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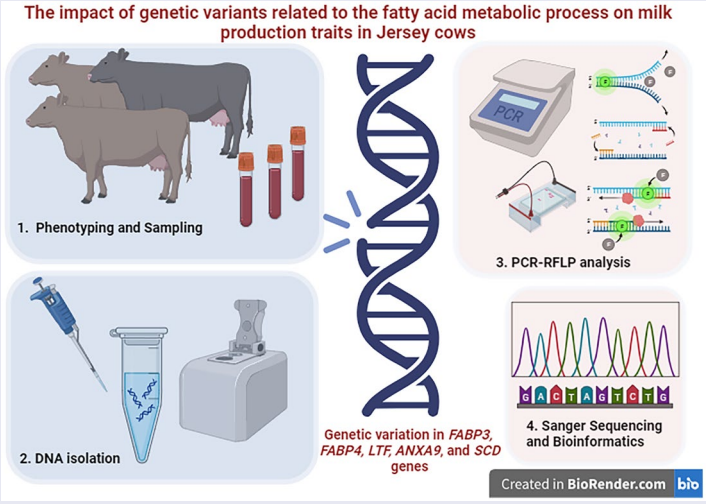
Sena Ardicli[a,b], Nursen Senturk[a], Berkay Bozkurt[c], Huseyn Babayev[b], Tuğçe Selvi[d], Stephen Skolnick[b,e], Hivdanur Ter[f], Beyza Aktas[f], Ayse Isık[f], Ozgur Toprak Ay[g], Ozge Ardicli[b,h] and Ozden Cobanoglu[a]

[a]Department of Genetics, Faculty of Veterinary Medicine, Bursa Uludag University, Bursa, Turkiye; [b]Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Switzerland; [c]Department of Biotechnology and Bioengineering, Graduate School of Science and Engineering, Izmir Institute of Technology, Izmir, Turkiye; [d]Department of Animal Science, Faculty of Veterinary Medicine, Bursa Uludag University, Bursa, Turkiye; [e]SEED Inc. Co, Los Angeles, CA, USA; [f]Department of Molecular Biology and Genetics, Faculty of Science and Arts, Bursa Uludag University, Bursa, Turkiye; [g]Department of Molecular Biology and Genetics, Faculty of Science, Izmir Institute of Technology, İzmir, Turkiye; [h]Division of Food Processing, Milk and Dairy Products Technology Program, Karacabey Vocational School, Bursa Uludag University, Bursa, Turkiye

ABSTRACT

The synthesis of fatty acids plays a critical role in shaping milk production characteristics in dairy cattle. Thus, identifying effective haplotypes within the fatty acid metabolism pathway will provide novel and robust insights into the genetics of dairy cattle. This study aimed to comprehensively examine the individual and combined impacts of fundamental genes within the fatty acid metabolic process pathway in Jersey cows. A comprehensive phenotypic dataset was compiled, considering milk production traits, to summarize a cow's productivity across three lactations. Genotyping was conducted through PCR-RFLP and Sanger sequencing, while the association between genotype and phenotype was quantified using linear mixed models. Moderate biodiversity and abundant variation suitable for haplotype analysis were observed across all examined markers. The individual effects of the *FABP3*, *LTF* and *ANXA9* genes significantly influenced both milk yield and milk fat production. Additionally, this study reveals novel two-way interactions between genes in the fatty acid metabolism pathway that directly affect milk fat properties. Notably, we identified that the GGAAGG haplotype in *FABP3*×*LTF*×*ANXA9* interaction may be a robust genetic marker concerning both milk fat yield and percentage. Consequently, the genotype combinations highlighted in this study serve as novel and efficient markers for assessing the fat content in cow's milk.

GRAPHICAL ABSTRACT



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Milk fat content; jersey breed; genetic marker; fatty acid biosynthesis

CONTACT Sena Ardicli sardicli@uludag.edu.tr, sena.ardicli@siaf.uzh.ch Department of Genetics, Bursa Uludag University, Faculty of Veterinary Medicine, 16059 Nilufer, Bursa, Turkey

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Introduction

Milk constituents, derived from substrates present in blood plasma, undergo synthesis within the epithelial cells of the mammary gland. This intricate biological process involves the transformation of specific substances circulating in the blood into various milk components. The process of milk production is complicated, involving a multitude of metabolic activities and, consequently, engaging numerous molecular pathways. Aligned with this, the complex genetic control of milk production is subject to polygenic inheritance. Genomic selection enables the identification of genetically superior animals at an early age by providing accurate genomically enhanced breeding values through DNA testing before reaching sexual maturity. This approach, involving the heavier use of young, genetically superior males and females, contributes to a shortened generation interval and increased selection intensity as breeders leverage genomic testing to identify a larger pool of potentially superior animals. Thus, increased precision and accelerated genetic improvement can be achieved.[1–5]

Milk serves as a rich source of protein, fats, carbohydrates (primarily lactose), and micronutrients. While it is a biological product shaped by the collaboration of various hormones, enzymes, and genes,[1,6] specific pathways are essential for both milk production and, notably, its nutritional content. In this context, the fatty acid metabolic process pathway stands out as one of the most crucial. Essentially, numerous genes are directly or indirectly involved in this pathway, with certain genes prominently situated at the foundation, especially in the context of studies within dairy cow farming. Of these, fatty acid-binding proteins (FABPs) are the most prominent and are members of the superfamily of lipid-binding proteins.[7] They form reversible bonds with long-chain fatty acyl-coenzyme A (LCFA-CoA) or free LCFAs, facilitating their transportation into the cell and directing them to various sites for β-oxidation or triglyceride and phospholipid biosynthesis.[8] In doing so, FABPs play a role in modulating fatty acid concentrations within cells influencing lipid metabolism.[9–11] Widely distributed, they are found in tissues with high fatty acid demands, such as the mammary gland during lactation.[12,13] Research indicates that among all the FABP proteins, FABP3 plays the most crucial role in fat production in the mammary gland of cattle.[13,14] However, the extent of FABP3's involvement in the signalling pathway for milk fat synthesis requires additional investigation. Fatty acid protein serves as the primary protein facilitating swift diffusion and selective targeting of long-chain fatty acids toward specific organelles for metabolism.[8] The bovine *FABP4* gene, engaged in lipid catabolism, contributes to lipid hydrolysis and intracellular fatty acid trafficking through its interaction with hormone-sensitive lipase.[11,15]

Lactotransferrin (*LTF*) belongs to the transferrin gene family, whose members encode a variety of highly bioactive protein products. Among these, *LTF* exhibits immunomodulatory, antimicrobial, antiviral, antitumoral and anti-inflammatory functions.[2,16] Furthermore, this gene impacts plasma lipid concentrations.[17] LTF protein can bind two iron molecules, rendering them inaccessible to pathogenic organisms, and exhibits bacteriostatic, antifungal and bactericidal properties. It interacts with lipid A of lipopolysaccharide (LPS) in bacterial membranes and may serve as a potential indicator of mastitis.[18] It is important to note that Arnould et al.[18] showed a positive correlation between LTF content predicted using mid-infrared spectrometry and milk fat percentage. Another vital gene in the fatty acid metabolic process pathway, Annexin A9 (*ANXA9*), encodes the Ca^{+2}-dependent phospholipid-binding protein, which is a fatty acid transporter linked to the cytoplasmic membrane.[2,12,19,20] Annexins play various roles within the cell, participating in signal processing, endocytosis, membrane trafficking, calcium channel formation, and the regulation of inflammatory responses throughout the cell cycle. The bovine chromosome 3 hosts the mapped gene for *ANXA9*. Various studies have also identified QTLs associated with certain milk yield and composition traits on this chromosome.[12,19,21] Stearoyl-CoA desaturase (*SCD*), a gene involved in fatty acid synthesis, is crucial for the lipogenesis.[22,23] It acts as a rate-limiting enzyme in the synthesis of monounsaturated fatty acids (MUFAs) from saturated FAs (SFAs). High levels of *SCD* expression are observed in lactating ruminants, and its significance is well recognized in relation to the composition of milk fat in these animals.[24]

Fatty acids are crucial contributors to the nutritional quality of milk, making the fatty acid metabolic process critical in the biology of milk production. While individual gene effects within this pathway have been documented in various studies, there is a lack of research investigating the collective impact of genes in the pathway and their interplay. Additionally, limited knowledge exists regarding the association of this pathway with milk content traits in Jersey cattle, an important dairy breed. In recent years, Jersey milk has gained popularity, particularly due to its high-fat content and beta-casein characteristics (increased frequency of

beta-casein A2A2 genotype). Many studies focus on a singular lactation period of a cow, neglecting the analysis of longitudinal records. The aim of the study was to assess significant genes, such as *FABP4*, *FABP3*, *LTF*, *ANXA9* and *SCD*, that play crucial roles in the genetic regulation of metabolic pathways, specifically focusing on fatty acid biosynthesis. Taken together, this study, based on comprehensive data spanning three lactations from Jersey cows exposed to identical environmental conditions, presents entirely novel and crucial insights into genotype-phenotype relationships and gene interactions.

Materials and methods

Animal material and data collection

The subjects in the current study comprised 314 pure-bred Jersey cows, all belonging to the same commercial dairy herd located in the South Marmara region of Turkiye. These cows were subjected to identical feeding and environmental conditions. The animals' diet consisted of a mixed ration comprising corn and vetch silage, concentrated feed, grass and wheat straw. Additionally, during the dry season (late June to early September), they had the opportunity to graze on pasture for approximately 8–10 hours following the morning milking. The pasture type varies seasonally. During this period, natural grasslands predominate, characterized by a mix of Mediterranean grasses such as tall fescue, ryegrass and clovers. These are supplemented with locally sourced hay and concentrates to ensure nutritional adequacy when pasture growth is insufficient due to the dry weather. Cows calved year-round. The management of the farm remained unchanged during the sampling period.

All animals were housed in open-stall barns and underwent two milking per day. The mean results from two consecutive PM and AM milkings were used. Individual milk samples were collected monthly during milk test days over the course of three lactations. Cows were milked in milking parlours equipped with electronic devices that autonomously recorded the milk quantity. Thus, milk yield for each cow at each milking was recorded using DeLaval's Alpro Herd Management System (DeLaval International, Tumba, Sweden). In this context, the calculation of the 305-day milk yield (305-DMY) was based on the dataset containing individual daily milk yield records. Thus, records for both test-day milk yield (TDMY) and 305-DMY were obtained. Milk protein and fat content were analyzed using an ultrasonic milk analyzer (MilkoScan™ FT1, Foss, Hillerød, Denmark). Protein and fat yields were computed using records related to milk traits. Data on composition traits, including milk fat percentage (MFP), milk protein percentage (MPP), test-day fat yield (TDFY), test-day protein yield (TDPY), 305-day fat yield (305-DFY), and 305-day protein yield (305-DPY), were obtained from the milk control samples. All test day milk records and samples were collected monthly from each cow over the lactation period, spanning from 30 to 300 days, with a total of 10-20 collections per cow. Figure S1 illustrates the descriptive characterization of the phenotypic traits within the population.

A large phenotypic dataset of milk production traits was constructed (total herd size was 550), aiming to provide a comprehensive and reliable overview of a cow's productive history over three lactations. As such, cows lacking or unorganized pedigree ($n = 140$) and production records ($n = 88$) were not included in the study. Furthermore, cows with body condition scores (BCS, 1 to 5 scale with half-point increments) that were either too low or relatively high were omitted from the association analysis. In this context, cows with BCS <2 and >4 were excluded ($n = 8$). The animals included in the study were healthy, without any known chronic diseases or signs of mastitis. Their lactation ranks varied between 1 and 5. Ultimately, analyses were performed on a final cohort of 314 cows that met the inclusion criteria for the research population.

Genetic analysis

In this study, the polymorphisms of five fatty acid metabolic process-related genes (Figs. 1 and 2) were genotyped, including *FABP4*, *FABP3*, *LTF*, *ANXA9* and *SCD* in Jersey dairy cattle. DNA extraction was carried out using both the phenol-chloroform-isoamyl alcohol method (25:24:1) and Roche's High Pure PCR Template Preparation Kit (Cat No. 11796828001, Roche Applied Science, Germany) from approximately 4 mL of whole blood obtained from the *vena jugularis* of each cow. DNA concentration (ng/mL) and the absorbance ratio at 260/280 were determined using a Nanodrop microvolume spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, Wilmington, DE, USA). Samples at the acceptable amount and purity ranges (50–100 ng/µL of pure DNA with a 260/280 value between 1.7 and 1.9) were used for subsequent analysis.

The detection of nucleotide substitutions in the chosen five genes was carried out using PCR-RFLP and Sanger sequencing. Table 1 presents the primer sequences and PCR conditions for each locus.

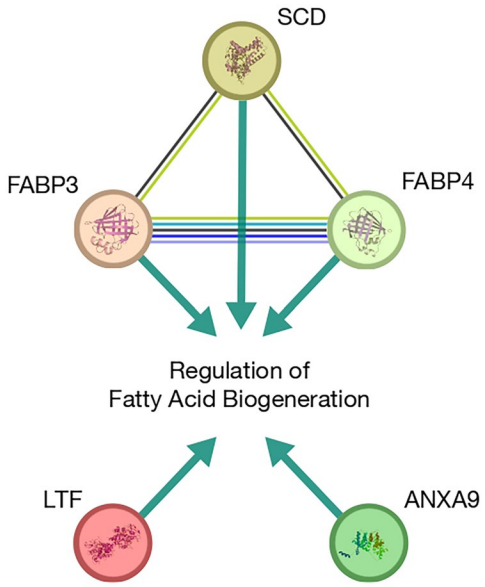


Figure 1. Pathway analysis of the markers in the study. A mechanistic approach was employed to examine the regulation of fatty acid biosynthesis and its associated marker effects. All genetic markers examined in this study play a crucial role in the formation of fatty acids, highlighting the significant value of cow's milk due to its high fat content. The STRING database (version 12) was utilized for the construction of biological pathway and subsequent interpretations. https://string-db.org/.

The specificity of the primer sequences was verified through BLAST searches conducted on the NCBI GenBank database. PCR amplifications were performed in a total volume of 25 or 50 µL using 2-3 µL of total purified DNA. PCR mix also contained 1 µL (0.025 mM) of each primer (Macrogen Company, Seoul, South Korea), 12.50 mL PCR master mix (OneTaq Quick-Load 2× MM, New England BioLabs (NEB) Inc., Ipswich, MA, USA, Cat# M0486S or Thermo scientific PCR master mix (2×), Cat# K0171, NY, USA), and 8 mL nuclease-free water (Thermo Scientific, Cat#R0581). Amplifications were conducted using the MyGenie 96 thermal block from Bioneer Corporation (South Korea), Palm Cycler GC1-96 from Corbett Research (Australia), or T100 Thermal Cycler from Bio-Rad (USA).

Digestion of the PCR product was performed by incubating 15 µL with 0.50–1 mL of the corresponding restriction enzyme (Table 1), obtained from either New England Biolabs (NEB), Ipswich, MA, USA, or Thermo Fisher Scientific. The enzyme-buffer mix, comprising 1 µL of the restriction enzyme, 8 µL of the corresponding buffer, and 10 µL of the PCR product, was prepared. The incubation period was adjusted from 3 hours to overnight. The thermal cyclers mentioned above were also utilized for the incubation of restriction enzyme digestion. Gel electrophoresis (90 V for approximately 1 hour) with 2–3% agarose gels (Sigma–Aldrich, Steinheim, Germany) was employed to confirm the quality of both PCR amplification and RFLP products for the SNPs. Gel images were captured under UV light and were evaluated for genotyping using a gel documentation system (DNR-Minilumi, DNR Bio-Imaging Systems, Israel). The PCR-RFLP electrophoresis patterns for each genetic marker are shown in Fig. S2A–J.

For additional validation, Sanger sequencing was performed on representative samples (n = 20) from each genotype, targeting the specified SNPs at the bovine *FABP4*, *FABP3*, *LTF*, *ANXA9*, and *SCD* genes (Fig. 3A–E). DNA samples were analysed using primers designed for the specific SNP regions and the FastStart High Fidelity PCR System, DNTPack Kit from Roche Diagnostics, Mannheim, Germany. The resulting PCR amplicons were purified through the Zymo DNA Clean and Concentrator-5 kit by Zymo Research. After purification, the samples underwent sequencing using an ABI 3500 automated sequencer from Applied Biosystems. The sequencing data were scrutinized and edited using BioEdit (v8.1.0) and Benchling softwares (https://benchling.com/). Finally, the findings were cross-verified and confirmed by referencing the cattle genome available on the Ensembl genome browser[28] and the results obtained from real-time qPCR assays. Lastly, the outcomes were cross-referenced and validated using the cattle genome available on the Ensembl genome browser.[28] We used the STRING database (v12)[29] to construct biological pathways and further interpretations.

Statistical analysis

The genotypic and allelic frequencies were computed following the methodology outlined by Falconer and Mackay.[30] Population genetic indices, such as gene homozygosity (Ho), gene heterozygosity (He), effective allele numbers (Ne), and the polymorphism information content (PIC), were computed based on allele frequencies according to Nei and Roychoudhury[31] and Botstein et al.[32] The Fixation Index (Fıs) was estimated from the values of theoretical and experimental heterozygosities. We used the Shannon-Weaver index (*H'*) value as a parameter of genetic diversity.

Linear mixed models were chosen according to the coefficient of determination (R^2) values, in the ANOVA to evaluate the association between genotype and phenotype. The ultimate statistical model included the fixed effect of calving season, the regression effect of days in lactation, and the fixed effect of lactation

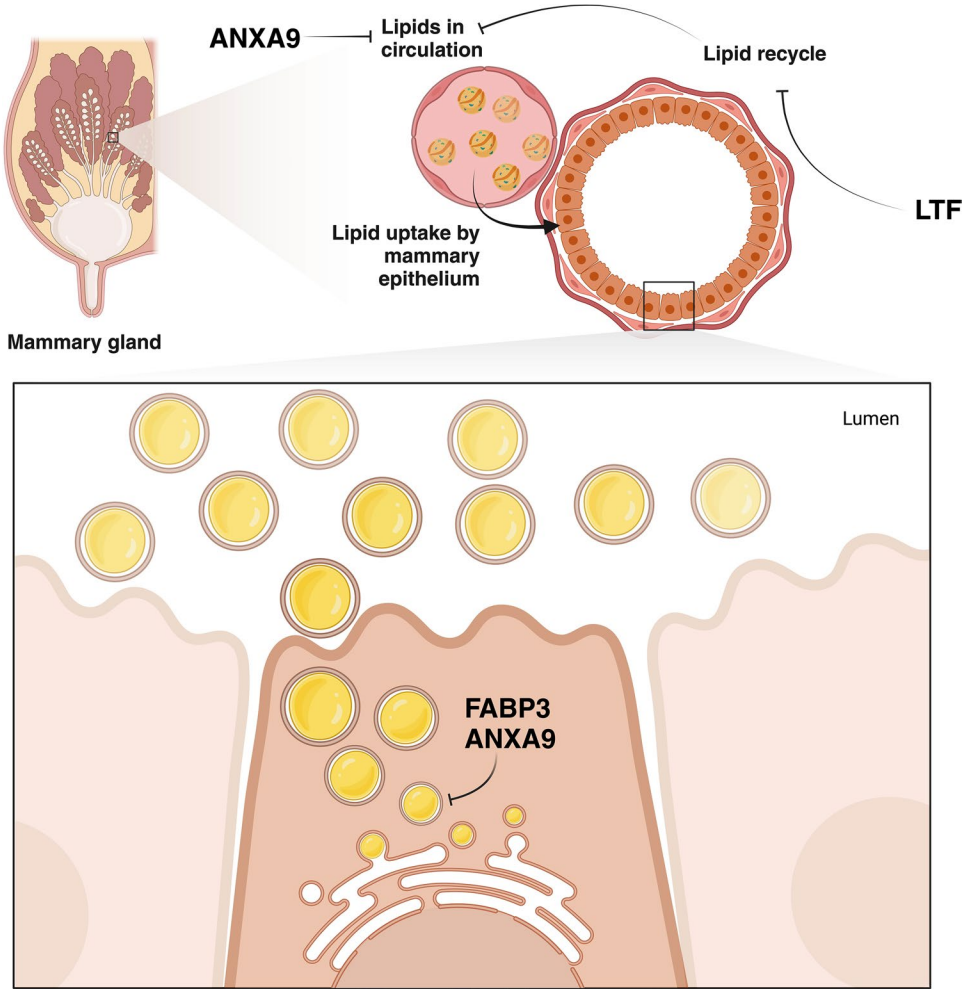


Figure 2. The proteins described herein form the basis of the fatty acid metabolic pathway. Understanding the genetic architecture of this pathway is crucial for elucidating the diversity in fatty acid composition of cow's milk. Key players in the genetic regulation of this metabolic pathway, particularly in fatty acid biosynthesis, include the genes *FABP4*, *FABP3*, *LTF*, *ANXA9*, and *SCD*. Fatty acids significantly influence the composition of milk fat, playing a vital role in energy metabolism during lactation and determining the nutritional value of milk. As a result, the metabolic pathway of fatty acids occupies a prominent position in milk production biology.

Table 1. Primer sequences (from 5′ to 3′), PCR conditions, and restriction enzymes used for genotyping the polymorphisms in the current study.

Genetic marker	Amplicon size, bp	Primer sequence (5′ to 3′)	Annealing temperature, C°	Restriction enzyme	Reference
FABP4 V110M[1]	565	F: ACCCCTATGATGCTATTCCACA R: ATACGGTTCACATTGAGAGGGA	60	*Nla*III	Shin et al. (2012)[11]
FABP3	438	F: GTGAGTTGAGGAAGGGGCTGTG R: TAGGTCTCCACCTCTTGTCCTTCAG	63	*Aci*I	Wu et al. (2005)[25]
LTF	301	F: GCCTCATGACAACTCCCACAC R: CAGGTTGACACATCGGTTGAC	58	*Eco*RI	Wojdak-Maksymiec et al., (2006) [16]
ANXA9 H84R[2]	241	F: TCCCAGACCTTGTCATFTCC R: CTCCTGGGAATCAGTGTGGT	55	*Nla*III	Kulig et al. (2010)[26]
SCD[3]	197	F: GTGTCCTGTTGTTGTGCTTCATCCTGCC R: AATATTCTCTCGGGGGTTGATGGTCTTG	63	*Nco*I	Taniguchi et al. (2004)[27]

FABP4: fatty acid-binding protein; *FABP3*: fatty acid-binding protein; *LTF*: lactoferrin; *ANXA9*: annexin A9; *SCD*: stearoyl CoA desaturase.
[1]Valine to methionine substitution at amino acid position 110.
[2]Histidine to arginine substitution at amino acid position 84.
[3]A synonymous mutation (p.Pro234=).

rank, as well as the impact of animal factor (performance metrics of an individual cow across three lactations). Initially, the model included the sire effect; however, it was subsequently excluded due to the limited number of genotyped bulls that were progenies of the same sire, in accordance with the suggestions

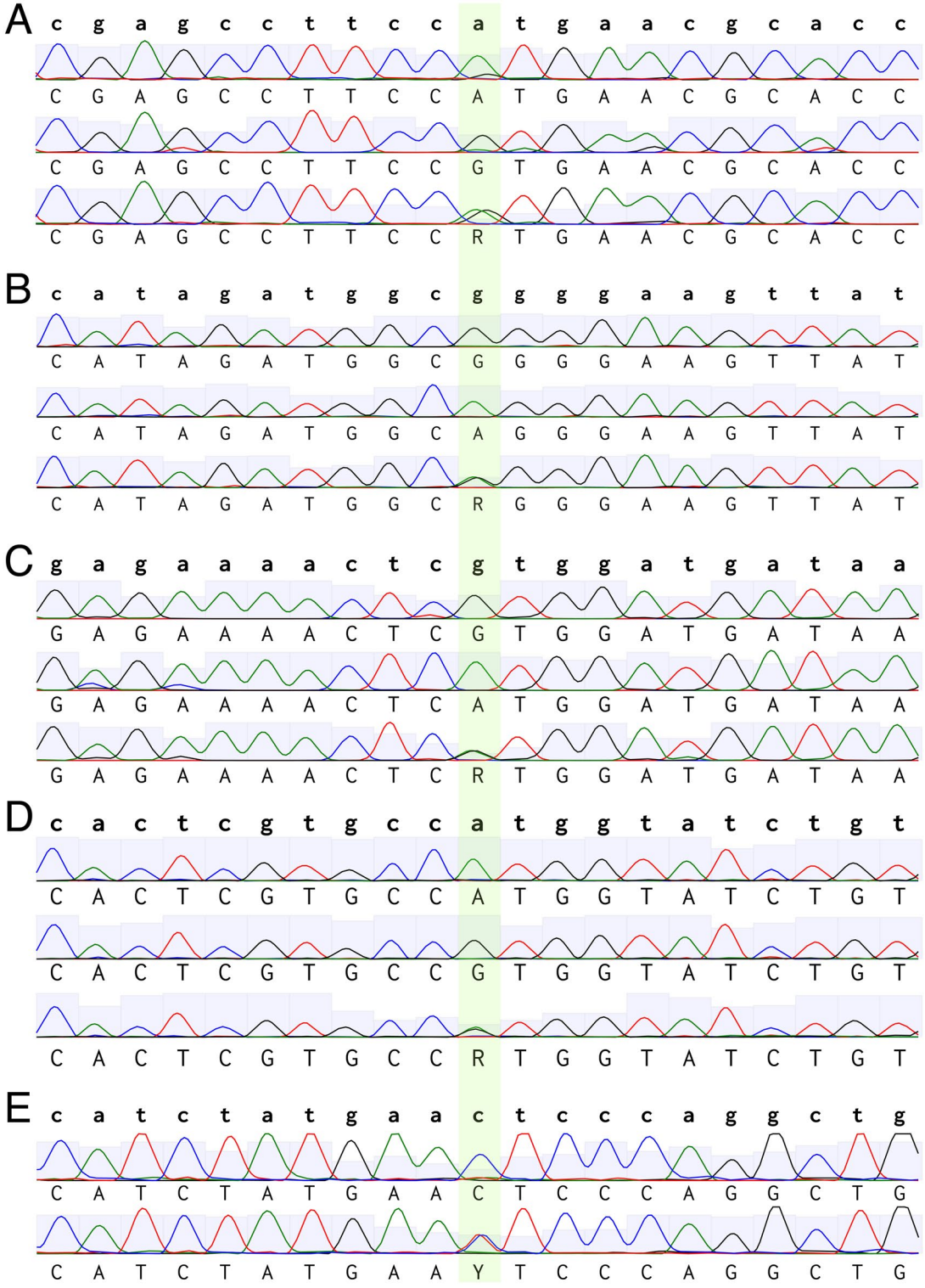


Figure 3. Sanger sequencing analysis of representative samples from each genotype, targeting specified SNPs in bovine *ANXA9*, *FABP3*, *FABP4*, *SCD*, and *LTF* genes. In every section (A-E), the wild type appears first, the sequences of the 20 bp regions flanking the relevant variant are displayed. The sequence shown in lowercase letters at the top corresponds to the reference genome, followed by the sample sequences of homozygous and heterozygous individuals. The benchling (https://benchling.com/) program was used for sequence alignment operations. The reference genome infomration is available online at; https://www.ensembl.org/cattle/search/results?q=;site=ensembl;facet_species=cattle. The green band indicates both the wild type and mutant nucleotide alterations for each gene.

(A) rs135700617 (g.19784652T > C; c.302A > G; p.His101Arg) polymorphism in the *ANXA9* gene **(B)** rs210042291 (g.122291188G > A; c.246 + 323G > A) polymorphism in the *FABP3* gene **(C)** rs110652478 (g.44677259C > T; c.328G > A; p.Val110Met) polymorphism in the *FABP4* gene **(D)** rs41255691 (g.21272246A > G; c.702A > G; p.Pro234=) polymorphism in the *SCD* gene **(E)** rs109623119 (g.52963859C > T; c.703 + 1216C > T; c.700 + 1216C > T; c.715 + 1216C > T) polymorphism in the *LTF* gene. No homozygous mutant type was detected within the Jersey population under examination for the *LTF* gene.

of Curi et al.[33] Utilizing comprehensive phenotypic data and an ample sample size enabled us to assess genotypic interactions. This approach facilitated the effective evaluation of both individual and combined effects of genetic markers. Initially, two- and three-way interactions were incorporated into the model, and their statistical significance was subsequently assessed. Thus, every conceivable genotypic interaction was examined. Genotype–environment interactions were either negligible or, in certain instances, challenging to assess, given the predominant influence of the environment. Consequently, they will not be pursued for further consideration. Association analysis was conducted using the statistical software Minitab (version 21.1.0) from PA, USA. The selected mixed model was as follows:

$$Y_{ijklmn} = \mu + S_i + R_j + G_k + A_l + \beta X_m + I_n + e_{ijklmn}$$

where

Y_{ijklmn} = phenotypic traits; μ = the overall mean; S_i = the fixed effect of calving season; R_j = the fixed effect of lactation rank; G_k = the fixed effect of genotypes; A_l = random effect of animal factor (l = varying performance values of an individual cow in different lactations; β X_m = the regression effect of days in lactation; I_n = is the fixed effect of the genotypic interactions; $e_{ijklmnop}$ = the random residual effect.

In cases where significant associations were detected, distinctions between groups were examined through Tukey's post hoc test.

We next computed additive and dominance effects using a reparameterized model, as outlined by Falconer and Mackay.[30] Consequently, the additive effect was calculated as the difference between the means of two homozygotes divided by two (a), while the dominance effect represented the deviation of the heterozygote from the mean of the two homozygotes (d). The degree of dominance was assessed based on the ratio d/a. If d exceeded + a or fell below -a, it indicated overdominance.

Results

Genotypic distribution and population genetics evaluation

This study identified three potential genotypes at all markers except for the *LTF* gene. Table 2 displays the allele frequency and genotype frequency of nine selected polymorphisms. A more evenly distributed genotypic pattern was noted in *FABP4* and *ANXA9* compared to other genes, suggesting that the distribution of these markers adhered to Hardy-Weinberg equilibrium (HWE) recommendations. The *FABP3*-A allele displayed the highest allelic frequency, with the AA genotype from this gene also standing out as the most prevalent. Among the entire population, 226 animals were found to have this particular genotype (Table 2).

As shown in Table 3, the selected markers demonstrated moderate levels of population genetics indices and genetic diversity when assessing the population genetics properties. However, it is noteworthy that the even distribution of genotypes in *FABP4* and *ANXA9* contributed to the favourable outcomes in population genetic parameters. For these markers, Ne surpassed 1.95. Likewise, *FABP4* and *ANXA9* genes exhibited the highest values for He, PIC, and *H'*. Fis displayed negative values across all markers (Table 3).

Individual impacts of the markers

Significant effects were identified in *FABP3*, *LTF* and *ANXA9* markers. The SNP at the *FABP3* gene

Table 3. Population genetics parameters observed in the present study.

Locus	Ho	He	PIC	Ne	Fis	*H`*
FABP4	0.5128	0.4872	0.3685	1.9501	−1.9146	0.6803
FABP3	0.7178	0.2822	0.2424	1.3932	−1.4805	0.4558
LTF	0.6250	0.3750	0.3047	1.6000	−3.1333	0.5623
ANXA9	0.5050	0.4950	0.3725	1.9802	−1.9091	0.6881
SCD	0.6152	0.3848	0.3108	1.6255	−1.4688	0.5731

Ho: gene homozygosity; He: gene heterozygosity; PIC: polymorphism information content; Ne: effective allele number; Fis: fixation index; *H'*: Shannon–Weaver diversity index.

Table 2. Genotypic and allelic distribution of the studied gene loci and Hardy-Weinberg equilibrium (HWE) test results in jerseys (*n* = 314). The symbols, p and q, indicate the corresponding alleles for each locus.

	Allele		Genotype count			Genotypic frequency (%)			Allelic frequency		
Locus	p	q	pp	pq	qq	pp	pq	qq	p	q	χ^2 HWE[1]
FABP4	A	G	60	142	112	19.11	45.23	35.66	0.42	0.58	1.540
FABP3	A	G	226	70	18	71.98	22.29	5.73	0.83	0.17	13.263***
LTF	A	B	159	155	0	50.64	49.36	0	0.75	0.25	33.718***
ANXA9	A	G	101	144	69	32.17	45.86	21.97	0.55	0.45	1.681
SCD	A	G	185	95	34	58.92	30.25	10.83	0.74	0.26	14.229***

FABP: fatty acid binding protein, *LTF*: lactoferrin, *ANXA9*: annexin A9, *SCD*: Stearoyl CoA desaturase.
χ2 (HWE): Hardy–Weinberg equilibrium χ2 value.
[1]Fisher's exact test was used to evaluate the HWE because of the low number of individuals per genotype (genotype counts below 5).
***$p < 0.001$–not consistent with HWE.

was recognized as a robust candidate marker for Jersey milk production, demonstrating highly significant associations in the assessment of TDMY ($p < 0.001$), 305-DMY ($p < 0.001$), and 305-DFY ($p < 0.001$). The AA genotype exhibited higher means for the mentioned traits. The association between *LTF* and 305-DFY was found to be significant ($p < 0.05$), while *ANXA9* showed a significant relationship with MFP ($p < 0.001$). Animals with a heterozygous genotype for *LTF* and *ANXA9* exhibited lower means for milk fat traits than those with other genotypes (Table 4). Table 5 illustrates the contributions of specific gene actions, as indicated by the additive and dominance effects estimation. Notably, overdominance was observed in the *ANXA9* marker.

Genotypic interactions

The study revealed noteworthy and novel interactions within the fatty acid metabolic process pathway through genotypic interaction analysis. We identified five significant two-way interactions for TDMY, MFP and 305-DFY in the association analysis (Table 6). But, two of them (*FABP4*×*ANXA9* and *FABP3*×*ANXA9*) were insignificant in Tukey's post hoc comparison (Table 6). The GGGG, AGGG, and AAGG genotypes seem favorable in the *FABP4* × *ANXA9*, *FABP4* × *SCD*, and *LTF* × *ANXA9* interactions, respectively. Table 7 presents detailed information on the effects of significant haplotypes. Additional details regarding the influence of two-way genotypic interactions on milk production traits are provided in Table S1. The GGAAGG

Table 4. The significance levels, least-squares means, and standard errors associated with the impact of studied genotypes on milk production traits in Jersey cows.

Genotype	*n*	TDMY (kg/d)	305-DMY) gk (	MFP)%(	MPP)%(	TDFY (kg/d)[1]	TDPY (kg/d)[1]	305-DFY (kg)[2]	305-DPY (kg)[2]
FABP4									
AA	60	14.25 ± 0.51	4629 ± 214	4.91 ± 0.09	3.34 ± 0.05	0.69 ± 0.09	0.45 ± 0.12	226.71 ± 11.2	149.40 ± 25.70
GA	142	14.51 ± 0.41	4626 ± 172	4.98 ± 0.08	3.38 ± 0.03	0.70 ± 0.08	0.43 ± 0.09	231.29 ± 8.98	139.10 ± 28.60
GG	112	14.79 ± 0.42	4624 ± 175	5.06 ± 0.08	3.40 ± 0.03	0.82 ± 0.08	0.60 ± 0.09	234.69 ± 9.12	185.70 ± 29.10
FABP3									
AA	226	**16.21[a] ± 0.29**	**5334[a] ± 120**	5.05 ± 0.05	3.38 ± 0.02	0.84 ± 0.06	0.54 ± 0.07	**266.62[a] ± 6.26**	176.50 ± 20.00
GA	70	**13.97[b] ± 0.46**	**4332[b] ± 194**	4.91 ± 0.09	3.37 ± 0.03	0.67 ± 0.09	0.54 ± 0.11	**214.70[b] ± 10.20**	169.30 ± 22.40
GG	18	**13.37[b] ± 0.74**	**4213[b] ± 309**	4.99 ± 0.14	3.37 ± 0.05	0.69 ± 0.14	0.40 ± 0.18	**211.30[b] ± 16.20**	128.50 ± 21.50
LTF									
AA	159	14.30 ± 0.40	4737 ± 167	5.02 ± 0.08	3.37 ± 0.03	0.77 ± 0.08	0.54 ± 0.10	**239.23[a] ± 8.73**	176.30 ± 27.80
BA	155	14.74 ± 0.38	4516 ± 158	4.95 ± 0.07	3.38 ± 0.03	0.70 ± 0.07	0.45 ± 0.093	**222.55[b] ± 8.25**	139.80 ± 26.30
ANXA9									
AA	101	14.73 ± 0.41	4566 ± 171	**5.04[a] ± 0.08**	3.41 ± 0.03	0.73 ± 0.08	0.54 ± 0.09	229.86 ± 8.96	167.80 ± 28.60
GA	144	14.67 ± 0.40	4668 ± 167	**4.75[b] ± 0.08**	3.34 ± 0.03	0.76 ± 0.08	0.51 ± 0.09	221.27 ± 8.73	160.40 ± 27.80
GG	69	14.15 ± 0.51	4645 ± 212	**5.16[a] ± 0.09**	3.37 ± 0.04	0.72 ± 0.09	0.44 ± 0.12	241.60 ± 11.11	146.11 ± 25.40
SCD									
AA	185	14.79 ± 0.35	4720 ± 145	4.94 ± 0.07	3.38 ± 0.02	0.78 ± 0.07	0.56 ± 0.08	234.40 ± 7.57	178.41 ± 24.10
GA	95	14.89 ± 0.43	4750 ± 182	5.03 ± 0.08	3.40 ± 0.03	0.74 ± 0.09	0.48 ± 0.10	239.43 ± 9.50	155.81 ± 30.30
GG	34	13.87 ± 0.61	4409 ± 257	4.99 ± 0.12	3.34 ± 0.04	0.69 ± 0.12	0.43 ± 0.15	218.81 ± 13.40	140.11 ± 22.80

[a,b]Different superscripts within a column indicate significant differences. The *P* values of the significant associations are presented in Table 5.
TDMY: test day milk yield; 305-DMY: 305-day milk yield; MFP: Milk fat percentage; TDFY: Test day fat yield; MPP: Milk protein percentage; TDPY: Test day protein yield; 305-DFY: 305-day fat yield; 305-DPY: 305-day protein yield.
[1]Fat yield and protein yield were calculated based on test day milk yield.
[2]Fat yield and protein yield were calculated based on 305-day milk yield.

Table 5. Additive and dominance effects of markers with significant associations.

Trait	Significant Marker	Additive Effect[1,2]	Dominant Effect[1,3]	Existence of overdominance[1,4]	Overall *p*-value[1,5]
Test day milk yield, kg/d	*FABP3*	1.42***	−0.82		0.000
305-day milk yield, kg	*FABP3*	560.50***	−441.50		0.000
Milk fat percentage, %	*ANXA9*	−0.06	−0.35***	+	0.000
305-day fat yield, kg	*FABP3*	27.66***	−24.26		0.000
305-day fat yield, kg	*LTF*[6]				0.039

[1]Table 4 displays the least squares means for each genotype.
[2]The additive effect is calculated as the difference between the two homozygous means, divided by 2, following the method of Falconer and Mackay.[29].
[3]The dominant effect is estimated as the nonadditive genetic effects, representing the deviation of the heterozygote from the mean of the two homozygotes, according to Falconer and Mackay.[29].
[4]The degree of dominance is expressed as d/a. In the case of over-dominance, d is greater than +a or less than −a.[29].
[5]The overall *p*-value for the marker serves as a fixed genotype effect.
[6]Additive and dominance calculations are not applicable in this case as only two genotypes were observed.
***$p < 0.001$.

Table 6. Summary of the associations observed (genotypic interactions) in the present study.

Genotype	TDMY (kg/d)	305-DMY) gk (	MFP)%(	MPP)%(	TDFY (kg/d)[1]	TDPY (kg/d)[1]	305-DFY (kg)[2]	305-DPY (kg)[2]
FABP4*×*FABP3	0.072	0.195	0.537	0.774	0.986	0.709	0.092	0.595
FABP4*×*LTF	0.389	0.373	0.211	0.071	0.400	0.187	0.459	0.268
FABP4*×*ANXA9	**0.032**‡	0.943	**0.003**	0.915	0.746	0.983	0.678	0.998
FABP4*×*SCD	0.118	0.813	**0.029**	0.883	0.760	0.816	0.929	0.854
FABP3*×*LTF	0.270	0.919	0.422	0.797	0.823	0.463	0.878	0.509
FABP3*×*ANXA9	0.948	0.518	0.114	0.934	0.983	0.753	**0.022**‡	0.744
FABP3*×*SCD	0.137	0.146	0.176	0.366	0.981	0.999	0.573	0.999
LTF*×*ANXA9	0.867	0.225	0.262	0.762	0.754	0.961	**0.001**	0.970
LTF*×*SCD	0.166	0.365	0.802	0.984	0.672	0.883	0.612	0.941
ANXA9*×*SCD	0.973	0.792	0.996	0.697	0.989	0.978	0.830	0.998

‡Not significant following Tukey's *post hoc* comparison.
TDMY: test day milk yield; 305-DMY: 305-day milk yield; MFP: Milk fat percentage; TDFY: Test day fat yield; MPC: Milk protein percentage; TDPY: Test day protein yield; 305-DFY: 305-day fat yield; 305-DPY: 305-day protein yield.
[1]Fat yield and protein yield were calculated based on test day milk yield.
[2]Fat yield and protein yield were calculated based on 305-day milk yield.

Table 7. The significance levels, least-squares means, and standard errors associated with the impact of significant two-way genotypic interactions on milk production traits in Jersey cows. Only significant haplotype associations are represented.

Genotype	*n*	TDMY (kg/d)	305-DMY) gk (	MFP)%(	MPP)%(	TDFY (kg/d)[1]	TDPY (kg/d)[1]	305-DFY [2]) gk (	305-DPY (kg)[2]
FABP4*×*ANXA9***									
AAAA	13	12.33 ± 1.17	4064 ± 504	**5.15^{abc} ± 0.22**	3.36 ± 0.09	0.66 ± 0.25	0.38 ± 0.09	207.40 ± 25.41	145.60 ± 24.41
AAAG	28	13.79 ± 0.97	4299 ± 417	**4.48^{c} ± 0.18**	3.32 ± 0.07	0.63 ± 0.20	0.42 ± 0.08	192.40 ± 21.60	136.40 ± 21.72
AAGG	19	15.50 ± 1.33	4516 ± 571	**4.64^{c} ± 0.25**	3.33 ± 0.09	0.77 ± 0.18	0.50 ± 0.09	207.40 ± 29.51	147.22 ± 28.00
AGAA	53	15.99 ± 0.75	4557 ± 322	**4.97^{abc} ± 0.14**	3.36 ± 0.05	0.81 ± 0.16	0.53 ± 0.09	224.60 ± 16.60	153.30 ± 25.21
AGAG	65	15.38 ± 0.82	4615 ± 352	**4.86^{abc} ± 0.16**	3.36 ± 0.06	0.76 ± 0.17	0.45 ± 0.11	223.30 ± 17.90	145.90 ± 29.61
AGGG	24	14.45 ± 1.19	4825 ± 513	**5.52^{ab} ± 0.23**	3.38 ± 0.09	0.85 ± 0.05	0.46 ± 0.04	274.30 ± 26.50	160.60 ± 28.12
GGAA	35	14.68 ± 0.960	4749 ± 413	**5.09^{abc} ± 0.18**	3.44 ± 0.07	0.70 ± 0.20	0.62 ± 0.05	242.00 ± 21.30	202.30 ± 20.92
GGAG	51	15.38 ± 0.89	4966 ± 386	**4.89^{bc} ± 0.17**	3.43 ± 0.07	0.91 ± 0.19	0.60 ± 0.11	243.00 ± 19.70	198.00 ± 25.51
GGGG	26	15.07 ± 1.25	4834 ± 537	**5.71^{a} ± 0.24**	3.50 ± 0.09	0.89 ± 0.26	0.55 ± 0.08	280.50 ± 27.60	184.20 ± 21.94
FABP4*×*SCD****									
AAAA	31	12.49 ± 0.81	4125 ± 350	**4.93^{ab} ± 0.15**	3.29 ± 0.06	0.64 ± 0.17	0.39 ± 0.12	203.50 ± 18.00	137.53 ± 19.80
AAGA	20	14.53 ± 1.27	4306 ± 545	**4.97^{ab} ± 0.24**	3.38 ± 0.09	0.72 ± 0.17	0.46 ± 0.12	216.90 ± 27.90	148.21 ± 12.71
AAGG	9	14.60 ± 1.61	4449 ± 691	**4.38^{b} ± 0.30**	3.35 ± 0.11	0.70 ± 0.14	0.45 ± 0.11	186.90 ± 25.60	144.00 ± 18.00
AGAA	88	15.24 ± 0.64	4891 ± 275	**4.86^{ab} ± 0.12**	3.33 ± 0.05	0.76 ± 0.13	0.47 ± 0.16	243.20 ± 14.10	157.90 ± 16.90
AGGA	44	15.07 ± 0.81	4731 ± 347	**4.99^{ab} ± 0.15**	3.39 ± 0.06	0.76 ± 0.17	0.48 ± 0.16	237.70 ± 17.70	156.32 ± 18.91
AGGG	10	15.51 ± 1.52	4375 ± 653	**5.49^{a} ± 0.29**	3.39 ± 0.11	0.89 ± 0.12	0.49 ± 0.17	241.20 ± 23.70	146.00 ± 12.00
GGAA	66	15.89 ± 0.77	5035 ± 330	**5.09^{ab} ± 0.15**	3.42 ± 0.05	0.92 ± 0.16	0.71 ± 0.19	259.20 ± 16.80	231.90 ± 15.80
GGGA	31	14.79 ± 0.87	4730 ± 374	**5.24^{ab} ± 0.17**	3.43 ± 0.06	0.70 ± 0.18	0.46 ± 0.22	252.10 ± 19.20	158.82 ± 13.82
GGGG	15	14.43 ± 1.61	4784 ± 693	**5.35^{ab} ± 0.31**	3.52 ± 0.11	0.87 ± 0.14	0.60 ± 0.11	254.30 ± 25.70	194.00 ± 19.11
LTF*×*ANXA9*****									
AAAA	55	14.16 ± 0.77	4485 ± 330	5.15 ± 0.15	3.38 ± 0.05	0.70 ± 0.16	0.58 ± 0.16	**231.40^{ab} ± 18.50**	193.40 ± 25.84
AAAG	73	14.56 ± 0.76	4672 ± 329	4.69 ± 0.15	3.36 ± 0.05	0.80 ± 0.16	0.55 ± 0.15	**207.60^{b} ± 20.30**	178.50 ± 26.40
AAGG	31	14.99 ± 1.14	5110 ± 489	5.35 ± 0.22	3.41 ± 0.08	0.81 ± 0.24	0.53 ± 0.12	**323.40^{a} ± 32.00**	186.40 ± 23.70
ABAA	46	14.51 ± 0.80	4429 ± 343	4.99 ± 0.15	3.41 ± 0.06	0.75 ± 0.17	0.44 ± 0.12	**218.00^{ab} ± 17.80**	140.80 ± 28.40
ABAG	71	15.14 ± 0.75	4581 ± 320	4.79 ± 0.14	3.39 ± 0.05	0.73 ± 0.16	0.43 ± 0.19	**225.30^{ab} ± 16.90**	141.80 ± 24.10
ABGG	38	15.03 ± 1.06	4340 ± 458	5.23 ± 0.20	3.39 ± 0.08	0.86 ± 0.22	0.47 ± 0.12	**199.30^{b} ± 25.90**	141.60 ± 28.50

TDMY: test day milk yield; 305-DMY: 305-day milk yield; MFP: Milk fat percentage; TDFY: Test day fat yield; MPC: Milk protein percentage; TDPY: Test day protein yield; 305-DFY: 305-day fat yield; 305-DPY: 305-day protein yield.
[1]Fat yield and protein yield were calculated based on test day milk yield.
[2]Fat yield and protein yield were calculated based on 305-day milk yield.
[a,b]Different superscripts within a column indicate significant difference.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

haplotype was identified as favourable for 305-DFY and MFP in the context of the *FABP3*×*LTF*×*ANXA9* interaction (Table 8).

Discussion

Bovine milk fat is distinguished by its composition, containing a significant portion (50–70%) of SFA, 20–40% MUFA, and a minimal proportion (1–5%) of polyunsaturated fatty acids (PUFA).[34] A comprehensive understanding of genomic regions, particularly individual genes, associated with genetic variation in milk fatty acid composition will deepen our knowledge of the biological pathways involved in fatty acid synthesis (Fig. 1). This insight may also reveal potential avenues for influencing and modulating milk fat composition.[35]

Table 8. The least-squares means and standard errors for the associations of *FABP3*×*LTF*×*ANXA9* haplotypes with the 305-day fat yield and milk fat percentage ($n = 314$).

Genotype	n	305-day fat yield, kg	Milk fat percentage, %
AAAAAA	37	$267.70^{abcd} \pm 11.90$	$5.25^{ab} \pm 0.11$
AAAAAG	51	$264.30^{abcd} \pm 10.30$	$4.89^{bc} \pm 0.09$
AAAAGG	20	$307.40^{ac} \pm 15.00$	$5.38^{ab} \pm 0.14$
AAABAA	28	$265.60^{abcd} \pm 13.00$	$5.01^{abc} \pm 0.12$
AAABAG	54	$247.80^{abcd} \pm 10.20$	$4.85^{bc} \pm 0.09$
AAABGG	27	$258.40^{abcd} \pm 13.30$	$5.21^{ab} \pm 0.12$
AGAAAA	14	$217.00^{abcd} \pm 19.00$	$5.12^{abc} \pm 0.17$
AGAAAG	18	$211.40^{bd} \pm 18.40$	$4.75^{bc} \pm 0.17$
AGAAGG	5	$219.60^{abcd} \pm 29.40$	$4.91^{bc} \pm 0.27$
AGABAA	15	$216.60^{bd} \pm 17.40$	$4.96^{bc} \pm 0.16$
AGABAG	12	$216.90^{bd} \pm 19.90$	$4.91^{bc} \pm 0.18$
AGABGG	6	$164.80^{d} \pm 32.80$	$4.97^{bc} \pm 0.29$
GGAAAA	4	$211.30^{abcd} \pm 32.70$	$4.96^{bc} \pm 0.29$
GGAAAG	4	$162.90^{d} \pm 37.70$	$4.70^{c} \pm 0.34$
GGAAGG	6	$451.80^{a} \pm 65.30$	$6.15^{a} \pm 0.59$
GGABAA	3	$179.10^{cd} \pm 37.60$	$5.15^{abc} \pm 0.34$
GGABAG	5	$213.30^{abcd} \pm 29.30$	$4.58^{c} \pm 0.27$
GGABGG	5	$173.30^{cd} \pm 46.00$	$5.69^{ab} \pm 0.42$
Significance		$p < 0.05$	$p < 0.05$

[a,b,c,d]Different superscripts within a column indicate significant difference.

Fatty acids play an active role in moulding the composition of milk fat, contributing crucially to energy metabolism during lactation and defining the nutritional quality of the milk. Therefore, the metabolic process of fatty acids holds a significant position in the biology of milk production. Bovine milk fat contains over 400 fatty acids with varying carbon chain lengths and unsaturation levels, including short-chain fatty acids (2-6 carbon atoms), medium-chain fatty acids (7–12 carbon atoms), and long-chain fatty acids (more than 12 carbons).[35,36] FABPs and SCD are important proteins for fatty acid biosynthesis. FABP3 supplies stearoyl-CoA to SCD for desaturation. The resulting endogenous fatty acids (mainly oleic acid) are then transported by FABP4 to other enzymes for milk lipid synthesis.[37] Both FABP3 and FABP4 have been observed to impact the content of different medium- and long-chain fatty acids in bovine milk.[38] A previous work has identified a robust connection between SCD1 and the content of monounsaturated fatty acids (MUFA) in bovine milk.[39] SCD is accountable for the production of over 70% of cis-9, trans-11-, and trans-7, cis-9-conjugated linoleic acids (CLA) found in ruminant milk.[40] The presence of bovine LTF has been observed to have positive effects on plasma lipid concentrations. LTF directly engages with modified LDL, hindering its interaction with scavenger receptors.[17] The ANXA9 has been identified in various tissues, with a notable presence in tissues characterized by a high rate of metabolism of fatty acids. This is particularly evident in mammary tissue during lactation, where the *ANXA9* gene is prominently expressed.

In brief, the proteins discussed above serve as the foundation for the fatty acid metabolic process pathway. The genetic framework of this pathway is pivotal in comprehending the variability in cow's milk fatty acid content (Fig. 2). Essential roles in the genetic regulation of this metabolic process pathway, specifically in fatty acid biosynthesis, are played by *FABP4*, *FABP3*, *LTF*, *ANXA9* and *SCD* genes. While the literature has explored the individual gene compositions in cow's milk, there is a notable absence of studies investigating the combined impacts of genes within this pathway with comprehensive data (especially milk composition data collected from different lactations of a cow). To the best of our knowledge, this study is the first of its kind to identify vital haplotypes for milk yield and composition traits in dairy cattle.

A primary prerequisite for genotype–phenotype assessments is an adequate variation and distribution of genotypes. The evaluation of genetic variation at specific loci serves not only to ascertain population dynamics but also to determine the suitability of employing the corresponding genetic markers in association analyses.[2] A genetic marker with a minor allele frequency below 5% should be disregarded because it may hinder the generation of reliable results in the analysis of the genotype-phenotype relationship.[41] In this study, minor allele frequency is calculated within the range of 0.17–0.45, indicating sufficient variation for subsequent association analyses. We noted deviations from HWE for the *FABP3*, *LTF* and *SCD* markers. These deviations could potentially be explained by population substructure. Specifically, such disequilibrium may stem from the unique characteristics of dairy cow breeds like Jerseys, which undergo intense selection and are commonly bred through artificial insemination, although not as pronounced as in the Holstein-Friesian breed. This breeding strategy involves a limited number of sires fathering a large number of offspring, which could contribute to the observed genetic discrepancies.[42] Population genetic parameters, including He, Ne and PIC, assess the population structure based on the genetic variation of specific genes. For example, low heterozygosity values suggest potential inbreeding issues at the population level, emphasizing the need for consideration in pedigree evaluation. Additionally, Ne has been utilized to express the impact of loci alleles in populations.[42,43] He values neared 0.50, while Ne values approached 2.00 in this study. The PIC values serve as an indicator of marker quality in genetic studies.[32] Based on the classification criteria proposed by Botstein et al. (1980),[32] there are three primary levels of informativeness for the polymorphisms as follows: low

polymorphism for PIC values <0.25, moderate polymorphism for PIC values between 0.25 – 0.50, and high polymorphism for PIC values >0.50. Except for *FABP3*, all the studied markers can be regarded as having moderate informativeness. PIC values are widely used indices for assessing a marker's polymorphism extent. The utility of a marker in segregation analysis is directly linked to its degree of polymorphism. According to the *H*' values, markers also demonstrated sufficient genetic diversity. Altogether, the genotypic informativeness and variation competence in this study provide a dependable source for the discussed phenotypic data.

ANOVA results indicated significant influences of *FABP3*, *LTF*, and *ANXA9* on milk production traits. The *FABP3* AA genotype exhibited higher means for both TDMY and 305-DMY compared to other genotypes. Specifically, the AA genotype produced 1002 kg and 1121 kg more 305-DMY than the heterozygous and GG genotypes, respectively. This superiority of the AA genotype was also evident in TDMY values. Additionally, animals with the AA genotype were significantly associated with higher 305-DFY means, producing 51.92 kg more milk fat than heterozygous animals and 55.32 kg more milk fat than GG animals. Our analysis partially aligns with the conclusions drawn in the study by Kulig et al.[26] in Jersey cows. These researchers indicated that the A allele was the most prevalent and linked to elevated fat and protein percentages in the milk. On the other hand, Kulig et al.[13] reported an association between *FABP3* polymorphism genotypes and the estimated breeding values (EBV) for fat yield in Polish Holstein-Friesian cows. In contrast to the findings of this study, the GG cows exhibited a notably higher EBV for this trait compared to the AG cows, with a statistically significant difference averaging 2.43 kg. An important consideration is that the limited number of samples in many studies results in certain *FABP3* genotypes being either very few in number or entirely absent. Consequently, the reliability of results in association analyses tends to be low. In our study, even though the G allele was found at a low frequency (0.17), we observed sufficient variation for meaningful evaluations in association analyses and genotypic interactions. This stands as an advantage of our study compared to similar studies in the literature. *FABP3*, along with *FABP4*, holds a central position in lipid metabolism and the biosynthesis of fatty acids. Upon entering the cell, LCFAs form complexes with FABPs, predominantly FABP3 and FABP4. Subsequently, these complexes are transported to the active metabolic enzymes located in the endoplasmic reticulum or mitochondria.[38,44] The *FABP3* gene has been assigned to BTA2, a chromosome where QTLs impacting both the fat yield and fat content of milk have been pinpointed.[45] Identifying variations in this gene and exploring its associations with genes in the fatty acid metabolic process pathway will yield valuable data for making genetic selections related to the composition of cow's milk.

Within the *LTF* marker, the AA genotype exhibited more favourable outcomes, showcasing higher 305-DFY means compared to heterozygotes. Notably, the BB genotype was absent in the examined population. However, the significant milk fat production difference observed between AA and heterozygous genotypes (~17kg) underscores the need for further investigation into this relationship. Bovine *LTF* is inherently linked to mastitis and has been a subject of genetic studies in this context.[16] However, this gene is also a significant component of fatty acid biosynthesis. Hence, it is evident that it might influence the composition of cow's milk. Nevertheless, the existing understanding of the connection between *LTF* and milk content is notably inadequate, emphasizing the need for further examination of variants of this gene in this context. Another notable gene in our study is *ANXA9*. The protein product of this gene functions as a membrane transport channel and belongs to the annexin family of proteins that bind to Ca^{+2} and phospholipids.[2,12,19] From a broad perspective, this gene assumes crucial functions in signal transduction, cell proliferation, and regulation of cellular transport, making it a potential candidate as a significant marker in assessing bovine milk content. In our study, *ANXA9* had a significant impact on MFP means, with the heterozygous genotype exhibiting less desirable qualities in this regard. In this context, the GG and AA genotypes demonstrated higher MFP means ($5.16 \pm 0.09\%$ and $5.04 \pm 0.08\%$, respectively). Kulig et al.[13] reported that the *ANXA9* H84R marker significantly influenced the breeding value for fat content in Polish Holstein-Friesian cows. These researchers indicated that the GG cows exhibited a notably reduced EBV for MFP compared to heterozygous and AA animals. However, it is important to note that Kulig et al.[13] observed no significant differences among *ANXA9* genotypes regarding MFP in Polish Jersey cows. This finding contrasts with the results presented in this study, which suggest that the AG genotype of *ANXA9* is associated with a lower milk fat percentage in a population of Turkish Jerseys. The discrepancy between these outcomes highlights potential variability in genetic effects across different animal populations or breeds, possibly due to interactions with other gene variants. Furthermore,

Martínez-Royo et al.[46] found a significant impact of *ANXA9* polymorphisms on the relationship between milk-fat yield EBVs and *ANXA9* genotypes, indicating an influential effect on this trait in Spanish Holstein-Friesians. Notably, these researchers observed that the heterozygous genotype exhibited intermediate values for milk-fat content and estimated additive effect of allele G was 12.06 kg for milk-fat yield. The outcomes from *ANXA9* may lend support to the previously identified QTL for milk-fat yield in the chromosomal region housing *ANXA9*.[21] Nevertheless, additional studies are required to validate these findings using an experimental design better-suited for detecting genetic associations.

This study delves into genetics with extensive support from comprehensive phenotypic data concerning selected pathways and genes. After evaluating the individual effects of genes, we delved into exploring genetic interactions. We hereby present novel two- and three-way genetic interactions revealed through the association analysis of milk productivity and fat-related traits. The GGGG genotype showed the highest MFP in the *FABP4*×*ANXA9* interaction, while the AGGG genotype exhibited the highest MFP in the *FABP4*×*SCD* interaction. Furthermore, the AAGG genotype of the *LTF*×*ANXA9* interaction was significantly associated with the highest means for 305-DFY in the studied Jersey cows. We identified a specific novel haplotype, *FABP3*×*LTF*×*ANXA9* GGAAGG, that significantly and positively influences both milk fat yield and percentage. Animals with this haplotype produced ~452 kg 305-DFY with ~6% MFP. Most economic traits are influenced by polygenic inheritance and are more complex than anticipated. Consequently, single DNA markers can only explain a limited portion of the genetic variance.[2,47] Many effects are typically too small to achieve statistical significance and are therefore overlooked.[48],[25] Analysis of interactions among marker loci can uncover valuable genotypic data, as these interactions may explain variations in genotype responses across different populations and genetic backgrounds.[42,47] Therefore, it is essential to conduct a more detailed investigation of interactions between marker loci to offer more accurate and effective recommendations for optimal breeding decisions. Here we postulate that selecting GGAAGG animals for the *FABP3*×*LTF*×*ANXA9* interaction may provide superior cows the 305-day fat yield and milk fat percentage. These traits are pivotal in dairy cattle breeding, and thus, focusing on this haplotype may facilitate cost-effective selection strategies to enhance milk fat yield.

The genes included in this study were chosen due to their previously established associations with fatty acid biosynthesis. They all play essential roles in the cattle fatty acid metabolic pathway (Figs. 1 and 2). Thus, all genetic markers examined in this study impact the formation of fatty acids, underscoring the significant value of cow's milk owing to its high-fat content. These findings confirmed by the STRING database (https://string-db.org/) emphasize the importance of elucidating the intricate pathways involved in fatty acid metabolism for better understanding the mechanisms underlying milk production and composition. Molecular mechanisms are related to lipid uptake by mammary epithelium, lipid recycle and circulation, and the regulation of fatty acid synthesis (Fig. 2). On the other hand, this study has certain limitations. The traits of interest in livestock production are considerably more complex than initially anticipated. These economically vital quantitative traits are determined by thousands of genes, each contributing minor effects to the phenotype.[48] Hence, genome-wide data will offer researchers a more comprehensive set of information. It is also important to acknowledge that other members of the *FABP* gene family (specifically, *FABP1* and *FABP6*), along with additional genes such as elongation of very long chain fatty acids protein 6 (*ELOVL6*), butyrophilin subfamily 1 member A1 (*BTNA1A*), and lipoprotein lipase (*LPL*), which could indirectly contribute to the fatty acid biosynthesis. To confirm the results and their applicability within the dairy industry, further analyses should be conducted on a larger scale, encompassing numerous animals and multiple herds, with attention to comparing effects across breeds. Further investigations utilizing RNA-based approaches are also needed to deepen our comprehension of these biological processes and their regulation.

Milk fat properties play a crucial role in dairy cattle breeding. The first step in enhancing the prevalence of superior animals through the genetic selection of dairy cattle involves identifying genetic structures that positively influence milk fat. Haplotype analyses and targeted evaluations of genetic markers within specific pathways, in particular, offer robust information for animal breeding.[2,42,47] The haplotype analysis results could offer more dependable insights into a specific biological process. In the majority of QTL studies, association analyses have consistently examined each locus independently, overlooking potential interactions between different loci. Exploring interactions among marker loci requires careful investigation, as these interactions could explain variations in genotype responses among populations and genetic backgrounds.[42,47] The data derived from this study, along with the newly established relationships, are evidently valuable for prospective investigations in the domain of genetic selection for dairy cattle.

Conclusions

A comprehensive genetic assessment of milk production traits was conducted in Jersey cows, focusing on genes associated with fatty acid biosynthesis. Firstly, we observed adequate genetic distribution and diversity to gather reliable data to analyse genotype–phenotype relationships. Upon assessing the individual effects of genes, specifically *FABP3*, *LTF* and *ANXA9*, their notable impact on milk fat yield and percentage was observed. Furthermore, *FABP3* demonstrated a solid relationship to both test day and 305-day milk yield, with a strong preference for the AA genotype. This study unveiled novel and highly significant genotypes associated with the examined milk characteristics in dairy cows. In particular, this study marks the first identification of the pivotal role played by the *FABP3*×*LTF*×*ANXA9* interaction in bovine milk fat content regulation. Utilizing a comprehensive dataset spanning three lactations, the study delved into fundamental genes associated with the fatty acid metabolic process pathway, uncovering novel relationships in Jerseys. Consequently, the acquired data is poised to contribute both theoretically and practically to advancements in the field of dairy cattle breeding.

Author contributions

Sena Ardicli: genetics methodology, conceptualization, and supervision, laboratory experiments, statistical analysis, writing the manuscript, review, and editing. Nursen Senturk: data collection, laboratory experiments. Berkay Bozkurt: genetics methodology, laboratory experiments. Huseyn Babayev: bioinformatics. Tugçe Selvi: data collection, laboratory experiments. Stephen Skolnick: a critical review of the manuscript and editing. Hivdanur Ter: laboratory experiments. Beyza Aktas: laboratory experiments. Ayse Isık: laboratory experiments. Ozgur Toprak Ay: laboratory experiments. Ozge Ardicli: methodology, writing the manuscript, review, and editing. Ozden Cobanoglu: methodology, data collection, conceptualization and supervision, review, and editing.

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ORCID

Sena Ardicli http://orcid.org/0000-0003-2758-5945
Nursen Senturk http://orcid.org/0000-0003-1975-0103
Berkay Bozkurt http://orcid.org/0000-0002-5536-9898
Huseyn Babayev http://orcid.org/0000-0001-6985-9436
Tuğçe Selvi http://orcid.org/0000-0003-3425-3201
Stephen Skolnick http://orcid.org/0000-0001-9968-3793
Hivdanur Ter http://orcid.org/0009-0006-9173-6567
Beyza Aktas http://orcid.org/0009-0002-7899-6373
Ayse Isık http://orcid.org/0009-0007-5403-1880
Ozgur Toprak Ay http://orcid.org/0009-0000-3462-344X
Ozge Ardicli http://orcid.org/0000-0001-6077-0478
Ozden Cobanoglu http://orcid.org/0000-0001-9633-634X

Data availability statement

The data used to support the findings of this study are included in the paper.

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