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The impact of oligosaccharide content, glycosidic linkages and lactose content of galacto-oligosaccharides (GOS) on the expression of mucus-related genes in goblet cells†

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Galacto-oligosaccharides (GOS) have been reported to modulate the function of intestinal goblet cells and to improve mucus barrier function. However, GOS is available in many structurally different compositions and it is unknown how GOS structural diversity impacts this modulation of goblet cells. This study aims to investigate the effects of oligosaccharide content and glycosidic linkages of GOS on expression of genes associated with the secretory function of goblet cells. To investigate the effect of oligosaccharide content, LS174T cells were incubated with (β1 → 4)GOS of variable transgalactosylated oligosaccharides and lactose (Lac) composition. To investigate the effect of glycosidic linkages, we compared the effects of (β1 → 4)GOS with (β1 → 3)GOS, and with a mixture of α-linked oligosaccharides (lactose-derived oligosaccharides-LDO). The changes in mRNA expression of mucus-related genes were assessed by RT-PCR. GOS containing Lac significantly enhanced the expression of *MUC2*, *TFF3* and *RETNLB* but not of Golgi sulfotransferases genes. In contrast, GOS without Lac did not impact these genes. Lac alone significantly enhanced *MUC2*, *TFF3*, *RETNLB*, *CHST5*, and *GAL3ST2* genes suggesting that Lac might be responsible for goblet cell modulation in (β1 → 4)GOS preparations. (β1 → 3)GOS induced the expression of *MUC2* and *TFF3*, and downregulated the *RETNLB* gene. Compared with the (β1 → 3) and GOS (β1 → 4) GOS, the α-linked LDO significantly upregulated the expression *MUC2*, *TFF3*, *RETNLB* and the Golgi sulfotransferases genes. We identify structural features of GOS that contribute to enhanced mucus integrity. Our study might lead to better GOS formulations for foods to prevent or treat different types of intestinal disorders.

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1. Introduction

Non-digestible carbohydrates (NDCs) are carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine.¹ These NDCs include compounds, such as galacto-oligosaccharides

(GOS) and human milk oligosaccharides (hMOS).² NDCs are important for gut health as they are fermented in the colon by the intestinal microbiota,³ which subsequently produce health-promoting degradation products.⁴

Cow-milk derived infant formulas contain fewer oligosaccharides compared with human milk. Therefore, GOS is added to substitute some of the hMOS functions.⁵ GOS support the growth of beneficial bacteria and their metabolic products, such as short-chain fatty acids (SCFAs), improve the function of the mucosal barrier and gut-associated immunity,^{6,7} and serve as a decoy for pathogens to prevent infection.^{8,9} The reason behind such effect is that GOS resemble hMOS structures and as a consequence, mimic the mucosal cell-surface receptors used by pathogens to invade their host.⁹ In addition to supplementation in infant formula, GOS support gut-health in elderly, where GOS enriches beneficial gut microorganisms and stimulates colonic transit.^{10,11}

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GOS supports directly the intestinal barrier function by impacting intestinal host cells and indirectly by stimulating gut microbiota and intestinal homeostasis.⁶ Recently, it has been reported that GOS enhance gut barrier function directly by stimulating goblet cells to produce more mucus and regulate genes involved in lowering the permeability of the mucus layer.^{12,13} Although valuable, this observation was done with one type of commercial GOS and did not provide insight into the chemical characteristics of GOS responsible for such an effect. GOS is produced from lactose (Lac) by β -galactosidase enzymes and composed of galactose (Gal) linked to a terminal glucose (Glc) or Gal *via* various glycosidic bonds; *i.e.* ($\beta 1 \rightarrow 2$), ($\beta 1 \rightarrow 3$), ($\beta 1 \rightarrow 4$), ($\beta 1 \rightarrow 6$).¹⁴ GOS vary in oligosaccharide content and type of linkages.¹⁵

The degree of GOS polymerization (DP) commonly ranges between 2 and 5 units,¹⁶ but higher DP has been reported.¹⁷ Most GOS also contain Lac as the enzymatic conversion of this starting material into GOS is not complete. Lac content may vary per commercial sample. Besides the differences in the content of GOS mixtures, differences in the position of the glycosidic linkages occur, because different β -galactosidase enzymes have different linkage specificity.^{18,19}

It is unknown whether these variations in chemical structures of GOS impact mucus production by goblet cells and thus, its barrier reinforcing capacity. In the present study, we compared the effect of different GOS preparations on the mRNA expression of goblet cells secretory products genes. The changes in the mRNA expression of the genes that encode the proteins mucin 2 (*MUC2*), trefoil factor 3 (*TFF3*), resistin-like molecule beta (*RETNLB*) and the Golgi sulfotransferases galactose-3-O-sulfotransferase 2 (*GAL3ST2*) and the carbohydrate (*N*-acetylglucosamine-6-O) sulfotransferase 5 (*CHST5*) were measured in LS174T cells.

MUC2 is the main secretory product of goblet cells. Its main function is to prevent the contact between luminal bacteria and the host epithelium.²⁰ *MUC2* is co-secreted and synergized by the presence of *TFF3*, which maintains the integrity of the mucus layer and favors epithelial repair.²¹ Another abundant secretory product is the *RELM β* encoded by the gene *RETNLB*. *RELM β* is known to have antiparasitic activity²² and to mediate insulin resistance.²³ *GAL3ST2* and *CHST5* are essential sulfotransferases located in the Golgi apparatus, which add sulfonyl groups to mucin. The sulfate groups add negative charge to the glycoprotein and as a result alter the protective properties of the mucus barrier and clearance of parasites and pathogens.^{24,25}

Here, we compared the impact of GOS types of different composition on mucus-related genes in goblet cells. As Lac is a major remaining component in the GOS preparations, we also studied the impact of this disaccharide on goblet cell function. Additionally, we synthesized a novel Lac-derived oligosaccharide (LDO) mixture with different structural make-up and glycosidic linkages in order to study whether glycosidic-linkage differences impact goblet cell function.

2. Materials and methods

2.1. High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD)

We characterized GOS-60.6, GOS-63.5, GOS-71, and GOS-97. The amount of Glc, Gal, Lac, and transgalactosylated oligosaccharides in the samples was studied by HPAEC-PAD analysis. The profiles of the oligosaccharide products were analyzed on a Dionex ICS-3000 work station (Dionex, Amsterdam, The Netherlands) equipped with an ICS-3000 pulse amperometric detection (PAD) system and a CarboPac PA-1 column (250 $\times$ 2 mm; Dionex), using a gradient adapted from van Leeuwen *et al.*¹⁴

2.2. Analytical size-exclusion chromatography

Analytical size-exclusion chromatography was performed as described by van Leeuwen *et al.*¹⁴

2.3. Cell culture and reagents

Human colorectal cancer cell line LS174T was cultured as described by Ren *et al.*²⁶ Cells were incubated with different GOS mixtures: GOS-60.6, GOS-63.5, GOS-71, GOS-97, Lac monohydrate and LDO.

GOS-63.5 was produced by *Bacillus circulans* wild type B-galactosidase BgaD and the R484H mutant derived GOS-60.6 as reported by Yin *et al.*²⁷ The commercial GOS-71 and GOS-97 were provided by FrieslandCampina, The Netherlands. The GOS content reported by the manufacturers was measured according to AOAC 2001.02 methods. Lac monohydrate was purchased from Sigma Aldrich, The Netherlands. LDO was produced with the glucansucrase Gtf180- ΔN using sucrose as donor substrate, as described previously.²⁸

2.4. Cell treatment

The LS174T cells were re-suspended in fresh culture medium at 3×10^5 cells per mL, after which 1 mL of cell suspension was seeded per well in 24-well plates (Corning, New York, USA). Cells were then cultured until reaching 70–80% confluence. Culture medium was replaced by fresh medium containing Lac monohydrate, LDO, GOS-60.6, GOS-63.5, GOS-71, and GOS-97, or no treatment as control. GOS, Lac, and LDO were first dissolved with cell culture medium at a concentration of 5, 10 and 20 mg mL⁻¹ and after complete dissolution they were filtered through a 0.2 μ m filter (Corning, New York, USA). Cells were incubated with the different treatments for 24 and 48 h.

2.5. RNA extraction and real-time reverse transcription polymerase chain reaction (RT-qPCR)

After the incubation times, 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). RT-qPCR was performed with primer and probe sets (TaqMan Gene Expression Assays) for different genes (*MUC2*, *TFF3*, *RETNLB*, *CHST5*,

GAL3ST2, *GUSB*) provided by Applied Biosystems (Foster City, USA) as previously described²⁹ and qPCR Mastermix Plus (Eurogentec, Seraing, Belgium). Reactions were carried out in 384-well PCR plates (Thermo Scientific, UK) using 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 housekeeping gene β -glucuronidase (*GUSB*), and comparative quantification of gene expression was analyzed using the $2^{-\Delta\Delta C_t}$ method.²⁹

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7. Normal distribution of the data was tested using the Shapiro–Wilk test. The results are presented as mean $\pm$ SEM. Statistical comparisons were performed by one-way ANOVA with Tukey's *post-hoc* test when multiple comparisons per gene and among treatments were performed. A *p*-value < 0.05 was considered to be statistically significant.

3. Results

3.1. GOS composition and DP profile

We compared four different GOS types which are categorized according to its GOS content but are different in more aspects as outlined below, *i.e.* GOS-60.6, GOS-63.5,²⁷ GOS-71, and GOS-97. The amount of Glc, Gal, Lac, and transgalactosylated oligosaccharides in the samples was studied by HPAEC-PAD analysis.

The GOS mixtures differed not only in the content (%) of transgalactosylated oligosaccharides but also in the content of unreacted Lac (Table 1). The GOS-60.6 and GOS-63.5 present a similar profile, whereas the GOS-71 contained the highest

amount of Lac (21.9%). Compared to the GOS-60.6 and GOS-63.5, the content of Glc and Gal in the GOS-71 (4%, respectively) was low. GOS-97 is a purified mixture (transgalactosylated oligosaccharide content >90% (ref. 30)), which contains 0.2% of Lac and traces of Gal and Glc.

The DP composition of the GOS mixtures was analyzed by HPSEC-RI. The DP profile of the GOS showed a distribution between DP2 and DP6+ for the different samples (Table 2). While GOS-60.6, GOS-63.5, and GOS-71 contained mostly DP2 and DP3 structures, the GOS-97 consisted mainly of DP3 and DP4. GOS-97 contained higher percentages of DP5 and DP6.

While GOS-63.5 was synthesized by the wild-type BgaD enzyme from *Bacillus circulans* and contained mainly ($\beta 1 \rightarrow 4$) elongations, the GOS-60.6 was produced by using a mutant derived from the wild type enzyme (ESI Fig. 1A†). The HPAEC-PAD chromatogram of GOS-60.6 showed a different structural composition, containing similar amounts of ($\beta 1 \rightarrow 3$) and ($\beta 1 \rightarrow 4$) elongations as previously described²⁷ (ESI Fig. 2†).

The production of GOS from Lac by β -galactosidase yields mixtures with β -linkages only. We also compared these types of GOS with LDO which mainly has α -linkages. The LDO product was produced by the transglucosylation activity of glucanase Gtf180- ΔN , using Lac as acceptor substrate and sucrose as donor substrate. As described by Pham *et al.*,²⁸ the enzymatic reaction of Lac with a glucanase produced a mixture of α -gluco-oligosaccharides. This LDO mixture consisted of five glucosylated products with a DP3–4. The profile of glycosidic linkages is characterized by [$(\alpha 1 \rightarrow 2)$, ($\alpha 1 \rightarrow 3$), ($\alpha 1 \rightarrow 4$) and ($\beta 1 \rightarrow 4$)] bonds (ESI Fig. 1B†).

3.2. Effect of GOS mixture content and structural diversity on the expression of goblet cell-like associated gene products

GOS has been reported to enhance MUC2 production and to regulate several genes related to mucus structure and composition.³¹ In the study by Bhatia *et al.*¹³ a GOS mixture with 90% purity was found to have a direct modulatory effect on barrier function through the modulation of goblet cells. However, there are several GOS mixtures on the market with differences in GOS content and differences in DP distribution.¹⁴ To determine whether differences in transgalactosylated oligosaccharide composition and DP of GOS might impact mucus synthesis we performed this study.

First, we studied three mixtures of ($\beta 1 \rightarrow 4$)GOS with a variable content of transgalactosylated oligosaccharides in order

Table 1 Quantitative description of GOS composition^a

GOS (wt% of transgalactosylated oligosaccharides)	Glc (wt%)	Gal (wt%)	Lac (wt%)	Main linkages
60.6	18.3	1.5	15.2	($\beta 1 \rightarrow 3$)/($\beta 1 \rightarrow 4$)
63.5	19.0	1.2	16.3	($\beta 1 \rightarrow 4$)
71	4.0	4.0	21.9	($\beta 1 \rightarrow 4$)
97	1.6	0.7	0.7	($\beta 1 \rightarrow 4$)

^a Values are expressed as wt%, weight/percentage. Gal, galactose; Glc, glucose; Lac, Lactose.

Table 2 Degree of polymerization (DP) of GOS mixtures^a

GOS	Gal	Glc	DP2	DP3	DP4	DP5	DP6+
60.6	2.8 $\pm$ 0.05	26.1 $\pm$ 0.13	31.3 $\pm$ 0.11	20.2 $\pm$ 0.05	11.5 $\pm$ 0.04	6.5 $\pm$ 0.03	1.8 $\pm$ 0.12
63.5	1.6 $\pm$ 0.05	21.5 $\pm$ 0.13	41.9 $\pm$ 0.11	21.9 $\pm$ 0.05	9.5 $\pm$ 0.04	3.3 $\pm$ 0.03	0.2 $\pm$ 0.12
71	2.1 $\pm$ 0.77	5.7 $\pm$ 0.89	45.5 $\pm$ 0.35	26.6 $\pm$ 0.15	12.5 $\pm$ 0.03	5.3 $\pm$ 0.09	2.8 $\pm$ 0.22
97	2.3 $\pm$ 0.25	3.2 $\pm$ 0.24	12.9 $\pm$ 0.21	40.7 $\pm$ 0.40	23.9 $\pm$ 0.24	10.1 $\pm$ 0.16	6.8 $\pm$ 0.19

^a Values are averages of triplicate measurements, expressed with their corresponding standard deviation (SD). DP, degree of polymerization; Gal, galactose; Glc, glucose; Lac, Lactose.

to determine whether this parameter influences the expression of goblet cell-associated genes. LS174T cells were cultured with the GOS-63.5, GOS-71, and GOS-97. The changes in gene expression of the secretory products *MUC2*, *TFF3*, *RETNLB*, and the Golgi sulfotransferases (*CHST5* and *GAL3ST2*) were evaluated after 24 and 48 h of treatment.

Prior to the experiments, we determined the optimal GOS dose for cell stimulation. LS174T cells were cultured for 48 h with 5, 10 and 20 mg mL⁻¹ of the different GOS. The gene expression of *MUC2*, *TFF3*, *RETNLB*, and Golgi sulfotransferase genes increased proportionally to the concentration of GOS (ESI Fig. 3†). Because the mRNA expression of the tested genes did not change in a dose-response fashion but was most pronounced with the highest concentration we decided to use 20 mg mL⁻¹ for further experiments. Cells were incubated for 24 and 48 h with 20 mg mL⁻¹ of GOS.

After 24 h of incubation, the treatments did not induce significant changes in the gene expression of *MUC2*, *CHST5*, and *GAL3ST2* when compared to the non-treated cells. GOS-63.5, GOS-71, and GOS-97 induced a slight increase in *TFF3* mRNA expression (1.4-fold, $p < 0.01$, respectively). *RETNLB* was not altered significantly by GOS-63.5. However, this gene was slightly induced by GOS-71 (1.5-fold, $p < 0.05$) and GOS-97 (1.5-fold, $p < 0.05$) (Fig. 1A). Most effects were observed after 48 h of incubation with the different treatments (Fig. 1B). The GOS-63.5 mixture enhanced the gene expression of *MUC2* 2.9-fold ($p < 0.0001$), *TFF3* with 1.9-fold ($p < 0.01$), and *CHST5* 1.3-fold ($p < 0.05$). This mixture reduced the expression of *RETNLB* 0.4-fold ($p < 0.01$) and it did not affect the mRNA of the *GAL3ST2* gene. The GOS-71 mixture induced upregulation of *MUC2* (1.6-fold, $p < 0.05$), *TFF3* (2.3-fold, $p < 0.0001$), *RETNLB* (1.7-fold, $p < 0.0001$), and *CHST5* ($p < 0.05$). This mixture did

Fig. 1 Effect of different types of ($\beta 1 \rightarrow 4$)GOS on the mRNA expression of mucus-related genes in LS174T goblet cells. Cells were treated for (A) 24 h and (B) 48 h with 20 mg mL⁻¹ of the GOS. The data is represented as fold-change of MRNA expression normalized to GUSB. Data were analyzed with one-way ANOVA followed by Tukey's *post hoc* test. Values are expressed as means $\pm$ SEM, $n = 4$. Significant differences were indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. *MUC2*, Mucin2; *TFF3*, Trefoil factor 3; *RETNLB*, resistin-like molecule beta; *CHST5*, carbohydrate (*n*-acetylglucosamine-6-*o*) sulfotransferase 5; *GAL3ST2*, galactose-3-*O*-sulfotransferase 2.

not induce changes in the expression *GAL3ST2*. The purest GOS (GOS-97) did not have an effect on *MUC2* and Golgi sulfo-transferases genes after 48 h of incubation. However, it did induce gene expression of *TFF3* (2.1-fold, $p < 0.0001$), *RETNLB* (1.6-fold, $p < 0.01$), and *CHST5* (1.4-fold, $p < 0.001$).

3.3. Lac in GOS preparations modulates gene expression of LS174T secretory products and Golgi-sulfo-transferases

GOS content was shown to be an important factor in the modulation of the gene expression of goblet cells and since we found that the purest form of GOS, *i.e.* GOS-97, did not enhance *MUC2* production, we questioned whether Lac, which is the most abundant other molecule in GOS preparations, might be responsible for modulation of mucus-related genes in goblet cells. GOS is prepared from Lac by enzymatic transgalactosylation, where synthesis and hydrolysis occur simultaneously. For this reason, a significant amount of unreacted Lac remains at the end of the reaction.^{19,32} In order to study whether the Lac content influences the secretory products of goblet cells, LS174T cells were stimulated with this compound.

The goblet-cells were treated with 20 mg mL⁻¹ of Lac for 24 and 48 h. No time-dependent effect was observed. After 48 h the expression of *MUC2*, *TFF3* and *RETNLB* was significantly enhanced 1.8-fold ($p < 0.001$), 1.4-fold ($p < 0.05$), and 1.4-fold ($p < 0.05$), respectively. The Lac treatment enhanced by 1.8-fold ($p < 0.01$) the expression of *CHST5* and by 1.3-fold ($p < 0.05$) the expression of *GAL3ST2* (Fig. 2).

3.4. Glycosidic linkage diversity effects in the modulation of the secretory function of LS174T cells

The type of β -galactosidases employed for the GOS production has a strong impact on the final structural make-up, specifically in the glycosidic linkage profile.^{14,19} To investigate the effects of glycosidic linkages in GOS on the regulation of mucus-related genes in goblet cells, we compared the above

mentioned GOS-60.6 and GOS-63.5, as they differ in β -glycosidic linkages. GOS-63.5 is elongated by ($\beta 1 \rightarrow 4$) linkages, while GOS-60.6 is mainly composed of ($\beta 1 \rightarrow 3/4$) elongations.²⁷ Additionally, we compared the effects of LDO, since its oligosaccharides are elongated by α -linkages. We aimed to study whether α - and β -glycosidic linkages or their DP have a differential influence on the expression of goblet cell secretory-associated genes.

LS174T cells treated for 24 h with the GOS-60.6 and GOS-63.5 did not enhance the expression of the studied genes (ESI Fig. 4†). After 48 h, both GOS types impacted mucus-related genes in an identical fashion. No significant differences were found between them. GOS-60.6 upregulated *MUC2* 2.6-fold ($p < 0.0001$), *TFF3* 2.2-fold ($p < 0.01$), and downregulated *RETNLB* 0.4-fold ($p < 0.0001$). The GOS-63.5 effects on *MUC2*, *TFF3*, and *RETNLB* were presented in subsection 3.2.

The LDO product mixture (α -linkages) had a significantly different effect on goblet cells. This was not evident yet at 24 h (ESI Fig. 5†). After 48 h LDO upregulated the gene expression of *MUC2* 2.7-fold ($p < 0.001$) and *TFF3* 1.8-fold ($p < 0.05$). The expression of the *RETNLB* gene was not significantly increased by LDO treatment, while it was significantly downregulated by GOS-63.5 ($\beta 1 \rightarrow 4$) linkages. The most important difference was that α -linked LDO in contrast to β -linked GOS, significantly enhanced the expression of *CHST5* (1.8-fold, $p < 0.0001$) and *GAL3ST2* genes (1.8-fold, $p < 0.05$) (Fig. 3).

4. Discussion

To the best of our knowledge, we show here for the first time that the structural make up of GOS impacts the mRNA expression of genes associated to the secretory function of goblet cells. We observed that GOS-63.5 and GOS-71 enhanced *MUC2*, *TFF3*, and *RETNLB* mRNA expression, whereas the

Fig. 2 Effect of Lac at 20 mg mL⁻¹ on the mRNA expression of goblet cell-like secretory products after incubation for 24 and 48 h. Values are means $\pm$ SEM, $n = 4$. The data is represented as fold-change of mRNA expression normalized to *GUSB*. Data were analyzed with one-way ANOVA followed by Tukey's *post hoc* test. Significant differences compared to the negative control were indicated by * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. *MUC2*, Mucin2; *TFF3*, Trefoil factor 3; *RETNLB*, resistin-like molecule beta; *CHST5*, carbohydrate (*n*-acetylglucosamine-6-*O*) sulfotransferase 5; *GAL3ST2*, galactose-3-*O*-sulfotransferase 2.

Fig. 3 mRNA expression changes in LS174 cells induced by the different treatments: ($\beta 1 \rightarrow 3$) linked GOS-60.6 at 20 mg ml⁻¹, ($\beta 1 \rightarrow 4$) linked GOS-63.5 at 20 mg ml⁻¹, or α -linked LDO at 10 mg ml⁻¹ for 48 h. Values are means $\pm$ SEM, $n = 4$. The data is represented as fold-change of mRNA expression normalized to *GUSB*. Data were analyzed with one-way ANOVA followed by Tukey's *post hoc* test. Significant differences were indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. *MUC2*, Mucin2; *TFF3*, Trefoil factor 3; *RETNLB*, resistin-like molecule beta; *CHST5*, carbohydrate (*n*-acetylglucosamine-6-*O*) sulfotransferase 5; *GAL3ST2*, galactose-3-*O*-sulfotransferase 2.

purest counterpart (GOS-97) did not enhance *MUC2*. The GOS-types altered the mRNA expression of *CHST5* but not the expression of *GAL3ST2*, as suggested previously.¹³ As GOS-97 did not enhance *MUC2* while the Lac containing GOS (GOS-63.5, GOS-71) did, we tested whether Lac might be involved in the observed changes in gene expression. Our findings show that not only GOS but also Lac is involved in the induction of *MUC2*, *TFF3*, *RETNLB*, *CHST5*, and *GAL3ST2* genes. The effect of Lac could be partially explained by the presence of galectin-3 (Gal-3), expressed on the LS174T cells.³³ Both Lac and mucin are known ligands for this receptor.^{34,35} The strong affinity of Gal-3 for modified Lac structures and disaccharides other than Lac has been supported *via* affinity chromatography studies.³⁶

The enhancing effect of Lac on mucin mRNA expression in goblet cells has not been described before but it has been found to be induced by other compounds found in milk, such as α -lactalbumin,³⁷ β -casomorphins,³⁸ and β -casein.³⁹ Lac is considered sometimes as an unwanted component of GOS mixtures because the concerns it arises for Lac-intolerant consumers and to avoid product crystallization.⁴⁰ Nonetheless, Lac has been found to exert other non-nutritional beneficial health effects. For instance, Lac present in human milk or infant formulas upregulates the expression of cathelicidins antimicrobial peptide (CAMP) in human colon carcinoma cells lines (HT29, Caco-2, and T84) *via* the MAPK pathway.⁴¹ *In vivo* Lac is hydrolyzed by lactase into monosaccharides which are absorbed in the small intestine. In normolactasia, this is when the activity of lactase is high, Lac can reach the colon, where it can be fermented by the colonic microbiota.⁴² While the consumption of Lac do not change the activity or efficiency of lactase, clinical studies have shown that the intake of lactose alone or intake of GOS stimulates the growth of Lac-fermenting microbes, which improves the digestion and tolerance for Lac.^{43–45}

The enhancement of mucus-related genes by Lac present in GOS mixtures can contribute to gut barrier protection or to repair defects of the intestinal mucus layer. Our data also suggest that Lac removal by ultracentrifugation, size-exclusion chromatography, selective fermentation, or *in situ* adsorption,^{46,47} which raises the production costs of GOS, might not be necessary. Also, other health parameters may benefit as GOS with different content of transgalactosylated oligosaccharides and its secondary reaction products, namely, Gal, Glu, and Lac have beneficial impact on the growth of intestinal bacteria such as *Bacteroides*, *Protobacteria*, and *Lactobacillus*.^{48–50} Together, with the findings from this study, it can be suggested that GOS with varying transgalactosylated oligosaccharide content and some Lac might have a broader spectrum of indirect and direct effects on the intestinal barrier.

The GOS preparations tested in the current study are also found in consumer products. We selected GOS with different composition to determine how their structural differences influence mucus-related genes in goblet cells. Most commercial GOS preparations are enzymatically produced. The remaining amounts of Gal, Glu, and Lac at the end of the enzymatic reaction depend on the type of β -galactosidase used for the GOS production⁵¹ and on the initial substrate, pH, temperature, origin of the β -galactosidase, and the presence of inhibitors/activators of the reaction. All these parameters determine the yield and composition of the GOS obtained.^{52,53} For this reason, the GOS preparations were investigated without altering their content of Glc, Gal or Lac, which gave us in the presented experimental design insight in how the composition of GOS impacts mucus-related genes in human goblet cells.

The presence of ($\beta 1 \rightarrow 4$) linkages in the GOS-63.5 or ($\beta 1 \rightarrow 3/4$) linkages in the GOS-60.6 affected the expression of *MUC2*, *TFF3*, and *RETNLB* in a similar fashion. Neither GOS mixture altered the expression of *CHST5* and *GAL3ST2*. In this study we

did not find a benefit of these different β -linkages on the expression of Golgi sulfotransferase genes. However, the LDO data suggests that a significant enhancement on mucus-related gene expression is obtained by oligosaccharides with α -linkages. LDO enhanced the expression of *MUC2*, *TFF3*, and *RETNLB*, and also induced the expression of Golgi sulfotransferases mRNA. All genes were strongly upregulated when compared to the effects of the GOS ($\beta 1 \rightarrow 3/4$) or ($\beta 1 \rightarrow 4$) samples. α -GOS were recently introduced in the market but unlike β -GOS, the former are obtained from non-dairy sources, such as raffinose oligosaccharides.⁵⁴ The LDO tested in this study differs from these commercial α -GOS by the presence of glucosylated-Lac structures, with predominantly α -glycosidic linkages, with no traces of disaccharides or monosaccharides.

Given that breast milk hMOS contain both α -linked and β -linked structures, a GOS/LDO mixture with both α - and β -linkages may better mimic the hMOS biological activity as illustrated by our observation that the LDO mixture exhibited stronger modulatory effects on the expression of mucus-related genes and Golgi sulfotransferases genes compared with the β -linked GOS. The effects of α -linked LDO and β -linked GOS mixtures have the potential to improve the production of secretory products of goblet cells such as of *MUC2*, *TFF3*, and *RETNLB* that are necessary for the maintenance of the intestinal barrier integrity in individuals with increased intestinal permeability and colitis.²⁰

In addition to the genes we would have preferred to quantify the final products of these genes which are mainly glycoproteins contributing to enforcement of mucus function. Our group is working on measurements of mucus function by quantifying the monosaccharide composition but unfortunately there are no technologies available yet that can adequately translate the gene changes in mucus function changes. This requires often full intestines and high amounts of mucus which cannot be obtained in these *in vitro* studies with so many compounds. Despite this, we are able to show with gene transcripts profound differences between the different lactose and lactose-derived oligosaccharides and GOS products demonstrating clear structure-effector relationships. This was the main aim of the current study.

Interestingly, both GOS and LDO molecules upregulated *TFF3*. This protein plays a role in the changes in monosaccharide composition of mucin.⁵⁵ Changes in the expression of the Golgi sulfotransferases affect mucin sulfonation, which in turn impact directly the mucus integrity,⁵⁶ and may be instrumental in preventing or lowering the frequency and symptoms of colitis, and bacterial dysbiosis.⁵⁷ Therefore, an increase in the expression of the proteins involved in mucin sulfonation can reduce the risk of bacterial infections and reduce gut barrier permeability. The modulation of the mucin *O*-glycosylation with specific types of GOS or LDO may open venues to formulate effective food ingredients for the prevention or the cure of different types of intestinal disorders.

The observation that Lac and LDO had a more profound effect on goblet cells implies that the current view that GOS alone is responsible for the effects on goblet cells¹³ should be

revisited. If gut barrier function by stimulation of mucus production is the goal, it is according to our findings, advisable to use GOS preparations with Lac or to design combinations of β -GOS with α -linkages. Therefore, the differences in effects observed with the diverse preparations may have impact on both manufacturing procedures and application of GOS in nutrition. The enzymes required for GOS preparation and the purification process are the main factors responsible for high production cost of GOS.⁵² By understanding the effects of enzyme specificity on the structure of GOS, and the impact of the final product on biological activity, the development of more specific β -galactosidases and GOS mixtures can target different types of individuals and conditions.

5. Conclusions

In summary, ($\beta 1$ -3/4) GOS induce the mRNA expression of *MUC2*, *TFF3*, and *RETNLB*. In contrast, Lac and the α -linked oligosaccharides display modulatory effects on the expression of all genes of interest expressed by goblet cells. The findings reported here not only contribute to the understanding of function-effector relationships of Lac and Lac-based oligosaccharides on gut barrier function by modulation of goblet cell-function, it opens also new venues to create more effective formulations to enhance gut barrier function. This for example, can be instrumental in the management of some diseases such as inflammatory bowel disease and metabolic disorders in which barrier function is impaired⁵⁸ but also for gut barrier dysfunction in ageing humans.⁵⁹

Abbreviations

<i>CHST5</i>	Carbohydrate (<i>N</i> -acetylglucosamine-6- <i>O</i>) sulfotransferase 5
Fuc	Fucose
Gal	Galactose
<i>GAL3ST2</i>	Galactose-3- <i>O</i> -sulfotransferase 2
GalNAc	<i>N</i> -Acetylgalactosamine
Glc	Glucose
GlcNAc	<i>N</i> -Acetylglucosamine
GOS	Galacto-oligosaccharides
hMOS	Human milk oligosaccharides
Lac	Lactose
LDO	Lactose-derived oligosaccharides
<i>MUC2</i>	Mucin2
NDCs	Non-digestible carbohydrates
Neu5A	Sialic acid
<i>RETNLB</i>	Resistin-like molecule beta
<i>TFF3</i>	Trefoil factor 3

Conflicts of interest

There are no conflicts to declare.

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H. Y. prepared and characterized the wild type and mutant type GOS and H. P. prepared and characterized the LDO. S. F. L. and C. R. performed the cell-based experiments and data analysis. S. F. L. and P. d. V. outlined and wrote the manuscript. S. v. L. performed the chemical characterization of the GOS mixtures and the critical review of the manuscript together with L. D. and P. d. V. All authors have revised and approved the manuscript.

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