Journal of Functional Foods 48 (2018) 507–514

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Temperature-treated gluten proteins in Gluten-Friendly™ bread increase mucus production and gut-barrier function *in human intestinal goblet cells*

Carmela Lamacchia[a,*], Daniela Musaico[a,b], Mary E. Henderson[b], Triana Bergillos-Meca[b], Morgane Roul[b], Loretta Landriscina[a], Ivana Decina[a], Giulia Corona[b,1], Adele Costabile[b,1]

[a] Department of the Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy
[b] Life Sciences Department, Whitelands College, University of Roehampton, London, United Kingdom

ARTICLE INFO

Keywords:
Gluten-Friendly bread
HT29 cell line
Intestinal permeability
Mucus production
Trans-epithelial electrical resistance

ABSTRACT

The effects of a control bread (CB) and a Gluten Friendly™ bread (GFB) on intestinal epithelium mucus production and barrier function in healthy human mucus-secreting goblet cells HT-29-16E were investigated. Mucus production in cells exposed to digested breads (GFB and CB) was preliminarily investigated using staining techniques, Periodic Acid-Schiff (PAS) and Alcian blue (AB), and MUC2 and MUC3 were also quantified by ELISA assay. The barrier function of the cell monolayer was evaluated by trans-epithelial electrical resistance (TEER) measurements. GFB increased the secretion of mucins, expressed as the level of PAS and AB staining in comparison with the control. MUC3 levels were not affected, whereas higher MUC2 concentrations ($P < 0.01$) were found on cells treated with GFB compared to the control. Additionally, significantly higher TEER values were observed after treatment with both CB and GFB in comparison with the control ($P < 0.01$), with GFB having a significantly higher effect than CB ($P < 0.01$).

1. Introduction

The intestinal epithelial layer is a physical and biochemical barrier, formed by a monolayer of cells that define the boundary between tissues of the intestine and the external environment. Intestinal epithelial layer integrity confers protection against luminal antigens (Lundin & Sollid, 2014). The intestinal goblet cells are specialized secretory cells that are found in the epithelial layer. They are responsible for the production of mucins, which create a first layer of defence (Kim & Ho, 2010; Peterson & Artis, 2014) against intrusion of large particles and bacteria into the epithelial cell layer (Turner, 2009). The most external part, the non-adherent mucus layer, predominantly contains glycoproteins, including the gel-forming mucins MUC2, MUC5AC, MUC6 and MUC5B (Pelaseyed et al., 2014). The inner adherent layer contains transmembrane mucins, predominantly MUC3, MUC1, and MUC17 (Pelaseyed et al., 2014). Goblet cells secrete both the main gel-forming mucins, MUC2, and the transmembrane-bound mucins (Kim & Ho, 2010). These mucins are thought to additionally play an active role in regulating the porosity and permeability of the epithelial membrane (Peterson & Artis, 2014).

Below the mucous layers, the intestinal epithelial cells form a tight, continuous physical barrier, where adjacent epithelial cells are sealed together by tight junctions and adherens junctions (Schumann, Siegmund, Schulzke, & Fromm, 2016). These multi-protein tight junction complexes that join together the intestinal epithelial cells create a selectively permeable seal, marking out the divide between apical and basolateral membrane domains (Kim & Ho, 2010; Lee, 2015). By regulating the intestinal epithelial barrier function and its selective permeability, tight junction complexes determine the rate of flux of molecules across the epithelium (Wells et al., 2016).

A disturbed intestinal epithelial barrier function has been found in coeliac disease (CD) patients (Schulzke, Bentzel, Schulzke, Riecken, & Fromm, 1998), even after treatment, due to both reduced mucus secretion and increased intestinal cell permeability (Smecuol et al., 1997). This weakened gut barrier is more susceptible to pathogens (Schulzke et al., 1998) and potentially antigenic macromolecules, such

Abbreviations: CD, coeliac disease; CB, control bread; GFB, Gluten-Friendly™ bread; GFD, Gluten-free diet; PAS, Periodic Acid-Schiff; AB, Alcian Blue; TEER, Trans-Epithelial Electrical Resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IBD, Inflammatory Bowel Diseases; IBS, (Irritable Bowel Syndrome); *nd*, *not determinable*; *ds*, *dry substance*; W, *dough strength*; *P*, *dough toughness*; *L*, *dough extensibility*; *A*, *water absorption*; *B*, *dough developing time*; *CD*, *dough stability*

* Corresponding author.
E-mail address: carmela.lamacchia@unifg.it (C. Lamacchia).
[1] These authors share co-senior authorship.

https://doi.org/10.1016/j.jff.2018.07.047
Received 3 April 2018; Received in revised form 21 July 2018; Accepted 23 July 2018
Available online 27 July 2018

as gluten (Alaedini & Green, 2005). Although disruption of intestinal barrier function in CD has been established, the precise nature of this dysfunction as both a cause and effect of CD is not clear (Schumann et al., 2016). Mechanistic evidence suggests that gluten increases intestinal cell permeability in sensitive individuals, and significantly reduces epithelial barrier function in CD patients compared to healthy controls. In patients adhering to a gluten-free diet (GFD), disruption of epithelial barrier function is partially reversed and intestinal permeability is improved, when compared to active CD patients not following a GFD (Fromm et al., 2009; Schumann et al., 2012). Currently, a lifelong GFD remains the only available treatment. However, achieving a lifestyle completely free of gluten is in reality often impossible, due to practical and social challenges, which include the cross-contamination of foods (Hall, Rubin & Charnock, 2013; Thompson & Simpson, 2015). Furthermore, the effectiveness of a GFD varies among patients, whereas in non-responsive and refractory CD patients, GFD does not improve symptoms at all (Shannahan & Leffler, 2017). Moreover, the GFD is often low in vitamins and minerals, such as B vitamins, iron, calcium and zinc (Bascunan, Vespa & Araya, 2017; Olivares et al., 2015; Shannahan & Leffler, 2017), and is deficient in fiber (Hallert et al., 2002; Shepherd & Gibson, 2013). In recent years, a novel temperature-based method called Gluten Friendly™ has been developed (Lamacchia, Landriscina & D'Agnello, 2016), where *in vitro*, the gluten becomes unrecognizable by gluten-specific antibodies, without removing gluten from the flour (Lamacchia et al., 2016). While the temperature-treated gluten loses its immunogenic properties *in vitro*, it does not lose its technological properties and viscoelasticity (its ability to create a matrix and form dough) (Lamacchia et al., 2016). Further *in vitro* studies conducted on wheat kernels treated with the Gluten Friendly™ method also link a reduced cross-reactivity of antibodies recognizing almost the entire range of gluten proteins, to conformational changes of gluten protein structure in the kernel (Landriscina et al., 2017). The effects of Gluten Friendly™ bread or flour on healthy subjects, celiac patients or individuals suffering from gluten-related disorders have not yet been investigated. However, it has been postulated *in vitro* that consuming GFB could contribute to maintaining the microbial balance in the gut (Bevilacqua et al., 2016; Costabile et al., 2017). Therefore, we aim to investigate the effects of the novel GFB, from temperature-treated wheat kernels, on mucus production and gut barrier function in *cell culture* experiments, using mucus-secreting intestinal goblet cells (HT29-16E) from healthy human models.

The reason why we selected bread instead of temperature-treated wheat kernels or flour is that we envisioned our study as a model which could explore a potential real-world application. In fact, in 2017 wheat was the second most common food crop produced in the world, after maize, as reported by the Food and Agriculture Organization (FAO). Bread is one of the most common ways in which wheat is processed for human consumption.

2. Materials and methods

2.1. Bread substrates and simulated in vitro gastrointestinal digestion

The wheat kernels were supplied by Casillo Group S.p.a (Corato, Italy) and the chemical/rheological of the flour (Table 1) and nutritional characteristics of the bread (Table 2) are provided. The kernels underwent the Gluten Friendly™ temperature-based process as previously cited (Lamacchia et al., 2016; Landriscina et al., 2017). The caryopses were then milled into flour and baked into Gluten Friendly™ bread (GFB). A control bread (CB) was also baked using untreated kernels. Both GFB and CB were prepared according to the same bread-making process (100 g wheat flour, 66 mL water, 1.33 g yeast, 1 g salt) (Bevilacqua et al., 2016). GFB and CB were digested *in vitro* under appropriate conditions according to the procedures previously described (Maccaferri et al., 2012) in order to simulate mouth, stomach and small intestinal conditions. In particular, breads were homogenized with sterile distillated water in a stomacher. Then, digestive enzymes like α-amylase, pepsin, bile and pancreatine were added in due course under appropriate pH conditions in order to simulate mouth, stomach and small intestinal conditions, respectively. Once digested, the breads were then centrifuged at 2000*g* for 15 min at room temperature to remove large particles and supernatants were filtered through a 0.2-μm sterile syringe filter in preparation for the cell culture experiments.

Table 1
Chemical and rheological properties of soft wheat used in the study.

Parameter	Amount
Moisture %	12.10
Protein %ds	11.50
Gluten %ds	7.50
Gluten index	80
Ash %ds	0.76
Falling number	403
Yellow index	Nd
Alveograph	W: 97–P/L: 0.73
Farinograph	A: 59.8–B: 2.1–CD: 2.5

nd = not determinable; ds = dry substance W = dough strength; P = dough toughness; L = dough extensibility; A = water absorption; B = dough developing time; CD: dough stability.

Table 2
Nutritional content of soft wheat bread per 100 g.

Energy	271.00/1134.00 (kcal/kj)	
Total fat	3.50 g	
	Saturated fat	0.85 g
	Polyunsaturated fat	1.39 g
	Monounsaturated	0.81 g
Total carbohydrate	50 g	
	Dietary fiber	2.70 g
	Sugars	0.83 g
Protein	9.6 g	
Cholesterol	0 mg	
Calcium	48 mg	
Sodium	530 mg	
Potassium	110 mg	
Iron	2.10 mg	
Magnesium	27 mg	
Thiamin (Vit. B1)	0.47 mg	
Riboflavin (Vit. B2)	0.15 mg	
Niacin (Vit. B3)	1.6 mg	

2.2. Cell lines

Human intestinal cells, HT29-19A (non-mucus-secreting) and HT29-16E (mucus-secreting) clones (Jarry, Merlin, Hopfer & Laboisse, 1994), were cultured using standard procedures with Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, UK) supplemented with foetal bovine serum (10%), 100 × non-essential amino acids (1%), L-glutamine (1%) and penicillin/streptomycin (1%) (Sigma-Aldrich, UK) and maintained in a 5% CO_2 incubator at 37 °C. All the assays were performed between the passages 12 and 20 (Davis, 2002).

2.3. Cytotoxic activity

The cytotoxic effect of CB and GFB breads was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (method (Schiller, Klainz, Mynett & Gescher, 1992). The HT29-16E and HT29-19A cells were seeded at a density of 2×10^5 cells/mL in 96-well plates. Monolayers for both cell lines were incubated for 5 days, until the subconfluence (at least 80%) was reached, and were fed on alternate days. In order to establish the optimal concentration to be used in the

experiments, different concentrations of the digested breads (1%, 5%, 10%) were diluted in DMEM, and were then added to the cells and incubated for 24 h at 37 °C and 5% CO_2. An equivalent volume of DMEM was added to HT29-16E and HT29-19A control monolayers, which were also incubated for 24 h at 37 °C and 5% CO_2. MTT solution (0.5 mg/mL) was then added and left for 3 h at 37 °C. The medium was then aspirated, and 100 µL of DMSO was added to the cells. Colour development was measured at 570 nm with a Multiskan EX spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). The cytotoxicity activity data were expressed as a percentage of viable cells, in relation to the control wells, which represent 100% viability.

2.4. Assessment of cell mucin production by Periodic acid Schiff (PAS) and Alcian blue staining

Mucus production was examined using both Periodic acid–Schiff (PAS) (Diebel, Liberati & Hall-Zimmerman, 2011; Schiller et al., 1992) and Alcian-blue (AB) (Steedman, 1950) staining techniques. PAS is used to detect mucosubstances containing a high proportion of carbohydrate macromolecules, as well as polysaccharides, the basal laminae and the glycocalyx of cells. A positive PAS reaction indicates the presence of neutral mucins. A positive Alcian Blue reaction at pH 1.0 and 2.5 indicates the presence of acidic sulphated and acidic carboxylated mucins, respectively (Schumacher, Duku, Katoh, Jörns, & Krause, 2004; Tootian et al., 2013).

Transmembrane mucins MUC3, MUC12 and MUC17 are a main component of the enterocyte glycocalyx, whereas MUC2 is the most important gel-forming mucin that is secreted by goblet cells (Pelaseyed et al., 2014). MUC3 can be considered as a neutral mucin, while MUC2 can be considered as an acidic cysteine-rich mucin, due to their different aminoacid composition in proline, threonine and serine, (Kim, Gum Jr., Byrd, & Toribara, 1991). Thus, MUC3 stains positive for PAS, and MUC2 stains positive for AB. For the experiments, HT29-19A and HT29-16E cells were cultured without passaging for at least 12 days and then seeded into wells of 24-well plates at 2×10^5 cells/mL. After 2 weeks, the digested breads, diluted at 5% in DMEM (Maccaferri et al., 2010, 2012), were added to the cells and incubated for different times (2h, 4 h and 24 h). PAS staining system (Sigma, Inc) was used to evaluate neutral mucin (Bouhet & Oswald, 2005; Steedman, 1950). Briefly, cell monolayers were immersed in PAS solution for 5 min at room temperature followed by several rinses in dH_2O. Cells were immersed subsequently in Schiff's reagent for 15 min at room temperature, and washed in tap water. Cells were then counterstained in Gill's haematoxylin solution no. 3 (Sigma, UK) for 90 s and again washed in tap water. Cell monolayers were allowed to dry and PAS staining assessed under the microscope. For the Alcian blue staining (Sigma, UK), the HT29-19A and HT29-16E cells were first fixed with chilled 95% ethanol/5% glacial acetic acid for 10 min, and then incubated with 1% Alcian blue/3% acetic acid for 5 min at room temperature. The cells were subsequently washed three times with phosphate buffer (Sigma, UK) to remove any residual stain. Mucin production, assessed by the level of blue staining, was examined by light microscopy using an OLYMPUS BX43F microscope (Olympus Life Science, Tokyo, Japan). Images were acquired using Infinity Capture software. In addition, the pictures were also analysed by means of Photoshop and Image Tool software, which convert the images to binary mode: the dark blue stained cells turn black, and the light blue unstained cells become white. The percentage of black and white pixels is then calculated in order to compare the effect of the different treatments. Mucin production by Alcian blue staining was expressed as a percentage (%) of black pixels obtained from Image Tool software.

2.5. Measurement of Trans-Epithelial Electrical Resistance (TEER) in cell monolayers

In order to evaluate the effect of GFB and CB on the cell monolayers, specifically its integrity and permeability, TEER measurements were taken using a Millicell-ERS meter (Millipore, Billerica, MA, USA) connected to a pair of electrodes, following the manufacturer's standard procedure. HT29-16E cells were seeded on microporous Transwell® 6-well plates (Sigma, UK) at 2×10^5 cells/mL for 3 weeks at 37 °C and 5% CO_2. HT29-19A non-mucus-secreting cells were used as a control. Cells were grown into polarized monolayers and TEER was measured at regular intervals (7, 10, 14, 18, 21 days). Monolayers with a TEER $> 250\ \Omega\,cm^2$ were used for exposure to digested bread products. HT29-16E cell monolayers were exposed to digested breads (5%) for 24 h at 37 °C and 5% CO_2. The 24-hour time frame was established as the optimal time measurement to evaluate *in vitro* the effects of substances on cell barrier integrity of the small intestine mucosa (Barnett, Roy, McNabb, & Cookson, 2016; Damiano et al., 2018). The integrity of the polarized cell monolayers was determined before and after treatments by measuring the TEER. TEER values were standardized and reported as percent changes relative to the TEER at the beginning of the experiment. After the last TEER measurement, all supernatants and cell lysates were collected and processed for further analysis of MUC2 and MUC3 levels.

2.6. Human MUC2 and MUC3 quantification by ELISA assays

Human MUC2 (Abbexa, UK, cat abx055282) and MUC3 ELISA (Abbexa, UK, cat abx152398) kits were used to determine the level of these two types of mucins in the supernatants and cell lysates. Concentrations of MUC2 in cell culture supernatants and MUC3 in cell lysates were analysed by Human Mucin enzyme linked immunosorbent assays (ELISA) Kits (Abbexa Ltd, Cambridge, UK). Briefly, HT-29 16E cell supernatants were taken; cells were washed with PBS in order to take all non-adherent mucins and they were finally centrifuged at 6000 rpm for 20 min to remove the precipitant. Non-adherent MUC2 production was measured in this fraction. Regarding the MUC3 quantification, HT-29 16E cell monolayers free of non-adherent mucins were detached with trypsin (1.5 mL) and collected by centrifugation at 4000 rpm for 10 min to remove the supernatant. Then the cells were washed three times in ice-cold PBS, lysed by ultra-sonication and centrifuged to remove cellular debris. MUC3 sandwich ELISA was carried out on this fraction. Control analyses were also carried out.

2.7. Statistical analysis

For the MTT, Mucin2, Mucin3 and TEER assays, statistical analysis was performed using one-way ANOVA with a significance level set at 0.05, followed by *Tukey* multiple comparison test, in order to assess the effect of the different treatments. For the Alcian blue staining at different time-points, the statistical analysis was performed using two-way ANOVA, comparing the simple effects of treatment within each time-point. All analyses were performed using GraphPad Prism Software (version 7.0, Inc., San Diego, CA, USA).

3. Results

3.1. Cytotoxic activity

Cytotoxicity of CB and GFB was evaluated by MTT assay in order to establish the optimal concentration to be used in the following experiments. Fig. 1 shows the percentage of viability of HT29-16E cells from healthy human models, after 24 h incubation in the presence of the two different types of digested bread (1%, 5% and 10% v/v) compared to the control. The cytotoxicity activity data were expressed as a percentage of viable cells, in relation to the control wells, which represent 100% viability. It was observed that the treatments were not cytotoxic for the cells at any of the concentrations tested.

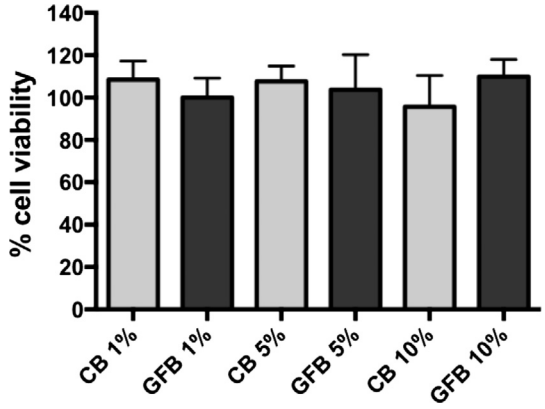

Fig. 1. Cell viability measured by MTT assay in HT29-16E cellsafter 24 h of incubation with different concentrations of control bread (CB) and Gluten Friendly™ bread (GFB). Data are means of 3 separate experiments, each performed in 4 replicates (n = 12), and presented as mean ± SD. Statistical analysis was conducted by One-way ANOVA with Tukey post-hoc test ($P > 0.05$ vs. control).

3.2. Assessment of mucin production by Periodic acid Schiff and Alcian-blue staining

Fig. 2 shows representative images of Alcian blue (Fig. 2A) and PAS (Fig. 2B) stainings of the HT29-16E cells (mucus-secreting) and of the HT29-19A cells (non-mucus-secreting) after 4 h of incubation with the digested breads. The stainings depict common morphological patterns of mucus production by these cells. The HT29-19A non-mucus-secreting cell line did not present relevant levels of PAS and AB staining as expected. With regards to the HT29-16E cells (mucus-secreting) we can observe that in the GFB group, the levels of staining with AB (Fig. 2A) are higher than in both the CB group and the control. With regards to PAS staining (Fig. 2B) of HT29-16E cells (mucus-secreting), control cells (exposed to control solution resulting from *in vitro* digestion) showed levels of staining similar to the levels in CB treated cells. CB treated cells and control cells showed lower levels of PAS staining than GFB treated cells. Additionally, Fig. 2C provides a semi-quantitative analysis using Photoshop and Image Tool software, of the AB staining at 3 time-points (2h, 4 h, and 24 h), showing that the cells treated with CB and GFB had significantly higher levels of staining, expressed as percentage of pixels, in comparison with the control, after 4 h and 24 h treatments (see Fig. 2C).

3.3. Assessment of cells mucin production by ELISA

Human MUC2 (Abbexa, UK, cat abx055282) and MUC3 ELISA (Abbexa, UK, cat abx152398) kits were used to determine the level of these two types of mucins in the supernatants and cell lysates, respectively (Fig. 3). The cells treated with GFB had a significantly higher level of MUC2 (Fig. 3A) than the ones incubated with the Control ($P < 0.001$) or CB ($P < 0.01$). No significant differences were observed in MUC3 levels (Fig. 3B) among all different treatments.

3.4. Assessment of cells monolayer integrity by TEER measurement

The integrity of the cells monolayer before and after treatment with the digested breads was evaluated by the TEER. Fig. 4 reports the TEER values, expressed as % of the initial TEER. Both CB and GFB treated cells had significantly higher TEER values compared to control cells ($P < 0.01$ and $P < 0.001$, respectively). In addition, the cells treated with GFB had significantly higher TEER values than CB treated cells.

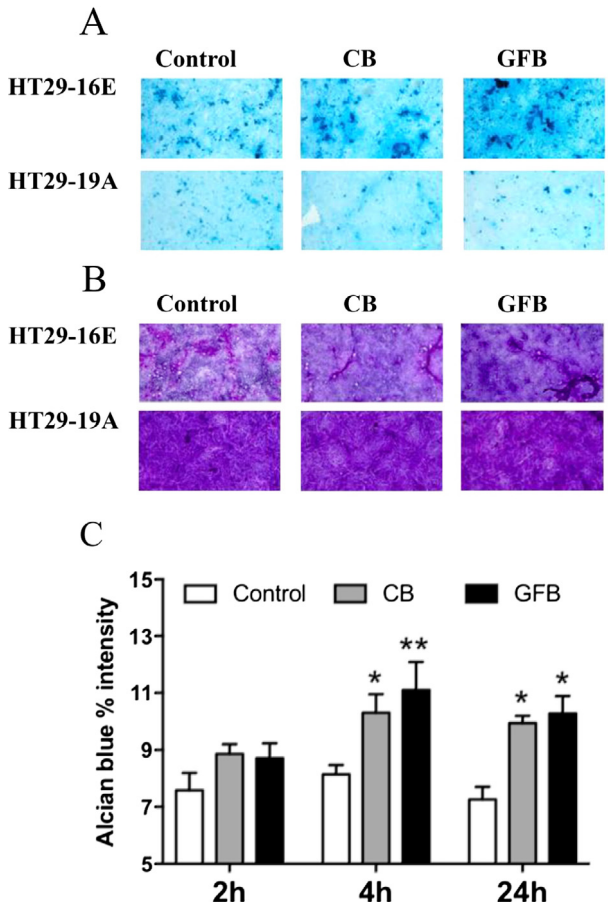

Fig. 2. Representative images of mucin staining in HT29-16E (mucus-secreting) and HT29-19A (non-mucus-secreting) cell lines after 4 h of incubation with the digested breads: control bread (CB) and gluten-friendly bread (GFB) compared to a control cell monolayer. Acid mucins were visualized by Alcian Blue (AB) staining (x10 objective) (A) and neutral mucins were visualized by Periodic acid-Schiff (PAS) staining (×10 objective) (B). 3 separate experiments were performed, with 5 replicates for each experimental group (n = 15). Acid mucin production semi-quantitative analysis after 2 h, 4 h and 24 h incubation of HT29-16E cells with control bread (CB) and gluten-friendly bread (GFB), compared to a control cell monolayer (C). The staining was performed with Alcian Blue and images were analysed using Photoshop and Image Tool. Data are means of 3 separate experiments, each performed in 5 replicates (n = 15), and presented as mean ± SD. Statistical analysis was conducted by One-way ANOVA with Tukey post-hoc test (* = $P < 0.05$ vs. control; ** = $P < 0.01$ vs. control).

4. Discussion

It is well-known that the intestine is the first organ exposed and affected by nutrients or antigens, following their ingestion (Maresca & Fantini, 2010; Scaldaferri, Pizzoferrato, Gerardi, Lopetuso, & Gasbarrini, 2012). Whereas specific membrane transporters allow absorption of beneficial compounds, the tight junctions and the mucus layer are responsible for the barrier function of the gut (Brandtzaeg, 2013; Pinton et al., 2015). Mucus plays major roles in the intestinal barrier function, and in the symbiosis with the microbiota. Perturbations of the composition and/or secretion of mucus are associated with diseases in animals and humans (Johansson, Sjövall & Hansson, 2013; Kim & Deng, 2008). Many studies have reported quantitative and qualitative abnormalities, of mucin gene expression in gastrointestinal diseases (Hafez, 2012). Previous studies have shown that GFB, produced from flours treated with the novel, patented temperature-based process (Lamacchia et al., 2016) is able to modulate *in vitro*, the gut microbiota function in both healthy and coeliac donors (Bevilacqua

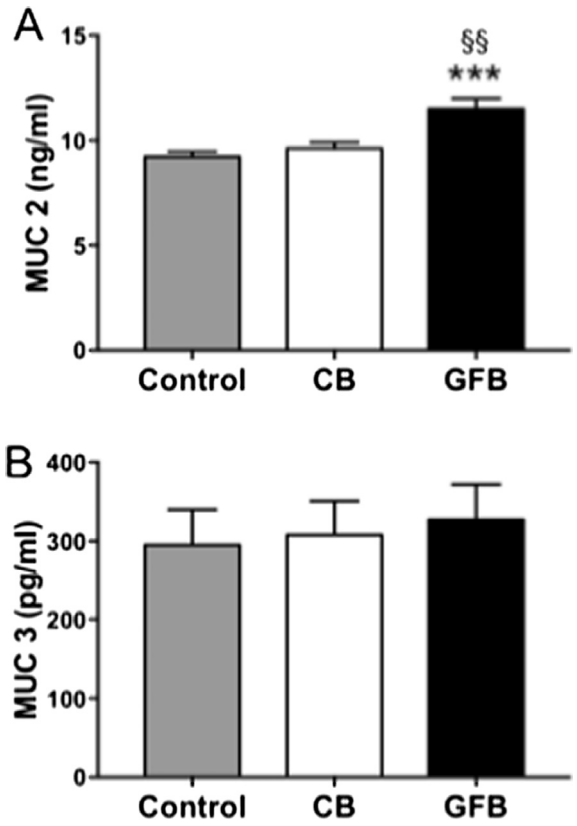

Fig. 3. Quantification of specific mucin production by HT29-16E cells after 24 h incubation with control bread (CB) and gluten-friendly bread (GFB), compared to a control cell monolayer. MUC2 levels (A) and MUC3 levels (B) were determined by ELISA. Data are means of 3 separate experiments, each performed in 4 replicates (n = 12), and presented as mean ± SD. Statistical analysis was conducted by One-way ANOVA with Tukey post-hoc test (*** = $P < 0.001$ vs. control; §§ = $P < 0.01$ vs. CB).

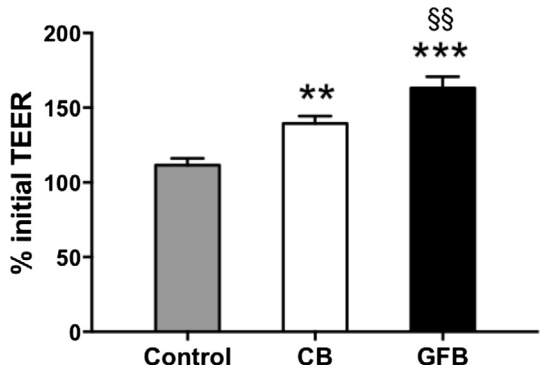

Fig. 4. Epithelial barrier function measured as Trans Epithelial Electrical Resistance (TEER). Data are shown as the % of initial TEER, in HT29-16E cells after 24 h incubation with control bread (CB) and gluten-friendly bread (GFB), compared to a control cell monolayer. Data are means of 3 separate experiments, each performed in 3 replicates, 2 measurement for each replicate (n = 18), and presented as mean ± SD. Statistical analysis was conducted by One-way ANOVA with Tukey post-hoc test (** = $P < 0.01$ vs. control; *** = $P < 0.001$ vs. control; §§ = $P < 0.01$ vs. CB).

et al., 2016; Costabile et al., 2017). The main hypothesis of this study was to determine whether GFB could have beneficial effects on mucus production and intestinal epithelial barrier *in vitro*. To study the effect of GFB on intestinal mucus, we used the HT29-16E mucus-secreting cells that, like normal goblet cells, produce mucins (Augeron & Laboisse, 1984; Maoret et al., 1989). Confluent HT29-16E monolayers secrete a dense mucus gel and represent an established healthy human model to study goblet cell mucus secretion *in vitro*. Importantly, *ex vivo* experiments performed on intestinal explants from pigs confirmed *in vitro* data, demonstrating the high predictive value of the HT29-16E cells as a health model of intestinal goblet cells (Pinton et al., 2015). It has been previously reported that granules positive for Alcian blue contain (Behrens, Stenberg, Artursson & Kissel, 2001) non-sulfated acidic mucins or sialomucin (pH 2.5), as well as sulphated mucins or sulphomucins (pH 1.0), and also that PAS-positive goblet cells secrete neutral mucins (Trevizan et al., 2016). Studies about mucin secretion have proven challenging in the past, as mucins are difficult to purify. At the same time, both chemical or radioactive labelling have limitations because labelling is restricted to a single attribute, such as a monosaccharide or amino acid, which is not exclusive to mucins, or necessarily evenly distributed among them (Forstner, 1995). The data presented here demonstrated that GFB does not affect cell viability, as indicated by the MTT assay, but does increase the secretion of mucins as observed after PAS and Alcian blue staining, in comparison with the control cells.

Whereas a rise in mucus production is indicative of a healthy response of the intestinal epithelia (Forstner, 1995), reduced mucus production is consistently observed in Inflammatory Bowel Diseases, such as ulcerative colitis and Crohn disease (Lammers et al., 1994; Makkink et al., 2002). Alteration of mucin production has also been reported in CD patients (Bischoff et al., 2014; Einerhand et al., 2002; Strugala, Dettmar & Pearson, 2008) and has been linked to the onset of CD (Boltin, Perets, Vilkin & Niv, 2013).

To better investigate the effect of GFB on mucin production, we used two specific MUC2 and MUC3 Human enzyme linked immunosorbent assays (ELISA) that allowed us to quantify the two mucins, and discriminate the inner layer attached firmly to the epithelial surface, and the outer layer non-adherent mucus (Augeron & Laboisse, 1984; Crabtree, Heatley & Losowsky, 1989). MUC3 is a membrane bound adherent mucin, while MUC2 is a gel-forming mucin secreted on the outer or non-adherent mucus layer (Augenlicht, Augeron, Yander & Laboisse, 1987).

Thus, MUC2 ELISA data support the finding that GFB induces higher production of MUC2 than the control and CB. No significant difference was observed in MUC3 secretion throughout different experiment conditions. Arike and Hansson (2004) explain that MUC2 mucins have a protective role owing to their o-glycosylation density, forming a hydrophilic diffusion barrier with its bound water. Furthermore, MUC2 also provides food for commensal bacteria (Byrd & Bresalier, 2004) and prevents pathogens from reaching the glycocalyx beneath, which is devoid of microbes (Arike & Hansson, 2004; Johansson et al., 2008). It is important to highlight that MUC2 does not only constitute a non-specific physical barrier, but also significantly affects the immunogenicity of gut antigens by delivering tolerogenic signals to dendritic cells, via the intestinal epithelial cells, and therefore enhancing gut homeostasis (Johansson et al., 2013; Shan et al., 2002). A reduced MUC2 production has indeed been associated to a range of gastrointestinal inflammatory states, including ulcerative cholitis (Makkink et al., 2002; Shan et al., 2013). The data presented here support the potential of GFB to maintain and/or enhance gut homeostasis and intestinal barrier function, by maintaining higher mucin levels than control and CB treated cells. Indeed, GFB triggers MUC2 production to a higher extent, potentially contributing to create a stronger intestinal barrier. It is also worth underlining that innate immunity has been considered as another possible key element in the development of CD (Van Klinken, Van der Wal, Einerhand, Büller, & Dekker, 1999), which is characterized by imbalances of the intestinal microbiota composition, thus suggesting a role of intestinal microbiota in this pathology. The mode by which GFB stimulates mucin production requires further investigation. Results collected so far suggest that exposure of hydrated wheat caryopses to the Gluten Friendly™ technology induces a different spatial conformation of the amino acid sequences and a rearrangement

of the secondary and tertiary structure of gluten proteins (Lamacchia et al., 2016). Furthermore, it has been postulated that such rearrangement of the gluten protein structure exposes positive charges, namely cationic residues, and could explain the novel effects of GFB on bacteria and probiotics (Bevilacqua et al., 2016). Given that secretion is triggered by a wide array of bioactive factors, including cholinergic agonist, hormones (neuropeptides), microbes and microbial products (peptides), inflammatory cytokines, and reactive oxygen and nitrogen species (Kim & Ho, 2010), the cationic peptides in GFB could also be acting as mucin segretagogues on HT29-16 E mucus-secreting cells.

TEER values for human small intestine vary from 50 to 100 Ω/cm^2. However, in this study, the integrity of the monolayer was confirmed by TEER $> 200\,\Omega/cm^2$ (Martínez-Maqueda et al., 2015, chap. 11). Variations in TEER measurements may arise due to differing culture parameters, such as the passage number of the cells; the age and the stage of differentiation of the cells; the type of culture medium used; the seeding density of the cells and the type of support the cells are cultured on (Sambuy, 2005). Therefore, in spite of some limitations of the *in vitro* cell model (Martínez-Maqueda et al., 2015, chap. 11; Sambuy, 2005), this study indicated the potential for GFB to improve the intestinal cellular barrier integrity, as indicated by the significantly higher TEER increase, compared to both control and CB. Even though the exact interactions and contributions of the intestinal mucosa are not clearly defined, an increased TEER signal indicates increased intestinal barrier function. Mucins are a key extracellular component of the intestinal barrier (Rossi & Schwartz, 2010). It could be assumed that the increased trans-epithelial electrical resistance is mainly due to the increase in the gel-forming MUC2 secretion. However, it should be noted that the glycocalyx and tight junction proteins are also known to be key contributors (Lee, 2015).

Abnormal bacterial adherence and internalization by epithelial cells have been reported in CD Patients (Drago et al., 2006; Rossi & Schwartz, 2010). *In vitro* studies using Caco-2 cells and IEC6 have shown that tight junction protein interactions are compromised by gluten (Rossi & Schwartz, 2010). This leads to the rearrangement of the cytoskeleton, and increases monolayer permeability (Drago et al., 2006). With recent knowledge that adherent mucosa-associated bacteria play a critical role in IBS (Irritable Bowel Syndrome) and colitis-associated colorectal cancers, there is increased understanding that an abnormal interaction between epithelium and bacteria also exists. There is now scientific consensus regarding the importance of gut microbiome in health and disease (Byrd & Bresalier, 2004; Ulluwishewa et al., 2011) and there are many factors that influence its composition (Nicholson et al., 2012; Ulluwishewa et al., 2011).

The gut microbial dysbiosis seen in CD patients has been found to dramatically affect the host physiology (Yatsunenko et al., 2012). Recently, the effect of GFB on the gut microbiota of both healthy and CD patients has been investigated (Bevilacqua et al., 2016; Costabile et al., 2017) and it has been shown that GFB positively modulated the complex bacterial ecosystem with an increase in numbers of health-promoting beneficial bacteria (Costabile et al., 2017).

Additionally, GFB prolonged the survival of *Lactobacillus acidophilus* and had antibacterial effects towards *Staphylococcus aureus* and *Salmonella* Typhimurium (Bevilacqua et al., 2016). Other studies have found that *L. acidophilus* counteracts inhibition of butyrate uptake in intestinal epithelial cells by enteropathogenic *E. coli* (Krishnan, Alden & Lee, 2015).

It has been reported that the intestinal microbiota may modulate goblet cell function and the intestinal mucus layer (Kumar, Rajendran, Kumar, Hamwieh, & Baum, 2015). Therefore, taken together with the outcome of the present study, intestinal epithelium mucus production may be increased not only by a direct effect of GFB itself, but also as a consequence of gut microbiota modulation triggered by GFB. Further research is, however, required to assess whether such functionality is also maintained *in vivo*, particularly in coeliac subjects. It could be concluded that GFB has the potential to induce mucin secretion by intestinal epithelial cells and to improve intestinal epithelial barrier function. Thus *in vivo* studies are recommended to confirm the *in vitro* outcome presented in this study. If confirmed, such observed potential may effectively contribute to consequent benefits, such as higher gut barrier defense, decreased susceptibility to infections and better absorption regulation, thus helping to redress such disturbances in chronic inflammatory intestinal diseases.

Author contributions

CL has conceptualized the study; CL, AC and GC have designed the study; CL, LL and ID applied the Gluten Friendly™ temperature treatment on wheat kernels for the production and characterization of GFB; TBM, DM and MEH have carried out the cell culture experiments; GC and AC carried out the statistical analysis; CL obtained funding; CL, AC and GC have supervised the study and have edited and validated the data analysis; CL has interpreted the data; AC and GC drafted the manuscript; CL has critically revised the manuscript for important intellectual content; CL, AC and GC have edited and approved the final manuscript.

Conflict of interests

Carmen Lamacchia declares to be the inventor of the following patents “Method for the detoxification of gluten proteins from grains of cereals. Patent Cooperation Treaty PCT/IB2013/000797” and “Methods for the detoxification of gluten proteins from grains of cereals and related medical uses. Italian priority patent n° 102015000084813 filed on 17.12.15.”

The authors declare no conflict of interest. Casillo Group was a commercial source; however, it had no role in the design of the basic patent of this research (Gluten Friendly™ temperature-based process), and did not play any role in the design of this research.

Ethics statement

The cellular *in vitro* model used in this study was a commercially available model. In this study we did not used any kind of human samples to require approval of Ethics Committee.

Acknowledgments

This work was performed with the financial support of Casillo Group S.p.a. (Corato, Italy). This project has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No. 732640. Disclaimer: This publication reflects only the author’s view and the Agency is not responsible for any use that may be made of the information it contains.

References

Alaedini, A., & Green, P. H. (2005). Narrative review: Celiac disease: Understanding a complex autoimmune disorder. *Annals of Internal Medicine, 142*, 289–298.

Arike, L., & Hansson, G. C. (2004). The densely O-glycosylated MUC2 mucin protects the intestine and provides food for the commensal bacteria. *Journal of Molecular Biology, 428*, 3221–3229.

Augenlicht, L. H., Augeron, C., Yander, G., & Laboisse, C. (1987). Overexpression of ras in mucus-secreting human colon carcinoma cells of low tumorigenicity. *Cancer Research, 47*, 3763–3765.

Augeron, C., & Laboisse, C. L. (1984). Emergence of permeability differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. *Cancer Research, 44*, 3961–3969.

Barnett, A. M., Roy, N. C., McNabb, W. C., & Cookson, A. L. (2016). Effect of a semi-purified oligosaccharide-enriched fraction from caprine milk on barrier integrity and mucin production of co-culture models of the small and large intestinal epithelium. *Nutrients, 8*, 267.

Bascunan, K. A., Vespa, M. C., & Araya, M. (2017). Celiac disease: Understanding the gluten- free diet. *European Journal of Nutrition, 56*, 449–459.

Behrens, I., Stenberg, P., Artursson, P., & Kissel, T. (2001). Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells.

Pharmacology Research, 18, 1138–1145.
Bevilacqua, A., Costabile, A., Bergillos-Meca, T., Gonzalez, I., Landriscina, L., Ciuffreda, E., ... Lamacchia, C. (2016). Impact of gluten-friendly bread on the metabolism and function of in vitro gut microbiota in healthy human and coeliac subjects. *PloS One, 11*, e0162770.
Bischoff, S. C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J. D., Serino, M., ... Wells, J. M. (2014). Intestinal permeability – A new target for disease prevention and therapy. *BMC Gastroenterology, 14*, 189.
Boltin, D., Perets, T. T., Vilkin, A., & Niv, Y. (2013). Mucin function in inflammatory bowel disease: An update. *Journal of Clinical Gastroenterology, 47*, 106–111.
Bouhet, S., & Oswald, I. P. (2005). The effects of mycotoxins, fungal food contaminants, on the intestinal epithelial cell-derived innate immune response. *Veterinary Immunology and Immunopathology, 108*, 199–209.
Brandtzaeg, P. (2013). Gate-keeper function of the intestinal epithelium. *Beneficial Microbes, 4*, 67–82.
Byrd, J. C., & Bresalier, R. S. (2004). Mucins and mucin binding proteins in colorectal cancer. *Cancer Metastasis Review, 23*, 77–99.
Costabile, A., Bergillos-Meca, T., Landriscina, L., Bevilacqua, A., Gonzalez-Salvador, I., Corbo, M. R., ... Lamacchia, C. (2017). An in vitro fermentation study on the effects of Gluten Friendly™ bread on microbiota and short chain fatty acids of fecal samples from healthy and celiac subjects. *Frontiers in Microbioliogy, 8*, 1722.
Crabtree, J. E., Heatley, R. V., & Losowsky, M. S. (1989). Glycoprotein synthesis and secretion by cultured small intestinal mucosa in coeliac disease. *Gut, 30*, 1339–1343.
Damiano, S., Sasso, A., De Felice, B., Di Gregorio, I., La Rosa, G., Lupoli, G. A., ... Santillo, M. (2018). Quercetin increases MUC2 and MUC5AC gene expression and secretion in intestinal goblet cell-like LS174T via PLC/PKCα/ERK1-2 pathway. *Frontiers in Physiology, 9*, 357.
Davis, J. M. (2002). *Basic cell culture: A practical approach* (2nd ed.). Oxford, UK: University Press381.
Diebel, L. N., Liberati, D. M., & Hall-Zimmerman, L. (2011). H2 blockers decrease gut mucus production and lead to barrier dysfunction in vitro. *Surgery, 150*, 736–743.
Drago, S., El Asmar, R., Di Pierro, M., Grazia Clemente, M., Sapone, A. T. A., Thakar, M., ... Fasano, A. (2006). Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scandinavian Journal of Gastroenterology, 41*, 408–419.
Einerhand, A. W., Renes, I. B., Makkink, M. K., van der Sluis, M., Buller, H. A., & Dekker, J. (2002). Role of mucins in inflammatory bowel disease: Important lessons from experimental models. *European Journal of Gastroenterology & Hepatology, 14*, 757–765.
Forstner, G. (1995). Signal transduction, packaging and secretion of mucins. *Annual Review of Physiology, 57*, 585–605.
Fromm, M., Krug, S. M., Zeissig, S., Richter, J. F., Rosenthal, R., Schulzke, J. D., & Günzel, D. (2009). High-resolution analysis of barrier function. *Annals of the New York Academy of Sciences, 1165*, 74–81.
Hafez, M. M. (2012). Upregulation of intestinal mucin expression by the probiotic bacterium E. coli Nissle 1917. *Probiotics and Antimicrobial Proteins, 4*, 67–77.
Hall, N. J., Rubin, G. P., & Charnock, A. (2013). Intentional and inadvertent non-adherence in adult coeliac disease. A cross-sectional survey. *Appetite, 68*, 56–62.
Hallert, C., Grant, C., Grehn, S., Granno, C., Hulten, S., Midhagen, G., ... Valdimarsson, T. (2002). Evidence of poor vitamin status in coeliac patients on a gluten-free diet for 10 years. *Alimentary Pharmacology & Therapeutics, 16*, 1333–1339.
Jarry, A., Merlin, D., Hopfer, U., & Laboisse, C. L. (1994). Cyclic AMP-induced mucin exocytosis is independent of Cl- movements in human colonic epithelial cells (HT29-Cl.16E). *Journal of Biochemistry, 304*, 675–678.
Johansson, M. E. V., Gustafsson, J. K., Holmén-Larsson, J., Jabbar, K. S., Xia, L., Xu, H., Hansson, G. C., et al. (2013). Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut, 63*, 281–291.
Johansson, M. E., Phillipson, M., Petersson, J., Velcich, A., Holm, L., & Hansson, G. C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proceedings of the National Academy of Sciences of the United States of America, 105*, 15064–15069.
Johansson, M. E., Sjövall, H., & Hansson, G. C. (2013). The gastrointestinal mucus system in health and disease. *Home: Nature Reviews Gastroenterology & Hepatology, 10*, 352–361.
Kim, Y. S., & Deng, G. (2008). Aberrant expression of carbohydrate antigens in cancer: The role of genetic and epigenetic regulation. *Gastroenterology, 135*, 305–309.
Kim, Y. S., Gum, J. R., Jr., Byrd, J. C., & Toribara, N. W. (1991). The structure of human intestinal apomucins. *The American Review of Respiratory Disease, 144*, S10–S14.
Kim, Y. S., & Ho, S. B. (2010). Intestinal goblet cells and mucins in health and disease: Recent insights and progress. *Current Gastroenterology Reports, 12*, 319–330.
Krishnan, S., Alden, N., & Lee, K. (2015). Pathways and functions of gut microbiota metabolism impacting host physiology. *Current Opinion in Biotechnology, 36*, 137–145.
Kumar, S., Rajendran, K., Kumar, J., Hamwieh, A., & Baum, M. (2015). Current knowledge in lentil genomics and its application for crop improvement. *Frontiers in Plant Science, 6*, 78.
Lamacchia, C., Landriscina, L., & D'Agnello, P. (2016). Changes in wheat kernel proteins induced by microwave treatment. *Food Chemistry, 197*, 634–640.
Lammers, K. M., Jansen, J., Bijlsma, P. B., Ceska, M., Tytgat, G. N., Laboisse, C. L., & van Deventer, S. J. (1994). Polarised interleukin 8 secretion by HT 29/19A cells. *Gut, 35*, 338–342.
Landriscina, L., D'Agnello, P., Bevilacqua, A., Corbo, M. R., Sinigaglia, M., & Lamacchia, C. (2017). Impact of gluten-friendly™ technology on wheat kernel endosperm and gluten protein structure in seeds by light and electron microscopy. *Food Chemistry, 221*, 1258–1268.
Lee, S. H. (2015). Intestinal permeability regulation by tight junction: Implication on inflammatory bowel diseases. *Intestinal Research, 13*, 11–18.
Lundin, K. E., & Sollid, L. M. (2014). Advances in coeliac disease. *Current Opinion in Gastroenterology, 30*, 154–162.
Maccaferri, S., Klinder, A., Cacciatore, S., Chitarrari, R., Honda, H., Luchinat, C., ... Costabile, A. (2012). In vitro fermentation of potential prebiotic flours from natural sources: Impact on the human colonic microbiota and metabolome. *Molecular Nutrition and Food Research, 56*, 1342–1352.
Maccaferri, S., Vitali, B., Klinder, A., Kolida, S., Ndagijimana, M., Laghi, L., ... Costabile, A. (2010). Rifaximin modulates the colonic microbiota of patients with Crohn's disease: An in vitro approach using a continuous culture colonic model system. *Journal of Antimicrobial Chemotherapy, 65*, 2556–2565.
Makkink, M. K., Schwerbrock, N. M., Mähler, M., Boshuizen, J. A., Renes, I. B., Cornberg, M., ... Dekker, J. (2002). Fate of goblet cells in experimental colitis. *Digestive Diseases and Sciences, 47*, 2286–2297.
Maoret, J. J., Font, J., Augeron, C., Codogno, P., Bauvy, C., Aubery, M., & Laboisse, C. L. (1989). A mucus-secreting human colonic cell line. Purification and partial characterization of the secreted mucins. *Journal of Biochemestry, 258*, 793–799.
Maresca, M., & Fantini, J. (2010). Some food-associated mycotoxins as potential risk factors in humans predisposed to chronic intestinal inflammatory diseases. *Toxicon, 56*, 282–294.
Martínez-Maqueda, D., Miralles, B. & Recio, I. (2015). HT29 cell line. In K. Verhoeckx et al. (Eds.), The impact of food bio-actives on gut health (pp. 117–118).
Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host-gut microbiota metabolic interactions. *Science, 336*, 1262–1267.
Olivares, M., Neef, A., Castillejo, G., Palma, G. D., Varea, V., Capilla, A., ... Sanz, Y. (2015). The HLA-DQ2 genotype selects for early intestinal microbiota composition in infants at high risk of developing coeliac disease. *Gut, 64*, 406–417.
Pelaseyed, T., Bergstrom, J. H., Gustafsson, J. K., Ermund, A., Birchenough, G. M. H., Schatte, A., ... Hansson, G. C. (2014). The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunological Reviews, 260*, 8–20.
Peterson, L. W., & Artis, D. (2014). Intestinal epithelial cells: Regulators of barrier function and immune homeostasis. *Nature Review and Immunology, 14*, 141–153.
Pinton, P., Graziani, F., Pujol, A., Nicoletti, C., Paris, O., Ernouf, P., ... Maresca, M. (2015). Deoxynivalenol inhibits the expression by goblet cells of intestinal mucins through a PKR and MAP kinase dependent repression of the resistin-like molecule. *Molecular Nutrition and Food Research, 59*, 1076–1087.
Rossi, M., & Schwartz, K. B. (2010). Editorial: Celiac disease and intestinal bacteria: Not only gluten? *Journal of Leukocyte Biology, 87*, 749–751.
Sambuy, Y. (2005). The Caco-2 cell line as a model of the intestinal barrier: Influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology, 21*, 1–26.
Scaldaferri, F., Pizzoferrato, M., Gerardi, V., Lopetuso, L., & Gasbarrini, A. (2012). The gut barrier: New acquisitions and therapeutic approaches. *Journal of Clinical Gastroenterology, 46*, S12–S17.
Schiller, C. J., Klainz, A., Mynett, K., & Gescher, A. (1992). Assessment of viability of hepatocytes in suspension using the MTT assay. *Toxicolology in Vitro, 6*, 575–578.
Schulzke, J. D., Bentzel, C. J., Schulzke, I., Riecken, E. O., & Fromm, M. (1998). Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. *Pediatric Research, 43*, 435–441.
Schumacher, U., Duku, M., Katoh, M., Jörns, J., & Krause, W. J. (2004). Histochemical similarities of mucins produced byBrunner's glands and pyloric glands: A comparative study. *The Anatomical Record Part A, Discoveries in Molecular, Cellular, and Evolutionary Biology, 278*, 540–550.
Schumann, M., Günzel, D., Buergel, N., Richter, J. F., Troeger, H., May, C., ... Schulzke, J. D. (2012). Cell polarity-determining proteins Par-3 and PP-1 are involved in epithelial tight junction defects in coeliac disease. *Gut, 61*, 220–228.
Schumann, M., Siegmund, B., Schulzke, J., & Fromm, M. (2016). Celiac disease: Role of the epithelial barrier. *Cellular and Molecular Gastroenterology and Hepatology, 3*, 150–162.
Shan, M., Gentile, M., Yeiser, J. R., Walland, A. C., Bornstein, V. U., Chen, K., ... Cerutti, A. (2013). Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science, 342*, 447–453.
Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G. M., ... Khosla, C. (2002). Structural basis for gluten intolerance in celiac sprue. *Science, 297*, 2275–2279.
Shannahan, S., & Leffler, D. A. (2017). Diagnosis and updates in celiac disease. *Gastrointestinal Endoscopy Clinics of North America, 27*, 79–92.
Shepherd, S. J., & Gibson, P. R. (2013). Nutritional inadequacies of the gluten-free diet in both recently-diagnosed and long-term patients with coeliac disease. *Journal of Human Nutrition and Dietetics: The Official Journal of the British Dietetic Association, 26*, 349–358.
Smecuol, E., Bai, J. C., Vazquez, H., Kogan, Z., Cabanne, A., Niveloni, S., ... Meddings, J. B. (1997). Gastrointestinal permeability in celiac disease. *Gastroenterology, 112*, 1129–1136.
Steedman, H. F. (1950). Alcian blue 8GS: A new stain for mucin. *The Quarterly Journal of Microscopical Science, 91*, 477–479.
Strugala, V., Dettmar, P. W., & Pearson, J. P. (2008). Thickness and continuity of the adherent colonic mucus barrier in active and quiescent ulcerative colitis and Crohn's disease. *International Journal of Clinical Practice, 62*, 762–769.
Thompson, T., & Simpson, S. (2015). A comparison of gluten levels in labeled gluten-free and certified gluten-free foods sold in the United States. *European Journal of Clinical Nutrition, 69*, 143–146.
Tootian, Z., Sadeghinezhad, J., Sheibani, M. T., Fazelipour, S., De Sordi, N., & Chiocchetti, R. (2013). Histological and mucin histochemical study of the small intestine of the Persian squirrel (Sciurus anomalus). *Anatomical Science International, 88*, 38–45.

Trevizan, A. R., Vicentino-Vieira, S. L., da Silva Watanabe, P., Góis, M. B., de Melo Gde, A., Garcia, J. L., ... Sant'Ana Dde, M. (2016). Kinetics of acute infection with Toxoplasma gondii and hispopathological changes in the duodenum of rats. *Experimental Parasitology, 165*, 22–29.

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nature Review and Immunology, 9*, 799–809.

Ulluwishewa, D., Anderson, R. C., McNabb, W. C., Moughan, P. J., Wells, J. M., & Roy, N. C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. *The Journal of Nutrition, 141*, 769–776.

Van Klinken, B. J., Van der Wal, J. W., Einerhand, A. W., Büller, H. A., & Dekker, J. (1999). Sulphation and secretion of the predominant secretory human colonic mucin MUC2 in ulcerative colitis. *Gut, 44*, 387–393.

Wells, J. M., Brummer, R. J., Derrien, M., MacDonald, T. T., Troost, F., Cani, P. D., ... Garcia-Rodenas, C. (2016). Homeostasis of the gut barrier and potential biomarkers. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 312*, G171–G193.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., ... Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature, 486*, 222–227.