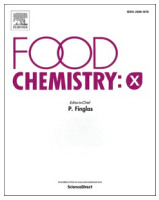
Food Chemistry: X 22 (2024) 101263

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Common proteins analysis of different mammals' mature milk by 4D-Label-Free

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ARTICLE INFO

Keywords:
Proteomics
Lactadherin
Lactoperoxidase
Infant
Goat

ABSTRACT

The milk proteins from samples of 13 different animals were identified utilizing 4D-Label-Free proteomics technology, leading to the identification of a substantial number of proteins. Among the various samples, Chinese people (CHP) milk proteins exhibited the highest count, with 1149 distinct proteins. Simultaneously, we identified common proteins present in these animal milk. It's notable presence in goat milk contributes to enhancing infant infection resistance, showcasing the beneficial role of lactoperoxidase. Galectin-3 binding protein (Gal-3BP) and tetraspanin in human milk are significantly higher than those in other animals, which determine the prominent antiviral effect of human milk and the important processes related to cell transduction. Furthermore, human milk, camel milk, goat milk and sheep milk proved to be rich sources of milk fat globule membrane (MFGM) proteins. The insights obtained from this study can serve as a foundational framework for exploring the role of different animal milk proteins in disease treatment and the composition of infant formula.

Introduction

Human milk is characterized by its high nutritional value and a rich array of components, encompassing proteins, fats, lactose, minerals, vitamins, among others. Human milk protein can be divided into casein, whey protein and mucin, which contain bioactive components that promote infant growth, such as lactoferrin and immunoglobulin (Andreas et al., 2015). Studies have shown that infants fed by breast milk are better than those fed by formula milk in terms of growth, development and immune function(Andreas et al., 2015). It is believed that the bioactive components in human milk are partly responsible for these developmental differences. Milk proteins exhibit capabilities in blood pressure reduction, antibacterial properties, and mineral binding (Lucey et al., 2017). The variations in milk composition among different animals have implications for their diverse effects. In contrast, camel milk uniquely boasts antioxidant and anti-diabetic attributes(Swelum et al., 2021). Remarkably, research conducted by Behrouz S has highlighted camel milk's potential role in anti-aging(Behrouz et al., 2022). In a comparative analysis with human milk, buffalo milk stands out due to its elevated content of proteins, fats, and vitamins, as well as the heightened activity and resilience of its probiotics(da Silva et al., 2020). A study has provided evidence suggesting that donkey milk may contain crucial compounds with potential applications in the treatment of anxiety-related disorders(Araújo et al., 2023). Additionally, donkey milk has demonstrated notable efficacy against a diverse range of bacteria, viruses, and fungi(Spada et al., 2021), which is consistent with subsequent analysis. Notably, horse milk and donkey milk possess lower levels of fats and proteins, preventing coagulation upon the addition of chymosin(Bittante et al., 2022). Donkey milk exhibits a greater similarity to human breast milk proteins than cow milk(Souroullas et al., 2018). However, substituting cow milk with sheep milk can provide a higher protein and lower fat intake, optimizing overall energy

Abbreviations: ALB, albumin; ALC, Alxa camels; BCA, bicinchoninic acid assay; BP, biological process; CC, cellular component; CHP, Chinese people; DZD, Dezhou donkeys; DTT, dithiothreitol; ER, endoplasmic reticulum; Gal-3BP, galectin-3-binding protein; GO, Gene Ontology; GXB, Guangxi buffaloes; HST, Holstein cows; IAA, Iodoacetamide; KAAS, KEGG Automatic Annotation Server; KEGG, Kyoto Encyclopedia of Genes and Genomes; LCG, Liaoning Cashmere goats; MF, molecular function; MFGM, milk fat globule membrane; MGH, Mongolian horses; MS, mass spectrometry; NBY, Nubian goats; OAS, Small Tail Han sheep; PASEF, parallel cumulative sequential fragmentation; PPI, protein-protein interaction; QHY, Qinghai yaks; SNG, Saanen dairy goats; TFA, trifluoroacetic acid; UA, Urea lysis buffer; TGB, Turpan goats; YXP, Large White pigs.

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https://doi.org/10.1016/j.fochx.2024.101263
Received 10 January 2024; Received in revised form 19 February 2024; Accepted 28 February 2024
Available online 1 March 2024

consumption(Hansen et al., 2021).

Moreover, we conducted a preliminary survey of the milk fat globule membrane proteins (MFGM) in various animals, revealing a substantial presence of MFGM proteins. Notably, Cavaletto et al. identified eight MFGM proteins with the highest abundance, including mucin I, xanthine oxidoreductase, mucin 15, CD36, lactotropic lipoprotein, lactadherin, adipose differentiation-related proteins and fatty acid-binding proteins (Cavaletto et al., 2022). Research findings have demonstrated that MFGM fosters metabolism, supports the advancement of brain function and cognitive abilities, and preserves intestinal health and its associated microbiota. Infant formula predominantly incorporates MFGM proteins, which play a dual role in nurturing infant growth and development while also safeguarding infants against bacterial and viral infections (Cavaletto et al., 2022). Moreover, MFGM proteins contribute to the establishment of the infant's neuroendocrine system, which is crucial for fostering infant growth(Hernell et al., 2016). These findings underscore the intricate nature of MFGM proteins and their multifaceted functionality.

Proteomics entails the systematic application of techniques aimed at the identification and quantification of the complete protein repertoire within cells, tissues, or organisms. It has evolved into a valuable methodology for characterizing the protein profiles within tissues and biological fluids, including emulsions and blood. Through proteomics analysis, a deeper comprehension of the biochemical processes associated with complex diseases can be achieved. This approach primarily relies on mass spectrometry (MS)-based methods for protein identification(Noor et al., 2021). The Label-Free technique is not susceptible to technical errors and is highly sensitive to MS analysis, so it is able to identify thousands of proteins from complex samples and is therefore widely used in proteomics analysis(Anand et al., 2017). However, it has a poor ability to detect low abundance proteins. In recent years, 4D-Label-Free technology has emerged as a significant MS-based quantitative approach. Building upon the initial three-dimensional separation of proteomics (retention time, *m/z*, ion intensity), 4D-Label-Free introduces a new dimension of separation, ion mobility, enhancing the capability of detecting low-abundance proteins and proteome coverage across a dynamic range(C. N. Wang et al., 2023). In this experiment, 4D-Label-Free technology was employed for the quantitative analysis of the proteomes from 13 mammals, allowing for an in-depth investigation of the impact of protein composition and component variances on functionality.

Currently, the focus of research centers on animal milk proteins. Previously, the majority of studies centered on the analysis of milk protein components within the same animals, with limited exploration into milk protein components from various animals. This study employed 4D-Label-Free proteomics technology to identify 13 types of animal milk proteins and conducted comprehensive functional analyses. Furthermore, building upon prior research, we performed a statistical analysis of common proteins in these 13 mammals. The purpose is to find functional proteins with high content in different animal milk, and to provide a theoretical basis for the study of infant milk powder components.

Materials and methods

Samples collection

In this study, Chinese people (CHP) samples were collected from Shenyang Maternal and Child Care Center. Holstein cows (HST) samples were collected from Huishan Dairy Group. Qinghai yaks (QHY) samples were collected from Lanzhou Institute of Husbandry and Pharmaceutical Science of CAS. Guangxi buffaloes (GXB) samples were collected from Nanjing Agricultural University and Guangxi Buffalo Research Institute. Large White pigs (YXP) samples were collected from Yangxiang Pig Group. Mongolian horses (MGH) samples were collected from Inner Mongolia Xilin Gol Xilin Gol League Science and Technology Association. Alxa camels (ALC) samples were collected from Alxa Alpaca Farm in Inner Mongolia. Small Tail Han sheep (OAS) samples were collected from Inner Mongolia and Lingle sheep farms. Liaoning Cashmere goats (LCG) samples were collected from Liaoyang National Core Sheep Breeding Farm. Turpan goats (TGB) samples were collected from Liaoyang dairy goat farm. Nubian goats (NBY) samples were collected from Liaoyang dairy goat farm. Saanen dairy goats (SNG) samples were collected from Liaoyang dairy goat farm. Dezhou donkeys (DZD) samples were collected from East Ajiao Donkey Farm in Fumeng County. We sampled in the fall, and milk samples were collected two weeks after the birth of the second litter, with three samples of each of the 13 mammals mentioned above. Veterinary examination did not detect any disease in the animals. Each time the experimental animals were milked, the milk was placed in a 10 mL container and immediately transferred to a refrigerator at − 20 °C for a short period of time, no more than two weeks, and then the sample was placed in dry ice for transportation. Long-term storage in a refrigerator at − 80 °C, but not more than 4 months.

Ethical Statements: The humans and animals in this study were approved by the Animal Welfare and Ethics Committee of Shenyang Agricultural University (Animal Welfare Number: 202,106,015). Human milk collection is conducted with the consent of the donors.

Protein extraction

Milk samples were centrifuged at 1006.2 × *g* for 15 min, the upper fat was discarded, and skim milk was collected. The skim milk was centrifuged at 1118000 × *g* for 1 h (casein was removed), and the supernatant was collected as whey sample. Proteins were extracted by urea lysis buffer (UA) cleavage and quantified by bicinchoninic acid assay (BCA). Specifically, the appropriate amount of UA lysate was added, and the precipitate was resuspended by Votex, it was crushed by ultrasonic cell crusher, with the ultrasonic time of 30 s, the intermittent time of 30 s, and the working time of 40 min, and then it was centrifuged at 17468.75 × *g* for 20 min at 21 °C, and the supernatant was taken and transferred to a new centrifuge tube. 20μg protein from each sample was mixed with 5X loading buffer, boiled for 5 min, and separated on 12.5 % SDS-PAGE gel (constant current 14 mA, 90 min). Coomassie brilliant blue R-250 staining showed protein bands.

Enzymatic hydrolysis in solution

Each sample was taken 200 μg protein, added 8 M UA, at room temperature for 1 h, added dithiothreitol (DTT) to the final concentration of 100 mM, shaked at 40.2 × *g* for 1 min, and incubated at room temperature for 1 h. Added Iodoacetamide (IAA) buffer (50 mM IAA in UA), oscillate at 40.2 × *g* for 1 min, and reacted at room temperature in dark for 30 min. Added 100 mM NH_4HCO_3 solution and diluted 8 M UA to 2 M. Added 40 μL Trypsin buffer (4 μg Trypsin in 40 μL 25 mM NH_4HCO_3), shaked at 40.2 × *g* for 1 min, and placed at 37 °C for 16–18 h. Added trifluoroacetic acid (TFA) acidification, and adjusted the pH to about 3. MCX (Oasis® MCX uElution Plate 30 μm) was used to desalt the peptides. After lyophilization, 40 μL 0.1 % formic acid solution was added to redissolve the peptides, and the peptides were quantified (OD280).

Mass spectrometry on machine and searching database

The separation was carried out by the nano-upflow HPLC system Easy nLC. Buffer: Solution A was 0.1 % formic acid aqueous solution, and Solution B was 0.1 % formic acid acetonitrile aqueous solution (84 % acetonitrile). The chromatographic column was balanced with 95 % A liquid. The sample was loaded onto a Thermo scientific EASY column (2 cm*100 μm 5 μm-C18) and separated on a Thermo scientific EASY column (75 μm*100 mm 3 μm-C18) at a flow rate of 300nL/min. The relevant liquid phase gradient is as follows: 1-hour gradient: 0 min-50

min, B liquid linear gradient from 0 % −35 %; 50 min-55 min, B liquid linear gradient from 35 % to 100 %; 55 min-60 min, B liquid maintained at 100 %. The peptides were separated by chromatography and analyzed by timsTOF Pro mass spectrometer. The MS detection time on the machine is 60 min and the detection mode is positive ion. The MS scan range is set to 100–1700 *m/z*. The data acquisition mode was parallel cumulative sequential fragmentation (PASEF) mode. After the first-order MS acquisition, the parent ion was collected in 10 PASEF mode, and the period window time was 1.17 s. The original data of mass spectrometry analysis were.D files, and Peaks software was used for database identification and quantitative analysis.

Bioinformatic analysis

Single-pool analysis in animals: CELLO (https://cello.life.nctu.edu.tw/) is a multi-class SVM classification system for predicting protein subcellular localization. Searched for protein sequences using Inter Pro Scan software and identify protein domains from the Inter Pro member database Pfam. The Gene Ontology (GO) terms were located and the sequences were annotated by Bblast2GO software. The GO annotation results were drawn by R script. Using KAAS (KEGG Automatic Annotation Server) software, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations were performed on the target protein set. The protein–protein interaction (PPI) networks were constructed using cytoscape software based on the proteins interaction relationship in the

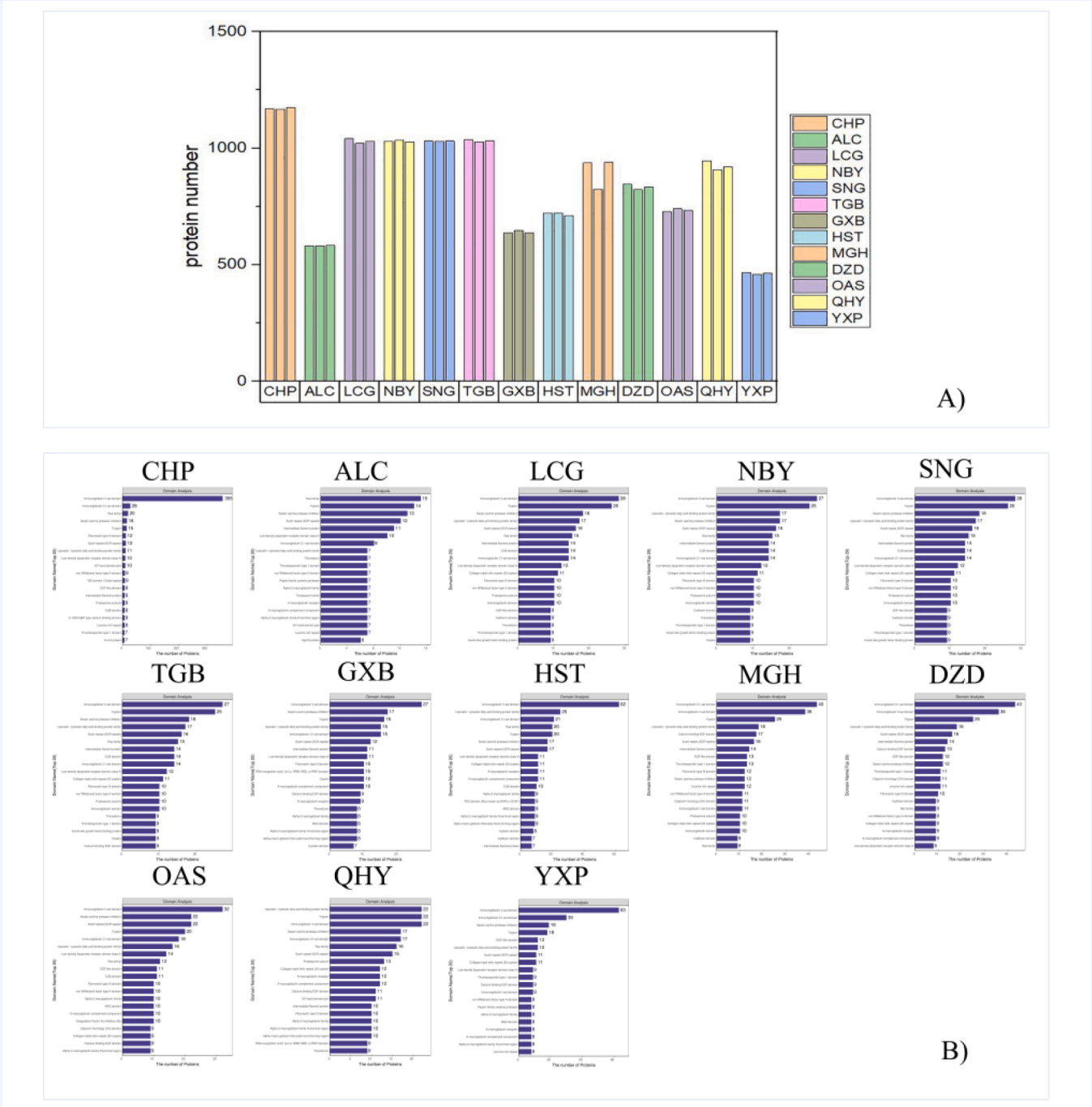

Fig. 1. A) Histogram representing the distribution of milk proteins from 13 different animals; B) Domain analysis of milk from 13 animals.

string database. A medium confidence (0.4) is set in the string database. Difference analysis: Cluster heatmap analysis using the Complexheatmap R package (R Version 3.4). Multi-group difference scatterplot, available on omicshare (https://www.omicshare.com/), has been created. Utilizing Fisher's exact test, enrichment analysis was conducted for protein domains, various GO categories, KEGG pathways.

Results

Histogram representing the distribution of milk proteins from 13 different animals and milk proteins venn diagram

As depicted in Fig. 1A, the CHP sample exhibited the highest count of milk proteins, with 1,149 proteins being universally shared across all three samples. In contrast, the YXP milk sample displayed the lowest protein count, identifying only 442 common proteins. This observation underscores the remarkable consistency in protein composition within the same animal and underscores the credibility of the collected samples.

Analysis of subcellular localization in the milk of 13 different animals

In this study, a comprehensive subcellular localization analysis was performed on all expressed proteins, with the findings illustrated in Table 1. Notably, CHP exhibited the highest total protein content. The QHY milk sample displayed the highest concentration of cytoplasmic proteins, while the milk samples from other animals demonstrated greater extracellular protein content. It was observed that milk proteins were prominently present in both the cytoplasm and nucleus. Cytoplasmic proteins were primarily localized within cellular components such as the cell membrane, mitochondria, lysosomes, and endoplasmic reticulum (ER).

Domain analysis of milk proteins from 13 different animals

Domain prediction was conducted on the expressed proteins, and the results, displayed in Fig. 1B (top 20), revealed noteworthy trends. In the milk from CHP, LCG, NBY, SNG, TGB, GXB, HST, OAS, and YXP, the most prevalent domain was the immunoglobulin V-set domain. Both CHP and YXP milk exhibited a significantly higher count of immunoglobulin V-set domain proteins compared to other domains. On the contrary, MGH and DZD milk contained the highest number of immunoglobulin C-set domain proteins. ALC milk was characterized by a predominant presence of Ras family domain proteins, whereas QHY milk displayed a high prevalence of lipocalin/cytosolic fatty-acid binding protein family, trypsin, and immunoglobulin V-set domain proteins. The serpin (serine protease inhibitor) and sushi repeat (SCR repeat) proteins were also notably abundant in the milk protein domains.

Gene Ontology analysis of milk proteins from 13 different animals

GO analysis, categorized into biological process (BP), molecular function (MF), and cellular component (CC), was performed at the secondary functional annotation level, as depicted in Fig. 2A. The functions, localization, and biological pathways of proteins in CHP, LCG, NBY, SNG, TGB, GXB, HST, MGH, DZD, OAS, and QHY exhibited similar patterns. In BP, the protein content related to cellular process, biological regulation, and metabolic process was the highest. In MF, the protein contents associated with binding, catalytic activity, and molecular function regulator were notably prominent. CC showcased the highest protein contents for cell, cell part, and organelle. However, ALC demonstrated higher protein content in the membrane and membrane part in CC, and YXP displayed a higher protein content in the extracellular region, ranking first, aligning with the findings from the previous subcellular localization analysis. GO analysis further revealed the enrichment of proteins involved in various GO pathways, including receptor tyrosine kinase, aminopeptidase, proteasome subunit alpha, proteasome subunit beta, rab GDP dissociation inhibitor, and protein disulfide-isomerase, predominantly in pathways related to cellular processes, binding, and cellular components.

Kyoto Encyclopedia of Genes and Genomes analysis of milk proteins from 13 different animals

According to the KEGG annotation pathway results (Fig. 2B), several pathways were common to milk of most animals, including the PI3K-Akt signaling pathway, complement and coagulation cascades, endocytosis, and phagosome. However, CHP milk displayed enrichment in a wide range of pathways, including the coronavirus disease-COVID-19 pathway, pathways related to neurodegeneration and multiple diseases, human immunodeficiency virus 1 infection, amoebiasis, salmonella infection, tuberculosis, lipid metabolism, atherosclerosis, cancer-related pathways, and viral carcinogenesis. ALC milk exhibited enrichment in the phospholipase D signaling pathway and vasopressin-regulated water reabsorption. GXB milk was enriched in pathways associated with the biosynthesis of cofactors and cell cycle. HST milk had the cGMP-PKG signaling pathway and the estrogen signaling pathway as enriched pathways. DZD milk was enriched in the pathway related to other types of o-glycan biosynthesis. YXP milk showed enrichment in pathways such as spliceosome, ECM-receptor interaction, viral protein interaction with cytokine and cytokine receptor, porphyrin and chlorophyll metabolism, sphingolipid metabolism, and the ferroptosis pathway. The KEGG analysis revealed that various pathways contained relevant proteins. For example, the PI3K-Akt signaling pathway was enriched with proteins such as osteopontin, heat shock protein HSP 90-alpha, 14–3-3 protein, cAMP responsive element binding protein 3 like 1, endoplasmin, fibronectin, and thrombospondin-1. The complement and coagulation cascades pathway included proteins like clusterin, complement component C6, complement component C9, Plasminogen, C3/C5 convertase, and CD59 glycoprotein. The lysosome pathway showed enrichment of alpha-galactosidase, asparaginyl endopeptidase, and cathepsin. The protein processing in the endoplasmic reticulum pathway was enriched with proteins such as calreticulin, glucosidase 2 subunit beta, protein disulfide-isomerase, 78 kDa glucose-regulated

Table 1
The number of proteins in each mammalian subcellular organelle.

SubLocation	CHP	ALC	LCG	NBY	SNG	TGB	GXB	HST	MGH	DZD	OAS	QHY	YXP
Extracellular	587	225	354	351	351	352	256	309	324	308	295	258	261
Cytoplasmic	349	207	326	325	326	324	209	216	319	295	245	413	101
Nuclear	348	158	311	309	309	311	197	200	289	268	220	314	116
Mitochondrial	109	47	90	89	90	88	64	55	107	91	59	84	25
PlasmaMembrane	72	67	105	107	106	105	44	75	77	70	65	70	52
Lysosomal	28	27	33	33	33	33	21	24	33	33	22	26	23
ER	9	9	14	14	14	14	11	10	12	12	10	12	3
Cytoskeletal	5	1	5	5	5	5	1	1	5	5	3	4	1
Golgi	3	1	3	3	3	3	3	4	3	2	2	5	0
Peroxisomal	2	1	2	2	2	2	2	1	2	2	1	2	2

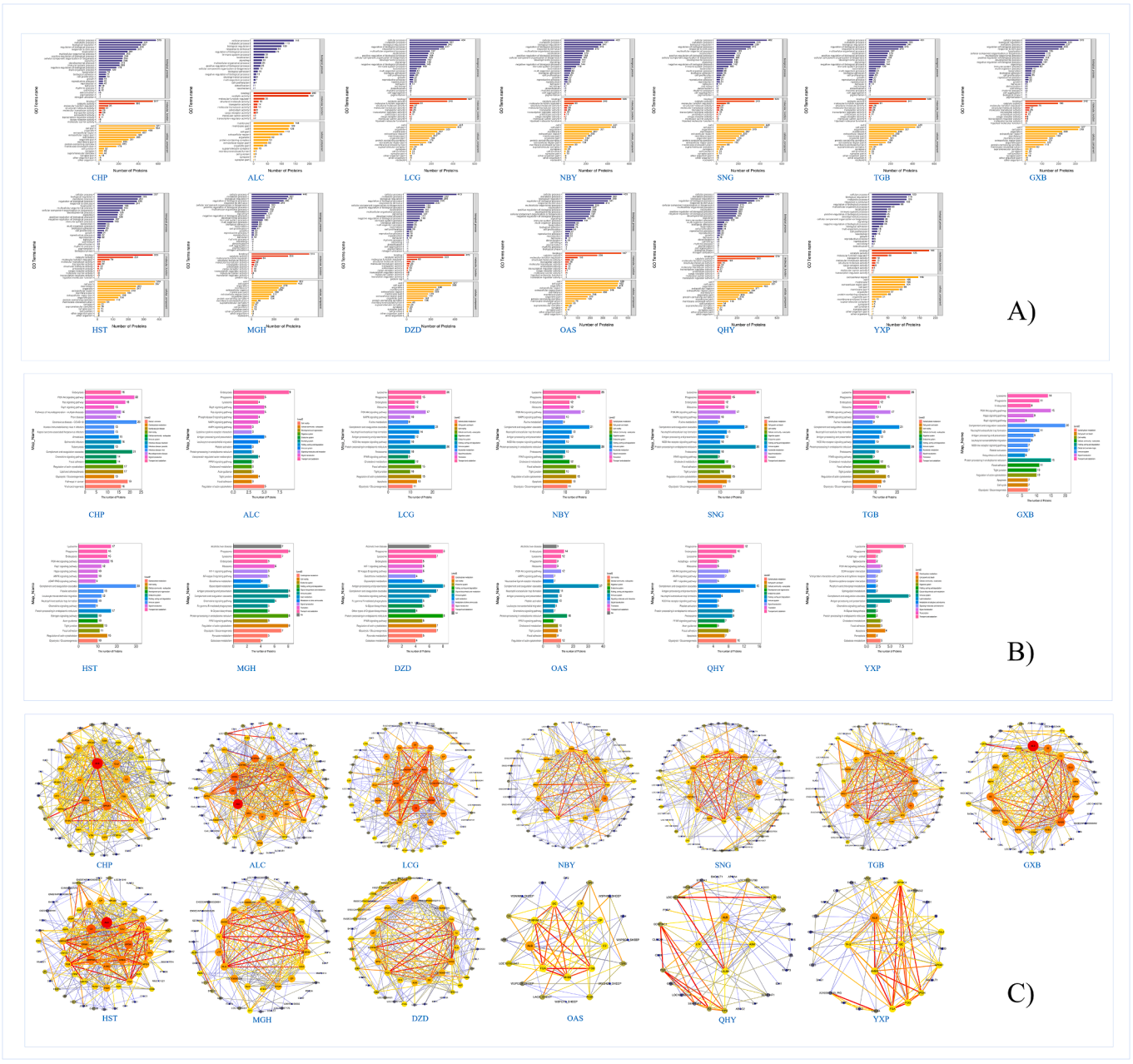

Fig. 2. A) GO functional annotation analysis of milk from 13 mammalian animals; B) Analysis of KEGG annotation pathway in milk of 13 mammals; C) Protein-protein interaction networks of the top 100 expressed milk proteins in 13 mammalian animals. In the networks, the size of the point is determined by the degree, the greater the degree, the greater the point; the color of the point is also determined by the degree, from small to large, showing a gradual change from blue to yellow to red; the thickness of the line is determined by co-expression. The larger the co-expression, the thicker the line; the color of the line is determined by co-expression, from small to large, showing a gradual change from blue to yellow to red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

protein, glucosidase II alpha subunit, and heat shock protein H.

Protein-protein interaction networks in the milk of 13 different animals

In the protein–protein interaction networks of milk proteins from 13 mammals (Fig. 2C), several key proteins such as ALB, APOA1, APOH, SERPINA1, SERPINC1, AHSG, C3, F2, GC, FGA, FGB, FGG, and more exhibited higher degrees and stronger co-expression with most milk proteins. Notably, ALB had the largest number of direct interacting proteins among other proteins in the milk of CHP, ALC, GXB, HST, OAS, QHY, and YXP.

Analysis of common proteins in milk across different animals

Here are the common proteins found in 13 animal milk (Table 2), and their corresponding expression levels (Fig. 3A). A total of 21 common proteins were identified, with Lactoperoxidase, Clusterin, and Alpha-2-HS-glycoprotein exhibiting the highest overall content among the 13 types of animal milk. In the provided data, 'Sample1' corresponds to 'LFQ intensity sample1' and 'ALC' represents 'LFQ intensity ALC' and so forth.

Table 2
Common proteins of 13 animals.

Protein name	CHP	ALC	LCG	NBY	SNG	TGB	GXB	HST	MGH	DZD	OAS	QHY	YXP
Clusterin	618865.07	175254.94	322912.16	486294.95	325705.95	380096.97	195754.70	117357.19	905286.52	25944.70	818510.54	452592.06	57306.79
Lactoperoxidase	73865.70	241566.95	1022851.12	1075357.82	1135723.47	1048854.75	21441.47	818825.17	5914.99	26878.33	89434.56	636835.53	1614.82
2-phospho-D-glycerate hydro-lyase	28348.20	4894.75	7435.56	13328.14	7476.44	15803.70	3420.07	6907.65	42488.32	800.50	3157.49	20874.03	532.70
Monocyte differentiation antigen CD14	6200.54	538290.15	31036.69	49232.51	39448.60	38747.03	221347.08	225958.61	23092.87	70459.33	107881.14	139606.49	7748.39
Galectin-3-binding protein	392188.77	6020.73	8486.37	16322.68	30830.70	27887.80	8289.49	6055.23	48894.03	3383.60	8967.25	13463.18	1259.58
45 kDa calcium-binding protein	19376.29	11010.43	41093.46	79533.34	59323.99	67061.97	68803.08	83771.11	61426.17	37242.79	114604.84	120369.59	40069.45
Aminopeptidase	3617.98	3143.15	2243.50	2543.95	1895.10	1483.24	571.63	12329.31	31929.29	14527.13	15462.34	6170.14	11058.66
Transthyretin	97941.58	17172.75	13307.25	11397.64	186812.21	153162.53	125231.68	35317.63	12948.57	2522.08	23144.05	135554.56	19313.81
Alpha-2-HS-glycoprotein	76822.21	500481.83	396912.99	238748.78	308822.45	296997.46	81107.77	228117.72	182122.14	120178.30	491099.54	401657.90	317164.60
Chitinase-3-like protein 1	12561.35	574.70	2802.16	5631.76	3231.74	5342.23	3719.91	13839.10	33789.44	5657.48	16519.20	7417.19	16288.23
Receptor protein-tyrosine kinase	24588.78	1841.25	16487.83	14215.50	21592.14	19007.90	3370.29	432.22	4148.39	1990.87	1949.69	16445.62	653.06
Alpha-dystroglycan	19051.74	34301.76	10745.62	24684.86	23939.01	20942.51	15124.73	38223.85	18115.28	7143.61	18708.12	48115.53	63983.75
Asparaginyl endopeptidase	7184.18	64296.14	508.88	1005.62	412.40	941.47	44823.31	29191.14	17160.26	3998.37	6909.96	9499.76	68858.54
Complement component 8 subunit beta	3303.79	13365.27	9442.68	5527.69	4835.22	4088.09	3291.14	8209.07	7494.11	692.20	10071.01	5725.85	1828.41
Na(+)-dependent phosphate cotransporter 2B	10573.21	95176.76	8305.60	20742.61	11591.11	15106.42	85248.88	150090.92	5223.10	6259.82	536.56	22027.63	4444.73
Fibrinogen beta chain	2968.96	123001.33	96324.84	143173.38	63456.15	153712.51	33218.95	107226.41	24608.55	11521.18	80409.46	47451.45	20561.28
Cathepsin B	6652.08	50714.89	20076.36	48081.57	12328.70	35179.97	19162.25	35618.47	288041.70	84499.87	90075.83	66529.22	23170.53
60S acidic ribosomal protein P2	6262.06	15955.24	274339.58	288480.23	465987.22	500408.02	6419.64	241.13	1911.91	81.78	15098.91	23107.84	141.57
Alpha-mannosidase	2900.91	1107.66	4630.96	9050.09	6090.70	13151.59	9026.24	3818.86	16099.68	3075.51	27229.97	4347.38	3438.69
Superoxide dismutase [Cu-Zn]	22055.54	45115.55	27497.06	32184.19	29873.06	31884.82	7050.44	51209.98	40744.95	3140.14	110777.27	6471.01	16156.59
Tetraspanin	169935.87	30663.83	36087.13	59620.92	50339.63	39806.81	794.03	4185.70	3307.00	710.53	18665.22	22104.93	14881.41

Statistical analysis of milk fat globule membrane proteins in 13 different types of animal milk

The manuscript provides a comprehensive overview of the main MFGM proteins in the milk of 13 animals, showcasing variations in protein types and quantities (Fig. 3B). The chart illustrates that CHP milk has the most diverse array of protein types, followed by MGH, DZD, and HST. In terms of MFGM protein content, CHP, ALC, HST, and OAS milk exhibit the highest levels, with SNG, TGB, NBY, and LCG following closely. Xanthine dehydrogenase is most abundant in CHP and HST milk. Fatty acid binding protein, on the other hand, is highest in LCG, SNG, TGB, NBY, CHP, and OAS milk. Lactadherin takes the lead in ALC, OAS, QHY and HST milk.

Difference analysis of mature milk in 13 animals

In the UniProt multi-species integrated database, a comprehensive identification yielded a total of 4,445 proteins, comprising 2,245 proteins characterized by differential expression. The differential quantification of proteins was assessed through One Way ANOVA analysis, wherein a significance level of $p < 0.05$ was deemed indicative of noteworthy disparities in protein expression levels.

Cluster analysis

Fig. 4A illustrates a notable similarity in the expression trends among the three samples within each animal group. There is a resemblance in the expression patterns of milk proteins in the SNG, TGB, NBY, and LCG groups. Similarly, a concordance is observed in the expression trends of milk proteins in the QHY and HST groups. Noteworthy is the similarity in protein expression trends between the MGH and DZD milk groups.

Multi-group difference scatterplot

To elucidate the substantial variances in protein expression between the compared groups, a multi-group difference scatterplot was employed, incorporating two key factors: the fold change in expression and the P value derived from a T-test. In Fig. 4B, proteins exhibiting a noteworthy down-regulation were denoted in green ($FC < 0.5$ and $p < 0.05$), those with a significant up-regulation were highlighted in orange ($FC > 2.0$ and $p < 0.05$), while proteins manifesting no significant difference were depicted in gray.

Domain analysis

In the diagram of domain enrichment analysis, the pathways associated with Immunoglobulin C1-set domain, Lipocalin/cytosolic fatty-acid binding protein family, and Cystatin domain exhibited a notably higher significance (Fig. 4C).

Gene Ontology functional enrichment analysis

GO functional enrichment bubble diagrams are employed to illustrate the enrichment of GO entries within each of the three GO categories. There is significant enrichment observed in the BP category for pathways related to negative regulation of protein metabolic process and negative regulation of cellular protein metabolic process. Within the biological process, these pathways exhibit significant enrichment and are encompassed within cellular process, biological regulation, and metabolic process pathways. Moving to the MF category, pathways associated with enzyme inhibitor activity, peptidase inhibitor activity, endopeptidase regulator activity, and endopeptidase inhibitor activity demonstrate significant enrichment. A majority of these pathways are included in the molecular function regulator. Within the CC category, pathways such as extracellular region, extracellular space, and extracellular region part exhibit significant enrichment (Fig. 4D-F).

Kyoto Encyclopedia of Genes and Genomes functional enrichment analysis

In the KEGG pathway enrichment bubble diagram (Fig. 4G), it is evident that complement and coagulation cascades, hematopoietic cell

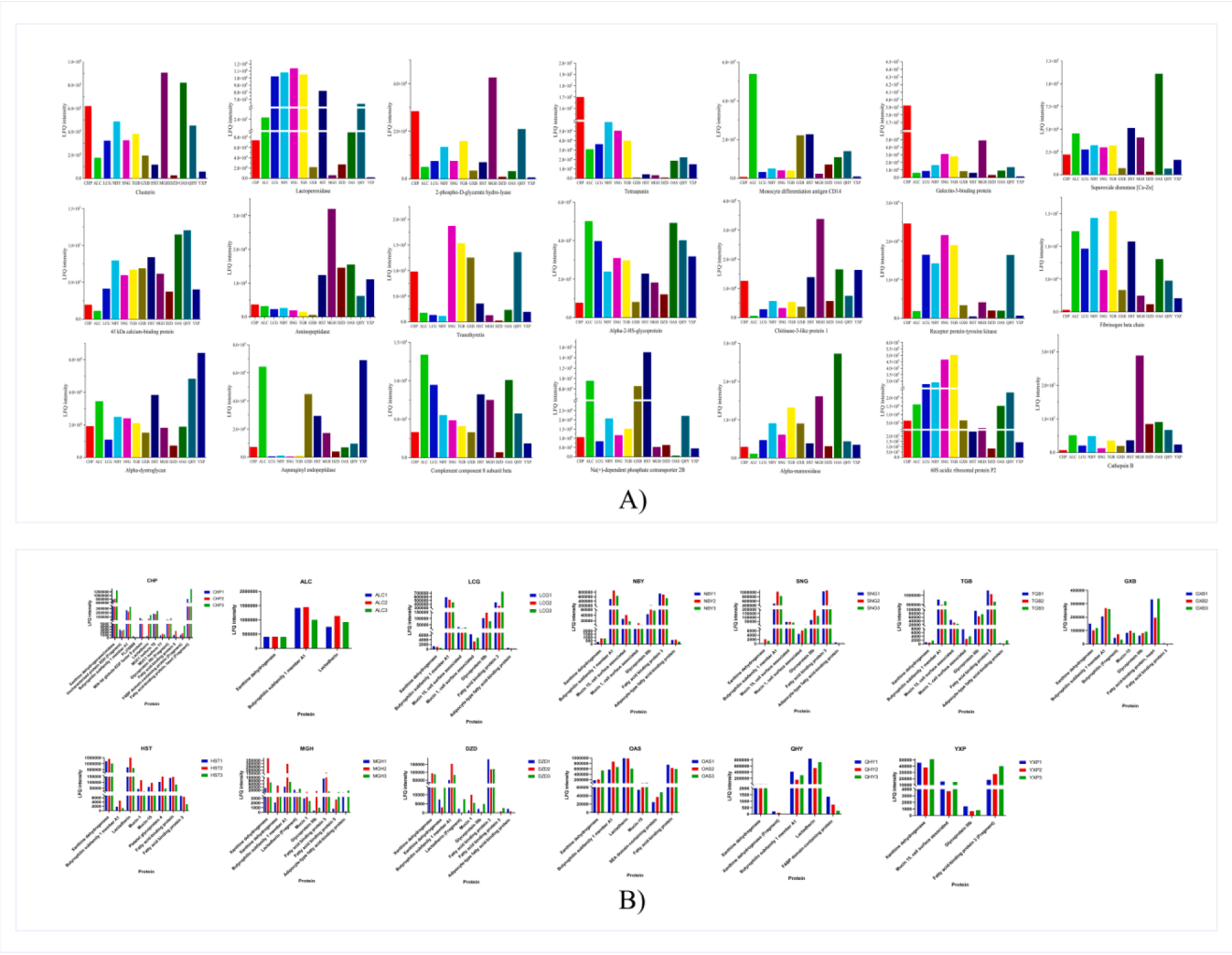

Fig. 3. A) Common proteins of 13 animals; B) MFGM proteins of 13 animals.

lineage, and intestinal immune network for IgA production pathway were notably significant.

Discussion

In previous studies, 237 proteins were detected in human milk and 843 proteins were detected in goat milk by Label-Free quantification (Chen et al., 2019; Dekker et al., 2022). In this study, advancements in technology have enhanced detection sensitivity, allowing for the identification of a broader range of animal milk proteins. Specifically, 4D-Label-Free technology revealed 1193 proteins in CHP milk. Goat milk exhibited over 1,000 detected proteins. In addition to this study, 4D-Label-Free technology was also applied to the detection of human whey proteins and MFGM proteins(C. N. Wang et al., 2023; Xia et al., 2024). Meanwhile, UA cleavage was chosen in this study, the separation was carried out by the nano-upflow HPLC system Easy nLC. Applying an acetonitrile system and adding the appropriate acid to change the pH can help minimize matrix effects(Du & White, 2008). Through localization analysis, it was evident that the majority of milk proteins in each animal were extracellular proteins, followed by a significant number of proteins in the cytoplasm and nucleus. Extracellular proteins are typically secreted after cellular synthesis, including hormones and antibodies, and play various roles in the body. The chemical reactions within cells primarily occur in the cytoplasm, while nuclear proteins are predominantly composed of genetic material. Domain analysis revealed the presence of serpin in all animals, a protein that regulates critical proteolytic cascades, including the mammalian coagulation pathway. Additionally, the sushi repeat domain was identified, promoting angiogenesis, a vital process for animals. These findings contribute to a deeper understanding of the complexity of milk protein composition across various animals(Miljkovic-Licina et al., 2009). In comparison with other domains, the number of immunoglobulin V-set domains in CHP, HST, and YXP milk was notably higher. This observation indicates that these milk possess enhanced immune functionality and exhibit substantial similarities, potentially contributing to overall health improvement.

In the analysis of GO functional annotations, it was discerned that within the Biological Process category, the protein content associated with cellular processes, biological regulation, and metabolic processes exhibited the highest representation. Within the Molecular Function category, a conspicuous prevalence of proteins linked to binding, molecular function regulation, and catalytic activity was observed. Moreover, within the Cellular Component category, the preeminent protein content was attributed to the categories of cell, cell part, and organelle. These outcomes align harmoniously with prior research findings, further solidifying the precision of our experimental approach and emphasizing the robustness and reliability of our experimental methodology(Zhao et al., 2023). Based on the KEGG pathway annotation results, the pathways shared across the majority of milk encompass the PI3K-Akt signaling pathway, endocytosis, phagosome, complement and

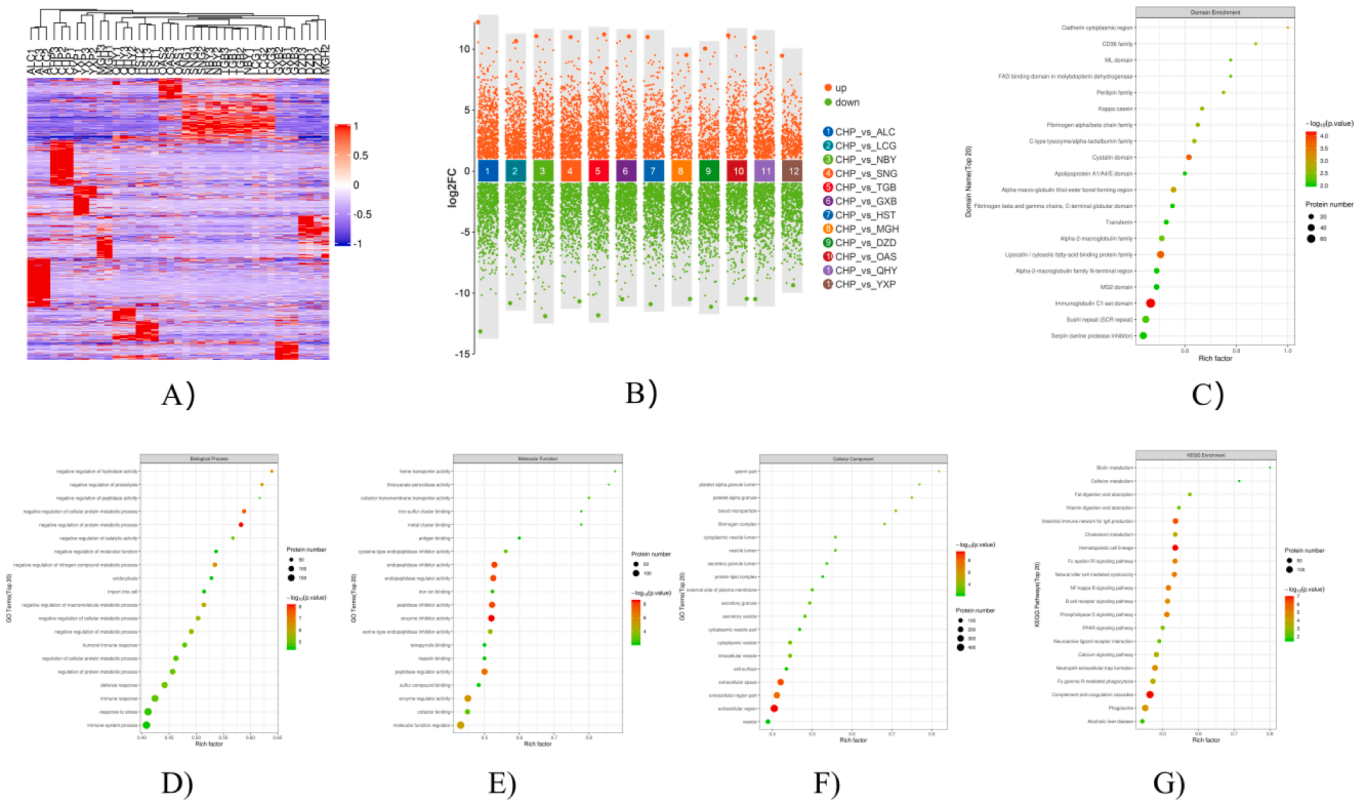

Fig. 4. A) Cluster analysis of differentially expressed proteins; B) Multi-group difference scatterplot; C) Structural domain enrichment bubble diagram; D ~ F) GO Function Enrichment Bubble Charts (D: BP; E: MF; F: CC); G) KEGG functional enrichment bubble map.

coagulation cascades, antigen processing and presentation, lysosome, protein processing in the endoplasmic reticulum, and glycolysis/gluconeogenesis. The coronavirus disease-COVID-19 pathway is exclusive to CHP milk proteins. Notably, the proteins within these pathways are implicated in cancer, anti-inflammatory responses, and immune functions. ALB (albumin) emerges as a central protein with the highest degree and co-expression in the PPI networks of CHP, ALC, GXB, HST, OAS, QHY, and YXP milk. It is conceivable that ALB plays a multifaceted role in immune regulation, lipid metabolism, calcium metabolism, antioxidant activities, and nutrient delivery for infants, potentially in synergy with other milk proteins(Jahanban-Esfahlan et al., 2019). Temperature and pH can affect the degree of protein aggregation. In a related study, it was found that heat treatment and addition of calcium ions to whey proteins to denature them and cause protein aggregation increases protein–protein interactions, and its application in infant formulas can increase their viscosity for processing(Joyce et al., 2017).

The levels of MFGM proteins exhibit their highest concentrations in CHP, ALC, HST, and OAS milk, followed by SNG, TGB, NBY, and LCG milk. Certain MFGM proteins play a crucial part in human health. The content of lactadherin was the highest in ALC, OAS, HST and QHY milk. Lactadherin enhances the development of the immune system of pups and maintains intestinal health. This adaptive response helps pups improve their viability. At the same time, it can help prevent rotavirus infection, and it has been speculated that it may be related to infant intestinal development(Brink & Lönnerdal, 2020). Fatty acid binding protein, an antimicrobial protein in the gut of infants, also works in concert with lactadherin against rotavirus(Mwape et al., 2017). However, it also serves other functions, fatty acid binding protein 3, which assumes a pivotal role in the PPAR signal transduction pathway. This role involves the regulation of key molecules within the fatty acid metabolism pathway(Jia et al., 2021). Fatty acid-binding protein, heart, serves as a biomarker for various heart diseases, it may worsen the symptoms in patients with COVID-19(Yin et al., 2020). This underscores the significant role that fatty acid binding proteins play in the detection of animal health. Research has reported a potential link between the deficiency of xanthine dehydrogenase and infant molybdenum cofactor deficiency, suggesting that the normal expression of xanthine dehydrogenase is a crucial factor for the healthy growth of infants(Per et al., 2007). Similarly, xanthine dehydrogenase deficiency results in hypouricemia, as this enzyme is responsible for the conversion of xanthine to uric acid(Köksoy et al., 2023). The key MFGM proteins are abundant in CHP, ALC, HST, OAS, LCG, NBY, SNG, TGB milk, which could potentially be beneficial for the update of infant formula composition.

The pathways identified as significant through single-pool analysis in animals and difference analysis across multiple species pools encompass cellular process, biological regulation, metabolic process, and molecular function regulator. This further confirms the high significance of these biological process pathways, indicating substantial biological differences in the aforementioned aspects. Utilizing the KEGG enrichment bubble diagram, notable distinctions in the modulation of immune response, coagulation function, and hematopoietic function among 13 varieties of animal milk have been identified. The specific proteins associated with these pathways may exert pivotal roles.

Cathepsin B is a papain-like cysteine peptidase protein that regulates the autophagy process in adipocytes to treat metabolic diseases such as obesity and diabetes(Araujo et al., 2018). Asparaginyl endopeptidase is beneficial in protecting podocytes from damage and in the treatment of diabetic nephropathy(Lei et al., 2022). Cathepsin B and asparaginyl endopeptidase in OAS milk, QHY milk and YXP milk exist in both the antigen processing and presentation pathway and the lysosome pathway. It reflects the importance and commonality of these proteins in immune response, and the potential molecular mechanism still needs to be studied. In related metabolomics studies, the presence of erythritol in the milk of these animals was found to play an active role in the treatment of diabetes(Z. Y. Wang et al., 2023), possibly synergizing with the above proteins. The data presented clearly demonstrates that

lactoperoxidase exhibited the highest expression levels in goat milk, followed by HST milk, QHY milk and ALC milk. It suggested a potential correlation between lactoperoxidase levels in cow and goat milk and breast infection. EL-Fakharany's study validated that the concentration of lactoperoxidase in cow, goat, and camel milk surpassed that in human milk(El-Fakharany et al., 2017). Concurrently, it was observed that the lactoperoxidase content in horse and donkey milk is relatively low. In the cow mammary gland, lactoperoxidase exhibits a synergistic antibacterial effect alongside lingual antibacterial enzymes(Isobe et al., 2009). Research findings indicate that lactoperoxidase in Sannen goat milk possesses robust bactericidal properties against Staphylococcus aureus strains(Novac & Andrei, 2020). This suggests that goat milk may be more beneficial in enhancing the infant's resistance to infections. The up-regulated proteins, galectin-3-binding protein (Gal-3BP) and tetraspanin of CHP, were identified in both the volcano plot results of ANOVA analysis and the common proteins across individual libraries. Tetraspanin is a remarkably conserved protein family that holds a pivotal role in various cellular processes, including cell migration, signal transduction, and protein transport(Susa et al., 2023). Galectin-3-binding protein, a multifunctional glycoprotein, assumes a pivotal role in antiviral defense and cancer. As a biologically active substance within the immune system, it exerts an impact on the human immune system, further serving as an indicator of maternal health, with elevated levels observed in the breast milk of AIDS patients(Chan et al., 2013). At the same time, Gal-3BP has an effect on adipose tissue, mainly involving the proliferation and differentiation of fat, which is increased in adolescent obesity and metabolic syndrome(Zhen et al., 2021). Furthermore, research indicates that Gal-3BP and its receptor Gal-3 are secreted proteins serving as initiators of signaling cascades in various diseases (DeRoo et al., 2015). Ana Mendes-Frias et al. proposed that Gal-3BP is a marker of the COVID-19 virus and has the potential to treat critically ill patients with COVID-19(Mendes-Frias et al., 2022).

Conclusion

In this study, we employed 4D-Label-Free proteomics to comprehensively investigate the diversity of milk protein components in 13 different animal species. We identified a total of 1,149 common proteins in human milk, which was the highest among the examined species, while pig milk exhibited the lowest number with 442 common proteins. The domain analysis revealed that across all types of animal milk, a notable number of domain proteins were associated with immune-related functions. Among these, ALB protein emerged as the key protein with the highest interaction frequency in the PPI network diagram. Difference analysis indicated significant enrichment of KEGG pathways in the 13 types of animal milk, particularly in complement and coagulation cascades, hematopoietic cell lineage, and the intestinal immune network for IgA production pathway. A comprehensive examination of data from both the individual species library and the UniProt multi-species common library revealed that Galectin-3 binding protein (Gal-3BP) and tetraester protein in human milk exhibited significantly higher levels compared to other animal milk. Moreover, we identified both the common proteins in the milk of these 13 animal species. Lactoperoxidase was found to be abundantly present in goat milk, suggesting its potential role in enhancing infants' resistance to infections. The abundant presence of MFGM proteins in human milk, camel milk, goat milk, and sheep milk suggests their potential to provide additional nutrition, immune support, and bioactive compounds, offering significant benefits for the brain development and overall health of infants and children. Future research can explore the potential applications of these proteins as milk alternatives and investigate their utility as cancer biomarkers in milk sample screening.

Fundings

This work was supported by National Key Research and Development Program of China [2023YFD1300204]; National Natural Science Foundation of China [No. 32272836]; and Postdoctoral Science Foundation of China [2021 M693859].

CRediT authorship contribution statement

Rui Chen: Writing – original draft, Formal analysis. **Yinggang Sun:** Data curation, Conceptualization. **Yanzhi Wu:** Software, Methodology. **Yanjun Qiao:** Supervision, Investigation. **Qiu Zhang:** Supervision, Investigation. **Qian Li:** Supervision, Investigation. **Xiaowei Wang:** Supervision, Investigation. **Yuan Pan:** Supervision, Investigation. **Siyi Li:** Supervision, Investigation. **Yining Liu:** Supervision, Investigation. **Zeying Wang:** Writing – review & editing, 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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101263.

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