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Proteomic profiling of ovine milk after grading up

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Research Article

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Abstract

We have previously bred Chinese local dairy sheep through grading up with local Small-Tailed Han (STH) sheep as female parent and DairyMeade (DM) sheep as male parent. In this research communication we characterize the whey protein profile of STH sheep and their offspring (F1, F2) to reveal physiological differences and variation in milk traits. A total of 1032 whey proteins were identified through tandem mass tag labeling (TMT) proteome profiling. Three proteins were significantly differentially abundant between F1 and STH milk, six between F2 and STH milk and five between F1 and F2 milk. In terms of differential changes between generations, WASHC4 and CUTA of F1 and Ig-like domain-containing protein of F2 milk were dominant whey proteins. Overall, the results showed that the whey protein profiles of different generations varied little. The crossbreeds of STH and DM sheep would be suitable for the development of the Chinese local sheep milk industry, and the F2 may be a better population for sheep milk production.

Bovine milk predominates in the global dairy industry. However, some small ruminants such as goat and sheep have been developed to dairy breeds. Research has shown that ovine milk typically possesses higher concentration of protein, fat and lactose than either bovine or caprine (Ferro *et al*., 2017; Pietrzak-Fiećko and Kamelska-Sadowska, 2020). To satisfy increased consumer demand for healthier products, the further development of ovine milk may be a suitable option for the dairy market.

There is no registered dairy sheep breed in China until now. The Small-Tailed Han (STH) sheep is a Chinese local dual-purpose (meat and wool) breed with long legs and large body also known for its high fecundity. In addition, it has good adaptability for various climatic conditions. These attributes would be useful in a dairy breed, however, the average milk yield (0.645 l/day) of STH ewes is lower compared with Katahdin (1.38 l/day) and Saint Croix (1.26 l/day) ewes (Wang *et al*., 2020). The DairyMeade (DM) sheep, introduced from New Zealand in 2015, with high milk yield inheritance (King *et al*., 2014) is a suitable male parent for Chinese dairy sheep breeding.

Milk proteins are generally categorized into three major groups: caseins, whey proteins and milk fat globule membrane proteins (Lönnerdal, 2014). There are a lot of proteins and peptides in whey that could be identify and quantified by proteomics technology. Some proteomic studies of bovine milk have characterized its bioactive profile and verified various biological functions (Lönnerdal, 2014; Reznikov *et al*., 2014). Also, there are some studies that described the difference between ovine and other species milk (Anagnostopoulos *et al*., 2016; Chen *et al*., 2016; Greco *et al*., 2018; Tomazou *et al*., 2019). However, few studies focused on cross breeding effects on milk proteomics. Previously, we obtained F1 and F2 offspring through grading up strategy with the STH sheep as female parent and DM sheep as male parent. We hypothesize that the proteome profile of different generations' milk may have special properties due to its different genetic background and milk producing ability. Therefore, the present study aimed to characterize the whey protein profile of STH sheep and its offspring which could reveal the physiological differences and variation in milk traits. The results would be a reference for ovine milk selection and for local new dairy sheep breeding.

Materials and methods

All procedures involving animals were approved by the Ethical Principles for the Use of Animals for Scientific Purposes of the Inner Mongolia University of China. All experiments were performed according to Chinese laws and institutional guidelines.

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Table 1. Significantly differential abundant whey proteins between different generations

Comparation	Accession	Gene name	Description	Fold change	*P* value
F1 *vs.* STH	W5Q6S3	WASHC4	Uncharacterized protein OS = *Ovis aries* OX = 9940 GN = WASHC4 PE = 4 SV = 1	7.77	4.52×10^{-5}
	W5PJA0	CUTA	Uncharacterized protein OS = *Ovis aries* OX = 9940 GN = CUTA PE = 4 SV = 1	3.72	0.020548
	W5Q3X2		Peptidylprolyl isomerase OS = *Ovis aries* OX = 9940 PE = 4 SV = 1	2.02	0.042887
F2 *vs.* STH	W5NXL7		Ig-like domain-containing protein OS = *Ovis aries* OX = 9940 PE = 4 SV = 1	3.38	0.000231
	P68240		Hemoglobin subunit alpha-1/2 OS = *Ovis aries* OX = 9940 PE = 1 SV = 2	3.16	0.013446
	W5QCY8	LOC101105437	GLOBIN domain-containing protein OS = *Ovis aries* OX = 9940 GN = LOC101105437 PE = 3 SV = 1	2.32	0.013173
	W5NXH0	LOC101117231	Uncharacterized protein OS = *Ovis aries* OX = 9940 GN = LOC101117231 PE = 4 SV = 1	2.29	0.042306
	W5PZJ1	SCGB2A2	Uncharacterized protein OS = *Ovis aries* OX = 9940 GN = SCGB2A2 PE = 4 SV = 1	0.50	0.018415
	W5PZD0	LOC101110099	Uncharacterized protein OS = *Ovis aries* OX = 9940 GN = LOC101110099 PE = 4 SV = 1	0.47	0.018224
F1 *vs.* F2	W5Q6S3	WASHC4	Uncharacterized protein OS = *Ovis aries* OX = 9940 GN = WASHC4 PE = 4 SV = 1	6.63	4.69×10^{-5}
	W5PJA0	CUTA	Uncharacterized protein OS = *Ovis aries* OX = 9940 GN = CUTA PE = 4 SV = 1	2.36	0.032462
	P42819	SAA1	Serum amyloid A protein OS = *Ovis aries* OX = 9940 GN = SAA1 PE = 1 SV = 1	2.26	0.02737
	W5NQW4	LOC101104482	Uncharacterized protein OS = *Ovis aries* OX = 9940 GN = LOC101104482 PE = 4 SV = 1	0.44	0.023071
	W5NXL7		Ig-like domain-containing protein OS = *Ovis aries* OX = 9940 PE = 4 SV = 1	0.43	0.001573

STH, Small-Tailed Han sheep; DM, DairyMeade sheep; F1, DM (♂) × STH (♀); F2, DM (♂) × F1 (♀).

Animals and sample preparation

At 90 d of lambing (peak lactation period), four 2–4 years and parous STH, F1 (STH (♀) × DM (♂)) and F2 (F1 (♀) × DM (♂)) ewes with similar body weight of 55–60 kg were selected for milk sampling. The ewes were fed at the standard conditions of Mengtianran Dairy Co. Ltd. (Ulanqab, Inner Mongolia autonomous region, China). Samples were aseptically collected before feeding in the morning for three days, then placed in liquid nitrogen and immediately transported to the laboratory for further analysis.

Analysis of whey proteins

The analysis of whey proteins of milk was processed by LC-bio Co. LTD. (Hangzhou, Zhejiang, China). The three milk samples of each ewe were mixed together and prepared by centrifugation at $4000 \times \boldsymbol{g}$ for 30 min, filtering and ultracentrifugation at $100\,000 \times \boldsymbol{g}$ for 60 min. After ultra-isolation, the supernatant containing whey protein was collected and stored at −80°C for later use. The protein concentration was determined by BCA method.

Protein digestion and tandem mass tag labeling

The proteins were digested with trypsin. Firstly, proteins were incorporated into SDT buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0). The UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.5) was used to remove the impurities. Then iodoacetamide was added and incubated for 30 min in darkness. The filters were washed with UA buffer and 0.1 M TEAB buffer. Finally, the protein suspensions were digested with 4 trypsin overnight at 37°C, and the resulting peptides were collected as a filtrate. The peptide content was estimated by UV light spectral density. The peptide mixture of each sample was labeled with TMT reagent according to the manufacturer's instructions (Thermo Fisher Scientific).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

TMT labeled peptides were fractionated by RP chromatography using the Agilent 1260 infinity II HPLC. Firstly, the peptide mixture was diluted with buffer A (10 mM $HCOONH_4$, 5% ACN, pH 10.0) and loaded onto a XBridge Peptide BEH C18 Column. Then peptides were eluted at with a gradient of buffer B (10 mM $HCOONH_4$, 85% ACN, pH 10.0). The fractions were collected every 1 min during 5–50 min. The collected fractions were dried down *via* vacuum centrifugation at 45°C.

The peptide mixture was loaded onto the C18-reversed phase analytical column (Thermo Fisher Scientific) in buffer A (0.1% Formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% Formic acid). LC-MS/MS analysis was

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performed on a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) that was coupled to Easy nLC (Thermo Fisher Scientific). The mass spectrometer was operated in positive ion mode. MS data was acquired over a range of 350–1800 *m*/*z* survey scan for HCD fragmentation. Survey scans were acquired at a resolution of 70 000 at *m*/*z* 200 and resolution for HCD spectra was set to 17 500 at *m*/*z* 200, and isolation width was 2 *m*/*z*. Normalized collision energy was 30 eV.

Data analysis

The high resolution mass spectrometer Q Exactive plus (Thermo Fisher Scientific) was used for TMT quantitative proteomics analysis. Proteome Discoverer 2.1 (Thermo Fisher Scientific) software and MASCOT 2.6 (Matrix Science) were used to analysis and identification of proteins. Differential analysis based on the FDR < 0.01 or $P < 0.05$ standard was used to obtain highly reliable qualitative results.

Results and discussion

The proteomes of STH, F1 and F2 ovine milk were profiled through TMT combined with LC-MS/MS proteomic technology. Four ewes' milks of each group ($n = 4$) were collected. In total, 1032 proteins were identified (online Supplementary Table S1). The abundance of most proteins showed no significant difference ($P > 0.05$) between the three groups. Three proteins with significantly different abundance were found between F1 and STH milk, two of which were annotated as WASHC4 and CUTA (Table 1). Six proteins with significantly different abundance were found between F2 and STH milk, of which four were annotated asLOC101105437, LOC101117231, SCGB2A2 and LOC101110099 (Table 1). Five proteins with significantly different abundance were found between F1 and F2 milk, of which four were annotated as WASHC4, CUTA, SAA1 and LOC101104482 (Table 1). In terms of differential changes between generations, WASHC4 and CUTA of F1 and Ig-like domain-containing protein of F2 milk were dominant whey proteins.

Proteome profiling showed most of whey proteins to have a similar abundance among three generations' milk, which indicated that three generations' milks were similar and the grading up strategy maintained the majority characteristics of female parent. The protein of WASHC4 is an interactor of valosin containing protein, the latter is a key regulator of cellular proteostasis (Kustermann *et al*., 2018). Loss of WASHC4 is associated with myopathy of muscle cells (Kustermann *et al*., 2018) but has no known functional significance in the mammary gland. We speculate that a high level of WASHC4 may be helpful for milk proteostasis and maintenance of high level of protein content. CUTA is a ubiquitous trimeric protein, homologous to the bacterial CUTA1 protein that belongs to an operon involved in resistance to divalent ions ('copper tolerance A') (Hou *et al*., 2015). High level of CUTA in F1 milk may be related to an unusual amount of divalent ions in their environment.

Serum amyloid A1 (SAA1) is an acute phase protein of inflammation (Lu *et al*., 2019). In our study SAA1 was differentially expressed in F1 relative to F2. A previous study showed that high level of SAA1 would dramatically impair the nutritional value of the milk of females and cause the retarded growth and development of the pups, even to death (de Groot *et al*., 2001). However, there is no similar report in livestock. Another research of dairy cattle revealed that the *SAA1* gene was differentially expressed in the mammary glands of lactating Holstein cows with extremely high *vs*. low phenotypic values of milk protein and fat percentage (Yang *et al*., 2016). Based on these observations, we can propose that the F2 generation may be a better population for milk production.

In conclusion, our results showed that the whey protein profiles of different generations varied little, which indicated that the crossbreeds of STH and DM sheep would be suitable for the development of the Chinese local sheep milk industry.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000455

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