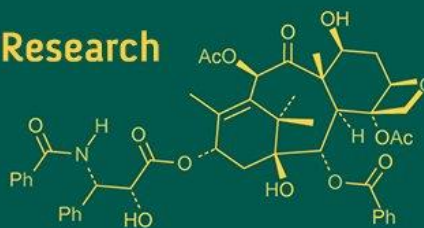


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Influence of seasonal changes on the positional arrangement of fatty acids in milk fat triglycerides of indigenous cow and indigenous buffalo

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Abstract

Milk fat triglycerides (TG) is the primary repository of fatty acids (FA) in bovine (cow) and bubaline (buffalo) milk, and the positional arrangement of FA within the glycerol backbone has profound implications for fat digestion, calcium absorption, and the physical properties of fat. The present study investigated the seasonal variation in intra-positional arrangement of FA in TG of indigenous cow (*Bos indicus*) and indigenous buffalo (*Bubalus bubalis*) milk fat collected over three seasons (summer, rainy and winter). Milk fat was extracted as TG-rich butter fat, and stereospecific positional analysis was performed using pancreatic lipase hydrolysis followed by preparative thin-layer chromatography (PLC) and gas chromatography (GC). The results revealed a significant seasonal influence ($p < 0.05$) on the positional arrangement of FA in both species. Palmitic acid (C16:0) was the predominant FA at the sn-2 position in both cow (~31-35 mol %) and buffalo (~29-35 mol %) milk fat TG, with the highest concentration observed in summer. Buffalo milk fat TG exhibited significantly higher ($p < 0.05$) oleic acid (C18:1cis) at the sn-2 position (21-27 mol %) compared to indigenous cow (16-20 mol %), particularly in rainy and winter seasons. Total unsaturated fatty acids (UFA) at sn-2 were significantly higher in buffalo milk fat (~28-34%) relative to indigenous cow (~21-25%), suggesting a structurally distinct TG architecture. Short-chain fatty acids (SCFA) were predominantly esterified at sn-1(3) positions in both species, consistent with the previously established non-random arrangement of FA in milk fat TG. These findings provide novel insights into the species-specific and season-dependent structural arrangement of FA in native Indian cow and buffalo milk fat TG, with implications for human nutrition and fat processing industries.

Keywords: Triglycerides, regiospecific arrangement, *Bos indicus*, *Bubalus bubalis*, seasonal variation, positional arrangement

1. Introduction

Lipid/fat is a major constituent of the human diet, exerting direct and indirect effects on human health through modulation of lipid metabolism, intestinal absorption, and systemic inflammatory pathways. Triglycerides (TG) account for approximately 98% of total lipids in bovine and bubaline milk fat ^[1]. In TG, three FA of variable chain lengths and degrees of unsaturation are esterified to the stereospecific positions sn-1, sn-2, and sn-3 of a glycerol backbone. The positional placement of FA in TG is not random; rather, it is regulated by metabolic enzymes ^[2, 3].

During digestion, lingual lipase initiates hydrolysis at the sn-3 position of TG, releasing short-and medium-chain fatty acids (SCFA and MCFA) into the aqueous environment of the intestinal lumen. Pancreatic lipase, the principal enzyme of fat digestion, preferentially cleaves ester bonds at the sn-1 and sn-3 positions, leaving the sn-2 monoacylglycerol (2-MAG) intact due to its positional specificity ^[4]. The 2-MAG is subsequently absorbed intact by enterocytes and re-esterified into TG through chylomicron pathway and lymphatic transport. Consequently, FA at the sn-2 position have distinctly superior absorption efficiency compared with FA released as free fatty acids from the sn-1(3) positions ^[5].

The sn-2 position is nutritionally critical for multiple reasons. Higher concentrations of palmitic acid (C16:0) at sn-2 have been shown to reduce formation of insoluble calcium soaps and thereby enhance calcium and fat absorption in neonates ^[2]. Conversely, FA occupying the sn-2 position of TG in human breast milk, such as docosahexaenoic acid (DHA, C22:6n-3), are more efficiently transferred to the developing neonatal brain ^[2].

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The sn-2 position also governs the melting and crystallization behavior of fats; TG bearing stearic acid (C18:0) at sn-2 tend to have elevated melting points, as exemplified by cocoa butter [6].

The FA composition and its positional arrangement in milk fat TG are governed by multiple intrinsic and extrinsic factors including genetics, breed, species, stage of lactation, feeding regimen, and seasonal variation [7, 8]. Indigenous Zebu (*Bos indicus*) cattle, such as the Sahiwal breed, are well adapted to tropical and subtropical climates and have a distinct de novo fatty acid synthesis profile compared with exotic breeds [9, 10]. Compared with bovine species, indigenous buffaloes (*Bubalus bubalis*) produce milk with higher fat content (~7-8%), a higher proportion of long-chain FA (LCFA), and greater concentrations of C18:0 and conjugated linoleic acid (CLA), but lower amounts of SCFA such as C4:0 and C6:0 [11, 12].

Despite the substantial body of literature on FA composition of bovine milk fat, investigations specifically addressing the stereospecific positional arrangement of FA in buffalo milk fat TG are comparatively sparse [1, 11]. Blasi *et al.* (2008) [1] reported that approximately 33-37% of C16:0 in buffalo milk fat is esterified at the sn-2 position, a proportion lower than reported for certain bovine breeds. Furthermore, seasonal changes in feeding practices, from dry fodder in winter to fresh green forage in rainy and summer seasons, shows significant influence on the FA profile and de novo synthesis in the mammary gland [13-15]. Yet the consequences of these feeding-induced compositional changes on the stereospecific arrangement of FA in TG of indigenous cow and buffalo milk fat have not been systematically compared. The present study was therefore designed to investigate the effect of season on the intra-positional arrangement of FA in TG of indigenous cow (*Bos indicus*) and indigenous buffalo (*Bubalus bubalis*) milk fat, collected over one complete year at ICAR-NDRI, Karnal, India. Such information is of fundamental importance for understanding fat digestibility, nutritional value, and the physical properties of milk fat from these two economically important indigenous species.

2. Materials and Methods

The seasonal effect on intra-molecular arrangement of fatty acids in TG of indigenous cow and buffalo milk fat was investigated using milk samples collected from April 2022 to March 2023 at ICAR-NDRI, Karnal, Haryana, India.

2.1 Materials

Pancreatic lipase (≥ 125 units/mg protein; EC No. 232-619-9), monoglyceride (MG) standard (1-oleoyl-rac-glycerol), diglyceride (DG) standard (1, 2-dioleoyl sn-glycerol), TG standard (glycerol trioleate), fatty acid standard (oleic acid), preparative thin-layer chromatography (PLC) plates (Silica gel 60, F [254], 2 mm thickness), and analytical thin-layer chromatography (TLC) plates were procured from Sigma-Aldrich, USA. All other solvents and chemicals (AR grade) were obtained from Thermo-Fisher Scientific, India.

2.2 Feed Composition in Different Seasons

Animals were maintained on a defined feeding regimen across seasons at the Livestock Research Centre of ICAR-NDRI, Karnal. During the summer season, a mixture of green fodder, dry fodder, and concentrate in a ratio of 95:1:4 was provided. The winter ration comprised green fodder, dry fodder, and concentrate in a ratio of 93:3:4. During the

rainy season, a mixture of green fodder, dry fodder, and concentrate in a ratio of 96:0:4 was offered. Feed composition differed significantly between seasons primarily in the availability and proportion of fresh green forage, which is highest during rainy season and lowest during winter.

2.3 Sample Collection

Indigenous cow (*Bos indicus*) and indigenous buffalo (*Bubalus bubalis*) maintained at the Livestock Research Centre (LRC), ICAR-NDRI, Karnal, were selected for the study. Pooled milk samples (500 mL) from ten animals of each species in mid-lactation were collected separately at four time points within each season. The sampling period was divided into three seasons: summer (April-June), rainy (August-November), and winter (December-March). Collected samples were processed on the same day for milk fat extraction.

2.4 Preparation of TG-Rich Milk Fat

The sample preparation was carried out according to the method of Rout *et al.* (2025) [16]. Milk samples were skimmed using a laboratory-scale cream separator (Kamdhenu, KD-60E, Benny Impex, New Delhi, India). The cream was subsequently churned into butter using a hand blender (Orpat HHB-100E, Ajanta LLP, Morbi, Gujarat, India). Obtained butter was washed twice with chilled water. The butter sample (~15 g) was placed in a beaker, heated to 50 °C, and filtered through 0.5 g sodium sulphate in an oven maintained at 50 °C as per the ISO/IDF method for TG analysis [17]. The moisture-free clarified butter fat, enriched in TG, was collected in 15 mL centrifuge tubes. TLC plates were used to verify that the milk fat was free from phospholipids and contained only TG, following the method of Blasi *et al.* (2008) [11]. The TG-rich milk fat was stored at -20 °C until further analysis.

2.5 Analysis of Positional Arrangement of Fatty Acids

2.5.1 Hydrolysis of Triglycerides

The FA composition at the sn-2 position was determined by enzymatic hydrolysis according to the method of Chen *et al.* (2020) [18] with minor modifications. Briefly, 120 mg of TG-enriched butter fat was transferred to a round-bottom tube containing 2.4 mL tris buffer (1 M, pH 8.0), 0.24 mL aqueous CaCl₂ [2] (22%), and 0.6 mL bile salt solution (0.1% aqueous). The contents were warmed to 45 °C, and 24 mg of powdered pancreatic lipase was added. The tube was incubated in a shaker at 45 °C for 10 min at 160 rpm. The reaction was quenched by addition of 0.8 mL of 5 M HCl and 0.8 mL diethyl ether. The contents were centrifuged at 3000 rpm for 3 min, and the upper ether layer containing hydrolysed fat fractions was collected and evaporated under nitrogen at 50 °C in a water bath.

2.5.2 Isolation of 2-Monoglyceride by Preparative Thin-Layer Chromatography

The dried hydrolysis residue was reconstituted in 200 μ L n-hexane. The 2-monoacylglycerol (2-MAG) fraction was isolated by PLC using a slightly modified method of Chen *et al.* (2020). An aliquot of 30 μ L of the reconstituted hydrolysate, along with MG, DG, TG, and FA standards, was applied to the PLC plate. Hexane:diethyl ether:acetic acid (59:50:4, v/v/v) was used as the developing solvent. Visualisation was performed with 2, 7-dichlorofluorescein

indicator (0.1% in ethanol). The 2-MAG band was scraped and extracted with chloroform: methanol (2:1, v/v), vortexed for 3 min, centrifuged at 3000 rpm for 5 min, and the clear supernatant was dried under nitrogen.

2.5.3 Fatty Acid Profiling by Gas Chromatography

Fatty acid methyl esters (FAME) of total milk fat and 2-MAG fractions were prepared according to ISO 15884 (2002) [17]. FAME analysis was performed on a Shimadzu GC-2010 Plus gas chromatograph equipped with a flame ionisation detector (FID) and a fused silica capillary column SLB®-IL 111 (100 m × 0.25 mm I.D., 0.2 µm film thickness; Supelco, Sigma-Aldrich, USA). The analytical method followed the technical report of Buchanan *et al.* (2022) [19].

The intra-positional FA composition was expressed as mol %. The FA concentration at sn-1(3) positions was calculated using the equation of Tzompa-Sosa *et al.* (2014) [20]:

$$\text{sn-1(3)}_{\text{FA}} = [3 \times \text{TG}_{\text{FA}} - \text{sn-2}_{\text{FA}}] / 2 \text{ (I)}$$

where sn-2_{FA} is the percentage of FA at the sn-2 position determined by GC analysis, and TG_{FA} is the total FA percentage at all three sn positions.

2.6 Statistical Analysis

All experiments were performed in quadruplicate (n = 4 per season per species). One-way ANOVA with Tukey's post hoc comparison was performed using SPSS v27.0 (IBM, Chicago, IL, USA).

3. Results

3.1 Total Fatty Acid Composition of Indigenous Cow and Buffalo Milk Fat Triglycerides

The total FA composition (mol %) of TG of indigenous cow and buffalo milk fat across three seasons is presented in Table 1. It can be seen from the data that the indigenous cow milk fat, palmitic acid (C16:0), was the most abundant FA (27.46-28.49 mol %), followed by oleic acid (C18:1 cis)

(19.54-22.56 mol %), stearic acid (C18:0) (10.50-12.06 mol %), and myristic acid (C14:0) (11.86-12.67 mol %). C18:1 cis was significantly higher ($p < 0.05$) in the rainy season (22.56±0.20 mol %) than in summer (20.98±0.22 mol %) and winter (19.54±0.19 mol %). Total SFA in cow TG was significantly higher ($p < 0.05$) in winter (75.40±0.15 mol %) and lowest in the rainy season (72.09±0.21 mol %), with summer occupying an intermediate position (73.95±0.14 mol %). Total MUFA showed a reverse trend, being highest in the rainy season (25.76±0.25 mol %) and lowest in winter (22.83±0.19 mol %).

In indigenous buffalo milk fat TG, C16:0 was similarly the dominant FA (28.98-30.04 mol %), with a significant ($p < 0.05$) increase from summer to winter. C18:1cis was highest in rainy (21.42±0.22 mol %) and lowest in winter (17.99±0.51 mol %) seasons. C18:0 ranged from 10.48±0.21 mol % (winter) to 12.10±0.17 mol % (summer), with a significant decrease from summer to winter. Total SFA in buffalo TG was significantly highest in winter (77.52±0.47 mol %) and lowest in the rainy season (73.31±0.27 mol %). Total MUFA was highest in rainy (24.71±0.22 mol %) and lowest in winter (20.85±0.53 mol %). Total PUFA was significantly ($p < 0.05$) lower in winter (1.63±0.07 mol %) than in summer (1.97±0.07 mol %) and rainy (1.98±0.06 mol %) seasons.

Butyric acid (C4:0) content in cow TG increased significantly ($p < 0.05$) from summer (5.62±0.12 mol %) to rainy (6.47±0.18 mol %) and winter (7.33±0.06 mol %). Similarly, in buffalo, C4:0 was significantly highest in winter (8.92±0.60 mol %) compared with rainy (7.04±0.12 mol %) and summer (7.79±0.27 mol %) seasons. C10:0 in cow was significantly higher in summer (3.97±0.27 mol %) and winter (4.13±0.23 mol %) than in the rainy season (3.07±0.08 mol %). In buffalo, C10:0 showed an increasing trend from summer to winter. C18:0 declined progressively from summer to winter in both species. C18:3 did not differ significantly across seasons in either species.

Table 1: Total fatty acid composition (mol %) of triglycerides (TAG) of indigenous cow (*Bos indicus*) and indigenous buffalo (*Bubalus bubalis*) milk fat across seasons (Summer, Rainy and Winter).

Fatty Acids	Indigenous Cow (<i>Bos indicus</i>)			Indigenous Buffalo (<i>Bubalus bubalis</i>)		
	Summer	Rainy	Winter	Summer	Rainy	Winter
C4:0	5.62±0.12 ^b	6.47±0.18 ^a	7.33±0.06 ^a	7.79±0.27 ^b	7.04±0.12 ^b	8.92±0.60 ^a
C6:0	3.93±0.11 ^a	3.79±0.16 ^a	4.27±0.17 ^a	4.26±0.09 ^a	4.18±0.08 ^a	4.93±0.25 ^a
C8:0	1.97±0.04 ^a	1.90±0.03 ^a	2.28±0.12 ^a	2.00±0.04 ^a	1.64±0.08 ^b	2.13±0.15 ^a
C10:0	3.97±0.27 ^a	3.07±0.08 ^b	4.13±0.23 ^a	2.06±0.07 ^b	2.46±0.07 ^b	2.95±0.21 ^a
C12:0	3.27±0.01 ^a	3.12±0.04 ^a	3.10±0.01 ^a	3.19±0.02 ^a	3.07±0.01 ^b	2.98±0.05 ^b
C14:0	11.93±0.09 ^a	11.86±0.14 ^a	12.67±0.15 ^b	12.27±0.09 ^a	12.08±0.20 ^a	12.71±0.30 ^a
C14:1	1.32±0.06 ^{ab}	1.26±0.03 ^b	1.42±0.02 ^a	1.19±0.01 ^a	1.24±0.02 ^a	1.04±0.03 ^b
C15:0	1.24±0.03 ^a	1.23±0.03 ^a	1.29±0.04 ^a	0.76±0.05 ^a	0.52±0.07 ^b	0.81±0.15 ^a
C16:0	28.49±0.18 ^a	27.46±0.20 ^b	28.39±0.35 ^a	28.98±0.21 ^b	29.76±0.38 ^{ab}	30.04±0.54 ^a
C16:1	0.51±0.03 ^b	0.71±0.03 ^a	0.64±0.01 ^a	0.36±0.01 ^b	0.83±0.03 ^a	0.63±0.03 ^b
C17:0	1.39±0.01 ^a	1.33±0.02 ^a	1.32±0.01 ^a	1.36±0.01 ^a	1.31±0.00 ^a	1.27±0.02 ^a
C18:0	12.06±0.19 ^a	11.76±0.76 ^a	10.50±0.09 ^b	12.10±0.17 ^a	11.11±0.05 ^b	10.48±0.21 ^b
C18:1t	1.30±0.01 ^a	1.24±0.01 ^b	1.23±0.00 ^b	1.27±0.01 ^a	1.22±0.00 ^b	1.18±0.02 ^b
C18:1c	20.98±0.22 ^b	22.56±0.20 ^a	19.54±0.19 ^c	20.31±0.10 ^b	21.42±0.22 ^a	17.99±0.51 ^c
C18:2	0.80±0.03 ^a	0.81±0.05 ^a	0.46±0.02 ^b	0.71±0.05 ^a	0.69±0.01 ^a	0.58±0.05 ^a
C18:3	1.15±0.03 ^a	1.34±0.08 ^a	1.31±0.11 ^a	1.26±0.06 ^a	1.29±0.05 ^a	1.05±0.04 ^a
C22:0	0.10±0.02 ^a	0.11±0.01 ^a	0.12±0.03 ^a	0.14±0.03 ^a	0.16±0.03 ^a	0.30±0.02 ^b
SFA	73.95±0.14 ^b	72.09±0.21 ^c	75.40±0.15 ^a	74.90±0.18 ^b	73.31±0.27 ^b	77.52±0.47 ^a
MUFA	24.10±0.14 ^b	25.76±0.25 ^a	22.83±0.19 ^c	23.13±0.12 ^b	24.71±0.22 ^a	20.85±0.53 ^c
PUFA	1.95±0.01 ^b	2.15±0.07 ^a	1.78±0.09 ^b	1.97±0.07 ^a	1.98±0.06 ^a	1.63±0.07 ^b
UFA	26.05±0.14 ^b	27.91±0.21 ^a	24.60±0.15 ^c	25.10±0.18 ^b	26.69±0.27 ^a	22.48±0.47 ^c

Data are presented as means ± S.E; n = 4. a-c Means within rows for each species with different superscripts differ significantly at $p < 0.05$ (one-way ANOVA, Tukey's post hoc).

3.2 Seasonal Variation in Intra-Positional Distribution of FA in Indigenous Cow Milk Fat TG

The intra-positional FA distribution in TG of indigenous cow milk fat across seasons is presented in Table 2. The major FA esterified at the sn-2 position of cow TG were C16:0 (30.9-35.5 mol %), C18:1cis (16.1-19.6 mol %), C14:0 (15.4-18.3 mol %), C18:0 (9.0-10.6 mol %), and C12:0 (5.3-7.8 mol %). At the sn-1(3) positions, the same FA were present but at different levels: C16:0 was reduced to 24.8-26.6 mol %, C14:0 to 8.6-10.2 mol %, while C18:1cis and C18:0 increased to 20.6-23.4 mol % and 9.9-13.5 mol %, respectively.

The C16:0 content at the sn-2 position was significantly highest ($p<0.05$) during summer (35.46 ± 0.30 mol %),

followed by winter (31.02 ± 0.45 mol %) and rainy (30.91 ± 0.50 mol %) seasons (Table 2). C18:1cis at the sn-2 position was significantly ($p<0.001$) higher in rainy season (19.64 ± 0.50 mol %) compared to summer (18.04 ± 0.50 mol %) and winter (16.12 ± 0.48 mol %). C18:0 at the sn-2 position was significantly ($p<0.05$) higher in summer (10.57 ± 0.43 mol %) and winter (11.04 ± 0.34 mol %) compared with rainy (8.98 ± 0.86 mol %) season.

Minor odd-chain FA (C15:0 and C17:0) were detected at the sn-2 position in the range of 1.42-1.84 mol % and 1.04-1.74 mol %, respectively. SCFA (C4:0-C8:0) were not detected at the sn-2 position, or were present in trace quantities (C6:0, C8:0).

Table 2: Intra-positional fatty acid distribution (mol %) at sn-2 and sn-1(3) positions in cow milk fat TG

Fatty Acid	sn-2 Position			sn-1(3) Position		
	Summer	Rainy	Winter	Summer	Rainy	Winter
C4:0	ND	ND	ND	8.48 ± 0.18^c	9.59 ± 0.30^b	10.61 ± 0.05^a
C6:0	1.57 ± 0.19^a	0.84 ± 0.10^c	1.14 ± 0.12^b	5.12 ± 0.15^a	5.20 ± 0.30^a	5.64 ± 0.26^a
C8:0	1.13 ± 0.04^a	0.90 ± 0.02^b	0.65 ± 0.07^c	2.39 ± 0.05^b	2.37 ± 0.04^b	2.99 ± 0.16^a
C10:0	4.44 ± 0.07^b	5.27 ± 0.02^a	5.52 ± 0.24^a	3.70 ± 0.43^a	1.98 ± 0.11^b	3.75 ± 0.12^a
C12:0	5.34 ± 0.05^a	6.14 ± 0.53^b	7.81 ± 0.22^a	2.17 ± 0.09^b	2.37 ± 0.24^a	2.60 ± 0.19^a
C14:0	15.44 ± 0.35^b	18.29 ± 0.19^a	17.52 ± 0.34^a	10.07 ± 0.19^a	8.59 ± 0.14^b	10.18 ± 0.39^a
C14:1	1.32 ± 0.07^b	1.61 ± 0.16^a	1.25 ± 0.04^b	1.31 ± 0.10^a	0.98 ± 0.04^b	1.47 ± 0.04^a
C15:0	1.42 ± 0.01^b	1.54 ± 0.04^b	1.84 ± 0.03^a	1.14 ± 0.05^a	1.07 ± 0.03^b	1.00 ± 0.05^b
C16:0	35.46 ± 0.30^a	30.91 ± 0.50^b	31.02 ± 0.45^b	24.76 ± 0.40^b	25.27 ± 0.21^b	26.64 ± 0.32^a
C16:1	0.64 ± 0.03^b	0.82 ± 0.03^a	0.64 ± 0.05^a	0.44 ± 0.04^b	0.64 ± 0.04^a	0.62 ± 0.02^a
C17:0	1.04 ± 0.11^c	1.37 ± 0.15^b	1.74 ± 0.12^a	1.56 ± 0.05^a	1.18 ± 0.08^b	0.96 ± 0.03^c
C18:0	10.57 ± 0.43^a	8.98 ± 0.86^b	11.04 ± 0.34^a	12.77 ± 0.36^b	13.51 ± 0.96^a	9.85 ± 0.15^c
C18:1t	1.22 ± 0.04^a	0.92 ± 0.10^b	1.28 ± 0.05^a	1.51 ± 0.08^b	1.74 ± 0.05^a	1.12 ± 0.14^c
C18:1c	18.04 ± 0.50^a	19.64 ± 0.50^a	16.12 ± 0.48^b	22.40 ± 0.15^b	23.39 ± 0.21^a	20.57 ± 0.24^c
C18:2	0.98 ± 0.12^b	1.24 ± 0.06^a	1.16 ± 0.03^a	0.71 ± 0.11^a	0.55 ± 0.06^a	0.13 ± 0.04^b
C18:3	1.15 ± 0.02^b	1.25 ± 0.02^a	0.98 ± 0.08^c	1.14 ± 0.03^a	1.35 ± 0.11^a	1.63 ± 0.06^a
C22:0	0.26 ± 0.03^a	0.28 ± 0.03^a	0.30 ± 0.05^a	0.34 ± 0.06^a	0.21 ± 0.02^b	0.22 ± 0.02^b
SFA	76.66 ± 0.57^a	74.52 ± 0.59^b	78.57 ± 0.49^a	72.49 ± 0.17^b	71.34 ± 0.23^b	74.46 ± 0.41^a
UFA	23.34 ± 0.57^b	25.48 ± 0.58^a	21.43 ± 0.49^c	27.51 ± 0.17^b	28.66 ± 0.23^a	25.54 ± 0.41^c
MUFA	21.21 ± 0.49^b	22.99 ± 0.53^a	19.29 ± 0.48^c	25.66 ± 0.16^a	26.76 ± 0.29^a	23.79 ± 0.35^b
PUFA	2.13 ± 0.14^b	2.48 ± 0.08^a	2.14 ± 0.08^b	1.85 ± 0.08^a	1.90 ± 0.09^a	1.76 ± 0.07^a

Values are Mean $\pm$ SE; n = 4 replicates per season. ND = not detected. SFA = saturated fatty acids; MUFA = monounsaturated; PUFA = polyunsaturated; UFA = total unsaturated.

3.3 Seasonal Variation in Intra-Positional Distribution of FA in Indigenous Buffalo Milk Fat TG

The distribution of FA in TG of indigenous buffalo milk fat at sn-2 and sn-1(3) positions showed significant ($p<0.05$) seasonal variation (Table 3). The major FA at the sn-2 position of buffalo TG were C16:0 (29.0-34.9 mol %), C18:1cis (21.5-27.2 mol %), C14:0 (15.2-18.4 mol %), C18:0 (10.1-10.7 mol %), and C12:0 (3.8-4.4 mol %). C16:0 at the sn-2 position was significantly ($p<0.001$) highest in summer (34.90 ± 1.81 mol %), followed by rainy (30.48 ± 0.23 mol %) and winter (28.96 ± 0.29 mol %) seasons.

The most notable finding in buffalo milk fat was the significantly ($p<0.001$) higher C18:1cis content at the sn-2 position in rainy (27.16 ± 0.29 mol %) and winter (25.90 ± 0.59 mol %) seasons compared with summer (21.54 ± 2.10 mol %). The inter-positional distribution confirmed that the proportion of C18:1cis at the sn-2

position in buffalo milk fat was 35.4% (summer), 42.1% (rainy), and 46.4% (winter) (Table 2). C18:0 at the sn-2 position did not differ significantly ($p>0.05$) across seasons in buffalo (10.1-10.7 mol %). Total MUFA at sn-2 increased from summer (25.05 mol %) to rainy (31.28 mol %) season, and the proportion of MUFA at sn-2 reached 44.74% in winter buffalo milk fat. C4:0 was found exclusively at the sn-1(3) positions (100%) across all seasons in buffalo TG.

The C17:0 at sn-2 position of buffalo milk fat was significantly ($p<0.05$) higher in summer (0.55 ± 0.03 mol %) than in rainy (0.38 ± 0.04 mol %) and winter (0.34 ± 0.02 mol %) seasons. Total SFA in buffalo TG was predominantly at sn-1(3) (68-70%) across all seasons, with total UFA at sn-2 being significantly higher in rainy (33.91 ± 0.33 mol %) and winter (31.56 ± 0.56 mol %) seasons than summer (27.67 ± 2.33 mol %).

Table 3: Intra-positional fatty acid distribution (mol %) at sn-2 and sn-1(3) positions in buffalo milk fat TG

Fatty Acid	sn-2 Position			sn-1(3) Position		
	Summer	Rainy	Winter	Summer	Rainy	Winter
C4:0	ND	ND	ND	11.31±0.41 ^c	10.20±0.17 ^b	13.51±0.52 ^a
C6:0	0.85±0.15 ^a	1.22±0.04 ^b	1.44±0.01 ^b	5.51±0.13 ^b	5.50±0.12 ^b	6.40±0.33 ^a
C8:0	0.64±0.03 ^a	0.63±0.03 ^a	0.60±0.05 ^a	2.28±0.21 ^a	2.05±0.12 ^a	2.69±0.19 ^b
C10:0	2.59±0.08 ^c	2.80±0.07 ^b	3.12±0.12 ^a	1.96±0.19 ^a	2.27±0.11 ^a	2.54±0.09 ^a
C12:0	4.36±0.22 ^b	3.94±0.03 ^b	3.84±0.02 ^a	2.85±0.15 ^b	3.35±0.17 ^{ab}	3.86±0.13 ^a
C14:0	16.70±0.42 ^a	15.20±0.32 ^b	18.42±0.26 ^c	10.26±0.32 ^b	10.58±0.26 ^b	9.80±0.41 ^a
C14:1	1.55±0.07 ^{ab}	1.68±0.03 ^a	1.45±0.02 ^b	1.07±0.03 ^a	1.03±0.03 ^a	0.89±0.04 ^b
C15:0	0.73±0.08 ^b	0.98±0.00 ^b	0.92±0.05 ^a	0.81±0.09 ^a	0.63±0.04 ^b	1.15±0.08 ^c
C16:0	34.90±1.81 ^a	30.48±0.23 ^b	28.96±0.29 ^c	27.66±0.93 ^b	29.23±0.53 ^{ab}	29.69±0.55 ^a
C16:1	1.21±0.13 ^b	1.58±0.02 ^a	1.11±0.07 ^b	0.34±0.07 ^a	0.35±0.07 ^a	0.41±0.04 ^a
C17:0	0.55±0.03 ^a	0.38±0.04 ^b	0.34±0.02 ^b	1.62±0.09 ^a	1.59±0.18 ^a	0.97±0.13 ^b
C18:0	10.65±0.51 ^a	10.10±0.25 ^a	10.51±0.72 ^a	11.45±0.38 ^a	11.50±0.07 ^a	9.54±0.19 ^b
C18:1t	0.75±0.05 ^a	0.86±0.01 ^{ab}	0.82±0.03 ^b	1.48±0.06 ^a	1.24±0.09 ^b	1.84±0.24 ^a
C18:1c	21.54±2.10 ^b	27.16±0.29 ^a	25.90±0.59 ^a	19.50±0.55 ^a	18.67±0.36 ^b	14.95±0.16 ^c
C18:2	1.15±0.09 ^a	1.49±0.05 ^a	1.43±0.03 ^a	0.42±0.03 ^a	0.35±0.03 ^a	0.32±0.09 ^a
C18:3	1.47±0.10 ^a	1.14±0.02 ^b	0.85±0.02 ^c	1.28±0.07 ^a	1.35±0.06 ^a	1.14±0.03 ^a
C22:0	0.37±0.02 ^a	0.37±0.03 ^a	0.29±0.02 ^b	0.18±0.01 ^b	0.12±0.01 ^b	0.30±0.03 ^a
SFA	72.33±2.33 ^a	66.09±0.33 ^b	68.44±0.56 ^b	75.90±0.56 ^b	77.01±0.40 ^b	80.46±0.42 ^a
UFA	27.67±2.33 ^b	33.91±0.33 ^a	31.56±0.56 ^a	24.10±0.56 ^a	22.99±0.40 ^a	19.54±0.42 ^b
MUFA	25.05±2.34 ^b	31.28±0.29 ^a	29.28±0.59 ^a	22.40±0.60 ^a	21.29±0.33 ^a	18.09±0.37 ^b
PUFA	2.62±0.06 ^a	2.63±0.06 ^a	2.28±0.03 ^b	1.70±0.05 ^a	1.70±0.09 ^a	1.46±0.13 ^a

Values are Mean±SE; n = 4 replicates per season. ND = not detected. SFA = saturated fatty acids; MUFA = monounsaturated; PUFA = polyunsaturated; UFA = total unsaturated.

3.4 Comparison of Positional Distribution between Indigenous Cow and Buffalo Milk Fat

A direct comparison of the FA distribution at the sn-2 position between indigenous cow and buffalo milk fat revealed several important species-specific differences. Across all three seasons, the concentration of total MUFA at sn-2 was significantly higher ($p<0.05$) in buffalo milk fat than in indigenous cow, with the greatest difference in rainy season (buffalo: 31.28±0.29 mol % vs cow: 22.99±0.53 mol %). Total SFA at sn-2 was consistently higher in cow milk fat (74.5-78.6 mol %) than in buffalo (66.1-72.3 mol %) across all seasons. The SCFA content at sn-2 was significantly ($p<0.05$) higher in indigenous cow milk fat (4.87-5.62 mol %) compared to buffalo (3.48-4.16 mol %) across all seasons. C10:0 at sn-2 was notably higher in cow (4.44-5.52 mol %) than in buffalo (2.59-3.12 mol %) across seasons.

4. Discussion

4.1 Total Fatty Acid Composition and Seasonal Effects

The total FA composition of TG of indigenous cow and buffalo milk fat obtained in the present study is broadly in agreement with earlier reports for bovine and bubaline milk fat [3, 11, 12]. The predominance of C16:0, C18:1cis, C18:0, and C14:0 in TG of both species reflects the combined contribution of de novo synthesis (for SCFA and MCFA) and uptake of preformed LCFA from blood lipids. The seasonal increase in total SFA in winter and its decline in rainy season, observed for both species, is consistent with earlier reports under Indian feeding conditions^{14, 15} and it is attributed to the higher availability of fresh green forage during the rainy season, which promotes mammary uptake of C18:1cis and stimulates Δ^9 -desaturase activity, thereby increasing UFA proportions in milk fat TG.

The significantly higher C18:1cis in rainy season TG of both species is consistent with the well-established role of pasture-based feeding in elevating oleic acid through rumen biohydrogenation-desaturation pathways^[7]. The progressive increase in C4:0 from summer to winter in both species, and its higher absolute concentration in buffalo than cow TG, is attributable to the greater dependence on concentrate-based rations in winter, providing more acetate substrate for mammary de novo synthesis^[13]. The decline in C18:0 from summer to winter in both species may reflect reduced flux of stearic acid from rumen biohydrogenation of dietary polyunsaturated FA when green fodder availability decreases. Comparatively, total SFA in both species was consistently higher in winter, which aligns with cold-season shifts in mammary metabolism documented by Pacheco-Pappenheim *et al.* (2021)^[7].

4.2 Intra-Positional Distribution in Indigenous Cow Milk Fat

The predominance of C16:0 at the sn-2 position (30.9-35.5 mol %) in indigenous cow milk fat TG is in consonance with earlier reports for bovine milk fat [3, 20, 21]. The significantly higher C16:0 at sn-2 in summer suggests that summer milk fat from indigenous cow may facilitate superior calcium and FA absorption, particularly in infant nutrition^[2]. This seasonal variation in C16:0 at sn-2 is associated with the shift in the de novo FA synthesis pathway in the mammary gland, influenced by the proportion of acetate and β -hydroxybutyrate available as precursors^[7, 20].

The elevated C18:1cis at sn-2 in rainy season is attributed to Δ^9 -desaturase activity in the mammary gland, driven by higher intake of C18:0 and unsaturated C18 precursors from fresh green forage (Pacheco-Pappenheim *et al.*, 2021). The

higher C18:0 at sn-2 in summer and winter milk fat suggests a slightly higher melting point for these seasonal ghee fractions, as TG bearing C18:0 at sn-2 is characterised by elevated melting temperatures, as exemplified by cocoa butter^[6]. The C15:0 preference for sn-2 over sn-1(3) in cow TG is consistent with findings of previous studies^[8, 22].

The exclusive esterification of C4:0 at sn-1(3) and the preferential location of SCFA at sn-3 are well-established features of bovine milk fat TG structure^[3]. The positional proportion of C16:0 at sn-2 is slightly lower than values reported for Jersey and HF crossbred animals^[7, 23], suggesting a breed-specific gradient in C16:0 preference for the sn-2 position. The marked increase in total MCFA at sn-1(3) in winter is consistent with the shift towards de novo FA synthesis when green forage is limited^[15].

4.3 Intra-Positional Distribution in Indigenous Buffalo Milk Fat

The overall higher proportion of C18:1cis at the sn-2 position in buffalo TG (21-27 mol %) compared with indigenous cow (16-20 mol %) across seasons is a structurally significant finding with nutritional implications. This pronounced preference of C18:1cis for the sn-2 position in buffalo TG may be related to higher activity of specific acyltransferases, particularly sn-2 acyl-CoA:1-acylglycerol-3-phosphocholine acyltransferase (LPCAT2), in the bubaline mammary gland, although further enzymatic studies are warranted. The overall higher oleic acid content of buffalo milk fat compared with bovine^[2] appears to be positionally amplified at the sn-2 site.

The stable C18:0 at sn-2 in buffalo across seasons (10.1-10.7 mol %), contrasting with the significant seasonal dip seen in cow milk fat during rainy season, may partly explain the more stable melting behaviour of buffalo ghee relative to bovine. The significantly higher proportion of MUFA at sn-2 in winter buffalo milk fat (44.74%) has implications for the physical properties of buffalo ghee: TG structures with more UFA at sn-2 tend to exhibit lower melting points and better spreadability^[24]. The preferential localisation of C4:0 exclusively at sn-1(3) (100%) and C6:0 and C8:0 predominantly (87-96%) at sn-1(3) in buffalo TG is consistent with the established positional preference of SCFA at sn-3 in ruminant milk fat^[3].

4.4 Species-Specific Comparison and Nutritional Implications

The consistently higher total SFA at sn-2 in indigenous cow milk fat (74.5-78.6 mol %) relative to buffalo (66.1-72.3 mol %) and the higher total UFA at sn-2 in buffalo are the most important species-specific structural differences identified in this study. These findings align with the review by Rout *et al.* (2024), who highlighted that bubaline milk fat TG generally exhibit a greater proportion of long-chain unsaturated FA at sn-2 compared with bovine. From a nutritional standpoint, the higher UFA content at sn-2 in buffalo milk fat suggests that buffalo TG may yield a 2-MAG fraction richer in oleic acid upon intestinal lipase action, which could have beneficial effects on postprandial lipid metabolism and cardiovascular health^[25].

Conversely, the higher C16:0 at sn-2 in cow summer milk fat is particularly advantageous for infant formula applications, as this structural arrangement mirrors that of human breast milk and improves calcium and fat absorption in neonates^[2]. The lower SCFA at sn-2 in buffalo across all

seasons may reflect the intrinsically lower short-chain acyl-CoA thioesterase activity of the bubaline mammary gland^[11, 14]. The higher C10:0 at sn-2 in cow than buffalo across seasons is consistent with the lower overall decanoic acid content in buffalo milk fat TG^[12].

5. Conclusion

The present study demonstrates a strong and significant seasonal effect on the intra-positional arrangement of FA in TG of both indigenous cow (*Bos indicus*) and indigenous buffalo (*Bubalus bubalis*) milk fat. Palmitic acid (C16:0) was the predominant FA at the sn-2 position in both species, with summer milk fat having the highest proportion of C16:0 at sn-2, which is nutritionally advantageous for calcium and fat absorption. A striking species-specific difference was found in oleic acid (C18:1cis) arrangement: buffalo milk fat consistently exhibited significantly higher C18:1cis at the sn-2 position (21-27 mol %) than indigenous cow (16-20 mol %), particularly in rainy and winter seasons. This structural difference renders buffalo TG with a more unsaturated sn-2 composition, which may have distinct implications for fat digestibility and cardiovascular health. Short-chain fatty acids (C4:0-C8:0) were exclusively or predominantly esterified at the sn-1(3) positions in both species. The overall SFA content at sn-2 was consistently higher in indigenous cow milk fat than in buffalo. Seasonal changes in the sn-2 C18:0 content suggest differences in melting behaviour between seasons and species, with summer and winter milk fat having a potentially higher melting point. These findings offer comprehensive, species-specific insights into the stereospecific FA architecture of Indian native cow and buffalo milk fat TG, laying a foundation for targeted nutritional recommendations, infant formula design, and quality-graded processing of milk fat from these economically important species.

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7. CRediT Authorship Contribution Statement

Pavel Rout: Writing-original draft, Validation, Software, Methodology, Visualization, Investigation, Formal analysis, Data curation.

Vivek Sharma: Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Shreya Saha: Software, Methodology, Writing-review & editing.

8. Declaration of Competing Interest

The authors declare no conflict of interest.

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