Experimental studies on anti-pathogenic and gut barrier enforcing effects of dietary fibers and human milk oligosaccharides
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Chapter 9

General discussion

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Human milk oligosaccharides (hMOs) and non-digestible carbohydrates (NDCs) are a group of dietary fibers that resist digestion in the upper gastrointestinal (GI) tract. A wide range of beneficial functions have been reported with the administration of hMOs and NDCs, including enhancement of the gut epithelial barrier, inhibition of the pathogens infection, improvement of the gut microbiota compositions, and regulation of the gut mucosal immune responses. Despite these advantages, there are currently only two hMO and several NDC molecules applied in infant formula. In order to more closely mimic the functions of mother milk, one major obstacle is to identify more effective hMO and NDC molecules, the other is upscaling production of effective hMO or NDC molecules with high purity to make them applicable in infant formula. In this thesis, a wide range of hMOs and NDCs of different structures were investigated to reveal how and which molecule impact the intestinal epithelial barrier function using different cellular systems. The aim was to study the interaction and impact on both pathogenic and commensal bacteria, and to explore the possible underlying mechanisms. All these experimental outcomes contribute to predicting the functions in vivo and provide suggestions for designing tailored infant formula.

1. hMOs and NDCs differently modulate the intestine epithelial barrier

In mother milk, a large family of over 200 oligosaccharide structures of hMOs has been identified, which are dominated by two main types, i.e. neutral fucosylated and acidic sialylated. The most widely applied hMOs (2'-FL and LNnT) and GOS/FOS belong to neutral molecules. To better mimic the components and functions of hMOs in mother milk, in this thesis, we evaluated individual molecules including both neutral hMOs, NDCs, and acidic pectins.

We started with the direct effect of these molecules in modulating the intestine epithelial glycocalyx, which has not been done before. The glycocalyx, also known as “sugar coat”, covering the intestinal epithelium is an important component of the intestine epithelial barrier. In chapter 2, we demonstrate that hMOs, inulins, and pectins were able to stimulate the maturation of the intestine epithelial glycocalyx in Caco-2 cells (Figure 1). hMOs and inulins showed a stronger effect than pectins in the glycocalyx maturation, since hMOs and inulins not only increased protein albumin, but also enhanced the glycosaminoglycan side chains HS and HA. Pectins only increased
albumin. Effects were highly dependent on the structures, since hMO 3-FL but not 2'-FL increased the average thickness of HS and average area coverage of HS and HA. Short chain inulin DP3-DP10 increased the average thickness of HS, while the long chain inulin DP30-DP60 selectively increased the average area coverage of HA. Although HS and HA both provide anchoring points for gut bacteria and are important for maintaining gut barrier integrity\textsuperscript{11,12}, HS is structurally more complex than HA\textsuperscript{13}. hMOs, inulins, and pectins differently modulated HS and HA, which may induce different binding capacity for the gut bacteria\textsuperscript{11,14}.

**Figure 1.** The intestinal epithelial glycocalyx development is crucial for the colonization by gut microbiota and the establishment of the intestinal barrier. The effects of different carbohydrates applied or suggested for infant formula, i.e. human milk oligosaccharides (hMOs), inulins and pectins of different compositions were studied for their impact on development of heparan sulfate (HS) and hyaluronic acid (HA) as well as for adsorbed albumin on the intestine Caco-2 epithelial glycocalyx *in vitro*. Effects were dependent on the chemistry of the carbohydrates.

Glycocalyx is mainly influenced by hMOs and NDCs in the small intestine as this part of the intestine is covered with a thin layer of mucus, allowing for the direct contact with the molecules. Differently, in the large intestine where the majority of gut microbiota colonize, is covered with a thick layer of mucus and form a protective barrier for pathogens invasion. In chapter 5, we show that hMOs directly enhanced mucosal barrier function of goblet cells under both homeostatic and inflammatory conditions. Effects were structure dependent, as 3-FL, LNT2, and GOS, but not 2'-FL increased the secretory genes and also MUC2 protein expression under homeostatic condition. Structure dependent effects were also observed under inflammatory
conditions. During different stressor challenges, goblet cells responded differently to specific hMOs, i.e. 3-FL, LNT2, and GOS but not 2'-FL up regulated MUC2 with TNFα challenge, while only LNT2 up regulated TFF3, RETNLB, and CHST5 with Tm challenge. These findings are very interesting since they give suggestions for choosing specific hMO molecule toward specific groups with predictable beneficial effects.

2. Novel mechanisms for the anti-adhesion effects of hMOs and NDCs

Inhibition of the pathogens infection in early life is an important function of hMOs and NDCs 15. As we observed that hMOs and NDCs differently modulated the epithelial glycocalyx in chapter 2, which is taken as the anchoring points for gut bacteria, we further studied whether pre-incubating Caco-2 cells with hMOs and NDCs inhibit pathogens adhesion in chapter 3. Also we studied how cell membrane glycosylation and inflammatory-associated genes were regulated in response to the individual hMO and NDC molecule during exposure to pathogens.

We observed that pre-incubating Caco-2 cells with hMOs, inulins, and pectins all inhibited the adhesion of pathogens, but the effects were dependent on the molecule structure, pathogen strain and growth phase. This might be a reason for the presence of a wide variety of hMO structures in mother milk 9 that collectively inhibit a wide spectrum of pathogens 16. Pre-incubating Caco-2 cells with pectins differently remodeled the cell membrane glycosylation, i.e. DM7 pectin up regulated EXT1 (HS), DM55 pectin down regulated LGALS9 (galectin 9) and GPC1 (glypican 1), DM69 pectin selectively up regulated LGALS1 (galectin 1) and HAS3 (HA), as such they may inhibit specific pathogen adhesion. Besides, we also observed DM7, DM55, and DM69 pectins all significantly up regulated ICAM1 (inflammation) in response to different pathogens.

What is even more intriguing, in chapter 3, we observed the lung pathogen K. pneumoniae differently regulated the inflammatory genes compared to gut pathogens. This is not surprising, as K. pneumoniae demonstrates a different biosynthesis and transport pathway for inflammation and virulence factors, and is evolved with a strong resistance to antibiotics treatment 17. This has been known as the immune evasion properties of Klebsiella strains in recent years 18, but the underlying mechanisms are still largely unknown. One proposed strategy for the clearance of K. pneumoniae from
the human host is by stimulating pro-inflammatory signaling \textsuperscript{18}, which well explained our observations that, pre-incubating Caco-2 cells with pectins, especially DM69 pectin restored the inflammatory genes that were down regulated by \textit{K. pneumoniae}. This illustrates an immune activating function by DM69 pectin in reducing the \textit{K. pneumoniae} infection.

Another mechanism by which hMOs and NDCs can reduce pathogen invasion is by decoy effects. By resemblance of the intestine epithelial glycosylation structures, hMOs have been reported to block the pathogens before they adhere to the intestinal epithelium \textsuperscript{19}. In \textit{chapter 4}, we tested the possible decoy effects of hMOs and NDCs by pre-incubating the pathogens before infection with Caco-2 cells. Pre-incubation with 2'\textsuperscript{-}FL, inulins, and low DM pectins reduced pathogen adhesion and similar to what we observed in \textit{chapter 3}. The effects were molecule structure, pathogen strain and growth phase dependent. But 3-FL and DM69 pectin were found to increase the adhesion of certain pathogens, which raised our interest to further investigate the underlying mechanisms of this increasing effect.

We performed comparison of transcriptomics of the pathogens for which enhanced adhesion was observed. These were 3-FL and DM69 pectin. 3-FL did not influence gene expression of \textit{E. coli} WA321 from log phase. This observation was in correspondence with the utilization of 3-FL by the gut microbiome of infant fecal samples in \textit{chapter 8}. 3-FL was only slightly degraded after 14 hours, which may suggest that 3-FL is not easily accessible to most of the infant gut bacteria. Compared to the regulation effect on the bacteria, 3-FL might be more involved in the interaction with gut epithelial cells as observed in \textit{chapter 2}. DM69 pectin strongly changed the gene expression of both \textit{E. coli} ET8 from log phase and \textit{E. coli} WA321 from stationary phase. It is clear that the genes involved in upbuilding of cell membrane proteins and biofilm formation were up regulated, which might be the reason for the increased adhesion to Caco-2 cells. However, the increased adhesion induced by DM69 pectin may not directly mean a higher infection or virulence, as many genes involved in bacterial virulence were dramatically down regulated with DM69 pectin pre-incubation.

3. Linkage rather than the backbone of hMOs influences the functions

Due to the limit availability of hMOs, efforts are being made to improve the strategies
for hMO production \(^{20}\). In recent years, only specific hMO molecules can be produced in sufficient amounts by bio-engineered microorganisms \(^{20}\). Meanwhile, the emerging enzymatic and chemical methods for the synthesis of novel hMO analogues are under active development \(^{21}\). In this thesis, we evaluated the bioactivity of a newly synthesized hMO analogue obtained with chemical methodologies.

From the former chapters, we observed a structure dependent effects of hMOs 2'-FL and 3-FL, \(i.e.\) 3-FL but not 2'-FL enhanced intestinal epithelial barriers (chapter 2 and chapter 5). 3-FL but not 2'-FL increased the commensal bacterium adhesion under physiological peristaltic shear force (chapter 7). 3-FL and 2'-FL also differently regulated the adhesion of gut pathogens (chapter 3 and chapter 4). These observations are very interesting, as 2'-FL and 3-FL both have lactose as the backbone, and only differ in the linkage of the fucose residue \(^{9}\). It seems that the linkage pattern of fucose influences the effects of hMOs. For these reasons, through the chemical method, a 3-FL mimicked hMO analogue named di-fucosylated \(\beta\)-cyclodextrin (DF\(\beta\)CD) was chemically synthesized based on a different backbone from the lactose backbone of 3-FL, \(i.e.\) \(\beta\)-cyclodextrin (\(\beta\)CD). This molecule contains two fucose residues that are \(\alpha\)-1,3 linked to glucose as the linkage pattern of 3-FL \(^{22}\).

The impacts of the fucose residue and the linkage pattern on the anti-pathogen effect of hMO analogue have been previously demonstrated \(^{23}\). In chapter 6, we studied the possible digestion and fermentation of DF\(\beta\)CD by 9-month-old infant gut microbiota, as well as the anti-adhesive properties against gut pathogen enterotoxigenic \(Escherichia\ coli\) (ETEC) O78:H11 to Caco-2 cells. 2'-FL, 3-FL, and \(\beta\)CD were also included for comparison. We observed that DF\(\beta\)CD could resist the digestive enzymes present in the upper GI, which indicate the characteristic of dietary fiber that reach the colon intact. Afterwards, however, different from 2'-FL, 3-FL, and \(\beta\)CD, which can be efficiently fermented by the gut microbiome of the 9-month-old baby fecal inoculum, DF\(\beta\)CD was shown non-fermentable. The observation might be a result of the specific fucose linkage positions of DF\(\beta\)CD that blocked the microbial enzymatic fermentation \(^{22}\). Meanwhile, DF\(\beta\)CD exhibited a significant inhibition effect on the adhesion of ETEC to Caco-2 cells, through a possible decoy effect with direct interaction with the bacteria, as observed with hMOs 2'-FL and 3-FL. Therefore, the findings expand the knowledge on the anti-adhesive effect of fucosylated structures on gut pathogens.
4. Factors that influence hMOs and NDCs on the adhesion of commensal bacterium

One important function of hMOs and NDCs supplementation in infant formula is to support the colonization of beneficial bacteria in the gut. In addition to supporting their growth, enhancing the adhesion of beneficial bacteria to intestinal epithelial cells are particularly of significance to exclude pathogen infection and to exert long-term benefits. However, the possible mechanisms of how hMOs influence the adhesion of commensal bacteria during passage through the GI tract are still largely unknown. In this thesis, we revealed the effects of intestinal peristaltic shear force exposure and gut microbiota fermentation of hMOs on the adhesion of a commensal bacterium to intestinal epithelial Caco-2 cells.

The intestinal epithelial cells, gut microbiota, and the food components in the gut are continuously exposed to intestinal peristaltic shear force. In chapter 7, we studied the effects of 2'-FL, 3-FL, and LNT2 on the adhesion of *Lactobacillus plantarum* WCFS1 to Caco-2 cells when they were exposed to shear force. We observed that 3-FL and LNT2 enhanced the adhesion of *L. plantarum* WCFS1 under both static and with shear force exposure, while the enhancing effect of 2'-FL on the adhesion of *L. plantarum* WCFS1 was only observed under static culture. In chapter 8, we studied the effects of the fermentation digesta of GOS/inulin, 3-FL, and LNT2 by infant fecal gut microbiome, on the adhesion of *L. plantarum* WCFS1 to Caco-2 cells. We observed that the fermentation by gut microbiome improved the effects of GOS/inulin and LNT2 on the adhesion of *L. plantarum* WCFS1 to Caco-2 cells, while the fermentation did not affect 3-FL. Our observations contribute to a better understanding about the impact of shear force and gut microbiome fermentation in the GI tract and our data suggest that this should be taken into account for evaluation of hMOs and NDCs effects.

5. *In vitro* models to evaluate the functions of hMOs and NDCs

Due to absence of predicting *in vitro* evaluating system, translation of *in vitro* results to *in vivo* are not always easy and responsible for lower efficacy of components when tested in clinical trials. One reason might be the intestinal peristaltic shear force that was not involved in most of the current *in vitro* models. In chapter 7, we applied a so-called ibidi system to generate shear force on intestinal epithelial Caco-2 cells.
We observed that shear force did impact the intestinal epithelial barriers, including gene expression of the glycocalyx associated molecules, an antimicrobial peptide, as well as protein expression of tight junctions. Shear force also changed the impact of hMOs on the intestinal epithelial barriers, i.e. LNT2 increased the gene expression of glycocalyx associated HAS3 of Caco-2 cells under static culture, but this effect was not observed when the cells were exposed to shear force. For the first time, we also exposed intestinal epithelial cells, hMOs, and a commensal bacterium to physiological shear force. With the presence of the commensal bacterium L. plantarum WCFS1, shear force exposure improved the effects of hMOs on the tight junction protein ZO-1, but failed to influence Claudin-3. All these observations demonstrate the important role of physiological shear force for evaluation of hMOs.

The beneficial effects of hMOs and NDCs on gut microbiota development have been demonstrated, but how individual hMO and NDC molecules are utilized are impossible to investigate through in vivo studies. Thus, an in vitro fermentation model has been established to visualize the fermentation activities of hMOs and NDCs by the gut microbiome. In chapter 8, we fermented 3-FL and LNT2, and GOS/inulin with pooled fecal samples from 12-week-old babies. Fermentation digesta was collected at different time points of 0 (T0), 14 (T14), 24 (T24), and 36 (T36) hours. We observed different fermentation patterns for 3-FL, LNT2, and GOS/inulin, of which, LNT2 and GOS in the GOS/inulin mixture were fastly utilized starting already at T14, while 3-FL and inulin in the GOS/inulin mixture showed a gradual degradation until T36. The degradation of these molecules was associated with gut microbiome changes and production of SCFAs and organic acids. LNT2 specifically increased the relative abundance of Collinsella and Bifidobacterium, and induced production of acetic acid, succinic acid, lactic acid and butyric acid. The age group studied here, i.e. 12-week-old is a common time point for the transition from breastfeeding to infant formula feeding. Therefore, our observations provide information for the benefits of 3-FL and LNT2 to improve the gut microbiota composition and organic acids production when supplemented in infant formula for babies as of the age of 12-weeks.

6. Conclusions and future perspectives

In this thesis, we investigated the regulatory mechanisms of individual hMOs and NDCs. We provide new insight in how they can have direct effects on the intestinal
epithelial barrier function. We also provide new data on how the molecules mediate crosstalk between gut pathogens or gut commensal bacterium with intestinal epithelial cells. We specifically explored the effects on the intestinal epithelial glycocalyx components, which were differently modulated by hMOs and NDCs, differently remodeled by the gut bacteria, and differently expressed under the physiological shear force. We thus demonstrate the importance for choosing the optimal in vitro models by including the necessary physiological factors for the functions evaluation of hMOs and NDCs. The different mechanisms described in this thesis give suggestions to strengthen the gut barrier functions of infants through infant formula supplemented with hMOs and NDCs.

Figure 2. Summary of the new insights about the effects of dietary fibers on the gut immune barriers in this thesis. (a) Dietary fibers as well as their fermentation metabolites stimulate the colonization of commensal bacterium; (b) The fermentation of dietary fibers by infant fecal inoculum modulate the production of SCFAs; (c) Dietary fibers reduce the adhesion of pathogens by serving as decoy receptors or through regulation of the gut epithelial cells; Dietary fibers increase the production of mucus of goblet cells (d) and glycocalyx of the epithelial cells (e); (f) Dietary fiber can regulate the gut permeability; (g) Physiological shear force shows importance in regulating the effects of dietary fibers.

The individual hMO and NDC molecules studied in this thesis as well as molecules studied by other researchers have proven a highly structure- and molecular pattern-dependent effect on biological processes (Figure 2). Combinations of the neutral hMO or NDC molecules with the acidic NDC molecules, eg. pectins are suggested to be tested for future application as they together will have a broader efficacy in stimulating beneficial host effects. Our data suggest that combinations as the typical GOS/FOS mixture (9:1), the ratio and dosage of other neutral and acidic hMOs or NDCs should be tested and will have other, may be even stronger health effects. In this thesis, we specifically studied the effects of individual hMO and NDC.
molecules on the intestine epithelial glycocalyx components. Glycocalyx is important for the colonization of gut bacteria and for maintaining gut barrier function. However, which and how glycocalyx components are responsible for the bacteria colonization are not elucidated yet. Approaches such as enzymatic removal or gene mutation of the specific component can be applied in future studies, and will give further insight in how hMOs and NDCs contribute via glycocalyx stimulation adhesion of commensal bacteria.

Also, we demonstrate the effects of physiological shear force on the function of hMOs and NDCs. During recent years, the development of such in vitro models, have considered inclusion of several physiological factors. A so-called “microfluidic device”, allowing the co-culture of gut bacteria, epithelial cells, and immune cells under shear force can be used to test hMOs and NDCs. However in such system the anaerobic commensals cannot survive. Another device named ‘human-microbial co-culture model’, allowing the culture of anerobic bacteria with epithelial cells, could be an option to investigate the interaction of hMOs or NDCs with anerobic commensals and epithelial cells. These “gut-on-a-chip” devices may be developed as a newly designed testing platform, which can integrate more gastrointestinal conditions to better understand the functions and mechanisms of hMOs and NDCs in vitro.

All the observations in this thesis contribute to the development of infant formula containing specific hMO and NDC molecules. With better understanding of the functions of individual hMO and NDC molecules, the effective molecules are screened out. Based on these observations, we expect to provide suggestions to design personalized infant formula for specific age group and health status, supplemented with specific or mixtures of hMOs and NDCs.
References


