ORIGINAL ARTICLE

Contribution of the mutation rs8193069 in *TLR4* to mastitis resistance and performance in Holstein cows in southern China

Hongbo Chen[1] ⬤ | **Chenhui Liu**[2] | **Min Xiang**[2] | **Jie Yu**[2] | **Yu Xia**[2] | **Xiuzhong Hu**[2] | **Dingfa Wang**[2] | **Bifei Tao**[2] | **Yongjin Zhang**[1] | **Lei Cheng**[2]

[1] Hubei Provincial Center of Technology Innovation for Domestic Animal Breeding, School of Animal Science and Nutritional Engineering, Wuhan Polytechnic University, Wuhan, P.R. China

[2] Institute of Animal Science and Veterinary Medicine, Wuhan Academy of Agricultural Sciences, Wuhan, P.R. China

Correspondence
Lei Cheng, Wuhan Academy of Agricultural Sciences, Institute of Animal Science and Veterinary Medicine, Zhijinchangshan Road No.2, Jinkou, Jiangxia District, Wuhan 430208, Hubei Province, P.R. China.
Email: chenglei_011@126.com

Present address
Yongjin Zhang, Innovation Team of Pig Genome Design and Breeding, Agricultural Genome Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, P. R. China.

Hongbo Chen and Chenhui Liu contributed equally to the work.

Abstract

Bovine mastitis has become increasingly important issues for farmers and consumers, leading to large economic losses in the dairy industry worldwide. Because treatment of mastitis is difficult and costly, improved mastitis resistance through selective breeding would be advantageous. The toll-like receptor 4 (*TLR4*) is an important player in recognising pathogens and activating immune responses. However, its roles in mastitis occurrence and the underlying molecular mechanisms are unclear. In this study, a single nucleotide polymorphism, rs8193069 (T → C) in *TLR4* gene was detected in a Holstein cow resource population in southern China. Association analysis with 5-year production traits, haematology, and biochemistry parameters revealed that individuals with genotype CC had significantly lower somatic cell counts (SCC), lower fat percentage, but higher 305-day milk ($p < 0.05$) and total milk yield ($p < 0.01$). Both genotypes CC and CT had lower lymphocyte counts (#LYMPH) ($p < 0.01$) and basophil counts (#BASO) ($p < 0.05$) than TT. Genotype CC had a less level of triglyceride ($p < 0.01$) and creatine kinase ($p < 0.05$) than CT. Further analysis based on the production data revealed significant positive correlations between SCC and #LYMPH. Analysis of TLR4 protein structure and properties suggested that the missense mutation on the 674th amino acid from Thr to Ile reduced the flexibility and hydrophilicity of TIR domain, implying a weakened binding ability of TLR4 to its adaptors. In conclusion, allele C of rs8193069 was the major allele in Holstein cows that indicated a greater genetic potential to mastitis resistance and milk yields, probably via the LPS-TLR4 inflammatory signalling. This study offers a marker to improve mastitis resistance in the dairy cow population in southern China.

KEYWORDS
association analysis, mastitis, single nucleotide polymorphism, somatic cell count, toll-like receptor 4

1 | INTRODUCTION

In dairy cattle production, the most common disease is mastitis, which is characterised by inflamed mammary tissue due to physical trauma or microorganism infection (Miles & Huson, 2020). Many factors, including herd and farm management, milking management, the milking machine and the cow itself, contribute to mastitis development (Miles & Huson, 2020), while pathogen infections, including pathogenic fungi, bacteria, mycoplasma and viruses are main causative factors. The populations of mastitis pathogens and/or risk of mastitis increase in summer season as ambient temperature and moisture increases (Hogan & Smith, 2012; Fukushima et al., 2020). In southern China, provinces such as Hunan, Hubei, Shanghai, Guangdong, the cow's environment belong to subtropical monsoon climate. Therefore, circumstances in these areas are detrimental to dairy cattle production due to the heat stress and high humidity. As a result, the treatment of mastitis is difficult and costly. Although much research has gone into its aetiology and diagnosis, there are still large economic losses every year.

White blood cells or leukocytes constitute most somatic cells in milk samples from cows with mastitis. The somatic cell count (SCC) increases sharply in response to pathogenic bacteria such as *Streptococcus agalactiae* and *Staphylococcus aureus*, which are causative agents of mastitis (Ruegg & Petersson-Wolfe, 2018). Therefore, SCC is not only an indicator of the quality of milk but is also used for mastitis diagnosis in dairy cows (Schukken et al., 2003). Generally, the acceptable SCC is less than 100,000 cells/ml, which means that cows with a SCC over 100,000 can be judged as having a higher prevalence of mastitis pathogens (Schwarz et al., 2010).

The toll-like receptor 4 (TLR4) is a type I transmembrane protein, a member of the toll-like receptor (TLR) family, which is recognised as the pattern recognition receptor (PRR). It is involved in the recognition of pathogens, as well as host danger signals that are released during tissue damage (Wang et al., 2018). It is expressed by cells of the innate immune system when stimulated by structural motifs, known as pathogen-associated molecular patterns (PAMPs), from bacteria, viruses and fungi (Lu et al., 2008). TLR4 recognises lipopolysaccharide (LPS), a PAMP present in many Gram-negative bacteria and some Gram-positive bacteria. A dramatic increase in TLR4 protein has been detected in the mammary gland during mastitis (Wang et al., 2018), indicating that the *TLR4* may be associated with mastitis in the dairy cow. Coinciding with this, one single nucleotide polymorphism (SNP) in TLR4 gene, rs8193069, has been found to be significantly associated with SCC in commercial dairy cattle populations (Wang et al., 2008). Nevertheless, contrary results have been shown in other studies (Wang et al., 2018). In addition, roles of rs8193069 in mastitis are not underpinned merely by association analyses using production traits, and there was little information between rs8193069 and health traits. At last, effects of rs8193069 on bovine TLR4 protein properties in terms of immune crosstalk have not been discussed before. To better understand roles of *TLR4* in the risk of mastitis, here our objectives

A single nucleotide polymorphism, rs8193069 (T → C) in TLR4 gene was detected in 785 Holstein cows from southern China. The rs8193069 of TLR4 is significantly associated with both performance traits and blood parameters in Holstein cows. The mutation of rs8193069 from T to C contributes the mastitis resistance with decreased SCC in milk, probably by weakening the binding ability of the TIR domain to TLR4 adaptors and LPS/TLR4-NF-κB signalling mediated inflammation.

were to investigate the associations of rs8193069 in *TLR4* with production traits in addition to haematology and biochemistry parameters in a southern China Holstein resource population, and to investigate the potential molecular mechanisms of rs8193069 affecting mastitis. Based on statistical model construction and TLR4 protein structure and properties predictions, several associations were identified and the potential mechanism of rs8193069 affecting mastitis resistance of dairy cows through LPS-TLR4 inflammatory signalling was proposed.

2 | MATERIALS AND METHODS

2.1 | Animals and production phenotypes

The resource population from a large-scale Holstein dairy farm located in the district of Wuhan City, P.R. China comprised of 785 Holstein cows, which were fed with TMR diet, milked three times daily and implemented regular and standard performance testing by dairy herd improvement (DHI).

A total of 1255 sets of production phenotypic data were collected in different lactations from 2015 to 2020, including 777 in parity 1 (P_1), 386 in P_2 and 92 in P_3. Each set comprised of eight production traits from a cow in a parity, including lactation days (d), 305 d milk yield (kg), total milk yield (kg), peak milk (kg), peak day (d), fat percentage (%), protein percentage (%) and somatic cell (10^4/ml).

2.2 | Blood sample collection and DNA extraction

Blood samples were collected via coccygeal vein in EDTA tubes (Wuhan Zhiyuan Medical Treatment Technology Co., Ltd., China). Samples for haematology were refrigerated at 4°C before detections, all samples were detected within 8 h following collections. Samples for DNA extraction were stored at −80°C until used. Genomic DNA was extracted from blood samples with a TIANamp Blood DNA kit [Tiangen Biochemical Technology (Beijing) Co. Ltd., China]. DNA

TABLE 1 Details of rs8193069 in bovine TLR4 gene

SNP		Amino acid	
Location on chromosome	8:107067611	Location on protein	674
Location on CDS	2021		
Alleles	C/T	Amino acid variation	T(Thr)/I(Ile)
Codons	ACC/ATC		
Mutation type	Missense variant		

CDS: coding sequence.

concentration and purity were measured in a Nanodrop™ 2000 spectrophotometer. Serums for biochemistry were obtained by centrifuging coagulated blood at 3000 rpm for 15 min at 4°C. The samples were then equated and stored at −80°C until used.

2.3 | Snapshot micro-sequencing

The snapshot micro-sequencing technology was used to for genotyping the rs8193069 in bovine *TLR4* gene (Table 1). One pair of amplification extension primer was designed using online software Primer 3.0 (http://bioinfo.ut.ee/primer3-0.4.0/) based on the bovine *TLR4* sequence (GenBank accession number: NC_037335.1). The forward primer was 5′- GTGGGAGCTGAGCAAATGATG-3′, and the reverse primer was 5′-CGCMCCCAGTCTTCATCCTG-3′, where M denotes the annex base (A/C). The length of the amplified product was 266 bp.

The PCR amplification was performed using 20 μl of the reaction mixture containing 1 × GC-I buffer (Takara), 3.0 mM Mg^{2+}, 0.3 mM dNTP, 1 U HotStar Taq polymerase (Qiagen Inc.), 1 μl genomic DNA (5-10 ng/μl), 1 μl (1 μM) pooled primers and PCR grade water. The PCR amplification conditions consisted of preformation at 95°C for 2 min; 11 cycles of denaturation at 94°C for 20 s, annealing at 65°C for 40 s with every cycle involving a decrease in temperature of 0.5°C and an extension at 72°C for 1.5 min; 24 cycles of denaturation at 94°C for 20 s, annealing at 59°C for 30 s and extension at 72°C for 1.5 min; 72°C for 2 min and storage at 4°C.

A total of 10 μl PCR products were purified with SAP (5 U) and exonuclease I (2 U) at 37°C for 1 h, followed by inactivation at 75°C for 15 min. The single base extension reaction was then conducted in 10 μl reaction system containing 5 μl SNaPshot™ Multiplex Kit (ABI), 2 μl purified PCR products, 1 μl extension primer (1.6 μM) and 2 μl PCR grade water. The cycling conditions consisted of preformation at 96°C for 1 min, 28 cycles at 96°C for 10 s, at 55°C for 5 s and at 60°C for 30 s, and storage at 4°C. The extension products were purified the same as the amplified products. Then, the purified product was denatured for 3 min and tested by sequencing machine ABI3730XL. Raw data were analysed using GeneMapper 4.1 (Applied Biosystems Co., Ltd., USA).

2.4 | Bioinformatics analysis of TLR4 protein structure and properties

The conservative domain and three-dimensional structure were performed with the sequence of bovine TLR4 protein (NC_037335.1). The InterPro server (http://www.ebi.ac.uk/interpro/scan.html) was used to predict the conservative domain (Hunter et al., 2009). Three-dimensional structures were predicted with online server I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Yang & Zhang, 2015). The PyMOL v1.6.x was used to visualise and edit the 3D structure (Shringi, 2005). The effect of rs8193069 on hydrophilicity of TLR4 proteins were predicted by Protscale online tools (https://web.expasy.org/protscale/) (Gasteiger et al., 2005). The effect of rs8193069 on flexibility was predicted by PredyFlexy online (https://www.dsimb.inserm.fr/dsimb_tools/predyflexy/) (Bornot et al., 2011).

2.5 | Haematology and serum biochemistry assays

The haematological parameters were determined using an ADVIA® 2120i Hematology System with the standard reagents (Siemens Healthcare Diagnostics Inc., USA). A total of 24 parameters for each animal were collected in bovine mode, including total red blood cell count (RBC, 10^6/μl), haemoglobin concentration (HGB, g/dl), haematocrit (HCT, %), mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, pg), mean corpuscular haemoglobin concentration (MCHC, g/dl), red blood cell haemoglobin (CH, pg), red blood cell distribution width (RDW, %), haemoglobin distribution width (HDW, g/dl), platelet count (PLT, 10^3/μl), mean platelet volume (MPV, fL), white blood cell count (WBC, 10^3/μl), total neutrophil count (#NEUT, 10^3/μl), eosinophils (#EOS, 10^3/μl), basophils (#BASO, 10^3/μl), lymphocytes (#LYMPH, 10^3/μl), monocytes (#MON, 10^3/μl), large unstained cells (#LUC, 10^3/μl) and the proportion of neutrophils (%NEUT), eosinophils (%EOS), basophils (%BASO), lymphocytes (%LYM), monocytes (%MON) and large unstained cells (%LUC).

Biochemical parameters were determined using an automatic biochemistry analyser HITEC 7100 (Hitachi, Ltd., Japan) with the matching commercial kits (Wako Pure Chemical Industries, Ltd., Japan). A total of 17 biochemical parameters for each animal were collected, including total bilirubin (TB, mg/dl), total protein (TP, g/dl), Albumin

(ALB, g/dl), aspartate transaminase (AST, U/L), alanine aminotransferase (ALT, U/L), alkaline phosphatase (ALP, U/L), total cholesterol (TC, mg/dl), triglyceride (TG, mg/dl), glucose (GLU, mg/dl), calcium (Ca, mg/dl), phosphorus (P, mg/dl), creatinine (CREA, mg/dl), low-density lipoprotein (LDL, mg/dl), high-density lipoprotein (HDL, mg/dl), urea nitrogen (BUN, mg/dl), gamma-glutamyl transpeptidase (GGT, U/L) and creatine kinase (CK, U/L).

2.6 | Statistical analysis

A total of 785 cows from the resource population were involved in association analysis. The allele and genotype frequency of rs8193069 in *TLR4* gene were estimated using EXCEL. The Hardy–Weinberg equilibrium was determined using a chi-squared (χ^2) test ($p < 0.05$). A normality test for each trait was performed using univariate progress of SAS online program (https://welcome.oda.SAS.com/). Data were transformed by BOX–COX method, and the outliers were eliminated by 'mean $\pm$ 3 $\times$ standard deviation (SD)'. General Linear Model (GLM) progress was used to compare variation in all traits between different genotypes. The genetic effects of rs8193069 on production traits, SCC, haematology and biochemical traits were analysed with the linear model of SAS with the following equation:

$$Y_{ijklmno} = \mu + Year_i + Season_j + Phase_k + Sire_l + Parity_m + Genotype_n$$

$$+e_{ijklmno},$$

where $Y_{ijklmno}$ is the dependent variable; μ is the overall mean; $Year_i$ is the fixed effect of calving year (i = 2014 to 2020); $Season_j$ is the fixed effect of calving season (j = spring, summer, autumn or winter); $Phase_k$ is the fixed effect of lactation stage ($k \leq$ 100 d, 100–200 d or >200 d); $Sire_l$ is the fixed effect of the sire (l = 1, 2, 3, to 48); $Parity_m$ is the fixed effect of parity (m = 1, 2, or 3); $Genotype_n$ is the fixed effect of SNP (n = CC, CT, or TT), $e_{ijklmno}$ is the random residual effect (Wang et al., 2018). The differences among the effects of different genotypes on each trait were compared with Duncan method. Finally, CORR progress was used to analyse the correlation among the traits.

3 | RESULTS

3.1 | Allele and genotype frequencies of rs8193069

The allele and genotype frequencies for rs8193069 are shown in Table 2. The genotypes included CC (n = 665), CT (n = 118) and TT (n = 2), giving frequencies of 92.2% and 7.8% for C and T, respectively. Individual frequencies of the genotypes are in Hardy–Weinberg equilibrium (p = 0.497).

3.2 | Effect of rs8193069 on bovine TLR4 protein structure and properties

Prediction of conserved domains of TLR4 (Figure 1a) showed that the 674th amino acid corresponding to rs8193069 was located at the start of the Toll/interleukin-1 receptor (TIR) domain. The 3D-structure prediction showed that missense mutation from T (Thr) to I (Ile) of rs8193069 induced a slight conformational change of the nearby amino acids and added a β-fold downstream (Figure 1b and c), but neither of them had significant effect on the whole structure of the protein.

Further protein property analysis of TLR4 showed that the mutation of rs8193069 from C to T increased the hydrophilicity values (Figure 1d), indicating an increased hydrophobicity of nearby amino acids. The mutation also decreased the B-factor values (Figure 1e), suggesting that the flexibility of nearby amino acids was reduced because of the mutation.

3.3 | Associations of rs8193069 with production traits

Associations of rs8193069 with 8 production traits are presented in Table 3. Significant effects ($p < 0.05$) of rs8193069 were detected on 305 d milk yield, SCC and Total milk yield. In comparison with genotype CT, individuals with genotype CC had higher 305 d milk yield and lower SCC. Total milk yield and fat percentage were extremely significantly associated with rs8193069 ($p < 0.01$). Individuals with genotype CC had higher total milk yield but lower fat percentage compared to genotypes CT. No significant differences were found for lactation days, peak milk, peak day and protein percentage among the three genotypes.

3.4 | Association of rs8193069 with haematology and biochemistry parameters

The estimated effects of rs8193069 on haematology and biochemistry parameters are shown in Table 4. rs8193069 had extremely significant effects on #LYMPH and TG ($p < 0.01$) and significant effects on MCV, MCHC, #BASO and CK ($p < 0.05$). Individuals with genotypes CC and CT had lower MCV, #LYMPH, #BASO and higher MCHC than genotype TT. Individuals with genotypes CC had lower TG and CK than genotype CT. Details of haematology and serum biochemistry parameters that not significantly associated with rs8193069 are shown in supplementary tables (Tables S1 and S2).

3.5 | Correlation analysis between SCC and haematology or serum biochemistry

The relationship between SCC and haematological or biochemical parameters were further analysed. As shown in Table 5, there

TABLE 2 Distribution of rs8193069 in the studied resource population ($n = 785$)

Genotype	Number	Genotype frequency	Allele	Allele frequency	χ^2	p Value
C/C	665	0.847	C	0.922	1.399	0.497
C/T	118	0.150	T	0.078		
T/T	2	0.003				

TABLE 3 Association of rs8193069 with production traits

Production traits	Genotype	Phenotypic valueMean ± SD[†]	F	p Value
Lactation day	C/C	309.096 ± 17.489	2.280	0.103
	C/T	297.408 ± 18.412		
	T/T	246.127 ± 50.837		
305 d milk yield	C/C	10073.005 ± 324.166[b]	3.840	0.022*
	C/T	9765.951 ± 346.743[a]		
	T/T	8310.936 ± 1035.947[ab]		
Total milk yield	C/C	10324.004 ± 596.00[B]	5.360	0.005**
	C/T	9667.778 ± 627.447[A]		
	T/T	7557.115 ± 1732.460 [AB]		
Peak milk	C/C	42.851 ± 1.368	2.860	0.058
	C/T	41.704 ± 1.440		
	T/T	38.892 ± 3.977		
Peak day	C/C	119.770 ± 10.550	0.230	0.795
	C/T	118.126 ± 11.106		
	T/T	103.356 ± 30.668		
Fat percentage	C/C	4.245 ± 0.098[A]	6.940	0.001**
	C/T	4.377 ± 0.103[B]		
	T/T	4.596 ± 0.283[AB]		
Protein percentage	C/C	3.235 ± 0.048	2.500	0.083
	C/T	3.272 ± 0.050		
	T/T	3.372 ± 0.139		
Somatic cell count	C/C	20.816 ± 2.718[a]	4.340	0.013*
	C/T	22.001 ± 2.864[a]		
	T/T	41.800 ± 7.900[b]		

[†]All values are expressed as mean ± SD.
The upper capitals (A, B, C)/double asterisk and (a, b, c)/signal asterisk were used when the difference is highly significant ($p < 0.01$) and significant ($p < 0.05$), respectively.

were extremely significant correlations between SCC and RBC, HGB, #LYMPH and TP ($p < 0.01$), and significant correlations between SCC and %EOS, #BASO, ALB, ALT, CA and P ($p < 0.05$). There were positive correlations between SCC and #LYMPH, #BASO and TP, and negative correlations between SCC and RBC, HGB, %EOS, ALB, ALT, CA and P.

4 | DISCUSSION

4.1 | Effects of rs8193069 on TIR domain stability

The TLR4 protein consists of three parts: an extracellular domain composed of many leucin-rich repeats (LRRs) that mediate LPS
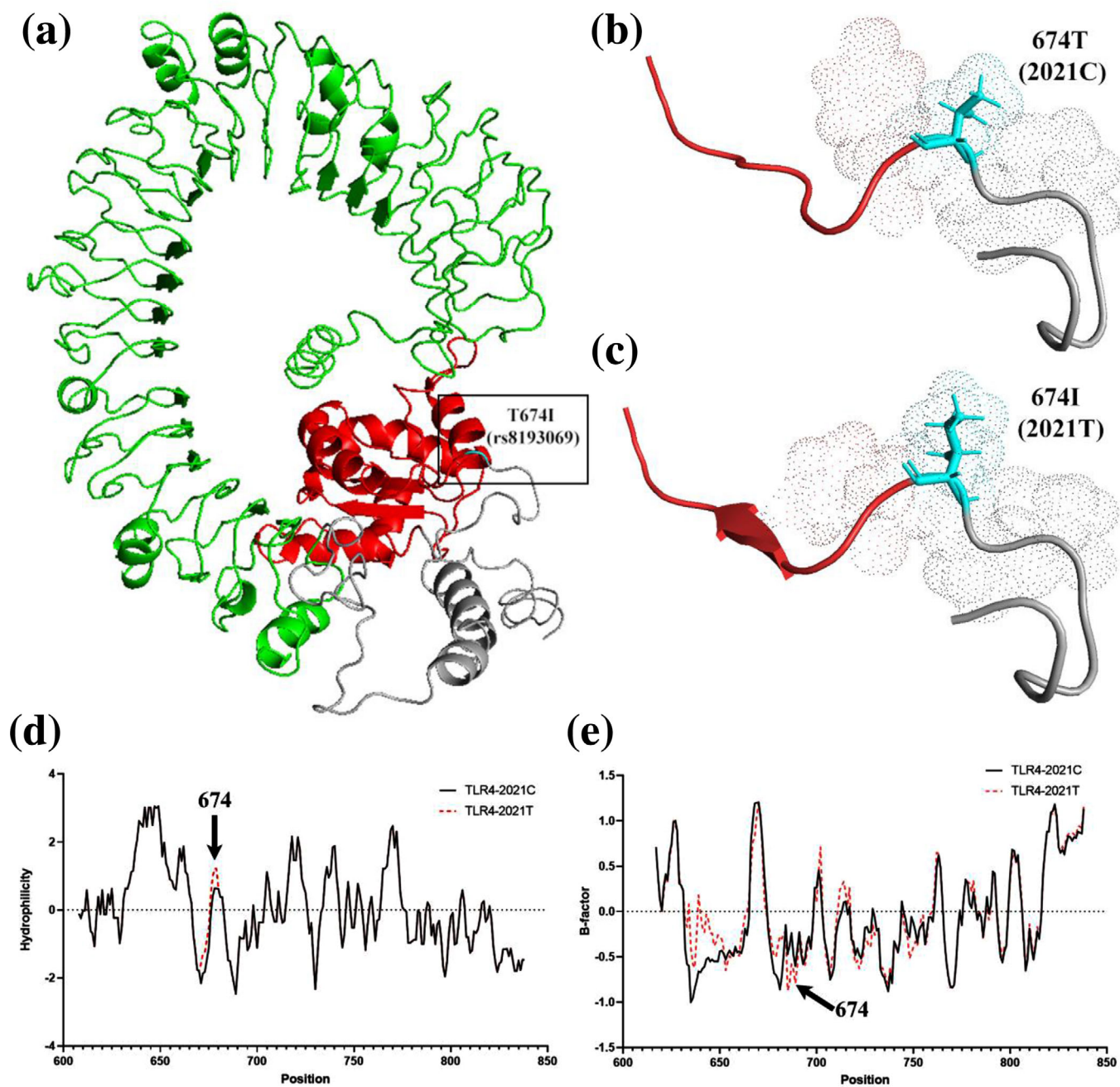
FIGURE 1 Effects of rs8193069 on TLR4 protein structure and function. (a) The location of amino acid variation corresponding to rs8193069 on theoretical three-dimensional model of TLR4 protein. The variation is marked with cyan. The TIR domain (marked with red) is located in the membrane and contains amino acids 674–814. The extracellular part of TLR4 is indicated with green. (b), (c) The predicted three-dimensional structure of TLR4 when the rs8193069 was C and T, respectively. (d) Effects of rs8193069 on hydrophilicity of TLR4. It shows the region at amino acid 603–841. The mutation of rs8193069 from C to T results in increased hydrophilicity values of the nearby amino acids. Higher positive values indicate stronger hydrophobicity and lower negative values indicate stronger hydrophilicity. (e) Effect of rs8193069 on flexibility of TLR4. It shows the region at amino acid 603–841. The mutation of rs8193069 from C to T results in decreased B-factor values of the nearby amino acids, which indicate the reduced flexibility

recognition and receptor dimerisation (Ohto et al., 2012); a transmembrane domain composed of an α-helix and the TIR domain, which is in the cytosolic face. The TIR domain, which is unique to the TLR system, is essential for downstream signal transduction (O'Neill & Bowie, 2007) and is responsible for mediating the protein-protein interac-

tions between the TLR4 and adaptor proteins (Mahita & Sowdhamini, 2017), including myeloid differentiation primary-response gene 88 (MyD88), MyD88-adaptor-like (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF; also known as TICAM1), TRIF-related adaptor molecule (TRAM; also known as

TABLE 4 Association of rs8193069 with haematology and biochemistry parameters

Indexes[†]	Genotype	Phenotypic valueMean ± SD[‡]	F	p Value
MCV	C/C	48.458 ± 0.450[a]	4.370	0.013*
	C/T	48.996 ± 0.641[a]		
	T/T	60.694 ± 4.375[b]		
MCHC	C/C	356.378 ± 1.269[b]	3.640	0.027*
	C/T	355.157 ± 1.808[b]		
	T/T	324.450 ± 12.342[a]		
#LYMPH	C/C	5.917 ± 0.325[A]	5.320	0.005**
	C/T	5.830 ± 0.464[A]		
	T/T	16.157 ± 3.163[B]		
#BASO	C/C	0.135 ± 0.008[a]	3.060	0.048*
	C/T	0.132 ± 0.011[a]		
	T/T	0.319 ± 0.076[b]		
TG	C/C	12.704 ± 0.501[A]	5.36	0.005**
	C/T	14.373 ± 0.696[B]		
	T/T	18.867 ± 4.719[AB]		
CK	C/C	82.084 ± 0.361[a]	3.03	0.049*
	C/T	84.338 ± 0.845[b]		
	T/T	83.550 ± 6.177[ab]		

[†]Meanings of all abbreviations have been indicated in Section 2.

[‡]All values are expressed as Mean ± SD.

The upper capitals (A, B, C)/double asterisk and (a, b, c)/signal asterisk were used when the difference is highly significant ($p < 0.01$) and significant ($p < 0.05$), respectively.

TICAM2) and sterile α- and armadillo-motif containing protein (SARM) (O'Neill & Bowie, 2007). The nuclear factor κB (NF-κB) is activated after a series of signal transduction chains (Fitzgerald et al., 2001), followed by translocation into nucleus, inducing the synthesis and secretion of proinflammatory cytokines and type I interferons, initiating an inflammatory response.

The amino acid 674 corresponding to rs8193069 is the beginning of the TIR region and located at the junction between the N-terminal and the TIR domain. Poltorak et al. (1998) found a missense mutation Pro712His in the TIR domain of TLR4 in C3H/HeJ mice, which led to the tolerance to LPS. The homology modelling of TLR4 showed that hydrophobicity played a crucial role in the binding of TLR4 protein to its adaptor proteins (Gu et al., 2002), while Pro712His changed the hydrophobicity and charge of the corresponding position, resulting in a decreased activity of LPS/TLR4 signal transduction pathway. Similarly, our analysis showed that the mutation of rs8193069 from C to T increased the hydrophobicity of the 674th amino acid and the strength of the hydrophobic interactions, which is obviously beneficial to the binding of TLR4 to its adaptors. The change of hydrophobicity also affects the stability of protein. When the hydrophilic residue mutates to the hydrophobic one, the stability of protein will increase (Gallardo et al., 2010).

The B-factor (atomic displacement parameter) in protein crystal structures is regarded as an important indicator of the protein structure, reflecting its flexibility and dynamics (Zheng et al., 2005). A large B-factor indicates high mobility of individual atoms and side chains, while a small B-factor indicates high stability (Bornot et al., 2011). Our prediction results of B-factor showed that the flexibility of area near the 674th amino acid reduced when rs8193069 mutated from C to T, implying the stability of this region was improved. The TLR4 protein with CC genotype may therefore has a weakened binding ability to adaptors, leading to reduced or even impaired activity of the LPS/TLR4 signal transduction pathway.

4.2 | Effects of rs8193069 on the mastitis resistance in Holstein cows through LPS/TLR4-NF-κB signal transduction pathway

The rs8193069 is a functional SNP in *TLR4* gene which has been widely examined in dairy cows. Previous studies have found that rs8193069 was associated with milk yields, fat percentages and protein percentages (Zhou et al., 2017; Wang et al., 2018). However, the association between this SNP and SCC is still undetermined among different populations (Wang et al., 2018). In this study, rs8193069 was found to be significantly associated with SCC in the trialled Holstein cow population, showing that individuals with genotype TT had higher SCC. As increased SCC in the milk indicates long term intra-mammary

TABLE 5 Correlation between SCC and haematology and biochemistry

Indexes[†]	R value	p Value	Indexes	R value	p Value
WBC	−0.016	0.713	TB	−0.021	0.635
RBC	−0.134	0.002**	TP	0.163	0.000**
HGB	−0.137	0.001**	ALB	−0.088	0.050*
HCT	0.035	0.418	AST	−0.082	0.059
MCV	0.050	0.244	ALT	−0.088	0.041*
MCH	−0.003	0.938	ALP	−0.044	0.318
MCHC	−0.041	0.342	TC	−0.046	0.288
CHCM	−0.037	0.398	TG	−0.012	0.776
CH	0.048	0.271	GLU	−0.079	0.074
RDW	−0.040	0.352	CA	−0.111	0.012*
HDW	−0.021	0.635	P	−0.113	0.011*
PLT	0.027	0.530	CREA	0.022	0.616
MPV	0.003	0.944	HDL	−0.056	0.193
%NEUT	−0.036	0.403	LDL	−0.044	0.306
%LYMPH	0.069	0.108	BUN	0.010	0.823
%MONO	−0.057	0.185	GGT	−0.009	0.838
%EOS	−0.097	0.025*	CK	0.024	0.580
%BASO	−0.020	0.645			
%LUC	−0.017	0.692			
#NEUT	0.080	0.063			
#LYMPH	0.126	0.004**			
#MONO	0.056	0.197			
#EOS	−0.023	0.593			
#BASO	0.086	0.047*			
#LUC	0.034	0.434			

[†]Meanings of all abbreviations have been indicated in Section 2. The double asterisk and signal asterisk indicate the difference is highly significant ($p < 0.01$) and significant ($p < 0.05$), respectively.

inflammation (Roldan-Montes et al., 2020), a lower SCC is free of, or at least, low infection (Pighetti & Elliott, 2011). Therefore, the allele C of rs8193069 is the major allele, in other words, individuals with genotype CC would have a greater genetic potential for mastitis resistance under the similar environment.

To better elucidate the underlying molecular mechanism on the contribution of rs8193069 to mastitis resistance in dairy cows, further associations of rs8193069 with haematology and biochemistry parameters were investigated. Our results showed that individuals with genotypes CC and CT had lower #LYMPH and #BASO than genotype TT, indicating mutation from C to T promoted the production of leukocytes. Lymphocytes and basophils are essential in provoking inflammation (Bartoli-Leonard et al., 2021; Hou et al., 2021), thus individuals with genotype CC will have a healthier status with low inflammatory stress during milk production. Further analysis based on the actual production data and health data from the same resource population validated the above results, revealing that SCC was significantly positively correlated with both #LYMPH and #BASO. It is to say that the number of lymphocytes and basophils in blood may be important fac-

tors affecting SCC. Based on these results, a mechanism was proposed that the binding ability of TLR4 to its adaptors in individuals with genotype CC was weaker than genotypes TC and TT (Figure 2). Individuals with genotype CC could be stimulated by PAMPs such as LPS, but with weakened ability to activate the LPS/TLR4-NF-κB pathway. As a result, cytokine expressions and inflammatory response were inhibited, and subsequently, the reduced proliferations of lymphocytes and basophils in the blood and less SCC in milk, which has been used to measure the risk of mastitis (Scarsella et al., 2021). So, individuals with genotype CC manifest greater capacity of resistance to LPS-induced mastitis or tolerance. To further verify this, additional function validation experiments will be needed in the future.

5 | CONCLUSION

The rs8193069 in bovine *TLR4* is significantly associated with both production traits and blood parameters in Holstein cows, where the allele C of rs8193069 is the major allele. The mutation of rs8193069
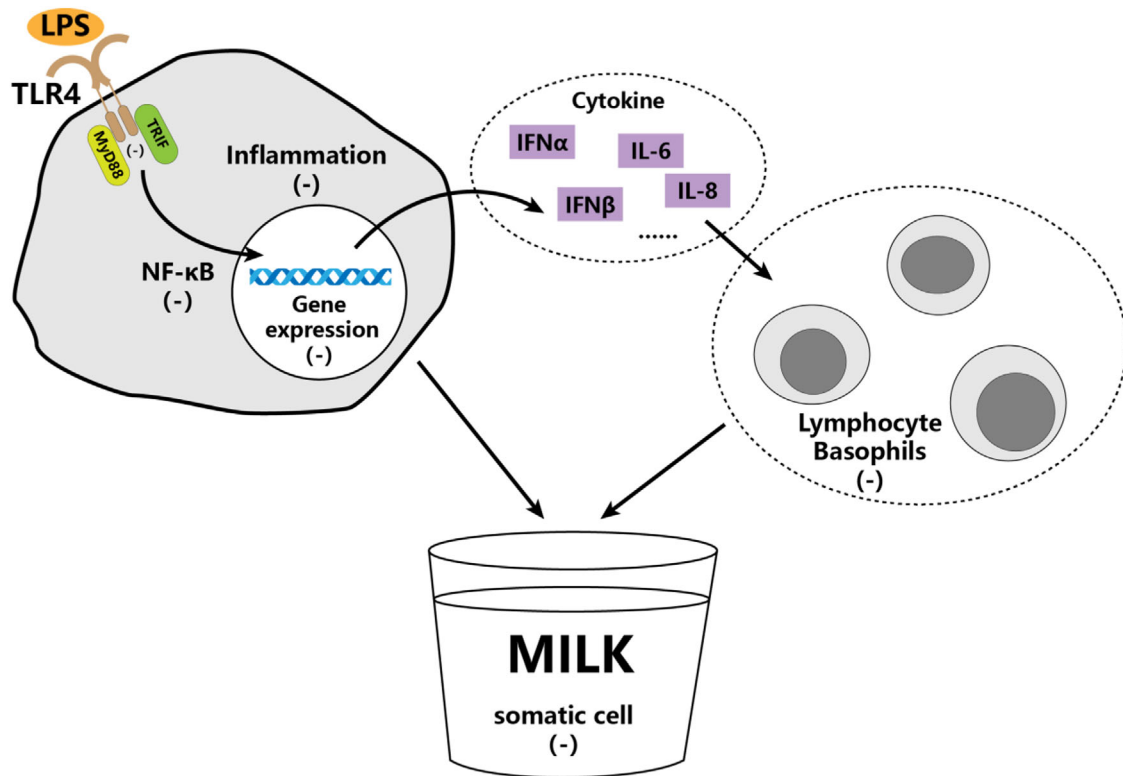
FIGURE 2 Molecular mechanism hypothesis of rs8193069 on SCC in milk or its contribution to mastitis resistance based on results in this study. The major allele C of rs8193069 may reduce the binding ability between TLR4 protein and its adaptors. This leads to the reduced activity of NF-κB, decreased cytokines expression and lymphocyte/basophils proliferation, resulting in reduced SCC in milk. The pathway has been described in detail in the text. IFN-α, Interferon-α; IFN-β, Interferon-β; IL-6, interleukin-6; IL-8, interleukin-8

from T to C contributes the mastitis resistance with decreased SCC in milk, probably by weakening the binding ability of the TIR domain to TLR4 adaptors and LPS/TLR4-NF-κB signalling mediated inflammation.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Hongbo Chen: conceptualisation (supporting); methodology (equal); writing – original draft (equal); writing – review and editing (equal); visualisation (lead); funding acquisition (equal). **Chenhui Liu**: Methodology (equal); software (lead); formal analysis (equal); writing – original draft (equal). **Min Xiang**: Data curation (equal); investigation (equal); resources (equal). **Jie Yu**: Investigation (equal); resources (equal). **Yu Xia**: Investigation (equal); resources (equal). **Xiuzhong Hu**: Data curation (equal); investigation (equal). **Dingfa Wang**: Resources (equal). **Bifei Tao**: Resources (equal). **Yongjin Zhang**: Data curation (equal). **Lei Cheng**: Conceptualisation (lead); methodology (equal); writing – review and editing (equal); visualisation (equal); funding acquisition (equal); project administration (lead).

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and all animal procedures were performed according to protocols approved by the Hubei Province for Biological Studies Animal Care and Use Committee. Protocols were carried out in accordance with Hubei Provincial Regulation on Administration of Laboratory Animals (10/1/2005). The US National Research Council's guidelines for the Care and Use of Laboratory Animals were followed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at https://publons. com/publon/10.1002/vms3.671

ORCID

Hongbo Chen 🔟 https://orcid.org/0000-0002-6051-3321

REFERENCES

Bartoli-Leonard, F., Zimmer, J., & Aikawa, E. (2021). Innate and adaptative immunity: The understudied driving force of heart valve disease. *Cardiovascular Research*, cvab273.

Bornot, A., Etchebest, C., & de Brevern, A. G. (2011). Predicting protein flexibility through the prediction of local structures. *Proteins*, 79(3):839–852.

Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T., McMurray, D., Smith, D. E., Sims, J. E., Bird, T. A., & O'Neill, L. A. (2001). Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature*, 413(6851):78–83.

Fukushima, Y., Kino, E., Furutani, A., Minamino, T., Mikurino, Y., Horii, Y., Honkawa, K., & Sasaki, Y. (2020). Epidemiological study to investigate the incidence and prevalence of clinical mastitis, peracute mastitis, metabolic disorders and peripartum disorders, on a dairy farm in a temperate zone in Japan. *BMC Veterinary Research*, 16(1), 389.

Gallardo, O., Pastor, F. I., Polaina, J., Diaz, P., Łysek, R., Vogel, P., Isorna, P., González, B., & Sanz-Aparicio, J. (2010). Structural insights into the specificity of Xyn10B from Paenibacillus barcinonensis and its improved stability by forced protein evolution. *The Journal of Biological Chemistry*, 285(4), 2721–2733.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., & Bairoch A. (2005). Protein identification and analysis tools on the ExPASy server. In: John, M. Walker (Ed.), *The Proteomics Protocols Handbook* (pp. 571–607). Humana Press.

Gu, C. G., Hu, Y. D., Li, S., & Li, L. (2002). Homology modeling for cytoplasmic region of toll-like receptor 4. *Journal of Immunology*, 18(2), 95–98.

Hunter, S., Apweiler, R., Attwood, T., Bairoch, A., Bateman, A., Binns, D., Bork, P., Das, U., Daugherty, L., Duquenne, L., Finn, R. D., Gough, J., Haft, D., Hulo, N., Kahn, D., Kelly, E., Laugraud, A., Letunic, I., Lonsdale, D., & Yeats, C. (2009). InterPro: The integrative protein signature database. *Nucleic Acids Research*, 37(Suppl 1), D211–D215,

Hogan, J., & Smith, K. L. (2012). Managing environmental mastitis. *Veterinary Clinics of North America: Food Animal Practice*, 28(2), 217–224.

Hou, T., Tsang, S. M., Kan, L. Y., Li, P., Chu, I. M., Lam, C. W., & Wong C. K. (2021). IL-37 targets TSLP-primed basophils to alleviate atopic dermatitis. *International Journal of Molecular Sciences*, 22(14), 7393.

Lu, Y. C., Yeh, W. C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine*, 42(2), 145–151.

Mahita, J., & Sowdhamini, R. (2017). Integrative modelling of TIR domain-containing adaptor molecule inducing interferon-β (TRIF) provides insights into its autoinhibited state. *Biology Direct*, 12(1), 9.

Miles, A. M., & Huson, H. J. (2020). Time-and population-dependent genetic patterns underlie bovine milk somatic cell count. *Journal of Dairy Science*, 103(9), 8292–8304.

Ohto, U., Yamakawa, N., Akashi-Takamura, S., Miyake, K., & Shimizu, T. (2012). Structural analyses of human toll-like receptor 4 polymorphisms D299G and T399I. *The Journal of Biological Chemistry*, 287(48), 40611–40617.

O'Neill, L. A., & Bowie, A. G. (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nature Reviews Immunology*, 7(5), 353–364.

Pighetti, G. M., & Elliott, A. A. (2011). Gene polymorphisms: The keys for marker assisted selection and unraveling core regulatory pathways for mastitis resistance. *Journal of Mammary Gland Biology and Neoplasia*, 16(4), 421–432.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., & Beutler, B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. *Science*, 282(5396), 2085–2088.

Roldan-Montes, V., Cardoso, D. F., Hurtado-Lugo, N. A., do Nascimento, A. V., de Abreu Santos, D. J., Scalez, D. C. B., de Freitas, A. C., Herrera, A. C., Albuquerque, L. G., de Camargo, G. M. F., & Tonhati, H. (2020). Polymorphisms in TLR4 gene associated with somatic cell score in Water buffaloes (Bubalus bubalis). *Frontiers in Veterinary Science*, 7, 568249.

Ruegg, P. L., & Petersson-Wolfe, C. S. (2018). Mastitis in dairy cows. *Veterinary Clinics of North America: Food Animal Practice*, 34(3), ix–x.

Scarsella, E., Zecconi, A., Cintio, M., & Stefanon, B. (2021). Characterization of microbiome on feces, blood and milk in dairy cows with different milk leucocyte pattern. *Animals (Basel)*, 11(5), 1463.

Schukken, Y. H., Wilson, D. J., Welcome, F., Garrison-Tikofsky, L., & Gonzalez, R. N. (2003). Monitoring udder health and milk quality using somatic cell counts. *Veterinary Research*, 34(5), 579–596.

Schwarz, D., Diesterbeck, U. S., Failing, K., König, S., Brügemann, K., Zschöck, M., Wolter, W., & Czerny, C. P. (2010). Somatic cell counts and bacteriological status in quarter foremilk samples of cows in Hesse, Germany—A longitudinal study. *Journal of Dairy Science*, 93(12), 5716–5728.

Shringi, R. P. (2005). Pymol software for 3d visualization of aligned molecules. *Biomaterials*, 26(1), 63–72.

Wang, M., Song, H., Zhu, X., Xing, S., Zhang, M., Zhang, H., Wang, X., Yang, Z., Ding, X., Karrow, N. A., König, S., & Mao, Y. (2018). Toll-like receptor 4 gene polymorphisms influence milk production traits in Chinese Holstein cows. *Journal of Dairy Research*, 85(4), 407–411.

Wang, X., Xu, S., Gao, Z., Li, J., Ren, H., & Luoren, Z. (2008). Cloning and SNP screening of the TLR4 gene and the association between its polymorphism and somatic cell score in dairy cattle. *South African Journal of Animal Science*, 38(2), 101–109.

Yang, J., & Zhang, Y. (2015). I-TASSER server: New development for protein structure and function predictions. *Nucleic Acids Research*, 43(W1), W174–W181.

Zheng, Y., Bailey, T. L., & Teasdale, R. D. (2005). Prediction of protein B-factor profiles. *Proteins*, 58(4), 905–912.

Zhou, H., Cheng, L., Gong, H., Byun, S. O., Edwards, G. R., & Hickford, J. G. (2017). Variation in the Toll-like Receptor 4 (TLR4) gene affects milk traits in dairy cows. *Journal of Dairy Research*, 84(4), 426–429.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.