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IMPACT OF GLUTEN-FRIENDLY™ TECHNOLOGY ON WHEAT KERNEL ENDOSPERM AND GLUTEN PROTEIN STRUCTURE IN SEEDS BY LIGHT AND ELECTRON MICROSCOPY

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Short title: Gluten-friendly technology and wheat endosperm and gluten protein structure

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ABSTRACT

The main aim of this paper was to assess the impact of Gluten-Friendly™ (GF) technology (Italian priority patent n° 102015000084813 filed on 17th December 2015) on wheat kernel endosperm morphology and gluten protein structure, using SEM, light and immunofluorescent microscopy. Microscopy was combined with immunodetection with specific antibodies for gliadins, _-gliadins, LMW subunits and antigenic epitopes to gain a better understanding of the technology at molecular level. Results showed significant changes to gluten proteins after GF treatment; cross-reactivity with antibodies that recognize both the almost entire range of gluten proteins and antigenic epitopes through the sequences QQSF, QQSY, PEQPFPQGC and QQPFP was significantly reduced. The present study confirms the results from our previous and showed,

for the first time, the mechanism by which a chemical-physical treatment abolishes the antigenic capacity of gluten.

Keywords: gluten-friendly, SEM, immunofluorescent microscopy, antigenic epitopes

1. INTRODUCTION

Wheat endosperm contains 8–15% protein, 80% of which is made up of gluten. Gluten consists of aqueous alcohol-soluble gliadins and insoluble glutenins. Gliadin is a mixture of heterogeneous monomeric proteins, divided into _/_, γ and ω-gliadins, whereas _/_, γ-gliadins are low-molecular-weight proteins (MW 28–35 kDa) with six and eight cysteine residues, respectively. ω-gliadins (MW 40–75 kDa) do not contain cysteine. Glutenin is a polymeric protein composed of high-molecular-weight (HMW-GS, MW 65–90 kDa) and low-molecular-weight glutenin subunits (LMW-GS, MW 30–45 kDa) linked by inter-chain disulphide bonds (Wieser, 2007). Gluten's unique physico-chemical properties make wheat suitable for preparing a wide range of food products, including breads, noodles, pastas and biscuits. Although gluten is used extensively in food products, it has been associated with coeliac disease (CD) in some individuals. Coeliac disease is a chronic immune-mediated enteropathy triggered by the ingestion of gluten in HLA-DQ2- or HLA-DQ8-positive subjects, mainly resulting in small-intestinal mucosal injury and nutrient malabsorption in susceptible individuals (Rossi & Schwartz, 2010). Approximately 30% of the general population carry the HLA-DQ2/8 coeliac disease susceptibility genes; however, only 2–5% of these individuals will go on to develop coeliac disease, suggesting that additional environmental factors contribute to disease development (Rossi & Schwartz, 2010). The only effective treatment available for CD patients is strict exclusion of gluten from their diet. The detrimental consequences of consuming gluten and/or analogous proteins (present in rye, barley and oats) are well-documented, showing that noncompliance with a gluten-free diet is associated with increased risk of anaemia, infertility, osteoporosis and intestinal lymphoma (Maki, & Collin, 1997).

An alternative way to exclude gluten is to detoxify it without affecting the technological performances of the resulting flour and dough. Recently, we have developed a new and innovative detoxification method of gluten proteins from cereal grains (Italian patented method n°:

0001414717, also filed under the Patent Cooperation Treaty, application no. PCT/IB2013/000797) (Lamacchia, Di Luccia, & Gianfrani, 2015a; Lamacchia, Di Luccia, & Gianfrani, 2013), aiming to combine the nutritional and technological properties of wheat proteins with safety for coeliac sufferers and other gluten-sensitive subjects. This innovation is usually referred to as "gluten friendly™"and relies on the application of microwave energy for a few seconds to hydrated wheat kernels before milling to reach a high temperature for a short amount of time and induce a structural change in gluten proteins (Lamacchia, Landriscina,& D'Agnello, 2016). This modification abolishes the antigenic capacity of gluten (Lamacchia et al., 2016) and reduces *in vitro* the immunogenicity of the most common epitopes involved in coeliac disease (Lamacchia, Di Luccia, & Gianfrani, 2015b), without compromising the nutritional or the technological properties necessary to process semolina in pasta and flours in bread and other baked goods (Lamacchia et al., 2016). Proteins are present in the wheat in their native form, as they are located in protein bodies, thus preventing microwave treatment from causing protein denaturation. However, the treatment was able to reduce gluten's antigenicity and this effect was attributed to a structural change in the protein (Lamacchia et al., 2016) Moreover, Gluten Friendly^{TM} bread was also able to partly correct and positively modify the quali-quantitive composition of the microbiota of coeliac people in a model system (Bevilacqua et al. 2016a). However, no data are available on the change occurring in grain seeds after Gluten Friendly^{TM} processing. Therefore, this paper addresses the effect of Gluten-Friendly™ technology on wheat endosperm morphology and gluten protein structure in seeds, by SEM, light and fluorescence microscopy. Immunodetection experiments with specific antibodies for gliadins, _-gliadins and LMW subunits were carried out to gain a better understanding of the technology at molecular level and the mechanism by which it abolishes the antigenic capacity of gluten.

2. MATERIALS AND METHODS

2.1 Raw materials and microwave treatment.

The wheat kernels (mixtures of soft wheat Canadian grains) used in this study were supplied by the Casillo group S.p.A. (Corato, Italy). Grains treated with microwaves were called Gluten-Friendly Grains (GFG) and were obtained by treating the caryopses, previously harvested and

threshed, with microwave energy (Italian patented method n°: 0001414717 also filed under the Patent Cooperation Treaty, application no. PCT/IB2013/000797). The technology has since been further improved (Italian priority patent n° 102015000084813. Method for the detoxification of gluten proteins from grains of cereals and related medical uses filed on 17th December 2015. Inventor: Lamacchia C.). Specifically, 100 g of cleaned wheat grains were dampened to 15-18% moisture; moisture was evaluated using a Halogen Moisture Analyzer (Mettler Toledo HB43-S, Switzerland). The seeds were heated with microwaves (DeLonghi, Italy, for about 1 min. between 1000 and 750 watt), followed by a phase of slow evaporation of the water content. Rapid heating and slow evaporation was repeated up to a final temperature of 80-90°C, as measured with a thermal camera (FLUKE i 20, Italy), and a moisture level of 13-13.5%.

After microwave treatment, the wheat kernels were cooled and dried at room temperature (24 °C) for 12-24 h. Seeds were left at room temperature until use. Grains before treatment were considered Control Grains (CG). After the treatment both CG and GFG were analysed using different approaches: a) SEM (Critical Point Drying and Immunogold) (section 2.2); b) immunofluorescence (section 2.3); c) light microscopy (section 2.4). The last step was the digital analysis of the data (section 2.5) and the use of different multivariate approaches (section 2.6) to pinpoint significant differences between CG and GFG.

2.2 SEM (Scanning Electron Microscopy) assays

The preliminary step for SEM assays is the freeze fracturing; it was performed by dropping seeds (six per treatment, CG and GFG, respectively) into liquid nitrogen for thirty seconds and a single, sharp tap was administered to the grain in order to produce a fracture. Samples were then sputter-coated with gold and imaged in the FEI Quanta FEG 600 Environmental Scanning Electron Microscope equipped with a Quorum PP2000T Cryo Stage.

For Critical-Point drying (CPD) analysis, the grains (six per treatment, CG and GFG, respectively) were fixed for 3h in 2.5% glutaraldehyde in 25 mM HEPES buffer at pH 6.9, 4°C, and then rinsed in HEPES buffer and distilled water for 15 min. The specimens were then dehydrated through a 10% ethanol series at room temperature (10% to 100% ethanol series) and dried in the critical-

point dryer (31°C/ 73.8 bar). Samples were then sputter-coated with gold and imaged in the JEOL JSM-6360 LV using a dry stage setting at a high voltage of 20.0 kV.

For immunogold analysis, freeze-fractured grains (five per treatment, CG and GFG, respectively) were fixed in formalin vapour for 96 hours by placing them in sealed Petri dishes containing a smaller open dish with 1 ml formalin. Grain pieces were stored in a desiccator containing silica gel until use, to prevent absorption of water from the air. Grains were briefly rinsed in PBS (Phosphate-buffered saline: 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) and then blocked in a 3% (w/v) solution of Bovine Serum Albumin (BSA) in PBST for 1 hour at room temperature on an orbital shaker. Grains were briefly rinsed in PBST (PBS, 0.1% Tween-20) and then incubated with mouse monoclonal antibodies in PBST for 1h at room temperature with agitation.

The antibodies used and their dilutions were as follows: i) 1:100 IFRN 0610 mouse monoclonal antibody which recognizes an epitope (QQSF) common to many gliadins and to LMW-GS but not to HMW-GS; ii) 1:100 S3B512 mouse monoclonal antibody, raised against a peptide from the _-gliadin repetitive domain (PEQPFPQGC) specific for _-gliadins (INRA, Nantes, France).

Grains were washed in PBST and then incubated for 1 hour with an anti-mouse IgG gold conjugate (2 nm) diluted 1:50 from goat, used at 1:5000 dilution. After rinses in PBST and distilled water, the gold signal was silver-enhanced using the Aurion silver enhancement kit for 30 min at 22°C. After repeated rinsing with distilled water, the samples were dried in a desiccator. Dried samples were coated with carbon, using a carbon rod evaporator. The grains were then imaged in the JEOL JSM-

6360 LV using a dry stage setting.

2.3 Immunofluorescence

The samples were prepared as reported by Palmer, Cornuault, Marcus, Knox, Shwery & Tosi (2016), slightly modified as follows. Transverse sections (approx. 1 mm thick) were cut from the middle of each seed. Sections were fixed for 8 h at room temperature in 2.5 % (w/v) paraformaldehyde and 0.5 % (w/v) glutaraldehyde in 0·1 M Sorenson's phosphate buffer (prepared with $NaH_2PO_4.2H_2O$ and $Na_2HPO_4.12H_2O$), pH 7.2. After three rinses in buffer, the

specimens were dehydrated in an ethanol series, infiltrated with LR White Resin (medium grade, TAAB L012) for several days and polymerized at 55 °C.

Fixed and resin-embedded grain sections were briefly rinsed with PBST [PBS, Sigma A4417, 0·1 % (v/v) Tween 20, pH 7·4] and incubated in blocking solution [5 % (w/v) BSA (Sigma A7638) diluted in PBST] for 40 min at room temperature. This step was followed by incubation in the primary antibodies diluted in 1 % BSA (w/v) in PBST for 2 h at room temperature. Mouse monoclonal anti-IFRN 0610 (1:100), and mouse monoclonal anti-_-gliadin (1:100) were individually tested. The unbound primary antibodies were removed by several rinses with PBST for a period of 20 min. The sections were then incubated for 1 h at room temperature in the dark with the secondary antibodies (Alexa Fluor 568 goat anti-mouse IgG; Invitrogen A-11001, A-11004) diluted 1:250 in 1% BSA in PBST. Finally, the slides were rinsed twice with PBST and three times with PBS.

Sections were examined with a Zeiss Axiophot epifluorescence microscope. A Retiga Exi CCD digital camera (QImaging, Surrey, BC, Canada) and MetaMorph software version 7·5·5 (Molecular Devices, Sunnyvale, CA, USA) were used to acquire the images.

2.4. Colorimetric assay for light microscopy

Semi-thin (1 μm) sections were cut using a Reichert-Jung Ultracut ultramicrotome, collected on drops of distilled water on multi-well slides coated with poly-L-lysine hydrobromide (Sigma P1399) and dried on a hot plate at 40 °C. Sections for general morphology and observation of protein bodies were stained with 0·01 % (w/v) Toluidine Blue in 1 % (w/v) sodium tetraborate, pH 9, and examined with bright-field optics on a Zeiss Axiophot microscope. Three independent batches (each batch was a seed from a different variety) were evaluated; for each seed, three different sections were analysed.

Grain sections collected on poly-lysine coated slides were pre-incubated for 30 min in a blocking solution made up of 0.3% Tween20 (Sigma-Aldrich) and 3% BSA (Sigma-Aldrich) in phosphate-buffered saline (PBS) (Sigma-Aldrich) solution at pH 7.4. Samples were then incubated for 2 h in primary R5-HRP conjugated monoclonal antibody, and diluted 1:100 in antibody buffer (1% BSA,

0.05% Tween 20 (Sigma-Aldrich) PBS solution). After three washes in a 0.3% Tween 20 PBS solution, samples were incubated in 1-Step™ Ultra TMB-Blotting revelation solution for 2 minutes and then analyzed with bright-field optics on a Zeiss Axiophot microscope. Three independent batches (each batch was a seed from a different variety) were tested; for each seed, three different sections were analysed.

2.5 Image Processing assay

Three images from each sample in immunofluorescence and light microscopy (colorimetry) were converted into 8-bit grey-scale images; then, the average MGV (Mean Grey Value) was evaluated by using a Digital Image Processing and Analysis ImageJ, an open source java program.

2.6 Statistical analysis

Statistics were performed in immunogold, colorimetric and immunofluorescence experiments.

Immunogold analysis: five grains from each treatment (CG and GFG) were considered;for each seed, three different area were analysed. Mean differences were compared by unpaired Student's t-test. A p-value < 0.01 was considered significant.

Colorimetric and immunofluorescence experiments: three grains from each treatment (CG and GFG) were considered; for each seed, three different sections were analysed. These data were used to run a Monte Carlo simulation and build a statistical population with at least 300 data points.

The results from antibody assays were preliminarily tested to verify the normal distribution of the population and then analysed through a multifactorial ANOVA using treatment (CG or GFG) and variety (variety 1, variety 2 or variety 3) as categorical predictors. Tukey's test with $P<0.05$ was used as the *post-hoc* comparison test to pinpoint the significant differences. The significance of each predictor and their interaction (variety *vs* treatment) was evaluated through the Fisher test and p-value: the quantitative effect (i.e. how and when the response of the antibody assays increased or decreased) was assessed by the graphs of the decomposition of the statistical hypothesis. A graph of hypothesis decomposition shows the effect of each predictor *per* time as

an XY plot, where X is the predictor (for this analysis treatment or variety) and Y the response (mean values ± 95% confidence intervals) (Bevilacqua et al., 2016).

The results from image processing were converted into an arbitrary scale from 0 to 4 to assess the presence of protein body type 1 (CG) or type 2 (GFG). These results were analysed through the Friedman's nonparametric test ($P<0.05$) and reported as median, minimum, and maximum values, 1^{st} and 3^{rd} quartiles of the statistical population. Finally, control and gluten-friendly image processing data were analysed through a Principal Component Analysis. Statistics were performed using the Statistica for Windows software ver. 12.0 (Statsoft, Tulsa, OK, USA).

3. RESULTS AND DISCUSSION

3.1 Effect of Gluten-Friendly™ technology on wheat kernel endosperm morphology

Gluten-Friendly™ technology could induce a significant change in gluten proteins (Lamacchia et al., 2016), thus endosperm structure was analyzed by SEM (Scanning Electron Microscopy) to better understand the effect of this technology. SEM provides unique three-dimensional views of membranes and cytoplasm, otherwise not available with thin-sectioned endosperm. Several samples of CG (control grains) and GFG (gluten-friendly grains) were cleaved transversally into two halves and prepared for CPD (Critical-Point Drying) and freeze-fracturing analysis, and SEM-analyzed at a high voltage of 20.0 kV. CPD preserves the structure of cells with minimum alteration from the natural state with regard to volume, morphology and spatial relationships of organelles and macromolecules, minimum loss of tissue contents, and protection for samples against subsequent treatments including rinsing, dehydration, vacuum and exposure to the electron beam (Hayat, 1989). Fig. 1 shows representative micrographs of CG and GFG with textural patterns of the pericarp, aleurone, subaleurone and starchy endosperm in the mature grain. GFGs did not present differences from CGs showing to be very well preserved after microwave treatment.

The aleurone layer is the outermost cell layer of the endosperm tissue; its cells appear square or rectangular and are characterized by thick cell walls. Subaleurone cells, which are peripheral endosperm cells, constitute one cell layer, made up of cells that are smaller than the subsequent inner endosperm cells. The endosperm cells below the subaleurone cells are prismatic, while central cells are more variable in shape. Fig. 1 pinpoints the differentiation of starch granules into

two main size categories: type A (large granules) which are typically 10-25 μm in size, and type B (small granules), ranging between 2-10 μm. Starch granules did not differ in number and shape in GFG and CG samples; moreover, they were dispersed into the continuous protein matrix (mainly proteins and residual cytoplasmic constituents).

The profiles of the freeze-fractured cleaved seeds were also studied, as shown in Fig. 2. Freeze-fracturing provided a technique to study the morphology of endosperm tissue in GFG samples, without many of the problems associated with fixed tissues. In fact, structural features may represent artefacts in chemically-fixed material (Bechtel & Barnett 1986; Bechtel 1983; Miflin, Field & Shewry, 1983). Currently, the most effective alternative to chemical fixation is cryofixation. All freezing methods have the common aim of preserving the sample in its native/original state without artefacts. Samples can be visualized in their native hydrated state without pre-treatment, preserving the natural morphology of cells and tissues. This effect is obtained by removing heat at such a rapid rate that water molecules form amorphous vitreous ice, thus avoiding the destructive effects of crystalline ice on cellular ultrastructure. When rapid freezing attains the vitrification temperature, viscosity reaches a level which prevents movement, thus immobilizing all the molecules in a cell within milliseconds (Moor, 1987). Thus, a freeze-fracturing technique was used and replicas of unfixed wheat endosperm revealed a different pattern of protein matrix in endosperm tissue. Specifically, the results showed (Fig. 2) a different protein matrix deposition pattern in GFG compared to CG samples. Micrographs of GFG clearly demonstrate the presence of a thick, confluent protein matrix in prismatic endosperm cells, with complete embedding of the starch granules. These results are in accordance with previous works that suggest that under heat stress, the endosperm of the kernels might appear increasingly aggregated, with the starch granules embedded in the protein matrix and a dense cellular structure (Pyler, 1988, Dias & Lidon, 2009). The different results obtained with CPD and freeze-fracturing may be explained by the fact that CPD treatment is more invasive, exposing samples to chemical reagents and dehydrating forces, that could break the interactions among aggregated proteins.

3.2 Effect of Gluten-Friendly™ technology on gluten protein structure

Gluten contains hundreds of proteins, which are present either as monomers or as oligomers/polymers, linked by inter-chain disulphide bonds (Wieser, 2007; Shewry & Halford, 2002) and characterized by high levels of glutamine and proline (namely prolamin) residues, clustered in either polyglutamine sequences or in repeating glutamine/proline sequences. The effects of Gluten-Friendly™ technology on gluten protein structure was evaluated by *SEM –Immunogold* and *Immunofluorescence* experiments with two monoclonal antibodies specific for repeated sequences common to gliadin and LMW fractions. We used the IFRN 0610 monoclonal antibody, that recognizes epitopes QQSF, QQSY which are common to many gliadins and LMW-GS, but not HMW-GS, and the mouse _-gliadin monoclonal antibody, that recognizes the repetitive domain PEQPFPQGC present in the _-gliadin fraction. The protocol was carried out on five CG and GFG samples to evaluate the differences in the amount of specific labelling.

Fig. 3 shows the results from *SEM-Immunogold* analysis. Fig. 3A shows CG and GFG samples after specific labelling with the two antibodies. Energy Dispersive Spectroscopy (EDS) confirmed that it was silver-enhanced gold labelling (and not an artefact). This technique works by detecting an alternative signal given off by the sample (X-rays) rather than those usually detected for producing high-quality images (secondary electrons). The X-rays emitted by a sample after irradiation with the electron beam are unique to each element, and therefore elemental identification is possible by detecting these X-rays' energy and comparing them to known standards. A typical EDS trace is shown in Figure 3B. The sections labelled in Fig. 3A indicate a point on the surface of the endosperm which was chosen to be analyzed with EDS. The trace in the same figure shows the elements present in the labelled area (65x51nm). This trace shows many elements are present, including carbon, oxygen and silver, confirming that the flecks previously assumed to be silver-enhanced gold labelling were indeed so. EDS was used for each sample shown in the following result to confirm the suspected labelling. Fig. 3A shows that labelling can be observed within the protein matrix situated between the starch granules, but not on the starch granule surface itself, nor anywhere else in the grain, indicating that specificity has been maintained. Labelling present on the protein matrix of CG samples could be defined as "heavy" compared to GFG samples. The results are summarized in Fig. 3C and show a significant ($p<0.01$) decrease in silver concentration in GFG seeds compared with CG, after labelling with the two

antibodies. The decrease was about 89% for monoclonal antibody 0610, and 87% for the _-gliadin antibody, respectively.

Fig. 4 A shows the results from *Immunofluorescence* experiments. In GFG samples, a strong and significant decrease in signal intensity was observed after labelling with antibody 0610 and the antibody specific for _-gliadin compared to the CG samples, in agreement with the *SEM* results. Labelling was measured by quantifying the light signal and correlating it to a greyscale, through the ImageJ software. Data reported in Fig.4B expressed as MGVs (mean grey values) summarize this reduction: 91.71% ($p < 0.001$) and 90.61% ($p < 0.001$) for 0610 and _-gliadin antibodies, respectively. Fig 4B shows the hypothesis decomposition trend and, for each treatment, three seeds from three different varieties are reported (sample 1, sample 2, sample 3). Only the predictor "treatment" (control or gluten-friendly) played a significant role in the decrease in MGV, whereas the effect of variety was not significant.

These results confirm that Gluten-Friendly™ technology induces significant changes in gluten proteins, thus reducing cross-reactivity with antibodies that recognize almost the entire range of gluten proteins through the sequences QQSF, QQSY, PEQPFPQGC. Lamacchia et al. (2016) suggested that the brief high temperatures generated by microwaves applied to the hydrated caryopses lead to conformational and/or structural changes in the proteins, as evidenced by the increase in total cysteine levels. Furthermore, the analysis by SDS-PAGE of protein fractions under reducing and non-reducing conditions showed that microwave treatment causes neither a decrease in the number of the bands nor a decrease in their intensity (Lamacchia et al., 2016); thus, a rearrangement of the secondary and/or tertiary structure in gluten proteins with a different spatial conformation of the aminoacid sequences could be suggested.

Enlargement of part of the micrographs (Fig. 5) highlighted another interesting feature, i.e. a strong merging of protein bodies in the CG and GFG samples. Gluten proteins comprise the major grain storage proteins, which are deposited in protein bodies in the developing starchy endosperm. The individual protein bodies range in diameter up to about 20 μm. However, as the endosperm cells fill with starch, the protein bodies should become disrupted and finally coalesce to form a matrix of storage proteins surrounding the starch granules in the mature dry tissue (Shewry, Tatham, Barro, Barcelo, & Lazzeri, 1995). Indeed, micrographs of the samples revealed that the

coalescence of protein bodies in mature wheat grains was complete and resulted in a continuous proteinaceous matrix surrounding the starch granules. Nevertheless, it was possible to still distinguish protein bodies (Fig. 5 A) and highlight differences in the merging level of protein bodies in GFG with respect to CG samples (Fig. 5 A). In the GFG samples, protein bodies (PB-type2) were completely merged in the protein matrix, which appeared more homogeneous and confluent, showing a higher level of aggregation when compared to CG samples (PB-type1). Data were collected on four semi-thin sections (1µm) of six different CG and GFG samples. The differences were pointed out through Friedman's nonparametric test, because a preliminary statistic revealed that the scores in the arbitrary scale did not follow a normal trend. The Friedman test was run on the median value, as reported in Fig 5B and highlighted a strong increase in PB-type 2 in GFG. This result was also confirmed by using variety as an input value, thus suggesting that technology and not variety plays a fundamental role in protein aggregation (data not shown). A multivariate approach (PCA) confirmed that PB-type 1 and PB-type 2 were negatively related.

These results suggest that aggregation may be the primary cause for the reduced cross-reactivity of proteins, rather than a change in secondary and/or tertiary structure. In any event, these findings are in accordance with analyses performed with SEM using freeze-fracturing techniques and with the fact that gluten proteins are susceptible to heat treatment and polymerize during heating (Schofield, Bottomley, Timms, & Booth, 1983). Gluten protein behaviour when subjected to relatively high temperatures has been studied by a number of workers and indeed the incorporation of gliadin monomers in the glutenin network through covalent bonds has been highlighted (Singh& MacRitchie, 2004; Redl, Morel, Bonicel, Vergnes, & Guilbert, 1999). However, Lamacchia, Baiano, Lamparelli, La Notte & Di Luccia (2010) showed, for the first time, that the application of high temperature to wheat grains generated protein polymerization in which gliadins did not cross-link with glutenins. The explanation for this phenomenon was that, within the kernel, the gluten protein network has not yet formed and gluten proteins are deposited in different protein bodies. Furthermore, Lamacchia et al., (2016) confirmed these results, showing that the application of a short burst of high temperature to mature hydrated wheat kernels using microwave energy leads to protein polymerization between proteins of the same class (i.e. gliadin-gliadin, glutenin-glutenin, albumin-albumin) and only through disulphide bonds (evidenced by the SE-HPLC chart and SDS-PAGE analysis). However, this does not seem to be in accordance with

the findings from the SEM and immunofluorescent experiments where images show a strong aggregation among all wheat kernel proteins. One explanation for this could be that the strong aggregation between different classes of proteins in seeds is due to ionic and/or hydrophobic interactions, caused by a rearrangement of the secondary and/or tertiary structure of the gluten protein molecules during heating. Such aggregation was not visible through SE-HPLC and SDS-PAGE (Lamacchia et al., 2016) because of the use of sodium dodecyl sulphate-denaturing conditions in the extraction buffer.

3.3 Effect of Gluten-Friendly™ technology on gluten protein antigenicity

The short sequence of amino acids that binds with the antibody to elicit immune reaction is called the antigenic epitope. The most common antigenic epitopes of gluten proteins are HLA DQ2 (Human Leukocyte Antigen), characterized by multiple proline (Pro) and Gln residues (Kim, Quarsten, Bergseng, Khosla, & Sollid, 2004). Tanabe (2008) also reported Gln-Gln-Gln-Pro-Pro as the major motif of wheat gluten protein to act as an antigenic epitope.
Currently thirty-one aminoacid peptide sequences in the prolamins of wheat and related species have been defined as being coeliac-toxic: these are often referred to as “coeliac epitopes”. However, mapping is incomplete and the number of distinct epitopes is a matter of ongoing discussion (Sollid, Qiao, Anderson, Gianfrani, & Konig, 2012).

These epitopes are located in the repetitive domains of the prolamins, which are proline- and glutamine-rich, and the high levels of proline in their sequences may reduce their susceptibility to protease activity in the GI tract. The prolamin-reactive T-cells (T-lymphocytes) of CD patients also recognize these epitopes to a greater extent when specific glutamine residues in their sequences have been deamidated to glutamic acid by a tissue transglutaminase (tTG2). This binding enables the formation of a stable peptide-MHC complex, which is important in the anti-prolamin T-cell response (Sollid et al., 2012).

The effect of Gluten-Friendly™ technology on gluten protein antigenicity was evaluated by *Light Microscopy* using the R5 monoclonal antibody which recognizes the most repetitive potential coeliac-toxic pentapeptide epitope, glutamine-glutamine-proline-phenylalanine-proline (QQPFP) in gluten proteins and the epitopes LQPFP, QLPYP and PQPFP in the sequence of the recently-described potent inducer of gut-derived human T-cell lines in coeliac patients, the A gliadin 33-

mer peptide of residues 57–89 (LQ LQPFPQPQLPYPQPQLPYPQPQL–PYPQPQPFP) (Arentz-Hansen, Korner, Molberg, Quarsten, Vader, & Kooy, 2000). Light microscopy was chosen because the R5 monoclonal antibody was provided horseradish peroxidase-conjugated. A colorimetric assay was developed, where the antibody was revealed with a specific enhanced single-component horseradish peroxidase (HRP) substrate for immunohistochemistry. The revealing solution, which contains soluble TMB (3,3',5,5'-tetramethylbenzidine), reacts very quickly with horseradish peroxidase enzyme to produce an insoluble dark blue precipitate.

Results are shown in Fig. 6A, where it was possible to detect a decrease in the signal emitted in GFG samples, compared to CG, both in the subaleurone and the crease portion of the seeds. The colour intensity was then measured through the ImageJ software. Data reported in Fig. 6B, expressed as mean grey values, summarize this reduction: 89.19% ($p<0.001$) for the subaleurone layer and 81.6% ($p<0.001$) for the crease tissue. The reduced gluten antigenicity in GFG samples observed with the R5 colorimetric assay was in accordance with the *SEM–Immunogold* and *Immunofluorescence* experiments and with Lamacchia et al. (2016) that showed a drastic reduction (99%) in the levels of detectable proteins, treated with Gluten-Friendly™ technology, tested for their ability to bind the specific monoclonal antibody R5 in a sandwich Elisa test. R5-Elisa includes an extraction procedure allowing aggregates of gliadins generated by the heat process to be solubilized (Valdés, García, Llorente, & Méndez, 2003). On this basis, a rearrangement of the secondary and tertiary structure in gluten proteins, with a different spatial conformation of the toxic sequences, induced by Gluten-Friendly™ technology, and not a strong aggregation among proteins, could explain the significant reduction in antigenicity in GFG samples and therefore a significant reduction in cross-reactivity of gluten proteins with their own antibodies. This is well supported by the fact that the availability of antigenic fragments in gluten depends on the secondary and tertiary structure of the protein as well as on the SS bonds that stabilize particular conformations of epitopes to bind with the antibody (Waga, 2004). The reduced gluten antigenicity observed in this study also confirms preliminary results showing that flours from grains treated with Gluten-Friendly technology did not induce production of the inflammatory cytokine, interferon gamma, when tested, after digestion and deamidation, on gut-derived human T-cell lines of coeliac patients highly reactive to 33-mer, omega and gamma peptides (Lamacchia et al., 2015).

5. CONCLUSION

The present study allows to gain a better understanding of the Gluten-Friendly™ technology at molecular level and the mechanism by which it abolishes the antigenic capacity of gluten.

Different microscopy techniques used in this study show that Gluten-Friendly™ technology induced a significant aggregation of proteins in seeds and deep changes to the gluten protein structure. Protein bodies were completely merged in the protein matrix, which appeared more homogeneous and confluent when compared with control samples. The cross-reactivity of gluten proteins with antibodies that recognize both the almost entire range of gluten proteins and antigenic epitopes was significantly reduced.. Data collected in this study suggest that chemical changes and reduced cross-reactivity of gluten proteins with their specific antibodiesare caused by a rearrangement of the secondary and tertiary structure, involving a different spatial conformation of the sequences, also of the so-called antigenic ones. These changes may allow a new kind of aggregation among different classes of wheat endosperm proteins, only through hydrophobic and/or ionic interactions.

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FIG 1

FIG 2

FIG 3

FIG 4

FIG 5

FIG 6

FIGURE CAPTIONS

1. SEM of transversally-cleaved CPD-treated Control Grain (CG) and Gluten-Friendly Grain (GFG) samples, (a, b) showing the pericarp and aleurone, subaleurone and endosperm cells x50; (c,d) Aleurone cells (Al) and the outermost endosperm cells (subaleurone layer) (x500); (e,f) Large starch granules (LS) and small starch granules (SS) as well as protein matrix (PM) in endosperm tissue (x1000); (g,h) A higher magnification of large and small starch granules and protein matrix (x2000).

2. SEM of transversally-cleaved freeze-fractured Control Grain (CG) and Gluten Friendly Grain (GFG) samples; (a) CG subaleurone cells rich in protein matrix (1500x); (b) (c) (d), GFG subaleurone cells completely embedded into a confluent protein matrix (1500x). Bars in grain samples correspond to 50.0µm.

3. (A) SEM-Immunogold labelling in Control Grain (CG) and Gluten Friendly Grain (GFG) samples with corresponding Energy Dispersive Spectroscopy (EDS) trace. (1) CG subaleurone immunogold labelled with IFRN 0610 antibody; (2) CG subaleurone immunogold labelled with

_-gliadin antibody; (3) GFG subaleurone immunogold-labelled with IFRN 0610 antibody; (4) GFG subaleurone immunogold labelled with _-gliadin antibody. Bars in grain samples correspond to 50 μm. **(B)** Energy Dispersive Spectroscopy (EDS) trace corresponding to SEM-Immunogold labelling in CG and GFG samples. **(C)** Histogram summarizing the atomic silver % (mean ± SD) detected with Energy Dispersive Spectroscopy (EDS) in CG and GFG samples after the labelling with 0610 and _-gliadin antibodies. A p-value < 0.01 was considered significant.

4. (A) Micrographs of Control Grain (CG) and Gluten Friendly Grain (GFG) semi-thin slices immunolabelled with 0610 and _-gliadin monoclonal antibody in immunofluorescence experiments. (1) CG subaleurone immunolabelled with IFRN 0610 antibody; (2) GFG subaleurone immunolabelled with IFRN 0610 antibody; (3) CG subaleurone immunolabelled with _-gliadin

antibody; (4) GFG subaleurone immunolabelled with _-gliadin antibody; (5) CG starchy endosperm immunolabelled with IFRN 0610 antibody; (6) GFG starchy endosperm immunolabelled with IFRN
0610 antibody.
(B) Two-way ANOVA: Hypothesis decomposition for the interaction kind of sample (1, 2, or 3) and treatment (CG and GFG samples) on the MGV (mean grey value). Bars denote 95% -confidence intervals.

5. (A) Enlargement of part of micrographs of Control Grain (CG) and Gluten-Friendly Grain (GFG) semi-thin slices immunolabelled with 0610 and _-gliadin monoclonal antibody in immunofluorescence experiments showing a strong merge of protein bodies in CG and GFG samples, respectively. (1,2) Protein bodies type 1 (PB-type1) from CG samples; (3,4) Protein bodies type 2 (PB- type2) from GFG samples. **(B)** Box-Whisker plots on the protein bodies. The differences were pointed out through Friedman's nonparametric test, which highlighted significant differences between CG and GFG samples for both PB-type1 and PB-type 2. PB-type1 and PB-type 2 showed discontinuous scores, ranging from 0 to 5.

6. (A) Micrographs of Control Grain (CG) and Gluten Friendly Grain (GFG) semi-thin slices immunolabelled with R5 monoclonal antibody. (1) CG subaleurone layer; (2) GFG subaleurone layer; (3) CG crease portion; (4) GFG crease portion. Bars in cross-sections correspond to 100 μm.

(B) Two-way ANOVA: Hypothesis decomposition for the interaction kind of sample (1, 2, or 3) and treatment (CG and GFG samples) on the MGV (mean grey value). Bars denote 95% -confidence intervals.