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Investigation of Gas Chromatography-Mass Spectrometry (GC-MS) Differentiated Fatty Acids in Negative Energy Balanced Cows Prepartum- and Postpartum

Ali Naer Aljumaili [1] , Miaad Jabbar Alhilali[2]

[1,2]Department of Physiology, Biochemistry and PharmacologyCollege of Veterinary Medicine,Al-Qadisiyah University, Diwaniyah, Iraq

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Correspondence
Ali Naer Aljumaili
hedralsaede@gmail.com

Miaad Jabbar Alhilali
Miaad.sahib@qu.edu.iq

Abstract Metabolic changes occur in mammary gland cells of dairy cows with negative energy balance (NEB) may alter milk fatty acid profile. The aim of study is assessment the concentrations of fatty acids in milk of dairy cows exploring gas chromatography- mass spectrophotometry (GC-MS) analysis. Twenty milk samples had collected from ten dairy cows, two samples from each cow 3weeks prepartum and 3weeks postpartum. All milk samples were lyophilized and analyzed using GC-MS to extinguish their fatty acid metabolites. Energy status of dairy cows either at normal-status or NEB had determined according to serum concentrations of beta-hydroxybutyric acid (β-HB). The results of the study revealed that palmitic acid (C16:0) is the prominent fatty acid in cow milk with a highest percentage of fatty acids in all groups of the study followed by oleic acid (C18:1 cis-9), stearic acid (C18:0), and myristic acid (C14:0), respectively. Except in normal-status cow milk prepartum which has no differentiated GC-MS stearic acid. In addition, palmitic acid decreased in NEB compared to normal-status cow milk both pre- and postpartum. Oleic acid and stearic acid decreased in NEB compared to normal-status group postpartum only. While myristic acid increased pre- and postpartum in NEB group compared to normal-status cows. However, all these differences in fatty acid concentrations did not reach significant levels ($p>0.05$). It can be concluded that NEB-associated metabolic disturbances in lipolysis and lipogenesis led to alterations in cow milk fatty acid profile and the long-chain fatty acids are most affected both pre- and postpartum.

Keywords: *Gas chromatography- mass spectrophotometry, metabolites, palmitic acid, lipolysis NEB*

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Introduction Milk is an enriched fluid produced by the mammary glands and meets the nutritional needs of newborn mammals during the early critical period of physical development providing essential nutrients for growth and development (1). Cow milk is a rich source of essential nutrients such as lipids, proteins, lactose, minerals and vitamins, and is known as "white blood". So, it represents a significant component in diet of many individuals. Furthermore, cow milk has unique composition of amino acid makes its protein provide different bioactive

peptides (2). One of the major milk components are milk lipids, about 98% of these lipid compositions consist of triacylglycerols and compromise fatty acids (FAs) of different saturations and lengths (3). Many factors affecting cow milk composition involving age, breed, feed, stage of lactation period, season, and cow's health condition (4). In dairy cows, the term of energy balance points to the difference between the energy intake and the energy demand for milk production and maintenance (5). In most dairy cows especially in early lactation, energy intake cannot meet the energy requirement for milk synthesis, leading to negative energy balance (NEB). Many detrimental effects of NEB on cow had established and severe NEB makes cows more susceptible to oxidative stress, incidence of metabolic disorders, and impaired their fertility (6, 7), decreased conception rate and increased early embryo fertility (8). For instance, the study of Macrae et al. (2019) found that within the first 20 days of milking, about 75.2% of cows in the UK suffering from NEB (9).

Being in NEB status for dairy cows is a complex physiological metabolic condition including extensive changes in energy sources and metabolic pathways, modifying blood constituents of non-esterified fatty acid (NEFA) or β-hydroxybutyrate acid (β-HB) (8, 10, 11). Therefore, testing of NEB in farms by veterinarians is classically depending on direct measure of these two biomarkers (9). However, direct measure of these biomarkers is always based on blood samples that are obtained in an invasive manner (12). The composition of FA concentrations in milk can be used as a biomarker for NEB. Milk biomarkers are more useful for sever NEB prediction than plasma biomarkers. It is difficult to routinely collect blood from each animal on a farm, while milk data may be readily available to daily farmers. This makes milk biomarkers potentially applicable for screening the energy balance of individual dairy cows (13).

Many noninvasive methods had used to predict NEB in milk samples such as Liquid-chromatography-Mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) to determine FAs, triglycerides and diglycerides in milk samples (10), mid-infrared (MIR) spectrometry (12), and also Fourier- transform infrared spectroscopy (FTR) analysis (13). FAs profile is typically analyzed in milk using gas chromatography as a precise method, but the expense makes it only suitable for limited numbers of samples, therefore, this analysis can be used in scientific researches (13). So, the aim of the current study is to assess concentrations of FAs in milk of dairy cows exploring GC-MS analysis. In addition, to examine whether these FAs affected by different energy balance states both pre- and postpartum.

Material and methods

Ethical Approval

This study was conducted in accordance with the ethical guidelines of the University of Al-Qadisiyah, College of Veterinary Medicine, and was approved by the institutional ethics committee (Approval No. 547, 2025).

Study design

Twenty milk samples were collected from ten dairy cows, two samples from each animal were collected three-weeks pre-and three-weeks postpartum. At the same time, blood samples from all study animals were drawn from the jugular vein and put in gel tube (Afco, Jordan). Then samples were centrifuged to collect serum for β-BH evaluation using the Sandwich bovine β-BH ELISA kits (SunLong, China). The preparation of β-HB standard solution and assay procedures was performed following the manufacturer's instructions. Cows were considered at normal status or in NEB when β-HB is <1.4 mmol/L or β-HB >1.4mmol/L, respectively according to Warma et al. (14). Animals were divided according to their energy balance status in to two groups including normal status cows (n=4) and negative energy balanced cows (n=6). Animals samples collection lasts from December 2024 to April 2025 at Taj Al-Nahrain for Plant Production and Animal Wealth in Diwaniyah Governorate. Milking time was 6:00 A.M and about 20 ml of milk from the four quarters of udder had collected in each sampling. Milk samples had preserved into clean and sterile plastic containers and frozen in -30 C˚ until analysis.

GC-MS condition

The GC-MS examination of twenty milk samples was conducted following the methods of Wang et al. (15). In summary, 5 mg of fat was mixed with 1 mL of n-hexane and treated with 0. 5 ml of KOH–CH3OH (2 mol/L) for methylation. This was then examined using the Agilent 7820A gas chromatography (GC) spectrometer (5977E), which had a hydrogen flame ionization detector from Agilent in Santa Clara, CA, USA, and a DB-Fast FAME column (see Table 1). The fatty acid methyl esters were recognized by comparing their retention times to standard mixtures of fatty acid methyl esters, and their amounts were reported as weight percent.

Analysis of sn-2 fatty acids was done based on the method by Qi et al. (16). To summarize, 80 mg of fat were broken down into sn-2 monoacylglycerol (MAG) using 80 mg of pancreatic lipase (porcine pancreatic lipase, L3126) while being kept in a water bath at 37 °C. The resulting products from the hydrolysis were separated on a thin layer chromatography plate using a developing solution of n-hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The section of the plate that contained sn-2 MAG was scraped off and extracted with diethyl ether. After removing the solvent using nitrogen gas evaporation, the sn-2 MAG was collected. Finally, the sn-2 fatty acids were methylated and analyzed by GC-MS as previously described.

Compounds were determined according to the NIST library, that is each GC-MS differentiated compound/metabolites has special library ID number and chemical service number (CAS number) for increasing accuracy of compounds determination. Match quality/similarity index (0-100) and refers to matching rate of the compound mass spectrum in the sample with respective mass spectrum in the library. Very strong matching, good and probable matching, weak or in definite matching were considered when match quality/similarity index more than 90, 70-89, and less than 60, respectively. The amount of certain compound in the sample was respective to its area % in GC-MS analysis at certain retention time.

Table 1. Characteristics of GC-MS conditions

Parameter	Condition
GC-Mass system	Spectrometer HP.5977E
Column	Packed column (30m length * 250µm inner diameter * 0.25µm film thickness)
Carrier gas	Helium 99.99%
Scan range	m/z 25-1000
Injection type	Spitless
Pressure	11.933 psi
Analytical column	Agilent
Injector temperature	250 C˚
Oven temp. programmed	60C for 7 min to the final Temperature 300 C˚
Injection volume	1µl
GC inlet line temp.	250 C˚
Aux heaters temp.	320 C˚

Statistical analysis

The statistical analysis of GC-MS differentiated fatty acids in milk samples of normal status and NEB cows pre- and postpartum had done. We used Graph Pad Prism (Version 8.4.3) (GraphPad Software Inc., LaJolla CA, USA) to analyze these data statistically and data were expressed as means ± standard errors of means (M± SEM). For accurate results, the statistical analysis involved the compounds that definitely diagnosed with GC-MS at match quality/similarity index about 98% only. Whereas other compounds with less quality/similarity index were excluded from the statistical analysis. Differences considered statistically significant when p-value is less than 0.05.

Results

GC-MS differentiated fatty acids prepartum

The chromatograph of all GC-MS detected metabolites in normal status cow milk prepartum was presented in (figure-1) as an example for this group of the study. GC-MS differentiated fatty acids/ fatty acid esters prepartum in normal-status had determined at retention time ranged between 4.775 for acetic acid

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ethoxyhydroxy-, ethyl ester and 29.338 for 2-ethylbutyric acid, 2,7-dimethyloct-5yn-7en-4-yl ester. According to the chromatogram data, many compounds were the more confident diagnosed, i.e. (matching quality was about 98%) based on their respective area % to the total milk compounds in GC-MS chromatogram at certain retention time. These compounds include: no free short-chain fatty acid (SCFAs) had differentiated in milk samples of this group prepartum. Medium-chain fatty acid (MCFA) was myristic acid (C14:0) 1.35%±0.39. Two long-chain fatty acids (LCFAs) had detected with GC-MS analysis in this group prepartum. One saturated LCFA fatty acids palmitic acid (C16:0) 9.78% ±3.16 and the unsaturated LCFA oleic acid (C18:1 cis-9) 3.12 ±0.86. Two other FAs were confident diagnosed in this group were lauric acid (C12:0) 1.54% and linolenic acid (C18:2) 4.05%, but they diagnosed in one sample only. The higher percentage of the confident GC-MS differentiated FAs in this group was palmitic acid followed by oleic and myristic acids, respectively (figure-2).

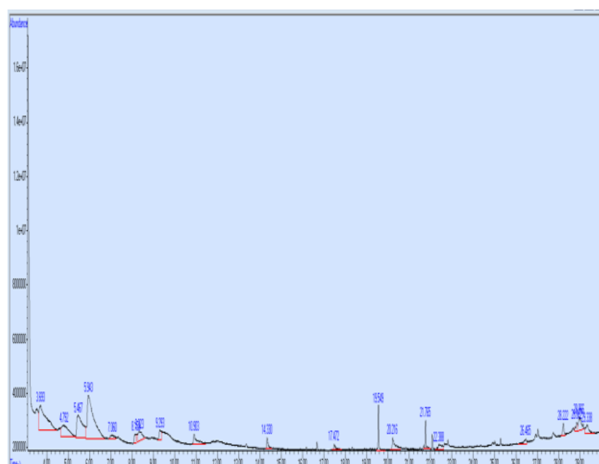

Figure 1. GC-MS differentiated metabolites in normal status cow milk prepartum.

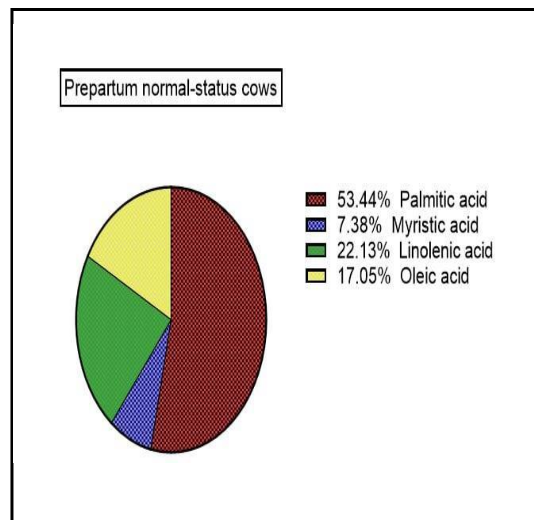

Figure 2. Pie chart showing GC-MS confident diagnosed fatty acids in normal status cow milk prepartum.

The chromatograph of all GC-MS detected metabolites in NEB cow milk prepartum was presented in (figure-3) as an example for this group of the study. GC-MS differentiated fatty acids/ fatty acid esters prepartum in NEB milk samples had determined at retention time ranged between 5.578 for butanoic acid, 2-oxo, methyl ester and 29.321 for capric acid-thio, S-butyl ester.

Like prepartum, many compounds were more confident diagnosed with their respective GC-MS area %, these compounds in NEB group prepartum include: no free SCFA had differentiated in milk samples of this group. The only MCFA was myristic acid (C14:0) 1.35%±0.39. Three LCFAs had detected with GC-MS analysis in this group prepartum. One saturated LCFA was palmitic acid (C16:0) 9.78% ±3.16 and two unsaturated LCFAs including oleic acid (C18:1 cis-9) 3.12 ±0.86 and linolenic acid 4.05%±0.71. In addition, the medium-chain capric acid (C10:0) 14.43% was confidently diagnosed, but in one sample only. The highest percentage of the confident GC-MS differentiated FAs in this group was palmitic acid followed by oleic acid, stearic acid, and myristic acid, respectively (figure-4). No significant differences ($p>0.05$) were appeared between the GC-MS area % of these FAs of normal-status and NEB milk samples prepartum.

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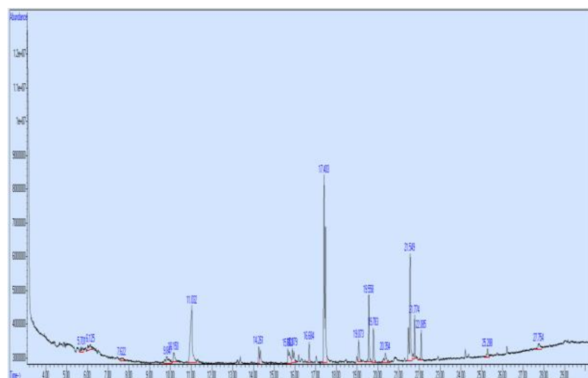

Figure 3. GC-MS differentiated metabolites in NEB cow milk prepartum.

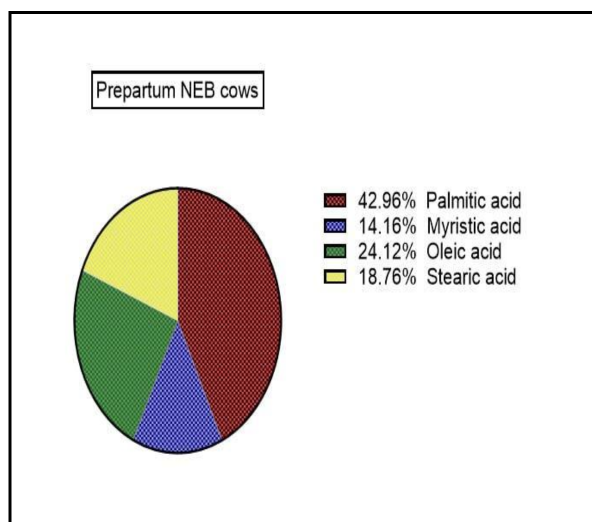

Figure 4. Pie chart showing GC-MS confident diagnosed fatty acids in NEB cow milk prepartum.

GC-MS differentiated fatty acids postpartum

The chromatograph of all GC-MS detected metabolites in normal status cow milk postpartum was presented in (figure-5) as an example for this group of the study. GC-MS differentiated fatty acids/ fatty acid esters postpartum in normal-status were determined at retention time ranged between 5.432 for propanoic acid, 2-(hydroxyimino)-, methyl ester and 29.546 for caproic acid butenyl ester. According to the chromatogram data, the more confident diagnosed compounds with their respective GC-MS area % in normal-status group prepartum include: no free SCFA had differentiated in milk samples of NEB cows prepartum. The only MCFA in NEB cow milk was myristic acid (C14:0) 3.60%±1.20. Three LCFAs had detected with GC-MS analysis in this group prepartum. Two saturated LCFAs were palmitic acid (C16:0) 14.04% ±6.35 and stearic acid 7.02%±2.93 and one unsaturated LCFAs was oleic acid (C18:1 cis-9) 13.13 ±5.30. The higher percentage of the confident GC-MS differentiated FAs in this group was palmitic acid followed by oleic acid, stearic acid, and myristic acid, respectively (figure-6).

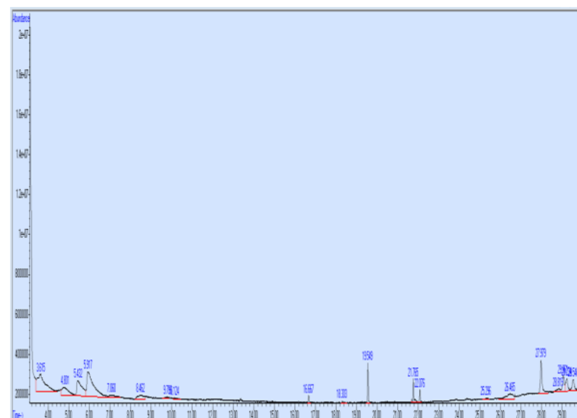

Figure 5. GC-MS differentiated metabolites in normal status cow milk postpartum.

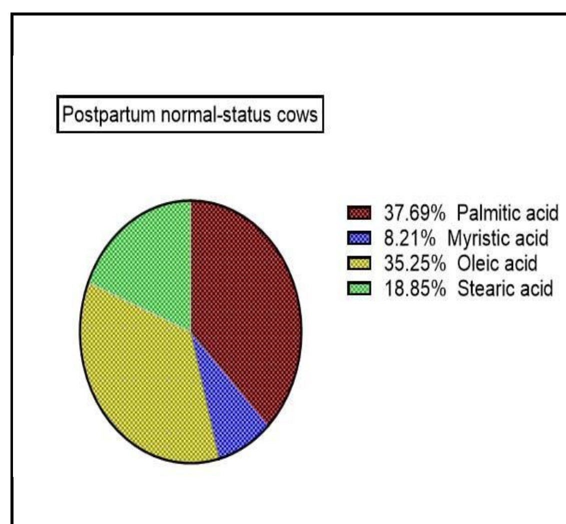

Figure 6. Pie chart showing GC-MS confident diagnosed fatty acids in normal status cow milk postpartum.

The chromatograph of all GC-MS detected metabolites in normal status cow milk postpartum was presented in (figure-7) as an example for this group of the study. GC-MS differentiated fatty acids/ fatty acid esters postpartum in NEB milk samples were determined at retention time ranged between 3.468 for acetic acid, oxo-, methyl ester and 22.085 for methyl isostearate. According to the chromatogram data, the more confident diagnosed compounds with

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their respective GC-MS area % in normal-status group postpartum include: no free SCFA had differentiated in milk samples of NEB cows postpartum. The only MCFA in NEB cow milk was myristic acid (C14:0) 3.51%±1.55. Three LCFAs had detected with GC-MS analysis in this group postpartum. Two saturated LCFAs were palmitic acid (C16:0) 10.93% ±3.56 and stearic acid (C18:0) 4.28%±1.51 and one unsaturated LCFA was oleic acid (C18:1 cis-9) 6.67 ±3.77. The higher percentage of the confident GC-MS differentiated FAs in this group was palmitic acid followed by oleic acid, stearic acid, and myristic acid, respectively (figure-8). No significant differences (p>0.05) were appeared between the GC-MS area % of these fatty acids of normal-status and NEB milk samples postpartum.

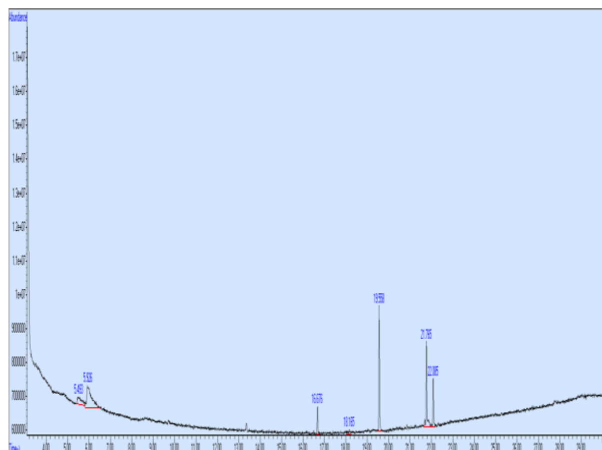

Figure 7. GC-MS differentiated metabolites in normal status cow milk postpartum.

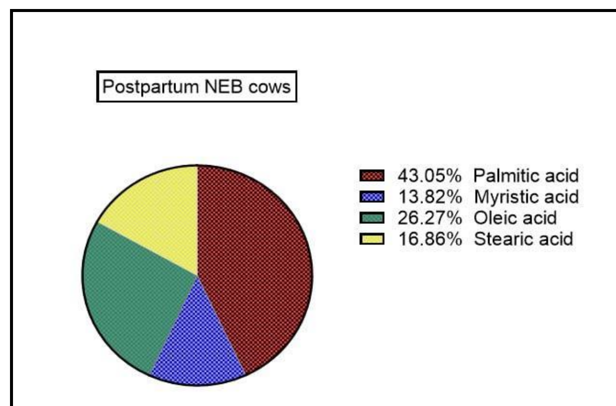

Figure 8. Pie chart showing GC-MS confident diagnosed fatty acids in NEB cow milk postpartum.

Discussion

The FAs of cow milk fat come from two major sources: synthesis de novo in the mammary gland and the plasma lipids originating from the diet (17). The FA that are produced de novo are mainly SCFAs and MCFAs from C4:0 to C14:0, whereas C18:0 and some C16:0 come from plasma lipids (18). In transition cows from late pregnancy to early lactation that mobilize excess FA postpartum, plasma levels of C14:0-, C16:0-, and C18:0- are increased relative to lean cows (19). The main substrate for de novo synthesis of FAs in the dairy cow is acetyl-CoA originating from acetate. Acetate provides the majority of the carbon and approximately one-half of the reducing equivalents needed for de novo lipogenesis (20). The NEB-associated ketosis resulted in alteration of the FAs profile, appeared as reduction in SCFA and MCFA and elevation in LCFA (21). Furthermore, L-aspartate and L-glutamate inhibit fat synthesis by fatty acid de novo and triacylglycerol synthesis pathways according to ultra-high performance liquid chromatography (HPLC-MS/MS) results (22).

The three most prominent FAs found in blood of dairy cows are saturated palmitic and stearic acid (23, 24) and the mono-unsaturated fatty acid oleic acid, which are indeed the most prominent FAs present in adipose tissue of cows (24). This consistent with our finding regarding myristic acid, palmitic acid, and oleic acid, however, stearic acid did not appear prepartum in normal-status cow milk in our study. The study of Churakov et al. (2021) revealed strong negative correlation between C18:1 Cis-9 and C18:0 in milk with energy balance according to FTR-analysis, at first six weeks post-calving (13). Oleic acid C18:1, a predominant FA in adipose tissue and is increased significantly within NEB-associated lipolysis. The increased levels of LCFA diverted into the plasma and added to the milk fat, in turn, these LCFAs implicate in inhibition of SCFA and MCFA de novo synthesis in mammary gland cells. Furthermore, milk FAs are correlated with methane production since they have common biochemical pathway in rumen (25, 26). Reduced milk C4–C14 concentrations along with elevated milk 18:0 and 18:1 Cis 9 concentrations indicate a sever mobilization of body reserve in early lactation stage (27).

Saturated/ unsaturated FAs act as bioactive signaling molecules able to modulate insulin production and sensitivity in addition to the modulation of FAs metabolic fate (28). In vivo, saturated and unsaturated fat induce hepatic insulin resistance independently of ceramide synthesis and TLR-4 signaling (29). This reduction of insulin sensitivity caused by FA may affect lipolysis and glucose

utilization in the mammary gland to synthesize milk. Regulation of insulin signaling depends on the degree of FA saturation, chain length and concentration, and whether the cow is under homeorhetic control (30). In addition, metabolic changes occur in mammary gland cells of NEB cows and increase in lipolysis and triglycerides anabolism according to the study of Xu et al. (2020) who revealed that FAs, triglycerides and diglycerides have pivotal role in membrane biosynthesis according to LC-MS and NMR analyzed milk samples (31). Furthermore, the study of Huang et al. (2024) identified the sphingolipid metabolism and FA degradation as important pathways in cows with subclinical ketosis using LC-MS/MS analyzed milk samples (32).

Conclusion

From the findings of current study, we can conclude that NEB-associated metabolic disturbances in lipolysis and lipogenesis lead to alterations in cow milk fatty acid profile and the long-chain fatty acids are the more affected ones, both pre- and postpartum. However, the small sample size may be the major limitation of this study. So, we recommended further studies with more sample size includes multiple time points during the transition period to enhance the accuracy of tracking metabolic changes.

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Conflict of interest

No conflict of interest was declared by the authors.

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