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Perspective on calf and mammary gland development through changes in the bovine milk proteome over a complete lactation

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ABSTRACT

Milk contains all the nutrients for the growth and development of the neonate. However, milk composition is not constant during lactation. To study the changes of the milk proteome over lactation, filter-aided sample preparation combined with dimethyl labeling followed by liquid chromatography tandem mass spectrometry was used to identify and quantify milk proteins from 4 cows. A total of 229 proteins were identified, of which 219 were quantified. An 80% overlap was found in identified and quantified proteins between the 4 individual cows during lactation. Over lactation, the number of quantified proteins changed slightly (less than 10%), whereas the concentration of proteins changed considerably. Transport proteins involved in lipid synthesis (fatty acid–binding protein, perilipin-2, butyrophilin) increased, whereas proteins related to cholesterol transport (apolipoprotein E) decreased. The changes of lipid-synthesis proteins are in accordance with the increased milk fat yield over lactation, indicating the increase of de novo mammary fatty acid synthesis as lactation advances. The high abundance of immune-related proteins in early lactation indicates the important role of these proteins for immune-system development of calves. The increase in immune-related proteins (immunoglobulins, osteopontin, lactoferrin) and the decrease of proteins related to milk-component synthesis (α-lactalbumin, β-lactoglobulin, fatty acid–binding protein, perilipin-2, butyrophilin) in late lactation can be associated with the protection of the mammary gland. In conclusion, the changes of proteins with different biological functions reflect not only the changing needs of calves but also the development and protection of the mammary gland over lactation.

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INTRODUCTION

Milk provides complete nutrition and bioactive proteins, which are essential for not only the development but also the health benefits of newborns (German and Dillard, 2006; Casado et al., 2009). Breast milk has been considered as the best food for infants (Lonnerdal, 2010). Because of a variety of reasons, a certain number of babies will not get breast milk and will therefore rely on infant formula for survival. Infant formula is developed with bovine milk as a protein source for mimicking human milk (Hernell, 2011). The differences in composition between human milk and bovine milk (D'Auria et al., 2005) have been shown to result in different health benefits of infants fed with breast milk or infant formula; for example, breastfed infants have fewer infections (gastrointestinal infections, acute otitis media) and reduced risk for celiac disease, obesity, and diabetes compared with formula-fed infants (Dewey, 2001; Hernell, 2011).

Recent developments in proteomic techniques have led to an interest in the bovine milk proteome. Previous research identified milk proteins in bovine colostrum and mature milk (D'Alessandro et al., 2011; Nissen et al., 2012; Sacerdote et al., 2013), and the quantitative differences in the milk proteome between bovine colostrum and mature milk (Stelwagen et al., 2009; Zhang et al., 2015). So far, however, variation has not been studied in these low-abundant proteins in mature bovine milk over the whole lactation (from early to the end of lactation). In addition, previous studies on the bovine milk proteome did not take into account individual differences nor was variation of milk proteome between individual cows examined over a full lactation period.

Such a comprehensive study of the variation in low-abundant proteins of bovine milk over lactation is ex-

pected to not only contribute to our understanding of the needs of calves over lactation but also to increase our understanding of the involution of the mammary gland and mastitis. Therefore, the objective of this study was to determine the qualitative and quantitative variation in the milk proteome from 4 individual cows from early to late lactation using proteomic techniques that combine filter-aided sample preparation and dimethyl labeling followed by liquid chromatography tandem mass spectrometry.

MATERIALS AND METHODS

Materials

Bovine milk was collected from 4 healthy, primiparous, Holstein-Friesian cows in a farm in Zaffelaere, Belgium, from August 2012 to August 2013. The cows were milked using an automatic milking system, with an average milk yield of 27.5 ± 6.5 kg/d. No specific permissions were required for this sample collection, because samples were taken from the milk collected during regular milking. A 100 mL sample, which was pooled milk from all 4 quarters representing an average of the whole milking, was collected at each time point. The samples were frozen immediately at −20°C after collection and transferred frozen to the laboratory for protein, fat, lactose, and proteomic analysis. Samples collected at 0.5, 1, 2, 3, 6, and 9 mo and the latest time point of the lactation (10 mo for cow 1, 11 mo for cow 2, and 12 mo for cow 3, the latest time point was missed for cow 4) were used for this study. The colostrum samples have been analyzed and published already (Zhang et al., 2015).

Milk Composition Analysis and Proteomics Techniques

Milk samples were analyzed for SCC and protein, fat, and lactose contents with a CombiFoss 5000 at Qlip (Foss, Zutphen, the Netherlands).

The proteomics methods used in this study were based on previous articles (Hettinga et al., 2011; Lu et al., 2011; Zhang et al., 2015).

Milk Serum Separation

Milk samples collected at different time points from 4 individual cows were centrifuged at 1,500 × *g* for 10 min at 10°C (Beckman Coulter Avanti J-26XP centrifuge, rotor JA-25.15, Beckman Coulter Inc., Brea, CA). The fat was removed and the obtained supernatant was transferred to the ultracentrifuge tubes followed by ultracentrifugation at 100,000 × *g* for 90 min at 30°C (Beckman L-60, rotor 70 Ti, Beckman Coulter Inc.). After ultracentrifugation, samples were separated into 3 phases. The top layer was milk fat, the middle layer was milk serum, and the bottom layer (pellet) was casein. Milk serum was used for bicinchoninic acid (BCA) assay and filter-aided sample preparation as described below.

BCA Assay

A BCA Protein Assay Kit 23225 (Thermo Scientific Pierce, Rockford, IL) was used for protein concentration determination, according to the manufacturer's instructions. Bovine serum albumin was used as standard for making a calibration curve. The standard curve covers the protein concentration from 0.02 to 2 μg/μL. Subsequently, the milk serum protein concentration was determined.

Filter-Aided Sample Preparation

Milk serum samples (20 μL), including samples of each time point and pooled samples of all time points from each cow, were diluted in 0.1 *M* Tris/HCl pH 8.0 + 4% sodium dodecyl sulfate + 0.1 *M* dithiotreitol (SDT-lysis buffer) to obtain a 1 μg/μL protein solution. Samples were then incubated for 10 min at 95°C and centrifuged at 18,407 × *g* for 10 min after cooling down to room temperature. Twenty microliters of sample was directly added to 180 μL of 0.05 *M* iodoacetamide/0.1 *M* Tris/HCl pH 8.0 + 8 *M* urea (**UT**) in a low-binding Eppendorf tube (LoBind tubes, Hamburg, Germany) and incubated for 10 min while mildly shaking at room temperature. All of the sample was transferred to a Pall 3K omega filter (10–20 kDa cutoff, OD003C34; Pall, Washington, NY) and centrifuged at 15,871 × *g* for 30 min. A total of 100 μL of iodoacetamide (0.05 *M* iodoacetamide in UT) was added and incubated for 10 min at room temperature and then centrifuged at 15,871 × *g* for 30 min. Three repeated centrifugations at 15,871 × *g* for 30 min were carried out after adding 100 μL of UT 3 times. After that, 110 μL of 0.05 *M* NH_4HCO_3 in water (**ABC**) was added to the filter unit, and the samples were centrifuged again at 15,871 × *g* for 30 min. Then, the filter was transferred to a new low-binding Eppendorf tube. A total of 100 μL of ABC containing 0.5 μg of trypsin was added followed by overnight incubation at room temperature. Finally, the sample was centrifuged at 15,871 × *g* for 30 min, and 3.5 μL of 10% trifluoroacetic acid was added to the filtrate to adjust the pH value of the sample to around 2. These samples with protein >10 to 20 kDa were ready for dimethyl labeling.