International Journal of Veterinary Sciences and Animal Husbandry 2025; SP-10(7): 101-105

ISSN: 2456-2912
NAAS Rating (2025): 4.61
VET 2025; SP-10(7): 101-105

www.veterinarypaper.com
Received: 17-06-2025
Accepted: 18-07-2025

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Fatty acid profile of Vechur cow milk at different stages of lactation

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DOI: https://www.doi.org/10.22271/veterinary.2025.v10.i7Sb.2427

Abstract
The purpose of this study was to determine the fatty acid profile of Vechur cow milk at different stages of lactation using gas chromatography equipped with a flame ionization detector (GC-FID). Milk contains a variety of fatty acids, including short-chain, medium-chain, and long-chain fatty acids, as well as saturated, unsaturated, odd-chain, cis, and trans fatty acids. Vechur cattle, an indigenous breed of Kerala, are well known for their disease resistance and high adaptability to the hot and humid conditions of the region. Six milk samples each were collected from early, mid, and late stages of lactation from Vechur cows maintained at the University Vechur Cattle Farm, Mannuthy. The milk samples were subjected to lipid extraction, converted into fatty acid methyl esters, and analyzed by GC-FID. The study analyzed 13 fatty acids across 18 samples from different lactation stages. Although variations in fatty acid concentrations were observed among the samples, statistical analysis revealed no significant differences between early, mid, and late lactation stages. The GC-FID method used in this study proved to be a simple, rapid, sensitive, and highly reproducible technique for analyzing the fatty acid profile of milk samples.

Keywords: GC FID, Vechur, milk, fatty acids, milk samples

1. Introduction
Milk is a secretory product from mammary glands of mammals and is a rich source of various nutrients especially fat-soluble vitamins, omega-3 fatty acids and conjugated linolenic acid. Milk contains about 3-4% of fat and the percentage of fat depends on factors like breed, season, region, nutrition, and period of lactation. Milk fat is a complex molecule containing triacylglycerol, as the major part which in turn composed of glycerol esterified with different fatty acids. In ruminants the milk fatty acids are derived from the microbial activity of ruminant microbes and from dietary sources. Majority of even numbered fatty acids are synthesized in the mammary gland itself, whereas odd chain fatty acids are the contribution of microbial activity in the rumen. Milk fat is said to have 400 different fatty acids of unique properties which make it more complex among natural fat. Besides, their role as source of energy, fatty acids act as an important constituent in cellular membranes as building blocks and helps in regulating membrane fluidity. In membrane phospholipids they assure flexibility, stability, signaling and permeability of membranes. Among the different types of fatty acids omega 3 and omega 6 polyunsaturated fatty acids seems to be the most important, as they help in reducing oxidative stress, neuroprotection and cardiovascular protection Nagy *et al.* (2017) [16]. Vechur cattle, the indigenous breed of Kerala is well known for its disease resistance and high adaptability to hot, humid conditions of Kerala. The determination of fatty acid profile in milk of Vechur cow will give an idea about the quality of milk with respect to fatty acid composition at different stages of lactation. Vechur cattle, the breed yields about 1.5-2 kg milk per day with fatty acid content of 4.7-5.8%. Due to smaller size of fat globule and rich fat content compared to crossbred cows, Vechur cow milk is beneficial for health in consumers especially infants and aged people. The purpose of identifying the fatty acid profile of indigenous cattle at different stages of lactation is to understand the compositional changes in milk fat throughout the lactation cycle.

This information can help assess the nutritional quality and health benefits of the milk, optimize feeding and management practices, and promote the value of indigenous breeds known for their unique milk composition and adaptability to local environments. Gas chromatography (either GC-FID or GC-MS) is a convenient method to analyze the fatty acids in milk because it is highly accurate, simple, safe, and rapid. The present study was carried out with an objective of determination of fatty acid profile in Vechur milk at different stages of lactation.

2. Materials and Methods

2.1 Sample collection

Six milk samples each from early, mid, and late lactation were collected from lactating Vechur cows maintained at University Vechur Cattle Farm, Mannuthy. A total of 18 morning milk samples were collected and subjected for lipid separation and then converted it into the fatty acid methyl esters (FAME) for GC analysis.

2.2 Chemicals and Reagents

Standard FAME Mix C_{14}-C_{22} (CRM18917-USA) 100mg (Table 1) was purchased from M/S Sigma-Aldrich. Sodium chloride and Isooctane, Methanol and Sodium sulfate (dry purified) were purchased from M/S Merck Life Science Pvt. Ltd. Mumbai. Sodium methoxide (25-30%) and Butylated hydroxy toluene were bought from M/S Himedia Laboratories Pvt. Ltd. Mumbai. N-Hexane (GC-Graded) 95% and Diethyl ether were obtained from M/S Spectrochem Pvt. Ltd. Mumbai.

2.3 Sample preparation

Ten mL of fresh milk sample were centrifuged at 12,000 rpm for 30 min at 4 °C. Fat layer of about 1 g was collected from the top and placed into a 15mL solvent resistant tube which was rinsed with hexane. Hexane: isopropanol (3:2 v/v) containing 50 mg of butylated hydroxytoluene was added at the rate of 18 mL/g of fat and vortexed. After vortexing, added 6.7% sodium sulfate solution at the rate of 12 mL/g of fat again vortexed for 1min and allowed to stand for layer separation to complete. After the layer separation, the upper hexane layer was transferred into a 15ml test tube containing 1 g anhydrous sodium sulfate and vortexed for 1 min and allowed to stand for 45 min. The upper hexane layer was transferred into a vial and then subjected for evaporation by placing it in a vacuum concentrator for 2 hours at 2000 rpm. Leftover unevaporated lipid in the vial was transferred into a 50 mL standing tube and 5 mL of 0.25 mol/ L sodium methoxide in methanol: Diethyl ether (1: 1 v/v) was added and vortexed for 1 min. Three mL isooctane and 15 mL saturated NaCl were added and vortexed for phase separation The top layer containing fatty acids was collected in labelled Eppendorf tubes and stored in refrigerator, until analysis.

2.4 Preparation of working standard solutions

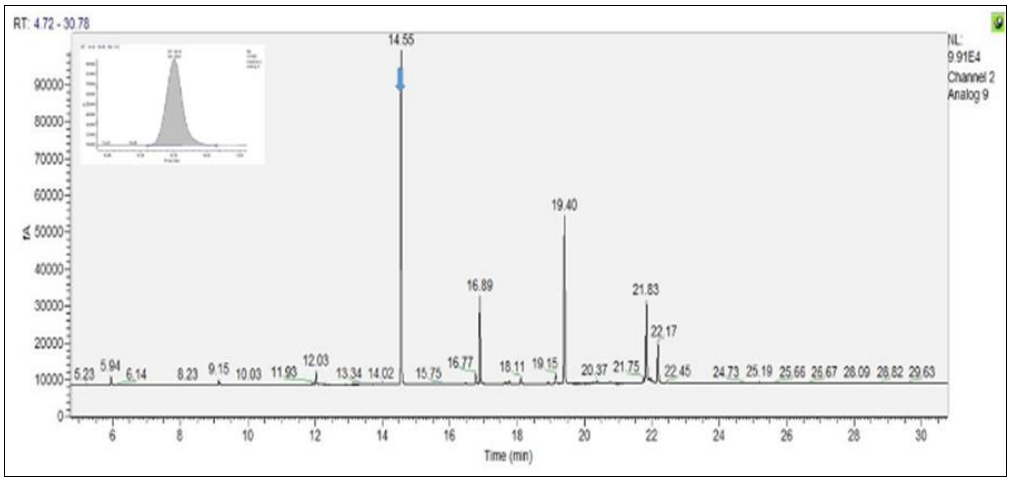

Fig 1: Representative chromatogram of sample V716 (early lactation), featuring myristic acid (C14:0) Downward arrow indicates retention time of 14.55.

Four different standard solutions of FAME mix C_{14}-C_{22} was prepared. The standard (100 mg in 1 mL n-hexane) was used as stock standard to prepare the working standard solution of 50 mg/mL, 25 mg/mL and 12.5 mg/mL All standards were kept in amber colored bottles at 4 °C to avoid any ill effects.

2.5 Chromatographic Condition

One µL of blank (hexane), standards and samples were injected separately into the 5 MS column using an auto sampler. The components were separated by elution using carrier gas helium at a flow rate of 30 mL/min. The Flame Ionization detector and injection temperature were set at temperature 250 °C. Air and H_2 gas flow rate of FID were 300 mL/min and 30 mL/min respectively. Helium gas was used as makeup gas with a flow rate of 30 ml/min. Chromatogram was generated for each injected solution.

2.6 Detection

The eluted fatty acids in the Vechur cow milk samples were detected by flame ionization detector.

2.7 Statistical analysis

Statistical analysis were done to compare the data at different stages of lactation using post hoc test namely duncan's multiple range test. Results were considered statistically significant for $p<0.05$ and the resulted presented as mean values ± standard deviation.

2.8 Analysis of fatty acids by GC-FID

The fatty acid methyl esters of milk sample of early, mid and late lactation stages were analyzed and chromatograms are shown in the Figures 1 to 3 with respect to representative sample used in each lactation stage. The data acquisition time for each sample were set for 30 minutes

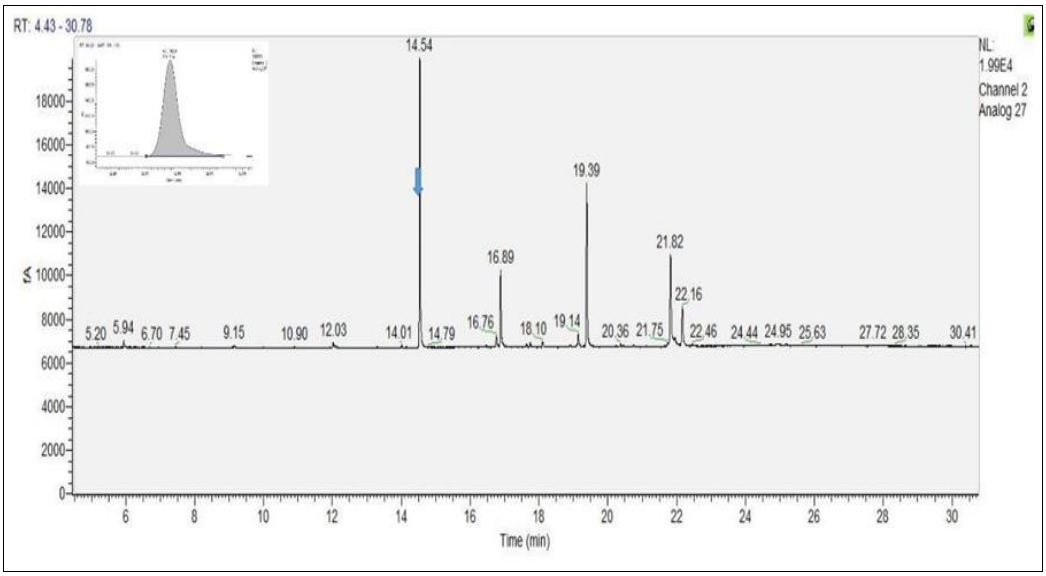

Fig 2: Representative chromatogram of sample V1048 (mid lactation), featuring myristic acid (C14:0) Downward arrow indicates retention time of 14.54

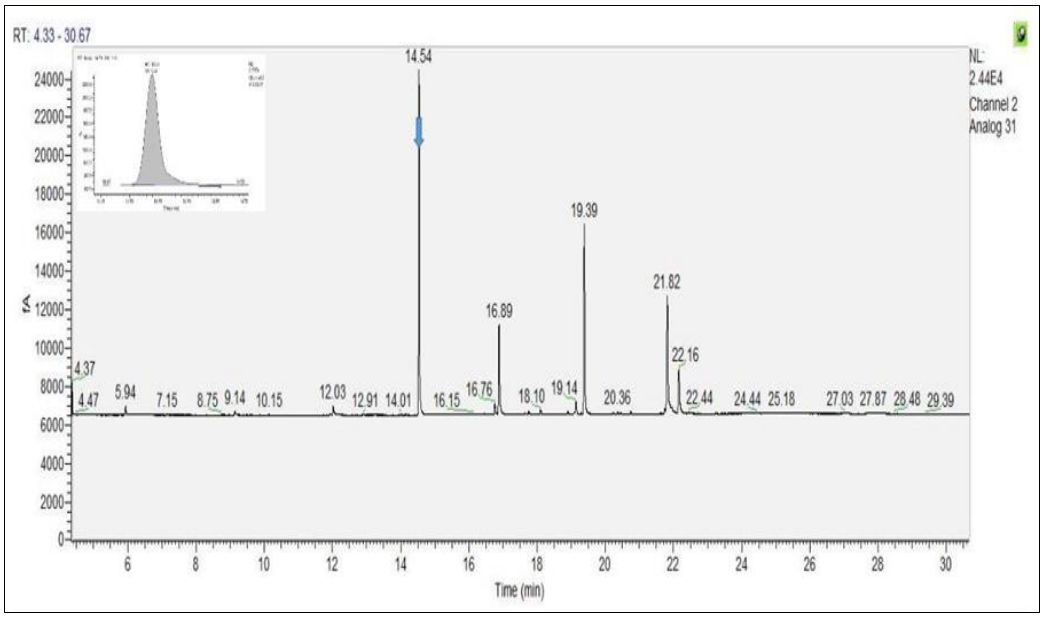

Fig 3: Representative chromatogram of sample V996 (late lactation), featuring myristic acid (C14:0) Downward arrow indicates retention time of 14.54.

Table 1: Fatty acid composition of milk fatty acids during lactation stages, each with six samples (Mean ± S.E)

SL No.	Fatty acids (mg/g)	Early lactation (Mean ± S.E)	Mid lactation (Mean ± S.E)	Late lactation (Mean ± S.E)	P-Value
Saturated fatty acids					
1	Caprylic acid (C8:0)	0.04±0.006	0.01922±0.00339	0.024±0.004	0.611
2	Capric acid (C10:0)	0.008±0.0014	0.00534±0.00088	0.010±0.002	0.664
3	Lauric acid (C12:0)	0.202±0.04	0.82±0.0004	0.70±0.133	0.603
4	Myristic acid (C14:0)	84.9±21.0345	90.88±39.3	58.8±10.1	0.877
5	Palmitic acid (C16:0)	2±0.4	9.06±4	10.05±3	0.515
6	Stearic acid (C18:0)	0.02±0.03	0.0344±0.03	0.08±0.02	0.517
7	Arachidic acid (C20:0)	0.0068±0.001	0.04±0.11	0.026±0.005	0.424
8	Behenic acid (C22:0)	0.002±0.0002	0.0024±0.0004	0.0004±0.0008	0.43
Mono unsaturated fatty acids					
9	Myristoleic acid (C14:1)	0.0268±0.0041	0.12±0.05	0.2±0.04	0.383
10	Palmitoleic acid (C16:1)	0.4±0.1	2.21±1	2.6±0.9	0.556
11	Oleic acid (C18:1)	0.0044±0.0006	0.0062±0.0016	0.0084±0.0025	0.723
Poly unsaturated fatty acids					
12	Linoleic acid (C18:2)	0.2±0.0224	0.50±0.201	0.6±0.2	0.605
13	Linolenic acid (C18:3)	0.008±0.0012	0.032±0.013	0.048±0.0132	0.432

Significant (p<0.05)
Highly significant (p<0.01)
Not-Significant (p>0.05)

Table 2: Average fatty acid content of samples during entire lactation

S. No	Fatty Acids	Chemical Formula	Average Fatty Acid Content (mg/g)
Saturated fatty acids			
1	Caprylic acid (C8:0)	C8H16O2	0.025
2	Capric acid (C10:0)	C10H20O2	0.007
3	Lauric acid (C12:0)	C12H24O2	0.573
4	Myristic acid (C14:0)	C14H28O2	68.754
5	Palmitic acid (C16:0)	C16H32O2	6.932
6	Stearic acid (C18:0)	C18H36O2	0.056
7	Arachidic acid (C20:0)	C20H40O2	0.022
8	Behenic acid (C22:0)	C22H44O2	0.003
Mono unsaturated fatty acids			
9	Myristoleic acid (C14:1)	C14H26O2	0.099
10	Palmitoleic acid (C16:1)	C16H30O2	1.730
11	Oleic acid (C18:1)	C18H34O2	0.006
Poly unsaturated fatty acids			
12	Linoleic acid (C18:2)	C18H32O2	0.392
13	Linolenic acid (C18:3)	C18H30O2	0.029

3. Results and Discussion

The present study analysed 18 Vechur cow milk samples at different stages of lactation for 13 fatty acids by GC-FID. Among 13, we analysed 8 saturated, 3 monounsaturated and 2 poly unsaturated fatty acids. The table 1 shows the fatty acid composition of milk during different lactation stages. The milk samples showed no significant difference in the fatty acid composition (p>0.05) from early to late lactation stages. Lauric acid and myristic acid showed significant increase during early and mid-lactation stages, then decreased during late lactation. As the lactation progressed palmitic acid and stearic acid showed gradual increase in the fatty acid content. Similarly, myristoleic acid, palmitoleic acid and linoleic acid progressively increased during the lactation stages. Caprylic acid, capric acid, arachidic acid, behenic acid, oleic acid and linolenic acid did not show any significant variation as lactation progressed.

The average composition of fatty acids during entire lactation is shown in table 2 and it showed that myristic acid ($C_{14:0}$) is the major fatty acid found in Vechur cow milk during all stages of lactation followed by palmitic acid ($C_{16:0}$). Their average concentration was 68.754 mg/g and 6.932 mg/g of total fatty acids respectively and least represented fatty acids were behenic ($C_{22:0}$) and oleic acid. ($C_{18:1}$). The content of behenic is 0.003 mg/g and oleic is 0.006 mg/g of total fatty acids. Among mono unsaturated fatty acids, palmitoleic acid has highest concentration of 1.730 mg/g compared to myristoleic acid (0.009 mg/g) and oleic acid (0.006 mg/g). The average content of poly unsaturated fatty acids like linoleic acid and linolenic acid was found to be 0.392 mg/g and 0.029 mg/g respectively. The average content of saturated fatty acids were 76.372 mg/g, mono unsaturated fatty acids 1.835 mg/g and poly unsaturated fatty acids 0.421 mg/g of total fatty acids during the entire period.

The present study analysed three medium chain and ten long chain fatty acids. The concentration of medium chain fatty acids namely caprylic and capric were found to be decreased during mid-lactation compared to early and late lactation, whereas lauric acid concentration was high in early and mid-lactation and showed a tendency to decrease towards late lactation. Long chain fatty acids like palmitic, stearic, behenic, myristoleic, palmitoleic, oleic, linoleic and linolenic acids were found to be increased as lactation progress while, arachidic acid concentration found decreased during the late lactation compared to early, mid and late lactation. Myristic acid get decreased in the late lactation. These findings are in accordance with the findings of (Craninx *et al*., 2008) [4] who reported short, medium, and long chain fatty acids were low in the beginning of lactation.

Current study observed an increase in concentration of saturated fatty acids like palmitic, stearic, arachidic and behenic during the progression of lactation. This finding was in agreement with Stoop *et al*., 2000 [19] who reported an increase in the content of stearic acid in late lactation. Saturated fatty acids such as lauric, myristic and palmitic fatty acids are highly associated with the risk of cardiovascular diseases in human beings (Baer, 1991) [2]. Palmitic acid influences the decreased level of insulin in our body and stearic acid provides a protection against cardiovascular diseases (Bainbridge *et al*., 2016) [3].

Quantity of medium chain saturated fatty acids such as caprylic and capric acid were found to be higher in early and late lactation compared to mid-lactation whereas lauric acid showed a decrease from early to late lactation. A study by Baer, 1990 reported increased amount of saturated fatty acid (capric to myristic) during early lactation and get declined in late lactation. The change reported in our study may be due to variation in the breed, season, feed, and environmental condition.

The current study analysed and quantified monounsaturated fatty acids (MUFA) like oleic, palmitoleic and myristoleic acids. These three fatty acids showed an increase in their concentration from early to late lactation. A study conducted by Duchacek *et al*., 2011 [6] in crossbreds observed a decrease in concentration of oleic and palmitoleic and increase in concentration of myristoleic as lactation advances. No significant difference in the concentration of MUFA was noticed by Baer 1990 at different stages of lactation. The variation of the present study may be due to change in the environmental condition, feed, breed, and nutrition at different stages of lactation.

Polyunsaturated essential fatty acids (PUFA) namely linoleic (ω 6) and linolenic (ω 3) acids were also quantified during the study. Both the fatty acids cannot be synthesised in body, but it has to be supplied through diet. Even though there is no significant difference in the concentration of linoleic and linolenic acid at different stages of lactation, both acids showed progressive increase in their concentration as lactation advances. The findings are in accordance with Bainbridge 2016 [3] who reported no significant difference in the concentration of ω 3 and ω 6 fattyacids at different stages of lactation.

4. Conclusion

The present work provides an overall GC analysis of the fatty acid profile in milk from Vechur cows. Not only the fatty acid groups or the most abundant and the ones that found in small quantities and showing benefic outcomes on human health are also included. Lactation stages highly influenced in the fatty acid content, medium chain fatty acids showed no significant differences in the fatty acid composition, while most of the long chain fatty acids shown a considerable increase as the lactation progressed. These differences in the fatty acid content in milk are mainly due to season, breed, environmental changes, and feed. The use of GC FID technique resulted in highly specific separation of fatty acid methyl esters, and it was a simple, rapid, sensitive, and highly reproducible technique for the analysis of fatty acid profile of milk samples.

5. Conflict of Interest

The authors in this article have no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

6. Acknowledgements

The work was carried out at Central Instruments Laboratory and Department of Veterinary biochemistry, College of Veterinary and Animal Sciences, Mannuthy, Kerala. The support and assistance given by all the staff members in the above-mentioned departments are duly acknowledged.

Financial Support

Not available

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How to Cite This Article

Nath AG, Chinnu MV, Vidhya KT, Saratht M. Fatty acid profile of Vechur cow milk at different stages of lactation. International Journal of Veterinary Sciences and Animal Husbandry. 2025;SP-10(7):101-105.