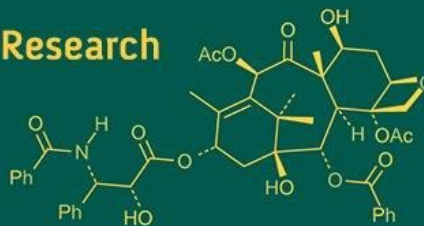


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Seasonal changes in major phospholipid composition of indigenous cow milk

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Abstract

The present study investigated the seasonal variation in total phospholipid content and major phospholipid classes, namely phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM), in the milk fat of indigenous Gir and Sahiwal cows. Milk samples were collected from Gir and Sahiwal cows ($n = 15$ of each season) throughout the winter, spring, summer, and rainy seasons. These samples were analyzed using a switchable hydrophilicity solvent (SHS) for fat extraction and silica-based solid-phase extraction methods. In Gir cows, the concentration of total phospholipids (mg/100 mg fat) showed a significant increase ($p < 0.05$) from winter (0.86 ± 0.10) to spring (0.95 ± 0.10). It peaked in summer (1.10 ± 0.13) and remained elevated during the rainy season (1.03 ± 0.11). Conversely, Sahiwal cows did not exhibit significant seasonal variations, with phospholipid levels consistently ranging from 0.91 ± 0.14 (in winter) to 1.00 ± 0.10 (in summer). Phosphatidylethanolamine (PE) was the primary element influencing seasonal variations across the various phospholipid classes. The majority of the seasonal rise in total phospholipids in Gir milk was due to a considerable increase in PE content, which increased from 0.28 ± 0.05 mg/100 mg fat in winter to 0.39 ± 0.05 mg/100 mg fat in summer ($p < 0.05$). Conversely, sphingomyelin (SM) and phosphatidylcholine (PC) levels were consistent across breeds and seasons, ranging from 0.25 to 0.37 mg/100 mg fat for SM and from 0.29 to 0.36 mg/100 mg fat for PC. Phosphatidylethanolamine, especially in Gir cows, is primarily responsible for breed-specific seasonal variations in milk phospholipids.

Keywords: Milk fat, phospholipid, HPLC, seasonal variation

Introduction

Milk fat is an important component of milk with high nutritional and physiological value (Lambrini *et al.*, 2021; Sonvanshi *et al.*, 2024) ^[1, 2]. It contains various lipids and is mainly present as milk fat globules surrounded by the milk fat globule membrane (MFGM), which is rich in polar lipids. Milk is a significant source of polar lipids such as glycerophospholipids and sphingophospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM). Phospholipids account for about 0.5-1.0% of total milk fat, with 60-65% located in the MFGM, where they form 15-30% of its structure (Pan *et al.*, 2023) ^[3]. The major phospholipids in the MFGM are PC, PE, and SM. Sphingomyelin is highly saturated, while PE has the lowest saturation among glycerophospholipids. During milk processing, phospholipids are unevenly distributed in different dairy fractions. Buttermilk and skim milk are especially rich in phospholipids due to their higher MFGM content, whereas butter, cream, and buttermilk contain the highest overall amounts.

The industrial importance of phospholipids has increased markedly in recent years, largely due to their widespread use as natural emulsifiers in food and dairy systems. Beyond their technological functions, phospholipids are of growing scientific interest because of their essential roles in cellular structure, metabolism, neural development, cognitive function, and other health-related processes (Liu *et al.*, 2013) ^[4]. Furthermore, phospholipids act as secondary messengers in cell signaling pathways and exhibit antioxidant, antimicrobial, and antiviral activities (Abd El Baky *et al.*, 2014) ^[5]. Milk-derived phospholipids, particularly those from buttermilk, have shown protective effects against gastric ulcers and the adhesion of pathogenic bacteria such as *Listeria monocytogenes*. Sphingomyelin and other sphingolipids are also known for their anti-tumor potential, as their metabolites can inhibit

cell proliferation and induce apoptosis (Li *et al.*, 2022) [6]. Additionally, sphingolipids can protect against infections by binding to bacterial toxins and viruses, thereby preventing their interaction with intestinal epithelial cells.

Seasonal variation is a major determinant of milk composition, as changes in feed availability, environmental stress, photoperiod, and metabolic status of dairy animals directly influence milk yield and nutrient profile (Mudavadi *et al.*, 2020) [9]. Although indigenous cows are adapted to local climatic conditions, seasonal fluctuations can still induce physiological adjustments that affect milk lipid metabolism; while seasonal effects on milk fat, protein, and lactose are well documented, the phospholipid fraction has received comparatively limited attention (Timlin *et al.*, 2021) [8]. Seasonal shifts in diet, particularly the consumption of fresh pasture rich in unsaturated fatty acids during the wet season versus dry forages in the dry season, can modify the fatty acid composition of phospholipid classes—especially polar lipids that are highly responsive to dietary precursors, thereby influencing milk quality, nutritional value, and dairy processing properties (Tocher *et al.*, 2015) [7].

Quantitative analysis of phospholipids remains challenging due to their low concentration in milk and amphiphilic nature, and thin-layer chromatography (TLC) has long been employed for phospholipid profiling because of its simplicity and cost-effectiveness, although it is primarily limited to qualitative or semi-quantitative assessments (Pimentel *et al.*, 2016) [10]. High-performance liquid chromatography (HPLC) is widely regarded as a robust and versatile technique for phospholipid determination, utilizing silica or chemically modified silica columns under isocratic or gradient elution, with hydrophilic interaction liquid chromatography (HILIC) gaining prominence for polar lipid separation owing to increased retention at higher organic solvent levels. Despite its advantages, the application of HPLC coupled with ultraviolet (UV) detection is constrained by solvent absorbance in the low UV range (200–210 nm), where phospholipids exhibit maximum absorbance, necessitating careful optimization of the mobile phase composition to achieve reliable sensitivity (Restuccia *et al.*, 2012) [11]. Nevertheless, several solvents, including acetonitrile, methanol, ethanol, n-hexane, isopropanol, and water, have been successfully employed in HPLC-UV methods, and the integration of HILIC columns with gradient HPLC-UV detection has emerged as an effective strategy for accurate phospholipid quantification (Rehman *et al.*, 2017; Ramani *et al.*, 2024) [12, 13].

Furthermore, understanding seasonal phospholipid variation has practical implications for dairy product formulation and human health. Phospholipids are involved in the formation of liposomes and nano-emulsions used in functional foods and nutraceuticals. Seasonal shifts in their abundance may impact the functional properties of milk and dairy derivatives such as cheese, butter, yoghurt, and infant formula. In infant nutrition, for example, sphingomyelin has been associated with cognitive development outcomes, making its seasonal modulation relevant for targeting nutritional interventions. In this context, the present study focuses on evaluating the seasonal variation and trends in milk fat and major phospholipid classes in milk obtained from indigenous cattle in India.

Materials and Methods

Chemicals and Materials

HPLC grade acetonitrile, methanol, hexane, ammonium acetate, and water were purchased from Sigma Aldrich, St. Louis, Mo, USA. AR grade Ammonium hydroxide (NH₄OH), petroleum ether, ethyl ether, trichloroacetic acid. Supelclean™ LC-Si SPE Tube (bed wt. 500 mg, volume 6 mL) for phospholipid isolation was purchased from Sigma Aldrich, St. Louis, Mo, USA. The certified reference standards of phospholipids L phatidylcholine (≥99 %, egg yolk), L α α -lysophos--phosphatidylethanolamine (≥99 %, egg yolk) were purchased from Sigma Aldrich, St. Louis, Mo, USA. Sphingomyelin (≥99 %, milk/bovine) from Avanti Polar Lipids, Alabaster, USA

Milk Sample Collection

Fresh milk samples were obtained from individual cows belonging to different breeds, including Gir and Sahiwal (indigenous breeds), as well as from pooled cow milk representing all breeds. Sampling was carried out at the Livestock Research Centre (LRC), National Dairy Research Institute (NDRI), Karnal. Immediately after collection, the milk samples were stored under refrigerated conditions at 4 °C until further analysis.

To study the influence of seasonal variation, the entire sampling period was categorized into four distinct seasons: Winter (October to January), Spring (February to March), Summer (April to July), and Rainy (August to September). Throughout the study, all milch animals were maintained under uniform feeding and management practices. Concentrate feed was provided consistently in all seasons, while green fodder such as berseem, oats, and alfalfa was additionally supplied during the winter months.

Extraction of Milk Fat

Milk fat extraction was carried out using N, N-dimethylcyclohexylamine following the switchable hydrophilicity solvent (SHS) approach described by Cheng *et al.* (2019) [14]. This method enables efficient separation of the lipid fraction through reversible changes in solvent polarity, facilitating selective recovery of milk fat. Subsequently, phospholipids were separated from the extracted milk fat by applying a solid-phase extraction (SPE) procedure based on the method reported by Avalli and Contarini (2005) [15], with suitable modifications where required. This step allowed selective isolation of the phospholipid fraction from neutral lipids and other interfering components.

Instrumentation Methodology

The chromatographic analysis was performed on a Dionex HPLC system (Ultimate 3000, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a UV detector, Chromeleon software for data acquisition and processing, and a manual injector fitted with a 20 μ L sample loop. Separation of phospholipids was achieved using a ReproSil Pur 200 diol HILIC column (150 mm $\times$ 4.6 mm, 5 μ m particle size, 200 Å pore size). A HILIC HPLC-UV analytical method developed by Ramani *et al.* (2024) was used for the analysis. A linear binary gradient system was employed with acetonitrile as mobile phase A and water as mobile phase B, maintained at a flow rate of 1.5 mL/min. The column temperature was controlled at 45 °C throughout the analysis. The initial mobile phase composition was set at

5% B and held for 2 minutes, after which it was linearly increased to 45% B over a period of 10 minutes. This was followed by a gradual decrease of mobile phase B by 5% over the next 15 minutes. Detection of phospholipids was carried out using UV absorbance at 203 nm.

Statistical Analysis

Statistical analysis was conducted using SPSS 20 software (IBM Corporation, USA). Two-way ANOVA and Tukey's HSD post-hoc test ($p < 0.05$) were employed to evaluate differences in phospholipid classes between breeds and seasons. A general linear model with season, breeds, and their interaction set as fixed effects was utilized for data analysis. Results were presented as mean with standard deviation.

Results and Discussion

In these studies, the influence of seasonal variation, the entire sampling period was categorized into four distinct seasons: Winter (October to January), Spring (February to March), Summer (April to July), and Rainy (August to September). Milk fat extraction was carried out using N, N-dimethylcyclohexylamine following the switchable hydrophilicity solvent (SHS) approach described by Cheng *et al.* (2019) [14]. This method enables efficient separation of the lipid fraction through reversible changes in solvent

polarity, facilitating selective recovery of milk fat. Subsequently, phospholipids were separated from the extracted milk fat by applying a solid-phase extraction (SPE) procedure based on the method reported by Avalli and Contarini (2005) [15].

Table 1 shows the effect of season on the total fat percentage of milk in Gir and Sahiwal cows. The results indicate that both breed and season significantly influenced milk fat content ($p < 0.05$). In Gir cows, the highest fat percentage was observed during winter (4.51 ± 0.22) and rainy season (4.41 ± 0.28), followed closely by spring (4.30 ± 0.28). The lowest fat content was recorded in summer (4.02 ± 0.18), which was significantly lower than other seasons ($p < 0.05$), due to heat stress and reduced feed intake. In Sahiwal cows, a similar seasonal trend was observed, but with overall lower fat percentages compared to Gir cows. The highest fat content was recorded in winter (4.30 ± 0.24) and spring (4.05 ± 0.30), while the lowest was observed in summer (3.55 ± 0.21). The summer value was significantly lower than all other seasons ($p < 0.05$). During the rainy season, fat percentage improved (4.02 ± 0.29) but remained significantly lower than winter and spring. Comparison between breeds within the same season shows that Gir cows consistently produced milk with higher fat content than Sahiwal cows, particularly in summer and rainy seasons, where the differences were statistically significant.

Table 1: Seasonal variation in total fat percentage of Gir and Sahiwal cows

Breed of cow	Winter	Spring	Summer	Rainy
Gir	4.51 ± 0.22^{aA}	4.30 ± 0.28^{aA}	4.02 ± 0.18^{aB}	4.41 ± 0.28^{aA}
Sahiwal	4.30 ± 0.24^{aA}	4.05 ± 0.30^{aA}	3.55 ± 0.21^{bC}	4.02 ± 0.29^{bB}
Data is presented as Mean $\pm$ SD (n = 15)				
a-c Mean values within a column with different superscripts differ significantly at $p < 0.05$				
A-C Mean values within a row with different superscripts differ significantly at $p < 0.05$				

Phospholipids were isolated from milk samples using the LC-SI silica method, while milk fat was obtained through the SHS extraction technique. Table 2 illustrates the seasonal influence on total phospholipid content (mg/100 mg fat) in milk of Gir, Sahiwal, and Karan Fries cows. The results show that season significantly affected phospholipid concentration in Gir cows, whereas Sahiwal and Karan Fries cows exhibited relatively stable phospholipid levels across all seasons. In Gir cows, phospholipid content increased significantly from winter (0.86 ± 0.10) to spring (0.95 ± 0.10) and reached the highest value during summer (1.10 ± 0.13). The summer and rainy season values (1.03 ± 0.11) were

statistically similar and significantly higher than winter and spring ($p < 0.05$). In Sahiwal cows, phospholipid content remained relatively stable throughout all seasons, with values ranging from 0.91 ± 0.14 in winter to 1.00 ± 0.10 in summer. Across all seasons indicates no significant seasonal effect on phospholipid levels in this breed. Comparison among breeds within the same season showed no significant differences. Overall, the findings demonstrate that phospholipid content in milk fat is more sensitive to seasonal changes in Gir cows, showing higher levels during summer and rainy seasons.

Table 2: Seasonal variation in total phospholipids of Gir and Sahiwal cows

Breed of cow	Phospholipids (mg/100 mg fat)			
	Winter	Spring	Summer	Rainy
Gir	0.86 ± 0.10^{aC}	0.95 ± 0.10^{aB}	1.10 ± 0.13^{aA}	1.03 ± 0.11^{aA}
Sahiwal	0.91 ± 0.14^{aA}	0.94 ± 0.12^{aA}	1.00 ± 0.10^{aA}	0.97 ± 0.12^{aA}
Data is presented as Mean $\pm$ SD (n = 15)				
a-c Mean values within a column with different superscripts differ significantly at $p < 0.05$				
A-C Mean values within a row with different superscripts differ significantly at $p < 0.05$				

Table 3 shows seasonal variation in total phospholipids and their major classes; phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM) in milk fat of two breeds (Gir and Sahiwal). All values are expressed as mg per 100 mg fat. The total phospholipid content of milk fat showed a clear seasonal variation in Gir

cows, whereas Sahiwal cows exhibited relatively stable values across all seasons. In Gir, total phospholipids increased significantly from winter (0.86 mg/100 mg fat) to summer (1.10 mg/100 mg fat), with summer and rainy seasons recording the highest levels. The significant difference among seasons, as indicated by different

uppercase superscripts, suggests that environmental conditions and seasonal changes in feeding and physiological stress strongly influence phospholipid synthesis and incorporation into the milk fat globule membrane (MFGM) in Gir cows. In contrast, Sahiwal milk showed no significant seasonal variation in total phospholipids (0.91-1.00 mg/100 mg fat), indicating greater stability and adaptability of this breed to seasonal fluctuations. Although breed-wise differences were mostly non-significant within the same season, Gir cows generally exhibited slightly higher total phospholipid values during summer and rainy seasons, implying a comparatively higher enrichment of MFGM components under warmer conditions.

Among the individual classes of phospholipids, phosphatidylethanolamine (PE) was the most responsive to seasonal changes and largely contributed to the observed variation in total phospholipids. In Gir cows, PE content increased significantly from winter (0.28 mg/100 mg fat) to summer (0.39 mg/100 mg fat), indicating enhanced

membrane lipid synthesis during warmer periods. This increase in PE is mainly responsible for the higher total phospholipid concentration observed in summer and rainy seasons. In Sahiwal cows, although a slight increase in PE was observed from winter to summer, the changes were not statistically significant, further confirming the breed's stability. Phosphatidylcholine (PC) remained relatively constant across all seasons and in both breeds, showing no significant seasonal or breed-dependent variation. This stability suggests that PC forms a core structural component of the MFGM that is less affected by environmental factors. Similarly, sphingomyelin (SM) showed only minor, non-significant seasonal variations in both breeds, though Gir cows tended to have marginally higher values, particularly during summer and rainy seasons. Overall, the results indicate that seasonal changes in total phospholipid content are primarily driven by fluctuations in PE, especially in Gir cows, while PC and SM remain comparatively stable, maintaining the fundamental structural integrity of the milk fat globule membrane.

Table 3: Seasonal variation in individual phospholipid concentration from Gir and Sahiwal cows

Breed	Season	Total Phospholipid	PE	PC	SM
		(mg/100 mg fat)			
Gir	Winter	0.86±0.10 ^{aC}	0.28±0.05 ^{aA}	0.29±0.08 ^{aA}	0.29±0.06 ^{aA}
	Spring	0.95±0.10 ^{aB}	0.31±0.07 ^{bA}	0.32±0.06 ^{aA}	0.32±0.06 ^{aA}
	Summer	1.10±0.13 ^{aA}	0.39±0.05 ^{bB}	0.34±0.06 ^{aA}	0.37±0.08 ^{aA}
	Rainy	1.03±0.11 ^{aA}	0.33±0.08 ^{bA}	0.34±0.05 ^{aA}	0.36±0.08 ^{aA}
Sahiwal	Winter	0.91±0.14 ^{aA}	0.32±0.06 ^{bA}	0.34±0.05 ^{aA}	0.25±0.06 ^{aA}
	Spring	0.94±0.12 ^{aA}	0.34±0.07 ^{aA}	0.33±0.04 ^{aA}	0.27±0.06 ^{aA}
	Summer	1.00±0.10 ^{aA}	0.38±0.07 ^{aA}	0.32±0.05 ^{aA}	0.30±0.08 ^{aA}
	Rainy	0.97±0.12 ^{aA}	0.33±0.08 ^{aA}	0.36±0.05 ^{aA}	0.28±0.07 ^{aA}

Conclusion

The seasonal variation significantly influences the phospholipid composition of milk fat in indigenous cows, with a more pronounced effect observed in Gir cows than in Sahiwal cows. Total phospholipid content increased significantly in Gir milk during summer and rainy seasons, while Sahiwal milk showed relative stability throughout the year. Among the major phospholipid classes, phosphatidylethanolamine (PE) was the most season-sensitive component and was mainly responsible for the increase in total phospholipids, particularly under warmer conditions. In contrast, phosphatidylcholine (PC) and sphingomyelin (SM) remained largely unchanged across seasons and breeds, indicating their stable structural role in the milk fat globule membrane. Overall, the study highlights that PE is the key driver of seasonal changes in milk phospholipids and that Gir cows are more responsive to environmental and climatic variations. These findings are important for improving milk quality, dairy processing, and the targeted utilization of milk phospholipids in functional and nutraceutical products.

Declaration of Competing Interest

The authors declare no conflict of interest in the presented research work.

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