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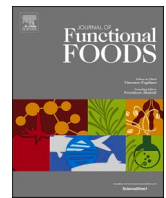
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2'-Fucosyllactose impacts the expression of mucus-related genes in goblet cells and maintains barrier function of gut epithelial cells

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ABSTRACT

Scope: Cost-effective microbial biosynthesis of 2'-fucosyllactose (2'-FL) allows its application in infant formula. The specific effects of 2'-FL on the gastrointestinal immune barrier are still largely unknown.

Methods/results: Here, we quantified and compared the effects of HMOs isolated from human milk, 2'-FL/lactose (Lac), and 2'-FL on the expression of the mucus associated genes *MUC2*, *TFF3*, *RET1B* and the Golgi sulfotransferases, *CHST5*, and *GAL3ST5*, in human goblet cells. We also determined whether these compounds have protective effects on A23187-induced barrier disruption of human T84 gut epithelial cells *in vitro*. The impact of isolated HMOs and 2'-FL/Lac on the mRNA expression of the mucus-related genes was minor while pure 2'-FL significantly induced *GAL3ST2* and *CHST5*. Isolated HMOs, 2'-FL/Lac and 2'-FL all prevented A23187-induced barrier disruption in human T84 cells.

Conclusion: Our findings indicate that 2'-FL modulates the secretory function of goblet cells and protects gut epithelial cells.

1. Introduction

The WHO recommends mothers to exclusively breastfeed infants for the first six months to reach optimal growth, development, and health (Kramer & Kakuma, 2012). Human milk (HM) contains human milk oligosaccharides (HMOs) that serve as a source of nutritional and bioactive components that contribute to the maintenance of the immune response by hindering pathogen adhesion in the intestine, and by modulating the epithelial response of intestinal cells and their production of cytokines (Triantis, Bode, & van Neerven, 2018; Cheng, Kong et al., 2019; Kong et al., 2019). These benefits of HMOs vary during lactation and are directly linked to changes in composition of HMOs which is suggested to be adapted to the needs of the offspring during lactation (Ballard & Morrow, 2013). At least 162 oligosaccharides found in human milk have been characterized (Urashima, Hirabayashi, Sato, &

Kobata, 2018). Based on the charge conferred by the type of monosaccharides that forms the HMOs, they are categorized as neutral (fucosylated and non-fucosylated) and acidic (sialylated) oligosaccharides. Neutral fucosylated HMOs constitute 35–50% and non-fucosylated constitute 42–55%, whereas acidic HMOs contribute to 12–14% of the total amount of oligosaccharides in milk (Smilowitz, Lebrilla, Mills, German, & Freeman, 2014).

When breastfeeding is not possible, infants receive cow milk-based infant formulas that largely lack HMOs (Zivkovic and Barile, 2011). To mimic the functionalities of HMOs, cow milk formulas are supplemented with other oligosaccharides (Borewicz et al., 2019). Up to now this has been achieved by the addition of non-digestible carbohydrates (NDCs), such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) (Matsuki et al., 2016; Skórka, Pieścik-Lech, Kołodziej, & Szajewska, 2018). However, this has recently changed as

Abbreviations: 2'-fucosyllactose, 2'-FL; *CHST5*, Carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 5; FOS, Fructo-oligosaccharides; *GAL3ST2*, Galactose-3-O-sulfotransferase 2; GOS, Galacto-oligosaccharides; HMOs, Human milk oligosaccharides; IECs, Intestinal epithelial cells; Lac, Lactose; *MUC2*, Mucin2; NDCs, Non-digestible carbohydrates; *RET1B*, Resistin-like molecule beta; TEER, Transepithelial electrical resistance; *TFF3*, Trefoil factor 3; TLR, Toll-like receptor.

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the development of fermentation-based manufacturing methods has enabled large-scale production of HMOs, such as 2'-fucosyllactose (2'-FL) (Hegar et al., 2019). 2'-FL is the most abundant neutral oligosaccharide. It constitutes almost 30% of the HMOs in breast milk (Vandenplas et al., 2018). 2'-FL is not primarily absorbed or metabolized by the infant and it can reach the lower intestinal tract intact (Bode, 2015). Although recent studies have shown that 2'-FL has many beneficial effects, such as the support of development of the glycocalyx (Kong et al., 2019), immune modulation via Toll-like receptors (TLRs) (Cheng, Kiewiet et al., 2019), and rescuing effects from endoplasmic reticulum stress induced by tunicamycin and IL-13 (Cheng, Kong et al., 2019), it is unknown how commercially available 2'-FL preparations that in some cases contain lactose (Lac) impact gut barrier function during homeostasis and during exposure to stressors.

The gut barrier has to develop early after birth as it is the gate-keeper between the content of the gut lumen and the host (Bering, 2018; Figueroa-Lozano & de Vos, 2019). The intestinal barrier is a multi-layer system. One of these layers is formed by the mucus secreted by goblet cells. Another layer is represented by the intestinal epithelial cells (IECs) which are tightly connected by tight junctions and desmosomes (Okumura & Takeda, 2017). The mucus layer is predominantly composed of mucin2 (*MUC2*) which is a gel-forming glycoprotein produced by intestinal goblet cells (Kim & Ho, 2010). The barrier function of the mucus layer is also regulated by other goblet cell proteins, such as the trefoil-3-factor (*TFF3*) and, resistin-like molecule beta (*RELMβ*) encoded by the gene *RETNLB*. Although their exact role in regulating permeability of the mucus layer is unknown, they both maintain the gut barrier integrity and regulate susceptibility to colonic inflammation (Marchbank et al., 2001; Hogan et al., 2006). Besides these secretory products of goblet cells, proteins involved in the glycosylation and sulfotransferases responsible for the sulfation of mucin, including the *CHST5* and *GAL3ST2* (Croix, Bhatia, & Gaskins, 2011), are also crucial for the barrier function of mucus. Sulfation of mucin confers resistance to enzymatic digestion and induces additional negative charge to the mucin, which makes it more viscous. This is essential to prevent pathogen adhesion and to maintain the physical barrier that protects the underlying epithelium (Cornick, Tawiah, & Chadee, 2015).

Underneath the mucus layer, the intestinal epithelium is bound together by the tight junctions, which regulate paracellular transport of macromolecules, and are key for the integrity of the intestinal barrier (Wang et al., 2016). An undisrupted gut epithelial layer does not allow entry of large antigenic molecules and specific paracellular or transcellular transport systems are present to transport larger compounds based on their charge and size (Vancamelbeke & Vermeire, 2017). The regulation of tight junction structures is influenced by endogenous stimuli but is also affected by stressors from the intestinal lumen (Anderson & Van Itallie, 2009). These exogenous stressors may activate TLR-2, which subsequently activate protein kinase C (PKC) and mitogen-activated protein kinase pathways (Ulluwishewa et al., 2011). As a consequence, these stressors decrease the transepithelial electrical resistance (TEER) and disrupts the distribution of tight junction proteins such as zonula occludens-1 (*ZO-1*). This translates into an increase in intestinal permeability and thus loss of barrier function (Van Itallie, Fanning, Bridges, & Anderson, 2009). Loss of epithelial barrier function in infants has been associated with an increased risk for food allergy, type I diabetes, inflammatory bowel disease, and irritable bowel syndrome (Halpern & Denning, 2015).

Many NDCs applied in infant formula, such as GOS and FOS have been shown to positively impact gut barrier function by either enhancing TEER of IECs (Akbari et al., 2017; Vogt et al., 2014) or by enhancing mucus function and mucus production (Bhatia et al., 2015). It has been observed that 2'-FL can protect goblet cells from endoplasmic reticulum stress. It is, however, unknown whether 2'-FL sources have the same effects on the secretory function of goblet cells and/or influences intestinal epithelial permeability. Therefore, in this study, we compared the effects of commercially available pure 2'-FL and 2'-FL with Lac (2'-

FL/Lac) on the expression of essential genes for mucus barrier function, i.e. *MUC2*, *TFF3*, *RETNLB*, *CHST5*, and *GAL3ST2* (Bhatia et al., 2015; Barnett, Roy, McNabb, & Cookson, 2012) in human goblet cells. In addition, we studied the protective effects of these two 2'-FL sources on TEER in human gut epithelial cells. This was done in the presence and absence of the epithelial permeability disruptor A23187. Effects of 2'-FL on both, goblet cells and IECs gut epithelial cells, were compared to effects of a mixture of HMOs isolated from breast milk.

2. Materials and methods

2.1. LS174T cell culture and reagents

The human colorectal cancer cell line LS174T was obtained from American Type Culture Collection (ATCC) and maintained in eagle MEM (EMEM) medium (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Lonza, Verviers, Belgium), 60 µg/ml gentamicin sulfate (Lonza, Verviers, Belgium). Cells were cultured at 37 °C in 5% CO₂ as recommended by the manufacturer. Cells were incubated for 24 and 48 h with HMOs oligosaccharides isolated from human milk from a Secretor+, Lewis + (Se⁺Le⁺) donor provided by SvL. The 2'-FL/Lac mixture-2'-FL/Lac (25:75) was obtained as tester sticks of Mum's Sweet Secret™ from Jennewein Biotechnologie GmbH (Rheinbreitbach, Germany). Purified 2'-fucosyllactose (2'-FL) was produced and supplied by Glycosyn LLC (Woburn, MA, USA). Purified lactose (Lac) was used as a control sample to exclude effects of lactose present in the 2'-FL/Lac mixture.

2.2. Preparation of HMOs from a secretor (Se⁺) and Lewis (Le⁺) donor

The infant was born at term >37 weeks post menstrual. After weaning, milk was extracted with a breast pump and stored immediately at -20 °C at home. After transport to the laboratory, the milk was stored at -80 °C until use. It was defrosted at 4 °C overnight and samples were pooled yielding ~650 mL of mixed milk. The milk was obtained between 1- and 6-months post-partum, pooled and processed to isolate the HMOs. The milk was homogenized and centrifuged at 3000g, at 4 °C for 30 min. The fat layer was skimmed off with a spatula and the skimmed milk was applied in 25 mL batches to a 10 g column of Carbotrap® B 20-40 mesh graphitized carbon black (Sigma-Aldrich) in an XK 16/20 column (GE-Lifesciences), using an ÄTKA-FPLC (Pharmacia) coupled to a UV-detector with a 280 nm filter for detection of *N*-acetyl sugars. The column was preconditioned with 30 mL 80% acetonitrile: 0.1% TFA prior to each batch, followed by washing with 30 mL Milli-Q water containing 0.1% TFA and 4% acetonitrile: 0.1% TFA. The HMOs were eluted with 30 mL 40% acetonitrile: 0.1% TFA. After elution the samples were reduced under N₂ stream overnight to eliminate acetonitrile and TFA. The remaining water was removed by lyophilization.

2.3. LS174T cells treatment

LS174T cells were cultured as described by Ren et al. (Ren et al., 2018). Wells were rinsed with 1 mL of PBS and culture medium was replaced by 1 mL of fresh medium containing different treatments. LS174T cells were stimulated with these treatments for 24 and 48 h.

2.4. RNA extraction and quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

At the end of stimulation, LS174T cells were homogenized with TRIzol reagent (Life Technologies, Carlsbad, USA). Total RNA was isolated following the manufacturer's instructions, and was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, USA). qPCR was performed with primer and probe sets for different genes (*MUC2*, *TFF3*, *RETNLB*, *CHST5*, *GAL3ST2*, *GUSB*) provided by

Applied Biosystems (Foster City, USA) as previously described [9] and qPCR Mastermix Plus (Eurogentec, Seraing, Belgium). Reactions were carried out in 384-well PCR plates (Thermo Scientific, UK) using a ViiA7 Real-Time PCR System (Applied Biosystems), and threshold cycle (Ct) values were calculated by ViiA7 software. Expression levels of target genes were normalized to that of the housekeeping gene glucuronidase alpha (*GUSB*), which provides instruction for the production of β -glucuronidase was used as housekeeper. This gene has been shown to be suitable for expression analysis in LS174T cells (Ren et al., 2018; Bhatia et al., 2015). Comparative quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Croix et al., 2011).

2.5. T84 cell culture

The human colon carcinoma T84 (Sigma-Aldrich, The Netherlands) cells were cultured in medium consisting of 1:1 Ham's F-12 and DMEM (Gibco). The medium was supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (ThermoFisher Scientific, USA), Gibco® HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (ThermoFisher Scientific) and 50 mg/mL of gentamicin (Life Technologies Europe). Cells were maintained in T25 flasks at 37 °C in a humidified environment containing 5% CO₂ and 95% O₂. Cells were grown until they reached 80% of confluence. For cell passage, cells were treated with trypsin/EDTA.

2.6. ECIS

In order to measure the Trans Epithelial Electrical Resistance (TEER) of confluent monolayers of T84 cells, we used an electric substrate impedance sensing (ECIS) incubator (Z-Theta model, Applied Biophysics). T84 cells were grown in 96-well array plates with 20 electrodes per well (96W20idfPET, Ibidi via Applied Biophysics). Before cells were seeded, the 96-well plate was coated with 300 μ L/well of 2 mg/mL L-cysteine in PBS. Afterwards, the plate was incubated for 30 min at room temperature and washed with 300 μ L DMEM. The plate remained incubated overnight with a solution of 0.1% Bovine Serum Albumin (Sigma Aldrich, Germany) and 1% collagen (PuerCol bovine tail collagen, Nutacon, The Netherlands). After incubation, plates were washed twice with supplemented medium and T84 cells were seeded at 1×10^4 cells/well in 300 μ L medium. Medium was changed every other day and the cell culture maintained for 21 days before measurement.

At day 21 the TEER was measured at a frequency of 400 Hz, which reflects tight junction resistance (Wegener, Keese, & Giaever, 2000). A stabilization period of 6 h was used before measurements were started. Cells were incubated with the different treatments at different concentrations and after 24 h, the disruptor calcimycin (A23187, Sigma-Aldrich, Germany) was added at 4 μ M. Measurements were performed for another 24 h. Data shown represent 8 independent experiments with 3 technical replicates for each condition. To analyze the protective effects of the treatments, the TEER values were analyzed from the administration of A23187. The potential protective effects of the treatments used were determined by calculating the area under the curve.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7. The data was not normally distributed according to the D'Agostino & Pearson normality test. Changes in mRNA expression values are represented with median with interquartile range. The data is represented as fold-change of mRNA expression normalized to the housekeeper *GUSB* ($n = 2-4$). Statistical significance was analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Values of $p < 0.05$ were considered to be statistically significant.

For the ECIS experiments, the data sets passed the D'Agostino & Pearson normality test. The results are presented as means \pm SEM. Statistical comparisons were performed using One-way ANOVA

followed by Tukey's post-hoc test when multiple comparisons were performed. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

HMOs support maturation and maintain barrier function of the gastrointestinal tract (Donovan & Comstock, 2017). Both mucus and the integrity of the tight junctions between epithelial cells are determinants of gut barrier function (Capaldo, Powell, & Kalman, 2017). In order to assess the effects of 2'-FL on intestinal gut barrier function, we investigated the changes in expression of the mucus associated genes induced by 2'-FL in human goblet cells and the impact on TEER of human gut epithelial cells in the presence and absence of the stressor A23187. HMOs isolated from human milk from a Se⁺Le⁺ donor were investigated in order to evaluate the possible effect of a natural and complex source of 2'-FL. The effects of 2'-FL were studied by stimulation of LS174T cells with a mixture of 2'-FL/Lac (25:75), 2'-FL, and Lac as these compounds are used to enrich infant milk formulas.

3.1. Effects of 2'-FL on goblet cell function

Fig. 1 shows the impact of a mixture of HMOs derived from a human donor on the expression of mucus-associated genes in LS174T cells at 24 and 48 h of exposure. Non-treated cells served as control. No significant differences were observed between the mRNA expression of the non-treated cells and the cells stimulated with HMOs.

Incubation for 48 h with a mixture of 2'-FL/Lac at a concentration of 5 mg/mL (Fig. 2) induced ($p < 0.05$) expression of *MUC2*. The genes *TFF3*, *RETNLB*, *CHST5*, and *GAL3ST2* were also increased but did not reach statistical significance. When 2'-FL and Lac were studied as separated and pure compounds, it was observed that pure 2'-FL significantly induced ($p < 0.05$) the mRNA expression of *CHST5* and *GAL3ST2*. Although Lac at 3.75 mg/mL displayed inductive effects for these Golgi sulfotransferases, the differences were not statistically significant.

3.2. Protective effects of 2'-FL on epithelial barrier as quantified in ECIS

Next, we studied the impact of 2'-FL on the epithelial barrier in the presence and absence of A23187, a stressor that disrupts gut epithelial barrier function by increasing intracellular Ca²⁺ (González-Mariscal, Tapia, & Chamorro, 2008). T84 cells were incubated for 24 h with HMOs, 2'-FL/Lac, and 2'-FL. Afterwards, the barrier disruptor A23187 was added. The addition of A23187 induced a reduction of TEER to a minimum mean value of $85.6 \pm 6.7\%$ ($p < 0.0001$) compared to the non-treated control (Fig. 3).

As shown in Fig. 3, A23187-induced reduction of TEER can be prevented by pre-incubation with HMOs isolated from HM, 2'-FL/Lac, and 2'-FL. The HMOs pretreatment strongly reduced the A23187-induced barrier disruption. While A23187 exposure reduced TEER to $85.6\% \pm 6.7\%$ ($p < 0.0001$), pre-exposure to isolated HMOs rescued the barrier function as the reduction was limited to $98.7\% \pm 2.62\%$ ($p < 0.0001$) implying a very minor impact of A23187 (Fig. 3 A/B). 2'-FL/Lac had similar effects. Following preincubation with 2'-FL/Lac the A23187 barrier disruption was only $94.6\% \pm 3.04$ ($p < 0.0001$) instead of $85.6\% \pm 6.65\%$ ($p < 0.0001$) (Fig. 3C/D). Following preincubation with pure 2'-FL the A23187 induced TEER reduction was only $93.4\% \pm 2.56$ ($p < 0.0001$) (Fig. 3E/F). There were no statistically significant differences in the magnitude to which HMOs isolated from HM, 2'-FL/Lac, and 2'-FL rescued the A23187-induced TEER loss.

4. Discussion

In this study we evaluated the effects of 2'-FL in pure form and in combination with Lac. The effects of 2'-FL contained in a mixture of

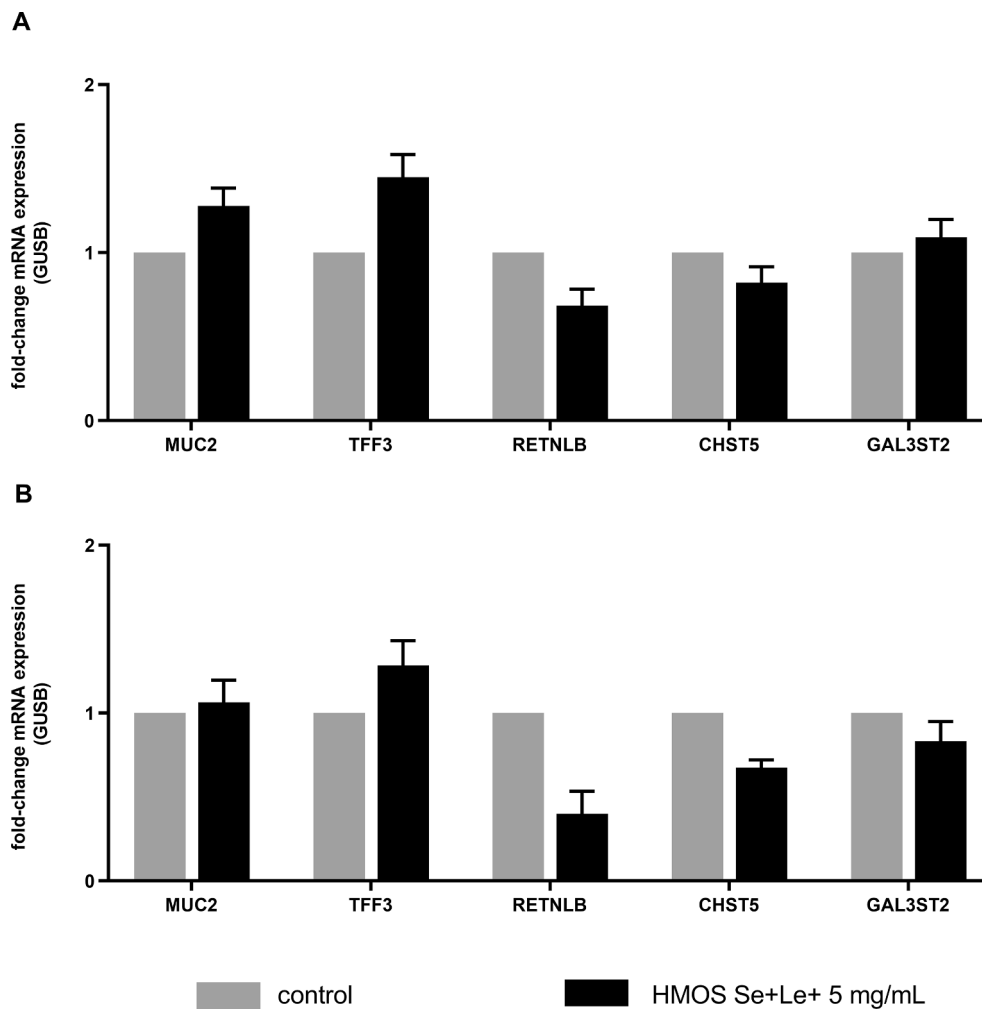


Fig. 1. Changes in the mRNA expression of the secretory proteins of goblet cells stimulated with HMOs isolated from human milk. LS174T cells were cultured for (A) 24 h or (B) 48 h with 5 mg/mL of HMOs. Values represent median with interquartile range. The data is represented as fold-change of mRNA expression normalized to the housekeeper *GUSB* ($n = 2-4$). Statistical significance was tested by using Kruskal-Wallis test followed by Dunn's multiple comparison test.

HMOs obtained from a Se^+Le^+ donor were also assessed. We show here that the two sources of 2'-FL impact the gut barrier function by preventing epithelial barrier disruption or by regulating specific mucus associated genes in goblet cells. We observed that the mixture of 2'-FL/Lac had upregulating effects on the mRNA expression of *MUC2*, *TFF3*, *RETNLB*, *CHST5*, and *GAL3ST2*. Lac addition can have additive effects as we observed only with the combination of 2'-FL/Lac *MUC2* upregulation. An enhanced *MUC2* expression was also observed with higher concentrations of pure Lac in absence of 2'-FL but this did not reach statistical significance. 2'-FL did have an effect on Golgi sulfotransferases, *CHST5* and *GAL3ST2* in goblet cells.

We choose to test HMOs isolated from a Se^+Le^+ milk donor because this is the most prevalent phenotype expressed in human milk (Tonon, de Moraes, Abrão, Miranda, & Moraes, 2019). Approximately 70–80% of human milk samples contain 2'-FL (Castanys-Muñoz et al., 2013). Secretor-milk contains higher amounts of oligosaccharides compared to non-secretor-milk (9.27 g/L vs 5.44 g/L, respectively) (Kunz et al., 2017). The amount of 2'-FL present in secretor-colostrum is around 3.99 g/L and 2.76 g/L in mature milk (Kunz et al., 2017). Our results showed that HMOs at 5 mg/mL, had no significant effects on the mRNA expression of *MUC2*, *TFF3*, *RETNLB*, *CHST5*, and *GAL3ST2* genes. Although HMOs have a beneficial impact on the intestinal barrier function, it is unclear whether this effect is microbiota-dependent (Cornick et al., 2015) or attributable to a given fraction of

oligosaccharides (Elwakiel et al., 2018).

We observed that HMOs isolated from HM did not alter the expression of *MUC2* and *TFF3* in goblet cells. However, Wu et al. observed that HMOs can induce the expression of *Muc2* and *Tff3* genes in mice with necrotic enterocolitis fed with HMOs (Wu et al., 2019). The protective effects of HMOs observed in this murine necrotic enterocolitis model was attributed to the ability of HMOs to restore the number of goblet cells in the intestine (Wu et al., 2019). Therefore, the absence of effects we see in human of goblet cells might be explained by species differences in response to HMOs between mouse and human as suggested before (Ermund, Gustafsson, Hansson, & Keita, 2013). However, the expression of mucus-related genes involved in inflammation has been suggested to be influenced by the presence of the acid fraction of HMOs that induces cell differentiation, whereas the neutral fraction does not exert such effect (Kuntz, Rudloff, & Kunz, 2008; Plaza-Díaz, Fontana, & Gil, 2018).

There are few studies about the effects of specific HMOs. For instance, the protection against NEC has been attributed to presence of disialyllacto-*N*-tetraose (Jantscher-Krenn et al., 2012; Autran, Schoterman, Jantscher-Krenn, Kamerling, & Bode, 2016). While isolated HMOs, such as 3'-fucosyllactose and lacto-*N*-triose II can induce mRNA expression of mucus-related genes under homeostatic conditions (Cheng, Kong et al., 2019), 2'-FL can induce the expression of *MUC2* after 72 h of exposure to TNF- α , IL-13, and tunicamycin (Cheng, Kong et al., 2019). Our findings indicate that 2'-FL affect the intestinal barrier

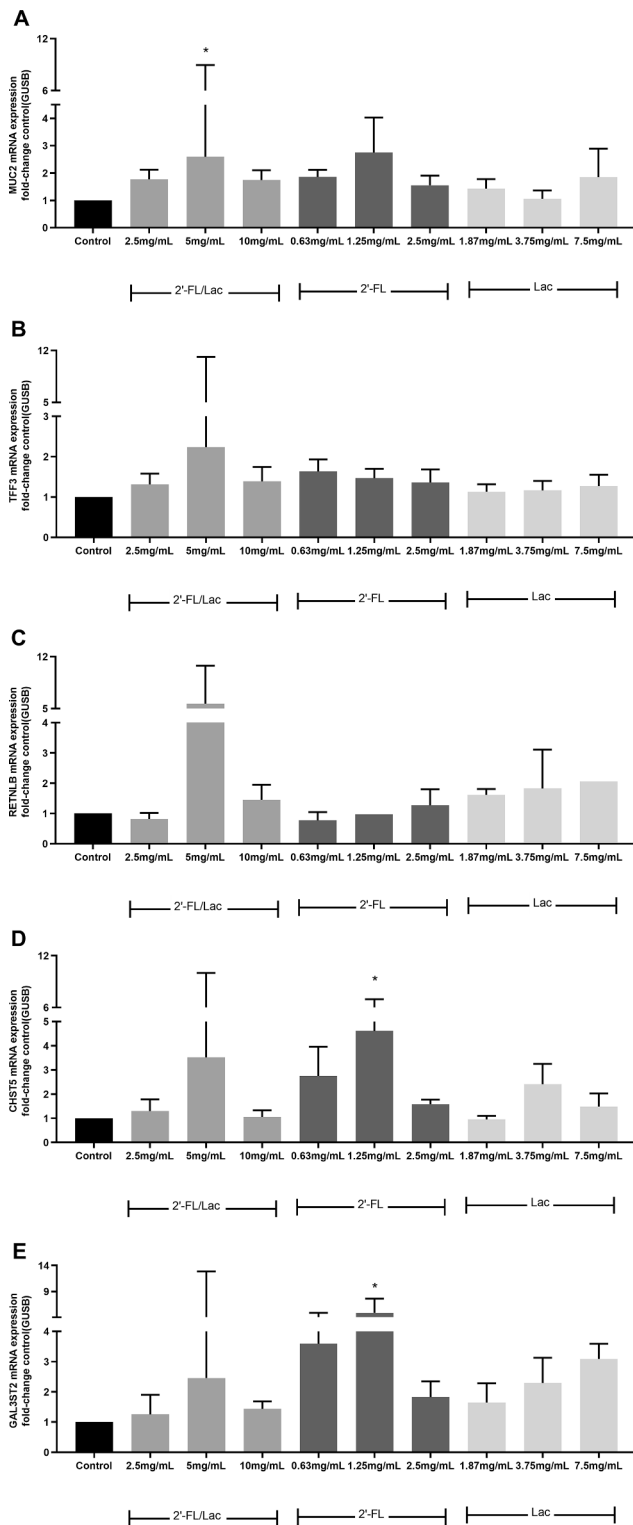


Fig. 2. Changes in the mRNA expression of the secretory proteins of goblet cells. The changes in the mRNA expression of (A) MUC2, (B) TFF3, (C) RETNLB, (D) CHST5, and (E) GAL3ST2 are presented. LS174T cells were cultured for 48 h with 2.5, 5, and 10 mg/mL of 2'-FL/Lac, 0.63, 1.25, 2.5 mg/mL of 2'-FL and 1.87, 3.75, and 7.5 mg/mL of Lac. Values represent median with interquartile range. The data is represented as fold-change of mRNA expression normalized to the housekeeper GUSB (n = 2-4). Statistical significance was tested by using Kruskal-Wallis test followed by Dunn's multiple comparison test. Significant differences compared to the control group (untreated cells) are indicated by * $p < 0.05$.

by promoting the mRNA expression of secretory products such as TFF3 and proteins involved in mucin sulfation after 48 h of incubation. These differences in gene activity have been described to occur due to the temporal dynamics in gene transcription (Yosef & Regev, 2011). In animal models, the overexpression of rat TFF3 resulted in increased resistance to ulceration, whereas mice with deleted TFF3 displayed higher susceptibility to gastrointestinal injury (Marchbank et al., 2001). TFF3 is not only essential for mucus integrity, it is also involved in the regulation of claudin-1 and Zo-1 in epithelial cells, which are proteins central to the formation of tight junctions (Zhou, Moodie, Blanchard, Leygue, & Myal, 2015) however it might in malignant cells also contribute metastatic processes. In the human epithelial cell lines, such as HT29/B6 and MDCK cells, TFF3 upregulates claudins in the tight junctions, enhancing the intestinal barrier (Meyer zum Büschenfelde et al., 2006). Upon recognition of pathogens via TLRs or nucleotide dimerization domain-like receptors, goblet cells increase the secretion of RELM β upregulating thus the expression of tight junctions in epithelial cells (Morampudi et al., 2016; Wells et al., 2017).

We compared a pure 2'-FL product, Lac and a mixture of 2'-FL/Lac. This was based on previous work from our group in which we demonstrated that Lac have a direct impact on the mucus associated genes in goblet cells (Figueroa-Lozano et al., 2020). Here, however, at the low Lac concentration used (7.5 mg/mL) we did not see an effect of this disaccharide on goblet cell function; in our previous study we only found an effect at a Lac concentration of 20 mg/mL (Figueroa-Lozano et al., 2020). Lac is added to infant formula as a source of energy (Martin, Ling, & Blackburn, 2016). Furthermore, Lac is involved in the absorption of calcium and it has been shown to induce antimicrobial peptides known as cathelicidins via p38 and JNK pathways in epithelial and monocytic cells (Cederlund et al., 2013). Our data suggest that Lac may have a supportive effect on 2'-FL induced changes in goblet cell function. Although we observed that the pure sample of 2'-FL had an inducing effect on the expression of CHST5 and GAL3ST2 at 1.25 mg/mL, this effect was not observed for the other three genes.

Although the impact of HMOs on the secretory function of goblet cells was minimal, the HMOs protected the intestinal barrier in T84 cells when exposed to the strong barrier disruptor A23187, which is an ionophore that increases intracellular Ca^{+} . A possible explanation for this observation is that some HMOs are known to stimulate TLRs. TLRs are involved in tight junction upregulation and enhancement of TEER (Podolsky, Gerken, Eyking, & Cario, 2009). Specifically, the activation of TLR-2 has been shown to directly enhance TEER via protein kinase C α/δ of enterocytes *in vitro* (Loegering & Lennartz, 2011). Oligosaccharides such as 3-fucosyllactose (3'-FL) and lacto-N-triose (LNT2) present in secretor-milk activate TLR-2 (Cheng, Kiewiet et al., 2019).

Also, both synthetic molecules, pure 2'-FL and 2'-FL/Lac prevented A23187-induced damage to the epithelial barrier function. TLR pathways have been suggested to be involved in the protection of the intestinal barrier against stressors. 2'-FL is reported to influence TLR-5 activity (Cheng, Kiewiet et al., 2019). TLR-5 has been shown to participate in the maintenance of gut permeability but the mechanism is unclear. Lower expression of TLR-5 is associated with low gut permeability in patients with ulcerative colitis (Stanislawowski et al., 2009) but the elevated expression of TLR-5 in animal models of Crohn's diseases ileitis is associated with inappropriate maintenance of the epithelial barrier integrity (Lopetuso et al., 2017). The protective effects of 2'-FL could also be partially explained by the induction of TFF3. TFF3 is involved in the modulation of the complex E-cadherin/catenin in the tight junctions. By regulating this complex this peptide promotes cell migration *in vitro* and healing of mucosal damage *in vivo* (Buda, Jepson, & Pignatelli, 2012).

TEER of the epithelial cells and the integrity of the mucus layer contribute to gut barrier function. However, they independently contribute to gut barrier function but some goblet cells products can support the epithelial barrier and the other way around (). The understanding of the epithelial barrier and its interplay with dietary

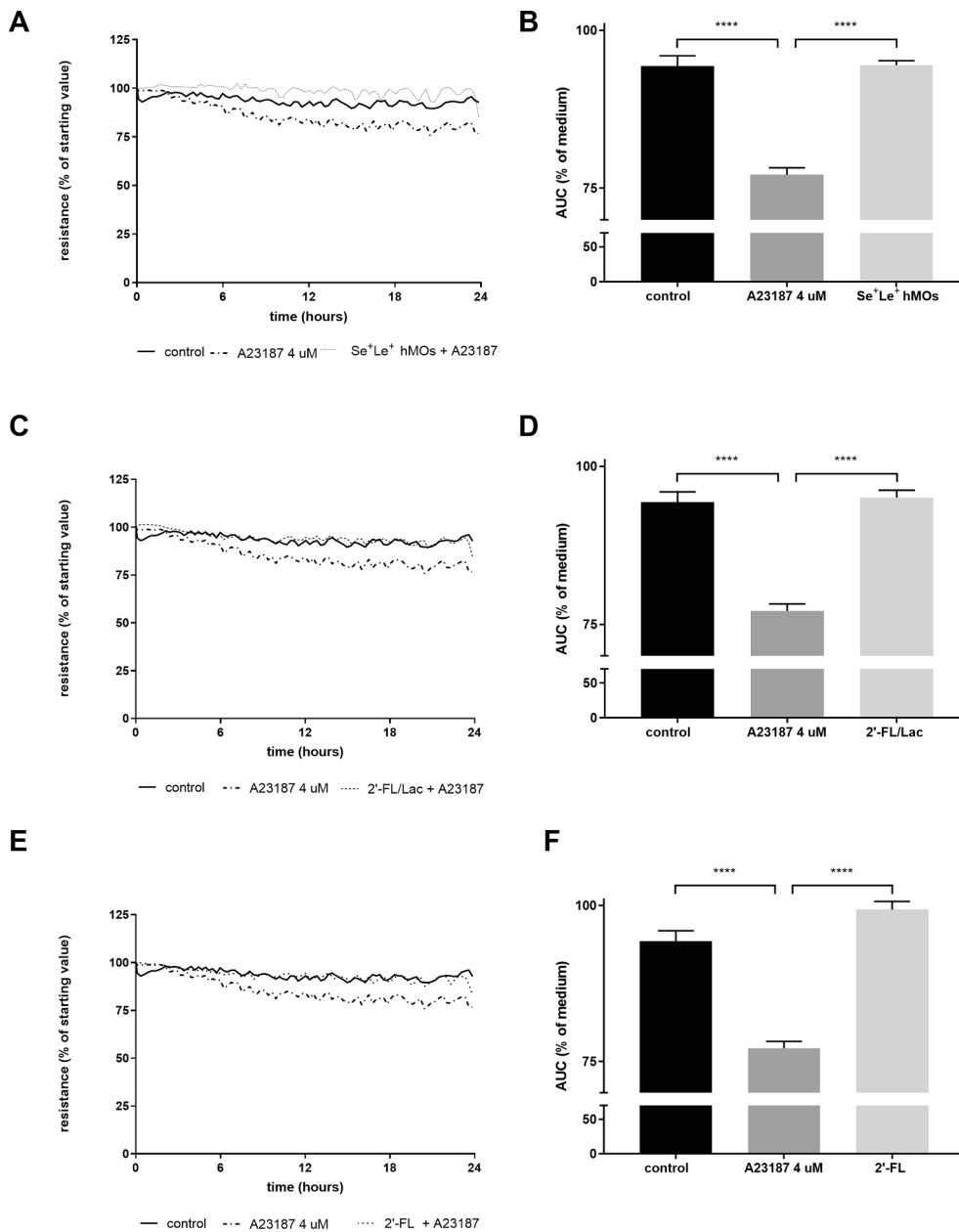


Fig. 3. HMOs, 2'-FL/Lac and 2'-FL suppressed A23187-induced barrier disruption in T84 cells. TEER was measured at different time points within a confluent layer of T84 cells. Cells were treated with 2 mg/mL of HMOs, 2'-FL/Lac, and 2'-FL for 24 h, after which the barrier disruptor A23187 (4 μ M) was added. Values represent means \pm SEM. Graphs A, C, E, show TEER values at different time points from the moment the disruptor was added. Graphs B, D, F, show the corresponding calculated AUC. AUC data are presented as percentage of the untreated control. Statistical significance was tested by using One-way ANOVA with Tukey's multiple comparisons test ($n = 4$). Significant differences compared to A23187 are indicated by (**** $p < 0.0001$).

components such as HMOs that are supplied in infant formula could provide means to prevent health issues in children, such as NEC, type I diabetes, and food allergy that are related to gut barrier dysfunction (Saleem et al., 2017; Sorini et al., 2019; Yu, 2012; König et al., 2016). At the same time, we show that 2'-FL in the presence and absence of Lac has an impact on both mucus-associated genes in goblet cells and on gut epithelial barrier function, while in the presence of Lac the impact is mainly on epithelial barrier function. Although *in vitro* models have clear limitations, and more studies are required to confirm the effects of these compounds on infant health, our findings suggest that 2'-FL may strengthen gut barrier function by both impacting the mucus layer and TEER in gut epithelial cells.

5. Conclusion

Here we show that pure 2'-FL alone and in combination with Lac impacts the secretory function of goblet cells. On gut epithelial cells, 2'-FL, the mixture 2'-FL/Lac and the HMOs all conferred protective effects.

The addition of 2'-FL to infant formulas or products for infant nutrition has the potential to promote the development and maintenance of the gut intestinal barrier. These findings also open new venues to use 2'-FL for the management of diseases in adults, such as inflammatory bowel disease, irritable bowel syndrome, or celiac disease and to rescue the intestinal barrier from the adverse effects of non-steroidal drugs and proton-pump inhibitors, in which barrier disruption has been shown to play an essential role.

Author contributions

S.F.L designed the experiments. S.F.L, R.A, and M.B performed the cell-based experiments and S.F.L performed the data analysis. S.F.L and P.d.v outlined and wrote the manuscript. S.v.L, L.D, and P.d.v. critically reviewed the manuscript.

Ethics statement

This work did not include any human subjects or animal experiments.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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