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THE EFFECTS OF *LTF* AND *TNFα* GENES ON MILK PRODUCTION ARE DEPENDENT ON LACTATION STATE

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Abstract. So far, a lot of studies have focused on the genetic background of bovine milk production traits and applied heritability models which assumed that the effects of individual genes add up in a simple way and remain unchanged throughout a cow's life, independently of external and internal factors. The aim of this research study was to verify this assumption by checking if the effects of individual alleles of *LTF* (substitution C/T in exon 3) and *TNFα* (substitution T/C in intron 6) are variable and depend on a cow's internal environment (physiological state). The genetic material used in the study was collected from black-and-white Holstein-Friesian cows kept in a single barn in Poland. Daily milk yield and percentage content of fat, protein and lactose were examined once a month on the basis of test-day milkings. The study results show that the effects of individual *LTF* and *TNFα* gene alleles vary throughout lactation, that is they depend on a cow's physiological state.

Key words: allele effect, mastitis, milk composition, cattle.

INTRODUCTION

Average milk production per cow is growing consistently (Wolf 2003). In response to the changing needs of consumers and producers, cow breeders have concentrated their efforts on increasing milk yield and improving milk quality traits (fat, protein and lactose content), which are now commonly considered for genetic selection in dairy cattle. To improve the effectiveness of breeding work, numerous research studies are carried out into the genetic background of economically crucial production and performance traits in dairy cattle (Miglior et al. 2017). The main focus is on identifying genetic markers for these traits. In our research, the genes coding for tumor necrosis factor-α (*TNFα*) and lactoferrin (*LTF*) were selected as potential markers for *mastitis* immunity due to the complex functions they play. The aim of the research was to examine the possible variable effects of the alleles of these genes depending on the day in milk (DIM).

TNFα is a multifunctional cytokine which e.g. stimulates the proliferation, differentiation and activity of a number of immune cells, including in particular lymphocytes B, lymphocytes T, natural killer (NK) cells and lymphokine-activated killer (LAK) cells, and regulates

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lipid metabolism. The effects of TNFα on lipid metabolism include inhibition of free fatty acids uptake, promotion and induction of lipolysis, reduction of the activity of enzymes involved in lipid metabolism, and regulation of cholesterol metabolism. *TNFα* gene was mapped to bovine chromosome 23 and is located in locus 23q22 within the major histocompatibility complex gene cluster *BoLA20*. The gene consists of four exons and three introns (Chen et al. 2009; Wojdak-Maksymiec et al. 2013; Kochnev et al. 2015). Kochnev et al. (2015) reported associations between a polymorphism of *TNFα* gene and milk production traits, and Muhaghegh-Dolatabady and Rezaei (2018) found a statistically significant link between different haplotypes of *TNFα* gene fragment and average daily milk production.

LTF is a milk whey protein with important physiological and biological functions. It has antimicrobial properties and due to its ability to bind iron, phosphorus and zinc ions, LTF makes these elements unavailable to pathogens. This protein is coded for by *LTF* gene located on bovine chromosome 22q24 and composed of 17 exons. *LTF* gene expression is regulated in different ways, e.g. by steroid hormones, the growth hormone, or the kinase cascade pathway (Teng 2002; Jenssen and Hancoock 2009). A study by Nanaei et al. (2016) showed statistically significant differences in milk fat content across cows with different *LTF* gene variants.

Most association studies only contain simple additive models which do not fully reflect the heritability mechanisms actually found in nature. The accuracy of estimates of an individual's breeding value can be improved by including non-additive effects in the heritability model. Non-additive gene effects result from interactions between genes in the same locus (dominance) or in different loci (epistasis). Research shows that inclusion in the model of both additive and non-additive effects increases the accuracy of breeding value prediction even by 17%. What is more, gene effects vary depending on the environment and internal factors such as parity and lactation stage (Yang et al. 2006; Su et al. 2012). This research focused on analysing the variable effects of selected genes depending on DIM.

MATERIAL AND METHODS

Research material

The research was carried out on 1,025 cows kept on a farm in the north-western part of Poland. All the animals were of the same breed (Holstein-Friesian, black and white variety) and belonged to one herd. The cows were kept in similar conditions in the same free-range barn. They had ad libitum access to water from individual automatic drinking vessels and were fed throughout the year according to the TMR (total mixed ratio) system. In addition, they received specially, individually selected rations with the addition of concentrated feeds. The animals were milked in a fish-bone parlour. Daily milk yield and percentage content of fat, protein and lactose were examined once a month on the basis of test-day milkings performed during first lactation.

Laboratory methods

DNA isolation was performed in a Zymo-Spin™ IC Fast-Spin column, using a ZymoResearch Genomic DNA Kit™ (ZymoResearch, USA). Two substitution polymorphisms were analysed: *TNFα* C/T in exon 3 and *LTF* T/C in intron 6. The analysis of *TNFα* polymorphism was performed using real-time PCR with a SimpleProbe, and *LTF* polymorphisms were identified using asymmetric real-time PCR. The latter method involves increasing the amount of primer that is on the same strand as the molecular probe while reducing the amount of molecular probe.

Real-time PCR reactions were carried out using a LightCycler 2.0 instrument (Roche Molecular Systems Inc., Pleasanton, USA). The results were analysed by real-time fluorescence reading. A melting curve analysis enabled detection of mutations and description of the product. A Qiagen® Multiplex PCR Kit (Qiagen GmbH, Hilden, Germany) containing a mixture of Q-solution – a strong coagulant that allows better denaturation of the template DNA and thus eliminates the formation of secondary structures due to primers – was used for amplification. The reagents used in the laboratory analyses were manufactured by Fermentas (Fermentas International INC, Burlington, Canada) except for the primers, which were manufactured by Proligo (Proligo France SAS). PCRs were carried out in thermal cyclers by Whatman Biometra (Whatman Biometra GmbH, Göttingen, Germany). The resultant restriction fragments were separated on agarose gel stained with ethidium bromide and visualized in UV light using an electrophoresis gel documentation and imaging system by Vilber Lourmat (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany).

Statistical analysis

The SNPs effects throughout lactation period were estimated on the basis of the model described by Yang et al. (2006) and taking into account the random additive polygenic effect and additional fixed effects:

$$y_{jklm} = \mu + td_m + c\alpha_l + \sum_{i=0}^{3} \varphi_{ikm}^{cs} cs_{ik} + \sum_{i=0}^{n\,LTF} \varphi_{im}^{LTF} LTF_i + \sum_{i=0}^{n\,TNF} \varphi_{im}^{TNF} TNF_i + \sum_{i=0}^{2} \varphi_{ijm}^{\alpha} \alpha_{ij} + \sum_{i=0}^{2} \varphi_{ijm}^{p} p_{ij} + e_{iklm}$$

where:

y_{jklm} – the traits phenotypic value of cow *j* (milk yield, protein, fat and lactose content) at *m* – milking day, *l* – age at calving and *k* – calving season,

μ – the population mean of analyzed trait,

td_m – the fixed *effect* of the *m*-th day in milking,

ca_l – cow's calving age grouped into four classes (less than 700 days, between 700 and 800 days, *between* 801 and 900 days, and older than 900 days),

cs_{ik} – *i*-th fixed *regression* coefficient for the two calving season class: the cold (October–April) and warm (May–September),

LTF_i, TNF_i – regression coefficients for the SNPs additive effects, specified as a half of the difference *between* the values of the two homozygous genotypes and establish as (−1) or (1) for the two *opposed* homozygotes and (0) for the heterozygote,

α~N(0,IÄA) – the cow random polygenic additive effect, and where I is the matrix of identity and A is the matrix of additive polygenic relationship coefficients between cows,

p~N(0,IÄI) – the random permanent environmental effect,

φ_* – the covariate *corresponding* to the day of milking.

In the model, the variance components corresponding to random effects were not estimated because it were assumed to be known.

For each effect, the coefficients of the Legendre polynomial (φi) were defined as follows:

$$\varphi_0 = 1\varphi_1, \varphi_1 = \tau, \varphi_2 = \frac{1}{2}(3\tau^2 - 1), \varphi_3 = \frac{1}{2}(5\tau^2 - 3), \varphi_4 = \frac{1}{3}(34\tau^4 - 10\tau^2 + 1)$$

where τ corresponding with standardised number of days in milking (*d*):

$$\tau = \frac{2(d-1)}{305-1} - 1$$

respectively to each test day. All effects not connected with analyzed genes were estimated with a predefined order of the polynomial, whereas for the SNP effects the best order of fit was determined on the basis of comparison of the fit of models with polynomial orders (maximally 5) by means of the likelihood ratio test:

a) at the beginning was fitted the full model with all SNPs using the 5th order polynomial;
b) if model a. provided a significantly better fit than the most parsimonious model without SNP effects, then, reduced models for each analyzed SNP were fitted with 5th order polynomials, in this model the other SNPs were omitted;
c) finally, for those SNPs which appeared to be significant for the description of a trait variation, various combinations with different orders of polynomial were fitted to determine the most parsimonious model which provides a fit not worse than the full model with 15 polynomial coefficients;
d) the sequence of likelihood ratio tests which allowed to evaluate the models with and without the SNP effect was used to indicate the most effective SNP effect parameterisation; since five tests were tried for each SNP, the nominal P values were adjusted for multiple testing with the Bonferroni approach and a significantly better fit at a 5% level.

RESULTS

The research results are presented in Figs. 1–4. Only those results which showed a statistically significant association between a gene effect and DIM are included. Results which were not confirmed statistically are not included in the graphs. All the presented results refer to the allele with a higher frequency in the studied population, that is allele T in the case of *TNFα* and allele C in the case of *LTF*.

Fig. 1 presents the variable effect of *TNFα* allele T on daily milk yield throughout lactation. The results show that from the start of lactation to day 20, *TNFα* allele T had an unfavourable effect on milk yield. However, from lactation day 21 to 86, the effect of allele T on milk yield was favourable. The peak value of allele T effect in this period was observed at day 48 (+4.5296 kg). After lactation day 86 up to day 214, the effect of allele T was unfavourable, adversely affecting milk yield. The lowest value of allele T effect was observed at lactation day 149, when it amounted to −7.5148 kg. From day 21 until day 270, the effect of allele T demonstrated favourable values for milk yield. The highest value for this period was recorded at day 246 (+3.2803 kg). Between days 270–305, the effect of allele T took values that were unfavourable for milk yield. This trait reached its peak at the beginning of lactation, starting to fall at day 48 (about 7 weeks) after calving. The lowest milk yield was recorded on the last day of lactation (Fig. 1). No statistically significant association was found between daily milk yield and the genetic variant of the *LTF* gene.

Fig. 2 shows the variable effects of *LTF* allele C and *TNFα* allele T on milk fat content throughout lactation (DIM). As for fat content, the effect of *LTF* gene allele C had an unfavourable effect on this trait from the beginning of lactation to day 134. Afterwards, between days 135–237, the effect of allele C had favourable values for fat content. The highest value in this period was recorded at day 175 (+0.0103%). From lactation day 238 to 305, allele C had an adverse effect on fat content. Throughout the lactation period, the effect of *TNFα* allele T took positive values that were favourable for fat content. From day 1 to day 146, the value of allele T effect kept falling, and the lowest values were recorded between 146–153 days of lactation (+0.0213%). After day 153, the effect of allele T gradually increased, reaching its peak value at day 305 (+0.1064%).

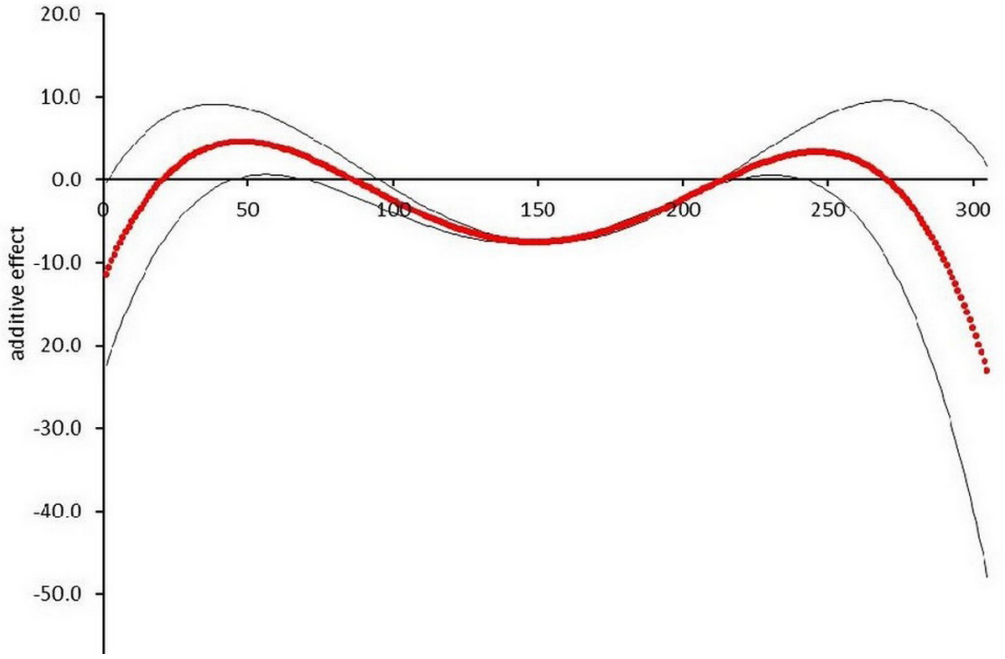

Fig. 1. Variable effect of *TNFα* on daily milk yield during lactation. Red curve – effect of *TNFα* allele T modelled by the 5th degree polynomial with 95% confidence intervals (black curves)

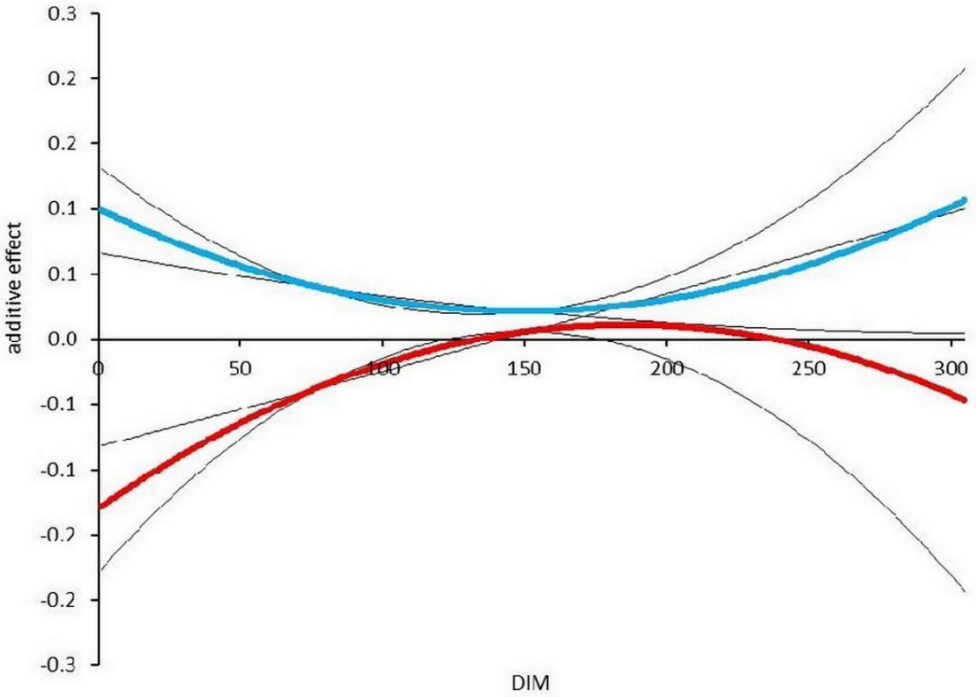

Fig. 2. Variable effect of *LTF* and *TNFα* on fat content during lactation. Red curve – effect of *LTF* allele C; blue curve – effect of *TNFα* allele T; both modelled by the 3rd degree polynomial with 95% confidence intervals (black curves)

Fig. 3 presents the variable effects of *LTF* allele C and *TNFα* allele T on milk protein content. As far as the effect of *LTF* allele C is concerned, it showed values that were favourable for milk protein content from the beginning of lactation to day 11. Between days 12–91, the effect of allele C had unfavourable values for this trait, with the lowest value in this period observed at days 43, 44 and 45 (–0.0329%). From day 92 to 224, the effect of allele C took favourable values for protein content, with the highest value recorded at day 155 (+0.0395%). Between lactation day 225 and 275, the effect of allele C had unfavourable values for protein content, with the lowest value in this period recorded from day 252 to 254 (–0.0130%). After day 275 until the end of lactation, the effect of allele C had favourable values.

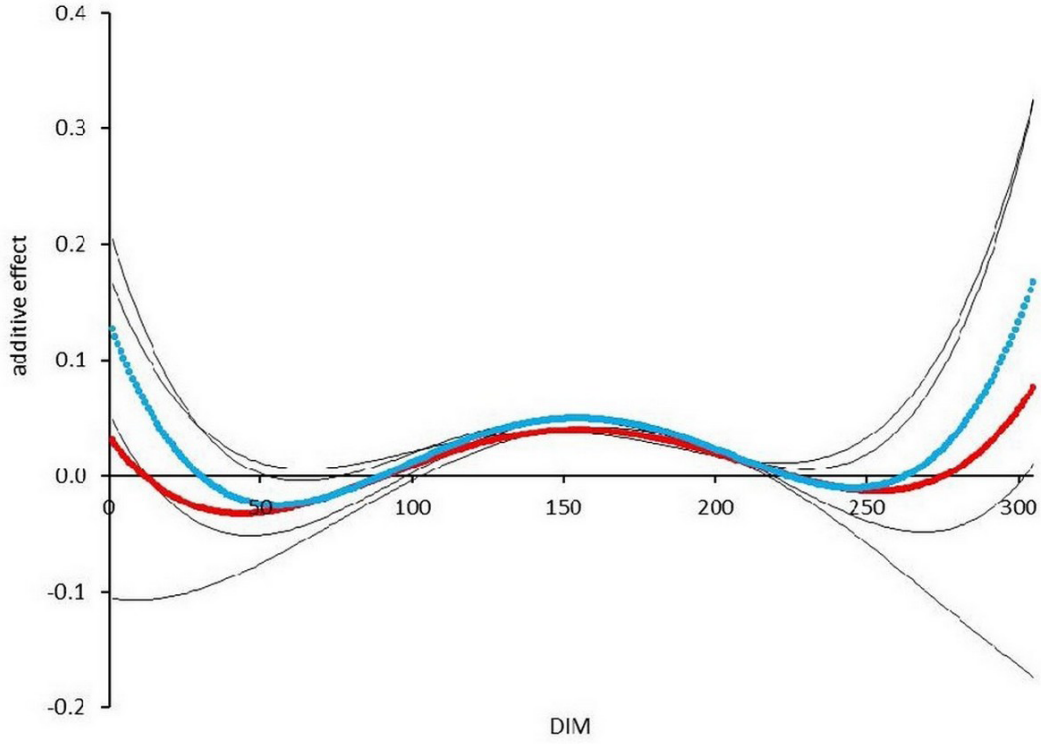

Fig. 3. Variable effect of *LTF* and *TNFα* alleles on protein content during lactation. Effect of *LTF* allele C – red curve; effect of *TNFα* allele T – blue curve; modelled by the 5th degree polynomial with 95% confidence intervals (black curves)

As can be seen in Fig. 3, in the case of *TNFα* gene allele T, its effect had values that were favourable for protein content from the beginning of lactation to day 30. At lactation day 31, the effect of allele T showed values that were unfavourable for protein content, with the lowest value in this period recorded at days 55, 56 and 57 (–0.0255%). Between days 91–223, the effect of allele T took values favourable for protein content, with the highest value recorded between days 153–156 (+0.0497%). From day 224 to 267, the effect of allele T took values that were unfavorable for protein content, with the lowest value in this period recorded between days 244–247 (–0.0105%). From day 267 till the end of lactation, the effect of allele T had favourable values.

Fig. 4 shows the variable effect of *TNFα* allele T on lactose content. From day 1 to day 19 of lactation, this trait was adversely affected by the effect of *TNFα* allele T. From day 20 to 95, the effect of this allele was favourable for lactose content, with the highest value observed

at day 50 (+2.1641%). From day 95 until day 227, the effect of allele T was unfavourable, adversely affecting lactose content. The lowest value of allele T effect was recorded at lactation day 158 (–2.7171%). Starting from day 228 till day 273, the effect of allele T took values that were favourable for lactose content. The highest value for this period was recorded at day 254 (+0.7288%). Between days 274–305, the effect of allele T showed values that were unfavorable for lactose content. The highest lactose content was recorded at the beginning of lactation and the trait reached its peak value at day 48 (about 7 weeks) after calving. The lowest lactose content was recorded on the last day of lactation.

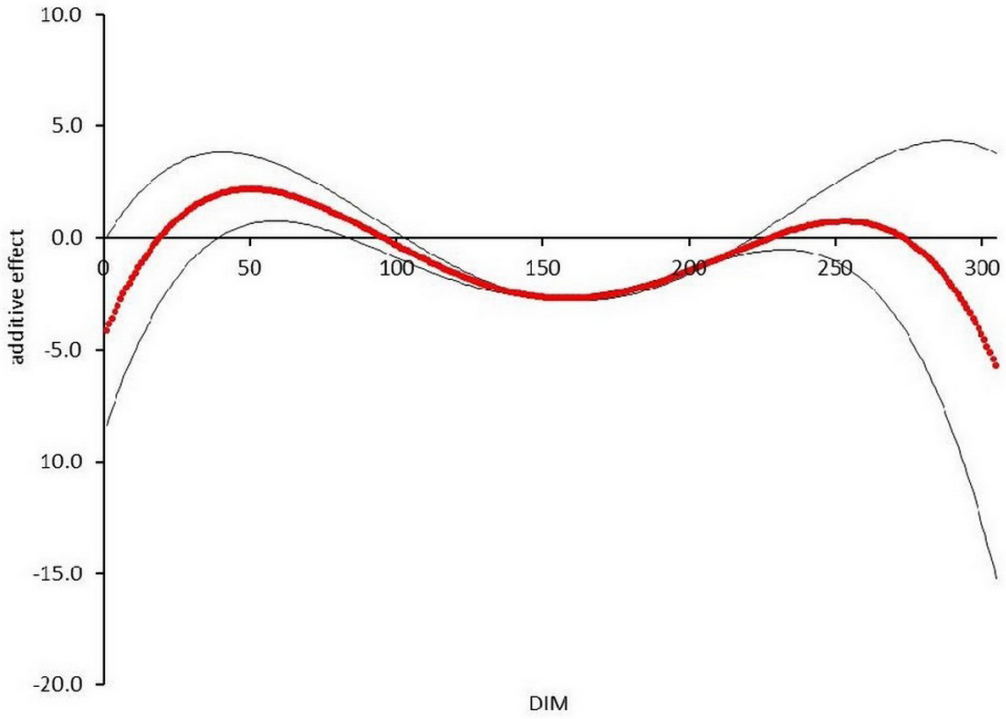

Fig. 4. Variable effect of *TNFα* on lactose content during lactation. Red curve – effect of *TNFα* allele T; modelled by the 5th degree polynomial with 95% confidence intervals (black curves)

No statistically significant association was found between lactose content and the genetic variant of *LTF* gene.

DISCUSSION

It is generally assumed in trait inheritance models that the effect of a gene allele has a permanent value. However, the results of this study show that the effect of alleles is not constant. It can be observed that, over the course of lactation, the effect varies depending on DIM. The variable effect of alleles should be included in statistical models due to its importance in assessing the usefulness of a genetic marker in dairy cattle selection programmes.

In our research, it was assumed that the effects of the selected alleles vary over the lactation period and this hypothesis has been confirmed. The recorded variable values might result from various interactions between genes (epistasis, pleiotropy, the complementing effect of genes, and epigenetic modifications). It is known that the value of the allele effect depends on numerous factors (e.g. hormone concentrations) (Su et al. 2012; Varona et al.

2018). In the case of our research, the variability of gene effects might result from interactions between the studied genes and the products of other genes, e.g. hormones, whose levels vary constantly during lactation and gestation. Especially bovine pregnancy is a highly dynamic process that results in rapid changes in the blood levels of hormones like estrogen and progesterone. These steroid hormones can modulate the response of the immune system by the presence of specific receptors in immune cells and can have an epistatic effect on the *LTF* gene. As LTF occurs in neutrophil granules, these hormones can regulate the functions of neutrophils e.g. by modulating *LTF* gene transcription (Kyurkchiev et al. 2011; Kovats 2015; Kadel i Kovats 2018). The activity of estrogen and progesterone is mediated by their specific hormonal receptors, which function as nuclear transcription factors. When the receptor binds to the appropriate hormone, it can bind to a specific cis-regulatory element acting in the gene promoter region and regulating the transcription of the gene. In the transcription regulation process, these receptors interact with specific cofactors to activate the transcription mechanism (DeMayo et al. 2009; Kyurkchiev et al. 2011; Kovats 2015).

The classic estrogen signalling pathway is mediated by the nuclear estrogen receptor alpha (ERα) and the nuclear estrogen receptor beta (ERβ). Progesterone signalling pathway is mediated by the progesterone receptor (PR), which has been found to have two isoforms, PRA and PRB. These isoforms are formed in the process of alternative mRNA splicing (DeMayo et al. 2009). Another research demonstrated that progesterone as a steroid hormone can reduce the production of mRNA and TNFα protein via an NF-κB-dependent mechanism. The NF-κB transcription factor is an important component of several signal transduction pathways, including those leading to TNFα synthesis (Miller and Hunt 1998).

The immunomodulatory activity of progesterone consists in direct or indirect inhibition of Th-1 cellular immune responses. Direct inhibition occurs on a receptor-dependent pathway through the participation of a progesterone-induced blocking factor (PIBF). PIBF reduces the activity of NK cells and as a result it decreases the production of TNFα (Szekeres-Bartho 2018). Thus, a low level of PIBF correlates with an increase in the concentration of cytokines like TNFα. Indirect inhibition consists in the induction of synthesis of placental protein 14 (PP14), which inhibits T and NK cells activity, and reduction of mitogenic reactivity of lymphocytes (Teng et al. 2002b; Ochanuna et al. 2010; Szekeres-Bartho 2018). Teng et al. (2002b) proved that progesterone inhibits *LTF* gene expression while estrogen induces it.

In T cells of pregnant patients with multiple sclerosis, estriol (E3) has been shown to stimulate the secretion of IL-10 and inhibit TNFα due to inhibition of the nuclear factor κB (NF-κB), which controls many immunity-related genes (Zang et al. 2002). In other studies, estrogen has been shown to inhibit the production of many cytokines, e.g. TNFα. Inhibition of cytokine production by estrogen has been associated with a decreased activity of NF-κB (Arlıer et al. 2018).

Other studies indicate a relationship between estrogen and *LTF* gene expression (Hajibemani et al. 2012). An analysis of the *LTF* gene promoter sequence showed that there is an estrogen response element (ERE) within the promoter (Moriuchi and Moriuchi 2006). Walmer et al. (1992) examined the expression of mRNA and LTF protein during the natural estrous cycle under physiological conditions in mice. The study showed a positive correlation between estradiol (E2) serum concentration and the level of *LTF* gene mRNA expression. During proestrus, estradiol reached the highest values and thus an increase in the level of mRNA and LTF protein was observed. However, during estrus, both estradiol and progesterone levels were high. The level of LTF mRNA also increased, while the level of LTF protein decreased. Protein expression may have been made difficult due to high levels of proges-

terone or some other local factor (Walmer et al. 1992). Teng et al. (2002a) showed that the estrogen receptor (ERα) binds to the ERE region within the *LTF* gene. This study confirmed that estrogen affects the expression of the human *LTF* gene (Teng et al. 2002a). Kelver et al. (1996) conducted studies with the participation of women which showed that estradiol can regulate the level of LTF. Based on the studies quoted above, it can be presumed that progesterone and estrogen have an epistatic effect on the *LTF* and *TNFα* genes.

Another example of studies which indicate complex interactions between gene products are studies on prolactin, which regulates milk secretion during lactation and is also linked to the immune system. Due to the presence of specific receptors within the cells of the immune system (lymphocytes, granulocytes, T and B cells, monocytes, macrophages, NK cells, thymus epithelial cells), prolactin can modulate the immune response. It stimulates the secretion of TNF by dendritic cells, T cells, monocytes and macrophages (Borba et al. 2018). Green and Pastewka (1978) showed that prolactin regulates the expression of LTF in the mammary gland of mice, which might indicate that it has an epistatic effect on *LTF* gene. Other studies also confirm that prolactin affects *LTF* expression. Nakajima et al. (2008) conducted a study which showed that prolactin influences the expression of *LTF* in the JAK2-dependent mechanism. The study indicated that the promoter region of the bovine *LTF* gene may presumably contain a binding site for the signal transducer and activator of transcription 5 (STAT5), i.e. a further mediator of JAK2 activation in mammary epithelial cells.

Another hormone that can modulate the expression of the *LTF* gene is oxytocin (OXT). It mobilizes the immune defence potential and suppresses excessive pathogen responses from innate immunity (Ndiaye et al. 2008; Deing et al. 2013; Wang 2016). A study conducted by Thomas and Fell (1985) in dairy cows showed that OXT had an effect on citrate to LTF ratio in milk.

In the light of the foregoing, the impact of gestation stage and lactation on the effects of *TNFα* and *LTF* genes deserves a special attention because it is a dynamic process that results in rapid changes in the blood levels of numerous hormones. In addition, there are a lot of other hormonal factors whose levels vary depending on a cow's physiological state, which might influence *TNFα* and *LTF* gene expression and as a result affect the value of the allele effect.

CONCLUSIONS

The results of the present study indicate that the effects of allele C of the *LTF* gene and allele T of the *TNFα* gene on milk production traits are variable and depend on a cow's physiological state (lactation and gestation). The *TNFα* gene has been found to have a variable effect on daily milk yield, fat content, protein content, and lactose content, while the *LTF* gene has been demonstrated to have a variable effect on milk and lactose content. Our hypothesis assumed that the value of the analysed gene allele effects might presumably depend on interactions between these genes and others genes (for example genes encoded various hormones whose concentration levels change during lactation and gestation).The reason behind the differences in the association patterns (or the lack of such associations) between the studied genes and milk production traits might be the different mechanisms in which the analysed traits and the expression of the two genes are regulated, or different epistatic effects of other genes. Being a proinflammatory factor, *TNFα* is regulated differently from *LTF*, which might account for the differences. The findings of this study seem to be an interesting topic for further and more detailed research.

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EFEKT GENÓW *LTF* I *TNF*α W ODNIESIENIU DO SKŁADU MLEKA JEST ZMIENNY W RÓŻNYCH STADIACH LAKTACJI

Streszczenie. Dotychczas wiele badań skupiało się na genetycznym podłożu cech produkcyjnych mleka bydła i stosowano w nich modele odziedziczalności, które zakładały, że efekty poszczególnych genów sumują się w prosty sposób i pozostają niezmienione przez całe życie krowy, niezależnie od czynników zewnętrznych i wewnętrznych. Celem pracy było zweryfikowanie tego założenia poprzez sprawdzenie, czy działanie poszczególnych alleli genów *LTF* (podstawienie C/T w eksonie 3) i *TNF*α (podstawienie T/C w intronie 6) jest zmienne i zależne od środowiska wewnętrznego krowy (stan fizjologiczny). Materiał genetyczny wykorzystany w badaniach pobrano od czarno-białych krów rasy holsztyńsko-fryzyjskiej utrzymywanych w jednej oborze na terenie Polski. Raz w miesiącu na podstawie próbnych udojów badano dobową wydajność mleka oraz procentową zawartość tłuszczu, białka i laktozy. Wyniki badań pokazują, że działanie poszczególnych alleli genów *LTF* i *TNF*α jest zmienne w trakcie laktacji, czyli zależy od stanu fizjologicznego krowy.

Słowa kluczowe: efekt allelu, mastitis, skład mleka, bydło.