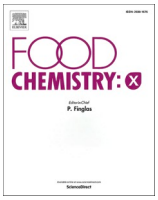
Food Chemistry: X 22 (2024) 101384

Contents lists available at ScienceDirect

Food Chemistry: X

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



Novel insights into whey protein among Yak, Yellow Cattle, and Cattle-Yak milk

Yufang Li [a,1], Shijun Li [a,1], Xingwen Zhao [b], Chongying Shi [a], Yunmei Chai [a], Aixiang Huang [a,*], Yanan Shi [a,*]

[a] College of Food Science & Technology, Yunnan Agricultural University, Kunming 650201, China
[b] College of Food Engineering, Dali Vocational and Technical College of Agriculture and Forestry, Dali 671003, China

ARTICLE INFO

Keywords:
Yak milk
Proteomics
Characteristic whey protein
Bioactive peptides
Immune activity

ABSTRACT

This study identified characteristic whey proteins from Zhongdian Yak (ZY), Diqing Yellow Cattle (DYC), and Cattle Yak (CY), revealing insights into their potential functions and released peptides. A total of 118 whey proteins were quantified in milk obtained from the three breeds of cattle, including seven characteristic proteins (IGL@ protein, 40S ribosomal protein S9, calreticulin, etc.) in CY milk and two characteristic proteins (RNA helicase and uncharacterized protein (A0A3Q1LFQ2)) in ZY milk. These characteristic proteins are involved in the phagosome and Fc gamma R-mediated phagocytosis pathways, exhibiting immunoprotective activities, verified through molecular docking. Furthermore, the molecular docking results showed five whey proteins (IGL@ protein, rho GDP-dissociation inhibitor 1, small monomeric GTPase, action-like protein 3, and adenylyl cyclase-associated protein) interacted with TLR4 through multiple hydrogen and hydrophobic bonds. Therefore, these proteins may exert immunomodulatory functions by inhibiting TLR4. Meanwhile, whey proteins produced bioactive peptides, such as antioxidant peptides and ACE inhibitory peptides after simulated gastrointestinal digestion (SGID). The whey proteins and bioactive peptides from CY exhibited more types and activities than the ZY and DYC whey proteins. This study provides a theoretical basis for promoting formula milk powder production.

1. Introduction

Milk is a major infant food, providing primary nutrition for infants and newborn mammals (Yang et al., 2013). As one of the prime proteins in milk with high nutritional value, whey proteins have received widespread attention, including immunoglobulins, β-lactoglobulin, α-lactalbumin, lactoperoxidase, and lactoferrin (Pereira, 2014). Whey proteins account for 20% of the total milk protein and possess many functional factors beneficial to the human body, such as promoting growth and development, enhancing immunity, and regulating intestinal flora (Maity & Ambatipudi, 2019). Moreover, milk-derived immunoglobulins are crucial in infant immunity (Hurley & Theil, 2011). Lactoferrin regulates immunological, antimicrobial, and antioxidant activity. However, several studies have reported that the whey protein components are influenced by the breeds, health status, and diet of the cattle (Roin et al., 2022). Additionally, gastrointestinal digestion may induce the release of bioactive peptides from whey protein sequences, which exert different biological functions towards different target organs in the body (Aspri, Leni, Galaverna, & Papademas, 2018). Therefore, it can be inferred that the whey proteins and their released bioactive peptides are complex and diverse.

China has the largest yak population, with 14 million, accounting for 95% of the global yak population. Yak milk is well known for its specific composition, making it a premium raw resource for producing foods for infants and the elderly, meeting the particular needs of certain populations (He et al., 2011). So far, many studies have been conducted on yak milk in the main yak-producing areas, including the studies on the nutritional components of yak milk at different altitudes of the Qinghai-Tibet Plateau (Fan, Wanapat, & Hou, 2020). However, very few studies focused on whey protein and its bioactive peptides. Yang, Zhao, Yu, and Cao (2015) identified 183 whey proteins in the colostrum and mature milk of yak from Qinghai province using quantitative iTRAQ-labeling proteomics; of which, 86 proteins showed differential expression in colostrum and mature milk. Zhongdian yak (ZY), widely distributed in

* Corresponding author.
E-mail addresses: aixianghuang@126.com (A. Huang), yananshihaha@126.com (Y. Shi).
[1] These authors contributed equally to this work and should be regarded as co-first author.

https://doi.org/10.1016/j.fochx.2024.101384
Received 18 December 2023; Received in revised form 8 April 2024; Accepted 9 April 2024
Available online 16 April 2024

Xianggelila, Yunnan Province, is one of the primary yak breeds in China, with excellent milk quality (Liu et al., 2021). Diqing yellow cattle (DYC), one of the local varieties in Yunnan Province, is a plateau cattle with strong adaptability to the ecological environment in high altitude areas. Cattle-yak (CY) is a crossbreed of ZY (female parent) and DYC (male parent) (He et al., 2011; He et al., 2015). ZY, DYC, and CY milk are the essential raw materials for Xianggelila farmers and herders to produce ghee, milk residues, and other necessities. Nevertheless, there are very few studies on these three kinds of milk. He et al. (2015) studied the basic nutrient contents of ZY, DYC, and CY milk. However, the composition and function of whey proteins in ZY, DYC, and CY remain is still unclear. Although whey protein is an essential component of milk and a by-product of cheese and ghee production with many functional activities, there has been a gradual waste of whey protein due to the lack of comprehensive research. Therefore, studying the whey protein components of ZY, CY, and DYC milk and their functions has great significance.

Mass spectrometry-based proteomics technology is mainly used to systematically identify and characterize the structure, function, activity, and quantity of proteins (Rajczewski, Jagtap, & Griffin, 2022). So far, many studies have assessed whey proteins in milk from various species using proteomics. Proteomics includes non-standard quantitative proteomics and labeled quantitative proteomics. Quantitative proteomics mainly includes label-free quantitative proteomics and tandem mass tag proteomics. Tandem Mass Tags (TMT) technology uses various isotope tags, which can react with the amino groups to establish a connection. Besides, high-precision mass spectrometry analysis can simultaneously achieve qualitative and quantitative analyses of multiple sample proteomes with high sensitivity and accurate results (Guo, Yu, Cupp-Sutton, Liu, & Wu, 2022). A total of 989 and 1534 whey proteins were quantified in donkey and bovine milk using HPLC-MS/MS-based proteomics (Li et al., 2021a; Li et al., 2021b). Sun, Wang, Sun, and Guo (2020) quantified 165 whey proteins in Guanzhong goat milk and Holstein cow milk using iTRAQ proteomics, of which 114 were significantly different in abundance. Han, Zhang, and Zhou (2023) analyzed whey proteins in bovine, goat, and camel milk using label free proteomics. The proteins, such as PAEP, CST3, SERPING1, CTSB, and GLG1, serve as important markers in the classification of these three species. Li et al. (2022) identified and quantified 981, 988, 1127, and 1534 whey proteins in donkey colostrum, donkey mature milk, bovine colostrum, and bovine mature milk using proteomics. Whereas the differences in the proteomes of milk whey proteins in Murrah, Nili-Ravi, and Mediterranean buffaloes were identified using TMT proteomics (Li et al., 2018). Abdel-Hamid et al. (2022) analyzed and quantified 638 whey proteins in Mediterranean and Murrah mature milk using TMT proteomics, of which lactoferrin and ferritin showed higher levels in Mediterranean and Murrah mature milk.

Therefore, the present study aimed to identify, characterize, and analyze the whey proteins in milk from ZY, DYC, and CY using TMT proteomics. The potential physiological function of whey proteins from ZY, DYC, and CY was explored. Additionally, the bioactive peptides released from whey proteins after simulated gastrointestinal digestion were also analyzed. The results provide insights into specific compositions of whey proteins (ZY, DYC, and CY) and a theoretical basis for developing and utilizing characteristic milk in the Yunnan plateau. Moreover, a better understanding of the unique characteristics of the protein composition of native Yunnan Province cattle breeds might instigate better utilization and development of dairy products with specific properties and avoid the waste of whey protein resources.

2. Materials and methods

2.1. Sample collection and whey protein preparation

Twelve milk samples were collected from ZY, DYC, and CY simultaneously reared on a dairy farm in Xianggelila City, Yunnan Province, China, respectively. Twelve milk samples from different breeds (ZY, DYC, and CY groups) were divided into three groups as three replicates, respectively. Basic information on the cattle breeds from which the milk samples were collected is shown in Table S1. All cattle breeds (aged between four and ten years) were in good health, under the same feeding environment, and receiving the same feed daily.

Three milk samples from each cattle group were centrifuged at 4 °C and 4000*g* for 15 min to remove fat. Briefly, the pH of the skim milk samples was adjusted to 4.6 using 33% acetic acid and kept at 25 °C for 15 min. Later, the mixture was centrifuged at 4000*g* for 25 min at 20 °C to remove casein. Afterward, the supernatant containing the whey proteins was collected, and the whey protein was stored at −80 °C until further analyses.

2.2. SDS profile of whey proteins

The whey proteins (20 μg) from each sample were mixed with a loading buffer at a ratio of 1:4, respectively, and boiled for 10 min. The proteins were resolved and separated on 12% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were visualized using Coomassie Blue R-250 staining.

2.3. Protein digestion and TMT labeling

A total of 100 μL of protein and 400 μL of pre-cooled acetone were mixed and precipitated at 20 °C overnight. Then, the sample was carefully transferred to a new centrifuged Eppendorf (EP) tube after centrifugation at 12,000 rpm and 4 °C for 10 min. Later, 200 mL of pre-cooled 80% acetone was added to the EP tube; the mixture was rinsed twice and then centrifuged at 12,000 rpm and 4 °C for 10 min. Afterward, the supernatant was carefully transferred to a new EP tube, and the protein precipitation was dissolved in an ultrasound water bath for 10 min. The disulfide bond was reduced by adding 1 μL of dithiothreitol to obtain a final concentration of 5 mM and incubating at 55 °C for 10 min. The sample was cooled to room temperature and the reduced disulfide bonds were alkylated by adding 2 μL of iodoacetamide to obtain a final concentration of 10 mM and avoiding a light reaction for 15 min. After alkylation, trypsin was dissolved using 0.5 μg/L of resuspension buffer and incubated at room temperature for 5 min. The sample was ultra-centrifugated and the proteins were incubated overnight at 1000 rpm and 37 °C.

Finally, the sample was centrifuged at high speed for 10 min, and the supernatant was transferred to a new EP tube. Equal amounts of samples were labeled following the instructions of the Thermo's TMT labeling kit (Thermo Fisher Scientific, Waltham, MA, USA). The labeled samples of each experiment were transferred into a centrifuge tube after labeling. After drying, it was divided into 12 components by reverse phase chromatography. Each fraction was fully dissolved in formic acid (FA, 0.1%) after drying and then analyzed by LC-MS.

2.4. LC-MS/MS identification and quantification of TMT labeled peptides

2.4.1. LC-MS/MS analysis

The LC-MS/MS analysis was performed according to the procedure described by Zhang et al. (2021) with some modifications. A total of 1 μg of protein in each sample was analyzed using the EASY-nLC1200 nano-ultra performance liquid chromatography (UPLC) system (Thermo Fisher Scientific, MA, USA) equipped with a Q Exactive HFX Orbitrap instrument (Thermo Fisher Scientific, MA USA) and a nano-electrospray ion source (Thermo Fisher Scientific, MA USA). The peptides were separated by a reversed-phase column (100 ID×15 cm, Reprosil-Pur 120 C18-AQ, 1.9, Dr. Maisch). The mobile phases were as follows: 0.1% formic acid and 2% acetonitrile as phase A and 80% acetonitrile and 0.1% formic acid as phase B. The sample was separated using a linear gradient of 5–95% gradient B for 90 min at a constant flow rate of 300 nL/min. The gradient B was 2–5% for 2 min, 5–22% for 68 min, 22–45% for 16 min, 45–95% for 2 min, and 95% for 2 min. The data were

obtained in a data-dependent acquisition mode (DDA) in a positive mode using an orbitrap analyzer. The six most intense ions in each survey scan were subjected to MS/MS analysis within the *m/z* range of 350–1600 (MS1) and 110 (MS2). The single-charged peaks and the peaks with a charge exceeding six were excluded from the DDA procedure.

2.4.2. Database search and quantification of raw data files

The raw MS files were processed using the Proteome Discoverer (PD) software (Version 2.4.0.305) and the built-in Sequest HT search engine. Next, the MS spectra lists were searched against their species-level UniProt FASTA database 9 aurusiprot-Bos taurus_9913-2021-9.fasta), with Carbamidomethyl [C], TMT 6 plex (K), and TMT 6 plex (N-term) as the fixed modification, Oxidation (M), and Acetyl (Protein Nterm) variable modifications, respectively. A maximum of 2 missed cleavage(s) were allowed. The false finding rate was established at 0.01 for the PSM and peptide levels. The peptides were identified using an initial precursor mass deviation of up to 10 ppm and a 0.02 Da particle mass deviation. The differentially expressed whey proteins were selected using *t*-test at $P < 0.05$ and cut-off points > 1.2 and < 0.83 for increased and decreased proteins, respectively.

2.5. Multivariate and bioinformatic analyses

The cluster heatmap analysis was performed using Heml_1.0 software (CUCKOO Workgroup, Wuhan, China; http://hemi.biocuckoo.org/). The functional categories, gene ontology (GO; http://geneontology.org/) classification, and Kyoto encyclopedia of genes and genomes (KEGG; https://www.genome.jp/kegg/) pathways were analyzed using the online database for annotation, visualization, and integrated discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp). The protein-protein interaction network was analyzed using STRING software.

2.6. Molecular docking study

The crystal structures of IGL@ protein (Q3T101), rho GDP-dissociation inhibitor 1 (A0A452DJ82), small monomeric GTPase (A6QLB1), action-like protein 3(A0A3Q1MXB7), adenylyl cyclase-associated protein(A0A3Q1LJQ7), and Toll-like receptor 4(Q8SQ55) were deduced from the Protein Structure Database (https://www.alphafold.ebi.ac.uk/). Finally, molecular docking analysis was performed on the HDOCK server (http://hdock.phys.hust.edu.cn/). The peptide-protein binding was visualized using LigPlot (Krobthong et al., 2023).

2.7. In vitro digestion of whey protein samples

Simulated in vitro gastrointestinal digestion of whey protein from ZY, DYC, and CY milk was conducted following the method of Liu et al. (2020) with some modifications. Briefly, the samples of ZY, DYC, and CY milk were preheated at 37 °C, and their pH values were adjusted to 1.5 with 1.0 M HCl. Then, pepsin was added (2%, *w*/w of protein), and the mixture was incubated at 37 °C for 2 h with continuous shaking. The pH was adjusted to 7.5 using 1.0 M NaOH. Subsequently, pancreatin was added (2%, w/w of protein) and the mixture was incubated at 37 °C for 2 h with continuous shaking. The reaction mixture was kept in boiling water at 95 °C for 10 min to stop the digestion. The experiment was performed in triplicate. The supernatant was collected by centrifugation (8000 ×*g* for 15 min, 4 °C) and sieved through a 3 kDa ultrafiltration membrane to obtain the peptides with molecular weights (MWs) <3 kDa. The peptides were freeze-dried by a rotary evaporator using liquid nitrogen and stored at −80 °C until further analysis.

2.8. Peptidomics profiling by liquid chromatography-mass spectrometry (LC-MS/MS)

The samples (MWs <3 kDa) were separated by UPLC, and LC-MS/MS identified the peptides. Briefly, LC-MS/MS analysis was performed on a Q Exactive mass spectrometer coupled to Easy nLC (Thermo Fisher Scientific, Germany). The peptide mixtures were loaded onto a C18-reversed phase column (15 cm long, 75 μm inner diameter) packed in-house with RP-C18 5 μm resin of buffer A (0.1% Formic acid in HPLC-grade water) and separated with a linear gradient of buffer B (0.1% Formic acid in 84% acetonitrile) at a flow rate of 250 nL/min. Later, the reaction was controlled by IntelliFlow technology for over 60 min. The MS data were acquired using a data-dependent top 10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 *m/z*) for HCD fragmentation. The target value was determined based on predictive Automatic Gain Control (pAGC). Dynamic exclusion duration was 20 s. Survey scans were acquired at a 70,000 resolution, 200 *m/z*, and 17,500 HCD resolution spectra, 200 *m/z*. Normalized collision energy was 27 eV, and the underfill ratio specifying the minimum percentage of the target value likely to be reached at the maximum fill time was defined as 0.1%. The instrument was run with peptide recognition mode enabled.

The MS/MS raw files were processed using MaxQuant software (version 1.5.5.1). The processed MGF files were searched against the UniProt_camelidae_89471_20201019 (containing 89, 471 sequences, downloaded on Oct 19, 2020) without specifying the enzyme cleavage rules. The search parameters, including fragment mass tolerance, were 20 ppm and 0.02 Da. The cutoff value of the global false discovery rate (FDR) for peptide identification was set to 0.01. The identified peptides were scored using the PeptideRanker (https://distilldeep.ucd.ie/PeptideRanker/) database. The potential peptide candidates were searched against the BIOPEP database (https://www.uwm.edu.pl/biochemia/index.php/en/biopep).

2.9. Statistical analysis

Statistical analysis was performed using one-way and two-way analysis of variance (ANOVA). The values of $P \leq 0.05$ were considered statistically significant. Statistical analyses were performed using GraphPad Prism 9.4.1, SPSS 25.0 software, and Origin 2021 software. The data were represented as means ± SD.

3. Results and discussion

3.1. Major whey proteins and their potential functions in milk

The identification of the significant bands in the whey proteins of ZY, DYC, and CY milk via SDS-PAGE (Fig.1A) indicates the effectiveness of the whey proteins extraction. The TMT proteomics approach identified 996 peptides corresponding to 514 proteins in this study. Fig.1B shows the protein polypeptide identification and distribution. Most peptides contain one unique protein peptide. The distribution of protein molecular weight is shown in Fig.1C. The protein molecular weight was mostly within the range of 20–40 KDa. The distribution of polypeptide length is shown in Fig. 1D, and the length of the peptide chain was within the range of 8–16 amino acids. Of these, 118 proteins were quantitatively analyzed in milk from ZY, DYC, and CY (Table S2).

Of the 118 identified proteins, about 28 differently expressed proteins were identified in milk whey proteins from ZY, DYC, and CY. Of the common whey proteins, β-lactoglobulin (P02756), albumin (P02769), and transferrin (G3X6N3) were highly abundant in the whey proteins of milk from the three breeds. The present study provides insights into milk composition with plateau characteristics in the Yunnan province, laying a theoretical basis for further research of milk proteins in other species.

The top 20 abundant proteins in the three breeds are shown in Table S3. Of the high-abundance whey proteins, albumin had the highest expression in whey protein from the three breeds of cattle milk. Albumin is highly expressed in horse colostrum, is central in regulating colloid osmolality in the blood (Chang, 2021). Previous studies have identified albumin in the whey proteins of human, goat, cow, yak, and

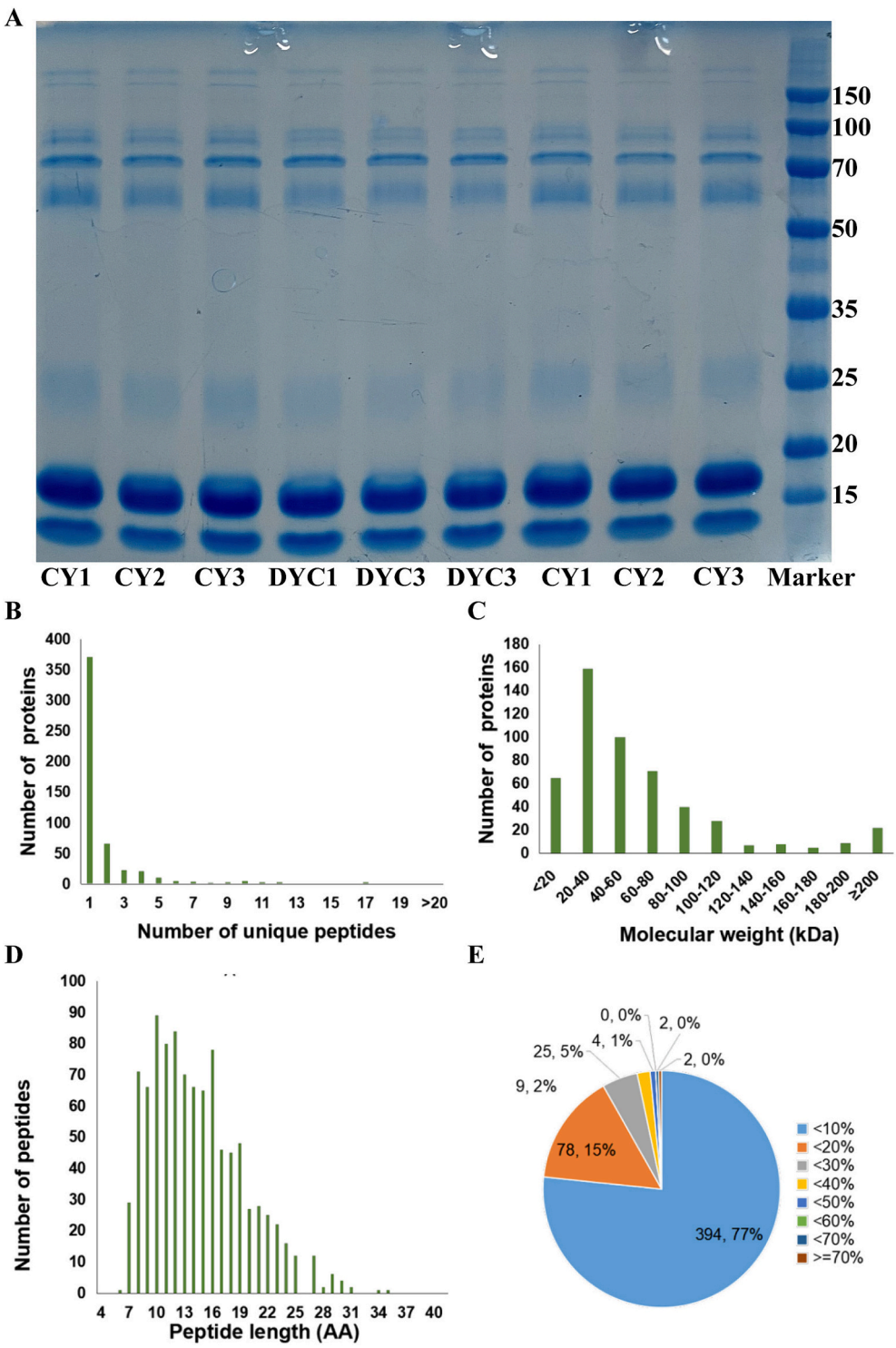


Fig. 1. SDS-PAGE of milk whey proteins in ZY, DYC, and CY (A). Identification number distribution of protein peptides in milk whey protein of ZY, DYC, and CY (B). Protein molecular weight distribution in milk whey protein of ZY, DYC, and CY (C). Peptide length distribution in milk whey protein of ZY, DYC, and CY (D). Peptide coverage distribution of protein identification (E).

camel milk whey proteins using LC-MS/MS (Li et al., 2018; Yang et al., 2013). Thus, albumin is a component of whey proteins during milk formation in the mammary gland (Yang et al., 2015b). Notably, the high-abundance proteins, including the endoplasmic reticulum (ER) chaperone binding immunoglobulin protein (BiP, Q0VCX2), alpha-2-HS-glycoprotein (P12763), β-lactoglobulin (P02754), and transferrin (G3X6N3), were associated with the immune system. Studies have reported that the upregulation of BiP in the cellular microenvironment could induce an anti-inflammatory response in the immune cells by upregulating interleukin 10 (IL-10) synthesis (Yoshida, Ochiai, Matsuno, Panayi, & Corrigall, 2011). Extracellular BiP is a highly abundant protein in the ZY, CY, and DYC milk, indicating that these three types have anti-inflammatory activity. Alpha-2-HS-glycoprotein can promote endocytosis and regulate the inflammatory response. It is reported that alpha-2-HS-glycoprotein as a booster antigen is beneficial for immune activities associated with Abs generation against CCR5 and ENV to

confer HIV-protective immunity (Otsubo et al., 2014). β-lactoglobulin, a primary whey protein fraction in cow milk, is crucial in transferring passive immunity to newborns and regulating phosphorus metabolism in the mammary gland (Xing, Giosafatto, Fusco, Dong, & Mariniello, 2021). Transferrin is essential for healthy growth, and a transferrin level below 0.1 g/L is associated with an increased incidence of infection, growth retardation, and anemia (Julia, Roberto, & Ayelen, 2023). In summary, the major whey proteins of milk from CY, ZY, and DYC showed excellent biological functions of enhancing immunity, anti-inflammation, promoting growth, and preventing viral infection. Therefore, the three milk types are characteristic advantageous resources from Yunnan Province, potential raw materials for infant formula.

3.2. Characteristic whey proteins in the three breeds of cattle milk

The threshold for screening the differentially expressed proteins was set to $P < 0.05$ with a fold change of either >1.2 or < 0.83. Of the 118 identified proteins, 28 differentially expressed whey proteins were identified in ZY, DYC, and ZY (Table S4). The differential expression of the whey proteins from the three breeds is graphically represented in Fig. 2 using the volcano plots −log10 (*P* value) vs. log2 (fold change) to

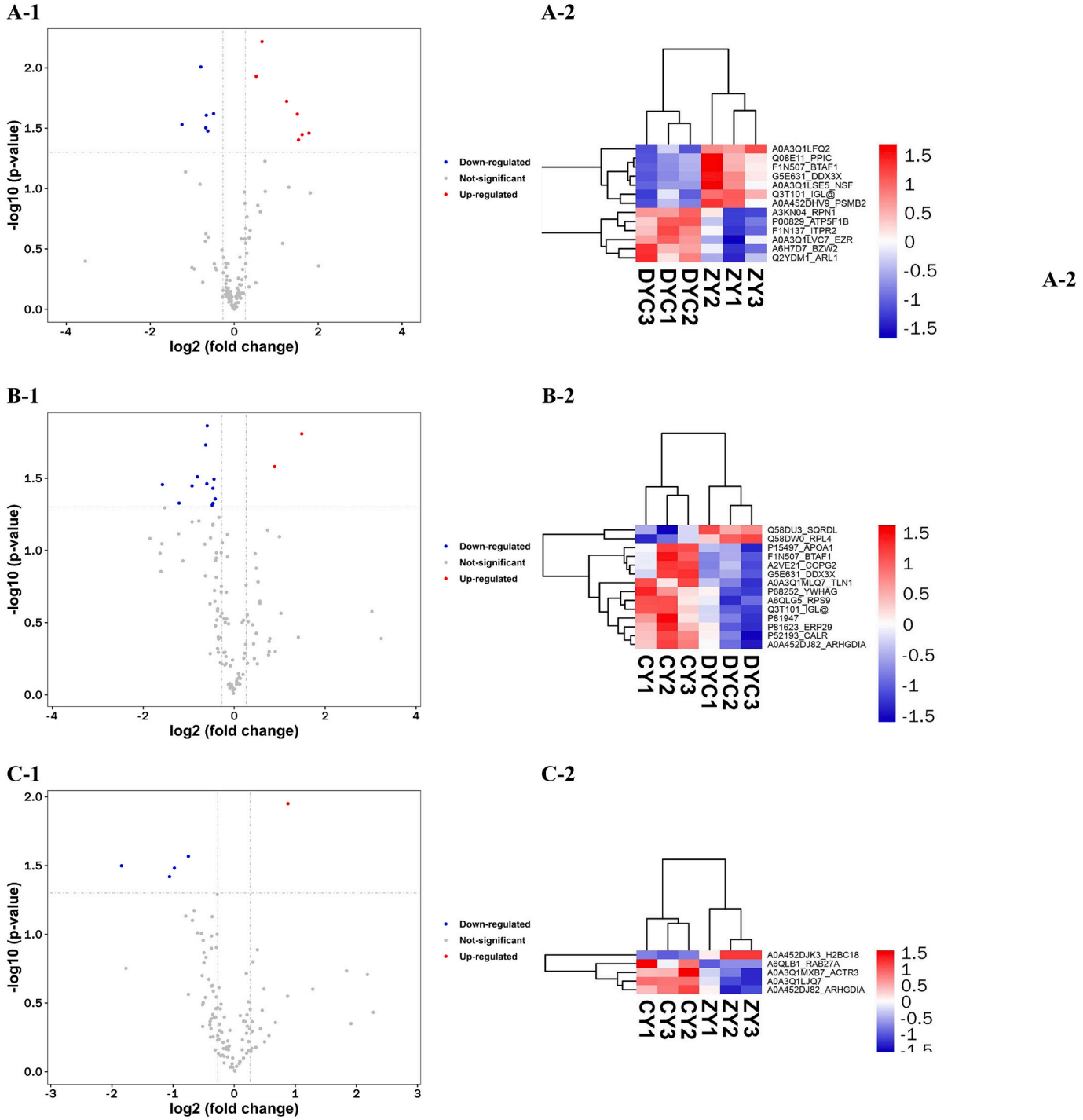


Fig. 2. Differential expression of proteins across groups. Volcano plot and hierarchical clustering showing the significance versus fold change in ZY vs DYC (A-1, A-2), DYC vs CY (B-1, B-2), and ZY vs CY.

display the quantitative data. As shown in Fig. 2A, 13 differentially expressed whey proteins were identified in ZY than DYC, of which seven were upregulated, and six were downregulated. In DYC and CY, 14 proteins were differentially expressed (Fig. 2B-1, B-2), while only five proteins were differentially expressed in ZY and CY (Fig. 2C-1, C-2). Twelve proteins were upregulated in CY compared to DYC, while four proteins were upregulated in CY compared to ZY. The actin-like protein 3 (A0A3Q1MXB7), a major cytoskeletal component related to the immune system process (Tanaka et al., 1992), was more expressed in CY than ZY. The whey proteins in milk from CY showed better immune function compared to those from DYC.

Of the 28 differentially expressed proteins, seven characteristic whey proteins in milk from CY and two characteristic proteins of ZY were identified by one-way analysis of variance using Tukey's post hoc test. The characteristic whey proteins of CY milk are IGL@ protein (Q3T101), 40S ribosomal protein S9 (A6QLG5), rho GDP-dissociation inhibitor 1 (A0A452DJ82), small monomeric GTPase (A6QLB1), calreticulin (P52193), action-like protein 3 (A0A3Q1MXB7), and adenylyl cyclase-associated protein (A0A3Q1LJQ7). RNA helicase (G5E631) and uncharacterized protein (A0A3Q1LFQ2) were the characteristic whey proteins of ZY milk. RNA helicases are vital regulators at the nexus of multiple pathways of RNA metabolism and in the complex cellular environment (Sloan & Bohnsack, 2018). The present study results suggest that the whey proteins in ZY milk could regulate the metabolism better.

Additionally, immunoglobulin proteins are significant in the immune system (Li et al., 2020. Each immunoglobulin molecule consists of two identical heavy chains and two identical light chains, and the IGL@ protein is one of the light chains. The IGL@ protein, one of the differentially expressed whey proteins in Guanzhong goat milk and Holstein cow milk, is associated with the immune system (Sun et al., 2020). The ribosomal Protein S9 is a B23/NPM binding protein essential for normal cell proliferation; it is also essential for normal embryonic development and plays an important role in genomic stability, ribosome biogenesis, and antiapoptotic signaling (Lindström & Zhang, 2008). Rho GDP-dissociation inhibitor 1, one of the key regulators of Rho family GTPases, possesses complex functions, such as regulating cell proliferation, apoptosis, adhesion, migration, and polarity (Wei et al., 2019). According to Venkatasubramanian et al. (2015), the Rho GDP-dissociation inhibitor 1 could inhibit the growth of *Mycobacterium tuberculosis* and promote immune defenses by improving antimicrobial effectiveness. Small GTPase modulates various cellular effects, such as cell survival, cytoskeletal rearrangements, apoptosis, growth control, and intracellular traffic (Umbayev et al., 2023) For instance, RasL10B is a new member of the Ras superfamily with tumor suppressor potential (Zou, Hu, Li, Zhan, & Cao, 2006). Calreticulin (P52193), highly expressed in CY than ZY and DYC, is essential for heart development. Calreticulin is a heart embryonic gene essential for heart development and has an immunomodulatory effect (Fucikova, Fucikova, Spisek, Spisek, & Kroemer, 2020; Jody, WenAn, & Alison, 2022). The whey protein components of milk in CY were more conducive to embryonic heart development than those in DYC and ZY milk. Mammalian adenylyl cyclase-associated protein has the functions of regulating cofilin function, actin cytoskeleton, and cell adhesion. These findings indicated that whey proteins of CY milk had more potential immune-promoting and tumor-suppressor functions than those of ZY and DYC milk. McClearn et al. (2022) improved milk quality, reproductive performance, and the health of Holstein cow through heterosis, and the results were consistent with the present study results, showing a hybrid advantage.

3.3. Functional classification of characteristic whey proteins

The characteristic whey proteins were analyzed using GO annotations and categorized as biological processes, cell composition, and molecular function (Fig. 3). As shown in Fig. 3A, most characteristic whey proteins (60%) were involved in the biological process of

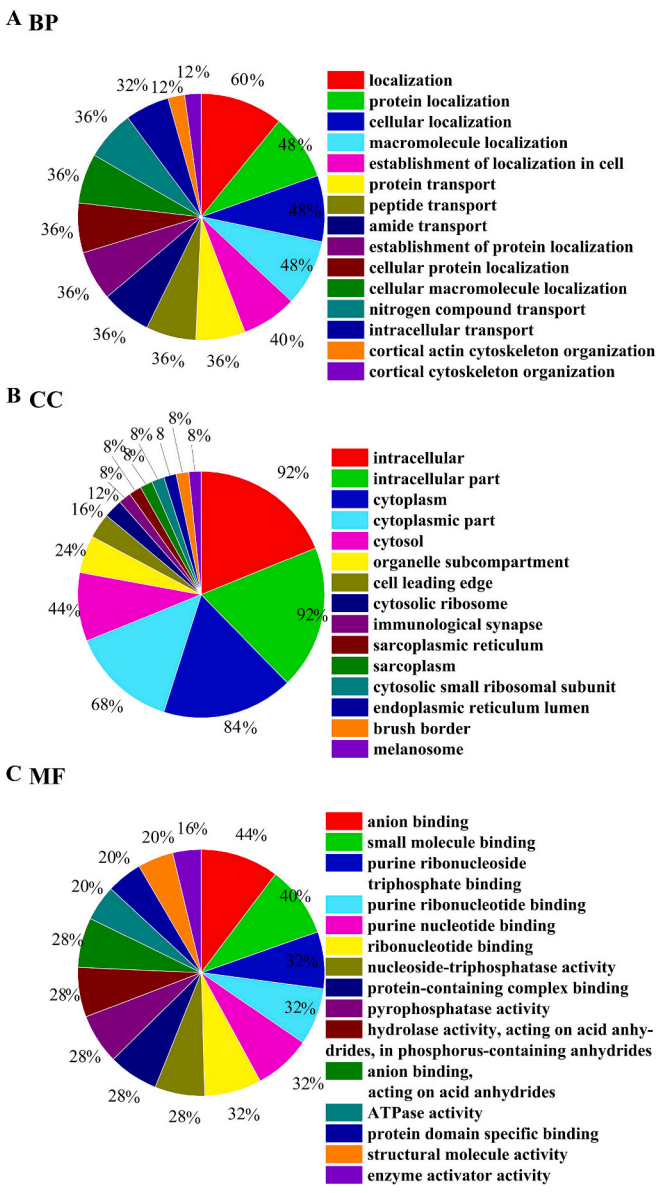


Fig. 3. GO annotation of characteristic milk whey proteins in ZY, DYC, and CY. Biological processes (A); cellular components (B); molecular functions (C).

localization, including protein localization, cellular localization, and macromolecule localization, which were enriched with 48% proteins. Previous studies have shown that the biological processes of buffalo and donkey whey proteins are associated with localization (Li et al., 2018; Wu et al., 2017).

The most common cellular component was an intracellular and intracellular part, accounting for 92% of the characteristic whey proteins (Fig. 3B). The second category was the cytoplasm (84%) and the third was the cytoplasmic part (68%). Furthermore, immunological synapse (8%) was involved in the cellular components. Sun et al. (2020) reported that the categories of the extracellular region, extracellular exosome, and extracellular space were the top three differentially expressed whey proteins in goat and cow milk. Li et al. (2021a, 2021b)) reported that the extracellular exosome was the most common differentially expressed cellular component of whey proteins in donkey and bovine milk. However, the present study results showed that intracellular component was found in the differentially expressed whey protein in Yunnan plateau cattle (CY, ZY, and DYC) milk, probably due to the unique geographical environment of Yunnan.

As for the classification of molecular functions (Fig. 3C), anion binding accounted for 44% of proteins, being the largest molecular functional category. Small molecule binding was the second largest category, involving 40% of proteins. Purine ribonucleoside triphosphate, purine ribonucleotide binding, purine nucleotide binding, and ribonucleotide binding accounted for 32% of proteins. Binding is the most prevalent molecular function of milk fat globule membrane proteins in many species, such as humans, goats, yak, and horses (Yang et al., 2015c).

3.4. Metabolic pathway of characteristic whey proteins

The whey proteins were further evaluated using KEGG pathway analysis (Fig. 4). As shown in Fig. 4A, the differentially expressed proteins in DYC and CY milk were involved in the pathways concerning the ribosome, protein processing in the ER, human T-cell leukemia virus 1 infection, focal adhesion, phagosome, and human immunodeficiency virus 1 infection. The differentially expressed proteins in ZY and CY milk were involved in nine pathways, including endocytosis, tight junction, Fc gamma R-mediated regulation the actin cytoskeleton, and bacterial invasion of epithelial cells (Fig. 4B).

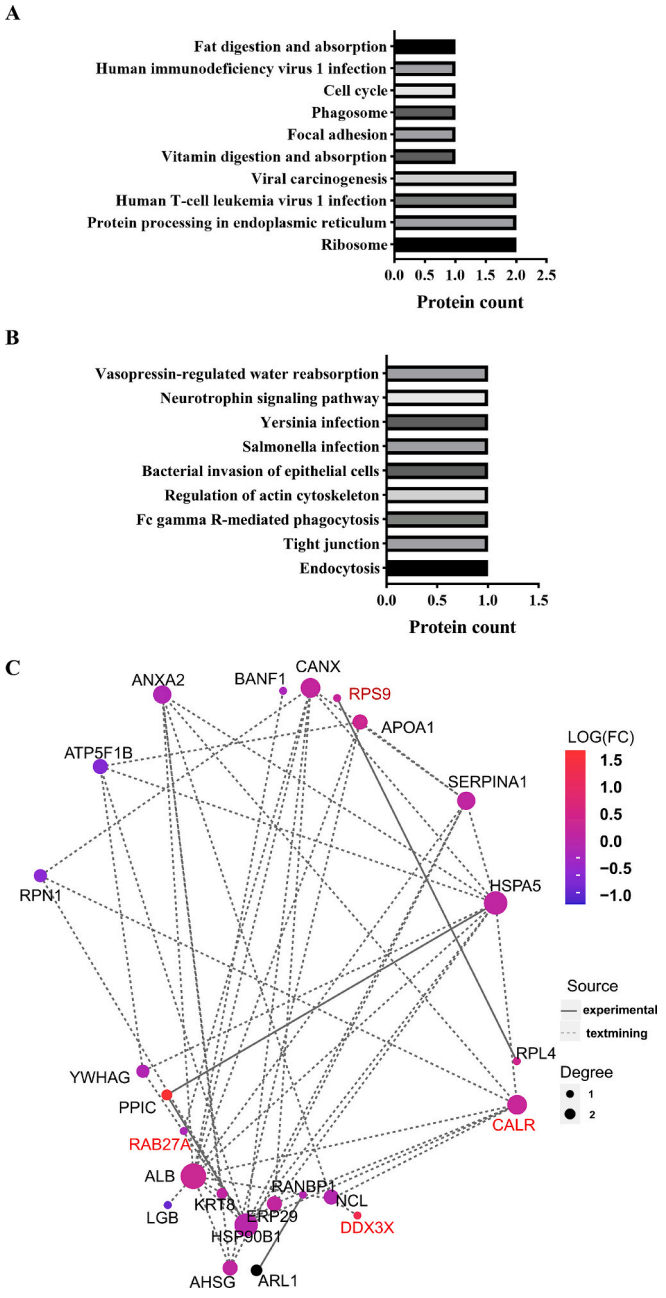


Fig. 4. KEGG pathway analysis of differentially expressed milk whey proteins in DYC vs CY (A) and ZY vs CY (B). Protein-protein interactions of milk whey proteins in ZY, DYC, and CY (C).

The characteristic whey proteins were found in the ribosome, Fc gamma R-mediated phagocytosis, and phagosome. The ribosome is the enrichment pathway, including one small subunit of ribosomal proteins (RP-S) and one large subunit of ribosomal proteins (RP-L), with the functions of protein synthesis in cells and regulation of many processes in organisms. Camel milk contains beneficial proteins mainly found in the ribosome (Li, Li, et al., 2020; Li, yue, et al., 2020). Ribosomal proteins play a significant role in protein translation processes related to cell growth and diseases (Li et al., 2019). Previous studies have reported that human milk whey proteins mainly involve focal adhesion, ribosome, phagosome, and human immunodeficiency virus 1 infection (Jin et al., 2021). Yang et al. (2017) found that human and bovine milk whey proteins were mainly involved in two immune-related pathways: complement and coagulation cascade and phagosome. Phagosomes play a crucial role in eliminating apoptotic cells, host defense, inflammation, and tissue remodeling and repair. Actin-like protein 3 was more abundant in CY milk than DYC and ZY milk, which is involved in Fc gamma R-mediated phagocytosis and host defense during infection (Zhang, Pu, Gu, & Mao, 2020). Thus, the characteristic proteins were mainly involved in the metabolism and immune-related pathways, indicating that whey proteins could enhance the immunity.

The analysis of protein interaction network provides insights into the difference in components and functions of proteins between different species (Li et al., 2021a, 2021b). The high-abundance whey proteins and the characteristic whey proteins in ZY, DYC, and CY milk were investigated using the STRING database to understand the relation between ZY further and develop a protein-protein interaction network map. As shown in Fig. 4C, the protein-protein interaction network included 24 proteins and 96 interactions. Albumin (ALB), interacting with 12 proteins, represented the node with the most interactions, while endoplasmin (HSP90B1) and endoplasmic reticulum chaperone BiP (HSPA5) interacted with 10 proteins, followed by calreticulin (CALR) and calnexin (CANX) with 7 interactions. Of them, the endoplasmic reticulum chaperone BiP (HSPA5) might exert immune function by interacting with other proteins. Calreticulin, the characteristic protein of CY and conducive to the normal development of embryos, was associated with the seven high-abundance proteins (HSPA5, ALB, NCL, HSP90B1, RPN1, ERP29, ANXA2). Additionally, there were more interactions between cattle and yak, indicating a close association.

3.5. Molecular docking of the immune activity of characteristic whey proteins

Molecular docking has been widely used to evaluate the interaction and recognition between proteins and ligands (Zhang et al., 2022). Toll-like receptor 4 (TLR4) is a transmembrane protein that plays a crucial role in the immune response. TLR4 regulates the signaling pathways associated with many diseases, such as intestinal inflammation, sepsis, and drug addiction (Tam, Coller, Hughes, Prestidge, & Bowen, 2021). Therefore, the inhibition of TLR-4 might improve the body's immune regulation and prevent certain diseases. Therefore, the interaction between the characteristic whey proteins and TLR4 was analyzed by molecular docking to investigate the immune activity of the characteristic whey proteins further.

Fig. 5 and Table 1 show the potential interactions of characteristic whey proteins with TLR4. Five characteristic whey proteins (Q3T101, A0A452DJ82, A6QLB1, A0A3Q1MXB7, and A0A3Q1LJQ7) interacted with TLR4 through multiple hydrogen bonds and hydrophobic bonds (Fig. 5). Additionally, the docking energies between the characteristic whey proteins (Q3T101, A0A452DJ82, A6QLB1, A0A3Q1MXB7, and

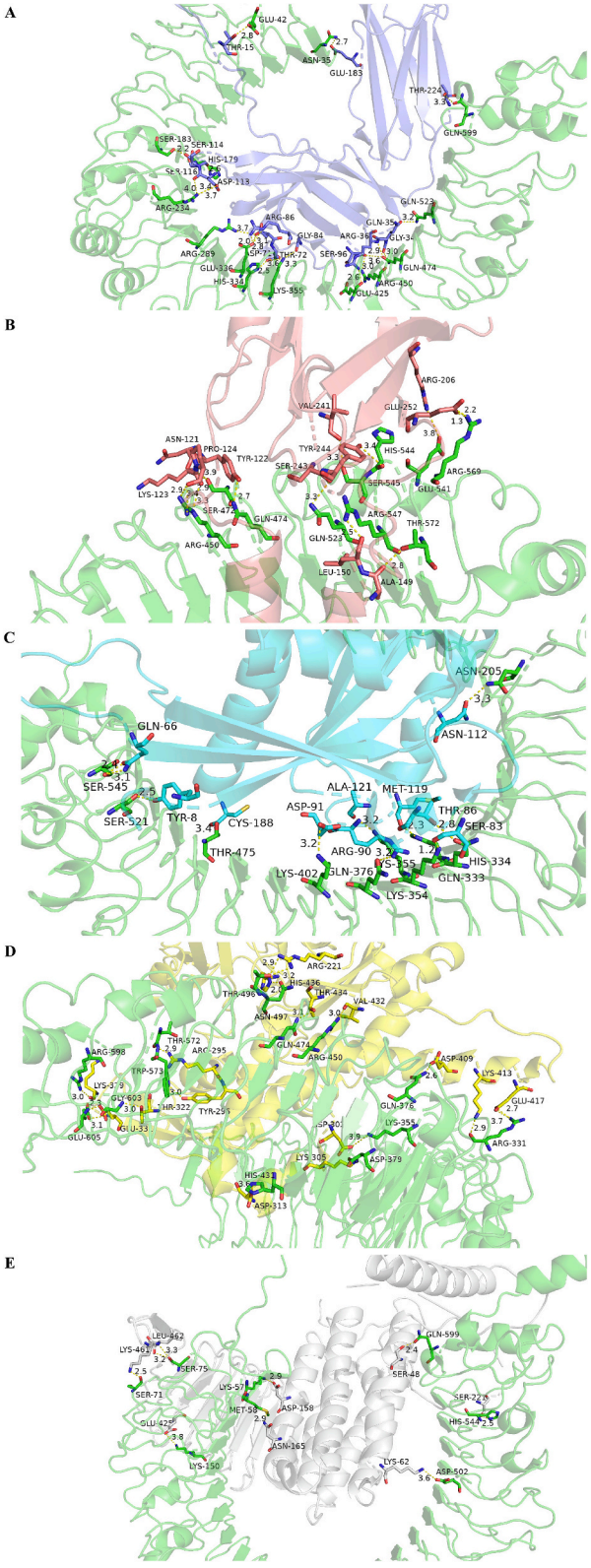


Fig. 5. Molecular docking analysis of the binding mode of five characteristic whey proteins. The interactions of IGL@ protein with TLR4 (A); the interactions of rho GDP-dissociation inhibitor 1 with TLR4 (B); the interactions of small monomeric GTPase with TLR4 (C); the interactions of ction-like protein 3 with TLR4 (D); the interactions of adenylyl cyclase-associated protein with TLR4 (E). The green dotted line indicates hydrogen bonding, and the red dotted line indicates hydrophobic interaction binding. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Docking binding energy between Characteristic whey proteins and theTLR4.

Target protein	Docking_score (kcal/mol)	Hydrogen Bond Binding Site	Hydrophobic Interaction Binding Site
IGL@ protein (Q3T101)	−331.07	THR-224,SER-116,AGR-36, SER-96,GLY-34, GLN-35,SER114, GLY-84,THR-72, ASP-113,ASP-71, AGR-86,GLU-183,THR-15	GLY-222,LYS-133, GLU-221,LEU-129, SER-97,SER-32, SER-115,SER-85, GLY-49,ASN-50, ARG-180,ASN-117, ALA-118,GLY-51, TYR-52,TRP-18, SER-87,ASN-181, GLY-16,SER-17, VAL-182,GLY-88, TYR-69,GLN-20
rho GDP-dissociation inhibitor 1 (A0A452DJ82)	−272.88	VAL-241,ARG-206,TYR-244, ALA-149,LEU-150,SER-243, GLU-252,TYR-122,ASN-121, LYS-123,PRO-124	GLY-242,PHE-253, LEU-254,TYR-146, GLY-245,TYR-251, LEU-151,LYS-208, ARG-247,MET-240, VAL-120,PRO-125
small monomeric GTPase (A6QLB1)	−322.44	ASP-91,CYS-188, TYR-8,MET-119, ALA-121,ARG-111,GLN-66, ASN-112,ARG-90,THR-86,SER-83	ARG-64,GLU-186, LYS-11,ARG-54, ALA-55,MET-93, LEU-9,MET-185, ASP-7,SER-115,HIS-120,TYR-122,MET-182,PHE-107,GLU-147,GLU-104,GLY-150,LEU-108,ALA-87,LEU-84,PHE-88, ASN-56
action-like protein 3 (A0A3Q1MXB7)	−293.67	THR-322,LYS-329,GLU-331, ARG-295,TYR-296,VAL-432, ARG-221,HIS-436,THR-434, ASP-409,ASP-302,LYS-305, LYS-413,GLU-417,ASP-313	ARG-394,TRP-317, ASP-395,PRO-301, ARG-398,GLN-401, LYS-330,GLN-222, GLY-397,LYS-309, THR-312,ASN-308, PRO-355,LYS-405, PRO-282,ARG-439, MET-392,ARG-406, ARG-402,ALA-410, GLN-26,LYS-316, ARG-274
adenylyl cyclase-associated protein (A0A3Q1LJQ7)	−305.66	SER-48,GLU-425,SER-221, LYS-461,LEU-462,ASP-158, ASN-165,LYS-62	ASP-33,ALA-55, LEU-50,ALA-24, THR-404,PRO-403, GLN-44,GLN-69, PRO-220,LEU-219, LYS-80,LYS-154, ARG-166,ALA-51, MET-161,PHE-162, ASN-157,LYS-172, ARG-83,ARG-13, ALA-20,ARG-17, GLY-16

A0A3Q1LJQ7) and TLR4 were − 331.07 kcal/mol, −272.88 kcal/mol, −322.44 kcal/mol, −293.67 kcal/mol, and − 305.66 kcal/mol, respectively (Table 1). Previous studies have reported that the lower the binding energy of the two proteins, the better the binding stability (Han, Liu, Wen, & Ni, 2021). The binding energies observed in this study were less than −5 kcal/mol, indicating the formation of a stable complex between the five characteristic whey proteins and TLR4. Liang et al. (2016) found that inhibiting of the TLR4 receptor could induce the inhibition of downstream signals, significantly reducing in the inflammatory response. Hou et al. (2020) found that Rutin could inhibit the TLR-4 receptor, inhibiting downstream signals, thereby reducing the inflammatory response and achieving the protective effect of liver fibrosis. Similarly, the characteristic whey proteins (Q3T101, A0A452DJ82, A6QLB1, A0A3Q1MXB7, and A0A3Q1LJQ7) may also

regulate immunity by inhibiting TLR4, thereby reducing the occurrence of inflammation.

3.6. Bioactive properties of whey proteins from ZY, DYC, and CY milk after in vitro simulated gastrointestinal digestion

Bovine whey proteins are rich in high-quality proteins with a high nutritional value and contain bioactive peptides encrypted in their sequences. Bioactive peptides are protein fragments with a positive impact on human function and health. These peptides exert their biological functions after being released by proteases, microbial fermentation, or natural gastrointestinal digestion. In recent years, milk and dairy products have received extensive attention as sources of bioactive peptides. The gastrointestinal digestion process has a major influence on the release of peptide sequences encrypted in food proteins. The whey proteins of ZY, DYC, and CY milk were subjected to simulated gastrointestinal digestion using pepsin and pancreatin to release the bioactive peptides and study the biological function of these whey proteins further. The digests were ultrafiltered with a 3 kDa membrane, and the <3 kDa fraction was subjected to LC-MS/MS to identify the amino acid sequences of the peptide. A total of 660 peptides were identified from the <3 kDa fraction of ZY, DYC, and CY milk whey protein digests (Table S5). A total of 442, 376, and 477 peptides were identified in the whey protein of milk from ZY, DYC, and CY, respectively (Fig. 6A).

A total of 1, 115, and 326 peptides were identified in the ZY whey protein digestion samples, with molecular weights in the range of 2000–3000 Da, 1000–2000 Da, and < 1000 Da, respectively (Fig. 6B). Similarly, 1, 57, and 318 peptides were identified in the DYC whey protein digestion samples, with molecular weights in the range of 2000–3000 Da, 1000–2000 Da, and < 1000 Da, respectively (Fig. 6D). A total of 3, 125, and 349 peptides were identified in the CY whey protein digestion samples, with molecular weights in the range of 2000–3000 Da, 1000–2000 Da, and < 1000 Da, respectively (Fig. 6E). Amino acid composition and molecular weight distribution reflect the degree of proteolysis and peptide bond cleavage. Meanwhile, molecular weight is closely related to the biological activity and bioavailability of digestion products (Luan, Feng, & Sun, 2021). After simulated gastrointestinal digestion of three types of bovine whey protein, the molecular weight of most peptides was <2000 Da. Small molecular weight peptides are reported to be more easily digested and absorbed by the human body (Dai, Zhang, He, Xiong, & Ma, 2017; Shi, Ahtesh, Mathai, Mcainch, & Su, 2016). The results of this study indicate that the whey protein of ZY, DYC, CY releases more small molecular weight peptides after passing through the gastrointestinal tract, which are easily absorbed and utilized by the human body.

Among them, 79 unique bioactive peptides were identified in ZY, 43 unique bioactive peptides were identified in DYC, and 146 unique bioactive peptides were identified in CY. The total number of bioactive peptides and unique active peptides released by CY whey protein were more than those in ZY and DYC after simulated gastrointestinal

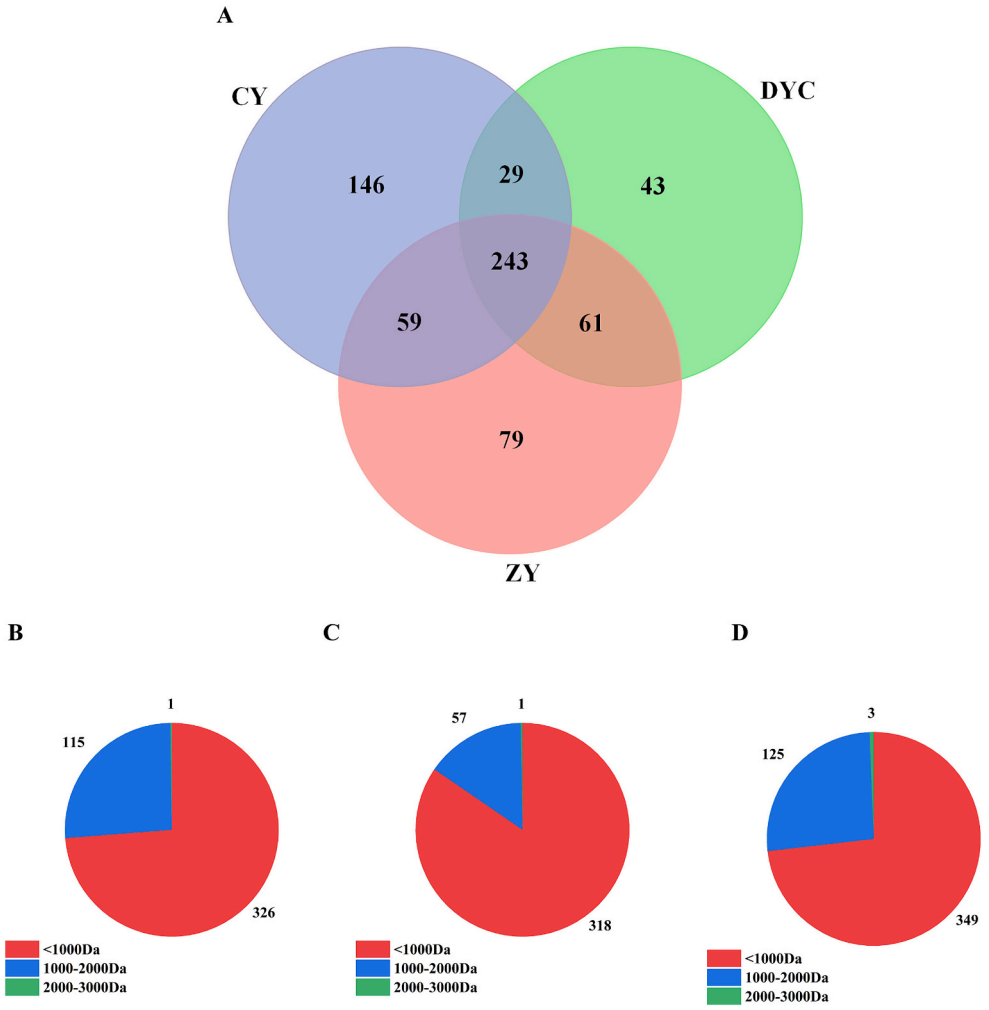


Fig. 6. Venn diagram of the peptides detected in whey protein from ZY, DYC, and CY SGID (A); Molecular weight distribution of the identified peptides from ZY milk whey after SGID (B); Molecular weight distribution of the identified peptides from DYC milk whey after SGID (C); Molecular weight distribution of the identified peptides from CY milk whey after SGID (D).

digestion. These results indicate that CY whey protein might possess more biological activity, which is consistent with the results of TMT proteomics.

Furthermore, the activity of peptides was analyzed using the BIOPEP database. ACE inhibitory tripeptides exert a better antihypertensive effect on the body, probably because they can be absorbed from the intestine to reach their target organ (Nishithkumar, Gangadharappa, & Ishani, 2021). A total of 26 ACE inhibitory peptides were released from three breeds of whey proteins after simulated gastrointestinal digestion, most of which were tripeptides. This result indicates that the whey proteins of ZY, DYC, and CY milk have a certain antihypertensive effect after digestion and absorption by the human body. Some reported active peptides were also identified in the digests of whey protein from ZY, DYC, and CY, including 22 antioxidative peptides, two antibacterial peptides, night dipeptidyl peptidase IV inhibitors peptides, two immunomodulatory peptides, and so on. Previous studies have shown that the antioxidant activity and antibacterial activity of bovine whey protein and camel whey protein were enhanced after simulated gastrointestinal digestion (Corrochano, Sariay, Arranz, Kelly, & Giblin, 2018; Hina et al., 2022). In Magouz et al. (2023) and Jin et al. (2016), gastrointestinal digestion increased the biological activities of peptides, such as iron reduction, free radical scavenging, ACE inhibition, and DPP-IV inhibition. Therefore, simulated gastrointestinal digestion possibly increases activity. This result suggested that the whey protein of ZY, DYC, and CY milk released more functional active peptides after simulated gastrointestinal digestion, with a health promoting effect. The score of the identified peptides was predicted using the Peptide Ranker database. Of the 660 peptides with molecular weights ranging from 0 to 3000 Da, 135 peptides showed scores >0.8. There were many new bioactive peptides without excavation, which can be further excavated and analyzed to obtain new bioactive peptides, laying a foundation for the development of functional products based on ZY, DYC, and CY whey proteins.

4. Conclusions

In summary, a total of 118 whey proteins were quantified, and the major whey proteins showed the functions of enhancing immunity, anti-inflammation, promoting growth, and preventing virus infection in the three cattle breeds. IGL@ protein (Q3T101), 40S ribosomal protein S9 (A6QLG5), rho GDP-dissociation inhibitor 1 (A0A452DJ82), small monomeric GTPase (A6QLB1), calreticulin (P52193), action-like protein 3 (A0A3Q1MXB7), and adenylyl cyclase-associated protein (A0A3Q1LJQ7) were identified as the characteristic whey proteins in CY milk. RNA helicase (G5E631) and uncharacterized protein (A0A3Q1LFQ2) were the characteristic whey proteins in ZY milk. These characteristic proteins had the potential for immunological, antibacterial, and antitumor activities and were mainly associated with the phagosome and Fc gamma R-mediated phagocytosis pathways. The whey proteins of CY milk showed more potential for immunoprotection and tumor suppression than those of ZY and DYC milk. The characteristic whey proteins (Q3T101, A0A452DJ82, A6QLB1, A0A3Q1MXB7, and A0A3Q1LJQ7) interacted with TLR4 via multiple hydrogen bonds and hydrophobic bonds and regulated immunity by inhibiting TLR4. Overall, these results indicate that milk quality can be improved through hybridization advantages to obtain higher cultivation efficiency. The bioactive peptides, such as anti-inflammatory peptides and ACE inhibitory peptides, were released from whey proteins after simulated gastrointestinal digestion. These results provide insights into the characterization and potential activities of whey proteins and a reference for developing infant formula milk powder.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101384.

CRediT authorship contribution statement

Yufang Li: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Data curation. **Shijun Li:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Xingwen Zhao:** Project administration, Methodology, Investigation. **Chongying Shi:** Visualization, Validation, Software, Resources. **Yunmei Chai:** Visualization, Resources, Data curation, Conceptualization. **Aixiang Huang:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Yanan Shi:** Writing – review & editing, Writing – original draft, Supervision.

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.

Acknowledgments

This work was supported by the Project of Yunnan Provincial Department of Education (2023Y1009), the Major Science and Technology Project in Yunnan Province of China (202102AE090027-1), and the Scientific Research Initiation Project of Yunnan Agricultural University (KY2022-43). We acknowledge the Shangri-La Kangmei Dairy Development Co., Ltd. for providing us with the milk samples and "BIOTREE" for assisting us with the data analysis.

References

Abdel-Hamid, M., Yang, P., Mostafa, I., Osman, A., Romeih, E., Yang, Y., … Li, L. (2022). Changes in whey proteome between Mediterranean and Murrah Buffalo colostrum and mature Milk reflect their pharmaceutical and medicinal value. *Molecules, 27*(5), 1575.

Aspri, M., Leni, G., Galaverna, G., & Papademas, P. (2018). Bioactive properties of fermented donkey milk, before and after in vitro simulated gastrointestinal digestion. *Food Chemistry, 268*.

Chang, H. X. (2021). *Differential proteomics of protein and milk fat globular membrane in different lactation stages of Horses*. Xinjiang Agricultural University.

Corrochano, A. R., Sariay, Y., Arranz, E., Kelly, P. M., & Giblin, L. (2018). Comparison of antioxidant activities of bovine whey proteins before and after simulated gastrointestinal digestion. *Journal of Dairy Science, 102*(1), 54–67.

Dai, C., Zhang, W., He, R., Xiong, F., & Ma, H. (2017). Protein breakdown and release of antioxidant peptides during simulated gastrointestinal digestion and the absorption by everted intestinal sac of rapeseed proteins. *LWT, 86*.

Fan, Q., Wanapat, M., & Hou, F. (2020). Chemical composition of Milk and rumen microbiome diversity of Yak, impacting by herbage grown at different Phenological periods on the Qinghai-Tibet Plateau. *Animals, 10*(6), 1030.

Fucikova, J., Fucikova, J., Spisek, R., Spisek, R., & Kroemer, G. (2020). Calreticulin and cancer. *Cell Research, 31*(1), 5–16.

Guo, Y., Yu, D., Cupp-Sutton, K. A., Liu, X., & Wu, S. (2022). Optimization of protein-level tandem mass tag (TMT) labeling conditions in complex samples with top-down proteomics. *Analytica Chimica Acta, 1221*, Article 340037.

Han, B., Zhang, L., & Zhou, P. (2023). Comparative proteomics of whey proteins: New insights into quantitative differences between bovine, goat and camel species. *International Journal of Biological Macromolecules, 227*, 10–16.

Han, Q., Liu, F., Wen, X., & Ni, Y. (2021). Kinetic, spectroscopic, and molecular docking studies on the inhibition of membrane-bound polyphenol oxidase from Granny Smith apples (*Malus domestica* Borkh.). *Food Chemistry, 338*, Article 127928.

He, S. H., Ma, Y., Wang, J. Q., Li, Q. M., Yang, X., Tang, S. H., & Li, H. M. (2011). Milk fat chemical composition of yak breeds in China. *Journal of Food Composition and Analysis, 24*(2), 223–230.

He, Z. X., Wang, X. D., Huang, M. F. H., Zhao, G., Cheng, Y. M., & Zhou, Y. P. (2015). Comparison of the major nutritional components of Milk from Zhongdian Yak, Diqing Yellow Cattle and Cattle-Yak. *Journal of Food Biotechnology, 34*(12), 1294–1301.

Hina, K., Sabika, J., Priti, M., Marwa, H., Akli, A. M., & Sajid, M. (2022). Camel whey protein with enhanced antioxidative and antimicrobial properties upon simulated gastro-intestinal digestion. *Nutrition and Health, 108*.

Hou, L., Cui, Z., Sun, P., Piao, H., Han, X., Song, J., Wang, G., Zheng, S., Dong, X., Gao, L., Zhu, Y., Lian, L., Nan, J., & Wu, Y. (2020). Rutin mitigates hepatic fibrogenesis and inflammation through targeting TLR4 and P2X7 receptor signaling pathway in vitro and in vivo. *Journal of Functional Foods, 64*, Article 103700.

Hurley, W. L., & Theil, P. K. (2011). Perspectives on immunoglobulins in colostrum and Milk. *Nutrients, 3*(4), 442–474.
Jin, D. P., Liu, H., Bu, L. L., Ke, Q. H., Li, Z. Y., Han, W. N., … Liu, C. H. (2021). Comparative analysis of whey proteins in human Milk using a data-independent acquisition proteomics approach during the lactation period. *Journal of Agricultural and Food Chemistry, 69*(14), 4319–4330.
Jin, Y., Yu, Y., Qi, Y., Wang, F., Yan, J., & Zou, H. (2016). Peptide profiling and the bioactivity character of yogurt in the simulated gastrointestinal digestion. *Journal of Proteomics, 141*, 24–46.
Jody, G., WenAn, W., & Alison, R. (2022). Calreticulin and the heart. *Cells, 11*(11).
Julia, M. P., Roberto, T. C., & Ayelen, P. C. S. D. (2023). Transferrin enhances neuronal differentiation. *ASN Neuro, 15*.
Krobthong, S., Yingchutrakul, Y., Wongtrakoongate, P., Chuntakaruk, H., Rungrotmongkol, T., Chaichana, C., … Aonbangkhen, C. (2023). Proteomics and molecular docking analyses reveal the bio-chemical and molecular mechanism underlying the Hypolipidemic activity of Nano-liposomal bioactive peptides in 3T3-L1 adipocytes. *Foods, 12*(4), 780.
Li, B., Ge, Y., Xu, Y., Lu, Y., Yang, Y., Han, L., … Le, G. (2019). Spatial learning and memory impairment in growing mice induced by major oxidized tyrosine product Dityrosine. *Journal of Agricultural and Food Chemistry, 67*(32), 9039–9049.
Li, M., Dong, Y., Li, W., Shen, X., Abdlla, R., Chen, J., Cao, X., & Yue, X. (2022). Characterization and comparison of whey proteomes from bovine and donkey colostrum and mature milk. *LWT, 158*, Article 113113.
Li, M., Yu, H., Chen, J., Abdlla, R., Liu, A., Song, W., Zhang, J., Zhang, X., Yue, X., & Li, Q. (2021b). Novel insights into whey protein differences between donkey and bovine milk. *Food Chemistry, 365*, Article 130397.
Li, M. H., Yu, H. K., Chen, J. L., Abdlla, R., Liu, A. C., Song, W. Y., … Li, Q. L. (2021a). Novel insights into whey protein differences between donkey and bovine milk. *Food Chemistry, 365*, Article 130397.
Li, R. R., Yue, H. T., Shi, Z. Y., Shen, T., Yao, H. B., Zhang, J. W., … Yang, J. (2020). Protein profile of whole camel milk resulting from commercial thermal treatment. *LWT, 134*, Article 110256.
Li, S. S., Li, L., Zeng, Q. K., Liu, J. X., Yang, Y. X., & Ren, D. X. (2018). Quantitative differences in whey proteins among Murrah, Nili-Ravi and Mediterranean buffaloes using a TMT proteomic approach. *Food Chemistry, 269*, 228–235.
Li, W. X., Li, M. H., Cao, X. Y., Han, H. J., Kong, F. H., & Yue, X. Q. (2020). Comparative analysis of whey proteins in donkey colostrum and mature milk using quantitative proteomics. *Food Research International, 127*, Article 108741.
Liang, G., Fu, W., Chen, L., Wang, Z., Zhao, C., Chen, G., Liu, X., Cai, Y., Zhou, J., Dai, Y., & Li, C. (2016). *Determination of the binding mode for anti-inflammatory natural product xanthohumol with myeloid differentiation protein 2* (p. 455). Development and Therapy: Drug Design.
Lindström, M. S., & Zhang, Y. (2008). Ribosomal protein S9 is a novel B23/NPM-binding protein required for Normal cell proliferation. *Journal of Biological Chemistry, 283*(23), 15568–15576.
Liu, D., Guo, Y., Wu, P., Wang, Y., Kwaku Golly, M., & Ma, H. (2020). The necessity of walnut proteolysis based on evaluation after in vitro simulated digestion: ACE inhibition and DPPH radical-scavenging activities. *Food Chemistry, 311*, Article 125960.
Liu, W. W., Wang, Q., Song, J. J., Xin, J. W., Zhang, S. S., Lei, Y. H., … Suo, H. Y. (2021). Comparison of gut microbiota of yaks from different geographical regions. *Frontiers in Microbiology, 12*.
Luan, X., Feng, M., & Sun, J. (2021). Effect of Lactiplantibacillus plantarum on antioxidant activity in fermented sausage. *Food Research International, 144*, Article 110351.
Magouz, O., Mehanna, N., Khalifa, M., Sakr, H., Gensberger-Reigl, S., & Dalabasmaz, S. (2023). Profiles, antioxidative and ace inhibitory activity of peptides released from fermented buttermilk before and after simulated gastrointestinal digestion. *Innovative Food Science & Emerging Technologies, 84*, Article 103266.
Maity, S., & Ambatipudi, K. (2019). Quantitative proteomics of milk whey reveals breed and season specific variation in protein abundance in Holstein Friesian cow and Murrah buffalo. *Research in Veterinary Science, 125*, 244–252.
McClearn, B., Delaby, L., Gilliland, T. J., Galvin, N., Guy, C., Dineen, M., … McCarthy, B. (2022). The effect of Holstein-Friesian, Jersey × Holstein-Friesian, and Norwegian Red × (Jersey × Holstein-Friesian) cows on dry matter intake and production efficiencies in pasture-based systems. *Journal of Dairy Science, 105*(1), 242–254.
Nishithkumar, J., Gangadharappa, U. Y., & Ishani, B. (2021). Antihypertensive activity of orally consumed ACE-I inhibitory peptides. *Critical Reviews in Food Science and Nutrition, 62*(32).
Otsubo, Y., Yashiro, S., Nozaki, K., Matsuura, K., Kiyonaga, K., Mitsumata, R., Takahashi, Y., Masuyama, M., Muneoka, A., Takamune, N., Shoji, S., & Misumi, S. (2014). Bovine alpha-2-HS-glycoprotein functions as a booster antigen for efficiently stimulating humoral immune responses to CCR5 and SIVmac239 envelope glycoprotein. *Biochemical and Biophysical Research Communications, 443*(1), 301–307.
Rajczewski, A. T., Jagtap, P. D., & Griffin, T. J. (2022). An overview of technologies for MS-based proteomics-centric multi-omics. *Expert Review of Proteomics, 19*(3), 165–181.
Roin, N. R., Larsen, L. B., Comi, I., Devold, T. G., Eliassen, T. I., Inglingstad, R. A., … Poulsen, N. A. (2022). Identification of rare genetic variants of the αS-caseins in milk from native Norwegian dairy breeds and comparison of protein composition with milk from high-yielding Norwegian red cows. *Journal of Dairy Science, 105*(2), 1014–1027.
Shi, M., Ahtesh, F., Mathai, M., Mcainch, A. J., & Su, X. Q. (2016). Effects of fermentation conditions on the potential anti-hypertensive peptides released from yogurt fermented bylactobacillus helveticusand flavourzyme®. *International Journal of Food Science and Technology, 52*(1), 37–145.
Sloan, K. E., & Bohnsack, M. T. (2018). Unravelling the mechanisms of RNA helicase regulation. *Trends in Biochemical Sciences, 43*(4), 237–250.
Sun, Y. X., Wang, C. N., Sun, X. M., & Guo, M. R. (2020). Proteomic analysis of differentially expressed whey proteins in Guanzhong goat milk and Holstein cow milk by iTRAQ coupled with liquid chromatography-tandem mass spectrometry. *Journal of Dairy Science, 103*(10), 8732–8740.
Tam, J. S. Y., Coller, J. K., Hughes, P. A., Prestidge, C. A., & Bowen, J. M. (2021). Toll-like receptor 4 (TLR4) antagonists as potential therapeutics for intestinal inflammation. *Indian Journal of Gastroenterology, 40*(1), 5–21.
Tanaka, T., Shibasaki, F., Ishikawa, M., Hirano, N., Sakai, R., Nishida, J., … Hirai, H. (1992). Molecular cloning of bovine actin-like protein, actin2. *Biochemical and Biophysical Research Communications, 187*(2), 1022–1028.
Umbayev, B., Yantsen, Y. S., Yermekova, A., Nessipbekova, A., Syzdykova, A., & Askarova, S. (2023). Role of a small gtpase cdc42 in aging and age-related diseases. *Biogerontology, 24*(1), 27–46.
Venkatasubramanian, S., Dhiman, R., Paidipally, P., Cheekatla, S. S., Tripathi, D., Welch, E., … Vankayalapati, R. (2015). A rho GDP dissociation inhibitor produced by apoptotic T-cells inhibits growth of mycobacterium tuberculosis. *PLoS Pathogens, 11*(2), Article e1004617.
Wei, Z. Q., Xu, H., Zhang, Y., Yi, X., Yang, X. Y., Chen, Y. Y., … Yang, F. (2019). Rho GDP dissociation inhibitor α silencing attenuates silicosis by inhibiting RhoA/Rho kinase signalling. *Experimental Cell Research, 380*(2), 131–140.
Wu, L. Y. M., Wang, M. X., Mi, S., Wu, Y. F., Wu, J. R., & Yue, X. Q. (2017). Comparative analysis of composition and function of whey proteins in bovine colostrum and bovine Milk. *Modern Food Science & Technology, 33*(5), 58–63.
Xing, G. L., Giosafatto, C. V. L., Fusco, A., Dong, M. S., & Mariniello, L. (2021). Combined lactic fermentation and enzymatic treatments affect the antigenicity of β-lactoglobulin in cow milk and soymilk-cow milk mixture. *LWT, 143*, Article 111178.
Yang, M., Cao, X. Y., Wu, R. N., Liu, B., Ye, W. H., Yue, X. Q., & Wu, J. R. (2017). Comparative proteomic exploration of whey proteins in human and bovine colostrum and mature milk using iTRAQ-coupled LC-MS/MS. *International Journal of Food Sciences and Nutrition, 68*(6), 671–681.
Yang, Y., Zhao, X., Yu, S., & Cao, S. (2015). Quantitative proteomic analysis of whey proteins in the colostrum and mature milk of yak (*Bos grunniens*). *Journal of the Science of Food and Agriculture, 95*(3), 592–597.
Yang, Y. X., Bu, D. P., Zhao, X. W., Sun, P., Wang, J. Q., & Zhou, L. Y. (2013). Proteomic analysis of cow, yak, Buffalo, goat and camel Milk whey proteins: Quantitative differential expression patterns. *Journal of Proteome Research, 12*(4), 1660–1667.
Yang, Y. X., Zheng, N., Zhao, X. W., Zhang, Y. D., Han, R. W., Ma, L., … Wang, J. Q. (2015b). Data from proteomic characterization and comparison of mammalian milk fat globule proteomes by iTRAQ analysis. *Data in Brief, 3*, 12–15.
Yang, Y. X., Zheng, N., Zhao, X. W., Zhang, Y. D., Han, R. W., Ma, L., … Wang, J. Q. (2015c). Proteomic characterization and comparison of mammalian milk fat globule proteomes by iTRAQ analysis. *Journal of Proteomics, 116*, 34–43.
Yoshida, K., Ochiai, A., Matsuno, H., Panayi, G. S., & Corrigall, V. M. (2011). Binding immunoglobulin protein resolves rheumatoid synovitis: A xenogeneic study using rheumatoid arthritis synovial membrane transplants in SCID mice. *Arthritis Research & Therapy, 13*(5), R149.
Zhang, F. L., Pu, J. H., Gu, Z. B., & Mao, H. M. (2020). DIA proteomics reveals hypotensive and immune-enhancing constituents in buffalo whey from different altitudes. *International Journal of Biological Macromolecules, 164*, 4146–4154.
Zhang, X., Wang, R., Cheng, C., Zhang, Y., Ma, Y., & Lu, W. (2022). Identification of two novel dipeptidyl peptidase-IV inhibitory peptides from sheep whey protein and inhibition mechanism revealed by molecular docking. *Food Bioscience, 48*, Article 101733.
Zhang, Y., Min, L., Zhang, S., Zheng, N., Li, D., Sun, Z., & Wang, J. (2021). Proteomics analysis reveals altered nutrients in the whey proteins of dairy cow Milk with different thermal treatments. *Molecules, 26*(15), 4628.
Zou, H. F., Hu, L. E., Li, J. X., Zhan, S. X., & Cao, K. M. (2006). Cloning and characterization of a novel small monomeric GTPase, RasL10B, with tumor suppressor potential. *Biotechnology Letters, 28*(23), 1901–1908.
Pereira, P. C. (2014). Milk nutritional composition and its role in human health. *Nutrition, 9*(1), 6.