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Research article

Polymorphism of bovine lipocalin-2 gene and its impact on milk production traits and mastitis in Holstein Friesian cattle

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ABSTRACT

Background: Mastitis is one of the most serious diseases of dairy cattle, causing substantial financial losses. While predisposition to reduced somatic cell count in milk has been considered for in cattle breeding programs as the key indicator of udder health status, scientists are seeking genetic markers of innate immune response, which could be helpful in selecting cows with improved immunity to mastitis. Lipocalin-2 (LCN2) is a protein involved in the response of the immune system by eliminating iron ions which are necessary for the growth of pathogenic bacteria, so LCN2 may be considered as a natural bacteriostatic agent and could become a marker of infection.

Results: A total of five SNPs were identified in LCN2 gene (one in the promoter, three in exon 1, and one in intron 1). A single haplotype block was identified. The locus g.98793763G>C was found to have a significant impact on protein levels in milk, and alleles of this locus were identified to have a significant positive dominance effect on this trait. None of the four analysed loci had a statistically significant impact on the milk yield, fat levels in milk or the somatic cell score. LCN-2 gene had no significant impact on the incidence of mastitis in the cows.

Conclusions: Although the identified SNPs were not found to have any impact on the somatic cell count or the incidence of mastitis in cows, it seems that further research is necessary, covering a larger population of cattle, to confirm the association between lipocalin-2 and milk production traits and mastitis.

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1. Introduction

Lipocalin-2 (LCN2) is classified into lipocalins, a large group of small extracellular human, animal and plant proteins of about 18–20 kDa [1,2], which belongs to the calycin superfamily [3]. Animal lipocalins are a group of highly diversified proteins which have a similar tertiary structure and highly conserved SCR (short conserved region) motifs in their amino acid sequences [4]. Lipocalins can bind and transport small hydrophobic particles, and they are characterised by considerable functional diversity. They participate in the regulation of cellular ageing and differentiation processes, and in modelling the immune response [5]. Lipocalin-2 (LCN2, also known as neutrophil gelatinase-associated lipocalin, NGAL) can exist as a monomer or be joined by a covalent bond with metalloproteinase (MMP-9) in granules of neutrophils [6]. LCN2 can bind enterochelin, a high affinity siderophore [7]. The iron-binding capacity of LCN2 is highly important in the immune processes. During an infection, bacteria synthesise the siderophores that capture iron from the extracellular space (it is necessary to bacterial growth). This causes iron deficiency which prevents the activation of cells of the innate immune response. Siderophores are produced by nearly all bacteria, especially those most common, e.g. *Escherichia coli* or *Staphylococcus aureus* [8,9]. In response to the iron deficiency caused by the infection, the organism synthetises e.g. LCN2 which binds and eliminates siderophores, thus reducing the accessibility of iron to bacteria and inhibiting their growth. Therefore LCN2 acts as a natural bacteriostatic agent [10]. Clinical studies have shown that LCN2 level rapidly increases in mammals during acute bacterial infections and inflammatory conditions [11,12]. Bacteria such as *E. coli*, *S. aureus* or *Streptococcus agalactiae* are the most critical etiological factor for mastitis in dairy cattle herds [13]. For years, this disease has been the major cause of financial losses in the dairy cattle sector. The antibiotic therapies currently in use, especially for treating clinical mastitis, vary in their efficacy (which is related to the increase in bacterial resistance to antibiotics). The elimination of bacteria requires apart from an effective antibiotic, also a well-functioning defence mechanism in the

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mammary gland. Therefore immunity to mastitis has been included in cattle breeding programs and intense research is being conducted into new molecular markers which could support the breeding process aimed at reducing mastitis in dairy cattle herds. It appears that LCN2 could become such a marker on account of its significant role in the immune response to pathogens. The aim of this study was to identify a polymorphism in bovine LCN2 gene and to establish the relationship between the identified polymorphisms and milk production traits and mastitis in Holstein Friesian cattle.

2. Materials and methods

2.1. Animals and genomic DNA sampling

The test material comprised milk samples collected from 214 Polish black-and-white Holstein-Friesian cows from one of the factory cattle farms in south-western Poland. The cows originated from 103 bulls. They were kept in a free stall system, received a total mixed ration (TMR) and remained under constant veterinary supervision. Milk samples of 100 ml were collected from the cows into sterile containers in accordance with the ethical principles approved by the National Ethical Committee for Experiments on Animals at the Ministry of Science and Higher Education in Poland. DNA was isolated from the milk samples using the method described by Pokorska et al. [14]. The quantity and purity of the resulting DNA was evaluated based on UV absorption of the wavelengths 260 nm and 280 nm using NanoDrop 2000 (ThermoScientific) spectrophotometer.

2.2. SNP genotyping

Primers were designed based on the reference sequence in GenBank (Accession No. AC_000168.1) using Primer3plus software [15] to investigate the polymorphism in a 592-bp segment of the bovine lipocalin-2 gene, which included a fragment of the promoter, exon 1 and a fragment of intron 1 (primer sequences: F: 5′-ACCACCAACCATGGACAG-3′, R: 5′-AGGTTTGCTAAGAGCCTTCA-3′). The amplification was performed in 25 μl of the total reaction mixture which contained 100–120 ng of genomic DNA, 1 × PCR buffer with 200 μM of each dNTP, 0.7 μM of each primer, 1% DMSO and 0.04 U/μL of Phusion High-Fidelity DNA Polymerase (Thermo Scientific). The amplification reactions were performed in C-100 thermocycler (BioRad) in accordance with the following thermal cycle procedure: initial denaturation at 98°C for 90 s, 34 cycles of denaturation at 98°C for 10 s, annealing at 64°C for 20 s, elongation at 72°C for 16 s, and final elongation at 72°C for 7 min. To investigate the *LCN2* polymorphism, the PCR products was sequenced. First, the PCR products were electrophoresed by agarose gel electrophoresis (2%, Sigma) with pUC19/MspI DNA Ladder (Thermo Scientific) and stained with ethidium bromide (Sigma). Next, the PCR products were purified enzymatically using EPPiC (A&A Biotechnology) in accordance with the manufacturer's protocol. The purified PCR products were sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the same primers as for the PCR reaction (separately for each primer). The thermal profile of the PCR sequencing reaction included denaturation at 96°C for 10 min, 25 cycles of 10 s each, denaturation at 96°C/5 s, annealing at 55°C/4 min, elongation at 60°C, and a hold stage at 4°C. The products of the sequencing process were purified using BigDye XTerminator™ Purification Kit (Applied Biosystems) in accordance with the attached protocol. The sequences were then read by capillary electrophoresis using 3500xl Genetic Analyser (Applied Biosystems) and POP-7 polymer (Applied Biosystems). All the results were analysed using Variant Reporter Software 2 (Applied Biosystems), with reference to AC_000168.1, *Bos taurus* breed Hereford chromosome 11, Bos_taurus_UMD_3.1.1, whole genome shotgun sequence. The SNP location was identified based on the reference sequence. We also used BioEdit Sequence Alignment Editor [16].

2.3. Data collection and statistical analyses

Statistical calculations were performed based on clinical mastitis reports which had been obtained from a veterinary doctor attending the cow herd, as well as using the information contained in the records kept by the farm. The data on milk performance (daily milk yield, fat, protein and lactose levels, and somatic cell count, SCC) of the analysed cows were acquired based on monthly milk yield reports compiled in accordance with the recommendations of the International Committee for Animal Recording (ICAR).

The frequencies of alleles and genotypes were calculated in accordance with the guidelines developed by Frankham [17]. The level of conformity with the Hardy–Weinberg principle was assessed for all the analysed loci using the calculator proposed by Rodriguez et al. [18]. The level of genetic diversity of the population was characterised using the following parameters: effective allele numbers (Ne), gene heterozygosity (He) and polymorphic information content (PIC) [19]. To determine whether the analysed segment of the LCN2 gene contained haplotypes formed by SNP variants, Haploview 4.2 software package [20] was used. The frequency of the resulting haplotypes and the linkage disequilibrium (LD) level, given by the parameters D′ (so-called standardised disequilibrium coefficient) and r^2, were calculated using the same software.

The association between genotypes and production traits of cows (averaged over the first three lactation periods), such as average daily milk yield, average fat, protein and lactose levels in milk, and average somatic cell count in milk, was assessed using the Proc Mixed procedure, SAS® 9.4 software, with Scheffe's test (SAS Institute, Cary NC, USA), based on the following model:

$$Y_{ij} = \mu + a_i + b_j\, \varepsilon_{ijk}$$

where: Y_{ij} — milk traits (average daily yield, average fat/protein/lactose levels, average somatic cell score (SCS); μ — overall mean; a_i — fixed effect of the ith single SNP genotype of LCN2,b_j — bull's effect, ε_{ijk} — random error.

To normalise the distribution of somatic cell count (SCC) in milk, the GLM model included the somatic cell score — a log-transformed somatic cell count in milk [21]:

$$SCS = \log_2(SCC/100,000) + 3$$

The dominance and the additive effects of the identified SNPs on the milk traits (average daily yield, average fat/protein/lactose levels, average SCS) were analysed using the PROC GLM procedure, SAS® 9.4 software, in accordance with the following model:

$$Y_i = \mu + b_1 A_1 + b_2 D_2 + \varepsilon_i$$

where: μ — overall mean, b_1 and b_2 — additive and dominance regression coefficients, A_1 — additive effect, D2 — dominance effect, εi — random error.

To determine the impact of the single SNPs identified in the LCN2 gene on the incidence of mastitis in cows (as a marker of susceptibility/immunity to mastitis), a logistic regression model (PROC LOGISTIC, SAS Institute, version 9.4) was used. This model included two classes of cows according to the incidence of clinical mastitis: class I ('resistant to mastitis' – no mastitis or only a single episode of mastitis observed during the first 3 lactation periods) and class II ('susceptible to mastitis' – more than 2 episodes of clinical mastitis observed during the first 3 lactation periods).

Table 1
+ SNP polymorphisms of bovine LCN2 gene identified in the study.

Locus	Region of the gene	Polymorphism	Type of mutation	SNP references number[a]
g.98793626	Promoter	A>G	Transition	rs 436,227,463
g.98793763	Exon 1	G>C	Synonymous	rs 133,552,604
g.98793812	Exon 1	C>T	Synonymous	rs 135,269,094
g.98793889	Exon 1	G>A	Missense, change of arginine (AGG) into lysine (AAG)	rs 132,900,291
g.98793951	Intron 1	G>A	Transition	rs 383,878,049

[a] The numbers are derived from the data in the database Ensembl.

The clinical mastitis model was defined as follows:

$$\text{logit P} = \ln\frac{P}{(1-P)} = b_0 + b_1 L$$

where: P = p(Y = 1); Y – occurrence of disease denoted as 1 – zero episodes or a single episode of clinical mastitis and 2 – at least two episodes of clinical mastitis during the first three lactation periods; b_0 – intercept; b_1 – regression coefficients of the disease occurrence on fixed effects; L – individual LCN-2 SNP genotype.

3. Results

3.1. SNP identification

A total of five SNPs were identified in the analysed cattle herd, one in the promoter (g.98793626A>G), three in exon 1 (g.98793763G>C, g.98793812C>T, g.98793889G>A), and one in intron 1 (g.98793951G>A) of the LCN2 gene (Table 1). It was found that one of the three SNPs identified in exon 1 (g.98793889G>A) resulted in a change of the amino acid at position 36 of the LCN2 protein (Table 1). Two of the three possible genotypes (G/G and G/A) were observed for the SNP g.98793951G>A, and all the possible genotypes were identified for the other SNPs (Table 2). Additionally, the locus g.98793951G>A was least polymorphic as the majority of cows had the GG (212) genotype in this locus, and therefore the locus was not taken into account in the subsequent statistical analysis. The locus g.98793763G>C was determined to be most genetically diversified as evidenced by the highest values of such parameters as effective allele numbers, gene heterozygosity and polymorphic information content. Additionally, based on χ^2 test results, the herd was found to have reached genetic equilibrium with respect to all the identified loci of SNPs, except the locus g.98793763G > C. This shows that the LCN2 gene is hardly susceptible to selective pressure which is characteristic of Holstein-Friesian cattle subjected to continuous genetic improvement oriented towards achieving the highest milk yield.

Table 2
The frequency of alleles and genotypes and genetic diversity parameters of genotyped SNPs within the bovine LCN2 gene.

Loci	Genotype frequencies			MAF	χ^2 (HWE)	Genetic diversity parameters		
						He	Ne	PIC
g.98793626A>G	AA	AG	GG	G	0.02	0.365	1.575	0.298
	0.575	0.369	0.056	0.241				
g.98793763G>C	GG	GC	CC	C	**8.81***	0.482	1.923	0.365
	0.407	0.383	0.210	0.404				
g.98793812C>T	CC	CT	TT	T	0.02	0.365	1.575	0.298
	0.575	0.369	0.056	0.241				
g.98793889G>A	GG	GA	AA	A	0.23	0.348	1.522	0.284
	0.607	0.337	0.056	0.224				
g.98793951G>A	GG	GA	AA	A	0.00	0.009	1.010	0.010
	0.991	0.009	0.000	0.005				

MAF – minor allele frequency, HWE – Hardy–Weinberg equilibrium, He – gene heterozygosity; Ne – effective allele numbers; PIC – polymorphism information content. The bold numbers indicate significant differences between genotypes.
* $P < 0{,}05$.

3.2. Haplotype analyses

Based on the information on the identified SNP variants in the LCN2 gene, an analysis of haplotypes was performed using Haploview software package. A single haplotype block was identified, composed of the loci g.98793812C>T and g.98793889G>A (Fig. 1). The frequencies for the identified three haplotypes are shown in Table 3. The CG haplotype was the most frequent haplotype. The analysis of the linkage disequilibrium (LD) demonstrated a 100% linkage between the analysed SNPs (D' = 1, r^2 = 1). Additionally, a 100% linkage was identified between the loci g.98793626A>G and g.98793812C>T, and therefore only one of them needs to be genotyped to determine genotypes in both loci (Fig. 1). As a result, it is sufficient to assess one of the two loci to characterise both loci as well as the above haplotype block. This association was also observed in further statistical analysis.

3.3. Association of LCN2 SNPs with milk production traits

The locus g.98793763G>C was found to have a significant impact on protein levels in milk. Milk from cows with the GC genotype had higher protein levels as compared to milk from cows with the GG genotype (Table 4). The alleles of this locus were found to have a positive dominance effect which was statistically significant (Table 5). However, the loci involved in the formation of the haplotype block had a highly significant impact on lactose levels in milk. Milk from cows with the heterozygous loci g.98793626A>G, g.98793812C>T

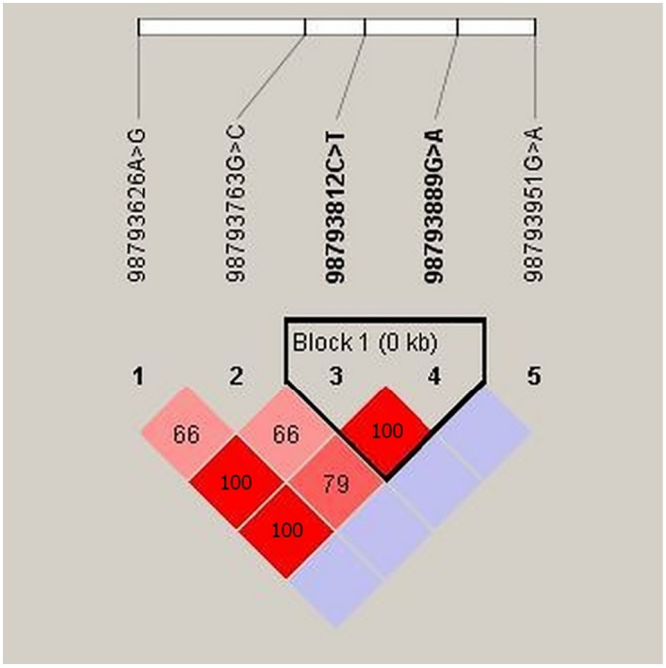

Fig. 1. Linkage disequilibrium (LD) plot in Holstein Friesian cattle. Colour scheme is according to Haploview r^2 scheme. *Number in red* cell stand for pairwise r^2-value (%) between all identified SNPs in LCN2 gene.

Table 3
Frequencies of identified LCN-2 haplotypes in Holstein-Friesian cattle.

Haplotypes	g.98793812C>T	g.98793889G>A	Frequencies
CG	C	G	0.760
TA	T	A	0.224
TG	T	G	0.016

and g.98793889G>A had higher lactose levels as compared to milk from cows with the most frequent homozygotes identified in those three loci (Table 4). It was also confirmed that the loci g.98793626A>G, g.98793812C>T and g.98793889G>A had a significant positive dominance effect on lactose levels, while the additive effect was negative (Table 5).

None of the four analysed loci had a statistically significant impact on the milk yield, fat levels in milk or the somatic cell score.

3.4. Association of LCN2 SNPs with mastitis occurrence

The logistic regression analysis demonstrated that the SNPs identified in the LCN-2 gene had no significant impact on the incidence of mastitis in the analysed herd of Holstein-Friesian cows (Table 6).

4. Discussion

In many countries, years of improving dairy cattle towards achieving higher milk yield caused increase in the amount of milk and dairy products. In highly-developed countries, the consumer's is now focused more on healthy foods of good quality. To obtain milk of high quality, it is necessary to keep cows in an appropriate environment, as well as to ensure proper milking hygiene. Non-compliance with these requirements frequently causes udder inflammation, which affects the quality of milk. Apart from environmental factors, the improvement of cows' immunity to infection depends on genetic factors. Therefore intense research is being conducted to identify genetic markers for udder health status, and in particular for immunity to mastitis, as well as other markers which could have a positive impact on milk quality parameters in addition to the improvement of udder health status [22, 23,24]. LCN2 could become such a marker as it has an active role in the improvement of immunity to infection on account of its bacteriostatic effects. The fact that it inhibits growth of the bacteria causing mastitis in cows (in particular *E. coli*, *S. aureus* or *S. agalactiae*) is most likely associated with its capacity to bind bacterial siderophores, which reduces the capture of iron necessary for bacterial growth [10]. To the best of our knowledge, this study is the first analysis of the polymorphism within the lipocalin-2 gene in Holstein-Friesian cows, which attempted to associate the identified SNPs with milk production traits and the incidence of mastitis in cows. The significant impact of four out of the five analysed LCN2 loci on the average protein and lactose levels in milk, as demonstrated in this study, contributes to the research into genetic markers, in particular markers associated with the improvement of milk quality parameters [22], all the more so given that the analysed loci, except the locus g.98793763G>C, were found to be in a state of genetic Hardy–Weinberg equilibrium. This suggests that the LCN2 gene is hardly susceptible to selective pressure. It should be noted that milk from cows with the homozygous loci g.98793626A>G, g.98793812C>T and g.98793889G>A (AA, CC and GG respectively) had the lowest lactose levels. This information can be useful for cattle breeding programs, given the high percentage of people with lactose intolerance nowadays [25].

Table 4
Effects of the LCN2 SNPs on milk production traits of Holstein-Friesian cattle.

SNPs	Genotype/(N)	Milk yield kg	Protein† %	Fat %	Lactose† %	SCS
g.98793626A>G	**AA** (123)	31.68 ± 0.40	3.41 ± 0.02	3.84 ± 0.04	4.79 ± 0.01^{A}	5.17 ± 0.07
	AG (79)	32.01 ± 0.50	3.46 ± 0.02	3.96 ± 0.05	4.85 ± 0.01^{A}	5.00 ± 0.09
	GG (12)	32.68 ± 1.29	3.41 ± 0.06	3.82 ± 0.14	4.88 ± 0.04	5.13 ± 0.22
	P value	0.714	0.281	0.178	**0.001****	0.332
g.98793763G>C	**GG** (87)	31.96 ± 0.48	3.39 ± 0.02^{a}	3.84 ± 0.05	4.80 ± 0.02	5.14 ± 0.08
	GC (82)	31.49 ± 0.49	3.47 ± 0.02^{a}	3.91 ± 0.05	4.83 ± 0.01	5.07 ± 0.08
	CC (45)	32.32 ± 0.67	3.42 ± 0.03	3.92 ± 0.07	4.83 ± 0.02	5.10 ± 0.11
	P value	0.589	**0.03***	0.597	0.235	0.843
g.98793812C>T	**CC** (123)	31.68 ± 0.40	3.41 ± 0.02	3.84 ± 0.04	4.79 ± 0.01^{A}	5.17 ± 0.07
	CT (79)	32.01 ± 0.50	3.46 ± 0.02	3.96 ± 0.05	4.85 ± 0.01^{A}	5.00 ± 0.09
	TT (12)	32.68 ± 1.29	3.41 ± 0.06	3.82 ± 0.14	4.88 ± 0.04	5.13 ± 0.22
	P value	0.714	0.281	0.178	**0.001****	0.332
g.98793889G>A	**GG** (130)	31.60 ± 0.39	3.42 ± 0.02	3.85 ± 0.04	4.79 ± 0.01^{A}	5.16 ± 0.07
	GA (72)	32.19 ± 0.53	3.45 ± 0.02	3.95 ± 0.06	4.85 ± 0.02^{A}	5.01 ± 0.09
	AA (12)	32.68 ± 1.29	3.41 ± 0.06	3.82 ± 0.14	4.88 ± 0.04	5.13 ± 0.22
	P value	0.539	0.564	0.376	**0.003****	0.439

The values in the columns are given as Least Squares Mean with a Standard Error.
The bold numbers indicate significant differences between genotypes.
† Means with the same superscripts with the same column are different at ** $P < 0.01$ (capital letter) or * $P < 0.05$ (small letter).

Table 5
The additive and dominant effects of the LCN2 SNPs on milk production traits of Holstein-Friesian cattle.

SNPs	Effect†	Milk yield kg	*P* value	Protein %	*P* value	Fat %	*P* value	Lactose %	*P* value	SCS	*P* value
g.98793626A>G	A	−0.50 ± 0.64	0.62	−0.005 ± 0.06	0.93	0.01 ± 0.15	0.92	**−0.045 ± 0.04**	0.02*	0.02 ± 0.23	0.88
	D	−0.17 ± 0.63	0.71	0.05 ± 0.03	0.11	0.13 ± 0.07	0.06	**0.01 ± 0.02**	0.004**	−0.15 ± 0.11	0.13
g.98793763G>C	A	−0.18 ± 0.82	0.67	0.02 ± 0.04	0.44	−0.04 ± 0.09	0.42	−0.02 ± 0.03	0.18	0.02 ± 0.14	0.76
	D	−0.65 ± 0.63	0.34	**0.06 ± 0.03**	0.011*	0.03 ± 0.07	0.54	0,02 ± 0.02	0.29	−0.05 ± 0.11	0.62
g.98793812C>T	A	−0.50 ± 0.64	0.62	−0.005 ± 0.06	0.93	0.01 ± 0.15	0.92	**−0.045 ± 0.04**	0.02*	0.02 ± 0.23	0.88
	D	−0.17 ± 0.63	0.71	0.05 ± 0.03	0.11	0.13 ± 0.07	0.06	**0.01 ± 0.02**	0.004**	−0.15 ± 0.11	0.13
g.98793889G>A	A	−0.54 ± 1.35	0.42	0.005 ± 0.06	0.98	0.02 ± 0.15	0.83	**−0.045 ± 0.04**	0.02*	0.02 ± 0.23	0.93
	D	0,05 ± 0.65	0.44	0.03 ± 0.03	0.28	0.11 ± 0.07	0.17	**0.02 ± 0.02**	0.008**	0.14 ± 0.11	0.20

The values in the columns are given as Least Squares Mean with a Standard Error.
The bold numbers indicate significant differences between additive and dominant effects.
† A means additive effect, D means dominant effect. Asterisks indicate statistical significance levels ** $P < 0.01$, * $P < 0.05$.

Table 6
Evaluation of the LCN2 SNPs impact on the occurrence of cattle mastitis using Logistic regression.

SNPs	Genotyp	N	N in particular classes		*P* value
			I	II	
g.98793626A>G	**AA**	123	65	58	0.27
	AG	79	49	30	
	GG	12	5	7	
g.98793763G>C	**GG**	87	51	36	0.76
	GC	82	44	38	
	CC	45	24	21	
g.98793812C>T	**CC**	123	65	58	0.27
	CT	79	49	30	
	TT	12	5	7	
g.98793889G>A	**GG**	130	70	60	0.37
	GA	72	44	28	
	AA	12	5	7	

Even though we assumed that lipocalin-2 would contribute to a significant extent to the immune response, we were unable to demonstrate such contribution based on a statistical analysis, attempting to associate the identified SNPs with the somatic cell count in milk (which is the primary indicator of udder health status).

It should be emphasised that the study included a small population of cattle without an in-depth analysis of their origin. Therefore the study results can only be interpreted as an association between a marker and production traits for this specific population of cows. Further research is necessary, covering a larger population of cattle of various breeds, to confirm the association between lipocalin-2 and production traits and udder health status.

Conflict of interest

The authors have no conflicts of interests to declare.

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