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# Pasta Made from Durum Wheat Semolina Fermented with Selected Lactobacilli as a Tool for a Potential Decrease of the Gluten Intolerance

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A pool of selected lactic acid bacteria was used to ferment durum wheat semolina under liquid conditions. 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. Pasta without prefermentation was used as the control. Ingredients and pastas were characterized for compositional analysis. As shown by two-dimensional electrophoresis, 92 of the 130 durum wheat gliadin spots were hydrolyzed almost totally during fermentation by lactic acid bacteria. Mass spectrometry matrix-assisted laser desorption/ionization time-of-flight and reversed phase high-performance liquid chromatography analyses confirmed the hydrolysis of gliadins. As shown by immunological analysis by R5-Western blot, the concentration of gluten decreased from 6280 ppm in the control pasta to 1045 ppm in the pasta fermented with lactic acid bacteria. Gliadins were extracted from fermented and nonfermented durum wheat dough semolina and used to produce a peptic-tryptic (PT) digest for in vitro agglutination tests on cells of human origin. The whole PT digests did not cause agglutination. Affinity chromatography on Sepharose-6-B mannan column separated the PT digests in three fractions. Fraction C showed agglutination activity. The minimal agglutinating activity of fraction C from the PT digest of fermented durum wheat semolina was ca. 80 times higher than that of durum wheat semolina. Pasta was subjected to sensory analysis: The scores for stickiness and firmness were slightly lower than those found for the pasta control. Odor and flavor did not differ between the two types of pasta. These results showed that a pasta biotechnology that uses a prefermentation of durum wheat semolina by selected lactic acid bacteria and tolerated buckwheat flour could be considered as a novel tool to potentially decrease gluten intolerance and the risk of gluten contamination in gluten-free products.

**KEYWORDS: Celiac disease; lactobacilli; gliadins; pasta**

## INTRODUCTION

Celiac Sprue (CS) is a genetically determined chronic inflammatory intestinal disease induced by an environmental precipitant, gluten. The mechanism of the intestinal immune-mediated response is not completely clear, but it involves an HLA-DQ2 or HLA-DQ8 restricted T-cell immune reaction in the lamina propria as well as an immune reaction in the intestinal epithelium (*1*). During endoluminal proteolytic digestion, mainly prolamins of wheat (α-, *β*-, *γ*-, and *ω*-gliadin subgroups), 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-mediated immune response (*2*). Although still debated, it seems that fragments 31−43 of A-gliadin (*3*), 62−75 of α2-gliadin (*4*), 33-mer epitope, corresponding to 57−89 of α2-gliadin (*4*), 134−153 of *γ*-gliadin (*5*), and 57−68 of α9-gliadin (*6*), are potent inducers of CS. Glutenin seemed to have a minor role in CS: T-cells within celiac lesions frequently recognize deaminated glutenin proteins, thus probably excluding the toxicity of this cereal protein component (*7*).

Recent serologic screening studies (*8*−*10*) have shown the CS worldwide prevalence to be one in 266 people in most European countries, South America, and the United States. Reports from North Africa, Iran, and India indicate the widespread occurrence of CS also (*1*). The clinical classification of CS distinguishes the symptomatic CS, which refers to presentation with diarrhea, with or without malabsorption, whereas in asymptomatic or silent CS, gastrointestinal symptoms are absent or not prominent even though the patients might report other nonintestinal symptoms. The number of diagnoses

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of silent forms continues to rise worldwide, and most people are subjected to risk of long-term complications such as osteoporosis, infertility, or cancer when the diet with gluten is prolonged (*11*).

The current treatment for CS is a strict gluten-free diet (GFD) for life. In the GFD, wheat, barley, and rye are avoided. The International Food Authority has recently redefined the term "gluten-free", which now means absolutely no gluten, whereas the Codex Alimentarius tolerates 200 ppm of gluten per food (*7*). As recently shown (*12*, *13*), a GFD completely devoid of gluten is in most of the cases unrealistic. Both naturally gluten-free and wheat starch-based gluten-free products may contain gluten, in some circumstances, over 200 ppm.

Pasta is regularly consumed in most Mediterranean countries and in most of Europe and the United States. The weekly consumption of pasta is spreading in North Africa also (*14*). The annual per capita consumption of pasta around the world varied from ca. 28.3 kg/person/year in Italy to 1.0 kg/person/year in Ireland. The annual quantity of pasta sold in the United States was ca. 404 million kg (*15*). For several CS patients, GFD is, therefore, much more restrictive for pasta than for bread, biscuits, or other wheat-made foods (*16*).

Recently, a sourdough made from wheat flour alone was fermented in semiliquid conditions (dough yield, 220) with selected lactobacilli for 24 h at 37 °C (*17*). Following fermentation, the wheat sourdough was mixed (dough yield, 150) with tolerated flours such as oat, millet, and buckwheat flours (BFs) and baker's yeast, allowed to ferment for 2 h at 37 °C, and baked at 220 °C for 20 min. The bread, containing ca. 2 g of gluten, was tolerated by 100% of CS patients as shown by acute intestinal permeability challenge. After this publication (*17*), one of the major questions raised has concerned the possibility of using a similar protocol for the manufacture of pasta. The standard manufacture of pasta does not include a fermentation step. Technological efforts that are addressed to decrease the contamination of wheat in gluten-free products and, in general, to decrease the potential toxicity of cereal-based products have a medical, nutritional, and economic interest.

This paper describes a technological approach for the manufacture of pasta made of a mixture of prefermented durum wheat semolina (DWS) and BF, which may decrease the concentration of nontolerated gliadins. Hydrolysis of gliadins was determined by complementary techniques such as two-dimensional electrophoresis (2DE), mass spectrometry matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), reversed phase high-performance liquid chromatography (RP-HPLC), and immunological analysis by R5-Western blot. Agglutination tests on K 562(S) subclone cells of human myelogenous leukaemia origin, which were well-correlated with medical trials in CS patients (*2*, *18*, *19*), were used in vitro to show the potential tolerance by CS patients.

## MATERIALS AND METHODS

**Microorganisms and Culture Conditions.** *Lactobacillus alimentarius* 15M, *Lactobacillus brevis* 14G, *Lactobacillus sanfranciscensis* 7A, and *Lactobacillus hilgardii* 51B were selected previously (*17*, *20*) on the basis of their ability to hydrolyze gliadin fractions and various Pro-rich oligopeptides, including the 33-mer epitope, and were used in this study. The strains were routinely propagated for 24 h at 30 (*L. alimentarius* 15M, *L. brevis* 14G, and *L. sanfranciscensis* 7A) or 37 (*L. hilgardii* 51B) °C in modified MRS broth (Oxoid, Basingstoke, Hampshire, England) with the addition of fresh yeast extract (5%, vol/vol) and 28 mM maltose at a final pH of 5.6. When used for the fermentation of DWS, cells were incubated until the late exponential phase of growth (optical density at 620 nm, ca. 2.5) was reached (ca. 12 h).

**Pasta Making.** Pasta was manufactured at the pilot plant of the Divella industry (Rutigliano, Bari, Italy). Divella is the second largest producer of pasta in Italy. The characteristics of DWS (*Triticum durum*) and buckwheat (*Fagopyrum esculentum*) flour (BF), respectively, were the following: moisture, 12.1 and 13.2%; protein ($N \times 5.70$), 13.2 and 9.0% of dry matter (d.m.); fat, 1.8 and 2.3% of d.m.; and ash, 0.8 and 1.5% of d.m. Eighty grams of DWS, 160 mL of tap water, and 30 mL of a mixed cellular suspension containing $10^9$ colony-forming units (CFU) of each lactic acid bacterial strain/mL (ca. $10^8$ CFU/g of dough) were used to produce 270 g of dough (dough yield, 337) with a continuous high-speed mixer (60*g*; dough mixing time, 5 min). The dough was incubated for 24 h at 37 °C under stirring (ca. 200 rpm). After fermentation, sodium citrate (2.5%, wt/vol) was added to the dough as the buffering agent currently used for the manufacture of fresh pasta (www.professionalpasta.it). The dough was freeze-dried to remove the excess of water, and the powder was gently milled to get an homogeneous size and mixed with BF at a ratio of 3:7, respectively. The moisture content of the pasta was adjusted on manufacture, accounting for the water absorption of the mixture, to produce a visually optimum dough prior to extrusion (ca. 25% of tap water). After it was mixed, the dough was extruded using a benchtop pasta maker (Monteferrina, Asti, Italy) to produce the Italian pasta named "fusilli" (0.7 cm diameter and 5.0 cm length). The fusilli were placed into pasta frames and allowed to dry for 24 h at 25 °C. This type of pasta was defined as the fermented pasta (FP). A dough made of nonfermented DWS and BF at a ratio 3:7 was also used to produce fusilli under the same conditions described above and used as the control pasta (CP). One hundred grams of pasta, which approximately corresponds to the daily portion, contained ca. 3 g of gluten. Another dough made of 80 g of DWS and 190 mL of tap water, without microbial inoculum, was chemically acidified to pH 3.2 with a mixture of lactic and acetic acids at a molar ratio of 4:1, which corresponds to that usually found after fermentation by facultatively and obligately heterofermentative lactic acid bacteria (*21*). It was incubated for 24 h at 37 °C and defined as the chemically acidified dough (CAD) to be used for comparative 2DE and agglutination tests only.

**Compositional Analyses.** DWS, BF, freeze-dried fermented durum wheat semolina (FDWS), and pastas were subjected to several compositional analyses. Moisture, ash, protein, and acidity were determined as described by AACC methods (*22*). The Gluten System GL (Esetek Instruments Srl, Roma, Italy) was used to determine the concentration of gluten by the AACC method (*22*). The polarimetric method was used to determine the concentration of starch (*23*).

**Protein Extraction.** Protein fractions were extracted from DWS, FDWS, and CAD by the method originally described by Osborne (*24*) and modified by Weiss et al. (*25*). An aliquot of dough (16.6 g, corresponding to 5 g of semolina) was diluted with 20 mL of 50 mM Tris-HCl (pH 8.8), held at 4 °C for 1 h with vortexing at 15 min intervals, and centrifuged at 20000*g* for 20 min. The supernatant contained albumins and globulins. To minimize cross-contamination among albumins, globulins, and gliadins, the pellets were further extracted with twice with 50 mM Tris-HCl (pH 8.8), and supernatants were discarded. After it was washed with distilled water to remove buffer ions, the pellets were diluted with 20 mL of ethanol (75%, vol/vol), stirred at 25 °C for 2 h, and centrifuged as described above. The supernatant contained gliadins. The extraction with ethanol was repeated twice. Residual ethanol was eliminated by resuspending the pellets with distilled water and centrifugation. Finally, the pellets were diluted with 20 mL of sodium dodecyl sulfate (SDS)−dithiothreitol (DTT) buffer (50 mM Tris-HCl [pH 8.8], 1% SDS, and 0.5% DTT), held for 2 h at room temperature with occasional vortexing, and centrifuged. The supernatant contained glutenins. All extracts were stored at −80 °C until they were used.

For RP-HPLC analysis, the extraction of gliadins was carried out as reported by Krugher et al. (*26*) also. An aliquot of dough (10 g) was diluted with 30 mL of 0.5 M NaCl in 150 mM sodium phosphate, pH 6.8, and the suspension was thoroughly mixed and incubated for 30 min at room temperature in an overhead mixer. The buffering capacity of the solvent sufficed to compensate for the differences in pH between FDWS and CAD. After centrifugation at 20000*g* for 20 min, the supernatant contained albumins and globulins. After they

were washed with 20 mL of distilled water to remove salt and residual organic acids, the pellets were diluted with 30 mL of 1-propanol (50%, v/v) and stirred at room temperature for 30 min. After centrifugation, gliadin extracts were stored at −20 °C in the dark until analysis by RP-HPLC. Protein concentrations of the fractions were determined by the Bradford method (*27*) by using bovine serum albumin as standard.

**2DE.** 2DE was performed with the immobiline-polyacrilamide system as described by Bjellqvist et al. (*28*). Aliquots of 30−50 μL (30 μg of protein) of gliadin fraction were used for the electrophoretic run. Isoelectric focusing was carried out on immobiline strips, providing a pH gradient of 6−11 and 3−10 (IPG strips; Amersham Pharmacia Biotech, Uppsala, Sweden) by IPG-phore, at 15 °C. The voltages were the following: 0−300 V for 1 h, 300−500 V for 3 h, 500−2000 V for 4 h, and a constant 8000 V for 4 h. Following electrophoresis, IPG strips were equilibrated for 12 min against buffer A (6 M urea, 30% [vol/vol] glycerol, 2% [wt/vol] iodoacetamide, and 0.5% bromophenol blue). The second dimension was carried out in a Laemmli system (*29*) on 12% polyacrilamide gels (13 cm × 20 cm × 1.5 mm) at a constant current of 15 mA/gel and at 10 °C for approximately 5 h, until the dye front reached the bottom of the gel. Gels were calibrated with two molecular mass markers: comigration of the extracts with human serum proteins for a molecular mass range of 200 to 10 kDa (*19*) and markers for 2DE (pI range of 7.6 to 3.8; molecular mass range, 17−89 kDa) from Sigma Chemical Co. (Milan, Italy). The electrophoretic coordinates used for serum were described by Bjellqvist et al. (*28*). Gels were silver stained as described by Hochstrasser et al. (*30*). The protein maps were scanned with an Image Scanner and analyzed with Image Master 2D v.3.01 computer software (Amersham Pharmacia Biotech). Four gels, corresponding to two independent samples (e.g., FDWS) subjected to extraction twice, were analyzed. Spot intensities were normalized as reported by Bini et al. (*31*). Only statistically significant hydrolysis factors, where the $P$ value was $<0.05$, are reported.

**Mass Spectrometry MALDI-TOF.** Gliadins of CAD and FDWS were subjected to mass spectrometry MALDI-TOF analysis on a Voyager De Pro Workstation (Perseptive Biosystems, United Kingdom). This determination was carried out at the Centro National de Biotecnologia, Gluten Unit, CNB (28049 Madrid, Spain). One-hundred microliters of ethanol extract of gliadins was added to 8 μL of 50 mM octyl-D-glucopyranoside detergent and to 25 μL of saturated sinapinic acid in 30% (v/v) acetonitrile solution, containing 0.1% (v/v) trifluoroacetic acid (TFA), used as the matrix solution. The matrix−sample mixture was dried in a Speed-Vac centrifuge (30−35 min), and the residue was dissolved in 6 μL of 60% ethanol, containing 0.1% TFA. One microliter of the sample−matrix mixture was placed on a 100 sample stainless steel probe and allowed to dry at room temperature for 5 min. Mass spectra were recorded in the linear positive mode at an acceleration voltage of 25 kV with a grid voltage of 93%, 0.25% guide wire, and 700 ns delay time by accumulating 100 spectra of single laser shots under threshold irradiance. A standard of European gliadins was also included in the analysis (*32*).

**RP-HPLC.** The separation of gliadin extracts from CAD and FDWS was performed using an ACTÁ Basic Instrument (Amersham Pharmacia Biotech, Milan, Italy) and a Source 5RPC ST 4.6/150 column (Amersham Pharmacia Biotech). The elution system consisted of (A) TFA (0.1%, v/v) and (B) acetonitrile/TFA (99.9/0.1%, v/v) (*33*). For each sample, 100 μL of gliadin extract (ca. 490 μg of protein) was injected. Further HPLC conditions were as follows: linear gradient of 0 min 25% B, 90 min 37% B; flow rate of 1 mL/min; column temperature set at 70 °C; and UV detection at 210 nm. The column was cleaned with 80% B (10 min) and equilibrated with 25% B (40 min). The experimental error of dough extraction, chromatographic separation, and integration was less than 5%; two independent FDWS samples yielded qualitative and quantitative consistent results.

**Immunological Analysis by R5-Western Blot.** This analysis was carried out at the Centro National de Biotecnologia, Gluten Unit, CNB. About 50 g of FP and CP pastas was placed on aluminum foil, allowed to dry in a vacuum-connected oven at 37 °C overnight, and milled. The water content was determined by weighing the sample before and after drying. An aliquot (0.25 g) of each sample was placed in a 10 mL propylene tube, added to 10 mL of 60% (v/v) ethanol, and incubated for 1 h at room temperature in a rotary shaker at 45 turns/min. After centrifugation at 2500*g* for 10 min, supernatants were transferred to other 10 mL polypropylene tubes and used for sandwich enzyme-linked immunosorbent assay (ELISA). European gliadin standards were used as the control. The R5 monoclonal antibody and the horseradish peroxidase (HRP)-conjugated R5 antibody (R5-HRP) were used for gluten analysis. Polystyrene enzyme immunoassay (EIA/RIA) flat-bottomed plates (Corning Inc., Acton, MA) were coated overnight at 4 °C with 0.5 μg of R5 monoclonal antibody in 100 μL of 50 mM sodium carbonate/bicarbonate, pH 9.6. Plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 80 (Sigma) (PBS-T) and blocked with PBS-T plus 10% bovine sera albumin (Roche Diagnostics, Mannheim, Germany) (blocking solution) for 1 h at 37 °C. Plates were washed again three times in PBS-T and incubated for 1 h with 100 μL of gliadin sample extracts or gliadin standards diluted in PBS-T from 100 to 0.78 ng/mL, using 1:2 serial dilutions. After washing, 100 μL of R5-HRP (1:20000 in PBS-T) was added, and plates were incubated for 1 h at room temperature. Plates were washed again six times with PBS-T, and 100 μL of K-blue 3′,3′,5′,5′-tetramethylbenzidine Max (Neogen, Lexington, KY) was added. The reaction was stopped 10 min later with 50 μL of 2.5 M sulfuric acid. The absorbance at 450 nm was measured in a microplate reader.

**Determination of Free Amino Acids.** Free amino acids were determined in the water extracts of DWS, FDWS, and CAD. Total and individual free amino acids were analyzed by a BioChrom 30 series amino acid analyzer (BioChrom Ltd., Cambridge Science Park, England) with an Na cation exchange column (20 cm × 0.46 cm [inside diameter]). A standard amino acid mixture (Sigma Chemical Co.) made up of cysteic acid, methionine sulfoxide, methionine sulfone, tryptophan, and ornithine was used. Proteins and peptides were precipitated by addition of 5% (vol/vol) cold solid sulfosalicylic acid, holding at 4 °C for 1 h, and centrifugation at 15000*g* for 15 min. The supernatant was filtered through a 0.22 μm pore size filter (Millex-HA; Millipore S. A., Saint Quentin, France) and diluted (1:5) with sodium citrate loading buffer (0.2 M, pH 2.2). Amino acids were postcolumn derivatized with ninhydrin reagent and detected by absorbance at 440 (proline and hydroxyproline) or 570 (all the other amino acids) nm.

**Sensory Analysis of the Cooked Pastas.** The sensory analysis of pastas was carried out after cooking the fusilli for 5 min, which was determined as the optimum cooking time. Sensory characteristics of the cooked pastas were evaluated by six trained panellists according to the method of D'Egidio et al. (*33*) under test conditions of the International Standard 7304 (*34*). Stickiness (material adhering to the surface of cooked pasta), firmness (resistance to bite through the cooked pasta with the incisors), and odor and flavor were determined. These characteristics were evaluated by a score of 20−100 (*35*), and each panelist analyzed the same sample twice. Evaluation scores for stickiness were assigned as: <20 = very high; 20−40 = high; 41−60 = average; 61−80 = almost absent; and 81−100 = absent. Evaluation scores for firmness were assigned as: <20 = very low; 20−40 = low; 41−60 = sufficient; 61−80 = good; and 81−100 = very good. Evaluation scores for odor and flavor were assigned as: <20 = very unpleasant; 20−40 = unpleasant; 41−60 = sufficient; 61−80 = good; and 81−100 = pleasant. The data were statistically evaluated by a one-way analysis of variance procedure using the Statistica 6.0 program (Staftware Software Package, Tulsa, OK).

**Peptic-Tryptic (PT) Digest, Affinity Chromatography, and Agglutination Test.** Gliadins were extracted from DWS, CAD, and FDWS. Fifty milligrams of the gliadin fractions was subjected to sequential PT digestion to produced the corresponding PT digest. Following production, the PT digest was heated at 100 °C for 30 min to inactivate enzymes. This peptide preparation was used directly for the agglutination test or subjected to affinity chromatography.

Fifteen milligrams of PT digest was loaded at a flow rate of 10 mL/h on a Sepharose-6-B-mannan column (3 cm × 8 cm) and equilibrated with 0.02 M ammonium acetate (pH 7.2) buffer (*36*). The column was washed with the above buffer until no absorbance at 278 nm was found in the effluent. Then, elution was with 0.1 M acetic acid (pH 2.8). The fractions eluted with 0.02 ammonium acetate were freeze-dried, whereas those eluted with 0.1 M acetic acid were neutralized with 0.5 M

**Table 1.** Compositional Characteristics (% of Dry Matter) and Acidity[a] of DWS, BF, Freeze-Dried FDWS, CP, and FP

| parameters | DWS | BF | FDWS | CP | FP |
|---|---|---|---|---|---|
| moisture | 12.1 c | 12.2 c | 8 e | 11.1 d | 11 d |
| ash | 0.8 g | 1.5 f | 7.5 c | 2.4 e | 3.9 d |
| protein | 13.2 c | 9 g | 11.1 d | 10.3 e | 9.8 f |
| starch | 72 c | 71 c | 60 e | 70 c | 68 d |
| gluten | 12.1 c | 8.2 d | 0 e | ND | ND |
| acidity[b] | 2.4 g | 2.8 f | 3.5 e | 5.2 d | 6.8 c |

[a] Each value is the average of three assays. [b] Acidity, expressed as mL of 1 N NaOH/100 g of d.m. (*19*). [c−g] Values in the same row with different superscript letters differ significantly ($P < 0.05$); ND, not determined.

$NH_4OH$ and freeze-dried. All freeze-dried fractions were stored at −20 °C. The concentration of peptides in the fractions was determined by the method of Oyama and Eagle (*37*).

K 562(S) subclone of human myelagenous leukaemia origin form the European Collection of Cell Cultures (Salisbury, United Kingdom) was used (*38*) for testing agglutination of PT digest and peptide fractions separated by affinity chromatography. The cells were grown in RPMI medium (HyClone, Cramlington, United Kingdom) supplemented with 0.2 mM L-glutammine, 50 U penicillin/mL, 50 mg of streptomycin/mL, and 10% (vol/vol) fetal calf serum (Flow Laboratories, Irvine, Scotland) at 37 °C in a humidified atmosphere of 5% $CO_2$ in air for 96 h. After cultivation, the human cells were harvested by centrifugation at 900*g* for 5 min, washed twice with 0.1 M PBS solution ($Ca^{2+}$ and $Mg^{2+}$ free; pH 7.4), and resuspended at a concentration of $10^8$ CFU/mL in the same buffer. Twenty-five microliters of this cell suspension was added to wells of a microtiter plate containing serial dilutions (0.013 to ca. 7.0 g/L) of PT digest or related fractions. The total volume in the well was 100 $\mu$L, and the mixture was held for 30 min at room temperature. Following incubation, a drop of the suspension was applied to a microscope slide to count clumped and single cells. Agglutination tests were carried out in triplicate, and photographs were taken with a Diaphot-TMD inverted microscop (Nikon Corp., Tokyo, Japan).

## RESULTS

**Compositional Characteristics.** BF was chosen as an ingredient since it is tolerated by CS patients and since it is used for the manufacture of pasta in Italy (pizzoccheri); in Japan, as soba; and in Korea and China, as extruded noodles and cats' ears (*39*).

The moisture content of the two types of pasta (FP and CP) did not differ significantly ($P < 0.05$) (**Table 1**) and was within the maximum limit (12.5%) established by the Italian law for pasta made of durum wheat alone (*40*). As expected, BF had a higher and lower content of ash and protein than DWS, respectively. The concentration of ash of FDWS and related pasta increased due to the addition of sodium citrate as buffering agent. The concentration of protein of the two types of pasta was slightly lower than the minimal concentration of protein fixed by the Italian law (*40*) for pasta made of durum wheat alone (10.5% of dry matter [d.m.]). The concentration of protein of FP was also significantly lower than CP. The lower concentration of protein (9.8% of d.m.) of FP could be attributed either to mixing with BF or to hydrolysis during DWS fermentation. As determined by Gluten System GL analysis, the concentration of gluten of DWS was 12.1%. After fermentation and freeze drying, the same analysis carried out on FDWS (moisture 8%) revealed the absence of gluten. As compared to DWS (72% of d.m.), freeze-dried FDWS showed a decrease of the concentration of starch (60% of d.m.) also. The activity of wheat durum endogenous amylases may be expected before reaching very acidic values of pH. After 24 h at 37 °C, the pH of FDWS was ca. 3.2. Before freeze drying, sodium citrate was added as a buffering agent. Nevertheless, the acidity increased slightly during pasta manufacture and, especially, during drying at 25 °C for 24 h. As a consequence, CP and FP pastas had acidity values of 5.2 and 6.8 mL of 1 N NaOH/100 g of d.m., which exceeded slightly the maximum limit (4.0 mL of 1 N NaOH/100 g) fixed by the Italian law for pasta made of durum wheat alone (*40*).

**Table 2.** Properties of Gliadin Polypeptides Hydrolyzed by Selected Lactic Acid Bacteria during Fermentation of DWS[a]

| range spot[b,c] | range estimated pI | range estimated molecular mass (kDa) | range hydrolysis factor (%) |
|---|---|---|---|
| 1–6 | 6.31–7.12 | 59.50–68.00 | 95.0–97.0 |
| 12–17, 19, 21 | 8.50–9.87 | 55.30–54.90 | 72.1–98.0 |
| 11, 20 | 7.37, 7.75 | 55.30, 54.7 | 72.1, 73.0 |
| 22–26 | 6.37–7.12 | 54.60–54.70 | 90.4–98.0 |
| 27 | 6.75 | 54.58 | 62.0 |
| 28–30, 32–36 | 6.87–10.12 | 45.50–45.80 | 95.0–98.0 |
| 31 | 9.75 | 45.85 | 70.8 |
| 38–39 | 6.82–7.00 | 45.42–45.43 | 97.0–98.0 |
| 41–47, 49 | 6.22–8.37 | 45.00–45.20 | 95.0–97.0 |
| 48 | 9.62 | 45.00 | 74.5 |
| 50–51 | 7.62–7.69 | 44.78 | 55.0–56.4 |
| 52–53, 55–57 | 6.22–10.06 | 44.75–44.80 | 83.8–97.0 |
| 60 | 7.06 | 44.70 | 70.9 |
| 61–65 | 6.72–9.55 | 44.70 | 89.5–98.0 |
| 67–68, 71–76, 78–79 | 6.25–10.12 | 41.25–42.72 | 95.0–98.0 |
| 77 | 9.30 | 41.74 | 57.1 |
| 81–83, 85 | 6.37–9.87 | 40.00–40.65 | 76.3–97.0 |
| 84, 86–87 | 9.37, 7.05–9.87 | 40.65, 40.00–40.00 | 76.8, 59.6–76.3 |
| 88–90 | 6.37–6.75 | 38.00–39.50 | 95.0–96.0 |
| 91–92 | 6.61, 8.15 | 38.00 | 53.5, 35.4 |
| 93–103, 105–107 | 76.84–9.75 | 38.00–36.25 | 71.4–97.0 |
| 104, 108–109 | 9.30, 7.04–7.06 | 37.50, 36.00 | 71.4, 53.2–63.0 |
| 110, 113 | 8.26, 8.68 | 33.00 | 70.6, 74.1 |
| 111–112, 119, 121, 123–130 | 8.29–10.24 | 33.00–30.00 | 99.4–98.0 |
| 118, 120, 122 | 6.62, 7.06, 7.50 | 32.5 | 56.5, 51.3, 72.3 |

[a] Analyses were performed with Image Master software (Pharmacia). Four gels of independent replicates were analyzed. For spot quantification and hydrolysis factor calculation, see the Materials and Methods. All of the hydrolysis factors were calculated on the basis of the average of the spot intensities of each of four gels. [b] Spot designations correspond to those of the gels in **Figure 1B**,**C**. [c] Spots were clustered in function of the range estimated molecular mass.

**Proteolysis During DWS Fermentation by Lactic Acid Bacteria.** After fermentation of DWS for 24 h at 37 °C, the cell concentration of lactobacilli was ca. $10^9$ CFU/g, presumably of each species used in the mixture. Before freeze drying, gliadin fractions were selectively extracted from FDWS and further analyzed by 2DE. The same was carried out from DWS and CAD, before and after incubation for 24 h at 37 °C. The total bacterial count of CAD was constant at $10^3$ CFU/g during 24 h of incubation at 37 °C. As shown previously (*17*, *20*), biological or chemical acidification caused a marked modification of the 2DE polypeptide pattern as compared to the nonacidified dough; therefore, the FDWS was compared to CAD to find variations due to bacterial proteolysis mainly (**Figure 1A−C** and **Table 2**). By this comparison, changes due to proteolysis by flour endogenous enzymes were also excluded in part (*41*). Overall, 2DE analysis is currently used for quantification of human (*42*), vegetable (*43*), and microbial (*44*) proteins.

Gliadins are very heterogeneous proteins subdivided in α-, $\beta$-, $\gamma$-, and $\omega$-fractions. A total of 130 polypeptides was identified by 2DE analysis of CAD after 24 h of incubation at 37 °C, which was widespread throughout the pI from 6 to 11

(**Figure 1B**). The major part of gliadin spots (ca. 60) had molecular masses of 40−46 kDa, ca. 40 spots had 30−38 kDa, and some (ca. 25) spots were clustered in the range of 55−70 kDa. As expected, this polypeptide profile was only in part similar to that of the DWS (data not shown). As compared to CAD before incubation (**Figure 1B** vs **A**), the profile showed a decrease of the intensity or the disappearance of some proteins (ca. 22 spots), probably due to an activation of flour endogenous enzymes during 24 h at 37 °C. As compared to our previous 2DE gels on wheat bread flour (*Triticum aestivum*) (*17*, *20*), a higher number of gliadin spots (130 vs 29) were identified. These results could be due either to a more heterogeneous gliadin composition of DWS or, especially, to the use for 2DE of the pH gradient 6−11, which had a better resolution than the pH gradient 3−10 (data not shown) (*17*, *20*). Most of the gliadin polypeptides almost disappeared after 24 h of fermentation of DWS with selected lactobacilli (**Figure 1C** vs **B**). Of the total 130 polypeptides identified in CAD, 92 were characterized by hydrolysis factors higher than 80% and 22 had hydrolysis factors, which ranged from 50 to 80% (**Table 2**). Lower hydrolysis factors were not considered. 2DE analysis of FDWS by using the pH gradient 3−10 excluded the significant presence of polypeptide below pH 6.0 (data not shown).

Currently, mass spectrometry MALDI-TOF is the only nonimmunological technique for monitoring gliadins and related peptides in foods (*32*). Despite the great heterogeneity and the high genetic variability of the α-, *β*-, *γ*-, and *ω*-gliadin fractions, the MALDI-TOF analyses of gliadin ethanol extracts of the European standard and CAD showed characteristic protonated mass patterns around 33−55 kDa (**Figure 2A**,**B**). Polypeptides included in this molecular mass range are commonly used for the identification of gliadins in wheat-contaminated food samples. All of the gliadin peaks detected in CAD markedly decreased in FDWS (**Figure 2B**,**C**). Hydrolysis gliadin fragments, especially in the range of 21000−28000 mass *m*/*z*, were detected in FDWS.

The quantification of gliadins in CAD and FDWS was carried out by RP-HPLC also. Chromatograms of 1-propanol soluble gliadins are shown in **Figure 3A**,**B**. As compared to CAD, the amount of gliadins considerably decreased (ca. 70%) in FDWS, especially in the hydrophilic part of the gradient (65−80 mL). Protein peaks of FDWS (range 40−65 mL) were not specifically assigned to individual gliadin polypeptides of CAD but might derive either from glutenin contaminants (*45*) or from fragments of gliadin hydrolysis. A similar hydrolysis profile was found using ethanol soluble gliadins (data not shown).

CAD showed a slight increase of the concentration of free amino acids with respect to DWS (252 vs 191 mg/kg). An endogenous proteolytic activity could be suspected under acidic conditions (*41*). The concentration of free amino acids of the FDWS was 595 mg/kg. The major increases were for the concentrations of Gly, Val, Ile, Leu, Phe, Lys, and Pro.

**Gluten Concentration of Pastas.** The concentration of gluten in FP and CP pastas was determined by sandwich R5 ELISA. This analysis permits the quantification of prolamins in wheat, barley, and rye flours, and it is insensitive to prolamins of celiac-tolerated cereals (oats, maize, and rice) (*46*). Both types of pasta were manufactured mixing fermented or nonfermented DWS and BF in a ratio of 3:7. CP contained 6280 ppm of gluten. The R5 ELISA of FP showed a decrease of the gluten concentration to 1045 ppm.

**Sensory Analysis of the Cooked Pastas.** After cooking for 5 min, FP and CP fusilli type were subjected to sensory analysis by a trained panel test (**Table 3**). The sensory characteristics

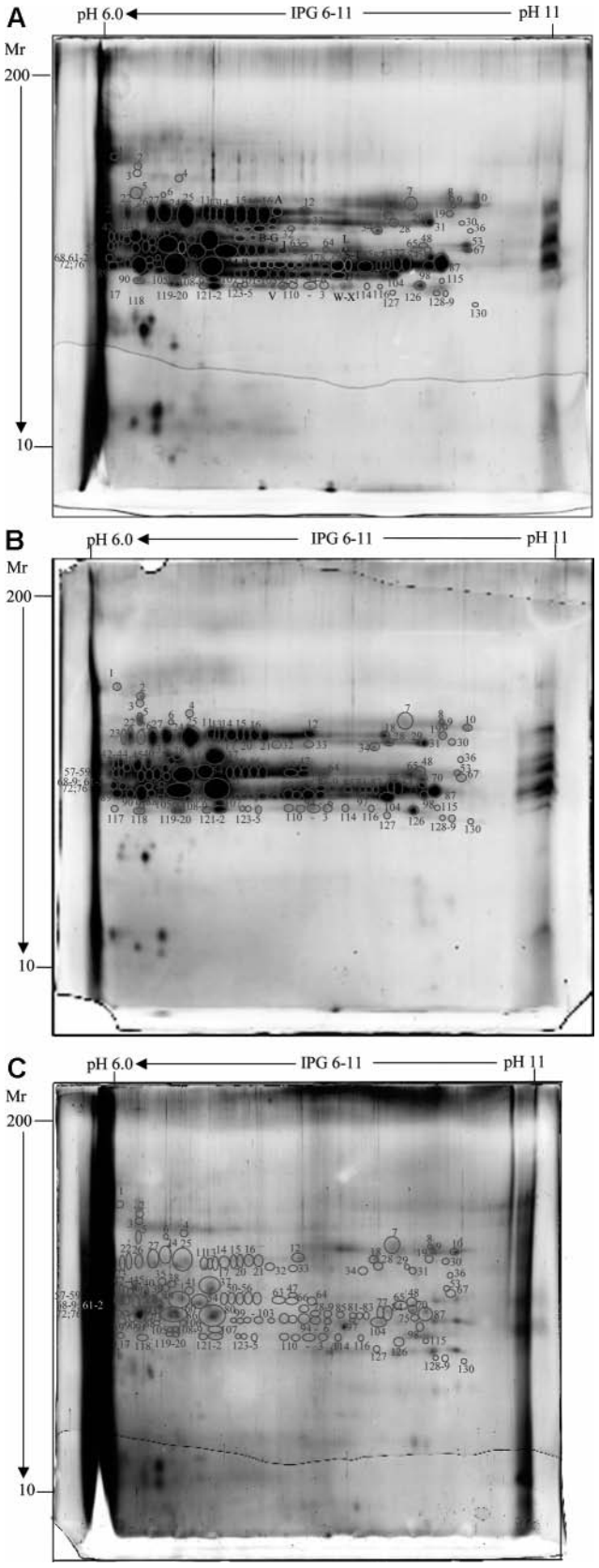


**Figure 1.** 2DE analysis of the gliadin protein fractions of different doughs made of DWS. CAD before (**A**) and after (**B**) incubation for 24 h at 37 °C and FDWS with the mixture of selected lactic acid bacteria for 24 h at 37 °C (**C**) were used. Gliadin polypeptides are shown by ovals. The numbered spots indicate gliadins degraded during fermentation by lactic acid bacteria, and lettered spots indicate gliadins degraded during incubation of CAD. Clustered spots are shown by a dash. Mr, molecular mass.

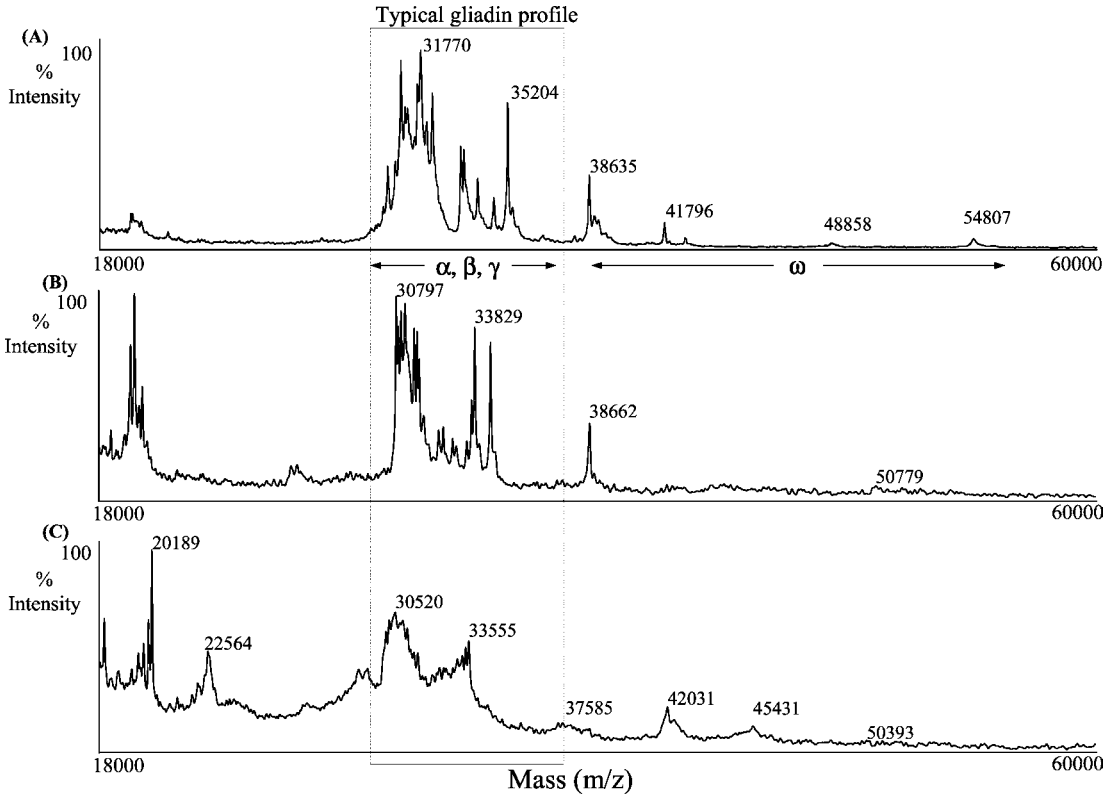


**Figure 2.** Mass spectrometry MALDI-TOF analysis of ethanol extract of wheat durum gliadin: (**A**) European gliadin standard showing the α-, β-, γ-, and ω-gliadin ranges; (**B**) CAD incubated for 24 h at 37 °C; and (**C**) FDWS with the mixture of selected lactic acid bacteria for 24 h at 37 °C. The typical α-, β-, and γ-gliadin profiles are displayed in the box.

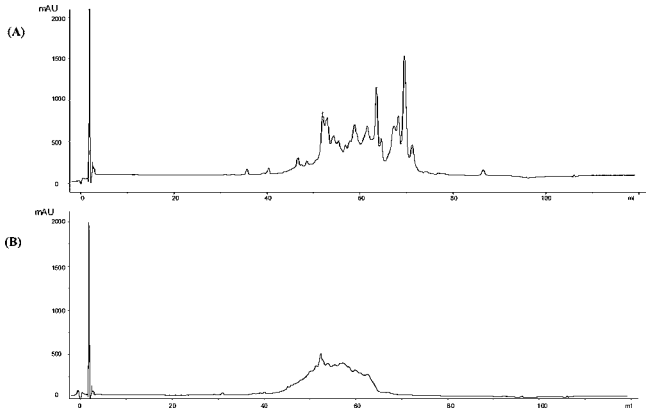


**Figure 3.** RP-HPLC analysis of 1-propanol extract of wheat durum gliadin: (**A**) CAD and (**B**) FDWS with the mixture of selected lactic acid bacteria for 24 h at 37 °C.

**Table 3.** Sensory Characteristics[a] of CP and FP

| pastas | stickiness | firmness | odor and flavor |
|---|---|---|---|
| CP | 70 ± 10.9 a | 63.3 ± 15 a | 60 ± 12.6 a |
| FP | 52.6 ± 7.5 b | 50.1 ± 11.3 b | 56. 7 ± 8.2 a |

[a,b] Average values ($n = 6$) ± standard deviations in the same column with different superscript letters differ significantly ($P < 0.05$).

were evaluated by a score of 20−100. FP received lower scores for stickiness (52.6 ± 7.5, average) and firmness (50.1 ± 11.3, sufficient) than those of CP, 70 ± 10.9 (almost absent) and 63.3 ± 15 (good), respectively. Nevertheless, odor and flavor of FP and CP did not significantly differ ($P < 0.05$) and were judged as sufficient.

**Agglutination Test.** Fifty milligrams of gliadin was extracted from DWS, FDWS, and CAD and subjected to PT degradation to simulate in vivo protein digestion (*47*). PT digests of DWS and FDWS did not agglutinate the K 562(S) subclone cells of human myelagenous leukaemia origin even at a concentration of 14 g/L. Therefore, PT digests were fractionated on a Sepharose-6-B mannan column. As shown in **Figure 4**, three polypeptide fractions were separated by affinity chromatography either from DWS or FDWS. Fraction A, eluted in the void volume of the column with 0.02 M ammonium acetate (pH 7.2) buffer, corresponded to ca. 1.5% of the peptides loaded; fraction B, eluted immediately afterward, accounted for ca. 92% of the total amount; and fraction C, eluted with 0.1 M acetic acid (pH 2.8), corresponded to ca. 1.5%. Individual fractions were then assayed for agglutinating activity. Fractions A and B were not active in agglutinating K 562(S) cells. On the contrary, fraction C of DWS had a minimal agglutinating activity (MAC) of 0.019 g/L. The same results were found by fraction C of CAD (data not shown). The MAC of fraction C from the PT digest of FDWS increased markedly to 1.5 g/L. The MAC of fraction C from DWS and FDWS is shown in **Figure 5**. Agglutinated cells showed a peculiar appearance, with a tendency toward the formation of a continuous layer and, when pipetted several times, had a distinct high resistance to shearing and whirling forces.

## DISCUSSION

A strict GFD is the current treatment for CS patients. Nevertheless, GFD seemed to be difficult if not impossible to maintain due to gluten contamination. The Committee for nutrition and foods for special dietary uses in the Codex Alimentarius allows a maximal content of 20 ppm gluten in products naturally free from gluten and 200 ppm in products

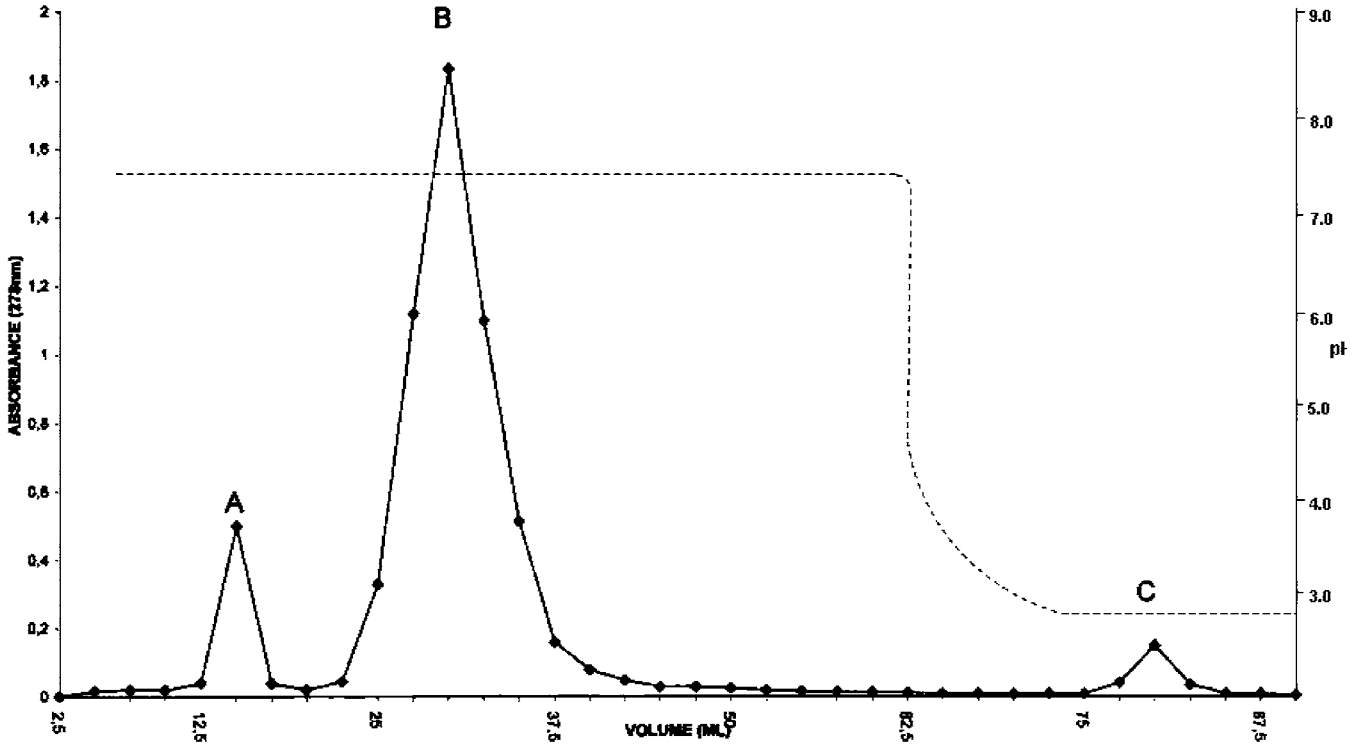


**Figure 4.** Fractionation of PT digest from DWS and FDWD by affinity chromatography with epoxy-activated Sepharose-6-B coupled with mannan. The elution profile shows that fractions A and B are eluted at a pH value of 7.2, whereas fraction C is only eluted at pH values between 5 and 3. The pH value was reported (- - -).

based on wheat starch, rendered gluten-free (*48*). This safe threshold is still debated and needs to be consolidated by emerging techniques for gluten quantification. A recent study by Størsrud et al. (*13*) showed that 30% of oat products in the market had a gluten content over 200 ppm. Most of the wheat starch-based gluten-free flours seemed to be contaminated with a concentration of gluten ranging from 10 to over 200 ppm (*12*). Although a single intake of small amounts of gluten may not cause damage to the intestinal mucosa of CS patients under GFD, care should be taken if gluten is ingested more frequently. Silent forms of the disease, which may expose people to long-term complications (*11*), and refractory sprue of a small percentage of patients, which continue to have symptoms and histologic abnormalities despite treatment with a GFD (*49*), are certainly influenced by a strict and sure GFD. In this context, research that is focused on the biological/biochemical reduction of a large part of gliadin polypeptides during food processing may represent one of the tools for increasing the human tolerance to gluten and for reducing the risk of gluten contamination.

To our knowledge, no studies have been carried out under this approach on DWS. This work was aimed at showing the ability of selected lactic acid bacteria to hydrolyze durum wheat gliadins and propose a novel protocol for manufacturing pasta, which may contain a certain level of durum wheat potentially more tolerated.

The role of bacteria in CS is still debated. Rod-shaped bacteria attached to the small intestinal epithelium of some untreated and treated CS patients ignited the notion that bacteria may be involved in the pathogenesis of celiac disease (*50*). Shan et al. (*4*) showed that the 33-mer peptide could be hydrolyzed by exposure to a prolyl-endopeptidase of *Flavobacterium meningosepticum*, suggesting a strategy for an oral peptidase supplement therapy. Later, Matysiak-Budnik et al. (*51*) showed that the hydrolysis of the 33-mer by the 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 the immune system. Previously (*17*), we showed that the pool of sourdough lactic acid bacteria used in this study, *L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A, and *L. hilgardii* 51B, had a pattern of specialized intracellular peptidases capable, after transport into the cytoplasm, of hydrolyzing all of the different bonds that potentially include the imino acid proline. The hydrolysis by the four lactobacilli also concerned the complete hydrolysis of polypeptides such as fragments 62−75 of A-gliadin and the 33-mer peptide.

Complementary techniques were used to detect the level of gluten contamination and the presence of gliadin epitopes. After fermentation by selected lactic acid bacteria, 2DE showed that 92 of the 130 identified gliadin spots identified in DWS had hydrolysis factors higher than 80%. Mass spectrometry MALDI-TOF and RP-HPLC analyses substantiated the considerable hydrolysis of the gliadin polypeptides. After hydrolysis, the liquid dough preferment was freeze-dried and mixed with BF (ratio of 3:7) to produce fusilli type Italian pasta. As compared to CP, the sandwich R5 ELISA analysis showed that the fermentation with selected lactobacilli caused a decrease of the gluten concentration of ca. 80%. A major advantage of the R5 monoclonal antibody is its ability to recognize the consensus amino acid sequence QXPW/FP (*52*) corresponding to multiple immunoreactive epitope repeats, which occur in α-, γ-, and ω-gliadins. The greatest reactivity has been associated with the QQPFP amino acid sequence, but homologous repeats such as LQPFP, QLPYP, QLPTF, QQSFP, QQTFP, PQPPP, QQPYP, and PQPFP are also recognized with a weaker reactivity by the R5 antibody (*52*). Three of these epitopes (LQPFP, QLPYP, and PQPFP) are placed in the sequence of the 33-mer peptide (*4*). Although the pasta fermented with lactobacilli still contained 1045 ppm of gluten, which may trigger CS, the use of a mixture,

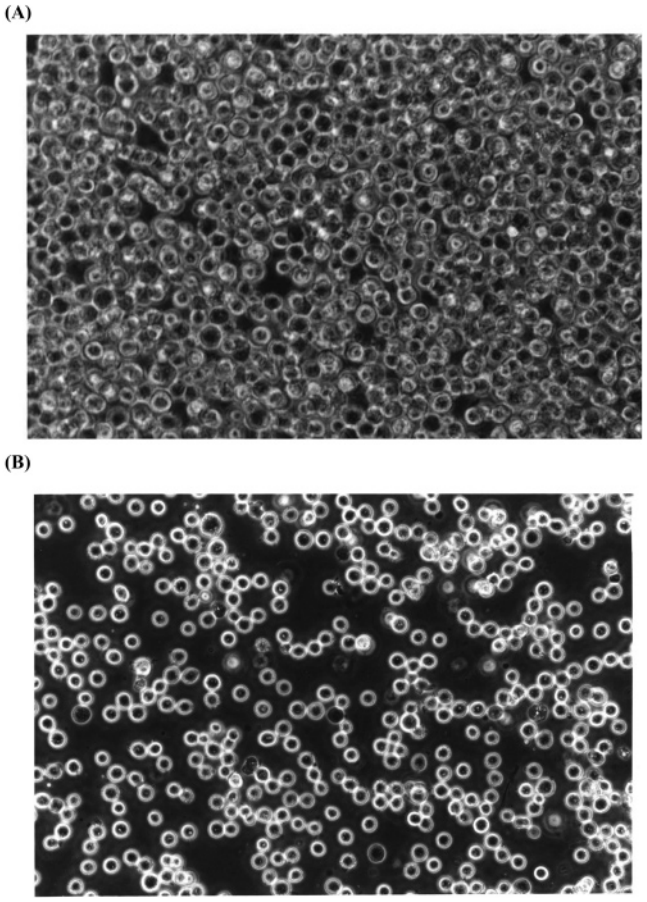


**Figure 5.** Agglutination test with K 562(S) cells. (**A**) Cells treated with the PT digest of the DWS at a concentration of 0.019 g/L. (**B**) Cells treated with the PT digest of the fermented durum wheat dough (FDWD) incubated for 24 h at 37 °C at a concentration of 1.5 g/L.

which includes 20% of FDWS in the pasta formulas may theoretically lead to a novel pasta product within the safe threshold for CS.

The commercially available gluten-free pasta is of a low sensory and cooking quality and, in Italy, ca. five times more expensive than normal pasta. We proposed a mixture of FDWS and BF. BF is known for its resistant starch and as an important source of antioxidative and dietary fiber substances (*39*). Buckwheat proteins have a high biological value and are suitable for increasing the low concentration of methionine and lysine in wheat flour (*53*). As shown by sensory analysis, the cooking properties (stickiness and firmness) of FP were lower but still appreciable with respect to CP. Preliminary results at an industrial plant showed that the cooking properties of FP were obviously lower than pasta made from DWS alone but slightly higher than commercially available gluten-free pasta. FP was palatable as CP without statistically significant differences in odor and flavor. Besides, compositional analyses did not show marked differences with respect to the limits fixed by Italian law for pasta made of durum wheat alone.

Most investigators would agree that in vivo testing such as direct instillation/biopsy is the "gold standard" for assessing the celiac toxicity of proteins or peptides (*1*, *11*), but ethical reasons are the most crucial limiting factors. Although we cannot substitute an ultimate in vivo test, in vitro tests such as the culture of tissue from human cells are a valuable aid in the search for potentially toxic or nontoxic factors. Overall, a relatively high correlation was found between the agglutination activity of cereal components against K 562(S) subclone cells of human myelagenous leukaemia origin and their toxicities in clinical and in vitro trials on the basis of biopsy samples of intestinal mucosa from CS patients (*2*, *18*, *19*). The same correlation was found in our previous report on wheat bread, which considered in vivo challenges also (*17*). Contrary to wheat bread flour, the whole PT digest of DWS and FDWS did not agglutinate K 562(S) cells. This confirmed the previous findings of De Vincenzi et al. (*36*) on 12 different varieties of durum wheat, which showed the presence of a 1157.5 Da peptide, which had the capacity to prevent the agglutination by PT digests of cereals (e.g., bread wheat, rye, and barley) not tolerated in CS patients (*47*). This peptide, LGQQQPFPPQQP, prevented the agglutination of K 562(S) cells by the fragment 31−43 of A-gliadin also (*19*). The results of this study showed that the protective peptide was also present in the PT digest of DWS, which had been subjected to fermentation by lactic acid bacteria. Digestive enzymes (pepsin and trypsin) are probably responsible for its generation. Affinity chromatography on Sepharose 6-B-mannan separated the fraction C of both DWS and FDWS, which caused agglutination. The MAC of the fraction C from the PT digest of FDWS was ca. 80 times higher than that of DWS. Although the presence of nontolerated polypeptides cannot be excluded in both fractions A and B of the Sepharose 6-B-mannan separation of DWS and FDWS, this study and previous findings (*19*, *36*) showed that their eventual effect on agglutination test is attenuated by the LGQQQPFPPQQP peptide. On the other hand, sandwich R5 ELISA showed a significant decrease of the gluten concentration in the pasta fermented with lactobacilli.

Most of the medicine specialists such as gastroenterologists and pediatricians are rightly focused on epidemiology, clinical significance, genetics, diagnosis, and innovative treatments of CS (*1*, *11*). Nevertheless, it should be considered that bread, pasta, or other wheat-made products are manufactured following different biotechnological options, which may influence differently the tolerance to CS. First, these results showed that a biotechnology, which uses selected lactic acid bacteria to ferment durum wheat semolina and tolerated BF, could be considered a novel tool for the manufacture of pasta, which may decrease the level of intolerance to gluten and reduce the risk of gluten contamination.

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