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Influence of *Bifidobacterium longum* CECT 7347 and Gliadin Peptides on Intestinal Epithelial Cell Proteome

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ABSTRACT: Celiac disease is an enteropathy caused by an abnormal immune response to cereal gluten proteins (gliadin). To unravel the possible role of the interactions between gliadin peptides and specific intestinal bacteria, the response of intestinal epithelial (Caco-2) cells to gliadin subjected to gastrointestinal digestion in the presence or absence of *Bifidobacterium longum* CECT 7347 has been studied. Changes in the proteome of Caco-2 cells were determined by 2DE and MALDI-TOF. Gliadins digested without *B. longum* altered the expression of a higher number of proteins than in the presence of the bacterium (21 versus 9), and these proteins were involved in disorganization of cell cytoskeleton, inflammation, and apoptosis. Gliadins digested in the presence of the bacterium influenced the production of proteins involved in calcium homeostasis and cell survival and function. Therefore, *B. longum* CECT 7347 might ameliorate gliadin toxicity and modify the responses of intestinal epithelial cells to the gliadin challenge.

KEYWORDS: *Bifidobacterium longum*, celiac disease, Caco-2 cells, probiotic, proteome

INTRODUCTION

Cereal gluten proteins (gliadin α, β, γ, and ϖ) are the main environmental factors causative of celiac disease (CD) pathogenesis. The incomplete hydrolysis of gliadin by human enzymes during gastrointestinal digestion leads to the generation of peptides able to trigger cytotoxic and inflammatory signals in intestinal epithelial cells of CD patients.[1] Clinical manifestations of the disease often include intestinal symptoms and nutrient malabsorption associated with severe mucosal damage.[2] Currently, the only available therapy for CD patients is the adherence to a strict life-long gluten-free diet. However, compliance with this dietary recommendation is complex, and other alternative or adjuvant strategies are needed.[3]

Alterations in the composition of the gut microbiota of CD patients are characterized by decreased *Bifidobacterium* numbers.[4,5] The association of imbalances in the intestinal microbiota and the positive reported roles that bifidobacteria play on intestinal health have led to the proposed use of probiotics as part of additional and alternative nutritional strategies for improving the quality of life of CD patients. Certain strains of the *Bifidobacterium* genus effectively reduced in vitro the toxicity and inflammatory potential of gliadin-derived peptides.[6] The positive effect of bifidobacteria was attributed to their proteolytic capacity on gliadin peptides; however, the effects produced by different bifidobacteria seemed to be strain-dependent, demonstrating the most positive effect produced by *Bifidobacterium longum* CECT 7347 strain, which motivated the present study.

Gliadin-derived peptides are thought to stimulate enterocytes[7,8] and macrophages[9] via specific surface receptors such as the chemokine CXCR3, a 7-transmembrane G-protein, present in the brush border membrane, causing the up-regulation of pro-inflammatory cytokine expression.[10,11] These effects have been linked to the activation of the nuclear transcription factor κ-B (NF-κB) pathway in the small intestinal mucosa of CD patients.[12] After sensing an inflammatory stimulus, the inhibitor IκB is phosphorylated, triggering its degradation, thereby allowing the translocation of NF-κB to the nucleus, where it induces the expression of a variety of genes involved in inflammation, immune regulation, and cell survival and proliferation.[13] Furthermore, alterations in the integrity and function of the intestinal barrier have also been associated with gliadin toxicity.[14] Gliadin-derived peptides can mediate the disorganization of tight junction-related proteins leading to increased intestinal permeability[15] and expression (mRNA) of ionic channels and the chemokine CXCR3 receptor.[6,7] However, there is limited knowledge of the possible role of the interplay between gliadin peptides and intestinal bacteria in the activation of pathogenic or protective responses in the intestine.

The objective of this study was to evaluate the changes in the proteome of Caco-2 cells, widely used as a human intestinal model, exposed to gliadin digested in vitro, in the presence or absence of *B. longum* CECT 7347 and in response to the bifidobacteria alone. The ultimate goal of the study was to gain a better understanding of early alterations induced by gliadin in enterocytes and their possible modulation by components of the intestinal microbiota.

MATERIALS AND METHODS

Bacterial Cultures. *B. longum* CECT 7347 was isolated from feces of healthy infants as described elsewhere.[15] The bacterial cultures were grown in Man−Rogosa−Sharpe broth and agar (Scharlau, Barcelona, Spain) supplemented with 0.05% (w/v) cysteine (MRSC) (Sigma, St. Louis, MO) and incubated at 37 °C under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, U.K.) for 24 h. The studies employed bacterial cell suspensions with absorbance of 0.5 (λ, 600 nm) corresponding to 10^8 colony-forming units (CFU)/mL.

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Cell Culture Conditions. The human colon carcinoma (Caco-2) cell line was obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passages 33−42. Caco-2 cells were grown in DMEM (AQ-Media, Sigma) containing 4.5 g L^{-1} glucose and supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Biowhittaker, Schiedam, The Netherlands), 10% (v/v) fetal bovine serum (Sigma), 1% (v/v) nonessential amino acids (Biowhittaker), 1% (v/v) L-glutamine (Sigma), and 1% (v/v) antibiotic solution (penicillin, streptomycin, and fungizone) (Sigma). Cells were maintained at 37 °C in 5% CO_2/95% relative humidity, and culture medium was changed every 2 days.[6]

For experimental studies, Caco-2 cells were seeded at densities of 50000 cells/cm^2 onto 6-well plates (Costar, Corning, NY). Cell cultures were grown with DMEM, and culture medium was changed every 2 days. Experiments were performed 5 days postseeding. Cell cultures exposed to DMEM were used as controls.

In Vitro Digestion of Gliadin. The gastrointestinal digestion process was simulated as previously described,[16] using porcine pepsin (P-7000, Sigma) (800−2500 units/mg protein), pancreatin (P1750, Sigma) (activity 4 × USP specifications), and bile (B3883, Sigma). Three milliliters of a saline solution (140 mM NaCl, 5 mM KCl adjusted to pH 3) was added to aliquots (150 mg) of a commercially available gliadin extract (G3375, Sigma). The mixture was immersed in a water bath (60 °C) for 30 min with gentle agitation. Once the samples were at room temperature, gastric and intestinal digestions were conducted on a rocking platform shaker placed in an incubator (37 °C/5% CO_2/95% relative humidity). After the gastric digestion (pepsin in 0.1 M HCl/pH 3/1 h), the intestinal digestion (pancreatin−bile in 0.1 M $NaHCO_3$/pH 6.8−7.0/2 h) was carried out in the upper chamber of a two-chamber system in 6-well plates formed by fitting the bottom of a Transwell insert ring (Costar) with a 15000 molecular mass cutoff dialysis membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). Aliquots (1.5 mL) of the gastrointestinal digests, inoculated or not with bacterial cell suspensions (10^8 CFU/mL), were loaded into the upper chambers, and 1 mL of saline solution (pH 7) was added to the bottom chamber. Afterward, the inserts were removed and total protein content in the dialysates was quantified using a Lowry method based on a commercial kit (TP0200, Sigma). After in vitro digestion, bacterial counts were confirmed in the digests by plate counting on MRSC and were approximately 10^6−10^7 CFU/mL.

Toxicity Experiments. Caco-2 cell viability in cultures exposed (6 h) to digested gliadin (0.25 mg/mL), inoculated or not with *B. longum* CECT 7347, were determined by the Neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) (Sigma) uptake assay.[6] The culture medium was removed, and cells were washed twice with phosphate buffer saline (PBS) at pH 7.2 (Sigma). NR uptake was measured using a commercial kit (no. 7H092, Sigma) at 540 nm with background subtraction at 690 nm. Control cells exposed to DMEM were analyzed in every assay.

Protein Extraction and 2-DE Separation. Caco-2 cells were exposed for 6 h to digested gliadins (0.25 g/mL), inoculated or not with *B. longum* CECT 7347, or to the bifidobacteria (10^8 CFU/mL). The culture supernatant was removed and kept to determine cytokines as described below, and cells were washed twice with PBS for proteome analyses (pH 7.2) (Sigma).

Protein extraction was performed as described elsewhere[17] by pipetting with lysis buffer [7 M urea (GE Healthcare, Princeton, NJ), 2 M thiourea (GE Healthcare), Nonidet p-40 (USB Corp., Cleveland, OH) (1% v/v), and protease inhibitors (Roche, complete 04 693 116 001)]. After centrifugation at 4 °C and 11000*g* for 10 min, total protein content was determined as previously described. Aliquots (200 μg of protein/2 μL) were mixed with rehydration solution (340 μL) [7 M urea, 2 M thiourea, dithiothreitol (DTT) (Fluka, Arjeplog, Sweden) (0.2%, v/v), Nonidet p-40 (1%, v/v), Pharmalyte 3−10 (GE Healthcare) (1%, v/v), and bromophenol blue] and loaded in the dry polyacrylamide gel strips with an immobilized pH gradient (IPG) (GE Healthcare, Immobiline DryStrip pH 3−10 NL, 18 cm). The IPG strips were covered with mineral oil (GE Healthcare) and kept at room temperature overnight. Protein separation according to the isoelectric point (p*I*) was conducted with an Ettan IPG-Phor II cuvette, and the program was visualized through Ettan control software 1.0. Isoelectric focusing (IEF) was carried out under the following conditions: step 1, increase from 0 to 500 V in 5 h; step 2, 500 V for 5 h; step 3, increase from 500 to 3500 V in 9.5 h; and step 4, 3500 V for 4 h. Focused IPG strips were equilibrated with equilibration buffer [50 mM Tris-HCl (pH 8.8) (Sigma), SDS (2%, v/v), 6 M urea, glycerol (30%, v/v), DTT (1%, v/v), and iodoacetamide (IAA) (Sigma) (2.5%, w/v)]. The second dimension was run (Ettan Dalt Six cuvette) in pairs side by side. The strips were sealed with an agarose solution 0.5% (w/v) and run overnight at a constant voltage in a polyacrylamide gel (Bio-Rad, Hercules, CA) (acrylamide−bisacrylamide, 12.5%−0.27%, 2.2 C). The analyses were performed in triplicate for each treatment.

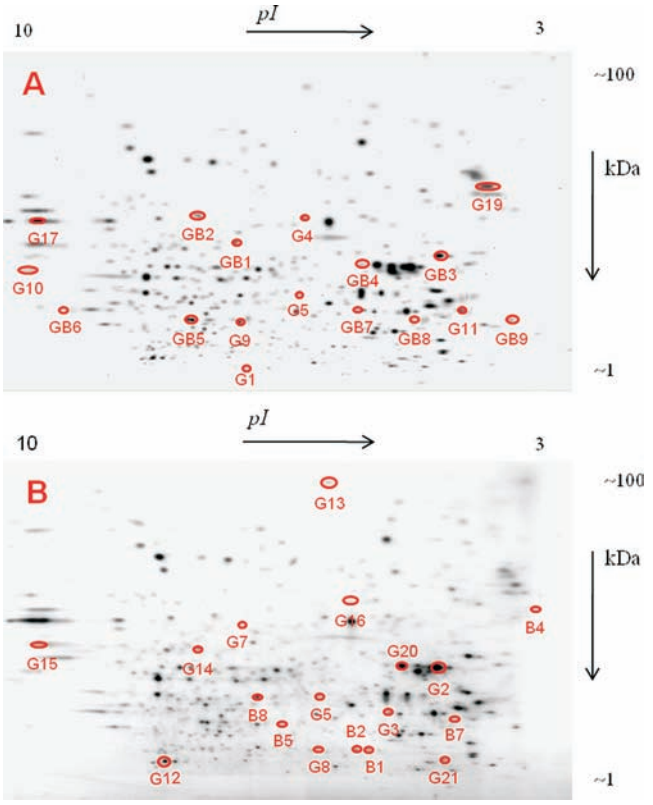

Figure 1. 2-D gel images of the proteins extracted from the Caco-2 cells exposed to gliadin (A) and gliadin inoculated with *B. longum* CECT 7347 (B). The first dimension was run on an inmobilized pH gradient (3−10) and the second dimension resolved in a SDS-PAGE 18 cm gel. Proteins were visualized by silver staining. Images show one representative gel of three independent samples. Arrows refer to spots that have been identified by MALDI-TOF MS. The names of the spots correspond to the nomenclature used in Table 1.

Analysis of Cytokines. Tumor necrosis factor (TNF)-α (eBioscience Inc., San Diego, CA), interleukin (IL)-1β (eBioscience), and IL-10 (eBioscience) were determined in the supernatant from cell cultures by ELISAs according to the manufacturers' instructions. The results of the ELISAs are expressed as picograms per milliliter (pg/mL).

Tryptic Digestion and Analysis of Protein Spots. Two-dimensional (2-D) gels of independent triplicates of each treatment were revealed by silver staining as described elsewhere.[18] The stained gel

Table 1. Identified Protein Spots of Caco-2 Cultures Exposed to Digested Gliadin, Inoculated (GB) or Not (G) with *B. longum* CECT 7347, and of *B. longum* CECT 7347 (10^8 CFU/mL) Alone (B) Showing Differential Production Compared to Control Cultures

spot	protein identified (M_r/pI)[a]	accession no.[b]	score/peptides matched	sequence coverage (%)	expression relative to controls[c]
G1	AN1-type zinc finger protein 3 (26023/7.42)	Q9H8U3	28/2	14	7.72
G2	coiled-coil domain-containing protein 29 (32003/6.41)	Q4UJ79	41/3	12	0.01
G3	DNA topoisomerase 3-alpha (114239/8.69)	Q13472	57/9	8	48.69
G4	keratin, type I cytoskeletal 17 (48361/4.97)	Q04695	72/8	19	0.06
G5	kinetochore protein Spc25 (26194/7.71)	Q9HBM1	47/4	15	5.73
G6	microtubule-actin cross-linking factor 1 (623626/5.27)	Q9UPN3	52/8	1	1.31
G7	myosin-Vc (203978/7.69)	Q9NQX4	51/7	4	19.81
G8	NEDD4-binding protein 3 (60889/8.33)	O15049	50/6	12	1.91
G9	nesprin-3 (112774/5.88)	Q6ZMZ3	61/9	12	1.25
G10	probable global transcription activator SNF2L1 (123211/8.26)	P28370	43/7	5	22.40
G11	protein SSX5 (21647/9.35)	O60225	37/2	14	0.66
G12	regulator of G-protein signaling 5 (21104/6.85)	O15539	27/3	8	1.50
G13	RhoGEF and PH domain-containing prot 1 (107691/6.14)	P98174	36/3	4	5.80
G14	secretagogin (32190/5.25)	O76038	47/4	17	1.65
G15	sorting nexin-20 (36668/8.94)	Q7Z614	27/2	8	3.60
G16	tetratricopeptide repeat protein 38 (53267/5.61)	Q5R3I4	44/3	7	2.20
G17	T-cell receptor α chain V region CTL-L17(15545/8.54)	P04437	41/3	14	4.71
G18	ubiquitin-protein ligase E3A (101593/5.12)	Q05086	42/4	5	0.31
G19	UV excision repair protein RAD23 homologue A (39642/4.56)	P54725	57/7	17	0.24
G20	uveal autoantigen with coiled-coil domains (163545/6.60)	Q9BZF9	33/5	3	3.90
G21	V-set and transmembrane domain-containing prot 1 (26405/4.98)	Q6UX27	27/2	17	0.57
B1	cell death activator CIDE-A (24899/9.34)	O60543	41/3	21	1.67
B2	docking protein 6 (38692/8.72)	Q6PKX4	31/3	10	1.41
B3	elongation factor 2 (96246/6.41)	P13639	31/3	3	75.70
B4	leucine-rich repeat-containing prot 45 (76475/5.95)	Q96CN5	50/6	10	0.08
B5	SH_3 domain-binding glutamic acid-rich-like protein 2 (12375/6.29)	Q9UJC5	21/1	16	3.26
B6	spectrin α chain (280884/4.96)	P02549	42/5	2	0.09
B7	U6 snRNA-associated Sm-like protein LSm4 (15511/10.02)	Q9Y4Z0	38/3	17	2.68
B8	zinc finger protein 540 (79325/9.58)	Q8NDQ6	54/4	5	0.72
GB1	dynein heavy chain 10, axonemal (517677/5.64)	Q8IVF4	50/10	3	1.38
GB2	EF-hand domain-containing (24092/8.95)	A8MZ26	44/3	14	3.68
GB3	EF-hand domain-containing (67022/9.51)	Q6NXPO	41/4	8	1.68
GB4	golgin subfamily A member 4 (261892/5.33)	Q13439	85/17	8	0.61
GB5	myosin-4 (223844/5.67)	Q9Y623	50/8	6	0.33
GB6	RWD domain-containing protein 4A (21238/5.24)	Q6NW29	36/2	14	23.27
GB7	signal recognition particle 54 kDa protein (55953/8.87)	P61011	51/4	10	229.80
GB8	trafficking protein particle sub 26 (18199/8.88)	Q86SZ2	29/2	13	4.39
GB9	zinc finger protein 64 homologue (74565/8.80)	Q9NTW7	46/5	9	472.01

[a] Theoretical molecular mass (M_r, Da) and theoretical pI of the corresponding protein based on the SwissProt database. [b] SwissProt accession number. [c] The relative expression to controls was determined by comparing the mean value of each spot to that of the corresponding spots of control cultures and expressed as a ratio. Significant differences in protein expression were calculated from triplicate analyses applying the Student's t test ($p < 0.05$).

was digitally scanned, and spot detection, quantification, and analysis were managed using PDQuest software (Bio-Rad). Two-dimensional gel data were normalized by dividing each spot quantity by the total of all of the valid spots in the 2-D gel image to obtain a normalized spot quantity value. For each matched spot, the mean of the values from three 2-D gels was calculated. The differences in protein expression between controls and treated groups were considered to be statistically significant at $P < 0.05$ by applying Student's t test. Gel spots were excised from the gels using biopsy punches. Proteins selected for analysis were in-gel reduced, alkylated, and digested with trypsin (Promega, Madison, WI). Briefly, spots were washed twice with water, shrunk for 15 min with acetonitrile (ACN) 50% (v/v), and dried in a vacuum centrifuge for 30 min. After reduction with 10 mM DTT in 25 mM NH_4HCO_3 for 30 min at 55 °C, the samples were alkylated with 55 mM IAA in 25 mM NH_4HCO_3 in ACN 50% (v/v) for 20 min. Then, samples were dehydrated with ACN 100% for 5 min and digested with 12.5 ng/mL sequencing grade trypsin (Promega) in 25 mM NH_4HCO_3 (pH 8.5) for at least 16 h at 37 °C.

Identification of Peptides and Database Search. Peptide analyses were performed using an Applied Biosystems 4700 Proteomics

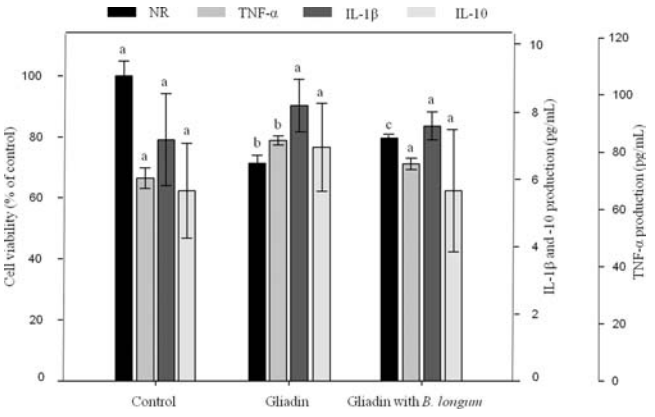

Figure 2. Effect of digested gliadin, inoculated or not with *B. longum* CECT 7347, on cell viability (Neutral red, NR uptake) and production (pg/mL) of tumor necrosis α (TNF-α), interleukin (IL)-1β, and IL-10. Different letters indicate significant ($p < 0.05$) differences for each treatment.

Analyzer mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a 355 nm pulsed nitrogen laser and operated in reflectron positive ion mode as previously reported.[19] Briefly, the digested samples were mixed with a 5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution and dissolved in ACN 50% (v/v) with formic acid 0.1% (v/v). The indicated collision cell pressure was increased from 4.0×10^{-8} mbar (no collision gas) to 6.6×10^{-7} mbar. At a resolution above 10000 in MS mode, the monoisotopic peptide signals could be accurately mass measured (<50 ppm). The mass spectrometry (MS) and MS/MS spectrum data of tryptic peptides were obtained from MALDI-TOF MS in the reflector mode and used to search for protein candidates in SwissProt and/or National Center for Biotechnology Information (NCBI) databases using MASCOT v 2.3. Only those spots that were found in all of the independent replicates were considered for further protein fingerprinting and database interrogation. We determined that a protein was correctly identified if the search results satisfied the following criteria: (i) protein is from the correct species (*Homo sapiens*); (ii) peptide mass tolerance of 50 ppm; (iii) MS/MS ion mass tolerance of 0.25 Da; (iv) up to one missed cleavage allowed; (v) variable modifications considered were Cys as *S*-carbamidomethyl derivative and Met in oxidized form.

Statistical Analysis. One-way analysis of variance (ANOVA) and the Tukey post test were applied. Statistical significance was established at <0.05 for all comparisons. SPSS v.15 software (SPSS Inc., Chicago, IL) was used for statistical analysis.

RESULTS AND DISCUSSION

A recent proteomic approach demonstrated that Caco-2 cells, although having a tumoral origin, express proteins that are characteristic of human intestinal epithelium at levels comparable to small intestinal scrapings, supporting their use to mimic the small intestine.[20] Representative 2-D gels of Caco-2 cell proteomes after the different treatments are shown in Figure 1. The proteome of cell cultures exhibited different protein expression patterns after different treatments (Table 1).

In Caco-2 cultures exposed to gliadin digested without *B. longum* CECT 7347 the spots that were up-regulated included proteins that participate in the cellular signaling of gliadin (G12), proteins of the actin filaments and cycle progression (G13), proteins of the outer nuclear membrane that are linked to intermediate filaments (G9) and proteins associated with microtubules (G6 and G5), proteins that regulate apoptosis via apoptotic protease activating factor 1 (APAF-1) and stress-induced apoptosis (G20), and proteins that participate in the cross-talk between intestinal and immune cells (G15 and G17).

The up-regulation detected in regulator G-protein signaling 5 (G12) could reflect the cellular response to counteract the cytotoxic effect of gliadin through their interaction with the CXCR3, a 7-transmembrane G-protein coupled receptor.[7] This result is concordant with the significant inhibition of NF-κB activation mediated by both G-protein coupled receptor kinase-2 and -5 reported in other studies.[21] It has also been reported that gliadin induced deterioration of actin cytoskeleton and function of intestinal proteins in Caco-2 cells.[22] The up-regulation of proteins such as sorting nexin-20 (G15) in response to the gliadin challenge could be related to the activation of interactions between intestinal epithelial cells and immune cells. This protein cycles P-selectin glycoprotein ligand 1 (PSLG1) into endosomes, via high-affinity calcium-dependent interactions with E-, P-, and L-selectins.[23] Enterocytes, which express human leukocyte antigen (HLA)-DR proteins, are capable of processing transcytosing and presenting food antigens to T-lymphocytes of the lamina propria.[24] PSGL1 is critical to initial leukocyte capture[25] and could mediate the interactions between enterocytes and T-lymphocytes in response to gliadin. Gliadin exposure also caused up-regulation of the T-cell receptor α chain V region CTL-L17 (G17) in Caco-2 cells. Accordingly, the presence of β/α T-cell receptors has been identified in large vacuoles and Golgi complexes of enterocytes from patients with active CD, but not in those from patients on a gluten-free diet.[26]

The exposure of Caco-2 cells to digested gliadin also caused the down-regulation of proteins involved in structural functions such as forming stiff bundles of fibres (G2), in DNA-dependent transcription (G11), in postreplication repair functions (G19), in protein synthesis and epithelial cell growth through binding to the adapter protein stratifin and stimulation of the protein kinase B/mammalian target of rapamycin (Akt/mTOR) pathway (G4), and in the cell cycle (G18).

Cytoskeletal (CK) proteins are major structural proteins in eukaryotic cells, which are usually overexpressed in colon carcinoma[27] and gastric cancer cells.[28] However, some CK proteins (CK-18 and -19) are down-regulated during apoptosis.[29] Apoptosis has been proposed as a major event underlying villous atrophy in CD.[30] In the present study, the gliadin-mediated down-regulation of keratin type I cytoskeletal 17 protein expression (G4) in Caco-2 cells would suggest a pro-apoptotic effect of gliadin. This suggestion is also supported by the up-regulation of uveal autoantigen with coiled-coil domains (G20), which promotes apoptosis by causing the up-regulation of apoptosome, the down-regulation of lectin galactoside-binding soluble 3 (LGALS3)/galectin-3, and the inactivation of NF-κB. However, different in vitro studies have reported conflicting results regarding gliadin-induced apoptosis in intestinal cells.[6,31] These studies suggest the likely existence of a threshold for gliadin to cause toxicity. Wheat gliadin (0.5−1.5 mg/mL for 48 h) induced apoptosis of not confluent intestinal cells (Caco-2) by a receptor-mediated (Fas−Fas ligand) pathway[31] similarly to the results reported in the present study. In contrast, in confluent Caco-2 cultures (5 days post-seeding) exposed to digested gliadin (0.25 mg/mL for 15 h), apoptosis was not detected.[6] All of the aforementioned changes detected in the proteome of Caco-2 cells after gliadin exposure were associated with an inflammatory response, as reflected by

increased TNF-α production (Figure 2); however, the exposure to digests of gliadin did not cause statistically significant ($p > 0.05$) differences in IL-1β and IL-10 production, although the results showed a similar trend.

In cell cultures exposed to digested gliadin inoculated with *B. longum* CECT 7347, changes were detected in nine proteins (Table 1). The seven up-regulated proteins might participate in vesicular transport (GB8), in calcium homeostasis (GB2), in DNA binding (GB9), and in ubiquitination in the case of proteins associated with ubiquitin−protein ligase activity (GB6). Ubiquitination is important not only in cellular homeostasis but also in human disease, ensuring controlled degradation of proteins. Even if the up-regulation of proteins involved in this pathway was able to induce ubiquitination and degradation of the (IκBα) inhibitor and activate inflammation via the NFκB pathway, the exposure of Caco-2 cells to gliadin digested with the bifidobacteria was not associated with an inflammatory response (Figure 2). Previous data also demonstrate that NF-κB is not activated in intestinal cells exposed to digests of gliadin inoculated with *B. longum* CECT 7347.[6] These results could be explained, at least in part, by the fact that ubiquitin ligases become highly specific by post-translational modification of the substrate proteins, and targets of the ubiquitin ligase also include many other types of proteins, such as cell cycle regulatory proteins and proteins unable to fold properly at the endoplasmic reticulum.

EF-hand domain-containing proteins (GB2), which participate in calcium homeostasis, were also up-regulated under the effects of gliadin digested in the presence of the bifidobacterium. This response could result from the activation of molecular systems controlling cytoplasmic Ca^{2+} levels into cells, which could contribute to stabilizing cytoskeletal alterations caused by gliadin in intestinal cells. It has been reported that gliadin-like peptides binding to CXCR3 receptor induced the overexpression of the glutamate receptor[7] coupled to ion channels controlling Ca^{2+} influx into cells,[32] playing a critical role in the cellular resistance to apoptosis.[33] This might suggest that *B. longum* CECT 7347 could reduce gliadin-induced toxicity to intestinal epithelial cells by regulating Ca^{2+} homeostasis. This hypothesis could explain the increased viabilities of Caco-2 cells exposed to gliadin digested in the presence of *B. longum* CECT 7347 compared to those not inoculated with the bacterium (Figure 2).

It has been suggested that *Bifidobacterium* strains play an important role in health by influencing the host's intestinal barrier functions and the epithelial and immune cell response(s).[15,34] To test this hypothesis, the influence of *B. longum* CECT 7347 alone on Caco-2 cell proteome was also evaluated. *B. longum* CECT 7347 exposure induced significant changes in eight proteins, five of which were up-regulated and three down-regulated (Table 1). *B. longum* CECT 7347 induced proteins involved in ribosomal translation (B3 and B7), in cell proliferation (B1), and in bacterial recognition (B4). The up-regulation of proteins involved in ribosomal translation in the presence of *B. longum* CECT 7347 could contribute to the adequate synthesis of proteins in intestinal epithelial cells and thus their rapid turnover. As such, protein elongation factor 2 (B3) promotes the guanosine triphosphate (GTP)-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome, and the protein U6 snRNA-associated Sm-like protein LSm4 (B7) binds specifically to the 3′-terminal U-tract of U6 snRNA. Up-regulation of protein leucine-rich repeat-containing protein 45 (B4) could be involved in the bacterial recognition by Toll-like receptors as part of their leucine-rich domains.[35]

The exposure of Caco-2 cells to *B. longum* CECT 7347 also led to the down-regulation of the SH3 domain-binding glutamic acid-rich-like protein 2 (B5), zinc finger protein 540 (B8), and spectrin α chain (SPTA) (B6). SPTA is a membrane protein that plays a role in signal transduction, regulating voltage-dependent anion channels,[36,37] which could contribute to gut physiology by modulating electrolyte transport processes through the apical side of enterocytes.

The results from the present study reveal marked changes in the proteome of intestinal epithelial cells in response to their interaction with gliadin-derived peptides. Caco-2 cell cultures exposed to gliadin digested in the absence of *B. longum* CECT 7347 showed alterations of key proteins involved in CK integrity, cell apoptosis, and interactions with immune cells and significant increases in TNF-α production. In contrast, when gliadin was digested in the presence of *B. longum* CECT 7347, its effect on the proteome was ameliorated, inducing alterations in a lower number of proteins. These proteins were involved in controlling ionic homeostasis, CK integrity, and signal transduction. Altogether, the results suggest that *B. longum* CECT 7347 reduces the toxic and inflammatory effects of gliadin-derived peptides and could contribute to improving the survival and physiological function of intestinal epithelial cells. The significance of these proteomic changes in vivo deserves further investigation.

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ABBREVIATIONS USED

2-D, two dimensional; ACN, acetonitrile; Akt/mTOR, protein kinase B/mammalian target of rapamycin; APAF-1, apoptotic protease activating factor 1; CD, celiac disease; CECT, Coleccion Española de Cultivos Tipo; CFU, colony-forming units; CHCA, α-cyano-4-hydroxycinnamic acid; CK, cytoskeletal; DTT, dithiothreitol; G, proteins modified by gliadin digested without *B. longum* CECT 7347; GB, proteins modified by digested gliadin inoculated with *B. longum* CECT 7347; B, proteins modified by *B. longum* CECT 7347 suspensions; GTP, guanosine triphosphate; HLA, human leukocyte antigens; IAA, iodoacetamide; IL, interleukin; IPG, immobilized pH gradient; LGALS3, lectin galactoside-binding soluble 3; MRSC, Man−Rogosa−Sharpe cysteine; MS, mass spectrometry; NCBI, National Center for Biotechnology Information; NR, Neutral red; NF-κB, nuclear factor κ-B; PBSphosphate-buffered salinep*I*, isoelectric point; PSLG1, P-selectin glycoprotein ligand 1; SPTA, spectrin α chain; TNF-α, tumor necrosis factor-α.

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