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CICLO: XXXIV

INFLUENCE OF GENOTYPE, ENVIRONMENT AND CROP MANAGEMENT ON GLUTEN PROTEIN COMPOSITION AND IMMUNOTOXICITY IN WHEAT AND TRITORDEUM

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Preface

This Ph.D. dissertation is the result of experimental work carried out in the Department of Agricultural, Forest and Food Sciences at the University of Turin (Italy) and in the Department of Food Science, at the "Federico II" University of Naples (Italy).

This Ph.D. project was carried out under the supervision of Prof. Massimo Blandino. The research period at the "Federico II" University of Naples was made possible thanks to a collaboration with Prof. Pasquale Ferranti, Dr. Maria Adalgisa Nicolai and Dr. Chiara Nitride.
The main themes of this Ph.D. dissertation concern the importance of studying the contribution of the genotype and the agronomic and environmental management practices to changes in the protein composition of cereal gluten in relation to its technological and potentially immuno-toxic properties, and they are organized in six chapters as described below.

- **Chapter I** introduces the importance of gluten protein for the end-use value of t wheat and other small cereal bread considering such new species as amphiploid tritordeum, and addresses the importance of the cultivation management practices and the environmental effects on the content and rheological properties of such grains. This chapter also highlights some possible negative traits of gluten, as far as the safety of consumers is concerned, focusing on disease related to gluten digestion;
- **Chapter II** outlines the aims of the experimental activities that were performed within the Ph.D. project;
- **Chapters III to VI** present the original papers and book chapters that have been published in International Peer-Reviewed Journals during the Ph.D. formation period:

- **Chapter III** – “Effect of Nitrogen Fertilization and Fungicide Application at Heading on the Gluten Protein Composition and Rheological Quality of Wheat” Landolfi, V., Visioli, G., & Blandino, M. (2021). Agronomy, 11(9), 1687. https://doi.org/10.3390/agronomy11091687;

- **Chapter IV** – “The effect of nitrogen fertilization on the expression of protein in wheat and tritordeum varieties using a proteomic approach”. Landolfi, V., D’Auria, G., Nicolai, M. A., Nitride, C., Blandino, M., & Ferranti, P. (2021). Food Research International, 148, 110617. https://doi.org/10.1016/j.foodres.2021.110617;

- **Chapter V** – “Tritordeum as an Innovative Alternative to Wheat: A Comparative Digestion Study on Bread.” Nitride, C., D’Auria, G., Dente, A., Landolfi, V., Picariello, G., Mamone, G., Blandino, M., Romano, R., Ferranti, P. (2022). Molecules 27 (4): 1308. https://doi.org/10.3390/molecules27041308;

- **Chapter VI** – “Minor Cereals and new crops: tritordeum: Sustainable Food Science - A Comprehensive Approach.” Landolfi, V. and Blandino, M. (2023) in Reference Module in Food Science, Elsevier; DOI: 10.1016/B978-0-12-823960-5.00023-8;

- **Chapter VII** – Conclusions and Perspective – this chapter summarizes the new information that has been obtained within the Ph.D. studies, highlights the need for further future research, and suggests some possibile activities that need to be investigated in more depth.

ABSTRACT

Wheat is the most widespread cultivated cereal in the world, and it is used to produce a large variety of baked foods, thanks to its particular rheological and bread-making properties, which mainly depend on its gluten-forming proteins. In the last few years, the presence of particularly short aminoacidic sequences in the wheat gluten network has been linked to several adverse immunotoxic reactions. Consumers, and the food supply chain in general, are requiring more and more information about the factors that can exert an effect on the peptides that trigger gluten-related diseases. Therefore, the goal of this research has been to investigate the possible role of the genotype, considering different wheat cultivars and a new crop, the tritordeum amphiploid hybrid, as well as different environmental conditions and crop practices on the gluten composition and occurrence of potential toxic epitopes. As expected, N fertilization, both as far as the rate and the application timing are concerned, affects the grain protein content and dough strength to a great extent, but has a limited effect on the relative gluten fraction and no effect on the potential immunotoxicity of such grains. The gluten composition has been found to mainly be affected by the genotype, in particular when modern and old cultivars are compared, but also by the meteorological conditions of the growing season. Tritordeum has shown a lower content of the peptides that trigger gluten-related diseases than bread and durum wheat cultivars in two proteomic studies that were carried out on *in vitro* digested flours and breads. The potential of tritordeum, in terms of crop management, and nutritional and health aspects, has been thoroughly investigated, through a review of the existing bibliography, and the presence of particular nutritional traits, such as high contents of carotenoids and soluble and insoluble fibers, in addition to a potential interesting gluten profile, has been confirmed. Furthermore, this crop has

shown a yield gap, compared to wheat, in several production situations, as well as a higher risk of mycotoxin contamination in temperate growing areas.

This study highlights the need to obtain more detailed knowledge on gluten in order to address breeding and agronomic strategies and to set up integrated strategies to obtain the healthier baked goods that are currently requested by the supply chain.

Chapter I – Gluten in wheats and other small cereals: the relationship with cultivation management practices and the effect on the technological and health properties of the derived flours and baked products.

The importance of wheat and other cereals as staple food

Human nutrition started to change about 10,000 years ago when, with the development of agriculture, there was a transition from a diet based on hunting products to one based on crop products, and cereals in particular (Kimber and Sears, 1987). Since then, the consumption of cereals, albeit with considerable differences, has continued to be the basis of the diet of the world's population, and it is expected to increase by 21% over the next 10 years, reaching 542 million tons (Mt) by 2030 (OECD-FAO Agricultural Outlook, 2021). Among the crops that are cultivated the most worldwide, wheat stands out as the first crop, in terms of overall cultivated area, and the second crop in the world, in terms of production, with about 777 Mt being produced in the 2020-2021 period. Moreover, its consumption is rising together with the increasing growth in population. Europe produces the most wheat, with about 138 Mt/year, followed by China and India with 137 and 110 Mt/year, respectively (European Commission, 2021).

This consistent demand and supply have certainly led to a marked increase in agricultural production since the initial Neolithic domestication process of diploid *Triticum* species, and the subsequent cultivation over the years of tetraploid and hexaploid species (Charmet, 2011).

Hexaploid bread wheat (*Triticum aestivum* spp. *aestivum L.*) is the result of two stages of a breeding process, which confers 3 different complete genetic pools (AABBDD) to it, each composed of 2 couples of 7

chromosomes. *Aegilops speltoides* wild grasses (BB, 2× = 14), naturally crossed with *Triticum urartu* (AA, 2x = 14) between 500 and 800 thousand years ago, originated the *Triticum turgidum* genotype (AABB, 4× = 28), which includes such species as emmer and durum wheat. In the next step, hybridization of the tetraploid wheat with *Aegilops tauschii* (DD; 2× = 14) resulted in the emergence of hexaploid bread wheat (AABBDD; 6× = 42) (van Ginkel and Ogbonnaya, 2007).

The modern bread wheat that is commonly cultivated today is relatively young, because it is the result of only 8000 generations of evolutions, and it can be defined as the most important cereal that has ever existed because of its particular growing adaptativety to various soil conditions. It can in fact grow in lands ranging from the northern polar circle to the equator latitude, and from areas below sea level to regions that reach 3000 m of altitude (Van Ginkel and Ogbonnaya, 2007).

Bread wheat has been the subject of the main crop domestication events, where the main objective has been to increase the yield traits, firstly by achieving reduced spike shattering at maturity, that is, by changing from a brittle rachis to a non-brittle rachis, with a consequent lower loss of grains at harvest time. Secondly, the passage between strong glumes, which envelop and protect the seed, to a hulled kernel shape, has facilitated the threshing process (Shewry, 2009). Furthermore, farmers conduct domestication processes by selecting varieties with a larger grain size in order to increase the yield and facilitate the milling transformation process (Fuller, 2007).

This cereal crop is of fundamental importance at a global level. In fact, there are numerous reasons why we should continue studying how to improve its weaknesses in order to strengthen its development. Nowadays, the request for an ever-increasing grain production has not diminished, and it has perhaps become even more necessary, given the future forecasted increase in the world population. According to the most recent UN estimates, there will be about 8.5 billion people on the Earth in

2030, and this number will continue to grow. In 2050, our planet will reach about 9.15 billion inhabitants (Alexandratos and Bruinsma, 2012).

In order to satisfy the increasing need for greater quantities of grain wheat, it is necessary to act by considering the need for a “sustainable intensification” of production. At the same time, in the next few decades, we will have to face the great challenge imposed by climate change, in terms of the rising CO_2 atmospheric concentration, and increasing temperatures, which will induce a rising photorespiration rate and night respiration (Godfray et al., 2010), as well as a lower availability of water that will vary for different crops and different Mediterranean areas. Any technical improvements of wheat will have to take into account these impending climate changes (Saadi et al., 2015), for example, by improving the water use efficiency and nitrogen sources, while maintaining the yield and quality, even by means of a reduction in their use (Martre et al., 2015).

In addition to research into the agronomical and productive traits, it will be necessary to focus on the quality of cereals. The *Triticum* species, which has recently witnessed a greater commercial interest, constitute the bread wheats which, due to thier fineness and ease of processing, are used the most for bread-making and baking for the production of leavened doughs or egg pasta, together with durum wheat, which occupies only 6% of the world’s wheat production, but is very important as a basic ingredient for dry pasta-making and for some types of bread. However, each single product needs different technological and rheological flour properties for the success of the final product, thus considerably expanding the concept of flour quality.

Genetic intervention by breeders to create new cultivars with desired characteristics seems to be one of the most promising ways of satisfying the ever-increasing need of new products, which may be achieved with the wheat genome sequencing techniques that are now available (Consortium IWGSC, 2018), thus constituting a very important step

forward in research, given the enormous size and complexity of the wheat genome, which is almost five times larger than the human one.

It is therefore necessary to continue the domestication process that has taken place for millennia in order to select new varieties that are more resistant to pests and environmental changes, as well as to possible biotic stresses during the growth phases, in order to keep wheat crops healthy and free of natural contaminants, such as mycotoxins, which are generally more concentrated in wholemeal flour (Zhang et al., 2019). As wheat is a basic crop in the world's diet, it is important to ensure a minimum of contaminants inside the grain in order to minimize the consequente risks to human health.

Furthermore, whole wheat-based foods are an important source of bioactive components, such as dietary fiber, vitamins, minerals, and phytochemicals (including antioxidants), which lead to several beneficial effects on human health, for example, by playing a protective role against chronic diseases related to metabolic syndromes, or type 2 diabetes and against cardiovascular disease (Ye et al., 2012; Shewry et al., 2010). The consumption of wheat flour has a regulative function in the digestive system, which may also increase the absorption of micronutrients, stabilize blood glucose and lower serum lipids (Slavin et al., 2009), thus resulting in a phytochemical content that is complementary to that present in fruit and vegetables (Adom et al., 2003). In addition to the positive role of fiber, bioactive compounds and micronutrients, the consumption of wheat products mainly results in satisfying the energy needs, thanks to the high starch content. Moreover, wheat baked products, which have a medium protein content of 12%, but which can reach up to 17% in certain production situations, are an important source of proteins (Young and Pellett, 1985).

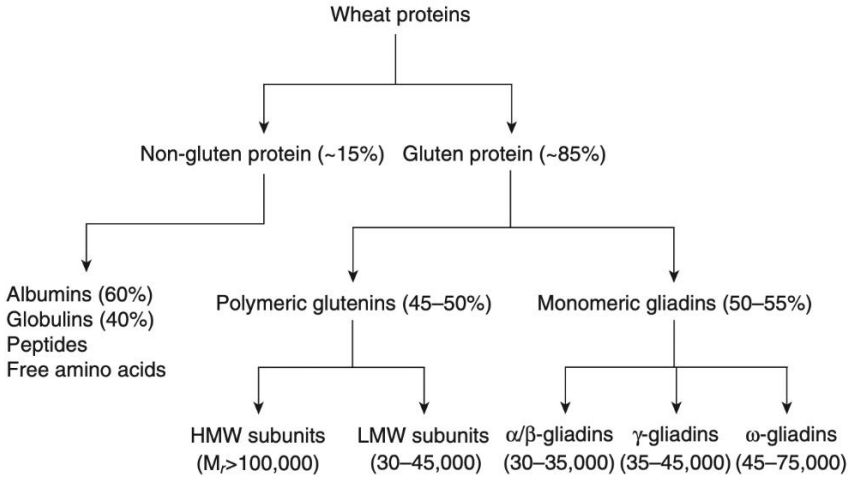
A huge variety of baked foods is currently produced using wheat flour, thanks to its rheological and bread-making properties, which are caused by a variety of gluten proteins.

Wheat gluten proteins, gliadins and glutenins all confer various quality properties to the dough and the related leavened wheat products, such as elasticity, gas retention, expansion properties and compactness. Any modification of their amounts and their ratios affects the rheological properties of the dough (Khatkar et al., 2013; Payne et al., 1987).
The protein content of wheat flour, and the associated technological and nutritional properties of such proteins could be influenced by different factors, for example, the genotype, the environment, including both excessive temperatures and precipitation frequencies, and the agronomic management practices.

Gluten content and its composition in wheat

Wheat proteins constitute up to 15-17% of the kernel, and gluten proteins constitute 85 % of these. One of the most important and common quality elements for wheat flours is the grain protein content (GPC), a desirable trait because of its nutritional power and for improved bread-making (Foca et al., 2007). The wheat gluten protein classification shown in Figure 1 presents the common percentages of the different protein fractions.

Figure 1 Classification of wheat gluten proteins (Day, 2011)



Non-gluten proteins, albumins and globulins, which are also called soluble proteins because water and sodium chloride solutions can easily be used for their extraction, play a structural and metabolic role.

Gliadins and glutenins are prolamin storage proteins that contain large amounts of non- essential amino acid glutamine, and apart from their mechanical and physical roles in dough formation, they are an important resource for the synthesis of non-essential amino acids and other physiologically important nitrogen-containing compounds that are necessary to strengthen and repair body muscles (WHO/FAO/UNU Expert Consultation, 2007).

In addition to the nutritional role, gluten endows dough with a unique viscoelastic property, due to the network of non-covalent hydrogen bonds and hydrophobic interactions between gliadins and glutenins, as well as the ability to retain gas during proof and baking (Dobraszczyk, 2004).

In general, monomeric protein confers viscosity and extensibility to dough, while the polymeric one gives elasticity and tenacity (Day, 2011). An optimal bread making gluten has to be capable of withstanding the pressure exerted by gas, thanks to the presence of a high number of

interactions between the protein network, but on the other hand, it also has to show good biaxial elongation. A large number of glutenins, especially those with a higher molecular weight, is an indispensable condition for the formation of a gluten which has a higher number of covalent interactions (disulfide bonds) than non-covalent ones (entanglements), a necessary condition to endow the dough with endurance and strength features (Dobraszczyk, 2004).

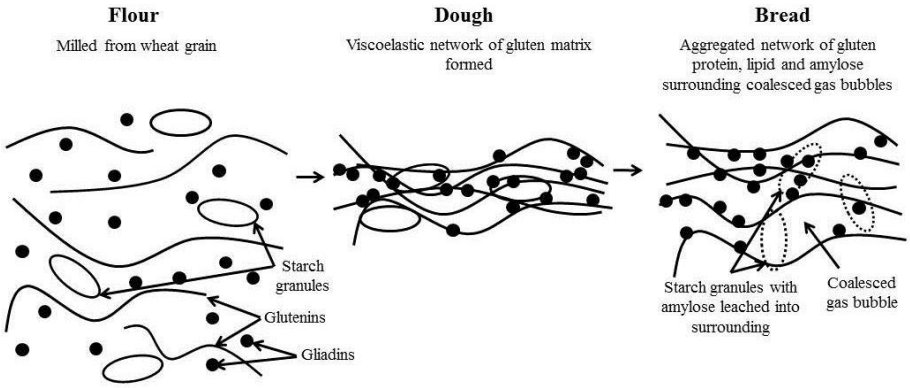
An elevated presence of a β-sheet structure has been found in the insoluble glutenin fraction, while a predominance of β-turn has been verified for gliadin and soluble glutenin fractions over the β-sheet content (Li et al., 2006)

The composition of these proteins and their proportions confer characteristics that are of fundamental importance for their end-use.

Technological role of gluten

The bio-accessibility of molecules to the flour, dough and final bread product is very different, and a more aggregated gluten structure may reduce access to a potential enzyme cleavage site, thereby conferring minor digestibility (Bustos et al., 2017) (Figure 2).

Figure 2 The gluten network structures of flour, dough and bread, respectively (Smith, 2017)



Alveograph indices, which are derived from the physical properties of dough through an evaluation that involves inflating the dough by blowing air into it until the bubble wall that simulates leavening breaks, are used to indicate the strength of a dough, with the alveograph W parameter, and the ratio between dough tenacity and extensibility, with the alveograph P/L ratio (Foca et al., 2007). Bread wheat flours can be grouped into five categories, on the basis of their protein content and different technological parameters, by means of the Synthetic Index of Quality proposed in 1997 (Borasio, 1997), considering their end use (Table 1).

In general, a flour with a high protein content can be classified as an improver wheat, which is preferably used for long and sustained leavening of baked products, thanks to its high W value, which is normally accompanied by long stability times that help to maintain a good dough consistency. The P/L parameter, which is independent of the alveograph W one, is considered an indication of a good extensibility degree, when in the 0.5-1 range (Payne et al., 1987), while a medium-low P / L value is preferable for an elastic dough preparation for such products as pizza.

Table 1 Reference ISQ table used for the classification of the different quality categories of wheats and their corresponding chemical and rheological parameters (Foca et al., 2007)

Parameter	**FF**	**FPS**	**FP**	**FB**
Protein content (%, dry matter)	>14.5	>12.5	>11.0	<9.0
Alveograph W	>340	250	>200	<80
Alveograph P/L	<0.7	<0.8	<0.7	<0.5
Farinograph stability (min)	>16	>11	>6	<4
Hectolitre weight (Kg $hL^{-}1$)	>75	>75	>75	>75
Falling number (s)	>250	>220	>220	>220

FF = improver wheat; FPS = superior bread making wheat; FP = ordinary bread making wheat; FB = wheat for biscuits.

Bread making wheat flours include flours with a wide GPC range, that is, from 10-12,5 %, and a wide range of alveograph parameters, and they are considered the perfect flours for the realization of a wide range of bread making transformations. Depending on the type of bread chosen, it is necessary to orient the choice considering the corresponding flour properties, as highly leavened breads, characterized by large air pockets, require a wide range of values of W and medium values of P/L, while more compact breads may have lower gluten strength values and less elasticity of the dough. The main variations up to 60% in the bread-making qualities may be ascribed to the characteristic effect of each HMW subunits of glutenin (HMW-GS), forming several possible structural backbones with highly resistant disulfide bonds (Lindsay and Skerritt, 1998).

Generally, lower W values than 150 indicate weak flours that are unsuitable for bread making, sticky and difficult to work doughs and bread with irregular alveoli, but flours with a GPC under 11% is perfect for biscuits, as they do not require a strong gluten mesh that is resistant to massive extensions (Farrer et al., 2006; Foca et al., 2007). This happens because there is a positive correlation between the variations in W and GPC, as has been reported in various works (López-Bellido et al., 2001, 1998).

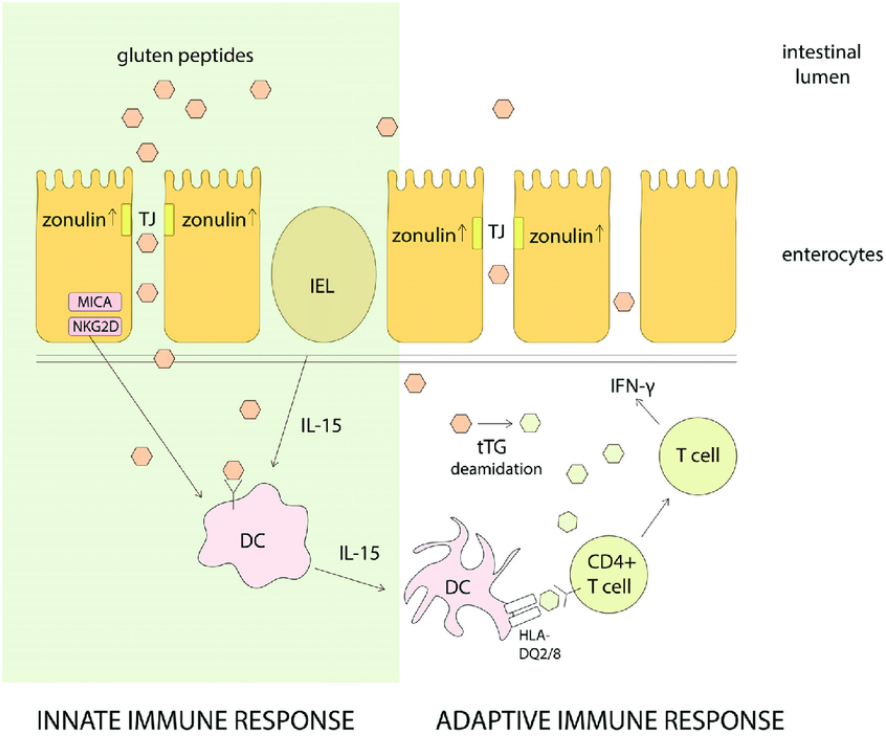
A wheat dough in which the gluten matrix is sufficiently thick and well developed, even under non-optimal hydration conditions, and with a high GPC is particularly suitable for pasta-making. The distinguishing gluten feature chosen by pasta producers pertains to the higher protein content and a particular protein composition of glutenin allele Glu-B3 locus for LMW-GS and Glu-B1 for LMW-GS, which is found in durum wheat semolina (Mastrangelo and Cattivelli, 2021). Another important aspect is the density of the cross links between the shorter chains of the LMW-GS formed in good durum wheat gluten (Edwards et al., 2003). Conversely, some HMW-GS of the Glu-D1 locus, which is essential to obtain a proper

bread making dough, are present in bread wheat and lacking in durum wheat (Mastrangelo and Cattivelli, 2021).

Disease associated with gluten consumption

Apart from the characteristic technological role of wheat gluten protein, studies began, at the beginning of the 1970's, to support the role of gluten in triggering immune toxic reactions in humans, related to what was then defined as celiac disease (Fasano and Catassi, 2012), an autoimmune enteropathy with complex mechanisms involving the innate and adaptive immune systems (Figure 2), and later on related to food allergies (Biesiekierski, 2017).

Figure 4 Mechanisms of the innate and adaptive immune systems involved in the celiac disease pathology (Balakireva and Zamyatnin, 2016). HLA, human leukocyte antigen; IEL, intraepithelial lymphocytes; IFN, interferon; Ig, immunoglobulin; IL-15, interleukin 15; T cell receptor; tTG, tissue transglutaminase; MICA, NKG2D, stress molecules on enterocytes, DC, dendritic cell.



The involvement of the innate immunity and adaptive immunity systems is the basis of the pathogenesis of celiac disease. The "innate" immunity system has the ability to deliver a prompt and rapid protective response, prior to antibody synthesis or a cellular T response. On the other hand, T cells and B cells are part of the adaptive component that is characterized by the ability to develop a memory towards previous immunological experiences and to adapt the immunological response over time (Ciccocioppo et al., 2005). The triggering of these human immune

mechanisms depends on the presence of T-cell stimulatory gluten peptides, which are called epitopes (Van den Broeck et al., 2010).

The T cell–stimulatory activity in the intestinal mucosa is stimulated by the presence of gluten epitopes that mostly interact with HLA-DQ2 and HLA-DQ8 heterodimer loci, that is, specific CD alleles of MHC class II present in CD genome patients (Kagnoff, 2007).

These epitopes are short amino acid sequences, located in prolamin repetitive domains, which have a high content of glutamines and prolins (Cebolla et al., 2018). The latter amino acid acts by moderating the susceptibility to the enzymatic conditions of the gastrointestinal tract (Shewry and Tatham, 2016).

The main concentration of gluten epitopes has been observed in the α-gliadin fraction, mainly due to the presence of 33-mer, a strongly immune dominant peptide. The 33-mer peptide contains immunodominant overlapping epitopes, with repetitive units, and it is a particular feature of bread wheat because it is encoded in α2-gliadin, exclusive of the D-genome, which is absent in durum wheat (Ozuna et al., 2015).

Despite the improved knowledge of the wheat genome after its sequencing, these chromosomal regions have enormous repetitions of domains that make the selection of the valuable characteristics for bread-making and reduced immunotoxicity very difficult, also considering that the mapping of the immunotoxic epitope is still incomplete (Sollid et al., 2012).

At the moment, the Codex Alimentarius has decreed that for a food to be considered gluten free and therefore to be consumed without problems by subjects suffering from celiac disease and wheat allergies, it has to contain below 20 ppm of gluten for the Food Standards Agency (FSA) and the European Food Safety Authority (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2014).

However, more than 10% of the Italian population suffers from a pathology called Non-Celiac Gluten Sensitivity (NCGS), with the same symptoms as

those found in celiac patients, but with the absence of atrophied intestinal villi and microvilli (Aufiero et al., 2018). Although the identification of NCGS patients is still unreliable and it is probably an underestimated pathology, it is a real disease that shows fewer effects if a diet with a low gluten content is followed (Dieterich and Zopf, 2019).

For this reason, it is important to investigate the effects of the immunogenic epitope sequences on the digestion products, and, thanks to *in vitro* digestion, it is easier to standardize the numerous conditions and variability of this complex physiological process, thereby allowing the differences between the different wheat varieties and food products to be recognized.

The recently revised INFOGEST in vitro static method, which has achieved international consensus (Brodkorb et al., 2019), has been applied to several cereal-based raw material and final products in order to conduct a proteomic analysis of different cereal varieties after simulated digestion.

This kind of approach permits knowledge about the real capacity of digestion to alter the final peptides that reach the gut mucosa to be increased, and the differences in the protein expression between new cereals and more common varieties of bread and durum wheat to be investigated.

Factors that affect the gluten content and its composition

The improvement of the end-use quality of gluten wheat depends on having a complete understanding of the effects of several factors, such as the environment, the genotype, their interaction and the crop management practices.

The protein content and the grain protein composition are influenced to a great extent by different factors, such as the environmental conditions and the cultivation management practices (Shewry, 2018).

Environmental influence

Among the variables that affect the quality of gluten wheat, climatic factors can play a considerable role by influencing the GPC and the gluten composition, acting directly on the plant physiology and indirectly on the efficiency of the agronomic practices. For example, rainfall is certainly an important climatic element from sowing to harvesting, because, under extreme conditions, it can exert stress on crops and alter the normal physiological processes (Asseng et al., 2019; Byamukama et al., 2019; Villegas et al., 2008) of the plant or modify the availability of nutritional elements in the soil, for example, through the phenomenon of N leaching (Vos and van der Putten, 2004). These climatic variables act among each other and influence the crop agronomic management practices, thus it is important to analyse the whole crop system as an interaction of metereological traits, soils and agronomic practices on gluten content and flour quality of the wheat (Kakabouki et al., 2020; Saadi et al., 2015; Shewry et al., 2010; Visioli et al., 2018). The great influence exerted by different wheat cultivation environments on the bread-making quality features is particularly enhanced in Mediterranean areas. This happens because of the more marked effects of drought conditions and excessive temperatures during important physiological growth steps, such as in the grain filling process (Borghi et al., 1997).

Fertilization

Among the elements that are essential for the plant, nitrogen (N) is of primary importance for the crop management of this cereal and it is almost always necessary in order to obtain high product yields. However, the right N rate varies from plant to plant, and depends on several factors, such as the necessity of each genetic variety, the N soil content and dynamics, the climatic conditions, which affect the activity of the soil microorganisms and the loss of N due to leaching, and the interaction with other agronomical

trials, such as the availability of water for the functionality of the plant physiology (Diacono et al., 2013).

Since the 1960s, there has been a massive increase in the use of N fertilizers for wheat crops, in order to maximize grain yields (Litke et al., 2018). Moreover, the gluten protein content seems to have been affected by the increased use of N fertilization (De Santis et al., 2020b), and there are also controversial opinions about its influence on the protein composition (Johansson et al., 2001; Yu et al., 2018).

As previously mentioned, the end use value of baked products depends on the GPC.

A high protein content is generally required for improver wheat and durum wheat, and it is important to investigate the applicable strategies for these qualitative categories to better exploit N fertilization without only increasing the N fertilization rate in the soil. For example, a late N fertilization strategy could ensure better results, in terms of qualitative requirements. (Blandino et al., 2020, 2015; Ottman et al., 2000).

Late N fertilization plays an increasing role in GPC and the abundance of HMW-GS, and also leads to an improvement in the loaf volume of wheat flour, and with greater effects when applied as nitrate rather than as urea (Xue et al., 2016a, 2016b). N fertilization has been found to be a useful tool to modulate such quality features of bread as the gluten content, and to increase the W technological parameter (Guerrini et al., 2020). A high N-fertilizer application has been shown to increase the β-sheet structure by 10.4% (Liu et al., 2022), thereby influencing dough elasticity (Belton, 1999). Another N fertilization strategy is its split application, which permits the same gluten quality level to be maintained while reducing the N rates (Fuertes-Mendizábal et al., 2010), thus lowering its impact on the environment (Kabir et al., 2021).

Furthermore,a synergistic effect between N and S fertilizations has been discovered, whereby the parallel assumption of N and S improves the GPC and the gluten network, in terms of strength and extensibility (Tea et

al., 2007), and potentially improves the baking quality (Guerrini et al., 2020).

Other agronomic practices

Among the other agrotechnique strategies that could influence the gluten content and composition, the tillage system can also be expected to play a certain role.

No-tillage systems seem to obtain a slightly higher content of gluten and grain quality than conventional tillage, but with a negative impact on the wheat yield (Amato et al., 2013; Grigoras et al., 2012; Mazzoncini et al., 2008), even though this influence can be mediated by certain weather conditions (Hernanz et al., 2002; Montemurro, 2009).

At the same time, it is important to set up a plant defense program to limit parasitic attacks and prevent the plants from undergoing stress, which alters their correct growth (Dimmock and Gooding, 2002). Therefore, it is important to consider the use of fungicide applications to protect the late stages of plant growth. A slight diluting effect of the protein concentration may occur in wheat grain after a fungicide application (Ruske et al., 2003), although there is not necessarily a clear and general effect of this practice on the gluten content and bread-making quality (Blandino and Reyneri, 2009; Castro et al., 2018; Wang et al., 2004).

Cultivar selection of the wheat

Considering environmental and management factors, the importance of the genetics of the variety in question is the most important factor that influences the gluten content (Gil et al., 2011).

In the genetic studies that have been conducted in recent years, it has been found that grains are first selected for their final processing on the basis of their rheological properties.

The next step involves selecting the grain on the basis of the gluten content and composition, considering it as an ingredient for the most suitable diet for those suffering from NCGS disorders.

As far as the milling, bread-making and pasta-making practices are concerned, the tendency to return to tradition has also been investigated through a re-evaluation of the genetic heritage of cereal biodiversity, that is, passing from new selections to the recovery of "ancient" or landrace wheat. Landrace wheats include populations of both bread and durum wheat, which were developed before the green revolution that took place in the second half of the 19th century (Dinu et al., 2018). After that moment in time, bread wheat lines can be called “modern”, and are distinguished by a lower susceptibility to diseases and characteristics that are more suitable for harvesting than the landrace ones (Gélinas and McKinnon, 2016).

Modern wheats also differ because of their different gluten contents and the different compositional characteristics of gluten. Varieties with stronger and more tenacious gluten are generally selected to supply the bakery industry with flours characterized by a high strength (Geisslitz et al., 2018). Landrace wheat lines instead present a weaker gluten and lower bread-making capacity (Ghiselli et al., 2016) than modern ones.

As far as the presence of gluten disorder-triggering epitopes in gluten is concerned, there has been a great deal of debate on the contribution that the genomes of landrace or modern cereal varieties can make. Although an early current of investigation attempted to support the healthy gluten composition of landraces, it was found that wheat breeding could play a role in increasing the prevalence of celiac disease (Van den Broeck et al., 2010), although this opinion has often been contradicted in the literature. According to Ribeiro et al. (2016), the bread wheat cultivar, which is not subjected to breeding practices, shows larger amounts of potential celiac disease immunostimulatory epitopes than modern varieties. Prandi et al. (2017) and Pronin et al. (2021) demonstrated that modern wheat does not

have higher contents of immunogenic peptides than landrace wheat. Similar results have been reached for landrace and modern varieties of durum wheat, where a greater concentration of allergenic epitopes and of celiac disease was found in the older cultivars (De Santis et al., 2020a). Other studies have not verified any particular differences in the reactivity of landrace and modern wheat varieties (Escarnot et al., 2018; Schalk et al., 2017).

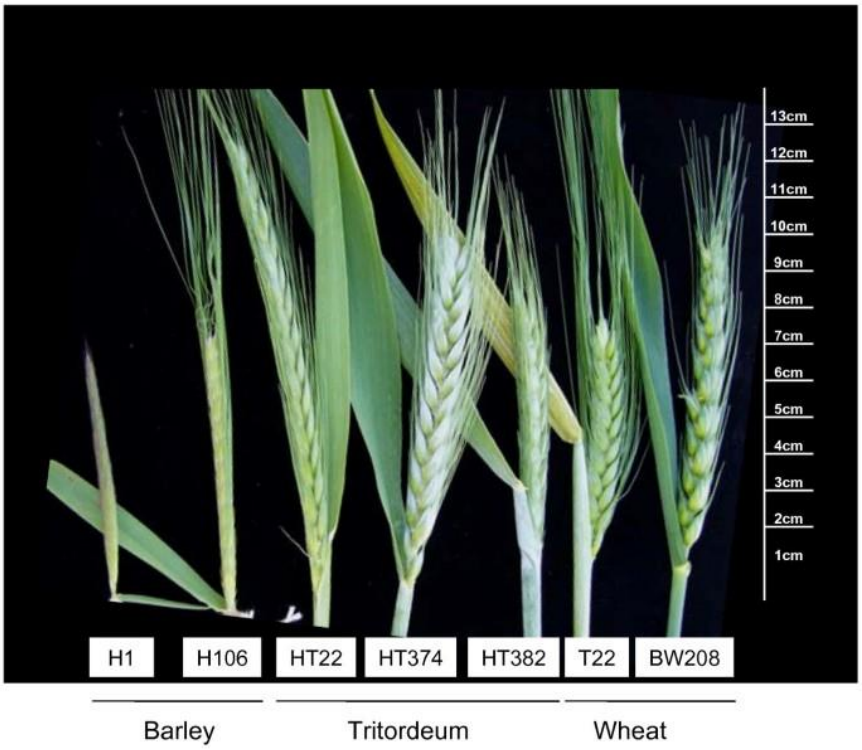
A new hybrid: the interest in tritordeum

Agricultural modernization processes have been directed towards achieving notable improvements in the cultivation and quality of wheat grains, while investigating the numerous varieties that currently exist and possible future ones for new crossings.

In recent years, the interest of the food industry in the use of cereal species with excellent organoleptic and nutritional characteristics has increased the desire to create a new cereal that can be widely used.

Attention has been focused on the study of a new species of cereal called tritordeum (x *tritordeum martinii* A. Pujadas, nothosp. nov.), which was created with the aim of combining, in its genome, the properties of a wild relative of *Hordeum vulgare* (*H. chilense* Roem. et Schultz.) with those of the parental durum wheat (Figure 5). Although the first hexaploid hybrids derived from backcrossing date back to the 1970s, only in the last 15 years have very promising varieties of tritordeum been genetically selected as a source of high protein in diets.

Figure 5 Pictures of barley, tritordeum, and durum wheat ears (Gil-Humanes et al., 2009).



Although numerous studies have investigated some of the characteristics of this new hexaploid species, the scientific literature has still not provided a clear and complete picture of this crop. The only possible starting point to set up innovative studies aimed at obtaining complete knowledge of this new species is the collection and reorganization of the present knowledge. For this reason, a review (Chapter V) has been carried out on the state of the art concerning the origin, characteristics and future challenges of tritordeum varieties.

According to recent scientific works, one of the most interesting potentials of tritordeum is the low immunogenicity of the flour (Sánchez-León et al., 2021; Vaquero et al., 2018). This has aroused great interest in comparing it with other varieties of bread and durum wheat, and of also looking at the

agronomic and technological performances and its wide environmental adaptability.

Considering the first indications about tritordeum flour, in terms of immunotoxicity, it was considered necessary to investigate it following the bread-making product from the flour to its digestion in order to obtain a more complete vision. In fact, studies on single protein sequences in the raw material do not correspond to those of a food structure, which are more multifarious.

Perspectives: how to produce gluten bakery products with a low infiammatory effect.

Gluten formation, from the union of an appropriate hydration and mixing action of wheat flour, confers the characteristic structure that gives a wide variability of processing to all of its processed goods. However, it seems that the only possibility to fight some diseases, such as CD and gluten allergy, is for the patients to completely avoid the ingestion of gluten in their diets throughout their lives. In the 1980s, CD was considered a rare childhood disease, and gluten restriction was relatively unknown to medical professionals and the general public, while now there is a consistent global demand for gluten-free products as a result of the growth of patients with gluten-related diseases, and an increase in the demand for gluten-free foods, even for people without any such health problems (El Khoury et al., 2018).

In the last 5 years, the global market has observed an increase of 10.4% in gluten-free product sales, and for which the market expects an even greater increase in the future (Gluten-Free Products Market by Type, 2020). Consumers now believe that there is an association between limiting the onset of diet-related health problems with the adoption of a gluten free diet.

However, some evidence is available regarding the far from beneficial effects of the adoption of gluten free diets in people who do not suffer from

gluten-related diseases, such as problems related to the incidence of cardiovascular diseases (Potter et al., 2018), the accumulation of heavy metals (Raehsler et al., 2018) and a more general deficiency of some nutritional elements (Mariani et al., 1998), such as the lack of bioactive compounds of which whole wheat grain is rich (Shewry et al., 2010). This aspect demonstrates that the adoption of gluten free diets in people who do not suffer from gluten-related diseases is not a good practice, because such a diet presents too many limits.

At the same time, cereal-based, gluten-free baked products have a lower quality than those based on wheat, even from the technological potential point of view (Janssen et al., 1996). For this reason, the trend towards a gluten-free diet for everyone does not seem to be beneficial. In such a scenario, it would be interesting to obtain a better understanding of the role of the genotype, and of the environmental and the crop management conditions on the content of the gluten fraction that is potentially responsible for the health risk for consumers.

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Chapter II – Aims

This thesis project aimed to carry out a broad investigation on the protein quality of the grain of bread and durum wheat and of a new amphiploid hybrid called tritordeum. In this context, the main factors that influence the variation of the protein composition of the kernel were questioned, looking for them among the main agrotechnical practices and environmental conditions of cultivation. In particular, the role of genetics in the most used varieties, from the oldest to the modern ones and for new promising hybrids, was questioned.

The study of all these factors also want to focus more specifically on their impact on the composition of gluten and on the potential relationship with gluten-related pathologies triggering sequences.

Since the food sector of baked goods, in response to the demand from consumers of healthier products, is asking for raw materials and ingredients with low immunogenic potential, this research was designed for searching the possible field strategies (e.g. genotype, crop practices, environmental conditions) able to guarantee gluten flours and related processed products with a lower toxic immunoreactivity.

Chapter III – "Effect of Nitrogen Fertilization and Fungicide Application at Heading on the Gluten Protein Composition and Rheological Quality of Wheat"

Landolfi, Viola, Giovanna Visioli, and Massimo Blandino. 2021. "Effect of Nitrogen Fertilization and Fungicide Application at Heading on the Gluten Protein Composition and Rheological Quality of Wheat." Agronomy 11 (9): 1687. https://doi.org/10.3390/agronomy11091687.

Abstract

Optimizing the bread-making quality properties of flour is currently one of the main aims of the bakery industry. Therefore, this study has investigated the effects of N fertilization and fungicide application at wheat heading on the protein content (GPC), gluten composition and rheological properties of wheat. Field experiments were carried out in North-West Italy over a 3-year period, on a high protein cultivar of soft winter wheat. Grain samples were collected for each agronomic treatment at four ripening timings, from the milk stage to the final combine harvesting, and the contents of the different gluten fractions were quantified. The late N fertilization increased the GPC (+1.2%) and dough strength (W) (+22%) as a result of a similar enhancement of all the gluten protein fractions, while the fungicide application slightly reduced the GPC (−0.3%) and W (−4%), mainly because of a dilution of the gliadin content, due to the significantly higher grain yield (+8.6%) and thousand kernel weight (+5.5%). These agronomic practices did not modify the gluten composition significantly, expressed as the relative ratio between the gliadins (glia) and the high (HMW) and low (LMW) molecular weight glutenins (gs), and confirmed by the accumulation trend of the different

protein fractions during ripening. The year resulted to have the most marked effect on the gluten protein fraction ratios and alveographic parameters. The lowest W was observed in 2015, and although the highest GPC was recorded for the same year, the lowest gs/glia ratio was also observed. Instead, 2016 showed the highest gs/glia and HMW-gs/LMW-gs (H/L) ratios, and also the highest P/L value (2.2). In 2015, a slightly higher temperature during the ripening stage resulted in a greater increase in the γ-gliadin enriched fraction than the α/β gliadin ones, and marked differences were noted in the rheological traits. This field experiment has highlighted the interactive role of environmental and agronomic factors on the content and quality of gluten proteins and their bread-making ability, thus making a further contribution to the development of an integrated crop strategy for the cultivation of high protein wheat in humid Mediterranean areas.

Keywords
high protein wheat; gliadins; glutenins; flour quality; humid Mediterranean area.

Introduction

Wheat is the crop that is cultivated the most throughout the world and it is a staple food in many countries. The flour obtained from common wheat (*Triticum aestivum* spp*. aestivum* L.) has excellent bread-making properties for most leavening and baking processes. Wheat foods, which are a major component of most diets, are particularly important for their protein intake in the human diet (Ma, 2019), and even more for vegetarians and vegans. Moreover, the dough obtained, from the mixing of flour with water, has unique elasticity and tenacity characteristics, thanks to the content and the types of wheat grain storage proteins (gluten proteins) which accumulate in the late ripening seed stage (Ma, 2019).

Gluten proteins are made up of gliadins, that is, monomers that confer viscous properties and dough extensibility, and glutenins, that is, protein aggregates of high (HMW) and low molecular weight (LMW) glutenin subunits (gs), which give the dough elasticity (Shewry and Halford, 2002). The rheology of the elongation and disruption of the gluten reticulum varies to a great extent, depending on the protein content, on the types of HMW-gs and on the variations in the glutenin/gliadin ratio (gs/glia) (Uthayakumaran et al., 2000). As far as the quality of flour is concerned, it is also important to consider the balance between HMW and LMW-gs in the polymeric fraction, which can significantly alter the strength (W) and extensibility (L) of the dough. Numerous factors, such as the genotype, the agronomic management practices and other environmental factors, such as the temperature and precipitation during grain filling, may play important roles in determining the bread-making quality of flour (Denčić et al., 2011). The important role of nitrogen (N) fertilization on the synthesis and accumulation of storage proteins is well known (Blandino et al., 2015; Blandino et al., 2020; Martre et al., 2003; Fuertes- Mendizábal et al., 2013). The top-dressed distribution of a late N fertilization, from booting to the heading growth stage (GS), leads to a clear increase in the grain protein content (GPC) in climatic zones with adequate spring rainfall or in irrigated cropping systems (Blandino et al., 2015). This late application of N fertilizer, in addition to a sufficient N application in the vegetative GS, is required in temperate growing areas to satisfy the quality requests of high protein wheat, which requires high GPC (>14%) and dough strength (W > 350 J × 10^{-4}) (Foca et al., 2007). The improvement in the GPC content after a late fertilizer application is due to a higher N availability, which, considering the mobility of this element in the soil, is limited by the source and the timing of the N supply (Martre et al., 2003). Both the N uptake in vegetative organs in the pre-anthesis period, which is then remobilized during grain filling, and the amount of N absorbed during ripening and directly transported to the kernels, provide an accumulation of grain

storage gluten proteins (Fuertes-Mendizábal et al., 2018). The contribution of post-anthesis N uptake, in comparison with the pre-anthesis stored N, which is subsequently translocated to the spikes during late leaf senescence (Fuertes-Mendizábal et al., 2018), can vary from 5–40% of the final total grain N (Bogard et al., 2010). The clear effect of a late N fertilization on the increase in the amount of grain protein quantity has been tested and verified in several works (Blandino et al., 2015; Blandino et al., 2020; Xue et al., 2016; Rossmann et al., 2020), while its influence on the change in the gluten protein composition and dough properties seems to be less significant, although opposing opinions are reported in the literature (Martre et al., 2003; Rekowski et al., 2019; Triboï et al., 2003). Martre et al. (2003) and Triboi et al. (2003) claimed that variations in the gluten protein composition are not influenced by the timing or rate of fertilization, and that increases in all the gluten protein fractions can be observed for a higher N supply. However, few studies have considered the potential effects of the timing of N uptake and the role of late N fertilization, applied close to the ripening stages, on the accumulation of the different gluten protein fractions during grain filling (Martre et al., 2003). In fact, the timing of the accumulation of the different gluten protein fractions during grain development seems to be fundamental to determine the structural thermal gluten properties and the wheat quality, and thus to provide useful indicators of the technological quality of dough (Song et al., 2020).

Among the other crop practices that could influence wheat physiology during the wheat ripening stages, the application of a fungicide to control head and foliar diseases plays an important role. It has been shown that fungicides increase the duration of the green leaf area and grain yield, compared to untreated crops (Castro et al., 2018). In fact, the duration of the green leaf area has a direct effect on the accumulation of grain starch as a consequence of a prolonged photosynthesis (Blandino and Reyneri, 2009), but it also indirectly influences the capacity of a crop to assimilate

and translocate nutrients from soil and the N remobilization from the stem and leaves to the grain (Gaju et al., 2014). A fungicide application at the heading stage is a key practice in temperate growing areas to control Fusarium head blight, which has an impact on the grain yield and on the accumulation of mycotoxins. Moreover, this timing of application, according to the agronomic and pedo-climatic conditions, may contribute to delaying canopy senescence (Blandino and Reyneri, 2009; Blandino et al., 2011) The influence of the fungicide on the protein content has been discussed widely in the literature (Gooding et al., 2005), where a decrease in GPC has been observed (Ruske et al., 2003; Motta-Romero et al., 2021; Dimmock and Gooding, 2002; Matzen et al., 2019), in conjunction with high N doses (Castro et al., 2018), while other researchers have observed a significant increase in the protein content (Fleitas et al., 2018) or even no change (Blandino and Reyneri, 2009). Furthermore, to the best of the authors' knowledge, no results on the effect of a late fungicide application to the gluten protein composition have been reported.

In addition to the management of crop practices, in order to guarantee a more constant level of wheat quality along the supply chain, the meteorological trend over different growing seasons could also have an important effect on the gluten properties and composition. For this purpose, many studies have been carried out to assess the variations in gluten protein and its composition under continental environmental conditions, such as those of North Europe and North America, where there is regular rainfall and prolonged ripening (Triboï, 2003; Malik et al., 2011), or in Mediterranean growing areas, where there is a short duration of the grain filling stages as a consequence of high spring temperatures and frequent drought (Garrido-Lestache et al., 2004). However, only a few studies have been carried out in humid Mediterranean environments (Fuertes-Mendizábal et al., 2018; Aranguren et al., 2021), such as in the North of Italy, with a medium duration of the ripening stages, which generally occur without any significant heat or drought stress. Moreover,

the interaction of crop practices occurring late during crop cultivation, such as N fertilization and fungicide application at heading, and the meteorological trends during wheat ripening have not yet been thoroughly investigated in the literature. The hypothesis of the present research is that in humid Mediterranean climate condition the agronomic practices that influence the cultivation conditions during ripening may affect, in addition to the gluten content, also its composition and therefore the rheological characteristics of the flours. The objective of this research has been to study the influence of a late N fertilization and fungicide application at wheat heading and their interaction on the alveographic traits and gluten composition in a high protein wheat cv, grown in the same field over a 3-year period under a typical humid Mediterranean.

Materials and Methods

Experimental Site and Treatments

The effects of the considered factors (N fertilization, fungicide application and meteorological conditions) were investigated by considering the accumulation of gluten protein fractions for 4 different wheat maturation timings.

The study was carried out over 3 years (2014, 2015 and 2016) at Carmagnola in the North-West of Italy (N 44°53’10.6” E 7°41’11.8”; elevation 245 m), in the experimental fields of the University of Turin. The daily temperatures and precipitations were measured by a meteorological station near the experimental area.

The experimental trial was carried out on a deep silty-loam soil (sand 28.7%, silt 64.6% and clay 6.7%, pH 8.0) with a medium cation-exchange capacity (12.2 meq 100 g^{-1}) and organic matter content (1.45%). The soils were sampled at a depth of 0.30 m and 30–60 cm each growing season, using Eijkelkamp cylindrical augers just before crop sowing. The average assimilable phosphorus and exchangeable potassium contents were 7 mg

kg^{-1} and 49 mg kg^{-1}, respectively, and the total N, analyzed using a CHN elemental analyzer (Flash EA 1112, Thermoquest) in 0–30 cm and 30–60 cm layers at the beginning of spring, is reported for each year in Table 1.

Table 1. Total nitrogen content in different soil layers at the tillering growth stage for each year of the experiment.

Year	0–30 cm	30–60 cm
2014	0.109%	0.086%
2015	0.102%	0.082%
2016	0.113%	0.092%

Soil was sampled at depths of 0–30 and 30–60 cm using Eijkelkamp cylindrical augers.

The compared treatments in each year were factorial combinations of:
late-season N fertilization:

- Unfertilized control, with a total of 130 kg N ha^{-1} top-dressed applied as a granular ammonium nitrate (AN) fertilizer, split into 50 kg N ha^{-1} at tillering (GS23) and 80 kg N ha^{-1} at the beginning of stem elongation (GS32), without any late fertilization at the middle of heading (GS55);
- N application at heading (GS55), with the addition of 30 kg N ha^{-1} top-dressed, applied as AN, to the previously reported fertilization during the vegetative stages; the total N rate was 160 kg N ha^{-1}.

fungicide application:

- Untreated control;
- Heading application (GS55), with the application of a mixture of triazole fungicide prothioconazole + tebuconazole applied as 0.125 kg

+ 0.125 kg of the active ingredient (AI) ha^{-1} (Prosaro®, Bayer, Italy).

All the top-dressed ammonium nitrate fertilizers were applied by hand as solid prills (27% N w/w). The fungicide was applied at the manufacturer's recommended field rates using a 4-nozzle precision sprayer (T-Jeet 110/04) with a fine mist at a slow walk to ensure effective coverage. The delivery pressure at the nozzle head was 324 KPa. No other fungicide was applied to any other GS to control foliar diseases.

The commonly adopted agronomic growing area practices were applied. Briefly, the previous crop was wheat, the field was ploughed each year, incorporating the debris into the soil, and this was followed by disk harrowing to prepare a seedbed. Planting was conducted in 12 cm wide rows at a seeding rate of 450 seeds m^{-2} in the last decade of October, using the fungicide fludioxonil as seed dressing to prevent root and stem diseases. The weed control was conducted with mesosulfuron-metile and iodosulfuron-metil-sodium at wheat stem elongation (GS 31). The selected winter wheat cv was Rebelde (Apsovsementi, Voghera, Italy), which is classified, according to the Italian Synthetic Index of Quality (Foca et al., 2007), as an improver wheat because of its high protein content. This cultivar is classified as medium-tolerant to both Fusarium head blight and Septoria Tritici Blotch diseases. The sowing and harvest dates, as well as the N fertilization and fungicide application dates are reported in Table 2 for each year. The treatments were assigned to experimental units using a completely randomized block design with four replicates. The plot size was 7 × 1.5 m.

Table 2. Main trial information and the date of N fertilization in the field experiments conducted over the 2013–2016 period in North-West Italy.

Crop Techniques	Growth Stage [a]	2014	2015	2016
Sowing		25 Oct. 2013	23 Oct. 2014	23 Oct. 2015
N fertiliz.	tillering (GS23)	10 March 2014	6 March 2015	25 Febr. 2016
	stem elong. (GS32)	11 April 2014	9 April 2015	29 March 2016
	heading (GS55) [b]	5 May 2014	7 May 2015	5 May 2016
Harvest		16 July 2014	6 July 2015	8 July 2016

[a] according to the Zadoks growth scale (Zadoks, Chang, e Konzak 1974); [b] the fungicide was applied at heading just after N fertilization.

Fifty ears were manually sampled from each plot at four different GS during ripening: the milk stage (GS75), soft dough stage (GS85), physiological maturity (GS91) and commercial maturity (GS99). The dates of the ear collection for each year are reported in Table 3. The ears collected at each ripening stage and for each plot were manually shelled, and the kernels were mixed thoroughly to obtain a random distribution, and samples (10–30 g) were then frozen at a temperature of –18 °C for the analysis of the different gluten protein fractions.

Table 3. Dates of the ear collection in the experimental plot for the analysis of the gluten protein fractions.

Years	Milk Stage GS75	Soft Dough Stage GS85	Physiological Maturity GS91	Commercial Maturity GS99
2014	3 June 2014	13 June 2014	23 June 2014	16 July 2014
2015	28 May 2015	11 June 2015	22 June 2015	6 July 2015
2016	10 June 2016	20 June 2016	30 June 2016	7 July 2016

Crop Assessments

Grain Yield

The grain yields were obtained by harvesting the whole plot at commercial maturity (GS99), using a Walter Wintersteiger cereal plot combine-harvester. A Dickey-John GAC2100 grain analyzer (Auburn, IL, USA) was utilized to measure the grain moisture. All the grain yield results were adjusted to a 13% moisture content. The grains were carefully mixed, and 1 kg of each sample was taken for the qualitative analyses.

Kernel Quality Traits and Rheological Properties

The test weight (TW), thousand kernel weight (TKW) and grain protein content (GPC; Kjeldahl N x 5.7, on a dry matter basis) were determined according to Blandino et al. (2015) on grains collected at the commercial maturity stage. Three kg of kernels were taken from each plot and milled using a Bona 4RB mill (Bona, Monza, Italy) to obtain refined flour. The alveograph test was carried out on the refined flour according to ICC-121 (The International Association for Cereal Science and Technology 1992).

Gluten Protein Quantification

Ten g of seeds taken at the milk stage and 10 g at the soft dough stage were ground in a mortar previously cooled in liquid nitrogen; 30 g of seeds, taken at physiological and commercial maturity, was milled into fine flour using a Knifetec™ 1095 (Foss, Hillerod, Denmark). Gliadins, HMW-gs and LMW-gs were extracted from 30 mg of each grain sample with a sequential extraction protocol described by (Visioli et al. 2016); in brief, the alcohol soluble gliadin fraction was extracted with 1.5 mL of 55% (v/v) propan-2-ol with continuous mixing at 65 °C for 20 min, followed by centrifugation at 10,000× *g* for 5 min. The supernatant was dried in a vacuum centrifuge to obtain the majority of gliadins. The procedure was repeated twice to wash the pellet from residues of gliadins. The remaining pellet containing the alcohol insoluble fraction of both HMW-gs and LMW-gs was dissolved in a 400 µL solution of 55% (v/v) propan-2-ol, 0.08 M

tris(hydroxymethyl) aminomethane hydrochloric acid (Tris–HCl, pH 8.3) and 10 mM 1,4-dithiothreitol (DTT, as reducing agent), and incubated for 30 min at 60 °C with continuous mixing. After centrifugation at 14,000× *g* for 5 min, the supernatant containing HMW-gs and LMW-gs was collected. After the addition of 40% (v/v) acetone, incubation at 15 min at RT, centrifugation at 14,000× g for 5 min the HMW-gs fraction was precipitated. At the remaining supernatant 80% (v/v) acetone was added and after incubation 15 min at RT, centrifugation at 14,000× g for 5 min the LMW-gs fraction was precipitated. Three technical replicates were performed for each sample (Blandino et al., 2020). The pellets of gliadins, HMW-gs and LMW-gs were dissolved in 50% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid and relative quantification was determined by colorimetric Bradford assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as protein standard. Data were reported considering 1 mg of extracted proteins for 1 g of dry weight (DW), as detected by means of a precision laboratory thermo balance. Ratios of the glutenin-to-gliadin fractions (gs/glia), HMW-gs-to-LMW-gs (H/L) and HMW-gs By-to-HMW-gs Bx fractions (Y/X (H)) were also calculated. The extracted fractions were then dried in a Savant SpeedVac SPD1010 device (Thermo Fisher Scientific, Walthman, MA, USA) at 45 °C and the gliadin fractions were then utilized for SDS-PAGE.

Gliadin Separation by Means of SDS-PAGE and Densitometric Analyses

SDS-PAGE was performed in a Mini-PROTEAN Tetra Cell (Bio-Rad) on 12% acrylamide gel in order to analyze the different sub-units of the gliadin fraction. An aliquot of 7 µg of gliadins was suspended in 20 µL of loading buffer containing 2% (w/v) SDS, 0.02% (w/v) bromophenol blue, 0.1% ß-mercaptoethanol, 0.05 M Tris-HCl pH 6.8 and 10% (v/v) glycerol, and boiled at 95 °C for 5 min before loading onto the gel. A Molecular-Weight Marker (Mw ® 14,000–66,000; Sigma Aldrich, St. Louis, MO, USA) was used to detect the gliadins of different molecular weights. After electrophoretic separation at 40 mA, the gels were stained with a brilliant

blue G-colloidal solution (Sigma Aldrich) fixed in 7% (v/v) acetic acid and 40% (v/v) methanol, and de-stained in 25% (v/v) methanol. The gliadins were analyzed in 3 technical replicates for each plot sample. IMAGE lab 4.5.1 (Bio-Rad) software was used for the relative quantification of the representative gliadin protein subunits on each gel. Exact masses of the different members of the gliadin fraction obtained in MALDI-TOF/MS analysis linear mode were previously reported (Visioli et al., 2016).

Statistical Analysis

The Kolmogorov–Smirnov normality test and the Levene test were carried out to verify the normal distribution and homogeneity of variances. The gluten and alveographic parameters of samples collected at ordinary maturity were compared by means of an analysis of variance (ANOVA), in which the late-season N fertilization, the fungicide application and the year were the independent variables. The effect of late-season N fertilization and fungicide application on the relative abundances of the gluten fractions for each single ear collection timing during ripening was analyzed through ANOVA, in which the agronomic factor was the independent variables. Multiple comparison tests were performed with the Ryan-Einot-Gabriel-Welsh F (REGW-F) test, on the treatment means. Statistical data analysis was carried out with the SPSS software package, version 26.0.

Results

Weather Conditions

Results from 2015 had the largest total amount of precipitation (>850 mm) in the period between wheat sowing (November) and flowering (May). Very little rainfall occurred in the winter and spring periods in 2016 (<300 mm) (Table 4), thus resulting in the highest availability of N in the soil at the beginning of spring (Table 1) for that year. As far as the ripening period

(May–June) is concerned, the 2015 season had the highest growing degree days (GDD).

Table 4. Monthly rainfall and growing degree days (GDDs) from the sowing stage (November) to the end of ripening stage (June) in the 2013–2016 period in North-West Italy.

Month	2014		2015		2016	
	Rainfall (mm)	GDDs [a] (°C-Day)	Rainfall (mm)	GDDs (°C-Day)	Rainfall (mm)	GDDs (°C-Day)
November	81	244	271	289	3	284
December	64	133	92	185	2	182
January	67	158	36	139	4	159
February	87	181	206	143	128	190
March	89	328	188	300	71	286
April	75	442	67	416	80	428
May	49	538	86	581	112	517
June	50	659	55	666	37	642
November–June	562	2683	1000	2720	436	2689
November–April	463	1485	859	1473	288	1530
May–June	99	1198	141	1247	149	1159

[a] Accumulated growing degree days were calculated for each month as: ∑ (daily mean temperature−base temperature), using a 0 °C base (Visioli et al., 2016).

Grain Yield and Kernel Traits

The grain yield at commercial maturity was significantly higher in 2016 (7.29 t ha^{-1}) than in 2014 (6.16 t ha^{-1}) or 2015 (6.28 t ha^{-1}), probably because of the higher N availability in the soil (Table 1), as a consequence of smaller amounts of rainfall during the winter and spring periods. The TKW was significantly higher in 2016 than in 2014, while the grain harvest in 2015 resulted in the smallest dimensions of the seeds (Table 5). The fungicide application significantly increased grain yield (+8.6%), as a result of a clear increase in the seed weight (+5.1%). The late N

fertilization did not influence either the TKW or the grain yield to any great extent.

Table 5. Effect of the late N fertilization and fungicide application at heading on the thousand kernel weight (TKW), grain protein content (GPC), high molecular weight glutenins (HMW-gs), low molecular weight glutenins (LMW-gs) and gliadins at the commercial wheat harvest stage in the field experiment carried out over 3 years in North-West Italy.

Factor	**Source of Variation**	**TKW**	**GPC**	**Gluten Extractable Fractions (mg g^{-1} Flour)**		
		g	**%**	**HMW-gs**	**LMW-gs**	**Gliadins**
Late N	unfertilized control	34.60 a	14.41 b	5.27 b	6.26 b	14.23 b
fertilization (N)	heading application	34.85 a	15.64 a	5.83 a	7.21 a	15.79 a
	p-value	0.682	<0.001	<0.001	<0.001	<0.001
Fungicide	untreated control	33.88 b	15.19 a	5.60 a	6.67 a	15.32 a
application (F)	heading application	35.61 a	14.86 b	5.50 a	6.80 a	14.66 b
	p-value	<0.001	0.034	0.281	0.205	0.014
Year (Y)	2014	34.55 b	14.96 b	4.86 b	6.50 b	13.60 b
	2015	33.87 c	15.43 a	4.50 c	5.42 c	18.69 a
	2016	36.01 a	14.69 b	7.29 a	8.34 a	12.43 c
	p-value	<0.001	0.003	<0.001	<0.001	<0.001
N * F	*p*-value	0.953	0.096	0.006	0.087	0.034
N * Y	*p*-value	0.925	0.204	0.181	0.019	0.124
F * Y	*p*-value	0.907	0.235	0.277	0.258	0.170
N * F * Y	*p*-value	0.996	0.136	0.047	0.443	0.797

Means followed by different letters are significantly different for each parameter (the level of significance, the p-value, is shown in the table). The reported values for the late season N fertilization are based on 18 replicates (2 fungicide applications × 3 years × 3 repetitions). The reported values for the fungicide application are based on 18 replicates (2 late-season N fertilizations × 3 years × 3 repetitions). The reported values for the year are based on 12 replicates (2 late-season N fertilizations × 2 fungicide application × 3 repetitions).

Gluten Fractions and Alveographic Parameters of the Refined Flour at Harvest

The effect of the late N fertilization and fungicide application at heading on the total protein content and the gluten protein extractable fractions is reported in Table 5.

The late N fertilization significantly (*p*-value < 0.001) increased the GPC (+1.23%) and the content of all the gluten protein fractions, compared to the control, without any application at heading. On the other hand, the fungicide application led to a significant reduction in GPC (−0.33%), because of a smaller gliadin content, while no significant difference was detected for the glutenins. The GPC content was higher in 2015 than in 2014 and 2016. The year 2016 had the highest HMW-gs and LMW-gs, but the smallest gliadin content. The largest and smallest contents of gliadins and glutenins were instead reported for 2015.

The year resulted to have the greatest effect on the gluten protein fraction ratios (gs/glia; H/L, Y/X) and alveographic parameters (Table 6). The dough strength (W) was significantly lower in 2015, which, although recording the highest GPC, had the lowest gs/glia ratio. On the other hand, 2016 had the highest gs/glia, H/L and Y/X ratios, thereby also resulting in a higher P/L ratio than 2014 or 2015. The interaction between the compared factors was never significant for GPC. The interaction between late N fertilization and fungicide application was significant for HMW-gs and gliadins. Despite the clear effect of the late N fertilization and fungicide on GPC and the gluten extractable protein fractions, these agronomic practices did not significantly impact their ratio. Thus, the dough strength (W) was significantly increased by the late N fertilization (+22%) and reduced by the fungicide application (−4%). In addition, only the N fertilization significantly reduced the P/L ratio.

Table 6. Effect of the late N fertilization and fungicide application at heading on the gluten fraction ratios and alveographic parameters at the commercial wheat harvesting stage in the field experiment carried out over 3 years in North-West Italy.

Factor	**Source of Variation**	**Gluten Protein Fraction Ratio**			**Alveographic Parameters**	
		gs/glia	**H/L**	**Y/X (H)**	**W**	**P/L**
					J * 10^{-4}	
Late N	unfertilized control	0.85 a	0.84 a	0.58 a	337 b	1.99 a
fertilization (N)	heading application	0.88 a	0.80 a	0.58 a	414 a	1.74 b
	p-value	0.210	0.103	0.956	<0.001	0.004
Fungicide	untreated control	0.86 a	0.84 a	0.59 a	383 a	1.89 a
application (F)	heading application	0.87 a	0.80 a	0.57 a	365 b	1.85 a
	p-value	0.071	0.046	0.576	0.045	0.574
Year (Y)	2014	0.83 b	0.75 b	0.41 c	390 a	1.75 b
	2015	0.53 c	0.84 a	0.61 b	322 b	1.64 b
	2016	1.26 a	0.88 a	0.72 a	409 a	2.24 a
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001
N * F	*p*-value	0.006	<0.001	0.027	0.099	0.338
N * Y	*p*-value	0.084	0.356	0.823	0.156	0.039
F * Y	*p*-value	0.404	0.214	0.571	0.640	0.754
N * F * Y	*p*-value	0.373	0.163	0.580	0.754	0.742

Means followed by different letters are significantly different for each parameter (the level of significance, the p-value, is shown in the table). The reported values for the late season N fertilization are based on 18 replicates (2 fungicide applications × 3 years × 3 repetitions). The reported values for the fungicide application are based on 18 replicates (2 late-season N fertilizations × 3 years × 3 repetitions). The reported values for the year are based on 12 replicates (2 late-season N fertilizations × 2 fungicide applications × 3 repetitions).

No significant interaction between the considered factors was recorded for the alveographic parameters, except for late N fertilization X year,

because of a lower effect of this practice in 2015. Conversely, the interaction between N fertilization and fungicide application was significant for all the considered gluten protein fraction ratios. The late N fertilization in the plots without any fungicide application resulted in a more marked effect on the LMW-gs, gliadins and H/L glutenin ratios than under the fungicide treated conditions (Table 7). On the other hand, a more marked effect of late N fertilization on the HMW-gs and gs/glia ratios was observed when the fungicide was applied at wheat heading.

Table 7. Effect of the combination of late N fertilization and fungicide application at heading on the qualitative gluten protein ratios and alveographic parameters at the commercial wheat harvesting stage in the field experiment carried out over 3 years in North-West Italy.

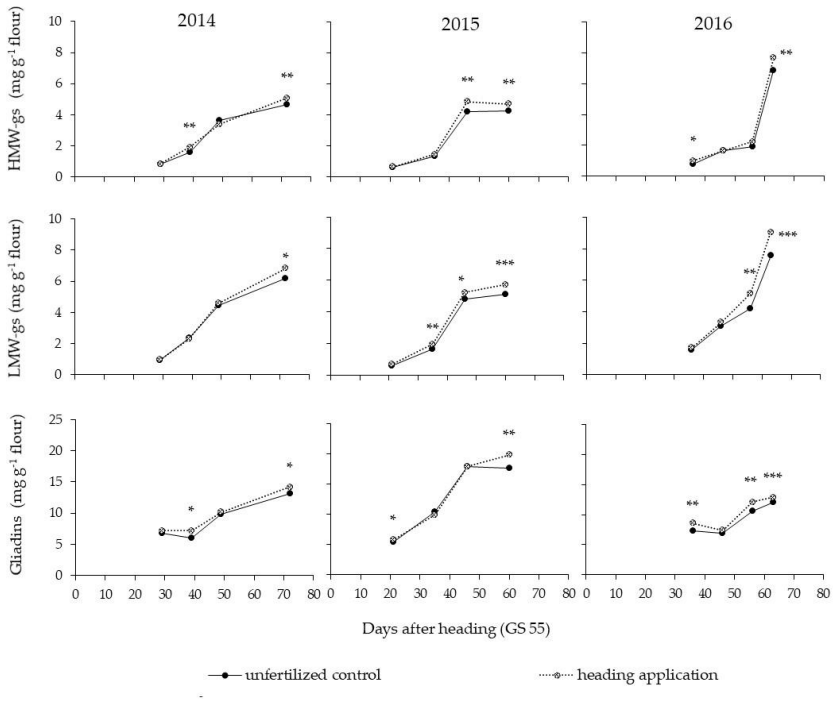
Fungicide	**Late N**	**Gluten Fractions**			**Gluten Fraction Ratio**		
Application	**Fertilization**	**HMW-gs**	**LMW-gs**	**Gliadins**	**gs/glia**	**H/L**	**Y/X (H)**
untreated control	unfertilized control	5.46 b	6.12 b	14.28 bc	0.87 ab	0.91 a	0.62 a
	heading application	5.74 ab	7.23 a	16.48 a	0.85 ab	0.78 b	0.55 a
heading application	unfertilized control	5.08 c	6.41 b	14.19 c	0.84 b	0.76 b	0.53 a
	heading application	5.91 a	7.19 a	15.15 b	0.90 a	0.83 b	0.61 a
p-value		<0.001	<0.001	<0.001	0.009	<0.001	0.137

Means followed by different letters are significantly different (the level of significance, the p-value, is shown in the table). The reported values are based on 9 replicates (3 years × 3 repetitions). The interaction between the agronomic treatment and year was never significant.

Accumulation of the Gluten Fractions during Ripening

The effect of N fertilization at heading on the accumulation of HMW-gs, LMW-gs and gliadins during ripening is reported in Figure 1.

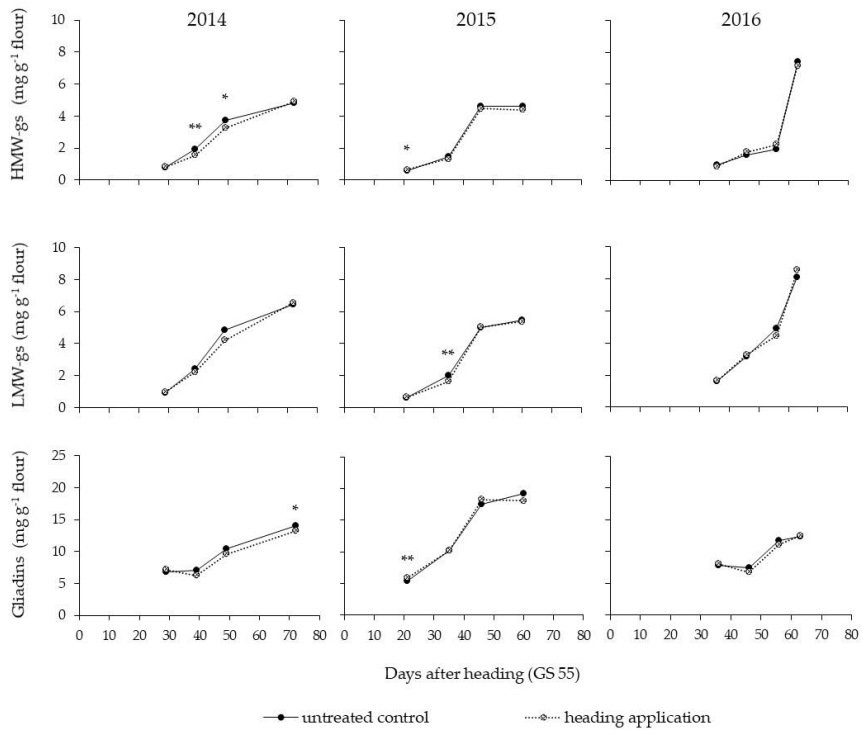
Figure 1. Effect of late N fertilization on the high molecular weight glutenin (HMW-gs), low molecular weight glutenin (LMW-gs) and gliadin contents during wheat ripening; field experiment carried out in Carmagnola (North-West Italy) over 3 different years. The reported values are based on 6 replicates (2 fungicide treatments × 3 replicates). ANOVA was performed for each timing of the sample collection: * significant difference at the <0.05 level; ** significant difference at the 0.01 level; *** significant difference at the <0.001 level.



All the gluten extractable protein fractions increased progressively from the milk stage (GS85) to commercial maturity (GS99). The late N fertilization significantly increased the HMW-gs, LMW-gs and gliadins at the last harvesting time, compared to the control, for all 3 years, but significant benefits of this practice were also observed within the 3 years

for earlier maturity stages. The rise in the glutenin and gliadin fractions during ripening showed a similar trend in 2014 and 2015. However, a greater increase of glutenins was observed in 2016, the growing season with the highest soil N availability, in the last ripening stages than in the gliadin fraction. Compared to the role of late season N, the fungicide application at heading led to a lower impact on the contents of the different gluten protein fractions during ripening (Figure 2).

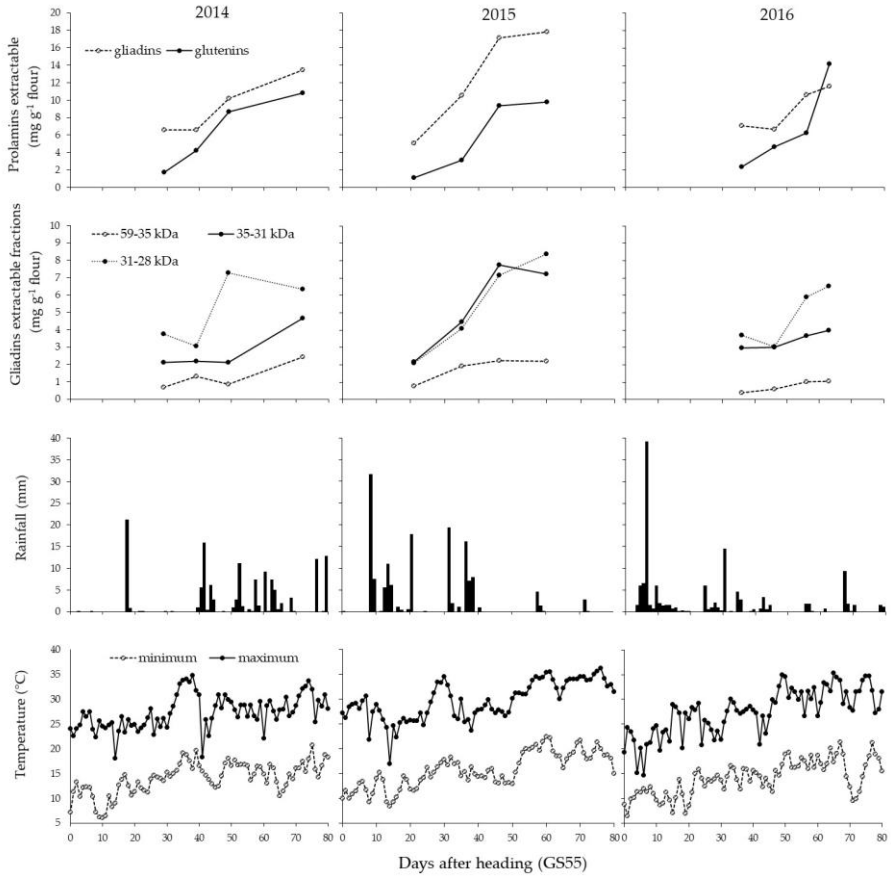
Figure 2. Effect of the fungicide application on the high molecular weight glutenin (HMW-gs), low molecular weight glutenin (LMW-gs) and gliadin contents during wheat ripening; field experiment carried out in Carmagnola (North-West Italy) over 3 different years. The reported values are based on 6 replicates (2 fungicide treatments × 3 replicates). ANOVA was performed for each timing of the sample collection: * significant difference at the <0.05 level; ** significant difference at the 0.01 level.



Thus, the increase in the gluten protein fractions occurred similarly in the wheat treated with fungicide and in the untreated control. Overall, a significantly lower concentration of glutenins was detected at the earlier ripening stages after a fungicide treatment. Only in 2014 were the gliadins at the commercial harvesting stage significantly higher in the untreated control than under the fungicide treatment conditions. The effect of the meteorological trend during the ripening stages on the gluten protein fractions, on the accumulation profile of the total extractable glutenins and gliadins and on the latter one in particular, as well as on their separation into different sub-units was specifically investigated and reported for the untreated control over the 3 years, without a late N fertilization or fungicide application at heading (Figure 3). The gliadins showed a higher content than the glutenins for all the maturity stages, except for the commercial maturity stage in 2016. The largest difference in the gliadin and glutenin contents was observed at the milk stage in 2014 and 2016, while the gliadin content was more than double that of the glutenins at the physiological and commercial maturity stages in 2015. The growth of both gliadins and glutenins in 2015 was particularly quick from the soft dough stage (35 days after heading) to the physiological maturity stage (46 days after heading). The α/β gliadin enriched fraction (31–28 kDa) maintained a constant trend for all the years and started to accumulate at the soft dough maturation stage. The γ-gliadin enriched fraction (35–31 kDa), an intermediate fraction rich in sulfur (S), resulted in a continuously high increase from the milk stage to the physiological maturity stage, and reached contents of 270% and 110% at this stage in 2015, which were higher than 2014 and 2016. The poor sulfur-gliadin fraction (59–35 kDa) showed a slow tendency to increase over all the years, mainly in the last ripening stages. As previously reported, the greatest differences, in terms of GPC, alveographic parameters and gluten composition, were observed in 2015 and 2016. As far as the meteorological trend is concerned, 2015 had the highest amount of rainfall (860 mm) during the winter and early

spring months (from November to April), thus resulting in a lower availability of N (0.102%) in the soil than the other years, and in particular compared to 2016.

Figure 3. Evolution of the prolamin fractions during wheat ripening; field experiment carried out in Carmagnola (North-West Italy) over 3 years. The reported values are based on 3 replications of the untreated control, without a late N fertilization or fungicide application.



Rainfall was well distributed, from heading to the end of ripening, in all the considered years, thus no drought stresses were observed before harvesting for any year and the duration of ripening was similar. As far as the temperature is

concerned, the average minimum and maximum temperatures from the heading phase to the physiological maturity stage in 2015 (13.6 °C and 27.4 °C, respectively) were higher than in 2016 (12.5 °C and 25.3 °C). Moreover, there were 10 days of high temperatures (more than 32 °C) during this growth stage in 2015, while only 4 days with the same conditions occurred in 2016.

Discussion

The protein value of wheat on the global market has been estimated to be 2.4 billion dollars in 2021, and it is expected to increase to 3.1 billion dollars by 2030 (Wheat Protein Market Industry Growth, Trends, and Forecasts 2021). Despite the rise in gluten-free products, the proteins of wheat, a staple worldwide food, are suitable alternatives to non-animal proteins, considering the growing popularity of plant-based foods. Moreover, the rheological and technological key roles of gluten protein in bread-making are well known. It is known that high levels of grain yield may dilute the amount of protein in the grain (Yu et al., 2018), thus it is necessary to balance the crop management practices for the production of high protein wheat in order to maximize the grain yields while maintaining a high content of functional proteins to obtain suitable bread-making qualities. In addition to the key role of the wheat genotype, both the environmental conditions (Bashir et al., 2013) and the agronomic practices (Fuertes-Mendizábal et al., 2018) have a clear influence on N uptake and its translocation to the kernel during the vegetative and reproductive stages.

Effect of Late-Season N Fertilization

The results of our study confirm that late N fertilization could be an efficient agronomic practice to increase the GPC of high protein wheat cvs. An additional 30 kg N ha^{-1} applied at heading in humid Mediterranean conditions (Fuertes-Mendizábal et al., 2018; Blandino., 2015) or in

temperate continental growing areas (Rossmann et al., 2020; Saint Pierre et al., 2008) is an excellent source of N during grain filling, even in soil with a high N content (Martre et al., 2003).

In this research, the late N fertilization led to a clear increase in the GPC and dough strength for all 3 years, although this practice had no effect on the grain yield in the considered growing area (Bogard et al., 2010). The first effect of late N fertilization is a slight increase in the total N rate, which determines an increase in the crop N uptake during the accumulation process of the storage proteins in the grain (Xue et al., 2016). During grain filling, the total N available for protein synthesis is derived from both the direct uptake of N from the soil and from the remobilization of N from leaves and stems. This latter process primarily depends on the N content stored in the vegetative organs during the vegetative stages (Gaju et al., 2014). A late source of N permits a rapid synthesis of the amino acids and allows them to move faster through the phloem and reach the kernel during the grain filling process than those obtained from the remobilization of the N source previously taken up by the plants (Xue et al., 2016), which can explain the significant increase in the GPC value. The late N fertilization clearly increased the W value in the high protein wheat, mainly as a result of an enhancement of the dough extensibility (L) value (Peña et al., 2005). However, the late N availability did not affect dough tenacity (P) and resulted in a significant lowering of the P/L ratio, as required for a high protein wheat cv, which is often characterized by an excessive tenacity.

As far as the effect on gluten composition is concerned, the late N application induced a significant and similar increase in all the prolamin fractions, thus confirming a few other recent studies (Zheng et al., 2018; Zhen et al., 2019). Martre et al. (2003) conducted a modelling of the accumulation of N in the grain following different N fertilization inputs. They showed that the ratios between the different prolamin fractions remain constant over all the growth stages during ripening, because the

different fertilization strategies only influence the total availability of N and the total N accumulated in the grain. Xue et al. (2016) claimed that late N fertilizations resulted in a greater accumulation of gluten proteins in some cvs, and in particular of x-type glutenins (HMW-gs Bx7), but the ratios between gliadins and glutenins remained unchanged, thus no improvement in the baking properties of the flour was verified (Bogard et al., 2010). Rekowski et al. (2019) observed that late N fertilization increased GPC in both ordinary bread-making and high protein cvs, while significant changes in gluten fractions were only observed as a consequent of a N fertilization strategy for the higher protein cv. The latter study found an increase in the storage protein fractions with a low-to-medium sulfur content, HMW-gs and ω-gliadins, while a slight decrease was observed for LMW-gs, with a consequent reduction in the gs/glia ratio. In short, the collected data highlighted that a late N fertilization, although accounting for only 15–25% of the total applied N, led to a significantly greater accumulation of all the different gluten protein fractions in the kernels, thus resulting in positive benefits for the alveographic traits of high protein wheat.

Effect of a Fungicide Application

Fungicides applied at heading increase the grain yield through the direct control of leaf and head diseases (Blandino et al., 2011). Moreover, since this practice enhances the plant stay green during ripening and the extension in the duration of grain filling (Gooding et al., 2005), it could also determine an effect on both the uptake of N from soil and its translocation from the leaves and stem to the kernel. Moreover, without an appropriate leaf protection from fungal diseases, the late N fertilization effect on increasing the GPC could be less effective, due to a premature plant senescence under high disease pressure conditions (Fleitas et al., 2018). In our experiment, the fungicide application at heading caused a slight but significant lowering of the GPC, compared to the untreated grains. The

fungicide treatment under the considered conditions probably resulted in a higher photosynthetic activity and starch accumulation in the grain, as demonstrated by the final higher TKW (Ruske et al., 2003), through a process that just overcome that of N translocation during ripening and thus it marginally reduced the dough strength. Considering the influence of a fungicide application on the gluten protein fractions, a significant effect was only observed for gliadins, probably because these fractions resulted in a higher content in the grain at maturity. Despite the weak reduction of the protein content in the kernel at maturity, as also confirmed by a significantly lower gluten protein fraction content at the early ripening stages, no significant change in the ratios between the different gluten fractions was detected after the fungicide application. The fungicide treatment had thus a minimal effect on rheological parameters, thus confirming information reported in similar environments with the application of triazole fungicide at heading to control head and foliar diseases (Castro et al., 2018; Blandino and Reyneri, 2009; Wang et al., 2004).

A significant interaction between fungicide and N fertilization at heading has been observed in our study: a late N application determined a more marked increase in gliadins in the untreated crop, while fertilization led to greater benefits for HMW-gs, as a result of the prolonged crop stay green induced by the fungicide, but less significant benefits for LMW-gs, which are the last storage gluten proteins to be accumulated (Shewry, 2009). The increase in glutenins, due to the combined application of fungicide and late N fertilization at heading, resulted in a final higher gs/glia ratio, although no significant interaction or effect was observed for the alveographic parameters. The application of the fungicide with a higher N availability could have caused a high uptake of this element from the soil (Gooding et al., 2005), as well as its translocation from the vegetative organs (Dimmock and Gooding, 2002), in the last part of ripening, when glutenins in particular were accumulated in the grains. Thus, although

these crop practices have a very limited effect on the composition of gluten when applied individually, their interaction could determine variations regarding the N availability for the plant in the different phenological stages, which seem to not only lead to a minimal effect on the accumulated protein quantity, but also on its composition.

Effect of the Year on Gluten Protein Accumulation during Ripening

Variations in the temperature and rainfall during wheat ripening can influence the grain protein composition more than the N application timing and fungicide treatments.

The effect of environmental conditions during ripening on the content and quality of gluten proteins in typical Mediterranean environments characterized by frequent drought or heat stresses during grain filling has been studied extensively in the literature (Garrido-Lestache et al., 2004; Peña et al., 2005; Blumenthal et al., 1993; Hernández-Espinosa et al., 2018), and these stress conditions have been found to act positively on the protein content of wheat and on the dough strength (Peña et al., 2005). Several studies have also been carried out in continental climates (Triboï et al., 2003; Malik et al., 2011; Shewry 2009), generally characterized by a long ripening period, which sometimes could be shortened by biotic and abiotic stresses, thus resulting in a lower yield potential and a less dilution of the proteins in the grains. A clear and negative relationship between the grain yield potential and GPC has been established in both of these environments, since the meteorological trend has a marked impact on the wheat yield potential under both conditions. Less information is available in the literature on the role of the meteorological trend on the gluten content and composition for the humid Mediterranean climate, where the length of ripening and the grain yield could be more constant across the growing seasons.

Our study, which was carried out on the same field, with the same genotype and crop management practices over a 3-year period, has highlighted how the meteorological conditions influenced the GPC content and, above all, the gluten protein composition to a great extent, and resulted in marked differences in the alveographic parameters.

It is largely reported that although genotype had a major influence in the performance of wheat in a specific end-use quality application, environmental effects also strongly impact on yield and qualitative properties of a given cultivar (Malik at al., 2013; Souza et al., 2004). Furthermore, within high protein cultivars, Peterson et al. (1992) highlighted that variance of grain yield, GPC and rheological traits associated with environmental effects were generally larger than those for genetic factors. Additionally, Panozzo and Eagles (2000) reported that environmental variation was greater than cultivar variation for the dough rheological characters. As far as the composition of gluten is concerned, the proportion of glutenin in flour protein was mainly dependent on cultivar, whereas environmental variation was greater than cultivar variation for gliadin (Panozzo and Eagles, 2000).

In our study, it is interesting to note that the recorded meteorological trends for all 3 years were representative of a humid Mediterranean environment, that is, with well distributed rainfall during the growing season and thus no significant drought stress for the crops. The greatest differences in the gluten protein composition and alveographic parameters were observed when comparing 2015 and 2016. The wheat flour had the highest W and dough tenacity (high P/L ratio) in 2016, probably as the result of the higher availability of N in the soil throughout the entire growing season, and therefore showed the highest gs/glia and y/x HMW-gs ratios. Although the highest GPC content was detected in 2015, the lowest value of W was also observed, as a consequence of the lower gs/glia ratio. The higher GPC recorded in that year is probably a consequence of the lower kernel dimension (TKW) than in the other years

(Panozzo and Eagles, 2000). The larger total amount of rainfall observed during the winter and spring months in that year (>850 mm), reduced the N availability at the late maturity stages because it has led to greater soil leaching, thus negatively affecting the grain filling process and reducing the TKW, since no drought stress was recorded and the physiological maturity of the crop was not anticipated. Moreover, the minimum temperatures from the heading stage to the physiological maturation stage (13.6 °C) were higher in 2015 than in 2016 (12.6 °C). Higher nocturnal temperatures increased the wheat transpiration rate, which may have decreased the starch accumulation in the grain (Wang and Liu, 2021), thus resulting in a lower dilution of grain proteins.

In addition, 2015 showed a markedly different trend in gluten fraction accumulation during ripening: the gliadins increased quickly, compared to the glutenins, from the first stages of grain maturation. The overall higher increase in the gliadin fraction in 2015 mainly depended on the marked rise in the γ-gliadin enriched fraction, which showed similar amounts as the α/β gliadin enriched fraction. The lower late N availability of 2015 could be the cause of the lower synthesis of the glutenins, since this nutrient is mainly dispensed for the accumulation of gliadins, that is, monomeric proteins of early and rapid synthesis (Abonyi et al., 2007; Johansson et al., 2013). However, another role could be played by the incidence of the temperature peaks above 32 °C on gliadin and glutenin accumulation at the dough stage. The occurrence of days with high temperatures during the early ripening could have impaired the protein folding processes and the formation of disulfide bridges between the different gluten proteins (Lafiandra et al., 1999), with a consequent reduction in the average molecular weights of the gluten network. It has been reported that temperature peaks above 32 °C after anthesis may decrease the gs/glia ratio, thereby leading to a weaker dough and consequent decrease in wheat flour quality (Blumenthal et al., 1993). This aspect is also highlighted by the presence of heat-shock elements, located upstream of

the promoter region of genes encoding gliadins, but absent in glutenin genes, which also leads to a constructive mechanism for gliadin synthesis with temperature fluctuations (Blumenthal et al., 1990). Malik et al. (2011), in an experiment carried out in Sweden, instead demonstrated that high temperatures during ripening resulted in a higher content of total polymeric proteins (% UPP) than the monomeric ones, which are associated with a longer period of grain maturation. Hurkman et al. (2013) investigated changes in the gluten composition under moderate and high temperatures in a greenhouse experiment. The authors showed an increase in the proportions of low S-proteins (ω-gliadins) and low to-medium S-proteins (HMW-gs and α-gliadins) and a decrease in the high S-proteins (LMW-gs and γ-gliadins). Such an alteration of the gluten protein composition is determined by a shorter grain filling period and a premature leaf senescence, as a result of high temperatures.

Our study, which was carried out maintaining the same wheat cv nd crop technique and in the same field over a 3-year period, made it possible to analyze the significant effect of the meteorological trend on the composition of gluten proteins and the relative qualitative parameters of dough. This result, thus, opens the door to an important range of effects generated by the interaction between genetic effects (G) and their growth conditions (growth environment = E) (G×E effect) (Johansson et al., 2013).

Conclusions

The continuous search for the optimal conditions to maximize grain yield and obtain satisfactory bread-making properties in wheat makes the study of the role of agronomic practices in a specific environment of fundamental importance.

In this study, a late N rate applied at heading clearly increased the GPC and all the gluten protein fractions, increased W and reduced P/L, without

affecting the grain yield. On the other hand, the fungicide treatment significantly increased grain yield, resulting in an indirect and slightly lower GPC. Both these agronomic practices, which are the most up-to-date agronomic strategies applied before harvesting, had an impact on the protein content in the grain, but when applied on their own did not result in a significant impact on the gluten composition, although the interaction of these two factors at heading led to a higher content in glutenins, thus increasing the gs/glia ratio.

Overall, the greatest influence on the rheological parameter was that of the year. The growing season, which has a direct effect on the plant physiology during ripening, but also indirectly by influencing the N availability in the soil, significantly influenced the GPC, but also resulted in a marked impact on the alveographic parameters as a result of the change in the gluten fraction. Although late fertilization and fungicide application at heading can influence the protein content and the grain yield to a great extent, small meteorological variations during grain filling in a production situation without any overt stress have also demonstrated important variations in the accumulation of gluten protein fractions, thereby highlighting a clear influence on the rheological traits. These data underline that the rheological traits of wheat flour are influenced by genetic, agronomic and environmental factors, and it is therefore necessary to consider the whole cropping system as well as the pedo-climatic conditions to achieve wheat with specific end-use features.

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Chapter IV – "The effect of nitrogen fertilization on the expression of protein in wheat and tritordeum varieties using a proteomic approach"

Landolfi, Viola, Giovanni D'Auria, Maria Adalgisa Nicolai, Chiara Nitride, Massimo Blandino, and Pasquale Ferranti. 2021. "The Effect of Nitrogen Fertilization on the Expression of Protein in Wheat and Tritordeum Varieties Using a Proteomic Approach." Food Research International 148 (October): 110617. https://doi.org/10.1016/j.foodres.2021.110617.

Abstract

Wheat, an essential ingredient for several bakery preparations, is also responsible for gluten-related diseases in sensitive subjects. The effect of the N fertilization rate (80 vs 160 kg N ha^{-1}) on gluten protein expression profile has been evaluated considering two soft wheats (landrace and modern) and one tritordeum cultivar (*cv*), grown in the same experimental field in North Italy. The proteins of refined flour were characterized through advanced proteomic approaches, including chromatography (RP-HPLC) and electrophoresis. A static model system was used to simulate i*n vitro* digestion and the digestome peptides were examined by mass spectrometry and *in silico* approaches, to investigate the celiac and allergenic sequences. The CD-toxic epitopes in the digested samples were quantified by means of a R5 ELISA assay. The N fertilization rate increased the grain protein content, but it did not lead to any difference in gluten composition, with exception of glu/glia ratio in the modern wheat *cv*. Moreover, the gluten composition and the occurrence of toxic/allergenic epitopes varied to a great extent, according mostly to the genotype. A lower immunoreactivity, determined using R5 ELISA, was

detected for the digested tritordeum flours than for the landrace (-51%) or modern (-58%) cvs, while no significant difference was observed for the N rates between each genotype. *In silico* analysis showed that tritordeum has fewer CD epitopes belonging to the ω-gliadins and a lower LMW-GS than the landrace or modern *cv*. Tritordeum presented fewer α-gliadin allergenic epitopes than the modern wheat *cv*. The lower frequency of celiac epitopes in tritordeum, compared to the old and the modern wheat, is probably due to the absence of a D genome.

Keywords: Soft wheat, Tritordeum, N rate, Gliadins, Glutenins, Toxic epitopes, Allergens.

Abbreviations: cvs, cultivars; glu, glutenins; glia, gliadins; HMW-GS, high molecular weight glutenins; LMW-GS, low molecular weight glutenins; GS, glutenin subunits; GPC, grain protein content; CD, celiac disease; NCGS, Non-Celiac Gluten Sensitivity; CPVO, Community Plant Variety Office; TW, test weight; TKW, thousand kernel weight; NIR, Near Infrared Reflectance; ACN, acetonitrile; 1-PrOH, propan-1-ol; EtOH, ethanol; AmBic, ammonium bicarbonate; SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TCA, trichloroacetic acid; DTT, dithiothreitol; IAA, iodoacetamide; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl) aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; TAME, p-toluenesulfonyl-L- arginine methyl ester; TPI, total protein isolates; 2-DE, 2-sulfanylethanol, two-dimensional electrophoresis; IPG, immobilized pH gradient; IEF, Isoelectrofic focusing; LIT, nano-ESI-linear ion trap; GID, gastrointestinal digested; Gln, glutamine; MW, molecular weight; OGD, oral, gastric and duodenal phases; WDEIA, wheat- dependent exercise-induced anaphylaxis.

Introduction

The quality of soft wheat (*Triticum aestivum* L. subsp. *aestivum*) flour influences the organoleptic and structural properties of the final prod- ucts to a great extent. Modern hexaploid wheats (AABBDD) are products of breeding processes that were aimed at producing cultivars (cvs) with an improved bread-making potential, a more balanced glutenin/gliadin (glu/glia) ratio and a higher high molecular weight glutenin (HMW-GS) / low molecular weight glutenin (LMW-GS) ratio (Dhaka and Khatkar, 2015) than wheat landraces (AABBDD) (local ecotype) or old wheat varieties, which have been cultivated since before the second half of the XX◦ century (Migliorini et al., 2016). A new cereal, tritordeum (x *Tritordeum martinii* A. Pujadas, nothosp. nov.) (AABBHchHch), has recently received a great deal of scientific and commercial attention (Ribeiro et al., 2016; Vaquero et al., 2018). This amphiploid species, which is derived from the crossing of a South American wild barley (*Hordeum chilense* Roem. et Schultz.) and a durum wheat (*Triticum tur- gidum* L. subsp. *durum* Desf.), has been described as a good bread-making flour, with similar performances to those of soft wheat, and it has found its way into the baking industry (Martıń et al., 1999).

Gluten, from a food technology perspective, is the main player in bread making, as it represents around 60% of the wheat proteome (Fiedler et al., 2014). Gluten can be subdivided, according to the Osborne classification, into 2 main fractions with the following sub- fractions: water-soluble non-prolamins, albumins and globulins; and prolamins, alcohol-soluble gliadins and polymeric alcohol-insoluble glutenins (Mamone et al., 2000). Monomeric gliadins are classified, on the basis of their electrophoretic mobility, into 3 fractions: α-, γ- and ω-gliadins. The polymeric glutenin subunits (GS) cover a wide range of molecular weights3 and are conventionally classified as LMW-GS (12–60 kDa) or HMW-GS (up to 120 kDa) (Mamone et al., 2000). The rheo- logical

properties of the flour depend on the quality and the quantity of the gluten and in particular on the glu/glia and HMW-GS/LMW-GS ratios. A large amount of gliadins increases the viscosity of a dough, thereby influencing its extensibility, while a greater quantity of glutenins enhances the strength of the dough, thereby influencing its elasticity (Plessis et al., 2013). Gluten strength is defined by considering the composition of the gluten proteins, the quantity of the HMW glutenin subunits and the glu/glia ratio (Johansson et al., 2001).

The meteorological and soil conditions, the type of cv and the different nitrogen rates are all factors that qualitatively and quantitatively modulate the flour protein profile (Godfrey et al., 2010). The environmental conditions can influence the grain protein content (GPC) to a greater extent than the cv (Wan et al., 2013). Moreover, N fertilization is a crop technique that is expected to quantitatively change the GPC and the rheological properties of the derived flour (Garrido-Lestache et al., 2004). The mapping of gluten expressed proteins is fundamental to assess the technological quality of different cereal species and cvs (Cho et al., 2018). Gluten proteins can cause inflammation of the small intestine in subjects affected by celiac disease (CD), which is a common autoimmune enteropathy of the small intestine, whose prevalence has risen in the last 50 years and today affects about 1% of Western populations (Lebwohl et al., 2015). The pathogenesis of CD begins with the gluten digestion derived peptides, which are transported throughout the microvilli and trigger an immune response of the T-cells (Dunne et al., 2020). CD disease has multifactorial etiologies, including the interaction of genetic factors in susceptible individuals (HLA-DQ2 or -DQ8 haplotype), environmental components and immunological mechanisms (Kagnoff, 2007). Non-Celiac Gluten Sensitivity (NCGS) is a “new” condition that has emerged in recent years, and it affects up to 6% of the world’s population (Casella et al., 2018). Its diagnosis is a challenging task as it requires the clinical exclusion of CD and wheat allergies, and the symptoms are hard

to distinguish from those of the irritable bowel syndrome (Casella et al., 2018). The triggering causes are not clear and require further investigation. Tritordeum bread was recently found to be tolerated by subjects with non-coeliac wheat sensitivity (NCWS) to a similar extent to gluten free bread (Sànchez-Leòn et al., 2021). Extensive characterization studies, including proteomics and immunobased ap- proaches, aimed at identifying the epitopes and toxic motifs responsible for triggering allergic and toxic reactions, are necessary. An extensive collection of known epitopes is available in the ProPepperTM database (Juhász et al., 2015). A deep understanding of the role of the environmental and agro- nomical conditions, specifically the choice of cv and N fertilization, on the gluten composition and expression of specific epitopes, could pro- vide useful technological information for bread making. In this respect, the type of selected wheat cv can also make a great difference in the consumers' acceptance of bakery products. In this paper, we present a proteomic characterization of three types of cereal grains: landrace wheat, modern wheat and tritordeum. Proteins isolated from grain flours were profiled using advanced proteomic approaches, including chromatography and electrophoresis. The flours were digested using an in vitro batch model to simulate the oral, gastric and duodenal com- partments of humans. Mass spectrometry was used to create a picture of the obtained digestome. The collected peptide sequences were analyzed *in silico* for the presence of toxic and allergenic sequences. Additionally, a competitive ELISA analysis was performed on the digestome to evaluate any diversification of the immunoresponse related to fertilization and to the genotype.

Materials and methods

Experimental site and treatments

The effect of the N fertilization rate was evaluated by means of a full factorial experimental design, considering 2 soft wheats and 1 tritordeum *cvs*, characterized by different gluten compositions. Two N fertilization treatments were compared: a low N rate (80 kg N ha^{-1}, N80) and a high N rate (160 kg N ha^{-1}, N160). The total N rate for each treatment was top-dressed applied as a granular ammonium nitrate fertilizer, split equally between tillering (growth stage 23) and the beginning of stem elongation (growth stage 32).

The considered genotypes were:

- Landrace, *cv* Andriolo, which was mainly cultivated in mountain areas in the Tuscany region (Italy) in the XIX◦ century, and is a soft wheat *cv* (hexaploid AABBDD) (Migliorini et al., 2016);
- Modern, *cv* Bologna (S.I.S., San Lazzaro di Savena, Bologna, Italy; genealogy (H89092 X H89136) X Soissons), is a soft wheat (hexa- ploid AABBDD) *cv*, classified as improver wheat according to the Synthetic Index of Quality (Foca et al., 2007), which was registered in the Italian varietal list in 2002 (https://www.sian. it/mivmPubb/listeVarieta.do);
- Tritordeum, *cv* Bulel (Agrasys S.L., Barcelona, Spain), which was registered in the CPVO List (Community Plant Variety Office) in 2011 (hexaploid AABBHchHch).

A field study was carried out in the Po plain at Carmagnola in North West Italy (44◦ 50′ N, 7◦ 40′ E; elevation 245 m), in the 2016–17 growing season. The experiment was performed on the University of Turin experimental farm in a deep silty-loam soil (Typic Udifluvents), characterized by a medium cation-exchange capacity and organic matter content. The same agronomic techniques were adopted for all the *cvs*. Briefly, the previous crop was soft wheat, and planting was performed in 12 cm wide rows at a seeding rate of 400 seeds/m^2 on 27 October 2016, following an autumn plowing (30 cm) and disk harrowing to prepare a proper seedbed. The weed control was in accordance with the agronomic management

practices usually carried out in the North of Italy for the cultivation of soft wheat, while no fungicide or insecticide was applied. Harvesting was carried out with a combine-harvester on 13 July 2017. The N rates (in three replicates) were assigned to experimental units, using a completely randomized block design. The plot size was 7 ×1.5m.

Grain yield and kernel quality traits

The grain yields were obtained by harvesting the whole plot with a Walter Wintersteiger cereal plot combine-harvester. Grain moisture was analyzed using a Dickey-John GAC2100 grain analyzer (Auburn, IL, The USA). The grain yield results were adjusted to a 13% moisture content. Three kg grain samples, which were milled (Bona 4RB mill) to obtain refined flour, were obtained from each plot.

The test weight (TW), thousand kernel weight (TKW) and grain protein content (GPC; N × 5.7, on a dry matter basis) were determined according to Blandino et al. (2015). GPC was determined on whole grains (1-mm-sieve Cyclotec mill), by means of NIR (Near Infrared Reflectance) spectroscopy, according to AACC 39–10 (AACC, 2000).

Materials and chemical reagents

All the reagents used in this study were of analytical or higher grade. The HPLC-grade solvents: water, acetonitrile (ACN), propan-1-ol (1- PrOH) and ethanol (EtOH) were from Carlo Erba (Milan, Italy). Sodium phosphate, ammonium bicarbonate (AmBic), acetic acid and the other chemicals, used to produce the simulated salivary fluid (SSF), the simulated gastric fluid (SGF) and the simulated intestinal fluid (SIF), were also provided by Carlo Erba. The enzymes used for in vitro human digestion were purchased from Sigma (St Louis, MO, USA), in line with those indicated by the Infogest protocol (Brodkorb et al., 2019). Tri-chloroacetic acid (TCA), dithiothreitol (DTT), iodoacetamide (IAA), sodium

dodecyl sulfate (SDS), glycerol, tris(hydroxymethyl) amino- methane hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), guanidine chloride, trifluoroacetic acid (TFA), 2-vinylpyridine monomer and *p*-toluenesulfonyl-L-arginine methyl ester (TAME) were purchased from Sigma-Aldrich (St Louis, MO, USA). Egg lecithin was purchased from Lipid Products (Redhill, UK). The electrophoresis re- agents were all from Bio-Rad (Milan, Italy).

Protein extraction

Production of the total protein isolate

The total protein isolate (TPI) of the wheat samples was produced according to the procedure of (Dupont et al., 2011). An aliquot of 1 ml of SDS buffer (2% SDS, 10% glycerol, 50 mM DTT, 40 mM Tris-HCl, pH 6.8) was added to the flour samples (50 mg), which were then incubated at room temperature with discontinuous mixing for 1 h. The obtained pellets were removed by centrifugation at 7,900 g for 10 min (Micro-centrifuge Multispin 12, Steroglass, Perugia, Italy). The proteins in the supernatant were precipitated with 4 vol cold (-20 ◦C) propan-2-one, following incubation overnight at – 20 ◦C and centrifugation at 7,900 g for 10 min. The pellets were dried under a nitrogen stream and stored at − 20 ◦C.

Selective extraction of the gliadins and glutenins

The protein fractions were extracted according to Mamone et al. (2000), with slight modifications. The albumins and globulins were removed from non-defatted flour (1 g) by adding a solution (10 ml) containing 100 mM KCl, 50 mM Tris-HCl pH 7.8 and 5 mM EDTA, and centrifuging for 15 min at 3,433 g. After removal of the supernatant, the gliadins were extracted with 10 ml of 70% (v/v) ethanol (twice) and the glutenins with 50% v/v 1-PrOH; 50 mM Tris-HCl (pH 8.5); 1% (w/v) DTT. Glutenin extraction was performed at 60 ◦C for 30 min. The solutions were mixed for 2 min and stirred on a magnetic plate for 10 min. The cysteine residues of the

glutenin extracts were pyridylethylated (VP glutenins) at 60 °C for 15 min with 2-vinylpyridine.

HPLC analysis

Reversed phase (RP)-HPLC analysis of the gliadins and glutenins was carried out in an HPLC chromatograph (HP 1100 Agilent, Palo Alto, CA, USA) modular system equipped with a diode array detector. The column effluent was monitored, by means of UV detection, at λ = 220 and 280 nm. The separation was performed using a C8 Vydac, 208TP52, 2.1 × 250 mm, 5 µm column (Hesperia, CA, The USA). Solvent A was 0.1% TFA v/v in water, while solvent B was 0.1% TFA in ACN. After 5 min of isocratic elution, using 25% solvent B (0.1% TFA in ACN, v/v), a 25–60% gradient ramp was applied for 60 min at a flow rate of 0.200 ml/min. This procedure was performed according to the work of Mamone et al. (2000) with some modifications. The peaks were then integrated using Agilent ChemStation software to obtain the relative quantity of both the gliadins and glutenins in each sample.

SDS–PAGE of the gliadins and glutenins

The glutenins and gliadins obtained from the selective extraction (section 2.4.2.), were precipitated in cold propan-2-one (1:4 v:v), sus- pended in an SDS-PAGE Laemmli Buffer [0.125 M Tris–HCl pH 6.8, 5% SDS, 20% glycerol, 5% (w/v) 2-sulfanylethanol, 0.02% bromophenol blue and boiled in a water bath for 5 min. The proteins (10 mg/ml) were loaded onto a 12% polyacrylamide gel in a final volume of 3 µL. Analysis was carried out at room temperature and constant voltage (100 V). After migration, the proteins were fixed overnight with TCA (24%) and stained with Coomassie G-250. The electrophoretic profiles were analyzed using GelAnalyzer 19.1 software (www.gelanalyzer.com by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD, CSc).

Two-dimensional electrophoresis analysis of the total protein isolate (TPI)

The TPI was dissolved (3 mg/ml) in an immobilized pH gradient (IPG) strip rehydration buffer, containing 8 M urea, 2% (w/v) CHAPS, 2% (v/v) Pharmalytes pH 3.0–10.0, 20 mM DTT and traces of bromo- phenol blue, for two-dimensional electrophoresis (2-DE). The isoelectric focalisation was performed using pH 3–10 Immobiline Dry Strips (11 cm) linear gradient, from Bio-Rad Laboratories. The strips were rehy- drated overnight in an Immobiline Dry-Strip Reswelling Tray (Amer- sham Pharmacia). Isoelectrofic focusing (IEF) was carried out using the Multiphor II system (Pharmacia Biotech, Uppsala, Sweden). The proteins were focused on up to 15,000 Vh at a maximum voltage of 6000 V at 20 ◦C. After focusing, the proteins were reduced for 15 min in an equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 2% DTT), and alkylated for 15 min with 2.5% IAA. SDS-PAGE was performed in the second dimension on a 10% polyacrylamide gel, using a Protean II system (BioRad Laboratories, Hercules, California, USA). The run was performed at a constant voltage of 220 V. After migration, the proteins were fixed overnight with TCA (24%) and stained with Coomassie G- 250.

In batch gastroduodenal digestion of flour

Flour samples were subjected to in vitro simulated human digestion, using the static model system optimized in the framework of the Infogest COST Action project (Brodkorb et al., 2019). Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized conditions (Brodkorb et al., 2019). Mastication was simulated using a manual mincer. One g of flour was mixed with 1 ml of SSF and minced for 2 min at 37 ◦C. The SSF included 1500 U ml^{-1} human salivary amylase. SGF was added to the bolus (50:50

v/v); a concentration of up to a 0.17 mmol/L of gastric liposome (egg lecithin prepared in a vesicular form) and 2000 U/ml of SGF porcine pepsin was added to this mixture. The pH of the digesta was adjusted to 2.7 and the incubation was carried out for 2 h at 37 ◦C under magnetic stirring. After adding SIF (50:50 v/v) to the gastric digesta, the pH was adjusted to 7.0, using NaOH 1 M. Ten mmol/L of bile salts (5 mmol/L sodium taurocholate, 5 mmol/L sodium glycodeoxycholate and 1.8 mmol/L egg lecithin in the final concentration), and pancreatin were added to the intestinal mixture. The amount of pancreatin was calcu- lated on the basis of the measured trypsin activity, and a final volume of up to 100 U of trypsin/ml was added. Trypsin activity was determined by means of a TAME assay (Brodkorb et al., 2019). Each sample was incubated for 2 h at 37 ◦C under magnetic stirring. The enzymatic ac- tivity was stopped by boiling the samples for 5 min in a water bath. The samples were then centrifuged at 3,433 g for 15 min to collect the su- pernatants, which were then stored at − 20 ◦C until use. The digestion was monitored by means of SDS-PAGE analysis. A part of the superna- tant of each sample (1 ml) was desalted using a Phenomenex (Torrance, CA, USA) Strata-X SPE cartridge following the manufacturer instruction and then concentrated with gaseous nitrogen before the LC-MS/MS analysis was performed.

R5 competitive ELISA assay on the digested samples

RIDASCREEN® Gliadin competitive (Art. No. R7021, R-BIOPHARM AG, Darmstadt, Germany) was used according to the manufacturer's instructions. This Competitive ELISA relies on the use of the R5 mono- clonal antibody (Di Stasio et al., 2020). The gastroduodenal digestion products were analyzed in duplicate.

LC-MS/MS analysis

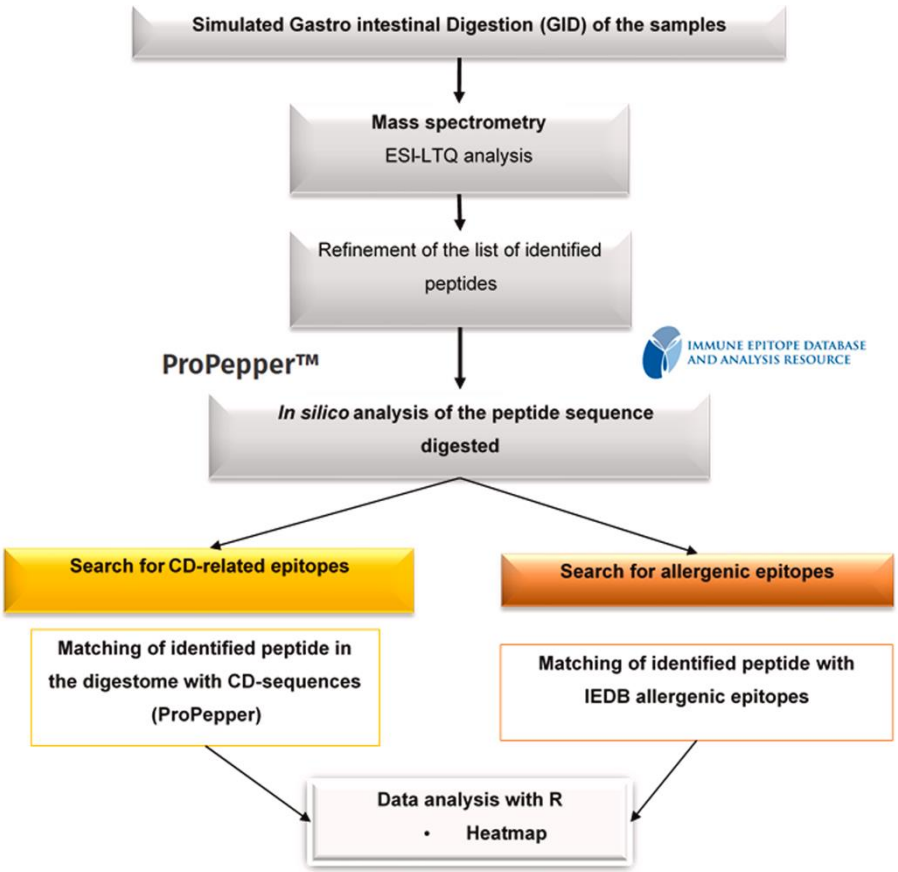
LC-MS analysis was performed using a Dionex UltiMate 3000 nano-UHPLC system coupled with a nano-ESI-linear ion trap (LIT) Thermo XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, The USA). Samples were resuspended in a 0.1% (v/v) formic acid solution, loaded through a 5 mm long, 300 µm id pre-column (LC Packings, The USA) and separated in an AcclaimTM PepMapTM C18 column (150 mm × 75 µm, 3 µm) at a flow rate of 0.200 µL/min. Eluent A was 0.1% formic acid (v/v) in Milli-Q water; eluent B was 0.1% formic acid (v/v) in ACN. The column was equilibrated at 5% B. Peptides were separated by applying a 5–40% gradient of B over 40 min. MS data were obtained over the 200 to 2000 *m*/*z* mass range. Data-dependent MS/MS spectra were collected from the five most abundant precursor ions upon frag- mentation (charge state ≥ 2; isolated width: 2 Da; min. signal required: 500), using CID activation with 35.0% normalized collision energy, an activation Q of 0.25, and an activation time of 30 ms. The spectra were processed using Xcalibur Software, 3.1 version (Thermo Scientific). The mass spectra were then analyzed using Protein Prospector software. The GluPro v 1.2 database of wheat gluten protein sequences (Daly et al., 2020) was used as background database for the analysis of the mass spectrometry data. The database searching parameters used for the identification of the peptidomes of the simulated gastrointestinal digested (GID) flour included “no enzyme” specification, and Met-oxidation and pyroglutamic acid for N-terminus glutamine (Gln) as variable protein modifications, with a mass tolerance value of 1 Da for the precursor ion and 0.6 Da for the MS/MS fragments. Analyses were carried out in triplicate.

Searching criteria for the celiac disease related epitopes, allergic epitopes and biopeptides

The identified peptides were searched manually for the presence of described toxic (CD epitopes), allergenic (IgE-binding epitopes) and

bioactive amino acid sequences. The applied bioinformatic pipeline is described in Figure 1.

Figure 1. Flow chart of the *in silico* analysis after in vitro digestion and Mass spectrometry analysis (ESI-LTQ). The identification of the CD and allergen epitopes obtained thanks to the already indexed ones. *Peptides were defined unique within the given database. Uniqueness was assessed using Skyline software and matching the list of identified peptides with the reference database as background in the “import peptide list” window.



The Propepper database (Juhász et al., 2015) and the CD epitope table presented in Mamone et al. (2011) were used as the sources of the CD-

toxic motifs. Only toxic motifs that appeared in at least 3 identified peptides were selected for comparison.

The IgE binding sequences were retrieved from the Immune Epitope Database (IEDB) (Vita et al., 2019), a repository of peer-reviewed epitopic sequences that have shown proved ability to trigger immune responses. The following search parameters were applied to extract IgE epitopes from the database: substring, allergy disease, and Triticum: ID 4564. RStudio® (64-Bit-R-3.6.3) was used to create a heatmap to represent the density of the verified epitopes per cv/N rate. The use of grayscale heatmaps permitted the distribution of the analyzed samples of the CD and IgE-binding epitopes to be visualized.

The bioactive peptides were retrieved from a literature review (Babini et al., 2017; Liu & Udenigwe, 2019; Suetsuna & Chen, 2002).

Statistical analysis

The agronomic parameters (grain yield, TW, TKW), the GPC, the glu/ glia and HMW/LMW-GS ratios and the percentage of single gliadin and glutenin fractions were compared by means of an analysis of variance (ANOVA), in which the combination of genotypes and N fertilization were the independent variables. Multiple comparison tests were per- formed, according to the Ryan-Einot-Gabriel-Welsh F (REGW-F) test, on the treatment means. Statistical data analysis was carried out with the SPSS software package, version 26.0.

Result and discussion

Agronomical and productive parameters

Due to the good soil fertility of the site where the experiment was carried out, the N rate did not result in any significant increase in grain yield, TW or TKW for any of the compared *cvs* (Table 1). Clear differences were

observed in the productive parameters and grain traits of the compared *cvs*. The grain yield of modern *cv* was 57% and 33% higher than that of landrace and tritordeum, respectively. Modern wheat showed the highest test weight value and the lowest TKW, the opposite of the landrace *cv,* which showed the highest TKW and lower values of TW, as a consequence of strong lodging under both N fertilizations.

Protein content

Although in the considered productive conditions, N fertilization did not have a significant impact on the grain yield, the GPC was clearly influenced by the combination of *cv* and the N application (Table 1).

Table 1. Effect of the cultivar and N rate on the grain yield, test weight (TW), thousand kernel weight (TKW) and grain protein content (GPC).

Cultivar	N rate (kg N/ha)	Grain yield t ha^{-1}	TW kg hl^{-1}	TKW g	GPC %
landrace	N80	2.9 c	73.8 b	45.0 a	17.1 b
	N160	3.0 c	74.5 b	45.6 a	18.2 a
modern	N80	6.8 a	77.3 a	33.0 c	14.5 d
	N160	7.0 a	76.2 a	31.9 c	15.2 cd
tritordeum	N80	4.8 b	70.3 c	36.8 b	15.8 c
	N160	4.2 b	69.4 c	36.1 b	17.3 b
p-value		< 0.001	< 0.001	< 0.001	< 0.001

Means followed by different letters are significantly different (the level of significance, *p*-value, is reported in the table), according to the REGW-F test.

On average, the highest GPC was recorded for landrace (17.7%) and tritordeum (16.6%), followed by the modern wheat *cv* (14.9%). A significant increase in the protein content was recorded in the landrace (+1.1%) and tritordeum (+1.5%) *cv* kernels for with a double N rate. The

GPC increased in N160 for the modern wheat, albeit not signifi- cantly (+0.7%). This increase confirms previous results in literature (Garrido-Lestache et al., 2004; Godfrey et al., 2010; Johansson et al., 2001). To better understand the nutritional value of tritordeum, it will be interesting in future works to verify the increase in essential amino acids related to the N fertilization (Zhang et al., 2016).

Proteomic analysis of the whole grain protein and gluten fractions

DE of the whole grain proteins

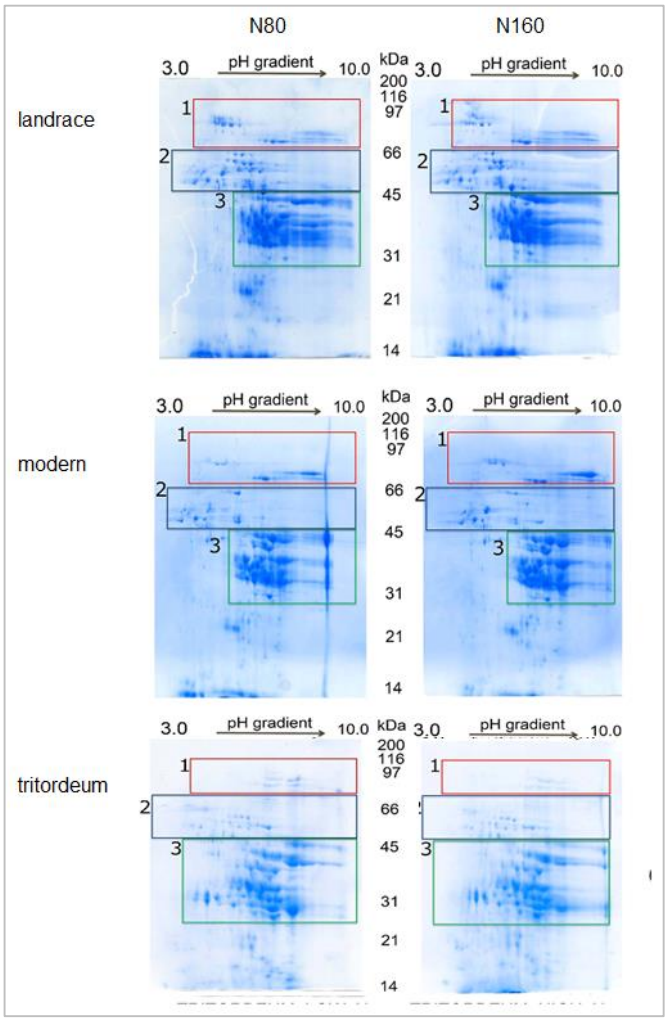
The 2-DE separation of crude extracted proteins for all the *cvs*/N rates allowed maps to be generated to show the typical migration patterns of the main cereal protein families (Figure 2). Distinctive spots were observed for each subfraction with corresponding molecular weight (MW) ranges of 80–120 kDa for HMW-GS, 60–68 kDa for the ω5-glia- dins, 43–60 kDa for the ω1,2-gliadins and 32–45 kDa for the α- and γ–gliadins and LMW-GS (Mamone et al., 2005).

The analysis revealed differences in the protein expression across the considered *cvs* but did not show macroscopic qualitative differences that could be attributed to the N rates.

Tritordeum flour appeared to have the proteome with the largest qualitative differences in the presence of protein spots in the acid region around 31 kDa, corresponding to the LMW glutenins and the gliadin region (α, γ).

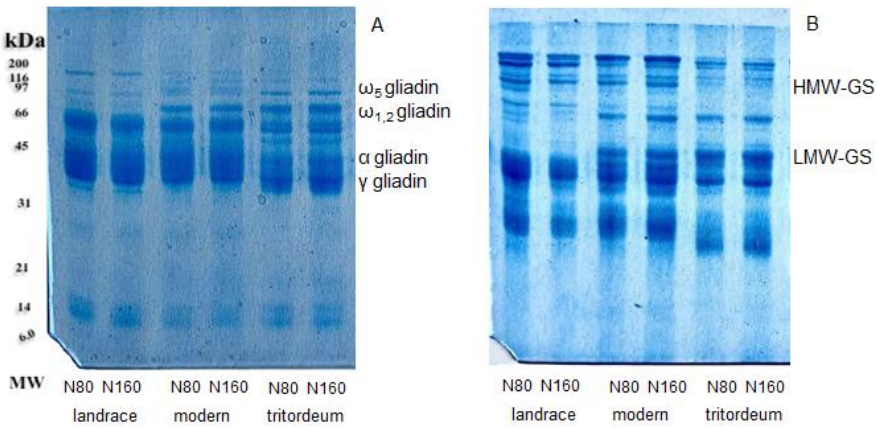
These differences were also clear in the SDS-PAGE profile of the isolated gliadin fraction (Figure 3) and can be attributed to the nature of the tritordeum cv, which is an amphiploid of T. turgidum and H. chilense (Alvarez et al., 1995, 1999; Sillero et al., 1999).

Figure 2. Two-dimensional electrophoresis analysis of the extracted flour proteins in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha^{-1}) and N160 (160 kg N ha^{-1}) fertilization rates.



The protein patterns, as shown in the regions between 20 and 200 kDa, are underlined by the red, blue and green colored rectangular areas, in which spots of the same fractions are indicated for HMW-GS, ω5, ω1,2 gliadins and LMW-GS, α-gliadin and γ-gliadin, respectively.

Figure 3. SDS-PAGE analysis of the gliadins (A) and glutenins (B) of the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha^{-1}) and N160 (160 kg N ha^{-1}) fertilization.



SDS-PAGE and analysis of the Osborn fractions

The gliadin and glutenin Osborne extracts were separated by means of SDS-PAGE. The glutenins (Figure 3_B) showed the typical separation of the HMW-GS (80–140 kDa) and LMW-GS (10–70 kDa) families. The glutenin expression in the analyzed *cvs* clearly differed. The gel analysis highlighted 8 bands in the landrace samples, while only 6 bands were observed in the modern wheat and tritordeum. Unlike the modern and landrace samples, tritordeum presented a less complex profile in the HMW glutenin region. This observation is in line with the calculation of HMW/LMW ratios retrieved from the HPLC profile (Table 2). The tritordeum gliadins showed a more complex profile, which results in a higher number of bands than the modern and landrace *cvs* (Figure 3_A). Interestingly no qualitative diversification, as induced by the N effect, was observed among the samples.

Table 2. Effect of the cultivar and N rate on the glutenin/gliadin (glu/glia) and HMW/LMW-GS ratios and the percentage of the single fractions of gliadin and glutenin, as obtained from the HPLC-MS analysis.

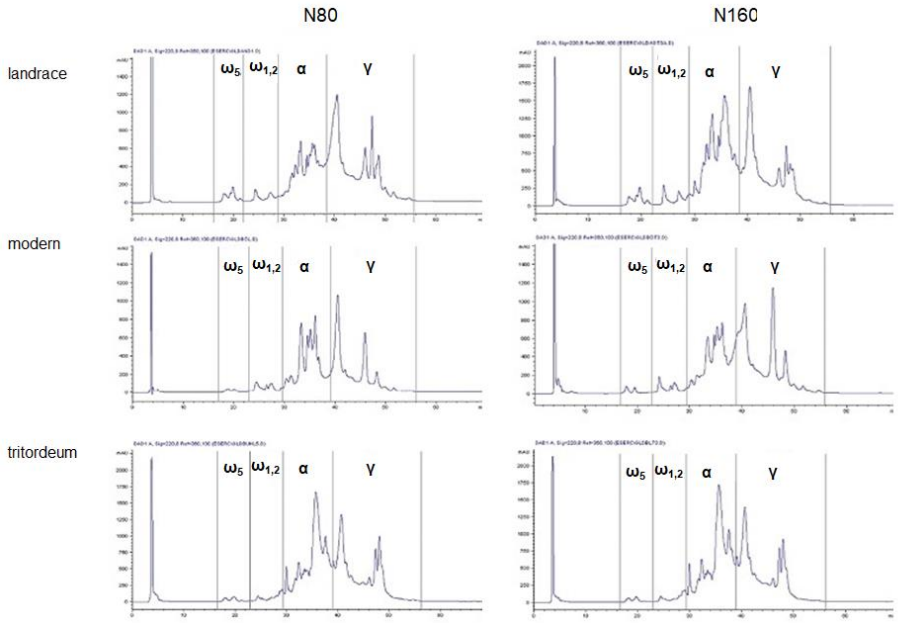
Cultivars	N rate (kg N/ha)	glu/ glia	HMW/ LMW-GS	Gliadins				Glutenins	
				ω5	ω1,2	α	γ	HMW-GS	LMW-GS
landrace	N80	0.84 b	0.70 a	2.8 a	5.8 a	43.9 a	47.5 a	41.2 a	58.8 b
	N160	0.88 b	0.83 a	2.7 a	4.2 a	45.4 a	47.7 a	45.5 a	54.5 b
modern	N80	0.89 b	0.79 a	1.4 bc	5.8 a	44.7 a	48.1 a	44.1 a	55.9 b
	N160	1.16 a	0.82 a	2.1 ab	5.5 a	42.3 a	50.1 a	45.0 a	55.0 b
tritordeum	N80	0.96 b	0.29 b	1.1 c	4.3 a	53.4 a	41.2 a	22.6 b	77.4 a
	N160	0.99 b	0.30 b	0.9 c	5.5 a	52.3 a	41.3 a	22.9 b	77.1 a
***p*-value**		0.001	< 0.001	0.001	0.251	0.119	0.063	<0.001	0.002

Means followed by different letters are significantly different (the level of significance, p-value, is reported in the table), according to the REGW-F test.

HPLC analysis

The RP-HPLC gradient separation of the gliadin and glutenin frac- tions was optimized on a RP C8 column to achieve high resolution profiles. The chromatographic profile of the gliadins (Figure 4) showed the typical peaks attributed to ω5, ω1,2, α- and γ-sub fractions, and allowed the relative amounts of the expressed gliadin subfractions to be determined (Table 2) (Mamone et al., 2000).

Figure 4. HPLC-UV-DAD chromatograms of the gliadins from the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha-1) and N160 (160 kg N ha-1) fertilization rates.



In all the genotypes, the α- and γ-fractions had similar values and were more abundant than the ω-gliadins. Both *cv* and N rate factors did not influence the relative amount of α- and γ-gliadins fractions. Also Vaquero et al. (2018) reported a stable behaviour of these gliadin fractions across different tritordeum and wheat genotypes. Conversely, the ω5 fraction was significantly higher in the wheat landrace compared to the tritordeum, while the modern cv had an intermediate content. This evidence is interesting, considering the potential IgE-binding capacity associated with this gliadin fraction (Matsuo et al., 2004).

The same approach was used to analyze the glutenin isolated fraction, and the determined HMW/LMW-GS ratios for each cv/N rate are reported in Table 2. Unlike the gliadin fraction, the glutenin fraction in tritordeum differed significantly from the other two wheat *cvs* for both glutenin subunits, leading to a HMW/LMW-GS ratio significantly lower than both the landrace and modern wheat *cvs*.

As far as the gluten fraction composition, the N rate significantly influenced only the glu/glia ratio in the modern wheat *cv*. Otherwise, N

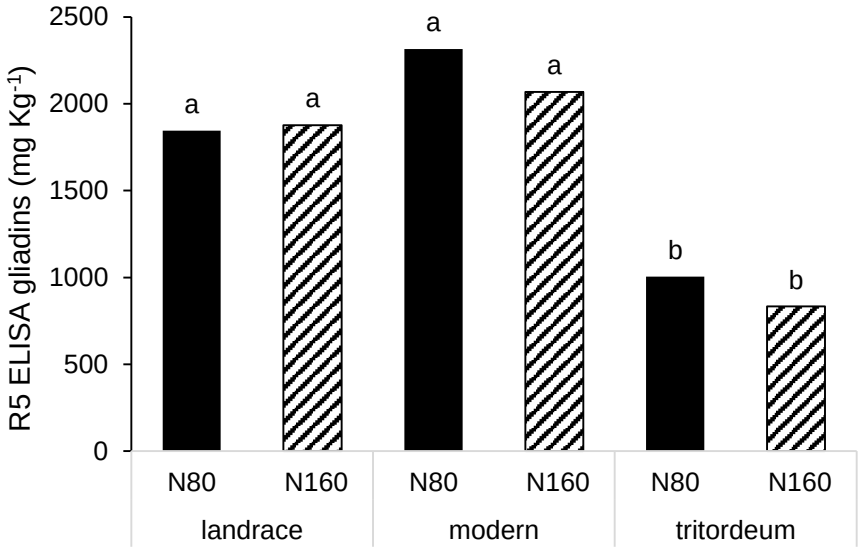
fertilization did not affect the prolamin ratio in landrace and tritordeum *cvs*, confirming results of Johansson et al. (2001). For these cvs, it is supposed that the higher N rate could have increase to a similar extent all the single individual storage protein components (Zhen et al., 2020), and they have equally contributed to the rise of GPC (Martre et al., 2003).

Residual immunoreactivity of the digested flours by means of ELISA

The immunoreactivity of the peptides generated during the gastro-duodenal digestion of flours was tested using a competitive enzyme-linked immunosorbent assay (Di Stasio et al., 2020). The assay uses a monoclonal antibody to target the “QQPFP“ repeated sequence found in prolamins. Digested tritordeum flour showed a lower immunoreactivity than that of the landrace (-50%) and modern (-58%) *cvs*. No significant differences were identified for the samples with different N rates for any of the *cvs*. The lower expression of the above-mentioned celiac-toxic epitopes in tritordeum, which could be attributed to the absence of genome D, is shown in Figure 5.

This evidence prompted us to conduct a more detailed structural analysis of the protein gastrointestinal digests of the flour by means of mass spectrometry.

Figure 5. Quantification of the “QQPFP” celiac toxic motif recognized by the R5 monoclonal antibody in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha^{-1}) and N160 (160 kg N ha^{-1}) fertilization rates.



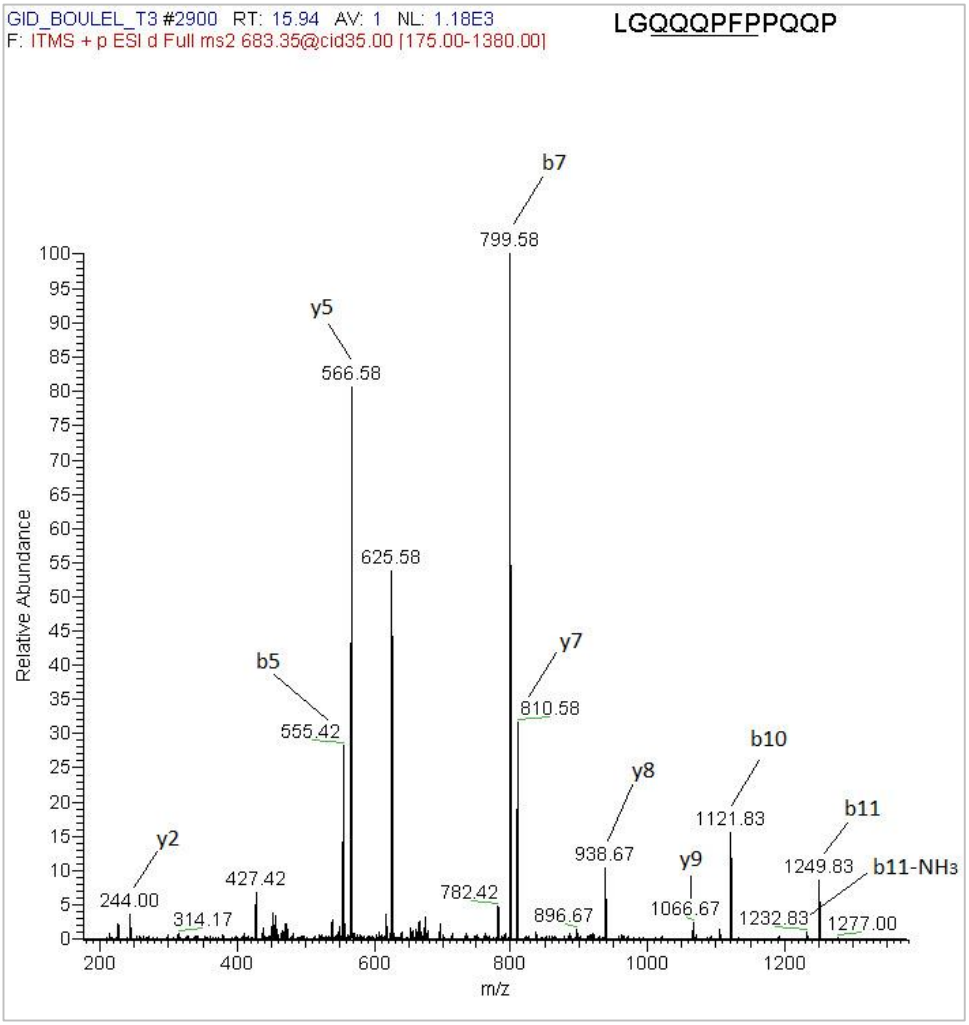
Bars with different letters are significantly different (*p*-value <0.05), according to the REGW-F test.

Mass spectrometry profiling of the gastrointestinal digested flours

In CD, the peptides derived from the digestion of food transmigrate through the intestinal epithelium. They are then deamidated in the subepithelial lamina propria by the tissue transglutaminase and pre- sented to the T-cells. An already validated static and multi-phasic in vitro digestion model (Brodkorb et al., 2019), comprising oral, gastric and duodenal sequentially simulated phases (OGD), was applied to collect sequence level information on the peptides resistant to OGD proteolysis. The mass spectrometry analysis allowed several flour derived peptides to be identified which indicated a resistance to diges- tive enzymes, as already described in literature (Mamone et al., 2015). The natural presence of protease and amylase inhibitors in the grain flours may have

contributed to the reduced digestibility. However, being pepsin the first proteolytic enzyme in the stomach environment where the pH is below 3.0, their inactivation is possible. The analysis of metabolic proteins has not been objective of the study and may require a dedicated investigation. The main peptides that were identified belonged to the digestive enzymes that were undergoing a natural and expected autolysis (Mamone et al., 2015). The grain derived identified peptides are listed in tables S1-6. The peptides were inferred to a single protein or to protein families when no unique peptide could be identified. We identified CD peptide sequences, derived from the γ-hordein and B1-hordein fractions, in the digestome of the tritordeum flour. Although the *p*-value of the identified peptides, which reflects the probability of finding a random peptide, was acceptable (*p*-value < 0.05), the high number of repeated regions and the high homology sequences, which are typical of wheat gluten proteins, led to high E-values, a parameter that describes the number of hits one can “expect” to see by chance when searching a database of a particular size. The E-value is obtained by multiplying the *p*-value by the total number of qualified peptides in the searched data- base, the masses of which fall into the precursor ion mass range, plus/ minus the specified tolerance (Alves & Yu, 2015). Since the LTQ mass spectrometer works at a low resolution, a tolerance of 1 Da was applied, and this caused the E value to be large. The peptides were validated manually, to confirm the software identifications, and exemplar spectra are provided in the supplementary material section (Figure 6).

Figure 6. Example of a chromatogram of the LGQQQPFPPQQP GID peptide of the tritordeum N160 sample (fertilization rate of 160 kg N ha^{-1}).



In silico evaluation of the in vitro gastrointestinal identified peptides

The digestion-derived peptides were *in silico* assessed to evaluate sequences known to potentially trigger CD, and allergic reactions. We also evaluated positive sequences with potential bioactivity.

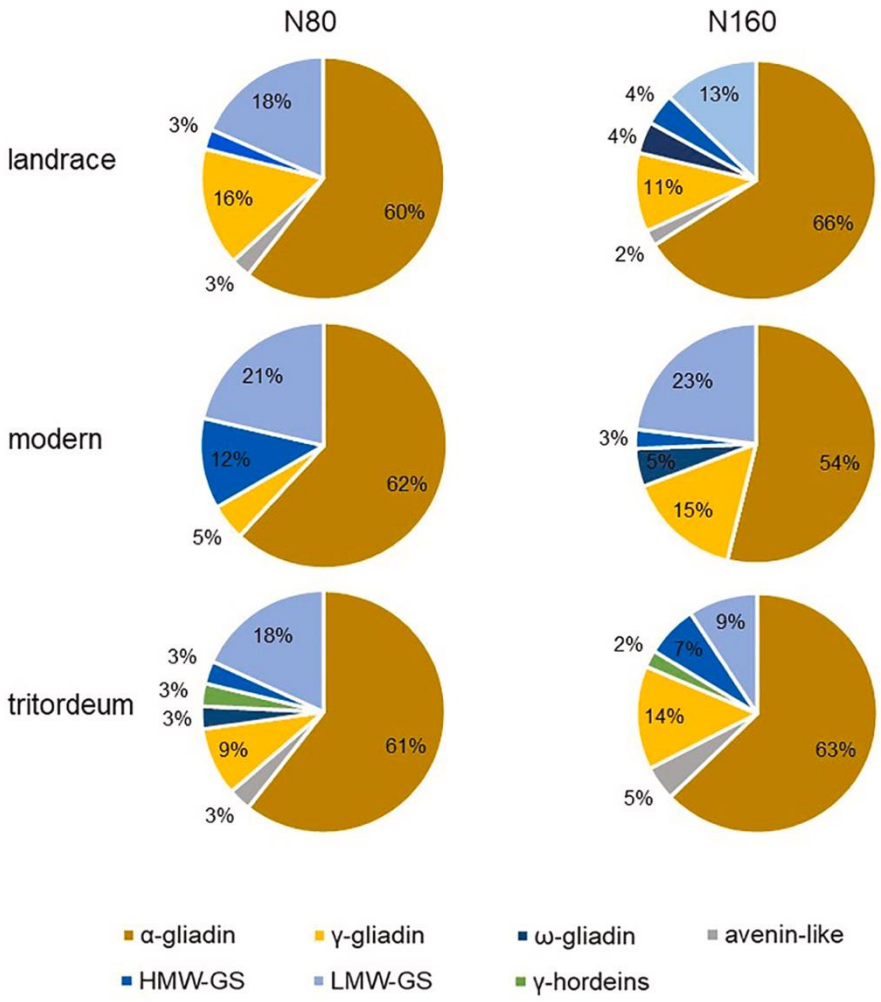
Celiac toxic motifs

The toxic motifs of CD predominantly arise from α-gliadins (Ozuna et al., 2015), which were found to be the most abundant gliadin fraction in our experiment (about 60%), followed by γ-type gliadins (Figure 7).

The same number of epitopes belonging to the α-gliadins was observed for modern and tritordeum, while landrace showed 15% more. Landrace also showed the highest numbers of identified epitopes for the γ-gliadins, followed by tritordeum (-18%) and then modern (-27%). Tritordeum showed 50% fewer CD epitopes belonging to the ω-gliadins and 23% and 44% lower epitope numbers of the LMW-GS class than the landrace and modern *cvs*.

The greatest number of CD epitopes was found in the α-gliadin class in all three varieties. On the basis of our observations, it can be seen that the most frequently identified toxic motifs (*i.e.* IPEQ, PQQLPQ, QPQQPF, QPQPFP and VRVPVPQL) are similar to those identified in previous studies and they were identified in all the studied samples (Figure 8) (Osman et al., 2000). For example, the presence of the CD epitope IPEQ was observed in all the digested flours, and it has already been described in the consensus sequences recognized by IgG human antibodies in a Pepscan experiment (Osman et al., 2000). Similarly, QQQPFP and PQQLPQ, which were also identified in all the flours, were recognized by human IgG and IgA antibodies. The QGSFQP sequence was only identified in landrace and modern at a high N rate and in both the tritordeum samples (Osman et al., 2000).

Figure 7. Gluten sources of the celiac epitopes in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha^{-1}) and N160 (160 kg N ha^{-1}) fertilization rates.



Truncated versions of the 33-mer (Glia-α 57–89, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) and the 25-mer (Glia-α 31–55, LGQQQPFPPQQPYPQPQPFPSQQPY) gliadin derived peptides, which have been described as being resistent to digestion, and are known to be strong stimulators of the T-cell response, were identified in all the analyzed samples (Table S7) (Ozuna et al., 2015).

Figure 8. Heatmap of the number of CD epitopes in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha^{-1}) and N160 (160 kg N ha^{-1}) fertilization rates

A matrix that presents the list of epitopes was obtained for each sample divided by gluten protein fractions (the asterisk marks the epitopic sequences present in all the samples).

The full length 33-mer peptide contains three repeated regions (p62–67, PQPQLPY) and is exclusively present in a D-genome encoded α-gliadin (Camarca et al., 2009). The D-genome in tritordeum is replaced by the Hch genome of *Hordeum chilense*. A single fragment of 33-mer (LQPFPQPQLPYPQPH) was unexpectedly identified in the tritordeum digested flour. Although the breeding program developed crosses between soft wheat and tritordeum in order to improve its yield and bread-making quality (Ávila et al., 2021), and a small introgression from D genome into tritordeum cannot be ruled out, our experiment was carried out using a tritordeum *cv* carefully selected for the absence of any soft wheat chromosomes. However, the *Hordeum chilense* genome has not yet been sequenced, and the natural presence of the peptide in the tritordeum proteome cannot therefore be excluded. Alternatively, a cross-contamination might also have occurred. A targeted approach would be required to confirm the presence of partial 33-*mer*-like sequences in tritordeum.

Allergenic epitopes

Wheat-dependent, exercise-induced anaphylaxis (WDEIA) is a serious allergy in which the combination of wheat ingestion and phys- ical exercise leads to anaphylaxis. Patients with WDEIA have IgE anti- bodies against ω5-gliadin, one of the major gluten allergens (Lehto et al., 2003). Together with the evaluation of the CD sequences, the presence of IgE-binding sequences was also evaluated (Vita et al., 2019). The IEDB epitopic sequence was manually searched in the pool of digested peptides. An epitope was considered as present when at least one pre- cursor peptide was identified in the digestome. In order to compare the samples, we only took into consideration the presence of the epitopic sequence, and not the number of times the sequence was identified. A graphic example of the search for GID sequences with a known allergen IEDB sequence is shown in supplementary Table S8.

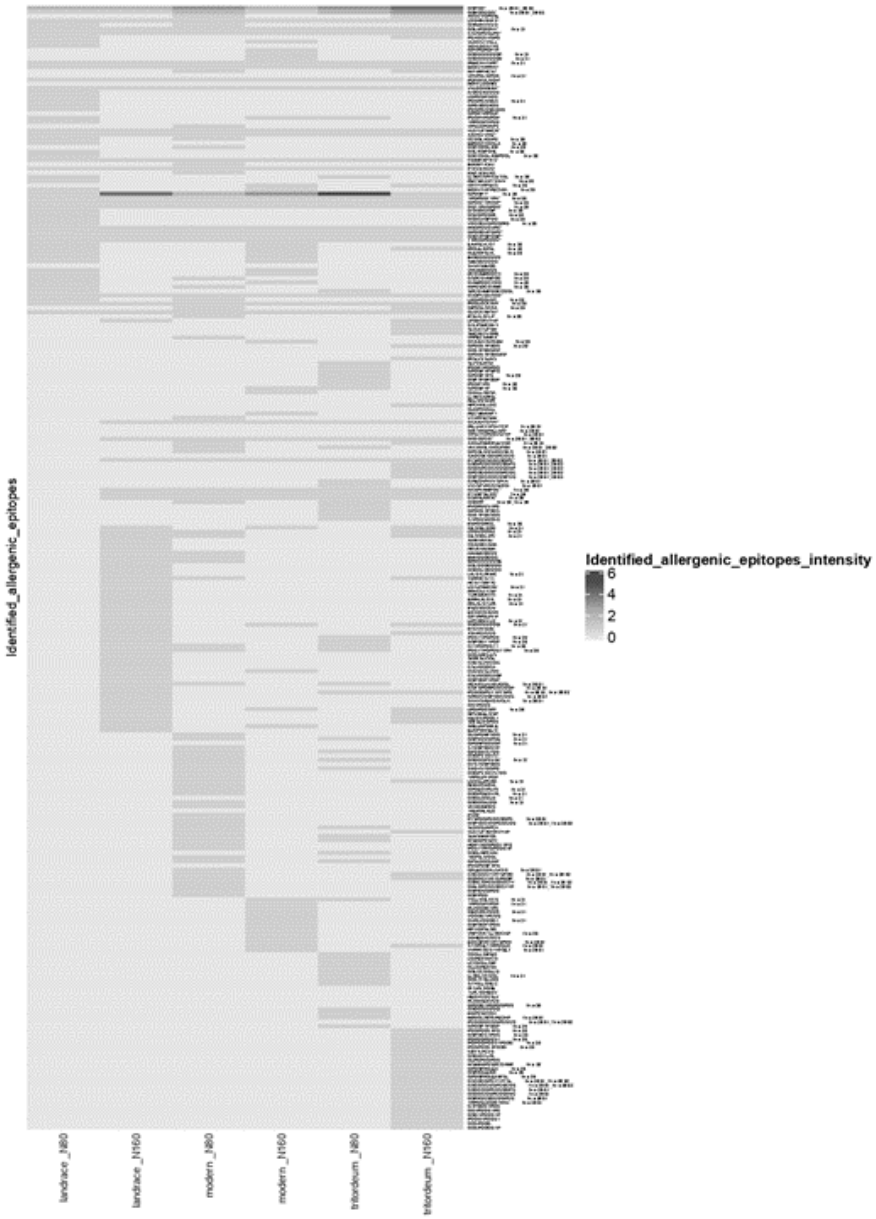
The digestive derived peptides were only considered for a compar- ative evaluation when 100% sequence homology was shared with the described IgE binding epitope. The relative abundance of the identified epitopes (Figure 9) was used to qualitatively compare the different cultivars and N rates. The largest number of allergenic epitopes belonged to the γ-gliadin fraction (about 40%), followed by epitopes of HMW-GS and α-gliadins prolamins for all the digested samples (Figure 10). The modern *cv* was distinguished by a greater percentage (30%) of α-gliadin allergenic epitopes, but showed a lower percentage for HMW-GS, LMW-GS and the ω-gliadins than landrace and tritordeum.

A limited number of the identified epitope sequences which belong to the 5 main gluten allergens, according to the World Health

Organization and the International Union of Immunological Societies (WHO/IUIS), were identified in all the *cv*s and at both N rates. This allergenic wheat gluten proteins list includes (http://www.allergen. org): Tri a 19 (ω5), Tri a 20 (γ), Tri a 21 (α), Tri a 26 (HMW-GS) and Tri a 36 (LMW-GS) (Figure 11). Tri a 26 (HMW-GS) was the protein with the highest number of identified epitope sequences in all the *cv* and N rate combinations. HMW-GS presents disulfide-bridge linked aggregates, which induce a high resistance to in vitro enzymatic digestion (Anderson et al., 1984).

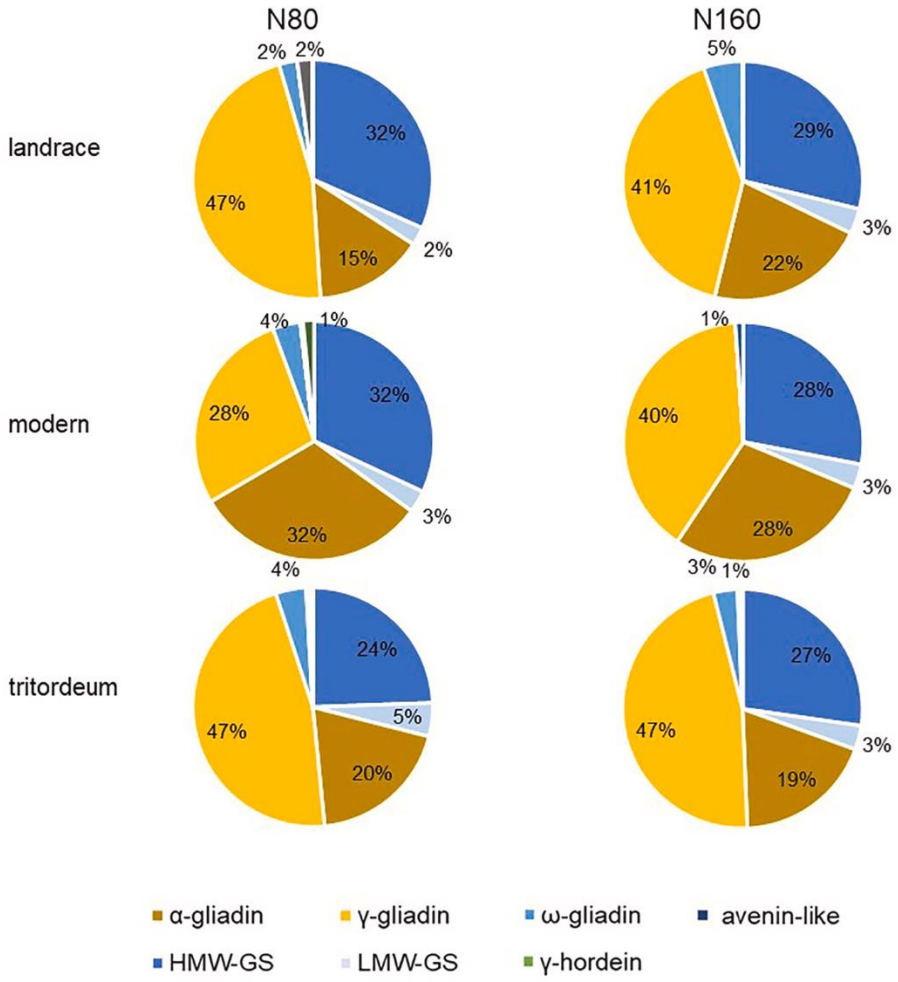
The second allergen with the highest number of epitope sequences identified was Tri a 20, belonging to the γ-gliadins, which has been identified as a strong trigger of WDEIA symptoms after ingestion of wheat protein (Yokooji et al., 2013). For the Tri a 21, the α-gliadin allergen, only a few peptide sequences were identified in the digested samples. This protein is responsible for the bakers' allergy (Sander et al., 2015).

Figure 9. Heatmap of the number of the allergenic epitopes in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha^{-1}) and N160 (160 kg N ha^{-1}) fertilization rates**.**



This heatmap was built using a matrix that reports the list of epitopes obtained for each sample divided by gluten protein fractions (the asterisk marks the epitopic sequences present in all the samples).

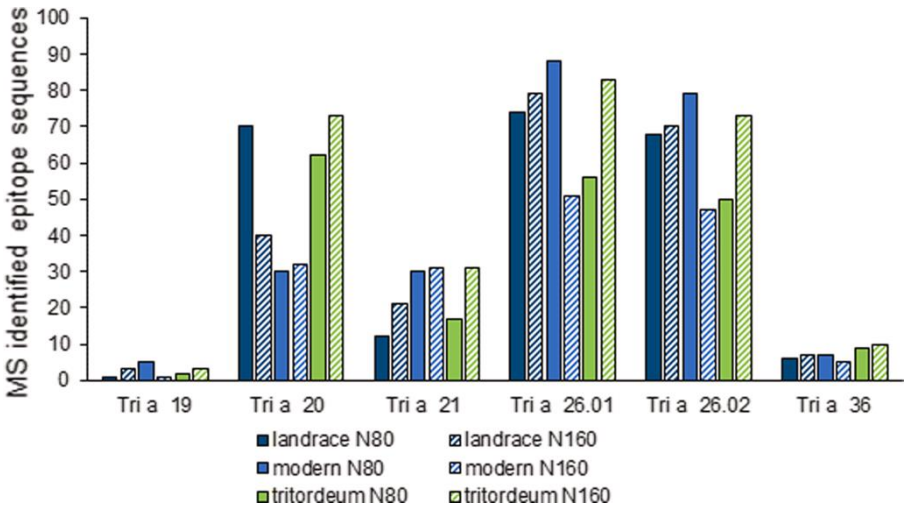
Figure 10. Gluten sources of the allergenic epitopes in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha− 1) and N160 (160 kg N ha− 1) fertilization rates.



Epitopes with a lower frequency matched with Tri a 19 and Tri a 36, show an occurrence that may be ascribed to a high susceptibility to enzymatic digestion. Although, Tri a 36 allergen (LMW-GS) is known to retain IgE reactivity, since it survives the extensive oral, gastric and duodenal in vitro digestion (Baar et al., 2012), a very small number of allergenic epitopes was found in all the considered *cvs* and N rates, thus suggesting that the amount of Tri a 36 that forms and accumulates in mature seeds was

probably quite low for each cv at the time of the collection (Baar et al., 2012). The occurrence of epitope sequences, determined by means of in silico analysis, should be confirmed by means of an appropriate immunological test using the sera of wheat allergic subjects.

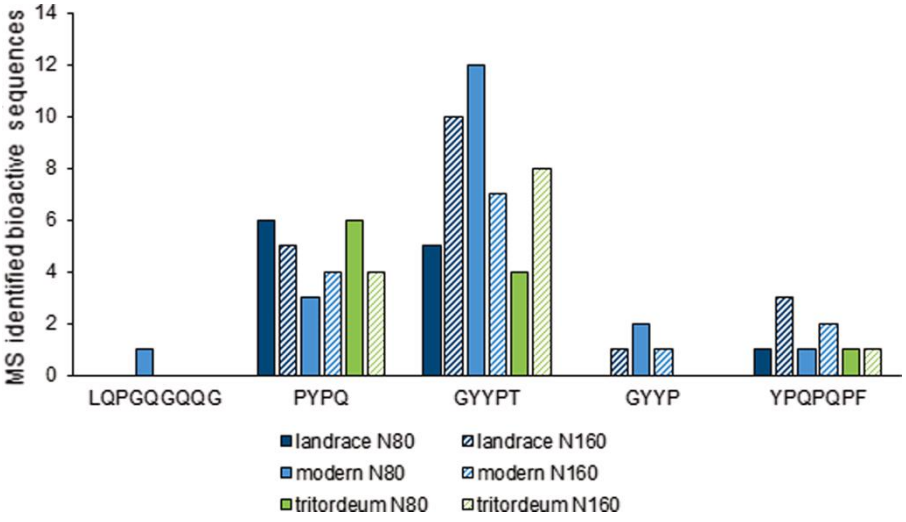
Figure 11. A graphical qualitative representation of the MS identified epitope sequences inferred to the five main 5 main wheat allergens (tri a 19, tri a 20, tri a 21; tri a 26.01; tri a 26.02; tri a 36) in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha^{-1}) and N160 (160 kg N ha^{-1}) fertilization rates.



Bioactive peptides

Together with the negative effects on human health, we evaluated any potential positive effects derived from the ingestion of the considered wheat and tritordeum *cvs*. Numerous digestion derived peptides are known to be precursors of peptides with described antioxidant and opioid effects. The bioactive peptides found in all the digested samples are shown in Figure 12.

Figure 12. A graphical qualitative representation of the MS identified epitope sequences inferred to a selection of bioactive peptide in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha^{-1}) and N160 (160 kg N ha^{-1}) fertilization rates.



The “GYYPT” and “YPQPQPF” opioid sequences were identified in all the samples. Gliadorphin 7 (also known as glu- teomorphin), a δ-exorphin with a YPQPQPF sequence, which is formed during the digestion of α-gliadin, deserves particular attention because its presence could be related to neurodevelopmental disease and psy- chotic disorders (Liu and Udenigwe, 2019). “GYYP” was identified in digested flour from the modern *cv*. For both N rates, and in landrace for the high N application. Peptides containing the “PYPQ” antioxidant sequence were identified in all the samples, while “LQPGQGQQG” was only identified in the modern *cv*.

Conclusions

We have investigated the effect of N fertilization on the gluten profile of three genotypes with different gluten compositions. The study has shown that increasing the N rate influenced the GPC to a great extent, while it

was found to have less impact on the gluten composition and on the type of celiac and allergenic epitopes after in vitro digestion. On the other hand, these parameters resulted in a greater variation only according to the genotype.

A strong genetic effect was observed with regard to the composition of gluten, especially for glutenins. Tritordeum showed the lowest levels of HMW-GS/LMW-GS and was also distinguished by lower values of ω5, compared to wheat *cvs*. Competitive R5 ELISA analysis highlighted that tritordeum may be regarded as a variety with a lower presence of highly CD epitopes than the old and modern wheat varieties that were here considered. The absence of the D genome in tritordeum could be one of the reasons for the lower immunodominant toxicity of this amphiploid.

The study on the expression of celiacogenic and allergenic sequences in different genomes remains of fundamental importance to provide scientific information. This proteomic study paves the way toward a more inclusive study with a larger number of genotypes, grown under the same environmental and agronomic conditions, to better understand their potential immunotoxicity. The qualitative analysis of the epitopes made it possible to observe that in all the studies *cv* and agronomic conditions (e.g. fertilization rate) the largest number of sequences identified belonging to the class of α- and γ-gliadins, respectively for celiac and allergenic epitopes. The CD epitopes showed a greater pres- ence in the α-gliadin fraction, where a 33-mer is present as strong stimulators of the T-cell response resistent to digestion. All the compared *cv* and N rate combinations showed quite concordantly a high presence of epitopes belonging to the allergens Tri a 26 and Tri a 21, of HMW-GS and γ-gliadins respectively, and very low for Tri a 19 and Tri a 36, belonging to ω5 and LMW-gs respectively. These results showed an interesting in-depth proteomic profile of the conditions studied. To verify the quantitative effect of a N rate and its interaction with the genotype, strictly at the epitope level, it would require to build a method to quantify robust information

considering the heterogeneity of the genome and the presence of repeated regions in the starting matrix. This will be a future goal of great interest.

The protein profile of tritordeum, compared to the two soft wheat cvs, supports its application as an interesting diet ingredient, given its lower R5 immunogenicity and high GPC content. However, the use of this new amphiploid species, as a promising alternative to soft wheat, will require specific breeding programs to enhance the end-use quality and immunogenic, nutritional and agronomic traits of such a crop.

Supplementary tables

All the supplementary tables are available online at https://www.sciencedirect.com/science/article/pii/S0963996921005160

Table S1 GID SEQUENCES for tritordeum for N80

Table S2 GID SEQUENCES for tritordeum for N160

Table S3 GID SEQUENCES for modern for N80

Table S4 GID SEQUENCES for modern for N160

Table S5 GID SEQUENCES for landrace for N80

Table S6 GID SEQUENCES for landrace for N160

Table S7 Fragmentation spectrum of the 25-mer peptide (LGQQPFQQPYPQPQPFPSQQPY) and 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) sequences with the GID celiac peptides of the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha-1) and N160 (160 kg N ha-1) fertilization rates. The letters in red represent mismatches in the alignment.

Table S8. Example of the alignment of one IEDB allergenic peptide (IEDB epitope sequence: QQPGQGQQ in red) with the GID celiac peptides of the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha-1) and N160 (160 kg N ha-1) fertilization rates.

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Chapter V – "Tritordeum as an innovative alternative to wheat: a comparative digestion study on bread"

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Abstract

Tritordeum results from the crossbreeding of a wild barley (Hordeum chilense) species with durum wheat (Triticum turgidum spp. turgidum). This hexaploid crop exhibits agronomic and rheological characteristics like soft wheat, resulting in an innovative raw material to produce baked goods. We applied a gel-based proteomic approach on refined flours to evaluate protein expression differences among two widespread tritordeum cultivars (Aucan and Bulel) taking as the reference semolina and flour derived from a durum and a soft wheat cvs, respectively. The products of in vitro digestion of model breads were analyzed to compare bio-accessibility of nutrients and mapping tritordeum bread resistant peptides. Significant differences among the protein profiles of the four flours were highlighted by electrophoresis. The amino acid bio-accessibility and the reducing sugars of tritordeum and wheat breads were comparable. Tritordeum cvs had about 15% higher alpha-amino nitrogen released at the end of the duodenal simulated digestion than soft wheat ($p < 0.05$). Bulel tritordeum flour, bread and digested bread had about 55% less R5-epitopes compared to the soft wheat. Differences in protein expression found between the two tritordeum cvs reflected in diverse digestion products and allergenic and celiacogenic potential of the

duodenal peptides. Proteomic studies of a larger number of tritordeum cvs may be successful in selecting those with good agronomical performances and nutritional advantages.

Keywords: Tritordeum; in vitro digestion; peptidomics; alpha amino nitrogen; R5; wheat allergy; celiac disease

Introduction

Wheat grains are the world's most important staple food crop. The derived flour is a key ingredient in the preparation of bakery and pasta products, accounting for 20% of the total dietary calories and proteins in the human diet (Poole et al., 2021). Throughout the centuries, the natural selection and hybridization among different wheat varieties, aimed at obtaining species easy to harvest and high in yield, have led to the modern tetraploid durum (*Triticum turgidum spp. durum*, AABB) and hexaploid bread wheat (*Triticum aestivum spp. aestivum,* AABBDD) (Örgeç et al., 2021). The fast global changes of the last decade have made agricultural productivity more uncertain. Particularly, rising temperatures and decreased water availability are primary reasons for crop yield losses and reductions in the area harvested (Bento et al., 2021; Daloz et al., 2021; Obembe et al., 2021). Barley is a crop that is adapted to a wide range of environmental landscapes, including high altitude and high latitude regions and to saline and dry conditions. Furthermore, barley flour has low machinability and bread-making performance compared to wheat. It was found that barley breads have an increased viscosity of the bolus, due to the presence of resistant starch and fibers, that reduced enzymes' accessibility and therefore slowed the in vitro static and dynamic starch digestion (Sagnelli et al., 2018). Since the beginning of the twentieth century, cereal breeders have focused their efforts on the development of interspecific wheat hybrids to obtain new cereals with increased phytochemical content, improved agronomic performances and

technological qualities. The hexaploid hybrid tritordeum (x *tritordeum martini*, AABBHchHch) is the product of cross-breeding *Hordeum chilense,* a South American wild barley species, and durum wheat. This hybridization aimed to combine the excellent traits of the *Hordeum*, such as high endosperm carotenoid content and higher tolerance to biotic and abiotic stress, with the technological qualities of wheat (Martín et al., 2000). Tritordeum is today commercialized as an innovative alternative to conventional small cereal crops (www.tritordeum.com) (Alvarez et al., 1992; Vaquero et al., 2018), with rheological and baking performances similar to bread wheat (Martín et al., 1999). Interestingly, in a clinical study involving subjects with non-celiac gluten sensitivity (NCGS), tritordeum breads were sensorially more appreciated than the gluten-free counterpart, showing good gastrointestinal tolerance (Sánchez-León et al., 2021; Vaquero et al., 2018).

A few recent studies in Europe have been focused on the agronomical traits, looking at yield performance of tritordeum cultivars (cvs) over conventional soft and durum wheats (Kakabouki et al., 2020; Martín et al., 2000; Visioli et al., 2020a). Scientific works have so far mainly focused on the bioactive compounds' content: tritordeum, in fact, has higher levels of carotenoids and arabinoxylans than wheat, and these result in a greater total antioxidant activity. Tritordeum has twice the amount of β-glucans compared to durum wheat, although a similar amount to soft wheat, but significantly higher arabinoxylans (Giordano et al., 2019) with prebiotic, immunomodulatory, antitumor, and anti-inflammatory activities (Jayachandran et al., 2018; Mendis and Simsek, 2014). Grain protein content (GPC) and gluten composition, in addition to the nutritional traits, play a major role in conferring the technological properties of wheat and other small cereals to the dough. By comparing the GPC of tritordeum with that of durum and soft wheat in different climatic conditions, tritordeum results in a higher GPC, ranging between 11% and 17%. Tritordeum produced by organic farming showed higher GPC than durum

wheat with a larger number of high-molecular-weight glutenin subunits (Visioli et al., 2020b). Although one of the parental lines of tritordeum is a durum wheat cultivar (cv), the similarity, in terms of derived flour quality, is much closer to that of hexaploid soft wheat, with specific interest for bread-making and baking processes (Visioli et al., 2020b).

Furthermore, little is known about the protein level differences across cvs or regarding the tritordeum protein digestibility.

This study aims at comparing the protein profile of two tritordeum cvs, Bulel and Aucan, with soft wheat cv Altamira and a durum wheat cv, Antalis. Model breads were used to compare the digestion products (free-amino nitrogen and free glucose) using an in vitro digestion that included a standardized oral, gastric, and duodenal model simulating the physiological conditions of a healthy adult (Brodkorb et al., 2019).

This work represents the first molecular characterization by advanced mass spectrometry of the peptides resistant to digestion of tritordeum bread, which was prepared with the two most common cvs, namely Aucan and Bulel. We mapped the contribution of the parent *H. chilense* to the tritordeum proteome and evaluated in silico the presence of peptides related to celiac toxicity and allergenicity. The immunoreactivity of the R5 monoclonal antibody targeting the celiacogenic sequence “QQPFP” was studied in the flours, as well as in undigested and digested bread.

Materials and Methods

Grains and Flours Production

A field study was carried out on the north-west Italian plain at Cigliano (45°18’N, 08°1’ E; elevation 237 m), in the 2019–2020 growing season. The experiment was performed on a silty-loam soil sub acid, characterized by a medium cation-exchange capacity and organic matter content. In the same experimental field, the following genotypes have been cultivated side by side:

- a soft wheat (hexaploid AABBDD), cv named Altamira (seeds provided by Limagrain Italia S.p.A., Busseto, Italy) classified as ordinary bread-making wheat (Blandino et al., 2015) registered in the Italian varietal list in 2009 (https://www.sian.it/mivmPubb/listeVarieta.do; Sian code: 11239; consulted on the 20/12/2021) and widely cultivated in Italy;
- a durum wheat (tetraploid AABB), cv named Antalis (seeds provided by CGS Sementi S.p.A., Acquasparta, Italy), characterized by medium-high GPC and gluten index; registered in the Italian varietal list in 2014 and widely cultivated in Italy;
- tritordeum (hexaploid AABBHchHch), cv named Bulel (seeds provided by Arcadia S.p.A., Pamplona, Spain), which was registered in the CPVO (Community Plant Variety Office) List in 2015;
- tritordeum (hexaploid AABBHchHch), cv named Aucan (seeds provided by Arcadia S.p.A., Pamplona, Spain), which was registered in the CPVO List in 2013.

The treatments were assigned to experimental units using a completely randomized block design with four replicates. The plot size was 7 × 1.5 m.

The same agronomic techniques have been adopted for all cvs. Briefly, the previous crop was maize and planting was performed in 12 cm wide rows at a seeding rate of 400 seeds m^{-2} on November 6th 2019, following an autumn plowing (30 cm) and disk harrowing to prepare a proper seedbed. A N fertilization treatment of 130 kg N ha^{-1} was used on all the cultivated samples. The total N rate for each treatment was top-dressed applied as a granular ammonium nitrate fertilizer, split 50 kg N ha^{-1} at tillering (growth stage, GS23) and 80 kg N ha^{-1} at the beginning of stem

elongation (GS32). The foliar diseases were controlled by applying a fungicide (pyraclostrobin 150 g ha^{-1} and fluxapyroxad 75 g ha^{-1}, Priaxor®, BASF Agricultural Solutions) at booting stage (GS45). Harvesting was carried out with a Walter Wintersteiger cereal plot combine-harvester on June 29th, 2020.

Grains (2 kg) from each plot and cv were milled using the Bona 4RB mill (Bona, Monza, Italy) to obtain refined flour, (tritordeum and soft wheat) and semolina (durum wheat). GPC (Kjeldahl N × 5.7, on a dry matter basis) and ash content were determined according to Blandino et al. (2015) on grains collected at the commercial maturity stage. Grains (2 kg) from each plot and cv were milled using the Bona 4RB mill (Bona, Monza, Italy) to obtain refined flour.

Materials

All the reagents used in this study were of analytical or higher grade. Sodium phosphate, ammonium bicarbonate (AmBic), acetic acid and the other chemicals used to produce the simulated salivary fluid (SSF), the simulated gastric fluid (SGF) and the simulated intestinal fluid (SIF), were provided by Carlo Erba (Milan, Italy). The enzymes used for in vitro human digestion were purchased from Sigma (St Louis, MO, USA), in line with those recommended by the INFOGEST protocol (Foca et al., 2007). Trichloroacetic acid (TCA), sodium dodecyl sulfate (SDS), glycerol, tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), guanidine chloride, trifluoroacetic acid (TFA), formic acid (FA), acetonitrile (ACN), 2-vinylpyridine monomer, and *p*-toluenesulfonyl-l-arginine methyl ester (TAME) were also from Sigma-Aldrich. Egg lecithin was purchased from Lipid Products (Redhill, UK). The electrophoresis reagents were all obtained from Bio-Rad (Milan, Italy).

The protein-free bread (< 0.1%, *w*/*w*) (Amino' pane le rosette produced by Antica Farmacia Orlandi) was purchased from a local pharmacy.

Quantification of Protein in Flour and Semolina

The Kjeldahl analysis was performed as described by Abrams et al. (2014), with some modifications. Two grams of each flour were weighed in a Kjeldahl tube, in which a mixture of copper and potassium sulphate (0.5 g and 12 g, respectively) and 20 mL of 96% sulfuric acid were added. The mineralization was performed following a thermal ramp: 230 °C for 20 min, 290 °C for 45 min, 320 °C for 35 min, and 420 °C for 60 min. The sample was diluted with 50 mL of deionized water, and 90 mL of 45% NaOH were added. The solution of the ammonia was distilled over steam and collected in a flask containing 50 mL of 4% boric acid. The total nitrogen was determined by titration with 0.1 N HCl, after adding a mixed indicator (methyl red 0,1% and bromocresol green 0,2% in ethanol). A conversion factor of 5.7 was used to convert the total nitrogen to total protein and the results were expressed as g of total protein over 100 g of sample. Samples were analyzed in biological duplicates.

Gliadin Quantification with R5 Commercial ELISA

Flour and bread samples were analyzed with the RIDASCREEN® Gliadin (Art. No. R7001, R-BIOPHARM AG, Darmstadt, Germany), which is a sandwich enzyme immunoassay (ELISA) based on R5 monoclonal antibody recognising the “QQPFP” celiac toxic motif. Proteins were extracted in the Cocktail (patented) recommended by Codex Alimentarius for the optimized extraction of gliadin from heat-processed and non-heated food samples (Art. No.: R7006 / R7016, patent WO 02/092633, R-BIOPHARM AG, Darmstadt, Germany), which was used according to the manufacturer’s instructions, and according to the AOAC Official Method of Analysis for gluten detection (OMA 2012.01).

RIDASCREEN® Gliadin competitive (Art. No. R7021, R-BIOPHARM AG, Darmstadt, Germany) was used to analyse the products of in vitro bread digestion, according to the manufacturer’s instructions.

The gastroduodenal digestion products were analyzed in duplicate.

3.5. Osborne Fractionation

The Osborne fractionation was performed as previously described in Landolfi et al. 2021. The albumins and globulins were solubilized from the non-defatted flour (1:10, *w*/*v*) in 100 mM KCl, 50 mM Tris-HCl pH 7.8, and 5 mM EDTA for 4 h at room temperature (−20 °C). The solution was centrifugated for 15 min at 3500× *g* and the supernatants from two consecutive extractions were pooled. The gliadins were extracted 1:10 *w*/*v* with 70% (*v*/*v*) ethanol for twelve hours at room temperature (−20 °C). Glutenin extraction was performed at 60 °C for 30 min, in 50% *v*/*v* 1-PrOH + 50 mM Tris-HCl (pH 8.5) + 1% (*w*/*v*) 1,4-Dithio-d-threitol (DTT). The cysteine residues of the glutenin extracts were pyridyl-ethylated at 60 °C for 15 min with 2-vinylpyridine.

1-Dimensional Electrophoresis (SDS-PAGE)

Purified protein fractions were separated by SDS-PAGE under reducing conditions, using a Mini-PROTEAN cell systems (Bio-Rad). To this purpose, proteins were precipitated in cold (−20 °C) propan-2-one (1:4, *v*/*v*), suspended in the SDS-PAGE Laemmli Buffer (0.125 M Tris–HCl pH 6.8, 5% SDS, 20% glycerol, 5% (*w*/*v*) 2-sulfanylethanol, 0.02% bromophenol Blue) and boiled in a water bath for 5 min. After quantification with a micro-Lowry kit (Sigma-Aldrich, Saint Loius, Missouri 63103 USA), 25 µg of gliadins, 75 µg of glutenins, and 75 µg of albumins/globulins were loaded onto a 12% acrylamide gel (Bio-Rad). Migration of proteins was conducted at 120 V for 10 min and 220 V for 35 min. Afterwards, gels were fixed with TCA (24%) overnight (16 h) and stained with Coomassie® Brilliant Blue R-250 (gliadins and glutenins) and G-250 (albumins and globulins).

Preparation of the Model Breads

Model breads were prepared using commercial baker's yeast (*Saccharomyces cerevisiae*). The same recipe was used for all the flours

from different cvs. Flour (50 g) was mixed with 33 g of water and 5 g of yeast and 7 g of salt were added. The mixture was allowed to stand at 20 °C for 12 h. Loaves were baked at 230 °C for 40 min. After cooling, loaves were cut into slices and subjected to in vitro digestion within a few hours to avoid any alteration of the digestibility due to storage conditions (e.g., starch retrogradation due to freezing) (Burton and Lightowler, 2008).

Static Oral-Gastric-Duodenal Digestion of Model Breads

In vitro oral and gastroduodenal digestion was carried out using the harmonized and standardized INFOGEST method (Foca et al., 2007). The trypsin activity of the porcine pancreatin was determined using the *p*-toluene-sulfonyl-l-arginine methyl ester (TAME) as the substrate according to Brodkorb et al. (2019) and measuring the absorbance at 247 nm for 10 min. The trypsin activity was found to be 9.5 U/mg of powder. At the end of the duodenal digestion, samples were boiled for 5 min to interrupt the enzymatic digestion and centrifuged at 7900× *g*, for 30 min. The supernatant containing digestion products, which are likely to be absorbed by enterocytes, was collected, and processed for further analysis, including peptidomics and α-amino nitrogen determination. Aliquots of duodenal digests were collected every 30 min.

Preparation of Samples for the Alpha Amino Nitrogen Determination

The solubilization of proteins from the cooked bread samples and protein-free bread was performed in the SSF without enzymes (1:12, *w*/*v*) for 3 h at 37 °C. Prior to α-amino nitrogen determination, all sample were de-proteinized TCA up to a final concentration of 20% (*w*/*v*). After the protein precipitation was conducted for 30 min at 20 °C, the solution was centrifuged at 4000× *g* for 30 min, 4°C and neutralised to a pH 7 with 1 N NaOH prior to analysis.

The content of free α-amino nitrogen in the samples was determined using the EnzytecTM Alpha-amino Nitrogen kit by R-Biopharm (E2500 R-Biopharm AG, 64297 Darmstadt Germany) following the manufacturer's instructions. The iCubio i-Magic M9 (Origlia S.r.L, 20007 Cornaredo (MI), Italy) was set to perform the enzymatic reaction in full automatization and the absorbance was read at 340 nm. All samples were assayed in triplicate and absorbance values were averaged.

Free Glucose Quantitative Determination

D-glucose was quantified directly in the soluble digest using the Enzytec™ Liquid D-Glucose kit by R-Biopharm (E8140 R-Biopharm AG, Germany), following the manufacturer's instructions. The analyses were performed on the iCubio i-Magic M9 (Origlia S.r.L, Italy) as described for the α-amino nitrogen (Section 3.9. All samples were assayed in triplicate and absorbance values were averaged.

Preparation of Peptides for Mass Spectrometry Analysis

Peptide digests were desalted using C18 Sep-Pak 360 mg sorbent weight (WAT051910, particle size 55–105 µm, pore size 125 Å) (Waters Co., Milford, MA, USA). The equilibration and cleaning phases were carried with a 0.1% TFA in water. Peptides were eluted with 70% acetonitrile (*v*/*v*) containing 0.1% TFA (*v*/*v*).

Liquid Chromatography-Tandem Mass Spectrometry (LC/MSMS) Analysis

LC-MS/MS analysis was performed by using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), online coupled with Ultimate 3000 ultra-high performance liquid chromatography equipment (Thermo Scientific, 95134 San Jose, CA, USA)). Samples were loaded through a 5mm long 300 µm id pre-column (LC Packings, 95134 San Jose, CA, USA) and separated by an EASYSpray™ PepMap

C18 column (2 µm, 25 cm × 75 µm) 3 µm particles, 100 Å pore size (Thermo Scientific, 95134 San Jose, CA, USA). Eluent A was 0.1% formic acid (FA) (*v*/*v*) in water; eluent B was 0.1% FA (*v*/*v*) in 80% (*v*/*v*) ACN. The column was equilibrated at 5% B. Peptides were separated applying a 5–40% gradient of B over 60 min. The flow rate was 300 nL/min. The mass spectrometer operated in data-dependent mode and all MS1 spectra were acquired in the positive ionization mode with an *m*/*z* scan range of 350 to 1600. Up to 10 of the most intense ions in MS1 were selected for fragmentation in MS/MS mode. A resolving power of 70,000 full width at half maximum (FWHM), an automatic gain control (AGC) target of 1×10^6 ions and a maximum ion injection time (IT) of 120 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. To prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 10s was applied. Ions with one or over six charges were excluded.

Spectra were processed using Peaks Studio (Bioinformatics Solutions, Waterloo, ON, Canada). A specific database was generated for the analysis of the MS/MS data. The database included UniprotKB entries for *Triticum turgidum* spp *durum*, *Hordeum chilense* and *Hordeum spontaneum*, downloaded on the 01/06/2021. The DB Toolkit was used to customize the database and remove redundant sequences (Bromilow et al., 2017; Martens et al., 2005). The *Sus scrofa* protein sequences, downloaded on the 16/10/2019 from UniprotKB, were also included in the database to detect contaminants, thus increasing the confidence of identification of the Tritordeum-derived peptides.

PEAKS Studio (version 6.0, Bioinformatics Solution Inc., 202-140 Columbia St W, Waterloo, Ontario N2L 3K8, Canada) was used for database searching, applying the following parameters: oxidation on methionine, deamidation on the glutamine and asparagine, and pyroglutamic for N-terminus glutamine as variable modifications; mass tolerance value of 8 ppm and 0.02 Da for precursor and MS/MS fragment

ions, respectively; no cleavage specificity. The peptide-level false discovery rate (FDR) was set at 0.1%
. Proteins with score -10LgP > 20 were accepted.

In silico Analysis of Peptides Resistant to Digestion

Peptides identified at the end of the gastroduodenal digestion were in silico evaluated for their celiacogenic potential and IgE capacity. IgE binding sequences were retrieved from the free Immune Epitope Database (IEDB) (https://www.iedb.org/, downloaded on the 20/09/2021). The celiac toxic motif was retrieved from the ProPepper database (https://www.propepper.net/, downloaded on the 20/09/2021) (Juhász et al., 2015). These epitopic/celiacogenic sequences were manually searched in the pool of resistant peptides identified by MS in the duodenal digests of Bulel and Aucan breads.

To increase the confidence of identification and the strength of the *in-silico* analysis, the epitopic/celiacogenic sequences were searched, only considering the peptides identified in both technical replicates. The analyses were performed with the peptides common to the tritordeum cvs and those uniquely identified in Bulel and Aucan. Peptides resulting from digestion that belonged to the same protein were aligned with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/, 20/12/2021) and graphically evaluated using the WebLogo software (, 20/12/2021) to highlight the recurring regions. This analysis was aimed at aligning in silico the surviving peptides to their toxic-allergenic potential.

Statistical Analysis

Statistical analysis was performed using SPSS statistic v.27 Chicago: SPSS Inc. Data were compared by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer post hoc test ($\alpha = 0.05$) for all analysis and Ryan–Eino–-Gabriel–Welsch F test ($\alpha = 0.05$) for the ELISA analysis.

Results and Discussion

Flour Protein Characterization

Flour from two tritordeum cvs, Aucan and Bulel, which are the most widely cultivated in Europe and agronomically characterized, were compared to flour obtained from two wheat cvs, Altamira (soft wheat) and Antalis (durum wheat), for mapping differences in protein content and quality.

The durum wheat, Antalis cv, and tritordeum, Aucan and Bulel cvs, had a total protein content (TPC) higher than the soft wheat, Altamira cv ($p < 0.05$) (Table 1).

Aucan also had the highest ash amount. These data are in line with a previous study looking at the adaptability of tritordeum cvs in the east Mediterranean region, showing Aucan with a higher protein content compared to Bulel and to the soft wheat cv Falado (Sánchez-León et al., 2021).

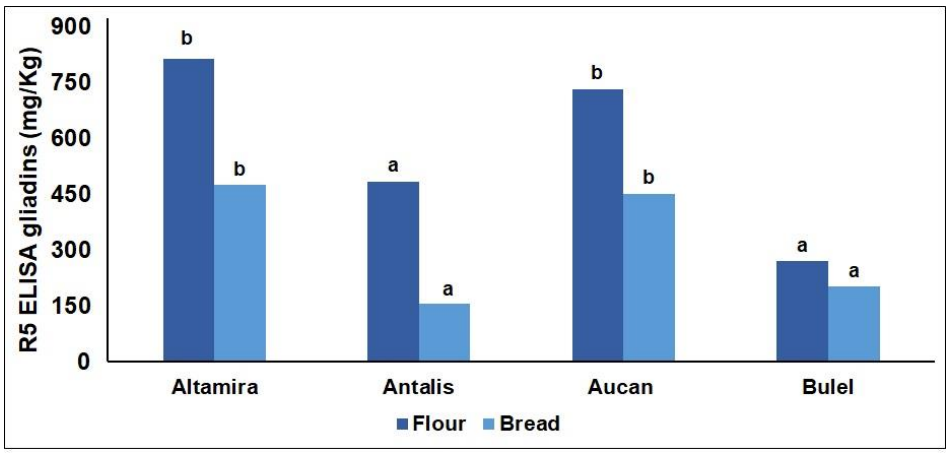
Table 1. Ash and grain protein content (GPC) and total protein content of the flour (TPC).

Species	Cv	Ashes (%)	GPC (%)	TPC (%)
Soft wheat	Altamira	1.89 ± 0.04^{b}	11.17 ± 0.23^{a}	8.65 ± 0.01^{a}
Durum wheat	Antalis	1.82 ± 0.02^{a}	11.66 ± 0.35^{b}	9.19 ± 0.21^{ab}
Tritordeum	Aucan	1.96 ± 0.02^{c}	13.43 ± 0.15^{c}	10.63 ± 0.33^{c}
Tritordeum	Bulel	1.86 ± 0.04^{ab}	11.97 ± 0.07^{b}	9.84 ± 0.3^{bc}

Values followed by different letters are significantly different ($p < 0.05$).

The concentration of gliadins in the flours was determined using a commercial sandwich ELISA test kit with the R5 monoclonal antibody to target the QQPFP celiac toxic motif (Figure 1) and the homologous LQPFP, QLPYP, QQSFP, QQTFP, PQPFPF, QQPYP, and PQPFP to a lower degree (Kahlenberg et al., 2006). The toxic sequence appears repeatedly in the ω-, γ-, and α/β-gliadins (Immer and Haas-Lauterbach, 2009).

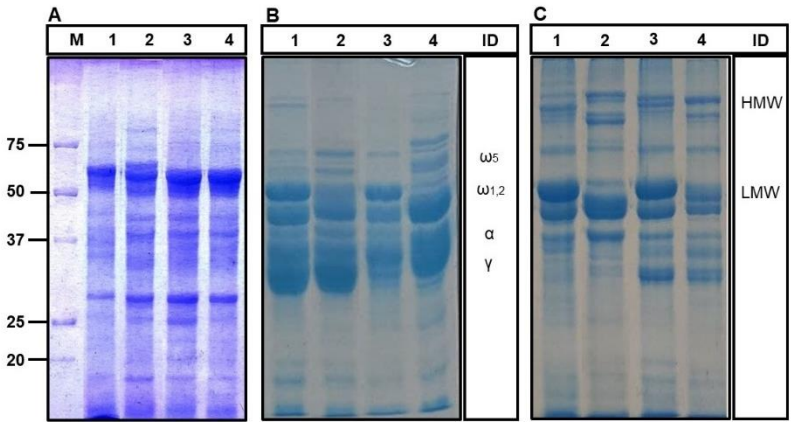
Figure 1. Quantification of the “QQPFP” celiac toxic motif recognized by the R5 monoclonal antibody of proteins extracted from the flours and the model bread obtained from different cereals. Within each product, flour and bread, bars with different letters are significantly different ($p < 0.05$) and the REGW-F test.



The data are expressed as mg of R5 gliadin per kg of flour and the mg of gluten can be extrapolated using a conversion factor of two as suggested by the ELISA manufacturer. Despite the fact that the Altamira cv showed the lowest TPC, this soft wheat had the highest R5-gliadin concentration, which was comparable to Aucan, which had a significantly higher protein content. The durum wheat cv Antalis had a 40% lower content of R5-gliadin concentration per kg of flour compared to soft wheat cv Altamira. These results were in line with literature data showing the gluten content in tritordeum to be comparable or even higher to bread wheats, with Aucan higher in gluten compared with Bulel by four percentage points (Sánchez-León et al., 2021). Interestingly, Bulel showed a 66% lower R5 immunoreactivity per kg of flour compared to Aucan. The reduced immunoreactivity of Bulel cv underlies important differences in terms of R5-gliadin sequences between the two tritordeum cvs. The analytical methodologies available for gluten determination in wheat may not be appropriate for all tritordeum cvs, since they may lead to an underestimation of the gluten content due to structural differences of the protein sequences.

The protein profile under reducing conditions of the Osborne fractionated proteins is presented in Figure 2.

Figure 2. Electrophoresis of Osborne fractions: albumins and globulins (Panel A); gliadins (Panel B) and glutenins (Panel C). Panel A was stained in Brilliant Blue Coomassie R250; Panel B and C with G250. M: Molecular markers (Precision plus Protein – Biorad); Lane 1: durum wheat cv Antalis; Lane 2: soft wheat cv Altamira; Lane 3: tritordeum cv Bulel; Lane 4: tritordeum cv Aucan; ID: Identification based on Landolfi et al. (2021). ω5: omega 5 gliadins; ω1,2: omega 1,2 gliadins; α: alpha gliadins; γ: gamma gliadins; HMW: high molecular weight glutenins; LMW: low molecular weight glutenins.



Tritordeum cv Bulel showed an electrophoretic profile of all fractions being less complex compared to both Aucan and durum wheat. The salt soluble protein profile (albumins and globulins) (Figure 2A) of both the tritordeum cvs appeared comparable to that of soft wheat, with a higher number of bands than the durum wheat flour. These differences were more pronounced in the lower-molecular-weight region (Mr < 30 kDa) and may be attributed to proteins encoded by the *H. chilense* inherited genome.

The electrophoretic profile of gliadins can be divided into four zones representing the typical regions of ω-, γ-, β-and α-gliadins (Figure 2, panel b). A greater protein variability was detected in Bulel compared to Aucan and the two wheat flours. The lowest number of bands were identified in the Bulel's gliadins, particularly in the high molecular mobility region (Mr

> 50 kDa) where the ω-gliadins migrate. This profile is consistent with the ELISA data showing Bulel characterized by the lowest concentration of gliadin detectable with the R5 antibody (Figure 1 and Figure 2). Aucan showed a greater complexity in the same region even when compared to the two reference wheat flours.

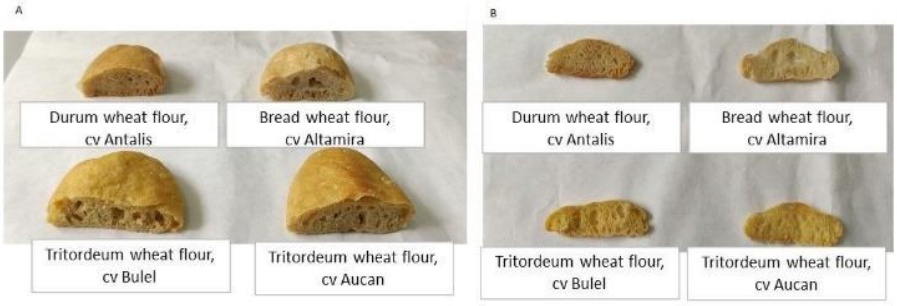
The electrophoretic profile of glutenin fractions varied across the four cvs (Figure 2, panel c) in terms of the number of electrophoretic bands detected and electrophoresis mobility. The high-molecular-weight glutenin subunits (HMW-GSs) are responsible for the gluten supramolecular structure, providing the cysteins involved in the formation of the disulfide-bonded backbone in gluten network, affecting the rheological properties of dough (Li et al., 2021). The presence of HMW-GS in tritordeum is due to the contribution of *H. chilense* locus "Glu-Hch1" gene expression on the chromosome 1Hch (Alvarez et al., 1999). This locus is homologous of the wheat Glu-1 locus and to the barley Hor-3 locus (Tercero et al., 1991). The *H. chilense* genome promotes a similar effect on gluten strength as the D genome inherited by the wheat species from *Aegilops tauschii* (Alvarez et al., 2001).

The region of the low-molecular weight glutenin subunits (LMW-GSs) in the two tritordeum cvs appeared similar between each other and to the durum wheat with a higher number of bands with faster molecular mobility compared to soft wheat. Unlike the control wheat, both the tritordeum cvs showed the presence of two main bands in the region of Mr < 30 kDa, which likely are expressed by the *H. chilense* inherited genome (mother). Once again, the electrophoresis showed substantial differences in terms of the overall protein expression between the two tritordeum cvs under evaluation. Since the two tritordeum cultivars share the same *H. chilense* line as mother, while they differ in the line of *T. turgidum* spp. *durum* used as father, these differences should be attributed primarily to the durum wheat inherited genome (Arcadia S.p.A., personal communication).

Digestomics

The simulated gastroduodenal digestion was performed on model breads prepared using refined flours to a 35% starting hydration (Figure 3).

Figure 3: A) Breads prepared with 100% reference flour; B) Sliced bread. Tritordeum bread appeared yellower than soft wheat. Tritordeum Baked breads showed a comparable alveolation to soft wheat bread, cv Altamira.

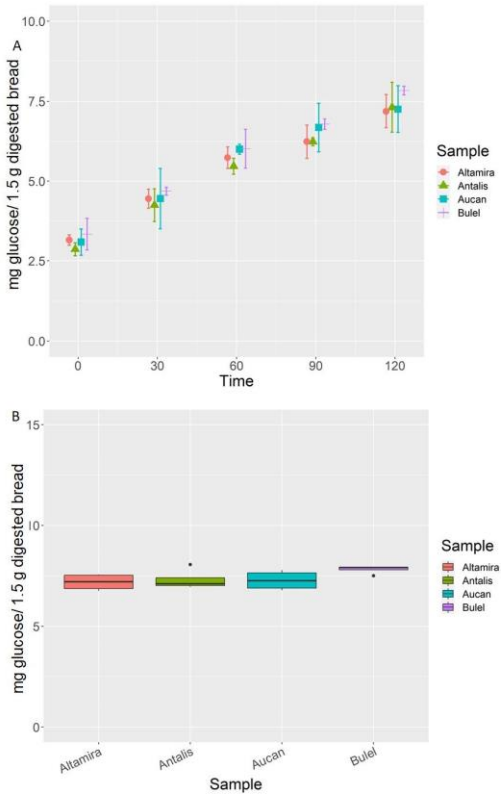


Breads were subjected to simulated digestion within a few hours from cooking to avoid any alteration of the starch that would have impaired (affected) the digestion. The R5 immunoreactivity of the four kinds of bread was measured and reported in Figure 1. In all cases, the R5 gliadin content of the bread samples was lower than the respective flour, although being comparable in terms of order of magnitude. This was somewhat expected, due to both the formation of the gluten network and baking-induced protein modifications, which may have partly impaired the protein extraction.

Quantitative Analysis of the End Products of Digestion

The digestion products of bread were quantitatively evaluated. The reducing sugar release (RSR) over duodenal digestion was measured by the enzymatic-spectrophotometric method (Figure 4).

Figure 4: Reducing sugar release (expressed as mg of glucose) from 1.5 g of digested breads. Error bars represent the variability over two breads digested in two days and two technical replicates. Panel A) kinetic of breads duodenal digestion (0-30-60-90 and 120 min); Panel B) mg of reducing sugar released after 4 h of gastroduodenal digestion of 1.5 g of bread.



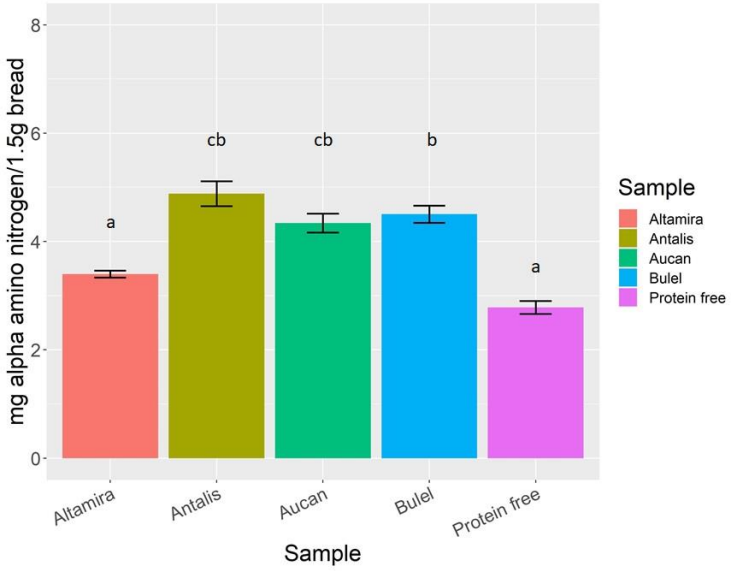
The RSR curves of the four bread samples were comparable ($p > 0.05$). Total starch content in tritordeum is knowingly higher than barley and lower than wheat, with a content in resistant starch similar to barley (Mikulíková et al., 2006). In the stomach and in the intestine, resistant starch, together with the higher viscosity (due to the presence of soluble fibers), makes the chyme of the barley bread less accessible to the enzymes, reducing the glycemic index compared with the reference wheat bread (Sagnelli et al., 2018).

The content of β-glucans in tritordeum was found to be five times lower than barley (Visioli et al., 2020b). The quantification of reducing sugars at

the end of digestion has only been a side part of this study. We are planning a forthcoming investigation aimed at quantifying the end products of starch digestion to confirm a different behavior for tritordeum bread than from its wheat counterpart.

Free amino acids, di- and tripeptides are the products of protein digestion that can be transported across the intestinal barrier. The starting content of free amino acids released during bread preparation was measured in the undigested cooked breads (Figure 5).

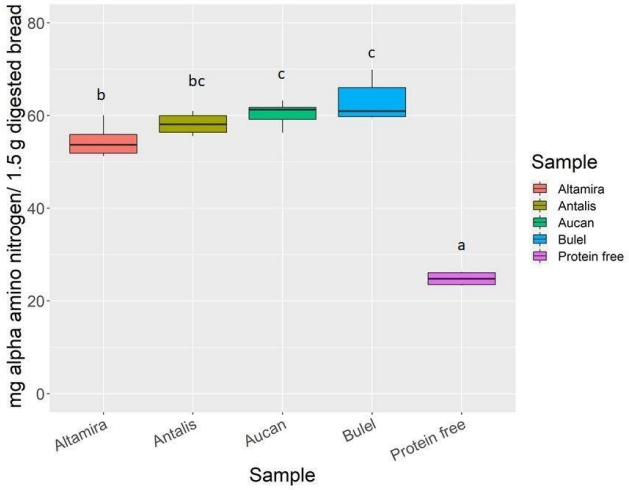
Figure 5: mg of alpha amino nitrogen determined in 1.5 g of the cooked breads. Bars with different letters are significantly different (p-value < 0.05) and the REGW-F test.



Bread made with the two tritordeum cvs and the durum wheat cv had a starting content of α-amino nitrogen of 0.35% (*w*/*w*), the soft bread was 0.25% and the protein-free bread was 0.15%. The commercial protein-free bread, used as background reference to quantify the endogenous amino acids products of natural gastroduodenal enzymes turnover, had an α-amino nitrogen content < 0.1%, in line with what was declared on the food package label.

The level of α-amino nitrogen determined in the digested protein-free bread accounted for half of the content of the analyzed bread samples on average. This background level is most likely due to the autoprotolysis of the digestive enzymes. This underlines the importance of having protein-free reference matrices to be used as background reference samples. The breads baked with the soft and the durum wheat showed a comparable digestibility (Figure 6).

Figure 6. Alpha-amino nitrogen released at the end of the duodenal simulated digestion of 1.5 g of bread obtained from the different cereals. Bars with different letters are significantly different ($p < 0.05$) and the Tukey-test.



The two tritordeum cvs showed the highest release ($p > 0.05$) of α-amino nitrogen related to the protein concentration of the flour determined by Kjeldahl.

Qualitative Evaluation of the Peptides Resistant to Digestion

The availability of well annotated and curated protein sequence databases is essential for inferring relevant information from mass spectrometry data. The analysis of cereal seed storage proteins is challenging because of the natural polymorphism, with a high number of protein isoforms differing by point mutations, and the homology across cvs and species (Daly et al., 2020). Tritordeum, being a novel crop, lacks

a protein database. Therefore, the identification of the proteins was performed using a combined database of the two parent proteomes, *H. chilense* and *Triticum turgidum* spp. *durum*, and of *H. vulgare*.

The tritordeum bread derived peptides, resistant to gastroduodenal digestion primarily belonging to α-amylase inhibitors (AAI) and to the glutenin family (Table 2 and Tables S1 and S3).

The AAI are knowingly resistant to gastroduodenal digestion, mainly due to the presence of disulphide bridges that stabilize the polypeptide chain (Nguyen et al., 2014; Salcedo et al., 2003), and are involved in IgE-mediated wheat (Tri a 28–39) and barley food allergies (Hor v 15) (Armentia et al., 1993; Koehler et al., 2014).

Several peptides were identified as derived from *Triticum* proteins, and fewer were associated with the *Hordeum* (Tables S1 and S3). Peptides belonging to the γ-3-ordeins (Uniprot ID: Q6EEY5) and the D-hordein (Uniprot ID: B0L965) from *H. chilense* could be identified in both digests of tritordeum bread. Unique proteins to tritordeum cv Aucan and Bulel were identified.

AAI and glutenin subunits may be suggested as suited species markers for discriminating between the Bulel and the Aucan varieties.

Table 2. LCMSMS identified proteins in tritordeum bread digests. Only proteins identified in both technical replicates were taken in consideration to increase confidence in identification. Isoforms were removed and the extensive list of identified proteins is available as tables S1 and S3.

	Accession	Species	10lgP	Coverage (%)	Peptides	Description
	Q9XGF0	TRITD	74.72	20	12	LMW-GS
	A0A446W0B5	TRITD	72.20	14	7	AAI
	K4N1X7	TRITD	74.67	10	8	HMW-GS
	A0A446W0A1	TRITD	76.67	12	9	AAI
	H8Y0D1	TRITD	68.82	15	9	Alpha prolamin
	A0A446W0B4	TRITD	63.66	12	4	UNP
Proteins identified in both tritordeum digests	A0A446W085	TRITD	71.15	11	7	AAI
	A0A446TL77	TRITD	39.57	5	2	rRNA N-glycosidase
	A0A446W0C7	TRITD	51.01	9	3	AAI
	A0A446V2J2	TRITD	42.65	4	2	AAI
	A0A446V2Q9	TRITD	45.34	8	3	AAI
	Q6EEY5	HORCH	40.78	8	3	Gamma 3 hordein
	B0L965	HORCH	31.52	2	1	D-hordein
	A0A446YMF0/M0WF36	TRITD/HORVV	21.54	4	1	UNP
	A0A287EEX5	TRITD	40.07	6	3	UNP
	A0A446JGR8	TRITD	63.12	8	5	AAI
Protein identified only in tritordeum cv Bulel	A0A0E4G9A4	TRITD	48.57	6	5	HMW-GS
	H8Y0M9	HORBR	37.19	12	3	Gamma prolamin
	A0A7H1K1W3	TRITD	31.27	7	2	AAI
	A0A446IHD3	TRITD	20.67	6	1	AAI
	A0A446IHC0	TRITD	31.50	4	1	AAI
Proteins identified in tritordeum cv Aucan	A0A2L1K3K6	TRITD	77.43	12	11	HMW-GS
	Q41603	TRITD	44.31	9	3	LMW-GS

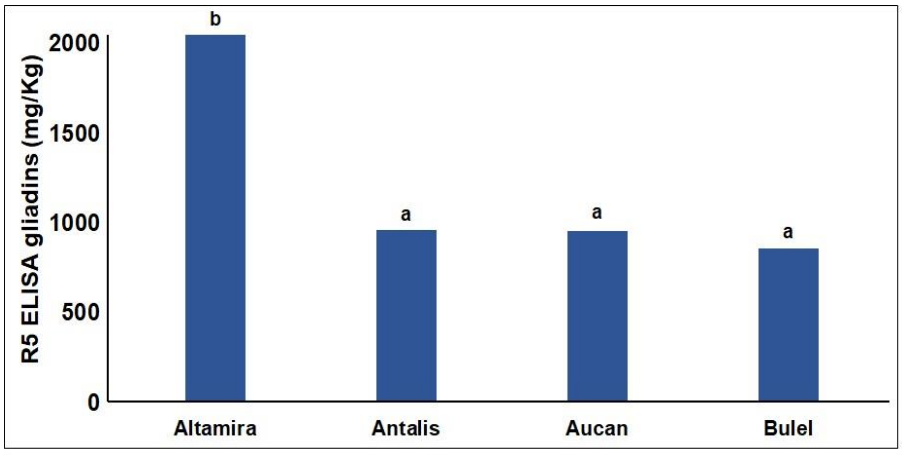
TRITD= *Triticum turgidum* subsp. *durum*; HORCH= *Hordeum chilense*; HORBR= *Hordeum brachyantherum* subsp. *brachyantherum*; HORVV= *Hordeum vulgare*; UNP= uncharacterized protein; LMW-GSs = low molecular weight-glutenin subunits; HMW-GSs= high molecular weight-glutenin subunits; AAI= α-amylase inhibitors.

As expected, the unique proteins were expressed from the *Triticum* father line differing between the two tritordeum. Interestingly, despite the common *Hordeum* mother, a gamma prolamin of the *Hordeum brachyantherum* subsp. *brachyantherum* (mother) could be uniquely identified by homology in cv Bulel.

The immunoreactivity of duodenal digests determined using a competitive R5-competitive ELISA was 50% lower in digested tritordeum and durum wheat bread samples compared with the soft wheat bread ($p < 0.05$) (Figure 7).

While the R5 immunoreactivity of Aucan flour and bread was comparable with the soft wheat flour, the digests of tritordeum cv Aucan bread had an immunoreactivity comparable with that of tritordeum cv Bulel and the durum wheat digests. The analyses were performed on the soluble digest, which is likely the fraction to be taken up in the gut. The lower R5-immunoreactivity of Bulel digest is in line with previous literature data (Landolfi et al., 2021).

Figure 7. Quantification of the "QQPFP" celiac toxic motif recognized by the R5 monoclonal antibody in duodenal digests (mg of gliadins per kg of the soluble duodenal digest) of bread obtained from different cereals. Bars with different letters are significantly different ($p < 0.05$) and the REGW-F test.



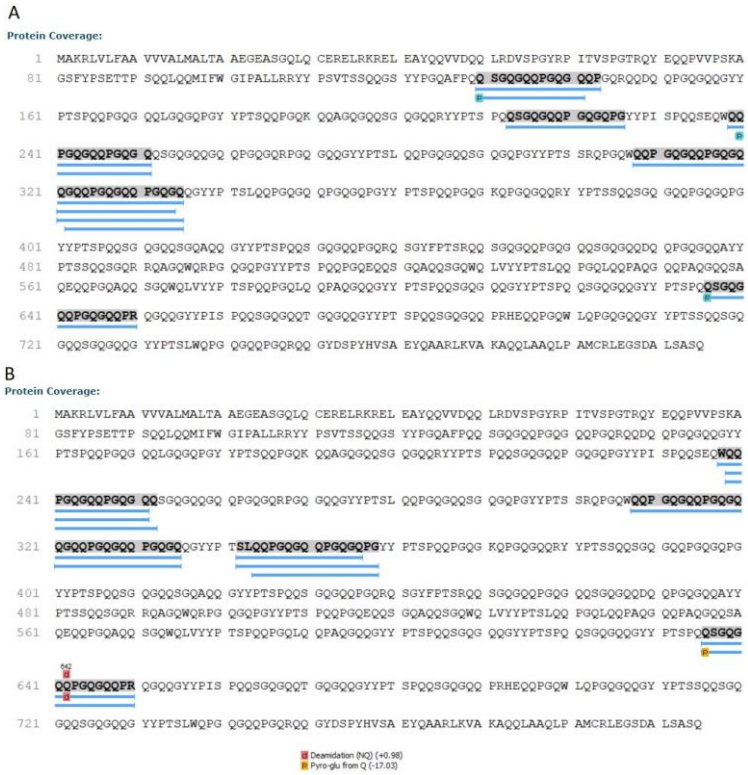
The reduced immunoreactivity of the Aucan bread duodenal digest may be explained by a low digestion level that could have spared large protein fragments carrying the R5-epitope(s) trapped in the insoluble fraction. This fraction is not taken up by enterocytes and represents the primary fermentation substrate of gut microbiota. A recent *in-vivo* study, showed a significant decrease of gluten intestinal peptides, determined by ELISA, in the stool of subjects fed with tritordeum bread compared with wheat bread-fed subjects (Vaquero et al., 2018). The bread produced with Bulel flour, under our analytical conditions, showed similar results as the in vivo study presented by Vaquero et al., 2018. The Aucan bread instead behaved in a completely different way suggesting future in vivo studies may need to be designed to include different tritordeum cvs to confirm their suitability for subjects affected by non-celiac gluten sensitivity, especially in consideration of the relative stability of AAI.

Due to the complexity of the mass spectrometry data an *in-silico* evaluation was carried out only on those peptides identified in both technical replicates, to enhance confidence. Overall, 93 peptides resistant to gastroduodenal digestion identified by mass spectrometry were common to the digests of Aucan and Bulel bread (Tables S3 and S4); 38 and 59 peptides were uniquely identified in the Bulel and Aucan bread digests, respectively (Table S5). Many of the unique peptides were inferred to the unique proteins previously listed in Table 1. Interestingly, one HMW-GS protein (Uniprot accession K4N1X7) was common to the two tritordeum cvs. The majority of the peptides identified in both digests mapped to the same protein regions, however, they had different N- and C-terminal trimming, therefore were assigned as unique (Figure 8).

Two peptides with sequences, 130-QSGQGQQPGQGQQP-143 and 213-QSGQGQQPGQGQPG-226 were uniquely identified in Aucan and were located in the N-terminal region of the protein. No peptides were identified in the same protein region among the Bulel-derived peptides. In contrast,

the peptide with sequence 342-SLQQPGQGQQPGQGQPG-358 was identified only in Bulel.

Figure 8: Coverage of the Triticum durum HMW GS (K4N1X7) by digestion-resistant peptides from Aucan bread (panel A) and Bulel bread (panel B). The alignment highlights the uniqueness in several cases is due to the hydrolysis of peptides differing by few amino acids (Supplementary Table 5).

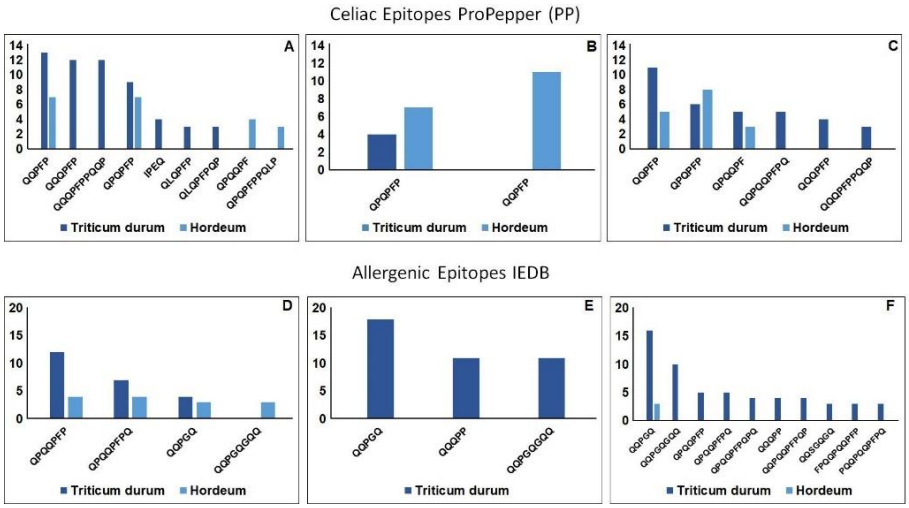


These misidentifications may be due to the bioinformatic protein inferring process, that would only list peptides with 100% identity. Wheat proteins are characterized by high polymorphism and the presence of several protein sequences differing by few amino acids (Daly et al., 2020). The protein assignment informs about the presence of a protein family rather than a specific protein, especially for gluten proteins. In this case, it may indicate the presence of two isoforms of the HMW-GS (K4N1X7) expressed in the two tritordeum cvs, carrying mutations in the two

identified regions. Two studies previously attempted to map the products of tritordeum cvs that had undergone simulated digestion, working either on isolated proteins (Vaquero et al., 2018) or the flour (Landolfi et al., 2021). This is the first study mapping the digestion products of model bread prepared with tritordeum flour, using the INFOGEST standardized model (Brodkorb et al., 2019).

The *in-silico* epitope analysis showed a larger number of peptide precursors of celiac toxic motifs and IgE binding peptides for the Aucan bread than for the Bulel counterpart (Figure 9).

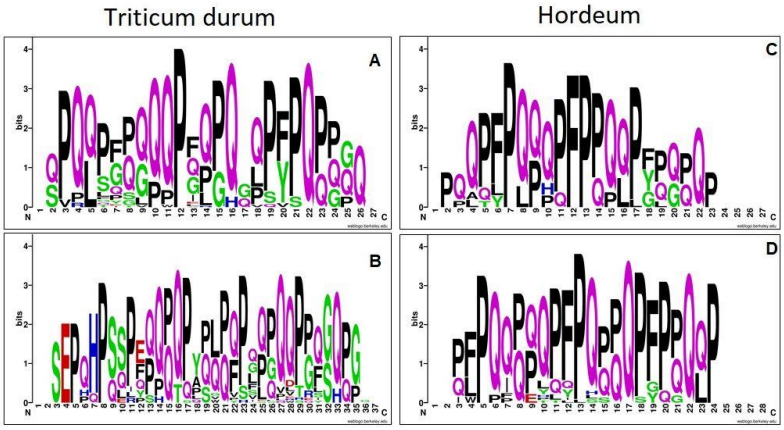
Figure 9. Graphical representation of the peptides surviving the digestion with potential adverse effects on human health. A) number of CD epitopes (ProPepper) common epitopes found in digests of bread baked with the two tritordeum cvs;). ; B) number of CD epitopes (ProPepper) found uniquely in digests of tritordeum cv Bulel bread; C) number of CD epitopes (ProPepper) found uniquely in digests of tritordeum cv Aucan bread; D) number of allergenic epitopes (IEDB) common epitopes found in digests of bread baked with the two tritordeum cvs ; E) number of allergenic epitopes (IEDB) found uniquely in digests of tritordeum cv Bulel bread; F) number of allergenic epitopes (IEDB) found uniquely in digests of tritordeum cv Aucan bread. Only epitopes identified in at least 3 precursor peptides were reported.



The analysis of epitopes also showed the prevalent contribution of *Triticum* in the overall allergenicity/celiacogenic potential of tritordeum bread.

The sequence analysis (Figures 9 and 10) of digestion-resistant peptides showed the high frequency among others of QQPFP, QQPYP, PQPFP sequences, which are targets of the R5 competitive ELISA. The mapping of the R5-epitopes within the protein sequences from the two tritordeum cvs highlighted the prevalent contribution of *Triticum*-derived sequences in Aucan and *Hordeum*-derived sequences in Bulel.

Figure 10: Graphical representation of the peptides surviving digestion. Peptides belonging to the same protein region were aligned and the height of the amino acid reflects its abundance in the sequences. The sequence R5-QQPFP sequence was highly repeated in Aucan bread Triticum derived peptides, while in Bulel was found highly repeated in Hordeum-derived peptides. Sequence logo of unique peptides from Aucan (Panel A, C) and Bulel (Panel B, D) bread duodenal digestomes. The frequency of the sequences is expressed in bits (Schneider and stephens, 1990).



Conclusions

This study examined for the first time the complex proteome of the tritordeum, highlighting the subtle but technologically and nutritionally relevant differences in the protein set of two commercially mainstream tritordeum cvs, namely Aucan and Bulel. The inter-cvs differences

observed may be attributable to the different contributions of the *Triticum turgidum spp. durum* genome. Our results suggest that attention should be paid in considering all the tritordeum cvs as a *unicum* in terms of protein expression, since in some cases the protein contribution can vary along the genomic characteristics of the *Hordeum* and *Triticum* parents.

In the same way, the first *in vitro* digestomic analysis carried out on bread baked with tritordeum flour in the present study evidenced that the process of digestion produced different peptidomes, with possible different outcomes in terms of immunoreactivity and allergenicity.

Supplementary tables

All the supplementary tables are available online at https://www.mdpi.com/1420-3049/27/4/1308/htm#app1-molecules-27-01308

Table S1: Nano-LC MS/MS identification of the proteins resistant to the in vitro gastroduodenal digestion of Aucan bread

Table S2: Nano-LC MS/MS identification of the proteins resistant to the in vitro gastroduodenal digestion of Bulel bread

Table S3: list of LCMS/MS identified peptides resistant to in vitro digestion of Aucan bread

Table S4: list of LCMS/MS identified peptides resistant to in --vitro digestion of Bulel bread

Table S5: list of LCMS/MS identified peptides resistant to digestion uniquely identified in Aucan and Bulel breads. This list of peptides was used for the in silico evaluation.

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Chapter VI – “Minor Cereals and new crops: tritordeum”

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Abstract

Tritordeum is an amphiploid that originates from the backcrossing of a wild barley, *Hordeum chilense,* with durum wheat. Tritordeum thus represents a “bridge” between the desirable genetic traits of its parent lines, but its end-use is comparable with that of bread wheat. Commercial tritordeum cultivars are now available and have begun to be cultivated in different growing areas. This crop could easily be inserted into cereal cropping system, since it has a similar crop cycle and agronomic management to wheat. Tritordeum has a lower grain yield but higher protein content than wheat; it has inherited a good tolerance to abiotic stress and diseases from *H. chilense*, but it has a high susceptibility to Fusarium head blight and mycotoxin accumulation. Scientific works have so far mainly focused on the bioactive compound content: tritordeum in fact has higher levels of carotenoids and arabinoxylans than wheat, and these result in a greater total antioxidant activity. Moreover, a lower presence of immunotoxic peptides has been observed in tritordeum than in wheat, although the benefits for whose people who suffer from non-celiac gluten sensitivity still need to be studied in depth. If breeders can provide tritordeum cultivars with a lower yield gap and a better end-use value, it could represent an interesting alternative to wheat for the baked goods supply chain.

Keywords: x *tritordeum martinii;* bread wheat; durum wheat; *Hordeum chilense;* grain yield; abiotic tolerance; fungal disease; grain protein content; rheological properties; bioactive compounds; fibers; carotenoids; total antioxidant activity; gluten immunotoxic peptides

Introduction

Food cereal-based products play a significant role in human diets throughout the world. Wheat is the second most important crop at the worldwide level, in terms of global production, after maize, but it is the first in terms of cultivated surface, and has shown a wide environmental adaptability, ranging from the tropics to the upper edges of temperate zones (FAO 2021). Moreover, wheat is the most important staple crop for food in developed countries, and the increasing global demand for this commodity is a consequence of industrialization and westernization processes that are also now taking place in developing countries. The first element of success in the use of wheat is related to its ability to be used to make unique food products. According to the distinctive technological properties of gluten protein, wheat flour can be used for a wide range of food products with unique organoleptic and texture traits: bread, pizza, cookies and other baked goods, but also pasta, noodles, couscous, porridge, pearled grain, flakes and a variety of other foods and ingredients (Day et al., 2006).

As far as the nutritional intake is concerned, in addition to being a key source of starch and energy, wheat-based foods also provide an important amount of protein, with a higher intake and also slightly higher biological values than those derived from the consumption of other cereals, such as maize or rice (Bekes and Wrigley 2004). Moreover, they also contain significant amounts of other important nutrients, particularly when wholegrain ingredients are used, including several non-digestible carbohydrates and such minor components as lipids, vitamins (particularly

B group vitamins), minerals and phytochemicals, which may contribute to a healthy diet (Shewry and Hey, 2015). It has widely been reported that a diet containing cereals, and wheat in particular, improves the contents of dietary fiber, micronutrients and a wide range of bioactive compounds. Some of these phytochemicals, such as phenolic compounds (phenolic acids, lignans and flavonoids) and carotenoids, show a marked antioxidant activity (Van Hung 2016) and their consumption has been associated with a reduced risk of degenerative and chronic diseases (Luna-Guevara et al., 2018).

Consumers' awareness about high-fiber diets and food naturally rich in beneficial components for the human diet is increasing, especially in developed countries (Ktenioudaki and Gallagher, 2012), as is the market demand for alternative and special food products. Improving the nutritional profile of baked foods, through supplementation with flour or ingredients of different origins, is therefore an important market requirement. Within this context, and according to a multigrain approach, the use of other minor cereals is a recent trend in the baking industry to obtain multiple functional benefits of bakery products (Torbica et al., 2021). The application of alternative cereal types for the production of special foods rich in bioactive compounds, obtained from innovative crops, has drawn the attention of both researchers and industrialists in the last few years (Donkor et al., 2012; Giordano et al., 2019; Zielinski et al., 2001).

These requests have again highlighted the need for the cultivation of minor cereals, such as barley, rye and oats, but also old types of cereals, such as einkorn, emmer, spelt, khorasan, old wheat cultivars and landraces, or pseudocereals, i.e., amaranth, quinoa, buckwheat and chia (Alvarez-Jubete et al., 2010). However, the use of flours and ingredients obtained from these crops in order to increase the nutritional value of baked products could be associated with some detrimental traits that could negatively impact their competitiveness on the market. First, these

ingredients could cause a deterioration of the technological properties, due to both the absence or a different composition of gluten and the high fiber content, which interact with the gluten-starch matrix (Mihai et al., 2017). When developing functional bakery products, it is in fact important to realize a food which delivers the appropriate level of bioactive compounds, but which also meets the consumers' requirements, in terms of appearance, taste and texture (Bender and Schönlechner, 2021; Meral and Köse, 2019). In addition, the lower productivity and some difficulties connected to the agronomic and mechanical management of these alternative crops, make the production of these raw materials, with the exception of barley, less sustainable than that of wheat in several temperate growing areas (Fabio and Parraga, 2017).

Since the beginning of the twentieth century, cereal breeders have focused their efforts on the development of interspecific wheat hybrids in order to obtain new cereals with increased phytochemical contents and improved agronomic performances and technological qualities. In this context, tritordeum (x *tritordeum martinii* A. Pujadas, nothosp. nov.) is a new crop that is derived from the crossing of a South American wild barley with wheat, and it may be considered a promising alternative to wheat as a basic ingredient for a wide range of foods. At present, tritordeum is cultivated in the European Union over an area of approximately 600 ha, of which 70% is in Spain, 17% in North Italy and 12% in Greece, although its cultivation has also recently started in the Netherlands and Australia (Arcadia Spa, personal communication).

As previously reported, there is a progressive process of specialization in cereal production, with the aim of obtaining raw materials with a higher end-use value, which are therefore more remunerative on the market, and for the supply chain. Thus, a new crop, such as tritordeum, could be an interesting alternative for the preparation of baked products, because, if the yield and qualitative gap are not too wide, compared to wheat, there will be no particular constraints concerning the agronomic technique or its

management along the supply chains. The aim of this chapter is to summarize the already available information on the cultivation of this new cereal, to make a comparison of its agronomic behaviors with those of wheat and other small cereals and to analyze the potential qualitative benefits of using tritordeum-based ingredients in the production of baked products and other foods. This framework may allow the strengths and weaknesses of this new crop, which is starting to spread in cereal cropping systems and on the market of several countries, to be outlined and the next objectives of genetic improvement to be addressed in a more effective way.

Origin and breeding of amphyploid tritordeum

Tritordeum is an amphiploid cereal which is the result of a series of artificial hybridizations, starting from a wild barley species, *Hordeum chilense* Roem. et Schultz. (2n = 2x, $H^{ch}H^{ch}$) and bread wheat (*Triticum aestivum* L. subsp. *aestivum,* which is also called common or soft wheat) (2n = 6x, AABBDD) or durum wheat (*Triticum turgidum* L. subsp. *durum* Desf.) (4x, AABB), whereby octoploid tritordeum (2n = 8x, $AABBDDH^{ch}H^{ch}$) and hexaploidy tritordeum (2n = 6x, $AABBH^{ch}H^{ch}$), respectively (Martin and Chapman, 1977; Martin and Sanchez-Mongelaguna,1982), are obtained.

Apart from being used as a genetic bridge to transfer the useful traits of barley to wheat, tritordeum has also been subjected to a breeding program to become a new, hulless, small cereal crop (Martín et al., 1999). Interest in breeding experiments between the two genera, *Hordeum* and *Triticum,* has been of great interest since the beginning of the 20th century (Martín et al., 1999). This interest has led to the successful development of a hybrid between wheat (*Triticum spp*) and rye (*Secale cereale L.*), that is, triticale (x *Triticosecale* Wittmack) (Cubero et al., 1986). The initial breeding aim of triticale was that of obtaining a promising wheat-rye from a crossing that would introgress the desirable genetic traits of the parents as a “bridge” to realize the typical high productivity and improved grain

quality of the wheat genotype, together with a wider adaptation for a stronger resistance to disease and a tolerance to environmental stresses, as conferred by rye (Estrada-Campuzano et al., 2012). The first triticale was obtained almost one century ago, after the spontaneous chromosome doubling of hybrids from crosses between wheat and rye (Rimpau, 1891); a vigorous F1 was produced, but it presented sterile offspring (Guedes-Pinto et al., 2012). The triticale breeding process started to advance after the development of the embryo rescue and colchicine-induced chromosome doubling approach (Blakeslee and Avery, 1937; Laibach, 1925). The obtainment of the triticale that is currently on the market has been possible thanks to breeding studies that have been carried out over a century to optimize the agronomic and quality performance of this amphiploid. Since the mid-1970s, more than 200 cultivars have been released, and the reference center for major discoveries and productions is the International Maize and Wheat Improvement Center (CIMMYT) in Mexico. Although octoploid (AABBDDRR, obtained from a chromosomal doubling of hybrids of hexaploidy wheat) and hexaploid (2n=42=AABBRR, obtained from a crossing of durum wheat with rye) hybrids were the first products, there has mainly been an evolution and improvement of hexaploid genotypes over the years, because of their greater vigor and major reproductive stability (Mergoum et al., 2009). However, octoploid triticale cultivars have shown a higher incidence of decreased fertility (Fernández, 1989), meiotic instability and a strong aneuploid frequency (Guedes-Pinto et al., 2012). Thus, although different ploidy levels have been developed for triticale, only the hexaploid one (Mergoum et al., 2009) is commercialized.

The prodigious success of triticale breeding has led to interest in obtaining a valid and interesting crossing of barley and wheat, from both the agronomic and nutritional perspectives. Several preliminary attempts to cross two different cereal genera of *Hordeum* and *Triticum* were not successful, but then Kruse (1973) was able to achieve the first fertile

success. This first important result was obtained using a wild relative of barley, *H. chilense,* as the female line for crop breeding, which permitted a good compatibility with the wheat cultivar to be obtained for the first time (Martin and Sanchez-Mongelaguna, 1982). *H. chilense* is a wild species that originated from South American. It is commonly found in Argentina and Chile, and is considered the *Hordeum* genus species with the highest breeding efficiency, thanks to its high crossability with the *Triticeae* species (Fernández, 1989). Furthermore, this plant has an interesting agronomical potential, due to its strong resistance to abiotic stress and its tolerance to diseases, such as brown rusts (*Puccinia hordei, P. recondite tritici*), powdery mildew (*Erysiphe graminis*), the Septoria leaf blotch complex (*Mycosphaerella graminicola)*, common bunt (*Tilletia caries*), smuts (*Ustilago nuda* and *U. tritici)* and to root-knot nematodes (*Meloidogyne naasi)* (Martín et al., 2000). Other beneficial effects of transferring *H. chilense* genes include the qualitative traits of the grain, such as the high genetic variability of expression of the storage protein composition, and the high content of bioactive compounds, such as carotenoids, in the kernel (Martín and Cabrera, 2005; Palomino and Cabrera, 2019).

Numerous tritordeum hybrids have been obtained since 1970 with the aim of creating a new crop that would be of interest to food supply chains, but all of them have been accompanied by sterility problems and low seed fertility (Martín et al., 1999). The first obtained tritordeum cultivars were octoploids, which were obtained using a hexaploid bread wheat, as a pollen donor (Martin and Chapman, 1977). Furthermore, the thus obtained octoploid tritordeum showed a poor early growth, a high frequency of aneuploids and high chromosome instability, all of which are not optimal for the development of a potential and sustainable crop (Martín et al., 1996). A few years after having obtained the first fertile octoploid amphiploid, a durum wheat line was used instead of the bread one, and thus a hexaploid tritordeum was generated (Martin and Sanchez-

Mongelaguna, 1982). Hexaploid tritordeum soon emerged because of its better initial growth, higher fertility and lower frequency of aneuploids than the octoploid ones, and it showed an overall better agronomic performance (Martín et al., 1996; Martín and Cubero 1981; Martín et al., 1999), even though octoploid amphiploids remain of great interest, considering their good bread-making parameters, which are comparable with those of bread wheat (Alvarez and Martín, 1994; Alvarez et al., 1994). Although durum wheat is the male parent of hexaploid tritordeum, the grain texture and the rheological traits of tritordeum are similar to those of bread wheat (Martín et al., 1999).

Despite the first good results obtained from the amphiploid primary lines of tritordeum, some agronomic parameters, such as the grain yield, did not reach the same values as the parental lines, and breeding studies are therefore now under way using backcrossing techniques (Martín et al., 1999). The attention of the breeders was directed toward studying the role of different nuclear genomes and cytoplasms as well as their interaction in amphiploid lines (Ekiz and Konzak, 1991a, 1991b, 1991c). Although it has been shown that the male line chosen for the nucleus has a predominant effect on the transmission of the characteristics, the cytoplasm, which is inherited from the female parent, also has a considerable influence (Hernández et al., 2001a; 2001b). Several studies have shown significant effects of the nucleus-cytoplasm interaction on agronomic and qualitative aspects, as for the amino-acid synthesis pathways (Atienza et al., 2008; Atienza et al., 2007a). However, even though very few data are available on the specific case of tritordeum, it seems that cytoplasm exploitation could be a source of variability for wheat breeding (Rodríguez-Suárez et al., 2011). Looking at the effects of using different cytoplasms as different sources of genetic variability to further improve agronomic performance (Millán and Martín, 1992) has led to studies targeted on obtaining a line with the best genetic stability, and the transfer and maintenance of the characteristics of interest from both

parental lines, through different combinations of cytoplasm x nuclear variability (Atienza et al., 2007c; Hernández et al., 2001a, 2001b; Millán and Martín, 1992; Rodríguez-Suárez et al., 2011). However, the alloplasmic lines obtained as result of several back-crossings with the same nucleus, but with cytoplasms from different species, such as *T. aestivum*, *T. turgidum* or *H. chilense*), did not show any clear differences, except for the grain yield, which resulted to be lower for the *T. turgidum* cytoplasm (Millán and Martín, 1992).

The genetic improvement of tritordeum is still in continuous evolution: according to Kakabouki et al. (2020), more than 250 tritordeum primary lines are available at present. Some lines that show a good yield performance, such as Aucan (2013) and Bulel (2015), have already been registered in the European Community Plant Variety Office (CPVO; www.cpvo.europa.eu) and have been commercialized in Europe as tritordeum Vivagrain since 2013 by Agrasys S.L., (Barcelona, Spain) and today by Arcadia Spa S.L. (Pamplona, Spain). Furthermore, recent studies have shown that the generation lines show even more interesting results for some agronomic aspects (Kakabouki et al., 2020) and new and better commercial cultivars are expected in the near future.

Agronomic management

Phenological and morphological traits

Since tritordeum has a comparable crop cycle and agronomic management with that of wheat, it could easily be inserted into the cereal cropping system as an alternative to other small cereals, without the need of any specific adaptation for its cultivation (Martín et al., 1999; Millán et al., 1988). Both spring- and winter-type tritordeum genotypes exist, according to the form of the wheat genotypes used for crossing. The duration of the growth stages and the development of tritordeum are similar to those of wheat plants in different growing areas. Martinek et al.

(2003) reported that spring tritordeum cultivars cultivated under Central-European conditions had a clear lateness and non-uniform maturation, as a consequence of a high level of rejuvenation of the tillers that characterize the perennial nature of *H. chilense.* Furthermore, these problems have successfully been avoided by means of a period of vernalization, together with an autumn sowing, which also result in a better development and higher yield of tritordeum in these environments. Moreover, Millán et al. (1988) and Villegas et al. (2010; 2008) reported that abiotic stresses during the vegetative stages in Mediterranean environments with frequent drought stress during ripening led to a longer delay in the anthesis moment for tritordeum than for wheat, and thus resulted in a shorter grain filling period.

The root system architecture of tritordeum is the typical one of small cereals. Furthermore, the root length, surface area and tips of tritordeum have been found to be lower than those of wheat (Visioli et al., 2020), although tritordeum has shown a greater root thickness. Furthermore, these authors highlighted that tritordeum had a root microbiome that was richer in species, particularly of *Bacterioides* species, than wheat cultivars.

The susceptibility of this crop to lodging has been debated in literature, in consideration of the production situations that could determine the manifestation of this agronomic problem. Several researches have reported a lower height for tritordeum, on average 20%, than for wheat, and thus a lower susceptibility to lodging (Francisco Barro et al., 1996; Martín et al., 1996; Visioli et al., 2020). However, Montesano et al. (2021) and Yousfi et al. (2010) reported a higher susceptibility of lodging for tritordeum than for wheat. Kakabouki et al. (2020) highlighted the key role of the genotype in influencing the plant height and showed clear intra-varietal differences within both the compared tritordeum and wheat. Visioli et al. (2020) also reported a clear variability of lodging for different tritordeum cultivars. The higher predisposition of tritordeum to lodging in

high fertility growing areas may be linked to its greater tillering capacity than that of wheat (Pinto et al., 2002) and a higher culm weakness (Lima-Brito et al., 2006).

As far as the ear is concerned, tritordeum has a brittle rachis and harder glumes than wheat, which cause a higher physical impediment to threshing, which in turn leads to a slightly more difficult cleaning of the kernel and/or greater grain losses at harvest (Ávila et al., 2021). In addition to a possibly higher number of ears per plant, as a consequence of a superior tillering capacity, the number of spikelets per ear may also be higher than that of wheat, although a higher incidence of sterile spikelets has been recorded (Pinto et al., 2002).

The caryopsis of tritordeum has an ellipsoidal form of around 9 cm, with a diameter of 3 mm, and has thin hair on the top and a floury endosperm (Salvá, 2016).

Tolerance to abiotic stress

Tritordeum had shown a widespread adaptability to different environmental growth conditions. It is mainly cultivated in South Europe, but its cultivation has also been reported in several continental production situations in Central and North Europe.

Several researches, carried out in Mediterranean growing areas, have reported the good agronomic response of this crop in semi-arid environments, thanks to its adaptability to drought stress conditions (Kakabouki et al., 2020; Martín et al., 2000; Martín et al., 1999; Villegas et al., 2010), and a general better drought tolerance than bread wheat (Martín et al., 1999), but sometimes also than durum wheat (Gallardo and Fereres, 1989; Simane, 1993). Lower stomatal conductance and transpiration under water-deficit conditions have been reported for tritordeum than for wheat during the pre-anthesis period (Villegas et al., 2010). Tritordeum has shown a high generation rate of molecular oxygen, under high irradiance conditions, but also a lower dark respiration rate and

light compensation, which help to explain the lower grain yield of tritordeum than that of wheat (Francisco Barro et al., 1996).

Tolerance to salinity is another ecological trait of *H. chilense* that also seems to be expressed in tritordeum (Villegas et al., 2010; Martín et al., 2000) and it is probably connected to a lower stomatal conductance of tritordeum than that of wheat (Aranjuelo et al., 2009). Yousfi et al. (2010), studying a combination of water deficiency and salinity concentration in the cultivation of durum wheat, triticale and tritordeum, confirmed a higher tolerance of this crop to moderate salinity, due to a lower stomatal conductance, a lower leaf accumulation of Na+ and a higher K+/Na+ ratio, all of which delay plant senescence and help maintain a plant's capacity to assimilate nitrogen. Martinek et al. (2003) highlighted that the winter-hardiness of tritordeum is clearly related to the freezing tolerance of the wheat genotypes used for the crossing with *H. chilense*. The tritordeum cultivars that are currently cultivated in central Europe in fact demonstrate a low risk to damage from winter frost.

Nutrient absorption and Nitrogen Use Efficiency (NUE)

The possibility of limiting the use of fertilizers in modern cereal cropping systems, and nitrogen (N) in particular, could lead to a significant increase in environmental sustainability. Thus, the aim of researchers is to optimize the crop management practices and improve the *Nitrogen use efficiency* (NUE) (Congreves et al., 2021) of the crop, without any excessive decrease in the cereal yields or quality (Lea and Azevedo, 2007; Lea and Miflin, 2010).

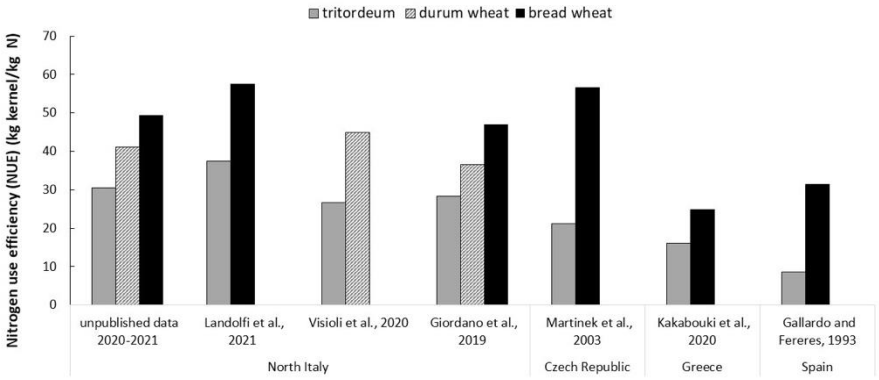
As tritordeum is a new cereal, it could be of particular interest to verify its capacity to maximize its nutrient use efficiency, in those areas that are the most suitable for its cultivation, through a correct management of such a cultivation and of its management, in fertilization terms. The first experiments in this direction, which were carried out in growth chambers (Aranjuelo et al., 2013; Barro et al., 2003; 1994; 1991), highlighted a

higher NUE in tritordeum than in wheat. Barro et al. (1994) showed that tritordeum has a higher affinity to nitrate than durum wheat and, consequently, a greater capacity to absorb it from soil. Tritordeum has shown higher levels of nitrate reductase activity at the leaf level than durum (Barro et al., 1991) and bread wheat (Wallace, 1986), which could lead to a high remobilization and translocation capacity of this nutrient to the kernel during ripening. Aranjuelo et al. (2013) found that tritordeum resulted in a higher N uptake than wheat in a controlled growth chamber, albeit only under low N rate conditions.

The efficiency in the use of N by tritordeum, and by durum and bread wheat, has been compared, in field production situations, with ordinary N rate fertilizations of between 120 and 230 kg N ha^{-1} (Figure 1).

The NUE of tritordeum was found to be 40% and 30% lower than that of bread and durum wheat, respectively. The NUE values for tritordeum were generally higher in growing areas with temperate climatic conditions than in Mediterranean areas. Unlike the positive physiological traits observed for tritordeum under controlled conditions, the field comparison of the tritordeum cultivars available on the market has highlighted a lower performance than wheat for the NUE, expressed as yield per unit of fertilization applied. According to the fertilized-based NUE reported in Figure 1, the low efficiency shown by tritordeum is mainly a consequence of an already existing yield gap with wheat (see section 2.4). Furthermore, since tritordeum has a higher N content in the grain at harvest than wheat (see section 3.2), the recovery efficiency of a fertilizer, which is used to indicate the apparent increase in plant N uptake in response to the N input (Congreves et al., 2021), is expected to be similar to that of wheat. In order to increase the nutrient efficiency of tritordeum and its sustainability, it will first be necessary to increase the yield potential of the next generation of commercial cultivars.

Figure 1. Comparison of fertilized-based nitrogen use efficiency (NUE) between tritordeum, durum and bread wheat for different production situations.



Fertilized-based nitrogen use efficiency (NUE) expressed as partially factor productivity, calculated according to (Congreves et al. 2021) as the ratio between the grain yield, expressed in kg/ha, and the total nitrogen applied with fertilization expressed in kg N/ha. The data sources are reported in the figure.

Disease tolerance and the related sanitary traits

The *H. chilense* genome presents numerous types of resistance to many foliar diseases which could be transferred to tritordeum (Martín et al., 1996). According to the date available in the literature, tritordeum shows a good tolerance to barley brown rusts (*Puccinia hordei*), while its susceptibility to wheat brown rusts (*P. recondita* f.sp. *tritici*) is similar to that of wheat (Rubiales et al., 1991). Prats et al. (2006) observed that tritordeum genotypes are sometimes more resistant to powdery mildew (*Erysiphe graminis tritici*) than wheat, while Martinek et al. (2013) reported a good tolerance to Septoria leaf blotch (caused by *Mycosphaerella graminicola)*. Rubiales et al. (2001) suggested that the better tolerance of tritordeum to Septoria leaf blotch is due to genes located on chromosome 4Hch and which are inherited by *H. chilense*. However, the typical avoidance mechanism of the *Hordeum* genome, that is, an appressorium

formation to overstep the wax barrier on the stomata, has not been identified for tritordeum (Martín et al., 1996).

Hexaploid tritordeum is less susceptible to common bunt (*Tilletia caries)* than wheat, although the tolerance to this disease was observed to decrease in octoploid lines (Rubiales and Martın, 1999). On the other hand, a high susceptibility to Fusarium head blight (FHB), mainly caused by *Fusarium graminearum* and *F. culmorum*, has been reported for tritordeum cultivars when grown in temperate areas prone to this disease (Spaggiari et al., 2019). The occurrence of FHB in tritordeum, as in other small cereals, leads to a high yield loss (Duffeck et al., 2020), a reduction in the test weight and, consequently, in the milling rate, and the accumulation of mycotoxins, that is, toxic compounds for which legislative thresholds have been established in several countries (European Commission, 2006). Tritordeum grown in North Italy has shown a higher content of trichothecenes, the most frequent class of mycotoxins found in small cereals, than bread wheat, and a similar content to durum wheat, a species considered highly prone to this sanitary risk (Spaggiari et al., 2019).

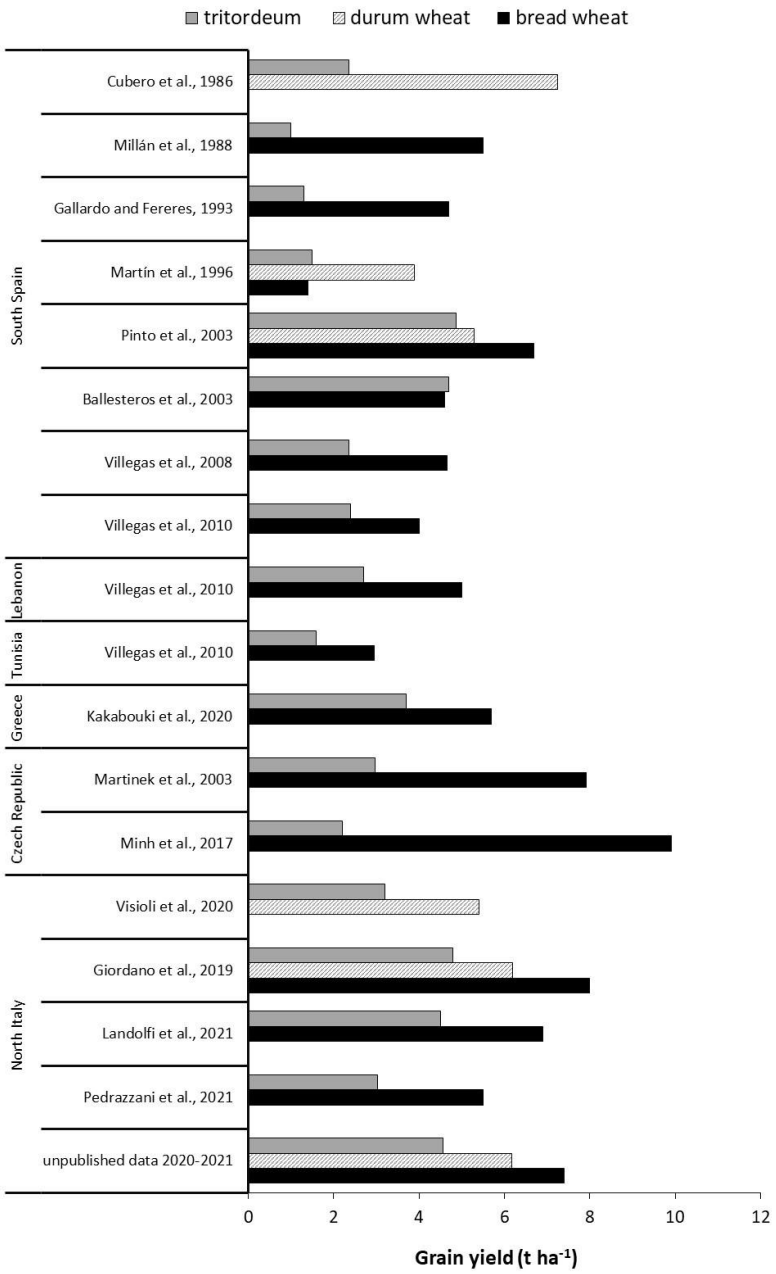
Although the tolerance of durum wheat to FHB is very low and the variability for this trait in the tetraploid gene pool is very limited (Haile et al., 2019), the development of tritordeum cultivars that are less prone to the occurrence of mycotoxins for Central-Europe growing areas would require the use of durum parental lines specifically selected for this trait. Moreover, after screening some accessions of *H. chilense* and detecting a certain variability of reaction to FHB, Fedak (2017) crossed the best accessions with a durum cultivar that resulted in a clear variability to disease tolerance in the obtained tritordeum lines. In addition to FHB, Martinek et al. (2003) also reported a higher infection by ergot (*Claviceps purpurea,* another fungal specie able to produce mycotoxins) for several tritordeum genotypes.

With the exception of the clearly higher susceptibility of FHB, the tritordeum presented in the literature shows a similar or better tolerance to foliar diseases than wheat, thus generally making the disease control of this crop simple. Furthermore, the majority of field experiments reported in the literature have been carried out in growing areas with a low disease pressure, and the tolerance of tritordeum to these biotic adversities still needs to be verified in production situations more prone to the diseases. Field experiments carried out in North Italy have highlighted that an application of fungicide at heading results in a 10% increase in grain yield, which is similar to that recorded for bread wheat (unpublished results). Thus, in temperate growing areas, tritordeum, as well as wheat, could benefit from control strategies that are able to prevent the infection of fungal diseases, while the cropping system primarily needs to be designed to limit the contamination of kernels by mycotoxins.

Grain yield

Tritordeum is a new species that has been the subject of limited breeding activities, compared to the reference small cereal species. Yield comparisons have been carried out between the first available genotypes of tritordeum and the conventional and widely cultivated bread and durum wheat cultivars over the last 35 years, and this crop has been confirmed to have an interesting yield potential. On average, the yield gaps with bread wheat and durum wheat fall between 43% and 40% respectively (Figure 2).

Figure 2. Comparison of the grain yields of tritordeum, durum and bread in different environments.



The data sources are reported in the figure.

The reported tritordeum grain yields show averages of 2.6 t/ha and 3.6 t/ha for temperate Mediterranean and Central-Europe continental

environments, respectively. As far as the interaction with the growing areas is concerned, the yield performance of tritordeum in such Mediterranean regions as South Spain, Tunisia, Lebanon, Greece and South Italy, is quite close to that of wheat and other species, such as triticale (Villegas et al., 2010). In such environments, the yield gap between tritordeum and durum and bread wheat is on average 46% and 39%, respectively.

The reported difference, in terms of grain yield, between tritordeum and wheat in continental and more fertile growing areas, such as the Czech Republic and North Italy, falls between 23% and 45%, respectively.

Although inferior to modern wheat cultivars, tritordeum has shown a 34 % higher grain yield than the wheat landraces that were cultivated in the last century (Landolfi et al., 2021), and for which there has been a relaunching for the production of special baked goods in developed countries.

There is a consistent difference in the kernel productive traits between the thousand kernel weight (TKW) of tritordeum and that of wheat: tritordeum on average results in a lower TKW than durum (-48%) and bread wheat (-38%) (Table 1). The lower TKW reported in Mediterranean growing areas could explain the lower yield potential of the crops in these production situations. Villegas et al. (2008) demonstrated that the low TKW of tritordeum could be due to a delay in reaching the anthesis stage, compared to other small cereals, which would result in a shorter period for the accumulation of starch in the grains, thus lightening their weight.

Table 1. The thousand kernel weight (TKW) and test weight (TW) of tritordeum, and of durum and bread wheat.

Location	Reference	TKW (g)			TW (kg/hL)		
		tritordeum	durum wheat	bread wheat	tritordeum	durum wheat	bread wheat
South Spain	Alvarez et al., 1992	35.4	54.3	44.6	75.3	81.2	80.34
South Spain	Gallardo and Fereres, 1993	28.3		48.7			
South Spain	Martín et al., 1996	33.0	56.0	39.0			
South Spain	Pinto et al., 2002	21.6	39.9	36.5			
South Spain	Pinto et al., 2003	21.0	39.9	36.5			
South Spain	Villegas et al., 2008	21.3		34.9			
South Spain	Villegas et al., 2010	33.4		51.7			
South Italy	Villegas et al., 2010	41.0	53.1				
Lebanon	Villegas et al., 2010	27.2		42.0			
Tunisia	Villegas et al., 2010	23.5		36.0			
South Spain	Ballesteros et al., 2003	46.5		49.7	74.0		81.0
South Spain	Atienza et al., 2007	26.0		34.5			
Greece	Kakabouki et al., 2020	29.8		29.0	67.4		72.7
South Italy	Montesano et al., 2021	36.3	35.4		73.9	74.8	
North Italy	Giordano et al., 2019	39.4	47.9	46.8	72.7	72.9	81.2
North Italy	Landolfi et al., 2021	36.5		38.9	69.9		75.5
North Italy	Unpublished data 2020-2021	37.7	51.1	45.0	72.9	80.1	81.3

The data sources are reported in the table.

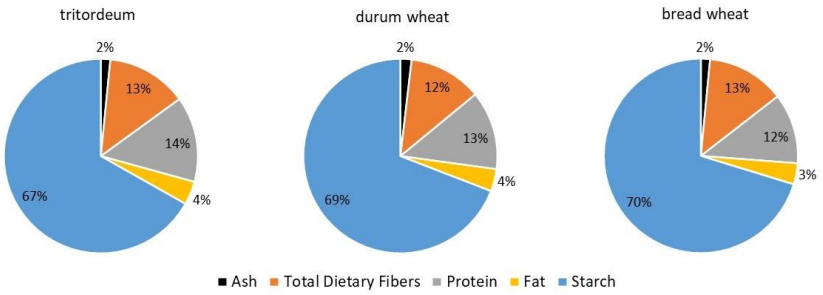
The test weight (TW) is a representative index of the yield capacity of a crop, as a result of a correct ripening stage. The TW is important from a nutritional point of view, because of the direct relationship between this

parameter and the energy content and feeding value of the grain. Moreover, it is an important qualitative index, since it is directly related to the milling rate and thus to the profitability of the transformation of grains into refined flour for millers. Hulled barley is characterized by a much lower TW than hulled durum or bread wheat, as the hull, which is rich in fiber, remains attached to the seed at maturity. Although the hull of tritordeum is removed during the harvesting operations, the reported TW has always been much lower than that of wheat. In the reported production situations, the TW of tritordeum has always been lower than 76 kg/hL, a value that is often considered the minimum threshold for the purchasing of wheat by mills. No information is available in the literature on the milling rate of tritordeum, compared to wheat. However, an improvement in the ability of this crop to accumulate starch during ripening and increase the TW may be a desirable trait for future breeding programs, in order to increase its productivity and the potential milling use of this cereal.

Nutritional traits

A few works in the literature have dealt with the characterization of the nutritional composition of tritordeum and compared it with bread and/or durum wheat. The tritordeum grain is overall very similar in proximal composition to that of wheat (Figure 3). The protein content and, albeit to a lesser degree, the fat concentration in the whole grain, which increase from bread to durum wheat to tritordeum, represent the main differences. Thus, tritordeum has a lower starch content than bread or durum wheat, while no difference has been observed in the kernel content of the total dietary fiber (TDF) or ash.

Figure 3. Comparison of the proximal composition (%) of tritordeum, and of durum and bread wheat.



Data sources: Cubero et al. (1986), Giordano et al. (2019), Mikulíková et al. (2011).

Carbohydrates

Starch

Cereals generally mainly consist of carbohydrates, mostly starch, which give the crop a particular source of energy, and this allows them to play a central role in human nutrition. Starch is the main component of grain and it is a digestible polysaccharide composed of amylose and amylopectin, whose properties are fundamental for the quality of cereal and, consequently, for numerous food formulations that are studied carefully by food processing industries (Khatkar et al., 2009).

Erlandsson (2010) and Mikulíková et al. (2011) reported slightly lower starch values in tritordeum (65% dry weight) than in bread and durum wheat (70%), thus confirming previously reported data obtained in other growing areas. However, there is a lack of studies on the starch content and above all on the starch properties of tritordeum.

As far as the starch composition is concerned, Alvarez et al. (2019) investigated the waxy (Wx) gene responsible for amylose synthesis, and demonstrated a greater similarity of the gene structure of tritordeum with the barley structure than with the wheat one. Any mutation of this gene determines a decrease in the amylose and amylopectin ratio, which

results in a higher flour swelling power and, consequently, a higher loaf volume (Martín et al., 2008). Although various differences have been observed in the position and presence of some amino acids in one transit-peptide of 70 amino acids belonging to the Wx gene, the enzymatic function seems to be almost the same (Alvarez et al., 2019). Further investigations are needed to select new tritordeum cultivars with different starch properties in order to satisfy the specific demands of the food industry.

Fibers

The TDF of grain includes insoluble fiber (cellulose, hemicelluloses as arabinoxylans, lignin) and soluble fiber (fructans, galactose, resistant starch, β-glucans). The intake of the dietary fiber of cereals has been shown to confer several beneficial effects to the health of individuals as it reduces the risk of developing numerous diseases, such as coronary heart disease, type II diabetes, obesity, several gastrointestinal disorders, and cancer, and it also promotes immunomodulatory activity, cholesterol lowering activity and several prebiotic effects (Anderson et al., 2009; Mendis and Simsek, 2014).

Although Giordano et al. (2019) did not observe any difference in the occurrence of the TDF of tritordeum, a few studies have reported that this crop could have a higher TDF (Table 2) than bread (+10%) or durum wheat (+10%), even though hulled barley still presents considerably higher amounts (on average +30%, compared to tritordeum) (Cubero et al., 1986; Erlandsson, 2010). This promising potential is stimulating a great deal of interest in developing food products rich in fiber, using tritordeum as a raw material (Dreher et al., 2001). An improvement in the TDF content of pasta has recently been verified, using not only brewers' spent grain, but also a portion of flour made from tritordeum (Nocente et al., 2021), without compromising the sensorial quality of the final product. Although knowledge on the distribution of the fiber components in the tritordeum kernel has not yet been explored in any great detail, Giordano

et al. (2019) reported that the content of the TDF can reach up to 80%, albeit only in the most external kernel layers, in analogy with wheat and barley.

Among the various types of soluble fiber, fructans are considered to be very important compounds that have a characteristic heathy prebiotic function which helps to preserve the correct functioning and balance of the intestinal bacterial flora. Åman (1988) observed their presence in higher concentrations in tritordeum (1.9 % of dry matter) than in bread wheat (0.8%). At the same time, the presence of these soluble compounds may not always be a desired element in a flour or food, because they also constitute one of the major fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) reported in cereals (Pejcz et al., 2019). FODMAP has been shown to trigger carbohydrates and cause the characteristic symptoms of celiac disease and irritable bowel syndrome (IBS). Recent studies have recommended a low FODMAP diet to reduce these disorders (Fraberger et al., 2018).

Certain components of dietary fiber, such as arabinoxylans (AX), present numerous interesting structural and functional properties at both the cellular and *technological levels, and these properties influence the* chemical-physical properties *of the derived dough and food. AX* structurally strengthens plant cells (Saulnier et al., 2007) and acts as a storage element for phenolic acids, e.g. ferulic acid. From the technological point of view, these polysaccharides confer physic-chemical properties that are particularly interesting for the food industry, both for bread-making (Courtin and Delcour 2002) and for brewing use (Nocente et al., 2021). In addition, important evidence has emerged of beneficial effects on human health, because of their role in the prebiotic function (Grootaert et al., 2007), immunomodulatory activity (Samuelsen et al., 2011), the control of postprandial glucose and the insulin level (Lu et al., 2000), as well as antitumor activity (Cao et al., 2011). The total AX amount

(Table 2) in tritordeum whole grain has shown higher values (+26%) than bread wheat (Giordano et al., 2019), with the highest concentration having been quantified in the Aucan variety (2.15% dry weight). Rakha et al. (2012) also confirmed a higher content of AX in tritordeum whole grain than in bread wheat (+11%) in colder and continental environments. Giordano et al., (2019) demonstrated a homogeneous distribution of these hemicellulose polysaccharides in all the grain pearled fractions.

Another soluble fiber class with an interesting health claim is β-glucan, which contributes to preventing type 2 diabetes, by improving the postprandial glucose and lipid control (Andrade et al., 2015; Henrion et al., 2019), and cardiovascular diseases (Henrion et al., 2019). Its capability to alter the gut microbiota profile has been associated with a decreasing impact of cardiovascular risk markers (Y. Wang et al. 2016). According to EFSA (2009), a regular consumption of this element, that is, at least 3 grams per day, helps to maintain a normal blood cholesterol concentration and maintain or achieve a normal body weight. Although developed from a wild barley species, tritordeum does not result in an enrichment of β-glucan, compared to wheat. On average, barley results in 33% more β-glucan than tritordeum (Table 2). Furthermore, as can be seen in the table, the β-glucan content of tritordeum (0.65% of dry matter) is very similar to that of the reference wheat cultivar (0.75%) (Giordano et al., 2019; Rakha et al. 2012), while tritordeum has a larger portion of β-glucan (+ 43%) than durum wheat (Giordano et al., 2019). The β-glucan in tritordeum is principally distributed in the intermediate layers of the kernel (10-25% of grain kernel), with a very similar distribution to that of durum wheat, while the bread wheat variety presents the largest content of β-glucan in the most external layer (5-10% pearled fraction) (Giordano et al., 2019).

Table 2. Comparison of the total dietary fibers, arabinoxylan and β-glucan for tritordeum, durum and bread wheat, and for barley.

Dietary compound	Study	tritordeum	durum wheat	bread wheat	barley
Total dietary fibre (%) (TDF)	Cubero et al., 1986	3.9	3.5	-	-
	Erlandsson, 2010	14.3		12.8	17.6
	Giordano et al., 2019	13.5	12.2	11.7	25.2
Arabinoxylans (%) (AX)	Giordano et al., 2019	1.9	1.1	1.4	1.3
	Rakha et al., 2012	6.9		6.2	8.1
β-glucans (%)	Giordano et al., 2019	0.7	0.4	0.9	3.5
	Rakha et al., 2012	0.6		0.6	5.9

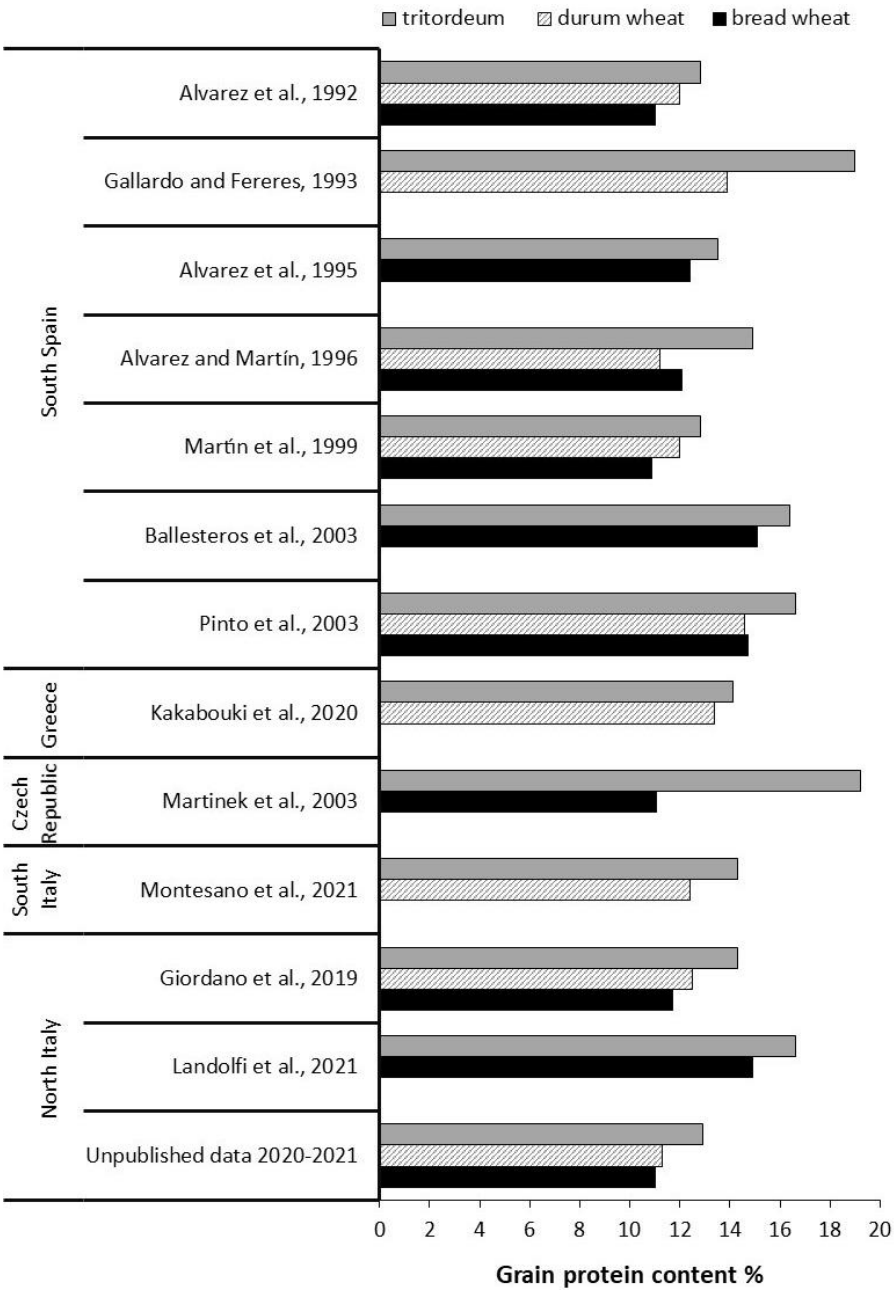
The data sources are reported in the table.

Grain protein content

Cereals play a major role in human nutrition, with the highest consumption of wheat products being observed in Mediterranean diets, and this consumption is increasing throughout the world (Shewry, 2009). For this reason, a cereal with a higher protein content and a good protein composition can be considered an advantage for a healthy diet, even though it remains a food source with a lower protein content than other sources. Since the first stages of tritordeum breeding, the grain protein content has been reported to be higher than that of durum or *bread* wheat (Alvarez et al., 1995; Cubero et al., 1986; Folina et al., 2020).

Several field studies have compared the grain protein content (GPC) of tritordeum with that of durum and bread wheat in different climatic areas over the last two decades (Figure 4).

Figure 4. Comparison of the grain protein content (GPC) in tritordeum, durum and bread wheat for different environments.



The data sources are reported in the figure.

On average, tritordeum results in a GPC of between 11 and 17%, and it reaches higher values than bread (+22%) and durum (+16%) wheat. This advantage, in terms of the GPC of tritordeum, has been confirmed in both continental and Mediterranean environments.

The large amount of grain protein in the kernel could be related to the negative correlation with the typical grain yield of Mediterranean environments, where the short duration of the grain filling stages results in a lower dilution of protein with starch, which is the main grain component (Garrido-Lestache et al., 2004). Different tritordeum cultivars have been studied in Greece to investigate their adaptation to a dry and warm climate, and the negative correlation between grain yield and grain protein was confirmed (Kakabouki et al., 2020). Furthermore, the large difference in GPC between tritordeum (+42%) and bread wheat cultivars was also recorded under central European climatic conditions (Martinek et al., 2003), even though tritordeum presented the same growth and development timings as the wheat varieties. However, under certain conditions, such as those characterized by a warm and dry Mediterranean climate, higher levels of GPC than those of durum (+12.5%) and bread wheat (+ 27%) have been confirmed, without showing any significant differences in grain yield (- 2% and -7%, respectively) (Ballesteros et al., 2003; Antonio Martín et al., 1996). Tritordeum seems to uptake N fertilisation more efficiently during post-anthesis than wheat and triticale varieties (Aranjuelo et al., 2013).

It is well known that GPC, in addition to the genetic properties, is influenced to a great extent by the crop management practices. Visioli et al. (2020) observed that tritordeum showed a higher GPC than durum wheat under organic management, while they observed no differences between the species under a conventional system. Folina et al. (2020) verified that tritordeum played a major role in increasing GPC after inorganic fertilization, compared to an organic one. Landolfi et al. (2021) compared a tritordeum cultivar with a landrace and a modern wheat, and

reported GPCs of 16.6%, 17.7% and 14.9%, respectively. They showed that the kernel gluten content of tritordeum and landrace, which showed the highest values of GPC, was influenced by increases in the total N fertilization rate.

Tritordeum seems to have a lower essential amino acid content of these compounds (66 g amino acid/l6 g of N) than durum wheat (69.8 g amino acid/l6 g of N) (Cubero et al., 1986). Furthermore, a slightly higher essential amino acid content in tritordeum has been reported in Central-Europe, and of phenylalanine, valine and cysteine in particular, than in bread wheat (Martinek et al., 2003).

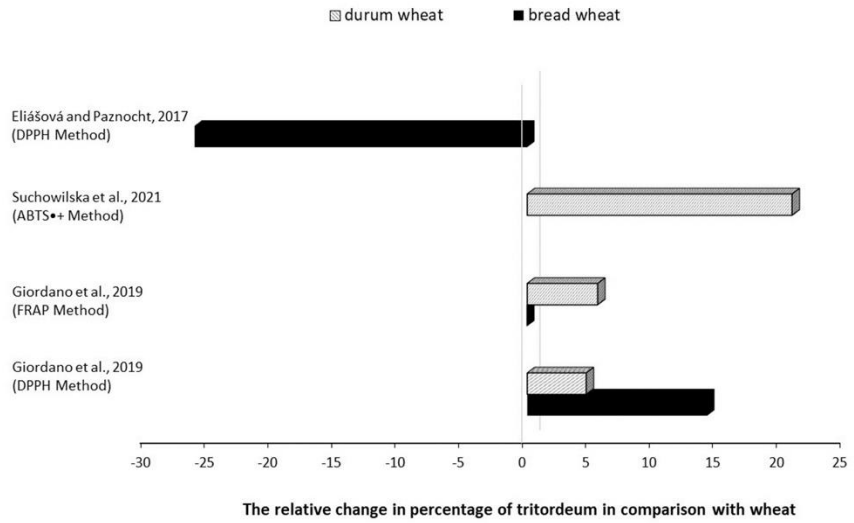
Bioactive compounds with antioxidant activity

Since the primary tritordeum lines were first developed, their phytochemical composition has shown its great potential as a raw ingredient for the production of healthy food (Alvarez et al., 1995). In fact, *H. chilense* is an interesting source of phytochemicals that exert antioxidant activity (Eliášová and Paznocht, 2017). The total antioxidant capacity depends on the synergistic interaction of several phytochemical compounds that are present in cereals, whole grains and flours (Adom and Liu, 2002). Although phenolic acids are generally the main compounds of cereals with antioxidant activity, other grain phytochemicals could also contribute to their overall antioxidant potential (Cömert and Gökmen, 2017).

The tritordeum kernel has shown an overall higher content and activity of antioxidant compounds than bread or durum wheat (Figure 5). Most of the available data, which have been obtained considering different methods to compare the antioxidant activity (DPPH, ABTS•+, FRAP), have highlighted better results for tritordeum than for bread or durum wheat. Only the study of Eliášová and Paznocht (2017) reported a clearly better total phenolic content and total antioxidant activity for bread wheat than for tritordeum. Furthermore, Eliášová and Paznocht (2017) and Giordano

et al. (2019) reported that barley kernels had an even higher total antioxidant activity than tritordeum and wheat.

Figure 5. Comparison of the percentage difference in the total antioxidant activity of tritordeum, durum and bread wheat.



The data sources are reported in the figure.

Giordano et al. (2019) verified that the compounds that confer an antioxidant capacity are mainly concentrated in the outer layer (10−20% external pearling layers) of tritordeum, durum and bread wheat, and of barley, thus also confirming the importance of the use of the pericarp fraction of tritordeum to obtain flour with a high health value. Furthermore, in their study on tritordeum, they identified a greater antioxidant activity (+50%) in the inner layers of the grain than for the other cereals. Thus, refined tritordeum flours represent a promising healthy raw food material and have a better photochemical profile than wheat. The bioactive compound content of tritordeum with antioxidant activity is analyzed in depth in the following sections.

Carotenoids

Carotenoids are isoprenoid compounds that can be divided into hydrocarbon carotenes (i.e. α and β-carotene and lycopene) and their oxygenated derivatives, which are called xanthophylls (i.e. lutein and zeaxanthin) (Paznocht et al., 2018). These elements are the characteristics that to give endosperm grains and, consequently, flour, their yellow color. The assumption of carotenoids in a diet confers several beneficial aspects because of their function of protecting individuals from photo-oxidative damage and limiting membrane damage (Atienza et al., 2007a), as well as of reducing the risk of developing certain types of cancer and other degenerative and/or chronic diseases (Al-Delaimy et al., 2005). Moreover, β-carotene presents provitamin A activity, which is responsible for maintaining certain properties against ocular degeneration, for protecting the skin from sunlight as well as being involved in healthy bone development and strengthening the immune system (Olson, 1989). Carotenoids have a great health potential, which is an important target for cereal bio-fortification (Farré et al., 2010). They are also of great interest because of their visual role in the consumers' choice of derived food products.

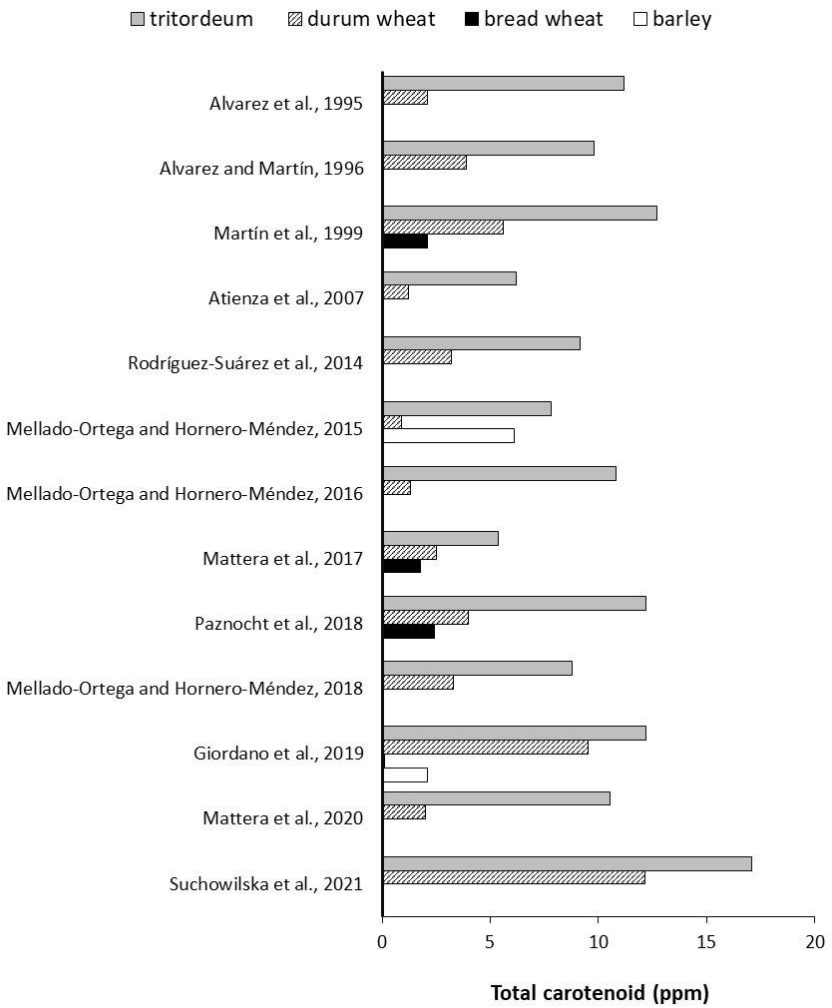
As far as the carotenoid profile is concerned, lutein is the most represented compound (>90%) in tritordeum (Mellado-Ortega and Hornero-Méndez 2015). With an average of 7 mg/kg of lutein, tritordeum presents much higher values than durum (+53%) and bread wheat (+67%), or barley (+37%). Durum wheat does not contain any lutein in esterified form and this aspect weighs negatively on its final amount, compared to tritordeum, which, on the other hand, accumulates most of the luteins in this more stable form (Mellado-Ortega and Hornero-Méndez 2018). Another important factor is related to the weather conditions during grain filling: it has been reported that high temperatures during ripening could lead to a decrease in the total carotenoid and free lutein contents of tritordeum (Mattera et al., 2017). Zeaxanthin compounds, even though in

a smaller number than luteins, are the second xanthophyll in number in tritordeum. This compound is principally concentrated in the intermediate part of the kernel (Giordano et al., 2019), and it has a similar content to that of wheat, although even 100 times greater concentrations have been found in barley (Table 3).

Tritordeum and bread wheat show similar amounts of α- and β-carotenes (almost 0.1 ppm) to durum wheat, while no carotenes have been identified in barley (Table 3). An extremely low level of carotene compounds has been detected in all of the so far studied cereal cultivars, and this is probably due to the rapid hydroxylation and formation of xanthophylls, followed by different esterification reactions (Paznocht et al., 2018). The esterification process in tritordeum is caused by the *Hordeum* genome (Mellado-Ortega and Hornero-Méndez, 2015). This process, which is clearly present in both tritordeum and durum wheat, permits the accumulation of carotenoids to be preserved at the endosperm grain level, thus transforming them into esterified forms during both the storage period and the flour baking process (Atienza et al., 2007a; Mattera et al., 2020; Mellado-Ortega and Hornero-Méndez, 2016) (figure 6), but also their bioavailability to be improved and their uptake and transport to be facilitated (Pérez-Gálvez and Mínguez-Mosquera, 2005).

As far as the distribution of carotenoid compounds in the kernel is concerned, there are conflicting results in the literature: Mellado-Ortega and Hornero-Méndez (2018) observed a homogeneous distribution between the outermost and innermost layers, while Giordano et al. (2019) verified a marked decrease from the external to the inner pearled fractions, similar to the behavior of durum and bread wheat observed by Mellado-Ortega and Hornero-Méndez (2015).

Figure 6. The total carotenoid content in tritordeum, durum wheat, bread wheat and barley



The data sources are reported in the figure.

Table 3. Concentration (ppm) of the main carotenoid compounds in tritordeum, durum and bread wheat, and in barley.

Carotenoid component		Study	tritordeum	durum wheat	bread wheat	barley
Xantophill	Lutein	Atienza et al., 2007	2.75	0.9		
		Giordano et al., 2019	5.3	4.6	2.2	2.1
		Mattera et al., 2017	0.2	0.2	0.1	
		Mattera et al., 2020	7.6		1.0	
		Mellado-Ortega and Hornero-Méndez, 2018	7.9	1.8		
		Mellado-Ortega and Hornero-Méndez, 2015	7.7	0.7		5.4
		Mellado-Ortega and Hornero-Méndez, 2016	7.5	0.9		
		Paznocht et al., 2018	10.5		2.9	0.9
		Rodríguez-Suárez et al., 2014	9.1	2.9		
		Suchowilska et al., 2021	12.0	9.3		
	Zeaxanthin	Giordano et al., 2019	0.5	0.5	0.6	1.4
		Mattera et al., 2017	5.13	2.31	0.5	
		Mattera et al., 2020	0.19		0.14	
		Mellado-Ortega Hornero-Méndez, 2018	0.5	0.9		
		Paznocht et al., 2018	0.9		0.7	1.3
		Suchowilska et al., 2021	2.02	1.58		
Carotene	α-carotene	Paznocht et al., 2018	0.1		0.1	
	β-carotene	Mattera et al., 2017	0.1	0.02	0.03	
		Mattera et al., 2020	0.4		0.2	
		Mellado-Ortega and Hornero-Méndez, 2018	0.1	0.1		
		Mellado-Ortega and Hornero-Méndez, 2015	0.1	0.02		0.02
		Mellado-Ortega and Hornero-Méndez, 2016	0.1	0.03		
		Paznocht et al., 2018	0.04		0.1	0.03
		Rodríguez-Suárez et al., 2014	0.1	0.1		
		Suchowilska et al., 2021	2.5	1.0		

The data sources are reported in the table.

Phenolic compounds

Numerous studies have demonstrated the presence of a wide range of antioxidant compounds in cereal grains, mainly concentrated in the bran tissues, but among these, phenolic compounds certainly stand out because of their greater beneficial effect on human health (Beta et al., 2005). Phenolic compounds include all the molecules that have one (phenolic acids) or more (polyphenols) aromatic rings with hydroxyl groups. These elements are responsible for the distinctive antioxidant activity of these compounds, which are able to inactivate free radicals and prolong the quality and shelf-life of food (Minatel et al., 2017). Phenolic acids are important elements for a healthy diet because of their antioxidant and anti-inflammatory properties (Sosulski et al., 1982) as well as their antitumor preventive effect (Riahi-Chebbi et al., 2019). Furthermore, phenolic acids are known to give excellent organoleptic characteristics, such as the flavor, taste, and color of the raw flour material used for the final baking of foods, to dough and, consequently, to processed products.

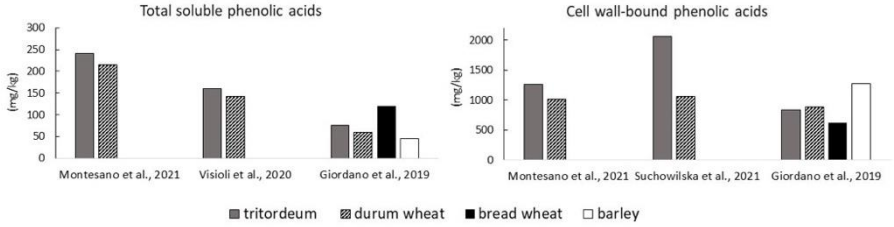
The main grain antioxidant compounds in tritordeum are phenolic acids, as in wheat and barley (Navas-Lopez et al., 2014). A relationship between the presence of phenolic acids and antioxidant activity has been reported for both tritordeum and bread wheat (Eliášová and Paznocht, 2017).

Phenolic acids are divided into soluble (SPA) and cell-wall bound (CWBPA) types, according to their solubility.

The total phenolic acids measured in a few studies (Figure 7), which compared tritordeum with durum and bread wheat, as well as with barley, showed that tritordeum grains had a greater soluble phenolic acid content than bread wheat (+25%) and barley (+46%), respectively. Giordano et al. (2019) confirmed the high content of SPA in tritordeum, compared to bread wheat, although it resulted in a 11% lower content than a durum wheat cultivar. Montesano et al (2021), Suchowilska et al. (2021) and

Giordano et al. (2019) also reported a higher CWBPA content in tritordeum than in durum wheat.

Figure 7. The total soluble and cell-wall bound phenolic acids in tritordeum, durum wheat, bread wheat and barley



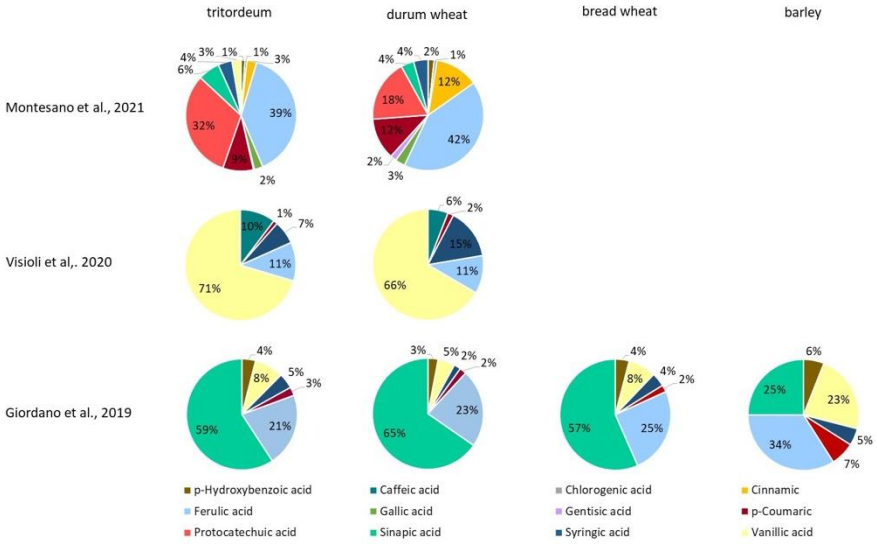
The data sources are reported in the figure.

Furthermore, Giordano et al. (2019) found that the content of these compounds was the highest in barley, followed by bread wheat. Giordano et al. (2019), studying the distribution of these bioactive compounds in grain pearled fractions, highlighted that the CWBPAs in whole grain were even lower in tritordeum than in bread wheat, and that tritordeum presented a 30% higher content at the inner level of the kernel than bread wheat.

Several differences in the phenolic acid composition in tritordeum have been pointed out in the available studies (Figure 8 and Figure 9), mainly on SPAs. Montesano et al. (2021) have underlined a greater difference in the composition of tritordeum than that of durum wheat, while Giordano et al. (2019) observed a clearly different SPA profile of tritordeum, albeit only in barley.

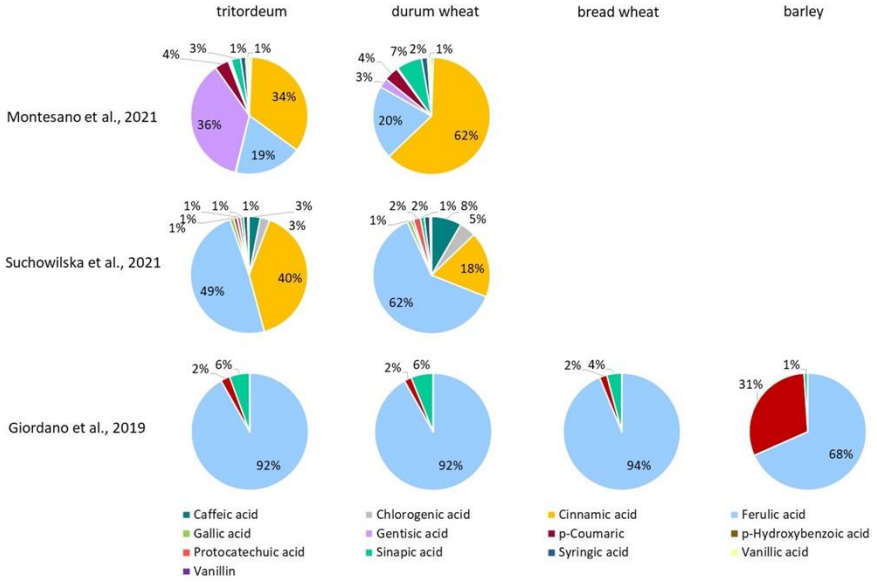
Among the various CWBPAs, ferulic acid is always the most widely concentrated phenolic acid, and it is followed by sinapic acid (Giordano et al., 2019) or cinnamic acid (Montesano et al., 2021; Suchowilska et al., 2021). These studies reported a similar composition of CWBPAs in tritordeum and durum wheat.

Figure 8. Soluble phenolic acid composition in tritordeum, durum wheat, bread wheat and barley



The data sources are reported in the figure.

Figure 9. Composition of the cell wall-bound phenolic acid in tritordeum, durum wheat, bread wheat and barley



The data sources are reported in the figure.

Other compounds

Among the other compounds that play an antioxidant role, selenium, which is essential for the antioxidant function of the glutathione peroxidase enzyme (Haug et al., 2008), has been receiving increasing attention because of the natural fortification of foods with this element. Tufarelli et al. (2016) demonstrated that an increased fertilization of selenium in tritordeum grain used for feeding hens can improve egg quality, without any collateral effects on their productive performance. Furthermore, Minh et al. (2017) found that in natural conditions the selenium content was lower in tritordeum kernels (0.08 mg Se/kg DM) than in wheat (1.6 mg Se/kg DM).

Pedrazzani et al. (2021) quantified 5-n-alkylresorcinol (AR) for the first time, in saturated and unsaturated AR homologues, and in the oxidized forms (2′-oxo) of different small cereals. Although they reported a large between cultivar variability for bread wheat, tritordeum showed a higher AR content than bread wheat (+11%). ARs have a high antioxidant activity and play many different biological roles, such as that of a cholesterol-like effect (Zawilska and Cieślik-Boczula, 2017), Bordiga et al. (2016) found that these compounds are mainly located in the external layer of cereal kernels.

Tritordeum kernels have shown higher vitamin E and tocol contents than bread wheat (+5%), although they are lower than in barley (-4%). Lachman, (2018) reported that tritordeum contains fewer tocopherols (-36%) but more tocotrienols (+ 30%) than bread wheat, and these are mainly located in the endosperm.

An interesting oleic acid content (14% of the total fatty acids) is present in tritordeum, in particular in the outermost layers of the kernel, and this is one of the most important fats in the Mediterranean diet because of its characteristic beneficial effects on the cardiovascular system (Mellado-Ortega and Hornero-Méndez, 2012).

The wholemeal flours obtained from tritordeum provide several other important nutrients that are fundamental for metabolic processes, such as calcium, potassium, sulfur, iron and zinc, and in higher concentrations than in durum wheat (Visioli et al., 2020).

Technological aspects

Rheological and baking quality

Gluten, which is a complex matrix composed of endosperm storage proteins (gliadins and glutenins), represents 60% of the grain proteome and has the function of supporting seedling growth (Shewry, 2009). From a food technology point of view, gluten plays a major role in the formation of the technological properties of dough and, consequently, the potential bread making use and dough rheological properties are based on the gliadin/glutenin ratio and the amount and composition of prolamins (Ronga et al., 2020). According to the information that is currently available, the bread-making aptitude of tritordeum seems to be closely related to the gluten composition inherited from *H. chilense* (Sillero et al., 1995). In fact, the high storage protein content of tritordeum is derived from both the *Hordeum* and *Triticum* genomes, since *H. chilense* is also characterized by a high aptitude to accumulate seed storage proteins (Alvarez et al., 2004; Martín et al., 1999).

The *Hordeum* genome is a good source of genetic variability of prolamin proteins, and it could be an excellent source of interesting technological and bread-making characteristics for tritordeum (Martín et al. 1999a). These quality traits are linked to the puroindoline genes (Pina-D1 and Pinb-D1), which are located on wheat chromosome 5D, and the homologue hordoindoline genes Hina and Hinb in barley (Guzmán e Alvarez 2014). The Hina-Hch1 and Hinb-Hch1 genes in *H. chilense* are very similar to the puroindoline genes of bread wheat, which may help to explain the soft grain texture of tritordeum. In fact, the addition of

chromosome 5Hch to durum wheat resulted in the enhancement of grain softness (Yanaka et al. 2011). Thus, although one of the parental lines of tritordeum is a durum wheat cultivar, the similarity, in terms of derived flour quality, is much closer to that of bread wheat (Alvarez and Martin, 1994; Pinto et al., 2002) and it is interesting for bread-making and baking processes (Alvarez et al., 1994; Alvarez et al., 1995; Alvarez and Martín, 1996) in addition to pasta-making use (Martín et al., 1999).

In order to compare the bread-making traits of tritordeum and bread wheat, the flour gluten content and composition, that is, the gliadin/glutenin ratio (glia/gs), the HMW-GS/LMW-GS (H/L), the alveographic parameters (dough strength, P/L ratio), the farinographic stability, the dough development time (DDT) and the loaf volume are reported in Table 4. It can be seen that the gluten content of tritordeum is higher than that of *bread* wheat (+14%), in agreement with the higher GPC. As far as the gluten composition is concerned, Landolfi et al. (2021) reported that tritordeum had a 10% higher glia/gs ratio than bread wheat. Moreover, tritordeum has a clearly lower occurrence of high-molecular-weight glutenin subunits (HMW-GS), which are closely related to the dough strength, than low-molecular-weight glutenin subunits (LMW-GS). Thus, the *H/L ratio of tritordeum is clearly lower (-170%).* Ballesteros et al. (2003) demonstrated how an artificial introgression of HMW glutenin subunits 1Dx5 + 1Dy10 from a bread wheat into a tritordeum cultivar can improve its bread-making quality, without significantly changing its agronomic traits.

Another particular difference in the gluten composition of tritordeum is its lower levels of ω-gliadins than wheat, which have been verified in both flour (Landolfi et al., 2021) and bread (Vaquero et al., 2018). Landolfi et al. (2021) investigated the effect of a higher N fertilization on the gluten composition of old and modern varieties of bread wheat, compared with tritordeum, but they did not observe any significant effect.

Table 4. Comparison of the rheological and technological properties of tritordeum and bread wheat

Rheological and technological parameter	**Reference**	**tritordeum**	**bread wheat**	
Gluten	Gluten (% dry weight)	Alvarez et al., 1992	11.2	8.9
		Alvarez et al., 1995	12.5	11.0
		Martín et al., 1999	11.2	8.9
		Landolfi et al., 2021	16.6	16.3
Gluten composition [1]	Glia/GS	Landolfi et al., 2021	1.0	0.9
	H/L	Landolfi et al., 2021	0.3	0.8
Chopin alveographs [2]	W ($J*10^{-4}$)	Alvarez et al., 1992	121	330
		Alvarez et al., 1995	111	290
		Pinto et al., 2003	77	283
		Ballesteros et al., 2003	84	267
	P/L	Alvarez et al., 1992	0.6	0.9
		Alvarez et al., 1995	0.3	0.6
		Ballesteros et al., 2003	0.7	1.0
Brabender farinograph [3]	Stability (min)	Alvarez et al., 1995	5.1	15
		Martinek et al., 2003	2.7	9.3
	DDT (min)	Alvarez et al., 1995	2.5	7.3
		Martinek et al., 2003	2.7	1.0
Baking properties	Loaf volume (cm3)	Alvarez et al., 1995	441	579
		Martinek et al., 2003	260	397

1 Relative quantification of the gluten fraction on the basis of an RP-HPLC analysis; Glia: gliadins, GS: glutenins; H: High molecular weight glutenin; L: low molecular weight glutenin.
2 Alveographic parameters W: dough strength; P/L: ratio between tenacity (P) and extensibility (L)
3 Farinographic parameter, dough stability and dough development time (DDT).
The data sources are reported in the table.

These data also confirm that the crop management of this new species clearly does not affect the composition, but instead affects the gluten

content (Johansson, Prieto- Linde, e Jönsson 2001), while the relative percentage of the gluten fraction is mainly a consequence of the genotype (Landolfi et al., 2021).

Thus, mainly as a consequence of a lower HMW-glutenin occurrence in gluten, tritordeum overall results in lower bread-making and baking qualities than bread wheat (Alvarez et al., 1995; Alvarez and Martín, 1996; Martinek, 2003; Martín et al., 1999).

Tritordeum has shown a lower W (-200%) than bread wheat, and also a lower or similar tenacity (P), but a generally higher extensibility (L), thus resulting in a decrease in the P/L ratio (-60%). The lower P/L ratio could make tritordeum dough rather too extensible and sticky for the bread-making process (Martinek et al., 2003). According to these authors, the high extensibility of tritordeum dough could be a consequence of the higher content of sulfur-containing amino acids, such as cysteine, which are involved in the formation of intra- and inter-disulfide bonds among individual groups of prolamin.

Overall, on the basis of the rheological quality of the flour, tritordeum can be used as a raw material for bread-making, although not for prolonged leavening, and appears to be more suitable for bread-making than for pasta-making, due to the lower HMW-GS content (Alvarez et al., 1995; Martín et al., 1999). Furthermore, apart from the positive and distinctive characteristics of tritordeum flour, concerning its extensibility of the dough, the eye-catching color of the bread and other baked products, which is obtained thanks to the high carotenoid content of the flour, is of particular interest.

Gluten related diseases

In addition to its technological role, the complex network of gluten protein and peptide sequences has been investigated in depth in recent years, in relation to its role in gluten disorders. Gluten is in fact the primary cause of celiac disease (CD) but is also considered to be responsible for other gluten-related diseases, such as non-celiac gluten sensitivity (NCGS),

and this topic needs a greater understanding of how it acts as a trigger in these widespread diseases.

Tritordeum contains glutens, and it is therefore not suitable for people who suffer from celiac intolerances/allergies, just like bread and durum wheat, spelt, emmer, einkorn, barley and rye. Furthermore, the consumers and food supply chain are now demanding clearer information about the factors that could exert an effect on the peptides that are responsible for different gluten disorders from CD (Jouanin et al., 2018). Numerous fragments of peptides with CD-toxic effects, which are called epitopes, may also be responsible for other gluten-related diseases, such as NCWGS. CD-toxic epitopes can be quantified using an ELISA immune-enzymatic assay with antibody R5, which recognizes the QQPFP, LQPFP, QQQFP and QLPFP amino acid sequences in cereal flour or digested food samples (Kahlenberg et al. 2006). The R5 ELISA assay has demonstrated that tritordeum flour could have fewer highly celiac epitopes than bread wheat (Landolfi et al., 2021; Vaquero et al., 2018). Landolfi et al., (2021) verified this significant difference through a comparison with landrace (-51%) and modern wheat (-58%) cultivars and suggested that a link exists between the lack of the D genome in this amphiploid and a lower immunodominant toxicity. Sánchez-León et al. (2021) have also recently studied tritordeum bread, in terms of its potential triggering of celiac disease (CD) and non-celiac wheat-sensitivity (NCWS). They studied the resistance of immunogenic peptides to digestion and the structure of the intestinal microbiota after tritordeum bread ingestion. They also studied the response of NCWS patients, either non-celiac or affected by a wheat allergy, but with health symptoms related to the ingestion of gluten-containing foods, and they reported no significant changes between gluten-free bread and tritordeum bread. They analyzed the bacterial gut microbiota, showed that the ingestion of tritordeum bread does not modify the overall composition of the intestinal microbiota, and observed only a few changes of some butyrate-producing bacteria. This

result suggests that some patients with NCWS might find tritordeum more tolerable than gluten-free bread, while the same good quality levels of bread could be maintained, in terms of the organoleptic and nutritional properties.

Conclusion

Tritordeum has been studied for more than 40 years, although its cultivation for commercialization in the baked goods supply chain is a recent achievement. This crop could be an interesting alternative to wheat for producers and consumers, since it shows a high adaptability to different growing areas, the possibility of being used in the production of a large number of baked and non-baked food products, and many notable and captivating features, from the health and organoleptic points of view.
This review has shown that genetic studies and breeding are crucial to increase the overall competitiveness of tritordeum, considering the possible agronomic, yield, sanitary, nutritional and technological improvements. The necessity of addressing the specific traits of new tritordeum cultivars, considering the environmental conditions of different growing areas, has been pointed out, as well as the need of a greater ability to adapt to climate change in order to increase the sustainability of this species. In addition to fundamental genetic improvements, it will be necessary to set up cropping system and agronomic practices for this species, in order to achieve a substantial increase in the yield and an improvement of the qualitative traits, as well as to minimize the sanitary risk related to contamination by mycotoxins, particularly in Central-European areas.
Considering the interest of consumers and industries in special baked products, obtained from different raw materials, and the interesting nutritional profile of both refined and whole grain flours, tritordeum could successfully be used as an ingredient for food production purposes. In

such a context, more information is necessary on the technological properties of flours derived from this cereal and the end-use values necessary to obtain food products, in order to allow the raw materials to be fully exploited and the management of the technological processes and the future breeding activities to be correctly addressed.

In short, the research activity that will be conducted in the coming years will be fundamental for the development of the cultivation of tritordeum and its supply chains, in order to obtain an agronomical and economical interesting alternative to the present cropping systems, as well as high innovative food products with health and organoleptic added value.

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Chapter VII – Conclusion and perspectives

This PhD project has involved the investigation of several factors that are able to influence the gluten content and composition of cereal grains and flours that can be used for baked goods, by taking into account different approaches and methodological analysis. As far as cereals are concerned, the wheat species shows one of the highest capacities of guaranteeing a high yield, resistance to diseases, a good food protein source, and an optimal rheological performance of flours, according to the end-use, in different environments and at a low cost. In fact, wheat is one of easiest crops to cultivate, and it has relatively easy agronomic management practices, and a great possibility of being processed in different derivative products. Thus, wheat is one of the main crops used throughout the world as a basic ingredient in human diets. However, a negative aspect of this crop is related to the possibility of its triggering intolerances and allergies in adults and children (Casella et al., 2018; Fasano and Catassi, 2012; Matsuo et al., 2004). Such kinds of health disorders are gluten related, and for this reason gluten has been widely studied to broaden the knowledge of its complex and partly redundant nature (Tye-Din et al., 2010).

The variation of the protein content in this viscoelastic network and in the ratios of these proteins was first investigated in bread wheat to establish the effects induced by crop management practices and by the environment. Late N fertilization at heading is a crop fertilization strategy that can significantly increase the GPC content of flour, increase all the gluten protein fractions and enhance different rheological parameters, such as dough strength (W), without altering the ratio between different gluten prolamins.

Moreover, the use of a fungicide at heading has a secondary impact on bread wheat gluten; although the key role of this crop protection practice is to achieve a substantial increase in grain yield, this late season

application does not involve any significant changes in the gluten content or in its composition.

On the other hand, the only differences that have been highlighted, in terms of prolamin composition and concerning the rheological properties of flour, are related to the meteorological trends observed in different growing seasons during the grain filling period. Studies in North Italy have shown how high temperatures during grain ripening lead to a greater protein accumulation, but also to an inversion in the protein ratio: a greater percentage of gliadins emphasizes the viscosity of the dough, at the expense of more elastic glutenins, and results in a lower overall strength of the flour and extensibility of the dough (P/L).

In addition to the role of N fertilization, the genetic wheat traits have been investigated considering different varieties cultivated in the same environmental and under the same agronomic conditions: landrace and modern wheat cultivars, as well as a new wheat hybrid called tritordeum.

The study of the genetic wheat traits have highlighted a similar positive response to the N fertilization rate of the cultivars, in terms of increasing GPC, and a constant ratio of the gluten protein fractions, even though the genotype is certainly the factor that influences the content of the different fractions of gluten proteins and their relationship the most. Tritordeum has shown a lower content of ω5-gliadin and LMW-GS than bread wheat, for both landrace and modern cultivars.

Another important difference that has been observed in the different genotypes is the presence of immunogenic and allergenic epitopes in flour after digestion. The resistance of numerous fragments containing epitopes, which have been identified as being harmful for CD or WDEIA patients, has been studied, through a qualitative approach, using untargeted mass spectrometry analysis, after a simulated gastro-duodenal digestion of the refined flours and their bread-making products (Brodkorb et al., 2019).

The analysis of flour and processed products in which *in vitro* digestion is simulated has led to a great innovation and to a greater level of complexity in the characterization of their peptidomes, their digestibility and inflammatory capacity, especially given the high digestion resistance of the so far discovered gluten proteins (Hausch et al., 2002). Most CD epitopes belong to the α-gliadin fraction, with almost 60% of all the epitopes having been identified by means of *in silico* analysis, and this fraction is followed by γ-gliadins. The opposite situation has been observed for allergenic epitopes; both situations have been confirmed in landrace, modern wheat and in tritordeum.

The results show that no data are available in favor of the hypothesis that landrace genotypes contain smaller numbers of immuno-toxic epitopes for varieties grown in the same environments and with the same crop techniques. ELISA quantitative tests, conducted on digested flours, have confirmed there are no differences, in terms of CD-immunogenic epitopes, between landrace and modern wheat varieties, while the only significantly lower values have been quantified for tritordeum flours. The same result pertaining to the low immunotoxic ability of tritordeum has been confirmed in another experiment carried out in another location and for another year, by comparing tritordeum breads and flours, even in a digested matrix, with a bread wheat hexaploid line and durum wheat tetraploid line.

A possible explanation for the lower toxic immunoreactivity of the tetraploid (AABB) and tritordeum amphiploid (AABBHchHch) observed in the aforementioned study, compared to that of hexaploid bread wheat (AABBDD), is the lack of a majority of immunotoxic sequences of the D genome (Spaenij–Dekking et al., 2005), as derived from *T. tauschii* sequences.

These data suggest that tritordeum is an interesting candidate for cereals that can be used in diets characterized by a minimal activation of the innate immunity and a reduced number of toxic gluten peptides, a result that has also been supported by a recent study in which a tritordeum-

based diet for patients with irritable bowel syndrome led to a significant reduction in symptoms (Russo et al., 2022).

Although this study shows an interesting proteomic characterization of cereal flours and breads, after simulated digestion, it is still necessary to study the peptidomic profile in depth to obtain a more complete gluten disorder related epitope characterization, while considering the requests of the supply chain.

Since a complete and exhaustive list of all HLA-DQ restricted gluten T cell epitopes does not yet exist, a targeted mass spectrometry approach remains somewhat limiting, and an untargeted approach is still too dispersive, as it results in the redundancy of the fragments with potential gluten epitopes. For this reason, more studies are necessary in this field to increase our knowledge of immunotoxic epitopes and their possible causes.

Until now, all the collected data place tritordeum species in a position of great commercial and productive interest and consider it a potentially healthier cereal than common wheat for the realization of common baking goods.

The amphiploid presents several interesting positive aspects, in particular a lower potential immunotoxicity than bread and durum wheat, and the possibility of similar processing to obtain baking goods. The baking of tritordeum flour has led to good results and compared to bread wheat, it has the added benefit of a bright yellow color, due to the high concentration of carotenoids, which constitutes an added value, thanks to the variety health advantages of such substances. Another positive health aspect of tritordeum grain is the potentially higher content of insoluble and soluble fibers, which could confer several beneficial effects.

At the same time, the agronomic performances of tritordeum, mainly in terms of the control of FHB diseases and grain yield, are lower than those of commercial bread and durum wheat cultivars grown in temperate areas. However, it is expected that breeding could lead to a significant genetic

improvement of this new species in the next few years, so that tritordeum cultivars that will be able to guarantee a greater agronomic competitive capacity will be commercialized.

The solution in terms of minor immunogenic potential cannot be related to the use of the current gluten-free flours used for common wheat baking preparations, as these products suffer from excessive limitations, due to the need to include numerous additives, such as thickeners and emulsifiers, which allow a good consistency and plasticity of the dough to be obtained (Blanco et al., 2011; Collar et al., 2015). However, these needs lead to products being obtained that are less healthy than the gluten-based equivalents, and which are also more expensive, and therefore it is still of great importance to obtain alternatives to offer to patients suffering from gluten-related diseases.

A possible fertilization approach, which has the aim of modifying the protein biosynthesis and, consequently, the number of gliadin proteins, and of potentially acting on the human pathologies induced by wheat, could be the use of a sulfur fertilization on wheat crops (Yu et al., 2018). In fact, the addition of a small amount of sulphur, with the aim of eluding sulphur soil limitations, generates an increase of NUE for the metabolic pathway glutamine synthetase regulation, which also induces a reduction in the gliadin protein content (Yu et al., 2021).

Gene editing, a targeted mutagenesis approach, is a very promising new method that can be used instead of the various approaches adopted to reduce wheat gluten immunogenicity, such as plant breeding strategies. Gene editing in fact makes it possible to remove, silence or modify gliadin genes and CD epitopes in a precise manner, and this may overall reduce the exposure of patients to CD epitopes. Several studies have obtained interesting results using knock out and the deletion of gluten genes with the CRISPR/Cas9 technology and RNAI silencing experiments on gliadin genes (Gil-Humanes et al., 2010; Jouanin et al., 2020; Sánchez-León et al., 2018). The first results of *in vivo* studies with non-celiac wheat

sensitivity (NCWS) patients showed that the consumption of bread made with low gliadin line genetically edited flour induced positive changes in the gut microbiota composition and no significant differences, in terms of aroma, flavor, texture, or overall acceptability (Haro et al., 2018). Furthermore, these applications of gene editing technologies should be considered within the context of the food sector, and in view of the present and forthcoming national and international regulatory outlines, where, for example, the position of the Italy concerning the use of these technologies has not yet been defined.

The understanding and results obtained in this PhD work provide useful information that can be used to obtain a good and balanced management of cereal in order to achieve a positive relationship between the grain protein content and gluten composition. Moreover, this work has highlighted the importance of resorting to consolidated *in vitro* digestion to represent a realistic situation of resistant peptides containing toxic and immunogenic CD related epitopes in flours and bakery product derivatives.

Overall, this study has increased the understanding of the importance of the utilization of plant genetic resources for the development of viable food processing options, such as the potential positive traits of amphiploid tritordeum. In the aforementioned scenario of climate change and population increase, the use of new genetic varieties should also take into consideration the need to produce new products that satisfy the preferences and the demand for healthier foods of consumers.

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