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RESEARCH ARTICLE

Interactions between *TNF-α*, *LTF* and *mLYZ* Gene Variants in Determining Somatic Cell Count in Jersey Cows

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ABSTRACT

The aim of the research was to establish if there are any statistically significant associations between the polymorphisms of the selected genes (*TNF-α*, *LTF*, *mLYZ*) and natural log-transformed SCC (LnSCC) and search for possible interactions between the genetic variants of the selected genes in determining various SCC values in milk. The study included 171 Jersey cows. Two alternative approaches were compared: the standard simple model accounting for the additive effect of a single *locus* only, and the full model including both additive and dominance effects of *TNF-α*, *LTF* and *mLYZ* as well as the interactions between them. The results obtained with the full model are different from those obtained with the standard model including only the additive effects of individual genes. The results presented above prove the existence of complex functional interactions between lactoferrin, lysozyme and tumor necrosis factor and suggest that the alleles of the genes that encode them might interact with each other too.

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INTRODUCTION

Mammary infections are one of the most serious problems in dairy cow farming. One method to reduce udder infection rate is to base the breeding process on individual differences in genetic susceptibility to mastitis. Therefore, it seems reasonable to employ marker assisted selection.

Tumor necrosis factor (TNF) is one of the main pro-inflammatory cytokines. Thanks to its pleiotropic properties, TNF activates the entire immune system (Parameswaran and Patial, 2010). It increases endothelium permeability, which facilitates diapedesis, i.e. the process of leukocyte extravasation. Then, TNF acts as a chemoattractant and induces chemotaxis, i.e. the migration of leukocytes (mainly neutrophils) towards the inflammatory focus (Bradley, 2008).

Lactoferrin (LTF) is a multifunctional protein with antimicrobial properties: it is active against many Gram-negative and Gram-positive bacteria (González-Chávez *et al*., 2009), enveloped and non-enveloped viruses, and various types of fungi and parasites (Jenssen and Hancock, 2009; Legrand and Mazurier, 2010). Due to its properties, lactoferrin is one of the major preventive factors against mastitis in dairy cows.

Lysozyme (LYZ) plays an antibacterial role primarily due to its destructive effect on the bacterial cell wall. Lysozyme's antibacterial effect is the strongest against Gram-positive bacteria (Steinstraesser *et al*., 2009). Gram-negative strains are less susceptible to lysozyme due to their cell walls having a more complex structure. The antimicrobial effect of lysozyme is also probably due to its non-enzymatic activity related to lysozyme's cationic and hydrophobic properties (Palumbo *et al*., 2010; Steinstraesser *et al*., 2011).

It is known that one multigene cluster within chromosome BTA5q23 contains 10 different bovine lysozyme genes. The structure of the immune-dependent bovine *mLYZ* gene (macrophage-expressed lysozyme gene) as well as its organization indicates that lysozyme found in milk is the product of this particular gene. In many populations a characteristic bimodal distribution of lysozyme activities has been observed. Pareek *et al*. (2003) found that en intron mutation in *mLYZ* gene and this SNP (single nucleotide polymorphism) mutation is segregated with high lytic activity.

In the case of resistance to mastitis as measured by SCC, it has been demonstrated that this trait varies with lactation number (i.e. SCC increases with each successive lactation); it is therefore possible that there are certain

interactions between the genotype and lactation perceived as an inner environment that changes over time. What is more, SCC is different at different stages of lactation, which implies that gene effects might also vary across the stages of lactation.

In a healthy udder, the concentrations of TNF, lactoferrin and lysozyme are very low but they may increase several times within a short time span as a result of infection (Schmitz *et al*., 2004). If the selected genotypes are significantly correlated with SCC in milk, then theoretically their effect should be particularly noticeable during an infection. It therefore seems advisable to investigate the associations between the selected genes and SCC separately in healthy cows and in infected ones (Urioste *et al*., 2010).

The aim of the research was to establish if there are any statistically significant associations between the polymorphisms of the selected genes (*TNF-α, LTF, mLYZ*) and immunity to mastitis expressed as natural log-transformed SCC (LnSCC), and to search for possible interactions between the genetic variants of the selected genes in determining various SCC values in milk in the population of cows under study.

MATERIALS AND METHODS

The study included 171 Jersey cows. The cows were milked in their stalls, watered *ad libitum* and fed standard rations. In the spring and summer, the animals were put out to pasture. The only animals selected for the analyses were those whose first three full lactations lasted 265−365 days. This procedure was chosen due to the fact that the final days of lactation, i.e. the drying-off period, is a critical moment as it is related to a higher incidence of mastitis and an increased SCC in milk. The cows were born of 14 different sires, the average number of daughters per one sire being 12.21. The data concerning SCC were derived from the results of monthly test milkings.

Laboratory methods: The polymorphism of the selected genes was identified by PCR-RFLP method using primers described by Higuchi *et al*. (1999) for *TNF-α*, Seyfert and Kühn (1994) for *LTF*, and Paarek *et al*. (2003) for *mLYZ*. Restriction analysis of the amplified fragment was performed with the RFLP method using *RsaI* enzyme for *TNF-α* gene, *Eco*RI for *LTF* gene and *Sau*3A I for *mLYZ* gene.

Statistical analysis: The laboratory results were then analyzed statistically. Computations were made separately for the 1st, 2nd and 3rd lactations, and for all the lactations combined together. Also examined were the effects of individual genes as variables in the lactation period. Therefore, separate estimations were applied to analyze data only from selected lactation stages. Three DIM (days in milk) ranges were used in the calculations: stage 6 – 21 days (I stage), 150 – 165 days (II stage), and the last test milking before the dry-off (III stage). Moreover, separate calculations were made for cows with SCC in milk within the broadly defined norm of ≤ 400,000/ml, and for cows with SCC > 400,000/ml.

Two alternative approaches were compared: approach I – the standard simple model accounting for the additive effect of a single *locus* only, and approach II – the full model including both additive and dominance effects of *TNF-α, LTF* and *mLYZ* as well as the interactions between them. In the latter case, the natural and orthogonal interactions model (NOIA) developed by Alvarez-Castro and Calbourg (2007) was used. As no orthogonal distribution of the genotypes investigated in this study was found in the herd in question, a statistical formula was applied at the first stage of the analysis. Afterwards, the effects of the genes were estimated according to a functional formula.

Functional estimators were denoted as: *a* – functional additive effect (effect of an allele in a homozygous set), and *d* – functional dominance effect (effect of an allele in a heterozygous set), whereas the symbols *aa*, *ad*, *da* and *dd* represent respective types of interactions between the alleles from different pairs in homo- and heterozygous sets.

To estimate the genetic effects of the examined *loci*, single-trait mixed multiple-regression models were applied. A general formula for the selected models is as follows:

$$Y = \mu + G + TD + BYS + DIM + A + PE + e$$

Where:
Y − matrix of observation;
μ − mean value of the analyzed trait in the population;
G_i − approach I: additive (*a*) effect of *TNF-α* (1) or *LTF* (2) or *mLYZ* (3) *loci*:

$$\sum_{i=1}^{1} G_i = a_1 \ (\text{or } a_2 \text{ or } a_3)$$

−approach II: additive (*a*), dominance (*d*) and epistatic (*aa*, *ad*, *da* and *dd*) effects between *TNF-α* (1), *LTF* (2) and *mLYZ* (3) *loci*:

$$\sum_{i=1}^{19} G_i = a_1 + a_2 + a_3 + d_1 + d_2 + d_3 + a_1a_2 + a_1d_2 + d_1a_2 + d_1d_2 +$$
$$+ a_1a_3 + a_1d_3 + +d_1a_3 + d_1d_3 + a_2a_3 + a_2d_3 + d_2a_3 + d_2d_3$$

TD − effect of test milking year/month;
BYS − effect of birth year/season;
DIM – effect of day in milk;
A − random additive polygenic effect;
PE − random permanent environmental effect;
e − random residue effect.

RESULTS

Allele and genotype frequencies: With the primers used, a 1233 bp long fragment of *TNF-α* gene was amplified, containing exons 2, 3 and 4.. In the case of allele B, this amplification product is cut with the restriction enzyme *Rsa*I into 928 bp and 305 bp long fragments while there is no restriction site for allele A (Fig. 1). Although the polymorphic site (a C/T transition) located in exon 4 corresponds to the third letter of the codon, no amino-acid substitution occurs (Higuchi *et al*., 1999). The alleles identified in the analysis resulted in three genotypes: AA, AB and BB. The allele frequencies were as follows: A – 0.447 and B – 0.553. The most frequent genotype in the

studied population was the heterozygous genotype AB (0.552) followed by genotype BB (0.227), while genotype AA was least frequent (0.171).

The amplified fragment of *LTF* gene is located on chromosome 2q24 (synteny group U12) within intron 6 and is 301 bp long. The polymorphism of this fragment is recognized by the restriction enzyme *Eco*RI. Allele A has no restriction site for this enzyme while allele B is cut by it into two fragments of 201 bp and 100 bp (Fig. 2). Allele A was found to be more frequent (0.627) than allele B (0.373) and the genotype frequencies were as follows: AA – 0.334, AB – 0.586, and BB – 0.080.

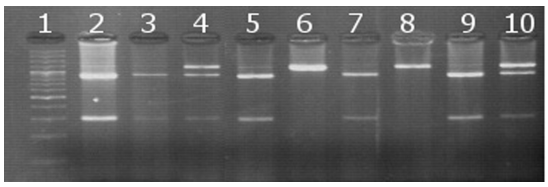

Fig. 1: Restriction fragment length polymorphism of *TNF-α* gene. Path 1 – GeneRuler™ 100 bp Plus DNA Ladder, 100-1500 bp; paths 6, 8 – AA genotype; paths 2, 3, 5, 7, 9 – BB genotype; paths 4, 10 – AB genotype.

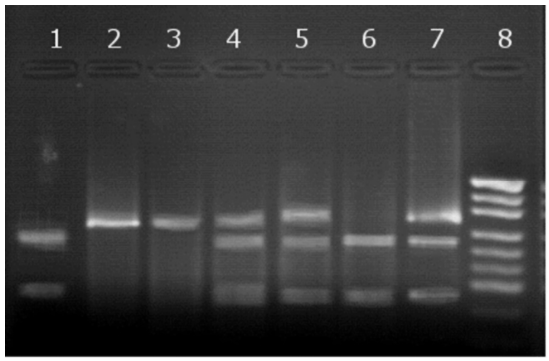

Fig. 2: Restriction fragment length polymorphism of *LTF* gene. Paths 1, 6 – BB genotype; path 2, 3 – AA genotype; paths 4, 5, 7 – AB genotype; path 8 – pUC 19/Msp I DNA Marker.

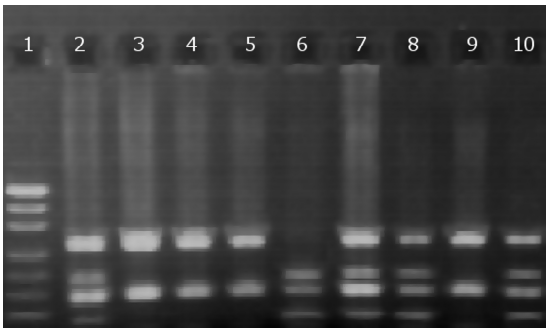

Fig. 3: Restriction fragment length polymorphism of *mLYZ* gene. Path 1 – pUC 19/Msp I DNA Marker; paths 3, 4, 5, 9 – CC genotype; path 6 – TT genotype; paths 2, 7, 8, 10 – CT genotype.

The primers complementary to the *mLYZ* gene fragments examined in this study make it possible to amplify a 395 bp sequence within intron 2 and exon 3. The polymorphic site recognized by enzyme *Sau*3AI (a C/T transition at position 8603) is located in the intron. Allele C is cut into two fragments: one is 260 bp long and the other is 135 bp long. In the case of allele T, the 260 bp sequence is further cut into two fragments of 171 bp and 89 bp (Pareek *et al*., 2003). In the population under study, allele C occurred with a frequency of 0.831 whereas allele T had a frequency of 0.169. There were more cows with genotype CC than those with genotypes CT and TT, the genotype frequencies being 0.720, 0.222 and 0.058, respectively.

Approach I: When investigating the associations between SCC in milk and *TNF-α* allele A, *LTF* allele A and *mLYZ* allele C, it was found that single effects of the genes under analysis were statistically non-significant both in the first three lactations and in the three selected stages of lactation as well as in the classes of SCC ≤ 400,000/ml and SCC > 400,000/ml. Only the effect of *mLYZ* allele C (equated 0.1699±0.0821) was significantly associated with a higher LnSCC in the third stage of lactation.

Approach II: The results obtained with the full model which accounted for both dominance and epistatic effects are different from those obtained with the standard model including only the additive effects of individual genes (Table 1 to 3).

The analysis showed a significant additive effect of *TNF-α* gene in lactations I and II, with allele A being associated with a lower SCC. The favorable effect of allele A was also observed in the third, i.e. last, stage of lactation, in test milkings with SCC over 400,000/ml, and in the whole herd under study. The results also indicate that allele A exhibits recessiveness as far as SCC is concerned. This effect was found to be statistically significant in lactations II and III as well as in the third stage of lactation and in the class of test milkings with SCC > 400,000/ml. Positive values of this effect reflect unfavorable deviations in SCC in the milk of heterozygous cows compared with the values expected in the case of no dominance or recessiveness.

The presented results show that *LTF* gene is involved in regulating SCC in milk, as well. There is a statistically significant association between allele A of this gene and a lower SCC in the last stage of lactation, in the class of test milkings with SCC < 400,000/ml, and in the group of all test milkings in total. However, *LTF* allele A did not have any statistically significant dominance effect.

The additive effect of *mLYZ* allele C was found to be statistically significant in the third lactation, in the first stage of lactation, in test milkings with SCC ≤ 400,000/ml, and in the entire test milkings analyzed jointly. This effect proved to be unfavorable as it was accompanied by a lower SCC.

It is symptomatic that there were practically no significant associations between the studied *loci* (except for *mLYZ* in the third stage of lactation) and SCC in the case of tests performed with a simple single-gene model. When analyzed, the effects of interactions between *TNF-α* and *LTF* genes showed mostly negative values and accordingly were reflected by an SCC that was lower than the expected one resulting from a simple additive gene action (in the case of no epistasis). Thus, combined *TNF-α/LTF* genotypes proved to be even more favorable than it might have been expected from their additive effects. The following statistically significant effects with the desirable negative values were found: following statistically

significant effects with the desirable negative values were found:
$\alpha\alpha$ – in the first and third stages of lactation and in test milkings with SCC exceeding 400,000/ml;
$d\alpha$ – in lactations I and III and in the class of test milkings with SCC over 400,000/ml;
αd – in lactations II and III and in test milkings with SCC over 400,000/ml;
dd – like for αd interactions: in lactations II and III and in test milkings with SCC over 400,000/ml.
Statistically significant epistatic effects were also detected between *TNF-α* and *mLYZ* genes in the following classes:
$\alpha\alpha$ – in lactations I and III and in the class of test milkings with SCC over 400,000/ml;
da – in lactation III, in the first and third stages of lactation, and in the class of test milkings with SCC below 400,000/ml;
αd – in lactation III and in test milkings with SCC over 400,000/ml.

The effects of *dd* interactions did not have any significant impact on the phenotypic values of the traits under study.

It is difficult to make an unequivocal interpretation of the epistatic effects of *TNF-α* and *mLYZ* genes as the effects of their interactions assumed both positive and negative values in different classes. Statistically significant interactions of the $\alpha\alpha$ type had positive values, thereby being unfavorably associated with SCC which was higher than expected in the case of no epistasis. On the other hand, $d\alpha$ and αd interactions had desirable negative values and were associated with a lower SCC in milk.

Finally, the following significant epistatic effects between *LTF* and *mLYZ* genes were observed:
$\alpha\alpha$ – in the first and third stages of lactation and in test milkings with SCC exceeding 400,000/ml;
αd – in the first stage of lactation and in test milkings with SCC up to 400,000/ml;
$d\alpha$ – in lactations I, II and III, in the class of test milkings with SCC below 400,000/ml, and in all test milkings combined together;
dd – in lactations I and III.

All the statistically significant interactions involving homozygous *LTF* genotypes AA (α_{LTF}) proved to be beneficial since they had negative values and were therefore associated with a lower SCC in milk. By contrast, the interactions between heterozygous *LTF* genotypes (α_{LTF}) and homozygous *mLYZ* genotypes (α_{mLYZ}) were deemed undesirable.

DISCUSSION

The lack of any significant effect of *TNF-α* gene on the somatic cell count in the milk of cows with SCC ≤ 400,000/ml in the present study may be explained by a very low expression of this gene in a healthy udder (Schmitz *et al*., 2004). The results can also explain why statistically significant effects of *TNF-α* gene and its interactions with other genes were observed in this study in test milkings with an elevated SCC probably resulting from the mammary gland inflammation. The present results might support the thesis that the associations between *TNF-α* and SCC in milk are of a functional nature since they do not occur when the gene is not expressed (Compton *et al.*, 2009).

What is more, Alluwaimi and Cullor (2002) found that the expression profiles for the cytokines they studied, including *TNF-α*, was significantly different in the middle and final stages of lactation, which was related to the dramatic changes taking place in the mammary gland. The findings cited above may explain the diversity of *TNF-α* gene effects in different stages of lactation observed in this study.

The absence of a statistically significant effect of *LTF* gene in the group of cows with SCC ≤ 400,000 in the present study may result from a very low expression of the gene in a healthy udder as compared with an infected one (Schmitz *et al*., 2004) and from a low concentration and activity of leukocytes, which are the main source of lactoferrin in an infected udder (Yen, 2011).

The products of the selected genes interact with each other in the non-specific phase of the immune response. The expressions of *TNF-α*, *LTF* and *LYZ* during the ongoing inflammatory process are precisely synchronized (Mitterhuemer *et al.*, 2010). An increase in the concentration of *TNF-α* mRNA is always followed by an increase in the concentration of *LTF* and *LYZ* mRNA. The highest expression of the genes encoding natural antibiotics occurs approximately 6 hours after pathogen infusion, that is 3 hours after the peak expression of *TNF-α* (Bruckmaier, 2005; Wellnitz *et al*., 2006).

TNF, as a major pro-inflammatory cytokine, stimulates neutrophil migration to the infection site, with neutrophils being the principal source of lactoferrin (Ito *et al*., 2010). They are also an important – though not the only one–source of lysozyme: it has been found that during infection *mLYZ* gene, that is the macrophage-expressed lysozyme encoding gene, is also active in neutrophils and mammary gland cells stimulated by TNF (Ernandez and Mayadas, 2009; Wiesner and Vilcinskas, 2010).

Moreover, a negative feedback can be observed between the products of the genes in question. Lactoferrin and lysozyme–besides having bacteriostatic and bactericidal properties are also characterized by their anti-inflammatory and antiallergic effects, and their presence inhibits the expression of *TNF-α* (Palumbo *et al*., 2010; Legrand and Mazurier, 2010).

It must be noted that lysozyme and lactoferrin have been shown to act in a close synergy. An *in vitro* study on the antimicrobial activity of lysozyme showed that its efficacy in destroying bacteria was markedly lower compared with an *in vivo* situation. This is because in a live tissue lysozyme interacts with various cofactors such as lactoferrin and the antibody – complement or hydrogen peroxide-ascorbic acid complexes. These cofactors damage the outer membranes of Gram-negative bacteria and facilitate the penetration of lysozyme into the more sensitive layers. Further research demonstrated that lysozyme itself has a bacteriostatic effect and it gains its bactericidal activity in combination with lactoferrin. It has also been demonstrated that the antibacterial properties of lysozyme are related to the presence of lactoferrin (García-Montoya *et al*., 2011).

Table 1: Additive and dominance effects of the genes under study and the effects of their interactions for the first three lactations with regard to LnSCC

Effects		Lactation I		Lactation II		Lactation III	
		Effect	SE	Effect	SE	Effect	SE
Additive and dominance effects of the alleles under analysis	$\alpha_{TNF\text{-}\alpha}$	-0.0877	0.0979	-0.2734	0.1020	-0.5433	0.1531
	$d_{TNF\text{-}\alpha}$	0.1602	0.1229	0.2073	0.1262	0.3165	0.1824
	α_{LTF}	-0.0277	0.1260	-0.0385	0.1327	-0.0626	0.0862
	d_{LTF}	-0.0079	0.1387	-0.0681	0.1500	-0.0559	0.2069
	α_{mLYZ}	0.0170	0.0788	0.0277	0.0898	0.7593	0.2336
	d_{mLYZ}	-0.0241	0.0780	-0.1992	0.0859	-0.3577	0.1756
TNF-α x *LTF* interactions	$\alpha_{TNF\text{-}\alpha}\ \alpha_{LTF}$	0.0093	0.1171	-0.0619	0.1382	0.0511	0.0922
	$d_{TNF\text{-}\alpha}\ \alpha_{LTF}$	-0.2098	0.1348	-0.3470	0.1568	-0.3098	0.1223
	$\alpha_{TNF\text{-}\alpha}\ d_{LTF}$	-0.2605	0.1660	-0.5574	0.1933	-0.4147	0.1295
	$d_{TNF\text{-}\alpha}\ d_{LTF}$	-0.0529	0.1861	-0.4217	0.2127	-0.0285	0.1766
TNF-α x *Mlyz* interactions	$A_{TNF\text{-}\alpha}\ \alpha_{mLYZ}$	0.3324	0.2681	0.2310	0.2128	0.7400	0.4649
	$d_{TNF\text{-}\alpha}\ \alpha_{mLYZ}$	-0.4366	0.2898	-0.3547	0.2429	-0.6758	0.5025
	$\alpha_{TNF\text{-}\alpha}\ d_{mLYZ}$	-0.4723	0.2667	-0.0284	0.2138	-0.8441	0.3881
	$d_{TNF\text{-}\alpha}\ d_{mLYZ}$	0.0763	0.3085	-0.0700	0.2641	0.1696	0.5421
LTF x *mLYZ* interactions	$\alpha_{LTF}\ \alpha_{mLYZ}$	-0.0364	0.2442	-0.2952	0.1636	-0.2354	0.1448
	$d_{LTF}\ \alpha_{mLYZ}$	0.3578	0.2864	0.2226	0.2387	0.0336	0.1849
	$\alpha_{LTF}\ d_{mLYZ}$	-0.4198	0.2608	-0.7643	0.2891	-0.8024	0.5088
	$d_{LTF}\ d_{mLYZ}$	0.4246	0.3115	-0.0290	0.2053	0.7320	0.4495

The effects in bold are significant at P≤0.05, and those additionally underlined are significant at P≤0.01.

Table 2: Additive and dominance effects of the genes under study and the effects of their interactions for three different stages of lactation with regard to LnSCC

Effect		Stage I		Stage II		Stage III	
		Effect	SE	Effect	SE	Effect	SE
Additive and dominance effects of the alleles under analysis	$\alpha_{TNF\text{-}\alpha}$	-0.0373	0.0480	0.0037	0.0452	-0.3300	0.1033
	$d_{TNF\text{-}\alpha}$	0.1003	0.0660	-0.0160	0.0638	+0.2464	0.1369
	α_{LTF}	0.0517	0.0630	-0.0245	0.0611	-0.2234	0.0982
	d_{LTF}	-0.1591	0.1136	-0.0499	0.1108	-0.0240	0.0826
	α_{mLYZ}	0.3054	0.0672	0.0389	0.0616	0.0244	0.1537
	d_{mLYZ}	-0.2204	0.1152	-0.1950	0.1026	-0.0549	0.0871
TNF-α x *LTF* interactions	$\alpha_{TNF\text{-}\alpha}\ \alpha_{LTF}$	-0.2516	0.0860	0.0964	0.0852	-0.3844	0.1106
	$d_{TNF\text{-}\alpha}\ \alpha_{LTF}$	0.1840	0.1347	-0.1063	-0.1319	-0.1403	0.0671
	$\alpha_{TNF\text{-}\alpha}\ d_{LTF}$	0.0694	0.1366	0.0769	-0.1401	-0.1715	0.0763
	$d_{TNF\text{-}\alpha}\ d_{LTF}$	-0.0335	0.2779	-0.1222	-0.2934	0.1125	0.0474
TNF-α x *mLYZ* interactions	$\alpha_{TNF\text{-}\alpha}\ \alpha_{mLYZ}$	0.0092	0.1039	0.0002	0.0956	0.0598	0.0973
	$d_{TNF\text{-}\alpha}\ \alpha_{mLYZ}$	-0.4318	0.2394	-0.2362	0.2231	-0.4748	0.0423
	$\alpha_{TNF\text{-}\alpha}\ d_{mLYZ}$	-0.0819	0.1332	-0.0032	0.1240	-0.1104	0.0747
	$d_{TNF\text{-}\alpha}\ d_{mLYZ}$	0.1649	0.1796	0.0545	0.1619	0.0413	0.0554
LTF x *mLYZ* interactions	$\alpha_{LTF}\ \alpha_{mLYZ}$	-0.3993	0.1222	-0.1037	0.1175	-0.0674	0.0955
	$d_{LTF}\ \alpha_{mLYZ}$	0.4350	0.2137	-0.2734	0.1964	-0.0800	0.0544
	$\alpha_{LTF}\ d_{mLYZ}$	-0.0723	0.2338	0.0544	0.2299	0.0932	0.0702
	$d_{LTF}\ d_{mLYZ}$	-0.1486	0.3518	-0.0739	0.3087	0.0643	0.0393

The effects in bold are significant at P≤0.05, and those additionally underlined are significant at P≤0.01.

Table 3: Additive and dominance effects of the genes under study and the effects of their interactions for classes of cows with SCC ≤ 400,000/ml and SCC > 400,000/ml and for a group of all cows in total with regard to LnSCC

Effect		SCC ≤ 400,000/ml		SCC > 400,000/ml		Total	
		Effect	SE	Effect	SE	Effect	SE
Additive and dominance effects of the alleles under analysis	$\alpha_{TNF\text{-}\alpha}$	0.0003	0.0268	-0.4251	0.2000	-0.3045	0.0302
	$d_{TNF\text{-}\alpha}$	0.0215	0.0367	0.3981	0.1396	0.1066	0.0427
	α_{LTF}	-0.1977	0.0346	-0.0150	0.1535	-0.2121	0.0407
	d_{LTF}	-0.0913	0.0608	-0.0938	0.0962	-0.0951	0.0752
	α_{mLYZ}	0.3937	0.0349	0.5154	0.1443	0.0007	0.0416
	d_{mLYZ}	-0.1713	0.0584	0.0422	-0.0405	-0.0637	0.0695
TNF-α x *LTF* interactions	$\alpha_{TNF\text{-}\alpha}\ \alpha_{LTF}$	-0.0150	0.1141	-0.2666	0.0494	-0.0242	0.0556
	$d_{TNF\text{-}\alpha}\ \alpha_{LTF}$	0.0057	0.0777	-0.2461	0.0791	0.1047	0.0897
	$\alpha_{TNF\text{-}\alpha}\ d_{LTF}$	0.0229	0.0796	-0.1847	0.0779	-0.0712	0.0929
	$d_{TNF\text{-}\alpha}\ d_{LTF}$	0.1202	0.0546	-0.3856	0.1569	-0.1393	0.1081
TNF-α x *mLYZ* interactions	$\alpha_{TNF\text{-}\alpha}\ \alpha_{mLYZ}$	0.3370	0.0922	0.1349	0.0547	-0.0172	0.0638
	$d_{TNF\text{-}\alpha}\ \alpha_{mLYZ}$	-0.1620	0.0507	-0.0554	0.0918	0.0024	0.1083
	$\alpha_{TNF\text{-}\alpha}\ d_{mLYZ}$	-0.1745	0.0711	-0.3174	0.0698	0.0044	0.0831
	$d_{TNF\text{-}\alpha}\ d_{mLYZ}$	-0.0616	0.0400	0.2232	0.1258	0.0073	0.1499
LTF x *mLYZ* interactions	$\alpha_{LTF}\ \alpha_{mLYZ}$	-0.0315	0.0641	-0.1319	0.0976	-0.0176	0.0790
	$d_{LTF}\ \alpha_{mLYZ}$	0.2342	0.0968	0.1484	0.0540	0.2035	0.1323
	$\alpha_{LTF}\ d_{mLYZ}$	-0.4380	0.1222	0.0503	0.0865	0.3341	0.1560
	$d_{LTF}\ d_{mLYZ}$	-0.1053	0.1709	0.0361	0.0479	0.0374	0.2113

The effects in bold are significant at P≤0.05, and those additionally underlined are significant at P≤0.01.

Conclusion: The results presented above prove the existence of complex functional interactions between lactoferrin, lysozyme and tumor necrosis factor and suggest that the alleles of the genes that encode them might interact with each other too. When conducting studies on associations between candidate genes and phenotypic traits, it is generally assumed that genes interact in a simple additive manner. However, no such assumption should be made *a priori* and dominant end epistatic allele effects should not be ruled out.

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