

## Recent Advances in Biotechnological Methods for Wheat Gluten Immunotoxicity Abolishment – a Review

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**Key words:** celiac disease, wheat immunogenicity, detoxified wheat, gluten hydrolysis

Due to the increasing incidence of gluten intolerance, researchers are focusing on finding ways to eliminate immunotoxicity of wheat, this would allow the use of wheat products for gluten-intolerant consumers. The article reviews recent studies on biotechnological methods to eliminate and reduce the immunogenicity of wheat products. So far, many gluten removal methods have been proposed, but their efficacy levels were quite different. Enzymatic treatment of gluten fragments can be considered the simplest and non-invasive tool to eliminate the toxicity of gliadins and glutenins. For this purpose, various endogenous enzymes derived from cereals, and also those of bacterial, fungal, plant, and animal origin can be used in food processing. Some of the enzymes hydrolyze gluten, others block the action of toxic protein fragments. The majority of studies were carried out using lactic acid bacteria cultures, as single strains or in consortia. Satisfactory results have been achieved using bacterial and plant enzymes, but the complete elimination of gluten immunogenicity is still possible by using fungal proteases, engineered enzymes or combining several treatments, for example, by using lactic acid fermentation or germination with fungal proteases. However, the question of how degradation of gluten affects the quality of flour (dough) in practice remains unanswered. It is not clear whether the products of such wheat flour are better and safer than those made from starches and whether their price and quality are acceptable to consumers. The insights presented in this review will be helpful to other researchers and cereal-based food producers in choosing ways to reduce gluten immunogenicity.

### ABBREVIATIONS

CD: coeliac disease; GF: gluten-free; GFD: gluten-free diet; HLA: human leukocyte antigen; LAB: lactic acid bacteria; NCGS: non-coeliac gluten sensitivity; PEP: prolyl endopeptidase; WA: wheat allergies.

### INTRODUCTION

Wheat and other grains are one of the most important components of human nutrition on a global scale, and total wheat consumption has increased in recent years [FAOSTAT: Production/Yield quantities of Wheat in World, 2020]. However, for a large number of consumers, wheat gluten proteins cause severe intolerance, manifested by allergic reactions. Gliadins and glutenins are fractions of the immunogenic gluten protein [Giménez *et al.*, 2019]. These toxic proteins can

cause wheat allergies (WA) and development of coeliac disease (CD) in some consumers [Navarro *et al.*, 2017]. CD is one of the most common diseases associated with food intolerance and its prevalence in the world is increasing [King *et al.*, 2020]. CD is more pronounced for genetically predisposed people as an inflammatory disease of the upper small intestine. The manifestation of the disease is determined by glutamine- and proline-containing cereal gluten peptides, most of them with a minimum length of nine amino acids [Bromilow *et al.*, 2017]. During digestion, these peptides are not sufficiently digested by digestive enzymes (pepsin, chymotrypsin, trypsin); they reach lymphatic tissue, meet antigenic cells HLA-DQ2 and HLA-DQ8, specific for coeliac disease, and stimulate intestinal T-cells. Typical clinical signs for CD are flat intestinal mucosa and malabsorption [McAllister *et al.*, 2019]. In addition, an increasing number of cases of non-coeliac gluten sensitivity (NCGS) have been reported in consumers without CD or WA (gliadin does not cause mucosal inflammation of mucous membrane) [Catassi *et al.*, 2015; Lionetti *et al.*, 2017]. These cases are manifested by irritation

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of the intestines and other symptoms associated with the use of gluten-containing foods [Tanveer & Ahmed, 2019].

According to the latest scientific data, the life-long (permanent) gluten-free diet (GFD) is an effective treatment for CD. This diet is popular among consumers with NCGS, and also among people who do not experience CD, but are seeking favorable health effect from consumption of a GFD [Kriegl & Lebwohl, 2016]. Consumers seeking GFD face many challenges associated with cross-contamination, lack of clarity in food labeling policies, poor quality of gluten-free (GF) products, and higher prices compared to gluten-containing foods [Do Nascimento *et al.*, 2017; Estévez *et al.*, 2016]. Nutrition of GFD-compliant consumers is unbalanced, leading to a higher percentage of calories from fat and less from carbohydrates. In addition, there was found a deficiency of non-starch polysaccharides [Hopkins & Soon, 2019], which are very important for reducing the risk factors for developing chronic diseases and certain types of oncological diseases [Lovegrove *et al.*, 2017]. GF products introduced in the market have a poorer taste than regular products, and may cause a nutritional deficiency among consumers due to the unbalanced composition of nutrients [Stantiall & Serventi, 2018]. Examination of patients with CD showed that those with the same energy intake as the control group had a lower intake of fiber, vitamin A, B-group vitamins: B<sub>6</sub>, B<sub>12</sub>, folic acid, thiamine and minerals: calcium, phosphorus, magnesium, and iron [Pellegrini & Agostoni, 2015; Vici *et al.*, 2016]. Zinc and selenium deficiency may be also associated with the elimination of cereals from the diet [Stazi & Trinti, 2008; Tran *et al.*, 2011]. Bread and other products made from naturally gluten-free raw materials such as: buckwheat, rice, corn, quinoa, sorghum, or teff flours, often have lower textural and sensory properties compared to the corresponding gluten-containing bread products [Naqash *et al.*, 2017]. The GF products usually have a high glycemic index [Vici *et al.*, 2016], which is associated with an increased risk of obesity among consumers with CD. The increasing demand for high-quality GF bakery products leads to the search for new approaches in GF food producing. The production of nutritionally-balanced GF products is an important social and economic issue. To solve it, new strategies are being searched for to remove immunogenicity from wheat and other cereals and produce balanced, sensorially-acceptable to consumer products.

The diet should not only be gluten-free but also health-friendly to avoid nutritional imbalances [Chishty & Singh, 2017]. The setup of application of biotechnological tools based on enzyme treatments is an active field of research that may provide new possibilities to GF wheat product development. For products used in GFD, it is necessary to remove or degrade wheat prolamins that are harmful to gluten-non-tolerant users. Studies related to the elimination of gluten from wheat processing products by using biological methods have been performed [Scherf *et al.*, 2018]. Unfortunately, there is a lack of scientific information about the efficiency of biological measures on different conditions, as well as on the possibilities of implementing these methods in the production of wheat products in order to modify their chemical composition and effectively eliminate gluten residues to improve product absorption.

The aim of this review article is to analyze the biotechnological methods for the elimination / reduction of immunogenicity of wheat products and evaluate the possibilities of their implementation. The insights of this review will be helpful to other researchers and wheat producers to choose ways for gluten immunotoxicity abolishment. The threshold set by the Codex Alimentarius [Standard 118–1979] for a gluten-free food claims at 20 mg gluten/kg product. However, despite the fact that manufacturers are subject to strict regulations, even consumers, who adhere to GFD, were reported to consume more than the tolerable amount of gluten because of the contamination of products and inaccurate labeling [Bruins Slot *et al.*, 2015]. Therefore, it is desirable to provide safe GF foods with a gluten level as low as possible. A reduction of the immunoreactivity of food proteins can be achieved by proteolysis occurring in food and degrading the immunoreactive protein fragments [El-Ghaish *et al.*, 2011].

### CLASSIFICATION OF BIOTECHNOLOGICAL MEASURES FOR GLUTEN HYDROLYSIS

Over the last decade, the use of biological measures in wheat products to eliminate or reduce the immunotoxicity of gluten proteins is being actively studied. Various enzymes such as those of bacterial, fungal, plant, and animal origin as well as recombinant enzymes expressed in microbial systems were investigated. The scheme of the sources of enzymes used for gluten hydrolysis, thus eliminating or reducing the gluten immunotoxicity, is shown in Figure 1.

Gluten can be hydrolyzed either by individual enzymes or by combining different biological measures [Scherf *et al.*, 2018]. Enzymatic cleavage of gluten fragments is the easiest and non-invasive way to eliminate the toxicity of gliadins and glutenins. It can be applied in two ways. Firstly, in order to reduce the negative effects of gluten on patients with CD, enzymes should be taken with food [Janssen *et al.*, 2015]. Secondly, the toxic effects of gluten can be eliminated before consumption during food processing [Jouanin *et al.*, 2018].

### POTENTIAL OF DIFFERENT ENZYMES TO ELIMINATE GLUTEN IMMUNOGENICITY

Different peptidases can be used to degrade gliadins and glutenins in food products [Wieser & Koehler, 2012]. Endoproteases attack internal peptide bonds, while exoproteases attack only the N-terminal (aminopeptidases) or C-terminal (carboxypeptidases) forms.

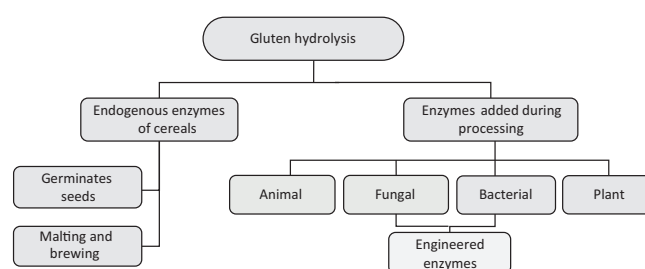


FIGURE 1. Sources of enzymes used for gluten hydrolysis.

TABLE 1. Cereal endogenous enzymes used for gluten hydrolysis.

Source of enzymes	Results	References
Germinating grains	Hydrolysis of prolamines.	Hartmann <i>et al.</i> [2006]
Proteases from germinating cereals	Hydrolysis of prolamines.	Stenman <i>et al.</i> [2009]
Wheat grains germinated for 8 days	Reduction of peptides eliciting immune response.	Boukid <i>et al.</i> [2017b]
Wheat kernels after 7-day germination	Degradation of gluten.	Michalcová <i>et al.</i> [2019]
Germination of wheat kernels	Gluten content in wheat germ (8–10 days) was lower than the limit of detection.	Adrianos <i>et al.</i> [2017]
Recombinant wheat cysteine protease Triticain- $\alpha$	Triticain- $\alpha$ activated proteolytic enzymes <i>in vitro</i> .	Savvateeva <i>et al.</i> [2015]
Cysteine protease EP-B2 from germinated barley	EP-B2 has good specificity for the immunotoxic wheat gluten amino acid sequences.	Diaz-Mendoza <i>et al.</i> [2019]

### Endogenous enzymes from cereals

Proteases are often synthesized as inactive specific proteases which are activated under the appropriate conditions of temperature, humidity, *etc.* One way to activate endoprotease is grain germination. Although this measure is mainly applied to barley – in the malt preparation and beer production [Guerdrum & Bamforth, 2012; Kerpes *et al.*, 2016; Knorr *et al.*, 2016], the immunogenicity of wheat can also be reduced in a similar way. Studies in which gluten is hydrolyzed by endopeptidases during grain germination are systematized in Table 1.

Hartmann *et al.* [2006] proved that after 2-h incubation (37°C, pH 6.5), the toxic wheat peptides were intensively degraded – peptides with more than eight amino acid residues were not detected. As Stenman *et al.* [2009] proved, the gluten content in specially prepared germinated wheat can be minimal. Michalcová *et al.* [2019] analyzed the conditions for digesting wheat gluten proteins by endoproteases during germination. In their experiment, the wheat kernels were germinated for up to 7 days at temperatures of 15–30°C (pH 3.0–8.0). The authors observed gluten degradation that started after 3 days and lasted for up to 7 days. The lowest content of gluten proteins was measured on the 7<sup>th</sup> day at a temperature of 20°C (pH 5.5). In turn, Adrianos *et al.* [2017] showed that the gluten content of wheat sprouts (8–10 days) in all preparations was lower than the limit of detection for both ELISA (4 mg/kg) and the thresholds established by Codex Alimentarius [Standard 118 – 1979]; therefore, the sprouts could be classified as GF. Wheat sprouts are rich in nutrients that might be beneficial for consumers with malabsorption disorders. They can be used for juicing, added to cocktails, or consumed as wheat grain dietary supplements in tablets, capsules, and in the liquid form. An isolate from germinated cereals or purified enzymes can also be used for gluten hydrolysis. Experiments showed that germinated cereal proteases were capable of cleaving intact gluten proteins [Geßendorfer *et al.*, 2011]. Schwalb *et al.* [2012] conducted studies on different germinated grain crops and identified their specific peptidase activity. The studies were performed with peptidase-enriched solutions. They were obtained by extraction from germinated, lyophilized, and milled grains. A synthetic  $\alpha$ -gliadin peptide was used as a peptide substrate. It was degraded under differ-

ent conditions: 60–90 min at 40–50°C (pH 4.0; 6.5). Peptide degradation was found to be more intense at pH 4.0 than at pH 6.5. Peptide was degraded slowly and only about 30% was left after 90 min. Germinating barley (*Hordeum vulgare*) seeds secrete an enzyme, cysteine protease, which hydrolyzes hordein, *i.e.* the barley analog of wheat gluten. This glutenase, named EP-B2, has good specificity for the immunotoxic wheat gluten amino acid sequences. Besides, it was found to be most active at low pH, resistant to pepsin but digested at physiological concentrations of trypsin; therefore, it was proved to be suitable for gluten hydrolysis [Diaz-Mendoza *et al.*, 2019]. Kiyosaki *et al.* [2009] and Savvateeva *et al.* [2015] studied abilities of recombinant wheat cysteine protease – Triticain- $\alpha$  to activate proteolytic enzymes (glutenase and collagenase) *in vitro*, which are optimally active at 37°C. Mass-spectrometry analysis showed that Triticain- $\alpha$  degraded immunotoxic peptides. Studies have shown that Triticain- $\alpha$  has a high glutenase activity under normal human physiological conditions (37°C) and can, therefore, be used in CD treatment.

Generally, proteases from germinated cereals can significantly reduce the amounts of toxic gluten proteins or peptides. Therefore, they may be used in a variety of areas: food supplements that help the body digest gluten without allergic reactions, as well as in the production of special foods for CD patients [Adrianos *et al.*, 2017]. The quality and nutritional value of wheat products can be improved by choosing the optimal duration and conditions of grain germination [Ding *et al.*, 2018], because by germinating wheat grains for up to 48 h, it is possible to bake improved-quality bread that will not be GF even though the rheological properties of the dough are poorer [Baranzelli, 2018; Cardone *et al.*, 2020]. Long-term germination increases the activity of  $\alpha$ -amylase (breaks down starch), can significantly reduce the quality of flour and the baking process: lower falling numbers can affect mixability, crumb strength, and loaf specific volume and sliceability [Thomason *et al.*, 2019]. Therefore, germination of wheat is a complex way to eliminate gluten from baked goods. Such detoxified wheat can only be used to supplement GF products, while in the production process it is easier to use the isolate from sprouted cereal grains or purified enzymes.

## Bacterial enzymes

### Fermentation with probiotic strains

The most common way of applying bacterial enzymes is fermentation with *Lactobacillus* strains. Lactic acid bacteria (LAB) fermentation can improve the texture and palatability of various types of foods (whole grains, fiber-rich, gluten-free), stabilize or increase the amount of various biologically-active compounds, retard starch bioavailability, and improve mineral bioavailability [Katina *et al.*, 2006]. LAB produce a variety of enzymes that degrade anti-nutritional compounds, thereby improving the texture of baked goods, ensuring the development of palatability and the formation of aromatic compounds, and prolonging the shelf life [Luz *et al.*, 2019; Gobetti *et al.*, 2019; Sun *et al.*, 2020]. Many studies have focused on the possibilities of probiotic strains to decrease the immunotoxicity of wheat products (Table 2). The main idea of those studies was to use bacterial enzymes during food processing to eliminate immunotoxic gliadins. Fermentation with LAB decreases the number of disulfide bonds in the gluten network, which causes an immune response in people with sensitivity to gluten [Gänzle *et al.*, 2008]. Individual LAB species produce specific peptidases that are capable of hydrolyzing hardly degradable, immunoreactive, and celiac disease-causing peptides [Vukotić *et al.*, 2016]. It is important to select LAB strains with specific proteolytic effects for the successful breakdown of the gliadin complex structure [Stefańska *et al.*, 2016].

The use of lactic acid cultures for the first time in the 21<sup>st</sup> century aimed to remove traces of gluten fragments from processed foods. Di Cagno *et al.* [2002] showed that selected LAB with proteolytic activity can effectively hydrolyze the toxic gliadin peptides in wheat sourdough. As Di Cagno *et al.* [2004] proved, *L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A, and *L. hilgardii* 51B strains have peptidases capable of hydrolyzing all the different peptide bonds present in prolamins. The next study of Di Cagno *et al.* [2008] proved the effectiveness of selected LAB cultures to remove gluten residues and enhance the nutritional value of GF bread. Forty-six strains of LAB were tested for evaluating the proteolytic activity and medium acidification rate. Cultures of *L. sanfranciscensis* LS40 and LS41, and *L. plantarum* CF1 were selected as the most suitable for the production of GF bread from pseudocereals with gluten addition. During fermenting the bread, the initial gluten content of 400 mg/kg was degraded to below 20 mg/kg, and the content of free amino acids increased.

Several studies have also been carried out to assess the effectiveness of individual lactic acid cultures. Fermentation using *L. sanfranciscensis* [Thiele *et al.*, 2004; Vermeulen *et al.*, 2006] or *L. plantarum* [Gerez *et al.*, 2008; Rollan *et al.*, 2005; Yin *et al.*, 2015] promoted hydrolysis and increased solubility of wheat proteins. It has been shown that protein hydrolysis in sourdough is predominantly associated with pH-dependent activity of cereal enzymes and corresponding changes in proteolytic activity.

Several studies have been carried out to assess the effectiveness of the pool of selected probiotic lactobacilli. De Angelis *et al.* [2006] found the capacity of probiotic VSL#3 prepara-

tion to intensively hydrolyze wheat gliadins. Probiotic product VSL#3 consisting of strains of *Streptococcus thermophilus*, *L. plantarum*, *L. acidophilus*, *L. casei*, *L. delbrueckii* spp. *Bulgaricus*, *Bifidobacterium breve*, *B. longum*, and *B. infantis* was used in the fermentation of wheat flour dough to reduce the content of immunotoxic fragments, to hydrolyze gliadin peptides, and to achieve almost complete hydrolysis of gliadin. Patent application WO2006 / 097415 [2006] describes a method for gluten degradation using a complex mixture of at least six lactic acid bacterial cultures and / or bifidobacteria and long fermentation time (24–31 h). After hydrolysis, non-degraded gliadins remained, some gliadins were partially hydrolyzed, and others were insensitive hydrolyzed; therefore, this method is not the most suitable for complete gluten degradation. *L. plantarum* CRL 775 and *Pediococcus pentosaceus* CRL 792 also hydrolyzed gliadins during the fermentation of wheat dough. The cleavage of gliadins obtained using cell extracts was higher than using cell suspensions [Gerez *et al.*, 2012]. Romanová & Urminská [2017] investigated growth characteristics of *L. plantarum* CCM 3627 and *L. brevis* CCM 1815 and the activity of aminopeptidases. In turn, Stefańska *et al.* [2016] investigated 11 LAB cultures that can hydrolyze gluten in baked goods. All sourdoughs have been found to contain some polypeptides with reactive epitopes. Two strains: *Enterococcus mundtii* and *Wickerhamomyces anomalus*, can be used as probiotics for leavening. *E. mundtii* QAUSD01 and *W. anomalus* QAUWA03 demonstrated the ability to tolerate low pH, resistance to bile salts, and hydrophobicity compared to other gluten-degrading yeast and bacterial strains. It is suitable to use them in cereal fermentation and, therefore, they can be used to produce bakery products for consumers with NCGS [Sakandar *et al.*, 2018].

Sourdough-based biotechnology could contribute to the quality of life improvement in consumers suffering from CD [Nionelli & Rizzello, 2016]. However, this method is not suitable for complete gluten degradation. It is very important to select the optimally parameters of the fermentation process while preparing GF products. The results achieved in various studies showed that the proteolytic activity of the selected LAB strains was not high enough [Stefańska *et al.*, 2016], long fermentation time worsened the technological properties of wheat bread [Katina *et al.*, 2006], and that baked wheat products fermented with LAB were not safe for consumers with CD [Laatikainen *et al.*, 2017]. Therefore, it is advisable to use these LAB to break down allergenic proteins in bakery products for consumers with CD in combination with other measures, and also to produce bakery products for consumers with NCGS [Sakandar *et al.*, 2018].

### Prolyl endopeptidases from microorganisms

A relatively new trend is gluten detoxification by breaking peptide bonds with prolyl endopeptidases (PEPs). PEPs are endoproteolytic enzymes secreted by microorganisms and plants. They hydrolyze gluten into smaller peptides that can be digested by intestinal enzymes or to amino acids [Heredia-Sandoval *et al.*, 2016]. PEPs can be used as a dietary therapeutic tool for CD patients. The mechanism of their action is as follows: gluten is hydrolyzed by co-ingested peptidases in the consumer's stomach and stops (prevents) CD specific

TABLE 2. Enzymes of bacterial origin for reducing gluten immunogenicity.

Enzyme source	Enzyme/bacterial strain	Results	References
Fermentation with LAB cultures	<i>L. alimentarius</i> , <i>L. brevis</i> , <i>L. sanfranciscensis</i> , <i>L. hilgardii</i>	Gluten degradation.	Di Cagno <i>et al.</i> [2002]; Di Cagno <i>et al.</i> [2004]
	Probiotic product VSL # 3	Intense gliadin degradation.	De Angelis <i>et al.</i> [2006]; WO2006/097415
	<i>L. sanfranciscensis</i>	Gluten degradation.	Thiele <i>et al.</i> [2004]; Vermeulen <i>et al.</i> [2006]
	4 strains of <i>L. plantarum</i>	Gluten degradation – hydrolysis of wheat proteins.	Gerez <i>et al.</i> [2008] Rollan <i>et al.</i> [2005]
	<i>L. plantarum</i>	Hydrolysis and solubilization of wheat proteins.	Yin <i>et al.</i> [2015]
	47 strains of LAB were tested for evaluating proteolytic activity	Removed gluten residues in GF bread.	Giuliani <i>et al.</i> [2016]
	<i>L. plantarum</i> and <i>L. brevis</i>	Active proline aminopeptidase was produced.	Romanová <i>et al.</i> [2017]
	13 strains of probiotic bacteria	Partial gluten degradation.	Stefanska <i>et al.</i> [2016]
	18 commercial strains of LAB	The pool of LAB strongly hydrolyzed gluten in bread.	Francavilla <i>et al.</i> [2017]
	<i>Enterococcus</i> <i>Enterococcus</i> and <i>Wickerhanomyces</i> strains	LAB tolerated low pH and bile salts.	Sakandar <i>et al.</i> [2018]
Enzymes of bacterial origin	12 strains of LAB and yeasts isolated from Chinese traditional sourdough	Its ability to degrade wheat protein was limited.	Fu <i>et al.</i> [2020]
	<i>Bifidobacterium</i> species: <i>B. bifidum</i> , <i>B. longum</i> , <i>B. breve</i> and <i>B. animalis</i>	Proteolysis of intact gluten proteins, gliadins, and glutenins.	de Almeida <i>et al.</i> [2020]
	Prolylendopeptidase (PEP) from <i>Myxococcus xanthus</i>	PEP reduces the amount of immunoreactive gliadin peptides.	Alvarez-Sieiro <i>et al.</i> [2014]
	Proteases: <i>Bacillus stearothermophilus</i> , <i>B. thermoproteolyticus</i> , <i>Streptomyces</i> <i>griseus</i> , <i>B. licheniformis</i>	<i>B. licheniformis</i> and <i>B. thermoproteolyticus</i> work very effectively.	Socha <i>et al.</i> [2015]
	PEP from <i>F. meningosepticum</i> , <i>S. capsulata</i> , <i>M. xanthus</i> and <i>E. coli</i>	PEP partially degraded gliadin peptides.	Moreno Amador <i>et al.</i> [2019]
	Alcalase 2.4 L	Effectively removes gluten from unpurified starch.	Bassi [2016]
	Alcalase 2.4 L	Reduced the antigenicity of wheat gluten hydrolysates.	Cruz-Chamorro <i>et al.</i> [2020]
	New E40 glutenase from <i>Actinomycete</i> strain <i>Actinoallomurus A8</i>	Efficiently degrades the most immune-toxic gluten.	Cavaletti <i>et al.</i> [2019]
	<i>Bacillus</i> spp. isolated from sourdough	Reduced gluten content in wheat sourdough.	Rashmi <i>et al.</i> [2020]
	Engineered enzymes	PEP enzyme of <i>Sphingomonas capsulate</i>	Effectively degraded CD-active peptides.
Peptidase Kuma030		High activity of 99.97% gluten degradation.	Wolf <i>et al.</i> [2015]
Peptidase KumaMax or Kuma010		116 times higher proteolytic activity compared to native.	Yoosuf & Makharia [2019]
Transglutaminase (mTG) of microbial origin	Transamidation with mTG in the presence of methyl ester of lysine	Reduced the ability to induce an immune response <i>in vitro</i> .	Luongo <i>et al.</i> [2020]
	MTG and chymotrypsin used to bind lysine or valine to gluten proteins	Reduced the specific immune response of gliadin.	Zhou <i>et al.</i> [2017]

immune responses in the small intestine. Likewise, PEPs may be used to produce GF products from gluten-containing raw materials. Enzymatic therapy for coeliac disease is promising; however, it is important to select the most active enzymes [Boukid *et al.*, 2017a]. Matysiak-Budnik *et al.* [2005] found that PEPs partially degraded gliadin peptides at low concentrations (20 mU/mL) both *in vitro* and *ex vivo*, but could not protect the intestines from immunotoxic metabolites. High levels of PEPs and a longer time (at least 500 mU/mL for 3 h) were required for the complete detoxification of peptides. As Socha *et al.* [2015] proved, all the bacterial proteases of various origin were slightly different in the degree of

proteolysis. *B. licheniformis* and *B. thermoproteolyticus* acted very effectively: molecular weight of fermentation products was low. The use of PEPs from *L. casei* with conventional food was proposed as a treatment method for CD patients [Alvarez-Sieiro *et al.*, 2014]. Bassi [2016] described a method to produce GF starch, where the mass of unpurified starch is treated with an agent to degrade gluten. It should be noted that the effective removal of gluten (even up to 470 mg/kg) from starch can be achieved using Alcalase. Prolonged exposure to high concentrations of PEPs was shown to reduce the amount of immunoreactive gliadin peptides in wheat flours [Rashmi *et al.*, 2020].

### Engineered enzymes

An ideal peptidase for use in the oral enzymatic therapy should possess many qualities to meet high requirements of its application; therefore, it is unlikely that a single naturally occurring enzyme can afford this to the full extent. In a way to solve the problem, the computational protein design allows introducing new traits or significantly improve functional properties of native enzymes [Gordon *et al.*, 2012]. Newly constructed enzymes, gluten hydrolases among them, exhibit higher specificity, much higher activity, solubility, and better performance in the required medium (for example, highly acidic environment of the stomach after meal) [Yoosuf & Makharia, 2019].

First works were carried out in this respect by Ehren *et al.* [2009]. They aimed to develop PEPs with higher activity and stability under gastric conditions, taking a PEP of *Sphingomonas capsulate* as a native template enzyme. The enzyme with as much as 20% enhanced specific activity at pH 4.5 and 200-fold greater resistance to pepsin was created. However, this peptidase was reported to have low to negligible levels of catalytic activity in a lower pH (in the actual pH range of the stomach) and was, thus, expected to be effective only in the small intestine region.

Best described engineered enzymes for gluten hydrolysis are peptidases KumaMax and Kuma030. KumaMax was designed as the improvement of kumamolisin-As (KumaWT, EC 3.4.21), an acidic serine endopeptidase of an acidophilic bacterium *Alicyclobacillus sendaiensis* [Gordon *et al.*, 2012]. The cleavage specificity of the catalytically-active site of KumaWT was shifted to CD-active peptides. The KumaMax has more than 100-fold increased activity on the gluten tetrapeptide substrate. The next step in kumamolisin-As improvement was carried out using the same Rosetta Molecular Modeling Suite, which allowed redesign the active site of KumaMax [Wolf *et al.*, 2015] to reach >99% activity of the enzyme. The new enzyme was referred to as Kuma030. It is 44-fold more active against peptides containing PQQ, and 11-fold more active against peptides containing PQL, than KumaMax. Kuma030 effectively (99.97%) degraded CD-active peptides in 30 min. This enzyme could also be applicable for gluten removal during food processing. Though the newly designed enzymes are still waiting for the clinical studies, they look as promising measures for preventing CD effects.

### Transamidation with transglutaminase

Enzymatic hydrolysis (during the production of wheat flour dough) involves the degradation of the wheat proteins, including highly immunotoxic ones, to those with CD. It completely destroys the gluten structure, and reduces the technological properties (elasticity) of the dough and baked goods. In manufacturing practice, these problems are dealt with by using flour structure enhancers (gelatinized starch), emulsifiers, and hydrocolloids. Therefore, the scientific community has a strong interest in finding alternative methods for gluten degradation: strategies are being developed to eliminate harmful gluten peptides from gluten-free products to counteract the immunogenic effects of gluten fragments, as well as strategies to block gluten-induced inflammatory response [Heredia-Sandoval, 2016]. The essence of this gluten-block-

ing method is the specific blocking of toxic gluten fragments by microbial tissue-transglutaminase (*Streptomyces mobaraensis*) using lysine methyl ester. It has a great advantage because blocking the immunogenicity of T-cell epitopes does not damage the gluten network and preserves the technological properties of flour. Marino *et al.* [2017] assessed the safety of mTG-modified wheat flour for patients with CD and found that transamidated gluten reduced the number of clinical relapses to a limited extent. There would be a need for more extensive clinical trials to confirm the safety of enzymatic treatment of wheat flour for individuals with CD, including further investigation involving a large number of volunteers who consumed products from detoxified wheat flour.

Wheat flour transamidation can be a good alternative to a GFD, but there is not enough scientific evidence that this method is 100% safe. Scientists are worried and warned against the transglutaminase added to the industrially-processed food, doubting its safety and bringing up its putative role in CD pathogenesis [Aaron & Torsten, 2019]. There are ample studies that have shown that transglutaminase causes some neurodegenerative diseases, such as Alzheimer disease and Huntington disease [Amirdivani *et al.*, 2018]. Furthermore, it is supposed that industrial processing of food using microbial transglutaminase to improve its properties has a negative effect on the gluten-sensitive population: it creates new immunogenic gluten peptides [Lerner & Matthias, 2015; Matthias *et al.*, 2016] and may affect autoantigen occurrence in patients with CD.

### Enzymes of fungal origin

Fungal proteases, commonly used as quality enhancers for baked goods, can cause primary proteolysis of gluten. Results of studies have shown that endoproteases from *Aspergillus niger* (AnP2), (AN-PEP) effectively degrade gluten proteins. Stepniak *et al.* [2006] noticed that *Aspergillus niger* (AnP2) prolyl endoprotease had optimal activity at pH 4–5 and remained stable at pH 2. Furthermore, the *A. niger*-derived enzyme effectively degraded all the T-cell stimulants and non-degraded gluten molecules. On average, the AnP2 degraded gluten 60 times faster than prolyl oligopeptidases. Low doses of pure AN-PEP can effectively reduce the amount of gluten fragments [Janssen *et al.*, 2015; Kumar Mohan *et al.*, 2019]. Toft-Hansen *et al.* [2014] tested 25 proteases and investigated 10 of them for their potential to degrade gluten *in vitro*. They proved that the protease from *Aspergillus niger* (AnP2) completely degraded gluten. In turn, Socha *et al.* [2015] tested the proteolytic activity of fungal proteases isolated from *Aspergillus sp.*, *Aspergillus oryzae*, and *Aspergillus niger*. The most effective proteolytic activity was also observed with the use of acid proteinase (AN-PEP) from *A. niger*, since wheat gliadins and peptides of low molecular weight were completely hydrolyzed. This prolyl endoprotease can also be used as an oral supplement to reduce the effect of gluten on consumers. AN-PEP can accelerate the digestion of gluten in the gastrointestinal tract [Salden *et al.*, 2015]. This means that using AN-PEP in combination with gluten-containing food can eliminate the toxic effects of gluten, thus offering the possibility to refuse (occasionally) a strict GFD [Mitea *et al.*, 2008; Montserrat *et al.*, 2015]. Fungal proteases have the highest activity in

degrading gluten immunotoxic compounds compared to other proteases [Socha *et al.*, 2015]. The composition of food affects the amount of AN-PEP required to eliminate gluten [Montserrat *et al.*, 2015]. Studies have shown that single fungi-derived proteases can be effective for gluten degradation and that the combination of several fungal proteases allows a faster reduction in the content of toxic gluten fragments using only one enzyme [Ehren *et al.*, 2009]. The ability of Flavourzyme (an enzyme complex from *Aspergillus oryzae*) to hydrolyze the prolamins of wheat was investigated. The results showed that Flavourzyme effectively degraded gliadins and could significantly reduce their immunotoxicity [Mickowska *et al.*, 2018]. Schultz *et al.* [2018] investigated the ability of prolyl endopeptidases extracted from *Flammulina velutipes* (FvpP) to hydrolyze gluten. The FvpP hydrolyzed  $\alpha$ -gliadin into small, less hydrophobic peptides after 20-h incubation. It was active at different pH values and higher salt concentrations, *i.e.*, under similar conditions as in grain products, showed a moderate temperature stability, and slight thermal inactivation after use.

Studies have shown that fungi-derived proteases could be considered the most effective for gluten degradation. Further research should focus on using AN-PEP-treated starch in GF bakery products, and on degrading gluten in wheat bran or fermented food products to maintain a high nutritional value, and good technological and organoleptic properties, for example, in leavened products. However, doubts remain as to whether the use of these proteases is completely safe in the production of wheat products and acceptable to consumers.

### Enzymes of plant origin

The use of plant enzymes is not a new concept in the baking industry. These enzymes play an important role in the production of some foods: syrups, alcoholic beverages, dairy products, bakery products, *etc.* [Meshram *et al.*, 2019]. Plant proteases are enzymes that are commonly found in fruits, such as papaya, pineapples, figs, and kiwifruit. Sun *et al.* [2016] evaluated protease activity in 90 species of plants, including fruit and vegetables. Ten types of fruit and thirteen vegetables possessed high protease activity. Pineapples, figs, and papaya used to produce commercial proteases showed a high level of protease activity. In addition, high protease activity was detected in kiwifruit, broccoli, ginger, leek, and red pepper. Based on data above, it can be concluded that plants have high untapped potential as candidates for plant protease production.

Papain, bromelain, and actinidin belong to the cysteine protease family and exhibit a high hydrolytic potential. Papain was used to produce wheat gluten hydrolysates, a by-product of wheat starch production. During treatment with papain, the low molecular weight peptides were released from proteins [Wang *et al.*, 2007]. Papain destroyed allergenic epitopes by hydrolyzing gliadins into small peptides [Buddrick *et al.*, 2015; Xue *et al.*, 2019] and exhibited great effects on gliadin hydrolysis even at its very low concentration [Li *et al.*, 2016]. Bromelain was used to produce hypoallergenic flour suitable for patients with wheat allergies. It can hydrolyze peptide bonds in proline residues and thereby alter the structure of gluten fragments [Watanabe *et al.*, 2000].

Kiwifruit proteases are enzymes belonging to the cysteine protease family of papain. An *in vitro* study [Kaur *et al.*, 2010] showed that actinidine from a green kiwifruit affected protein digestion in the small intestine. Various food proteins, including cereal gluten, have been incubated with or without green kiwifruit extract using a two-stage *in vitro* digestive system consisting of an incubation with pepsin at stomach pH (mimicking gastric digestion) and then with pancreatin at low intestinal pH, imitating the human digestive tract. The kiwifruit extract affected gluten absorption. Actinidin has been shown to improve gluten digestion in experimental rats [Rutherford *et al.*, 2011]. Jayawardana *et al.* [2019] analyzed the possibility of minimizing gluten intolerance by co-consumption of some fruits: papaya, pineapple and green kiwifruit, and highlighted the potential of green kiwifruit for consumption as a means of minimizing adverse effects of dietary gluten.

As Taga *et al.* [2017] proved, ginger protease can also hydrolyze gluten to peptides with an average molecular weight of <600 Da under weak acidic conditions. Data obtained from the studies performed by Bellir *et al.* [2014] and Gabr [2018] have confirmed that *Nigella sativa* (also known as blackseed or black caraway) had proteases that could be used in the food industry. The protease from the seeds of *N. sativa* can hydrolyze the gluten protein; therefore, it can be used to treat coeliac disease. *N. sativa* seed proteases, due to their ability to detoxify gluten, may offer an alternative treatment for CD in the future. Less common plants also have active proteolytic enzymes. Considerable attention was drawn to the study of digestive enzymes of an exotic fleshy plant *Nepenthes pitcher*, including protease nepenthesin [Ravee *et al.*, 2018]. The *Nepenthes pitcher* fluid has a particularly strong ability to detoxify gluten, which can be associated with the formation of a new generation of prolyl endopeptidases [Rey *et al.*, 2016; Schröder *et al.*, 2017]. Cumin (*Cuminum cyminum* L.) water extracts have high protease activity too. Cumin seed peptides significantly increased the proteolytic activity of pepsin (up to 400%) [Siow *et al.*, 2016]; however experiments with gluten have not been performed.

### Enzymes of animal origin and human digestive enzymes

Wheat gluten can be hydrolyzed using various enzymes of animal origin. A number of studies have been published in which wheat gluten has been degraded by commercial proteases of animal origin (pepsin, pancreatin, trypsin and chymotrypsin). All enzymes cleaved gliadin to peptides with a lower molecular weight (10–15 kDa), and chymotrypsin was the most effective; however, their effects have been found to be limited [Cao *et al.*, 2020; Giorgi *et al.*, 2020].

Insects and larvae have many strong proteolytic enzymes in the digestive tract [Grover *et al.*, 2018; Pilon *et al.*, 2017]. Insect enzymes have been shown to be specially adapted for the efficient hydrolysis of wheat proteins. A proline specific serine peptidase from the midgut of the yellow mealworm (*Tenebrio molitor*) can actively hydrolyze wheat gluten to polypeptides [Tereshchenkova *et al.*, 2016]. Proteolytic bacteria from the gut of the velvetbean caterpillar (*Anticarsia gemmatalis*) showed increased activity at 40°C, and were active at pH 7.5–10 [Pilon *et al.*, 2017]. From among all the tested insects, *Rhizopertha dominica* showed the highest activity of

prolyl peptidase [Mika *et al.*, 2015]. The proteolytic activity in *R. dominica* is owed to the trypsin-type enzymatic activity. This enzyme has been cloned and characterized but has not been used in the gluten-free food industry. Gutierrez *et al.* [2017] showed that enzymes of the gastrointestinal elastase 3B, elastase 2A, and carboxypeptidase A1 from human digestive tract could also degrade gluten. The investigation of the ability to degrade typical gluten peptides showed that, although they all were cleaved by proteases to a certain extent, the proteolysis products remained immunoreactive to coeliac T-cells and were likely to induce signs of CD [Tavano *et al.*, 2018].

### COMBINED APPROACH FOR WHEAT GLUTEN ALLERGENICITY ABOLISHMENT

Even if the enzyme treatment alone is unable to fully eliminate the immunogenicity of wheat products, the combined approach would allow applying these products in GF diet. There are various options for combining different methods. The results of studies on the combination of several biological treatments to eliminate the immunogenicity of wheat are presented in Table 3.

#### Combination of cereal germination with LAB fermentation

As reported by Lopenen *et al.* [2007] and Montemurro *et al.* [2019], almost all wheat prolamins (gliadins and glutenins) were degraded during sourdough fermentation of sprouted wheat flour. Prolamin hydrolysis in sourdough of germinated wheat was more intense, possibly due to the high activity of cysteine proteinase in germinated wheat. Both of these methods are natural, and products made in this way are likely to be attractive to consumers, but they are not 100% effective.

#### Combination of cereal germination with fungal proteases treatment

Fungal proteases are often used to produce GF beer [Guerdrum & Bamforth, 2012]. Malt hydrolysis with AN-PEP resulted in a significant reduction in the residual prolamins content. There are previous studies [Walter *et al.*, 2014] that fungal proteases successfully hydrolyzed gluten residues in sprouted wheat bran, but there have been no recent studies in this area.

#### Combination of selected *Lactobacillus* cultures with different proteases treatment

It has been shown that using selected LAB together with different proteases over a long time can reduce the residual content of gluten immunogenic sequences. The required fermentation time for gluten degradation was significantly reduced (up to 12–20 h at 30–37°C) using fungal proteases [Giuliani *et al.*, 2016]. This effect was obtained using only two selected lactic acid bacteria (*L. sanfranciscensis* DPPMA12 and *L. plantarum* DPPMA125) in combination with fungal proteases (isolated from *Aspergillus oryzae*, *A. niger*, or mixtures thereof). It has been found that LAB and fungal proteases can degrade the gluten of different wheat varieties, and the good tolerance to such treated wheat in coeliac patients has been demonstrated.

Curiel *et al.* [2014] have developed a technology for producing gluten-free pasta using entirely hydrolyzed wheat flour with good organoleptic characteristics and nutritional value. Wheat flour fermentation with LAB and fungal proteases reduces the content of gluten. The study by Arte *et al.* [2015] revealed the effects of various biological treatment methods, such as activation of endogenous bran enzymes, addition of an enzyme mixture, and microbial fermentation on wheat bran protein modification. The biological treatment in acidic media significantly increased the solubility of wheat bran protein. The study by Di Cagno *et al.* [2010] has shown that young coeliac patients are safe to eat sweet pastries made from wheat flours that have become GF during the fermentation. After fermentation, the wheat flour was dried in a spray dryer and used to produce sweet pastries. Selected LAB cultures and fungal proteases, commonly used for bakery products, degraded gluten to <10 mg/kg. Greco *et al.* [2011] have also proved that there is no immune response in CD patients who daily consume baked goods produced from hydrolyzed wheat flour. The tolerance to such treated wheat in coeliac patients has been proven on short-term consumption [Mandile *et al.*, 2017]. This patented method encourages the already applied practice in Italy to produce GF bakery products with sourdough and pasta. However, more detailed clinical surveys are needed to demonstrate its long-term safety as well the technological properties of wheat flours with hydrolyzed gluten, and to elucidate its impact on the baking process. Whether the broken gluten network is replaced by hydrocolloids, the question arises – whether it is expedient to use such flour after gluten hydrolysis for baking, or might it be better to use wheat starch for bread making.

#### Combination of enzymes of different origins

Studies have shown that concomitant use of several enzymes leads to more efficient degradation of gluten than the use of individual enzymes. The use of several different enzymes together (of plant, bacterial, animal, or fungal origin), that cleave different peptide bonds has improved the efficiency of gluten hydrolysis [Brzozowski *et al.*, 2020; Janssen *et al.*, 2015]. Li *et al.* [2016] found that the sequential hydrolysis of wheat flour using several different enzymes was more effective in reducing the amount of gliadin than the hydrolysis by every individual enzyme. The sequential fermentation of wheat flour with Alcalase and papain almost completely eliminated the gluten under optimal conditions. It is obvious that, under suitable conditions, such hydrolysis is a promising way of producing low-allergenic wheat products. However, the organoleptic and functional characteristics of the Alcalase-papain-treated product have to be examined to determine the feasibility of this method for production. Although wheat gluten can be hydrolyzed using enzymes of different origins, it is necessary to carefully select them and manage the hydrolysis process to achieve the desired effect, since improper organization of the process can have the opposite effect [Tavano *et al.*, 2018]. Therefore, some GF food additives can have a negative effect on consumers associated with wheat intolerance and even on patients with coeliac disease. The manufacturing process of GF products should be carefully designed and managed to avoid complications. It remains the challenge to



TABLE 3. Combined approach of several biological treatments to eliminate the immunogenicity of wheat.

Combined treatment	Enzyme source	Results	References
Malting or germinating + sourdough fermentation	<i>Lactobacillus rossiae</i> , <i>L. plantarum</i> and <i>L. sanfranciscensis</i> were used for fermentation of flours from germinated grains	Fermentation enhanced the nutritional and functional features of sprouted flours.	Montemurro <i>et al.</i> [2019]
Malting or germinating + fungal proteases	Bran extracts from germinated cereals + <i>Aspergillus niger</i> prolyl endopeptidase	AN-PEP exceeded the activities of germinated cereal in gluten degradation.	Walter <i>et al.</i> [2014]
Sourdough fermentation + fungal proteases	<i>L. sanfranciscensis</i> and <i>L. plantarum</i> in combination with fungal proteases from <i>A. oryzae</i> and <i>A. niger</i>	The required fermentation time for gluten degradation significantly reduced.	Giuliani <i>et al.</i> [2016]
	White bread was prepared from wheat flour pre-treated with selected lactobacilli and fungal proteases	Such bread did not affect an immune response in patients with CD on short-term consumption.	Mandile <i>et al.</i> [2017]
Sourdough fermentation + proteases of animal origin	Mixture of 5 different bacterial strains by a combination of pepsin, trypsin or chymotrypsin	Probiotic strains reduced the toxicity of gliadin that remains after peptic-tryptic digestion.	Giorgi <i>et al.</i> [2020]
Enzymes of different origin	Activation of endogenous wheat bran enzymes in addition of microbial fermentation	Biological treatment significantly increased solubility of wheat bran proteins.	Arte <i>et al.</i> [2015]
	Pepsin and other digestive supplements + AN-PEP enzyme	AN-PEP enzyme enhanced the effects of digestive supplements.	Janssen <i>et al.</i> [2015]
	Chymotrypsin, Flavourzyme, Trypsin, Pepsin or Alcalase + Papain	Alcalase-papain significantly reduced allergenicity of wheat flour.	Li <i>et al.</i> [2016]
	The mixture of peptidases from <i>L. acidophilus</i> + <i>A. niger</i>	Coeliac-toxic peptides were hydrolyzed effectively after 3 h.	Brzozowski <i>et al.</i> [2020]

manage process factors such as enzyme type and its activity, content of proteins, pH, action mechanism, reaction time, and others. These conditions do not always correspond to the real conditions of wheat products manufacture in practice. Besides, additional measures are necessary to compensate for the reduction or elimination of gluten in order to ensure high product quality.

### Combined approach of biotechnological and non-biotechnological measures

A number of studies have been performed to degrade gluten-immunotoxic compounds by physical treatments. Lamacchia *et al.* [2016] studied the effects of microwaves on soaked wheat kernels and reported that this modification could reduce the immunotoxic effects of wheat proteins by up to 99%, which would allow them to produce low-gluten bread. However, subsequent studies [Gianfrani *et al.*, 2017] had used more accurate methods for the determination of gluten residues and observed that the microwave treatment did not reduce the celiac immunogenicity of gliadins. As Mahroug *et al.* [2019] confirmed, despite the significant changes observed in the gluten secondary structure, the microwave treatment was ineffective in decreasing the amount of potential celiac-toxic epitopes in wheat flour but even increased it when the flour was exposed to the low doses of energy [Leszczynska *et al.*, 2003]. The reduction of the residual antigenicity of wheat proteins can be achieved during thermal treatment, by using high pressure, extrusion, and spray drying [Stănciuc *et al.*, 2018]; however, the effect was not sufficient to make wheat products suitable for gluten-sensitive people.

Another physical method – ultrasound treatment, can significantly improve emulsifying, foaming, and rheological properties of wheat gluten proteins, but slightly decreases

their molecular weight [Zhang *et al.*, 2011]. The ultrasound effects on the immunogenicity of wheat gluten are insignificant, but it can be used to activate enzymatic hydrolysis. Controlled ultrasound pretreatment can alter the microstructure, nano-mechanical properties, and secondary structures of wheat gluten, to increase the content of free amino acids and to improve the effects of enzymolysis [Zhang *et al.*, 2015; Yang *et al.*, 2017]. Combined enzyme/ultrasound bioprocessing produces cavitation effects that enhance the transport of enzyme macromolecules to the surface of the substrate and thus activates the action of enzymes [Delgado-Povedano & De Castro, 2015; Kwiatkowska *et al.*, 2011]. However, time and power control is very important in this process, because choosing the wrong cavitation parameters can reduce the degree of enzymatic hydrolysis [Islam *et al.*, 2014; Yu *et al.*, 2014].

Thus, although physical methods would be more accessible and do not require much energy and time, the enzymatic hydrolysis appears to be a more effective approach in minimizing allergenicity of wheat proteins [Rahaman *et al.*, 2016]. However, the possibility of combining physical and biotechnological measures should be further explored.

### CONCLUSIONS

The current gluten-free products available on the market have technological and sensory drawbacks. Due to the growing trend of gluten-free market in the last years, technologies for the production of gluten-free or reduced-gluten wheat products are being developed. Research on the use of the biological approach in wheat products in order to eliminate or reduce the immune toxicity of gluten proteins is being actively undertaken. Various enzymes, such as those of bacte-

rial, fungal, plant and animal origin, can be used to this end. Most of the studies were carried out using cultures of lactic acid, including their individual strains or various combinations. Selected strains that exhibit proteolytic activity, which reduces the allergenicity of wheat sourdough, can be used as specific starter LAB cultures to prepare foods for special purposes. Satisfactory results are achieved by using bacterial and plant enzymes, but the complete elimination of gluten immunogenicity in wheat products is still possible only by using fungal proteases, engineered enzymes or combining several treatments, for example, by using LAB fermentation or germination with fungal proteases. Despite numerous research in scientific laboratories, it is still impossible to offer patients with CD an alternative diet based on highly nutritious and tasty cereal GF products in practice.

The applicability of the used techniques in bread and bakery production is uncertain. Complete degradation of gluten requires long fermentation times, often in combination of several strains of different lactic acid bacteria. Moreover, with the addition of peptidases, gluten degradation must be controlled. This long and complicated process also leads to higher production costs of the final product. Furthermore, when wheat gluten is completely degraded, the viscoelastic properties are lost, which reduces the benefits of the process [Engström *et al.*, 2015]. Without the use of additives, the optimal dough and cereal product cannot be prepared from such altered and processed wheat grains or flours. The safety of such products has not been fully proven. It has not been established whether the products of such wheat flour are better than those made from starches and whether their price and quality are acceptable to consumers. Furthermore, the question remains unanswered: Is it beneficial to degrade gluten in wheat flours?

As the majority of the studied biotechnological tools readily remove small amounts of gluten, it would be appropriate to use wheat by-products after physical removal of gluten by wet fractionation. However, further research should focus on using enzymatically-treated wheat starch and bran in gluten-free bakery products or fermented food products with a high nutritional value, and good technological and organoleptic properties, that can be consumed not only by people suffering from gluten intolerance, but also by other personalized groups of consumers.

#### RESEARCH FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

#### CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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Submitted: 20 November 2020. Revised: 12 January 2021.  
Accepted: 26 January 2021. Published on-line: 12 February 21.