FOOD PROTEOMES: BEYOND THEIR NUTRITIONAL VALUE

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FOOD PROTEOMES: BEYOND THEIR NUTRITIONAL VALUE

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EDITORIAL
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Editorial: Food Proteomes: Beyond Their Nutritional Value

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Editorial on the Research Topic

Food Proteomes: Beyond Their Nutritional Value

This Research Topic collects diverse studies focused on the in-depth characterization of dietary proteomes by evaluating their singular features, possible roles in health and disease conditions; as well as exploring the changes caused by industrial processing to food proteomes. Of note, characterization of dietary proteomes has been performed by using high throughput mass spectrometry strategies in all compiled studies.

The first article of this topic entitled "Structural Changes and Evolution of Peptides During Chill Storage of Pork" (Zou et al.) is focused on the investigation of post-mortem aging of dietary proteins in fresh pork meat (*Longissimus dorsi*) during chill-storage and their relevance in protein digestibility. The authors reported that protein denaturation and unfolding occurs in <3 days of chill-storage. The ordered and stable structures of meat proteins were gradually destabilized during chill-storage to become a mesh of loose and disordered protein fragments due to the action of endogenous enzymes. This basal proteolysis increased the number of exposed tyrosine and tryptophan residues as well as the number of exposed digestion sites. Myofibrillar proteins followed by sarcoplasmic proteins were the families of proteins more degraded in meat during chill-storage. Authors also demonstrated how the new hydrophobic status and the altered protein structure increases protein digestibility. Proteomics characterization of proteolytic peptides demonstrated that although long storage times led to degradation of proteins into amino acids or small peptides, moderate chill storage increased the production of antioxidant, ACE inhibitors and DPP-IV inhibitory bioactive peptides.

Moreover, it has to be emphasized that human milk (HM) is the optimal milk source for newborns due to its nutritional composition and non-nutritive bioactive fraction, specially when they are born prematurely (1). HM composition is highly complex, and although milk proteome only represents around 0.9–1.2 g/dL (1), this fraction is not only considered a macronutrient fraction, as it is rich in innate-immune proteins that contribute to the development of the infant's innate immunity (2). In this line, Sari et al. performed a comparative study of human milk

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proteomes obtained from donors from eight cities of China during the first 6 month of lactation as part of the Chinese Human Milk Project. This study entitled "Comparative proteomics of human milk from eight cities in China during 6 months of lactation in the Chinese Human Milk Project (CHMP) study" (Sari et al.) reinforces the fact that HM composition is influenced by endogenous and exogenous factors. In this proteomics comparative study Sari et al. reported differences in the HM proteomes across individuals and along the lactation period depending on the geographic localization. As proposed by the authors these findings account for a potential dynamic mechanism that may fulfill the changing infant needs alongside their development. The geographic differences observed by the authors were mainly explained by 12 immune-related proteins, including different lactalbumins, lactoferrins, and immunoglobulins. These differences may be the response toward the pathogen pressure of the environment [(3), Sari et al.]. In-depth analysis of differentially expressed proteins also demonstrated their close relation with infant host defense. Remarkably, a significant subset of triglycerides metabolic process-associated proteins were differentially enriched depending on the location between the first and fifth month of lactation. Nonetheless, authors indicate that the global differences observed in HM compositions along the lactation period were the result of a more complex and subtle modulation mechanism mediated by a higher number of proteins.

When HM is not an option, the selection of a good HM substitute with adequate nutritional quality is crucial for the newborn development. In this line, the other two studies compiled in this Research Topic investigate the composition and characteristics of milk proteomes from different species. Chopra et al. with the study entitled "High-Resolution Mass Spectrometer–Based Ultra-Deep Profile of Milk Whey Proteome in Indian Zebu (Sahiwal) Cattle" reported the first in-depth characterization of the low abundant proteins fraction present in milk whey of indian zebu (Sahiwal) cattle, being sahiwal a more disease and heat resistant cattle compared to most farmed *Bous taurus* cattle. In this study Chopra et al. optimized the protein extraction procedure and by combining different in gel and in solution strategies identified more than 6200 proteins from bovine milk whey (Chopra et al.). A high proportion of these low abundant proteins were found to display an immune regulation and host defense role in bovine. This subset of proteins included lactoglobulins, lactoperoxidases, caseins, and other immune-related proteins such as complement C3. The chromosomal mapping of the identified proteins demonstrated an uneven contribution of all chromosomes in the translation of the identified bovine whey proteome.

Continuing with the search of mammalian milks with high similitudes with HM, Zhang et al. in the study entitled "Quantitative Label-Free Proteomic Analysis of Milk Fat Globule Membrane in Donkey and Human Milk," included in this Research Topic, characterized the specific and crucial fraction of milk fat globule membrane (MFGM) proteins of donkey milk (DM) (Zhang et al.). It is known that DM is a mammalian milk that highly resembles HM as it displays similar protein and lactose contents, and fatty acids and protein profiles compared to HM (4). Additionally, MFGM proteins of DM are smaller and therefore show high digestibility in infants (5). Due to these reasons, the interest in DM from the dairy and infants-food industries is growing. The detailed characterization of MFGM performed by Zhang et al. demonstrated the significant presence of common MFGM proteins in both analyzed milks, representing the 43% of the total MFGM proteome in DM. Besides, the fraction of uniquely identified proteins from DM included proteins such as semaphoring 7A, complement C3, proteins from the solute carrier (SLC) superfamily or multiple apolipoproteins (Apos), among others. Similarly to what was reported by Chopra et al. for sahiwal bovine milk (Chopra et al.), the gene ontology and KEGG pathway enrichment analysis demonstrated that the uniquely identified MFGM proteins from DM were involved in immune response, participating in complement activation, defense response and positive regulation of B cell activation, fact that may be positive when using DM in infant formulas.

With all this, we believe that this Research Topic puts together a representative collection of studies that reflect the state of the art in this interesting and emerging field aimed to decipher the secrets and biological role(s) of dietary proteomes and their potential implications in health and disease conditions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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High-Resolution Mass Spectrometer–Based Ultra-Deep Profile of Milk Whey Proteome in Indian Zebu (*Sahiwal*) Cattle

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Milk serves as a mode of protection to neonate through transferring the host defense proteins from mother to offspring. It also guards the mammary gland against various types of infections. Along with the presence of six vital proteins, bovine milk (whey) contains a massive class of minor proteins, not all of which have been comprehensively reported. In this study, we performed an LC-MS/MS-based ultra-deep identification of the milk whey proteome of Indian zebu (*Sahiwal*) cattle. Three independent search engines that are Comet, Tandem, and Mascot-based analysis resulted in the discovery of over 6,210 non-redundant proteins commonly identified. Genome-wise mapping revealed that chromosome 1 showed a minimum expression of 14 proteins, whereas chromosome 19 expressed 250 maximum proteins in milk whey. These results demonstrate that milk proteome in *Sahiwal* cattle is quite complicated, and minor milk fractions play a significant role in host defense.

Keywords: host defense, Indian zebu, mass spectrometer, milk whey, proteomics, *Sahiwal*

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INTRODUCTION

Milk synthesis is an absolute feature that defines the mammalian class. It has received considerable interest because of its nutritional and functional properties to newborn and young offspring (1). It majorly acts as a vital source for the transfer of defense molecules against pathogens from mother to child. Worldwide, humans consume bovine milk in their diet, and a regular diet accounts for 80–90% of total bovine milk intake and acts as an essential source of nutrition to them. Milk acts as a medium for the transfer of host defense proteins, yet its repertoire of minor proteins has been only partly characterized (2). In addition, bovine milk is exceedingly being utilized for dairy products, including yogurt, curd, cheese, butter, and, to some extent, as bioactive peptides (3).

Milk from different species was previously studied as a substitute for human milk in infant food. Several studies were mainly focused on major milk components (4). The low abundant proteins in milk could be explored with the development of analytical methods. The comprehensive understanding of milk of different species could be beneficial in promoting the utilization of milk as a source of nutrition. Attaining a complete knowledge of the milk whey proteome could serve as a significant foundation for the production of functional foods and infant products.

More recently, proteomic approaches based on mass spectrometry have been used for farm animal milk proteome research (5). Various proteomic approaches have been identified for exploring the molecular pathways and cellular functions of the complex milk proteins in caseins, whey protein, and milk-fat globular membrane (6). Proteomic studies have been conducted for exploring the milk whey proteome in humans and bovine for the identification of proteins related to host defense and immune system (7).

Sahiwal (*Bos indicus*) cattle breed is the dominant milch breed of Indian origin having native tract in North-Western region of India but a much broader breeding tract in the country. In terms of milk production, it is a high milk-producing breed (8); it is known to be genetically more thermotolerant and less disease susceptible (9). In the tropics, *Indicine* cattle have lesser disease susceptibility for reproductive and productive disorders and parasitic infections compared to *taurine* cattle (10). Also, Indian native zebu cattle (*Sahiwal*) can survive comparatively well on low input production systems with moderate milk production but yield much more when maintained on the intensive system of production. Therefore, it is crucial to generate the proteome profile of Indian zebu cattle with the aim of identifying low abundant proteins involved in the host defense mechanism of *Indicine* cattle. The protein composition of milk varies with genetic and non-genetic factors (11, 12). In cow milk, the significant determinants of milk proteome include breed, feed conversion, stage of lactation, parity of production, and environmental determinants (13). The foremost fraction of milk includes caseins, and ∼20% of milk composition is the whey proteome comprising alpha-lactalbumin and beta-lactoglobulin. Bioactive proteins and peptides in cow milk were reported to play a different role in cellular and physiological functions. It acts as a significant determinant in the development of immune response, protective functions against fungal, bacterial, and viral infections, and milk also plays a vital role in the maintenance of intestinal microbiota (14). Most of the reported proteins responsible for high biological activity are immunoglobulins, lactoferrin, α-lactalbumin, β-lactoglobulin, αS1-, αS2-, β-, k-caseins, and lactadherin (15). The presence of immunoglobulins in whey confers the initial line of passive defense to neonates, and in adults, it regulates a person's immune system (16). β-Lactoglobulin and lactoferrin fractions of milk proteome exhibit anticarcinogenic, immunomodulatory, antimicrobial, and antioxidant responses (17). Therefore, it is essential to perform the in-depth comparison of whey proteome of different species for unraveling the diversity in genetics and biological traits for a given species. Previously, a study identified the limited number of 211 proteins but demonstrated the uniqueness of a few proteins in the given species (1). Recent reports suggest the comparison of milk proteins within Holstein and Jersey breeds of dairy cattle (18). They reported differences in the low abundant proteins present in the skimmed milk fractions of Holstein and Jersey breeds fed on the same diet with similar management conditions. A report on comparison of different lactation stage proteins was conducted in Malanad Gidda (*Bos indicus*) cattle (19); however, to date, no such report is available in Indian zebu (*Sahiwal*) cattle. The present study was conducted to explore the milk whey proteome of Indian zebu cattle. As per our knowledge, it is the first report of milk whey proteome of *Sahiwal* cattle of Indian origin and can be used as a comprehensive reference database for cattle genetic resources of India.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals, reagents, and organic solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Ultra-pure grade water was used throughout. All reagents used for this study were of molecular grade.

Selection of Animals for Milk Collection

Indian zebu (*Bos indicus*) cattle, *Sahiwal* breed ($n = 10$), in different significant lactation phases were used for the current study. The animals were reared in the animal herd under uniform feeding and breeding practices at the ICAR–National Dairy Research Institute, Karnal, India. Before the study, all animals were screened for the absence of mastitis by the California mastitis test. Only the healthy cows with somatic cell count ≤200 lakhs per cell without any sign of mammary gland infection were included in the study, and it was ascertained that the milk is free from bacteria. Before the collection of milk, the rectal examination for the absence of high body temperature was obtained twice a day, and cows having normal body temperature range were used for milk collection. All cows had free access to water and offered the same *ad libitum* diet.

The ICAR–National Dairy Research Institute, Karnal, India, animal care, and the committee approved all procedures used in this study. The milk samples were immediately transported to the laboratory under an icebox within 1 h and pooled before the experiment. The pooled samples aim to reduce the animal-to-animal variations.

Sample Preparation

Raw milk samples were defatted by centrifugation at 4,000 rpm for 15 min to obtain skimmed milk samples. Subsequently, the skimmed milk samples were ultracentrifuged at 65,000×*g* for 2 h at 4°C for separating casein, and the liquid portion was referred to as milk whey. Phenyl methyl sulfonate was added at a concentration of 0.01% to prevent proteolytic degradation of milk whey samples. The samples were stored at −80°C for further experimentation and analysis.

Optimization by Various Protein Extraction Methods

Various protein extraction procedures were followed and optimized, which includes (a) ultracentrifugation followed by acetone precipitation, (b) $CaCl_2$ precipitation methods at different concentrations (60, 90, and 120 mM), and (c) TCA/acetone precipitation methods, respectively.

a. **Ultracentrifugation followed by acetone precipitation**

The milk was subjected to ultracentrifugation at 65,000×*g* for 2 h for whey preparation with minor modifications (7). Whey samples were further precipitated by adding chilled acetone and

incubated at −20°C for 14–16 h followed by protein precipitation at 13,000 rpm at 4°C for 10 min (20). The supernatant was discarded, and the pellet was reconstituted with 1× PBS by adding 0.2% DEA (diethylamine) and 20% 0.5 M Tris of pH 6.8. Subsequently, the sample was dried in the vacuum-sealed concentrator and processed for tryptic digestion and mass spectrometric analysis.

b. **$CaCl_2$ precipitation**

$CaCl_2$ precipitation method was optimized at different concentrations (60, 90, and 120 mM) of $CaCl_2$, respectively, for precipitating milk whey proteins from defatted milk samples followed by SDS-PAGE profiling of the whey precipitate.

c. **TCA/acetone precipitation**

TCA/acetone precipitation method was used for precipitating protein samples. Then 1.5 ml of 10% TCA/acetone was added to the whey sample and stored at −20°C overnight (21). The sample was centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was discarded. The pellet was further re-suspended in 1× PBS and 2% DEA (diethylamine) for pellet dissolution.

Protein Quantitation

2D-Clean Up kit (GE Healthcare, USA) was used for removal of interfering substances from precipitated milk protein preparation, and total protein concentration was estimated using Bradford protein estimation kit (Bio-Rad) as per the manufacturer's instruction.

SDS-PAGE

Twenty-five micrograms (25 μg) of pooled Proteo-miner enriched milk whey sample (pooled from 10 animals of different lactation stages, $n = 10$), respectively, were subjected to 12% SDS-PAGE (10 × 10.5 cm) in a Mini VE complete gel electrophoresis system (GE Healthcare, USA). The gel was stained with colloidal Coomassie brilliant blue dye for in-gel digestion.

In-gel Tryptic Digestion

The gel lane with the enriched sample was cut into 12 equal pieces, which was further de-stained using 40% ACN (acetonitrile) and 40 mM NH_4HCO_3 (ammonium bicarbonate) at a ratio of 1:1 (*v*/*v*), respectively. The de-stained gel bands were reduced with 5 mM dithiothreitol (DTT) in 40 mM NH_4HCO_3, followed by alkylation with 20 mM iodoacetamide in 40 mM NH_4HCO_3. Overnight digestion of gel bands was carried out using 12.5 ng/μl trypsin (modified sequencing grade; Promega, USA) at 37°C. Further, peptide extraction was done from gel pieces, lyophilized, and desalted using zip tip (Millipore, Germany) following the manufacturer's instruction and stored at −80°C for peptide identification by LC-MS/MS.

In-solution Tryptic Digestion

For in-solution digestion, 500 μg of pooled protein samples (50 μg each from 10 animals pooled from different stages of lactation) from TCA/acetone precipitated protein samples were processed separately. Further, 45 mM DTT (dithiothreitol) was dissolved in 50 mM NH_4HCO_3 (ammonium bicarbonate) to reduce disulfide bonds followed by alkylation of cysteine residues using 10 mM IAA (iodoacetamide) in 50 mM NH_4HCO_3 (ammonium bicarbonate). Digestion was carried out overnight using trypsin (1:100) at 37°C. The reaction was subsequently stopped with 10% TFA (trifluoroacetic acid), and peptides were further vacuum dried. Samples were fractionated into 24 fractions, further desalted by zip tip, and further subjected for peptide identification by LC-MS/MS (22).

Electrospray Ionization Tandem Mass Spectrometry LC-MS/MS Analysis

The lyophilized peptide fractions were reconstituted in 0.1% formic acid in LC-MS-grade water and subjected to nano-LC (Nano-Advance; Bruker, Germany) followed by identification in captive ion source (Bruker Captive Spray tip) spray-in Maxis-HD qTOF (Bruker) mass spectrometer (MS) with high mass accuracy and sensitivity. The peptides were enriched in nano-trap column (Acclaim Pep Map, particle size 5 μm, pore size 100 Å; Thermo Scientific) and eluted on to nano-analytical column (Kaya Tech HIQ SIL C_{18}HS/3, 0.1 × 150 mm, 3 μm particle size, and 200 Å pore size). The peptide elution was carried out using a linear gradient of 5–45% acetonitrile at 400 nl/min flow rate in a total run time of 135 keeping the solvent system as follows: solvent A, 100% water in 0.1% formic acid; and solvent B, 100% acetonitrile in 0.1% formic acid. Positive ions were generated by electrospray, and the q-TOF was operated in data-dependent acquisition mode to automatically switch between MS and MS/MS acquisition. Precursor ion TOF MS survey scan was acquired with a range of 300–1,800 m/z with resolution R = 75,000. Q1 sequentially selects six most intense precursor ions for fragmentation using collision-induced dissociation for MS-MS analysis with a fixed cycle time of 3 s along with 2 min of release for exclusion filter (Data acquisition otof software, version 24.8; Bruker Daltonics).

Data Processing and Analysis

The vendor-created.d file format was analyzed with three different search engines that are Mascot, Comet, and Tandem for high confidence identification of the profiled whey proteome. For Mascot (Matrix Science, UK, version 2.4.1), we used the ProteinScape platform (version 2.0). Initially, the peak lists were generated through Hyster (Bruker Daltonics) to make spectral data in the form of mgf format (Mascot Generic Files). Resultant mgf files were used for the identification of proteins using the UniProt database downloaded April 3, 2019 along with usual contaminant proteins for spectra examination. The parameter for MS/MS ion search contains tryptic digested sites with two missed cleavage allowed, flexible modification on amino acids—methionine for oxidation, N-terminal acetylation, Gln-pyro Glu, and Glu-pyro Glu, while static modification of cysteine as carboxy amidomethylation or propionamide. The peptide precursor ion tolerance was 20 ppm, with MS/MS tolerance of 0.01 Da. The "ion score cutoff" was set to 30, thereby eliminating the lowest quality matches and minimum peptide length as six amino acid residues. To increase the confidence and remove the false-positive identification, a 1% false discovery rate (FDR) was used at equal peptide and protein levels. The decoy reversed sequences database was included for the calculation of the FDR.

The data were also re-searched in Trans-Proteomic Pipeline (version 5.1.0) for Comet and Tandem search engines. The in-depth procedure for performing the analysis was reported in Suhail et al. (23). Briefly, in the first step, the raw data were transformed to open format mzML files. The converted files were searched using the TPP pipeline keeping the aforementioned common database and the search parameters. However, on a different note, TPP initially performs the peptide assignments using Tandem and Comet search engine. In addition, PeptideProphet and ProteinProphet algorithms were employed in the pipeline to calculate the probability values for both independently examined peptides and the corresponding proteins. The exact mass model for high probability in PeptideProphet was utilized to boost the confidence for peptide associations and also to promote the definite possibility of peptides. Extra protein validation level was performed applying both PeptideProphet and ProteinProphet scores, where the protein was confirmed if it includes at least two top-ranked peptides with each peptide probability score above 95% (**Supplementary Table 1**). iProphet algorithm validated and merged all search engine results. This method takes as the input of PeptideProphet spectrum-level results from multiple LC-MS/MS runs and then computes a unique probability at the level of a unique peptide sequence (or protein sequence). This structure provides for the incorporation of outcomes from various search engines. It brings into account additional promoting determinants, including the number of sibling experiments distinguishing the equivalent peptide ions, the number of replicate ion identifications, sibling ions, and sibling modification states. A model of iProphet performance with regard to the abundance of correct entries vs. error is provided in **Supplementary Table 1**. An iProphet probability of higher than 0.9999 was accepted as the cutoff for the conclusive identification of the protein. For protein quantitation, $\geq$2 unique peptides per protein were estimated to ensure high-quality quantitation.

Bioinformatics Analysis

All the graphical analyses were performed in the R environment using respective tools. Histograms, density scatter plots, and principal component analysis (PCA) were generated using the ggPlot2 package. All the identified proteins were mapped with UniProt bovine chromosomal proteome information and parsed to create files appropriately formatted for input to Circos. The Gene Ontology (GO) categories were analyzed using the DAVID bioinformatics resources, and only the genes with a *p* adjusted value (FDR) of $<$0.05 were included and subsequent GO term plotted.

RESULTS

Assessment of Different Methods for the Quality of Aqueous Milk Whey Protein Extraction

The present study aims to determine the comprehensive profile of bovine milk whey proteome in Indian zebu (*Sahiwal*) cattle. The method optimization using three different procedures (ultracentrifugation-acetone precipitation, $CaCl_2$ precipitation at different concentrations, and TCA/acetone precipitation methods) for the isolation of the aqueous whey fraction showed that TCA/acetone precipitation performed best and resulted in the identification of maximum peptides/proteins in quality check LC-MS/MS runs (data not shown). The SDS-PAGE profiles for different protein extraction procedures and method optimization for milk whey samples also showed a better profile of the TCA/acetone precipitation method in comparison with others (**Supplementary Figures 1A,B**). Before running the whole experiment, we performed the quality assurance test; we ran the 2D gel for the TCA/acetone precipitated whey and identified the clear sorted pattern of spots (**Supplementary Figure 2**), suggesting its suitability for further in-depth proteome analysis.

Protein Identification by LC-MS/MS

We employed the optimized bovine milk whey extraction workflow for the identification of ultra-deep proteome (**Supplementary Figures 1**, **2**). We ran the SDS-PAGE whey proteome profile of 18 animals separately (**Supplementary Figure 3**) and selected 10 animals whose profile was matching almost similar for sample pooling. In this way, we can determine the whey proteome profile of the population and neglected the animal-to-animal variations. The isolated proteome of indigenous zebu (*Sahiwal*) milk whey samples were fractionated using a combined approach: (1) *in-gel digestion* LC-MS/MS methods and (2) *in-solution digestion* LC-MS/MS methods. Samples prepared from *in-gel* and *in-solution* tryptic digestion were subjected to high-resolution qTOF, nano-LC-MS/MS (**Figure 1**). An outstanding feature of analyzing this dataset is the conversion of open file format and its interchange data processing between modules (i.e., comet, tandem, SpectraST) under the umbrella of Trans Proteomics Pipeline (TPP). Finally, the total combined run of 36 fractions resulted in the identification of 1,154,123 spectra, 57,286 peptides, and 6210 proteins. The authenticity and confidence of the identified proteome were also confirmed through cross-mapping the proteome information using three independent proteomics software (TPP and Protein Scape) (**Supplementary Tables 1**, **2**). Only the 6,210 common proteins identified in all the three platforms were selected for further analysis. All the proteins were selected at $<$1% FDR and having the protein prophet and iProphet value of 0.9999 and 0.95, respectively. Next, we chose the MS2-based Normalized Spectral Index for the quantification of individual proteins using the StPeter algorithm implemented in TPP for whey proteome quantitation. It allows the identification of individual proteins in the sample.

To date, this is the largest comprehensive whey proteome dataset. Our next aim was to determine the quality pattern of the proteins present in the whey. We plotted density plots to identify the information; most of the densely populated proteins were determined in the concentration range of 0.0156–0.0625 ng (**Figures 2A,B**) with the identified high-density percentage coverage of 20–65% (**Figures 2C,D**) in whey proteome. The protein length is ranging in between 500 and 800 amino acid

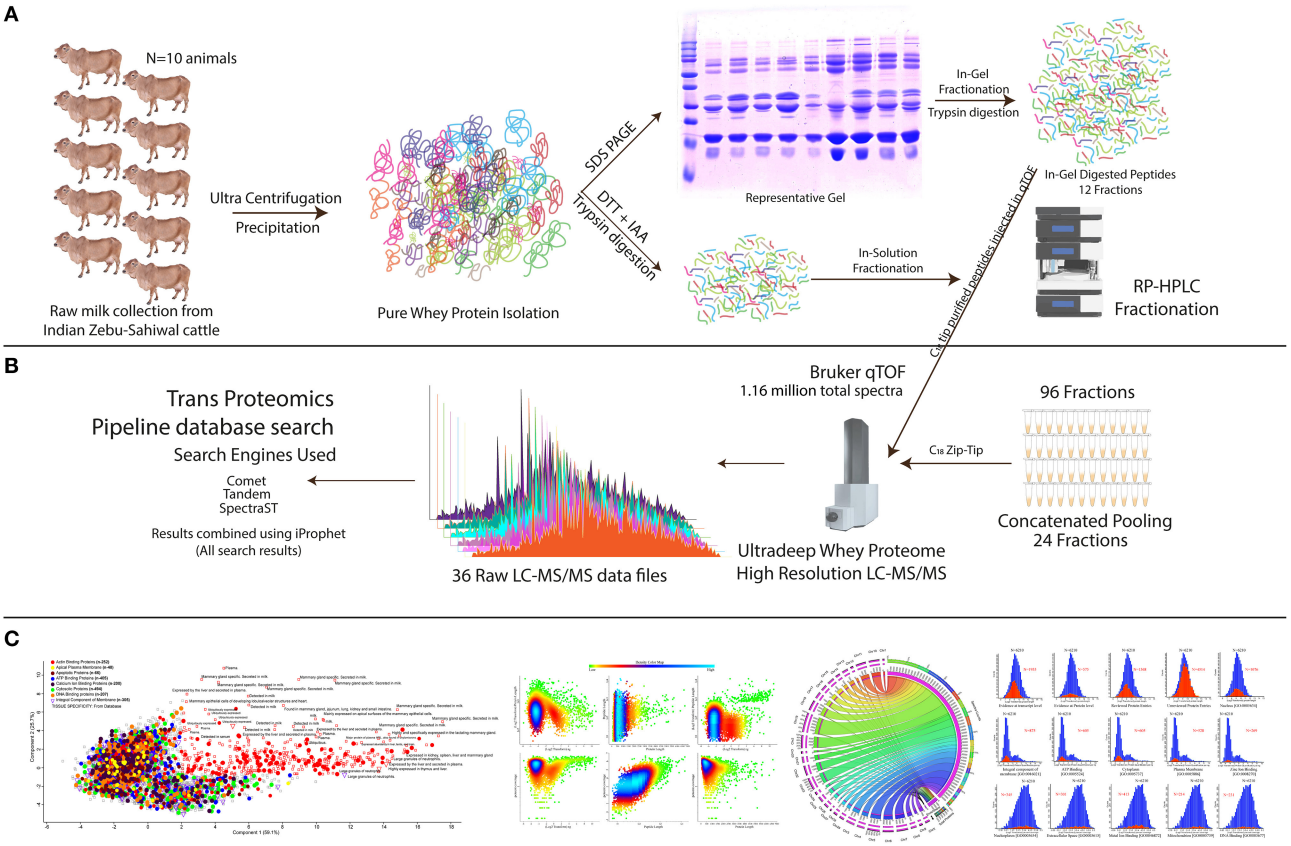

FIGURE 1 | Experimental setup and systemic proteomics workflow for the comprehensive discovery of bovine milk whey proteome. **(A)** From 10 individual animals and pooled milk samples, the milk whey proteome is isolated and purified using ultracentrifugation and TCA/acetone precipitation strategy. In-gel fractionation through SDS-PAGE and in-sol digested peptides were subjected to C18 chromatography for long gradient 96 fractionation (see Materials and Methods). **(B)** C18 separated fractions were concatenated, mixed, and analyzed using a high-resolution qTOF instrument. All the raw data were extracted, and the resulting peptides and proteins were analyzed using the TPP pipeline utilizing Comet, Tandem, and SpectraST search engines, and the obtained results were compiled using iProphet algorithm as described in Materials and Methods section. **(C)** Brief description of the data analysis; detailed information is provided in the Materials and Methods section. For determination of the high confidence bovine whey proteome, data were filtered using the stringent parameter. Evidence of the proteome at the different levels was determined along with the identification of the different Gene Ontology (GO) classes in it.

residues (**Figure 2F**) with the identification of tryptic peptides ranging between 7 and 15 amino acid residues (**Figure 2E**).

Chromosomal Mapping of Bovine Whey Proteome

Next, we sought to determine the expression of the proteins from the individual chromosomes of the genome. We performed the chromosomal mapping analysis creating a Circos plot based on the UniProt data. Total spectra and protein information fetched from the search engine for identification of the proteins were mapped using the UniProt database. We found the contribution of all the 29 + X chromosomes in the whey proteome, but the analysis resulted in interesting facts such as the uneven distribution of the protein expression in the chromosome-wise manner. Chromosome 1 showed a minimum expression of 14 proteins, whereas chromosome 19 expressed 250 maximum proteins (**Figure 3**); the average expression number of proteins per chromosomes is 123. To make the analysis unbiased, we calculated the enrichment percentage of all the chromosomes which is the division of the total proteins assigned in the database to the actual number of proteins identified in the mapping analysis; however, the results are the same (**Supplementary Table 3**). The factors responsible for such high expression of chromosome 19 and very low expression of chromosome 1 contributing to the whey proteome are unknown. Nonetheless, further studies on genome-wide proteome analysis of mammary gland cells are required to answer these questions.

Functional Significance of Bovine Milk Whey Proteins

To understand the overall function of bovine whey protein/proteome, we characterize the subcellular localization using the program ngLOC utilizing the n-gram-based Naïve Bayesian classification model for the fixed-length peptide sequences density distributions over each distinct subcellular

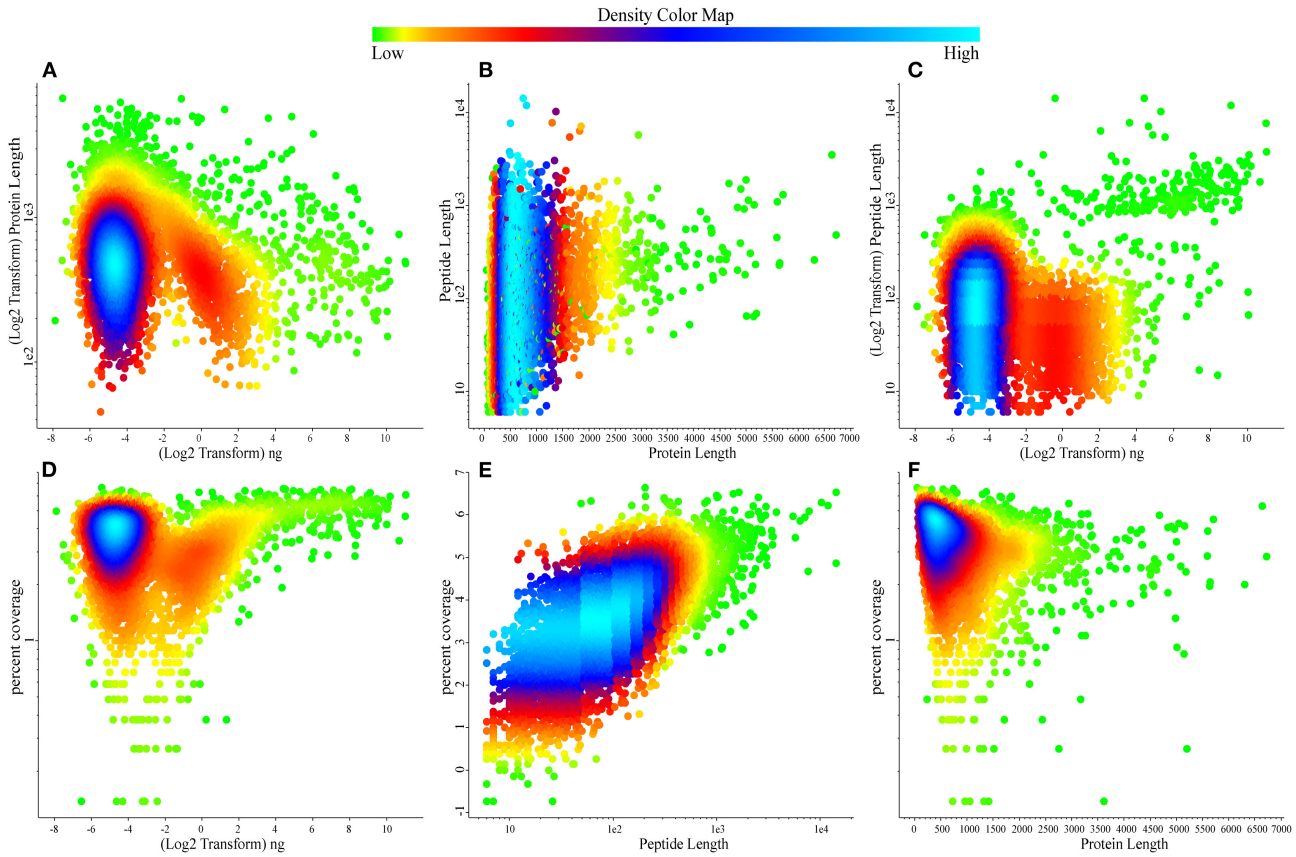

FIGURE 2 | Scatter relationship plots describing the density of attributes over the bovine whey proteome data. **(A)** Protein lengths over quantification values. **(B)** Peptide length over protein length. **(C)** Peptide length over quantification values. **(D)** Protein percentage coverage over quantification values. **(E)** Protein percentage coverage over peptide length. **(F)** Protein percentage coverage over protein length. Color map reports the density of the values in the data, green reports the low, dense data points while sky blue reports highly dense data points.

location (**Supplementary Table 4**). The results showed the identification of 11 different organelle classification while maximum proteins 46.13% (3,012 proteins) in whey are annotated to come from nucleus followed by cytoplasm 18.63% (1,220 proteins) and plasma membrane 17.20% (1,123 proteins). Further, we performed the ontological gene classification and found the majority of proteins in functional GO terms were nuclear proteins (46.13%) followed by proteins present in the cytoplasm (18.68%), placenta (17.2%), exosomes (7.08%), and Golgi bodies (1.18%). The cellular component GO enriched term reveals the identification of 18.92% proteins in the cytoplasm, 17.41% in the nucleus, 16.5% proteins were phosphoproteins, and 15.5% were present in extracellular exosomes. The majority of proteins had a functional role in metal ion binding (9.88%) and ATP binding (9.5%) (**Supplementary Table 5**).

Molecular functions in terms of protein percentage reveal the involvement of the majority of proteins in ATP binding (9.5%) followed by metal ion binding, RNA binding, zinc ion binding, DNA binding, and transcription factor activity. The proteins in biological process class were majorly identified in transcription, DNA template, oxidation–reduction process, intracellular signal transduction, and regulation of transcription. In similar terms, the majority of proteins in the KEGG pathway were present in metabolic pathways, PI3K, Akt, MAPK, and RAS signaling pathways. Also, several proteins have a role in pathways related to cancer, focal adhesion, regulation of actin cytoskeleton, and endocytosis. In the SMART term, the majority of proteins are SMW382: AAA. In Interpro term counts, the majority of protein percentage was found in P-loop containing NTP hydrolase, Protein kinases like domain, Homeodomain, and Serine/threonine–protein kinase active site. Clustering the proteins in terms of tissue specificity, milk proteins revealed expression in the mammary gland, milk, fetal skin and muscle, brain, and oviduct.

In Reactome term counts, 1.09% proteins were involved in platelet degranulation, followed by ubiquinilation and proteasome degradation, anchoring of the basal body to the plasma membrane, cell adhesion, *in utero* embryonic development, oxidation–reduction process, and positive regulation of gene expression (**Supplementary Figure 4**).

Bioactive proteins present in the whey fraction are involved in a wide range of physiological activities, including anti-inflammatory effects and protection against pathogen-induced intestinal inflammation (24). The relationship between counts vs.

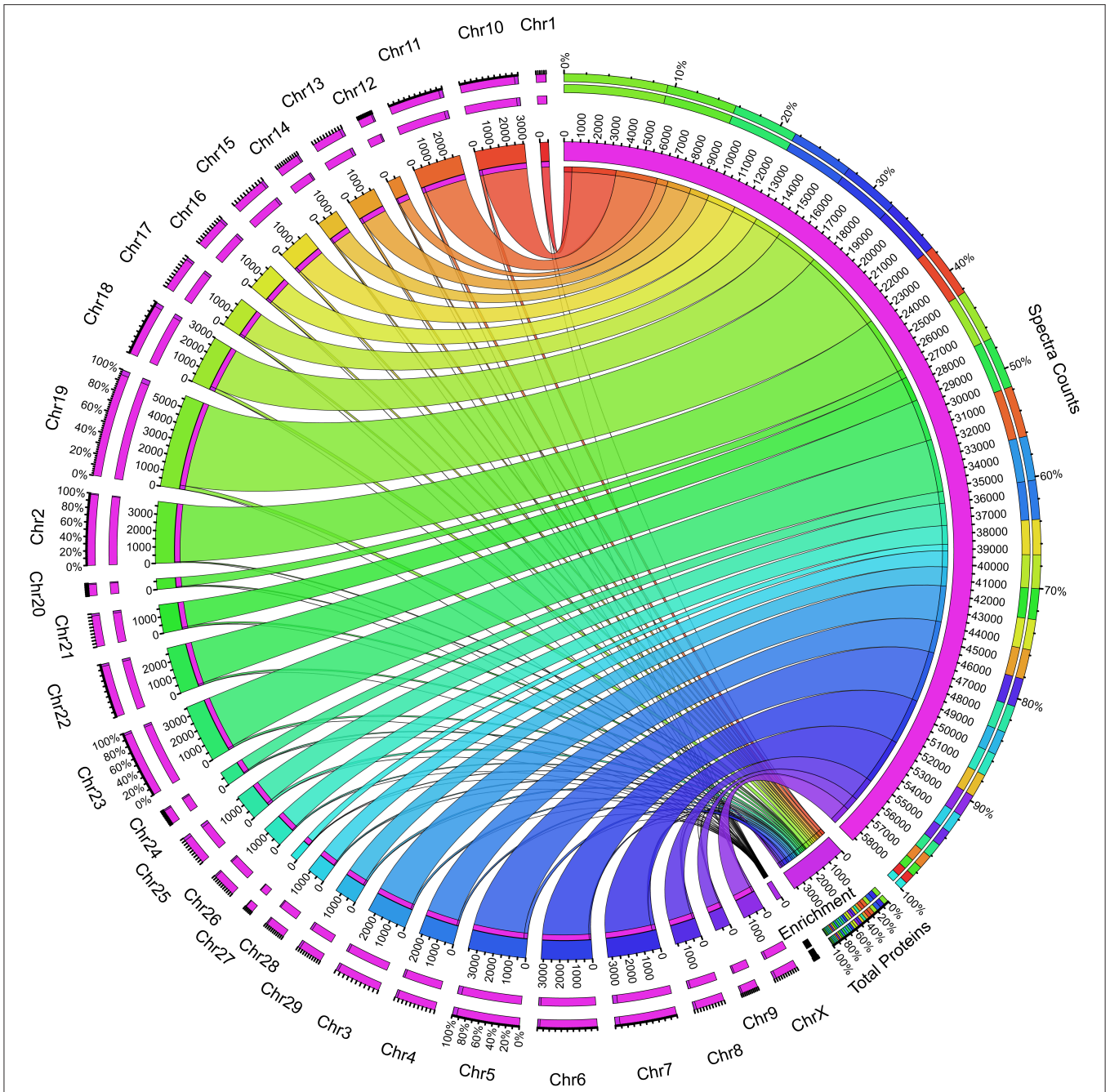

FIGURE 3 | Circos plot showing the whey protein expression mapped to individual chromosomes in the bovine genome. The right inner pink ring indicates the total spectra collected during the proteome analysis. The connecting set of ribbons describes the collection of spectra identified from individual chromosomes, and its width showed the number of spectra assigned. The higher the number spectra, the broader the ribbon width and vice versa. The bottom of the plot describes the total proteins assigned and the enrichment value based on the protein coded for the respective chromosome. The left outer ring describes the chromosomes and the number of protein assignment, which expresses bovine whey.

log_2-transformed protein length reveals that out of 6,210 proteins, 1,955 proteins had evidence at transcript levels; however, 575 proteins have evidence at the protein level. Out of 6,210 entries, 1,568 protein entries are reviewed, and 4,514 entries are unreviewed (**Figure 4**). The relationship between counts vs. log_2 protein quantification per ng reveals 875 proteins as integral components of the membrane (GO: 0016021). The GO terms reveal that 1076 proteins (GO: 0005634) were present in the nucleus, 605 in the cytoplasm (GO: 0005737), 528 in the plasma membrane (GO: 0005886), 345 proteins

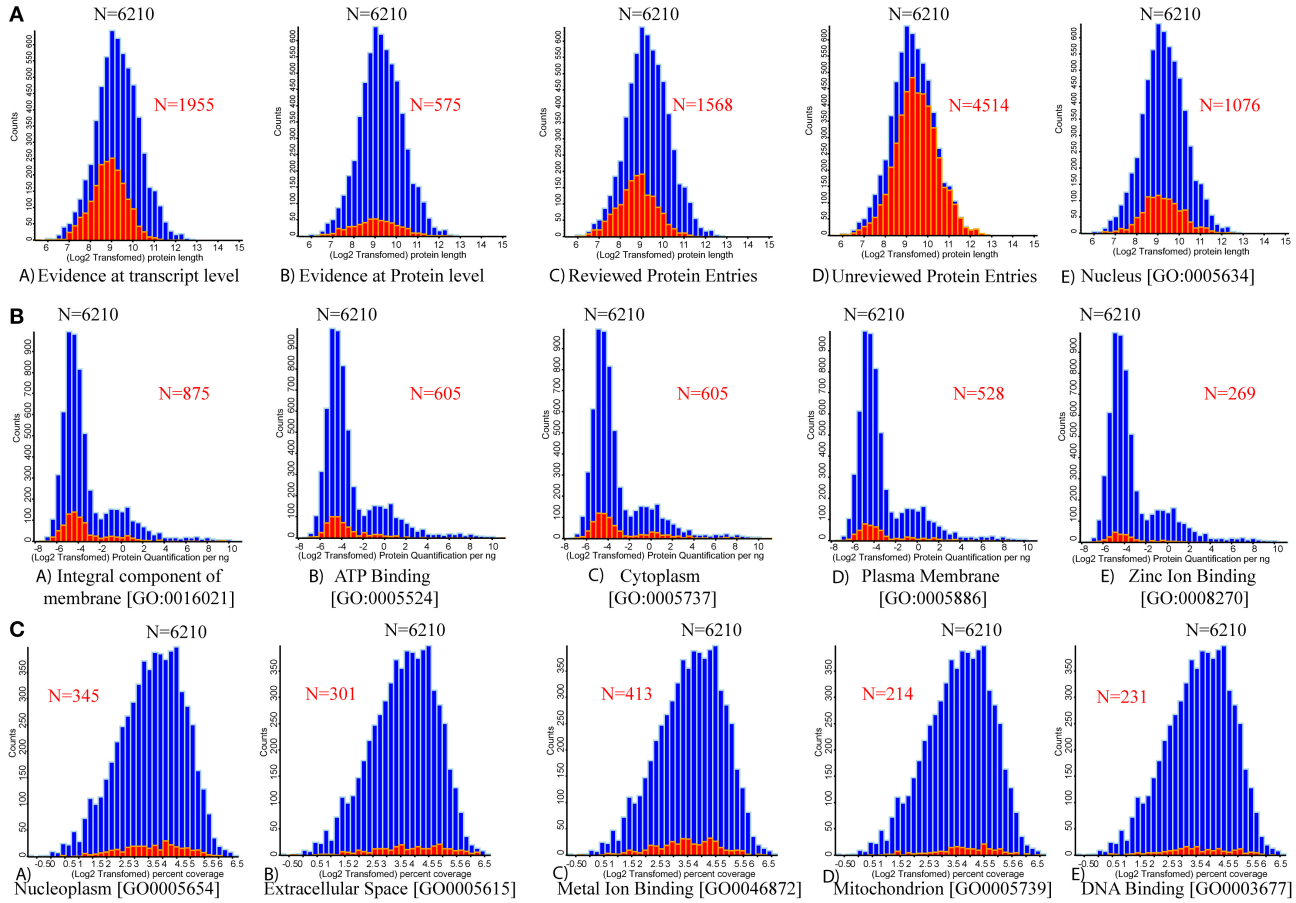

FIGURE 4 | Histograms for the distributions of the protein lengths, protein quantification, and percentage coverage are presented. The vertical axis of the histogram presents the number of each categorical protein in each bin. **(A)** Distribution of varying protein length (full length) counts identified through database mapping to the number of proteins. (A) Evidenced at transcript level. (B) Evidenced at protein level. (C) Reviewed UniProt database protein entries. (D) Un-reviewed UniProt database protein entries. (E) Protein associated with nucleus origin. **(B)** Mapping the number of protein quantification values for five high abundant UniProt-based GO terms. (A) Integral component of membrane. (B) ATP binding. (C) Cytoplasm. (D) Plasma membrane. (E) Zinc ion binding proteins. **(C)** Mapping the number of individual protein percentage coverage for the next five high abundant UniProt-based GO terms. (A) Nucleoplasm. (B) Extracellular space. (C) Metal ion binding. (D) Mitochondrion. (E) DNA binding. Red color overlapped histogram presents the respective mapped attribute over the whole bovine whey proteome data (n = 6,210).

in nucleoplasm (GO: 0005654), 301 in extracellular space (GO: 0005615), and 214 in mitochondria (GO: 0005739). Some other proteins involved in different metabolic processes include 605 proteins in ATP binding (GO: 0005524), 269 proteins in zinc ion binding (GO: 0008270), 413 proteins in metal ion binding (GO: 0046872), and 231 proteins in DNA binding (GO: 0003677).

PCA Score Plots for Milk Whey Proteome

Milk whey proteins can be graphically represented using a PCA score plot where two components explain maximum variability. It is used as a data reduction tool where the score plot represents a dataset of 6,210 proteins. These were assigned as actin-binding proteins (n = 252), apical plasma membrane (n = 48), apoptotic protein (n = 66), ATP binding protein (n = 405), Ca ion binding protein (n = 200), cytosolic protein (n = 494), DNA binding protein (n = 207), and integral component of membrane (n = 305). The score plot classifies the whey proteins based on their involvement in several metabolic, biochemical, and cellular processes. Actin-binding proteins are maximally distributed, and the majority of proteins were mammary gland specific, secreted in milk. Some of the integral components are found in the large granules of neutrophils with their possible role in immunoregulation (**Figure 5**).

Many of the milk bioactive proteins and peptides are also known to exhibit multifunctional physiological properties. In the present study, the majority of proteins with a high score as beta-lactoglobulin, lactoperoxidase, caseins, alpha-lactalbumin, alpha-2 macroglobulin, complement C3, lactotransferrin, alpha-S2 and S1 casein, xanthine oxidase, serotransferrin, and lactadherin were found to play a role in immune regulation and host defense. The top 60 lowest abundant proteins identified with high confidence levels and significant scores are shown in **Table 1**. The majority of the high score proteins have a role in immune status and host defense against invading microbes. However, the high abundant proteins like complement C3, lactoferrin, lactoperoxidase, and

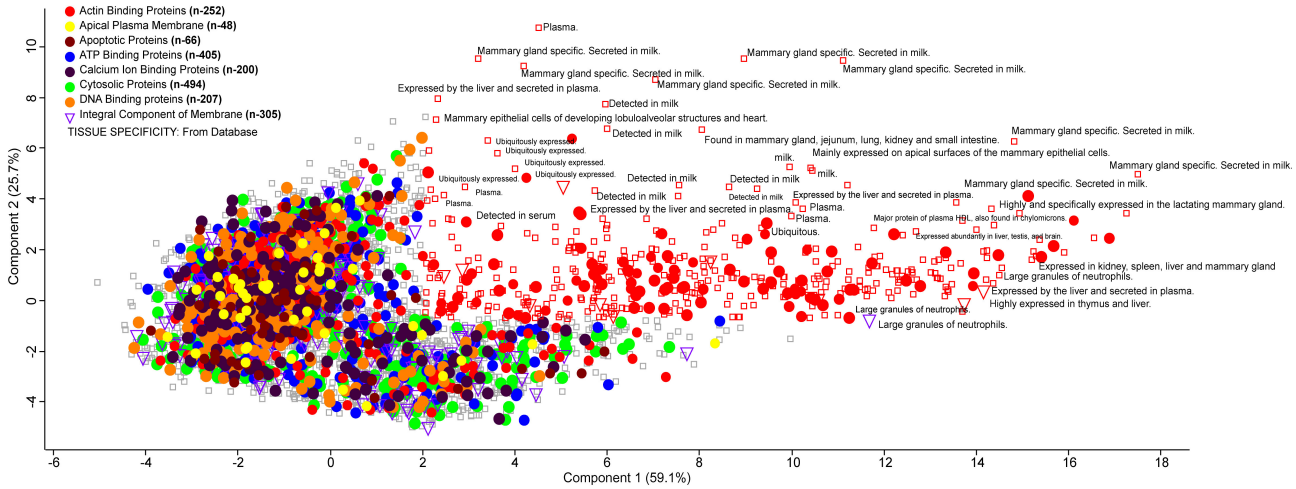

FIGURE 5 | PCA plot describing the orthogonal dispersion of the proteins reported in the deep whey proteome analysis. Color coding indicates the different class of major proteins identified in the whey proteome data. All the proteins identified to specific tissue from the UniProt database are reported with a rectangle.

xanthine oxidase proteins exhibit immunomodulatory and anti-inflammatory properties. Polymeric immunoglobulin receptor (PIGR) and osteopontin proteins play a role in the innate immune system (25). Heat shock proteins (HSP 70) were found to be involved in pathways related to thermotolerance and help in maintaining homeostasis against adverse climatic conditions in livestock (26).

DISCUSSION

To date, to the best of our knowledge, the current report is the most comprehensive characterization of minor bovine milk proteome. Bovine milk is a complex body fluid containing various secreted proteins and is an important source of human nutrition. However, the minor immune-related host defense proteins have not been wholly characterized and need to be explored exhaustively. Milk-derived bioactive peptides present in whey and casein have potential roles as health-promoting agents against cancer, diabetes, hypertension, osteoporosis, and several other disorders (27). Whey proteins can serve promising health benefits in terms of their ability to act as antioxidant, antihypertensive, anti-tumor, anti-viral, anti-bacterial, and hypo-lipidemic (28). Bioactive peptides present in milk exosomes, colostrum, milk fat globular membrane, skimmed milk, and whey provide information on generating large bovine milk proteome datasets.

The present study was designed with the aim to explore the in-depth milk whey proteome of Indian zebu (*Sahiwal*) cattle, which was not studied earlier. Indian zebu (*Sahiwal*) cattle have been acclaimed for the potential to disease resistance and adaptability for heat stress as compared with exotic *Bos taurus* cattle. For proteomics analysis purpose, different protocols for milk protein extraction were attempted including acetone precipitation of full cream milk (20), ultracentrifugation to pellet caseins (7), ammonium sulfate precipitation of caseins to isolate serum (29), and acetic acid removal of caseins to isolate whey proteins (30). Further, optimization of protein extraction methods in skimmed milk of Holstein and Jersey breeds was studied (21), where they compared three protein extraction procedures that were urea-based buffer, TCA/acetone, and methanol/chloroform. The results reported the methanol/chloroform method as the best-optimized method for peptide identification in cattle breeds. In our study, we optimized three methods for protein extraction, which includes ultracentrifugation followed by acetone precipitation, $CaCl_2$ precipitation at different concentrations, and TCA/acetone precipitation method. Out of these three methods, TCA/acetone precipitation was found to be the best-optimized method with maximum peptide identification in comparison with other protein extraction methods.

In an attempt to generate the proteome map, Smolenski et al. used bovine skimmed milk, whey, and milk fat globular membrane during peak lactation, colostrum, and mastitis stages. The results identified only 53 minor milk host defense proteins with 2,903 peptides (2). The latest report on differentially expressed changes in lactation and parity stage-specific whey proteome determined 103 proteins on primiparous cows (31). According to earlier reports, milk proteins from exosomes, milk fat globular membrane, skimmed milk, and whey have a nutraceutical role (32). Putative biomarkers of negative energy balance from milk proteins of early lactation in dairy cows were identified (33). The study found six milk proteins out of 59 proteins linked to metabolism and mammary gland cell proliferation. Another study was conducted on the characterization of bovine milk whey proteome during early lactation in Holstein and Jersey breeds by Tacoma et al. (18). They identified 935 low abundant proteins in skimmed milk fractions of the two breeds and highlighted the breed difference between proteome of Jersey and Holstein Friesian

TABLE 1 | Top 60 low abundant proteins identified in whey proteome with respective database information, molecular function, cellular component, biological process, Prosite, Pfam, InterPro, SMART, and SUPFAM.

Protein	Protein names	Gene symbol	ng	Molecular function	Cellular component	Biological process	Prosite	Pfam	InterPro	SMART	SUPFAM
F1MCK2	AHNAK nucleoprotein	AHNAK	0.074		Costamere	Regulation of RNA splicing	PS50106		IPR001478	SM00228	SSF50156
E1BPD7	Marker of proliferation Ki-67	MKI67	0.066				PS50006	PF00498	IPR000253	SM00240	SSF49879
F1N415	Piccolo presynaptic cytomatrix protein	PCLO	0.075	Metal ion binding	Presynaptic active zone	Synapse assembly	PS50004	PF00168	IPR000008	SM00239	SSF50156
E1BGB0	Kinesin family member 13B	KIF13B	0.054	14-3-3 protein binding	Axon	Microtubule-based movement	PS00845	PF01302	IPR036859	SM01052	SSF49879
F1MV51	APC regulator of WNT signaling pathway	APC	0.094	Beta-catenin binding	Beta-catenin complex	Cell cycle arrest	PS50176	PF05972	IPR026836	SM00185	SSF48371
E1BLA0	Shugoshin 2	SGOL2	0.057		Centromeric region	Meiotic sister chromatid cohesion			IPR026706		
E1BKT9	Desmoplakin	DSP	0.018	Cell adhesion molecule binding	Plasma membrane	Adherens junction organization	PS50002	PF00681	IPR028462	SM00250	SSF75399
E1BPB1	Kinesin family member 24	KIF24	0.093	ATPase activity	kinesin complex	Microtubule-based movement	PS00411	PF00225	IPR027640	SM00129	SSF47769
L8IEV2	Dynein heavy chain 1, axonemal	M91_21125	0.078	ATPase activity	Dynein complex	Cilium movement		PF12774	IPR035699		SSF52540
E1BHP0	Alpha-mannosidase	MAN2B2	0.095	Alpha-mannosidase activity	Vacuolar membrane	Mannose metabolic process		PF09261	IPR011013	SM00872	SSF74650
L8IV13	Leucine-rich repeat-containing protein KIAA1731	M91_18003	0.083	Microtubule binding	Centriole	Positive regulation of centriole elongation		PF15309	IPR029299		
L8HSL9	WD repeat-containing protein 87	M91_17430	0.085				PS50082	PF00400	IPR015943	SM00320	SSF50978
E1BKZ5	Golgin B1	GOLGB1	0.084		cis-Golgi network	Protein localization to pericentriolar material			IPR026202		
F1N2K7	Phosphoinositide kinase, FYVE-type zinc finger containing	PIKFYVE	0.033	1-Phosphatidylinositol-3-phosphate 5-kinase activity	Cell–cell junction	Intracellular signal transduction	PS50186	PF00118	IPR002423	SM00049	
E1BC24	Midasin	MDN1	0.062	ATPase activity	Cytosol	Ribosomal large subunit assembly	PS50234	PF07728	IPR003593	SM00382	SSF52540
E1BKC4	Nipped-B protein	NIPBL	0.071	Chromatin binding	Chromatin	Brain development		PF12765	IPR011989		SSF48371
L8IT59	Vacuolar protein sorting-associated protein 13A	M91_05527	0.081					PF09333	IPR015412		
E1BKI3	Nitric oxide synthase (EC 1.14.13.39)	NOS1	0.065	Calmodulin binding	Cytosol	Arginine catabolic process	PS51384	PF00667	IPR003097	SM00228	SSF50156
E1B9N6	Envoplakin	EVPL	0.035	Intermediate filament binding	Cornified envelope	Epidermis development		PF00681	IPR041615	SM00250	SSF75399

(Continued)

TABLE 1 | Continued

Protein	Protein names	Gene symbol	ng	Molecular function	Cellular component	Biological process	Prosite	Pfam	InterPro	SMART	SUPFAM
E1BPX1	Vacuolar protein sorting 13 homolog C	VPS13C	0.103		Cytosol	Mitochondrion organization		PF09333	IPR015412		
O46382	Brefeldin A-inhibited guanine nucleotide-exchange protein 1	ARFGEF1	0.091	ARF guanyl-nucleotide exchange factor activity	Cytosol	Endomembrane system organization	PS50190	PF16213	IPR016024	SM00222	SSF48371
F1MC51	ATP binding cassette subfamily B member 5	ABCB5	0.067	ATPase activity	Plasma membrane		PS50929	PF00664	IPR003593	SM00382	SSF52540
E1BHT5	Ubiquitin protein ligase E3 component n-recognin 4	UBR4	0.065	Ubiquitin-protein transferase activity	Centrosome	Ubiquitin-dependent protein catabolic process	PS51157	PF13764	IPR016024	SM00396	SSF48371
E1BCI2	E3 ubiquitin protein ligase (EC 2.3.2.27)	RNF40	0.061	Metal ion binding	HULC complex	Histone H2B ubiquitination	PS00518		IPR013956	SM00184	
E1BL95	Centromere protein J	CENPJ	0.037	Identical protein binding	Centriole	Microtubule polymerization		PF07202	IPR033068		
E1B7W1	Thyroid hormone receptor associated protein 3	THRAP3	0.092			RNA splicing		PF15440	IPR026667		
F1N137	Inositol 1,4,5-trisphosphate receptor type 2	ITPR2	0.094	Inositol 1,4,5 trisphosphate binding	Endoplasmic reticulum		PS50919	PF08709	IPR014821	SM00472	SSF100909
F6RF21	Structural maintenance of chromosomes flexible hinge domain containing 1	SMCHD1	0.047	ATPase activity	Barr body	Double-strand break repair		PF06470	IPR036890	SM00968	SSF55874
F1MHT1	Amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	AGL	0.047	4-Alpha-glucanotransferase activity	Cytoplasm	Glycogen biosynthetic process		PF06202	IPR008928		SSF48208
L8J1D8	Eukaryotic translation initiation factor 3 subunit B (eIF3b)	EIF3B	0.072	Translation initiation factor activity	43S complex	Cytoplasmic translation initiation complex	PS50102	PF08662	IPR011400	SM00360	SSF54928
F1MJ95	Unc-80 homolog, NALCN channel complex subunit	UNC80	0.081	Cation channel activity	Axon	Cation homeostasis		PF15778	IPR031542		
E1B9N8	Lysine methyltransferase 2D	KMT2D	0.033	Histone binding	MLL3/4 complex	RNA polymerase II	PS51805	PF05965	IPR034732	SM00542	SSF47095
F1MWK8	Protein tyrosine kinase 7 (inactive)	PTK7	0.089	ATP binding	Cell–cell junction	Actin cytoskeleton reorganization	PS50835	PF07679	IPR007110	SM00409	SSF48726
E1BCV4	Nucleoporin 98	NUP98	0.059	mRNA binding	Kinetochore	Nuclear pore complex assembly	PS51434	PF04096	IPR037665		SSF82215
A4IFK4	Synaptopodin 2	SYNPO2	0.076	Actin binding	Cytoskeleton	Actin bundle assembly	PS50106	PF00595	IPR001478	SM00228	SSF50156
A6QLI5	Melanoma-associated antigen D4 (MAGE-D4 antigen)	MAGED4	0.094				PS50838	PF01454	IPR037445	SM01373	

(Continued)

TABLE 1 | Continued

Protein	Protein names	Gene symbol	ng	Molecular function	Cellular component	Biological process	Prosite	Pfam	InterPro	SMART	SUPFAM
E1BLB6	Protein kinase, DNA-activated, catalytic subunit	PRKDC	0.086	ATP binding	DNA-dependent kinase- ligase 4 complex	Activation of innate immune response	PS51189	PF02259	IPR016024	SM01343	SSF48371
F1MGR3	Arginine-glutamic acid dipeptide repeats	RERE	0.100	Chromatin binding	Histone deacetylase	Branching of a nerve	PS51038	PF03154	IPR002951	SM00439	SSF46689
A6H737	Lysyl oxidase homolog 2 (Lysyl oxidase-like protein 2)	LOXL2	0.080	Calcium ion binding	Basement membrane	Cellular protein modification process	PS00926	PF01186	IPR001695	SM00202	SSF56487
A6QQJ9	GPRASP1 protein	GPRASP1	0.085					PF04826	IPR006911		SSF48371
F1MZM7	Synapse defective Rho GTPase homolog 2	SYDE2	0.054			Signal transduction	PS50238	PF00620	IPR035892	SM00324	SSF48350
A0JN33	Kin of IRRE like 3	KIRREL3	0.083		Plasma membrane		PS50835	PF07679	IPR007110	SM00409	SSF48726
F1MNU5	Phospholipase A2	PLA2G4F	0.078	Calcium-dependent phospholipase A2	Cytoplasm	Arachidonic acid secretion	PS50004	PF00168	IPR016035	SM00239	SSF52151
E1B949	FAT atypical cadherin 4	FAT4	0.086	Calcium ion binding	Apical part of cell	Branching involved in ureteric bud	PS00010	PF00028	IPR002126	SM00112	
F1MZU6	Collagen alpha-3(IV) chain	COL4A3	0.048	Extracellular matrix	Collagen	Apoptotic process	PS51403	PF01413	IPR008160	SM00111	SSF56436
E1BEB3	Histone acetyltransferase	KAT6A	0.102	DNA binding	Cytosol	Cellular senescence	PS51504	PF01853	IPR016181	SM00526	SSF46785
E1BKZ9	Sortilin	SORT1	0.070		Cytosol	Endocytosis		PF15902	IPR031777	SM00602	
L8HZQ1	Kielin/chordin-like protein	M91_00882	0.063				PS01208	PF00093	IPR036084	SM00832	SSF57567
F1MG90	WDFY family member 4	WDFY4	0.023				PS50197	PF02138	IPR016024	SM01026	
L8J3H5	Sperm-associated antigen 1	M91_19629	0.096				PS50005	PF13877	IPR025986	SM00028	SSF48452
E1BHN4	RING-type domain	RNF213	0.063	ATPase activity	Cytoplasm	Wnt signaling pathway	PS00518	PF00097	IPR003593	SM00382	SSF52540
F1MFU7	Zinc finger protein 862	ZNF862	0.069	Nucleic acid binding		Transcription	PS50805	PF05699	IPR008906	SM00349	SSF10964
E1BNA3	Adaptor related protein complex 4 subunit epsilon 1	AP4E1	0.064		AP-4 adaptor complex	Vesicle-mediated transport		PF01602	IPR017109	SM01356	SSF48371
P98167	SCO-spondin	SSPO	0.044	Peptidase inhibitor	Extracellular	Cell adhesion	PS01225	PF08742	IPR006207	SM00832	KOG1215
E1BEJ9	NLR family apoptosis inhibitor	NAIP	0.056	ATP binding	Plasma membrane	Apoptotic process	PS50143	PF00653	IPR003593	SM00382	SSF52540
F1MFH3	FRAS1 related extracellular matrix protein 2	FREM2	0.055		Basement membrane	Cell communication	PS51854	PF03160	IPR038081	SM00237	SSF141072
E1B754	Microtubule associated serine/threonine kinase 2	MAST2	0.086	ATP binding	Microtubule cytoskeleton	Cytoskeleton organization	PS51285	PF08926	IPR000961	SM00228	
E1BP05	Usherin	USH2A	0.092	Collagen binding	Apical plasma membrane	Animal organ morphogenesis	PS01248	PF00041	IPR013320	SM00180	SSF49265
A3KN18	CEBPZ protein	CEBPZ	0.067			Ribosome biogenesis		PF03914	IPR016024		SSF48371

cows. However, no reports are available to date on milk whey profiling in Indian zebu cattle. Thus, the present study focuses on the global proteomic profiling of Indian zebu (*Sahiwal*) cattle, an important milch breed of the Indian subcontinent, to generate the proteomic profiling data for the first time. The findings suggest the identification and role of pathways related to activated immune response and stress tolerance homeostasis mechanisms. However, comparative proteomic analysis across different lactation stages was conducted in Malnad Gidda cattle (19) and Jersey and Kashmiri cattle (34). A recent report compiled an online database with 3,100 proteins from milk whey, milk fat globular membrane, and exosomes (35). Similarly, another separate study reported 4,654 cow milk proteins in five lactation stages to create the database. The stages were colostrum, early lactation, mid-lactation, peak lactation, and milk fractions consisting of exosomes, skimmed milk, and milk fat globular membrane (36).

Altogether, small sets of bovine milk proteome have been studied earlier, but none of them was able to provide the exhaustive proteome profile. The current report is the first time identification of 6,210 proteins in milk whey of Indian zebu (*Sahiwal*) cattle. It provides an in-depth identification of milk whey proteome to understand the role in human health and its biological significance. We determined proteome with the role in metal ion binding, zinc binding, and many more. Notably, from 6,210 proteins, a total of 1,568 proteins have UniProt reviewed protein entries, but the large majority (4,514) are unreviewed protein entries that have been validated by our data and could be further explored. Our bioinformatics analysis found that identified proteins are involved in various activities as ATP binding, DNA binding, metal ion binding, and zinc ion binding. Our fuzzy clustering analysis identified the presence of metal ion binding proteins, clusters in two independent databases Pfam and Panther, suggesting the ample presence of these proteins in whey (**Supplementary Figure 5**). The PCA revealed that actin-binding proteins are maximally distributed, and the majority of proteins were mammary gland specific, secreted in milk. Some of the integral components are found in the large granules of neutrophils with their possible role in immunoregulation.

The cellular component–centered gene ontology analysis reveals the involvement of major identified proteins in the nucleus, cytoplasm, plasma membrane, nucleoplasm, and mitochondria. Based on the interactive studies of whey proteins with metal ions, our whey proteome dataset proposed that it has potential applications in nutraceuticals and food industry (37). The metal ion binding properties of whey proteins could be highly useful and provide a way for designing functional foods. Purification of whey proteins such as lactoferrin which is known for its anti-bacterial and antimicrobial role can be utilized as a nutritional and dietary supplement as well as synergetic treatment in patients with anemia, kidney disease (38), and also in cancer (39). The low abundant minor milk proteins have a potential role in host defense and immune regulation. Several studies reviewed the role of immunoglobulins in milk for health and immunity (40–42). The presence of antimicrobial proteins and peptides such as beta-defensin, cathelicidins, complement proteins, and several other minor milk whey proteins were reported (42–46). In our study, we identified several minor proteins as cathelicidin 1 and 2, gelsolin, interferon 12 subunit alpha, fatty acid-binding protein, nucleobindin 2, transthyretin, claudin, haptoglobin, MFGE8 protein, polymeric immunoglobulin receptor, ubiquilin 4, and collectin 46 with different roles and biological significance (**Table 1**).

The complete proteome advocates that milk acts as an excellent medium for microbial growth that is evident from the enormous diversity of different high concentration peptides and proteins. It is the best source of nitrogen content for microbial growth. It allows the natural growth and habitat of beneficial microbes. Recently, we identified the occupancy of probiotics in fermented milk products and found a constant abundance (47). The determination of different attributes from the isolated microorganism showed positive results to classify them in the probiotics category (48). Combined genomics and proteomics analysis provide the landmarks for several genes and proteins coded in the genome for the probiotic attributes (49, 50). Hence, it strongly suggests that the whole milk supplementation allows the nourishment and abundant growth of healthy gut microbiota.

Apart from the significant milk proteins with a role in the innate immune system and host defense, numerous minor proteins were found to be overwhelming and found to be involved in response to regulation of immunity and inflammation. Quantification of the protein amount in ng reveals that significant whey proteins as alpha-lactalbumin, beta-lactoglobulin, beta-casein, ribonuclease pancreatic, albumin, and immunoglobulin chain proteins were identified in higher protein concentrations. Several other low abundant proteins (**Table 1**) as gelsolin, tetranectin, haptoglobin, cathepsin z, lysozyme, brain ribonuclease, peptidoglycan recognition protein 1, cathelicidin 2, vimentin, and others have been identified with a possible role in biological and physiological mechanisms.

CONCLUSION

Milk proteome profiling was reported for the first time in Indian zebu (*Sahiwal*) cattle. Although studies were conducted in Holstein and Jersey (*B. taurus*) cattle, there is a lack of milk proteome profiling data in Indian zebu (*Sahiwal*) cattle. In this study, different protein extraction procedures as ultracentrifugation followed by acetone precipitation, $CaCl_2$ precipitation at different concentrations, and TCA/acetone precipitation were optimized where TCA/acetone precipitation gave maximum peptide identification by LC-MS/MS. This study led to the identification of 6,210 proteins from *in-gel digestion* and *in-sol digestion*. All the generated proteomics raw files were analyzed with three different search engines, namely, Mascot, Comet, and Tandem, for high confidence identification of the profiled whey proteome. The majority of the proteins

identified with a high score were found to play a role in immune regulation and host defense system implicating lesser disease susceptibility and better adaptability of Indian zebu (*Sahiwal*) cattle. The present study represents the first time reported *Sahiwal* milk proteome data in Indian zebu cattle, which could serve as a database for different cattle genetic resources of India.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AC and SA equally designed, conducted data analysis, and wrote the article. AC, SA, SB, PR, and VV helped in designing and performing the experiments. SA performed the data analysis and created the illustrations for the article. SK and AM supervised the whole project and reviewed the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.00150/full#supplementary-material

Supplementary Figure 1 | (A) Comparison of SDS-PAGE patterns of milk whey samples for optimization of different extraction procedures. 1D image of milk whey protein extraction procedures followed for optimization. (a) Ultracentrifugation followed by acetone precipitation. (b) $CaCl_2$ precipitation method at different concentrations: 60, 90, and 120 mM. (c) TCA/acetone precipitation method. **(B)** Comparison of different methods of preparation of bovine milk whey in Indian *Sahiwal* cattle. (a) Acid precipitation at different pH. (b) Ultracentrifugation at 65,000×*g* for 2 h. (c) Ultracentrifugation followed by acid precipitation.

Supplementary Figure 2 | Two-dimensional gel electrophoretogram of bovine milk whey in Indian *Sahiwal* cattle.

Supplementary Figure 3 | Animal-to-animal proteome SDS profile of 18 different animals shown on two individual gels **(A,B)**. Ten best sharing proteome profiles were selected for the determination of deep whey proteome.

Supplementary Figure 4 | All the different Gene Ontological Information determined for the whey proteome data shown in **(A–J)**. **(A)** ngLog mapped total proteome. **(B)** Functional annotation terms specified. **(C)** Complete cellular component counts. **(D)** The total molecular function counts. **(E)** The total biological function counts. **(F)** Full KEGG pathway annotation. **(G)** Total SMART term counts. **(H)** Total InterPro terms counts. **(I)** Tissue-specific proteins determined. **(J)** Total reactome term counts.

Supplementary Figure 5 | Fuzzy C-means clustering of proteins identified with the metal ion binding ability.

Supplementary Table 1 | Final complete table for identifications of total whey proteins in Trans Proteomics Pipeline (TPP) using Comet and Tandem search engine through *in-gel* and *in-solution* digestion.

Supplementary Table 2 | Final complete table for identifications of total whey proteins in ProteinScape using Mascot search engine through *in-gel* and *in-solution* digestion.

Supplementary Table 3 | Total number of chromosome-associated spectra counts, total proteins mapped, and enrichment values.

Supplementary Table 4 | ngLOC-based classification of whey proteome in respective cellular organelle classification.

Supplementary Table 5 | DAVID-based classification of all the proteins mapped in whey proteome.

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Structural Changes and Evolution of Peptides During Chill Storage of Pork

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In this work, we investigated changes in protein structures in vacuum-packed pork during chill storage and its impact on the *in vitro* protein digestion. *Longissimus dorsi* muscles were vacuum packed and stored at 4°C for 3 days. Samples were subjected to Raman spectroscopy, *in vitro* digestion and nano LC-MS/MS. The 3 d samples had lower α-helix content, but higher β-sheet, β-turn, and random coil contents than the 0 d samples ($P < 0.05$). SDS-PAGE revealed significant protein degradation in the 3 d samples and the differences in digested products across the storage time. Proteome analysis indicated that the 3 d samples had the higher susceptibility to digestion. Increasing protein digestibility was mainly attributed to the degradation of myofibrillar proteins. Thus, exposure of more enzymatic sites in loose protein structure during chill storage could increase protein degradation in meat.

Keywords: pork, chill storage, *in vitro* digestion, Raman spectroscopy, LC-MS/MS

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INTRODUCTION

Postmortem aging and its impact on eating quality of fresh meat has been widely concerned (1–3). Physicochemical and structural changes in meat proteins occurring in postmortem aging and processing may affect the protein digestion and nutritional value of meat (4–6). Postmortem aging for relatively long time may produce bioactive peptides in beef that exhibit DPPH radical scavenging, ACE- and renin-inhibitory activities (7). However, little is known about whether bioactive peptides are produced in a short-term aging of pork.

At early postmortem time, the dephosphorylation of several metabolic enzymes has been shown to affect the rate of glycolysis and pH decline, and subsequently the activation of μ-calpain, the release of lysosomal enzymes and meat tenderization (8). In addition, protein oxidation may occur in fresh meat during chill storage (9), which may cause the formation of disulfide bonds, and a decrease in protein hydrophilicity and water holding capacity. To avoid such changes, vacuum packaging and chill storage have been widely applied (10). However, protein oxidation and structural changes may still occur in vacuum-packed and chilled meat because radical oxygen species widely existing in living muscle tissues could be retained in postmortem muscles (11). Furthermore, protein oxidation also alters the protein structures and digestibility of meat (5). An increase in digestion can be attributed to protein unfolding and increased susceptibility to digestion (4). Previous studies have focused how processing and physicochemical changes affect protein structure and digestion (12, 13). However, few data are available on how structural changes

of meat proteins enhanced digestibility of fresh meat from a proteomic perspective, in terms of the evolution of peptides from the stomach to the small intestine. In this study, we investigated the structural changes of meat proteins in pork during chill storage and their impacts on protein digestibility and the release of bioactive peptides.

MATERIALS AND METHODS

Reagents

Ellman's reagent (4 mg/mL 5,5′-dithiobis-2-nitrobenzoic acid in Tris-Gly) and Tris-Gly (10.4 g Tris, 6.9 g glycine, and 1.2 g EDTA per liter, pH 8.0) were obtained from Sigma Aldrich (St. Louis, MO, USA), as were porcine gastric pepsin (Cat. No. P7125) and porcine pancreatic trypsin (Cat. No. T7409). BCA protein assay kit (No. 23225) and protein marker (No. 26619) were obtained from Thermo Scientific (Rockford, IL, USA). Amicon Ultracel-3 membrane (UFC500396) and Zip Tip C18 pipette tips (ZTC18S096) were obtained from Millipore (Billerica, MA, USA).

Sample Preparation

Longissimus dorsi muscles (size: 10 × 10 × 5 cm) were obtained from 8 native Suhuai pig carcasses at the same line in a commercial slaughterhouse. Each muscle was cut into four 2.5 × 10 × 5 cm pieces (weight: 50 to 65 g each), vacuum-packed and stored at 4°C for 3 days. Samples were taken on 0, 1, 2, and 3 d for further analyses.

Raman Spectroscopy

Raman spectroscopy was performed to evaluate changes in protein secondary structure (LabRAM HR Evolution, Horiba/Jobin, Yvon, Longjumeau, France) as previously described (5). Laser (excitation wavelength: 785 nm, power: 100 mW) was applied and the backscattering Raman signals ranging from 400 to 3,200 cm^{-1} were collected. Raman spectra were normalized against the band at 1,003 cm^{-1}. The 1,685–1,645 and 1,309–1,229 cm^{-1} bands correspond to amide I and III vibrational modes of α-helix, random coil, and β-sheet. The 1,341 and 940 cm^{-1} bands reflect CH-bending and C-C stretching respectively. The range of 1,658–1,650 cm^{-1} in the amide I vibrational mode mainly reflects C=O stretching vibrations, C-N stretching, and N-H in-plane bending of peptide groups. The 1,003, 830–850, and 760 cm^{-1} bands correspond to phenylalanine, tyronsine, and tryptophan, respectively. The α-helix, β-sheet, β-turn, and random coil were quantified (14).

In vitro Digestion

The protein digestibility of cooked meat from 0, 1, 2 to 3 d samples was assessed as described by Zou et al. (15). Briefly, meat pieces were packed in plastic bags and cooked in a 72°C water bath until the core temperature reached 70°C. The cooking time was around 20 min. The core temperature was tracked by a portable thermal probe (Pt 100, Testo AG, Mönchaltorf, Schweiz, Germany). After cooking, meat samples were cooled for 2 h to room temperature (22°C). Then, samples (1.00 g) were homogenized (9,600 rpm, 30 s, twice; 13,400 rpm, 30 s, twice) in PBS (10 mmol/L, pH 7.0). The homogenate was digested by gastric pepsin (32 mg/mL in 0.1 mol/L HCl, pH 1.0) at 37°C for 2 h and the digestion was stopped by adjusting the pH to 7.5 with 1 mol/L NaOH. Then the resulting mixture was digested by trypsin (24 mg/mL in 0.1 mol/L PBS, pH 7.0) at 37°C for 2 h and stopped by heating the system at 95°C for 5 min.

The undigested proteins were precipitated by adding three volumes of ethanol at 4°C for 12 h. Then the samples were centrifuged at 10,000 × g at 4°C for 20 min. Protein contents in the whole meat samples and the precipitates were quantified by a BCA protein assay kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Protein digestibility was calculated as follows:

$$\text{Protein digestibility } (\%) = \frac{W0 - W1}{W0} \times 100\%$$

Where W_1 is the content (g) of undigested proteins precipitated by ethanol. W_0 is the total protein content (g) in the whole meat before digestion.

The supernatant containing digested products that are ethanol soluble was subjected to nano LC-MS/MS analysis and the precipitate including undigested proteins was separated on the SDS-PAGE gels.

SDS-PAGE

The whole proteins were extracted according to the method of Zarkadas and Maloney (16) with some modifications. Briefly, cooked meat samples (0.3 g) were homogenized in 4.5 mL 2% SDS with 3 × 30 s at 10,000 rpm and centrifuged at 4,000 × g for 20 min at 4°C. Two milliliters of the supernatant were dialyzed for 48 h in 1 L distilled water to remove SDS. The dialyzed samples were transferred to new tubes and stored at −80°C for further analyses.

SDS-PAGE was performed to separate proteins or their fragments under reducing conditions (5). Appropriate volumes of samples were mixed with 12.5 μL sample buffer (4×) and 5 μL reducing sample agent (10×) and made up to a total volume of 50 μL with ultrapure water. The final protein concentrations were 0.50 μg/μL for all samples. The samples were heated at 70°C for 10 min. Twelve microliters of samples were loaded in triplicate into the wells of 4–12% precast gels (GenScript, Piscataway, NJ, USA). The gels were run in a total 800 mL of SDS running buffer at 150 V till the blue dye front disappeared. The gels were stained with colloidal Coomassie brilliant blue R250 (CBB) for 30 min and then destained for 20 min three times. CBB staining solution contained 0.1% CBB/45% acetic acid/10% ethanol/45% ultrapure water. Destaining buffer contained 10% acetic acid/10% ethanol/80% ultrapure water. The gel images were acquired by an image scanner (GE Healthcare, Little Chalfont, Uppsala, Sweden) and the band intensities were quantified with the Quantity One image analysis software (Bio-Rad, Hercules, CA). The relative intensities of bands were calculated by the actual band intensity divided by that of the 150 kDa band in the calibration marker lane.

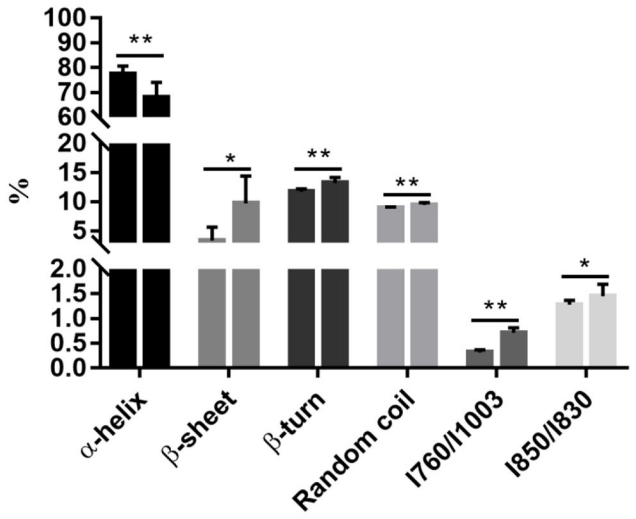

FIGURE 1 | Postmortem aging caused a reduction of the α-helix content but an increase in the β-sheet, β-turn, and random coil contents, accompanying with the exposure of tryptophan. All data were presented as means and standard deviations. Asterisks denote that the contents of α-helix, β-sheet, β-turn, random coil, and the ratios of $I_{760cm}^{-1}/I_{1003cm}^{-1}$ and $I_{850cm}^{-1}/I_{830cm}^{-1}$ differ significantly between the two time points. $^{*}P < 0.05$; $^{**}P < 0.05$.

Protein Identification of Digested Products by Nano LC-MS/MS

The ethanol-soluble fractions of the digested products were identified by *Nano LC-MS/MS* system as previously described (17) with minor modifications. Briefly, ethanol in the supernatant was removed by a vacuum concentrator (ZXJY, Beijing, China), and dried in a freeze dryer (Christ, Osterode, Germany). The dried samples were dissolved in 0.2% formic acid in ultrapure water and then centrifuged (15,000 × g, 15 min, 4°C) in ultra-0.5 mL filter tubes and desalted in ZipTip C18 tips (Millipore, Billerica, MA). Peptides (1.5 μg) were separated in a C18 column (2 cm × 200 μm, 5 μm, Thermo Fisher Scientific, Palo Alto, CA) and then sequentially a C18 chromatographic column (75 μm × 100 mm, 3 μm, Thermo Fisher Scientific, Palo Alto, CA). Elution was applied by running a mixture of buffer A (0.2% formic acid in 60% acetonitrile) and buffer B (0.2% formic acid in ultrapure water) at 300 nL/min. The elution buffers were changed by 97%A from 0 to 10 min, 92%A from 10 to 70 min, 62%A from 70 to 72 min, 2%A from 72 to 82 min, and 97%A from 82 to 90 min. The eluted peptides were identified by a hybrid quadrupole orbitrap mass spectrometer equipped with a nanoelectrospray ionization source (Thermo Fisher Scientific, Palo Alto, CA). A full-scan mode was selected from 300 to 1,800 amu.

MS/MS spectra were matched using the Proteome Discoverer-1.4 (Thermo Fisher Scientific, Palo Alto, CA). Pepsin and pepsin/trypsin were selected in peptic and peptic/tryptic peptides database search, respectively. The parameters for searching were set as follows: MS/MS tolerance: 10 ppm; main search: 4.5 ppm; missed cleavage: 2; searching database: Sus scrofa under Uniprot (Uniprot-Sus scrofa.fasta, www.uniprot.org/); de-isotopic: TRUE; fixed modification: carbamidomethyl (cys); variable modification: oxidation (met), acetyl (protein N-term); label free quantification (LFQ): TRUE; decoy database pattern: reverse; LFQ min ratio count: 1; match between runs: 2 min; peptide false discovery rate (FDR): 0.01; and protein FDR: 0.01. Proteins that could not be annotated were not further used. BIOPEP database was used in the search of similar sequences previously identified showing ACE inhibitory and DPPIV inhibitory activity (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

Statistical Analysis

The effects of storage time on measured variables (α-helix, β-sheet, β-turn, random coil, $I_{760cm}^{-1}/I_{1003cm}^{-1}$ and $I_{850cm}^{-1}/I_{830cm}^{-1}$) were evaluated by Student's *t*-test using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla). The effects of storage time on other measured variables were evaluated by analysis of variance (ANOVA) and the least significant means were compared by Tukey's *post-hoc t*-test. Data were presented as means and standard deviations. The means were considered significantly different if the *P*-value was smaller than 0.05. Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/) were applied to analyze the differences of matched proteins among the four time points.

RESULTS AND DISCUSSION

Changes in Raw Meat Protein Structures Detected by Raman Spectroscopy

Raman spectroscopy provides powerful information on secondary structural modifications in proteins (18). The α-helix content decreased from 77.41% on 0 d to 67.89% on 3 d ($P < 0.05$, **Figure 1**), while β-sheet, β-turn, and random coil increased from 3.39, 11.72, and 8.92% on 0 d to 9.75, 13.21, and 9.49% on 3 d, respectively ($P < 0.05$, **Figure 1**). This indicates that denaturation and unfolding occurred in meat proteins from 0 to 3 d. During aging, meat proteins were degraded by endogenous enzymes into fragments. Degradation of myofibrillar proteins is modulated by protein oxidation and nitrosylation (19).

The side chains of tryptophan and tyrosine residues may change under a polar microenvironment, which results in modifications in the tertiary structure of proteins. The ratios of $I_{760cm}^{-1}/I_{1003cm}^{-1}$ and $I_{850cm}^{-1}/I_{830cm}^{-1}$ were related to the exposed or buried status of tryptophan and tyrosine residues, respectively (20). A substantial increase in the ratio of $I_{760cm}^{-1}/I_{1003cm}^{-1}$ representing changes of tryptophan residues was observed during chill storage ($P < 0.05$, **Figure 1**), indicating transformation of the tryptophan residues from a buried, hydrophobic state to a polar aqueous state (20). The ratio of $I_{850cm}^{-1}/I_{830cm}^{-1}$ significantly increased from 0 to 3 d ($P < 0.05$, **Figure 1**), indicating an increasing number of exposed tyrosine residues and tryptophan residues. In addition, the ratio of $I_{850cm}^{-1}/I_{830cm}^{-1}$ is also a good indicator for the microenvironment and hydrogen bonds of the ionization of the phenolic hydroxyl group (20). It could be the

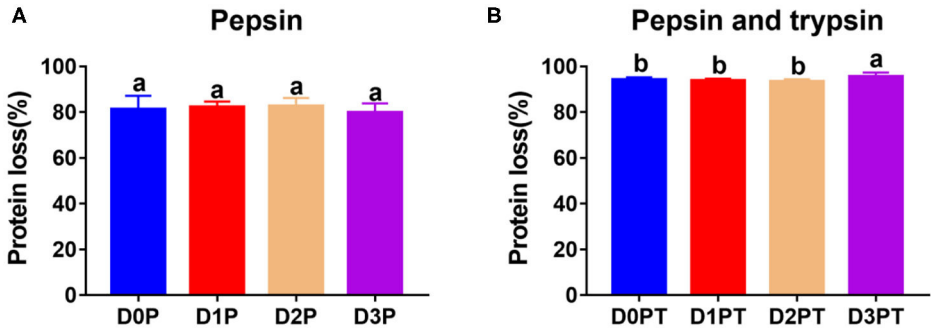

FIGURE 2 | Protein digestibility did not differ after pepsin treatment by differ significantly after pepsin and trypsin treatments. **(A)** D0P, D1P, D2P, and D3P represent 0, 1, 2, and 3 d samples treated by pepsin, respectively. No significant difference was observed among groups ($P > 0.05$). **(B)** D0P/T, D1P/T, D2P/T, and D3P/T represent 0, 1, 2, and 3 d samples treated by pepsin and trypsin, respectively. a, b denotes that protein digestibility differ significantly ($P < 0.05$, $n = 8$ each).

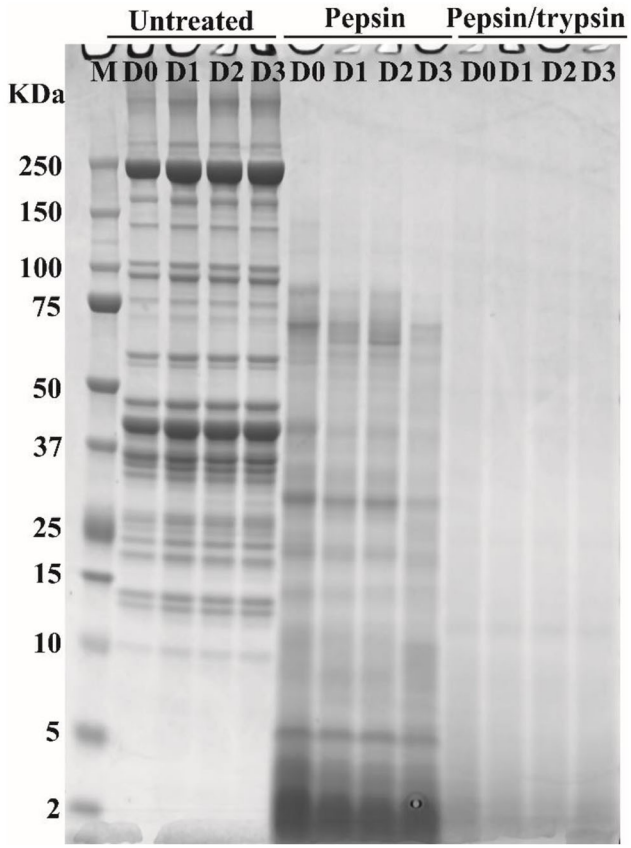

FIGURE 3 | Typical SDS-PAGE patterns of pork proteins before and after digestion. "Untreated" represents total proteins in cooked pork before digestion. Pepsin represents ethanol-insoluble fraction after pepsin digestion. Pepsin/trypsin represents ethanol-insoluble fraction after pepsin and trypsin treatments. Lanes M, D0, D1, D2, and D3 represent calibration marker, 0, 1, 2, and 3 d samples.

exposure of polar amino acid residues at the surface of the protein molecules. The ordered and steady structure of the protein was broken into disordered and loose fragments during storage, from a buried status of tryptophan and tyrosine residues to an exposed status, which exposed cleavage sites of digestive enzymes. Thus, we postulate that this disordered and hydrophobic status would increase the degree of protein degradation by exposing more cleavage sites to digestive enzymes.

The Degree of Protein Degradation in Cooked Meat

No significant difference was observed in the degree of protein degradation after pepsin digestion among the four time points ($P > 0.05$, **Figure 2A**). However, the degree of protein degradation increased after two-step digestion, and the 3 d samples had the highest values ($P < 0.05$, **Figure 2B**). In a previous study, postmortem storage did not improve the *in vitro* digestion parameters of pork (4), in which the structural organization of the muscle cell and the extracellular matrix were not taken into account. To a certain extent, the storage-induced differences in the degree of protein degradation could be attributed to changes in protein secondary or tertiary structures. Such a structural change may further affect protein digestion after meat cooking. Different cooking conditions may affect the structure of meat protein, and the digestibility of meat (17). To avoid it, we cooked meat samples with a similar size for the same time in a water bath. The proteolysis during chill storage may increase protein unfolding and expose more sites to bind to pepsin and trypsin. This is in agreement with the Raman data that reflected structural changes in side chains of proteins, including hydrophobic and electrostatic interactions.

SDS-PAGE of Proteins in Cooked Meat and Their Digested Products

SDS-PAGE was applied to separate soluble meat proteins or their digested products before or after pepsin and trypsin treatments (**Figure 3**). Before digestion (untreated samples), a reduction in band intensity of several protein bands was observed in cooked pork as the storage time of raw meat increased, indicating fragmentation and breakdown of meat proteins. This result was in agreement with our previous study (15). The appearance of the 30 kDa component (Troponin-T fragment) has been considered

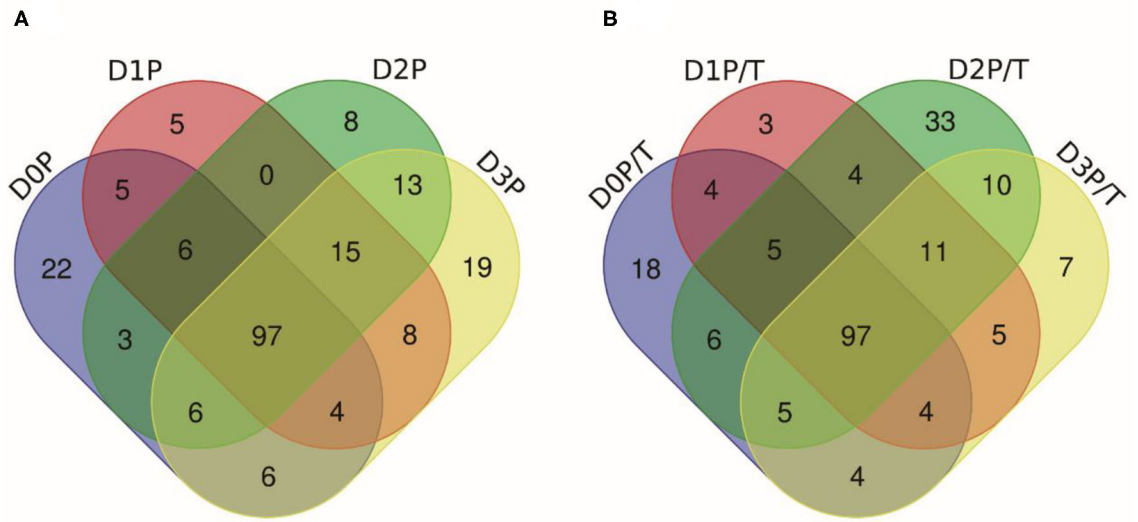

FIGURE 4 | The numbers of unique peptides after pepsin and trypsin treatments. **(A)** D0P, D1P, D2P, and D3P represent 0, 1, 2, and 3 d pepsin treated samples, respectively; **(B)** D0P/T, D1P/T, D2P/T, and D3P/T represent 0, 1, 2, and 3 d pepsin and trypsin treated samples, respectively.

a good indicator of meat tenderization (21). During postmortem storage, high molecular weight proteins (65–200 kDa) may be degraded into smaller fragments (<25 kDa) (22).

In pepsin treated samples, a substantial decrease in band intensity was observed for some large-molecular-weight protein bands (**Figure 3**), which could be due to degradation of proteins into smaller peptides or free amino acids. In addition, some insoluble proteins could become soluble after enzymatic digestion (23). Intensity loss or even disappearance of bands was observed for pepsin and trypsin treated samples (**Figure 3**). Most of large molecular weight bands disappeared because proteins or peptides were further degraded into smaller ones after two-step digestion (**Figure 3**). Similar phenomena have been observed in different pork cuts and pork products (14, 24). However, SDS-PAGE could not separate smaller peptides well that were characterized by nano LC-MS/MS (15).

Unique Peptides Could Be a Good Indicator for Chill Storage Time

Pepsin or two-step digestion products were identified by nano LC-MS/MS and matched to proteins. All matched proteins are listed in **Supplementary Files 1, 2**. Venn diagrams revealed that 97 peptides were matched in all pepsin digested samples and pepsin and trypsin digested samples, respectively (**Figures 4A,B**). Most of these peptides came from myofibrillar proteins (myosin-1, myosin-7, actin) and sarcoplasmic proteins (phosphoglycerate kinase, creatine kinase M-type). In general, peptide abundances decreased as the storage time of raw meat increased, indicating that these peptides were degraded into smaller ones during digestion. Furthermore, 9 peptides matched to myofibrillar proteins, and 52 peptides matched to sarcoplasmic proteins in pepsin-treated samples (**Supplementary File 1**). More peptides matched to myofibrillar proteins (48/97) than to sarcoplasmic proteins (22/97) (**Supplementary File 2**). Taken together, the increased protein degradation could be mainly attributed to the degradation of myofibrillar proteins.

Several common peptides appeared in 1, 2, and/or 3 days samples, which were derived from large molecular weight peptides or proteins. Specifically, 22, 5, 8, and 19 peptides were uniquely matched in pepsin-treated samples on 0, 1, 2, and 3 days respectively, and 18, 3, 33, and 6 peptides were unique for pepsin/trypsin-treated samples at the four-time points (**Figures 4A,B**). Unique peptides could be a good indicator for chill storage time.

Digested Products From Myofibrillar Proteins in Pork Evolved With Storage Time

All peptides matched to myofibrillar proteins were listed in **Table 1**. Previous studies have shown that actin, myosin-1, myosin-4, myosin-7, and many glycolytic enzymes are highly abundant proteins in pork muscle (15, 25). These proteins are involved in muscle contraction and energy production. In the present study, most of differently abundant peptides are derived from these proteins (**Tables 1**, **2**).

Oxidative modification happened in four peptides (MNVKH WPWMKL, EAFVKHIMSI TVKEDQVFPMNPPKF, MSVK NWPWMKL) originating from myosin. Long-chain peptides (e.g., EEAEASLEHEEGKILRIQL) were less abundant than short-chain peptides (e.g., EEAEASLEHEEGKIL) and some long-chain peptides disappeared in 2nd days samples (**Table 1**). After gastric digestion, unique peptides were further hydrolyzed by trypsin. Abundances of common peptides (ALIHYAGT VDY, TVKEDQVFPMNPPKF, LEQQVDDLEGSLEQEK, VKL EQHVDDLEGSLEQEK, KPAAAAAPAPAPAPAPAPAPPK, EDQVFPMNPPK) decreased as the storage time of raw meat increased, indicating that these peptides were digested by pepsin or/ and trypsin (**Tables 1**, **2**). A total of 16 peptides, including

TABLE 1 | Dynamic evolution of peptides derived from myofibrillar proteins after gastric digestion.

	Origin	Accession[a]	Sequence[b]	Modifications	MH+ [Da]	Area[c]			
						D0P	D1P	D2P	D3P
Myosin	Myosin-1	F1SS64	MNVKHWPWMKL		1469.75				
			MNVKHWPWMKL	M9(Oxidation)	1485.74				
			TVKEDQVFPMNPPKF		1776.90				
			TVKEDQVFPMNPPKF	M10(Oxidation)	1792.90				
			EEAEASLEHEEGKIL		1683.81				
			EEAEASLEHEEGKILRIQL		2194.13				
			ETLKRENKNLQQEISDL		2058.08				
			IRIHFGTTGKL		1242.73				
			EHEEGKILRIQL		1464.82				
			SLIHYAGTVDY		1238.60				
			EDQIISANPLL		1212.65				
			MLTDRENQSIL		1319.66				
			ITGESGAGKTVNTKRVIQYF		2169.16				
			WMVTRINQQL		1288.68				
	Myosin-1 (Fragment)	F1SS62	SELKTKEEEQQRLINDL		2073.08				
			KTKEEEQQRLINDLTAQRARL		2540.39				
	Myosin-7	F1S9D6	MSVKNWPWMKL		1419.73				
			MSVKNWPWMKL	M9(Oxidation)	1435.72				
			ALIHYAGTVDY		1222.61				
			IDSRKGAEKLLGSL		1486.86				
			EEAEASLEHEEGKILRAQL		2152.09				
			RQRYRILNPAAIPEGQF		2029.11				
	MLC2v (Fragment)	A1XQV9	NAFKVFDPEGKGVL		1520.81				
			DYKNLVHIITHGEEKD		1910.96				
			AAFPPDVTGNL		1101.56				
	Myosin light chain	Q29069	RALGTNPTNAEVKKVLGNPSNEEMNAKKIEF		3399.77				
			EAFVKHIMSI	M8(Oxidation)	1190.62				
			EAFVKHIMSI		1174.63				
Troponin	Troponin T fast skeletal muscle type	Q75NG6	YQLEIDKF		1055.54				
Tropomyosin	Tropomyosin TM30-pl (Fragment)	P79309	LEEELKNVTNNL		1415.74				
	Beta-tropomyosin (Fragment)	Q8MKF3	LEEKLKEAETRAEF		1692.88				

[a]Protein database ID (Uniprot-Sus scrofa). [b]Peptides obtained by MS/MS analysis. [c]D0P, D1P, D2P, and D3P, 0, 1, 2, and 3 d samples treated by pepsin. Different color indicates different relative abundance of peptides. Red color represents the highest abundance, and the green color represents the lowest abundance.

KPAAAAAPAPAPAPAPAPAPPK, KPAAAAAPAPAPAPAPAPPKEEK, TKLEQQVDDLEGSLEQEK, and TKLEQQVDDLEGSLEQEKK were cleaved into 1 to 4 amino acids peptides by trypsin (**Table 2**).

From the digested products, 117 peptides were selected to match the BIOPEP database showing ACE-inhibitory (angiotensin-converting-enzyme inhibitory) and dipeptidyl peptidase IV (DPPIV) inhibitory activities (**Table 2**). Eighteen peptides were estimated to have ACE-inhibitory and DPPIV inhibitory activities, which have been identified in the database (26). ACE inhibitors are known to beneficial to treat arterial hypertension (27), diabetes mellitus, ischemic heart disease, chronic heart failure, angioedema, chronic kidney disease, and Parkinson's disease (28, 29). DPPIV inhibitor may be beneficial for NASH subjects (30). Four peptides (TKLEQQVDDLEGSLEQEKK, LEQQVDDLEGSLEQEKK, VKLEQHVDDLEGSLEQEKK, LEQHVDDLEGSLEQEKK) were identified, which contain sequences VDDLEGSLEQEKK and DDLEGSLEQEKK that were previously reported to have antioxidant peptides (31). It is of note that some identified peptides maintain a long sequence after the two-step digestion. However, almost all the peptides would be hydrolyzed by peptidases into smaller fragments (dipeptides and tripeptides) locating the outer layer of intestinal epithelium before reaching the blood stream (32). Thus, moderate chill storage may enhance the production of antioxidant, ACE inhibitor and DPP-IV inhibitory peptides.

Table 2 illustrates dynamic evolution of 117 peptides derived from cooked meat samples. The potential bioactive peptides were predominantly originated from myosin, actin, tropomyosin and troponin. Peptides EDQVFPMNPPK (Oxidation) and IEDEQALALQLQK appeared in 0 day samples, whereas peptides EFEMSNLQSKIEDEQALAMQLQK, IEDEQALAMQLQK, and SKQLEDELVSLQK appeared only in 2 day samples. Abundances of LAQESIMDIENEK, LAQESTMDIENDKQQLDEK, and their

TABLE 2 | Dynamic evolution of peptides derived from myofibrillar proteins after two-step digestion.

Origin	Accession[a]	Sequence[b]	Modifications	MH+ [Da]	Area[c]				BIOSEP sequences	Activity
					D0P/T	D1P/T	D2P/T	D3P/T		
Myosin										
Myosin-1	F1SS64	EDQVFPMNPPK		1301.62					MNPPK	ACE-inhibitory
		EDQVFPMNPPK	M7(Oxidation)	1317.61					MNPPK	ACE-inhibitory
		LAQESIMDIENEK		1519.73					EK	ACE-inhibitory
		LAQESIMDIENEK	M7(Oxidation)	1535.72					EK	ACE-inhibitory
		GQTVEQVTNAVGALAK		1585.85						
		TVKEDQVFPMNPPKF		1776.90						
		EDQIISANPLL		1212.65						
		IEDEQALALQLQK		1498.81					QK	ACE-inhibitory
		NDLQLQVQAEAEGLADAEER		2199.06						
Myosin-1 (Fragment)	F1SS62	VVESMQSMLDAEIR	M5(Oxidation)	1623.77						
		VVESMQSMLDAEIR		1607.77						
		LAQESTMDIENDKQQLDEK	M7(Oxidation)	2251.04					EK	ACE-inhibitory
		LAQESTMDIENDKQQLDEK		2235.05					EK	ACE-inhibitory
		NLTEEMAGLDETIAK	M6(Oxidation)	1650.79						
		NLTEEMAGLDETIAK		1634.79						
		AGLLGLLEEMR		1201.66						
		AGLLGLLEEMR	M10(Oxidation)	1217.66						
		LQNEVEDLMIDVER	M9(Oxidation)	1718.83						
		LQNEVEDLMIDVER		1702.84						
		LQNEVEDLMLDVER	M9(Oxidation)	1718.83						
		LQNEVEDLMLDVER		1702.83						
		KLETDISQIQGEMEDIIQEAR	M13(Oxidation)	2462.20						
		KLETDISQIQGEMEDIIQEAR		2446.21						
		KKLETDISQIQGEMEDIIQEAR		2574.31						
		NAYEESLDQLETLK		1652.79						
		NAYEESLDQLETLKR		1808.90						
		TKLEQQVDDLEGSLEQEK		2089.03					EK	ACE-inhibitory
		TKLEQQVDDLEGSLEQEKK		2217.13						
		DIDDLELTLAK		1245.66						
		DIDDLELTLAKVEK		1601.86					EK	ACE-inhibitory
		NLQQEISDLTEQIAEGGK		1972.99						
		NLQQEISDLTEQIAEGGKR		2129.08						
		LEQQVDDLEGSLEQEK		1859.89					EK	ACE-inhibitory
		LEQQVDDLEGSLEQEKK		1987.98						
		AITDAAMMAEELK		1393.67						
		AITDAAMMAEELKK		1521.77						
		EFEMSNLQSKIEDEQAL AMQLQK		2710.31					QK	ACE-inhibitory
		EFEMSNLQSKIEDEQALA MQLQKK		2838.40						
		AEDEEEINAELTAK		1561.72						
		ALQEAHQQTLDDLQAEE DKVNTLTK		2838.41						
		ANLLQAEIEELR		1398.76						
		IQLELNQVK		1084.63						
		KDIDDLELTLAK		1373.75						
		NDLQLQVQAEADSLADAEER		2215.05						

(Continued)

TABLE 2 | Continued

Origin	Accession[a]	Sequence[b]	Modifications	MH+ [Da]	Area[c]				BIOSEP sequences	Activity
					D0P/T	D1P/T	D2P/T	D3P/T		
		SEIQAALEEAEASLEHEEGK		2170.02						
		SQEDLKEQLAMVER		1675.83						
		VAEQELLDASER		1359.68						
		SLIHYAGTVDY		1238.60						
		DTQLHLDDALR		1296.66						
		DTQIHLDDALR		1296.65						
		IEELEEEIEAER		1488.71						
		IEELEEELEAER		1488.71						
		MEGDLNEMEIQLNHANR		2013.91						
		RANLLQAEIEELR		1554.86						
		TLEDQLSELK		1175.62						
		MNVKHWPWMKL		1469.76						
		IEKPMGIF		934.51						
		NTQGILKDTQIHLDDALR		2051.08						
		TNEKLQQFF		1154.58						
		ENKNLQQEISDLTEQIAEGGKR		2500.27						
		IEDEQALAMQLQK		1516.77					QK	ACE-inhibitory
		KAITDAAMMAEELKK	M9(Oxidation)	1665.85						
		KALQEAHQQTLDDLQAEE DKVNTLTK		2966.51						
		KMEGDLNEMEIQLNHANR		2142.01						
Myosin-7	F1S9D6	NLTEEMAGLDEIIAK		1646.83						
		NLTEEMAGLDEIIAK	M6(Oxidation)	1662.83						
		LEDEEEMNAELTAK		1621.72						
		LEDEEEMNAELTAK	M7(Oxidation)	1637.72						
		VKLEQHVDDLEGSLEQEK		2096.06					EK	ACE-inhibitory
		VKLEQHVDDLEGSLEQEKK		2224.15						
		LEQHVDDLEGSLEQEK		1868.89					EK	ACE-inhibitory
		LEQHVDDLEGSLEQEKK		1996.98						
		ALQEAHQQALDDLQAEE DKVNTLTK		2808.40						
		DFELNALNAR		1162.59						
		LAEQELIETSER		1417.72						
		LTQESIMDLENDKQQLDER		2305.10						
		NDLQLQVQAEQDNLADAEER		2299.08						
		NLQEEISDLTEQLGSSGK		1947.95						
		NNLLQAELEELR		1441.77						
		LQDLVDKLQLK		1312.78						
		DTQIQLDDAVR		1273.64						
		LQNEIEDLMVDVER		1702.83						
		LELQSALEEAEASLEHEEGK		2212.06						
Myosin light chain 1/3	A1XQT6	KPAAAAAPAPAPAPAPA PAPAPPK		2098.18					PK	DPPIV inhibitory
		KPAAAAAPAPAPAPAPAP APAPPKEEK		2484.36					EK	ACE-inhibitory
		ITLSQVGDVLR		1200.71						
		DQGSYEDFVEGLR		1514.68						
Myosin heavy chain	Q95249	KLETDISQIQGEMEDIVQEAR		2432.20						

(Continued)

TABLE 2 | Continued

Origin	Accession[a]	Sequence[b]	Modifications	MH+ [Da]	Area[c]				BIOSEP sequences	Activity
					D0P/T	D1P/T	D2P/T	D3P/T		
		KLETDISQIQGEMEDIVQEAR	M13(Oxidation)	2448.19						
Myosin, heavy chain 7	H6SHX6	LGSLDIDHNQY		1274.60						
Actin										
Actin (Fragment)	B2ZFN7	IGMESAGIHETTY	M3(Oxidation)	1424.64						
		IGMESAGIHETTY		1408.64						
		MKILTERGYSF		1344.70						
		MKILTERGYSF	M1(Oxidation)	1360.69						
		FQPSFIGMESAGIHETTY		2014.92						
		QPSFIGMESAGIHETTY		1867.85						
		SYELPDGQVITIGNER		1790.89						
Tropomyosin										
Tropomyosin 4	D0G7F7	MEIQEMQLK	M6(Oxidation)	1165.56						
		MELQEMQLK		1149.56						
		LVILEGELER		1170.67						
		KLVILEGELER		1298.77						
Tropomyosin alpha-1 chain	F2Z5B6	AISEELDHALNDMTSI	M13(Oxidation)	1774.82						
		AISEELDHALNDMTSI		1758.82						
		MELQEIQLK	M1(Oxidation)	1147.60						
		MELQEIQLK		1131.61						
		KLVIIESDLER		1314.76						
		LVIIESDLER		1186.67						
		IQLVEEELDR		1243.65						
		IQLVEEELDRAQER		1727.89						
		QLEDELVSLQK		1301.70					QK	ACE-inhibitory
		SKQLEDELVSLQK		1516.82					QK	ACE-inhibitory
		KAISEELDHALNDMTSI		1886.91						
Tropomyosin 3	Q2XQY5	LVIIEGDLER		1156.66						
Troponin										
Troponin I	B3VI70	SVMLQIAATELEK		1432.77					EK	ACE-inhibitory
troponin C	A1XQV5	AAFDMFDADGGGDISVK		1715.76						
		SYLSEEMIAEFK		1446.68						
Troponin T	Q75NG6	YQLEIDKF		1055.54						

[a]Protein database ID (Uniprot-Sus scrofa). [b]Peptides obtained by MS/MS analysis. [c]D0PT, D1PT, D2PT, and D3PT, 0, 1, 2, and 3 d samples treated by pepsin and trypsin. Different color indicates different relative abundance of peptides. Red color represents the highest abundance, and the green color represents the lowest abundance.

oxidative peptides and KPAAAAAPAPAPAPAPAPAPPKEEK were the lowest in 2 day samples. Abundances of SVMLQIAA TELEK, DIDDLELTLAKVEK, and TKLEQQVDDLEGSLEQEK were the highest in 2 day samples. This could be mainly attributed to the degradation of proteins and accumulation of peptides in the first stage, which would be further degraded into smaller peptides that have ACE and DPPIV inhibitory bioactivities. Thus, 2 days could be a key time point for chill storage of pork in terms of meat nutrition.

Two peptides were observed to have the same sequence but their molecular weights were different, which is attributed to protein oxidation based on the database. Eighteen peptides from two-step digestion were observed to have oxidation modification. It is known that protein oxidation occurs during storage and the extent of protein oxidation may be enhanced as meat storage time increases. In the present study, pork samples were vacuum packed and stored at low temperature (4°C) for 3 days. In such a condition, protein oxidation could be alleviated. And thus more cleavage sites could be exposed in loose protein structure and protein degradation increased. The number of bioactive peptides may increase after the two-step digestion.

The above results could be associated with rigor mortis and subsequent aging of muscle (33). It is well-known that glycolysis occurs in skeletal muscles after slaughter (34, 35). This process is accompanied by the dephosphorylation of energy metabolic enzymes, the formation of actomyosin,

and the shortening of sarcomeres (6, 36). Such activities may make some energy metabolic enzymes and myofibrillar proteins less susceptible to pepsin and trypsin digestion under *in vitro* condition on day 1. However, prolonged storage can tenderize meat, and make the myofibrillar proteins and bound energy metabolic enzymes more digestible. This is because the endogenous enzymes, e.g., μ-calpain and cathepsins, catalyze the degradation of myofibrillar proteins into smaller fragments (33), which will expose the cleavage sites for digestive enzymes. It is notable that, too long-time storage of fresh meat may have the problems of food spoilage, discoloration, and the decreased digestibility of meat proteins due to oxidation (37).

CONCLUSIONS

In this study, postmortem aging was shown to have significant impacts on the structural characteristics and *in vitro* digestion of pork proteins. It is observed that the 3 d samples had lower α-helix content and the peak intensity at 760 cm^{-1} (tryptophan residues), but higher β-sheet, β-turn, and random coil contents than the 0 d samples. This is accompanied by significant protein degradation and increased susceptibility to digestion. The structural changes of proteins altered the accessibility of proteolytic enzymes to the cleavage sites, and consequently the protein digestion. Further work is necessary to evaluate the effect of postmortem aging on *in vivo* digestion of pork protein.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in Proteome Xchange, Accession No. PXD020595.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of Experimental Animal Center of Nanjing Agricultural University.

AUTHOR CONTRIBUTIONS

CL designed the experiments. XZ, JH, DZ, MZ, YX, and CW conducted the experiments. XZ, JH, and CL wrote the paper. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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Quantitative Label-Free Proteomic Analysis of Milk Fat Globule Membrane in Donkey and Human Milk

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Previous studies have found donkey milk (DM) has the similar compositions with human milk (HM) and could be used as a potential hypoallergenic replacement diet for babies suffering from cow's milk allergy. Milk fat globule membrane (MFGM) proteins are involved in many biological functions, behaving as important indicators of the nutritional quality of milk. In this study, we used label-free proteomics to quantify the differentially expressed MFGM proteins (DEP) between DM (in 4–5 months of lactation) and HM (in 6–8 months of lactation). In total, 293 DEP were found in these two groups. Gene Ontology (GO) enrichment analysis revealed that the majority of DEP participated in regulation of immune system process, membrane invagination and lymphocyte activation. Several significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were determined for the DEP, such as lysosome, galactose metabolism and peroxisome proliferator-activated receptor (PPAR) signaling pathway. Our study may provide valuable information in the composition of MFGM proteins in DM and HM, and expand our knowledge of different biological functions between DM and HM.

Keywords: human, donkey, quantitative proteomic, comparison, milk fat globule membrane

INTRODUCTION

Donkey milk is more similar to human milk because of its total protein and lactose contents, similar fatty acid and protein profiles (1). It has been indicated to be more suitable for children and elderly people due to its remarkable nutritional value and less allergenic. Besides, milk fat globules in DM are smaller and more easily digested and absorbed by infants (2). With its obvious advantages, the demand of direct consumption for DM has increased.

Milk fat globule membrane (MFGM), a three-layer membrane, covers on the surface of the milk fat globule (3). MFGM proteins make up only 1–2% of the total milk proteins, but they are thought to play important roles in biological processes, including cell growth promotion, cell activity regulation and defense mechanisms against bacteria and viruses in infants (4). Investigations on MFGM proteome have primarily focused on profiling analyses of MFGM fractions from different mammals. For example, Yang et al. identified 232 differentially expressed MFGM proteins in HM

and CM across different lactation stages using the iTRAQ proteomic approach (5). To reveal the differences in the formation of MFGM in different mammals, the MFGM proteins of cow, yak, buffalo, goat, horse, camel and human were also compared by iTRAQ proteomics (6). Recently, Li et al. hoped to explore the changes in the regulation mechanism of different lactation stages by analyzing the differences of MFGM proteins between donkey colostrum and mature milk (2). However, studies of the DM MFGM proteome are relatively sparse and less comprehensive, especially fully comparative analyses of MFGM protein compositions and potential biological activities between DM and other species.

The aim of our study was to compare the expression of MFGM proteins between DM and HM by label-free quantification and to explore the biological processes they were involved in. The results are helpful for us to better understand the differences between DM and HM in the composition of MFGM and provide strong support for the future development of formula milk using donkey milk as nutritional provider.

MATERIALS AND METHODS

Sample Collection and Treatment

HM samples were donated by twelve healthy mothers with the lactation of 6–8 months with written informed consent which indicated that the milk would be used in research. Twelve DM samples were obtained from a local farm breeding Dezhou donkeys (6–9 years old) with the lactation of 4–5 months in Liaocheng City of Shandong province, China. All procedures involving donkeys were performed by the Shandong Agricultural University Animal Care and Use Committee (approval number, SDAUA-2020-053). Four samples of each group were randomly mixed and stored at −80°C.

MFGM proteins separation was done as described by previous report (2). All samples were centrifuged at 4°C and 10,000 g for 15 min to obtain the upper fat portion containing MFGM. The upper fat layer was washed with 10 ml cold phosphate-buffered saline (0.24 g KH2PO4, 1.44 g Na2HPO4, 8 g NaCl and 0.2 g KCl were dissolved in 800 ml deionized water, the PH was adjusted to 7.4 and the volume was fixed to 1,000 mL) and homogenized by ultrasound (80 W, 15 s) for 3 times. The mixture was centrifuged at 4°C and 10,000 g for 1 h. Then, acetone was added to the collected supernatant to segregate the fat globules overnight. After centrifugation at 15,000 g at 4°C for 30 min, the supernatant was poured away. Finally, lysate was added to the protein particle sample and ultrasonicated for 15 s at 80 W for 10 cycles.

MFGM Protein Digestion

Thirty microliters protein solution were taken from each sample and dithiothreitol (DTT) were added to the final concentration of 100 mM. The samples were incubated in boiling water for 5 min and cooled to room temperature. Then, 200 μL UA buffer (8 M urea, 150 mM TrisHCl, pH 8.0) was added and mixed thoroughly. After that, each sample was transferred into a 10 kDa ultrafiltration centrifuge tube (Sartorius, Germany) and centrifuged at 14,000g for 15 min, then discarded the filtrate (repeat this step once). 100 μL IAA buffer (100 mM IAA in UA) was added to the protein mixture in the tube and shaken at 600 rpm for 1 min. The mixture was left at room temperature in dark for 30 min and centrifuged at 14,000 g for 15 min and the process was repeated twice. Hundred microliters 25mm NH4HCO3 solution was added to the tube and centrifuged at 14,000 g for 15min and repeated the procedure twice. Subsequently, 40 μL trypsin buffer (4 μg trypsin in 40 μL 100mM NH4HCO3) were added to the mixture and sample was shaken at 600 rpm for 1 min. The mixture was left at 37°C for 16–18 h and then transferred to a new collecting tube and centrifuged at 14,000 g for 15min. Then, 40 μL 25 mm NH4HCO3 was added in the mixture and sample was centrifuged at 14,000 g for 15 min to collect the filtrate. The peptide was desalted by C18 cartridge (Sigma, USA). After freeze-drying, the peptide was dissolved in 40 μL 0.1% formic acid solution.

LC- MS/MS Experiments

Each sample was separated by the Easy-nLC^{TM} (Proxeon Biosystems, Thermo Fisher Scientific) orbitrap HPLC system with nano-flow. Buffer A was 0.1% formic acid, and buffer B was 84% acetonitrile in 0.1% formic acid. The chromatographic column was equilibrated with 95% buffer A. The sample was loaded by an automatic sampler to the sample column (Thermo Scientific Acclaim Pepmap 100, 100 μm × 2 cm, nanoViper C18), and separated by the analytical column (Thermo scientific EASY column, 10 cm, ID75 μm, 3 μm, C18-A2). The flow rate was 300 nL/min. Two-hour gradient: 0–110 min, buffer B linear gradient from 0 to 55%; 110–115 min, buffer B linear gradient from 55 to 100%; 115–120 min, buffer B maintained at 100%.

The samples were analyzed on a Q Exactive^{TM} mass spectrometer (Thermo Fisher Scientific). Parameters were set as follows: analysis time 120 min; detection mode: positive ion; scanning range of parent ion: 300–1,800 m/z; first-order mass spectrometry resolution: 70,000 at 200 m/z; AGC (automatic gain control) target: 1e6; maximum IT (inject time): 50 ms; dynamic exclusion: 60 s. The mass charge ratio of polypeptide and polypeptide fragments was collected according to the following methods: after each full scan, 20 fragments (MS2 scan) were collected. The MS2 activation type was HCD, the isolation width was 2 m/z, the secondary mass spectrum resolution was 17,500 at 200 m/z. Normalized collision energy was 30 eV, and the underfill ratio was defined as 0.1%.

Sequence Database Searching

Relative intensity-based label-free quantification (LFQ) was analyzed by MaxQuant version 1.5.3.17 (Max Planck Institute of Biochemistry in Martinsried, Germany) and searched against the UniProtKB *Equus asinus* database (47,825 total entries, downloaded on August 12, 2019) and *Homo sapiens* (included 20,422 series, downloaded on May 22, 2019). Proteins with $p < 0.05$ and fold change >2 or <0.5 were deemed significantly expressed between groups by *t*-test. The false discovery rate (FDR) for peptide and protein identification was set to 1%.

Bioinformatics Analysis

Quantified MFGM samples were used to performing hierarchical clustering analysis. For this purpose, Cluster3.0 (http://bonsai.

hgc.jp/$\sim$mdehoon/software/cluster/software.htm) and the Java Treeview software (http://jtreeview.sourceforge.net) were used. GO enrichment on three ontologies (biological process, molecular function, and cellular component) and KEGG pathway enrichment analyses were applied based on the Fisher' exact test, considering the whole quantified protein annotations as background dataset using DAVID (https://david.ncifcrf.gov/). Benjamini-Hochberg correction for multiple testing was further applied to adjust derived *P* -values, and only functional categories and pathways with *P*-values under a threshold of 0.05 were considered as significant.

RESULTS

Quantitative Overview of Identified MFGM Proteins in DM and HM

As shown in **Figures 1A**, 454 MFGM proteins were identified in DM and HM. Proteins having at least two replicates and fold changes >2.0 or <0.5 and $P < 0.05$ were defined as differentially expressed proteins (DEP). In addition, the proteins with at least two replicates in one group and in another group with null values were also defined as DEP. A volcano plot was used to show significant differences between the two groups based on the fold change and *P*-value (**Figure 1B**). Compared with MFGM proteins in HM, 204 MFGM proteins in DM were upregulated and 89 MFGM proteins were downregulated. DEP detailed information is shown in **Supplementary Table 1**.

Cluster Analysis

Meanwhile, a hierarchical cluster analysis of MEGM proteins in DM and HM group was shown in **Figure 2**. The upregulated MFGM proteins in DM compared with HM mainly included alpha-s2-casein, lysozyme, alpha-s1-casein and secreted phosphoprotein 1 (SPP1), and the downregulated MFGM proteins were lipoprotein lipase, fatty acid-binding protein 3 and nucleobindin 1.

GO Analysis of DEP in DM Compared With HM and CM

DEP were then classified into the GO enrichment analysis of three distinctive functional sets, cellular component, molecular function, and biological process. In terms of molecular function, DEP in DM and HM group were primarily related to immunoglobulin receptor binding, protein homodimerization activity and antigen binding (**Figure 3**). In the category of biological process, DEP in these two groups were mainly involved in regulation of immune system process, membrane invagination and lymphocyte activation. GO enrichment of DEP information is listed in **Supplementary Table 2**.

KEGG Pathway Analysis of DEP in DM Compared With HM and CM

In **Figure 4**, the DEP were mainly involved in lysosome, galactose metabolism and PPAR signaling pathway. KEGG pathway enrichment of DEP information was listed in **Table 1**.

DISCUSSION

In this study, 293 MFGM proteins were found to be significantly different between DM and HM. These DEP were involved in regulation of immune system process, complement activation and integral component of membrane. These results may provide valuable information in the MFGM composition of DM and HM, especially for low abundant components, and expand our knowledge of different biological functions between DM and HM.

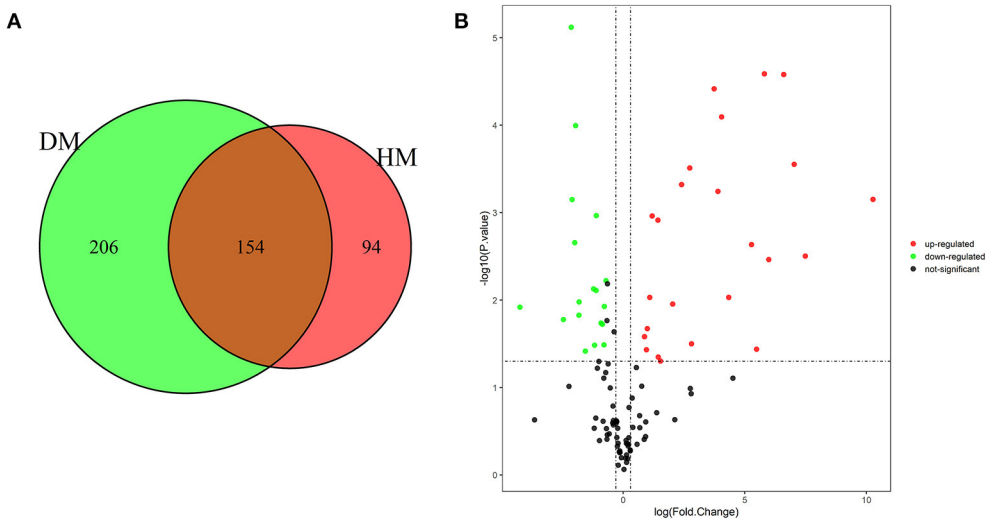

FIGURE 1 | Venn diagram of MFGM proteins identified from DM and HM group **(A)**; Volcano plot of proteins identified from DM and HM group **(B)**. Red dot, up-regulated proteins in DM and HM MFGM; Green dot, up-regulated proteins in DM and HM MFGM; Black dot, not significant different proteins between DM and HM. MFGM, milk fat ball membrane; DM, donkey milk; HM, human milk.

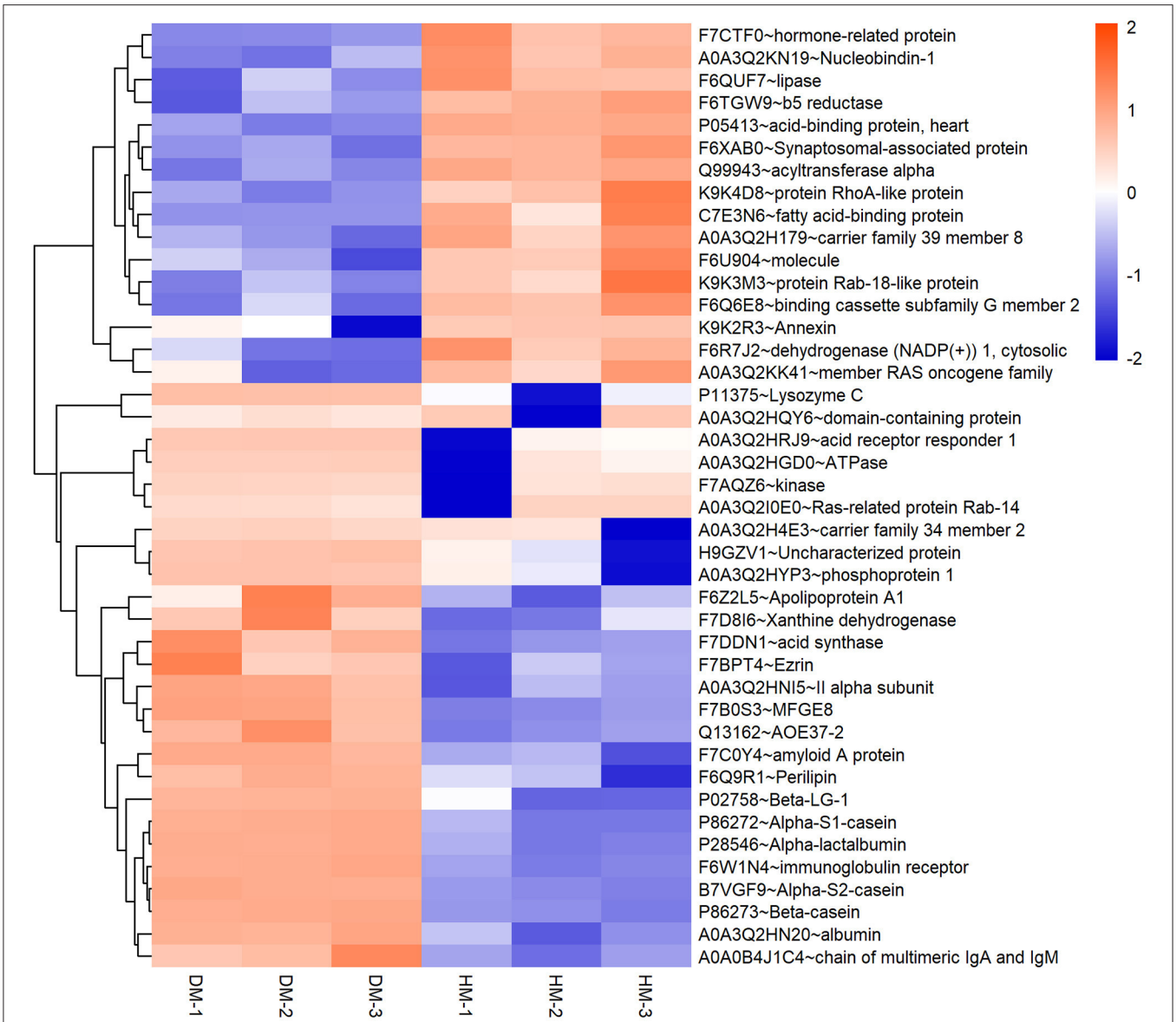

FIGURE 2 | Hierarchical clustering of DEP in DM and HM group. Bar color represents a logarithmic scale from −2 to 2. DEP, differentially expressed proteins; DM, donkey milk. HM,: human milk.

Caseins in milk exert multifunctional effects including amino acid and calcium supply (5), cellular immune functions stimulation (7, 8), and chemotactic properties (9). Studies of human, bovine, goat, and camel MFGM proteomes identified milk caseins in the MFGM fractions (2, 6, 10). In our data, four caseins (α-s1-casein, α-s2-casein, β-casein, κ-casein) were detected in DM MFGM fractions and significantly higher than that in HM. The main role of human α-s1-casein is to serve as an amino acid source to the newborns. Beyond nutritional aspects, α-s1-casein could contribute to the development of immune system. Moreover, α-s1-casein in nursed individuals gives rise to sustained specific IgG production (8). Cocco et al. found that single amino acid substitution in the specific linear epitopes of alpha s1-casein can significantly reduce the binding ability of serum IgE in patients with CM allergy (11). Donkey and cow milk alpha s1-casein share a low sequence homology, and particularly their IgE binding linear epitopes have remarkable differences in amino acid sequences (12). Moreover, Bertino et al. found that donkey alpha-s1-casein appears as both phosphorylated and glycosylated forms, but neither human nor bovine alpha-s1-caseins have been reported to be glycosylated (13). Studies have shown that in many cases milk from donkey represents a safe and alternative food in both IgE-mediated and non-IgE-mediated cow's milk protein allergy (14, 15). These differences in alpha s1-casein amino acid sequence or post-translational modifications might be related to the low allergenic properties

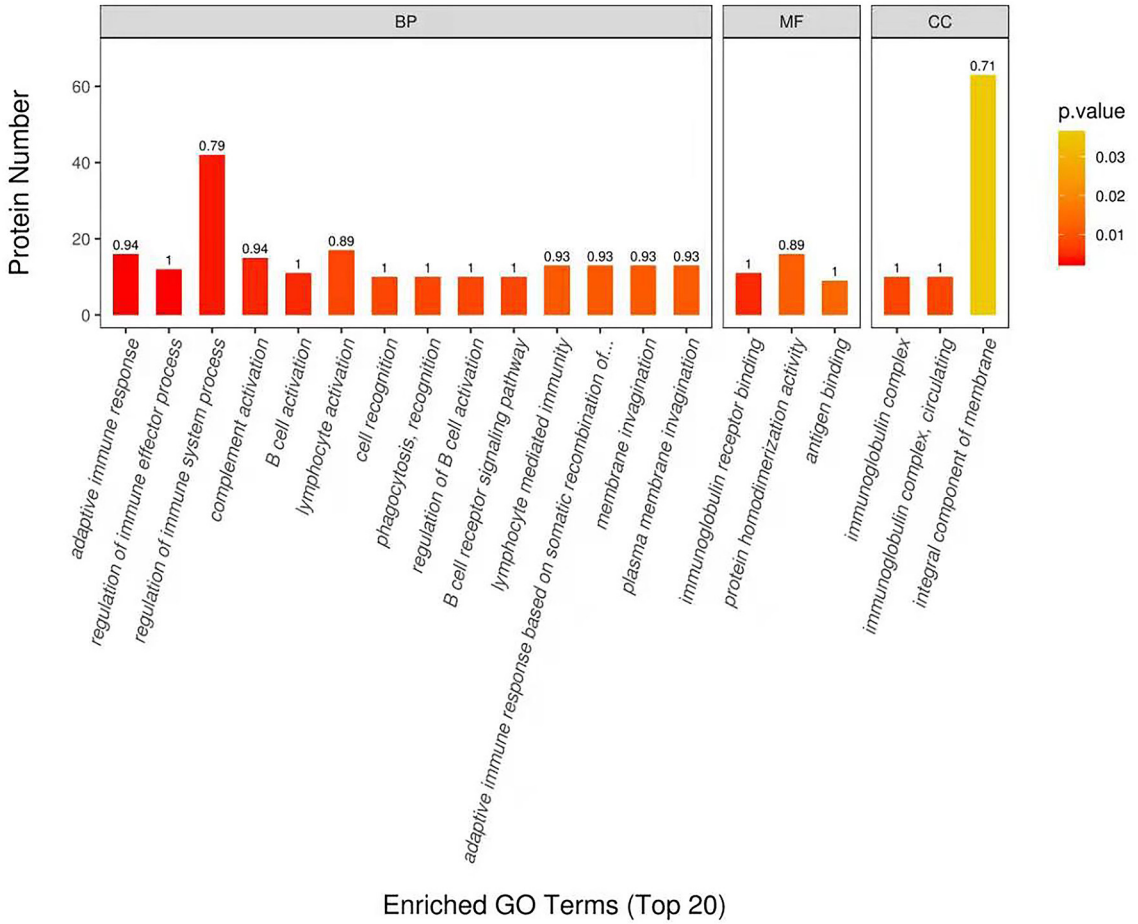

FIGURE 3 | Enriched Gene Ontology (GO) Terms of the DEP in DM and HM group. GO enrichment of DEP on three categories. BP, biological processes; MF, molecular functions; CC, cellular components; DEP, differentially expressed proteins; DM, donkey milk; HM, human milk.

of donkey milk. As for β-casein and kappa-casein, both of them from HM and DM are more closely related to each other than the cow counterparts (16). β-casein is the major casein constituent in human MFGM and generates smaller casein phosphopeptides upon digestion to aid the absorption of calcium (17). κ-casein is the only glycosylated in the four casein families. DM κ-casein carries a higher number of potential *O*-glycosylation than that cow milk κ-casein. Therefore, DM similar to HM may contribute to inhibit the adhesion of *Helicobacter pylori* to gastric mucosa in infant (18).

In general, β-lactoglobulin can be found in the majority of milks, but not in human's milk. However, in this study we detected β-lactoglobulin in HM. It has been found that beta-lactoglobulin can be detected in the human milk within 7 days after ingestion of milk (19). We hypothesized that the mothers who donated breast milk in this study were likely to have taken other milks before providing milk samples.

According to the GO enrichment analysis, DEP in DM vs. HM group were mainly involved immune response, such as complement activation, defense response or positive regulation of B cell activation (**Supplementary Table 2**). It is well-known that milk could provide large amounts of bioactive components to the infants in the critical phase of immunological immaturity. Mature breast milk could enhance B cell proliferation and antibody secretion (20). Among the DEP involved in the positive regulation of B cell activation, 9 proteins were all upregulated in DM, including immunoglobulin heavy constant gamma 3 (IGHG3) and immunoglobulin kappa constant (IGKC), which all function in B cell selection or antigen recognition (21). In addition, 49 DEP were involved in defense response, among which 32 proteins were upregulated in the DM MFGM, namely semaphorin 7A, complement 3 (C3), joining (J) chain. Semaphorin 7A (also known as CD108) plays a key role in innate immune regulation. Semaphorin 7A could induce proinflammatory cytokines production (22). Semaphorin 7A-deficient mice are defective in T cell-mediated inflammatory responses, indicating the role of semaphorin 7A in evoking inflammatory immune reactions (23). In addition to its role in the immune response, semaphorin 7A also functions as a chemoattractant and stimulates neuronal migration, which is an essential process in central nervous system development (24). The defect of neuron migration may lead to nervous system

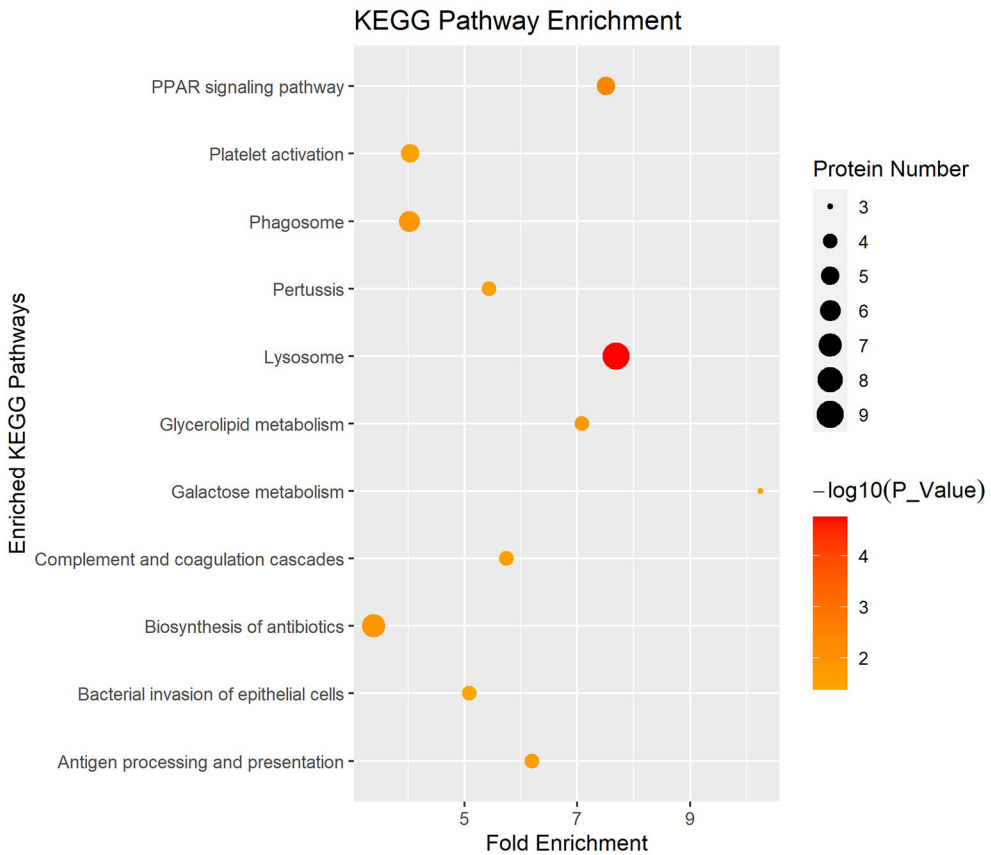

FIGURE 4 | Enriched KEGG pathway analysis of the DEP in DM and HM group. KEGG, Kyoto Encyclopedia of Genes and Genomes pathways; DEP, differentially expressed proteins; DM, donkey milk; HM, human milk.

disorder (25). J chain is a small polypeptide contained in dimeric IgA and pentameric IgM, which plays an important role in the generation of secretory antibodies (26). This peptide can be produced by immunocytes of all Ig isotypes, but it becomes incorporated only into IgA and pentameric IgM (27). Moreover, the expression of J chain may be a marker of B cell clone in mucosa associated lymphoid tissue, as there is a positive correlation between the production of polymeric IgA, IgG or IgD-producing cells and J chain (28). C3 is an important part of the innate immune system. It combines with other complement proteins to form the main host mechanism for detecting and eliminating potential pathogens (29). Complement proteins contribute to the establishment of natural immune system in newborns (2). The presence of abundant immunological factors in DM MFGM proteins are helpful for the newborns to establish an immune system against microbial infection to adapt to the new environment to prevent diseases.

In this study, a solute carrier (SLC) superfamily, namely, SLC34A2, SLC36A1, SLC4A9, and SLC9A3R1, was more abundant in DM MFGM proteins. This superfamily is a major membrane transporter group that controls the uptake and excretion of nutrients, neurotransmitters, metabolites, drugs and toxins (30). SLC34A2 is a member of SLC34 family, a group of phosphate transporters, which are responsible for transporting inorganic phosphate. Phosphate is an essential nutrient for life and a key component of bone formation (31). Recently, it was concluded that SLC34A2 was responsible for the sodium-dependent component of intestinal phosphate absorption (32). SLC36A1, an amino acids transporter in small intestinal enterocytes, could regulate cell growth and sense the availability of amino acids in other cell types. SLC36A1 also could be a target for rapamycin complex 1 (TORC1) activation (33). TORC1 regulates some metabolic pathways and adapts cells to applied bioenergy and anabolic conditions (34). SLC9A3R1 is a multifunctional scaffold protein, which is involved in cell activities and affects many protein functions, including ion channels, receptors, signaling and nuclear proteins. In addition, SLC9A3R1 has potential antitumor effects in breast cancer (35). SLCs also play an important role in the function of the central nervous system. A total of 287 SLC genes were identified in the brain, especially in the barrier cells. SLCs expressed in neurons and glial cells play irreplaceable roles in maintaining brain homeostasis (36). Moreover, we also found some other abundant proteins in DM MFGM that are expressed in the brain, such as neuro plastin (NP). NP is a cell adhesion molecule rich in synaptic membranes and belongs

TABLE 1 | Detailed information of KEGG pathway-based enrichment of the differentially expressed proteins between DM and HM.

Pathway_ID	KEGG pathways	Protein number	Genes	Fold enrichment	*P*-value
ecb04142	Lysosome	9	F6YQM5, F7BPX8, F6YNH6, F6V812, F7B6D0, F7DG10, F6V3X9, F7BV85, F6VUW2	7.69	1.72E-05
ecb03320	PPAR signaling pathway	5	F7AYT5, F6Z2L5, F6RM73, F6QUF7, F6U904	7.51	4.07E-03
ecb04145	Phagosome	6	Q95M34, Q5XWB8, F7BPX8, F7DG10, F6U904, F6VUW2	4.02	1.56E-02
ecb01130	Biosynthesis of antibiotics	7	F6X8Q2, F6TL52, F6SX98, F6Y688, F6RI26, L7MRN0, F6R7J2	3.38	1.58E-02
ecb00561	Glycerolipid metabolism	4	F6TN81, F6QUF7, F7B6D0, F6RI26	7.08	1.79E-02
ecb04612	Antigen processing and presentation	4	Q9GKX8, F7BPX8, F6YNH6, F6VUW2	6.19	2.55E-02
ecb04610	Complement and coagulation cascades	4	F6USP9, F6XSF7, F6PH38, F6XGE0	5.74	3.10E-02
ecb00052	Galactose metabolism	3	F6X8Q2, F7B6D0, F6SUZ2	10.25	3.33E-02
ecb04611	Platelet activation	5	F6QMB8, F6XAB0, F7AQZ6, K9K4D8, F6PH38	4.03	3.36E-02
ecb05133	Pertussis	4	Q6TGR2, Q5XWB8, F6XSF7, K9K4D8	5.43	3.57E-02
ecb05100	Bacterial invasion of epithelial cells	4	F6VZN7, F6Y0D9, K9K4D8, F7BV85	5.08	4.22E-02

to immunoglobulin superfamily. Np plays a role in synaptic plasticity and neurite growth (37, 38). In recent years, NP has attracted much attention because of its correlation with adolescent cortical thickness and intelligence (39). Therefore, the abovementioned functional components in DM MFGM may play a more important role in promoting the growth and learning of newborn infants.

Among these DEP, a family of apolipoproteins (Apos), namely, apolipoprotein A1 (ApoA1), ApoA2, ApoC3, ApoD, and ApoE, were significantly upregulated in DM MFGM. ApoA1 and ApoA2 are the major proteins in high-density lipoproteins (HDL) (40). ApoA1 has multiple beneficial functions, including potent antioxidant, anti-inflammatory, antiviral and antibacterial activities in blood (41, 42). Recently, ApoA1 was found in HM and DM. Kim et al. identified that ApoA1 interacts with cholesterol in HM, provides antioxidant activity and improves embryo survivability (43). In DM MFGM, ApoA1 was upregulated in colostrum compared with mature milk (2). ApoD is a glycosylated protein involved in lipid transport, food intake, inflammation, antioxidant response and development. In humans, ApoD levels rise considerably in association with aging possibly in response to accumulated damage (44). Overexpression of human ApoD could protect drosophila against various extrinsic stresses and extend its normal lifespan (45). ApoE is an important element in the lipoprotein metabolism and cholesterol transport (46). Cholesterol plays a key role in vitamin D and steroid hormones synthesis, which is critical to the development of the newborns (47). In our study, DM MFGM provides a higher level of cholesterol transporters, which may help the newborns acquire a large amount of cholesterol.

In conclusion, 204 up-regulated proteins were identified in the lipid globules of donkey milk. Through GO functional annotation and KEGG pathway enrichment analysis, we found that these upregulated proteins not only have nutritional effects, but also promote the improvement of the immune system, cognitive learning and anti-oxidation of newborns. Therefore, donkey milk might be used as a nutritional provider in infant formula. In addition, the comparison of the fat globular membrane protein between donkey milk and cow milk was missing in this study, which was a limitation of our research and will be carried out in the future.

CONCLUSION

A quantitative proteomic method was used to investigate the MFGM proteins proteome in DM and HM. DEP were analyzed by multivariate statistical methods and found mainly involved in regulation of immune system process, membrane invagination and lymphocyte activation. Our findings also provided more in-depth reference for the dairy food industry and infant health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Animal Care and Use Committee of Shangdong Agricultural University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Shangdong Agricultural University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

FZ designed the experiment and revised the manuscript. XZ completed the experiment and drafted the manuscript. BJ and CJ participated in improving the experiment. HL, LY, GJ, YW, GuaL, and GuiL participated in collecting and preparing the samples. LM helped to revise the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.670099/full#supplementary-material

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Comparative Proteomics of Human Milk From Eight Cities in China During Six Months of Lactation in the Chinese Human Milk Project Study

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Human milk (HM) is the golden standard of infant nutrition that can protect immature body function and enhance nutrition metabolism to ensure infant growth. Region specificity and lactation period could change the protein composition in HM. In this research, proteomics analysis was used to compare proteomes across eight cities, namely Harbin, Lanzhou, Guangzhou, Chengdu, Jinhua, Weihai, Zhengzhou, and Beijing, which represented the northeast, northwest, southeast, southwest, east, and north and central regions of China,. Proteins varied significantly among the cities. These different proteins were mainly involved in the process of platelet degranulation, innate immune response, and triglyceride metabolic process, which might be due to different living environments. These differences also lead to variation in protection and fat metabolism from mothers to infants in different cities. Four proteins were expressed differently during 6 months of lactation, namely Dipeptidyl peptidase 1, Lysozyme C, Carbonic anhydrase 6, and Chordin-like protein 2. The changes in these proteins might be because of the change of growth needs of the infants. The findings from our results might help to improve the understanding of HM as well as to design infant formula.

Keywords: Chinese human milk, human milk, cities, lactation period, proteomics

INTRODUCTION

Human milk (HM) is a complex liquid that contains variative compositions among mothers. It consists of true solutions, colloids, membranes, membrane-bound globules, and cells (1). The exclusive breastfeeding period of the first 6 months then continued up to 2 years is considered as the ideal standard for infant feeding (2). During the exclusive breastfeeding period, HM acts as the single source of nutrients that supports the immature immunity and metabolism of the infant (3). Meanwhile, the composition of HM is altered by many factors such as maternal factors, lactation stages, environmental exposures, regions, ethnicities, handling, and storage (1, 4).

Human milk contains myriad proteins that play a role in the biological process of infants such as immunological, antimicrobial, and developmental functions (5, 6). Recently, proteomics has become a robust approach to explore the overall biological function of HM proteins (7). Previously, proteomes variation of HM serum and milk fat globule membrane of individual mothers and

variation of HM and ruminant's milk have been observed (3, 8–10). Those prior studies found that HM proteome contributed to various functions including immune protection, biological growth, and maturation of the digestive tract. HM profile, including lipid, oligosaccharides, and distinct patterns of microbiota varied across the population (11, 12). However, not so many studies emphasized the variation of protein composition, especially low abundant ones in the different regions. The variation of quantitative proteomes between Chinese and Dutch HM serum and the different proteomes across Chinese ethnicity and geographic location has been revealed (13, 14). They studied milk serum proteome of four different regions in China, mainly focused on the western region of China, namely Yunnan, Gansu, Xinjiang, and Inner Mongolia. To our knowledge, the milk proteins, especially low abundant proteins were not well-investigated in the eastern region of China.

Thus, HM proteomes in eight cities of China, which represented northeast, northwest, southeast, southwest, east, north, and central China, during the first 6 months of lactation were investigated in the present study by using proteomics methods with a bigger number of samples than prior studies. The result of this research will build in-depth understanding of proteomes in each city for further use, for instance, the development of infant formula for the infants that could not access sufficient HM.

MATERIALS AND METHODS

Materials

All the reagents were provided in analytical grade and suitable for liquid chromatography–mass spectrometry (LC-MS/MS). The Bicinchoninic acid assay (BCA) kit was purchased from Biodee, China. Ammonium bicarbonate, dithiothreitol (DTT), and iodoacetamide (IAA) were obtained from Sigma, USA. Acetonitrile was obtained from Thermo Fisher, USA. Spectrometry grade of trypsin was purchased from Promega, USA.

Sample Collection

The Chinese Human Milk Project (the CHMP study) recruited 1,800 participants from eight cities of Mainland China (Beijing, Guangzhou, Chengdu, Weihai, Lanzhou, Jinhua, Zhengzhou, and Harbin) to evaluate HM composition in the Chinese population. This present study used samples during 1–6 months of lactation and had been clinically registered in ClinicalTrials.gov with registration identifier NCT03675204.

The present study used cross-sectional sample by using one sample from one individual analyzed for one time. HM was collected at 9:00–11:00 in the morning from 15 to 180 days after delivery. The samples were collected from both the right and left sides of fully pumped breasts. The minimum size for each sample was 60 ml. The inclusion criteria were lactating mothers 25–35 years old, breast-fed infants 15–180 days old, physically healthy, non-smoking and non-alcoholic consumers, given birth to physically healthy infants, and signed informed consent forms. The detailed number of the samples is figured in **Table 1**. The obtained samples were frozen and transported to the lab and stored at a temperature of −80°C until further analysis.

TABLE 1 | Number of samples obtained from the hospitals in eight cities in China.

Cities	Total samples	Number of samples in month					
		1	2	3	4	5	6
Beijing	30	1	9	4	8	5	3
Guangzhou	24	3	4	5	4	4	4
Chengdu	24	4	4	5	3	5	3
Weihai	16	2	2	4	3	2	3
Lanzhou	31	3	5	9	5	9	0
Jinhua	34	5	5	5	5	5	5
Zhengzhou	30	3	7	5	5	5	5
Harbin	18	3	3	3	3	3	3

Protein Digestion

The procedure of protein digestion was described as previously (15). In brief, the HM samples were centrifuged at 3,000 rpm at 10°C for 30 min. The fat layer was removed and the remaining parts of protein concentration were measured using the BCA kit. Based on the results of the BCA, 10 μl proteins (1 μg/μl) was diluted with 100 μl 0.05 M ammonium bicarbonate in 0.5 ml Eppendorf tube. Approximately, 10 μl 0.1 M DTT was added. The mixture was kept in 56°C water-bath for 30 min, followed by adding 15 μl 0.5 M Iodoacetamide (IAA). The mixture was incubated at room temperature for 30 min in the dark. The protein digestion was performed by adding a mass ratio of 1:100 trypsin/protein and mildly shook at room temperature overnight. The digestion was stopped by adding 1% formic acid. Before running in LC-MS, the samples were desalted using C-18 column.

Liquid Chromatography–Mass Spectrometry

The proteomics was performed using EASY-nLC 1200 coupled with Q Exactive HF. Generally, the samples were separated using a C18 analytical column (150 μm inner-diameter, outer-diameter 15 cm−1.9 μm, 120Å pore size, ReproSil-Pur C18-AQ). The mobile phase was constituted by 0.1% formic acid in water and 0.1% formic acid as solution A; and 19.9% water mixed with 80% acetonitrile as solution B. The flow rate was 0.6 μl/min, the column temperature was 50°C, the gradient was 4–7% solution B at the initial 1 min, 7–13% solution B for 1–7 min, 13–25% solution B for 7–47 min, 25–40% solution B for 47–68 min, 40-955 solution B for 68–69 min, and keeping 95% solution B for 69–75 min. The MS setting was 2.1 kV spray voltage. MS data were acquired by using data-dependent acquisition mode which dynamically chose the top-30 most abundant precursor ions from survey scan (300–1,400 *m/z*) for high energy collisional dissociation (HCD) fragmentation with a resolution of 120,000 (200 *m/z*). The MS/MS spectra were acquired in the HF normal scan mode.

Data Analysis

The results from LC-MS/MS raw files were analyzed by using Maxquant 1.6.3.4 (9, 16). The *Homo sapiens* proteome database was downloaded from Uniprot (https://www.uniprot.org) and configured by Maxquant. In addition, the contamination database of Maxquant was adopted.

The carbamide-methylation of cysteine was set as fixed modification, and oxidation of methionine, N-terminal acetylation, and deamidation of asparagine or glutamine was set as variable modifications. Mass tolerance was set as 20 ppm for MS peaks and 0.5 Da for MS/MS peaks. The false discovery rates (FDR) was set as 1% and at least 1 peptide was required for identification. Label-free quantification (LFQ) and intensity-based absolute quantification (iBAQ) values were selected for relative protein quantification across all the samples and comparing the levels of different proteins from the same sample, respectively.

Statistical Analysis

The significant differences were analyzed using SPSS 22 (IBM, USA), and the test used was one-way ANOVA with *post-hoc* Tukey's HSD ($P < 0.05$). Protein differentiation and principal component analysis (PCA) were performed by XLSTAT 2021 with the Benjamini-Hochberg test ($P < 0.05$).

Cluster and Gene Ontology Enrichment Analysis

The GO enrichment of protein was performed by using DAVID Bioinformatics resources 6.8 (https://david.ncifcrf.gov) (9, 17). The protein–protein interaction was figured out by using STRING 9 (18).

RESULTS

Identified Proteins in Eight Cities

In the present study, 860, 760, 1,426, 1,298, 1,029, 1,029, 1,022, and 960 proteins were identified in Beijing, Guangzhou, Chengdu, Weihai, Lanzhou, Jinhua, Zhengzhou, and Harbin, respectively. The list of identified proteins can be seen in **Supplementary Table 1**.

Major GO biological processes from the overall identified protein (**Figure 1**) showed that translational initiation had the dominant function in HM proteomes with a *p*-value of 4.52×10^{-86}. It was followed by viral transcription, cell-cell adhesion, Fc-epsilon signaling pathway, translation, complement activation, and receptor-mediated endocytosis. The metabolism function was represented by terms of proteolysis and carbohydrate metabolic process. Several immune related functions showed to be significant in the GOBP enrichment, such as antigen processing and presentation, immune response, and innate immune response with a *p*-value of 1.28×10^{-28}, 4.42×10^{-11}, and 1.40×10^{-7}, respectively.

Protein Variation Across the Cities and Lactation Periods

The PCA showed from principal component 1 (PC1) and principal component 2 (PC2) could explain 99.37% of intensity-based protein variance among eight cities. From PC1 and PC2, the first pool consisted of Weihai, Chengdu, and Jinhua, which could be discriminated from the second pool that consisted of Zhengzhou, Beijing, Guangzhou, Lanzhou, and Harbin (**Figure 2A**). Those discrimination were based on several protein including LALBA, CSN2, and IGKC that were higher in the second pool. Meanwhile, CSNS1, CSN3, LTF, CEL, IGHA1, CLU, B2M, LYZ, and XDH were abundant in the first pool (**Figure 2B**).

Proteins among different lactation periods were figured out by using PC1 and PC2 that could explain 99.95% of intensity-based protein variation (**Figure 3**). The first pool consisted of the 1st, 2nd, and 5th months of the lactation period, while the other groups consisted of the 3rd, 4th, and 5th months of the lactation period (**Figure 3A**). These groups were discriminated by several proteins, including LALBA, CSN1S1, CEL, LTF, B2M, SPP1, and IGHA1 that were abundant in the first pool. The second pool had a higher abundance of CSN2, CSN3, LYZ, IGKC, CLU, and PIGR (**Figure 3B**). The seven major proteins in all the cities were LALBA, CSN2, CSN1S1, CSN3, LTF, ALB, and CEL. The average intensity-based absolute quantification (iBAQ) value and the putative function of each protein based on the DAVID Bioinformatics were available in **Table 2**. Among the identified proteins, LALBA shared the highest percentage than the rest of the proteins. Zhengzhou had the highest LALBA concentration. LALBA and CSN2 in Weihai were below the other provinces on average, while the kappa-casein was the highest. Bile salt-stimulated lipase (CEL), as the seventh major protein in HM across the provinces, was noticed as the highest in Jinhua.

The intensity of 184 proteins was significantly different among the cities (**Supplementary Table 2**), including several abundant proteins: CSN1S1, CSN2, CSN3, LTF, CEL, IGKC, IGHA1, B2M, and PIGR. Four proteins were significantly different during 6 months of lactation, namely CTSC, LYZ, CA6, and CHRDL2 (**Supplementary Table 3**). As the lactation prolonged, the intensity of CTSC, CA6, and CHRDL2 decreased, while the intensity of LYZ increased.

Gene Ontology Enrichment of Significantly Different Proteins

Gene ontology biological process (GOBP), cellular (GOCC), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were enriched from the significantly different proteins across the cities (**Figure 4**). Platelet degranulation was the most noticeable GOBP observed that had a *p*-value of 3.66×10^{-27}, and it was followed by innate immune response and triglyceride metabolic process with a *p*-value of 1.87×10^{-5} and 5.38×10^{-4}, respectively. The white bar showed the extracellular exosome as the major GOCC, and the KEGG pathway was dominated by complement and coagulation cascades, fat digestion and

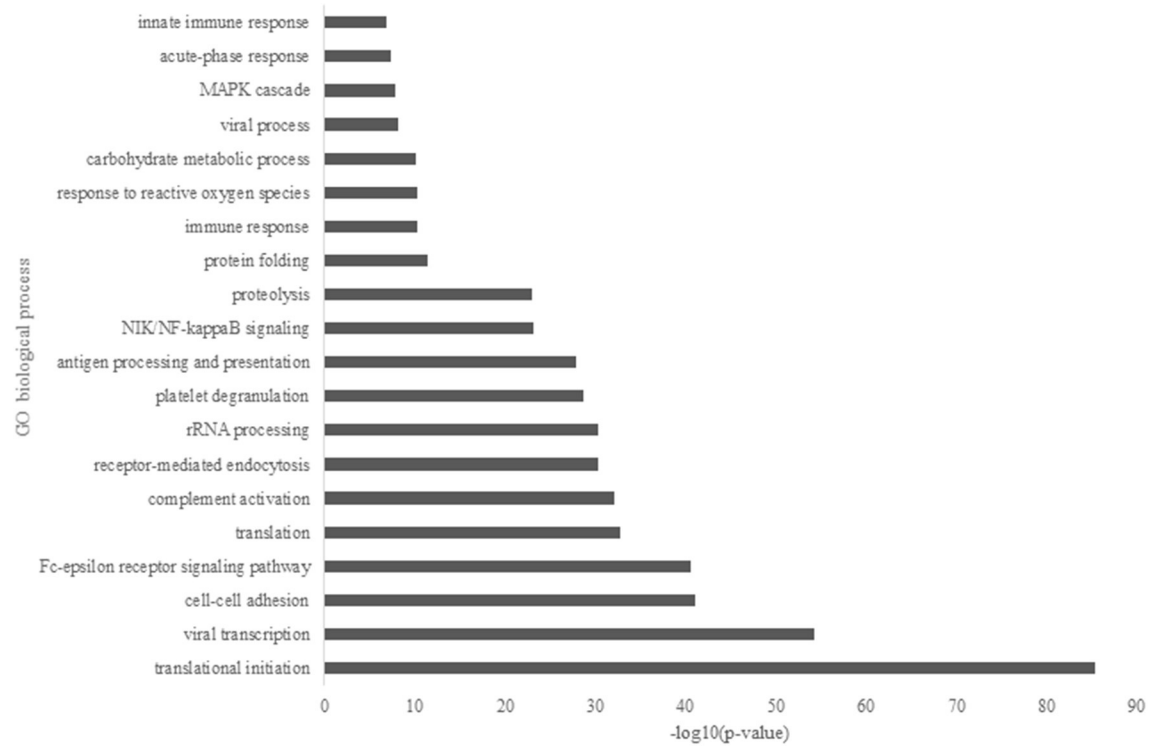

FIGURE 1 | Major gene ontology (GO) biological process of overall identified proteins in Chinese human milk (HM) enriched by the DAVID bioinformatics.

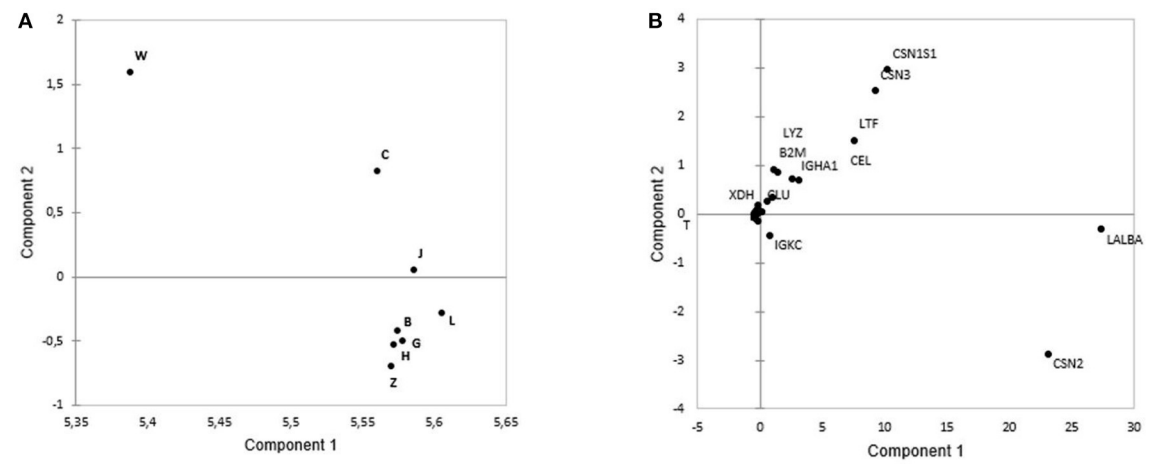

FIGURE 2 | The principal component analysis (PCA) plots based on protein intensity of: **(A)** B, Beijing; G, Guangzhou; C, Chengdu;W, Weihai; L, Lanzhou; J, Jinhua; Z, Zhengzhou, and H, Harbin. Weihai, Chengdu, and Jinhua are located in the same pool, while Lanzhou, Beijing, Guangzhou, Harbin, and Zhengzhou are located in the second pool. **(B)** Loading plots based on the intensity of the proteins showed that CSN2, LALBA, and IGKC were the abundant proteins in the second pool, while the first pool had abundance of CSN1S1, CSN3, LTF, LYZ, B2M, IGHA1, CEL, CLU, B2M, and XDH.

absorption, and peroxisome proliferator-activated receptors (PPAR) signaling pathway.

The intensity of proteins enriched in platelet degranulation, triglyceride metabolic process, and innate immune response is summed in **Figure 5**. **Figure 5A** shows the obvious different intensities in platelet degranulation across eight cities except in the 1st month of lactation which ranged from 5 to 11% of the iBAQ values. Weihai had the highest iBAQ values in the term of platelet degranulation during the 1st to 5th months of the lactation period. Meanwhile, in the 6th month Weihai and Chengdu were not significantly different based on the HSD *post-hoc* test. Zhengzhou consistently had the lowest intensities during

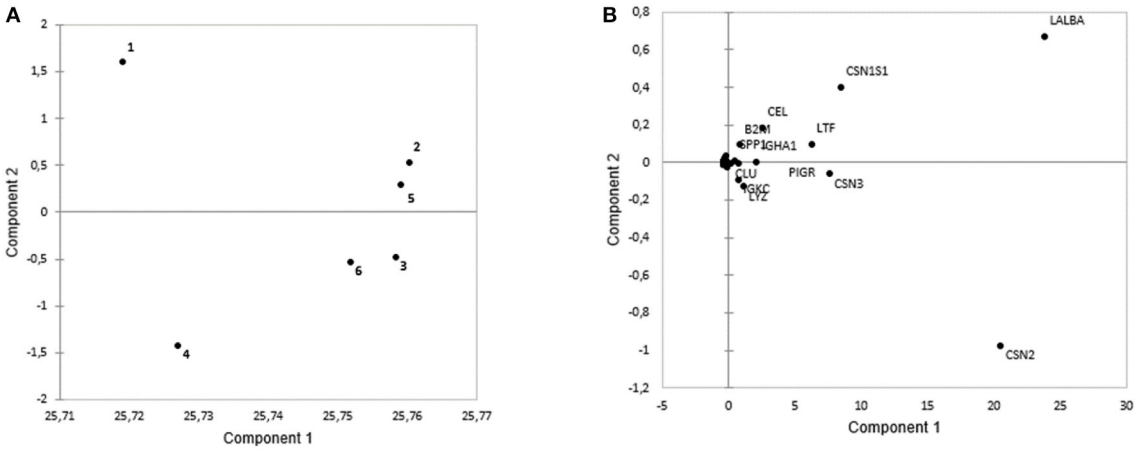

FIGURE 3 | The PCA plots based on protein intensity of: **(A)** Different lactation periods during the 6 months of lactation. The first pool includes the 1st, 2nd, and 5th month of lactation, while second pool includes the 3rd, 6th, and 4th month of lactation. **(B)** Loading plots based on intensity of the proteins based on the different lactation periods. The first pool was abundant in LALBA, CSN1S1, CEL, LTF, B2M, SPP1, and IGHA1. Meanwhile, the second pool was abundant with CLU, PIGR, IGKC, LYZ, and CSN.

TABLE 2 | Most abundant protein identified in eight cities.

Gene names	Average iBAQ (%)[a] across the cities[b]								Putative function
	B	G	C	W	L	J	Z	H	
LALBA Alpha-lactalbumin	23.61 ± 3.36	24.71 ± 3.82	21.43 ± 3.81	18.07 ± 4.90	25.11 ± 4.59	27.73 ± 4.71	30.20 ± 2.86	29.68 ± 2.22	Enzyme: apoptotic process, cell-cell signaling, defense response to bacterium, lactose biosynthetic process, signal transduction
CSN2 Beta casein	25.83 ± 6.63	26.75 ± 6.20	15.65 ± 6.56	12.27 ± 4.20	22.39 ± 8.04	19.08 ± 5.58	26.69 ± 4.66	22.70 ± 2.64	Transport: calcium ion transport, lactation, negative regulation of-cystein type endopeptidase
CSN1S1 Alpha casein S1	10.41 ± 1.90	8.71 ± 2.73	10.96 ± 1.93	12.02 ± 2.70	8.42 ± 1.82	8.37 ± 1.56	8.66 ± 1.35	7.25 ± 1.82	Transport: response to dehydroepiandrosterone, response to estradiol, response to progesterone, transmembrane transport
CSN3 Kappa casein	7.80 ± 1.21	8.67 ± 1.35	9.51 ± 2.50	10.50 ± 3.12	7.41 ± 1.50	9.36 ± 1.55	6.94 ± 1.09	7.81 ± 1.29	Transport: lactation, protein stabilization, transmembrane transport
LTF Lactotransferrin	6.40 ± 1.35	6.76 ± 1.45	7.22 ± 1.15	7.91 ± 1.35	7.52 ± 1.64	7.14 ± 1.41	6.66 ± 1.03	6.75 ± 0.86	Immune: antibacterial humoral response, innate immune response, cellular protein metabolic process, retina homeostasis
ALB Albumin	4.32 ± 0.82	5.05 ± 0.80	6.92 ± 1.19	7.32 ± 1.64	3.35 ± 1.28	3.88 ± 1.13	4.73 ± 0.72	5.23 ± 0.60	Transport: platelet degranulation, cellular protein metabolic process, receptor-mediated endocytosis
CEL Carboxyl-ester-lipase	3.36 ± 1.17	2.90 ± 0.78	3.44 ± 1.27	3.28 ± 0.92	3.35 ± 1.27	3.88 ± 1.13	2.08 ± 0.66	2.61 ± 2.90	Enzyme: fatty acid catabolic process, chemical synaptic transmission, lipid digestion, lipid metabolic process

[a] *Accounted as average of iBAQ during 6 months of lactation.*
[b] *B, Beijing; G, Guangzhou; C, Chengdu; W, Weihai; L, Lanzhou; J, Jinhua; Z, Zhengzhou; H, Harbin.*

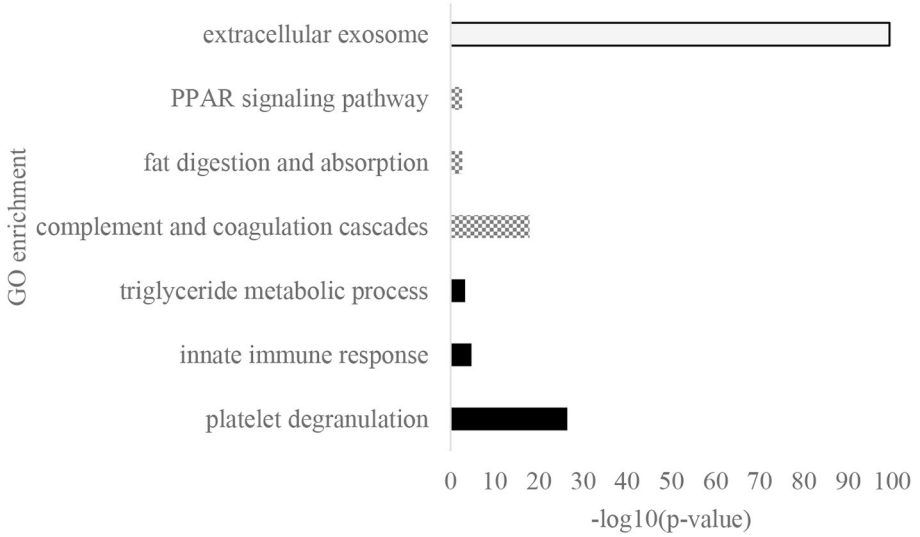

FIGURE 4 | The GO enrichment of significantly different protein across eight cities. White: GO cellular location, dominated by extracellular exosome; Gray: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that consisted of complement coagulation cascades, fat digestion and absorption, and Peroxisome proliferator-activated receptors (PPAR) signaling pathway; and Black: GO biological process, dominated by platelet degranulation, innate immune response, and triglyceride metabolic process.

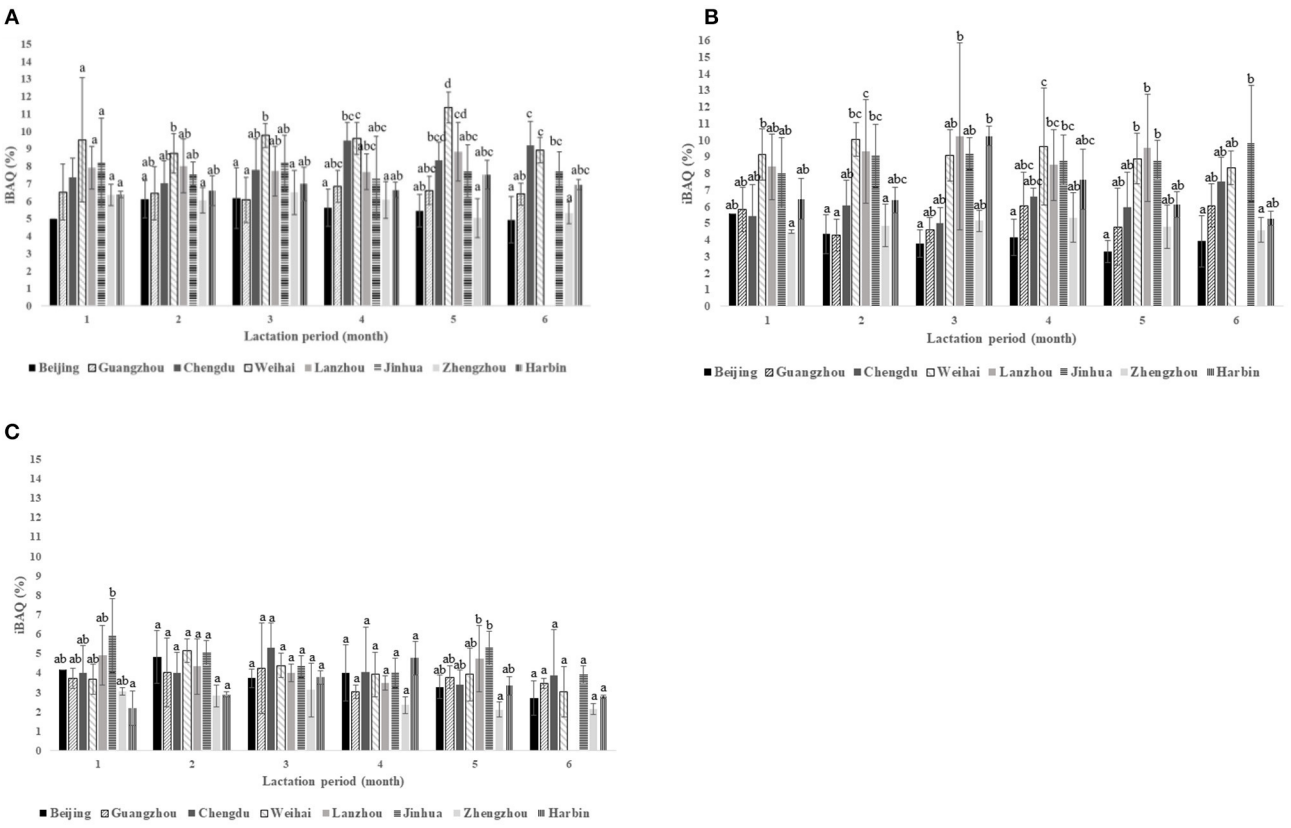

FIGURE 5 | The summed intensities of GO biological process in the terms of: **(A)** platelet degranulation; **(B)** innate immune response, and **(C)** triglyceride metabolic process periods showed the most varied intensities values among the cities.

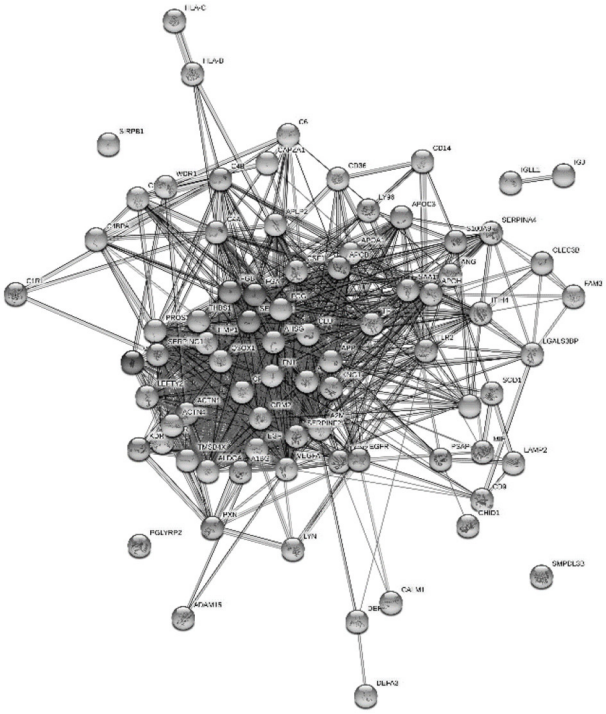

FIGURE 6 | Interaction of platelet degranulation and innate immune response protein in Chinese HM were enriched with DAVID Bioinformatics and visualized using STRING 9.

the periods, while Harbin also shared the insignificant difference with Zhengzhou except in the 5th month of lactation.

Remarkable different protein intensities were found during 6 months of the lactation period in terms of innate immune response that ranged from 4 to 11% of the iBAQ values (**Figure 5B**), especially in Weihai, Lanzhou, and Jinhua, which had higher intensities among the other cities. In contrast, Beijing and Zhengzhou were lower in iBAQ values compared with the others. In this term, the significant variation was observed along the lactation period in each city. The 2nd and 4th months had more variation intensities compared to that of the other periods.

The triglyceride metabolic process, which ranged from 2 to 6% of the iBAQ values, revealed a significant difference in the 1st and 5th months of the lactation period (**Figure 5C**). Meanwhile, no significant difference was observed during the rest of the lactation period. Harbin had the lowest intensities in the 1st month of the lactation period; in contrast, Jinhua was the highest. However, in the 5th month, Zhengzhou had the lowest iBAQ values, and Jinhua still had the highest.

The proteins from platelet degranulation and innate immune response were illustrated by STRING 9 to see the protein–protein interactions since both the terms were enriched under the complement and coagulation cascade in the KEGG pathways (**Figure 6**). Almost all of the proteins were connected except the SMPDL28, SIRPB1, IGLL, and IGJ.

DISCUSSIONS

Human Milk Proteome Varied Across the Cities and Lactation Periods

Human milk composition is suited for infant needs and growth. However, the profile of HM is crucial for developing products for infants who either cannot access their mother's milk or whose mothers' milk production volume was insufficient. The HM variation in the different continents had been reported previously using the metabolomics method (11). The present method found a higher number of identified proteins than the previous study (13). It means our proteomics method was comparable to the other methods and could be deeper in the bioinformatics enrichment since more proteins were identified to interpret its biological function.

Twelve proteins were the discriminants of the cities, namely LALBA, CSN2, IGKC, CSN1S1, CSN3, LTF, CEL, IGHA1, B2M, LYZ, CLU, and XDH. These discriminant proteins were mainly contributed as host defense in the biological process of an infant. It was agreed with a previous research that found the immune protein including lactalbumin, lactoferrin, and IgA were varied among the population as a function of pathogen pressure of the environment (19).

In the most abundant protein in HM, this study found that CSN1S1, CSN2, and CSN3, which belonged to casein, were statistically different across the cities. Casein plays the role of primary source of phosphate and calcium in HM due to its function in casein-micelle aggregates in the calcium transport process (20, 21). This finding was in line with a prior study that reported the remarkable difference of kappa and beta casein in HM serum of Chinese and Dutch mothers (14). The LTF variations across the cities were in line with previous findings in Chinese HM across distinct regions (22).

Based on differential expression tests, variation of significant proteins across the cities was very high that presented in **Supplementary Table 2**. A total of 184 proteins were significantly different across the city with a *p*-value of below 0.05 that was followed by enrichment in **Figure 4**. Meanwhile, only four proteins were statistically different during the 6 months of lactation, namely CTSC, LYZ, CA6, and CHRDL2. LYZ was upregulated while CTSC, CA6, and CHRDL2 were downregulated. The downregulated CHRDL2 was in line with a prior proteomics research in HM over lactation (3). Since the function of CHRDL2 is related to ossification, it might be possible that the expression was downregulated by organ maturation throughout the 6 months. The increase of LYZ in the human mature milk was previously described by Montagne et al. as the passive protective agents of breast-fed infants during mature lactation (23).

In **Figure 2**, the first pool consisted of the 1st, 2nd, and 5th months of the lactation period that had a high abundance of LALBA, CSN1S1, CEL, LTF, B2M, SPP1, and IGHA1. The high abundance of LALBA and CSN1S1 in the beginning months of the lactation had been previously reported in bovine milk (24). B2M, IGHA1, and LTF as the proteins related to antibacterial humoral response were logical to have a high abundance in the 1st and 2nd months of lactation since newborns had

little protection against bacteria from the environment. CEL, as the main protein related to fat metabolism, was reported to be highly abundant during the initial months (3) since it functions as an immature pancreatic lipase substitution. Meanwhile, the highly percentage of aforementioned proteins in the fifth month needs to be further investigated. We speculated it was because of the highly individual variation in the 5th month samples.

Significant Different Proteins Across the Cities Were Linked to Newborn Protection

The significantly different proteins across the cities were involved in platelet degranulation, and those proteins were mainly located in the extracellular exosome (**Figure 4**). It was agreed with a previous finding in proteomics of bovine milk exosome that showed platelet degranulation was the most significant biological process (25). Beyond its primary role in thrombus or plug formation in wound healing, the platelet also worked in tissue repair, angiogenesis, inflammation, and host defense (26–30). In newborns, overall platelet degranulation released during platelet activation was lower compared to adults (31). Based on our results, there was no significant difference in platelet degranulation in the 1st month across the cities (**Figure 5A**). It might indicate that the newborns demanded HM proteins to regulate platelet degranulation for completing platelet function since platelets activation was low in the first few days of life (32).

Neonates were generally believed to have partially immunological incompetence and were susceptible to infections. Platelets also played a role in the host defense of the newborns. By the present result, platelet degranulation shared similar proteins with the innate immune response in GOBP, including FGA, FGB, CLU, SERPING1, and CD36. All proteins contributed in both terms were figured out in protein network connection (**Figure 6**). Platelet glycoprotein 4 (CD36) had a responsibility in the phagocytosis process (31). Phagocytosis was an important innate immune response process to protect infants from microbes. CLU is a highly glycosylated protein that was linked to cell damage and apoptosis. This protein was found to be overexpressed at stressed tissues to provide a chaperone-like activity and prevent other proteins from damage (33). FGA is known as the main protein in handling fibrin production as one of the primary components of blood clots. It also acts as a fibrin deposition process that is associated with infection, where it protects against IFNG-mediated hemorrhage. The link of platelet degranulation and immune response as the host defense were regulated in complement and coagulation cascades, which were enriched in major KEGG pathway (**Figure 4**).

Immune response plays important roles in infants, especially innate immune response in newborns since adaptive immune response is not well-developed yet. The function of HM as a complementary immune system for newborns has been previously reported (34). From our identified protein (**Supplementary Table 1**), above 54 proteins were enriched as innate immune response in biological process with *p*-value 1.6×10^{-10}. It was higher than previous findings that used a similar GO enrichment tool in HM (8). That research found the *p*-value of the immune system to be 1.8×10^{-7}. The reason might be because this study had higher identified proteins. Based on the significant expressed protein enrichment, the *p*-value of the innate immune response was 1.87×10^{-5},which consisted of 17 proteins, namely FGB, FGA, IGHM, CFI, CLU, IGHG4, C4A, IGHG1, IGHG2, IGKC, IGLL1, SERPING1, CD14, IGHA1, SMPDL3B, IGHA2, and B2M. These 17 proteins brought variation to the quantitative amount of innate immune response across the cities (**Figure 5B**) that showed significant variations in each period of the lactation. The differences of immune-related protein intensity were also previously found in the different geographic location and ethnicity in China because the environment could influence the pathogen (13). However, other factors such as the health condition or infection of the infant should be further observed, since the infant infection could upregulated immune proteins in the HM, especially in the first year of lactation (35, 36).

Triglyceride Metabolism Variations Across the Cities

Breastfeed infants have better lipid utilization compared to formula-feed infants (37–39). Since delivery, infants used fat as the major energy source that contributed about 40–55% of the total energy and could obtain 5.5 kg fat intake during 6 months of lactation (40, 41). Triglycerides represented 98–99% of total fat in HM (42). Fat metabolism and fat absorption were noticed as the enriched KEEG pathway from the significantly expressed proteins (**Figure 4**). The crucial protein that handled fat metabolism in HM was CEL, which was found to be the seventh abundant protein (**Table 2**) and also significantly expressed protein across the cities (**Supplementary Table 2**). CEL from HM played a major role in infant lipid utilization by replacing pancreatic triglycerides lipase (PTL), which is secreted by the pancreas, while the pancreas condition is immature (38, 43). The present research found five significantly expressed proteins in different cities, namely CEL, APOH, APOA2, LPL, and APOE which were enriched in the triglycerides metabolic process. These proteins brought remarkable variation in the triglyceride metabolic process (**Figure 5C**), particularly in the 1st and 5th months of the lactation period. This could be noteworthy because the lower abundance of the triglyceride metabolic process proteins might lead to lower weight gain of the infant since fat should be the major energy intake. In addition, the immature digestion tract of the infant needs HM protein to aid fat digestion (43).

However, bigger samples in a longitudinal study during the lactation stages will give more benefit to fill the gaps in this research as well as decrease the variations among individual mothers. It will bring a deeper quality of biological role interpretation as the basic data for developing the most suitable infant formula for babies who cannot obtain HM in the exclusive breastfeeding period.

CONCLUSION

This present research found a noticeable variation of HM proteins across eight cities in China and four significantly expressed proteins during the 6 months of lactation. The 184 significantly expressed proteins across the cities mainly influenced the infant biological process in terms of platelet degranulation, innate immune response, and triglyceride metabolic process. This research could be a good recommendation for developing specialized region infant formula or HM fortifiers during the exclusive breastfeeding period.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Library of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JLu: conceptualization and validation. WZ and JLu: methodology. RS and WZ: software and investigation. WZ: formal analysis. JLu, JP, and YL: resources. RS: data curation, writing—original draft preparation, and visualization. HZ, XP, SZ, and JLu: writing—review and editing. JLu and JLv: supervision. SJ, JLu, and JLv: project administration. JP, SJ, JLu, and JLv: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.682429/full#supplementary-material

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