ABSTRACT

Casein micelles (CM) play an important role in milk secretion, stability, and processing. The composition and content of milk proteins are affected by physiological factors, which have been widely investigated. However, the variation in CM proteins in goat milk throughout the lactation cycle has yet to be fully clarified. In the current study, milk samples were collected at d 1, 3, 30, 90, 150, and 240 of lactation from 15 dairy goats. The size of CM was determined using laser light scattering, and CM proteins were separated, digested, and identified using data-independent acquisition (DIA) and data-dependent acquisition (DDA)-based proteomics approaches. According to clustering and principal component analysis, protein profiles identified using DIA were similar to those identified using the DDA approach. Significant differences in the abundance of 115 proteins during the lactation cycle were identified using the DIA approach. Developmental changes in the CM proteome corresponding to lactation stages were revealed: levels of lecithin cholesterol acyltransferase, folate receptor α, and prominin 2 increased from 1 to 240 d, whereas levels of growth/differentiation factor 8, peptidoglycan-recognition protein, and 45 kDa calcium-binding protein decreased in the same period. In addition, lipoprotein lipase, glycoprotein IIIb, and α-lactalbumin levels increased from 1 to 90 d and then decreased to 240 d, which is consistent with the change in CM size. Protein–protein interaction analysis showed that fibronectin, albumin, and apolipoprotein E interacted more with other proteins at the central node. These findings indicate that changes in the CM proteome during lactation could be related to requirements of newborn development, as well as mammary gland development, and may thus contribute to elucidating the physical and chemical properties of CM.

Key words: goat milk, casein micelle, data-independent acquisition, lactation, proteomics

INTRODUCTION

Goat milk is a hypoallergenic food material with potential advantages for infants and patients with milk allergies (Zenebe et al., 2014). Goat milk protein is small and easily digested by pepsin, making it easy for humans to quickly absorb high-quality protein (Ye et al., 2019). Caseins are the major components (70–80%) of goat milk protein and can provide abundant amino acids and active peptides, including calcium phosphate nanocrystals, which contribute to various biological functions (O’Mahony and Fox, 2013; Rezaei et al., 2016). Casein gene expression in the mammary gland directly determines protein synthesis and content, as well as milk quality (Nongonierma and Fitzgerald, 2016). The composition of milk proteins is affected by several factors, including genetics, lactation, parity, and health status (Yang et al., 2020; Zhang et al., 2020a). Among these, the components of casein micelles (CM) in milk have received increased attention because of their structure and function (Holt et al., 2013).

Casein micelles exhibit an open, dynamic structure and a hydrophobic state in milk, allowing the incorporation of whey proteins into micelles. With the development of molecular biology techniques, proteomics approaches have considerably expanded the range of detectable proteins. For example, a previous study used a data-dependent acquisition (DDA)-based proteomics method to identify and quantify 82 proteins from 1-, 2-, 3-, 6-, and 12-month human CM (Liao et al., 2011). In another study, 193 distinct proteins were identified among ruminants (Holstein and Jersey cows, buffaloes, yaks, and goats) and nonruminants (camels and horses) using the liquid chromatography-tandem MS (LC-MS/MS) proteomics method (Wang et al., 2017).
addition, protein components of CM affected by heat treatment have been investigated (Han et al., 2021). Heat-dependent changes in the goat milk proteome were also investigated in a previous study (Chen et al., 2019). Although the specific proteins present in CM have been identified, variation in the CM proteome of dairy goats throughout the lactation period remains to be clarified.

Recently, a data-independent acquisition (DIA)-based proteomics approach was developed to fragment, detect, and acquire all precursor ions after the first-level full scan, compared with the DDA-based approach. The DIA method facilitates in-depth proteomics data with higher reproducibility and sensitivity, as well as a more extensive dynamic range (Bichmann et al., 2021). Data-independent acquisition–based proteomics has been widely applied to characterize protein components in several biological samples. For example, the whey proteins of buffaloes from different altitudes have been investigated, and several proteins related to altitude have been identified (Zhang et al., 2020a). In addition, changes in whey proteins incolostrum and mature milk from humans have been investigated using a DIA-based proteomics approach (Jin et al., 2021).

Therefore, we hypothesized that the proteomic characteristics of CM can be used to analyze the CM proteome and contribute to identifying proteins related to the lactation cycle of goats. To investigate changes in protein components of goat CM and the pattern of particle size throughout the lactation cycle, milk samples were collected from dairy goats, and the size and proteins of CM were identified using laser light scattering and DIA- and DDA-based quantitative proteomics approaches. The results of this study expand our knowledge of the milk proteome of goat CM and protein biosynthesis in the mammary gland during lactation and provide clues for exploring the specific physiological health requirements of the mother and offspring during lactation.

MATERIALS AND METHODS

Ethical approval was not required for this study because only routine animal procedures (milking) were conducted.

Sample Collection

In this study, goat milk samples were collected from the Qingdao Aotei goat farm in China in 2019. Fifteen dairy goats (parity 2) without clinical diseases were selected according to their veterinary records. The feed comprised whole peanut seedlings, corn, soybean meal, wheat bran, corn silage, stone powder, dicalcium phosphate, salt, and a premix. Morning milk samples were collected at 6 stages: 1 (within 24 h), 3, 30, 90, 150, and 240 d postpartum (Qin et al., 2021), generally corresponding to colostrum (1 d), transition milk (3 d), early (30 d), middle (90 and 150 d), and late (240 d) lactation stages. A total of 86 samples were obtained (samples from 4 goats were not collected because of insufficient milk at 240 d). Collected samples were stored at −20°C, transferred to the laboratory, and finally stored at −80°C.

Preparation of Skim Milk

After the milk samples were thawed, 5 individual milk samples from each lactation stage were combined into one fraction. This yielded 3 biological replicates for each lactation stage and 18 pooled samples for the 6 lactation timepoints. Whole milk was centrifuged at 4,000 × g at 4°C for 30 min. The top layer of milk fat was removed, and the liquid phase of skim milk was collected.

Milk CM Separation

Skim milk was ultracentrifuged at 100,000 × g for 60 min at 4°C to separate the caseins from the supernatant using a model L-80XP centrifuge (Beckman). The bottom of the casein precipitate was collected, washed 3 times with simulated milk ultrafiltrate to obtain CM, and stored at −80°C.

Size Distributions of CM

The particle sizes of CM were measured using the laser light scattering method. Skim milk samples obtained from different lactation periods were diluted with simulated milk ultrafiltrate at a volume ratio of 1:10. The CM particle size of the diluted milk samples was analyzed using a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd.), and 3 replicates were prepared for each sample.

SDS-PAGE

The CM were thawed and dissolved in 50 mM Tris-HCl (pH 7.4), with 4% SDS, sonicated for 5 min, and incubated in a 95°C water bath for 5 min. After the sample was dissolved entirely, a 12% separation gel and a 5% concentration gel were prepared; 20 μg of protein sample was mixed with a loading buffer and then placed in a 95°C water bath for 5 to 10 min. After the samples were cooled, the sample and protein marker (14.4–97.4 kDa) (Solarbio) were loaded and electrophoresed. Electrophoresis was performed at 80 V for 20 min and then
at 120 V for 60 min. The gels were then placed on a plate, fixed with 40% methanol and 10% ethanol, and stained with Coomassie brilliant blue G-250 solution. Finally, the gels were decolorized and imaged.

**Protein Digestion**

Casein micelle proteins (30 μg) from each lactation period were heated in a 50°C water bath for 30 min with 100 mM SDS-Tris-HCl and 100 mM dithiothreitol. After the samples were cooled, they were mixed with 200 μL of UT buffer (8 M urea and 100 mM Tris-HCl, pH 8.5), transferred into a filter tube (10-kDa cut-off, Sartorius), and centrifuged at 14,000 × g for 25 min. Subsequently, the samples were washed with UT buffer, mixed with 100 μL of 50 mM iodoacetamide solution, and incubated for 45 min in the dark at 25°C. After incubation, samples were centrifuged and washed. Finally, the samples were mixed with 100 μL of trypsin buffer (1 μg of sequencing-grade trypsin in 50 mM NH₄HCO₃) and incubated for 16 to 18 h at 37°C. The reaction was stopped by adding formic acid (FA). The filter was transferred to a new tube, centrifuged at 120 V for 60 min, and washed twice with 50 mM NH₄HCO₃. The filter was transferred to a new tube, centrifuged at 120 V for 60 min, and washed twice with 50 mM NH₄HCO₃. The eluates containing the peptides were pooled and desalted using a C18 column (60108-303; Thermo Fisher Scientific). The samples were dried in a speed vacuum and stored at −80°C.

**DDA and DIA Analyses by Nano-Liquid LC-MS/MS Analysis**

The dried tryptic peptides were resuspended in 0.1% FA and subjected to EASY-nLC 1000 coupled with Orbitrap Fusion Lumos (Thermo Fisher Scientific). The column was equilibrated with buffer A (0.1% FA). The peptides were loaded onto a C18 trap column (100 μm × 20 mm, 5 μm; Thermo Fisher Scientific) using an autosampler and separated using a C18 analytical column (75 μm × 150 mm, 3 μm; Thermo Fisher Scientific) at a flow rate of 300 nL/min. The separation gradient was as follows: buffer B (80% acetonitrile and 0.1% FA) from 4 to 10% within 5 min, from 10 to 30% within 58 min, from 30 to 40% within 9 min, from 40 to 100% within 8 min, and finally, holding at 100% for 10 min.

For DDA analysis, mass spectrometry was performed in positive ion mode with a parent ion scanning range of 395 to 1,205 m/z. The MS parameters were as follows: (1) MS: resolution, 60,000; AGC target, 2e6; and maximum injection time, 100 ms; (2) high-energy collisional dissociation-MS/MS: resolution, 15,000; AGC target, 50,000; maximum injection time, 50 ms.

For DIA analysis, MS was performed in positive ion mode with a parent ion scanning range of 395 to 1,205 m/z. The MS parameters were as follows: (1) MS: resolution, 60,000; AGC target, 2e6; and maximum injection time, 100 ms; (2) high-energy collisional dissociation-MS/MS: resolution, 15,000; AGC target, 1e6; collision energy, 30 eV; (3) a DIA using an isolation width of 26 Da (containing 1 Da for the window overlap) and 32 overlapping windows was constructed covering the precursor mass range of 400 to 1,200 Da for DIA acquisition according to a previous study (Jin et al., 2021).

**Protein Identification and Quantification**

The DDA raw files were analyzed using MaxQuant software (version 2.0.3.0; https://www.maxquant.org/) to search the UniProt database (46,754 entries of Bos taurus; 35,479 entries of Capra hircus; downloaded in December 2020). The relevant parameters were as follows: digestion mode, trypsin/P specificity; maximum missed cleavages, 2; fixed carbamidomethyl modification of cysteine; and variable modifications of N-terminal acetylation and methionine oxidation. Protein and peptide identifications were achieved at a false discovery rate and peptide spectrum matching of 0.01. The conditions for matching between the runs were set as 0.7, match time window; 0.05, ion mobility; 20 alignment time window; and 1, alignment ion mobility. The identified proteins were quantified based on the abundance of razor peptides and unique peptides using a label-free quantitation workflow. The DIA raw files were also searched against the downloaded database using the MaxQuant software, as mentioned above. In addition, the spectral library was obtained according to the results of the DDA, and the other parameter settings were the same as those applied in the DDA procedure.

**Bioinformatics and Statistical Analysis**

The CM proteins with at least 2 identified peptides and all 3 runs of each studied group were selected and then imported into Perseus software (www.maxquant.org/perseus/). Hierarchical clustering, volcano plot analysis, principal component analysis (PCA), and statistical analysis of the quantified proteins among the studied groups were performed. Differentially abundant proteins were determined according to |log2fold change|
RESULTS

Size Distribution of CM in Goat Milk

As shown in Supplemental Table S1 (https://doi.org/10.17632/zrwnyjn5z9.1), CM size significantly increased from 1 (168.27 ± 3.43 nm) to 90 d (205.53 ± 2.37 nm; P < 0.05) and then significantly decreased at 240 d (155.13 ± 1.80 nm; P < 0.05). The average size of CM peaked at 90 d of lactation and was smallest at 240 d.

Statistical Analysis of CM Proteins

The SDS-PAGE gel with Coomassie blue staining is shown in Supplemental Figure S1 (https://doi.org/10.17632/zrwnyjn5z9.1). The bands of stained proteins were found to be consistent in the CM samples. Based on the DDA proteomics method, 253 proteins were identified in the CM fraction of goat milk throughout the lactation cycle, whereas 303 CM proteins were identified using DIA proteomics (Supplemental Table S2; https://doi.org/10.17632/zrwnyjn5z9.1). For the DIA data, 115 proteins were significantly different across lactation stages (Supplemental Table S3; https://doi.org/10.17632/zrwnyjn5z9.1). The abundance of several proteins, such as olfactomedin-like protein 3 (OLFML3), apolipoprotein A-I (APOA1), and apolipoprotein E (APOE) significantly decreased (P < 0.05) from 1 to 240 d of lactation. Lecithin-cholesterol acyltransferase (LCAT), folate receptor α (FOLR3), lactotransferrin, and fibronectin significantly increased from 1 to 240 d. In addition, we found that the abundance of lipoprotein lipase (LPL), glycoprotein IIIb (CD36), and α-LA increased from 1 to 90 d and then decreased to 240 d, which is consistent with the change in CM size.

To visualize the changes in CM proteins during the lactation stages, volcano plots comparing d 1 to d 3, 30, 90, 150, and 240 are shown in Figure 1. Of these, the number of differentially abundant proteins (35) was lowest between 1 and 3 d. The number of these proteins then increased by approximately 100 in the comparison of 1 and 30 d versus 240 d. We found that several proteins, such as APOA1, APOE, and apolipoprotein A-IV (APOA4), were decreased, whereas fibronectin, LCAT, and FOLR3 were increased on 1 d compared with those at 3 to 240 d. In addition, changes in the abundance of most CM proteins in goat milk throughout the lactation stages obtained from the DDA data were consistent with those obtained from the DIA data. Several of these differentially abundant proteins, such as ceruloplasmin, albumin, APOE, growth/differentiation factor 8 (MSTN), and fructose-bisphosphate aldolase (ALDOA) are shown in Figures 2a and b.

Clustering and PCA of Differentially Abundant CM Proteins

Clustering analysis of differentially expressed CM proteins in goat milk collected during the entire lactation stage is shown in Supplemental Figure S2 (https://doi.org/10.17632/zrwnyjn5z9.1). Samples at 30, 90, 150, and 240 d formed one subcluster, and those at 1 and 3 d formed another subcluster. The results of the clustering analysis of the DDA proteomics approach were consistent with those of the DIA proteomics approach.

The PCA of the quantified CM proteins from the 6 lactation stages in goat milk using the DIA method is shown in Figure 3a. According to the score plots, the protein profiles of CMs at 1, 3, 30, and 240 d were clearly separated from each other, whereas those at 90 and 150 d were not distinguished. Apparent changes in the CM proteome profile during the lactation stages were evident among the 1, 3, 30, 90/150, and 240 d groups. Principal component (PC)1 and PC2 could explain the difference in protein intensity at different lactation stages, as well as the total variance of 70%. The score plots of PCA based on the DDA data are shown in Figure 3b. In addition, loading plots of PCA from DIA and DDA data are presented in Figure 3, in which each plot shows a quantified protein. We found that several proteins of cysteine-rich secretory protein 3, biglycan, MSTN, and α-LA in the direction of PC1, and osteopontin, complement C3, and APOE in the direction of PC2 contributed to separating the different studied milk groups on the score plots.

GO and KEGG Analysis of Differentially Abundant CM Proteins

According to protein annotations, differentially abundant CM proteins identified in goat milk at 3 d, 30 d, 90 d, 150 d, and 240 d versus 1 d were classified according to biological processes, cellular components, and molecular functions. As shown in Figure 4, the most common biological processes were localization, developmental processes, response to stress, regulation
of biological quality, and immune system processes. The differential proteins were mainly located in the extracellular region and membrane-bound vesicles, and other proteins were located in the extracellular vesicle, cytoplasm, and extracellular exosome. The main molecular functions of the differentially expressed proteins were protein binding, calcium ion binding, and enzyme regulatory activity. In addition, the number of proteins related to most GO terms increased in the comparison of 3 d versus 1 d with 240 d versus 1 d.

The significantly enriched differentially abundant CMs proteins at 3, 30, 90, 150, and 240 d versus 1 d are shown in Table 1. The results showed that most of the differentially abundant CM proteins were involved in glycolysis/gluconeogenesis, carbon metabolism, biosynthesis of antibiotics, biosynthesis of amino acids, and

Figure 1. Volcano plots of differentially abundant casein micelle proteins from 1, 3, 30, 90, 150, and 240 d of dairy goats using data-independent acquisition (DIA)-based proteomics approach. FC = fold change.
phagosome. Several differentially abundant proteins were also related to vitamin digestion and absorption, regulation of the actin cytoskeleton, and bacterial invasion of epithelial cells.

**PPI of Differentially Abundant CM Proteins in Goat Milk**

The PPI networks of the differentially abundant CM proteins identified in goat milk during the lactation cycle were predicted using STRING software (Figure 5). The evidence of active PPI sources was selected from experiments, curated databases, and text mining, and the minimum required interaction score was set as a higher confidence (0.700). In the protein network of comparison of 1 and 3 d, APOA1, with more interactions than the other proteins, was considered a hub node. In the comparison network of 1 and 30 d, fibronectin and albumin interacted with 9 proteins, followed by APOE and ALDOA, which had more interactions than other proteins and were considered the central hub nodes. Most of the interactions presented were considered hub nodes. In the comparison between 1 and 90 d, fibronectin interacted with 12 proteins, followed by APOE, LPL, and α-1-antiproteinase, which had more interactions than other proteins and were considered central hub nodes. In the comparison between 1 and 150 d, fibronectin, APOE, and LPL were considered the central hub nodes. Albumin and APOE were considered the central hub nodes in the comparison between 1 and 240 d. Collectively, fibronectin, LPL, and APOE were considered the central proteins among the comparisons.

*Figure 2. Clustering analysis of differential abundant casein micelle proteins from 1, 3, 30, 90, 150, and 240 d of dairy goats using (a) data-independent acquisition (DIA), and (b) data-dependent acquisition (DDA)-based proteomics approaches.*
that were associated with ECM-receptor interaction, regulation of actin cytoskeleton, and lipid and cholesterol metabolism pathways.

**DISCUSSION**

In the current study, we mapped the proteome profile and size of CM in goats during lactation using DIA-based proteomics and laser light scattering approaches. In addition to caseins, many proteins, such as peptidoglycan-recognition protein (PGLYRP1), APOA1, and lactotransferrin, have been identified in the CM of goats. Several studies have investigated the protein components of CM in humans and dairy animals. More than 70 specific proteins present in CM have been found to depend on the type of milk, sample preparation, and detection technology (Wang et al., 2017). In addition, changes in several proteins in accordance with specific lactation stages have been reported (Liao et al., 2011). Of these, the levels of albumin, 45 kDa calcium-binding protein (SDF4), and APOA1/A4/E decreased from 1 to 240 d, whereas the levels of LCAT, FOLR3, and lactotransferrin increased from 1 to 240 d. In addition, levels of LPL, CD36, and α-LA increased from 1 to 90 d and then decreased to 240 d, which is consistent with the change in CM size. These findings provide a better understanding of the proteome of CM, which could be related to the requirements of newborns and formation of CM in the mammary glands.
Sun et al.: CHANGES IN CASEIN MICELLE PROTEINS DURING LACTATION

Figure 4. (a) Biological processes, (b) cellular components, and (c) molecular functions of differential abundant casein micelle proteins among the comparisons of d3, 30, 90, 150, and 240 versus d1 of dairy goats using a data-independent acquisition (DIA)-based proteomics approach.
We found that the levels of several proteins involved in transport, such as LCAT, FOLR3, and prominin 2, increased from 1 to 240 d. Lecithin-cholesterol acyltransferase catalyzes the transfer of fatty acids from 2n-lecithin to the 3-hydroxy group of free cholesterol to form cholesteryl esters and lysolecithin (Saeedi et al., 2015). A previous study found a significant elevation in LCAT on d 7 and 30 compared with the values obtained on d 0 in bovine serum (Khalphallah et al., 2016). High-density lipoprotein plays a pivotal role in reversing cholesterol transport to the liver. Folate receptor α (FRα) transports folate to the cytoplasm via endocytosis. The expression of FOLR1, the gene encoding FRα, is consistent with folate secretion into milk (Aboulhagag et al., 2018). In a previous study, a 5-fold increase in FRα from colostrum to mature milk was identified in yak samples using the iTRAQ-labeled proteomics approach (Yang et al., 2015). According to microarray analysis, FOLR1 gene expression was 12.7-fold higher in lactation than in late pregnancy in the bovine mammary gland and 2.4-fold higher in early lactation than in late lactation in the wallaby mammary gland. Folate receptor α is considered a regulator of milk protein synthesis (Menzies et al., 2009). As previously discussed, we speculated that FRα could be an important regulatory point of folate metabolism for the biosynthesis of milk protein in the mammary gland.

In addition, APOA1/A4/E, LDL receptor-related protein 2 (LRP2), and the nucleotide exchange factor SIL1 were found to decrease from colostrum to mature milk. The DIA proteomics approach has revealed that in human milk, APOA4 levels decreased from 1 to 6 mo (Jin et al., 2021). Using the LC-MS/MS proteomics approach, APOA4 and APOA1 were found to be higher in colostrum than in mature milk in cows (Zhang et al., 2015; Honan et al., 2020); APOA1 and APOA4 are involved in lipid metabolism. They are the main components of high-density lipoproteins along with APOA1, which can increase insulin synthesis. In addition, APOA4 is involved in many aspects of lipid metabolism, including the intestinal absorption of lipids, antioxidation of plasma lipoproteins, and enhanced cellular reverse cholesterol transport (Kohan et al., 2015; Hou et al., 2019). A previous study using a glycoproteomics approach found that LRP2 is higher in colostrum than in mature milk in human and bovine milk fat globule membrane (Cao et al., 2017); LRP2 can bind and internalize various ligands to systemic vitamin homeostasis and plays important roles in lipoprotein metabolism, cholesterol homeostasis, embryonic development, and immune response control (Aunderset et al., 2016). The high abundance of these proteins in colostrum involved

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### Table 1. Pathway analysis of differentially abundant casein micelle proteins from 3, 30, 90, 150, and 240 d versus d 1 of lactation in dairy goats using a data-independent acquisition-based proteomics approach

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<th>Percent</th>
<th>P-value</th>
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Figure 5. Protein–protein interactions of differential abundant casein micelles proteins from (a) 3 d versus 1 d, (b) 30 d versus 1 d, (c) 90 d versus 1 d, (d) 150 d versus 1 d, (e) and 240 d versus 1 d of dairy goats using a data-independent acquisition (DIA)-based proteomics approach. Each node represents a protein, and each edge represents the interaction between proteins. Red boxes represent increased central proteins; blue boxes represent decreased central proteins.
in transport might be related to the physiological needs and functions of goats and their newborns.

Other transport-related proteins such as CD36 and LPL increased from 1 to 90 d and then decreased to 240 d, which is consistent with the change in CM size. Lipoprotein lipase plays a central role in triglyceride metabolism, facilitates triglyceride utilization, and regulates the concentrations of triglyceride and triglyceride-rich lipoproteins (Arora et al., 2019). In small ruminants, the profiles of LPL gene and mRNA expression were found to be markedly high at 1 mo, decreasing thereafter in goat mammary glands (Zhao et al., 2014). Lipoprotein lipase has been identified in Holstein, buffalo, yak, goat, and camel, and its abundance is associated with the size of CM affected by heat treatment (Han et al., 2021). In addition, using the STRING software, we found that LPL interacted with APOE, APOA4, and APOA1. Interestingly, the inhibition of LPL to hydrolyze low density lipoprotein triglycerides was strongly correlated with APOE and, to a lesser extent, with APOA2. In addition, exogenous APOE inhibits LPL lipolysis in a dose-dependent manner (Whitacre et al., 2022). Thus, we suggest that changes in the abundance of LPL correlated with APOE for milk stability could be modulated by intrinsic physiological functions in the mammary gland. For example, CD36 is an intracellular docking site for fatty acid–binding proteins and facilitates membrane fatty acid transport. Apart from its role in lipid transport, CD36 is involved in phagocytosis, antigen presentation, and clearance of apoptotic cells (Cifarelli and Abumrad, 2018). A previous study found that CD36 increased from 0 to 30 d and then decreased to 250 d in yak milk (Lee et al., 2017). Another study found that CD36 can bind to casein using an inhibition ELISA approach (Zenker et al., 2021). In addition, CD36 has been identified as an N-glycoprotein in human and bovine colostrum and milk MFGM (Cao et al., 2019). As discussed previously, we suggest that these proteins increased from 1 to 90 d and then decreased to 240 d, which may be related to the formation of CM by interacting with caseins; however, this observation requires further investigation using a labeled protein assay.

**Differentially Abundant CM Proteins Involved in Developmental Processes**

Our study found that several proteins were related to growth and development, including MSTN, ALDOA, actin-depolymerizing factor (GSN), OLFML3, PGLYRP1, and α-actinin-4, which decreased from colostrum to mature milk. Growth/differentiation factor 8 or myostatin (MSTN) is a negative regulator of muscle growth that can increase the mass of mammalian skeletal muscle and play a role in regulating tissue glucose uptake (Meloux et al., 2019). According to dimethyl labeling with the LC-MS/MS proteomics approach, bovine milk was rich in MSTN for the first 9 d (Zhang et al., 2015). Fructose-bisphosphate aldolase (ALDOA) can promote the conversion of glucose to pyruvate, thereby providing energy for the growth and development of newborns (Chen et al., 2019). We also found that ALDOA was a hub node in PPI. A previous study found that ALDOA decreased in bovine milk from the early to the middle stage of lactation using a tandem mass tag–labeled proteomics approach (Mol et al., 2018). Interestingly, colostrum with a high abundance of ALDOA was observed in humans using filter-aided sample preparation and dimethyl-labeled proteomics technology (Zhang et al., 2016). Actin-depolymerizing factor (GSN) is a major constituent of embryonic and adult tissues and plays an important role in regulating actin filaments (Ono, 2018); GSN decreases from the early to late stages in the milk of the Indian indigenous cattle breed Malnad Gidda (Mol et al., 2018). Another study found that GSN levels decreased from 0 to 9 d in bovine milk (Zhang et al., 2015). Olfactomedin-like protein 3 (OLFML3) plays a special physiological role in embryonic development and can increase growth rate and modulate cytoskeletal organization, cell adhesion, and migration (Jin and Li, 2019). A study using tandem MS analysis showed that OLFML3 is expressed in goat milk (Pisanu et al., 2020). A study conducted using label-free proteomics technology found that, in bovines, OLFML3 increased from the end of lactation through d 21 of the dry period (Reinhardt and Lippolis, 2020). The high abundance of these proteins in colostrum may contribute to the growth and development of newborns.

**Differentially Abundant CM Proteins Involved in Metal Ion Binding**

The mineral fraction of milk is distributed between the soluble and colloidal phases of milk, where it interacts with caseins to form CM (Holt et al., 2013). Our study found that several proteins involved in metal ion binding, such as ceruloplasmin, albumin, calmodulin, and SDF4, decreased from 1 to 240 d. Ceruloplasmin is the main extracellular ferroxidase and Cu transporter. It plays an important role in the absorption, distribution, and accumulation of Cu in newborns. Ceruloplasmin was higher in colostrum (26.5 mg/L) than in mature milk (6.6 mg/L) in pigs using SDS-PAGE and immunoblotting (Cerveza et al., 2000). Moreover, the concentrations of ceruloplasmin were 150 ± 30 mg/L in human colostrum, decreasing to 40 ± 20 mg/L in mature milk (Puchkova et al., 2018). We also found
that albumin, with more interactions than other proteins in PPI, is a crucial protein for metal ion binding. Albumin is the major transporter of zinc, calcium, and magnesium in plasma. According to a study conducted using SDS-PAGE, albumin was identified in bovines and decreased by 12% from 0 to 30 d (Raimondo et al., 2019). In addition, 2-dimensional gel electrophoresis and MALDI TOF/TOF MS approaches show that albumin abundance decreased from colostrum to mature milk in sheep (Zhang et al., 2020b). Milk can provide all the calcium required to support rapid bone growth in neonates (Grinman et al., 2020). Most milk calcium is bound to CM and forms large, highly organized macromolecular micelles (VanHouten et al., 2007). In bovine milk, SDF4 was found in colostrum and mature milk in sheep (Zhang et al., 2020b). The high abundance of these proteins in colostrum, related to calcium and other metal ion transfers, could be beneficial to the health of neonates.

CONCLUSIONS

Changes in CM proteins during the lactation cycle in dairy goats were investigated for the first time using DIA-based proteomics approaches. Several proteins, such as APOA1/A4/E, MSTN, and SDF4 had the highest abundance in colostrum, and decreased from 1 to 240 d in lactation. Abundances of FOLR3, fibronectin, LCAT, κ-casein, and lactotransferrin increased from 1 to 240 d. Abundances of CD36 and LPL increased from 1 to 90 d and then decreased to 240 d. According to PPI analysis, fibronectin, LPL, albumin, and APOE, having more interactions than other proteins, were considered central hub proteins related to ECM–receptor interaction, regulation of actin cytoskeleton, and lipid and cholesterol metabolism pathways. These findings provide insight into the CM proteome and temporary changes in CM components that occur during the lactation cycle of dairy goats, and contribute to a better understanding of the physical and chemical properties of these specific proteins in CM.

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