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ARTICLE

Mimicking Human Milk Functions: The Human Milk Oligosaccharide Building Block GlcNAc in Combination with GOS Protects the Intestinal Barrier

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The intestinal barrier plays a crucial role in infant health by regulating nutrient absorption and limiting exposure to pathogens. Its development is rapid and stimulated by compounds like hMOs found in breastmilk. However, where breastfeeding is not possible, infants rely on infant formula which generally lacks hMOs. hMOs are expensive and difficult to synthesize and instead infant formula is often supplemented with other non-digestible carboydrates such as GOS. In contrast, the building blocks that compose hMOs are more easily accessible. In this study, we investigated whether the building blocks GlcNAc and Neu5Ac in combination with the non-digestible carbohydrate GOS can protect the intestinal barrier function. An in vitro model using T84 intestinal epithelial cells exposed to the disruptors A23187 and Tunicamycin after a pre-treatment with GlcNAc or Neu5Ac and/or GOS was used. Our findings demonstrated that GlcNAc in combination with GOS was able to modulate the intestinal barrier function, supporting their potential use in promoting barrier development in early life while also being economical and sustainable.

Introduction

Exclusive breastfeeding is recommended for all infants by the WHO, especially for the first six months of life [1]. These first months of an infant's life are characterized by rapid growth and development and require a solid source of nutrition to support these changes. Naturally, breast milk provides infants with balanced nutrients and various bioactive ingredients, one of which are human milk oligosaccharides (hMOs) [2]. hMOs are a type of non-digestible carbohydrate (NDC) that has been found to steer many health related processes [3,4]. Over 200 different types of hMOs have been discovered to date, and they make up the third most abundant component in breastmilk [5]. All hMOs are composed of a mix of monosaccharide building blocks namely glucose, galactose, N-Acetylglucosamine (GlcNAc), fucose, and sialic acid (Neu5Ac) [6]. The diverse combinations and linkages between these building blocks give rise to the wide array of hMOs with varying structures and functional complexities.

One of the most abundant hMO found in breastmilk is 2'-fucosyllactose (2'FL) [7]. It is composed of fucose, galactose and glucose and one of the most extensively studied hMO to date [8]. 2'FL has been found to support the development of a healthy gut microbiota, strengthen the intestinal barrier, modulate immune responses, and protect against pathogen infections in early life [7]. However, infants who are not breastfed rely on infant formula to meet their nutritional needs, which often do not contain more than a few hMOs. To ameliorate this, they are supplemented with other oligosaccharides, such as galacto-oligosaccharides (GOS) [9] that also provide infants with health benefits such as promoting growth of beneficial gut bacteria [10]. Although in recent years the large-scale productions of a few hMOs, including 2'-FL, have been made possible, they remain expensive to synthesize [11]. This limits the accessibility of hMO supplemented formulas. For that reason, the search for NDCs supporting infant development in a more cost-effective manner continues.

One of the main targets of hMOs is the regulation of the intestinal barrier. The intestinal barrier develops quickly after birth as it is not only crucial for the digestion and the uptake of nutrients, but also has a critical role in maintaining immune homeostasis [12]. This barrier is composed of several layers, including the epithelial layer, which contains tight junctions to limit the paracellular transport of bacteria and/or their products into systemic circulation [13]. Regulation of molecule transport across the barrier is crucial to ensure efficient nutrient uptake and the controlled delivery of macromolecules to the immune system, while preserving barrier integrity [14]. This regulation is influenced both by endogenous stimuli as well as those from the external environment. Various stressors have been found to decrease the transepithelial electrical resistance (TEER) through various pathways [15]. This leads to the disruption of the distribution of tight junction

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proteins such as zonula occludens-1 (ZO-1) [16]. These tight junction associated protein disruptions translate into an increase in intestinal permeability resulting in a loss of barrier function. In infants, this has been associated with increased risk of developing diseases such as food allergy, inflammatory bowel disease and irritable bowel syndrome [17].

Among the individual building blocks of human milk oligosaccharides, GlcNAc and Neu5Ac stand out as two critical structural components that define both the architecture and biological function of many complex hMOs. GlcNAc forms the backbone of numerous oligosaccharides and participates in N- and O-linked glycosylation [18,19], which are essential for the synthesis of larger glycans that interact with epithelial and immune receptors in the gut. Its biological relevance extends beyond structural assembly, as GlcNAc-containing glycans have been implicated in supporting epithelial differentiation [20], maintaining barrier integrity [21], and modulating innate immune signaling [22]. Neu5Ac, on the other hand, is typically found as a terminal sugar on hMOs and confers negative charge, influencing microbial adhesion, immune recognition, and mucosal protection [23]. Sialylated oligosaccharides are known to serve as decoy receptors for pathogens, modulate immune cell signaling, and contribute to brain development through their role in ganglioside synthesis [24]. To approximate the natural complexity of breastmilk, we combined these building blocks with GOS, the most widely used prebiotic substitute in infant formulas. GOS are safe, cost-effective, and promote the growth of beneficial gut bacteria [25], yet they lack the structural diversity and receptor-binding potential of hMOs [26]. The rationale for combining GlcNAc and Neu5Ac with GOS lies in the possibility to bridge this functional gap, creating carbohydrate mixtures that not only stimulate a healthy microbiota but also mimic key epithelial and immune interactions characteristic of human milk. Such combinations may offer a practical and biologically meaningful strategy to enrich infant formula with bioactive carbohydrate structures that better support gut maturation, immune balance, and resilience in early life when breastfeeding is not possible.

The aim of this study was to explore whether individual hMO building blocks, when combined with established substitutes such as with GOS or 2'FL, could reproduce some of the beneficial effects of full-length hMOs on intestinal barrier function. We specifically focused on GlcNAc and Neu5Ac as key structural units of hMOs and examined their interaction with GOS, which are commonly incorporated in infant formulas. Using T84 epithelial monolayers, we evaluated how these building blocks and their mixtures modulate transepithelial electrical resistance, tight-junction gene expression, and inflammatory responses under controlled barrier disruption. By integrating these mechanistic readouts, this work aimed to identify whether simplified oligosaccharide structures, alone or in combination, could be a step closer to mimicking the barrier-protective actions of complex hMOs. Ultimately, this approach contributes to the broader goal of developing more affordable, functionally enriched infant formulas that better approximate the biological functions of human milk for infants who cannot be breastfed.

Materials and Methods

hMOs, building blocks, and GOS

2'-FL and Vivinal GOS used in this study was provided by FrieslandCampina (Amersfoort, the Netherlands). GlcNAc and Neu5Ac (Sigma-Aldrich, Zwijndrecht, The Netherlands) were used as hMO building blocks in this study. An overview of the structures is found in Table 1. All NDCs were dissolved in cell culture medium at a concentration of 2 mg/mL for use during the experiments, calculated using total dry weight while mixtures of hMO building blocks (GlcNAc and Neu5Ac) and GOS were prepared at a 1:4 ratio of dissolved NDCs, as established in earlier studies[27].

The concentration of 2 mg/mL used in this study was selected because it falls within the physiological range of 2'FL concentrations reported in human milk[28], as well as based on our prior experience with intestinal cell models. During the development of several studies using these systems, we consistently observed substantial biological responses within the concentration range of 0.5–2 mg/mL, whereas responses tended to plateau at higher concentrations. Therefore, 2 mg/mL was chosen as a biologically active yet physiologically relevant concentration that has previously yielded optimal responses in similar experimental settings[29,30,31,32].

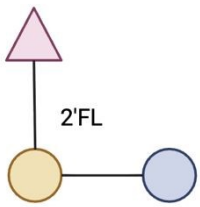
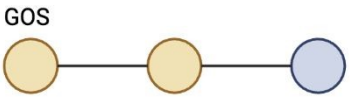
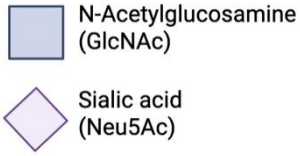
NDC	Structure
2'-fucosyllactose (2'-FL)	
Galacto-oligosaccharide (GOS)	
Building blocks	

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Table 1: NDC structures used in this study

Cell culture

The T84 human colorectal carcinoma cell line (Sigma-Aldrich, Zwijndrecht, The Netherlands) between passages 8-16 were used in this study. T84 cells were cultured at 37 °C with 5% CO_2 and grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Gibco, Grand Island, NY USA) supplemented with 10% heat-deactivated fetal bovine serum (Serana Europe GmbH, Pessin, Germany), 15 mM HEPES buffer solution (Gibco, Paisley, UK) and 60 µg/ml gentamicin solution (Capricorn Scientific GmbH, Ebsdorfergrund, Germany). The culture medium was refreshed every other day. Upon reaching 80% confluency, the cells were passaged after being treated with 0.1% trypsin-EDTA (Gibco, Grand Island, NY USA).

Trans-epithelial electrical resistance (TEER) measurement

Plates containing 96 gold electrode plated wells (96W20idf PET, Applied Biophysics) were treated with 300 µL/well of a 2 mg/mL solution of L-cysteine (Merck, Darmstadt, Germany) dissolved in PBS for 30 minutes at room temperature. The plates were then washed with Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate (Gibco, Grand Island, NY USA). The plates were then left to coat overnight at room temperature with 300 µL per well 1% type I bovine collagen (PureCol®, Advanced BioMatrix, San Diego, CA USA) and 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO USA) in DMEM. Plates were then washed twice with complete medium, and T84 cells were seeded at a density of 10,000 cells per well at a final culture volume of 300 µL per well. The plates were then cultured at 37 °C with 5% CO_2 for 21 days to form a confluent monolayer with a stable TEER. Until the cells reached confluency, the culture medium was changed every other day.

Once the cells reached confluency, the TEER of the T84 monolayer was continuously measured in a real-time electric cell-substrate impedance sensing system (ECIS, Applied BioPhysics TM model Zθ, Troy, NY USA) at a frequency of 400Hz, reflecting tight junction resistance. Before experimental conditions were added, the plates were placed in the ECIS reader, and the resistance was monitored overnight to confirm stability.

During the experiment, the T84 cells were pre-incubated with specific hMOs, specific hMO building blocks, and GOS at a concentration of 2mg/mL. Where a combination of a building block and GOS was added, this was done at a 1:4 ratio (1 building block/2 FL : 4 GOS) established in earlier studies, while maintaining a concentration of 2mg/mL. The cells were pre-incubated for 24 hours, after which they were exposed to a disruptor. Cells were treated with either calcimycin (A23187, Sigma-Aldrich, Germany) at a concentration of 4µM, or Tunicamycin (TUN, Sigma-Aldrich, Germany) at 10µM. These concentrations were based on previous studies[33] and validated in a dose-response curve (see Figure S1, ESI†). TEER measurements were performed for another 24 h.

To analyze the protective effects of the treatments, the TEER values were analyzed from the moment of exposure to A23187. The potential protective effects of the treatments used were determined by calculating the area under the curve (AUC). Each experiment had 3 technical replicates and was performed at least 6 times.

RNA extraction, cDNA synthesis, and quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

At the end of the TEER measurements, the supernatant was removed from the 96 well ECIS plates, leaving the T84 monolayer. The wells were washed with cold PBS and subsequently homogenized with TRIzol reagent (Invitrogen, USA). Total RNA was isolated according to the manufacturer's instructions and was quantified using a Nanophotometer N60 (Implen, Germany). Subsequently, cDNA was synthesized using DNase Treatment and RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, USA) according to manufacturer's instructions. cDNA samples were stored at -20°C before use.

RT-qPCR was performed in 384-well PCR plates with the ViiA7 Real-Time PCR system (Applied Biosystems, Foster City, CA USA). Primer sequences used for this study are listed in Table 2. Gene expression was quantified with FastStart Universal SYBR® Green Master Mix (Roche Diagnostics GmbH, Germany). The expression levels of target genes were normalized to the housekeeping gene *GAPDH*. The fold changes in gene expression of each group were determined through $2^{-\Delta\Delta CT}$.

Primer	Forward (5' → 3')	Reverse (5' → 3')
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
Occludin (OCLN)	CTATAAATCCACGCCGGTTC	TATTCCTGTAGGCCAGTGTC
Claudin-1 (CLDN-1)	GATGAGGTGCAGAAGATGAG	GGACAGGAACAGCAAAGTAG
Claudin-2 (CLDN-2)	CAGTGCAATCTCCTCCCTGG	GTCTTTGGCTCGGGATTCCT

Claudin-3 (CLDN-3)	CTGCTCTGCTGCTC GTGTC	CGTAGTCCTTGCGG TCGTAG
Claudin-4 (CLDN-4)	GCTGCTTTGCTGCA ACTGTC	CTTGGCGGAGTAA GGCTTGT
Zonula occludens-1 (ZO-1)	TGCCTCTGAGAGA GACGACA	TCTCTACTCCGGAG ACTGCC

Table 2: Primer sequences used for RT-qPCR

ELISA

After the TEER measurements, interleukin-8 production of the T84 cells was measured in the supernatant. This was done using a Human IL-8/CXCL8 DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions. Samples were diluted 20x for all measurements.

Statistical analysis

GraphPad Prism version 10.6.0 was used for data analysis. Normal distribution of data was examined by the Shapiro-Wilk test, which concluded the data is normally distributed. Data are represented as the mean ± standard error of the mean (SEM). Statistical significance was determined through one-way ANOVA with Holm-Šídák's multiple comparison test. Significance was defined as p-value ≤ 0.05. # or * ($p \leq 0.05$), ## or ** ($p \leq 0.01$), ### or ***($p \leq 0.001$), #### or **** ($p \leq 0.0001$). A priori comparisons between medium control and positive control were performed using an unpaired t-test.

Results

GlcNAc in combination with GOS prevents calcium ionophore–induced barrier disruption in T84 epithelial cells

To determine the potential protective effect of hMO building blocks alone, and in combination with GOS, the impact of calcium ionophore (A23187) and tunicamycin (TUN) treatments on transepithelial electrical resistance (TEER) in differentiated T84 cell monolayers were initially evaluated. Additionally, we investigated if a 24-hour pre-incubation with hMO building blocks and GOS mixtures could reduce this barrier disruption.

Cells exposed to A23187 (4µM) had significantly reduced TEER by an average of 24.31% compared to untreated controls ($p \leq 0.001$) (Figure 1). A 24-hour pre-incubation with 2mg/mL of hMO building blocks alone did not mitigate this disruption. However, when combined with GOS, GlcNAc completely prevented the TEER decline and even produced a modest increase of approximately 3 percent relative to the untreated control (Figure 1). Though not statistically significant, GOS, Neu5Ac, and the combination of Neu5Ac and GOS did lessen the effect of the disruptor.

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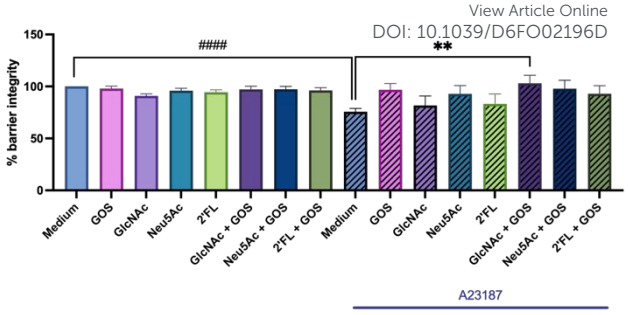

Figure 1: T84 cell monolayers were challenged with A23187 (4µM) after a 24 hour pre-incubation with hMO building blocks with/without GOS at a concentration of 2mg/mL. The figure shows the effects of this pre-treatment on barrier function through the area under the curve (AUC) of TEER data. AUC values are presented as means ± SEM. Statistical significance was determined using an ordinary one-way ANOVA with Šidák's post hoc test (** $p \leq 0.01$). Comparison between the A23187 control and untreated medium control was assessed using an unpaired t-test (#### $p \leq 0.0001$).

Cells exposed to TUN (10µM) also had significantly reduced TEER, by an average of 9.21% ($p \leq 0.05$) (Figure 2). Similarly to cells exposed with A23187, a 24-hour pre-incubation with 2mg/mL of hMO building blocks did not mitigate this disruption. However, this mitigation could also not be seen in mixtures with GOS. Thus, there does not seem to be a rescuing effect on the TEER of T84 cells when treated with TUN (Figure 2).

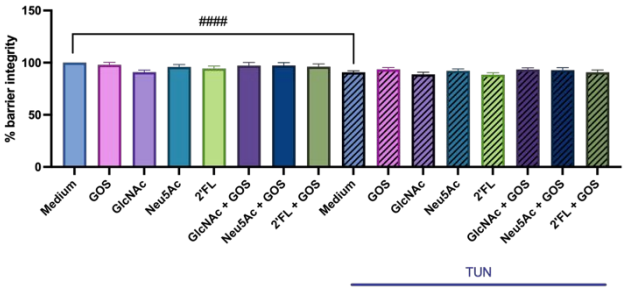

Figure 2: T84 cell monolayers were challenged with TUN (10µM) after a 24 hour pre-incubation with hMO building blocks with/without GOS at a concentration of 2mg/mL. The figure shows the effects of this pre-treatment on barrier function through the area under the curve (AUC) of TEER data. AUC values are presented as means ± SEM. Statistical significance was determined using an ordinary one-way ANOVA with Šidák's post hoc test. Comparison between the TUN control and untreated medium control was assessed using an unpaired t-test (#### $p \leq 0.0001$).

GlcNAc–GOS mixtures influence tight-junction gene regulation depending on disruptor type

Subsequently, we investigated how exposure to A23187 and TUN affects expression of tight junction genes when pre-treated with hMO building blocks and GOS. Claudin-1 (*CLDN1*), Claudin-2 (*CLDN2*), Claudin-3 (*CLDN3*),

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Claudin-4 (*CLDN4*), Occludin (*OCLN*), and Zonula occludens-1 (*ZO-1*) were investigated as markers of tight junction formation and integrity.

When treated only with hMO building blocks and GOS, significant changes in *CLDN2* expression were observed (Figure 3). The mixture of GlcNAc + GOS reduced gene expression 0.48-fold ($p \leq 0.01$). The mixture of Neu5Ac + GOS reduced gene expression 0.37-fold ($p \leq 0.001$). The mixture of 2FL + GOS reduced gene expression 0.42-fold ($p \leq 0.001$). Interestingly, this effect cannot be observed when looking at the individual components, only as a mixture with GOS (Figure 3). However, no statistically significant changes were observed in expression of other genes investigated.

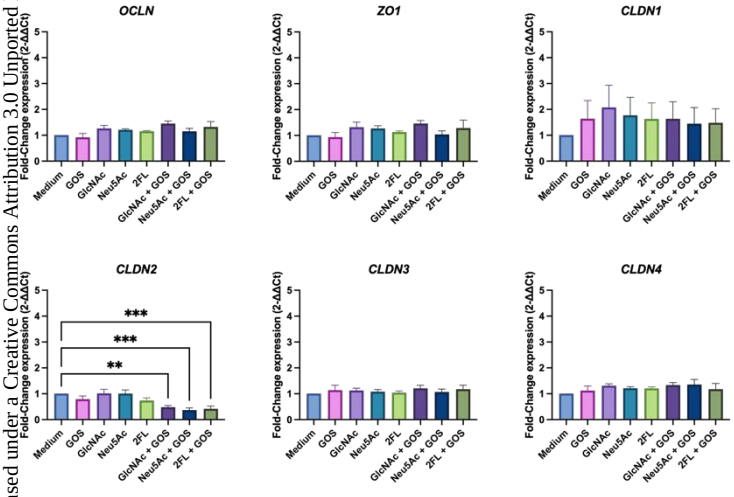

Figure 3: Changes in gene expression after incubation with hMO building blocks and/or GOS. Expression of *CLDN1*, *CLDN2*, *CLDN3*, *CLDN4*, *OCLN*, and *ZO1* in T84 cells. Data are normalized to the housekeeping gene *GAPDH* and expressed as fold change using the 2^−ΔΔCt method. Values are presented as means ± SEM. Statistical significance was determined using an ordinary one-way ANOVA with Šidák's post hoc test (** $p \leq 0.01$, *** $p \leq 0.001$).

When treated with A23187, there are significant differences in fold change expression of *OCLN*, *ZO1*, *CLDN2*, and *CLDN3* (Figure 4). Additionally, pretreatment with NDCs resulted in changes of *CLDN3* expression compared to the disruptor control. GlcNAc and Neu5Ac increased expression of *CLDN3* 2- and 2.18-fold respectively ($p \leq 0.05$, $p \leq 0.01$) (Figure 4). When treated with TUN, fold change expression was significantly increased in *OCLN*, *ZO1*, *CLDN1*, and decreased in *CLDN2* (Figure 5). The combinations of GlcNAc and GOS and Neu5Ac and GOS increased the expression of *OCLN* and *ZO1* compared to the disruptor control, however, this did not reach statistical significance.

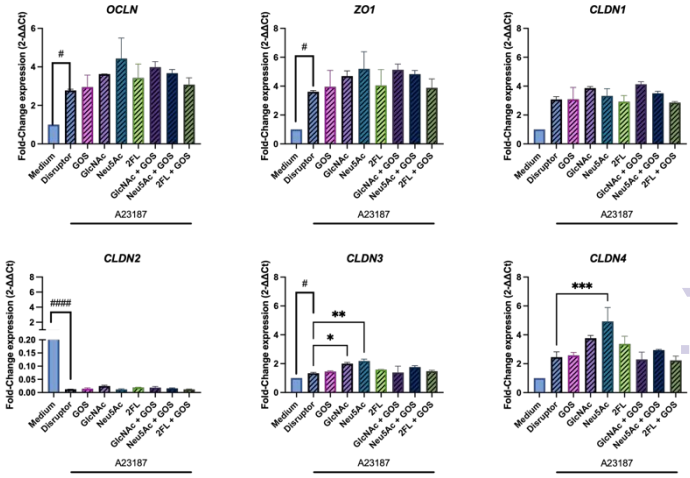

Figure 4: Changes in gene expression after pre-treatment with hMO building blocks and/or GOS and A23187. Expression of *CLDN1*, *CLDN2*, *CLDN3*, *CLDN4*, *OCLN*, and *ZO1* in T84 cells. Data are normalized to the housekeeping gene *GAPDH* and expressed as fold change using the 2^−ΔΔCt method. Values are presented as means ± SEM. Statistical significance was determined using an ordinary one-way ANOVA with Šidák's post hoc test. Comparisons between the A23187 control and medium control were evaluated using an unpaired t-test (# $p \leq 0.05$, #### $p \leq 0.0001$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

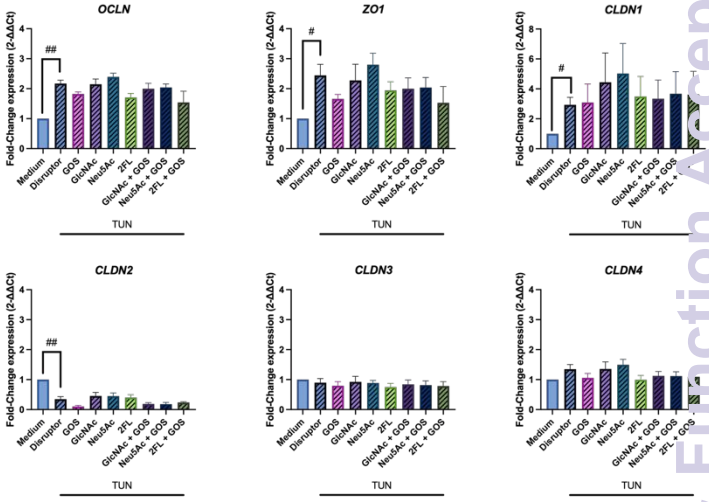

Figure 5: Changes in gene expression after pre-treatment with hMO building blocks and/or GOS and TUN. Expression of *CLDN1*, *CLDN2*, *CLDN3*, *CLDN4*, *OCLN*, and *ZO1* in T84 cells. Data are normalized to the housekeeping gene *GAPDH* and expressed as fold change using the 2^−ΔΔCt method. Values are presented as means ± SEM. Statistical significance was determined using an ordinary one-way ANOVA with Šidák's post hoc test. Comparisons between the TUN control and medium control were evaluated using an unpaired t-test (# $p \leq 0.05$, ## $p \leq 0.01$).

IL-8 secretion of T84 cells after exposure to A23187 and TUN with and without pretreatment of hMO building blocks and GOS

Interleukin-8 (IL-8) secretion was measured to investigate the immune modulatory potential of hMO building blocks. The levels of IL-8 secreted are strongly stressor dependent, treatment with A23187 resulted in an IL-8 concentration on average 3.85 times that of TUN.

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Following treatment with A23187, Neu5Ac shows decreased IL-8 secretion in the supernatant compared to the disruptor control (Figure 6A). However, this decrease did not reach statistical significance. Treatment with TUN did not show significant effects of hMO building block with or without GOS (Figure 6B). Similarly, no effects were observed without a disruptor treatment (Figure 6A, 6B).

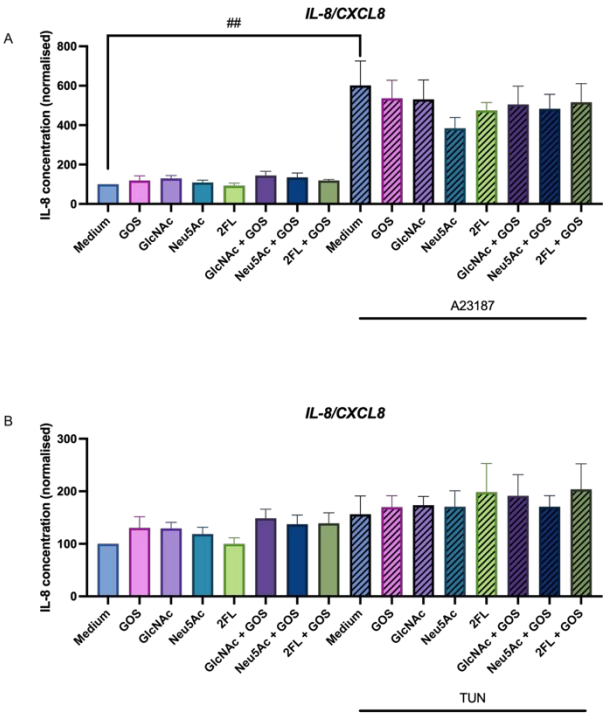

Figure 6: IL-8 secretion from T84 cells pre-treated with hMO building blocks with/without GOS and a disruptor. IL-8 concentrations in the supernatant of ECIS samples that were treated with A23187 (A) or TUN (B). Values are presented as means ± SEM. Statistical significance was determined using an ordinary one-way ANOVA with Šidák's post hoc test. Significance was tested between the disruptor controls and medium control using an unpaired t-test (## $p \leq 0.01$).

Discussion

Breastmilk remains the gold standard of infant nutrition [34], providing an optimal balance of nutrients and bioactive compounds that guide immune and intestinal development [35]. For infants who cannot be breastfed, infant formulas serve as an essential alternative, yet they still lack the diversity and biological activity of human milk oligosaccharides (hMOs). To move closer to mimicking the protective and modulatory functions of breastmilk, this study explored whether individual hMO building blocks could contribute to epithelial protection when combined with established prebiotics. Specifically, we examined *N*-acetylglucosamine (GlcNAc) and sialic acid (Neu5Ac), two abundant structural units of hMOs, and evaluated their effects, alone and in combination with galacto-oligosaccharides (GOS), on intestinal barrier integrity and gene regulation in T84 epithelial cells.

The choice of GlcNAc and Neu5Ac follows from the following reasoning. GlcNAc is a fundamental constituent of many hMOs and of intestinal glycoproteins such as mucins that form the first line of defense in the gut [36]. It has been associated with epithelial differentiation, maintenance of tight-junction integrity, and immune modulation through pattern recognition receptors [37]. Neu5Ac, a terminal residue in many sialylated hMOs, provides negative charge and plays a key role in preventing pathogen binding, modulating immune cell activation, and supporting neural development during early life [23]. Both molecules are thus not only structurally representative of hMOs but also biologically active at the host–microbe interface.

Our results show that both GlcNAc and Neu5Ac alone did not protect the epithelial barrier when exposed to disruptors such as the calcium ionophore (A23187) or the ER disruptor tunicamycin (TUN). However, when GlcNAc was combined with GOS, the mixture completely prevented the TEER decline induced by A23187, indicating a synergistic barrier-protective effect. GOS has been previously shown to decrease *CLDN2* expression in intestinal epithelial cells when treated with a stressor[38]. These observations were accompanied by selective modulation of tight-junction gene expression, particularly of *CLDN2* and *CLDN3*, suggesting that combinations of building blocks and GOS may influence epithelial junction dynamics in a disruptor-dependent manner. While the effects on cytokine secretion were limited, the combined data provide a clear indication that GlcNAc, especially when paired with GOS, can mitigate barrier stress through mechanisms likely involving calcium-regulated signaling and protein kinase C (PKC)–dependent tight-junction regulation [39]. Protein kinase C–dependent tight-junction dysregulation is a hallmark of A23187 induced barrier disruption [40].

GlcNAc and Neu5Ac represent highly attractive targets for inclusion in next-generation infant formulas as they are not only practical, but also sustainable. GlcNAc can be obtained from chitin, an abundant polysaccharide present in crustacean shells such as shrimp and crab, which are currently underutilized side-streams of the seafood industry [41]. Meanwhile, Neu5Ac can be derived enzymatically from glycoprotein-rich dairy or fermentation residues [42]. Valorizing these materials aligns with circular economy principles and reduces dependency on costly chemical or microbial synthesis of complex hMOs. Thus, developing formulations that combine inexpensive, bio-based sources of hMO building blocks with existing prebiotics like GOS offers not only functional but also environmental and economic benefits.

Taken together, our findings highlight that mimicking breastmilk functionality may not require reproducing entire hMO structures but rather capturing their key building block interactions. The observed disruptor-dependent protection by GlcNAc–GOS mixtures provides proof-of-concept that simplified, sustainable

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oligosaccharide combinations can exert meaningful biological effects on epithelial resilience. The protective effects of GlcNAc and Neu5Ac were tested on intestinal barrier integrity using T84 cells. T84 cells were exposed to disruptors calcium ionophore (A23187) or tunicamycin (TUN), which reduced transepithelial electrical resistance (TEER) indicating a compromised barrier. T84 cells were used in this study as they are well established for use in the ECIS system and have a stable colonic phenotype [43].

At a transcriptional level, significant changes of tight junction protein gene expression were observed. In cells that were not exposed to disruptors, significant decreases in *CLDN2* expression were observed. Mixtures of GOS with GlcNAc, Neu5Ac and 2FL all reduced *CLDN2* gene expression significantly. These effects were not observed without the addition of GOS. When treated with A23187, significant increases in gene expression of *OCLN*, *ZO1*, *CLDN2*, and *CLDN3* were observed. This disruption in *CLDN3* expression was mediated by pre-treatment with GlcNAc or Neu5Ac. No significant mediation was seen in gene expression for *OCLN*, *ZO1* or *CLDN2.* Upon treatment with TUN, changes in expression were observed in *OCLN*, *ZO1*, *CLDN1* and *CLDN2*. However, these changes were not mediated by any of the NDC pretreatments.

In some previous literature, a downregulation of TJ protein gene expression is observed contrary to our findings[44]. However, this is due to differences in experimental design. In models where cytokines are used as stressors, an inflammatory phenotype is often induced. This often leads to an increased expression of pro-inflammatory mediators and pore forming claudins (e.g. CLDN2) as well as decreased expression of TJ proteins[45]. This results in barrier disruption and increased permeability. However, calcium induced epithelial stress, like from A23187, disrupts barrier function primarily through cytoskeletal mechanisms while simultaneously inducing transcriptional programs aimed at restoring tight junction integrity through AMP-activated protein kinase[46].

The selective regulation of CLDN2 and CLDN3 may reflect their distinct physiological roles in gut barrier control. CLDN2 forms pore-like channels that increase paracellular ion permeability and is typically upregulated during inflammation or calcium imbalance [47,48]. A reduction in CLDN2 expression, as observed here, is generally associated with tightening of the epithelial barrier and restoration of ion selectivity. CLDN3, in contrast, is considered a sealing claudin that strengthens tight-junction integrity and limits paracellular leakage [49]. Its upregulation by GlcNAc and Neu5Ac under calcium-induced stress suggests that these molecules may support recovery of barrier cohesion through modulation of calcium-dependent signaling pathways such as PKC activation [50]. Additionally, these changes in CLDN2 and CLDN3 suggest these molecules can support maturation of the intestinal barrier, which is especially relevant in infancy.

In early life, the intestinal barrier is immature and has a higher baseline permeability. This is often associated with a higher expression of CLDN2 [47], while the maturation of the barrier is associated with increased CLDN3 [51]. However, these effects were not observed with Tunicamycin, further underlying that the regulation of CLDN2 and CLDN3 is closely linked to calcium signaling rather than to endoplasmic-reticulum stress or glycosylation inhibition.

Additionally, T84 cells, as well as other intestinal cells, express lectins such as galectins intracellularly and on the cell surface [52]. Galectins bind to carbohydrates containing β-galactoside links, otherwise known as terminal galactose residues [53]. Both 2'FL and GOS contain β-galactoside links, which may make them of interest to galectins. However, Neu5Ac and GlcNAc alone are not. In fact, Neu5Ac has been found to block galectin binding as it could bind a galactose residue and therefore prevent it from binding to galectins. Though not statistically significant, this trend can also be observed in the IL-8 secretion of cells pre-treated with Neu5Ac. Binding to galectins can further modulate the barrier by altering PCK activation – the mechanism through which A23187 induces barrier disruption [54]. hMOs like 2'FL have been found to prevent reactive oxygen species (ROS) accumulation [55], which would decrease protein kinase C activation and dampen downstream effects. Additionally, the prevention of PCK translocation or activation would not alter the effect of Tunicamycin as it targets the ER. These pathway specific changes in barrier function also suggest that targeted or tailored carbohydrate combinations may be more effective than a single universal solution for all infants.

Although the effects of individual hMO building blocks on epithelial barrier function appear limited, this finding remains highly informative. It highlights that NDCs act in a disruptor-dependent and context-specific manner rather than through uniform structural effects. This observation suggests that the biological activity of hMOs may rely not only on their individual monosaccharide components but also on their three-dimensional configuration, branching, and synergistic interactions within complex molecular networks. Of the more than 200 hMOs identified in human milk, only a handful, mostly short and simple structures, are currently included in infant formulas. Yet, evidence increasingly indicates that more structurally diverse and higher-order combinations are necessary to fully support intestinal and immune development in early life [56]. In this context, this study is, to our knowledge, the first to examine the functional potential of hMO building blocks in relation to intestinal barrier protection.

Our results provide an essential starting point to unravel how minimal oligosaccharide units such as GlcNAc and Neu5Ac might mimic or complement the effects of full-length hMOs when combined with accessible prebiotics like GOS. Subsequent work should focus on the potential dose effect of NDCs and protein-level validation of these findings to allow for stronger conclusions.

Currently, individual hMO building blocks have not been extensively studied in vivo. Additionally, the effects of hMO building blocks and GOS, as studied in this paper, on the microbiome are unknown and would need to be studied for potential future applications.

Future research should extend beyond epithelial models to include host–microbe co-cultures and in vivo systems that better capture the interplay between dietary glycans, microbial metabolism, and mucosal immune responses. These studies may reveal that even simple building blocks can exert profound effects once metabolized by commensal bacteria, generating secondary metabolites such as short-chain fatty acids that reinforce barrier and immune function. Exploring such integrative pathways will be key for translating these mechanistic insights into sustainable, affordable nutritional strategies that promote healthy gut and immune development in infants who cannot be breastfed.

Conclusions

To conclude, our findings suggest that there is promise to using combinations of hMO building blocks and other NDCs to help bridge the gap between breast milk and infant formula. By capturing critical functional interactions through various building blocks and combining them with established prebiotics, infant formula may guide infant development in a manner more similar to that of breast milk. This study focuses on the direct effects that hMOs and other NDCs can exert on intestinal epithelial cells and serves as a starting point for understanding how NDCs may influence these effects. Further understanding of how different NDC combinations may directly and indirectly influence the intestinal barrier as well as the microbiota may offer the possibility for more personalized formulations. As an added benefit, some hMO building blocks can be obtained from industry byproducts, supporting sustainable and affordable functional nutrition for infants.

Author Contributions

S.K and P.D.V. designed the study. S.K performed the experiments and data analysis. P.D.V supervised and administered the project. The manuscript was written by both S.K and P.D.V.

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Disclosure Statement

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting the findings of this study are available within the article.

Notes and References

Electronic supplementary information (ESI) available

Supplementary figures and tables supporting this article are available in the ESI.

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Data Availability Statement

All data supporting the findings of this study are available within the article.