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Detection of two SNPs of the *LIPE* gene in Holstein–Friesian cows with divergent milk production

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The *LIPE* gene (*lipase E*, hormone-sensitive type), also known as *hormone-sensitive lipase*, acts as a primary regulator of lipid metabolism during lactation in cows. We studied a total of two hundred Holstein–Friesian cows and performed sequencing analysis that revealed two synonymous nucleotide changes within the *LIPE* gene: a transition change, c.276 T > C in exon 2 (g.50631651 T > C; position 351 of GenBank: ON638900) and a transversion change, c.219C > A in exon 6 (g.50635369C > A; position 1070 of GenBank: ON638901). The observed genotypes were TC and CC for the c.276 T > C SNP and CC and CA for the c.219C > A SNP. Notably, the heterozygous TC genotype of the T351C SNP exhibited a significant association with high milk yield. Furthermore, the T351C SNP displayed significant associations with various milk parameters, including temperature, freezing point, density and the percentages of fat, protein, lactose, solids and solids-not-fat, with the homozygous CC genotype showing higher values. The c.219C > A SNP also demonstrated a significant association with milk composition, with heterozygous genotypes (CA) exhibiting higher percentages of fat, protein, and lactose compared to homozygous genotypes (CC). This effect was consistent among both high and low milk producers for fat and lactose percentages, while high milk producers exhibited a higher protein percentage than low milk producers. These findings highlight the importance of considering the detected SNPs in marker-assisted selection and breeding programs for the identification of high milk-producing Holstein–Friesian cows and potentially other breeds. Moreover, this study strongly supports the fundamental role of the *LIPE* gene in milk production and composition in lactating animals.

Research Article

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Milk is a crucial component of the human diet and serves as a rich source of various nutrients. According to FAO reports (2019), approximately 81% of consumable milk is derived from cows, which significantly impacts human health due to its constituents. One such constituent is fat content, which varies not only across different species but also within the same species. The composition of milk is a complex trait that is influenced by multiple factors, including genetic regulation and environmental conditions (such as lactation stage, milk yield, season, herd and diet: Singh and Gupta, 2016) and it is worth noting that there exists a negative correlation between milk yield and fat content. High milk-producing cows tend to exhibit low-fat content in their milk, whereas cows with low milk yield typically possess high fat content (Pizarro *et al.*, 2020).

In dairy animals, dietary fatty acids together with fatty acids released from adipose tissue through lipolysis are taken up by the lactating udder for milk fat synthesis (Zidi *et al.*, 2010). Therefore, lipolytic enzymes like *LIPE* (*lipase E*, hormone-sensitive type, also known as *hormone-sensitive lipase*) play a vital role in regulating the hydrolysis of triacylglycerol, diacylglycerol and monoacylglycerol molecules, as well as the release of free fatty acids (Fang *et al.*, 2014). LIPE is expressed in various tissues, including the kidney, adipose, spleen, rumen and lung, with the highest expression level found in fat tissues (Fang *et al.*, 2017). The bovine *LIPE* gene is located on chromosome 18 (Fang *et al.*, 2017) and comprises 10 exons, as stated by the NCBI Reference Sequence: NM_001080220.1. Previous studies have reported associations between polymorphisms in the *LIPE* gene and meat fatty acid composition traits (Fang *et al.*, 2014, 2017). Furthermore, a missense polymorphism in exon 6 of the goat *LIPE* gene has been found to be associated with milk yield and composition (Zidi *et al.*, 2010). While the structural and transcriptional characteristics of the *LIPE* gene have been investigated in sheep (Lampidonis *et al.*, 2008) and cattle (Yonezawa *et al.*, 2008), its

association with milk composition traits in cows has not been thoroughly examined. Hence, the objective of this study was to identify potential SNPs in the *LIPE* gene that may influence bovine milk yield and composition traits, such as protein and fat percentages, lactose content and solid-not-fat (SNF) contents, in animals with high and low milk production.

Materials and methods

The current study was conducted at the Department of Animal Wealth Development, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt. The experimental protocol was approved by the Animal Care and Ethics Committee at Kafrelsheikh University with an ethical approval number of KFS 2017/9.

Animal source and categorization, sampling and milk composition analysis

A total of 200 pure Holstein–Friesian cows were involved in this study. These cows were sired through artificial insemination and were raised at the Animal Production Research Institute (APRI), Ministry of Agriculture, Al Qarada, Kafrelsheikh, Egypt. The cows were divided into two groups based on their milk yield. Cows producing milk above the herd average were categorized as high milk yield (HMY) animals, while those with milk yields below the herd average were classified as low milk yield (LMY) cows. To select the cows for each group, we considered the extreme values of the milk yield distribution. Specifically, the top 100 animals were included in the high milk yield group, while the bottom 100 animals were included in the low milk yield group. Before categorization, the milk yield records were adjusted to the 305-day milk yield. This value was obtained from the farm records, where it was calculated using the following equation: 305-day milk yield = [total milk yield/(lactation period + 100)] × 405 (Abou-Bakr, 2009).

All cows received the same management practices in terms of feeding and housing. They were milked twice daily, with a 12-h interval, and were fed concentrate mixtures. For data collection, we gathered information on the 305-day total milk yield, lactation length and seasons of lactation from official farm records for each cow. This data spanned the period from January 2017 to December 2020. We analyzed approximately 10,140 milk records from the 1st to 5th lactations (categorized as 1–5, with parities ranging from 1 to 5) of all selected cows. However, for this study, we only considered milk records ($n = 800$) from cows in the second lactation and in the early stage of lactation (2 months post-parturition) since the expressions of most lipolytic enzymes, including LIPE, are modulated during early lactation (Khan *et al.*, 2013; Contreras *et al.*, 2017).

A total of 200 milk samples were collected from the cows following strict aseptic conditions for milk composition analysis. The analysis included determining the percentage of fat, protein, lactose, total solids, water content, milk temperature, milk freezing point and density. These analyses were performed using a Milko-Scope at the Animal Health Research Institute in Egypt. The milk composition parameters were adjusted to account for the 305-day milk yield of the animals.

For the extraction of genomic DNA, 200 blood samples were collected from the jugular vein of the cows, with a volume of 5 ml per cow. EDTA was used as an anticoagulant in the collection process. The blood samples were collected on ice and subsequently stored at a temperature of −20°C until they were used in the DNA extraction process.

Genomic DNA extraction, SNP detection and genotyping

Three fragments of the bovine LIPE gene, specifically containing exon 2, exons 3–5, and exon 6, were amplified through PCR using specific primers and annealing temperatures as provided in Supplementary Table S1. Further details can be found in the online Supplementary File. The PCR products ($n = 25$, each group) were sequenced. The resulting sequences were confirmed and subsequently submitted to GenBank. Sequence analysis and alignment were conducted using Geneious Prime software version 2022.2.2 (Biomatters, Ltd, Auckland, New Zealand). For sequence comparisons, BLASTN available on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) was employed. The alignment of sequences was performed using CLUSTAL-W version 1.8.

Statistical analysis

The association between the identified SNPs and phenotypic traits was analyzed using the GLM procedures of SPSS version 22, employing the following model: $Y_{it} = \mu + S_r + G_i + M_t + GM_{it} + e_{ik}$. In this model, Y_{it} represents the percentages of fat, protein, lactose, total solids, water content, milk temperature, milk freezing point, and density. The symbol μ denotes the overall population mean, S_r donates the random effect of rth sires, G_i represents the effect of the reported SNPs in each animal population, including the subsequent genotypes (homozygous or heterozygous), M_t represents the effect of animal groups based on their milk yield (high and low milk production), GM_{it} tests the interaction between genotype and milk yield level, and e_{ik} accounts for the residual effect. Regarding LIPE 1, since no variations were detected at the genotype level within each group, no interaction was reported, and comparisons were only performed separately for milk yield and genotypes. Genotypic and allelic frequencies were calculated using PopGene32 software. The Hardy–Weinberg equilibrium was assessed using the chi-square test (χ^2) for population comparison. Milk yield and composition are presented as least-squares means ± standard errors.

The polymorphic information content (PIC) was calculated using the GenCal online tool (https://gene-calc.pl/). Linkage disequilibrium (LD) was estimated by calculating the LD coefficient (D') and the absolute association (r^2) between two reported SNPs (LIPE1 and LIPE3). Additionally, the minor allele frequency (MAF) was estimated using Haploview version 4.2 (Cambridge, MA, USA).

Results

PCR successfully amplified different sizes of PCR products, specifically 669 bp for exon 2 with partial sequences from the flanking introns, fragments spanning exons 3, 4, and 5, and 506 bp for exon 6 of the LIPE gene (Fig. 1A, online Supplementary Fig. S1, and Fig. 2A, respectively). The PCR products from various animals, including high and low milk producers, were sequenced and deposited in the GenBank database under the accession numbers ON638900 and ON638901 for exon 2 (LIPE 1) and exons 3–6 (LIPE 2 and LIPE 3, using overlapped primers), respectively.

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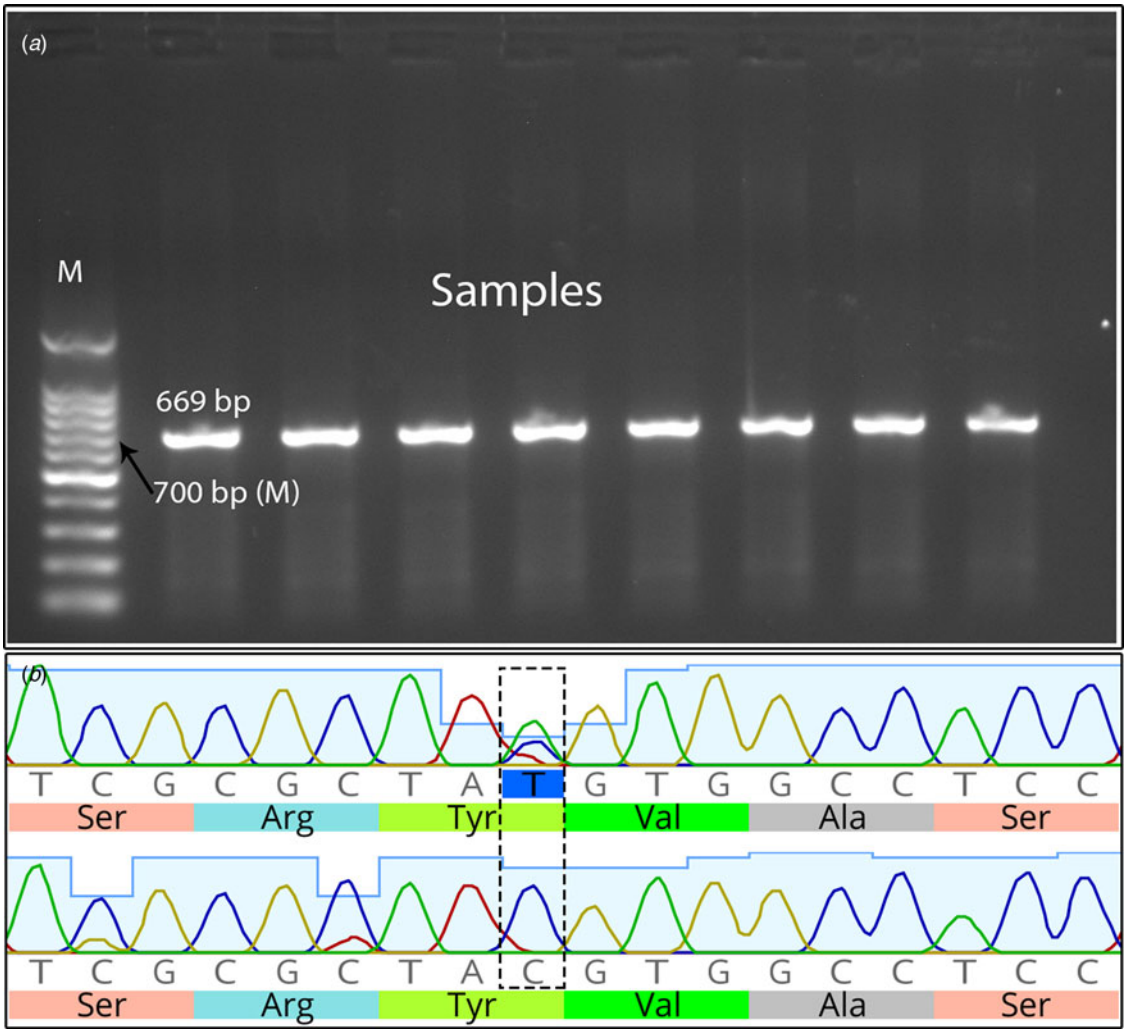

Figure 1. (A) Ethidium bromide-stained 1.5% agarose gel of PCR products from LIPE 1 (exon 2 with partial sequence from the flanking introns) of the cattle *LIPE* gene (*lipase E*, hormone sensitive type); target size 669 bp. M represents the DNA marker. (B) Sequence of exon 2 of the cattle *LIPE*, which indicates the synonymous SNP c.276 T > C (g.50631651 T > C; position 351 of LIPE 1) (dashed box).

Comparison of the nucleotide sequences between HMY and LMY animals revealed two SNPs with different allele frequencies. At nucleotide position 351 of LIPE 1 (exon 2; ON638900), T was replaced with C (c.276 T > C; g.50631651 T > C, based on GenBank sequence ID: CM038096.1, Holstein–Friesian) (Fig. 1B). Similarly, at nucleotide position 1070 of LIPE 3 (exon 6; ON638901), C was replaced with A (c.219C > A; g.50635369C > A, based on GenBank sequence ID: CM038096.1, Holstein–Friesian) (Fig. 2B).

The detected SNPs, genotyping and linkage disequilibrium analysis

The genotype and allele frequencies of the identified SNPs were calculated (online Supplementary Table S2). Upon sequencing the samples, we observed the presence of both heterozygous genotypes (CT in exon 2 and AC in exon 6) and homozygous genotypes (CC for both SNPs). The genotypes of the reported SNPs in LIPE 3 for high milk yield cows were consistent with the Hardy–Weinberg equilibrium ($P > 0.05$), Notably, however, the genotypes of the detected SNPs in LIPE 1 (found in both HMY and LMY cows) and LIPE 3 (specific to LMY cows) deviated from it ($P < 0.05$). Additionally, we calculated the genetic indices of the reported SNPs in LIPE 1 and LIPE 3 (exon 2 and 6, respectively) (Table 1). The pair-wise LD analysis between the LIPE1 and LIPE3 SNPs in the HMY and LMY samples demonstrated the absence of LD. This conclusion is supported by the values of the linkage disequilibrium coefficient and the absolute association, which were $D' = 0.00$ (0%) and $r^2 = 0.00$, respectively (online Supplementary Fig. S2). These results suggest that there is no relationship between the two SNPs.

Association analysis

Association analyses of the SNP c.276 T > C in exon 2 (g.50631651 T > C; position 351 of LIPE 1) revealed that animals with the TC genotype exhibited higher milk yield compared to those with the CC genotype (Table 2). Furthermore, this SNP demonstrated associations with various milk composition traits, including milk temperature, milk freezing point, density, and percentages of fat, protein, lactose, ash, and solids-not-fat. The homozygous CC genotype displayed higher values for these traits.

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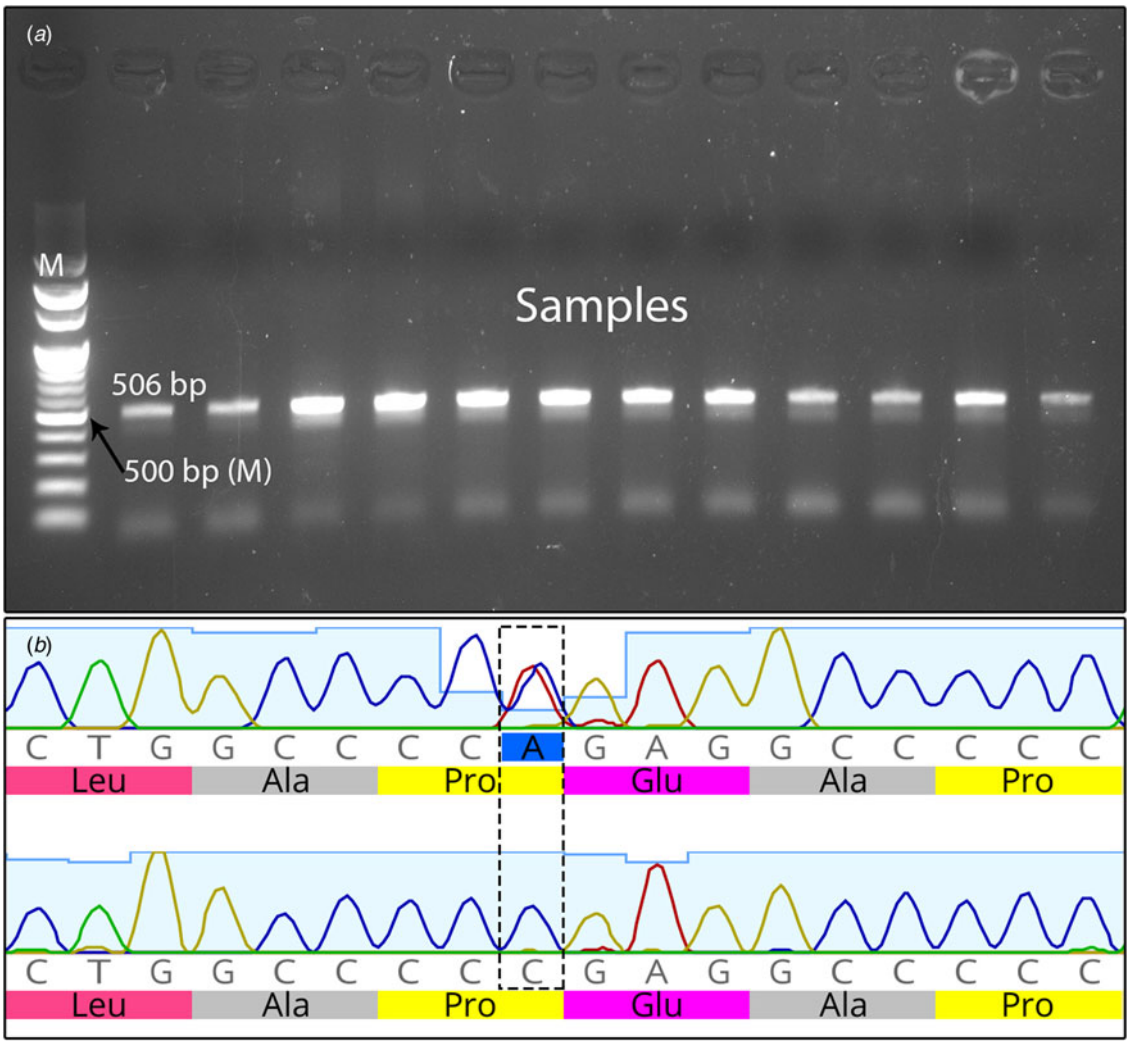

Figure 2. (A) Ethidium bromide-stained 1.5% agarose gel of PCR products from LIPE 3 (exon 6 with partial sequence from the flanking introns) of the cattle *LIPE* gene (*lipase E*, hormone sensitive type); target size 506 bp. M represents the DNA marker. (B) Sequence of exon 6 of the cattle *LIPE* gene, which indicates the synonymous SNP c.219C > A in exon 6 (g.50635369C > A; position 1070 of LIPE3) (dashed box).

In the case of LIPE 2 (exons 3–5), no SNPs were detected, indicating that the observed variations between high- and low-milk producers can be attributed solely to differences in milk yield (online Supplementary Table S3). However, the SNP c.219C > A in exon 6 (g.50635369C > A; position 1070 of LIPE 3) exhibited a significant association with milk composition. Specifically, the CA genotype demonstrated higher percentages of fat, protein and lactose compared to the CC genotype. While the CA and CC genotypes exhibited similar percentages of fat and lactose ($P > 0.05$), the protein percentage was significantly higher in the CA genotype than the CC genotype. Notably, this effect was more pronounced in high milk producers compared to low milk producers (Table 3).

Discussion

Although the identified SNPs, c.276 T > C in exon 2 (g.50631651 T > C; position 351 of LIPE 1) and c.219C > A in exon 6 (g.50635369C > A; position 1070 of LIPE 3) of the cattle *LIPE* gene, are located at the third position of genetic codons and do

Table 1. Genetic indices of the identified SNPs

	Ho		He		Ne		PIC	
	HMY	LMY	HMY	LMY	HMY	LMY	HMY	LMY
LIPE1	1.00	0.00	0.5	0.0	2	0.00	0.375	0.0
LIPE3	0.25	0.75	0.219	0.469	1.28	1.88	0.195	0.359

LMY, low milk yield; HMY, high milk yield.
Ho & He donate observed and expected heterozygosity, respectively. Ne represents the effective number of alleles. PIC means polymorphic information content.

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Table 2. Association of different LIPE 1 (exon 2) genotypes with milk characteristics in Holstein Friesian cows

Items	Animal's production	Genotype	Mean ± SE	*P* value*
Milk yield (kg)	High milk yield	TC	3853.81 ± 82.32[a]	< 0.001
	Low milk yield	CC	2060.68 ± 80.24[b]	
Milk Temp. (°C)	High milk yield	TC	32.52 ± 0.31[b]	0.001
	Low milk yield	CC	34.21 ± 0.32[a]	
Fat (%)	High milk yield	TC	3.03 ± 0.180[b]	0.041
	Low milk yield	CC	3.58 ± 0.184[a]	
SNF (%)	High milk yield	TC	7.92 ± 0.167[b]	0.023
	Low milk yield	CC	8.49 ± 0.172[a]	
Density (g/l)	High milk yield	TC	28.01 ± 0.312[b]	0.025
	Low milk yield	CC	29.05 ± 0.320[a]	
Protein (%)	High milk yield	TC	2.99 ± 0.023[b]	< 0.001
	Low milk yield	CC	3.12 ± 0.024[a]	
Lactose (%)	High milk yield	TC	4.42 ± 0.045[b]	0.001
	Low milk yield	CC	4.67 ± 0.049[a]	
Ash (%)	High milk yield	TC	0.67 ± 0.005[b]	< 0.001
	Low milk yield	CC	0.70 ± 0.005[a]	
Milk Freezing point (°C)	High milk yield	TC	−0.51 ± 0.005[b]	< 0.001
	Low milk yield	CC	−0.54 ± 0.005[a]	

SNF, solids-not-fat; data are presented as least squares means ± standard errors
**P* values denote the effect of genotype and animal groups (according to milk yield: high or low) on milk quality. The *P* values were the same for the two factors because each animal group had only one genotypic form. No interaction was reported because there are no variations within the same group that make the *P* values for genotype and milk yield the same. The lowercase letters denote the statistical significances between the two categories of milk yield.

not result in amino acid changes, they show associations with milk yield and composition. The precise impact of such SNPs on gene expression is still unclear (Robert and Pelletier, 2018). It is possible that these SNPs may influence translation efficiency, thereby affecting the levels of protein production or mRNA half-life. It is important to consider synonymous SNPs, also known as 'silent' SNPs, as they have been found to modulate translation efficiency and are strongly recommended for interpretation in association studies (Waldman *et al*., 2011). Factors such as rare tRNAs encoding specific anticodons complementary to synonymous codons on mRNAs, wobble base pairing and adjacent codon interactions can influence translation rates (Plotkin and Kudla, 2011; Brule and Grayhack, 2017). Synonymous codons can affect ribosome occupancy time, leading to changes in translation elongation rates and subsequent modulation of co-translational protein folding (Plotkin and Kudla, 2011; Brule and Grayhack, 2017). Gustafsson *et al*. (2004) demonstrated that specific codons can significantly upregulate heterologous expression by more than 1000-fold. Moreover, SNPs within genes can result in posttranscriptional changes, including alterations in mRNA splicing and stability, nucleocytoplasmic export, and translation processes (Robert and Pelletier, 2018). This information underscores the crucial role of SNPs in shaping associated phenotypes.

Generally, the majority of SNPs do not lead to changes in gene expression and, consequently, they do not affect the function and structure of the resulting protein (Karki *et al*., 2015). Nonetheless, ongoing research aims to identify SNPs and explore their potential associations with production- and health-related traits. For instance, Fang *et al*. (2017) detected variants in the 5′-terminal sequence of the cattle *LIPE* gene, which exhibited a strong association with fat deposition traits and the fatty acid composition of the fatty tissue. Furthermore, a population genetic analysis of four missense mutations in exon 8 and a synonymous mutation in exon 9 of the cattle *LIPE* gene revealed a significant association of the three missense mutations with intramuscular fat content (Gui *et al*., 2020).

Our results demonstrate a significant association between the TC genotype of the SNP c.276 T > C in exon 2 (g.50631651 T > C; position 351 of LIPE 1) and the CC genotype of the SNP c.219C > A in exon 6 (g.50635369C > A; position 1070 of LIPE 3) with high milk yield and favorable milk composition. However, the LD analysis and r^2 did not show any association between the two detected SNPs. In a study involving goats, Zidi *et al*. (2010) identified a triallelic polymorphism in exon 2 (c.327C > A > T) and polymorphisms in exon 3 (c.558C > T and exon 6 (c.1162G > T) of the *LIPE* gene. Association analysis revealed that the *LIPE* genotypes in goats are linked to milk yield and composition. These findings, combined with our results, raise the question of whether exons 2 and 6 play a functional role in LIPE expression, which necessitates further studies and analyses, such as haplotype associations between different haplotypes and milk traits and genome-wide association studies (GWAS).

Our results also highlight the association between the c.276 T > C in exon 2 (g.50631651 T > C; position 351 of LIPE 1) and milk composition. Similar studies have demonstrated that synonymous polymorphisms in the *LIPE* gene are significantly associated with lipid metabolism, fatty acid composition, fat deposition and milk composition (Zidi *et al*., 2010; Fang *et al*.,

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Table 3. Association of LIPE 3 (exon 6) genotypes with milk characteristics in Holstein Friesian cows

				P value		
Items	Animal's population	Genotype	Mean ± SE	Animal's production	Genotype	Interaction
Milk yield (kg)	High milk yield	CC	3879.34 ± 94.77^{Aa}	<0.001	0.567	0.857
		CA	3777.20 ± 164.14^{Aa}			
	Low milk yield	CC	2100.00 ± 164.74^{Ba}			
		CA	2046.64 ± 98.09^{Ba}			
Milk Temperature (°C)	High milk yield	CC	32.36 ± 0.36^{Ba}	0.002	0.877	0.168
		CA	33.00 ± 0.62^{Ba}			
	Low milk yield	CC	34.80 ± 0.63^{Aa}			
		CA	34.00 ± 0.37^{Aa}			
Fat (%)	High milk yield	CC	3.04 ± 0.162^{Ab}	0.921	0.031	0.769
		CA	3.62 ± 0.281^{Aa}			
	Low milk yield	CC	3.13 ± 0.279^{Ab}			
		CA	3.58 ± 0.168^{Aa}			
SNF (%)	High milk yield	CC	7.85 ± 0.197^{Aa}	0.097	0.503	0.771
		CA	8.124 ± 0.341^{Aa}			
	Low milk yield	CC	8.412 ± 0.347^{Aa}			
		CA	8.519 ± 0.204^{Aa}			
Density (g/l)	High milk yield	CC	28.137 ± 0.366^{Ba}	0.047	0.930	0.364
		CA	27.612 ± 0.635^{Ba}			
	Low milk yield	CC	28.730 ± 0.630^{Aa}			
		CA	29.163 ± 0.379^{Aa}			
Protein (%)	High milk yield	CC	3.46 ± 0.174^{Ab}	0.014	0.012	0.287
		CA	4.38 ± 0.302^{Aa}			
	Low milk yield	CC	3.09 ± 0.300^{Bb}			
		CA	3.47 ± 0.180^{Ba}			
Lactose (%)	High milk yield	CC	4.545 ± 0.093^{Ab}	0.756	0.048	0.374
		CA	4.922 ± 0.161^{Aa}			
	Low milk yield	CC	4.622 ± 0.093^{Ab}			
		CA	4.762 ± 0.096^{Aa}			
Ash (%)	High milk yield	CC	0.668 ± 0.006^{Ba}	0.004	0.569	0.569
		CA	0.668 ± 0.011^{Ba}			
	Low milk yield	CC	0.690 ± 0.010^{Aa}			
		CA	0.700 ± 0.006^{Aa}			
Milk Freezing point (°C)	High milk yield	CC	-0.515 ± 0.005^{Ba}	0.004	0.575	0.670
		CA	-0.516 ± 0.009^{Ba}			
	Low milk yield	CC	-0.535 ± 0.008^{Aa}			
		CA	-0.543 ± 0.006^{Aa}			

SNF stands for solids-not-fat, and the data are presented as least squares means ± standard errors. Different uppercase letters indicate statistical significance between the high and low milk yield groups, while lowercase letters denote statistical significance between different genotypes within each milk yield category.

2014, 2017; Kong *et al.*, 2022). These findings emphasize the regulatory role of LIPE in lipolytic function. These synonymous coding SNPs can potentially lead to changes in protein folding, binding, cellular localization and RNA secondary structure (Komar, 2007; Shatoff and Bundschuh, 2020). Such molecular alterations can impact protein function, as well as the pathways and expressed traits that depend on it. Consequently, modifications in the genomic structure of LIPE can modulate its function, thereby influencing milk production and composition. However, the limited number of samples in our study restricted the

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detection of certain genotypes, and further investigations should explore the potential effects of these remaining genotypes.

LIPE is a crucial enzyme responsible for lipolysis, which involves the release of free fatty acids from adipose tissue, and it plays an essential role in lipogenesis and adipose metabolism (Kraemer and Shen, 2006). This enzyme is predominantly expressed in adipose tissues and other tissues, such as macrophages, muscle, adrenal gland, testes and pancreatic islets, all of which have significant roles in mediating lipid metabolism and related pathways like steroidogenesis, insulin secretion and sensitivity and spermatogenesis (Saltiel, 2000; Kraemer and Shen, 2002, 2006). The interaction between LIPE and polymerase I and transcript release factor (PTRF), a major caveolae-associated protein highly expressed in adipocytes, is suggested to be regulated by insulin during lipolysis (Aboulaich *et al.*, 2006, 2011; De Koster *et al.*, 2018). In bovine mammary epithelial cells, *LIPE* was downregulated by insulin or dexamethasone and upregulated by saturated long-chain fatty acids (Yonezawa *et al.*, 2008). Notably, during late pregnancy and early lactation, levels of fatty acids and insulin increase to meet the requirements of mammogenesis and lactation (Greenfield *et al.*, 2000a, 2000b). During this time, *LIPE* appeared to be downregulated (Martín-Hidalgo *et al.*, 2005), while it was later upregulated during lactation (i.e., during peak and midlactation) (McNamara *et al.*, 1987; Zachut, 2015), suggesting its essential roles in milk production. Additional support for these findings comes from studies in dairy cattle, where basal lipolytic activity was lower during lactation compared to the dry period, and the use of an LIPE inhibitor (CAY) significantly reduced the basal lipolytic activity in fresh and lactating animals, with minimal or negligible effects in dry animals (De Koster *et al.*, 2018).

In conclusion, our results demonstrate that the *LIPE* gene plays a significant role in modulating milk production and composition. Specifically, the SNPs c.276 T > C (g.50631651 T > C) in exon 2 and c.219C > A (g.50635369C > A) in exon 6 hold promise as potential indicators for selecting superior milk producers in animal breeding strategies. To the best of our knowledge, this study represents the first evidence of a correlation between LIPE gene SNPs and milk production and composition in cattle. The identified markers exhibited associations with important milk traits, such as fat percentage, protein content, and lactose levels. These findings have significant implications for livestock improvement programs. By utilizing these favorable SNPs, it becomes possible to selectively breed animals with superior production traits, facilitating marker-assisted selection and the development of a high-quality genetic profile in livestock populations.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S002202992300050X

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