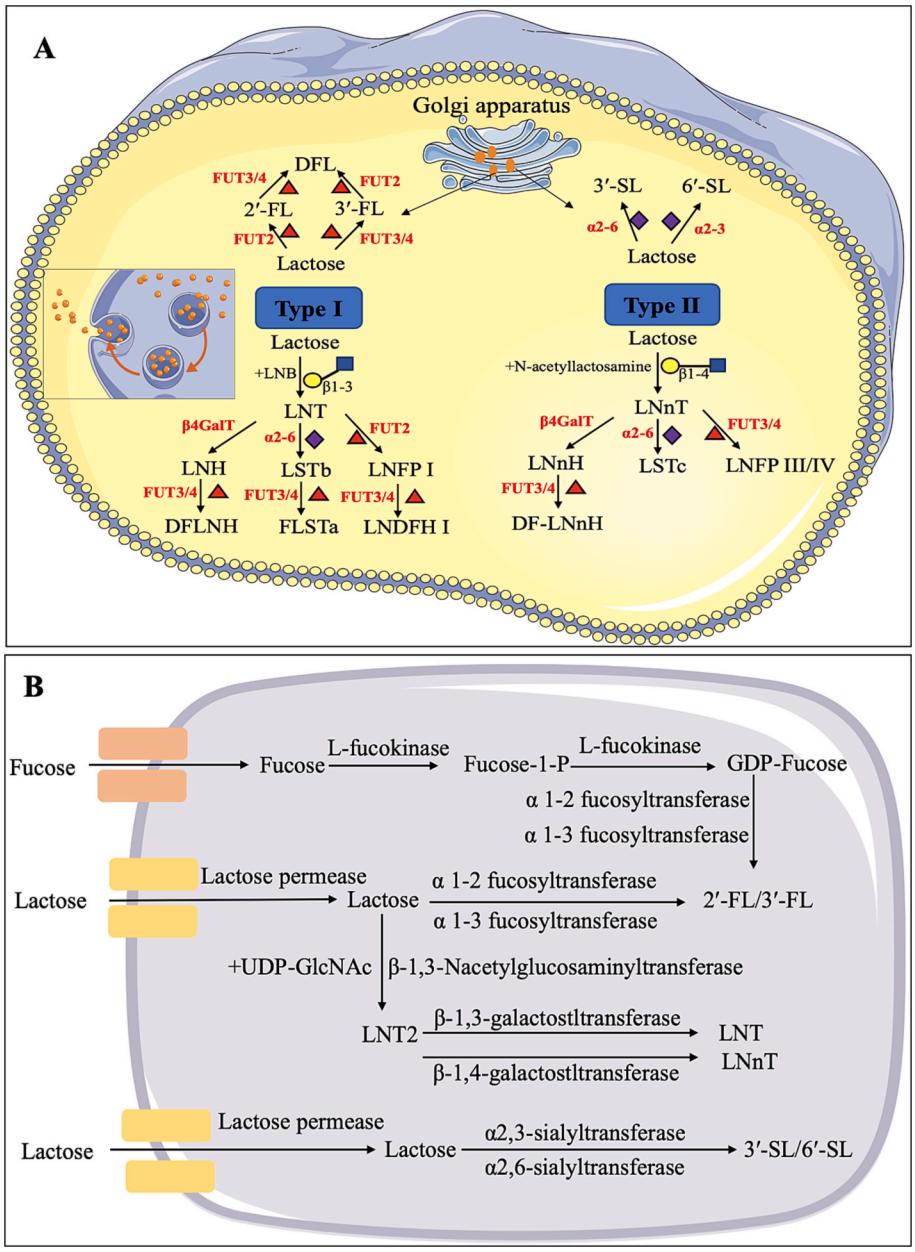

Fig. 8. Biosynthetic pathway of main MOs in the human mammary gland (A) and *E. coli* (B).

infants who are not breastfed. Two specific HMOs, namely 2′-FL and LNnT, have been recognized as novel foods by the European Union (EU) (Commission Implemented Regulation (EU) 2017/2470). In 2015, the European Food Safety Authority (EFSA) affirmed the safety of adding 2′-FL and LNnT to infant and follow-on formulae, at concentrations up to 1.2 g/L of 2′-FL and up to 0.6 g/L of LNnT, in a 2:1 ratio in reconstituted formula, for infants up to one year of age (EFSA-Q-2015-00052, EFSA Journal 2015; Román et al., 2020; Lasekan et al., 2022). As the repertoire of available HMOs expands, formulations containing various short-chain MOs have been developed. Parschat et al. (2021) devised a MOs-mix infant formula integrating the five most abundant MOs from different types (2.99 g/L 2′-FL, 0.75 g/L 3-fucosyllactose (3-FL), 1.5 g/L LNT, 0.23 g/L 3′-sialyllactose (3′-SL), and 0.28 g/L 6′-sialyllactose (6′-SL)). They observed that this 5 MOs-Mix was well tolerated and led to softer stools at a higher frequency compared to the control formula group (without MOs). Another MOs-mix comprising 2′-FL, difucosyllacto-N-hexaose (DFL), LNT, 3′-SL, and 6′-SL, exhibited the potential to bolster the development of the intestinal immune system and shift the gut microbiome closer to that of breastfed infants, particularly by promoting the growth of *Bifidobacterium infantis* (Bosheva et al., 2022). The intricate composition of MOs guides the selection of specific combinations in these MOs mixes. Their concentrations are based on reported physiological HMO levels in milk, with slight adjustments to emphasize certain MOs. These adjustments aim to harness synergistic and complementary effects, contributing to optimal infant development. However, substantial data regarding the application of the 5 MOs-mix remains limited, necessitating comprehensive preclinical models and clinical intervention studies to yield conclusive results.

Research on the application of MOs in adults, while not as developed as in infant nutrition, is a burgeoning field with significant potential. Šuligoj et al. (2020) demonstrated the ability of MOs to modulate immune function and enhance the gut barrier in adults. In a study by Smilowitz et al. (2017), it was found that supplementation with bovine MOs was well-tolerated by healthy adults and could potentially steer fecal microbiota toward more beneficial strains, especially when used in conjunction with probiotic cultures in a synbiotic approach. Although

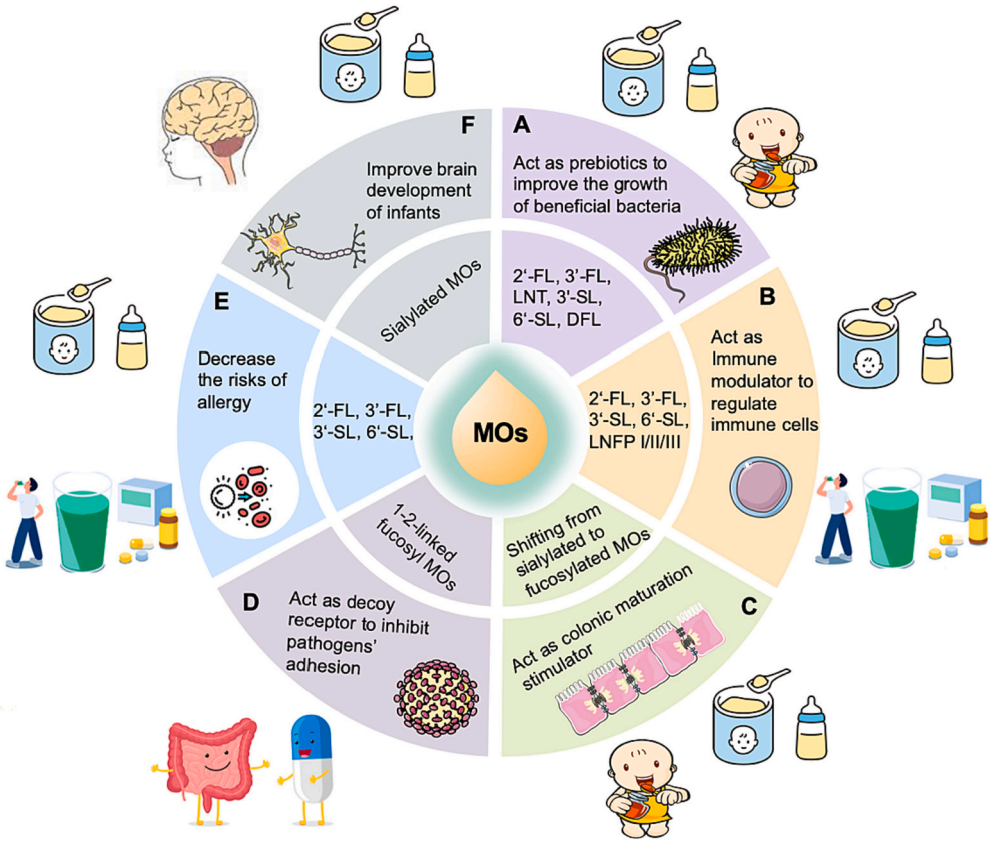

Fig. 9. Potential applications of MOs in the future. MOs have the potential applications in infant formulas, children functional food supplements, adult functional food supplements and pharmaceutical products based on their roles in regulating gut microbiota (A), modulating immune system (B), stimulating colonic maturation (C), inhibit the adhesion of pathogens (D), decreasing risks of allergy (E) and improving the brain development (F).

there have been indications from studies by Iribarren et al. (2021, 2020) that MOs might offer targeted therapeutic options for patients with irritable bowel syndrome (IBS), the need for more extensive and comprehensive studies is evident to fully elucidate the range and mechanisms of MOs' benefits in adult health.

This development is not just a leap forward in infant nutrition but also represents a broader application of biotechnological advancements in food science, potentially leading to new, health-promoting food products for various age groups.

7. Perspectives

Although significant strides have been made in understanding MO, challenges persist in this field. One major challenge is achieving cost-effective large-scale synthesis and purification methods to scale up the production of specific MOs for potential applications in infant formula and functional foods. MOs are complex molecules that require intricate synthesis processes, often resulting in low yields. Additionally, there is a need for the development of purification techniques to obtain highly pure MOs products. Recent advances have been focusing on improving the efficiency of synthetic methods, such as enzymatic approaches utilizing glycosyltransferases from bacteria. Purification techniques, including HPLC and membrane-based processes, have also been explored. Future breakthroughs may involve the development of novel enzymatic or chemical synthetic strategies, as well as innovative purification techniques.

While the composition of MOs is well-documented, their specific functions and mechanisms of action in infant health and development are not yet fully understood. Recent studies have made significant progress, linking certain MOs to benefits such as prebiotic effects, antimicrobial activity, and influencing the infant gut microbiota. Advances in high-throughput sequencing and metabolomics technologies have facilitated comprehensive profiling of microbial communities and metabolites, aiding in the elucidation of underlying mechanisms. These tools, combined with in vitro and in vivo models, are instrumental in uncovering the roles of specific MOs. Future breakthroughs may involve a deeper understanding of host-microbe interactions, long-term health outcomes, and personalized nutrition strategies. The ongoing research in this field holds the promise of unlocking new insights and applications of MOs, potentially revolutionizing infant nutrition and health.

CRediT authorship contribution statement

Qianqian Yao: Writing – original draft, Visualization. **Yanan Gao:** Visualization. **Nan Zheng:** Visualization, Writing – review & editing. **Veronique Delcenserie:** Supervision, Writing – review & editing. **Jiaqi Wang:** Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests.

Data availability

No data was used for the research described in the article.

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