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Comparative analysis of red deer milk proteins throughout lactation using quantitative proteomics

Catherine Ann Maidment,[1,2] Jessica Gathercole,[1] Stephen Haines,[1] Siqi Li,[2]* and Aiqian Ye[2]†
[1]AgResearch Ltd., Christchurch, 7608, New Zealand
[2]Riddet Institute, Massey University, Palmerston North, 4442, New Zealand

ABSTRACT

Red deer milk is known for its high nutritional quality, containing elevated levels of protein, fat, and essential minerals compared with other ruminant milks. This study investigates the protein profile of red deer milk across various lactation stages, using advanced liquid chromatography-MS/MS techniques to enhance understanding of its nutritional composition. In this study, milk samples were collected from 120 lactating does at 8 distinct points during the lactation period, ensuring consistent dietary conditions. Through this comprehensive approach, a total of 73 proteins were identified, with 9 previously known in deer milk. Significant variations in protein concentrations were observed, highlighting 27 proteins with substantial changes throughout lactation. These proteins are crucial for supporting the physiological needs of the fawn. Key findings revealed the roles of specific proteins, such as osteopontin and lactotransferrin, in immune function, alongside transport proteins involved in nutrient delivery, reflecting the dynamic requirements during lactation. Bioinformatics analysis indicated significant quantitative changes in protein expression, with regression analysis confirming these findings. Gene Ontology analysis was conducted; however, limitations in genomic data for red deer necessitated reliance on related species for functional annotation. The results underscore the complex biochemical changes in deer milk, establishing a foundational understanding of its unique proteome. In conclusion, despite identifying fewer proteins than observed in studies of other ruminants, this research represents the most thorough analysis of proteins in red deer milk to date. It emphasizes the dynamic nature of milk composition throughout lactation and its implications for nutritional and functional attributes in cosmetic products and food, thereby contributing valuable insights into the dairy potential of red deer.

Key words: red deer milk, protein profile, lactation stages, LC-MS/MS, nutritional composition

INTRODUCTION

Milk is a rich source of nutrients and bioactive compounds, enhancing the functional diversity of dairy products (Qin et al., 2021). Notably, red deer milk is characterized by higher levels of protein, fat, calcium, zinc, iodine, branched-chain fatty acids, and α-linolenic acid compared with other ruminant milks (Li et al., 2023). Red deer milk has been used as an ingredient in food, personal care, supplement, and nutritional product categories for consumers in the commercial market. For example, deer milk has been dried into commercial deer milk powder, which is used in supplement products and has been featured on the menu in fine dining restaurants in New Zealand and Australia. Research on deer milk has predominantly addressed overall compositional features (Landete-Castillejos et al., 2000; Malacarne et al., 2015; Wang et al., 2017), with limited focus on specific protein characteristics or their variations throughout lactation (Ha et al., 2014; Wang et al., 2021; Li et al., 2023).

Red deer milk contains approximately 8.8% total protein content. The predominant CN is thought to be β-CN (Opatha Vithana, 2012), although some studies report α_{s1}-CN as the major component (Park, 2004; Ha et al., 2015). Challenges in protein separation have made quantification of β-CN difficult using reversed-phase HPLC (Ha et al., 2015). Studies have also noted relatively high levels of β-LG and elevated lactoferrin content, with lower levels of α-LA (Wang et al., 2017).

The proportions of nutritive constituents, especially fat and protein, in milk vary throughout lactation (Boland and Singh, 2019). The lactation period of red deer is relatively short, lasting approximately 4 to 5 mo. Studies have shown that over this period, milk volume

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*Current address: Fonterra Research and Development Centre, Palmerston North 4442, New Zealand.
†Corresponding author: a.m.ye@massey.ac.nz

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-26. Nonstandard abbreviations are available in the Notes.

decreases while protein and fat content increase in both red deer and roe deer (Landete-Castillejos et al., 2000; Malacarne et al., 2015). Variations in lactose levels throughout lactation have been reported, with some studies indicating stability (Landete-Castillejos et al., 2000; Berruga et al., 2021) and others showing a decrease (Li et al., 2023). Similar trends in protein and fat increases, along with lactose decreases, have also been observed in other ruminant species such as cows, sheep, and goats (Li et al., 2019, 2022).

Limited information is available on how individual proteins in red deer milk vary throughout lactation. Conflicting results have been reported regarding CN changes: Some studies have reported an increase in CN concentration during lactation (Malacarne et al., 2015; Berruga et al., 2021), and others suggest stability (Li et al., 2023) or variability depending on the individual deer (Arman et al., 1974). A previous study conducted by our group used SDS-PAGE and HPLC to investigate protein composition changes in red deer milk across lactation stages (Li et al., 2023). This work revealed trends such as stability in β-CN, increases in κ-CN, and decreases in α_{s2}-CN, along with some unidentified whey proteins, throughout lactation. The limited ability of HPLC to detect low-abundance proteins or resolve complex mixtures highlighted the need for more advanced analytical methods.

To address these limitations, liquid chromatography (**LC**)-tandem mass spectrometry (**LC-MS/MS**) was employed in the present study. This technique offers higher sensitivity and specificity, enabling the identification of less abundant proteins and providing detailed structural insights into complex protein profiles (Cunsolo et al., 2011). Liquid chromatography-MS/MS has been used to characterize milk proteins from different species, including cows (Nissen et al., 2012; Vincent et al., 2016), sheep (Ha et al., 2015), and goats (Chen et al., 2019; Sun et al., 2023). It has also been used to study how individual proteins vary throughout lactation in cow (Delosière et al., 2020), goat (Qin et al., 2021; Sun et al., 2023), and sheep milk (Zhang et al., 2020). These protein changes affect the digestibility and overall quality of the milk products consumed. For instance, Sun et al. (2019) identified differences in protein hydrolysis and peptide composition between colostrum and mature goat milk during digestion, particularly in the duodenum.

The objective of this study was to identify the proteins in red deer milk using LC-MS/MS and characterize their profiles throughout lactation (3–16 wk). The same milk samples were studied by Li et al. (2023) for their macronutrient and protein composition using SDS-PAGE and HPLC. Insights from the present study will enhance understanding of the production, processing, and commercialization of red deer milk across lactation stages.

MATERIALS AND METHODS

Reagents

All reagents used in this study were of LC-MS grade unless otherwise stated. Water, acetonitrile, methanol, formic acid (**FA**), trifluoracetic acid (**TFA**), and urea were sourced from ThermoFisher Scientific (Waltham, MA). Thiourea was purchased from Acros organics (China). Chloroform and ammonium bicarbonate were acquired from BDH Prolabo (Poole, UK). DL-Dithiothreitol (**DTT**), iodoacetamide, and 37% hydrochloric acid were supplied by Sigma-Aldrich (St. Louis, MO). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI).

Sample Collection

Bulk deer milk samples were collected from a Pāmu deer milk supply partner farm in Gore, New Zealand. The herd consisted of 120 lactating does. Throughout the milking season, the does were pasture-fed while also provided a grain-based supplement. There was no systematic change in the diet over the season.

Bulk tank deer milk from the 120 does was collected at 8 different time points throughout the lactation period from August 23, 2020 (winter), corresponding to 3 wk of lactation, to February 22, 2021 (summer), corresponding to 16 wk of lactation. Week 3 of lactation was the start of commercial milk supply. Milk was sampled every 2 wk from wk 3 to wk 15, and one more sample was collected in wk 16, before the does were dried off. At each of the 8 collections, 3 subsamples were taken for analysis in triplicate. Subsamples were stored at −80°C before analysis.

Separation of Whey Proteins

To remove the cream fraction, 1 mL of the sample was centrifuged at 11,500 × *g* for 45 min at 4°C, after which the cream was carefully scraped off. For whey and CN fraction separation, 500 µL of the skim milk fraction was acidified to a pH of ~4.5 using 0.1 *M* HCl and incubated at room temperature for 1 h. Samples were then centrifuged at 14,000 × *g* for 25 min at 4°C. The whey fraction (supernatant) was transferred to a clean Eppendorf tube. Protein concentrations of the whey fraction was estimated at 280 nm using the built-in protein assay on a Nanophotometer NP80 (Implen, Munich, Germany). The pellet containing CN was retained and processed in parallel for protein extraction.

Protein Extraction

Both whey and CN fractions were subjected to protein extraction. For each, 250-μg aliquots were dried using a CentriVap vacuum centrifuge (Labconco, Kansas City, MO) at 40°C. To each sample, 100 μL of urea buffer (7 *M* urea, 2 *M* thiourea, 50 m*M* DTT) was added, followed by overnight incubation at 25°C. Chloroform and methanol extraction was performed according to Wessel and Flügge (1984). The supernatant was discarded, and the precipitated protein was air dried and resuspended in 50 μL of 50 m*M* ammonium bicarbonate by sonicating for 5 min.

Protein Reduction, Alkylation, and Digestion

The extracted protein samples were reduced by addition of 10 m*M* DTT and incubation at 56°C for 45 min and then alkylated using 20 m*M* iodoacetamide for 30 min in the dark at room temperature (21°C). Trypsin digestion was achieved by adding 2 μg of trypsin (1:50 enzyme) and mixing at 37°C overnight (Ning et al., 2023). Desalting was performed using Pierce C18 pipette tips (Thermo Scientific, Waltham, MA) following the manufacturer's instructions, and eluted peptides were dried in a CentriVap vacuum.

Chromatography and MS

Samples were resuspended in 50 μL of 0.1% TFA and diluted 10-fold with 0.1% FA before LC-MS analysis. Nanoflow LC-MS and LC-MS/MS were conducted on an Ultimate 3000 HPLC system (Thermo Scientific, Waltham, MA) interfaced with a CaptiveSpray ion source connected to an Impact HD quadrupole time-of-flight (**Q-TOF**) mass spectrometer (Bruker Daltonik, Bremen, Germany). The CaptiveSpray was equipped with a nanoBooster device to enhance sensitivity. Each sample was injected (1 μL) onto a PepMap100 C18 Nano-Trap column (Thermo Scientific) for peptide trapping, followed by elution onto a Pronto-SIL C18AQ analytical column (150-mm × 100-μm i.d., 3-μm particle size, 20-nm pore size; nanoLCM Solutions, Oroville, CA). Both columns were maintained at 50°C. Peptide separation was achieved at a flow rate of 1 μL/min using a multistep linear gradient of solvent A (water with 0.1% FA) and solvent B (acetonitrile with 0.1% FA), as follows: 2% B for 4 min, increasing to 20% B over 45 min, then to 45% B over an additional 15 min. The column was then cleaned and re-equilibrated.

During LC-MS/MS runs, the mass spectrometer operated using collision-induced dissociation and automated data-dependent acquisition. Two acquisition methods optimized peptide identification, including full scan MS spectrum (*m*/*z* 50–2,200) and a maximum of 10 MS/MS of precursors (*m*/*z* 150–1,200) or (*m*/*z* 350–2,200).

Sample protocol lists were generated for each pooled sample using ProteinScape version 4.0 (Bruker, Billerica, MA), and subsequently, pooled samples, consisting of all whey samples, were rerun to identify lower abundance peptides.

Protein Identification and Quantification

Peptides and proteins were identified using PEAKS Studio version 10.6 (Bioinformatics Solutions Inc., Toronto, ON, Canada). Database searches were conducted using an in-house deer FASTA database consisting of 278,740 nonredundant sequences. Parameters included a precursor ion mass error tolerance of 10 mg/kg, fragment mass error tolerance of 0.05 Da, semispecific trypsin as the enzyme with a maximum of 2 missed cleavages, and a peptide false discovery rate (**FDR**) of 1%. Proteins were accepted based on −10log P-values above 20, at least 1 unique peptide, and additional supporting peptides. Fixed and variable modifications were applied as specified.

Label-Free Quantitation

The label-free quantitation search used MS data with peptide identifications transferred to MS features, using a mass error tolerance of 20 mg/kg and retention shift tolerance of 2 min. The FDR threshold was set at 1%. An in-house R script was employed for normalizing peptide intensity by total ion chromatogram until the acetonitrile gradient reached 50% B.

Bioinformatics and Statistical Analysis

Gene ontology (**GO**) enrichment analysis was performed using both DAVID Bioinformatics Resources 6.8 (https://davidbioinformatics.nih.gov/) and PANTHER Classification System (version 19.0; https://pantherdb.org/), with a significance threshold set at a *P*-value <0.05. Quantified proteins were analyzed for differences throughout lactation using regression analysis. Proteins consistently observed at all 8 time points were fitted with regression lines, and significant changes were identified with *P*-values <0.05. Additionally, fold change and *t*-tests were calculated between wk 3 and 16 of lactation for each protein, with significance based on *P*-values <0.05. Heatmaps to visualize quantitative changes were created using Prism version 10.2.1 (GraphPad Software, Boston, MA).

RESULTS

Identification and Quantification of Red Deer Milk Proteins

In this study, a total of 73 milk proteins were identified in red deer milk using LC-MS/MS. The identified

Table 1. Proteins identified in deer milk using LC-MS/MS

UniProt ID	Species	Protein	Gene
A0A6J0VYA4	*Odocoileus virginianus texanus* *Muntiacus reevesi*	Actin	*ACTB*
Q4TU70	*Cervus canadensis canadensis*	α Hemoglobin chain	*HBA*
A0A6J0WBI5	*Odocoileus virginianus texanus*	α-2-HS-glycoprotein	*AHSG*
A0A212DFY0	*Cervus elaphus hippelaphus* *Cervus elaphus xanthopygus*	α-LA	*LALBA*
A0A6J0VVP2	*Cervus elaphus hippelaphus* *Muntiacus muntjak* *Odocoileus virginianus texanus*	α-S1-CN	*CSN1S1*
A0A212D6E7	*Odocoileus virginianus texanus* *Cervus elaphus hippelaphus*	α-S2-CN	*CSN1S2*
A0A6J0XJ59	*Muntiacus muntjak* *Muntiacus reevesi* *Odocoileus virginianus texanus* *Cervus nippon hortulorum*	Annexin	
Q2Q1M6	*Cervus elaphus*	Annexin A2	*ANXA2*
A0A212DI82	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus*	Apolipoprotein AI	*APOA1*
A0A6J0VYG	*Odocoileus virginianus texanus* *Alces alces* *Muntiacus reevesi*	β-CN	*CSN2*
Q00P86	*Rangifer tarandus tarandus*	β-LG	*LGB*
A0A212D5D9	*Cervus elaphus hippelaphus*	Butyrophilin subfamily 1 member A1	*BTN1A1*
A0A6J0WL20	*Cervus elaphus hippelaphus*	Collagen α-1(I) chain-like	*COL1A1*
A0A6J0Z0K5	*Odocoileus virginianus texanus*	Collectin-46	*CL46*
A0A5J5MNL6	*Muntiacus reevesi* *Muntiacus muntjak*	C-type lectin domain-containing protein	*CLEC*
A0A6J0Y3C4	*Odocoileus virginianus texanus* *Muntiacus muntjak*	Cytoplasmic dynein 2 heavy chain 1	*DYNC2H1*
A0A212CAL2	*Cervus elaphus hippelaphus* *Muntiacus reevesi* *Muntiacus muntjak* *Odocoileus virginianus texanus*	Elongation factor 1-α	*EEF1A1*
A0A6J0WPP2	*Odocoileus virginianus texanus*	Fatty acid-binding protein	*FABP3*
A0A220IGB8	*Cervus elaphus*	Fetal β-globin	
A0A5N3VSU4	*Muntiacus muntjak* *Muntiacus reevesi*	Folate receptor-like domain-containing protein	
A0A5N3UKR3	*Muntiacus reevesi*	Globin domain-containing protein	*HBA1*
A0A212DFU4	*Cervus elaphus hippelaphus*	Glycosylation-dependent cell adhesion molecule 1	*GLYCAM1*
A0A5N3W0N5	*Muntiacus reevesi* *Muntiacus muntjak*	H15 domain-containing protein	*H1–5*
B6D985	*Cervus elaphus*	Haptoglobin	*HP*
A0A6J0YGS2	*Odocoileus virginianus texanus*	Hemoglobin fetal subunit β	
A0A6J0Y2T5	*Rangifer tarandus* *Odocoileus virginianus texanus*	Hemoglobin subunit α	*HBA*
A0A6J0Y2J9	*Odocoileus virginianus texanus*	Hemoglobin subunit α-like	*HBA*
A0A6J0WWZ2	*Odocoileus virginianus texanus*	Histone H1.3 isoform X1	*H1–3*
A0A6J0WX52	*Odocoileus virginianus texanus*	Histone H1.3 isoform X2	*H1–4*
A0A833SCJ5	*Cervus hanglu yarkandensis* *Muntiacus reevesi* *Muntiacus muntjak* *Odocoileus virginianus texanus*	Histone H2A	*H2A*
A0A6J0WXB3	*Odocoileus virginianus texanus*	Histone H3.1-like	*H3–1*
A0A6J0WX68	*Odocoileus virginianus texanus*	Histone H4	*H4*
A0A212CM59	*Cervus elaphus hippelaphus* *Muntiacus reevesi*	Ig-like domain-containing protein	
A0A212D5R7	*Cervus elaphus hippelaphus*	Ig J	*JCHAIN*
A0A5N3V8L0	*Muntiacus muntjak* *Muntiacus reevesi*	Joining chain of multimeric IgA and IgM	*JCHAIN*
Q95149	*Cervus elaphus* *Cervus nippon*	κ-CN	*CSN3*
A0A212CT53	*Cervus elaphus hippelaphus*	Lactadherin	*MFGE8*
A0A212C9V5	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus* *Muntiacus muntjak*	Lactotransferrin	*LTF*

Continued

Table 1 (Continued). Proteins identified in deer milk using LC-MS/MS

UniProt ID	Species	Protein	Gene
A0A5N3WTM7	*Muntiacus muntjak*	Lipocalin/cytosolic fatty acid binding domain-containing protein	
A0A212C9X7	*Cervus elaphus hippelaphus* *Muntiacus reevesi* *Muntiacus muntjak* *Odocoileus virginianus texanus*	L-Lactate dehydrogenase	*LDHB*
A0A5N3XIV9	*Muntiacus reevesi*	L-Lactate dehydrogenase A chain	*LDHA*
A0A212CR41	*Cervus elaphus hippelaphus*	Msx2-interacting protein	*SPEN*
A0A212CF87	*Cervus elaphus hippelaphus*	Mucin 1	*MUC1*
A0A5N3V0T7	*Muntiacus muntjak*	Nonspecific serine/threonine protein kinase	*PINK1*
A0A212CLU0	*Cervus elaphus hippelaphus* *Cervus nippon*	Osteopontin	*SPP1*
A0A212DE23	*Cervus elaphus hippelaphus*	Peptidoglycan-recognition protein	*PGRP*
A0A212CKA1	*Cervus elaphus hippelaphus* *Muntiacus reevesi* *Muntiacus muntjak* *Odocoileus virginianus texanus*	Peptidyl-prolyl cis-trans isomerase	*PPIA*
A0A212CQF6	*Cervus elaphus hippelaphus* *Muntiacus reevesi* *Muntiacus muntjak* *Odocoileus virginianus texanus*	Phosphopyruvate hydratase	*ENO1*
A0A212CL42	*Cervus elaphus hippelaphus*	Platelet glycoprotein 4	*CD36*
A0A212CRH2	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus*	Polymeric immunoglobulin receptor	*PIGR*
A0A6J0XNE6	*Odocoileus virginianus texanus*	Polyubiquitin	
A0A6J0WMU2	*Odocoileus virginianus texanus*	Polyubiquitin-B	*UBB*
A0A6J0XG22	*Odocoileus virginianus texanus*	Polyubiquitin-C	*UBC*
A0A5N3WBS0	*Muntiacus muntjak* *Muntiacus reevesi*	Protein S100	*S100*
A0A6J0X6C4	*Odocoileus virginianus texanus*	Putative elongation factor 1-α-like 3	
A0A6J0XJA7	*Odocoileus virginianus texanus*	Pyruvate kinase	*PKM*
A0A6J0Z7G8	*Odocoileus virginianus texanus*	Ribosomal protein S6 kinase	*RPS6KB1*
A0A5J5MZ62	*Muntiacus muntjak* *Muntiacus reevesi*	SEA domain-containing protein	
A0A5N3XVI2	*Muntiacus muntjak* *Muntiacus reevesi*	Secretoglobin family 1A member 1	*SCGB1A1*
A0A6J0ZFK	*Odocoileus virginianus texanus*	Secretoglobin family 1D member-like	*SCGB1D*
A0A6J0ZDI0	*Odocoileus virginianus texanus*	Serotransferrin	*TF*
A0A6J0XZZ8	*Odocoileus virginianus texanus*	Serpin A3–3 isoform X1	*SERPINA3–3*
A0A6J0XZL1	*Odocoileus virginianus texanus*	Serpin A3–3 isoform X2	*SERPINA3–4*
A0A5N3XDE4	*Muntiacus reevesi*	Serpin domain-containing protein	
A0A212D5P0	*Cervus elaphus hippelaphus* *Cervus nippon*	Serum albumin	*ALB*
A0A5N3VKC1	*Muntiacus muntjak*	Tr-type G domain-containing protein	
A0A5N3WWM1	*Muntiacus muntjak* *Odocoileus virginianus texanus*	Ubiquitin-ribosomal eS31 fusion protein	*RPS27A*
A0A6J0WU44	*Muntiacus muntjak* *Odocoileus virginianus texanus* *Cervus hanglu yarkandensis*	Ubiquitin-ribosomal protein eL40 fusion protein	*UBA52*
A0A5N3WF64	*Muntiacus muntjak* *Muntiacus reevesi*	Ubiquitin-like domain-containing protein	
A0A5N3WQE0	*Muntiacus muntjak*	Ubiquitinyl hydrolase 1	*TNFAIP3*
A0A5N3WFI1	*Muntiacus muntjak* *Muntiacus reevesi*	Uncharacterized protein	
A0A5N3V3L3	*Muntiacus muntjak* *Muntiacus reevesi* *Odocoileus virginianus texanus*	Vitamin D-binding protein	*GC*
A0A212CXS3	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus*	Xanthine dehydrogenase/oxidase	*XDH*

proteins are listed in Table 1, alongside their UniProt identification (**ID**), species, and gene names. Notably, among the identified proteins, 9 were established components of deer milk (CN, α-LA, β-LG, serum albumin, lactotransferrin, and Ig) as referenced in previous studies (Ha et al., 2014; Wang et al., 2017; Li et al., 2023). The remaining 88% of proteins had not previously been reported in deer milk proteomic studies; however, they

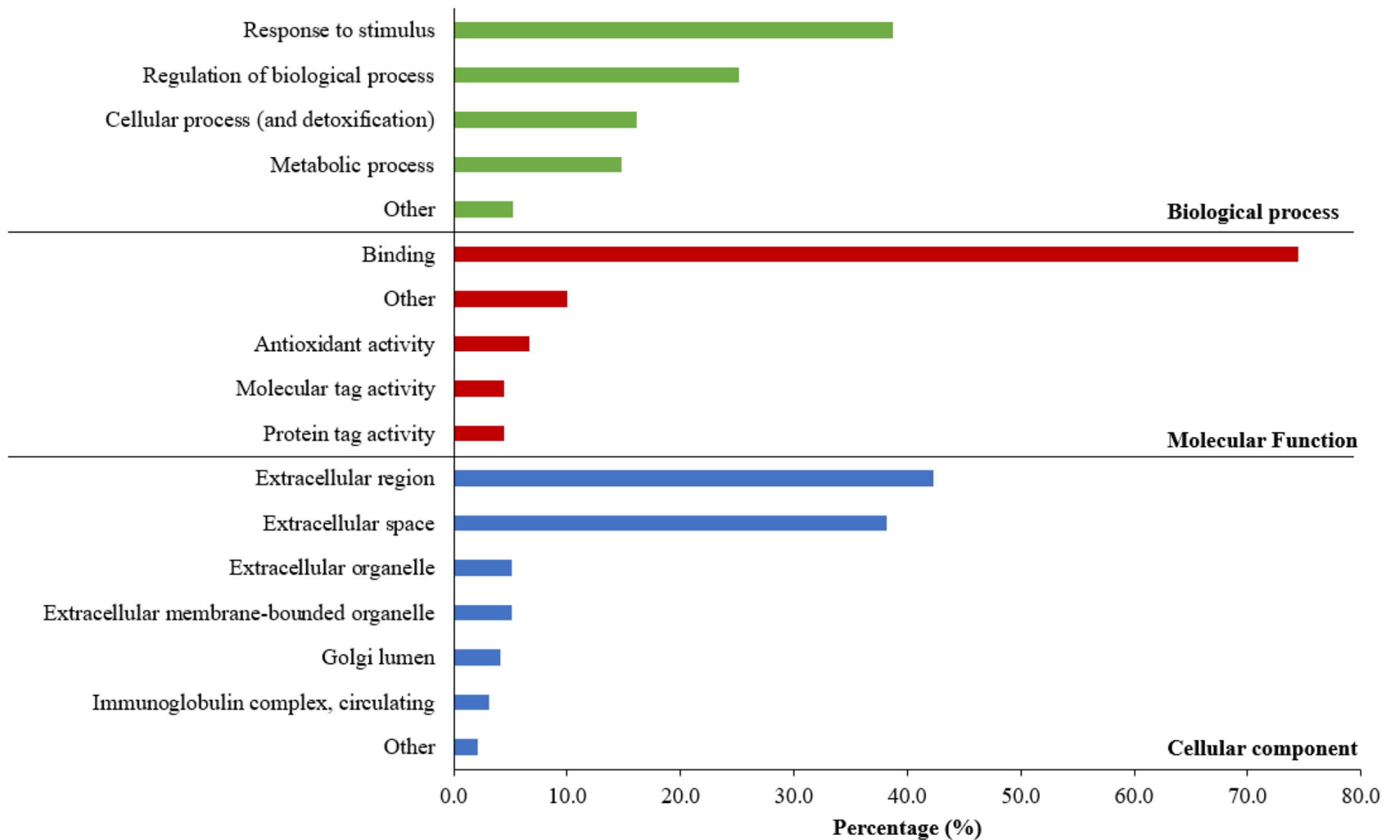

Figure 1. Gene Ontology annotation of proteins in red deer milk via GO enrichment analysis powered by Panther using *Bos taurus* as the species selected.

have been identified in cow milk (Delosière et al., 2020; Das et al., 2022).

GO Analysis of Proteins in Deer Milk

Proteins were categorized based on biological processes, molecular functions, and cellular components. Although the red deer genome has been sequenced (Bana et al., 2018), it is not a model organism, and publicly available information is limited. Therefore, proteins could only be classified against *Cervus elaphus* in the DAVID database, and only some proteins had relevant data. The analysis revealed that biological processes related to metabolism (e.g., carboxylic acid metabolism) and immune responses (e.g., defense against bacteria) were annotated. Key molecular functions included GTP binding and enzyme activities, and the extracellular region was noted as a cellular component.

Further analysis using *Bos taurus* revealed similar results. The top biological processes included response to stimuli, regulation of biological processes, and cellular processes. Main molecular functions involved binding and various activities, with cellular components identified as extracellular region and organelles. Figure 1 and Figure 2 summarize the top functional annotations from the GO ontology enrichment and DAVID analysis, respectively using *Bos taurus*.

Both tools showed similarities. The top 3 biological processes in both searches were response to stimuli, regulation of biological processes, and cellular processes (including detoxification). Main molecular functions were consistent, focusing on binding and various activities. The GO enrichment analysis identified the top 3 cellular components as the extracellular region, extracellular space, and extracellular organelle, whereas DAVID highlighted the nucleus, nucleosome, and extracellular space.

The differences in annotations may stem from the distinct classes of enrichment algorithms used by the tools (Huang et al., 2009). The GO enrichment tool is classified as class I (singular enrichment analysis), and DAVID falls under class III (modular enrichment analysis). Despite these differences, we found an overlap in annotations when comparing *Cervus elaphus* and *Bos taurus* for the identified proteins.

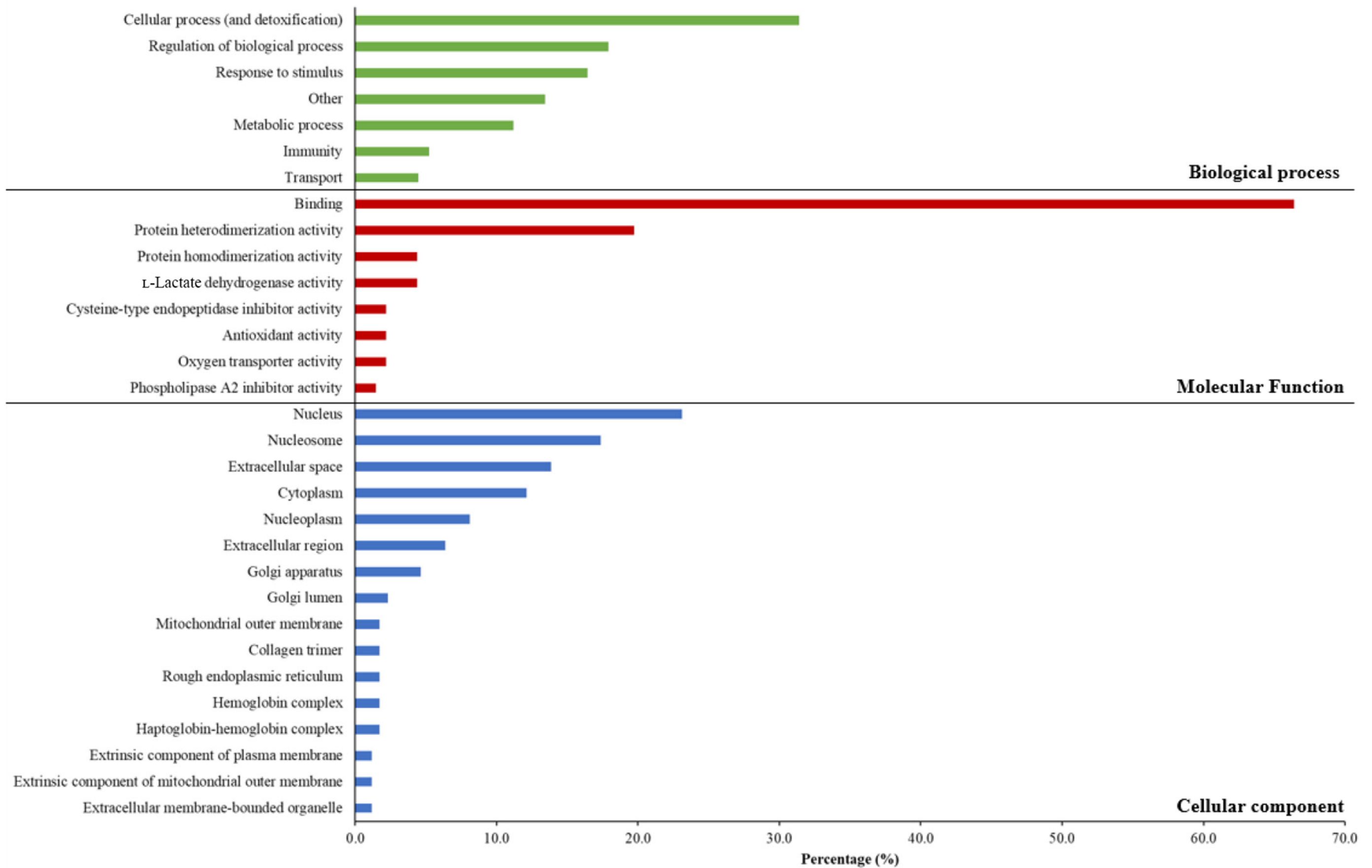

Figure 2. Gene Ontology annotation of proteins in red deer milk via DAVID analysis using *Bos taurus* as the species selected.

Quantitative Changes of Whey Proteins in Red Deer Milk over Lactation

Of the quantified proteins, 51 proteins were consistently present in at least 70% of the samples regardless of time point (8 time points, 3 replicates). Figure 3 illustrates the quantitative changes in the whey fraction throughout lactation. To determine which proteins significantly differ throughout lactation 2 different tests were used. Simple linear regression calculations were performed for each of the 51 proteins. A total of 27 proteins showed a significant increase or decrease. The differences between the samples at wk 3 and wk 16 were analyzed using log fold change. A total of 21 proteins showed a significant increase or decrease. To improve the accuracy of the study, proteins were only deemed significantly different if they met the criteria of both tests. Seventeen proteins met this criterion and are outlined in Table 2, with 6 proteins being upregulated and 11 proteins being downregulated throughout lactation.

The most prevalent biological function of the 17 proteins that significantly differed over lactation annotated in the UniProt knowledgebase was immune-related functionality (35.3%). Other differentially expressed proteins were related to transport (17.6%), protease inhibition (17.6%) and enzyme activity (5.9%). Analysis of the proteins that changed throughout lactation using GO annotation illustrated that the top biological processes were response to stimulus and regulation of biological processes, the top molecular functions were binding and enzyme inhibitor activity, and the top cellular component was extracellular space and organelle (Figure 4). The UniProt annotations are descriptors of the protein functionality, and the GO annotation further describes the biological, molecular, and cellular functions of the protein.

DISCUSSION

Deer Milk Proteome

The number of proteins identified in this study (73) is lower than in similar studies of milk from other species. For instance, donkey milk studies reported 106 unique gene products via LC-MS/MS (Cunsolo et al., 2011) and 216 proteins using LC-MS/MS (Zhang et al., 2019). Goat

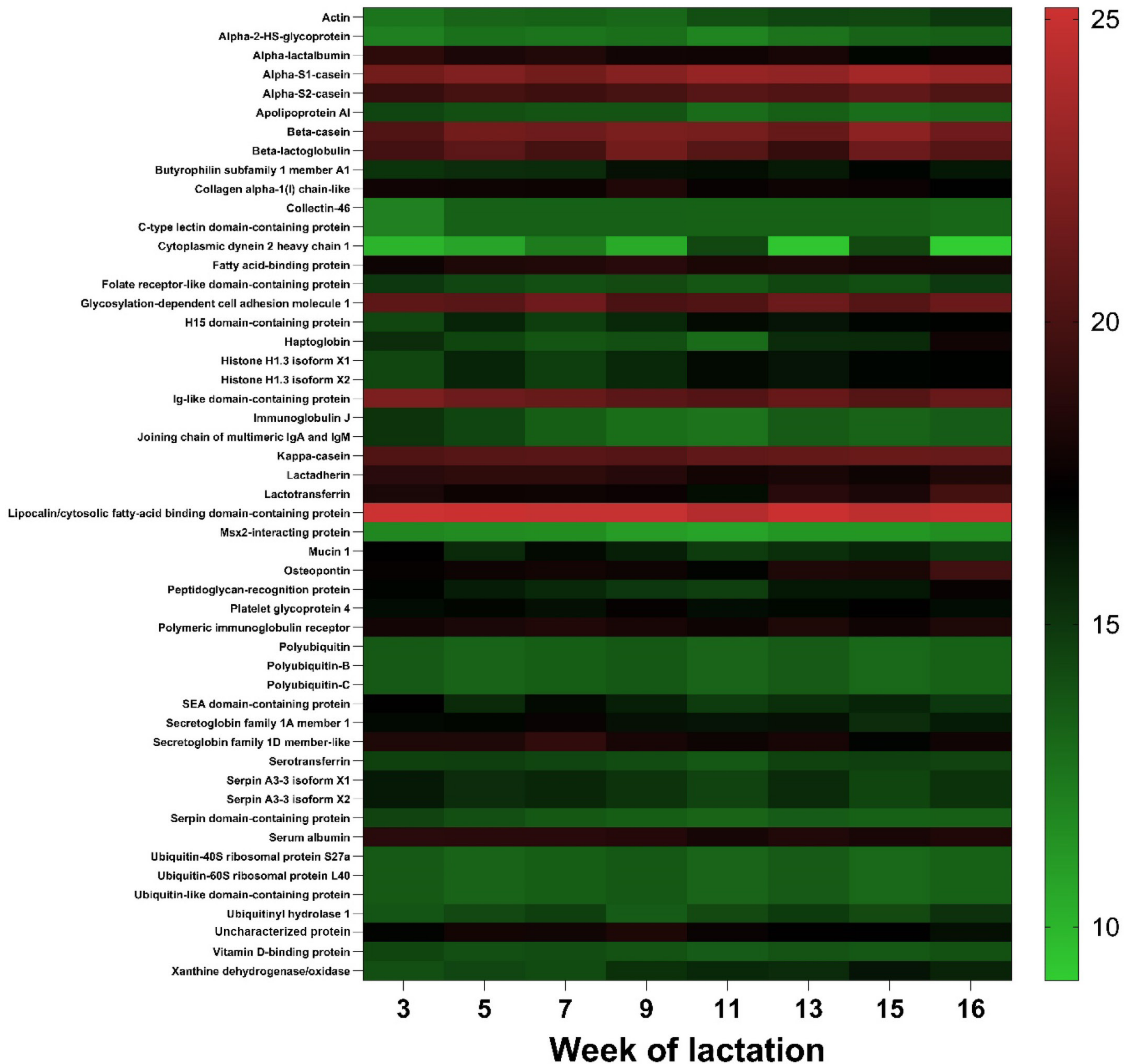

Figure 3. Quantitative changes ($\log_2$) of whey proteins in deer milk from wk 3 to 16 of lactation. The color of the scale bar indicates the abundance ($\log_2$) of the protein, with red being the most abundant and green being the least abundant.

milk studies identified 331, 314, and 524 proteins in whey, colostrum, and mature milk, respectively (Sun et al., 2020, 2023). An extensive analysis of sheep milk whey found 669 proteins (Ha et al., 2015), and a recent study on Indian Zebu cow milk identified more than 6,000 nonredundant proteins using LC-MS/MS (Chopra et al., 2020).

The lower protein count in this study may result from insufficient separation of whey and CN proteins. Previous proteomic studies of red deer tissues, such as antlers and meat, identified 259 and 320 proteins, respectively, suggesting methodological limitations rather than a lack of available data (López-Pedrouso et al., 2019). Despite attempts to separate whey and CN fractions, CN proteins dominated the whey, with α_{s1}-CN being the fifth most abundant protein identified. This dominance likely reduced the detection of low-abundance proteins because their MS/MS spectra may not have been captured because of the long duty cycle of the Q-TOF mass spectrometer.

Table 2. Red deer milk proteins from the whey fraction that significantly differed over lactation

Protein name	UniProt ID	Species	Gene	Biological function
Upregulated throughout lactation				
Actin	A0A6J0VYA4	*Odocoileus virginianus texanus* *Muntiacus reevesi*	*ACTB*	Cell
α-2-HS-glycoprotein	A0A6J0WBI5	*Odocoileus virginianus texanus*	*AHSG*	Immunity
κ-CN	Q95149	*Cervus elaphus* *Cervus nippon*	*CSN3*	Transport
Lactotransferrin	A0A212C9V5	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus* *Muntiacus muntjak*	*LTF*	Immunity
Osteopontin	A0A212CLU0	*Cervus elaphus hippelaphus* *Cervus nippon*	*SPP1*	Immunity
Ubiquitinyl hydrolase 1	A0A5N3WQE0	*Muntiacus muntjak*	*TNFAIP3*	Other
Downregulated throughout lactation				
α-LA	A0A212DFY0	*Cervus elaphus hippelaphus* *Cervus elaphus xanthopygus*	*LALBA*	Milk component
Apolipoprotein AI	A0A212DI82	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus*	*APOA1*	Transport
Ig-like domain-containing protein	A0A212CM59	*Cervus elaphus hippelaphus* *Muntiacus reevesi*		Immunity
IgJ	A0A212D5R7	*Cervus elaphus hippelaphus*	*JCHAIN*	Immunity
Joining chain of multimeric IgA and IgM	A0A5N3V8L0	*Muntiacus muntjak* *Muntiacus reevesi*	*JCHAIN*	Immunity
Secretoglobin family 1A member 1	A0A5N3XVI2	*Muntiacus muntjak* *Muntiacus reevesi*	*SCGB1A1*	Other
Serpin A3–3 isoform X1	A0A6J0XZZ8	*Odocoileus virginianus texanus*	*SERPINA3–3*	Protease inhibitor
Serpin A3–3 isoform X2	A0A6J0XZL1	*Odocoileus virginianus texanus*	*SERPINA3–4*	Protease inhibitor
Serpin domain-containing protein	A0A5N3XDE4	*Muntiacus reevesi*		Protease inhibitor
Serum albumin	A0A212D5P0	*Cervus elaphus hippelaphus* *Cervus nippon*	*ALB*	Other
Vitamin D-binding protein	A0A5N3V3L3	*Muntiacus muntjak* *Muntiacus reevesi* *Odocoileus virginianus texanus*	*GC*	Transport

Additionally, red deer milk has a higher CN-to-whey ratio than other ruminants such as cows and goats (Opatha Vithana et al., 2012), indicating a need for more rigorous fractionation methods for improved protein identification. Studies that report larger numbers of proteins typically use multiple extraction and fractionation techniques and more sensitive mass spectrometers, such as Orbitraps, which offer higher resolution (Chopra et al., 2020; Ha et al., 2015).

To our knowledge, this is the first study analyzing the red deer milk proteome using LC-MS/MS. Previous studies within our group analyzed deer milk using SDS-PAGE and HPLC. The SDS-PAGE results showed a profile largely resembling other ruminant milks, and HPLC analysis was used to show stability in β-CN, increases in κ-CN, and decreases in α_{s2}-CN, along with some unknown whey proteins throughout lactation. However, fewer than 10 proteins were identified using these techniques (Ha et al., 2014; Li et al., 2023). Thus, although the number of proteins identified in this study is not large, it represents the most comprehensive list of proteins found in red deer milk to date.

Variations in the Proteome over Lactation

Despite a limited sample size, we demonstrated the lactational trends of different types of protein based on the available data on bulk deer milk sampled from a commercial deer milk farm. These trends can be verified in future studies on a larger sample size of individual deer milk.

Immune-Related Proteins

This study observed an increase in proteins associated with immune system processes, such as osteopontin, lactotransferrin, and α-2-HS-glycoprotein as lactation progressed. Osteopontin is a secreted phosphorylated glycoprotein that is involved in diverse biological functions such as immune activation, wound healing, angiogenesis, bone remodeling, cell migration, and invasion of mammary epithelial cells (Lund et al., 2009; Hubbard et al., 2013). It has also been observed to increase throughout lactation in both cow milk (Zhang et al., 2015b) and goat milk (Sun et al., 2023). Lactotransferrin is an iron-binding glycoprotein with antimicrobial activity (Riley et al., 2008). The increase in lactotransferrin in this study, particularly toward the end of lactation, has also been observed in cow milk (Riley et al., 2008; Zhang et al., 2015b) and goat milk (Sun et al., 2023). It is thought that lactotransferrin has a regulatory role during early involution of the mammary gland, decreasing CN expression and reducing bovine

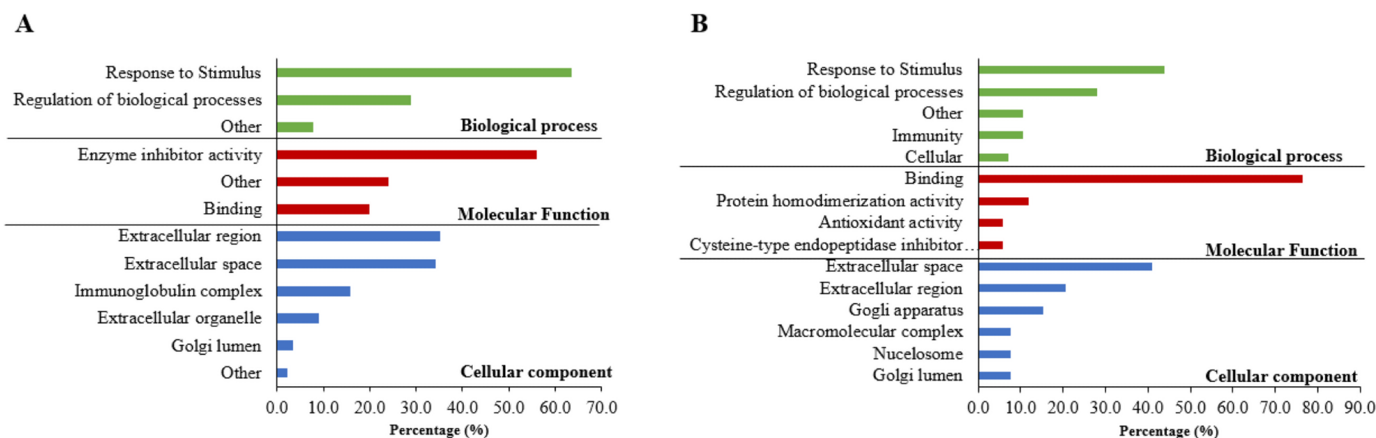

Figure 4. Comparison of GO annotation of differentially expressed proteins in red deer milk throughout lactation via (A) GO enrichment analysis or (B) DAVID analysis using *Bos taurus* as the species selected.

mammary epithelial cell viability (Riley et al., 2008). Alpha-2-HS-glycoprotein, also known as fetuin-A, is a free fatty acid transporter and an acute-phase protein that enhances cellular lipid uptake and lipogenesis (Strieder-Barboza et al., 2018; Ning et al., 2023). Prior studies have shown that although α-2-HS-glycoprotein is higher in bovine colostrum than in mature milk (Ning et al., 2023), an overall increase is observed throughout lactation (Zhang et al., 2015b).

Meanwhile, immunoglobulin-related proteins such as IgJ decreased throughout lactation. Immunoglobulin J is a joining protein that links monomers of antibodies IgM and IgA to form polymeric antibodies capable of secretion (Johansen et al., 2000). Although immunoglobulins such as IgM and IgA were not detected in this study, the decrease in IgJ suggests that they are present in deer milk and that they decrease throughout lactation. A decrease in Ig throughout lactation has also been reported in both goat and cow milk (Zhang et al., 2015b; Sun et al., 2023). The decrease in Ig throughout lactation is thought to be the result of a decreased ability to transfer immune-related proteins from mother to offspring (Zhang et al., 2015a).

This study also observed a decrease in the serine protease inhibitor serpin A3–3. Previous studies have also observed a decrease in serpins throughout lactation in cow milk, which is strongly correlated with the decrease in Ig (Zhang et al., 2015b). Thus it is thought that serine protease inhibitors have a role in protecting the Ig and promoting the maturation of the newborns immune system (Zhang et al., 2015a).

Overall, the difference in immune-related proteins throughout lactation is likely driven by the needs of the fawn (particularly during early to midlactation) and protection of the mammary gland during involution (toward the end of lactation; Zhang et al., 2015b).

Transport-Related Proteins

This study observed a decrease in transport-related proteins, such as apolipoprotein E and vitamin D-binding protein throughout the lactation period of wk 3 to 16. Apolipoprotein E is responsible for transporting cholesterol. Cholesterol plays an important role in the synthesis of vitamin D and the steroid hormones, which are critical to the development of the newborn (Berg et al., 2002). In cow milk apolipoprotein E decreased from 2 wk to the middle of the lactation cycle (Zhang et al., 2015b), and in goat milk it is decreased from d 1 to 240 of lactation (Sun et al., 2023). Vitamin D-binding protein is primarily responsible for preventing vitamin D from biodegradation (Bouillon et al., 2020). Sun et al. (2023) also found a decrease in vitamin D-binding protein between d 1 and 240 of lactation in goat milk.

Other Differentially Expressed Proteins

A decrease in α-LA was observed from wk 3 to 16 of lactation. α-Lactalbumin is a regulatory component of the lactose synthase heterodimer and plays a role in the synthesis of lactose (Boland and Singh, 2019). This coincides with the decrease in lactose over the lactation found in the same deer milk samples observed by Li et al. (2023). Other studies have shown that lactose remains constant throughout lactation (Landete-Castillejos et al., 2000; Berruga et al., 2021). In cow milk studies an increase in α-LA has been observed between 2 wk and midlactation, which coincides with a higher synthesis of lactose over that period (Zhang et al., 2015b). The same study shows a decrease in α-LA between 9 mo and the last time point of late lactation. Due to this it is believed that the decrease of α-LA may aid or even accelerate mammary gland involution (Zhang et al., 2015b).

CONCLUSIONS

This study used LC-MS/MS to identify proteins in red deer milk and investigate the quantitative changes of these proteins throughout lactation. Although from a limited dataset of 8 bulk tank milk samples, we identified clear trends over the lactation that are in line with those previously reported for milk of other ruminants. Proteins related to immunity (such as osteopontin, lactotransferrin, α-2-HS-glycoprotein, and Ig), transport (such as apolipoprotein E and vitamin D-binding protein), and enzyme activity (such as α-LA) are key proteins that change throughout lactation and reflect not only the changing needs of the newborn but also the changing needs of the mammary gland. These results suggest that lactation stage influences individual proteins and that these proteins change according to biological function to reflect the development and protection of the mammary gland over lactation.

NOTES

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Nonstandard abbreviations used: DTT = dithiothreitol; FA = formic acid; FDR = false discovery rate; GO = Gene Ontology; ID = identification; LC = liquid chromatography; LC-MS/MS = liquid chromatography tandem MS; Q-TOF = quadrupole time of flight; TFA = trifluoracetic acid.

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ORCIDS

Jessica Gathercole, https://orcid.org/0000-0001-7780-3168
Stephen Haines, https://orcid.org/0000-0002-6652-1568
Siqi Li, https://orcid.org/0000-0003-4971-9705
Aiqian Ye https://orcid.org/0000-0003-1048-8858