

Green Tea Polyphenols Mitigate Gliadin-Mediated Inflammation and Permeability in Vitro

Charlene B. Van Buiten, Joshua D. Lambert, and Ryan J. Elias*

Scope: Green tea, a polyphenol-rich beverage, has been reported to mitigate a number of inflammatory and hypersensitivity disorders in laboratory models, and has been shown to moderate pathways related to food allergies in vitro. The present study investigates the impact of decaffeinated green tea extract (GTE) on the digestion of gliadin protein in vitro and the effect of physical interactions with GTE on the ability of gliadin to stimulate celiac disease-related symptoms in vitro.

Methods and results: Complexation of GTE and gliadin in vitro is confirmed by monitoring increases in turbidity upon titration of GTE into a gliadin solution. This phenomenon is also observed during in vitro digestion when gliadin is exposed to the digestive proteases pepsin and trypsin. SDS-PAGE and enzymatic assays reveal that GTE inhibits digestive protease activity and gliadin digestion. In differentiated Caco-2 cell monolayers as a model of the small intestinal epithelium, complexation of gliadin with GTE reduces gliadin-stimulated monolayer permeability and the release of interleukin (IL)-6 and IL-8.

Conclusion: There are potential beneficial effects of GTE as an adjuvant therapy for celiac disease through direct interaction between gliadin proteins and green tea polyphenols.

1. Introduction

Celiac disease (CD) is an autoimmune enteropathy which affects approximately 1% of the world's population and is triggered by ingestion of gluten, a protein found in wheat, barley, and rye. Rich in glutamine and proline residues, gluten comprises two main subunits, glutenins and gliadins.^[1] CD is characterized by formation of lesions in the small intestinal mucosa, hyperplasia of the intestinal crypts, and villous atrophy. Impaired nutrient absorption as a result of CD can lead to malnutrition and extraintestinal manifestations including anemia and reduced bone density.^[2]

Dr. C. B. Van Buiten, Prof. J. D. Lambert, Prof. R. J. Elias
Department of Food Science
Pennsylvania State University, University Park
PA 16802, USA
E-mail: elias@psu.edu

Prof. J. D. Lambert
Center for Molecular Toxicology and Carcinogenesis
Pennsylvania State University, University Park
PA 16802, USA

 The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/mnfr.201700879>

DOI: 10.1002/mnfr.201700879

CD pathogenesis begins with incomplete digestion of gluten proteins in the small intestine, which triggers an increase in intestinal permeability via the enterocytic release of zonulin, an endogenous tight junction inhibitor.^[3] Increased permeability of the intestinal epithelium allows paracellular transport of gliadin across the brush border in addition to transcellular transport, creating an influx of undigested gliadins to the lamina propria.^[4,5]

The influx of gliadin to the lamina propria stimulates monocytes to release interleukin (IL)-15, a proinflammatory cytokine that signals the infiltration of intraepithelial lymphocytes (IELs) to the submucosa. IELs kill the epithelial cells producing stress signals, leading to the macroscopic changes in the small intestinal mucosa described previously as well as the secretion of tissue transglutaminase (TG2) by epithelial cells. This enzyme is normally associated with wound healing; however, in the context

of CD, TG2 deamidates glutamine residues on the gliadin fragments in the lamina propria, increasing their affinity to bind with CD-specific antigen presenting cells.^[6,7] Antigen presentation of gliadin and gliadin bound to TG2 results in the activation of CD4⁺ T cells, which produce inflammatory cytokines and stimulate plasma cells to produce anti-gliadin and anti-TG2 antibodies, propagating the immune response.^[8]

The only reliable treatment currently available to patients is lifelong adherence to a gluten-free diet, which is both expensive and difficult to maintain.^[9–12] For this reason, there is an urgent need for development of adjuvant therapies for CD. Among the proposed therapies are enzyme supplements that can be taken orally to further digest immunostimulatory epitopes,^[13] antagonists of intestinal permeability^[14] and a synthetic polymer that binds to gluten in the lumen.^[15–17] The polymer, composed of hydroxyethyl methacrylate and sodium-4-styrene sulfonate (poly(HEMA-co-SS)), has been shown to be a potentially effective strategy for mitigating CD symptoms. Poly(HEMA-co-SS) binds to gluten proteins in the lumen, making them resistant to digestion and absorption. This prevents the initiation of the CD symptoms and the previously described immune response, supporting gliadin sequestration as a possible adjuvant therapy for CD.^[15–17]

Polyphenols are secondary metabolites produced by a wide variety of plants, and have been demonstrated to bind

proteins with varying affinity. Protein-polyphenol interactions have been studied in terms of their role in the tactile oral sensation of astringency. Protein structure has been shown to be critical to these interactions, with proline-rich proteins favoring interactions with polyphenols via hydrogen bonding and van der Waals interaction.^[18] Protein-polyphenol interactions can affect digestibility and bioavailability of proteins directly by sequestering proteins, making them inaccessible to digestive proteases, and indirectly by inhibiting digestive protease activity.^[19–21]

A number of laboratory and epidemiological studies have suggested that green tea (*Camellia sinensis*, Theaceae) and its major polyphenolic component, (-)-epigallocatechin-3-gallate (EGCG) may prevent chronic inflammatory conditions including metabolic syndrome and inflammatory bowel diseases (IBD).^[22,23] Animal model studies have shown that green tea polyphenols can mitigate damage to the small intestinal mucosa,^[23,24] and reduce production of inflammatory cytokines, decreasing oxidative stress in epithelial cells.^[25–27]

IBD shares a number of pathologies with CD.^[22,28] However, CD is unique in its requirement for exposure to gluten antigens, the specific environmental trigger for the disease.^[1] Given the overlap in inflammatory signaling between these two conditions, the putative anti-inflammatory effects of green tea polyphenols, and the ability of green tea polyphenols to bind to and sequester dietary proteins, we hypothesized that green tea catechins can ameliorate the inflammatory pathologies associated with CD.

In the present study, our objectives were to characterize the formation of gliadin-green tea polyphenol complexes in vitro, to determine the impact of green tea polyphenols on gliadin digestion and the activity of small intestinal protease function, and to determine the efficacy of green tea extract (GTE) in reducing gliadin-mediated increases in intestinal permeability and inflammation.

2. Experimental Section

Gliadin, pepsin, trypsin, and Folin–Ciocalteu reagent were obtained from Sigma-Aldrich (St. Louis, MO). Decaffeinated GTE was kindly donated by Nature's Sunshine Products, Inc. (Spanish Fork, UT). The GTE used in this study contained 806 mg g⁻¹ total polyphenols, 653 mg g⁻¹ of which were catechins including 413 mg g⁻¹ EGCG, as reported by the manufacturer based on HPLC analysis. Pepsin/trypsin-digested gliadin (PT-gliadin) was prepared as described previously.^[29] In brief, gliadin (0.1 g mL⁻¹) was suspended in 0.2 N HCl and stirred continuously at 37 °C. Pepsin (2.0 g L⁻¹) was added to the suspension after 10 min, and the solution was stirred for 2 h, at which point, the pH was adjusted to 7.4 and trypsin (2.0 g L⁻¹) was added. Following an additional 4 h of continuous stirring at 37 °C, the enzymes were inactivated by boiling (30 min) and the solution was lyophilized.

2.1. In Vitro Digestion

The impact of GTE on the small intestinal digestion process was tested by dissolving gliadin in 0.2 N HCl (final concentration = 20 mg mL⁻¹) to which GTE was added. Pepsin was added (final concentration = 0.3 mg mL⁻¹) and the samples were digested at

37 °C for 2 h with stirring. The pH of the reaction was subsequently adjusted to 7.4 with 2 N NaOH, trypsin (final concentration = 0.3 mg mL⁻¹) was added, and the reaction was incubated at 37 °C for 4 h with stirring. At the end of the incubation, the reaction was boiled for 30 min. In order to evaluate the extent of digestion and the degree of gliadin precipitation in the presence of GTE, the samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In brief, aliquots were mixed with Laemmli sample buffer (Bio-Rad, Hercules, CA) and electrophoresed on 4–20% polyacrylamide gels using a constant voltage of 80 V for 15 min followed by 100 V for 45 min. Gels were stained with Coomassie R-250 dye (Sigma-Aldrich, St. Louis, MO) and imaged using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

2.2. Digestive Protease Activity

Inhibition of pepsin by GTE was measured using a commercially-available fluorimetric method (Molecular Probes, Eugene, OR). Pepsin (final concentration = 2.7 μg mL⁻¹ in 10 mM HCl, pH 2.0) was combined with GTE (final concentrations = 0–500 μg mL⁻¹). The reaction was started by addition of a fluorogenic substrate (BODIPY FL casein, final concentration = 10 μg mL⁻¹). After incubation at ambient temperature in the dark for 1 h, the fluorescence of the reaction was measured (λ_{ex} = 485 nm and λ_{em} = 530 nm) using a Fluoroskan Ascent FL (Thermo Scientific, Ashville, NC).

The inhibitory activity of GTE against trypsin was measured using a modified version of the method of Liu and Markakis.^[30] In brief, *N*-α-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA, 40 mg mL⁻¹ in DMSO) was diluted to a final concentration of 400 μg mL⁻¹ in pre-warmed assay buffer (50 mM Tris buffer, pH 8.2, 10 mM CaCl₂). Two volumes of GTE were then added to four volumes of the BAPNA solution to final GTE concentrations of 0–500 μg mL⁻¹. The enzyme reaction was initiated by addition of trypsin (stock solution = 0.2 mg mL⁻¹ in 1 mM HCl containing 2.5 mM CaCl₂, pH 2.5) to achieve a final concentration of 2.7 μg mL⁻¹ trypsin. The reaction was incubated for 10 min at 37 °C before being stopped by the addition of one volume of 30% acetic acid. The trypsin-mediated release of *p*-nitroaniline was determined by measuring the absorbance at 385 nm using a Multiskan Go UV/Vis plate reader (Thermo Scientific, Ashville, NC).

Mechanisms of pepsin and trypsin inhibition by GTE were measured by performing experiments analogous to those previously described, but with modifications. GTE was held at concentrations of 0, 50, or 100 μg mL⁻¹ while substrate concentrations were varied from 0 to 2 μM BODIPY FL casein for pepsin and from 0 to 2 mM BAPNA for trypsin.

2.3. Co-precipitation Profiling

Samples in the following experiments were prepared in 10 mM phosphate buffered saline (PBS) at pH 6.8. All experiments were completed in triplicate. PBS, PT-gliadin or native gliadin, and GTE were all used as controls.

To analyze the precipitation of gliadins by GTE, gliadin (PT and native gliadin, final concentrations of 0–4 mg mL⁻¹) was added to GTE (0–1.5 mg mL⁻¹). Samples were prepared in 96-well plates and shaken at 300 rpm for 2 h prior to analysis.

Turbidity was measured at 400 nm using a Multiskan Go UV/Vis Plate Reader (Thermo Scientific, Ashville, NC). Control absorbencies were subtracted from each corresponding turbidity reading as a blank. After turbidity readings, samples were removed from wells and centrifuged (10 min at 1300 × g). The supernatant was used for further analysis.

Percent precipitation of total polyphenols was measured using the Folin-Ciocalteu method. Soluble protein remaining in the supernatant was measured using the EZQ protein quantitation kit (Life Technologies, Grand Island, NY). Percent protein precipitated was calculated by comparing the soluble protein content of the controls to the protein content of each supernatant.

2.4. Cell Culture

Caco-2 cells from the BBE subclone line (C2BBE1, ATCC CRL-2102; American Type Tissue Collection, Rockville, MD) were kindly donated by Lankenau Institute for Medical Research (Wynnewood, PA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with sodium pyruvate supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 50 U mL⁻¹ penicillin and 50 µg mL⁻¹ streptomycin (Corning Life Sciences, Corning, NY). Cells were maintained at 37 °C in a 5% CO₂, 95% air humidified atmosphere. Cells were passaged twice weekly at 70% confluence. Passages 54–64 were used for the following experiments. The cytotoxicity of the treatments used in the following experiments was assessed as described in Supporting Information.

2.4.1. In Vitro Intestinal Permeability

A transwell monolayer model was used to measure paracellular permeability as a function of transepithelial electrical resistance (TEER). Cells were seeded on polycarbonate Transwells (0.33 cm², 0.4 µm pore size; Corning Life Sciences, Corning, NY) at a density of 2 × 10⁵ cells mL⁻¹. Media was refreshed every 2–3 days after confluence for 21 days until cells were fully differentiated and TEER values, measured every 2 days for the last week of growth, were stabilized.^[31] Only monolayers with TEER values above 300 Ω·cm² were used for permeability experiments. On day 21, cells were treated with 1 mg mL⁻¹ PT-gliadin, 1 mg mL⁻¹ GTE or a combination of the two treatments, delivering 1 mg mL⁻¹ of each. Treatment concentrations were chosen based on previous studies using in vitro models of celiac disease^[29] and a physiologically relevant dose of GTE, as calculated by a stomach volume of 0.25–1.7 L in a fasted versus fed state^[32] and a 250 mL cup of tea delivering approximately 900 mg of water extractable solids.^[33] This results in a concentration of 500–1000 mg mL⁻¹ in the stomach. DMEM was used as a negative control. TEER was measured using a Millicell ERS-2 Epithelial Volt-Ohm Meter (Millipore Corp, Billerica, MA) prior to, and immediately after, treatment as well as 1, 3, 4, and 24 h after treatment.

2.4.2. Inflammatory Markers

Cells were plated in 24-well plates at 4 × 10⁴ cells per well and allowed to attach for 48 h. Cells were then treated with 1 mg mL⁻¹ PT-gliadin, 1 mg mL⁻¹ GTE or the combination. DMEM was used as a negative control. Media was aliquoted at 30 min, 1 h, 4 h, and 24 h, then stored at –80 °C until analysis. Levels of IL-6 and IL-8 were assayed in duplicate by electrochemiluminescence using a multiplex V-Plex kit from Meso Scale Discovery (MSD; Meso Scale Discovery, Rockville, MD). Sample concentrations were determined based on a standard curve generated by MSD Workbench software. After 24 h of the aforementioned treatments, cells were trypsinized and counted using a hemocytometer to determine the impact of each treatment on the number of viable cells over the 24 h period.

2.5. Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM) and completed in triplicate unless otherwise indicated. One-way analysis of variance (ANOVA) was used for protease inhibition assays and two-way ANOVA was used for cell culture experiments, each with Tukey's Multiple Comparison Test to compare samples. Values of *p* < 0.05 were considered significant.

3. Results

3.1. Gliadin Digestion is Inhibited by GTE

In the absence of GTE, pepsin and trypsin hydrolyze gliadin, resulting in the formation of lower molecular weight peptides (Figure 1A). GTE inhibits pepsin/trypsin-mediated digestion in vitro as indicated by the greater levels of higher molecular weight gliadins present in incubations containing increasing amounts of GTE relative to gliadin (Figure 1B). We hypothesized that inhibition of gliadin digestion resulted from physical interaction between GTE and gliadin, leading to precipitation and decreased digestibility of gliadin, direct inhibition of the digestive proteases pepsin and trypsin, or a combination of these mechanisms.

Using in vitro enzyme activity assays, we found that GTE inhibits both pepsin (IC₅₀ = 29 µg mL⁻¹) and trypsin (IC₅₀ = 88 µg mL⁻¹) in a dose-dependent manner (Figure 2A,B). Kinetic analysis revealed that GTE exerts mixed mode of inhibition against pepsin with respect to substrate concentration, and uncompetitive mode of inhibition against trypsin with respect to substrate concentration (Figure 2B,C, Table 1).

3.2. GTE Binds to Native Gliadin and PT-Gliadin

The ability of GTE to bind to and precipitate gliadin was measured by examining the turbidity of gliadin solutions in combination with various amounts of GTE (Figure 3). Both native and PT-gliadin interact with GTE, resulting in the formation of insoluble aggregates. Infrared imaging of the GTE/gliadin complexes indicated that while GTE/native gliadin formed large, dense

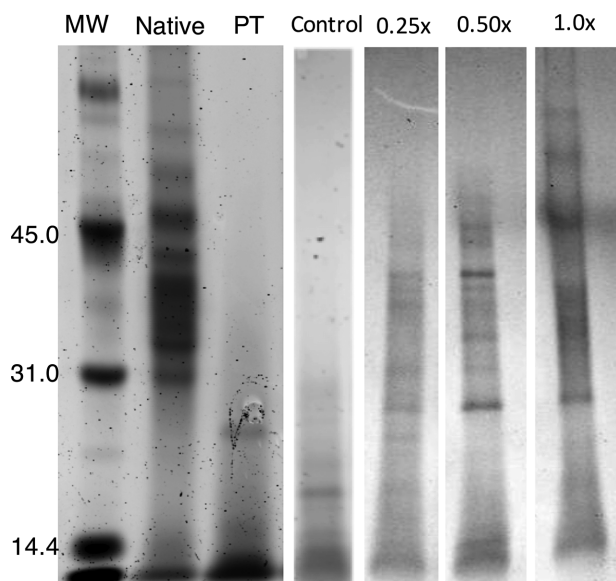


Figure 1. Molecular weight profiles of native and pepsin-trypsin digested (PT) gliadin. Dose-dependent inhibition of gliadin digestion. Control gliadin shows complete digestion of the protein. Supplementation of GTE caused decreased formation of low MW digestion products.

aggregates (Figure 3A), GTE/PT-gliadin resulted in the formation of haze (Figure 3B). These conformational differences contribute to the differences in measured turbidities of the solutions. The turbidity of GTE/native gliadin increases as a function of GTE concentration regardless of gliadin concentration (Figure 3C), while the turbidity of GTE/PT-gliadin depends on the concentrations of both GTE and PT-gliadin (Figure 3D).

As expected, soluble GTE polyphenols were also reduced in the co-incubations with gliadin and PT-gliadin. In the GTE/native gliadin samples, maximum polyphenolic precipitation was achieved at a 1:1.5 ratio of GTE:gliadin (w/w), with 33.35% of total polyphenols being removed from solution (Figure 3E). This is in contrast to GTE/PT-gliadin samples, where phenolic precipitation was greatest at the lowest GTE concentration, with 72.53% of total polyphenols precipitated when the sample contained a ratio of 0.05:1 GTE:PT-gliadin (w/w) (Figure 3F). Analysis of the supernatant showed that GTE is an effective sequestrant of PT-gliadin, precipitating $93.4\% \pm 9.2$ of protein at an addition ratio of 1:1. (Figure 3G).

Table 1. Kinetic analysis of GTE inhibition of digestive proteases.

GTE [$\mu\text{g mL}^{-1}$]	Pepsin		Trypsin	
	K_m [$\mu\text{mol L}^{-1}$]	$V_{\max}$ [$\text{pmol min}^{-1} \mu\text{g}^{-1}$ protein]	K_m [mmol L^{-1}]	$V_{\max}$ [$\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein]
0	0.49 ± 0.07^a	6.48 ± 0.33^a	4.15 ± 0.30^a	15.93 ± 0.87^a
50	0.74 ± 0.14^b	4.59 ± 0.36^b	0.61 ± 0.07^b	3.04 ± 0.15^b
100	0.82 ± 0.16^b	3.15 ± 0.27^c	0.57 ± 0.08^b	2.12 ± 0.12^c

Concentration of GTE is expressed as micrograms per milliliter. The units of K_m are expressed as millimole per liter *N*- α -benzoyl-DL-arginine for trypsin and micromole per liter BODIPY FL casein for pepsin. The units of $V_{\max}$ are expressed as micromole *p*-nitroaniline per minute per milligram protein for trypsin and pmol BODIPY FL per minute per microgram protein for pepsin. Values are expressed as mean $\pm$ the standard deviation of the mean of three independent experiments and were analyzed using one-way ANOVA. Values in the same column not sharing common letters are significantly different ($p < 0.05$).

3.3. GTE Exerts a Protective Effect on Intestinal Epithelial Cells

GTE exerts a protective effect against gliadin-mediated intestinal permeability and inflammation (Figure 4 and 5). The treatments used were found to have no significant effect on cell viability under the experimental conditions used based on the MTT assay (Figure S1, Supporting Information).^[34]

The protective effect of GTE against gliadin-mediated intestinal permeability was observed as the preservation of TEER (Figure 4). This effect can be observed as early as 1 h into the treatment, with both the GTE and combination treatments demonstrating similar increases in TEER, approximately 12% greater than baseline. Gliadin-mediated permeability appears at the 1 h time point and is maintained through 24 h, but the protective effects of GTE are sustained.

Secretion of proinflammatory cytokines IL-6 and IL-8 by Caco-2 cells was also mitigated by GTE (Figure 5). Over the course of 4 h, PT-gliadin stimulated a significant increase in IL-6 production, which has been mediated by the addition of GTE at a 1:1 ratio. The protective effects of GTE against IL-6 production are sustained over 24 h, even as IL-6 production by the control cells increases to similar levels as those treated with PT-gliadin. IL-8 production, which is elevated by PT-gliadin within the first 0.5 h of treatment, was suppressed in the presence of GTE throughout the course of the experiment. The PT:GTE-treated cells produced significantly less IL-8 than the PT-gliadin and control treatments. The protective effect of GTE alone is apparent as well, based on similarly suppressed IL-8 production throughout the 24 h experiment.

With respect to the elevated levels of IL-6 and IL-8 in the control samples, it is important to note that each of the applied treatments affects cell proliferation and, thus, the apparent concentrations of inflammatory cytokines shown are likely affected by the number of cells. Cell proliferation experiments demonstrated that cell growth rate was modified by the presence of the treatment groups; however, the cell counts at the time of the experiment were not different from one another, validating comparisons between experimental groups (Figure 6).

4. Discussion

Adherence to a gluten-free diet is currently the only widely accepted therapy for individuals with CD; however, many individuals are exposed to gluten proteins inadvertently. For this

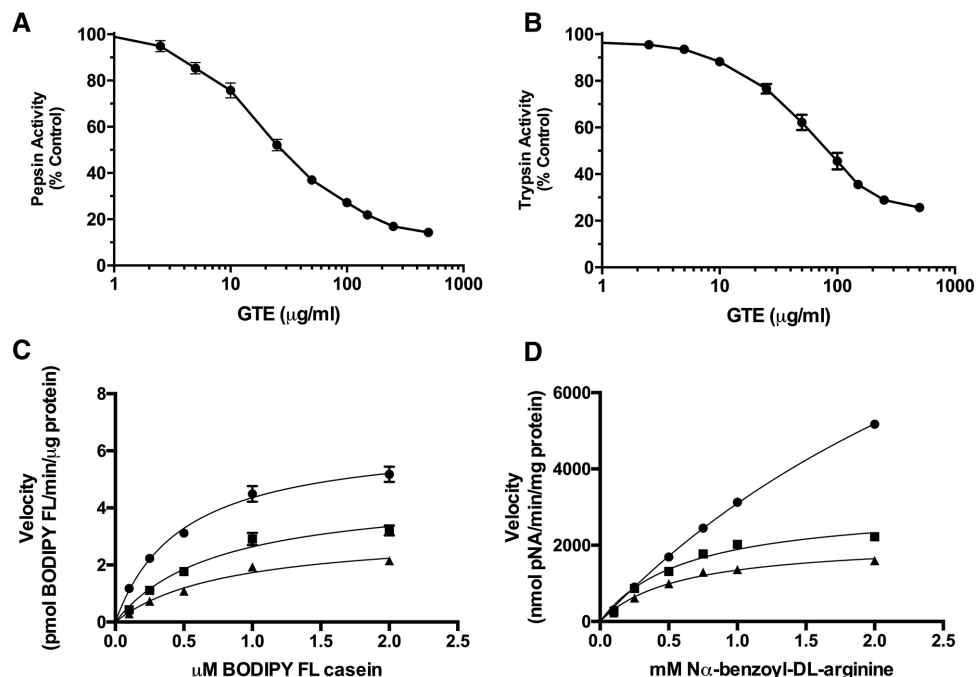


Figure 2. GTE dose dependently inhibits pepsin and trypsin in vitro. A) Pepsin is inhibited by GTE with an IC_{50} of $29 \mu g mL^{-1}$ while B) trypsin is inhibited with an IC_{50} of $88 \mu g mL^{-1}$. Mechanisms of inhibition were determined using Michaelis-Menten analysis, showing GTE as C) a mixed inhibitor of pepsin and D) an uncompetitive inhibitor of trypsin. All values are expressed as mean $\pm$ SEM from three independent experiments.

reason, adjuvant therapies are needed to protect individuals with CD from the deleterious effects of gluten exposure. Data suggest that sequestration of gliadin may be an effective prevention strategy for CD by inhibiting digestion and absorption of the protein. Oral administration of the synthetic polymer poly(HEMA-co-SS) prevents gliadin-mediated toxicity in vivo.^[16] Green tea polyphenols have been shown to interact with a number of proteins, and to mitigate inflammation and oxidative stress associated with IBD, a condition that has pathologic similarities to CD.^[25–27] The present study explored the potential protective effect of GTE in CD through physical interaction with gliadin, inhibition of gliadin digestion, and prevention of gliadin-mediated damage in an in vitro model of the small intestine.

CD pathogenesis begins when partially digested gliadin peptide crosses the brush border as a result of protected transcytosis^[5] or paracellular transport, which is increased as a result of zonulin-mediated intestinal permeability.^[4,35] Gliadin molecular weights fall within the range 28–55 kDa,^[36] but hydrolysis by pepsin and trypsin yields lower molecular weight digestive products (Figure 1). Upon addition of GTE to the in vitro digestion, the production of low molecular weight digestive products is decreased. Our findings suggest two separate mechanisms of digestion inhibition: 1) direct inhibition of digestive proteases and 2) substrate sequestration, wherein gliadins bound by GTE are protected from enzymatic hydrolysis.

Pepsin and trypsin were both dose-dependently inhibited by GTE by mixed and uncompetitive mechanisms, respectively. The inhibition of digestive proteases by dietary polyphenols has been studied with respect to the impact on digestibility and accessibility of proteins in the human diet, and this inhibition has been shown to occur primarily through non-covalent interactions.^[37]

The structure of the polyphenols has been shown to play an important role with respect to inhibitory activity, with greater affinity between polyphenols and trypsin observed as a function of increasing hydroxyl groups on the phenolic rings.^[38] As a result, dietary polyphenols are likely to exhibit varying degrees of inhibitory activity due to variability in polyphenolic profile (i.e., hydroxyl group substitution). In general, GTE is associated with trypsin inhibitory activity,^[20,21] although conflicting data exist as to whether GTE inhibits or activates pepsin activity.^[21,39]

While GTE formed insoluble complexes with both native and PT-gliadins, physical differences were observed in the particles formed via the measured turbidity and infrared imaging of samples, as GTE/native gliadin complexes demonstrated lower overall turbidity and larger, more dense particles in comparison to GTE/PT-gliadin, where greater overall turbidity was observed in combination with the formation of haze rather than aggregates. These conformation differences are likely due to the differences in the molecular weight and particle size of the protein in solution prior to the addition of GTE, as turbidity measurements are affected by concentration and geometry of particles. Although the overall mass protein concentration is the same between samples, PT-gliadin features a greater number of individual molecules per milligram as a result of enzymatic hydrolysis.^[40]

CD is characterized by intestinal barrier permeability and the formation of mucosal lesions upon exposure to gliadin.^[1] During the initial stages of pathogenesis, gliadin causes the disruption of tight junction proteins and polymerization of cytoskeletal actin in the intestinal epithelium, resulting in permeability of the brush border.^[3] When plated on Transwells and grown into a confluent monolayer, Caco-2 cells serve as a simple model for the intestinal brush border. The biological implications of complexation with

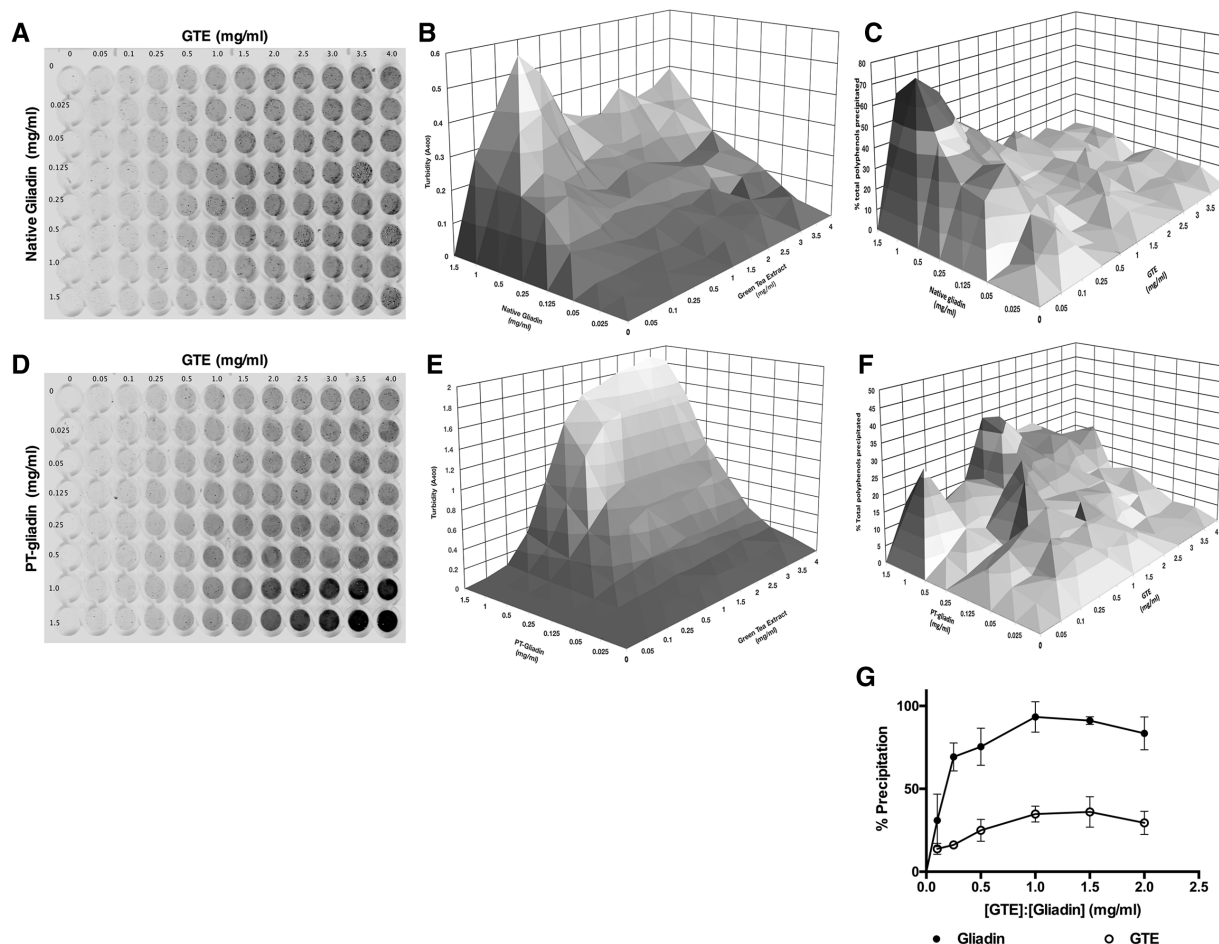


Figure 3. GTE interacts with gliadins both before and after enzymatic hydrolysis. Precipitation profiles of A–C) native gliadin and D–F) PT-gliadin with GTE. Native gliadin was precipitated by GTE to form A) larger dense particles compared to D) the haze formed by PT-gliadin, resulting in lower overall turbidity (B vs. E). GTE interaction with PT-gliadin results in C, F, G) co-precipitation of both the protein and polyphenols.

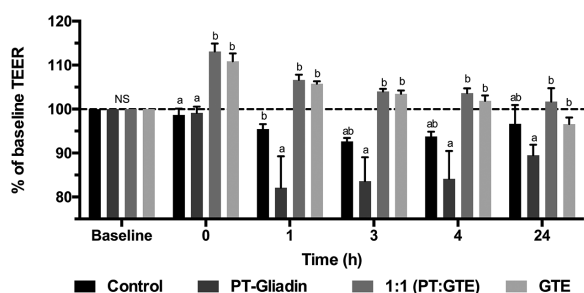


Figure 4. Changes in TEER as a result of gliadin and GTE addition. Gliadin-mediated permeability is demonstrated by decreases in TEER. GTE supplementation increases TEER in the presence and absence of gliadin, suggesting an improvement in barrier integrity. Values are expressed as mean $\pm$ SEM from two independent experiments with three replications per treatment. Values in the same time point not sharing common letters are significantly different ($p < 0.05$).

GTE on gliadin-mediated permeability and inflammation of the small intestine were tested using pre-digested PT-gliadin mixed with GTE at a 1:1 (w/w) ratio and the Caco-2 cell line. Previous studies have shown that inhibiting the formation of immunos-

timulatory and cytotoxic digestive fragments of gliadin may be an effective strategy for preventing CD symptoms^[16,17]; therefore, we chose to investigate the system from the perspective of toxic gliadin fragments having already been produced by digestive enzymes and GTE administered as a therapy post-ingestion.

In the absence of GTE, PT-gliadin elicited an increase in permeability, as demonstrated by decreasing TEER. The pretreatment of PT-gliadin with GTE at a 1:1 (w/w) ratio prevented this gliadin-mediated permeability, instead promoting barrier integrity as noted by increasing TEER. GTE and its polyphenolic components have previously been associated with improved barrier function in non-CD gastrointestinal diseases. EGCG, the most prominent catechin in green tea, has been shown to improve barrier function in DSS-induced colitis in vivo via upregulation of GLP-2, a peptide hormone associated with protection against mucosal injury.^[27] In addition to preventing gliadin-mediated permeability, GTE mitigated the production of proinflammatory cytokines IL-6 and IL-8 by Caco-2 cells. IL-6 is associated with the aforementioned intestinal permeability observed upon gliadin challenge in CD, and it has been found to be expressed in both acute and chronic inflammatory processes.^[26] IL-8, stimulated in CD pathogenesis by the binding

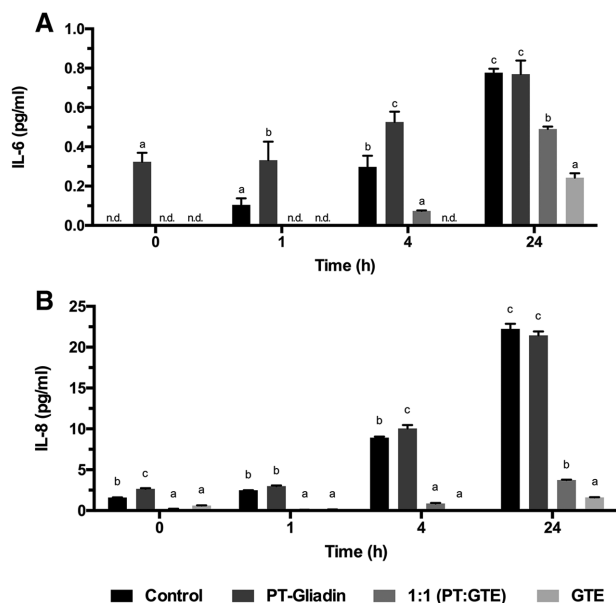


Figure 5. A) Gliadin-mediated IL-6 production is downregulated by GTE. Experiments completed in duplicate, expressed as mean $\pm$ standard deviation. Values in the same time point not sharing common letters are significantly different ($p < 0.05$). B) IL-8 production is downregulated by GTE in the presence and absence of gliadin. Values are expressed as mean $\pm$ SEM from two independent experiments with three replications per treatment. Values in the same time point not sharing common letters are significantly different ($p < 0.05$).

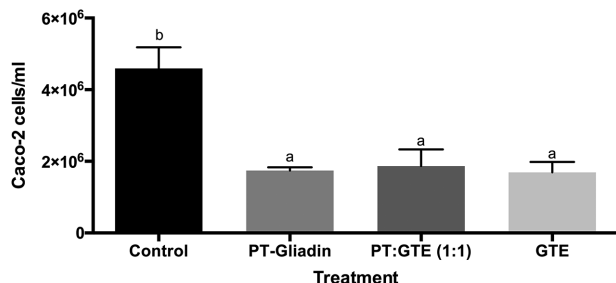


Figure 6. PT-gliadin and GTE modify cell growth rates over the course of 24 h in comparison to the untreated control. Cells were initially plated with a seeding density of 2×10^5 cells mL^{-1} . Values are expressed as mean $\pm$ SEM from three independent experiments. Values in the same time point not sharing common letters are significantly different ($p < 0.05$).

of PT-gliadin to the CXCR3 receptor, is the cytokine responsible for the recruitment and activation of IELs in the small intestinal mucosa, a diagnostic characteristic of the disease.^[1,41]

Mediation of proinflammatory cytokines such as IL-6 and IL-8 has been investigated as a treatment for IBD. Green tea polyphenols and purified EGCG have been shown to mediate TNF- α -stimulated IL-8 release by IEC-6 cells^[42] as well as prevent IL-6 and TNF- α secretion, and ameliorate colonic lesions in a murine model of ulcerative colitis.^[24] Our findings suggest that similar benefits may exist in the application of green tea polyphenols as a treatment for CD via similar mechanisms of direct influence on cytokine production by intestinal cells as observed in IBD models, or by our demonstrated mechanism of gliadin sequestration. Further studies are required to determine the extent of each of

these contributing mechanisms to the observed protective effects of GTE against gliadin-mediated inflammation and permeability.

The results of this study support the potential for GTE as an adjuvant therapy for controlling CD. The therapeutic mechanism of GTE appears to be similar to that of poly(HEMA-co-SS), in that both can sequester gliadin. We have shown that GTE may also confer protection against gliadin by preventing protein digestion through protease inhibition and can mitigate gliadin-mediated inflammatory responses. Though the in vitro model used in this study simplifies the reaction conditions for digestion and CD pathogenesis, the findings provide proof of the concept that inhibition of gliadin digestion may be beneficial in ameliorating CD-related intestinal permeability and inflammation. One limitation of the model used is the absence of non-gliadin proteins which would likely also bind to polyphenols in vivo.^[43] However, dietary polyphenols have previously been shown to inhibit protein digestion in vivo and it has also been determined that proteins with high frequencies of proline have increased likelihood to interact with polyphenols.^[44] Future studies should focus on the translation of this treatment approach to a more complex in vitro or ex vivo system in order to determine the impact of GTE/gliadin interactions on the CD response to gliadin as a whole, including the activation of gluten-sensitive immune cells.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgments

C.B.V. designed the study, performed the experiments, and wrote the manuscript. J.D.L. assisted with experimental design. R.J.E. supervised the study. Caco-2 cells were donated by Dr. James Mullin from the Lankenau Institute for Medical Research (Wynnewood, PA). This project was supported by a National Institute of Food and Agriculture Predoctoral Fellowship awarded to C.B.V. under Grant no. 2016-67011-24702, USDA Agriculture and Food Research Initiative.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

Caco-2, celiac disease, gliadin, gluten, green tea

Received: October 19, 2017

Revised: April 9, 2018

Published online: April 28, 2018

[1] A. Fasano, C. Catassi, *New Engl. J. Med.* **2007**, 367, 1731.

[2] D. A. Leffler, P. H. R. Green, A. Fasano, *Nat. Publ. Gr.* **2015**, 12, 561.

[3] S. Drago, R. El Asmar, M. Di Pierro, M. Grazia Clemente, A. Tripathi, A. Sapone, M. Thakar, G. Iacono, A. Carroccio, C. D'Agate, T. Not,

- L. Zampini, C. Catassi, A. Fasano, *Scand. J. Gastroenterol.* **2006**, 41, 408.
- [4] K. M. Lammers, R. Lu, J. Brownley, B. Lu, C. Gerard, K. Thomas, P. Rallabhandi, T. Shea-Donohue, A. Tamiz, S. Alkan, S. Netzel-Arnett, T. Antalis, S. N. Vogel, A. Fasano, *Gastroenterology* **2009**, 135, 194.
- [5] T. Matysiak-Budnik, C. Candalh, C. Dugave, A. Namane, C. Cellier, C. Cerf-Bensussan, M. Heyman, *Gastroenterology* **2003**, 125, 696.
- [6] A. Sapone, K. M. Lammers, V. Casolaro, M. Cammarota, M. T. Giuliano, M. De Rosa, R. Stefanile, G. Mazzarella, C. Tolone, M. I. Russo, P. Esposito, F. Ferraraccio, M. Carteni, G. Riegler, L. de Magistris, A. Fasano, *BMC Med.* **2011**, 9, 23.
- [7] S.-W. Qiao, E. Bergseng, Ø. Molberg, J. Xia, B. Fleckstein, C. Khosla, L. M. Sollid, *J. Immunol.* **2004**, 173, 1757.
- [8] M. Fleur, L. M. Sollid, *Best Pract. Res. Clin. Gastroenterol.* **2015**, 29, 413.
- [9] L. M. Sollid, C. Khosla, *J. Intern. Med.* **2012**, 269, 604.
- [10] A. R. Lee, D. L. Ng, J. Zivin, P. H. R. Green, *J. Hum. Nutr. Diet.* **2007**, 20, 423.
- [11] G. M. Forbes, *United Eur. Gastroenterol.* **2016**, 4, 6847.
- [12] B. Lebowitz, J. A. Murray, A. Rubio-Tapia, P. H. R. Green, J. F. Ludvigsson, *Aliment Pharmacol Ther* **2014**, 39, 488.
- [13] M. Siegel, M. E. Garber, A. G. Spencer, W. Botwick, P. Kumar, R. N. Williams, K. Kozuka, R. Shreenivas, V. Pratha, D. C. Adelman, *Dig. Dis. Sci.* **2012**, 57, 440.
- [14] S. Khaleghi, J. M. Ju, A. Lamba, J. A. Murray, *Therap. Adv. Gastroenterol.* **2016**, 9, 37.
- [15] J. L. McCarville, Y. Nisemblat, H. J. Galipeau, J. Jury, R. Tabakman, A. Cohen, E. Naftali, B. Neiman, E. Halbfinger, J. A. Murray, A. N. Anbazhagan, P. K. Dudeja, A. Varvak, J.-C. Leroux, E. F. Verdu, *PLoS One* **2014**, 9, 1.
- [16] M. Pinier, E. F. Verdu, M. Nasser-Eddine, C. S. David, A. Vézina, N. Rivard, J.-C. Leroux, *Gastroenterology* **2009**, 136, 288.
- [17] M. Pinier, G. Fuhrmann, H. J. Galipeau, N. Rivard, J. A. Murray, C. S. David, H. Drasarova, L. Tuckova, J.-C. Leroux, E. F. Verdu, *Gastroenterology* **2012**, 142, 316.
- [18] A. J. Charlton, N. J. Baxter, M. L. Khan, A. J. G. Moir, E. Haslam, A. P. Davies, M. P. Williamson, *J. Agric. Food Chem.* **2002**, 50, 1593.
- [19] R. J. Green, A. S. Murphy, B. Schulz, B. A. Watkins, M. G. Ferruzzi, *Mol. Nutr. Food Res.* **2007**, 51, 1152.
- [20] S. Naz, R. Siddiqi, T. P. Dew, G. Williamson, *J. Agric. Food Chem.* **2011**, 59, 2734.
- [21] Q. He, Y. Lv, K. Yao, *Food Chem.* **2006**, 101, 1178.
- [22] Y. Zhang, Y. Li, *World J. Gastroenterol.* **2014**, 20, 91.
- [23] T. Vezza, A. Rodríguez-Nogales, F. Algieri, M. P. Utrilla, M. E. Rodríguez-Cabezas, J. Galvez, *Nutrients* **2016**, 8, 211.
- [24] H. S. Oz, T. Chen, W. J. S. De Villiers, *Front. Immunol.* **2013**, 4, 1.
- [25] M. Brückner, S. Westphal, W. Domschke, T. Kucharzik, A. Lügering, *J. Crohn's Colitis* **2012**, 6, 226.
- [26] F. Yang, W. J. S. De Villiers, C. J. McClain, G. W. Varilek, *J. Nutr.* **1998**, 128, 2334.
- [27] Z. T. Bitzer, R. J. Elias, M. Vijay-Kumar, J. D. Lambert, *Mol. Nutr. Food Res.* **2016**, 60, 2267.
- [28] L. M. Sollid, K. E. Lundin *Mucosal Immunol.* **2009**, 2, 3.
- [29] K. E. Thomas, A. Sapone, A. Fasano, S. N. Vogel, *J. Immunol.* **2006**, 176, 2512.
- [30] K. Liu, P. Markakis, *Cereal Chem.* **1989**, 66, 415.
- [31] M. Natoli, B. D. Leoni, I. D'Agnano, F. Zucco, A. Felsani, *Toxicol. Vitro* **2012**, 26, 1243.
- [32] M. Einhorn, *Diseases of the Stomach: A Text-Book for Practitioners and Students*, 2nd ed., Ithaca, NY, USA printed, Cornell Univ. Library, **2009**.
- [33] C. S. Yang, P. Maliakal, X. Meng, *Annu. Rev. Pharmacol. Toxicol.* **2002**, 42, 25.
- [34] A. A. van de Loosdrecht, R. H. J. Beelen, G. J. Ossenkoppele, M. G. Broekhoven, M. M. A. C. Langenhuijsen, *J. Immunol. Methods* **1994**, 174, 311.
- [35] A. Fasano, *Physiol. Rev.* **2011**, 91, 151.
- [36] H. Wieser, *Food Microbiol.* **2007**, 24, 115.
- [37] A. Martinez-Gonzalez, A. Diaz-Sanchez, L. de la Rosa, C. Vargas-Requena, I. Buston-Jaimes, E. Alvarez-Parrilla, *Molecules* **2017**, 22, 1.
- [38] Q. Li, Q. Wei, E. Yuan, J. Yang, Z. Ning, *Int. J. Food Sci. Technol.* **2014**, 49, 1063.
- [39] Z. Tantoush, D. Apostolovic, B. Kravic, I. Prodic, L. Mihajlovic, D. Stanic-Vucinic, T. C. Velickovic, *J. Funct. Foods* **2012**, 4, 650.
- [40] H. H. Kleizen, A. B. de Putter, M. van der Beek, S. J. Huynink, *Filtr. Sep.* **1995**, 32, 897.
- [41] K. M. Lammers, S. Khandelwal, F. Chaudhry, D. Kryszak, E. L. Puppa, V. Casolaro, A. Fasano, *Immunology* **2011**, 132, 432.
- [42] H. S. Oz, J. Ebersole, *J. Cancer Ther.* **2010**, 1, 105.
- [43] L. Hussein, H. Abbas, *Nutr. Rep. Int.* **1985**, 31, 67.
- [44] V. De Freitas, N. Mateus, *Curr. Org. Chem.* **2012**, 16, 724.

Supporting Information

The MTT assay was used to test the cytotoxicity of each treatment on Caco-2 cells after 4 hours of exposure. Cells were seeded in 96-well plates at 10×10^3 cells per well using the culture methods described in the manuscript and allowed to grow to confluence. On the day of experiments, medium was aspirated and 100 μ L of new medium containing 1 mg/mL PT-gliadin, 1 mg/mL GTE or 1 mg/mL PT:GTE (1:1) was added to the wells. The control was treated with the DMEM culture medium. The MTT assay was carried out as described in previously published literature [1]. Viability of treated cells was normalized to that of the control. It was determined that the treatments had no significant impact on metabolic activity.

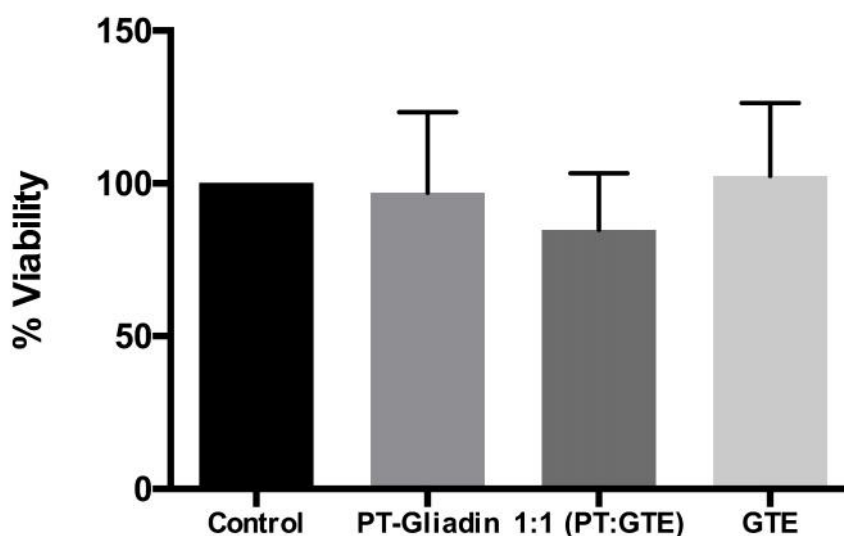


Figure S1. Viability of Caco-2 cells after 4 h of exposure to 1 mg/ml PT-gliadin, 1 mg/ml PT:GTE (1:1) or 1 mg/ml GTE. Values are expressed as mean $\pm$ SD. No statistically significant differences were detected between groups using one-way ANOVA with a Tukey's multiple comparisons post-test.

- [1] van de Loosdrecht, A.A., Beelen, R.H.J., Ossenkoppele, G.J., Broekhoven, M.G., et al., A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *J. Immunol. Methods* 1994, 174, 311–320.

