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Abstract

Coeliac disease is an autoimmune enteropathy that develops in genetically predisposed subjects after the ingestion of gluten or related proteins. Coeliac disease has an increasing incidence in the last years in western countries and it has been suggested that wheat breeding might have contributed to select more toxic forms of gluten. In this work, we analysed gluten peptides generated by *in vitro* digestion of different old and modern *Triticum* varieties, using LC-MS. We concluded that old varieties analysed produced a higher quantity of peptides containing immunogenic and toxic sequences than modern ones. Thus old wheat lines are not to be considered “safer” for subjects that are genetically predisposed to celiac disease.

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Keywords

Wheat breeding, coeliac disease, gluten peptides, *in vitro* digestion, old wheat

1. Introduction

Old cereal species and varieties have been widely promoted in the last years in order to exploit their characteristic organoleptic properties, the (real or alleged) nutritional properties and the intrinsic cultural and historic value. These species and varieties are increasingly in demand by consumers and the agro-food industry is strongly investing in the study of innovative products based on these cereals. Indeed, several studies focused on the phenolic components of old common wheat lines, finding good basis for antioxidant and chemo protective properties (Leoncini et al., 2012). Old wheat varieties contain indeed a wider spectrum of phenolic compounds, derived from the higher genetic biodiversity, compared to the modern one (Dinelli et al., 2011), even if the content of health beneficial substances can be influenced by the sowing season (Rascio et al., 2015). Other studies on durum wheat found good prebiotic activity of soluble fibre extracted from old durum-type wheat grains, so they could be used as a source for prebiotic formulations (Marotti et al., 2012). An extensive database of phytochemicals and dietary fibers was done comparing landraces and modern wheat varieties, demonstrating that wheat breeding did not affect these wheat components (Shewry et al., 2011). Other studies demonstrated that ancient wheat is not healthier than modern wheat, in terms of bioactive compounds present (Shewry et al., 2015). However, old wheat varieties are more adaptable to adverse environments. Thus, they could represent a solution for local communities, were the commonly grown wheat varieties are not cultivable (Migliorini et al., 2016).

In the last years, the increasing incidence of coeliac disease has shifted the focus also to the protein fraction of old wheat varieties. Coeliac disease is an autoimmune enteropathy that develops in genetically predisposed subjects after the ingestion of gluten or related proteins of barley (hordeins) and rye (secalins) (Kagnoff, 2007). The possibility to eat oats by celiac patients is

still debated, since different oat varieties showed different activation of the gluten-induced transglutaminase-2 (TG2)-dependent events (Silano et al., 2014). During gluten digestion, some resistant peptides are formed due to the high proline content of prolamins (Hausch, Shan, Santiago, Gray, Khosla, 2002); some of these peptides contain sequences (epitopes) able to trigger the immunological reaction in coeliac patients. Beside the immunological implications, gluten is very important also for the technological point of view, because it determines the viscoelasticity of the dough in bread making and in the production of wheat derived products (Goesaert, Brijs, Veraverbeke, Courtin, Gebruers, Delcour, 2005). The varietal selection undertaken by breeders in the last decades in order to achieve the desired rheological properties has led to a decrease in the genetic biodiversity of wheat varieties present nowadays on the market (Fu et al., 2009). So, the higher biodiversity of old wheat lines was studied in order to identify genotypes encoding a lower number of coeliac disease epitopes (van den Broeck et al., 2010). However, T-cell proliferation assays demonstrated that all strains of wheat, independent of ploidy or old/modern origin, triggered heterogeneous responses covering wide ranges of stimulation indices. So, according to the reported data, old strains of wheat, although previously suggested to be low or devoid of coeliac toxicity, should be avoided by coeliac patient and should not be considered “safe”.

Nevertheless, old varieties, even if dangerous for coeliac patients, could actually be producing, upon gastrointestinal digestion, less immunotoxic peptides, thus actually helping to prevent the spread of the disease among predisposed subjects. If a lower content of toxic peptides after digestion would be proven true for old varieties, it would indeed be a strong indication that modern varieties have somehow a role in the current increase of the coeliac disease. However, there are only few studies comparing old and modern *Triticum* varieties, representing a knowledge gap.

In this paper, we compared the peptide profile of different *Triticum* species (old and modern), generated after simulated gastrointestinal digestion. In this way, it was possible to evaluate differences in the peptide pattern generated from the *in vitro* digestion of the different species, with particular focus on peptides containing sequences known in literature to be implicated in coeliac disease.

2. Materials and methods

2.1.1 Samples.

Triticum samples analysed are reported in Table 1, together with the protein content on dry matter. Protein content is expressed as a range (minimum and maximum content found), taking into account for each variety the three different harvesting conditions (Parma/organic, Parma/conventional, Bologna/conventional). All the samples were harvested in two different Italian regions (Parma and Bologna) in the conventional way. To investigate a possible effect of organic farming, in the Parma area conventional and organic farming were compared. Three biological replicates (harvested in three different independent blocks) were taken for all the samples. Samples were ground with an analytical mill (A11 basic, IKA, Staufen, Germany). Flour was not defatted prior to digestion.

2.2 Reagents and solvents.

Deionised water was obtained from a Millipore Alpha Q-Waters purification system (Billerica, MA, USA). Pepsin from porcine gastric mucosa, trypsin from porcine pancreas, α-chymotrypsin from bovine pancreas, α-amylase from barley malt (type VIIIa), uric acid, mucin from porcine stomach (type III), glucose, glucuronic acid, glucosamine hydrochloride, albumin bovine, pancreatin from porcine pancreas, lipase from porcin pancreas (type II), bile from bovine and ovine, sodium dihydrogen phosphate, potassium chloride, urea, acetonitrile, ethanol, Fmoc-glutamine(Trt)-OH, piperidine, diisopropylethylamine, dichloromethane, triisopropylsilane, tyrosine, phenylalanine, tris(hydroxymethyl)-aminomethane, urea, dithiothreitol and diethyl ether were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (37% V/V), sodium hydroxide, sodium hydrogen carbonate, calcium chloride, ammonium chloride and dimethylformamide were purchased from Carlo Erba (Milan, Italy). Fmoc-leucine-OH, Fmoc-

proline-OH, HBTU and Fmoc-tyrosine(tBu)-Wang resin were purchased from Novabiochem (Darmstadt, Germany). Formic acid and trifluoroacetic acid were purchased from Acros Organics (Geel, Belgium). Sodium chloride was purchased from analaR Normapur (Milan, Italy). Potassium thiocyanate was purchased from Riedel De Haen (Seelze, Germany). Magnesium chloride was purchased from Merck (Darmstadt, Germany).

2.3 *In vitro* digestion.

The samples were digested as described by Versantvoort, Oomen, van den Kamp, Rompelberg and Sips (2005) with the volume of digestive juices scaled to the smallest amount of sample. Briefly, 450 mg of sample were incubated 5 minutes with 600 µl of saliva (ratio α-amylase:matrix 1:1300); after this phase, 2.4 ml of gastric juice (ratio pepsin:matrix 1:38) were added and the sample was incubated for 2 hours. To set the pH for the intestinal phase, 400 µl $NaHCO_3$ 1 mol L^{-1} were added; after, 2.4 ml of duodenal juice (ratio pancreatin:matrix 1:10, ratio lipase:matrix 1:63) and 1.2 ml of bile (ratio bile:matrix 1:6) were added and the sample was incubated for 2 hours. All the digestion steps were carried out at 37°C. At the end of the digestion, 58.3 µl of HCl 37% were added to inactivate enzymes and the sample was centrifuged at 8965 *g* at 4°C for 45 minutes, in order to precipitate insoluble compounds and undigested proteins. No dialysis of the sample was performed. Prior to LC-MS analysis, all samples were filtered with a cut off of 0.45 µm. For the quantification, 295 µl of digested sample were spiked with 5 µl of standard peptide solution (LQLQPF(d_5)PQPQLPY, 0.477 mmol L^{-1}). Since in this digestion model also exopeptidases are used and different truncated of its analogue natural peptide are found in the digested sample, the internal standard was added at the end of the digestion (after enzyme inactivation) in order to avoid its digestion or degradation.

2.4 Synthesis of the internal standard.

The internal standard was synthesized in our laboratory as previously described by Prandi et al. (2012). Briefly, the peptide LQLQPF(d_5)PQPQLPY was synthesized on solid phase according Fmoc/t-butyl strategy using a Syro I Fully Automated Peptide Synthesizer (Biotage, Uppsala, Sweden). The ring of phenylalanine was labeled with five deuterium atoms (Mr +5). The peptide was cleaved from the Wang-resin using a trifluoroacetic acid with 2.5% of triisopropylsilane and 2.5% of water as scavengers. Peptide purification was carried out on a semipreparative RP-HPLC-UV (detection wavelength: 280 nm, where tyrosine absorbs). The separation was obtained using a Jupiter 5 µm, C18 300 Å column (250×10 mm, Phenomenex) using a gradient elution. Eluent A was water and eluent B was acetonitrile. Both A and B eluents were acidified with 0.1% of formic acid. The flow was 4 ml/min and the gradient was: 0-5 min 100% A, 5-15 min from 100% to 60% A, 15-25 min 60% A, 25-30 min from 60% A to 0% A, 30-35 min 0% A, 35-40 min from 0% to 100% A, 40-55 min 100% A. The purified product was quantified with a spectrophotometer at a wavelength of 280 nm using an external calibration curve made with an equimolar solution of tyrosine and phenylalanine.

2.5 UPLC/ESI-MS analysis.

UPLC/ESI-MS method for peptide separation and quantification was previously developed in our laboratory as described by Prandi et al. (2012). Briefly, the digested samples were separated by a RP column (ACQUITY UPLC BEH 300 C18 1.7 µm 2.1*150 mm) in an UPLC/ESI-MS system (UPLC Acquity Waters with a single quadrupole mass spectrometer Waters Acquity Ultraperformance) using a gradient elution. Eluent A was water and eluent B was acetonitrile. Both A and B eluents were acidified with 0.1% of formic acid; gradient: 0-7 min 100% A, 7-50 min from 100% A to 50% A, 50-52.6 min 50% A, 52.6-53 min from 50% A to 0% A, 53-58.2 min

0% A, 58.2-59 min from 0% A to 100% A, 59-72 min 100% A. The digested samples were analysed with UPLC/ESI-MS in the Full Scan mode (flow 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; ionization type positive ions; 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 characteristic ions of every peptide were extracted, obtaining eXtract Ion Chromatograms (XICs), in which the identified peptides and internal standard LQLQPF(d_5)PQPQLPY were integrated with the MassLynx software. The quantification value was obtained as the ratio peptide area/internal standard area multiplied by the moles of internal standard assuming the same response factor for all peptides. The actual response factors of all the peptides could not be determined given the unavailability of all standard necessary. The use of the isotopically labeled peptide chosen (already used in previous works, [Prandi et al., 2012 and 2014]) however allows the comparison among the different varieties tested, that is the aim of the work.

2.6 HPLC/ESI-MS/MS analysis.

HPLC/ESI-MS/MS method for gluten peptides identification was previously developed in our laboratory as described by Prandi et al. (2012). Briefly, the digested samples were separated by a RP column (JUPITER 5 µm C18 300 Å 250*2 mm) in an HPLC/ESI-MS/MS (HPLC Waters Alliance 2695 with a triple quadrupole mass spectrometer Waters 4 Micro), using a gradient elution. Eluent A was water and eluent B was acetonitrile. Both A and B eluents were acidified with 0.1% of formic acid; gradient: 0-12 min 100% A, 12-77 min from 100% A to 50% A, 77-81 min 50% A, 81-82 min from 50% A to 0% A, 82-90 min 0% A, 90-91 min from 0% A to 100% A, 91-110 min 100% A. The samples were first analyzed in Full Scan mode, to identify the

characteristic ions and the retention time of the unknown compounds, and then in product ion mode using a variable collision energy on the basis of the mass and charge of the ion to be fragmented. HPLC/ESI-MS/MS parameters were: flow 0.2 ml/min; analysis time 110 min; column temperature 35°C; injection volume 40 µl; acquisition time 7-90 min; ionization type positive ions; scan range 100-2000 m/z; capillary voltage 3.2 kV; cone voltage 30 V; source temperature 100°C; desolvation temperature 150°C; cone gas flow 100 l/h; desolvation gas flow 650 l/h. The peptide sequences were assigned on the basis of the mass spectra obtained. Briefly, the software FindPept (http://web.expasy.org/findpept/) was used to find the peptide sequences whose molecular weight matched with the experimental data. Then, the software Proteomics Toolkit (http://db.systemsbiology.net/proteomicsToolkit/FragIonServlet.html) was used to verify the correspondence between the theoretical MS/MS fragmentation and the obtained spectra.

2.7 Statistical analysis.

Immunogenic peptides: the normality of the data was checked with the Shapiro-Wilk test. Three grouping of the data were taken into account: cultivation area, species and type of breeding (old/modern). Data followed a normal distribution for what concerned cultivation area and species grouping, while for the type of breeding data did not follow a normal distribution. The homogeneity of variance was checked with the Levene test: data variances were homogeneous for cultivation area and type of breeding grouping, but not for species grouping. Thus, significant differences among the different regions were assessed with one way ANOVA using LSD post-hoc test, while differences among the various species were assessed using one-way ANOVA followed by Tamhane post-hoc test. Differences between old and modern varieties were evaluated with the Mann-Whitney test. Toxic peptides: the

normality of the data was checked with the Shapiro-Wilk test. Three grouping of the data were taken into account: cultivation area, species and type of breeding (old/modern). Data followed a normal distribution. The homogeneity of variance was checked with the Levene test: data variances were homogeneous. Thus, significant differences were assessed with one way ANOVA using LSD post-hoc test. Differences between old and modern varieties were evaluated with the student t test.

2.8 Total amino acids determination.

Three milliliters of the digestate were transferred into a 18 ml Pyrex glass tube fitted with teflon-lined screw caps. 6 ml of hydrochloric acid (6 mol L^{-1}) were added and mixed. The tube was flushed with nitrogen for 1 min in order to remove air. Hydrolysis was then carried out at 110°C for 23 h. After letting the tubes cool at room temperature, the internal standard (7.5 ml of Nor-leucine 5 mmol L^{-1} in water) was added; the mixture was filtered through paper filter and collected into a 250 ml volumetric flask. In order to prepare a calibration standard solution, 40 µl of Nor-leucine (2.5 mmol L^{-1} in HCl 0.1 mol L^{-1}), 40 µl of Amino Acid Hydrolyzate Standard Mixture (Sigma-Aldrich) and 920 µl of deionised water were mixed. Then 10 µl of hydrolyzate sample or standard solution were transferred into a 1.5 ml tube, 70 µl of borate buffer were added, in order to keep the optimal pH range for derivatization (8.2–9.7), and the solution was briefly vortexed. Twenty microliters of reconstituted AccQ.Fluor reagent were finally added and the mixture was immediately vortexed for several seconds. The tube was closed and left to stand for one minute at room temperature, then heated in a heating bath at 55°C for 10 min. The resulting derivatized standard solution was diluted with 400 µl of deionised water before injecting in the HPLC system.

All samples and standard solutions were analysed by using an Alliance 2695 separation system with a Waters AccQ.Tag amino acid analysis column (3.9mm×150 mm). The column was

213 thermostated at 37°C and the flow rate was set at 1.0 ml/min. The injection volume of samples
214 was 5 µl, while standard calibration solution was injected at several volumes: 5, 10, 15, 20, 25 and
215 30 µl, corresponding to 10, 20, 30, 40, 50, 60 pmoles injected. Mobile phase A consisted of
216 AccQ.Tag eluent A (100 ml AccQ.Tag A concentrate+1 L deionised water). Mobile phase B was a
217 60:40 (V/V) solution of acetonitrile and deionised water respectively. Gradient elution was
218 performed according to the following steps: 0 min 100%A, 1 min 97%A, 13 min 93%A, 18 min
219 90%A, 38 min 67%A, 51 min 67%A, plus washing step and reconditioning. Detection was carried
220 out by Waters 470 Fluorescence detector (λ excitation = 250 nm and λ emission = 395 nm).

221

3. Results and discussion

3.1 Simulated gastrointestinal digestion of old and modern wheat varieties

A range of *Triticum* species were chosen, with different ploidy levels (Table 1). Besides the more diffused common and durum wheat (covering most of the *Triticum* market), also samples of einkorn, emmer and spelt were analysed. Since there is no generally accepted rule on the terms "ancient" and "modern" as related to wheat varieties, for the aim of this study we chose to use the terms "old" and "modern", considered as related to the heavy breeding developments which started after the First World War. Thus with the term "old" we refer to a variety already existing before the first world war, and with the term "modern" to a variety first developed after the first world war. The history and morphology of the studied varieties are detailed in the Supplementary on line material. The wheat varieties analysed were present on the Italian market, but it is recognized that the use of wheat varieties can be extremely variable in the different regions of the world. The prevalence of coeliac disease is on the rise in Italy as in other countries: the number of celiac patient increased of more than 15% in only two years, from 2012 to 2014 (Annual report of Italian parliament, 2014).

The protein content of the different species was measured according to the Kjeldahl method (Table 1). Protein content was found to vary between 11.2% and 17.3%. As expected, protein content of common wheat was lower than that of durum wheat and emmer. The lowest value was observed for the modern common wheat variety Blasco, while the highest protein content was found for ID331, a modern einkorn variety. No trends were thus observed for protein content between old and modern wheat varieties.

243 Human metabolism and digestion of foods, from saliva to gut, is a very complex process and *in*
244 *vitro* models are necessary to study this process, even if they cannot be fully representative of
245 human physiology and coeliac physiopathology.
246 Samples were digested following the model of Versantvoort et al (2005). This method has been
247 previously applied to wheat products in our laboratory (Prandi et al., 2014). Briefly, the kernels
248 were ground and submitted to simulated gastrointestinal digestion using artificial fluids (saliva,
249 gastric juice, duodenal juice and bile), whose chemical composition strictly reflects the
250 physiological fluids. The peptides generated from *in vitro* digestion were then analysed by means
251 of liquid chromatography coupled to mass spectrometry (UPLC/ESI-MS). In Figure 1A, two overlaid
252 chromatograms (Total Ion Current) of a common wheat sample (senatore Cappelli) and of an
253 einkorn sample (ID331) are shown. The two chromatograms are apparently very similar, showing
254 the same peak profile, even if the two samples are very different from a genetic point of view
255 (hexaploid vs diploid). The first 7 min of chromatographic run (containing mostly free amino acids)
256 were not considered due to the high salt and sugars content. From 12 min to 30 min peptides
257 ranging from Mr 200 to 3600 are eluted, while after 35 min essentially bile salts are present.

258 All the chromatographic peaks were analysed and the molecular weight of the peptides was
259 annotated for each sample. Peptide identification was performed using multiple stage mass
260 spectrometry, as previously described (Prandi et al., 2014). Briefly, all the peptides were submitted
261 to tandem MS experiments using a triple quadrupole instrument and the amino acid sequence
262 was assigned on the basis of the fragmentation pattern. No automated database searching was
263 employed, because the peptides generated after gastrointestinal digestion of wheat were
264 previously characterized (Prandi et al., 2014). Thus, only confirmation by means of low resolution
265 tandem mass spectrometry was necessary. Annotated spectra of the peptides containing
266 immunogenic or toxic sequences can be found in supplementary material (S1). Then, the

chromatographic areas of all the 77 identified peptides were integrated using MassLynx software and used for Principal Component Analysis (PCA), using SPSS 17.0 software. The total explained variance was 60%, of which 34% determined by component 1. As shown in Figure 2, all the tetraploid and hexaploid varieties are grouped together, while ID331 (*T. monococcum*, diploid) can be easily distinguished. ID331 showed a markedly different peptide production after digestion compared to the tetraploid and hexaploid varieties: probably the presence of only two copies of each chromosome (AA) leads to a different protein expression, compared to durum and common wheat (respectively AABB and AABBDD). The latter, in contrast, despite the genomic differences, lead to a similar peptide profile upon *in vitro* digestion. Thus, in terms of peptide production during gastrointestinal digestion, we can approximate that the outcome of the digestion for the samples analysed is quite similar for durum and common wheat, emmer and spelt, while it is markedly different for einkorn. For what concerns old and modern wheat varieties, a discriminant analysis was performed, and the discriminating peptides were 9 in total, of which one contained immunogenic sequences (PQTQQPQQPFPQFQQPQQPFPQPQQP) and two peptides contained toxic sequences (RPQQPYPQPQPQ and QQPPFSQQQPPPFS). The former, in particular, is markedly high in old wheat varieties.

The amino acid profile of the digestates is reported in Figure 3. As it was largely expected, the most abundant amino acid is Glx (sum of glutamic acid and glutamine), due to its wide presence in gluten proteins. Total amino acid content is comprised between 3344 ppm (Farro della Garfagnana, emmer) and 5319 ppm (Grano del Miracolo, common wheat). Comparing Figure 3 and Figure 4, it is clear that there is no correlation between the amino acid content of the digestates and the amount of toxic and immunogenic peptides. This suggests that it is not the total protein content that determine the amount of toxic and immunogenic peptides, but the different distribution of gluten proteins.

3.2 Celiac disease related peptides

To further investigate this matter, we quantified immunotoxic peptides related to coeliac disease (Figure 4). Peptides related to coeliac disease were identified by mean of low resolution mass spectrometry and the sequences were in agreement with previously reported peptides (Prandi et al., 2014) and they are reported in Table 2, together with the protein of origin. More information about HPLC/ESI-MS/MS identification, such as the fragmentation pattern and the annotated spectra, are available in the supplementary on line material, S1 and S2. These sequences were also previously confirmed by high resolution mass spectrometry using an Orbitap instrument in a previous work (Prandi et al., 2014). Two different categories of celiac disease related peptides were taken into account: those containing sequences known in literature to elicit an adaptive immune response (called immunogenic peptides; Schuppan, 2000) and those that stimulates an innate immune response (called toxic; Cornell et al., 2001). This means that among all the peptides identified, only those containing sequences known in literature to elicit coeliac disease were taken into account (extract ion chromatograms are shown in Figure 1B and 1C). Samples were compared from a chemical point of view, quantifying celiac disease related peptides. The aim of the work was in fact to compare the immunogenic potential of old and modern wheat varieties, basing on sequences previously characterized in literature. Obviously, the complexity of celiac disease pathology makes necessary future *ex vivo*/*in vivo* tests to better support these findings. However, it is remarkable that peptides containing immunogenic and toxic sequences were found in all the sample analysed, thus none of the *Triticum* variety analysed has to be considered safe for celiac patients. The amount of each immunogenic and toxic peptide detected, together with the amino acid sequence, is reported respectively in Table 3 and 4. As shown in Table 2, all the peptides that contained sequences known in literature to elicit an adaptive response in celiac patient derived from γ-gliadin, while toxic peptides derived from a wider range of proteins: α-gliadin, γ-gliadin and

315 LMW-glutenins. The 33-mer immunogenic peptide previously identified in gliadin digests (Molberg
316 et al., 2005; Shan et al., 2002 and 2005) was not detectable in our samples. Probably the higher
317 complexity of the digestion model used (Versantvoort et al., 2005) allows to digest this peptide
318 into shorter sequences. Coeliac disease is a complex pathology, with a lot of epitopes that are not
319 equally recognized by all the subjects, and the reactions are different from patient to patients
320 (Hischenhuber et al., 2006). This work does not purport to be exhaustive for all the possible
321 immunogenic and toxic peptides generated, but takes into account all the most studied epitopes
322 found in literature, taking into account also their relative abundance. No standards were available
323 for all the identified peptides, thus the quantification was achieved with the use of an isotopically
324 labelled peptide standard. Even if small differences in ionization efficiency can be present due to
325 structural dissimilarity, the use of the isotopically labelled peptide allows comparing the amount
326 of the same peptide in all the samples. In all cases high intra-category variability was observed,
327 due to the greater number of isoforms of gluten proteins. In fact these proteins have a storage
328 role, and thus they suffered only a limited evolutionary pressure.

329 We did not observe significant differences in the content of immunogenic peptides according to
330 organic or traditional cultivation. As it can be observed in Figure 4A, there are no statistically
331 significant differences in the content of immunogenic peptides between the Parma harvesting
332 area and the organic farming, that was also performed in Parma (in the same farm). These data
333 are in agreement also with the protein content data shown in Figure 5, where protein content is
334 compared between samples cultivated in organic and conventional farming. Data distribution was
335 normal and variance was homogeneous (checked by Shapiro-Wilk and Levene tests, respectively)
336 and from the statistical analysis (student t test) emerged that no significant differences were
337 present between the two groups. Also looking to the single immunogenic peptides, most of them
338 did not show significant differences, only peptide PQTQQPQQPFPQFQQPQQPFPQPQQP and

PQQPQLPFPQQPQQPFPQPQQPQ showed a lower content in Parma location, while peptide PFPQPQQPQQPFPQSQQPQQPFPQP was higher in Bologna farming. Despite the possible shortage of nitrogen and phosphate nutrients in organic cultivation, no differences in the protein content were detected. For what concerns toxic peptides (Figure 4D), a slightly higher amount of toxic peptides was detected in Bologna compared to Parma. No significant difference was found among the toxic peptides between organic and conventional farming. So, for what concerns coeliac disease, from our results organic farming gives the same amount of immunogenic and toxic peptides compared to the traditional one.

Focusing on immunogenic peptides, among the different species common wheat gave the highest results (average 524 mg/kg, ranging from 240 mg/kg of Blasco harvested in Parma to 829 mg/kg of Grano del Miracolo harvested in Bologna), followed very closely by the other two tetraploid species, durum wheat (average 457 mg/kg, ranging from 208 mg/kg of Odisseo harvested in Parma to 840 mg/kg of Senatore Cappelli harvested in Bologna) and emmer (average 421 mg/kg, ranging from 247 mg/kg of Farro della Garfagnana harvested in organic way in Parma to 572 mg/kg of the same variety harvested in Bologna), as shown in Figure 4B. No statistically significant differences were observed among these three species. Einkorn (average 166 mg/kg, ranging from 137 mg/kg of ID331 harvested in Parma to 236 mg/kg of ID331 harvested in Bologna) and, to a lesser extent, spelt (average 225 mg/kg, ranging from 63 mg/kg of Rouquin harvested in organic way in Parma to 436 mg/kg of the same variety harvested in Parma in conventional way) showed a lower amount of immunogenic peptides, but notwithstanding this they cannot be considered safe for coeliac patients. From these data emerged that the total quantitative amount of immunogenic peptides is not directly related to the ploidy level: for example emmer and durum wheat (tetraploids) showed the same amount of immunogenic peptides than common wheat (hexaploid). In contrast, the diploid einkorn showed a significantly lower amount of immunogenic peptides. Some studies in

363 the past showed a lacking of toxicity of *Triticum monococcum* on cells from intestinal biopsies of
364 coeliac patients (Pizzuti et al., 2006), but data related to the immunogenic peptide content on the
365 opposite showed that it should not be eaten by coeliac patients, according to the general
366 guidelines for coeliac patients (Fasano and Catassi, 2001). This is somehow different from the
367 literature data demonstrating that diploid wheat gluten has a reduced number of immunogenic
368 peptides (Molberg et al., 2005 and Spaenij-Dekking et al., 2005) and elicits a low immune response
369 upon the extensive gastrointestinal proteolytic digestion (Gianfrani et al., 2015 and 2012). This
370 contrasting data could be explained by the large inter-cultivar difference in the γ-gliadin genes of
371 the domesticated einkorn, which then can elicit in a different way different type of T cell clones
372 (Molberg et al., 2005). In our study, the lower amount of immunogenic peptides of ID331 can be
373 explained by the lower amount of all the immunogenic peptides identified with the exception of
374 the peptide TQQPQQPFPQ, that is significantly higher in *T. monococcum*.

375 Toxic peptides show the same trend, with the exception of einkorn ID331 showing the highest
376 content (Figure 3E). As it can be observed from the graph, no statistically significant differences
377 were found among common wheat, durum wheat, einkorn and emmer (average 129 mg/kg,
378 ranging from 51 mg/kg of Bologna harvested in organic way in Parma to 230 mg/kg of Virgilio
379 harvested in Bologna). A lower amount of toxic peptides was found in spelt (average 79 mg/kg,
380 ranging from 26 mg/kg of Rouquin harvested in organic way to 117 mg/kg of Rouquin harvested in
381 conventional way). The peptide LQPQNPSQQQPQ was significantly higher in emmer, common and
382 durum wheat than in einkorn and spelt. The peptide RPQQPYPQPQPQ indeed is significantly
383 higher in ID331 (einkorn). Finally, the peptide QQPPFSQQQPPPFS is significantly higher in common
384 and durum wheat. These data are partially in contrast with a previous observation that ID331 has
385 a reduced immunogenicity on celiac disease T cells after hydrolysis with intestinal brush border
386 enzyme BBM enzymes (Gianfrani et al., 2015). A possible explanation could be the differences in

gliadin profile between samples harvested in different conditions and also possible crop year variability. For this reasons samples here analysed were harvested in the same regions and in the same years. However, data are in agreement with the recent study of Šuligoj et al. (2013), where *T. monococcum* triggered positive T-cell responses of most T cell lines tested. In this case we found peptides containing sequences that are considered toxic (PSQQ, QQQP, QPYP) (so able to elicit the innate response), but we found a significantly lower amount of immunogenic peptides, that should induce a lower stimulation of the adaptive system. Anyway, even if this amount is lower, it does not mean that it is absent, and the level of 20 mg/kg of gluten fixed to declare a food as glutenfree is enough to consider einkorn not safe for celiac patients. Common wheat, durum wheat, einkorn and emmer were not statistically different in this case. Spelt, beside the lower amount of immunogenic peptides, showed also the lower amount of toxic peptides.

It is sometimes believed that old wheat varieties are “safer” and “more healthy” compared to the modern wheat lines. On the opposite, in this study emerged that old *Triticum* varieties generate a higher amount of immunogenic peptides (Mann-Whitney test, $p<0.05$) as shown in Figure 4C, thus causing an even higher exposure to immunogenic sequences. Looking at the single peptide amount reported in table S3, we can observe that there is no single peptide responsible for this higher amount of immunogenic peptides in old wheat varieties. Indeed, all the immunogenic peptides (with the only exception of TQQPQQPFPQ and PFPQPQQPQQPFPQSQQPQQPFPQP) were higher in old wheat samples ($p<0.05$). The same behaviour is observed for toxic peptides, with old wheat varieties displaying a slightly higher content (Figure 4F). As we look at single toxic peptides amount, most of them are not statistically significant between old and modern wheat varieties, so the higher content of toxic peptides showed by the old wheat varieties can be ascribed mostly to peptides LQPQNPSQQQPQ ($p<0.05$), deriving from α-gliadin. Several studies correlated the increase in the prevalence of coeliac disease with an increased gluten consumption (Catassi et al.,

411 2010; Gobbetti, Rizzello, Di Cagno and De Angelis, 2007; Ivarsson et al., 2000). Wheat flour
412 consumption had indeed a steady increase from 1970 to 2000 (Kasarda, 2013). Thus, it has been
413 hypothesized that a certain responsibility could be ascribed to the wheat breeding, oriented to
414 increase amylopectin, protein and gluten. But literature data are in contrast with that, because
415 wild tetraploids were found to have protein contents in the range of 16−27% (Ciaffi, Dominici,
416 Lafiandra and Porceddu, 1992), whereas domesticated tetraploids usually have lower protein
417 contents of about 10−12%. According to these data, we found an even higher amount of
418 immunogenic peptides in the old wheat varieties compared to the modern ones. Thus, it is unlikely
419 that modern wheat varieties are the responsible of the current coeliac disease increase. Beside
420 changes in the wheat consumption habits, also possible modification in the immune system of the
421 population in the last decades should be considered to explain the rise in celiac disease cases.
422 Moreover, since gluten epitopes are many and diverse and deriving from different proteins, also
423 possible modification in the response of the immune system to gluten peptides may have
424 changed. Incidentally, it is also quite obvious that no wheat species, or cultivar, is safe for patients
425 with a diagnosis of celiac disease, accordingly with recent literature (Šuligoj et al., 2013). We
426 concluded that old varieties are producing a higher quantity of peptides containing immunotoxic
427 sequences upon digestion than modern ones, thus are not to be considered “safer” for subject
428 that are genetically predisposed to celiac disease.

429

430 **4. Abbreviations used**

431 ANOVA: analysis of variance

432 Fmoc: fluorenylmethyloxycarbonyl

433 LC/MS or LC-MS: liquid chromatography coupled to mass spectrometry

PCA: principal component analysis

RP-HPLC-UV: reversed phase high performance liquid chromatography coupled to ultraviolet detector

tBu: tert-butyl

TFA: trifluoroacetic acid

TIS: triisopropylsilane

Trt: trityl

UPLC/ESI-MS: ultra high performance liquid chromatography coupled to electrospray ionization mass spectrometry

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6. Conflict of interest

The authors declare that they have no conflict of interest.

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8. Figure captions.

Figure 1. Panel A: overlaid UPLC/ESI-MS chromatogram (total ion current) of a common wheat sample (senator Cappelli) and a einkorn variety (ID331) submitted to simulated gastrointestinal digestion. Panel B: extract ion chromatogram of immunogenic peptides. Panel C: extract ion chromatogram of toxic peptides.

Figure 2. Principal Component Analysis (PCA) of the samples, using as variables the peptide content after gastrointestinal digestion, classified for variety.

Figure 3. Amino acid profile of the digestates, expressed as ppm (μg of amino acid per ml of digestate). Asx: sum of Asp and Asn; Glx: sum of Glu and Gln. Amino acid analysis was carried out on samples harvested in Parma (conventional farming).

Figure 4. Box and whiskers plots of total immunogenic peptides content for harvesting area (A, n=26 for Parma, n=27 for Bologna, n=29 for organic), species (B, n=36 for *Triticum aestivum*, n=20 for *Triticum durum*, n=9 for einkorn, n=8 for emmer and n=9 for spelta) and old/modern (C, n=37 for old and n=45 for modern). Different letters mean statistically significant different values ($p<0.05$, one-way ANOVA for A, B, D and E, Student t for C and F). Organic farming was performed in the Parma area.

Figure 5. Protein content expressed as % of protein on dry weight in the organic and conventional cultivation conditions.

Tables.

Table 1. Old and modern[a] wheat varieties studied.

SPECIES	VARIETY	TYPE	PROTEIN CONTENT (% on dry matter)
Triticum aestivum L.	Grano del Miracolo	Old	11.8-13.2
	Virgilio	Old	12.0-13.7
	Blasco	Modern	11.2-12.8
	Bologna	Modern	11.7-12.4
Triticum turgidum var. *durum* Desf.	Senatore Cappelli	Old	14.1-15.0
	Timilia	Old	12.3-13.9
	Odisseo	Modern	11.5-13.1
Triticum monococcum L. (einkorn)	ID331	Modern	13.3-17.3
Triticum turgidum var. *dicoccum* L. (emmer)	Farro della Garfagnana	Old	11.9-13.4
Triticum spelta L. (spelt)	Rouquin	Modern	12.4-13.7

[a]The term "old" and "modern" in this context are considered as related to the heavy breeding developments which started after the first world war.
Thus with the term "old" we refer to a variety already existing before the first world war, and with the term "modern" to a variety first developed after the first world war.

Table 2. List of the identified peptides containing sequences known in literature to elicit the adaptive immune response (immunogenic peptides) or the innate immune response (toxic peptides).

Adaptive immune response – immunogenic peptides	Protein	HLA locus
TQQPQQPFPQ	γ-gliadin	DQ2 (DQA1*05:01/DQB1*02:01) (http:www.iedb.org/)
SQQPQQPFPQPQ	γ-gliadin	DQ2 (DQA1*05:01/DQB1*02:01) (http:www.iedb.org/)
TQQPQQPFPQQPQQPFPQ	γ-gliadin	DQ2 (DQA1*05:01/DQB1*02:01) (http:www.iedb.org/)
PQTQQPQQPFPQFQQPQQPFPQPQQP	γ-gliadin	DQ2 (DQA1*05:01/DQB1*02:01) (http:www.iedb.org/)
PFPQPQQPQQPFPQSQQPQQPFPQP	γ-gliadin	DQ2.5 (Juhàsz, Gell, Bèkès, Balàzs, 2012); DQ2 (DQA1*05:01/DQB1*02:01) (http:www.iedb.org/)
PQQPQLPFPQQPQQPFPQPQQPQ	γ-gliadin	DQ2 (DQA1*05:01/DQB1*02:01) (http:www.iedb.org/)
QQPQQPFPQPQQTFPQQPQLPFPQQPQQPFP	γ-gliadin	DQ2 (DQA1*05:01/DQB1*02:01) (http:www.iedb.org/); DQ2 (Shan et al., 2005)
Innate immune response – toxic peptides	Protein	
LQPQNPSQQQPQ	α-gliadin	
RPQQPYPQPQPQ	α-gliadin	
QQPPFSQQQPPFS	LMW-glutenin	

Table 3. Amount (in mg/kg) of each immunogenic peptide in the different varieties tested. Values are the average of three biological replicates, coming from three different parcels.

wheat variety	cultivation area	farming	TQQPQQPFPQ	SQQPQQPFPQPQ	TQQPQQPFPQQPQQ PFPQ	PQTQQPQQPFPQFQ QPQQPFPQPQQP	PFPQPQQPQQPFPQS QQPQQPFPQP	PQQPQLPFPQQPQQP FPQPQQPQ	QQPQQPFPQPQQTFP QQPQLPFPQQPQQPF
grano del miracolo	Parma	conventional	41 ±7	38±6	217±58	116±28	28±7	12±5	33±11
Virgilio	Parma	conventional	43±8	52±4	294±62	98±24	9±4	8±6	3±2
Blasco	Parma	conventional	41±9	32±6	181±43	45±20	15±4	5±3	15±9
Bologna	Parma	conventional	28±8	43±11	156±70	47±19	27±15	8±6	25±18
senatore Cappelli	Parma	conventional	30±0	43±1	166±8	93±6	22±2	16±2	30±4
Odisseo	Parma	conventional	27±4	30±4	122±33	67±19	11±7	11±5	26±6
ID331	Parma	conventional	78±17	18±5	6±5	5±3	13±6	15±4	2±1
farro della Garfagnana	Parma	conventional	41±16	28±11	218±81	113±21	10±8	11±1	36±10
Rouquin	Parma	conventional	29±5	27±	166±67	49±24	23±6	7±4	16±6
grano del miracolo	Bologna	conventional	48±6	51±12	275±51	176±41	48±10	21±3	60±16
Virgilio	Bologna	conventional	61±7	81±7	453±15	159±11	10±1	15±5	6±5
Blasco	Bologna	conventional	38±6	34±3	244±48	73±4	20±4	10±3	22±6
Bologna	Bologna	conventional	36±9	52±11	194±44	62±13	33±14	7±3	24±10

senatore Cappelli	Bologna	conventional	43±2	62±9	285±65	174±58	53±19	25±10	70±24
Odisseo	Bologna	conventional	37±2	42±9	203±65	119±58	29±19	13±10	44±24
ID331	Bologna	conventional	87±17	22±4	31±12	15±8	25±6	27±13	7±2
farro della Garfagnana	Bologna	conventional	39±4	32±9	213±52	139±27	16±6	11±3	37±12
Rouquin	Bologna	conventional	31±10	30±4	157±52	42±20	17±11	7±2	10±6
grano del miracolo	Parma	organic	48±4	51±6	288±30	192±17	44±3	14±2	41±8
Virgilio	Parma	organic	69±4	84±13	446±27	154±8	12±5	14±1	3±1
Blasco	Parma	organic	48±3	39±4	266±17	89±6	18±6	9±0	23±3
Bologna	Parma	organic	29±10	47±14	175±79	47±21	29±19	11±	28±15
senatore Cappelli	Parma	organic	32±8	45±15	185±40	94±26	29±9	19±6	34±12
Timilia	Parma	organic	43±6	42±2	237±24	149±11	32±7	17±3	37±5
Odisseo	Parma	organic	27±8	31±	149±33	82±32	23±7	11±1	21±6
ID331	Parma	organic	82±5	18±2	6±4	5±2	14±3	17±4	4±2
farro della Garfagnana	Parma	organic	33±4	21±0	155±37	89±19	8±4	5±3	21±14
Rouquin	Parma	organic	19±6	25±9	71±43	21±16	11±11	5±3	4±1

Table 4. Amount (in mg/kg) of each toxic peptide in the different varieties tested. Values are the average of three biological replicates, coming from three different parcels.

wheat variety	cultivation area	farming	LQPQNPSQQQPQ	RPQQPYPQPQPQ	QQPPFSQQQPPPFS
grano del miracolo	Parma	conventional	51±13	27±4	19±8
Virgilio	Parma	conventional	73±8	60±11	3±0
Blasco	Parma	conventional	33±4	36±9	13±2
Bologna	Parma	conventional	38±11	41±12	11±7
senatore Cappelli	Parma	conventional	50±6	42±1	10±2
Odisseo	Parma	conventional	56±13	48±11	7±1
ID331	Parma	conventional	32±9	93±23	2±1
farro della Garfagnana	Parma	conventional	66±11	62±16	2±1
Rouquin	Parma	conventional	43±7	44±15	6±4
grano del miracolo	Bologna	conventional	67±16	37±9	23±5
Virgilio	Bologna	conventional	111±11	104±7	5±1
Blasco	Bologna	conventional	42±7	43±5	15±1
Bologna	Bologna	conventional	42±9	44±11	12±4
senatore Cappelli	Bologna	conventional	75±12	55±11	23±10
Odisseo	Bologna	conventional	63±0	61±4	18±2
ID331	Bologna	conventional	38±6	123±34	3±1
farro della Garfagnana	Bologna	conventional	68±13	74±14	3±0
Rouquin	Bologna	conventional	43±9	43±13	7±2
grano del miracolo	Parma	organic	74±5	38±4	25±1
Virgilio	Parma	organic	109±9	95±9	5±3
Blasco	Parma	organic	49±3	46±5	19±3
Bologna	Parma	organic	35±16	45±15	12±5
senatore Cappelli	Parma	organic	64±15	46±15	13±4
Timilia	Parma	organic	77±4	71±4	10±0
Odisseo	Parma	organic	58±16	55±14	12±7

ID331	Parma	organic	39±1	104±	3±2
farro della Garfagnana	Parma	organic	46±8	51±7	2±0
Rouquin	Parma	organic	22±10	27±12	3±2