Dynamics of the proteome in human and farm animal milk

Lina Zhang

Thesis committee

Promotor

Prof. Dr A.C.M. van Hooijdonk
Professor of Dairy Science and Technology
Wageningen University

Co-promotors

Dr K.A. Hettinga
Assistant professor, Food Quality and Design
Wageningen University

Dr J.J.M. Vervoort
Associate professor, Laboratory of Biochemistry
Wageningen University

Other members

Prof. Dr H.F.J. Savelkoul , Wageningen University
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Dr E.A.F van Tol, MeadJohnson Nutrition, Nijmegen

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Thesis

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Chapter 1

General introduction

1.1 Introduction

Milk is a complete and complex food suited to the requirements for the growth and development of the neonate. It contains lipids, carbohydrates, proteins, minerals, and vitamins. Currently, the milk proteins are attracting more and more attention because of their importance in the development of the immune system and gastrointestinal tract of the neonate (Casado et al., 2009).

Human milk can reduce the risk of sepsis and necrotizing enterocolitis for neonate, especially for very low birth weight babies (Meier and Bode, 2013). In the United States, more than 900 infants can be saved per year if 90% of mothers would exclusively breastfed for 6 months. In developing countries, more than 1 million infants can be saved per year with the same period of breastfeeding (Breastfeeding, 2012). Therefore, the WHO recommends to exclusively breastfeed infants for the first 6 months of life (Walker, 2010). Although human milk is considered as the best food for the neonate (Molinari et al., 2012), still a certain number of infants need to rely on infant formula, because of insufficient milk production, medical reasons, and mothers who choose not to breastfeed.

In the 19th century, human milk substitutes started to be developed. From the early 20th century, infant formula was developed using human milk as reference and bovine milk as major source (Hernell, 2011). Despite the fact that infant formula can provide sufficient nutrients for the growth of infants (Ladomenou et al., 2010), formula feeding has been shown to lead to different growth patterns in infants compared to that of breastfed infants. Studies have shown that breastfed infants require less doctor visits for acute otitis media, acute respiratory infection, and gastroenteritis compared to formula-feed infants (Ladomenou et al., 2010). Nutritional status and intestinal microbiota between breastfed infants and formula-fed infants were also reported to be different, which may be associated with different disease risk later in life (Lönnerdal, 2014). The short- and long-term medical and developmental advantages of breastfeeding relative to formula feeding are likely related to differences in bioactive components, such as proteins which are involved in the development of the immune and digestion system of the neonate (Newburg, 2001, Lönnerdal, 2014). The milk proteome not only differs qualitatively and quantitatively between species (Hettinga et al., 2011) but also changes due to factors, such as lactation, health status, and individual differences (Gustafsson

et al., 2005, Safi et al., 2009, Liao et al., 2011a, c, Hinz et al., 2012b, Le Parc et al., 2014). A comprehensive understanding of differences in milk protein composition within and between species, as well as the effect of processing, will lead to a better understanding of the gap between bovine milk (the major supply of infant formula) and the needs of infants (breast milk). The aim of the research described in this thesis is therefore to study the differences in proteins from human and dairy animal for a better understanding of their biological functions in growth and development of the neonate.

1.2 Milk proteins

The milk protein content ranges from 12 to 34 g/l from human to bovine, camel, and caprine. Milk contains a variety of proteins and these proteins are considered to be highly digestible, resulting in a high amino acids uptake (Nilsson et al., 2007). Generally, milk proteins can be grouped into three major classes: caseins, milk fat globule membrane (MFGM) proteins, and whey proteins (also called milk serum proteins). Caseins, the most abundant milk proteins in the milk of most animals, are a valuable source of amino acids, phosphate, and calcium, used for the growth of the neonate. MFGM proteins account for only 1–2% of the total milk protein content. Although MFGM proteins have small contributions in nutritional value, they have been reported to play an important role in various cellular processes and defense mechanisms in the newborn (Cavaletto et al., 2008, Liao et al., 2011a). Milk serum comprises 20% to 60% of all proteins in animal and human milk. It not only has a high nutritional value but also contains many bioactive proteins, such as immunologically-active proteins (Hettinga et al., 2011, Liao et al., 2011b).

1.2.1 Milk serum proteins involved in the transport of nutrients

Milk serum proteins have diverse functional properties. The principal function of milk proteins is providing essential amino acids and nitrogen, but they are also involved in additional biological functions. For instance, β-lactoglobulin functions in transporting fatty acid and possibly also vitamin A; α-lactalbumin provides a high proportion of Tryptophan and is also coenzyme for biosynthesis of lactose; serum albumin plays a role in transporting fatty acids (Korhonen, 2009). In addition to these relatively high abundant proteins, low abundant proteins in milk serum also participate in transporting nutrients.

Lipoprotein lipase, platelet glycoprotein 4, and fatty acid-binding proteins function in transporting fatty acids thereby accelerating the up-take of long chain fatty acids by epithelial cells; perilipin-2, butyrophilin, lactadherin, and xanthine dehydrogenase/oxidase, are related to milk lipid droplet formation and secretion; apolipoprotein E, apolipoprotein A-I, apolipoprotein A-IV, and apolipoprotein C-III are involved in cholesterol transport & synthesis (Lu et al., 2014).

1.2.2 Milk serum involved in the immune system

Milk serum proteins are also involved in the protection against pathogens and development of the immune system in neonates (Haug et al., 2007, Kanwar et al., 2009). The function of immune proteins can be divided into innate immunity and adaptive immunity. Innate immunity acts as the first line of defense and reacts with infectious pathogens before adaptive immunity plays its role. Complement proteins (including C1R, C3, C4A, C5, and C7) play a central role in the innate immune system. Antibacterial proteins are also related to innate immunity, including lactoferrin, glycosylation-dependent cell adhesion molecule 1, lactoperoxidase, osteopontin, CD14, and alpha-1-acid glycoprotein 1. Their antibacterial effects could be enhanced by interactions with other immune-active proteins (Wheeler et al., 2007a, Hettinga et al., 2011, Zhang et al., 2013). The adaptive immune system slowly responds to novel threats but it will trigger the memory of T and B cells to quickly eliminate the threats if the host is exposed to the threat again. Immunoglobulins, including immunoglobulin G (IgG) and immunoglobulin A (IgA), are the main immune components of the adaptive immune system (Stelwagen et al., 2009). IgG functions in the protection against pathogens, whereas IgA plays a critical role in the mucosal immune system (Kovar et al., 1984, Stelwagen et al., 2009). Blood coagulation proteins (fibrinogen alpha chain and fibrinogen beta chain) and protease inhibitors (alpha-1-antitrypsin, antithrombin-III, serpins, and inter-alpha trypsin inhibitor heavy chain 1) are also important for the immune system. They participate in activation and inactivation of proteins involved in innate and adaptive immune pathways (Gao et al., 2012) .

1.3 Variability of milk serum proteins

Milk serum proteins differ both qualitatively and quantitatively due to many factors, such as species differences, lactation stage, and animal health.

1.3.1 Species differences

The differences in milk serum proteins between species have been previously reported, as shown in Table 1.1. β-lactoglobulin is the most abundant protein in bovine and caprine milk serum (Zervas and Tsiplakou, 2013), whereas it is absent in human and camel milk serum (El-Agamy, 2008, Hinz et al., 2012b). IgG is the predominant antibody in bovine, caprine, and camel milk, while it is IgA in human milk (Stelwagen et al., 2009, Sánchez-Macías et al., 2014). IgG can be transferred to the foetus prior to birth in humans but not in several animals (e.g. cattle and sheep) (Stelwagen et al., 2009). The relatively low abundant proteins, such as complement proteins, antibacterial proteins, acute phase proteins, blood coagulation proteins, and protease inhibitors, are also different in concentration between species (D'Auria et al., 2005, Hettinga et al., 2011).

Table 1.1 The milk serum protein content in human and farm animal milk

Content (mg/mL)	Human	Bovine	Camel	Caprine
Milk serum	12	9	5.6	6
β-lactoglobulin	NP	3.5	NP	2.4
α-Lactalbumin	2.45	1.3	1.84	0.9
Immunoglobulin G	0.04	0.7	0.75	1
Immunoglobulin A	0.7	ND	ND	ND
Lactoferrin	1.4	0.12	0.6	0.12
Osteopontin	0.14	0.02	ND	ND
Bile salt-activated lipase	0.1	NP	NP	NP
Lactoperoxidase (μg/mL)	1.5	30	ND	ND
Lysozyme C (μg/mL)	300	0.4	0.73	0.25
Pancreatic Ribonuclease (μg/mL)	ND	14	ND	4.25

Note: NP is not present; ND is not detected. Human milk data are from (Sanchez-Hidalgo et al., 1998, Araujo et al., 2005, Gapper et al., 2007, Park, 2009, Castellote et al., 2011, Queiroz et al., 2013); bovine milk data (Watanabe et al., 2000, Gapper et al., 2007, Korhonen, 2009); camel milk data (Zhang et al., 2005, El-Hatmi et al., 2006, El-Hatmi et al., 2007, Konuspayeva et al., 2007, El-Agamy, 2008); caprine milk (Csapó et al., 1994, Levieux et al., 2002, Hernández-Ledesma et al., 2011, Zervas and Tsiplakou, 2013).

1.3.2 Lactation stage

Lactation starts from colostrum and changes to mature milk (including early, middle, and late lactation stage), and then stops at involution of the mammary gland. Colostrum is the milk collected in the first few days after the neonate is born. Its composition is remarkably different compared to later in lactation (Lönnerdal et al., 1987, Blum et al., 1997). Immunoglobulins decrease remarkably from colostrum to mature milk in both human and bovine milk (Liao et al., 2011b, Senda et al., 2011). In addition to these well known proteins, other low abundant immune-related proteins also decrease from early lactation stage to middle lactation stage, such as complement proteins, lactoferrin, osteopontin, glycosylation-dependent cell adhesion molecule 1, alpha-1-acid glycoprotein 1, and protease inhibitors (Korhonen et al., 2000, Senda et al., 2011, Zhang et al., 2013). On the other hand, lipid transport proteins, including apolipoprotein A-I, A-IV, and C-III, were shown to increase from early to middle lactation (Zhang et al., 2013). In late lactation, proteins related to milk fat synthesis (adipophilin, fatty acid binding protein, butyrophilin), and proteins related to lactose synthesis (α-lactalbumin and β-1,4-galactosyltransferase 1) decline (Piantoni et al., 2010b, Boutinaud et al., 2013), whereas the immune-related proteins increase at this stage (Riley et al., 2008, Wickramasinghe et al., 2012, Zhang et al., 2013).

1.3.3 Effect of animal health on milk serum proteins

Animals with health issues results in increased expenditures for veterinary services, drug supplies, as well as reduced milk output (Wells et al., 1998). Of these health issues, mastitis ranks the highest in the estimated costs and animal welfare (Kaneene and Scott Hurd, 1990). It induces changes in milk appearance and milk quality, as well as the increase of somatic cell count (SCC) (Erb et al., 1985, Forsbäck et al., 2010). Although milk from cows with subclinical mastitis does not show visible changes in milk appearance, it normally results in a significant loss of milk production (Koldeweij et al., 1999). In addition, changes in SCC and milk proteins occur in the milk from cows with subclinical mastitis. For example, the concentrations of α-lactalbumin and β-lactoglobulin were reported to be different between infected cows and healthy cows (Batavani et al., 2007, Yang et al., 2009). Proteolysis related proteins such as, plasmin, cathepsin B and D, elastase, and amino- and carboxypeptidases, were increased in bovine milk following infusion with lipoteichoic acid isolated from

Staphylococcus aureus (Larsen et al., 2010), which has been shown to lead to protein breakdown. Moreover, mastitis, either clinical or subclinical, leads to changes in both innate and adaptive immune-related proteins (Safi et al., 2009, Turk et al., 2012) and proteins related to milk fat synthesis and secretion (Massart-Leen et al., 1994).

1.4 Stability of milk serum proteins during processing

Milk serum proteins are usually globular proteins, that can be modified during processing (Miyamoto et al., 2010). The general changes of protein structure is followed by unfolding of secondary and tertiary structures, breaking of disulphide bonds, new intra/intermolecular interactions, rearrangements of disulphide bonds, and finally aggregation (Bu et al., 2009). The aggregation of proteins may lead to the decrease in proteins' solublility and changes in their bioactives (Miyamoto et al., 2010, Ewaschuk et al., 2011).

1.4.1 Stability of milk serum proteins during heat treatment

The thermal process is designed to eliminate pathogens and spoilage microorganisms from raw milk with minimal chemical, physical, and organoleptic changes in the milk. Pasteurization is a process in which milk should be heated to at least 62.8 ℃ for 30 min (batch pasteurization) or 72 ℃ for 15 s (continuous pasteurization) (Hammershøj et al., 2010). Drying is another method for preserving milk through depriving microorganisms of the water necessary for their growth. Spray drying can extend the shelf life of skim milk powder up to three years. Heat treatment is an essential step applied in producing dairy products in general and infant formula in particular, which may directly influence the nutritional and biological activity of milk proteins. The effect of heat treatment has been frequently studies in the last decades. The heat-induced changes of high abundant milk serum proteins, such as α-lactalbumin and β-lactoglobulin, have been reported for human, bovine, camel, and caprine milk (Schwarcz et al., 2008, Akinbi et al., 2010, Atri et al., 2010). The heat stability of major serum proteins are in the order α-lactalbumin>β-lactoglobulin>serum albumin (Lin et al., 2010). Moreover, the heat treatment also influences immune-related proteins in milk serum. Bovine immunoglobulins were reported to be denatured during heat treatments that were more intense than regular pasteurization (72˚C for 15 s) and their heat

stability is IgG>IgA>IgM (Mainer et al., 1997). Several immunologically active compounds in camel, caprine, and human milk were also found to be reduced in concentration after pasteurization (Li-Chan et al., 1995, Elagamy, 2000, Laleye et al., 2008, Ewaschuk et al., 2011). The heat stability of milk serum proteins is also influenced by pH. For instance, basic pH reduced the heat stability of human milk lysozyme, whereas acidic pH increased its heat stability (Castillo et al., 2011). In addition, due to the differences in the amino acid sequence, protein structure, and post-translation modifications between species, the stability of milk proteins may differ between them. However, no comparison has been conducted on the influence of heat treatment on the low abundant proteins in milk serum between species.

1.4.2 Stability of milk serum proteins during freezing

In addition to thermal processes, freezing is a technique used to preserve and extend the shelf life of milk (Voutsinas et al., 1995). Although commercially frozen bovine milk is not an important commodity, freezing has been used frequently in processing camel milk. Furthermore, it is also an essential step in handling breast milk for human milk banks around the world (Tyson et al., 1980, Ogundele, 2000) as well as in commercial human milk production. The concentration of immune-related proteins, especially lactoferrin, secretory immunoglobulin A, and lysozyme, in human milk were shown to be changed after freezing and pasteurization (Chan et al., 2011, Chang et al., 2013). However, less is known on the influence of freezing on animal milk protein concentrations.

1.5 Proteomics techniques

Proteomics was used as a fundamental research tool for life scientists in protein characterization and biomarker discovery (Bourry and Poutrel, 1996). It has been applied in the development of diagnostics (Roberts et al., 2004), which emerged as a great promise of medicine. Recently, proteomics was also applied in the area of animal welfare, to improve production and enhance quality and safety of foods (Boehmer et al., 2010, Alonso-Fauste et al., 2012). Nowadays, proteomics is frequently used to understand the complexity of the milk proteome between different species (Hettinga et al., 2011, Liao et al., 2011b, Yang et al., 2013). Proteomics can be divided into two groups namely: non-targeted and targeted proteomics.

1.5.1 Non-targeted proteomics techniques

Non-targeted proteomics techniques refer to the measurement of as many proteins as possible in a sample. Next to identification, they are frequently used to detect differences in the abundance of proteins between multiple samples (Nesvizhskii, 2007). There are two important steps: protein separation and protein characterisation. Protein separation includes isoelectric-focusing electrophoresis, ultracentrifugation, and chromatographic separation, etc. (Lottspeich, 2009). Protein characterization includes protein digestion, protein identification, and protein quantification based on mass spectrometric analysis in comparison with database (Nesvizhskii, 2007).

Gel-based proteomics techniques, including one dimensional and two dimensional gel electrophoresis have been used for protein identification and quantification (Reinhardt and Lippolis, 2006, Boehmer et al., 2010). Such techniques may lead to high identification rates, but will result in less precise quantification because of the drawback of cutting gels (Bantscheff et al., 2007). In the past few years, Lu and colleagues used a gel-free shot-gun proteomic techniques, filter aided sample preparation (FASP) and dimethyl labelling followed by nanoLC-Orbitrap-MS/MS approach, to identify and quantify the milk proteome (Lu et al., 2011), as shown in Figure 1.1. This shot-gun proteomic technique can overcome both the time and accuracy issues simultaneously. Moreover, dimethyl labelling is a cheap and simple approach for quantification (Boersema et al., 2009). The FASP-dimethyl labelling-LC-MS/MS has a good repetition in quantification as shown in Figure 1.2. The ratios of milk serum proteins in five replicates are in the range of 0.5–1.0 showing the accuracy of quantification. This is well below the threshold (15%) for quantitative bioanalysis (Ji et al., 2005). Although dimethyl labelling increases the time for sample preparation and the complexity of chromatography for protein identification, it can give precise protein quantification and pick up small changes (>1.5 fold) in protein concentration (Lu et al., 2011).

Milk serum proteins contain many low abundant bioactive proteins, which play important roles in the growth and development of the neonate, especially for the immune system. Identification and quantification of low-abundant serum proteins is also of great interest due to their role as potential modulators for various other processes in the body (Wheeler et al., 2007b). However, milk serum is a complex system with many low abundant proteins. For these

reasons, non-targeted proteomics techniques, FASP and dimethyl labelling combined with LC-MS/MS was chosen to identify and quantify the milk serum proteome as well as determine the differences in protein concentrations between different samples in this thesis.

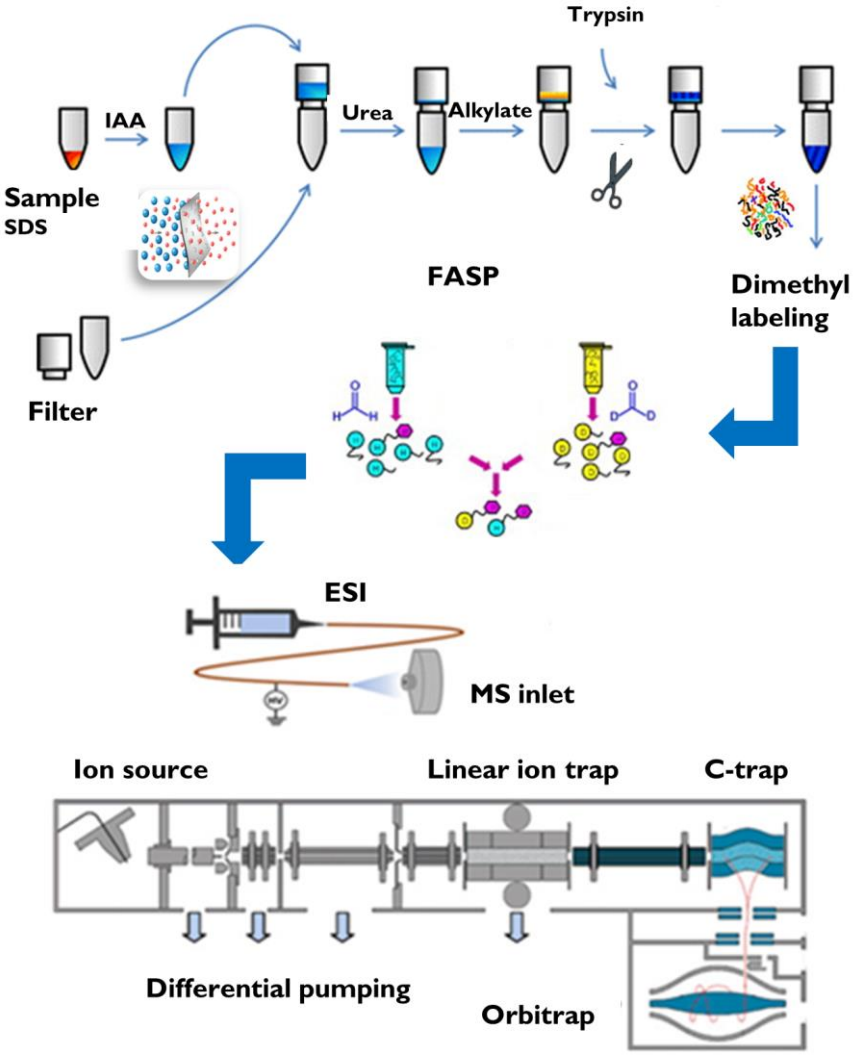


Figure 1.1 Schematic view of FASP, dimethyl labelling, and nanoLC-ESI-Orbitrp-MS/MS based proteomics techniques (Liang et al., 2013, Erde et al., 2014, Webdesign, 2009).

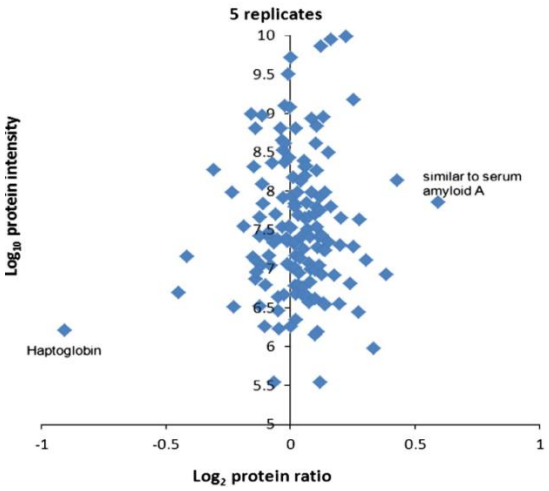


Figure 1.2 Heavy to light protein abundance ratios of 5 replicates. Both protein abundance ratio (x-axis) as well as the intensity of each protein (y-axis) was calculated by MaxQuant (Lu et al., 2011).

1.5.2 Targeted proteomics techniques

Although FASP, dimethyl labelling combined with LC-MS/MS can precisely quantify the milk proteome, its quantification refers to the relative concentration. In order to know more about the absolute concentration of milk protein, targeted proteomics techniques should be applied in the future studies. Targeted proteomics techniques are typically designed to quantify less than one hundred proteins with very high precision, sensitivity, specificity and throughput (Lottspeich, 2009). Examples of such techniques are enzyme-linked immunosorbent assay (ELISA), western blotting, protein chip-based technology, and selected reaction monitoring (SRM). Antibody based techniques represent the current gold standard for protein quantification. However, they require extensive time, resources and effort, and have been a bottleneck in protein quantification. SRM utilises LC-MS with three quadrupoles as mass spectrometer, representing a significant technical breakthrough for reducing this bottleneck (Picotti and Aebersold, 2012). This approach enables quantification of several proteins simultaneously, by selectively monitoring a specific peptide molecular ion (Q1), one or several fragment ions generated from the peptide by collisional activated dissociation (Q2), and then measure the fragment ions uniquely derived from the targeted

peptide (Q3) (Lange et al., 2008, Picotti and Aebersold, 2012), as shown in Figure 1.3. However, targeted proteomics strategies limit the number of proteins that can be monitored simultaneously.

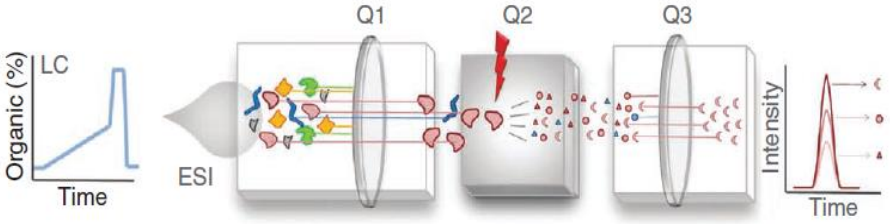


Figure 1.3. Schematic view of SRM-based proteomics technique (Picotti and Aebersold, 2012).

1.6 Aim and outline of this thesis

The aim of the research described in this thesis is to use shot-gun proteomics techniques to 1) study qualitative and quantitative changes in both human and bovine milk proteome related to lactation stage and health status, and 2) investigate the influence of processing (freezing, pasteurization, and spray drying) on the milk proteome from bovine, camel (*dromedary*), and caprine origin.

Firstly, the studies described in **Chapter 2, 3, and 4** investigate the qualitative and quantitative changes of both human and bovine milk proteome over lactation. DAVID GO and Uniprot were used to classify the biological functions and pathway analysis of identified proteins. Heat maps were used to visualize the changes in the milk proteome over lactation. The results were placed in perspective by reflecting on the needs of infants and calves thereby highlighting the importance of milk proteins in the development of the immune and digestion system of the neonate.

Secondly, the changes in the milk proteome from cows with high SCC compared to that of healthy cows are studied in depth, as described in **Chapter 5**. The significant changes of proteins in the milk with different SCC levels were described and the correlation between quantified milk proteins and SCC was calculated. A high correlation may result in a potential biomarker for the detection of bulk milk with high SCC thereby predicting the bulk milk quality for further processing in the industry.

Thirdly, **Chapter 6** describes the protein stability in bovine, camel, and caprine milk after freezing, pasteurization, and spray drying. The significant changes of milk proteins in frozen, pasteurized, and spray dried milk were analyzed in comparison to fresh milk. The differences in the protein stability between the three species were compared as well. This may provide guidance on the improvement of infant formula from both milk source and processing perspective.

Chapter 7 is a general discussion how all results described in this thesis contribute to our understanding of the dynamics of milk proteins associated with lactation stage, health status, species differences, and their stability after processing. The results found in the studies may not only contribute to the understanding of the importance of milk proteins in the development of the neonate, but also provide guidance on the improvement of dairy products.

Chapter 2

Bovine milk proteome in the first 9 days: protein interactions in maturation of the immune and digestive system of the newborn

Zhang, L., Boeren, S., Hageman, J.A., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. (2015). Bovine milk proteome in the first 9 days: protein interactions in maturation of the immune and digestive system of the newborn. **PLoS ONE** 10(2):e0116710.

Abstract

In order to better understand the milk proteome and its changes from colostrum to mature milk, samples taken at seven time points in the first 9 days from 4 individual cows were analyzed using proteomic techniques. Both the similarity in changes from day 0 to day 9 in the quantitative milk proteome and the differences in specific protein abundance, were observed among four cows. One third of the quantified proteins showed a significant decrease in concentration over the first 9 days after calving, especially in the immune proteins (as much as 40-fold). Three relative high abundant enzymes (XDH, LPL, and RNASE1) and cell division and proliferation protein (CREG1) may be involved in the maturation of the gastro-intestinal tract. In addition, high correlations between proteins involved in complement and blood coagulation cascades illustrates the complex nature of biological interrelationships between milk proteins. The linear decrease of protease inhibitors and proteins involved in innate and adaptive immune system implies a protective role for protease inhibitor against degradation. In conclusion, the results found in this study not only improve our understanding of the role of colostrum in both host defense and development of the newborn calf but also provide guidance for the improvement of infant formula through better understanding of the complex interactions between milk proteins.

2.1 Introduction

Milk is the most important food for the growth and development of the neonate because of its unique nutrient composition combined with the presence of many bioactive components, especially proteins. Human milk is considered as the most suitable food for the infant because it contains proteins which have significant beneficial effects for the babies from both a short-term and a long-term point of view (Lönnerdal, 2010). Although the proteome of bovine milk shows important differences with human milk (Hettinga et al., 2011), bovine milk and bovine colostrum have received considerable attention, as they are an important source for the production of ingredients for infant formula and protein supplements (Le et al., 2011).

Bovine colostrum contains a wide range of proteins, including high abundant proteins, like αs_1-casein, αs_2-casein, β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin (Farrell et al., 2004), and low abundant proteins, such as monocyte differentiation antigen CD14 (CD14), xanthine dehydrogenase/oxidase (XDH/XO), glycosylation-dependent cell adhesion molecule 1 (GLYCAM1), lactadherin (MFGE8), and clusterin (CLU) (Le et al., 2011). These proteins not only provide nutrition for the neonates during the initial phase of their lives, but also modulate their immune system to secure healthy growth (Wheeler et al., 2007a, Stelwagen et al., 2009). Apart from the immune function mentioned above, bovine colostrum also contains enzymes involved in digestion, and proteins related to maturation of the neonatal gastrointestinal tract (Godlewski et al., 2005, Brooks et al., 2006, Hammon et al., 2013).

Despite a large number of studies concerning the properties of bovine colostrum, the in-depth study of bovine colostrum proteins was accelerated by the application of proteomic techniques (Le et al., 2011). However, previous proteomic studies mainly focused on the identification of the colostrum proteome (Nissen et al., 2012) and the comparison in the proteome between pooled colostrum and mature milk (Le et al., 2011). No quantitative proteomics studies have been reported that study the change from colostrum to transition milk, using multiple time points from individual cows. A comprehensive understanding of the bovine colostrum proteome and the quantitative changes in time may not only contribute to our knowledge on the needs of the calves, but may also contribute to our understanding of biological functions of milk proteins.

Therefore, the objective of this study is to apply advanced proteomic techniques, the combination of filter aided sample preparation (FASP) and dimethyl labelling followed by LC-MS/MS, to explore the bovine milk serum proteome during the transition from colostrum to milk in the first 9 days after calving. During this period, the low abundant proteins present in colostrum and transition milk will be identified and quantified from four individual cows.

2.2 Materials and methods

2.2.1 Materials

Bovine milk was collected from 4 healthy, first-parity, Holstein-Friesian cows from a farm in Zaffelare, Belgium. After the first day, all cows had a somatic cell count lower than 100,000. In order to exclude the influence of diet and management effects, we collected milk from cows on the same farm being managed (including feed) in the same way, with calves born within a short time frame (between 20th August and 27th September 2012). No specific permissions were required for this sample collection, as samples were taken from the milk collected during regular milking. The cows were milked using an automatic milking system, and samples were collected every milking from day 0 to day 9. A total of 100 mL milk was collected at each time point. Samples of each time point were frozen immediately at -20°C after collection. After finishing sample collection, samples collected after 0, 0.5, 1, 2, 3, 5, and 9 days were transferred frozen to the laboratory for further analysis.

2.2.2 Milk serum separation

The samples collected at each time point of each individual cow were centrifuged at 1500×g for 10 min at 10°C (Beckman coulter AvantiJ-26 XP centrifuge, rotor JA-25.15). The pellet 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 70Ti). After ultracentrifugation, samples were separated into three 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 BCA assay and filter aided sample preparation (FASP) as described below.

2.2.3 BCA assay

BCA Protein Assay Kit 23225 (Thermo Scientific Pierce®) 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 0.02-2 μg/μL. Subsequently, the milk serum protein concentration was determined.

2.2.4 FASP-dimethyl labelling–LC-MS/MS

The method used to prepare milk serum samples for LC-MS/MS analysis was based on FASP (Wisniewski et al., 2009) and dimethyl labelling (Lu et al., 2011). Then, the prepared samples were analyzed using nanoLC-Orbitrap-MS/MS.

Milk serum samples (20 μL), including samples of each time point and pooled samples of all the time points from each cow, were diluted in SDT-lysis buffer (0.1 M Tris/HCL pH 8.0+4% SDS+0.1 M dithiotreitol (DTT)) to get 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. 20 μL of sample was directly added to the middle of 180 μL 0.05M iodoacetamide (IAA)/urea (0.1 M Tris/HCL pH 8.0+8 M UT) in a low binding Eppendorf tube 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, USA) and centrifuged at 15,871×g for 30 min. 100 μL of 0.05 M IAA 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 three times 100 μL UT. After that, 110 μL 0.05 M ABC (NH_4HCO_3 in water) was added to the filter unit and the samples were centrifuged again at 15,871×g for 30min. Then, the filter was transferred to a new low-binding Eppendorf tube. 100 μL ABC containing 0.5 μg 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 10% trifluoroacetic acid (TFA) was added to the filtrate to adjust the pH value of the sample to around 2. These samples were ready for dimethyl labelling.

The trypsin digested samples of pooled milk serum from each individual cow were labelled with the light reagent (using normal unlabelled formaldehyde and cyanoborohydride), whereas trypsin digested samples of milk serum collected at each time points of each individual cow were labelled with the heavy reagent (using deuterated formaldehyde and normal cyanoborohydride). The dimethyl labelling was carried out by on-column dimethyl labelling

according to (Boersema et al., 2009). Stage tips containing 2 mg Lichroprep C18 (25 um particles) column material (C18+ Stage tip) were made in-house. The C18+ Stage tip column was washed 2 times with 200 µL methanol. The column was conditioned with 100 µL of 1 mL/L formic acid (HCOOH) and then samples were loaded on the C18+ Stage tip column. The column was washed with 100 µL 1 mL/L HCOOH, and then slowly flushed with 100 µL labelling reagent (0.2% CH_2O or CD_2O and 0.03 M cyanoborohydride in 0.05 M phosphate buffer pH 7.5) in about 10 min. The column was washed again with 200 µL 1 mL/L HCOOH. Finally, the labelled peptides were eluted with 50 µL of 70% acetonitrile/30% 1 mL/L HCOOH from the C18+ Stage tip columns. The samples were then dried in a vacuum concentrator (Eppendorf Vacufuge®) at 45°C for 20 to 30 min until the volume of each sample decreased to 15 µL or less. The pairs of light dimethyl label and heavy dimethyl label were then mixed up and the volume was adjusted to exactly 100 µL by adding 1mL/L HCOOH. These samples were ready for analysis by LC-MS/MS.

18 µL of the trypsin digested milk fractions was injected on a 0.10×30 mm Prontosil 300-5-C18H (Bischoff, Germany) pre-concentration column (prepared in house) at a maximum pressure of 270 bar. Peptides were eluted from the pre-concentration column onto a 0.10×200 mm Prontosil 300-3-C18H analytical column with an acetonitrile gradient at a flow of 0.5 µL/min, using gradient elution from 9% to 34% acetonitrile in water with 0.5 v/v% acetic acid in 50 min. The column was washed using an increase in the percentage acetonitrile to 80% (with 20% water and 0.5 v/v% acetic acid in the acetonitrile and the water) in 3 min. A P777 Upchurch micro-cross was positioned between the pre-concentration and analytical column. An electrospray potential of 3.5 kV was applied directly to the eluent via a stainless steel needle fitted into the waste line of the micro-cross. Full scan positive mode FTMS spectra in LTQ-Orbitrap XL (Thermo electron, San Jose, CA, USA) were measured between an m/z of 380 and 1400. CID fragmented MS/MS scans of the four most abundant multiply charged peaks in the FTMS scan were recorded in data-dependent mode in the linear trap (MS/MS threshold=5.000).

2.2.5 Data analysis

Each run with all MSMS spectra obtained was analyzed with Maxquant 1.3.0.5 with Andromeda search engine (Cox and Mann, 2008). Carbamidomethylation of cysteine was set as a fixed modification (enzyme=trypsin, maximally 2 missed cleavages, peptide tolerance 20 ppm, fragment ions tolerance 0.5 amu).

Oxidation of methionine, N-terminal acetylation and de-amidation of asparagine or glutamine were set as variable modification for both identification and quantification. The bovine reference database for peptides and protein searches was downloaded as fasta files from Uniprot (http://www.uniprot.org/ accessed March 2012) with reverse sequences generated by Maxquant. A set of 31 protein sequences of common contaminants was added including Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human), and Keratin K1C1 (P35527, human). A maximum of two missed cleavages were allowed and mass deviation of 0.5 Da was set as limitation for MS/MS peaks and maximally 6 ppm deviation on the peptide m/z during the main search. A 1% false discovery rate (FDR) was set to on both peptide and protein level. The length of peptides was set to at least seven amino acids. Finally, proteins were displayed based on minimally 2 distinct peptides of which at least one unique.

Dimethyl labelling was based on doublets with dimethLys0 and dimethNter0 as light, dimethLys4 and dimethNter4 as heavy labels. Razor and unique peptides were used for quantification. Normalized H/L ratios were used for further statistical analysis. Also the intensity based absolute quantification (iBAQ value) algorithm was used in this research. It estimates absolute protein concentration as the sum of all peptide intensities divided by the number of theoretically observable tryptic peptides. The iBAQ value has been reported to have a good correlation with known absolute protein amounts over at least four orders of magnitude (Schwanhausser et al., 2011).

The function of the identified proteins was checked in the UniprotKB database released April 2012 (http://www.uniprot.org/). To select the proteins that significantly decrease over time, proteins were analyzed univariate. For each protein and per cow, a regression line was fitted on the protein concentrations measured at time points 0, 0.5, 1, 2, 3, 5, and 9 days. To reliably estimate a regression line, only proteins with at least 4 observed time points per cow were considered. The regression line summarizes per cow the concentration profiles for each protein into four intercepts and four slopes. The intercepts are the protein concentration at time 0, and the slopes indicate the decrease in concentration per day. By using hypothesis tests on the slopes it can be determined if the decrease in concentration is significant. The Lilliefors normality test (Lilliefors, 1967) was used to test if the four slopes were normally distributed. Proteins for which the four slopes were not normally

distributed were discarded, since the non-parametric Wilcoxon signed rank test cannot establish a significant decrease with only four observations with α=0.05. Proteins with normally distributed slopes were subjected to a one-sided t-test to test if the slopes were significantly decreasing (with α=0.05). Gene Ontology (GO) enrichment analysis was done using DAVID bioinformatics Resources 6.7 (Dennis et al., 2003). SPSS (Version 21, IBM Corp.) was used to calculate correlation coefficients among quantified proteins. The linear regression and subsequent hypothesis tests between proteins related to complement and coagulation system was performed in Matlab R2012A and Microsoft Excel (2010).

2.3 Results

2.3.1 Protein concentrations determined by BCA

The protein concentrations of milk serum from four cows collected at different time points are shown in Table 2.1. There was roughly 10-fold decrease in the protein concentrations from day 0 to day 9 and the rate of change was especially high in the first three days. The total protein content among these four individual cows at day 0 were approximately 2-fold different, whereas the protein content decreased to comparable levels at day 9.

Table 2.1 Protein concentrations determined by BCA assay

Time point (day)	Protein concentration (μg/μL)			
	Cow1	Cow2	Cow3	Cow4
0	85.28	114.96	141.21	169.55
0.5	53.18	73.78	51.42	78.60
1	22.18	19.44	22.31	29.62
2	14.38	17.02	18.44	20.40
3	12.76	14.20	17.16	19.92
5	12.20	11.26	16.93	20.17
9	15.25	13.39	16.05	15.33

2.3.2 The number of identified and quantified proteins

A total of 212 proteins were identified in all the samples, of which 208 proteins were quantified. In the sample of the four individual cows, around 200 proteins were detected respectively. Of these identified proteins, approximately 98% could be quantified using dimethyl labelling. Moreover, as can be seen in Figure 2.1, 80% of identified and quantified proteins were detected in the milk of all individual cows.

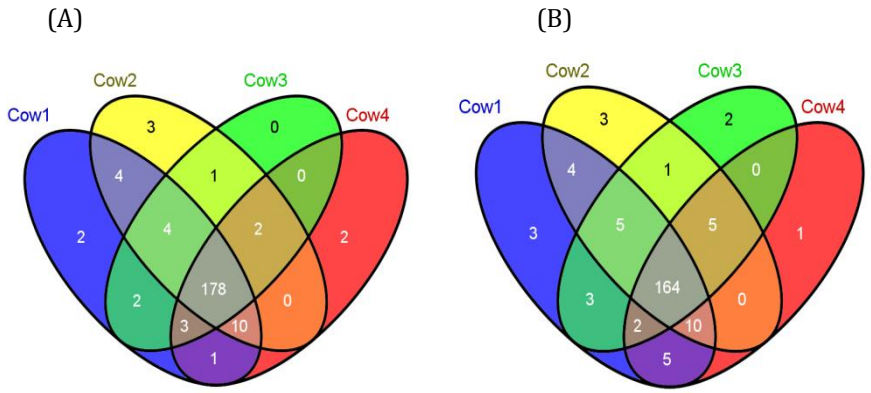


Figure 2.1 Number of identified (A) and quantified proteins (B) in four biological duplicates.

2.3.3 The distribution of biological functions and subcellular locations of identified proteins

The identified proteins were grouped based on their biological function and subcellular location according to Uniprot as shown in Figure 2.2. Immune-related protein appeared to be the dominant biological function group (25%). The detailed functions of these immune-related proteins are shown in Table 2.2 according to the classification of DAVID gene ontology. Enzymes (15%) ranked second, tied with transport proteins (15%). Also, the proportion of protease inhibitors (10%) was relatively high. With respect to subcellular location distribution, 50% of the identified proteins were secreted proteins, followed by cytoplasm (15%) and membrane proteins (12%). Proteins originating from mitochondrion, endoplasmic reticulum (ER), lysosome, Golgi apparatus, and nucleus accounted for about 15% in total.

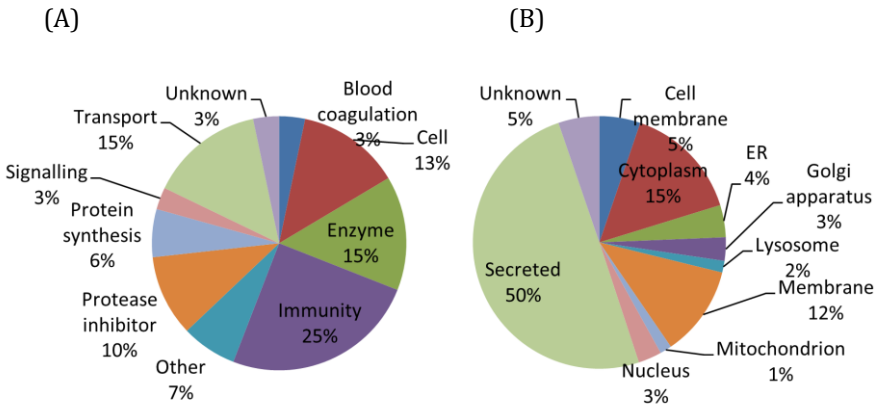


Figure 2.2 The distribution of biological functions (A) and subcellular location (B) of identified proteins (Cell includes cell motility, cell apoptosis, cell growth, cytoskeleton, cell adhesion, cell differentiation, cell proliferation, and cell shape; immunity includes immune response, complement, antibacterial, host defense, acute phase, and antigen binding; other includes calcium homogenises, hormone, and cytokine growth; ER is endoplasmic reticulum).

Table 2.2 The number of significant proteins with time-depended changes analyzed by t-test

Category of immune-related proteins	Gene name
Complement proteins	C1R, C1S, C3, C6, C7, C8B, C9, CFB, CFD, CFH, CFI
Antibacterial proteins	CATHL1, CATHL2, CATHL3, CATHL4, CATHL5, CATHL6, CATHL7, LPO, PGLYRP1
Immunoglobulin-like proteins	A1BG, AHSG, B2M, BoLA, PIGR, IGJ, IGK , IGLL1
Acute phase proteins	ORM1, F2, C3, FN1, SERPINF2, ITIH4, SAA1, SAA3
Other immune-related proteins	AZGP1, B4GALT1, BOLA-NC1, MUC15, CHI3L1, CLU, CRISP3, GLYCAM1, GP2, HP, RNASE4

2.3.4 The qualitative and quantitative changes of protein between day 0 and day 9 based on biological functions

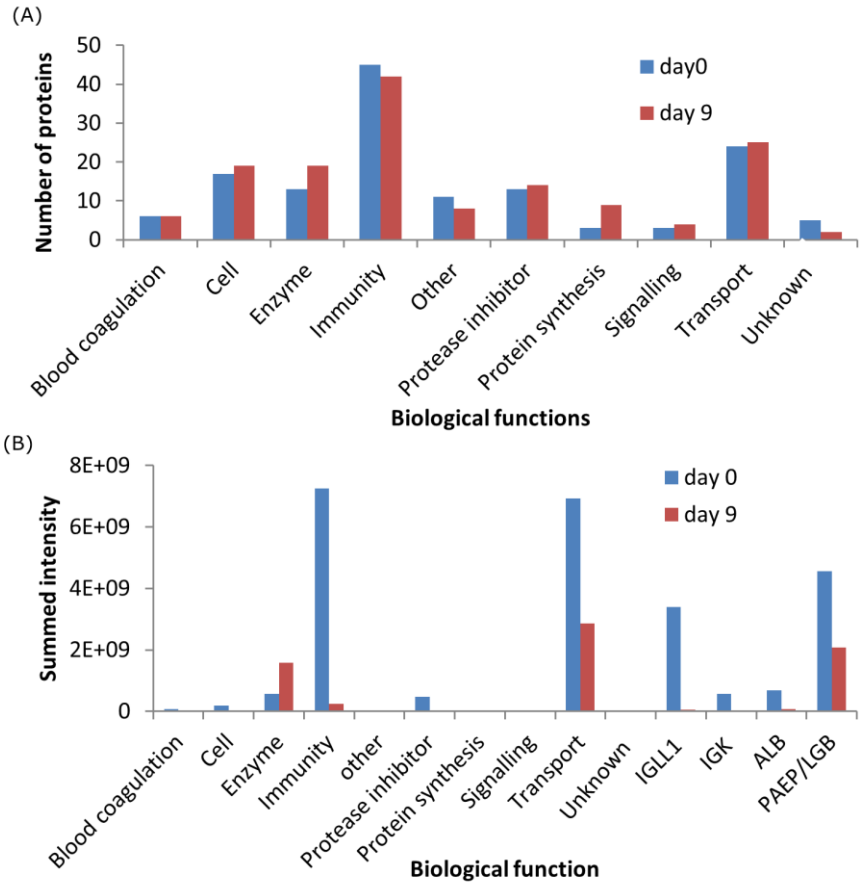


Figure 2.3 The comparison of biological function distribution of identified proteins (A) and their summed intensities (B) in the milk collected at day 0 and day 9.

The qualitative and quantitative changes of proteins classified by biological function at day 0 and day 9 are shown in Figure 2.3. Enzyme is the most different group. Both the number (1.46-fold) and intensity (2-fold) of enzyme show increase from day 0 to day 9. Immune-related proteins showed a slight decrease (10.8%) in the number of identified proteins and a large decrease (96.6%) in their summed intensities. The total number of transport proteins increased slightly (7.1%) whereas the total summed intensities decreased by

60%. The protease inhibitors showed a different pattern, the number of identified proteins did not change from day 0 to day 9 but the intensities decreased remarkably (96.7%); for cell related proteins, the number of identified proteins increased slightly (13%), whilst the intensities decreased remarkably (85%), a similar decrease was also found for the proteins grouped under "other".

2.3.5 The quantitative variation of proteins in the milk collected at the first 9 days

The Log_2 ratio of proteins present in at least 14 samples out of 28 samples collected at different time points are shown in a heat map (Figure 2.4). The four individual cows show a similar pattern of changes over the first 9 days of lactation. The Log_2 ratio of the majority proteins showed a rapid decrease from day 0 to day 9; a few proteins, however, increased in concentration during the same period (marked with a blue rectangle). This was for instance the case for ribonuclease pancreatic (RNASE1), xanthine dehydrogenase/oxidase (XDH), and lipoprotein lipase (LPL) as shown in Table 2.3.

Table 2.3 The variation in the average intensities (log_{10} iBAQ value) of four abundant enzymes over the first 9 days

Enzyme	Day 0	Day	Day 1	Day 2	Day 3	Day 5	Day 9
LALBA	8.74	8.74	9.57	9.07	9.03	9.26	9.20
RNASE1	7.10	7.14	7.95	7.49	7.43	7.44	7.07
XDH	6.04	5.71	6.71	6.41	6.34	6.49	6.45
LPL	0.00	4.92	6.31	6.34	4.25	6.14	5.97

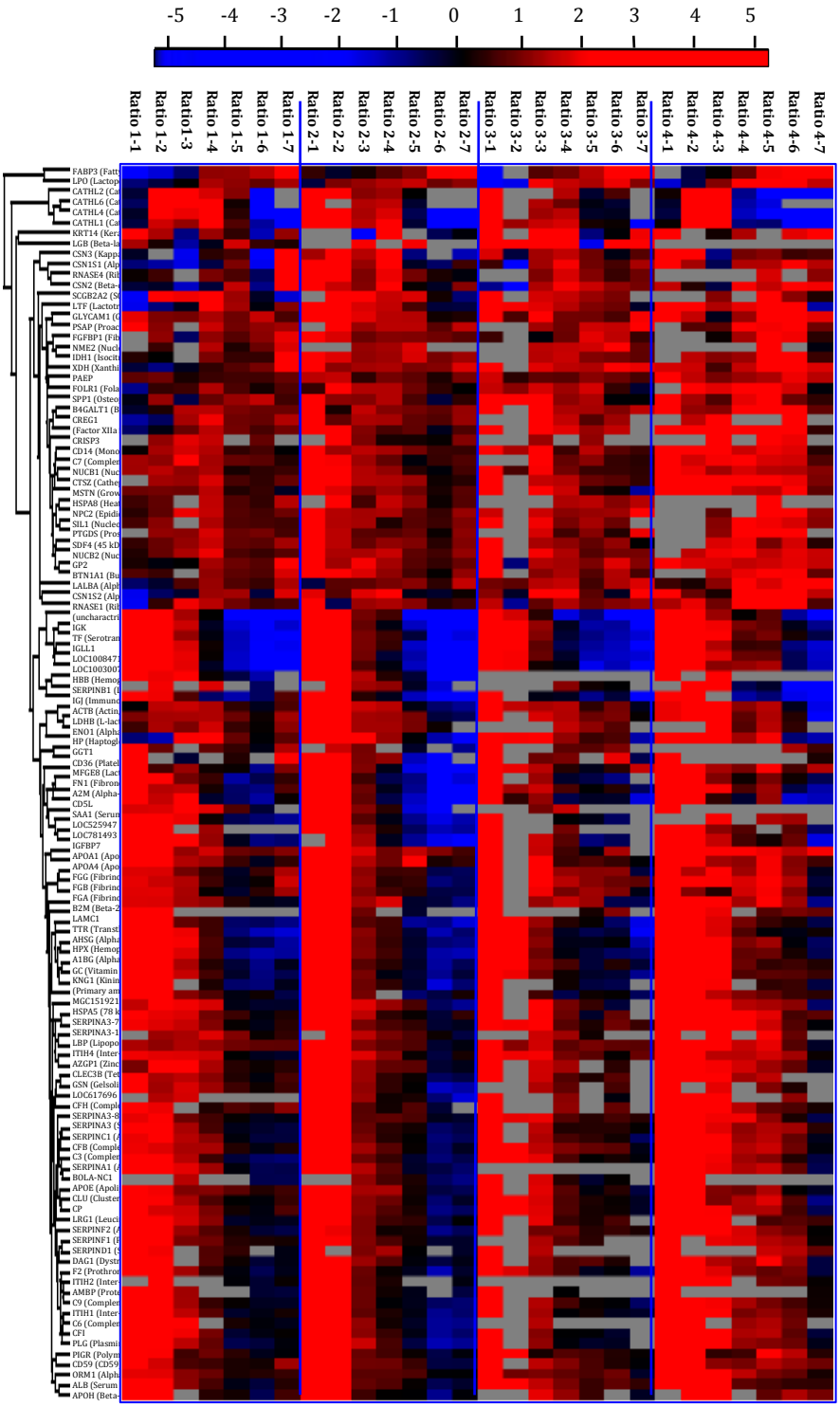

-5
-4
-3
-2
-1
0
1
2
3
4
5
Ratio 1-1
Ratio 1-2
Ratio1-3
Ratio 1-4
Ratio 1-5
Ratio 1-6
Ratio 1-7
Ratio 2-1
Ratio 2-2
Ratio 2-3
Ratio 2-4
Ratio 2-5
Ratio 2-6
Ratio 2-7
Ratio 3-1
Ratio 3-2
Ratio 3-3
Ratio 3-4
Ratio 3-5
Ratio 3-6
Ratio 3-7
Ratio 4-1
Ratio 4-2
Ratio 4-3
Ratio 4-4
Ratio 4-5
Ratio 4-6
Ratio 4-7
FABP3 (Fatt
LPO (Lactop
CATHL2 (Ca
CATHL6 (Ca
CATHL4 (Ca
CATHL1 (Ca
KRT14 (Ker
LGB (Beta-la
CSN3 (Kappa
CSN1S1 (Alp
RNASE4 (Rib
CSN2 (Beta-
SCGB2A2 (S
LTF (Lactotr
GLYCAM1 (G
PSAP (Proac
FGFBP1 (Fib
NME2 (Nucl
IDH1 (Isocit
XDH (Xanthi
PAEP
FOLR1 (Fola
SPP1 (Osteo
B4GALT1 (B
CREG1
(Factor XIIa
CRISP3
CD14 (Mono
C7 (Complem
NUCB1 (Nuc
CTSZ (Cathe
MSTN (Growt
HSPA8 (Heat
NPC2 (Epidi
SIL1 (Nucleo
PTGDS (Pros
SDF4 (45 kD
NUCB2 (Nuc
GP2
BTN1A1 (Bu
LALBA (Alph
CSN1S2 (Alp
RNASE1 (Rib
(uncharacteri
IGK
TF (Serotran
IGLL1
LOC100847
LOC100300
HBB (Hemog
SERPINB1 (
IG] (Immuno
ACTB (Actin
LDHB (L-lac
ENO1 (Alpha
HP (Haptogl
GGT1
CD36 (Platel
MFGE8 (Lact
FN1 (Fibron
A2M (Alpha-
CD5L
SAA1 (Serum
LOC525947
LOC781493
IGFBP7
APOA1 (Apo
APOA4 (Apo
FGG (Fibrino
FGB (Fibrino
FGA (Fibrino
B2M (Beta-2
LAMC1
TTR (Transt
AHSG (Alpha
HPX (Hemop
A1BG (Alpha
GC (Vitamin
KNG1 (Kinin
(Primary am
MGC151921
HSPA5 (78 k
SERPINA3-7
SERPINA3-1
LBP (Lipopo
ITIH4 (Inter
AZGP1 (Zinc
CLEC3B (Tet
GSN (Gelsol
LOC617696
CFH (Comple
SERPINA3-8
SERPINA3 (S
SERPINC1 (A
CFB (Comple
C3 (Complem
SERPINA1 (A
BOLA-NC1
APOE (Apoli
CLI (Cluster
CP
LRG1 (Leuci
SERPINF2 (A
SERPINF1 (P
SERPIND1 (S
DAG1 (Dystr
F2 (Prothro
ITIH2 (Inter
AMBP (Prote
C9 (Complem
ITIH1 (Inter
C6 (Complem
CFI
PLG (Plasmi
PIGR (Polym
CD59 (CD59
ORM1 (Alpha
ALB (Serum
APOH (Beta-

Figure 2.4 The ratio of identified proteins in the milk collected in the first 9 days with biological duplicates. The red color shows proteins with a Log_2 ratio more than 2, while blue color shows proteins with a Log_2 ratio less than -2. The stronger the color is, the larger the value is. Proteins that couldn't be quantified are labelled gray.

In total, 94 proteins were determined at least at four time points per cow. This was deemed as a minimal requirement to reliably estimate the trend over time. The concentration profiles of these 94 proteins were summarized into intercepts and slopes as described in the methods section. The Lilliefors test indicated that 8 proteins had not normally distributed slopes, so these proteins were discarded. From the 86 proteins with normally distributed slopes, a total of 64 proteins showed a significant decrease and they are listed in Table 2.4. For some individual proteins, the decrease in concentration from day 0 to day 9 was as high as 40-fold. The changes in protein ratios over time of quantified proteins (gray) and the significantly different proteins (red) are shown in Figure 2.5. For instance, IGJ, decreased by 65% in the first 12 h and 85% after 1 day; IGK by 32% in the first 12 h and 69% after 1 day. SERPINA1, GSN, and ITIH1 decreased by 88%; PLG by 95% and KNG1 by 93% after three days. Although the four individual cows showed the same pattern in the reduction of low abundant proteins, the concentration of certain proteins at day 0 could differ as much as 5-fold. The concentrations of most proteins in the milk of cow 1 were lower compared to the other three cows, which agrees with the BCA results as shown in Table 2.1.

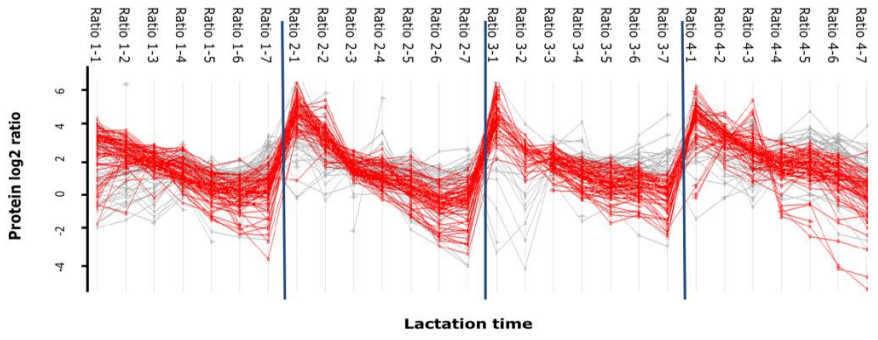


Figure 2.5 The variation of significantly different proteins in the milk collected in the first 9 days in four individual cows.

Table 2.4 Significant different proteins with time series (one-sided t-test, α=0.05)

Protein IDs	Protein name	Gene name	Biological function	Subcellular location	p value
P01044	Kininogen-1	KNG1	Blood coagulation	secreted	0.000
P02672	Fibrinogen alpha chain	FGA	Blood coagulation	secreted	0.004
P02676	Fibrinogen beta chain	FGB	Blood coagulation	secreted	0.014
P06868	Plasminogen	PLG	Blood coagulation	secreted	0.009
P12799	Fibrinogen gamma-B chain	FGG	Blood coagulation	secreted	0.000
P17690	Beta-2-glycoprotein 1	APOH	Blood coagulation	secreted	0.006
P02769	Serum albumin	ALB	Cell	secreted	0.013
Q2KIF2	Leucine-rich alpha-2-glycoprotein 1	LRG1	Cell	secreted	0.005
F1N076	Uncharacterized protein	CP	Cell	secreted	0.003
F1MPP2	Insulin-like growth factor-binding protein 7	IGFBP7	Cell adhesion	secreted	0.014
P31096	Osteopontin	SPP1	Cell adhesion	secreted	0.037
F1N514	CD5L protein	CD5L	Cell apoptosis	membrane	0.002
P02702	Folate receptor alpha	FOLR1	Cell death	cell	0.042
O18738	Dystroglycan	DAG1	Cell-Cytoskeleton	secreted	0.000
F1N4M7	Complement factor I	CFI	Enzyme	membrane	0.020
P05689	Cathepsin Z	CTSZ	Enzyme	lysosome	0.001
Q29437	Primary amine oxidase, liver isozyme		Enzyme	secreted	0.016

Protein IDs	Protein name	Gene name	Biological function	Subcellular location	p value
Q5E9B1	L-lactate dehydrogenase B chain	LDHB	Enzyme	Secreted	0.013
F1MZ96	IGK protein	IGK	Immunity	Secreted	0.001
P00735	Prothrombin	F2	Immunity	Secreted	0.000
P01888	Beta-2-microglobulin	B2M	Immunity	Secreted	0.000
P07589	Fibronectin	FN1	Immunity	Secreted	0.019
P12763	Alpha-2-HS-glycoprotein	AHSG	Immunity	Secreted	0.021
P17697	Clusterin	CLU	Immunity	Secreted	0.002
P19660	Cathelicidin-2	CATHL2	Immunity	Secreted	0.011
P22226	Cathelicidin-1	CATHL1	Immunity	Secreted	0.003
P28800	Alpha-2-antiplasmin	SERPINF2	Immunity	Secreted	0.002
P33046	Cathelicidin-4	CATHL4	Immunity	Secreted	0.001
P54228	Cathelicidin-6	CATHL6	Immunity	Secreted	0.005
P81187	Complement factor B	CFB	Immunity	Secreted	0.002
P81265	Polymeric immunoglobulin receptor	PIGR	Immunity	Secreted	0.001
Q29RQ1	Complement component C7	C7	Immunity	Secreted	0.010
Q2KJF1	Alpha-1B-glycoprotein	A1BG	Immunity	Secreted	0.000
Q2TBU0	Haptoglobin	HP	Immunity	Secreted	0.023

Protein IDs	Protein name	Gene name	Biological function	Subcellular location	p value
Q2UVX4	Complement C3	C3	Immunity	Secreted	0.001
Q32PA1	CD59	CD59	Immunity	Membrane	0.017
Q3MHN2	Complement component C9	C9	Immunity	Secreted	0.007
Q3SYR8	Immunoglobulin J chain	IGJ	Immunity	Secreted	0.012
Q3SZR3	Alpha-1-acid glycoprotein	ORM1	Immunity	Secreted	0.002
Q3T052	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	Immunity	Secreted	0.012
Q3ZCH5	Zinc-alpha-2-glycoprotein	AZGP1	Immunity	Secreted	0.001
Q7SIH1	Alpha-2-macroglobulin	A2M	Immunity	Secreted	0.004
Q95122	Monocyte differentiation antigen CD14	CD14	Immunity	Cell	0.001
Q0P569	Nucleobindin-1	NUCB1	Other	Golgi	0.002
Q3SX14	Gelsolin	GSN	Other	Cytoplasm	0.015
Q3ZBZ1	45 kDa calcium-binding protein	SDF4	Other	Golgi	0.034
A2I7N1	Serpin A3-5	SERPINA3	Protease inhibitor	Cytoplasm	0.002
F1MSZ6	Antithrombin-III	SERPINC1	Protease inhibitor	Secreted	0.001
P34955	Alpha-1-antiproteinase	SERPINA1	Protease inhibitor	Secreted	0.000
Q0VCM5	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	Protease inhibitor	Secreted	0.005
Q9TTE1	Serpin A3-1	SERPINA3-1	Protease inhibitor	Cytoplasm	0.001

Protein IDs	Protein name	Gene name	Biological function	Subcellular location	p value
P60712	Actin, cytoplasmic 1	ACTB	Protein synthesis	Cytoplasm	0.010
Q0VCX2	78 kDa glucose-regulated protein	HSPA5	Protein synthesis	ER	0.025
A6QPK0	SCGB2A2 protein	SCGB2A2	Signalling	Secreted	0.030
O46375	Transthyretin	TTR	Transport	Secreted	0.009
P15497	Apolipoprotein A-I	APOA1	Transport	Secreted	0.017
Q03247	Apolipoprotein E	APOE	Transport	Secreted	0.008
Q0IIA2	Odorant-binding protein-like	MGC151921	Transport	Secreted	0.006
Q29443	Serotransferrin	TF	Transport	Secreted	0.000
Q32KV6	Nucleotide exchange factor SIL1	SIL1	Transport	ER	0.032
Q3MHN5	Vitamin D-binding protein	GC	Transport	Secreted	0.000
Q3SZV7	Hemopexin	HPX	Transport	Secreted	0.000
F1MLW8	Uncharacterized protein	LOC100847119	Unknown	Unknown	0.000
G3N1R1	Uncharacterized protein	LOC100300716	Unknown	Unknown	0.000

*Note: ER is endoplasmic reticulum

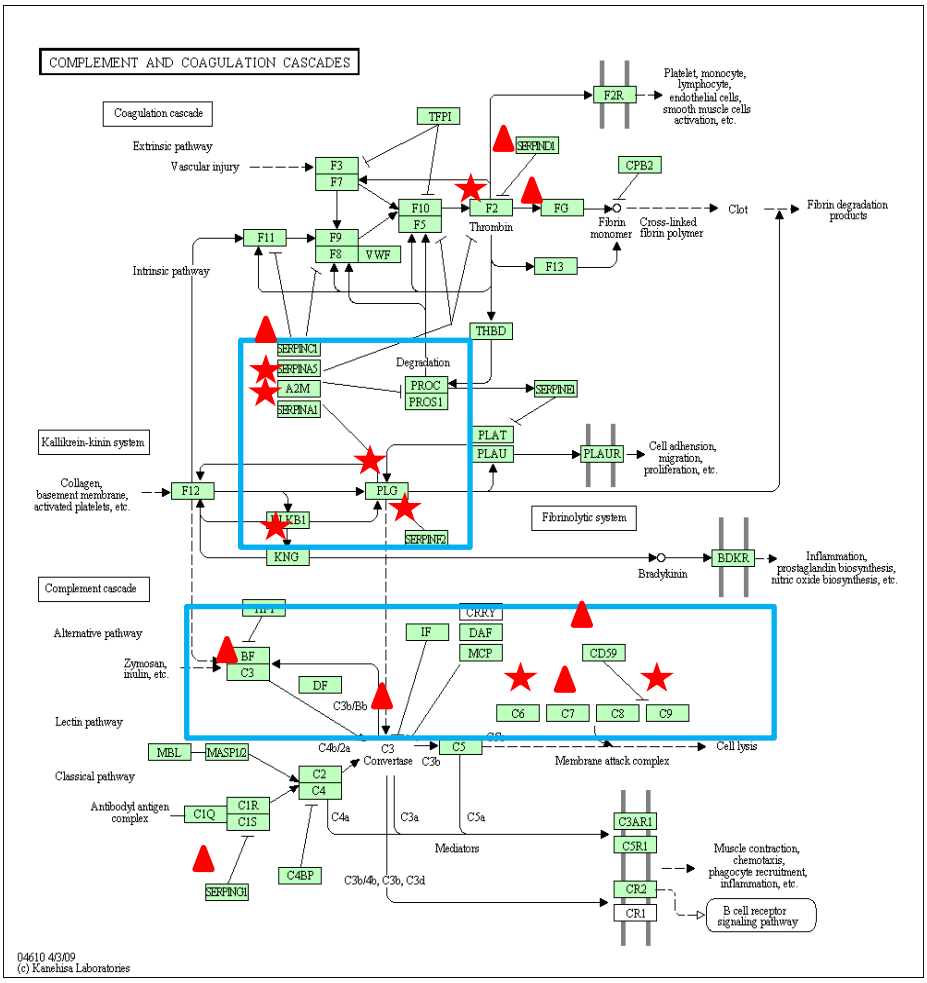
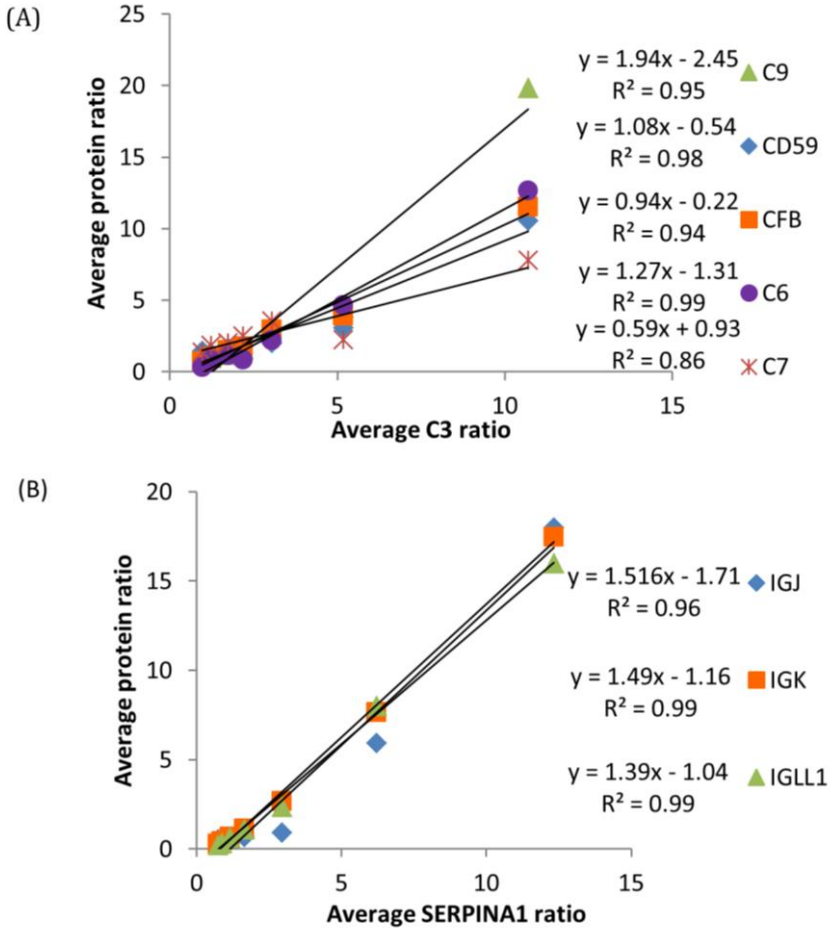


Figure 2.6 Significant different proteins involved in complement and coagulation cascades.

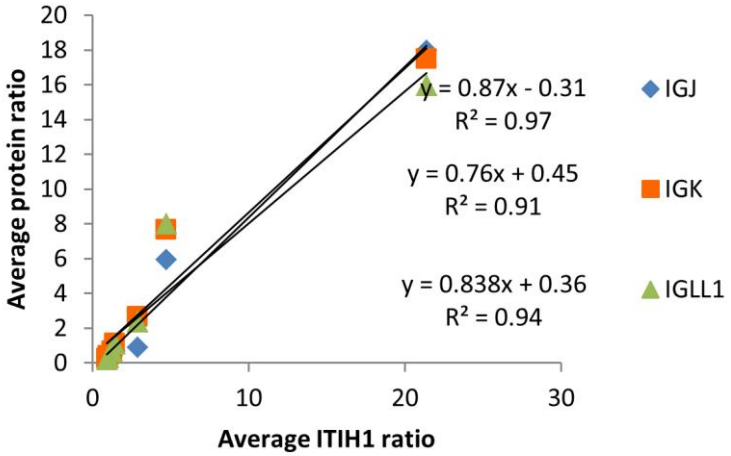
2.3.6 Correlation analysis of proteins

Correlation analysis was also conducted among proteins (140), which can be identified and quantified in at least half of the samples. Several immune-related proteins, coagulation-related proteins, and protease inhibitors were found to be highly correlated (r^2>0.80). Based on the DAVID gene ontology analysis, we found that most of the highly correlated proteins participate in the complement system and coagulation cascade as shown in Figure 2.6. The significantly different proteins are mainly related to two specific parts of these pathways,

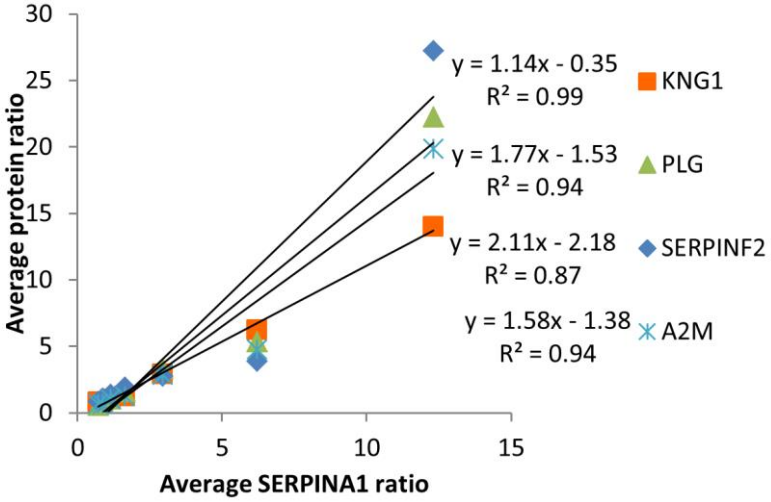
the complement cascade and the kallikrein-kinin system. These two pathways were reported to have many similarities because both cascades utilize multi-domain serine proteases with a similar domain structure as catalysts. Correlation coefficients of proteins involved in these two specific pathways (SERPINA1, A2M, PLG, KNG1, C3, C6, C7, C9, CFB, CD59, and SERPINF2) were also between 0.815 to 0.997 as shown in Figures 2.7A, 2.7D, and 2.7E). In addition, protease inhibitors were found to be highly correlated with complement proteins and immunoglobulins. Two examples (SERPINA1 and ITIH1) are shown in Figure 2.7B and 2.7C.



(C)


Average protein ratio
Average ITIH1 ratio
y = 0.87x - 0.31
R² = 0.97
y = 0.76x + 0.45
R² = 0.91
y = 0.838x + 0.36
R² = 0.94
IGJ
IGK
IGLL1

(D)


Average protein ratio
Average SERPINA1 ratio
y = 1.14x - 0.35
R² = 0.99
y = 1.77x - 1.53
R² = 0.94
y = 2.11x - 2.18
R² = 0.87
y = 1.58x - 1.38
R² = 0.94
KNG1
PLG
SERPINF2
A2M

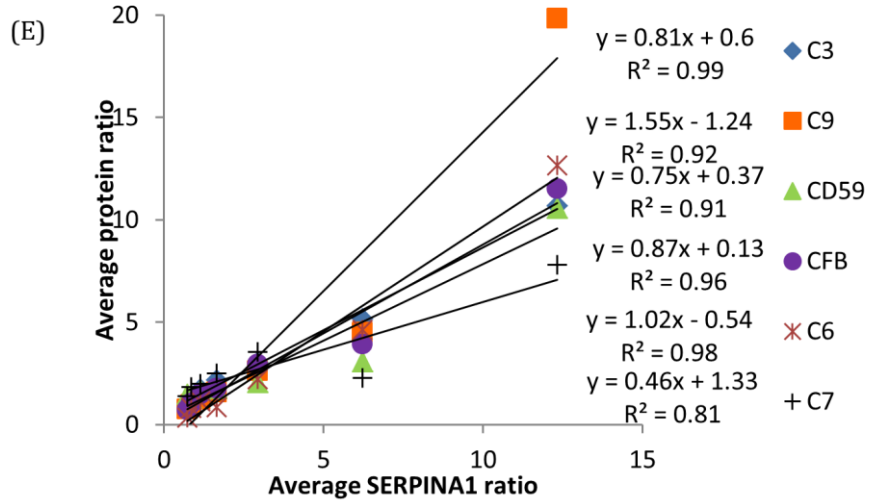


Figure 2.7 The correlation of significantly different proteins involved in complement, coagulation pathway, and immunoglobulins (A is proteins involved in complement cascade; B is the correlation between SERPINA1 and immunoglobulins; C is the correlation between ITIH1 and immunoglobulins; D is proteins involved in kallikrein-kinin system; E is the correlation between SERPINA1 and complement proteins).

2.4 Discussion

2.4.1 Overview of the whole proteome

This study investigated the time-dependent changes of low abundant proteins in bovine milk in the first 9 days of lactation, using dimethyl labelling for quantification. For optimal quantification results, we mixed the sample collected at each time point labelled with heavy reagent with a single pooled sample labelled with light reagent as reference. All analysis were thus done relative to the same reference. A total of 212 proteins were identified in bovine milk sample, of which 208 proteins were quantified. Most of the low abundant proteins reported in proteomics studies of bovine colostrum and milk (Smolenski et al., 2007, Stelwagen et al., 2009, Senda et al., 2011, Wheeler et al., 2012) were also found to be present in the current study. In addition, we also identified and quantified many proteins, which have not been reported by Zhang, et al. (2011) who used 2-D gel separation combined with LC-MS/MS

(Zhang et al., 2011). The relatively higher number of identified and quantified proteins found in this study confirms the suitability of FASP and dimethyl labelling combined with LC-MS/MS on identifying and quantifying low abundant proteins in milk (Lu et al., 2011). The proteins, identified or quantified for the first time in the current study, were often identified in only a few time points in individual cows. This may be due to the sensitivity of the instrument used in this study because the concentration of these proteins were all around the instrument detection limit. The overlap between the four individual cows in both identified (83% found in all four cows) and quantified proteins (78%) gives an indication of the similarity of the milk proteome among individual cows. In addition, all four cows showed similar changes in protein concentrations over time as shown in Table 2.1 and Figure 2.5. Approximately one-third of all proteins changed significantly (p<0.05, up to 40-fold) over the first 9 days as shown in Table 2.4. At the same time, there were also differences in protein concentrations between individual cows. These differences may be caused by individual differences as a more than 20-fold difference in concentration was found to have within a herd of 189 healthy cows (Stelwagen et al., 2009).

The classification of identified proteins as shown in the Figure 2.2A depicts the different biological functions of the bovine milk proteome. Based on the comparison of identified proteins and their intensities between day 0 and day 9 (Figure 2.3A and 2.3B), we may conclude that the variation in the milk proteome is determined by concentration rather than by composition. The remarkable decrease in the summed intensities of immune-related protein was mainly attributed to immunoglobulins (IGJ and IGK), which will be discussed further below. The large decrease in the summed intensities of transport proteins was mainly caused by the decrease of the major proteins β-lactoglobulin (LGB) and serum albumin (ALB), as shown in Figure 2.3B. The rapid decrease of LGB and ALB in the first few days is in agreement with the results of previous studies (Levieux and Ollier, 1999).

2.4.2 Proteins involved in the development of the gastro-intestinal tract

Next to transport proteins, enzymes also showed an increase in intensities from day 0 to day 9. The increase in intensities is attributed to four high abundant enzymes, which contributed to LALBA, XDH, LPL, and RNASE1 (Table 2.3).

LALBA regulates subunit of lactose synthase (Piantoni et al., 2010a), but it doesn't have a catalytic activity. The up-regulation of XDH was previously reported for bovine milk serum (Le et al., 2011) during the transition from colostrum to mature milk. This increase may be related to mitigation of the oxidative stress in newborns, because it exerts an antimicrobial activity through inducing reactive oxygen species (ROS) generation (Harrison, 2002). XDH may function in the digestion system of calves as it has been reported to play a key role in blood-meal digestion in flies (Sant'Anna et al., 2008). LPL is an enzyme that is secreted from the pancreas into the digestive tract but also transferred from the lactating mammary gland into the milk (Lidmer et al., 1995). In the gastrointestinal tract, bovine LPL functions in digestion of triglycerides and absorption of lipid nutrients in newborns (Hui and Howles, 2002). RNASE1, is another pancreatic enzyme that plays a major role in digestion of nucleic acids of microorganisms in the lumen of calves (Guilloteau et al., 2009). RNASE1 has been thought to play a role in the nutrient uptake in the gut and in the degrading of bacterial RNA in the intestinal tract (Barnard, 1969), which is especially important in plant eating animals like cows.

Although digestive functions of calves develop during fetal life, the gastrointestinal tract cannot be fully developed in 2-3 days after birth, and this development continues until the calf fully transitioned to solid food (Zabielski et al., 2002). As the intestine and the pancreas of calves are not mature at the age of 7 days (Guilloteau et al., 2009), the digestion process probably depends also on the enzymes transferred from colostrum or milk to the calf. The increase of XDH, LPL, and RNASE1 over the first 9 days suggests the important roles in the digestion processes of the newborn calf .

Whereas the enzymes involved in digestion increased in the first 9 days, protein related to development of the gastrointestinal tract showed a decrease. Cell division and proliferation protein CREG1, a secreted glycoprotein, has been reported as cellular repressor inhibiting cell proliferation and enhancing cell differentiation in human embryonic carcinoma cells (Sacher et al., 2005). Growth cytokine MSTN is a member of the transforming growth factor (TGF)-β family, which is one of the predominant growth factors present in bovine milk (Rogers et al., 1996). These growth cytokines have been reported to promote the growth and development human intestine (Purup et al., 2007). This is to be expected, as colostrum and milk has been reported to provide proteins related to maturation of the neonatal gastrointestinal tract (Godlewski et al., 2005, Brooks et al., 2006, Hammon et al., 2013).

2.4.3 Proteins involved in development of the immune system

Based on biological functions the dominant groups of proteins are the immune-related proteins, for which both the number and summed intensities decreased considerably over the first 9 days. The decrease of intensities is mainly driven by a decrease of the immunoglobulins as is shown in Figure 2.3B. Immunoglobulins showed a high abundance in the first two days and then decreased steeply afterwards. Also other immune-related proteins such as A2M, C9, A1BG, AHSG, and CLU, decreased significantly during these 9 days. A decrease in immune-related proteins in bovine milk has been reported before (Le et al., 2011, Senda et al., 2011, Zhang et al., 2011). The relative higher concentration of immune-related proteins in colostrum compared to mature milk was also reported in yak milk (Yang et al., 2014). The high concentration of immunoglobulins in the first two days is mainly due to the important role in the build-up of the adaptive immune system of calves, since they don't get any immunoglobulins from the mother cow before they are born (Larson et al., 1980). The significant decrease of immune-related proteins in the first 9 days is probably related to the decrease in the ability to transfer immune-related proteins from cows to calves (Cortese, 2009). This transfer during the first two days has been linked to the immature digestion system of calves, the high pH of abomasum content, and the relatively low pH in the proximal duodenum, which are all favorable conditions to reduce enzyme action and thus allow sufficient absorption of intact immune-related proteins during the first days of live (Guilloteau et al., 2009). After the fast development of the calves' immune system during the first two days, the reliance of calves on immune proteins from colostrum also reduces (Moore et al., 2005).

In addition to these major proteins of the adaptive immune system, we also found a range of complement components (A2M, C3, C6, C7, C9, CFB, and CD59) that are important for the innate immune system (Figure 2.6). These components are also present in high concentrations in the first two days and decrease at comparable rates during the following days (Figure 2.7A). CD59 is one of the main regulators of the complement pathway (Bjorge et al., 1996), which limits C9 input and prevents the polymerization of C9 during the final step of membrane attack complex (MAC) formation on the cell membrane (Rollins and Sims, 1990). When the cow is infected by pathogens, the level of CD59 drops and therefore its inhibitory role in the complement system will be reduced, thereby allowing the complement system to function during the

inflammation (Kimberley et al., 2007). The high abundance of complement proteins indicates that colostrum not only confers components from the adaptive immune system to the newborn but that it also transfers proteins of the innate immune system (Jensen et al., 2012).

Although protease inhibitors have been previously reported to be present in milk, their potential function in milk is still unclear. In this study, we not only found high abundant protease inhibitors but also found a high correlation of the protease inhibitors with other immune-related proteins (Figure 2.7). Several protease inhibitors, such as SERPINA1, GSN, AMBP, ITIH1, and ITIH2, decreased significantly (Table 2.4). The decrease of these protease inhibitors was highly correlated with a similar decrease in immunoglobulins. Two examples are shown in Figure 2.7B and 2.7C. The high correlation between protease inhibitors and immunoglobulins agrees with a previous study which reported that SERPINA1 can protect IgG (Quigley et al., 1995) and lactoferrin (Chowanadisai and Lönnerdal, 2002) from proteolytic degradation. Therefore, protease inhibitors in milk may help protecting immune-related proteins. The same reduction of protease inhibitors and immunoglobulins may also be caused by protein-protein interactions within complex (Jackson, 1999).

In addition, protease inhibitors are also involved in the blood coagulation cascade and complement pathway (Krarup et al., 2007) as shown in Figure 2.6. The participation of proteases and protease inhibitors in the immune response and blood coagulation (Ward and German, 2004) can be attributed to the complexity and interactions of milk proteins in biological functions. As an example, the activity of PLG, which is a protease that functions as blood coagulation protein, has also been shown to increase during severe mastitis (Pareek et al., 2005). PLG can act as a cofactor in adhesion, or, following activation to plasmin, provide a source of potent proteolytic activity of bacterial cells (Sanderson-Smith et al., 2012). Proteins A2M, AHSG, C3, ITIH4, and SERPINF2, which are classified as immune-related proteins, also function as protease inhibitor according to DAVID Gene Ontology (Dennis et al., 2003), whereas complement pathway proteins F2, C1S, CFB, CFD, and HP, have serine-type endopeptidase activity (Dennis et al., 2003). SERPINA1 has been previously reported to regulate leucocyte-released serpin proteinase activity during complement activation and inflammation, and it was also shown to be involved in the blood coagulation system through inhibiting coagulation pathway enzymes (Law et al., 2006). These proteins related to the complement and coagulation pathways were also reported in yak milk (Yang et al., 2014).

The correlated changes of immune-related proteins, protease inhibitors and blood coagulation proteins (Figure 2.7D and 2.7E) agree with the result from previous studies (Fritz, 1979, Kanost, 1999). This phenomenon is probably related to the balance between proteases and protease inhibitors that are involved in not only blood clotting, but also cytokine activation and inflammation.

In conclusion, this study for the first time shows the quantitative changes of the milk proteome from four individual cows at 7 time points between day 0 and day 9 after calving. Non-targeted proteomics analysis combined with time series study contributes to our understanding of the needs of the calf in the first days of life, as well as the complex biological interactions of milk proteins in the growth and development of newborns. This study also indicates which proteins may be of importance to the newborn and therefore warrant further targeted investigations.

Chapter 3

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

Zhang, L., Boeren, S., Hageman, J.A., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. (2015). Perspective of calf and mammary gland development via changes in the bovine milk proteome over 12 months lactation period. **Journal of Dairy Science** 98(8):5362-5373.

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 (FASP) combined with dimethyl labelling followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to identify and quantify milk proteins from four cows. A total of 229 proteins were identified, of which 219 were quantified. A 80% overlap was found in identified and quantified proteins between the four 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, and 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, and lactoferrin) and the decrease of proteins related to milk component synthesis (α-lactalbumin, β-lactoglobulin, fatty acid-binding protein, perilipin-2, and 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.

3.1 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 (Lönnerdal, 2010). Due to 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 protein source for mimicking human milk (Hernell, 2011). The differences in composition between human milk and bovine milk (D'Auria et al., 2005) has 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), reduced risk for celiac disease, obesity, and diabetes compared to 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, Senda et al., 2011, Zhang et al., 2015). So far, however, there has been no study about the variation in these low abundant proteins in mature bovine milk over the whole lactation (from early to the end of lactation). In addition, previous bovine milk proteome studies 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 expected to not only contribute to our understanding of the needs of calves over lactation but also to better understanding of the involution of the mammary gland and mastitis. Therefore, the objective of this study is to determine the qualitative and quantitative variation in the milk proteome from four individual cows from early to late lactation using proteomic techniques that combine filter aided sample preparation (FASP) and dimethyl labelling followed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

3.2 Materials and Methods

3.2.1 Materials

Bovine milk was collected from four 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/day. No specific permissions were required for this sample collection, as samples were taken from the milk collected during regular milking. 100 mL sample, which is from pooled milk from four udders throughout the milking, was collected at each time point. The samples were frozen immediately at -20℃ after collection and transferred frozen to the laboratory for protein, fat, lactose, and proteomic analysis. Samples collected at 0.5 month, 1 month, 2 months, 3 months, 6 months, 9 months, and the latest time point of the lactation (10 months for cow 1, 11 months for cow 2 and 12 months 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).

3.2.2 Milk Composition Analysis

Milk samples were analyzed for somatic cell count (SCC), protein, fat, and lactose contents by CombiFoss 5000 by Qlip.

3.2.3 Milk serum separation

Milk samples collected at different time points from four individual cows were centrifuged at 1500×g for 10 min at 10 °C (Beckman coulter Avanti J-26 XP centrifuge, rotor JA-25.15, USA). 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, USA). After ultracentrifugation, samples were separated into three 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 BCA assay and FASP as described below.

3.2.4 BCA assay

BCA Protein Assay Kit 23225 (Thermo Scientific Pierce®, USA) 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 0.02-2 μg/μL. Subsequently, the milk serum protein concentration was determined.

3.2.5 FASP-dimethyl labelling–LC-MS/MS

The method used to prepare milk serum samples for LC-MS/MS analysis was based on FASP (Wisniewski et al., 2009) and dimethyl labelling (Lu et al., 2011). Then, the prepared samples were analyzed using nanoLC-Orbitrap-MS/MS (Zhang et al., 2015).

3.2.6 Data analysis

Each run with all MS/MS spectra obtained was analyzed with Maxquant 1.3.0.5 with Andromeda search engine (Cox and Mann, 2008, Cox et al., 2011). Carbamidomethylation of cysteines was set as a fixed modification (enzyme=trypsin, maximally 2 missed cleavages, peptide tolerance 10 ppm, fragment ions tolerance 0.5 amu). Oxidation of methionine, N-terminal acetylation and de-amidation of asparagine or glutamine were set as variable modification for both identification and quantification. The bovine reference database for peptides and protein searches was downloaded as fasta files from Uniprot (http://www.uniprot.org/ accessed Dec 2013) with reverse sequences generated by Maxquant. A set of 31 protein sequences of common contaminants was added including Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human), and Keratin K1C1 (P35527, human). A maximum of two missed cleavages were allowed and mass deviation of 0.5 Da was set as limitation for MS/MS peaks and maximally 6 ppm deviation on the peptide m/z during the main search. A 1% false discovery rate (FDR) was set to on both peptide and protein level. The length of peptides was set to at least seven amino acids. Finally, proteins were displayed based on minimally 2 distinct peptides of which at least one unique and unmodified.

Dimethyl labelling was based on doublets with dimethLys0 and dimethNter0 as light, dimethLys4 and dimethNter4 as heavy labels. Razor and unique peptides were used for quantification. Normalized H/L ratios of proteins from samples collected over time (H) to the pooled sample of each cow (L) were used for further statistical analysis. Also the intensity based absolute quantification (iBAQ value) algorithm was used in this research. Because this study was carried out as a follow-up to the earlier published study describing the changes in colostrum proteome (Zhang et al., 2015), the summed iBAQ value of proteins based on biological function in colostrum (average of three time points from day 0 to day 2) based on that study were also included. It estimates absolute protein concentration as the sum of all peptide intensities divided by the number of theoretically observable tryptic peptides. The iBAQ value has been reported to have a reasonable correlation with known absolute protein amounts over at least four orders of magnitude (Schwanhausser et al., 2011).

The function of the identified proteins was checked in the UniprotKB database released March 2014 (http://www.uniprot.org/). The correlation between the amount of total immunoglobulins and total protease inhibitors was calculated using SPSS (Version 21, IBM Corp.). To select the proteins that significantly decrease over time, proteins were analyzed univariately. For each protein and per cow, a regression line was fitted on the protein concentrations measured at time points 14 days, 1 month, 2 months, 3 months, 6 months, 9 months, and the latest time point of the lactation. To reliably estimate a regression line, only proteins with at least 4 observed time points per cow were considered. The regression line summarizes per cow the concentration profiles for each protein into four intercepts and four slopes. The intercepts are the protein concentration at time 14 days and the slopes indicate the decrease or increase in concentration between time points. By using hypothesis tests on the slopes it can be determined if the decrease or increase in concentration is significant. The Lilliefors normality test (Lilliefors, 1967) was used to test if the four slopes were normally distributed. Proteins for which the four slopes were not normally distributed were discarded, since the non-parametric Wilcoxon signed rank test cannot establish a significant decrease with only four observations with $\alpha=0.05$. Proteins with normally distributed slopes were subjected to a two-sided t-test to test if the slopes were significantly decreasing or increasing (with $\alpha=0.05$). This significant analysis has been used in our previous bovine colostrum study (Zhang et al., 2015).

3.3 Results

3.3.1 The Changes of milk composition over lactation

Figure 3.1 shows the changes of protein, fat, and lactose content of milk samples as well as milk yield over lactation. Milk yield and lactose increased from colostrum to 3 months and slightly decreased afterwards. Milk protein and fat content decreased from colostrum to 0.5 month. Both components were relatively constant in mid lactation before a slightly decrease in late lactation (Figure 3.1). The average SCC of milk samples was relatively higher (60,000 cells/mL) in early and late lactation, compared to mid lactation (37,000 cells/mL). The SCC of milk samples used in this study were all below 130,000 cells/mL. The udder health in terms of microbiological milk analysis was not performed.

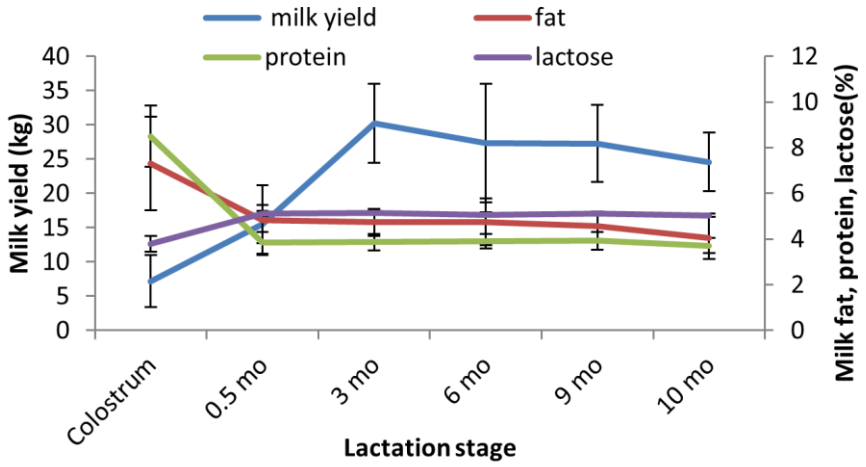


Figure 3.1 The milk yield, and fat, protein, and lactose percentage over lactation.

3.3.2 Overview of the identified proteins and quantified proteins in four individual cows

A total of 229 proteins were identified in this study, of which 219 could be quantified. For the four individual cows, around 220 proteins were identified in each individual cow and 80% of these proteins could be identified and quantified in all four cows. Besides these similar identified and quantified

proteins, there were also differences between the four individual cows. For example, some proteins could only be identified and quantified in one cow as shown in the Venn diagram (Figure 3.2). In addition, the number of identified and quantified proteins at early and late lactation stage are also different among the four individual cows, whereas they are quite similar at middle lactation stage (3 and 6 months).

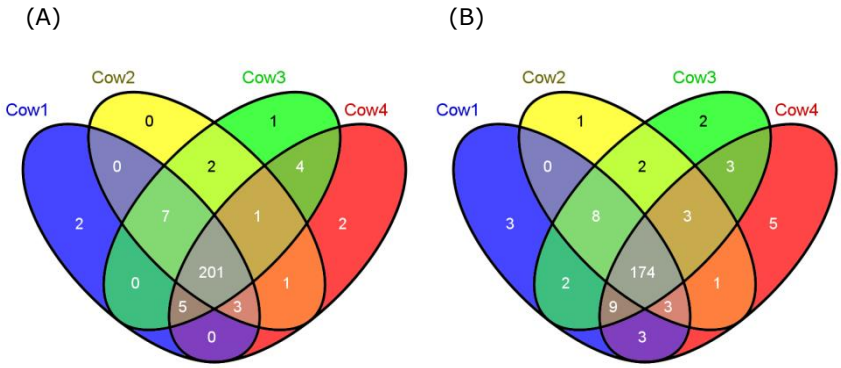


Figure 3.2 The number of identified (A) and quantified (B) milk serum proteins in four individual cows.

3.3.2 Distribution of identified and quantified proteins over biological functions

Table 3.1 shows the distribution of identified proteins based on their biological functions according to Uniprot. Enzymes (19%), immune-related proteins (18%), and transport proteins (15%) were the three dominant groups. There were slight changes in the distribution of biological functions over lactation (less than 10%, data not shown). The summed iBAQ value of quantified proteins based on the biological functions changed to a much larger extent than the number of proteins from colostrum to late lactation (Figure 3.3). Because this study was carried out as a follow-up to the earlier published study describing the changes in colostrum proteome (Zhang et al., 2015), the summed iBAQ value of proteins based on biological function in colostrum (average of three time points from day 0 to day 2) based on that study were also included in Figure 3.3. Transport and immune-related proteins decreased remarkably from colostrum to two weeks, followed by an approximately 1.5-fold increase from 2 weeks to 6 months. Similar trends could be found for protease inhibitors (Figure 3.3). On the contrary, enzymes increased from colostrum to mature milk. The three groups, immunity, transport, and enzymes all increased from

two weeks to 6 months of lactation. The increase of transport proteins from week 2 to month 3 is comparable to the change in milk yield during that period (Figure 3.1). In fact, the changes in the summed iBAQ value during that period were almost completely caused by the three most abundant proteins of each biological function group, as shown in Table 3.1. For instance, the increase of two dominant groups, enzymes and transport proteins, were mainly due to the increase of α-lactalbumin (LALBA), ribonuclease pancreatic (RNASE1), beta-1,4-galactosyltransferase 1 (B4GALT1), and β-lactoglobulin (LGB). The three most abundant proteins represented 99.6% of summed iBAQ value in the enzyme group and 90.7% of summed iBAQ value in the transport group.

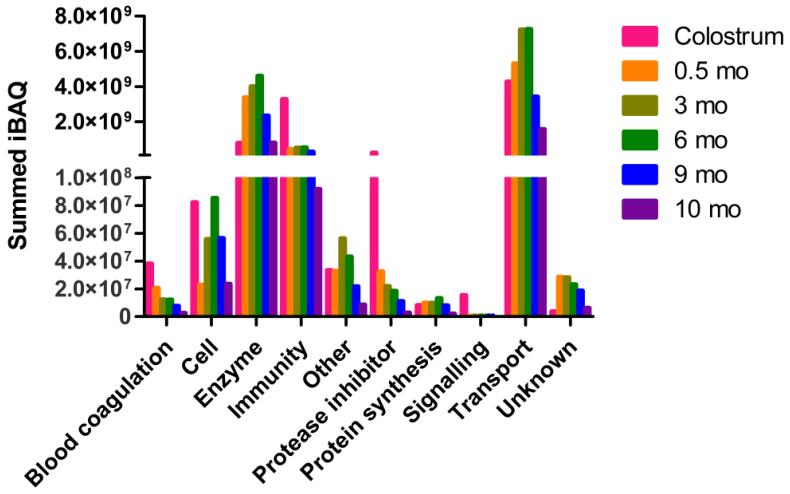


Figure 3.3 Quantitative changes of milk serum proteins based on biological functions over lactation stage (Cell includes cell motility, cell apoptosis, cell growth, cytoskeleton, cell adhesion, cell differentiation, cell proliferation, cell shape; immunity includes immune response, complement, antibacterial, host defense, acute phase, antigen binding; ER is endoplasmic reticulum).

Table 3.1 Distribution of identified milk serum proteins over biological functions, and intensity based absolute quantification (iBAQ value) of the three most dominant proteins per biological function

Biological function	Num (%)[1]	Top3 (%)[2]	Gene name	Protein name	iBAQ value
Enzyme	19	99.6	LALBA	Alpha-lactalbumin	5.7×10^{9}
			RNASE1	Ribonuclease pancreatic	3.5×10^{7}
			B4GALT1	Beta-1, 4-galactosyltransferase 1	1.4×10^{7}
Unknown	5	98.1	Bt.57604	Uncharacterized protein	4.1×10^{7}
			CRISP3	Uncharacterized protein	4.0×10^{6}
			GP2	Uncharacterized protein	3.7×10^{6}
Transport	15	90.7	PAEP	Beta-lactoglobulin (99% similarity)	7.5×10^{9}
			CSN2	Beta-casein	1.1×10^{9}
			CSN1S1	Alpha-S1-casein	5.2×10^{8}
Protein synthesis	5	87.2	CREG1	Protein CREG1	1.2×10^{7}
			SEP15	15 kDa selenoprotein	1.3×10^{6}
			HSPA5	78 kDa glucose-regulated protein	7.6×10^{5}
Protease inhibitor	8	86.3	SERPINA3-5	Serpin A3-5	1.4×10^{7}
			SERPINA1	Alpha-1-antitrypsin	1.4×10^{7}
			SERPINA3-7	Serpin A3-7	3.5×10^{6}
Cell	12	85.0	SPP1	Osteopontin	8.1×10^{7}
			CD36	Platelet glycoprotein 4	9.6×10^{6}
			ACTB	Actin, cytoplasmic 1	4.9×10^{6}

Biological function	Num (%)[1]	Top3 (%)[2]	Gene name	Protein name	iBAQ value
Signalling	4	80.1	YWHAZ	14-3-3 protein zeta/delta	1.8×10^{5}
			GNG12	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12	1.2×10^{5}
			GNB1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	1.1×10^{5}
Other	10	74.0	NUCB1	Nucleobindin-1	2.5×10^{7}
			SCGB1D	Secretoglobin family 1D member	9.9×10^{6}
			FGFBP1	Fibroblast growth factor-binding protein 1	9.4×10^{6}
Immunity	18	60.0	IGLL1	IGLL1 protein	1.7×10^{8}
			GLYCAM1	Glycosylation-dependent cell adhesion molecule 1	1.6×10^{8}
			LTF	Lactoferrin	8.9×10^{7}
Blood coagulation	4	56.7	Factor XIIa inhibitor	Factor XIIa inhibitor	4.1×10^{6}
			FGA	Fibrinogen alpha chain	4.5×10^{6}
			FGB	Fibrinogen beta chain	4.0×10^{6}

[1]Num(%): the percentage of identified proteins per biological function out of the total number of identified proteins;

[2]Top 3(%): the summed iBAQ value of the three most abundant proteins per biological function as a percentage of the total summed iBAQ value of all protein per biological function

3.3.3 The quantitative changes of proteins in four individual cows over lactation

Next to the changes of summed iBAQ values based on each biological function, Figure 3.4 shows changes of proteins related to lipid synthesis and transport, and proteins related lactose synthesis in four individual cows over lactation, such as fatty acid-binding protein (FABP3), epididymal secretory protein E1 (NPC2), perilipin-2 (PLIN2), butyrophilin subfamily 1 member A1 (BTN1A1), apolipoprotein E (APOE), LALBA, and B4GALT1. And for the changes of blood coagulation proteins, protease inhibitors as well as both innate and adaptive immune-related proteins, including polymeric immunoglobulin receptor (PIGR), immunoglobulins (IGLL1), lactoperoxidase (LPO), alpha-1-acid glycoprotein (ORM1), lactoferrin (LTF), and complement proteins, were shown in Figure 3.4. Proteins participating in the complement and coagulation cascade showed a similar trend over lactation (blue box in Figure 3.4). Also the immunoglobulins and protease inhibitors changed comparable over lactation (green box in Figure 3.4). A linear regression between the amount of total immunoglobulins and total protease inhibitors was established after testing for heteroscedasticity and normal distribution. The result showed that immunoglobulins and protease inhibitors were highly correlated ($r=0.69$, $p< 0.05$).

Of the quantified proteins, 95 proteins were determined at least at four time points per cow. This was deemed as a minimal requirement to reliably estimate the trend over time. The concentration profiles of these 95 proteins were summarized into intercepts and slopes as described in the methods section. The Lilliefors test indicated that 5 proteins had not normally distributed slopes, so these proteins were discarded. From the 90 proteins with normally distributed slopes, a total of 33 proteins showed a significant increase or decrease and they are listed in Table 3.2. Table 3.2 also shows the biological functions and subcellular locations of these significantly different proteins. Immune-related proteins were the largest group among the changed proteins (33% of all significantly different proteins) and they decreased rapidly from early lactation to middle lactation. The significantly changed immune-related proteins are shown in Figure 3.4. Transport proteins followed, accounting for 27% of the significantly changed proteins. Except for caseins, most of them were related to lipid secretion and transport (NPC2, BTN1A1, PLIN2, and APOE, Figure 3.4). However, the changes of these proteins over lactation were

different between the individuals animals. Cow 1 and cow 4 had high concentrations of complement proteins at two weeks, which decreased rapidly in middle lactation (blue box in Figure 3.4). Cow 2 and cow 3, on the other hand, had low concentrations of complement proteins at two weeks, which increased gradually afterwards. Although immunoglobulins decreased from early to middle lactation in all four cows, the rate of this decrease was much faster in cow 1 and cow 4 compared to cow 2 and cow 3, as shown in green box in Figure 3.4.

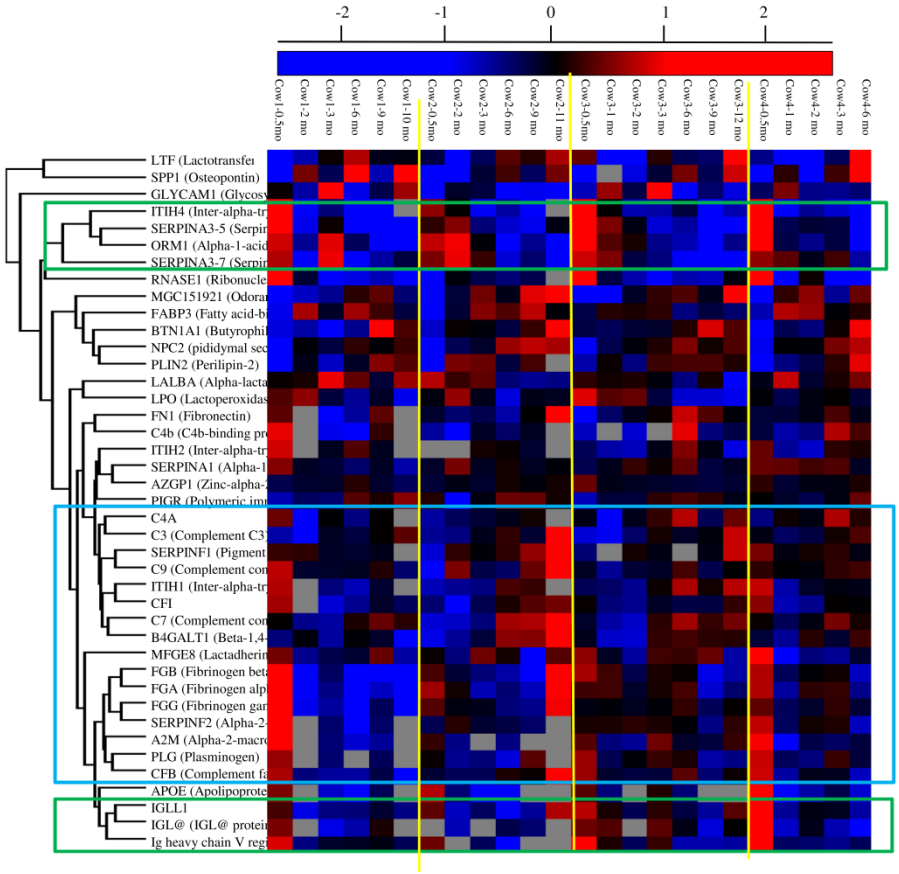


Figure 3.4 Quantitative changes (Log_2 ratio) of milk serum proteins in four individual cows over lactation, showing proteins related to milk synthesis and secretion, lactose synthesis as well as complement proteins, blood coagulation and protease inhibitors. The blue box includes proteins participating in the complement and coagulation cascade, and the green box includes immunoglobulins and protease inhibitors.

Table 3.2 Significantly different milk serum proteins over lactation

	Uniprot ID	Protein name	Gene name	Biological function	Subcellular location	*p* value
Increased Proteins	P18892	Butyrophilin subfamily 1 member A1	BTN1A1	Transport	Membrane	0.022
	F6R3I5	Uncharacterised	CRISP3	Unknown	Secreted	0.048
	G3N2D8	Uncharacterised	GGT1	Enzyme	Unknown	0.023
	Q0IIA2	Odorant-binding protein-like	MGC151921	Transport	Secreted	0.001
	A6QR11	Protein kinase C-binding protein NELL2	NELL2	Other	Secreted	0.005
	P79345	pididymal secretory protein E1	NPC2	Transport	Secreted	0.014
	Q0IIH5	Nucleobindin 2	NUCB2	Other	Endoplasmic reticulum	0.025
	P81265	Polymeric immunoglobulin receptor	PIGR	Immunity	Cell	0.008
	Q9TUM6	Perilipin-2	PLIN2	Transport	Membrane	0.040
	P62935	Peptidyl-prolyl cis-trans isomerase A	PPIA	Enzyme	Secreted	0.041
	Q27960	Sodium-dependent phosphate transport protein 2B	SLC34A2	Transport	Membrane	0.008
	P31096	Osteopontin	SPP1	Immunity	Secreted	0.021
DP	P12763	Alpha-2-HS-glycoprotein	AHSG	Immunity	Secreted	0.014
	Q03247	Apolipoprotein E	APOE	Transport	Secreted	0.046
	E1BH06	Complement component C4A	C4A	Immunity	Secreted	0.041

Uniprot ID	Protein name	Gene name	Biological function	Subcellular location	*p* value
Q29RQ1	Complement component C7	C7	Immunity	Secreted	0.036
P17697	Clusterin	CLU	Cell	Secreted	0.032
F1N076	Uncharacterised protein	CP	Transport	Secreted	0.025
P02663	Alpha-S2-casein	CSN1S2	Transport	Secreted	0.030
P02666	Beta-casein	CSN2	Transport	Secreted	0.045
P00735	Prothrombin	F2	Blood coagulation	Secreted	0.010
Q9MZ06	Fibroblast growth factor-binding protein 1	FGFBP1	Other	Cell membrane	0.024
P07589	Fibronectin	FN1	Immunity	Secreted	0.044
P80195	Glycosylation-dependent cell adhesion molecule 1	GLYCAM1	Immunity	Membrane	0.009
Q0VCX2	78 kDa glucose-regulated protein	HSPA5	Protein synthesis	Endoplasmic reticulum	0.018
Q3T052	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	Immunity	Secreted	0.015
P80025	Lactoperoxidase	LPO	Immunity	Secreted	0.048
Q2KIF2	Leucine-rich alpha-2-glycoprotein 1	LRG1	Cell	Secreted	0.002
Q95114	Lactadherin	MFGE8	Immunity	Secreted	0.016
Q3SZR3	Alpha-1-acid glycoprotein	ORM1	Immunity	Secreted	0.006

Uniprot ID	Protein name	Gene name	Biological function	Subcellular location	*p* value
Q3ZBZ1	45 kDa calcium-binding protein	SDF4	Other	Golgi	0.024
G8JKW7	Serpin A3-5	SERPINA3-5	Protease inhibitor	Cytoplasm	0.028
A2I7N3	Serpin A3-7	SERPINA3-7	Protease inhibitor	Cytoplasm	0.010

3.4 Discussion

This study assessed the qualitative and quantitative variation of the bovine milk proteome in four individual cows from two weeks to the end of lactation. The small differences found in the number of identified and quantified proteins among the four individual cows (Figure 3.2) are in accordance with data we previously collected for colostrum (Zhang et al., 2015), using samples from the same cows. The apparent presence of unique proteins in individual cows (Figure 3.2) is not caused by differences in udder health, according to SCC because they are much lower than the threshold (250,000 cells/mL) of subclinical mastitis (Turk et al., 2012). But could be due to both the detection limit of the instrument or differences between individual cows (e.g. age/parity). Since most unique proteins have only two identified peptides at relatively low intensity, we assume the unique proteins are due to the detection limit. The summed iBAQ values of proteins for each biological function changed considerably from early to late lactation (Figure 3.3), especially for the functions of enzyme, transport, and immunity, showing that variation was more on a quantitative than a qualitative level. At the same time, we need to acknowledge that the data interpretation is limited by the small sample size (n=4), and it is unknown how well these four cows represent the full variation that can be expected between cows. Nevertheless the data gives a first insight in the qualitative and quantitative changes between cows over lactation.

The increase of iBAQ value in enzyme group is mainly caused by the large increase in α-lactalbumin (LALBA) and β-1,4-galactosyltransferase 1 (B4GALT1) (Table 3.1). Both these proteins play an important role in the synthesis of lactose (Landers et al., 2009), which coincides with higher synthesis of lactose in middle lactation compared to week two. RNASE1, which is another high abundant enzyme, is involved in degrading bacterial RNA and nutrient uptake in the intestinal tract, and is especially important for plant eating animals like cows (Liu et al., 2014). The relative high concentration of RNASE1 in this early lactation stage may be related to the immature digestion system of calves in the early lactation.

3.4.1 Lipid synthesis and transport proteins

In addition to the changes of dominant transport proteins, other transport proteins also changed as lactation advanced (Figure 3.4). FABP3 has been known to be highly abundant in the bovine mammary gland (Bionaz and Loor,

2008b). It transports endothelial long-chain fatty acids to the endoplasmic reticulum for the synthesis of triglycerides that are eventually incorporated into lipid droplets for secretion (Bionaz and Loor, 2008b). FABP3 increased from two weeks to middle lactation (Figure 3.4), which agrees with the gene expression level of FABP3 during lactation (Bionaz et al., 2012). BTN1A1 and PLIN2, which play an important role in the milk fat synthesis (Bionaz and Loor, 2008b), were both significantly increased (p<0.05) (Table 3.2). The increase of FABP3, BTN1A1, and PLIN2 in bovine milk (Figure 3.4) suggests an increased de novo mammary fatty acid synthesis as lactation advances, which is similar to the change of these proteins in human milk (Zhang et al., 2013). The increase of these proteins from two weeks to middle lactation correlates with milk fat yield (Figure 3.1), and may therefore be a potential indicator for metabolic activity in the mammary gland. On the contrary, APOE significantly decreased over lactation (p<0.05), which is a cholesterol transporter (Friedland et al., 2003). Cholesterol plays an important role in the synthesis of vitamin D and the steroid hormones (Berg et al., 2002), which is critical to the development of the newborn. The decrease of APOE from two weeks to middle lactation in this study is in agreement with what was found in human milk (Zhang et al., 2013). The high concentration of APOE in the early lactation (Figure 3.4) indicates the importance of cholesterol in the development of the neonate.

3.4.2 Immune-related proteins

Next to the changes in proteins related to milk synthesis and secretion, immune-related proteins also showed large changes over lactation (Figure 3.4). The high concentration of immune-related proteins is consistent with the result in bovine colostrum study (Zhang et al., 2014), which can be related to the role of these proteins in the maturation of immune system of calves. The steady increase of these proteins during late lactation (Figure 3.4) is also in line with other studies (Lefevre et al., 2007). The correlation between immunoglobulins and protease inhibitors is in line with the correlation between immune-related proteins and protease inhibitors as described before for colostrum (Zhang et al., 2015). This corresponds with the hypothesis given in that paper that protease inhibitors play a role in the protection of immunoglobulins and thus promote maturation of the newborn's immune system. Our results, however, show different changes over lactation of proteins related to the innate immune system and the adaptive immune system. The differences between the two groups of immune proteins among four individual cows from 2 months to 6

months (as shown in Figure 3.4, innate immune proteins in the blue box and adaptive immune proteins in the green box) may be mainly associated with differences in their immune response phenotypes caused by genotype, and age. Gamma delta T-cell receptor lymphocyte subpopulations have been reported differentially distributed in blood and spleen between young and adult cattle (Wyatt et al., 1994). Individual cows have been shown to respond differently in adaptive immune response (B-cell regulated) and in innate immune response (T-cell regulated) after immunization (Hine et al., 2012). The changes of innate immune proteins may be related to the regulation of T cells during the immune response (Kwan et al., 2012), whereas the changes of adaptive immune proteins is related to the secretion of B cells (Mauri and Bosma, 2012).

With respect to the specific immune-related proteins, glycosylation-dependent cell adhesion molecule 1 (GLYCAM1, $p<0.05$), decreased significantly (Table 3.2). It is the most abundant protein after exclusion of major milk proteins, which is consistent with a previous proteomics studies of bovine milk (Hettinga et al., 2011). The high abundance of GLYCAM1 was suggested to be related to its important role in the host defense of the neonate. LTF, another relative high abundant proteins, is an iron binding protein with antimicrobial activity (Riley et al., 2008). The concentration of LTF was low at two weeks and progressively increased as lactation advanced (Figure 3.4), which was also previously reported (Cheng et al., 2008). The relative high concentration of LTF has been related to its protective role in the host defense of the neonate (Riley et al., 2008). Osteopontin (SPP1) changed similarly as LTF over lactation (Figure 3.4). The increase of SPP1 from two weeks to middle lactation found in this study is similar to what was found in human milk (Lönnerdal, 2014). SPP1 is a phosphorylated acidic glycoprotein that has been implicated in several physiological and pathological processes, including immune activation, wound healing, angiogenesis, bone remodelling, cell migration, and invasion of mammary epithelial cells (Ashkar et al., 2000, Denhardt et al., 2001, Hubbard et al., 2013). The addition of bovine SPP1 to infant formula changed expression of genes related to galactose metabolism, immune response, growth, and development toward a profile more similar to that in breastfed infants (Lönnerdal, 2014). As the decrease of immunoglobulins, the increase of these immune-related proteins from early lactation to middle lactation reflect their protection on the neonate as well as their importance in the maturation of the immune system of the neonate.

3.4.3 Preparation for involution of mammary gland

From 9 months to the last time point of late lactation, milk yield declined during the preparation for the involution of mammary gland as shown in Figure 3.1, which agrees with a previous study of Piantoni et al. (2010). A rapid decline in proteins related to milk fat synthesis (PLIN2, FABP3, and BTN1A1), as shown in Figure 3.4, corresponds to the decrease in milk fat content, as shown in Figure 3.1. The decrease of PLIN2, FABP3, BTN1A1, and LALBA, are in line with what was found in studies on the gene expression (Piantoni et al., 2010b) and transcripts level (Boutinaud et al., 2013) in the bovine mammary gland during involution. The decrease of these proteins may aid or even accelerate mammary gland involution (Ollier et al., 2013).

In addition, mammary gland involution also requires the remodelling of mammary epithelial cells of all mammary tissue (Piantoni et al., 2010b). Since mammary gland remodelling is a challenging process, during which the mammary gland is highly susceptible to new intramammary infections (Dingwell et al., 2003), protection of the mammary gland through secretion of immune-related proteins is expected. The present study indeed shows such an up-regulation of immune-related proteins in late lactation, including complement C3 (C3), IGLL1, SPP1, and LTF (Figure 3.4), which is also in accordance with results of previous studies (Riley et al., 2008, Wickramasinghe et al., 2012, Zhang et al., 2013). This increase in immune-related proteins may be caused by the pro-inflammatory state of the mammary epithelial cells during preparation for involution (Riley et al., 2008). In the classic component pathway, complement C5 is formed by C3 and it can recruit complement C9 to form the membrane attached complex on the microbial surfaces during cytolysis, which plays an important role in complement cascade (Zhang et al., 2013). IgG is the strongest mediator of the classical complement pathway via increased formation of the pathogen-IgG-C1 complex (Zhang et al., 2013). The increase of IGLL1 and C3 in late lactation indicates their joint role in protecting the mammary gland against infections. SPP1 could act as a macrophage chemotactic factor for recruitment at the inflammatory sites (O'Brien et al., 2011) and as a candidate mediator of wound-healing during mammary gland involution in rat mammary glands (O'Brien et al., 2012). LTF was reported to assist in the involution process by reducing bovine mammary epithelial cells' viability and inhibiting synthesis of caseins by a mechanism involving interleukin-1 beta (Riley et al., 2008). The increase of LTF in late lactation may

also be related to its protective role in the bovine mammary gland against infections during early involution because of its known antibacterial role (Riley et al., 2008). The up-regulation of immune-related proteins and down-regulation of proteins involved in milk component synthesis and secretion correspond to the physiological changes that are expected to occur in the bovine mammary gland during preparation for involution. In addition, the changes in the concentration of immune-related proteins over the course of lactation as presented here could also be useful for distinguishing proteins that are modulated in response to disease. This may thereby aid in developing biomarker for diseases, as the natural variability of these low-abundant proteins has not been described before.

3.5 Conclusion

Although there are some qualitative and quantitative differences in the milk proteome between individual cows, their changes over lactation are similar between cows. The changes of proteins involved in lipid synthesis are similar to the changes in lipid yield over lactation, and the differences in the changes of lipid synthesis and transport proteins suggest different sources of milk fat are used at different lactation stages. Moreover, the relative high abundant immune-related proteins also reinforces the important role of immune-related proteins in the growth and development of calves. In addition, the increase of immune-related proteins and the decrease of proteins related to milk synthesis and secretion in late lactation not only suggests their role in protection of the mammary gland but also indicates physiological changes that occur in the bovine mammary gland during preparation for involution.

Chapter 4

The quantitative changes of functional proteins in human milk during six month lactation period

Zhang, L., Waard, de M., Verheijen, H., Boeren, S., Hageman, J.A., Hooijdonk, van A.C.M., Vervoort, J.J.M., Goudoever, van J.B., Hettinga, K.A. The quantitative changes of functional proteins in human milk during six month lactation period. **Submitted**.

Abstract

Breast milk is the preferred nutrition for infants, but not all infants receive human milk. A comprehensive understanding of the human milk proteome may lead to the improvement of infant formula. To quantify functional protein changes over a 6 month lactation period, milk samples from four individual women collected at seven time points in the first six months after delivery were investigated by filter aided sample preparation and dimethyl labelling combined with liquid chromatography tandem mass spectrometry. 247 individual proteins were identified, of which 200 could be quantified. The milk proteome showed a high similarity (80% overlap) between women and the number of quantified proteins didn't change over time. The quantitative changes in milk proteins were mainly caused by the three groups, enzymes, transport, and immunity. The dominant proteins, bile salt-activated lipase in enzyme group, immunoglobulins, and lactoferrin in immunity group decreased as lactation advances; whereas serum albumin in transport group increased. In total, 21 proteins were found to change significantly over lactation, and of which 30% were transport proteins, such as serum albumin and fatty acid binding protein transporting nutrients for the infant. Human milk proteome doesn't differ qualitatively but quantitatively both as lactation advances and between mothers.

4.1 Introduction

Breast milk is the preferred nutrition for all newborn infants. It provides essential nutrients and bioactive and immunologic constituents that support optimal growth (Agostoni et al., 2009, Liao et al., 2011b). Infants who are exclusively breastfed for at least six months have a lower morbidity from gastrointestinal and allergic diseases, and from acute otitis media and respiratory infections (Kramer and Kakuma, 2004, Agostoni et al., 2009, Ladomenou et al., 2010). Additionally, breast milk reduces the risk of sepsis, necrotizing enterocolitis (NEC) and death, especially in very low birth weight infants (Meier and Bode, 2013). Breastfed infants have a higher IQ than formula fed infants, even when corrected for all known con-founders. (Anderson et al., 1999, Kramer et al., 2008). These short and long term advantages of breastfeeding may, amongst other reasons, be related to bioactive proteins in human milk, although other factors such as fatty acid quality and quantity may play a role as well.

Human milk proteins comprise caseins, milk fat globule membrane (MFGM) proteins and milk serum proteins. Of these proteins, milk serum proteins account for 60% of the proteins (Lien, 2003) and is therefore the dominant group in human milk proteins. In addition to a high proportion of essential amino acids and coenzymes for biosynthesis of lactose (Korhonen, 2009), milk serum has many bioactive proteins (Hettinga et al., 2011). These bioactive proteins play important roles in regulating the maturation of the immune and digestive system of infants (Ambroziak and Cichosz, 2014) and they change over lactation. Immunoglobulins A (IgA) is the dominant immunoglobulin in human milk and is much higher in colostrum as compared to mature milk (Politis and Chronopoulou, 2008, Zhang et al., 2013). Also α-lactalbumin (LALBA), lactotranferrin (LTF), and some low abundant proteins change over lactation as have previously been discussed (Liao et al., 2011b). The understanding of the human milk proteome is still not complete (Ballard and Morrow, 2013). Less is known on the changes of milk proteome in individual mothers.

Although breast milk is considered to be the best nutrition for all infants, it is unfortunately not always available for all infants due to for instance insufficient production, or maternal use of medication. The alternative source of breast milk, infant formula, should ideally have similar nutritional value and bioactive

functions as breast milk to benefit growth and development of infants (Ladomenou et al., 2010). A comprehensive understanding of the human milk proteome may lead to improve infant formula to become more similar to human milk. The objective of this study is therefore to investigate the changes of the milk proteome from four individual mothers over a six month lactation period by shotgun proteomic techniques and to compare this to the bovine colostrum study as previously described (Zhang et al., 2015).

4.2 Materials and methods

4.2.1 Sample collection

Human milk samples were collected from women who gave birth at the obstetric department in VU University medical center (VUmc) in Amsterdam. All women who delivered singleton term infants (gestational age 37-42 weeks) were eligible for this study. Women with haemolysis elevated liver enzymes, low platelet syndrome, history of breast surgery, and (gestational) diabetes mellitus were excluded. The institutional medical ethical review board approved the study and written informed consent was obtained from all participants. Participants were asked to donate milk samples during six months after delivery. The samples were collected weekly in the first month, every two weeks in the second and third month, and monthly afterwards. Approximately 5-10 ml was collected in a polypropylene bottle after one minute of pumping for every sample and stored at -18°C immediately afterwards.

Table 4.1 Characteristics of participants and their infants

Mother	Maternal age	Gestation age	Parity	Delivery mode	Gender of infant	Birth weight (g)
1	30	263	3	Vaginal	Boy	3450
2	33	273	2	C-section	Boy	4064
3	39	270	2	Vaginal	Girl	3348
4	35	288	1	Vaginal	Girl	2705

In total, 28 women were recruited between September 2013 and June 2014, of whom four mothers continued breastfeeding and thus collected milk samples for six months. The other women dropped out from the second month onwards

due to several reasons, such as insufficient milk production, mastitis or work related stress. Detailed information of the four included women and their infants is shown in Table 4.1. Since previous studies on distinct proteins showed a difference especially in early lactation, samples were assessed weekly in the first month and every two months (at week 8, 16, and 24) thereafter.

4.2.2 Milk serum separation (Lu et al., 2011)

The samples were centrifuged at 1500×g for 10 min at 10 °C (Beckman coulter Avanti J-26 XP centrifuge, rotor JA-25.15). The milk fat was removed and the obtained supernatant was transferred to the ultracentrifuge tubes followed by ultracentrifugation at 100,000×g for 90 min at 4°C (Beckman L-60, rotor 70 Ti). After ultracentrifugation, samples were separated into three phases. The top layer was remaining milk fat, the middle layer was milk serum, and the bottom layer (pellet) was casein. Milk serum was used for FASP as described below after the measurement of protein content by the BCA protein assay (Fisher Scientific) .

4.2.3 FASP-dimethyl labelling–LC-MS/MS

The method used to prepare milk serum samples for LC-MS/MS analysis was based on FASP (Wisniewski et al., 2009) and dimethyl labelling (Lu et al., 2011). Then, the prepared samples were analyzed using nanoLC-Orbitrap-MS/MS (Zhang et al., 2015).

4.2.4 Data analysis

Each run with all MS/MS spectra obtained was analyzed with Maxquant 1.3.0.5 with Andromeda search engine (Cox and Mann, 2008). Carbamidomethylation of cysteines was set as a fixed modification (enzyme=trypsin, maximally 2 missed cleavages, peptide tolerance 20 ppm, fragment ions tolerance 0.5 amu). Oxidation of methionine, N-terminal acetylation and de-amidation of asparagine or glutamine were set as variable modification for both identification and quantification. The human reference database for peptides and protein searches was downloaded as fasta files from Uniprot (http://www.uniprot.org/ accessed September 2014) with reverse sequences generated by Maxquant. A set of 31 protein sequences of common contaminants was added including Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human),

Keratin K2C1 (P04264, human), and Keratin K1C1 (P35527, human). A maximum of two missed cleavages were allowed and mass deviation of 0.5 Da was set as limitation for MS/MS peaks and maximally 6 ppm deviation on the peptide m/z during the main search. A 1% false discovery rate (FDR) was set to on both peptide and protein level. The length of peptides was set to at least seven amino acids. Finally, proteins were displayed based on minimally 2 distinct peptides of which at least one unique and unmodified.

Dimethyl labelling was based on doublets with dimethLys0 and dimethNter0 as light, dimethLys4 and dimethNter4 as heavy labels. Razor and unique peptides were used for quantification. Normalized H/L ratios were used for further statistical analysis. Also the intensity based absolute quantification (iBAQ value) algorithm was used in this research. It estimates absolute protein concentration as the sum of all peptide intensities divided by the number of theoretically observable tryptic peptides. The iBAQ value has been reported to have a reasonable correlation with known absolute protein amounts over at least four orders of magnitude (Schwanhausser et al., 2011).

The function of the identified proteins was checked in the UniprotKB database released Sep 2014 (http://www.uniprot.org/). To select the proteins that significantly change over time, proteins were analyzed univariate. For each protein and per mother, a regression line was fitted on the protein concentrations measured at time points 1, 2, 3, 4, 8, 16, and 24 weeks. To reliably estimate a regression line, only proteins with at least 4 observed time points per mother were considered. The regression line (Matlab, R2012A) summarizes per mother the concentration profile for each protein into an intercept and slope. The intercepts are the protein concentration at time 0, the slopes indicate the decrease or increase in concentration per week. By using hypothesis tests on the slopes it can be determined if the change in concentration is significant. The Lilliefors normality test (Lilliefors, 1967) was used to test if the four slopes were normally distributed. Proteins for which the four slopes were not normally distributed were discarded, since the non-parametric Wilcoxon signed rank test cannot establish a significant change with only four observations with α=0.05. Proteins with normally distributed slopes were subjected to a one sample t-test to test if the slopes were significantly changing (with α=0.05). The Gene Ontology (GO) enrichment analysis of significantly changed proteins was done using DAVID bioinformatics Resource 6.7 (http://david.abcc.ncifcrf.gov/). The GO terms shown in the results section were significantly enriched compared to the Go terms of all proteins identified

in this study. Significance was defined as $p<0.05$ where the Fisher Exact test was adopted to measure the gene-enrichment in Go terms. A linear regression between the amount of total immunoglobulins and total protease inhibitors was also conducted using SPSS (Version 21, IBM Corp.). Heteroscedasticity test was conducted to check whether "regression standardized residuals" remain constantly spread across "regression standardized predicted value" and histogram plot was used to check the normal distribution of standardized residuals before linear regression analysis.

4.3 Results

4.3.1 Number of identified and quantified proteins

A total of 247 proteins were identified in this study (Figure 4.1A) of which 200 proteins were quantified (Figure 4.1B). The overlap of identified and quantified proteins in the four individual mothers accounted for 72.5% and 82.5% respectively. The differences in the number of identified proteins and quantified proteins are shown in Figure 4.1.

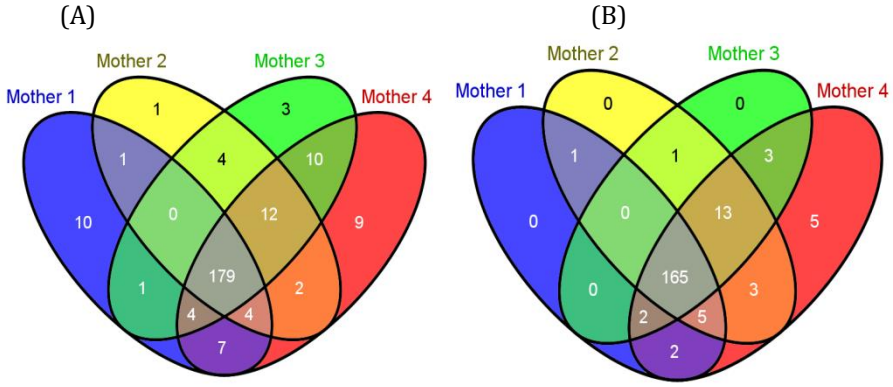


Figure 4.1 The number of identified (A) and quantified (B) proteins.

4.3.2 Distribution of biological functions of milk proteins

Table 4.2 shows the distribution of identified proteins based on their biological functions. Immune-related proteins (26%), enzymes (21%), and transport proteins (14%) were relatively the dominant groups. The number of identified proteins within these groups ranged from 204 to 229 over the first six months (data not shown), whereas the summed iBAQ value of quantified proteins

based on the biological functions changed to a much larger extent (Figure 4.2). The relatively dominant proteins mainly caused the changes of these biological groups as shown in Table 4.2, such as LALBA and bile salt-activated lipase in the enzyme group.

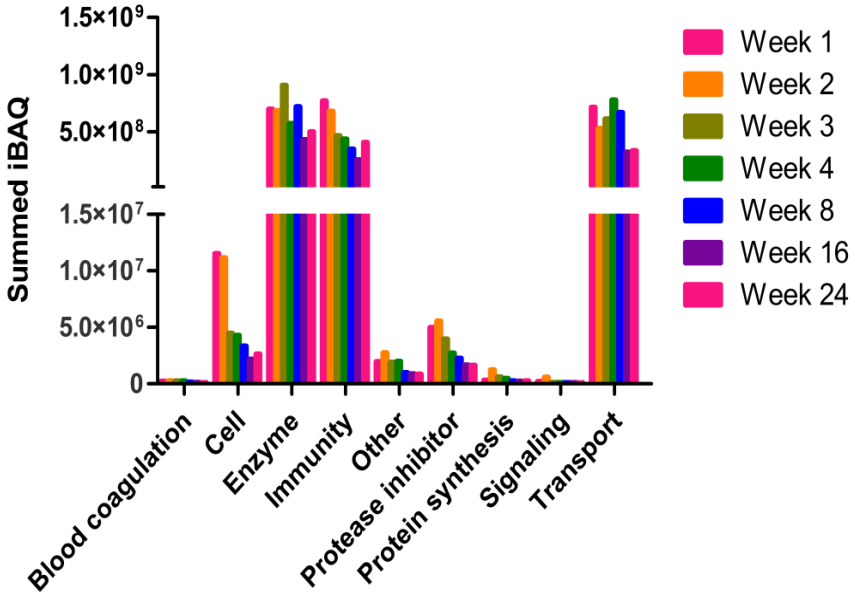


Figure 4.2 The quantitative changes of proteins based on biological functions over 6 months lactation.

Table 4.2 Distribution of identified proteins based on biological functions and iBAQ value of three dominant proteins per biological function

Biological function	Num (%)	Top3 [%]	Gene name	Protein name	iBAQ value
Blood coagulation	2	91.83	SERPING1	Plasma protease C1 inhibitor	2.83×10^{5}
			APOH	Beta-2-glycoprotein 1	7.20×10^{5}
			KNG1	Kininogen-1	3.39×10^{5}
Cell	18	63.20	LRG1	Leucine-rich alpha-2-glycoprotein 1	5.84×10^{6}
			ACTB	Actin, cytoplasmic 1	1.56×10^{7}
			MFGE8	Lactadherin	3.51×10^{6}
Enzyme	21	99.43	LALBA	Alpha-lactalbumin	4.14×10^{9}
			CEL	Bile salt-activated lipase	1.06×10^{8}
			ENO1	Alpha-enolase	7.81×10^{6}
Immunity	26	59.31	IGKC	Ig kappa chain C region	4.51×10^{8}
			IGHA1	Ig alpha-1 chain C region	4.74×10^{8}
			LTF	Lactoferrin	9.74×10^{8}
Other	7	60.08	GSN	Gelsolin	8.04×10^{5}
			MRC1	Macrophage mannose receptor 1	5.09×10^{6}
			MARCKS	Myristoylated alanine-rich C-kinase substrate	7.27×10^{5}
Protease inhibitor	3	98.38	CST3	Cystatin-C	2.83×106
			PEBP1	Phosphatidylethanolamine-binding protein 1	3.03×10^{6}
			SERPINA1	Alpha-1-antitrypsin	1.55×10^{7}

Biological function	Num (%)	Top3 [%]	Gene name	Protein name	iBAQ value
Protein synthesis	4	97.54	EEF2	EEF2 protein	9.10×10^{4}
			HSPA8	Heat shock cognate 71 kDa protein	9.05×10^{5}
			PPIA	Peptidyl-prolyl cis-trans isomerase A	1.90×10^{6}
Signalling	5	65.50	YWHAZ	14-3-3 protein zeta/delta	8.07×10^{5}
			YWHAB	14-3-3 protein beta/alpha	3.08×10^{5}
			RIC8B	Synembryn-B	2.28×10^{5}
Transport	14	93.25	CSN2	Beta-casein	2.17×10^{9}
			ALB	Serum albumin	6.38×10^{8}
			CSN1S1	Alpha-S1-casein	7.15×108

*Note: Num%, means the percentage of identified proteins based on each biological function out of the total identified proteins; Top 3% means the iBAQ value percentage of three dominant proteins in one biological function out of the summed iBAQ value of protein in this biological function

4.3.3 The quantitative changes of proteins over six months lactation

Figure 4.3 shows the changes of quantified proteins in breast milk from the four individual mothers from week 1 to week 24. Two major clusters were classified by hierarchical clustering as delimited by yellow boxes in the heat map (Figure 4.3). The first cluster (Cluster A, Figure 4.4) comprised proteins, like phosphatidylethanolamine-binding protein 1, UTP-glucose-1-phosphate uridylyltransferase, triosephosphate isomerase, and alpha-enolase, related to energy metabolism, which had relative low concentration at the first week and reached a peak at the second or third weeks before decreasing (Figure 4.3) again. However, the changes of these energy metabolisms related proteins (Cluster A) over lactation were different between the individual mothers. They showed relative high abundance at week 2 in mother 1 and 3, week 3 in mother 2, and week 16 in mother 4.

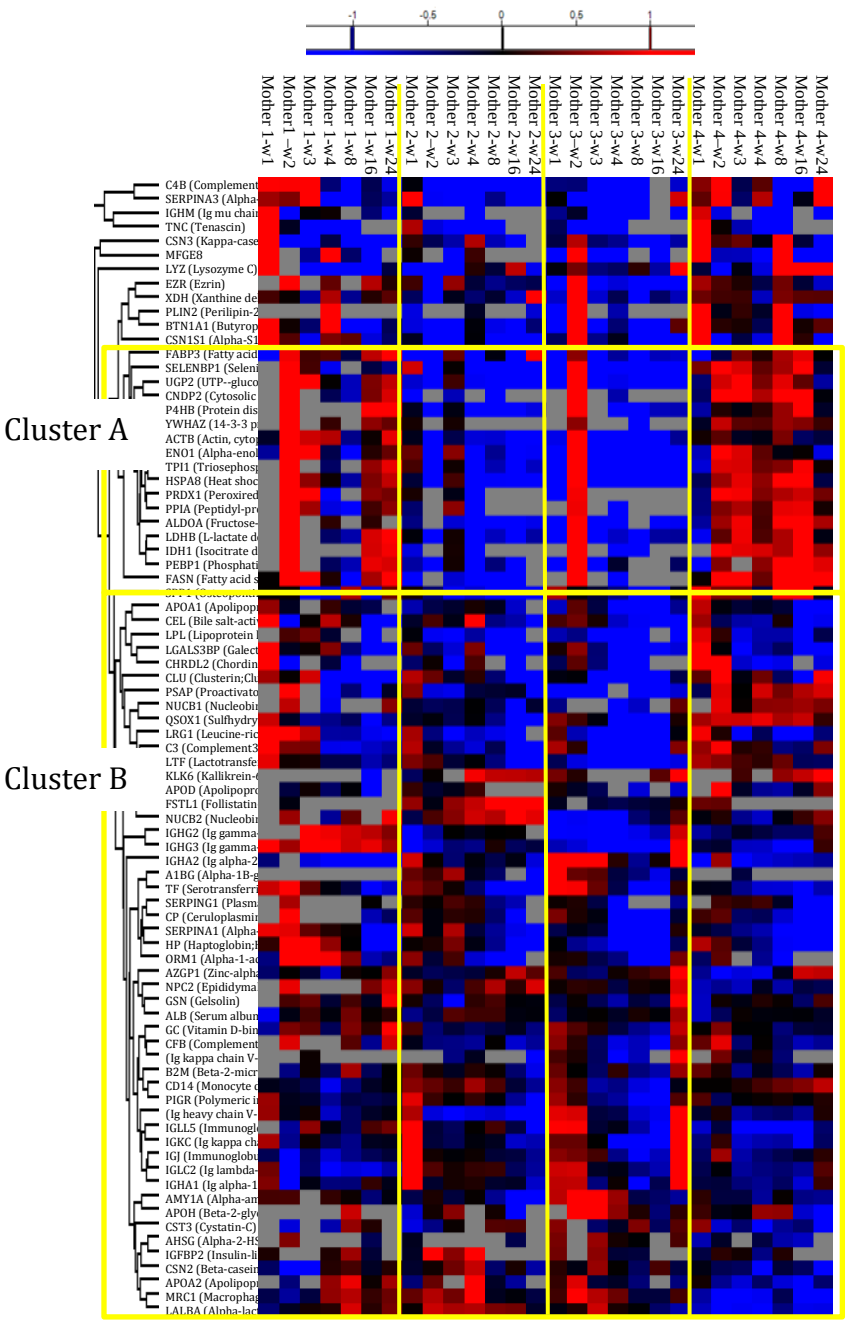


Figure 4.3 Quantitative changes (Log_2 ratio) of milk proteins in four individual mothers over 6 months lactation.

The second cluster (Cluster B) comprised defense response proteins (Figure 4.3), which were relatively high abundant in the first two weeks and changed similarly over lactation in all four mothers (Figure 4.3). These proteins include, IgA and immunoglobulins M (IgM), complement component 3 (C3), complement component 4B (C4B), complement factor B (CFB), LTF, monocyte differentiation antigen CD14 (CD14), alpha-1-antitrypsin (SERPINA1), plasma protease C1 inhibitor (SERPINC1), clusterin (CLU), haptoglobin (HP), alpha-1-acid glycoprotein 1 (ORM1). In addition, the changes of immunoglobulins significantly related to the changes of protease inhibitors (r^2=0.61, p<0.0005, SPSS), as shown in Figure 4.5.

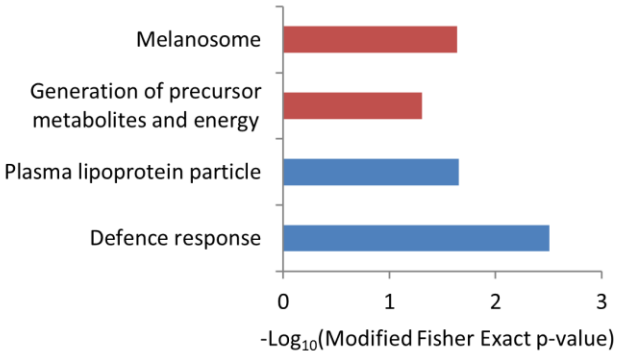


Figure 4.4 GO enrichment for subcellular location and biological process of cluster A (red) cluster B (blue). Comparison was made to GO terms of all identified proteins in the present study (DAVID bioinformatics Resources 6.7) (p<0.05).

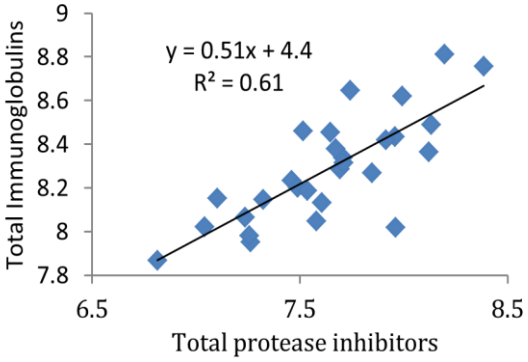


Figure 4.5 Linear correlation between immunoglobulins and protease inhibitors.

4.3.4 Significantly different proteins

Table 4.3 Significant different proteins with time series (one sample t-test, α=0.05)

	Protein ID	Protein name	Gene name	Biological function	*p* value
DP[1]	P02750	Leucine-rich alpha-2-glycoprotein	LRG1	Cell	0.039
	P80303	Nucleobindin-2	NUCB2	Cell	0.003
	Q08380	Galectin-3-binding protein	LGALS3BP	Cell	0.013
	P24821	Tenascin	TNC	Cell	0.018
	P19835	Bile salt-activated lipase	CEL	Enzyme	0.010
	P07195	L-lactate dehydrogenase B chain	LDHB	Enzyme	0.000
	O00391	Sulfhydryl oxidase 1	QSOX1	Enzyme	0.031
	Q16851	UTP--glucose-1-phosphate uridylyltransferase	UGP2	Enzyme	0.012
	P61626	Lysozyme C	LYZ	Immunity	0.034
	P06396	Gelsolin	GSN	Other	0.011
	Q6WN34	Chordin-like protein 2	CHRDL2	Other	0.034
	P07900	Alpha-1-antitrypsin	SERPINA1	Protease inhibitor	0.046
	P30086	Phosphatidylethanolamine-binding protein 1	PEBP1	Protease inhibitor	0.015
	P47710	Alpha-S1-casein	CSN1S1	Transport	0.006
	P07498	Kappa-casein	CSN3	Transport	0.010
	P02787	Serotransferrin	TF	Transport	0.018
	Q13228	Selenium-binding protein 1	SELENBP1	Transport	0.025
IP[2]	P00738	Haptoglobin	HP	Immunity	0.031
	P25311	Zinc-alpha-2-glycoprotein	AZGP1	Immunity	0.002
	P05413	Fatty acid-binding protein, heart	FABP3	Transport	0.012
	P02768	Serum albumin	ALB	Transport	0.001

[1] Decreased proteins; [2] Increased proteins

Of the quantified proteins, 60 proteins were determined at least at four time points in each participating woman. This was deemed as a minimal requirement to reliably estimate the trend over time. The concentration profiles of these 60 proteins were summarized into intercepts and slopes as

described in the methods section. The Lilliefors test indicated that 5 proteins did not have normally distributed slopes, so these proteins were discarded. From the 55 proteins with normally distributed slopes, a total of 21 proteins showed a significant increase or decrease as shown in Table 4.3, which accounted for 10% of the total quantified proteins. Transport proteins accounted for the largest percentage (30%) of the total significantly different proteins (Table 4.3). Of these significantly changed transport proteins, fatty acid-binding protein (FABP3), and serum albumin (ALB) increased, whereas serotransferrin (TF) and selenium-binding protein 1 (SELENBP1) decreased.

4.4 Discussion

This study investigated the time-dependent changes of the milk proteome in individual women over a six months lactation period. The overlap in both identified proteins (72.5%) and quantified proteins (82.5%) (Figure 4.1) indicates the similarity of the milk proteome in individual mothers. This is consistent with what has been reported in the milk proteome in other mammalian species such as cows (Zhang et al., 2015). Although milk proteins show high similarity in the qualitative level between individual women (Figure 4.1), milk proteins differed in amount and in their changes over lactation from one mother to the other. The variability in changes of energy metabolism proteins between mothers (Figure 4.3) may be associated with differences in milk production as described previously (Mitoulas et al., 2002). The great quantitative changes over lactation (Figure 4.2) confirms that changes in the milk proteome are not related to the qualitative level but involved in the quantitative level as shown in our previous study (Zhang et al., 2015).

The quantitative changes of milk proteins during lactation period could be attributed to the three dominant groups, enzymes, transport, and immunity, according to the biological classification (Figure 4.2). A large amount of proteins in human milk were biologically classified as enzymes (Table 4.2). The changes over lactation in this group of proteins could be ascribed to the two relatively high abundant proteins, LALBA, and bile salt-activated lipase (CEL) (Table 4.2). LALBA is a subunit of lactose synthase without catalytic activity by itself (Piantoni et al., 2010a). It contains a relatively high content of tryptophan as well as lysine and cysteine (Lönnerdal, 2014). CEL is one of the key enzymes secreted by the pancreas, which in cooperation with gastric lipase operates to achieve efficient fat degradation and absorption during infancy (Lindquist and

Hernell, 2010). It is inactive in milk and in the stomach of the neonate, however, it can survive the stomach's acidic and proteolytic environment to be activated by bile in the small intestine (Abrahamse et al., 2012). CEL may facilitate the digestion and absorption capacity of lipids, which would otherwise might become a limited factor in energy delivery to infants (Lindquist and Hernell, 2010). The relative high abundance of CEL in the first two weeks (Figure 4.3) may help to digest nutrients into small particles, and accelerate their absorption in the gastrointestinal tract. The decrease of CEL at week 24 (Figure 4.3) may be associated with the maturation of the liver and digestion system that will take over the triacylglycerol digestion, and with the change in nutritional needs of the infant who will by then be fed with solid foods as well.

Proteins related to nutrient transport contributed most to the overall significant difference in proteins over lactation (Table 4.3). The dominant transport protein ALB (Table 4.2) increased significantly in the first 2 weeks (Figure 4.3), which contradicts with previous study (Lönnerdal et al., 1976). This may suggest that changes in milk composition were more complex than was currently explained by the permeability of the paracellular pathway from blood to milk (Fetherston et al., 2006). The relative high abundance of ALB in human milk is related to its importance in providing amino acids to infants. Human milk ALB has been reported to bind zinc, copper (Lönnerdal et al., 1982), and thyroxine (Etling and Gehin-Fouque, 1984), suggesting its potential role in nutrient binder or supplier for infants. The significant increase in FABP3 (Table 4.3), is consistent with the results of a previous human milk study (Zhang et al., 2013) and our own bovine colostrum study (Zhang et al., 2015). In addition to transporting long-chain fatty acids, FABP3 also play an important role in the mammary lipid synthesis through channeling long chain fatty acid toward esterification into milk triglyceride (Bionaz and Loor, 2008a). Its increase indicates the increase of copious milk fatty acid synthesis in the mammary with the progression of lactation (Zhang et al., 2013). These two binding proteins in human milk may be related to promote the nutrients absorption of infants through transporting to intestinal cells.

In contrast, TF and SELENBP1 decreased significantly over six months lactation (Table 4.3). TF delivers iron to cells via a receptor-mediated endocytotic process to provide antibacterial activity (Wally and Buchanan, 2007). SELENBP1 has been shown to mediate the intracellular transport of selenium (Porat et al., 2000), which is a trace element that functions as a cofactor for reduction of antioxidant enzymes, such as glutathione peroxidases. The higher

concentration of transferrin and selenium-binding protein 1 in the early lactation stage reflects that human milk not only provides nutrients but also provide protections on infants.

The change of proteins in the immunity group could again be attributed to two dominant proteins: immunoglobulins and LTF (Table 4.2). The relatively high abundant IgA and LTF found in this study (Figure 4.3) are in accordance with previous studies (Hettinga et al., 2011, Gao et al., 2012). The relatively high concentration of IgA in early lactation was linked to the protection in the intestine against infection of the newborn infant (Hettinga et al., 2011). However, the protection by IgA is non-existing when the infant gets exposed to a novel antigen (Newburg and Walker, 2007) because the adaptive immune system has antigen specificity, immunological memory which requires some time to develop with subsequently a delayed reaction. Innate immune system, which plays a rapid or ongoing protection against broad groups of pathogens for newborns, is highly needed in early lactation.

Innate immune-related proteins accounted for a larger percentage in host defense proteins (Cluster B, Figure 4.4). Their relative high concentration in early lactation (Figure 4.3), is in line with previous studies (Gao et al., 2012, Zhang et al., 2013). This may be due to their importance in the protection of mucosal surfaces and also the complementary role of these proteins to immunological protection of the newborn (Ogundele, 2001). LTF can inhibit the bacterial growth by chelating free iron (Newburg and Walker, 2007) and reduce the production of inflammatory cytokines in monocytes by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation for the innate immune response (Walker, 2010). CD14 may sensitize the innate mucosal immune system to Gram-negative bacteria in the immature gut of the infant (Hoffmann et al., 1999). Complement proteins not only play important roles in immune bacteriolysis, neutralization of viruses and immune adherence but also in protection of the mucosal surfaces of the newborn (Ogundele, 2001). Protease inhibitors, including inter-alpha-trypsin inhibitor heavy chain H2 (ITIH2), SERPINA1, are key components in regulating the complement system (Gao et al., 2012). They could also protect immunoglobulins against degradation (Quigley et al., 1995), as serine proteases and trypsin were reported to rapidly degrade immunoglobulins (Gardner et al., 1993, Hurley and Theil, 2011). The linear correlation between protease inhibitors and immunoglobulins (Figure 4.5) was in line with what we have reported in our bovine colostrum study (Zhang et al., 2015). This agrees with

our hypothesis that protease inhibitors may protect immunoglobulins against degradation in the digestive tract. The involvement of protease inhibitors in both innate and adaptive immune-related proteins indicate that milk proteins interact with each other and function as a complex.

When comparing the quantitative changes of milk proteome over lactation between individuals, the variation was greater between individual mothers (Figure 4.3) than between individual cows (Zhang et al., 2015). However, the decrease of immune-related protein concentration was slower in human milk than bovine milk. The slower decrease of immune-related proteins in human milk than bovine milk was consistent with previous study (Korhonen et al., 2000). This may be related to the slower maturation of the immune system in infants as compared to calves (Hettinga et al., 2011). The differences in the digestive enzymes between human milk (CEL, Table 4.2) and bovine milk (pancreatic ribonuclease-RNASE1) (Zhang et al., 2015) may be related to the differences in the dietary needs of the offspring.

This study showed the qualitative and quantitative changes in the human milk proteome over lactation with a focus on specific proteins that showed large variability over the six months lactation period. The result found in this study may give some indications on the potential candidates to be enriched in infant formula. For instance, CEL, LTF, CD14, complement proteins, and protease inhibitors, which are probably more important in the first couple of months than later based on this study. There is a limitation of sample size in this study. Although sample size with four is a trustworthy number to show the changes in the milk proteome over lactation according to the normally distributed analysis in this study, it would still be better to have more participants. Large sample size study with a longer lactation period as well as complete information on both mothers and infants study may give an even more comprehensive reference of the needs of the infants at different lactation stages. This may lead to a better understanding of the importance of human milk in health and development of infants.

4.5 Conclusion

Human milk proteome doesn't differ qualitatively but quantitatively both as lactation advances and between mothers. As lactation advanced, the milk proteins with different biological functions changed in different directions with regards to their concentration, especially for transport, enzyme, and immune-

related proteins. The changes of milk proteins also differed between individual mothers although their milk proteome was similar in qualitative level. The decrease of immunity proteins over lactation was much slower in human milk compared to bovine milk. The results found in this study give a clue on the needs of infants in the first six months and the biological function of human milk proteins in the development of infants, which can be used as reference in improving infant formula.

Chapter 5

A proteomic perspective on the changes in milk proteins due to high somatic cell count

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Abstract

Although cows with subclinical mastitis have no difference in the appearance of their milk, milk composition and milk quality is altered because of the inflammation. In order to know the changes in milk quality with different somatic cell count (SCC) levels, five pooled bovine milk samples with SCC from 10^5 to 10^6 cells/mL were analyzed qualitatively and quantitatively using both one-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (1-D SDS-PAGE) and filter aided sample preparation (FASP) coupled with dimethyl labelling, both followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Minor differences were found on the qualitative level in the proteome from milk with different SCC levels, whereas the concentration of milk proteins showed remarkable changes. Not only immune-related proteins (cathelicidins, IGK protein, CD59 molecule, complement regulatory protein, and lactadherin), but also proteins with other biological functions (e.g. lipid metabolism: platelet glycoprotein 4, butyrophilin subfamily 1 member A1, and perilipin-2) were significantly different in milk from cows with high SCC level compared to low SCC level. The increased concentration of protease inhibitors in the milk with higher SCC levels may suggest a protective role in the mammary gland against protease activity. Prostaglandin-H2 D-isomerase (PTGDS) showed a linear relation with SCC, which was confirmed with an enzyme-linked immunosorbent assay (ELISA). However, the correlation coefficient was lower in individual cows compared to bulk milk. These results indicate that PTGDS may be used as an indicator to evaluate bulk milk quality, and thereby reduce the economic loss in the dairy industry. The results from this study reflect the biological phenomena occurring during subclinical mastitis and in addition provide a potential indicator for the detection of bulk milk with high SCC.

5.1 Introduction

Mastitis, an inflammation of the mammary gland, is one of the most devastating diseases affecting dairy cows, which results in changes of milk appearance, milk composition, and somatic cell count (SCC) (Forsbäck et al., 2010, Awale et al., 2012). A decrease in casein and whey protein concentrations has been reported to occur during mastitis (Hogarth et al., 2004). The lactose, sodium, and potassium contents were also found to be changed in concentration in milk from cows with mastitis due to leaky tight junctions (Hagiwara et al., 2003, Lindmark-Månsson et al., 2006). The inflammation of the mammary gland during mastitis easily develops in response to infection and can also lead to severe damage to the milk secretory tissue of the udder, resulting in a reduction in milk production and deteriorated milk quality (Hogarth et al., 2004). Mastitis is therefore considered as a major source of economic losses on dairy farms and also a serious burden on the dairy producers.

Although milk from cows with subclinical mastitis does not have visible changes in the appearance of their milk, as mentioned above, changes in milk composition and high SCC do occur. Milk from quarters with subclinical mastitis showed elevated levels of sodium, chloride, albumin, lactate dehydrogenase activity, and immunoglobulins as well as reduced levels of α-lactalbumin, β-lactoglobulin, calcium, inorganic phosphorus, and potassium (Batavani et al., 2007). It also contributes to an average milk production loss of 470 kg per primiparous dairy cow and 740 kg per multiparous dairy cow, during the full lactation with each unit increase in Log_{10} (SCC) (Koldeweij et al., 1999). The greater the SCC increase, the greater the production loss. In addition, subclinical mastitis causes a similar reduction of reproductive performance as mastitis (Schrick et al., 2001). High SCC in milk has been considered as the only evidence that helps in the diagnosis of subclinical mastitis (Turk et al., 2012). Therefore, identifying new biomarkers for subclinical mastitis may help to develop an easy test aiming at predicting milk suitability for further milk processing. Consequently, it is interesting to study the differences of the milk proteome between healthy cows and cows with high SCC for understanding the variations of proteins expressed in milk from cows with subclinical mastitis. This may contribute to potential biomarker discovery for the detection of bovine subclinical mastitis.

Compared to the effect of subclinical mastitis, the effect of clinical mastitis has been studied much more widely. For instance, Yang, et al (2009) detected differently expressed proteins in the mammary gland from mastitis cows and healthy cows, such as hemoglobin, β-casein, κ-casein, and tryptophanyl-tRNA-synthetase, which all showed lower concentrations in milk from mastitis cows, whereas cytochrome C oxidase and Annexin V showed higher abundance in milk from mastitis cows (Yang et al., 2009). Proteases, including plasmin, cathepsin B and D, elastase, and amino- and carboxypeptidases, were identified in bovine milk following infusion with lipoteichoic acid isolated from *Staphylococcus aureus* (Larsen et al., 2010). Low-abundant inflammation markers like serotransferrin, fibrinogen β chain, S100 calcium-binding protein A12, and the antimicrobial cathelicidins were shown to be present in relative high amounts in milk 12 h after infusion with *Escherichia coli* lipopolysaccharide (Hinz et al., 2012a). Only few studies have been carried out on the milk proteome of cows with subclinical mastitis. Safi, et al. (2009) found that acute phase proteins (haptoglobin and amyloid A) increased in milk from cows with subclinical mastitis (Safi et al., 2009). Serpin A3-1, vitronectin-like protein, and complement factor H were shown to be up-regulated in milk from subclinical mastitis cows in comparison with healthy cows (Turk et al., 2012).

The objective of the present study is to investigate the influence of high SCC (up to 10^6 cells/mL) on the milk proteins in bovine milk by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1-D SDS-PAGE) and also by filter aided sample preparation (FASP) combined with dimethyl labelling, both followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Using shotgun proteomics techniques to determine variation in protein levels in milk from infected cows will increase our understanding of the influence of high SCC levels on the protein composition of milk, thereby providing potential biomarkers for detecting milk with a high SCC level.

5.2 Materials and methods

5.2.1 Sample collection

A total of 100 cows were used in this study. Milk samples were a mixture from all four quarters of each cow. Five groups were made, consisting of pooled samples from 20 cows with similar SCC, which are SCC1 (<10^5 cells/mL); SCC2 (2.25-2.75×10^5 cells/mL); SCC3 (4.8-5.3×10^5 cells/mL); SCC4 (7-8×10^5 cells/mL); SCC5 (9.25-10.75×10^5 cells/mL). The sample with lowest SCC (SCC1)

will be referred to as low SCC. All other samples (SCC2-SCC5) represent samples with increased cell counts and will be referred to as high SCC, because SCC 2.5×10^5 cells/mL (SCC2) is considered as a threshold for subclinical mastitis (Turk et al., 2012).

Sodium azide (0.02% m/m) and bronopol (0.0005% m/m) were added to prevent bacterial growth in these pooled milk samples supplied by Qlip (Dutch milk controlling station, Zutphen, The Netherlands). A number of 28 individual samples with SCC ranging from 1×10^5cells/mL to 8.5×10^5cells/mL were also collected from Qlip for determining the relation between PTGDS and SCC in the milk serum of individual cows.

5.2.2 Milk composition analysis

Milk samples were analyzed for somatic cell count, dry matter, protein, fat, and lactose contents by CombiFoss 5000 by Qlip.

5.2.3 Milk serum separation

To separate milk serum, the pooled samples were centrifuged at 1500 g for 10 min at 10°C (Beckman coulter Avanti J-26 XP centrifuge, rotor JA-25.15, USA). The pellet 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, USA). After ultracentrifugation, samples were separated into three phases. Milk serum, in the middle layer, was separated and used for the proteomics sample preparation, as described below.

5.2.4 SDS-PAGE

SDS-PAGE was used to further separate milk proteins. Samples were subjected to 1-D SDS-PAGE using pre-cast 12% Precise Protein Gels with HEPES buffer (Thermo Fisher Scientific Inc., USA). The thawed protein samples were mixed 1:1 with 2x sample buffer (125mM Tris-HCL (pH 6.8), 4% SDS, 20% glycerol, 0.01% bromophenol blue in water); just before use, 5% β-mercaptoethanol was added and the samples were heated for 5 min at 95°C. Gels were loaded with approximately 30 μg of protein per well. The gels were run for 45 min at 130 V, and then fixed and stained with the Colloidal Blue Staining Kit (LC6025, Invitrogen, Carlsbad, CA, USA) for 4 h, and finally destained overnight in water.

5.2.5 In gel digestion

Except when stated otherwise, all NH_4HCO_3 (ABC, 0.05M) buffers used in this section were pH 8. After each step, the samples were sonicated for one minute followed by spinning down using a centrifuge. For each sample, the gel lane was cut into eight slices. Each slice was cut into pieces of maximum $1mm^3$ size using a scalpel and transferred to a low-binding microcentrifuge tube (0030 108.094, Eppendorf, Hamburg, Germany). The gel pieces were washed twice with water. The proteins were reduced by incubation in 0.05 M dithiotreitol (DTT) for one h at 60°C followed by incubation in 0.1 M iodoacetamide (IAA) for one h at room temperature in the dark. After carboxamidomethylation, the gel pieces were washed three times with 0.05 M ABC buffer. The gel pieces were then frozen and thawed 3 times to increase the accessibility for trypsin. Next, the samples were rehydrated in 20 µL freshly prepared trypsin solution (10 ng/µL). Extra 0.05 M ABC buffer was added to completely cover the gel pieces before they were incubated overnight at room temperature. The next day, the basic supernatant of digest solution was transferred to a clean low-binding microcentrifuge tube. The gel pieces were extracted twice with 10 µL 5% trifluoroacetic acid (TFA) in water and 10 µL 10% acetonitrile/1% TFA respectively with sonication after each extraction step. The supernatants after every sonication were added to the same low-binding microcentrifuge tube. Final pH of the peptide mixture was checked using pH paper.

5.2.6 FASP-dimethyl labelling–LC-MS/MS

The method used to prepare milk serum samples for LC-MS/MS analysis was based on FASP (Wisniewski et al., 2009) and dimethyl labelling (Lu et al., 2011). Then, the prepared samples were analyzed using nanoLC-Orbitrap-MS/MS (Zhang et al., 2015).

5.2.7 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to confirm the correlation between prostaglandin-H2 D-isomerase (PTGDS) and SCC that was found in the LC-MS/MS analysis. The samples were diluted 5000-fold aiming at the final concentration of PTGDS ranged from 93.75 to 6000 pg/mL. The concentration of PTGDS in the milk serum was detected in duplicates by the ELISA kit (bovine PTGDS, MBS942806) bought from EMELCA Bioscience Company (Breda, the Netherlands). The ELISA test, including creation of a standard curve, was performed according to the

manufacturer's protocol. A four-parameter logistic model, y=((d+((a-d)/(1+(x/c)^b)), was used for calibration by Masterplex readerfit. The standard curve y=2.69-2.65/(1+x/673.33)^-1.41 (r^2=0.988) was used to calculate the concentration of PTGDS in the samples based on the response value from the micro plate reader.

5.2.8 Data analysis

Each run with all MSMS spectra obtained was analyzed with Maxquant 1.3.0.5 with Andromeda search engine (Cox and Mann, 2008, Cox et al., 2011). Carboxamidomethylation of cysteines was set as a fixed modification (enzyme=trypsin, maximally 2 missed cleavages, peptide tolerance 20 ppm, fragment ions tolerance 0.5amu). Oxidation of methionine, N-terminal acetylation and de-amidation of asparagine or glutamine were set as variable modifications for both identification and quantification. The bovine reference database for peptide and protein searches was downloaded as fasta file from Uniprot (http://www.uniprot.org/ accessed March 2012) with reverse sequences generated by Maxquant. A set of 31 protein sequences of common contaminants was added including Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human), and Keratin K1C1 (P35527, human). A maximum of two missed cleavages were allowed and mass deviation of 0.5 Da was set as a limitation for MS/MS peaks and maximally 6 ppm deviation on the peptide m/z during the main search. A 1% false discovery rate (FDR) was set to on both peptide and protein level. The length of peptides was set to be at least six amino acids. Finally, proteins were displayed based on minimally 2 distinct peptides of which at least one unique.

Dimethyl labelling was based on doublets with dimethLys0 and dimethNter0 as light, dimethLys4 and dimethNter4 as heavy. Razor peptides (peptides that are non-unique, which are assigned to the protein with the most other peptides) and unique peptides (peptides that are unique to one protein) were used for quantification. Normalized H/L ratios calculated as the median of all dimethyl labelled peptide-pair ratios that belong to one protein were used for further statistical analysis. In each LC-MS run, peptide ratios were normalized, so that the median of their logarithms is zero, which corrects for unequal protein loading (Cox and Mann, 2008, Cox et al., 2011). Also the intensity based absolute quantification (iBAQ value) algorithm was used, which calculates absolute protein amounts as the sum of all peptide intensities divided by the

number of theoretically observable tryptic peptides. The iBAQ value has been reported to have a good correlation with known absolute protein amounts over at least four orders of magnitude (Malmström et al., 2009, Schwanhausser et al., 2011).

The function of the identified proteins was checked in the UniProtKB database released April 2012 (http://www.uniprot.org/). Gene Ontology (GO) enrichment analysis was done using DAVID bioinformatics Resources 6.7 (Dennis et al., 2003) (http://david.abcc.ncifcrf.gov/). The significant analysis in the changes of milk composition was conducted by one-sample t-test in SPSS (Version 21, IBM Corp.), which has been used in the significant analysis of microarray-based gene expression (Patterson et al., 2006). For the changes of individual proteins, a 2-fold change was used as the significant cut-off in this study, which has been reported in the previous milk proteomics studies done by FASP and dimethyl labelling (Lu et al., 2011, Lu et al., 2013). The linear correlation analysis between SCC and the relative concentration of milk proteins was performed in SPSS (Version 21, IBM Corp.).

5.3 Results

5.3.1 Identification of the milk serum proteome

Table 5.1 shows the composition of the milk samples of the five groups (20 cows per group) with different SCC levels. No significant differences were found in milk composition among these five groups (p>0.05). After proteomic analysis using 1D SDS-PAGE combined with nanoLC-Orbitrap-MS/MS, a total of 273 proteins from the five pooled samples were identified. There were minor differences (less than 10%) observed in the number of identified proteins between the groups. From the group with the lowest SCC to the group with the highest SCC, 217, 187, 201, 208, 217 proteins were identified, respectively. The proteins identified were grouped based on their biological functions and subcellular locations according to GO using DAVID (Dennis et al., 2003) as shown in Figure 5.1 and 5.2. Most of the proteins identified were linked to immunity, transport, cell functions (including cell adhesion, cell mobility, cell growth, and cell apoptosis) or enzymes. Based on the subcellular location, the three major groups, secreted, membrane and cytoplasm, constituted over 90% of the total proteins. Secreted was the dominant subcellular location and accounted for around 45% of the total proteins. Even though proteins originating from endoplasmic reticulum, Golgi, lysosome, and nucleus

accounted for a very little part of identified proteins, they were present in all of these samples as shown in Figure 5.2. The number of identified proteins based on their biological functions and subcellular locations didn't change with the increase of SCC.

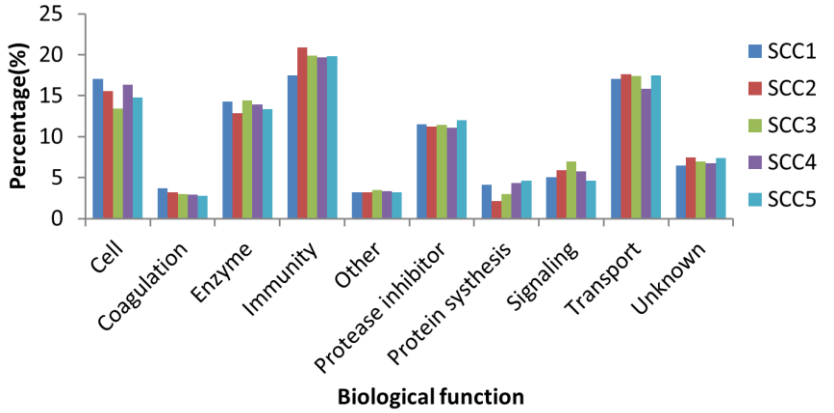


Figure 5.1 Functional ontology classification of proteins in bovine milk with different level of SCC (SCC1 is<10^5 cells/mL; SCC2 is 2.5×10^5 cells/mL; SCC3 is 4×10^5 cells/mL; SCC4 is 7.5×10^5 cells/mL; SCC5 is 10^6 cells/mL; cell includes cell adhesion, cell mobility, cell growth, and cell apoptosis).

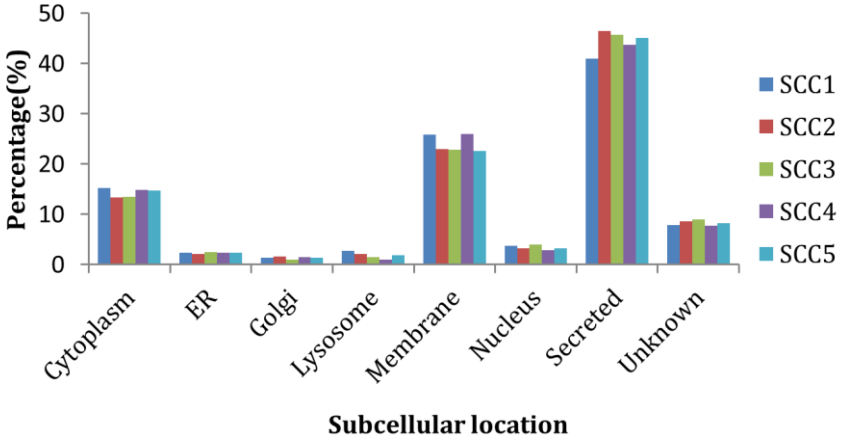


Figure 5.2 Subcellular location classification of proteins in bovine milk with different level of SCC (SCC1 is <10^5 cells/mL; SCC2 is 2.5×10^5 cells/mL; SCC3 is 4×10^5 cells/mL; SCC4 is 7.5×10^5 cells/mL; SCC5 is 10^6 cells/mL; ER =endoplasmic reticulum).

Table 5.1. Composition of the milk samples with different SCC (SCC1 is $<10^5$ cells/mL; SCC2 is 2.5×10^5 cells/mL; SCC3 is 4×10^5 cells/mL; SCC4 is 7.5×10^5 cells/mL; SCC5 is 10^6 cells/mL; significant analysis was done by one-sample test analysis in SPSS)

Milk composition	SCC1	SCC2	SCC3	SCC4	SCC5	One-sample test (Test value=0)	
						Sig. (2-tailed)	Mean difference
Dry matter (%)	13.8	13.46	13.24	13.42	13.22	0.943	0.008
Protein(%)	3.72	3.65	3.91	3.68	3.54	1	0.000
Fat(%)	4.42	4.48	4.24	4.52	4.46	1	0.000
Lactose(%)	4.84	4.49	4.16	4.34	4.37	0.973	0.004

5.3.2 Quantification of the milk serum proteome

In order to know the influence of high SCC on the expression level of the milk proteome, FASP combined with dimethyl labelling, followed by nanoLC-Orbitrap-MS/MS was used for quantitative analysis. A total of 100 proteins were quantified. The biological functions and relative concentration of the 56 common proteins are shown in Table 5.2. A specific group of immune-related proteins, including all cathelicidins, PTGDS, Ig gamma-2 chain C region (IGHG2), and inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), showed an increase in the milk samples with high SCC compared to low SCC.

Next, a comparison was made between milk from cows with highest SCC (SCC5, 10^6 cells/mL) and milk with lowest SCC (SCC1<10^5 cells/mL), as shown in Figure 5.3. These significantly different proteins had more than 2-fold changes (Log_2 ratio beyond -1 and 1), which was reported as cut-off level by previous studies (Lu et al., 2011, Lu et al., 2013). Nine of quantified proteins showed a more than 2-fold increase in milk with highest SCC, as shown in Figure 5.3, whereas 12 of them showed a more than 2-fold decrease. Among these up-regulated proteins, most are related to the immune system, such as cathelicidins, alpha-1-microglobulin (AMBP), Ig gamma-2 chain C region (IGHG2), inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), and S100 calcium-binding protein A8 (S100A8). The most significantly changed protein (cathelicidin 4-CATHL4) showed an almost 10-fold increase in the milk from cows with the highest SCC. The up-regulation of these proteins was similar to what we found in a preliminary experiment conducted by the same techniques (data not shown). Of the down-regulated proteins, platelet glycoprotein 4 (CD36), butyrophilin subfamily 1 member A1 (BTN1A1), and perilipin-2 (PLIN2) are related to lipid synthesis and secretion, ATP-binding cassette sub-family G member 2 (ABCG2) is related to milk secretion, lactadherin (MFGE8) is involved in cell apoptosis, and CD59 glycoprotein (CD59) is related to the immune system. The concentration of CD36 was about 20-fold lower in milk from cows with highest SCC, as shown in Figure 5.3.

Table 5.2. The ratios of 56 common proteins and immune-related proteins between samples from subclinical mastitis cows with different SCC (SCC2 is 2.5×10^5 cells/mL; SCC3 is 4×10^5 cells/mL; SCC4 is 7.5×10^5 cells/mL; SCC5 is 10^6 cells/mL) and sample from healthy cow (SCC1 is $<10^5$ cells/mL)

Uniprot ID	Protein name	Gene name	Biological function	SCC2/ SCC1	SCC3/ SCC1	SCC4/ SCC1	SCC5/ SCC1
P33046	Cathelicidin-4	CATHL4	Immunity	3.64	NQ	NQ	9.82
O02853	Prostaglandin-H2 D-isomerase	PTGDS	Enzyme	1.98	3.163	5.849	7.144
P22226	Cathelicidin-1	CATHL1	Immunity	3.302	7.441	6.148	7.012
P19660	Cathelicidin-2	CATHL2	Immunity	NQ	4.96	4.75	4.2
G3N0V0	Ig gamma-2 chain C region	IGHG2	Immunity	2.119	2.755	1.77	2.638
Q3T052	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	Immunity	NQ	3.12	1.36	2.19
P02754	Beta-lactoglobulin	LGB	Transport	0.601	0.456	4.033	2.092
G3N1U4	Serpin A3-8	SERPINA3-8	Protease inhibitor	1.18	1.446	1.152	2.042
P61823	Ribonuclease pancreatic	RNASE1	Enzyme	1.309	1.069	2.429	1.929
P17697	Clusterin	CLU	Immunity	1.764	5.09	3.621	1.733
P60712	Actin, cytoplasmic 1	ACTB	Protein synthesis	1.756	1.198	2.224	1.63
Q0IIA2	Odorant-binding protein-like	MGC151921	Transport	1.597	3.067	1.353	1.507
P24627	Lactoferrin	LTF	Immunity	2.082	2.735	2.717	1.494

Uniprot ID	Protein name	Gene name	Biological function	SCC2/ SCC1	SCC3/ SCC1	SCC4/ SCC1	SCC5/ SCC1
A7YWR0	Apolipoprotein E	APOE	Transport	1.006	1.577	2.019	1.473
B0JYP6	IGK protein	IGK	Immunity	1.289	2.021	1.864	1.351
P79345	Epididymal secretory protein E1	NPC2	Transport	1.737	1.016	1.332	1.348
P00735	Prothrombin	F2	Immunity	NQ	0.95	NQ	1.34
G3N1R1	Hyaluronan and proteoglycan link protein 1	HAPLN1	Immunity	1.193	2.557	2.248	1.328
P80195	Glycosylation-dependent cell adhesion molecule 1	GLYCAM1	Immunity	0.83	1.021	1.599	1.326
Q29443	Serotransferrin	TF	Transport	1.344	1.549	1.752	1.308
Q2UVX4	Complement C3	C3	Immunity	1.565	1.502	1.589	1.283
P80025	Lactoperoxidase	LPO	Immunity	1.078	0.865	1.271	1.279
P31096	Osteopontin	SPP1	Cell	1.603	2.982	0.948	1.276
P02663	Alpha-S2-casein	CSN1S2	Transport	0.982	1.163	2.193	1.259
A5D7Q2	TAP binding protein	TAPBP	Immunity	2.064	1.53	2.339	1.211
Q3ZCH5	Zinc-alpha-2-glycoprotein	AZGP1	Immunity	1.138	1.045	0.986	1.195
P81265	Polymeric immunoglobulin receptor	PIGR	Immunity	1.212	1.09	1.327	1.167
Q3T101	IGL@	IGL@	Immunity	1.606	2.202	1.887	1.151
Q2KJF1	Alpha-1B-glycoprotein	A1BG	Immunity	0.843	1.08	1.644	1.111

Uniprot ID	Protein name	Gene name	Biological function	SCC2/ SCC1	SCC3/ SCC1	SCC4/ SCC1	SCC5/ SCC1
P02666	Beta-casein	CSN2	Transport	0.423	1.516	3.407	1.084
P02662	Alpha-S1-casein	CSN1S1	Transport	0.458	0.88	2.284	1.062
P01044	Kininogen-1	KNG1	Coagulation	1.057	1.222	1.113	1.053
P08037	Beta-1,4-galactosyltransferase 1	B4GALT1	Immunity	1.258	1.148	2.382	1.039
Q0IIH5	Nucleobindin 2	NUCB2	Transport	1.08	0.885	0.774	1.033
P34955	Alpha-1-antiproteinase	SERPINA1	Protease inhibitor	1.773	1.105	0.982	1.01
P41361	Antithrombin-III	SERPINC1	Coagulation	1.131	0.915	0.98	0.991
B5B0D4	Major allergen beta-lactoglobulin		Transport	0.619	0.948	0.952	0.989
Q2KIS7	Tetranectin	CLEC3B	Cell	0.909	0.88	0.889	0.978
Q95122	Monocyte differentiation antigen CD14	CD14	Immunity	1.078	1.014	0.983	0.962
Q3SZR3	Alpha-1-acid glycoprotein	ORM1	Transport	1.132	0.913	0.733	0.94
Q9TRB9	Enterotoxin-binding glycoprotein PP20K		Unknown	1.073	1.05	0.874	0.933
Q3MHN5	Vitamin D-binding protein	GC	Transport	1.151	0.992	1.175	0.925
Q3ZCL0	Cysteine-rich secretory protein3	CRISP3	Immunity	1.228	1.647	1.279	0.919
Q28049	Alpha lactalbumin	LALBA	Enzyme	0.726	0.77	1.118	0.917
P17690	Beta-2-glycoprotein 1	APOH	Coagulation	0.528	1.019	1.638	0.891

Uniprot ID	Protein name	Gene name	Biological function	SCC2/ SCC1	SCC3/ SCC1	SCC4/ SCC1	SCC5/ SCC1
Q29RQ1	Complement component C7	C7	Immunity	NQ	1.38	0.99	0.89
Q0P569	Nucleobindin-1	NUCB1	Protein synthesis	1.252	0.998	0.945	0.881
Q9MZ06	Fibroblast growth factor-binding protein 1	FGFBP1	Cell	1.197	0.758	0.826	0.852
P02702	Folate receptor alpha	FOLR1	Protein synthesis	1.124	1.049	0.839	0.772
Q0IIH5	Nucleobindin 2	NUCB2	Transport	1.08	0.885	0.774	1.033
P34955	Alpha-1-antiproteinase	SERPINA1	Protease inhibitor	1.773	1.105	0.982	1.01
A8YXY3	15 kDa selenoprotein	SEP15	Protein synthesis	1	0.996	0.835	0.696
P10790	Fatty acid-binding protein, heart	FABP3	Transport	0.942	0.606	1.205	0.687
F1MUT3	Xanthine dehydrogenase/oxidase	XDH	Enzyme	1.341	0.528	1.079	0.638
P12799	Fibrinogen gamma-B chain	FGG	Coagulation	1.207	1.308	1.678	0.547
Q0IIA4	Glycoprotein 2 (Zymogen granule membrane)	GP2	Transport	0.871	0.551	0.906	0.457
P02672	Fibrinogen alpha chain	FGA	Coagulation	1.34	1.181	1.577	0.417
Q32PA1	CD59 molecule, complement regulatory protein	CD59	Immunity	NQ	0.26	1.35	0.35
Q9TUM6	Perilipin-2	PLIN2	Transport	0.906	0.214	0.778	0.262

Uniprot ID	Protein name	Gene name	Biological function	SCC2/ SCC1	SCC3/ SCC1	SCC4/ SCC1	SCC5/ SCC1
P02668	Kappa-casein	CSN3	Transport	0.108	0.281	1.065	0.233
Q95114	Lactadherin	MFGE8	Cell	1.003	0.24	1.323	0.179
P18892	Butyrophilin subfamily 1 member A1	BTN1A1	Transport	0.829	0.135	1.375	0.156
Q27960	Sodium-dependent phosphate transport protein 2B	SLC34A2	Transport	1.153	0.107	1.135	0.106
P26201	Platelet glycoprotein 4	CD36	Cell	0.946	0.027	1.143	0.046
P12763	Alpha-2-HS-glycoprotein	AHSG	Immunity	NQ	0.72	1.01	NQ
Q32PJ2	Apolipoprotein A-IV	APOA4	Immunity	0.99	NQ	NQ	NQ

*Note: Values more than 1 mean up-regulated in milk with high SCC compared to milk with low SCC, whereas the values less than 1 mean down-regulated in milk with high SCC compared to milk with low SCC; NQ means not quantified; the biological functions were found using Uniprot (http://www.uniprot.org/) and DAVID (Dennis et al., 2003)]; cell includes cell adhesion, cell mobility, cell growth, and cell apoptosis.

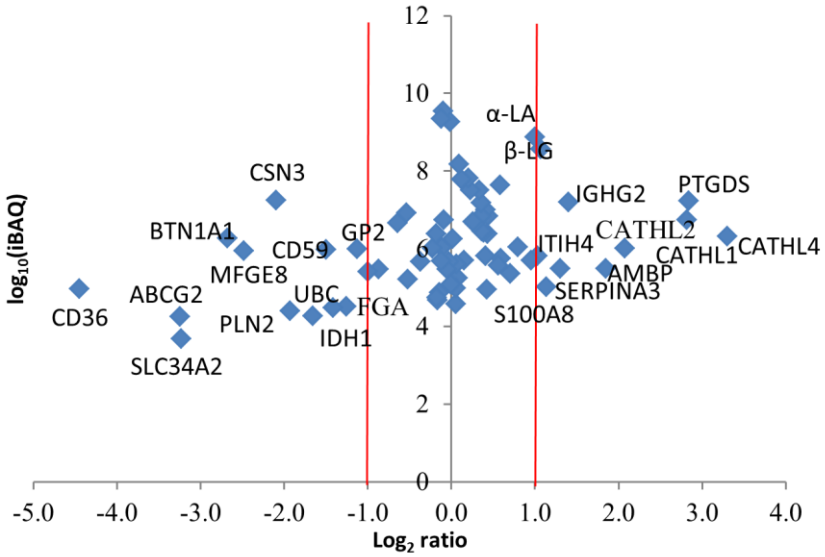


Figure 5.3 Log_2 ratio of protein concentration in milk with the highest SCC (SCC5, 10^6 cells/mL) compared to milk with the lowest SCC (SCC1<10^5 cells/mL).

In addition, the correlation between the concentration of proteins and SCC was calculated to find potential biomarkers for subclinical mastitis. Among the immune-related proteins, the concentration of PTGDS showed the strongest linear relation with SCC (Figure 5.4A). The increase in both PTGDS and SCC had the same order of magnitude, showing a 10-fold increase in PTGDS with a 10-fold increase in SCC. A similar correlation between PTGDS and SCC was also found in a preliminary experiment (data not shown). Next, this correlation was confirmed by an ELISA assay (Figure 5.4B). Finally, in order to know whether the amount of PTGDS correlates with SCC in individual cows, the concentration of PTGDS from 28 individual milk samples with SCC ranging from 1×10^5 cells/mL to 8.5×10^5 cells/mL were determined using the same ELISA assay, as shown in Figure 5.4C. Even though the correlation between PTGDS and SCC in the individual cows was not very strong, there was a similar direction in the relation, as well as similar absolute values, in the individual cows.

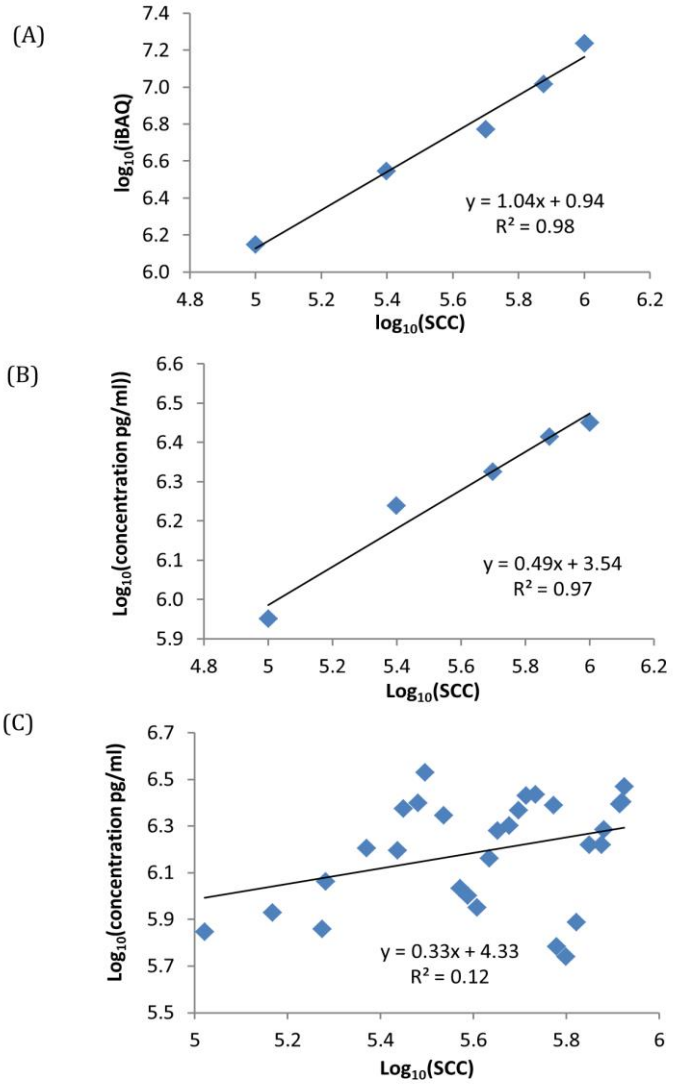


Figure 5.4. Relation between SCC and PTGDS amount (A-The relation between SCC and the concentration of PTGDS in five bulk milk from 100 cows determined by nanoLC-Orbitrap-MS/MS; B-The relation between SCC and the concentration of PTGDS in five bulk milk from 100 cows determined by ELISA; C-The relation between SCC and the concentration of PTGDS in the milk from 28 individual cows determined by ELISA).

5.4 Discussion

5.4.1 Protein identification

In the current study, a total of 273 proteins were identified in bovine milk serum by 1-D SDS-PAGE followed by nanoLC-Orbitrap-MS/MS. A similar number of proteins, 269 (including 232 in bovine milk fat globule membrane and 192 in bovine milk serum), were identified in a previous study using the same proteomics techniques (Hettinga et al., 2011). We found several low abundant immune-related proteins (S100 calcium-binding protein A2-S100A2, S100A8, S100 calcium-binding protein A9-S100A9, serum amyloid A-1 protein-SAA1, serum amyloid A-3 protein-SAA3, and cathelicidins) in milk from both healthy cows and cows with high SCC (up to 10^6 cells/mL), whereas these proteins were previously only identified in bovine milk from cows with clinical mastitis (Alonso-Fauste et al., 2012). This may be attributed to the high sensitivity of the proteomics technique applied in this study.

The absence of changes in protein and dry matter contents (Table 5.1) in milk with high SCC is in accordance with an earlier study (Ogola et al., 2007). The decreasing trend in lactose (Table 5.1) agrees with what has been reported before (Ogola et al., 2007). Moreover, the number of identified proteins and their distribution over biological functions and subcellular locations was similar in milk samples at different SCC levels (Figure 5.1 and 5.2). Milk at all SCC levels contained a large number of immune-related proteins as well as secreted proteins (Figure 5.1 and 5.2). The data shows that the difference between milk from healthy cows and cows with subclinical mastitis is not related to the presence or absence of proteins, but is caused by their expression levels, as will be discussed next.

5.4.2 Protein quantification

To study differences in expression levels of milk proteins, FASP combined with dimethyl labelling was used for quantitative analysis. The results of this quantitative analysis show many differences in the concentration of proteins, including both immune-related proteins and proteins with other biological functions, such as cellular functions, transport, enzymes, and protease inhibitors. The increase of free casein (or casein peptides) in the milk serum with high SCC samples (Table 5.2) is consistent with the decrease of micellar casein described in a previous study (Forsbäck et al., 2010). As SCC increased,

proteolysis also increased (Larsen et al., 2010), which thereby results in the increase of free casein, or casein peptides, in the milk serum. A more comprehensive study of these casein peptides would, however, require a different technological approach than used in this study. The concentration of specific immune-related proteins (all cathelicidins, IGHG2, and ITIH4) increased in all samples with high SCC, but not proportionally with SCC (Table 5.2). Chiaradia, et al. (2013) reported that also in ovine milk, the proteins upregulated with mammary gland infection did not increase proportionately with SCC (Chiaradia et al., 2013). They believed this could be due to different processes occurring during subclinical mastitis because the increase of SCC in milk is not only caused by the bacterial infection but also by the stressful conditions and a probable metabolic impairment of the mammary gland (Chiaradia et al., 2013). The non-linear increase of cathelicidins with the increase of SCC (Table 5.2) was also reported by Smolenski et al (2011) who suggested that it might be caused by a variation in neutrophil concentration and activity at different stages of subclinical mastitis.

5.4.3 Up-regulated proteins in milk from cows with high SCC

There were 10 proteins that showed a more than 2-fold increase in milk serum with an average SCC of 10^6 compared to milk serum with an average SCC of 10^5, as shown in Figure 5.3. Of these up-regulated proteins, three proteins were cathelicidins. Their concentration increased significantly in milk serum from cows with subclinical mastitis (Figure 5.3), which was also found in milk from cows with mastitis after *E. coli* or *Streptococcus uberis* invasion (Lippolis and Reinhardt, 2005, Boehmer et al., 2008, Smolenski et al., 2011). This may be related to the antimicrobial activity of cathelicidins as well as its role in modulation of the innate and adaptive immune response (Tecle et al., 2010). The significant increase in immunoglobulins (IGHG2 and IGK) and acute phase proteins (S100A8 and ITIH4) in the milk with high SCC (Figure 5.3) has been discussed frequently in the previous studies (Caffin and Poutrel, 1988, Mitterhuemer et al., 2010, Alonso-Fauste et al., 2012, Huang et al., 2014). Protease inhibitors play a crucial role in the regulation of protease activity originating from neutrophils (Owen and Campbell, 1999). Serine protease inhibitors have been shown to protect tissue against damage that can be caused by proteases secreted by neutrophils during an infection (Thompson and Ohlsson, 1986). For this reason, the observed increase in SERPINA3 in the milk

with high SCC (Figure 5.3) might be related to the protection of mammary gland cells.

5.4.4 Down-regulated proteins in milk from cow with high SCC

As shown in Figure 5.3, 12 proteins were more than 2-fold down-regulated in milk with the highest versus the lowest SCC. Most of the down-regulated proteins were related to other biological functions than immune protection, for example, lipid metabolism (CD36: 20-fold, BTN1A1: 4-fold, and PLIN2: 6-fold). The down-regulation of all these proteins has been reported before in milk from cows with mastitis (Moyes et al., 2009, Huang et al., 2014). These changes in concentration of cellular function proteins were related to the immune response to mastitis infection (Genini et al., 2011). Since the complement regulatory protein CD59 can protect the mammary gland from over-activated complement mediated cell lysis and tissue damage (Bjorge et al., 1996), the decrease of CD59 in milk with high SCC (Figure 5.3) may indicate its protection on the mammary gland during inflammation. MFGE8 mediates phagocytosis of apoptotic epithelial cells. Down-regulated MFGE8 can result in inefficiently engulfment of dead cells by phagocytes (Boutet et al., 2004) and this delayed apoptosis would lead to necrotic cell death, which is believed to induce inflammation. Therefore, the decrease of cellular function proteins CD59 and MFGE8, as found in this study, indicates a stimulation of the immune response against inflammation.

5.4.5 Linear correlation between PTGDS and SCC

The same linear correlation between PTGDS and SCC obtained from both LC-MS/MS results and ELISA assay (Figure 5.4A and 5.4B) shows the quantitative precision of LC-MS/MS approach used in this study. The increase of PTGDS in the milk with high SCC was consistent with its increase in the milk from mastitis cows reported by Beaker et al (2002). They hypothesized that the up-regulation of PTGDS in milk was either the result of overexpression or damage of the blood-milk barrier caused by mastitis (Baeker et al., 2002). In addition, prostaglandins have been shown to induce chemokines resulting in infiltration of inflammatory cells, such as neutrophils, eosinophils, and macrophages (Aoki and Narumiya, 2012). This reflects that epithelial cells may produce PTGDS during mastitis to recruit the necessary immune cells through converting prostaglandin H synthase 2 (PGH2) to prostaglandin-H2 D-isomerase (PGD2)

(Figure 5.5), resulting in an increase in SCC. Although cathelicidins have been reported to be a biomarker for the mastitis or subclinical mastitis, the correlation between cathelicidins and SCC was lower than the correlation between PTGDS and SCC. The non-linear increase of cathelicidins was also reported by Smolenski et al (2011) who suggested that it might be caused by a variation in neutrophil concentration and activity at different stages of subclinical mastitis. The higher correlation between PTGDS and SCC indicates that PTGDS may be a more suitable indicator for bulk milk SCC. However, it seems to be not suitable for testing of milk from individual cows (Figure 5.4C). This inability to predict SCC level of individual cow's milk may be due to variation in the immune response between cows at different stages of subclinical mastitis, which has been described before by Smolenski et al. (2011).

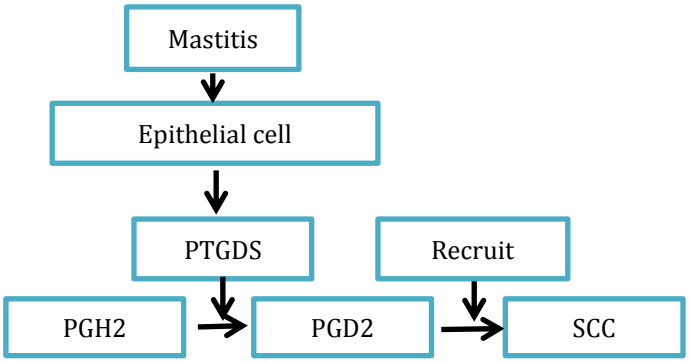


Figure 5.5 The role of PTGDS in the recruitment of SCC in the mammary gland from cows with mastitis inflammation (PGH2: Prostaglandin H2, PTGDS: Prostaglandin-H2 D-isomerase, PGD2: Prostaglandin D2, L-PGD2: Lipocalin-type prostaglandin), based on (Bonnefoy and Legrand, 2000, Baeker et al., 2002, Aoki and Narumiya, 2012).

Bulk milk is used in dairy industry for commercial production of dairy products. Therefore, a simple and easy test could be beneficial to distinguish bulk milk with high versus low SCC. PTGDS may thus be a potential candidate for such an easy test, which could for example be a color test or lab-on-a-chip assay. Such tests for enzymes in milk have previously already been developed for other enzymes like the alkaline phosphatase assay for the validation of correct milk pasteurization (Rankin et al., 2010). According to the ELISA assay, the concentration of PTGDS in milk is above the detection limits of alkaline phosphatase assay, suggesting the feasibility of developing an easy color test. Such a test may be much more cheap and simple than advanced instruments

like the Fossomatic. In addition, PTGDS may give more insight on the protection mechanisms of mammary gland during infections than SCC, if it turns out to give additional information related to stage of mastitis.

The impact of high SCC of cows on changes in their milk composition and subsequent impacts on the quality of dairy products have been previously reported to be associated with increased proteolysis. For instance, milk with a high SCC level can lead to the development of off-flavors in pasteurized milk, UHT milk, yogurts, and cheese due to lipolysis and proteolysis (Maréchal et al., 2011). Elevated levels of free fatty acids have been associated with rancidity and extensive proteolysis in milk could produce more hydrophobic peptides resulting in bitterness and astringency (Maréchal et al., 2011). Moreover, the shelf life of these dairy products was also shortened due to relatively high proteolytic activity in the milk with high SCC (Datta and Deeth, 2001, Santos et al., 2003, Maréchal et al., 2011). In addition, high SCC in milk also influences the coagulation properties, leads to increased moisture content and lower cheese yield (Barbano et al., 1991), resulting in economic losses. An easy and cheap test based on PTGDS could help separate milk with high SCC and thus avoid these negative influences on dairy product quality.

5.5 Conclusion

This is the first study investigating the qualitative and quantitative differences of the milk proteome from cows with different levels of SCC (range: 10^5-10^6). The results from this study indicate that the influence of high SCC is not related to the presence or absence of specific proteins, but mainly involves the expression level of proteins. These results thereby contribute to better understanding of the influence of subclinical mastitis on the changes of the milk proteome, as well as the biological phenomena occurring in the mammary gland. The strong linear relation between PTGDS and SCC indicates that PTGDS may be a good candidate for developing a simple and easy test to evaluate bulk milk quality, by separating milk with high SCC to increase milk suitability for further milk processing. To understand the possible recruitment role of PTGDS on SCC, further studies on the mechanism underlying the relation between PTGDS and SCC may be helpful.

Chapter 6

Proteomic study on the stability of immune proteins in bovine, camel, and caprine milk after processing

Zhang, L., Boeren, S., Smits, M., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. (2015). Proteomic study on the stability of immune proteins in bovine, camel, and caprine milk after processing. **Submitted**.

Abstract

Milk is a complete and complex food suited to the requirements of newborns with regard to growth and development. One of the most important components in milk is protein. However, milk proteins, especially immune-related proteins, have been shown to be very sensitive to processing. This study aims to investigate the changes of the bovine, camel and caprine milk proteins after freezing, pasteurizing (62°C-63°C, 30min), or spray drying by proteomic techniques, filter-aided sample preparation (FASP) and dimethyl labelling followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A total of 129, 125 and 74 proteins were quantified in bovine, camel, and caprine milk serum. The protein content decreased significantly after freezing, pasteurization, or spray drying, which can be ascribed to the removal of denatured proteins (aggregates) by pH adjustment and ultracentrifugation. Most of the proteins decreased in amount were immune-related proteins, such as lactoferrin (LTF), glycosylation-dependent cell adhesion molecule 1 (GLYCAM1), and lactadherin (MFGE8). These proteins decreased in the amount present by 25% to 85% after pasteurization and 85% to 95% after spray drying. On the other hand, α-lactalbumin (LALBA), osteopontin (SPP1), and whey acidic protein (WAP) were relatively heat stable with a 10% to 50% reduction during pasteurization and 25% to 85% during spray drying. The increase of some individual protein in concentration after freezing is probably caused by the protein transportation from damaged milk fat globules and somatic cells to milk serum. The changes of milk proteins also differed between species after processing. For example, GLYCAM1 decreased significantly during pasteurization in camel (*dromedary*) and caprine milk but this protein is relatively stable in bovine milk, whereas MFGE8 changed the other way around.

6.1 Introduction

Milk proteins can be divided into three phases, lipid (milk fat globule membrane, MFGM), colloidal (caseins), and soluble (whey proteins) phase proteins. MFGM proteins are the smallest in amount of the protein fractions, accounting for only 1-2% of the total milk protein content (Lu et al., 2011). Caseins (αs_1, αs_2, β, and κ), on the other hand, are the major milk proteins in commercial dairy species, like cow, camel, and goat (Moatsou et al., 2008). Whey protein accounts for 20% in commercial species, whereas it accounts for 40% in human milk. Because of the high content of casein in animal milk relative to human milk, adding whey protein to infant formula is one important step to make it more comparable to breast milk (Grant et al., 2005).

Whey proteins, however, are much more sensitive than caseins to heat treatments, such as pasteurization and spray drying (Nabhan et al., 2004). Pasteurization is an essential step used by the dairy industry for removing pathogens from milk. Spray drying is also frequently used in dairy industry for producing many different powders, including infant formula. However, these two type of processes can modify the structure of milk proteins and thereby lead to changes of protein function (Li et al., 2013). Several immunoactive compounds in bovine, camel, caprine, and human milk were found to be reduced in amount after pasteurization (Li-Chan et al., 1995, Elagamy, 2000, Laleye et al., 2008, Ewaschuk et al., 2011). Intensive heat treatment was shown to affect both the functional properties (Bu et al., 2009, Zúñiga et al., 2010) and soluble properties of milk serum proteins (deWit and Klarenbeek, 1984, Miyamoto et al., 2010).

In addition to pasteurization and spray drying, freezing is a technique used to preserve and extend the shelf life of milk (Voutsinas et al., 1995). Although freezing is commercially applied only for the widespread and constant supply of unheated milk on a year-round basis (Sun, 2006), it has been frequently used in producing ice cream in the industry, preserving donated milk in human milk banks (Ogundele, 2000), and for scientific research purposes. Recently, researchers have investigated the stability of major proteins from bovine, camel, and caprine milk during heat treatment (Li-Chan et al., 1995, Elagamy, 2000, Laleye et al., 2008, Ewaschuk et al., 2011). However, less is known about the stability of the whole proteome of bovine, camel, and caprine milk during pasteurization and spray drying, and even less is known for the stability during

freezing. In addition, the worldwide consumption of camel or caprine milk is increasing (El-Agamy, 2008, Zervas and Tsiplakou, 2013) and consequently there is a need to know to what extent freezing, pasteurization, and spray drying influence the protein composition and thus possible health aspects of these milks.

The objective of this study is to investigate the sensitivity of soluble milk serum proteins in bovine, camel, and caprine milk to freezing, pasteurization, and spray drying. Using shotgun proteomics techniques, filter aided sample preparation (FASP), and dimethyl labelling combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS), we were able to measure the qualitative and quantitative changes of milk serum proteins between bovine, camel, and caprine during processing.

6.2 Material and Methods

6.2.1 Material

Fresh camel milk samples were obtained from 16 healthy camels (*Camelus dromedarius*) with the lactation period from day 2 to day 292 from Kamelenmelkerij Smits camel farm (Berlicum, The Netherlands). Fresh bovine and caprine milk samples were obtained from tank milk from several farms provided by Lyempf company (Kampen, the Netherlands). Bovine samples were all collected from Holstein Friesians cows and goat samples were all collected from Saanen goats. All the spray dried milk powders were processed in Lyempf company. The samples of each species, including fresh and processed samples, were collected from the same batch. Fresh samples were transported using an ice box to our lab within 2 h for further analysis. Ethical approval for sample collection was not required because the milk used for this study was from bulk milk.

6.2.2 Pasteurization, freezing, and spray drying milk

Fresh milk samples from each species were divided into three portions (50 ml of each portion). One portion was used as control (unheated milk). The second portion was poured into glass tubes sealed with aluminium foil and heated for 30 min in a heating block at 62°C-63°C, which is well-established condition for batch pasteurization (Food and Drug Administration (FDA),1988). After heat treatment, the milk was immediately cooled to room temperature for

centrifugation. The third portion was stored at -20°C for three weeks. We choose three weeks because changes in concentration of milk proteins were previously reported after 15 days of frozen storage (-20°C) (Garcia-Lara et al., 2012) and the freeze-thaw processing was shown to influence the milk protein concentrations more than duration of freezing (Takahashi et al., 2012). The frozen milk was thawed at room temperature for centrifugation. Spray dried milk powder was obtained by first pasteurizing and defatting at maximum approximately 80°C, and then evaporating at maximum approximately 90-95°C, and lastly, drying at maximum approximately 80°C. Spray dried milk powder was dissolved in Milli-Q water, at a weight ratio of 1:10. To obtain a well-dissolved milk sample, Milli-Q water was slowly added to the milk powder under continuous manual stirring.

6.2.3 Centrifugation

We prepared triplicates for each sample and did sample preparation and analysis separately for all samples. All samples were centrifuged at 1500×g for 10 min at 10°C (with a rotor 25.15, Avanti Centrifuge J-26 XP, Beckman Coulter, USA) to remove the fat.

6.2.4 pH adjustment

After centrifugation, all skimmed milk samples were acidified by drop-wise addition of 1 M HCL under stirring, until a pH of 4.6 was reached. The samples were then kept at 4°C for 30 min to equilibrate. When needed, pH was adjusted before the final pH reading. This pH adjustment was done to separate the denatured serum proteins from the native serum proteins during ultracentrifugation, as previously described (Spiegel, 1999b).

6.2.5 Ultracentrifugation

The acidified skim milk was transferred to ultracentrifuge tubes followed by ultracentrifugation at 100,000×g for 90 min at 30°C (Beckman L-60, rotor 70 Ti). After ultracentrifugation, samples were separated into three 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 BCA assay and filter aided sample preparation (FASP) as described below.

6.2.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Milk serum samples with different treatment were subjected to 1-D SDS-PAGE using pre-cast 12% Precise Protein Gels with HEPES buffer (Thermo Fisher Scientific Inc., USA). The milk serum samples were mixed 1:1 with 2 x sample buffer (125 mM Tris-HCL (pH 6.8), 4% SDS, 20% glycerol, 0.01% bromophenol blue in water); just before use, 5% β-mercaptoethanol was added and the samples were heated for 5 min at 95°C. Gels were loaded with approximately 30 μg of protein per well. The gels were run for 45 min at 130 V, and then fixed and stained with the Colloidal Blue Staining Kit (LC6025, Invitrogen, Carlsbad, CA, USA) for 4 h, and finally destained overnight in water.

6.2.7 BCA assay

BCA Protein Assay Kit 23225 (Thermo Scientific Pierce®) was used to determine protein concentration, according to the manufacturer's instructions. Bovine serum albumin was used as the standard to make a calibration curve, covering the protein concentration from 0.02-2 μg/μL, and the milk serum protein concentration was determined.

6.2.8 FASP-dimethyl labelling–LC-MS/MS

The method used to prepare milk serum samples for LC-MS/MS analysis was based on FASP (Wisniewski et al., 2009) and dimethyl labelling (Lu et al., 2011). Then, the prepared samples were analyzed using nanoLC-Orbitrap-MS/MS (Zhang et al., 2015).

6.2.9 Data analysis

Each run with all MSMS spectra obtained was analyzed with Maxquant 1.3.0.5 with Andromeda search engine (Cox and Mann, 2008). Carbamidomethylation of cysteines was set as a fixed modification (enzyme=trypsin, maximally 2 missed cleavages, peptide tolerance for the first search 20 ppm, fragment ions tolerance 0.5 amu). Oxidation of methionine, N-terminal acetylation, and de-amidation of asparagine or glutamine were set as variable modification for both identification and quantification. The bovine reference database for peptides and protein searches was downloaded as fasta file from Uniprot with reverse sequences generated by Maxquant. The camel reference database for the

peptides and proteins searches were downloaded as fasta files from both Uniprot and from National Center for Biotechnology Information (NCBI). A database containing goat and sheep proteins was created by combining databases of Ovine, Ovis aries, and Capra hircus from Uniprot (All fasta files downloaded from http://www.uniprot.org/ accessed Dec 2013). A set of 31 protein sequences of common contaminants was used as well, which included Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human), and Keratin K1C1 (P35527, human). A maximum of two missed cleavages were allowed and mass deviation of 0.5 Da was set as limit for MS/MS peaks and maximally 6 ppm deviation on the peptide m/z during the main search. The false discovery rate (FDR) was set to 1% on both peptide and protein levels. The length of peptides was set to at least seven amino acids. Finally, proteins were displayed based on minimally 2 distinct peptides of which at least one unique and at least one was unmodified.

Dimethyl labelling was based on doublets with dimethLys0 and dimethNter0 as light, and dimethLys4 and dimethNter4 as heavy labels. Razor and unique peptides were used for quantification (Cox and Mann, 2008). Peptide ratios were normalized based on setting the median of their logarithms at zero, which corrects for unequal protein loading, assuming that the majority of proteins show no differential regulation (Cox and Mann, 2008). Normalized H/L ratios were used for further statistical analysis. Also the intensity based absolute quantification (iBAQ value) algorithm was used in this research. It estimates absolute protein concentration as the sum of all peptide intensities divided by the number of theoretically observable tryptic peptides. The iBAQ value has been reported to have a good correlation with known absolute protein amounts over at least four orders of magnitude (Schwanhausser et al., 2011).

The function of the identified proteins was checked in the UniprotKB database released Feb 2014 (http://www.uniprot.org/). One-way ANOVA (post hoc LSD test) was performed in SPSS (Version 21, IBM Corp.) to determine significant differences in total milk serum protein content during frozen, pasteurization and spray drying milk compared to unheated milk. The differences in protein concentration between freezing, pasteurization, and spray drying (three replicates) were then statistically tested using two-tailed one-way ANOVA (p=0.01, S0=1), correcting for multiple testing using permutation-based FDR (Tusher et al., 2001), followed by post-hoc LSD analysis (SPSS, version 21).

6.3 Results

6.3.1 SDS-PAGE

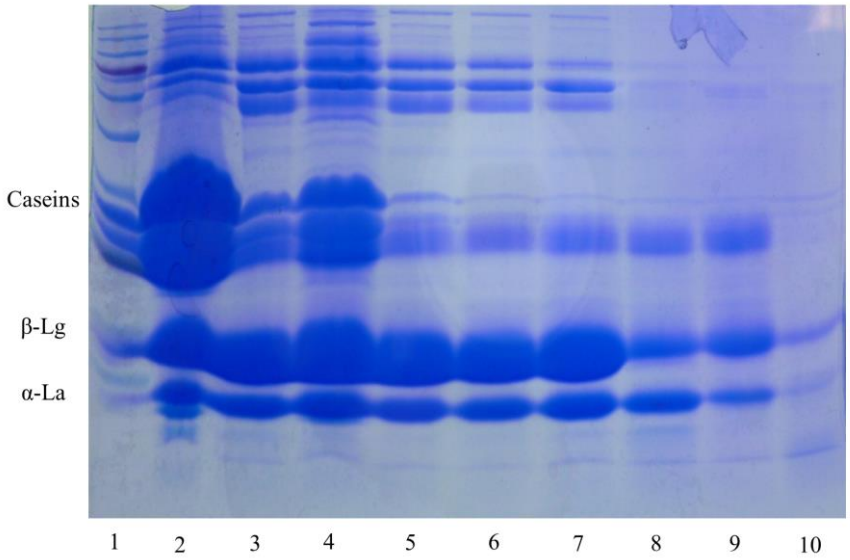


Figure 6.1 SDS-PAGE gel of bovine milk with and without separation by acidification and ultracentrifugation (Lane 1: molecular weight marker, lane 2: raw bovine milk without pH adjustment and ultracentrifugation, lane 3: unheated bovine milk serum with pH adjustment and ultracentrifugation, lane 4: unheated bovine milk serum after ultracentrifugation without pH adjustment, lane 5-8: bovine milk serum heated for 10 min at (5) 50°C, (6) 60°C, (7) 70°C, (8) 80°C after pH adjustment and ultracentrifugation, lane 9: bovine milk serum heated for 5 min at 90°C after pH adjustment and ultracentrifugation, lane 10: Trypsin digested bovine milk serum after pH adjustment and ultracentrifugation).

SDS-PAGE shows the changes of milk proteins after ultracentrifugation and pH adjustment (Figure 6.1). Bovine casein band decreased in the intense after ultracentrifugation (lane 4) and pH adjustment (lane 3) compared to that in raw bovine milk (lane 2). No changes were observed in the intensity of milk serum proteins after ultracentrifugation (lane 4 vs lane 2) and pH adjustment (lane 3 vs lane 2). However, bovine milk serum proteins decreased in intensity with the increase of heating temperature as shown from lane 5 to 9 in Figure

6.1. Therefore, we assume that ultracentrifugation and pH adjustment have no influence on native bovine milk serum proteins but could remove casein micelles and aggregates of whey proteins after heat treatment.

6.3.2 BCA assay

The decrease of bovine milk serum proteins with the increase of heating temperature and heating times determined by BCA assay (Figure 6.2), also support our assumption that the decrease of bovine milk serum proteins is caused by heat treatment and the reduction changes with increasing heat intensity. Therefore, we decided to use ultracentrifugation and pH adjustment to separate native milk serum proteins from denatured milk serum proteins in order to determine the changes of native milk serum proteins after processing with proteomics techniques.

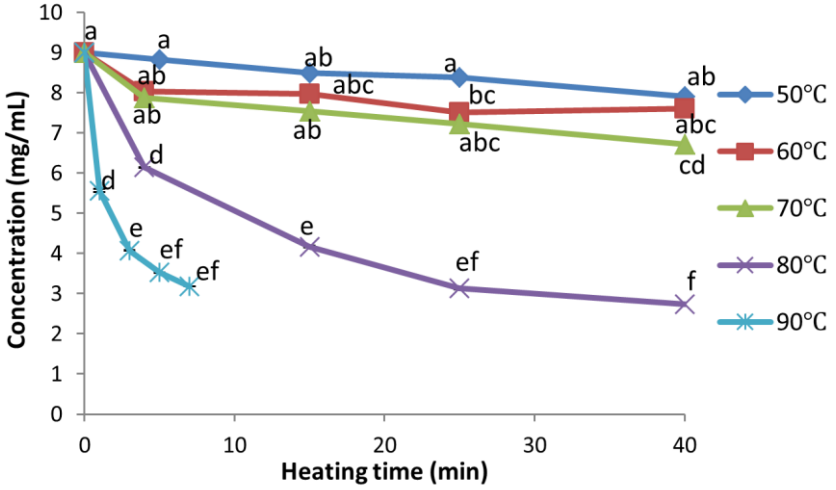


Figure 6.2 The protein concentration of bovine milk serum heated at different heating temperatures and heating times.

Figure 6.3 shows the protein content of milk serum with ultracentrifugation and pH adjustment treatment from unheated, frozen, pasteurized and spray dried samples. The protein content changed significantly after freezing, pasteurization, and spray drying compared to unheated milk (Figure 6.3). The protein content decreased by around 10% after freezing and pasteurization, whereas they decreased by 30-50% in three species.

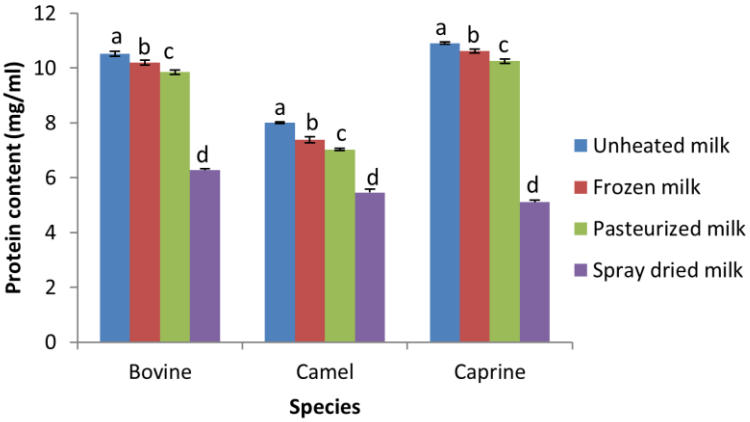


Figure 6.3. Serum protein content in unheated, frozen, pasteurized, spray dried bovine, camel, and caprine milk (p<0.05, SPSS).

6.3.3 Identified and quantified proteins in different processed samples

A total of 149, 143, and 79 proteins were identified in bovine, camel, and caprine milk. Of these proteins, 129, 125, and 74 were quantified in bovine, camel, and caprine milk respectively. The number of quantified proteins in these three species decreased with the intensity of processing as shown in Figure 6.4. After spray drying, around 50%, 30%, and 65% of proteins could still be detected in bovine, camel, and caprine milk.

6.3.4 Biological function of identified and quantified proteins

According to classification of identified proteins based on their biological functions, immune-related proteins, enzymes, and transport proteins were the three groups affected most in all three species. The number of quantified proteins for each biological function decreased with the intensity of processing. Figure 6.5 shows the changes in relative concentration (summed iBAQ value) between unheated samples and processed samples for the different biological activities. In all milk samples, the summed iBAQ value of transport proteins was the highest, followed by enzymes and immune-related proteins. With respect to processing, although the number of quantified proteins was lower in frozen milk than in unheated milk, the iBAQ value for the biological groups was higher in frozen milk compared to unheated milk in all three species. Differences

between species in summed iBAQ value of each biological function group were found (Figure 6.6). Transport proteins had the highest summed iBAQ value in caprine milk, enzymes had the highest summed iBAQ value in bovine milk, and protease inhibitors had the highest summed iBAQ value in camel milk. Summed iBAQ value of proteins in each biological function group also decreased differently in these three species during pasteurization and spray drying. For example, protease inhibitors were reduced much less in camel milk compared to bovine and caprine milk during spray drying.

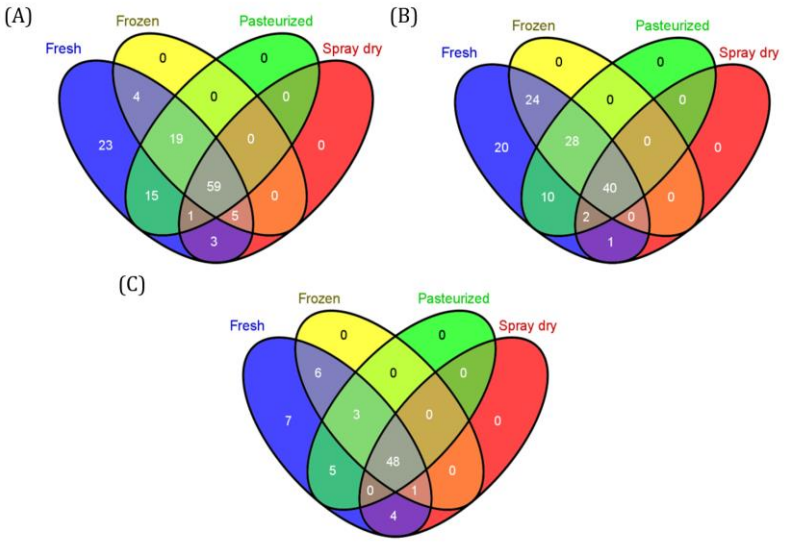
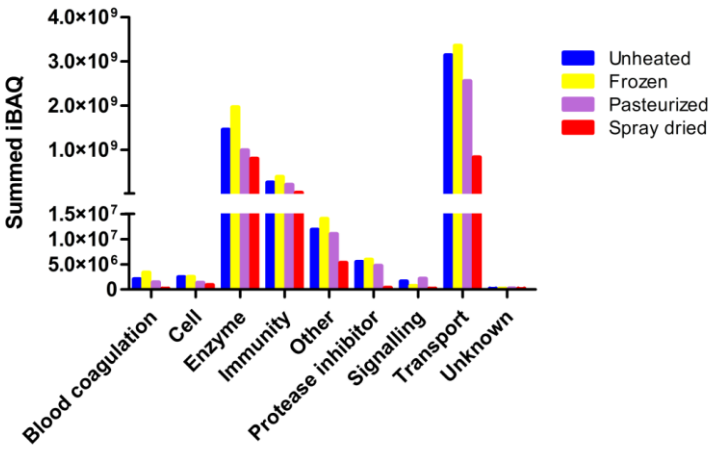
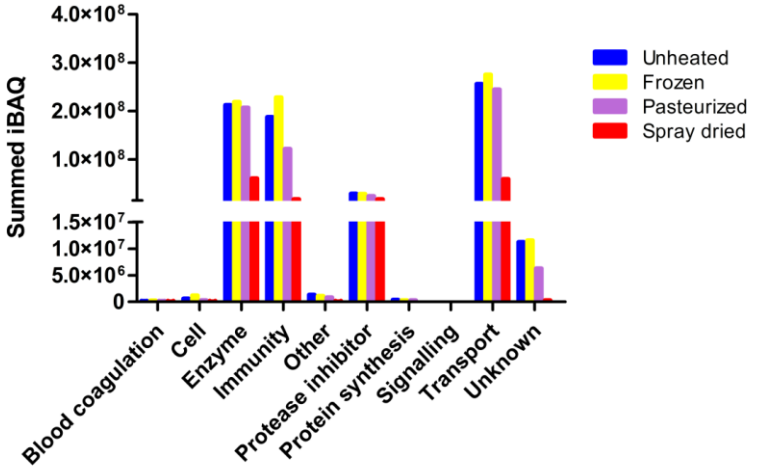


Figure 6.4 The number of quantified proteins in fresh, frozen, pasteurized and spray dried milk (A -Bovine; B- Camel; C- Caprine).

(A)



(B)



(C)

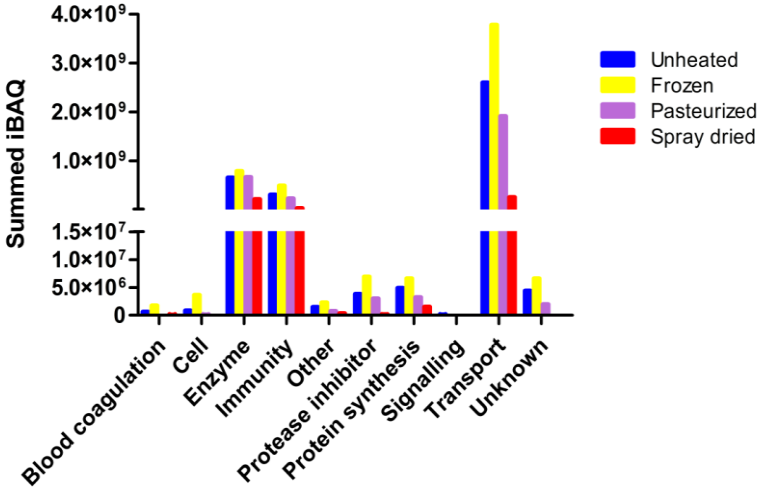
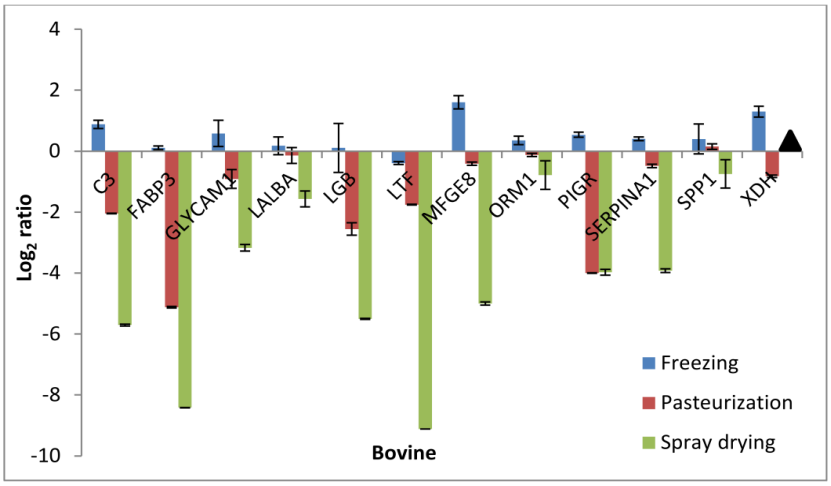
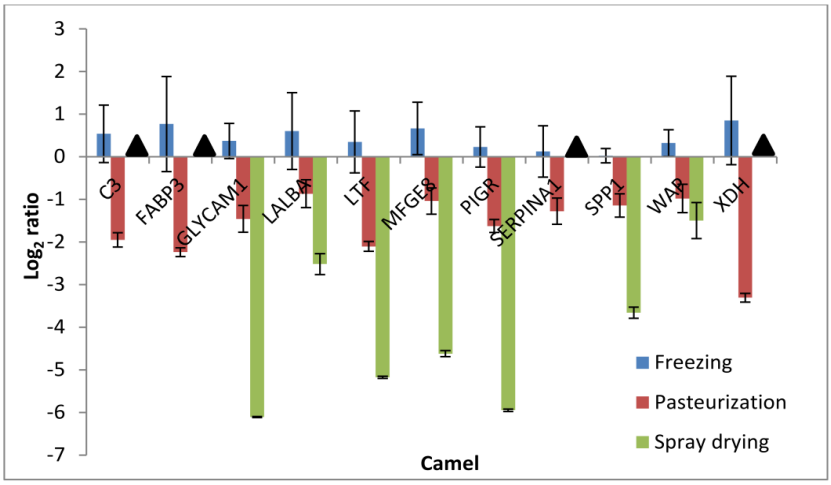


Figure 6.5. The summed iBAQ value of quantified proteins in unheated, frozen, pasteurized, and spray dried milk based on biological function group (A-Bovine; B-Camel; C-Caprine).

(A)


4
2
0
-2
-4
-6
-8
-10
Log2 ratio
C3
FABP3
GLYCAM1
LALBA
LGB
LTF
MFGE8
ORM1
PIGR
SERPINA1
SPP1
XDH
Freezing
Pasteurization
Spray drying
Bovine

(B)


3
2
1
0
-1
-2
-3
-4
-5
-6
-7
Log2 ratio
C3
FABP3
GLYCAM1
LALBA
LTF
MFGE8
PIGR
SERPINA1
SPP1
WAP
XDH
Freezing
Pasteurization
Spray drying
Camel

(C)

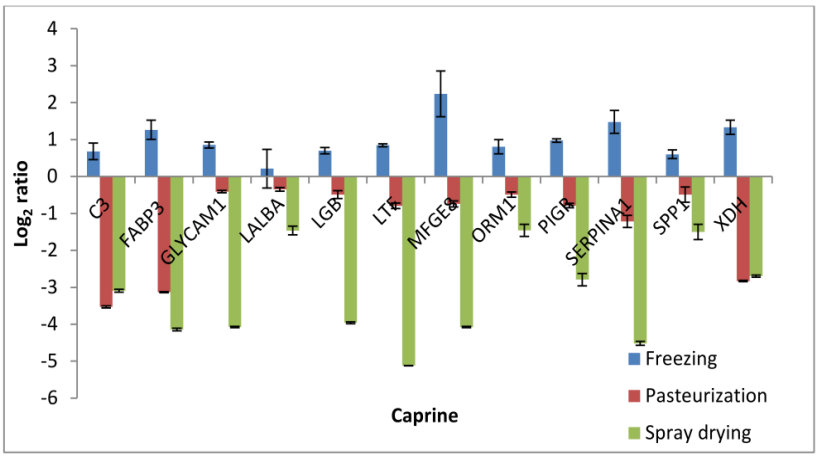


Figure 6.6. The changes of common proteins in bovine (A), camel (B), and caprine milk (C) during freezing, pasteurization, and spray drying (The concentration of proteins with ▲ are below the instrument detection limits).

6.3.5 The changes of relative protein concentration during freezing, pasteurization, and spray drying

In addition to the changes of summed iBAQ value of each biological function group, the changes in relative concentration (the ratio between processed milk and unheated milk based on dimethyl labelling) of individual proteins during processing was also determined. Most of these quantified proteins were significantly changed by performing one-way ANOVA (p=0.01, Perseus). In order to know more about the changes of proteins during freezing, pasteurization, and spray drying, proteins present in all three species (common proteins) were selected for Post Hoc LSD test (one-way ANOVA, SPSS) (Table 6.1, 6.2, and 6.3). Figure 6.6 shows changes of the common protein during freezing, pasteurization, and spray drying. Most of these common proteins were present at higher concentration in frozen milk than unheated milk, whereas they all decreased after pasteurization and spray drying. Significantly changed proteins, including complement C3 (C3), osteopotin (SPP1), glycosylation-dependent cell adhesion molecule 1 (GLYCAM1), lactoferrin (LTF), lactadherin (MFGE8), polymeric immunoglobulin receptor (PIGR), alpha-1-antiproteinase (SERPINA1), xanthine dehydrogenase/oxidase (XDH), and fatty acid-binding protein, heart (FABP3), decreased by 25%-85% after pasteurization and 85%-

95% after spray drying. The relatively stable proteins, such as α-lactalbumin (LALBA), alpha-1-acid glycoprotein (ORM1), SPP1, and whey acidic protein (WAP), reduced by 10%-50% during pasteurization and 25%-85% during spray drying. Although the common proteins were all quantified in three species, their amount were different between species.

Figure 6.7 shows the relative concentration (Log_{10} iBAQ value) of significantly changed proteins in unheated bovine, camel, and caprine milk. In addition, the changes of proteins with same treatment were also differently between three species. For instance, GLYCAM1 decreased significantly during pasteurization in camel and caprine milk but relatively stable in bovine milk, whereas MFGE8 changed the other way around (Table 6.1, 6.2, and 6.3). The relative concentration of these common proteins in bovine, camel, and caprine milk was shown in Figure 6.7.

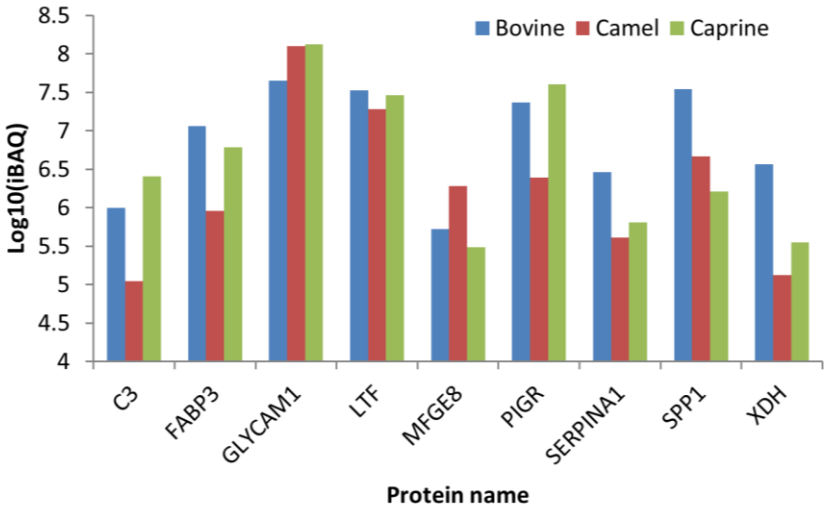


Figure 6.7. The relative concentration of common proteins in unheated bovine, camel, and caprine milk.

Table 6.1. Average dimethyl labelling ratios of processed to unheated bovine milk (superscript letters denote significant differences between groups)

Gene name	Unheated	Frozen	Pasteurized	Spray-dried
C3	1.00^{a}	1.84±0.11^{b}	0.24±0.01^{c}	0.02±0.03^{d}
FABP3	1.00^{a}	1.08±0.48^{b}	0.03±0.02^{c}	0.00±0.00^{cd}
GLYCAM1	1.00^{ab}	1.50±0.35^{a}	0.53±0.25^{bc}	0.11±0.09^{c}
LALBA	1.00^{a}	1.13±0.24^{a}	0.91±0.22^{a}	0.34±0.21^{b}
LGB	1.00^{a}	1.08±0.65^{a}	0.17±0.16^{b}	0.02±0.02^{b}
LTF	1.00^{a}	0.76±0.04^{b}	0.30±0.01^{c}	0.00±0.00^{d}
MFGE8	1.00^{a}	3.04±0.18^{b}	0.75±0.04^{c}	0.03±0.04^{d}
ORM1	1.00^{ab}	1.28±0.11^{ac}	0.92±0.04^{ab}	0.58±0.38^{b}
PIGR	1.00^{a}	1.45±0.07^{b}	0.06±0.01^{c}	0.06±0.08^{c}
SERPINA1	1.00^{a}	1.32±0.05^{b}	0.71±0.05^{c}	0.07±0.05^{d}
SPP1	1.00^{ab}	1.32±0.40^{ac}	1.11±0.08^{ab}	0.60±0.38^{b}
XDH	1.00^{a}	2.46±0.15^{b}	0.57±0.03^{c}	0.00±0.00^{d}

Table 6.2. Average dimethyl labelling ratios of processed to unheated camel milk (superscript letters denote significant differences between groups)

Gene name	Unheated	Frozen	Pasteurized	Spray-dried
C3	1.00[a]	1.38±0.29[b]	0.26±0.18[c]	0.00±0.00[c]
FABP3	1.00[a]	1.70±0.63[a]	0.21±0.09[b]	0.00±0.00[b]
GLYCAM1	1.00[a]	1.29±0.20[a]	0.36±0.26[b]	0.01±0.01[b]
LALBA	1.00[ab]	1.52±0.75[b]	0.55±0.27[a]	0.17±0.19[a]
LTF	1.00[a]	1.27±0.38[a]	0.23±0.09[b]	0.03±0.02[b]
MFGE8	1.00[a]	1.58±0.40[b]	0.49±0.25[ac]	0.04±0.06[c]
PIGR	1.00[a]	1.17±0.17[a]	0.32±0.13[b]	0.02±0.02[c]
SPP1	1.00[a]	0.68±0.48[ab]	0.45±0.22[ab]	0.08±0.10[b]
WAP	1.00[ab]	1.25±0.20[a]	0.51±0.27[bc]	0.35±0.34[c]
XDH	1.00[a]	1.81±0.73[a]	0.10±0.08[b]	0.00±0.00[b]

Table 6.3. Average dimethyl labelling ratios of processed to unheated caprine milk (superscript letters denote significant differences between groups)

Gene name	Unheated	Frozen	Pasteurized	Spray-dried
C3	1.00[a]	1.60±0.18[b]	0.09±0.02[c]	0.12±0.03[c]
FABP3	1.00[a]	2.40±0.21[b]	0.11±0.01[c]	0.06±0.03[c]
GLYCAM1	1.00[a]	1.81±0.07[b]	0.75±0.03[c]	0.06±0.02[d]
LALBA	1.00[a]	1.16±0.43[a]	0.79±0.04[ab]	0.36±0.10[b]
LGB	1.00[a]	1.62±0.07[b]	0.71±0.09[c]	0.06±0.02[d]
LTF	1.00[a]	1.80±0.03[b]	0.58±0.06[c]	0.03±0.00[d]
MFGE8	1.00[a]	4.71±0.50[b]	0.60±0.07[ac]	0.06±0.01[c]
ORM1	1.00[a]	1.75±0.16[b]	0.78±0.04[a]	0.36±0.13[c]
PIGR	1.00[a]	1.96±0.04[b]	0.58±0.05[c]	0.14±0.14[d]
SERPINA1	1.00[a]	2.78±0.25[b]	0.43±0.13[c]	0.04±0.04[d]
SPP1	1.00[a]	1.52±0.10[b]	0.71±0.17[a]	0.35±0.17[c]
XDH	1.00[a]	2.52±0.16[b]	0.14±0.02[c]	0.15±0.02[c]

6.4 Discussion

6.4.1 Optimization of separation method of the native–denatured bovine milk serum proteins

In order to validate pH adjustment and ultracentrifugation on separating native milk serum proteins from denature milk serum proteins, SDS-PAGE and BCA assay were used to check efficiency of this approach. Through comparison between lane 2 and 4 in Figure 6.1, we can see that the ultracentrifugation step hardly reduced milk serum proteins. The less intense band in lane 3 (with pH adjustment to 4.6) compared to lane 4 (without pH adjustment) indicates the importance of pH adjustment in assisting the separation of denatured serum proteins and casein micelles from native milk serum proteins. This is because isoelectric points of milk serum protein aggregates and caseins are approximately 4.5-4.6 (Jean et al., 2006). The similar intensive band of bovine milk serum proteins with (lane 3) and without (lane 4) pH adjustment (Figure 6.1) suggests that pH adjustment has no influence on the milk serum protein concentration, which is consistent with previous studies (Spiegel, 1999a, Law and Leaver, 2000). The decrease of milk serum protein concentration with increased heating temperatures and times, as shown in Figure 6.1 and 6.2, confirms the efficiency of pH adjustment and ultracentrifugation on separating native milk serum proteins from denatured milk serum protein.

6.4.2 Qualitative differences between bovine, camel, and caprine milk proteome

This study used the combination of pH adjustment, ultracentrifugation, and shot-gun proteomics techniques to study the remaining soluble milk serum proteins from bovine, camel, and caprine after freezing, pasteurization, and spray drying. As the intensity of processing increased, the native protein concentration (after removal of denatured proteins by pH adjustment and ultracentrifugation) in milk decreased in bovine, camel, and caprine (Figure 6.3). This finding agrees with the decrease in the number of identified and quantified proteins, as shown in Figure 6.4. The slight decrease in the protein content (5%) during freezing indicates that small influence of freezing on the total milk serum protein content. Although caprine milk had the highest protein content of the three species (Figure 6.3), it had the lowest number of identified and quantified proteins. This can be related to the incomplete database of the

goat species in Uniprot. The relative lower number of identified and quantified proteins in bovine milk compared to the previous study (Hettinga et al., 2011) can be attributed to the use of labelling techniques because it is more precise than label free quantification methods (Lu et al., 2011, Hustoft et al., 2012).

6.4.3 Quantitative differences among biological functions

The decrease in the number of quantified proteins in each biological function group with the increasing processing intensity can be related to the aggregation of proteins after processing, which were removed by the sample preparation. Proteins present at low concentrations in unheated milk may disappear during pasteurization and spray drying, thereby not only contributing to a decrease in the quantity but also in the number of identified proteins as shown in Figure 6.4.

The changes of summed iBAQ value in each biological function group (Figure 6.5) is comparable to the overall change of protein content (Figure 6.3), however, the changes of some proteins based on the ratio obtained from dimethyl labelling (Figure 6.6) do not agree with the total protein content (Figure 6.3). For instance, the amount of C3, FABP3, and XDH decreased by more than 90% after spray drying (Figure 6.6); whereas the total protein content decreased by 40% (Figure 6.3). This is because the changes in the total protein content is dominated by the major proteins, such as α-lactalbumin (LALBA) and β-lactoglobulin (LGB) (Figure 6.6).

The higher iBAQ value of proteins in frozen milk compared to unheated milk (Figure 6.6) may be related to the release of proteins from the milk fat globule membrane (MFGM) (Takahashi et al., 2012), such as FABP3, MFGE8, XDH, and GLYCAM1 (Figure 6.6), which have previously been detected in milk fat globule membrane (Le Provost et al., 2003, Bionaz and Loor, 2008b, Zheng et al., 2013). The release of proteins from damaged somatic cells may also contribute to the increase of iBAQ value after the freeze-thaw process (Pittard Iii and Bill, 1981). For example, an increase of antibacterial proteins C3 and SPP1 is observed (Figure 6.6).

The differences in the reduction of the summed iBAQ values between biological function groups were mainly due to the differences in the denaturation of major milk serum proteins after pasteurization and spray drying. For instance,

LALBA, LGB, the dominant proteins of enzymes, transport group showed different heat stability after pasteurization and spray drying (Figure 6.6).

6.4.4 Comparison of the influence of freezing, pasteurization and spray drying on the milk proteins between the three species

Due to the transfer of proteins from MFGM and somatic cells after freeze-thaw process, the larger increase in the summed iBAQ value in caprine than in bovine and camel milk after freezing can probably be attributed to their differences in both milk fat globule size and somatic cell count. Caprine milk fat globules are much smaller than those in bovine and camel milk (El-Zeini, 2006), which leads to a much larger amount of MFGM to cover all these globules. In addition, the somatic cells in caprine milk is much higher than in bovine milk (Droke et al., 1993).

After heat treatment, although MFGE8, LTF, and GLYCAM1 changed (Table 6.1, 6.2, and 6.3), they decreased differently between different heat intensities and species (Figure 6.6). The slight decrease of bovine MFGE8 (around 25%) during pasteurization is consistent with a previous study (Nilsson and Dobson, 2003). The significant decrease of MFGE8 during spray drying showed small differences between species (Figure 6.6). This may be related to the differences in the isoforms of MFGE8 caused by the post-translational modification. The different MFGE8 isoforms in heat stability still need to be examined in these three species.

Similar to MFGE8, LTF decreased much more remarkably after spray drying than during pasteurization in milk of all three species (Figure 6.6). The less reduction of bovine milk LTF after pasteurization corresponds to its intact secondary structure during pasteurization (72°C for 20s) reported previously (Schwarcz et al., 2008). However, its structure changed drastically and irreversibly with the increase of temperature and heating time (Schwarcz et al., 2008), which is consistent with its large decrease after spray drying (Figure 6.6). Another study also showed that LTF was completely denatured after 30 min at 72°C (Kulmyrzaev et al., 2005). The greater decrease of LTF in bovine milk than camel and caprine milk (Table 6.1, 6.2, and 6.3) may be related to the difference in C-lobe LTF and N-lobe LTF percentage, iron saturation, and glycosylation between species (Conesa et al., 2008). Because of the comparable denaturation temperature of C-lobe and N-lobe as well as the similar degree of

iron saturation (Conesa et al., 2008), the differences in the heat stability of LTF between bovine, camel, and caprine may be due to the difference in glycosylation patterns. Caprine milk LTF has 24 N-glycans in common with bovine milk, and 15 N-glycans with human milk (Le Parc et al., 2014). In addition, milk environment, such as pH and salts may cause differences in the heat stability of LTF among species (Steijns and van Hooijdonk, 2000, Claeys et al., 2014).

The non-significant decrease of bovine GLYCAM1 during pasteurization (Table 6.1) is in line with its high thermo-stability due to the stable tetrameric structures formed by hydrophobic interactions via the C-terminal amphipathic helix (Pedersen et al., 2012). The different changes of GLYCAM1 after spray drying between three species (Figure 6.6) may be attributed to the differences in their primary and secondary structures (Kappeler et al., 1999, Girardet et al., 2000, Le Provost et al., 2003) and in the extent of post-translational modification. GLYCAM1 is a phosphorylated glycoprotein and exhibits 55% similarity between bovine and camel (Groenen et al., 1995). Although GLYCAM1 has high similarity (88%) between bovine and caprine, its sequence contains an insertion of a serine residue at position 25 in caprine, which is absent in bovine (Lister et al., 1998). In addition, the concentration of GLYCAM1 in unheated milk was also different between species (Figure 6.7). The higher concentration of GLYCAM1 in camel milk is consistent with the result found in a previous study (Yang et al., 2013).

LALBA, SPP1, and WAP are relatively stable proteins during processing (Figure 6.6). The slight reduction of LALBA during pasteurization is related to the breakdown of disulfide bonds in both bovine and caprine milk (Vanhooren et al., 2002, Chedad and Van Dael, 2004). A similar secondary structure for camel and bovine LALBA was reported from circular dichroism experiments in unheated milk (Atri et al., 2010), explaining their comparable decrease during pasteurization (Figure 6.6). The heat stability of SPP1 in bovine (Table 6.1), camel (Table 6.2) and caprine milk (Table 6.3) agrees with a previous study carried out by Yamniuk, et al. (2009) (Yamniuk et al., 2009). The difference in heat stability of SPP1 between these three species may be related to differences in post-translational modification. This will need to be confirmed by further studies on the post-translational modification of SPP1 in three species. WAP, one of the major proteins in camel milk, contains multiple cysteine-rich regions, known as four-disulfide core domains (Simpson and Nicholas, 2002), which may be associated with its heat stability.

6.4.5 The nutritional value and biological function of milk proteins changed during processing

The proteins discussed above have different nutritional values and biological functions for the growth and development of the neonate. LTF, GLYCAM1, and MFGE8, can exert antibacterial activities (Groenen et al., 1995, Girardet et al., 2000, Spitsberg, 2005, Conesa et al., 2008). Although LTF shows high heat sensitivity in all three milks, its antibacterial activity was not affected by pasteurization (Paulsson et al., 1993). GLYCAM1 also decreased remarkably during spray drying in all three species (Figure 6.6), however the anti-viral activity of GLYCAM1 has been shown to remain after heating at 95°C for 30 min in bovine milk (Inagaki et al., 2010). No information on stability of function is available for camel and caprine GLYCAM1.

With respect to the heat stable proteins, LALBA not only contains a high content of tryptophan, lysine, and cysteine, which can provide nutritional needs for babies (Lönnerdal, 2014), it also can release bioactive peptides during digestion, which may exert antibacterial activity (Jaziri et al., 1992). LALBA has therefore been found to be a valuable constituent to enrich the infant formula (Velusamy and Palaniappan, 2011, Lönnerdal, 2014). SPP1, highly glycosylated and phosphorylated protein, has multiple functions, including immune activation, wound healing, angiogenesis, and bone remodelling (Ashkar et al., 2000). The addition of bovine SPP1 to infant formula changed gene expressions in the intestine related to galactose metabolism, immune response, growth, and development toward a profile more similar to that in breastfed infants (Lönnerdal, 2014). WAP participates in diverse physiological processes, including protease inhibition, bacterial killing, and inhibition of calcium transport (Smith, 2011). A possible role for WAP as a protease inhibitor is to provide protection against antibody degradation at a time of passive immunological support for the marsupials young (Simpson and Nicholas, 2002). The secretion of WAP is also essential for the adequate nutrient supply of the growing young (Triplett et al., 2005). Therefore, the presence of WAP in camel milk may be involved in the growth and development of the newborn from both a nutritional and immunological point of view. The stability of LALBA, SPP1, and WAP during heat treatment provides great advantage for their usage in food products.

In conclusion, camel and caprine milk contain many proteins with comparable immunological and nutritional properties as bovine milk, such as caseins, milk serum proteins, including GLYCAM1, LTF, MFGE8, SPP1, and LALBA, which were discussed in this study. The influence of freezing on the milk serum proteome found in this study suggests that the protein transition from MFGM and somatic cells to milk serum need to be taken into account after freezing. The quantitative changes of milk serum proteins during pasteurization and spray drying show the importance of heat-induced denaturation of many milk serum proteins during processing. The differences in the changes of milk serum proteins upon processing between the three species may be related to the differences in milk environment, the amino acid sequence, and post-translational modifications of the proteins involved. In the current study, we only compared the concentration of soluble proteins in milk serum from processed milk, after removal of aggregated proteins by acidic precipitation combined with ultracentrifugation (Spiegel, 1999b), to that of unheated milk serum. However, proteins that are not removed using this procedure are not necessarily in their native state anymore, so our approach may underestimate the loss of native proteins. Also, glycation of proteins due to the Maillard reaction may lead to a lower abundance of proteins, as determined using our proteomics approach. Furthermore, the bioactivity of milk proteins may be impaired after heat treatment. A previous study has reported that heat treatment applied during processing could result in lower digestibility of LALBA and LGB through the changes in their structure (Chatterton et al., 2006). UHT treatment could affect the ability of native and iron-saturated LTF to bind various bacterial species (Paulsson et al., 1993). The major challenge however is to determine for many and perhaps even for most proteins in milk the native biological function. It will be a challenging task to determine these biological activities before and after processing of milk.

Chapter 7

General discussion

The objective of the work described in this thesis was to investigate the differences in the dynamics of proteins between human milk and bovine milk. In addition, the stability of proteins from bovine, camel, and caprine milk during processing was investigated. This may contribute to provide guidance on the improvement of infant formula. Untargeted shotgun proteomics techniques (discussed in section 7.1), consisting of FASP and dimethyl labelling combined with LC-MS/MS, were applied for characterization of changes in the milk proteome associated with lactation stage, individual variation, health status, and processing (**Chapter 2-6**). A number of proteins were found to change significantly over lactation in both bovine and human milk (**Chapter 2, 3, and 4**). Digestive enzymes in bovine and human milk were present in different concentrations from early to middle lactation. These enzymes, which are supposed to support the development of the gastrointestinal tract, are discussed in section 7.2.1. Immune-related proteins decreased significantly, and in concert with protease inhibitors, over lactation (**Chapter 2, 3, and 4**). In **Chapter 5** the up-regulation of immune-related proteins and protease inhibitors in the milk with high somatic cell count (SCC) is described, with a focus on prostaglandin-H2 D-isomerase (PTGDS), which linearly increased with SCC. The importance of the immune-related proteins and protease inhibitors for the protection of the neonate and mammary gland is discussed in section 7.2.2 and 7.2.3. Subsequently, section 7.3 shows the differences in the changes of the bovine and human milk proteome over lactation, the differences in the milk proteome between individuals, and the differences in the milk proteome between four species. The stability of the milk proteome in bovine, camel, and caprine samples after freezing, pasteurization and spray drying is described in **Chapter 6**. This is further discussed in section 7.4. Based on the results found in this thesis, some recommendations on the improvement of infant formula are given in section 7.5. Finally, the main conclusions from the work described in this thesis are given in section 7.6.

7.1 Proteomic-based analytical approaches

In total, we identified 430 different proteins in the four species: 299 proteins in bovine milk, 247 in human milk, 143 in camel milk, and 79 in caprine milk (**Chapter 2-6**). Some other studies report slightly higher number of identified proteins (Gao et al., 2012, Yang et al., 2013). The differences between our

study and these studies is associated with the differences in the sample preparation and identification criteria. FASP and dimethyl labelling used in our study is a method for optimal quantification (Lu et al., 2011), but leads to lower number of identified proteins due to the absence of protein fractionation. The effect of lactation (**Chapter 2-4**), subclinical mastitis (**Chapter 5**), and processing (**Chapter 6**) on the milk proteome reflect the ability to detect small changes in protein concentrations, indicating the powerful capability of FASP and dimethyl labelling in the quantification of the milk proteome. In addition, stricter identification criteria (e.g. a false discovery rate (FDR) of 0.01) was used in this thesis compared to a previous study (e.g. FDR of 0.02-0.05) (Gao et al., 2012).

Although the non-targeted proteomics technique used in this study is very precise for relative quantification of milk proteins, it is unable to give absolute concentrations. Targeted proteomics techniques are more capable of quantifying the absolute concentration of specific proteins, for instance ELISA and selected reaction monitoring (SRM). Such targeted techniques can bridge the gap between large- and small-scale protein analysis and provide insight into the roles of protein systems in several biological processes (Hause et al., 2011). The ELISA analysis described in **Chapter 5** was used to determine the correlation between PTGDS and SCC. The strong correlation between the results of FASP with dimethyl labelling and ELISA also confirms the precision of FASP with dimethyl labelling in quantifying milk proteins.

However, ELISA is not optimal for analysing many protein abundances simultaneously, which could be obtained with SRM. SRM can quantify dozens of proteins but not hundreds of proteins in one run. Therefore, non-targeted proteomics, FASP with dimethyl labelling, is still the best approach to select the interesting proteins from the whole milk proteome and SRM-based targeted proteomic techniques can then be applied to absolutely quantify the interesting milk proteins and determine their differences between different samples.

7.2 Milk proteins and their biological functions

7.2.1 Proteins supporting the development of the gastrointestinal tract of the neonate

The development of gastrointestinal digestive tract starts from fetus, but is not completed at birth. The digestive system of infants experiences a dramatic

switch in the nutrients from amniotic fluid before birth to colostrum after birth and the energy supply switches from glucose-dominated to lipid-dominated (Lindquist and Hernell, 2010). This transition requires the digestion of lipids prior to their absorption in the gastrointestinal tract (Abrahamse et al., 2012). Human milk provides both nutrients and enzymes. Human milk itself also has digestive capacity and can therefore more effectively deal with immature luminal digestion (Lindquist and Hernell, 2010). The well-known digestive enzyme, lipoprotein lipase, hydrolyzes triglycerides and is involved in promoting the cellular uptake of chylomicron remnants, cholesterol-rich lipoproteins, and free fatty acids (Mead et al., 2002). It was found in bovine, human, camel, and caprine milk (**Chapter 2, 3, and 6**). The presence of lipoproteins lipase in the milk of all these four species indicates its importance in promotion of nutrient digestion and absorption.

The most abundant digestive enzyme in human milk is bile salt-activated lipase, which has been discussed in **Chapter 4**; whereas it is pancreatic ribonuclease in bovine milk as described in **Chapter 2**. Bile salt-activated lipase plays an important role in lipid digestion and uptake in newborn infants (Lindquist and Hernell, 2010). Pancreatic ribonuclease plays a major role in digestion of nucleic acids of microorganisms in the rumen of calves and thereby promote the nutrient uptake in the intestine (Barnard, 1969), which is especially important in plant eating animals like cows, camels, and goats. The differences in the dominant digestive enzymes found in human milk (bile salt-activated lipase) and bovine milk (ribonuclease pancreatic) (**Chapter 2 and 4**) reflects the differences in the developmental needs of the digestion system between human and cows. Although cow, camel, and caprine are all herbivores, the concentration of digestive enzymes differ between these species. A previous study reported that camel has a relatively lower rumen dry-matter digestibility of grass than cow and goat (Migongo-Bake, 1992), indicating that the camel has a lower bacterial digestion in the rumen compared to cow and goat, which is consistent with the relative low concentration of pancreatic ribonuclease in camel milk (**Chapter 6**). Essentially, the differences in digestive enzymes between species seem to be caused by differences in the diet.

7.2.2 Proteins related to the development of the immune system of the neonate

The transfer of immunity from mother to infant through milk was reported since 1892 and this immunity has been reported to be mainly related to immunoglobulins (Newburg, 2000, Newburg and Walker, 2007). The high abundant immunoglobulins in early lactation found in both bovine milk (**Chapter 2**) and human milk (**Chapter 4**) and their decrease from early to middle lactation stage suggests their importance in the protection of the neonate (Stelwagen et al., 2009). Next to adaptive immunity transfer, innate immune-related proteins, including complement proteins (complement 3, 4, 7, and 9, complement factor B, and complement factor I), antibacterial proteins (lactoferrin, monocyte differentiation antigen CD14, alpha-1-acid glycoprotein, lactadherin, and osteopontin) also showed a high abundance in early lactation in both bovine and human milk (**Chapter 2-4**). The high abundance of innate immune-related proteins in early lactation may be due to its rapid reaction against broad groups of pathogens in the gastrointestinal tract of the neonate (Hettinga et al., 2011, Jensen et al., 2012), especially just after birth. The relatively high amount of both innate and adaptive immune-related proteins in early lactation (**Chapter 2 and 4**) suggests the importance of both the innate and adaptive immune system in the protection of the neonate.

Protease inhibitors participate in the regulation of the complement and coagulation system (Gao et al., 2012). They could also protect the immune-related proteins against degradation during digestion as discussed in **Chapter 2-4,** especially immunoglobulins. Whey acidic protein, a protease inhibitor found in camel milk (**Chapter 6**), was also reported to provide protection against antibody degradation at a time of passive immunological support for the marsupials young (Simpson and Nicholas, 2002). The relative high concentration of protease inhibitors in early lactation and their high correlation with immunoglobulins (**Chapter 2-4**) indicates that protease inhibitors indirectly contribute to the development of the immune system of neonates. The involvement of protease inhibitors in both innate and adaptive immune system also reflects the complex nature of biological interrelationships between milk proteins in the protection of the newborn. Hettinga and colleagues also found that protease inhibitors may be linked to allergy and asthma because of their much higher concentrations in the breast milk of allergic than non-allergic mothers (Hettinga et al., 2015). This may indicate that protease inhibitors may play even more complex roles in development of the immune system. Further studies on the role in resistance against digestion of protease inhibitors and their protection of immunoglobulins would need to be

conducted through combination of digestion models and peptidomics analysis. To more precisely determine the absolute concentration of protease inhibitors in milk, targeted proteomics techniques like SRM could be used.

7.2.3 Proteins related to the protection of mammary gland

In addition to the protection on the neonate, immune-related proteins also protect the mammary gland against infections. The significant increase in immune-related proteins, such as immunoglobulins, cathelicidins, lactoferrin, lactadherin, and alpha-1-acid glycoprotein, as described in **Chapter 5**, indicates the activation of both the innate and adaptive immune system for protecting the mammary gland. Serpin A3-8 was also increased in the milk with high SCC level, which is in line with its increase during an infection in both bovine (Thompson and Ohlsson, 1986) and human mammary gland (Semba et al., 1999). The increase of serpin A3-8 found in milk with high SCC, is probably related to the protection of the mammary gland through inhibiting proteases secreted by neutrophils during inflammation, because these proteases not only kill pathogens but can also cause tissue damage. PTGDS was hypothesized to be a biomarker for SCC level in bulk milk, due to its high correlation with SCC (**Chapter 5).** However, the PTGDS concentration in the milk from individual cows did not correlate well with the SCC (**Chapter 5**), which might be caused by different stages of subclinical mastitis (Smolenski et al., 2011). The metabolites of PTGDS (PGD2, PGH2, and PGE2) were previously reported to play multiple roles during different stages of infection (Sandig et al., 2007). Moreover, differences in the response to infections may also contribute to the low correlation between PTGDS and SCC in individual cows. ELISA and SRM would be good options for the detection of PTGDS in a larger number of samples to confirm the result found in **Chapter 5**. In addition, further studies on the immune response by the mammary epithelial cells at different PTGDS levels would be useful to elucidate the function of PTGDS. Monitoring the secretion of PTGDS at different subclinical stages may help in getting insight in the mechanisms used by the mammary gland to protect itself.

The increase of immune-related proteins in bovine milk from late lactation (**Chapter 3**) may be related to the protection of the mammary gland. When the mammary gland is preparing for involution, it is a challenging period in which it is highly susceptible to new intra-mammary infections (Dingwell et al., 2003). A similar increase of immune-related proteins at week 24 was found in human milk (**Chapter 4**). As the decrease in the reliance of the neonate on the

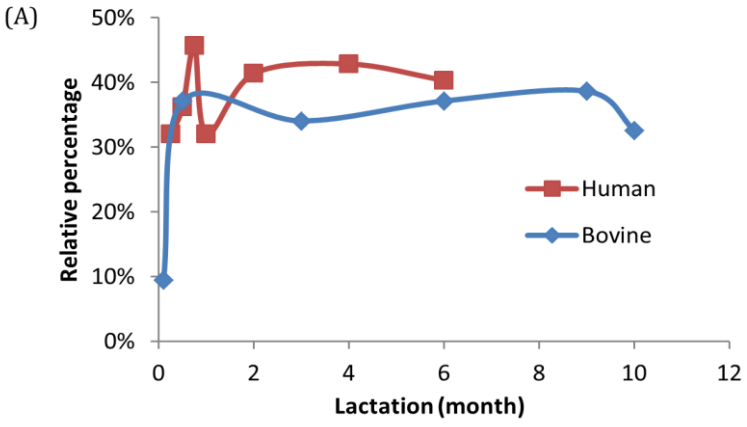
immune-related proteins from early to middle lactation (Hurley and Theil, 2011, Gao et al., 2012), the increase of immune-related proteins in week 24 may also be associated with the protection of the mammary gland.

Overall, the changes of immune-related proteins (both decreases and increases, depending on stage of lactation) may thus be related to the protection of both the neonate and the mammary gland (**Chapter 2-4**). Therefore, further studies on a one year or even longer lactation period, or studies during involution itself, could be helpful to better understand which immune-related proteins are predominantly responsible for protecting the mammary gland.

7.3 Milk proteome in bovine, human, camel, and caprine

7.3.1 The differences in the changes of bovine and human milk proteome over lactation

In addition, transport, enzymes, and immunity proteins changed differently over lactation between bovine milk and human milk, especially the immunity proteins, which decreased more rapidly in bovine milk than in human milk (Figure 7.1). The differences in the changes of these three groups of proteins over lactation can be related to the differences in the needs between infants and calves (Hettinga et al., 2011). The investigation of the changes of the milk proteome over lactation helps us to better understand the role of milk proteins in the development of the immune system of the neonate and also the differences in the needs of the neonate between different species.



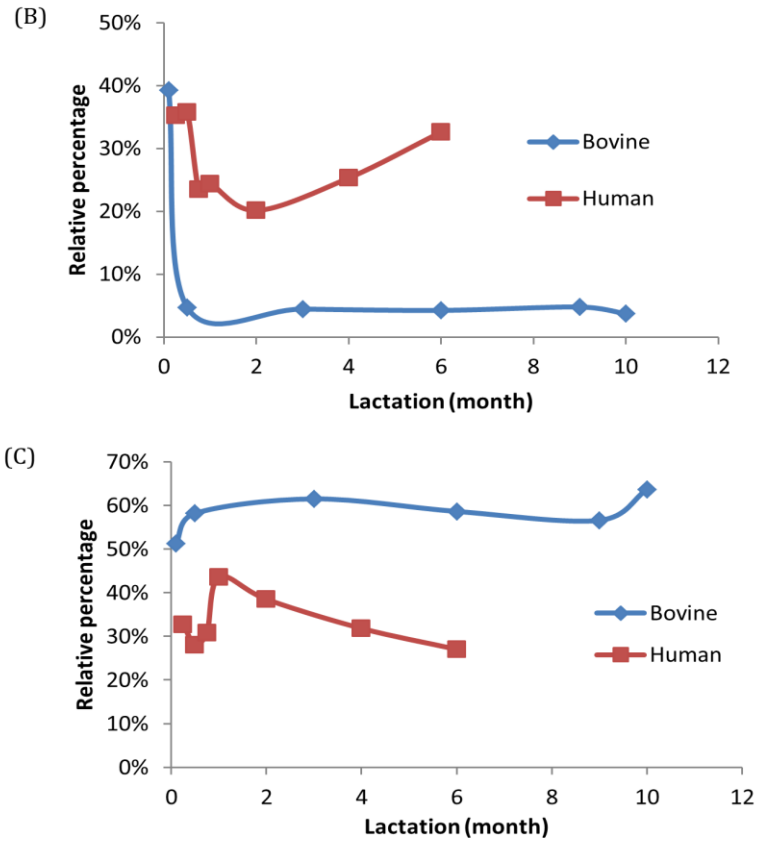


Figure 7.1 The changes of enzymes (A), immune proteins (B), and transport proteins (C) in both human and bovine milk over lactation.

7.3.2 The differences in the milk proteome between individuals

The milk proteome not only differed over lactation, but also differed between individuals, as discussed in **Chapter 2-4**. It is rather constant from a qualitative perspective as around 80% overlap was found in the milk proteome between both individual cows and individual mothers (**Chapter 2-4)**. However, the protein concentration differed to some extent at the same lactation stage between individual cows and mothers (**Chapter 2-4)**. Figure 7.2 shows the variation of protein concentration between individual cows, based on a study of 17 cows in the middle lactation. This quantitative difference in the milk

proteins is probably due to a multiple factors. Parity/age of cows may result in changes of milk serum proteins. Bovine β-lactoglobulin and immunoglobulins were positively correlated with cow's age, and bovine serum albumin increased from the first to fourth parity followed by a decline as cows became older (Ng-Kwai-Hang et al., 1987). Generally, concentrations of all milk serum proteins were negatively correlated with milk production (Ng-Kwai-Hang et al., 1987).

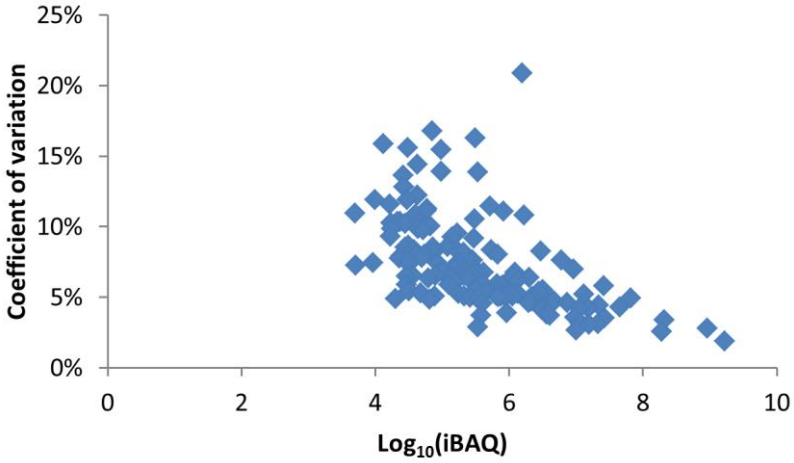


Figure 7.2 The variation of protein concentration in the milk serum from 17 cows in the middle lactation.

The frequency of suckling and the volume of milk taken by infants varies greatly from breast-feed to breast-feed (Dewey and Lönnerdal, 1986) whereas the variation in the milk yield of cows varied from 18-31 kg/day (Ikonen et al., 2004). SCC also influenced the concentration of milk proteins, as discussed in **Chapter 5**. Furthermore, the individual differences in the protein concentration may be caused by (epi)genetic differences between individuals. Genotypes of β-lactoglobulin were reported to influence the concentration of a_s-casein concentration in bovine milk (Ng-Kwai-Hang et al., 1987). The β-casein genotype was associated with milk yield and protein yield (Heck et al., 2009). Human milk α-lactalbumin was also found to be different between Chinese, Japanese, and Philippines (Chowanadisai et al., 2005). Therefore, the individual differences in protein concentration could be attributed to parity number, SCC, milk yield, and genotypes, and possible more, yet unknown, parameters.

7.3.3 The differences in the milk proteome between four species

The milk proteins identified and quantified in bovine, human, camel, and caprine milk have been described in **Chapter 2-6**. The Venn diagram (Figure 7.3) shows the overlap of identified proteins between four species. The low overlap found in these four species may be related to the low number of proteins identified in camel and caprine due to the incomplete databases compared to the bovine and human milk databases. Alternatively, proteomic techniques and identification criteria could also contribute to the low number of identified proteins in camel and caprine milk (as discussed in section 7.2). The one-third overlap between bovine and human milk (Figure 7.3) is similar to what has been previously reported by Hettinga and colleagues (2011). The low overlap reflects the differences in the milk proteome between species.

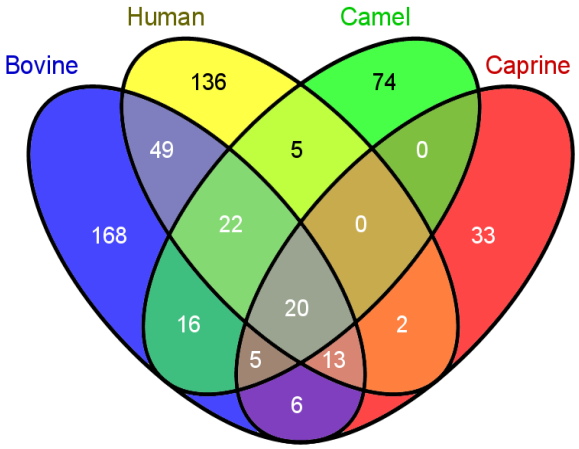


Figure 7.3 Number of identified proteins in four species.

The differences in the milk proteome can be associated with the presence or absence of milk proteins in different species. For example, β-lactoglobulin was absent in human and camel milk (**Chapter 4 and 6**), whereas the whey acidic protein was not identified in bovine and caprine milk (**Chapter 6**). Moreover, the differences in the dominant immunoglobulins between human and animal milk reported previously were also found in this study (**Chapter 2-6**). Except for these well-known differences in the milk proteins between species, some low abundant proteins were also different. The number of blood coagulation

proteins and serpin protease inhibitors were much higher in bovine milk compared to the other three species (**Chapter 2-6**), which is in agreement with previous studies (Hettinga et al., 2011, Zhang et al., 2013). Cathelicidins (**Chapter 2**) were detected in bovine milk with seven isoforms whereas only one or two were found in the other three species (**Chapter 2-6**). This corresponds to the genome level of cathelicidins, which have at least 10 cathelicidin copies in the bovine genome and only one in the human genome (Lemay et al., 2009). The expansion in the immune-related proteins in animal milk, including blood coagulation proteins, protease inhibitors, and cathelicidins may be related to a high exposure to bacteria at the epithelial surface of the mammary gland as their living conditions contain a lot of bacteria.

In addition to the differences in the diet as discussed in section 7.2.1, the presence of lipid digestion enzymes in human milk may be associated with higher digestibility of human milk compared to animal milk. Other enzymes, like plasmin, trypsin, elastase, cathepsin D, pepsin, chymotrypsin, proline endopeptidase, and glutamyl endopeptidase-like enzyme have been reported to actively take part in the digestion of human milk proteins within the mammary gland (Khaldi et al., 2014). This may indicate that human milk itself may have digestive capacity in the baby's gastrointestinal tract, accelerating the absorption of nutrients, thereby dealing more effectively with the immature digestive system of the newborn.

The differences in the milk proteome may also be related to quantitative differences. As shown in Figure 7.1, the amount of enzymes and immunity proteins is much higher in human milk than in bovine milk, whereas for transport proteins it is the other way around (Figure 7.1). The relative high abundance of immunity proteins in human milk is in line with previous studies (Hettinga et al., 2011, Liao et al., 2011b, Zhang et al., 2013). The relatively high amount of transport proteins in bovine milk is related to the presence of β-lactoglobulin, which is one of the most abundant proteins in bovine milk, but is absent in human and camel milk. In addition, individual milk proteins also differ in concentration (based on iBAQ value) between species, as shown in Figure 7.4. Lactoferrin shows the highest concentration as discussed in **Chapter 4**, however, lactoperoxidase shows the lowest concentration in human milk (Figure 7.4). This is in agreement with previous studies (Kussendrager and van Hooijdonk, 2000, Fonteh et al., 2002, Hettinga et al., 2011, Gao et al., 2012). Lactoperoxidase can convert SCN^- into $SCNO^-$, inhibiting bacterial growth (Fonteh et al., 2002). Since plant material in cow's diet is a good source of SCN-,

the lower concentration of lactoperoxidase in human milk than bovine milk may be related to the differences in their diet. Another antibacterial protein, lysozyme also differed in concentration between species. It was quantified in human milk but not in bovine, camel, and caprine milk in this study. This can be related to the relative high concentration of lysozyme in human milk (around 200-400 μg/mL) and the trace amount in milk of ruminants (Chandan et al., 1964, Yang et al., 2011). In addition to these well-known immune-related proteins, other low abundant immune-related proteins also differed between species (Figure 7.4). The concentration of camel milk proteins differs most from human, bovine, and caprine milk proteins (Figure 7.4), although the camel milk proteome would be expected to be much more close to humans, according to the previous study (Yang et al., 2015). This may be related to the climate (hot and dry) in which camels are living, which is very different from cows, human, and goats. Since the comparison in proteins' amount between species is conducted based on the relative concentration, it could be improved by using other mass spectrometry based methods, such as SRM, to establish absolute concentrations.

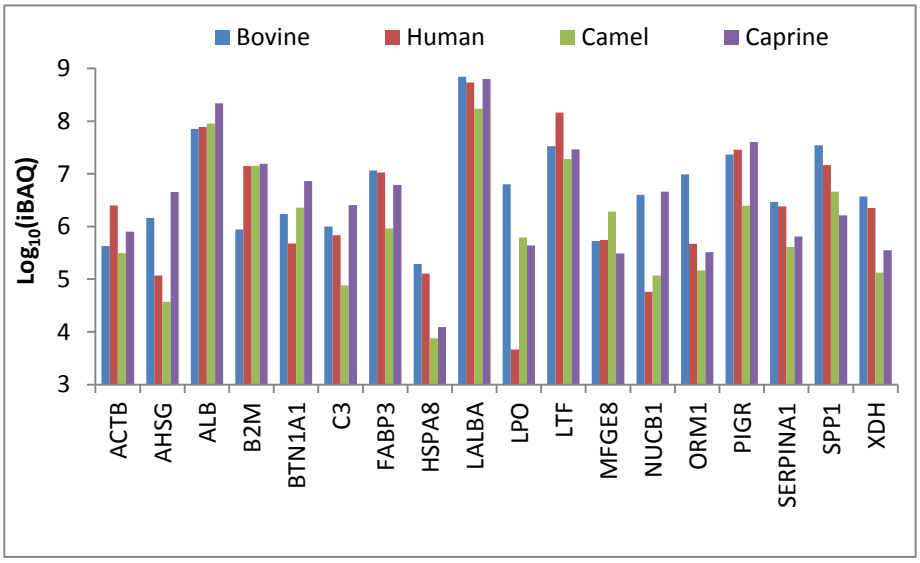


Figure 7.4 The distribution of common proteins from human, bovine, camel, and caprine milk.

Although some milk proteins were different in concentration and their changes over lactation were also different between species, proteins related to milk

synthesis and secretion were relatively constant. This is to be expected, as the function of these milk proteins is for providing nutrients to the offspring, which is required in all species. The similar changes in the proteins related to lipid synthesis and transport in bovine and human milk from colostrum to mature milk (**Chapter 2-4**) also indicates the similarity in the milk synthesis mechanism between bovine and human milk. In addition, the dominance of enzymes, transport, and immune-related proteins in bovine, human, camel, and caprine milk (**Chapter 2-6**) demonstrates the importance of these proteins in the growth and development of the neonate. Especially the relatively high abundant immune-related proteins in early lactation of both bovine and human milk (**Chapter 2-4**) illustrates their importance in the protection and development of the neonate just after birth. In all mammals, milk supply is a complex process with changes in milk composition and interactions between parent and young beyond the straightforward nutritional function (Lefèvre et al., 2010). The differences in the milk proteome between species may be due to their unique lactation strategies to accommodate reproductive success and adapt to their specific environment.

7.4 The influence of processing on the milk proteome

Thermal processes are designed to eliminate pathogens and spoilage microorganisms from raw milk. However, these processes may also denature milk proteins depending on heating intensity. The denaturation of milk proteins is attributed to the changes of protein structure by unfolding of tertiary and secondary structures, breakdown of disulphide bonds, interactions within the protein and with other components, rearrangements of disulphide bonds, and finally aggregation (Bu et al., 2009). In the study described in **Chapter 6**, we accelarated the aggregation of denatured protein by pH adjustment and then removed these aggregates by subsequent ultracentrifugation. Milk proteins differed in their heat stability depending on heating intensity as well as species from which the milk originated (**Chapter 6**).

The differences in the heat stability of milk proteins during processing described in **Chapter 6** may be attributed to the differences in their structure (S-S and -SH) and post-translational modification (phosyphorylation, glycosylation). For instance, the heat sensitivy of β-lactoglobulin is caused by its free -SH group, which is easily accesible due to unfolding during mild heat

treatment. The relatively high heat stability of osteopontin may be attributed to its higly phosphorylated and glycosylated protein structure (Sorensen et al., 1995). Differences in the post-translational modification of the same protein between species could induce differences in heat stability. Difference in N-glycosylation of lactadherin between species result in two different polypeptides in bovine, human, and camel milk and a single polypeptide chain in caprine milk lactadherin (Cebo et al., 2012), which is probably related to the differences in the stability of lactadherin between species after pasteurization. In addition, difference in the milk environment, e.g. pH, fat content, casein micellar structure, salts, and Maillard reaction has been reported between species (Luz Sanz et al., 2007, Raynal-Ljutovac et al., 2007, Claeys et al., 2014), which may induce differences in the stability of milk proteins between species.

The heat treatment may not only induce the decrease in soluble proteins, but it may also decrease their bioactivity (Kher et al., 2007, Miyamoto et al., 2010, Ewaschuk et al., 2011). For example, the antibacterial activity of lactoferrin decreased in bovine and camel milk after heat treatment (Elagamy, 2000). The biological activity of the remaining soluble proteins after heat treatment discussed in **Chapter 6** still needs to be investigated, as our approach was only to determine the remaining soluble protein concentration.
Protein concentrations not only changed after heat treatment but also after freezing. The significant increase of proteins after freezing was reported for the first time (**Chapter 6**). This increase of milk proteins is though to be associated with the damage of MFGM and somatic cells during the freeze-thaw process, resulting in the release of proteins in milk serum. The increase of milk serum proteins after freezing (**Chapter 6**) suggests that we may overestimate the concentration of milk serum proteins from frozen samples, due to this effect of the freeze-thaw process. Therefore, the transition of milk proteins from somatic cells and MFGM to milk serum after freezing-thaw process needs to be taken into account for scientific research. Moreover, comparisions between sample makes sense only when they undergone the same treatment. Further studies on the influence of freezing temperature, freezing period, and freeze-thaw cycles on the milk proteome is needed to better understand the effect of MFGM and somatic cell damage on the proteome of milk serum.

7.5 Recommendations on how to improve infant formula to be more similar to human milk

As discussed above, milk proteins play multiple roles in the health and general development of newborn infants. However, differences in the milk proteome between species make it almost impossible to produce infant formula with exactly the same composition as human milk. In order to close the gap between human milk and infant formula, and thereby improve the infant formula to be more similar as human milk, we believe both enriching interesting components and keeping milk components intact during processing are potential directions to do this.

Milk proteins, including lactoferrin, α-lactalbumin, osteopontin, mucins, and lactadherin, have been commercially used as ingredients for application in infant formulas (Lönnerdal, 2014). Bovine lactoferrin is commercially available and shows similar bioactivities to human lactoferrin (Lönnerdal et al., 2011). The addition of proteins to infant formula has been shown to change gene expressions in the intestine related to galactose metabolism, immune response as well as intestinal microbiota toward a profile more similar to that in breastfed infants (Donovan et al., 2012, Lönnerdal, 2014). In addition, alpha-1-antitrypsin (**Chapter 2-4**) protects the immune-related proteins against degradation during digestion (as discussed in 7.2.2), which may have beneficial effect for the protection of the neonate. Bile salt-activated lipase (**Chapter 4**) is involved in lipid degradation and absorption in the gastrointestinal tract and thereby improves the nutrient availability for the neonate. Alpha-1-acid glycoprotein participates in both immunomodulatory and anti-inflammatory processes because of its sialylated and fucosylated glycans (Orczyk-Pawilowicz et al., 2014), which could protect the neonate, as well as mammary gland against inflammation or infection. Moreover, it possesses relatively high heat stability (**Chapter 6**). Therefore, these proteins could be used as potential ingredients to enrich infant formula as well as other food or pharmaceutical applications to meet the different requirement of humans depending on their age and health status.

Although it is not possible to add proteins to infant formula structurally and quantitatively completely identical as human milk due to its complexity, researchers have evaluated the feasibility and desirability of adding proteins to partially mimic the functional properties of human milk. Isolating proteins from

bovine colostrum and mature milk has been used in the industry, for example for producing health-promoting dairy-based ingredients (Korhonen and Pihlanto, 2007). Lönnerdal discussed the potential of human milk proteins expression by inserting human milk protein genes in animals and edible plants (Lönnerdal, 2013). However, the recombinant human milk proteins don't have the same post translational modification as human milk proteins and the genomic modification cannot mimic the complexity of milk proteins.

Keeping milk proteins intact during heating may be a useful way to maintain the complexity and bioactivity of milk proteins in dairy products, like infant formula. Consumption of raw bovine milk was able to reduce the risk of manifest respiratory infections and fever by about 30% (Loss et al., 2015), as well as the incidence of allergic disease (Neerven et al., 2012) compared to consumption of processed milk. However, raw milk contains bacteria, which are a safety concern. Although heating is one of the essential steps to kill pathogens in dairy processing, it could denature milk proteins as discussed in section 7.4, resulting in the loss of protein and probably biological activity. Heat processing is about the balance between safety and biological activity. Therefore, the mild processing approaches could be very useful in the production of infant formula. Further studies, for example developing kinetic models can be used to understand the key processing step involved in inactivation of immunologically-active milk proteins. Such research will give possible directions for adjusting the processing in the dairy industry, as well as other food industries. Enriching the key components, and keeping these components intact during processing, are two important possible directions for increasing the quality of dairy products in general and infant formula in particular.

7.6 Conclusions

1. Immune-related proteins decreased much more rapidly in bovine milk compared to human milk in early lactation.
2. Although the milk proteome differs qualitatively and quantitatively between species, the function of milk proteins is probably the same for all newborns, in providing nutrients and immunity for their growth and development.
3. Since freezing influences the milk serum proteome, sample comparison in scientific research only makes sense when samples undergo the same treatment.

4. The heat stability of milk proteins not only differed between different processing intensities but also differed between species.
5. Both enriching specific milk proteins and mild processing of milk proteins should be considered for the improvement of infant formula.

Abbreviations

1-D SDS-PAGE	one-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis
ABC	NH_4HCO_3 in water
BCA Assay	bicinchoninic acid assay
CD_2O	deuterated formaldehyde
CH_2O	formaldehyde
DTT	dithiotreitol
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FASP	filter aided sample preparation
FDA	food and drug administration
FDR	false discovery rate
GO	gene ontology
HCOOH	formic acid
IAA	iodoacetamide
iBAQ value	intensity based absolute quantification
LC-MS/MS	liquid chromatography tandem mass spectrometry
MAC	membrane attack complex
MFGM	milk fat globule membrane
NEC	necrotizing enterocolitis
ROS	reactive oxygen species
SCC	somatic cell count
SDS	sodium dodecyl sulfate
SRM	selected reaction monitoring
TFA	trifluoroacetic acid
UT	8 M urea in a tris/HCl solution

Note: Gene names are not included in the abbreviations list.

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Summary

The qualitative and quantitative changes in milk proteome have been determined over lactation, between individuals and species, under different health status and during different processing (freezing, pasteurization, and spray drying). The influence of these factors on milk proteins has been performed in relative high abundant proteins in the last few decades. As the development of proteomics techniques, the investigation has been extended to relative low abundant proteins. However, still a lot is unknown about the dynamics of low abundant proteins, especially immune-related proteins. The objective of the work described in this thesis was to extensively understand the dynamics of the milk proteome from milk source to processing, and provide guidance on the improvement of infant formula through selection of proteins source and enriched proteins, and adjust the processing conditions.

The study in **Chapter 2 and 3** describe the changes of milk proteome from the first day after the calves were born until the end of the lactation. Samples from four individual cows were analyzed by filter aided sample preparation (FASP) and dimethyl labelling combined with LC-MS/MS. The differences in the milk proteome among four individual cows were discussed. Significant changes of milk proteome were determined and the correlation analysis was performed between proteins involved in blood coagulation and complement cascades. The results demonstrated that there was a big overlap in the milk proteome among individual cows. Immune-related proteins (immunoglobulins, complement proteins, osteopontin, polymeric immunoglobulin receptor, lactadherin, alpha-1-acid glycoprotein 1, and alpha-1-antitrypsin), changed significantly over lactation and their relatively high abundance in colostrum indicates their importance for the protection of calves. The high abundant enzymes related to the nutrients digestion in colostrum also reflect the importance of milk for the maturation of the gastrointestinal tract of calves. The high correlation between protease inhibitors (alpha-1-antitrypsin and inter-alpha-trypsin inhibitor heavy chain H1) and immunoglobulins suggests the involvement of protease inhibitors on the protection of the neonates as well as the complex of milk proteins functioning in host defense system. In addition to the needs of calves, the changes of proteins with different biological functions (β-1,4-galactosyltransferase 1, α-lactalbumin, fatty acid-binding protein, heart, butyrophilin subfamily 1 member A1, and perilipin-2) over lactation also reflect milk synthesis and secretion and the development of the mammary gland over lactation.

The changes of human milk proteome over lactation from four individual mothers over six months lactation were described in **Chapter 4**. The relatively high abundant immune-related proteins, including immunoglobulins, complement proteins, antibacterial proteins, and protease inhibitors, as well as nutrient digesting enzymes were also found in early lactation stage of human milk. It indicates the importance of breast milk proteins for the protection of the infant and the promotion of the development in both immune system and digestive system. Differences in the dominant digestive enzymes were found between human and bovine milk. This may be related to the differences in the needs between infants and calves. The variation in milk protein concentrations between individual mothers was found to be relatively greater than between individual cows, which may be due to the diversity of diet consumed by humans.

In **Chapter 5**, the influence of high somatic cell count (SCC) on the bovine milk proteome was investigated. The results showed that high SCC not only induced changes in immune-related proteins (cathelicidins, immunoglobulins, inter-alpha-trypsin inhibitor heavy chain H4, CD59 glycoprotein, and prostaglandin-H2 D-isomerase-PTGDS, etc.) but also resulted in changes of proteins with other biological functions (platelet glycoprotein 4, butyrophilin subfamily 1 member A1, perilipin-2, serpin A3-8, and lactadherin) in milk. The increase of protease inhibitor (serpin A3-8) with the increase of SCC may be related to its protective role in the mammary gland against protease activity. The linear relation between PTGDS and SCC (r^2=0.98) obtained from LC-MS/MS was confirmed by enzyme-linked immunosorbent assay (ELISA). This suggests that PTGDS may play an important role in SCC recruitment at the inflammation site. It also underlines that PTGDS may be a potential indicator for the detection of bulk milk with high SCC.

The study described in **Chapter 6** was aimed to compare the differences in the stability of milk proteins from cows, camels, and goats during freezing, pasteurization, and spray drying. The results showed that the influence of processing on the milk protein concentration increased with the intensity of processing in all three species. It was discussed that the increase of protein concentration during freezing was associated with the damage of SCC and milk fat globule membrane during frozen-thaw processing. The differences in the heat stability of milk proteins, such as such as latoferrin, glycosylation-dependent cell adhesion molecule 1, lactadherin, α-lactalbumin, osteopontin, and whey acidic protein may be related to their different structures. The decreases of these milk proteins during heat treatment were also different

between cows, camels, and goats, which is probably attributed to the differences in amino acid sequence, post-translational modification of proteins, and the differences in milk environment, such as pH, SCC, and fat content between three species.

Chapter 7 discussed how the results in this thesis increase our understanding of the dynamics of low abundant proteins and contribution to the improvement of infant formula. This study shows that the importance of immunoglobulins, lactoferrin, osteopontin, lactadherin, complement C3, alpha-1-acid glycoprotein, alpha-1-antitrypsin, bile salt-activated lipase, ribonuclease pancreatic, xanthine dehydrogenase/oxidase, and PTGDS on the protection of the neonate and the mammary gland. PTGDS could be used as a biomarker for detection of milk quality and suitability for further processing. However, the changes of these proteins were different between human milk and bovine milk over lactation. Furthermore, the dominant digestive enzymes were also different between human milk (bile salt-activated lipase) and bovine milk (pancreatic ribonuclease). In addition, the stability of these proteins in bovine, camel, and caprine milk was also different. It is concluded that differences in the changes of human and bovine milk proteome over lactation is caused by the differences in the need of infants and calves, the different stability of milk proteins are related to the differences in proteins structures and post-translational modification between species, as well as the different intensities of processing. In order to bridge the gap between human milk and infant formula, both enriching interesting proteins and keeping milk proteins intact during processing should be considered to improve the infant formula.

List of publications

Zhang, L., Boeren, S., Heck, J.M.L., Hageman, J.A., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. The variation in milk serum proteome within and between individual cows. (In preparation)

Zhang, L., Boeren, S., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. The differences between human and bovine milk serum proteome over lactation. (In preparation)

Zhang, L., Boeren, S., Hooijdonk, van A.C.M., Schols, H.A., Vervoort, J.J.M., Hettinga, K.A. Comparison of immune-related components in human and farm animal milk. (In preparation)

Zhang, L., Waard, de M., Verheijen, H., Boeren, S., Hageman, J.A., Hooijdonk, van A.C.M., Vervoort, J.J.M., Goudoever, van J.B., Hettinga, K.A. The quantitative changes of functional proteins in human milk during six months lactation period. (*Submitted)*

Zhang, L., Boeren, S., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. Proteomic study on the stability of immune proteins in bovine, camel, and caprine milk after processing. (*Submitted)*

Wheeler, T. T., Boggs, I., Hine, B., Smolenski, G., Hettinga, K..A, **Zhang, L**. Changes in the repertoire of bovine milk proteins during mammary involution. (*Submitted)*

Zhang, L., Boeren, S., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. (2015). A proteomic perspective on the changes in milk proteins due to high somatic cell count. *Journal of Dairy Science* 98(8):5339-5351.

Zhang, L., Boeren, S., Hageman, J. A., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. (2015). Perspective of calf and mammary gland development via changes in the bovine milk proteome over 12 months lactation period. *Journal of Dairy Science* 98(8):5362-5373.

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Conference abstract

Zhang, L., Boeren, S., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. Proteomic perspective on mammary gland and calf development over a 12 month lactation period. 11th International Symposium on Milk Genomics & Human health (2014), Aarhus, Denmark.

Zhang, L., Boeren, S., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. Proteomic perspective on mammary gland and calf development over a 12 month lactation period. 11th IMGC Workshop (2014), Aarhus, Denmark.

Zhang, L., Boeren, S., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. Comparison of characterization in the proteome of human and bovine milk. 9th International Symposium on Milk Genomics & Human health (2012), Wageningen, the Netherlands.

Zhang, L., Boeren, S., Hooijdonk, van A.C.M., Vervoort, J.J.M., Hettinga, K.A. Different proteomics techniques for identifying milk proteins. 9th IMGC Workshop (2012), Wageningen, the Netherlands.

About the author

Lina Zhang was born on 9th November 1986, in Zaozhuang, Shandong Province, China. She grew up in Shandong Province until finishing her high school in Zaozhuang third middle school in 2005. During 2005-2009, Lina started her study on Food Science and Technology at Hebei Economic and Business University in Shijiazhuang, Hebei Province. Lina continued her Master programme of agricultural products processing and preservation engineering in China Agricultural University from 2009. She did research on the changes of freshness of grass carp during storage and obtained Master degree in 2011. In the same year, she got scholarship from China Scholarship Council and enrolled in the PhD project in Wageningen University at Dairy Science and Technology group, FQD. The topic of the PhD thesis was dynamics of the proteome in human and farm animal milk. Lina graduated her PhD in 2015. She has been appointed as a Post-doc in Center for Translational Biomedical Research, University of North Carolina at Greensboro, USA.

Overview of completed training activities

Discipline specific activities

International conferences

- 9th International Symposium on Milk Genomics & Human health (2012), Wageningen, The Netherlands (Poster)
- 9th Netherlands Proteomics Centre (2012), Utrecht, The Netherlands
- 10th Netherlands Proteomics Centre (2013), Utrecht, The Netherlands
- 11th International Symposium on Milk Genomics & Human health (2014), Aarhus, Denmark (Poster)

Workshop &Training

- Techniques for measuring milk phenotypes (2012), Wageningen, The Netherlands (Oral presentation)
- Bioinformatics tools training (2012), Swiss Institute of Bioinformatics, Geneva, Switzerland
- Proteomics techniques training (2013), Universität Erlangen-Nürnberg Erlangen, Nürnberg, Germany (Oral presentation)
- Tools and possibilities for optimized milk (2014), Aarhus, Denmark (Oral presentation)

Courses

- Advanced visualisation, integration, and biological interpretation of -omics data (2011), VLAG/WGS, Wageningen
- Advanced food analysis (2013), VLAG/Food Chemistry, Wageningen
- Advanced proteomics (2013), VLAG/Biochemistry, Wageningen

- Training school on proteome bioinformatics (2013), Bassols Anna, Universitat Autonoma de Barcelona, Barcelona, Spain
- Summer Course Glycosciences (2014), VLAG, Wageningen

General courses

- Information Literacy PhD including EndNote Introduction (2011), WGS, Wageningen
- VLAG PhD Week (2012), VLAG, Baarlo
- Project and Time Management (2012), WGS, Wageningen
- Philosophy and Ethics of Food Science and Technology (2012), VLAG, Wageningen
- Interpersonal Communication for PhD Students (2012), WGS, Wageningen
- Competence Assessment (2012), WGS, Wageningen
- Applied Statistics (2012), VLAG, Wageningen
- Techniques for Writing and Presenting a Scientific Paper (2013), WGS, Wageningen
- Scientific Writing (2014), WGS, Wageningen

Optional courses and activities

- Preparation of Research Proposal (2011)
- DST meetings and Seminars in Food Quality and Design (2011-2015), Wageningen
- PhD Excursion (2012), Food Quality and Design, United Kingdom
- PhD Excursion (2014), Food Quality and Design, Singapore and Thailand

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