Chapter 15

Celiac Disease: Gluten Peptides Characterization after *In Vitro* Digestion

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Additional information is available at the end of the chapter

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Abstract

Gluten proteins are characterized by the high glutamine and proline content; thus, during gluten digestion, several resistant peptides are produced. Some of them contain sequences that, in celiac patients, are able to trigger an immunological reaction. The prolamin fraction of different wheat samples was submitted to *in vitro* digestion, and the peptides generated were analysed using liquid chromatography coupled to mass spectrometry techniques. Several wheat varieties were analysed, showing large differences in the production of immunotoxic peptides on digestion. After simulated gastrointestinal digestion of wheat, emerged that peptides containing sequences known to elicit the adaptive immune response derived mainly from γ-gliadin, whereas peptides containing sequences involved in the innate immune response were distributed among α-gliadin and γ-gliadin and low-molecular-weight glutenins. From the results, no major differences due to the different cultivation places were observed. On the other hand, statistically significant differences are present among the genotypes tested, especially for the immunogenic peptides. The possible development would be the selection of wheat genotypes with reduced amount of immunogenic sequences, to reduce the exposure of people and decrease the risk of new cases of disease.

Keywords: *In vitro* digestion, gluten peptides, celiac disease, LC-MS, wheat protein

1. Introduction

Approximately 8% of children and 1–2% of adults suffer from food allergy worldwide, and the perceived prevalence is even much higher, up to 22% of the population, constituting a fast growing health problem [1, 2]. The prevalence of food allergies is continuously increasing in the last decades, especially in the developed countries. The Big-8 of food allergens, namely the foods that are mainly involved in these immunological reactions, are milk, eggs,

fish, crustaceans, peanuts, tree nuts, soybeans and wheat. These foodstuffs can be eaten by most of the population without problems, but they can give a strong immunological reaction with topic and systemic consequences in sensitive people [3, 4]. Thus, the only therapy available for patients suffering from food allergy is the strict avoidance of the offending food. This means that allergic consumers must absolutely avoid eating foods that could provoke potentially life-threatening reactions. Successful avoidance depends on having complete and accurate information on food labels. Thus, huge efforts are made by regulatory agencies, with the collaboration of food industry, to protect allergic consumers, to ensure that all food allergens present in the food are declared on the label and that effective controls are used to prevent the presence of unintended allergens [5]. In the case of children, dietary elimination of nutrient-dense foods may result in inadequate nutrient intake and impaired growth: children with multiple food allergies have a higher risk of impaired growth and may have a higher risk of inadequate nutrient intake than children without food allergies. In addition to this, the social lifestyle of individuals with food allergy and of their families can be severely disadvantaged, since they need to constantly avoid the allergenic ingredient [6]. This task becomes more difficult to manage when people do not eat at home but in restaurants, canteens and other food chains, even if a list of the ingredients of all the dishes must be provided. Moreover, the repercussions of food allergy are not only limited to individuals or households: the food industry must also sustain a lot of extra costs due to food allergy. Primarily, legislative changes, such as the new EU-legislation on food labelling (EU Directive 2003/89/EC amending Directive 2000/13/EC), force the industry to adapt productive processes, food labelling and monitoring to improve allergic consumer protection. The burden of responsibility falls to the food manufacturer, who is required to manage production processes to ensure allergenic ingredients are labelled [7]. Up to now, the potential social impact and economic costs of food allergy on the individual, families, health-related services and food industry are relevant.

Wheat is in the list of the eight main allergenic foods, because the gluten contained in it is the main external trigger of celiac disease. Celiac patients eat several types of gluten-free products, some of them are naturally gluten-free foods (fruits, vegetables and unprocessed meat, fish and poultry) but some others are gluten-free substitute foods (pasta, bread, cereals, crackers and snack foods) where wheat flour is replaced by gluten-free flours. Gluten-free products can be purchased at general and specialty food stores as well as via Internet. Several studies demonstrated that gluten-free food is not always readily available, and it is considerably more expensive than regular, gluten-containing foods [8]. The increasing incidence of celiac disease in the population has negative effects not only on the quality of life but also on the health care system: it has been estimated that the average annual health care costs per-patient in primary care significantly increased by 91% for CD patients after they had been diagnosed with the disease [9]. The impact is also evident for the agricultural and food sectors: wheat is one of the first three cereals for diffusion and cultivation for human nutrition. Gluten, the main trigger of celiac disease, is at the basis of rheological properties of wheat-based products. In fact, the formation of a gluten network in the dough is of outmost importance for air bubbles and starch retention (respectively for leavened products and pasta). A low gluten content of the flour leads to loss of product shape in the case of leavened products and to soft

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and mushy pasta. The consequence is that wheat breeding has been, during the last decades, oriented toward increasing yield and the amounts of amylopectin, gluten and protein [10].

At the moment, no therapies are available for people that are already celiac, so the only treatment is the gluten-free diet. But, on the other hand, efforts can be made in the direction of decreasing celiac disease incidence. Different hypotheses have been made on the reasons of the increased incidence of celiac disease. Since celiac disease affects the gastrointestinal tract, the gut microflora can play a key role in the loss of the immunological tolerance. For example, rod-shaped bacteria in the upper small bowel are present in one-third of the children with CD but in less than 2% of the controls [11]; another study showed that the species *Bacteroides fragilis* is more abundant in the intestinal microbiota of CD patients, whereas *Bacteroides ovatus* is less abundant in comparison to healthy controls [12]. Besides usual microflora, also viral and bacterial gastroenteritis may have a role in celiac disease pathogenesis; in fact, it has been previously demonstrated that a high frequency of rotavirus infections may increase the risk of celiac disease autoimmunity in childhood in genetically predisposed individuals [13]. For what concerns gluten, timing of gluten introduction into the infant diet is associated with risk of celiac disease autoimmunity [14]. Recent studies demonstrated that the oral tolerance to gluten can be lost also in the elderly [15]; the study was conducted after a cohort from 1974 up to now. In parallel, it appears that vital gluten consumption has tripled since 1977. This increase is of interest because it is in the time frame that fits with the predictions of an increase in celiac disease [16]. It seems that massive and early exposure to gluten can be one of the causes of the switch from oral tolerance to celiac disease. Another cause that it has been hypothesized is the transition from sourdough fermentation of bread and baked products to yeast fermentation. So, the bacterial proteolytic activity is rather promising not only as currently demonstrated for eliminating traces of contaminant gluten but probably also in perspective for the manufacture of tolerated baked goods [17].

Thus, trying to decrease these risk factors could help to stop the rising of celiac disease incidence. It is known for a long time that breast feeding has a protective effect against the development of celiac disease, especially when it is still ongoing during gluten introduction in the diet. Also the improvement of infant milk formula, decreasing protein content and osmolarity, has helped to reduce celiac disease incidence [18]. Obviously, the easiest way to reduce the amount of gluten ingestion is the reduction of wheat-derived products consumption, but this would mean a kind of "preventive gluten-free diet", with all the problems and limitations previously described (first of all the decrease in life quality). An alternative way could be the reduction of gluten content in wheat (in contrast with what has been done in the last decades), but this would mean a dramatic decrease in the texture quality of baked products and pasta. Since gluten proteins have a reserve role (nitrogen stock), they underwent to a limited evolutionary pressure, thus showing a high-sequence variability with a lot of different isoforms. This lays the groundwork for a possible varietal selection aimed to have the same total gluten amount (maintaining the same rheological properties) but expressing protein isoforms with a reduced content of sequences involved in celiac disease. In this way, the exposure of the population to immunotoxic sequences will be reduced and, possibly, also the incidence of the disease.

2. Characterization of the peptides deriving from gluten digestion

Differences in gluten coding genes have been extensively characterized, besides for their technological and functional implication in baked products, also for evaluating how much the wheat genetic characteristics can impact on the final immunotoxicity of gluten. One of the most studied immunogenic peptides, the 33-mer LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, has been demonstrated to be encoded by the 6D chromosome, thus being absent in diploid and tetraploid *Triticum* species. The lacking of genes encoding for some immunogenic sequences in diploid/tetraploid varieties has also found evidences in T-cell assays performed on gluten chymotryptic digests that gave different results among the species tested [19]. A lot of efforts have been made in the last years in the direction of decreasing wheat toxicity for celiac patients, for example, making use of *ex vivo* organ cultures and immunohistochemistry assays [20]. The K562 cell agglutinating activity of wheat has been known for many years [21] to screen cereal toxicity for celiac patients [22]. The majority of the studies performed in the past to assess gluten toxicity with immunochemical methods, the main immunotoxic peptides were identified, and in some cases, also a complete characterization of the digestion mixture was obtained [23]. In the recent years, the advances in the field of mass spectrometry allowed more accurate peptides identification, especially in the case of recombinant gliadin and synthetic peptides [24–27]. Other studies took in consideration purified gliadins, to identify the target peptides of tissue transglutaminase [28]. Peptide mixtures obtained by gluten digests are very complex, since than more than 40 components are present both for gliadins and glutenins [29]. The composition of peptide mixtures derived from simulated gastrointestinal digestion of the whole gliadin fraction of wheat was recently investigated [30]. The gliadin fraction was extracted with 70% ethanol in water and then desiccated. The *in vitro* digestion was performed using the three main proteases of the gastrointestinal tract: pepsin in the gastric phase (3 h) and chymotrypsin/trypsin in the intestinal phase (4 h). All the enzymes were used at their optimal temperature (37°C) and pH (respectively 2 and 7.2), using an enzyme:substrate ratio of 1:100. The determination of the amino acid sequence of the gluten-derived peptides was achieved using reverse phase chromatography (High Performance Liquid Chromatography [HPLC], Ultra Performance Liquid Chromatography [UPLC] and Micro-High Performance Liquid Chromatography [μHPLC]) coupled with different types of mass spectrometer (both high and low resolution, e.g., Linear Trap Quadrupole (LTQ) OrbiTrap, single and triple quadrupoles) to achieve the desired resolving power.

This extensive characterization (**Table 1**) gives useful information for a better understanding of the peptides that presumably come in contact with the intestinal mucosa, triggering the immunological response in celiac patients. Immunogenic peptides are those containing sequences known in literature to elicit the adaptive immune response, through recognition by the HLA-DQ2 (or DQ8) of the antigen presenting cells (APC), leading to stimulation of T cell response. As it is shown in **Table 1**, the identified immunogenic peptides derive mainly from α-gliadin, in particular from the region between the 56th to the 88th amino acid. Toxic peptides are those containing sequences known in literature to elicit the innate immune response: their interaction with epithelial cells, macrophages and dendritic cells leads to the up regulation of different cell mediators, the most important one being interleukin-15. Also in this case,

most of the peptides were identified as deriving from α-gliadin, more specifically from the N-terminal region. Using the isotopically labelled internal standard method, peptides containing sequences involved in celiac disease can be quantified: these data can be very helpful for interpretation of the results of immunological assays, since the different response can be due both to different epitopes generation in terms of amino acid sequence and to a different relative amount of pathogenic peptides.

	Gliadin type	Relative amount (durum)	Relative amount (common)
Immunogenic peptides identified			
QLQPFPQPQLPY	α-Gliadin	+++	+
QLQPFPQPQLPYPQPQPF	α-Gliadin	+	+
LQLQPFPQPQLPY	α-Gliadin	+	+
LQLQPFPQPQLPYPQPQPF	α-Gliadin	++	+
QLQPFPQPQLPYPQPQLPYPQPQPF	α-Gliadin	nd	+
QLQPFPQPQLPYPQPHLPYPQPQPF	α-Gliadin	nd	++
LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	α-Gliadin	nd	+++
LPFPQQPQQPFPQPQ	γ-Gliadin	Trace	Trace
Toxic peptides identified			
SHIPGLEKPSQQQPLPL	LMW-glutenin	+	+
VRVPVPQLQPQNPSQQQPQEQVPLVQQQQF	α-Gliadin	+	+
QNPSQQQPQEQVPLVQQQ	α-Gliadin	+	+
VPVPQLQPQNPSQQQPQEQVPL	α-Gliadin	++	++
VRVPVPQLEPQNPSQQQPQEQVPL	α-Gliadin	+	+
VRVPVPQLQPQNPSQQQPQEQVPL	α-Gliadin	+++	+++
VRFPVPQLQPQNPSQQQPQEQVPL	α-Gliadin	+	+
PSSQVQWPQQQPVPQ	γ-Gliadin	+	+
NMQVDPSGQVQWPQQQPF	γ-Gliadin	+	+

Adapted with permission from Ref. [30]
+++, very abundant; ++, abundant; +, detectable; nd, not detectable.

Table 1. Most abundant immunogenic and toxic peptides identified in the digested prolamin extracts (known immunogenic and toxic sequences are underlined), together with an indication of the protein of origin and of their relative abundance in the different types of wheat (durum wheat: *Triticum durum*; common wheat: *Triticum aestivum*).

3. Wheat screening through *in vitro* digestion and LC-MS analysis

The *in vitro* digestion of the prolamin extract described in Section 2 was performed on a wide spectrum of wheat samples, to observe eventual differences in the production of immunogenic

and toxic sequences after gastrointestinal digestion. Briefly, the prolamin fraction was extracted with 70% ethanol and submitted to simulated *in vitro* digestion using the three main proteases of the gastrointestinal tract: pepsin in the gastric phase (3 h) and chymotrypsin/trypsin in the intestinal phase (4 h). All the enzymes were used at their optimal temperature (37°C) and pH (respectively 2 and 7.2), in an enzyme:substrate ratio of 1:100. The quantification of the gluten-derived peptides was achieved using reverse-phase ultra-high-performance liquid chromatography (UPLC) coupled with single quadrupole mass spectrometer (SQD), using the isotopically labelled internal standard method (the peptide LQLQPF(d_5)PQPQLPY, one of the most abundant, was used as standard). In this way, it has been possible to evaluate the influence of the cultivation area (i.e. the soil—climatic conditions) and of the genotype of the wheat (genetic influence).

3.1. Influence of the cultivation region

To investigate the role of soil and climatic conditions on the total amount of toxic and immunogenic sequences, durum wheat samples harvested in three different Italian regions were submitted to prolamin extraction and *in vitro* digestion. The three harvesting area were chosen to maximize the soil and climatic differences. Argelato is located in Northern Italy, in the Po plain with a temperate sub-oceanic climate: mean annual temperature is 11.0–13.0°C (months with mean temperature below 0°C: January) and precipitations are 690–1200 mm (May and October are the rainy months, whereas July and August are the driest months). Falconara is located in the hills of the Central Italy, with Mediterranean sub-oceanic climate: mean annual temperature is 12.5–16.0°C (no months with mean temperature below 0°C) and precipitations are 700–1000 mm (November is the rainy month, whereas July and August are the driest). Lucera is located in the Capitanata area, with Mediterranean subtropical climate: mean annual temperature is 12.0–17.0°C (no months with mean temperature below 0°C) and precipitations are 400–800 mm (October and November are the rainiest month, May to September are the driest).

The total amount off peptides containing immunogenic and toxic sequences is reported in **Figure 1**, mediated for each harvesting area. Statistically significant differences were determined with analysis of variance (two ways ANOVA), with $p < 0.05$. Immunogenic and toxic peptides show the same trend: there were no statistically significant differences among the three different locations, with the exception of Argelato (BO), that show a slightly higher content of peptides containing sequences involved in celiac disease. Thus, the soil and climate conditions do not have a determining role on the amount of immunogenic and toxic gluten sequences. The high intra-region variability, indeed, suggests that there are other factors that are playing an important role.

3.2. Influence of the genotype

The accurate molecular characterization of the *in vitro* digested prolamin mixtures is an interesting tool for the screening of different wheat lines aimed to identify those producing a smaller amount of pathogenic peptides [31]. Genetic selection operated by breeders, to achieve the desired rheological properties, has led to a decrease in genetic biodiversity of wheat varieties nowadays present on the market. Thus, 25 accessions from a durum wheat

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panel were chosen to maximize genetic biodiversity of the samples: the prolamin fraction was extracted with 70% ethanol and submitted to *in vitro* digestion. From a molecular point of view, the results obtained confirm what has been previously assessed using genetic and immunologic approaches, that there is a strong influence of the genotype in the final amount of peptides containing sequences involved in celiac disease.

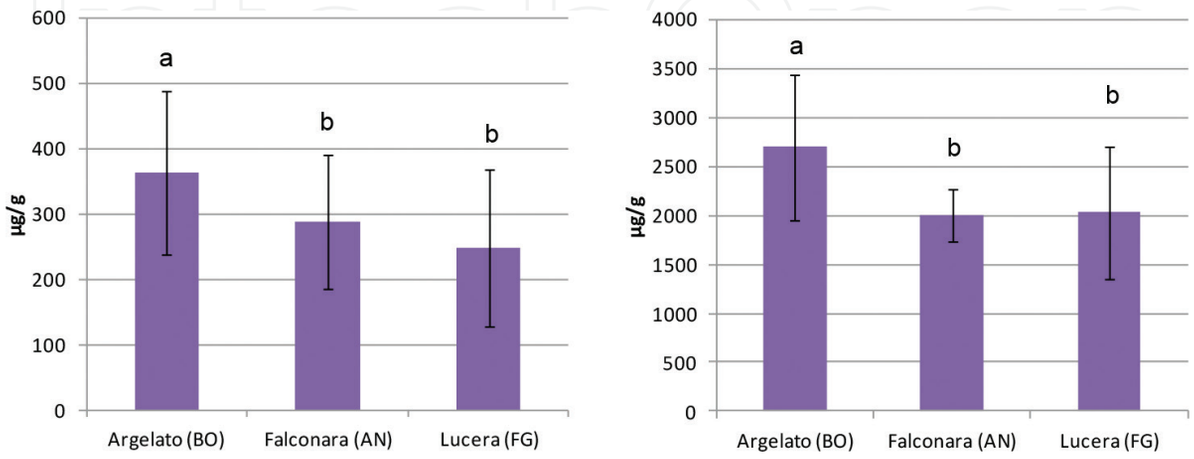

Figure 1. Total average content of immunogenic (left panel) and toxic peptides (right panel) of wheat samples harvested in three different regions (Argelato, Falconara and Lucera). Total amount of peptides is expressed in µg of peptide for gram of sample. Bars with different letters mean statistically different samples. Adapted with permission from Ref. [31].

As shown in **Figures 2** and **3**, there are great differences among the different samples. More specifically, the peptides that are more affected by genetic features are those eliciting the adaptive immune system (immunogenic peptides). This relies on the fact that toxic peptides derive from the N-term region of gliadins, which is much more conserved than the region that originates immunogenic peptides. In the latter case, the difference is surprisingly high: there is a 6-fold difference between the highest and the lowest scoring sample (600 µg/g vs 100 µg/g). These data confirm the huge variability in gluten-coding genes, since also among accessions of the same genetic group, there are noticeable differences, for example, in the first group. Recent studies demonstrated that number of subjects that lost the immunological tolerance to gluten in their adulthood is increasing and among the possible causes there is also the amount and the quality of ingested gluten [15]. This means that the use of less immunogenic wheat varieties (especially in the preparation of baby foods) can reduce the exposure to gluten, possibly decreasing the incidence of the disease. And, moreover, it would be possible to operate a varietal selection aimed to have the same gluten content (thus comparable rheological properties), but expressing different gliadin isoforms, with a reduced content of immunogenic and toxic peptides, to reduce the exposure of genetically predisposed subjects, and possibly to reduce the risk of celiac disease development. These data take in consideration the molecular point of view, so it would be really interesting to cross the data with immunological tests (such as T cell proliferation assays or K562 cells agglutination) on the samples to verify the quality of the correlation between pathogenic peptides content and immune response.

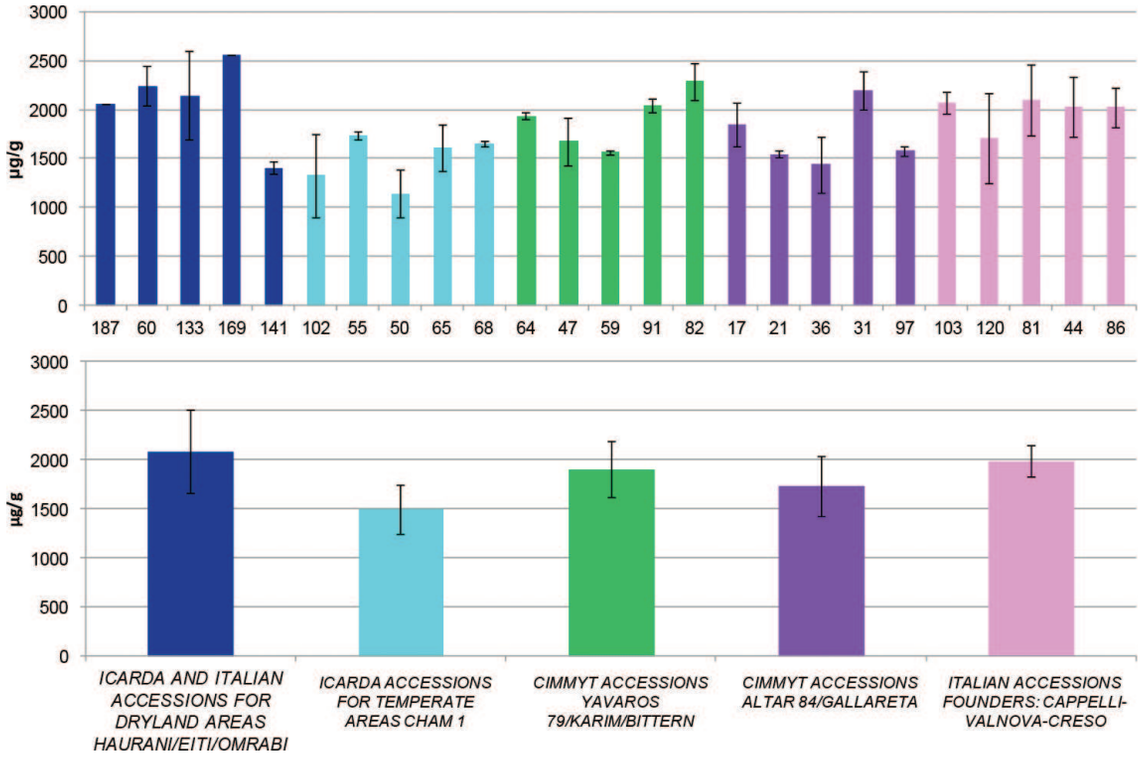

Figure 2. Total content of toxic peptides (expressed in µg of peptide for gram of sample) in 25 samples from a Durum Panel collection (upper panel). Samples were grouped on the bases of phylogenetic affinity on dendrogram (lower panel). Adapted with permission from Ref. [31].

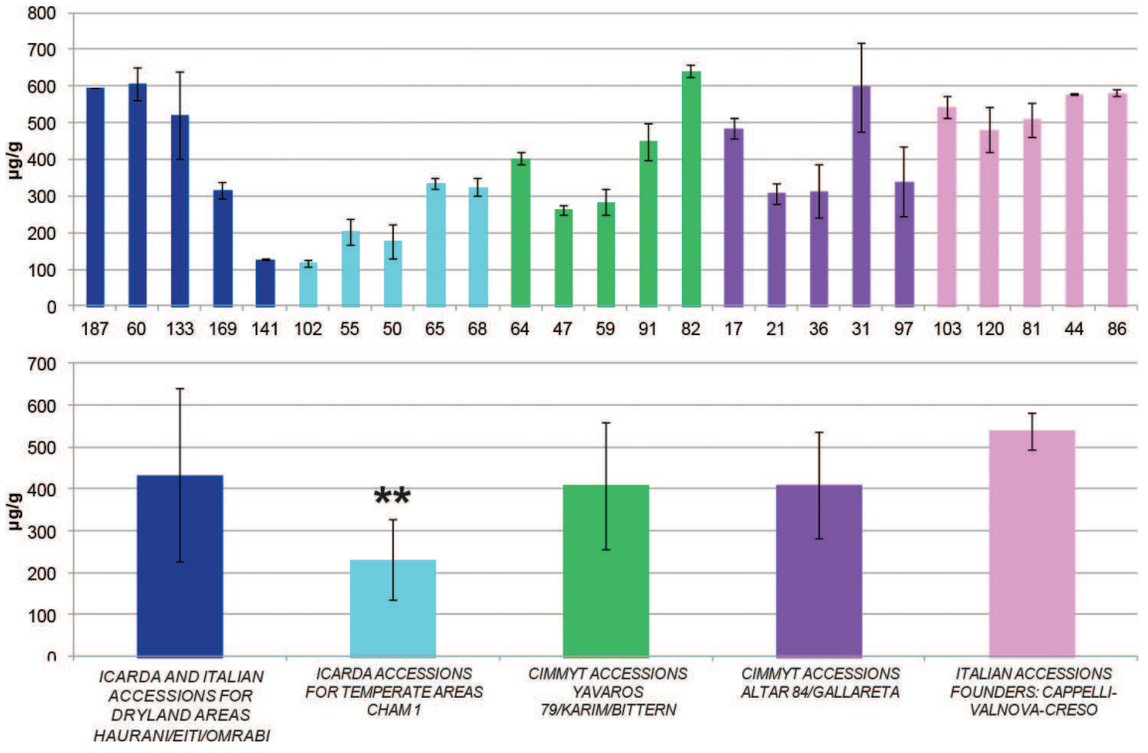

Figure 3. Total content of toxic peptides (expressed in µg of peptide for gram of sample) in 25 samples from a Durum Panel collection (upper panel). Samples were grouped on the bases of phylogenetic affinity on dendrogram (lower panel). ** Statistically different group. Adapted with permission from Ref. [31].

4. Wheat digestion: comparison between two different models

To perform immunological assays on gluten peptides, it is necessary to simulate the human gastrointestinal digestion on gliadin/gluten/wheat samples. In literature, *in vitro* digestion models were amply used to study gluten peptides. But the weakness of these approaches is that they are all different and there was no standard method. Previously used models are not consistent with each other for the type of enzyme used (peptic/tryptic digests, peptic/chymotryptic, pancreatin, eventual exoproteases), for the digestion times of the gastric and intestinal phase (from 20 min to several hours), for the buffering agents used (hydrochloric acid, formic acid, bicarbonate, or phosphate buffer), for the protein:enzyme ratio used and so on. All these factors could have a strong influence on the outcome of the digestion, both in terms of peptide sequence and amount, which can be reflected also on subsequent analysis on the gluten digest. In view of this, a qualitative and quantitative comparison of the peptides generated was performed [32] applying two extremely different digestion models: a very simple peptic/tryptic-chymotryptic digestion of a gliadin ethanol extract and a more complex and more physiological method involving the use of artificial digestive juices [33]. These juices strictly reflect the physiological composition of salts and enzymes, involving the use of α-amylase, pepsin and pancreatin (a mixture of α-amylase, lipase, trypsin, chymotrypsin, elasase and carboxypeptidase). Peptides generated were identified using LC-MS/MS techniques both at high and low resolution and quantified using the isotopically labelled internal standard method.

A qualitative comparison of the peptides generated with the two models is reported in **Table 2**, together with the protein of origin of the peptide and the retention time. Results clearly showed that the peptide composition obtained is completely different. While with the simplified digestion model, quite all the peptides derive from α-gliadins; using the physiological digestion model, they are equally distributed among α-gliadins, γ-gliadin and low-molecular-weight glutenins. This fact can be ascribed to the different solubilisation power of the two methods. Ethanol extraction of the prolamin fraction probably leads to a better extractability of α-gliadins; on the opposite, in the physiological digestion model, the presence of additional enzymes other than proteases (such as α-amylases and lipases), together with the bile salts, contributes to matrix degradation, improving the extractability and digestibility of higher molecular weight proteins such as γ-gliadins and low-molecular-weight glutenins. Another important difference among the two models is the presence of specific cleavage sites for the enzymes used. In the simplified digestion model, all the peptides show specific cleavage sites for the three enzymes used (pepsin, chymotrypsin and trypsin), for example, tyrosine, phenylalanine and leucine. In the physiological model indeed, in most cases, there are no specific cleavage sites, due to the action of the exoproteases present in pancreatin. Thus, changing the *in vitro* digestion model, the peptide profile is completely different. Using the isotopically labelled internal standard method, the peptides were quantified for both digestion models, and their total amount was plotted to obtain a quantitative comparison. Despite the different identified peptide sequences, the two *in vitro* digestion methods showed a good correlation in terms of immunotoxic sequences. This means that, despite the different amino acid sequence of the peptides generated, the immunotoxicity of a wheat variety is an own intrinsic characteristic of its gluten. In **Figure 4**, the total amount of immunogenic and toxic peptides obtained with the physiological digestion is plotted with those arising from the simplified digestion model.

Simplified digestion			Physiological digestion		
Adaptive immune response	**Prot**	**Rt**	**Adaptive immune response**	**Prot**	**Rt**
QLQPFPQPQLPY	α	30.5	TQQPQQPFPQ	γ	20.5
QLQPFPQPQLPYPQPQPF	α	32.7	SQQPQQPFPQPQ	γ	21.3
LQLQPFPQPQLPY	α	32.6	QAFPQQPQQPFPQ	γ	24.4
LQLQPFPQPQLPYPQPQPF	α	34.1	TQQPQQPFPQQPQQPFPQ	γ	24.9
QLQPFPQPQLPYPQPQLPYPQPQPF	α	34.1	PQTQQPQQPFPQFQQPQQPFPQPQQP	γ	26.8
QLQPFPQPQLPYPQPHLPYPQPQPF	α	33.3	FPQQPQLPFPQQPQQPFPQPQQPQ	γ	29.3
LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	α	26.5	PFPQPQQPQQPFPQSQQPQQPFPQP	γ	29.3
LPFPQQPQQPFPQPQ	γ	29.6	QPQLPFPQQPQQPFPQPQQPQQPSPQSQQPQQPFPQ	γ	29.8
			QQPQQPFPQPQQTFPQQPQLPFPQQPQQPFP	γ	30.7
Innate immune response	**Prot**	**Rt**	**Innate immune response**	**Prot**	**Rt**
VRVPVPQLQPQNPSQQQPQEQVPLVQQQQF	α	28.2	LQPQNPSQQQPQ	α	16.6
QNPSQQQPQEQVPLVQQQ	α	26.8	RPQQPYPQPQPQ	α	18.0
VPVPQLQPQNPSQQQPQEQVPL	α	28.0	LQPQNPSQQQPQEQVPL	α	23.9
VRVPVPQLEPQNPSQQQPQEQVPL	α	26.6	LGQQQPFPPQQPYPQPQPFPS	α	27.3
VRVPVPQLQPQNPSQQQPQEQVPL	α	28.8	SQQQQPV	γ	14.5
VRFPVPQLQPQNPSQQQPQEQVPL	α	29.8	QQQPL	LMW	16.5
PSSQVQWPQQQPVPQ	γ	23.0	QQQPPFS	LMW	19.8
NMQVDPSGQVQWPQQQPF	γ	28.1	PQQPPFSQQQQPV	LMW	22.0
SHIPGLEKPSQQQPLPL	LMW	25.6	QQPPFSQQQPPPFS	LMW	25.5
			QQQPLPL	LMW	25.4

Adapted with permission from Ref. [32]
α, α-gliadin; γ, γ-gliadin; LMW, low-molecular-weight glutenin; Rt, retention time expressed in minutes. Epitopes and toxic sequences are underlined.

Table 2. Pathogenic peptides identified in the digested samples obtained with the two different models, with the protein of origin.

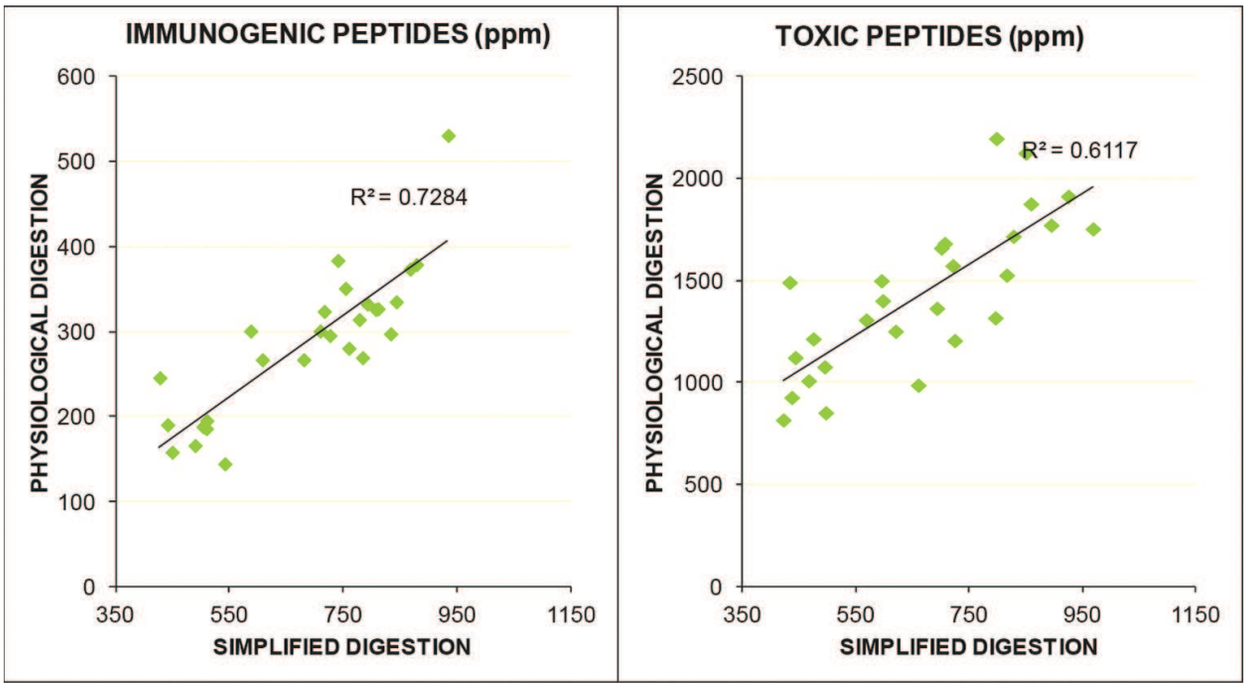

Figure 4. Correlation between the amount of immunogenic and toxic peptides generated with the two digestive models. Peptide amounts are expressed as parts per million (ppm).

A good correlation was found using the Pearson test ($p < 0.05$, coefficient of correlation = 0.73 for immunogenic peptides and 0.61 for toxic peptides), as shown in **Figure 4**. This means that for biological experiment, the physiological systems should be suitable, because the peptides generated are similar to those that really come in contact with intestinal cells. Of course, the limit of these models is the lacking of brush border membrane enzymes (that can further proteolyse the peptides) and of the intestinal microflora. A possible interesting continuation of the work could be the use of a physiological model taking into account also these latter variables and studying the effects in terms of peptides produced. However, the good correlation between the total amount of immunogenic and toxic peptides, would suggest to use the simplified method for varietal screening or comparison purposes, where a high throughput, low cost and simple analysis is required.

5. Gluten peptides' fate in the pasta production chain

The quantification of gluten peptides reported in **Table 2** was carried out for six steps of pasta production (involving only durum wheat), to verify if some technological treatment have an influence on protein extractability/digestibility. Three different varieties were analyzed to exclude variations exclusively due to the genotype. Results are shown in **Figure 5** for whole wheat, flour, dough, extruded pasta, dried pasta and cooked pasta.

As observed in **Figure 5**, after the physiological digestion method, the amount of toxic peptides is approximately a half than with the simplified method, whereas immunogenic peptides are approximately twice, due to the different type of peptides generated. This should

be taken into account when biological tests are done to assess immunological responses. It can be noted that the amount of toxic and immunogenic peptides remains largely constant along the pasta production chain, so none of the processing steps of pasta is at the moment able to decrease wheat immunogenic potential for celiac patients. In other words, if a varietal screening has to be performed, there is no need to use the end product; it is sufficient to test the basic wheat variety. The difference between the two digestion methods becomes more evident after pasta cooking; in fact, heat causes polymerization of gliadins through intermolecular disulphide bridge formation and to a lesser extent for dehydroalanine formation [34]. Thus, the heat treatment leads to the loss of gliadin extractability, which is the reason of the high underestimation of peptides generated after digestion of the ethanolic extract. It is interesting to note that independently from the method adopted, the differences between varieties maintain the same trend at all the steps of processing. This is an ulterior confirmation that traditional pasta processing leaves gluten immunogenic and toxic peptides unaffected.

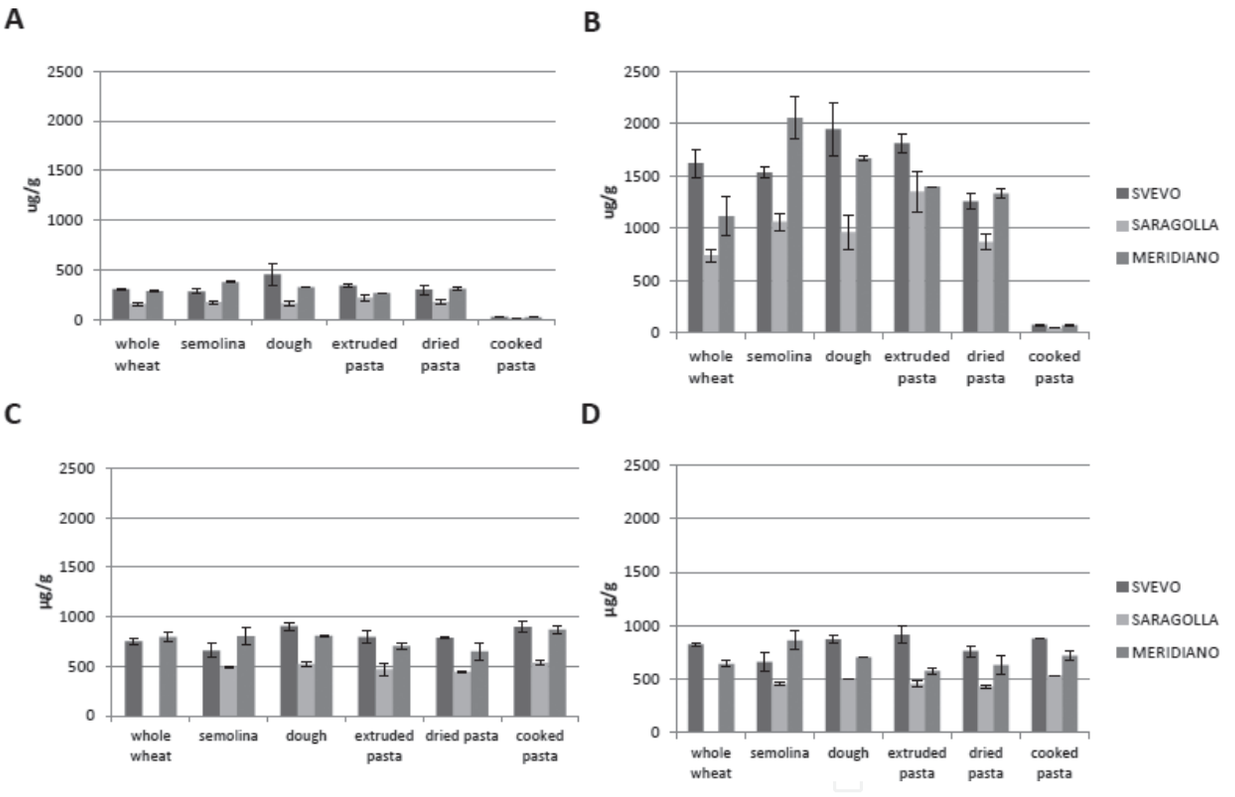

Figure 5. Total amount of toxic and immunogenic peptides (upper and lower plots, respectively) quantified after the simplified and the physiological digestion model. Adapted with permission from Ref. [32].

6. Conclusion

Several digestion methods applied to gluten proteins are reported in literature. Generally, these models are very simple involving only the use of the main gastric and pancreatic proteases (pepsin, trypsin and chymotrypsin). A buffering agent is also used, to keep the correct pH value at each phase. However, a physiological digestion procedure was previously used

in literature to assess the release of mycotoxins and heavy metals from food matrices. This method involves the use of digestive juices whose chemical composition strictly reflects the physiological one. These two methods were compared to assess gluten peptides generated. In both cases (simple and complex model), the peptides generated from the digestion were characterized using liquid chromatography coupled with mass spectrometry. In these *in vitro* experiments, the processes occurring in the human gastrointestinal tract during food digestion were simulated, and the outcome of the digestion was assessed by LC-MS techniques. With the use of tandem mass spectrometry, the exact amino acid sequence of the peptides generated by the digestion was determined. Among all the peptides, the ones containing sequences known to be implied in celiac disease were identified. Strong differences were present between the two digestion models. First, with the simplified model almost all the peptides derive from α-gliadin, whereas with the physiological method, they are equally distributed among α-gliadins and γ-gliadins and LMW glutenins. This can be explained by the observation that α-gliadin-derived peptides of the simplified method are further proteolyzed into shorter peptides in the physiological model and often these shorter peptides did not contain immunotoxic sequences anymore. Moreover, in the physiological model are present enzymes other than proteases (like amylase and lipase) that, even if not directly implied in protein cleavage, can contribute (together with bile salts) to matrix degradation, thus improving the extractability and digestibility of higher molecular weight proteins such as γ-gliadins and glutenins.

Thus, in the case, a subsequent immunological experiments or biological trials have to be performed, the more physiological method is more suitable than the simplified one, because the peptides generated are really different and the complex method is more similar to what really happens in the human gastrointestinal tract.

The peptides containing immunotoxic sequences were quantified for both the *in vitro* digestion models along the pasta production chain, to evaluate also the suitability of the two methods for processed foods. The samples (kernels, semolina, dough, extruded pasta, dried pasta and cooked pasta) were obtained from three different durum wheat varieties (Svevo, Meridiano and Saragolla). The physiological digestion method produced lesser amount of toxic and a higher amount of immunogenic peptides compared to the simplified one, probably due to the different molecular weight of the peptides generated. A noticeable result is that the difference among the varieties tested remains unchanged, with Saragolla showing a lower content of peptides involved in celiac disease compared to Svevo and Meridiano. Another remarkable result is that the simplified method cannot be applied to thermally treated foods, because heating induces gluten polymerization leading to poor proteins extractability. The two different models are very well correlated in terms of total amount of immunotoxic peptides generated. So, to perform a varietal screening, the simplified method is suitable when a large amount of samples has to be analyzed.

In vitro digestion of the prolamin extract was then applied to 45 durum wheat samples belonging to five different varieties and harvested in three different Italian regions (Argelato in the North of Italy, Falconara in the Centre and Lucera in the South). These findings showed no major differences due to the different cultivation place, consistently with the reserve role of that class of proteins (that thus is not affected by environmental factors).

For what concerns genotype influence, since the cultivar selection operated by breeders in the last years to achieve the desired rheological properties has led to a decrease in the genetic biodiversity of durum wheat varieties present nowadays on the market, 25 durum wheat accessions were selected from a durum wheat panel in order to maximize the genetic biodiversity of the samples (and thus eventual differences in immunotoxic peptides production upon digestion). Results obtained from every single accession were mediated in five groups on the basis of phylogenetic affinity on dendrogram.

For toxic peptides, no significant differences were present while strong variability emerged for immunogenic peptides, with accessions of the second groups (International Center for Agricultural Research in the Dry Areas (ICARDA) accessions for temperate areas) showing a significantly lower content of peptides eliciting adaptive immune response.

The higher variability of immunogenic peptides compared to toxic peptides can be explained on the basis of gliadins sequence variability; in fact, toxic peptides usually derive from the N-term region of the protein, which is the most conserved. On the contrary, immunogenic peptides derive from a region of the protein showing a much higher variability. So, different wheat genotypes can express different gliadins isoforms thus showing a different final content of immunogenic sequences.

Then, it is possible to select wheat varieties with good gluten content (and good rheological properties) but with a reduced amount of immunogenic sequences in order to reduce the exposure of people to a possible trigger for celiac disease.

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