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FERMENTATION WITH *LACTOBACILLUS* STRAINS FOR ELIMINATION OF GLUTEN IN WHEAT *(TRITICUM AESTIVUM)* BY-PRODUCTS

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Abstract

Recently there is an increase in the number of consumers with gluten intolerance that causes expanding of the demand for gluten-free products. Gluten-free diet is unbalanced and usually has a higher percentage of calories from fat, less of carbohydrates, as well as low intake of non-starch polysaccharides. To solve this problem, new strategies are looked for to eliminate gluten in products of wheat and other cereals and to make them more balanced. Fermentation with lactic cultures and/or enzymes enables to reduce the gluten content in wheat flour. However, this process takes a long time, it is complicated to control, and hydrolysed gluten loses its technological properties. The purpose of this work was to find another way of removing gluten residues: first remove gluten from wheat by wet fractionation, then hydrolyse gluten residues in the remaining fractions by using biotechnological measures. The fractions of starch, fiber and bran had an initial gluten concentration of 85–33750 mg kg^{-1}. For eliminating the gluten residues, they were fermented with four probiotic strains separately: *Lactobacillus plantarum* P-1, *Lactobacillus brevis* R-1, *Lactobacillus acidophilus* 308, *Lactobacillus acidophilus* 336. Short (12 hours) and long fermentation (24 hours) at 30 and 37 °C was used. Gluten was degraded in wheat starch to below 20 mg kg^{-1} using *Lactobacillus plantarum* in short time, other strains performed better using long fermentation. In conclusion, it could be stated that sourdough-based biotechnology could eliminate the immunogenicity of wheat by-products and to improve the quality of life of celiac patients.

Keywords: wheat, gluten, hydrolysis, sourdough, *Lactobacillus*

Introduction

Wheat is one of the most popular cereals in the world, however, gluten proteins of wheat are responsible for very common allergic reactions in populations, leading to immune disorder and non-celiac gluten sensitivity (Gujral et al., 2012; Kang et al., 2013; Catassi et al., 2014). Currently, the only therapy is a strict, lifelong gluten-free diet (GFD). Compliance with a GFD is an extremely challenging task, given a number of problems related to poor quality of gluten-free products compared to their gluten-rich counterpart (Do Nascimento et al., 2017) as well as these products are more expensive (Stevens, Rashid, 2008). Patients with celiac disease are looking for alternatives and are using products from gluten free materials such as corn, rice, millet, buckwheat, amaranths and potatoes. The diet of these patients is unbalanced and have a higher percentage of calories from fat and less from carbohydrates, also in GFD was obtained low intakes of non-starch polysaccharides (Thompson et al., 2005; Wild et al., 2010). Products made from naturally gluten-free raw materials resulted in breads often having inferior textural and sensory properties compared to the corresponding gluten-containing products (Hager et al., 2012; Miranda et al., 2014; Pellegrini, Agostoni, 2015). To resolve this socioeconomic problem, new strategies are looked for to eliminate harmful gluten from wheat and other cereals and to produce balanced products with good sensory properties (Greco et al., 2011; Nionelli, Rizzello, 2016).

Wheat gluten fragments (peptides) remain intact during digestion. They penetrate through the small intestine wall and initiate antigenic cellular immune responses. There is no immune response if the gluten is hydrolysed to peptides, which contain less than nine amino acid residues. Research on the use of biological measures in wheat products to eliminate or reduce the immune toxicity of gluten proteins is being actively pursued in the last decade. Numerous studies (Di Cagno et al., 2008; Giuliani et al., 2016; Gerez et al., 2012; Loponen et al., 2007; Romanová, Urminská, 2017; De Palma et al., 2010; Stefanska et al., 2016) were carried out using lactic cultures - their individual strains or various combinations. They focus on probiotic strains' possibilities to decrease the toxicity of wheat flour, but there is a lack of informationon on biological measures to completely eliminate gluten from wheat processing products.

Sourdough fermentation with lactic acid bacteria (LAB) can improve the texture, palatability, aroma, shelf life and nutritional value of wheat breads (Guerzoni et al., 2011), texture and palatability of whole grain, fiber-rich or gluten-free products, stabilise or increase levels of various bioactive compounds, retard starch bioavailability and improve mineral bioavailability (Katina et al., 2005; Moroni et al., 2009). LAB degrade celiac active gluten peptides, because some species of LAB produce specific peptidases during growth, which are capable to hydrolyse hardly cleavable bonds between amino acids in proline-rich peptides (Vukotić et al., 2016). Selecting strains of LAB with targeted proteolytic effects is vital important (Stefańska et al, 2016).

The use of sourdough LAB was at first proposed with the aim of eliminating traces of gluten epitopes in 2002. Di Cagno et al. (2002) showed that selected LAB, possessing proteolytic activities, could efficiently hydrolyse the toxic peptides of gliadin in wheat sourdough. The pool of *L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A, and *L. hilgardii* 51B has a

pattern of specialized peptidases capable of hydrolysing different peptide bonds that potentially include the proline (Di Cagno et al., 2004). Study of Di Cagno et al. (2008) highlighted the use of selected LAB consisted of *Lactobacillus sanfranciscensis* LS40 and LS41 and *Lactobacillus plantarum* CF1 to eliminate risks of contamination by gluten and to enhance the nutritional properties of GF bread.

Several studies were carried out using individual strains of LAB also. Sourdough fermentation using *Lactobacillus sanfranciscensis* (Thiele et al, 2004; Vermeulen et al, 2006) or *Lactobacillus plantarum* (Yin et al, 2015) showed a decrease in pH and resulted in hydrolysis and solubilization of wheat proteins.

De Angelis et al. (2006) showed the capacity of probiotic VSL#3 preparation to hydrolyse extensively wheat flour. Probiotic product VSL#3 including *Streptococcus thermophilus, L. plantarum, L. acidophilus, L. casei, L. delbrueckii spp. bulgaricus, Bifidobacterium breve, B. longum and B. infantis* strains was used in the fermentation of a mass with wheat flour in order to hydrolyse gliadin peptides and promoted almost complete hydrolysis of gliadin peptides. Patent Application WO2006/097415 (2006) describes a process for gluten degradation by means of the use of a complex mixture consisting of at least six lactic acid bacteria and/or bifidobacteria and long fermentation times (24–31 hours) also. After hydrolysis some gliadins were partially hydrolysed, but others were not susceptible to hydrolysis process. *Lactobacillus plantarum* CRL 775 and *Pediococcus pentosaceus* CRL 792 also hydrolysed gliadins during wheat dough fermentation (Gerez et al., 2012).

Romanová, Urminská (2017) described growth characteristics and intracellular aminopeptidases activities of *Lactobacillus plantarum* CCM 3627 and *Lactobacillus brevis* CCM 1815. The results confirm production of active proline aminopeptidase, which is important for cleavage of proline rich-peptides. Two strains: *Enterococcus mundtii* and *Wickerhamomyces anomalus* exhibited the potential to be used as probiotic for sourdough fermentation: they have shown the ability to tolerate low pH, bile salt properties and hydrophobicity compared to other gluten-degrading yeast and bacterial strains (Sakandar et al., 2018).

Stefańska et al. (2016) have selected 11 LAB strains capable of hydrolysing gliadin in bakery sourdoughs. However, in all sourdoughs were found some polypeptides with IgE-reactive epitopes. Previous research has shown that fermentation with LAB reduces the amount of reactive gluten fragments, but does not reach the safe limit for gluten free products, which is 20 mg kg^{-1} (Standard 118-1979, 2015).

Fermentation with mixtures of selected lactic acid cultures in combination with fungal enzymes enable to reduce the gluten content in wheat flour to gluten free limit (Rizzello et al., 2007; 2014). However, this process takes a long time, it is necessary to control it in several stages, but hydrolysed gluten still loses its technological properties: enzymatic hydrolysis destroys the gluten network, reduces the elasticity of the dough and baked goods (Van Den Broeck et al., 2009).

In this work, it would be advisable to combine physical and biotechnological measures for the preparation of raw materials for the production of gluten-free wheat products: at first remove gluten from wheat by wet fractionation, then hydrolyse gluten residues in the remaining fractions by using LAB fermentation. Whereas the levels of gluten are low in the wheat by-products, it can be expected that the effect of LAB will be sufficient to eliminate it.

Wet fractionation of wheat could be done by centrifuging of the flour-water mixture in the laboratory according to Czuchajowska and Pomeranz (1993) as well as at industrial plants producing gluten and starch from wheat that are widely used in the food industry, meanwhile the fractions of fiber and bran are diverted to feed production.

Eliminating of gluten residue in wheat processing products allows produce gluten free starch and gluten free fraction of arabinoxylan and other non-starch polysaccharides, suitable for flour mixtures or bakery production for users intolerant to gluten or celiac sufferers.

The aim of the research was to use fermentation with *Lactobacillus* strains for elimination of gluten in wheat by-products.

Materials and Methods

Materials

Investigations were carried out at the Kaunas University of Technology, Food Institute, Lithuania. Samples of wheat fractions after dry and wet fractionation: starch, fiber and bran were provided by Roquette Amilina, AB, Lithuania.

A fraction of wheat bran was obtained as a by-product during the dry milling of wheat grain and was composed of outer layers of wheat kernel, mainly pericarp. A fraction of fiber was obtained as a by-product in the wet processing of the flour for starch and gluten separation, and was mainly composed of seed coat and aleurone residues.

Physical-chemical analysis of composition of wheat by-products

Moisture content of wheat by-products was measured by humidity measuring device Kern MLS 50-3HA 160N.

pH was measured by pH-meter ORION 3STAR.

Determination of protein content was done by Kjeldahl method (LST EN ISO 20483).

Research on the selection of Lactobacillus strains

Research to remove wheat gluten was carried out experimenting with microorganisms of the collection from the KTU Food Institute. Four probiotic strains: *Lactobacillus plantarum* P-1, *Lactobacillus brevis* R-1, *Lactobacillus acidophilus* 308, *Lactobacillus acidophilus* 336 were used.

The LAB cultures were stored for the study at –72–74 °C in the VIABANK (MWE medical wire) system. Cultures revived in MRS broth (Biolife, Italy):

an initial LAB suspension was prepared by seeding of the initial culture on MRS agar (Biolife, Italy) and incubating at 30 °C *(L. plantarum* P-1, *L. brevis* R-1) and at 37 °C (*L. acidophilus* 308, *L. acidophilus* 336) for 24 h. Each LAB culture was transformed then into sterile milk and incubated at an appropriate temperature for 72 h under anaerobic conditions (aerostat with oxygen sorbent).

The number of lactic acid bacteria was determined by the method of seeding in Petri dishes by incubation on MRS agar under anaerobic conditions for 72 h at 30 or 37 °C.

Total plate count of samples performed according to standard procedures LST EN ISO 4833:2003, found 2.1×10^2 CFU g^{-1}, count of yeast and mold 1.2×10^1 CFU g^{-1}, aerobic and anaerobic spore forming bacteria was not found. The amounts of these microorganisms in the samples were small and the samples were suitable for consumption and biotechnological work without sterilization from the microbiological safety point of view.

Wheat starch or fiber samples (5–10 g) were weighed into glass tubes (30 mL), distilled water (10 mL) and LAB suspension (7 mL) at an active concentration of $1.7–2.8\times10^6$ was added (Table 1) and mixed well.

Table 1

Preparing of samples of wheat product with different LAB strains

Sample	Wheat by-product		LAB strains	Active concentration, CFU g^{-1}
	Starch, g	Fibre, g		
SLA1	5	–	*L. acidophilus* 308	2.0×10^6
SLA2	5	–	*L. acidophilus* 336	2.1×10^6
SLB	5	–	*L.brevis* R-1	1.7×10^6
SLP	5	–	*L.plantarum* P-1	2.8×10^6
FLA1	–	10	*L. acidophilus* 308	2.0×10^6
FLA2	–	10	*L. acidophilus* 336	2.1×10^6
FLB	–	10	*L.brevis* R-1	1.7×10^6
FLP	–	10	*L.plantarum* P-1	2.8×10^6

LAB – lactic acid bacteria, SLA1 – starch with *L. acidophilus* 308*;* SLA2 – starch with *L. acidophilus* 336*;* SLB – starch with *L. brevis* R-1*;* SLP – starch with *L. plantarum* P-1*;* FLA1 – fiber with *L. acidophilus* 308*;* FLA2 – fiber with *L. acidophilus* 336*;* FLB – fiber with *L. brevis* R-1*;* FLP – fiber with *L. plantarum* P-1

Samples were incubated at 30 and 37 °C, pH and gluten content in sourdough were measured after 12 and 24 hours.

Gluten quantitation by ELISA

Gluten residues in wheat products were quantitated by competitive ELISA using G12 antibody AACCI 38-52.01 (Romer Labs, UK Ltd) according to the manufacturer's instructions. Gluten concentrations were established based on calibration function provided by Romer Labs. Multiscan EX microplate reader with a 450 nm filter was used for the reading of the strips.

Results and Discussion

The fractions of starch, fiber and bran had different moisture content (10.78–72.01%), different amount of total protein (0.32–16.9%) an initial gluten concentration of 85–33750 mg kg^{-1} (Table 2).

For eliminating of gluten residues starch and fiber were fermented with four probiotic strains separately: *Lactobacillus plantarum* P-1, *Lactobacillus brevis* R-1, *Lactobacillus acidophilus* 308, *Lactobacillus acidophilus* 336. Short (12 hours) and long fermentation (24 hours) at 30 and 37 °C was used.

Table 2

Characteristics of samples

Wheat by-products	Moisture, %	Total protein, %	Gluten content, mg kg^{-1}
Fiber*	72.01±0.20	3.80±0.02	7800.00±218.00
Bran**	13.43±0.20	16.90±0.02	33750.00±945.00
Starch	10.78±0.20	0.32±0.02	85.00±2.00

* Outer layers of wheat kernel, mainly pericarp.
** Seed coat and aleurone residues.

The reducing of gluten content depending on the decrease of pH was observed. Gerez et al. (2008), Rollan et al. (2016) demonstrated also that protein hydrolysis in sourdough were partially caused by pH-dependent activation of cereal enzymes according to change in proteolytic activity. Di Cagno et al. (2002) proved also, that primary proteolysis is exerted by wheat endogenous enzymes, which are activated by the low pH. Among the selected LAB cultures, the lowest pH was achieved by using *L. acidophilus* 308, the least acidic sourdough was obtained with *L. brevis* R-1. The pH of the fermentation of starch was also lower than sourdough of fiber (Figure 1).

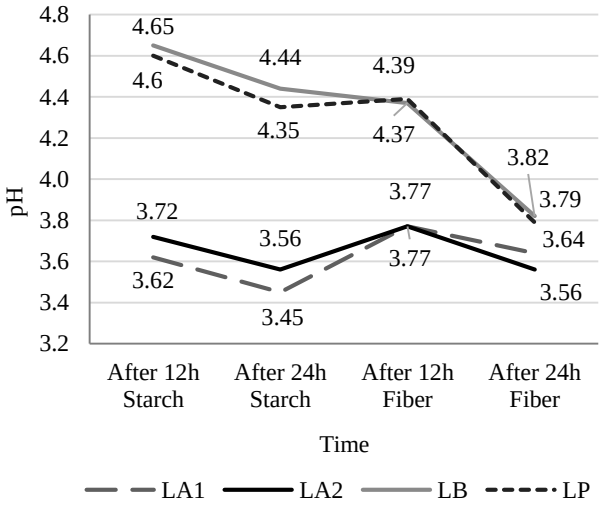

Figure 1. Decreasing of pH in different sourdough
LA1 – *L. acidophilus 308*; LA2 – *L. acidophilus 336;* LB – *L. brevis R-1*; LP – *L. plantarum P-1*

Further hydrolysis of peptides was exerted by intracellular peptidases of LAB in a strain-specific manner: the type and amount of released amino acids depend on the fermenting strain (Di Cagno et al., 2002).

Table 3

pH and gluten content in samples of wheat starch after fermentation

Samples	Temperature, °C	Duration of fermentation, h			
		12		24	
		pH	Gluten content, mg kg^{-1}	pH	Gluten content, mg kg^{-1}
SLA1	37	3.62±0.02	25.00±0.60	3.45±0.02	12.00±0.30
SLA2	37	3.72±0.02	28.00±0.70	3.56±0.02	26.00±0.70
SLB	30	4.65±0.02	36.00±0.90	4.44±0.02	30.00±0.80
SLP	30	4.60±0.02	20.00±0.50	4.35±0.02	15.00±0.40

SLA1 – starch with *L. acidophilus* 308*;* SLA2 – starch with *L. acidophilus* 336*;* SLB – starch with *L. brevis* R-1*;* SLP – starch with *L. plantarum* P-1

Table 4

pH and gluten content in samples of wheat fiber after fermentation

Samples	Temperature, °C	Duration of fermentation, h			
		12		24	
		pH	Gluten content, mg kg^{-1}	pH	Gluten content, mg kg^{-1}
FLA1	37	3.77±0.02	4500.00±126.00	3.64±0.02	2450.00±67.00
FLA2	37	3.77±0.02	4650.00±130.00	3.56±0.02	2200.00±62.00
FLB	30	4.37±0.02	5100.00±143.00	3.82±0.02	2810.00±79.00
FLP	30	4.39±0.02	4700.00±132.00	3.79±0.02	2500.00±70.00

FLA1 – fiber with *L. acidophilus* 308*;* FLA2 – fiber with *L. acidophilus* 336*;* FLB – fiber with *L. brevis* R-1*;* FLP – fiber with *L. plantarum* P-1

The selected strains exhibited different proteolytic activity in this research, which leads to a reduction of gluten content in wheat sourdoughs. The amount of non-digestible gluten peptides decreased in all fermented starch samples after 12 h, but the most pronounced proteolytic effect was observed in sourdough with *L. Plantarum* P-1 (Table 3).

After 24 hours the lowest amount of immunoreactive gluten peptides was found in sourdough with *L. acidophilus* 308 and *L. Plantarum* P-1. Sourdough fermentation decreases the disulphide bonds in gluten network, which influence its digestibility in people with gluten sensitivity (Gänzle et al, 2008). Although fermentation of starch with *L. acidophilus* 336 most reduced pH, failed to reduce the gluten content to 20 mg kg^{-1}. Fiber of wheat (after wet fractionation) had a high initial content of gluten (up to 33750 mg kg^{-1}).

Despite a significant decrease in pH after fermentation with LAB the amount of gluten was reduced but remained still high (Table 4). The results showed that LAB fermentation can only eliminate small amounts of gluten while content of gluten at high concentrations still remains high after hydrolysis. Similar results were obtained by hydrolysis of fermentation of wheat flour. In the wheat flour 74590–80127 mg kg^{-1} of gluten were found (Greco et al., 2011). Results achieved in the studies (De Angelis et al., 2006; Stefańska et al., 2016) demonstrate that the proteolytic activity of the selected LAB strains is not high enough to allow their use for the degradation of allergenic proteins in bakery products made from wheat flour intended for patients with food allergy to gluten. Fermentation with LAB, however, could be used in production from raw materials with low gluten content and, at high concentrations, their effects must be combined with proteolytic effects of the enzymes.

Conclusions

The role of a fermentation process for improving the quality of GF products and developing a new concept of GF products is very important, however, this method is not suitable to completely degrade gluten, when its initial amount in the raw material is high.

Gluten could be degraded in wheat starch to levels below 20 mg kg^{-1} by application of *Lactobacillus plantarum* P-1 in a short time period, and *L. acidophilus* 308 after a long fermentation. These selected LAB strains may be applied as the specific starter cultures to prepare bakery products of special nutritional use from wheat starch, but do not reduce the amount of gluten in fiber and bran to a safe limit for gluten-free diet.

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