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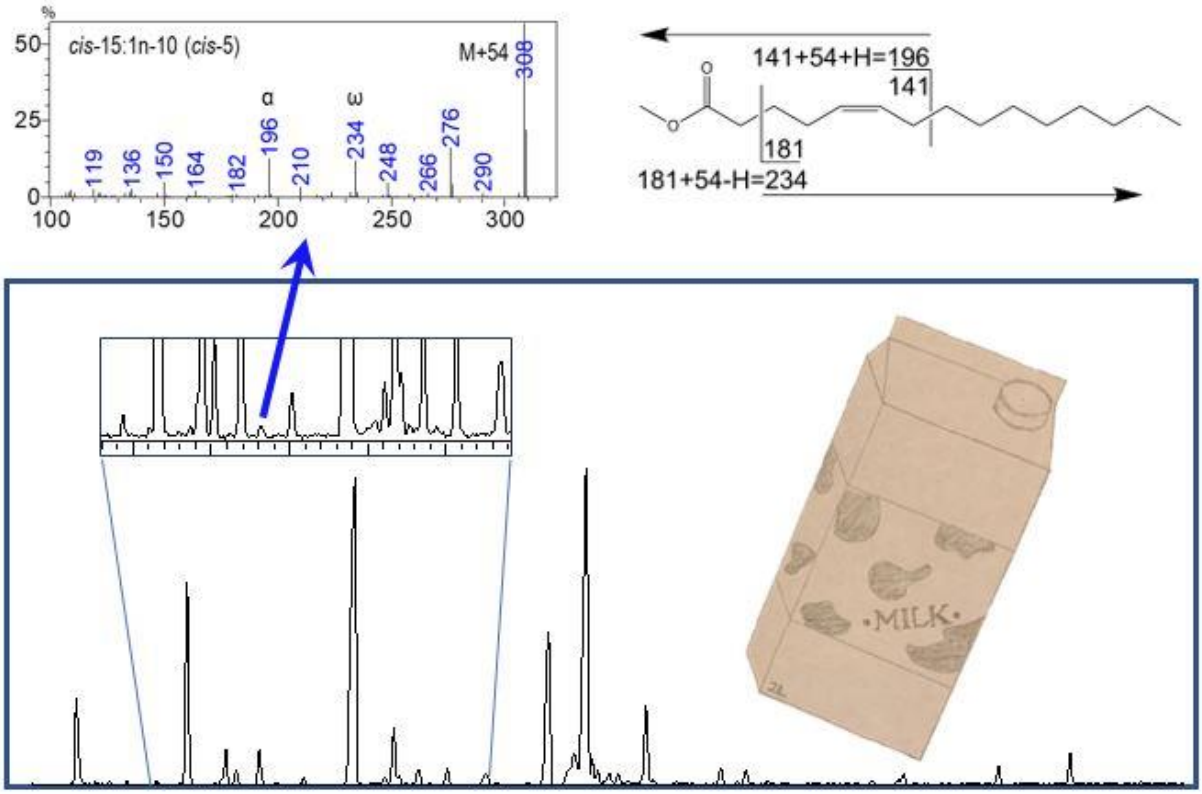
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Lipid Profiling and Microstructure Characteristics of Goat Milk Fat from Different Stages of Lactation

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ABSTRACT: Goat milk at different lactations show varied lipids distributions, which are potentially dietary influencing factors for the health of human consumers. Herein, the effects of lactation stages (colostral, transitional and mature stages) on lipid profiling and microstructure of goat milk fat (GMF) were investigated. A total of 359 species of triacylglycerols (TAGs), 27 species of diacylglycerols (DAGs) and 10 classes of phospholipids (PLs) were identified using high resolution tandem mass spectrometry (HR-MS/MS). Of importance, goat transitional milk presented the highest levels of MUFA (29.51%) and lyso-phospholipids (7.95% of total PLs) among these three different lactations. A lactation-dependent attenuation was found at the level of PUFA in goat milk, particularly long-chain PUFA ω-6. Similar behavior was observed in the total proportions of POO (16:0/18:1/18:1) and PSL (16:0/18:0/18:2), presenting a decrease from 3.70% to 3.23% as the proceeding period from colostrum to mature. The relative contents of sphingomyelin and cholesterol in goat colostrum were approximately twice and three times that in mature milk, respectively. Unlikely, both PMCy+MCaM (16:0/14:0/8:0+14:0/10:0/14:0) and BuPO (4:0/16:0/18:1) TAGs, the foremost saturated and monounsaturated TAGs in goat colostrum, respectively, behaved upward trends over the period from colostrum to mature. Interestingly, no significant variation in milk fat globule morphology was monitored at different lactation periods. Therefore, all our results demonstrated that the main influences of lactation stages on GMF were the lipid profiling, providing a theoretical guidance for rational implement of lipids in goat milk.

KEYWORDS: Goat milk; Lactation; Lipid composition; Microstructure; HR-MS/MS

1. Introduction

As one of the major contributors to non-bovine milk production, goat milk has drawn widespread attention in recent years. The global market revenues in 2024 are estimated to be approximately $15 billion with a compound annual growth rate of more than 7% from 2018 to 2024, indicating the increasing consumption of goat milk and dairy products [1-2]. It is well acknowledged that goat milk is an attractive substitute to cow milk because of its higher digestibility, less inflammation and fewer allergens [3]. More intriguingly, goat milk can improve the physiological functions of humans, particularly for infants and elderly [4]. However, these health benefits are closely related with the structural components of goat milk, such as types and contents of lipids. Accordingly, it is essential to explore the lipid profiling of goat milk for making the most of its potential.

Lipid composition of goat milk plays a key role in its technological and nutritional quality. As the most dominant component (about 97%) of goat milk fat (GMF), triacylglycerols (TAGs) are the core of the milk fat globules and covered with the membrane containing polar lipids, such as phospholipids (PLs) and cholesterol [5], which have been found to have strong anti-atherogenic activities [6]. Furthermore, goat milk is superior to cow milk in its great concentrations of fatty acids with short and medium chains, and large proportions of small milk fat globules, favoring for treating metabolic disorders, low bone mineral density and anaemia [7-8]. With the development of analytical techniques in recent years, mass spectrometry as a new method has been applied for analyzing the lipids (TAGs, PLs, etc.) of human and cow milk, as well as other mammalian milk such as camel, donkey and goat milk [9-11]. As for the goat milk, dozens of TAGs were identified by means of high-performance liquid chromatography (HPLC) with atmospheric-pressure chemical

ionization mass spectrometry [8], whereas 165 TAGs were characterized using ultra-HPLC with atmospheric-pressure chemical ionization ion trap-time of flight-mass spectrometry [12]. Similarly, 35 species of PLs molecules were detected in GMF using matrix-assisted laser-desorption/ionization-time of flight mass spectrometry [13], while 68 species of PLs were determined in goat milk powder using ultra-HPLC combined with electrospray ionization-quadrupole-time of flight-mass spectrometry [14]. Comparatively speaking, high resolution tandem mass spectrometry (HR-MS/MS) is a more effective tool for accurate lipid molecules identification of goat milk in comparison to monopole mass spectrometric approach.

Lipid profiles are closely associated with the lactation stages, varying the nutritional values of mammalian milk. Colostrum intake could promote the growth of postnatal body and organ development in neonatal calves [15], as well as ameliorate the unfavorable effects on systemic immunity and intestinal health of premature infants caused by formula feeding [16]. On the other hand, Lugonja et al. [17] demonstrated that mature breast milk provided better antioxidant protection and exerted direct pharmacologic relaxation effects in comparison to formula milk. Nevertheless, Minic et al. [18] found that transitional milk from mothers of premature newborns had higher antioxidant capacity than colostrum or mature milk. Taken all together, each lactation stage of mammalian milk exerts its specific nutritional value, which may be attributed to its own components. Of importance, lipid compositions of goat milk are the most variable components among the whole lactation period [19]. For instance, Kuchtik et al. [20] observed a decreasing tendency of polyunsaturated fatty acids (PUFA) during lactation and the lowest level of conjugated linoleic acid (CLA) at the end of lactation. However, in the work of Curro et al. [21], the amount of n-3, n-6, monounsaturated fatty acids (MUFA), PUFA and CLA were lower at the beginning than at the

end of lactation. Accordingly, these lipid patterns of goat milk mentioned above are controversial, and further detailed research should be concerned about characterizing goat milk with different lactation stages.

To our knowledge, there is limited information about the systematic exploration for the characteristics of GMF during different stages of lactation. Herein, this study was aimed to study the effects of lactation stages on lipids profiling and microstructure of GMF. Specifically, goat milk from colostrum, transitional, and mature lactation was investigated for the lipid compositions, including glycerides, PLs, cholesterol, fatty acid composition and positional distribution. Besides, the microstructure of goat milk fat globules was observed by confocal laser scanning microscopy (CLSM) for evaluating the physical properties evolution during lactation periods.

2. Materials and methods

2.1 Materials

Lipase from porcine pancreas (type II), the 37-component fatty acid methyl esters (FAMEs) mixture standard, and the fluorescent dye 9-diethylamino-5H-benzoalpha-phenoxazine-5-one (Nile red) were purchased from Sigma Chemical Co. (St. Louis, USA). N- (Lissamine rhodamine B sulfonyl) dioleoyl-phosphatidylethanolamine fluorescent dye (Rh-DOPE) was purchased from Avanti Polar Lipids, Inc. (Alabaster, USA). Sodium cholate (CAS:361-09-1, 98%) was obtained from J&K Scientific Ltd. (Beijing, China). Cholesterol standard (CAS: 57-88-5, ≥99.5%) was obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). All other chemicals used were of analytical reagent grade.

2.2 Samples

Goat milk samples from three lactation stages were provided by Ausnutria Hyproca Dairy

Group BV (Changsha, China). Colostrum (1-7 days postpartum), transitional milk (8-14 days) and mature milk (15-30 days) were collected from Saanen goats fed daily with fresh grass and hay in Netherlands farm. Milk samples from same lactation were pooled and then freeze-dried. All freeze-dried powder was stored at -20°C. Each goat milk sample for further analysis was dissolved into ultrapure water (1 g of powder to 10 mL of water at 40°C).

2.3 Lipid extraction

Lipid in goat milk was extracted by the Folch method [22] with slight modification. Goat milk solution was mixed with $CHCl_3$: MeOH (2:1, v/v), followed with 30 minutes of ultrasonic treatment at 30℃. The organic phase was transferred and the remaining fractions were extracted repeatedly. The merged organic phase was evaporated to 5 mL under vacuum, and further dried by a stream of nitrogen to a constant weight. The milk lipid obtained was stored at -20°C for further lipid analysis.

2.4 Fatty acid and *sn*-2 fatty acid analysis

Fatty acid compositions were investigated in the form of methyl esters under gas chromatography system (Nexis GC-2030, Shimadzu, Kyoto, Japan) as described by Ye et al. [23], using a TR-FAME capillary column (60 m×0.25 mm×0.25 μm). Milk lipid extract was mixed with KOH-CH_3OH (0.5 M, 2 mL) and BF_3-CH_3OH (1:3, v/v), successively. The generated FAMEs were extracted with n-hexane (HPLC grade) and then purified before gas chromatography analysis. Fatty acids identification was accomplished by matching the retention time with that of FAMEs standards.

The hydrolysis of TAGs to *sn*-2 monoacylglycerol was achieved by the method of Sun et al. [24] The concentrated hydrolyzate was applied to a thin-layer chromatographic plate, and diethyl

ether/hexane/acetic acid (50/50/1, v/v/v) was the developing solvent. The *sn*-2 monoacylglycerol obtained was methylated like fatty acid analysis. As described by Sun et al. [24], *sn*-2 fatty acid×100%/ (3×total fatty acid) was used to calculate the relative proportion of each fatty acid at the *sn*-2 position.

2.5 Glycerides analysis

Glycerides profiles were analyzed as described by Li et al. [25] with some modifications. Lipid samples were injected into an Exion LC™ AD UPLC system (AB Sciex, Redwood City, CA, USA) coupled to the X500R QTOF high-resolution mass spectrometer. The Kinetex C18 column (100×2.1 mm×2.6 μm, Phenomenex, USA) was used to achieve chromatographic separation. Mobile phases employed consisted of: (A) water/acetonitrile (4/6, containing 10 mM ammonium formate) and (B) acetonitrile/isopropanol (1/9, containing 10 mM ammonium formate). The 30 min gradient of phase B was 0% at 0 min, 20% at 0.5 min, 60% at 4 min, 98% at 25 min, 20% at 26.1 min and 0% at 30 min. The flow rate of mobile phase was 0.3 mL/min. The column chamber and sample tray were respectively held at 55°C and 4°C. The injection volume was 1.0 μL. Information dependent acquisition and sequential window acquisition of all theoretical fragment ions modes were used to acquire data with mass ranges of m/z 200-1300 and m/z 100-1300, under the following optimized conditions: ion source gas, 60 psi; curtain gas, 35 psi; spray voltage, 5500 V; declustering potential, 80 V; collision energy, 45±20 V; desolvation temperature, 550°C. Data acquisition and processing were carried out by means of the SCIEX OS software.

2.6 Phospholipids analysis

PLs purification in the lipid extract was realized by solid phase extraction (SPE) according to Donato et al. [26] Briefly, lipid extract (100 mg) was dissolved in 1 mL mixture of $CHCl_3$: MeOH

(2:1, v/v). The SPE cartridge (ANPEL, Shanghai, China) was conditioned with n-hexane firstly, and then 3 mL of diethyl-ether/hexane (2:8, v/v) and diethyl-ether/hexane (1:1, v/v) were employed to elute the non-polar lipids. Methanol (4 mL) and 3 mL of water/methanol/chloroform (2:5:3, v/v/v) were used to recover PLs from the cartridge. The recovered PLs were dried under nitrogen and then injected in the above UPLC-MS/MS system. PLs species were determined under the same conditions as glycerides analysis, except the 30 min gradient of mobile phase B: 0 min, 0%; 0.5 min, 20%; 4 min, 60%; 10 min, 70%; 17 min, 98%; 17.1 min, 20%; 30 min, 0%.

2.7 Cholesterol analysis

Cholesterol in goat milk was determined according to Albuquerque et al. [27] with some modifications. Goat milk was saponified in ethanol and KOH solution, and extracted with anhydrous ether/petroleum ether (1:1, v/v). The residue obtained by evaporating to dryness under vacuum, was dissolved in ethanol and then determined by HPLC (Shimadzu, Kyoto, Japan) equipped with an SPD-20A detector, using a Venusil MP-C18 column (4.6×250 mm×5 μm, Bonna-Agela, China). Methanol was employed as mobile phase with flow rate at 1.0 mL min^{-1}. The settings of injection volume and column temperature were 10 μL and 38°C, respectively. The detection was achieved at 205 nm. Cholesterol was quantified by the external standard method.

2.8 Particle size and zeta-potential analysis

The average diameter of goat milk fat globules was observed by dynamics laser scattering technique with Nano Brook Omni instrument (Brookhaven, USA) according to Liang et al. [28] To avoid the deviation as a result of multiple light scattering, goat milk samples were diluted 100 times by water prior to each analysis. The globule size was expressed as the intensity-weighted average particle diameters with the average of five measurements.

The zeta-potential of goat milk was evaluated by phase analysis light scattering with a Zeta Potential Analyzer (Nano Brook Omni, Brookhaven, USA) at 25°C referred to the method of Shao et al. [29] All samples were diluted 100 times to avoid multiple scattering effects firstly and then each sample was measured three times.

2.9 Confocal laser scanning microscopy (CLSM) analysis

CLSM images of goat milk fat globules were monitored by using an LSM 710 Meta confocal microscope (Zeiss, Jena, Germany) with 40× objective. Samples preparation were realized by means of Yao et al. [8] 100 μL of Nile Red (dissolving in acetone at 42 μg/mL) was mixed with 500 μL of milk solution to dye the neutral lipids. The polar lipids in fat globule membrane of goat milk (500 μL) were marked with 20 μL of Rh-DOPE fluorescent stain (dissolving in chloroform at 1 mg/mL). 5 μL of prepared samples were dropped on the glass slide and observed on the microscope.

2.10 Statistical analysis

The measurements were executed in duplicate at least, and final results were presented as mean ± standard deviation. The SPSS 20.0 software (IBM, USA) was applied for statistical treatment. The level of $P<0.05$ indicated significant difference. Figures involved in this study were obtained by using Origin 9.5 software (Origin Lab, USA).

3. Results and discussion

3.1 Fatty acid composition

The composition of fatty acids in goat milk is listed in Table 1. A total of 33 fatty acids were identified and quantified, including 9 major fatty acids (relative content >1%). Saturated fatty acids (SFA) accounted for 66.86-72.79% of total fatty acids in GMF with C16:0 being the most abundant, which was consistent with the previous findings [14, 30-32]. Both caprylic acid (C8:0) and capric acid

(C10:0), production of de novo synthesis in the mammary gland [33], showed a gradual growth over the lactation periods and reached the maximum at mature stage, indicating the special aroma of goat milk given by C8:0 and C10:0 might be enhanced during lactation [31].

Goat transitional milk presented the greatest level of MUFA (29.51%) among three different lactations, mainly due to the change of C18:1 ω-9. The relative contents of PUFA ω-3 and PUFA ω-6 changed insignificantly with prolonged lactation, while an obvious decrease in LC-PUFA ω-6 was observed from colostrum to mature milk. Some important fatty acids including C18:3 ω-6, DHA and EPA, also behaved downward trends over lactation periods. As the essential fatty acid for human body, neither C18:2 ω-6 nor C18:3 ω-3 were influenced by lactation stages. Additionally, to achieve a proper balance, the ratio of C18:2 ω-6 and C18:3 ω-3 (LA/LNA) was recommended to be between 5:1 and 15:1 in infant formulas on account of their competition for the same enzymatic systems [34]. However, in this study, the LA/LNA ratio of goat milk exceeded this range (>20:1). Thus, it is crucial to adjust this ratio specifically, when goat milk is used as raw materials for infant formulas.

Odd-numbered saturated fatty acids (ONSFA) might be used to discriminate the fat source of infant formulas, on account of its much higher contents in milk fat of ruminants than most plant oil [35]. Five kinds of individual ONSFA were detected in GMF as shown in Table 1. As the main ONSFA, the levels of C15:0 (0.61%~0.84%) and C17:0 (0.51%~0.65%) were obviously decreased from goat colostrum to mature periods. *Trans* fatty acids (TFA), generated from biological hydrogenation of rumen bacteria, naturally occurred in goat milk [35]. The individual TFA like C18:1 (T) and C18:2 (T) were less than 1%, which had a negligible change as the proceeding lactation. Therefore, most fatty acids in goat milk showed conspicuously different characteristics during

lactation, mainly presenting as the variations in their relative contents.

3.2 Fatty acid positional distribution

The specific distribution of different fatty acid on the glycerol backbone played a decisive role in absorption and metabolism as well as practical applications of milk fat [10, 36]. As shown in Table 1, the *sn*-2 fatty acid in GMF was also principally SFA with C16:0 being the most copious, which was in accordance with the previous results [32, 37]. Most of individual saturated and monounsaturated fatty acids at the *sn*-2 position varied slightly as a function of lactation. Notably, the levels of *sn*-2 LC-PUFA ω-6 and LC ω-6/ω-3 ratio evidently decreased from goat colostrum to mature milk, while the *sn*-2 LC-PUFA ω-3 showed no obvious variation. Similar behavior was also observed in human milk [36]. Unlikely, the relative percentage of C16:0 at the *sn*-2 position kept constantly (37.18-41.49%) throughout the proceeding lactation, indicating that C16:0 in GMF was mainly distributed at the s*n*-2 position. It was reported that the fatty acid and calcium absorption in infants would be improved, if the relative proportion of C16:0 at the *sn*-2 position in infant formulas was greater than 40% [38]. The individual LC-PUFA including C20:3 ω-3 and C20:4 ω-6 were primarily located at the *sn*-2 position in GMF (the calculated relative percentage >35%), whereas C20:3 ω-6 was primarily at the *sn*-1,3 position (the calculated relative percentage <30%). The absorption of LC-PUFA and essential fatty acids could be ameliorated when they distributed at the *sn*-2 position and MC-SFA at the *sn*-1,3 positions, in infants with malabsorption and cystic fibrosis syndromes [24].

3.3 Glycerides profiles

Precursor ion $[M+NH_4]^+$ and fragment ion $[M+H-R_{1,2,3}COOH]^+$ with accurate mass were used to calculate glyceride molecular formula and infer individual fatty acid on the glyceride

molecules, respectively. As presented in Table S1 and Table 2, glycerides molecular species in GMF were found to be 359 TAGs and 27 diacylglycerols (DAGs), corresponding to the documented glycerides numbers [8, 25, 39]. There was no distinction between the positions of *sn*-1, *sn*-2, and *sn*-3 in the identified glycerides molecules.

As shown in Table S1, the total acyl carbon number (ACN) of the TAGs identified in goat milk ranged at 36 to 62, and the double bond (DB) number was at 0 to 8. Goat colostrum, transitional and mature milk had the same TAG species but with different relative contents. PMCy+MCaM (ACN:DB being 38:0) was the most abundant saturated TAGs in goat milk, and its content increased significantly with prolonged lactation from 5.63% to 7.37% of total TAGs. Some previous studies on GMF also observed the highest percentage when the ACN of the TAGs was 38 or 40 [8, 30, 39-40]. However, Marziali et al. [33] observed the maximum content when the ACN was 52 in goat milk. A total of 57 TAGs containing short-chain SFA (C4:0 or C6:0) were detected in GMF, and BuPO (4:0/16:0/18:1) was the richest monounsaturated TAGs in goat colostrum and mature milk. Moreover, Bu (C4:0) was mainly distributed at the positions of *sn*-1,3 according to the compositions and distribution of fatty acid (Table 1). The DAGs in goat milk were determined with ACN at 20~38 and DB at 0~5, and there were 19 species of DAGs formed of palmitic acid or oleic acid (Table 2). There were 7 species of main DAGs (>5% of total DAGs) in goat colostrum, of which P-O (16:0-18:1 diacylglycerols) was the most copious and its relative content displayed a lactation-dependent attenuation.

As shown in Figure 1A, monounsaturated TAGs were rich in GMF and increased obviously from 39.68% to 44.70% during lactation. The maximum contents of unsaturated TAGs were achieved at the transitional stage. Figure 1B shows the molecular weight distribution of the

256 dominant TAGs in goat milk. All individual saturated or monounsaturated TAGs containing 38~44
257 even-numbered acyl carbon and 0~1 double bond, took the dominant proportions in goat milk.
258 Furthermore, the levels of those TAGs were increased markedly from goat colostrum to mature
259 milk. Meanwhile, the TAGs constituted with 38~46 even-numbered acyl carbon and two double
260 bonds reached the highest contents in transitional milk, but there was no significant difference
261 between colostrum and mature milk. As the major TAGs in Finnish and Chinese human milk [41],
262 TAGs with ACN:DB being 52:2 was the foremost polyunsaturated TAGs in goat milk, accounting
263 for more than 3% of total TAGs. Specifically, the total contents of POO (16:0/18:1/18:1) and PSL
264 (16:0/18:0/18:2) in goat milk showed few fluctuations from colostrum to transitional period,
265 following whereas an obvious decrease from transitional to mature stage, which went along with
266 the trends of total polyunsaturated TAGs in goat milk (Table S1 and Figure 1A). Interestingly, the
267 content of main individual saturated TAGs gradually reduced as the ACN from 38 to 50 in goat
268 colostrum as well as transitional and mature milk, demonstrating that goat milk contained more
269 saturated TAGs with low molecular weights. This corresponded to the high levels of short- and
270 medium-chain fatty acids in goat milk, favoring the treatment of metabolic disorders, bone
271 demineralization and anaemia [7].

272 **3.4 Phospholipids profiles**

273 The method commonly applied for PLs evaluation in mammalian milk was HPLC in
274 combination with either an evaporative light-scattering detector or mass spectrometry. Studies on
275 the dynamic changes of the goat milk PLs during lactation analyzed by HR-MS/MS were still
276 scarce. Table S2 shows the types of PLs and their relative contents measured according to the
277 corresponding extracted ions in GMF. A total of 10 classes of PLs were detected in negative ion

mode in this study (detected as $[M+FA-H]^-$ or $[M-H]^-$ ions, where FA is formic acid), including 50 phosphatidylethanolamine (PE), 46 phosphatidylcholine (PC), 32 sphingomyelin (SM), 8 phosphatidylserine (PS), 27 phosphatidylinositol (PI), 3 phosphatidylglycerol (PG), 3 phosphatidic acid (PA), 8 lyso-phosphatidylcholine (LPC), 8 lyso-phosphatidylethanolamine (LPE), and 4 lyso-phosphatidylinositol (LPI). The main PLs species in the respective classes in goat colostrum were PE (18:1-18:1), PC (16:0-18:1), SM (d34:1), PS (18:0-18:1), PI (18:0-18:1), PG (16:0-18:2), PA (18:0-18:1), LPC (16:0), LPE (18:1) and LPI (18:2), respectively. This correlated well with the results that C16:0 and C18:1 were the foremost saturated and unsaturated fatty acids in GMF separately, corresponding to the findings of Russo et al. [42] Figure 2A shows the changes of those main PLs species with lactation stages except PG (16:0-18:2). Most of PLs showed obvious variation during the lactation. For instance, the relative level of SM (d34:1) in goat colostrum was more than 1.5 times that in mature milk, while PE (18:1-18:1) showed the opposite changing trend.

PLs compositions in goat colostrum, transitional and mature milk are displayed in Figure 2B. Similar to other mammalian milk lipids, five major categories of PLs in GMF were identified as PE, PC, SM, PI and PS. These accounted for more than 90% of the total PLs and PE was with the highest proportion, followed by PC, which was in agreement with the previous studies [30, 43-44]. However, some researchers observed that PC was predominant in PLs in GMF [8, 33, 45]. This discrepancy might be relevant to factors such as goat breeds and diets. The relative contents of PC and PS were not affected by lactation periods, while PE and PI increased remarkably from goat colostrum to mature milk. SM in goat colostrum accounted for 21.2% of total PLs, and then decreased to 13.1% and 11.4% in transitional and mature milk, respectively. The high proportion

of SM in colostrum implied its potential on promoting brain myelination and neurotransmitter generation at early infancy as well as anti-cancer, bacteriostatic and cholesterol-lowering [46]. In addition, three types of lyso-phospholipids (LPC, LPE and LPI), served as intracellular signaling molecules and membrane phospholipid metabolites [47], reached the maximum levels (4.42%, 3.09% and 0.44%, respectively) at transitional stage. In a word, the phospholipids profiles showed an obvious lactation-dependence, presenting as the highest contents of PE and PI but the lowest proportion of SM and lyso-phospholipids at mature stage.

3.5 Cholesterol comparation

Cholesterol is not only an essential component for cell membranes, steroid hormones and bile acids synthesis, but also a key factor for the levels of other lipids like sphingomyelin, and the development of the central nervous system [27, 48]. Thus, the levels of cholesterol and total lipids of goat milk were detected and listed in Table 3. Notably, the cholesterol levels in goat milk decreased sharply from 171.68±10.80 to 64.20±7.14 μg/mL throughout the lactation periods (P<0.05), corresponding to the downward trends of SM contents presented in Figure 2B. This consistency might be interpreted by the presence of lipid domains, formed tightly by cholesterol and SM in the liquid-ordered phase (lipid raft) in biological membranes [49]. Interestingly, the contents of total lipids in goat milk were also observed to descend markedly from colostrum to mature milk. These downtrends might be relevant to the dilution effect caused by increased milk volume and the decreased fat mobilization that reduced the availability of plasma non-esterified fatty acid for mammary lipid synthesis [33]. On the other hand, the highest proportion of cholesterol in total lipids (6.69±0.42 mg g^{-1}) occurred at colostrum stage, which might be explained by the elevated expression of mammary gland enzymes related to the synthesis and transport of cholesterol and

lipid [50]. Certainly, the high level of cholesterol in infants was beneficial to lessen the risk of cardiovascular disease in future adult life via regulating long-term cholesterol metabolism [51].

3.6 Milk fat globules

It has been reported that goat milk has larger scale of small milk fat globules compared with other mammalian milk [3]. Herein, we also investigated the structural characteristics of milk fat globules in goat milk under different lactations. As shown in Table 3, the average diameter of goat milk fat globules maintained at approximately 800 nm during all investigated lactation (P>0.05), but the size was smaller than the results of Yao et al. [8] This might be attributed to the difference of the milk samples properties (such as breed and genetics, etc.) and drying treatment, as well as the measurement and calculation employed for the fat globules size. The neglectable fluctuation of milk fat globules diameter among lactation stages might be associated with the stable ratios of lipid and protein, signifying the balance between protein secretion and lipid secretion [52]. Notably, the change of lactation periods also had slight effect on the surface charge of fat globule, demonstrating its good electrical stability in a colloidal system.

The microstructure of goat milk fat globules was further observed by CLSM. As depicted in Figure 3a-c, regardless of the varied lactation, fat globules dispersed uniformly in goat milk with a spherical structure. Also, no obvious variation of particle size was observed in the fat globules during lactation periods, which was in accordance with above particle size results. Additionally, after labelling the polar lipids in membrane of fat globules with Rh-DOPE fluorescence, we found the emission fluorescence was distributed at fat globules periphery in the form of green rings, and the interior (mainly TAGs) was not marked by the probe (Figure 3a'-c'). In goat milk from the same lactation stage, some fat globules were integrally dyed by Rh-DOPE, while the non-

fluorescent domains were also observed around other fat globules (as indicated by white arrows). These areas were associated with preferential accumulation of SM resulting in its lateral segregation from the glycerophospholipids in the plane of fat globule membrane [49]. Furthermore, in milk from three different lactations, different size of SM-rich domains in circular and irregular shapes were observed. This could be due to the difference in SM and cholesterol contents as well as SM/cholesterol ratio between goat colostrum, transitional and mature milk [8, 49]. More importantly, those SM-rich domains could potentially influence digestion of milk fat, and the interaction with gut pathogens and viruses [8].

4. Conclusions

The lactation stages had considerable effects on the physicochemical characteristics of goat milk, particularly the lipid profiling. We identified a total of 359 species of TAGs, 27 species of DAGs and 10 classes of PLs in goat milk by HR-MS/MS. Among three different lactation stages (colostral, transitional and mature stages), goat transitional milk showed the highest levels of MUFA (29.51%) and lyso-phospholipids (7.95% of total PLs), as well as the individual polyunsaturated TAGs containing 38~46 even-numbered acyl carbon and two double bonds. Unlikely, the levels of all individual TAGs composed of 38~44 even-numbered acyl carbon and 0~1 double bond (such as PMCy+MCaM and BuPO), PE and PI increased significantly over the investigated period from goat colostrum to mature stage. On the contrary, a lactation-dependent attenuation was found at the levels of PUFAs in goat milk, especially LC-PUFA ω-6 and the PSL+POO TAGs, which decreased obviously from colostrum to mature periods. Moreover, the relative contents of SM and cholesterol in goat colostrum were approximately twice and three times that in mature milk, respectively. Interestingly, the fat globule morphology of goat milk

showed no obvious variation during different lactation periods. Therefore, our results demonstrated the physicochemical changes of goat milk during three stages of lactation, particularly the lipid profiles and structure, providing a vital supplement for the lipid database of goat milk and guiding significance for its appropriate implement in infant and elderly nutrition.

ASSOCIATED CONTENT

Supporting Information

Composition and relative content (%) of TAGs identified in goat colostrum, transitional and mature milk; Identified PLs molecular species and their relative content (% of total PLs) in goat colostrum, transitional and mature milk.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ACN, acyl carbon number; BuPO, 4:0/16:0/18:1 triacylglycerols; CLA, conjugated linoleic acid; CLSM, confocal laser scanning microscopy; DAGs, diacylglycerols; DB, double bond; FAMEs, fatty acid methyl esters; GMF, goat milk fat; HR-MS/MS, high resolution tandem mass spectrometry; HPLC, high-performance liquid chromatography; LC-PUFA, long-chain

393 polyunsaturated fatty acids; LC ω-6/ω-3, LC-PUFA ω-6/ LC-PUFA ω-3; LA/LNA, C18:2 ω-
394 6/C18:3 ω-3; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; LPI, lyso-
395 phosphatidylinositol; MUFA, monounsaturated fatty acids; MC-SFA, medium-chain saturated
396 fatty acids; MCaM, 14:0/10:0/14:0 triacylglycerols; ONSFA; odd-numbered saturated fatty acids;
397 PUFA, polyunsaturated fatty acids; PLs, phospholipids; PMCy, 16:0/14:0/8:0 triacylglycerols;
398 PSL, 16:0/18:0/18:2 triacylglycerols; POO, 16:0/18:1/18:1 triacylglycerols; P-O, 16:0-18:1
399 diacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol;
400 PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; Rh-DOPE, N-
401 (Lissamine rhodamine B sulfonyl) dioleoyl-phosphatidylethanolamine; SPE, solid phase
402 extraction; SFA, saturated fatty acids; SM, sphingomyelin; TAGs, triacylglycerols; TFA, *trans*
403 fatty acid; UPLC-MS/MS, ultraperformance liquid chromatography-tandem mass spectrometry.

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Table 1 Fatty acid composition and positional distribution of goat milk fat from colostrum, transitional and mature milk [a]

Fatty acids [b]	TAGs			*sn*-2		
(%)	Colostrum	Transitional	Mature	Colostrum	Transitional	Mature
C4:0	0.06±0.05	0.02±0.01	0.04±0.01	ND	ND	ND
C6:0	1.11±0.51	1.50±0.09	1.44±0.18	1.38±0.12	1.36±0.02	1.42±0.11
C8:0	2.47±0.03 b	2.43±0.04 b	2.68±0.00 a	2.42±0.62	2.38±0.12	2.62±0.02
C10:0	4.95±1.17	5.14±0.31	6.23±0.16	4.19±0.45	5.00±0.17	4.92±1.59
C11:0	0.06±0.01 ab	0.04±0.00 b	0.07±0.00 a	0.09±0.00 B	0.10±0.00 A	ND C
C12:0	4.73±0.53 ab	4.25±0.19 b	5.62±0.11 a	2.94±1.02	4.28±0.10	4.27±0.12
C13:0	0.07±0.01 a	0.05±0.00 b	0.07±0.00 a	0.03±0.01 B	0.04±0.00 B	0.09±0.02 A
C14:0	14.54±0.52 a	10.30±0.30 c	12.06±0.18 b	12.26±4.11	13.81±0.44	12.18±0.94
C14:1 ω-5	0.31±0.02 a	0.19±0.01 b	0.23±0.01 b	0.19±0.06	0.19±0.01	0.15±0.01
C15:0	0.84±0.01 a	0.61±0.02 c	0.77±0.01 b	0.56±0.17	0.64±0.02	0.57±0.07
C15:1 ω-5	0.19±0.00 a	0.13±0.00 c	0.18±0.00 b	0.43±0.05	0.08±0.01	0.28±0.22
C16:0	32.64±0.90 a	28.98±0.41 b	32.18±0.20 a	36.93±4.34	35.97±0.31	35.76±0.51
C16:1 ω-7	0.75±0.20	0.62±0.01	0.64±0.24	0.44±0.02	0.60±0.00	0.46±0.10
C17:0	0.61±0.02 a	0.65±0.03 a	0.51±0.02 b	0.31±0.06 B	0.64±0.07 A	0.35±0.04 B
C17:1 ω-7	0.39±0.00	0.43±0.09	0.34±0.03	1.09±0.09 A	1.05±0.01 A	0.50±0.12 B
C18:0	7.87±0.72 c	12.91±0.10 a	11.26±0.05 b	11.44±0.08	12.93±0.33	10.74±2.14
C18:1(T)	0.60±0.15	0.29±0.01	0.37±0.15	0.50±0.12	0.33±0.03	0.30±0.03
C18:1 ω-9	23.85±1.50 b	27.80±0.35 a	22.28±0.82 b	20.42±2.23	17.45±0.07	20.47±4.42
C18:2(T)	0.30±0.10	0.18±0.01	0.17±0.01	0.06±0.05	0.04±0.02	0.05±0.02
C18:2 ω-6	2.53±0.17	2.62±0.57	2.29±0.06	2.94±0.10	2.22±0.16	4.10±1.40
C20:0	0.07±0.00	0.08±0.00	0.06±0.00	ND	ND	ND
C18:3 ω-6	0.46±0.03 a	0.37±0.02 b	0.28±0.01 c	0.12±0.03	0.11±0.01	0.09±0.03
C20:1 ω-9	0.17±0.03	0.13±0.06	0.14±0.06	0.37±0.11	0.32±0.01	0.31±0.01
C18:3 ω-3	0.12±0.05	0.10±0.04	0.10±0.00	0.10±0.02	0.11±0.08	0.08±0.02
C20:2 ω-6	0.02±0.01	0.02±0.00	0.02±0.01	0.02±0.01 B	ND C	0.04±0.00 A

C22:0	0.03±0.01 a	0.02±0.00 b	0.03±0.00 ab	ND	ND	ND
C20:3 ω-6	0.29±0.01 a	0.17±0.01 b	0.14±0.00 c	0.22±0.02 A	0.11±0.01 B	0.08±0.01 B
C20:3 ω-3	0.06±0.02	0.05±0.02	0.05±0.00	0.11±0.06	0.07±0.01	0.10±0.02
C20:4 ω-6	0.34±0.19	0.11±0.05	0.05±0.01	0.42±0.09 A	0.18±0.03 B	0.06±0.00 B
C23:0	0.08±0.01 a	0.04±0.01 b	0.02±0.00 b	0.03±0.01 A	ND B	ND B
C24:0	ND b	0.02±0.00 a	0.03±0.00 a	ND	ND	ND
C20:5 ω-3	0.05±0.00 a	0.01±0.00 b	0.01±0.00 b	ND	ND	ND
C22:6 ω-3	0.03±0.00 a	0.02±0.00 ab	0.01±0.00 b	ND	ND	ND
SFA	69.72±1.09 ab	66.86±1.18 b	72.79±0.65 a	72.58±2.16	77.15±0.10	72.92±5.58
SC-SFA	1.16±0.55	1.52±0.08	1.48±0.19	1.38±0.12	1.36±0.02	1.42±0.11
MC-SFA	26.66±2.22 a	22.14±0.75 b	26.63±0.48 a	21.93±4.40	25.61±0.63	24.09±2.69
LC-SFA	41.89±1.68	43.20±0.51	44.68±0.35	49.27±2.96	50.18±0.55	47.42±2.77
MUFA	26.11±1.18 b	29.51±0.50 a	24.08±0.59 b	23.43±2.41	20.03±0.03	22.48±4.14
PUFA	4.20±0.09	3.65±0.68	3.14±0.06	3.99±0.25	2.83±0.07	4.60±1.44
PUFA ω-3	0.25±0.07	0.18±0.06	0.18±0.00	0.21±0.08	0.18±0.09	0.18±0.04
LC-PUFA ω-3	0.14±0.03	0.08±0.02	0.08±0.00	0.11±0.06	0.07±0.01	0.10±0.02
PUFA ω-6	3.65±0.06	3.29±0.63	2.79±0.07	3.73±0.22	2.61±0.18	4.37±1.43
LC-PUFA ω-6	0.65±0.21 a	0.31±0.04 ab	0.21±0.02 b	0.67±0.09 A	0.29±0.02 B	0.18±0.00 B
ω-6/ω-3	15.01±3.23	18.89±2.79	15.73±0.25	19.48±4.71	17.10±7.04	24.30±1.88
LC ω-6/ω-3	4.93±1.73	3.70±0.32	2.71±0.28	7.29±2.42 A	4.09±0.91 AB	1.87±0.29 B
LA/LNA	22.01±1.51	27.40±5.97	23.15±0.16	30.25±3.42 B	30.18±1.41 B	50.02±2.14 A
ARA/DHA	10.89±0.37 a	6.76±0.12 b	3.63±0.15 c	ND	ND	ND
TFA	0.89±0.25	0.47±0.00	0.54±0.14	0.55±0.12 A	0.37±0.03 B	0.35±0.04 B

[a] Different lowercase letters (a, b, c) and uppercase letters (A, B, C) in the same row, represent significant differences in fatty acid of TAGs and *sn*-2 fatty acid, respectively, among three lactation stages ($P<0.05$). ND is not detected.

[b] Abbreviations are: SFA, saturated fatty acids; SC-SFA, short-chain SFA; MC-SFA, medium-chain SFA; LC-SFA, long-chain SFA; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain PUFA; ω-6/ω-3, PUFA ω-6/PUFA ω-3; LC ω-6/ω-3, LC-PUFA ω-6/ LC-PUFA ω-3; LA/LNA, C18:2 ω-6/C18:3 ω-3; ARA/DHA, C20:4 ω-6/C22:6 ω-3; TFA, *trans* fatty acid.

Table 2 Composition and relative content (%) of diacylglycerols (DAGs) identified in goat colostrum, transitional and mature milk [a]

Num-ber	Retention time (min)	$[M+NH_4]^{+}$; [b]	Formula	ACN: DB	DAGs [c]	Mass Error (ppm)	Colostrum	Transitional	Mature
1	4.10	442.3532	$C_{25}H_{44}O_5$	22:2	Bu-L	1.2	0.09±0.00 c	0.29±0.02 a	0.26±0.01 b
2	4.35	418.3521	$C_{23}H_{44}O_5$	20:0	Ca-Ca	-1.3	0.08±0.01 b	0.31±0.03 a	0.28±0.02 a
3	4.59	444.3683	$C_{25}H_{46}O_5$	22:1	Bu-O	-0.1	0.32±0.03 c	1.15±0.10 a	0.81±0.02 b
4	4.67	470.384	$C_{27}H_{48}O_5$	24:2	Co-L	-0.1	0.13±0.00 b	0.29±0.03 a	0.27±0.03 a
5	4.94	446.3837	$C_{25}H_{48}O_5$	22:0	Ca-La	-0.7	0.39±0.01 b	0.95±0.03 a	0.99±0.02 a
6	5.09	472.3998	$C_{27}H_{50}O_5$	24:1	Co-O	0.2	0.47±0.01 c	1.07±0.01 a	0.80±0.01 b
7	5.18	498.4154	$C_{29}H_{52}O_5$	26:2	Cy-L	0.3	0.20±0.01 c	0.29±0.01 a	0.26±0.01 b
8	5.48	474.4148	$C_{27}H_{52}O_5$	24:0	Ca-M	-1.0	0.89±0.01 c	1.36±0.07 b	1.54±0.01 a
9	5.59	500.4306	$C_{29}H_{54}O_5$	26:1	Cy-O	-0.7	0.78±0.01 c	1.29±0.05 a	1.08±0.02 b
10	5.71	526.4462	$C_{31}H_{56}O_5$	28:2	Ca-L	-0.7	0.59±0.02 b	0.80±0.05 a	0.85±0.03 a
11	6.09	502.4469	$C_{29}H_{56}O_5$	26:0	Ca-P	0.6	7.57±0.20 a	4.61±0.26 c	5.77±0.11 b
12	6.19	528.462	$C_{31}H_{58}O_5$	28:1	Ca-O	-0.4	2.36±0.02 c	3.46±0.04 a	3.20±0.07 b
13	6.82	530.4781	$C_{31}H_{60}O_5$	28:0	La-P	0.5	3.19±0.05 b	2.95±0.10 c	3.84±0.10 a
14	6.92	556.493	$C_{33}H_{62}O_5$	30:1	La-O	-0.9	3.01±0.03 b	3.48±0.04 a	3.46±0.04 a
15	7.10	582.5089	$C_{35}H_{64}O_5$	32:2	M-L	-0.5	1.62±0.02 a	1.43±0.04 b	1.57±0.03 a
16	7.34	608.5253	$C_{37}H_{66}O_5$	34:3	P-Ln	0.8	0.72±0.05 a	0.47±0.05 b	0.44±0.05 b
17	7.67	558.5093	$C_{33}H_{64}O_5$	30:0	M-P	0.2	8.39±0.01 a	6.04±0.16 c	7.90±0.10 b
18	7.79	584.5249	$C_{35}H_{66}O_5$	32:1	M-O	0.1	8.35±0.05 a	8.06±0.13 b	7.88±0.19 b
19	7.86	660.556	$C_{41}H_{70}O_5$	38:5	P-Dp	-0.2	0.98±0.04 a	0.60±0.03 b	0.40±0.03 c
20	8.00	610.5407	$C_{37}H_{68}O_5$	34:2	P-L	0.4	5.03±0.07 a	4.46±0.16 c	4.69±0.08 b
21	8.11	636.5566	$C_{39}H_{70}O_5$	36:3	O-L	0.7	3.01±0.06 b	3.27±0.04 a	2.75±0.15 c
22	8.16	572.525	$C_{34}H_{66}O_5$	31:0	Pa-P	0.3	0.61±0.04 b	0.58±0.00 b	0.70±0.02 a
23	8.64	586.5406	$C_{35}H_{68}O_5$	32:0	P-P	0.1	13.05±0.38 a	10.14±0.32 b	13.74±0.53 a
24	8.76	612.5564	$C_{37}H_{70}O_5$	34:1	P-O	0.5	20.30±0.16 a	19.15±0.27 b	18.14±0.31 c
25	8.89	638.5724	$C_{39}H_{72}O_5$	36:2	O-O	1.0	8.36±0.34 b	11.14±0.17 a	7.62±0.11 c
26	9.70	614.5726	$C_{37}H_{72}O_5$	34:0	P-S	1.3	4.76±0.07 a	5.89±0.15 b	5.89±0.07 b
27	9.83	640.588	$C_{39}H_{74}O_5$	36:1	S-O	0.8	4.73±0.12 b	6.49±0.39 a	4.85±0.17 b

[a] Values are presented as mass% in the form of means ± standard deviation. Different letters in the same row indicate significant differences (P<0.05). ACN is acyl carbon number; DB is double bond.

[b] Values are experimental m/z data;

[c] Abbreviations are: Bu, butyric acid (C4:0); Co, caproic acid (C6:0); Cy, caprylic acid (C8:0); Ca, capric acid (C10:0); La, lauric acid (12:0); M, myristic acid (C14:0); Pa, Pentadecanoic acid (C15:0); P, palmitic acid (C16:0); S, stearic acid (C18:0); O, oleic acid (C18:1); L, linoleic acid (C18:2); Ln, linolenic acid (C18:3); Dp, docosapentaenoic acid (C22:5).

Table 3 Physico-chemical properties of goat colostrum, transitional and mature milk [a]

Parameters	Colostrum	Transitional	Mature
Total lipids (mg/mL)	25.67±1.50 a	24.98±0.16 a	22.38±0.14 b
Cholesterol (μg/mL)	171.68±10.80 a	116.62±4.85 b	64.20±7.14 c
Cholesterol/total lipids (mg/g)	6.69±0.42 a	4.67±0.19 b	2.87±0.32 c
Diameter (nm)	800.79±27.38	757.50±45.20	795.87±53.28
Zeta-potential (mV)	-26.84±0.96	-26.15±1.81	-26.15±2.85

[a] Values are presented as means ± standard deviation. Different letters in the same row indicate significant differences (P<0.05).

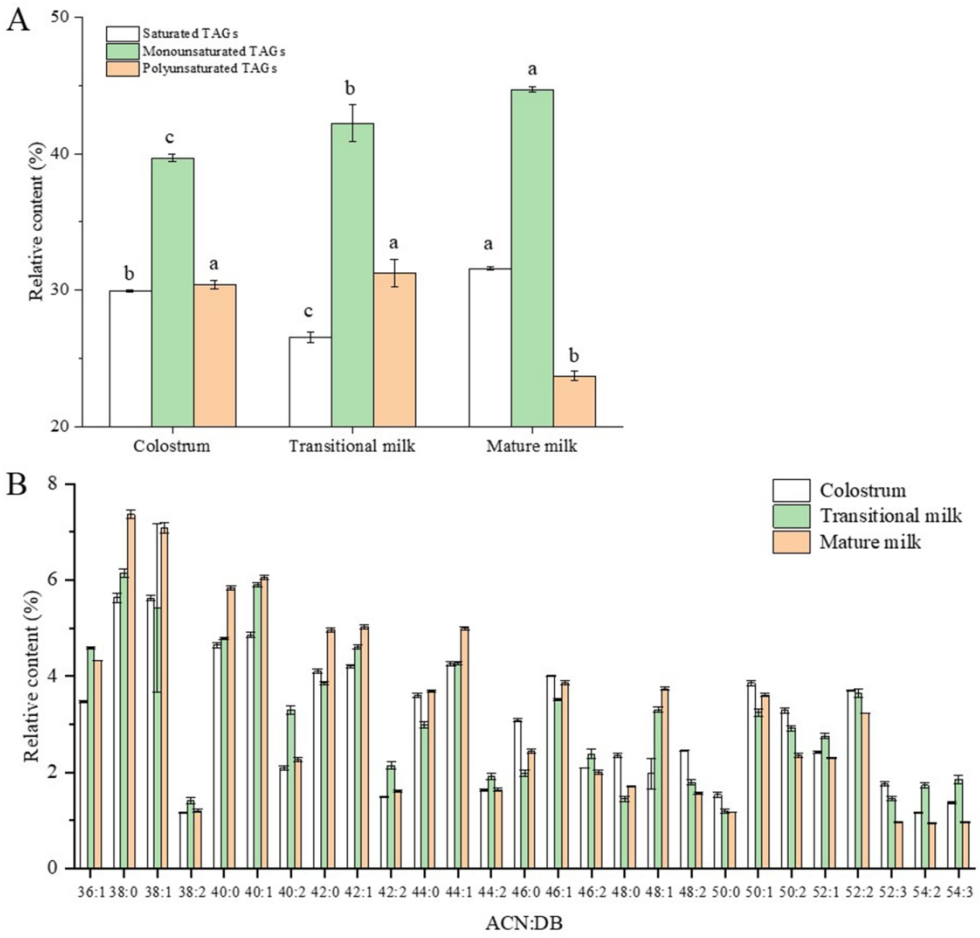

Figure 1. TAGs analysis of goat milk fat from different lactation stages. (A) Saturation of TAGs in goat milk. Different letters indicate significant differences at different lactation periods (P<0.05); (B) Molecular weight distribution of main TAGs (>1% of total TAGs) in goat milk, presented as ACN:DB (acyl carbon number: number of double bonds).

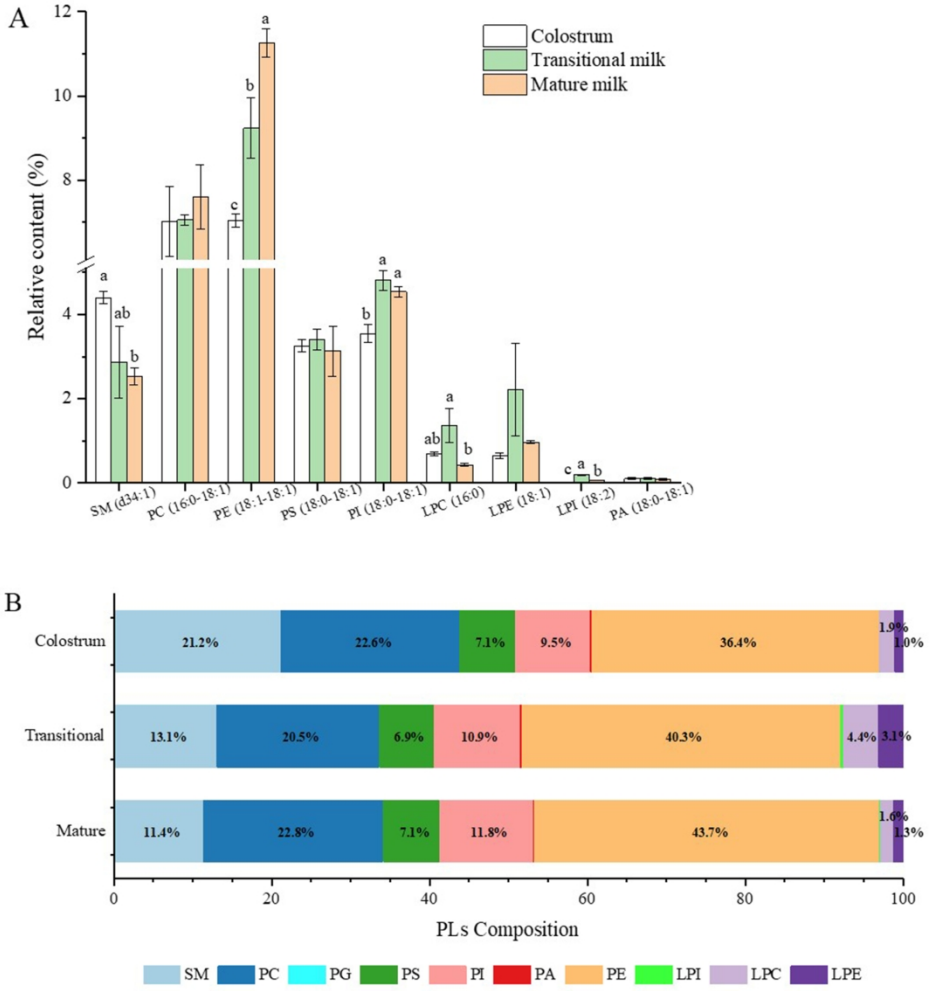

Figure 2. PLs analysis of goat milk fat from different lactation stages. (A) The main species in the respective PLs classes in goat colostrum. Different letters represent significant differences at different lactation periods (P<0.05); (B) PLs composition (%) in goat colostrum, transitional and mature milk.

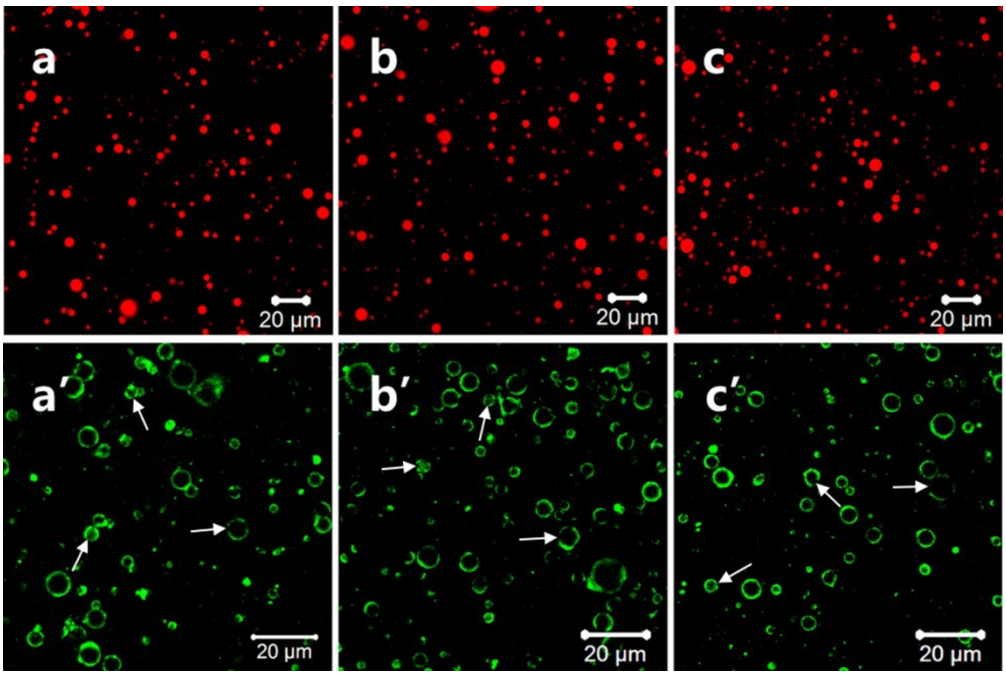

Figure 3. Microstructure of fat globules labelled by Nile red fluorescent (red) and Rh-DOPE fluorescent (green) in goat colostrum (a, a'), transitional (b, b') and mature milk (c, c') observed by CLSM (objective ×40; zoom ×1 and ×2).