QUANTITATIVE ANALYSIS OF WHEY PROTEINS IN RELATION TO HEALTH STATUS OF THE UDDER QUARTERS AND SEASON

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Abstract. The aim of the study was to evaluate the amount of chosen whey proteins, i.e. lactoferrin (LF), immunoglobulin G (IgG), alpha-lactalbumin (alpha-LA), beta-lactoglobulin (beta-LG) and bovine serum albumin (BSA) in cow milk in relation with somatic cell count (SCC) and pathogenic bacteria presence in quarter milk at different seasons. The quarters health status was set on the base of SCC and microbiological analysis. The diseased quarters (DQ) showed increased concentration of all proteins analysed, except alfa-LA, in compare to healthy quarters (HQ) ($p<0.001$). Significant differences of LF, IgG and beta-LG were observed between quarters with presence of bacterial growth (BG), nonspecific mastitis (NM), subclinical mastitis (SM) and healthy quarters (HQ) ($p<0.05$). In our research data, significant effect of season was estimated on LF ($p<0.001$), IgG ($p<0.001$), alfa-LA ($p<0.01$), beta-LG ($p<0.001$) and BSA ($p<0.05$) contents.

Keywords: whey proteins, udder, milk, mastitis

Introduction

Mastitis has been and continues to be recognized as one of the major disease problems concerning the dairy industry. The prevention and treatment of mastitis represent a serious burden to producers. Innate immunity is a target of choice for selection against infectious diseases (Rainard and Riollet, 2006). Immune factors in colostrum and milk play an important role in the host defense of the mammary gland itself, protecting it from pathogenic organisms (Sordillo et al., 1997; Oviedo-Boyso et al., 2007). Milk contains many peptides and proteins, which exhibit bacteriostatic and bactericidal properties in their intact form (Bagnicka et al., 2010). Especially the whey proteins contain peptides that improve the immunomodulatory activity (stimulating defence mechanisms of the body) and antibacterial activity (Sobczuk-Szul et al., 2010). The concentration of the whey proteins varies not only depending on the degree of infection of the udder but it is also affected by the keeping conditions (Kocina et al., 2012).

In order to understand and evaluate the role of LF, IgG, alpha-LA, beta-LG and BSA in maintaining the udder health, the dynamics of their amount in cow milk in relation with SCC and pathogenic bacteria at different seasons was investigated.

Materials and methods

The tested dairy herd consisted of 30 Lithuanian Black-and-White crossbreed with Holstein dairy cows. In total 120 milk samples were collected individually from normal lactating dairy cows. Milk samples from individual quarters were collected once during the spring, summer and autumn (10 cows/40 quarter samples/each season). All cows were clinically healthy without any signs of udder infection at the sampling time. Milk samples were collected for laboratory examination aseptically in accordance with the method recommended by the „Milk and milk products. Guidance on sampling (ISO 707:2008)“ standard.

Microbiological examination for identification of pathogenic microorganisms was made as soon as the milk samples were delivered to the accredited central milk testing laboratory. Standard procedures for identifying pathogenic microorganisms in milk were performed in compliance with the laboratory criteria (General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025:2005) and according the method „Estimation of the main pathogenic microorganisms causing mastitis in milk SDP 5.4.4.B.6“(2009).

For determination of SCC milk samples were preserved with bronopol in microtabs and analysed with the flow cytometric analysis method using a Somascope cell counter (Foss, 3400 Hillerød, Denmark).

ELISA used to determine LF and IgG concentration in bovine milk (Biopanda Reagents, UK). Determinations were carried out in whey obtained by centrifugation of 50 ml of fresh milk for 20 minutes at a temperature of 4°C and the speed of 3,000 rpm (Sobczuk-Szul et al. 2014). The resultant whey was stored frozen at a temperature of -20°C until analysed. The concentration of LF and IgG in quarter milk samples was assayed using ready kits, following the procedure recommended by the manufacturer.

To evaluate the content of whey proteins, i.e. alpha-LA, beta-LG and BSA, samples from cow's milk were prepared according to Romero et al. (1996). Separation of whey proteins was performed by high performance liquid chromatography (HPLC) method described by J. Krol (2012). For this purpose, a high pressure gradient HPLC system Varian ProStar (Varian Corp., USA) was used, consisting of two ProStar 210 pumps, automatic sampling module Prostar 410 and Prostar 363 fluorescence detector. The separation of the alpha-LA, beta-LG and BSA content was performed by HPLC using a 5 μm particle size, 250 mm long and 4.6 mm internal diameter Nucleosil C18 chromatographic column equipped with guard column containing the same packing material (Macherey-Nagel, Düren, Germany). Each protein was calibrated individually by injecting solutions of the standards (20 μl). Purifield proteins from bovine milk (alpha-LA, beta-LG and BSA) were puchased from Sigma (Germany). All chemicals were HPLC analytical grade. The separation was carried out at 25 ºC using the gradient system. The mobile phase was solvent A: 90% water, 10% acetonitrile and 0.1% triflouroacetic acid (TFA) and solvent B: 90% acetonitrile, 10% water and 0.1% TFA. alpha-LA, beta-LG and BSA content was identified and quantified by measuring the fluorescence at 220 nm wavelengths. The sample injection volume was of 20 μl. Data collection and evaluation was performed by using LG Solution (Shimadzu Corp., Kyoto, Japan) operating system.

Grouping of the numerical material for statistical analysis

The health status of the udder quarters was assessed according to microbiological analysis and SCC in milk (according to Chaneton et al., 2013). Quarters with presence of bacterial growth, nonspecific mastitis, or subclinical mastitis were classified as diseased quarters (DQ) (Table 1).

Table 1. **Grouping of the udder quarters according to the microbiological analysis and SCC in milk**

Group	Specification	Pathogen isolation	SCC (10^3/ml)	Health status of the quarters	Number of samples
BG	Presence of bacterial growth	+	< 200	diseased	42
NM	Nonspecific mastitis	-	≥ 200	diseased	13
SM	Subclinical mastitis	+	> 200	diseased	16
HQ	Healthy quarters	-	< 200	healthy	49
(+/-) presence /absence of pathogenic microorganisms in milk sample					

All the data obtained were fractionated into spring (n=40), summer (n=40) and autumn (n=40) for the analysis of seasonal effect on whey proteins.

Statistical analysis

The obtained data were statistically processed by using SPSS program 20.0 for Windows using a one-way ANOVA procedure. The results are presented as mean and standard error of mean (mean±SEM). The significance of differences between mean values of the evaluated groups was determined with the *post-hoc* Fisher *LSD* criterion (α=0.05) (Juozaitienė et al., 2014).

Results

The DQ showed statistically significant increased concentrations of LF ($p<0.001$) 46%, IgG ($p<0.001$) 46%, beta-LG ($p<0.05$) 4% and BSA 6% compared with HQ, while alfa-LA concentration was 2% higher in HQ milk samples (Table 2).

Table 2. **Mean values of whey proteins (mg/ml) in healthy and diseased quarters according to health status of the udder**

Quarters	LF	IgG	alfa-LA	beta-LG	BSA
Healthy	0.03±0.01***	0.12±0.02***	0.93±0.03	3.14±0.11*	0.31±0.03
Diseased	0.08±0.01	0.32±0.03	0.89±0.02	3.47±0.11	0.35±0.02
Differences significant at * $p<0.05$; ** $p<0.01$;*** $p<0.001$					

Under the udder health assessment 33% of DQ corresponded to the group BA, 15% - to SM and 11% corresponded to NM and the rest part 41% corresponded to HQ. The highest mean value of LF (0.1±0.02 mg/ml), IgG (0.41±0.06 mg/ml) and beta-LG (3.88±0.24 mg/ml) were observed in quarters with SM. It was determined higher LF (72.7%) IgG (74.5%), beta-LG (50.8%) and BSA (57.3%) concentration in quarters with BG compared to HQ. The peak of alfa-LA was observed in HQ. Significant differences of LF, IgG and beta-LG mean values between groups were determined ($p<0.05$) (Table 3).

Further seasonal effect on whey proteins content in milk was evaluated. LF, IgG and BSA peak concentrations were clearly observed in spring, alfa-LA in summer and beta-LG in autumn. LF content remained stable in summer and autumn and 60% were lower compared to content in springtime. The lowest levels of IgG, beta-LG and BSA (0.08 mg/ml, 2.94 mg/ml and 0.27 mg/ml, respectively) were in summer in compare to content with other seasons. The final results of one-

way analysis of variance (summarized in Table 4) indicate a significant effect of season on the content of all the proteins analysed.

Table 3. **Distribution of whey proteins mg/ml (mean±SEM) in quarters according to the microbiological analysis and SCC groups**

Groups	n	LF	IgG	alfa-LA	beta-LG	BSA
BG	42	0.08±0.01 a	0.35±0.04 a	0.89±0.03	3.24±0.13 abd	0.36±0.03
NM	13	0.04±0.01 b	0.14±0.03 b	0.89±0.03	3.74±0.21 bc	0.33±0.03
SM	16	0.10±0.02 a	0.41±0.07 a	0.87±0.06	3.88±0.24 c	0.35±0.04
HQ	49	0.03±0.01 b	0.12±0.02 b	0.92±0.03	3.14±0.11 d	0.31±0.03
a,b,c,d - differences signifcant at $p<0.05$						

Table 4. **Variability of whey proteins mg/ml (mean±SEM) depending on season**

Season	n	LF***	IgG***	alfa-LA**	beta-LG***	BSA*
Spring	40	0.12±0.01 a	0.54±0.03 a	0.90±0.04	3.33±0.12 a	0.37±0.02 a
Summer	40	0.03±0.01 b	0.08±0.01 b	0.98±0.03 a	2.94±0.10 b	0.27±0.03 b
Autumn	40	0.03±0.01 b	0.11±0.01 b	0.84±0.02 b	3.74±0.16 c	0.35±0.03 a
a,b,c -differences signifcant at $p<0.05$ and significant effect at * $p<0.05$; ** $p<0.01$;*** $p<0.001$						

Discussion and conclusion

The SCC in milk constitutes a good diagnostic tool that allows early detection of either subclinical or acute form of mastitis (Cheng at al. 2008, Krol et al. 2012). In the present research, the concentration of LF, IgG, alpha-LA, beta-LG and BSA in the cow's milk related to health status of udder quarters was analysed. Two combined variables were used to determine the quarter health status: the SCC value and the isolation of pathogenic bacteria. Similar criteria have been used by other authors (Chaneton et al. 2013). In the present study 59% corresponded to DQ and 41% to HQ. In Chaneton et al. (2013) research the most part of analyzed milk samples consisted of HQ (86.8%) and of the DQ 62% corresponded to NM. Most of analysed milk samples with SCC levels lower than 200,000 cells/ml were positive for bacterial isolation. This observations means that some specific bacterial species could be associated with weak immune responses (Schwarz et al. 2010) and it is possible that the physiological status of the mammary gland could induce a diminished immune response upon bacterial invasion. Chaneton et al. (2013) have established most of the samples with SCC level higher than 200,000 cells/ml and were negative for bacterial isolation. This factor researchers explained that an inflammation process can occur without detectable bacteria in milk. The present research demonstrated higher levels of all proteins analysed, except alfa-LA, in DQ compared to HQ ($p<0.001$). Significant differences of LF, IgG and beta-LG were observed between quarters with BG, NM, SM and HQ ($p<0.05$). The same tendency of LF was also shown by Chaneton et al. (2013), but no significant differences were observed between quarters. Korcina et al. (2012) reported IgG mean values in milk samples with pathogenic agents and without pathogens differed slightly and insignificantly.

The present studies on seasonal effect on analyzed whey proteins indicated the significantly higher LF, IgG and BSA values in spring season ($p<0.05$). This is in agreement with Conesa et al. (2005), who reported that IgG values from the whole population of analysed samples were found to be significantly higher also in the spring. It can be assumed that the bioactive compounds contained in green forages (at pasture period), which have immunomodulatory properties, have an indirect effect on LF levels (Brodziak et al. 2014). In another study, the seasonal effect was observed, with the highest values in the spring for LF and in the winter for IgG (Konuspayeva et al. 2007). In our research data, significant effect of season was estimated on LF ($p<0.001$), IgG ($p<0.001$), alfa-LA ($p<0.01$), beta-LG ($p<0.001$) and BSA ($p<0.05$) contents. These results are in agreement with the findings of Korcina et al. (2012), who noted significant effect of the seasonal keeping of cows on the concentration of IgG in cows milk ($p<0.001$) and Brodziak et al. (2014) research results with the significant effect of the season on LF and alfa-LA contents in goats milk ($p<0.01$). A higher percentage of whey proteins in the spring–summer season compared with the autumn–winter season was also found in research performed on cow's milk by Litwinczuk et al. (2011).

It can be concluded that LF, IgG and BSA concentration in milk were elevated during subclinical mastitis as well as in quarters positive for bacterial growth. It should be assumed that the increased concentrations of analysed proteins (except alfa-LA) in the diseased quarters showed their immune response activity. Furthermore, the findings showed the high contribution of season of the year on milk antimicrobial proteins concentrations.

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