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ORIGINAL CONTRIBUTION

Effects of galacto-oligosaccharide ingestion on the mucosa-associated mucins and sucrase activity in the small intestine of mice

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Abstract
Background Galacto-oligosaccharides (GOS) are non-digestible oligosaccharides with short galactosyl chain units produced by lactose fermentation which are considered as prebiotics. Only few studies have investigated the effects of GOS medium-term ingestion on the small intestinal epithelium characteristics.
Aim of the study In this study, we evaluated the consequences of GOS ingestion on small intestinal mucosal morphology, on brush-border membrane enzyme activities and on mucin content in BALB/c mice.
Methods Mice received the experimental diets for 4 weeks and then the small intestine was collected to measure sucrase, lactase and alkaline phosphatase activities, to study the villus heights in the jejunum mucosa and to determine mucosal mucin content as well as MUC-2 and MUC-4 mRNAs expression by qRT-PCR.
Results Our results showed that GOS has no detectable effect on the intestine villus height but increased the total protein content by twofold. Sucrase activity was significantly increased in the intestinal mucosa recovered from animals fed the GOS diet without any detectable modification of lactase and phosphatase activities. Interestingly, GOS was also able to increase sucrase activity in cultured Caco-2 cells raising the view that they likely act directly on these cells. Furthermore, GOS was found to markedly increase O-linked glycoproteins associated with the intestinal mucosa without modifying MUC-2, MUC-4 mRNAs expression. Lastly, TNF-α mRNA expression was also not modified after GOS ingestion.
Conclusions These results suggest that, in BALB/c mice, 4-week GOS ingestion is able to increase the small intestinal mucosa-associated mucin content and enterocyte-associated sucrase activity without modifying villus height.

Keywords Galacto-oligosaccharides · Intestinal mucosa · MUC genes · Mucins

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Introduction

Dietary fibers (DF) have been defined by the American Association of Cereal Chemists (AACC) as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" [1]. Depending on their solubility, DF have numerous but different effects on the gastrointestinal tract. In particular, soluble DF such as pectins or guar gum are fermented by the colonic microflora [10, 30], with production of short-chain fatty acids (SCFAs) [46]. This phenomenon has several consequences on the colonic functions, like a decrease of the luminal pH [34], a stimulation of colonic epithelial cell proliferation [21, 24, 37] and some modifications in the composition and thickness of the mucosal layer [38, 40].

Galacto-oligosaccharides (GOS) are a class of non-digestible dietary carbohydrates defined as prebiotics. To

be considered as a prebiotic, a food ingredient has to be resistant to gastric acidity and to hydrolysis by mammalian enzymes, but fermented by the intestinal microflora, and to allow specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host well-being and health [17, 35].

Galacto-oligosaccharides (GOS) are short-chain carbohydrates commercially produced from lactose using the galactosyltransferase activity of β-galactosidase [8]. It has been shown that they are able to reach the large intestine where they are metabolised by the indigenous microflora. Moreover, GOS are able to enhance some health-promoting microbial populations [12, 20, 29, 31, 36, 44]. This property has led to consider GOS as prebiotics [17]. Since they are non-digestible and fermentable by the colonic microflora and also can modulate intestinal barrier function [49] GOS may therefore be considered as DF.

Although the effects of DF have been studied in several studies on the small intestinal mucosa [6, 15, 18, 21, 24, 38], experimental works on GOS have mainly focused on their prebiotic effects on the large intestine [5, 12, 22, 27, 48] and on their immunomodulatory effects [28, 42, 50]. Few studies have been conducted on a specific mixture of GOS with long chain fructo-oligosaccharides (FOS) [22, 27, 50]. A recent study has shown that GOS milk formula increases fecal secretion of immunoglobulin A (IgA) suggesting a positive effect on mucosal immunity [39]. However, to our knowledge, only one study has been conducted to evaluate their effects on mucins [26]. This work has shown that GOS modified the mucin cell distribution in the colon, but secretion and thickness of the mucus layer were not evaluated in this study. The mucus layer covering the gastrointestinal mucosa is considered to represent the first line of defense against mechanical, chemical or microbiological aggression of luminal compounds in excessive concentrations [33].

The working hypothesis used in the present study was that the ingestion of a GOS-containing fraction may modify mucosal morphology, the mucosal enzymatic activities and the mucosa-associated mucins of the small intestine. To test this hypothesis, we used BALB/c mice as an in vivo model. We also used Caco-2 cells to directly test the GOS-containing fraction on intestinal epithelial cells.

Materials and methods

Composition of the GOS fraction

The GOS-containing fraction was obtained from lactose by fermentation with *Streptococcus thermophilus* St065. The lyophilized powder was analyzed by HPLC as described previously [32]. This fraction contained mono-, di-, tri- and tetrasaccharides as follows (%w/w): galactose, 3.8; glucose, 15.3; lactose, 51.2; β-Gal(1,6)-Glc, 4.3; β-Gal(1,3)-Glc, 0.7; β-Gal(1,2)-Glc, 0.4; β-Gal(1,3)-β-Gal(1,4)-Glc, 18.3; β-Gal(1,6)-β-Gal(1,4)-Glc, 2.2; β-Gal(1,3)-β-Gal(1,6)-Glc, 0.9; β-Gal(1,3)-β-Gal(1,3)-β-Gal(1,4)-Glc, 1.5; β-Gal(1,6)-β-Gal(1,6)-β-Gal(1,4)-Glc, 1.4. In this fraction, GOS per se, i.e., except glucose, galactose and lactose represent 30% by weight.

In vivo study

Animals and treatments

These experiments were conducted on 54 BALB/c female mice obtained at 6 weeks of age from Harlan (The Netherlands). The mice were allocated to 3 groups ($n = 18$) a control group (no oligosaccharide), a gos5.0% group [5% (w/w) of GOS-containing fraction] and as a matter of comparison a lac5.0% group [5% (w/w) of lactose], respectively. Mice were housed in plastic cages in a temperature-controlled room with a 12-h light/dark cycle. Water and food were freely available throughout the experiment. After 6 days of acclimatization they were given the experimental diets containing either lactose or GOS-containing fraction for 4 weeks (Table 1). At the end of the experiments, mice were killed by decapitation and their small intestine was isolated. The intestine was washed with sterile 0.9% (w/v) NaCl. In some mice ($n = 10$), after removal of the first 50 mm duodenum, 20 mm of jejunum were excised for histological study, the remainders being scrapped to collect the intestinal mucosa. This latter was frozen for further measurement of enzymatic activities, DNA and mucin contents. The whole small intestine of some mice ($n = 8$) was removed as described previously, diluted in 1 ml Trizol and frozen until RNA extraction for MUC gene expression measurements.

Histological study

For the measurement of villus height, jejunum was used since villus height is known to be higher in this anatomical part than in the ileum [7]. The jejunal samples were fixed in Bouin's solution for 24 h, immersed twice in 95% (v/v) ethanol to remove the picric acid and afterwards dehydrated in increasing butanol bathes. Then samples were embedded in paraffin and cut in 5 µm longitudinal sections. Sections were mounted on gelatine-chrome slides and stained in hematoxyline and eosine. Briefly, slides were unembedded in xylene, rehydrated in decreasing ethanol bathes and finally in water for 10 min. Slides were immersed in hematoxyline (5 g/L) for 3 min, washed in water, immersed in eosine (20 g/L in ethanol) for 5 min, rinsed with 70% (v/v) ethanol and finally immersed in

Table 1 Composition of the experimental diets (g/kg)

Ingredient	Control	Lac5.0%	gos5.0%
Milk protein LR85F[a]	140	140	140
Soy bean oil	40	40	40
Mineral mix[b]	35	35	35
Vitamin mix[b]	10	10	10
Choline	2.3	2.3	2.3
Starch	622.4	622.4	622.4
Cellulose	50	50	50
Sucrose	100.3	50.3	50.3
Lactose added	0	50	0
From GOS-containing fraction			
Total GOS-containing fraction	0	0	50.0
Glucose	0	0	7.6
Galactose	0	0	1.9
Lactose (β-Gal(1,4)-Glc)[c]	0	0	25.6
β-Gal(1,6)-Glc	0	0	2.1
β-Gal(1,3)-Glc	0	0	0.3
β-Gal(1,2)-Glc	0	0	0.2
β-Gal(1,3)-β-Gal(1,4)-Glc	0	0	9.1
β-Gal(1,6)-β-Gal(1,4)-Glc	0	0	1.1
β-Gal(1,3)-β-Gal(1,6)-Glc	0	0	0.4
β-Gal(1,3)-β-Gal(1,3)-β-Gal(1,4)-Glc	0	0	0.7
β-Gal(1,6)-β-Gal(1,6)-β-Gal(1,4)-Glc	0	0	0.7

gos, galacto-oligosaccharides; *lac*, lactose

[a] From Armor Protéines, France

[b] AIN-93 M

[c] Lactose from GOS-containing fraction

toluene. To determine villus height, 10 villi were measured on 5 mice per group. Villus height is the distance from the crypt-villus junction to the tip of the villus. The measurements were made using an optical microscope Axio Imager Z1 equipped with a camera and coupled with an image-analyzing system (Zeiss, Le Pecq, France).

Protein and DNA contents

Mucosa scrapped from the jejunum (excluding the first 20 mm) and ileum was pooled, diluted and homogenized to obtain a final concentration of 100 mg/ml in 0.9% (w/v) NaCl.

Total protein contents were measured according to the manufacturer's instructions with the BCA protein assay kit (Pierce Biotechnology, Brebières, France). Results were expressed as milligrams proteins per gram (mg proteins/g) mucosa.

Total DNA contents were determined using the Fluo-Reporter® Blue Fluorometric dsDNA Quantitation Kit (Molecular Probes), according to the manufacturer's instructions.

Enzymatic measurements

Sucrase activity was measured in the pooled jejunal and ileal mucosa and in Caco-2 cells according to the method of Dahlqvist [11] modified by Lloyd and Whelan [23]. Briefly, 50 µl of cell lysates was kept at 37 °C; 50 µl of sucrose solution (0.056 M) was added and tubes were kept at 37 °C for 1 h. Then 1.5 ml of tris-glucose oxydase buffer (TGO) was added and tubes were placed for 1 h at 37 °C. Finally, aliquots of 100 µl of reaction mixture were transferred to a 96-well plate and the plate was read at 405 nm. For lactase activity, sucrose (0.056 M) was replaced by lactose (0.056 M). Sucrase and lactase activities were expressed as unit per milligram (U/mg) mucosa.

Alkaline phosphatase activity was measured in the pooled jejunal and ileal mucosa using the fluorogenic substrate 4-methylumbelliferyl phosphate (4-MUP) (Sigma, St-Quentin-Fallavier, France). Hundred microliters (µl) of cell lysates was transferred to a 96-well plate and 50 µl of ready-to-use 4-MUP was added. Plates were immediately put in a microplate reader (Cytofluor 4,000, Applied Biosystems, Courtaboeuf, France) with excitation at 360 nm and emission at 440 nm, and read every 5 min during 30 min. The intensity of fluorescence increased linearly during this period. Results are expressed as relative fluorescence intensity (RFU) per minute and mg mucosa (RFU/min mg mucosa).

Preparation of mucin fraction

The mucin fraction was isolated from the pooled jejunal and ileal mucosa according to the method of Tanabe et al. [43]. Mucosa samples were suspended in 0.15 M NaCl with 0.02 M sodium azide. Samples were homogenized using a polytron and centrifuged at 10,000*g* for 30 min at 4 °C. The mucins dissolved in the supernatant were then precipitated with 60% (v/v) ethanol and recovered by a second centrifugation (5,000*g* for 5 min at 4 °C). Mucins were finally dissolved in 100 µl water.

Mesurement of O-linked glycoprotein chains (mucins)

Mucins were determined according to Crowther and Wetmore [9] using a fluorometric assay that discriminates O-linked glycoproteins (mucins) from N-linked glycoproteins. Briefly, alkaline cyanocetamide (CNA) was prepared by adding 200 µl of 0.6 M cyanoacteamide to 1 ml of 0.15 M NaOH. Then 100 µl of mucin solution was mixed with 120 µl of alkaline CNA and incubated at 100 °C for 30 min. After incubation, 1 ml of 0.6 M borate buffer (pH 8.0) was added. After allowing the solution to cool to room temperature, 200 µl of the final solution was transferred to a 96-well plate and the fluorescence was measured

in a microplate reader (Cytofluor 4,000, Applied Biosystems, Courtaboeuf, France) with excitation at 336 nm and emission at 383 nm. The quantity of O-linked glycoproteins was determined using a standard curve of N-acetylgalactosamine treated as the samples. Results are expressed as micromol per gram (μmol/g) mucosa.

RT-PCR analysis of MUC and TNF-α gene expression

For the measurement of MUC and TNF-α gene expression, we used ileum since thickness of mucus gel is higher in ileum than in jejunum [3]. Tissues from ileum were homogenized in 1 ml Trizol (Invitrogen) with a Turrax homogenizer. Total RNA was extracted according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 400 ng of total RNA in 40 μl, using random primers and reverse transcriptase (Applied Biosystems, Courtaboeuf, France). At the end of the reaction, each tube was completed with sterile H_2O qsp 200 μl and frozen until the completion of realtime PCR. Realtime quantitative PCRs for each gene were performed using 5 μl of the reverse transcripted total RNA solution, with 0.015 mM concentrations of both sense and antisense primers (Applied Biosystems) [19] in a final volume of 20 μl, using Power SYBR Green PCR Master Mix (Applied Biosystems) in the thermocycler (Applied biosystems). PCR efficiencies were determined using the formula $E = 10^{(1/\text{slope})} - 1$. Relative quantification for any given gene was calculated after determination of the difference between the C_t of the given gene and that of the calibrator gene 18S using the formula $2^{-\Delta C_t}$.

In vitro study

Measurement of Caco-2 cell proliferation

Caco-2 cells were obtained from the American Tissue Culture Collection and seeded at 2×10^4 cells/cm^2 in Dulbecco's modified Eagle's Medium (DMEM, Gibco) containing 15% (v/v) fetal bovine serum, 25 mM D-glucose, 6 mM L-glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin. The cells were grown under a 5% CO_2–95% air atmosphere. The culture medium was changed every two days before confluency and daily afterwards. Cells were used between the 38th and the 45th passages. For the assay, four 24-well plates were used and GOS-containing fraction was added in culture wells at day 3 after seeding in order to reach a final concentration of 0, 0.1, 1 or 5 g/L. From day 3 to day 6 after seeding, one plate was stopped every day: culture medium was removed and cells were rinsed with PBS. DNA was evaluated using a FluoReporter® Blue Fluorometric dsDNA Quantitation Kit (Molecular Probes, Cergy-Pontoise, France), according to the manufacturer's instructions.

Protein(s) content

Total protein contents were measured on Caco-2 cell lysates according to the manufacturer's instructions with the BCA protein assay kit (Pierce Biotechnology, Brebières, France).

Enzymatic measurements

To evaluate GOS effect on Caco-2 cell differentiation, GOS-containing fraction was added in culture wells at day 3 after seeding, in order to reach a final concentration of 0, 0.1, 1 or 5 g/L. At day 7 culture medium was removed, cells were rinsed with PBS and homogenized before enzymatic activity measurement. Sucrase and alkaline phosphatase activities were measured as described above. Sucrase activity was expressed as milliunits per milligram (mU/mg) proteins and alkaline phosphatase activity was expressed as relative fluorescence intensity (RFU) per minute and milligram proteins (RFU/min mg proteins).

Statistical analysis

Data are expressed as mean ± SD. Statistical analyzes were performed using SAS software. Results were compared using a one-way analysis of variance (ANOVA) to assess the effect of diets. When significant effects were observed, a Tukey's test was applied a posteriori to determine significant differences between groups. Significance was established at $P < 0.05$.

Results

In vivo study

Histological study

Jejunal villus height was measured on five mice of each group. The results expressed in micrometers (μm) are the following (mean ± SD): 320 ± 44 for control mice, 298 ± 37 for lac5.0% mice and 313 ± 9 for gos5.0% mice. Statistical analysis did not show any significant difference between groups ($P = 0.671$).

Enzymatic activities and DNA content of the intestinal mucosa

Enzymatic activities and DNA quantification were measured in the small intestine mucosa recovered after

consumption of the different experimental diets. Alkaline phosphatase and lactase activities were not significantly modified by GOS consumption (data not shown). Sucrase activity (U/mg mucosa) was: 11.4 ± 2.2, 11.4 ± 1.9 and 13.6 ± 2.3 in the mucosa of control, lac5.0% and gos5.0% mice, respectively ($n = 10$). This activity was significantly increased in the small intestine of GOS-consuming mice ($P < 0.05$). DNA content (RFU/mg mucosa) was similar in the control, lac5.0% and gos5.0% groups representing, 942 ± 252, 1169 ± 356 and 1206 ± 285, respectively ($n = 10$ in each experimental group).

Protein and mucin contents of the intestinal mucosa

Total protein content of the small intestine mucosa (Fig. 1a) recovered after the consumption of the different experimental diets was twofold higher in GOS-consuming mice as compared to the protein content of the mucosa recovered from the control mice ($P < 0.001$) and from the lac5.0% mice ($P < 0.001$).

GOS ingestion also significantly increased the mucin content associated with the intestinal mucosa as compared to either control mice ($P < 0.05$) or lactose-consuming mice ($P < 0.05$), as shown in Fig. 1b.

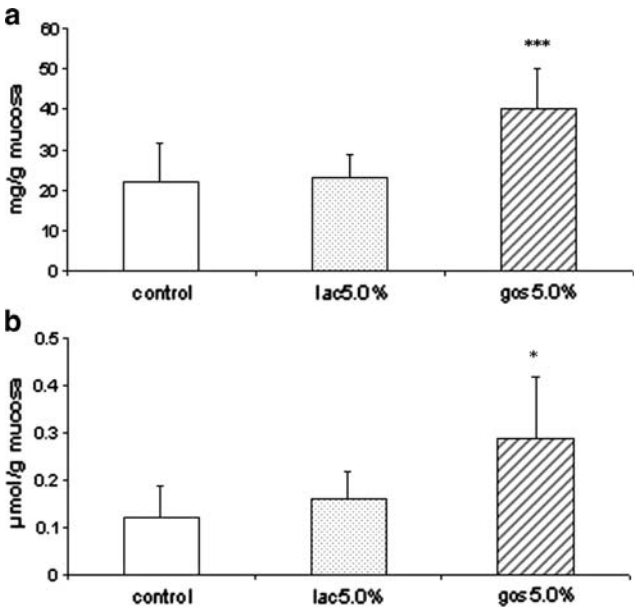

Fig. 1 Total protein content (**a**) and mucin (**b**) content in intestinal mucosa of mice consuming a control diet, a lactose-containing diet (lac5.0%) or a GOS-containing diet (gos5.0%) for 4 weeks (mean ± SD, $n = 10$). ***Mean values were significantly different from those of the control and the lac5.0% groups ($P < 0.001$). *Mean values were significantly different from those of the control and the lac5.0% groups ($P < 0.05$)

MUC and TNF-α gene expression

Mucus is mostly composed of mucins whose protein backbones are encoded by MUC genes. We analyzed MUC-2, which encodes mucins specifically synthesized and secreted by the goblet cells to form the gel that covers and protects the mucosal surface, and MUC-4, which encodes membrane-bound mucins. MUC and TNF-α gene expression in the ileal part of the small intestine, according to the different experimental diets is shown in Fig. 2. No significant difference was observed for MUC-2 and MUC-4 expression between the experimental groups. When TNF-α mRNA were measured as an index of intestinal inflammation, no difference was recorded between the three experimental groups.

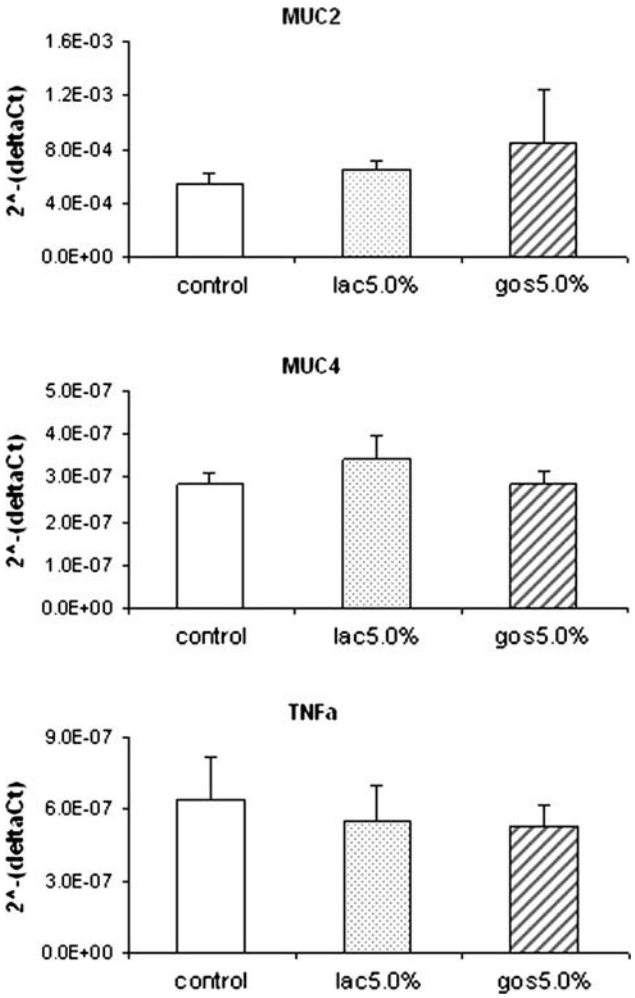

Fig. 2 MUC and TNF-α gene expression in the ileum of mice consuming a control diet, a lactose-containing diet (lac5.0%) or a GOS-containing diet (gos5.0%) for 4 weeks (mean ± SEM, $n = 8$). Mean values were significantly different from those of the control and the gos5.0% groups ($P < 0.05$)

In vitro study

Proliferation of Caco-2 cells in the presence of GOS

The growth curve of the Caco-2 cells was not modified by the addition of GOS in culture medium (data not shown). Statistical analysis did not reveal any significant difference between GOS-containing wells and control wells ($P = 0.143$).

Enzymatic activities in Caco-2 cells

Sucrase activity was measured in Caco-2 cells grown for 4 days in the presence or absence of GOS-containing medium. At day 7, the sucrase activity was measured in Caco-2 cell homogenates, values of 1.6 ± 0.3, 2.5 ± 0.6, 2.1 ± 1.0, and 3.5 ± 1.3 mU/mg proteins were reported when they were grown in the presence of 0, 0.1, 1 and 5 g/L of GOS-containing fraction, respectively. Caco-2 cells expressed a significantly higher sucrase activity when they were grown in the presence of the highest concentration of GOS ($P < 0.05$) as compared to the control experiments (no GOS). In contrast, alkaline phosphatase activity was not modified by the presence of GOS in the culture medium (data not shown).

Discussion

Our study clearly shows that GOS are able to modify some characteristics of the intestinal mucosa in BALB/c mice. First, GOS consumption strongly increased the mucosal protein and mucin content when compared to the values obtained in control and lactose-consuming animals. However, GOS consumption did not significantly affect the DNA content of the intestinal mucosa. Furthermore, mice consuming the GOS-enriched diet exerted a higher sucrase specific activity associated with the intestinal mucosa, but had similar alkaline phosphatase and lactase activities indicating that the GOS-enriched diet exerted a rather specific effect on mucosal epithelial cell. This increase in sucrase activity was however limited (1.2-fold), suggesting a modest increased capacity for sucrose hydrolysis by enterocytes.

Despite the increase in protein content, the jejunal villus height did not show any modification when GOS were added to the diet. These apparently contradictory results may be reconciled if we take the increase mucosa-associated mucins in animals receiving the GOS-enriched diet into account. Moreover, total mucosal DNA content was similar in GOS-consuming mice when compared with both the control and lactose-fed animals. This result is in accordance with the results obtained in the in vitro study since Caco-2 cell proliferation was unaffected by the presence of GOS. In addition, the consumption of GOS was associated with a specific higher sucrase activity in the small intestine mucosa. Such an increase of sucrase activity was also measured when Caco-2 cells were grown in presence of GOS. However, it is worth to underline that this latter effect was observed only at a high GOS concentration (5 g/L). With this reservation in mind, the results obtained with Caco-2 cells indicate that GOS may exert an effect on enterocytes which is not mediated by microflora. Although the mechanisms involved in the GOS effect remains unknown, we propose that GOS may act—at least in part—through a direct effect on intestinal epithelial cells. Due to their oligosaccharidic structure, GOS may activate sucrase activity in intestinal epithelial cells. It is not possible, however, to exclude a SCFA-mediated effect on both the enterocyte differentiation and on the amount of mucins associated with the intestinal mucosa since SCFAs are present in significant concentrations in the small intestine [41]. SCFAs are known to be increased in the large intestine following GOS administration [12, 13, 26, 29, 48] and are able to increase differentiation of intestinal colonic-absorptive-epithelial cells [16, 46, 51].

The strong increase in the mucin content of the intestinal mucosa is in accordance with the observations on total protein content. However, evaluation of two groups of MUC genes, i.e., secreted mucins (MUC-2) and membrane-bound mucins (MUC4) showed that GOS-consuming mice display similar MUC mRNA levels in the small intestine mucosa when compared with control mice. Dietary fibers had different effects on mucus production depending on their nature. For instance, butyrogenic fibers like FOS or inulin can enhance the production of colonic mucins [14, 47], whereas non-butyrogenic fibers have no effect on mucin production or secretion [38]. In contrast, GOS have been shown to decrease the number of acid mucin-containing cells in the cecum of rats [26]. However, these studies performed with FOS, inulin or GOS used histological or biochemical methods instead of MUC mRNA level measurement in the intestinal mucosa. De novo mucin synthesis (basal and induced) is controlled primarily at the transcriptional and post-transcriptional levels [4] but much of the works on mucin biosynthesis have been devoted to the transcriptional control [45]. Our present work indicates an increased mucosa-associated mucin after GOS consumption without detectable changes of MUC mRNA level. This result is in favor of the hypothesis that in our experimental situation, GOS are acting mainly at the post-transcriptional level. However, it is not possible to exclude an effect of GOS on the level of mucin glycosylation. Moreover, knowing that modification of MUC gene-associated mRNA levels can be used as an early marker of inflammation [26], it is worth to note that

no change was observed in either MUC or the proinflammatory cytokine TNF-α mRNA levels.

Further work is obviously required to decipher the mechanisms involved in the GOS effect and to determine if GOS could act in the small intestine as a mucin exocrine secretagogue. It has been demonstrated that GOS are able to increase the proportion of some SCFAs [12, 26, 41, 48] and to lower the colonic luminal pH. Moreover, GOS are considered as prebiotics and have been shown to modify the composition of the intestinal microflora [5, 12, 48]. Although these experiments were performed using the large intestine, they indicate that GOS are able to induce modifications of the luminal environment and to reinforce the structure of the intestinal mucus gel. The effect of the luminal pH on mucus structure and thickness has already been shown in the upper part of the intestinal tract [2, 25] and this may explain at least in part the marked effect of GOS consumption on the mucosa-associated mucus.

In terms of intestinal physiology, the increase of intestinal mucosa-associated mucins after GOS consumption may be viewed as a beneficial effect of GOS since mucins are known to protect the intestinal epithelial cells towards deleterious luminal compounds when present in excess. The fact that after GOS consumption, mucosa-associated TNF-α mRNA was not modified and that the histological aspect of the mucosa remained unchanged suggests that, based on such parameters, GOS did not exert any major deleterious effect. Inversely, from the data recorded in the present study, it appears that GOS consumption in our rodent model is likely to exert protective effects on the small intestine mucosa.

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