Bifidobacteria reduce gliadin-induced toxicity

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Running title: Bifidobacteria attenuate gliadin-induced inflammation

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ABSTRACT

Celiac disease (CD) is a chronic enteropathy triggered by intake of gliadin, the toxic component of gluten. This study aims at evaluating the capacity of different *Bifidobacterium* strains to counteract the inflammatory effects of gliadin-derived peptides in intestinal epithelial (Caco-2) cells. A commercial extract of several gliadin (Gld) types (α, β ,γ ,ϖ) was subjected to *in vitro* gastrointestinal digestion (pepsin at pH 3, pancreatin-bile at pH 6), inoculated or not with cell suspensions (10^8 colony forming units/ml) of either *B. animalis* IATA-A2, *B. longum* IATA-ES1, or *B. bifidum* IATA-ES2, in a bicameral system. The generated gliadin-derived peptides were identified by reverse phase-HPLC-ESI-Ms/Ms. Caco-2 cell cultures were exposed to the different gliadin peptide digestions (0.25 mg protein/mL), and the mRNA expression of NF-κB, TNF-α, and chemokine CXCR3 receptor were analysed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) in stimulated cells. The production of the pro-inflammatory markers NF-κB p50, TNF-α, and IL-1β by Caco-2 cells was also determined by ELISA. The peptides from gliadin digestions inoculated with bifidobacteria did not exhibit the toxic amino acid sequences identified in those non inoculated (α/β-Gld [158-164] and α/β-Gld [122-141]). The RT-PCR analysis evidenced a down-regulation in mRNA expression of pro-inflammatory biomarkers. Consistent with these results the production of NF-κB, TNFα and IL-1β was reduced (18.2-22.4%, 28.0-64.8% and abolished, respectively) in cell cultures exposed to gliadin digestions inoculated with bifidobacteria. Therefore, bifidobacteria change the gliadin-derived peptide pattern and, thereby, attenuate their pro-inflammatory effects on Caco-2 cells.

Keywords: Celiac disease, gliadin, *Bifidobacterium*, Caco-2, cytokines.

INTRODUCTION

Celiac disease (CD) is an autoimmune enteropathy caused by a permanent intolerance to cereal gluten proteins. Gliadins (α, β, γ and ϖ types), are the main toxic components of gluten. In CD patients, gliadin-derived peptides generally induce intestinal symptoms and severe mucosal damage due to an abnormal immune response to the incomplete digested gliadin peptides by human digestive enzymes [Wieser and Koehler, 2008]. Currently, the only available therapy for CD patients is the adherence to a strict life-long gluten free diet; however, the compliance with this dietary recommendation is complex and other alternative strategies are needed [Sanz, 2009].

It has been reported that gliadin-derived peptides stimulate not only immunocompetent cells [Thomas et al., 2006], but also enterocytes [Lammers et al., 2008] via the myeloid differentiation factor (MyD88) and the chemokine receptor CXCR3 associated to Toll-like receptor signaling pathways. Different gliadin-derived peptides with specific amino acid sequences have proven to trigger pro-inflammatory cell responses. These involve activation of the nuclear factor kappa-B (NF-κB) in small intestinal mucosa of celiac patients [Chiara et al., 2003] and increased expression of pro-inflammatory cytokines related to the innate immune response, such as tumor necrosis factor α (TNFα) [Nilsen et al., 19989] and interleukine (IL) 1β [Beckett et al., 1999].

Scientific evidence supports the hypothesis that not only genetic, but also environmental factors other than gluten intake may play an important role in CD pathogenesis [Fergusson et al., 2007]. Imbalances in the gut microbiota of CD patients have been previously reported [Nadal et al., 2007; Sanz et al., 2007]. In particular, the abundance of *Bifidobacterium* species tended to be reduced in feces and biopsies of CD patients [Nadal et al., 2007]. *Bifidobacterium* species are thought to positively influence the host-immune response(s) in a species and strain-specific manner [Young et al., 2004; Roselli et al., 2006; Medina et al., 2008]. Specific *Bifidobacterium* strains have been shown to counteract the pro-inflammatory response induced by the fecal microbiota of CD patients in peripheral blood mononuclear cells [Medina et al., 2008]. A *Bifidobacterium animalis* subsp. *lactis* strain was also shown to inhibit the increased epithelial permeability induced by gliadin [Lindfors et al., 2008].

The association of alterations in the gut microbiota composition with CD, and the recognized roles played by probiotic bacteria on host's health have led to propose the

use of probiotics as an additional nutritional strategy to improve the quality of life of CD patients [Sanz, 2009]. Increasing efforts are being made to elucidate the interactions among bacteria, the enterocytes and the immune system [Troncone et al., 2008]. In this context, diverse *in vitro* models have been designed to evaluate and/or predict the effect that potentially probiotic bacterial strains might exert to reduce the toxicity and inflammatory response(s) at intestinal level. The use of these models is critical in view to developing future clinical trials in humans.

The objectives of this study have been to identify the effects of bifidobacteria on the peptide sequences generated during the gastrointestinal digestion of gliadins and to compare their toxicity and pro-inflammatory effects on Caco-2 cell cultures, used as model of intestinal epithelia.

MATERIAL AND METHODS

Bacterial cultures. *Bifidobacterium* strains (*B. bifidum* IATA-ES2 [CECT 7365, *B. longum* IATA-ES1 [CECT 7347], and *B. animalis* IATA-A2) were isolated from faeces of healthy infants as described elsewhere (Medina et al., 2008). The bacterial strains were grown in Man-Rogosa-Sharpe broth and agar (Scharlau, Barcelona, Spain) supplemented with 0.05% (w/v) cysteine (Sigma, St. Louis, MO), and incubated at 37ºC under anaerobic conditions (AnaeroGen; Oxoid, Basingstoke, UK) for 24h.

Cell culture conditions. The human colon carcinoma Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17 and used in experiments at passage 25-33. Caco-2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM Glutamax, Gibco) containing 4.5 g L^{-1} glucose, 25 mM HEPES buffer, and 10% fetal bovine serum (Gibco). The cells were maintained at 37°C in 5% CO_2, 95% air and the culture medium was changed every 2 days [Laparra et al., 2008].

For experimental studies Caco-2 were seeded at a density of 50,000 cells cm^{-2} onto 6 well plates (Costar, Cambridge, MA, USA). Cell cultures were grown with DMEM, and culture media was changed every two days. Experiments were performed 5 days post seeding.

***In vitro* digestion of gliadins.** The gastrointestinal digestion process was simulated as previously described [Laparra et al., 2009], using porcine pepsin (P-7000, Sigma) (800-2500 units/mg protein), pancreatin (P1750, Sigma) (activity, 4×USP specifications) and bile (B3883, Sigma). Aliquots (150 mg) of a commercially available extract of gliadin (G3375, Sigma) were weighted in centrifuge tubes (50 ml), and 3 ml

of a saline solution (140 mM NaCl, 5 mM KCl adjusted a pH 3) was added to each sample. The mixture was immersed in a water bath (60 ºC) for 30 minutes with gentle agitation. Briefly, 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 0.1M HCl/pH 3/1h), the intestinal digestion (pancreatin-bile extract in 0.1 $NaHCO_3$/pH 6.9-7/2h) was carried out in the upper chamber of a two-chamber system in 6-well plates. The upper chamber was formed by fitting the bottom of an appropriately sized Transwell insert ring (Corning) with a 15,000 molecular mass cut-off dialysis membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA, USA). Aliquots (1.5 ml) of the intestinal digest, inoculated or not with bacterial cell suspensions (10^8 colony forming units [CFU]/ml), were loaded into the upper chambers and incubated for 2 h. Afterwards, the inserts were removed and an additional 1 mL of DMEM was added to each well. Cell cultures were returned to the incubator for additional 12 hours.

Total protein concentration in both dialyzates and retentates were determined using a Lowry method based commercial kit (TP0200, Sigma). After *in vitro* digestion, bacterial cell growth ability was confirmed by plate count and ranged between 10^{6}-10^{7} CFU/mL.

Reverse phase-HPLC and tandem Mass spectrometry (Ms/Ms) analysis (RP-HPLC-ESI-Ms/Ms). Aliquots (1 mL) of the dialyzates from the gliadin digestions, inoculated or not with *Bibifidobacterium* strains, were filtered through a nylon membrane (13 mm 0.22 µm Millex GN, Millipore) before the analysis. The separation and identification of gliadin-derived peptides was performed on an Agilent HPLC system connected on line to an Esquire-LC electrospray system equipped with a quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The HPLC system was equipped with a quaternary pump, an in line degasser, an automatic injector, and a variable wavelength absorbance detector set at 214 nm (1100 Series, Agilent Technologies, Waldbronn, Germany). The column used in these analyses was a BioBasic C18 5 µm 4.6x250 mm (Thermo, Waltham, MA, USA). The elution phases consisted of (A) Acetonitrile (ACN) 15% (v/v)/Trifluoroacetic acid (TFA) 0.1% (v/v), and (B) ACN 80% (v/v)/TFA 0.1% (v/v). Aliquots (100 µl equivalents to 116 µg protein) of the dialyzates resulting from digestions of the commercially available extract of gliadin types were injected in each analysis. The gradient program started with 95% of solvent A and 5% of solvent B, and changed linearly to reach 10% of solvent A and

90% of solvent B in 30 min. The column was cleaned with 90% of solvent B (5 min) and equilibrated with the initial conditions for 5 minutes. UV absorbance was recorded at 214 nm. Nitrogen was used as the nebulizing and drying gas, and the helium collision gas pressure was approximately 5 x 10^{-3} bar. The capillary was held at 4 kV. Mass spectra were recorded over the mass/charge (*m*/*z*) range 100-3500. About 15 spectra were averaged in the Ms analyses and about five spectra in the Ms/Ms analyses. The signal threshold to perform auto-Ms/Ms analyses was 5000, and the precursor ions were isolated within a range of 4.0 *m*/*z* and fragmented with a voltage ramp from 0.39 to 2.6 V. The *m*/*z* spectral data were processed and transformed to spectra representing mass values using the program Data Analysis version 3.0 (Bruker Daltonics). BioTools version 2.1 (Bruker Daltonics) software was used to process the Ms/Ms spectra and to perform peptide sequencing by comparing with the different gliadin sequences (accession number: α/β, AAZ94420; γ, AAQ63856; ϖ, AAT74547). Three independent samples were injected in each analysis.

Toxicity experiments. Cell culture viabilities were determined by the toluylene red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) uptake assay [Borenfreund and Puerner, 1985]. The medium was removed and cells were washed twice with phosphate buffered saline (PBS, P4417, Sigma). The uptake of toluylene red was measured using a commercial kit (No. 7H092, Sigma) at 540 nm with background subtraction at 690 nm. *In vitro* digests of bovine serum albumin (BSA) (0.25 mg protein/ml), were used as negative controls for toxicity. Control cells exposed to DMEM were analyzed with every assay.

Analysis of pro-inflammatory markers. To evaluate Nuclear factor kappa B (NF-κB) production the nuclear extract from Caco-2 cell cultures was obtained using a commercial kit according to the manufacturer's instructions (Active Motif, Cat. No. 40010). Afterwards, the commercial TransAM™ kit (Active Motif, Cat. No. 43296) that contains antibodies directed against the NF-κB p65 subunit was used (TransAM™, Cat. nº. 43296).

Tumor necrosis factor-α (TNF-α, eBioscience; Cat. nº. 88-7346), and interleukine (IL)-1β (eBioscience; Cat. nº. 88-7010) were determined by ELISAs according to the instruction of the manufacturers. The results of the ELISA assay for NF-κB are expressed as percentage of the control, and TNF-α and IL-1β as pico-grams per mL (pg/ml) of media.

Nucleic acid distribution in Caco-2 cultures. Cell cycle analysis was performed by propidium iodide (PI) staining of DNA content in exposed cultures [Laparra et al., 2008]. Cells were washed with PBS and resuspended in 1 ml of lysis buffer [1 mg ml^{-1} of trisodium citrate, 1 µl ml^{-1} of sodium dodecyl sulphate (0.5% w/v), 0.05 mg ml^{-1} PI, and 1 mg ml^{-1} of RNase A (R4875, Sigma)]. After incubation overnight at 4ºC, the released nuclei were re-suspended by agitation with a Pasteur pipette, and the fluorescence was analyzed by flow cytometry (Coulter, EPICS XL-MCL, USA) at λ_{exc} = 536 nm and λ_{em} = 617 nm. Control cells exposed to DMEM were analyzed in each assay.

To evaluate total RNA distribution in cell cycle phases, simultaneous Hoechst 33342 and pyronin Y staining was performed [Laparra et al., 2008]. Cells were harvested and resuspended in 700 µL of fixation solution (PBS:Ethanol, in 1:3 v/v ratio) for 5 minutes. Then, cells were centrifuged (1200 rpm/10 minutes) and resuspended in 700 µl PBS containing Hoechst 33342 (30 µg/mL, final concentration) during 20 minutes at room temperature. Afterwards, 700 µL PBS containing pyronin Y (2 µg/mL, final concentration) were added and incubated for 10 minutes at room temperature. Samples were analyzed on a Modular Flow Cytometer Cell Sorter (MoFlo Sorter, Dakocytomation, USA). Hoechst was excited with the UV line of an argon laser (λ_{exc}: 346 nm - λ_{em}: 460 nm), and pyronin Y was excited at λ_{exc}: 555 nm and the fluorescence collected at $_{\lambda em}$: 580 nm.

Analysis of the mRNA expression of proinflammatory biomarkers. Total RNA was isolated from cell cultures (Qiagen, Cat. No 74104, USA) and first strand cDNAs were synthesized from 0.5 µg of total RNA. Polymerase chain reaction (PCR) was carried out with primers designed for TNFα (NM003842.4) (TNFα superfamily, member 2) (forward: 5'-AGG GTA CCA CAG AAA GAT GC-3'; reverse: 5'-GCA GAT GAG ACC CTT AGG TT-3'), NF-κB (NM003998.2) (forward: 5'-CTT CTC GGA GTC CCT CAC TG-3'; reverse: 5'-CCA ATA GCA GCT GGA AAA GC-3') and chemokine CXCR3 receptor (NM001142797.1) (forward: 5'-AAG AAT GCG AGA GAA GCA GC-3'; reverse: 5'-AAG AGG AGG CTG TAG AGG GC-3'). β-actin gene (NM000251.1) was used to normalize the results (forward: 5'-CTC TTC CAG CCT TCC TTC CT-3'; reverse: 5'- TAG AGC CAC CAA TCC ACA CA-3'). Amplifications of the TNFα, NF-κB and β-actin genes were performed in 35 cycles, which consisted of a first cDNA synthesis stage (45 ºC, 1 h), AMV-reverse transcriptase (RT) inactivation

(95 ºC, 2 min), denaturation (94 ºC, 30 s), annealing (60 ºC, 1 min), and extension (78 ºC, 2 min). RT-PCR products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and quantified using a Image Gauge version 4.0 (Media Cybernetics, LP).

Statistical analysis. Each of the experiments were conducted in triplicate during two different days. One-way analysis of variance (ANOVA) and the Tukey *post hoc* test were applied [Box et al., 1978]. Statistical significance was established at $p<0.05$ for all comparisons. SPSS v.15 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

RESULTS

Gliadin-derived peptides. The total protein content of the dialyzates from *in vitro* gliadin digestions without bifidobacteria constituted up to 37.8 ± 3.8% of the protein content loaded in the upper chamber of the *in vitro* system. Only samples inoculated with *B. longum* IATA-ES1 produced a statistically significant ($p<0.05$) reduction (by 5.3 ± 0.9%) in the dialyzable total protein content, suggesting a change in the degree of gliadin peptide hydrolysis.

The amino acid sequences of gliadin-derived peptides present in the dialyzates and generated in the presence or absence of bifidobacteria, were analyzed by RP-HPLC-ESI-Ms/Ms (**Table 1**). Different peptide patterns were detected in samples inoculated with bifidobacteria compared to those non-inoculated. Most peptides generated in samples inoculated with bifidobacteria showed lower molecular mass than those generated in non inoculated samples (~2500 Da) during intestinal digestion.

Toxicity of gliadin digests. The cytotoxic effect of proteolytic resistant gliadin-derived peptides was evaluated by using the neutral red uptake assay, which is based on the activation of endosomal/lysosomal activities [Borenfreund and Puerner, 1985] (**Figure 1**). The peptides from samples non-inoculated with bifidobacteria and from those inoculated with *B. animalis* and *B. bifidum* were cytotoxic for intestinal epithelial cells, as concluded from the decreased neutral red uptake percentages (by 5%) compared to the controls. In contrast, digestions inoculated with *B. longum* produced higher neutral red uptake percentages, which reflect an activation of endosomal/lysosomal activities and absence of toxicity.

None of the dialyzates from the different digestions assayed caused alterations in cell cycle phases population compared to the controls (sub-G1, 6.58 ± 2.43; G0/G1, 63.51 ± 1.02; S, 12.56 ± 1.37; G2/M, 14.23 ± 2.87). Regarding total RNA content in cycle phases, only cell cultures (G1, 70.4 ± 0.1%; G2, 14.6 ± 4.6%) exposed to digests from gliadins and with those (G1, 70.3 ± 3.7%; G2, 19.1 ± 5.5%) digests inoculated with *B. bifidum* caused a reduction of total RNA content in G2 phase compared to the levels in Caco-2 cultures control (G1, 70.9 ± 2.9%; G2, 28.8 ± 1.7%).

Pro-inflammatory cytokine production. The activation of NF-κB and TNFα production was induced in intestinal epithelial cell cultures by gliadin digestions non-inoculated with bifidobacteria (**Figure 2**). In all cases NF-κB activation was significantly ($p<0.05$) reduced when the gliadin digestions were inoculated with all bifidobacterial strains, compared to non inoculated digestions. TNFα production was reduced in cultures exposed to gliadin digestions inoculated with all bifidobacterial strains ($p<0.05$) and, especially, in those exposed to digests inoculated with *B. longum*, which were reduced to basal levels. In addition, IL-1β production was 2.7-fold higher (6.17 ± 0.98 ng/ml) in gliadin exposed culture supernatants than in controls (2.27 ± 0.51 ng/ml). There was no statistically significant differences on IL-1β production (1.87-2.34 ng/ml) in cultures exposed to gliadin digestions inoculated with bifidobacteria relative to the controls.

mRNA expression of pro-inflammatory markers. The reverse transcriptase-PCR analysis (**Figure 3**) revealed that the studied bifidobacterial strains reduced ($p<0.05$) the gliadin induced up-regulation of TNFα mRNA expression to different extent; the inhibitory effects of gliadins inoculated with *B. longum* were the highest. Furthermore, only inoculation of gliadin digestions with *B. longum* caused a down-regulation of NF-κB mRNA expression. According to these results together with those related to TNFα production (Fig. 1), a post-transcriptional control of this pro-inflammatory biomarker seems to occur. In addition, an increased CXCR3 mRNA expression in cell cultures exposed to gliadin digestions non inoculated with bifidobacteria was noted. Interestingly, inoculation of gliadin digests with *B. longum* and *B. bifidum* lowered the CXCR3 mRNA expression to similar levels ($p>0.05$) as in controls.

DISCUSSION

The gastrointestinal digestion of gliadins leads to the generation of peptides, which have been shown to exert cytotoxic and inflammatory effects on intestinal epithelial cells [Shan et al., 2005]. The concentration (0.25 mg/ml) of gliadin-derived peptides of the dialyzate samples used for the Caco-2 cells assays is achievable in the small intestine after consumption of a gluten-containing meal [Chartrand et al., 1997], and has been previously used in *in vitro* experiments on Caco-2 cell cultures as model of intestinal epithelia [Giovannini et al., 2000; 2003]. Herein, it has been demonstrated for the first time that the presence of the studied bifidobacterial strains during the intestinal digestion led to the generation of different gliadin peptide sequences *in vitro*, which could modify their toxic effects. In peptides derived from digestions non inoculated with bifidobacteria, amino acid sequences such as α/β-Gld [122-141] and α/β-Gld [158-164] similar as those proven to interact with the chemokine receptor CXCR3 [Lammers et al., 2008] have been identified. However, the aforementioned amino acid sequences were not detected in the gliadin digestions inoculated with bifidobacteria. This is particularly important since it has been suggested that gliadin-derived peptides stimulate enterocytes via the transmembrane G-protein-coupled chemokine CXCR3 receptor, which is involved in cytoskeleton rearrangement into inflamed tissues and the release of zonulin [Lammers et al., 2008]. In this context, the digestions inoculated with *B. bifidum* and *B. longum* did not up-regulate CXCR3 mRNA expression in contrast to the other digested gliadin samples, which could contribute to maintain the intestinal barrier integrity (Fig. 3).

Gliadin digestions, inoculated or not, with *B. animalis* and *B. bifidum* were cytotoxic for intestinal epithelial cells while not those inoculated with *B. longum*. However, the non-increased sub-G1 cell population in cell cultures challenged with every digestion, suggest that apoptosis processes are not involved in gliadin digests-mediated toxicity. Only, a reduction of total RNA content in G2 phase population was induced by gliadin digestions non inoculated and inoculated with *B. bifidum*, which could reflect alterations in the cell biology. Apoptosis has been suggested to be a major event that explains the villous atrophy in celiac disease [Moss et al., 1996] and, *in vitro*, wheat gliadins were shown to induce apoptosis of intestinal cells (Caco-2) [Giovannini et al., 2000; 2003] via an autocrine mechanism mediated by a receptor-mediated (Fas-Fas ligand) pathway [Giovannini et al., 2003]. In the present study, the use of confluent Caco-2 cultures, a lower protein concentration (0.25 mg/ml versus 0.5-1.5 mg/ml) to

challenge the cell cultures, and shorter exposure time (15h versus 48h) may be responsible for the differences observed in cell cycle progression between our results and those obtained by other authors [Giovannini et al., 2000; 2003]. It also should be taken into account that immature enterocytes present licking junctions causing an easier passage of gliadin-derived peptides through licking jucntions producing stronger toxic effects than in mature cells.

In the present study, gliadin-derived peptides have been shown to trigger the activation of pro-inflammatory pathways (NF-κB) and the production of pro-inflammatory cytokines (TNFα and IL-1β) (Fig. 2). The reduced NF-kB production in cultures exposed to digests of BSA may be explained by the significant down-regulation in nitric oxide synthase activity caused by albumin-derived glycosilation products [Rojas et al., 2000]. In contrast, NF-κB is known to be activated in small intestinal mucosa of CD patients [Chiara et al., 2003] and gluten peptides have been shown to up-regulate the expression of cytokines such as TNFα [Nilsen et al., 1998] and IL-1β [Beckett et al., 1999], related to the innate immune response, in previous studies. The presence of bifidobacteria during intestinal digestion of gliadins reduced the ability of gliadin peptides to induce NF-κB (nuclear p65 subunit) and TNFα production, especially in the case of *B. longum*, and completely abolished the IL-1β production. Into cells, TNFα and IL-1 actions converge over the IκB kinase complex, which control the phosphorylation-regulated activation of NF-κB [Schmitz et al., 2001; Leeman and Gilmore, 2008]. However, it has been demonstrated that NF-κB is a negative regulator of IL-1β and that inhibition of IκKB causes an increased IL-1β production in myeloid cells and neutrophils [Greten et al., 2007]. Thus, it seems likely that the use of probiotic bacteria could reduce the gliadin-induced NF-κB activation without disconnecting the NF-κB mediated regulatory effect on IL-1β production.

The extent of inhibitory effects produced by bifidobacteria on the pro-inflammatory response(s) to gliadins by Caco-2 cells seemed to depend on the strain considered. Of the tested strains, *B. longum* exerted the strongest inhibitory effects in both the NF-κB activation and TNFα production induced by gliadin-derived peptides in intestinal epithelial cells. The latter observations were correlated not only to the total proteins in dialyzates but also to the smaller molecular masses of peptides generated during *in vitro* digestion. These effects could have important consequences on the intestinal barrier function because TNFα increases tight junction dependent permeability, which

induction involves NF-κB activation [Ma et al., 2004]. The reduction of TNFα production by gliadin digestions inoculated with *B. longum* might also have important physiological implications for CD since TNFα in conjunction with IL-1β are the most important cytokines involved in nitric oxide synthase (NOS) activation [Hoffman, 2000]. NOS has been reported to act as a mediator to facilitate the interaction of intraepithelial lymphocytes and intestinal epithelial cells promoting tissue inflammation [Hoffman, 2000]. In addition, TNFα also has a positive effect on IL-8 production, which is a prototypic chemokine that attracts inflammatory cells such as neutrophils. A prolonged infiltration of neutrophils would perpetuate the inflammatory responses and contribute to cell damage, and epithelial barrier dysfunction.

In summary, the assayed bifidobacteria bacteria can cleave gliadin peptides during intestinal digestion, originating different peptide patterns that would reach the intestinal epithelia. In this way, bifidobacterial strains can inhibit the gliadin induced cytotoxic and pro-inflammatory responses in intestinal epithelial cells. Important inflammatory markers such as NFκB, TNFα and IL-1β were significantly reduced as a result of the proteolytic capacity of bifidobacteria on gliadin peptides. The reported data extend the spectrum of beneficial effects that probiotic bacteria might exert on intestinal epithelial cells function in CD and justify their possible evaluation in these patients.

Acknowledgements

This work was supported by grants AGL2008-01440/ALI and Consolider Fun-C-Food CSD2007-00063 from the Spanish Ministry of Science and Innovation (MICINN, Spain) and PIF08-010-4 form CSIC. J. M. Laparra has a postdoctoral contract of the programme “Juan de la Cierva” (MICINN, Spain).

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Figures' legend

Figure 1. Neutral red uptake percentages in Caco-2 cell cultures exposed to the dialyzable fraction (0.25 mg/mL) from digests of gliadins (Gld), inoculated or not with bifidobacteria. A digest of bovine serum albumin (BSA) was used as negative control of toxicity. Results are expressed as mean ± standard deviation (n=5). Different superscript letters indicate statistically significant ($P<0.05$) differences.

Figure 2. Nuclear factor kappa-B (NF-κB) and tumor necrosis factor α (TNF-α) production in Caco-2 cell cultures exposed to the dialyzable fraction from digests of gliadins (Gld), inoculated or not with bifidobacteria. A digest of bovine serum albumin (BSA) was used as negative control of cytokine-induced production. Results are expressed as mean ± standard deviation (n=5). Different superscript letters for each biomarker indicate statistically significant ($P<0.05$) differences.

Figure 3. mRNA expression of Pro-inflammatory biomarkers (TNFα, NF-κB, and chemokine CXCR3 receptor) in Caco-2 cell cultures exposed to the dialyzable fraction (0.25 mg/mL) from digests of gliadins (Gld), inoculated or not with bifidobacteria. Results are expressed as mean ± standard deviation (n=5). Different superscript letters indicate statistically significant ($P<0.05$) differences for each of the biomarkers analyzed.

Table 1. Gliadin-derived peptides in the dialyzates from different gastrointestinal digestions of gliadins, inoculated or not with bifidobacteria.

Sample	Peptide	Amino acid sequence	Observed m/z	Calculated m/z	Ion (m/z) selected for Ms(n) (charge)
Gliadins	α/β-Gld [158-164]	QVLQQST	802.9	803.4	802.9 (1)
	γ-Gld [145-162]	VSSLWSIILPPSDCQVMR	2031.6	2032.4	2031.6 (1)
	α/β-Gld [122-141]	QQQQQQQQILQQILQQQILP	2460.6	2459.3	1230.3 (2)
	γ-Gld [222-243]	QGIIQPQQPTQLEVFRSLVLQT	2489.3	2489.4	2489.3 (1)
	γ-Gld [90-110]	QPFPQSKQPQQPFPQPQQPQQ	2487.7	2488.2	2487.7 (1)
Gliadins + *B.animalis*	γ-Gld [201-212]	QILVPLSQQQQV	1381.0	1381.6	1381.0 (1)
	ϖ-Gld [270-285]	SFPQQPQQPFPPTTTK	1829.0	1830.0	1829.0 (1)
	ϖ-Gld [71-386]	QPQQPYPQQQPYGSSL	1845.6	1846.9	1845.6 (1)
	ϖ-Gld [310-330]	SQQSFLQPQPQQPQQPSILQP	2404.4	2405.6	1202.2 (2)
	α/β-Gld [233-269]	GFFQPSQNPQAQGSFQPQQLPQFEAIRNLALQTLPA	4125.3	4127.56	1375.1 (3)

Table 1. Continuation.

Sample	Peptide	Amino acid sequence	Observed m/z	Calculated m/z	Ion (m/z) selected for Ms(n) (charge)
Gliadins + *B. bifidum*	ϖ-Gld [37-44]	FSHQQQPF	1018.5	1018.4	1018.5 (1)
	ϖ-Gld [310-318]	SQQSFLQPQ	1063.2	1063.1	1063.2 (1)
	α/β-Gld [52-62]	GQQQPFPPQQP	1252.3	1252.4	1252.3 (1)
	α/β-Gld [235-246]	FQPSQQNPQAQG	1329.5	1330.4	1329.5 (1)
	ϖ-Gld [358-375]	SQQPQQPFPQQPHQPQQP	2125.0	2126.3	1062.5 (2)
	γ-Gld [68-87]	QPQQPYPQQPQQPFPQTQQP	2390.2	2391.6	1195.1 (2)
Gliadins + *B. longum*	ϖ-Gld [62-68]	SQQPFPT	803.4	804.4	803.4 (1)
	ϖ-Gld [253-259]	QPQQLPQ	838.8	838.4	838.8 (1)
	γ-Gld [91-97]	QPQQPFP	841.6	841.4	841.6 (1)
	α/β-Gld [195-202]	IILHQQQQ	1007.1	1007.6	1007.1 (1)
	γ-Gld [123-132]	SLQQQLNPCK	1154.8	1158.6	577.0 (2)
	α/β-Gld [193-211]	QQPLSQVSFQQPQQQYPSG	2178.2	2178.0	1089.1 (2)
	ϖ-Gld [131-150]	LQPQQPFPQQPQQPFQPQLP	2470.0	2468.3	1235.0 (2)