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Comparative proteomics of milk fat globule membrane in different species reveals variations in lactation and nutrition

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ABSTRACT

In present study, 312, 554, 175 and 143 proteins were identified and quantified by label-free quantitative proteomics in human, cow, goat and yak milk fat globule membrane (MFGM), respectively. Fifty proteins involved in vesicle mediate transport and milk fat globule secretion were conserved among species. Moreover, proteins involved in lipid synthesis and secretion (xanthine dehydrogenase/oxidase, stomatin and CD36), showed different expression pattern and the host defense proteins exhibited various profiles within species. Notably, the content and activity of lipid catabolic enzymes were significantly higher in human MFGM, which could be indicative of the superior fat utilization in breast fed infants. Our findings unraveled the significant differences in protein composition of human milk and conventionally used substitutes of it. The in-depth study of lipid metabolic enzymes in human MFGM will probably contribute to the improvement of the fat utilization through modulation of lipid catabolic enzymes in infant formula.

1. Introduction

Milk is consumed worldwide with bovine milk accounting for 80–90% of total consumption. Due to regional and cultural varieties, milk from other species, such as goat, yak and camel milk, is also well used as food resources. As the substitute of human milk in infant food, milk from different species has been studied extensively to address their nutritional value. However, most studies mainly focused on the major components (Hinz, O'Connor, Huppertz, Ross, & Kelly, 2012; Spertino et al., 2012). As the development of analytical methods, the low abundant components in milk are emerging and found to be of great importance. The comprehensive understanding of variations between milk from different species could help promote the utilization of various milk sources as nutrition provider especially in infant food.

Milk fat globule membrane (MFGM) is a 3-layer membrane covering on milk fat globule, which consists of proteins and lipids. Although MFGM proteins account for only 1–4% of total milk proteins, more than two hundred proteins with diverse functions have been identified by proteomics analysis (Reinhardt & Lippolis, 2006).

MFGM is originated from endoplasmic reticulum membrane, cytoplasm and apical plasma membrane of secretory cells in mammary gland (McManaman & Neville, 2003). Thus, MFGM proteins are considered as representatives of the secretory cells (Cebo, 2012) and have been used to study the milk synthesis and secretion by using proteomics techniques, which promoted the understanding of lactation biology in bovine. Lu et al. have identified hundreds of proteins involved in lipid synthesis and secretion in bovine MFGM and applied proteomic and metabolic analysis on MFGM proteins to explain the lactation physiology in cows with negative energy balance and different DGAT1 genotypes (Lu, Boeren, Van Hooijdonk, Vervoort, & Hettinga, 2015; Lu, van Hooijdonk, Boeren, Vervoort, & Hettinga, 2014; Lu et al., 2013). The MFGM proteome was also used to elucidate the mechanism of mastitis in lactating cows (Reinhardt, Sacco, Nonnecke, & Lippolis, 2013).

The nutritional value of MFGM proteins on human health cannot be neglected. The health benefits of some major MFGM proteins have been investigated. Butyrophilin, which is the most abundant MFGM proteins, has been reported to modulate the encephalitogenic T-cell response to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis related to human multiple sclerosis (Guggenmos et al., 2004; Stefferl et al., 2000). Fatty acid binding protein has been found to have anticancer effect on breast cancer cell line (Spitsberg & Gorewit, 2002; Spitsberg, Matitashvili, & Gorewit, 1995). Mucins are well known for their anti-adhesive property due to their high glycosylation which can

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protect the attack of bacteria and enzyme on epithelial cells (Parker et al., 2010; Sando et al., 2009). In addition to the major proteins, minor proteins play their biological function at low concentration. The blue print of MFGM proteome is thus on the spotlight in the last decades by identifying hundreds of bioactive proteins.

In present paper, advanced proteomics analysis was applied to elucidate the different and conserved proteins in MFGM from human milk and three conventionally consumed milk source in China, including cow milk, goat milk and yak milk. The reasons for the variations and their influence on the nutritional value were dissected.

2. Material and methods

2.1. Sample collection

Human milk was donated by 7 lactating women with written informed consent which indicated that the milk will be used in research. Cow milk was the tank milk obtained in Sino farm with the permission of farm manager (Beijing, China). Goat milk was the tank milk obtained in the farm of Shengtang Dairy Co. Ltd with the permission of farm manager (Shanxi Province, China). Yak milk was the tank milk obtained in the farm of Yake Dairy Co. Ltd with the permission of farm manager (Qinghai Province, China).

2.2. Separation of MFGM proteins

The separation of MFGM proteins was based on the paper of Lu et al. (Lu et al., 2013).The pooled milk samples were centrifuged at 1500 g (10 min) to obtain cream (top layer). The cream (0.5 mL) was separated and washed with 5 mL of PBS (0.1 M, pH 6.8), and centrifuged at 1500 g (10 min). Thereafter, the washing solution was discharged. This step was repeated three times. The washed milk cream was mixed with 0.4% SDS (1:1, v/v), sonicated for 1 min, and centrifuged at 1500*g* (10 min). Subsequently, the MFGM proteins (bottom layer) were separated from the remaining fat. The protein concentration was determined using the BCA assay (Thermo Scientific Pierce BCA protein assay kit, USA).

2.3. Protein digestion

The protein digestion procedure was described before (Ding et al., 2013) with modifications: 10 μl protein were dissolved in 100 μl 0.05 M NH_4HCO_3 and reduced by adding 10 μl 0.1 M dithiothreitol for 30 min at 56 °C. Subsequently, the sample was alkylated by adding 15 μl of 0.5 M iodoacetamide for 30 min at room temperature in the dark and then digested by adding trypsin at a mass ratio of 1:100 enzyme/protein overnight at 37 °C and stopped by addition of 1% formic acid (FA). The sample was desalting by a C18 column.

2.4. EASY-nLC-Orbitrap LTQ VELOS

The settings of LC–MS was based on the paper of Ding et al. (Ding et al., 2013). Digested protein samples were injected to a homemade C18 pre-column (100 μm inner diameter, 360 μm outer diameter × 2 cm, 5 μm 150 Å pore size, Durashell C18 particle) followed by separating over a C18 analytical column (75 μm inner-diameter, 360 μm outer-diameter × 15 cm, 3 μm150 Å pore size, Durashell C18). Mobile phase A consisted of 0.2% formic acid in water, and mobile phase B consisted of 0.2% formic acid in acetonitrile; a series of adjusted linear gradients according to the hydrophobicity of fractions eluted in LC with a flow rate of 380 nL/min. The MS conditions are as the followings: The source was operated at 2.0 kV, with no sheath gas flow and with the ion transfer tube at 350 °C. The mass spectrometer was programmed to acquire in a data dependent acquisition mode. The survey scan was from *m*/*z* 375 to 1300 with resolution 60,000 at *m*/*z* 400. The 25 most intense peaks with charge state 2 and above were acquired with collision induced dissociation with normalized collision energy of 35%, activation time of 10 ms, one microscan and the intensity threshold was set at 50 counts. The MS^2 spectra were acquired in the LTQ normal scan mode.

2.5. Lipase activity analysis

The lipase activity of MFGM proteins was analyzed by Quantichrom™ Lipase Assay Kit with or without 0.7% Bile salts (Bioassay system, USA).

2.6. Data Analysis

Twelve LC-MS/MS raw files were obtained with three replicates for MFGM of each species. All MS/MS spectra obtained in each run were analyzed by MaxQuant 1.5.0.12, with Andromeda as peptide search engine (Cox et al., 2011). The database for peptide/protein searches was a concatenated homo sapiens, bos taurus, bos mutus and capra genus database downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) with reverse sequences generated by MaxQuant. The contaminants database of MaxQuant was also used for peptide/protein searches including sequences of trypsin and human keratins. Identification and quantification of proteins were simultaneously performed in MaxQuant. Carbamidomethylated cysteine was set as fixed modification; oxidation of methionine, N-terminal acetylation, and deamidation of asparagines or glutamine were set as variable modifications for both identification and quantification. A mass deviation of 0.5 Da was set as maximum allowed for MS/MS peaks, and a maximum of two missed cleavages was allowed. Maximum false discovery rates (FDRs) were set to 1% both on peptide and protein levels. Minimum required peptide length was 6 amino acids for both identification and quantification. A minimum of 2 peptides for each protein were required for reliable identification and quantification. Protein intensity calculated by Maxquant was used for the quantification analysis. The abundance of each protein were calculated as following: protein intensity/summed all identified protein intensity × 100%.

2.7. Statistical analysis

The calculated abundance of each protein was used in statistical analysis. Student *t*-test were performed with PASW statistics 18 (SPSS Inc., USA) to visualize the significant different proteins in MFGM of each species ($p < 0.05$). Pearson correlation analysis was performed using Origin 9.1(OriginLab Corporation, USA). Principle component analysis was performed with Matlab 7.0 (Mathworks, USA).

2.8. Cluster analysis and Gene Ontology enrichment analysis

Clusters of proteins were obtained according to the protein abundance with complete Euclidean as algorithm in MultiExperiment Viewer (MeV) package31 (http://www.tm4.org/mev/). The gene ontology (GO) enrichment of proteins was performed by using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) (Huang, Sherman, & Lempicki, 2008).

3. Results

3.1. Identification and quantification of MFGM proteins in different species

By using proteomics analysis, 312, 554, 175 and 143 proteins were identified and quantified in human, cow, goat and yak MFGM