Comprehensive REVIEWS in Food Science and Food Safety

How Looking for Celiac-Safe Wheat Can Influence Its Technological Properties

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Abstract: Because of the continuous increase in the prevalence of gluten-related disorders, selection of wheat with a low content of immunogenic gluten epitopes could be an innovative alternative for prevention. In this review, the focus is on literature data concerning the deallergenization tools of wheat, which are mainly related to breeding approaches (classic and advanced) and processing operations (germination and fermentation). Until now, no safe wheat genotype has been identified, whereas decreasing wheat allergenicity is possible. On the other hand, the decrease of gluten or some of its epitopes can strongly affect technological properties. Thus, obtaining celiac-safe gluten without affecting the technological properties of wheat could be considered as a new challenge that scientists will be facing. Celiac-safe wheat-based product development could be a great revolution in the market of foods for special medical purposes. The present paper is aiming to: (a) review the strategies and the approaches used, or that can be used, for developing low allergenic wheat: their utilities and limits were also discussed and (b) screen the impact of gluten reduction or removal on the quality of wheat end-use products.

Keywords: celiac disease, gluten, wheat safety, wheat quality, wheat technology

Background

Celiac disease

Celiac disease (CD) is among of the best understood autoimmune diseases, given the advancement of the knowledge of the environmental, genetic, and immunologic factors associated with this disorder (Kabbani and others 2014). This pathogenesis involves gluten as an external trigger and genetic predisposition (Mamone and others 2013). For CD cases, after gluten deamidation by the tissue transglutaminase, the modified peptides bind to human leucocyte antigen (HLA) molecules, DQ2 and DQ8, resulting in the destruction of intestinal CD4+ T-cell (Real and others 2012; Mamone and others 2013; Ribeiro and others 2015). The gluten peptides are involved in 2 immunological pathways. The innate immune response is activated by toxic peptides, which can damage the small-intestinal mucosa, whereas the adaptive immune response is triggered by immunogenic peptides, which can interact with HLA-DQ2/8 (Caputo and others 2012; Ferretti and others 2012).

Prevalence of CD keeps increasing drastically

In recent years, CD has gained much attention due to its rapidly increasing prevalence (Manti and others 2017). The incidence of CD in the U.S. was reported to be about 1% (Ludvigsson and others 2013a). However, a survey conducted from 2009 to 2012 showed that potentially 0.79% of the general U.S. population demonstrates serologic evidence of CD autoimmunity (Mardini and others 2015). In Europe, it was 2.4% in Finland, 0.3% in Germany, and 0.7% in Italy (Mustalahti and others 2010). In north China, it reached 0.76% (Yuan and others 2017). However, reliable data are absent in sub-Saharan Africa and in the Asia-Pacific region (Castillo and others 2015). Likewise, in North Africa, the diagnostic rate is still very low, mostly due to low availability of diagnostic facilities and poor disease awareness (Catassi and others 2014). CD, if undiagnosed and untreated, is associated with many complications including, hematological, metabolic, obstetric, gynecological, and neurological diseases, as well as enteropathy-associated T-cell lymphoma, small-bowel adenocarcinoma, and esophageal and oropharyngeal carcinomas (Miśkiewicz and others 2012; Rand and others 2013; Mahmud and others 2015).

Gluten is the major external trigger of CD

Gluten is a collective term for several seed storage proteins in wheat (gliadins and glutenins), barley (hordeins), rye (secalins), and some oat varieties (avenins) that are harmful to CD patients (Ludvigsson and others 2013b; Moreno and others 2015). Gluten, representing up to 80% of total grain proteins, is present as monomers, oligomers, and polymers (Kroghsbo and others 2013). Oligomers and polymers are linked by disulfide bonds between the S-containing amino acid cysteine, which make up approximately 2% of gluten (Kroghsbo and others 2014). Gluten is rich in glutamine, proline, and small amounts of lysine, methionine, threonine, and other amino acids (Gasbarrini and Mangiola 2014).

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Indeed, the gluten high content of glutamine (30% to 35%) and proline (10% to 15%) residues makes it resistant to complete proteolysis digestion, ensuring the survival of immunostimulatory epitopes to digestion (Ribeiro and others 2015). For people with a CD genetic susceptibility, these peptides are considered the main triggers of the immune reaction.

Several researchers have studied wheat epitopes triggering CD. Gliadins are considered the major factor causing issues for CD subjects. Gliadins are divided into 4 monomeric classes: α-, β-,γ- and ω-gliadins. The most allergenic sequences occur in the repetitive N-terminal domain of α-/β-gliadins, which mainly consist of glutamine, proline, and aromatic amino acids, such as phenylalanine and tyrosine (Malalgoda and Simsek 2017). Notably, the α-gliadins are suggested to be the primary initiator of the inflammatory response to gluten in CD patients (Zörb and others 2013). The 31 to 49 α-gliadin peptide is the most implicated in the innate immune activation in the mucosa of CD patients (Mamone and others 2013). Furthermore, the *in vitro* digestion of wheat has revealed that major immune epitopes also belong to γ-gliadins (Prandi and others 2014). Low-molecular-weight (LMW)-glutenin proteins have also been associated with the induction of CD (Mamone and others 2015).

Gluten is a key ingredient for the quality of wheat and derived products

Wheat is the raw material for a wide range of food products like pasta, bread, bourghul, couscous, freekeh, cakes, and cookies. Wheat protein composition and characteristics are some of the determining factors for high-quality products. Based on their solubility, wheat protein could be subdivided into 2 groups: water/salt-soluble proteins and water/salt-insoluble ones or gluten (Scherf and others 2016). Gluten proteins, glutenins, and gliadins, are of great importance in dough processing conferring its unique baking quality involving water absorption capacity, cohesiveness, viscosity, and elasticity (Wieser, 2007; Tosi and others 2011). Glutenins are associated with dough elasticity, while gliadins are associated with viscosity and extensibility. Gluten proteins encoded by the genes located in the D genome are reported to make a profound impact on dough rheology, and hence bread-making characteristics of wheat flour (Delcour and others 2012).

Taking all the above into consideration, it is difficult to ensure high quality of baking for gluten-free product. Gluten-free dough lacks cohesiveness and elasticity, which makes it harder to handle (Houben and others 2012). To overcome the issues associated with the absence of gluten, bakers and cereal researchers have focused on improving batter consistency to achieve greater gas retention during proofing and baking (De la Hera and others 2013).

The challenge: a celiac-safe wheat preserving its technological quality

Until now, celiac patients had no option but they must follow a strict gluten-free diet. Despite the fact that gluten-free products are relatively expensive, CD is often not covered by health insurance programs. On the other hand, managing a dietary-controlled health condition, such as CD, creates pressures that may raise the risk of developing disordered eating behaviors (Satherley and others 2016). Gluten is also a widely used additive in foodstuffs, such as sauces, soups, and canned meals (Mejías and others 2014). Consequently, up to 50% of subjects do not comply properly with the gluten-free diet, which results into developing an active symptomatology (Bernardo and Peña 2012). Thus, CD patients are facing a scary imposed situation. Nutritional therapy for a lifelong time requires extensive care and attention. Food safety requirements should be respected to avoid any complications. Additionally, gluten-free food provides inadequate supplies of fibers, minerals, and vitamins and excess calories in the diet and exhibits poor sensory properties (De Angelis and others 2010). Moreover, the gluten-free diet excludes many cereal-based staple foods, which are important sources of energy, protein, carbohydrate, iron, calcium, niacin, and thiamine (Kinsey and others 2008). Removing these staple foods and their derived products from the diet can affect the nutritional status of individuals with CD. Although it is still difficult to draw a conclusion about the nutritional adequacy of a gluten-free diets because of conflicting results reported in available studies, several ones have found that the celiac population does not include the recommended intake of energy (Kinsey and others 2008), and amounts of fiber, several minerals, and certain vitamins (Kinsey and others 2008; Dall'Asta and others 2012). According to the *Codex Alimentarius* (CODEX STAN 118–1979, 2008), gluten-free foods are dietary foods consisting of/or made only from one or more ingredients that do not contain wheat or genetically related cereals and in which the gluten level does not exceed 20 mg/kg in total. Many immunological, proteomic and peptidomic methods have been developed as analytical tools to validate accurate gluten detection tests (Gianfrani and others 2016). Standardized ELISA-based assays for IgA autoantibodies against tissue transglutaminase remain the serologic test of choice for most populations (Leffler and Schuppan 2010). The availability of analytical methods to detect and determine levels of markers of priority allergens in foods is also of the outmost importance to support standard setting initiatives (Weber and others 2009). These standards are mainly quantitative with cut-offs to honor; but to ensure more reliability and effectiveness it might be necessary to set a database for toxic and immune sequences.

Besides CD, there is a multitude of diseases related to gluten, such as wheat allergy and non-celiac gluten sensitivity (NCGS). Indeed, nowadays, differentiating between CD and NCGS is often challenging, as there is a lack of evidence-based recommendations for the evaluation of patients reporting gluten-responsive symptoms (Kabbani and others 2014). In addition, a new trend of a gluten-free lifestyle is mounting among nonceliac consumers. Close to 1% of the population is electively following a gluten-free diet despite having no evidence of the disease (Mardini and others 2015). As result, the gluten-free market is expanding because of therapeutic food and lifestyle choices. The global market for gluten-free products was valued recently at 4.63 billion USD in 2015 and is projected to reach 7.59 billion USD by 2020, at a compound annual growth rate (CAGR) of 10.4% from 2015 to 2020. To respond to this rising demand, improving gluten-free technological quality is of high priority to satisfy consumer expectations. Due to the absence of a quality standards manual, gluten-free products with stable quality levels are still complicated to handle. Development of new technologies, mainly additives such as starches, hydrocolloids, enzymes, and fats, will make it possible to find alternatives for the traditional bakery products (Houben and others 2012).

Beyond a strict gluten-free diet, developing safe wheat varieties with a gluten content lower than 20 ppm and/or low immunogenic wheat might mean a revolution in the market of preventive and therapeutic gluten-free foods.

Several tools for the deallergenization of wheat are the subjects of numerous works (Figure 1). Although genetically manipulated wheat is neither marketed nor likely to be, given the opposition to GMO products in Europe, the selection of new varieties with

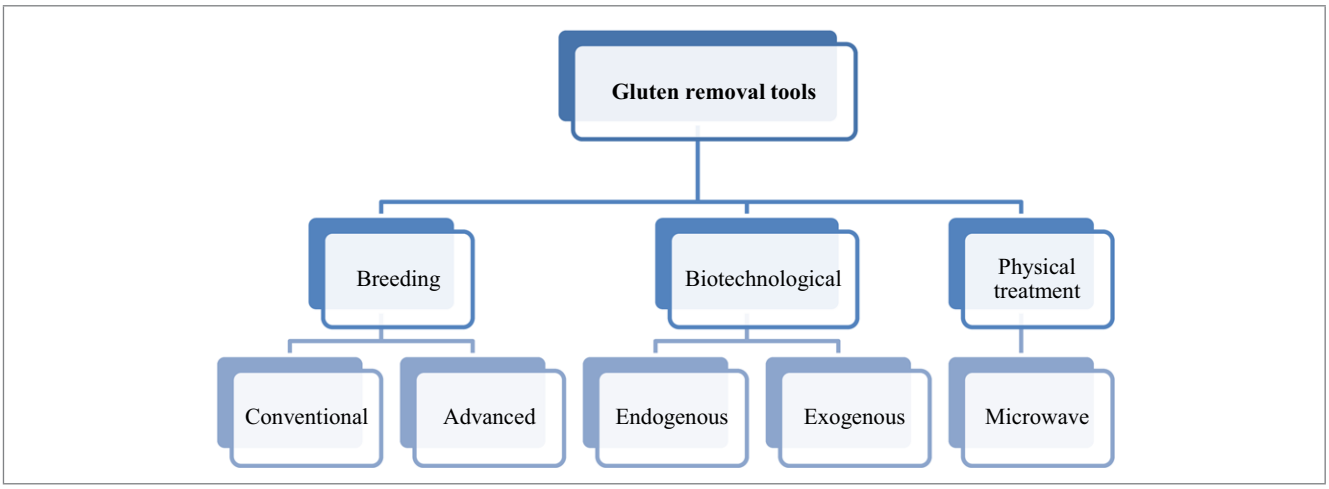

Figure 1–Wheat gluten removal tools. Several approaches are suggested with potential in gluten allergenicity removal. In this work, the focus is on genetic approaches, biotechnological tools and physical treatment (microwave heat).

low gluten is seemingly possible. Beside the genetic transformation, biotechnological approaches are also used as a treatment to reduce gluten. For instance, Gobbetti and others (2007) investigated the potential of selected sourdough lactic acid bacteria during fermentation; Stenman and others (2009) assessed germinating wheat proteases and, more recently, Landriscina and others (2015) brought a new method, called gluten friendly, which is based on microwave heat to remove the antigenic capacity of gluten. These approaches might offer a safe and good-quality product, as compared to gluten-free products thereby an official label for the products with up to 100 mg/kg as low in gluten could be launched (Walter and others 2014a). If removing the allergenicity of gluten peptides related to CD seems feasible, it still must be considered its effect on the technological properties of wheat-derived products.

Recent Advances in Wheat Gluten Allergenicity Abolishment Tools

Is the "ancient" wheat less allergenic than the modern one?

Since wheat domestication, several wheats (*Triticum* spp.) were selected namely, *Triticum monococcum* (AA genome), *Triticum speltoides* (BB genome), wild emmer (2n = 4× = 28; genome BBAA), which is the progenitor of domesticated tetraploid wheat (*Triticum durum*; 2n = 4× = 28; BBAA), and the hexaploid wheat (*Triticum aestivum* L.; 2n = 6× = 42; BBAADD) (Peleg and others 2011). Wheat, one of the Neolithic founder crops, now occupies 215 million hectares (16% of all cropland), providing about one-fifth of the calories and protein consumed by humans (Golan and others 2015). Despite being the world staple food, the genomes evolution versus wheat safety was often questioned, more particularly its implication in CD prevalence. Efforts have been made to investigate if there is a difference between ancient and modern wheat allergenicity through a screening of the evidence dealing with wheat genome progress influence on genes encoding gluten epitopes. Recent reports have suggested that ancient wheat cultivars are less allergenic and safer than modern wheat. *Triticum monococcum* does contain gluten, but it is different from most wheat because it contains only 14 chromosomes as opposed to 28 in emmer or 42 in modern wheat (Cooper 2015). Similarly, Lombardo and others (2015) indicated that it is possible to use *Triticum monococcum* in the production of hypoallergenic foods because it is ancestral wheat lacking B chromosomes. Further, Kucek and others (2015) suggested that D genome can be the responsible for the expression of the major part of CD epitopes because the genes encoding gliadin proteins are located on the short arms of chromosomes at 3 homologous loci-Gli-A1, Gli-B1, and Gli-D1 (Group 1) and loci-Gli-A2, Gli-B2, and Gli-D2 (Group 6) genes (Balakireva and Zamyatnin 2016). Some previous works (Molberg and others. 2005; Van Herpen and others 2006) indicated that genes encoding αG-33 mer proteins, α-gliadins most triggering epitopes, are absent in A genome and even certain cultivars of AB genome. It was suggested that the full immunodominant 33-mer fragment was only present in hexaploid wheat at low abundance, probably due to allohexaploidization events (Ozuna and others 2015).

A detailed analysis of the γ-gliadin transcripts in bread wheat revealed that almost half of the γ-gliadin transcripts (49%) were assigned to locus Gli-D1 (Salentijn and others 2012). Despite the advancement in genetic and molecular tools, there are no definitive data indicating that genome D is at 100% responsible for genes encoding CD epitopes because gliadins are encoded by a complicated multi-gene (Goryunova and others 2012). Regarding glutenin, the high-molecular-weight (HMW)-GS are encoded by loci on the long arm of group 1 chromosomes (Glu-A1, -B1, and -D1), while the LMW-GS are mainly encoded by the Glu-3 loci on the short arms of group 1 chromosomes (Glu-A3, -B3, and -D3) (Van den Broeck and others 2009). Šuligoj and others (2013) assessed the safety of a collection made up of *Triticum monococcum*, *Triticum speltoides*, *Triticum durum* (old and modern accessions), and *Triticum aestivum* using small intestinal gluten-specific T-cell lines generated from CD patients by proliferation assays. The results indicated that all strains of wheat independent of ploidy or ancient/modern origin were able to trigger heterogeneous responses. Further, Ribeiro and others (2016) found that epitopes were higher in spelt followed by wheat landraces and modern wheat varieties. Likewise, it was demonstrated that gastrointestinal digesta coming from old and new varieties of wheat contain the same amount of CD-related peptides, with even a slight prevalence of old varieties, concluding that old lines are not to be considered "safer" for subjects that are genetically predisposed to CD (Prandi and others 2017). Thus, the assumption that the progress in wheat genomic promoted allergenicity is not fully sustained, clearly because gluten epitopes are encoded by 15 major loci in the A, B, and D genomes of hexaploid wheat (Van den Broeck and others 2011). Indeed, the majority of the commercialized wheat species shares the A or B

Table 1–Gluten removal through genetic tools. It focused on the genetic tools used to remove or to reduce gluten in wheat. These approaches are either a target or untarget. The effectiveness of these methods is highly associated with the method adopted.

Tool	Approach	Method	References
Untarget target	Mutagenesis	Radiation or chemical agent	Chen and others (2014)
	Transgenesis	Down-regulation or shutdown of the expression of several gluten epitopes	Gil-Humanes and others (2008, 2011); Piston and others (2011)
Target tools	ZFNs	Zinc finger protein (ZFP) domain and a Fok I endonuclease cleavage domain	Carroll (2011); Xiao and others (2013). Chen and others (2014)
	TALENs	TALE-DNA-binding domains fused with nonspecific Fok I cleavage domains	Jankele and Svoboda (2014); Liang and others (2014)
	CRISPR	Combination of Cas9 genes and sequences for small trans-encoded CRISPR RNA.	Miao and others (2013); Shan and others (2014); Lawrenson and others (2015); Zang and others (2016).

genomes or both and consequently, putting forward D genomes exclusively as the allergens factory is not sustained by the available data.

Abolishment of gluten allergenicity through advanced genome editing approaches

Genetic editing of crops is an extremely versatile tool for providing sustainable productive agriculture to better feeding of a rapidly growing population (Khatodia and others 2016). Table 1 summarized the methods currently available for gluten removal or reduction (Table 1). Mutagenesis and transgenic approaches have been widely used to obtain wheat mutants. Nevertheless, these methods cannot target specific genes and require laborious work to identify the determined phenotype (Chen and others 2014). Remarkably, RNA interference technology proved quite an important success in the down-regulation or shutdown of the expression of several gluten epitopes offering low-gluten wheat (Barro and others 2016).

As for targeting approaches, sharp tools are developed to generate site-specific breaks for DNA binding domains including, Zinc Finger Nucleases (ZFNs), Transcription-Activator Like Effector Nucleases (TALENs), and, more recently, the Cas9 protein associated with Type II Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) (Lawrenson and others 2015). ZFN-induced double-strand breaks are subject to cellular DNA repair processes that lead to both targeted mutagenesis and targeted gene replacement at remarkably high frequencies (Carroll 2011). ZFNs contain a zinc finger protein (ZFP) domain and a Fok I endonuclease cleavage domain (Xiao and others 2013). TALENs consist of TALE-DNA-binding domains fused with nonspecific Fok I cleavage domains (Jankele and Svoboda 2014). TALEs is comprised of a series of 34-amino-acid repeats, each with a repeat-variable di-residue (RVD) at positions 12 and 13 that can be used for recognizing a single target nucleotide (Liang and others 2014). CRISPR consists of an array of repeat sequences separated by "spacer" sequences that belong to the targeted gene/genome (Upadhyay and others 2013). The CRISPR loci contain a combination of Cas9 genes which are sequences for noncoding RNA elements called CRISPR RNA (crRNA) and sequences for small trans-encoded CRISPR RNA, namely trans-activating crRNA (tracrRNA) (Wiles and others 2015; Khatodia and others 2016).

Overall, the CRISPR/Cas9 system has the advantages of simplicity and high efficiency as compared to ZFNs and TALENs (Xingliang and Yaoguang 2016). The CRISPR/Cas system provides a straight forward method for rapid gene-targeting with a high degree of certainty (Shan and others 2014). The CRISPR-Cas9 system has been successfully used in cereals such as rice, sorghum, wheat, and maize (Jiang and others 2013; Miao and others 2013; Shan and others 2014; Wang and others 2015). This technique was used to generate mutations in target genes of barley resulting in a successful and stable transmission of these mutations (Lawrenson and others 2015). More recently, Zhang and others (2016) developed a protocol able to edit genes in hexaploid bread wheat and tetraploid durum wheat, and to generate mutants with no detectable transgenes. Bortesi and Fischer (2015) reported that targeted gene knockouts might be used to eliminate genes that negatively affect food quality.

To date, no data have been published on the application of advanced molecular tools in the shutdown of genes encoding gluten. The use of these approaches might enable the regulation of gluten epitopes without a quality genes alteration. Probably, given the large number of researchers working with CRISPR/Cas9 and the speed at which it has been developed, new generations of genomes tools will be designed for nutritional purposes in the future (Bortesi and Fischer 2015). Genetic editing and wheat safety are anyway still a controversial subject, particularly in EU countries where public acceptance of GM organisms is basically opposed.

Abolishment of gluten allergenicity through biotechnological tools

Numerous biotechnological methods are emerging with a high efficiency in abolishing gluten allergenicity (summarized in Figure 2 and Table 2). These approaches are based on proline- or glutamine-specific enzyme degradations or modification of gluten epitopes structure, which is recognized by the cells of the immune system (Brzozowski 2016). In particular, germinated cereal grains, as well as bacteria and fungi, are known to be suitable sources for proline-specific peptidases (Walter and others 2014a).

During germination, besides starch, storage proteins are used to nourish the embryo. Because these proteins are insoluble in water, their utilization by the growing embryo is possible only

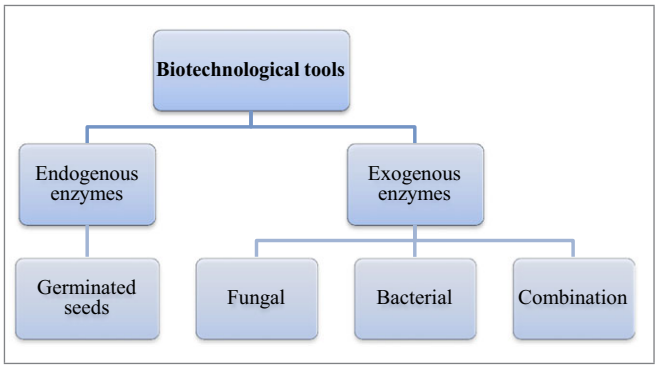

Figure 2–Biotechnological approaches for gluten removal. Biotechnological methods are based on the proteolytic activity of enzymes either endogenous or exogenous or mix of both.

Table 2–Potential of endogenous and exogenous enzymes in gluten removal. It summarized the enzymes and processes involved in the gluten removal or reduction from wheat. Endogenous, and exogenous enzymes, and their combinations are the agents responsible of gluten hydrolysis.

Agent	Approach	Mechanism of action	References
Endopeptidases	Germinating grains	Hydrolysis of prolamins	Hartmann and others (2006); Stenman and others (2009, 2010); Schwalb and others (2012).
	Malting and brewing	Malt enzymes induced the degradation of celiac-active peptides	Van Landschoot (2011) Knorr and others (2016); Kerpes and others (2016).
Combination between endogenous and exogenous enzymes	Proline-specific AN-PEP and malt autolysates (wheat and rye)	The combination reduced prolamin below the gluten limit of 100 mg/kg.	Stepniak and others (2006); Comino and others (2012); Guerdrum and Bamforth (2012); Luoto and others (2012); Janssen and others (2015).
	Germinated cereals and *Aspergillus niger* prolyl endopeptidase	AN-PEP activity exceeded the activities of germinated cereal in gluten degradation	Walter and others (2014a).
Fermentation with sourdough lactobacilli and fungal proteases	Sourdough lactobacilli-derived peptidases: *Lactobacillus plantarum* and *Pediococcus pentosaceus*)	Gluten degradation	Gerez and others (2012); Gobbetti and others (2007); Loponen and others (2007).
	Sourdough lactobacilli-derived peptidases in combination with fungal proteases	Total degradation of gluten	Di Cagno and others (2010); Giuliani and others 2010).
Transamidation	microbial transglutaminase (mTG)	Degradation of α-gliadin-derived peptides	Gianfrani and others (2007); Mazzarella and others (2012)

after their degradation to soluble products (Capocchi and others 2000). The proteolytic activity increase was due to the synthesis and secretion of endoproteases (Dominguez and Cejudo 1996). Gluten hydrolysis by endoprotease might result in an alteration of gluten epitopes immunogenic potential. According to Hartmann and others (2006), germinated cereals led to α-gliadin degradation into nonallergenic small peptide fragments. Later, *in vitro* cell trials showed that protease isolated from naturally germinating wheat did not stimulate T-cell proliferation to the same extent as unprocessed gliadin, concluding that germinating wheat enzymes are able to alter gliadin immunological potential (Stenman and others 2009).

Several outstanding research efforts have been made to produce safe beers using the endogenous protease to abolish gluten epitopes. Indeed, malting and brewing are suggested to have abolishment capacities. A preliminary study of Van Landschoot (2011) revealed that malting and brewing are able to reduce gluten epitopes much lower than the threshold of 20 ppm for food products. In fact, the addition of 10% concentrated endogenous malt peptidases extract from wort for an incubation of 24 h at 50 °C resulted in a gluten-free wort (Knorr and others 2016). Enzymes from barley malt showed high degradation efficiency of celiac-active peptides PQPQLPYPQPQLPY and SQQQFPQPQQPFPQQP (Kerpes and others 2016). Likewise, Luoto and others (2012) demonstrated that the level of allergenic prolamin epitopes in the malt autolysates (wheat, barley, rye) was substantially lower than in the native malts, but too high to be allowed for CD patients. Despite the high degradation rate of gluten, beers based on cereal containing gluten cannot be commercialized as a safe product for CD patients.

Because wheat bran is characterized by a high content of bioactive compounds such as dietary fiber, minerals, and folic acid (Walter and others 2014a), several studies have focused on germinated wheat bran as a source for peptidase. Indeed, it has been indicated that there is CD-specific peptidase activity in the bran of sprouted cereals (Gessendorfer and others 2011; Schwalb and others 2012). It was observed that active extracts of bran recorded high degradation rates of gliadin and the celiac-allergenic peptide (PQPQLPYPQPQLPY) (67% and 100%, respectively) (Schwalb and others 2012). Further, several comparative studies of different germinating cereals versus their proteolytic activities were conducted. In the case of barley, germinating enzymes were more efficient in the degradation of rye secalin than other cereal enzymes (oat and wheat) (Stenman and others 2010). Another study showed that emmer peptidase activity was most active in the degradation of the peptide PQPQLPYPQPQLPY after 90 min, followed by spelt, common wheat, and einkorn (Gessendorfer and others 2011). Thus, peptidase activity was strongly correlated to cereal species, cultivar, germination temperature, and pH value of the application (Schwalb and others 2012). Industrial use of germinating cereal peptidases is a simple, biological, stable, and safe method (Wieser and others 2012). *In vitro*, gluten can be degraded by several exogenous enzymes, such as a cysteine endoprotease isolated from barley, a prolyl endopeptidase from *Sphingomonas capsulata*, and a prolyl endoprotease from *Aspergillus niger* (Koning 2012). However, most of these enzymes are irreversibly inactivated in the stomach by pepsin and acidic pH, and do not reach the intestine in adequate concentration (Makharia and others 2014). Stepniak and others (2006) found that *A. niger* prolyl endoprotease (AN-PEP) works optimally at a pH ranging between 4 and 5, remains stable at 2, and is completely resistant to digestion with pepsin. Janssen and others (2015) reported that AN-PEP efficiently degrades gluten under the conditions mimicking the gastrointestinal tract as well as is safe in animal studies and humans.

During the brewing process, prolamin content of the barley malt was reduced from 6832 $\pm$ 61 to 131 $\pm$ 1 mg/kg, which is an outstanding rate of degradation, but it does not fit in with the regulations of gluten-free food (Guerdrum and Bamforth 2012). However, a prolyl endoproteinase supplementary addition enhanced the reduction of prolamin level below the reliable limit of detection (Van Landschoot 2011). Moreover, in a recent work, commercial proline endopeptidase increased the loss of a fragment of the 33-mer peptide, while HMW glutenins were quite resistant (Panda and others 2015). Consistently, Comino and others (2012) revealed that malting barleys were less immunogenic, with reduced levels of gluten, and were possibly less harmful to CD patients. Lab-scale brewing experiments and an industrial brewing case studies revealed that the gluten content in the final beer could clearly be reduced by either using prolyl endopeptidase and/or tannins

during the brewing processes (Van Landschoot 2011). According to Luoto and others (2012), the combination of proline-specific AN-PEP and malt autolysates (wheat and rye) induced the degradation of prolamin below the very low gluten limit of 100 mg/kg. Nevertheless, these foods should not be recommended for CD patients until a proper trial has been done.

Other experiments proved that enzymes from bran of germinated cereals and *A. niger* prolyl endopeptidase enhanced the production of a bread drink with a gluten level below the threshold for gluten-free foods (Walter and others 2014a). Bread drink gluten was easily degraded after a short incubation time (30 min) using AN-PEP. Furthermore, Walter and others (2014a) revealed that peptidase activity of AN-PEP exceeded the activities of bran from germinated cereals by a factor up to 690000 in the abolishment of wheat starch gluten. Therefore, these findings disagree with the hypothesis of Schwalb and others (2012) suggesting peptidase of germinated bran cereals with higher effectiveness than exogenous peptidases (fungi or bacteria). De Angelis and others (2010) indicated that at least 3 peptidases (general aminopeptidase type N, X-prolyl dipeptidyl aminopeptidase, and endopeptidase) were necessary to abolish the 33-mer allergenic peptide without generation of related immunogenic epitopes. *A. niger* prolyl endopeptidase is not only highly active towards celiac-active substrates, but also capable of eliminating gluten from wheat starch with contents up to 2000 mg gluten per kg (Walter and others 2014b). A short-term pilot-study was carried out to assess safety and efficacy of AN-PEP in mitigating the immunogenic effects of gluten on 16 celiac patients (Tack and others 2013). Although this trial revealed that patients reacted differently to this diet, no serious adverse events occurred during the trial and no patients withdrew during the trial (Tack and others 2013). It is also notable that the meal composition influences the amount of AN-PEP needed for gluten elimination; therefore, AN-PEP should not be used to replace a gluten-free diet, but rather to support the digestion of occasional and/or inadvertent gluten consumption (Montserrat and others 2015). A larger-scale trial with long-term duration is required to confirm the efficiency of AN-PEP.

On the other hand, the combination of 2 lactic acid bacteria (*Lactobacillus plantarum* and *Pediococcus pentosaceus*) supplemented to wheat doughs induced a gliadin decrease, but it remains relatively high to be recommended to CD patients (Gerez and others 2012). Gobbetti and others (2007) demonstrated that even fermentation for a long duration cannot eliminate gluten completely, consistently with the works of Loponen and others (2007). However, the lactobacilli and fungal combination of proteases allowed a total abolishment of gluten in wheat flour (Di Cagno and others 2010; Giuliani and others 2010). A pilot study was conducted on the impact of gluten-free sourdough wheat intake made using a cocktail of lactobacilli with the addition of fresh yeast extract on 8 young celiac patients (Di Cagno and others 2010). The results revealed that, although 2 patients interrupted the trial (after 15 and 30 d, respectively); 6 patients had normal values of hematology, serology, and intestinal permeability during 60 d of challenge (Di Cagno and others 2010). This study showed that flour-fermented was not safe for all CD patients. A 2nd trial was conducted on 16 CD patients following 3 diets: 6 patients at a natural flour-baked goods (80127 ppm gluten), 2 patients at extensively hydrolyzed flour baked products (2480 ppm residual gluten) and 5 patients consumed fully hydrolyzed baked products (8 ppm residual gluten) for 60 d (Greco and others 2011). Regarding the first set, increased levels of antitissue transglutaminase antibodies and small bowel deterioration were observed, whereas the second set had only minor subtotal atrophy. However, the third set did not show any symptoms.

Another enzymatic pretreatment method used transamidation to render the α-gliadin-derived peptides nonimmunogenic (Stoven and others 2013). The transamidation activity of microbial transglutaminase (mTG), a transamidase of the endo-γ-glutamine:ε-lysine transferase type was used in a p56-68 or gliadin wheat flour treatment under alkaline conditions (Gianfrani and others 2007; Ribeiro and others 2015). They found that the enzyme was able to inhibit the interferon gamma expression in iTCLs as well as to reduce binding to DQ2 (Gianfrani and others 2007). Similarly, wheat flour transamidation inhibited IFN-γ secretion by intestinal T cells from CD patients (Mazzarella and others 2012). Nevertheless, the trial conducted on 47 patients revealed that the enzyme reaction was insufficient in removing the gluten (Mazzarella and others 2012). Furthermore, Brzozowski (2016) concluded that transglutaminase/peptidase LS and peptidase LS/transglutaminase combinations were the most effective, favoring the gluten epitopes reduction. Despite the promising results of enzyme therapy, limitations are mainly related to the validation of their safety mediating clinical trials. Wheat has to be fully tested for its safety before recommending to CD patients. The ideal standard would be to perform serology and histology on all patients, but it is an expensive and time-consuming approach (Stoven and others 2013).

Gluten-friendly technology

An innovative method for cereal gluten removal based on microwave heat was recently developed. Officially, this method is called Gluten-Friendly (GF) technology (Italian priority patent n° 102015000084813 filed on 17th December 2015). In 2010, Lamacchia and others reported that applying high microwave temperature for a short period to hydrated durum wheat kernels induced protein denaturation and polymerization. Interestingly, the R5-ELISA indicated a very high reduction (99%) in the levels of detectable gluten after the microwave treatment (Lamacchia and others 2016). SE-HPLC revealed a new peak originated from the aggregates formed by soluble proteins (albumins and globulins) and gliadins suggesting that microwave treatment affected proteins solubility and size distribution. Later, scanning electron microscopy pinpointed that microwave treatment also induced a significant change in the protein structure (Lamacchia and others 2016). Although it is suggested that this treatment reduced the antigenic capacity of gluten, enabling to obtain wheat with low gluten content (21 to 100 ppm), further *in vitro* and *in vivo* studies are needed to explain the mechanism behind gluten removal. Indeed, more recently, Gianfrani and others (2017) went deeper investigating the efficiency of this method, in the case of durum and common wheat, under a wide range of variables and using several detection techniques. R5 sandwich ELISA and T cell assays of gliadin extracts showed a drastic reduction of gluten in microwave-treated wheat compared to untreated kernels, in agreement with the previous findings of Lamacchia and others (2016). However, after performing the same tests on the digests of unextracted gluten proteins, they concluded that the microwave treatment did not modify the antigenic properties of gluten because allergens were simply not extracted and remained bound to the starchy pellet. Further, G12 immunoassay and mass spectrometry analysis confirmed that the gluten chemical composition was left unaffected after microwave treatment and consequently, CD allergenic peptides retain full activity. Therefore, before drawing

any conclusions, a comprehensive assessment of gluten destruction tools is, certainly, mandatory to ensure their efficacy and safety.

Influence of Removal of Gluten Allergenic Epitopes on Wheat Technological Properties

Despite the considerable progress in products made from naturally gluten-free ingredients, their quality is often inferior to their gluten-containing counterparts regarding taste, flavor, color, texture, and mouth-feel (O'Shea and others 2015). So, if gluten removal or reduction could be a strategy to produce safe wheat-based products in terms of protein composition and quality attributes, its effect should be deeply considered.

Bread making, baked goods, and pasta

Down-regulation of gluten epitopes using the RNA interference technology was used to study the technological properties of wheat transgenic genotypes. Indeed, Becker and others (2012) deduced that silencing of α-gliadin genes in hexaploid wheat resulted in a strong reduction in the content of α-gliadin, which was compensated for by an increase of albumins/globulins, ω-gliadins, γ-gliadins and HMW glutenin subunits. With regard to wild lines, dough resistance and extensibility were not affected, thus highlighting that these quality attributes are not related to α-gliadin. In contrast, gluten resistance increase was probably attributed to a HMW-GS fraction increase due to the shutdown of α-gliadin (Xu and others 2006). Although the silencing of γ-gliadins in hexaploid wheat resulted in an increase in the content of all other gluten protein, the γ-gliadin amino acid content was quite similar to the wild lines (Pistón and others 2011). It is suggested that the quality of genotype in terms of dough strength and extensibility are associated with HMW-GS and LMW-GS alleles (Bekes and Wrigley 2013). Thus, the compensatory effect on the synthesis of the other prolamins, including glutenin, resulted in stronger doughs (Gil-Humanes and others 2008). Interestingly, Tosi and others (2005) decreased the amount in LMW glutenin subunit of durum wheat cultivar Ofanto. As a result, the notable increase in the amount of large glutenin polymers HMW-GS might be responsible for the strong and stable dough obtained. Likewise, it was reported that HMW-GS determines as much as two-thirds of the bread-making quality of wheat flour (Liu and others 2016). Therefore, the down-regulation of γ-gliadins resulted in stronger doughs and a better tolerance to overmixing in some transgenic lines (Gil-Humanes and others 2008). RNAi technologies were used to down-regulate groups of proteins encoded by multigene families (Travella and others 2006). The down-regulation of gliadins by RNAi provides wheat lines with all the gliadin fractions strongly down-regulated accompanied by an increase in other storage proteins or nongluten proteins (Gil-Humanes and others 2014a; Rosell and others 2013). Gil-Humanes and others (2011) reported that transgenic wheat lines with suppressed gliadins had protein bodies of irregular shape and formation. The technological properties of dough prepared from the low-gliadin lines indicated a general weakening effect, while the stability was increased significantly in some of the transgenic lines, indicating better tolerance to overmixing (Gil-Humanes and others 2014a). Baking characteristics, sensory properties, and overall acceptance of reduced-gliadin flour were similar to those of normal flour, but with up to 97% lower gliadin content. Moreover, the low-gliadin flour improved nutritional properties, since its lysine content is significantly higher than that of regular flour (Gil-Humanes and others 2014b). As well as the omega-5 gliadins, gene silencing enabled an over 80% decrease in omega-5 gliadins, with slight changes in the levels of other gluten proteins, but functionally it improved dough time and mixing tolerance (Altenbach and others 2014).

Fermentation with selected lactobacilli and fungal proteases allowed dough gluten decrease to below 10 ppm suggesting its suitability for making sweet baked goods as well as bread and pasta if supplemented with gluten-free structuring agents (Greco and others 2011). The gluten-free sourdough wheat baked bread protocol is based on wheat flour fermentation with a pool of lactobacilli with baker's yeast (Di Cagno and others 2004). Technologically, the obtained bread had comparable texture and flavor to those of traditional wheat sourdough bread. The specific loaf volume of this bread was slightly lower than that of baker's yeast bread, and the bread had the typical flavor of sourdough wheat bread as judged by an internal taste panel (Rizzello and others 2007). Heredia-Sandoval and others (2014) showed that gluten in wheat was reduced up to 71% using microbial chymotrypsin, but only 42% was degraded using microbial transglutaminase. Moreover, treated breads with chymotrypsin presented better appearance, homogeneous crumb, and specific volume values than the transglutaminase-treated ones. Besides, transglutaminase has been shown to improve the dough viscoelasticity and to decrease crumb hardness (Ngemakwe and others 2015). Indeed, transpeptidation during bread-making seems to contribute to the improvement of bread quality, but its potential in gluten removal is insufficient (Ngemakwe and others 2015).

Italian pasta was prepared using freeze-dried fermented durum wheat semolina with sourdough lactobacilli (Di Cagno and others 2005). The sensory scores of stickiness and firmness were more appreciable and preferable with respect to the conventional gluten-free pasta (De Angelis and others 2010). It was observed also that odor and flavor did not differ between gluten-free pasta and low-gluten wheat pasta (Di Cagno and others 2005). Long-time fermentation of dough was shown to be a potential tool to abolish gluten epitopes (De Angelis and others 2007). However, by fully degrading wheat gluten, the viscoelastic properties are lost, which reduces the benefit of the process (Engström and others 2015).

A recent study by Walter and others (2015) investigated the gluten content of rye sourdough during fermentation. They showed that the *A. niger* prolyl endopeptidase extensively degraded gluten concentrations in rye flour, rye sourdough, and sourdough starter with lactobacilli. The obtained gluten-free bread recorded poor sensory attributes as compared to a conventional rye bread, but it was superior to bread made from naturally gluten-free raw materials. A possible alternative to overcome these quality problems is considering the addition of hydrocolloids.

Beverages

Unlike with baking, only a few studies have been performed on gluten reduction influence for beverage technological assessment. As a matter of fact, Walter and others (2014a) evaluated quality attributes of a bread drink made using a combination of bran of germinated wheat with enzymatic treatment (proline-specific peptidase preparation from *A. niger*). Sensory scores revealed that the mix containing the gluten-free bread drink was slightly more acidic and marginally, but less, bitter than the gluten-containing mix. It is also notable that dietary fiber and folates values of wheat bran can be increased by germination offering a product with improved nutritional composition.

On the other hand, beers made using prolyl endopeptidase from *A. niger* and the malt enzymes extract showed similar quality parameters, namely, density and pH values, as compared to a

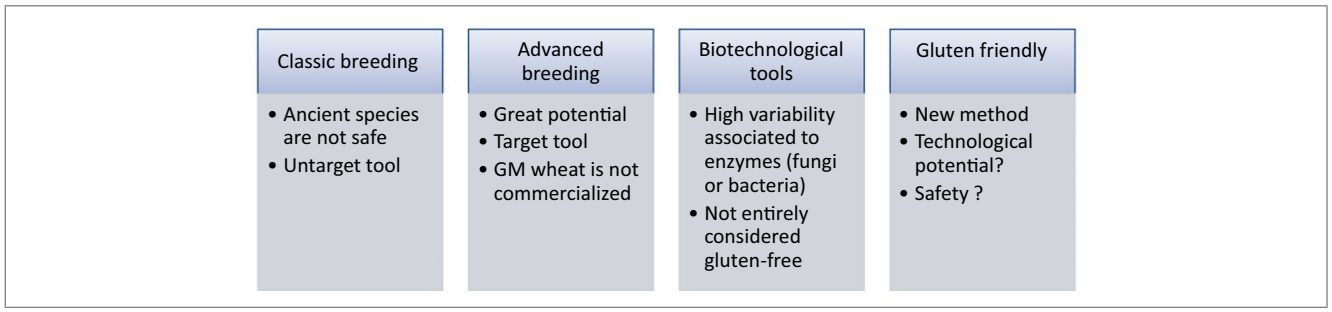

Figure 3–Overall assessment of gluten removal tools. This figure presented the advantages and downsides of breeding, biotechnological and physical strategies followed to abolish gluten allergenicity.

commercial beer (Knorr and others 2016). However, the extract-treated beer with commercial enzyme was classified as a strong beer due to its high alcohol content, which explains its appreciation by the taste panel, but it had less foam, which was less stable, than the other 2 types of beers (Knorr and others 2016).

Concluding Remarks and Future Challenges

Many approaches for gluten removal have been suggested, all with different levels of effectiveness (Figure 3), and the concept of complete elimination of gluten is still debatable (Gerez, and others 2012).

To start breeding nonallergenic wheat or wheat with lower levels of immunogenic polypeptides is premature (Rasheed and others 2014). Nevertheless, the high speed of progress in genetic editing is promising. Besides, enzymatic therapy is an expanding field, but it remains a challenge to manage the several factors such as enzyme origin, concentration, pH, action mechanism, reaction time, and others. Further, more complementary strategies are considered necessary to compensate for the reduction or the removal of gluten to ensure a high-quality product. The shelf-life, the approximate nutritional composition and the digestibility are, indeed, crucial factors to be taken into consideration.

Despite the controversial subject of genetically modified wheat, the identification of low-gluten varieties could be the starting point. Then, finding a good match between natural low-gluten wheat and enzymatic treatment might put forward a new line of therapeutic food. However, to ensure safety and efficiency, eligibility criteria of large-scale *in vivo* trials are required.

References

Altenbach SB, Tanaka CK, Allen PV. 2014. Quantitative proteomic analysis of wheat grain proteins reveals differential effects of silencing of omega-5 gliadin genes in transgenic lines. J Cereal Sci 59:118–25.

Balakireva AV, Zamyatnin AA. 2016. Properties of gluten intolerance: gluten structure, evolution, pathogenicity and detoxification capabilities. Nutrients 8:644–71.

Barro F, Iehisa JC, Giménez MJ, García-Molina MD, Ozuna CV, Comino I, Sousa C, Gil-Humanes J. 2016.Targeting of prolamins by RNAi in bread wheat: effectiveness of seven silencing-fragment combinations for obtaining lines devoid of coeliac disease epitopes from highly immunogenic gliadins. Plant Biotechnol J 14:986–96.

Becker D, Wieser H, Koehler P, Folck A, Muhling KH, Zorb C. 2012. Protein composition and techno-functional properties of transgenic wheat with reduced a-gliadin content obtained by RNA interference. J Appl Bot Food Qual 85:23–33.

Bekes F, Wrigley CW. 2013. Gluten alleles and predicted dough quality for wheat varieties worldwide: a great resource-free on the AACC International Website. Cereal Foods World 58:325–8.

Bernardo D, Peña AS. 2012. Developing strategies to improve the quality of life of patients with gluten intolerance in patients with and without coeliac disease. Eur J Intern Med 23:6–8.

Bortesi L, Fischer R. 2015. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv 33:41–52.

Brzozowski B. 2016. Immunoreactivity of wheat proteins modified by hydrolysis and polymerization. Eur Food Res Technol 242:1025–40.

Capocchi A, Cinollo M, Galleschi L, Saviozzi F, Calucci L, Pinzino C, Zandomeneghi M. 2000. Degradation of gluten by proteases from dry and germinating wheat (*Triticum durum*) seeds: an in vitro approach to storage protein mobilization. J Agric Food Chem 48:6271–9.

Caputo I, Secondo A, Lepretti M, Paolella G, Auricchio S, Barone MV, Esposito C. 2012. Gliadin peptides induce tissue transglutaminase activation and ER-Stress through Ca^{2+} mobilization in Caco-2 cells. PLoS One 7:e45209.

Carroll D. 2011.Genome engineering with zinc-finger nucleases. Genetics 188:773–82.

Castillo NE, Theethira TG, Leffler DA. 2015. The present and the future in the diagnosis and management of celiac disease. Gastroenterol Rep (Oxf) 3:3–11.

Catassi C, Bai JC, Bonaz B, Bouma G, Calabrò A, Carroccio A, Castillejo G, Ciacci C, Cristofori F, Dolinsek J. 2014. Non-celiac gluten sensitivity: the new frontier of gluten related disorders. Nutrients 5:3839–53.

Chen W, Qian Y, Wu X, Sun Y, Wu X, Cheng X. 2014. Inhibiting replication of begomoviruses using artificial zinc finger nucleases that target viral-conserved nucleotide motif. Virus Genes 48:494–501.

Codex alimentarius commission 2008. Codex standard for foods for special dietary use for persons intolerant to gluten. CODEX STAN 118–1979. Rome: FAO/WHO.

Comino I, Real A, de Lorenzo L, Cornell H, López-Casado MÁ, Barro F, Lorite P, Torres MI, Cebolla A, Sousa C. 2012. Diversity in oat potential immunogenicity: basis for the selection of oat varieties with no toxicity in coeliac disease. Gut 60:915–22.

Cooper R. 2015. Re-discovering ancient wheat varieties as functional foods. J Tradit Complement Med 5:138–43.

Dall'Asta C, Scarlato AP, Galaverna G, Brighenti F, Pellegrini N. 2012. Dietary exposure to fumonisins and evaluation of nutrient intake in a group of adult celiac patients on a gluten-free diet. Mol Nutr Food Res 56:632–40.

De Angelis M, Cassone A, Rizzello CG, Gagliardi F, Minervini F, Calasso M, Di Cagno R, Francavilla R, Gobbetti M. 2010. Mechanism of degradation of immunogenic gluten epitopes from *Triticum turgidum* L. var. durum by sourdough lactobacilli and fungal proteases. Appl Environ Microbiol 76:508–18.

De Angelis M, Rizzello CG, Scala E, De Simone C, Farris GA, Turrini F, Gobbetti M. 2007. Probiotic preparation has the capacity to hydrolyze proteins responsible for wheat allergy. J Food Prot 70:135-44.

De la Hera E, Talegón M, Caballero P, Gómez M. 2013. Influence of maize flour particle size on gluten-free breadmaking. J Sci Food Agric 93:924–32.

Delcour JA, Joye IJ, Pareyt B, Wilderjan E, Brijs K, Lagrain B. 2012. Wheat gluten functionality as a quality determinant in cereal-based food products. Annu Rev Food Sci Technol 3:501–10

Di Cagno R, Barbato M, Di Camillo C, Rizzello CG, De Angelis M, Giuliani G, De Vincenzi M, Gobbetti M, Cucchiara S. 2010. Gluten-free sourdough wheat baked goods appear safe for young celiac patients: a pilot study. J Pediatr Gastroenterol Nutr 51:777–83.

Di Cagno R, De Angelis M, Alfonsi G, De Vincenzi M, Silano M, Vincentini O, Gobbetti M. 2005. Pasta made from durum wheat semolina fermented with selected lactobacilli as a tool for a potential decrease of the gluten intolerance. J Agric Food Chem 53:4393–402.

Di Cagno R, De Angelis M, Auricchio S, Greco L, Clarke C, De Vincenzi M, Giovannini C, Archivio MD, Parrilli G, Minervini F. 2004. Sourdough bread made from wheat and nontoxic flours and started with selected lactobacilli is tolerated in celiac sprue patients. Appl Environ Microbiol 70:1088–96.

Dominguez F, Cejudo FJ. 1996. Characterization of the endoproteases appearing during wheat grain development. Plant Physiol 112:1211–7.

Engström N, Sandberg AS, Scheers N. 2015. Sourdough fermentation of wheat flour does not prevent the interaction of transglutaminase 2 with α2-gliadin or gluten. Nutrients 7:2134–44.

Ferretti G, Bacchetti T, Masciangelo S, Saturni L. 2012. Celiac disease, inflammation and oxidative damage: a nutrigenetic approach. Nutrients 4:243–57.

Gasbarrini GB, Mangiola F. 2014.Wheat-related disorders: a broad spectrum of 'evolving' diseases. United Eur Gastroenterol J 2:254–62.

Gerez CL, Dallagnol A, Rollan G, de Valdez GF. 2012. A combination of two lactic acid bacteria improves the hydrolysis of gliadin during wheat dough fermentation. Food Microbiol 32:427–30.

Gessendorfer B, Hartmann G, Wieser H, Koehler P. 2011. Determination of celiac disease-specific peptidase activity of germinated cereals. Eur Food Res Technol 232:205–9.

Gianfrani C, Maglio M, Rotondi Aufiero V, Camarca A, Vocca I, Iaquinto G, Giardullo N, Pogna N, Troncone R, Auricchio S, Mazzarella G. 2012. Immunogenicity of monococcum wheat in celiac patients. Am J Clin Nutr 96:1339–45.

Gianfrani C, Mamone G, la Gatta B, Camarca A, Di Stasio L, Maurano F, Picascia S, Capozzi V, Perna G, Picariello G, Di Luccia A. 2017. Microwave-based treatments of wheat kernels do not abolish gluten epitopes implicated in celiac disease. Food Chem Toxicol 101:105–11.

Gianfrani C, Siciliano RA, Facchiano AM, Camarca A, Mazzeo MF, Costantini S, Salvati VM, Maurano F, Mazzarella G, Iaquinto G, Bergamo P, Rossi M. 2007. Transamidation of wheat flour inhibits the response to gliadin of intestinal T cells in celiac disease. Gastroenterology 133:780–9.

Gil-Humanes J, Piston F, Altamirano-Fortoul R, Real A, Comino I, Sousa A, Rosell CM, Barro F. 2014a. Reduced-gliadin wheat bread: an alternative to the gluten-free diet for consumers suffering gluten-related pathologies. PLoS One 9:e90898.

Gil-Humanes J, Piston F, Barro F, Rosell CM. 2014b. The shutdown of celiac disease-related gliadin epitopes in bread wheat by RNAi provides flours with increased stability and better tolerance to over-mixing. PLoS One 9:e91931.

Gil-Humanes J, Piston F, Gimenez MJ, Martin A, Barro F. 2011.The introgression of RNAi silencing of g-gliadins into commercial lines of bread wheat changes the mixing and technological properties of the dough. PLoS One 9:e45937.

Gil-Humanes J, Piston F, Hernando A, Alvarez JB, Shewry PR, Barro F. 2008. Silencing of g-gliadins by RNA interference (RNAi) in bread wheat. J Cereal Sci 48:565–8.

Giuliani G, Benedusi A, Di Cagno R, Rizzello CG, De Angelis M, Gobbetti M, Cassone A. 2010. Process of microbic biotechnology for completely degrading gluten in flours. International patent WO2010073283A2

Gobbetti M, Rizzello GC, Di Cagno R, De Angelis M. 2007. Sourdough lactobacilli and celiac disease. Food Microbiol 24:187–96.

Golan G, Oksenberg A, Peleg Z. 2015. Genetic evidence for differential selection of grain and embryo weight during wheat evolution under domestication. Exp Bot 66:5703–11.

Goryunova SV, Salentijn EMJ, Chikida NN, Kochieva EZ, Meer IM, van der Gilissen LJWJ, Smulders MJM. 2012. Expansion of the gamma-gliadin gene family in Aegilops and Triticum. BMC Evol Biol 12:215. https://doi.org/10.1186/1471-2148-12-215.

Greco L, Gobbetti M, Auricchio R, Di Mase R, Landolfo F, Paparo F, Di Cagno R, De Angelis M, Rizzello CG, Cassone A. 2011. Safety for patients with celiac disease of baked goods made of wheat flour hydrolyzed during food processing. Clin Gastroenterol Hepatol 9:24–9.

Guerdrum LJ, Bamforth CW. 2012. Prolamin levels through brewing and the impact of prolyl endoproteinase. J Am Soc Brew Chem 70:35–8.

Hartmann G, Koehler P, Wieser H. 2006. Rapid degradation of gliadin peptides toxic for coeliac disease patients by proteases from germinating cereals. J Cereal Sci 44:368–71.

Heredia-Sandoval NG, Islas-Rubio AR, Cabrera-Chávez F, Calderón de la Barca AM. 2014. Transamidation of gluten proteins during the bread-making process of wheat flour to produce breads with less immunoreactive gluten. Food Funct 5:1813–8.

Houben A, Höchstötter A, Becker T. 2012. Possibilities to increase the quality in gluten-free bread production: an overview. Eur Food Res Technol 235:195–208.

Jankele R, Svoboda P. 2014. TAL effectors: tools for DNA targeting. Brief Funct Genom 13:409–19.

Janssen G, Christis C, Kooy-Winkelaar Y, Edens L, Smith D, van Veelen P, Koning F. 2015. Ineffective degradation of immunogenic gluten epitopes by currently available digestive enzyme supplements. PLoS One 1:e0128065.

Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 31:233–9.

Kabbani TA, Vanga RR, Leffler DA, Villafuerte-Galvez J, Pallav K, Hansen J, Mukherjee R, Dennis M, Kelly CP. 2014. Celiac disease or non-celiac gluten sensitivity? An approach to clinical differential diagnosis. Am J Gastroenterol 109:741–6.

Kerpes R, Knorr V, Procopio S, Koehler P, Becker T. 2016. Gluten-specific peptidase activity of barley as affected by malting and its impact on gluten degradation. J Cereal Sci 68:93–9.

Khatodia S, Bhatotia K, Passricha N, Khurana SMP, Tuteja N. 2016. The CRISPR/Cas genome-editing tool: application in improvement of crops. Front Plant Sci 7:506.

Kinsey L, Burden ST, Bannerman EA. 2008. Dietary survey to determine if patients with celiac disease are meeting current healthy eating guidelines and how their diet compares to that of the British general population. Eur J Clin Nutr 62:1333–42.

Knorr V, Wieser H, Koehler P. 2016. Production of gluten-free beer by peptidase treatment. Eur Food Res Technol 242:1129–40.

Koning F. 2012. Celiac disease: quantity matters. Semin Immunopathol 34:541–9.

Kroghsbo S, Andersen NB, Rasmussen TF, Jacobsen S, Madsen CB. 2013. Acid hydrolysis of wheat gluten induces formation of new epitopes but does not enhance sensitizing capacity by the oral route: a study in "gluten free" brown Norway rats. PLoS One 9:e107137.

Kroghsbo S, Rigby NM, Johnson PE, Adel-Patient K, Bøgh KL, Salt LJ, Mills EN, Madsen CB. 2014. Assessment of the sensitizing potential of processed peanut proteins in brown Norway rats: roasting does not enhance allergenicity. PLoS One 9:e96475.

Kucek LK, Veenstra LD, Amnuaycheewa P, Sorrells ME. 2015. A grounded guide to gluten: how modern genotypes and processing impact. Wheat sensitivity. Compr Rev Food Sci Food Saf 14:285–302.

Lamacchia C, Landriscina L, D'Agnello P. 2016. Changes in wheat kernel proteins induced by microwave treatment. Food Chem 15:634–40.

Landriscina L, D'Agnello P, Bevilacqua A, Corbo MR, Sinigaglia M, Lamacchia C. 2015. Impact of gluten-friendly™ technology on wheat kernel endosperm and gluten protein structure in seeds by light and electron microscopy. Food Chem 221:1258–68.

Lawrenson T, Shorinola O, Stacey N, Li C, Østergaard L, Patron N, Uauy C, Harwood W. 2015. Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. Genome Biol 16:258. https://doi.org/10.1186/s13059-015-0826-7.

Leffler DA, Schuppan D. 2010. Update on serological testing in celiac disease. Am J Gastroenterol 105:2520–4.

Liang Z, Zhang K, Chen K, Gao C. 2014. Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. J Genet Genomics 41:63–8.

Liu H, Ke W, Lele X, Shunli W, Lipu D, Xinyou C, Xiaoxiang Z, Yang Z, Yueming Y, Xingguo Y. 2016. Comprehensive identification and bread-making quality evaluation of common wheat somatic variation line AS208 on glutenin composition. PLoS One 11:e0146933.

Lombardo C, Bolla M, Chignola R, Senna G, Rossin G, Caruso B, Tomelleri C, Cecconi D, Brandolini A, Zoccatelli G. 2015. Study on the immunoreactivity of *Triticum monococcum* (einkorn) wheat in patients with wheat-dependent exercise-induced anaphylaxis for the production of hypoallergenic. J Agric Food Chem 63:8299–306.

Loponen J, Sontag-Strohm T, Venäläinen J, Salovaara H. 2007. Prolamin hydrolysis in wheat sourdoughs with differing proteolytic activities. J Agric Food Chem 55:978–84.

Ludvigsson JF, Leffler DA, Bai JC, Biagi F, Fasano A, Green PH, Hadjivassiliou M, Kaukinen K, Kelly CP, Leonard JN, Lundin KE, Murray

JA, Sanders DS, Walker MM, Zingone F, Ciacci C. 2013b. The Oslo definitions for coeliac disease and related terms. Gut 62:43–52

Ludvigsson JF, Rubio-Tapia A, van Dyke CT, Melton LJ 3rd, Zinsmeister AR, Lahr BD, Murray JA. 2013a. Increasing incidence of celiac disease in a North American population. Am J Gastroenterol 108:818–24.

Luoto S, Jiang Z, Brinck O, Sontag-Strohm T, Kanerva P, Bruins M, Edens L, Salovaara H, Loponen J. 2012. Malt hydrolysates for gluten-free applications: autolytic and proline endopeptidase assisted removal of prolamins from wheat, barley and rye. J Cereal Sci 56:504–9.

Mahmud FH, Melo EN, Noordin K, Assor E, Sahota K, Davies-Shaw J, Cutz E, Somers G, Lawson M, Mack DR, Gallego P, McDonald C, Beaton MD, Bax K, Saibil F, Gilbert J, Kirsch S, Perkins BA, Cino M, Szentgyorgyi E, Koltin D, Parikh A, Mukerji G, Advani A, Lou O, Marcon MA. 2015. The celiac disease and diabetes-dietary intervention and evaluation trial (CDDIET) protocol: a randomised controlled study to evaluate treatment of asymptomatic coeliac disease in type 1 diabetes. BMJ 5:e008097.

Makharia GK, Mulder CJ, Goh KL, Ahuja V, Bai JC, Catassi C, Green PH, Gupta SD, Lundin KE, Ramakrishna BS, Rawat R, Sharma H, Sood A, Watanabe C, Gibson PR. 2014. Issues associated with the emergence of coeliac disease in the Asia–Pacific region: a working party report of the World Gastroenterology Organization and the Asian Pacific Association of Gastroenterology. J Gastroenterol Hepatol 29:666–77.

Malalgoda M, Simsek S. 2017. Celiac disease and cereal proteins. Food Hydrocolloids. 68:108–13.

Mamone G, Camarca A, Fierro O, Sidney J, Mazzarella G, Addeo F, Auricchio S, Troncone R, Sette A, Gianfrani C. 2013. Immunogenic peptides can be detected in whole gluten by transamidating highly susceptible glutamine residues: implication in the search for gluten-free cereals. J Agric Food Chem 61:747–54.

Mamone G, Nitride C, Picariello G, Addeo F, Ferranti P, Mackie AJ. 2015. Tracking the fate of pasta (*T. durum* semolina) immunogenic proteins by in vitro simulated digestion. J Agric Food Chem 63:2660–7.

Manti S, Cuppari C, Tardino L, Parisi G, Spina M, Salpietro C, Leonardi S. 2017. Applied nutritional investigation: HMGB1 as a new biomarker of celiac disease in children: a multicenter study. Nutrition 37:18–21.

Mardini HE, Westgate AP, Grigorian RY. 2015. Differences in the prevalence of celiac disease in the US population: National Health and Nutrition Examination Survey (NHANES) 2009–2012. Dig Dis Sci 60:1738–42

Mazzarella G, Salvati VM, Iaquinto G, Stefanile R, Capobianco F, Luongo D, Bergamo P, Maurano F, Giardullo N, Malamisura B, Rossi M. 2012. Reintroduction of gluten following flour transamidation in adult celiac patients: a randomized, controlled clinical study. Clin Dev Immunol 2012:329150. https://doi.org/10.1155/2012/329150.

Mejías JH, Lu X, Osorio C, Ullman JL, von Wettstein D, Rustgi S. 2014. Analysis of wheat prolamins, the causative agents of celiac sprue, using reversed phase high performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). Nutrients 6:1578–97.

Miao J, Guo D, Zhang J, Huang Q, Qin G, Zhang X, Wan J, Gu H, Qu LJ. 2013. Targeted mutagenesis in rice using CRISPR-Cas system. Cell Res 23:1233–6.

Miśkiewicz P, Kępczyńska-Nyk A, Bednarczuk T. 2012. Coeliac disease in endocrine diseases of autoimmune origin. Endokrynol Pol 63:240–9.

Molberg O, Uhlen AK, Jensen T, Flaete NS, Fleckenstein B, Arentz-Hansen H, Raki M, Lundin KE, Sollid LM. 2005. Mapping of gluten T-cell epitopes in the bread wheat ancestors: implications for celiac disease. Gastroenterology 128:393–401.

Montserrat V, Bruins MJ, Edens L, Koning F. 2015. Influence of dietary components on *Aspergillus niger* prolyl endoprotease mediated gluten degradation. Food Chem 174:440–5.

Mustalahti K, Catassi C, Reunanen A, Fabiani E, Heier M, McMillan S, Murray L, Metzger MH, Gasparin M, Bravi E, Mäki M. 2010. Coeliac EU Cluster, Project Epidemiology. The prevalence of celiac disease in Europe: results of a centralized, international mass screening project. Ann Med 42:587–95.

Ngemakwe PH, Le Roes-Hill M, Jideani VA. 2015. Advances in gluten-free bread technology. Food Sci Technol Intl 21:256–76.

O'Shea N, Rößlea C, Arendt E, Gallaghera E. 2015. Modelling the effects of orange pomace using response surface design for gluten-free bread baking. Food Chem 166:223–230.

Ozuna CV, Iehisa JC, Giménez MJ, Alvarez JB, Sousa C, Barro F. 2015. Diversification of the celiac disease a-gliadin complex inwheat: a 33-mer peptide with six overlapping epitopes, evolved following polyploidization. Plant J 82:794–805.

Panda R, Zoerb HF, Cho CY, Jackson LS, Garber EA. 2015. Detection and quantification of gluten during the brewing and fermentation of beer using antibody-based technologies. J Food Prot 6:1064–243.

Peleg Z, Fahima T, Korol AB, Abbo S, Saranga Y. 2011. Genetic analysis of wheat domestication and evolution under domestication. J Exp Bot 62:5051–61.

Piston F, Gil-Humanes J, Rodrıguez-Quijano M, Barro F. 2011. Downregulating c-gliadins in bread wheat leads to non-specific increases in other gluten proteins and has no major effect on dough gluten strength. PLoS One 6:e24754.

Prandi B, Faccini A, Tedeschi T, Cammerata A, Sgrulletta D, D'Egidio MG, Galaverna G, Sforza S. 2014. Qualitative and quantitative determination of peptides related to celiac disease, in mixtures derived from different methods of simulated gastrointestinal digestion of wheat products. Anal Bioanal Chem 406:4765–75

Prandi B, Tedeschi T, Folloni S, Galaverna G, Sforza S. 2017. Peptides from gluten digestion: a comparison between old and modern wheat varieties. Food Res Intl 91:92–102.

Rand AJ, Cardona DM, Proia AD, Lagoo AS. 2013. Clinically undiagnosed enteropathy associated T-cell lymphoma type II presenting with prolonged lower gastrointestinal tract symptoms: report of an autopsy case and review of diagnostic challenges and clinicopathological correlation. J Gastrointest Oncol 4:103–8.

Rasheed A, Xia XC, Yan YM, Appels R, Mahmood T, He ZH. 2014. Wheat seed storage proteins: advances in molecular genetics, diversity and breeding applications. J Cereal Sci 60:11–24.

Real A, Comino I, de Lorenzo L, Merchán F, Gil-Humanes J, Giménez MJ, López-Casado MÁ, Torres MI, Cebolla Á, Sousa C, Barro F, Pistón F. 2012. Molecular and immunological characterization of gluten proteins isolated from oat cultivars that differ in toxicity for celiac disease. PLoS One 7:e48365.

Ribeiro M, Nunes FM, Guedes S, Domingues P, Silva AM, Carrillo JM, Rodriguez-Quijano M, Branlard G, Igrejas G. 2015. Efficient chemo-enzymatic gluten detoxification: reducing toxic epitopes for celiac patients improving functional properties. Sci Rep 5:18041. https://doi.org/10.1038/srep18041.

Ribeiro M, Rodriguez-Quijano M, Nunes FM, Carrillo JM, Branlard G, Igrejas G. 2016. New insights into wheat toxicity: breeding did not seem to contribute to a prevalence of potential celiac disease's immunostimulatory epitopes. Food Chem 213:8–18.

Rizzello CG, De Angelis M, Di Cagno R, Camarca A, Silano M, Losito I, De Vincenzi M, De Bari MD, Palmisano F, Maurano F. 2007. Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: new perspectives for celiac disease. Appl Environ Microbiol 73:4499–507.

Rosell CM, Altamirano-Fortoul R, Don C, Dubat A. 2013. Thermomechanically induced protein aggregation and starch structural changes in wheat flour dough. Cereal Chem J 90:89–100.

Salentijn EM, Esselink D, Goryunova S, van der Meer I, Gilissen L, Smulders M. 2012. Quantitative and qualitative differences in celiac disease epitopes among durum wheat varieties identified through deep RNA-amplicon sequencing. BMC Genomics 14:905. https://doi.org/10.1186/1471-2164-14-905.

Satherley RM, Howard R, Higgs S. 2016. The prevalence and predictors of disordered eating in women with coeliac disease. Appetite 107:260–7.

Scherf KA, Ciccocioppo R, Pohanka M, Rimarova K, Opatrilova R, Rodrigo L, Kruzliak P. 2016. Biosensors for the diagnosis of celiac disease: current status and future perspectives. Mol Biotechnol 58:381–92

Schwalb T, Wieser H, Koehler P. 2012. Studies on the gluten-specific peptidase activity of germinated grains from different cereal species and cultivars. Eur Food Res Technol 235:1161–70.

Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C. 2014.Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol 31:686–8.

Stenman SM, Lindfors K, Venäläinen JI, Hautala A, Männistö PT, Garcia-Horsman JA, Kaukovirta-Norja A, Auriola S, Mauriala T, Mäki M. 2010. Degradation of coeliac disease-inducing rye secalin by germinating cereal enzymes: diminishing toxic effects in intestinal epithelial cells. Clin Exp Immunol 161:242–9.

Stenman SM, Venäläinen JI, Lindfors K, Auriola S, Mauriala T, Kaukovirta-Norja A, Jantunen A, Laurila K, Qiao SW, Sollid LM. 2009.

Enzymatic detoxification of gluten by germinating wheat proteases: implications for new treatment of celiac disease. Ann Med 41:390–400.

Stepniak D, Vader LW, Kooy Y, van Veelen PA, Moustakas A, Papandreou NA, Eliopoulos E, Drijfhout JW, Papadopoulos GK, Koning F. 2006. T-cell recognition of HLA-DQ2-bound gluten peptides can be influenced by an N-terminal proline at p-1. Immunogenetics 57:8–15.

Stoven S, Murray JA, Marietta E. 2013. Celiac disease—advances in treatment via gluten modification. Clin Gastroenterol Hepatol 10:859–62.

Šuligoj T, Gregorini A, Colomba M, Ellis HJ, Ciclitira PJ. 2013. Evaluation of the safety of ancient strains of wheat in coeliac disease reveals heterogeneous small intestinal T cell responses suggestive of coeliac toxicity. Clin Nutr 32:1043–9.

Tack GJ, van de Water JM, Bruins MJ, Kooy-Winkelaar EM, van Bergen J, Bonnet P, Vreugdenhil AC, Korponay-Szabo I, Edens L, von Blomberg BM, Schreurs MW, Mulder CJ, Koning F. 2013. Consumption of gluten with gluten-degrading enzyme by celiac patients: a pilot-study.World J Gastroenterol 19:5837–47.

Tosi P, Gritsch CS, He J, Shewry PR. 2011. Distribution of gluten proteins in bread wheat (*Triticum aestivum*) grain. Ann Bot 108:23–35.

Tosi P, Masci S, Giovangrossi A, D'Ovidio R, Bekes F, Larroque O, Napier J, Shewry P. 2005. Modification of the low molecular weight (LMW) glutenin composition of transgenic durum wheat: effects on glutenin polymer size and gluten functionality. Mol Breeding 16:113–26.

Travella S, Klimm TE, Keller B. 2006. RNA interference-based gene silencing as an efficient tool for functional genomics in hexaploid bread wheat. Plant Physiol 142:6–20.

Upadhyay SK, Kumar J, Alok A, Tuli R. 2013. RNA-guided genome editing for target gene mutations in wheat. G3 (Bethesda) 3:2233–8.

Van den Broeck HC, Gilissen LJWJ, Smulders MJM, van der Meer IM, Hamer RJ. 2011. Dough quality of bread wheat lacking α-gliadins with celiac disease epitopes and addition of celiac-safe avenins to improve dough quality. J Cereal Sci 53:206–16.

Van den Broeck HC, van Herpen TWJM, Schuit C, Salentijn EMJ, Dekking L, Bosch D, Hamer RJ, Smulders MJM, Gilissen LJWJ, van der Meer IM. 2009. Removing celiac disease-related gluten proteins from bread wheat while retaining technological properties: a study with Chinese Spring deletion lines. BMC Plant Biol 9:41.

Van Herpen TWJM, Goryunova SV, van der Schoot J, Mitreva M, Salentijn E, Vorst O, Schenk MF, van Veelen PA, Koning F, van Soest LJM. 2006. Alpha-gliadin genes from the A, B, and D genomes of wheat containdifferent sets of celiac disease epitopes. BMC Genomics 7:1. https://doi.org/10.1186/1471-2164-7-1.

Van Landschoot A. 2011. Gluten-free barley malt beers. Cerevisia 36:93–7.

Walter T, Wieser H, Koehler P. 2014a. Degradation of gluten in wheat bran and bread drink by means of a proline-specific peptidase. J Nutr Food Sci 4:293–9.

Walter T, Wieser H, Koehler P. 2014b. Production of gluten-free wheat starch by peptidase treatment. J Cereal Sci 60:202–9.

Walter T, Wieser H, Koehler P. 2015. Degradation of gluten in rye sourdough products by means of a proline-specific peptidase. Eur Food Res Technol 240:517–24.

Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, Qiu JL. 2015. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol 32:947–51.

Weber D, Cléroux C, Godefroy SB. 2009. Emerging analytical methods to determine gluten markers in processed foods—method development in support of standard setting. Anal Bioanal Chem 395:111–7.

Wieser H, Konitzer K, Koehler P. 2012. Celiac disease multidisciplinary approaches. Cereal Foods World 57:215–24.

Wieser H. 2007. Chemistry of gluten proteins. Food Microbiol 24:115–9.

Wiles M, Wenning V, Albert Q, Cheng W, Wang H. 2015. CRISPR–Cas9-mediated genome editing and guide RNA design. Mamm Genome 26:501–10.

Xiao A, Wu Y, Yang Z, Hu Y, Wang W, Zhang Y, Kong L, Gao G, Zhu Z, Lin S, Zhang B. 2013. EENdb: a database and knowledge base of ZFNs and TALENs for endonuclease engineering. Nucleic Acids Res 41:415–22.

Xingliang M, Yaoguang L. 2016. CRISPR/Cas9-based genome editing systems and the analysis of targeted genome mutations in plants. Hereditas 38:118–25.

Xu P, Zhang Y, Kang L, Roossinck MJ, Mysore KS. 2006. Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. Plant Physiol 142:2429–40.

Yuan J, Zhou C, Gao J, Li J, Yu F, Lu J, Li X, Wang X, Tong P, Wu Z, Yang A, Yao Y, Nadif S, Shu H, Xu J, Wu Y, Gilissen L, Chen H. 2017. Prevalence of celiac disease autoimmunity among adolescents and young adults in China. Clin Gastroenterol Hepatol. S1542-3565(17)30468–8

Zhang Y, Liang Z, Zong Y, Wang Y, Liu J, Chen K, Qiu JL, Gao C. 2016. Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat Commun 7:12617. https://doi.org/10.1038/ncomms12617.

Zörb C, Becker D, Hasler M, Mühling KH, Gödde V, Niehaus K, Geilfus CM. 2013. Silencing of the sulfur rich α-gliadin storage protein family in wheat grains (*Triticum aestivum* L.) causes no unintended side-effects on other metabolites. Front Plant Sci 4:369. https://doi.org/10.3389/fpls.2013.00369.