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β(2 → 1)-β(2 → 6) branched graminan-type fructans and β(2 → 1) linear fructans impact mucus-related and endoplasmic reticulum stress-related genes in goblet cells and attenuate inflammatory responses in a fructan dependent fashion†

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Dietary fibers such as fructans have beneficial effects on intestinal health but it is unknown whether they impact goblet cells (GCs). Here we studied the effects of inulin-type fructans (ITFs) and graminan-type fructans (GTFs) with different molecular weights on mucus- and endoplasmic reticulum (ER) stress-related genes in intestinal GCs. To that end, GCs were incubated in the presence of ITFs or GTFs, or ITFs and GTFs + TNFα or the *N*-glycosylation inhibitor tunicamycin (Tm). IL-8 production by GCs was studied as a marker of inflammation. Effects between ITFs and GTFs were compared. We found a beneficial impact of GTFs especially on the expression of *RETNLB*. GTF II protects from the TNFα-induced gene expression dysregulation of *MUC2*, *TFF3*, *GAL3ST2*, and *CHST5*. Also, all the studied fructans prevented Tm-induced dysregulation of *GAL3ST2*. Interestingly, only the short chain fructans ITF I and GTF I have anti-inflammatory properties on GCs. All the studied fructans except ITF I decreased the expression of the ER stress-related *HSPA5* and *XBP1*. All these benefits were fructan-structure and chain length dependent. Our study contributes to a better understanding of chemical structure-dependent beneficial effects of ITFs and GTFs on gut barrier function, which could contribute to prevention of gut inflammatory disorders.

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1. Introduction

The intestinal epithelium is covered by a mucus layer which serves as the first barrier against the harmful content of the lumen.[1] This mucus is formed by a film mainly constituted by mucins.[2] Mucin-2 (encoded by *MUC2*) is the most abundant mucin in the gut. This large multimeric protein undergoes posttranslational modifications such as *O*- and *N*-glycosylation and sulfation, conferring MUC2 a highly viscous gel-like consistency.[3,4] MUC2, along with proteins such as trefoil factor-3 (encoded by *TFF3*),[5] resistin-like molecule β (encoded by *RETNLB*)[6] and other antimicrobial peptides form an organized structure similar to a net and provide a semi-permeable barrier between the intestinal epithelium and the lumen which allows the passage of small molecules while it hampers the translocation of microorganisms.[7]

Interweaved between enterocytes and the other components of the intestinal epithelium are goblet cells (GCs), which are mucus-synthesizing specialized cells.[8] Due to MUC2 dual expression (constitutive and regulated), GCs possess and require a highly active protein trafficking from the endoplasmic reticulum (ER) where its folding occurs, and where glycosylation and sulfation occurs, to its final site of action.[9] In these last posttranslational sulfation modifications galactose-3-*O*-sulfotransferase (encoded by *GAL3ST2*), and carbohydrate sulfotransferase (encoded by *CHST5*) are implicated.[10,11]

In GCs under stress, unfolded protein response (UPR) activation might occur due to inefficient protein folding in the ER.[12] The activation of this group of intracellular signaling pathways can drive GCs to stress responses or toward apoptosis when malfunction is irreversible.[13,14] Genes such as *HSPA5* and *XBP1* participate in this UPR.[15] *HSPA5* codes for the ER chaperone BiP, member 5 of the heat shock protein 70 (Hsp70) family. BiP acts mainly as a sensor of unfolded proteins activating the UPR and the subsequent downstream signaling.[16] The

XBP1 gene codes for the X-box binding protein 1, a transcription factor that belongs to the inositol-requiring enzyme 1α(IRE1α)-signaling axis.[17] Inflammation can also activate ER stress.[18] Intestinal inflammatory disorders such as inflammatory bowel disease (IBD) have been related to ER stress.[19] Under these inflammatory conditions, impairment of GCs function such as aberrant mucin folding has been observed.[20]

Chronic consumption of Western diet, characterized by low intake of dietary fibers, favor the development of intestinal inflammatory disorders,[21–24] whereas, regular consumption of dietary fibers has been associated to a low inflammatory state and to a strengthened barrier function of the mucus layer.[25–27]

An important group of dietary fibers are fructans. Fructans are mainly plant-derived water-soluble oligo and polysaccharides.[28,29] Fructans from chicory plant are broadly used in Europe in the food industry.[30] Chicory fructans are composed of fructofuranose molecules linked by β(2 → 1) bonds with a glucose moiety at the reducing end, which confers them a linear structure.[31] These chicory fructans are known as inulin-type fructans (ITFs).[32] In Latin America, fructans from agave plants are used as fiber supplements in foods.[33,34] These agave fructans are structurally different from ITFs since besides β(2 → 1) fructose chains, they also contain fructose units linked by β(2 → 6) bonds.[35] These β(2 → 1)-β(2 → 6) bonds gives them a branched complex structure.[36,37] This type of fructans are named graminan-type fructans (GTFs).[38]

It is unknown whether ITFs and GTFs have an impact in GCs on mucus-related genes in presence and absence of inflammatory or ER-stress. Therefore, in this study we aimed to determine whether ITFs and GTFs have an influence on the expression of mucus-related and ER stress-related genes in GCs. We also tested if ITFs and GTFs were capable to protect, and attenuate inflammatory responses of GCs exposed to disruptor agents.

2. Materials & methods

2.1 Fructans

To study the effect of fructans of different sizes with a branched or linear structure, on the expression of mucus-related and ER stress-related genes in gut GCs, β(2 → 1)–β(2 → 6) GTFs and β(2 → 1) ITFs were used. GTFs were extracted from *Agave tequilana* Weber blue variety and were provided by Nekutli™, Guadalajara, Mexico. ITFs Frutafit™ CLR and Frutafit™ TEX! were studied as well. Both ITFs were provided by Sensus™ (B.V. Roosendaal, The Netherlands). These fructans have been previously studied by our group.[39–42]

2.2 Cell culture

To study the effects of fructans on the expression of mucus-related and ER stress-related genes in gut GCs, the human colorectal adenocarcinoma-derived LS174T cell line, which exhibits a GC-like phenotype (ATCC® CL188™, Manassas, USA) was used. GCs were maintained in T-75 flasks at 37 °C in humidified air and 5% CO_2 and cultured in Eagle Minimum Essential Medium (EMEM, Lonza, MD, USA) supplemented with 10% heat-inactivated fetal bovine Serum (Sigma-Aldrich, Zwijndrecht, The Netherlands), 2 mM L-glutamine (Lonza, MD, USA) and penicillin/streptomycin 50 U mL^{-1} and 50 μg mL^{-1} (Gibco, Paisley, UK). When reached ~80% confluency and after treatment with 10% trypsin-EDTA (Sigma-Aldrich, Zwijndrecht, The Netherlands), GCs were passaged. GCs were used for experiments between passages 30–40.

2.3 Effect of ITFs and GTFs on the expression of intestinal mucus- and ER stress-related genes

Freshly resuspended GCs were seeded at a cell density of 3×10^5 cells per mL in standard 24-well plates (Corning, NY, USA) and cultured at 37 °C and 5% CO_2 until they reached ~80% confluency (2–3 days) (Fig. 1A). At the experiment day, the medium was replaced with 1 mL medium containing 10 mg mL^{-1} of endotoxin-free GTFs and ITFs, followed by incubation for 72 hours under homeostatic conditions (without any stressor) (Fig. 1B). This incubation time was selected based on previous observations by our group and others that the expression of the studied genes on GCs increased with incubation time up to 72 hours.[43,44] Untreated cells (only medium) were used as untreated controls. At the end of experiments, the morphology of controls as well as the cells under the different treatments were studied at light microscope to exclude possible morphological changes.

2.4 Protective effect of fructans from induced inflammation, mucus barrier disruption and ER stress on GCs

To study whether fructans can protect from induced inflammation, mucus barrier disruption and ER stress, GCs were exposed to 10 ng mL^{-1} of the pro-inflammatory cytokine TNFα

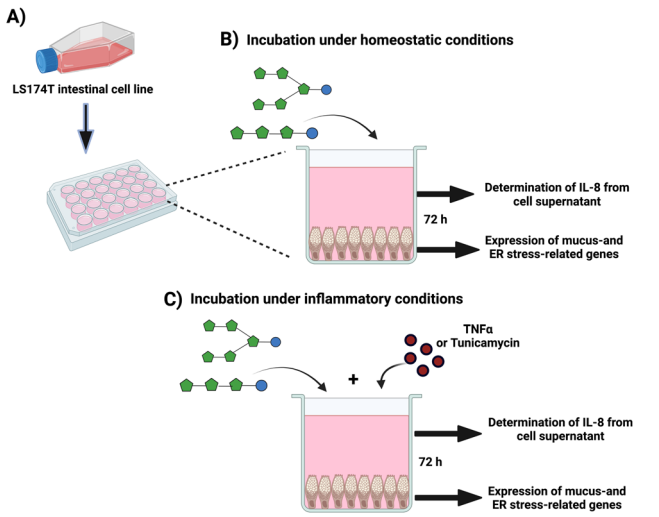

Fig. 1 Experimental design. (A) Confluent goblet cells were seeded in 24-well plates. (B) After a 72-hour incubation of cells in presence of ITFs or GTFs the expression of mucus- and ER stress-related genes was determined by qPCR. The production of IL-8 was quantified from GCs supernatants. (C) GCs were incubated for 72 hours with TNFα or Tm in presence of ITFs or GTFs. Afterwards, expression of mucus- and ER stress-related genes, as well as IL-8 production were determined.

(Immunotools, Friesoythe, Germany), or to 1 μg mL^{-1} of the *N*-glycosylation inhibitor tunicamycin (Tm) (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 72 hours, according to the previously standardized and described by our group.[44,45] These molecules are known to dysregulate the expression of mucus- as well as ER-stress related genes in gut GCs.[46,47] In a second set of experiments, GCs were incubated for 72 hours with TNFα or Tm in presence of 10 mg mL^{-1} of fructans (Fig. 1C). Untreated cells (only culture medium) were used as negative controls. To calculate the fold-change in these experiments, cells treated with disruptor (*i.e.*, Tm or TNF) where used as reference, thus data was normalized using the disruptors as reference conditions. At least five independent experiments were performed. After the experiments, cells were prepared for RNA isolation with trizol reagent.

2.5 RNA extraction, and quantitative reverse-transcription polymerase chain reaction PCR (qPCR)

We studied the relative mRNA expression of the mucus-related genes *MUC2*, *TFF3*, and *RETNLB*. We also studied the *CHST5* and *GAL3ST2* genes which are associated to posttranslational modification of mucin. Finally, on these GCs we studied the expression of the genes *HSPA5* and *XBP1*, which are related to control of protein maturation, ER-stress, and UPR.[48,49] After incubation under the different experimental conditions, RNA was isolated from GCs by the addition of 500 μL of TRIzol™ reagent (Life Technologies, Bleiswijk, The Netherlands) following the manufacturer's instructions. Followed by RNA quantitation, which was performed by spectrophotometry (Thermo Fisher Scientific, USA). The RNA integrity was determined by gel electrophoresis on a 2% agarose gel. Afterwards, 500 ng of total RNA were used for cDNA synthesis, which was performed with SuperScript II™ reverse transcriptase (Life Technologies, Bleiswijk, The Netherlands) according to the manufacturer's instructions. Briefly, after pre-treatment with DNAse I, RNA samples were used for a retro-transcription reaction with 300 ng of random hexamer primers (Life Technologies, Bleiswijk, The Netherlands),10 mmol L^{-1} of DNTPs (Thermo Fisher Scientific, USA), 5× first strand buffer, 0.1 mM of DTT, 40 units per μL of recombinant ribonuclease inhibitor (Thermo Fisher Scientific, USA) and 200 units of SuperScript II™ reverse transcriptase. This was followed by incubation at 25 °C for 10 minutes, 42 °C for 50 minutes and 70 °C for 15 minutes using a TProfessional Basic Thermocycler (Biometra, Göttingen, Germany). The total cDNA obtained was diluted 20x with DEPC-treated water (Life Technologies, Bleiswijk, The Netherlands) and 5 μL of this dilution were mixed with 5 μL of FastStart Universal SYBR Green Master (ROX) qPCR Master Mix 2× concentrated (Roche Diagnostics, Basel, Switzerland) including gene specific primers at a final concentration of 300 nmol L^{-1} (Table S1†).[44,45,50] The qPCRs were performed in a Viia 7 Real-Time PCR System (Applied Biosystems, CA, USA). The cycling protocol used is shown in Table S2.† The primer annealing temperature and efficiency were determined with a 5-point standard curve, which was prepared with a pool of all cDNA samples diluted 5–80×. A reaction efficiency of 90–110% was considered acceptable. B2M (beta-2-microglobulin) was used as housekeeping gene.[43,46] mRNA fold-change was calculated against untreated controls or against cells treated only with disruptors using the $2^{-\Delta\Delta Ct}$ method.[51]

2.6 Measurement of GCs IL-8 production

Interleukin-8 (IL-8) from cell supernatants was considered as a marker of inflammation of GCs challenged with the disruptor molecules TNFα and Tm. After the incubation of GCs under the above-mentioned conditions, cells supernatants were collected and centrifuged for removing death cells and cell debris. The IL-8 was quantitated by ELISA from GCs supernatants according to manufacturer's instructions (R&D Systems, Biotechne, Minneapolis, USA). Controls were those GCs without any treatment.

2.7 Statistical analyses

GraphPad Prism version 9.2 was used for statistical analyses. Normal distribution of data was examined by the Shapiro–Wilk test. These data were normally distributed. Therefore, data are expressed as mean ± standard deviation (SD) from least five independent assays. Untreated control and those cells incubated with fructans were compared. Untreated control and cells treated with TNFα or Tm were also compared. Finally, cells treated with TNFα alone and those treated with TNFα in presence of fructans were compared. In all experiments statistical significance was determined using ordinary one-way ANOVA with Dunnett's multiple comparison test. A p-value < 0.05 was considered significant. * or † ($p < 0.05$), ** or †† ($p < 0.01$), *** or ††† ($p < 0.001$), **** or †††† ($p < 0.0001$).

3. Results

3.1 Chemical characteristics of studied fructans

To investigate the effect of fructans with different structure and size on the expression of mucus-related and USP-related GCs genes, GTFs and ITFs were included. The presence of β(2 → 1) and β(2 → 6) linkages in GTFs confers them a complex branched structure.[37] GTF I is a mixture with mostly short chain fructans with a degree of polymerization (DP) between 3–4 but it also has fructans of DP 7–45. GTF II contains a mixture of longer chain fructans with an average DP of 17, but it also has longer chain fructans. These GTFs possess oligosaccharides of fructose (F_n) and glucose-fructose (GF_n) series.[41] ITFs are constituted only of fructans linked by β(2 → 1) bonds, this gives them a linear structure. ITF I is a short chain fructan mixture with a DP range of 3–10. It has fructans of the F_n and GF_n series. ITF II possess a mixture composed of longer chain fructans with a DP range of 10–60 and it only has fructans of the GF_n series (Table 1).[39]

3.2 Graminan-type fructans enhance mucus-related genes in GCs

To study the effects of ITFs and GTFs on the expression of mucus-related genes, GCs were incubated in presence of these

Table 1 Chemical and structural characteristics of fructans included in the study[39,41]

	Branched graminan-type fructan	Linear inulin-type fructan	DP range
ITF I	Absent	Present	3–10
ITF II	Absent	Present	10–60
GTF I	Present	Present	3–45
GTF II	Present	Present	3–60

● = glucose, ⬟ = fructose, *n* = number of fructose units. The schemes of fructans were made following the standardized symbol nomenclature for glycans (National Institute of Biotechnology, **https://www.ncbi.nlm.nih.gov/glycans/snfg.html**, consulted on December 16th, 2021).

fructans for 72 hours. This provoked a differential gene expression in a fructan structure dependent manner (Fig. 2). The expression of *MUC2* was 0.38 ± 0.3-fold decreased (p > 0.01) and 0.48 ± 0.1-fold decreased (p > 0.001) by ITF I and ITF II, while none of the GTFs influenced *MUC2* expression (Fig. 2A). This was different for *TFF3*, where an enhancement of 0.52 ± 0.3-fold (p < 0.05) and 0.52 ± 0.4-fold (p < 0.05) was observed in GCs treated with ITF I and GTF II, respectively (Fig. 2B). The *RETNLB* expression was strikingly enhanced only by the branched fructans. GTF I enhanced it 6.3 ± 2-fold (p < 0.01) and GTF II enhanced it 9.37 ± 1-fold (p < 0.0001) (Fig. 2C). The *CHST5* expression was only enhanced 0.25 ± 0.4-fold (p < 0.05) by GTF II (Fig. 2E). Conversely, the expression of *HSPA5* and *XBP1* were significantly reduced by all fructans except by ITF I (Fig. 2F and G).

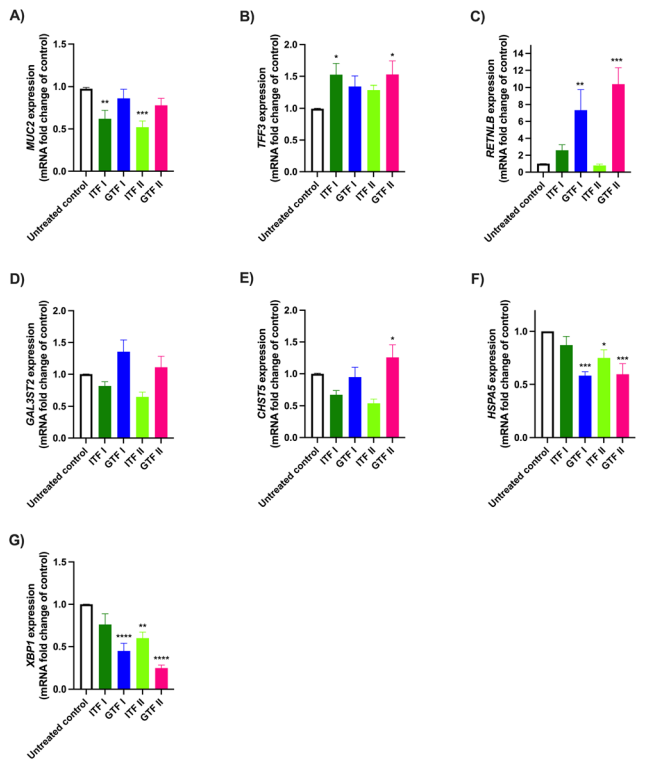

Fig. 2 Effect of ITFs and GTFs with different molecular weight on the expression of mucus- and ER stress-related genes in goblet intestinal cells. GCs were incubated for 72 hours with 10 mg mL^{-1} of ITFs and GTFs, followed by the determination of mRNA production of *MUC2* (A), *TFF3* (B), *RETNLB* (C), *GAL3ST2* (D), *CHST5* (E), *HSPA5* (F), and *XBP1* (G) by qPCR. At least five independent experiments were performed. These data were normally distributed. Therefore, data are expressed as mean ± SD. Statistical differences between untreated control and cells incubated with fructans were calculated by ordinary one-way ANOVA with Dunnett's multiple comparison test. * (p < 0.05), ** (p < 0.01), *** (p < 0.001), **** (p < 0.0001).

3.3 Long chain GTF prevents TNFα-induced dysregulation of mucus-related genes in GCs

We investigated the potential protective effect of ITFs and GTFs on mucus- and ER stress-related genes in GCs exposed to TNFα. It is known that TNFα alters the expression of secretory proteins in GCs.[46] To study the prevention of this impairment, GCs were incubated with TNFα in presence of the studied fructans for 72 hours. Compared with the untreated control *MUC2* expression was 1.7 ± 0.7-fold decreased by TNFα (p < 0.01) (Fig. 3A). In cells incubated with TNFα in presence of GTF II this increase was 0.3 ± 1.5-fold attenuated compared with cells challenged with TNFα (p < 0.05). The remaining fructans did not significantly prevent the reduction of *MUC2* expression.

Compared with the untreated control, TNFα significantly decreased the expression of *TFF3* 1.5 ± 0.9-fold (p < 0.01) (Fig. 3B). This decrease was 0.3 ± 0.9-fold lower and thus partly prevented by GTF II (p < 0.05). The other studied fructans did not prevent the reduction of *TFF3* expression.

The *RETNLB* expression was 4.6 ± 0.7-fold decreased in cells treated with TNFα compared with the untreated control (p < 0.001). All the studied fructans except ITF II prevented this decrease and they even enhanced *RETNLB* expression. Cells incubated with TNFα in presence of GTF II had a better prevention of TNFα-induced decrease of *RETNLB* expression as it was 13.2 ± 3-fold higher compared with cells challenged with TNFα (p < 0.0001) (Fig. 3C).

TNFα decreased the expression of *GAL3ST2* and *CHST5* 0.4 ± 0.1-fold (p < 0.01) and 2.8 ± 0.9-fold (p < 0.001) respectively (Fig. 3D and E). These genes are related to mucin posttranslational modifications. The only fructan that significantly prevented this decrease was GTF II. Neither GTF I nor ITFs prevented the decreased production of *GAL3ST2* and *CHST5* mRNA.

The expression of the ER stress-related genes *HSPA5* and XBP1 was significantly decreased in cells treated with TNFα 1.4 ± 0.5-fold (p < 0.0001) and 1.8 ± 0.4-fold compared with the untreated control (p < 0.0001) (Fig. 3F and G). None of the studied fructans prevented this induced decrease.

3.4 ITFs and GTFs do not prevent Tm-induced changes in mucus-related genes in GCs

The preventive effect of fructans from the deleterious action of the *N*-glycosylation inhibitor Tm was studied. To that end, GCs

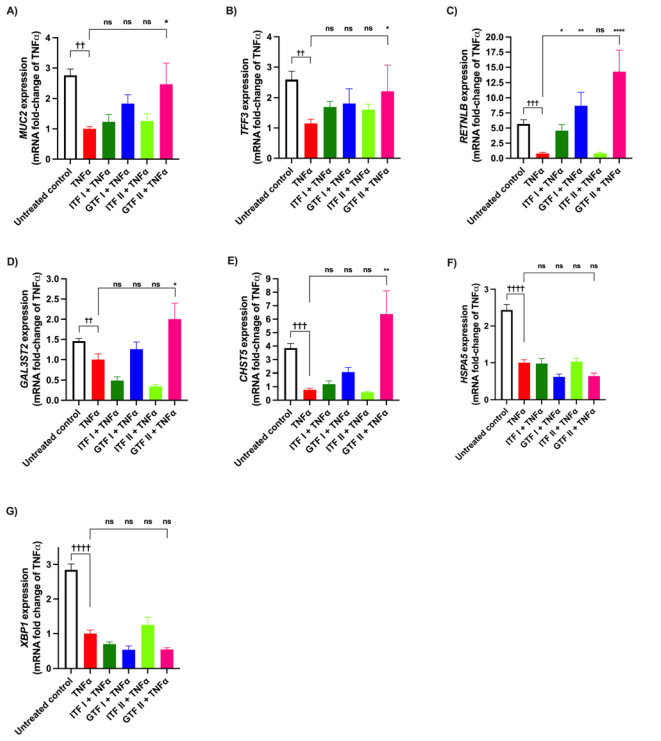

Fig. 3 Prevention by ITFs and GTFs of TNFα-induced mucus- and ER stress-related gene dysregulation in goblet cells. GCs were incubated for 72 hours with TNFα in presence of 10 mg mL^{-1} of ITFs or GTFs. Expression of *MUC2* (A), *TFF3* (B), *RETNLB* (C), *GAL3ST2* (D), *CHST5* (E), *HSPA5* (F), and *XBP1* (G) was determined by qPCR. At least five independent experiments were performed. These data were normally distributed. Therefore, data are expressed as mean ± SD. Statistical differences between untreated control and cells treated with TNFα were calculated by ordinary one-way ANOVA with Dunnett's multiple comparison test. Cells treated with TNFα alone and those treated with TNFα in presence of fructans were compared and statistical differences were calculated by ordinary one-way ANOVA with Dunnett's multiple comparison test. * or † ($p < 0.05$), ** or †† ($p < 0.01$), *** or ††† ($p < 0.001$), **** or †††† ($p < 0.0001$).

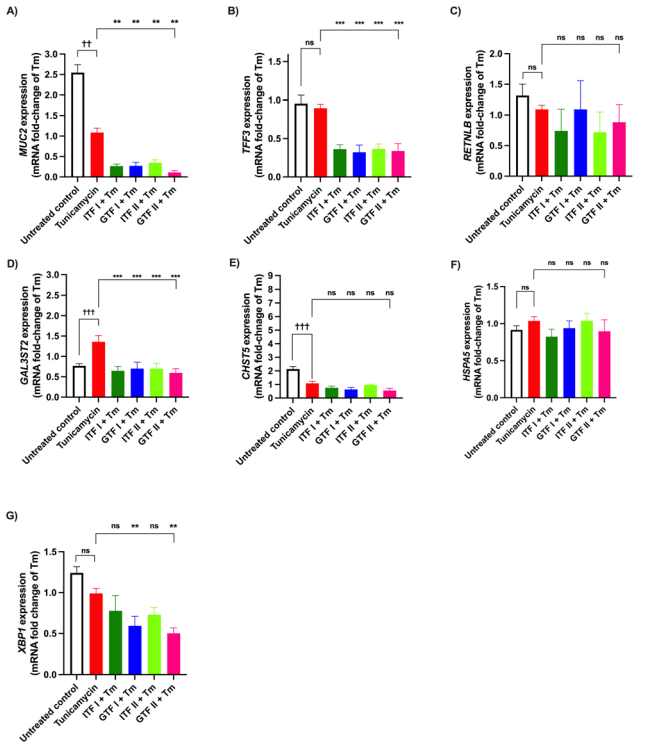

Fig. 4 Prevention by ITFs and GTFs of Tm-induced mucus- and ER stress-related gene dysregulation in goblet cells. GCs were incubated for 72 hours with Tm in presence of 10 mg mL^{-1} of ITFs or GTFs. Expression of *MUC2* (A), *TFF3* (B), *RETNLB* (C), *GAL3ST2* (D), *CHST5* (E), *HSPA5* (F), and *XBP1* (G) was determined by qPCR. At least five independent experiments were performed. These data were normally distributed. Therefore, data are expressed as mean ± SD. Statistical differences between untreated control and cells treated with Tm were calculated by ordinary one-way ANOVA with Dunnett's multiple comparison test. Cells treated with Tm alone and those treated with Tm in presence of fructans were compared. Statistical differences were calculated by ordinary one-way ANOVA with Dunnett's multiple comparison test. * or † ($p < 0.05$), ** or †† ($p < 0.01$), *** or ††† ($p < 0.001$), **** or †††† ($p < 0.0001$) (Tm = Tunicamycin).

were incubated with Tm for 72 hours in presence of fructans (Fig. 4). The *MUC2* expression was 1.5 ± 0.6-fold decreased compared with the untreated control ($p < 0.01$) (Fig. 4A). None of the studied fructans prevented this decrease.

Compared with the untreated control the expression of *TFF3* and *RETNLB* was not influenced by the Tm challenge (Fig. 4B and C).

Tm treatment provoked a 0.6 ± 0.1-fold increase of the *GAL3ST2* expression, compared with the untreated control ($p < 0.001$) (Fig. 4D). All of the studied fructans provoked a *GAL3ST2* expression more likely to that of the untreated control.

Compared with the untreated control a 1.0 ± 0.5-fold decrease of the *CHST5* expression was observed in cells treated with Tm ($p < 0.001$) (Fig. 4E). None of the studied fructans influenced this decrease.

The mRNA production of *HSPA5* remained without significant changes after the Tm challenge. The same occurred when incubating GCs with Tm in presence of fructans (Fig. 4F).

Compared with the untreated control the *XBP1* expression was not influenced by the Tm challenge alone or in presence of fructans (Fig. 4G).

3.5 GTF I prevent TNFα-induced IL-8 increase in GCs

Next, the effect of fructans on the IL-8 production by GCs was investigated. To that end, GCs were incubated with fructans for 72 hours. Fig. 5A shows that ITF I as well as GTF I significantly decreased the IL-8 production by GCs compared with the untreated control, from 1322 ± 107 pg mL^{-1} to 885 ± 176 pg mL^{-1} ($p < 0.05$) for those cells treated with ITF I and to 888 ± 278 pg mL^{-1} for GTF I treated cells ($p < 0.05$).

Next the protective effect of fructans on IL-8 production by GCs exposed to TNFα or Tm was studied. To this end, GCs were incubated for 72 hours with TNFα or Tm in presence of 10 mg mL^{-1} of fructans. The IL-8 production was quantified from the GCs supernatant. The presence of TNFα alone increased IL-8 production from 1322 ± 107 pg mL^{-1} in the

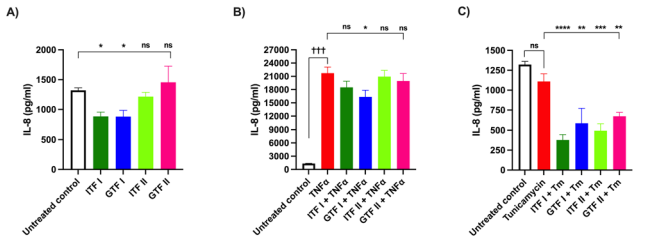

Fig. 5 Production of IL-8 by goblet cells. (A) Cells were incubated with either ITF I, ITF II, GTF I or GTF II for 72 hours, followed by quantification of IL-8 production from cell supernatants. At least five independent experiments were performed. These data were normally distributed. Therefore, data are expressed as mean ± SD. Statistical differences between untreated control and cells incubated with fructans were calculated by ordinary one-way ANOVA with Dunnett's multiple comparison test. (B and C) In a second set of experiments, GCs were incubated for 72 hours with TNFα or tunicamycin alone or with TNFα or tunicamycin in presence of 10 mg mL^{-1} of ITFs or GTFs. At least five independent experiments were performed. These data were normally distributed. Therefore, data are expressed as mean ± SD. Statistical differences between cells treated only with disruptors and cells incubated with TNFα or tunicamycin in presence of 10 mg mL^{-1} of ITFs or GTFs were calculated by ordinary one-way ANOVA with Dunnett's multiple comparison test. * or † ($p < 0.05$), ** or †† ($p < 0.01$), *** or ††† ($p < 0.001$), **** or †††† ($p < 0.0001$) (Tm = tunicamycin).

untreated control to 21 716 ± 3100 pg mL^{-1} ($p < 0.001$) (Fig. 5B). This increased IL-8 production was decreased to 16 336 ± 3383 pg mL^{-1} ($p < 0.05$) by GTF I. The other studied fructans did not influence the over IL-8 production of GCs under TNFα challenge.

The challenge of GCs with Tm did not influence the IL-8 concentration (Fig. 5C). However, incubation of cells with Tm in presence of fructans caused a significant decrease in IL-8 production to values even lower than those of the untreated control. The production of IL-8 decreased from 1109 ± 192 pg mL^{-1} in cells treated with Tm to 378 ± 135 ($p < 0.0001$), 588 ± 316 ($p < 0.01$), 499 ± 174 ($p < 0.001$) and 671 ± 97 ($p < 0.01$) in cells pre-incubated with ITF I, GTF I, ITF II and GTF II, respectively.

4. Discussion

In the present study we present, to the best of our knowledge, for the first time the *in vitro* effect of ITFs and GTFs on the expression of mucus- and ER stress-related genes under homeostatic and inflammatory conditions and their regulatory capacities in gut GCs. We found a remarkable beneficial impact of GTFs especially on the expression of *RETNLB*. It was unknown whether and to what extend these fructans could protect from the action of TNFα and Tm. Here we demonstrate that the branched long chain GTF II can protect from the gene expression dysregulation induced by TNFα. We also show that all the studied fructans prevent the Tm-induced dysregulation of *GAL3ST2*. Interestingly, only the short chain fructans have anti-inflammatory properties on GCs under homeostatic conditions. All these benefits found are fructan-structure and chain length dependent.

When tested under homeostatic conditions, ITF I and GTF II significantly increased the *TFF3* expression. TFF3 belongs to the trefoil family of secretory peptides whose main function is mucosal protection and repairment of damaged epithelium.[52] This integral and constituent mucus protein has shown to possess therapeutic potential, since its interaction with protease activator receptors (PAR) cause downregulation of proinflammatory cytokines such as IL-6 and IL-8 and upregulation of defensin expression in intestinal epithelial cells.[53] Moreover, when GCs were challenged with TNFα, the expression of *TFF3* significantly decreased. This effect, which is in line with others,[54] was prevented by GTF II. Therefore, the prevention of *TFF3* downregulation by GTF II could have an application under inflammatory conditions, such as IBD or gastrointestinal infections. Additionally, due to its protective and reparative functions, TFF3 has been proposed as a therapeutic agent for intestinal inflammatory disorders such as colitis and IBD.[54,55] Furthermore, the anti-inflammatory capacity of TFF3 goes beyond the gut since it has been found in microglia.[56]

Interestingly, the expression of *RETNLB* was the most up regulated among the studied genes, and this was observed only with the branched fructans GTF I and GTF II under homeostatic conditions. When GCs were stimulated with TNFα *RETNLB* expression downregulation was observed. This was prevented by all fructans, except ITF II. *RETNLB* codes for RELMβ protein which belongs to the wide repertory of intestinal antimicrobial peptides.[57] The intestinal secretion of this cysteine-rich 12.5 kDa protein increases in animal models during helminth infection[58,59] and other enteric pathogens[60] to promote their expulsion.[59] It is also known that RELMβ can inhibit parasite chemotaxis and interferes with their nutrition by directly binding to their chemosensory constituents.[61,62] Therefore, the increased *RETNLB* expression caused by GTFs could be considered as a defensive factor to aid during intestinal infections.

Under homeostatic conditions the *CHST5* expression was increased only by GTF II. This is one of the proteins that participate in the decoration of the mucin protein core by *O*-glycosylations.[63] The increased expression of *CHST5* has been previously associated with enhancement of the mucosal barrier function in LS174T cells.[10] Also, this may imply that cells with higher expression of *CHST5* may be able to deal better with ER stress, since an increase in this enzyme may help to relieve the proteins that, due to poor folding or altered posttranslational modifications, got stuck in the ER. This might explain too why cells challenged with Tm in presence of GTF II have lower markers of ER stress. Thus, GTF II could represent a potential molecule for strengthening mucus barrier function.

The expression of the ER stress-related gene HSPA5 was decreased only by GTFs under homeostatic conditions. During homeostasis the protein product of *HSPA5* BiP acts as a repressor of the UPR signal transducers inositol-requiring protein-1

(IRE1) and protein kinase RNA (PKR)-like ER kinase (PERK) by forming a complex. Upon ER stress BiP dissociates from the complex, provoking activation of the UPR with subsequent downstream signaling to restore ER homeostasis.[49,64] Since the activation of the UPR signal depends on the repressor action of BiP, then BiP overexpression keeps UPR repressed, whereas reduced BiP levels activate the UPR.[64] Thus, the decreased expression of *HSPA5* that we observed would be indicating a more efficient capacity of counteraction of ER stress. However, herein we are presenting preliminary data about the influence of GTFs on UPR at mRNA level, and additional assessments of BiP protein might further support a complete insight into the role of GTFs in this pathway.

Under homeostatic conditions the expression of the ER stress-related gene *XBP1* was decreased by all fructans, except ITF I. The downregulation of these ER-stress- and UPR-related gene has been previously reported by Engevik *et al.*, as an ER-stress modulatory beneficial effect, which was observed when incubating T84 human colonic cells with a probiotic organism.[65]

The same was observed by Xiong *et al.* when incubating GCs with a flavonoid molecule.[66] It is known that UPR activation is associated with inflammation[18] and to failure in immunotolerance with the concomitant development of autoimmune disorders such as IBD and rheumatoid arthritis.[67–69] Moreover, genetic sequencing of IBD patients revealed several *XBP1* variants as factors of susceptibility for IBD development.[19] Thus, the use of these fructans for the prevention of UPR activation could be valuable for the design of therapeutics against these disorders.

Incubation of GCs with Tm and TNFα-induced downregulation of the *XBP1* mRNA. Upon ER stress, *XBP1* mRNA suffers unconventional splicing to obtain an active *XBP1* spliced form.[17] Previous studies from Xue *et al.*, who studied the expression of *XBP1* on the murine fibrosarcoma L929 cell line, demonstrated that under homeostatic conditions there is a basal expression of both the unspliced and spliced forms of *XBP1*. When cells are exposed to ER stress by the action of the Tm, then the proportion of the spliced form increases until practically a complete predominance of this spliced form. This leads to the disappearance of the unspliced *XBP1* form.[70]

These findings of Xue, *et al.*, let us hypothesize that in our study we indirectly found that the *XBP1* spliced form increases as the observed unspliced form decreases. In other words, under homeostatic conditions we could expect the *XBP1* spliced form in the same proportion as the unspliced form (untreated control), and in those cells challenged with Tm we would expect the double proportion of the *XBP1* spliced form than the observed unspliced form. Finding the spliced form in major proportion would indicate a more efficient counteraction of ER stress. As to the cells challenged with TNFα, again based on Xue, *et al.* findings, the predominance of the unspliced and spliced forms of *XBP1* change practically in the same manner as the challenge with Tm. Therefore, our findings indicate a more efficient manner to counteract the ER stress induced by TNFα.

It is known that TNFα can induce the UPR in a reactive oxygen species (ROS)-dependent fashion.[70] In the present study the measurement of ROS was not determined, however, since it is known that fructans from chicory and from agave possess ROS scavenging capacities,[71–73] assessment of ROS after exposure to fructans, in presence or absence of TNFα, along with other inflammatory stressors deserves further studies.

Incubation of GCs in presence of Tm significantly downregulated the expression of *MUC2* and *CHST5 and* increased the expression of *GAL3ST2*. This is in line with our previous studies.[44,45] Tm is a well-known glycosylation inhibitor which induces ER stress.[47] None of the fructans protected GCs from the downregulating effect of Tm. This could be indicative of the null capacity of these fructans to protect from the deregulatory effects of Tm under the studied experimental conditions.

The production of IL-8 was considered to be an indicator of inflammation in GCs. When GCs were incubated in presence of fructans, the short chain ITF I and GTF I significantly decreased this IL-8 production. Thus, these fructans could be protecting GCs from inflammation. We have previously observed this protective effect in gut epithelial cells.[42] The challenge with TNFα alone caused a 16-fold increase of IL-8 production, while this TNFα challenge in presence of GTF I significantly decreased this elevation. IL-8 is a chemoattractant molecule for immune cells such as neutrophiles, whose excessive production is related to inflammatory disorders.[66] This, allow us to suggest that GTF I possess a potential anti-inflammatory effect, that could contribute to lowering symptoms of intestinal inflammatory disorders.

A possible explanation of all these findings (Fig. 6) could be related to the establishment of an interaction between the fructans applied and GCs just as observed in our previous studies,[41,42] which may lead to lowering permeability of the mucus layer, due to the saccharide nature of fructans, which resembles the glycans that are added to mucins.

However, a deeper study of the mechanisms by which the studied fructans influence GCs biology is guaranteed. In line with this is the fact that LS174T cells compared to normal GCs synthesize glycans that are truncated compared to normal MUC2 glycosylation.[74] This might make these cells prone to enteropathogenic infection, as has been reported in these cells.[75] Thus, we speculate that the studied fructans could have a beneficial influence on the barrier function of mucins with truncated glycosylation by replacing the lack of glycans.

Besides the direct protecting and regulatory effects of the studied fructans on GCs presented herein, it is important to acknowledge that there may also be substantial effects that are mediated by the intestinal microbiota and the metabolites they produce from fructans fermentation. This well-known prebiotic effect[76–78] remains to be elucidated on GCs.

One limitation of the present study is the absence of complementary assessment of the proteins coded by the studied genes. However, since the influence of fructans derived from agave on intestinal goblet cells has never been studied, we consider our preliminary data on mRNA data is still of value,

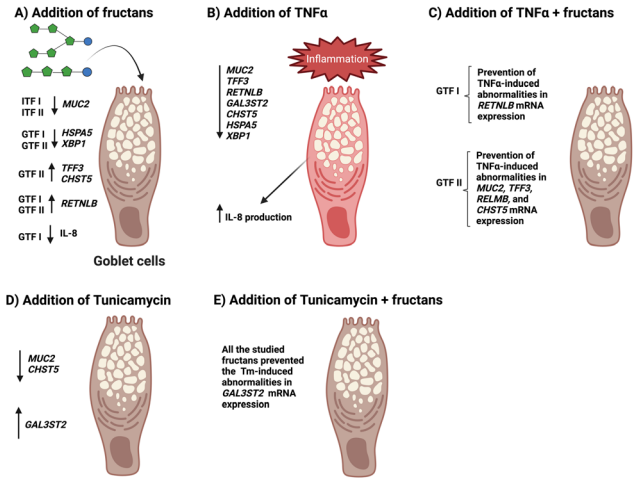

Fig. 6 Schematic summary of the observed effects of ITFs and GTFs and of fructans + disruptors on mucus- and ER stress-related GCs genes.

although further additional assessments of the protein products of the studied genes might provide a wider understanding of the potential beneficial effects of these dietary fibers on cellular homeostasis in GCs. Another limitation is the lack of assessment of the expression of other mucin coding genes than MUC2, such as MUC5AC, MUC6 and MUC1, MUC4 and MUC13, since LS174T cells do produce these mucins which also represent challenges for the ER secretory pathway.[20,79]

5. Conclusions

Findings of the present study contribute to enhanced knowledge on beneficial effects that dietary fibers such as ITFs and GTFs can exert on the preservation of GC-homeostasis. It is shown that the beneficial effects are dependent on the structure and chain length of the fructan applied. This, knowledge combined with finding from our previous studies[39–42] allow us to propose specific fructan molecules as potential candidates for the design of therapies for the reestablishment of health in gut inflammatory disorders.

Author contributions

C.F.L. and P.D.V. designed the study. C.F.L. and M.A.D.S. performed the experiments. L.A.S.L. conceptualization and assisted in the qPCR data analysis. G.L.V. revised and edited the manuscript. P.D.V. Supervised and Administered the project. C.F.L. and P.D.V. wrote the manuscript. All authors have revised and improved the manuscript.

Conflicts of interest

There are no conflicts to declare.

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