



Differences in the immunoreactivity of milk from local farms and from points of purchase

Aneta Tomczak¹ · Magdalena Zielińska-Dawidziak¹ · Dorota Piasecka-Kwiatkowska¹

Received: 21 August 2019 / Accepted: 2 November 2019 / Published online: 13 November 2019
© Springer-Verlag GmbH Germany, part of Springer Nature 2019

Abstract

Cow's milk proteins belong to food allergens. The composition of milk is influenced by many factors, among others by cows' feeding. Thus, the aim of the study was to check if introducing legumes seeds (soy meal and ground lupine) into fodder changes the composition of milk immunoreactive protein. 18 milk samples were compared, (UHT milks and samples obtained from local farms). The differences in the total protein content were not statistically significantly different, but the studied milk varied in the content of histidine, serine, aspartic acid, glutamic acid, lysine, phenylalanine, and tyrosine. To control immunoreactivity of milk proteins, extracts in PBS were prepared. Thus, the samples were deprived of most of casein—the main milk allergen. Immunoreactivity of the milk from the local farms and obtained from the cows fed with a fodder containing soy (FS) differed from the immunoreactivity of UHT milk and the milk from the cows fed with a fodder containing soy and lupine (FL) and without the addition of legumes (NL). It was proved by a slot-blot analysis, both with sera of the children allergic solely to milk, as well the children allergic solely to soy (not to milk). SDS-PAGE analysis of the prepared extracts showed the presence of additional peptide bands: ~ 18 kDa in case of the most of immunoreactive milks (FS).

Keywords Cow's milk allergy · Soybean · Lupine · Cross-reactivity · Slot blotting

Introduction

Cow's milk proteins are an important source of protein in human nutrition. The total protein content in bovine milk is approximately 3.5% by weight, and milk proteins are considered highly valuable and standard in child nutrition [1]. However, allergy to cow's milk protein (CMA) affects 2–3% of infants, because their intestinal barrier and the immune system are immature. CMA may persist into adulthood and affects 0.4–0.9% of adult human [2–4]. In some countries, it is the most common food allergy [4].

The mechanism of those undesirable and abnormal reactions of the immune system to milk proteins is recognized both as the IgE-mediated type as well as non-IgE mediated (i.e., IgA- or IgG-mediated) allergies [3, 6]. Among over 50 proteins involved in the allergic reaction to milk [6, 7],

the key fractions are phospho-glycoproteins, i.e., α 1-, α 2-, β -, γ -, κ -casein, and whey proteins such as β -lactoglobulin, α -lactoglobulin and BSA [8, 9]. The problem of milk allergenicity is not solved by thermal preparation, such as the UHT process (ultrahigh temperature). The thermostability of the immunoreactive properties of casein and partial stability of lactoglobulin is proved. In addition, during the heat treatment, disulfide bonds between casein-k and β / α -lactoglobulin may be formed, causing formation of thermostable neoallergens [10, 11]. In case of CMA, the only therapy that is completely safe is the complete elimination of allergens from the diet.

Simultaneously, substituting cow's milk by soy milk preparations or goat milk usually does not solve the problem. Soy proteins are also a food allergen and induce immune system reactions in some patients with recognized CMA, which result from cross-reactivity of milk and soy protein. Data show that between 10 and 44% of children with CMA also suffer from soy protein allergy [12]. Therefore, soy milk should not be usually used as a substitute of cow's milk for patients with diagnosed CMA. Bovine casein represents 50–70% homology to soy proteins and 87–98% to

✉ Magdalena Zielińska-Dawidziak
mzd@up.poznan.pl;
magdalena.zielinska-dawidziak@up.poznan.pl

¹ Department of Food Biochemistry and Analysis,
Poznan University of Life Sciences, ul. Mazowiecka 48,
60-623 Poznan, Poland

goat casein [8, 13, 14]. Only 2 in 26 children with CMA tolerate goat milk [8].

Since the composition of cow's milk is influenced by many factors: genetics, age, cattle breed, lactation period, health and diet [15], the diet composition may also change the milk protein composition. Different composition of fodder modifies the mixture of amino acids and short peptides transported to the milk gland, where milk proteins are formed from amino acids and peptides transported by blood. Thus, it can be expected that a modified composition of amino acid mixture delivered by blood may modify the composition of milk protein. A small amount of proteins with a distinct composition may be formed. The aim of the presented studies was to search the difference in the protein composition and their immunoreactivity in UHT cow's milk, local farms cow's milk, and goat's milk. Immunoreactivity of the samples was controlled with the sera of the children (with recognized CMA or soy allergy), as well as using commercially purchased anti-soy, anti-lupine, anti-lactoglobulin and anti-casein antibodies.

Materials and methods

Materials

18 samples of milk were used in the study (Table 1). The first group constituted popular UHT milk (Nos. 1–8), some of them were declared to be GMO free (Nos. 5–8). The second pool was dairy cattle milk obtained from local farms with a declared method of cows' feeding—with (Nos. 9, 11, 14, 15–18) or without (Nos. 10, 13) the addition of legumes into the fodder and also one goat's milk (No. 12) (Table 1). In case of the milk from local farms, the milk was taken after mixing the milk from one herd of Holstein Friesians cows from a single milk yield. There were also positive controls of proteins used in the study, i.e., solution of casein from bovine milk (SIGMA, C7078, USA), β -lactoglobulin

(SIGMA, 3908, USA), and extracts of soybean seeds (Augusta variety) and lupine seeds (Oskar variety) (seeds were obtained from Poznan University of Life Sciences).

Patients

Sera of ten children (in age between 3 and 13 years) diagnosed with an allergy solely to milk or solely to soy were obtained from SNOZ Allergology Plus Center for Diagnosis and Treatment of Allergy Therapy in Poznan (Poland). No serum was obtained from a child that was allergic to both soy and milk.

The method used to determine the class of antibody was the ELISA test. The serum characteristic is presented in Table 2. Ethical approval for the use of human sera was obtained from the local ethics committee (No. 671/17, 2017).

Methods

Protein extraction

Protein from the studied milk samples was extracted with a PBS (ratio 1:10 w/v). The samples were mixed in 4 °C for 17 h, then centrifuged three times (15 000×g, 10 min) [5, 16].

Protein concentration analysis

The total concentration of milk protein was determined with the Kjeldahl method [17]. The nitrogen content was recalculated from the protein content with the conversion factor 6.38.

Table 1 List of milk types used in the research

Nos.	Source of milk	Type of milk
1–4	Shop	UHT, lack of any declaration
5–8	Shop	UHT, declaration of being GMO free
10,13	Farm	COW's fodder without soy or lupine addition
9,11,14	Farm	COW's fodder containing 6% of soy addition
12	Farm	GOAT's fodder without added soy
15,16	Farm	COW's fodder containing 9% addition of lupine and soy (in rate 2:1)
17,18	Farm	COW's fodder containing 6% addition of lupine and soy (in rate 2.5:2)

Table 2 Presentation of the used sera

Serum	Allergy to			
	milk (lactoglobulin, casein, serum albumin)		Soy bean	
	Class	IgE concentration [kilounits/L]	Class	IgE concentration [kilounits/L]
I	2	0.7–3.5	–	–
II	5	50–100	–	–
III	3	3.5–17.5	–	–
IV	3	3.5–17.5	–	–
V	4	~50	–	–
VI	–	–	4	~50
VII	–	–	2	0.7–3.5
VIII	–	–	2	0.7–3.5
IX	–	–	2	0.7–3.5
X	–	–	6	> 100

The concentration of protein in the obtained milk extracts was determined by means of the Bradford method [18] using SP 8001 UV–Vis spectrophotometer, Metertech Inc. Taipei, Taiwan.

Amino acid composition

The amino acid composition, including L-histidine (His), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), L-methionine (Met), L-phenylalanine (Phe), L-threonine (Thr), L-valine (Val), L-tyrosine (Tyr), glycine (Gly), L-arginine (Arg), L-proline (Pro), L-aspartic acid (Asp), L-glutamic acid (Glu), L-alanine (Ala), L-cysteine and L-serine (Ser), was determined by means of ultra-performance liquid chromatography (UPLC, Shimadzu Nexera 2.0, Kyoto, Japan) equipped with a PDA and FL detector (Kyoto, Japan) after acidic hydrolysis (110 °C, 23 h) according to the method described by Tomczak et al. [19], while sulfuric amino acids were prepared by means of oxidative (4 °C, 16 h) and the following acidic hydrolysis (110 °C, 2.5 h). The PDA detector was set at 260 nm with a sampling rate of 20 points/s. The amino acid content was calculated in g/16 g N (which is equivalent to g/100 g of protein). Further, a percentage of the particular amino acid in the determined total amino acid content was calculated.

Slot-blot analysis

Immunoreactivity controlled with the sera

The prepared extracts were transferred to a polyvinylidene difluoride membrane (Immobilon-P 0.45 µm, Merck Millipore Ltd.), then 45 min blocked by 0.01 M TBS, pH 7.4 with 1% gelatin from porcine skin (Sigma G8150). The sera were diluted 20-fold (v/v) in TBS-PG-T [1% gelatin solution in the TBS with the addition of 0.05% Tween 20 (SIGMA P9416, USA)] and applied on the membrane for 1.5 h. After washing, the membranes were incubated for the following 1.5 h with a monoclonal anti-human IgE (AbII), labeled with alkaline phosphatase (SIGMA A3076, USA) diluted 1:1000 (v/v) with TBS-PG-T. Membranes were washed five times and then the substrate was applied for 20 min. BCIP/NBT (5-bromo-4-chloro-3'-indolyl phosphate and nitro-blue tetrazolium) was used as a substrate (Calbiochem). The reaction was stopped with water, and then the membranes were dried and analyzed.

Immunoreactivity controlled with the commercial antibodies

Four commercially purchased primary antibodies (AbI) produced in rabbit, diluted in TBS-PG-T were used instead

of sera: (1) polyclonal anti-soy protein antibody (SIGMA S2519, USA) diluted 1:500 (v/v); (2) polyclonal anti-conglutin γ-lupine specific antibody (Agrisera, AS08 335, Sweden) diluted 1:1000; (3) monoclonal anti-casein antibody (Abcam, ab166596, GB) diluted 1:7500; (4) monoclonal anti-lactoglobulin antibody (Abcam, ab112893, GB) diluted 1:10,000.

As the secondary (AbII) antibodies, anti-rabbit IgG (SIGMA, A3687, USA) was applied after dilution 1:20,000 (v/v) in TBS-PG-T. The rest of the procedure was as presented for studies with sera application (i.e., washing and BCIP/NBT application).

SDS-page analysis

The proteins' profile in the obtained extracts was examined using polyacrylamide gel electrophoresis. The samples (containing ~7 µg of protein) were analyzed under denaturing conditions [19], with the use of 12% polyacrylamide gel [with the application of a 20–120 kDa molecular marker (Thermo Scientific, 26612)] and a 16.5% gradient gel [with the molecular marker 1.4–26.6 kDa (Bio-Rad, 1610326)]. The gels were stained with Coomassie Brilliant Blue and documented using CLIQS (TotalLab Quant, Great Britain).

Results and discussion

To compare the quality of milk, fat content is usually used, and it is also presented on the label, while the protein content is not emphasized. However, this factor is very important in the immunoreactive studies. Thus, at first, the protein content in the studied milk samples was controlled. In the comparison, the milk samples were divided into four groups and compared in pairs: 1. UHT without declaration versus 2. UHT, GMO free; 3. cow's milk from local farms with soy feeding (FS), 4. cow's milk from local farms with soy and lupine feeding (FL) versus 5. cow's milk from local farms without any legume feeding (NL). The UHT milk that was bought (i.e., milk after ultrahigh temperature processing) had a declared fat concentration of 3%, but the total content of protein varied greatly and changed in the range 3.2–3.5% for the milk without any declaration (1) and 3.0–3.5% for the milk declared to be GMO free (2). This declaration present on the label suggests that the milk was obtained from the cows that were not fed with fodder containing imported soy meal, and even if, e.g., Polish soy meal was added to the fodder, its content probably was lowered.

In the samples obtained from the local producers, the protein content varied more (2.8–3.6%); however, no statistically significant differences were found between groups 3–5.

The differences in amino acids composition were also checked. Statistically significant differences in the studied

group of milk were observed in the content of: His, Ser, Asp, Glu, Lys, Phe, Tyr (Table 3). No significant differences were found between the groups of the UHT milks (1 and 2). When comparing milks from local farms, it was interesting that cow's milk with soy and lupine feeding (FL) contained less Ser, Phe and Tyr, but more His than milk samples obtained from local farms without any legume feeding (NL). The content of glutamic and aspartic acids in milk samples was significantly higher in FL milk compared to other samples tested. The differences between processed and unprocessed milks were not surprising. The content of the rest of the amino acids did not change statistically significantly in the studied groups. The content of Arg was 4.0 ± 0.4 , Gly 2.5 ± 0.5 , Thr 4.5 ± 0.1 , Ala 3.4 ± 0.2 , Pro 10.0 ± 0.2 , Val 0.5 ± 0.6 , Leu 10.0 ± 0.2 , Ile 4.6 ± 0.5 , Met 1.8 ± 0.5 and Cys 0.4 ± 0.3 (expressed as the % of the total amino acid content $\pm$ SD). The variability of the amino acid content due to feeding with a different type of fodder is interesting, but it must be confirmed in a definitely bigger number of samples. However, the observed variability of some amino acid content suggests that some differences in the formation of protein fractions could be noted. The goat milk was excluded here from the studied samples (although the amino acid content was comparable to the milk samples from the group NL), but introduced to the rest of the analysis—studies on immunoreactivity.

The main milk allergen, i.e., casein, is responsible for 57% of allergic reactions [20]. Simultaneously, cross-reactivity of soy peptides (30 kDa and 18.4 kDa) and α -, β - and κ -casein is confirmed and the organism deprived of any contact with soya reveals the response of the immune system after oral soy application [5]. However, in the presented experiment, the object of interest was not the observations of obvious cross soy–casein reactions, but the formation of specific immunoreactive peptides in response to cows feeding. Thus, to analyze the differences in the peptides' immunoreactivity, the milk samples were subjected to extraction

by means of an extrahent typically recommended for allergic proteins (PBS buffer). The method allowed to extract only traces of casein proteins; however, the aim of the study is to check the presence of other than casein allergenic proteins. The purpose of the study was to analyze the possibility of forming a modified protein fraction in response to adding soy or lupine to the fodder. In case of a human being, short peptide fractions, which are formed during the digestion of food eaten by the mother, are transported via the mother's blood from the food to the milk glands. The phenomenon in which peptides from food penetrate into blood [21] and the transfer of peptide female milk have already been known and widely described [22]. However, we do not have the knowledge or literature data on the transition of protein fractions from feed to milk of other mammals, even if it was proved that the activity of bovine oligopeptide transporter (bPepT1) depends on short peptide supply, which suggests influence of the activity of the transporter on the absorption of peptides from the feed into the blood [23]. Moreover, it is known that cow milk is produced 90% in amino acids and 10% in peptides circulating in the blood [24]. To analyze peptides and proteins in the studied samples, they were not digested in the in vitro experiment, because milk allergy affects mainly children with an immature immune system and the problem of allergy results also from incomplete digestion of proteins in their digestive tract. Furthermore, it has been proved that most epitopes of milk proteins are not digested. It applies to both casein [5] and proteins from the lipocalin family (β -lactoglobulin and α -lactoglobulin) [25]. These epitopes are resistant to proteolysis and are absorbed in the human intestine in an unchanged form.

Thus, the prepared extracts were transferred to the PVDF membrane and their immunoreactivity was compared. Figure 1 presents the results of slot-blot analysis of the milk extracts with the sera of the children allergic to milk.

The sera donors I–V were allergic, among others, to one of the main milk allergen, i.e., β -lactoglobulin, while the

Table 3 The percentage content of amino acids in the total amino acid content which varied statistically significantly in the studied groups of cow's milks

Milk groups	Content of amino acids (% in the total amino acids content)						
	His	Ser	Asp*	Glu**	Lys	Phe	Tyr
1	$2.6 \pm 0.2a,b$	$6.2 \pm 0.3a,b$	$8.6 \pm 0.3a,b$	$22.5 \pm 0.5a$	$6.4 \pm 0.2a,b$	$5.7 \pm 0.5c$	$0.9 \pm 0.2b$
2	$2.6 \pm 0.2a,b$	$6.3 \pm 0.3a,b$	$8.3 \pm 0.4a$	$22.0 \pm 1.0a$	$6.2 \pm 0.5a$	$5.9 \pm 0.5c$	$0.8 \pm 0.1b$
3	$2.6 \pm 0.2a,b$	$6.0 \pm 0.1a,b$	$9.1 \pm 0.1b,c$	$23.0 \pm 0.3a,b$	$6.7 \pm 0.4a,b$	$5.0 \pm 0.1b$	$0.9 \pm 0.2b$
4	$3.0 \pm 0.3b$	$5.8 \pm 0.1a$	$9.4 \pm 0.2c$	$24.4 \pm 0.1b$	$6.9 \pm 0.1b$	$4.5 \pm 0.1a$	$0.3 \pm 0.1a$
5	$2.4 \pm 0.2a$	$6.3 \pm 0.3b$	$9.1 \pm 0.5a-c$	$23.4 \pm 0.8a,b$	$6.8 \pm 0.0a,b$	$5.0 \pm 0.1b$	$0.8 \pm 0.1b$

1—UHT without declaration; 2—UHT, GMO free; 3—cow's milk, fodder containing 6% addition of soy; 4—cow's milk, fodder containing 6–9% addition of lupine and soy, 5—cow's milk, fodder without addition of legumes. Values denoted by different letters differ statistically significantly at the significance level $\alpha=0.05$

*Asp—the result is the sum of asparagine, aspartic acid and its salts

**Glu—the result is the sum of glutamine, glutamic acid and its salts

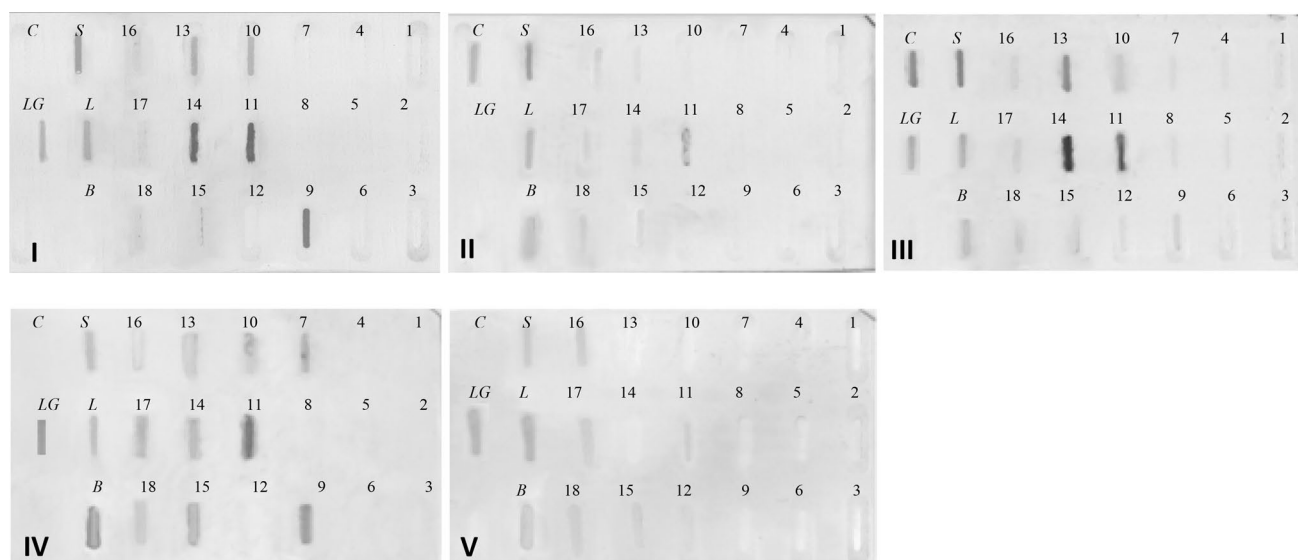


Fig. 1 Images of membrane obtained as a result of slot-blot analysis with the sera of patients with a milk/milk protein allergy: I–V. S—soy extract, L—lupine extract, B—beef extract, C—casein, LG—lactoglobulin. 1–18—milk samples numbered as in Table 1

donors II and III were allergic to casein, which was proved by means of the standard solution of lactoglobulin (LG) and casein (C) on the membranes. Simultaneously, the sera were selected from the children with a recognized allergy neither to soy nor lupine, but the antibodies present in each of the sera unexpectedly reacted with soy (S) and lupine (L) extracts. Not every serum also contained the same number of antibodies reacting with proteins present in beef extract (B)—the strongest signal was observed with serum IV. There was vestigial immunoreactivity of UHT milk (1–8) extract that was observed with the sera (except sample 7 and serum IV). However, it should be remembered that even if lactoglobulin is a thermolabile protein, casein (which has been almost completely removed from the samples) is thermostable, and thermal treatment may form neoallergens from β/α -lactoglobulin and casein [11]. Thus, the observed membranes did not inform us about the safety of UHT milk, but about the differences in the immunoreactivity of these milk samples and the milk samples obtained directly from the local farms. The reactivity of goat milk extract (No. 12) was not noted either, which resulted from removal of casein. Bovine and goat casein shows high homology, and even if the goat milk contains less casein [26, 27], only ~8% of children allergic to cow milk tolerate goat milk [8]. However, what was most interesting were the differences observed in the immunoreactivity of the milk obtained from the same local farms, but from cattle fed with the different fodder. The antibodies present in three out of five sera reacted stronger with the peptides/proteins present in the extract of the milk obtained from the cows fed a fodder containing soy (FS). Sera I, III and IV reacted with sample 9 and 14, while antibodies of sera I–IV reacted with sample 11. The reactions

were stronger when compared to the milk obtained from the same local farm without legume addition (NL) (compare the reaction of sample 11 and 10 with sera I–IV, and reaction of sample 14 with 13 with sera I and III, Fig. 1). Reactivity was also noted for sample 15–18 and serum IV, and the trace in other sera (milk from the cows fed lupine and soy—FL). It should be remembered here that the presented studies are preliminary studies and require continuation with a programmed nutritional experiment.

These extracts were analyzed with the sera of the children allergic to soy and not allergic to milk proteins. The results of the slot-blot analysis have been presented in Fig. 2. Even if children were not allergic to milk (lack of reactivity to casein and lactoglobulin standards was confirmed on the presented slot blots) and the extracts were deprived of casein (cross-reacting with soy allergens), the antibodies present in sera VI, VII and VIII reacted strongly with the milk proteins extracted from the FS samples (9, 11 and 14). Thus, the differences in the immunoreactivity of the FS milk when compared to the NL, FL and UHT milk samples were confirmed.

At the end of this part of the presented experiment, the prepared samples were analyzed using commercial antibodies (Fig. 3). Anti-soy protein antibodies reacted with soy and lupine extract (S, L) and samples 9–14. However, the strongest reactivity was noted for the sample 9 and 11, i.e., the extract obtained from the samples of milk with the biggest addition of soy into fodder. When comparing samples 13 (NL) and 14 (FS) obtained from the same local farm, sample 13 (soy-free fodder) proved to be less reactive than sample 14 (fodder with soy), even if the difference was not as big as the difference between sample 10 (NL) and 11 (FS) from the second farm.

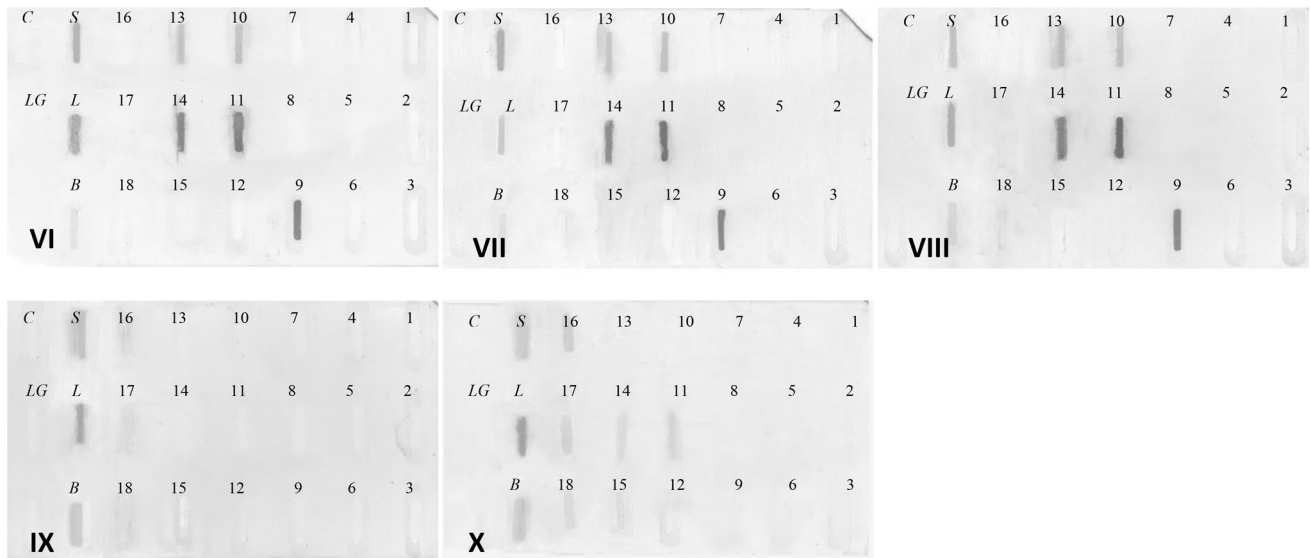


Fig. 2 Images of membrane obtained as a result of slot-blot analysis with the sera of patients with a soy allergy: VI–IX and X. S—soy extract, L—lupine extract, B—beef extract, C—casein, LG—lactoglobulin. 1–18—milk samples numbered as in Table 1

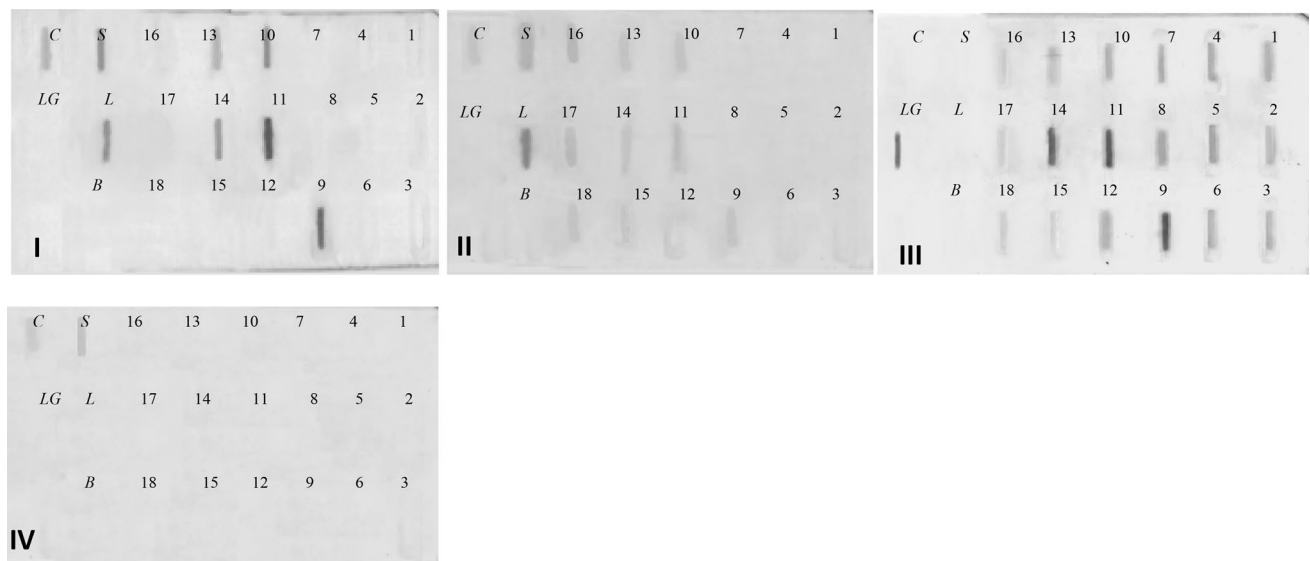


Fig. 3 Images of membrane obtained as a result of slot blot with commercial antibodies: I—anti-soy, II—anti-lupine, III—anti-lactoglobulin, IV—anti-casein. S—soy extract, L—lupine extract, B—beef, C—casein, LG—lactoglobulin. 1–18—milk samples numbered as in Table 1

Despite the fact that cross-reactivity of lupine and soy protein in the studied samples was confirmed, the reactivity of the milk samples with anti-lupine antibodies was only vestigial. These antibodies obviously reacted with soy and lupine extracts and vestigial reactivity was observed also for samples 16–18 (extract from the milk of the cows fed with lupine and soy—FL).

However, the most interesting results were obtained with anti-lactoglobulin antibodies. Here, the strongest reactivity was observed for samples FS (9, 11, 14). However, each

sample reacted with the antibody, even the UHT samples. Thus, even if it could be expected that FS samples were simply richer in β -lactoglobulin, it is not the only protein that is present in the sample and which was recognized by the sera of children allergic to milk (compare with Fig. 1 I–IV). The last commercial antibody informed us that these immuno-reactive proteins were not casein—the samples prepared in the described way contained probably only traces of casein.

To sum up, the results of the slot blots suggest that FS (9, 11, 14) samples contained a/some protein(s) which changed

immunoreactivity when compared to samples NL (10, 13). The protein(s) is(are) recognized by anti-soy, anti-lupine and anti-lactoglobulin antibodies. But there is no information in the literature about the cross-reactivity of BLG, soy and lupine. Thus, it may be expected that some modification in the structure of milk protein could take place when the cows were fed with a fodder containing soy. Because of some differences in amino acid content, some differences in the peptide profile could be expected.

The obtained extract was subjected to SDS-PAGE analysis, so that the protein and peptide fractions (Figs. 4 and 5) could be compared. The same protein fractions were detected in the range 20–120 kDa, although their percentage content and the number of fractions detected varied (Fig. 4). On the basis of the analysis, the identification of the main milk protein was possible: beta-lactoglobulin (BLG) (~ 19 kDa), trace amount of casein (because it was rejected at the extraction stage)— γ_1 - (~ 20.6 kDa), κ - (~ 27 kDa) β - (~ 24 kDa) and α_{s1} -casein (~ 23.6 kDa), respectively, α_{s2} -casein (~ 25.2 kDa), immunoglobulin G (~ 55 kDa), serum albumin (~ 67 kDa) and lactoferrin (~ 80 kDa) [28, 29].

However, the analysis of the smaller proteins indicate some profile differences in the milk obtained from the local farm when compared to UHT milk and the differences were also observed depending on the feeding system.

In the extract of the milk obtained from cows fed soy-containing fodder (samples Nos. 9, 11 and 14), and fodder containing soybean and lupine (samples Nos. 15–18), additional protein bands were observed (Fig. 5). These protein bands distinguished milk samples from cows fed legumes containing fodders from samples Nos. 10 and 13 (from the cows fed fodder not containing legumes). The band corresponds to a protein with a molecular weight of 18 kDa (in samples 9, 11, 14), and the densitometric analysis indicates that the protein makes up ~ 4–9% of all proteins present in the extracts and is not any fraction of the main milk proteins. Moreover, in some samples (9, 11, 15, 16), the presence of additional protein (~ 15 kDa) in its composition in relation to the other samples also was noted. The percentage of that band in the studied extract was 9–15%, and a much smaller (but identified in densitometric analysis) presence of this protein was noted in samples 17 and 18. These two proteins could modify the observed immunoreactivity of the milk of cows fed legumes (especially soy) containing fodder. The protein band with a molecular weight ~ 18 kDa common in FS samples (9, 11, and 14) could be immunoreactive both for the children allergic solely to milk and solely to soy.

However, these results should be confirmed in an experiment designed for more animals and designed fodder composition.

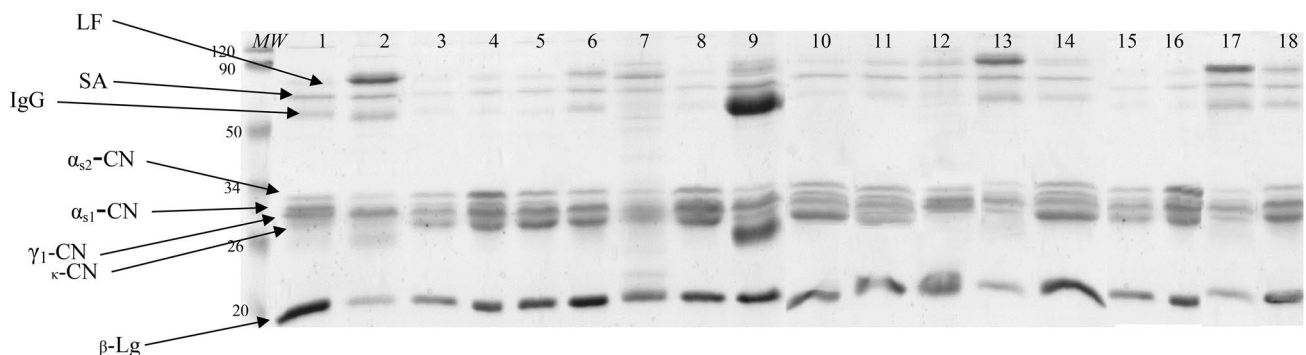


Fig. 4 SDS-PAGE separations peptide profiles with Coomassie blue staining: 1–18 milk samples (compare Table 1). MW molecular weight marker. The arrowhead additionally indicates the identified fraction. α -La alpha-lactalbumin, β -Lg beta-lactoglobulin

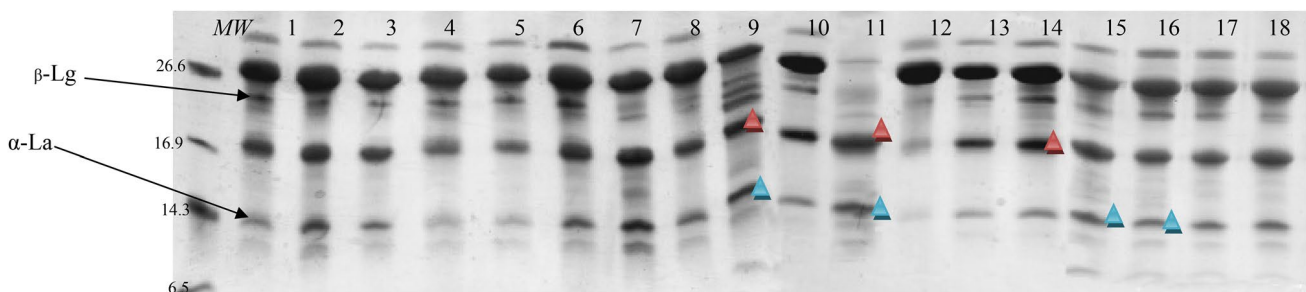


Fig. 5 SDS-PAGE separations peptide profiles with Coomassie blue staining: 1–18 milk samples (compare Table 1). The arrowhead additionally indicates the identified fraction. MW molecular weight marker, α -La alpha-lactalbumin, β -Lg beta-lactoglobulin

Conclusions

The presented results indicate a possible difference in milk protein composition, and—as a consequence—in immunoreactivity of milk obtained from the cows fed fodder of different compositions. The milk obtained from the cows fed a soy-containing fodder (FS) differed in their immunoreactivity from the milk obtained from the cows fed a fodder which did not contain legumes and from the UHT milks that were bought. The experiment carried out suggests that FS milk could contain additional proteins, cross-reacting with anti-soy and anti-BLG antibodies. The additional protein identified in those samples had molecular weight ~ 18 kDa, whereas additional proteins identified in FL samples had a molecular weight ~ 15.5 kDa. Moreover, the presence of the BLG fraction was confirmed in the studied extracts as the protein band, with molecular mass ~ 19.6 kDa, and α -lactalbumin with MW ~ 14.2 kDa; thus these additional proteins should not be one of these obvious milk allergens. Extracts from FS milks reacted with the sera of children allergic solely to milk, as well as with the sera of children allergic solely to soy (which is surprising). Samples obtained from the cows fed a legumes-containing fodder (both soy and lupine—FL) were significantly less immunoreactive to children allergic solely to soy, and only one of these samples contained protein with molecular weight ~ 18 kDa (recognized in all samples of FS). The observed differences in the immunoreactivity may be a consequence of different amino acid composition of the studied milk.

These results should be regarded as the preliminary studies and need confirmation, with more animals and variable fodder composition taken into account.

Acknowledgements The authors specially thank SNOZ Allergology Plus Center for Diagnosis and Treatment of Allergy Therapy in Poznan (Poland) for providing sera. The work was financially supported by the Polish Ministry of Science and Higher Education.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in publishing this research.

Compliance with Ethics Requirements Ethical approval for the use of human sera was obtained from the local ethics committee (No. 671/17, 2017).

References

1. FAO (2013) Food and Agriculture Organization of The United Nations. Milk and dairy products in human nutrition. Rome, Italy. <http://www.fao.org/3/i3396e/i3396e.pdf> Accessed 19 July 2019
2. Liu AH, Jaramillo R, Sicherer SH, Wood RA, Bock A, Bock A, Burks W, Massing M, Cohn RD, Zeldin DC (2010) National prevalence and risk factors for food allergy and relationship to asthma: results from the national health and nutrition examination survey 2005–2006. *J Allergy Clin Immunol* 126:798–806. <https://doi.org/10.1016/j.jaci.2010.07.026>
3. Lifschitz C, Szajewska H (2015) Cow's milk allergy: evidence-based diagnosis and management for the practitioner. *Eur J Pediatr* 172:141–150. <https://doi.org/10.1007/s00431-014-2422-3>
4. Navarrete-Rodriguez EM, Rios-Villalobos LA, Alcocer-Arreguin CR, Del-Rio-Navarro BE, Del-Rio-Chivardi JM, Saucedo-Ramirez OJ, Sienra-Monge JLL, Frias RV (2018) Cross-over clinical trial for evaluating the safety of camel's milk intake on patients who are allergic to cow's milk protein. *Allergol Immunopathol (Madr)* 46:149–154. <https://doi.org/10.1016/j.aller.2017.06.005>
5. Rozenfeld GH, Docena MC, Anon MC, Fossati CA (2002) Detection and identification of a soy protein component that cross-reacts with caseins from cow's milk. *Clin Exp Immunol* 130:49–58. <https://doi.org/10.1046/j.1365-2249.2002.t01-1-01935.x>
6. Vandenplas Y, De Greef E, Devreker T (2014) Treatment of cow's milk protein allergy. *J Pediatr Gastroenterol Nutr* 1:1–5. <https://doi.org/10.5223/pghn.2014.17.1.1>
7. Docena G, Fernandez R, Chirido F, Fossati C (1996) Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy* 51:412–416. <https://doi.org/10.1111/j.1398-9995.1996.tb04639.x>
8. Bellioni-Busino B, Paganelli R, Lucenti P, Giampietro PG, Perborn H, Busino L (1999) Allergenicity of goat's milk in children with cow's milk allergy. *J Allergy Clin Immunol* 6:1191–1194. [https://doi.org/10.1016/S0091-6749\(99\)70198-3](https://doi.org/10.1016/S0091-6749(99)70198-3)
9. Natale M, Bisson C, Monti G, Peltran A, Garoffo LP, Valentini S, Fabris C, Bertino E, Coscia A, Conti A (2004) Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Mol Nutr Food Res* 48:363–369. <https://doi.org/10.1002/mnfr.200400011>
10. Lewis M, Heppell N (2000) Effects on Milk Food Components. In: Litzenberg K (ed) Continuous thermal processing of foods: pasteurization and UHT sterilization. Aspen Publishers Inc., Gaithersburg
11. Jovanovic S, Barac M, Macej O, Vucic T, Lanjevac C (2007) SDS-PAGE analysis of soluble proteins in reconstituted milk exposed to different heat treatments. *Sensors* 7:371–383. <https://doi.org/10.3390/s7030371>
12. Kattan JD, Cocco RR, Javinen KM (2011) Milk and soy allergy. *Pediatr Clin North Am* 58:407–426. <https://doi.org/10.1016/j.pcl.2011.02.005>
13. Baumgartner S, Schubert-Ullrich P (2010) Soy (*Glycine max*) allergens. In: Jedrychowski L, Wichers HJ (eds) Chemical and biological properties of food allergens, 1st edn. CRC Press, Taylor and Francis Group, Boca Raton. <https://doi.org/10.1201/9781420058574>
14. Curciarello R, Smaldini PL, Candreva AM, Gonzalez V, Parisi G, Cauerhff A, Barrios I, Blanch LB, Fossati CA, Petruccioli S, Docena GH (2014) Targeting a cross-reactive Gly m 5 soy peptide as responsible for hypersensitivity reactions in a milk allergy mouse model. *PLoS One* 9(1):e82341. <https://doi.org/10.1371/journal.pone.0082341>
15. Tyasi TL, Gxasheka M, Tlabela CP (2015) Assessing the effect of nutrition on milk composition of dairy cows: a review. *Int J Curr Sci* 17:56–63
16. Peeters KA, Nordlee JA, Penninks AH, Chen L, Goodman RE, Bruijnzeel-Koomen M, Hefle SL, Taylor S, Knulst AC (2007) Lupine allergy: not simply cross-reactivity with peanut or soy. *J Allergy Clin Immunol* 3:647–653. <https://doi.org/10.1016/j.jaci.2007.05.032>

17. ISO 1871:2009 (2009) Food and feed products—general guidelines for the determination of nitrogen by the Kjeldahl method. ISO, Geneva
18. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72(1–2):248–254. <https://doi.org/10.1006/abio.1976.9999>
19. Tomczak A, Zielińska-Dawidziak M, Piasecka-Kwiatkowska D, Lampart-Szczapa E (2018) Blue lupine seeds protein content and amino acids composition. *Plant Soil Environ* 64:147–155. <https://doi.org/10.17221/690/2017-PSE>
20. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685. <https://doi.org/10.1038/227680a0>
21. Maciński J, Kowalski IM, Zwierzchowski G, Szarek J, Pierożyński B, Zabłocka E (2013) Characteristics of cow's milk protein including allergenic properties and methods for its reduction. *Pol Ann Med* 20:69–76. <https://doi.org/10.1016/j.poamed.2013.07.006>
22. Wang L, Wang Q, Liang Q, He Y, Wang Z, He S, Xu J, Ma H (2015) Determination of bioavailability and identification of collagen peptide in blood after oral ingestion of gelatin. *J Sci Food Agric* 95:2712–2717. <https://doi.org/10.1002/jsfa.7008>
23. Casas R, Böttcher MF, Duchén K, Björkstén B (2000) Detection of IgA antibodies to cat, β -lactoglobulin, and ovalbumin allergens in human milk. *J Allergy Clin Immunol* 105:1236–1240. <https://doi.org/10.1067/mai.2000.105805>
24. Xu Q, Liu Z, Liu H, Zhao F, Huang X, Wu Y, Liu J (2018) Functional characterization of oligopeptide transporter 1 of dairy cows. *J Anim Sci Biotechnol*. <https://doi.org/10.1186/s40104-017-0219-8>
25. Cichocki T, Litwin J, Mirecka J (2016) *Kompendium histologii (Histology compendium)*, 5th edn. Wydawnictwo Uniwersytetu Jagiellońskiego, Kraków (**in Polish**)
26. Mills C, Moreno FJ, Sancho AI, Jenkins JA, Wichers HJ (2004) Processing approaches to reducing allergenicity in proteins. In: Yada RY (ed) *Proteins and food processing*. Woodhead Publishing Limited, Cambridge
27. Wuthrich B, Johansson SGO (1995) Allergy to cheese produced from sheep's and goat's milk but not cheese produced from cow's milk. *J Allergy Clin Immunol* 96:270–273. [https://doi.org/10.1016/s0091-6749\(95\)70021-8](https://doi.org/10.1016/s0091-6749(95)70021-8)
28. Calvani M, Alessandri C (1998) Anaphylaxis to sheep's milk cheese in a child unaffected by cow's milk protein allergy. *Eur J Pediatr* 157:17–19. <https://doi.org/10.1007/s004310050759>
29. Islam MA, Ekeberg D, Rukke EO, Vegrud GE (2015) Ex vivo digestion of milk from red Chittagong cattle focusing proteolysis and lipolysis. *Asian Australas J Anim Sci* 28:559–567. <https://doi.org/10.5713/ajas.14.0269>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.