Research Article

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Dissection of proteome and transcriptome reveals advantages for antifungal activity and immunocompetence in horse milk.

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ABSTRACT

Horse milk is a highly valuable organic food that is a promising alternative to cow milk, whereas studies of horse milk and its protein constitution remain unclear. A comparative proteomic analysis of milk among cow, goat, camel and horse was developed. The composition of milk varied among four types of milk and horse milk contained the highest amounts of functional whey proteins. GO enrichment analysis showed that the proteins related to proteolysis, degradation and catalysis were highly expressed in horse milk compared to other three types of milk, resulting in easier assimilation and palatability. KEGG enrichment analysis suggested that the horse milk contained abundant antimicrobial proteins relevant to shigellosis, salmonella infection, pathogenic Escherichia coli infection, leading to the decreased risk of pathogenic diseases. Higher accumulation of proteins related to caffeine metabolism, biosynthesis of amino acids and glycolysis/gluconeogenesis in horse milk contributed to the flavor and taste of horse milk. Meanwhile, we also found that highly expressed proteins in horse milk were closely related to PI3K-Akt and MAPK signaling pathways, which suggested that horse milk could promote the immune response by interacting with both pathways. Importantly, we identified four highly expressed antimicrobial proteins in horse milk including PGL, B2M, CD14 and PGL, which conferred direct inhibitory effect to Pseudomonas aeruginosa, Escherichia coli, Candida albicans and Aspergillus niger. Transcriptome analysis demonstrated that exogenous treatment of PGL could inhibit growth of P. aeruginosa and E. coli by repressing the biosynthesis of secondary metabolites. Overall, our results highlighted the advantages of horse milk about immune enhancement and nutrient composition compared to cow, goat and camel milk. These findings contributed to a better comprehension of horse milk and expected to provide valuable insights for its promotion.

Keywords : horse milk; camel milk; goat milk; cow milk; proteomics; bowel disease; immune resistance

INTRODUCTION

Milk is a unique substance secreted by the mammary glands of mammals to feed their offspring, and is the perfect food for survival and development after birth.1 Besides human breastmilk, the human habit of consuming milk derived from animal like cow and sheep survived thousands of years and early documented at the Neolithic Age.2 With the progress of food technology, more and more dairy products generated by milk showed up which further improved the popularity of milk. Nowadays, milk is regarded as an essential beverage and food that widely attractive around the world. To date, the global milk production of dairy livestock has increased from 522 million tons to 937 million tons from 1987 to 2022, reaching an increase of about 80%.3 Despite the cow milk still had the predominant occupation in milk production, approximately 82%,4,5 increasing demands for different nutrient compositions in dairy products also facilitated the exploration of other animal derived milk like camel, buffalo, sheep and horse.6-9 Therefore, the researchers take an interest in finding out the value of nutrient and economic in other alternative milk products in current studies.

Horse milk (HM) is a promising and valuable dietary resource of dairy products. The inhabitants lived in Western Europe and Central Asia that consumed HM as daily food.10 The nutrient compounds and proportion among their compounds of HM were similar to that of human breastmilk, including the small proteins (β-lactoglobulins and α-lactoalbumin), lactose, minerals and microelements.11 Horse milk has an elevated lactose content, excellent palatability, and promotes intestinal calcium absorption, which is highly critical for bone mineralization in children.12 In terms of levels of protein and

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inorganic content, horse's milk has a kidney load comparable to that of human milk, suggesting that mare's milk is suitable for infant food.13 The activity and variety of prebiotics and probiotics in mare's milk are beneficial for infants and children with cow's milk protein allergies (CMPA) and intolerances to multiple food ingredients.9,14 These studies confirmed that HM can serve as an excellent alternative of breastmilk and beverage with low digestive burden.15 More nutrition and health studies have shown that moderate consumption of HM positively contributes to the health of individuals at all stages of their life cycle.16 Adults can also reduce the risk of chronic diseases such as metabolic syndrome,17 cardiovascular disease,18 type II diabetes19 by continuously taking appropriate amounts of HM. In addition, the elderly can improve their cognitive level, improve the quality of skeletal muscle, and reduce the risk of weakness and Sarcopenia by taking appropriate amounts of HM.20 Meanwhile, the replenishment of HM could enhance the glycogen level in liver and muscle contributing to the improvement of exercise-induced fatigue.13 Increasing documents revealed these healthful benefits of horse milk attributed to the higher concentrations of proteins like lactotransferrin (LTF), peptidoglycan recognition protein (PGRP1) and whey acidic protein (WAP) compared to other types of milk (goat, cow and buffalo).12 Despite such nutritional evidence emphasized the importance of HM consumption, limited cognition of HM containing unique proteins and flavor substance is known in current research.

Over the past years, substantial advances have been made in composition determination of various food stuff that is beneficial to human health by proteomic strategy. Proteomic technologies ensure the large-scale and in-depth investigation of proteins, in particular, the determination of potential bioactive substance in complex biological materials by means of a high-throughput manner.21 Renzone et al.22 detected the quality of advanced glycation end-products (AGE) in various infant formula milk by proteomics analysis which contributed to the risk assessment of infant formula milk. Proteomics analysis of Australian camel milk in different seasons elucidated that summer camel milk contained more abundant accumulation of whey proteins resulting in high nutritional value and easier assimilation.23 In this study, we used the label-free protein identification approach to quantify and assess difference in proteomes among horse milk, cow milk, goat milk and camel milk. We aimed to investigate major nutritional compounds and characterized chemical properties of horse milk compared to other types of animal milk. These proteins could be exploited as nutritional marks to highlight the value of horse milk and avail of HM promotion. Further identification of four antimicrobial proteins in horse milk illustrated their immune contributions on inhibitory effect for multiple pathogens and enhancement of host immunity. These findings provide a valuable insight to horse milk protein constitution and immune benefits for further promotion of horse milk.

MATERIALS AND METHODS

Sample collection and preparation

Four types of milk, including Horse (Equus caballus), cow (Bos taurus), goat (Capra hircus) and camel(Camelus bactrianus) were abstracted from Nanshan region in Xinjiang. These milk samples meet production-grade food requirements. Horse milk of colostrum and normal types were prepared to abstract 200 mL and was freeze-dried into powder by Freeze-Drying Digital Unit(MODULYOD-230). Each type of milk had six replicates. Milk samples of goat, horse, camel and cow milk were abstracted in six individuals of them, which were taken at 4°C, centrifuged at 11000 r/min for 20 minutes, and the upper fat part was carefully discarded. The whey solution was adjusted to pH 4.6 with 1 mol/L hydrochloric acid, left for 0.5 h, centrifuged at 11000 r/min for 20 minutes, then the supernatant was collected and freezable into powder. Take equal amount of horse milk, cow milk, goat milk and camel milk respectively. Then 0.1 mol/L hydrochloric acid was used to adjust the pH value to 4.6 with continuous stirring to prevent protein denaturation. The samples were placed for 10 minutes, and then were centrifuged for 10 minutes at 12000 r/min. The supernatant of milk was carefully transformed into glass culture dish, then put the glass dish in the freeze dryer for drying treatment, freezing temperature ≤-50°C for 24 hours. The lower casein is prepared into casein powder under the same conditions.

Sample preparation for label-free proteomic quantification

An appropriate amount of SDT cracking solution was added to samples of milk from four groups, boiling water bath for 5 min. Then, samples were centrifuged at 14000 g for 15 min, and supernatant was taken and stored at -80°C for proteome analysis. The protein quantification was detected using BCA method. Take 200 ug protein solution of each samples, add DTT to the final concentration of 100 mM, then boil in water for 5 min, cool to room temperature. Then the solution samples were mixed with 200 μL UA buffer, then transferred to the ultrafiltration centrifuge tube for centrifugation at 14000 g for 15 min(repeat this step once), and the filtrate was discarded. Subsequently, 100 μL IAA buffer (100 mM IAA in UA) was added to the solution and shake at 600 rpm for 1 min, then the samples were kept at 28°C for 30 min before centrifugation at 14000 g for 15 min. After centrifugation, 100 μL UA buffer was added, and the samples were centrifuged at 14000 g for 15 min, and the procedure was repeated twice. In total, 100 μL 25 mM NH4HCO3 solution was added,

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centrifuged at 14000 g for 15 min, and the procedure was repeated twice. Then, 40 µL Trypsin buffer (4 ug Trypsin in 40 µL 100 mM NH4HCO3) was added into each sample, shake at 600 rpm for 1 min, then the samples were kept at 37°C for 16-18 h. All samples were taken into another collection tube, and centrifuged at 14000 g for 15 min; Then 40 µL 25 mM NH4HCO3 was added and centrifuged at 14000 g for 15 min to collect filtrate. C18 Cartridge was used to desalinate the peptide. After lyophilization, the peptide was redissolved with 40 µL 0.1% formic acid solution, and the peptide was quantified (OD280).

Protein identification by LC-MS/MS

According to the quantitative results, 2 µg enzymolysis products were taken for LC-MS/MS analysis (Q-Exactive). HPLC system Easy nLC was used for separation. Solution Buffer A is 0.1% formic acid, and solution B is 0.1% formic acid acetonitrile aqueous solution (acetonitrile is 84%). The column was balanced with 95% solution A. The samples were loaded into the Thermo Scientific EASY Column (2 cm*100 µm 5 µm-C18), and then analyzed on a Thermo Scientific EASY Column (75 µm*100 mm) 3 µm-C18) at a flow rate of 300 nL/min. The solution gradient is as follows: 0 min-110 min, linear gradient of liquid B ranges from 0% to 55%; 110-115 min, the linear gradient of liquid B increased from 55%-100%; 115-120 min, liquid B was maintained at 100%. The peptides were separated by chromatography and analyzed by MASS spectrometry using Q-Exactive Mass spectrometer (Thermo Scientific). Detection method: positive ion; Scanning range of parent ion: 300-1800 m/z; Primary mass spectrometry resolution: 70,000 at 200 m/z; The peptide fragments was collected according to the following methods: 20 fragments were collected after each Full scan (MS2 Scan), and the resolution of MS was 17,500 at 200 m/z. Microscans: 1, Isolation Window: 2 m/z, Maximum IT: 60 ms, MS2 Activation Type:HCD, Normalized Collision Energy: 27 eV, Dynamic Exclusion: 60.0 s, Underfillratio: 0.1 %.

Data processing and enrichment analyses

The resulting MS/MS data were processed using MaxQuant search engine (vs 1.6.3.3, Cox and Mann, 2008). Tandem mass spectra were searched against the UniProt database concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to 2 max missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in First search and 6 ppm in Main search, and the mass tolerance was 20 ppm. Carbamidomethyl on Cys was specified as fixed modification and acetylation modification and oxidation on Met were specified as variable modifications. The LFQ was used to determine the differences of proteins. The threshold $P < 0.05$ and $|Log1.5Foldchange| > 1.0$ were set to identify the significantly differentially expressed proteins in each comparison. The differentially expressed proteins were classified by KEGG pathway and Gene Ontology (GO) annotation enrichment analyses. For each category, a two-tailed Fisher's exact test was employed to test the enrichment of the differentially expressed protein against all identified proteins. The GO terms and KEGG pathways with P value < 0.05 were considered significant.

Transcriptome analysis

The testing samples were delivered to Allwegene (Nanjing, China) for RNA isolation, and raw data processing was analyzed by Allwegene online cloud platform (http://218.2.224.234:8888). Differential expression analysiswas performed using the DESeq R package (1.10.1). DESeq provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq were assigned as differentially expressed. Gene Ontology (GO) consists of Molecular Function(MF)□ Biological Process(BP)□Cellular Component (CC) three parts. So protein or gene can through corresponding gene ID or the method of sequence annotation find the corresponding GO term that is function category or cell localization GO enrichment analysis of differentially expressed genes was implemented by the GOseq R package, in which gene length bias was corrected. GO terms with corrected Pvalue less than 0.05 were considered significantly enriched by differential expressed genes. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (http://www.genome.jp/kegg/). We used KOBAS software to test the statistical enrichment of differential expression genes in KEGG pathways.

Prokaryotic expression and protein purification

For prokaryotic expression assay, the coding sequences of PGL, CD14, B2M and LPO were fused with glutathione S-transferase (GST) tag in pGEX-4T-1 plasmid with double-digested by the BamH I and EcoR I sites. Then recombinant plasmids of four proteins were transformed into Escherichia coli Rosetta Gami 2 and the transformants were chosen to produce GST-fusion proteins. Recombinant protein expression was induced with 0.4 mmol L-1 isopropyl-β-D-1-thiogalactopyranoside (IPTG) and continuous cultivation in incubator at 18°C, 160 rpm. After 16 h, E. coli cells were collected and cleaned in suspension buffer (10 mmol L-1 Tromethamine-HCl, pH 7.4, 50 mmol L-1 NaCl). Samples of

the E. coli suspension culture was pulverized with ultrasound for 20 min and then centrifugated at 12,000 g, 1 h, 4°C. The collected supernatant was purified with GST resin (Sangon, C600912) at 4°C for 1 h in an overhead shaker. The supernatant was then discarded, and the remaining beads were rinsed with glutathione buffer three times before collection. Then sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify whether the target proteins were expressed. Purified recombinant proteins were added to SDS-PAGE loading buffer, boiled, and centrifuged. The supernatants were subjected to PAGE, and the proteins were transferred to polyvinylidene difluoride membranes. The membranes were incubated with the indicated antibodies in protocols.

Antimicrobial activity in vitro

Add 100 µl of the prepared pathogens solution onto an agar plate, spread it evenly with a spreader for 5 min. Using a pipette, draw 12 µl of protein and drug solution onto sterile paper discs. Sterilize the tweezers in the outer flame of an alcohol lamp and let them cool. Once cooled, use the tweezers to place the paper discs onto the agar plate, pressing lightly to ensure close contact with the medium. Prepare three plates for each type of bacteria, repeating the process three times. Place the plates with paper discs in the incubator, incubate bacteria at 37°C for 16-24 h, and fungi at 30°C for 36-48 h. Observe the antibacterial results and measure the diameter of the inhibition zones using a ruler.

RESULTS

Proteomic patterns of horse milk separated with other three kinds of mammal milk.

HW is characterized as abundant content of proteins, including caseins, whey proteins and lactoferrin, which is beneficial for human health and immunity.21 However, the detailed information about constitution and distribution of these proteins or other beneficial proteins among several mammal milk remains unclear. We then performed an in-depth proteome analysis on milk from horse, cow, goat and camel (HW, MW, GW and CW) to illustrate the difference of protein composition among them. we first developed the principal component analysis of proteomic profiles in four groups. The result showed that each group contained 3 aggregated samples that formed four distinct separations with the variations of PC1 (28.7%) and PC2 (21.4%), suggesting their variable proteomic compositions (Figure 1A). Intriguingly, we noticed that HW group was distinguished from other three groups with markedly separated distribution (Figure 1A). Consistently, Pearson correlation analysis displayed similar results that all samples from same group clustered together, and formed four clades including HW, MW, GW and CW, demonstrating the credibility of data and differences among samples of four types of milk (Figure 2B). Further detailed representative proteins were mapped in correlation analysis and showed that there were four sets of proteins among these samples, which reached a consensus with the number of experimental groups (Figure 1B-C). The correlation analysis confirmed the significantly different compositions of proteomic profiles among four types of animal milk which in favor of next work. Subsequently, we set threshold P value < 0.05 and |Log1.5Foldchange| > 1.0 to identified differentially expressed proteins. In HW vs. CW comparison, 34 proteins were identified to upregulate in HW, while 89 proteins were downregulated in HW (Figure1D). In parallel, there are 53 upregulated and 68 downregulated proteins in HW vs. GW comparison (Figure 1D). Then, compared with MW, the expression levels of 41 proteins were higher in HW, whereas the levels of 73 proteins were lower in HW (Figure 2D). And these proteins will be recognized as focus for further analysis to explain the advantages of horse milk in consumption.

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Figure 1

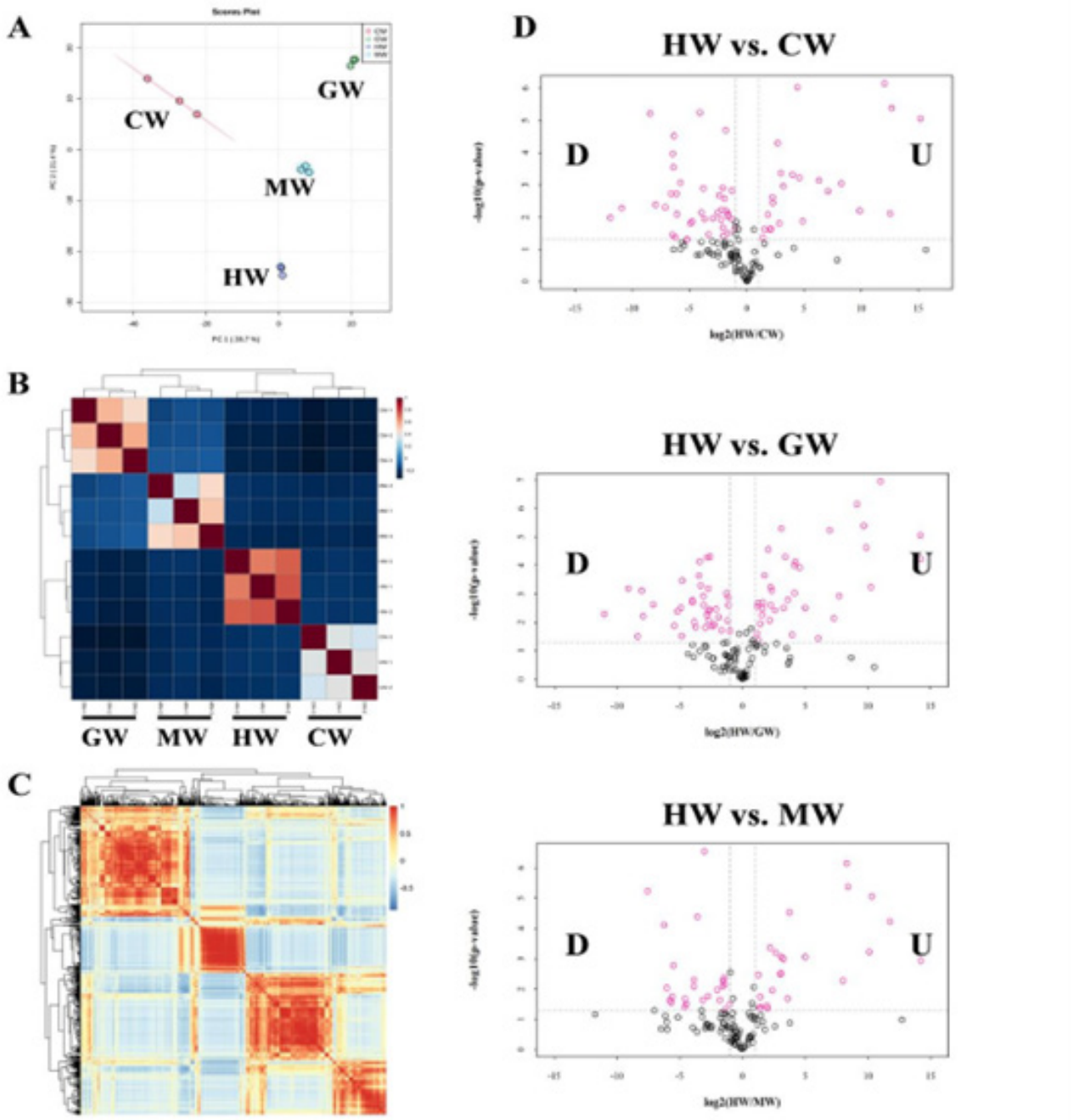

Figure 1. Overview of proteomic profiles from four organisms.

A: Score plot of principal component analysis (PCA) of the proteome datasets. Each plots represented a sample in experiment. B: Hierarchical clustering displayed the correlation among all samples. The samples from same organisms closely clustered together at one clade and clearly separated with other three groups of samples. C: Correlation analysis among all proteins in the proteomic profiles. Red and blue represented the high and low correlation relationship between two proteins, respectively. D: Volcano plot (p value versus fold change ratio) displayed the significantly differentially expressed proteins in each comparison (HW vs. CW, HW vs. GW and HW vs. MW). Red dots are significant at P value < 0.05.

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The protein component and function analysis between horse milk and cow milk

Horse milk is considered to be a promising alternative for cow milk, to investigate the difference of protein constitution between them, we performed Gene ontology analysis on the differentially expressed proteins in HW vs MW. GO analysis provides a dynamically updating controlled vocabulary set to describe genes and gene product attributes in an organism. Previous identification of 69 up-regulated and 72 down-regulated proteins (Figure 1D) were mapped into GO enrichment analysis. The result showed that these proteins were mainly enriched in 121 GO terms containing 96 BP terms, 9 CC terms and 16 MF terms. For terms of BP, we found that these proteins involving in protein proteolysis, peptidase activity, acute-phase response, hydrolase activity, regulation of proteolysis, regulation of endopeptidase activity, regulation of peptidase activity and catalytic activity were significantly enriched (P-value<0.05) (Figure 2A).This implied that the proteins relevant to proteolysis activity were more active in horse milk compared to cow milk, which caused the difference of digestive absorption between them. For terms of MF, we found these proteins were predominantly gathered inodorant binding, peptidase inhibitor activity, peptidase regulator activity, endopeptidase inhibitor activity, enzyme inhibitor activity, endopeptidase regulator activity, vitamin D binding, serine-type endopeptidase inhibitor activity, molecular function regulator, transporter activity, enzyme regulator activity, calcidiol binding, pheromone binding, D3 vitamins binding, vitamin binding and lipid binding (P-value<0.05) (Figure 2A). These results suggested that proteins associated protein degradation and activity varied between HM and MM, suggesting the advantages of HM may contained more proteins related to degradation. For terms of CC, we found these proteins were mainly enriched in organelle outer membrane, chromaffin granule, mitochondrial outer membrane, outer membrane, extracellular membrane-bounded organelle, mitochondrial membrane, mitochondrial envelope, apical dendrite and mitochondrial part (P-value <0.05) (Figure 2A). The results suggested the location of these proteins which function proteolysis and enzyme activity. We thus proposed that HW was rich in proteins functioning active proteolysis, degradation and catalysis compared to MW, which may contribute to absorbable and palatable feature of horse milk. As expected, expression pattern of these proteins was further abstracted in proteome file and depicted in heatmap (Figure 2B), showing most of proteins were from whey proteins including lactotransferrin, lysozyme C, lactoglobulin, immunoglobulin, macroglobulin, serum albumin and serotransferrin, which showed significant increase in content compared to MW. These results supported that horse milk contained more whey protein components that were easier to digest and absorb, and these whey proteins also showed beneficial goodness to human health and immunity.

Further KEGG enrichment pathway analysis was used to determine protein distribution in metabolic pathway. We noticed that a large number of DEPs was significantly gathered in immune signaling pathways or disease resistance pathways (Figure 2C). Especially 7 significantly enriched metabolic pathways including PI3K-Akt signaling pathway, MAPK signaling pathway, shigellosis, salmonella infection, Escherichia coli infection, legionellosis and endocytosis overrepresented the metabolic direction of most proteins (P-value<0.05) (Figure 2C and Table S1). These results suggested that horse milk contained more abundant proteins related to immune response that may contribute to trigger immune signaling pathways or reduce the risk for contaminates by such pathogenic microbes in horse milk. Additionally, these proteins were further used to construct protein-protein interaction network, the result showed that four hub proteins like HSP90AA1, YWHAE, APOE and ENO1 functioned pivotal roles in regulation of immune regulatory network in horse milk (Figure 2D). Taken together, these findings demonstrated that HW had advantages on elevated accumulation of whey proteins and immune-related proteins for human health and immunity compared to MW.

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Figure 2

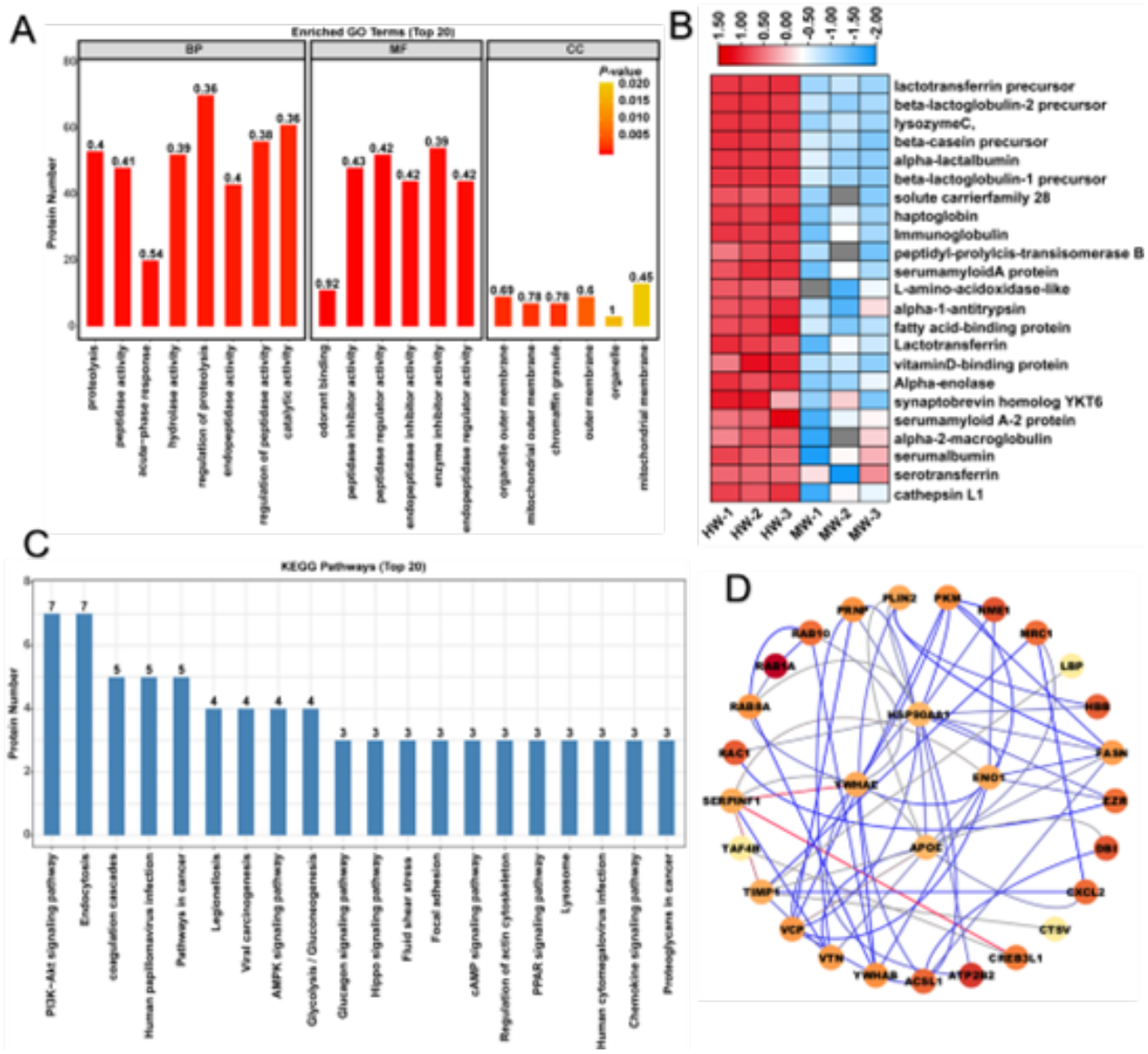

Figure 2. GO and KEGG enrichment analysis illustrated difference of protein function between horse milk and cow milk.

A: GO enrichment analysis on differentially expressed proteins in HW vs MW. Three main terms represented by molecular function, cellular component and biological process were shown the histogram.

B. Heatmap displayed the relative expression levels of proteins in HW and MW. The up- and downregulated proteins were shown in red and blue, respectively. The scale represented the normalized expression values of each proteins.

C. KEGG enrichment analysis on differentially expressed proteins in HW vs. MW. D. the construction of protein-protein interaction network for differentially expressed proteins in HW vs. MW.

The protein component and function analysis between horse milk and goat milk

In the comparison of HW vs. GW, 108 up-regulated proteins and 114 down-regulated DEPs were into GO enrichment analysis and found that these proteins were mainly enriched in 275 GO terms containing 230 BP terms, 9 CC terms and 36 MF terms. For terms of BP, we found that these proteins were remarkably clustered in BP term of the negative regulation of catalytic activity (P-value<0.01, FDR<0.05) (Figure 3A). Besides, we also noticed that proteins related to negative regulation of catalytic activity, negative regulation of molecular function and inflammatory response were significantly enriched (Figure 3A). The results implied that significant difference of protein components between both types of milk proteins was the variation of

catalytic activity and inflammatory response, which may account for different nutrition constitution and flavor between them. For terms of MF, we found these proteins were predominantly gathered in enzyme inhibitor activity, endopeptidase inhibitor activity, molecular function regulator, endopeptidase regulator activity, peptidase inhibitor activity and enzyme regulator activity (P-value<0.05, FDR<0.1) (Figure 3A). The result showed that proteins associated protein degradation and activity were significantly enriched in HW compared to GW, suggesting more active protein degradation and digestion events occurred in HW. Indeed, low molecular weight protein in milk composition was readily available to assimilation for human. We thus proposed their difference of absorption should be taken into consideration in further assessment between them. For terms of CC, we found these proteins were predominantly enriched in blood microparticle, nuclear outer membrane, high-density lipoprotein particle, basolateral plasma membrane, cell surface, nuclear envelope lumen, HFE-transferrin receptor complex, nuclear chromatin and phagocytic cup (P-value <0.05) (Figure 3A). The results suggested the location of these proteins which function regulator or enzyme activity. Expression of these proteins were further depicted in heatmap (Figure 3B), illustrating that most of whey protein components were up-regulated in HW compared to GW, leading to the higher benefits for human health and immunity.

Based on previous result, 108 up-regulated proteins and 114 down-regulated proteins were further mapped into KEGG enrichment pathway analysis in the comparison of HW vs. GW. The results showed that the DEPs linked in such immune signaling pathways representing main function of them, including PPAR signaling pathway, HIF-1 signaling pathway, ferroptosis, ECM-receptor interaction and complement and coagulation cascades were significantly enriched(Table S2). PPAR (peroxisome proliferators-activated recepotor) signaling pathway partook in the physiological processes of lipid metabolism, cell proliferation and differentiation.25 HIF-1 (hypoxia- inducible factor-1) signaling pathways promote the adaption to low oxygen tension in cells and organisms resulting in the transcriptional induction of a series of genes that participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival.26 It has been reported that the higher accumulation of proteins relevant to PPAR, HIF-1 signaling pathways and ferroptosis enhanced the risk of inflammatory bowel disease.27-29 Thus, these results manifested that HW could avoid to stimulate organic inflammatory response and reduce the risk of pathogenic microbes compared to GW. Meanwhile, most of proteins were gathered in such metabolic pathways, including phagosome, proteoglycans in cancer, regulation of actin cytoskeleton, tuberculosis pathogenic Escherichia coli infection and MAPK signaling pathway (Figure 3C). We noticed that these enriched proteins probably functioned as antibodies associated with the pathogenic bacterium species like shigellosis, salmonella and Escherichia coli that mainly caused bowel diseases. it was documented that horse milk exhibited the inhibitory effect to Salmonella Typhimurium by suppressing the virulence gene expression (hilA and ssrB2).30 Moreover, donkey belonged to Equus genus, was found to contained many antimicrobial factors by proteomic analysis, implying the antimicrobial activity of donkey's milk.31 We thus speculated that HW may contain some immune related proteins that exerted the positive therapy effect to multiple disease compared to GM. Further construction of protein interaction network showed five hub immune-related proteins such as HSP90AA1, ACTB, APOE, APP and EGFR functioned crucial roles in manipulation of host immune response in horse milk (Figure 3D), which may take part in prevention of pathogenic microbes or immune trigger in horse milk.

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Figure 3

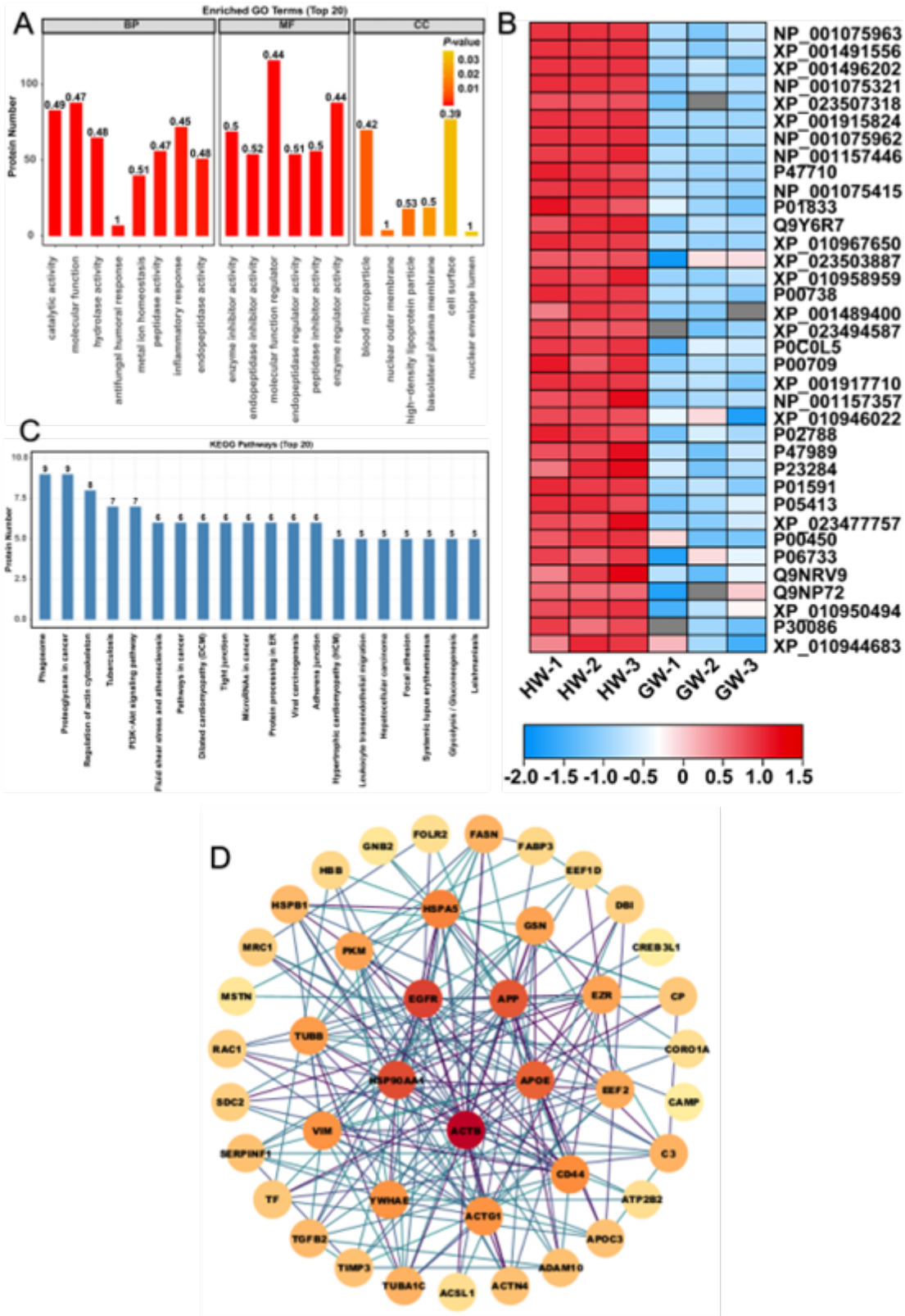

Figure 3. GO and KEGG enrichment analysis illustrated difference of protein function between horse milk and goat milk.
A: GO enrichment analysis on differentially expressed proteins in HW vs GW. Three main terms represented by molecular function, cellular component and biological process were shown the histogram.
B. Heatmap displayed the relative expression levels of proteins in HW and GW. The up- and downregulated proteins were shown in red and blue, respectively. The scale represented the normalized expression values of each proteins.
C. KEGG enrichment analysis on differentially expressed proteins in HW vs. GW. D. the construction of protein-protein interaction network for differentially expressed proteins in HW vs. GW.

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The protein component and function analysis between horse milk and camel milk

For differentially expressed proteins in HW vs. CW comparison, GO functional enrichment analysis was performed on all 34 upregulated and 89 downregulated proteins (Figure 4A). The results cover a wide range of terms relevant to molecular functions (MF), biological processes (BP) and cellular components (CC). There are 84 terms with $P < 0.05$ were significantly enriched against these 123 differentially expressed proteins (Figure 3A). For terms of cellular component (CC), these proteins mainly performed functions at high-density lipoprotein particle, postsynaptic specialization, neuron to neuron synapse, postsynaptic density, asymmetric synapse, protein-lipid complex, plasma lipoprotein particle, lipoprotein particle, preribosome, membrane microdomain and membrane raft (Figure 4A). And these proteins mainly performed 7 kinds of functions, including transferase activity, transferring aldehyde or ketonic groups, lipid binding, ATPase regulator activity, high-density lipoprotein particle binding, molecular function regulator, enzyme regulator activity and ATPase binding (Figure 4A). Additionally, for biological process category (BP), 66 related terms were significantly overrepresented among these proteins, including cellular copper ion, homeostasis, copper ion homeostasis, striated muscle adaptation, amyloid fibril formation, positive regulation of hemostasis, positive regulation of coagulation, positive regulation of blood coagulation, glyceraldehyde-3-phosphate metabolic process, glucose 6-phosphate metabolic process, pentose-phosphate shunt, muscle atrophy, striated muscle atrophy, sterol transport, cholesterol transport, regulation of lipid catabolic process, positive regulation of lipid catabolic process, regulation of cytokine production involved in immune response, protein kinase A signaling, muscle adaptation and negative regulation of fibrinolysis (Figure 4A). We noted various proteins which are beneficial to human health were identified, such as positive regulation of hemostasis, muscle atrophy, striated muscle atrophy and regulation of cytokine production involved in immune response (Figure 4A). Meanwhile, various proteins involved in primary metabolisms were enriched, such as regulation of lipid catabolic process, glyceraldehyde-3-phosphate and cholesterol transport (Figure 4A). Heatmap illustrated expression level of these associated proteins showing most of them belonged to whey protein components with significant up-regulation in HW compared to CW (Figure 4B). These results suggested the advantages of horse milk in improving immunity and chemicals which could be used as energy and nutrient for human, compared with CW.

Further pathway enrichment analysis on the these proteins showed that they were mapped onto 144 KEGG pathway, with 5 pathways were significantly enriched against these proteins, including phagosome, PI3K-Akt signaling pathway, pathways in cancer, rap1 signaling pathway, MAPK signaling pathway, pathogenic Escherichia coli infection and prion diseases (Figure 4C). Further construction of protein interaction network showed potential interacted and regulatory relationship among them, we noticed six hub proteins including HSP90AA1, EEF2, ACTG1, ACTB, YWHAE, PFN1 and HSPAB functioned essential roles in regulation of these proteins in immune response (Figure 4D). these proteins may act as triggers or antigens in response to pathogenic microbes invasion.

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Figure 4

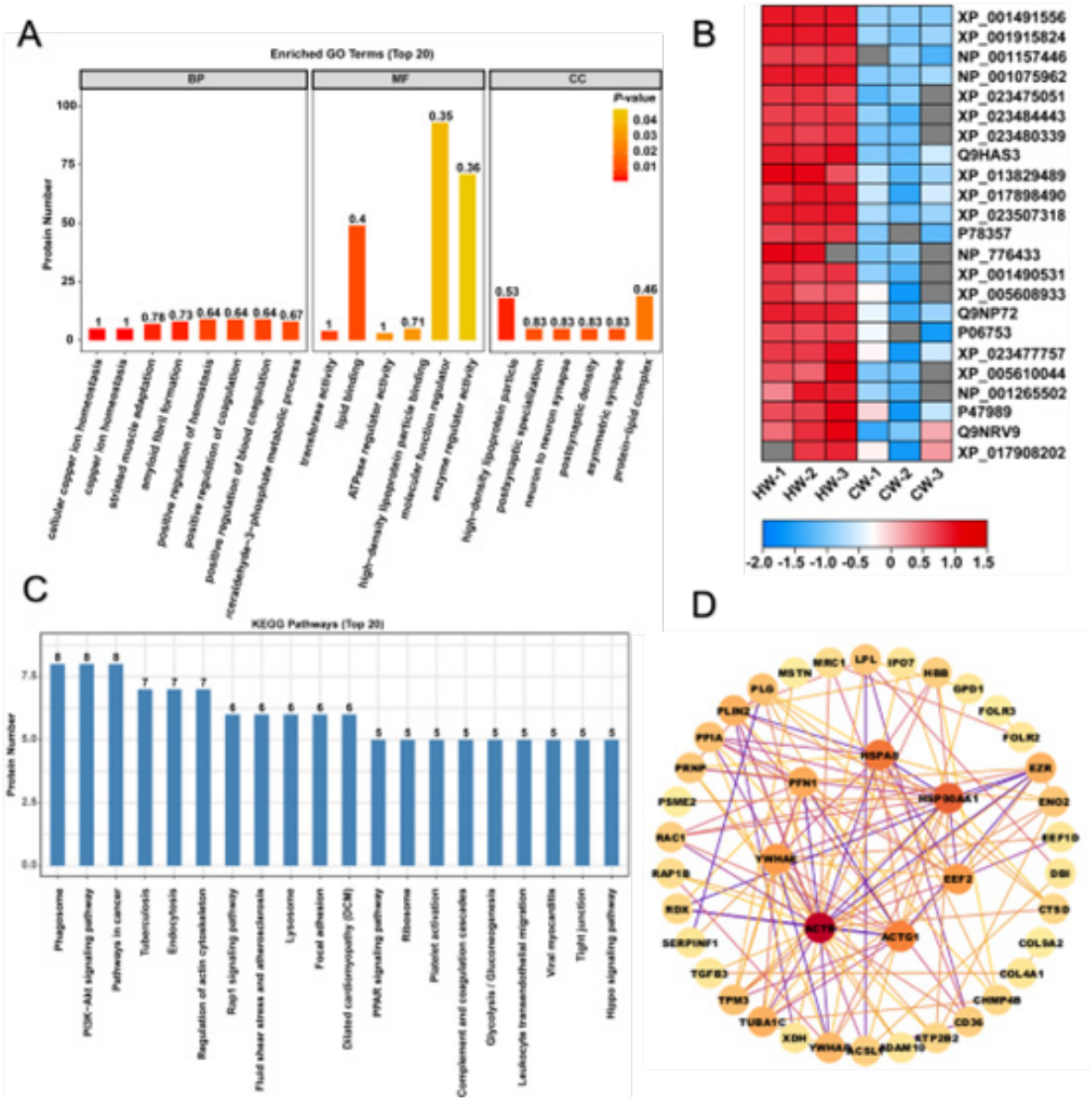

Figure 4. GO and KEGG enrichment analysis illustrated difference of protein function between horse milk and camel milk.
A: GO enrichment analysis on differentially expressed proteins in HW vs CW. Three main terms represented by molecular function, cellular component and biological process were shown the histogram.B. Heatmap displayed the relative expression levels of proteins in HW and CW. The up- and downregulated proteins were shown in red and blue, respectively. The scale represented the normalized expression values of each proteins. C. KEGG enrichment analysis on differentially expressed proteins in HW vs. CW. D. the construction of protein-protein interaction network for differentially expressed proteins in HW vs. CW.

Increased accumulation of immune-related proteins in horse milk conferred more healthy benefits

To identified the hub proteins that highly expressed in HW compared to other three organisms, we analyzed all upregulated proteins in HW vs. CW, HW vs. GW and HW vs. MW comparisons (Figure 5A). As shown in Fig. 5A, we identified 32 candidate proteins shared in at least two comparisons. Remarkably, in total 7 proteins were shared in three comparisons, suggesting the their levels were significantly higher in HW than other three organisms (Figure 5A, B). Then, we analyzed the expression levels of all 32 proteins among all samples, and identified 15 proteins that highly expressed in HW than other three organisms (Figure 5B). These 15 proteins were recognized as hub proteins which contributed to the particular advantages of horse milk. Especially four whey protein components including α-lactalbumin (XP_001915824), lysozyme C (XP_001491556), β-lactoglobulin-1 precursor (NP_001075962) and lactotransferrin precursor (NP_001157446) showed significant accumulation in HW compared to other three types of milk.

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The pathway enrichment analysis is the most intuitive way to understand the particular advantages of horse milk, thus KEGG pathway enrichment analysis was employed to reveal the functional roles of these 15 hub proteins (Figure 5C). The results showed the overrepresentation of 20 pathways among these 15 hub proteins associated with HW, including prolactin signaling pathway, HIF-1 signaling pathway, intestinal immune network for IgA production, Complement and coagulation cascades, RNA degradation, SNARE interactions in vesicular transport, caffeine metabolism, thyroid hormone synthesis, pertussis and drug metabolism (Figure 5C). IgA has been implicated in functioning center roles in the maintain of normal development and suppressing harmful bacterial microbes.9 We noted various pathways relevant to immunity were identified, including HIF-1 signaling pathway, Intestinal immune network for IgA production and complement and coagulation cascades (Figure 5C), suggesting that usage of horse milk could improve human immunity. Additionally, various metabolisms relevant to beneficial chemicals were found to be active in HW, including caffeine metabolism, biosynthesis of amino acids and glycolysis/gluconeogenesis (Figure 5C), indicating that horse milk is rich in these related proteins.

Figure 5

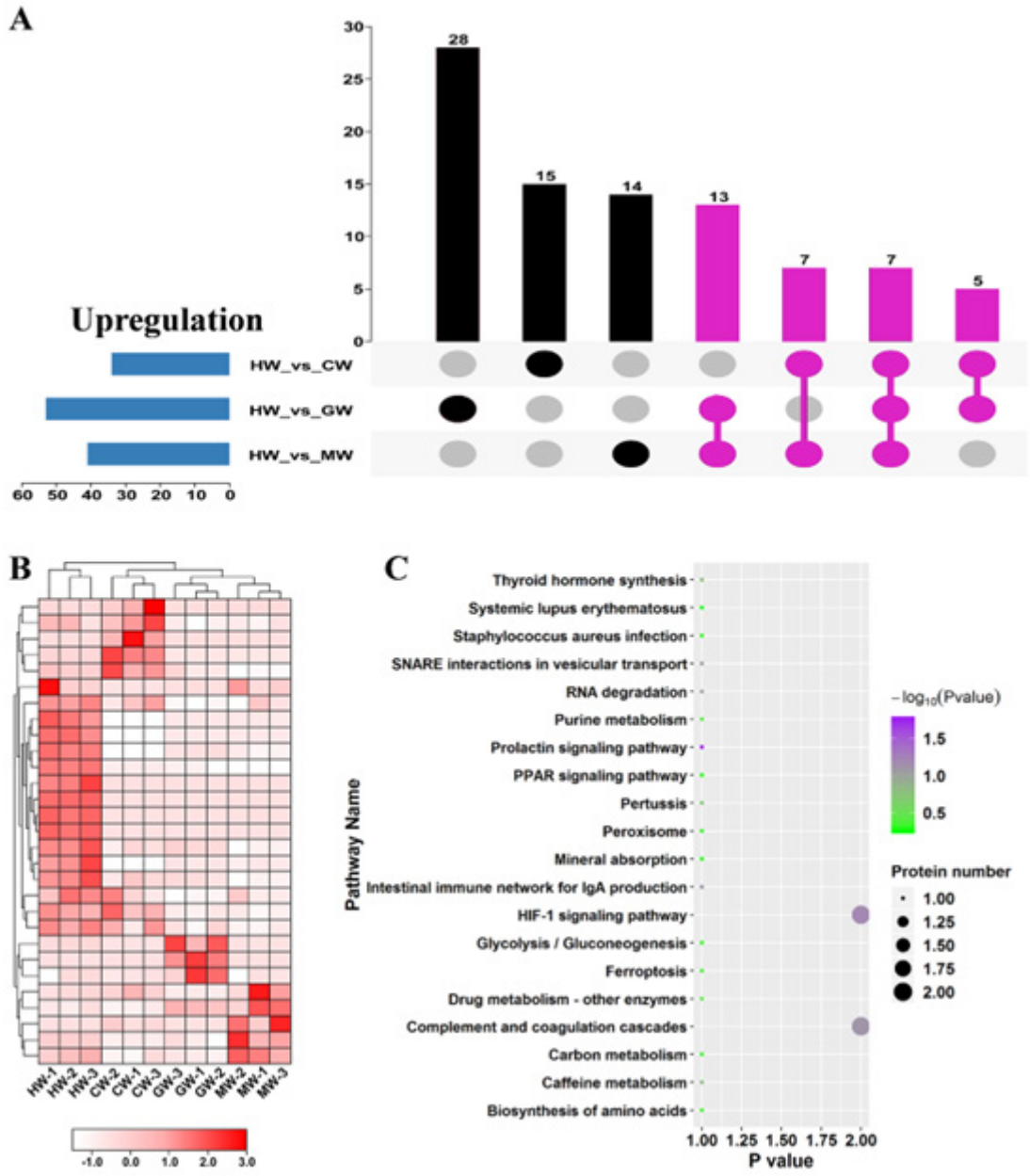

Figure 5. identification and function enrichment analysis on hub upregulated proteins in HW.
A: Upset diagrams representing the overlap of significantly upregulated proteins in HW vs. CW, HW vs. GW and HW vs. MW pairwise comparisons. The hub upregulated proteins of HW were labeled by purple. B: Clustering analysis of hub upregulated proteins in HW. The proteins with relative high expression level were shown in red, whereas the relative low expression level was shown in white. C: KEGG pathway classification of hub upregulated proteins.

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The identification of antimicrobial proteins in horse milk.

To further investigate the molecular function of these 7 shared proteins with high expression in horse milk, we examined the expression profiles and listed them in the table below (**Table 1**), including LPO, B2U, NAMLAA, CD14, TLR2, PG4 and LTFp. Notably, we found that the expression of LPO, B2U, NAMLAA and CD14 was significantly increased in three pairwise comparisons, suggesting the four candidate proteins may confer strong antimicrobial activity in horse milk.

Table 1. The expression profiles of unique antimicrobial proteins in HW

NCBI ID	Annotation	Abbreviation	Log1.5FC (HW/CW)	Log1.5FC (HW/GW)	Log1.5FC (HW/MW)
XP_014596904.2	lactoperoxidase	LPO	1.33**	1.91**	1.42**
XP_005602652.1	beta-2-microglobulin	B2U	1.21**	1.51**	1.32**
XP_023481087.1	N-acetylmuramoyl-L -alanine amidase	NAMLAA (PGL)	1.25**	1.1*	1.21**
XP_023490830.1	monocyte differentiation antigen CD14	CD14	1.52**	1.08*	1.18**
XP_023481835.1	toll-like receptor 2	TLR2	0.88	1.01*	1.21**
XP_023494587.1	platelet glycoprotein 4	PG4	0.74	0.87	1.19**
NP_001157446.1	lactotransferrin precursor	LTFp	0.84	1.01**	1.34**

P<0.05, *; P<0.01, **.

To explore the sequence characterization of these candidate proteins, a phylogenetic tree was constructed to evaluate their evolutionary relationships. As shown in Figure 6, we found that LPO was clustered with its homologous protein derived from Equus caballus, Sus scrofa, Equus asinus and Diceros bicornis. NAMLAA had an aggregation with proteins coming from Equus quagga and Equus asinus. CD14 had a high sequence conservation in multiple horse species including Equus caballus, Equus asinus and Equus quagga. And Equus-derived B2U proteins were also closely gathered in one clade. In common, the candidate proteins were sequence-conservative in Equus genus, suggesting their conservative molecular function in horse milk.

Based on AlphaFold protein structure database,24 we predicted probable protein structure of four candidate proteins, depicting them as Figure 7A. Among them, B2U protein showed relatively simple structure, containing a α-helix and five β-sheets. We then performed codon optimization of NAMLAA, CD14 and LPO for next protein purification. The optimized open reading frame (ORF) of candidate proteins was cloned and fused with pGEX-4T-1 for prokaryotic expression. Further SDS-PAGE and Western Blot assays authorized the successful expression of four candidate proteins (Figure 7B). Purified proteins fused with GST tag were lyophilized to condense at the concentration of 10 mg/ml. We then performed an in vitro antimicrobial activity assay to assess the capacity of inhibition against multiple pathogenic microorganisms. In total, six strains including Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Staphylococcus epidermidis, Aspergillus niger and Candida albicans were chose for testing object. As shown in Figure 7C, four proteins all exerted significantly inhibitory effect to Candida albicans growth (Figure 7C-f), and they also lost their inhibitory resistance to Staphylococcus epidermidis and Staphylococcus aureus (Figure 7C-d). Obviously, we noticed that NAMLAA and CD14 showed marked inhibition to Pseudomonas aeruginosa and Aspergillus niger growth even stronger than positive control. In anti-Escherichia coli assay, only application of NAMLAA could inhibit pathogen growth. In particular, we found that NAMLAA showed stronger inhibitory effect to P. aeruginosa and E. coli than other proteins. CD14 conferred significant resistance to fungal pathogen, including A. niger and C. albicans. Overall, our results suggested that the usage of horse milk improved human immunity attributing to abundant accumulation of proteins with direct antimicrobial activity.

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Figure 6

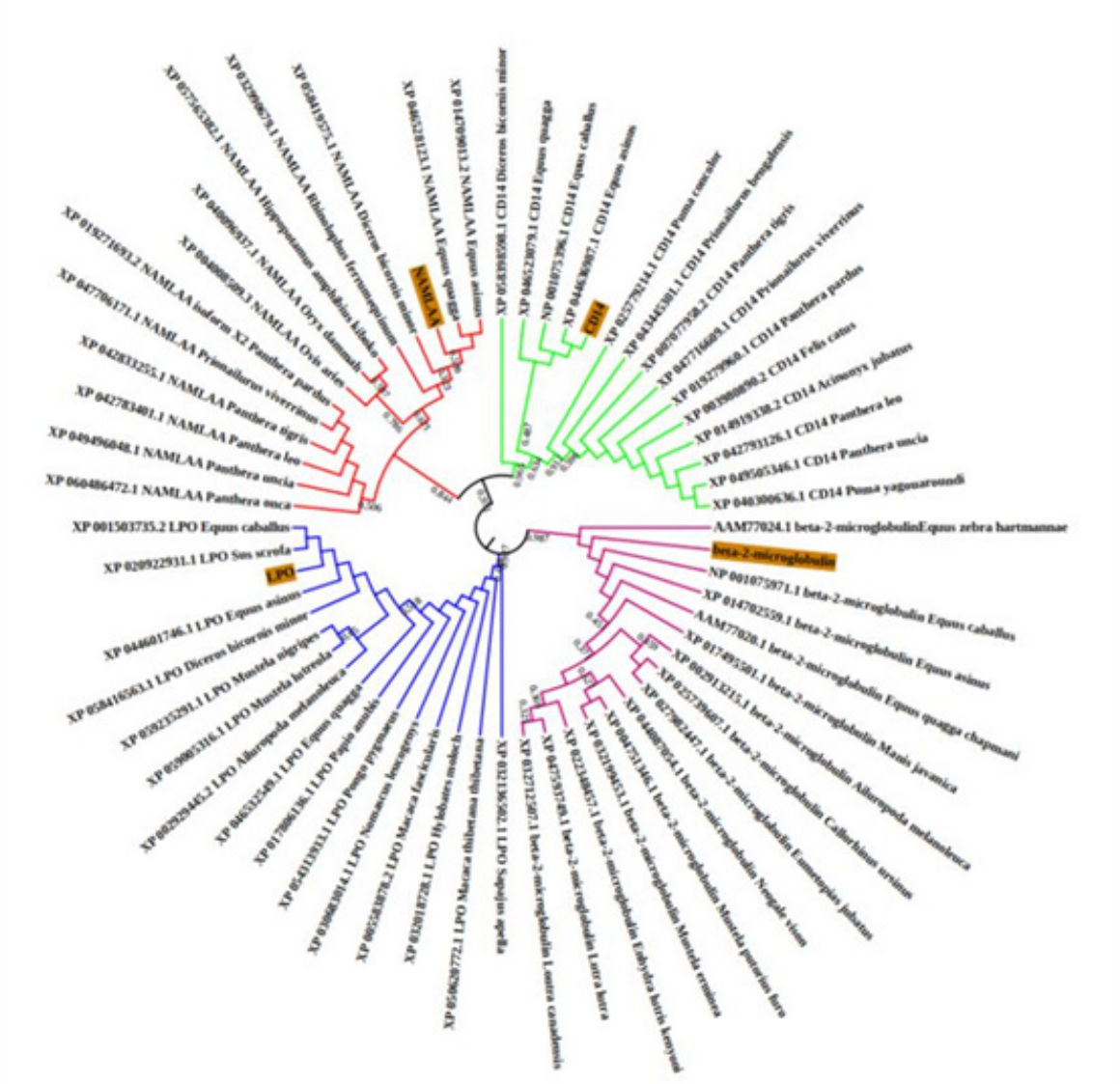

Figure 6. Phylogenetic tree of candidate proteins in horse milk.

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Figure 7

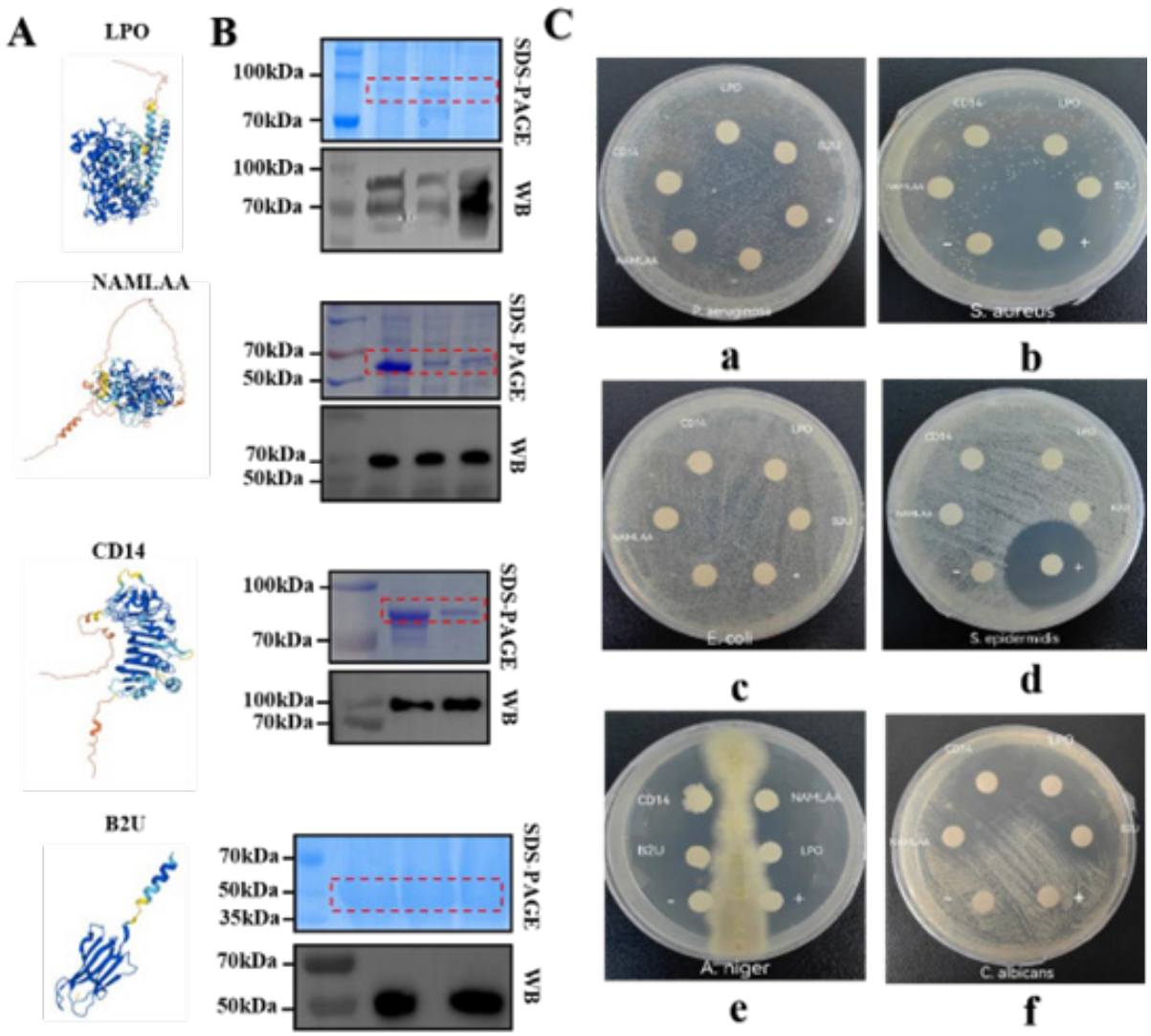

Figure 7. Candidate proteins in HW conferred antimicrobial activity for multiple pathogenetic microorganisms.
A: The probable protein structure model predicted by AlphaFold database. B: The authorization of SDS-PAGE and Western Blot for expression of candidate proteins. C: In vitro antimicrobial activity assay tested the inhibition effect of candidate proteins. Six testing strains were numbered, a, Pseudomonas aeruginosa; b, Staphylococcus aureus; c, Escherichia coli; d, Staphylococcus epidermidis; e, Aspergillus Niger; f, Candida albicans. The inhibitory effect of these pathogens was evaluated by paper disk method. GST protein was used as negative control and ampicillin sodium with the concentration of 50 μg/ml was used as positive control.

Elevated immunity in horse milk by four antimicrobial proteins

To further evaluate the resistant contributions of four antimicrobial protein in immunity, we exogenously treated RAW264.7 cells with four antimicrobial proteins. First, we examined the toxicity of four proteins on RAW264.7 cells. The results showed that exogenous treatment with antibacterial proteins at 5-20 mg/mL did not significantly inhibit cell viability, ensuring that none of the four antibacterial proteins were toxic to RAW264.7 cells (Figure S1A). H2O2 is a well-recognized inducer of apoptosis. Our research discovered that pre-treatment with the four antibacterial proteins reduced H2O2-induced apoptosis in RAW264.7 cells, with CD14 and PGL demonstrating significant inhibitory effects (Figure S1B). Further electron microscopy observation showed that PGL and CD14 proteins could inhibit H2O2-induced apoptosis, significantly reducing the apoptosis rate by approximately 25%~30% (Figure 8A-B). This supports the notion that PGL and CD14 proteins possess anti-apoptotic activity in RAW264.7 cells. To further investigate the immune effects of the core proteins LPO, PGL, CD14, and B2M on their host, we treated RAW264.7 cells with the core proteins LPO, PGL, CD14, and B2M, along with H2O2, for 1 hour. Samples were collected for RNA extraction and reverse transcription into cDNA. qRT-PCR was then used to measure the relative expression

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levels of immune-related genes AKT, BCL2, FOS, IL17, IL6, JAK2, NR3C1, PI3K, and TNFa (Figure 8C). These genes were relatively upregulated in RAW264.7 cells treated with H2O2. Importantly, CD14 and PGL notably induced the upregulation of immune-related genes, excluding TNFa, thereby enhancing the immune response in RAW264.7 cells.

Figure 8

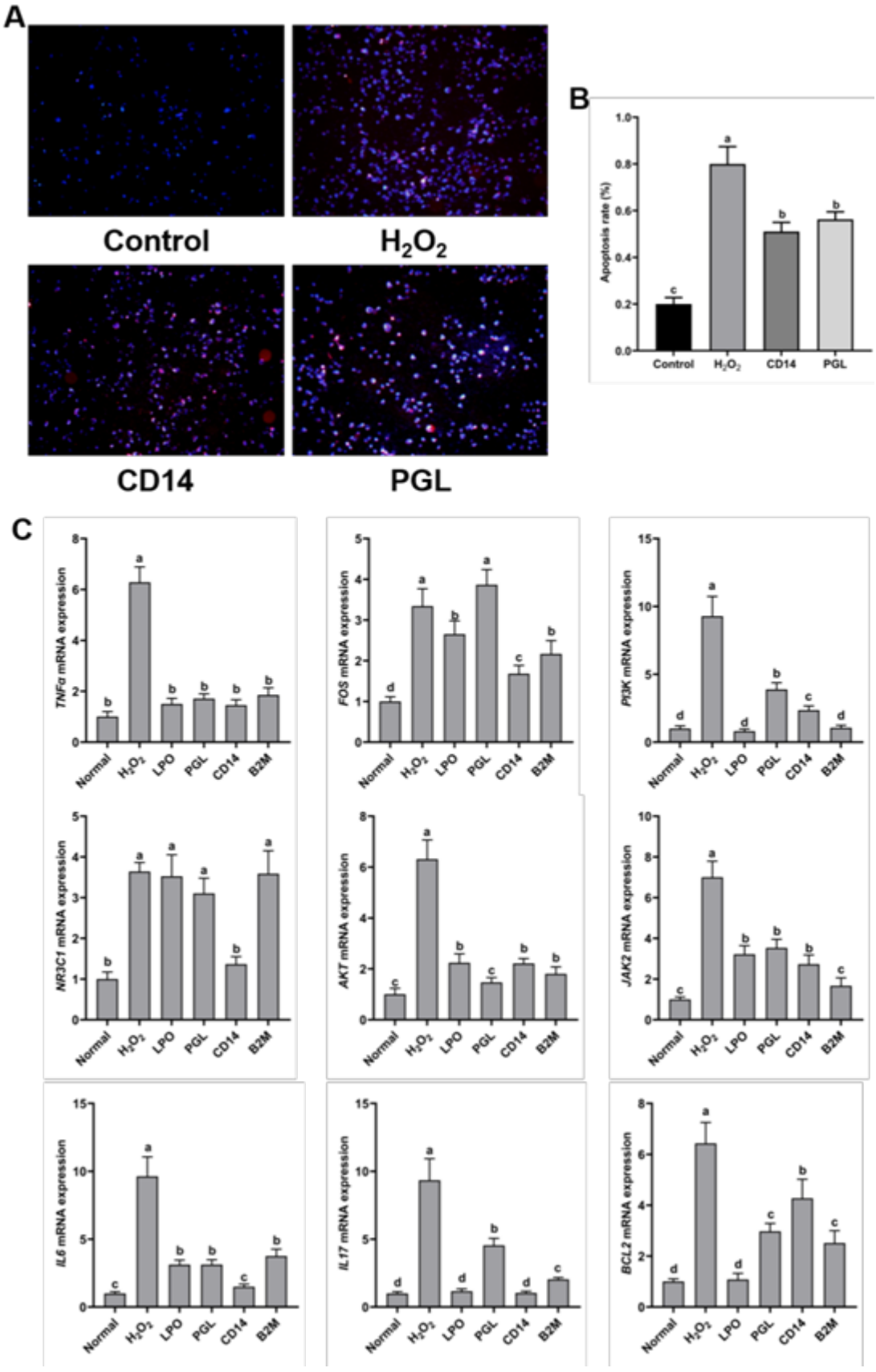

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Figure 8. Exogenous application of CD14 and PGL inhibited apoptosis and enhanced host immunity.
A: The apoptosis of horse milk treated with PGL and CD14 protein and hydrogen peroxide was observed, Bar=50 μM. B: Cell apoptosis statistics between treatments. Different letters represent significant differences between treatments. The relative expression levels of immune-related genes in LPO, PGL, CD14 and B2M treated horse milk were detected by qRT-PCR, normal indicates normalization. Error bars represent the standard deviation of the means. Different letters represent significant differences between treatments.

Exogenous treatment of PGL inhibited pathogen growth by interference of secondary metabolism.

Through scanning electron microscopy, we directly observed that PGL exogenous treatment for 30 minutes caused damage to the cell membrane structure of Escherichia coli and Pseudomonas aeruginosa (Figure 9A and 9D). However, the molecular mechanism by which it induces host immunity is still unclear. Therefore, we continued to explore the transcriptional changes in these two bacteria under PGL treatment by transcriptome analysis. First, PCA analysis revealed significant transcriptional changes in E. coli and P. aeruginosa after PGL treatment, resulting in 80.71% and 66.29% of transcriptomic differences, respectively (Figure 9B and 9E). This supports that PGL treatment caused major transcriptional changes in these bacteria. Volcano plots showed that, compared with the control, PGL treatment resulted in 258 upregulated and 392 downregulated differentially expressed genes in E. coli, and 825 upregulated and 954 downregulated differentially expressed genes in P. aeruginosa (Figure 9C and 9F). These differentially expressed genes were mapped to specific functional blocks through further GO and KEGG enrichment analysis, reflecting the transcriptional changes induced by PGL in host immunity.

Secondly, GO enrichment analysis indicated that the differentially expressed genes in E. coli were mainly distributed in biological process categories, with upregulated genes significantly enriched in intracellular biomolecule synthesis and downregulated genes mainly associated with transmembrane and localization (Figure 10A). These results suggest that PGL may interfere with the synthesis and transport of intracellular biomolecules, thereby affecting the growth of E. coli. In P. aeruginosa, the upregulated differentially expressed genes were predominantly enriched in cell metabolism and catalytic activity categories, while the downregulated genes were mainly related to intracellular components and membrane components (Figure 10B). This indicates that PGL might disrupt normal catalytic processes, affecting the integrity of intracellular components.

KEGG analysis revealed that the differentially expressed genes in E. coli were primarily enriched in pathways including oxidative phosphorylation, metabolic pathways, carbon metabolism, and biosynthesis of antibiotics. Notably, there was a majority of downregulated genes in pathways related to metabolism and antibody synthesis. These findings indicate that PGL inhibits metabolic processes and antibiotic synthesis in E. coli, leading to the suppression of pathogenic bacterial growth (Figure 11A). In P. aeruginosa, differentially expressed genes were significantly enriched in pathways related to biosynthesis of secondary metabolite, biosynthesis of amino acids and carbon metabolism, suggesting that PGL exogenous treatment disrupted the normal pathways of secondary metabolite synthesis, leading to inhibition of pathogenic bacterial growth (Figure 11B). In summary, these findings indicate that PGL suppresses the growth of pathogenic bacteria by disrupting the synthesis of secondary metabolites.

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Figure 9

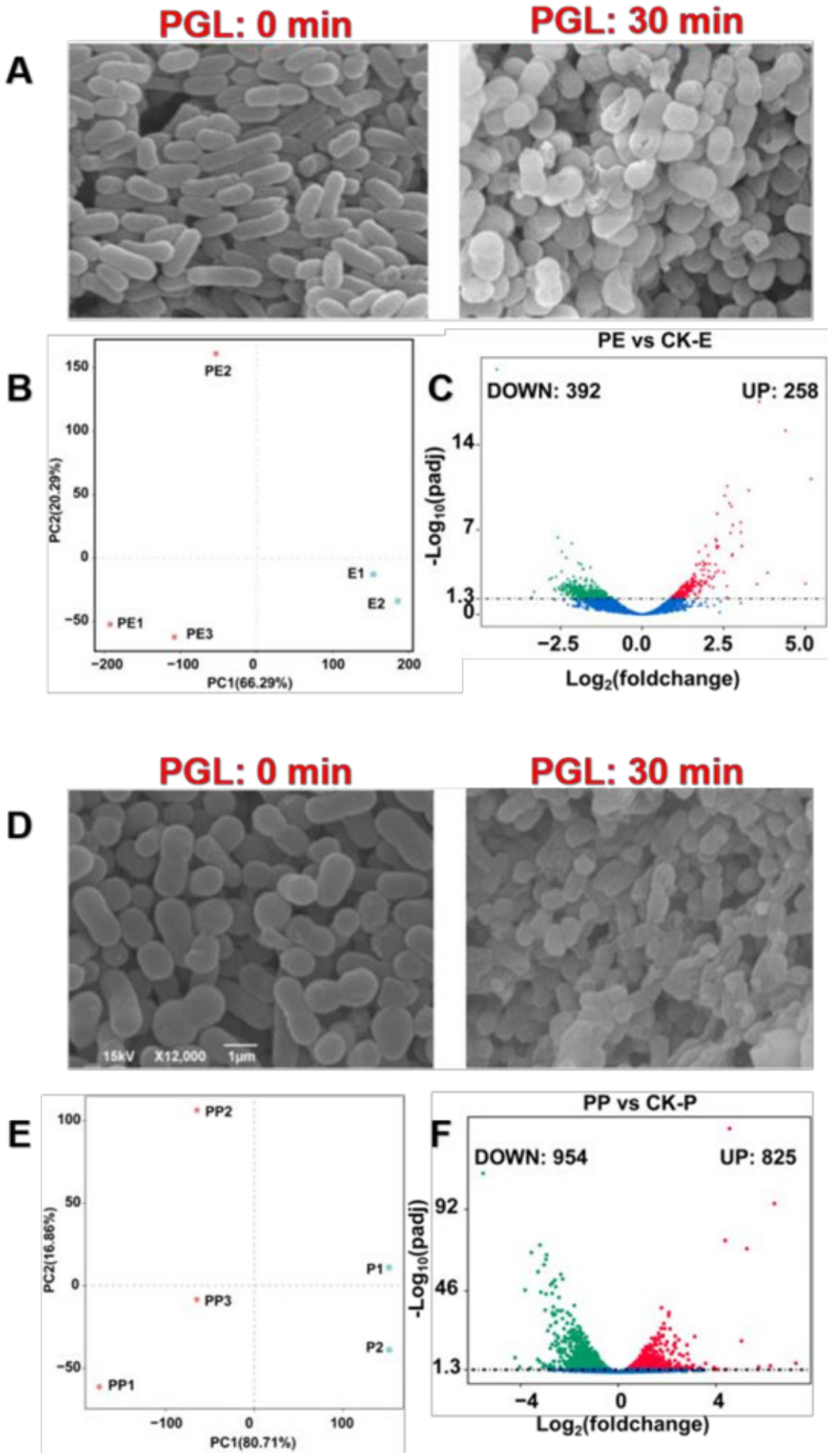

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Figure 9 A global view of transcriptome files of Escherichia coli and Pseudomonas aeruginosa treated by PGL.
A and D: Observations of the morphology of E. coli and P. aeruginosa after 30 minutes of PGL protein treatment under scanning electron microscopy. The left image shows the normal morphology of normal pathogens(× 12000), while the right image shows the morphology of pathogens(× 12000), after 30 minutes of protein PGL treatment. B and E: Principal component analysis of transcriptome files of Escherichia coli and Pseudomonas aeruginosa treated by PGL as well as controls. C and F: Identification of differentially expressed genes (DEGs) in PE vs CK-E and PP vs CK-P using volcano plots.

Figure 10

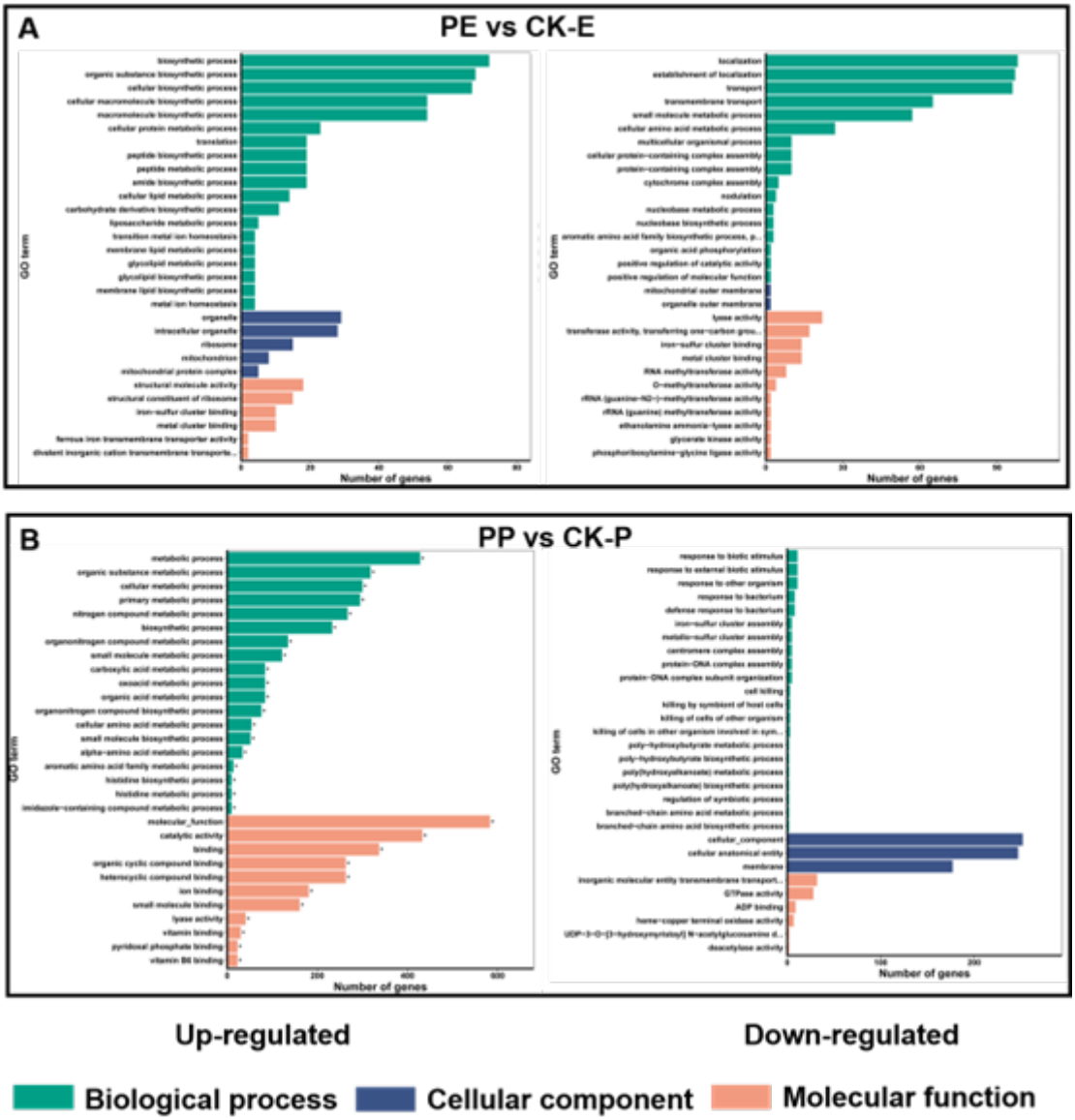

Figure 10. GO enrichment analysis illustrated the functional distribution of differentially expressed genes in PE vs CK-E and PP vs CK-P.
A: GO enrichment analysis of up-regulated and down-regulated DEGs in PE vs CK-E. B: GO enrichment analysis of up-regulated and down-regulated DEGs in PP vs CK-P.

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Figure 11

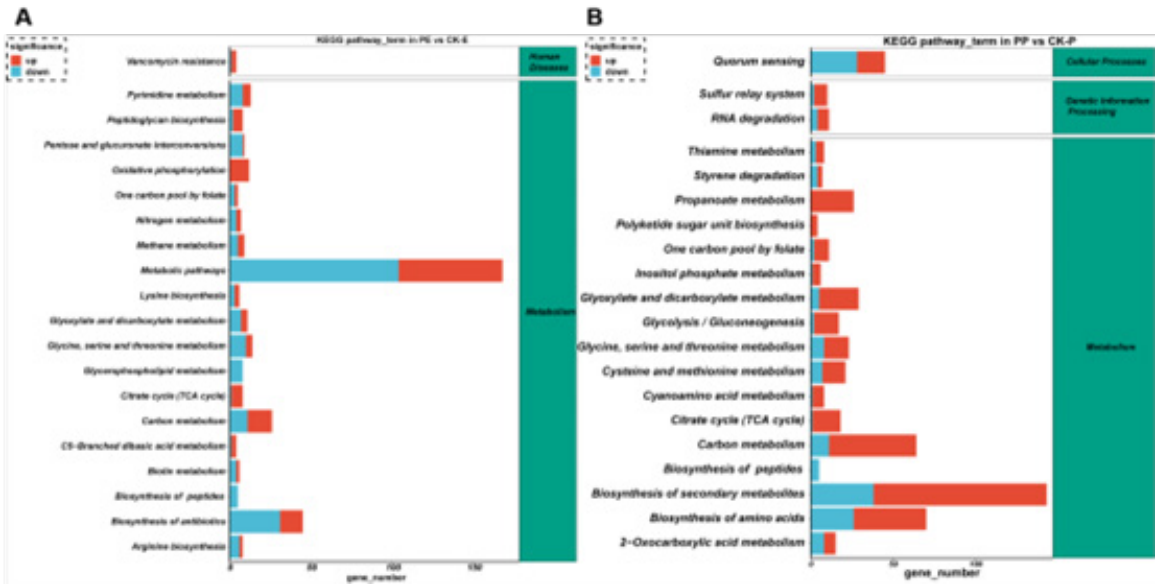

Figure 11. KEGG enrichment analysis illustrated the metabolic pathways of differentially expressed genes in PE vs CK-E and PP vs CK-P. A: KEGG enrichment analysis of DEGs in PE vs CK-E. B: KEGG enrichment analysis of DEGs in PP vs CK-P.

DISCUSSION

Researches on more nutritious and affordable milk source are of paramount importance. This study investigated the changes in protein and metabolites abundance in cow, goat, camel and horse milk by proteomic analysis. We summarized our acquired evidence depicting them as following conclusions. (a) Horse milk varied from other types of milk (cow, goat and camel) in protein composition. (b) Horse milk contained proteins distinguished from others with readily available for proteolysis, absorption and human immunity. (c) The increased content of proteins in horse milk contributed to elevate immune response and resistance to pathogenic disease. (d) The Identification of PGL protein conferred inhibitory effects to Escherichia coli and Pseudomonas aeruginosa by repressing biosynthesis of secondary metabolites.

Despite the low yield of horse milk because of lactation and longer gestation, its nutritional value was emphasized due to abundant whey protein and microelements.24 Our data verified that whey proteins occupied the most proportion in total proteins of horse milk among these four types of animal milk. Whey proteins were not only readily to digest and absorb, but also contained various amino acids that are essential for human, like lysine (Lys), leucine (Leu), glutamic acid (Glu) and aspartic acid (Asp), which attracted the preference of consumers.33 As a comparison, cow milk contained plentiful casein proteins compared to whey proteins (casein: whey =4: 1), especially A1-β casein, which has been reported to be directly associated with milk intolerance.34 Huge demands for cow milk around the world, though there still cannot be neglected the cow milk intolerance. The result highlighted the advantage of whey protein in horse milk compared to other types of milk. Besides, we also decipher the detailed protein compounds in four types of milk and found that horse milk contained more abundant proteins relevant to proteolysis, degradation and catalysis contributing to absorption and palatability of horse milk. Like casein proteins with large molecular weight were uneasy to degrade, which could be decomposed into small casein peptides by caseinase in milk.35 Our work proposed that horse milk was promising to be excellent alternative for easier assimilation in human daily diet.

Our data manifested that horse milk exerted the positive therapy effect to multiple human diseases. We noticed that the proteins relevant to shigellosis, salmonella infection, Escherichia coli infection, tuberculosis and thyroid cancer were highly expressed in HW among pairwise comparisons with other three types of milk (cow, goat and camel milk). Importantly, horse milk contained the largest amount of proteins involved multiple immune response signaling pathways, including PI3K-Akt signaling pathway, Rap1 signaling pathway, PPAR signaling pathway and MAPK signaling pathway, suggesting excellent immune benefits of horse milk. PI3K-Akt signaling pathway has been emphasized in many documents as essential hub governor to modulate apoptosis, inflammation and immunity events in organic development.38 Here, we found that the proteins involved in PI3K-Akt and MAPK signaling pathways were highly expressed in horse milk compared to other three types of milk. We suggested that some active

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protein ingredients in horse milk could trigger the immune response by targeting PI3K-Akt and MAPK signaling pathways. For instance, CD14 was consider to be important components that functioned as immune activation in PI3K-Akt and MAPK signaling pathways. Herein, our data highlighted that CD14 conferred the inhibitory effect to pathogens, implying its dual immune functions in response to pathogen infection. Besides, active proteins related to caffeine metabolism, biosynthesis of amino acids and glycolysis/gluconeogenesis in horse milk contributed to the flavor and taste of horse milk. Antifungal proteins play a vital role in horse milk, protecting newborn foals from infections and supporting the development of their immune systems through its strong antimicrobial and immunomodulatory effects. It not only directly kills pathogens but also enhances overall defense capabilities by regulating the host's immune response. In the early stages of life for newborn animals, antimicrobial peptides serve as critical natural defense mechanisms. Our data suggested that these proteins with highly expressed promoted the immune performance of horse milk and also proposed that some potential antimicrobial proteins functioned in therapy for bowel disease, which need to be further studied. Donkey belonged to Equus genus, previous studies demonstrated that donkey's milk contained many antimicrobial factors with inhibitory effect.31 Consistently, our work identified four candidate antimicrobial proteins with conservative evolutionary relationships in Equus genus, which conferred direct antimicrobial activity to inhibit the growth of Pseudomonas aeruginosa, Escherichia coli, Candida albicans and Aspergillus niger. These findings supported that milk derived from Equus genus possessed the advantage in the enhancement of immunity and wellness accounting for abundant antimicrobial proteins. Consistent with our finding,Guri et al.30 manifested that some active antibacterial proteins existed in horse milk that resulted in an inhibitory effect to Salmonella Typhimurium growth. It has been reported that the content of lysozyme protein in horse milk was higher than that in cow and human milk, which also implied higher antimicrobial activity in horse milk due to lysozyme capability.36 Additionally, whey protein, lactoferrin and casein protein have been implicated in functioning antimicrobial activity in confrontation with various pathogens. Enhancement of immune resistance by horse milk diet has been reported in recent years, especially in the treatment of tuberculosis and chronic ulcer.37 Our identification of CD14, LPO, PGL and B2M conferred directly inhibitory effect to multiple pathogens, like E. coli and P. aeruginosa. Moreover, exogenous application of them enhanced host immune response by elevating immune-related genes like AKT, PI3K, IL6, IL17 and so on, highlighting their contributions to host immunity in horse milk. Besides, These antimirobial proteins in horse milk work synergistically to effectively inhibit the growth and reproduction of various pathogenic microorganisms, prolong the shelf life of mare milk, and reduce the risk of pathogenic contamination. Because of the natural antibacterial activity of these proteins, mare milk can maintain a relatively long shelf life and a low rate of pathogenic contamination without excessive processing.

Taken together, our work highlighted the advantages of horse milk about immune improvement and nutrient composition, which would be useful for further medical application and alternative to cow milk allergy. Our findings expected to provide a valuable insight to horse milk compounds and contributed to the promotion of horse milk.

Author Contributions: Conceptualization, G.X.; validation, N.C, D.H. and K.G.; data curation, C.X.; writing—original draft preparation, C.X.; writing—review and editing, C.X. and G.X. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data used to support the findings of this study can be made available by the first author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

In this article, the abbreviation "horse milk" is used as "HM"; The abbreviation for "camel milk" is "CM"; The abbreviation for "goat milk" is "GM"; "Cow milk" is abbreviated as "MW".

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