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.

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

**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 [Jiménez 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 gluten- 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...
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 [Kriegel & 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, 2016].

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.

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**FIGURE 1.** Sources of enzymes used for gluten hydrolysis.
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 [Guerdum & 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 7th 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. Germinating of wheat kernels [Geßendorfer et al., 2011] 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 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-α to activate proteolytic enzymes (glutenase and collagenase) in vitro, which are optimally active at 37°C. Mass-spectrometry analysis showed that Triticain-α degraded immunotoxice peptides. Studies have shown that Triticain-α 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 α-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.

### Table 1. Cereal endogenous enzymes used for gluten hydrolysis.

<table>
<thead>
<tr>
<th>Source of enzymes</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germinating grains</td>
<td>Hydrolysis of prolamines.</td>
<td>Hartmann et al. [2006]</td>
</tr>
<tr>
<td>Proteases from germinating cereals</td>
<td>Hydrolysis of prolamines.</td>
<td>Stenman et al. [2009]</td>
</tr>
<tr>
<td>Wheat grains germinated for 8 days</td>
<td>Reduction of peptides eliciting immune response.</td>
<td>Boukid et al. [2017b]</td>
</tr>
<tr>
<td>Wheat kernels after 7-day germination</td>
<td>Degradation of gluten.</td>
<td>Michalcová et al. [2019]</td>
</tr>
<tr>
<td>Germination of wheat kernels</td>
<td>Gluten content in wheat germ (8–10 days) was lower than the limit of detection.</td>
<td>Adrianos et al. [2017]</td>
</tr>
<tr>
<td>Recombinant wheat cysteine protease Triticain-α</td>
<td>Triticain-α activated proteolytic enzymes in vitro.</td>
<td>Savvateeva et al. [2015]</td>
</tr>
</tbody>
</table>
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, immuno-reactive, and coeliac 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 21st 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 preprparation 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 & Urmínská [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 polyepitides with reactive epitopes. Two strains: Enterococcus mundtii and Wickerhamomyces anomalus, can be used as probiotics for leavening. E. mundtii QAUSD01 and W. anomalus QAUA03 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 [Laatkainen 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 therapeutical 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.

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

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]. Matsysak-Budnicky 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 hydrolyases 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 capulate* 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 designed engineered enzymes for gluten hydrolysis are peptidases KumaMax and Kuma030. KumaMax was designed as the improvement of kumamolinis-As (KumaWT, EC 3.4.21), an acidic serine endopeptidase of an acidophilic bacterium *Alcyclobacillus 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 kumamolinis-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-
digesting 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 [Montserrrat 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 α-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 actinidin 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 enzymatic enzymes of an exotic fleshy plant Nepenthes pitcher, including protease nepenthisin [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; Schräder 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 Lopenon 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 protease 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 [Guerdrom & Bamforth, 2012]. Malt hydrolysis with AN-PEP resulted in a significant reduction in the residual prolamin 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 papaain 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-papaain-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
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 [Leszcynska *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 bioprocesses produce 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 Castroč, 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-
rrial, 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.

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**CONFLICT OF INTERESTS**

Authors declare no conflict of interest.

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