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Characterization of Cow, Goat, and Water Buffalo Milk Fat Globule Lipids by High-Performance Thin Layer Chromatography

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Abstract: Ruminant milk is an essential part of the human diet and is widely accepted as a major nutrient source in developing countries. However, the polar and neutral lipid content variation in milk fat globules (MFG)among cow, goat, and water buffalo is poorly understood. This study used high-performance thin layer chromatography to identify and quantify five major polar (PL) and three neutral lipids (NL) from the MFG of cow, goat, and water buffalo. Optimal separation was achieved for PLs using chloroform: methanol: water (65:25:4), and hexane: diethyl ether: acetic acid (70:30:1) for NLs. The lower detectable (0.12 to 1.53 µg/mL) and quantification (0.12 to 1.53 µg/mL) limits indicated the high sensitivity of the method. Quantification at 540 nm showed the highest abundance of phosphatidylethanolamine and triglycerides. Fat globules were further characterized for size and microstructural properties, which revealed smaller globules in goats (0.99 ± 0.04 µm) than cows (1.85 ± 0.03 µm) and water buffaloes (2.91 ± 0.08 µm), indicating a negative correlation with PL but a positive correlation with NL. The variation in lipid quantity among different animal species suggests more research to support their selection as a suitable source for developing functional food to impact human health positively.

Keywords: milk fat globule membrane; high-performance thin-layer chromatography; polar lipid; neutral lipid; microstructure; lipid quantitation

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1. Introduction

Milk is a complex fluid composed of a diverse set of proteins, lipids, carbohydrates, and vitamins responsible for nourishing the neonate [1]. It is a natural emulsion present in the form of spherical droplets of milk fat globules (MFG) having triglycerides (TAG) at its core and surrounded by a tri-layered membrane called milk fat globule membrane (MFGM) [2]. The complex composition of MFGM includes cholesterol, glycerophospholipids, sphingolipids, glycoproteins, glycolipids, enzymes, etc. [3]. Major glycerophospholipids in MFGM are phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE). Sphingomyelin (SM), which contains the same head group as PC, is the dominant sphingolipid species present in milk and, therefore, is often referred to as a polar lipid (PL) [4]. Among these lipid classes, PC and SM are primarily present on the outer layer of the tri-layer membrane, whereas PS, PE, and PI are mainly located on the inner surface of MFGM [5,6].

TAG, the major neutral lipid (NL) that forms approximately 98% of MFG, is mainly responsible for providing energy [2]; diglycerides (DAG) and cholesterol (Chl) are other important NL present in milk lipids. Despite the low abundance of PL in MFG, it plays a crucial role in imparting beneficial health effects in cardiovascular diseases, cholesterol uptake, and neurodegenerative diseases [7,8]. Furthermore, studies have highlighted the discrete benefits of individual PL (e.g., SM) classes from different dietary sources to

play a crucial role in neonatal gut maturation during breastfeeding and neurobehavioral development [9,10].

As MFGM is considered one of the most promising food additives, PLs in the membrane and NLs in the core have also been extensively investigated for improving the efficacy of adult diet [11], infant formula [12], and infant gut microbiome [10]. Despite numerous health benefits of MFG and its membrane bioactives to improve health traits of milk and dairy products, surprisingly, there is limited information on the extent of natural variation in MFG lipid composition and its microstructural properties across different ruminants that could be used as milk sources for a targeted supplement to improve human health.

Several techniques have been reported to characterize milk lipids, including high-performance liquid chromatography, gas chromatography for fatty acid profiling, and 31P nuclear magnetic resonance [3,13,14]. However, there are certain disadvantages associated with these methods, which include limited sensitivity, use of large volume of expensive chemicals, time-consuming, and cost-ineffective. In contrast, thin layer chromatography has several advantages, such as speed of analysis, less solvent requirement, batch analysis, and cost-effectiveness [15]. Consequently, there has been a growing interest and momentum in using high-performance thin-layer chromatography (HPTLC) for wide applications in analyzing different components from biological samples. For instance, phospholipids from mammary tumor cell lines [16], bioactive compounds in tea [17], ergosterol in wheat [18], adulteration in honey [19], phytochemicals present in coffee [20], etc.

Amongst ruminants, cow, goat, and water buffalo milk are the most consumed milks globally, but surprisingly, the variation in MFG PLs and NLs has not been well documented. Thus, this study aims to identify and quantify the natural variations of PLs and NLs in cows, goats, and water buffaloes using HPTLC, including particle size, zeta potential, and microstructural variations.

2. Materials and Methods

2.1. Isolation of Milk Fat Globules

Milk samples were collected from healthy Holstein Friesian cows (n = 6), Murrah buffalo (n = 6), and Sirohi goats (n = 6) during the winter season (15th–30th December) in their first lactation stage from a local dairy farm situated in Roorkee (29.87111° N, 77.89243° E), India where all the animals were housed in the same farm and stall-fed. The age of animals at the time of milk sample collection ranged from 2 to 3 years for cow, water buffalo, and goat. After collecting milk samples from these animals in the evening, the fat content of the milk was determined by Gerber's method using a milk butyrometer. The average fat percentage for Holstein Friesian cows, Murrah buffalo, and Sirohi goats was 3.04%, 6.25%, and 4.2%, respectively, and were normalized in all the samples for further analysis. For MFG isolation, 50 mL of whole milk was centrifuged at 1500× g at 4 °C for 10 min. Following centrifugation, the cream was divided into two parts: one part to isolate and quantify PLs and NLs, and the second part was used to measure the particle size, zeta potential, and microstructure of all three animal groups.

2.2. Lipid Extraction

2.2.1. Total Lipid Extraction

The total lipids in milk samples were extracted by the Folch method [21]. Briefly, 1.5 g of cream was mixed with 30 mL of chloroform: methanol (2:1, v/v) containing 0.9% NaCl and 0.01% butylated hydroxytoluene as an antioxidant. The mixture was vortexed (Popular Traders, Ambala, India) for 5 min followed by centrifugation at 500× g for 10 min at 25 °C. The lipid-containing lower phase was collected and dried under a rotary evaporator (Popular Traders, Ambala, India) at 35 °C. Total lipids obtained were stored at −20 °C until further analysis.

2.2.2. Solid-Phase Extraction (SPE)

The PL and NL fractions were separated from total lipid by the SPE method using silica Sep-Pak cartridges (Waters Corporation, Milford, MA, USA)with 500 mg silica and a barrel size of 6 cubic centimeters. The PL extraction was done by dissolving 400 mg of total lipid extracts in 1 mL of chloroform: methanol (2:1, *v/v*). After conditioning the cartridges with hexane, the neutral lipids were eluted by adding 3 mL of hexane-diethyl-ether (8:2, *v*/*v*) followed by 3 mL of hexane-diethyl-ether (1:1, *v*/*v*). PL was recovered by two-step elusion, first with 4 mL of methanol and second with 2 mL of methanol plus 2 mL of chloroform: methanol: water (3:5:2, *v*/*v*/*v*). The recovered PL fraction was then dried under a rotary evaporator at 35 °C and re-dissolved in chloroform: methanol (2:1, *v*/*v*) at 100 mg/mL before using it for HPTLC [22].

For NLs, the cartridges were initially equilibrated with 2 mL of hexane: MBTE (96:4, *v*/*v*) followed by 3 mL of hexane. The total lipid extracts, dissolved in hexane: MBTE (200:3, *v*/*v*) at a 400 mg/mL concentration, were loaded into the cartridge. TAG was eluted by first loading 4 mL of hexane: MBTE (200:3, *v*/*v*) followed by hexane: MBTE (94:4, *v*/*v*). The cartridge was further acidified using 4 mL of hexane: acetic acid (100:0.2, *v*/*v*), and the obtained volume was then discarded. Four mL of hexane: MBTE: acetic acid (100:2:0.2, *v*/*v*/*v*) followed by 4 mL of MBTE: acetic acid (100:0.2, *v*/*v*) was then loaded to elute DAG and Chl fractions [23]. The obtained fractions were dried and re-dissolved in chloroform: methanol (2:1, *v*/*v*) at a 100 mg/mL concentration for HPTLC.

2.3. Lipid Analysis by High-Performance Thin Layer Chromatography

2.3.1. Preparation of Standard Solutions

Stock solutions of standard compounds were prepared in HPLC-grade chloroform at a 1 mg/mL concentration and stored at −20 °C until further use. Different concentrations of these standard solutions (1–10 µg/band) were applied on an HPTLC plate to obtain a five-point calibration curve to separate and quantify PL and NL.

2.3.2. Chromatographic Conditions

PLs and NLs were separated, identified, and quantified using the HPTLC system (CAMAG, Muttenz, Switzerland), equipped with LINOMAT V and CAMAG TLC Scanner 3. The integrated VisionCATS software was used for data acquisition, processing, and visualization. Separation of compounds was performed on silica-coated aluminium plates Si 60F_{254} (20×20 cm) using chloroform: methanol: water (65:25:4) as mobile phase for PL and hexane: diethyl ether: acetic acid (70:30:1) for NL. The lipid extracts (25 µL) and standards (3–12 µL) were applied on an HPTLC plate at room temperature (~25 °C) using an autosampler (LINOMAT V) under nitrogen flow and by using a 100 µL syringe. Application conditions were set as band length 8 mm, rate of application 1 µL/s, distance from the bottom of plate 8 mm, the distance between bands 11.4 mm, distance from plate side edge 20 mm, and the length of chromatogram run was fixed at 9 cm. The twin trough TLC chamber was initially saturated for approximately 30 min, and a saturation pad with a sample run time of 20 min. After completion of the run, the HPTLC plate was dried for 5 min by a hairdryer used at a distance of 30 cm. The derivatization of the HPTLC plate was done by keeping the plate in a saturated iodine chamber for 10 min, which consists of iodine crystals (10 g) and is saturated for about 1 h. The quantification of bands was done by scanning HPTLC plates using computerized TLC Scanner 3 and VisionCATS software, in absorption mode at 540 nm with slit width set at 6 × 0.45 mm, data resolution 100 µm per step, and scanning speed 20 mm/s. PL and NL in different samples were quantified by comparing the peak areas with linear regression obtained from the calibration curve of standards. All experiments were carried out in triplicate, and data were used to calculate the limit of detection (LoD) and quantification (LoQ). The LoD was calculated as LoD = 3SDblank/Slope and LoQ as LoQ = 10 SDblank/Slope, where SDblank represents the standard deviation of the blank signal, following the guidelines of the International Conference on Harmonization (ICH), 2005 [24].

2.4. Determination of Particle Size and Zeta Potential

MFG size distribution was measured using Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK), based on dynamic light scattering (DLS) with a 90° scattering detector angle, and results are expressed in terms of Z-average size. The globule size width distribution was reported as the polydispersity index (PDI), a dimensionless number ranging from 0 to 1.0, where values close to zero indicate narrow distribution. The cream was suspended in a buffer consisting of 20 mM imidazole, 50 mM NaCl, and 5 mM $CaCl_2$ at pH 7.0 [25]. Cream (0.1 g) was diluted 10-fold in the buffer and was measured where the average of three technical measurements was reported as particle size in micrometer (μm). Alternatively, the globule size was also measured from differential interferential contrast (DIC) images by converting pixel images into micrometers using Image J software version 1.64r (National Institutes of Health, Bethesda, MD, USA). Snapshots from four different fields with 25 globules in each field were selected (a total of 100 MFG per sample) to calculate the diameter of each sample [26].

The electrophoretic mobility of MFG was measured by electrophoretic light scattering at 25 °C using Zetasizer Nano ZS90, as described by Menard et al., 2010 [25]. The Smoluchowski equation was used to convert electrophoretic mobility to zeta potential:

$$\zeta = U\eta/\varepsilon$$

where ζ is zeta potential, U is electrophoretic mobility, η is medium viscosity, and ε is the dielectric constant. The sample preparation method was the same as that of particle size measurement by Zetasizer. The average of three technical measurements was reported as zeta potential in millivolts (mV).

2.5. Microstructural Analysis of Milk Fat Globule

The microstructure of MFG was analyzed by an imaging system (EVOS M7000, Thermo Fisher Scientific, Waltham, MA, USA) with Texas red light cube at a 60× lens. The staining of MFG was done as reported previously [6]. Briefly, the cream samples were suspended in 1× PBS buffer (pH 7) and stained with Rh-DOPE (1 mg/mL chloroform) in a ratio of 1:100 (v/v) to label the polar lipids. For neutral lipids (NL), Nile red solution (1 mg/mL acetone) was mixed with suspended cream samples. The stained cream samples were then incubated at room temperature for 15 min, and 10 μL of this stained cream solution was deposited onto the glass slide to analyze the microstructure.

2.6. Statistical Analysis

All the experiments, including HPTLC quantitative analysis, were performed in triplicate, and data are reported as mean ± standard error of the mean (SEM) of biological and technical replicates. Analysis of variance (ANOVA) was done using Graph Pad Prism, Version 6.01 GraphPad Software, Inc., La Jolla, CA, USA. The results are reported with 95% confidence intervals where p value ranged from 0.0001 to 0.01 for different experiments.

2.7. Chemicals and Reagents

Standard compounds of phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS),phosphatidylethanolamine (PE), sphingomyelin (SM), and diglycerides (DAG) were purchased from Avanti Polar Lipids Inc. (Birmingham, UK), triglycerides (TAG) from Cayman Chemical (Ann Arbor, MI, USA) and cholesterol (Chl) from Himedia Chemicals (Mumbai, India). The HPLC-grade chloroform, methanol, hexane, and silica pre-coated HPTLC plates (Si $60F_{254}$; 20 cm × 20 cm)were purchased from Merck (Darmstadt, Germany). The other chemicals, such as acetone, diethyl ether, acetic acid, and methyltertiary-butyl ether(MTBE), were all of the analytical grade (purity level > 99%). N-(Lissamine rhodamine B sulfonyl) di-oleoyl-phosphatidylethanolamine (Rh-DOPE) was purchased from Avanti polar lipids Inc., Birmingham, England, and the Nile red from Himedia.

3. Results

3.1. Separation and Identification of PL and NL Classes in Cow, Goat, and Water Buffalo Milk Fat Globules

The PL fraction was run along with five PL standards on HPTLC to separate and identify each PL class present in MFGM of goat, cow, and water buffalo milk. All the PL classes were distinctly separated in each sample, as observed in chromatographic bands (Figure 1A). The image indicates that the PL bands present in goat MFG were most intense, followed by cow and water buffalo. The PL class in samples was identified based on their retention factor (R_f) values corresponding to pure standards. However, PS and PI bands appear close but did not co-elute, confirmed by the difference in their R_f values and the chromatogram, as shown in Figure 1B–E.

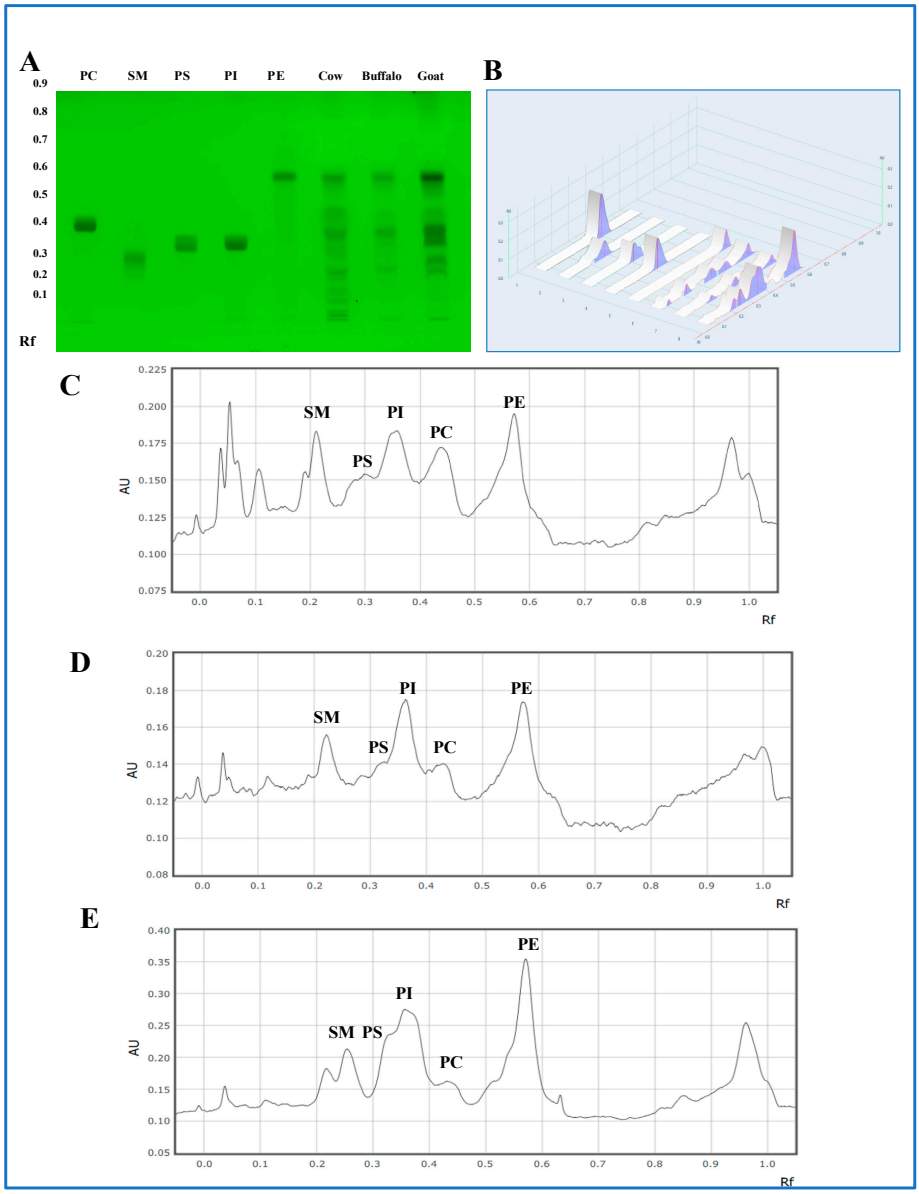

Figure 1. Analysis by high-performance thin-layer chromatography of (**A**) polar lipid standards and animal groups visualized under UV light at 254 nm after derivatization with iodine vapors. Polar lipid standards loaded at different volumes from track 1–5 and milk samples in track 6–8; (**B**) high-performance thin-layer chromatography 3-D densitogram; Chromatogram of (**C**) cow, (**D**) water buffalo, and (**E**) goat polar lipids. Representative image of three independent biological samples performed in triplicate.

Similarly, TAG, DAG, and Chl pure standards were separated and identified along with the NL fractions, as shown in Figure 2A. Water buffalo NL fractions were found to be more intense on the HPTLC plate than goat and cow. The effective separation of DAG and Chl was established by their different R_f values and separated peaks in the chromatogram, as shown in Figure 2B–E. The five-point calibration curve obtained was used to calculate the limits of detection (LoD) and quantification (LoQ) and quantify lipid classes. Table 1 shows HPTLC data, including R_f values of all samples corresponding to pure standards, LoD, LoQ, and correlation coefficient from the standard curve.

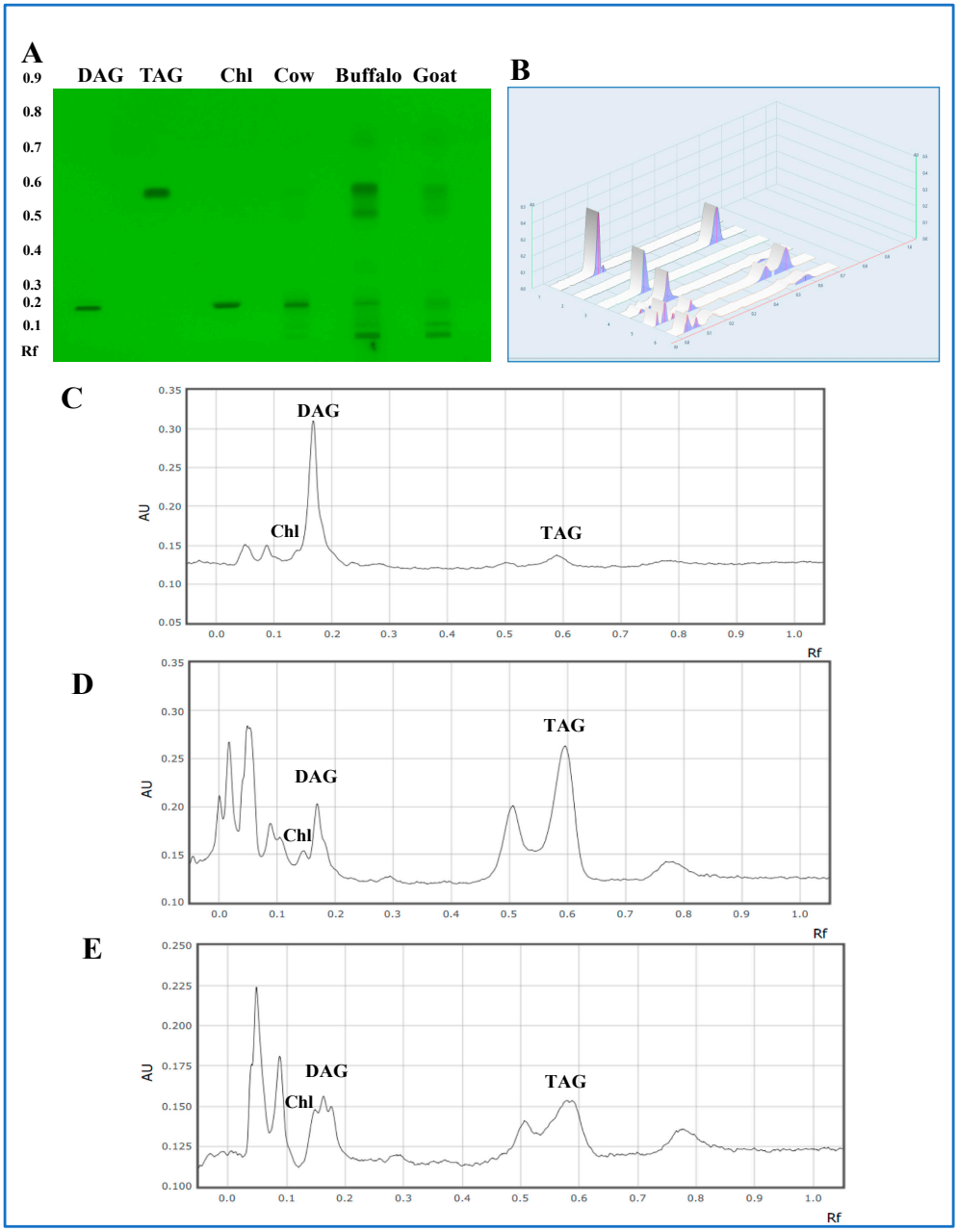

Figure 2. Analysis by high-performance thin-layer chromatography of (**A**) neutral lipid standards and animal groups visualized under UV light at 254 nm after derivatization with iodine vapors. Neutral lipid standards loaded at different volumes from track 1–3 and milk samples in track 4–6; (**B**) high-performance thin-layer chromatography 3-D densitogram; Chromatogram of (**C**) cow, (**D**) water buffalo, and (**E**) goat milk neutral lipids. Representative image of three independent biological samples performed in triplicate.

Table 1. HPTLC method parameters for PL and NL standards.

Parameters	Polar Lipids					Neutral Lipids		
	PC	**SM**	**PS**	**PI**	**PE**	**DAG**	**TAG**	**Chl**
R_f	0.40	0.27	0.31	0.33	0.58	0.17	0.60	0.16
Linearity Range (µg/band)	0.5–5.3	0.4–6.4	1–7	0.4–2	6–19	0.4–3.6	2–10	0.5–8.5
Correlation coefficient (r^2)	0.99	0.98	0.99	0.99	0.99	0.99	0.99	0.99
LOD (ng/mL)	554	933	437	120	1536	147	566	1036
LOQ (ng/mL)	1680	2828	1326	365	4654	446	1716	3140

3.2. Quantification of Polar Lipid and Neutral Lipid Classes by HPTLC in Cow, Goat, and Water Buffalo Milk Fat Globules

The five major polar lipid classes, PE, PC, PS, PI, and SM, extracted from MFGM were quantified using the HPTLC method. As shown in Figure 3A, the concentration of PE was found to be significantly higher ($p \leq 0.001$) in goat (1585.5 ± 26.50 µg/mL), followed by cow (538.46 ± 14.65 µg/mL) and water buffalo (344 ± 16.38 µg/mL). The choline-containing PC also followed similar trend with goat reportedly having 150.72 ± 4.78 µg/mL, followed by 96.89 ± 1.71 µg/mL in cow and 65.96 ± 2.64 µg/mL in water buffalo. In contrast, SM, another choline-containing PL present in MFGM, was present in significantly higher amount ($p \leq 0.01$) in cow milk (23.25 ± 1.01 µg/mL) compared to goat (11.30 ± 0.3 µg/mL) and water buffalo (7.79 ± 0.35 µg/mL). The PS concentration was significantly higher ($p \leq 0.001$) in goat (411.85 ± 3.23 µg/mL), than cow (37.94 ± 3.41 µg/mL) and water buffalo (32.12 ± 1.67 µg/mL) in water buffalo fat globule membrane. On the other hand, water buffalo milk had the lowest PI amount (12.03 ± 0.58 µg/mL). Notably, of five classes of lipids, four were detected higher in goat than cow and water buffalo.

TAG, DAG, and Chl were quantified using HPTLC as the major NL classes in goat, cow, and water buffalo MFG. Figure 3B shows significantly higher ($p \leq 0.01$) content of DAG in cow (540.35 ± 25.72 µg/mL), followed by water buffalo (32.42 ± 2.37 µg/mL) and goat (29.52 ± 0.36 µg/mL). A similar pattern was observed for Chl, with cow MFG containing a significantly higher ($p \leq 0.001$) amount (986.43 ± 15.70 µg/mL), followed by water buffalo (65.54 ± 20 µg/mL), and goat (25.14 ± 2.12 µg/mL). Water buffalo MFG contained a significantly higher ($p \leq 0.001$) amount of TAG (109.72 ± 5.10 mg/mL), followed by cow (56.29 ± 3.39 mg/mL) and goat (45.32 ± 1.11 mg/mL), as shown in Figure 3C. Of note, goat milk contained the least amount for all the three NL classes.

3.3. Particle Size Distribution of Milk Fat Globules in Cow, Goat, and Water Buffalo

The size distribution and spherical shape of MFG in cow, goat, and water buffalo milk characterized using DIC images is shown in Figure 4A–C. The images show variations in globule sizes between animal groups with significantly ($p \leq 0.001$) smaller MFG observed in goat, followed by cow and water buffalo. Similarly, particle size measurement by Zetasizer showed significantly ($p \leq 0.0001$) smaller globule size in goat (0.99 ± 0.04 µm), followed by cow (1.85 ± 0.03 µm) and water buffalo (2.91 ± 0.08 µm), as shown in Figure 4E. The PDI values of goat, cow, and water buffalo MFG was 0.186 ± 0.02, 0.165 ± 0.03, and 0.135 ± 0.02, respectively.

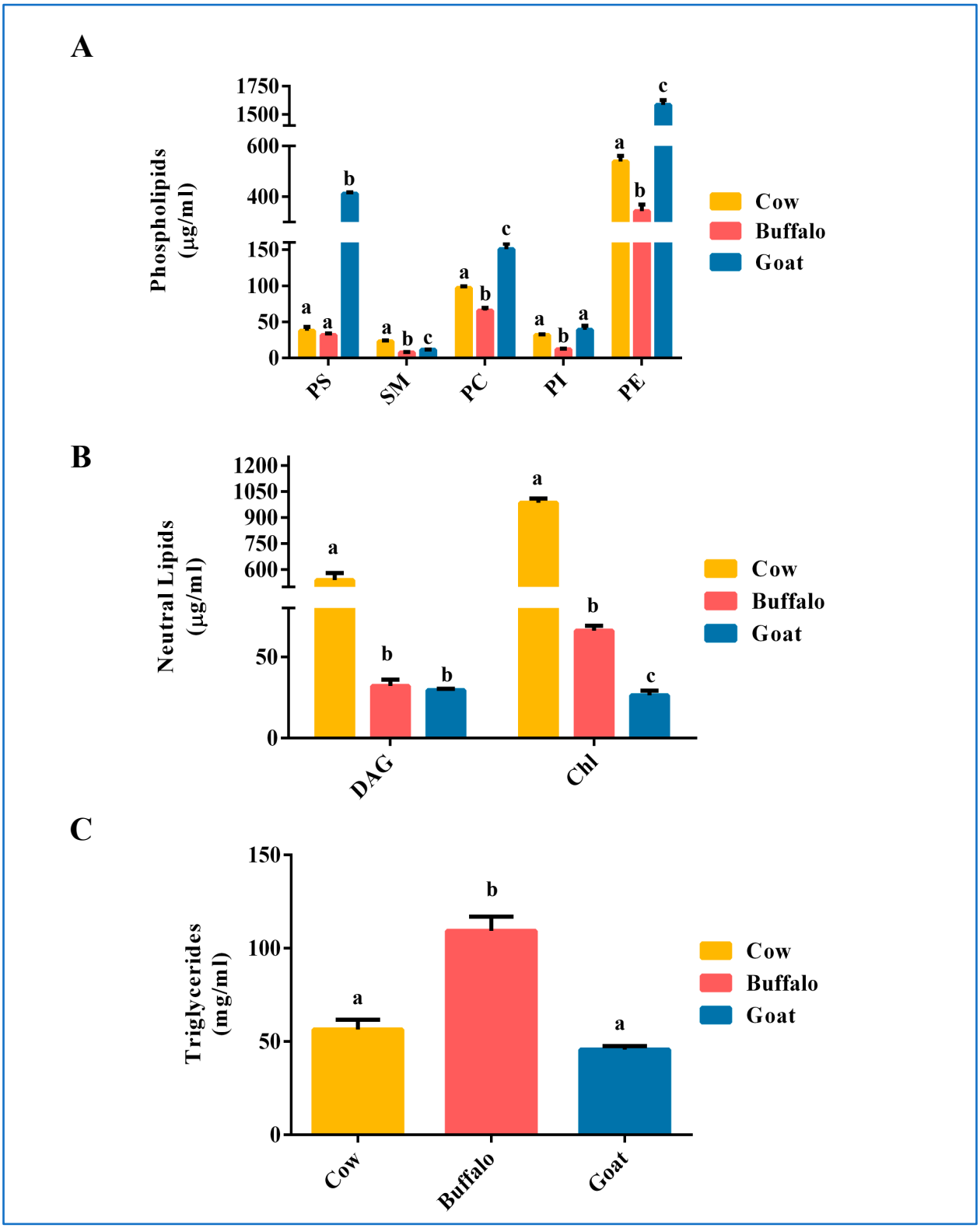

Figure 3. Concentration of lipids present in milk fat globules of cow, goat, and water buffalo; (**A**) Polar lipids, (**B**) Diglycerides and Cholesterol and (**C**) Triglycerides. Data indicate the mean values of lipid concentration ± standard error of the mean (SEM) of biological and technical replicates. Different letters indicate a significant difference ($p \leq 0.001$).

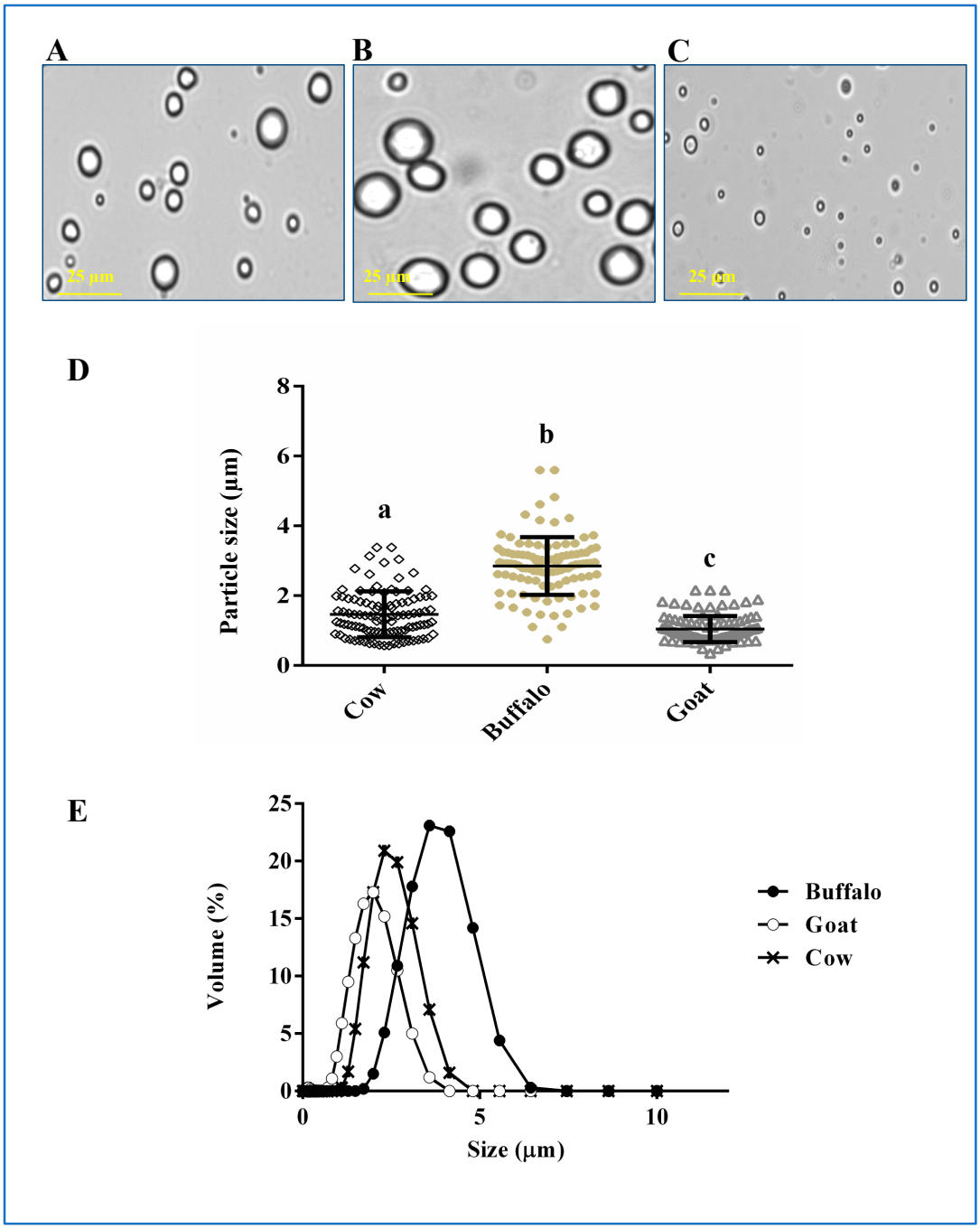

Figure 4. Milk fat globule shape and size distribution in (**A**) cow, (**B**) water buffalo, and (**C**) goat determined with differential interferential contrast; (**D**) Comparative analysis of size variation in milk fat globules calculated using differential interferential contrast images across the animals; (**E**) Fat globule size distribution based on dynamic light scattering. Representative data of three independent biological samples performed in triplicate. Different letters indicate a significant difference ($p \leq 0.001$). Scale bar = 25 µm.

3.4. Microstructure, Zeta Potential, and Correlation of Milk Fat Globule Size and Lipids Classes

The emission fluorescence of cow, goat, and water buffalo MFG stained with Rh-DOPE showed the distribution of polar lipid layers on the surface of MFG as indicated by yellow arrows in Figure 5A–C. The non-fluorescent, sphingomyelin-rich domains appear as black areas indicated by white arrows, as shown in Figure 5A–C. The emission fluorescence of

Nile Red stained MFG triglycerides in the three animal groups as dispersed in spherical shape as shown in Figure 5D–F.

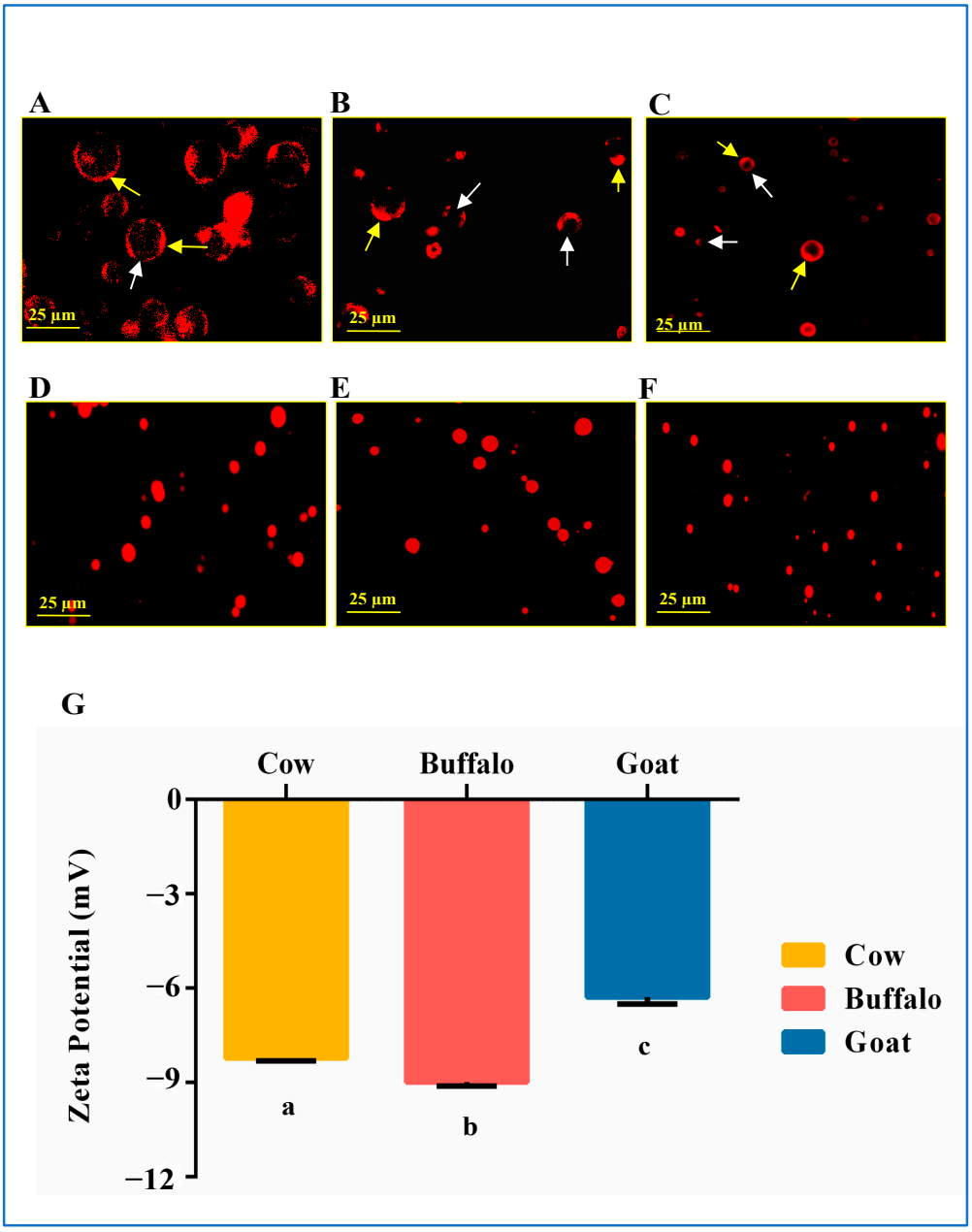

Figure 5. Microstructure analysis of fat globules stained with phosphatidylethanolamine-lissamine rhodamine (red) in (**A**) cow, (**B**) water buffalo, and (**C**) goat milk; Triglycerides were stained with the Nile red in fat globules of (**D**) cow, (**E**) water buffalo and (**F**) goat; (**G**) Zeta potential measurements between the animal groups. Representative image of three independent biological samples performed in triplicate. Different letters indicate a significant difference ($p \leq 0.0001$). Scale bar = 25 µm.

The zeta potential of fat globules was significantly higher ($p \leq 0.0001$) in goat (-6.28 ± 0.053 mV), followed by cow (-8.22 ± 0.022 mV) and water buffalo (-8.98 ± 0.030 mV), as shown in Figure 5G. Furthermore, the correlation between total PL (sum of PC, PS, PI, PE, and SM) and globule size measured by DLS ($r = -0.89$, $p \leq 0.001$) showed a negative relation, as shown in Figure 6A. Whereas, a positive correlation was found between total NL (sum of TAG, DAG, and Chl) and MFG size ($r = 0.93$, $p \leq 0.001$), shown in Figure 6B.

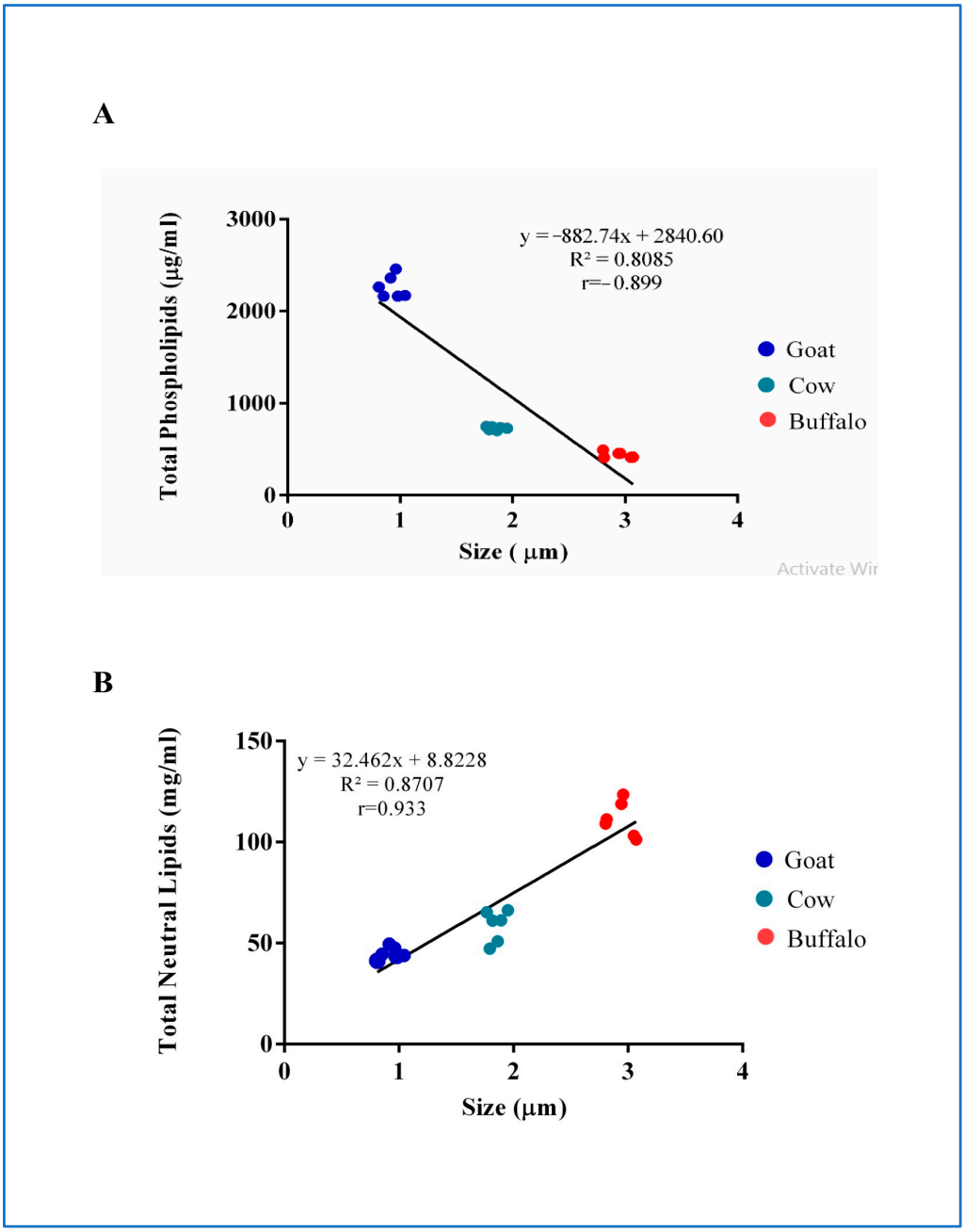

Figure 6. Correlation (*r*) between milk fat globule size of animal groups measured by dynamic light scattering and (**A**) total polar lipids and (**B**) total neutral lipids present in cow, water buffalo, and goat.

4. Discussion

Different lipids from MFG were characterized using HPTLC, and the resulting order of their appearance was consistent with previous reports of separation of polar lipids in egg yolk [27] and neutral lipids in food emulsifiers [28]. Notably, lower detection and quantification limits were achieved for all PL and NL across the animal groups, highlighting the efficiency and sensitivity of the method.

Using DIC images and DLS, the particle size measurement of cow, goat, and water buffalo MFG demonstrated significant size differences among the animal groups, with the smallest MFG observed in goat, followed by cow and water buffalo. This size distribution pattern was consistent with previous studies that measured MFG size based on an image analysis program and highlighted the importance of MFG geometry in determining the

quality of processed dairy products [29,30]. Subsequently, the uniform and unimodal globule size was confirmed by PDI values, and, not surprisingly, a narrow range of size distribution was observed in the three cow, goat, and water buffalo animal groups, indicating MFG belongs to one group. The same was verified through particle size distribution, where a single peak was detected in each animal group, similar to previous studies on cow, water buffalo, and goat globule size [24,31]. To obtain further information about MFG size distribution, the diameter was measured by DIC images and DLS, where the results of these experiments did not significantly differ among the three animal groups. However, the mean diameter of cow, goat, and water buffalo fat globules was lower than in previous reports, possibly due to variation in diet, lactation stage, genetic factors, and fat content [3,25,32]. Notably, several studies have highlighted the correlation between smaller globule size with higher digestibility of fat, such as in goat and camel [33], and have also reported a correlation between larger surface area and polar lipid content and larger liquid-ordered regions in MFG [31,33]. The smaller globules in goat or cow could find a suitable application in preparing infant formula [34] and dairy products such as cheese [33], while larger fat globules of water buffalo were reported to be suitable for manufacturing dairy products, including butter and ghee [25]. Consequently, it is critical to understand a species-specific variation of MFG sizes to screen and select small or large globules correlating with fat content for nutrition and manufacturing dairy products.

Zeta potential, indicative of electric charge interactions in food systems, was higher in goat MFG than in cow and water buffalo, possibly due to the differences in their MFGM composition, microstructure, and composition of their aqueous phase (especially the amount of mineral salts). Notably, the zeta potential pattern of the three animal groups was in accordance with the absolute values described in the literature [25,31]. Using fluorescent stains to analyze the microstructure of globules enabled the localization of polar lipids and thus revealed their non-homogenous distribution on the membrane. Such heterogeneities, suggestive of SM-rich domains, were observed in cow, goat, and water buffalo MFG and were consistent with previous studies [2,31,35]. Considering the presence of SM in the outer leaflet of the globule, these SM-rich domains correspond to 75% of the fat globule surface [2].

The PLs composition varied considerably among different animal groups, and as it positively impacts infant and human health, it is vital to know the exact amount from the preferred ruminant milk source for consumption. Our study using HPTLC demonstrated that each animal group has unique PL fingerprints, with a negative correlation between polar lipids and fat globule size, where a higher polar lipid content is associated with smaller fat globules. Interestingly, the majority of PLs classes were detected in significantly higher amounts in goat milk than in cow and water buffalo milk, consistent with previous studies [3,31]. The higher PL content in goat milk could be due to smaller MFG size and large surface area, resulting in higher MFGM content and PLs than in bovine milk. However, a relatively lower amount of choline-containing PC and SM was detected in all the animal groups attributed to the cream separation by centrifugation process, releasing PC and SM from the outer leaflet of MFGM [6]. The PLs content in milk from different ruminant sources is of practical interest to produce functional foods or dietary supplements to prevent or treat specific diseases, produce new formulae for infants (and premature infants), and dietary supplements for adults [36–38]. Additionally, MFG polar lipids have been studied to promote the adhesion and growth of gut lactic acid bacteria, highlighting the benefit of fat globules with higher PLs on dairy products and the human gut microbiome [39–41]. Thus, studying the exact amount of different PL species available from different milk sources is essential for their potential application in supplementing human milk fortifiers or developing new milk substitutes.

To obtain a comprehensive understanding of MFG lipid composition, the three major classes of NL (e.g., TAG, DAG, and Chl) were quantified. Apart from being the major NL class present in the globule, TAG plays a crucial role in lipid digestion and absorption efficiency due to the positioning of its fatty acids [42]. Interestingly, the highest TAG

amount was detected in water buffalo milk and showed a positive correlation between TAG quantity and MFG size. Along with PL and protein composition, globule size has also been reported to influence fatty acid bioavailability and lipid digestion [43]. For instance, compared to cow, goat MFG was reported to contain fewer long-chain fatty acids in its TAG molecule and, therefore, possessed easy-to-digest TAG, responsible for a higher digestion rate [31]. Similarly, DAG was detected as another predominant NL class present in the MFG of cow, goat, and water buffalo, which was reported to play a critical role in lowering serum TAG levels and exhibiting anti-obesity effects [44]. In our study, the highest amount of DAG was detected in cow milk, followed by water buffalo and goat, consistent with previous literature reporting a higher amount of DAG in cow milk (0.80% of lipid class) than in goat (0.25% of lipid class) [45]. Of note, the liquid-ordered region in the membrane, represented by Chl/SM ratio, was higher in goat MFG, possibly due to its smaller globule size [46]. The presence of Chl in the lipid bilayer has been reported to decrease mechanical resistance to rupture of SM-rich domains by inducing the liquid-ordered phase, highlighting the functional role of Chl in milk polar lipids [47]. The correlation between total NLs and globule size of cow, goat, and water buffalo indicated a negative association. Contrary to the paradigm, NLs present in fat globules are more than just a source of energy and are now being explored for their functional role, such as lipid digestion and absorption [42]. Therefore, a better understanding of its composition and MFG size from different ruminant sources will contribute to existing knowledge to study NLs as bioactive components.

5. Conclusions

In conclusion, the results of this study indicated considerable differences in the abundance of major PLs and NLs in three conventionally consumed milk sources in India: cow, water buffalo, and goat. The successful separation of lipid classes by HPTLC led to their identification and quantification, therefore establishing it as an efficient technique for milk fat globule lipid characterization. The correlation between globule size and lipid quantity highlighted that smaller globules have a larger surface area and thus more membrane material. The varying levels in lipid composition as a function of MFG size reflect changes in the mammary gland physiology and genetic characteristics of these ruminant species. Thus, it is imperative to understand and identify specific physiological and animal husbandry conditions that could enhance specific globule size distribution or desired lipids for their application to improve infant formula and dairy products as functional foods to impact human health positively.

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