Chapter 2

Modulation of intestinal epithelial glycocalyx development by human milk oligosaccharides and non-digestible carbohydrates

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Abstract

Scope: The epithelial glycocalyx development is of great importance for microbial colonization. Human milk oligosaccharides (hMOs) and non-digestible carbohydrates (NDCs) might modulate the glycocalyx development.

Methods and Results: We investigated effects of hMOs and NDCs on human gut epithelial cells (Caco-2) by quantifying thickness and area coverage of adsorbed albumin, heparan sulfate (HS) and hyaluronic acid (HA) in the glycocalyx. Effects of hMOs (2'-FL and 3-FL) and NDCs [inulins with degrees of polymerization (DP) (DP3-DP10, DP10-DP60, DP30-DP60) and pectins with degrees of methylation (DM) (DM7, DM55, DM69)] were tested using immunofluorescence staining at 1 and 5 days stimulation. HMOs showed significant enhancing effect on glycocalyx development but effects were structure dependent. 3-FL induced a stronger albumin adsorption and enhanced HS and HA stronger than 2'-FL. The DP3-DP10, DP30-60 inulins also enhanced glycocalyx development in a structure dependent manner as DP3-DP10 selectively increased HS, while DP30-DP60 specifically enhanced HA. Pectins had less effects, and only enhanced albumin adsorption.

Conclusion: Here we show that 2'-FL and 3-FL and inulins stimulate glycocalyx development in a structure dependent fashion. This might contribute to formulation of effective hMO and NDC formulations in infant formulas to support microbial colonization and gut barrier function.
1. Introduction

Breastfeeding is considered the golden standard for supply of nutrients and bioactive molecules to neonates. Important mother milk components for neonatal gastrointestinal development are the human milk oligosaccharides (hMOs). HMOs have been shown to shape the microbiome within the gastrointestinal tract;¹,² reduce pathogenic infections by serving as decoy for pathogens;³,⁴ stimulate the immune system of infants and enhance intestinal barrier function.⁵ However, there are still about 70% of the infants that cannot be solely fed with breastfeeding for a variety of reasons.⁶ These infants receive cow milk derived infant formulas, in which often non-digestible carbohydrates (NDCs) such as inulins and pectins are included to mimic some functions of hMOs.⁷ To even more closely mimic the compositions of mother milk, significant attempts have been made to synthesize hMOs for supplementation in infant formulas. Some of these molecules can be produced in a cost-effective way, making application in infant formulas a realistic option.⁸

One important function of hMOs and NDCs in infant formula is stimulation of colonization of the gastrointestinal tract by microbiota and promote development of gut barrier function.⁹,¹⁰ A possible way by which this is accomplished is by stimulating the development of the epithelial glycocalyx on gut epithelial cells. The glycocalyx on neonatal gut epithelium provides binding sites for the commensal microorganisms and a well-developed glycocalyx might prevent adhesion of pathogens and serve as a barrier for luminal toxins and enzymes.¹¹,¹² The glycocalyx is composed of glycans and proteins. Proteoglycans are generally considered the most important component of the glycocalyx and form the skeleton of the glycocalyx.¹³ In addition, the glycocalyx contains glycosaminoglycan chains which are linked to the core protein of the proteoglycans.¹³ Heparan sulfate (HS) and hyaluronic acid (HA) are the predominant glycosaminoglycan components in glycocalyx.¹⁴ HS is a linear highly sulfated polysaccharide that is composed of repeating disaccharide units that consist of an O-sulfated derivative of glucosamine and an uronic acid.¹⁵ HA is a non-sulfated disaccharide polymer, lacking the complex chemical structure of HS and is much longer than HS.¹⁶

If the glycocalyx components are not properly developed it might lead to enhanced chances for gastrointestinal disorders.¹⁷ The different glycocalyx molecules can contribute in different ways to gut homeostasis. HS can mediate cell
signaling by binding numerous extracellular ligands such as fibroblast growth factor and contributes to homeostasis of innate immunity.\textsuperscript{18,19} HA is highly viscous and forms a physical barrier on top of the gut epithelium.\textsuperscript{20,21} In dextran sodium sulfate induced colitis, HA expression is enhanced to support colonic epithelial repair.\textsuperscript{22} Inflammatory bowel disease can be caused by modification of the glycosaminoglycan composition and distribution on gut epithelium.\textsuperscript{17} Proteins adsorbed on glycosaminoglycan also play an important role in the stability of the glycocalyx. When protein adsorption is absent, the interactions within the glycocalyx collapse and barrier function is lost.\textsuperscript{14} Especially absence of albumin induces collapse and shedding of the glycocalyx.\textsuperscript{23}

As not much information is available about effects of hMOs and NDCs on glycocalyx development, we performed this study to determine whether and to what extent these molecules influence glycocalyx synthesis by human gut epithelial cells. To this end, we stimulated gut epithelial Caco-2 cells for 1 and 5 days with different hMOs and NDCs. Effects were tested on adsorbed albumin, HS, and HA and average thickness and average area coverage of the glycocalyx layer components on gut epithelial cells. We tested effects of the hMOs 2'-FL (2'-fucosyllactose; Fucα1, 2- Galβ1, 4Glc) and 3-FL (3-fucosyllactose; Galβ1, 4(Fucα1, 3) Glc). Effects were compared with molecules that are often used as substitutes for hMOs in infant formula. To this end we tested inulins with three different degrees of polymerization (DP) (DP3-DP10, DP10-DP60, DP30-60) and pectins with three different degrees of methylation (DM) (DM7, DM55, DM69) in order to gain insight in chemical structure-effects relationships.
2 Material and Methods

2.1 Carbohydrates
The human milk oligosaccharides (hMOs), 2′-FL was provided by FrieslandCampina Domo (the Netherlands) and 3-FL was provided by Glycosyn LLC (Woburn, MA, USA). Commercially extracted chicory inulins with different degree of polymerization range (DP3-DP10, DP10-DP60, and DP30-60) were provided by Sensus (the Netherlands). DP3-DP10 is of the highly soluble powdered Frutafit® CLR inulins, which was produced from partially hydrolyzed chicory inulin. DP10-DP60 and DP30-DP60 are of the moderate soluble powdered Frutafit® TEX! inulins. Lemon originated pectins with different degree of methylation (DM7, DM55, and DM69) were obtained from CP Kelco (Denmark). Endotoxin levels in the 8 samples were analyzed by endotoxin detection kit (Thermo Fisher Scientific), and all fell below the endotoxin detection level of 0.1 ng/ml. All of the carbohydrates were dissolved to 2 mg/ml in cell culture media before stimulation.

2.2 Cell culture
Human colon carcinoma Caco-2 cells were incubated with 5% CO₂ at 37 °C in Dulbecco’s Modified Eagle Medium (DMEM, Lonza), supplemented with 10% (v/v) fetal calf serum (FCS, Invitrogen), 1% (v/v) non-essential amino acid (NEAA, Sigma), 50 U/ml Penicillin (Sigma), 50 µg/ml Streptomycin (Sigma), and 2.5% (v/v) HEPES (Sigma) to maintain a stable pH environment. The cells density was adjusted to 1.6×10⁴/ml before seeded onto the 8-well Lab-Tek Chamber Slide (w/Cover, Nunc, Thermo Fisher Scientific). To improve the adhesion of cells to the slide, 200 µl of poly-L-lysine solution (Sigma) was applied to pre-coat the slide. After 2 days of growth, the cells were stimulated with the 8 types of carbohydrates for 1 or 5 days.

2.3 Immunofluorescence staining
For staining of albumin, the following procedure was applied. After stimulation with carbohydrates, Caco-2 monolayers were washed with 1×Dulbecco’s phosphate-buffered saline (DPBS), fixed with 2% paraformaldehyde/0.1% glutaraldehyde for 30 min, and blocked with 2% donkey serum for 30 min at room temperature. After overnight incubation with anti-albumin (rabbit IgG, 1:150, Invitrogen) at 4 °C, cells were washed with 1×DPBS for three times and incubated with Alexa Fluor 555
donkey anti-rabbit secondary antibody (1:400, molecular probes) in the dark for another 30 min, followed by washing three times with 1×DPBS.

**Figure 1. Glycocalyx thickness analysis.** The merged images of the maximum intensity from the Z projection of the FITC (green) and DAPI (blue) channels (A). The orthogonal views of the FITC channel (B). (C) We applied the threshold value determined from the corresponding control and negative control groups to include all of the green channel of the XZ axis of this plane. At least 10 XZ and YZ slices were chosen for quantification of each image. Scale bar = 20 μm.

The heparan sulfate (HS) staining procedure was the same as the staining for albumin, except that the cells were incubated overnight with Ab-Heparan Sulfate F58-10E4 (1:100, amsbio) at 4 °C, and after washing three times with 1×DPBS, the cells were incubated with Alexa Fluor 488 donkey anti-mouse secondary antibody (1:100, molecular probes) in the dark for 30 min.

Hyaluronic acid (HA) staining was also performed in virtually the same way as the staining for albumin, but the cells were blocked with 2% goat serum for 30 min and incubated overnight with hyaluronic acid binding protein (HABP, 50 μg/ml, Calbiochem) at 4 °C. After three times washing with 1×DPBS, the cells were incubated with Streptavidin FITC (1:100, eBioscience) in the dark for 30 min.

DAPI (1:5000, Sigma) staining in the dark for 10 min was applied to stain the nuclei. This was done after the staining of the glycocalyx layer components, followed by washing three times with 1×DPBS. Then the chamber frame was removed, cells on the slide were mounted with CitiFluor™ (Electron Microscopy Sciences) and covered with glass coverslip. For all of the three glycocalyx layer components, the control groups were incubated with 1×DPBS overnight at 4 °C
instead of the primary antibodies. For the negative control group, the primary and secondary antibodies were both replaced with 1×DPBS.

### 2.4 Confocal microscopy

All the images were captured with a Leica SP8 confocal laser microscope (Leica Microsystems, Wetzlar, Germany) with the 64×/1.4 oil DIC objective. Albumin was excited at 555 nm and emitted at 580-650 nm (red); HS and HA were excited at 488 nm, and emitted at 500-580 nm (green); DAPI was excited at 405 nm, and emitted at 420-460 nm (blue). Z-stack (512×512-pixel resolution×8 bit) images of each field of view (FOV, 246.51×246.51 µm²) were taken with a step length of 1.0 µm from the bottom to the top of the monolayer. At least 3 images were taken of each sample in one experiment.

The average thickness of the glycocalyx components was quantified according to the method of using the Image J software (Version 1.51n; National Institutes of Health, USA) as shown in Figure 1.24 The staining of HA is shown in Figure 1A as an example of merged images of the maximum intensity plane from the Z projection of the FITC (green) and DAPI (blue) channels. The green and blue channels were split, and then we turned to the orthogonal views of the green channel (Figure 1B). Along the XZ axis, the threshold value can be adjusted to cover the green channel with red color (Figure 1C). The threshold values for all the images quantification were determined from the corresponding control and negative control groups as follows: the pixel intensity histograms from the maximum intensity Z projections of the control and negative control were introduced to one curve, and the intersection point was defined as the threshold value that we applied for the data analysis. At least 10 XZ and YZ slices were chosen of each image to do the quantification. As for one experiment, at least 3 images were taken, and we did at least 6 individual experiments. There were at least 360 slices involved in the average thickness quantification. The average thickness of each slice of the green channel was calculated as Eq. (1):

\[
\text{Average thickness} = \frac{\text{Total area}}{\text{Total length}}
\]

All of the Z-stack images taken by the Leica SP8 confocal microscope (at least 18 images for each sample) were applied to quantify the average area of each glycocalyx layer component using IMARIS software (Version 8.0, Bitplane,
Chapter 2

Switzerland). There were two channels in total. The blue (DAPI) channel was for the quantification of the total number of the cells of each image; The green (FITC for example) channel can be split out to quantify the total area of the staining. The threshold value of the green channel was adjusted to include the whole staining. The average area was expressed as Eq. (2):

\[
\text{Average area} = \frac{\text{Total area}}{\text{Total number of cells}} \tag{2}
\]

2.5 Statistical analysis

Data analysis was done by GraphPad Prism 6 statistical software (GraphPad Prism Software Inc. San Diego, CA, USA). The Kolmogorov-Smirnov test was applied to determine normality of data distribution. Results were expressed as mean ± SD. All data were finally analyzed with Kruskal-Wallis test of One-way ANOVA. Significant difference level was defined as \( p < 0.05 \) (*\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \)), \( p < 0.1 \) was considered to be a statistical trend.
3. Results

The glycocalyx layer of gut epithelial cells is the primary site for adhesion of commensal bacteria. Its compositions and development are important for building up of the intestine barrier and a normal gut microbiota. As human milk oligosaccharides are involved in stimulating gut microflora, we tested whether this might happen via stimulation of the development of the glycocalyx on gut epithelial cells that form anchoring points for gut bacteria.

The glycocalyx major constituents are heparan sulfate (HS) and hyaluronic acid (HA) chains. In the human body albumin provides stability to the glycocalyx. Albumin is synthesized in the liver, and adsorbed on gut epithelial cells. It can be found in the small intestine and contributes to the structure of the glycocalyx layer. Albumin was also present in the culture medium and could therefore also be studied. We tested the effects of the hMOs 2'-FL and 3-FL, inulins with DPs of DP3-DP10, DP10-DP60, and DP30-60, and lemon pectins with DMs of DM7, DM55, and DM69. The average thickness and average area of albumin, HS and HA components around the cells that were covered with glycocalyx were quantified. This was done after 1 day and 5 days of culture to follow the development of the glycocalyx when the gut epithelial cells were exposed to the hMOs and NDCs.

3.1 The glycocalyx layer compositions of Caco-2 cells after 1 day and 5 days exposure to hMOs

The hMOs 2'-FL and 3-FL were tested first. After 1 day and 5 days stimulation with 2 mg/ml, the cells were stained for albumin, HS and HA on Caco-2 epithelial cells.

After 1 day of stimulation, both 2'-FL and 3-FL hMOs significantly enhanced the thickness of the albumin layer in the glycocalyx (p < 0.01, Figure 2A). The average area coverage by albumin was almost doubled by the hMOs but only 3-FL reached statistical significant levels (p < 0.01, Figure 2D).

There was a tendency that the average thickness of HS was also increased by the hMOs after 1 day of stimulation (p < 0.10, Figure 2B). The average area of cells that were covered with HS was enhanced by the hMOs, but the magnitude of enhancement was hMOs-type dependent and only reached statistical significance with 3-FL (p < 0.05, Figure 2E).
Chapter 2

There was no effect on the average thickness of HA of the two hMOs. However, the average area of cells covered with the HA-component was increased by both 2'-FL and 3-FL but only 3-FL reached statistical significance (p < 0.05, Figure 2F).

Figures 2. The glycocalyx layer compositions of Caco-2 cells were modified by hMOs at one day of culture. Caco-2 cells were stimulated with hMOs. These were 2'-FL and 3-FL tested at 2 mg/ml for 1 day. Untreated Caco-2 cells served as controls. After incubation, we applied immunofluorescence to label the glycocalyx layer components albumin, HS, and HA. Three dimensional (3D) images were taken with a Leica SP8 confocal laser microscope. The average thickness of albumin (A), HS (B), and HA (C) were quantified using Image J software of the 3D images. The average area of cells covered with albumin (D), HS (E), and HA (F) were measured with IMARIS software of the 3D images. All data was expressed as mean with standard deviation from six replicates. Statistical significance was tested using One-way ANOVA (*p < 0.05, **p < 0.001).

A duration of 5 days exposure to the hMOs resulted in a stable glycocalyx layer. The average thickness of albumin was significantly improved by the 2'-FL (p < 0.05, Figure 3A) and 3-FL (p < 0.01, Figure 3A). The average area of cells covered with albumin was also enhanced by the hMOs, while 2'-FL reached statistical significance (p < 0.01, Figure 3D).

Both 2'-FL and 3-FL enhanced the average thickness of the glycocalyx layer that contains HS, but only 3-FL hMO showed significant effects (p < 0.01, Figure 3B). The average area that was covered with HS component was not influenced by the hMOs.
The 3-FL almost doubled the average area of cells that were covered with HA but did not reach statistical significance.

Figure 3. The glycocalyx layer compositions of Caco-2 cells were modified after 5 days of exposure to hMOs. Caco-2 cells were stimulated with hMOs. These were 2'-FL and 3-FL tested at 2 mg/ml for 5 days. Untreated Caco-2 cells served as controls. After incubation, we applied immunofluorescence to label the glycocalyx layer components albumin, HS, and HA. Three dimensional (3D) images were taken with a Leica SP8 confocal laser microscope. The average thickness of albumin (A), HS (B), and HA (C) were quantified using Image J software of the 3D images. The average area of cells covered with albumin (D), HS (E), and HA (F) were measured with IMARIS software of the 3D images. All data was expressed as mean with standard deviation from six replicates. Statistical significance was tested using One-way ANOVA (*p < 0.05, **p < 0.001).

3.2 The glycocalyx layer compositions of Caco-2 cells after 1 day and 5 days exposure to inulins

Inulins are often used as substitute for hMOs in infant formulas. Inulins vary in composition in different infant formulations and were therefore tested in three different DP compositions. Inulins with DP3-DP10, DP10-DP60, and DP30-60 were incubated with Caco-2 cells at a concentration of 2 mg/ml. After 1 day and 5 days of culture, the cells were stained and analyzed for the average thickness and average area of the glycocalyx.

A pronounced development of the glycocalyx was already observed after day 1. After 1 day of stimulation, DP3-DP10, DP10-DP60, and DP30-60 inulins all significantly increased the average thickness of the glycocalyx layer that contains the
albumin (p < 0.05, Figure 4A). There was no DP dependent effect of the inulins on adsorbed albumin.

Figure 4. The glycocalyx layer compositions of Caco-2 cells were modified by inulins at one day of culture. Caco-2 cells were stimulated with different chain length of inulins. These were inulins with DP3-DP10, DP10-DP60, and DP30-60 tested at 2 mg/ml for 1 day. Untreated Caco-2 cells served as controls. After incubation, we applied immunofluorescence to label the glycocalyx layer components albumin, HS, and HA. Three dimensional (3D) images were taken with a Leica SP8 confocal laser microscope. The average thickness of albumin (A), HS (B), and HA (C) were quantified using Image J software of the 3D images. The average area of cells covered with albumin (D), HS (E), and HA (F) were measured with IMARIS software of the 3D images. All data was expressed as mean with standard deviation from six replicates. Statistical significance was tested using One-way ANOVA (*p < 0.05, **p < 0.001).

The average thickness of the HS component was also enhanced by the inulins but only reached statistical significance with inulins DP3-DP10 (p < 0.05, Figure 4B). The average area that was covered by HS was almost doubled by the inulins but was not more than a statistical trend (p < 0.1, Figure 4E).

The average thickness of the HA was not influenced by the inulins but the average coverage of the cells with HA was enhanced in a DP dependent manner. The long chain DP30-DP60 had the most pronounced effect and almost doubled HA on the cells (p < 0.05, Figure 4F).

A period of 5 days exposure resulted in a more pronounced effect of the inulins. DP3-DP10, DP10-DP60, and DP30-DP60 inulins all significantly increased
the average thickness of the albumin component \((p < 0.05, \text{Figure 5A})\), while they only showed a slight enhanced effect on the average coverage of the albumin.

![Figure 5A](image)

Figure 5. The glycocalyx layer compositions of Caco-2 cells were modified after 5 days of exposure to inulins. Caco-2 cells were stimulated with different chain length of inulins. These were inulins with DP3-DP10, DP10-DP60, and DP30-DP60 tested at 2 mg/ml for 5 days. Untreated Caco-2 cells served as controls. After incubation, we applied immunofluorescence to label the glycocalyx layer components albumin, HS, and HA. Three dimensional (3D) images were taken with a Leica SP8 confocal laser microscope. The average thickness of albumin (A), HS (B), and HA (C) were quantified using Image J software of the 3D images. The average area of cells covered with albumin (D), HS (E), and HA (F) were measured with IMARIS software of the 3D images. All data was expressed as mean with standard deviation from six replicates. Statistical significance was tested using One-way ANOVA (*\(p < 0.05\), **\(p < 0.001\)).

The average thickness of HS component was also enhanced by the inulins after 5 days stimulation, but it only reached statistical significance after DP3-DP10 \((p < 0.01, \text{Figure 5B})\) and DP10-DP60 \((p < 0.05, \text{Figure 5B})\) inulin exposure. The average thickness of HA was not influenced after 5 days exposure to inulin.

3.3 The glycocalyx layer compositions of Caco-2 cells after 1 day and 5 days exposure to pectins

Pectin-derived oligosaccharides are another NDC source used as supplement in infant formula. Since the biological functions are reported to be DM dependent, pectins with different methylation degrees of DM7, DM55, and DM69 were also
applied to stimulate Caco-2 cells at a concentration of 2 mg/ml. Cells were harvested and stained for glycocalyx layer components after 1 day and 5 days incubation.

After 1 day stimulation, the average thickness of the albumin was almost doubled, but this did not reach statistical significance. The average area of the cells covered with albumin was enhanced by pectins in a DM dependent manner, in which the low DM7 pectin significantly enhanced the average area of albumin in the glycocalyx layer ($p < 0.05$, Figure 6D).

Figure 6. The glycocalyx layer compositions of Caco-2 cells were modified by pectins at one day of culture. Caco-2 cells were stimulated with different methylation degrees of pectins. These were pectins with DM7, DM55, and DM69 tested at 2 mg/ml for 1 day. Untreated Caco-2 cells served as controls. After incubation, we applied immunofluorescence to label the glycocalyx layer components albumin, HS, and HA. Three dimensional (3D) images were taken with a Leica SP8 confocal laser microscope. The average thickness of albumin (A), HS (B), and HA (C) were quantified using Image J software of the 3D images. The average area of cells covered with albumin (D), HS (E), and HA (F) were measured with IMARIS software of the 3D images. All data was expressed as mean with standard deviation from six replicates. Statistical significance was tested using One-way ANOVA (*$p < 0.05$, **$p < 0.001$).

The average thickness of the glycocalyx layer containing HS was also increased but never reached statistical significance. The pectins didn’t influence the average thickness of HA. Although the average area of cells covered with HA was enhanced by pectins, we didn’t observe any significant difference.
Figure 7. The glycolcalyx layer compositions of Caco-2 cells were modified after 5 days of exposure to pectins. Caco-2 cells were stimulated with different methylation degrees of pectins. These were pectins with DM7, DM55, and DM69 tested at 2 mg/ml for 5 days. Untreated Caco-2 cells served as controls. After incubation, we applied immunofluorescence to label the glycolcalyx layer components albumin, HS, and HA. Three dimensional (3D) images were taken with a Leica SP8 confocal laser microscope. The average thickness of albumin (A), HS (B), and HA (C) were quantified using Image J software of the 3D images. The average area of cells covered with albumin (D), HS (E), and HA (F) were measured with IMARIS software of the 3D images. All data was expressed as mean with standard deviation from six replicates. Statistical significance was tested using One-way ANOVA (*p < 0.05, **p < 0.001).

After 5 days stimulation, the pectins all significantly enhanced the average thickness of albumin (p < 0.05, Figure 7A). However, the average area coverage of albumin was not influenced by the pectins. There were no effects on the development of HS and HA components.
4. Discussion

The intestinal epithelial glycocalyx is acknowledged for its function as initial adherence site for microbiota in infants and for its initial barrier function towards pathogen invasion and luminal toxins.\textsuperscript{11,12,31} Despite this pivotal function of the epithelial glycocalyx only minor information is available about the gut epithelial glycocalyx composition and possible effects of dietary components on the epithelial glycocalyx. Here we show, to the best of our knowledge for the first time, that hMOs as well as commonly used NDCs in infant formula (inulins and pectins) do impact the average thickness and average area coverage of the glycocalyx on intestinal epithelial cells.\textsuperscript{7,8}

The hMOs and NDCs investigated here do stimulate the development of the glycocalyx on intestinal epithelial cells. This might be a possible mechanism for the observed enhancing effects of hMOs and NDCs and its beneficial effects on gut microbiota that need the glycocalyx to adhere to the gut epithelium, with subsequent positive effects on fermentation products short chain fatty acids (SCFAs) and vitamins.\textsuperscript{32} Also, it might be a possible mechanistic explanation for the hMOs and NDCs induced enhanced gut barrier function.\textsuperscript{5,10,33} As shown in our current study the glycocalyx reinforcing effects of the tested food ingredients are due to direct effects on the epithelial cells and not induced by possible microbiota effects as all tests were performed in the absence of bacteria. Effects on the glycocalyx are dependent on the structure of the hMOs and NDCs as discussed below.

We show that the hMOs 2′-FL, 3-FL as well as inulins with DP3-DP10, DP10-DP60, and DP30-DP60 significantly increased the average thickness of adsorbed albumin on gut epithelial cells within 1 day of stimulation. These effects in albumin uptake in the glycocalyx are long lasting and still observed after 5 days. Pectins had less effects at day 1. Only after 5 days stimulation with DM7, DM55, and DM69 pectins, a significant effect on albumin adsorption was observed. An increase in albumin adsorption in the glycocalyx is considered to provide more stability of the glycocalyx as other components including HS and HA are better integrated into the glycocalyx structure by the adsorbed protein.\textsuperscript{14,24} Also enhanced albumin adsorption might contribute to the anti-pathogenic effects of the glycocalyx as it has been shown that a supplement of albumin results in less adhesion of e.g. \textit{Staphylococcus epidermidis}, \textit{Staphylococcus aureus}, \textit{Pseudomonas aeruginosa}.\textsuperscript{34,35} An enhanced
albumin adsorption also contributes to enhanced barrier function as it protects during volume resuscitation in hydroxyethyl starch solutions against adverse effects of hydroxyethyl starch on intestinal cells, metabolic functions, fluid shifts and epithelial barrier permeability. This suggests that the enhancing effects on albumin adsorption of hMOs and NDCs might not only strengthen the glycocalyx structure but also protect against pathogen adhesion and maintain under luminal changes the gut epithelial barrier.

HMO 3-FL but not 2'-FL had a thickness increasing effect on HS after 5 days of stimulation. Of the tested NDCs only the short chain inulin DP3-DP10 had such an effect on HS and significantly increased the average thickness of HS on both day 1 and 5. Even though only minor information is available on functional effects of HS enhancements on intestine epithelial cells, it has been shown for endothelial cells that a thicker HS layer provides stronger hydraulic resistance. HS and HA are the major components of glycosaminoglycan in the glycocalyx. Both of them can regulate mechanotransduction and maintain gut barrier integrity. Interestingly, just like with HS also HA in the glycocalyx was enhanced by 3-FL and not by 2'-FL after 1 day of stimulation. Of the NDCs tested only the long chain inulin DP30-60 enhanced HA on day 1. An enhanced HA expression is associated with a defense mechanism against intestinal injury and inflammation. In response to endoplasmic reticulum stress, HA accumulates to form cable-like structure which can serve as binding sites for leukocytes. Enhanced HA expression might also contribute to expedited signaling with gut microbiota, as HA binds to Toll-like receptors (TLR) 2 and 4, which are important receptors in regulation of host responses to both commensal and pathogenic bacteria within the gastrointestinal tract.

Thus, our data demonstrate that hMOs stimulate the glycocalyx development in a structure dependent manner. Even the structurally related 2'-FL and 3-FL had different effects in the glycocalyx. The 2'-FL hMO impacted the average thickness of the albumin layer at day 1 and 5 while 3-FL not only enhanced this average thickness of albumin but also improved the average area coverage of albumin after 1 day. This took more time with 2'-FL that needed 5 days to significantly increase the average area of albumin. The average area coverage of HS and HA were significantly enhanced by 3-FL on day 1, while there’s no effect after 5 days stimulation. The 3-FL also significantly increased the average thickness of HS, but no effects were observed with 2'-FL. Such a large difference in effect is rather surprising
as 2'-FL and 3-FL share the same core structure and only differ in that the L-fucose of 2'-FL is fucosylated to galactose, while the L-fucose of 3-FL is fucosylated to glucose. Our data suggest that this difference in fucosylation site has a significant impact on the glycocalyx synthesis machinery in epithelial cells.

The effects of inulins on the epithelial glycocalyx were similar to the effect of 3-FL. All three tested inulin types with DPs of DP3-DP10, DP10-DP60, and DP30-DP60 enhanced the average thickness of the albumin layer after 1 day and 5 days stimulation. The short chain DP3-DP10 inulin that is commonly applied in infant formula already increased the average thickness of HS within 1 day. Interestingly, DP30-DP60 inulin selectively increased the average area coverage of HA within 1 day. All these data suggest that inulins have a structure dependent effect on the compositions and development of the glycocalyx. Since the enhanced development of the intestinal epithelial glycocalyx layer is associated with expedited colonization with commensal microorganisms, our results suggest that short chain inulins might be instrumental for this effects and might be further supported with application of long chain inulins as this increases the average area coverage of HA.

The effects were different with pectins than with hMOs and inulins and effects were DM dependent. Only DM7 pectin significantly enhanced the average area coverage of albumin after 1 day of stimulation. After longer-term exposure, i.e. 5 days to DM7, DM55, and DM69 pectins a significant increased average thickness of albumin was observed. There was a tendency of pectins to increase the development of HS after 1 day, but this never reached statistical significance. Our observations indicate that the pectins can modulate protein adsorption but has only a minor impact on the carbohydrate components of the glycocalyx layer.

In summary, here we show that 2'-FL and 3-FL, and NDCs including inulins and pectins are able to stimulate maturation of the glycocalyx of intestine epithelial cells in a structure dependent fashion. Especially hMOs and inulins have such an effect while pectins were less effective. As enhanced maturation expedites colonization of the infant intestine with microbiota, our data suggest that hMOs and inulins seem both to be effective for glycocalyx development. Our data also contributes to a better understanding of how hMOs and dietary fibers contribute to a healthier gut in infants. It shows that it not only is beneficial for microbiota by serving as carbohydrate source but also directly stimulates maturation of the intestinal anchoring points for bacteria, i.e. the glycocalyx.
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