Immunoreactivity of transglutaminase cross-linked milk proteins in fermented milk product obtained with *Lactobacillus acidophilus*

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Abstract

Introduction: Milk fermented beverages, such as yoghurt, kefir or others obtained with probiotic cultures, are regarded as products with lower antigen potential than milk and therefore they can be included in the diet for cow's milk allergic patients. Lactic acid bacteria (LAB) are claimed to have proved immunomodulating effects. Several LAB strains possess the documented ability to modulate immune host responses, and can also stimulate antibody production and macrophage activity, inhibit intestinal inflammation, and alleviate allergic disease symptoms and autoimmune disorders. Particular microbial strains change the immunoreactivity of cow milk proteins to a varied extent. Addition of cross-linked microbial transglutaminase (m-TG) during production of a fermented milk beverage can make proteins and peptides linked engaging epitopes sites, thus reducing their immunoreactive potential.
Aim: The aim of the study was to reduce the immunoreactivity of milk proteins (β-lactoglobulin, α-lactalbumin, casein fractions) present in dairy products obtained with *Lactobacillus acidophilus* and improve the sensory quality of products fermented with lactic acid bacteria with the addition of microbial transglutaminase (m-TG).
Material and methods: Fermented dairy product samples, with and without m-TG, were produced in Danisco Biolacta Company (Olsztyn). Immunoreactivity was examined using polyclonal antibodies directed to α-casein, β-casein, κ-casein, α-la, and β-lg from our own collection by competitive ELISA. Immunoblot was performed with pooled human sera. The study design was approved by the Medical Research Ethics Committee (30/2009) from the Medical University of Warsaw, Poland. The overall sensory quality of the samples was estimated by the trained panellists of the Sensory Laboratory of the Institute of Animal Reproduction and Food Research of the PAS, Olsztyn.
Results: The application of *L. acidophilus* in fermented milk production allowed reduction of the immunoreactivity of major milk allergens, α-casein, β-lactoglobulin, which additionally was reduced as a result of cross-linked reaction with m-TG and the 30 days of storage. The immunoblot revealed the specific reaction between milk proteins with high molecular weight (66-80 kDa) and 29 kDa and specific IgE antibodies of cow milk allergic patients.
Conclusions: It can be concluded that application of *L. acidophilus* strain and addition of m-TG during the technological process can yield a cow milk beverage with low antigen potential. Moreover, the product with m-TG was characterized by a more palatable quality than those obtained without m-TG. The more allergenic proteins were proved to BSA, lactoferrin and α-casein.

Key words: food allergy, milk proteins, immunoreactivity, *Lactobacillus acidophilus*, transglutaminase.

Introduction

Food hyper-reactivity, also called a food allergy, is becoming a widespread disease. It is estimated that in well-developed countries over 6% of children and 3-4% of adults suffer from this ailment [1-3]. The allergy to milk proteins is the most common one within newborns and children up to two years old. Milk is a highly valuable food product in human nutrition and, thus, it is justified to search for technologies that would lower anti-

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genicity and allergenicity of particular milk proteins so that they could also be consumed by people with a diagnosed milk allergy.

Presence or absence of some bacteria species in the digestive tract is one of the factors that influence the emergence of a food allergy. It has been observed that the intestine is colonised faster by favourable bacteria species, enterobacterium, enterococci, eubacteria, as well as the bacteria of the *Lactobacillus* genus in groups of children from developing countries (e.g. Estonia) [4]. Food-allergic newborns in developed countries (e.g. Finland) were characterised by a higher number of clostridia while that of bifidobacteria was lower [5]. The efficient work of the digestive tract and the connected immune system (gut-associated lymphoid tissue – GALT) may be modulated by stimulation with the addition of microflora of a probiotic nature.

Lactobacillus acidophilus is one of the better known and described species. Some strains of *L. acidophilus* play a role in limiting the amount of intestinal infections resulting from both rotavirus and bacteria-based diarrhoeas; they also enhance digestion and lactose absorption. Their actions also include production of substances inhibiting the development of some types of neoplasms, stimulation of the immune response to undesired intestinal microorganism, and control of cholesterol in the serum. While competing with pathogens for receptors, they block the growth of pathogenic organisms and prevent their reproduction and colonisation of the intestine. It is this bacterium that produces natural antibiotics – acidophilin, lactacin, and lactocidin – that *in vitro* manifest very strong antagonistic actions towards such bacteria as *Escherichia coli*, *Klebsiella*, *Salmonella*, *Shigella*, *Staphylococcus*, and *Vibrio* [6]. Also, *L. acidophilus* has some antiproliferative properties that inhibit creation of a large populations of Th2 lymphocytes that are crucial in developing food allergies; it also significantly increases the secretion of the IL-12 cytokine, and, to a lesser degree, of the IL-10 one, tumour necrosis factor (TNF), and interferon (IFN)-γ. The latter two pro-inflammatory cytokines participate actively in sealing up the intracellular connections and are crucial for Th0 to Th1 cell differentiation [7]. Moreover, *L. acidophilus* decreases the secretion of IL-4 that stimulates the synthesis of E-class antibodies.

The *L. acidophilus* strain also has an influence on occurrences of non-specific immune reactions. It has been proved that consumption of fermented milk with *L. acidophilus* for three weeks entailed a decreased phagocytic activity of leukocytes in human peripheral blood. Granulocytes demonstrated a higher capacity of phagocytosis that monocytes. That increase was maintained for 6 weeks after the consumption of fermented milk was stopped. It is speculated that it is a result of modulation of surface molecules that participate in capture of bacteria by leukocytes, as well as a result of the creation of increased amounts of free oxygen radicals and lysosomal enzymes capable of fast destruction of microorganisms [8].

Addition of appropriate microflora is not the only factor that facilitates production of a low-antigen milk drink. It has been observed that addition of microbiological transglutaminase (m-TG) enables trans-protein and trans-peptide connections, which eliminates the places of allergenic epitopes [9]. In spite of its cross-linking properties, m-TG does not connect to human immunoglobulin E, and its daily human-safe dose may be 8-13 mg/day depending on its consumer's age. It has been shown that the hydrolysis of m-TG by pepsin in stomach conditions (pH 2) caused formation of peptides within one minute; yet, trypsin did not hydrolyse m-TG even after 48 h of the reaction. At the same time, when applying the research protocol that makes the guidelines of the FAO/WHO Decision Tree, no danger has been stated in terms of allergenic potential of m-TG [10].

Aim

The aim of this study was to propose a technology for production of a fermented milk drink using the *L. acidophilus* strain and a simultaneous addition of m-TG, which causes a reduction of the milk protein immunoreactivity (β-lactoglobulin, α-lactalbumin and casein fractions) and an improvement of the sensory qualities of the modified drink.

Material and methods

Research material

The examined samples consisted of a fermented drink produced using the microbiological *L. acidophilus* strain and simultaneous addition of m-TG.

Defining the amount of the antigen with the competitive ELISA

Microplates were coated with a solution of antigens (α-casein C-6780, β-casein C-6905, κ-casein C-0406, α-lactalbumin L-6010, β-lactoglobulin L-6879 by Sigma). The samples of properly diluted fermented drinks in the amount of 50 µl were incubated together with 50 µl of a rabbit serum solution containing specific IgG antibodies obtained and validated during our previous research. Then, 100 µl of goat's anti-rabbit immunoglobulin G conjugate marked with horseradish peroxidase (Sigma A-6154) were added. In the next phase a substrate was added: a 100% solution of 3,3',5,5'-tetramethylbenzidine (TMB Sigma 5525). After 30 min of incubation, the reaction was stopped with 2 M sulphuric acid (VI) solution and the absorbance was read at the wavelength of 450 nm with the SUNRISE reader by TECAN. The applied Competitive ELISA is a method developed in our department to validate food product groups.

Percentages of immunoreactivity changes for the proteins in the examined samples were calculated as the amount of the antigen observed at the 50% share of antibody bonds directed towards the standard protein.

Polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The electrophoresis was performed in denaturing conditions according to Laemmili's method using a 12% polyacrylamide gel [11]. The process was carried out at the direct current of 120 V. For immunoblotting, the separated proteins were transferred onto a nitrocellulose membrane at the current of 30 V for 20 h. Then, the membrane was incubated at 4°C for 10 h with human serum collected from patients with a food allergy to cow's milk proteins. After washing the membrane with phosphate buffer, it was incubated for 2 h with goat's anti-human E-class antibody (Sigma A-9667). The membrane was stained with a solution of 2-chloro-2-naphthol (Sigma, C-6788) with the addition of methanol and H_2O_2 and the reaction was stopped by washing the membrane in distilled water.

Sensory evaluation of products with and without transglutaminase addition

The sensory assessments of the fermented products were carried out by a panel consisting of 9 members selected and trained according to ISO guidelines [12]. All assessors have passed the basic taste test, the odour test and the colour vision test.

In the sensory evaluation, each panelist was asked to assess the samples for overall quality based on the overall colour, odour, taste and texture [13]. An unstructured graphical scale was 10 cm long and anchored on both ends: disliked (0) – extremely liked (10). Treatment means were compared using Fisher's protected least significant difference test (LSD, at $p \leq 0,05$).The assessments were carried out at a sensory laboratory room, which fulfils the requirements of the ISO standards [14]. The results were collected and calculated using software package FIZZ, Biosystemes, v. 2.45 A, France.

Results

Using the *L. acidophilus* strain to obtain a fermented milk product decreased the immunoreactive potential of particular milk proteins that belong to a group of major allergens. The addition of m-TG caused a further decrease of that property, particularly in relation to several proteins (α-casein, κ-casein, and β-lactoglobulin) (Table 1).

In the presented study, the products were analysed after 30 days of cooling storage that corresponded with the expiry date of the dairy products. It was observed that a further decrease of immunoreactivity occurred in all the proteins. The results obtained for β-lactoglobulin showed an immunoreactivity reduction of over 86%. The casein fractions turned out to be the most resistant ones and their immunoreactivity was about a dozen per cent. The whey proteins (α-lactalbumin and β-lactoglobulin) as well as κ-casein were detected in trace amounts (0.03-1.82%) (Table 1).

The immunoblot enabled tracing of the reaction between the antibodies in the sera of patients with the food allergy and the milk proteins. It was found that all the samples of the milk drinks produced using *L. acidophilus* were characterised by occurrences of a reaction between the patients' specific E-class antibodies and the milk proteins with molecular weight of 66-80 kDa, which may respond to the presence of bovine blood serum albumin (BSA = 66 kDa) and lactoferrin (LF = 80 kDa). Additionally, the presence of a strongly reactive fraction of α-casein in the fresh and stored drinks was found, regardless of the m-TG enzyme addition (Fig. 1).

The sensory evaluation of the fermented drinks was carried out directly after the completed production process and after 30 days of cool storage (Fig. 2). The m-TG-free product was characterised by some intensively palpable negative features, which included the smell and the bitter taste of 'burned milk'. Its aftertaste and coating of the mouth cavity were also strongly felt. The product, however, was also characterised by a high level of positive features such as its sour taste and the smell of kefir.

The addition of m-TG increased the palpability of the taste and the smell of soured milk, which is desired in

Table 1. Specific immunoreactivity of fermented milk products obtained with *Lactobacillus acidophilus*

Fermented product obtained with *Lactobacillus acidophilus*	Specific immunoreactivity of milk proteins [%]				
	α-casein	β-casein	κ-casein	α-la	β-lg
Fresh product					
Without m-TG	31.4	16.7	0.11	2.64	13.57
With m-TG	17.71	19.44	0.06	5.92	2.37
Storage product (30 days)					
Without m-TG	16.43	15.87	0.35	4.37	7.8
With m-TG	15.0	12.82	0.03	1.13	1.82

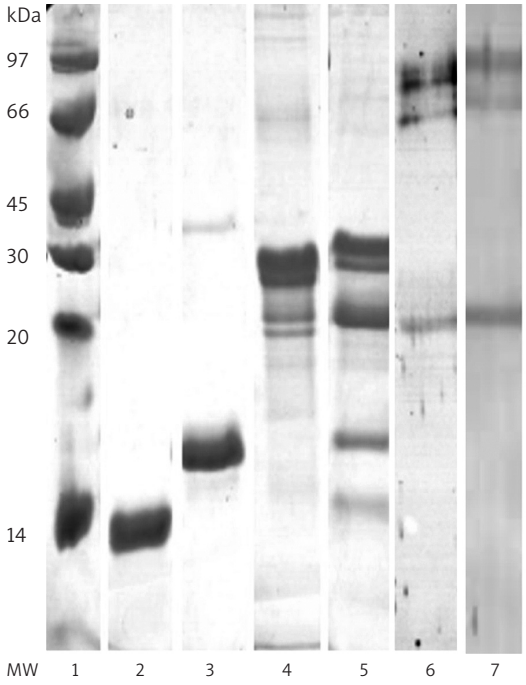

Fig. 1. SDS-PAGE electrophoresis: 1) low molecular markers (Sigma), 2) α-la, 3) β-lg, 4) α-casein, 5) milk, 6) immunoblot of beverage with *Lactobacillus acidophilus* and without m-TG, 7) immunoblot of beverage with *L. acidophilus* and with m-TG

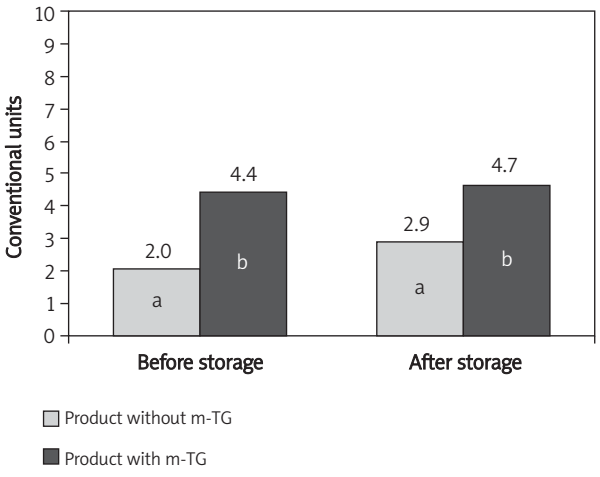

Fig. 2. Overall sensory quality of probiotic products obtained with *Lactobacillus acidophilus*

milk products of that type. Also, an improvement of visual properties of the drink occurred – its density and surface smoothness were increased and the taste and the smell of 'burned milk' were totally neutralised.

After 30 days of storing the drink that contained the addition of m-TG its properties of the sour taste and smell as well as the smell of soured milk were reduced. Also, the aftertaste and coating of the mouth cavity were less intensive. The obtained drink had the properties of a mild fermented product, which is favoured by its potential consumers. In both the analysed products, their remaining values for the organoleptic evaluation did not change significantly during the storage time.

The results showed that both the addition of the m-TG enzyme and storage had effects on the sensory quality of fermented products (Fig. 2). The samples with m-TG obtained higher scores for overall quality them the products without m-TG. The former obtained 4.4 and 4.7 points in the 10-unit conventional scale while the latter 2.0 and 2.9 points, respectively. It indicated that m-TG might contribute to improve the sensory properties of fermented products.

It was noted that addition of m-TG during the drink production increased the general quality of the product and nearly a twofold increase of the general sensory quality was noted after adding m-TG to the fresh drink. The storage time also contributed to the further increase of the general desirability of the products (Fig. 2).

Discussion

The basis of newborns' diet is usually their mothers' milk. There are, however, cases when due to various reasons a decision is made to include milk of different species, most often cow's milk and its products, into their nutrition faster that usual. Cow's milk contains on average 3-4% proteins in total, nearly 2.5% of which is made up of casein and the rest of whey proteins. Over 100 characterised proteins and peptides isolated from milk have allergenic properties. The most common are casein fractions (αs_1, αs_2, β, κ, γ), which are commonly referred to as allergen Bos d 8, as well as whey proteins α-lactalbumin (α-la, Bos d 4) and β-lactoglobulin (β-lg, Bos d 5). The IgE-dependent epitopes are presented in Table 2. A change to the peptide structures may be conducted by denaturation, hydrolysis, chemical modifications, and microbiological-culture-aided transformations.

Lactic acid fermentation bacteria may activate the immune system cells of the digestive tract as a result of direct contact with the whole bacterium cell, its fragment or metabolites, as a result of modifications to the intestinal flora and allergen permeability through the intestinal walls, or as a result of introduced adjuvants that influence the course of the immune response [15].

The *L. acidophilus* strain was used in the study as it naturally inhabits the digestive and genital tracts. It is also present in food products. As a result of the activities of microbiological strains of *Lactobacillus* sp., the milk proteins were hydrolysed under the influence of the secreted proteolytic enzymes. Due to that process, the antibodies previously identified by the epitopes, mostly

Table 2. Immunodominant IgE epitopes identified in milk proteins

Milk proteins	Amino acid sequences of IgE	Reference
αs_1-casein	19-30, 86-103, 141-150	Spuergin *et al*., 1997 [20]
	17-36, 39-48, 69-78, 93-102, 109-120, 123-132, 139-154, 159-174, 173-194	Chatchatee *et al*., 2001 [21] Elsayed *et al*., 2004 [22]
	173-194	Nakajima-Adachi *et al*., 1998 [23] Cerecedo *et al*., 2008 [24]
αs_2-casein	31-44, 43-56, 83-100, 93-108, 105-114, 117-128, 143-158, 157-172, 165-188, 191-200	Busse *et al*., 2002 [25]
	33-42, 87-96, 159-168, 145-154, 171-180	Järvinen *et al*., 2002 [26]
	1-20, 13-32, 67-82, 106-125, 122-141, 157-182, 181-207	Cerecedo *et al*., 2008 [24]
β-casein	1-16, 45-54, 55-70, 83-92, 107-120, 135-144, 149-164, 167-184, 185-208	Chatchatee *et al*., 2001 [21]
	25-50, 52-74, 154-173	Cerecedo *et al*., 2008 [24]
β-lg	1-16, 31-48, 47-60, 67-78, 75-86, 127-144, 141-152	Järvinen *et al*., 2001 [26]
	49-60, 119-128, 129-138, 143-152	Järvinen *et al*., 2001 [26]
	124-134	Adams *et al*., 1991 [27]
	97-108	Ball *et al*., 1994 [28]
α-la	1-16, 13-26, 47-58, 93	Järviven *et al*., 2001 [26]
Bovine serum albumin	524-542	Beretta *et al*., 2001 [29]

conformational ones, might have been destroyed. Occurrence of new amino acid sequences with allergenic properties, which had previously been bound in the protein structure, were also possible.

The addition of m-TG, an enzyme with cross-linking properties, was also used in the production of the milk drink, which made it possible to aggregate the obtained proteins and peptides, probably also in the places of the allergenic epitopes, thus decreasing the immunoreactivity. At the same time, the actions of m-TG caused building of the revealed antigen determinants into the newly-created protein agglomerates in a way that blocked their identification by the antibodies directed towards the main milk allergens.

It was observed that the addition of m-TG caused a significant decrease in the immunoreactivity of the two main milk allergens, α-casein (by 50%) and β-lactoglobulin (by over 80%), in relation to the fresh probiotic drink produced without m-TG. The time of cool storage had a negative influence on bonding of the antigen determinants characteristic for the presence of α-lactalbumin and κ-casein, causing an increase in the level of immunoreactivity by 65% and over 200% respectively in relation to the fresh drink (Table 1). It should be stressed, however, that those are not the immunodominant milk allergens. Yet, the allergenic potential of the remaining proteins kept decreasing. Data concerning characterized IgE-mediated epitopes are shown in Table 2.

The immunoblot analysis has provided some valuable information on the reaction between IgE from the sera of the people allergic to milk with the proteins in the ready-to-consume milk drink. Proving an explicit reaction between the antibodies and the milk proteins with higher molecular masses turned out to be crucial. Most likely, those included BSA (66 kDa), lactoferrin (70-80 kDa), and also α-casein (27-30 kDa) (Figure 1); however, some further research on the identification of reactive milk proteins is required. Those high-molecular proteins are much more resistant to high temperature, enzymes, and the influence of chemical compounds than the proteins of lower molecular masses, and therefore they remained in the environment of the milk product and their epitopes reacted with the patients' antibodies. However, no reaction of the E-class antibodies with the whey proteins (e.g. β-lactoglobulin) was observed though those proteins are often presented in the available literature as the basic allergens within milk proteins [16]. β-Lactoglobulin is destroyed during heating in the so-called low-temperature pasteurisation, from 74°C to 90°C [17]. Circular dichroism analysis has made it possible to identify that circa 50% of the changes in a β-lactoglobulin molecule occur during the first 15 s of heating the protein at the temperatures of 80-95°C [18]. Presently, high-temperature pasteurisation methods are used together with pasteurisation, which aims at obtaining fully sterile milk where labile proteins and their epitopes are denaturised easily. However, some chemical structures resistant to technological pro-

cessing remain, and at the same time, as a result of drastic production conditions, some new non-specific bonds are created (protein-sugar, protein-fat) that are difficult for the immune system to identify.

Proposing a mixed technology (hydrolytic and cross-linking) for the production of fermented milk drinks with m-TG seems to be reasonable for obtaining a low-antigen drink. The m-TG-modified food produced so far is perceived as non-allergic. No cases of IgE-dependent allergy due to the presence of m-TG in food have been recorded. Is has been demonstrated that the human body is capable of total hydrolysis of isopeptide cross-linked protein bonds in the digestive tract, which conditions the availability and digestibility of lysine. The enzymes responsible for cleavage of chemical bonds formed during cross-linking include γ-glutamine-cyclotransferase localised in the kidneys, and γ-glutamine-transpeptidase localised also in the kidneys and the intestinal brush border [19].

Conclusions

As a result of the performed research, it was found that the fermented milk drink produced using the selected *L. acidophilus* strain is a product with reduced immunoreactivity of all the main milk allergens. Adding the cross-linking enzyme m-TG during the technological process caused a further decrease in the antigen potential of the ready-to-consume drink, particularly that of α- and κ-casein a, as well as β-lactoglobulin. During the product storage, within 30 days guaranteed before its expiry date, a further reduction of immunoreactivity occurred. The immunoblot analysis proved that the proteins of the highest allergic potential consisted of the proteins resistant to high temperatures and proteolytic enzymes, i.e. the proteins with the highest molecular masses. Those most likely include BSA and LF but some further studies on their detailed identification are required. However, no reaction has been observed between E-class antibodies in the patients' sera and the proteins commonly recognised as the strongest allergens: β-lactoglobulin and α-casein.

Moreover, the obtained drinks are characterised by favourable sensory properties that have been enhanced as a result of the m-TG addition, which makes the products attractive for a potential customer.

Summing up, the simultaneous application of the *L. acidophilus* strain and m-TG may be used to produce a low-antigen milk product supplementing the diet of people with food allergies to milk proteins.

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