

Bifidobacteria Inhibit the Inflammatory Response Induced by Gliadins in Intestinal Epithelial Cells via modifications of Toxic Peptide Generation During Digestion

J.M. Laparra and Y. Sanz*

Microbial Ecophysiology and Nutrition Group, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Apartado 73, 46100 Burjassot (Valencia), Spain

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 , ω) 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 nuclear factor kappa-B (NF- κ B), tumor necrosis factor α (TNF- α), and chemokine CXCR3 receptor were analyzed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) in stimulated cells. The production of the pro-inflammatory markers NF- κ B p50, TNF- α , and IL-1 β (interleukine 1 β) 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 noninoculated (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- κ B, TNF- α , and IL-1 β 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. *J. Cell. Biochem.* 109: 801–807, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: CELIAC DISEASE; GLIADIN; BIFIDOBACTERIUM; CACO-2; CYTOKINES

Celiac disease (CD) is an autoimmune enteropathy caused by a permanent intolerance to cereal gluten proteins. Gliadins (α , β , γ , and ω 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- κ B) 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 (TNF- α) [Nilsen et al., 1998] and interleukine (IL) 1 β [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 [Ferguson 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

*Correspondence to: Dr. Y. Sanz, PO Box 73, 46100 Burjassot (Valencia), Spain. E-mail: yolsanz@iata.csic.es
Received 25 August 2009; Accepted 17 November 2009 • DOI 10.1002/jcb.22459 • © 2010 Wiley-Liss, Inc.
Published online 5 January 2010 in Wiley InterScience (www.interscience.wiley.com).

microbiota of CD patients in peripheral blood mononuclear cells [Medina et al., 2008]. A *Bifidobacterium animalis* ssp. *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.

MATERIALS AND METHODS

BACTERIAL CULTURES

Bifidobacterium strains (*B. bifidum* IATA-ES2, *B. longum* IATA-ES1, and *B. animalis* IATA-A2) were isolated from feces 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 37°C under anaerobic conditions (AnaeroGen; Oxoid, Basingstoke, UK) for 24 h.

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 25–33. Caco-2 cells were grown in Dulbecco's modified Eagle medium (DMEM, Glutamax, Gibco) containing 4.5 g/L glucose, 25 mM HEPES buffer, and 10% fetal bovine serum (Gibco). The cells were maintained at 37°C in 5% CO₂, 95% air, and the culture medium was changed for every 2 days [Laparra et al., 2008].

For experimental studies Caco-2 were seeded at a density of 50,000 cells/cm² onto 6-well plates (Costar, Cambridge, MA). Cell cultures were grown with DMEM, and the culture media was changed for every 2 days. Experiments were performed 5 days postseeding.

IN VITRO DIGESTION OF GLIADINS

The gastrointestinal digestion process was simulated as previously described [Laparra et al., 2009], using porcine pepsin (P-7000, Sigma) (800–2,500 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 to a pH 3) was

added to each sample. The mixture was immersed in a water bath (60°C) for 30 min with gentle agitation. Briefly, gastric and intestinal digestions were conducted on a rocking platform shaker placed in an incubator (37°C/5% CO₂/95% relative humidity). After the gastric digestion (pepsin in 0.1 M HCl/pH 3/1 h), the intestinal digestion (pancreatin-bile extract in 0.1 NaHCO₃/pH 6.9–7/2 h) 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). Aliquots (1.5 ml) of the intestinal digest, inoculated or not with bacterial cell suspensions (10⁸ colony forming units [CFU]/ml), were loaded into the upper chambers and incubated for 2 h. Afterward, 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 h.

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 10⁶ and 10⁷ 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 *Bifidobacterium* 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, 4.6 × 250 mm (Thermo, Waltham, MA). 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 min. 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 × 10⁻³ bar. The capillary was held at 4 kV. Mass spectra were recorded over the mass/charge (m/z) range 100–3,500. About 15 spectra were averaged in the MS analyses and about 5 spectra in the MS/MS analyses. The signal threshold to perform auto-MS/MS analyses was 5,000, 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; and ω , 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 NF- κ B 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). Afterward, the commercial TransAM™ kit (Active Motif, Cat. No. 43296) that contains antibodies directed against the NF- κ B p65 subunit was used (TransAM™, Cat. No. 43296).

TNF- α (eBioscience; Cat. No. 88-7346), and IL-1 β (eBioscience; Cat. No. 88-7010) were determined by ELISAs according to the instruction of the manufacturers. The results of the ELISA assay for NF- κ B are expressed as percentage of the control, and TNF- α and IL-1 β as picograms 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 of trisodium citrate, 1 μ l/ml of sodium dodecyl sulfate (0.5% w/v), 0.05 mg/ml PI, and 1 mg/ml of RNase A (R4875, Sigma)]. After incubation overnight at 4°C, the released nuclei were resuspended 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 μ l of fixation solution (PBS:ethanol, in 1:3 v/v ratio) for 5 min. Then, cells were centrifuged (1,200 rpm/10 min) and resuspended in 700 μ l PBS containing Hoechst 33342 (30 μ g/ml, final concentration) during 20 min at room temperature. Afterward, 700 μ l PBS containing pyronin Y (2 μ g/ml, final concentration) were added and incubated for 10 min at room temperature. Samples were analyzed on a Modular Flow Cytometer Cell Sorter (MoFlo Sorter, Dakocytometry, USA). Hoechst was excited with the UV line of an argon laser (λ_{exc} : 346 nm and λ_{em} : 460 nm), and pyronin Y was excited at λ_{exc} : 555 nm and the fluorescence collected at λ_{em} : 580 nm.

ANALYSIS OF THE MRNA EXPRESSION OF PRO-INFLAMMATORY BIOMARKERS

Total RNA was isolated from cell cultures (Qiagen, Cat. No. 74104, USA) and first strand cDNAs were synthesized from 0.5 μ g of total RNA. Polymerase chain reaction (PCR) was carried out with primers designed for TNF- α (NM003842.4) (TNF- α superfamily, member 2) (forward: 5'-AGG GTA CCA CAG AAA GAT GC-3'; reverse: 5'-GCA GAT GAG ACC CTT AGG TT-3'), NF- κ B (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 TNF- α , NF- κ B, and β -actin genes were performed in 35 cycles, which consisted of a first cDNA synthesis stage (45°C, 1 h), AMV-reverse transcriptase (RT) inactivation (95°C, 2 min), denaturation (94°C, 30 s), annealing (60°C, 1 min), and extension (78°C, 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 was 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) 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 $37.8 \pm 3.8\%$ 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 $5.3 \pm 0.9\%$) 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 I). Different peptide patterns were detected in samples inoculated with bifidobacteria compared to those noninoculated. Most peptides generated in samples inoculated with bifidobacteria showed lower molecular mass than those generated in noninoculated samples (~2,500 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] (Fig. 1). The peptides from samples noninoculated with bifidobacteria and from those inoculated with *B. animalis*

TABLE I. 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]	QVLQOST	802.9	803.4	802.9 (1)
	γ -Gld [145-162]	VSSLWSIILPPSDCQVMR	2031.6	2032.4	2031.6 (1)
	α/β -Gld [122-141]	QQQQQQQILQQILQQILP	2460.6	2459.3	1230.3 (2)
	γ -Gld [222-243]	QGHQPQPTQLEVFRSLVLQT	2489.3	2489.4	2489.3 (1)
	γ -Gld [90-110]	QFPQSKQPQFPQPQQPQQ	2487.7	2488.2	2487.7 (1)
Gliadins + <i>B. animalis</i>	γ -Gld [201-212]	QILVPLSQQQV	1381.0	1381.6	1381.0 (1)
	α -Gld [270-285]	SFPQQPQFPPTTK	1829.0	1830.0	1829.0 (1)
	α -Gld [71-386]	QPQQYPQQPYGSSL	1845.6	1846.9	1845.6 (1)
	α -Gld [310-330]	SQQSFLQPQFPQPSILQP	2404.4	2405.6	1202.2 (2)
	α/β -Gld [233-269]	GFFQPSQNPQAQGSFQPQL PQFEAIRNLALQTLPA	4125.3	4127.56	1375.1 (3)
Gliadins + <i>B. bifidum</i>	α -Gld [37-44]	FSHQQQF	1018.5	1018.4	1018.5 (1)
	α -Gld [310-318]	SQQSFLQPQ	1063.2	1063.1	1063.2 (1)
	α/β -Gld [52-62]	GQQQFPQQP	1252.3	1252.4	1252.3 (1)
	α/β -Gld [235-246]	FQPSQNPQAQG	1329.5	1330.4	1329.5 (1)
	α -Gld [358-375]	SQQPQFPQPHQPQQP	2125.0	2126.3	1062.5 (2)
Gliadins + <i>B. longum</i>	γ -Gld [68-87]	QPQQYPQQPQQFPQTQQP	2390.2	2391.6	1195.1 (2)
	α -Gld [62-68]	SQQPPT	803.4	804.4	803.4 (1)
	α -Gld [253-259]	QPQLPQ	838.8	838.4	838.8 (1)
	γ -Gld [91-97]	QPQQFP	841.6	841.4	841.6 (1)
	α/β -Gld [195-202]	IILHQQQ	1007.1	1007.6	1007.1 (1)
γ -Gld [123-132]	SLQQQLNPCK	1154.8	1158.6	577.0 (2)	
α/β -Gld [193-211]	QQPLSQVFSFQQQQYPSG	2178.2	2178.0	1089.1 (2)	
α -Gld [131-150]	LQPQQFPQFPQQLP	2470.0	2468.3	1235.0 (2)	

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, $70.4 \pm 0.1\%$; G2, $14.6 \pm 4.6\%$) exposed to digests from gliadins and with those (G1, $70.3 \pm 3.7\%$; G2, $19.1 \pm 5.5\%$) 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, $70.9 \pm 2.9\%$; G2, $28.8 \pm 1.7\%$).

PRO-INFLAMMATORY CYTOKINE PRODUCTION

The activation of NF- κ B and TNF- α production was induced in intestinal epithelial cell cultures by gliadin digestions noninoculated with bifidobacteria (Fig. 2). In all cases NF- κ B activation was significantly ($P < 0.05$) reduced when the gliadin digestions were inoculated with all bifidobacterial strains, compared to noninoculated digestions. TNF- α 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-1 β 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 difference on IL-1 β 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 RT-PCR analysis (Fig. 3) revealed that the studied bifidobacterial strains reduced ($P < 0.05$) the gliadin induced up-regulation of TNF- α 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- κ B mRNA expression. According to these results together with those related to TNF- α production (Fig. 1), a posttranscriptional control of this pro-inflammatory biomarker seems to occur. In addition, an increased CXCR3 mRNA expression in cell cultures exposed to gliadin digestions noninoculated with

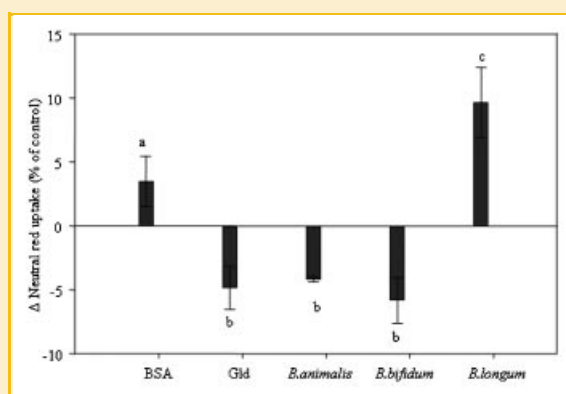


Fig. 1. Neutral red uptake percentages in Caco-2 cell cultures exposed to the dialyzable fraction (0.25 mg/ml) from digests of gliadins (Gld), inoculated or not with bifidobacteria. A digest of bovine serum albumin (BSA) was used as negative control of toxicity. Results are expressed as mean \pm standard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

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 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 noninoculated 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 nonincreased 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 noninoculated 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 CD [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 vs. 0.5–1.5 mg/ml) to challenge the cell cultures, and shorter exposure time (15 h vs. 48 h) 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 junctions 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- κ B) and the production of pro-inflammatory cytokines (TNF- α and IL-1 β) (Fig. 2). The reduced NF- κ B production in cultures exposed to digests of BSA may be explained by the significant down-regulation in nitric oxide synthase (NOS) activity caused by albumin-derived glycosylation products [Rojas et al., 2000]. In contrast, NF- κ B 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 TNF- α [Nilsen et al., 1998] and IL-1 β [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- κ B (nuclear p65 subunit) and TNF- α production, especially in the case of *B. longum*, and completely abolished the IL-1 β production. Into cells, TNF- α and IL-1 actions converge over the

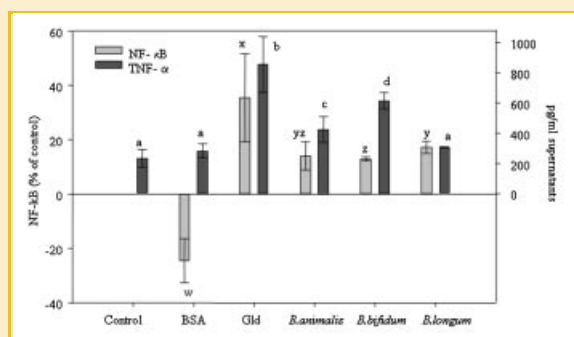


Fig. 2. Nuclear factor kappa-B (NF- κ B) and tumor necrosis factor α (TNF- α) production in Caco-2 cell cultures exposed to the dialyzable fraction from digests of gliadins (Gld), inoculated or not with bifidobacteria. A digest of bovine serum albumin (BSA) was used as negative control of cytokine-induced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

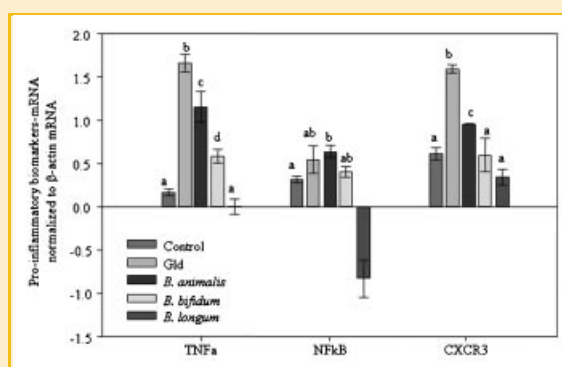


Fig. 3. mRNA expression of pro-inflammatory biomarkers (TNF- α , NF- κ B, and chemokine CXCR3 receptor) in Caco-2 cell cultures exposed to the dialyzable fraction (0.25 mg/ml) from digests of gliadins (Gld), inoculated or not with bifidobacteria. Results are expressed as mean \pm standard deviation ($n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

I κ B kinase complex, which control the phosphorylation-regulated activation of NF- κ B [Schmitz et al., 2001; Leeman and Gilmore, 2008]. However, it has been demonstrated that NF- κ B is a negative regulator of IL-1 β and that inhibition of I κ B causes an increased IL-1 β 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- κ B activation without disconnecting the NF- κ B-mediated-regulatory effect on IL-1 β 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- κ B activation and TNF- α 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 TNF- α increases tight junction dependent permeability, which induction involves NF- κ B activation [Ma et al., 2004]. The reduction of TNF- α production by gliadin digestions inoculated with *B. longum* might also have important physiological implications for CD since TNF- α in conjunction with IL-1 β are the most important cytokines involved in 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, TNF- α 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 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 NF κ B, TNF- α , and IL-1 β 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.

REFERENCES

Beckett CG, Dell'Olio D, Shidrawi RG, Rosen-Bronson S, Ciclitira PJ. 1999. Gluten-induced nitric oxide and pro-inflammatory cytokine release by cultured coeliac small intestinal biopsies. *Eur J Gastroenterol Hepatol* 5:529-535.

Borenfreund E, Puerner J. 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol Lett* 24:119-124.

Box GEP, Hunter NG, Hunter JS. 1978. *Statistics for experimenters. An introduction to design, data analysis and model building*. New York: John Wiley & Sons.

Chartrand LJ, Russo PA, Duhaine AG, Seidman EG. 1997. Wheat starch intolerance in patients with celiac disease. *J Am Diet Assoc* 97:612-618.

Chiara M, DeStefano D, Mele G, Fecarotta S, Greco L, Troncone R, Carnuccio R. 2003. Nuclear factor κ B is activated in small intestinal mucosa of celiac patients. *J Mol Med* 81:373-379.

Ferguson LR, Shelling AN, Browning BL, Huebner C, Petermann I. 2007. Genes, diet and inflammatory bowel disease. *Mutat Res* 622:70-83.

Giovannini C, Sanchez M, Straface E, Scazzocchio B, Silano M, De Vincenzi M. 2000. Induction of apoptosis in Caco-2 cells by wheat gliadin peptides. *Toxicology* 145:63-71.

Giovannini C, Matarrese P, Scazzocchio B, Vari R, D'archivio M, Straface E, Masella R, Malorni W, De Vincenzi M. 2003. Wheat gliadin induced apoptosis of intestinal cells via an autocrine mechanism involving Fas-Fas ligand pathway. *FEBS Lett* 540:117-124.

Greten FR, Arkan MC, Bollrath J, Hsu LC, Goode J, Miething C, Göktuna SI, Neuenhahn M, Fierer J, Paxian S, Rooijen NV, Xu Y, O'Cain T, Jaffee BB, Busch DH, Duyster J, Schmid RM, Eckmann L, Karin M. 2007. NF- κ B is a negative regulator of IL-1 β secretion as revealed by genetic and pharmacological inhibition of IKK β . *Cell* 130:918-931.

Hoffman RA. 2000. Intraepithelial lymphocytes coinduce nitric oxide synthase in intestinal epithelial cells. *Am J Physiol: Gastrointest Liver Physiol* 278:G886-G894.

Lammers KM, Lu R, Brownley J, Lu B, Gerard C, Thomas K, Rallabhandi P, Shea-donohue T, Tamiz A, Alkan S, Netzel-Arnett S, Antalis T, Vogel SN, Fasano A. 2008. Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3. *Gastroenterology* 135:194-204.

Laparra JM, Alegria A, Barberá R, Farré R. 2008. Antioxidant effect of casein phosphopeptides compared with fruit beverages supplemented with skimmed milk against H₂O₂-induced oxidative stress in Caco-2 cells. *Food Res Int* 41:773-779.

Laparra JM, Glahn RP, Miller DD. 2009. Assessing potential effects of inulin and probiotic bacteria on Fe availability from common beans (*Phaseolus vulgaris* L.) to Caco-2 cells. *J Food Sci* 74:40-46.

Leeman JR, Gilmore TD. 2008. Alternative splicing in the NF- κ B signaling pathway. *Gene* 423:97-107.

Lindfors K, Blomqvist T, Juuti-Uusitalo K, Stenman S, Venäläinen J, Mäki M, Kaukinen K. 2008. Live probiotic *Bifidobacterium lactis* bacteria inhibit the toxic effects induced by wheat gliadin in epithelial cell culture. *Clin Exp Immunol* 152:552-558.

Ma TY, Iwamoto GK, Hoa NT, Akotia V, Pedram A, Boivin MA, Said HM. 2004. TNF- α -induced increase in intestinal epithelial tight junction permeability requires NF- κ B activation. *Am J Gastrointest Liver Physiol* 286:G367-G376.

Medina M, De Palma G, Ribes-Koninckx C, Calabuig M, Sanz Y. 2008. Bifidobacterium strains suppress in vitro the pro-inflammatory milieu triggered by the large intestinal microbiota of coeliac patients. *J Inflamm (Lond)* 5:19. 10.1186/1476-9255-5-19.

Moss SF, Attia L, Scholes JV, Walters JRF, Holt PR. 1996. Increased small intestinal apoptosis in celiac disease. *Gut* 39:811-817.

Nadal I, Donat E, Ribes-Koninckx C, Calabuig M, Sanz Y. 2007. Imbalance in the composition of the duodenal microbiota of children with celiac disease. *J Med Microbiol* 56:1669-1674.

Nilsen EM, Jahnsen FL, Lundin KE, Johansen FE, Fausa O, Sollid LM, Jahnsen J, Scott H, Brandtzaeg P. 1998. Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology* 115:551-563.

Rojas A, Romay S, González D, Herrera B, Delgado R, Otero K. 2000. Regulation of endothelial nitric oxide synthase expression by albumin-derived advanced glycosylation end products. *Circ Res* 86: E50-4.

Roselli M, Finamore A, Britti MS, Mengheri E. 2006. Probiotic bacteria *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG protect intestinal Caco-2 cells from the inflammation-associated response induced by enterotoxigenic *Escherichia coli* K88. *Br J Nutr* 95:1177-1184.

Sanz Y. 2009. Novel perspectives in celiac disease therapy. *Mini Rev Med Chem* 9:359-367.

- Sanz Y, Sánchez E, Marzotto M, Calabuig M, Torriani S, Dellaglio F. 2007. Differences in faecal bacterial communities in celiac and healthy children as detected by PCR and denaturing gradient gel electrophoresis. *FEMS Immunol Med Microbiol* 51:562–568.
- Schmitz ML, Bacher S, Kracht M. 2001. I κ B-independent control of NF- κ B activity by modulatory phosphorylations. *Trends Biochem Sci* 26:186–190.
- Shan L, Qiao SW, Arentz-Hansen H, Molberg Ø, Gray GM, Sollid LM, Khosla C. 2005. Identification and analysis of multivalent proteolytically resistant peptides from gluten: Implications for celiac sprue. *J Proteome Res* 4:1732–1741.
- Thomas KE, Sapone A, Fasano A, Vogel SN. 2006. Gliadin stimulation of murine macrophage inflammatory gene expression and intestinal permeability are MyD88-dependent: Role of the innate immune response in celiac disease. *J Immunol* 176:2512–2521.
- Troncone R, Ivarsson A, Szajewska H, Mearins ML. 2008. Review article: Future research on coeliac disease—A position report from the European multistakeholder platform on coeliac disease (CDEUSSA). *Aliment Pharmacol Ther* 27:1030–1043.
- Wieser H, Koehler P. 2008. The biochemical basis of celiac disease. *Cereal Chem* 85:1–13.
- Young SL, Simon MA, Baird MA, Tannock GW, Bibiloni R, Spencely K, Lane JM, Fitzharris P, Crane J, Town I, Addo-Yobo E, Murray CS, Woodcock A. 2004. Bifidobacterial species differentially affect expression of cell surface markers and cytokines of dendritic cells harvested from cord blood. *Clin Diagn Lab Immunol* 11:686–690.