Proteomic approach-based comparison of metabolic pathways and functional activities of whey proteins derived from Guishan and Saanen goat milk

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ABSTRACT

Guishan goats, a unique goat breed in Yunnan Province, have a long history and representation, but their whey protein and function remain unclear. In this study, we carried out a quantitative analysis of the Guishan and Saanen goat whey proteome using a label-free proteomic approach. A total of 500 proteins were quantified from the 2 kinds of goat whey proteins, including 463 common proteins, 37 uniquely expressed whey proteins (UEWP), and 12 differentially expressed whey proteins (DEWP). Bioinformatics analysis indicated that UEWP and DEWP were mainly involved in cellular and immune system processes, membrane, and binding. In addition, UEWP and DEWP in Guishan goats participated primarily in metabolism and immune-related pathways, whereas Saanen goat whey proteins were associated mostly with environmental information processing-related pathways. Guishan goat whey promoted the growth of RAW264.7 macrophages more than Saanen goat whey, and significantly reduced the production of nitric oxide in lipopolysaccharide-stimulated RAW264.7 cells. This study provides a reference for further understanding these 2 goat whey proteins and finding functional active substances from them.

Key words: goat whey protein, label-free quantification, biological functions, immunomodulatory

INTRODUCTION

Goat milk, compared with cow milk, is getting more attention because of its digestibility, softer texture, and lower allergenicity (Ceballos et al., 2009; Izquierdo-González et al., 2019; Sun et al., 2019). Goat milk contains various nutrients, including protein, fat, lactose, vitamins, and minerals, with protein being the main component. Casein makes up 70% of the goat milk proteins, whereas whey and milk fat globule membrane proteins constitute 25 and 5%, respectively (Chen et al., 2019a). Although the whey protein has a lower proportion in goat milk, it is recognized as having a wide spectrum of biological functions, such as antibacterial, antioxidant, and immunomodulatory activities (Mann et al., 2014; Sun et al., 2020b; Campos et al., 2022). In addition, whey contains a variety of immune-related proteins, such as β-LG, α-LA, serum albumin, immunoglobulin, lactoferrin, and lactoperoxidase (Madureira et al., 2007; Kim et al., 2019). Lee et al. (2019) found that whole and whey bovine colostrum have anti-inflammatory, antiadipogenic, and antioxidation effects on LPS-stimulated 3T3-L1 cells. Interestingly, Yamaguchi et al. (2009) found that α-lactalbumin may contribute to its analgesic and anti-inflammatory effects by inhibiting IL-6, COX-2, and PLA2. β-Lactoglobulin peptide is digested further by intestinal symbiotic bacterial proteases to release anti-inflammatory IL-10 from mouse spleen cells (Prioult et al., 2004). Lactoferrin, a component of innate immunity, can reduce the expression of IL-6, MCP-1, and TNF-α on LPS-induced uveitis in mice, and prevent experimentally induced vascular leakage and inflammation (Talukder and Harada, 2007). In addition, human immunoglobulins can reduce oxidative rupture of human monocytes and downregulate TNF-α and IL-6 (Wolf et al., 1996).

The whey protein in goat milk is composed of different proteins, the amount and composition of which depend on a variety of factors, including species, lactation phase, region, and feeding conditions (Costa et al., 2014; Argenta and Scheer, 2019). Proteomics is based mainly on liquid chromatography (LC)-tandem mass spectrometry (MS/MS) technology to systematically identify and characterize the structure, function, activity, quantity, and molecular interactions of proteins (Gerber et al., 2003). In recent years, many studies have used proteomic methods to reveal the differences in whey proteins among different goat species, their lactation period, region, and feeding conditions. Anagnostopoulos et al. (2016) identified 486 and 595 proteins in the milk of 2 indigenous Greek goat breeds.
Sun et al. (2020b) used proteomics to analyze the differences in whey protein and found 314 and 524 whey proteins from Xinong Saanen goat colostrum and mature milk, respectively. Lu et al. (2018) reported 213 proteins using a label-free proteomic technique in the serum of goat tank milk and indicated a high level of complement proteins in the goat milk serum. A total of 550 whey proteins were quantified in Saanen goat milk from 3 provinces in China using a data-independent acquisition technique (Zhao et al., 2021). Sun et al. (2020a) used the isobaric tags for relative and absolute quantitation proteomic technique to analyze the differentially expressed whey proteins, and a total of 165 whey proteins were quantified, 114 of which differed significantly in abundance in Guanzhong goat milk and Holstein cow milk. Li et al. (2020) used a label-free proteomics approach to identify and quantify 288 and 287 whey proteins in the colostrum and mature milk of donkeys, respectively. Zhang et al. (2019) reported that 216 whey proteins were identified in different yields from donkeys using a label-free comparative proteomics approach.

Guishan goat is a unique breed in the Yunnan Province of China with a long history, black skin, strong stress resistance, less disease, coarse food tolerance, and moderate milk yield (Li et al., 2000). Guishan goat milk was often used to supplement nutrition for the weak. Saanen goats were introduced to Yunnan Province from Shaanxi Province in 2004; they inhabit the same environment as Guishan goats and show a greater milk yield (Ang and Li, 2004). Currently, researchers have done a lot of work on meat quality (Chen et al., 1996), genetics (Fan et al., 2013), breeding (Li, 2019), and reproduction (Guan et al., 2010; Shao et al., 2010) in Guishan goats; however, studies on their milk have not been reported. In addition, as a unique goat in Yunnan, it is unclear how the whey protein and partial biological functions of Guishan goats are different from those of Saanen goats. Thus, this study aimed to use a label-free proteomics approach to characterize and explore differences in whey proteins of Guishan and Saanen goats. Furthermore, bioinformatics was used to analyze the uniquely expressed whey protein (UEWP) and differentially expressed whey protein (DEWP) of these 2 kinds of goat milk. Finally, the effects of both goat whey proteins on nitric oxide (NO) production in LPS-stimulated RAW264.7 cells were determined.

**MATERIALS AND METHODS**

**Materials**

Acetic acid was purchased from Tianjin Fengchuan. SDS, dithiothreitol, and Tris were purchased from Solarbio. A bicinchoninic acid assay kit and NO determination kit were purchased from Beyotime Technology. Dulbecco’s Modified Eagle Medium, PBS (pH 7.4), fetal bovine serum, streptomycin, penicillin, and PBS (without calcium and magnesium) were purchased from VivaCell Biotechnology. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide, and LPS were obtained from Solarbio. Other liquid pure reagents were purchased from Merck.

**Sample Collection and Preparation of Whey Protein**

All experiments involving animals were performed in accordance with the Guidelines for the Care and Use of Experimental Animals of Yunnan Agricultural University and approved by the Animal Ethics Committee of Yunnan Agricultural University. No adverse events were observed.

Morning milk (at ~0730 h) samples were collected from 27 Guishan and 36 Saanen goats from a dairy farm in Shilin City, Yunnan Province, China. Twenty-seven Guishan goats were divided into 3 groups, with 9 goats in each group. Thirty-six Saanen goats were also divided into 3 groups, with 12 goats in each group. The goats were 1 to 1.5 yr old. They had similar growth conditions and good health. They were all milked mechanically at the same time. Approximately 500 mL of milk was collected from each goat. Three samples from each goat group were centrifuged at 4°C and 4,000 \( \times g \) for 15 min to remove fat (TGL20M, Changsha Maijiasen Instrument & Meter Co., Ltd.). Whey protein was obtained according to the procedure described by Huppertz et al. (2004). Briefly, the skim milk samples were adjusted to pH 4.6 with 33% acetic acid and kept at 25°C for 15 min. The mixture was subsequently centrifuged at 20°C and 4,000 \( \times g \) for 25 min to remove the casein. Thereafter, the supernatant containing the whey proteins was collected, and the protein concentration was determined using the bicinchoninic acid assay kit, with BSA as the standard. The whey proteins were stored at −80°C until analysis.

**Milk Composition Analysis**

The individual milk samples were analyzed using a previously described method by Verma et al. (2020). The fat, protein, lactose, and SNF of fresh goat milk (~5 mL) were determined using infrared spectroscopy (MilkoScan FT120, indiFOSS) at 37°C.

**SDS-PAGE**

Each sample was mixed with 5× loading buffer (Solarbio) at a ratio of 4:1 and boiled for 10 min. Proteins...
were loaded and separated on 12% SDS-PAGE gel (BioRad Mini protein) at 20 μg protein/well (50 V for 30 min and 120 V for 90 min). After electrophoresis, the gel was fixed with 30% methanol for 30 min, and the separated proteins were visualized by staining with Coomassie Brilliant Blue (CBB-R250, DA0201; Beijing Leagene Biotech).

**Trypsin Digestion of Whey Fractions**

A total of 200 μg of whey proteins was mixed with 30 μL of buffer consisting of 4% SDS, 100 mM dithiothreitol, and 150 mM Tris-HCl (pH 8.0). Small molecular impurities were removed by repeat ultrafiltration (microcontroller units, 10 kD). Next, 100 μL of iodoacetamide (100 mM iodoacetamide in uric acid buffer) was added to block reduced cysteine residues. The filters were washed 3 times with 100 μL of uric acid buffer and then twice with 100 μL of 25 mM NH₄HCO₃ buffer. Last, the protein suspensions were digested with 4 μg of trypsin (Promega) in 40 μL of 25 mM NH₄HCO₃ buffer overnight at 37°C, and the resulting peptides were collected as a filtrate. The peptides of each sample were desalted on C18 cartridges (standard-density Empore SPE Cartridges C18; bed i.d., 7 mm; volume, 3 mL; Sigma), concentrated using vacuum centrifugation, and reconstituted in 40 μL of 0.1% (vol/vol) formic acid. The peptide content was estimated using a UV light spectral density at 280 nm with an extinction coefficient of 1.1 of 0.1% (g/L) solution that was calculated.

**Identification and Quantitative Analysis of Whey Proteins Using LC-MS/MS**

The LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) coupled with Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 60 min. The peptides were loaded onto a reverse-phase trap column (Thermo Scientific Acclaim PepMap100, 100 μm × 2 cm, nanoViper C18) connected to a C18 reverse-phase analytical column (Thermo Scientific Easy Column; length, 10 cm; i.d., 75 μm; 3 μm resin) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (84% acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min controlled by IntelliFlow technology. The mass spectrometer was operated in a positive ion mode. The instrument was run by enabling the peptide recognition mode. The MS raw data for each sample were combined and searched using MaxQuant version 1.5.3.17 software for identification and quantitation analysis. Full-scan MS and MS/MS analyses allowed mass tolerances of 6 ppm and 20 ppm for fragment ions, respectively. The maximum error detection rate for proteins and peptides was set to 0.01 (Li et al., 2020). Whey protein was quantified using label-free quantitation (Luber et al., 2010). Protein abundance was calculated from normalized spectral protein intensity label-free quantitation (LFQ) intensity (Dozio and Sanchez, 2017). The abundance of each peptide was obtained by integrating the peak area. The abundance of identified peptides was normalized to the abundance of each unique peptide. The abundance of identified proteins was calculated by summing all ion abundances from the unique normalized peptides (Dozio and Sanchez, 2017).

**Multivariate and Bioinformatic Analyses**

Proteome Discoverer 2.2 software (Thermo Scientific) was used to analyze the raw data files of MS/MS by searching against the database (file: uniprot-capa hircus_35497_20210419_scj.fasta). The peptide and fragment mass tolerance values were ±20 ppm and ±0.1 Da, respectively. Trypsin was set as the cleavage enzyme with a maximum missed cleavage of 2. The peptides were identified at a false discovery rate of ≤0.01. The DEWP were selected by an absolute value of fold change in protein abundance of >1.5 or <0.66 (Guichon goat to Saanen goat). The online database for annotation, visualization, and integrated discovery (DAVID; https://david.ncifcrf.gov/; Sherman et al., 2022) was used for the analysis of functional categories, Gene Ontology (GO; http://geneontology.org/) classification, and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) pathways.

**RAW264.7 Cell Viability Assay**

The RAW264.7 murine macrophage cell line (an Abelson leukemia virus-induced tumor cell line) was purchased from Cellcook. RAW264.7 cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ (CB170, BINDER). Dulbecco’s Modified Eagle Medium with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin was used as the culture medium. Cell viability of RAW264.7 was determined using MTT colorimetry (Wen et al., 2021). Briefly, RAW264.7 cells were inoculated into 96-well plates at a density of 6 × 10⁴ cells per well and stabilized for 12 h. The medium was removed, and 100 μL of sample at different concentrations was added (dissolved in medium) for 20 h. Next, 20 μL MTT solution (dissolved in PBS at a concentration of 5 mg/mL) was added to each well and incubated for 4 h under the same conditions. After removing the medium, 150 μL of dimethyl sulfoxide
was added to each well and the whole plate was shaken at 25°C for 10 min until the solid material was dissolved completely. Absorbance was measured at 490 nm using a microplate reader (Multiskan MK3, Thermo). Cell viability was calculated as

\[ \text{cell viability (\%) = } \left( \frac{A_S}{A_C} \right) \times 100, \]

where \( A_S \) is the optical density of the sample and \( A_C \) is the optical density of the control.

**LPS Treatment and Determination of NO Production**

The LPS induction and determination of NO production were conducted as reported previously (Wen et al., 2021). In brief, RAW264.7 cells were seeded into 96-well plates (1.5 \( \times \) 10^4 cells per well) and stabilized for 12 h. The medium was removed, and 90 \( \mu \)L of sample at various concentrations was added for 12 h, followed by incubation in the absence or presence of LPS (1 \( \mu \)g/mL final concentration) for 8 h. The cells without treatment were used as the control; LPS solution was added as a negative control. The supernatants were collected, and NO was determined with Griess reagent (Schulz et al., 1999).

**Statistical Analysis**

All experiments had 3 repetitions. The data are expressed as mean ± standard deviation. SPSS statistics version 23.0 (IBM) was used for statistical significance (Tukey’s honestly significant difference test, \( P < 0.05 \)). Graphs were made using GraphPad Prism version 8.0.1 and Adobe Illustrator CS6.

### RESULTS AND DISCUSSION

#### Proximate Component Analysis of Guishan and Saanen Goat Milk

The fat, protein, lactose, TS, and SNF content of Guishan and Saanen goat milk were determined (Table 1). The mean values of fat, protein, lactose, TS, and SNF in Guishan goat milk were 4.05, 3.92, 4.73, 13.37, and 9.54%, whereas those in Saanen goat milk were 4.22, 3.39, 4.87, 13.03, and 9.04%, respectively. The lactose content of Guishan goat milk was similar to that of Saanen, and the fat content was less than that of Saanen. However, the protein, TS, and SNF contents in Guishan goat milk were greater than those in Saanen. This may be related to the high milk yield of Saanen goats and relatively lower content of nutrients in milk. Verma et al. (2020) found that the mean fat, SNF, and protein were 4.10, 9.06, and 3.65%, respectively, in the goat milk from diverse agroclimatic regions of India. The fat content of Guishan goat milk was similar, and the protein and SNF values were greater than those of Indian goat milk from diverse agroclimatic regions. Varieties such as Jamunapari, Jakrana, Surti, and Malabar are known for their high milk yield (Verma et al., 2020). Himalayan goat (4.31%), Ganya goat (4.01%), and Gadi goat (3.91%) milks have a greater protein content. It is worth noting that the protein content of Guishan goat milk was close to that of Gadi and Ganya goat milk. The SNF in the milk of Guishan and Himalayan goats (9.61%) and Gadi goat (9.53%) was similar.

#### Identification and Quantification of the Whey Proteome in Guishan and Saanen Goat Milk

The SDS-PAGE showed the major bands of whey proteins in Guishan and Saanen goat milk, which indicated the effective extraction of whey proteins (Figure 1). Moreover, there were a few other proteins such as casein. Furthermore, we used label-free proteomics to identify and quantify 481 and 482 whey proteins in Guishan and Saanen goat milk, respectively. As shown in Figure 2A, there were 463 common whey proteins in the 2 goat milk proteins, and there were 18 and 19 UEWP identified in Guishan and Saanen goat milk, respectively. Of the common whey proteins, we found that β-LG (P02756), glycosylation-dependent cell adhesion molecule 1 (A0A452EGX6), α-LA (A5JSS8), and serum amyloid A protein (A0A452ECD3) were highly abundant in Guishan and Saanen goat whey proteins. Notably, polymeric immunoglobulin receptor (A0A452EDG7) and lactotransferrin (A0A452EYH6) were also highly expressed in the whey proteins of both goats. Polymeric immunoglobulin receptors play a key role in the immune system (Li et al., 2020), which can bind to polymerized IgA and IgM, and transport them across cell membranes to carry out immune functions (Kaetzel, 2005). In addition, casein (αS1-CN, αS2-CN, β-CN, and κ-CN) and blood-derived proteins (albumin, serum transferrin, complement C3) were also found in the whey protein components of these 2 goats. Ji et al. (2017) also found residual casein from extracted bovine whey protein using the proteomic method, which might...
be related to casein residues during the extraction of whey protein.

**Uniquely Expressed Whey Proteins in Guishan and Saanen Goat Milk**

The label-free proteomic approach has high sensitivity in identifying proteins and can find unique proteins from samples. As shown in Table 2, 18 UEWP in Guishan goat milk mainly included vacuolar protein sorting 13 homolog C (A0A452FH58), heat shock-related 70 kDa protein 2 (A0A452E495), CCT-β (A0A452G4D6), and myosin light chain 6 (A0A452DSV3). Vacuolar protein sorting 13 homolog C (VPS13C, A0A452FH58) was a galectin-12 binding protein, which plays an important role in protein quality control (Yang et al., 2016). In the cell, VPS13C and galectin-12 colocalize, which is closely associated with lipid droplets and lysosomes. In addition to VPS13C, CCT1 and CCT8 subunits of protein TRiC/CCT, and HSP70 family member HSC70 are also known chaperones that promote galectin-12-binding protein folding (Rothman and Schekman, 2011). The extracellular heat shock-related 70 kDa protein has diverse immunomodulatory properties, can show different activities in airway inflammatory processes and asthma, and can be used as a proinflammatory trigger or anti-inflammatory agent (Shevchenko et al., 2021). In addition, heat shock related 70 kDa protein 2 played a tumor suppressive role in pancreatic cancer, played a regulatory role in ovarian follicle development, and served as a biomarker and potential drug target for the interaction between serum proteins and ischemic stroke (Turek-Jakubowska et al., 2017; Xu et al., 2017; Chen et al., 2019b). Myosin light chain 6 (A0A452DSV3, MYL6B) was expressed at greater levels in the homozygote favorable to tenderness (CC) for UOGCAST, which contributed to meat tenderness (Rosa et al., 2018). CCT-β (A0A452G4D6, CCT2) played an important role in regulating follicle development and ultimately oocyte quality (Douville and Sirard, 2014). Mimecan (G1DFQ3, OGN) was an appetite regulator and satiety hormone (Page, 2015; Su et al., 2021). The tumor suppressor activity of the first identified member of the family, TSC22 domain family member 1 (A0A452EBM0, TSC22D1), has been...
extensively studied (Dragotto et al., 2019). The UEWP in Saanen goat milk consisted primarily of the proteasome 26S subunit, ATPase 5 (A0A452FDS2), thrombomodulin (A0A452E649), NAD(P)(+)–arginine ADP-ribosyltransferase (A0A452DPV2), and transcobalamin 1 (A0A452F261). In addition to its anticoagulant effect, thrombomodulin had protective effects on many organs, such as anti-inflammatory, angiogenetic, and cell proliferative (Oda et al., 2022). CD5 antigen-like (A0A452EJM6) was involved in the apoptotic process and inflammatory response, and it is also detected in cow, sheep, and yak whey proteins (Lu et al., 2018). Thrombomodulin (A0A452E649) is a specific endothelial cell receptor that forms a complex with thrombin, which was responsible for the conversion of protein C to activated protein C (protein Ca). It has also been found in the whey protein of Murrah buffalo (Maity and Ambatipudi, 2019). Some unique and highly expressed whey proteins, especially in Guishan goats, are involved in providing immunity, antimicrobial, and anti-inflammatory effects, and in regulating some metabolic diseases.

### Differentially Expressed Whey Proteins in Guishan and Saanen Goat Milk

Differentially expressed whey proteins were selected from the 463 common whey proteins found in Guishan and Saanen goat milk. A threshold for screening the DEWP was set to $P < 0.05$, with a fold change of either >1.50 or <0.66. As shown in Table 3 and Figure 2B, 12 DEWP were identified in Guishan and Saanen goat milk, including 9 higher and 3 lower DEWP in Guishan goat milk compared with Saanen goat milk.

### Table 2. Uniquely expressed whey proteins in the whey of Guishan (G) and Saanen (S) goat milk

<table>
<thead>
<tr>
<th>UniProt accession no.</th>
<th>Description</th>
<th>Gene name</th>
<th>Coverage (%)</th>
<th>Molecular weight (kDa)</th>
<th>LFQ intensity</th>
<th>Breed</th>
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<tr>
<td>A0A452FH58</td>
<td>Vacuolar protein sorting 13 homolog C</td>
<td>VPS13C</td>
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<td>415.11</td>
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<td>A0A452E495</td>
<td>Heat shock–related 70-kDa protein 2</td>
<td>HSPA2</td>
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<td>69.824</td>
<td>124,439,500</td>
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<tr>
<td>A0A452G4D6</td>
<td>CCT-β</td>
<td>CCT2</td>
<td>4.1</td>
<td>57.461</td>
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<td>A0A452DSV3</td>
<td>Myosin light chain 6</td>
<td>MYL6B</td>
<td>25</td>
<td>17.98</td>
<td>75,280,500</td>
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<td>A0A452EB8</td>
<td>Mitochondrial carrier 2</td>
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<td>8.2</td>
<td>32.161</td>
<td>43,200,000</td>
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<tr>
<td>A0A452FJ8</td>
<td>Fibrillin 1</td>
<td>FBN1</td>
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<td>31.211</td>
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<td>G</td>
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<tr>
<td>G1DFQ3</td>
<td>Mmecan</td>
<td>OGN</td>
<td>5.7</td>
<td>34.042</td>
<td>34,755,000</td>
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<tr>
<td>A0A452EBM0</td>
<td>TSC22 domain family member 1</td>
<td>TSC22D1</td>
<td>14.6</td>
<td>15.68</td>
<td>29,199,500</td>
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<td>A0A452DZI2</td>
<td>Family with sequence similarity 234 member A</td>
<td>FAM234A</td>
<td>4.7</td>
<td>58.585</td>
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<td>A0A452E3F1</td>
<td>Histone H3</td>
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<td>A0A452F0H2</td>
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<td>25,500,150</td>
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<td>A0A452FRB9</td>
<td>Uncharacterized protein</td>
<td>HSPA1L</td>
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<td>70.732</td>
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<td>A0A452E573</td>
<td>ATP synthase subunit β</td>
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<td>A0A452EBK8</td>
<td>RAB11A, member RAS oncogene family</td>
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<td>A0A452F6J1</td>
<td>Adhesion G protein-coupled receptor L2</td>
<td>ADGRL2</td>
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<td>131.84</td>
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<td>Transmembrane p24 trafficking protein 7</td>
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<td>25.335</td>
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<td>A0A452FB52</td>
<td>DNAJ heat shock protein family (Hsp40) member B9</td>
<td>DNAJB9</td>
<td>6.3</td>
<td>25.814</td>
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<td>A0A452FDS2</td>
<td>Proteosome 26S subunit, ATPase 5</td>
<td>PSMC5</td>
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<td>45.933</td>
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<td>CD5 antigen-like</td>
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<td>42.74</td>
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<td>60.569</td>
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<td>TCN1</td>
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<td>51.223</td>
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<td>227.22</td>
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<td>Polypeptide N-acetylgalactosaminyltransferase</td>
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<td>101.6</td>
<td>38,671,000</td>
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<td>Postglycosylphosphatidylinositol attachment to proteins 6</td>
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<td>A0A452EQT0</td>
<td>Fc fragment of IgE receptor Ig</td>
<td>FCER1G</td>
<td>11.8</td>
<td>8.5091</td>
<td>36,357,666.67</td>
<td>S</td>
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<tr>
<td>A0A452DV97</td>
<td>Lymphocyte cytosolic protein 1</td>
<td>LCP1</td>
<td>5.7</td>
<td>70.178</td>
<td>27,470,000</td>
<td>S</td>
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<tr>
<td>A0A452E5S1</td>
<td>Tetraspanin</td>
<td>TSPAN6</td>
<td>5.7</td>
<td>27.544</td>
<td>26,375,000</td>
<td>S</td>
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<tr>
<td>A0A452G1E4</td>
<td>Bone morphogenetic protein 3</td>
<td>BMP3</td>
<td>4.7</td>
<td>52.374</td>
<td>19,796,000</td>
<td>S</td>
</tr>
<tr>
<td>A0A452FCW7</td>
<td>Neural proliferation, differentiation, and control 1</td>
<td>NPDC1</td>
<td>7.2</td>
<td>34.186</td>
<td>19,643,666.67</td>
<td>S</td>
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<tr>
<td>A0A452E545</td>
<td>Angiotensinogen</td>
<td>AGT</td>
<td>6.7</td>
<td>51.319</td>
<td>18,971,500</td>
<td>S</td>
</tr>
<tr>
<td>A0A452E3P2</td>
<td>Proteoglycan 4</td>
<td>PRG4</td>
<td>3.1</td>
<td>47.088</td>
<td>18,219,000</td>
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<tr>
<td>A0A452F73</td>
<td>Creatine kinase</td>
<td>CKM</td>
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<td>43.011</td>
<td>17,941,750</td>
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<tr>
<td>A0A452EC39</td>
<td>IL-6 receptor</td>
<td>IL6R</td>
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<td>48.043</td>
<td>16,220,550</td>
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</tr>
<tr>
<td>A0A452G696</td>
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<td>PROM1</td>
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<td>96.608</td>
<td>12,787,000</td>
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</tr>
<tr>
<td>A0A452G8E8</td>
<td>Protein–tyrosine–phosphatase</td>
<td>PTPRQ</td>
<td>1</td>
<td>258.06</td>
<td>8,509,850</td>
<td>S</td>
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</table>

1LFQ = label-free quantitation.
tor, acyl-CoA binding protein, CD320 antigen, and Na(+)−dependent phosphate cotransporter 2B were greater in Guishan goat milk, whereas the expression levels of Serpin family F member 2, leucine rich α-2-glycoprotein 1, delta/notch-like EGF repeat-containing protein were less in Guishan goat milk than in Saanen goat milk. Notably, SH3 domain-binding glutamic acid-rich–like protein was found to be upregulated 2.8-fold in Guishan whey proteins compared with Saanen. SH3 domain-binding glutamic acid-rich–like protein is important in maintaining cytoskeletal integrity and cellular viability of neonatal rat ventricular cardiomyocytes (NRVCM) through modulation of SRF/YAP signaling pathways (Deshpande et al., 2021). In addition, tetraspanin (A0A452FHI7) and leucine-rich α-2-glycoprotein (A0A452EZ30) have also been reported to be highly expressed in donkey colostrum and cow milk, respectively (Li et al., 2020; Sun et al., 2020a). Tetraspanin (A0A452FHI7) is involved in protein transport, promotion of cell survival, reorganization of the actin cytoskeleton, and cell adhesion, spreading, and migration via its role in the activation of AKT and FAK/PTK2. It plays a role in intracellular vesicular transport processes and is required for normal trafficking of the PMEL luminal domain, which is essential for the development and maturation of melanocytes. CD320 is a type I transmembrane protein responsible for the cellular uptake of vitamin B12 in peripheral tissues (Chen et al., 2022). Zhao (2014) found that CD320 is expressed in Dazu black goats and Nanjiang Yellow goats, and the CD320 antigen is involved in the immune response of the body, and can mediate the transfer and transport of substances and signals.

### Table 3. Differentially expressed whey proteins in Guishan and Saanen goat milk

<table>
<thead>
<tr>
<th>UniProt accession no.</th>
<th>Description</th>
<th>UniProt description</th>
<th>Molecular weight (kDa)</th>
<th>Coverage (%)</th>
<th>LFQ intensity in G</th>
<th>LFQ intensity in S</th>
<th>Fold change^2 (G/S)</th>
<th>P-value</th>
<th>Change^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0A452FTM7</td>
<td>SH3 domain-binding glutamic acid rich-like protein</td>
<td>SH3BGRL3</td>
<td>10.438</td>
<td>20.4</td>
<td>183,610,000</td>
<td>165,314,000</td>
<td>2.811</td>
<td>0.044</td>
<td>↑</td>
</tr>
<tr>
<td>A0A452EQX6</td>
<td>Diazepam binding inhibitor, acyl-CoA binding protein</td>
<td>DBI</td>
<td>10.04</td>
<td>75.9</td>
<td>8,878,533,333</td>
<td>3,666,866,667</td>
<td>2.421</td>
<td>0.04</td>
<td>↑</td>
</tr>
<tr>
<td>A0A452ERT5</td>
<td>Na(+)−dependent phosphate cotransporter 2B</td>
<td>SLC34A2</td>
<td>75.719</td>
<td>20.3</td>
<td>1,298,966,667</td>
<td>596,376,666</td>
<td>2.178</td>
<td>0.017</td>
<td>↑</td>
</tr>
<tr>
<td>A0A452FTM7</td>
<td>SH3 domain-binding glutamic acid rich-like protein</td>
<td>SH3BGRL3</td>
<td>10.438</td>
<td>20.4</td>
<td>183,610,000</td>
<td>165,314,000</td>
<td>2.811</td>
<td>0.044</td>
<td>↑</td>
</tr>
<tr>
<td>A0A452EJ36</td>
<td>Diazepam binding inhibitor, acyl-CoA binding protein</td>
<td>DBI</td>
<td>10.04</td>
<td>75.9</td>
<td>8,878,533,333</td>
<td>3,666,866,667</td>
<td>2.421</td>
<td>0.04</td>
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</tr>
<tr>
<td>A0A452ERT5</td>
<td>Na(+)−dependent phosphate cotransporter 2B</td>
<td>SLC34A2</td>
<td>75.719</td>
<td>20.3</td>
<td>1,298,966,667</td>
<td>596,376,666</td>
<td>2.178</td>
<td>0.017</td>
<td>↑</td>
</tr>
<tr>
<td>A0A452FNG1</td>
<td>Chromosome 7 C5orf15 homolog Secretoglobin family 1D member</td>
<td>LOC51180194</td>
<td>28.442</td>
<td>5.2</td>
<td>82,046,000</td>
<td>40,476,000</td>
<td>2.027</td>
<td>0.026</td>
<td>↑</td>
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<td>LOC51180194</td>
<td>28.442</td>
<td>5.2</td>
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<td>40,476,000</td>
<td>2.027</td>
<td>0.026</td>
<td>↑</td>
</tr>
<tr>
<td>A0A452DKP1</td>
<td>Lysosomal-associated membrane protein 1</td>
<td>LAMP1</td>
<td>43.961</td>
<td>10.8</td>
<td>283,480,000</td>
<td>151,766,666</td>
<td>1.868</td>
<td>0.017</td>
<td>↑</td>
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<tr>
<td>A0A452FZ38</td>
<td>Tetraspanin 1</td>
<td>TPD52L1</td>
<td>22.249</td>
<td>51</td>
<td>1,804,600,000</td>
<td>984,836,666</td>
<td>1.832</td>
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<tr>
<td>A0A452FHI7</td>
<td>Tetraspanin</td>
<td>CD63</td>
<td>25.725</td>
<td>12.7</td>
<td>193,236,666</td>
<td>114,276,666</td>
<td>1.691</td>
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<td>↑</td>
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<tr>
<td>A0A452FNA8</td>
<td>Serpin family F member 2</td>
<td>SERPINF2</td>
<td>45.802</td>
<td>36.2</td>
<td>682,573,333</td>
<td>1,541,766,666</td>
<td>0.443</td>
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</tr>
<tr>
<td>A0A452EJ36</td>
<td>Leucine-rich α-2-glycoprotein 1</td>
<td>LRG1</td>
<td>39.058</td>
<td>34.6</td>
<td>4,310,266,667</td>
<td>12,368,000,000</td>
<td>0.349</td>
<td>0.023</td>
<td>↓</td>
</tr>
<tr>
<td>A0A452FCV3</td>
<td>Delta/notch-like epidermal growth factor repeat containing protein</td>
<td>DNER</td>
<td>76.365</td>
<td>1.3</td>
<td>22,818,000</td>
<td>34,868,500</td>
<td>0.654</td>
<td>0.045</td>
<td>↓</td>
</tr>
</tbody>
</table>

^1LFQ = label-free quantitation; G = Guishan; S = Saanen.

^2Fold change (Guishan goat milk to Saanen goat milk) = ratio of protein abundance in Guishan milk to that in Saanen goat milk.

^3Upward arrows indicate significant upregulation of the protein; downward arrows represent significant downregulation of the protein.
To further understand the functional differences in the 2 kinds of goat whey proteins, we used GO to analyze the cellular components, biological processes, and molecular functions of UEWP and DEWP in Guishan and Saanen goat milk. As shown in Figure 3, in the classification of biological processes, the UEWP and DEWP in Guishan and Saanen goat milk were involved jointly in cellular processes, biological regulation, regulation of the biological process, response to stimulus, metabolic processes, and multicellular organic processes. Previous studies have shown that human and some mammalian whey proteins are mainly involved in biological regulation, response to stimulus, and immune system processes (Liao et al., 2011; Cao et al., 2017; Yang et al., 2017). Furthermore, the UEWP and DEWP of Guishan goat milk were involved in biological adhesion (A0A452E573, A0A452FIJ8), behavior (A0A452G5I4), and pigmentation (A0A452G5I4). A whey protein in Saanen goat milk was also involved in cell proliferation (A0A452EC39). In addition, ATP synthase subunit β (A0A452E573) promoted ATP production from ADP in the presence of a transmembrane proton gradient, and was associated with angiogenesis, positive regulation of blood vessel endothelial cell migration, and regulation of intracellular pH. It was also involved in major histocompatibility complex class I protein binding and was responsible for lymphocyte recognition and antigen presentation. Notably, goat skin color is associated with pigmentation, and Microrna-200A regulates goat pigmentation by targeting Wnt5A and Fzd4 (Li et al., 2022). The skin of the Guishan goats in this study was black, and that of Saanen goats was white, which might be associated with the expression of a pigmentation protein. From the perspective of the immune system processes, 7 whey proteins were involved in immune processes. Among them, the Fc fragment of the IgE receptor Ig (A0A452EQT0), lymphocyte cytosolic protein 1 (A0A452DVP7), proteoglycan 4 (A0A452E5P2), and tetraspan (A0A452E4S1) were UEWP in Saanen goat milk. Lysosomal-associated membrane protein 1 (A0A452DKP1), ATP synthase subunit β (A0A452E573), and fibrillin 1 (A0A452FIJ8) were highly expressed and unique in Guishan goat milk. It is noteworthy that the expression abundance of 2 proteins (A0A452DKP1 and A0A452FIJ8) in Guishan goats was greater than that in Saanen goats, especially the expression abundance of lysosomal-associated membrane protein 1 (A0A452DKP1). Fibrillin 1 limited TGF-β to the extracellular matrix, separating it from other components of the signaling pathway. TGF-β is thought to be primarily associated with immunosuppressive function and anti-inflammatory responses (Li et al., 2006, 2020). This result suggests that Guishan whey protein may be more active in building the immune system than Saanen milk.

Figure 3. Gene Ontology (GO) annotation of the uniquely expressed whey proteins and differentially expressed whey proteins in Guishan and Saanen goat milk.
In the classification of molecular functions, the UEWP and DEWP in Guishan and Saanen goat milk were involved jointly in binding, catalytic activity, molecular translator activity, and molecular function regulation. Highly expressed or UEWP in Guishan milk mainly included structural molecule activity (A0A452FIJ8), transporter activity (A0A452E573), and transcription regulator activity (A0A452EBM0). The highly expressed or UEWP in Saanen milk had, primarily, cargo receptor activity (A0A452E5P2, A0A452EJM6) and molecular function regulation (A0A452G1E4). In addition, coatomer complex (A0A452G5I4) was required for budding from Golgi membranes and is essential for the retrograde Golgi-to-endoplasmic reticulum transport of dilyseine-tagged proteins. In the UEWP of Saanen goat milk, angiotensinogen (A0A452E545) was an essential component of the renin–angiotensin system, a potent regulator of blood pressure, body fluid, and electrolyte homeostasis. A0A452EJM6 combined with any modified low-density lipoprotein or other polyanionic ligand and delivered the ligand into the cell via endocytosis; tetraspanin could prevent or reduce the frequency, rate, or extent of NIK/NF-κB signaling. Transcobalamin 1 bound to cobalamin (vitamin B₁₂), a water-soluble vitamin characterized by the possession of a corrin nucleus containing a cobalt atom. Once evolved, protein C scissors the activated cofactors of the coagulation mechanism, factor Va and factor VIIIa, thereby reducing the amount of thrombin generated. Polypeptide N-acetylgalactosaminyltransferase (A0A452DMV7) was involved in the pathway protein glycosylation, which was part of protein modification. It could be concluded that the highly expressed or UEWP of Guishan goat were mainly involved in metabolic-related functional activities whereas those of Saanen goats were related mainly to disease regulation.

According to the classification of cellular components, the UEWP and DEWP in Guishan and Saanen goat milk were involved jointly in the cellular part, membranous part, organellar part, and protein-containing complex. Two proteins from Guishan UEWP were involved in the membrane-enclosed lumen (A0A452E573, A0A452E495) and one in the nucleoid (A0A452E573). In addition, 11 UEWP in Guishan goat milk were involved in the organellar part. Organelles include mitochondria, endoplasmic reticulum, Golgi apparatus, and ribosome, which play an important role in infant growth and development (Ye et al., 2016). Four UEWP in Saanen goats were involved in the extracellular region. The extracellular region was the main cellular component of whey protein localization. Some serum-derived proteins can be transferred to whey through intercellular or transcellular transmission at the tight junction of breast cells (Zhang et al., 2018).

### KEGG Pathway Analysis of the UEWP and DEWP in Guishan and Saanen Goat Milk

The UEWP and DEWP in Guishan and Saanen goat milk were related to 12 KEGG pathways. As shown in Figure 4A, 12 highly expressed and UEWP in Guishan goat milk were involved in 7 pathways, including lysosome (A0A452DKP1, A0A452FH7), phagosome (A0A452DKP1), and oxidative phosphorylation (A0A452DKP1). Five highly expressed and UEWP in Saanen goat milk were involved in 5 pathways, including arginine and proline metabolism (A0A452FZ73), the phospholipase D signaling pathway (A0A452E545), neuroactive ligand–receptor interaction (A0A452E545), tight junction (A0A452FAW1), and regulation of actin cytoskeleton (A0A452FAW1) (Figure 4B). Previous studies have reported that human, cow, and buffalo milk whey proteins are mainly involved in 2 immune-related pathways: complement and coagulation cascade, and phagosome (Cao et al., 2017; Yang et al., 2017). We noted that lysosomal associated membrane protein 1 (A0A452DKP1) from the DEWP in Guishan goat milk also participated in phagosome pathways. Phagosomes are vacuoles in the plasma membrane that contain invasive microorganisms engulfed by innate immune cells (Botelho et al., 2004). As part of the key pathways in innate and adaptive immunity, phagosomes play a crucial role in the elimination of apoptotic cells, host defense, inflammation, and tissue remodeling and repair (Gordon, 2016). Thus, through KEGG pathway analysis, we had a preliminary understanding of the function of albumin in Guishan and Saanen goats. Guishan goat milk whey proteins were involved mainly in metabolism and immune-related pathways, whereas Saanen goat whey proteins were mostly associated with environmental information processing-related pathways.

### Protein–Protein Interaction Network Analysis of the UEWP and DEWP in Guishan and Saanen Goat Milk

Protein–protein interaction network analysis is of great significance to understanding the protein function and plays an important role in various biological processes (Li et al., 2020). To understand the relation between UEWP and DEWP, and their involvement in crosslinking different biological network, a protein–protein interaction network map of the UEWP and DEWP in Guishan and Saanen goat milk was analyzed using Cytoscape with protein–protein interactions being retrieved from the STRING database. In addition, the minimum required interaction score was set to 0.400. As shown in Figure 5, the network contained 49 proteins. Among these, there were 9 highly expressed and 18 uniquely expressed proteins in Guishan goat.
milk whey, and 3 highly expressed and 19 uniquely expressed proteins in Saanen goat milk whey. The protein, heat shock protein family A (HSP70) member 1 like (HSPA1L), from Guishan goat milk interacting with 4 proteins was the protein that was found to interact the most, followed by heat shock–related 70-kDa protein 2 (HSPA2) and lysosomal-associated membrane protein 1 (LAMP1) with 3 interacting proteins. Other proteins had less than 3 interacting partners. It is worth noting that most proteins interacting with other proteins come from Guishan goat whey. The protein (HSPA1L) from Guishan goat milk belongs to the heat shock protein 70 family. Heat shock–associated 70-kDa protein 2 was associated with protecting cellular proteins from oxidative stress and infection in animals. Greater concentrations of HSP70
also contribute to milk stability during pasteurization or sterilization, suggesting that it is easier to handle during processing in the dairy industry (Maity and Ambatipudi, 2019). The gene expression of the HSP70 family was different in goats in different seasons, and it had a greater expression in cold-adapted goats during the summer and heat-adapted goats during the winter (Banerjee et al., 2014). In addition, LAMP1 from the DEWP in Guishan goat milk was associated with 2 immune response-related proteins (CD63 and FCER1G).

**Effects of Whey Protein on Cell Viability and NO Production in LPS-Induced RAW264.7 Cells**

Whey protein itself has been reported to have anti-inflammatory effects in various cells, such as macrophages (Chatterton et al., 2013; Da Silva and Rudkowska, 2015). Macrophages play an important role in host defense as a part of nonspecific defense (innate immunity) and specific defense (adaptive immunity) (Yuan et al. 2015). Macrophages not only maintain homeostasis, but also provide defense against pathogens and invading cells such as cancer cells. Macrophage activity and viability in vitro can serve as an indicator of immune activation (Gamal-Eldeen et al., 2007). We used the MTT assay to investigate the effects of different concentrations of Guishan whey protein (GWP) and Saanen whey protein (SWP) on RAW264.7 cell proliferation. As shown Figure 6A, the effect of GWP in the concentration range of 1 to 8 mg/mL on RAW264.7 cell viability remained at more than 98%, reaching 135.29% at 2 mg/mL compared with the control. In contrast, the effect of SWP on RAW264.7 cell viability was not significant compared with the control ($P > 0.05$). To determine the relationship between concentrations and the anti-inflammatory effect of both goat whey proteins, their 4 concentrations (1, 2, 4, and 8 mg/mL) were selected for further study.

Lipopolysaccharide induction can activate macrophages, which subsequently overproduce proinflammatory mediators regulated by the NF-κB pathway, such as NO, TNF-α, IL-6, and IL-1β, thereby promoting the immune response (Toopcham et al., 2016). Nitric oxide is an important mediator of iNOS production during inflammation (Ndiaye et al., 2011). Moreover, NO changes microvascular contractility and epithelial barrier function by increasing permeability (Wallace and Miller, 2000). Low levels of NO enhance the immune response and regulate indoleamine 2,3-dioxygenase activity; however, its overproduction is associated with cyclooxygenase (COX) expression and the development of human cellular inflammation (Engin, 2011). As shown in Figure 6B, LPS treatment significantly increased the NO production of cells, ~5.51 times that of the control group ($P < 0.0001$). However, both goat milk whey-treated cells reduced NO production significantly in a dose-dependent manner. In particular, GWP reduced NO production significantly.

**CONCLUSIONS**

We analyzed comprehensively the UEWP and DEWP in Guishan and Saanen goat milk at a molecular level through LFQ proteomics. Guishan goat milk
ph whey proteins were involved mainly in metabolism and immune-related pathways, whereas Saanen goat milk whey proteins were involved primarily in environmental information processing-related pathways. Further analysis showed that both whey proteins could increase the viability of RAW264.7 cells further, and reduced significantly NO production in LPS-stimulated RAW264.7 cells, especially the whey protein of Guishan goats. These findings provide not only new insights into the characteristics and potential activity of whey proteins of Guichon and Saanen goat milk, but also provide possible directions for dairy-based functional food development. In subsequent research, we may verify this differential whey proteins and explore more functionally active substances, such as antioxidant and anti-inflammatory peptides, from these proteins.

ACKNOWLEDGMENTS

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Zhao et al.: PROTEOMIC ANALYSIS OF GOAT WHEY PROTEINS


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