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Dynamics of Lipogenic Genes and Milk Fatty Acid Indices Across Lactation in Pastoral Belahi Cattle

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ABSTRACT

Background: The fatty acid profile of milk is a key indicator of milk quality and holds considerable nutritional as well as economic importance. This profile is influenced by the coordinated action of several genes involved in fatty acid synthesis, transport and desaturation and varies with the stage of lactation. Despite this, information on the lactation related expression patterns of these genes in indigenous cattle breeds, such as Belahi, remains scarce. Therefore, the present investigation was undertaken to assess the expression dynamics of major lipogenic genes across different lactation stages in Belahi cattle and to associate these patterns with variations in milk fatty acid composition.

Methods: Milk samples were obtained from 18 clinically healthy Belahi cows representing early (15-90 days in milk), mid (>90-180 days in milk) and late (>180 days in milk) lactation stages. Milk somatic cells were isolated and total RNA was extracted using the Trizol method. After evaluating RNA quality, complementary DNA (cDNA) was synthesised and subjected to quantitative real time PCR analysis. The relative expression levels of four target genes ACACA (Acetyl-CoA carboxylase alpha), FABP3 (Fatty acid binding protein 3), SCD (Stearoyl-CoA desaturase) and FASN (Fatty acid synthase) were quantified using SYBR Green dye, with β actin employed as the internal reference gene. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, considering early lactation as the calibrator.

Result: Distinct stage specific expression patterns of the studied genes were observed across lactation. The expression of ACACA increased progressively, reaching its maximum level during late lactation (2.10 fold). In contrast, FABP3 expression declined substantially during mid lactation (0.26 fold) and remained suppressed in late lactation. A marked reduction in SCD expression was observed during mid lactation (0.11 fold), followed by a recovery in late lactation (1.09 fold), suggesting lactation-stage-dependent regulation. Similarly, FASN expression decreased during mid lactation (0.11 fold) and showed partial restoration in late lactation (0.76 fold), although it remained below early lactation levels. Overall, early lactation was associated with increased mobilisation of body fat reserves; late lactation exhibited enhanced expression of genes involved in de novo fatty acid synthesis and desaturation; and mid lactation was characterised by a general downregulation of lipogenic gene expression.

Key words: Belahi cattle, Desaturase indices, Gene expression, Milk fatty acids, RT-qPCR.

INTRODUCTION

The composition of milk fatty acids has become a key focus as an important economic trait, alongside milk fat percentage (Jensen, 2002). Milk yield and quality are vital traits for dairy cattle (Matsumoto *et al*., 2012). Breeding efforts aim to increase milk production per cow; other important traits include solids non-fat components like milk protein and sugar, as well as milk fat, since these are closely linked to milk flavor (Masuko *et al*., 1999). Milk fat is the main energy source in milk, making up over half of its total energy despite only comprising about 3-5% of milk by volume and recent research shows that various genes influence milk fatty acid composition (Soyeurt *et al*., 2007). Candidate genes involved in fat synthesis and metabolism pathways regulated by multiple genes likely control fatty acid profiles in milk and meat. For example, the fatty acid synthase gene (FASN) impacts milk fat content and the C14 Index in Holstein milk (Matsumoto *et al*., 2012a). Additionally, a nonsynonymous mutation in the stearoyl-CoA desaturase gene has been linked to MUFA (mono-unsaturated fatty acids) percentage and melting point in intramuscular fat of Japanese Black cattle (Taniguchi *et al*., 2004) and plays a role in the nutritional quality of milk fat (Moioli *et al*., 2007).

Milk fat composition varies across different stages of lactation. Karijord *et al*. (1982) observed, in a limited set of fatty acids (FA) from Norwegian cows, that levels of short

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and medium-chained saturated fatty acids from peaked around month three of lactation, while long-chain saturated fatty acid levels dipped to a minimum at this time (Palmquist and Beaulieu, 1993). Metabolic changes characterise early, mid and late lactation stages: early lactation often involves negative energy balance, high fat mobilization and increased expression of genes related to lipid mobilization (Bainbridge *et al*., 2016). Mid-lactation typically features a stable metabolism and heightened expression of genes involved in de novo fatty acid synthesis. The late stage shows a decline in milk yield and modified lipid metabolism. It is well established that milk's fatty acid profile shifts throughout lactation (Kęsek-Woźniak *et al*., 2023). Fatty acids influence various physiological processes in humans, making it crucial to understand the molecular mechanisms regulating fatty acid synthesis in the bovine mammary gland.

Studying gene expression can provide insights into stage-specific regulation of fatty acid metabolism. These studies help identify active and suppressed pathways and clarify whether changes in fatty acids during lactation are due to de novo synthesis in the mammary gland or mobilisation of body fat in early lactation. Improving milk quality traits through traditional breeding is limited by the cost of phenotyping. Discovering genes that influence composition traits could support the development of gene-assisted selection programs to partly overcome these challenges (Crepaldi *et al*., 2013). Key genes ACACA, a major regulator of fatty acid biosynthesis and SCD, involved in monounsaturated fatty acid production in the mammary gland. ACACA is the rate-limiting enzyme in de novo fatty acid synthesis. Bionaz and Loor (2008) identified a gene network involved in fatty acid synthesis and secretion, including ACACA, FASN and SCD, which were significantly upregulated throughout lactation.

This study involves Belahi cattle, which constitute a distinct indigenous cattle population primarily maintained by the Gujjar/Gurjar pastoralist community in Haryana, India. These cattle are mainly managed by the nomadic and semi-nomadic pastoralists of the Gujjar community, whose livelihoods are closely linked to seasonal mobility and shared grazing resources (Chavan *et al*., 2025). The objective of the present study was to look at the gene expression profile for these four genes across lactation in Belahi cattle and infer the importance of these genes and related pathways for fatty acids (FA) profiling of Belahi cattle.

MATERIALS AND METHODS

Animals and sample collection

This study focused on the Belahi cattle breed, native to the pastoral regions, to examine the changes in lipogenic gene activity and milk fatty acid profiles throughout lactation. Eighteen lactating cows were selected from a herd in Shahzadpur, Ambala, Haryana. The Belahi breed originates from the North Himalayan foothills, particularly the Shivalik hills extending into Haryana, Chandigarh and Punjab (Chavan *et al*., 2025). Milk samples were collected following the farm's usual milking routine during the winter of February 2026. All cows were healthy, with somatic cell counts under 400000 and each was tested for mastitis using the California Mastitis Test (CMT) to confirm there was no infection. Samples were taken at three lactation stages: early (15-90 days in milk), mid (>90-180 days) and late (beyond 180 days), with all cows in their 3rd parity. A 200 ml milk sample was immediately cooled to 4°C after collection to preserve somatic cell integrity.

Milk somatic cells (MSCs) were extracted from these samples. Approximately 45 ml of fresh milk was defatted by centrifugation at 2500 rpm at 4°C for 20 minutes in sterile, RNase-free 50 ml tubes. After centrifugation, the fatty layer on top was carefully removed with a sterile tip and the remaining skim milk was gently inverted to discard residual fat. This method followed the protocol of Choudhary and Choudhary (2019), with minor modifications. The tubes were then inverted onto blotting paper to drain excess liquid by gravity. The cell suspension was washed with 10 ml of DEPC-treated, autoclaved, chilled PBS, then centrifuged again at 2500 rpm at 4°C for 10 minutes. This washing process was repeated until the PBS was clear of fat, resulting in a transparent or slightly cloudy solution. Finally, the supernatant was discarded and the cell pellet was resuspended in 1 ml of Trizol and stored at -20°C for RNA extraction.

Isolation, quantification and cDNA synthesis of total RNA

Total RNA was isolated from milk somatic cells using Trizol Lysis Reagent (Sigma-Aldrich) with minor modifications in the protocol of Choudhary and Choudhary (2019). The milk somatic cell pellet was mixed with 1 ml of Trizol reagent in a DEPC-treated tube, homogenised and incubated at room temperature for 5 minutes at 20°C. After adding 200 µl of chloroform, the mixture was incubated for 10-15 minutes at room temperature and centrifuged at 12,000 rpm for 20 minutes at 4°C. The upper aqueous phase (200 µl) was transferred to a new tube, mixed with an equal volume of isopropanol and incubated at -20°C for 1 hour. Following centrifugation at 12,000 rpm for 20 minutes, the RNA pellet was washed twice with 70% ethanol (500 µl each), centrifuged at 7,500 rpm for 5 minutes per wash, air-dried and dissolved in 50 µl of nuclease-free water. RNA was stored at -20°C and its concentration and purity were measured by determining the optical density (OD 260/OD 280) ratio using spectrophotometry.

RNA integrity was evaluated using the RNA Integrity Number (RIN) (Schroeder *et al*., 2006), with only samples scoring ≥8 used for qPCR. Agarose gel electrophoresis displayed clear 28S, 18S and 5S rRNA bands, confirming RNA quality. Approximately 1 µg of total RNA was reverse transcribed into cDNA using the ThermoFisher U.S. Reverse Transcription System following the modified protocol of Pławińska-Czarnak *et al*. (2019) and the cDNA was stored at -20°C for future use.

Table 1: List of genes with forward and reverse primers used in the study.

Genes	Primers		NCBI reference sequence	Tm (°C)	Product size
Acetyl-CoA carboxylase alpha (ACACA)	F	CATCTTGTCCGAAACGTCGAT	AJ132890	58	101
	R	CCCTTCGAACATACACCTCCA			
Fatty acid binding protein 3 (FABP3)	F	GAACTCGACTCCCAGCTTGAA	DN518905	60	102
	R	AAGCCTACCACAATCATCGAAG			
Stearoyl-CoA desaturase (SCD)	F	TCCTGTTGTTGTGCTTCATCC	AY241933	58	101
	R	GGCATAACGGAATAAGGTGGC			
Fatty acid synthase (FASN)	F	GCATCGCTGGCTACTCCTAC	NM001012669	61	136
	R	GTGTAGGCCATCACGAAGGT			

Expression profile of candidate genes using qPCR

To optimize real-time quantitative PCR, initial testing focused on refining standard PCR with primer sets on a programmable thermocycler for amplification. Custom primers from Eurofins Genomics India Pvt. Ltd, Bangalore, were chosen for gene-specific amplification. Housekeeping genes served as internal controls for mRNA amplification, ensuring they remain unaffected by experimental conditions.

Real-time PCR employed SYBR Green dye, analyzing each gene ACACA, FABP3, SCD and FASN in triplicate. Non-template controls confirmed reaction specificity. The threshold cycle (Ct) was used to evaluate gene expression levels for each RT-qPCR sample. After each run, melt curve analysis verified product specificity by gradually increasing the temperature above the primer annealing temperature (60°C) while monitoring fluorescence. A single sharp peak indicated specific amplification.

Data analysis and statistics

The qPCR data were analysed for fold change using the $2^{-\Delta\Delta CT}$ method, normalised to the reference gene β-Actin. Relative quantification of the target gene to β-Actin followed the methodology described by Livak and Schmittgen (2001). The initial phase of lactation animals served as control samples for normalization and comparison and gene expression was estimated using the specified formula. Relative expression,

$$R = 2^{-\Delta\Delta CT}$$

Where,

$$\Delta\Delta CT = \Delta CT_{(sample)} - \Delta CT_{(control)}$$

$$\Delta CT_{(sample)} = CT_{(target\ gene)} - CT_{(reference\ gene)}$$

The association of gene expression (delta CT) values for the key genes with the three stages of the lactation was analysed using regression analysis.

To assess the relationship between gene expression and fatty acid composition, correlation analysis was performed between relative mRNA expression levels of ACACA, FABP3, SCD and FASN and major fatty acid traits. Gene expression was quantified using RT-qPCR and calculated by the $2^{-\Delta CT}$ method with β-actin as the reference gene. Fatty acids were grouped into short-, medium-and long-chain SFA, as well as MUFA, PUFA and desaturation indices (DIC14 and DIC16). Correlations were estimated separately for early, mid and late lactation stages using Spearman's rank correlation coefficient.

Phenotypic data and traits

Milk fat extraction was performed according to ISO/IDF (2001) and fatty acid methyl esters (FAME) were prepared following ISO/IDF (2002) using hexane and methanolic KOH. The FAME samples were analysed using a Shimadzu GC-2010 gas chromatograph equipped with a triple quadrupole mass detector under standard conditions. Fatty acids were identified by matching spectra with a 37-component FAME standard mixture and the NIST database (National

Institute of Standards and Technology). Only major fatty acids were considered, including short-, medium-and long-chain saturated fatty acids, along with mono-and polyunsaturated fatty acids. Results were expressed as grams per 100 grams of total fatty acids. Desaturation indices (C14, C16, C17 and C18) were calculated using standard formulas.

RESULTS AND DISCUSSION

During the winter season, fresh milk samples were collected from 18 Belahi cows, with 6 animals representing each of the early, mid and late lactation stages. All cows were maintained at the same parity (third parity) to ensure consistency. The quality of the synthesised cDNA was verified by amplifying the β-actin housekeeping gene, which produced clear bands of the expected size, confirming cDNA quality. Table 1 provides details of the gene-specific primers used for qPCR analysis, including forward and reverse sequences for ACACA, FABP3, SCD and FASN, along with their NCBI reference sequences. The primers were optimised at annealing temperatures between 54°C and 62°C, yielding specific amplicons of 101-136 bp, ensuring precise and efficient target gene amplification.

The relative expression of lipid metabolism genes showed distinct patterns across lactation phases, as shown in Fig 1. The ACACA gene expression increased progressively from the early to the late phase of lactation (Table 2), with the highest upregulation in the late phase (2.10-fold) compared to the early phase, which was taken as control. FABP3 expression decreased sharply in mid-phase (0.26-fold) and remained low in late-phase (0.29-fold), indicating downregulation during later lactation. SCD showed a strong decrease in mid-phase (0.11-fold), followed by a recovery in late phase (1.09-fold), suggesting a phase-specific expression pattern. FASN expression also declined in mid-phase (0.11-fold) and partially recovered in late phase (0.76-fold) but remained below baseline levels.

The study observed that gene expression profiling and FA indices in the mid phase of lactation were intermediate (Fig 1). The early stage was characterised by mobilisation-driven processes, whereas the last phase was synthesis-driven. The second phase of lactation is therefore a transitional metabolic stage, dominated by energy balance and the importance of metabolic priorities (Bauman and Griinari, 2003). The statistical analysis showed that the delta CT values were not significantly affected across the three stages of the lactation. However, we can see that the fold-change expression values reveal dynamic regulation of lipogenic genes across lactation.

The phenotype of interest, the fatty acid indices, demonstrated a dynamic shift in the milk fatty acid composition of Belahi cattle across lactation (Fig 2). This was characterised by a reduction in unsaturation and an increase in saturation during the last phase of lactation, alongside enhanced desaturation activity. Short-chain and medium-chain SFAs remained relatively stable, whereas

Table 2: The estimated fold change expression values ($2^{-\Delta\Delta CT}$) values for four key genes.

	$2^{-\Delta\Delta CT}$ values		
Genes	Early lactation stage	Mid lactation stage	Late lactation stage
ACACA	1	1.25	2.10
FABP3	1	0.26	0.29
SCD	1	0.11	1.09
FASN	1	0.11	0.76

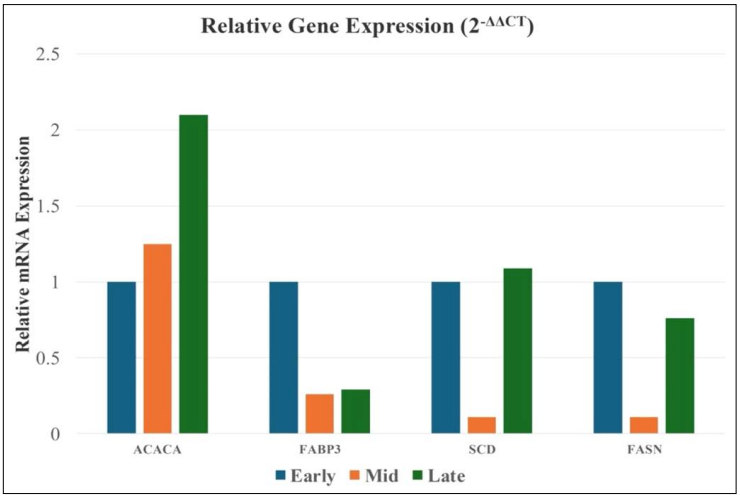
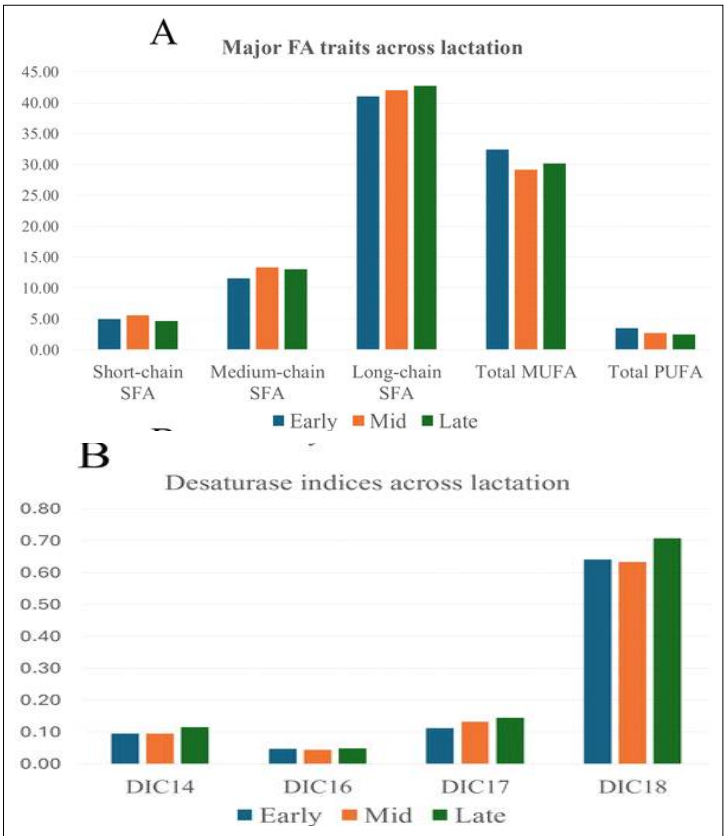


Fig 1: Comparative analysis of ACACA, FABP3, SCD and FASN gene expression during early, mid and late lactation.

Long-chain SFAs showed a progressive increase from Early to Late lactation, peaking during the final phase. Total MUFA and PUFA concentrations were highest during the Early phase, followed by a decline in the Mid phase. This trend aligns with the high rate of lipid mobilisation from adipose tissue typical of early lactation. The DIC18 index (representing the activity of Stearoyl-CoA Desaturase on C18 substrates) was significantly higher than the indices for shorter chains (DIC14, DIC16). A notable increase in DIC18 and DIC17 was observed in the Late phase, suggesting enhanced mammary desaturation activity as the animal nears the end of the lactation cycle.

The expression profiles of lipid metabolism genes showed phase-dependent variation, with ACACA and SCD exhibiting higher expression during late lactation, suggesting increased metabolic activity (Table 3). Correlation analysis revealed that ACACA and FASN were strongly associated with short-, medium- and long-chain saturated fatty acids as well as MUFA, indicating their central role in de novo fatty acid synthesis. In contrast, SCD, despite its increased expression, showed weak association with MUFA, suggesting that desaturation of fatty acids may be influenced by factors beyond gene expression, such as substrate availability or post-transcriptional regulation. FABP3 exhibited moderate correlations with long-chain fatty acids and PUFA, supporting its role in fatty acid transport. Overall, the combined analysis of gene expression and fatty acid profiles highlights coordinated regulation of lipid metabolism during different lactation stages.

This research offers insights into how lipogenic gene expression varies across different lactation stages and its connection to milk fatty acid profiles in Belahi cattle. Results show notable differences in the expression of key genes, including ACACA, FABP3, SCD and FASN, during lactation, underscoring the mammary glands' metabolic flexibility. These genes regulate fatty acid synthesis and metabolic pathways that shape milk's fatty acid composition. As reported by Bionaz and Loor (2008), a group of lipogenic genes, including ACACA, FASN and SCD, are upregulated during lactation, highlighting their critical roles in fatty acid creation and secretion. The SCD gene encodes an endoplasmic reticulum desaturase that converts saturated fats into monounsaturated fats, influencing milk fat quality (Ntambi and Miyazaki, 2004; Mele *et al*., 2007). FASN is essential for de novo lipogenesis,



Note: DIC14: Desaturase index of FA C14, DIC16: Desaturase index of FA C16, DIC17: Desaturase index of FA C17, DIC18: Desaturase index of FA C18.

Fig 2: A: Lactation stage wise estimates of saturated, monounsaturated and polyunsaturated fatty acids in belahi milk. B: Lactation stage wise estimates of desaturation indices.

producing long-chain fatty acids (Kumar *et al*., 2017). The FABP3 gene is associated with intracellular fatty acid transport and shows high expression in the mammary gland during early lactation and cellular differentiation (Yadav *et al*., 2019). Overall, these genes are integral to the molecular mechanisms governing lipid metabolism in the mammary gland. Additionally, transport-related genes like ABCG2 impact milk yield and composition by controlling secretion in mammary epithelial cells (Singh *et al*., 2020). Likewise, hormonal genes such as prolactin (PRL) are crucial in lactation, with PRL polymorphisms influencing test-day milk yield in Sahiwal cattle (Karuthadurai *et al*., 2023).

The study quantified the relative expression of key lipogenic genes which revealed distinct phase-wise expression across lactation stages. Similar results were reported by Kęsek-Woźniak *et al*. (2023), where ACACA, FASN and SCD expression varied significantly across lactation in Polish Holstein-Friesian cows, with generally higher expression during late lactation, indicating increased de novo fatty acid synthesis and desaturation activity. Janmeda *et al*. (2017) reported that in Surti and Jaffarabadi buffalo, lipogenic gene expression (SCD and ACACA) is highly regulated and maintained at a steady state during peak lactation, ensuring consistent milk fat synthesis. Overall, gene expression patterns corresponded well with changes in milk fatty acid composition during lactation.

A similar trend was noted for SCD, however, it has shown minimal expression during mid lactation and increased markedly in late lactation. SCD is primarily responsible for the desaturase activity of fatty acids. The gene expression results are supported by high Desaturase Index activity of C14, C16 and C18 in the last phase of lactation (Fig 2). Similar results were reported by Macciotta *et al*. (2008), that variation at the SCD locus significantly influenced milk fatty acid composition, particularly by increasing the proportion of monounsaturated fatty acids (MUFA) and affecting desaturation indices. Yadav *et al*. (2015) reported that, in buffaloes, SCD showed stage-dependent expression, reflecting its role in regulating fatty acid desaturation.

We also observed that FASN expression declined sharply in mid phase and partially recovered in last phase of lactation, although it remained lower than in early lactation. Expression of the FASN gene correlates well with long-chain saturated fatty acids in Fig 2. In contrast, FABP3 expression decreased significantly from the early phase through the remaining mid phases and remained low in the late phase.

Gene expression analysis revealed coordinated upregulation of de novo fatty acid synthesis genes (ACACA, FASN) and the desaturation gene (SCD) during late lactation. Similar patterns were reported by Pećina *et al*. (2023), where FASN contributed to fatty acid synthesis and

Table 3: Correlation between relative gene expression of candidate genes and fatty acid composition across different lactation stages.

Traits	Short-chain SFA			Medium-chain SF			A Long-chain SFA			MUFA			PUFA			DIC14			DIC16		
Phase of lactation	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
ACACA	0.15	0.20	0.41	0.56	0.65	0.24	0.56	0.83	0.67	0.72	0.98	0.18	0.55	0.73	0.43	0.76	0.60	0.69	0.66	0.87	0.48
FABP3	0.15	0.19	0.41	0.56	0.65	0.24	0.56	0.81	0.67	0.72	0.97	0.18	0.55	0.72	0.43	0.76	0.61	0.68	0.66	0.87	0.47
SCD	0.15	0.94	0.41	0.56	0.65	0.24	0.56	0.09	0.67	0.73	0.07	0.18	0.56	0.88	0.43	0.75	0.02	0.68	0.67	0.03	0.48
FASN	0.15	0.19	0.41	0.56	0.66	0.24	0.56	0.83	0.68	0.72	0.98	0.18	0.56	0.73	0.44	0.75	0.59	0.67	0.67	0.88	0.47

Note: SFA (Saturated fatty acids), 1: Early, 2: Middle, 3: Late phase of lactation.

SCD was associated with increased MUFA due to its desaturase activity. The GH gene was mainly linked to fat deposition traits, indicating an indirect role in lipid metabolism. In contrast, FABP3, involved in intracellular fatty acid transport, showed reduced expression after early lactation, corresponding with the observed decline in MUFA and PUFA during the late phase.

In Belahi cattle, higher MUFA and PUFA levels during early lactation suggest greater reliance on circulating lipids from body reserves and diet, reflecting the metabolic demands and possible negative energy balance (NEB) at this stage. In late lactation, increased expression of ACACA and FASN indicates enhanced de novo fatty acid synthesis, while SCD activity contributes to fatty acid desaturation, as supported by increased desaturation indices. However, the concurrent rise in SFA suggests the involvement of additional regulatory mechanisms maintaining milk fat composition. Studying these expression patterns in pastoral breeds like Belahi is important, as their extensive management and variable nutrition can influence metabolic regulation across lactation. These findings provide valuable insights into lipid metabolism in indigenous cattle under pastoral systems, which remain less explored compared to intensively managed dairy breeds.

CONCLUSION

This study showed that key lipogenic genes (ACACA, FABP3, SCD and FASN) in Belahi cattle are differentially regulated across lactation, reflecting dynamic changes in milk fatty acid metabolism. Late lactation is marked by upregulation of ACACA and SCD, indicating enhanced fatty acid synthesis and desaturation, whereas early lactation is dominated by lipid mobilisation and mid-lactation is a transitional phase. The sustained downregulation of FABP3 suggests reduced fatty acid transport in later stages. These results highlight stage-specific molecular regulation of milk fat composition and indicate that ACACA, FASN and SCD may be candidate genes for improving milk quality through marker-assisted or genomic selection. However, further validation in larger populations is necessary to confirm their utility in breeding programmes.

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Ethical approval

The collection of blood samples required for genotyping in this study was approved by the Institutional Animal Ethics Committee (IAEC) of ICAR-NDRI under proposal number 52/IAEC/24/28, dated 21 May 2024.

Conflict of interest

The authors declare that they have no conflicts of interest related to the publication of this article.

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