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Effectiveness of Germination on Protein Hydrolysis as a Way to Reduce Adverse Reactions to Wheat

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Abstract

In this work, the aim is to study the effectiveness of germination on wheat protein degradation, with a specific focus on proteins involved in adverse reactions to wheat. The effect of 8 days of germination at 25 °C on the chemical composition and the protein profile were determined. Germination showed a reducing effect on starch content, while it had not a significant effect on protein, lipid and ash contents. General protein profile, as indicated by gel analysis, revealed that germination induced a relevant degradation in protein fraction. After *in vitro* gastrointestinal digestion, gluten peptides involved in celiac disease (CD) were identified and quantified using UPLC/ESI-MS technique. Also, CM3 protein, involved in baker's asthma and intestinal inflammation, was quantified by measuring a marker peptide. Statistical analysis underlined that germination and genotype had significant impact on the amount of both components. Regarding gluten peptides related to CD, germination enabled an average reduction of 47% in peptides eliciting adaptive immune response and 46% in peptides eliciting innate immune response. CM3 protein showed also a high average reduction (56%). Thus, this study suggests that germination might be a good bio-alternative to provide a low "impact" raw ingredient for special wheat based foodstuffs.

Keywords: Germination, Wheat, Celiac disease, Gluten, CM3 α -amylase /trypsin inhibitor, Mass Spectrometry.

35 Introduction

36 The major wheat protein is gluten, which includes two classes of proteins, the monomeric
37 gliadins, soluble in aqueous alcohols, and the polymeric glutenins, insoluble in aqueous
38 alcohols^{1,2}. Gluten is a key functional parameter for wheat technological quality. Indeed, the
39 viscoelastic properties of doughs are largely determined by the gluten proteins, which form a
40 continuous proteinaceous network³.

41 Gluten, or rather the peptides arising from its incomplete digestion, is also the major trigger of
42 celiac disease (CD). CD is the most common autoimmune disease worldwide, affecting
43 approximately 1% of individuals⁴. Currently, CD is one of the most studied gluten related
44 disorders. Indeed, in celiac subjects, gluten ingestion induces chronic autoimmune responses
45 that can manifest in a variety of ways and affect multiple organ systems⁵. Genetically, CD is
46 strongly linked to the HLA locus, specifically HLA-DQ2 (A1*0501–B1*0201) and/or HLA-
47 DQ8 (A1*0301–B1*0302)⁶. To date, thirty-one sequences of nine amino acids have been
48 defined in the gluten (gliadins and glutenins) of wheat and related species (e.g., barley, rye,
49 oat...) as being celiac triggering peptides, often referred to as celiac “epitopes”⁷.

50 Besides CD, which is a genetic autoimmune disorder, wheat proteins might be involved in
51 IgE-mediated food allergies⁸. Protein allergens are found also in the soluble fraction
52 (albumins and globulins) such as α -amylase/trypsin inhibitors (ATIs)⁹. Indeed, wheat
53 amylase/trypsin inhibitors drive intestinal inflammation, via activation of the toll-like receptor
54 4 on myeloid cells^{10,11}. CM3 (chloroform/methanol soluble), a type of the wheat ATIs, is one
55 of the major wheat allergens, being implicated in both atopic dermatitis¹² and baker's asthma
56 ¹³.

57 According to the indication of *Codex Alimentarius* (CODEX STAN 118-1979)¹⁴, below the
58 level of 20 ppm gluten (an amount considered to be safe for celiac subjects), a material can be
59 labelled as “gluten-free”. Because wheat is a major staple food, several attempts have been
60 made to convert wheat flour into a gluten-free raw material¹⁵. Indeed, breeders have been
61 doing extensive works on gluten epitopes genes silencing^{16,17,18}. Research was also done for
62 studying biotechnological processes based on enzymatic degradation of gluten epitopes or
63 modification of their structure, such as sourdough fermentation using a selected pool of
64 *Lactobacilli*, malting and brewing^{19,20,21,22,23}. Several authors have also investigated the
65 proteolytic activity in germinating kernels and their effect on gluten abolishment^{24,25,26},
66 concluding that wheat enzymes involved in germination enabled the toxicity reduction of
67 prolamins^{24,25}. Anyway, in these previous studies, attention was mainly attributed to the
68 isolated protease from germinating wheat kernels as a potential enzymatic treatment to reduce
69 cereal allergenicity, rather than on the use of germination process itself. Regarding protein
70 soluble fraction, germination of brown rice grains induced the degradation of some allergens
71 (14–16 kDa and 26 kDa proteins) by endogenous proteolytic activity and heat-processing²⁷.
72 Furthermore, germinated wheat-based foodstuffs are an emerging trend in the food with
73 health benefits because germination might enhance the nutritional value, such as increasing
74 the folate content, as well as improving the flavour^{9,28,29,30}.

75 In the present work, the scope was to investigate germinated whole grain flour as a possible
76 low immunogenic raw ingredient. A comparative study was done between germinated and not
77 germinated wheat whole flours. First, changes induced in chemical composition by
78 germination were investigated in terms of starch, protein, lipid and ash contents. The
79 influence of the proteolytic activity on wheat protein profile was also studied by using SDS-
80 PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Then, the content of
81 CM3 protein and gluten peptides, were also investigated using *in vitro* digestion and ultra-

82 performance liquid chromatography/electrospray ionization-mass spectrometry (UPLC/ESI-
83 MS).

84 **Material and methods**

85 **Plant material**

86 Wheat samples were four tetraploid *Triticum* species: three modern and one ancient. The three
87 modern durum wheat genotypes were D240, Levante and Svevo, and their ancestors of origin
88 were Syndioux/Mahmoudi/Langdon 341, G80/Piceno/Ionio, and Linea Cimmyt/Zenit,
89 respectively. The ancient wheat was Kamut® (Khorasan wheat).

90 **Wheat kernels germination procedure**

91 Wheat kernels germination was conducted following the method of Schwalb and others²⁶
92 with slight modifications. Grains (20 g) were germinated for 8 days at 25 °C. Briefly, grains
93 were soaked in deionized water (100 mL) for 5 h, the water was decanted, and, after washing
94 with fresh water, the grains were left to equilibrate for 19 h at 100% humidity. Soaking and
95 equilibration were repeated for 5 and 20 h, respectively. The wet grains were finally soaked
96 for 10 min and transferred into a plastic container for 6 days. Germination was stopped by
97 pouring liquid nitrogen onto the grains, which were then crushed, lyophilized, and milled.
98 Germinated grains were stored at -20 °C until further studies.

99 **Chemical composition**

100 Starch, protein, lipid, ash and moisture contents were determined using near-infrared (NIR)
101 spectroscopy (TANGO, Bruker Optik GmbH, Ettlingen, Germany).

Before analysis, a calibration set of ground whole wheat samples was used. Then, the spectra were collected and analysed using the spectroscopic software Opus 5 (Bruker Optik GmbH, Ettlingen, Allemagne). Partial least square regression prediction models were developed for each wheat components using QUANT software (version 7, Bruker Optik GmbH, Ettlingen, Germany).

Four determinations were performed for each sample. Data were expressed as g/100 g dry basis (db).

Total protein extraction

Ground wheat (10 mg) (before and after germination) were extracted with 1 mL of extraction buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% w/v sodium dodecyl sulphate, 350 mmol/L dithiothreitol) and incubated for 1 h at room temperature. Samples were centrifuged at 23,897g for 10 min at room temperature and supernatants were stocked at -20 °C for the analysis. Two determinations were performed for each sample.

Protein sequential extraction

Wheat proteins were fractionated into albumin, globulin and gliadin, as described by Lookhart and Bean³¹ with slight modifications. Flour (100 mg) were extracted with deionized water (500 µL) for 30 min and centrifuged for 5 min at 479 g. The supernatant was saved and the pellet was vortexed with 400 µL of deionized water and centrifuged as before. Extraction was repeated two times with 400 µL water. The three supernatants were poured off and saved as albumin. The pellet from albumin was then extracted with an aqueous solution of 0.5 mol/L sodium chloride (400 µL) for 30 min and centrifuged for 5 min at 479 g. The supernatant was decanted and saved. This operation was repeated two times. The three supernatants were saved as globulin. The pellet from globulin was extracted with 70% v/v aqueous ethanol (400

μL) for 30 min and centrifuged for 5 min at 479 g. The supernatant was decanted and saved. This step was redone two times. The three supernatants were poured off and saved as gliadin. Glutenins were extracted as described by Wieser and others³² from the gliadin pellet with modifications (60 min instead of 30 min): 1 mL of a solution containing 50% (v/v) 1-propanol, 0.05 mol/L Tris-HCl (pH 7.5), 2 mol/L urea, and 1% (w/v) dithiothreitol and was added to the samples, which were kept for 60 min at 60 °C. The suspensions were centrifuged for 20 min at 15,000 g. This step was repeated and the two supernatants were poured off and saved as glutenin. Two determinations were performed for each sample.

SDS-PAGE

Protein contents were quantified using a Q-bit fluorometer (Invitrogen, Grand Island, NY, USA) according to the manufacturer instructions. The volume of gel-ready protein was computed considering that the quantity required in each well was 20 μg. If the required volume was higher than 10.5 μL, it was dried under nitrogen flux. Samples were reconstituted with 15 μL of reducing sample buffer (protein concentration in the sample buffer was 1.3 μg/μL); then, the extracts of the entire protein fraction and fractionated proteins (albumins, globulins, gliadins and glutenins) from wheat samples (before and after germination) were subjected to SDS-PAGE. SDS-PAGE analysis was performed according to the instruction by manufacturer (Bio-Rad). In brief, for each well of the Criterion XT 12% Bis-Tris (bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane) Precast gel (Bio Rad), 15 μL of sample (containing 20 μg of proteins, sample buffer, XT Reduction buffer) and 5 μL of protein standard were loaded. The running buffer was 2-(N-morpholino)ethanesulfonic acid (XT MES). The voltage applied to the Criterion Cell was 150 V and the run lasted 60 min. Later, gels were placed in plastic containers, covered with the staining solution, and allowed to soak for at least 3 hours. The staining solution was made up of 0.1% w/v of Coomassie brilliant

blue R-250 dissolved in 10% acetic acid, 40% methanol and 50% deionized water. For the destaining steps, gels were rinsed with a solution of 10% acetic acid, 40% methanol, 50% deionized water, until the desired contrast was achieved.

Quantification of gluten peptides associated with CD in digested samples

Synthesis of the standards used as internal calibration

For CD-related peptides, the chosen standard was the most abundant immunogenic peptide (TQQPQQPF(d_5)PQQPQQPF(d_5)PQ) labelled on the two phenylalanine residues. For CM3 protein, the marker peptide sequence was FIA(d_3)LPVPSQPVDPR. The standards were synthesized following the method of Prandi and others³³.

Standardized static *in vitro* digestion method

In vitro digestion was performed for wheat samples before and after germination, following the standard method of Minekus and others³⁴. Briefly, 1 gram of sample was weighted and placed for 2 min with 1 mL simulated saliva containing amylase (75 U/mL of digesta) in the incubator at 37°C; then, 2 mL of simulated gastric juice containing pepsin (2000 U/mL of digesta) were added, the pH was adjusted to 3 and the mixture was incubated for 2 h at 37°C. Afterward, 4 mL of duodenal juice containing pancreatin (100 U trypsin activity/mL of digesta) and bile (10 mmol/L in the total digesta) were added, the pH was adjusted to 7 and the mixture was incubated for 2 h at 37°C. To inactivate the enzymes, the digested samples were heated for 10 min at 95°C, and then centrifuged (3220g, 4 °C, 45 min). For the UPLC/ESI-MS analysis, 295 μ L of each sample supernatant were added to 5 μ L of the standard solution (TQQPQQPF(d_5)PQQPQQPF(d_5)PQ, 1.6 mmol L⁻¹). Two determinations were performed for each sample.

UPLC/ESI-MS analysis and data processing

According to Prandi and others³⁴, the complex mixture obtained from enzymatic cleavage was separated by a RP column (ACQUITY UPLC BEH 300, C18, 1.7 mm, 2.1×150 mm equipped with a ACQUITY UPLC BEH C18 VanGuard Pre-column, 130Å, 1.7 µm, 2.1 mm×5 mm, Waters, Milford, MA, USA) in a UPLC/ESI-MS system (Acquity Ultra-performance UPLC with a single quadrupole mass spectrometer SQD, Waters, Milford, MA, USA) using a gradient elution. Eluent A is a deionized water solution with 0.1% formic acid and 0.2% acetonitrile, and eluent B is an acetonitrile solution with 0.1% formic acid. Gradient elution was carried out as follows: 0-7 min 100% eluent A; 7-50 min from 100% to 50% eluent A; 50-52.6 min 50% eluent A; 52.6-53 min from 50% to 0% eluent A; 53-58.2 min 0% eluent A; 58.2-59 min from 0% to 100% eluent A; 59-72 min 100% eluent A. The samples are analysed with UPLC/ESI-MS in the Full Scan mode. Flow is 0.2 mL/min; analysis time 72 min; column temperature 35°C; sample temperature 18°C; injection volume 2 µL; acquisition time 7-58.2 min; ESI positive mode; scan range 100-2000 m/z; capillary voltage 3.2 kV; cone voltage 30 V; source temperature 150°C; desolvation temperature 300°C; cone gas flow 100 l/h; desolvation gas flow 650 l/h. The areas of the identified peptides and standards used as internal calibration (TQQPQQPF(*d*₅)PQQPQQPF(*d*₅)PQ and FIA(*d*₃)LPVPSQPVDPR) were integrated with the MassLynx software (Waters, Milford, MA, USA). The semi-quantification value was obtained as the ratio peptide area/standard area multiplied by the moles of standard. Two determinations were performed for each sample.

Quantification of CM3 in samples**Salt protein extraction**

Ground wheat samples before and after germination (0.5 gram) were extracted with 10 mL of sodium chloride aqueous solution (0.5 mol L^{-1}) for 2 h and 30 min at room temperature. The mixture centrifuged at 3320g for 15 min at 4°C . Then, the supernatant was stocked at -20°C .

Enzymatic cleavage

According to Prandi and others³⁵ method, 1 mL of each extract was dried under nitrogen flux and dissolved in 500 mL of hydrochloric acid 10 mmol/L (pH 2); then 20 μL of a 1 mg/mL pepsin solution (489 units/mL) were added and the mixture was incubated for 3 h at 37°C . Then, 300 μL of sodium dihydrogenphosphate 100 mmol L^{-1} (pH 7.2), 20 μL of a 1 mg/mL chymotrypsin solution (59 units/mL) and 20 μL of a 1 mg/mL trypsin (1749 BAEE/mL) solution were added and the mixture was incubated for 4 h at 37°C . The samples were boiled in a water-bath for 5 min at 95°C to inactivate the enzymes. Then, samples were dried under nitrogen flux and reconstituted with 300 μL of a 0.1% formic acid solution, centrifuged at 15093g for 10 min (4°C) and the supernatant was saved for UPLC/ESI-MS analysis. For the UPLC/ESI-MS, 250 μL of supernatant were spiked with 5 μL of labelled standard as an internal calibration (FIA(d_3)LPVPSQPVDPR) solution (1.28 mM)). The quantification value was obtained as the ratio peptide area/standard area multiplied by the moles of standard. Two determinations were performed for each sample.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS for windows version 11.0; SPSS Inc, Chicago, Illinois, USA) was used for the statistical analyses. The fixed effect Multivariate Analysis of Variance (MANOVA) model was conducted on 13 parameters (7 immunogenic, 3 toxic, total immunogenic, total toxic and total immune-toxic epitopes). The percentage of total variation was computed to explain the variance of each epitope as a function of the main and interaction effects. The level of significance was expressed as significant at $p < 0.05$ and

highly significant at $p < 0.001$. Significant differences among the mean values were calculated using Duncan's test.

Results and discussion

To the purpose of this work, first wheat kernels were germinated in a lab-scale incubator to maintain a stable germination process. Moreover, germination process was performed for a total duration of 8 days because it was previously reported that these conditions enabled important proteolytic activity thereby extensive gluten epitopes hydrolysis^{26,36}. Germination process was subdivided in two phases; steeping or soaking phase (48 h), where the grains were steeped at 25°C for 48 h (5 h wet, 19 h air, 5 h wet, 19 h air), and germination phase, where the grains were left to germinate for 6 days at 25°C, with watering once a day. In this study, besides germination, we assessed the effects of variety (old and modern) on quantitative features associated to chemical composition and immunogenic potential. For each parameter, non-germinated and germinated wheat were compared to underline the germination influence.

Effect of germination on the chemical composition

Chemical changes induced by wheat kernels germination were investigated in terms of starch, protein, lipid and ash contents (g/100g db) (Table 1). Multivariate analysis revealed that genotype had highly significant effect ($P < 0.001$) on the chemical composition of wheat samples, while germination effect was not significant. Results showed that germination induced a slight change in starch, protein, lipid and ash content due to the increasing activities of enzymes (e.g., amylases, proteases and lipases) during germination, to ensure nutrients the growth of the embryo^{37,38}.

Effect of germination on the protein profile

Whole protein extracts as well as protein fractions of germinated and non-germinated wheat were subjected to SDS–PAGE. Figure 1 evidenced that germination induced significant changes on the protein profile. Although the protein pattern was not altered, several bands were vanished or less intense after germination. Indeed, upon imbibition, the important secretion of hydrolytic enzymes drives the degradation of storage compounds, including starch, lipid, and protein, for seedling establishment³⁹. Gliadin and glutenin were remarkably reduced in terms of band intensity because storage proteins are partially hydrolysed to water-soluble polypeptides, and further degraded into amino acids⁴⁰.

Identification of gluten peptides related to CD

After the *in vitro* digestion, the generated peptides were analysed using UPLC/ESI-MS. The identified gluten peptides related to CD were presented in Table 2.

The identified peptides belonged to two categories namely, immunogenic (triggering the adaptive immunity) or toxic (triggering the innate immunity) peptides. These T-cell epitopes are derived from γ -gliadins and α -gliadins. Furthermore, the major part of the identified epitopes was HLA-DQ2.5-restricted gluten T-cell epitopes. Indeed, these sequences are rich in glutamine and proline, which make them more resistant to gastrointestinal digestion and consequently, induce the immune response in the case of CD subjects⁴¹. All the identified immunogenic peptides derived from γ -gliadin. It was also observed that the sequence QQPQQPFPQ, related to the response DQ2.5-glia- γ 4c, was found in all the identified immunogenic sequences. On the other hand, the toxic peptides derived from α -gliadin. The sequence QQQP was observed in TP1 and TP3, while the sequence PSQQ was only found in TP1 and TP3. These two sequences were exclusively identified in the toxic peptides but absent in the non-toxic peptides⁴².

Furthermore, it should be kept in mind that the peptides pattern generated is closely related to the method of digestion as previously demonstrated by Prandi and others³⁵. Indeed, the present work did not include all the possible immunogenic and toxic peptides generated. However, it took in consideration the most studied epitopes found in literature as well as their relative abundance.

Effect of germination on gluten peptides related to CD

Due to the unavailability of all standards necessary, a semi-quantification of gluten peptides, before and germination, was performed. Indeed, this approach was adopted to enable the comparison between the samples before and after germination, which was the aim of this work. The obtained results were in concordance with previous works^{35,43}.

Afterward, to assess the effectiveness of the proteolytic activity in gluten epitopes hydrolysis, the reduction factor was calculated based on the data reported in Table 3. As a result, 8 days of germination reduced total peptides, with a range from 35 % (Kamut®) to 62 % (Svevo). It was also found relevant reduction in terms of total toxic peptides, with a range from 30% (Kamut®) to 79% (Svevo). As well, the total immunogenic peptides were considerably reduced with a variability range from 36 % (Kamut®) to 58 % (Svevo). Consistently, experiments using isolated gliadins and glutenins or celiac-toxic peptides as substrates had showed in the past that these proteases enabled the degradation of toxic peptides into non-toxic fragments with less than nine amino acids²⁴. Further, gliadin treated by germinating wheat enzymes showed 83% degradation of the 33-mer immunodominant α -gliadin peptide²⁵. Schwalb and others²⁶ revealed also that the immunodominant ω -5 gliadin was almost totally degraded after 7 days of durum germination. Overall, also consistently with our results, the proteolytic activity allowed a drastic reduction in gluten peptides associated with CD, but

it remained unsafe for celiac patients. However, it might find applications as a food ingredient for market for partially detoxified foodstuffs.

Gluten peptide amounts of non-germinated and germinated kernels were statistically analysed using a multivariate analysis to determinate the effects of genotype, germination and their interaction. As a result, Pillai's trace test showed that studied parameters had significant effect on the identified peptide amounts (genotype: Pillai's trace value=2.97, $p<0.001$, germination: Pillai's trace value=1, $p<0.001$; their interaction: Pillai's trace value=2.98, $p<0.001$, respectively). The implication of genotype, germination and their interaction in the amounts of identified peptides related to CD was explained using the sum square percent (Table 4). Regarding immunogenic peptides, genotype and germination had the greatest impact (48.80% and 45.89%, respectively). On the other side, the proteolytic activity showed slightly superior effect on the immunogenic peptide IP4 (TQQPQQPFPQQPQQPFPQ) which was identified as the peptide with the highest relative abundance, consistently with previous work^{35, 41}. Otherwise, IP2 (SQQPQQPFPQPQ) was majorly controlled by genotype (84.43%). However, the interaction between germination and genotype had a weak influence on the total immunogenic (5.30%). Besides, this interaction had no significant impact on some immunogenic peptides namely, IP 1, IP2 and IP6. As for toxic peptides, genotype was the leading factor (61.17%) compared to germination (30.77%) and their interaction (8.06%). Overall, it could be concluded that the degradation of gluten epitopes was controlled at 46.60% by the proteolytic activity of germinated wheat and 47.20% by genotype effect. Only 6.20% was under the control of their interaction suggesting that germination effect on gluten epitopes reduction was not correlated to genotype.

Effect of germination on CM3 protein

Soluble protein extracts of germinated and non-germinated kernels were also digested *in vitro*, and then analysed by UPLC/ESI-MS to assess the amount of CM3 protein, following a published procedure³⁶. In this previous study³⁶, the production of the marker peptide during digestion reached a steady state, and consequently it might be considered that the amount of the peptide was quite similar to the amount of the protein. Therefore, for the purpose of this work, a marker peptide (FIALPVPSQPVDPR) deriving from the digestion of CM3 was quantified in each sample. Then, the obtained values were used as a semi-quantification of the CM3.

Results revealed that significant differences were observed in CM3 contents between wheat genotypes before and after germination. Further, the combined analysis of fixed effects showed that genotype, germination and their interaction had significant impact on CM3 protein. Indeed, the partitioning of corrected total sum of square (CTSS) revealed that the germination effect accounted for 58.52% of the CTSS, and genotype effect presented 32.96% of CTSS. Their interaction effect represented only 8.51% of CTSS, indicating that germination reducing-effect was not conditioned by certain genotype, and consequently these findings emphasised the relevant influence of germination on CM3 independently of the genotype choice. As a result, Figure 3 showed important reduction in CM3 protein after 8 days of germination for all the studied genotypes. Indeed, Levante recorded the highest reduction of CM3 protein (71%), followed by Svevo (61%), Kamut® (47%) and D240 (43%). These results might be attributed to the proteolytic activity; indeed, proteases were responsible of the degradation of ATIs into peptides and amino acids to be used in embryo development⁴⁴. Further, it should be taking into consideration that a marker peptide was used for the quantification of CM3 in the samples. Therefore, an overestimation of CM3 after germination might be possible, if protease degraded the protein CM3 in the sample, but the investigated peptide remained intact

The present study highlighted that germination appeared to decrease starch content, but no significant effect was found on protein, as and lipid contents. Moreover, SDS-page electrophoresis revealed that germination caused significant degradation of protein fractions. Interestingly, it was demonstrated that germination had relevant effectiveness on wheat components associated with CD, baker's asthma and intestinal inflammation. Indeed, the comparison of the identified gluten peptides related to CD, before and after germination, showed important reduction. Likewise, CM3 amounts were severely reduced after germination. These findings underlined the effect of proteolytic activity on proteins-derived epitopes hydrolysis.

Despite the important reduction of wheat immunogenic components, germinated kernels are not a safe product for celiac patients. However, it could be considered a good raw ingredient for genetically predisposed subjects or people following gluten-free lifestyle, not medically motivated. Further proper immunologic testes are required to confirm these *in vitro* findings such as CD4+ T cells clone lines, agglutinating activity K562, and cytokine production.

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Tables caption

Table 1: Changes in durum wheat composition induced by germination (expressed as g/100 g). Means of same column of the same section followed by different letters differ significantly ($p < 0.05$).

Table 2: List of the identified gluten peptides from wheat related to CD. The list of peptides is subdivided into classes immunogenic associated with the adaptive response and toxic associated with the innate response.

Table 3: A semi-quantification of each immunogenic, toxic and total peptides in the different varieties tested before and after germination. TIT: total immunogenic and toxic peptides. Results are indicated as average of two replicates. Values of same column of the same section followed by different letters differ significantly ($p < 0.05$).

Table 4: F significance level and sum square percent of genotype, germination and their interaction on wheat identified peptides related to CD. The results highlight that germination, genotype and their interaction had significant impact on the content of peptides associated with CD.

Figures caption

Figure 1: SDS-PAGE patterns of whole nitrogen extract and different fractions obtained by Osborne fraction of kernels before and after germination (M: Molecular weight marker, 1: D240, 2: Svevo, 3: Levante, 4: Kamut®, TP: total proteins, ALB: albumin, Glob: globulin, Gli: gliadin, Glu: glutenin, BG: before germination, G: germinated). The amount of protein in every lane is the same (1.3µg/µL).

Figure 2: Efficiency of germination on CM3 degradation. CM3 genotypic comparison between wheat kernels before and after germination. Data are expressed as average of two replicates. Bars with the same colour carrying different letters are significantly different ($p < 0.05$) from each other.

Table 1: Changes in durum wheat composition induced by germination (expressed as g/100g db).

Genotype	Starch	Protein	Lipid	Ash
<i>Before germination</i>				
D240	76.04±0.30 ^c	12.88±0.01 ^a	5.83±0.03 ^a	1.23±0.01 ^a
Svevo	73.78±0.06 ^a	17.94±0.26 ^c	5.39±0.21 ^a	1.17±0.08 ^a
Levante	77.79±0.70 ^c	15.05±0.06 ^b	7.19±0.39 ^b	1.46±0.03 ^b
Kamut®	75.14±0.22 ^b	19.72±0.13 ^d	5.54±0.45 ^a	1.09±0.04 ^a
<i>After germination</i>				
D240	77.87±0.37 ^a	12.93±0.19 ^a	6.61±0.37 ^{ab}	1.27±0.02 ^a
Svevo	74.85±0.16 ^b	18.22±0.14 ^c	6.07±0.03 ^a	1.26±0.07 ^a
Levante	79.51±1.18 ^a	15.19±0.05 ^b	7.30±0.40 ^b	1.45±0.03 ^b
Kamut®	77.44±1.01 ^a	20.07±0.14 ^d	6.52±0.08 ^{ab}	1.30±0.04 ^a

Means±SD of same column of the same section followed by different letters differ significantly (p < 0.05)

549

Table 2: List of the identified gluten peptides from wheat related to CD.

Code	Identified peptides related to CD	Protein
<i>Total immunogenic peptides (TI)</i>		
IP1	TQQPQQPFPQ	γ -gliadin
IP2	SQQPQQPFPQPQ	γ -gliadin
IP3	QAFPQQPQQPFPQ	γ -gliadin
IP4	TQQPQQPFPQQPQQPFPQ	γ -gliadin
IP5	PQTQQPQQPFPQFQQPQQPFPQPQQP	γ -gliadin
IP6	FPQQPQLPFPQQPQQPFPQPQQPQ	γ -gliadin
IP7	QQPQQPFPQPQQTFPQQPQLPFPQQPQQPFP	γ -gliadin
<i>Total toxic peptides (TT)</i>		
TP1	LQPQNPSQQQPQ	α -gliadin
TP2	RPQQPYQPQPQ	α -gliadin
TP3	LQPQNPSQQQPQEQVPL	α -gliadin

550 IP: Immunogenic peptide; TP: Toxic peptide

551

552 **Table 3:** A semi-quantification of each immunogenic, toxic and total peptides in the different varieties tested before and after germination.

	IP1	IP2	IP3	IP4	IP5	IP6	IP7	TP 1	TP 2	TP 3	TI	TT	TIT
<i>Before germination</i>													
D240	67 ^a	71 ^a	21 ^a	289 ^b	274 ^c	62 ^b	170 ^b	15 ^a	222 ^c	65 ^c	953 ^b	302 ^c	1255 ^b
Svevo	89 ^b	108 ^c	38 ^b	394 ^c	255 ^{ab}	91 ^c	319 ^c	33 ^c	207 ^b	31 ^b	1293 ^{bc}	270 ^b	1563 ^c
Levante	74 ^b	85 ^b	22 ^a	243 ^a	124 ^a	18 ^a	42 ^a	21 ^b	80 ^a	5 ^a	609 ^a	107 ^a	717 ^a
Kamut®	128 ^c	168 ^d	56 ^c	458 ^d	294 ^c	107 ^d	148 ^{ab}	53 ^d	235 ^{cd}	67 ^c	1359 ^c	356 ^d	1715 ^d
<i>After germination</i>													
D240	43 ^a	74	15 ^b	186 ^c	119 ^b	13 ^b	33 ^b	9 ^a	132 ^c	58 ^d	482 ^b	199 ^b	681 ^b
Svevo	43 ^a	87	7 ^a	78 ^a	218 ^c	32 ^c	82	10 ^{ab}	19 ^a	24 ^c	547 ^{bc}	53 ^a	600 ^b
Levante	48 ^a	45	15 ^b	133 ^b	64 ^a	8 ^a	11	19 ^{bc}	34 ^b	3 ^a	324 ^a	57 ^a	380 ^a
Kamut®	85 ^b	133	36 ^c	292 ^c	173 ^{bc}	62 ^d	92	33 ^c	198 ^d	13 ^b	874 ^c	244 ^c	1118 ^c

553 TIT: total immunogenic and toxic peptides

554 Results are indicated as average of two replicates. Values of same column of the same section followed by different letters differ significantly (p

555 < 0.05).

Table 4: F significance level and sum square percent of genotype, germination and their interaction on wheat identified peptides related to CD

	Germination		Genotype		Germination *genotype	
	Sig	SS (%)	Sig	SS (%)	Sig	SS (%)
IP1	***	40.74	***	55.91	Ns	3.35
IP2	**	10.31	***	84.43	Ns	5.27
IP3	***	28.44	***	59.65	**	11.91
IP4	***	52.96	***	34.16	*	12.89
IP5	***	35.96	***	54.83	**	9.21
IP6	***	34.17	***	58.80	Ns	7.03
IP7	***	37.80	***	43.91	***	18.29
TP1	***	20.60	***	68.91	*	10.49
TP2	**	30.84	***	55.71	**	13.45
TP3	***	12.17	***	69.51	***	18.33
TI	***	48.80	*	45.89	*	5.31
TT	***	30.77	***	61.17	**	8.06
TIT	***	46.60	***	47.20	*	6.20

ns= not significant. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$, SS: sum of squares, TIT: total immunogenic and toxic peptides

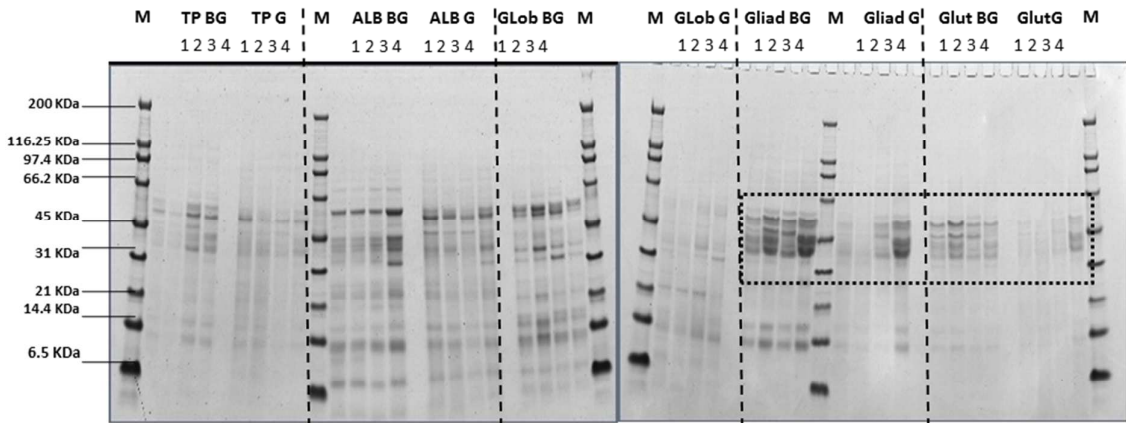


Figure 1: SDS-PAGE patterns of whole protein extract and different fractions obtained by Osborne fraction of kernels before and after germination (M: Molecular weight marker, 1: D240, 2: Svevo, 3: Levante, 4: Kamut®, TP: total proteins, ALB: albumin, Glob: globulin, Gli: gliadin, Glu: glutenin, BG: before germination, G: germinated). The amount of protein in every lane is the same (1.3µg/µL).

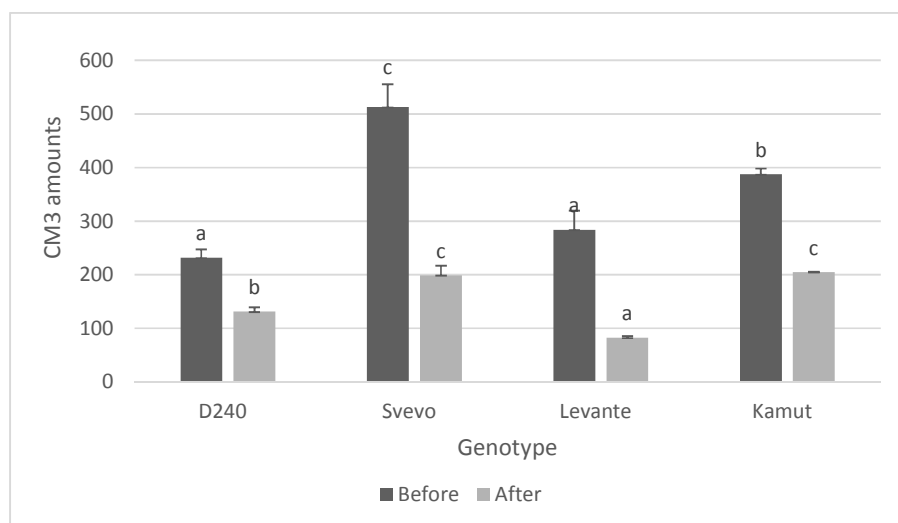
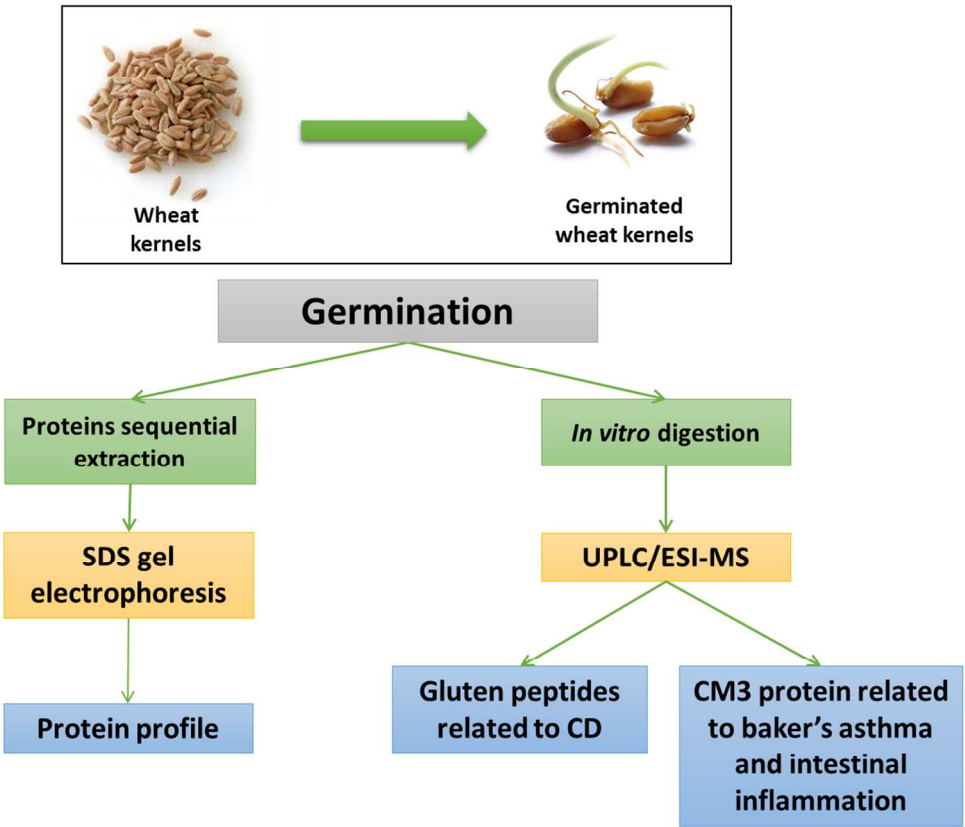
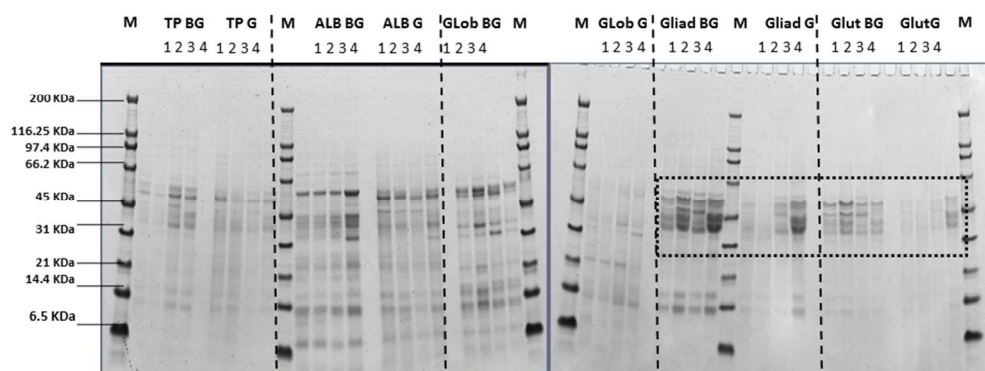


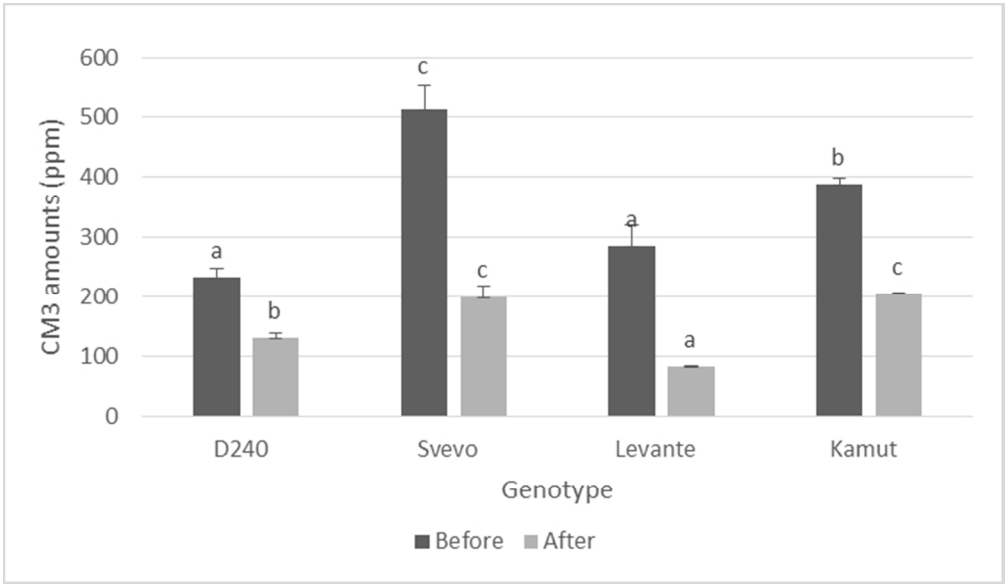
Figure 2: Efficiency of germination on CM3 degradation. Data are expressed as average of two replicates. Bars with the same colour carrying different letters are significantly different ($p < 0.05$) from each other.



209x182mm (150 x 150 DPI)



171x72mm (144 x 144 DPI)



125x72mm (150 x 150 DPI)