Detailed Materials & methods

Animal and milk production records

Mixed milk (volume in morning: noon: night = 4:3:3) samples were collected monthly from June 2010 to December 2014. Milk samples were treated with potassium bichromate (30 mg/tube) immediately after milking, and analyzed for SCC based on flow cytometry (Fossmatic 5000, Foss Electric, Denmark). The infrared technique (Milkoscan 6000, Foss Electric, Denmark) was used to determine concentrations of fat, protein, lactose, total solids **(TS)** and milk urea nitrogen **(MUN)**. Production traits included test-day milk yield **(TDMY)**, fat content **(FC)**, protein content **(PC)**, lactose content **(LC)**, MUN and somatic cell count **(SCC)**. Somatic cell score **(SCS)** was calculated using the formula: $SCS=\log_2^{(SCC/100\ 000)}+3$ (Wiggans et al. 1987). These records were obtained from the Dairy Herd Improvement **(DHI)** lab of the Shanghai Dairy Cattle Breeding Center. Editing of the data was performed to ensure both reliability and consistency for statistical analyses. Requirements were designed as follows: TDMY between 5-60 kg, FC between 2-7%, PC between 2-6%, LC between 2-5.5%, TS content between 9-18%, MUN between 5-30%, SCC between 1×10^3 - 9999×10^3, and SCS between 0 - 9. Only records from parities from 1 to 3, and from 5 to 305 days in milk (DIM) were included. For analyses, 20 556 records were retained and utilized in this study. The mean values and standard errors for the analyzed traits stratified by parity are summarized in Table 1.

DNA extraction

Blood samples were placed immediately on ice for subsequent DNA extraction. An Eppendorf Biophotometer (Berlin, Germany) was used to assess the DNA concentration and DNA quality based on absorbance of ultraviolet light at 260 and 280 nm. The ratio of OD_{260}/OD_{280} for the DNA samples ranged from 1.70 to 1.85, and the concentration of the DNA was above 50 ng μl^{-1}. Finally, the concentration of the DNA sample used for PCR was diluted to 50 ng μl^{-1} with ddH_2O and stored at -20

°C.

SNP genotyping and transcription-factor binding sites analysis

Primers for PCR and single base extension for the SNPs of *TLR4* c.-226 G>C and *TLR4* c.2021 C>T were designed using the Assay Designer software package (Sequenom Inc., San Diego, CA) (Table 2). As a quality control measure, genomic DNA from 40 animals was genotyped in duplicate for each SNP. Concordance across SNP and all duplicates was 100%.

SNP and Haplotype associations

The additive (a) and dominance (d) and effects of single SNPs were evaluated using the equations: a = (AA-BB)/2, and d = AB-(AA+ BB)/2, where AA and BB indicate the two homozygous genotypes, AB represents the heterozygous genotype (Falconer and Mackay 1996). Allele substitution effects (α) at each SNP-locus were estimated by regressing the milk production traits and SCS on the number of copies of one allele of a SNP (Sherman et al., 2008) using a linear model in SAS. For the analyses above, the Bonferroni method was adopted to correct for multiple t-tests according to the number of SNP loci or haplotype blocks detected. We declared a significant SNP or haplotype if a raw *P*-value is <0.05/N, where N is the number of SNP loci or haplotype blocks tested in the analyses.

Applied tables

Table 1 Descriptive statistics for milking traits by parity (means ± SE)

Parity	Records number	TDMY[a] (kg)	FC[a] (%)	PC[a] (%)	FPR[a]	SCS[a]	LC [a] (%)	TS[a] (%)	MUN[a] (g/100mL)
1	7 018	29 06±0 08	4 14±0 01	3 34±0 01	1 25±0 01	2 03±0 02	5 01±0 01	13 49±0 01	10 94±0 03
2	6 741	33 06±0 12	4 36±0 01	3 38±0 01	1 29±0 01	1 82±0 02	4 94±0 01	14 05±0 02	12 52±0 04
3	6 797	34 62±0 16	4 24±0 01	3 32±0 01	1 29±0 01	2 22±0 02	4 86±0 01	13 80±0 02	13 80±0 04
Total	20 556	32 21±0 07	4 25±0 01	3 35±0 01	1 27±0 01	2 02±0 01	4 95±0 01	13 77±0 01	12 17±0 02

Abbreviations:

TDMY: test-day milk yield

FC: fat content

PC: protein content

FPR: fat to protein ratio

SCS: somatic cell score

LC: lactose content

TS: total solid

MUN: milk urea nitrogen

Table 2 The primers sequence for TLR4 c -226 G>C and c 2021 C>T

Loci	Primer of sequences(5′→3′)
c -226 G>C	F:ACGTTGGATGGGTCTGCAGACGTTTTCTTC
	R:ACGTTGGATGTCTGGACTTTCGTTTCTCTG
	U: ATCCTCTAACTTCCCCTC
c 2021 C>T	F:ACGTTGGATGCTCGAGTAGATGACAAAGGC
	R:ACGTTGGATGTTCCACCTGATGCTTCTTGC
	U: GATGACAAAGGCATCATAG

Table 3 **G**enotypic and allelic frequency & values of X^2 test significance for *TLR4* c -226 G>C and c 2021 C>T in Holstein cows

Locus	Genotype	Genotypic frequency	Number	Allele	Allelic frequency	X^2-test （P value）
c -226 G>C	CC	0 199	172	C	0 447	0 017*
（rs 29017188）	CG	0 497	430	G	0 553	
	GG	0 305	264			
c 2021 C>T	CC	0 753	652	C	0 865	0 940
（rs 8193069）	CT	0 225	195	T	0 135	
	TT	0 022	19			

*: $P<0\ 05$

Table 4 Haplotype reconstruction for SNP in *TLR4* c -226 G>C and *TLR4* c 2021 C>T and their frequencies

Haplotypes	TLR4 c -226	TLR4 c 2021	Number	Frequencies
1	C	C	541	0 312
2	C	T	233	0 135
3	G	C	958	0 553
Total			1 732	1 000