Proteomic analysis of differentially expressed whey proteins in Saanen goat milk from different provinces in China using a data-independent acquisition technique

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ABSTRACT

Whey proteins of Saanen goat milk samples from 3 provinces in China (Guangdong, GD; Inner Mongolia, IM; Shaanxi, SX) were characterized and compared using data-independent acquisition quantitative proteomics technique. A total of 550 proteins were quantified in all 3 samples. There were 44, 44, and 33 differentially expressed proteins (DEP) for GD versus IM, GD versus SX, and IM versus SX, respectively. Gene ontology annotation analysis showed that the largest number of DEP for the 3 comparisons were as follows: for biological processes: response to progesterone, glyceraldehyde-3-phosphate metabolic process, and negative regulation of megakaryocyte differentiation; for molecular functions: antioxidant activity, binding, and peroxiredoxin activity; and for cellular components: the same category of extracellular regions for the 3 comparisons, respectively. Pathways for the DEP of 3 comparisons were (1) disease; (2) synthesis and metabolism; and (3) synthesis, degradation, and metabolism. Protein–protein interaction network analysis showed that DEP for GD versus SX had the most interactions. **Key words:** Saanen goat milk, whey protein, data-independent acquisition

INTRODUCTION

Goat milk is attracting increasing attention because of its low allergenicity and easy digestibility compared with cow milk (Izquierdo-González et al., 2019). The high nutritional value of goat milk is related to its protein (Costa et al., 2014), which can be classified into caseins, whey proteins, and milk fat globule membrane proteins. Whey protein accounts for about 20%

of the total protein (Rahmatalla et al., 2020) and is a high-quality protein source. Goat milk whey proteins are easily absorbed and utilized by the human body (Brodziak et al., 2014). The whey proteins in goat milk comprise various proteins, the number and composition of which depend on many factors, including goat breed and region (Costa et al., 2014). Cunsolo et al. (2015) identified 452 whey proteins in Camosciata goat milk using a proteomics technique. Sun et al. (2019) characterized 283 whey proteins in Guanzhong goat milk using EASY-nano liquid chromatography combined with orbitrap-tandem mass spectrometry. Sun et al. (2020) determined that there were 314 and 524 whey proteins in Xinong Saanen goat colostrum and mature milk, respectively. Diet also greatly influences goat milk composition (e.g., adding *Leucaena* to the TMR may reduce the urea content in Saanen goat milk; Leketa et al., 2019).

Proteomics is a basic technique for systematic identification and characterization of protein structure, function, activity, quantity, and molecular interactions (Gerber et al., 2003). Proteomics analysis mainly relies on liquid chromatography-MS/MS. The previous strategy developed for MS data acquisition is data-dependent acquisition (DDA; Fernández-Costa et al., 2020). However, tandem MS (MS/MS) spectra collected using DDA technology rarely sample the part with the strongest signal of the peptide elution profile (Egertson et al., 2013). As many as 15 to 20% of MS/MS spectra sampled from complex mixtures are chimeric. Low-intensity ions cannot be quantified in DDA technology because they are always ignored (Bourassa et al., 2015). To solve these problems, a high-throughput data-independent acquisition (DIA) mode has emerged in recent years (Lin et al., 2018). In DIA mode, all samples can be scanned and low-abundance protein can be detected (Lin et al., 2018). Data-independent acquisition can provide high accuracy, dynamic range, and reproducibility of selected reaction monitoring (Gillet et al., 2012; Liu et al., 2014).

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Data-independent acquisition is a popular and promising method for protein identification and quantification. In this study, we characterized and compared whey protein profiles of Saanen goat milk samples collected from Guangdong, Inner Mongolia, and Shaanxi in China using DIA quantitative proteomics technology. The data were compared for bioinformatics analysis of whole identified proteins and differentially expressed proteins (DEP) between goat milks from 2 regions at a time. The whole quantified whey proteins were analyzed using Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. We identified DEP between each pair of regions for GO and KEGG enrichment and conducted a protein-protein interaction (PPI) analysis.

MATERIALS AND METHODS

Materials and Sampling

Mature Saanen goat milk was sampled (30 samples each) from 3 different regions of China, including Guangdong (GD; Meizhou, 115.897567E, 24.572625N), Shaanxi (SX; Xi'an, 108.946166E, 34.349382N), and Inner Mongolia (IM; Hohhot, 111.755426E, 40.848119N). Mature milk was collected from 2- to 3-yr-old Saanen goats in third to sixth parity. Samples were collected in 50-mL centrifuge tubes in the morning from September 15 to 21, 2019. Samples were transported to the laboratory by cold chain and stored at -80°C until analysis. Protease inhibitors, urea, trypsin, acetone, bicinchoninic acid protein assay kit, dithiothreitol, acetonitrile, iodoacetamide, and ammonium bicarbonate were provided by Sigma-Aldrich Corp. Feed for Saanen goats in GD was composed of 60% mountain top grass, 20% mountain foot grass, and 20% rich and nourishing concentrated feed. Feed for Saanen goats in IM was a mixture of green oil wheat, silage, corn stover, soybean hulls, and rich and nourishing concentrated feed. The Saanen goat feed in SX was a blend of okara, silage, cauliflower leaves, sweet elephant grass, and rich and nourishing concentrated feed. Nourishing concentrated feed was composed of corn, soybean meal, distillers dried grains, cotton meal, corn husks, and so on, and provided by the local farm.

Preparation of Whey Protein

Goat milk samples (containing protease inhibitors) were centrifuged at $860 \times g$ at 4°C for 60 min. Two layers were obtained, including a fat layer and a skim milk layer (from top to bottom). The skim milk layer

was adjusted to pH 4.6 with 10% acetic acid and then centrifuged at $9,600 \times g$ at 4°C for 15 min. The supernatant (10 mL) was taken, and 4-fold volume of prechilled acetone was added. The mixture was left at -20°C overnight to precipitate the whey proteins. Whey protein was then obtained by washing the precipitate 6 to 9 times with cool acetone and then dried. The whey protein was quantified using a bicinchoninic acid kit.

Digestion of Whey Proteins

To 100 μg of protein sample, urea (8 M) was added to a volume of 200 μL , and dithiothreitol was added to a final concentration of 10 mM. The mixture was incubated at 37°C for 2.5 h, and then iodoacetamide was added to a final concentration of 50 mM. The mixture was incubated at room temperature for 40 min in the dark and then transferred to a 10-K tube (MRCPT010, Merck). After the mixture was centrifuged at $12,000 \times g$ at room temperature for 30 min, the filtrate was discarded. Urea (8 M, 400 μL) was added into the mixture and centrifuged again at $12,000 \times g$ at room temperature for 30 min and the filtrate was discarded. This step was repeated twice. Then, 400 μL of ammonium bicarbonate solution (50 mM) was added to the UF tube and centrifuged at $12,000 \times g$ at room temperature for 30 min, and the filtrate was discarded. This step was repeated 3 times, and the temperature was set at room temperature for the first time and 4°C for 30 min for the next 2 times. Ammonium bicarbonate solution (50 mM, 200 μL) was added to the UF tube, and then trypsin was added to the UF tube at a mass ratio of sample to enzyme at 50:1. The mixture was incubated at 37°C for 16 h with a shaker and centrifuged at $12,000 \times g$ at 4°C for 30 min. The peptides were obtained by freeze-drying the collected filtrate.

High pH Reversed-Phase Chromatography Separation

Methanol (1 mL) was used to activate the chromatographic column. Acetonitrile (80%) and liquid ammonia (pH 10) at a volume of 1 mL were used to clean the chromatographic column for 3 times. Liquid ammonia (pH 10, 1 mL) was used to equilibrate the chromatographic column. Freeze-dried peptides were dissolved in 200 μL of liquid ammonia (pH 10) and then loaded into the column. The peptides were separated into 3 fractions by eluting liquid ammonia (pH 10) with 6, 9, 12, 15, 18, 21, 25, 30, 35, and 50% acetonitrile in sequence. The separated fractions were lyophilized for use.

Liquid Chromatography-MS/MS Experiments

Data of all the peptides were obtained from Q ESXc-tive HF and Easy nanoLC 1000 (Thermo Scientific) for DIA analysis. Each peptide sample (3 µg) was loaded onto the chromatography column and separated with an aqueous solution with 0.1% (vol/vol) formic acid in buffer A and acetonitrile with 0.1% (vol/vol) formic acid in buffer B for 180 min at a flow rate of 600 nL/min. Then, it was connected to an oven at 60°C. The DDA analysis was performed with precursor MS spectral analysis at 400 to 1,200 m/z and analyzed at a resolution of 120 k at 400 m/z , and then the first 20 ions were selected with an isolation window of 2 Da. For dynamic exclusion time and maximum injection time, MS1 and MS2 of the tandem MS were at 20 and 50 ms, respectively. For DIA analysis, the m/z range was 400 to 1,200, and the resolution was 60,000, followed by 29 variable DIA scans with a resolution of 30,000.

DDA Data Search and DIA Data Analysis

All DDA raw data were uploaded to the Sequest HT node integrated in the Proteome Discoverer software (version 2.1, Thermo Fisher Scientific), and the Biognosys iRT peptide sequence was added through the Mouse Uniprot fasta database (83,625 entries, downloaded on March 18, 2019; <https://www.uniprot.org>). The miss cleavage site set was 2. Carbamidomethylation of cysteine was set as static modification, and oxidation of Met and deamidation of Asn and Gln were set as dynamic modifications. The false discovery rate (FDR) of peptides and proteins was 1%. The DDA search results were imported into Skyline (4.2.0.18305) to generate the library. Then, DIA raw data of 9 samples were imported for quantification. We used the mProphet (Reiter et al., 2011) peak scoring model (each data set and iteration were trained independently) to reintegrate all detected peaks, and q-value annotations were added to each peak. Then, MSstats (version 2.4.0, R package, <https://www.r-project.org/>) was used to output protein quantitative results for further bioinformatics analysis.

Bioinformatics Analysis

UniProt database (<https://www.uniprot.org/>) was used to obtain specific information on all proteins determined in this study. The CAZy annotations were obtained using the CAZy database (<http://www.cazy.org/>). We conducted GO and KEGG analysis of proteins using online David 6.8 software (<http://david.abcc.ncifcrf.gov/home.jsp>). The PPI analysis was per-

formed using STRING online database (version 11.0, <https://string-db.org/>).

RESULTS AND DISCUSSION

Characterization of Quantitatively Identified Proteins

The proteomics technique has been widely used to identify and characterize whey proteins in milk from different species. Liu et al. (2020) identified 161 serum proteins in raw bovine milk using a label-free proteomic approach. Li et al. (2018) quantified 580 whey proteins of Murrah, Nili-Ravi, and Mediterranean buffaloes through tandem mass tag proteomics. Scumaci et al. (2015) identified 342 colostrum whey proteins in 15 Apenninic sheep (3–4 yr old). In this study, we used the DIA proteomic technique to identify the whey proteins in Saanen goat milk in 3 different provinces in China. A total of 550 whey proteins were found, and the detailed information on these quantified proteins is shown in Supplemental Table S1 (<https://data.mendeley.com/datasets/rz3yzznv6s/1>, Zhao, 2021). More proteins were identified in this study compared with other studies (Anagnostopoulos et al., 2016; Sun et al., 2019, 2020), which may be due to the reversed-phase chromatography fractionation and DIA quantitative proteomics technology. According to the CAZy database, there were a total of 124 enzymes in 550 proteins. The enzymes were divided into 5 categories (i.e., auxiliary activities, carbohydrate-binding modules, carbohydrate esterases, glycoside hydrolases, and glycosyl transferases). Each category had 3, 54, 3, 28, and 36 enzymes, respectively (Figure 1A). Most of the proteins were involved in carbohydrate binding. Among the top 10 proteins with the highest abundances, 9 proteins were shared by the 3 regions, which were not listed in this study. β -Lactoglobulin and α -LA, which are common proteins, were the top 2 in abundance in all 3 regions, and albumin was third most abundant in milk from GD and SX. The detected differences in protein abundance may be due to the different feeds of the goat and location of the 3 provinces. For example, from north to south, IM and SX span 6 latitudes, and SX and GD span 16 latitudes.

The strongest correlation was observed between samples collected from GD and SX, with a correlation coefficient of 0.91. The correlation coefficients for samples between GD and SX and between IM and SX were 0.90. Figure 1B shows the density map of whey proteins in the 3 goat milk samples. Figure 1B suggests that protein abundances of GD and SX were closer than those of other location pairs.

Figure 1. Enzyme classification (A) and density map (B) of the whey protein identified in goat milk collected from 3 regions in China: Guangdong (GD), Inner Mongolia (IM), and Shaanxi (SX). The x-axis represents logarithm of the relative abundance, and the y-axis represents probability density.

GO Annotation and KEGG Pathway Analysis

The GO functional annotation is divided into 3 aspects: biological process (BP), cellular components (CC), and molecular function (MF). All identified proteins were annotated for GO function and the results are shown in Figure 2A. Regarding BP, most proteins acted on cellular processes. For CC, proteins were mainly involved in cell, cell part, and organelle

part. With respect to MF, most proteins had functions of binding, followed by catalytic activity and molecular function regulator. These results were similar to those of Anagnostopoulos et al. (2016), which showed that the top 3 MF categories for Greek goat milk were catalytic activity, nucleotide binding, and metal ion binding.

The KEGG pathway analysis is a primary analysis of biological pathways and can be divided into the following 5 branches: (1) cellular processes, (2) environmental

Figure 2. (A) Gene Ontology (GO) function annotation, and (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway map for whey proteins identified in goat milk collected from 3 regions in China. A, cellular processes; B, environmental information processing; C, genetic information processing; D, metabolism; and E, organismal systems, which respectively correspond to (1), (2), (3), (4), and (5) of the GO Annotation and KEGG Pathway Analysis in the paper.

information processing, (3) genetic information processing, (4) metabolism, and (5) organismal systems. All identified proteins were subjected to KEGG analysis, and the results are shown in Figure 2B. All proteins were involved in a total of 26 pathways, and most involved pathways were related to carbohydrate metabolism, cell transport, and catabolism. For cellular processes, there were 4 pathways, of which 26 of the proteins were mainly involved in transport and catabolism. For the environmental information processing section, 11 of the proteins were involved in signal transduction, and 5 proteins were involved in signaling molecules and interaction. For genetic information processing, 12 of the proteins were involved in folding, sorting, and degradation. For metabolism, the relevant pathway for whey protein participation was carbohydrate metabolism (30 terms), which reflected the metabolic performance, physiological and nutritional status of organisms, and ability to adapt to the external environment (Wang et al., 2017). In organismal systems, 15 proteins participated in the immune system, and among them, 11 types of proteins were involved in regulating complement and coagulation cascades. The complement and coagulation cascades were related to cell adhesion, migration and proliferation, B cell receptor signaling pathways, and phagocytosis (Falanga et al., 2013; Kourtzelis and Raffail, 2016; Afshar-Kharghan, 2017).

Characterization of DEP

Identified whey proteins of goat milk from each pair of regions were compared, and proteins with fold changes >2 or <0.5 and $\text{FDR} < 0.4$ were selected as DEP, which can be used to distinguish and classify Saanen goat milk whey proteins from the 3 regions.

A total of 44 DEP for GD versus IM, including 8 upregulated and 36 downregulated proteins, were found as shown in the volcano picture (Figure 3A). The top 3 significant DEP among upregulated proteins were the ones with the accession numbers of A0A452FMC2 ($\text{FDR} = 0.0006$), A0A452EJM6 ($\text{FDR} = 0.0049$), and A0A452E243 ($\text{FDR} = 0.0252$), which have not been characterized according to the Uniprot database. The protein with the largest difference among downregulated proteins was that with accession number A0A452EFJ3, which has also not been characterized; it is a protease-associated (PA) domain-containing protein.

A total of 44 DEP, 13 upregulated and 31 downregulated, were identified for GD versus SX (Figure 3B). The most significant one among the upregulated proteins was major facilitator superfamily (MFS) domain-containing protein ($\text{FDR} = 0.0041$), whereas that for the downregulated proteins was galectin. Galectin

constitutes a family of animal β -galactoside-binding lectins and contains few carbohydrate recognition domains (Chiariotti et al., 2002). Galectin is synthesized and stored in the cytoplasm and released or secreted by cells as a pattern recognition receptor when there is a long-term infection or tissue damage caused by infection. These infectious diseases act as immunomodulators or cytokine-like modulators in innate responses (Sato et al., 2009).

When samples from IM and SX were compared, 33 DEP (24 upregulated and 9 downregulated) were found (Figure 3C). The most significant upregulated proteins were the same as those for GD versus SX. The most significant downregulated protein has not yet been characterized in Uniprot ($\text{FDR} = 0$), and the second most significant protein is peroxiredoxin-6 (updated by Uniprot; $\text{FDR} = 0.0152$). Peroxiredoxin is a common family of nonselenium peroxidases with molecular weight ranging from 22 to 27 kDa. The main function of peroxiredoxin is to catalyze peroxide reduction and balance the hydrogen peroxide level in cells (Park et al., 2016). The peroxidase family is involved in invasion and metastasis of cancer cells, and it may be a new target for cancer radiotherapy (Zhang et al., 2009; Chen et al., 2018).

Analysis of Venn Diagram for DEP

The distribution of DEP between each comparison was studied and the results are shown in Figure 3D. The comparisons of GD versus IM and IM versus SX have 6 common DEP, 2 upregulated and 4 downregulated. There were 12 common DEP for GD versus IM and GD versus SX, of which 10 were downregulated and 2 were upregulated. The most different downregulated protein was alkaline phosphatase. Alkaline phosphatase exists in most human tissues, such as liver and kidney, blood cells, and bones (Rankin et al., 2010). Alkaline phosphatase is also present in milk (Shakeel-ur-Rehman et al., 2003), and it can be used to characterize the correct pasteurization in milk (Harding and Garry, 2005). Groups of IM versus SX, and GD versus SX have 6 common DEP, including 2 downregulated proteins and 4 upregulated proteins, of which the highly significantly upregulated protein is MFS domain-containing proteins.

The 3 compared pairs had 1 common DEP, histone H4, which had the highest abundance in samples collected from GD. Histones are involved in several cellular processes such as transcription, repair, and recombination (Marayati et al., 2020). A high level of histone H4 is one of the important risk factors leading to an increased probability of death in patients with

Figure 3. Volcano diagram (A, B, C) and Venn diagram (D) for differentially expressed proteins of whey proteins in goat milk for GD versus IM, GD versus SX, and IM versus SX. GD = Guangdong; IM = Inner Mongolia; SX = Shaanxi; FDR = false discovery rate.

sepsis, and it is also considered an effective biomarker for evaluating sepsis (Lu et al., 2020).

GO Enrichment Analysis of DEP

The DEP between each pair of regions underwent GO enrichment analysis, and the results are shown in

Figure 4. The 44 DEP for GD versus IM showed significant enrichment in 10 BP terms, 19 CC terms, and 1 MF term ($P < 0.05$; Figure 4A). With respect to BP, most DEP were involved in the category of response to progesterone, followed by participation in cellular detoxification and detoxification processes. For CC, DEP acted mainly on extracellular regions. The categories

with the largest numbers of DEP were antioxidant activity and extracellular region under MF, followed by membrane-bounded vesicle and vesicle. Goat milk with whey proteins is high in antioxidant activity and may be beneficial to consumers by reducing oxidative damage caused by free radicals (Lakram et al., 2019). Three proteins were involved in response to progesterone, all of which were downregulated. There were 23 proteins involved in the extracellular region, of which 2 were upregulated and 21 were downregulated. Four proteins were involved in antioxidant activity, of which 2 were upregulated, and 2 were downregulated.

The DEP for GD versus SX showed significant enrichment in 18 BP terms, 11 CC terms, and 1 MF term ($P < 0.05$; Figure 4B). For BP, most DEP were involved in the glyceraldehyde-3-phosphate and the glucose 6-phosphate metabolic processes. With respect to CC, DEP in the category of extracellular regions ranked first, followed by extracellular region parts, membrane-bounded vesicles, and vesicles. The DEP mainly had the molecular function of binding. There was a total of 3 proteins involved in the glyceraldehyde-3-phosphate metabolic process, all of which were downregulated proteins. There were 23 proteins involved in the extracellular region, of which 6 were upregulated and 17 were downregulated. Twenty-four were involved in binding, with 7 upregulated and 17 downregulated.

For IM versus SX, DEP had significant enrichment in BP (10 terms), CC (16 terms), and MF (4 terms; $P < 0.05$; Figure 4C). With respect to BP, DEP mainly acted on negative regulation of megakaryocyte differentiation. For CC, whey proteins were mainly in the extracellular region, followed by extracellular region part, extracellular exosome, and extracellular vesicle. Regarding MF, peroxiredoxin activity was the main function, and oxidoreductase activity and antioxidant activity also played important roles. Two proteins were involved in regulation of megakaryocyte differentiation, both of which were downregulated. Fifteen proteins were involved in the extracellular region, of which 10 were upregulated and 5 were downregulated. Two proteins were involved in peroxiredoxin activity, and both were downregulated.

KEGG Pathway Analysis of DEP

Forty terms were found with $P < 0.05$ for the DEP of GD versus IM, and among them, amoebiasis, phagosome, and systemic lupus erythematosus were the top significant 3 terms (Figure 4D). The pathway in which most DEP participated in was metabolic pathway, including 1 upregulated and 7 downregulated proteins.

Most DEP for GD versus SX were involved in synthetic and metabolic pathways, such as biosynthesis of AA, folate biosynthesis, and the pentose phosphate pathway (Figure 4E). One of the significant pathways was the renin-angiotensin system metabolic pathway (Figure 5A), which exists in all organs of the body and is closely related to regulation of blood pressure. It was reported that SARS-CoV-2 may be particularly harmful to patients with cardiovascular disease (South et al., 2020), perhaps because SARS-CoV-2 results in the imbalance of the renin-angiotensin system and can lead to a further increase in blood pressure. In this pathway, 2 DEP were detected, peptidase S1 domain-containing protein and ATPase H(+)-transporting lysosomal accessory protein 2, both of which were downregulated. The position of renin-angiotensin system metabolic pathway and protein function in the pathway are shown in Figure 5A (blue box represents protein position). Two DEP can regulate the biological effects of the pathway by regulating upstream and downstream proteins. For example, AGT can be directly changed to ANG2 under the action of peptidase S1 domain-containing protein. Also, AGT can also be transformed into ANG1 by REN and then change into ANG2 under action of peptidase S1 domain-containing protein. Both pathways may cause blood pressure to increase. Simultaneously, blood pressure can be lowered via hydrolyzing ANG1 and ANG2 by ACE2. Thus, the blood pressure balance can be regulated. Additionally, REN can act on PRR [corresponding to ATPase H(+)-lysosomal accessory protein 2] to promote protein synthesis on the cell membrane, resist apoptosis, and inhibit cell proliferation. It has been reported that ACE2 is the receptor for SARS-CoV-2 (Samavati and Uhal, 2020) and binding with SARS-CoV-2 resulted in the remarkably decreased ACE2 content in human cells. Subsequently, ANG2 expression increases relatively and blood pressure increases further. Moreover, SARS-CoV-2 has caused multiple organ damage in humans.

For DEP of IM versus SX, most proteins were involved in pathways of synthesis, degradation, and metabolism, and the results are shown in Figure 4F. The pathway for galactose metabolism is shown in Figure 5B. The red box represents DEP for the following pathway: β -galactosidase (β -gal) converts lactose into D-galactose or α -D-galactose, thereby regulating the pathway balance. The literature shows β -gal is a common enzyme that catalyzes hydrolysis of terminal β -galactosidic linkages in glycoproteins (Tanaka et al., 1975). The literature also shows that β -gal is highly active in patients with human glioma (Wielgat et al., 2006) and also related to rheumatoid arthritis inflammation (Su et al., 2020).

Figure 4 (Continued). Histogram of Gene Ontology (GO) enrichment classification (A, B, C) and statistics of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment (D, E, F) for differentially expressed proteins of whey proteins in goat milk for GD versus IM, GD versus SX, and IM versus SX. The asterisk on each bar in the bar chart represents statistical significance. The top 20 GO enrichment results are shown in order of *P*-value from smallest to largest. GD = Guangdong; IM = Inner Mongolia; SX = Shaanxi.

Figure 6. Protein–protein interaction network map for differentially expressed proteins of whey proteins in goat milk for (A) GD versus IM, (B) GD versus SX, and (C) IM versus SX. GD = Guangdong; IM = Inner Mongolia; SX = Shaanxi.

PPI Network Analysis of DEP

Studying the interactions between proteins helps to identify core regulatory proteins. There are many PPI databases; STRING is an online search database that covers the most species and has extensive interaction information. The DEP for each paired comparison across the 3 regions were analyzed for PPI using the STRING database, the data imported into Cytoscape v3.8.2 software, and the top 10 hub genes in PPI network selected using the CytoHubba plug-in. The results are shown in Figure 6, where circled nodes represent proteins and connections between nodes represent protein–protein interactions.

The PPI network analysis for DEP of GD versus IM (Figure 6A) showed that it contained a 26 nodes and 27 edges. The top 10 hub genes were, in order, *FN1*, *ORM1*, *CNN2*, *THBS1*, *PDIA3*, *BGN*, *PRDX6*, *ATP6AP1*, *RAB5B*, and *TTR*. Fibronectin 1 is a large adhesion glycoprotein in the extracellular matrix that has multiple binding domains with cell surface and extracellular ligands (Goossens et al., 2009). It has been reported to be a potential biomarker for radiation resistance in squamous cell carcinoma of head and neck (Jerhammar et al., 2010). One of the reasons for low survival rate of patients with nasopharyngeal cancer is that high concentration fibronectin 1 tumor cells are expressed, and fibronectin 1 may be an independent prognostic factor for nasopharyngeal carcinoma (Yu et al., 2020).

Figure 6B shows the PPI network for DEP of GD versus SX, which contained 27 nodes and 46 edges. The top 10 hub genes were *ORM1*, *BPI*, *CTSG*, *MMP8*, *FUCA1*, *PGLYRP1*, *SDCBP*, *CNN2*, *VAT1*, and *TIMP2*. ORM was first discovered more than 100 yr ago, and it has a variety of physiological functions, including immune regulation, drug binding, and transport (Luo et al., 2015). The protein BPI is a cationic protein with molecular weight of 55 kDa (Theprungsirikul et al., 2020) and has strong bactericidal activity against some gram-negative organisms (Gazzano-Santoro et al., 1992). CTSG is a serine protease (Meyer-Hoffert, 2009) that has a wide range of biological activities, including regulating functions and antibacterial properties (Zamolodchikova et al., 2020). delaCadena et al. (1996) proposed that the activity of α -L-fucosidase in tumor tissue is lower than that in healthy mucosa. Also, α -L-fucosidase may have a certain effect on patients with a high-risk of tumor recurrence of colorectal cancer (Fernández-Rodríguez et al., 2000).

The DEP for IM versus SX had 15 interacting proteins and 23 protein interactions, as shown in Figure 6C. The top 10 hub genes were *PRDX6*, *GLB1*, *TTR*, *SDCBP*, *CREG1*, *SNAP23*, *MANBA*, *STOM*, *MAN2A1*, and

CP. Peroxidase is a highly versatile protein that plays a vital role in regulating the level of peroxides in cells (Zeida et al., 2019). In particular, it is the center of cell redox homeostasis and signal transduction (Pastor-Flores et al., 2020). Also, *PRDX6* is highly expressed in lungs (Sundar et al., 2010) and is the only member of *PRDX* family that can move to damaged mitochondria (Yang et al., 2016), thus protecting nonalcoholic fatty liver disease by regulating mitochondria (Lee et al., 2019).

CONCLUSIONS

In this study, we characterized and compared Saanen goat milk samples collected from 3 provinces of China for whey proteins using a DIA quantitative proteomics technique. About 550 proteins were quantified in goat milk from each region. There were 124 enzymes out of 550 proteins, and most of the proteins were involved in carbohydrate-binding. The proteins of goat milk from GD and SX were most similar in composition. The DEP differed significantly in GO, KEGG pathways, and PPI. These difference may have been caused by different feeds of the goats and the location of the different provinces. Data of this study may provide useful information for use of Saanen goat milk in China, and the DEP can be used for discrimination and classification of Saanen goat milk of different geographic origins.

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