International Journal of Biological Macromolecules 283 (2024) 137866

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Astral-DIA proteomics: Identification of differential proteins in sheep, goat, and cow milk

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ARTICLE INFO

Keywords:
DIA proteomics
Dairy
Functional analysis

ABSTRACT

Dairy products are of great benefit to human health, and the nutritional differences between different dairy products have attracted attention. In this study, DIA proteomics technique, combined with parallel reaction monitoring (PRM) as a validation method, was used for the qualitative and quantitative analysis of proteins in sheep, goat, and cow milk. In total, 4316 proteins were identified. Beta-2-glycoprotein 1 and aminopeptidase can be used as potential biomarkers for sheep milk, fibrinogen alpha chain and Alpha-1-B glycoprotein can be used as potential biomarkers for goat milk, and angiogenin-1 and Serpin family G member 1 can be used as potential biomarkers for cow milk. Functional analysis showed that these different proteins were enriched through different pathways, such as complement and coagulation cascades. These data reveal the differences in protein content and physiological functionality and provide an important basis for the study of dairy nutrition and adulteration identification.

1. Introduction

Dairy products are rich in nutrients and an important protein source in the human diet [1]. At present, consumer demand for premium high-quality dairy products is growing, and the supply of high-quality small ruminant milk particularly from sheep and goats, has risen to meet this market demand [2]. Although cow milk currently has a large market share, most people in the world are lactose intolerant [3]. Compared to cow milk, goat and sheep milk have a higher nutritional value and relatively higher dry matter content, and the milk lipids occur in the shape of spherical globules of smaller diameters, which is conducive to digestion and metabolism [4]. However, the prices of sheep and goat milk are high, production is low, and some merchants choose to adulterate with cow milk. Therefore, the analysis of the differences in the ingredients of sheep, goat, and cow milk and the identification of potential biomarkers of various milk types will help improve the understanding of specialty milk and provide a theoretical grounding for monitoring milk adulteration.

There are about 80 % casein and 20 % whey protein in milk, including a variety of functional proteins, such as lactoferrin and immunoglobulin [5]. There are differences in the amounts of these proteins in different types of milk, and the resulting differences in dairy functions are of increasing concern. By comparing proteomic analysis of different types of milk, specific protein molecules can be identified, which can be potential biomarkers of milk from different species [6,7]. For the past few years, various proteomic technologies have been widely applied [8], and mainstream methods such as isobaric tags for relative and absolute quantitation/tandem mass tag (iTRAQ/TMT) and label-free methods have been developed based on data-dependent acquisition (DDA) to acquire protein profile data [9–11]. However, the DDA data collection mode has some shortcomings, such as poor repeatability, inaccurate quantification, missing data, and difficulty in detecting low-abundance proteins [12]. Consequently, data-independent acquisition (DIA) technology has advanced rapidly because it combines the advantages of high throughput and high repeatability. The principle of DIA technology is based on a mass spectrometer continuously scanning all ion fragments in a certain range of mass-charge ratios to obtain comprehensive mass spectral data [13]. New Orbitrap Astral mass spectrometer was released with the Asymmetric Track Lossless (Astral) analyzer [14]. Orbitrap Astral saves considerable time and effort by running Orbitrap Full Scan and Astral MS/MS independently, simultaneously producing high-resolution full-scans and high-quality secondary maps. DIA technology is also applied widely in the study of dairy products. Zhang et al. revealed blood pressure-lowering and immune components in the whey of buffalo at different altitudes using DIA proteomics and found that whey at high altitudes contained more

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https://doi.org/10.1016/j.ijbiomac.2024.137866
Received 20 October 2024; Received in revised form 13 November 2024; Accepted 17 November 2024
Available online 19 November 2024

immune components [15]. Sun et al. used DIA proteomics to analyze the changes in whey protein in goat milk during the entire lactation cycle [16]. The whey protein changes with lactation. From day 1 to day 240, levels of plasminogen, alpha-2-macroglobulin, and fipronectin decreased, while levels of polymerized immunoglobulin receptors, nuclear binding protein 2, fatty acid binding protein 3, and lactoperoxidase increased. These studies have profound implications for better understanding the potential roles of specific proteins. However, limited research has been conducted on protein composition comparisons between milk from different species to identify potential biomarkers.

To research the differences in protein composition in sheep, goat, and cow milk, and thus identify potential biomarkers, this study used Astral-DIA proteomics to analyze differentially expressed proteins and validate the results with parallel reaction monitoring (PRM) methods. Using bioinformatics methods, we predicted the functions of characteristic proteins and associated biological processes through gene ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. After identifying potential biomarkers for each species, corresponding antibodies can be prepared to detect adulteration. And to further investigate the unique functions of these proteins to produce high-quality dairy products tailored to individuals with lactose intolerance or those with specific dietary needs. These studies will help improve consumers' understanding of the ingredients and functions of various dairy products, strengthen the quality control of dairy products in the market, improve people's quality of life, and promote the healthy development of the global dairy industry.

2. Materials and methods

2.1. Sample and protein preparation

East Frierian sheep milk samples were obtained from the Yuansheng Ranch, (Jinchang City Gansu Province, China). Saanen goat and Holstein cow milk samples were obtained from Herds Dairy Company, Ltd. (Shaanxi Province, China). We selected healthy, disease-free, similarly growing individuals in each species. Forty samples were collected from each species approximately about 15–30, 60, 120 and 180 days after lambing. Original samples were randomly mixed into four bioreplicates (each containing 10 samples) for protein extraction. After freeze-drying, in order to efficiently extract the protein and keep it dissolved and stable, SDT cracking solution (4 % sodium dodecyl sulfate (SDS), 100 mM Tris-HCl, pH 7.6) was added to each sample. Samples were then transferred to eppendorf (EP) tubes, boiled in a water bath, subjected to ultrasonic crushing for 2 min. The supernatant was taken for quantitative analysis by bicinchoninic acid (BCA) Protein Assay Kit (BeyoTime, China).

2.2. Protein digestion

To each specimen, dithiothreitol (DTT) (100 mM) was added, boiled in a water bath for 5 min, and then cooled to 25 °C. Then 200 μL UA buffer (8 M Urea, 150 mM Tris-HCl, pH 8.0) was added, transferred to a 10 kDa ultrafiltration centrifuge tube. Next, 100 μL iodoacetamide (IAA) (50 mM IAA in UA) was added, shaken at 600 rpm for 1 min, stored at room temperature protected from light for 30 min. UA buffer (100 μL) and NH_4HCO_3 buffer (Sigma) (100 μL) were added, centrifuged at 14,000*g* for 10 min. The addition of 40 μL trypsin buffer (6 μg trypsin in 40 μL NH_4HCO_3 buffer) was performed at 600 rpm. The filtrate was collected, and appropriate 0.1 % trifluoroacetic acid (TFA) solution was added. After enzymatic hydrolysis, the peptide segments were desalted with C18 cartridges and freeze-dried under vacuum.

2.3. LC-MS/MS analysis

Liquid Chromatograph Mass Spectrometer/Mass Spectrometer (LC-MS/MS) was operated using an Orbitrap Astral mass spectrometer coupled to a Vanquish Neo UHPLC system (Thermo Fisher Scientific). The RP−HPLC mobile phase A was 0.1 % formic acid in water and B was 0.1 % formic acid in 80 % acetonitrile. Peptides were eluted over 8 min with a linear gradient of buffer B at 1.25 μL/min. The linear gradient was set as follows: 0–0.1 min, linear gradient from 4 % to 6 % buffer B; 0.1–1.1 min, linear gradient from 6 % to 12 % buffer B; 1.1–4.3 min, linear gradient from 12 % to 25 % buffer B; 4.3–6.1 min, linear gradient from 25 % to 45 % buffer B; 6.1–6.5 min, linear gradient from 45 % to 99 % buffer B; 6.5–8 min, buffer B maintained at 99 %. DIA MS/MS scans were acquired using Astral from 150 to 2000 *m/z* with a 2 *m/z* isolation window, AGC target of 500 %, and 3 ms injection time. The normalized collision energy was set to 25, and the cycle time was 0.6 s.

2.4. Bioinformatics analysis

Bioinformatics analysis was performed using Excel 2016 and R statistical computing software. We used Excel 2016 to arrange, classify and organize the identified protein data. Principal component analysis was performed on quantitative protein data to visualize the relationships between samples obtained from different heights (R: ggplot2 (3.5.10, ggrepel (0.9.3), ggsci (3.0.3), ggforce (0.4.1), pcaMethods (1.88.0))). GO and KEGG were performed using Fisher's exact test, and False Discovery Rate (FDR) correction for multiple testing was performed. The specific R package used by GO and KEGG is clusterProfiler 4.4.4. GO terms were grouped into three categories: biological processes (BP), molecular functions (MF), and cellular components (CC). The GO bubble diagram is R: topGO (2.48.0), qvalue (2.28.0), GO.db (3.15.0), stringr (1.5.0), xlsx (0.6.5) and the KEGG string diagram is Python: argparse (1.1), re (2.2.1). Protein–protein interaction (PPI) networks were constructed using the STRING database and Cytoscape software (Python: argparse (1.1)). The PPI mapping file is generated using perl, strict (1.11), Getopt::Long (2.5), FindBin (1.51), ppi range: 400–999.

2.5. LC-PRM/MS analysis

Peptides (2 μg) were subjected to LC-PRM/MS analysis. Chromatographic separation was performed using a nanoliter flow rate Easy nLC 1200 chromatography system (Thermo Scientific). Buffer A was a 0.1 % formic acid aqueous solution and buffer B was a 0.1 % formic acid in 95 % acetonitrile in water. The column was equilibrated with 95 % buffer A. The samples were poured into a Trap Column (100 μm * 20 mm, 5 μm, C18, Dr. Maisch GmbH) and then subjected to gradient separation on a chromatographic column (75 μm * 150 mm, 3 μm, C18, Dr. Maisch GmbH). The peptides were isolated and subjected to targeted PRM mass spectrometry using a QExactive HF-X mass spectrometer (Thermo Scientific). Analysis duration was 60 min, detection mode: positive ion, parent ion scanning range: 300-1200 *m/z*, primary mass spectrometry resolution: 60,000 @m/z200, AGC target: 3e6, primary mass spectrometry Maximum IT: 50 ms. Secondary mass spectrometry of peptides was performed: the resolution was 30,000@m/z200, AGC target: 1e6, secondary mass spectrometry Maximum IT: 100 ms, MS2 Activation Type: HCD, Isolation window: 1.6Th. The resulting raw mass spectrometry file was analyzed using the software Skyline 4.1 on the PRM data.

2.6. Data analysis

DIA-NN (version 1.8.1) software was used to combine all mass spectral data to complete the database retrieval of DIA mass spectral data and quantitative analysis of the protein DIA. The main software parameters were as follows: trypsin was used as the enzyme, the FDR for both Peptide-Spectral Matching (PSM) and protein levels was 0.01, and the fixed modification was made into aminomyl (C). The databases were Uniprotkb-*Ovis aries* (sheep) [9940], *Capra hircus* (goat) [9925], and *Bos taurus* (bovine) [9913] (https://www.uniprot.org/taxonomy/9904 (9925) (9913)). Pairwise comparison was performed using Student *t*-test

combined with Fold change (FC, ratio of mean expression between the two groups), and significantly different proteins were screened out if *p*-value <0.05 and FC ≥1.5 or ≤1/1.5. One-way ANOVA test method was used to compare multiple groups, and the protein with $p < 0.05$ was selected as the differentially expressed protein. All data visualization was performed using GraphPad Prism 8.

3. Results

3.1. Identification of proteins in sheep, goat, and cow milk

In total, 4316 proteins were identified in sheep, goat, and cow milk. A Venn diagram was used to analyze the differences in the identified proteins among the different species, as shown in Fig. 1. A total of 3310, 3859, and 2998 proteins were detected in sheep, goat, and cow milk, respectively. A total of 2373 common proteins (54.98 %) were found in sheep, goat, and cow milk. In total, 116, 458, and 264 unique proteins were identified in sheep, goat, and cow milk, respectively. Moreover, there were 744 common proteins between sheep and goat milk, 77 common proteins between sheep and cow milk, and 284 common proteins between both goat and cow milk. These data revealed differences in protein levels in sheep, goat, and cow milk, which lays the foundation for further screening of potential biomarkers.

3.2. Differentially expressed proteins (DEPs) in sheep, goat, and cow milk

Principal component analysis of the identified proteins revealed that the samples in this study were divided into sheep, goat, and cow milk. The PCA score plot in Fig. 2A shows that goat milk differs from the other two types of milk (sheep and cow milk) according to the PC1 direction. PC1 and PC2 accounted for 41.17 % and 23.97 % of the protein variation among the different species, respectively. In this study, proteins with *P* values <0.05 and FC ≥1.50 or ≤1/1.50 were considered significant DEPs. As shown in the clustering results in Fig. 2B, three major of different protein clusters were observed in the heat map, and there were differences in the patterns among the samples of the three species. LBP, TUBB4A, ATP1B1, TUBB2A, and TUBA1D are the most abundant proteins but are found in different proportions in sheep, goat, and cow milk. Fig. 2C-E volcano plots shows the differential expression of proteins in the sheep milk-goat milk group (S-G), sheep milk-cow milk group (S—C), and goat milk-cow milk group (G-C), with each dot representing a protein. These results provide a ground for further research on the functions of the differentially expressed proteins.

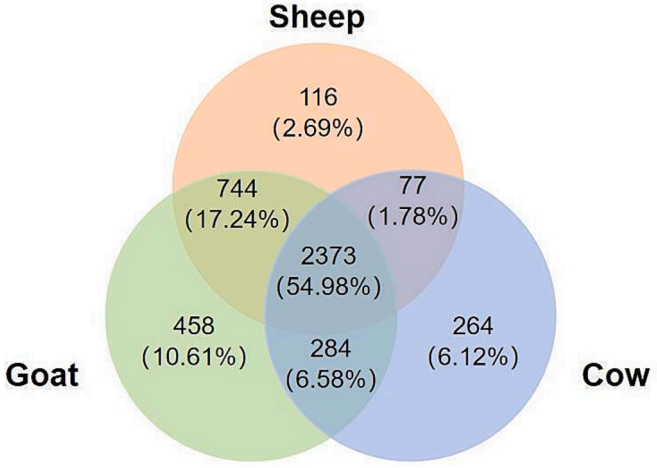

Fig. 1. The Venn diagram of proteins from sheep, goat, and cow milk.

3.3. GO enrichment analysis of DEPs in sheep, goat, and cow milk

Using GO enrichment analysis, DEPs in sheep, goat, and cow milk were divided into three categories: BP, CC, and MF. The results showed that the different proteins identified in S-G were significantly enriched in the peptide metabolic process and peptide biosynthetic process in BP, ribosome and intracellular anatomical structure in CC, and structural constituents of ribosome and ligase activity in MF (Fig. 3). The different proteins identified in S—C were significantly enriched in proteolysis, secretion in BP, extracellular region, and extracellular space in CC, and in endopeptidase regulator activity in MF. The different proteins identified in G-C were significantly enriched in translation, peptide biosynthetic processes in BP, ribosome and endopeptidase complexes in CC, and structural constituents of ribosomes in MF. These results predict the function of differential protein enrichment in sheep, goat, and cow milk.

3.4. KEGG pathway analysis of DEPs in sheep, goat, and cow milk

A chord diagram of the first 15 pathways associated with DEPs in the different types of milk is shown in Fig. 4. According to KEGG analysis, many proteins are involved in multiple disease pathways. In the S-G group, differential proteins mainly participated in Coronavirus disease-COVID-19, ribosome, and other processes, whereas In S—C group, differential proteins were mainly involved in complement and coagulation cascades, lysosomes, and other processes. In the G-C group, differentially expressed proteins were mainly involved in the Coronavirus disease-COVID-19, complement, and coagulation cascades. These differentially expressed proteins are mainly involved in signaling pathways involved in immunity and disease and play an important role in these fields.

3.5. PPI network analysis of DEPs in sheep, goat, and cow milk

Protein-Protein Interaction (PPI) analysis of differentially expressed proteins in the S-G group revealed 406 nodes. The top 50 differentially expressed proteins with the highest connectivity are shown in Fig. 5-A. Among them, the large ribosomal subunit protein eL19 (RPL19) interacted with 26 proteins and was the node with the highest degree of interaction. Small ribosomal subunit protein eS28 (RPS28), small ribosomal subunit protein uS3 (RPS3), large ribosomal subunit protein, differential proteins such as uL14 (RPL23), and elongation factor 2 (EEF2) are associated with multiple proteins. In the S—C group, 167 nodes of differentially expressed proteins were identified using PPI analysis, and the top 50 differentially expressed proteins with the highest connectivity are shown in Fig. 5-B. Alpha-2-HS-glycoprotein (AHSG) interacted with 16 proteins and was the node with the highest degree of interaction. Differential proteins such as plasminogen (PLG), Beta-2-glycoprotein 1 (APOH), apolipoprotein A-I (APOA1), and albumin (ALB) were associated with many proteins. PPI analysis of differentially expressed proteins in the G-C group revealed 279 nodes. The top 50 differentially expressed proteins with the highest connectivity are shown in Fig. 5-C. Among them, the large ribosomal subunit protein eL19 (RPL19) interacted with 17 proteins and was the node with the highest degree of interaction. The small ribosomal subunit protein uS3 (RPS3), large ribosomal subunit protein uL14 (RPL23), elongation factor 2 (EEF2), actin, and differential proteins, such as cytoplasmic 1 (ACTB), are associated with multiple proteins. These results reflect an intrinsic relationship in sheep, goat, and cow milk proteins.

3.6. PRM analysis of proteins in sheep, goat, and cow milk

In the PRM experiment, we verified two potentially functional proteins in each of the three groups and found that PRM analysis accurately characterized the differentially expressed proteins in sheep, goat, and cow milk. As shown in Figs. 6 and S1, DIA analysis shows that Beta-2-glycoprotein 1 and aminopeptidase were highly enriched proteins in

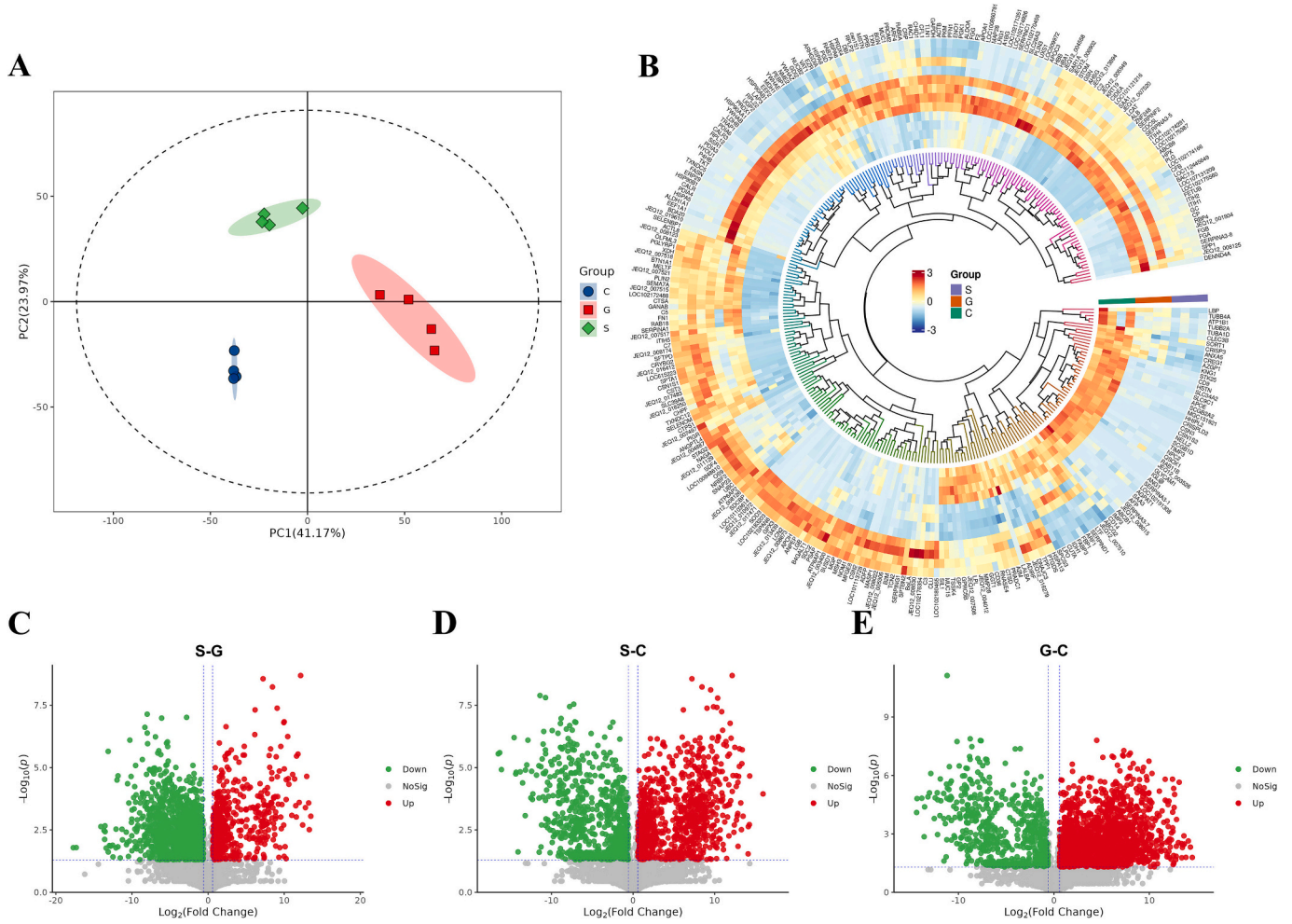

Fig. 2. PCA score plot (A) of the first two principal components. Hierarchical clustering (B) of significantly different proteins in sheep, goat, and cow milk. Volcano plots show the differences in different milk (C-E).

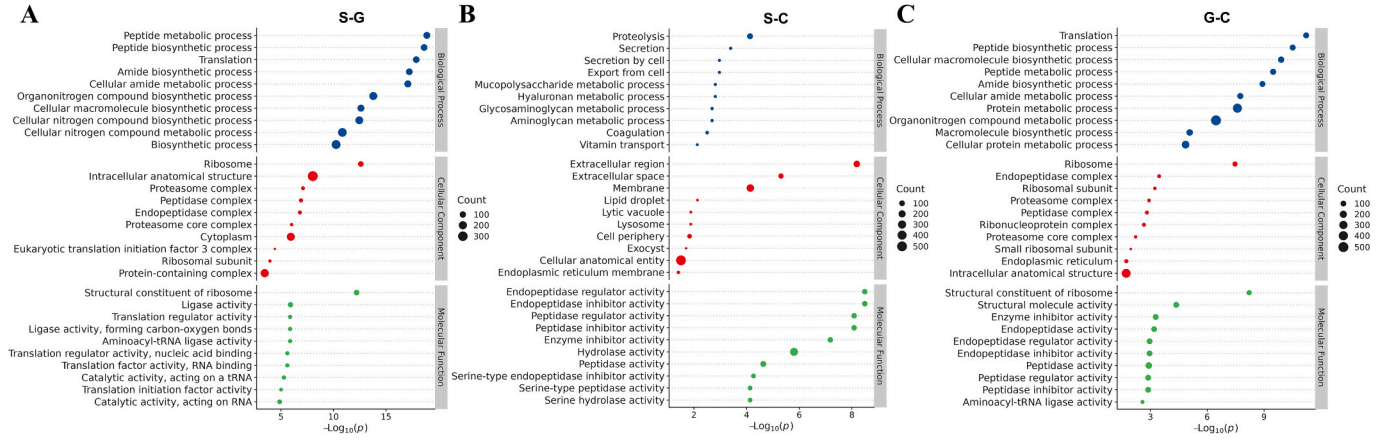

Fig. 3. GO enrichment analysis of differential proteins in sheep, goat, and cow milk based on biological process, cellular component and molecular function.

sheep milk. Fibrinogen alpha chain and alpha-1-b glycoprotein were highly enriched proteins in goat milk, and angiogenin-1 and Serpin family G member 1 were highly enriched proteins in cow milk. The results for the selected proteins analyzed by PRM were broadly similar to those analyzed by DIA, confirming the accuracy of the identification results.

4. Discussion

Milk proteins are the source of most nutrition for humans and have many biological functions. The proteomes of several mammalian milk samples have been studied [17–19], and provided a theoretical basis for our study. Han et al. compared the globule protein profiles of milk fat from goat, cow, and camel milk based on label-free proteomics, and

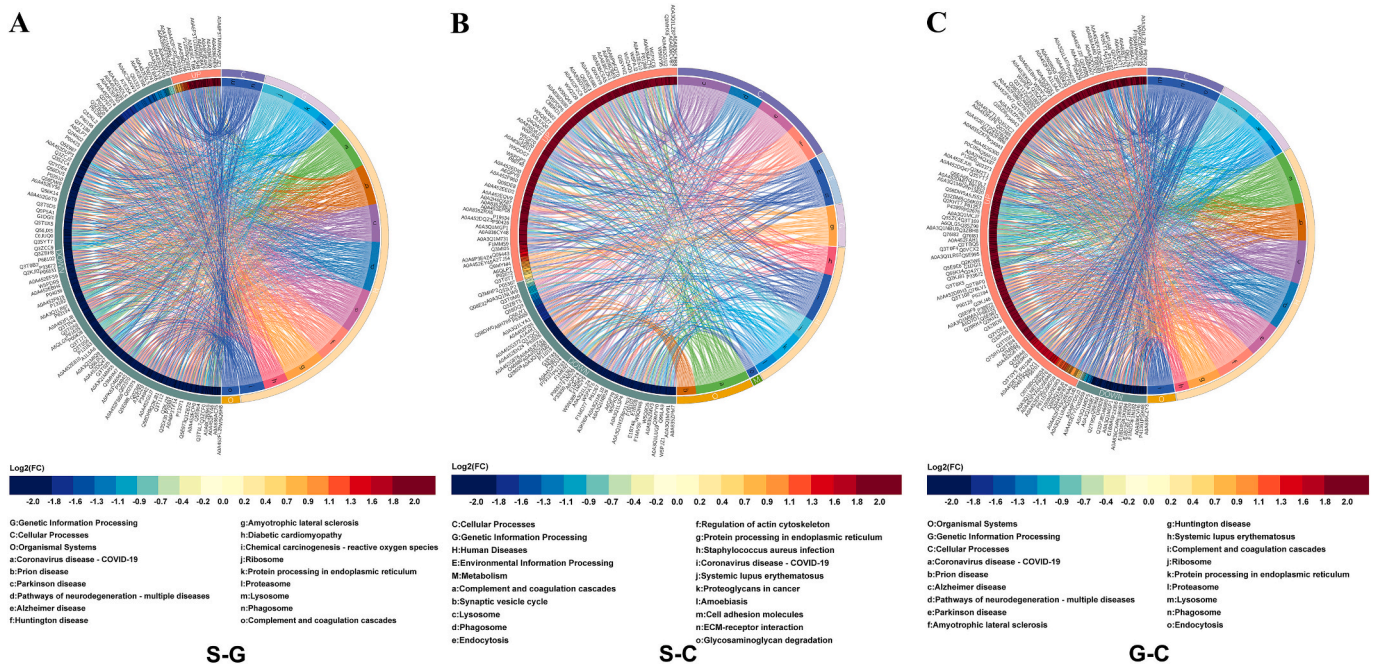

Fig. 4. KEGG pathway analysis of differentially expressed proteins in sheep, goat, and cow milk.

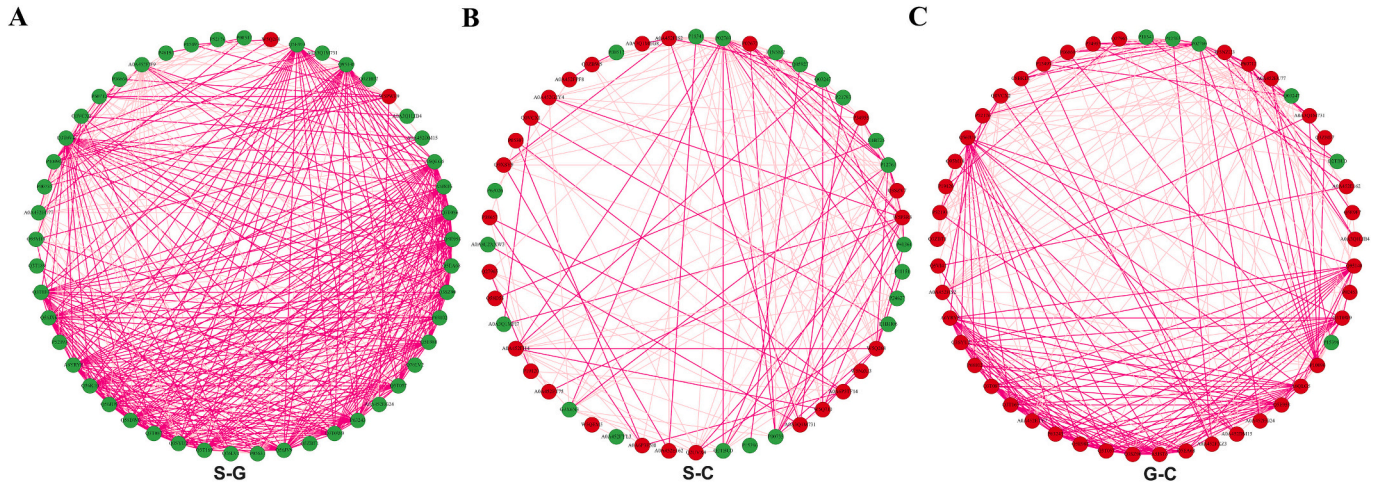

Fig. 5. PPI network analysis of differentially expressed proteins between sheep, goat, and cow milk. Red nodes represent upregulated proteins; green nodes represent downregulated proteins.

identified 1579 proteins, greatly paving the way for camel milk to be featured in the study [20]. In addition, there are several studies on the proteome of sheep milk, investigating sheep milk itself, such as different grades [21] and different periods [22]. Therefore, the research of sheep, goat, and cow milk proteomes has not been comprehensive. In this study, the astral-DIA proteomic method combined with PRM was taken to identify proteins in sheep, goat, and cow milk. We identified 3310, 3859, and 2998 proteins in the three milk types. Our research provides directions for identifying potential biomarkers and developing specialized dairy products.

By studying the differences in the protein composition of different types of milk, their unique functions can be better understood [23,24]. In this study, we found significant differences in the abundance of certain proteins in sheep, goat, and cow milks. Beta-2-glycoprotein 1 and aminopeptidase are highly enriched in sheep milk. Beta-2-glycoprotein 1 is a protein that circulates at high concentrations in the blood and is found to clear lipopolysaccharides, and removes excess anion cell residues from circulation [25]. Anti-beta-2-glycoprotein 1 antibodies play an irreplaceable role in antiphospholipid syndrome (APS) [26]. Aminopeptidase is one of the metalloenzymes, which can catalyze the cleavage of amino acids near the n terminal of peptide, so as to hydrolyze peptide bonds [27]. A recent research identified a novel brain aminopeptidase inhibitor as an antihypertensive treatment regimen that improved long-term cardiovascular disease morbidity and mortality [28]. Goat milk is highly enriched in fibrinogen alpha chain and alpha-1-b glycoprotein. The fibrinogen alpha chain is highly sensitive to proteolysis. Fibrinogens isolated from plasma consist of a set of molecules with different chain lengths called fibrinogens I-V [29]. Alpha-1-B glycoprotein is a functional plasma glycoprotein, and a study showed that cysteine-rich secreted protein 3 is a ligand for alpha-1b glycoprotein in human plasma [30]. They suggest that the A1BG-CRISP-3 complex has a similar effect in protecting blood circulation from the potentially harmful effects of free CRISP-3. Angiogenin-1 and Serpin family G member 1 were highly enriched in cow milk.

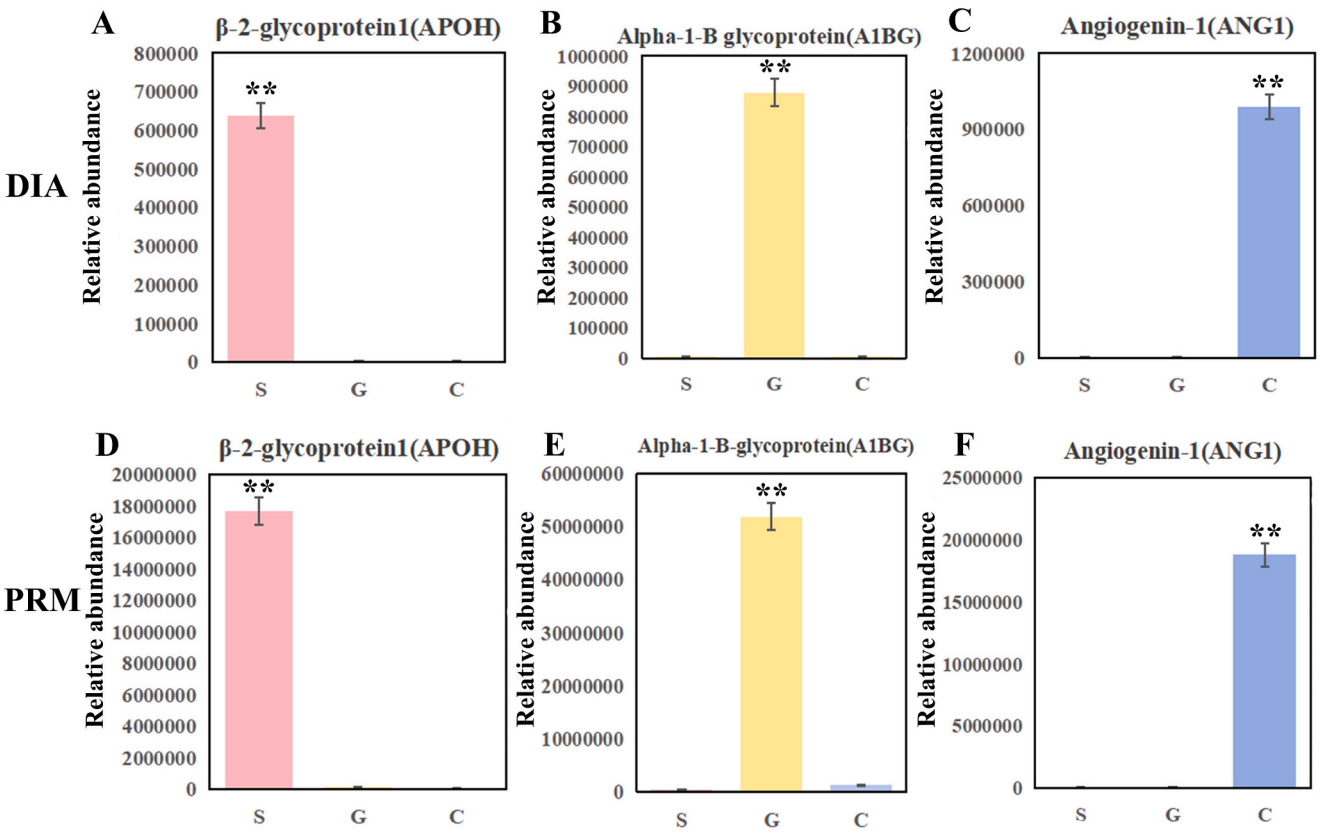

Fig. 6. Comparison between PRM verification results and DIA results.

Angiogenin-1, an enzyme belonging to the ribonuclease A superfamily, plays an important role in vascular biology [31]. A recent study found that plasma angiopoietin is associated with the risk of future cardiovascular events in patients with type 2 diabetes and may be a promising biomarker for identifying high-risk patients with type 2 diabetes for early management [32]. Serpin family G member 1 is a serine protease inhibitor that controls a range of processes involved in the maintenance of blood vessels, including inflammation [33]. These differences in protein abundances highlight the unique functionality of milk from different species.

Next, we conducted GO functional analysis of the differentially expressed proteins d, and the identified differential proteins showed multiple biological functions. GO enrichment results (Fig. 3) showed that these differentially expressed proteins were mainly concentrated in peptide biosynthesis and metabolism, and in the regulatory activity of endopeptidase. A recent study found that bioactive peptides derived from milk proteins (angiotensin-I-converting enzyme inhibition (ACEI) peptides) are of great scientific interest because of their beneficial properties [34]. Ziegenfuss et al. found that a proprietary milk protein concentrate reduced joint discomfort while improving motor performance in non-osteoarthritis patients [35]. Our results provide good predictors of the functionality of differentially expressed proteins in sheep, goat, and cow milk. KEGG pathway analysis showed that there were significant differences in the protein-enriched KEGG pathways among sheep, goats, and milk (Fig. 4). We found that many proteins are involved in various diseases, including the complement and clotting pathways. This is consistent with previous research showing that milk proteins play an important role in immunity [36]. Finally, interaction analysis of the significantly different proteins showed that the large ribosomal subunit protein eL19 (RPL19) was the node with the highest degree of interaction. Moreover, we verified these results using PRM and found that they were consistent, enhancing the reliability of the study.

5. Conclusion

In summary, astral-DIA proteomics combined with PRM verification was used to investigate differentially expressed proteins in sheep, goat, and cow milk. Based on the biological functional analysis, these differentially expressed proteins were enriched through different pathways, such as complement, coagulation cascade, and lysosomes. In addition, Beta-2-glycoprotein 1 and aminopeptidase are potential biomarkers for sheep milk, fibrinogen alpha chain and Alpha-1-B glycoprotein are potential biomarkers for goat milk, and Angiogenin-1 and Serpin family G member 1 are potential biomarkers for cow milk. These potential biomarkers can be used as the basis for the detection of adulteration, thus strengthening the quality supervision and control of dairy products in the market, safeguarding the legitimate interests of consumers, and are of great significance for the development and production of functional dairy products suitable for specific dietary needs. In addition, our research results provide a direction for the development of dairy products, provide a theoretical basis for the formulation of relevant dairy industry standards, and support the effective, rapid and healthy development of the dairy industry. However, the special functions of these proteins need to be further explored and verified in future studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.137866.

CRediT authorship contribution statement

Zhongshi Zhu: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Data curation, Conceptualization. **Tingting Chu:** Visualization, Resources, Methodology, Data curation, Conceptualization. **Chen Niu:** Writing – review & editing, Validation, Supervision, Data curation. **Hao Yuan:** Writing – review & editing, Formal analysis, Conceptualization. **Lei Zhang:** Writing – review & editing, Formal analysis, Conceptualization. **Yuxuan Song:**

Writing – review & editing, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by National Key Research and Development Program of China (2022YFD1600101-02), Shaanxi Livestock and Poultry Breeding Double-chain Fusion Key Project (2022GD-TSLD-46-0202), Rapid Expansion and Selective Breeding of High-Yield and High-Quality Dairy Sheep for Improvement (SMXMKJ001) and Shaanxi Provincial Central Government Guide Local Science and Technology Development Fund Project (2024ZY-CGZY-20).

We would like to thank Yangling Animal Husbandry Industry Innovation Center, Shanghai Bioprofile Technology Company, Ltd. and Editage (www.editage.cn) for English language editing.

Data availability

Data will be made available on request.

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