CHANGES IN THE PROPORTION OF PROTEINS FRACTIONS DEPENDING ON LACTOFERRIN POLYMORPHISM GENE AND THE SOMATIC CELLS COUNT IN THE MILK OF POLISH HOLSTEIN-FRISIAN AND POLISH RED-WHITE CATTLE

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Abstract. In dairy cattle, lactoferrin plays an important role in the control of mammary gland health, protecting it from mastitis, the most costly disease in milk production. The aim of the study was to analyze the relationship between genetic polymorphism of lactoferrin and somatic cells count and the proportion of certain milk protein fractions (serum albumin, α-casein, β-casein, κ-casein, α-lactalbumin). The research material was milk samples collected from cows of two breeds: Polish Holstein-Frisian (PHF) and Polish Red-White (PRW). Basic composition, proportion of protein fractions, lactoferrin concentration and genetic polymorphism of lactoferrin were determined in milk. Two genotypes of lactoferrin gene were found: AA and AB. In milk from PRW cows, a significantly ($P \leq 0.01$; $P \leq 0.05$) higher level of α-casein and lower level of β-casein was shown. The serum albumin level was higher in the milk from PHF cows with AB genotype. The milk from PRW cows with AA genotype was characterized by a higher level of α-lactalbumin. In milk from PHF cows an analogous relation was not observed. The concentration of lactoferrin in milk from cows with AA genotype was higher. The level of serum albumin, α-casein and β-casein was higher in milk from cows with SCC lower than 400 000 per ml, independently to the breed and to the genotype. This study showed that the polymorphism of lactoferrin gene does not affect fat level and composition of protein fractions in milk as expected. It was showed that SCC affects more the fat level and composition of protein fractions than the polymorphism of lactoferrin gene or the breed (PRW or PHF).

Keywords: bovine, milk, lactoferrin, polymorphism, SCC, protein fractions.

Introduction

At present, consumers are more aware of their health and more interested in slowing aging and preventing diseases. Therefore, a growing interest in food with directional pro-health activity is observed. There are over 560 biologically active substances in milk. Among them there is lactoferrin, which plays an important role in iron absorption and because of that influences indirectly the immunological answer of the body. Lactoferrin shows also bacteriostatic, bactericidal and anti-tumor activities (Lonnerdal *et al.* 1995, McIntosh *et al.* 1998, Adlerova *et al.* 2008).

In dairy cattle, lactoferrin plays an important role in the control of mammary gland health, protecting it from mastitis, the most costly disease in milk production. Inflammation of the mammary gland causes unprofitable changes in milk composition and its physico-chemical traits which lower its usefulness for processing and consumption value (Walawski *et al.* 1994, Urech *et al.* 1999). The main indicator of an inflammation of the mammary gland is the somatic cells count (SCC) in milk.

Several researche showed a dependence between polymorphic form of lactoferrin gene and SCC in milk. The milk from cows with allele B of lactoferrin gene is characterized by a higher SCC, which is related to a higher level of lactoferrin (Zahao *et al.* 2008, Changhong *et al.* 2009).

The Polish Holstein-Frisian (PHF) cattle, a modern dairy breed, and the Polish Red-White (PRW) cattle, which represent a double purpose breed and are included in polish animal genetic resources conservation program, were compared in this study. The aim of this research was to analyze the relationship between genetic polymorphism of lactoferrin and SCC and the proportion of certain milk protein fractions: serum albumin, α-casein, β-casein, κ-casein, α-lactalbumin in milk from PHF and PRW cows.

Materials and Methods

The research was conducted on 46 Polish Red-White (PRW) and 57 Polish Holstein-Frisian (PHF) cows during the first 3 months of lactation. Animals were chosen on the basis of analogy including age and milk yield. Cows were kept indoors in loose housing system. Milk samples were collected from cows during milk recording. In fresh milk, the basic composition was determined. Some of the milk from each sample (10 ml/sample) was defatted and frozen at -20°C, for the purpose of DNA extraction and determination of lactoferrin concentration and proportion of protein fractions.

Content of milk protein, fat, lactose and dry matter were determined using Infrared Milk Analyzer 150 (Bentley Instruments Inc, Chaska, MN, USA). SCC was determined on Somacount 150 apparatus (Bentley Instruments Inc, Chaska, MN, USA). The proportion of

protein fractions was determined by electrophoresis (Fig. 1) according to the method by Laemml (1970) on polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) as described previously by Pecka *et al.* (2011).

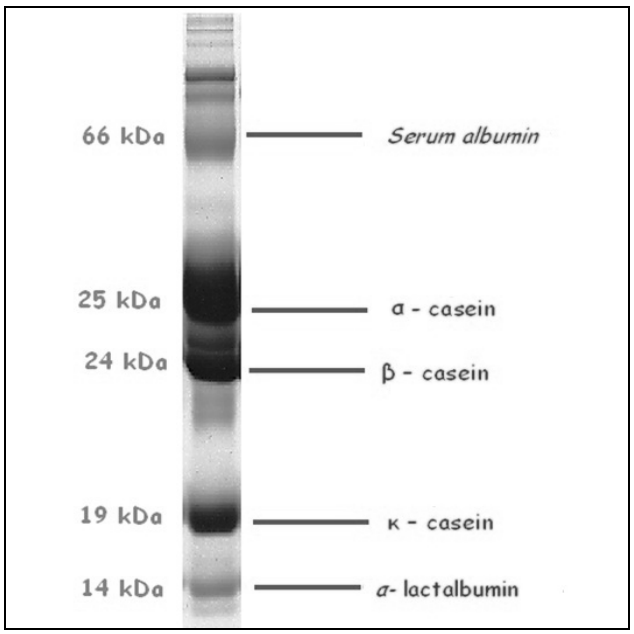

Fig. 1. **Electrophoresis separation of bovine milk protein fractions on polyacrylamide gel with sodium dodecyl sulfate** (the present study)

Genomic DNA was extracted from the skim milk samples using JETQUICK Blood & Cell Culture DNA Spin Kit (Genomed GmbH, Germany). Lactoferrin gene fragment of 301 bp was amplified by polymerase chain reaction (PCR) using two specific primers (forward: 5'-GCC TCA TGA CAA CTC CCA CAC-3' and reverse: 5'-CAG GTT GAC ACA TCG GTT GAC-3') as described previously by Wojdak-Maksymiec *et al.* (2006). The total volume of each reaction was 25 µl. The PCR began with a 2 minutes denaturation/activation step at 95°C. The cycling protocol was 30 cycles of denaturing at 95°C for 45 s, followed by annealing temperature of 58°C for 1 min and concluded with a 30 s elongation step at 72°C. Final extension temperature was 72°C for 5 min. PCR product was restricted with 10 units of restriction enzyme EcoRI (Fermentas, Life Sciences: www.fermentas.com) for 3 hours at 37°C. The EcoRI restriction enzyme digests PCR products of lactoferrin gene on fragments of 301 bp, 201 bp and 100 bp. The fragments of 301 bp were allele A, which was not recognized by EcoRI. The allele B consists of 201 bp and 100 bp fragments. Restriction fragments were loaded on a 4% agarose gel, with 5 µl of ethidium bromide and 100 mls of 1X TAE buffer. The gel was subjected to electrophoresis at 95 volts for approximately 20 minutes using a MP-300N horizontal gel electrophoresis apparatus and then photographed using UltraCam Digital Imaging System (www.ultralum.com).

Lactoferrin concentration was determined in the defatted milk samples using commercial ELISA kit (Bovine Lactoferrin ELISA Kit: Bethyl Laboratories, Montgomey, TX, USA). All procedures were performed according to the instructions of the producer. The milk samples were diluted 1:2.000 and a standard curve was generated for each set of samples. The absorbance of the samples in 96-well plate was recorded using Synergy ^{TM} BioTek plate reader (BioTek Inst, VT, USA) at 450 nm wavelengths. Measurement of each sample was run in duplicate.

The milk traits were analyzed according to the breed: PRW and PHF. The population was classified according to lactoferrin gene polymorphism (genotypes AA and AB) and SCC in milk (1st group up to 400 000 SCC/ml and 2nd group over 400 000 SCC/ml). Statistical analysis was done using multivariate analysis of variance with Statistica 9.0 software (StatSoft Poland, Krakow, Poland). Significant differences were determined by Duncan's test. Specified frequency of alleles and genotypes, and statistical evaluation of genotypes distribution were performed by χ^2 test according to Hardy-Weinberg equilibrium.

Results

The study showed two genotypes of lactoferrin gene: AA and AB. In the PRW population, the frequencies of these genotypes were respectively 32.61% and 67.39%, whereas in the PHF population, respectively 67.42% and 32.58%. The BB genotype was not shown. The frequency of allele A in the PHF population was 84.21%, while in the PRW population this frequency was 66.30%. On the contrary, the frequency of allele B was higher in PRW population (33.70%) than in the PHF population (15.79%). The differences between the observed frequencies of lactoferrin genotypes and their expected frequencies according to Hardy-Weinberg equilibrium are presented in Tables 1a and 1b.

Table 1a. **Frequency of lactoferrin genotypes in population of PRW cows**

Genotype	Observed frequency (%)	Expected frequency (%)	Chi-square
AA	32.61	43.96	1.35
AB	67.39	44.68	5.31
BB	0.00	11.35	5.22
Total	100.00	100.00	11.88

Chi-square = 11.88; *df* = 1; P ≤ 0.000567

Table 1b. **Frequency of lactoferrin genotypes in population of PHF cows**

Genotype	Observed frequency (%)	Expected frequency (%)	Chi-square
AA	67.42	70.91	0.05
AB	32.58	26.59	0.53
BB	0.00	2.49	1.42
Total	100.00	100.00	2.00

Chi-square = 2.00; *df* = 1; P ≤ 0.1569

The analysis of milk composition (Table 2) shows that milk from PRW cows and with AB genotype had a higher content of dry matter than milk from PRW cows with AA genotype. However, in milk from PHF cows such a relation was not found. The protein content in milk from cows with AA genotype and from the 1st group of SCC, in both breeds, was higher comparing to the milk from cows with AB genotype. In the 2nd group of SCC, an opposite tendency was shown. In the milk from PRW cows with AB genotype, a higher fat content was determined comparing to AA genotype. In the milk from PHF cows, a similar interaction was not detected. Whereas a significantly higher level of lactose was observed in the milk from PHF cows with AA genotype compared to the milk from cows with AB genotype. In milk from PHF cows higher protein and lactose levels and a lower fat content were determined, comparing to milk from PRW cows.

Table 2. **Composition of milk** (mean±SD) **from Polish Red-White** (PRW) **and Polish Holstein-Frisian** (PHF) **cows depending on lactoferrin gene polymorphism** (AA and AB) **and somatic cell count** (1st group up to 400.000 SCC/ml and 2nd group over 400.000 SCC/ml)

Breeds	Lactoferrin genotypes	SCC group	n	Percentage content (%)			
				Dry matter	Protein	Fat	Lactose
PRW	AA	1	9	12.73±1.31	3.10±0.26	4.20±1.17	5.04^{bc}±0.17
		2	6	12.71^{a}±1.95	3.08±0.14	4.32±2.03	4.91^{ab}±0.14
	AB	1	24	13.26b±1.27	3.12±0.23	$4,70^{a}$±1.27	5.05^{bc}±0.24
		2	7	12.84±0.65	3.06±0.34	4.57±0.82	4.84^{Aac}±0.06
PHF	AA	1	27	12.64±0.85	3.27±0.35	3.72±0.65	5.11^{Bc}±0.16
		2	12	13.06±1.03	3.27±0.42	4.29±1.00	5.00±0.18
	AB	1	12	12.76±1.52	3.32±0.35	3.85±1.42	5.06^{bc}±0.19
		2	6	12.29±0.57	3.21±0.26	3.65^{b}±0.42	4.95±0.20

$^{a-c}$ superscripts indicates differences (P<0.05); $^{A-B}$ superscripts indicates differences (P<0.01); n – number of animals

Table 3. **Proportion of protein fractions** (mean±SD) **in milk from Polish Red-White** (PRW) **and Polish Holstein-Frisian** (PHF) **cows depending on lactoferrin gene polymorphism** (AA and AB) **and somatic cell count** (1st group up to 400.000 SCC/ml and 2nd group over 400.000 SCC/ml)

Breeds	Lactoferrin genotypes	SCC group	n	Proportion of protein fractions (%)				
				α-casein	β-casein	κ-casein	α-lactalbumin	serum albumin
PRW	AA	1	9	37.97^{ACac}±8.17	12.64^{ACc}±3.53	13.44±1.58	14.14±2.46	10.51±6.28
		2	6	32.01^{ab}±6.99	12.25^{A}±3.34	15.45±4.46	14.50^{a}±2.75	8.45±1.97
	AB	1	24	35.37^{ACac}±4.73	13.33^{ACc}±2.88	15.65±1.56	14.12±1.94	11.23±3.73
		2	7	34.56^{Cc}±7.18	10.40^{Aac}±2.13	13.60±2.59	12.50±2.60	8.31^{a}±1.86
PHF	AA	1	27	25.24B±5.47	17.18^{Cab}±3.79	15.26±2.93	12.55±3.09	11.40±3.83
		2	12	23.64B±4.67	15.51^{C}±3.90	12.96^{a}±2.39	11.83±2.56	9.25±2.04
	AB	1	12	27.04^{ABbc}±4.45	17.94^{B}±3.53	16.10^{b}±3.74	11.84^{b}±3.68	12.63^{b}±2.89
		2	6	21.69^{Bc}±6.69	14.56^{bc}±3.35	14.96±3.62	13.93±3.07	11.46±3.63

$^{a-c}$ superscripts indicates differences (P<0.05); $^{A-C}$ superscripts indicates differences (P<0.01); n – number of animals

The statistical analysis of different protein fractions concentration (Table 3) showed that the part of serum albumin, α-casein and β-casein was higher in milk from cows from the 1st group of SCC, independently to the breed and to the genotype. In milk from PRW cows, a significantly (P≤0.01; P≤0.05) higher level of α-casein and lower level of β-casein was observed. The serum albumin level was higher in the milk from PHF cows with AB genotype. Moreover, a higher level of κ-casein was found in milk from these cows and its decreased level in the 2nd group of SCC. The milk from PRW cows with AA genotype was characterized by a higher level of α-lactalbumin compared to the milk from cows with AB genotype. Instead, in milk from PHF cows an analogous relation was not observed. Moreover, in milk from PHF cows a higher level of β-, κ-casein, serum albumin and lactoferrin was found, comparing to milk from PRW cows.

The concentration of lactoferrin (Fig. 2) in milk from cows with AA genotype, independently to the breed, was higher (middling 30%) comparing the milk from cows with AB genotype. Furthermore, a significantly (P≤0.05) higher level of lactoferrin was shown in the milk from PHF cows from the 2nd group of SCC, than in the milk from PRW cows.

Discussion

The concentration of lactoferrin in the analyzed milk samples ranged between 97.4 and 199.33 μg/ml, which is consistent with results of other authors (Neville 1998; Cheng 2008). An increasing content of lactoferrin with increasing SCC was determined. This lactoferrin raise in milk can be understood as an immunological response to bacterial infection. Hagiwara *et al.* (2003) observed similar changes in lactoferrin content depending on SCC.

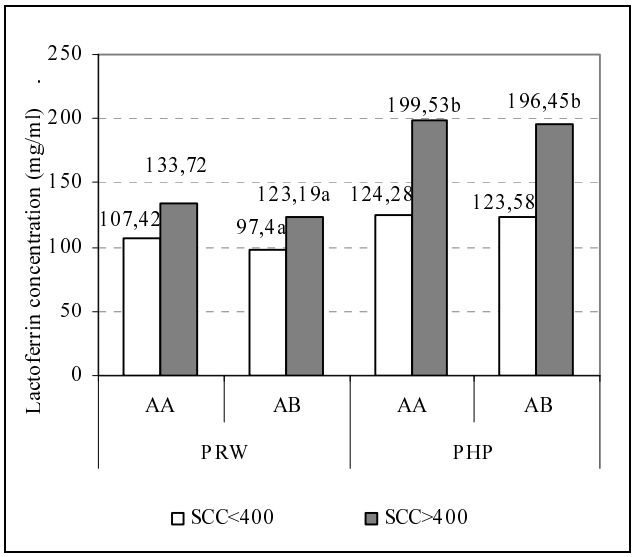

Fig. 2. **Lactoferrin concentration in milk from Polish Red-White** (PRW) **and Polish Holstein-Frisian** (PHF) **cows depending on lactoferrin gene polymorphism** (AA and AB) **and somatic cell count** (1st group up to 400.000 SCC/ml and 2nd group over 400.000 SCC/ml)

They determined that lactoferrin concentration depends on cell structures content in milk and that it is higher in milk from cows with symptoms of mammary gland inflammation than from healthy cows. The results obtained in the present study are consistent with the results of other authors (Harmon *et al.* 1975; Bernatowicz & Reklewska 2003; Cheng *et al.* 2008). Different researche (Schmitz *et al.* 2004; Chaneton *et al.* 2008) showed that milk from microbiologically infected quarters was characterized by a higher level of lactoferrin, compared to milk from healthy quarters. In milk from cows with subclinical mammary gland inflammation lactoferrin content was about 500 µg/ml and from cows with clinical inflammation reached 750 µg/ml (Kawai *et al.* 1999; Hagiwara *et al.* 2003). *Ipso facto*, in milk from cows with allele B, the lactoferrin level should be higher than in milk from cows with allele A (Wojdak-Maksymiec *et al.* 2006; Changhong *et al.* 2009; Sharifzadeh & Dostoi 2011). In our study, an opposite relation was observed. In milk from cows with AA genotype the lactoferrin level was higher compared to milk from cows with AB genotype.

No publications about the changes of milk composition and proportion of protein fractions in cattle milk depending on genetic polymorphism of lactoferrin and SCC were found. The most frequently analyzed effect is the effect of polymorphism of four protein fractions, α-, β-, κ-casein (α-, β-, κ-CN) and β-lactoglobulin (β-LG) on cattle milk traits (Paterson *et al.* 1999; Felenczak *et al.* 2006; Keating *et al.* 2007; Michalcová & Krupová 2007).

Seyfert and Kuhn (1994) analyzed the polymorphism of lactoferrin gene and described two genetic variants: A and B. These alleles encoded three genotypes: AA, AB and BB. The frequency of allele A was 0.755 and of allele B 0.245. Wojdak-Maksymiec *et al.* (2006) conducted a research on Black-and-White Holstein-Frisian cattle and identified the two alleles, A and B, in frequencies 67.74% and 32.56% respectively. Identified alleles encoded three genotypes: AA, BB and AB, occurring with frequencies 37.90%, 2.42% and 59.68% respectively. The results of many researche indicate a low frequency of BB genotype in dairy cattle herds (Wojdak-Maksymiec *et al.* 2006; Changhong *et al.* 2009; Sender *et al.* 2006 and 2012; Sharifzadeh & Dostoi 2011). The polymorphism of lactoferrin is inherited according to Mendel's Law of Segregation (the "First Law"). The connection of allele B with lower milk performance can be the cause of a progressive supersession of its genotype from herds due to selection for high milk performance (Litwińczuk *et al.* 2006; Sender *et al.* 2012). Moreover, homozygous cows with AA genotype are characterized by a lower SCC, thus they are kept in herd during selection. In turn, cows with BB genotype are more susceptible to inflammation of the mammary gland (Zhao *et al.* 2008; Changhong *et al.* 2009). Sender *et al.* (2012) disagree, suggesting that cows with BB and AB genotypes are characterized by a healthier mammary gland. In our study, a differentiation of lactoferrin genotypes in examined herds of PHF and PRW cattle was observed. In PRW population, the AB genotype was in majority. However, in the PHF population, most of cows had AA genotype. In both herds, no BB genotype was observed.

Significant differences between the SCC and the lactoferrin genotype were demonstrated. The highest SCC was determined in milk from cows with AB genotype and the lowest in milk from cows with AA genotype. The research carried out by Changhong *et al.* (2009) confirmed the relation between lactoferrin gene polymorphism and SCC. Although, they presented other results, significantly highest SCC was observed in milk from cows with BB genotype, in contrast to cows with AA genotype. Meanwhile, the frequency of AB genotype was not significant in any of analyzed groups of animals. Zahao *et al.* (2008) found the highest frequency of BB genotype in cows with subclinical mastitis.

Results presented by Sarikaya *et al.* (2006) demonstrate that the dry matter level in milk decrease with raising SCC. In our study, a similar relation was also observed, except PHF cows with AA genotype.

Cheng *et al.* (2008) found an important relation between lactoferrin and total protein content ($r=0.482$; $P\leq0.001$). A probable cause of such an interaction can be the fact, that during inflammations of the mammary gland the concentration of lactoferrin increases, as does the concentration of other protein related to immune response. Auldist *et al.* (1996) proved that milk from cows suffering mastitis was characterized by a higher concentration of total protein. Ogola *et al.* (2007) presented different conclusion, that there is no significant relation between inflammation of the mammary gland and total protein concentration. A decreased level of total protein in milk with increasing SCC can indicate that increasing SCC is not always connected to an inflammation, but can be caused by a mechanical irritation or a metabolic disease.

In our study a positive interaction between AA genotype and lactoferrin and fat content was determined, in both herds. In milk from cows with AB genotype an opposite tendency was observed. Results presented by other authors show that the level of fat decrease with raising SCC (Auldist *et al.* 1996). This interaction was observed in cows with AB genotype in present study. Cheng *et al.* (2008) showed that there is no significant relation between lactoferrin level and fat content in milk.

Cheng *et al.* (2008) found that the increase of lactoferrin level in milk is related with a decreased level of lactose (r=-0.183). Bernartowicz *et al.* (2004) esteemed that the cause of lactose level diminution and lactoferrin level raise in milk is an inflammation of the mammary gland. Other researchers (Auldist *et al.* 1995; Bansal *et al.* 2007; Ogola *et al.* 2007) showed that lactose level lowers with enhance of inflammation of the mammary gland. In our study, the described relation was also observed. Additionally, the milk from cows with AA genotype had a higher lactose level compared to milk from cows with AB genotype. Probably, the reason of such an interaction is the fact that the allele A of lactoferrin effects favourably on mammary gland health, which influences the composition of milk.

In polymorphism of lactoferrin gene, the allele B is connected with an increased SCC. In consequence, it affects protein fraction composition in milk (Urech *et al.* 1999; Wojdak-Maksymiec *et al.* 2006; Heck *et al.* 2008; Changhong *et al.* 2009; Sharifzadeh & Dostoi 2011). Different research showed that in milk from cows with a higher SCC the level of casein is significantly lower than whey protein level (Auldist *et al.* 1996; Urech *et al.* 1999; Heck *et al.* 2008). Thus, milk from cows with AB genotype should be characterized by a higher level of whey proteins comparing to milk from cows with AA genotype. However, in analyzed milk an unequivocal relation between genotypes and protein fractions was not found. αs1-, αs2-, β- and κ-casein are composed of calcium phosphate, magnesium and potassium salts, which affect, together with hydrophilic and hydrophobic groups, the stability of the sol structure of milk (McMahon & Oommen 2008; Summer *et al.* 2010). In consequence, the casein level determines the level of macroelements in milk and affects its nutritional value. For the dairy industry the most eligible is milk with higher κ-casein content, because of its better technological parameters and usefulness for processing (Ng-Kwai-Hang 1998).

In our study, in milk from cows with AB genotype and from the 1st group of SCC, a highest level of β- and κ-casein was found, independently to breed. This may suggest that this milk is more eligible for processing. A decrease of α-, β-, κ-casein percentage with increasing SCC was observed. The lowest casein percentage was determined in milk from PHF cows. Results of other researche show the positive relation between SCC and serum albumin content in milk (Urech *et al.* 1999; Lieskea *et al.* 2005; Zeng *et al.* 2009). In our study, such an interaction was not observed. The serum albumin content decreased with raising SCC.

Urech *et al.* (1999) found that during inflammation of the mammary gland, the level of α-lactalbumin decreases. In analyzed milk, an unequivocal effect of SCC on α-lactalbumin was not determined, which indicates that other factors affect its level.

Conclusions

It was showed that the fat level and composition of protein fractions are rather affected by SCC than by the polymorphism of lactoferrin gene or the breed (PRW or PHF). Milk from PHF cows with AA genotype and milk from cows with AB genotype and from the 1st group of SCC was characterized by higher κ-casein level, thus a higher technological quality. Ambiguous impact of genetic polymorphism of lactoferrin gene on the milk composition of the examined breeds of cattle may be due to a significant difference in the genotypes of animals, different backgrounds, different capacities and levels of production.

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