Comparative Whey Proteome Profiling of Donkey Milk With Human and Cow Milk

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Abstract

**Background:** Donkey milk (DM), similar to human milk (HM) in compositions, has been suggested as the best potential hypoallergenic replacement diet for babies suffering from cow milk (CM) protein allergens. Despite advances in proteomics technologies, studies of the DM whey proteome are relatively sparse. In this study, label-free mass spectrometry analysis was conducted to quantitatively identify the differentially expressed whey proteins (DEPs) in DM vs HM group and DM vs CM group.

**Results:** In total, 249 and 418 DEPs were found in these two groups respectively. DEPs were then subjected to intensive bioinformatic analysis. Results revealed that the majority of DEPs participated in lipid metabolic process, regulation of cytokine production, chemical homeostasis and catabolic process. Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways analysis found that these DEPs mainly participated in complement and coagulation cascades, and cholesterol metabolism.

**Conclusion:** These results may provide valuable information in the composition of milk whey proteins in DM, HM and CM, especially for low abundant components, and expand our knowledge of different biological functions between DM and HM or CM.

Background

Extensive studies on donkey milk (DM) nutritional properties and chemical composition have confirmed that DM differs from other dairy species employed for human feeding and has composition similar to human milk (HM). Rather than milks from ruminant species, DM has been indicated to be more suitable for human due to its remarkable nutritional value and good palatability. For example, DM shows potential for hypoallergenic diet replacement for babies suffering from cow milk (CM) protein allergies or food intolerance [1, 2]. DM is also a promising nutraceutical for the elderly because it can upregulate immune responses and prevent metabolic pathologies [3].

Milk contains about 3% protein, mainly whey, which has important bioactive properties and remarkably differs from proteins in different species, leading to changes in the composition and biological function of milk [4]. For example, the major whey proteins in DM are α-lactalbumin (α-LA), β-lactoglobulin (β-LG) and lysozyme (LYS), but β-LG which has been identified as the main allergen in CM does not occur in HM [5, 6]. And in mare's milk three molecular types of α-LA have been isolated while only one genetic variant was found in DM [7]. With the development of proteomic technologies, different fractions of the milk proteome, especially low-abundant proteins, can be analyzed in detail. In recent years, investigations on DM whey proteome are on the rise and many whey proteins have been identified using proteomic approaches. Previous study has reported the characterization of the protein profile of the DM whey fraction by direct RP-HPLC/electrospray ionization (ESI)-MS analysis [8]. Narrow pH (3.5 to 6) range two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry was applied to compare the whey proteins of DM, CM and HM, with particular focus on the major CM allergens [9]. Our previous study also investigated the DM whey proteome to explore the mechanism of influence on production traits of
DM by label-free based comparative proteomic analysis [10]. These studies provided useful information regarding the DM whey protein components, however, studies of the DM whey proteome are relatively sparse and less comprehensive, especially fully comparative analyses of differences between whey protein compositions and potential biological activities from DM and other species.

DM has been confirmed to be similar to HM and could be a better alternative to CM for infants [1, 11]. However, most studies mainly focused on the major components, the low abundant components in milk are emerging and found to be of great importance. The aim of this study was to characterize the differences in whey comparison and potential biological activities between DM and HM or CM, and more specifically focused on their relative abundance using label-free MS analysis which is a quantitative proteomic method with the advantage of identifying the entire expression profile of a sample of proteins with or without significantly different abundance between groups. Comprehensive analysis of these variations could gain a better understanding of the biological function and differences between DM and HM or CM, and promote the utilization of DM as nutrition provider.

**Results**

**Quantitative overview of identified whey proteins in different species**

By using a label-free proteomic approach, a total of 374 whey proteins were identified in DM vs HM group and 542 whey proteins in DM vs CM group, respectively (Fig. 1). Proteins identified in at least three replicates and having fold changes > 2.0 or < 0.5 and \( P < 0.05 \) were regarded as having undergone a significant change (defined as DEPs). In addition, the proteins identified in at least three replicates in one group and in another group with null values were defined as consistent presence/absence expression profile (DEPs). According to the fold change and \( P \) value between the two groups, a volcano plot was mapped and used to show significant differences (Fig. 2). Compared with those in HM, 154 whey proteins were upregulated and 95 whey proteins were downregulated in DM. Compared with those in CM, 134 whey proteins were upregulated and 284 whey proteins were downregulated in DM. The detailed information of DEPs is listed in Supplementary Table 1.

**Cluster analysis**

Meanwhile, a hierarchical cluster analysis was used to analyze the DEPs to compare the expression patterns of whey proteins in DM vs HM group and DM vs CM group (Fig. 3). The upregulated whey proteins in DM compared with HM mainly included vitamin D binding protein, joining chain of multimeric IgA and IgM, polymeric immunoglobulin receptor, lysozyme, and the downregulated whey proteins were peptidyl-prolyl cis-trans isomerase, elongation factor 1-alpha and lipoprotein lipase. The upregulated whey proteins in DM compared with CM mainly included vitamin D binding protein, serum albumin, joining chain of multimeric IgA and IgM, polymeric immunoglobulin receptor, and the downregulated whey proteins were peptidyl-prolyl cis-trans isomerase, nucleobindin 2 and so on.
GO analysis of DEPs in DM compared with HM and CM

According to the GO enrichment analysis of three distinctive functional sets, cellular component (CC), molecular function (MF), and biological process (BP) were applied (Fig. 4) with a corrected statistically significant level ($P < 0.05$) based on Fisher's exact test. In terms of molecular function, DEPs in DM vs HM group and DM vs CM group were both primarily related to lipid binding and steroid binding. In the category of biological processes, DEPs in these two groups were mainly involved in lipid metabolic process. Other major biological process categories included regulation of cytokine production, chemical homeostasis and catabolic process. The detailed information on the GO enrichment of DEPs is listed in Supplementary Table 2.

KEGG pathway analysis of DEPs in DM compared with HM and CM

KEGG pathway enrichment analysis was applied on the basis of Fisher's exact test, considering the whole quantified proteins as background dataset. Only pathways with $P < 0.05$ were considered as significant. In Fig. 5, the DEPs in DM vs HM group and DM vs CM group were mainly involved in complement and coagulation cascades, and cholesterol metabolism. The detailed information on the KEGG pathway enrichment of DEPs is listed in Supplementary Table 3.

Discussion

In this study, we applied a label-free mass spectrometry-based quantitative proteomics approach to compare the whey proteome profiling of DM with HM or CM. In total, 249 whey proteins were found to be significantly different between DM and HM; 418 whey proteins were significantly different between DM and CM. These DEPs were involved in lipid metabolic process, regulation of cytokine production, chemical homeostasis and catabolic process. These results may provide valuable information in the composition of milk whey proteins in DM, HM and CM, especially for low abundant components, and expand our knowledge of different biological functions between DM and HM or CM.

DM was characterized by a particularly high whey protein content which was rich in lysozyme, α-lactalbumin (α-La), β-lactoglobulin (β-Lg) and serum albumin (SA) [1]. In our data, we also found these whey proteins in DM were significantly higher than HM and CM. Among them, the amount of lysozyme in DM has proved to be higher with respect to that in CM, DM and goat’s milk [12]. As a powerful antibacterial protein, lysozyme plays an important role in the intestinal immune response. DM lysozyme belongs to C-type calcium-binding lysozyme and could bind calcium ions which leads to more stable complex with an enhanced antimicrobial activity [13]. β-Lg is the major whey protein in DM and CM, whereas in HM the β-Lg is absent [14]. However, in present study β-Lg was identified in HM with low abundance. The reason for this probably is that β-Lg can be detected in HM after dairy products ingestion [15, 16].
According to the GO and KEGG pathway enrichment analysis, DEPs in DM vs HM group and DM vs CM group were all mainly involved lipid binding, lipid metabolic process and cholesterol pathway. Among these DEPs, a family of apolipoproteins (Apos), namely, apolipoprotein A1 (ApoA1), ApoA2, ApoA4, ApoE and ApoH, were significantly upregulated in DM. ApoA1 and ApoA2 are the major proteins in high-density lipoproteins (HDL) [17]. ApoA1 has multiple beneficial functions, including potent antioxidant, anti-inflammatory, antiviral and antibacterial activities in blood [18] [19, 20]. Recently, ApoA1 was found in HM and DM. Kim et al. identified that ApoA1 interacts with cholesterol in HM, provides antioxidant activity and improves embryo survivability [21]. In DM milk fat globule membrane (MFGM), ApoA1 was upregulated in colostrum compared with mature milk [22]. ApoE and ApoH are also crucial elements in the lipoprotein metabolism and cholesterol transport [23, 24]. The higher concentrations of ApoE in CM and HM were found in early lactation, and significantly decreased over lactation, indicating the importance of cholesterol in the development of the neonate [25, 26]. Cholesterol plays an important role in the synthesis of vitamin D and the steroid hormones, which is critical to the development of the newborns [26]. In our study, DM provides a higher level of cholesterol transporters than HM and CM, which may help the newborns acquire a large amount of cholesterol. In addition to cholesterol transport function, recent work in both cell culture and in mice indicates that HDL (mainly ApoA1, and ApoA2) have anti-atherogenic effects and cause robust activation of endothelial nitric oxide (NO) synthase [17]. ApoE deficiency in mice results in a profound susceptibility to atherosclerosis [27]. Meanwhile, DM could induce human peripheral blood mononuclear cells (PBMCs) to release NO, which is very useful in the prevention of atherosclerosis [3]. Therefore, we hypothesized that these upregulated Apos might contribute the effect to atherosclerosis prevention induced by DM.

Milk provides large amounts of bioactive components to the infants in the critical phase of immunological immaturity of the newborn, particularly for the immune system of mucous membranes. Breastfeeding protects infants against infections mainly via secretory IgA (SIgA) antibodies. IgM antibodies, the second most abundant immunoglobulin in human colostrum, are also important in protecting the mucosal surfaces of infants through its reaction with viruses and bacteria [28]. Both dimeric IgA and pentameric IgM are transported across the epithelial cells into the milk by the polymeric Ig receptor (plgR), expressed on the basolateral surface of mammary epithelial cells [29, 30]. In addition to the heavy and light chains, dimeric IgA and pentameric IgM contain a small polypeptide known as the joining (J) chain, which plays an important role in the generation of secretory antibodies because it provides them with the capacity to bind the plgR [31]. This peptide can be produced by immunocytes of all Ig isotypes, but it becomes incorporated only into IgA and pentameric IgM [32]. Moreover, J chain expression may be a marker for B-cell clones derived from mucosa-associated lymphoid tissue, as there is a positive correlation between the production of polymeric IgA, IgG or IgD-producing cells and J chain [32, 33]. In our data, J chain were significantly upregulated in DM vs HM group (18.0-fold) and DM vs CM group (3.3-fold) respectively. We speculated that this high level of J chain production might reflects the abundant IgA, IgM or other immunoglobulins in DM and contributes to explain the previous studies indicating DM intake improves anti-inflammatory defenses in rats [34]. Other abundant whey proteins related to immune responses in DM included secreted phosphoprotein 1 (SPP1), complement C2 and
complement C3. SPP1, present in significant amounts in breast milk [35], is a multifunctional protein involved in cell-mediated immune responses and anti-inflammatory responses [36, 37]. Complement proteins are helpful for establishment of a natural immune system in newborns [38]. The presence of abundant immunological factors in DM are helpful for the newborns to establish an immune system against microbial infection to adapt to the new environment to prevent diseases.

**Conclusion**

In this study, a quantitative proteomic method was used to investigate the whey proteins proteome in DM, HM and CM, which will help us to understand the nutritional composition across different species comprehensively. DEPs were analyzed by multivariate statistical methods, and found mainly involved in lipid metabolic process, regulation of cytokine production, chemical homeostasis and catabolic process, which are of great significance to identify functional factors beneficial to infants. Our findings also provided more in-depth reference for the dairy food industry and for the health of infants.

**Methods**

**Sample collection and treatment**

Twelve human milk samples were donated by healthy mothers between 6 and 8 months’ lactation with written informed consent which indicated that the milk will be used in research. Twelve DM samples were obtained from a local farm breeding Dezhou donkeys in Liaocheng City of Shandong province, China. Twelve CM samples was the tank milk obtained in a local farm with the permission of farm manager (Liaocheng, China). Four samples of each group were randomly mixed and stored at −80°C until further analysis. The samples were thawed, pooled, and centrifuged at 10,000 × g for 10 min to remove the cream layer. A mammalian protease inhibitor cocktail was added to each pooled sample, which was then depleted of casein by using a previously described method [39]. In brief, 60 mM CaCl$_2$ was added, and pH was adjusted to 4.3. The samples were then centrifuged at 189,000 × g at 4 °C for 60 min, and the supernatant was collected.

**Protein extraction and digestion**

SDT (4% SDS, 100 mM Tris-HCl, 1 mM DTT, pH 7.6) buffer was used for sample lysis and protein extraction. The amount of protein was quantified with a BCA protein assay kit (Bio-Rad, USA), and the protein was digested by trypsin in accordance with filter-aided sample preparation (FASP) [40]. The digested peptides of each sample were desalted on C18 cartridges (Empore™ SPE C18 Cartridges [standard density], 7 mm bed I.D., 3 mL volume, Sigma), concentrated by vacuum centrifugation, and reconstituted in 40 µL of 0.1% (v/v) formic acid.

**Liquid Chromatography (LC)-Electrospray Ionization (ESI) Tandem MS (MS/MS) Analysis using Q Exactive Mass Spectrometer**
MS experiments were performed on a Q Exactive mass spectrometer coupled to Easy nLC (Proxeon Biosystems, Thermo Fisher Scientific). In this procedure, 5 µg of the peptide was loaded onto the C18-reversed phase column (Thermo Scientific Easy Column, 10 cm long, 75 µm inner diameter, 3 µm resin) in buffer A (2% acetonitrile and 0.1% formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) at a flow rate of 250 nL/min controlled by IntelliFlow for 120 min. MS data were acquired using a data-dependent top 10 method by dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. Target value was determined on the basis of predictive Automatic Gain Control (pAGC). The duration of dynamic exclusion was 25 s. Survey scans were acquired at a resolution of 70,000 at 200 m/z, and the resolution of the HCD spectra was set to 17,500 at 200 m/z and an isolation width of 2 m/z. Normalized collision energy was 30 eV, and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at the maximum fill time, was defined as 0.1%. The instrument was run in an enabled peptide recognition mode.

**Sequence database searching and protein quantification**

MS data were analyzed using MaxQuant version 1.3.0.5 and searched against the UniProtKB *Equus asinus* database (47825 total entries, downloaded on Aug.12, 2019), uniprot *Bos taurus* (included 45847 series, downloaded on May.7, 2019) and *homo sapiens* (included 20422 series, downloaded on May.22, 2019). An initial search was set at a precursor mass window of 6 ppm. The search was in accordance with an enzymatic cleavage rule of trypsin/P and allowed a maximum of two missed cleavage sites and a mass tolerance of 20 ppm for fragment ions. The carbamidomethylation of cysteines was defined as fixed modification, whereas protein N-terminal acetylation and methionine oxidation were described as variable modifications for database searching. The cutoff of the global false discovery rate (FDR) for peptide and protein identification was set to 0.01.

**Functional Analysis of DEPs**

The protein sequences of the selected DEPs were locally searched using NCBI BLAST + client (ncbi-blast-2.2.28+-win32.exe) and InterProScan to find homolog sequences. Gene ontology (GO) terms were mapped and sequences were annotated using Blast2GO. Afterward, DEPs were blasted against the online KEGG database (http://geneontology.org/) to retrieve their KEGG orthology identities and subsequently mapped to pathways in KEGG. Quantified whey samples were performed for hierarchical clustering by Cluster 3.0 software (USA). Enrichment analyses were applied on the basis of Fisher’s exact test in which the whole quantified protein annotations were considered as a background dataset, and only functional categories and pathways with $P < 0.05$ were recognized as significant.

**Abbreviations**

DM
donkey milk; HM:human milk; CM:cow milk; DEPs:differentially expressed whey proteins; α-La:α-lactalbumin; β-Lg:β-lactoglobulin; ApoA1:polipoprotein A1; MFGM:fat globule membrane; SPP1:secreted
Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of Shangdong Agricultural University. All procedures involving dairy cows and donkeys were performed in accordance with the Guide for Care and Use of Agricultural Animals of Shangdong Agricultural University. HM samples were donated by healthy mothers with written informed consent which indicated that the milk will only be used in research. DM and CM samples were obtained from two local farms with the permission of farm managers.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing financial interest.

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Authors’ Contributions

F.W.Z.: study design, article review, quality assessment, draft written; X.H.Z.: statistical analysis, draft written, manuscript revise; G.M.J., H.J.L., Y.T.W., C.L.J., L.Y., Q.Z.: recruited samples and interpreted the data. All authors have read and approved the manuscript”, and ensure that this is the case.

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