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A relationship between somatic cell count, polymorphic form of β4-defensin and susceptibility of cow milk fat to lipolysis*

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The aim of this study was to evaluate the effects of somatic cell count and the polymorphic form of β4-defensin on the concentration of free fatty acids (FFA) and physico-chemical characteristics of cow's milk. The study was carried out on 120 Polish Holstein-Friesian Black and White dairy cows. The animals were maintained in a loose barn and fed with the TMR system according to the INRA norm. The animals were divided into groups according to their polymorphic form of the defensin β4 gene: 1st – CT (def-1); 2nd – CC (def-2) and into two groups in terms of their somatic cell count: 1st - <3×10^5 (SCC-1) and 2nd – 3×10^5 – 6×10^5 (SCC-2) cell/ml. Milk samples were collected once a month during the whole lactation. Chemical composition and some physico-chemical parameters of milk were determined by automated infrared analysis with a Milkoscan FT2 instrument. SCC were evaluated using BactoCaunt IBCm. A relationship was found between polymorphic forms of the defensin gene and the level of FFA in milk directly after milking (CT<CC). The chemical composition of milk and its physicochemical parameters differed significantly between the identified genetic variants of defensin β4: fat (CT<CC), total protein (CT<CC), casein (CT<CC), total solids (CT<CC) and solids-non-fat (CT<CC). Cows from the SCC-1 group produced milk characterised not only by lower susceptibility to lipolysis, but also a higher concentration of the basic components. High positive correlations were found between the basic milk parameters (with the exception of lactose) and the FFA concentration. The results indicate that fat lipolysis of cows' milk is determined both by the somatic cell count and by the polymorphic form of β4-defensin.

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Milk of major bovine breeds contains 3.5-5.0% fat, which is composed of more than 400 different fatty acids, mainly present as triacylglycerols (TAGs) [Kantkanen *et al*. 2011]. Milk fat fractions are widely used in a variety of food products, due to the many favourable physical, chemical and nutritional properties of milk fat [Jóźwik *et al*. 2010b]. People mainly consume milk fat in different forms of traditional dairy products, such as liquid milk, butter, cream, cheese and ice cream.

The process of lipolysis, i.e. enzymatic hydrolysis of milk fat, leads to the release of free fatty acids as well as mono- and diacylglycerds from triacylglycerds. Free fatty acids lead to unfavourable organoleptic changes (taste, aroma) in fresh milk and lower its value as a raw material for the dairy industry [Antonelli *et al.* 2002, Collins *et al*. 2003].

Lipolysis of cow milk fat is a complex process affected by numerous factors, both genetic and environmental. The genetic factors include the structure and size of fat globules, as well as the fatty acid structure and profile of milk fat. The main environmental factors affecting the intensity and progress of lipolysis are connected with the health condition of the mammary gland, stage of lactation, nutrition, health condition of the animal, the temperature and time of storage of raw milk and the applied milking method [Deeth 2006].

Defensins are a part of the immunological system in animals. Results from investigations conducted over the recent years point to an important defensive role of cell peptides existing in various animal organs, including the mammary gland epithelium [Exner *et al.* 2000]. An increase in milk somatic cell count (SCC) has been negatively correlated with changes in milk components [Jóźwik *et al*. 2010a, Bagnicka *et al*. 2011]. Several studies have reported decreases in lactose, fat, cholesterol and casein contents in milk [Somers *et al.* 2003, Lindmark-Mansson *et al.* 2006, Barłowska *et al.* 2009, Strzałkowska *et al.* 2009a, Strzałkowska *et al.* 2009b, Strzałkowska *et al.* 2009c, Strzałkowska *et al.* 2010, Jóźwik *et al.* 2012]. These modifications in milk composition result in lower cheese yields and altered technological properties [Santos *et al.* 2003].

The aim of this study was to evaluate the effects of somatic cell count and the polymorphic form of β4-defensin on the concentration of free fatty acids and physico-chemical characteristics of cow's milk.

Material and methods

Animals

The study was carried out on 120 Polish Holstein-Friesian Black and White cows from the herd maintained at the Experimental Farm of the Institute of Genetics and Animal Breeding PAS, Jastrzębiec, Poland.

The mean yield was about 9600 kg milk per cow per lactation, containing 4.0% fat and 3.4% protein. The cows were in lactation I to III. The animals were maintained

in a loose barn, with free access to water and they were fed in the TMR system. Diets fed were formulated according to standards of the Institute National de la Recherche Agronomique adjusted by the National Research Institute of Animal Production [INRA, 2001], which met all their individual nutritive requirements. All animals were daily examined for clinical *mastitis*.

Sampling milk

The cows were machine-milking twice a day according to standard procedures. Prior to milking the teats were washed with clean tap water and dried with a single service paper towel. Before attaching the milking machine to the teats, the first three to four streams of milk from all teats of each cow were discarded onto a strip cup and examined for any sign of *mastitis*. Milk samples were taken every month during the whole lactation.

Analytical

Milk was analysed by the Laboratory of the Institute of Genetics and Animal Breeding PAS, Jastrzębiec. Immediately after milking each milk sample was analysed for free fatty acids (FFA), fat, protein, casein, total solids, solids-non-fat, lactose, citric acid, urea, freezing point (FDP), density and acidity. The composition and parameters of milk were estimated using an IR-spectrophotometer MilkoScan FT2 device (Foss Electric, Hillerod, Denmark). The somatic cell count (SCC) was determined using fluorescence microscopy (IBC_M Bentley Instruments, Inc., Chaska, USA).

A method was developed for the identification of enteric β4-defensin in the blood of milking cows. The PCR genomic matrix was the DNA isolated from blood samples of cows. The study was conducted on 120 samples of DNA. To establish polymorphic forms of β4-defensin primers were designed for the amplification of gene fragments from nt 1568 to 24902 GeneBank (AF008307). It was a part of an intron and a part of the second exon. The optimal annealing temperature of primers was 63.9°C. The SSCP reaction of DNA samples revealed polymorphism of the examined fragment of the β4-defensin gene. The PCR products with polymorphic forms were sequenced. The A→G 2239 substitution was identified. It was the additional restriction position of the *Nla*III enzyme. This enzyme divided the amplification fragment into two fragments 446 and 448. When the mutation appeared, the fragment of 448 was also divided into two fragments of nt 253 and 195. The frequency of mutation was 30%. Cows were divided into two groups as related to the polymorphic form of β4-defensin: 1st – CT and 2nd – CC.

Statistical

Depending on the somatic cell count milk samples were divided into two groups: 1^{st} – $<3\times10^5$ and 2^{nd} – 3×10^5 – 6×10^5 cell/ml. Depending on the polymorhpic form of the β4 defensin gene milk samples were divided into two groups: 1^{st} – CT (def-1), n=20 and 2^{nd} – CC (def-2), n=25.

Statistical inference was conducted on the basis of the GLM procedure of SAS Version 9.1 for Windows [SAS/STAT 2002-2003] using a model including fixed effects of SCC and the polymorhpic form of the β4 defensin gene. Prior to the statistical analysis the data for SCC were transformed to a logarithmic value (ln). Statistical package *SAS/STAT (2002-2003*, version 9.1.3*) CORR procedure* was used to calculate the *Pearson's correlation* coefficients

All procedures involving animals were in accordance with the Guiding Principles for the Care and Use of Research Animals and were approved by the Local Ethics Commission (Warsaw University of Life Sciences, Permission No. 56/2009).

Results and discussion

Table 1 presents data on the relationship between the β4 defensin genotype and the level of free fatty acids (FFA) and physico-chemical composition of milk. Cows with the β4-CT defensin genotype (def-1) produced milk with a lower FFA concentration (P≤0.01) when compared to animals with the β4-CC defensin genotype (def-2). Milk obtained from cows from the def-1 group showed lower susceptibility to the fat fraction degradation. In turn, cows from group def-2 produced milk characterised by a physico-chemical composition more favourable from the nutritional and technological point of view (Tab. 1). Milk of cows from group def-2 showed higher concentrations of fat (P≤0.01), total protein (P≤0.01), casein (P≤0.01) as well as milk solids (P≤0.05) and non-fat milk solids (P≤0.01). A somewhat lower somatic cell count was recorded for the milk of cows from group def-1(Tab. 1).

Table 1. Least squares means (LSM) and their standard errors (SE) for milk chemical composition and physical traits depending on the polymorphic form of β4-defensin.

Item	Def-1		Def-2	
	LSM	SE	LSM	Se
FFA (mmol/L)	0.88^{A}	0.03	0.98^{B}	0.04
Fat (%)	3.95^{A}	0.15	4.34^{B}	0.19
Protein (%)	3.60^{A}	0.05	3.84^{B}	0.06
Casein (%)	2.74^{A}	0.03	2.84^{B}	0.04
Total solids (%)	13.14^{a}	0.23	13.69^{b}	0.22
Solids-non-fat (%)	9.31^{A}	0.05	9.48^{B}	0.06
Lactose (%)	4.59	0.03	4.59	0.03
Urea (mg/L)	308	15.7	323	19.8
Citric acid (%)	0.14	0.004	0.13	0.005
FDP (-°C)	528.5	2.64	529.9	3.32
Density (g/L)	1.029	0.001	1.030	0.001
Acidity (SH)	16.8	0.26	6.57	0.14
SCC	6.57	0.88	6.77	0.14

$^{aA...}$Within rows means bearing different superscripts differ significantly at: small letters – P<0.05; capitals – P<0.01.
FFA – free fatty acids ; FDP – freezing point. Def-1 – CC; Def-2 – CT.

Differences were observed in the FFA concentration in the cows' milk depending on SCC (Tab. 2). Milk produced by cows from group SCC-2 was characterized by a higher FFA concentration (P≤0.01) when compared with that obtained from the SCC-1 cows. It needs to be stressed here that the health condition of the mammary gland and SSC may affect significantly the progress and level of the milk fat fraction degradation, as it is shown by the results reported by other authors [Ma *et al.* 2000, Santos *et al.* 2003, Bansal *et al.* 2005, Gargouri *et al.* 2008]. A high somatic cell count in milk is related to an increased permeability of the glandular tissue secreting epithelium, as result of which the factor activating spontaneous lipolysis passes from blood to milk [Deetha 2006].

Table 2. Least squares means (LSM) and their standard errors (SE) for milk chemical composition and physical traits depending on somatic cell counts

Item	SCC-1		SCC-2	
	LSM	SE	LSM	SE
FFA (mmol/L)	0.81^{A}	0.04	0.95^{B}	0.03
Fat (%)	4.33^{A}	0.15	3.87^{B}	0.14
Protein (%)	3.91^{a}	0.12	3.62^{b}	0.05
Casein (%)	2.91^{a}	0.08	2.68^{b}	0.03
Total solids (%)	13.73^{a}	0.63	13.10^{b}	0.23
Solids-non-fat (%)	9.66^{A}	0.13	9.13^{B}	0.05
Lactose (%)	4.72^{A}	0.07	4.46^{B}	0.02
Urea (mg/L)	312	40.9	285	15.5
Citric acid (%)	0.14	0.010	0.13	0.004
FDP (°C)	545.8^{A}	1.45	512.6^{B}	2.45
Density (g/L)	1.031	0.001	1.028	0.001
Acidity (SH)	6,84	0.67	6,72	0.24

$^{aA...}$Within rows means bearing different superscripts differ significantly at: small letters – P<0.05; capitals – P<0.01.
FFA – free fatty acids; FDP – freezing point; SCC-1 – <3 x 10^5; SCC-2 – <3 x 10^5 – 6 x 10^5 cell/ml.

Fernandes *et al.* [2011] stated that a high somatic cell count in the milk of cows affects the FFA level in dairy products obtained from this raw material. The authors cited demonstrated that yoghurt obtained from milk of low cytological quality is characterised by a short storage time. For this reason the authors recommended producing yoghurt from milk, in which the SCC does not exceed 40 x 103/ml.

Cows from group SCC-1 (SCC in milk lower than in the SCC-2 group) produced milk characterised not only by a lower susceptibility to lipolysis, but also by a higher concentration of the basic components, i.e. fat by 0.46 percentage points, total protein by 0.29 percentage points, casein by 0.23 percentage points and lactose by 0.26 percentage points, respectively (Tab. 2). The higher concentration of the basic components in the milk of SCC-1 cows led to a higher content of total milk solids (P≤0.05) and non-fat milk solids (P≤0.1).

During the development of the inflammation process in the mammary gland one may observe inhibition of synthesis and an increased degradation of milk components as well as an intensified permeability of blood vessels for many blood components. As a result of those processes the levels of lactose, fat, total casein, milk solids decrease, whereas somatic cell count and whey protein contents, including serum albumins and immunoglobulin, increase [Rouxa *et al.* 2003].

Table 3 presents data referring to Pearson's coefficients of a linear correlation between the physico-chemical composition of milk and the FFA level. A higher urea concentration in milk is accompanied by a growing FFA level (Tab. 3), which may be caused by the fact that the cows' diet is not balanced. Animal nutrition is one of the significant factors affecting the progress of lipolysis, as poor quality feeds may lead to the production of milk characterised by a fat fraction highly susceptible to lipolysis [Wiking *et al.* 2003]. Farlay *et al.* [2002] demonstrated in studies conducted on dairy cows that animals fed diets based on maize silage and concentrate produced milk less susceptible to lipolysis than did cows receiving diets based on pasture grass. According to the cited authors, grazing dairy cows may fully cover their nutritive requirements. The use of sunflower oil in diets for dairy goats results in a decrease of FFA in milk [Chilliard *et al.* 2003]. The research conducted by Hanusa

Table. 3. Pearson correlations coefficients of chemical composition and physical traits of cow milk

	Day in milk	Milk yield	Fat	Protein	Casein	Lactose	T. solids	SNF	Urea	C. acid	FDP	FFA	Density	Acidity	log
Day in milk	1.00	-	0.32**	0.51**	0.48**	-0.35**	0.34**	0.33**	-0.01	-0.04	-0.12	0.34**	0.01	0.18*	0.06
Milk yield		0.51** 1.00	-0.56**	-0.59**	-0.56**	0.37**	-0.52**	-0.43**	0.03	0.02	-0.04	- 0.25**	-0.12	-0.38**	-0.06
Fat			1.00	0.59**	0.59**	-0.40**	0.91**	0.42**	0.02	0.01	0.17*	0.32**	0.02	0.16*	-0.15
Protein				1.00	0.96**	-0.40**	0.68**	0.79**	0.31**	-0.08	0.29**	0.57**	0.36**	0.49**	0.10
Casein					1.00	-0.26**	0.71**	0.85**	0.24**	-0.04	0.31**	0.44**	0.46**	0.46**	0.05
Lactose						1.00	0.28*	0.18*	0.10	0.08	0.17*	-0.11	0.46**	-0.29**	-0.27**
Total solids							1.00	0.61**	0.06	0.03	0.21**	0.40**	0.17*	0.22*	-0.13
Snf								1.00	0.29**	-0.10	0.43**	0.52**	0.63**	0.39**	-0.09
Urea									1.00	-0.13	0.49**	0.38**	0.16	0.05	0.09
Citric acid										1.00	0.24**	-0.08	-0.04	-0.06	-0.12
FDP											1.00	0.11	0.30**	0.28**	-0.14
FFA												1.00	0.14	0.18*	0.10
Density													1.00	0.12	-0.20*
Acidity														1.00	0.21*
log SCC															1.00

*P<0.05; **P<0.01, Snf – solids-non-fat; FDP – freezing point.

et al. [2008] demonstrated that the FFA content in milk may constitute an indicator of balancing of the cows' diets, of the strain caused by milk production, of bacterial pollution and of milk storage conditions.

The positive correlation between the FFA level in milk and day of lactation ($P \leq 0.01$, Tab. 3) shows that with the progress of lactation the degradation processes in the milk fat fraction are intensified. Studies by other authors confirm the results recorded here, indicating that milk obtained during the final stage of lactation is especially susceptible to lipolysis, as manifested by the high FFA level [Cartier and Chilliard 1990, Wiking *et al.* 2003a, Evers *et al.* 2004, Deeth *et al.* 2006].

The negative correlation between the FFA concentration in milk and the daily milk yield ($P \leq 0.01$) confirms the previously discussed correlation with the stage of lactation. The milk yield decrease observed during the subsequent stages of lactation is accompanied by an increased FFA level in milk. This indicates that during the final stages of lactation milk is more susceptible to lipolysis (Tab. 3). According to Cartier and Chilliard [1990], the end of lactation is one of the principal factors causing spontaneous lipolysis. This form of lipolysis occurs only shortly after milking and may be especially intensive on farms, in which cows calve seasonally.

The positive correlation demonstrated here between fat concentration in milk and the FFA level ($P \leq 0.01$) indicated that milk characterised by a high fat concentration is more susceptible to lipolysis when compared with milk with a low level of this component.

One of the reasons for the correlation between fat concentration in milk and the FFA level may lie in the limited amount of components necessary for the synthesis of the milk fat globule membrane. The insufficient amount of components necessary for the membrane synthesis may result in a greater number of damaged fat globules, which in turn leads to an increased FFA concentration in milk [Wikinga *et al.* 2003a]. Similar problems with a lack of components for the production of fat globule membranes may occur in cows after calving in the case of a negative energy balance [Hanus *et al.* 2008].

The degradation processes in the milk fat fraction are caused among others by bacteria infecting milk during subsequent operations related to milking and storage. Those bacteria cause milk fat lipolysis and protein proteolysis, because they secrete enzymes participating in the degradation of the lipid and protein fractions in milk. As a result of the activity of enzymes one may observe a decrease in the concentration of casein α, casein-β and lipids, as well as an increase in the contents of casein-κ, proteozo-peptons and the FFA concentration in milk [Spitsberg 2005]. This is confirmed by the positive correlation observed in the present studies between the contents of total protein ($P \leq 0.01$) and casein ($P \leq 0.01$) and the FFA concentration in milk. Apart from enzymes secreted by micro-flora in milk one may find native enzymes which, similarly to bacterial ones, participate in the degradation of milk fat and protein [Antonelli *et al.* 2002, Deetha 2006, Martins *et al.* 2006, Kontkanen *et al.* 2011].

The high positive correlations between the basic milk parameters (with the exception of lactose) and the FFA concentration resulted in a similar correlation recorded between the FFA and milk solids ($P \leq 0.01$).

In turn, the high correlation between FFA and the basic milk components leads to a similar correlation between the FFA concentration and the content od non-fat milk solids, because the level of non-fat milk solids is determined principally by the concentrations of total protein and casein. Similar relationships between total and non-fat milk solids and the components of the protein fraction were also reported by Hanus *et al.* [2008].

The results obtained here indicate that the fat lipolysis of cows' milk is determined both by the somatic cell count and by the polymorphic form of β4-defensin.

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