Comparative analysis of changes in whey proteins of goat milk throughout the lactation cycle using quantitative proteomics

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ABSTRACT

The composition and content of goat milk proteins are affected by many factors and have been extensively studied. However, variation in whey protein composition in goat milk throughout the lactation cycle has not been clarified. In the current study, 15 dairy goats were selected, and milk samples were collected at 1, 3, 30, 90, 150, and 240 d after delivery. Whey proteins were separated and digested and then identified using data-independent acquisition (DIA) and data-dependent acquisition proteomics approaches. Protein profiles identified using DIA were consistent with those of the data-dependent acquisition proteomics approach according to clustering and principal component analyses. Significant differences in the abundance of 238 proteins around the lactation cycle were identified using the DIA approach. Developmental changes of the whey proteome corresponding to lactation stage were revealed: plasminogen, α-2-macroglobulin, and fibronectin levels decreased from d 1 to 240, whereas polymeric immunoglobulin receptor, nucleobindin 2, fatty acid-binding protein 3, and lactoperoxidase increased from d 1 to 240. Protein-protein interaction analysis showed that fibronectin with a higher degree of connectivity is a central node. The findings are of great significance to better understanding the potential role of specific proteins and the mechanism of protein biosynthesis or intercellular transport in the mammary glands related to the physiological changes of dairy goats.

Key words: goat, whey protein, data-independent acquisition, lactation, proteomics

INTRODUCTION

Milk is rich in nutrients, including proteins, fats, vitamins, and minerals. Although the essential components of protein, fat, and TS are similar in both goat and bovine milk, goat milk has lower αS1-casein content and is considered less allergenic than bovine milk (Duan et al., 2021). Several previous studies have shown that milk composition is affected by several factors, including species, health status, lactation, parity, and environment (Yang et al., 2020; Qin et al., 2021). Proteins, as key components of milk, and their physicochemical and functional properties have recently received increased attention (Qin et al., 2021). To investigate milk proteins’ physicochemical and functional properties, milk components should be explored. The results may further reveal the mechanism of protein biosynthesis and intercellular transport in the mammary glands (D’Alessandro et al., 2010).

With the development of molecular biology techniques, proteomics approaches provide a powerful tool for mapping protein profiles and investigating changes in colostrum and mature milk proteins (Yang et al., 2020). For example, several previous studies have outlined the changes in whey and milk fat globule membrane (MFGM) proteins during the lactation stages in human milk (Cao et al., 2017; Zhu et al., 2021), as well as the differences in whey proteomes from colostrum to mature milk in bovine milk (Yang et al., 2017; Mol et al., 2018). In addition, MFGM proteins from colostrum to mature milk in goats showed 189 proteins with significant differences, with the acute-phase proteins higher in colostrum than in mature milk (Lu et al., 2016). Subsequently, MFGM and whey from colostrum and mature goat milk were analyzed using LC-MS proteomics technology (Sun et al., 2020a,b). In another study, 2-dimensional gel electrophoresis MALDI-TOF MS technologies compared whey proteins from the first 56 d of lactation in Hu sheep. It was observed that 25 proteins were highly abundant in the first 7 d after lambing, and the expression level decreased to...
a minimum value at 56 d (Zhang et al., 2020c). However, the milk proteome during the lactation stage of dairy goats has received relatively little attention. It is worth noting that the proteome distribution is differently abundant in humans, cattle, camel, yak, and goat milk (Yang et al., 2013). It is important to explore the distribution, variation, and protein biosynthesis of the mammary gland of the milk proteome of dairy goats over a complete lactation cycle.

Recently, data-independent acquisition (DIA)-based proteomics has emerged as an alternative to data-dependent acquisition (DDA) in shotgun proteomics (Zhang et al., 2020a). Data-independent acquisition parallelizes the fragmentation of all detectable ions within a wide mass/charge (m/z) range, regardless of the intensity, thereby providing a broader dynamic range of the detected signals. This technology has improved the identification, reproducibility, sensitivity, and accuracy of such testing, potentially enhancing proteome-coverage capabilities (Bichmann et al., 2021; Weng et al., 2021). Data-independent acquisition-based proteomics has been widely applied to characterize protein components in blood, cells, and milk samples (Zhang et al., 2020b; Yu et al., 2021). For example, DIA-based proteomics has been used to investigate whey proteins from colostrum to mature milk in humans. Several proteins related to lactation stages, such as plasminogen, lactoferrin, and apolipoprotein A-IV have been identified (Jin et al., 2021).

Therefore, we hypothesized that proteomic characterization based on the DIA strategy would provide in-depth knowledge of the whey proteome throughout the lactation cycle of dairy goats. This study investigated the whey proteome of goat milk at 1, 3, 30, 90, 150, and 240 d using DIA- and DDA-based quantitative proteomics approaches. The results of this study contribute to enlarging the goat milk protein database, revealing the temporal changes in the goat whey proteome, and exploring the protein biosynthesis and transport of the mammary gland during various lactation stages.

**MATERIALS AND METHODS**

**Sample Collection**

The samples were collected from the Qingdao Aote Goat Farm of China. Fifteen healthy dairy goats with second parity and without clinical diseases were selected. The diet ingredients and components of goats during the lactation stages are listed in Supplemental Table S1 (https://doi.org/10.17632/n8n4s2sv9r.3; Sun, 2022). At 6 stages (1, 3, 30, 90, 150, and 240 d), 50 mL of milk was collected from the 15 goats. Eighty-six samples were obtained in total; samples from 4 goats were not collected due to insufficient milk on d 240. The dairy goats were housed freely, fed TMR diets twice daily at 0700 and 1630 h for ad libitum intake, and milked twice daily at 0600 and 1800 h. Samples on d 1 and 3 were obtained via manual milking, while the remaining samples were obtained via milking from both udders with a machine. After collection, the samples were stored at −20°C, transferred to the laboratory, and stored at −80°C. Only routine animal procedures (milking) were conducted in this study, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

**Separation of Milk Whey Proteins**

After the milk samples were thawed at 4°C, 5 individual milk samples from each lactation stage were combined into 1 fraction. This resulted in 3 biological replicates for each lactation stage and 18 pooled samples for the 6-lactation time point. The whole milk was first centrifuged at 4,000 × g and 4°C for 30 min. The top layer of milk fat was removed, and the liquid phase of skim milk was collected. The skim milk was then ultracentrifuged at 100,000 × g for 60 min at 4°C to separate the casein precipitates and the whey protein supernatant using the model L-80XP (Beckman Coulter). The supernatant containing whey proteins was collected and stored at −80°C. Protein concentrations in milk whey were determined using a BCA assay in which bovine serum albumin served as a standard.

**Polyacrylamide Gel Electrophoresis**

A 12% separation gel and a 5% concentration gel were prepared. Twenty micrograms of protein samples were mixed with loading buffer and placed in a 95°C water bath for 5 min. After the samples cooled, the sample and protein marker with 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. Next, the gel was placed on a plate, fixed with 40% methanol and 10% ethanol, and stained with Coomassie Brilliant Blue G-250 solution. Finally, the gels were incubated in distilled water until the background was colorless and imaged (Supplemental Figure S1; https://doi.org/10.17632/n8n4s2sv9r.3; Sun, 2022).

**Protein Digestion**

Thirty micrograms of whey proteins from each lactation time point were heated in 50°C water for 30 min with 30 mM 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 (Sartorius), and centrifuged at 14,000 × g and 25°C for 25 min. Subsequently, the samples were washed with UT buffer, mixed with 100 μL of 50 mM iodoacetamide solution, and then 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 tryptic buffer (1 μg sequencing grade trypsin in 50 mM NH₄HCO₃) and then incubated for 16 to 18 h at 37°C. Formic acid (FA) was added to stop the reaction. The filter was transferred to a new tube, centrifuged at 14,000 × g for 15 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.

Data-Dependent Acquisition and Data-Independent Acquisition Analysis

Dried tryptic peptides were resuspended in 0.1% FA and subjected to EASY-nLC 1,000 coupled with Orbitrap Fusion Lumos (Thermo Fisher Scientific). The column was equilibrated with buffer A (0.1% FA). Peptides were loaded onto a C18 trap column (100 μm × 20 mm, 5 μm; Thermo Fisher Scientific) using an autosampler and separated on 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 then 100% hold for 10 min.

For DDA analysis, MS was performed in positive ion mode with a parent ion scanning range of 300 to 1,800 m/z and automatic switching between MS and MS/MS acquisition. The parameters of MS were set as follows: (1) MS: resolution = 60,000, automatic gain control (AGC) target = 400,000, maximum injection time = 50 ms, and exclusion duration = 40 s. The top 20 most abundant precursor ions with a charge ≥2 from the MS scan were selected and fragmented by higher energy collisional dissociation with normalized collision energies of 27 eV. (2) high energy collisional dissociation (HCD)-MS/MS: resolution = 15,000, AGC target = 50,000, and maximum injection time = 50 ms.

For DIA analysis, MS was performed in positive ion mode with a parent ion scanning range of 395–1,205 m/z. The parameters of MS were set as follows: (1) MS: resolution = 60,000, AGC target = 2 × 10⁶, and maximum injection time = 100 ms; (2) HCD-MS/MS: resolution = 15,000, AGC target = 1 × 10⁶, and 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 were constructed covering the precursor mass range of 400 to 1,200 Da for DIA acquisition.

Protein Identification and Quantification

The DDA raw files were analyzed using MaxQuant software (version 2.0.3.0) to search against the database downloaded from UniProt (46,754 entries of Bos taurus; 35,479 entries of Capra hircus; downloaded in December 2020). The relevant parameters were set as follows: the digestion mode was set to trypsin/P specificity, maximum missed cleavages at 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 runs were set as 0.7 match time window, 0.05 ion mobility, 20 alignment time windows, and 1 alignment ion mobility. The identified proteins were quantified based on the abundance of razor and unique peptides using a label-free quantitation (LFQ) 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 established using DDA, and the other parameter settings were the same as those applied in the DDA procedure.

Bioinformatics and Statistical Analysis

The whey proteins with at least 2 identified peptides and all 3 runs of each studied group were selected and imported into the Perseus software (www.maxquant.org/perseus/). Hierarchical clustering, volcano plots, principal component analysis (PCA), and statistical analysis of the quantified proteins among the studied groups were performed. Quantified proteins among the studied groups were analyzed using ANOVA with Benjamin-Hochberg false discovery rate. Differentially abundant proteins were determined according to [fold-change] ≥2 and q-value <0.05. Gene Ontology (GO) enrichment and the Kyoto Encyclopedia of Gene and Genome (KEGG) pathway of these different proteins were analyzed using DAVID Bioinformatics Resources 6.8 software (david.ncifcrf.gov/summary.jsp), in which P-value <0.05 was considered to be significantly enriched. The protein-protein interactions (PPI) of differentially abundant proteins were predicted using STRING software (string-db.org) with 0.70 confidence and visualized using Cytoscape software.
RESULTS

Identification and Quantification of Whey Proteins

Clustering analysis of the quantified whey proteins in goat milk collected during the entire lactation cycle is shown in Supplemental Figure S2 (https://doi.org/10.17632/n8n4s2sv9r.3; Sun, 2022). Using the DIA proteomics method, 344 proteins were identified in the whey component of goat milk throughout the lactation cycle (Supplemental Table S2; https://doi.org/10.17632/n8n4s2sv9r.3; Sun, 2022). We found that the samples from d 90, 150, and 240 formed a subcluster, samples from d 30 joined them and formed a large cluster, and samples from d 1 and 3 formed another subcluster and then a cluster. Using the DDA proteomics method, 331 whey proteins were identified in goat milk throughout the lactation cycle (Supplemental Table S3; https://doi.org/10.17632/n8n4s2sv9r.3; Sun, 2022). The clustering analysis results using the approach based on quantified whey proteins and DDA proteomics were consistent with those of the DIA proteomics approach. The whey proteomes in dairy goats at d 90, 150, and 240 were more similar to one another than those at d 30, whereas the whey proteomes at d 1 and d 3 were similar.

The PCA of the quantified whey proteins from the 6 stages in the goat milk cycle using the DIA method is presented in Figure 1a. According to the score plots, the protein profiles of the 1-, 3-, 30-, and 240-d whey components were clustered, whereas the whey protein profiles at d 90 and 150 were not distinguishable from one another. Apparent changes in the whey proteome profile throughout the lactation stages were revealed, and there were apparent differences in the proteomes in the 1-, 3-, 30-, 90-, 150-, and 240-d groups. Principal components 1 and 2 accounted for 52.6% of the total variance at the different lactation stages. The score plots of the PCA based on the DDA data are shown in Figure 1b. We found that apparent changes in the whey proteome profiles throughout the lactation stages in the DDA data were similar to those in the DIA data.

Differentially Abundant Whey Proteins Throughout the Lactation Stages

For the DIA data, 238 proteins were considerably different throughout the lactation stages according to q-value and fold-change (Supplemental Table S4; https://doi.org/10.17632/n8n4s2sv9r.3; Sun, 2022). Several proteins, including IgG, lipopolysaccharide-binding protein (LBP), fibronectin, serotransferrin, melanotransferrin, inter-α-trypsin inhibitor, α-1-antiprotease, prothrombin, α-2 macroglobulin, complement C3, and growth/differentiation factor 8 were significantly decreased from d 1 to 240. Lipoprotein lipase, fatty acid-binding protein 3 (FABP3), lactoperoxidase, polymeric immunoglobulin receptor (PIGR), osteopontin, and endoplasmin were significantly increased from d 1 to 240. In addition, lactoferrin, actin-depolymerizing factor, and cysteine-rich secretory protein 3 levels decreased from d 1 to 30 and then increased until d 240.

To visualize the changes in whey proteins during the lactation stages, volcano plots comparing d 1 and d 3, 30, 90, 150, and 240 are shown in Figure 2 and Supplemental Table S5 (https://doi.org/10.17632/n8n4s2sv9r.3; Sun, 2022). Compared with the d 1 sample, significantly altered proteins increased from d 3 to d 90 and remained similar to those at d 240. Changes in the components of whey protein profiles in goat milk increased from d 1 to 90 during the lactation stage. Of these, we found that the levels of lactoperoxidase, syndecan, and FABP3 were significantly increased, whereas those of complement C3, complement subcomponent C1r, α-2 macroglobulin, matrix Gla protein, and IgG were significantly decreased from d 1 to 240. In addition, the changes in the abundance of most whey proteins in goat milk throughout the lactation stages from the DDA data were consistent with those from the DIA data. Several of the differentially abundant proteins from the studied groups, such as apolipoprotein A-IV, lactoperoxidase, FABP3, fibrinogen, complement C3, and vitamin D-binding protein, are shown in Figures 3a and 3b.

Bioinformatics Analysis of Differentially Abundant Whey Proteins

According to the protein annotations, the 238 differentially abundant whey proteins identified in goat milk were classified according to biological process, cellular component, and molecular function. As shown in Figure 4, most biological processes were a response to stimulus, localization, protein metabolic process, transport, and immune system processes. The most abundant cellular components were the extracellular region and membrane-bound vesicle, and other proteins were located in the extracellular vesicle, exosome, and extracellular space. The most common molecular functions of differential proteins are protein binding, carbohydrate derivative binding, molecular function regulation, and receptor binding. We also found that the number of differentially abundant proteins involved in most GO terms increased when comparing d 1 and 3 to d 1 and 240.

The protein networks of the differentially abundant whey proteins identified in goat milk throughout the lactation cycle were predicted using STRING software.
Active PPI sources from experiments, curated databases, and text mining were selected, and the minimum required interaction score was set at high confidence (0.70). In the protein network comparing d 1 and 3, fructose-bisphosphate aldolase with a higher degree of connectivity was considered a hub node. In the network comparing d 1 and 30, fibronectin with a higher degree of connectivity was identified as a hub node.

Figure 1. Principal components analysis of whey 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.
of connectivity was considered a hub node. In comparing d 1 and 90, endoplasmin, α-1-antiproteinase, and fibronectin with a higher degree of connectivity were considered central hub nodes. In comparing d 1 and 150, fibronectin, endoplasmin, α-1-antiproteinase, and endoplasmic reticulum chaperone BiP were considered the central hub nodes. In the comparison protein network of d 1 and 240, fibronectin and α-1-antiproteinase were considered central hub nodes. Collectively, fibronectin, endoplasmin, and α-1-antiproteinase were considered the central proteins among the comparisons at d 1 and 90, 1 and 150, and 1 and 240.

Differentially abundant whey proteins were classified into KEGG pathways (Table 1). The results showed that most of the differentially abundant whey proteins were involved in the complement and coagulation cascades, regulation of actin cytoskeleton, *Staphylococcus aureus* infection, and phagosomes. In addition,
several differentially abundant proteins, such as PIGR, fructose-bisphosphate aldolase, and connective tissue growth factor (CTGF), were related to the biosynthesis of antibiotics, AA, antigen processing and presentation, and Hippo signaling pathways.

**DISCUSSION**

**Characterization of the Identified Whey Proteins**

Previous studies utilized LC-MS/MS proteomics technology to investigate goat colostrum and mature whey, wherein 314 and 524 whey proteins were identified in goat colostrum and mature milk, respectively (Sun et al., 2020a). Another study that utilized Q-Orbitrap high resolution mass spectrometry (HRMS) analysis expanded the proteome to 400 whey proteins in goat colostrum and mature milk (Jia et al., 2021a). EASY-nLC-Orbitrap LTQ VELOS detected MFGM proteins from colostrum and mature milk in goat milk. A total of 423 proteins were identified, 189 of which were significantly different (Lu et al., 2016). Compared with previous studies, changes in whey proteins in goat milk throughout the lactation cycle were first investigated using a DIA-based proteomics approach that elucidated the main results of this study, whereas the results of the DDA proteomics method can be used to verify the accuracy of the DIA results. Among these, 92 proteins including IgG, melanotransferrin, apolipoprotein E, α-1-antiproteinase, and fibronectin decreased from d 1 to 240, whereas 85 proteins including lactoperoxidase, PIGR, and osteopontin increased from d 1 to 240. In addition, several differentially abundant proteins were involved in immune system processes, complement and coagulation cascades, protease inhibition, and transport.
Figure 4. (a) Biological processes, (b) cellular component, and (c) molecular functions of differentially abundant whey proteins among the comparisons of 3 d versus 1 d, 30 d versus 1 d, 90 d versus 1 d, 150 d versus 1 d, and 240 d versus 1 d of dairy goats using data-independent acquisition (DIA)-based proteomics approach.
Differentially Abundant Proteins Associated with Immune Function

According to GO analysis, several differentially abundant proteins involved in immune system processes, such as α-2-macroglobulin, immunoglobulin, peptidoglycan-recognition protein, LBP, and melanotransferrin were identified, and these proteins decreased from d 1 to 240. In a previous study, IgG was found to be higher in colostrum than in mature bovine and human milk using iTRAQ labeling proteomics (Yang et al., 2016). In small ruminants’ milk, IgG was highly abundant at 0 d and then declined, reaching the lowest level at 56 d in sheep milk using 2-dimensional gel electrophoresis and MALDI-TOF MS technologies (Zhang et al., 2020c). In nature, IgG is the highest in the maternal colostrum, which corresponds to the intestinal closure of neonatal ruminants. This result is related to lambs’ reliance on the timely ingestion of IgG via colostrum to acquire an initial passive immunity that protects them against the invasion of various pathogens during early life (Moore and Townsend, 2019). According to other immune-related proteins, α-2-macroglobulin can eliminate endogenous and exogenous proteases produced by invading pathogens and parasites, thereby acting as a humoral defense barrier against pathogens with a unique pan-protease inhibitor function (Rehman et al., 2013). Alpha-2-macroglobulin has been found to be approximately 7-fold higher in colostrum than in mature milk in goat whey using LC-MS/MS proteomics technology (Jia et al., 2021a). In goat MFGM, α-2-macroglobulin levels were approximately 4-fold higher in colostrum than in mature milk using a label-free proteomics approach (Jia et al., 2021b). In addition to playing a protective role, immune proteins can bind to receptors on intestinal epithelial cells to activate downstream signals. Lipopolysaccharide-binding protein can bind to bacterial lipopolysaccharides to initiate an immune response and catalyze the transfer of bacterial lipopolysaccharide to CD14. Interactions between CD14 and LBP are necessary to activate toll-like receptors 2 and 4, followed by the activation of signal transduction pathways and the production of cytokines in response to LPS (Meng et al., 2021). Lipopolysaccharide-binding protein was found in goat colostrum using Q-Orbitrap HRMS proteomics technology (Jia et al., 2021a). The LBP concentration during early lactation (63.3 μg/mL) significantly decreased to 27.5 μg/mL during late lactation in bovine milk when analyzed using an ELISA (Wenz et al., 2010). In the goat MFGM fraction, LBP in

![Protein-protein interactions (PPI) of differentially abundant whey 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, and (e) 240 d versus 1 d of dairy goats using data-independent acquisition (DIA)-based proteomics approach. Each node represents a protein, and each edge represents the interaction between proteins. Pink box means upregulated proteins. Blue box means downregulated proteins.](image-url)
Table 1. Pathway analysis of differentially abundant whey proteins from 1, 3, 30, 90, 150, and 240 d of dairy goats using data-independent acquisition (DIA)-based proteomics approach

| Term                                      | Count | Hits | Percentage | P-value   | Fold enrichment |
|-------------------------------------------|-------|------|------------|-----------|-----------------|
| **3 d vs. 1 d**                           |       |      |            |           |                 |
| Biosynthesis of antibiotics               | 6     | 206  | 11.32      | 1.37E-03  | 6.87            |
| Glycolysis/gluconeogenesis                | 5     | 63   | 9.43       | 1.17E-04  | 18.73           |
| Biosynthesis of AA                        | 5     | 71   | 9.43       | 1.87E-04  | 16.62           |
| Salmonella infection                       | 5     | 83   | 9.43       | 3.41E-04  | 14.21           |
| Carbon metabolism                         | 5     | 109  | 9.43       | 9.58E-04  | 10.82           |
| Phagosome                                 | 5     | 158  | 9.43       | 3.74E-03  | 7.47            |
| Regulation of actin cytoskeleton          | 5     | 212  | 9.43       | 1.05E-02  | 5.56            |
| Hippo signaling pathway                   | 4     | 151  | 7.55       | 2.34E-02  | 6.25            |
| PPAR signaling pathway                    | 3     | 70   | 5.66       | 3.31E-02  | 10.11           |
| **30 d vs. 1 d**                          |       |      |            |           |                 |
| Protein processing in endoplasmic reticulum | 8   | 169  | 10.53      | 4.06E-05  | 8.12            |
| Antigen processing and presentation       | 7     | 75   | 9.21       | 3.57E-06  | 16.02           |
| Phagosome                                 | 5     | 158  | 6.58       | 1.21E-02  | 5.43            |
| Proteoglycans in cancer                   | 5     | 203  | 6.58       | 2.76E-02  | 4.23            |
| Biosynthesis of antibiotics               | 5     | 206  | 6.58       | 2.90E-02  | 4.16            |
| Biosynthesis of AA                        | 4     | 71   | 5.26       | 7.51E-03  | 9.67            |
| Estrogen signaling pathway                | 4     | 98   | 5.26       | 1.80E-02  | 7.00            |
| Carbon metabolism                         | 4     | 109  | 5.26       | 2.38E-02  | 6.30            |
| Legionellosis                             | 3     | 57   | 3.95       | 4.15E-02  | 9.03            |
| **90 d vs. 1 d**                          |       |      |            |           |                 |
| Regulation of actin cytoskeleton          | 8     | 212  | 8.51       | 8.05E-04  | 5.09            |
| Biosynthesis of antibiotics               | 8     | 206  | 8.51       | 6.79E-04  | 5.24            |
| Biosynthesis of AA                        | 7     | 71   | 7.45       | 1.13E-05  | 13.29           |
| Carbon metabolism                         | 7     | 109  | 7.45       | 1.29E-04  | 8.66            |
| Phagosome                                 | 7     | 158  | 7.45       | 9.49E-04  | 5.97            |
| Protein processing in endoplasmic reticulum | 7   | 169  | 7.45       | 1.35E-03  | 5.58            |
| PI3K-Akt signaling pathway                | 7     | 347  | 7.45       | 3.93E-02  | 2.72            |
| Glycolysis/gluconeogenesis                | 6     | 63   | 6.38       | 8.69E-05  | 12.84           |
| Complement and coagulation cascades       | 6     | 74   | 6.38       | 1.87E-04  | 10.93           |
| Hippo signaling pathway                   | 6     | 151  | 6.38       | 4.66E-03  | 5.36            |
| Proteoglycans in cancer                   | 6     | 203  | 6.38       | 1.57E-02  | 3.98            |
| Antigen processing and presentation       | 5     | 75   | 5.32       | 2.09E-03  | 8.99            |
| Salmonella infection                      | 5     | 83   | 5.32       | 3.03E-03  | 8.12            |
| Tuberculosis                              | 5     | 181  | 5.32       | 4.23E-02  | 3.72            |
| Legionellosis                             | 4     | 57   | 4.26       | 8.11E-03  | 9.46            |
| Bacterial invasion of epithelial cells    | 4     | 77   | 4.26       | 1.93E-02  | 7.00            |
| Pertussis                                 | 4     | 77   | 4.26       | 1.83E-02  | 7.00            |
| Estrogen signaling pathway                | 4     | 98   | 4.26       | 3.42E-02  | 5.50            |
| Amoebiasis                                | 4     | 112  | 4.26       | 4.78E-02  | 4.82            |
| **150 d vs. 1 d**                         |       |      |            |           |                 |
| Regulation of actin cytoskeleton          | 8     | 212  | 8.42       | 5.70E-04  | 5.38            |
| Complement and coagulation cascades       | 7     | 74   | 7.37       | 1.03E-05  | 13.48           |
| Hippo signaling pathway                   | 7     | 169  | 7.37       | 9.99E-04  | 5.90            |
| Proteoglycans in cancer                   | 6     | 203  | 6.32       | 1.25E-02  | 4.21            |
| Biosynthesis of antibiotics               | 6     | 206  | 6.32       | 1.32E-02  | 4.15            |
| Biosynthesis of AA                        | 5     | 71   | 5.26       | 1.38E-03  | 10.03           |
| Antigen processing and presentation       | 5     | 75   | 5.26       | 1.70E-03  | 9.50            |
| Salmonella infection                      | 5     | 83   | 5.26       | 2.46E-03  | 8.58            |
| Carbon metabolism                         | 5     | 109  | 5.26       | 6.54E-03  | 6.53            |
| Hippo signaling pathway                   | 5     | 151  | 5.26       | 1.98E-02  | 4.72            |
| Phagosome                                 | 5     | 158  | 5.26       | 2.30E-02  | 4.51            |
| Glycolysis/gluconeogenesis                | 4     | 63   | 4.21       | 9.15E-03  | 9.04            |
| Bacterial invasion of epithelial cells    | 4     | 77   | 4.21       | 1.58E-02  | 7.40            |
| Pertussis                                 | 4     | 77   | 4.21       | 1.58E-02  | 7.40            |
| Estrogen signaling pathway                | 4     | 98   | 4.21       | 2.96E-02  | 5.51            |
| Amoebiasis                                | 4     | 112  | 4.21       | 4.15E-02  | 5.09            |
| **240 d vs. 1 d**                         |       |      |            |           |                 |
| Regulation of actin cytoskeleton          | 9     | 212  | 8.33       | 1.71E-04  | 5.53            |
| Biosynthesis of antibiotics               | 8     | 206  | 7.41       | 8.45E-04  | 5.06            |

Continued
colostrum was approximately 3-fold higher than that in mature milk as observed using LFQ-based proteomics. This observation was confirmed using ELISA (Lu et al., 2016). As previously discussed, the high abundance of LBP in colostrum helps neonates protect themselves from bacterial invasion. In addition, milk has considerable potential as a source of high-quality protein to produce healthy food products.

Several proteins, such as PIGR and lactoperoxidase, increased from d 1 to 240. Lactoperoxidase is one of the most prominent enzymes and is a component of natural antimicrobial systems in raw milk. Lactoperoxidase tended to increase from 0.5 to 2 mo and then decrease from 9 to 12 mo in bovine milk using filter-aided sample preparation combined with dimethyl labeling proteomics approach (Zhang et al., 2015a). Mol et al. (2018) used tandem mass tag labeling proteome technology and found that lactoperoxidase decreased from early to late lactation in indigenous Indian cattle. Our results are partly consistent with those of Zhang’s group (Zhang et al., 2015b). This observation could be related to the intrinsic characteristics of goat milk, wherein the lactoperoxidase system catalyzes the oxidation of thiocyanate to antibacterial hypothiocyanite and contributes to the conservation of the lactating mammary gland during involution (Zou et al., 2021). Polymeric immunoglobulin receptors contribute to bridging the innate and adaptive immune responses at mucosal surfaces. In dairy goats, PIGR was approximately 133-fold higher in colostrum than in the whey fraction of mature milk, and 26-fold higher in colostrum than in the MFGM fraction of mature milk using Q-Orbitrap HRMS-based proteomics techniques (Jia et al., 2021a,b). It was lower in colostrum than in mature milk in yaks using iTRAQ-labeled proteomics (Yang et al., 2015). Zhang et al. (2015a) used filter-aided sample preparation combined with dimethyl labeling followed by LC-MS/MS and found that PIGR increased from 0.5 to 12 mo in bovine milk. As discussed previously, we speculated that PIGR with high abundance in the late lactation stage of ruminant dairy animals might be related to the mammary gland immune response by transporting polymeric immunoglobulins, such as IgA and IgM, across mucosal epithelial cells (Matsumoto, 2022).

In our study, lactoferrin decreased from d 1 to 30 and then increased until d 240. Lactoferrin was approximately 51-fold higher in colostrum than in mature whey of goat (Jia et al., 2021a). A previous study found that lactoferrin in bovine milk was low at 0.5 mo and increased as lactation advanced (Zhang et al., 2015a). Lactoferrin was significantly influenced by lactation stages, with correlation coefficients of 0.557 that increased from early to late lactation in bovine milk. The late lactation with the highest lactoferrin concentration of 156.68 μg/mL was found by Cheng et al. (2008) using the ELISA method. In goat milk, lactoferrin was highest in the colostrum (387 ± 69 μg/mL), rapidly decreased in the following week (62 ± 25 μg/mL), and then increased in late lactation (107 ± 19 μg/mL; Hiss et al., 2008). Our results are similar to those of Hiss’s group. As discussed previously, the changes in LBP, lactoperoxidase, PIGR, and lactoferrin during the lactation stage may be related to their protective roles in neonatal and goat mammary glands against infections during involution.

| Term                                      | Count | Hits | Percentage | P-value    | Fold enrichment |
|-------------------------------------------|-------|------|------------|------------|-----------------|
| Biosynthesis of AA                        | 7     | 71   | 6.48       | 1.39E-05   | 12.83           |
| Complement and coagulation cascades       | 7     | 74   | 6.48       | 1.76E-05   | 12.31           |
| Carbon metabolism                         | 7     | 109  | 6.48       | 1.57E-04   | 8.36            |
| Hippo signaling pathway                    | 7     | 151  | 6.48       | 9.07E-04   | 6.03            |
| Phagosome                                 | 7     | 158  | 6.48       | 1.15E-03   | 5.77            |
| Viral carcinogenesis                       | 7     | 234  | 6.48       | 8.06E-03   | 3.89            |
| PI3K-Akt signaling pathway                 | 7     | 347  | 6.48       | 4.57E-02   | 2.63            |
| Glycolysis/gluconeogenesis                 | 6     | 63   | 5.56       | 1.03E-04   | 12.40           |
| Salmonella infection                       | 6     | 83   | 5.56       | 3.80E-04   | 9.41            |
| Protein processing in endoplasmic reticulum| 6     | 169  | 5.56       | 8.67E-03   | 4.62            |
| Proteoglycans in cancer                    | 6     | 203  | 5.56       | 1.81E-02   | 3.85            |
| Antigen processing and presentation        | 5     | 75   | 4.63       | 2.38E-03   | 8.68            |
| Bacterial invasion of epithelial cells     | 5     | 77   | 4.63       | 2.62E-03   | 8.45            |
| Tuberculosis                              | 5     | 181  | 4.63       | 4.72E-02   | 3.60            |
| Legionellosis                              | 4     | 57   | 3.70       | 8.95E-03   | 9.13            |
| Pertussis                                  | 4     | 77   | 3.70       | 2.01E-02   | 6.76            |

Table 1 (Continued). Pathway analysis of differentially abundant whey proteins from 1, 3, 30, 90, 150, and 240 d of dairy goats using data-independent acquisition (DIA)-based proteomics approach.
Differentially Abundant Proteins Related to Complement and Coagulation Cascades

We found that several proteins participate in complement and coagulation cascades, including complement C3, complement subcomponent C1r, fibronectin, and complement C5, which decreased from d 1 to 240. A previous study found that complement C3 in colostrum was higher than in mature human and bovine milk using an iTRAQ labeling proteomic method (Yang et al., 2017). In goat milk, complement C3 was also approximately 3-fold higher in colostrum than in the whey fraction of mature milk (Jia et al., 2021a). This observation may be related to complement C3 serving a central role in the complement system, in which processing is the central response in the classical and alternative pathways. A deficiency of complement C3 can lead to susceptibility to bacterial infections (Barbara et al., 2018).

Fibronectin was found in Saanen goat milk using DIA proteomics technology (Zhao et al., 2021) and decreased from d 1 to 9 in bovine milk using dimethyl labeling proteomic technology (Zhang et al., 2015b). In another study, fibronectin was only identified in goat colostrum and was not detected in mature milk based on an LC-MS/MS proteomics approach (Sun et al., 2020a). Fibronectin is an acute-phase reactive protein that protects the host from bacterial infections (Orczyk-Pawilowicz et al., 2015). In addition, fibronectin mediates cell interactions and plays an essential role in cell adhesion, migration, proliferation, and extracellular matrix remodeling (Zollinger and Smith, 2017). We also found that fibronectin, with more interactions than other proteins in PPI, could serve as a crucial protein contributing to the regulation of the complement and coagulation cascades. Complement and coagulation cascades are an essential part of the immune system, and the coexistence and interaction of complement and coagulation cascades in the same microenvironment generally ensures successful host immune defense in an impaired barrier environment (Oikonomopoulou et al., 2012).

Differentially Abundant Proteins Involved in Protease Activity

In our study, α-1-antiproteinase, inter-α-trypsin inhibitor, plasminogen, and antithrombin-III levels decreased from d 1 to 240. A previous study found that plasminogen, inter-α-trypsin inhibitor, and antithrombin-III levels were higher in bovine colostrum than in mature milk (Zhang et al., 2015b). In human milk, plasminogen levels were higher in colostrum than in mature milk using a DIA proteomics approach (Jin et al., 2021). In goat milk, plasminogen was increased by approximately 3-fold in the whey fraction of colostrum compared with that in mature milk (Jia et al., 2021a). Plasminogens can be converted into active plasmin by plasminogen activators. The plasmin system can interact with other milk components such as whey and casein proteins and promote proteolysis (Ismail and Nielsen, 2010). According to α-1-antiproteinase, it was decreased with prolonged lactation in human milk based on dimethyl labeling and LFQ proteomics approaches (Liao et al., 2011; Zhang et al., 2016). Alpha-1-antiproteinase was also higher in colostrum than in mature milk in humans and bovines using an iTRAQ-based proteomics approach (Yang et al., 2017). In goat milk, α-1-antiproteinase was increased by approximately 13-fold in the whey fraction of colostrum compared with that of mature milk (Jia et al., 2021a). Our results are similar to the results of above previous studies. The higher abundance of α-1-antiproteinase may contribute to the protection of immune-related proteins such as LBP, lactoferrin, and IgG against proteolysis, especially IgG, across the intestine in neonatal calves (Wang et al., 2020). Our results revealed that α-1-antiproteinase and plasminogen interact with several proteins, such as hemopexin, fibronectin, and CD59 glycoprotein, and may inhibit proteolysis to maintain milk protein stability. Serpins protect cells, resist proteases, and play an important role in regulating the proteolysis process (Spence et al., 2021). Several previous studies have indicated that serpins A3–5 and A3–7 decreased with prolonged lactation in human milk based on dimethyl labeling and LFQ proteomics approaches (Liao et al., 2011; Zhang et al., 2016). Therefore, we speculated that protease inhibitors with high abundance in colostrum might protect immune-related proteins against proteolysis, especially by transporting IgG across the intestine into the blood and promoting the maturation of the immune system in neonates.

Differentially Abundant Proteins Involved in Transport

Our study found that several proteins involved in transport, such as FABP3, nucleobindin 2, calgranulin-A, and calgranulin-B, increased from d 1 to 240. It has been reported that calgranulin-A and calgranulin-B are calcium-binding proteins involved in the antimicrobial functions and activation of cytokines and chemokines. However, Honan et al. (2020) found that calgranulin-A and calgranulin-B levels decreased from colostrum to mature milk in bovines (Zhang et al., 2015a,b). Therefore, we speculated that protease inhibitors with high abundance in colostrum might protect immune-related proteins against proteolysis, especially by transporting IgG across the intestine into the blood and promoting the maturation of the immune system in neonates.
to 12 mo after birth using the tandem mass tag labeled proteomics approach and found that FABP3 levels increased from 1 wk to 12 mo. In bovine, Bionaz et al. (2012) found that the expression level of FABP3 in mammary glands increased from 0 to 40 d after delivery using a transcriptomics approach. FABP3, through the uptake and transport of exogenous fatty acids into breast epithelial cells, plays a vital role in breast fatty acid transport (Ye et al., 2022).

In addition, several transport-related proteins, such as hemopexin, vitamin D-binding protein, apolipoprotein A-IV/H/E, and thrombospondin 1, decreased from d 1 to 240. In bovine milk, apolipoprotein E decreased from 0.5 mo to the middle of the lactation cycle (Zhang et al., 2015a). However, in human milk, apolipoprotein E decreased 30% from 1 wk to 12 mo (Zhang et al., 2013). Apolipoprotein E is involved in cholesterol transport. Cholesterol plays a vital role in the synthesis of steroid hormones and vitamin D, which is critical for the development of neonates (Xu et al., 2019). Zhang et al. (2013) did not find differences in the vitamin D-binding protein between samples collected at 1 wk and 1, 3, 6, 9, and 12 mo using a tandem mass tag labeling proteomics approach. Using a tandem mass tag labeling proteomics approach, Zhang et al. (2015a) found that in bovine milk, vitamin D-binding protein increased in whey from 0.5 to 3 mo and then decreased until 9 mo. In goat milk, Jia et al. (2021a) found that the vitamin D-binding protein level was approximately 2-fold higher in colostrum than in mature milk whey using HRMS-based proteomics techniques. It is well known that vitamin D-binding protein is primarily responsible for preventing vitamin D from biodegradation. Thus, we speculated that proteins related to nutrient transport could contribute to enhancing the immune system and promoting the development and growth of neonates.

**CONCLUSIONS**

Related proteins corresponding to specific stages of lactation were revealed using a DIA-based proteomics approach that was confirmed using a DDA quantitative proteomics strategy. The proteins IgG, vitamin D-binding protein, LBP, and fibronectin decreased from d 1 to 240, whereas lactoperoxidase, PIGR, calgranulin-A, and calgranulin-B increased from d 1 to 240. Fibronectin with a higher degree of connectivity was considered a central node. These findings provide new insights into the whey proteome profile and temporary changes in whey proteins throughout the lactation cycle, which may contribute to understanding the intrinsic physiological functions of goats.

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