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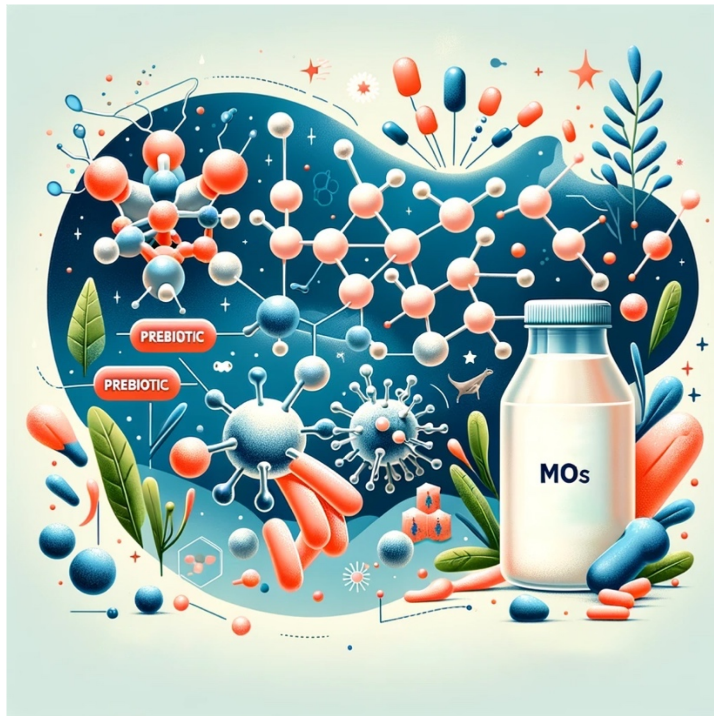
# Comparing milk oligosaccharides across various mammal species and assessing their impacts on intestinal health

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COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGR-BIO TECH

# Comparing milk oligosaccharides across various mammal species and assessing their impacts on intestinal health

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Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

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# Abstract

Qianqian Yao (2023). “Comparing milk oligosaccharides across various mammal species and assessing their impacts on intestinal health” (PhD Dissertation in English)

Gembloux Agro-Bio Tech, University de Liege, Belgium

133 pages, 11 tables, 28 figures.

Milk oligosaccharides (MOs), the third most abundant solid component of breast milk, are of great importance for infant growth. To date, knowledge on MOs profiles in different mammalian species is limited. MOs can alleviate colonic inflammation by shaping the composition of the gut microbiota. However, it remains unclear whether the gut microbiota mediates the protective activity of MOs and whether the beneficial effects change with different types of MOs.

To understand these questions, first, an LC-ESI-MS/MS method was developed to simultaneously analyze 11 MOs in milk samples from human, dairy cow, sheep, mare, camel, yak and buffalo. The results showed that human milk presented an abundance of MOs 7.6 to 15.8 times higher than in the milk of other animals. Fucosylated neutral forms were dominant. Dairy cow, camel, yak, sheep, buffalo and mare milks were similar in their MOs profiles and rich in sialylated forms. Compared to other animals, the composition of MOs in sheep is more similar to that of human milk. Moreover, heat treatment at 65 °C for 30 min had no effect on the concentration or distribution of MOs, whereas heat treatment at 135 °C for 60 s induced a decrease in their concentration and distribution.

Then the beneficial functions of 2′-fucosyllactose (2′-FL), the predominant MO in human breast milk, were assessed for alleviating colon inflammation, and the roles of gut microbiota in this process were further explored. The obtained results showed that 2'-FL improved colitis symptoms in a gut microbiota-mediated manner by reshaping the composition of gut microbiota, as well as a reorganization of mucin-utilizing bacteria. The underlying protective mechanism was associated with promoting an increase in goblet cell number and MUC2 secretion. Additionally, 2'-FL exerted anti-inflammatory effects by targeting the TLR4/MyD88/NF-κB-related inflammatory pathway.

Finally, to further compare the effect of individual MOs on enhancing mucin expression, the effects of 2'-FL (a representative fucosylated neutral MO), 3'-sialyllactose (3'-SL, a representative sialylated MO), galacto-oligosaccharide (GOS, commonly added into infant formula) and lactose (Lac, their structure core) on promoting MUC2 secretion in goblet cell models under homeostasis and inflammatory state were assessed. The results showed that 2′-FL and GOS, but not 3′-SL and Lac, were able to increase the mRNA expression of *MUC2*, *TFF3* and *CHST5* only in inflammatory conditions. Furthermore, 2′-FL was able to exert its function in goblet cells via several processes, such as promoting mucin secretion through NLRP6 and suppressing the TLR4 related inflammatory pathway.

Together, these results provide new insights into the functions of MOs from different species and could potentially help to better utilize them in infant formula.

**Key words:** milk oligosaccharides; human milk; animal milk; LC-MS/MS; 2′-FL; colonic inflammation; gut microbiota; MUC2; NLRP6

# Resume

Qianqian Yao (2023). “Comparaison des oligosaccharides complexes du lait de différents mammifères et évaluation de leur impact sur la santé intestinale” (thèse de doctorat en anglais)

Gembloux Agro-Bio Tech, Université de Liège, Belgium

133 pages, 11 tableaux, 28 figures.

Les oligosaccharides du lait (MOs) représentent dans leur ensemble le troisième composant solide le plus abondant du lait maternel, et sont d'une grande importance pour la croissance du nourrisson. A ce jour, les connaissances sur les profils des MOs chez différentes espèces de mammifères sont limitées. Par exemple, on ne sait toujours pas si le microbiote intestinal affecte l'activité protectrice des MOs, et si les effets bénéfiques changent en fonction des différents types de MOs.

Afin de répondre à ces questions, une méthode LC-ESI-MS/MS a été mise au point dans cette étude pour analyser simultanément 11 MOs dans des échantillons de laits humains, de vaches laitières, de brebis, de juments, de chamelles, de yacks et de bufflonnes. Les résultats ont montré que le lait humain présentait une abondance de MOs 7,6 à 15,8 fois plus élevée que dans les laits en provenance d’autres espèces animales. Les formes neutre et fucosylées étaient dominantes. Les laits de vache laitière, de chamelle, de yak, de brebis, de bufflonne et de jument étaient similaires dans leurs profils de MOs et tous étaient riches en formes sialylées. Comparée aux autres espèces, la composition des MOs chez les brebis est la plus proche de celle du lait humain.

Par ailleurs, un traitement thermique de 65 °C durant 30 min n'a eu aucun effet sur la concentration ou la distribution des MOs, alors qu’un traitement thermique de 135 °C durant 60 secondes les a fait chuter considérablement, suggérant qu’une plus grande attention doit être portée aux températures utilisées lors des traitements thermiques des produits laitiers.

Ensuite, les fonctions bénéfiques du 2′-FL, le MO prédominant dans le lait maternel, ont été évaluées sur la diminution de l'inflammation du côlon, et les rôles du microbiote intestinal dans ce processus ont été explorés plus en détail. Les résultats ont montré que les symptômes de colites étaient améliorés grâce au 2'-FL, et ce, de manière médiée par le microbiote intestinal via un remaniement de celui-ci, notamment un remaniement des bactéries utilisant la mucine. Le mécanisme sous-jacent était associé à une augmentation du nombre de cellules caliciformes et de la sécrétion de MUC2. De plus, le 2'-FL a pu exercer des effets anti-inflammatoires en ciblant la voie d'inflammation liée au TLR4/MyD88/NF-κB via la surexpression de NLRP6.

Enfin, pour comparer davantage l'effet des MOs individuels sur l'amélioration de l'expression de la mucine, une évaluation des effets du 2'-FL (un MO neutre fucosylé représentatif), du 3'-SL (un MO sialylé représentatif), du GOS (couramment ajouté dans les préparations pour nourrissons) et du lactose (Lac, leur noyau de structure) a été réalisée sur la promotion de la sécrétion de MUC2 dans des modèles de cellules caliciformes sous homéostasie et sous état inflammatoire. Les résultats ont montré

que le 2′-FL et le GOS, mais pas le 3′-SL et le Lac, étaient capables d'augmenter l'expression de l'ARNm de MUC2, TFF3 et CHST5 uniquement en cas d'état inflammatoire. En outre, le 2′-FL a pu exercer sa fonction dans les cellules caliciformes via plusieurs processus, tels que la promotion de la sécrétion de mucine via le NLRP6 et la suppression de la voie inflammatoire liée au TLR4.

Ensemble, ces résultats fournissent de nouvelles informations sur les fonctions des MOs en provenance de différentes espèces et pourraient potentiellement aider à mieux les utiliser dans les produits laitiers.

**Mots clés:** oligosaccharides de lait; Le lait maternel; Lait animal; LC-MS/MS; 2′-FL; Inflammation du côlon; Microbiote intestinal; MUC2; NLRP6

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Last, I want to express my gratitude to the broader academic community, whose body of knowledge provided the foundation for this thesis. It is an honor to contribute to the collective pursuit of understanding and knowledge.

As I conclude this chapter of my academic journey, I am filled with a mixture of emotions—accomplishment, humility, and excitement for what lies ahead. This thesis stands as a testament to the collaboration, dedication, and passion that define the scientific pursuit.

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Qianqian Yao
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Liege, Belguim

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# List of 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 |
| *B. infantis* | *Bifidobacterium longum* subsp. *infantis* |
| *B. longum* | *Bifidobacterium longum* subsp. *longum* |
| CE | capillary electrophoresis |
| CHST5 | carbohydrate sulfotransferase 5 |
| DAI | disease activity index |
| DSS | dextran sulfate sodium |
| ESI | electrospray ionization |
| FMT | fecal microbiota transplantation |
| FOS | fructooligosaccharide |
| Fuc | fucose |
| FUT | fucosyltransferase |
| Gal | galactose |
| GAL3ST2 | galactose-3-o-sulfotransferase 2 |
| 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 |
| IBD | inflammatory bowel disease |
| IL-1β | interleukin 1β |
| Lac | lactose |

| 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 |
| LOQ | limit of quantitation |
| LPS | lipopolysaccharide |
| MALDI | atrix assisted laser desorption/ionization |
| MOs | milk oligosaccharides |
| MRM | multi-reaction monitoring |
| MS | mass spectrometry |
| MUC2 | mucin 2 |
| MyD88 | myeloid differential protein-88 |
| Neu5Ac | N-acetylneuraminic acid |
| NeuAc | sialic acid |
| NF-κB | nuclear factor |
| NLRP6 | nod-like receptor 6 |
| NMR | nuclear magnetic resonance |
| PAD | photodiode array detector |
| PAS | periodic acid schiff |
| PGC | porous graphitized carbon |
| RETNLB | Resistin Like Beta |
| RPLC | reversed-phase LC |
| *Se* | secretor gene |
| SEC | size exclusion chromatography |
| TFF3 | trefoil factor 3 |
| TLRs | toll-like receptors |
| TNF-α | tumor necrosis factor α |
| UDP-Gal | uridine diphosphate-Gal |
| UV | ultraviolet |

# Chapter 1

## Objectives and outline of thesis

# 1 Background

Human milk is a nutritious source of proteins, fats, micronutrients, prebiotics and probiotics. As the initial food for neonates, it contributes significantly to their growth during the first several months of life. Milk oligosaccharides (MOs), the third most abundant solid component of breast milk, are of great significance for infant growth (Bode, 2012). They are a group of complex carbohydrates and made up of a unique combination of monosaccharide building blocks, such as glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (NeuAc) (Chaturvedi et al., 2001). According to the decoration with fucose, NeuAc or none of them, MOs are classified into three different groups: fucosylated, sialylated and non-fucosylated neutral ones (Ninonuevo et al., 2006).

The concentrations and patterns of MOs vary significantly among different species. The majority of studies focus on revealing the MOs concentration and composition in human milk. Currently, over 200 structures of MOs in breast milk have been identified with 70% fucosylated MOs and 20% sialylated ones (Ninonuevo et al., 2006). The concentration of total MOs is 5-20 g/L in mature breast milk with 2′-FL being the most abundant (Tao et al., 2011). Domestic animals’ MOs exhibit similar structural elements compared to those in human milk; however, they are 10-100 times less abundant (Wang et al., 2020; Boehm & Stahl, 2003). Currently, most research is focused on dairy cows and goats (Tao et al., 2009; van Leeuwen et al., 2020). Although there are some studies on MOs in other animal species, such as camel, yak, and donkey, they are mostly focused on elucidating MOs structures (Yan et al., 2018; Albrecht et al., 2014). Limited research has been conducted on the systematic comparison of MOs among different species, especially regarding absolute quantitation.

In breast-fed infants, more than 95% of MOs can pass through the gastrointestinal tract intactly and reach the intestine for microbes use (Goehring et al., 2014). About 1% of digested MOs can be absorbed into the blood circulation to further exert beneficial functions in other target organs (Bode, 2015). Intestine is the main target organ for MOs. On the one hand, MOs can be metabolized by *Lactobacillaceae*, *Bifidobacterium* and *Bacteroides* to promote their growth, as well as prevent some pathogenic bacteria such as *Campylobacter jejuni* and *Escherichia coli* (*E. coli*) from adhering to epithelial cells. On the other hand, in addition to regulating the composition of microbiota, MOs pool promotes the proliferation of crypt cells and then increases mucin secretion to enhance intestinal physical and chemical barrier function, to consequently alleviate the inflammation (Bering, 2018; Wu et al., 2018). Intestinal microbiota has been reported to mediate the function of biochemicals (Du et al., 2017). Thus, we wondered whether the gut microbiota could affect the protective activity of MOs. Moreover, the function of MOs depends on their unique structure, like their decorated groups. Therefore, the effects among different types of MOs should be compared to locate the most important fraction.

# 2 Problem definition

Currently, the majority of studies focus on revealing the MOs concentration and composition in human milk and how they vary during the lactation. For domestic animals, few researchers have analyzed and compared their MOs composition. These limited data hinder us from gaining a full understanding of MOs files among domestic animals.

Although MOs were reported to improve the mucin barrier in several mice inflammatory models, it remains unclear whether this effect is microbiota-mediated. Moreover, the beneficial effects among different types of MOs have not yet been compared.

To answer these questions, in vitro and in vivo studies have been performed. These experiments that make up the body of this thesis are briefly depicted in the following section.

# 3 Objectives

This thesis aims to compare the composition and pattern of MOs in 7 different mammalian species; to clarify the protective effects of 2′-FL, the predominant MO in human milk, on alleviating colon inflammation, and the roles of gut microbiota in this process; and to compare the function of different types of MOs in promoting mucin secretion.

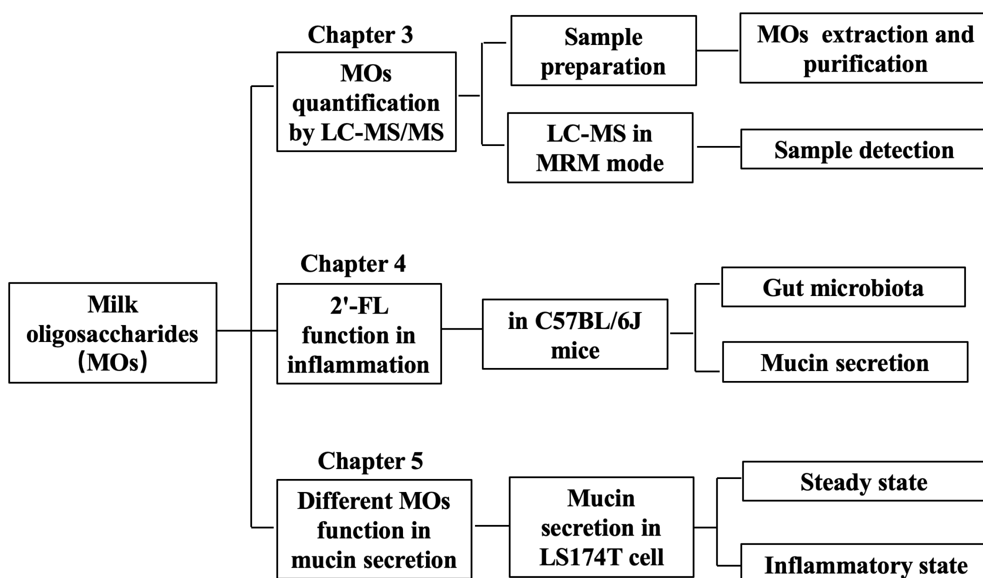


**Figure 1** Overall working scheme of the thesis.

# 4 Content of the thesis

The thesis is structured into 6 Chapters. After the introduction of the objectives of the thesis (Chapter 1), the literature review of the art about MOs is dedicated (Chapter 2).

In the experimental part, the composition and pattern of MOs in 7 different mammal species by LC-ESI-MS/MS method were compared in Chapter 3. In this chapter, an LC-ESI-MS/MS method was established to simultaneously analyze 11 MOs (2′-FL, 3′-FL, LNT, LNnT, 3-GSL, 3′-SL, 6′-SL, LNFP I, LNFP II, LNDFH I and LNDFH II) in milk samples from human, dairy cow, sheep, horse, camel, yak and buffalo. This study was published in Food Chemistry (2023). Then the beneficial functions of 2′-FL, the predominant MO in human milk, was assessed for alleviating colon inflammation, and the roles of gut microbiota in this process were further explored (Chapter 4). This study was published in Frontiers in Nutrition (2022). Then, to further compare the functions of individual MOs in enhancing mucin expression, the effects of 2'-FL (a representative fucosylated neutral MO), 3'-SL (a representative sialylated MO), GOS (commonly added into infant formula) and lactose (Lac, their structure core) on promoting MUC2 secretion in goblet cell models under homeostasis and inflammatory stages were assessed (Chapter 5). This study was published in Foods (2023). The thesis is then finalized by a general discussion leading to a conclusion and perspectives (Chapter 6).

# 6

# Chapter 2

## The overall review of MOs and their applications

# 1 MOs structure and composition

## *1.1 The finding of MOs*

The MOs were discovered when microbiologists and chemists were attempting to comprehend the apparent health advantages of human milk feeding. Nearly simultaneously in 1900, Moro and Tissier reported that infants who were breastfed as opposed to those who were given infant formula had different bacterial compositions in their feces (Moro, 1990; Tissier, 1990). In 1926, Schönfeld et al. found that human milk contained a crucial growth factor for *Lactobacillus bifidus* (now known as *Bifidobacterium bifidum*), whereas cow's milk showed a very low promotion of those strains' growth. However, which components in human milk influenced the fecal bacteria was not clear at that time.

Coincidentally, chemists discovered a unique unknown carbohydrate fraction in human milk, which then was characterized as "gynolactose" (Polonowski and Lespagnol, 1929, 1933). After decades of studying, these unclear "gynolactose" were successfully separated into individual MOs (Polonowski and Montreuil, 1954). And still, chemists did not know the functions of these oligosaccharides.

The story became clearer when György and Kuhn proposed a link between the research on bacteria by Moro (1900) and Tissier (1900) and the research on "gynolactose" by Polonowski and Lespagnol (1929, 1933). Then, their team carried out a series of experiments and finally identified the crucial growth factor for *Lactobacillus bifidus* as MOs, which are the key component in determining bacterial composition in babies' feces (György et al., 1954).

As research continues, researchers are discovering that MOs are actually a group of carbohydrates, which are the third most important nutritional component of human breast milk. Additionally, they exhibit several beneficial effects on children, including supporting their brain development and immune system maturation, as well as shaping the composition of the gut microbiota, which will be summarized in the following sections.

## *1.2 The structures of MOs*

In addition to lactose, the carbohydrate or carbohydrate containing components in milk include glycoconjugates and free MOs.

Regarding to glycoconjugates, according to their localization and function, protein glycans have been to be compartmentalized into three basic categories groups, i.e. N-Linked, O-Linked and proteoglycans (Lyons et al., 2015; Schnaar, 2015). In particular, a fraction of asparagine (ASP) residues in the Asn-X-Ser/Thr motif, are related to N-glycans, whereas a subset of serines and threonines are associated to O-glycans (Schachter, 2000, Yan and Lennarz, 2005). Proteoglycans are also linked to the serine or threonine residues with long disaccharide repeats (Esko and Selleck, 2002). For glycolipids, the glycans are linked to a ceramide lipid moiety (Schnaar, 2015). Altogether, these glycans make up the diverse cells surface structures that perform

various functions. Glycoconjugates are reported to play a vital role in regulating immunological responses and diseases are caused by mutations in glycosylation (Freeze et al., 2014), which, however, is not further discussed here.

For MOs, they are made up of a unique combination of monosaccharide building blocks, including glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (NeuAc) (Chaturvedi et al., 2001). Generally, lactose, composed of Gal and Glc, is the basic unit of MOs, and then it has been elongated in a straight or branched chain by different numbers of Galβ1-3GlcNAc (lacto-N-biose, type I chain) or Galβ1-4GlcNAc units (N-acetyllactosamine, type II chain) (Bode, 2015). When lacto-N-biose is added, the chain can no longer be extended, wherease, this is not the case with N-acetyllactosamine, which can allow the chain to be stretched further (Figure 2). With this system, disaccharides attaching themselves to the chain via β1-3 or β1-6 bonds lead to the formation of linear or branched chains. Linear or branched chains are further decorated with Fuc and NeuAc residues. These various links, branching patterns and decorative residues lead to diverse structures of MOs, and so far, more than 200 structures of human MOs (HMOs) have been identified (Leo et al., 2010; Ruhaak and Lebrilla, 2012).

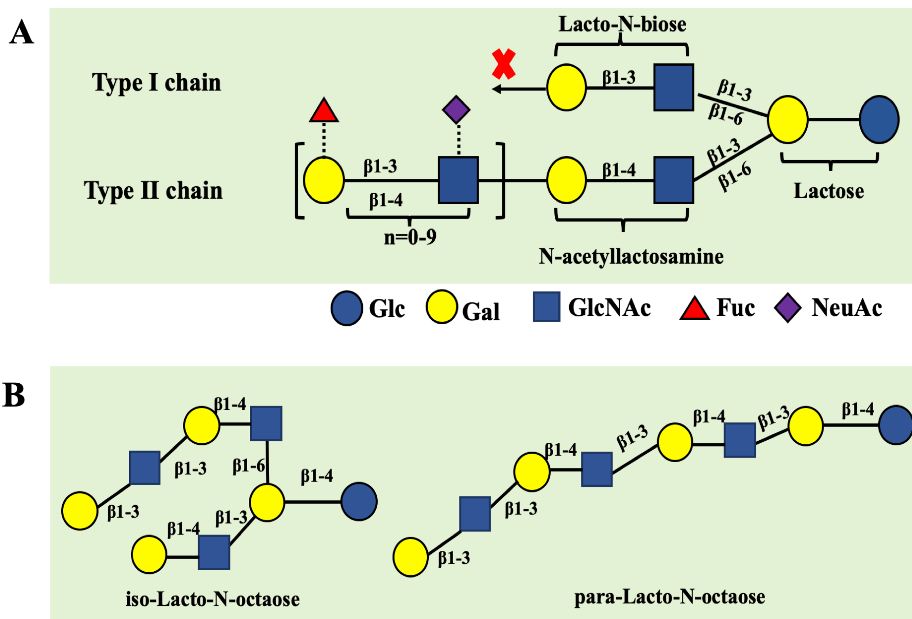


**Figure 2** Basic MO units and structure. **A**, Structural blueprint of MOs; **B**, An example of structural isomers generated by different linkages. The figure panels are adapted from Cheng et al., (2021).

## *1.3 The classification of MOs*

The MO backbone can be either sialylated in the a2-3 or a2-6 linkage or fucosylated in the α1-2, α1-3 or α1-4 linkage, and accordingly form different MOs groups, i.e. sialylated, fucosylated and non-fucosylated neutral (Totten et al., 2012).

The fucosylated MOs are decorated with at least one Fuc, and 2′-fucosyllactose (2′-FL) and 3′-fucosyllactose (3′-FL) are the presentative ones (Figure 3). Overall, about 70% of MOs in human milk are fucosylated and approximately 20% are sialylated (Ninonuevo et al., 2006). The sialylated MOs have at least one NeuAc. And the most abundant sialylated MOs are 3′-sialyllactose (3′-SL) and 6′-sialyllactose (6′-SL) (Figure 3). In animal milk, the sialylated MOs are dominant. There are also MOs without Fuc or NeuAc residues, which are called non-fucosylated neutral MOs, such as LNT and LNnT. Different types of MOs have different chemical properties, indicating that they might exert different functions, which will be summarized in the follow section.

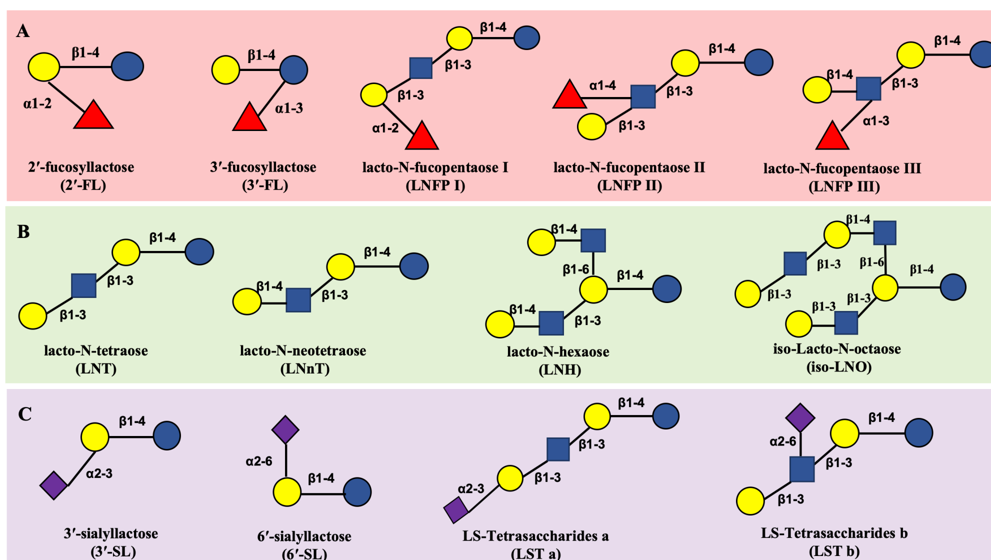


**Figure 3** Three classifications of MOs. **A**, Representative fucosylated neutral MOs; **B**, Representative non-fucosylated neutral MOs; **C**, Representative sialylated MOs. The figure panels are adapted from Cheng et al., (2021).

# 2 MOs quantification

The numerous coexisting isomeric structures and multiple connectivity sites make the analysis and quantification of MOs challenging. Nowadays, several methods have been developed to quantify MOs. Generally, MOs quantification includes three main steps: MOs preparation, separation of individual MO and quantification of individual MO.

## 2.1 MOs preparation

Milk is a complex matrix containing many nutrients, which 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 (Tonon et al., 2019; Porfirio et al., 2020). However, the crystallization of lactose and co-crystallization of MOs with lactose caused by organic solvents could hinder the detection of MOs (Tonon et al., 2019). After removing protein and lipids, crude MOs are then further purified using porous graphitized carbon (PGC) since a significant quantity of lactose and salts are included. The PGC works based on complex interactions between graphite and MOs, then the non-bonded lactose can be removed by wash liquid (Packer et al., 1998). In many researchers, MOs are usually converted to a more stable version, such as labeling with a fluorescent tag, or to a permethylated form (Tonon et al., 2019; Oursel et al., 2017; Ruhaak and Lebrilla, 2012). With the development of MOs quantification technology, however, the additional chemical step is controversial as it is time-consuming and not ideal for high-throughput analysis.

## 2.2 MOs separation and analysis

Once the pure MOs are prepared, the separation of individual MO can follow. Currently, high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are the two most frequently used techniques for MOs separation. In recent years, a wide range of HPLC methods have been established, such as reversed-phase liquid chromatography (RPLC), normal phase liquid chromatography (NPLC), hydrophilic interaction chromatography (HILIC), high pH anion-exchange chromatography (HPAEC) and size exclusion chromatography (SEC). Among them, RPLC, HILIC and HPAEC are currently widely used.

RPLC is the most common separation technique for no-polar carbohydrates. It works based on hydrophobicity of the solute against the stationary phase (C18 column) (Porfirio et al., 2020). The more hydrophobic the carbohydrate, the more it binds to the stationary phase, and the higher concentration of the organic solvent required to elute it, resulting in a different retention time. However, with this method, MOs must be derivatized with a hydrophobic tag, such as a methylated label, to retain hydrophobicity (Kurz et al., 2021). Hydrophobicity of tag and mobile phase components significantly influences MOs separation and elution order in RPLC (Vreeker and Wuhrer, 2017).

HILIC is a common method to separate polar carbohydrates, which relies on the hydrophilic interaction between MOs and stationary phase. The separation of MOs mainly depends on the number of polar groups (Pabst and Altmann, 2011). The higher the hydroxyl group contained in the MOs chain, the higher the hydrophilicity, and the later the appearance of the peak. Yan et al (2018) successfully explored sialylated MOs in human breast milk, as well as seven other animal milks (cow, yak, buffalo, camel, donkey, swine, and sheep), using online solid phase extraction (SPE)-HILIC. Li et al (2021) used the SPE-HILIC method to separate the acidic MOs in rats and mice and found twelve new ones. However, the derivatization of MOs (labeled with

a fluorescent tag) is also required in this method to provide an adequate response when the UV detection is used. On the other hand, HILIC works poorly for quite similar small carbohydrates, such as trisaccharides (Balogh et al., 2015).

Another well-established method for separating MOs is HPAEC, which requires no labeling. MOs have a differential ion exchange capacity with the stationary phase, which means that MOs can be eluted separately by appropriate buffer solutions (Cataldi et al., 2000). Neutral MOs (no charge) cannot be combined with a stationary phase by ion exchange, and, therefore, can be eluted with water. While the negatively charged acidic MOs can be bound to the stationary phase and must be eluted respectively by saline solution with an appropriate concentration (Kunz et al., 1996). One of the biggest drawbacks of HPAEC is that the eluted solution contains many structurally similar MOs, requiring the need of a further separation. Hence, in recent years, HPAEC has become a less widely applied separation method.

CE is a separation technique for charged MOs; therefore, pre-charged MOs must be prepared to ensure that electrophoresis works (Ruhaak et al., 2010). Generally, 2-aminobenzoic acid (2-AA), but also 2-aminoacridone (2-AMAC) are commonly used as the charge labels in this system, as well as 8-aminopyrene-1,3,6-trisulfonic acid (Galeotti et al., 2014). The fact that the migration rate across the column is inversely correlated to the size-charge ratio is one of the main obstacles in CE. This implies that structural isomers will elute together, such as 2′-FL and 3′-FL, and LNT and LNnT. Galeotti et al. (2014) analyzed 17 2-aminoacridone labeled MOs in human milk by CE, and they found that LNFP II cannot be separated from LNFP III, but both were sufficiently separated from LNFP I. The same study differentiated 2′-FL and 3′-FL, but not 3′-SL and 6′-SL. Co-elution also appeared in another study by Olivares et al. (2015), who observed that four pairs of isomers cannot separate from each other, i.e. 2′-FL and 3′-FL, 3′-SL and 6′-SL, LNT and LNnT, and LNFP I and LNFP III. Extensive co-elution in CE prevents accurate analysis of individual MO.

Generally, each MO can be identified according to the same retention time as its corresponding standards in LC and CE methods. When combined with PAD or UV detection techniques, quantification of individual MO is possible. However, there are several drawbacks. On the one hand, co-elution occurs in most separation methods, which means that accurate quantification is impossible. On the other hand, standards are usually not offered commercially, and if they are, they are either very expensive or of insufficient purity. In these cases, it is necessary to combine other analytical approaches, such as mass spectrometry (MS), to achieve MOs quantification.

## *2.3 MOs quantification and structural analysis*

Currently, MS-based identification and unclear magnetic resonance (NMR) are common tools for MOs analysis, which can not only identify MOs, but also analyze their specific structures through fragmentation studies. To date, more than 200 neutral and anionic (sialylated) MOs have been identified (Leo et al., 2010; Ruhaak and Lebrilla, 2012).

MS-based techniques can be applied separately or in conjunction with HPLC separations. Although the former may be faster as no separation is required, it may

still be a significant disadvantage if the samples are not pure. In addition, isomer separation cannot be achieved without first carrying out a column separation. Therefore, MS-based techniques are often used in conjunction with LC separation of MOs molecular species. The two most widely used MS-based ionization methods for MOs analysis are electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI). In MALDI ionization, a laser beam is used to ionize the sample after it has been coated or mixed with an energy absorbing matrix. With this technique, the analytes produce solitary protonated ions (Lai & Wang, 2017). In contrast, ESI uses an electric field to change analytes from the solution phase into numerous charged ions in the gas phase (Zhong et al., 2014). Porfirio et al (2020) identified 80 permethylated MOs (including sialylated and/or fucosylated MOs) in human milk samples by LC-ESI-MS/MS method, with the highest permethylated masses >5000 Da. Recently, Remoroza and coworkers (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. When standards are not available, this reference standard mixture provides a promising option.

NMR is another technique, which focuses on the analysis of specific combinations within MOs (Smilowitz et al., 2013; Vliegenthart and Kamerling, 2007). The most outstanding advantage of this method is that different MOs with different Lewis epitopes can be detected effectively (Van Leeuwen et al., 2018), with no clean-up steps or as few as possible. However, the similar structures among MOs result in an overlap signal, which makes it difficult to differentiate them. Additionally, MOs should be pre-derivated, such as by adding an isotope label, before further structural analysis (Hamagami et al., 2020).

The process of MOs quantification and analysis is shown in Figure 4. Taking all into account, there are several possibilities for improvement. First, sample preparation is complicated and time-consuming with the risk of losing MOs at low concentration. More importantly, when MOs are derivate, the difference in labeling efficiency must be taken into account (Austin & Bénet, 2018). For high-throughput analysis, easier sample preparation is needed to enable method development. Second, all methods can only provide relative quantification of MOs, if there are no standards. This means that higher quality standards are needed for production. Last but not the least, the comparison of MOs concentrations in different research are challenging because there are large differences in the techniques adopted to analyze MOs.

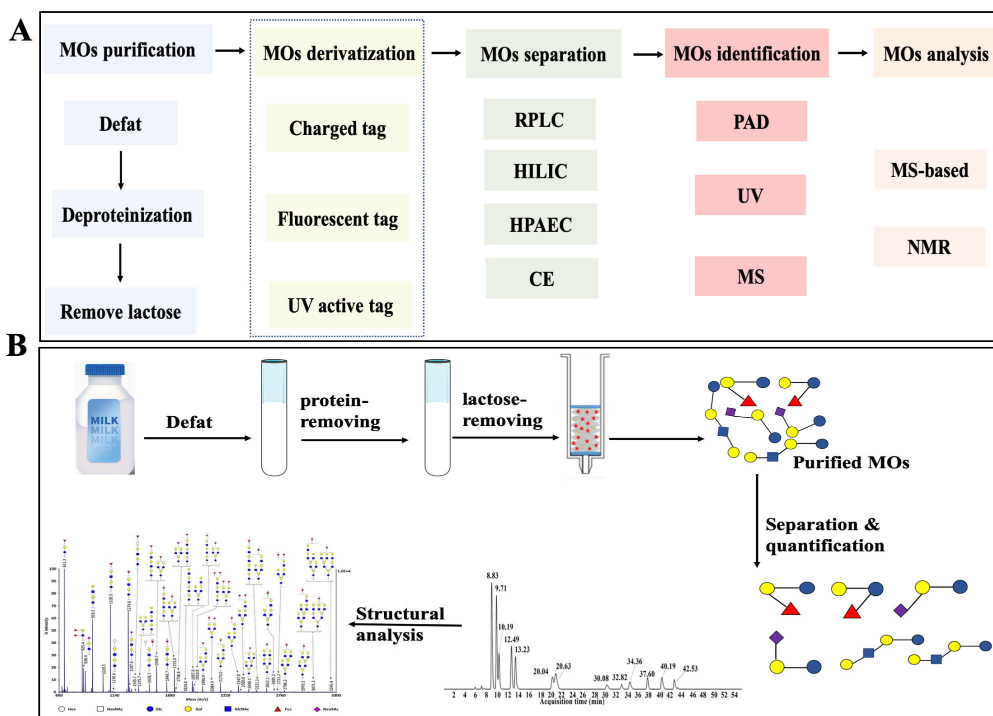


**Figure 4** Flow chart of MOs quantification and analysis. **A**, The technique used during the four main processes; **B**, The general flow chart. The figure panels are adapted from Porfirio et al. (2020) and Wang et al. (2022).

# 3 MOs concentration in various species

## *3.1 General comparison of MOs between human milk and animal milk*

The concentrations and patterns vary significantly among different species. Human milk had a completely different composition pattern of MOs when compared to other mammal milk, mainly manifested in two aspects. On the one hand, breast milk contains 10-100 times more total MOs than other mammal milks (Wang et al., 2020; Boehm & Stahl, 2003). On the other hand, the structures of human milk MOs are more complex and diverse. Currently, over 200 structures of MOs in breast milk have been identified, of which about 162 have been structurally characterized (Urashima et al., 2018). Among them, fucosylated neutral MOs are the most prevalent, accounting for 50-80% of the total; however, those in domestic animal milk are very rare (<1%) (Figure 5, Tao et al., 2011). It has been hypothesized that the system glycosidase/glycosyltransferases or the enzymatic activity of epithelial cells are

responsible for the variations in MOs patterns between human milk and other mammal species (Nwosu et al., 2012).

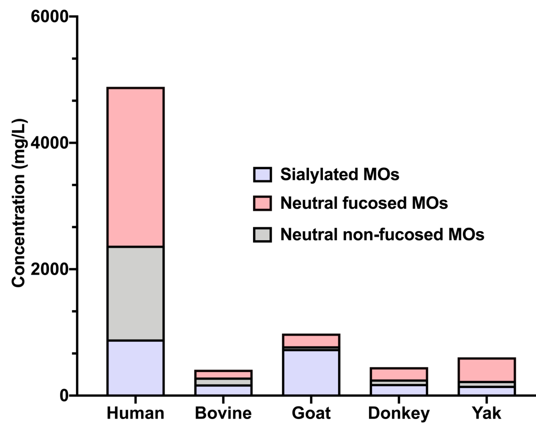


**Figure 5** Concentrations of different types of MOs in different mammalian species. Data are obtained from Wang et al., 2020.

## *3.2 MOs concentration and composition in human milk*

Currently, the majority of studies focus on revealing the MOs concentration and composition in human milk. Although more than 200 structures of MOs have been identified, few MOs can be quantified. Table 1 shows the concentrations of 16 MOs in breast milk samples for the whole lactation period (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). The mean total MOs content (16 MOs) is 7935 mg/L, ranging from 1773 to 21025 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 on average), widely ranging from 380 to 2872 mg/L. Human milk contains relatively few sialylated MOs, with 3′-SL, 6′-SL and DSLNT being the most prevalent ones. Additionally, the ratio of fucosylated MOs: non-fucosylated MOs: sialylated MOs in human milk is about 66%:16%:18%, as calculated by the data in Table 1.

The nutrient concentrations vary throughout lactation, so do MOs. To explore the influence of lactation time on MOs concentrations, the time postpartum are classified into 4 periods, i.e., 0-14 days (containing colostrum), 14-30 days, 30-90 days and ＞ 90 days. The data in Table 1 are reanalyzed according to the lactation periods, which is shown in Figure 6. The contents of majority of analyzed MOs are declined with prolongation of lactation. Compared to the first period, the concentrations of 2′-FL, LNFP I, LNT+LNnT, 6′-SL and DSLNT decreased 1.5-, 3.7-, 1.8-, 1.7- and 2.2-folds in the fourth period, respectively. However, the 3′-FL sees a 2.0-folds rise during the fourth period. The ratio of 2′-FL to 3′-FL at 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, respectively. And the concentrations of LNH, 3′-SL and LSTb seems to be stable during the whole lactation.

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

**Table 1** Examples of main MOs found in human milk and colostrum (mg/L).

| MOs | Mean | Minimum | Maximum |
|---|---|---|---|
| Fucosylated neutral MOs | | | |
| 2′-FL | 1990±998 | 410 | 4130 |
| 3′-FL | 766±487 | 147 | 2350 |
| LNFP I | 904±654 | 158 | 2250 |
| LNFP III | 364±331 | 60 | 1869 |
| LNDFH I | 859±404 | 156 | 1550 |
| LNDFH II | 326±319 | 64 | 960 |
| Non-fucosylated neutral MOs | | | |
| LNH | 95±50 | 17 | 170 |
| LNnH | 63±52 | 18 | 160 |
| LNT+LNnT | 1143±553 | 380 | 2972 |
| Sialylated MOs | | | |
| 3′-SL | 218±189 | 97 | 1125 |
| 6′-SL | 451±368 | 73 | 1420 |
| LSTa | 48±37 | 10 | 141 |
| LSTb | 100±56 | 33 | 193 |
| LSTc | 247±227 | 29 | 940 |
| DSLNT | 358±174 | 121 | 795 |
| Total | 7935 | 1773 | 21025 |

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).

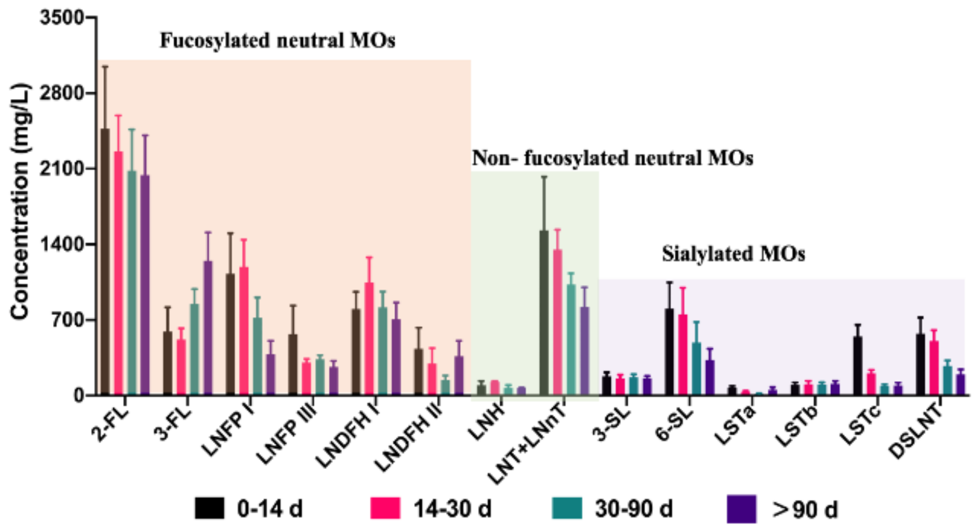


**Figure 6** Concentration changes of representative neutral and acidic MOs in human milk during lactation. Summarized from references of MOs quantification, including Asakuma et al., (2007), Bao et al., (2007, 2013), Borewicz et al., (2019), Leo et al., (2009, 2010), Ma et al., (2018), Samuel et al., (2019), Thurl et al., (2010), Zhang et al., (2019), Siziba et al., (2021), Alderete et al., (2015).

## *3.3 MOs concentration and composition in animal milk*

In addition to the breast milk, MOs also exist 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, much lower than that 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 2 shows the concentrations of main quantified MOs in domestic animal milk (Liu et al., 2014; McJarrow et al., 2004; Shi et al., 2021; Chatziioannou et al., 2021; Wang et al., 2020a; Difilippo et al., 2015, 2016; Licitra et al., 2019; Kunz et al., 1999; Albrecht et al., 2014; Yan et al., 2018a). Most of the time, the studies on MOs quantification from animal milk samples have focused on dairy cow, sheep and horse (colostrum), and their MOs concentration are around 414-1720, 562-1349 and 2420-13850 mg/L, respectively. Due to the lack of adequate data, it is difficult to summarize MOs concentration in came, monkey, yak, elephant and pig.

Different from human milk, the sialylated MOs are predominant in concentration (80% - 90% of the total MOs) in domestic animals, with 3′-SL and 6′-SL detected in almost all samples (Albrecht et al., 2014). However, the situation is slightly different in goat milk. Proportion 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 (Shi et al., 2021; Chatziioannou et al., 2021; Wang et al., 2020a).

It is worth to note that the quantity of MOs in animal milk is variable, making comparisons between research and species challenging. On the one hand, variations might be caused by different analytical techniques. On the other hand, the MOs composition is subject to genetic variation and course of lactation. Therefore, additional data are needed to obtain the reliable general understanding about MOs composition in animal milk.

**Table 2** Examples of main MOs found in milk and colostrum from domestic animals.

| Abb. | Concentration in milk samples (mg/L) | | | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|
| | Bovine | Goat | Camel | Monkey | Yak | Horse (colostrum) | Elephant | Swine | |
| 2′-FL | nd | 3.11-41.41 | 0.19 | nd | nd | nd | nd | nd | c,d,e,h,i,j |
| 3′-FL | 0.36-86.55 | 0.44-71.21 | 3.02 | 123.05 | 63.3 | nd | 160-310 | nd | c,e,h,i |
| LNFP I | 71.17 | 98.59 | nd | 34.84 | 99.89 | 30-110 | 370-126 | nd | e, f,h,i,k |
| LNFP II | nd | nd | — | nd | — | nd | — | nd | i |
| LNFP III | nd | nd | nd | nd | — | nd | — | — | i |
| LNDFH I | nd | nd | — | — | — | — | — | — | i |
| LNDFH II | nd | nd | — | — | — | — | — | — | i |
| 3′-GSL | 4.18 | 31.69-147.94 | 1.15 | nd | — | 40-230 | 1201-4000 | 190-1980 | c, d, f, h,k |
| 6′-GSL | nd | 8.05-65.67 | — | nd | — | 550-3150 | — | 640-1230 | d, f, k |
| LNH | — | — | — | nd | — | 10-70 | — | — | f |
| LNnH | — | 1-5 | nd | nd | — | nd | — | 100-430 | H,I,k |
| LNT+LNnT | 0.41-37.66 | 0-1.40 | 1.57 | 42.17 | 10.74 | 20-390 | 1160-2490 | — | c, e, f, g, h |
| 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 |
| 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 |

| Abb. | Concentration in milk samples (mg/L) | | | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|
| | Bovine | Goat | Camel | Monkey | Yak | Horse (colostrum) | Elephant | Swine | |
| DSL | 201-283 | 1.9 | — | nd | — | 20-400 | — | — | b, c, f , j |
| LSTa | nd | nd | — | nd | nd | 20-830 | 50-520 | 100-250 | e, f, j |
| LSTb | 15.39 | 30.69 | — | 49.16 | nd | — | nd | — | e,i |
| LSTc | nd | 9.92 | — | 0.30-0.77 | nd | — | 360-1090 | nd | e, g,h, j |
| DSLNT | nd | 284.80 | — | nd | nd | — | | — | e |
| 6-SLN | 0.27-220 | 7.36-107.2 | nd | nd | nd | 1420-6710 | nd | nd | b, d, f, j |
| Total | 414.78-1720.95 | 562.65-1349.06 | NED | NED | NED | 2420-13850 | NED | NED | |

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

# 4 Beneficial effects of MOs

MOs are not digestible. In breast-feeding infants, more than 95% of MOs can pass through the gastrointestinal tract intactly and reach the intestine where they regulate the microbiota population (Goehring et al., 2014). About 1% of digested MOs can be absorbed into the blood circulation to further exert beneficial functions in the target organs (Bode, 2015). Currently, the increasing evidence shows that there are multiple ways to achieve these beneficial effects: (1) regulate the composition of gut microbiota through selectively promoting the growth of beneficial bacteria, while suppressing the growth of pathogens; (2) directly interact with glycan binding proteins to promote gut maturation and improve gut barrier; (3) modulate immune system responses and development; and (4) provide with essential factors for brain development. The overall beneficial functions of MOs in breast milk are summarized in Figure 7, which will be further described below.

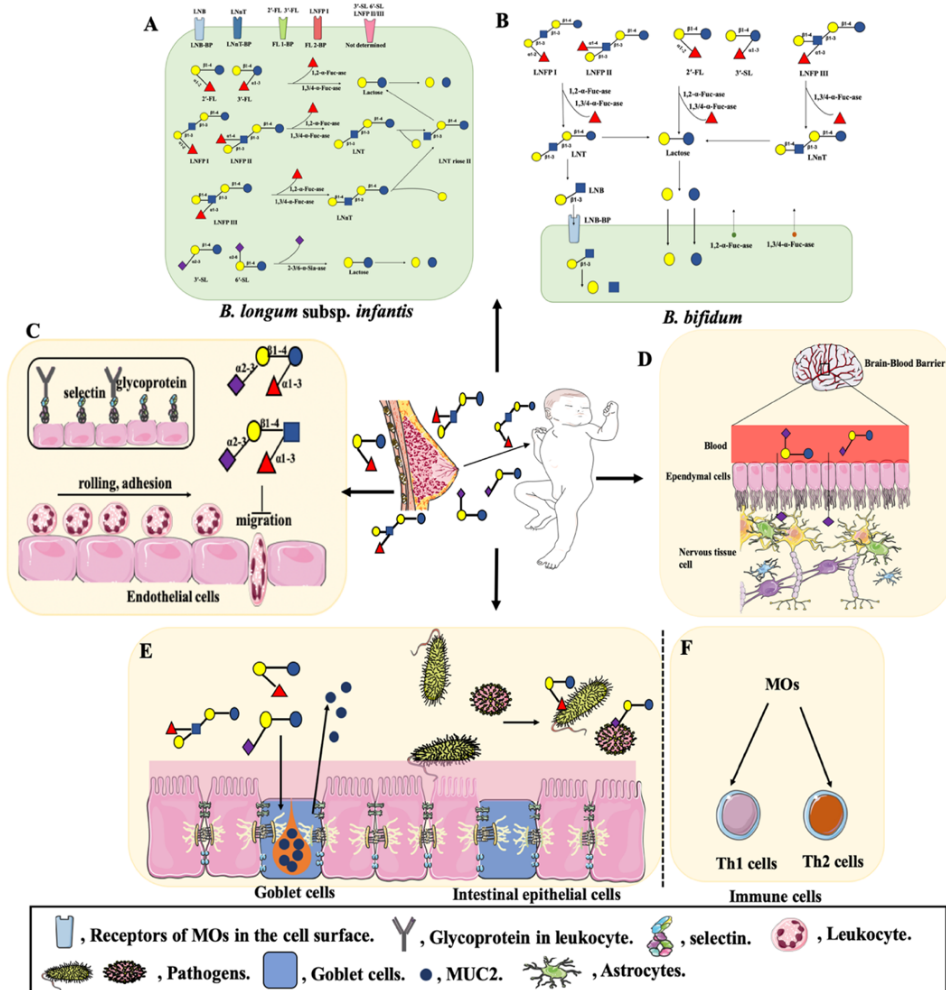


**Figure 7** Overview of beneficial functions of MOs in breast milk for the body. **A**, The process of *B. longum* subsp. *infantis* ferments MOs. **B**, The process of *B. bifidum* ferments MOs. **C**, MOs prevent leukocytes extravasation. **D**, MOs promote brain development. **E**, MOs enhance intestinal barrier functions. Pathogens attach to and continue to colonize through interaction between host cell surface glycans and protein-assembling adhesins of pathogen surface. MOs can bind to pathogens and then prevent them to colonize the intestine. **F**, 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. The figure is partially adapted from Bode and Jantscher-Krenn (2012) and Urashima et al. (2022).

## *4.1 Regulate the composition of gut microbiota*

### 4.1.1 Fermented by specific bacteria

Glycosidases are responsible for breaking down the glycosidic bonds between the sugar units in MOs. Several glycosidases are involved: lactase is an enzyme that breaks down lactose into glucose, and galactose; α-Galactosidase cleaves α-1,6 linkages in HMOs releasing individual sugar units; β-Galactosidase can cleave β-1,3 and β-1,4 linkages. Then sialidases and fucosidases can remove sialic acid and fucose residues from MOs, respectively (Katayama et al., 2004; Zivkovic et al., 2011). Some gut commensal bacteria can secrete specific enzymes to ferment different MOs.

*Bifidobacteria* are predominant in breastfed infants, including *Bifidobacterium longum* subsp. *longum* (*B. longum*), *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), *Bifidobacterium breve*, and *Bifidobacterium bifidum*. Different *Bifidobacteria* species have developed different strategies for utilizing MOs, especially fucosylated ones. For *B. bifidum* strains initiate MOs degradation extracellularly using 1,2-α-fucosidase (AfcA) and a 1-3/4-α-fucosidase (AfcB) to release fucose (Katayama et al., 2004). Next, lacto-N-biosidase from *B. bifidum* or *B. longum* subsp. *longum* liberates LNB from MOs chain containing no fucose or sialic acid residues (Wada et al., 2008). Those LNB are then transported into cells via ABC transporter to be used by *B. bifidum* (Suzuki et al., 2008). On the other side, *B. longum* subsp. *infantis* adopt a distinct approach to metabolize MOs. As possessing almost all essential sialidase, fucosidase, galactosidase, and hexosaminidase, as well as transporters, like ABC transporter (LoCascio et al., 2007), *B. longum* subsp. *infantis* can utilize a wide range of MOs. For them, small mass MOs are imported into cell via specific ABC or solute-binding proteins (SBP) transporters. Then, those MOs are further metabolized to monosaccharide by glycosidases (Zivkovic et al., 2011). Conversely, *B. longum* subsp. *longum* and *B. breve* only can consume non-fucoselyated neutral MO, LNnT and the latter can also ferment many other monosaccharide (Ward et al., 2007).

Besides *Bifidobacteria*, several other bacteria also can metabolize MOs. In Salli's study, the ability of 57 bacterial strains, from *Lactobacillaceae*, *Bifidobacterium*, *Bacteroides*, as well as potentially pathogenic bacteria, to utilize 2′-FL, 3′-FL and difucosyllactose (DFL) were compared. They found that in addition to *Bifidobacterium*, *Bacteroides fragilis*, *Bacteroides vulgatus*, and *Bacteroides thetaiotaomicron* utilized 2′-FL, 3′-FL and DFL as their sole carbon source, while none of *Lactobacillaceae* grew on 2′-FL or DFL, either for any pathogenic bacteria (Salli et al., 2021). Yu et al. (2013) got similar results, in which study, *Lactobacillus delbrueckii* ATCC7830, *Enterococcus faecalis* ATCC19433, and *Streptococcus thermophilus* ATCC19258 showed slight growth on 2′-FL or 3′-FL, but not LDFT. 3′-SL or 6′-SL promoted the growth of *Bifidobacterium longum* (JCM7007, 7009, 7010, 7011,1272, 11347, ATCC15708), *Bacteroides vulgatus* ATCC8482, and *B. thetaiotaomicron* ATCC29148. No *Clostridium* spp., *Staphylococcus* spp., *Enterobacter* spp., and *E. coli* K12 grew on MOs supplemented medium. While *Enterococcus* and *E. coli* species can ferment various carbohydrates, their ability to metabolize MOs specifically may be limited. They primarily ferment simpler

carbohydrates like lactose, glucose, and various polysaccharides, but their capacity to break down complex MOs structures are limited compared to some *Bifidobacteria* or other gut microbes (Marcobal et al., 2010).

#### 4.1.2 Antimicrobial activity

There are two main ways for MOs to exert antimicrobial activity. One is to prevent pathogen adhesion that is attributable to the structural similarities between mucosal cell surface of pathogen and MOs. Pathogens bind to MOs and will pass the intestinal tract without adhering to epithelial cells. Up to now, fucosylated and acidic MOs are reported to prevent the adhesion of pathogens, including *Campylobacter jejuni* and *E. coli* (Newburg et al., 2005). They are both the main cause of bacterial infection and poses a challenge to young children health. The mechanism of MOs inhibiting infection by *Campylobacter jejuni* has been fully studied. *Campylobacter jejuni* attaches to and continues colonize the host through interaction between host cell surface glycans and protein-assembling adhesins of pathogen surface (Morrow et al., 2005). Among the diverse glycans, fucosylated one is crucial for fucose binding of *Campylobacter jejuni*. *Campylobacter jejuni* pathogenesis, can indeed be inhibited by α 1-2-linked fucosyl MOs, like 2′-FL (Morrow et al., 2004).

Craft and Townsend (2019) found another approach of MOs to inhibit pathogen growth. MOs showed antimicrobial activity against *Acinetobacter baumannii* and Group B *Streptococcus* through increasing the membrane permeability of bacterial cells. However, they had no effects against *Staphylococcus aureus*. Moreover, the activity of MOs was strain-dependent, i.e. different bacteria showed varied susceptibilities to MOs (Craft and Townsend, 2019).

### *4.2 Enhance intestinal barrier function*

MOs not only alter the population of gut microbiota, but also can regulate intestinal barrier function, especially the physical and chemical barrier. The physical and chemical barrier, consisting of various intestinal epithelial cells and mucin layer, can hinder the transmission of bacteria to lamina propria. MOs are reported to directly influence the intestinal epithelial cells. According to Kuntz et al. (2008), MOs inhibited the growth of intestinal cells (HT-29 and HIEC) under homeostatic conditions by suppressing cell cycle progression through inducting differentiation and/or by regulating apoptosis. Detailly, only acidic MOs induced differentiation in HT-29 and HIEC cells, however, a strong apoptosis was induced by neutral MOs. Also, MOs contribute to the mucin secretion both in *in vivo* and *in vitro* models. In inflammatory model, pooled MOs promote the proliferation of crypt cells and then increase mucin secretion to alleviate inflammation in intestine (Wu et al., 2018). The above results suggest that MOs might have a crucial role in the maturation of the intestinal tract, which requires a shift from sialylation to fucosylation membrane-bound glycoproteins (Lenoir et al., 1995). The different types of glycoproteins lead to alteration in the ability of pathogens to bind the cell surfaces. It is verified by the research of Angeloni et al. (2005). In their research, 3′-SL treatment could decrease the expression of sialyltransferases ST3Gal1, ST3Gal2, and ST3Gal4 in Caco2 cells,

leading to a decrease of a2-3-, a2-6-sialylation glycans on the cell surface, and consequently sharply decline the *E. coli* adherence.

Besides the directly influence, MOs can improve intestinal barrier through its degraded products and/or through the secretion of MO-utilizing bacteria. On one hand, acetate is produced when *Bifidobacteria* ferment MOs, and it protects the host to defend itself against *E. coli* O157:H7 infection (Fukuda et al., 2011). On the other hand, cell-free spent media, containing the secretion from *Bifidobacterium infantis*, can protect intestinal epithelial barrier function. This is certified by Guo et al. (2017) and Lewis et al. (2017). They found that *B. infantis* conditioned media (BCM) protects Caco2 cells against IL-1β stimulation (Guo et al., 2017), and also increases claudin-1 and occludin protein synthesis, to promote the intestinal barrier integrity (Lewis et al., 2017).

## 4.3 Immunomodulatory effects

Although most of MOs are fermented by gut microbiota in colon, about 1% of MOs are absorbed into blood circulation and then exert functions at lymphoid tissue cells or/and at the systemic level (Goehring et al., 2014). Numerous immunological receptors are predominantly expressed on the surface of immune cells, which can recognize different types of MOs. Table 3 shows several kinds of lectins and toll-like receptors (TLRs) with MOs binding sites (Walsh et al., 2020). These receptors can identify glycoprotein ligands of MOs using their glycoprotein ligands, and the cell-cell communication, in turn, can be altered by MOs by changing the length, quality, or affinity of cell surface receptors and their ligands (Rabinovich et al., 2012; Rana and Haltiwanger, 2011). One convincing example is that MOs mediate leukocyte extravasation. Selectins, one class of cell-surface proteins, mediate the migration of leukocytes from the blood stream towards inflammatory tissue in the cellular immune response (Ley, 2003). As MOs have binding ligands for selectin, they may interfere with leukocyte enrollment and potentially reduce its extravasation. This hypothesis has been proven by Schumacher et al. (2006) who found that sialyl-Lewis x (3-sialyl-3-fucosyllactosamine) and also 3-sialyl-3-fucosyllactose could interact with selectins and then decrease leukocyte enrollment and adhesion which finally lead to the decline in leukocyte extravasation. However, this function is structure-depending as nonfucosylated MOs, like 3′-SL and 6′-SL, do not present this effect. Additionally, Sodhi et al. (2021) showed that 2′-FL and 6′-SL could directly dock into the LPS binding pocket of TLR4, and then alleviate necrotizing enterocolitis in mice and piglets (Sodhi et al., 2021). Additionally, 2′-FL inhibited LPS-mediated inflammation by reducing CD14 expression, a factor that mediates the interaction of LPS and TLR4, to inhibit inflammation (He et al., 2016). But it is unclear whether this function can apply to all Fuc-bone MOs.

On the other hand, MOs are involved in the systemic immune response by altering the expression of immune cell populations. An imbalance between Th1 and Th2 phenotypes exists in T-helper cell populations in newborns, with a shift toward the Th2 phenotype (Adkins, 2000). Th1 cells primarily release cytokines, including interferon (IFN)-γ and IL-2, to participate in the body's defense against viruses and intracellular bacterial infections. Th2 cells secrete cytokines such as IL-4 and IL-5,

which are involved in humoral immunity, contributing to immune responses against bacterial infections and immediate hypersensitivity reactions (Akbari et al., 2003). An imbalanced Th1/Th2 ratio may partially contribute to certain clinical conditions. For instance, excessive Th2 activity can lead to allergic diseases, while Th1 predominance is associated with inflammatory bowel disease (Nakayama et al., 2017). Eiwegger et al. (2004) first proved that sialylated MOs increased Th-1 type cytokine (IFN-γ) and Th2-cytokines as well as Treg cells. Then, they found sialylated MOs could relieve 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 modify immunological signaling in juvenile intestinal mucosa, reduce the production of acute phase inflammatory cytokines like IL-6 and IL-1β, and restrain the overexpression of Th17-polarizing cytokines like TNF-β, TGF-β, IL-4, and IL-17 (He et al., 2014).

**Table 3** Receptors on the surface of immune cells interact with MOs (Walsh et al., 2020).

| Type | Expression | Name | MO related ligands |
|---|---|---|---|
| Selectins | Leukocytes/ Endothelial cells | P-selectin, E-selectin | MO with sialyl Lewis X epitopes |
| TLRs | Macrophages and dendritic cells | TLR2 | 3′-FL，3′/6′-SL |
| | | TLR3 | 3′/4′/6′-GL |
| | | TLR4 | 2′-FL，LNFP I/III |
| C-type lectins | Antigen presenting cells | DC-SIGN | 2′/3′-FL，LNFP III/ IV，LNDFH I |
| Galectins | T cells/ Intestinal Epithelial cells | Galectin-3 | 3′-SL, LNFP I/II/II |
| Siglecs | Neutrophils, monocytes, dendritic cells | Siglec-4 | α 2-3 sialic acid |
| | | Siglec-2 | α 2-6 sialic acid |
| | | Siglec-5 | Sialyl-Lewis c |
| | | Siglec-9 | Sialylated MOs |

## *4.4 Improve brain development*

Promoting brain development is a unique function of sialylated MOs because of their key functional player sialic acids (Sia). Sia is a crucial part of sialylated glycoconjugates, including polySia and gangliosides, and it is essential for the transmission of synapses as well as learning, memory, and cognition (Becker et al., 1996). In Wang's study (2007), when compared with control group, newborn piglets supplemented with sialylated MOs performed better in the learning and memory task of 8-arm radial maze, with 2 learning-associated genes expression (*ST8SIA4* and *GNE*) increased. Additionally, in another research, the hippocampus of the sialylated MOs supplemented pre-term pigs showed enhanced expression of genes related to Sia metabolism, myelination, and ganglioside production (Obelitz-Ryom et al., 2019).

Interestingly, that ability varies between individual MOs, as rat consuming 6′-SL, not 3′-SL, improved cognitive ability, as well as enhanced polysialic acids-neural cell adhesion (PolySia-NCAM) expression (Oliveros et al., 2018).

However, the detailed metabolic pathway of dietary sialylated MOs *in vivo*, such as how it arrives to the brain to exert function is lacking. Researchers have made efforts to figure it out employing rats, mice and pigs models. Wang et al. (2007) sought to determine if sialic acid (N-acetylneuraminic acid 6 $^{14}$C) could pass the blood-brain barrier and whether cognition-related areas of the brain were more likely to absorb it. The results showed that 80% of the activity was removed from the piglet blood within 2 min of injection. At 120 min, only 8% activity in blood remained, while the brain contained significantly more activity. In mice model, oral administrated sialic acids were found to be well absorbed with a concentration of 3%-4% in the brain within 6 h after ingestion (Nohle et al., 1981), suggesting sialic acid can go through the blood-brain barrier.

# 5 Influencing factors of MOs

## *5.1 Species*

Just as descripted above, MOs are structurally diverse, and their concentration and composition greatly vary between species, especially between human and other animal milk. Human milk contains 10-100 times more total MOs than other mammal milk does and have a more complex MOs profile. Specifically, fucosylated neutral MOs are the most prevalent in human milk, accounting for 50%-80 % of the total, however, the sialylated MOs are predominant in domestic animals (80%-90% of the total MOs) (Albrecht et al. 2014). It is believed that the presence of MOs is determined genetically (Erney et al., 2001). Up to now, the reason why MOs in human milk differ from those of other animals is difficult to explain. There is a hypothesis resulting from evolutionary adaptations and the specific needs of each species (Urashima et al., 2022). According to Urashima et al. (2022) article, evolution has shaped the MOs composition in each species to support the specific needs of their offspring. For example, MOs provided evolutionary advantages on other animals as a significant energy source for nursing infants. Human infants, however, have a relatively undeveloped immune system and a rapidly growing brain. MOs therefore play a crucial role in supporting the immune system and promoting brain development, as well as establishing symbiosis with colonic bacteria. Researchers make lots of efforts to explore if the co-evolution of MOs with colonic bacteria can be found in other species (Akazawa et al., 2021; Li et al., 2021). *Enterococcus gallinarum* isolated from the feces of suckling neonatal rats could hydrolyze 3′-SL to Neu5Ac and lactose (Akazawa et al., 2021). As *Bifidobacteria* are not predominant in infant rats (Korgan et al., 2022), *Bifidobacteria*-type microbiota may not be applicable to rats but may still be relevant to other species.

From the perspective of MOs synthesis, some differences also exist between human milk and other animal milk. The synthetic pathways of MOs in human are much more

complex than those in animal milk, like bovine for example (Figure 8, Urashima et al., 2022). Kellman et al. (2022) integrated glycan and RNA expression data to construct an HMO biosynthetic pathway and found that several fucosyltransferase and sialyltrasferases were involved in biosynthesis, such as FUT1, FUT2, FUT3, FUT4, FUT5, FUT9, FUT11 and ST6GAL1, ST6GAL2, ST3GAL3. And some MOs in bovine milk are unique as β1-4N-acetylgalactosaminyltransferase (β4GalNAcT), is present in bovine, but not in humans.

**Figure 8** Biosynthetic pathway of the MOs in human milk (**A**) and bovine milk/colostrum (**B**, no sialylated MOs) (Urashima et al., 2022).

## 5.2 Lewis blood group of mothers

The MOs found in human milk exhibit significant variation in composition among individuals. While the overall structure and types of MOs are similar, the specific combinations and quantities of individual MOs can vary widely. The human MOs composition is drastically altered by a difference of 1 base pair (bp) out of the genome's approximately 3 billion base pairs, defining the so-called secretor and non-secretor lactotypes (Boad, 2020). In lactocytes, two different fucosyltransferases have been found: (1) α1-2- fucosyltransferase (FUT2), encoded by Secretor (*Se*) gene, is responsible for linking Fuc to terminal Gal through α 1-2 bonds, (2) α1-3/4-fucosyltransferase (FUT3), encoded by Lewis (*Le*) gene, which contacts Fuc to GlcNAc through α1-4 bonds (Bode, 2015). Therefore, the "secretors mothers", who contain *Se* gene, are able to produce MOs, such as 2′-FL (Fucα1-2Galß1-4Glc) and LNFP I (Fucα1-2Galß1-3GlcNAcß1-3Galß1-4Glc). Lack of FUT2 activity affects the concentration of nearly all other MOs, not only those that are 1-2-fucosylated, and has repercussions across the whole HMOs biosynthesis process. Non-secretors, on the other hand, have a non-functional or inactive FUT2, leading to the absence or reduced production of fucosylated MOs in their milk.

Similar, SNPs in the gene encoding the FUT3, which is connected to the Lewis blood group antigen, can be observed (Boad, 2020). Those that carry *Le* gene can produce α1-4-fucosylated MOs, such as LNFP II (Fucα1-4Galß1-3GlcNAcß1-3Glß1-4Glc) (Xu et al., 1996). MO containing Le b antigens are present in milk if both FUT2 and FUT3 are expressed. If only FUT3 is expressed, the milk includes MO with Le a antigens. MOs do not have Le a or b antigens when FUT3 is not expressed. 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. Significantly, the milk of mothers, who express neither FUT2 or FUT3, contains fucosylated HMOs such as 3′-FL or LNFP III. This fact suggests that other *Se*- and *Le*-independent FUTs (FUT4, 5, 6, 7 or 9) could be involved in the synthesis of fucosylated MOs (Newburg et al., 2005).

**Table 4** Fucosyltransferases determines the diverse structure of fucosylated MOs.

| Mother | Secretors | | Non-secretors | |
|---|---|---|---|---|
| Groups | *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-) |
| Percent (%) in European country* | 70% | 9% | 20% | 1% |

*, Data from Gabrielli et al., (2011) and Thurl et al., (1997).

### *5.3 Maternal diets*

Diet can have a significant impact on the composition of MOs, particularly in terms of MOs in human milk. It is reported that maternal diet predictably and significantly impacts specific MOs concentrations in human milk (Seferovic et al., 2020; Azad et al., 2018). The types and amounts of carbohydrates, fibers and fat consumed by the mother can directly influence the composition of MOs. Increased glucose intake leads to lower total fucosylated MOs compared to the galactose diet, whereas no individual fucosylated MOs were statistically different between the two diets (Seferovic et al., 2020). The concentration of total sialylated MOs was reduced in high fat diet relative to the carbohydrate diet after 8 days of intervention starting at 10 weeks postpartum, accompanied by the increased energy output and energy expenditure but not gluconeogenesis (Seferovic et al., 2020; Mohammad et al., 2009). In another study, there were no effect of a lipid-based nutrient supplement on MOs composition (Jorgensen et al., 2017). This is similar to the results from Azad et al. (2018), who found that total MOs concentration was not correlated with any type of maternal diets, although a few dietary components were associated with individual MOs. LSTb was inversely correlated with total protein and empty calories, whereas fucosyllacto-N-hexaose was positively correlated with whole grains. Positive correlations were found between LNT and difucosyllacto-N-hexaose (DFLNH) and overall energy consumption (Azad et al., 2018).

It is important to note that the extent of dietary influence on MOs composition may vary among individuals. Genetic factors, overall maternal health, and other physiological processes also play a role in determining the final composition of MOs in breast milk. Research on the relationship between diet and MOs composition is ongoing, and further studies are needed to fully understand the precise mechanisms and effects of different dietary components on MOs synthesis.

# 6 Application of MOs in infant formula

### *6.1 Artificial synthesis of MOs*

In human, MOs are synthesized in the mammary gland epithelial cells, where specific enzymes catalyze the step-by-step assembly of these complex HMOs. The synthesis of HMOs starts from lactose, generated by active UDP-Gal and Glc (Ramakrishnan et al., 2002). Then various enzymes participate in the process of chain lengthening of lactose. Glycosyltransferases, such as fucosyltransferases (FUT2, FUT3, FUT4, FUT5, FUT9 and FUT11), sialyltransferases (six different ST6GalNAc) and N-acetylglucosaminyltransferases (β3GlcNAcT (iGnT) for type I and β6GlcNAcT (IGnT) for type II), are responsible for catalyzing the glycosylation reactions that link the specific monosaccharide units with the growing MOs chain (Stahl et al., 2001; Tsuchida et al., 2003; Kobata, 2003). Among diverse glycosyltransferases, fucosyltransferases and sialyltransferases are responsible for adding fucose and sialic acid residues, leading to the very diverse fucosylated and sialylated MOs (Figure 9A). Once synthesized, the MOs are packaged into vesicles

and transported through the Golgi apparatus to the apical side of the mammary epithelial cells, and then released into the milk through a process called exocytosis (Sasaki et al., 1978).

The concentration of MOs in human milk is relatively low, therefore, several methods to produce MOs were developed, including chemical synthesis, enzymatic synthesis, chemoenzymatic synthesis and microbial fermentation (Perez-Escalante et al., 2020). Chemical synthesis involves stepwise assembly of the MOs chain through glycosylation reactions with precise control over stereochemistry. However, its usefulness in MOs production has been limited because of multi-step synthesis steps and reactants (Bode et al., 2016). Enzymatic synthesis uses glycosyltransferases with mild reaction conditions, and compatibility with aqueous environments, but enzyme availability can be limited (Zeuner et al., 2018; Schmölzer et al., 2015). Chemoenzymatic synthesis combines chemical and enzymatic methods to overcome limitations. Enzymes selectively modify or activate sugar molecules, which are then coupled using chemical reactions. But their synthesis usually yields low amounts of MOs at the milligram level (Muschiol and Meyer, 2019). To overcome those obstacles, an effective approach using microbial fermentation to generate MOs was established. *E. coli*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, and *Corynebacterium glutamicum* are the major strains that are used today as hosts for the engineered microbial pathway, and a total of 42 MOs have been produced (Faijes et al., 2019). Due to its quick growth, high level of expression, and effective DNA molecule introduction, *E. coli* has been utilized most frequently in the development of MOs. Up to now, 2′-FL, 3′-FL, LNT, LNnT, 3′-SL and 6′-SL have been produced successfully in *E. coli* with high yield at the Gram level (Figure 9B). The production of complex fucosylated MOs usually takes LNT and LNnT as the core structure and then connects to fucose by α glycosidic linkages, like LNFP I/II/II and LNDFH I/II production (Dumon et al., 2001, 2004). However, because it is endotoxin-carrying, *E. coli* is not thought to be the best host strain. Other strains, such as *S. cerevisiae*, *B. subtilis* and *Corynebacterium glutamicum* (*C. glutamicum*), are considered as safe candidates, but there are few reports on their use in the production of MOs (Lu et al., 2021).

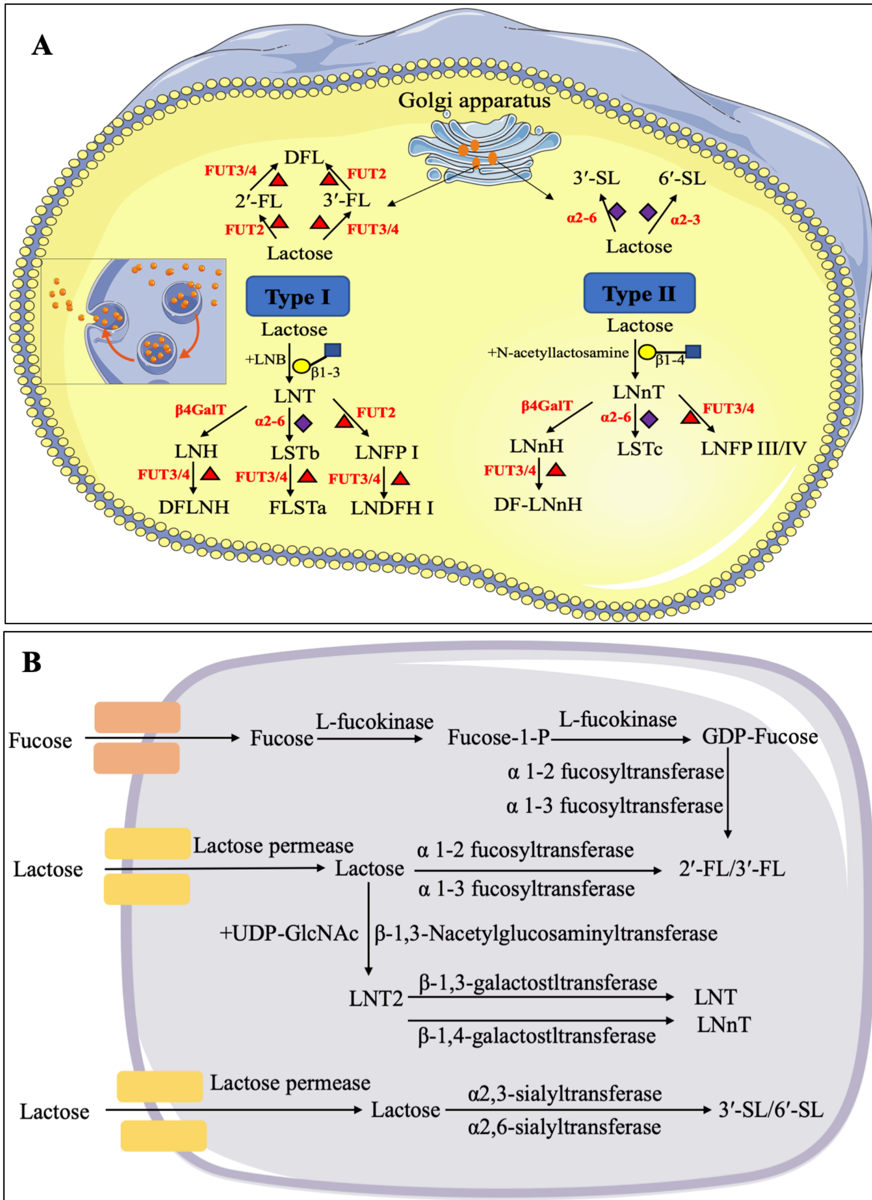


**Figure 9** Biosynthetic pathway of main MOs in the human mammary gland (**A**) and *E. coli* (**B**).

## 6.2 Application of MOs in infant formula

MOs are not present in baby formula, hence newborns who consume this formula do not get the health advantages of MOs. Infant formula is often made from bovine milk, which present significantly lower levels of prebiotics than human milk. The prebiotic is a substrate that target specific bacteria to give them a growth advantage. Furthermore, the majority of the MOs in bovine milk are sialylated, whereas few of them are fucosylated. All those lead to a big difference for babies between breast milk and infant formula (Klement et al., 2004). To narrow the gap between infant formula and breast milk, galactooligosaccharide (GOS) and fructooligosaccharide (FOS) are supplemented in infant formula to mimic the function of human MOs. However, GOS and FOS do not present any sialylated or fucosylated form. While GOS and FOS in infant formula can promote the increase of *Bifidodobacteria* and *Lactobacillus* numbers (Ladirat et al., 2014), they do not replicate the full spectrum of functions and structures observed in human MOs, such as the functions in immune system support and pathogen defense. Also, it has been discovered that infant formula containing GOS increases the growth of *Bifidobacteria* strains in a different rate compared to human milk (Macfarlane et al., 2008; Subramanian et al., 2015).

The unique complexity and diversity of MOs in breast milk contribute to their extensive physiological effects on infant health and development. Therefore, recently, MOs have been supplemented into infant formula. Two HMOs, 2′-FL and LNnT, are regarded as new foods by the European Union (EU) (Commission Implemented Regulation (EU) 2017/2470). In 2015, the European Food Safety Authority (EFSA) issued a statement stating that the addition of 2′-FL to infant and follow-on formulae in combination with LNnT at concentrations up to 1.2 g/L of 2′-FL and up to 0.6 g/L of LNnT, at a ratio of 2:1 in the reconstituted formula, is safe for infants up to one year of age. (EFSA-Q-2015-00052, EFSA Journal 2015; Roman et al., 2020). With more HMOs becoming available, the mixture containing several other short-chain MOs are developed (Table 5). Parschat et al. (2021) developed a MOs-mix infant formula combined the five most abundant MOs from all three types (2.99 g/L 2′-FL, 0.75 g/L 3′-FL, 1.5 g/L LNT, 0.23 g/L 3′-SL, and 0.28 g/L 6′-SL ) and they found that the 5 MOs-Mix was well tolerated, with 5 MOs-Mix and breastfed infants producing softer stools at a higher frequency than the control formula group (without MOs). Another MOs-mix, consisting in 2′-FL, DFL, LNT, 3′-SL and 6′-SL, were found to support the intestinal immune system development and shifts the gut microbiome closer to that of breastfed infants with higher *Bifidobacteria*, particularly *B. infantis* (Bosheva et al., 2022). The complex composition of MOs determines the different combinations in the MOs mix. But all these blends included the most common MOs in human milk. Their concentrations were chosen according to the average of reported physiological HMO concentrations in milk, with a slightly increased proportion of specific ones. This adjustment was made to promote synergistic and complementary benefits that may contribute to the optimal development of infants. However, for now, the data about 5 MOs-mix application is limited and suitable preclinical models and clinical intervention studies are needed to obtain convincing results.

# 7 Intestinal mucin secretions

The mucus in the colon is divided into two layers: the outer layer, which is typically inhabited by gut bacteria, and the inner layer, attached to intestinal epithelial cells, serves as a barrier to segregate bacteria (Tailford., et al., 2015). Mucins are large glycoproteins and highly glycosylated at serine and threonines residues with oligosaccharides consisting of N-acetylgalactosamine (GalNAc), GlcNAc, Fuc, Gal and NeuAc (Bansil & Turner, 2006). There are two types of mucins, membrane-bound and secreted. In human, two of them are membrane-bound (MUC1 and MUC4) and five are secreted (MUC2, MUC5AC, MUC5B, MUC6, MUC7) and MUC2 is the most predominant one (Hansson, 2020). Additionally, distinct mucin types are selectively expressed within specific segments of the gastrointestinal (GI) tract; for instance, MUC5AC mucin predominates in gastric glands, MUC6 is confined to Brunner's gland in the duodenum, and MUC5B exhibits marginal expression in the colon (Paone and Cani, 2020). Besides these mucins, the gut epithelial cells also express transmembrane mucins such as MUC1, MUC4, MUC13, and MUC16 (Cornick et al., 2015; Vancamelbeke and Vermeire, 2017). However, the precise functions of these mucins are yet to be fully comprehended.

Some commensal bacteria may attach to and consume mucin-associated oligosaccharides, which provide a rich supply of nutrition for the bacteria. The fucosidase and sialidase activity of certain bacteria allow them to liberate mucosal glycans for colonization (Wlodarska et al., 2017). To use mucin glycans, bacteria need to encode enzymes, like sulfatases, sialidases and fucosidases. *Ruminococcus gnavus* containing sialidases can grow on mucin through cleaving α2,3 linkages in mucin (Raba & Luis, 2023), while *Bacteroides thetaiotaomicron* encodes fucosidases to degradate mucin by removing of fucose (Sakurama et al., 2012). Another good example is *Akkermansia muciniphila*, which can cleave almost all kinds of linkages in mucin (Teze et al., 2020).

The microbiota is a crucial regulator of mucus barrier function. Johansson et al. (2015) found a declined of goblet cell abundance and thinner colonic mucus layers in germ-free mice when compared with normal mice. Mucosa-associated bacteria have been shown to expand excessively in IBD, and coculture of those bacteria with MUC2 increased their growth, indicating that mucin breakdown plays a role in the proliferation of certain bacteria during inflammation (Png et al., 2010).

**Table 5** Main studies about MOs application in the infant formula.

| MOs | Concentration | Subject | Intervention time | Main results | References |
|---|---|---|---|---|---|
| 2'-FL | 1.0 g/L 2'-FL *Limosilactobacillus reuteri* DSM 17938 (1 × $10^7$ CFU/g) | Healthy infants < 14 days old | 6 months | *L. reuteri*-containing infant formula with 2'-FL supports age-appropriate growth. | Alliet et al., 2022 |
| 2'-FL, LNnT | 1 g/L of 2'-FL and 0.5 g/L of LNnT | 159 infants in Spain | 8 weeks | Growth and tolerance outcomes of HMO-supplemented infants were similar to breast-feeding ones | Roman et al., 2020 |
| 5MOs-mix | 0.87 g/L 2′-FL, 0.10 g/L DFL, 0.29 g/L LNT, 0.11 g/L 3′-SL, 0.14 g/L 6′-SL | aged ≥ 7–≤21 days | 15 months | Microbiota compositions in MO-supplemented infants were close to breast-feeding ones. At 6 months, calprotectin in MO-supplemented infants was lower than breast-feeding ones. | Bosheva et al., 2022 |
| 5MOs-mix | 1.45 g/L 2′-FL, 0.14 g/L DFL, 0.48 g/L LNT, 0.18 g/L 3′-SL, 0.24 g/L 6′-SL | aged ≥ 7–≤21 days | 15 months | microbiota compositions in MO-supplemented infants were close to breast-feeding ones. | Bosheva et al., 2022 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | At 6 months, sIgA in MO-supplemented infants was higher than breast-feeding ones. | |
| 5MOs-mix | 3.0 g/L 2′-FL, 0.8 g/L 3'-FL, 1.5 g/L LNT, 0.2 g/L 3′-SL, and 0.3 g/L 6′-SL | 341 subjects aged ≤14 days | 4 months | 5MOs-Mix and breastfed infants producing softer stools at a higher stool frequency than the control formula group. | Parschat et al., 2021 |
| 5MOs-mix | 3.0 g/L 2′-FL, 0.8 g/L 3'-FL, 1.5 g/L LNT, 0.2 g/L 3′-SL, and 0.3 g/L 6′-SL | 363 0-14 days infants | 3 months | Five MOs benefit for normal growth and gastrointestinal tolerance in healthy infants | Lasekan et al., 2022 |

# 8 NLRP6 function

NLRP6, a new member of nod-like receptor family, is highly expressed in the intestinal tract and liver of mice and human (Elinav et al., 2011; Gremel et al., 2015). In the intestine, NLRP6 is a key factor to regulate IL-18 production, bacterial homeostasis and specially MUC2 expression in goblet cells (Levy et al., 2017; Birchenough et al., 2016). A decrease of MUC2 has been identified in colitis cases (Fyderek et al., 2009), which likely contribute to reduce commensal fitness and drive the microbial dysbiosis in colitis. Wlodarska et al. (2014) found that, in NLRP6$^{-/-}$ mice, the lack of NLRP6 greatly contributed to impairments of function in goblet cells and consequently lead to mucin secretion decrease.

The microbiota signal and metabolic signal, such as taurine, unsaturated fatty acid, histamine and spermine, act as the priming signal to regulate the expression of NLRP6 in the intestinal epithelial cells, and the microbiota component, such as family *Prevotellaceae*, act as the secondary signal to bind NLRP6 to induce the assembly and activation of inflammasome (Levy et al., 2015; Wang et al., 2020). Besides of that, TLR ligand activates MyD88-ROS pathway to activate NLRP6 inflammasome, and then influence the function of goblet cells (Yao et al., 2021).

# 9 Conclusion

MOs found abundantly in human breast milk, constitute a vital nutritional component. They function as prebiotics, hindering pathogen adherence, modulating the immune system, and improving newborn brain development. Remarkably, their concentrations and patterns vary significantly among different species. Currently, the majority of studies focus on revealing the MOs pattern in human milk. Limited research has been conducted on the quantitative comparison of MOs among domestic species, especially on camels, monkeys, yaks, elephants, and pigs.

MOs have been found to alleviate colonic inflammation in several neonatal cases and to regulate the composition of gut microbiota by promoting the growth of certain beneficial symbiotic bacteria, as well as by decreasing some pathogenic bacteria. However, it remains unknown if the protective activity is microbiota-mediated. Moreover, the function of MOs depends on their unique structures, like their decorated groups. Therefore, the effects among different types of MOs should be compared to locate the most important fraction.

# Chapter 3

## Label free absolute quantitation of MOs in milk from domestic animals

The work is an original contribution, adapted from：

Yao, Q., Gao Y., Zheng, N., Delcenserie, V., Wang, J. (2023). Label Free Quantitation of Milk Oligosaccharides from Different Mammal Species. *Food Chemistry*, **430**, 136977.

To compare the compositions and patterns of MOs in different mammal species, we established a LC-ESI-MS/MS method to simultaneously analyze 11 MOs (2′-FL, 3′-FL, LNT, LNnT, 3-GSL, 3′-SL, 6′-SL, LNFP I, LNFP II, LNDFH I and LNDFH II) in milk samples from human, dairy cow, sheep, mare, came, yak and buffalo.

**A**bstract: Milk oligosaccharides (MOs) exhibit significant variations in concentration and patterns among different species. However, there is limited knowledge about MOs in domestic animals and the impact of heat treatment on them. Here, we developed an LC-ESI-MS/MS method to analyze 11 MOs in 7 distinct species simultaneously. The results showed that human milk presented a completely different composition pattern of MOs from animals. In particular, animal milk predominantly contained sialylated MOs, and human milk had high levels of fucosylated neutral MOs. Notably, sheep milk exhibited similarities to human milk in terms of MOs composition. Then, the dairy cow milk samples were treated by two common industrial heat treatments, and 2′-FL, 3′-FL, LNT+LNnT, 3-GSL, 3′-SL, and 6′-SL concentrations were analyzed. We found that a heat treatment of 65 °C for 30 min had no significant effect on the concentration of milk oligosaccharides, whereas a heat treatment of 135 °C for 60 seconds was associated with their decline.

Key words: Milk oligosaccharides (MOs), LC-ESI-MS/MS, Human milk, Domestic animal milk, Heat treatment

# 1. Introduction

Breast milk is vital for the growth of newborns, serving as their primary source of nutrition (Azad et al., 2018). Human milk oligosaccharides (HMOs), the third most abundant solid component of breast milk, are complex carbohydrates (Bode, 2012). HMOs consist of various monosaccharide building blocks, including glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid (NeuAc), which determine their structure and function (Chaturvedi et al., 2001; Zivkovic et al., 2017). Based on the specific linkage and branching patterns of monosaccharides, HMOs are classified into fucosylated, sialylated, and non-fucosylated neutral HMOs (Ninonuevo et al., 2006).

These MOs are structurally diverse, and their concentration and composition can vary between species (Shi et al., 2021; Wang et al., 2020). Currently, previous research on milk MOs has mostly focused on a single breed of one species and its lactation regularity. Multiple studies have explored the concentration and composition of MOs in human milk, with fucosylated MOs being found to be the most abundant (Ma et al., 2018; Plows et al., 2021). As for domestic animals, sialylated MOs were identified as predominant in bovine and goat milk (Tao et al., 2009; van Leeuwen et al., 2020). Although there are some studies on MOs in other animal species, such as camel, yak, and donkey, they are mostly focused on elucidating MOs structures (Yan et al., 2018; Albrecht et al., 2014). Limited research has been conducted on the systematic comparison of MOs among different species, particularly with respect to absolute quantitation. Only a few examples of absolute quantitation are available, such as the studies by Shi et al. (2021) and Wang et al. (2020). Shi et al. (2021) quantified 7 MOs in milk from Chinese human, cow, goat, sheep, and camel by UPLC-MRM-MS, while Wang et al. (2020) compared the composition and concentration of 12 MOs between Chinese human and domestic animals (cow, goat,

yak, and donkey) using HPAEC-PAD. However, the different analytical methods employed in these studies hinder the comparative analysis of MOs profiles. Moreover, the composition and concentration of MOs in horse and buffalo remain largely unknown. Comparing the MOs profiles of different domestic animals can provide valuable insights into their nutritional value, including their potential as sources for infant formula.

Among domestic animals, milk from dairy cows is the most extensively consumed. Although milk is sometime still consumed as “untreated” or raw, the two mainly described heat treatments for milk are pasteurization (at 65 ºC) and sterilization (UHT, at 135 ºC) to reduce the microbial population (Lewis & Deeth, 2009). However, intense thermal processes can lead to severe degradation of water-soluble vitamins, certain fatty acids, and hormones present in milk (Kilic-Akyilmaz et al., 2022). But it is unclear whether MOs are sensitive to heat treatment.

Therefore, the study was carried out to obtain a comprehensive overview of MOs profiles in the milk of human, cow, sheep, horse, yak, buffalo, and camel using the LC-ESI-MS/MS method combined with MRM scanning. Additionally, the effects of heat treatment on MOs were explored to gain a better understanding of their characteristics.

# 2. Material and Methods

## *2.1 Chemicals*

Acetonitrile (LC-MS grade, purity≥99.9%) was purchased from Honeywell Research Company (Charlotte, North Carolina, US). Ammonium acetate (CAS No. 631-61-8, purity≥98.0%) was obtained from Merck Ltd (Beijing, China). Ultrapure water was purified from the Milli-Q124 purification system (Millipore, Bedford, MA, USA).

2′-fucosyllactose (2′-FL, Cat# 41263-94-9), 3′-fucosyllactose (3′-FL, Cat#41312-47-4), 3'-sialyllactose sodium salt (3′-SL, Cat#GY1143), 6′-sialyllactose (6′-SL, Cat#GY1144), 3′-galactosyllactose (3′-GSL, Cat#GY1093), lacto-N-tetraose (LNT, Cat#GY1145), lacto-N-neotetraose (LNnT, Cat#GY1146) and lacto-N-fucopentaose I (LNFP I, Cat#GY1147) were obtained from HuicH Biotech Co., Ltd. (Shanghai, China).The lacto-N-fucopentaose II (LNFP II, Cat#ZG-10049), lacto-N-difuco-hexaose I (LNDFH I, ZG-10060) and lacto-N-difuco-hexaose II (LNDFH II, Cat#ZG-10189) were purchased from Shanghai ZZBIO Co., Ltd.

## *2.2 Samples*

Human milk (n=50) was collected from mothers who gave birth to healthy full-term infants and had no metabolic disease, with a mean lactation value of day 90, the earliest at day 7, and the longest at day 270 (Table 6).

Milk (n=20, day 100-120 post-partum) from Holstein cows was obtained at Zhongdi Dairy Farm (Beijing, China). Camel milk (n=20, day 180 post-partum),

horse milk (n=10, day 120-150 post-partum), sheep milk (n=20, days 150 post-partum), yak milk (n=10, days 90 post-partum) and buffalo milk (n=30, days 120-150 post-partum) were obtained from Hebei, Xinjiang, Shanxi, Gansu, and Guangxi provinces, separately. All milk samples were stored at −80 °C. This study was approved by the Committee on the Ethics of Animal Experiments of the Chinese Academy of Agricultural Sciences (Beijing, China; permission number: IAS2022-124).

**Table 6** Information of mother volunteers

| Sample No. | Donor | Age of mother (year) | Gestational time (week) | Mode of delivery | Parity | Gender of infant | Days post-partum |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 32 | 41 | C-section | 1 | Male | 51 |
| 2 | | | | | | | 78 |
| 3 | | | | | | | 98 |
| 4 | | | | | | | 121 |
| 5 | 2 | 33 | 38+4/7 | C-section | 1 | Male | 64 |
| 6 | | | | | | | 95 |
| 7 | 3 | 28 | 39+4/7 | C-section | 1 | Male | 70 |
| 8 | 4 | 29 | 39+3/7 | Vaginal | 1 | Female | 57 |
| 9 | | | | | | | 72 |
| 10 | | | | | | | 95 |
| 11 | 5 | 33 | 40+1/7 | Vaginal | 1 | Female | 77 |
| 12 | | | | | | | 85 |
| 13 | 6 | 32 | 39+5/7 | C-section | 1 | Male | 68 |
| 14 | | | | | | | 97 |
| 15 | | | | | | | 116 |
| 16 | 7 | 33 | 37+5/7 | C-section | 1 | Male | 20 |
| 17 | | | | | | | 71 |
| 18 | | | | | | | 90 |
| 19 | | | | | | | 122 |
| 20 | | | | | | | 161 |
| 21 | | | | | | | 180 |
| 22 | | | | | | | 196 |
| 23 | 8 | 28 | 39+1/7 | Vaginal | 1 | Female | 13 |

| Sample No. | Donor | Age of mother (year) | Gestational time (week) | Mode of delivery | Parity | Gender of infant | Days post-partum |
|---|---|---|---|---|---|---|---|
| 24 | | | | | | | 26 |
| 25 | | | | | | | 43 |
| 26 | | | | | | | 79 |
| 27 | | | | | | | 99 |
| 28 | | | | | | | 127 |
| 29 | 9 | 33 | 37 | C-section | 1 | Female | 7 |
| 30 | | | | | | | 10 |
| 31 | | | | | | | 64 |
| 32 | 10 | 31 | 40+6/7 | Vaginal | 1 | Male | 27 |
| 33 | | | | | | | 39 |
| 34 | | | | | | | 60 |
| 35 | | | | | | | 80 |
| 36 | | | | | | | 98 |
| 37 | 11 | 32 | ＞37 | Vaginal | 1 | Female | 60 |
| 38 | | | | | | | 72 |
| 39 | | | | | | | 80 |
| 40 | 12 | 24 | ＞37 | Vaginal | 2 | Male | 94 |
| 41 | | | | | | | 120 |
| 42 | | | | | | | 150 |
| 43 | | | | | | | 180 |
| 44 | 13 | 20 | ＞37 | Vaginal | 1 | Male | 30 |
| 45 | 14 | 27 | ＞37 | C-section | 1 | Male | 35 |
| 46 | 15 | 30 | 38+1/7 | C-section | 1 | Female | 270 |
| 47 | 16 | 32 | 38+5/7 | Vaginal | 2 | Female | 270 |
| 48 | 17 | 27 | ＞37 | Vaginal | 1 | Male | 120 |
| 49 | 18 | 30 | ＞37 | C-section | 1 | Male | 74 |
| 50 | 19 | 28 | 39+3/7 | Vaginal | 1 | Male | 90 |

## 2.3 Oligosaccharide Extraction and Analysis

MOs were isolated from milk samples using a modified approach published before (Galeotti et al., 2012, Liu et al., 2014). Briefly, 1 mL milk samples were mixed with ultra-pure water (3 mL for animal milk and 9 mL for human milk) and then centrifuged at 12000 rpm for 20 min at 4 ℃. The 1 mL middle layer (containing oligosaccharides) was collected into a new 15 mL sterile polypropylene tubes and diluted 3 times by adding 2 mL pure water. After homogenizing, the PestiCarb graphitized carbon column (GCB) (500 mg/3 mL, S-GCB5003, Kangpu Xing Technology Co., Ltd, Beijing, China) was used to purify the rude oligosaccharides extraction. The GCB was activated by 10 mL acetonitrile, and then balanced with 10 mL pure water. After that, Oligosaccharide extraction was added into which would flow through the column under the action of gravity, and then the column was washed with 10 mL pure water to remove excess salt and small molecules. Finally, 1 mL of 20%, 40%, and 80% acetonitrile were used to elute the oligosaccharide. The filtrates were filtered through 0.22 mm membranes before MOs components analysis.

## 2.4 LC-ESI-MS/MS Analysis

The oligosaccharides in mammals were identified and quantified using a Waters UPLC XevoTQS (G-147) equipped with a ACQUITY UPLC BEH amide column (1.7 μm, 2.1×100 mm, Waters Corporation, Milford, MA). A 24 min LC separation was performed using a binary gradient at 0.15 mL/min flow rate: solvent A of 10 mmol/L ammonium acetate; solvent B of acetonitrile (100%) and the injection volume was 1 μL. The optimized elution gradient was 70%−56% solvent B for 0−21 min, 56%−70% solvent B for 21−21.2 min, 70% solvent B for 21.2−24 min.

The multi-reaction monitoring (MRM) mode of the mass spectrometry (MS) was operated in positive mode, which can reduce background matrix interference and provided the best quantification sensitivity and accuracy. The following parameters were optimized for oligosaccharide analysis: source temperature 150 °C, desolvation temperature 650 °C, cone gas flow 152 L/Hr, desolvation gas flow 640 L/Hr, collision gas flow 0.13 mL/min. Individual standard solutions of the 11 MOs (2′-FL, 3′-FL, 3′-SL, 6′-SL, 3-GSL, LNT, LNnT, LNH, LNnH, LNFP I, LNFP II, LNDFH I, LNDFH II) at 10 or 50 μg/mL in 30% acetonitrile and were directly injected into the MS at 20 μL/min. For each MO, the capillary and cone voltage, cone gas flow, and desolvation temperatures were optimized and recorded.

## 2.5. Method Validation

### 2.5.1 linear Regression Curves

For LC-MS detection, mixed standard solutions of 11 MOs with varied concentration gradients were created, and linear regression curves were established. The concentrations in the present study are from 0.4 to 40 mg/mL for 2′-FL, 3′-FL, 3-GSL, LNT, LNnT, from 0.4 to 10 mg/mL for 3′-SL, 6′-SL, LNFP I, LNFP II, LNDFH I and LNDFH II. The calibration curve's closeness of fit was calculated using the coefficient of determination ($R^2$). The limit of detection (LOD) and the limit of

quantitation (LOQ) of sensitivity was defined as the concentration with signal-to-noise ratio at 3 and 10. The value of S/N was calculated by MassLynx (Waters). The intra-day precision of the method was determined by testing 3 parallel samples within a day, while the inter-day precision was analyzed by testing those samples once a day for 3 consecutive days.

**2.5.2 Recovery Test**

The recovery experiment involved spiking 11 MOs standards into a 25× diluted extracted oligosaccharide sample at three concentration levels. Three concentration levels were 5, 20, and 40 mg/mL for 2′-FL and 3′-FL, 2.5, 10, and 20 mg/mL 3-GSL, LNT and LNnT, 1, 5, and 10 mg/mL for 3′-SL, 6′-SL, LNFP I, LNFP II, LNDFH I and LNDFH II. These concentrations were selected with the aims: a) to make the concentrations of middle levels closer to those of the real samples; b) to ensure those concentrations were within the linear ranges of corresponding standards.

## *2.6 Quantification of 11 MOs in Milk Samples*

The 11 MOs in human milk, as well as 6 domestic animals were identified and quantified according to the retention time and peak area. The corresponding concentration was calculated by standard curve. In human milk, the MOs concentrations were far above the detection range of the standard curves. Consequently, the samples were diluted 144 times and 72 times before the result was determined. Meanwhile, the other animal milk samples were diluted 8 times. Peak areas were obtained using Masslynx (Waters Corporation). The quantification results were analyzed using GraphPad Prism 9 (GraphPad Software) and expressed in mean.

## *2.7 Quantification of 7 MOs in Heated Dairy Cow Milk Samples*

Three parallel samples of Holstein cow's milk (n=30) were collected simultaneously from Zhongdi Dairy Farm (Beijing, China). One of the three samples was without any heat treatment (raw milk), while another one was heated at 65 ℃ for 30 mins (DK-S22 electric thermostatic water bath, Jinghong Co. Ltd., Shanghai, China), then rapidly cooling the samples by immersed into cold water for 10 min. The other one was heated at 135 °C for 60 s (LLZ-225B Immersion Oil Bath, Semis Instrument Equipment Co. Ltd., Beijing, China), then rapidly cooling for 30 min. In this study, the treatment time in UHT group was longer compared to previous studies, considering the lower thermal conductivity of oil. Moreover, the sterile centrifuge tubes containing milk were pre-heated.

MOs were extracted and analyzed by LC-ESI-MS/MS method. The quantification results were analyzed by Ordinary One-Way ANOVA using GraphPad Prism 9 (GraphPad Software) and expressed in mean ± standard deviation (SD).

# 3. Results and Discussion

## 3.1 Method Validation

To get stronger signals in the detector or increase ionization efficiency in MS, MOs are derivatized in some studies (Oursel et al., 2017; Plows et al., 2021). However, derivatization alters the natural structure of MOs resulting in the loss of their full biological activity. LC-MS/MS with MRM offers the possibility of a robust label-free method for quantifying MOs (Hong et al., 2014). In our study, the ionization and fragmentation conditions were developed for each MO compound, and the unique daughter ions were determined. After that, chromatographic separation of MOs was performed under optimal conditions. Each MO compound was identified based on its elution time and characteristic fragments.

### 3.1.1 Optimization of MS Conditions

To increase the detection's specificity and sensitivity, the MS conditions in positive ion mode were optimized. The high MRM sensitivity of precursor ions for each MO compound was obtained by optimizing the capillary and cone voltage. Due to the difficulty for oligosaccharides to fragment, collision energy was tuned to produce a stronger signal of daughter ions for each MO. Table 7 displayed the entire ion scans for the 11 standard oligosaccharides.

**Table 7** Optimal MRM conditions of each oligosaccharide.

| Name | Capillary (kv) | Cone (v) | Collision (v) | Parent (m/z) | Daughter iron 1 (m/z) | Daughter iron 2 (m/z) |
|---|---|---|---|---|---|---|
| 2′-FL | 3.0 | 30 | 15/15 | 511.2 | 365.2 | 346.8 |
| 3′-FL | 3.0 | 30 | 38/34 | 511.2 | 365.2 | 346.8 |
| 3′-SL | 3.0 | 58 | 34/34 | 656.3 | 365.1 | 314.2 |
| 6′-SL | 3.0 | 60 | 34/36 | 656.3 | 365.1 | 314.2 |
| 3-GSL | 3.0 | 60 | 45/35 | 527.0 | 202.4 | 346.8 |
| LNT | 3.0 | 70 | 20/20 | 730.1 | 550.0 | 388.0 |
| LNnT | 3.0 | 70 | 50/50 | 730.1 | 550.0 | 388.0 |
| LNFP I | 3.0 | 30 | 20/20 | 854.2 | 512.2 | 366.2 |
| LNFP II | 3.0 | 60 | 52/60 | 876.1 | 550.0 | 730.3 |
| LNDFH I | 3.0 | 100 | 58/68 | 1022.4 | 387.8 | 876.2 |
| LNDFH II | 3.0 | 100 | 58/78 | 1022.4 | 549.9 | 876.2 |

### 3.1.2 Unique Fragmentation-assisted MS for Isomer Analysis

The liquid chromatography equipped with an ACQUITY UPLC BEH amide column was used to separate the MOs. There are five pairs of isomers in our study, including 2′-FL and 3′-FL, 3′-SL and 6′-SL, LNT and LNnT, LNFP I and LNFP II, LNDFH I and LNDFH II. Due to their similar polarity, isomers are challenging to achieve chromatographic separation. After optimizing the separation condition, significant separation was achieved for three pairs of isomers: 2′-FL and 3′-FL, 3′-SL and 6′-SL, as well as LNFP I and LNFP II (Figure 10). LNT and LNnT did not exhibit separation and displayed the same retention time (8.6 min), and neither did LNDFH I and LNDFH II (13.47 min). In LC-MS/MS, retention time is not the only way to identify the compounds. Some MOs isomers produce characteristic fragments that facilitate their identification by MS (Yan et al., 2018, Li et al., 2022). For example, although no chromatographic resolution could be obtained for LNDFH I and LNDFH II, they could be successfully quantified using unique ion pair 1022→387 for LNDFH-I and 1022→550 for LNDFH-II. LNT and LNnT share the same precursor, iron products, and retention time due to their highly similar structure and polarity, making their differentiation challenging. Consequently, we quantified together LNT and LNnT (as LNT+LNnT) using LNT as a calibration standard, the same as Galeotti et al. (2012).

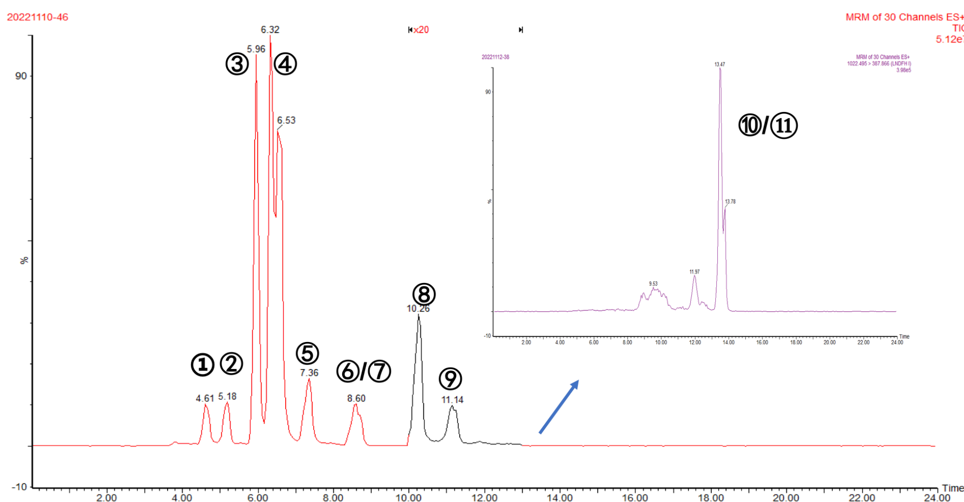


**Figure 10** Chromatogram of 11 MOs standards in MRM model. ① 3′-SL; ② 6′-SL; ③ 2′-FL; ④ 3′-FL; ⑤ 3-GSL; ⑥/⑦ LNT/LNnT; ⑧ LNFP I; ⑨ LNFP II; ⑩/⑪ LNDFH I/II

### 3.1.3 Regression Equations Identification

A wide dilution range was established to cover all 11 MOs within the monitoring range as their concentrations in milk varied considerably. As shown in Table 8, the $R^2$ for each regression equation was more than 0.99, indicating the dependability of

the equation to be suitably used for the calculation. Due to their great abundance, 2′-FL and 3′-FL required a wider linear range than the other MOs. The LODs and LOQs of 11 MOs in milk ranged from 0.94 μg/mL to 125 μg/mL and 3.13 μg/mL to 416 μg/mL, respectively. The more complicated structures of LNDFH I and LNDFH II make it challenging for them to ionize, resulting in higher LOQ of 416 μg/mL and 250 μg/mL, respectively.

**Table 8** Regression equations of 11 MOs.

| Compounds | Calibration curves | $R^2$ | Range (mg/L) | LOD (μg/L) | LOQ (μg/L) |
|---|---|---|---|---|---|
| 2′-FL | y=637356x-472869 | 0.993 | 0.4-40 | 1.04 | 3.49 |
| 3′-FL | y=1002576x-1342568 | 0.995 | 0.4-40 | 1.12 | 3.72 |
| 3′-SL | y=48567x+1575 | 0.998 | 0.1-10 | 100 | 333.3 |
| 6′-SL | y=231918x+46102 | 0.999 | 0.1-10 | 0.94 | 3.13 |
| 3′-GSL | y=178044x-212974 | 0.998 | 0.4-40 | 12.01 | 40.0 |
| LNT+LNnT | y=216190x-9862834 | 0.990 | 0.4-40 | 7.90 | 25.40 |
| LNFP I | y=13263x-1625 | 0.991 | 0.4-10 | 21.8 | 72.72 |
| LNFP II | y=35317x-1486 | 0.990 | 0.4-10 | 40.0 | 133.3 |
| LNDFH I | y=16057x-3714 | 0.996 | 0.4-10 | 125 | 416 |
| LNDFH II | y=12792x-4184 | 0.994 | 0.4-10 | 100 | 250 |

### 3.1.4 Recovery Tests

The recovery experiments were carried out to assess the accuracy of the method. The levels of the MOs detected in the non-spiked sample were subtracted from the amounts measured in the corresponding spiked sample before comparison with the amounts known for the added standards. As shown in Table 9, the recovery rate ranged from 75.6% to 107.4% for 11 MOs, with a few exceptions where the recoveries were below 80%. For example, in LNFP I and LNFP II, the recovery rate at low concentrations was 77.3% and 75.6%, respectively. This could be attributed to systemic losses, such as samples adhering to the tube walls during pre-treatment, which had a greater impact on low-level results compared to middle- and high-level results. For 3-GSL and 6′-SL, the CV at low concentrations was 15.42% and 13.42%, indicating that the operational errors (the noise) during pre-treatment processes had a greater influence on the S/N. Consequently, in sample testing, the concentrations of 3-GSL and 6′-SL should not be lower than 2.5 mg/L and 1 mg/L, respectively. For 3′-SL, the CV at high concentration was relatively high (14.40%) due to the possible matrix effect, indicating the high concentration of 3′-SL should be concerned in the sample test.

**Table 9** Recovery rate of 11 MOs.

| Compounds | Low | | Medium | | High | |
|---|---|---|---|---|---|---|
| | Recovery (%) | CV (%) | Recovery (%) | CV (%) | Recovery (%) | CV (%) |
| 2′-FL | 88.6-95.3 | 3.90 | 85.6-96.6 | 6.42 | 95.2-102.9 | 3.94 |
| 3′-FL | 78.3-87.3 | 5.74 | 75.97-90.6 | 9.05 | 81.2-84.9 | 2.47 |
| 3′-SL | 77.3-89.8 | 7.59 | 94.9-105.7 | 5.64 | 86.7-116.0 | 14.40 |
| 6′-SL | 79.1-102.1 | 13.42 | 76.7-92.9 | 9.68 | 73.3-79.1 | 3.92 |
| 3-GSL | 73.1-101.4 | 15.42 | 77.5-98.2 | 11.81 | 78.4-85.0 | 4.18 |
| LNT+LNnT | 81.3-104.9 | 12.13 | 84.9-86.8 | 1.68 | 93.8-99.0 | 2.79 |
| LNFP I | 74.0-81.2 | 3.60 | 78.5-83.9 | 3.68 | 88.8-100.9 | 6.59 |
| LNFP II | 71.3-78.5 | 3.77 | 72.3-91.6 | 11.84 | 89.9-102.8 | 6.70 |
| LNDFH I | 94.2-102.8 | 4.34 | 86.4-99.8 | 7.59 | 102.2-109.9 | 4.13 |
| LNDFH II | 100.6-105.1 | 2.49 | 76.9-82.5 | 3.84 | 85.1-103.7 | 10.68 |

## *3.2 MOs in Various Mammalian Milk*

Using the above method, 11 MOs were detected in the milk of humans, dairy cows, sheep, yaks, buffaloes, horses, and camels. According to Table 10 and Figure 11, human milk presented a distinct oligosaccharide composition pattern compared to milk from other animals, with a higher concentration and a greater number of detected MOs species (11 MOs). In general, the concentration of 11 MOs in human milk (6.285 g/L in total) is significantly higher than in other mammalian milk, with approximately 11.7,15.8, 10.7, 9.0, 7.6, 7.7 times more MOs than in dairy cow, camel, yak, buffalo, sheep, and horse milk, respectively. Studies have reported that the total MO concentration in mature human milk was approximately 5-15 g/L (Thurl et al., 2017). Among the MOs, 2′-FL was the most dominant component in human milk, consistent with previous findings (Li et al., 2022; Samuel et al., 2019).

However, not all samples of animal milk contained the 11 MOs. Among them, dairy cow and yak milk presented 7 MOs (2′-FL, 3′-FL, LNT+LNnT, 3-GSL, 3′-SL, and 6′-SL), horse milk contained 8 MOs (2′-FL, 3′-FL, LNT+LNnT, 3-GSL, LNFP II, 3′-SL, and 6′-SL), and camel, buffalo, and sheep contained 9 MOs (2′-FL, 3′-FL, LNT+LNnT, 3-GSL, LNFP I, LNFP II, 3′-SL, and 6′-SL). In contrast to human milk, 2′-FL concentration in animal milk was low, and 3′-SL was the most predominant MO, which has been confirmed by multiple studies (Liu, et al., 2014; Mariño et al., 2011). In sheep milk, the proportion of 3′-SL was approximately 30%, while in milk samples from the other 5 species, it exceeded 50% (Figure 11).

Additionally, 2′-FL in sheep milk accounted for about 30% of the total MOs, which was 20 times higher than in other animal milk. Compared to other animals, the MOs

composition in sheep is more similar to that of human milk.

**Table 10** Concentration of MOs in different species.

| MOs | Human | Dairy cow | Camel | Yak | Buffalo | Sheep | Horse |
|---|---|---|---|---|---|---|---|
| Fucosylated neutral MOs (g/L) | | | | | | | |
| 2′-FL | 2.705±1.108 | 0.010±0.005 | 0.012±0.002 | 0.009±0.001 | 0.011±0.003 | 0.244±0.061 | 0.010±0.001 |
| 3′-FL | 1.546±0.740 | 0.023±0.003 | 0.043±0.027 | 0.016±0.002 | 0.042±0.028 | 0.024±0.002 | 0.021±0.001 |
| LNFP I | 0.183±0.303 | ND | 0.003±0.001 | ND | 0.004±0.002 | 0.003±0.001 | ND |
| LNFP II | 0.416±0.497 | ND | 0.002±0.001 | ND | 0.003±0.005 | 0.001±0.001 | 0.001±0.004 |
| LNDFH I | 0.089±0.113 | ND | ND | ND | ND | ND | ND |
| LNDFH II | 0.098±0.116 | ND | ND | ND | ND | ND | ND |
| Non-fucosylated neutral MOs (g/L) | | | | | | | |
| LNT+LNnT | 0.628±0.321 | 0.049±0.008 | 0.050±0.074 | 0.050±0.002 | 0.054±0.008 | 0.050±0.004 | 0.049±0.001 |
| 3-GSL | 0.189±0.057 | 0.032±0.012 | 0.055±0.034 | 0.021±0.001 | 0.093±0.049 | 0.075±0.030 | 0.165±0.004 |
| Sialylated MOs (g/L) | | | | | | | |
| 3′-SL | 0.163±0.092 | 0.347±0.151 | 0.194±0.175 | 0.431±0.042 | 0.346±0.184 | 0.245±0.086 | 0.511±0.046 |
| 6′-SL | 0.268±0.264 | 0.075±0.032 | 0.040±0.021 | 0.061±0.003 | 0.146±0.089 | 0.190±0.124 | 0.062±0.005 |
| SUM | 6.285±1.554 | 0.535±0.187 | 0.399±0.132 | 0.589±0.043 | 0.699±0.043 | 0.832±0.247 | 0.818±0.05 |

ND, the concentration was lower than LOQ

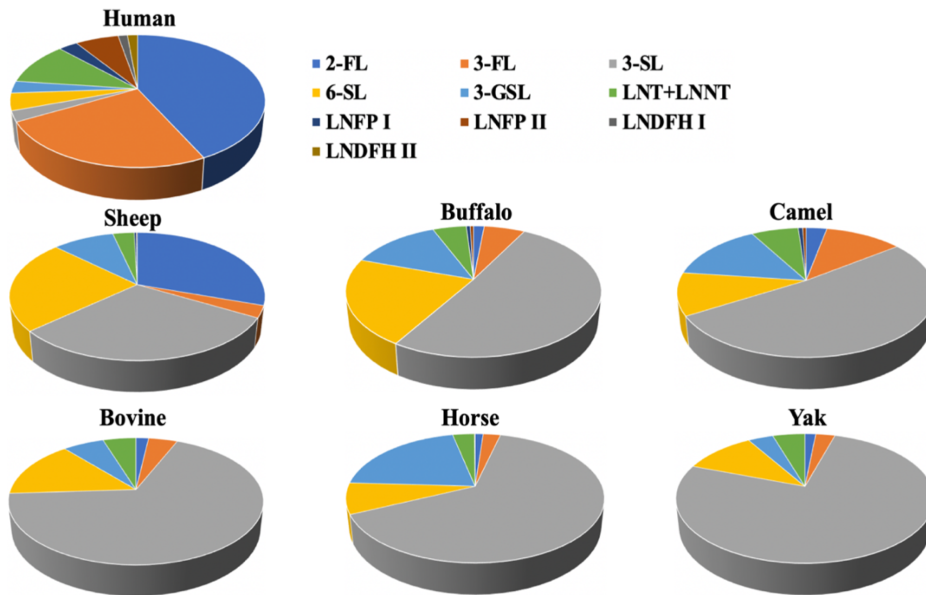


**Figure 11** Component pattern of 11 MOs in different species.

## *3.3 Different Types of MOs in Various Mammalian Milk*

As shown in Table 10 and Figure 12, there were 2 fucosylated neutral MOs detected in all milk samples: 2′-FL (2.705, 0.01, 0.012, 0.009, 0.011, 0.244 and 0.01 g/L in human, dairy cow, camel, yak, buffalo, sheep, and horse milk, respectively) and 3′-FL (1.546, 0.023, 0.043, 0.016, 0.042, 0.024 and 0.021 g/L in human, dairy cow, camel, yak, buffalo, sheep, and horses milk, respectively). Although all milk samples contained 3 non-fucosylated neutral MOs (3-GSL, LNT+LNnT) and 2 sialylated MOs (3′-SL and 6′-SL), their contents were much lower compared to human milk.

Furthermore, the ratio of different types of MOs in human milk and animal milk showed a significant difference. In detail, the fucosylated neutral MOs dominated in human milk, and the ratio of fucosylated neutral MOs: non-fucosylated neutral MOs: sialylated MOs was 80:13:7. Nevertheless, the sialylated MOs were the predominant type in animal milk, constituting more than 50% of the total MOs, which aligns with the previous studies (Shi et al., 2021; Albrecht et al., 2014).

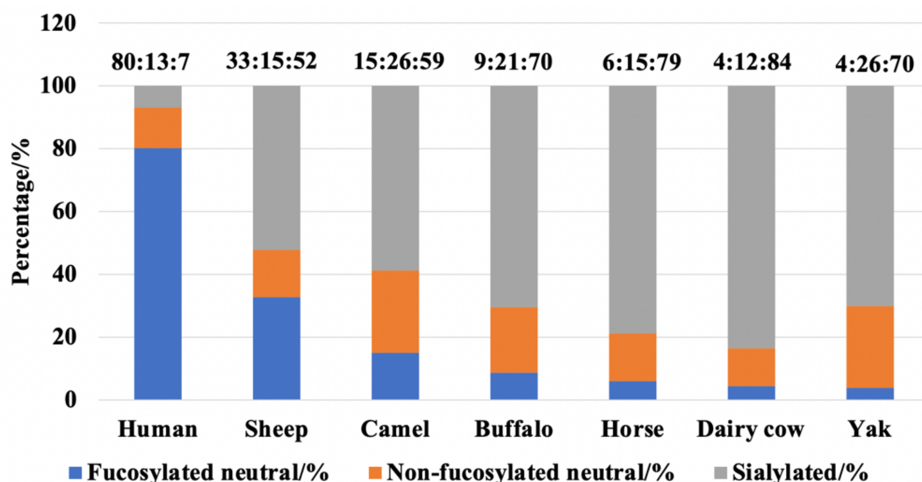


**Figure 12** Different types of MOs in 7 mammalian species.

## *3.4 Heat-treated Effects on Components of MOs*

In fact, for various reason, a considerable number of newborns are not available to breast milk. For them, instead of breast milk, the dairy milk-basis formula became the only source of nourishment. However, even though infant formulas are well-designed and recommended for early life, they do not provide an adequate quantity of desired components, such as cholesterol (Pietrzak-Fiećko and Kamelska-Sadowska, 2020), lipids (Sokol et al., 2015), and MOs (based on the above results). Therefore, supplemented MOs, including 2′-FL, 3′-FL, LNT, LNnT, 3′-SL and 6′-SL (approved by the United States Food and Drug Administration), are added to the formula. However, many procedures, including heat treatment, occur during the manufacturing process of infant formula, which may cause chemical changes in MOs. Currently, there is a lack of knowledge regarding the effects of heat treatment on the concentration and composition of MOs. Thus, in our study, we chose two common heat treatments in milk production to conduct preliminary research on the influence of industrial heat treatment on the concentrations of MOs (2′-FL, 3′-FL, LNT+LNnT, 3-GSL, 3′-SL and 6′-SL) in dairy milk, to provide data for further process of MOs-supplemented products.

According to Figure 13, the MOs composition in dairy milk after 65 °C heat treatment did not differ from that of raw milk samples, as indicated by the overlapping clusters in PCA analysis. In contrast, samples treated at 135 °C appear to separate from raw milk (Figure 13). The concentration of each MO was further analyzed to determine which one was sensitive to high temperatures, and $P$ values were calculated by analysis of variance (ANOVA).

It has been said that pasteurized donor breast milk (heated to approximately 65 °C) provides a safe alternative and is regarded as the next best option when mother's milk is unavailable (Arslanoglu et al., 2010). According to our results, 3′-FL was significantly decreased after heating at 65 °C ($P<0.05$, Figure 14), while the

concentration of other individual MO showed no significant changes ($P > 0.05$, Figure 14). Although fucosylated neutral MOs are the dominant type in human milk, they are present in minimal amounts in dairy milk, and 2′-FL was not present in all dairy milk samples measured in this study. Therefore, a significant alteration in 3′-FL had little impact on the entire picture, as indicated by no significant difference of fucosylated neutral MOs (2′-FL, 3′-FL) between the control group and 65 °C heating group ($P > 0.05$, Figure 14). Additionally, the other indices, including non-fucosylated neutral (3-GSL, LNT+LNnT) and sialylated MOs (3′-SL, 6′-SL), as well as total MOs, showed no remarkable changes in samples after heating at 65 °C ($P > 0.05$). Bertino et al. (2008) assessed how holder pasteurization (62.5 °C heating treatment for 30 min) affected the distribution of 24 MOs and found that the concentration or pattern of HMOs was not influenced by 62.5 °C treatment, which is consistent with the results of our study. Furthermore, other data supported our findings that pasteurization did not significantly influence the concentration of total HMO (Bertino et al., 2008; Hahn et al., 2019).

After heating at 135 °C, the most dominant MO in dairy cow milk, 3′-SL, (constituting over 70% of the total MOs), showed a significant decrease ($P<0.05$, Figure 14), while other individual MO did not exhibit significant changes ($P > 0.05$, Figure 14). And concentrations of sialylated MOs (3′-SL, 6′-SL), as well as total MOs, all declined ($P<0.05$; $P<0.01$). Meredith-Meredith-Dennis et al. (2018) compared the composition of MOs between fresh human milk samples and retort sterilized ones (121 ºC, 5 min) and observed a significant decrease in sialylated HMOs in the retort sterilized samples. One of the possible explanations for the low concentration of MOs in the retort sterilized samples could be attributed to the Maillard reaction, which involves a reaction between amino acids and reducing sugars at temperatures ≥ 120 ºC (Van Boekel et al., 1998).

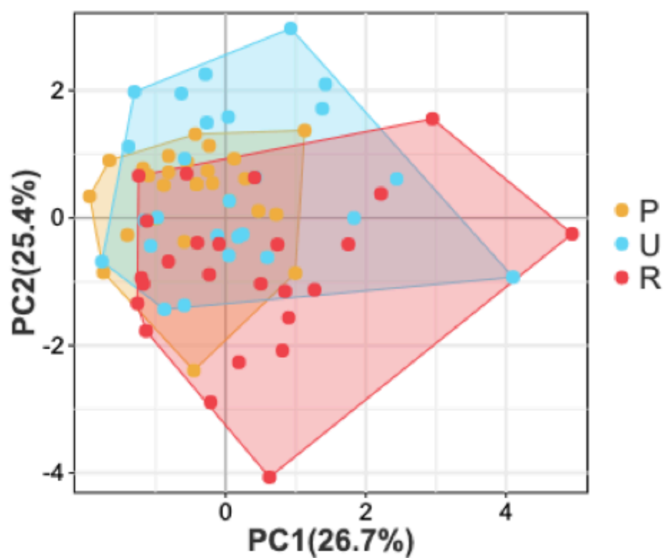


**Figure 13** Principal component analysis (PCA) diagrams of MOs composition in dairy cow milk after heat-treatments. R, Raw milk; P, Milk samples were treated by 65 °C for 30 min; U, Milk samples were treated by 135 °C for 60 s.

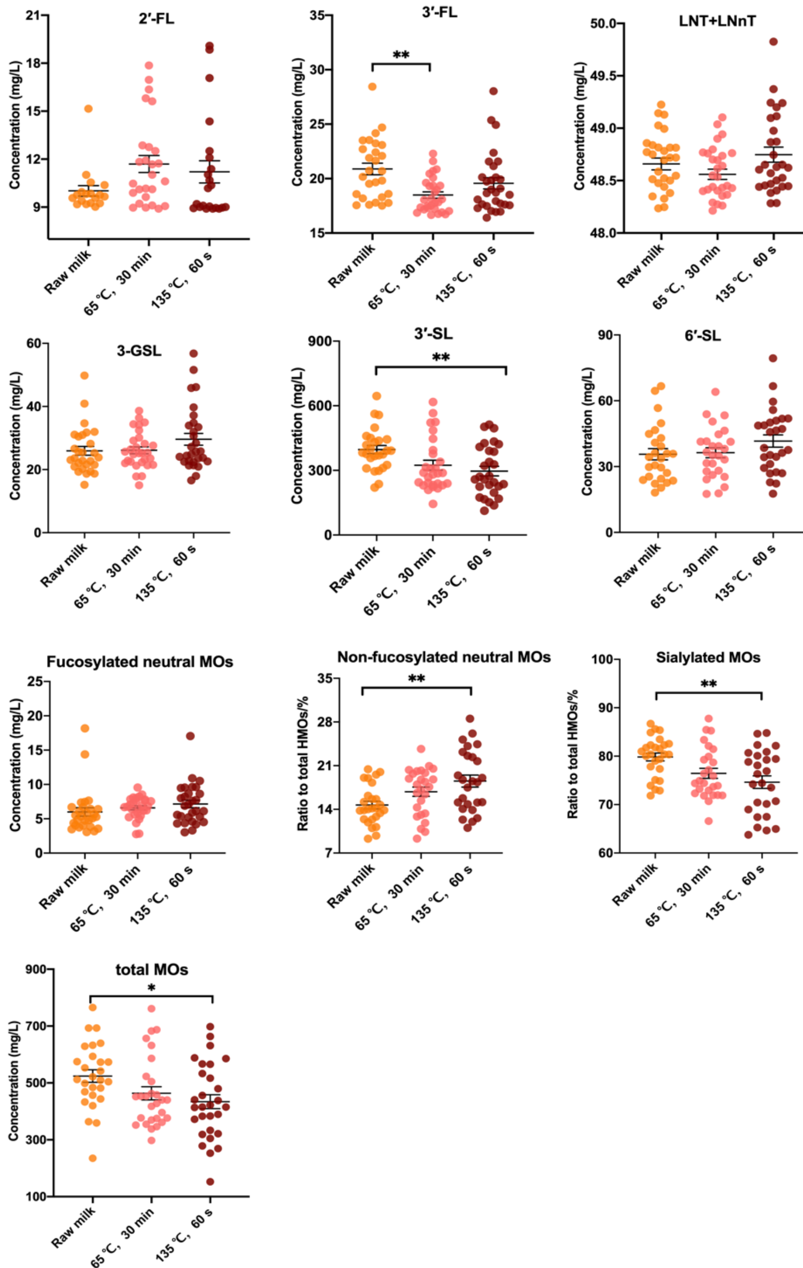


**Figure 14** Heat-treated effects on components of MOs in dairy cows milk. Significance determined using one-way ANOVA analysis and expressed as mean ± SEM. ∗, $P$ <0.05. ∗∗, $P$ < 0.01.

# 4. Conclusion

We employed the LC-ESI-MS/ME method to simultaneously quantify 11 MOs in milk samples from 7 different species. Human milk exhibited the highest abundance of MOs, with 7.6 to 15.8 times more than other animal milk, and the fucosylated neutral MOs were predominant. The milk from dairy cow, camel, yak, sheep, buffalo, and horse showed similar MOs profiles, characterized by a richness of sialylated MOs. Compared to other animals, the MOs composition in sheep milk more closely resembled that of human milk. Furthermore, treatment at 65 °C had no significant effect on the concentration or distribution of MOs, whereas 135 °C heating was associated with their decline, suggesting more attention to temperature control is needed in milk product processing.

# Chapter 4

## 2'-FL ameliorates colon inflammation by modulating gut microbiota and promoting MUC2 expression

The work is an original contribution, adapted from：

Yao, Q., Fan, L., Zheng, N., Blecker, C., Delcenserie, V., Li, H., & Wang, J. (2022). 2′-fucosyllactose (2′-FL) ameliorates inflammatory bowel disease by modulating gut microbiota and promoting muc2 expression. *Frontiers in Nutrition*, **9**, 822020.

According to chapter 3, 2′-FL is the most predominant MO in human milk. The aim of this study was to test the effects of MOs on remitting colonic inflammation in adult model. To meet this objective, the beneficial functions of 2′-FL was assessed, and the roles of gut microbiota in this process were further explored.

**Abstract:** Gut microbiota dysbiosis, together with goblet cells dysfunction has been observed in ulcerative colitis cases. In this study, 2′-FL was orally administered to C57BL/6J mice daily (400 mg/kg b.w.) for 21 days and 5% dextran sulfate sodium (DSS) was used to induce the colitis in the last 7 days. Meanwhile, fecal microbiota transplantation (FMT) was conducted to test the roles of gut microbiota in the remission of colitis by 2′-FL, and bacteria alteration was analyzed through 16S rRNA sequencing. The results showed that the DSS+2′-FL mice were found to have a slower rate of weight loss, lower disease activity index (DAI) scores, and longer colon lengths than the DSS group ($P<0.05$), so as in the FMT recipient mice which received fecal microbiota from the DSS+2′-FL group. In addition, the data revealed that 2′-FL relieved the disorder of DSS-induced gut microbiota, as well as altered mucin-utilizing bacteria. PAS and immunofluorescence staining showed that 2′-FL treatment promoted the recovery of goblet cells and enhanced MUC2 and NLRP6 expression, which was also observed in the FM (DSS + 2′-FL) group. Moreover, NLRP6, which is a potential negative regulator for TLR4/MyD88/NF-kB pathway, was upregulated by 2′-FL in colon tissue. In conclusion, this study suggests that 2′-FL ameliorates colitis in a gut microbiota-dependent manner. The underlying protective mechanism associates with promoting the recovery of goblet cells number and MUC2 secretion.

**Key words**: 2′-fucosyllactose, colitis, gut microbiota, anti-inflammation, MUC2

# 1. Introduction

Inflammatory bowel disease (IBD), a chronic, recurring inflammatory response, is rising in prevalence across the world and diagnosed with increasing frequency during adolescence and early adulthood (Loftus, 2004). Although the mechanism of IBD onset is still unclear, it is widely accepted that dysbiosis of intestinal microbiota is closely associated with its development (Seksik et al., 2003; Desai et al., 2016). Moreover, in clinical and animal experiments, a thinner mucus layer and lower level of glycosylation of Mucin 2 (MUC2) have been found in IBD (Fyderek et al., 2009; Larsson et al., 2011), which likely contribute to reduce commensal fitness and drive the microbial dysbiosis in colitis.

2′-FL has been found to play a crucial role in stimulating the growth of beneficial intestinal bacteria (such as *Bifidobacteria* and *Lactobacillus*), as well as inhibiting the adhesion of pathogens to the surface glycans of epithelial cells (Salli et al., 2021; Facinelli et al., 2019). However, it is still unclear if the gut microbiota is necessary for 2′-FL to exert physiological function and whether 2′-FL has any positive effect besides regulating the composition of microbiota.

To address the above questions, in this study, the colitis mice model was constructed to evaluate the potential of 2′-FL to prevent inflammation in IBD. Moreover, the responses of gut microbiota to 2′-FL during the anti-inflammatory process, as well as the MUC2 secretion were investigated, thus providing new insights to understand the relationship between MOs and intestine health.

# 2. Material and Methods

## *2.1 Chemicals*

Dextran sulfate sodium (DSS) [molecular weight (MW): 36,000–50,000, cat#CD4421] was obtained from Coolaber (Beijing, China). 2′-FL (purity ≥ 98%, cat#GY1141) was purchased from Huich Technology Corporation Ltd. (Shanghai, Beijing). Mouse interleukin-1 beta (IL-1b) (cat#85-BMS6002), interleukin-6 (IL-6) cat#85-BMS603-2), and tumor necrosis factor a (TNF-a) (cat#85-BMS607-3) ELISA kits were purchased from Thermo Fisher Scientific (Waltham, Mississippi, USA), while interleukin-10 (IL-10) (cat#ab255729) and interleukin-17 (IL-17) (cat#ab100702) were purchased from Abcam (Cambridge, England, UK). Ampicillin (cat#A105483-5g), vancomycin (cat#V105495-5g), and neomycin (cat#N109017-5g) were obtained from Aladdin (Shanghai, Beijing, China) and metronidazole (cat#443-48-1) was purchased from Solarbio (Beijing, China).

## *2.2 Animal Experiments*

A total of 18 C57BL/6J male mice (18–20 g, wide type, 6–8 weeks old) were purchased from Vital River Laboratory， Animal Technology Corporation Ltd., Beijing, China. The animals were kept in cages at a constant temperature of 25 °C and relative humidity of 50% under specific pathogen-free conditions. The mice were acclimated for 7 days before the formal experiment. The protocol applied in this study was approved by the Committee on the Ethics of Animal Experiments of the Chinese Academy of Agricultural Sciences (Beijing, China; permission number: IAS-2021-03), which conforms to internationally accepted principles in the care and use of experimental animals (NRC, 2011). Surgical procedures were performed under anesthesia and all the efforts were made to minimize the suffering of the mice.

## *2.3 DSS-Induced Colitis Model Construction*

The mice were randomly assigned into three groups: the control group, the DSS group, and the DSS + 2′-FL group (n=6). During 0–21 days, mice in the DSS + 2′-FL group were orally administrated with 0.3 mL 400 mg/kg b.w. 2′-FL, once daily, while the mice in the control group and the DSS group received equal volumes of PBS. The daily dose of 2′-FL was selected according to previous studies (Li et al., 2020) and based on the safe daily intake range per kg body weight in infants, which is equivalent to 20 g one day for an adult (Heinonen, 2015; Elison et al., 2016). Between days 14–21, mice in the DSS and DSS + 2′-FL groups were administrated with 5% (w/v) DSS in their drinking water ad libitum for 7 days to construct the colitis model (Figure 15). During the experiment, the DAI, consisting of weight loss, stool features, and stool bleeding, was assessed daily according to references (He et al., 2021). Scores were given as a summation of three indexes according to the severity of each parameter. At the end of the experiment, all the mice were killed under anesthesia and blood and colon tissue were collected.

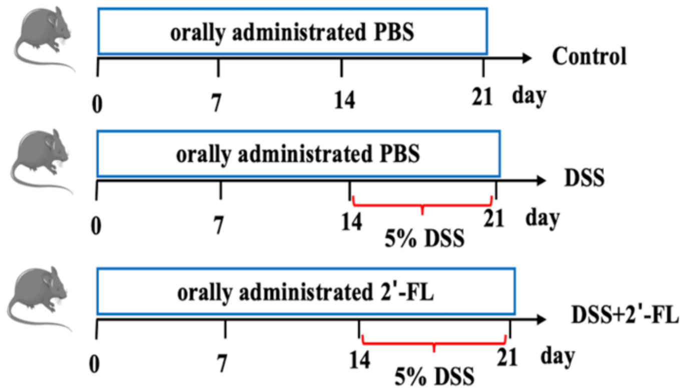


**Figure 15** Experimental workflow for evaluating the effects of 2′-FL on colitis.

## *2.4 Fecal Microbiota Transplantation (FMT)*

The mice were orally administered 0.3 mL antibiotic cocktails (200 mg/kg ampicillin, 200 mg/kg neomycin, 200 mg/kg metronidazole, and 100 mg/kg vancomycin) for 7 days, once a day, for intestinal flora depletion. Fresh feces samples were collected from donor mice on the 5th day after DSS treatment. The fresh samples were suspended in sterile PBS [1 pellet (with similar weight)/mL] and vortexed for 5 min. Then, the suspension was centrifuged at 800 g for 5 min at 4 °C to remove solid particles. The fecal suspension obtained by centrifugation was pooled. The unused fecal suspension is divided and frozen (Gregory et al., 2015). The bacterial suspension was prepared every 2 days and the recipient mice were intragastrically administered 0.3 mL of the bacterial suspension once a day for 7 days, as shown in Figure 16. All the operations were carried out on a clean bench and all the materials used were sterilized to prevent other bacterial contamination.

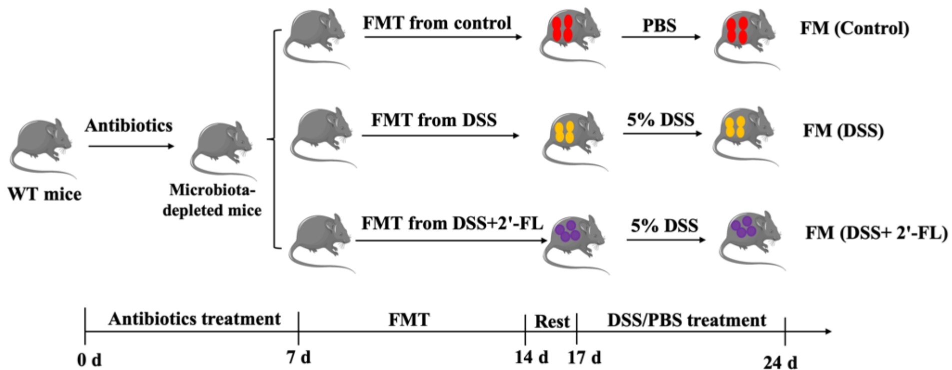


**Figure 16** Experimental workflow of FMT.

## 2.5 Histopathology

The cleaned distal colon segments were rinsed with pre-cooled PBS three times to remove the content. It was then placed into a 10% paraformaldehyde solution for 24 h, followed by dehydrating, paraffin embedding, sectioning, and staining (H&E, PAS). Finally, histological analysis was performed according to a previously reported method (Allen et al., 2012).

## 2.6 DNA Extraction and 16S Sequencing

The fecal DNA was extracted using the Magnetic Soil and Stool DNA Kit (Tiangen Biotechnology Corporation Ltd., Beijing, China), according to the instructions of the manufacturer. Its concentration was measured with the Qubit dsDNA HS Assay Kit and Invitrogen Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The universal primer set 27F: AGRGTTTGATYNTGGCTCAG and 1492R: TASGGHTACCTTGTTASGACTT was used to amplify the full-length 16S rRNA gene from the genomic DNA extracted from each sample. SMRTbell libraries were prepared from the amplified DNA by the SMRTbell Express Template Prep Kit 2.0 and then sequenced on a single PacBio Sequel II 8M cell using the Sequel II Sequencing kit 2.0 (Pacific Biosciences, Menlo Park, California, USA). The raw reads generated from sequencing were filtered and demultiplexed using SMRT Link software (version 8.0) with the minPasses $\geq$ 5 and minPredicted Accuracy $\geq$ 0.9, to obtain the circular consensus sequencing (CCS) reads. The UCHIME algorithm (version 8.1) was used to detect and remove chimera sequences to obtain clean reads. Sequences with similarities $\geq$ 97% were clustered into the same operational taxonomic unit (OTU) by USEARCH (version 10.0) (Edgar, 2013) and the OTUs with abundance <0.005% were filtered (Bokulich et al., 2013). Taxonomy annotation of the OTUs was performed based on the naive-Bayes classifier in QIIME2 using the SILVA database (Quast et al., 2013) with a confidence threshold of 70%. The alpha diversity was calculated and displayed by the QIIME and R software, respectively. Beta diversity was determined using QIIME to evaluate the degree of similarity in microbial communities from different samples. Principal coordinate analysis (PCoA), heat maps, unweighted pair group averaging (UPGMA), and non-metric multidimensional scaling (NMDS) were used to analyze the beta diversity.

## 2.7 Immunofluorescence Staining

Frozen sections were prepared and blocked with 10% sheep serum at 37 °C for 1 h and then were added primary antibodies including anti-MUC2 and anti-NLRP6 overnight at 4 °C; the next day, sections were incubated with fluorescent sheep anti-rat secondary antibody in 0.5% BSA-PBS with Tween (PBST) solution for 1 h at room temperature. The nucleus was stained with 4′, 6-diamidino-2-phenylindole (DAPI) dye (diluted with PBS at 1:1000) at room temperature for 8 min in the dark place. The slices were sealed with anti-fluorescence quenching solution and observed with a Zeiss Fluorescence Microscope (LSM 800, Germany, UK).

## 2.8 RNA Extraction and Quantitative RT-PCR

Colon tissues were collected and grounded in liquid nitrogen. RNA was extracted by the Trizol method. After being added with 75% ethanol, samples were centrifuged at 10 000 g for 10 min and then the supernatant was removed. RNA was purified by the following steps: 30 μL 8 mol/L LiCl and 270 μL RNase-free water were added at −20 °C standing for 30 min and then centrifuged at 10 000 g for 10 min. After the precipitate was dissolved by 90 μL Rnase-free water, 10 μL 3 mol/L $CH_3COONa$ and 200 μL absolute ethyl alcohol were added and incubated for 30 min at −20 °C and then centrifuged at 14 000 g for 30 min. After the supernatant was removed, 75% ethanol was added to precipitated RNA and samples were centrifuged at 8 000 g for 10 min. RNA samples were transcribed into complementary DNA (cDNA) (42 °C for 10 min, 65 °C for 10 s, stored at 4 °C) by the PrimeScript$^{TM}$ RT Reagent Kit (Takara Biotechnology Incorporation, Kusatsu, Shiga, Japan). The primers of MUC2 (F: AACACAGTCCTGGTGGAAGG; R: CATTGTCAGGTCCCACACAG); NLRP6 (F: AAGGT GAAGGAGAGGAATG; R: GAAGAGCCGATTGAAAGTG); intestinal trefoil factor (TFF3) (F: CATGTCACCCCCAAGGAGTG; R: AGGTGCATTCTGCTTCCTGC), and resistinlike beta (RETNLB) (F: CACCCAGGAGCTCAGAGATCTAA; R: ACGGCCCCATCCTGTACA) were synthesized and genes expression were detected, and glyceraldehyde phosphate dehydrogenase gene (GAPDH) were adopted as housekeeping gene. The results were calculated by $2^{-\Delta\Delta Ct}$ method.

## 2.9 Western Blot

In total, 0.1 g colon tissue was collected and grounded in liquid nitrogen. 100 μL radio immunoprecipitation assay (RIPA) buffer was added and then the tissue was processed by Ultrasonic Cell Pulverizer (JY88-IIN) for 5 min. After fully lysed, it was centrifuged at 12 000 g for 15 min, 1 μL supernatant was taken for quantitative determination using the bicinchoninic acid (BCA) Protein Assay Kit. For rest of the solution, a 200 μL 5×NuPage lithium dodecyl sulfate (LDS) loading buffer was added. After being boiled, equal amounts of protein from each sample were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, Massachusetts, USA). After being blocked in 5% skim milk dissolved in TBST, membranes were incubated with primary antibodies for 2 h at 25 °C and then probed with secondary antibodies conjugated with horse radish peroxidase (HRP) for 1 h at 25 °C. Primary antibodies used in this study were anti-NLRP6 (cat#SAB1302240), anti-Toll-like receptor 4 (TLR4) (cat#bs-1021R), antimyeloid differential protein-88 (MyD88) (cat#bs-1047R), antinuclear factor kappa-B (NF-κB) (cat#51-0500) and anti-β-actin (cat#A01011). All the signals were visualized and analyzed by Image J (1.53a).

## 2.10 Data Analysis

Animal study results were expressed as mean± SEM with six biological repeats. All the records were analyzed by ordinary One-Way ANOVA with Tukey's analysis to assess differences between the groups. Values of $P<0.05$ were considered as

statistically significant. The GraphPad Prism version 9.0 was applied to drawbar charts of the above data.

# 3. Results

## *3.1 2′-FL Remitted the Colitis Induced by DSS in C57BL/6J Mice*

The results showed colitis was successfully induced in the mice after the treatment with DSS for 7 days, as reflected in their significantly decreasing body weight, shorter colon lengths, and higher DAI scores in comparison to those of the control group ($P<0.05$; Figures 17A, B, D). Mice in the DSS group exhibited severe and diffuse destruction of the epithelial layer and extensive inflammatory cells infiltration in epithelium and lamina propria of the colon (arrows in Figure 17C). In contrast, mice in the DSS + 2′-FL group showed a less weight loss, lower DAI scores, and longer colon ($P<0.05$), as well as a lower degree of inflammation compared to the DSS group. The concentrations of proinflammatory cytokines, such as IL-6, IL-1β, TNF-α, and IL-17, were significantly increased in the DSS groups compared with those of the control group ($P<0.01$), while the level of anti-inflammatory cytokine, IL-10, was remarkably decreased ($P<0.01$, Figure 17E).

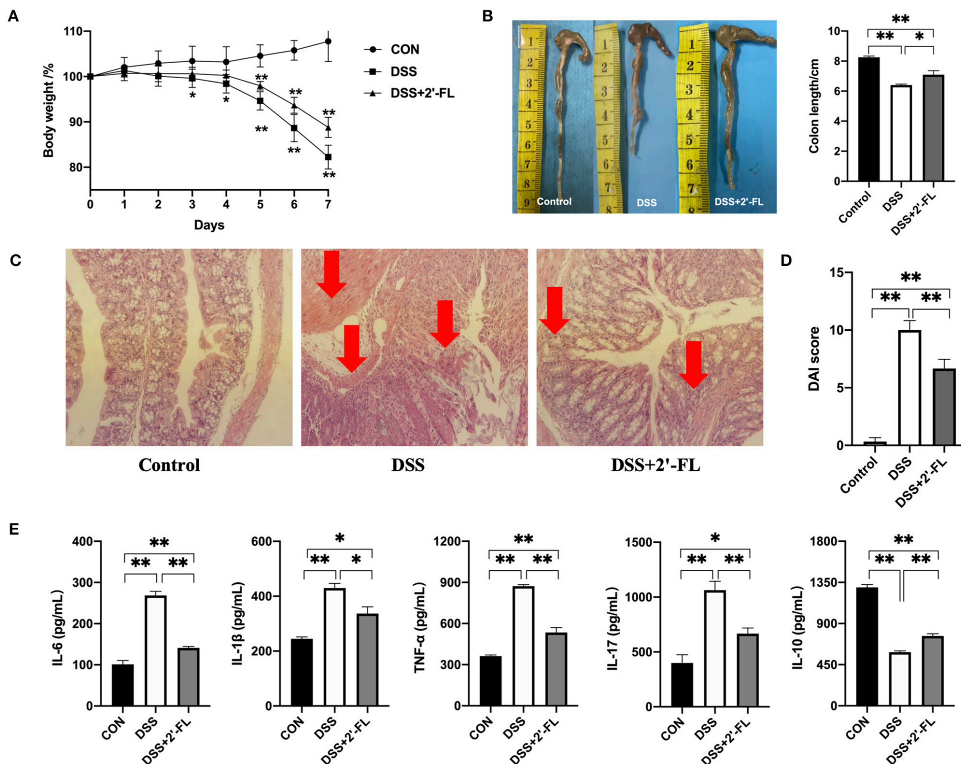


**Figure 17** 2′-FL remits DSS-induced colon inflammation in C57BL/6J mice. **A**, Changes in the body weight of mice after receiving 5% DSS water; **B**, The representative images (Left) and statistical analyses (Right) of colon length; **C**, Representative images of colon by H&E staining; **D**, DAI indices; **E**, Cytokine levels in serum of C57BL/6J mice. Significance was determined using One-Way ANOVA analysis and expressed as mean ± SEM. * *P*<0.05; ** *P*<0.01.

## *3.2 Gut Microbiota Involved in the process of 2'-FL Mitigating Colitis*

Figure 18 demonstrated that the recipient mice in the FMT groups showed similar pathological phenotypes with those of the donor mice, as evidenced by the slower weight loss, longer colons, lower DAI scores, alleviated pathology, and lower proinflammatory cytokine levels in the FM (DSS + 2′-FL) group compared with the FM(DSS) group. These results accumulatively indicated that gut microbiota was involved in the process in which 2′-FL mitigated colon inflammation.

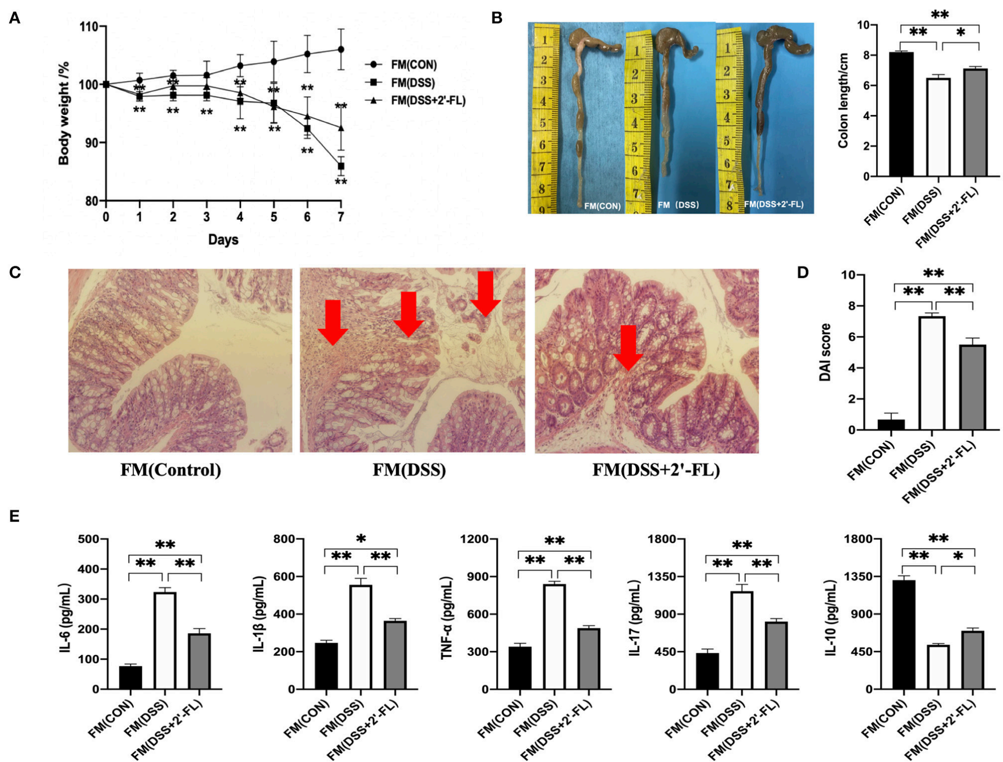


**Figure 18** Gut microbiota is involved in 2′-FL mitigation of colon inflammation. **A**, Changes in body weight of mice after receiving 5% DSS water; **B**, The representative images (Left) and statistical analysis (Right) of colon length; **C**, Representative images of colon by H & E staining; **D**, DAI indices. **E**, Cytokine levels in serum of FMT C57BL/6J mice. Significance was determined using One-Way ANOVA analysis and expressed as mean ±SEM. * $P<0.05$; ** $P<0.01$.

## *3.3 2'-FL Significantly Altered the Composition of Gut Microbiota*

As shown in Figure 19, an apparent cluster separation among the three groups was revealed in the PCoA of the Bray–Curtis distances. Alpha diversity was significantly declined (ACE and Chao1) in the DSS group compared with the control group ($P<0.05$). The relative abundances of microbes were further analyzed by One-Way ANOVA. At the phylum level, Firmicutes was the most predominant phylum in the DSS group, accounting for 53.0%, while Bacteroidetes were most abundant in the control and DSS+2′-FL groups, with proportions of 42.88% and 58.55%, respectively. The F/B ratio in the DSS group was significantly greater than that in the control and DSS+2′-FL groups ($P<0.01$). Specifically, the family *Muribaculaceae* (originally called S24-7), order Bacteroidales (and class Bacteroidia) displayed a relative richness in the DSS+2′-FL group. Nevertheless, *Lachnospiraceae NK4A136*,

*Rumincoccaceae_UCG-014*, and *Iiebacterium valens* were relatively overrepresented in the mice with colitis ($P<0.05$), while *Faecalibaculum rodentium*, *Bifidobacterium animalis*, and *Bacteroides caecimuris* were relatively enriched in the control and DSS + 2′-FL groups ($P<0.05$).

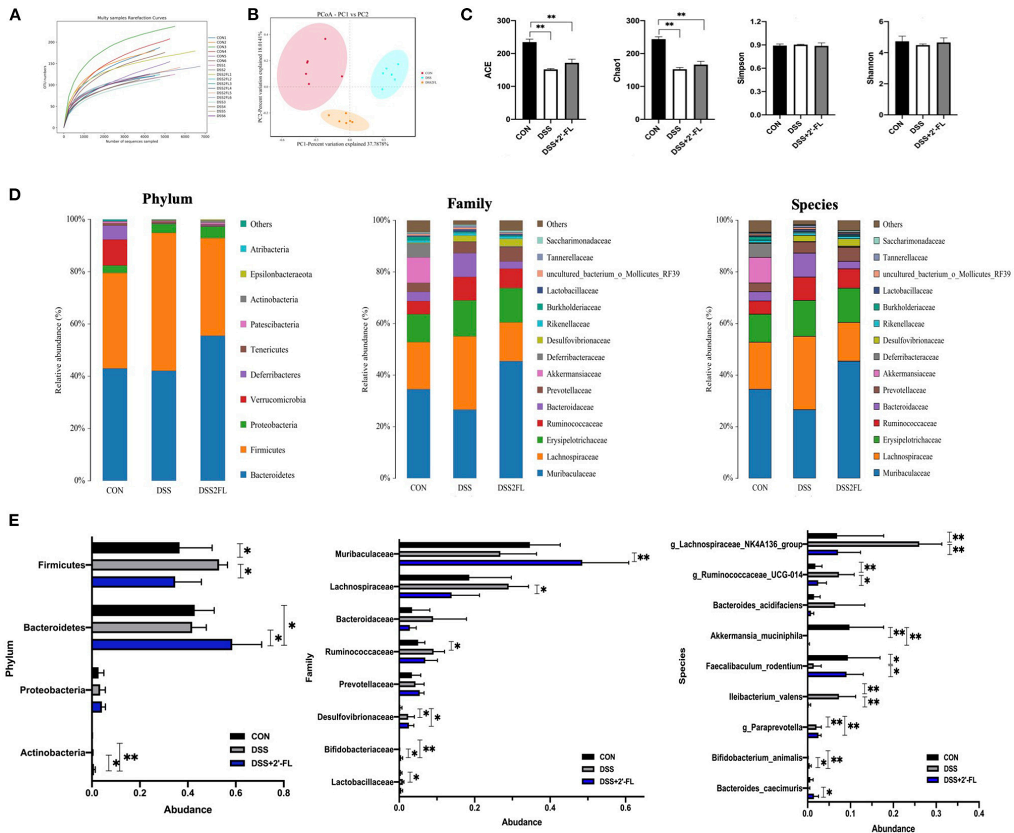


**Figure 19** Gut microbiota composition changes in response to DSS and 2′-FL. **A**, Multi samples rarefaction curve; **B**, Principal coordinate analysis (PCoA) using the Bray-Curtis metric distances of beta diversity. **C**, Alpha diversity; **D**, Stacked bar plots of species distribution at phylum, family, species levels, respectively. **E**, Representative significantly changed bacteria in phylum, family, species levels across groups. Significance was determined using One-Way ANOVA analysis and expressed as mean ± SEM. * $P<0.05$; ** $P<0.01$.

## *3.4 2′-FL Altered Mucin-Utilizing Bacteria*

The multi-layered mucus covering the colonic surface acts as a major defensive barrier and serves as “food” for some special bacteria, namely mucin-utilizing bacteria. Comparing colitis mice with or without 2′-FL intake, we found pronounced different abundances on mucin-utilizing bacteria. Specifically, *Paraprevotella*,

*Lachnospiraceae NK4A136*, *Lachnospiraceae*, and *Bacteroides* were found significantly enriched in the DSS group (*P*<0.05); however, *Odoribacter*, *Faecalibaculum*, and *Akkermansia muciniphila* were significantly decreased in the DSS group (*P*<0.05, Figure 20) compared with the control and DSS+2′-FL groups.

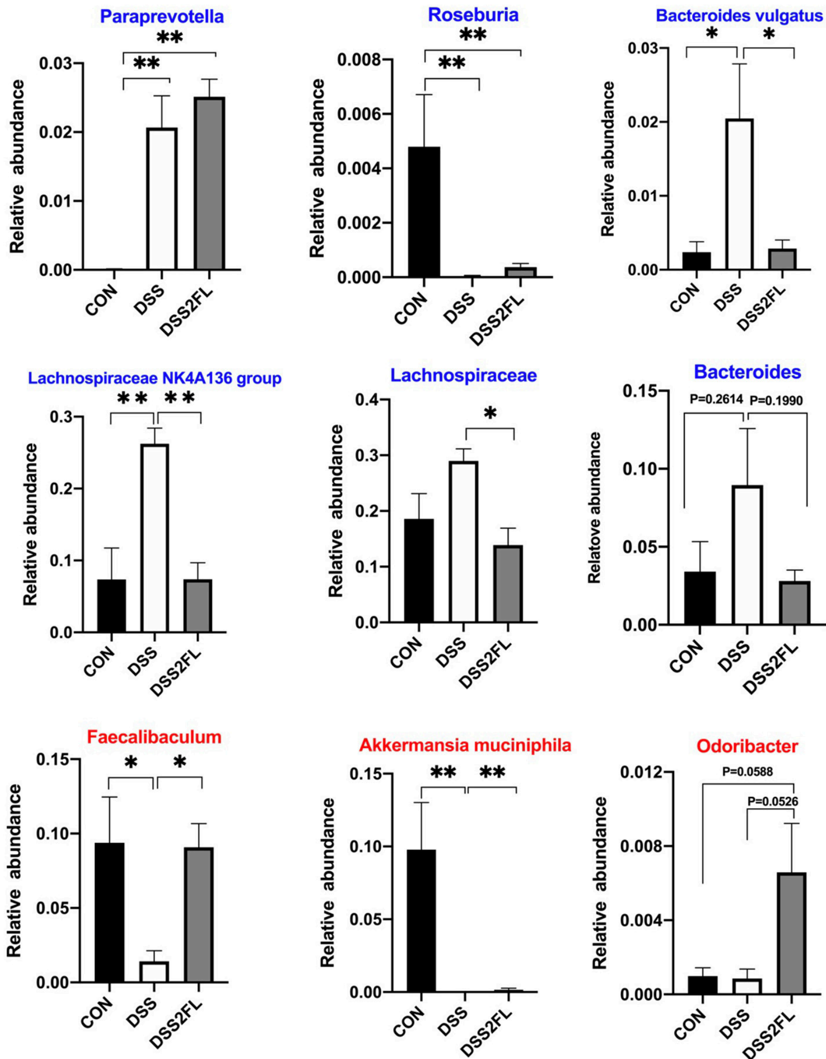


**Figure 20** 2′-FL alters mucin-utilizing bacteria. Blue font, the bacterial genus encodes cleavage genes and transporters of mucin-associated O-glycans; red font, the bacterial genus encodes cleavage genes of mucin-associated O-glycans. Significance was determined using one-way ANOVA analysis and expressed as mean ± SEM. * *P*<0.05; ** *P*<0.01.

## *3.5 2′-FL Enhanced Mucus Barrier in DSS-Induced Colitis Mice*

PAS and immunofluorescence staining results showed a significant depletion of goblet cells and MUC2 secretion in the DSS group. Moreover, colonic inflammation resulted in a decrease of TFF3 and RETNLB expression, two proteins closely related with MUC2 secretion, indicating a dysfunction in goblet cells. After 2′-FL ingestion, the number of goblet cells in colon was increased, so did as *MUC2*, *NLRP6*, *TFF3*, and *RETNLB* mRNA expressions (Figure 21).

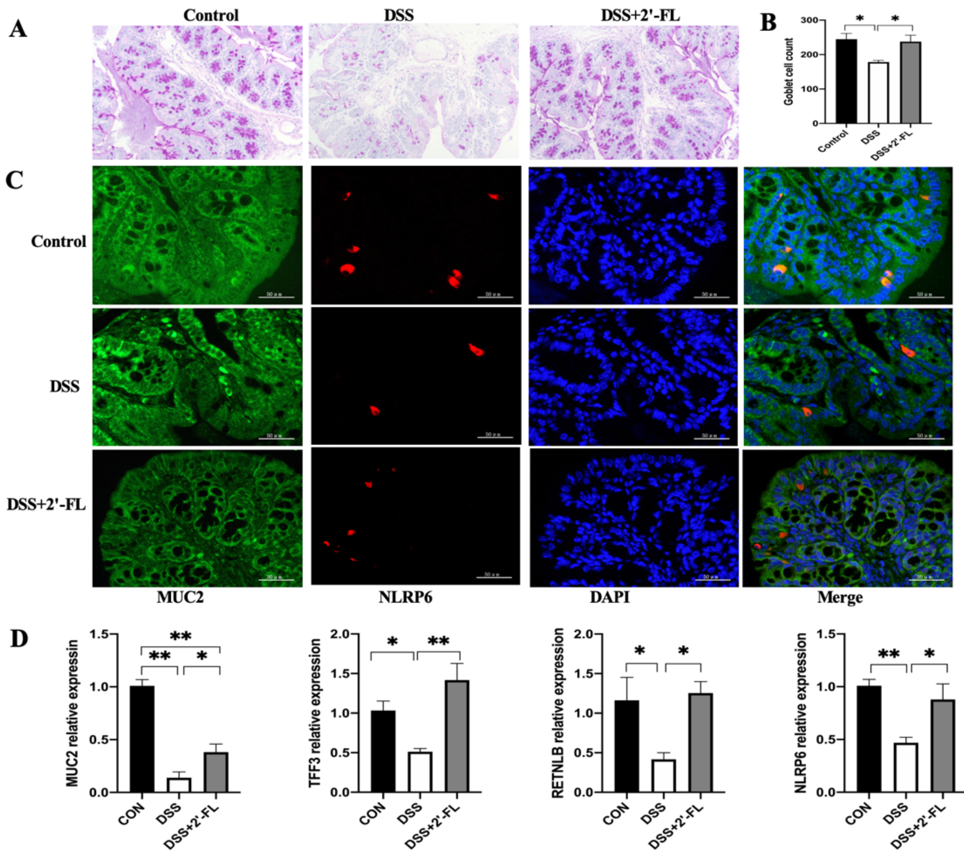


**Figure 21** 2′-FL increases the number of goblet cells and mucin expression. **A**, Representative PAS-stained distal colon sections showing goblet cells. **B**, The number of goblet cells in distal colon; **C**, Representative fluorescent images of MUC2 (green) and NLRP6 (red) in colon tissue. **D**, Quantitative RT-PCR showing expression of *MUC2*, *TFF3*, *RETNLB* and *NLRP6*, relative to GAPDH. Significance was determined using One-Way ANOVA and expressed as mean ± SEM. * $P<0.05$; ** $P<0.01$.

## *3.6 NLRP6 is a Potential Negative Regulator for TLR4 Pathway*

The levels of TLR4, MyD88, NF-κB, and TNF-a were significantly upregulated in the DSS group compared with the control ($P$<0.05), while NLRP6 levels were remarkably downregulated ($P$<0.05) (Figure 22). The tendency was totally reversed by 2′-FL, as reflected in significantly decreasing levels of TLR4, MyD88, NF-κB, and increasing levels of NLRP6 ($P$<0.05), indicating that NLRP6 might be a negative regulator for TLR4-related pathway.

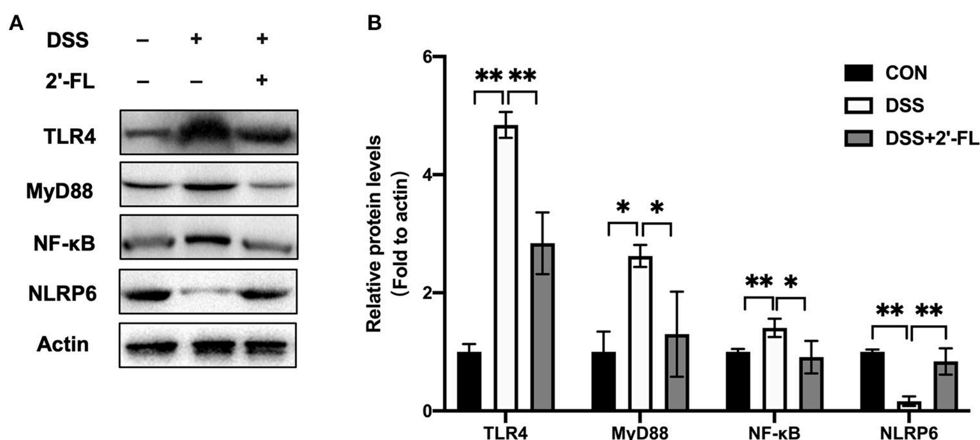


**Figure 22** Protein expression of factors in TLR4-related pathway in mouse colon tissue. **A**, Immunoblot bands of Actin, TLR4, MyD88, NF-kB and NLRP6 in colon tissue. Actin was regarded as an internal reference. **B**, Densitometric quantitation for normalized proteins relative to Actin (%) in colon tissue. * $P$<0.05; ** $P$<0.01.

# 4. Discussion

2′-FL cannot be digested by the stomach due to the absence of specific types of galactosidases and, consequently, it remains intact until reaching the intestine to be used by microorganisms. Intestinal microbiota has been reported to mediate the function of biochemicals (Du et al., 2017). Thus, the question arose whether the gut microbiota could affect the protective activity of 2′-FL against colitis. To test this, the feces of donor mice treated with DSS+2′-FL were transplanted to recipient mice, which had been excluded from 2′-FL treatment. As expected, after a continuous 7-day FMT and then a successive 7-day DSS treatment, the recipient mice showed a similar protective activity against colitis as the donor mice, despite them not being treated with 2′-FL. It suggested that 2′-FL could remit the colitis in a gut microbiota-mediated manner.

Further analysis revealed that 2′-FL reshaped the gut microbiota after dysbiosis. The level of inflammation-promoting bacterium Firmicutes was found to be significantly increased by DSS treatment, while 2′-FL prevented the induction. The highest F/B ratio in the DSS group, a distinctive dysbiosis sign of the intestinal

microbiota (Xie et al., 2020; Indiani et al., 2018), was significantly decreased by 2′-FL. The family *Muribaculaceae,* order Bacteroidales (and class Bacteroidia) displayed a relative richness in the DSS+2′-FL group.

On the other hand, 2′-FL also upregulated mucin-utilizing bacteria. Indeed, some commensal bacteria can utilize mucin as an energy source to support their growth (Shao et al., 2020). Family *Muribaculaceae* has been found to be the major mucin monosaccharide foragers, followed by members of *Lachnospiraceae*, *Rikenellaceae*, and *Bacteroidaceae* families (Martens et al., 2009). In this study, *Lachnospiraceae* was significantly upregulated by DSS; however, the *Muribaculaceae* were selectively increased by 2′-FL treatment, which is consistent with Li's study (Li et al., 2020). The abundances of mucin-utilizing bacteria containing cleaved mucin-associated sugars (cleavage genes) or cleaved sugar uptake genes (transporter genes) were then analyzed according to Wlodarska et al. (2017). The results showed that 9 mucin-utilizing bacteria were significantly modified in the presence of 2′-FL. Those alterations in mucin-utilizing bacteria, as well as depletion and NLRP6 decline in goblet cells of the DSS group, might lead to excessive consumption of MUC2, the decrease of the thickness of the mucus layer, and more severe inflammation in the colon. Recovery from those negative alterations might be the main mechanism by which 2′-FL attenuated the inflammation.

# 5. Conclusion

The findings of this study indicate that 2'-FL ameliorated colitis in a gut microbiota-mediated manner. The underlying protective mechanism is associated with promoting the recovery of goblet cells number and MUC2 secretion. Besides, 2'-FL exerts anti-inflammatory effects by targeting TLR4/MyD88/NF-κB-related inflammation pathway. Taken together, 2'-FL might be a valuable candidate for development of functional foods to protect from intestinal inflammation.

# Chapter 5

## 2'-FL inhibits inflammation and promotes MUC2 secretion in LS174T goblet cells

The work is an original contribution, adapted from：

Yao, Q., Li, H., Gao, Y., Zheng, N., Delcenserie, V. & Wang, J. (2023). The milk active ingredient, 2'-fucosyllactose, inhibits inflammation and promote MUC2 secretion in LS174T goblet cells *in vitro*. *Foods*, **12**, 186.

According to Chapter 2, there are different types of MOs with various functions. The aim of this study was therefore to compare the effects of different MOs on improving mucin secretion. The effects of 2'-fucosyllactose (2'-FL, a representative fucosylated neutral MO), 3'-sialyllactose (3'-SL, a representative sialylated MO), galacto-oligosaccharide (GOS, commonly added into infant formula) and lactose (Lac, their structure core) on promoting MUC2 secretion in goblet cell models were more particularly assessed.

**Abstract:** To compare the functions of individual human milk oligosaccharides (HMOs) in enhancing mucin expression, the effects of 2'-FL, 3'-SL, galacto-oligosaccharides (GOS) and Lactose (Lac) on goblet cells' functions were tested *in vitro*. Firstly, the appropriate dosage of the four chemicals was assessed in LS174T cells using the CCK-8 method. Then they were supplemented into a homeostasis and inflammatory environments, and the mucin secretion-related genes, including *MUC2*, *TFF3*, *RETNLB*, *CHST5* and *GAL3ST2*, in LS174T cells were detected using quantitative RT-qPCR. The results showed that 2′-FL (2.5 mg/mL, 72 h) was unable to increase *MUC2* expression in the steady-state condition. Comparatively, it exhibited a higher ability to improve mucin secretion under an inflammatory condition compared with GOS, demonstrated by a significant increase in *TFF3* and *CHST5* mRNA expression levels ($P$>0.05). However, 3′-SL and Lac exhibited no effects on mucin secretion. However, after silencing the *NLRP6* gene, the mRNA expression levels of *MUC2*, *TFF3* and *CHST5* in the (2′-FL+TNF-α+NLRP6 siRNA) group were significantly decreased compared to the (2′-FL+TNF-α) group ($P$<0.05), indicating that NLRP6 was essential for MUC2 expression in goblet cells. In addition, 2′-FL could significantly decrease TLR4 ($P$<0.055), MyD88 ($P$<0.05) and NF-κB ($P$<0.05) levels in LS174T inflammatory cells, even when the NLRP6 was silenced. Altogether, these results indicated that, in goblet cells, 2′-FL exerts its function via multiple processes, i.e., by promoting mucin secretion through NLRP6 and suppressing inflammation by inhibiting the TLR4/MyD88/NF-κB pathway.

**Key words**: 2'-FL, 3'-SL, GOS, lactose, goblet cell, MUC2, NLRP6

# 1. Introduction

Although HMOs were reported to improve the mucin barrier in several mice inflammatory models (Bering, 2018), it remains unclear whether this is due to the HMOs mixture or if a fraction of HMOs plays the main role. Moreover, the difference in improving mucin secretion between different types of HMOs has not yet been elucidated.

Based on their structure, HMOs can be classified as fucosylated HMOs (2′-FL as a representative), sialic acid HMOs (3′-SL as a representative) and non-fucosylated neutral HMOs (Hill et al., 2021). Currently, GOS are added to infant formula to mimic the function of HMOs (Ghosh et al., 2020). In addition, lactose (Lac) is the most abundant sugar carbohydrate in dairy products, and it is also the core structure of MOs and GOS.

MUC2, the main protein in the mucus layer, is primarily produced by goblet cells. It forms a single non-moving mucus layer that tightly adheres to colonic epithelia (Arike & Hansson, 2016; Blacher et al., 2017). Several genes are involved in the process of mucin secretion. Trefoil factor 3 (TFF3) is co-secreted and synergizes with MUC2 to maintain the integrity of the mucin barrier (Ge et al., 2015). Another two related products, Golgi sulfotransferases galactose-3-O-sulfotransferase 2 (GAL3ST2)

and carbohydrate sulfotransferase 5 (CHST5), are essential sulfur transferases located in the Golgi apparatus. It was found that adding sulfonyl groups to mucin improved the function of the mucus barrier and protected agains adherence of pathogens (Hasnain et al., 2017). The resistin-like beta (RETNLB) drives spontaneous colitis in MUC2-deficient mice by promoting commensal microbial dysbiosis (Morampudi et al., 2016). NLRP6 is essential for mucin secretion and related to mucus granule exocytosis (Wlodarska et al., 2014). Moreover, in chapter 4, NLRP6 was found to be involved in the process of 2′-FL promoting MUC2 expression in C57BL/6J mice.

In the present study, the potential of 2′-FL, 3′-SL, GOS and Lac to improve mucin secretion under homeostasis and inflammatory states was investigated by testing the expression of MUC2 secretion-related genes. NLRP6 siRNA was also investigated to further confirm the roles of NLRP6.

# 2. Material and Methods

## *2.1 Chemicals*

2′-FL, (cat# GY1141, 98%) and 3′-SL, (cat# GY1143, 98%) were purchased from HuicH Biotech Co., Ltd. (Shanghai, China). GOS, (cat#G9150, 70%) and Lac (cat#SL8740, 98%) were purchased from Solarbio Life Sciences (Beijing, China). LS174T cells (cat#CL-0145) were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Recombinant human tumor necrosis factor α (TNF-α, cat#200-13-2) was obtained from Peprotech (Rocky Hill, NJ, USA). Anti-MUC2 Polyclonal Antibody (cat#K106881P) and FITC Goat Anti-Rabbit IgG (cat#A22120) were purchased from Bioss (Beijing, China). Anti-NLRP6 Antibody (cat#ABF29) was obtained from Merch (Shanghai, China). The siRNA of NLRP6 was designed and produced from GenePharma (Shanghai, China). Lipofectamine™ 3000 transfection reagent (cat#L3000008) was purchased from ThermoFisher Scientific (Shanghai, China).

## *2.2 Cell Culture*

LS174T cells (human intestinal goblet cells) were grown using complete growth medium (MEM+10% FBS+1% P/S) at 37 °C in a cell incubator with 5% $CO_2$. The cells were plated into 6-well plates ($5×10^5$ cells/mL medium) until they reached 70%–80% adherence, then the old medium was replaced with 1 mL of fresh medium containing different concentrations of the four chemicals (0, 1.0, 1.5, 2.0, 2.5, 3.0 and 5.0 mg/mL of 2′-FL or 3′-SL; 0, 5, 7.5, 10, 12.5, 15 and 20 mg/mL of GOS or Lac). The selection of 2′-FL and 3′-SL dosages were based on the work of Figueroa-Lozano et al. (2021) and He et al. (2016), while those of GOS and Lac were based on studies by Ghosh et al. (2020). The cells were allowed to grow for another 24, 48 and 72 h, followed by a cell viability assessment using CCK-8 to identify the proper dosage and culture time.

## *2.3. Cellular Inflammatory Model Construction by TNF-α*

LS174T cells were seeded into 12-well plates and cultured to 70%–80% adherence. The proper dosage of 2′-FL, 3′-SL, GOS and Lac was applied for 48 h and then TNF-α (10 ng/mL) was added (Cheng et al., 2019). At the end of the stimulation, LS174T cells were homogenized with TRIzol reagent and stored at -20 °C for RNA extraction.

## *2.4. siRNA Gene Treatment*

LS174T cells were seeded into 6-well plates and cultured until they adhered to the wall. The medium was replaced by an FBS-free and A/P-free medium containing siRNA fragments of NLRP6 (designed by GenePharma) and then cells were continuously cultured for 24 h. Next, 2.5 mg/mL 2′-FL was added for 48 h followed by a TNF-α (10 ng/mL) challenge for 24 h. At the end of the experiment, 1 mL of TRIzol reagent was added into the wells and cells were harvested and stored at -20 °C for RNA extraction. The primers of NLRP6 siRNA are shown in Table 11.

## *2.5 Cell Immunofluorescence Staining*

LS174T cells were fixed in 4% (vol./vol.) paraformaldehyde for 15 min, followed by permeabilization with Triton-X-100 (2%, vol./vol.) for 10 min, blocked for 2 h at 4 °C in bovine serum albumin (BSA, 5%, wt/vol.). The cells were then labeled overnight at 4 °C using polyclonal anti-MUC2 (1:100) and anti-NLRP6 (1:100), respectively, followed by three washings with phosphate-buffered solution–Tween-20 (PBST) for 10 min. Then, the cells were stained for 1 h using FITC-conjugated anti-rabbit (1:200) and Cy5-conjugated anti-rabbit (1:200) secondary antibodies. After washing thrice with PBST for 5 min, the cells were cultured with DAPI cell nuclear dye for 5 min and then washed thrice with PBST for 3 min. Lastly, the cells were washed and mounted on glass slides, and images were acquired using a fluorescence microscope (Olympus DP71, Tokyo, Japan).

## *2.6 Total RNA Extraction and Gene Expression Detection*

Total RNA was extracted and then transcribed into cDNA (42 °C for 10 min, 65 °C for 10 s, stored at 4 °C) using the PrimeScript™ RT reagent Kit (TaKaRa, Osaka, Japan). The primers of MUC2 (GenBank: NG_051929), TFF3 (GenBank: NM_003226), RELMB (GenBank: NM_032579.3), CHST5 (GenBank: NG_029853) and GAL3ST2 (GenBank: NG_046977) are outlined in Table 11. The expressions of MUC2, TFF3, RELMB, CHST5 and GAL3ST2 gene were detected using reverse transcription-polymerase chain reaction (qRT-PCR), with GADPH as the reference gene. The reaction system was made to a total volume of 20 µL, comprising 1 µL template cDNA, 0.5 µL forward primer/reverse primer (10 µM), and 10 µL of TB Green® Fast qPCR Mix. Data were calculated using $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

**Table 11** Primers of targeted genes

| Genes | Primer sequence (5′→3′) |
|---|---|
| CHST5 | F: CCC AGT GAG GAA CTG GTC TTC |
| | R: ATC TGT GTT CCA GGA AAG CC |
| GAL3ST2 | F: TGG GCG GCT TGC AGA GAT A |
| | R: GCT CTA AGT CCG AGT GCA GGA |
| MUC2 | F: AAC ACA GTC CTG GTG GAA GG |
| | R: CAT TGT CAG GTC CCA CAC AG |
| RETNLB | F: CAC CCA GGA GCT CAG AGA TCT AA |
| | R: ACG GCC CCA TCC TGT ACA |
| TFF3 | F: CAT GTC ACC CCC AAG GAG TG |
| | R: AGG TGC ATT CTG CTT CCT GC |
| GADPH | F: AAG ATC ATC AGC AAT GCC TCC TGC |
| | R: ATG GAC TGT GGT CAT GAG TCC TTC |
| SiRNA-NLRP6 | F: CCU UCU UCA UCC ACU CUU UTT |
| | R: AAA GAG UGG AUG AAG AAG GTT |
| NLRP6 | F: TTC CGA GGA AAT GAT GAC GA |
| | R: ACA GTA GAC GAT GCG GTT GC |
| IL-6 | F: CCT TCC AAA GAT GGC TGA AA |
| | R: CAG GGG TGG TTA TTG CAT CT |
| TNF-α | F: CCT GTG AGG AGG ACG AAC AT |
| | R: AGG CCC CAG TTT GAA TTC TT |
| NF-κβ | F: GGC GTG GAG CTG AGA GAT AAC |
| | R: GGT GTG GGT GAG GAG CAC AT |
| IL-1β | F: GGG CCT CAA GGA AAA GAA TC |
| | R: TTC TGC TTG AGA GGT GCT GA |

### 2.7 Statistical Analysis

All data were analyzed using One-Way ANOVA multiple comparisons to assess differences between the groups and were expressed as mean ± SEM. $P< 0.05$ were considered statistically significant. GraphPad Prism v9.0 (GraphPad Software company, San Diego, California, USA) was applied to draw bar charts of the above data.

## 3. Results

### 3.1 Effects of the Four Chemicals on Cell Viability of LS174T Cells

As shown in Figure 23, compared with control group, the cell viability of LS174T cells treated with 1.0-2.5 mg/mL 2′-FL and 3′-SL showed no obvious changed. Moreover, 1.5, 2.0 and 2.5 mg/mL 2′-FL could slightly enhance cell viability when cultured for 72 h. While, the cells treated with 5.0 mg/mL 2′-FL and 3′-SL were exhibited significantly declined viability ($P<0.05$). The viability of cells in 15 and 20 mg/mL GOS and Lac groups were significantly decreased when compared with control group ($P<0.05$), meanwhile, other dosages exhibited no harm for cells survival when cultured for 72 h. The cell viability in 24 and 48 h showed a similar tendency with 72 h. Therefore, the doses 1.5, 2.0 and 2.5 mg/mL of 2′-FL and 3′-SL, and 7.5, 10 and 12.5 mg/mL of GOS and Lac were selected for subsequent experiment to determine the optimal dosage.

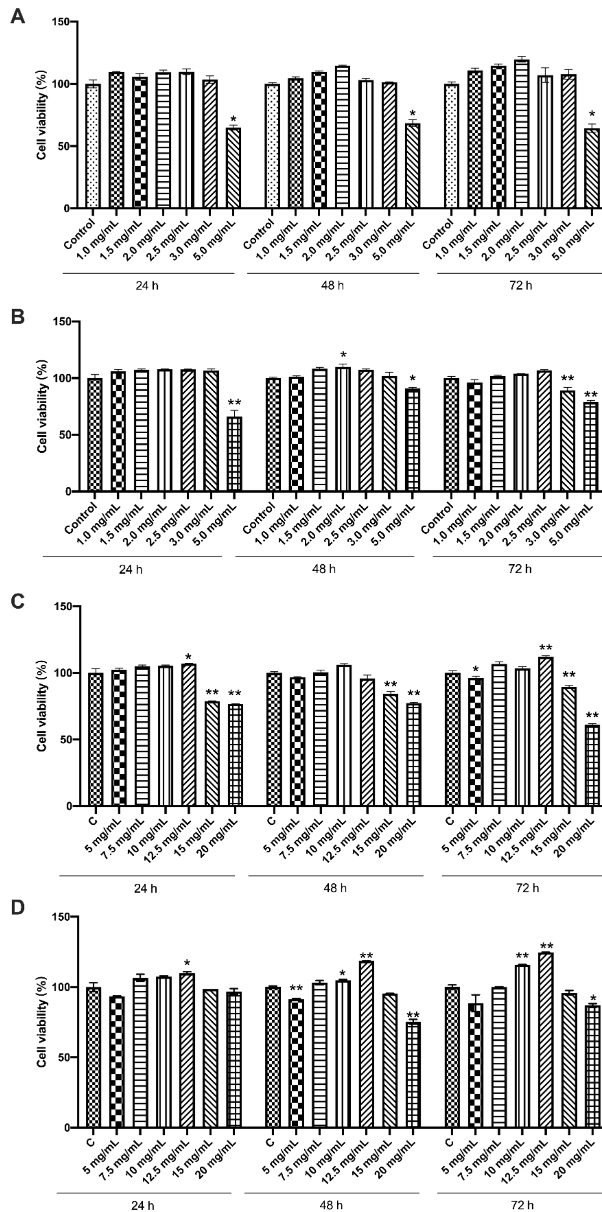


**Figure 23** Cell viability of LS174T cells with or without prebiotic treatment. **A**, 2′-FL; **B**, 3′-SL; **C**, GOS; **D**, Lac. Significance was determined using One-Way ANOVA and expressed as mean ± SEM (*n*=5 for each group). * *P*< 0.05; ** *P*< 0.01 when compared with control group.

## *3.2 Effects of the Four Chemicals on Mucin Secretion under Normal Condition*

As shown in Figure 24, there was no significant difference in *MUC2* gene expression among the different treatment groups (*P*>0.05). The expression of *RETNLB* gene in 2.5 mg/mL 2′-FL and/or 12.5 mg/mL GOS, as well as of *GAL3ST5* in 2.0 mg/mL 2′-FL and 12.5 mg/mL GOS were significantly up-regulated compared with the control group (*P*<0.05). The 3′-SL and Lac showed no obvious influence on mucin secretion in a homeostasis condition. Based on these results, the doses of 2.5 mg/mL 2′-FL and 3′-SL, and 12.5 mg/mL GOS and Lac were chosen for the following experiments.

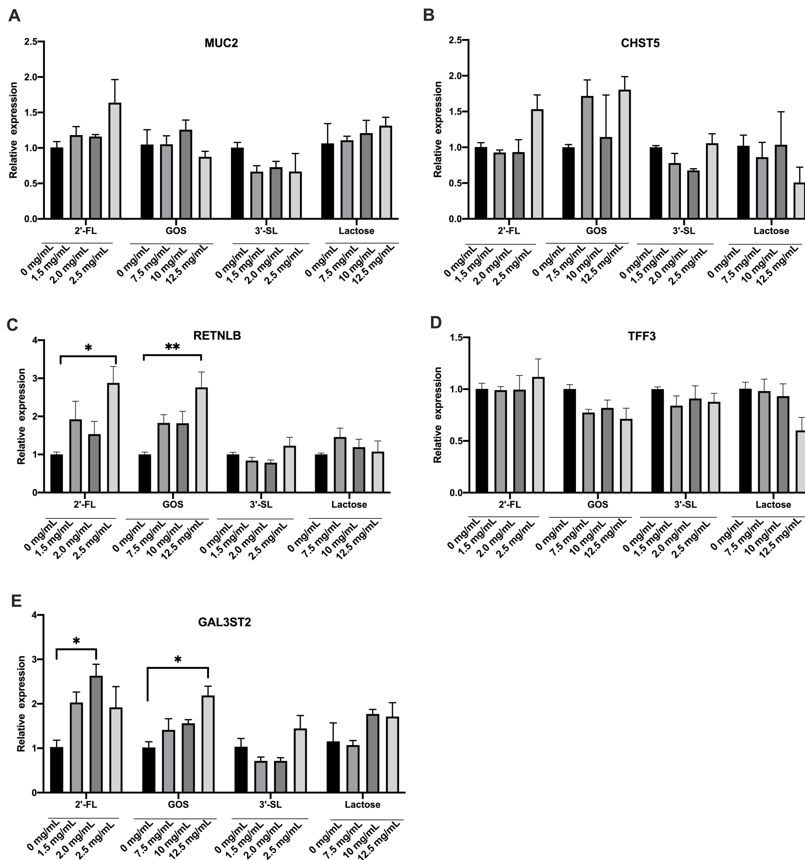


**Figure 24** mRNA expression levels of mucin secretion-related genes under steady-state condition. **A-E**, mRNA expression of *MUC2*, *CHST5*, *RETNLB*, *TFF3* and *GAL3ST2* genes. * *P*< 0.05; ** *P*< 0.01 when compared with control group.

### 3.3 Effects of the Four Chemicals on MUC2-Related Gene Expression under the Inflammatory Condition

TNF-α induced a sharp increase in the mRNA expression of TNF-α when compared with the control group ($P< 0.01$), which could be inhibited by 2′-FL and GOS. Regarding the mucin secretion indexes, the gene expression of MUC2, CHST5 and TFF3 was down-regulated in the TNF-α group compared with control group ($P< 0.05$, Figure 25). However, in the 2′-FL + TNF-α group, these genes were significantly up-regulated when compared with TNF-α group ($P<0.05$). GOS also showed the ability to enhance the expression of MUC2 and TFF3 ($P<0.05$). The ability of 2′-FL was 1.68 times higher than that observed for GOS. 3′-SL and Lac had no obvious effect on the expressions of MUC2 and mucin secretion related genes. Cell immunofluorescence staining confirmed that 2′-FL enhanced the expression of MUC2 protein under inflammatory condition.

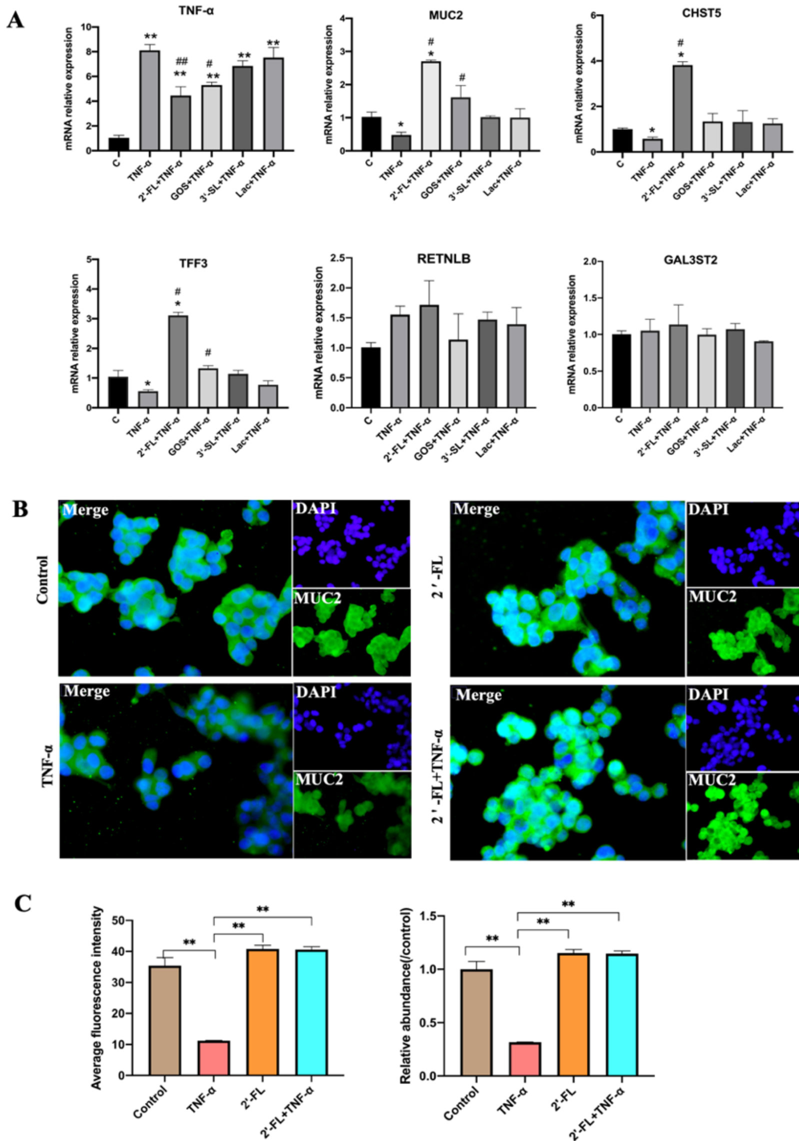


**Figure 25** mRNA expression levels of mucin secretion-related genes and cell immunofluorescence staining of LS174T cells under an inflammation condition. **A**, Mucin secretion-related genes; **B**, Cell immunofluorescence staining; **C**, Average fluorescence intensity of MUC2 and its relative abundance (compared to control group), calculated using Image J pro. * $P< 0.05$; ** $P< 0.01$ compared to the control group; # $P< 0.05$. ## $P< 0.01$ compared to the TNF-α group.

### *3.4 NLRP6 is Necessary for MUC2 Secretion*

To further validate the roles of NLRP6 in an *in vitro* inflammatory model, cell immunofluorescence staining was performed in this study. The results showed that, after TNF-α stimulation, NLRP6 expression was significantly decreased compared to the control group ($P<0.05$), showing as the decreased average fluorescence intensity (Figure 26A, B). However, 2′-FL treatment significantly improved its expression ($P<0.05$). We further performed an siRNA gene experiment to test the role of NLRP6. After siRNA-2172 treatment, the expression of *NLRP6* significantly decreased compared to the control group (Figure 26C, $P<0.05$), suggesting that *NLRP6* was silenced successfully by siRNA-2172. Compared to the 2′-FL+ TNF-α group, the *MUC2* and its related two genes (*TFF3* and *CHST5*) were all significantly decreased in the 2′-FL+TNF-α+ NLRP6 siRNA group (Figure 26D, $P<0.05$). This suggests that 2′-FL was unable to improve the MUC2 secretion under inflammatory condition after *NLRP6* gene silencing.

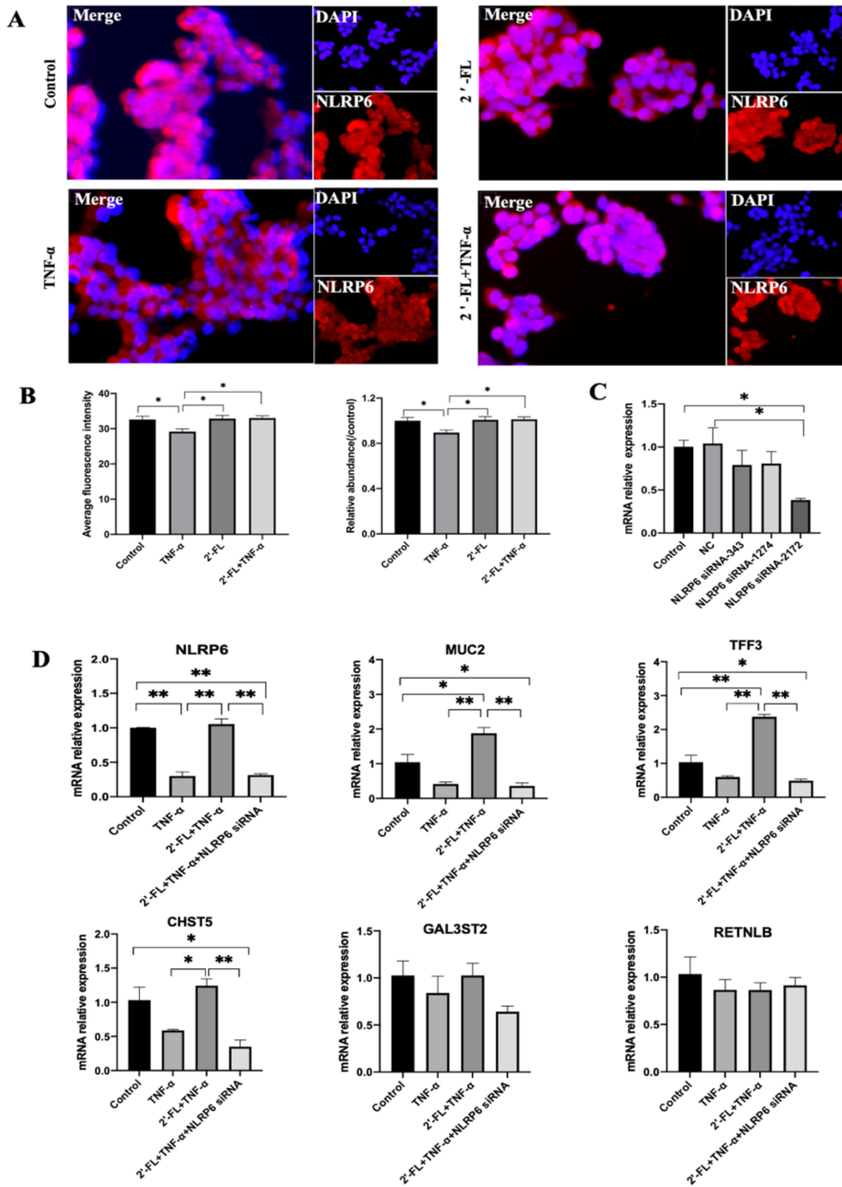


**Figure 26** Expression of mucin-related genes before and after *NLRP6* gene silencing. **A**, 2′-FL exposure (2.5 mg/mL) for 72 h increased NLRP6 expression in LS174T cells (representative images). **B**, Statistics analysis of average of fluorescence intensity and its relative abundance (compared to control group) using GraphPad Prism v9.0. **C**, The screening of *NLRP6* siRNA primers. **D**, mRNA expression levels of mucin secretion-related genes after *NLRP6* gene silencing. * $P< 0.05$; ** $P< 0.01$ compared to the control group.

## *3.5 2′-FL Suppressed the Inflammation via TLR4-related Pathway*

As shown in Figure 27, in the TNF-α group, the expression of TLR4, MyD88, NF-κB, and TNF-α levels were remarkably increased compared to the control ($P$<0.05). However, opposite results were observed in the 2′-FL+TNF-α group with a sharp decrease in the expressions of TLR, MyD88, NF-κB and increased levels of NLRP6 ($P$<0.05). More importantly, after *NLRP6* silencing, 2′-FL was still able to decrease the levels of the above inflammatory factors, suggesting that, in goblet cells, 2′-FL could not only promote MUC2 secretion, but also suppress inflammatory cytokine expression, to reduce inflammation.

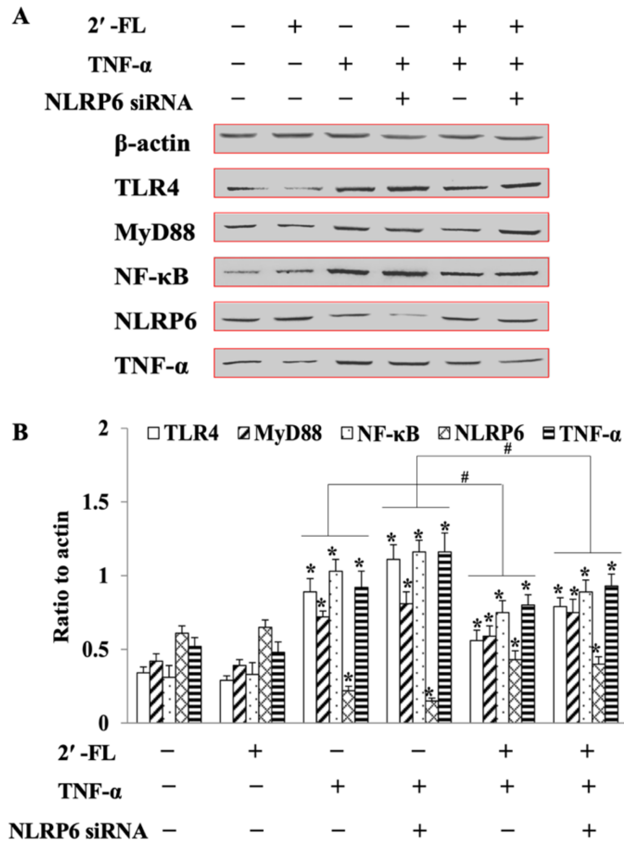


**Figure 27** Cytokines expression in TLR4/MyD88/NF-κB pathway in LS174T cells. **A**, Western blotting bands. **B**, Quantitation for normalized proteins relative to Actin. * $P$< 0.05; ** $P$< 0.01 compared to the control group; # $P$< 0.05. ## $P$< 0.01 compared to the TNF-α group.

# 4. Discussion

Both 3′-SL and Lac had little influence on MUC2 expression either in steady situation or under the TNF-α challenge. Currently, 3′-SL is known to improve cognitive ability (Pisa et al., 2021), so it is partially absorbed into the blood circulation and then arrives to the brain to exert its functions of neurotransmitter. Lac is highly present in milk and is also the core structure of HMOs and GOS. Lactase can hydrolyze Lac into monosaccharides, allowing the non-hydrolyzed Lac to reach the colon to be further fermented by microbiota. Figueroa-Lozano et al. (2020) demonstrated that Lac mixed with GOS improved the expression of genes related to mucus in goblet cells, which, however, might be due to GOS.

In contrast to 3′-SL and Lac, 2′-FL and GOS showed a strong effect in improving mucin barrier after TNF-α treatment, and the ability of 2′-FL was higher than GOS. However, they did not exert function in steady condition. Those results suggested that there might be chemical signals such as TLRs, to activate 2′-FL and GOS to exert their function in the inflammatory state.

In chapter 4, NLRP6 was found to be involved in the process of 2′-FL alleviating DSS-induced colitis and promoting MUC2 expression in C57BL/6J mice. Thus, the interactions between NLRP6 and 2′-FL in enhancing mucin expression in goblet cells (LS174T cells) were explored. It was found that *MUC2* and its related two genes (*TFF3* and *CHST5*) were all significantly decreased after *NLRP6* was silenced.

In protein level, TLR4, MyD88, NF-κB, and TNF-α levels were significantly decreased by 2′-FL treatment. After *NLRP6* was silenced, 2′-FL could still decrease the levels of the above inflammatory factors, confirming the function of 2′-FL in goblet cells via multiple processes, i.e., promoting mucin secretion through NLRP6 and suppressing inflammation through inhibiting the TLR4-related pathway. Sodhi et al. (2021) found that 2′-FL docked into the binding pocket of the TLR4-myeloid differentiation factor 2 (MD2) complex to inhibit TLR4 signaling without NLRP6 being involved. In the cytoplasm, the signal caused by the 2′-FL dissolution of the MyD88 adapter via TLR4 was then transmitted to the nucleus via the NF-κB signaling pathway to regulate the process of inflammation.

# 5. Conclusion

In summary, 2′-FL and GOS, but not 3′-SL and Lac, were shown to increase mRNA expression of MUC2, TFF3 and CHST5 only in an inflammatory state. Moreover, 2′-FL exerted its function in goblet cells via several processes, such as promotion of mucin secretion via NLRP6 and suppression of TLR4 related inflammation.

# Chapter 6

## General discussion, conclusion, and perspectives

# 1. General discussion

## *1.1. MOs quantification among different mammal species*

### 1.1.1 MOs extraction in LC-ESI-MS/MS method

Generally, MOs quantification includes three main steps: MOs preparation, separation and identification of individual MO, and quantification. Milk is a complex matrix containing many nutrients, which 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 (Tonon et al., 2019; Porfirio et al., 2020). Those processes are time-consuming and challenging and takes usually more than 16 hours. Thus, in this study, the previous strategy was improved in some ways. One of them was to remove the proteins by replacing the water with 100% ethyl alcohol, which eliminates the need for freeze drying or evaporation, and simplifies the procedure. The recovery rate of 11 MOs was in the range of 71.3%-116.0%, demonstrating that sample loss during the extraction process was acceptable.

Due to the lack of fluorescent labels, MOs are challenging to quantify using high sensitivity chromatography. In addition, MOs are hydrophilic, which makes them difficult to separate by LC. Therefore, in many research, MOs are usually converted to a more stable version to be able to perform quantification (Oursel et al., 2017; Ruhaak and Lebrilla, 2012). However, derivatization destroys the natural structure of MOs resulting in the loss of their full biological activity. Thus, in this research project, it was proposed to use the original form of MOs rather than its derived form. All these attempts have preserved the full functionality of MOs, as well as saving time. The results showed a good separation between the MOs isomers before their identification by MS.

### 1.1.2 MOs profiles in different mammal species

For human breast milk, the results showed that it contained 6.285 g/L of total MOs, and that 2′-FL was the most abundant (2.705 g/L). However, the concentrations of 2′-FL and LNFP I were lower than those obtained by Thurl et al., (2017). This could be since we did not assess the mothers' genotypes in our tests, which prevented us from processing the *Se*+ and *Se*- genotypes data independently and resulted in a lower average. Both 3′-SL and 6′-SL were abundant in acidic MOs, consistent with previous reports (Siziba et al., 2021; Alderete et al., 2015).

Regarding the other animal milks, they contained approximately 7.6 to 15.8 times less total MOs than human milk does and were richer in sialylated MOs. The results were somewhat different from those of earlier studies by Wang et al. (2020) and Shi et al., (2021), who discovered that breast milk contained 10-100 times more MOs than other animal milks. This may be because a smaller number of MOs was quantified in our experiment and resulted in a decrease in total MOs concentration in human breast

milk. In addition, many sialylated MOs (mainly 3′-SL and 6′-SL) may provide additional beneficial functions for the growth of infants consuming cow's milk-based infant formula. However, the efficacy of bovine MOs in infant formula needs to be evaluated.

## *1.2. Gut microbiota is involved in the process of 2'-FL mitigation of colon inflammation.*

2′-FL cannot be digested by the stomach due to the absence of specific types of galactosidases and, therefore, remains intact until the intestine where it is used by bacteria in the colon. Thus, we wondered whether the gut microbiota could affect the protective activity of 2′-FL against colitis. To test this, the feces of donor mice treated with DSS+2′-FL were transplanted to recipient mice, which had been excluded from 2′-FL treatment. As expected, recipient mice showed similar protective activity against colitis as donor mice, despite not being treated with 2′-FL, suggesting that 2′-FL may attenuate colitis in a gut microbiota-mediate manner.

2′-FL can reshape the gut microbiota in colitis. The level of inflammation-promoting bacterium Firmicutes was found to be significantly increased by DSS treatment, while 2′-FL prevented the induction of inflammation-promoting bacterium Firmicutes induced by colitis, as well as Firmicutes/Bacteroidetes ratio. 2′-FL also altered mucin-utilizing bacteria. Some commensal bacteria can utilize mucin as a source of energy to support their growth (Shao et al., 2020). Family *Muribaculaceae* and *Lachnospiraceae* are the main consumers of mucin monosaccharides (Martens et al., 2009). In this study, *Lachnospiraceae* were significantly upregulated by DSS; however, *Muribaculaceae* were selectively increased by 2′-FL treatment, consistent with Li's study (Li et al., 2020) and 8 other mucin-utilizing bacteria were significantly modified by 2′-FL (Wlodarska et al., 2017). Overall, in the colitis model, dysbiosis of microbiota could release some chemical signals to activate the NLPR6 inflammasome, leading to dysfunction in MUC2 secretion in goblet cells, thereby worsening inflammation. This speculation was validated by the decrease of NLRP6 in goblet cells and the decrease of MUC2 in the DSS group. Recovery of these negative alterations might be the main mechanism by which 2′-FL attenuated the inflammation.

## *1.3. 2'-FL promotes MUC2 secretion in vitro*

There is increasing evidence suggesting that milk oligosaccharides play a role in promoting mucin secretion and maintaining gut health. HMOs mixture was found to increase goblet cells in neonatal mice presenting necrotizing enterocolitis, upregulate MUC2 expression in cell models (Wu et al., 2019), as well as reduce the duration of diarrhea and improve intestinal functions in newborn piglets (Li et al., 2014; Rasmussen et al., 2017). Besides infant models, the function of HMOs is also illustrated in adult models. Daddaoua et al. (2006) found that goat MOs could decrease anorexia, body weight loss and the expressions of IL-1β and mucin 3, but enhance TFF3 production in adult colitis rats.

However, the MOs studied in above research are a mixture, consisting of more than 200 neutral and anionic (sialylated) ones (Leo et al., 2010; Ruhaak et al., 2012). However, understanding the effects of individual MOs on mucin secretion and gut health is of great importance. Thus, here we discussed the individual effects of 2'-FL,

3'-SL, GOS and Lac on promoting MUC2 secretion in goblet cell models. Both 3′-SL and Lac were found to have little influence on MUC2 expression either in steady situation or under the TNF-α challenge. Currently, 3′-SL is known to improve cognitive ability (Pisa et al., 2021). This means it is partially absorbed into the blood circulation and arrives to the brain to exert its functions of neurotransmitter. Figueroa-Lozano et al. (2020) demonstrated that Lac mixed with GOS improved the expression of genes related to mucus in goblet cells. This effect, however, might be due to GOS only. In this thesis work, in contrast to 3′-SL and Lac, 2′-FL and GOS presented a strong effect in improving mucin barrier after TNF-α treatment and the ability of 2′-FL was higher than GOS. Those results suggested that not all kinds of MOs, but a particular group of MOs exert the function to improve mucin secretion under inflammatory condition.

Intestinal mucin barrier is complex, consisting in various kinds of mucin proteins. Besides MUC2, there are many other types of mucin protein, including, MUC5AC (predominant in gastric glands), MUC6 (in the duodenum), MUC5B (in the colon) (Paone and Cani, 2020), and MUC1, MUC4, MUC13, and MUC16 (transmembrane mucins) (Cornick et al., 2015). Most of the research focus on exploring the influence of MOs on MUC2 production, however, its function on other mucin proteins remains unknown.

Additionally, while research on the effects of MOs on mucin secretion has advanced our understanding, translating these findings into practical clinical applications, such as therapeutic interventions for gastrointestinal disorders, is a significant challenge.

# 2. Conclusion

MOs, the third most abundant solid component of breast milk, are of great significance for infant growth. As far as we know, the knowledge about MOs in different mammal species are limited. Moreover, the role of the intestinal microbiota on the protective activity of MOs was not clear, as well as the variation of this protective activity according to the types of MOs and inflammation state.

In our study, we confirm and found that:

a. Human milk presents the greatest abundance of MOs with 7.6 to 15.8 times more than the milks of other animal species investigated in this work. Fucosylated neutral forms were dominant. Dairy cow, camel, yak, sheep, buffalo and mare milks had similar MO profiles and were rich in sialylated MOs. Compared to other animals, the MOs composition in sheep was most similar to that of human milk.

b. 2'-FL ameliorated colitis in a gut microbiota-mediated manner by reshaping the composition of gut microbiota, as well as by altering mucin-utilizing bacteria. The underlying protective mechanism was associated with promoting recovery of goblet cells number and MUC2 secretion. In addition, 2'-FL exerts anti-inflammatory effects by targeting TLR4/MyD88/NF-κB-related inflammation pathway.

c. 2′-FL and GOS, but not 3′-SL and Lac, were shown to increase the mRNA expression of MUC2, TFF3 and CHST5 only during inflammatory state. Besides, 2′-FL exerted its function in goblet cells via multiple mechanisms, such as promoting

mucin secretion through NLRP6 and suppressing TLR4 related inflammatory pathway.

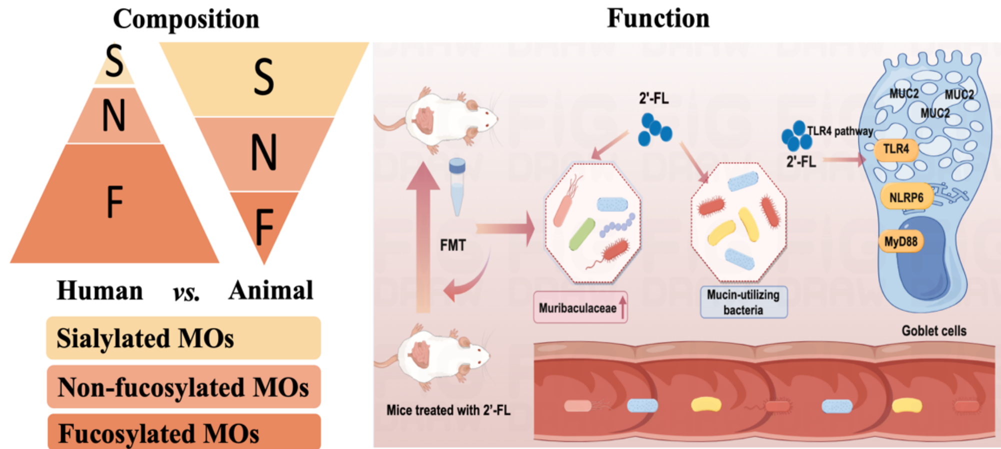


**Figure 28** General conclusion of the thesis

# 3. Perspectives

Despite the extensive information emerged from this thesis, certain issues were left unresolved and may be of interest for future research.

a. In chapter 3, we quantified 11 MOs in mammal species, with only 2 sialylated MOs (3′-SL and 6′-SL). Considering the sialylated MOs enriched in domestic animals, the few quantified members of sialylated MOs could lead to an underestimation of their total concentration. Therefore, more sialylated MOs with available standards should be considered in future studies.

b. According to the results of chapter 3, fucosylated MOs are predominant in human milk, while bovine milk contains more sialylated ones. As a result, less abundant and less complex fucosylated MOs are found in infant formula produced from cow’s milk. To optimize the possible health effects of MOs, we suggest developing an MOs blend contains different types of individual MO to add to infant formula, including more fucosylated MOs.

c. In chapter 4, we validated that gut microbiota was involved in the 2′-FL attenuating colitis process. However, after FMT, the colonization of bacteria in the recipient mice remains uncertain. Through analyzing the composition of gut microbiota in FMT mice, we might be able to identify the main bacteria that respond to 2′-FL in mitigating colitis. Therefore, further studies are still necessary to better understand how specific microbial communities influence health and disease.

d. Mucins are groups of high molecular weight glycoproteins that play important roles in preventing pathogens invasion. Besides MUC2, there are other important mucin proteins, like MUC5B, MUC4, MUC3 and MUC17 (Bansil & Turner, 2006).

In the present study, the expression files of other mucin protein remain unknown, which should be considered in future studies.

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# List of Publications

1. **Yao, Q**., Gao, Y., Wang, F., Delcenserie, V., Wang, J., & Zheng, N. (2023). Label-Free quantitation of milk oligosaccharides from different mammal species and heat treatment influence. *Food Chemistry*, **430**, 136977.

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3. **Yao, Q**., Li, H., Gao, Y., Zheng, N., Delcenserie, V. & Wang, J. (2023). The milk active ingredient, 2'-fucosyllactose, inhibits inflammation and promotes MUC2 secretion in LS174T goblet cells *in vitro*. *Foods,* **12(1)**, 186.

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