

VSL#3 probiotic preparation has the capacity to hydrolyze gliadin polypeptides responsible for Celiac Sprue

Maria De Angelis^a, Carlo G. Rizzello^a, Alessio Fasano^b, Maria G. Clemente^b,
Claudio De Simone^c, Marco Silano^d, Massimo De Vincenzi^d, Ilario Losito^e, Marco Gobetti^{a,*}

^a Department of Plant Protection and Applied Microbiology, University of Bari, 70126 Bari, Italy

^b Mucosal Biology Research Center and Center for Celiac Research, University of Maryland School of Medicine, Baltimore, MD 21201, USA

^c Department of Experimental Medicine, University of L'Aquila, 67100 L'Aquila, Italy

^d Istituto Superiore di Sanità, Reparto di Alimentazione, Nutrizione e Salute, I-00161 Roma, Italy

^e Dipartimento di Chimica, Università degli Studi di Bari, 70126 Bari, Italy

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Abstract

The native structure and distribution of gliadin epitopes responsible for Celiac Sprue (CS) may be influenced by cereal food processing. This work was aimed at showing the capacity of probiotic VSL#3 to decrease the toxicity of wheat flour during long-time fermentation. VSL#3 (10^9 cfu/ml) hydrolyzed completely the $\alpha 2$ -gliadin-derived epitopes 62–75 and 33-mer (750 ppm). Two-dimensional electrophoresis, immunological (R5 antibody) and mass spectrometry analyses showed an almost complete degradation of gliadins during long-time fermentation of wheat flour by VSL#3. Gliadins non-hydrolyzed during fermentation by VSL#3 were subjected to peptic-tryptic (PT) digestion and analyzed by CapLC-ESI-Q-ToF-MS (Capillary Liquid Chromatography-Electrospray Ionization-Quadrupole-Time of Flight-Mass Spectrometry). Search for several epitopes showed the only presence of $\alpha 2$ -gliadin-fragment 62–75 at a very low concentration (sub-ppm range). Compared to IEC-6 cells exposed to intact gliadins extracted from the chemically acidified dough (control), VSL#3 pre-digested gliadins caused a less pronounced reorganization of the intracellular F-actin which was mirrored by an attenuated effect on intestinal mucosa permeability. The release of zonulin from intestinal epithelial cells treated with gliadins was considerably lower when digested with VSL#3. Agglutination test on K 562 (S) cells showed that the PT-digest of wheat flour treated with VSL#3 increased the Minimal Agglutinating Activity of ca. 100 times. Wheat proteins were extracted from doughs and subjected to PT digestion. Compared to PT-digest from chemically acidified dough, celiac jejunal biopsies exposed to the PT-digest from the dough fermented by VSL#3 did not show an increase of the infiltration of CD3⁺ intraepithelial lymphocytes. Proteolytic activity by probiotic VSL#3 may have an importance during food processing to produce pre-digested and tolerated gliadins for increasing the palatability of gluten-free products.

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1. Introduction

Currently, Celiac Sprue (CS) prevalence has been estimated to be 1 in 266 people worldwide [1]. Such a rate establishes CS as one of the most common food intolerance. Similar figures have been reported in most European countries, South America and USA [2–4]. CS is now reported to be present in 0.5 to 1% of the USA population [5]. Reports

from North Africa, Iran and India indicate the widespread occurrence of CS [6]. CS is a genetically-determined chronic inflammatory intestinal disease induced by an environmental trigger, gluten. The clinical classification of CS includes symptomatic, classic forms with diarrhoea, weight loss and bloating, with or without malabsorption, atypical and asymptomatic or silent forms, where gastrointestinal symptoms are absent. The number of silent forms continues raising world-wide [7].

During endoluminal proteolytic digestion, mainly prolamins of wheat (α -, β -, γ - and ω -gliadin sub-groups), rye (e.g., secalin) and barley (e.g., hordein) release a family of Pro- and Gln-rich polypeptides that are responsible for the inappropriate

* Corresponding author. Dipartimento di Protezione delle Piante e Microbiologia Applicata, Facoltà di Agraria di Bari, Via G. Amendola 165/a, 70126 Bari, Italy. Tel.: +39 080 5442949; fax: +39 080 5442911.

E-mail address: gobetti@agr.uniba.it (M. Gobetti).

T-cell-mediated immune response [8]. Apparently, residues of the N-terminus of α -gliadin are immunologically active and probably toxic. Amino acids at position 55–75 induce immunologic activation via association with HLA DQ2, particularly when Q65 is deaminated by tissue Transglutaminase (tTG) to glutamic acid. This is known as the dominant epitope, although other regions, including 31–55 and parts of the C-terminus, are also toxic [9]. Although still debated, fragments 31–43 of α 2-gliadin [10], 62–75 of α 2-gliadin [11], 33-mer epitope, corresponding to 57–89 of α 2-gliadin [11], 134–153 of γ -gliadin [12] and 57–68 of α 9-gliadin seem to be potent inducers of CS [13]. It has been suggested that epitopes challenge may initiate two parallel pathways. First, epithelial cells over-express HLA-DR molecules within 2 h, and in the second stage, T-lymphocytes become fully activated. T-lymphocytes activation plays a pivotal role in the tissue destruction occurring in small bowel mucosa in coeliac disease. The infiltration of epithelium by CD3⁺ cells has been reported to be a signal of the T-cell activation towards gliadin peptides in coeliac disease [14]. The organ culture system has already been used to characterize gliadin epitopes able to active mucosal immunity and to test the toxicity of some cereals for celiac [15]. In fact, despite the intrinsic limitations of this technique, such as the loss of connection with the systemic immune network and the abnormal gliadin encounter, it is the best tool to study the effects of gliadin exposure and the pathogenic mechanism implicated in coeliac disease [16]. Besides, the intestinal epithelium with its intact intercellular tight junctions (TJ) serves as the main barrier to the passage of macromolecules, including gliadin polypeptides. When the integrity of the TJ system is compromised, an immune response to gliadin may develop. The up-regulation of zonulin, a recently described intestinal peptide involved in TJ regulation [17], seems to be responsible, at least in part, for the increased gut permeability which characterizes the early phase of CS [18]. Following binding to its surface receptor, zonulin induces a protein kinase C-mediated polymerization of intracellular actin filaments which are directly connected to structural proteins of the TJ system, hence regulating epithelial permeability [17–19].

The current treatment for CS is a strict gluten-free diet (GFD) for life, even though the Codex Alimentarius tolerates 200 ppm of gluten per food [1]. Nevertheless, a small percentage of patients diagnosed with CS fail to have a long-term favorable response to a strict GFD. These individuals continue to have symptoms and histologic abnormalities or have symptomatic relapse despite treatment with GFD and ultimately develop a potentially life-threatening disease known as refractory sprue. Since the burden of illness related to CS is doubtless higher than previously thought, efforts to reduce the human intolerance to cereals are of medical, nutritional and economic interest. Beyond genetic predisposition, several environmental factors influenced CS prevalence. Recent epidemiological studies show that, besides being frequently found in countries where individuals are mostly of European origin, CS is a common disorder in many areas of the developing world where agriculture started 10,000 years ago

[20]. More recently, cereal food technology has changed dramatically by influencing dietary habitudes of entire population previously naive to gluten exposure. Cereal baked goods are currently manufactured by very accelerated processes where long-time fermentations by sourdough, a cocktail of acidifying and proteolytic lactic acid bacteria with yeasts, were almost totally replaced by the indiscriminate use of chemical and/or baker's yeast leavening agents. Under these technological circumstances, cereal components (e.g., proteins) are subjected to very mild or absent degradation during manufacture, resulting, probably, in lower digestible foods compared to traditional and ancient sourdough baked goods [21].

A pool of sourdough lactic acid bacteria had a pattern of specialized peptidases capable of hydrolyzing all the different bonds that potentially include the imino acid proline [22]. As shown by in vivo acute challenges based on intestinal permeability, breads fermented by selected lactic acid bacteria and containing ca. 2% of gluten were tolerated by CS patients. Probiotics, defined as the viable microorganisms that exhibit a beneficial effect on the health of the host by improving its intestinal microbial balance, are, in most of the cases, parental strains of lactic acid bacteria starters used for food processing as in sourdough bread. To our knowledge, no reports have considered the potential role of probiotics in CS disease management. Among probiotic preparations, VSL#3 (VSL Pharmaceuticals, Gaithersburg, MD) is a highly concentrated (ca. 5×10^{11} cells/g) mixture of lactic acid and bifido-bacteria which has been shown to colonize the intestine and to promote several beneficial effects for the host [23–25].

This paper was aimed at exploring the capacity of VSL#3 preparation to hydrolyze wheat flour gliadins, including polypeptides responsible for CS, as a tool to increase tolerability in CS patients exposed to traces of gluten.

2. Materials and methods

2.1. Probiotic preparations

Freeze-dried preparation of VSL#3 (VSL Pharmaceuticals, Gaithersburg, MD) (ca. 450 billion cells/sachet), containing *Streptococcus thermophilus*, *Lactobacillus plantarum*, *L. acidophilus*, *L. casei*, *L. delbrueckii* spp. *bulgaricus*, *Bifidobacterium breve*, *B. longum* and *B. infantis* was used for dough fermentation and gliadin polypeptides hydrolysis. Other commercial freeze-dried probiotic preparations such as Oxadrop (*L. acidophilus*, *L. brevis*, *B. infantis* and *St. thermophilus*) (VSL Pharmaceuticals), Florisia (*L. brevis*, *L. salivarius* spp. *salicinius* and *L. plantarum*) (VSL Pharmaceuticals), and Yovis (*St. salivarius* spp. *thermophilus*, *B. breve*, *B. infantis*, *B. longum*, *L. acidophilus*, *L. plantarum*, *L. casei*, *L. delbrueckii* spp. *bulgaricus*, *St. faecium*) (Sigma Tau, Industrie Farmaceutiche Riunite S.p.a., Roma) were also used.

2.2. Sourdough fermentation

The characteristics of the wheat flour used were as follows: moisture, 12.8%; protein ($N \times 5.70$), 10.7%, of dry matter (d.m.); fat, 1.8% of d.m.; ash, 0.6% of d.m.; and total soluble carbohydrates, 1.5% of d.m. Eighty grams of wheat flour and 190 ml of tap water (containing a cell concentration of the probiotic preparations of ca. 10^9 cfu per g of dough) were used to produce 270 g of dough. The dough was incubated for 24 h at 37 °C under stirring conditions (ca. 200 rpm). Overall, the European daily diet includes 200 g or

elution program was: gradient from 0 to 70% B (v/v) in 35 min, isocratic at 70% B for 5 min. Positive ions MS full scan spectra were acquired in the 50–2000 Th m/z range using the ToF analyzer in the V-mode (resolution 10000). Before each set of MS acquisitions, the ToF m/z scale was calibrated using Glu-Fibrinopeptide fragment ions as calibrants. Epitopes such as 33-mer peptide, fragment 62–75 of the α 2-gliadin, fragment 134–153 of β -gliadin (Q–Q–L–P–Q–P–Q–Q–P–Q–Q–S–F–P–Q–Q–Q–R–P–F); fragment 57–68 of α 9-gliadin (Q–L–Q–P–F–P–Q–P–Q–L–P–Y), and fragment 31–43 of A-gliadin (L–G–Q–Q–Q–P–F–P–P–Q–Q–P–Y) (Neosystem Laboratoire) were used as standards at a concentration of 10 ppm.

2.7. IEC6 cell cultures

Rat intestinal epithelial cells (IEC-6 cells) were grown in cell-culture flasks (Falcon Labware) at 37 °C in an atmosphere of 95% air and 5% CO₂. The medium consisted of Dulbecco's modified Eagle medium (DMEM, GIBCO) containing 4500 mg/L D-glucose, pyridoxine hydrochloride, 5% heat inactivated (56 °C, 30 min) fetal bovine serum (FBS), 0.1 U/ml bovine insulin, 4 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin.

2.8. Fluorescence microscopic analysis of intracellular F-actin

Cells were washed in PBS and gently detached with 2–3 min exposure with 0.25% trypsin, 1 mM EDTA solution (Gibco brl). The cells (2×10^4 cells/ml) were suspended in medium and seeded onto 8 chamber slides (Nalge Nunc International) for 24 h. Gliadins extracted from 200 g dough were added at increasing concentrations and exposure times. Cells were then washed twice in phosphate-buffered saline (PBS), fixed in 3.7% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature, permeabilized with 0.5% TritonX-100 in PBS (Sigma) for 10 min at room temperature and stained by incubation with 0.3 µM fluorescein phalloidin (Sigma) in PBS at 37 °C for 30 min. After two additional washes, the cover slips were mounted with glycerol-PBS (1:1) at pH 8.0. The results were analyzed with a fluorescence microscope (ZEISS).

2.9. Zonulin quantitation by sandwich enzyme-linked immunosorbent assay (ELISA)

A sandwich enzyme-linked immunosorbent assay was developed in order to measure zonulin concentration in cell culture supernatants using affinity-purified anti-Zonula occludens toxin (Zot) antibodies, produced as previously described [17]. Five different serial dilutions of a 200 µg/ml Zot solution (0.7, 3.1, 12.5, 50 and 200 ng/ml) were prepared in PBS-T (0.05% Tween-20 in PBS) and used to generate the standard curve. First, a 10-µg/ml anti-Zot IgG solution in PBS was added to each well (100 µl/well) of a 96-well microplate. After incubation for 48 h at +4 °C, the plate was washed three times with PBS-T and blocked overnight with PBS-T (300 µl/well) containing 1% bovine serum albumin (BSA). After draining the blocking solution, five Zot serial standards and the cell culture medium samples were added in double (100 µl/well) and incubated for 2 h at RT in continuously shaking. Following a 3-time wash with PBS-T, 0.5 µg/ml biotinylated anti-Zot antibody solution in PBS–BSA1%–PEG 4%, was added to each well (100 µl/well) and incubated for 1 h at RT shaking. After washing six times in PBS-T, a 15-min incubation was performed with ExtrAvidin-Alkaline Phosphatase (Sigma) diluted 1:16,000 in 0.1 M Tris–HCl, 1 mM MgCl₂, BSA 1% at pH 7.3 at RT. The plate was washed again three times with PBS-T and then incubated for 30 min at 37 °C with 0.1 ml of p-nitrophenyl phosphate substrate in glycine buffer (pH 10.7, containing 0.1 M NaCl, 0.1 mM ZnCl₂, 1 mM MgCl₂). The absorbance at 405 nm was measured with a microplate auto-reader (Molecular Devices Thermo-max Microplate Reader, USA). To define the intra- and inter-assay precision of the ELISA-sandwich method, the coefficient of variation (CV) was calculated using three replicates from two samples with different concentrations of zonulin, on three consecutive days. The inter-assay test of the ELISA-sandwich method produced CV values of 9.8%. The CV of the intra-assay test was 4.2% at day 1, 3.3% at day 2 and 2.9% at day 3.

2.10. Intestinal permeability in the Micro-Snapwell system

Costar snapwells (Costar Corning Incorporated, NY USA) were modified to attain a reduced surface area (7 mm² vs. 113 mm²) of exposed mucosa in order to perform experiments on small intestinal mouse specimens and to reduce the overall volume of bathing medium. A 3-mm diameter central hole was cut in circular Plexiglas pieces with a 12-mm diameter. The Plexiglas inserts were washed in 100% ethanol, air dried, and sterilized overnight under UV. Segments of small intestine (jejunum) of Balb/c mice were removed, opened along the mesenteric border, rinsed free of the intestinal content using PBS, and unstripped pieces of 3.5 mm in diameter (7 mm² exposed surface area) were placed on snapwell filters with the mucosal side oriented upward under a dissecting microscope. Tissues so prepared were then sandwiched between two Plexiglas inserts, introduced into Costar snapwells, and placed in the incubator (37 °C, 5% CO₂) for 30 min to stabilize the pH.

Following equilibration in the incubator, the baseline transepithelial electrical resistance (TEER) was measured, gliadin preparations were added to the luminal aspect of the mucosa, and TEER measured at increasing time intervals. Tissue culture media samples were collected from both the mucosal and the serosal sides at 30-min intervals for zonulin analysis. PBS-exposed tissues were used as controls.

2.11. Agglutination test

Ethanol-extractable gliadins from wheat flour (S. Pastore variety) were submitted to peptic–tryptic (PT) sequential digestion to produce the corresponding PT digest by simulating the *in vivo* digestion [39]. After production, the PT-digest was heated at 100 °C for 30 min to inactivate enzymes. This peptide preparation was used directly for agglutination test or it was further digested with the VSL#3 cell preparation. The reaction mixture was as follows: 1000 µl of 5 mM phosphate buffer, pH 7.0, containing VSL#3 at a final cell concentration 10^9 cfu/g and 10 mg of PT digest. After incubation at 37 °C for 24 h under stirring conditions (150 rpm), the mixture was freeze dried and used for agglutination test. K 562 (S) subclone cells of human myelogenous leukemia origin from the European Collection of Cell Culture (Salisbury, United Kingdom) were used for the agglutination test as described previously [40].

2.12. Processing of jejunal biopsies and *in vitro* organ culture

Four female untreated CD patients (mean age: range 5–10 years) underwent gastrointestinal endoscopy for diagnostic purposes. All of them suffered from symptoms suggestive of celiac disease and resulted positive for serum Ab anti transglutaminase. During the endoscopy, performed with a gastroscope, for each patient, two samples of small intestine mucosa were obtained, one for diagnostic and one for the experiments. The histological observation confirmed the clinical suspect of CD for all the subjects, showing villous atrophy and crypt hyperplasia.

Albumins, globulins, prolamins and glutamins were extracted from doughs fermented by VSL#3 and chemically acidified, freeze-dried, and pooled. Before freeze-drying, each protein fraction was dialyzed for 12 h at 4 °C against distilled water (membrane cutoff, 1,000 Da) to remove substances (e.g., carbohydrates) that interfered with the immunohistochemical analysis. Fifty-milligram portions of the pooled protein fractions were subjected to sequential PT digestion as described elsewhere.

Each jejunal biopsy was sliced in two parts. The specimens were placed on a stainless steel grid positioned over the central well of an organ culture dish. The villous surface of the mucosa was placed upward. Jejunal biopsies were cultured for 24 h in RPMI supplemented with 10% FCS (fetal calf serum) and PT-digests (1 mg/ml). PT-digest from chemically acidified dough was used as the positive control. Incubation with RPMI medium alone was carried out as the negative control. After incubation, the specimens were harvested, embedded in optimal cutting temperature compound (Bioplica, Milano, Italy) and stored at –80 °C. The biopsy samples were sectioned into 4-µm slices, that were fixed in acetone for 20 min and incubated for 30 min with normal rabbit serum (1:200, Dako, Carpinteria, CA) in order to prevent non-specific antibody binding [41]. Afterwards, sections were incubated with anti CD3⁺ (1:200,

Dako) monoclonal antibody and exposed to rabbit anti-mouse immunoglobulin for 30 min. Monoclonal antibody were diluted in Ab dilution solution (Dako). After washing with Tris, pH 7.4, the sections were incubated with monoclonal mouse APAAP (Dako) for 30 min and New Fuchsin was used for staining. Finally, section were counterstained with Mayer's hematoxylin (Dako) and mounted in Aquamount (Sigma). All the procedure was carried out at room temperature and the incubation was performed in a humidity chamber.

The density of cells expressing CD3⁺ was determined counting the stained cells per mm of epithelium. The count was repeated in two different sections for each sample. The data were compared by the Student's *t* test. $P > 0.05$ was considered significant.

3. Results

3.1. Gliadins hydrolysis by probiotic preparations

After 24 h of fermentation, the pH of doughs fermented with the four probiotic preparations ranged from 3.7 to 4.0. As previously shown [40], biological or chemical acidification may cause a direct modification of the polypeptide pattern compared to non-acidic wheat flour. Therefore, fermented dough was always compared to a chemically acidified (pH 4.0) dough to find variations due to bacterial proteolysis only. By performing this comparison, changes due to proteolysis by flour endogenous enzymes were also excluded in part.

Fig. 1 shows the SDS-PAGE profiles of the gliadin polypeptides after dough fermentation with commercial probiotic preparations. The highest hydrolysis was found by VSL#3, while Florisia and Yovis were unable to cause an appreciable degradation. Oxadrop caused a very low degradation of gliadin polypeptides. Further evidence of the highest hydrolyzing activity of VSL#3 was provided by the RP-FPLC analysis of the 70% ethanol-soluble gliadin fraction which also contains polypeptides having lower apparent molecular masses than that detectable by electrophoresis (data not shown). When used individually, strains belonging to VSL#3 preparation were

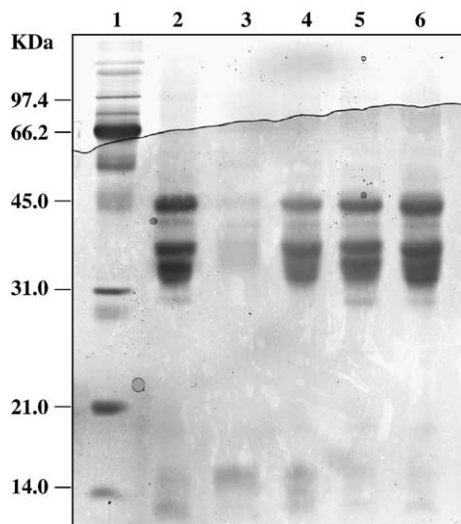


Fig. 1. SDS-PAGE analysis of gliadin polypeptides from wheat flour doughs incubated for 24h with the different probiotic preparations (10^9 cfu/ml). Lanes: standard (1); chemically acidified dough (control) (2); dough incubated with VSL#3 (3); Oxadrop (4); Florisia (5); and Yovis (6).

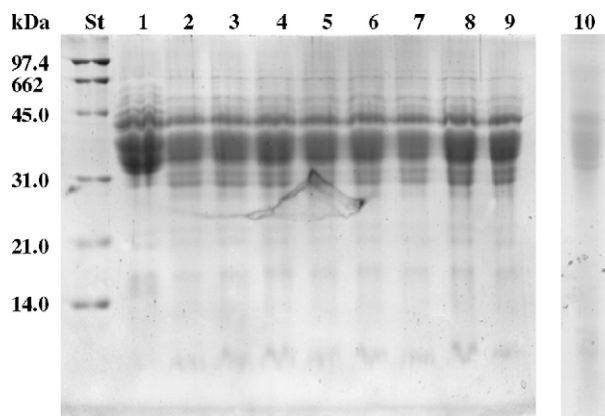


Fig. 2. SDS-PAGE analysis of gliadins polypeptides from wheat flour doughs incubated for 24 h with the different cell preparations (10^9 cfu/ml) which composed the VSL#3 preparation. Protein standard (St). Chemically acidified dough (1); doughs incubated with cells of *Bifidobacterium longum* (2); *Lactobacillus delbrueckii* subsp. *bulgaricus* (3); *L. plantarum* (4); *L. casei* (5); *B. infantis* (6); *L. acidophilus* (7); *Streptococcus thermophilus* (8); *B. breve* (9); and VSL#3 preparation (10).

less effective in causing hydrolysis as compared to the mixture (Fig. 2). Based on these preliminary results, the proteolytic activity of VSL#3 was further characterized.

3.2. Peptidase activity

Gliadins and related epitopes are characterized by a large proportion of proline residues [42]. To adequately deal with such peptides, a group of specific peptidases is necessary [43]. Initially, the proline specific peptidase and general aminopeptidase activities of VSL#3 were characterized by using synthetic substrates relatively specific for proline iminopeptidase, aminopeptidase type N and A, dipeptidase, prolinase, prolidase, dipeptidyl peptidase, tripeptidase, prolyl-endopeptidase and endopeptidase enzymes (Table 1). All these enzyme activities were found in the VSL#3 preparation. Peptidase activities under simulated gastro-intestinal conditions with peptic/tryptic casein digest (2 mg/ml), as competing substrate, showed only a moderate decrease with respect to the activities found under optimal conditions.

The peptidase activity of VSL#3 was further tested on previously reported toxic or immuno-modulatory peptides involved in CS pathogenesis [8,11]. After 24 h of incubation, the epitope 33-mer, at a concentration of 750 ppm, was completely hydrolyzed by 10^9 cfu/ml of VSL#3 (Fig. 3). Residual polypeptide peaks in the 33-mer digests could be attributed to microbial peptides/proteins. The above cell numbers are usually found for microbial starters during wheat dough fermentation. Similar results were obtained after 6 h of incubation with the fragment 62–75, at a concentration of 750 ppm (data not shown). The kinetics of hydrolysis of the 33-mer was determined by the Lineweaver–Burk plot. A $V_{\max}$ of 0.26 μmol per milliliter per min and a K_m of 216 μM were calculated. After 4 h of incubation, VSL#3 preparation had the capacity to completely hydrolyze a considerable concentration (150 ppm) of the 33-mer.

Table 1
Enzyme activity^a of VSL#3 on various substrates containing proline residues

Substrate	Type of enzyme	Substrate concentration (mM)	Unit of activity (U) ^b	Unit of activity (U) ^c
Pro- <i>p</i> -NA	Proline iminopeptidase	2	3.2±0.02	3.3±0.05
Leu- <i>p</i> -NA	Amino peptidase type N	2	8.4±0.04	7.9±0.03
Ala- <i>p</i> -NA	Amino peptidase type A	2	12.3±0.05	11.0±0.11
Leu-Leu	Dipeptidase	2	15.51±0.03	14.1±0.05
Val-Leu	Dipeptidase	2	17.22±0.07	18.0±0.13
Pro-Gly	Prolinase	3	8.0±0.02	7.70±0.21
Val-Pro	Prolidase	2	3.03±0.02	2.87±0.07
Gly-Pro-Ala	Dipeptidyl peptidase IV/ carboxypeptidase P	0.2	2.73±0.01	0.73±0.02
Leu-Leu-Leu	Tripeptidase	2	10.63±0.41	10.0±0.38
Z-Gly-Pro- <i>p</i> -NA	Prolyl-endopeptidase	2	1.3±0.01	1.0±0.02
NCBZ-Gly-Gly-Leu- <i>p</i> -NA	Endopeptidase	2	1.9±0.02	1.7±0.03

^a Each value is the average of three enzyme assays, and standard deviations were calculated. A unit of enzyme activity (U) on *p*-NA substrates was defined as the amount of enzyme which produced an increase in absorbance at 410 nm of 0.01/min. A unit on polypeptides was the amount of enzyme which liberates 1 micromole of substrates/min.

^b Unit of enzymatic activity under optimal conditions.

^c Unit of enzymatic activity under simulated gastro-intestinal conditions (see Materials and methods).

3.3. Proteolysis during dough fermentation

Two-DE analysis was used to characterize the hydrolysis of gliadin and glutenin polypeptides by VSL#3 during dough fermentation. Gliadins are very heterogeneous proteins subdivided in α -, β -, γ - and ω -fractions. A total of 84 polypeptides was identified by 2DE analysis of chemically acidified dough (control) (Fig. 4A). Although enzyme activities of VSL#3 preparation in complex protein matrix may be in part different from that found by using synthetic substrates, 79 of the 84 gliadin polypeptide spots were degraded after dough fermentation with VSL#3 compared to control (Fig. 4B). Table 2 refers to the hydrolysis factors of the spots identified by 2DE. Most of the polypeptides degraded (65 of the 79) had hydrolysis factors higher than 80% and only 8 showed hydrolysis factors lower than 40%. With respect to chemically acidified dough, the VSL#3 fermented dough showed partial hydrolysis of HMW glutenins (Fig. 4C and D).

Compared to European gliadin references, the Western blot by R5 monoclonal antibody of the chemically acidified dough had the typical profile of intact gliadins (Fig. 5). A major advantage of the R5 monoclonal antibody is its ability to recognize the consensus amino-acid sequences QXPW and QXPFP corresponding to multiple immuno-reactive epitope repeats, which occur in α -, γ - and ω -gliadins as well as in different wheat varieties and related barley and rye prolamins

[44,45]. Greatest reactivity has been associated with the QQPFP amino-acid sequence, but homologous repeats such as LQPFP, QLPYP, QLPTE, QQSFP, QQTFF, PQPPP, QQPYP and PQPFP are also recognized with a weaker reactivity by the R5 antibody [44]. Three of these epitopes (LQPFP, QLPYP and PQPFP) are placed in the sequence of 33-mer [11,13]. The Western blot of the dough fermented by VSL#3 showed an almost complete degradation of α -, β - and γ -gliadins recognized by R5 monoclonal antibody.

These results were mirrored by mass spectrometry MALDI-TOF analysis (Fig. 6). Currently, mass spectrometry MALDI-TOF is the only accepted, non-immunological technique for monitoring gliadins and related peptides in foods [38]. In spite of the great genetic heterogeneity of α -, β -, γ -, and ω -gliadin fractions, the MALDI-TOF analyses of gliadin ethanol extracts of the European standard and chemically acidified dough showed characteristic protonated mass patterns at 33–55 kDa. Polypeptides included in this molecular mass range are commonly used for the identification of gliadins in wheat-contaminated food samples. All the gliadin peaks detected in chemically acidified dough almost completely disappeared in the dough fermented with VSL#3. Fragments of gliadin hydrolysis, especially in the range 21 to 28 kDa mass $-m/z$, were detected.

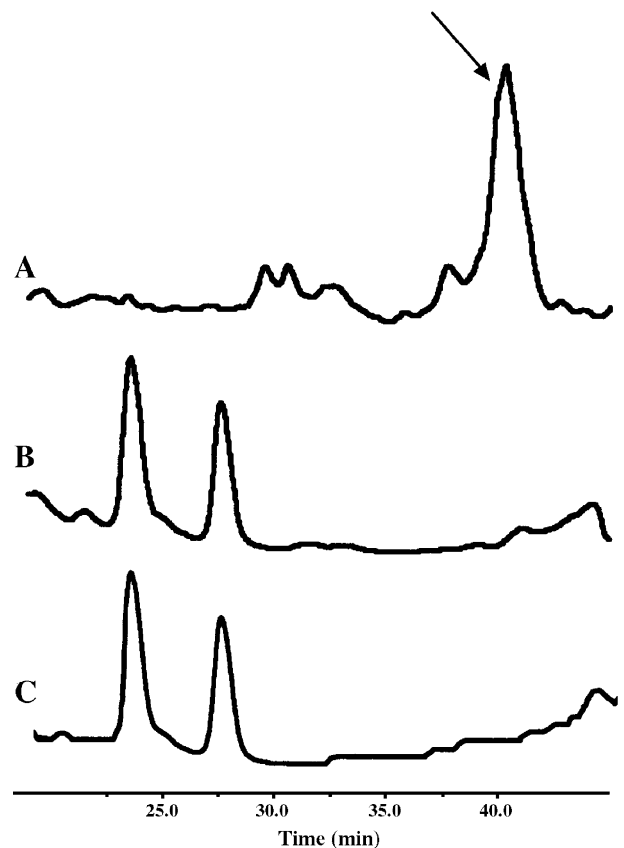


Fig. 3. Hydrolysis of 33-mer peptide by VSL#3 (10^9 cfu/ml). RP-FPLC at UV 214 nm trace of 750 ppm 33-mer after 24 h of incubation at 37 °C without microbial inoculum (A), after 24 h of hydrolysis by VSL#3 at 37 °C (B), and after incubation of VSL#3 without 33-mer (10^9 cfu/ml) for 24 h at 37 °C (C).

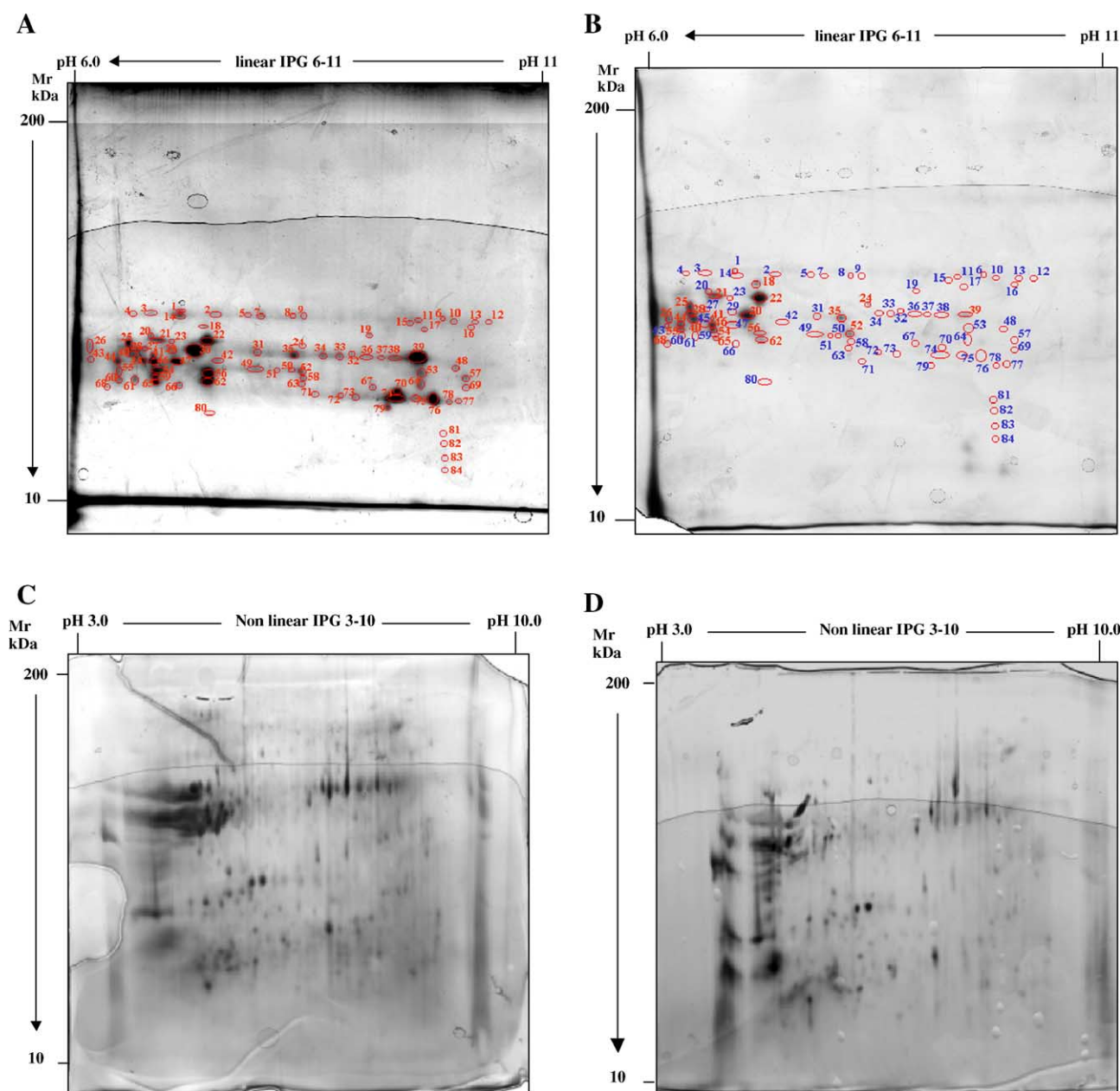


Fig. 4. 2DE analysis of protein fractions of different doughs made of wheat flour. (A) Gliadin fraction from chemically acidified dough (control) and (B) from dough incubated for 24 h at 37 °C with VSL#3. Prolamin polypeptides were indicated by numbered red ovals. Blue numbers refer to polypeptides, which were degraded more than 80%. Mr, molecular mass. (C) Glutenin fraction from chemically acidified dough (control) and (D) from dough incubated for 24 h at 37 °C with VSL#3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Gliadins non-hydrolyzed during fermentation by VSL#3 were used to produce the PT-digest and analyzed by CapLC-ESI-Q-ToF-MS (Fig. 7A). The chromatographic trace was quite complex, with a large band eluting between 15 and 35 min, and only a major peak observed at 29 min. Extraction of ion currents was adopted to search for peaks related to epitopes arising from gliadins. In particular, m/z ratios corresponding to mono, doubly and triply protonated ions of several known sequences were used for this elaboration but only traces with a poor signal/noise ratio were observed and distinct peaks were never found. The only positive signal was found for the m/z 841.9 ion, corresponding to the bi-

protonated form of α 2-gliadin-derived epitope 62–75 (Fig. 7B). In order to check the Q-ToF sensitivity towards gliadin peptides in the specific matrix, an aliquot of the same sample was then spiked with synthetic analogues of some gliadin peptides, at a final concentration of 10 ppm each. The extracted ion chromatogram for doubly charged epitope 62–75 obtained from this sample is shown in Fig. 7C. A much better peak was observed in this case, its intensity being more than 15 times higher than in chromatogram of Fig. 7B. Extracted ion chromatograms with excellent signal/noise ratios were also obtained for peptides 33-mer, fragment 62–75 of the α 2-gliadin, fragment 134–153 of α -gliadin; fragment 57–

Table 2

Properties of alcohol-soluble polypeptides hydrolyzed by VSL#3 after dough incubation at 37 °C for 24 h^a

Spot ^b	Range estimated pI	Range estimated molecular mass (kDa)	Range hydrolysis factor (%)
1	6.8	51.0	54.0
2–17	6.4–9.9	46.3–49.8	85.0–97.7
18	7.1	46.0	52.5
19–20	6.5–8.7	44.0–44.5	93.2–95.6
21–22	6.6–7.1	43.0–43.2	0.0–10.0
23	6.7	42.9	91.4
24	8.0	42.6	67.0
25–26	6.0–6.3	41.8–42.5	0.0
27	6.5	41.7	87.7
28	6.4	41.6	16.0
29	6.8	41.4	95.0
30	7.0	41.3	47.5
31–34	7.6–8.5	40.9–41.2	86.2–93.2
35	8.0	40.8	20.5
36–39	8.7–9.2	40.55–40.7	81.5–93.1
40	6.4	40.5	45.6
41–43	6.0–7.2	39.9–40.4	82.0–95.2
44	6.3	39.8	24.8
45	6.5	39.7	95.0
46	6.6	39.6	44.5
47–51	6.8–9.5	38.7–39.5	87.9–93.5
52	8.0	38.6	58.2
53	9.2	38.5	90.8
54	6.6	38.3	0.0
55–67	6.3–9.6	35.8–38.2	85.7–95.7
68	6.1	35.7	24.8
69	9.6	35.6	95.0
70	9.0	35.5	44.5
71–77	8.2–9.6	33.9–35.2	82.0–95.0
78	9.5	33.6	0.0
79–81	7.1–9.4	30.3–33.0	90.5–94.8
82	9.5	29.3	88.5
83–84	9.5	26.6–28.0	94.7–96.5

^a Analyses were performed with Image Master software (Pharmacia). Four gels of independent replicates were analyzed. For spot quantification and hydrolysis factor calculation, see Materials and methods. All of the hydrolysis factors were calculated based on the average of the spot intensities of each of four gels, and standard deviations were calculated.

^b Spot designation correspond to those of the gels in Figs. 4A and B.

68 of α 9-gliadin, and fragment 31–43 of α -gliadin. Except for the very low amount (<1 ppm) of α 2-gliadin fragment 62–75, these findings indicated that gliadins non-hydrolyzed by VSL#3 and subjected to PT digestion did not generate the above epitopes at a concentration detectable by CapLC-ESI-Q-ToF-MS analysis.

3.4. F-actin reorganization and zonulin release in rat intestinal epithelial cells (IEC-6 cells)

IEC-6 cells were used to determine the effect of gliadins on intracellular F-actin. Recent studies performed on intestinal cell lines and whole intestinal tissues from normal animals suggest that these models may be valuable tools for the determination of potentially toxic or non-toxic factors in gliadin preparations [46]. Incubation of IEC-6 cells with gliadins (250 μ g/ml) extracted from 200 g of the chemically acidified dough caused a reorganization of the intracellular

F-actin which was characterized by a redistribution of F-actin to the cell sub-cortical compartment. Less significant changes were found when IEC-6 cells were exposed to a similar concentration of gliadin hydrolyzed with VSL#3 preparation (data not shown). Since the effect of gliadins on the F-actin polymerization is mediated by zonulin [47], an intestinal peptide involved in the tight junctions (TJ) regulation [17], its release from IEC-6 cells treated with gliadins was determined also. The concentration of zonulin detected in media from cells exposed to untreated gliadin extracts resulted higher (7.8 ± 0.21 ng/mg protein) as compared to the amount secreted in media of cells exposed to gliadin extracts pre-treated with VSL#3 (5.0 ± 0.19 ng/mg protein) or bovine serum albumin (BSA)-treated cells (1.0 ± 0.03 ng/mg protein).

3.5. Intestinal permeability in Balb/c small intestine

Recently, it has been demonstrated that mammal intestinal tissues exposed to gliadin react by releasing zonulin with subsequent increase in intestinal permeability [46]. Addition of gliadins extracted from the chemically acidified dough to Balb/c mice small intestinal mucosa mounted on Microsnapwells [47] led to a reduction of the tissue trans-epithelial electrical resistance (TEER) that became significant after a few minutes of incubation (Fig. 8). The presence of heat inactivated VSL#3 cells in the chemically acidified dough did not modify this reduction (data not shown). Compared to baseline, the effect of gliadins on TEER was partially inhibited by hydrolysis with VSL#3. This decreased effect on TEER was related to a decreased amount of zonulin released by the tissue exposed to VSL#3-treated gliadins as compared to untreated gliadins (Fig. 9).

3.6. Agglutination test

Gliadins were extracted from wheat flour (S. Pastore variety) and subjected to peptic–tryptic (PT) degradation to

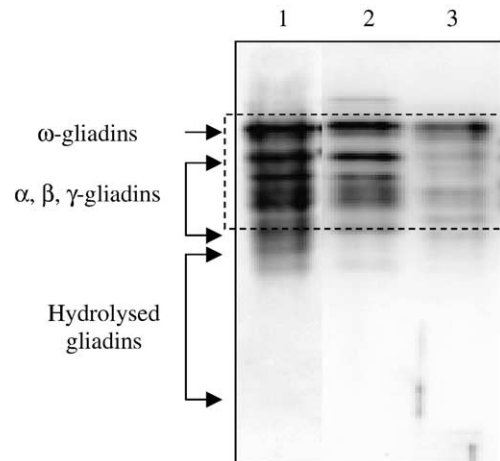


Fig. 5. Western blot/R5 analysis of European gliadin reference (1); chemically acidified dough (control) (2); dough incubated for 24 h at 37 °C with VSL#3 (3).

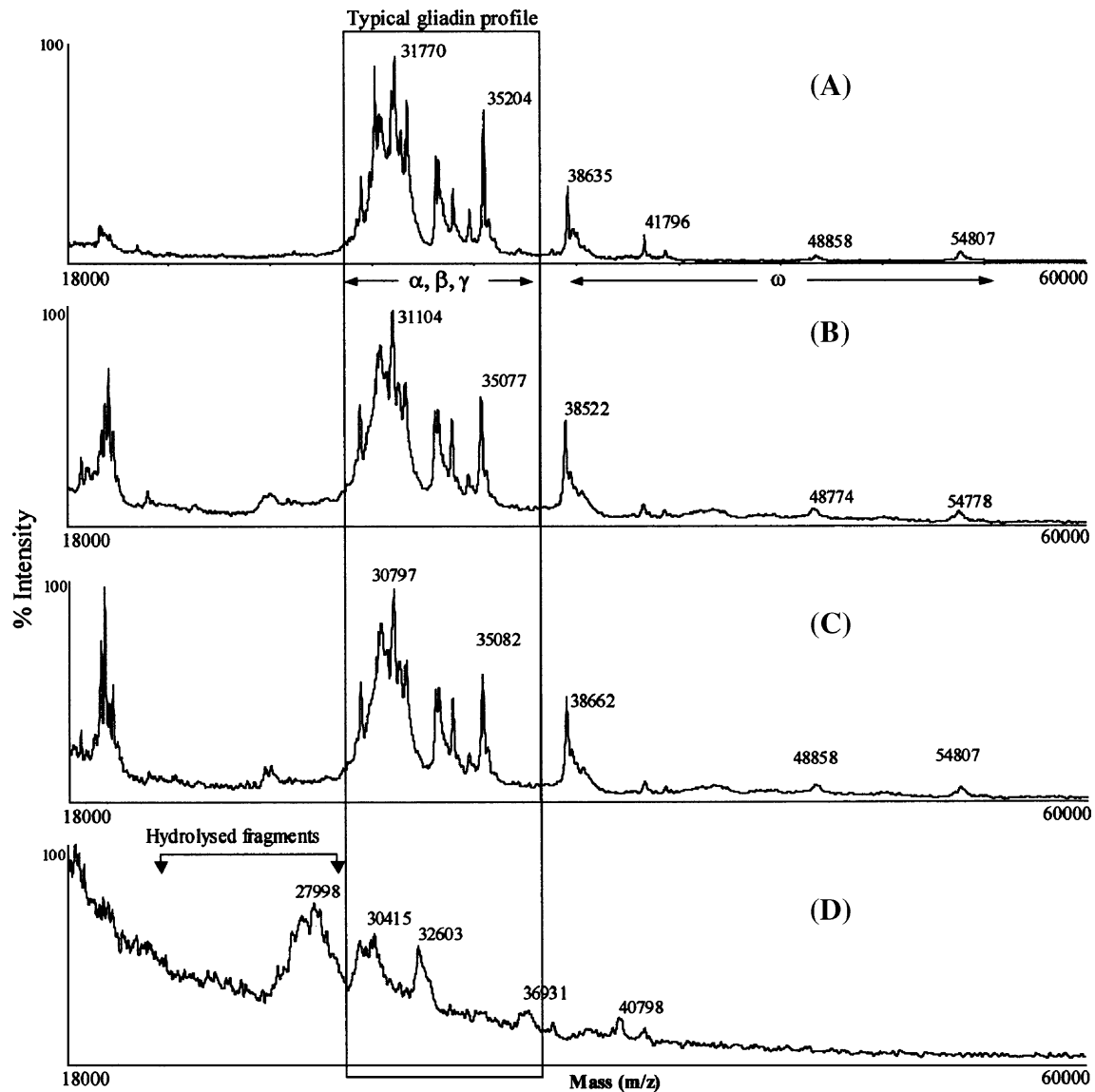


Fig. 6. MALDI-TOF mass spectra of aqueous ethanol extract of wheat gliadin: (A) European gliadin standard showing the α -, β -, γ -, and ω -gliadin ranges; (B) chemically acidified dough (control) incubated for 24 h at 37 °C; (C) chemically acidified dough with heat inactivated VSL#3 cells incubated for 24 h at 37 °C; and (D) fermented dough incubated with VSL#3 for 24 h at 37 °C. The typical α -, β -, γ -gliadin profile is displayed in a box.

mimic *in vivo* protein digestion [40]. A number of investigations have shown the ability of the wheat gliadin PT digest to prevent *in vitro* recovery of celiac mucosa biopsy specimens, thus causing disorganization of crypt architecture, reduced height, irregularities of enterocytes and crypt cells [8,39,48]. Overall, a relatively high correlation is found between the agglutination activity of cereal components against K 562(S) cells and their toxicities in clinical and *in vitro* trials on the basis of biopsy samples of intestinal mucosa from CS patients [8]. No significant evidence of cell clustering was found when the undifferentiated K562 (S) cells were not treated with the PT-digest. On the contrary, the PT-digest caused the 100% of the cell agglutination at the Minimal Agglutinating Activity (MAC) of 0.027 g/l (Fig. 10A). The agglutinated cells had a peculiar appearance, e.g., a tendency to form a continuous cell layer with high resistance to shearing and whirling

forces. Before use, the PT digest was further digested for 24 h at 37 °C with VSL#3. When assayed alone, the probiotic preparation was ineffective in causing cell agglutination (data not shown). The MAC of the PT digest treated with VSL#3 increased markedly. No agglutination was found even at a concentration of 1.89 g/l (Fig. 10B).

3.7. CD3⁺ cell infiltration of mucosa

Organ culture of the small intestine is a valuable model to study the immunological events occurring in the coeliac mucosa following contact with wheat epitopes. *In vitro* challenge systems reproduces many features of the mucosal immune response which occur in the established coeliac lesion [15]. Compared to cultivation in medium alone (negative control), celiac jejunal biopsies cultured with PT-digest of wheat proteins extracted from chemically acidified

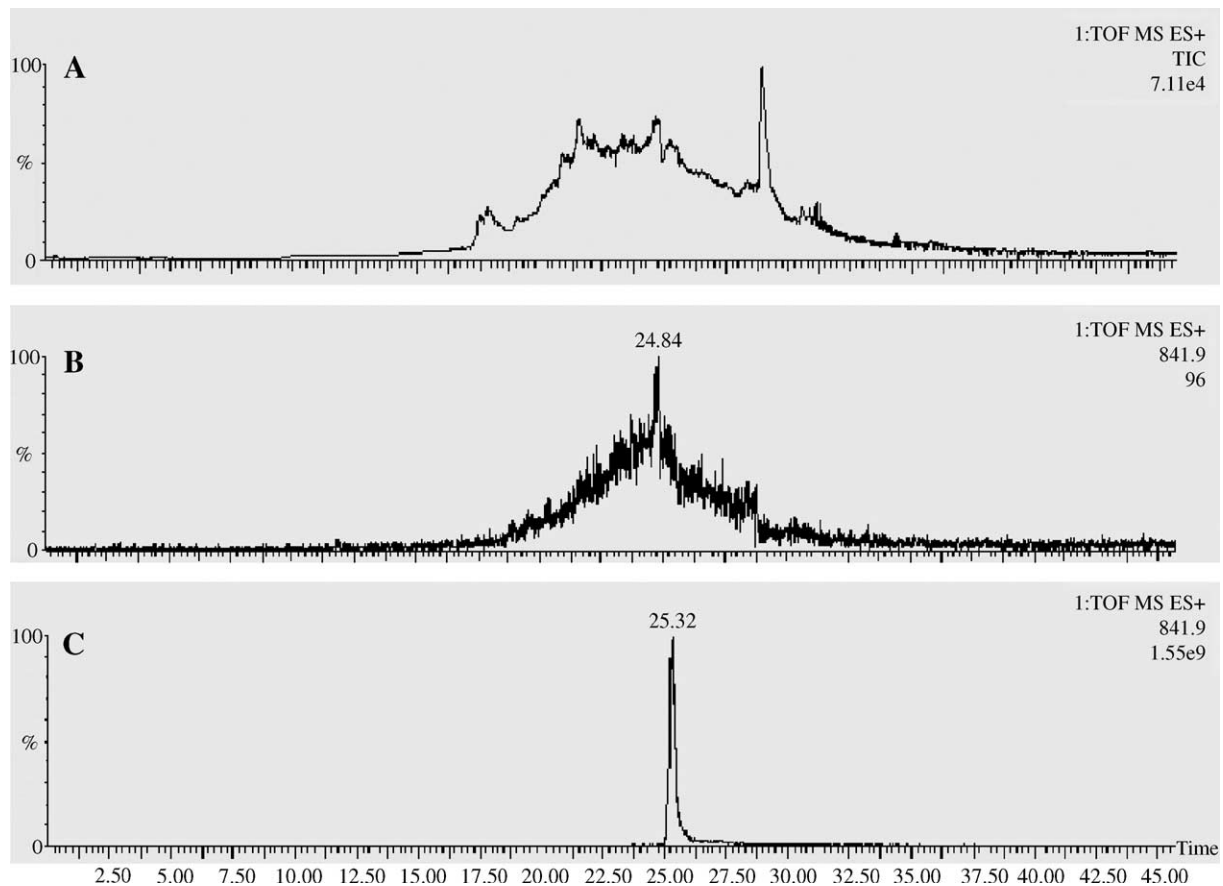


Fig. 7. TIC chromatogram obtained from PT digest of gliadins non-hydrolyzed during fermentation by VSL#3 (A); chromatogram obtained from trace (A) after extraction of the ion current for the m/z ratio 841.9, corresponding to the α 2-gliadin-derived epitope 62–75 (B); chromatogram obtained from trace of PT digest of gliadins non-hydrolyzed during fermentation by VSL#3 and spiked with synthetic analogue of α 2-gliadin-derived epitope 62–75 at a 10-ppm concentration level, after extraction of the ion current for the m/z ratio 841.9 (C).

dough (positive control) showed a significant increase of the CD3⁺ intraepithelial lymphocytes infiltrating the mucosa (Figs. 11 and 12). The PT-digest obtained from the dough

fermented with VSL#3 preparation showed a CD3⁺ response similar to that found by cultivating celiac jejunal biopsies in the medium alone.

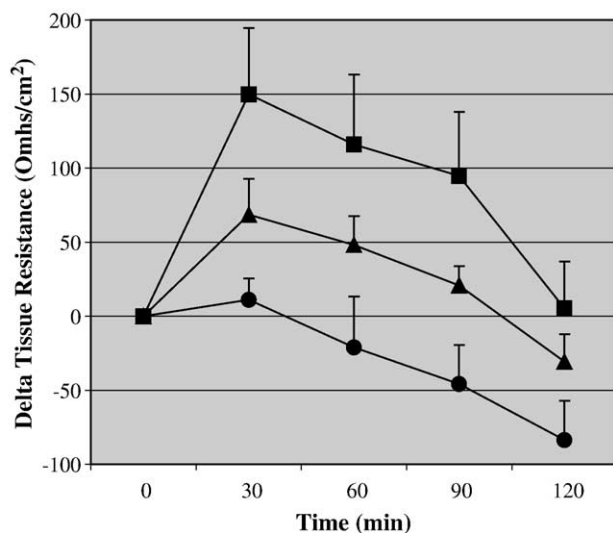


Fig. 8. Effect of wheat flour gliadin protein fraction on tissue epithelial electrical resistance (TEER) in mouse intestinal mucosa mounted in Micro-Snapwell System. Addition of non-hydrolyzed gliadins (chemically acidified dough, control) (●) and hydrolyzed gliadins by VSL#3 (▲). A normal profile of TEER was also included (■).

4. Discussion

This work was aimed at showing the capacity of probiotic VSL#3 preparation to extensively hydrolyze wheat flour gliadins as a tool for decreasing the level of toxic/immunogenic epitopes. CS is a very common disorder: the worldwide prevalence is increasing. Two major points are well established concerning the CS etiology and epidemiology: some disease-triggering epitopes (e.g., 33-mer) correspond mainly to gliadin polypeptides and most of the people have CS in the silent form. The protean clinical manifestation of the disease often causes delay in diagnosis, exposing affected individuals to possible long-term complications such as osteoporosis, infertility or cancer. Currently, new proteomic technologies, together with the development of possible animal models are suggested to be the most effective approaches to investigate the disease [7]. At the same time, food technology options to manipulate or eliminate toxic epitopes in gluten should also be pursued, especially by using microorganisms and related enzymes.

Shan et al. [11] showed that the 33-mer peptide could be hydrolyzed by exposure to a prolyl-endopeptidase of *Flavo-*

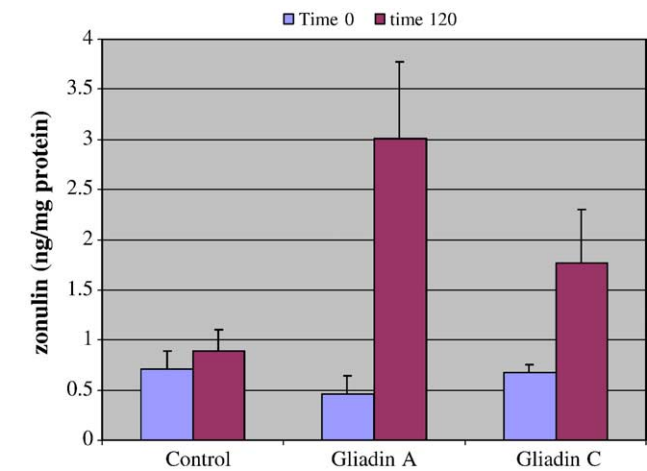


Fig. 9. Effect of wheat flour gliadin protein fraction on zonulin release from mouse intestinal mucosa. Non-hydrolyzed prolamins (chemically acidified dough, control) (Gliadin A) and hydrolyzed gliadins by VSL#3 (Gliadin C). A negative control using bovine serum albumin on zonulin release was also included (control).

bacterium meningosepticum suggesting a strategy for an oral peptidase supplement therapy. Nevertheless, Matysiak-Budnik et al. [49] showed that the hydrolysis of the 33-mer by the

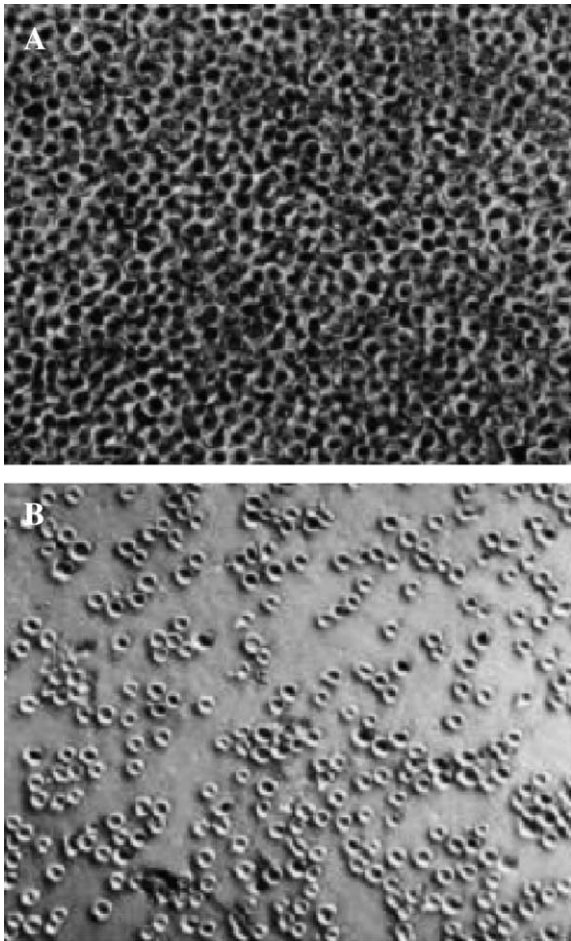


Fig. 10. Agglutination test on K 562 (S) cells. (A) Cells treated with the PT-digest (control) at a concentration of 0.027 g/l; (B) Cells treated with the PT-digest, hydrolyzed with VSL#3 for 24 h, at a concentration of 1.89 g/l.

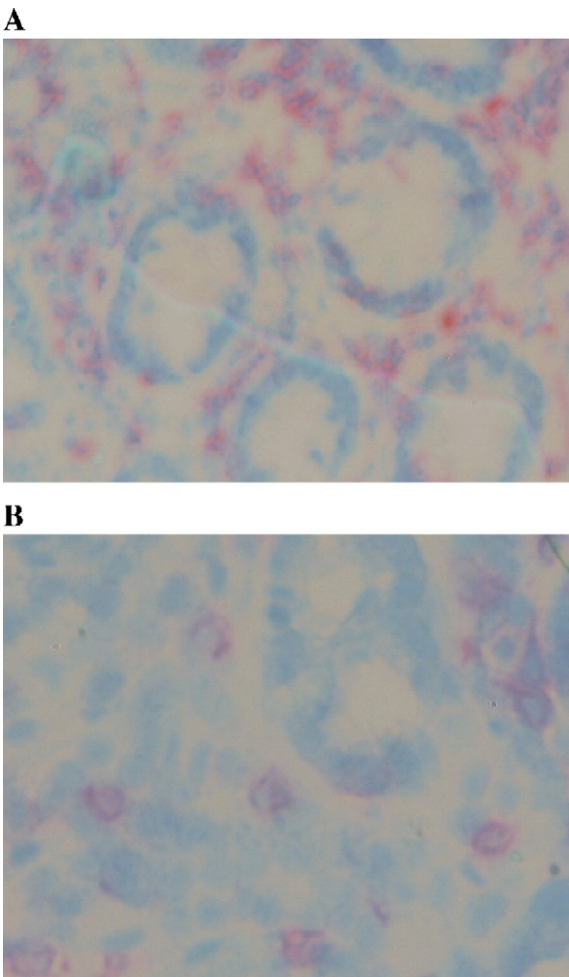


Fig. 11. Immunofluorescence staining of the jejunal mucosa from a coeliac patient after in vitro incubation with PT-digests from wheat proteins extracted from doughs chemically acidified (A) or fermented by VSL#3 (B). CD3⁺ intraepithelial lymphocytes are stained in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

prolyl-endopeptidase of *F. meningosepticum* in CS patients was not complete and led to the release of potentially immunogenic peptides, which after crossing the intestinal mucosa contacted

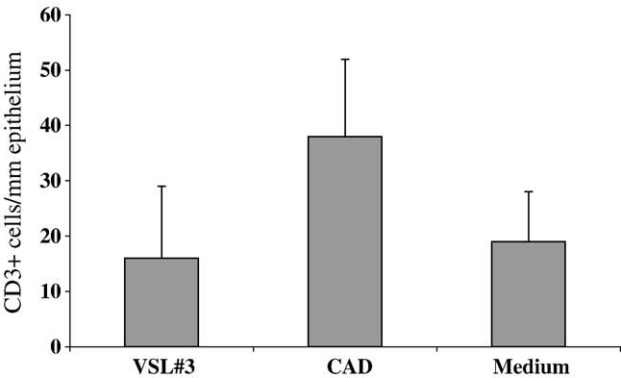


Fig. 12. Organ culture studies in coeliac patients: intraepithelial-CD3⁺ cells. Bars indicate the mean and SD in biopsies cultured with PT-digests from wheat proteins extracted from doughs chemically acidified (CAD) or fermented with VSL#3 (VSL#3). A control with RPMI medium alone was reported as the negative control (Medium).

the immune system. Piper et al., [35] have addressed this apparent controversy [11, 49] by performing dose-dependent intestinal perfusion experiments in rats. Prolyl-endopeptidase supplementation at more pharmacologically relevant enzyme doses (e.g., 190 mU/ml) substantially reduced the concentration of the 33-mer, confirming that this enzyme could be able to treat CS by decreasing or eliminating immunostimulatory peptides from the intestine [35,50]. Previously, a pool of sourdough lactic acid bacteria was shown to possess a pattern of specialized peptidases capable of hydrolyzing all the different bonds that potentially include the imino acid proline [22]. Overall, epitopes involved in CS contain a large proportion of Pro residues. Proline is unique among the 20 amino acids because of its cyclic structure. This specific conformation imposes many restriction on the structural aspects of peptides and proteins, making them extremely resistant to hydrolysis. To adequately deal with such peptides, a pattern of specific peptidases is necessary in order to hydrolyze all the peptide bonds in which a proline residue is present as potential substrate at different positions [43]. The hydrolyzing activities responsible for the degradation of CS epitopes are not widespread in sourdough lactic acid bacteria and, in general, it is very rare that a unique microbial strain may possess all these necessary enzymes [43,51]. Long-time fermentation in the presence of a mixture of selected lactic acid bacteria seemed to be indispensable to affect epitopes integrity and toxicity [22]. Industrial processing for cereal foods may influence the native structure and distribution of the above epitopes and/or of their precursors. The same baked goods (e.g., bread) may be manufactured under different technological conditions: long-time fermentation with a mixture of proteolytic lactic acid bacteria (sourdough bread) or short-time fermentation with chemical and/or baker's yeast (baker's yeast bread). Our experiments showed that enzyme activities specific for Pro-rich peptides and general aminopeptidases which determine the hydrolysis of gliadin epitopes are largely distributed in the probiotic VSL#3 preparation, while other commercially available probiotics seem to lack the same capacity to degrade gliadin polypeptides. The capability of VSL#3 to hydrolyze gliadins was lost when the individual strains which composed VSL#3 were tested, suggesting that no single probiotic strain contains the entire portfolio of peptidases necessary to degrade gliadins. The hydrolyzing activity of VSL#3 towards synthetic peptides was maintained relatively constant under simulated gastro-intestinal conditions. After 24 h of incubation at 37 °C, 10^9 cells/g of VSL#3 hydrolyzed totally the 33-mer epitope (750 ppm). Besides, considerable amount (150 ppm) of 33-mer were hydrolyzed totally after 4 h of treatment by VSL#3. Preliminary results on the identification of 33-mer hydrolysis end-products showed that only dipeptides and free amino acids remained after 24 h of digestion (data not shown). Notwithstanding the heterogeneity of T-cell epitope in gluten, the 33-mer is probably the most important for the following properties: (i) it remains intact despite prolonged exposure to gastric and pancreatic proteases; (ii) it shows a hydrolysis less than 20% over 20 h of incubation with small brush border membrane enzymes; and (iii) it remains intact for a long time in

the small intestine and even at low concentration acts as potential antigen for T-cell proliferation [11].

Hydrolysis of gliadin polypeptides during dough fermentation by VSL#3 was determined by several complementary techniques routinely used to assay gluten-free products. Compared to chemically acidified dough used as the control and to European gliadin standards, 2DE, immunological (R5 antibody) and mass spectrometry MALDI-TOF analyses showed univocally an extensive degradation of gliadins by VSL#3. Gliadins non-hydrolyzed during fermentation by VSL#3 were used to produce the PT-digest and derived epitopes were searched by CapLC-ESI-Q-ToF-MS analysis. Among the major epitopes reported as potent inducers of CS [13], only the α 2-gliadin-derived epitope 62–75 was found at a concentration in the sub-ppm range. This result might indicate that gliadins which persisted after VSL#3 treatment had very low or absent toxicity.

The lack of animal models of celiac disease and the ethical constraints to use human intestinal tissues, highly limit the capability to screen protein or peptide epitopes possible toxic to CS patients in a high throughput manner. Three different *in vitro* systems were used to estimate the toxicity of the wheat dough fermented by VSL#3 preparation. The use of intestinal cell lines from normal animals as a tool to investigate CS [46] was recently corroborated by studies performed *in vivo* on intestinal tissues obtained from both celiac patients in remission and healthy controls [52]. It was suggested that normal intestinal tissues react to exposure to gliadin with a transient secretion of zonulin [46], while in CS gut tissues the zonulin system is up-regulated at baseline and is abnormally activated when stimulated by gliadin exposure [52]. The zonulin-dependent sustained increase in intestinal permeability may cause passage of macromolecules (such as gliadins) from the intestinal lumen to the lamina propria. Gliadins are deaminated by tissue transglutaminase, located at the lamina propria and brush border of enterocytes [53,54], and then recognized by HLA-DQ2/DQ8 bearing antigen presenting cells, triggering the onset of the CS autoimmune reaction in genetically susceptible subjects. Our results indicated that gliadins treated with probiotic VSL#3 decreased the activation of the zonulin release, cytoskeleton reorganization and intestinal permeability with respect to non-hydrolyzed wheat gliadins. As recently reported, a mixture of probiotic bacteria enhanced T84 tight junctions (TJ) and prevents *Salmonella* Dublin-induced TJ dissolution and zonulin-1 redistribution [55,56]. Also, a factor present in the bacterial-conditioned media enhanced the function of TJ. Studies on intestinal T cell clones from the mucosa of CS patients have led to the identification of immunogenic gliadin epitopes. CD3⁺ intraepithelial lymphocytes increased after challenge of small intestine mucosa from CS patients with gluten [14,57]. Indeed, small intestine T cell activation by anti-CD3⁺ antibodies in explants of human fetal gut induced a significant increase in intraepithelial lymphocytes, suggesting that it might be a consequence of lamina propria T cell activation [58]. Wheat proteins were extracted from doughs and subjected to PT digestion. Compared to PT-digest from chemically acidified

dough, celiac jejunal biopsies exposed to the PT-digest from the dough fermented by VSL#3 did not show an increase of the infiltration of CD3⁺ intraepithelial lymphocytes. Agglutination test on K 562 (S) cells also showed a decreased toxicity of gliadins digested by VSL#3 preparation.

VSL#3 preparation was shown to have the capacity to largely colonize the intestine [23–25]. Overall, some other probiotic strains, belonging to the same species of those included in the VSL#3, had the capacity to colonize the human stomach and duodenum, where the hydrolysis of gliadin epitopes may be relevant for decreasing the abnormal secretion of zonulin and the initial step of immune response to gliadin [59–61]. It seemed that, previous exposure to low pH in the stomach might cause a transitory rise of bile resistance, increasing the survival of probiotics at high bile concentration in the duodenum [61]. The capacities to maintain peptidase activities under gastro-intestinal conditions and to hydrolyze 150 ppm of 33-mer in a rather short time (4 h) may deserve further investigation on the use of VSL#3 as probiotic preparation for CS therapy. Nevertheless, the most promising use of this preparation seemed to be as a starter for sourdough fermentation.

The compliance to a gluten-free diet is an extremely challenging task, given the problems related to cross-contamination, lack of clear food labeling policies, and poor information on minimal toxic amounts of gliadins in CS subjects. Therefore, a biotechnology like VSL#3 treatment that would eliminate any traces of toxic peptides in processed foods will minimize the long-term risks and improve the quality life of a multitude of individuals affected by CS worldwide.

References

- [1] A. Fasano, C. Catassi, Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum, *Gastroenterology* 120 (2001) 636–651.
- [2] C. Catassi, E. Fagiani, I.M. Ratsch, G.V. Coppa, P.L. Giorni, R. Pierdomenico, S. Alessandrini, G. Iwanejko, F. Viola, R. Lazzari, M. Vallini, G. Guariso, M. Plebani, F. Cataldo, G. Traverso, C. Ughi, G. Chiaravallotti, M. Baldassarre, P. Scarcella, F. Baschetto, L. Ceglie, A. Valenti, P. Paolucci, M. Caradonna, E. Bravi, A. Ventura, The coeliac iceberg in Italy: a multicentre antigliadin antibodies screening for coeliac disease in school-age subjects, *Acta Paediatr.* 412 (1996) 29–35.
- [3] L. Gandolfi, R. Pratesi, J.C. Cordoba, P.L. Tauil, M. Gasparin, C. Catassi, Prevalence of celiac disease among blood donors in Brazil, *Am. J. Gastroenterol.* 95 (2000) 689–692.
- [4] A. Fasano, I. Berti, T. Gerarduzzi, T. Not, R.B. Colletti, S. Drago, Y. Elitsur, P.H. Green, S. Guandalini, I.D. Hill, M. Pietzak, A. Ventura, M. Thorpe, D. Kryszak, F. Fornaroli, S.S. Wasserman, J.A. Murray, K. Horvath, Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study, *Arch. Intern. Med.* 163 (2003) 286–292.
- [5] W.F. Balestrieri, The Consequences of obesity and recognition of celiac disease—Two growing concerns worldwide, *Medscape Gastroenterol.* 6 (2004) 1.
- [6] H.R.P. Green, B. Jabri, Celiac disease, *Lancet* 362 (2003) 383–391.
- [7] G. Robbins, P.D. Howdle, Advances in celiac disease, *Curr. Opin. Gastroenterol.* 20 (2004) 95–103.
- [8] M. Silano, M. De Vincenzi, Bioactive antinutritional peptides derived from cereal prolamines: a review, *Nahrung* 43 (1999) 175–184.
- [9] J.S.R. Jennings, P.D. Howdle, New developments in celiac disease, *Curr. Opin. Gastroenterol.* 19 (2003) 118–129.
- [10] A. Picarelli, L. Di Tola, M. Sabbatella, R. Greco, M. Silano, M. De Vincenzi, 31–43 amino acid sequence of the α -gliadin induces anti-endomysial antibody production during in vitro challenge, *Scand. J. Gastroenterol.* 34 (1999) 1099–1102.
- [11] L. Shan, O. Molberg, I. Parrot, F. Hausch, F. Filiz, G.M. Gray, L.M. Sollid, C. Khosla, Structural basis for gluten intolerance in celiac sprue, *Science* 297 (2002) 2275–2279.
- [12] M. Aleanzi, A.M. Demonte, C. Esper, S. Garcilazo, M. Waggenger, Celiac disease: antibody recognition against native and selectively deaminated gliadin peptides, *Clin. Chem.* 47 (2001) 2023–2028.
- [13] H. Arentz-Hansen, R. Korner, O. Molberg, H. Quarsten, W. Vader, Y.M. Kooy, K.E. Lundin, F. Koning, P. Roepstorff, L.M. Sollid, S.N. McAdam, The intestinal T cell response to α -gliadin in adult celiac disease is focused on a single deaminated glutamine targeted by tissue transglutaminase, *J. Exp. Med.* 191 (2000) 603–612.
- [14] R. Troncone, G. Mazzarella, N. Leone, M. Mayer, M. De Vincenzi, L. Greco, S. Auricchio, Gliadin activates mucosal cell mediated immunity in cultured rectal mucosa from coeliac patients and a subset of their siblings, *Gut* 43 (1998) 484–489.
- [15] L. Maiuri, A. Picarelli, M. Boirivant, Definition of initial immunologic modifications upon in vitro challenge in the small intestine of celiac patients, *Gastroenterology* 110 (1996) 1368–1378.
- [16] L. Maiuri, C. Ciacci, I. Ricciardelli, L. Vacca, V. Raja, S. Auricchio, J. Picard, M. Osman, S. Quarantino, M. Londei, Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease, *Lancet* 362 (2003) 30–37.
- [17] W. Wang, S. Uzzau, S.E. Goldblum, A. Fasano, Human zonulin, a potential modulator of intestinal tight junctions, *J. Cell. Sci.* 113 (2000) 4435–4440.
- [18] A. Fasano, T. Not, W. Wang, S. Uzzau, I. Berti, A. Tommasini, S.E. Goldblum, Zonulin, a newly discovered modulator of intestinal permeability, and its expression in celiac disease, *Lancet* 335 (2000) 1518–1519.
- [19] H. Lu, C. Shen, R.C. Brunham, *Chlamydia trachomatis* infection of epithelial cells induces the activation of caspase-1 and release of mature IL-18, *J. Immunol.* 165 (2000) 1463–1469.
- [20] A. Fasano, Prevalence and genetics, AGA Clinical Symposium - Celiac Disease Clinical Symposium. Program and abstracts of Digestive Disease Week 2004. May 15–20, 2004. New Orleans, Louisiana, Sp4182004.
- [21] M. Gobetti, The sourdough microflora: interactions between lactic acid bacteria and yeasts, *Trends Food Sci. Technol.* 9 (1998) 267–274.
- [22] R. Di Cagno, M. De Angelis, S. Auricchio, L. Greco, C. Clarke, M. De Vincenzi, C. Giovannini, M. D'Archivio, F. Landolfo, G. Parrilli, F. Minervini, E. Arendt, M. Gobetti, Sourdough bread made from wheat and nontoxic flours and started with selected lactobacilli is tolerated in celiac sprue patients, *Appl. Environ. Microbiol.* 70 (2004) 1088–1096.
- [23] I.P. Kaur, K. Chopra, A. Saini, Probiotics: potential pharmaceutical applications, *Eur. J. Pharm. Sci.* 15 (2002) 1–9.
- [24] P. Gionchetti, M. Campieri, Probiotics in inflammatory bowel disease: new insight to pathogenesis or a possible therapeutic alternative? *Gastroenterology* 116 (1999) 1246–1249.
- [25] P. Gionchetti, F. Rizzello, A. Venturi, P. Brigidi, D. Matteuzzi, G. Bazzocchi, G. Poggioli, M. Miglioli, M. Campieri, Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial, *Gastroenterology* 119 (2000) 305–309.
- [26] T.B. Osborne, The proteins of the wheat kernel. 1907. Carnegie Institute of Washington publication 84. Judd and Detweiler, Washington, D.C.
- [27] W. Weiss, C. Volgelmeier, A. Gorg, Electrophoretic characterization of wheat grain allergens from different cultivars involved in bakers' asthma, *Electrophoresis* 14 (1993) 805–816.
- [28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [29] B. Bjellqvist, J. Hughes, C. Pasquali, N. Paquet, F. Ravier, J.C. Sanchez, S. Frutiger, G. Hughes, V. Pallini, D.F. Hochstrasser, P. Tosi, The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences, *Electrophoresis* 14 (1993) 1023–1031.

- [30] D.F. Hochstrasser, M.G. Harrington, A.C. Hochstrasser, M.J. Miller, C.R. Merrill, Methods for increasing the resolution of two dimensional protein electrophoresis, *Anal. Biochem.* 173 (1998) 424–435.
- [31] L. Bini, B. Magi, B. Marzocchi, F. Arcuri, S. Tripodi, M. Cintorino, J.C. Sanchez, S. Frutiger, D. Hochstrasser, Protein expression profiles in human breast ductal carcinoma and histologically normal tissue, *Electrophoresis* 18 (1997) 2832–2841.
- [32] D. Appel, D.F. Hochstrasser, Computer analysis of 2-D images, p. 431–443, in: A.J. Link (Ed.), *Proteome Analysis Protocols*, Methods in Molecular Biology, vol. 112-D, Humana Press, Totowa, NJ, USA, 1999.
- [33] M. Gobbetti, E. Smacchi, A. Corsetti, The proteolytic system of *Lactobacillus sanfrancisco* CB1: purification and characterization of a proteinase, a dipeptidase, and an aminopeptidase, *Appl. Environ. Microbiol.* 62 (1996) 3220–3226.
- [34] H. Lineweaver, D. Burk, The determination of enzyme dissociation constants, *J. Am. Chem. Soc.* 56 (1934) 658–666.
- [35] J.L. Piper, G.M. Gray, C. Khosla, Effect of prolyl endopeptidase on digestive-resistant gliadin peptides in vivo, *J. Pharmacol. Exp. Ther.* 311 (2004) 213–219.
- [36] G. Pasini, M. Simonato, M. Giannattasio, A.D.B. Peruffo, A. Curioni, Modification of wheat flour proteins during in vivo digestion of bread dough, crumb and crust: an electrophoretic and immunological study, *J. Agric. Food Chem.* 49 (2001) 2254–2259.
- [37] I. Valdés, E. Garcia, M. Lorente, E. Méndez, Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol, *Eur. J. Gastroenterol. Hepatol.* 15 (2003) 465–474.
- [38] A. Hernando, I. Valdes, E. Mendez, New strategy for the determination of gliadins in maize-or-based foods matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: fractionation of gliadins from maize or rice prolamins by acidic treatment, *J. Mass Spectrom.* 38 (2003) 862–871.
- [39] S. Auricchio, G. De Ritis, M. De Vincenzi, P. Occorsio, V. Silano, Effect of gliadin peptides prepared from hexaploid and tetraploid wheat on cultures of intestine from rat fetuses and coeliac children, *Pediatr. Res.* 16 (1982) 1004–1010.
- [40] R. Di Cagno, M. De Angelis, P. Lavermicocca, M. De Vincenzi, C. Giovannini, M. Faccia, M. Gobbetti, Proteolysis by sourdough lactic acid bacteria: effects on wheat flour protein fractions and gliadin peptides involved in human cereal intolerance, *Appl. Environ. Microbiol.* 68 (2002) 623–633.
- [41] R. Auricchio, F. Paparo, M. Maglio, A. Franzese, F. Lombardi, G. Valerio, G. Nardone, S. Percopo, L. Greco, R. Troncone, In vitro-derived intestinal immune response gliadin in type 1 diabetes, *Diabetes* 54 (2004) 1680–1683.
- [42] H. Wieser, Relation between gliadin structure and coeliac toxicity, *Acta Paediatr.* 412 (1996) 3–9.
- [43] D.F. Cunningham, B. O'Connor, Proline specific peptidases, *Biochim. Biophys. Acta* 1343 (1997) 160–186.
- [44] A.A. Osman, H.H. Uhlig, I. Valdés, M. Amin, E. Méndez, T. Mothes, Monoclonal antibody recognizing a potential celiac toxic repetitive pentapeptide epitope in gliadins, *Eur. J. Gastroenterol. Hepatol.* 13 (2001) 1189–1193.
- [45] P. Shewry, A. Tatham, D. Kasada, Cereal's proteins and celiac disease, in: M. Marsh (Ed.), *Coeliac Disease*, Blackwell Scientific Publications, Oxford, 1992, pp. 305–348.
- [46] M.G. Clemente, S. De Virgiliis, J.S. Kang, R. Macatagney, M.P. Musu, M.R. Di Pierro, S. Drago, M. Congia, A. Fasano, Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function, *Gut* 52 (2003) 218–223.
- [47] R.E. Asmar, P. Panigrahi, P. Bamford, I. Berti, I. Not, G.V. Coppa, C. Catassi, A. Fasano, Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure, *Gastroenterology* 123 (2002) 1607–1615.
- [48] Z.M. Falchuk, R.L. Gebhard, C. Sessoms, W. Strober, An in vitro model of gluten sensitive enteropathy: effect of gliadin on intestinal epithelial cells of patients with gluten sensitive enteropathy in organ culture, *J. Clin. Invest.* 53 (1974) 487–500.
- [49] T. Matysiak-Budnik, C. Candalh, C. Dugave, Is prolyl-endopeptidase effective in the detoxification of gliadin peptides in celiac disease? *J. Pediatr. Gastroenterol. Nutr.* 39 (2004) s53.
- [50] T. Marti, O. Molberg, Q. Li, G.M. Gray, C. Khosla, L.M. Sollid, Prolyl endopeptidase-mediated destruction of T cell epitopes in whole gluten: chemical and immunological characterization, *J. Pharmacol. Exp. Ther.* 312 (2005) 19–26.
- [51] E.R.S. Kunji, I. Mierau, A. Hagting, B. Poolman, W.N. Konings, The proteolytic systems of lactic acid bacteria, *Antonie Van Leeuwenhoek* 70 (1996) 187–221.
- [52] S. Drago, R.E. Asmar, C. D'Agate, G. Iacono, M. Di Pierro, C. Catassi, A. Fasano, Gliadin induces increased intestinal permeability, zonulin release, and occludin down-regulation in an ex-vivo human intestinal model of celiac disease, *Gastroenterology* 124 (2003) 658.
- [53] G. D'Argenio, I. Sorrentini, C. Ciacci, G. Mazzacca, Transglutaminase activity along the rat small bowel and cellular location, *Enzyme* 39 (1988) 227–230.
- [54] C. Esposito, F. Paparo, I. Caputo, R. Porta, V.M. Salvati, G. Mazzarella, S. Auricchio, R. Troncone, Expression and enzymatic activity of small intestinal tissue transglutaminase in celiac disease, *Am. J. Gastroenterol.* 98 (2003) 1813–1820.
- [55] J.M. Otte, D.K. Podolsky, Functional modulation of enterocytes by gram-positive gram-negative microorganisms, *Am. J. Physiol.: Gastrointest. Liver. Physiol.* 286 (2004) G613–G626.
- [56] Y. Fang, D.B. Polka, Commensal bacteria in the gut: learning who our friends are, *Curr. Opin. Gastroenterol.* 20 (2004) 565–571.
- [57] G. Mazzarella, M. Maglio, F. Paparo, G. Nardone, R. Stefanile, L. Greco, Y. Van de Wal, Y. Kooi, F. Koning, S. Auricchio, R. Troncone, An immunodominant DQ8 restricted gliadin peptide activates small intestinal immune response in in vitro cultured mucosa from HLA-DQ8 positive but not HLA-DQ8 negative celiac patients, *Gut* 52 (2005) 57–62.
- [58] T. Monk, J.O. Spencer, N. Cerf-Bensussan, T.T. MacDonald, Stimulation of mucosal T cells in situ with anti CD3 antibody location of the activated T cells and their distribution within the mucosal micro-environment, *Clin. Exp. Immunol.* 74 (1988) 216–222.
- [59] N. Valeur, P. Engel, N. Carbajal, E. Connolly, K. Ladefoged, Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract, *Appl. Environ. Microbiol.* 70 (2004) 1176–1181.
- [60] Y.K. Lee, P.S. Ho, C.S. Low, H. Arvilommi, S. Salminen, Permanent colonization by *Lactobacillus casei* is hindered by the low rate of cell division in mouse gut, *Appl. Environ. Microbiol.* 70 (2004) 670–674.
- [61] L. Noriega, M. Gueimonde, B. Sánchez, A. Margolles, C.G. de los Reyes-Gavilán, Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross-resistance to bile salts in *Bifidobacterium*, *Int. J. Food. Microbiol.* 94 (2004) 79–86.