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Sourdough lactobacilli and celiac disease

Marco Gobbetti*, Carlo Giuseppe Rizzello, Raffaella Di Cagno, Maria De Angelis

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari, Via G. Amendola 165/a, 70125 Bari, Italy

Abstract

Celiac disease (CD) is one of the most common food intolerance. The only effective treatment for CD is a strict adherence to a gluten-free diet throughout the patient's lifetime. Gluten-free products are not widely available and are usually more expensive than their gluten-containing counterparts. There is, therefore, an urgent need to develop safe and effective therapeutic alternatives, to develop high-quality gluten-free products and to investigate the potential of the bread making biotechnology following ancient protocols which include long-time fermentation by selected sourdough lactic acid bacteria.

This review describes the most relevant results related to biotechnologies that use selected sourdough lactic acid bacteria and probiotics as starters for sourdough fermentation to investigate their potential to decrease the risk of gluten contamination in gluten-free products. As shown by studies in vitro on celiac intestinal tissue and in vivo on CD patients, the bacterial proteolytic activity is rather promising not only as currently demonstrated for eliminating traces of contaminant gluten but probably also in perspective for the manufacture of tolerated baked goods.

Keywords: Celiac disease; Sourdough lactobacilli; Proteolytic activity; Sourdough bread and pasta

1. Introduction

Celiac disease (CD), also known as celiac sprue or gluten-sensitive enteropathy, is a food hypersensitivity disorder caused by an inflammatory response to wheat gluten and similar proteins of barley and rye (Maki et al., 2003). Genes encoding HLA-DQ2 and HLA-DQ8 molecules are the single most important predisposing genetic factor. However, although these polymorphisms are necessary, they are not sufficient for disease development. HLA-DQ2 and HLA-DQ8 predispose to disease development by preferential presentation to mucosal $CD4^+$ T cells of Pro-rich gluten peptides that have undergone deamidation by the enzyme tissue transglutaminase. Fewer details are known about the effector mechanisms that lead to the development of the typical celiac lesion (villous atrophy, crypt hyperplasia and infiltration of inflammatory cells), but, once activated, gluten-reactive $CD4^+$ T cells produce cytokines and are likely to control the inflammatory reactions that produce celiac lesions (Sollid and Khosla, 2005).

Reports of CD date back to the first century A.D. (Adams, 1856), but it was not until 1888 that Samuel Gee gave the classical description of the disease (Gee, 1888), and only in the 1950s, Dicke demonstrated that removal of wheat from diet alleviated symptoms and signs of CD (Van Berge-Henegouwen and Mulder, 1993). Currently, CD prevalence has been estimated to be 1 in ca. 100 people worldwide (Hamer, 2005; Sollid and Khosla, 2005). Such a rate establishes CD as one of the most common food intolerance (Fasano and Catassi, 2001). The only effective treatment for CD is a strict adherence to a gluten-free diet throughout the patient's lifetime, which in time results in clinical and mucosal recovery. Gluten is, however, a common and in many countries unlabeled ingredient in the human diet, presenting a big challenge for CD patients. Unsurprisingly, dietary compliance is, at the best, imperfect in a large fraction of patients, especially adolescents and adults. There is therefore an urgent need to develop safe and effective alternatives.

Beyond genetic predisposition, several environmental factors influenced CD prevalence. Recent epidemiological

*Corresponding author. Tel.: +39 080 5442949; fax: +39 080 5442911.
E-mail address: gobbetti@agr.uniba.it (M. Gobbetti).

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studies show that, besides being frequently found in countries where individuals are mostly of European origin, CD is a common disorder in many areas of the developing world where agriculture started 10,000 years ago (Fasano, 2004). More recently, cereal food technology has changed dramatically by influencing dietary habitudes of entire population previously naïve to gluten exposure. Baked cereal goods are currently manufactured by highly 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 (Gobbetti, 1998).

After a brief description of the new and most relevant therapeutic opportunities, and of foods allowed in the gluten-free diet, this review focuses on the recent biotechnologies that use selected sourdough lactic acid bacteria and probiotics to decrease the risk of gluten contamination.

2. Therapy and gluten-free diet

Recent advances have improved the understanding of the molecular basis of the CD disorder and several targets have been developed for new treatments. Several therapeutic options are currently under investigation with various promising potentialities: (i) supplying of microbial prolyl-endopeptidase which complements the gastrointestinal proteolytic processes; (ii) supplying of tissue transglutaminase inhibitors (e.g. cystamine) to block the proliferative capacity of gluten-responsive T cells; (iii) blocking the binding sites of HLA-DQ2 to prevent the presentation of disease-inducing gluten peptides; and (iv) supplying of therapeutic agents used for other chronic inflammatory diseases such as cytokine therapy and selective adhesion molecule inhibition (Sollid and Khosla, 2005).

Nevertheless, at present, the only available treatment for CD is a strict exclusion diet. Food disallowed in a gluten-free diet include: (i) breads, cereals or other foods made with wheat, rye, barley, triticale, spelt, oat and kamut flour or ingredients and by-products from those grains; (ii) processed foods containing wheat, barley and rye and gluten-derivatives as thickeners and fillers, e.g. hot dogs, salad dressing, canned soups/dried soup mixes, processed cheeses, cream sauces; and (iii) medications that use gluten or similar proteins as pill or tablet binders (Accomando and Cataldo, 2004). The Codex Standard for gluten-free foods was adopted by the Codex Alimentarius Commission of the World Health Organization (WHO) and by the Food and Agricultural Organization (FAO) in 1976. In 1981 and 2000 draft revised standards stated that so-called gluten-free foods are described as: (i) consisting of, or made only from ingredients which do not contain any prolamins from wheat or all *Triticum* species such as spelt, kamut or durum wheat, rye, barley, oats or their crossbred varieties with a gluten level not exceeding 20 ppm; (ii) consisting of ingredients from wheat, rye, barley, oat, spelt and their crossbred varieties, which have been rendered gluten-free, with a gluten level not exceeding 200 ppm; and (iii) any mixture of two ingredients as in (i) and (ii) mentioned with a level not exceeding 200 ppm (Gallagher et al., 2004).

Development of grains that have low or no content of immunotoxic sequences, but with reasonable baking quality, is also investigated by breeding of ancient varieties or transgenic technologies (Vader et al., 2003; Molberg et al., 2005; Sollid and Khosla, 2005). Using RNA-interference methodology it is possible to silence parts of the wheat genes and this way may lower the content of possible toxic peptides. Nevertheless, the large degree of sequence homology between the cereal protein family, and considering the fact that cereals like wheat are hexaploid, complete elimination should be considered unlikely. Research and development are currently focused in improving mouth-feel, flavour and rheology of gluten-free products. Gluten is often termed the structural protein for bread making; the properties of gluten become apparent when flour is hydrated, giving an extensible dough, with good gas holding properties, and a good crumb structure in baked bread. The absence of gluten often results in a liquid batter rather than a dough pre baking, and can result in a baked bread with a crumbling texture, poor colour and other post-baking quality defects. Also the preparation of gluten-free pasta is difficult, as gluten contributes to a strong protein network that prevents dissolution of pasta during cooking (Gallagher et al., 2004). In recent years there has been extensive work in using several ingredients to improve the structure, mouth-feel, acceptability and shelf-life of gluten-free bakery products. Dairy products, and many gum types including hydroxypropyl-methylcellulose (HPMC), locust bean gum, guar gum, carageenan, xanthan gum, and agar, may be used in gluten-free bread formulas to increase volume expansion, water absorption and, therefore, enhance the handling properties of the batter (Kang et al., 1997; Gan et al., 2001; Kenny et al., 2001). Rice, corn, soya, millet, buckwheat and potato starches with different fat sources were used in the formulation of gluten-free biscuits showing a comparable quality to wheat biscuits (Arendt et al., 2002).

In the above context, the biotechnology for bread making should also be exploited. During the last 5 years the authors' laboratory has developed a strategy which resembles the ancient tradition of using the sourdough for bread making in order to investigate its effect on CD.

3. Hydrolysis of Pro-rich gluten polypeptides

Overall, the lack of animal models for CD and the ethical constraints on the use human intestinal tissues, limit the capability of screening, with a high throughput, protein

or peptide epitopes potentially toxic to CD patients. A various level test systems are available: (i) in vivo test based on feeding or instillation, biopsy and morphometry; (ii) in vitro test based on organ culture and morphometry; and (iii) T cell stimulation. Other test systems (e.g. agglutination test on K562 or Caco-2-cell tests) might be also preliminarily used, but the results have to be confirmed by the tests mentioned above.

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 T-cell mediate immune response (Sollid and Khosla, 2005). Although still debated and monthly updated, several fragments (f) are defined as indubitably toxic; e.g. f31–43 of α2-gliadin (Picarelli et al., 1999), f31–49 of A-gliadin (Sturgess et al., 1994), f56–75 of α-gliadin (Fraser et al., 2003), f62–75 of α2-gliadin (Shan et al., 2002), 33-mer epitope, corresponding to f57–89 of α2-gliadin (Shan et al., 2002), f134–153 of γ-gliadin (Aleanzi et al., 2001) and f57–89 of α2-gliadin (Arentz-Hansen et al., 2000). Recently, it has been shown that glutenin may contain cryptic regions which originate toxic epitopes (Wieser, 1996; van de Wal et al., 1999; Molberg et al., 2003). Glutenins contain sequences (e.g., glt04 707–742) that activate T cells from the small intestine and result in the secretion of large amounts of interferon-γ (IFN-γ). The minimal T cell stimulatory core of the peptide (residues 724–734) is repetitively present in glutenin molecules and a large number of naturally occurring variants of this peptide are recognized by the T cells (van de Wal et al., 1999; Stepniak et al., 2005). High molecular weight glutenin subunits (HMW-GS) stimulate T cell lines from some celiac patients and exacerbate CD in vivo, inducing expression, within 2 h, of interleukin 15 (IL-15), suggesting an innate immune response to these proteins (Dewar et al., 2006). A prolamins group of research is coordinating the work on gluten analysis in food and clinical evaluation of patient sensitivity to prolamins (Stern et al., 2001). Based on the current findings, it was suggested that celiac toxicity and immunogenicity (humoral and cellular) of various prolamins are not identical in CD patients. The large proportion and location of proline residues in the amino acid sequences of these toxic peptides make them extremely resistant to further hydrolysis (Auricchio et al., 1978; Andria et al., 1980; Hausch et al., 2002). Proline is unique among the 20 amino acids because of its cyclic structure. This specific conformation imposes many restrictions on the structural aspects of peptides and proteins, and confers particular biological properties. To adequately deal with such peptides, a group of specific peptidases is necessary to hydrolyse peptide bonds in which a proline residue occurs as a potential substrate.

Prolyl endopeptidases (PEPs) of microbial origin are endoproteolytic enzymes which, in contrast to human gastrointestinal protease, can readily cleave Pro-rich immunostimulatory gluten peptides (Hausch et al., 2002). The use of PEPs in the medical therapy approach has been investigated. Every PEP-catalysed cleavage generates not only one new amino and carboxyl terminus, but it also truncates the long-peptide end-products of gastric and duodenal gluten metabolism. It has been proposed that oral administration of a therapeutic dose of suitably formulated PEP might counter the toxic effects of moderate quantities of ingested gluten (Hausch et al., 2002). This hypothesis is supported by extensive in vitro, in vivo (rats) and ex vivo (using biopsy-derived T cells) studies on synthetic gluten peptides, recombinant gliadin molecules and whole gluten as obtained in grocery store (Hausch et al., 2002; Shan et al., 2002, 2004; Piper et al., 2004; Marti et al., 2005). A recent study (Pyle et al., 2005) showed that pre-treatment of gluten with PEP from *Flavobacterium meningosepticum* avoided the development of fat or carbohydrate malabsorption in the majority of CD patients who ingested a low dose of a gluten supplement daily (5 g) during a challenge lasting 14 days. Nevertheless, Matysiak-Budnik et al. (2004) showed that the hydrolysis of the 33-mer by PEP of *F. meningosepticum* in CD patients was not complete and led to the release of potentially immunogenic peptides, which after crossing the intestinal mucosa contacted the immune system. Piper et al. (2004) have addressed this apparent controversy (Shan et al., 2002; Matysiak-Budnik et al., 2004) by performing dose-dependent intestinal perfusion experiments in rats. A homologous PEP from *Myxococcus xanthus* seems to be comparable to the *F. meningosepticum* enzyme with respect to gluten detoxification (Shan et al., 2004) and *Lactobacillus helveticus* has a zinc-dependent PEP that can also cleave long substrates with relatively broad sub-site specificity (Chen et al., 2003). Interestingly, a newly identified PEP from *Aspergillus niger* that is optimally active at pH 4-5, remains stable at pH 2 and is completely resistant to digestion with pepsin, efficiently degraded T cell stimulatory peptides as well as peptic/tryptic digest of gluten and intact gluten. Also this PEP is proposed as an oral supplement to reduce gluten intake in CD patients (Stepniak et al., 2006).

Enzyme therapy approach is at least theoretically related with the use of selected lactic acid bacteria for fermentation of wheat or rye dough, especially if these food grade microorganisms are used as cell factories for multiple and complementary enzyme activities. Lactic acid bacteria possess a very complex peptidase system (Kunji et al., 1996), although not a unique strain may posses the entire pattern of peptidases needed for hydrolysing all the potential peptides where Pro is involved. Recently, a large number of sourdough lactic acid bacteria was screened for proteolytic activity (Di Cagno et al., 2002) and a pool of lactobacilli was selected and used for further studies. This pool of sourdough lactobacilli had specialized peptidases capable of hydrolysing Pro-rich peptides, including the 33-mer peptide, the most potent inducer of gut-derived human T-cell lines in CD patients. This epitope was completely hydrolysed after treatment with cells and their cytoplasmic

extracts. Same results were observed by using a mixture of probiotic strains, i.e. VSL#3 (VSL Pharmaceuticals, Gaithesburg, MD) used as starter for fermentation (De Angelis et al., 2005). The VSL#3 preparation 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 (Gionchetti and Campieri, 1999; Gionchetti et al., 2000; Kaur et al., 2002). Preliminarily, 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 probiotic preparation (De Angelis et al., 2005). Nevertheless, the hydrolyzing capacity was lost when individual strains which composed VSL#3 were tested, confirming that no single strain contains the entire portfolio of peptidases necessary to degrade Pro-rich polypeptides. Furthermore, VSL#3 hydrolysed completely the epitope 33-mer and the f62–75 of α2-gliadin, previously reported as immuno-modulatory peptides involved in CD pathogenesis (Silano and De Vincenzi, 1999; Shan et al., 2002). An X-prolyl dipeptidyl aminopeptidase (PepX) from sourdough *Lactobacillus sanfranciscensis* was partially purified (Gallo et al., 2005). No hydrolysis of the Pro-rich 33-mer epitope was found when it was treated with PepX alone. When the general aminopeptidase type N was combined with PepX, the hydrolysis of 33-mer peptide (0.2 mM) was complete after 24 h of incubation at 30 °C. Leucine and glutamine residues were liberated from the Pro-rich 33-mer peptide by aminopeptidase type N, thus favoring the subsequent PepX activity.

Based on the above considerations, enzyme treatments of gluten epitopes may be considered the easiest and non-invasive tool to eliminate in a dose-dependent manner the toxicity of gliadins and glutenins. Two routes may be persuaded: one is concerning the use of enzymes as oral supplements to reduce gluten intake in CD patients and the other may consider their activities during food processing to eliminate the gluten toxicity before consumption.

4. Fermented cereal products

Aiming at investigating the potential of sourdough lactic acid bacteria as a source of proteolytic enzymes to eliminate gluten toxicity during processing, several attempts were done during last years for bread or pasta making.

4.1. Sourdough wheat bread

Two studies (Di Cagno et al., 2004; De Angelis et al., 2005) showed that pools of lactic acid bacteria (sourdough lactobacilli and commercial probiotic preparation) under specific processing conditions (long-time and semi-liquid fermentation) had the capacity to markedly hydrolysed the wheat gliadin fraction.

A sourdough made from a mixture of wheat (30%) and non-toxic oat, millet and buckwheat flours was started with the selected *Lactobacillus alimentarius* 15M, *Lactobacillus brevis* 14G, *L. sanfranciscensis* 7A and *Lactobacillus hilgardii* 51B (ca. 10^9 CFU/g) and subjected to long-time (24 h) fermentation at 37 °C (Di Cagno et al., 2004). The use of 30% of wheat flour seemed to be the dose compatible with the hydrolyzing activities of selected lactobacilli. As shown by two-dimensional electrophoresis, an almost complete hydrolysis of gliadins was achieved (Fig. 1), while prolamins from oats, millet and buckwheat were not affected during dough fermentation. A comparison with a chemically acidified dough or with a dough started with baker's yeast alone showed that the hydrolysis was due to the proteolytic activity of sourdough

Table 1
Enzyme activity[a] of VSL#3 on various substrates containing proline residues (from De Angelis et al., 2005)

Substrate	Type of enzyme	Substrate concentration (mM)	Unit of activity (U)[b]	Unit of activity (U)[c]
Pro-*p*-NA	Proline iminopeptidase	2	3.2±0.02	3.3±0.05
Leu-*p-NA*	Aminopeptidase type N	2	8.4±0.04	7.9±0.03
Ala-*p-Na*	Aminopeptidase 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–*p*–NA	Prolyl-endopeptidase	2	1.3±0.01	1.0±0.02
NCBZ–Gly–Gly–Leu–p–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 of 0.01/min. A unit on polypeptides was the amount of enzyme which liberates 1 μM of substrates/min.

[b]Unit of enzymatic activity under optimal conditions.

[c]Unit of enzymatic activity under simulated gastro-intestinal conditions.

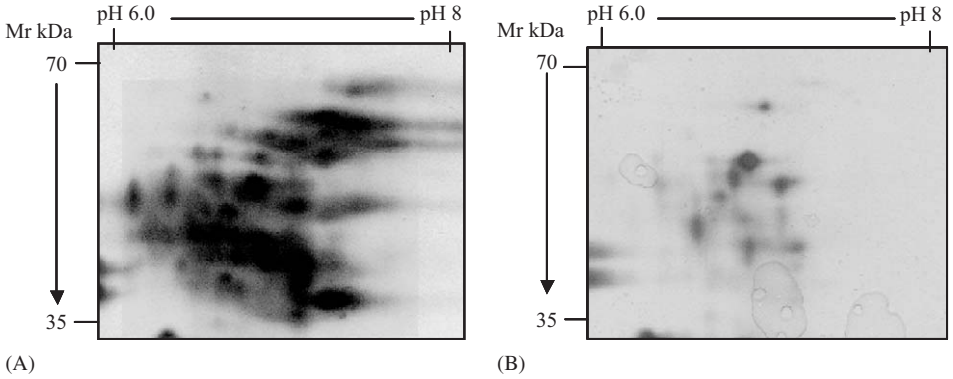

Fig. 1. 2DE analysis of the prolamin protein fractions of different doughs made of a mixture of wheat (30%), oat (10%), millet (40%), and buckwheat (20%) flours. Chemically acidified dough incubated for 24 h at 37 °C (A) and sourdough started with selected lactid acid bacteria for 24 h (B) at 37 °C were used. From Di Cagno et al. (2002).

lactobacilli. After performing a preliminarily toxicity screening based on agglutination test on K 562(S) cells, the bread making process was started. The protocol included (i) fermentation of wheat flour with selected lactobacilli for 24 h at 37 °C under semi-liquid conditions (30% wheat flour and 70% water); (ii) mixing with non-toxic flours (oat, millet and buckwheat) in the ratio 3:7; (iii) further fermentation for 2 h at 30 °C with baker's yeast; and (iv) baking at 220 °C for 20 min. The semi-liquid prefermentation of wheat flour was another indispensable condition to fully exploited the potential of sourdough lactobacilli enzymes. This type of bread was technologically suitable: the volume was ca. one half of that started with baker's yeast alone, and the texture and flavour were comparable to those of traditional wheat sourdough breads. Portion of this bread and a baker's yeast started bread, containing ca. 2 g of gluten, were used for an in vivo double-blind acute challenge of CD patients. Thirteen of the 17 patients showed a marked alteration of the intestinal permeability (Greco et al., 1991) after ingestion of baker's yeast bread. When fed the sourdough bread, the same 13 patients had values for intestinal permeability that did not differ significantly from the baseline values. The remaining 4 patients did not respond to gluten after ingesting baker's yeast or sourdough bread (Di Cagno et al., 2004).

The probiotic VSL#3 preparation also showed the capacity to decrease the toxicity of wheat flour during long-time fermentation (De Angelis et al., 2005). As shown by two-dimensional electrophoresis, gliadin polypeptide spots were almost completely hydrolysed after fermentation with the probiotic mixture. These results were mirrored by mass spectrometry MALDI-TOF and Western blot analyses (Fig. 2). Non-hydrolysed gliadins were subjected to peptic-tryptic (PT) digestion to mimic the gastrointestinal processes and analysed by CapLC-ESI-Q-ToF-MS (Capillary Liquid Chromatography-Electrospray Ionization-Quadrupole-Time of Flight-Mass Spectrometry). Search for the most known epitopes showed only the presence of α2-gliadin f62–75 at a very low concentration (sub-ppm range). Compared to rat intestinal epithelial cells

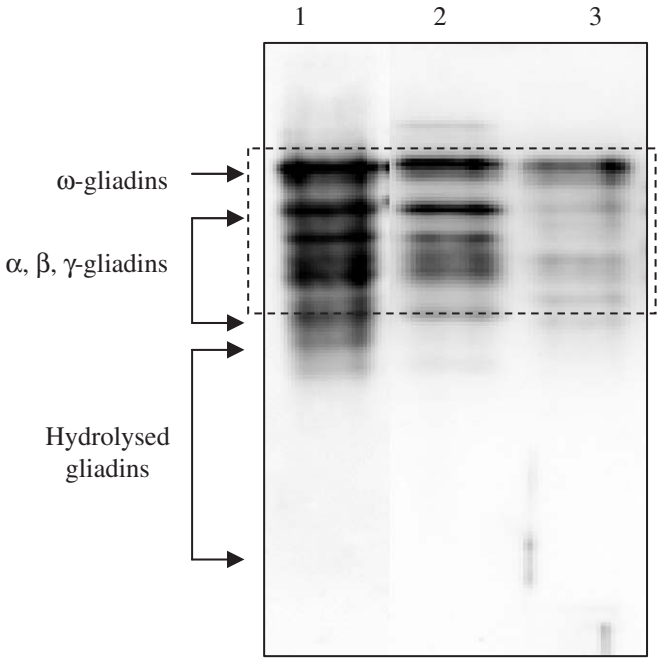

Fig. 2. Western blot/R5 analysis of European gliadin reference (1); chemically acidified dough (control) (2); dough incubated for 24 h at 37 °C with VSL#3. From De Angelis et al. (2005).

IEC-6 exposed to intact gliadins, VSL#3 predigested gliadins caused a less-pronounced reorganization of the F-actin which was confirmed by an attenuated effect on intestinal mucosa permeability. The release of zonulin, a molecule which sustains the increase of the intestinal permeability as a mechanism of response to toxic peptides (Clemente et al., 2003; Drago et al., 2003), from intestinal epithelial cells treated with gliadins was considerably lower when digested with VSL#3 (Fig. 3). 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. Overall, $CD3^+$ intraepithelial lymphocytes increased after challenge of small intestine mucosa from CD patients with gluten (Troncone et al., 1998; Mazzarella et al., 2005).

Although an acute in vivo test and in vitro tests based on organ cultures were carried out, the above results (Di Cagno et al., 2004; De Angelis et al., 2005) only showed a marked decrease of mainly the gliadin fraction and further studies have to be addressed for getting the complete detoxification of wheat flour. Practically, the use of two-steps fermentation resembled the ancient and traditional bread making and could be technologically suitable.

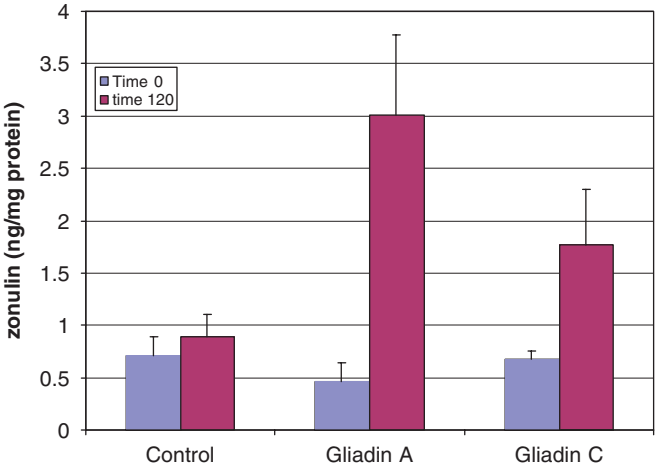

Fig. 3. Effect of wheat flour gliadin protein fraction on zonulin release form mouse intestinal mucosa. Non-hydrolysed prolamin (chemically acidified dough, control) (Gliadin A) and hydrolysed gliadins by VSL#3 (Gliadin C). A negative control using bovine serum albumin on zonulin release was also included (control). From De Angelis et al. (2005).

4.2. Pasta from fermented durum wheat semolina

The same approach as those described for sourdough wheat bread (Di Cagno et al., 2004; De Angelis et al., 2005) was adapted for pasta making. The same pool of selected sourdough lactobacilli (*L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A and *L. hilgardii* 51B) was used to preferment durum wheat semolina under semi-liquid conditions (Di Cagno et al., 2005). After fermentation, the dough was freeze-dried, mixed with buckwheat flour at a ratio of 3:7, and used to produce the "fusilli" type Italian pasta at an industrial level. Pasta without prefermentation was used as a control. As shown by two-dimensional electrophoresis and mass spectrometry MALDI-TOF analyses (Fig. 4), durum wheat gliadins were almost totally hydrolysed during fermentation by lactic acid bacteria. As determined by R5-Western blot and R5–ELISA, the concentration of gluten decreased from 6280 ppm in the

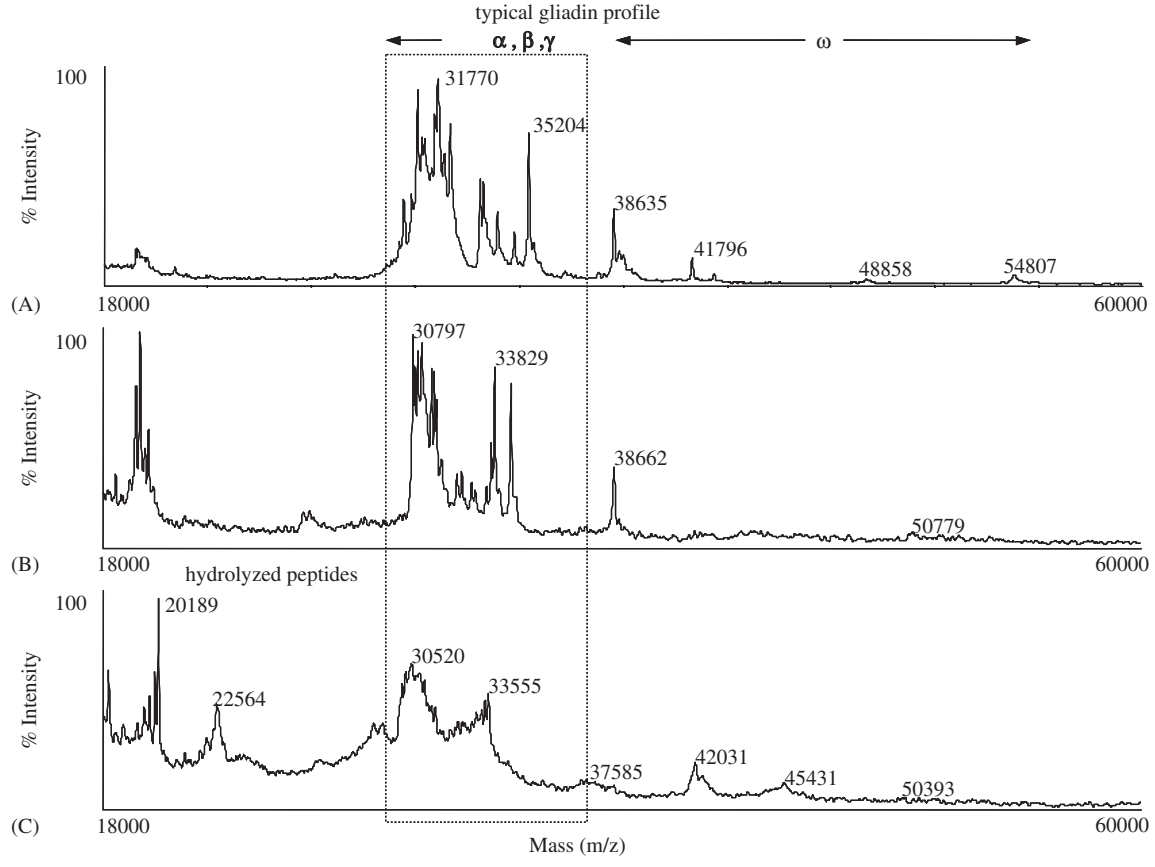

Fig. 4. Mass Spectrometry MALDI-TOF analysis of ethanol extract of wheat durum gliadin: (A) European gliadin standard showing the α-, β-, γ-, and ω-gliadin ranges; (B) chemically acidified dough incubated for 24 h at 37 °C and (C) fermented durum wheat dough with the mixture of selected lactic acid bacteria for 24 h at 37 °C. The typical α-, β-, γ-gliadin profile is displayed in the box. From Di Cagno et al. (2005).

control pasta to 1045 ppm in the fermented pasta. Although this type of pasta still contained 1045 ppm of gluten, which may trigger CD, the use of a mixture which includes 20% of fermented durum wheat semolina in the pasta formulas may theoretically lead to a novel pasta product within the safe threshold for CD (200 ppm), as stated in the Codex Standard of WHO and FAO. Gliadins were extracted from fermented and non-fermented durum wheat semolina dough, and used to produce the PT-digests for in vitro agglutination test on K 563(S) subclone cells of human myelagenous leukaemia origin. The whole PT digests did not cause agglutination. Contrarily to wheat, rye and barley bread, it seemed that durum wheat contained a dodecapeptide which had the capacity to prevent the agglutination by PT-digest and which may have a protective effect on CD (De Vincenzi et al., 1998). Affinity chromatography separated the PT-digests in three fractions. Only one of these fractions showed agglutination activity while the others did not interfere with K 563(S) cells. The minimal agglutinating activity of the active fraction from the PT-digest of fermented durum wheat semolina was ca. 80 times higher than that of durum wheat semolina, indicating a decreased toxicity.

The above results (Di Cagno et al., 2005) showed the suitability of the proposed protocol for the manufacture of pasta at an industrial plant. The scores for stickiness and firmness were slightly lower than for the control pasta but odour and flavour did not differ between the two types of pasta. Nevertheless, also in this case a decrease but not the complete elimination of toxicity was achieved.

4.3. Rye sourdough fermentation

The same pool of selected sourdough lactobacilli as described above (Di Cagno et al., 2004) was used for rye flour fermentation showing the potential towards ethanol-soluble rye polypeptides (De Angelis et al., 2006). Rye is the most important cereal crop after wheat, rice and maize. A substantial part of the cultivated rye is used for bread making, especially in Central, Northern and Eastern European countries where more than 3 million tonnes of rye per year are used for bread making (FAOSTAT 1998). Fermentation was carried out under semi-liquid (30% of wheat flour and 70% water) conditions for long-time (24–48 h). The same analytical approach based on two-dimensional electrophoresis, mass spectrometry MALDI-TOF and RP-HPLC analyses showed that the major part of the ethanol-soluble rye polypeptides were almost totally hydrolysed by the lactic acid bacteria. After 48 h of fermentation, no prolamin polypeptides were recognized by R5-Western blot analysis. Compared to secalins, HPLC analysis of glutelin polymers showed a very low bacterial proteolysis. Nevertheless, it was shown that acidification (chemical or biological) led to a substantial degradation of glutelins due to the probable activation of rye flour endogenous proteolytic enzymes (Thiele et al., 2004). Prolamins were extracted from rye flour and used to produce a PT-digest for in vitro tests with Caco-2/TC7 cells of human origin (De Angelis et al., 1998; Giovannini et al., 2003). Hydrolysis of rye PT-digest by selected sourdough lactic acid bacteria decreased the toxicity of PT-digest itself towards Caco-2/TC/cells as estimated by cell viability (evaluated through uptake of Neutral Red), caspase-3 activity (to quantify apoptosis) and release of nitrogen oxides (that are produced by Caco 2 cells exposed to gluten PT-digest). On the other side, prolamins and glutelins were extracted from fermented rye sourdough and subjected to PT-digestion. Compared to the PT-digest from chemically acidified dough, celiac jejunal biopsies exposed to the PT-digest from the dough fermented by lactic acid bacteria did not show an increase of the infiltration of $CD3^+$ intraepithelial lymphocytes (Fig. 5). Fas expression on epithelial cells is a measure of apoptosis (Maiuri et al., 2001). Gliadins inducing apoptosis of enterocytes play a pivotal role in causing villous atrophy, the main histological feature of the small intestine mucosa of CD patients (Maiuri et al., 2001). Fas expression, in biopsies cultured with PT-digest from rye sourdough did not show significant differences with respect to the chemically acidified control (De Angelis et al., 2006).

Overall, before to carry out extensive in vivo long-term challenge on CD patients several analytical techniques and, especially, in vitro and T cell stimulation assays are necessary to demonstrate the absence of toxicity for new treatments or foods. The extensive in vitro tests for toxicity showed that sourdough and probiotic microbial pools have a rater promising detoxifying potential (Di Cagno et al., 2004, 2005; De Angelis et al., 2005, 2006) but not enough to cause the complete absence of toxicity. It should be supposed that instead of an extensive peptidase activity, microbial pools have to be supplemented with proteinases that start the first step of proteolysis. Besides, the emerging toxicity of HMW glutenins (Dewar et al., 2006) has to be carefully considered. It limits the current diagnostic value of the R5 antibody for detecting toxic epitopes and imposes the need for in vitro (organ culture) and T cell stimulation

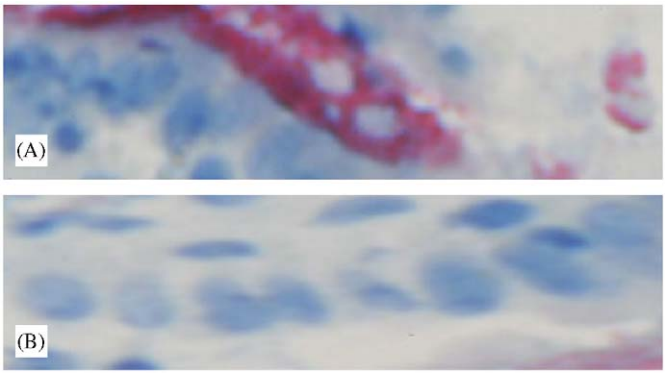

Fig. 5. Fas expression in a CD specimen treated with PT-digest from secalin and glutelin proteins extracted from doughs chemically acidified (A) or fermented with selected lactic acid bacteria for 24 h (B). Original magnification 200×; immunohistochemistry; Red Fuchsin APAAP staining technique. From De Angelis et al. (2006).

assays to show the degree of toxicity. Technologically, the main criticism to the strategy proposed is concerning the real advantages compared to gluten-free products and the suitability of the wheat flour with completely degraded gluten to be used for bread making. Gluten-free diet is expensive, hard to follow and needs continuous monitoring by dieticians (Diosdado, 2006). Unsurprisingly, it is, at the best, imperfect in a large fraction of patients (Sollid and Khosla, 2005) and gluten-free products generally have very poor sensory and shelf-life properties and can only be eaten fresh (Hamer, 2005). The manufacture of baked goods from detoxified wheat flour may dramatically reduce the costs, allow the large part of the bakeries to produce foods for CD patients and it resembles, in a more natural way, the genetic strategy for silencing wheat toxic sequences. Preliminarily, experiments in the authors' laboratory showed that the use of structuring agents (e.g. gums and modified starch) enabled the use of pre-hydrolysed wheat flour to obtain breads with almost the same rheology properties as the traditional and with sensory characteristics highly superior to gluten-free products.

5. Concluding remarks

The compliance to a gluten-free diet is an extremely challenging task, given the problems related to cross contamination, lack of clear food labelling policies, and poor information on minimal toxic amounts of prolamins in CD patients (De Angelis et al., 2006). It has been shown that 30% of oat products in the market have a gluten content over 200 ppm (Størsrud et al., 2003). Additionally, most starch-based, gluten-free wheat flours are contaminated with gluten at concentrations ranging from 10 to over 200 ppm (Collin et al., 2004).

Sourdough fermentation is a traditional process that retains its importance in wheat baking because it improves bread quality by prolonging shelf life, increasing loaf volume, delaying staling, as well as by improving bread flavour and nutritional properties (Thiele et al., 2004). Fermentation of wheat and rye flours with selected sourdough lactic acid bacteria following an ancient protocol which includes long-time fermentation may at the moment decrease the risk of gluten contamination in gluten-free products. Although for CD patients the goal is not to decrease the gluten toxicity but to eliminate it, the authors' laboratory is currently using a more complex microbial pool with the addition of fungal proteases which decreases the level of persistent gluten below 20 ppm and is optimizing the baking quality of the bread to get superior nutritional, sensory and rheological characteristics with respect to the current gluten-free products.

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