General discussion

Lianghui Cheng

Imunoendocrinology, Division of Medical Biology, Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands
Human milk oligosaccharides (hMOs) is the third-largest solid compound in human milk, which provides numerous health-promoting effects on the gastrointestinal immune system [1, 2]. The beneficial effects of hMOs in human milk include modulation of the colonization and activity of the resident microbiota [3, 4], modulate immune function in different ways [5, 6], enhancement of intestinal barrier function [7], as well as attenuation of systemic and intestinal inflammation [8].

Over the decades, it was not possible to produce hMOs in great amounts and used in infant formulas. Nondigestible fibers such as galacto-oligosaccharides (GOS) and inulins were, therefore, being used to substitute hMO function in infant feeding [9]. However, recently, major advances have been made in large scale production of hMOs and two isolated hMOs are now able to be used in infant formulas [10]. The beneficial effects of hMOs in human milk are indubitable, however, nowadays, hMOs are usually supplied in infant formula as single-molecule and there is little evidence available to support the beneficial function of individual hMOs. Besides that the low pH in the gastrointestinal tract may lead to the formation of the acid hydrolysate of the tetra and higher hMOs [11, 12], and how these acid hydrolysates impact the gastrointestinal immune barrier is barely studied. In order to optimize the future design of hMOs-containing infant formulas, more knowledge about different functions and the mechanisms involved in gastrointestinal immune effects of individual hMOs is needed. Therefore, the aim of the studies in this thesis was to investigate in vitro how different individual hMOs, as well as hMO's acid hydrolysis products, modulate gastrointestinal immune barriers on different cellular systems, unravel the structure-function relationships of hMOs, and explore the possible mechanisms of action.

The functions of hMOs are highly structure-dependent
hMOs are unique to humans and are not found in the same variety and composition in other mammals [13]. To date, approximately 200 different hMOs have been discovered and characterized in human milk [14]. In this thesis, we demonstrated that the individual hMOs have different effects and that the final outcome of a specific health benefit dependents on the composition of individual hMOs from multiple aspects.

The hMOs isomers with the same formula but in different structures can already have a major influence on its bioactivities. We observed that 3-FL shows anti-inflammatory
effects (chapter 3), enhances mucus barrier function (chapter 4), and increases tight junction of intestine epithelial cells (chapter 6). However, most of the beneficial effects are not observed for 2'-FL. Interestingly, the effective 3-FL and non-effective 2'-FL only differ in the attachment position of L-fucose (Fuc) residues on the lactose core region. We speculate that the terminal Fuc in 2'-FL masks the terminal Gal, and an uncovered β1-4 linked Gal terminal might be essential for the above functions. Besides the 2'-FL and 3-FL, we also observed another group of hMOs which have same molecular composition but different bioactivity due to its structure differences. In chapter 3, we showed that LNnT rather than LNT has strong anti-inflammatory effects, while the effective hMO LNnT and non-effective LNT only differ in their hMO type 1 chain (Galβ1-3GlcNAc-) or type 2 chain (Galβ1-4GlcNAc-) linkage on lactose. And again, LNnT contains a β1-4 linked Gal terminal while LNT does not, further proved that β1-4 linked Gal terminal may play an important role in anti-inflammatory effects. Infants solely fed with breast milk have a lower risk of infections and inflammatory diseases than formula-fed babies [15]. It has been reported that hMOs could directly interact with intestinal cells, attenuate systemic and intestinal inflammation [6]. In addition to our observations, we reasonably hypothesize that the β1-4 linked Gal terminal might be a specific effects site that is responsible for that. Including more hMOs contain a β1-4 linked Gal terminal to investigate their anti-inflammatory properties could be an interesting choice to confirm our hypothesis.

Not only structures make major differences, the monomers also play an important roles. In chapter 2, we showed that 6'-SL does not inhibit but augments TLR8 activation in the presence of the agonist at a concentration of 2 mg/mL while it has no activation effect as a single molecule on TLR8. And also, in chapter 6, we found that 6'-SL can promote the growth of B. longum subsp. infantis in B. longum subsp. infantis and F. prausnitzii co-culture. We deduce that the sialic acid in 6'-SL may play a role in these observations. More studies and different hMOs are needed to confirm the observed structure-specific effects as well as our hypothesis.

**The digestion in the gastrointestinal tract influence the bioactivity of hMOs**

Although the majority of hMOs can reach the intestine without being digested, some hMOs undergo hydrolyzation at low pH during transit through the gastrointestinal tract
This digestion may lead to the formation of LNT2, which is the acid hydrolysate of the tetra and higher hMOs such as LNT and LNnT [11, 12]. How these process influence the bioactivity of hMOs and how these acid hydrolysates may impact the gastrointestinal immune barrier are not been studied before.

While LNT2 is the acid hydrolysate of LNnT, opposite modulatory effects via TLRs were observed between them. In chapter 2, we demonstrated that LNT2 shows strong activating effects on all TLRs, but no inhibition effects are observed, while LNnT shows only inhibiting effects on TLRs. The different effects between LNT2 and LNnT are also observed in chapter 3, where LNnT shows strong anti-inflammatory effects, and LNT2 induces IL-8 secretion on intestine epithelial cells. Our observation indicates that digestion in the gastrointestinal tract could have strong influence on bioactivity of hMOs, and should be taken into consideration in the future hMOs functional study and product designs. LNnT is one of the hMOs that currently is being used in infant formula [16], considering the digestion in gastrointestinal tract, LNnT might reach in intestinal tract as a mixture of LNnT and LNT2. However, the effects of LNnT and LNT2 mixtures that mimic the possible ratio in the infant’s intestine are not investigated yet. Therefore, use of in vitro infant digestion models [17] to study the function of digestion products of hMOs would be an interesting option for further researches.

The interaction between hMOs and its receptors

Due to its special structural composition, the interaction between hMOs with receptors plays an important role. However, potential hMO receptors are still not all identified. In this thesis, our observations identified and provided several potential receptors for hMOs as well as the possible mechanism behind.

The immune effects of hMOs via Toll-like receptors (TLRs) have been previously described in literature [6], in chapter 2, we studied the direct interaction between different hMOs and individual TLRs. Both activation and inhibition effects are observed on TLRs signaling. We observed 3-FL activates TLR2, while LNT2 activates all TLRs, 2'-FL, 6'-SL, and LNnT inhibit TLR5 and 7, while 3-FL inhibits TLR5, 7, and 8. However, we only use reporter cells to screen the effects of hMOs and prove its immunomodulatory effects on THP1 macrophages, the potential the mechanisms that responsible for the effects are not addressed in this study. Therefore, in-depth studies on how these hMOs interact
with individual TLRs and the pathways involve in these would be the next step that provides more molecular insights. Besides the already studied receptors for hMOs, i.e. TLRs, we identified a new hMOs receptor, TNFR1. In chapter 3, we demonstrated that specific hMO types inhibit TNF-α induced inflammatory responses in fetal gut epithelial cells in a structure-dependent fashion by interacting with the TNFR1 receptor. Different mechanisms are observed, 3-FL, LNnT, and LDFT exert TNFR1 ectodomain shedding while LNnT also show binding affinity to TNFR1. The good follow up studies on our results would be explore the enzymes and molecular mechanisms that involve in TNFR1 ectodomain shedding, and use in silico way to identify the possible binding pocket and binding site of the hMOs and TNFR1, which might help to find the signaling pathways and functional sites of hMOs during this process.

Except for the identified receptors we mentioned above, our results also provide some potential receptors as candidate for future studies. In chapter 4, we showed that hMOs enhance mucus barrier function through direct modulation of intestinal goblet cells in structural and stressor-dependent way. For this observation, galectin-3 (Gal-3), a β-galactoside binding protein which modulates the expression of its major ligand MUC2 mucin in human colonic cells [18], might be a potential receptor involved in this process and a good candidate to investigate.

**Cross-talk and shear stress modulate the effects of hMOs**

Support of growth of beneficial bacteria is an important effect of hMOs [19]. In chapter 5, we studied the effects of individual hMOs and hMO’s acid hydrolysate on different beneficial bacteria in both monoculture and co-cultures. In the co-culture setup, we found that the possible cross-talk between different bacteria species plays an important role in microbiota colonization. In addition to our results, an interesting option for further research would be to investigate the effects of hMOs on multiple bacteria species. This could be done for example by isolating bacteria from infant feces and to study the effects of hMOs on growth and fermentation of those bacteria.

Moreover, in chapter 6, we observed again hMOs support the crosstalk between commensal bacteria and intestinal epithelial cells. Our observations indicate the cross-talk between different factors, both between different bacteria as well as bacteria and human cells, play important roles in gastrointestinal immune barrier
functions. The factors that are responsible for the observed effects on cross-talk are not well addressed in this thesis, in-depth investigations on identifying the responsible factors, would be the next step that provides more molecular insights in the effects of individual hMOs on crosstalk.

The mechanical force on epithelial cells during transit through the gastrointestinal tract could influence the adhesion of commensal bacteria [20]. Adhesion of commensal bacteria is the first step to the colonization of the intestine in early life. It also plays an important role in the prevention of pathogen adhesion and invasion in later life [21]. In chapter 6, we applied the Ibidi system to mimic the shear force during intestinal peristalsis and we demonstrate the impact of some hMOs can only be observed when a physiological shear force is applied. Most in vitro studies that investigate the function of hMOs were under static culture, without shear force as well as other members of the gut immune barrier experience during transition through the gastrointestinal tract [22]. From our in vitro data, it is clear that shear force as well as the co-culture of bacterium and intestine cells together, could influence the functions of hMO. This suggests that shear force and bacteria all play important roles and should be taken into account in hMOs function study.

Nowadays, most of the in vitro studies that explore hMO functions only include single factors, i.e. bacteria or cells, and almost all under static culture. Although exploring the effects of hMOs on individual elements of gastrointestinal immune barrier is important, which helps to screen promising individual hMOs for further study, studies include different factors should be considered for future investigations. Transwell is a good way to study the cross-talk between bacteria and cells, which can co-culture bacteria, intestine epithelial cells, and immune cells together [23]. But this set up is under static condition and hard to include anaerobic bacteria. To include aerobic cells, anaerobic microbiota, as well as dynamic conditions, more comprehensive devices should be considered [24]. For example, anoxic-oxic interface-on-a-chip (AOI Chip), a new device developed by Shin et al., which could co-culture anaerobic bacteria and epithelial cells in a dynamic condition [25], would be a promising device for functional study of hMOs.
Translation to nutritional applications and development

hMOs can have many beneficial effects, as individual hMOs have different effects and a specific health benefit is highly dependent on the composition or quantity of individual hMOs, there might be negative impacts when applied in the wrong conditions. The knowledge gained in this thesis is important for future product applications.

Nowadays, only 2'-FL and LNnT are used in infant formula [16]. However, according to our findings, 2'-FL may not always be the most effective one, in general, 3-FL could be a better candidate than 2'-FL for infant formulas, especially during the early stages of life. At early stages in life, the intestinal immune system is immature and it develops rapidly in the early postnatal period, infant’s intestine is relatively diffuse, and the maturation to an intact barrier is needed to prevent pathogens from entering the host [26]. Compared to 2'-FL, 3-FL shows a better effect on the enhancement of the intestine barrier by stimulating the mucin products, increasing the intestine tight junction, and increasing the thickness of the glycocalyx layer [27]. Premature neonates that are more prone to NEC or other inflammatory disorders than term-born babies, and TNF-α plays an important role in these inflammatory diseases [28]. 3-FL, LNnT, and LDFT, which effectively attenuate TNF-α induced inflammation by interacting with the TNFR1, are highly suggested to apply in infant formula for premature neonates. The hMO’s acid hydrolysate LNT2, which shows strong immune stimulation characteristic, might be avoided to include in formulas for infants at high risk of food allergy, but it shows that digestion in the gastrointestinal tract may enhance bioactivity of hMOs. However, the strong immune-boosting effects of LNT2 might help to enhance the vaccination efficacy. Therefore, we believe LNT2 could be a nice candidate as a dietary supplement for infants and children to support vaccination efficacy. However, besides our in vitro data, in vivo studies should be performed to confirm our findings.

Besides as supplementary for infant food, our data also suggest hMOs could be applied at a broader scale. Not only neonates, some patients, such as IBD and TLR dependent intestinal disorders, also have increased epithelial permeability. For those patients, hMOs with protective effects on intestinal barrier function, i.e. 3-FL and LNT2, could be an adequate supplement. For people suffering from immune-mediated inflammatory disorders, like systemic lupus erythematosus and rheumatoid arthritis [29], hMOs like 2'-FL, 6'-SL and LNnT could be provided as functional foods to dampen
the immune response. Although hMOs only found in human milk, rather than only add in infant formulas, our data suggest that hMOs may have beneficial properties that could be used more wildly. The studies designed for testing the functions of hMOs in specific target groups could lead to more effective use of hMOs.

**Conclusions and future perspective**

In this thesis, we explored the structure-function relationships of hMOs and hMO’s acid hydrolysate from different aspects and explore the possible mechanisms of action. hMOs contribute to the reinforcement of the gastrointestinal immune barrier via direct and indirect modulatory effects, but all in a structure-dependent way, slight differences in structure have a significant impact on the biological action of an hMO (Figure 1A). Our results also indicate that the function of hMOs and its acid hydrolysis product on gastrointestinal immune barrier could be very different (Figure 1B). We also identified a new potential receptor for hMOs (Figure 1C), and proved the importance of cross-talk.
and dynamic culture system (Figure 1D). Our data contribute to a better understanding of the structure-function relationship of hMOs which helps for the future design of hMO containing products, and also bring new insights for the future hMOs study.

It has been shown by us and others, that the interaction between hMOs with receptors play important roles in its functions due to its special structural composition. However, potential hMO receptors are still not all identified. Since hMOs are small ligand without specific antibodies, it is not easy to target its receptor by using biological methods only. Therefore, methods such as electro spray ionization mass spectrometry (ESI-MS) [30] or microscale thermophoresis (MST) [31] should be taken into consideration in future studies. Also, we demonstrate that the cross-talk between different factors play an important role in gastrointestinal immune barrier functions, therefore, an *in vitro* model which include anaerobic microbiota as well as aerobic epithelial and immune cells could bring a better and more comprehensive understanding of the underlying mechanisms of hMOs. Newly developed devices “anoxic-oxic interface-on-a-chip (AOI Chip)” [25], “apical anaerobic co-culture system” [32], and “human-microbial crosstalk (HuMiX)” [33] could be used in the future to investigate hMOs.

For optimal use of hMOs in the nutrition products, a high throughput method to screen which hMOs or hMOs mixtures have beneficial effects for specific target groups is needed. Based on this thesis, before animal and human studies, the cell-based *in vitro* assay is a good choice to screen the proper candidates. In this way, with a larger library of hMOs, the specific individual hMOs or hMOs mixtures might be identified for their specific functions and can be used as advising to design functional food products.
References

**Summary**

Human milk is the gold standard for newborn infants. Breast milk not only provides nutrients, it also contains bioactive components that guide the development of the infant’s intestinal immune system, which can have a lifelong effect. Human milk oligosaccharides (hMOs) is the third-largest solid compound and the most abundant bioactive molecules in human milk which have multiple beneficial functions on the gastrointestinal immune system. The beneficial effects of hMOs in human milk are indubitable, however, nowadays, hMOs are usually supplied in infant formula as single-molecule and there is little evidence available to support the beneficial function of individual hMOs. Therefore, more knowledge about different functions and the mechanisms involved in gastrointestinal immune effects of individual hMOs as well as hMO’s acid hydrolysis products is needed.

Human milk is a source of bioactive components such as complex proteins, lipids, and carbohydrates, which impact the infant’s metabolism and immune system. In chapter 1, the current insight into the benefits of bioactive molecules in mother milk that contribute to neonatal development, current knowledge of hMO-functions on microbiota and the gastrointestinal immune barrier are reviewed. We discuss the composition of intestinal immunity in the small and large intestine, critically review the current insight into the benefits of bioactive molecules in mother milk, and focus on the current knowledge of specific structure-activity relations of hMOs. In the end, we also discuss the currently applied hMOs in infant formula as well as their prospects and scientific challenges in the field of infant nutrition research.

To investigate the immune effects of different hMOs, immunomodulatory effects of different hMOs and its acid hydrolysate on TLR signaling are studied in chapter 2. We first measured the activation and inhibition effects of individual hMOs and hMO’s acid hydrolysate on TLR signaling pathways. 3-FL activated TLR2 and LNT2 activated all TLRs in a dose-dependent way. In an inhibition assay, 2’-FL, 6’-SL, and LNnT inhibited TLR5 and 7, while 3-FL inhibited TLR5, 7, and 8. 6’-SL showed a synergistic effect on ssRNA40-induced TLR8 activation. To test whether hMOs have immunomodulatory effects on TLR carrying cells such as macrophages, we measured hMO-induced cytokine production in THP1 macrophages. IL-10 and TNF-α were induced by LNT2, and the effects were NF-κB dependent, while the other hMOs had minor effects. These results suggest
that the effects of hMOs on TLR signaling and immunomodulation of macrophages are hMO-structure and dose-dependent.

hMOs can attenuate systemic and intestinal inflammation by modulating intestine epithelial cells, but the mechanisms of action are not well-understood. To gain more insight on capacity of hMOs to modulate gut epithelial cells under inflammatory stress we investigate, in chapter 3, the effects of different hMOs and hMO’s acid hydrolysate on TNF-α induced inflammatory events in fetal and adult gut epithelial cells, and the possible mechanisms of action. We found that 3-FL, LNNt, and LDFT significantly attenuate TNF-α induced inflammation in immature intestine epithelial cells, while LNT2 induce IL-8 secretion in mature intestine epithelial cells. The anti-inflammatory effects of 3-FL, LNNt, and LDFT were induced by interacting with the TNFR1 receptor which is highly expressed in the fetal cells compared to adult gut epithelial cells. Taken together, our data suggests that specific hMOs in infant formulas may attenuate TNF-α mediated inflammatory disorders.

Some studies report that hMOs are able to modulate intestinal mucus barrier function through supporting goblet cell functions and thereby improving gut health, but structure-dependent effects of individual hMOs on mucus production and the mechanisms involved are still not clear. Therefore, in chapter 4, we assessed the effects of different hMOs and hMO’s acid hydrolysate on mucus function-related genes in goblet cells under homeostatic conditions and when exposed to different stressors. Effects of hMOs were compared to effects of GOS, which is currently applied in infant formula as a substitute for hMOs. First, we examined gene expression alterations of the goblet cell secretory related genes in the human goblet cell line LS174T under homeostatic condition. 3-FL, LNT2, and GOS modulated LS174T gene expression profiles in a dose and time-dependent manner and resulted in a significant increase in MUC2 protein production under homeostatic condition. Effects of 2’-FL, 3-FL, LNT2, and GOS on gene transcription of LS174T were also assessed during exposure to TNF-α, IL-13, or tunicamycin (Tm). Different effects were observed during the different challenges. Our data indicate that the modulatory effects of hMOs on goblet cells are highly structure dependent and different during inflammation and under ER stress.

Support of growth of beneficial bacteria is an important effect of hMOs. To investigate how individual hMOs influence growth of several relevant intestinal
bacteria species we studied the effects of individual hMOs and hMO’s acid hydrolysate on different beneficial bacteria in both monoculture and co-cultures, in chapter 5. We observed that in monoculture, *B. longum* subsp. *infantis* could grow well on all hMOs but supported this in a structure-dependent way. *F. prausnitzii* reached a lower cell density on the hMOs in stationary phase compared to glucose, while *B. longum* subsp. *longum* and *B. adolescentis* were not able to grow on the tested hMOs. In a co-culture of *B. longum* subsp. *infantis* with *F. prausnitzii*, different effects were observed with the different hMOs. 6’-SL, rather than 2’-FL, 3-FL, and LNT2, was able to promote the growth of *B. longum* subsp. *infantis*. Our observations demonstrate that effects of hMOs on infant gut microbiota is hMO specific and provides new effective ways of supporting growth of specific beneficial microorganisms in the intestine.

In the previous chapters, we studied the effects of individual hMOs and a hMO’s acid hydrolysate on human cells or bacteria separately and under steady culture. In a physiological setting, the intestinal epithelial cells, food molecules, and gut microbiota are continuously experiencing intestinal peristaltic shear force, which may impact the crosstalk of hMOs with commensal bacteria and intestinal epithelial cells. Therefore, in chapter 6, we studied how the hMOs combined with intestinal peristaltic shear force impact intestinal epithelial cells behavior and crosstalk with a commensal bacterium. In this study, we applied a so-called Ibidi system to mimic the shear force during intestinal peristalsis. First, we studied the impact of shear force on intestine epithelial cells with or without the stimulation of individual hMOs and hMO’s acid hydrolysate. Shear forces had a large impact on intestinal epithelial cell characteristics such as glycocalyx gene expression, production of anti-microbial peptide, and expression of tight junction proteins, and also changed the impact of individual hMOs and hMO’s acid hydrolysate on gut epithelial cells. Then, the commensal bacteria *Lactobacillus plantarum WCFS1* (*L. plantarum WCFS1*) was introduced into the system and was studied in presence of hMOs and shear force. Profound changes were observed when crosstalk between a commensal bacterium and gut epithelial cells was allowed. We observed 3-FL and LNT2 enhanced commensal bacteria adhesion, and all tested hMOs up-regulated the expression of antimicrobial peptide and tight junction protein ZO-1. Overall, our results show that shear force as well as the co-culture of bacterium and intestine cells together, could influence the functions of hMOs.
Finally, in chapter 7, the results described in this thesis are discussed. It is concluded that minor differences in the molecular structure of hMOs, as well as digestion in the gastrointestinal tract, can have significant impact on their bioactivities. Possible mechanisms of action are proposed and new insights for future hMOs studies are proposed. In this thesis, we present new insight in structure-function relationships of hMOs and hMO’s acid hydrolysate on gastrointestinal immunity and present novel possible mechanisms of action.
NEDERLANDSE SAMENVATTING

Moedermelk is de gouden standaard voor pasgeboren baby’s. Het voorziet de baby niet alleen van nutriënten, maar bevat ook bioactieve componenten die helpen bij de ontwikkeling van het immuunsysteem van de darm van de baby. Deze effecten kunnen een levenslange invloed hebben. Humane melk oligosaccharides (hMOs) zijn de derde meest voorkomende vaste stof in moedermelk en de meest voorkomende bioactieve component. hMOs hebben verschillende functies in het maagdarm immuunsysteem. De voordelige effecten van hMOs in moedermelk zijn onbetwistbaar, maar tegenwoordig worden er slechts alleen individuele hMOs toegevoegd aan babyvoeding. Er is nog maar weinig bewijs beschikbaar die de voordelen van individuele hMOs onderbouwen. Daarom is er meer kennis nodig over de verschillende functies van zowel de individuele hMOs als de hMO zuur hydrolase producten en de mechanismen die betrokken zijn bij de maagdarm immuuneffecten.

Moedermelk is een bron van bioactieve componenten zoals complexe eiwitten, vetten en complexe suikers. Deze hebben allen een effect op het metabolisme en immuunsysteem van baby’s. In hoofdstuk 1 wordt de huidige kennis over de voordelige effecten van bioactieve moleculen in moedermelk die bijdragen aan de ontwikkeling van pasgeborenen en de huidige kennis over hMO-functies op microbiota en de maagdarm immuun barrière besproken. We bediscussiëren de compositie van het immuunsysteem in de dunne en dikke darm, bespreken de huidige inzichten in de voordelige effecten van de bioactieve moleculen in moedermelk en focussen op de huidige kennis van de structuur specifieke activiteit van hMOs. Ook worden de hMOs die momenteel worden toegepast in babyvoeding besproken evenals hun toekomstige toepassingen en de wetenschappelijke uitdagingen in het babyvoedingsonderzoeksveld.

Om de immuuneffecten van verschillende hMOs te onderzoeken, bestudeerden we de immuunmodulatoire effecten van verschillende hMOs en een hMO zuur hydrolysaat op TLR signalerings cascade in hoofdstuk 2. Als eerste hebben we de activerende en inhibiterende effecten van individuele hMOs en een hMO zuur hydrolysaat op TLR signalerings pathways onderzocht. 3-FL activeerde TLR2 en LNT2 activeerde alle TLRs in een dosisafhankelijke wijze. In een inhibitie analyse inhibeerden 2'-FL, 6'-SL en LNnT TLR5 en 7, terwijl 3-FL TLR5, 7 en 8 inhibeerde. Voor 6'-SL werd een synergetisch effect gevonden voor ssRNA-40 geïnduceerde TLR8 activatie. Om te onderzoeken of hMOs
een immuunmodulatior effect hebben op cellen die TLRs tot expressie brengen zoals macrofagen, hebben we de hMO-geïnduceerde cytokine productie in THP1 macrofagen gemeten. IL-10 en TNF-α werden geïnduceerd door LNT2. Deze effecten waren NF-κB afhankelijk. Andere hMOs hadden minimale effecten. Deze resultaten suggereren dat de effecten van hMOs op TLR signalering en de immuunmodulatie in macrofagen afhankelijk zijn van de hMO structuur en dosering.

hMOs kunnen systemische en intestinale ontstekingen remmen door modulatie van darm epitheel cellen, maar de mechanismen hierachter zijn onduidelijk. Om meer inzicht te krijgen in het vermogen van hMOs om het darmepithel te moduleren onder ontstekingsstress hebben we in hoofdstuk 3 de effecten van verschillende hMOs en een hMO zuur hydrolysaat op TNF-α geïnduceerde ontsteking in foetale en volwassen darm epitheel cellen onderzocht, evenals de mogelijke achterliggende mechanismen. We vonden dat 3-FL, LNNt en LDFT de TNF-α geïnduceerde ontsteking in immature epitheel cellen significant reduceerde, terwijl LNT2 IL-8 secretie in mature epitheel cellen induceerde. De anti-inflammatoire effecten van 3-FL, LNNt en LDFT werden geïnduceerd door een interactie met de TNFR1 receptor, welke hoog tot expressie komt in foetale cellen in vergelijking tot volwassen epitheel cellen. Samengenomen suggereert onze data dat de toevoeging van specifieke hMOs aan babyvoeding TNF-α gemedieerde ziektes kunnen afzwakken.

Sommige studies rapporteren dat hMOs de barrière functie van de mucus laag in de darm kunnen moduleren door de goblet cel functies te ondersteunen en daarmee de darmgezondheid te bevorderen, maar de structuur afhankelijke effecten van individuele hMOs op mucus productie en de daarbij betrokken mechanismen zijn nog niet duidelijk. Daarom hebben we in hoofdstuk 4 bepaald wat de effecten zijn van verschillende hMOs en een hMO zuur hydrolysaat op genen die gerelateerd zijn aan mucus functie in goblet cellen onder homeostatische omstandigheden en tijdens blootstelling aan verschillende stressoren. De effecten van hMOs werden vergeleken met de effecten van GOS, die momenteel worden toegevoegd aan babyvoeding ter vervanging van hMOs. Als eerste werden veranderingen in genexpressie van genen die gerelateerd zijn aan goblet cel uitscheiding bepaald in de humane goblet cellijn LS174T onder homeostatische condities. 3-FL, LNT2 en GOS moduleerden de genexpressie profielen van LS174T in een dosis- en tijdsafhankelijke wijze en ze induceerden een
significante toename in de productie van het MUC2 eiwit onder homeostatische condities. De effecten van 2'-FL, 3-FL, LNT2, en GOS op de gen transcriptie van LS174T werden ook bepaald na blootstelling aan TNF-α, IL-13 of tunicamycin (Tm). Er werden verschillende effecten geobserveerd na deze verschillende behandelingen. Onze data toont aan dat de modulerende effecten van hMOs op goblet cellen structuurafhankelijk zijn en dat ze verschillend zijn tijdens wanneer cellen worden blootgesteld aan onstekings signalen en tijdens ER stress.

De ondersteuning van de groei van gunstige bacteriën is een belangrijk effect van hMOs. Om te onderzoeken hoe invididuele hMOs de groei van verschillende relevante darmbacterie soorten beïnvloeden, hebben we in hoofdstuk 5 de effecten van individuele hMOs op de groei van gunstige bacteriën in zowel monoculturen als coculturen bestudeert. We tonen aan dat *B. longum* subsp. *infantis* in monocultuur goed groeide op alle hMOs, maar dat de groei structuur afhankelijk was. *F. prausnitzii* behaalde in monocultuur een lagere cel dichtheid in de stationaire fase vergeleken met glucose, terwijl *B. longum* subsp. *longum* and *B. adolescentis* niet groeiden op de geteste hMOs. In een co-culture van *B. longum* subsp. *infantis* en *F. prausnitzii*, werden verschillende effecten geobserveerd met de verschillende hMOs. 6'-SL stimuleerde de groei van *B. longum* subsp. *infantis* meer dan 2'-FL, 3-FL, en LNT2. Onze bevindingen laten zien dat de effecten van hMOs op de darm microbiota van babies hMO specifiek zijn. Ons onderzoek levert nieuwe effectieve manieren op om de groei van specifieke gunstige micro-organismen in de darm te stimuleren.

In de vorige hoofdstukken hebben we de effecten van individuele hMOs en een hMO zuur hydrolysaat op humane cellen en bacteriën apart bestudeerd onder stabiele kweekomstandigheden. In een fysiologische situatie worden de darm epitheelcellen, voedsmoleculen en de darmmicrobiota blootgesteld aan een continue peristaltische schuifbewegingen. Deze beweging kan van invloed zijn op de ‘crosstalk’ tussen hMOs, gunstige bacteriën en darm epitheelcellen. Daarom bestudeerden we, in hoofdstuk 6, hoe de hMOs gecombineerd met de schuifkracht in de darm een invloed kan hebben op het gedrag van darm epitheelcellen en de ‘crosstalk’ met gunstige bacteriën. In deze studie hebben we het zogenaamde Ibidi systeem gebruikt om de schuifkracht tijdens de peristaltiek na te bootsten. Eerst bestudeerden we de invloed van schuifkracht op darmepitheelcellen met of zonder de stimulatie van individuele hMOs of een hMO
zuur hydrolysaat. De schuifkracht had een grote invloed op karakteristieken van de darm epitheelcellen, zoals de glycocalyx genexpressie, de productie van antimicrobiële eiwitten en de expressie van tight junction eiwitten. De schuifkracht veranderde ook de invloed van individuele hMOs en een hMO zuur hydrolysaat op darm epitheelcellen. Vervolgens werd de gunstige bacterie *Lactobacillus plantarum WCFS1* (*L. plantarum WCFS1*) geïntroduceerd in het systeem en werd de ‘crosstalk’ tussen deze bacterie en epitheelcellen bestudeerd in de aanwezigheid van hMOs en schuifkracht. Er werden duidelijke veranderingen geobserveerd toen ‘crosstalk’ tussen een commensaal bacterie en darmepitheelcellen werd toegestaan. 3-FL en LNT2 verbeterden de adhesie van gunstige bacteriën en alle geteste hMOs verhoogden de expressie van antimicrobiële eiwitten en het ZO-1 tight junction eiwit. Onze resultaten laten zien dat zowel de schuifkracht als de co-culture van een bacterie samen met darmcellen de functie van hMOs kunnen beïnvloeden.

Tenslotte, in hoofdstuk 7, bediscussiëren we de resultaten die worden beschreven in dit proefschrift. We concluderen dat kleine moleculaire verschillen in hMOs, net als de digestie in het maagdarmkanaal, een sterke invloed hebben op de bioactiviteit van de moleculen. We stellen mogelijke werkingse mechanismen voor evenals nieuwe inzichten voor toekomstige hMOs studie. In dit proefschrift presenteren we nieuwe inzichten in de structuur-functie relatie van hMOs een hMO zuur hydrolysaat op het gastro-intestinale immuunsysteem en nieuwe mogelijke werkingse mechanismen.
Acknowledgements

After four years and three months, I finished this thesis. Looking back at the journey of my PhD, it was a great time to spend these four years in Groningen. Although there were some obstacles, with all the help and support, I managed to finalize this thesis. This thesis would not have been completed without the help of many people. Therefore, I would like to take this opportunity to thank each and every one of you.

First and foremost, I would like to acknowledge my dear promotor Prof. P. de Vos and co-promotor Dr. M. M. Faas. Dear Paul, it is an honor to work in this group. You are an enthusiastic scientist and a very responsible promotor, always have the big picture in your mind, which influenced me in many aspects. You not only taught me how to design and conduct the experiments, but also the how to presenting and writing clearly. Thank you for giving me the opportunities to improve. Dear Marijke, your wonderful ideas and profound statistical knowledge truly inspired me. I appreciate the advices you gave to me and discussions we had. Thank you for your contribution and devotion.

I would like to acknowledge the members of the reading committee Prof. G. Folkerts, Prof. T. Plosch and Prof. L. Dijkhuizen for reading and approving my thesis.

My thanks for to my office mates: Rui, Shuxian, Monique, Alberto, Daniela, Julian, Sophie, Yutong, Erika, and Koji, thank you for being the best office mates. Dear Rui, I feel lucky to have you as a senior fellow in the office. Thank you for your friendship and considerations. Wish you and your family all the best in China. Dear Shuxian, being not only office mate, but also neighbors, we spent so much happy time together. Our countless dinner, card game, and LOL with you and your boyfriend Jingrui are really relaxing and enjoyable time for me. Thanks for the unforgettable nights we spent together. Dear Yutong, thank you for your instruction at gyms and help take care of Coffee for such a long time, I am sure we will meet again when you back in China. Dear Erika, come to our office at the end of my PhD, you bring an active atmosphere in the office and thanks for your inspirations of positive attitude towards life, keep the spirit!

I would like to extend my thanks to my colleagues. Gea, Chengcheng, Yuanrui, Renate, Martin, Susana, Tamara, Alexia, Karlijn, Luis, Rei, Sandra, Marlies, Marjolein, Yu, Carlos, Cynthia, Anna, Tom, Taco, and Bart, thank you for the nice ideas on our group meetings joyful chat during our cake times. Bart, you can always find and fix everything in the lab, help us get access to all the places, and also give me lots of
nice advice on experiments during our weekly meeting. Gea, thank you for helping me during your postdoc time in our group, you are not only a good colleague, but also a very nice daily supervisor. Dear Renate and Martin, working in the similar field, we always meet each other in ML-2 and have lots of discussion on our experiments, thank you for being encouraging all the time, and of course thanks a lot for help me translate the summary. Dear Susana, doing similar experiments and being in the same page of our PhD, I joyed our chats a lot. Dear Yuanrui, I feel lucky to be your senior fellow. Good luck with your last year PhD, be strong, you can do it.

I would like to thank Jelleke, Peter, and Pytrick for your help in the lab. Jelleke, thanks for your help in ML-2 and cell culture. Peter and Pytrick, thank you for helping me order primers and discussing with me when I have questions on qPCR.

I would like to acknowledge Annet, Susan, Caroline, Petra, and Hans for helping arrange things and solving problems during these years, my life here became easier thanks to you.

My appreciation for the collaborators or their contributions. Madelon, thanks for your help on chapter 5 with all the sample analysis. Wenjia, it is a pity that we met late, you are always considerate and warm-hearted. I can’t finish chapter 3 without your help, good luck on with finalizing the thesis! Andre and Arjen, thanks for the samples you provided, and nice advices during our meetings. Marthe, thanks for your nice suggestions for chapter 1 and chapter 5.

Many thanks to my Chinese friends: Wanli, Zhibo, Qi Wang, Yuan He, Xue Liu, Qian Li, Jingjing, Yuanze, Chunxu, Yu Lu, Wei, Yizhou, Keni, Yana, Wenxuan, Meishan, Xinhong, Kai Gao and all others I don’t mention here. Wanli, you initiated and organized the DPSD project and give me the chance to come here, and also supported me a lot during these four years, you are like a teacher and a friend to me. Thank you for all your warm support, all the best to your career and your family. Zhibo, we three apply the project and come to Groningen together, support each other from the very beginning. Maybe we are not able to finish at same time, but I am sure you will create a wonderful lab in the future with your talent and hard work. Dear Qi, I’m glad to meet you at the beginning of my PhD, but also feel pity that I meet you in your last year in Groningen. I joyed every lunch and all the talks we had, your positive attitude towards life encouraged me all the PhD life. Dear Yuan, I’m lucky to have you as friend in
Groningen, you always share with me your experiences in life and research which helps me a lot. Wish you all the best in Nanjing and hope to meet you again in China. Dear Jingjing and Yuanze, I joyed time we spent together, you offered so much helped in life and research. Dear Yu Lu, it is so good to have an old friends you in Europe, you gave me so much encourages during my PhD, also thanks you for the company and guide in Paris every time.

Special thanks to my paranymphs: Chenxi and Chunli. It is a great pleasure to have you as my paranymphs. Dear Chenxi, we have known each other for more than ten years, and start to be roommate since 2013 during our master. We support each other on both life and research, apply PhD project together, come to Netherlands together, you always stand by my side, I don’t know whether I will be here without you. You are also at the end of your PhD, good luck with your thesis. I’m sure an open minded, fearless, and cleaver scientist like you could explore your future career no matter where you want in any field you interested. Dear Chunli, I’m so glad to be your senior fellow in the same field. We cooperate on chapter 4 and chapter 6, spent a lot of time together in the labs and discuss our project. I have to say that you are a really nice collaborator. Besides that, you are also a very good friend to me, I enjoy the time that we spent together during the experiments, courses, games, and of course our trip to London. Good luck for the rest of your PhD, and wish to meet you again in China.

Also special thanks to my boyfriend. Dear Tianqi, you did not back off when we encountered hard times. You let me pursue my dreams and supported me in every possible way. Although we have been in different place and time zone, you always courage me during good and bad times. Thank you from the bottom of my heart. Wish we will have tons of happy times together in the future.

To my family: 亲爱的爸爸妈妈, 这些年来都远离家乡, 没有办法陪伴在你们的身边也很少回家, 感谢你们多年来的支持和鼓励。是你们无条件的支持让我有力量走到今天。希望在今后的日子里, 可以常伴在你们的左右。
**Publication List**


