The biology of milk synthesis from a proteomics perspective

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The biology of milk synthesis from a proteomics perspective

Jing Lu

Thesis

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Chapter 1

Introduction

1.1 Milk

Bovine milk and its products are consumed daily by many people all around the world. It is considered as a rich source of different nutrients, such as proteins, calcium and B vitamins. Bovine milk contains around 3-5% fat, 3-4% protein, 4.8% lactose, and various vitamins and minerals. However, large variations in milk composition were observed (Stoop et al., 2007), e.g. the milk fat content of Dutch Holstein cows varied from below 2% to over 6%. Many factors influence milk composition, including genetic variation, environment (e.g. season), feed and health (Walstra et al., 2006). Hence, the alteration of milk synthesis and secretion by these factors in mammary gland cells leads to the variation in milk composition. Over the last few decades, milk production and nutritional value were improved due to the advances in knowledge on lactation biology. However, it is still far from to be fully understood, especially with regards to the milk synthesis and secretion processes.

1.2 Milk synthesis and secretion pathways

After more than 50 years of effort, several synthesis and secretion pathways have been proposed in mammary glands (Bauman et al., 2006) as shown in Fig 1.1. The major components of milk aqueous phase, including proteins, lactose, phosphate, citrate and calcium, are considered to be secreted through vesicular transport. They are packaged into vesicles in Golgi and transported to apical plasma membrane to be released to milk as same as the exocytotic process in other types of cells. Some of the components in milk are directly derived from blood or the stromal cells through membrane transport, including immunoglobulins, albumin, transferrin, ions, fatty acids and glucose. Normally, specific transporters are required for this process. In addition, during pregnancy, involution and inflammatory response, there is another transport pathway called the paracellular pathway that is mainly used for transportation of plasma components and leucocytes directly to milk through the junction between epithelial cells. Unlike the other components in milk, lipid has its own unique synthesis and secretion pathways in mammary gland (Mather and Keenan, 1998). As known, lipid exits as milk fat globules in milk. These globules consist of more than 98% of triglycerides, 1-2% phospholipids and 1-2% proteins (Walstra et al., 2006). In epithelial cells, triglycerides are synthesized in the membrane of smooth endoplasmic reticulum and then released to cytoplasm as micro lipid droplets (MLD) covered with one layer of ER membrane. These MLD either coalesce with each other to form cytoplasmic lipid droplets (CLD) or remain their size, followed by being transported to the apical membrane to be secreted as milk fat globules (MFG) (McManaman and Neville, 2003).

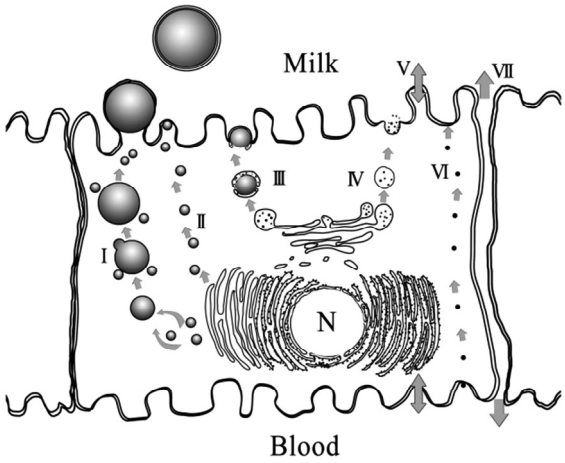

Figure 1.1 Summary of pathways for milk components synthesis and secretion from epithelial cells in mammary gland (Mather and Keenan, 1998, McManaman and Neville, 2003). Pathway I, micro lipid droplets (MLD) synthesized in smooth ER fuse with each other or with cytoplasmic lipid droplets (CLD) when moving to apical plasma membrane for secretion to milk. Pathway II, MLD remained their size and transported to apical plasma membrane for secretion to milk. Pathway III, cytoplasmic vesicles and CLD form vacuoles for secretion to milk. Pathway IV, exocytotic secretion of milk proteins, lactose, calcium and other components. Pathway V, direct transport of monovalent ions, water and glucose across plasma membrane. Pathway VI, transcytosis of macromolecules, such as immunoglobulins from blood to milk. Pathway VII, paracellular pathways for plasma components and leucocytes directly to milk during pregnancy, involution and inflammatory response.

There are several hypothesises about the secretion of lipid droplets to milk (Mather and Keenan, 1998). One of the most accepted models is that the lipid droplets are secreted through a budding process in the apical membrane region. But another hypothesis that lipids droplets are secreted together with vesicles has also been raised (Wooding, 1971, 1973). During the lipid droplets secretion process, the double-layer plasma membrane will cover the MFG with some cytoplasm trapped in between the membrane layers. However, many aspects still remain unclear about lipid synthesis and secretion, such as the regulation of lipid droplet transport in cytoplasm and proteins involved in lipid droplet secretion.

Epithelial cells work as a precise machine to produce milk. In the synthesis and secretion process of all milk components, many proteins are vital for the accurate production, such as

transporters of precursors (e.g. amino acids and fatty acids) in protein and lipid synthesis, enzymes catalyse steps of synthesis and membrane proteins for vesicle/lipid droplets fusion and fission. Thus, variation in the presence and amount of these proteins could influence the synthesis and secretion process and finally lead to variation in milk composition.

1.3 Proteomics analysis

Proteomics analysis was invented and fast developed over the last few decades. The proteomics analysis enables the detection, quantification and characterization of proteins in various types of samples (Steen and Mann, 2004). It often consists of three steps. Firstly, the protein-sample needs to be prepared for the measurement of mass spectrometry. It includes the isolation of proteins from the sample matrix, pre-separation of proteins (e.g. 2D-gel electrophoresis or 1D SDS-PAGE), and enzymatic protein digestion. Depending on the sample properties, the first two procedures can be different or omitted. 2D-gel and SDS-PAGE are both useful methods to pre-separate proteins. However, both of them have some shortages. The number of proteins identified after 2D-gel separation is lower (Cebo, 2012, Gygi et al., 2000, Vuong et al., 2000) than when using 1D SDS-PAGE. Using 1D SDS-PAGE, a large number of proteins can be identified, but the proteins are not easily quantified (Cebo, 2012). Another difficulty in proteomics analysis is identification of the membrane proteins. Since membrane proteins are hydrophobic, they normally have low solubility in water, which could induce insufficient enzymatic digestion of these proteins. Wiśniewski applied a filter aided sample preparation (FASP) on different tissues (Wśniewski et al., 2009). Membrane proteins accounted for more than 40% in identified proteins in their study. This showed the ability of FASP for studying milk membrane proteins. In this thesis, proteins in the milk fat globule membrane are of great interests. In order to identify and quantify them in proteomic analysis, FASP may be a useful sample preparation technique. Secondly, in proteomics analysis, the digested peptides will be analysed by using mass spectrometry sometimes coupled with liquid chromatography for pre-separation of peptides. The masses of the peptides and its fragments will be measured and the peptides will be identified. In this thesis, nanoLC-Orbitrap-MS/MS will be applied for analysing milk proteins. Thirdly, by using bioinformatics software, the amino acid sequence of peptides will be calculated based on the analysed mass. By using certain algorithms, the peptides will be matched to protein databases, leading to the identification of the proteins in a sample.

In addition to identification of proteins, quantification gets more attention nowadays. It enables a more in-depth study of differences between samples. Dimethyl labelling, which is thought to be an easy and inexpensive quantification method (Boersema et al., 2009), was

applied in this thesis to compare different milk samples. It is the first time that dimethyl labelling was used in studying bovine milk.

1.4 Proteomics analysis of milk proteins

In recent years, proteomics analysis has been applied in different milk fractions (MFGM, serum, buttermilk etc.). Several hundreds of low abundant proteins were identified in milk in addition to the well-known major proteins (Cebo, 2012, O'Donnell et al., 2004, Reinhardt and Lippolis, 2006). The function of these low abundant proteins were also analysed and discussed. Milk serum (whey) has been extensively studied to find biomarkers for mastitis in the mammary gland (Boehmer et al., 2010, Hogarth et al., 2004, Ibeagha-Awemu et al., 2010), because it contains many proteins related to host defense. The other fraction that became of more interest over the years is MFGM. As discussed in the previous section, MFGM is a 3-layer membrane structure originating from mono-layer ER membrane and double-layer plasma membrane covering milk fat globules with some cytoplasm trapped in between. MFGM was thus considered as partly representative for epithelial cells (Cebo, 2012). Even though MFGM proteins only accounts for 1-2% of total proteins in milk, it is the most diverse protein fraction. More than 100 proteins have been identified in MFGM, and these proteins were found to be involved in various cellular processes including fatty acid/lipid metabolism, protein metabolism/transport, membrane trafficking, cell signalling etc. (Reinhardt and Lippolis, 2006). MFGM was also used to study and explain the changes of colostrum to milk (Reinhardt and Lippolis, 2008). D'Alessandro et al. (2011) did a meta-analysis of milk proteins identified by a range of different proteomics analyses from different milk fractions. In total, 573 non-redundant proteins were shown to exist in bovine milk based on 40 different papers. Pathway and network analysis revealed that the proteins in milk are related to many cellular biological processes, which are not only known processes such as nutrient transport, lipid metabolism, immune response but also cellular proliferation and anatomical & haematological system development. Milk is the bio-fluid of cows and provides the nutrients and protection to calves, the change of proteins related to cellular process could reflect of the variation in cellular activities in mammary gland. The identification and quantification of low-abundant milk proteins, especially the ones in MFGM, can thus hopefully be used to explain the variation in milk composition.

1.5 Aim and outline of this thesis

The objectives of this thesis are first to identify and quantify low-abundant proteins in milk by using proteomic techniques and, second, to use these low-abundant proteins in explaining the variation in milk composition.

Chapter 2 describes the validation of the proteomic methods used in the thesis. Filter aided sample preparation coupled with dimethyl labelling was tested in studying milk fat globule membrane proteins. The validity of identification of membrane proteins and the accuracy of quantification of MFMG proteins were examined.

Sixty three out of 247 proteins identified in MFGM and milk serum by using FASP-dimethyl labelling-nanoLC-Orbitrap-MS/MS were related to milk synthesis and secretion pathways in mammary gland. The detailed function of proteins in lipid metabolism, protein metabolism, lactose synthesis, membrane trafficking, vitamin transport and mineral transport is discussed in **chapter 3**. The involvement of low-abundant milk proteins in all milk components synthesis and secretion pathways enables the use of these proteins in studying variation in milk composition.

Thus, the proteomic analysis of low-abundant milk proteins coupled with other analysis approaches (^{1}H-NMR for milk lipid composition and milk serum metabolites) were used to explain different biological questions related to milk synthesis and secretion in **chapter 4, chapter 5 and chapter 6**. In **chapter 4**, proteomic analysis of MFGM and NMR analysis of milk lipid composition and milk serum metabolites were applied to milk samples from cows with DGAT1 KK and DGAT1 AA genotypes to investigate the effect of the DGAT1 K232A polymorphism on milk production. DGAT1 is an enzyme catalysing the synthesis of triglycerides from diglycerides. The substitute of alanine to lysine in position of 232 was found to be associated with increased fat percentage, increased fat yield, increased protein percentage, decreased protein yield, decreased milk yield and different fatty acid composition (Grisart et al., 2002, Schennink et al., 2007). In **chapter 5**, different sizes of MFG were separated from milk of seven individual cows. Protein composition were analysed to investigate the differences in synthesis and secretion process of large and small milk fat globules. In **chapter 6**, the differences in proteins and metabolites in milk during negative energy balance and improved energy balance/positive energy balance of lactating cows were studied. The physiological differences in epithelial cells in mammary gland leading to variation in milk production and body performance were discussed and hypothesized.

Chapter 7 contains a general discussion of the results in this thesis and recommendations for further investigations.

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Chapter 2

Filter-aided sample preparation with dimethyl labelling to identify and quantify milk fat globule membrane proteins

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Filter-aided sample preparation with dimethyl labelling to identify and quantify milk fat globule membrane proteins
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Abstract

Bovine milk is a major nutrient source in many countries and it is produced at an industrial scale. Milk is a complex mixture of proteins, fats, carbohydrates, vitamins and minerals. The composition of the bovine milk samples can vary depending on the genetic makeup of the bovine species as well as environmental factors. It is therefore important to study the qualitative and quantitative differences of bovine milk samples. Proteins in milk can be present in casein micelles, in the serum (the water soluble fraction) or in fat globules. These fat globules have a triple membrane layer with proteins being bound to or being encapsulated in the membrane layer. The identification and molecular composition of the milk proteins have gained increased interest in recent years. Proteomic techniques make it now possible to identify up to many thousands of proteins in one sample, however quantification of proteins is as yet not straightforward. We analysed the proteins of the milk fat globule membrane using dimethyl labelling methods combined with a filter-aided sample preparation protocol. Using these methods, it is now possible to quantitatively study the detailed protein composition of many milk samples in a short period of time.

2.1 Introduction

Raw bovine milk contains on average 4%–5% fat, most of which is present in fat globules. Milk fat is synthesized in the endoplasmic reticulum in secretory cells in the mammary gland of the cow, and subsequently transported to the cytoplasm. During this transport, the fat droplets are covered with a layer of membrane (consisting of proteins and lipids) from the endoplasmic reticulum. The fat droplets coalescence in the cytoplasm and will be enveloped by the apical plasma membrane (double layer) during their extrusion from the cells. These fat droplets will then be released into the milk. During this secretion, components of the cytoplasm will be retained between the different membrane layers (McManaman and Neville, 2003). Hence in milk, fat globules are covered by 3-layers of membranes from the epithelial cells. This complex membrane system is called the milk fat globule membrane (MFGM). The MFGM protects the fat globule from aggregation and enzymatic degradation (Dewettinck et al., 2008). It consists of a mixture of lipids and proteins (Heid and Keenan, 2005), of which 25-70% is protein (Dewettinck et al., 2008). Although MFGM proteins account for only 1-2% of the total milk protein content, it is a protein group which shows a relative larger diversity than other milk phases. MFGM proteins have been studied extensively in different mammals (Fortunato et al., 2003, Pisanu et al., 2011, Wu et al., 2000). Firstly, because MFGM contains proteins with a wide range of bioactive functions (Hancock et al., 2002, Mana et al., 2004, Martin et al., 2004, Riccio, 2004, Spitsberg et al., 1995), and secondly, because of the origin of MFGM, it may help understand fat synthesis and secretion (Casado et al., 2009).

In the study of milk fat globule membrane proteins, a vast number of proteins have been identified by using proteomic techniques (Bianchi et al., 2009, Fong et al., 2007, Reinhardt and Lippolis, 2006, Smolenski et al., 2007, Vanderghem et al., 2008). Up to now, proteomic research on bovine milk fat globule membrane proteins mainly used 2D-gel electrophoresis followed by in-gel-digestion of spots of interest. As proteomic techniques developed, disadvantages of 2D-gel became more obvious. Firstly, by using 2D-gels, it is difficult to detect low abundant proteins which could be the more interesting ones (Gygi et al., 2000, Vuong et al., 2000). Secondly, many intrinsic membrane proteins may be lost by using 2D-gel electrophoresis (Reinhardt and Lippolis, 2006). These disadvantages make 2D-gel electrophoresis not the best method to study MFGM proteins, also because the 2D-gel separation creates numbers of fractions that have to be analysed individually which requires a large amount of measurement time (Nagaraj et al., 2008). More separation steps also may result in more variable results, especially when proteins have to be quantified. Instead of using 2D-gels, 1D-gel electrophoresis can be used for protein separation,

followed by fractionation of the 1D-gel into 5–25 slices and analysis of these slices by LC–MS/MS. When the proteomic analysis of the 1D-gel slices is performed with advanced MS instrumentation as for instance an Orbitrap-FTMS, the 1D-gel proteomics result turns out to be superior to 2D-gel electrophoresis techniques (Reinhardt and Lippolis, 2006). Nevertheless a substantial amount of time is needed for a holistic proteomic analysis even with 1D-gel electrophoresis.

In proteomics research, it is difficult to study membrane proteins because of the low aqueous solubility due to their hydrophobicity. The solubility of hydrophobic proteins can be enhanced by adding an ionic surfactant like SDS. The SDS will have to be removed when LC–MS is applied to analyse the peptides. This can be accomplished by using a so called filter-aided sample preparation method (FASP) (Wiśniewski et al., 2009). In addition, the FASP method can provide proteomic analysis of a sample in one run whereas a 1D-gel method still would require more LC-MS/MS runs to be performed.

Only a few bovine milk proteomic studies have applied quantitative proteomics (Affolter et al., 2010, Reinhardt and Lippolis, 2008). In recent years, quantification of proteins has gained attention (Elliott et al., 2009, Gygi et al., 1999, Hsu et al., 2003, Ong et al., 2002, Ross et al., 2004). In principle these quantification methods use relative quantification comparing samples to each other. One of the recent methods for quantification is dimethyl labelling. Dimethyl labelling was first applied in proteomics research by Hsu et al. (2003), but further improved by the group of Boersema et al. (2009). Dimethyl labelling gives a high and specific yield. It is a reliable, cheap, and relatively easy procedure that can be automated and applied in high-throughput proteomic experiments (Boersema et al., 2009).

In this paper, we validated the combined use of FASP and dimethyl labelling on the complex membrane system: the bovine milk fat globule membrane (MFGM). The MFGM proteins were studied both in a qualitative and in a quantitative manner. By combining these techniques, we show that the method gives a reliable and easy procedure for both identification and quantification of hydrophobic and hydrophilic proteins from a complex source.

2.2 Materials and methods

2.2.1 Milk sample preparation

Milk samples were collected and combined from 65 Holstein cows at Wageningen University farm. The milk samples were centrifuged for 10 min at 1500 g to obtain cream (top layer) and skimmed (bottom layer) milk. The cream (1 ml) was separated and washed

with 10 ml milli-Q water (Le et al., 2009) and centrifuged at 1500 g for 10 min. This washing step was repeated 3 times. The washing solution was removed after centrifugation. Finally, the washed milk cream was sonicated for 1 min and the protein concentration of washed cream was determined using the Dumas method with an NA 2100 Protein nitrogen analyzer (CE Instruments, Milan, Italy) (Dumas, 1831).

2.2.2 Filter-aided sample preparation (FASP)

The method used was based on FASP (Wiśniewski et al., 2009) with some modifications. Our procedure is as follows: Protein samples were diluted in SDT-lysis buffer (100mM Tris/HCl pH 8.0+4% SDS+0.1 M dithiotreitol) to get a 1 µg/µl protein solution. It was then incubated at 95°C for 5 min and cooled down to room temperature. 10 µl of sample was directly added to a Pall 3K omega filter and centrifuged at 20,000 g for 1 min. 100 µl of UT (100mM Tris/HCl pH 8.0+8M urea) was added to the filter and centrifuged at 20,000 g for 30 min. 100 µl of IAA (0.05M iodoacetamide in UT) was added and mixed followed by 10 min of incubation at room temperature and centrifuged at 20,000 g for 30 min. 110 µl UT was added to the filter and centrifuged at 20,000 g for 30 min. This step was repeated two times with 120 µl and 130 µl UT respectively. 140 µl 0.05M NH_4HCO_3 (ABC) was added to the filter unit and centrifuged at 20,000 g for 30 min. Then, 100 µl ABC containing 0.5 µg trypsin was added and incubated overnight at room temperature. Finally, the filter unit was centrifuged at 20,000 g for 30 min. The sample obtained was acidified with 10% TFA (trifluoroacetic acid) to pH 2-4. These samples were ready for either direct nanoLC-Orbitrap-MS/MS analysis or dimethyl labelling.

2.2.3 Dimethyl labelling

The peptide samples prepared with FASP were labelled according to the method described by Boersema et al. (2009). C18+ Stage tip columns were made in-house. The C18+ Stage tip column was washed 3 times with 200 µl methanol. It was then conditioned with 100 µl of 1ml/l HCOOH. The peptide samples were loaded on the C18+ Stage tip column followed by washing with 100 µl 1ml/l HCOOH. The columns were then slowly flushed with 100 µl labelling reagent (light: CH_2O or heavy: CD_2O) in about 10 min. The column was washed with 200 µl 1ml/l HCOOH. The labelled peptides were eluted with 50 µl of 70% acetonitrile/30% 1ml/l HCOOH from the columns. Two samples, one with light and one with heavy dimethyl labels, were then mixed together and analysed by using nanoLC-Orbitrap-MS/MS.

2.2.4 NanoLC- Orbitrap-MS/MS

2.2.4.1 NanoLC and Orbitrap-MS/MS quality checks.

The MS was checked weekly for proper functioning by direct infusion of a standard calibration mixture at a flow rate of 0.5 µl/min. A nanospray source setup was used. The MS method was programmed to measure four segments, each for 1 min. These consisted of: MS (Orbitrap, between m/z 150 and 2000, resolution 60.000, Profile), MSMS (Orbitrap, between m/z 150 and 600, resolution 60.000, Profile, CID with 35% normalized collision energy of m/z 524 with isolation width of 3 m/z), MS (Iontrap, between m/z 150 and 2000, Normal scan Rate, Centroid), MSMS (Iontrap, between m/z 150 and 600, Normal scan Rate, Centroid, CID with 35% normalized collision energy of m/z 524 with isolation width of 3 m/z). Observed were the intensities measured in MS mode of both Orbitrap and Iontrap of the peptide MRFA at m/z 524.26 as well as its b_2 fragmentation product at m/z 288.2 measured in MSMS mode again in both Orbitrap and Iontrap. The same setup was used to calibrate the Orbitrap every day.

The combination of nanoLC and MS was checked on a daily basis by injection of a standard BSA digest (18 µl of 130 fmol peptides) with an acetonitrile gradient of 9% to 34% in 50 min and measuring MS in both Orbitrap and Iontrap as mentioned above but using a narrower m/z range of m/z 380-1400 followed by 3 MSMS segments (threshold 500 counts): selection of the most abundant double or triple charged ion with an isolation width of 2 m/z without fragmenting (segment 1) and with fragmenting (segment 2) at default settings as well as selection and fragmentation of the second most abundant double or triple charged ion (segment 3).

The system check comprised of following the intensity of dioctylphthalate at m/z 391.29 during the columns void volume time around 5 to 6 min as measured in the Orbitrap MS together with the signal intensities of the lowest signals measured in both Orbitrap and Iontrap. Also, peak intensities and peak width at half the height of m/z 582.3 (LVNELTEFAK) were recorded. The peak intensities of m/z 582.32 together with those of the lowest measured signals were used to calculate a “Signal to Noise” value. Next to this, the first MSMS measurement of m/z 582.32 was checked for the presence of 4 low abundance signals at m/z's 213.2 (b_2), 218.2 (y_2), 327.2 (b_3) and 365.2 (y_3).

2.2.4.2 MFGM sample analysis.

The samples were analysed by injecting 18 µl sample (Proxeon nanoLC) over a 0.10×32mm 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 0.10×200mm Prontosil 300-3-C18H analytical column with an acetonitrile gradient at a flow rate of 0.5 µl/min. The gradient consisted of an increase from 9% to 34% acetonitrile in water with 1ml/l HCOOH in 200 min followed by a fast increase in the percentage acetonitrile to 80% (with 20%water and 1ml/l HCOOH in both the acetonitrile and the water) in 3 min as a column cleaning step.

Between the pre-concentration and analytical column, an electrospray potential of 3.5 kV was applied directly to the eluent via a solid 0.5mm platinum electrode fitted into a P777 Upchurch microcross. Full scan positive mode FTMS spectra were obtained between m/z 380 and 1400 on a LTQ-Orbitrap XL (Thermo electron, San Jose, CA, USA). MS/MS scans of the 10 most abundant doubly and triply charged peaks in the FTMS scan were recorded in data dependent mode in the linear trap (MS/MS threshold=5000, 60 s exclusion duration).

2.2.5 Data analysis

Identification. Each run with all MS/MS spectra obtained was analysed with Bioworks 3.3.1. A maximum of 1 differential modification per peptide was set for oxidation of methionines and de-amidation of N or Q. Carboxyamidomethylation of cysteine was set as a fixed modification (enzyme=trypsin, maximally 2 missed cleavages, peptide tolerance 10 ppm, fragment ion tolerance 0.5 amu).

A combined FASTA database was constructed from the bovine IPI database (downloaded from ftp.ebi.ac.uk/pub/databases/IPI/current August 2009) plus a set of 31 protein sequences of common contaminants including: Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human) and Keratin K1CI (P35527, human). A decoy database was created by adding the reversed sequences using the program Sequence Reverser from the MaxQuant 1.0 package (Cox and Mann, 2008). This gave a database containing 65,994 proteins in total.

The peptide identifications obtained were filtered in Bioworks with the following filter criteria: $\Delta Cn>0.08$, Xcorr>1.5 for charge state 2^+, Xcorr>3.3 for charge state 3^+ and Xcorr>3.5 for charge state 4^+ (Peng et al., 2003). Finally, protein filters used were: minimum of 2 distinct peptides of which at least 1 unique, a minimum Sf score of 1 and a protein probability <0.05. The false discovery rate, calculated as the number of hits against the inverted decoy proteins within filter settings divided by the total number of proteins within filter settings (<0.25% for all 5 experiments).

Quantification. Ratios of proteins identified by Bioworks were calculated by MaxQuant 1.1.1.21 for samples prepared using FASP combined with dimethyl labelling. For label-free quantification, the protein intensity was also calculated by MaxQuant 1.1.1.21 (Hubner et al., 2010).

2.3 Results and discussion

2.3.1 NanoLC and MS checks

The MS signal intensities and signal to noise values are compared in Tables 2.1 and 2.2. The standard deviations of both MS and MSMS signal intensities as measured in the Orbitrap and the LTQ iontrap, when the calibration mixture was directly infused, are below 50% of the average signal as calculated over 50 measurements (Table 2.1). With the nanoLC connected, the standard deviation of the Orbitrap and LTQ iontrap MS signal decreases to approximately 30% of the average signal (Table 2.2 A). Also, the Orbitraps

Table 2.1. Statistics over the last 50 measurements of infused calibration mixture for the Orbitrap (A) and the Iontrap (B).

A				
	Orbitrap Signal Intensity			Orbitrap
	Lowest MS signal	MS of m/z 524	MSMS of m/z 288	MS Signal to noise
Average value	537	9,507,380	1,412,800	22,478
Std. deviation	557	4,515,411	626,450	13,672
B				
	Iontrap Signal Intensity			Iontrap
	Lowest MS signal	MS of m/z 524	MSMS of m/z 288	MS Signal to noise
Average value	4,701	731,520	77,970	184
Std. deviation	3,401	279,191	32,509	68

Table 2.2. Statistics of the nanoLC–MS checks that were obtained during 50 weeks with an injection of a standard BSA digest of the MS (A) and nanoLC (B). All intensities in A are divided by 10^6.

A					
	391	Orbitrap lowest MS	Iontrap m/z 582.3	Orbitrap m/z 582.3	Signal to noise Orbitrap MS
Average	0.228	0.0000123	1.55	9.27	764,784
Std.	0.075	0.0000024	0.51	3.06	301,108
B					
	Retention time (min)		Retention time (min)		LC Peak width
Average	17.4		31.7		0.17
Std.	1.5		1.5		0.02

lowest MS signal measured decreases drastically which is the main reason of the increase of the calculated "signal to noise" value. To calculate this value, the "noise" is replaced by the lowest MS signal measured to get an indication of a "signal to noise" value. For the tryptic BSA peptide LVNELTEFAK as measured at m/z 582.3 at a retention time of 32 min, the calculated "signal to noise" ratio is approximately 765,000 when 130 fmol peptide was injected and when the lowest signal measured was used as the "noise" value. Table 2.2 also shows that the retention time standard deviation is 1.5 min. This can be considered as good since different pre-concentration columns as well as analytical columns and analytical columns of variable lengths and backpressures have been used during the time span of the calculation. The LC peak width, measured at half the peak height, is one of the main reasons of peak intensity variation. The peak width itself reflects the condition of both pre-concentration column and the analytical column as well as their connections.

2.3.2 Identification of MFGM proteins

Using FASP, a total of 169 proteins were identified with a false discovery rate <0.25% (Supplementary Table S2.1). The good reproducibility of our method (MFGM proteins isolation, FASP, and LC–MS analysis) is evident from the 94% overlap between two technical replicates. Table S2.1 also lists if the proteins were previously reported in literature. The major proteins in milk fat globule membrane, i.e. xanthine oxidase, butyrophilin, adipophilin and lactadherin were clearly observed. However, we also identified 55 proteins in bovine MFGM which were not reported before. Most of these newly identified proteins are low-abundant proteins according to the calculated MS intensities. An interesting example of a low-abundant protein we detected in our study is the mitochondrial enzyme glycerol-3-phosphate acyltransferase (GPAM). This is the first time this enzyme is reported in the bovine milk fat globule membrane fraction. GPAM catalyzes the first step in the *de novo* synthesis of neutral lipids (triglycerides) and glycerophospholipids (Coleman and Lee, 2004, Gimeno and Cao, 2008). The intensity of GPAM (in the quantitative analysis by MS) is approximately 1500 times lower than the most abundant proteins (see 2.3.3.2 on label-free quantification), showing that GPAM is indeed a very low abundant protein. In addition, a whole range of other low abundant proteins (e.g. RAB7A, GNAO1, CD14) were found. These results showed that FASP combined with LC–MS/MS is a useful method for detection of low-abundant proteins in a complex membrane system. The higher number of proteins identified in our study as compared to previous bovine MFGM proteomic studies may be caused by two effects. Firstly, it may be caused by the use of more advanced techniques, including FASP as sample preparation method, more sensitive equipment (nanoLC-Orbitrap-MS/MS), proper system setup and an experienced operator. Secondly, the information on the bovine genome

is more complete than before, so the protein databases are more complete. Because many of the newly discovered proteins were low-abundant proteins, the quality of the improved methods and hardware is likely to be more important than the improved genome information.

In the past few years, there were several papers describing the bovine MFGM proteome (Bianchi et al., 2009, Fong et al., 2007, Reinhardt and Lippolis, 2006, Smolenski et al., 2007, Vanderghem et al., 2008). These papers reported around 60–133 proteins for the MFGM, including many different modified forms of the same proteins. One hundred and twenty proteins were reported by using a 1D-gel together with LC–MS/MS by Reinhardt and Lippolis (2006), of which 48 proteins were also found by us. The differences between our results and the result of Reinhardt and Lippolis may be caused by the databases they used for protein identification. They used the non-redundant universal protein reference database UniRef 100 from Swiss-Prot, as a result of which proteins from human and mouse origin were included in their results. Also the separation procedure is different. They pelleted and extracted the MFGM proteins from washed milk cream, we used washed cream directly. The most recent paper describing MFGM proteins (Affolter et al., 2010) used a MFGM protein enriched buttermilk as study material. In this milk fraction, they identified 133 proteins, of which 78 proteins were also detected by us. The difference between our results and by Affolter was that they used industrial buttermilk and we used MFGM prepared from raw bovine milk samples.

The identified proteins were annotated according to function (Figure 2.1). An important, perhaps the most important class of proteins, was immunity and host defense. Because milk is the first food source for neonates, it is both safe for usage (host defense proteins) as well it provides immune proteins to the neonate. Also a large number of proteins of the Ras superfamily (Ras, Rab, Rho, Rap) are present in milk (Table S2.1). This family of proteins functions as regulator in molecular processes (molecular switches) (Roberts and Waelbroeck, 2004, Stenmark and Olkkonen, 2001). Another large group of proteins observed are involved in the lipid metabolic process. Most of the major (abundant) proteins (butyrophilin, xanthine oxidase, adipophilin, and CD36) in MFGM were classified in this group (Mather and Keenan, 1998). Butyrophilin, xanthine oxidase and adipophilin are thought to interact with each other in MFGM and play an important role during milk fat secretion (McManaman et al., 2002, Vorbach et al., 2002).

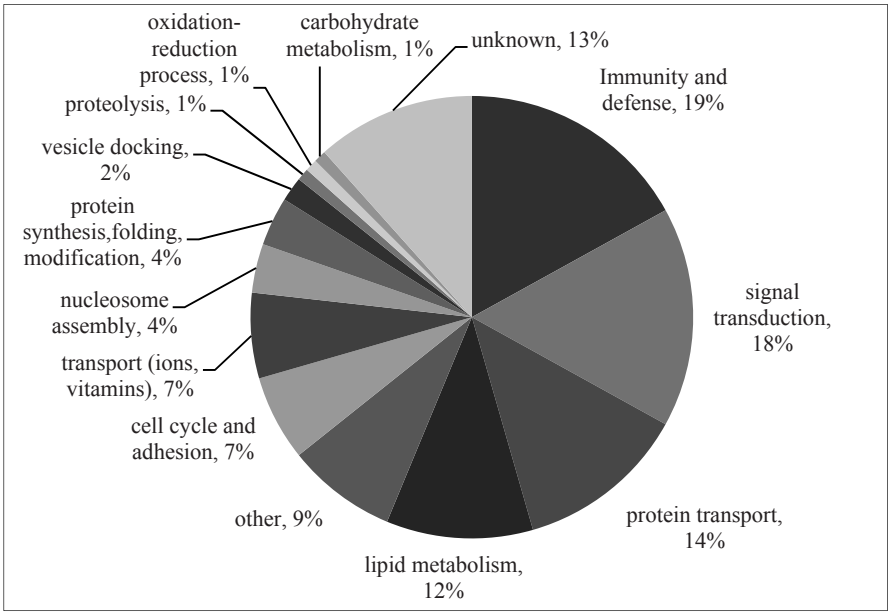


Figure 2.1. Gene ontology based functional classes of identified proteins. The gene ontology information was taken from Swiss-Prot database (www.uniprot.org). For proteins which function were not immediately clear from Swiss-Prot database, a BLAST search was applied at www.uniprot.org.

The subcellular location of the identified proteins is shown in Figure 2.2. Seventy six proteins with known subcellular location were reported to be membrane bound. In this category, most of the proteins are from the cell apical membrane, which is the primary membrane source of the MFGM (Heid and Keenan, 2005). There were also some membrane proteins from intracellular membrane sources like the endoplasmic reticulum, Golgi apparatus, mitochondrion and cytoplasmic vesicle. The next biggest group contains proteins from the cytoplasm. These proteins might originate from "cytoplasmic crescents" which is trapped between the membrane layers of the MFGM during the budding process when the fat globule leaves the epithelial cell (McManaman and Neville, 2003). Proteins from the nucleus, endoplasmic reticulum, Golgi apparatus, and mitochondrion were also found. All these proteins observed, originating from different intracellular sources, indicate that the MFGM is very interesting for studying proteins from the secretory cell. Thus, studying milk synthesis and secretion may even be possible by studying MFGM proteins, instead of taking biopsies, which is the most regular way for studying milk secretion. The high amount of membrane proteins we identified shows that FASP is a useful method for studying membrane proteins. The efficient solubilisation of the hydrophobic MFGM

proteins is a key step for their identification (Affolter et al., 2010). The use of SDS in the FASP method enables this critical step, and the sufficient removal of the SDS with urea and the filter provided good chromatography for peptide separation and identification.

Another advantage by using FASP is that one biological sample results in only one LC/MS run. This will help to decrease variation if quantitative analysis has to be obtained as no fractionation was applied before LC–MS measurements.

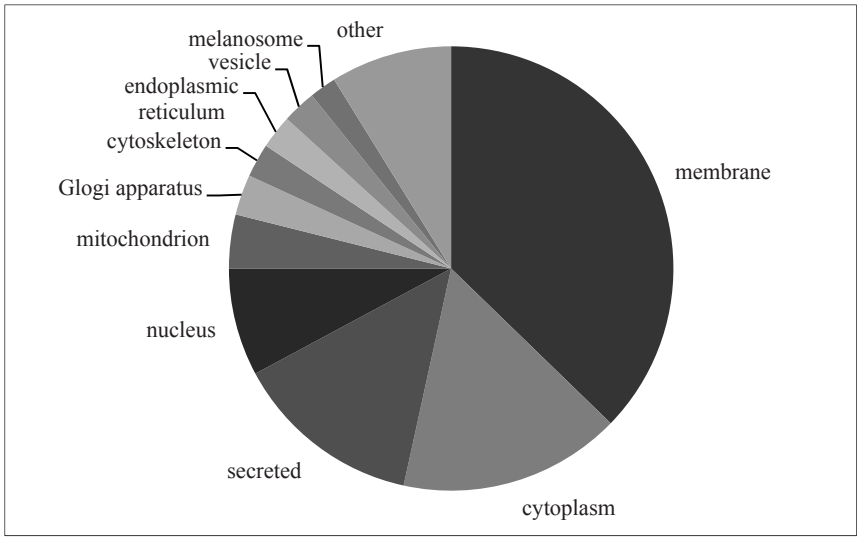


Figure 2.2. Subcellular locations of identified proteins. The subcellular location information was obtained from the Swiss-Prot database.

2.3.3 Quantification of MFGM proteins

2.3.3.1 Dimethyl labelling

Source of variance. For quantification of MFGM proteins in our study, a two-step procedure was used: first, MFGM was prepared from whole bovine milk by centrifugation and washing and second, FASP-dimethyl labelling was followed by LC-MS/MS analysis. Both of these procedures contribute to the variation in the quantification results. In order to see how much of the variation can be explained by the biological sample preparation and proteomic techniques, we did two types of experiments. First, two identical bovine milk samples were centrifuged and washed to obtain two MFGM samples. These two MFGM samples were diluted to 1 µg/µl (yielding a volume of about 5000 µl) and analysed using FASP-dimethyl labelling-LC–MS with light and heavy labels (using 10 µl from each

sample). Second, one of the MFGM samples was equally divided to two portions, and then these two portions (taking 10 µl from each sample) were analysed by using FASP-dimethyl labelling-LC–MS with light and heavy labels. This second experiment eliminated the variation of the biological sample preparation (preparing MFGM from bovine milk) and can be used to examine the variation of FASP-dimethyl labelling-LC-MS/MS. Each type of experiment was done in duplicate as shown in Table 2.3. The peak intensities of light and heavy labelled peptides, as well as protein ratios, were calculated by MaxQuant. From these results, it can be seen that the average ratio of all 4 analyses is around the theoretical value 1.0, but the coefficient of variation of experiment 1 is significant larger than for experiment 2. The coefficient of variation of FASP-dimethyl labelling-LC-MS/MS was 12%. This value is below 15%, which is normally considered as the threshold for quantitative bio-analysis (Ji et al., 2005). This indicates that the proteomic techniques we applied here were accurate for quantification of complex membrane protein samples. If the preparation of MFGM was included, the coefficient of variation was 20.7±3.5% for each analysis. The MFGM preparation therefore significantly contributes to the variation in the results. That may be related to the washing steps in preparation of MFGM, which was used to get rid of relative high abundant serum proteins and caseins. But loosely bound MFGM proteins could also have been washed away to some extend (Dewettinck et al., 2008). MFGM was also isolated without washing steps, but the number of proteins identified was much less than for washed cream, which is probably caused by the interference of high abundant serum proteins (data not shown). Since the washing steps could not be excluded, in order to get more accurate quantification for these isolated MFGM proteins, more replicates were needed for the quantification experiments.

Table 2.3. Source of variation in dimethyl labelling quantification.

	E1-1	E1-2	E2-1	E2-2
Number of protein quantified	118	121	133	128
Average of protein ratio	1.12	0.98	1.01	1.01
Std. Deviation of protein ratio	0.20	0.23	0.14	0.11
Coefficient of variation %	18.18[a]	23.20[a]	13.62[b]	11.01[b]

E1: Experiment 1, preparation of MFGM from whole milk+FASP-dimethyl labelling-LC-MS/MS; E2: Experiment 2, FASP-dimethyl labelling-LC-MS/MS. Each experiment is done in duplicate. a,b: significant level of $P<0.01$, F-test was applied to analyse difference of variance of 4 data sets.

Number of replicates needed. Quantification data were obtained by using MaxQuant on 3 and 5 replicates (MFGM isolation+FASP-dimethyl labelling-LC-MS/MS). Each protein ratios shown in Table S2.1 are the combined ratio calculated by MaxQuant of 3 replicates and 5 replicates. The 3 replicates consist of the first 3 runs of the 5 replicates. There were in total 139 proteins that could be quantified with an average ratio of 1.05±0.14 in 3 replicates

and 145 proteins with an average ratio of 1.02±0.11 in 5 replicates, both of which are close to the theoretical level of 1.0. As expected, the more replicates we applied the smaller the variance of the whole dataset. But from economic and workload point of view, 3 replicates can be considered to be accurate enough for quantification of the proteins in the MFGM.

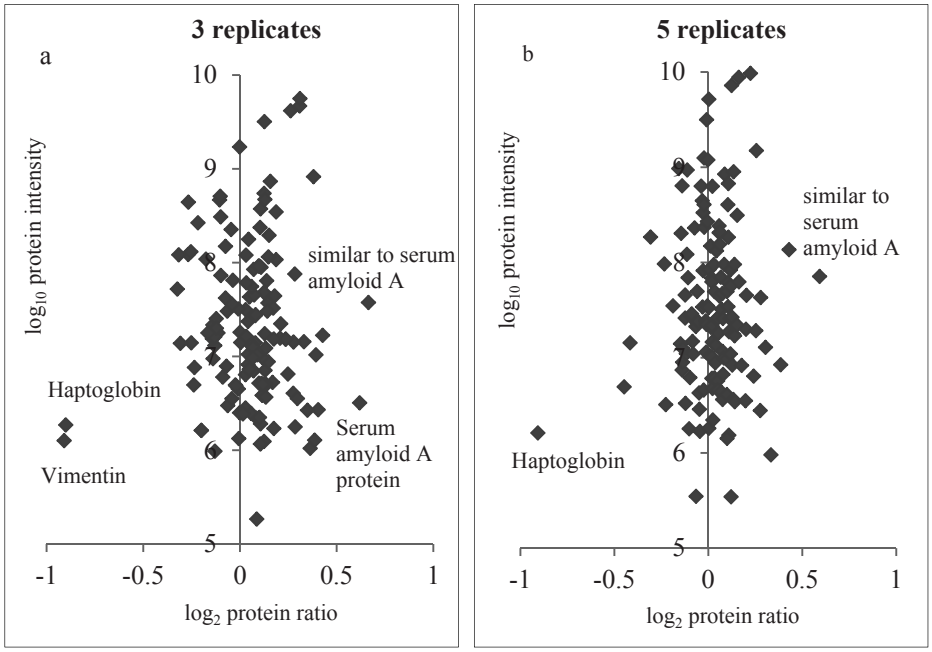


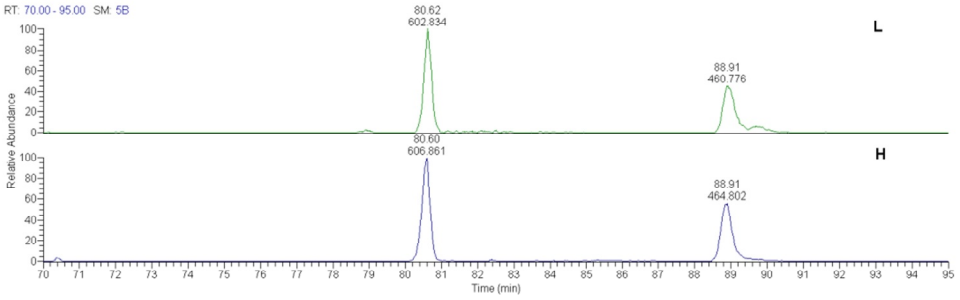
Figure 2.3. Heavy to light protein abundance ratios of 3 replicates (a) or 5 replicates (b). Both protein abundance ratio as well as the intensity of each protein as plotted on the y-axis was calculated by MaxQuant 1.1.1.21.

Accuracy of quantification. Figure 2.3 shows the relation between calculated protein ratios (heavy to light) and protein intensities (heavy+light). Figure 2.3 shows that all data points are within a ratio of 0.5 to 1.6 for the low intensity proteins. The ratio dataset of the 3 replicates was analyzed by the significant B test in MaxQuant. Several proteins were calculated to be significantly different from 1.0 and they are indicated in Figure 2.3. These outliers in the case of 3 replicates are shown to be outliers in the 5 replicate dataset as well, but with a smaller deviation from the theoretical value of 1.0. The outliers in both dataset are serum amyloid A protein, a protein similar to serum amyloid A, vimentin, and haptoglobin. The chromatograms and MS spectra were examined manually, and the ratios as calculated by MaxQuant were considered correct. Even though these proteins are outliers,

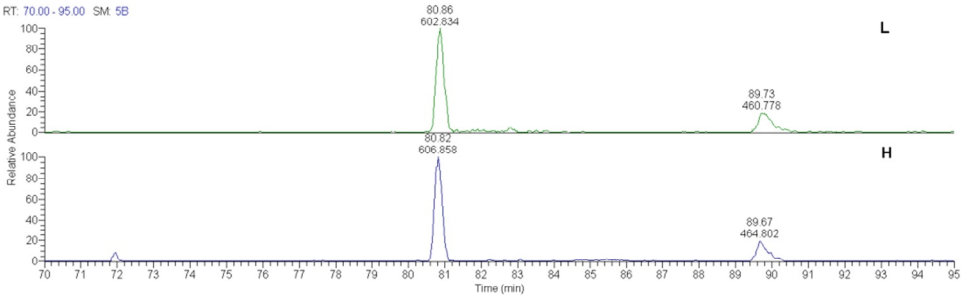
the ratios of these proteins are in the range of 0.5–1.6 which is considered quite acceptable in proteomics analysis.

As an example of the dimethyl labelling results, Figure 2.4 shows the chromatograms of two peptides of the protein CD14. Of the 5 replicates, samples a and b were measured on the same day, samples c, d and e were measured four months later. In Figure 2.4, for each run, in the upper chromatograms the light labelled peptides are shown and in the lower chromatograms the corresponding heavy labelled peptides. The difference in retention time and peak intensity of the upper two and the lower three runs could result from variation in sample preparation or from not completely identical chromatographic conditions. The ratios of the two light to heavy labelled peptides are 1.14±25% and 1.10±18%. This shows that it is beneficial to use dimethyl labelling for quantitative analysis by LC-MS/MS techniques. Finally, the sharp peak shapes of the chromatogram in Figure 2.4. showed that SDS, which can cause broad peak, was successfully removed.

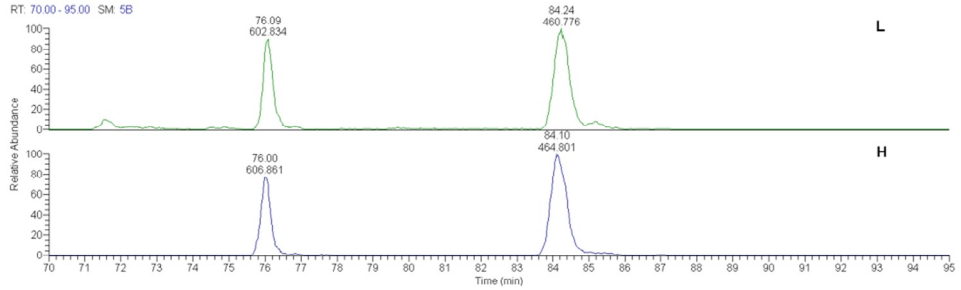
a



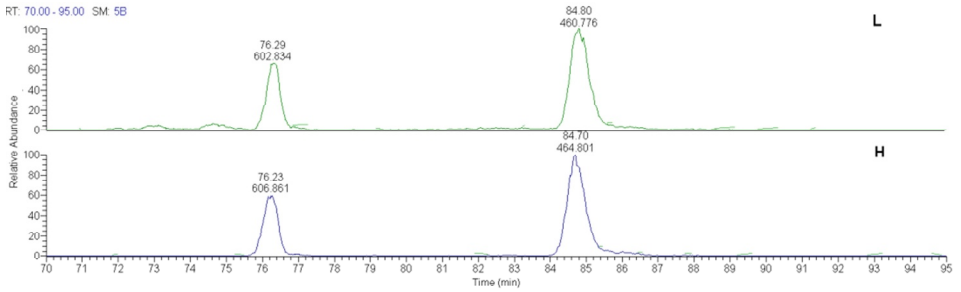
b



c



d



e

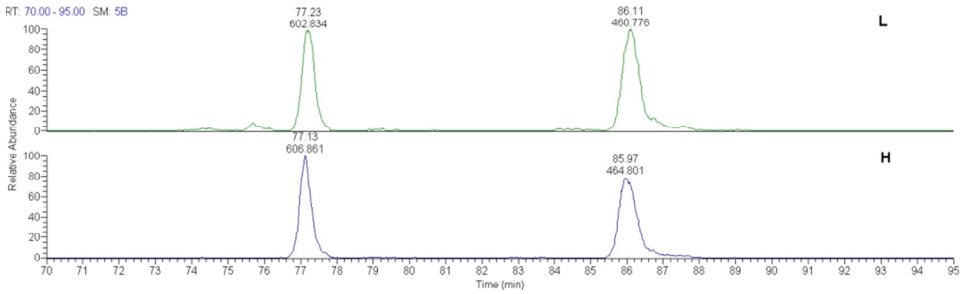


Figure 2.4. Chromatograms of two peptides (LSVLDLSCNK, m/z 574.803; SLEQFLK, m/z 432.745) of the protein CD 14. L: peptides labelled with the light dimethyl label (CH_2O). H: peptides labelled with heavy dimethyl label (CD_2O). Shown are five different runs (a–e) on the same day (a, b) or after four months (c, d, e).

2.3.3.2 Label-free quantification

In addition to labelled quantification, FASP together with nanoLC-Orbitrap-MS/MS can also be used for label-free quantification. Figure 2.5 shows $\log_{10}$ intensities of all proteins from two analytical runs (the same samples as were used in the qualitative data analysis). The Pearson correlation coefficient of the two datasets is 0.95, which shows the usefulness of label-free quantification based on FASP together with nanoLC-Orbitrap-MS/MS. Some outliers were present, which are circled in Figure 2.5. The outliers were annexin A5, Rab-7a, and haploglobin. For these proteins, the big difference occurred because one or two peptides showed a very deviating peak intensity or absence in one of the replicates. Though FASP has the potential for label free quantification, FASP together with dimethyl labelling is to be preferred. The fact that both the heavy and light labelled samples were analysed simultaneously in the dimethyl labelled sample improves clearly the quantification results.

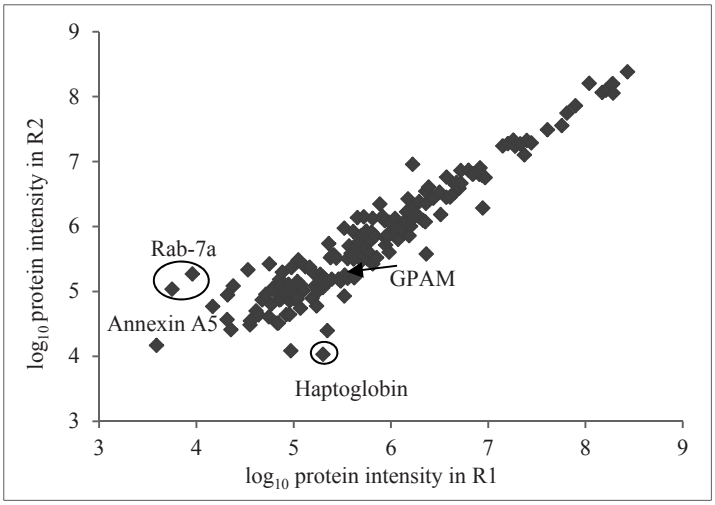


Figure 2.5 Replicate total peak intensity spread for all proteins identified. The outliers are circled and labelled with their protein name. For the total protein peak intensity, the intensity columns from the MaxQuant result table were used.

2.4 Conclusion

This is the first time dimethyl labelling and FASP were combined and applied to a complex protein system. The results show that FASP and dimethyl labelling are useful for identification and quantification of MFGM proteins. The high amount of membrane proteins identified in MFGM proteins showed that FASP is a suitable sample preparation

technique for studying membrane proteins. The low variation of protein ratios in quantification revealed that FASP combined with dimethyl labelling is useful for accurate quantification. FASP and dimethyl labelling are fast and easy techniques. Both methods can be applied for studying complex protein systems.

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Supplementary Table S2.1: Identified and quantified proteins list. Proteins were identified by Bioworks 3.3.1 with at least 2 peptides for each. The ratio was the normalized protein ratio calculated by Maxquant 1.1.1.21. Protein elimination : proteins identified by same peptides were grouped, the one with highest sf score was listed here. The list protein here are at least with 1 unique peptide identified in MS/MS analysis. Gene Ontology information were taken from Swiss-Prot database (www.uniprot.org). For proteins which function were not immediately clear from swiss-Prot database, a BLAST search was applied at www.uniprot.org.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
14-3-3 protein beta/alpha	IPI00760424	13.9	1	1.08	1.03	*1*	protein targeting	protein domain specific binding	cytoplasm
14-3-3 protein epsilon	IPI00696435	13.7	2	0.99	0.91		protein targeting, neuron migration	protein domain specific binding	cytoplasm
14-3-3 protein zeta/delta	IPI00703110	13.5	3	1.03	1.00	*4*	protein targeting	protein domain specific binding, transcription factor binding	nucleus, cytoplasm, melanosome, mitochondrion
17-beta hydroxysteroid dehydrogenase	IPI00686178	6.6	4	1.09	0.92	*5*	lipid metabolic process	oxidoreductase activity, binding	ER
Acetyl-CoA carboxylase 1	IPI00712907	3.9	11	1.11	1.10	*1,5*	lipid metabolic process, lipid biosynthesis process	ATP binding, acetyl-CoA carboxylase activity, biotin binding	
Acetyl-CoA synthetase 2	IPI00717515	4.1	2	1.08	1.08		fatty acid biosynthetic process	acetate-CoA ligase activity	cytoplasm, nucleus, membrane
Actin, cytoplasmic 1	IPI00698900	21.3	8	0.93	0.92	*1,2,3,4,5,6*	chaperone mediated protein folding independent of cofactor	ATP binding	cytoplasm, cytoskeleton
Acyl-CoA synthetase long-chain family member 1	IPI00729755	17.5	13	1.07	1.03	*5*	fatty acid biosynthetic process	catalytic activity	
Adipophilin	IPI00716572	65.1	34	1.09	1.00	*1,2,3,4,5*	long chain fatty acid transport		membrane, cytoplasm, nucleus, lipid particle
Aldehyde dehydrogenase 3B1 isoform 1	IPI00703351	4.7	3	1.00	1.04		cellular aldehyde metabolic process, oxidation-reduction process	oxidoreductase	
Aldose 1-epimerase	IPI00712164	9.9	2				carbohydrate metabolism	aldose 1-epimerase activity, carbohydrate binding	cytoplasm
Alpha-lactalbumin	IPI00717424	36.6	8	1.22	1.35	*5*	lactose biosynthesis	milk protein	secreted

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
Alkaline phosphatase, tissue-nonspecific isozyme	IPI00702768	9.9	5	1.09	1.09	*5*	metabolic process, response to vitamin D, dephosphorylation, response to antibiotic	hydrolase	membrane
Alpha-S1-casein	IPI00706094	22.0	8	0.93	1.19	*5*	transport	antioxidant, milk protein transporter activity	secreted
Alpha-S2-casein	IPI00698843	43.7	13	0.83	0.90	*5*	defense response to bacterium	antibiotic, antimicrobial, milk protein	secreted
Ankyrin repeat domain 22	IPI00732057	17.3	4	1.06	1.07	*1*			
Annexin A1	IPI00703345	26.0	7	1.02	1.06	*4,5*	cell cycle, signal transduction	phospholipase A2 inhibitor	cell projection, cilium, cytoplasm, membrane, nucleus
Annexin A2	IPI00706002	13.0	4	1.00	1.01	*3,4*	angiogenesis, collagen fibril organization, positive regulation of vesicle fusion, positive regulation of binding, cellular response to acid	calcium ion binding, cytoskeletal protein binding, phospholipase inhibitor activity, calcium-dependent phospholipid binding	basement membrane,
Annexin A11	IPI00695993	5.2	1				cell cycle, cell division	S100 alpha binding, calcium-dependent phospholipid binding	cytoplasm, cytoskeleton, nucleus
Annexin A4	IPI00686981	6.9	1	0.96	0.92			calcium ion binding, calcium-dependent phospholipid binding	cytoplasm
Annexin A5	IPI00692093	17.1	1	1.29	0.97	*2,5*	blood coagulation	calcium ion binding, calcium-dependent phospholipid binding	
Apolipoprotein E	IPI00712693	32.3	17	0.8	0.81	*1,5,6*	lipid transport	beta-amyloid binding, heparin binding	chylomicron, HDL, secreted, VLDL
ATP-binding cassette, sub-family G	IPI00690408	32.2	20	1.11	1.00	*5*	transport	ATP-binding, ATPase activity	membrane
Beta-casein	IPI00712994	22.3	1	0.81	0.91		transport	transporter activity, milk protein	secreted
Beta-lactoglobulin	IPI00699698	51.7	16	1.00	0.99	*2,3,5,6*	transport	retinol binding, transporter activity, milk protein	secreted
Butyrophilin	IPI00708535	57.2	5	1.24	1.17	*1,2,3,4,5,6*	lipid droplet secretion		membrane
C4b-binding protein alpha chain	IPI00702590	3.3	2				complement pathway, immunity, innate immunity,		secreted

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
Cathelicidin-1	IPI00718108	23.2	1	0.89	0.88	*4,5*	defense response to bacterium	antibiotic, antimicrobial	secreted
Cathelicidin-2	IPI00691669	13.6	1	0.87	0.92		defense response to bacterium	antibiotic, antimicrobial	secreted
Cathelicidin-7	IPI00694677	9.7	1	0.87	0.92	*5*	defense response to bacterium	antibiotic, antimicrobial	secreted
Cationic amino acid transporter	IPI00696048	5.9	4	1.11	1.01		amino acid transmembrane transport	amino acid transmembrane transporter activity	membrane
CD14 protein	IPI00686931	8.5	2	0.99	1.01	*1,3,5*	immunity, inflammatory response, innate immunity	lipopolysaccharide binding, lipoteichoic acid binding	membrane
CD5 molecule	IPI00867131	8.9	3	1.31	0.93	*5*		scavenger receptor activity	membrane
CD59	IPI00711804	20.7	3	1.02	1.04				
Cell death activator CIDEA	IPI00707616	18.3	6	0.97	0.98	*5*	apoptosis	protein homodimerization activity	mitochondrion, nucleus
Cell division control protein 42 homolog	IPI00704257	11.0	1	1.03	1.00	*5*	small GTPase mediated signal transduction	GTP binding	membrane
Clusterin	IPI00694304	7.3	2		1.26	*5,6*	cytoplasmic vesicle	cell death	chromaffin granule
Complement C3	IPI00713505	8.5	9	0.95	0.94	*5*	complement alternate pathway, complement pathway, immunity, inflammatory response, innate immunity	endopeptidase inhibitor activity	secreted
Dehydrogenase/reductase (SDR family) member 1	IPI00700781	24.4	6	0.97	0.98	*5*	oxidation-reduction process	binding, oxidoreductase activity	ER
Desmoplakin isoform II isoform 1	IPI00686447	1.0	3		1.94		cell adhesion		membrane, cytoskeleton, mitochondrion
DnaJ homolog subfamily A member 1	IPI00823816	8.8	2	0.97			androgen receptor signalling pathway, protein folding, response to heat sperm motility, spermatogenesis	chaperone	membrane
Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	IPI00712650	11.0	9	0.97	1.07	*1,3,5*	immune response	hydrolase	membrane
Elastase 2	IPI00867378	9.4	3				proteolysis		
Elongation factor 1-alpha 1	IPI00712775	15.8	5	1.06	0.99	*1,5*	protein biosynthesis	elongation factor	cytoplasm

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
Fatty acid synthase	IPI00712133	14.2	35			*1,3,5,6*	fatty acid biosynthesis	hydrolase, lyase, oxidoreductase, transferase	cytoplasm
Fatty acid-binding protein	IPI00691946	40.6	7	1.09	1.10	*1,2,3,4,5,6*	lipid transport	lipid binding, transporter activity	cytoplasm mitochondrion
F-box/LRR-repeat protein 20	IPI00718197	7.6	4	0.99	0.93		Ubl conjugation pathway		cytoplasm
Fibroblast growth factor-binding protein 1	IPI00704023	15.4	3	0.95	0.93	*5*	carrier protein that release fibroblast-binding factors	growth factor binding	membrane, secreted
Folate receptor alpha	IPI00708447	12.4	3	0.89	0.90	*3,5*		folic acid binding, receptor	
Gamma-soluble NSF attachment protein	IPI00711042	8.0	2	1.04	1.10		ER-Golgi transport, protein transport	binding	membrane
Glycerol-3-phosphate acyltransferase, mitochondrial	IPI00703084	3.7	5	1.23	1.18		phospholipid biosynthesis	acyltransferase, transferase	membrane, mitochondrion
Glycerol-3-phosphate acyltransferase 4	IPI00697212	9.0	5	1.19	0.91	*5*	Phospholipid biosynthesis	acyltransferase, transferase	ER, membrane
Glycoprotein GP2	IPI00695142	24.0	12	1.08	1.02	*5*			membrane
GTP-ase	IPI00696647	12.0	1	0.94	1.01		GTP catabolic process	GTP binding, GTPase activity, calcium ion binding	
GTP-ase_Ras	IPI00690367	27.2	2	0.91	0.98		small GTPase mediated signal transduction	GTP binding, GTPase activity	membrane, vesicle
GTP-binding protein SAR1a	IPI00717465	19.7	4	1.16	1.15	*2,5*	ER-Golgi transport, protein transport, transport	GTP binding	Golgi apparatus
GTP-binding protein SAR1b	IPI00716201	13.6	4	1.08	1.00	*1,3*	ER-Golgi transport, protein transport, transport	GTP binding, metal ion binding	endoplasmic reticulum, Golgi apparatus, membrane
GTP-binding regulatory protein Gi alpha-2 chain	IPI00718065	28.8	1	1.27	0.98	*1*			
Guanine nucleotide binding protein	IPI00825112	14.2	3	0.96	1.02	*5*	G-protein coupled receptor protein signalling pathway, protein ADP-ribosylation	GTP binding, signal transducer activity	

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	IPI00706869	23.2	2	1.13	1.09	*5*	G-protein coupled receptor protein signalling pathway	GTP binding, signal transducer activity	
Guanine nucleotide binding protein, alpha transducing 3	IPI00686836	5.4	3	1.08	0.98		G-protein signalling, coupled to cAMP nucleotide second messenger, detection of chemical stimulus involved in sensory perception of bitter taste, rhodopsin mediated photo transduction, sensory perception of sweet/umami taste	transducer	cytoplasm
Guanine nucleotide-binding protein alpha-11 subunit	IPI00704832	10.8	2	0.85	1.06	*1,3*	G-protein signalling, coupled to cAMP nucleotide second messenger, activation of phospholipase C activity by dopamine receptor signalling pathway, protein ADP-ribosylation, regulation of action potential	transducer	membrane
Guanine nucleotide-binding protein G(i), alpha-1 subunit	IPI00716567	16.6	1	1.06	0.96		inhibition of adenylate cyclase activity by G-protein signalling pathway	transducer	membrane
Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12	IPI00690761	15.3	1	1.13	0.99		G-protein coupled receptor protein signalling pathway	transducer	membrane
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	IPI00703160	7.4	5	1.13	1.06	*1,3,5*	sensory perception of taste, signal transduction, cell proliferation	signal transducer activity	heterotrimeric G-protein complex
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	IPI00712819	12.9	3	1.15	1.08	*1,2,3,4,5*	GTP catabolic process, signal transduction	transducer, GTPase activity	cytoplasm

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
Guanine nucleotide-binding protein G(o) subunit alpha 1	IPI00716843	6.8	3	1.05	1.05	*2,5*	G-protein signalling, coupled to cAMP nucleotide second messenger, dopamine receptor signalling pathway	transducer	heterotrimeric G-protein complex, plasma membrane
Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	IPI00695917	10.9	3	1.08	1.08	*1,3,5*	activation of adenylate cyclase activity by dopamine receptor signalling pathway, sensory perception of chemical stimulus	transducer	membrane
Guanine nucleotide-binding protein subunit alpha-13	IPI00702031	9.7	1				Rho protein signal transduction, activation of adenylate cyclase activity by G-protein signalling pathway, activation of phospholipase D activity, cellular component movement, platelet activation	transducer	membrane
Haptoglobin	IPI00705491	14.5	4	0.54	0.53	*5*	proteolysis	Serine protease homolog	secreted
Heat shock 70kDa protein 1B	IPI00686726	4.8	1	1.18	1.11	*5*	stress response	chaperone	
Heat shock cognate 71 kDa protein	IPI00708526	10.2	7	1.18	1.11	*2,3,4,5*	stress response	chaperone	cytoplasm
Heat shock protein HSP 90-alpha	IPI00699622	7.6	3	0.98	0.99	*1,5*	stress response	chaperone	cytoplasm
Heat shock protein HSP 90-beta	IPI00709435	5.4	1	1.00	0.97	*4,5*	stress response	Chaperone	cytoplasm
Histone H1.1	IPI00699808	25.8	2	0.84	0.75		nucleosome assembly	DNA binding	chromosome, nucleus
Histone H1.5	IPI00703727	14.6	1		0.67		nucleosome assembly	DNA binding	chromosome, nucleus
Histone H2A.J	IPI00708769	20.9	1		0.78		nucleosome assembly	DNA binding	chromosome, nucleosome core, nucleus
Histone H2B type 1-N	IPI00689632	20.6	7	0.83	0.99	*4*	nucleosome assembly	DNA binding	chromosome, nucleus
Histone H3.3	IPI00713695	19.9	2	0.80	0.85	*4*	nucleosome assembly	DNA binding	chromosome, nucleus
Histone H4	IPI00686060	40.8	1	0.84	0.95	*5*	nucleosome assembly	DNA binding	chromosome, nucleus
Immunoglobulin	IPI00691861	22.6	1		0.69	*5*	complement regulatory protein immune response		
Immunoglobulin heavy constant mu	IPI00718725	12.9	1	1.07	1.08	*1,5*	immune response		

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
Immunoglobulin light chain, lambda gene cluster	IPI00867205	25.2	1		1.07		immune response		
Immunoglobulin like	IPI00852509	11.5	5	1.06	1.09	*5*	immune response		
Kappa-casein	IPI00707811	10.0	3	1.11	1.00	*1,5*		milk protein	secreted
Lactadherin	IPI00689035	47.1	23	1.24	1.12	*1,2,3,4,5,6*	angiogenesis, cell adhesion, fertilization	phosphatidylserine binding, integrin binding, phosphatidylethanolamine binding	membrane, secreted
Lactoperoxidase	IPI00716157	8.0	8	0.92	1.00	*5,6*	defense response to bacterium, hydrogen peroxide catabolic process, oxidation-reduction process	antibiotic, antimicrobial, oxidoreductase, peroxidase	secreted
Lactophorin	IPI00716366	47.7	10	1.30	0.98	*1,4,5*			membrane
Lactotransferrin	IPI00710664	50.3	34	0.93	0.97	*3,4,5*	cellular iron ion homeostasis, defense response to bacterium, iron ion transport	antibiotic, antimicrobial, hydrolase, protease, serine protease	secreted
Lanosterol synthase	IPI00688804	16.1	10	1.04	1.02	*5*	lipid synthesis, steroid biosynthesis	Isomerase	ER membrane
Leucine rich repeat containing 8 family	IPI00686591	2.3	2				pre-B cell differentiation		
Leucine-rich repeat-containing protein 8C	IPI00685147	4.5	1	1.02	1.04	*1*			endoplasmic reticulum, membrane
Lipoprotein lipase	IPI00692291	10.0	3	1.31	1.08	*5*	lipid degradation	hydrolase	chylomicron, membrane, secreted, VLDL
Long-chain-fatty-acid--CoA ligase 3	IPI00702446	3.3	4	1.05	1.08	*5*	fatty acid metabolism, lipid metabolism	catalytic activity	
Mannose-6-phosphate receptor binding protein 1	IPI00700098	15.1	5	0.91	0.92	*5*		receptor	cytoplasm, membrane
Membrane cofactor protein / CD46	IPI00760397	11.7	3	0.91	0.94	*5*	complement pathway, fertilization, immunity, innate immunity		cytoplasmic vesicle, membrane
Methyltransferase-like protein 9	IPI00689143	5.7	2	0.91	0.97				
Mucin 16	IPI00695308	7.3	3	1.07	1.07	*5*	cell adhesion	protein binding	membrane, secreted

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
Mucin-15	IPI00716220	12.4	6	1.03	1.04	*1,5*			membrane, secreted
Na+/glucose cotransporter	IPI00717216	4.5	2	1.02	0.96	*1,5*	sodium/glucose transport	transporter activity	membrane
NAD(P) dependent steroid dehydrogenase	IPI00716133	7.3	5	1.02	1.03	*3,5*	cholesterol biosynthesis, sterol biosynthesis	oxidoreductase	membrane
NADH-cytochrome b5 reductase 3	IPI00868665	14.6	5	1.10	1.00	*1,5*	cholesterol biosynthesis, sterol biosynthesis	cytochrome-b5 reductase activity	cytoplasm, endoplasmic reticulum, membrane, mitochondrion
Neutrophil Gelatinase-Associated Lipocalin	IPI00685784	15.5	1	1.10	1.06	*5*	transport	binding, transporter activity	
Nucleotidase	IPI00698673	14.8	7	1.09	1.10	*1,3,5*	nucleotide catabolic process, negative regulation of inflammatory response	hydrolase	membrane
Olfactomedin 4	IPI00732608	5.2	2				cell adhesion		secreted
PDZ domain-containing protein	IPI00717723	7.3	2	0.91	0.96				cytoplasm
Peptidoglycan recognition protein	IPI00701640	23.7	3			*4*	immunity, innate immunity	antibiotic, antimicrobial, fungicide	secreted
Peptidyl-prolyl cis-trans isomerase A	IPI00697285	17.7	4	1.34	1.23	*1,3,5,6*	protein folding, regulation of viral genome replication	isomerase, rotamase	cytoplasm
Platelet glycoprotein 4	IPI00710204	10.8	6	1.09	1.06	*1,4,5*	cell adhesion, transport of long chain fatty acid	receptor	membrane
Polymeric immunoglobulin receptor	IPI00696714	27.3	22	1.14	1.08	*2,4,5,6*	transporter of polymeric IgA and IgM		membrane, secreted
PP1201	IPI00697330	4.9	2	1.26	1.19	*1*			membrane
Protein C1orf93 homolog	IPI00714621	10.0	2		0.91		fatty acid biosynthesis, lipid synthesis, prostaglandin biosynthesis	oxidoreductase	cytoplasm
Protein S100-A12	IPI00713229	9.8	1			*4*	inflammatory response	calcium ion binding, drug binding	nucleus, cytoplasm
Protein S100-A8	IPI00693583	34.8	4	0.48	0.82		chemotaxis	antimicrobial	cytoplasm, cytoskeleton, membrane, secreted

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
RAB-11A	IPI00695221	24.5	4	1.04	0.96	*1,3,5*	cell cycle, protein transport, small GTPase mediated signal transduction	GTP binding, GTPase activity	endosome, membrane
RAB-13	IPI00687642	20.2	2	1.09	1.01	*1,3*	protein transport, small GTPase mediated signal transduction	GTP binding	cytoplasmic vesicle membrane, tight junction
RAB-18	IPI00691826	58.7	10	1.07	1.07	*1,2,3,5*	protein transport, regulation of transcription, DNA-dependent, small GTPase mediated signal transduction	ATP/GTP binding, transcription factor binding	plasma membrane
RAB-1A	IPI00829520	26.3	3	1.14	1.04	*1,5*	protein transport, small GTPase mediated signal transduction	GTP binding	Golgi apparatus
RAB-1B	IPI00727050	20.9	2	1.1	1.21	*2,3,5*	protein transport, small GTPase mediated signal transduction	GTP binding	membrane
RAB-25	IPI00710347	17.4	2	1.04	1.09		positive regulation of cell proliferation, protein transport, pseudopodium organization, small GTPase mediated signal transduction	GTP binding	vesicle, membrane
RAB-2A	IPI00695881	12.2	1	1.01	1.02	*1,5*	protein transport, small GTPase mediated signal transduction	GTP binding	
RAB-35	IPI00852464	10.0	4	1.05	1.04		protein transport, small GTPase mediated signal transduction	GTP-binding	mitochondrion, membrane, endocytic vesicle
RAB-3D	IPI00709014	6.8	1	0.85	0.95	*5*	protein transport, small GTPase mediated signal transduction	GTP binding	
RAB-5B	IPI00694198	17.6	2	1.03	1.01		protein transport, small GTPase mediated signal transduction	GTP binding	early endosome membrane melanosome, plasma membrane
RAB-7A	IPI00704752	17.9	2	1.21	1.21	*5*	protein transport, small GTPase mediated signal transduction	GTP binding	late endosome, lysosome, melanosome, phagocytic vesicle
RAB-8A	IPI00718035	12.1	1	1.02	1.02		protein transport, small GTPase mediated signal transduction	GTP-binding	Golgi apparatus, membrane

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
RAP-1A	IPI00703665	21.2	2	1.08	1.01	*1,5*	small GTPase mediated signal transduction	GTP-binding, GTPase activity	membrane
Ras-related C3 botulinum toxin substrate 1	IPI00689229	24.0	2	1.06	1.05		small GTPase mediated signal transduction , cell adhesion	GTP-binding	membrane
Rho-related GTP-binding protein RhoC	IPI00712172	17.6	1	1.09	1.04	*1*	small GTPase mediated signal transduction	GTP-binding	membrane
Rho-related GTP-binding protein RhoF	IPI00695809	14.0	3	1.05	1.09	*1*	small GTPase mediated signal transduction	GTP binding	cytoplasm, cytoskeleton, plasma membrane
Ribonuclease/angiogenin inhibitor 1	IPI00712042	7.7	3	1.07	0.85				cytoplasm
Ribosomal protein S27a	IPI00709961	24.4	4	0.92	1.12	*5*	translation	ribonucleoprotein, ribosomal protein	cytoplasm, nucleus
Saccharopine dehydrogenase	IPI00706311	6.1	2	1.32	1.15	*1*	oxidation-reduction process, amino acid degradation	binding, saccharopine dehydrogenase (NAD+, L-glutamate-forming) activity	mitochondrion
Secretoglobin, family 2A, member 2	IPI00711254	23.9	2					steroid binding	
Secretory carrier-associated membrane protein 2	IPI00867311	7.9	2			*1*	post-Golgi vesicle-mediated transport, protein transport	protein binding	membrane, nucleus
Serotransferrin	IPI00690534	3.6	34			*5*	ion transport, iron transport	ferric iron binding	secreted
Serum albumin	IPI00708398	29.3	1	0.95	0.98	*3,5,6*	cellular response to starvation, hemolysis by symbiont of host erythrocytes, maintenance of mitochondrion location, negative regulation of apoptosis, transport	DNA binding, drug binding, fatty acid binding, metal ion binding, pyridoxal phosphate binding, toxin binding	secreted
Serum amyloid A protein	IPI00694402	31.5	2	1.54	1.31	*1*	Acute phase		HDL
Similar to Acetyl-CoA carboxylase 1	IPI00839110	4.2	2				lipid metabolic process, lipid biosynthesis process	ATP binding, acetyl-CoA carboxylase activity, biotin binding	
Similar to cation-transporting P5-ATPase	IPI00710377	3.3	9	1.10	1.05	*5*	ATP biosynthetic process, cation transport	hydrolase	membrane

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
Similar to ATP-binding cassette transporter C4	IPI00734394	2.0	1	1.13	1.05		transport	ATP-binding, ATPase activity, chloride channel activity,15-hydroxyprostaglandin dehydrogenase (NAD+) activity	membrane
Similar to concentrative Na+-nucleoside cotransporter hCNT3	IPI00720574	12.5	1			*5*			membrane
Similar to Gamma-glutamyltranspeptidase 1	IPI00705565	11.3	8	1.08	1.04	*5*	glutathione biosynthesis	acyltransferase, transferase	membrane
Similar to Immunoglobulin	IPI00693917	28.8	5				immune response		
Similar to Immunoglobulin heavy	IPI00867362	8.0	5				immune response		
Similar to immunoglobulin heavy constant mu	IPI00714264	11.5	1	1.07	1.08		immune response		
Similar to MyD-1 antigen	IPI00695334	5.8	1					SH3 domain binding	membrane
Similar to RAB-2A	IPI00842216	13.1	2	1.06	1.08		ER to Golgi vesicle-mediated transport, protein transport, small GTPase mediated signal transduction	GDP/GTP binding, GTPase activity	membrane, melanosome
Similar to Serum amyloid A protein	IPI00685653	27.7	4	1.58	1.51		acute phase		amyloid, HDL, secreted
Similar to Solute carrier family 15 member 2	IPI00701901	5.6	5				peptide transport, protein transport, symport, transport	peptide: hydrogen symporter activity, protein binding	membrane
Similar to vesicle amine transport protein 1 isoform 1	IPI00689789	6.5	3	1.03	1.03		oxidation-reduction process	oxidoreductase	cytoplasm
Similar to Xanthine dehydrogenase/oxidase	IPI00843038	32.3	4	0.86	0.98		oxidation-reduction process, xanthine catabolic process, lipid droplets secretion	oxidoreductase	peroxisome, membrane
Sodium-dependent phosphate transport protein 2B	IPI00703813	11.1	13	0.93	0.93	*1,4,5*	ion transport, sodium transport, symport, transport	phosphate binding, sodium ion binding, sodium-dependent phosphate transmembrane transporter activity	membrane

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
Solute carrier family 28 member 3	IPI00696906	6.6	8	1.11	1.11	*5*	nucleobase, nucleoside and nucleotide metabolic process	nucleoside binding	membrane
Solute carrier family 5 member 1	IPI00701822	5.30	4	1.22	1.10		ion/sodium transport	transporter activity	membrane
STOM protein	IPI00710834	35.2	10	1.05	1.01	*1*			membrane
Synaptobrevin homolog YKT6	IPI00708611	14.1	3	0.92	0.94	*1*	ER to Golgi vesicle-mediated transport, protein transport, vesicle docking involved in exocytosis, vesicle targeting	protein-cysteine S-palmitoleyltransferase activity	cytoplasm, cytoplasmic vesicle, Golgi apparatus, membrane
Synaptosomal-associated protein, 23kDa	IPI00718671	30.8	5	1.03	1.05	*1,5*			synaptosome
Syntaxin 3	IPI00867107	17.3	5	1.00	1.03	*5*	intracellular protein transport, neurotransmitter transport	SNAP receptor activity	membrane, cell junction
Syntaxin binding protein 2	IPI00734153	10.8	3	0.91	0.90		neutrophil degranulation, vesicle docking involved in exocytosis	syntaxin-3 binding	cytosol
Toll-like receptor 2	IPI00694401	5.6	4	1.09	1.15	*1,5*	immunity, inflammatory response, innate immunity	receptor	membrane
Type II cGMP-dependent protein kinase	IPI00714992	11.4	7	1.10	1.10	*5*	protein phosphorylation	kinase, serine/threonine-protein kinase, transferase,	
Tyrosine-protein kinase	IPI00714515	9.1	5	1.21	1.02		protein phosphorylation	kinase, transferase, tyrosine-protein kinase	
Tyrosine-protein kinase Lyn	IPI00686890	3.5	2	1.12	1.13	*5*	Host-virus interaction	kinase, transferase, tyrosine-protein kinase	cytoplasm, Golgi apparatus, membrane, nucleus
Tyrosine-protein phosphatase	IPI00708771	3.8	2			*5*		SH3 domain binding	membrane
Uromodulin	IPI00688780	1.6	2			*1*	excretion, tissue development, chemical homeostasis	calcium ion binding	cell projection, membrane ,secreted
Vimentin	IPI00689228	5.8	2	0.53	0.73	*4*	lens fibre cell development ,intermediate filament-based process	structural constituent of cytoskeleton	Intermediate filament

Table S2.1 Continued.

Protein name	Accession Number	Protein coverage %	Unique peptides identified	Ratio of 3 replicates	Ratio of 5 replicates	reference	GO Biological process	GO Molecular Function	GO cellular component
Xanthine dehydrogenase/ oxidase	IPI00695367	35.0	4	1.20	1.09	*1,2,3,4,5,6*	oxidation-reduction process, xanthine catabolic process, lipid droplets secretion	oxidoreductase	peroxisome, membrane
XRP2 protein	IPI00702677	8.1	2		1.02		CTP/GTP/UTP biosynthetic process	ATP binding, nucleoside diphosphate kinase activity	cytoplasm, membrane

Reference

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Chapter 3

Identification of milk synthesis and secretion related proteins in bovine milk using a proteomic approach

This chapter is submitted as:
Identification of lipid synthesis and secretion proteins in bovine milk
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Abstract

Lactation physiology is a process that is still partly understood. Proteomics techniques have been shown to be useful to help advance the knowledge on lactation physiology in human and rodent species but have not been used for dairy cows, except for mastitis. In this paper, advanced proteomics techniques (Filter aided sample preparation and nanoLC-Orbitrap-MS/MS) were applied to study the milk fat globule membrane and milk serum fraction, resulting in the identification of 246 proteins. The function of the proteins is discussed in detail. Sixty three proteins were related to milk synthesis and secretion. Within this group, the membrane trafficking proteins and lipid synthesis and secretion proteins are the most prominent proteins. The high number of milk synthesis and secretion proteins present in milk indicates that it is possible to study milk synthesis and secretion by using milk.

3.1 Introduction

Milk provides both nutrients and protection to neonates. It is also a food product consumed daily by many people. It supplies energy, essential amino acids, fatty acids and some key minerals and vitamins (German et al., 2002). During the last few decades, the yield and quality of bovine milk were improved due to the increased knowledge about the physiology and biology of lactation (Bauman et al., 2006). Nevertheless, the molecular mechanisms of synthesis and secretion of major milk components are not fully clear yet, and thus need further investigation (Neville, 2006).

Proteomics techniques were shown to be useful to gain knowledge on lactation physiology and biology (Reinhardt and Lippolis, 2008). Proteomics allows for the detection, identification and characterization of proteins involved in milk synthesis and secretion. This proteomics approach has been used to study rodent and human mammary gland and lactation (Aksu et al., 2002, Davies et al., 2006, Hadsell et al., 2011, Jacobs et al., 2004, Kim et al., 2008, Wu et al., 2000). Their results have advanced our knowledge of mammary gland function and milk synthesis and secretion, but may not fit the unique aspects of milk secretion in dairy cows (Reinhardt and Lippolis, 2006). For dairy cows, research on lactation and physiology was mainly done by using functional genomics and transcriptomics (Bionaz and Loor, 2007, 2008, Suchyta et al., 2004). In only one study proteomics was used as the primary tool to profile the metabolic proteome of bovine mammary tissue (Beddek et al., 2008). Several proteomic studies on mammary glands were related to mastitis (Boehmer et al., 2010, Ibeagha-Awemu et al., 2010, Yang et al., 2009). Lactation physiology in dairy cows based on proteomics information has hitherto limited as compared to other mammals. More recently, proteomic techniques have also been used to analyze different bovine milk fractions such as milk fat globule membrane (MFGM), buttermilk and whey (Affolter et al., 2010, Reinhardt and Lippolis, 2006, 2008, Smolenski et al., 2007). Especially in the MFGM fraction, some proteins involved in milk synthesis and secretion were detected. This can be explained by the fact that the MFGM proteins are thought to originate from the epithelial cells (apical plasma membrane, endoplasmic membrane and cytoplasm) of the mammary gland (Neville, 2006). Many proteins involved in synthesis and secretion may thus be secreted in milk as part of the MFGM fraction. This offers the possibility to use milk to obtain knowledge about milk synthesis and secretion. Unfortunately, these milk proteomic studies provide only an incomplete picture of the relation between these identified proteins and synthesis and secretion pathways, as either only one pathway or the functions of a few selected proteins were discussed. D'Alessando et al. (2011) reported 573 proteins in bovine milk and analyzed their biological functions.

However, these 573 proteins are combined from around 40 different proteomics analyses and also the milk synthesis and secretion pathways were not focused. So far, there is no comprehensive discussion about milk synthesis and secretion proteins in milk. Furthermore, the number of the proteins identified in bovine milk was limited because of limitations in techniques used. With recent improvements in proteomics techniques, it is now possible to obtain a much more refined picture in one proteomics analysis.

In this paper, the bovine MFGM and milk serum were studied by using advanced proteomics techniques (FASP-nanoLC-Orbitrap-MS/MS). A large number of proteins were detected related to milk synthesis and secretion and their functions in metabolic pathways will be discussed in detail. Bioinformatics analysis was performed to assist the understanding of the relation between these proteins and milk synthesis and secretion pathways. This paper presents a starting point for further investigation in bovine milk synthesis and secretion by using milk through proteomics techniques.

3.2 Materials and methods

All methods, except milk serum separation and GO annotation, are as previously described by Lu et al. (2011).

3.2.1 Milk sample preparation

Pooled milk samples of 65 clinically healthy Holstein cows were collected at Wageningen University farm. The MFGM preparation was performed as described before. The skim milk obtained was subsequently ultra-centrifuged at 100,000 g for 90 min at 30°C. The supernatant was collected as milk serum and the protein concentration was determined by using the BCA assay (Smith et al., 1985).

3.2.2 Filter-aided sample preparation (FASP)

The method used to prepare samples for LC/MSMS analysis was based on FASP as described by Wiśniewski et al. (Wiśniewski et al., 2009) with some modifications as shown in the paper of Lu et al. (2011)

3.2.3 NanoLC-Orbitrap-MS/MS

For each sample, 18 ul digested peptides were used for nanoLC- Orbitrap-MS/MS analysis.

3.2.4 Data Analysis

Identification. Each run with all MS/MS spectra obtained was analyzed with Bioworks 3.3.1. A combined fasta database was constructed from the bovine IPI database (downloaded from ftp.ebi.ac.uk/pub/databases/IPI/current in August 2009) plus a set of 31 protein sequences of common contaminants.

GO Annotation. The gene ontology (GO) analysis of identified proteins was performed by using DAVID bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009b). GOTERM_BP_FAT, GOTERM_MF_FAT and GOTERM_CC_FAT were applied as annotation category. Functional annotation clustering was performed with medium classification stringency. The GO clusters/terms shown here were significantly enriched compared to the complete bovine genome. Significance was defined as $p<0.05$ where the Fisher Exact test was adopted to measure the gene-enrichment in annotation terms.

3.3 Results

All the experiments were done in duplicates. In total, 247 proteins (94% overlap of proteins in duplicates) with at least two peptides per protein were identified in the bovine milk fat globule membrane and the serum fractions. Each protein contains at least one unique peptide in order to avoid redundant identifications. Forty-two proteins present in both MFGM and serum fraction. MFGM contains 127 unique proteins, as serum contains 78 unique proteins. The major proteins in serum and MFGM were all clearly observed (β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulin, xanthine oxidase, butyrophilin, adipophilin and lactadherin). Besides these major proteins, hundreds of other less abundant proteins were also identified. The function of proteins was categorized according to their gene ontology from Swiss-uniprot database (www.uniprot.org) as well as from published information. Sixty three out of 247 proteins were considered to be directly or indirectly related to milk components synthesis and secretion (Figure 3.1). Each category was further divided according to specific function in synthesis and secretion pathways (Tables 3.1 to 3.6). A large number of proteins (21) was directly associated with synthesis and secretion of lipids in epithelial cells. Twenty-three proteins were associated with membrane/protein trafficking. Protein transporters for other milk components (minerals, vitamins) and also protein related to protein and lactose synthesis and secretion were detected.

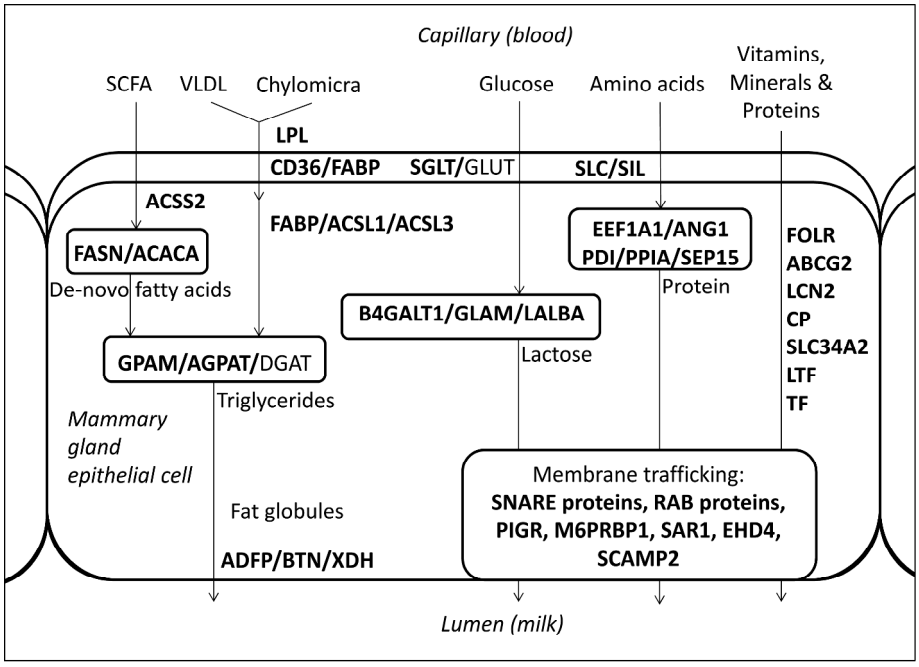
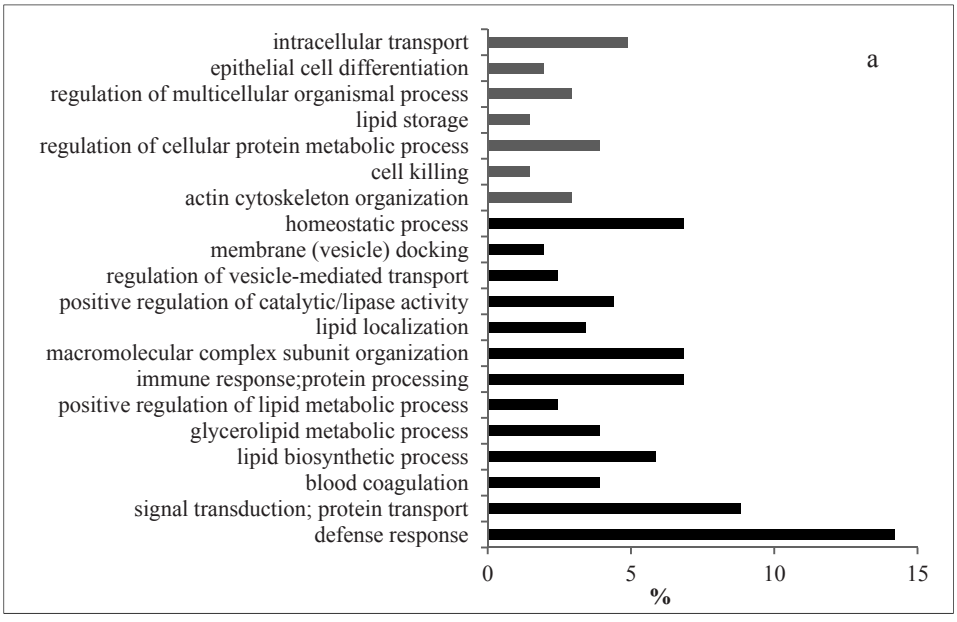


Figure 3.1. Schematic overview of identified proteins involved in milk synthesis and secretion pathways. Proteins identified in this study are shown in bold.



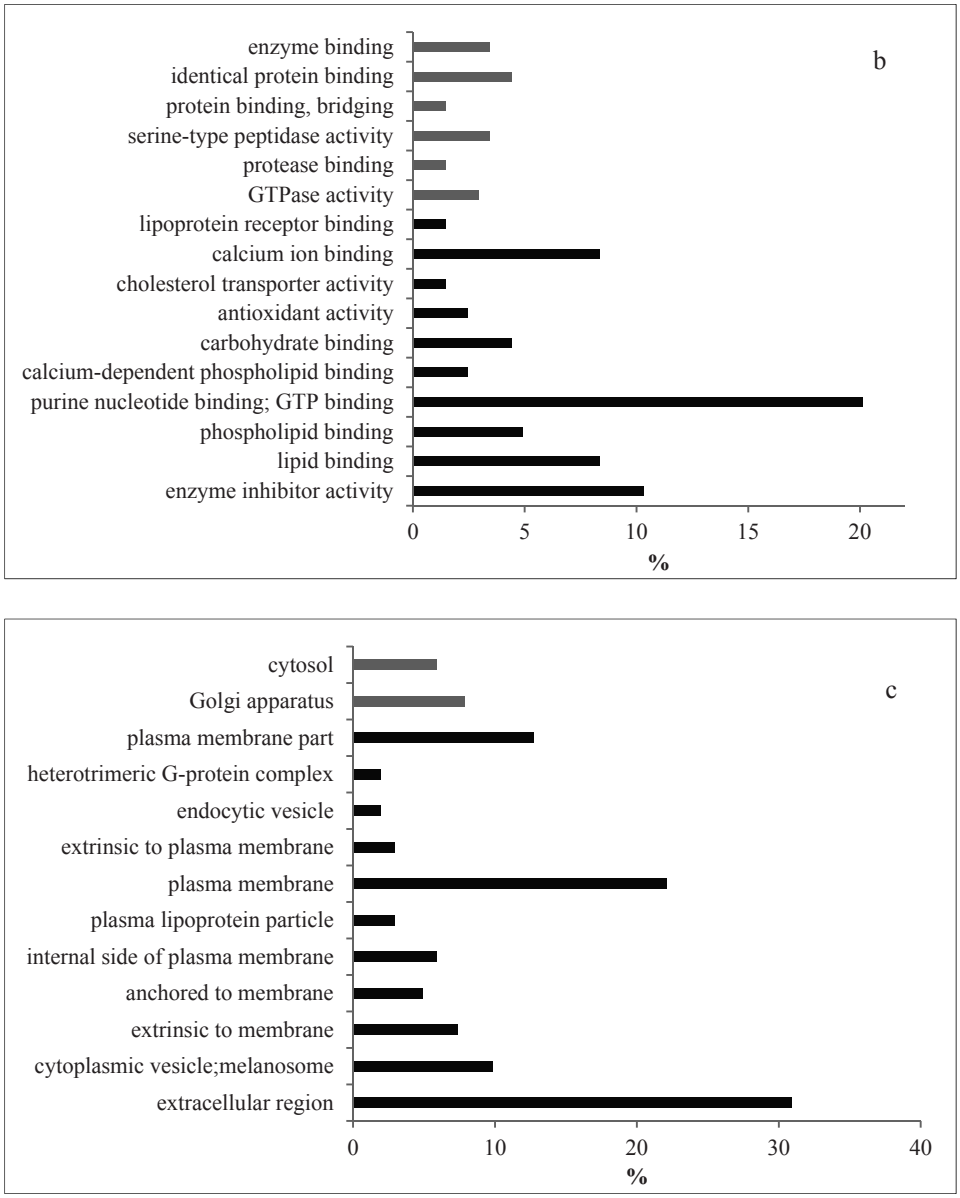


Figure 3.2. GO enrichment analysis by using DAVID bioinformatics resources 6.7 a. GO biological process enrichment. b. GO molecular function enrichment. c. GO cellular component enrichment. Significant terms with $0.01<P<0.05$ are light colour. The order of GO terms in y-axis depends on the descending p-value. X-axis: percentage of all identified proteins. Y-axis: GO term

Besides the manually derived functional annotation, a GO enrichment analysis was also done by using DAVID bioinformatics resources (Huang et al., 2009a, b) for biological process, molecular function and cellular components as shown in Figure 3.2. This analysis helps to understand the function of proteins and addresses them into different biological pathways as discussed below in detail. The GO terms shown in Figure 3.2 are significantly enriched in milk as compared to the whole bovine genome, which means that a relatively larger part of proteins was involved in these enriched GO groups than in the whole bovine genome. Twenty GO biological process terms, in which the identified proteins are involved, were enriched. The most enriched GO process is "defense response", implicating the importance that milk helps to protect calves as well as cows from infection. Half of these GO terms can be considered as being associated with milk synthesis and its secretion process (protein transport, lipid biosynthetic process, lipid metabolic process, protein processing, macromolecular complex subunit organization, lipid localization, positive regulation of lipase activity, regulation of vesicle mediated transport, membrane docking, regulation of cellular protein metabolic process, lipid storage and intracellular transport). For molecular function of identified proteins, the GO terms with binding functions of different substrates, such as protein, lipid and calcium, were highly enriched in milk. These substrate binding proteins are probably involved in transport and secretion of milk components. GO cellular component enrichment analysis infers the subcellular location of identified proteins. A large number of proteins are originating from the extracellular region, which can be considered as secretion from the epithelial cell to milk. The second largest group of proteins are originating from the plasma membrane, which is the primary source of MFGM (Heid and Keenan, 2005). Also the proteins from intracellular organelles (such as Golgi apparatus and cytosol) were enriched, which is consistent with the origin of MFGM as discussed in introduction (section 3.1).

3.4 Discussion

The number of proteins identified in this study is relatively large compared to the number in previous reports on the bovine proteome (Reinhardt & Lippolis, 2006, D'Amato et al., 2009). The higher number of low abundant proteins identified enhance the opportunity for revealing the crucial proteins which can affect milk synthesis and secretion. To our knowledge, the largest bovine milk proteome identified 269 proteins (Hettinga et al., 2011). The FASP preparation method used here resulted in a comparable number of proteins.

3.4.1 Lipid Synthesis and Secretion

As shown in Table 3.1, 21 proteins identified in milk have a function in lipid synthesis and secretion. These proteins are involved in different steps of lipid synthesis and secretion. In Fig 3.2a, the categories of lipid biosynthetic process, glycerolipid metabolic process, positive regulation of lipid metabolic process, lipid localization and lipid storage confirm the manually annotated function of proteins from Table 3.1. Most of the proteins in the category of lipid synthesis and secretion were detected in the MFGM fraction, which is a 3-layer membrane covering the milk fat globule. The MFGM is originating from the outer layer of the ER membrane and a double layer of apical membrane from the epithelial cell. During the milk fat globule budding process from the secretory cell, some cytoplasm is trapped between these two membrane layers. Proteins originating from cytoplasm are therefore also detected in the MFGM fraction. Since the compartments (ER, cytoplasm and apical plasma membrane) contributing to MFGM are the places where the milk fat globules are synthesized and secreted (McManaman and Neville, 2003), it is not surprising to find lipid synthesis and secretion related proteins in this protein fraction.

Bionaz et al. (2008) studied 45 genes associated with lipid synthesis and secretion in biopsies of bovine mammary gland. Thirteen protein products from these genes were observed by us in milk (LPL, CD36, ABCG2, ACSS2, ACSL1, FABP3, ACACA, FASN, ADFP, BTN1A1, XDH, GPAM, and AGPAT6). The absence of the other gene products, not identified in our results, is probably due to their relative low abundance or no translation. The mRNA amounts of these unidentified proteins are below 0.3% of the total mRNA amount. Even though the ratio between mRNA and proteins is hardly 1:1 (Bionaz and Loor, 2008), the mRNA amount will probably reflect to a certain extent the relative abundance of the corresponding protein products. All proteins with an amount above 0.3% of total mRNA, as calculated in the paper of Bionaz et al. (2008), were detected by us except stearoyl-CoA desaturase (SCD), possibly, because SCD is unstable and could have been degraded (Heinemann et al., 2003). Compared to Bionaz's study, a large group of protein products, not observed by us, are proteins that regulate the transcription of lipid synthesis proteins. The reason that these proteins were not detected probably is, besides their relative low abundance, the fact that these proteins function in the nucleus, which does not contribute to the MFGM fraction (McManaman and Neville, 2003). Another group of proteins which were not observed, as compared to Bionaz's study, are the sphingolipid synthesis proteins. The lower abundance of these proteins can possibly be explained by the much lower amount of sphingolipids, compared to triacylglycerols (TAG), in milk. Thus, the proteins required for sphingolipid synthesis are expected to have a relative lower concentration than those for TAG. Besides the 13 proteins, 9 additional proteins which may

have functions related to lipid synthesis and secretion were also identified. These proteins were mainly involved in cholesterol synthesis and transport. The presence of these extra 9 proteins in milk may reflect their importance during milk fat synthesis and secretion. It is therefore recommended to also include the corresponding genes of these proteins when studying milk fat synthesis and secretion when using transcriptomics analysis.

Fatty acid import into cells and intracellular transport. Fatty acids which form milk lipids (triglycerides, diglycerides, monoglycerides and phospholipids) are considered to originate from two sources. The short and medium-chain-fatty-acids (SCFA and MCFA) are synthesized *de novo* in the udder (Popjak et al., 1951), whereas the majority of the long-chain-fatty-acids (LCFA) are directly derived from the blood (Palmquist, 2006).

Lipoprotein lipase (LPL, Table 3.1), which hydrolyzes the TAG from chylomicron and VLDL in plasma to release the fatty acids for further utilization by mammary gland, were detected in both MFGM and serum. The LPL in bovine milk is assumed to originate from the leakage of epithelial cells (Jensen and Pitas, 1976). Bionaz et al. (2008) indicated that the bovine mammary LPL expression pattern is similar to the lactation curve. They therefore hypothesized that LPL has an important role in the maintenance of milk synthesis.

The up-take of LCFA by epithelial cells for utilization in the mammary gland still has to be elucidated. Two mechanisms have been considered: passive diffusion (flip-flop) and protein-mediated uptake. Both of the mechanisms are likely to occur, but protein-mediated uptake is thought to play a dominant role (Doege and Stah, 2006). Proteins such as CD 36, ACSLs, FABP and FAPTs are involved in the protein-mediated uptake (Schwenk et al., 2010). These proteins will regulate the fatty acid transport by absorbing FA from blood to membrane and segregating/organizing fatty acid for metabolism (Doege and Stah, 2006). In our results, we observed CD36, FABP, ACSL1 and ACSL3 but we did not detect FAPTs (Table 3.1). Similar to previous reports on the bovine milk proteome, the FAPTs could not be identified. This is probably related to their low abundance. The mRNA abundance of FAPTs was reported to be 150 times less than FABP (Loor and Bionaz, 2008). In one of the proposed models for FA transport, CD36 and FABP either collaborate or act alone to help LCFA transport. Then ACSLs rapidly esterified LCFA to be utilized further (Schwenk et al., 2010). Acetyl-CoA synthetase 2 (ACSS2, Table 3.1) which activates SCFA was also detected in MFGM. Although the exact mechanism of FA transport from blood to cell is still under investigation, the role of these transporters is considered important (Glatz et al., 2010). Besides being involved in trans-membrane transport, FABP also transports FA intracellular. FABP binds FAs and transports them to different cellular compartments.

FABP also functions as inhibitor of cell division and stimulates cell differentiation in the mammary gland (Mather, 2000).

Table 3.1. 21 proteins related to lipid synthesis and secretion. The proteins were grouped according to their function in lipid synthesis and secretion.

Protein name	Gene name	IPI number	Source[1]
FA transport			
Lipoprotein lipase	LPL	IPI00692291	M, S
Platelet glycoprotein 4	CD36	IPI00710204	M,S
Acetyl-CoA synthetase 2	ACSS2	IPI00717515	M
Long-chain-fatty-acid--CoA ligase 3	ACSL3	IPI00702446	M
Acyl-CoA synthetase long-chain family member 1	ACSL1	IPI00729755	M
Fatty acid-binding protein	FABP	IPI00691946	M,S
FA synthesis and desaturation			
Acetyl-CoA carboxylase 1	ACACA	IPI00712907	M
Fatty acid synthase	FASN	IPI00712133	M
NADH-cytochrome b5 reductase 3	CYB5R3	IPI00868665	M
Lipid/TAG synthesis			
Glycerol-3-phosphate acyltransferase, mitochondrial	GPAM	IPI00703084	M
Glycerol-3-phosphate acyltransferase 4	AGPAT6	IPI00697212	M
Lipid droplet formation and secretion			
Adipophilin	ADFP	IPI00716572	M
Butyrophilin	BTN	IPI00708535	M,S
Xanthine dehydrogenase/oxidase	XDH	IPI00695367	M,S
Lactadherin	MFGE8	IPI00689035	M,S
Cholesterol transport			
Apolipoprotein E	APOE	IPI00712693	M,S
Epididymal secretory protein E1 precursor	NPC2	IPI00711862	S
Apolipoprotein A-IV precursor	APOA4	IPI00695965	S
Apolipoprotein A-I	APOA1	IPI00715548	S
Cholesterol synthesis			
Lanosterol synthase	LSS	IPI00688804	M
NAD(P) dependent steroid dehydrogenase-like protein	NSDHL	IPI00716133	M

[1]S: milk serum; M: MFGM

Fatty acid synthesis and desaturation. As discussed above, SCFA and MCFA in milk fat are synthesized *de novo*. Several proteins that are involved in this process were identified. Two essential enzymes Acetyl-CoA carboxylase (ACACA, Table 3.1) and fatty acid synthase (FASN, Table 3.1) were found in the MFGM fraction. ACACA catalyzes the first committed step in fatty acid synthesis: from the acetate carbon source to malonyl-CoA (Palmquist, 2006). Subsequently, acetyl-CoA, malonyl-CoA and butyryl-CoA will be used

by FASN to synthesize FA. The main product of FASN is palmitate but in ruminants it also produces SCFA and MCFA (Bionaz and Loor, 2008). FASN has recently been demonstrated to have a close interaction with lipid raft domains which involves the membrane protein caveolin-1 (Di Vizio et al., 2008). This may explain the presence of FASN in MFGM (Cebo et al., 2010). Moriya et al (2011) also suggested that in mouse, ACACA and FASN were localized in the ER membrane and the cytoplasmic MFG precursor surface, and therefore secreted together with MFG to milk to increase the speed and efficiency of triglycerides synthesis.

NAPH-cytochrome b5 reductase (CYB5R3, Table 3.1) is a component of the FA-desaturase system. CYB5R3 functions as the electron transporter for the desaturase enzymes in the process of formation of double bonds in fatty acids. CYB5R3 is located on the ER membrane (Ollier et al., 2008). It could therefore be secreted in milk through the ER membrane.

Lipid synthesis. Glycerol-3-phosphate acyltransferase, mitochondrial (GPAM, Table 3.1) is observed to be present in the bovine MFGM fraction. GPAM catalyzes the first step in the *de novo* synthesis of neutral lipids (triglycerides) and glycerophospholipids. It catalyzes the acylation of glycerol-3-phosphate (GP) to 1-acyl-sn-glycerol-3 phosphate (AGP). The localization of GPAM is in the outer layer of the mitochondrial membrane (Coleman and Lee, 2004). The reason why it is secreted in MFGM is still unknown.

After the activation of GP, the product AGP will be catalyzed by acylglycerolphosphate acyltransferase (AGPAT) to synthesize 1,2-diacyl-sn-glycero-3-phosphate (DGP), which is the key intermediate for glycerolipid synthesis. There are six main AGPAT isoforms: AGPAT 1 to 6. AGPAT6 (Table 3.1) is the major isoform in bovine mammary tissue (Loor and Bionaz, 2008). AGPAT6 is exclusively expressed in the ER membrane. Beigneux et al. (2006) observed that AGPAT6 -/- mice had a dramatic decrease in the size and number of lipid droplets within the mammary epithelial cells and ducts. The milk was also markedly depleted in diacylglycerol and triacylglycerol. These observations indicate the critical role of AGPAT6 during the synthesis of triacylglycerol. The ER membrane localization of AGPAT6 and its crucial role during milk lipid synthesis probably explain its presence in MFGM. There are more enzymes involved in triacylglycerol biosynthesis, but either due to their low-abundance or absence in MFGM, they were not detected in this study.

Lipid droplet formation and secretion. Adipophilin, butyrophilin and xanthine dehydrogenase/oxidase (ADFP, BTN and XDH, Table 3.1) are the three most abundant proteins in MFGM. These three proteins are suggested to collaborate in a tripartite structure

to secret lipid droplets to milk (McManaman et al., 2007). Although, other mechanisms or models for lipid droplets secretion have also been proposed (Heid and Keenan, 2005), the importance of ADFP, BTN and XDH for their secretion is beyond doubt. Mather and Keenan (1998) stated that lactadherin (MFGE8, Table 3.1) could also play a role in MFG secretion. Though many models of milk lipid secretion have been raised and some of its elements being confirmed, the exact proteins and regulation factors are still ambiguous (Heid and Keenan, 2005).

Cholesterol transport. Transporters and enzymes for cholesterol synthesis and secretion were identified in milk too. Three apolipoproteins were found in milk (APOE, APOA1and APOA4, Table 3.1). APOE was found in both the MFGM and serum fraction, APOA1 and APOA4 were only identified in milk serum. Apolipoproteins bind lipids to form lipoproteins, which transport lipid/cholesterol in the bloodstream. APOE also distributes cholesterol into different cells. Apolipoproteins are also related to lipid metabolism by interacting with lipoprotein receptors (Mahley, 1988). These proteins could be secreted from blood to milk by transcytotic pathways in mammary epithelial cells (Monks et al., 2001). To our knowledge, there is no research done on the function of apolipoproteins in milk secretion, but based on the general function of these proteins in other tissues, they may play a role in milk lipid/cholesterol transport in the mammary gland.

Epididymal secretory protein E1 (NPC2, Table 3.1) was previously found in a high concentration in milk serum (Larsen et al., 1997). Why this protein is secreted into milk, and what its function is in milk is not yet known. NPC2 is a liposomal glycoprotein and binds cholesterol with sub micromole affinity (Stock et al., 2007). It is expressed in kidney, spleen, liver and mammary gland. Intracellular, NPC2 transports cholesterol out of lysosomes, after which, the cholesterol is utilized by different cell compartments (Friedland et al., 2003). Because of its high affinity to cholesterol, NPC2 is probably related to the transport of cholesterol to milk.

Cholesterol synthesis. Two enzymes related to cholesterol biosynthesis were also found in the MFGM fraction: lanosterol synthase (LSS, Table 3.1) and NAD(P) dependent steroid dehydrogenase-like protein (NSDHL, Table 3.1). LSS converts (S)-2,3-oxidosqualene to a protosterol cation and finally to lanosterol (Dean et al., 1967). Lanosterol is a key intermediate in cholesterol biosynthesis (Huff and Telford, 2005). Reinhardt et al (2008) found that LSS is 2.8 fold upregulated on day 7 MFGM compared to colostrum, but no explanation was offered. NSDHL is involved in the conversion of lanosterol into cholesterol. Besides in ER, NSDHL is also found in lipid droplets (Ohashi et al., 2003), which is consistent with our finding that NSDHL is present in MFGM.

3.4.2 Protein Synthesis, Folding and Modification

The milk protein synthesis and secretion is a complicated process. The basic aspects of protein synthesis are the transcription and translation of milk protein genes. This process is thought to be modulated by several different factors including hormonal control, mTOR (mammalian target of rapamycin) signaling regulation, and amino acid and glucose uptake from the blood (Bionaz and Loor, 2011). Due to the limited knowledge about the regulation of protein synthesis, it is still difficult to associate the identified proteins with the milk protein synthesis pathways. The proteins which could possibly function in protein synthesis are discussed here.

Transporters. Cationic amino acid transporter (SLC7A4, Table 3.2) was named as an amino acid transporter, but there are doubts that this is its real function. Wolf et al. (2002) suggested that it is either not an amino acid transporter or that it needs an additional protein to function as a transporter.

Another identified transporter is nucleotide exchange factor SIL1 (Table 3.2) which is a chaperone protein that acts with the SEC60 complex to transport synthesized protein to ER for post-processing. This transport is critical for the biosynthesis of most secretory and membrane proteins (Zimmermann et al., 2006).

Table 3.2. 7 proteins related to synthesis, folding and modification of proteins

Protein name	Gene Name	IPI number	Source[1]
Transport proteins			
Cationic amino acid transporter	SLC7A4	IPI00696048	M
SIL1 Nucleotide exchange factor	SIL1	IPI00703448	S
Protein synthesis			
Elongation factor 1-alpha 1	EEF1A1	IPI00712775	M
Angiogenin-1	ANG1	IPI00710136	S
Protein folding and modification			
Peptidyl-prolyl cis-trans isomerase A	PPIA	IPI00697285	M
Protein disulfide-isomerase	PDI	IPI00709465	S
15 kDa selenoprotein	SEP15	IPI00704729	S

[1]S: milk serum; M: MFGM

Protein translation. Elongation factor1-alpha 1 (EEF1A1, Table 3.2) is a protein involved in the recruitment of aminoacyl-tRNA to ribosomes (Menzies et al., 2009). Its subcellular location in cytoplasm explains its presence in MFGM. Angiogenin-1 (ANG1, Table 3.2) is highly expressed in milk serum. It has multiple biological functions. One of the functions is the abolishment of protein synthesis by catalyzing tRNA (Komolova and Fedorova, 2002).

Also its function of modulating the immune response could be a reason for the presence in milk serum.

Protein folding and modification. Protein disulfide-isomerase (PDI or P4HB, Table 3.2) has an important role during post translational modification of proteins (Wilkinson and Gilbert, 2004). Ghosal et al (1994) found that PDI was associated with intracellular lipid droplets. It was also found to be involved in the triglycerides transport complex protein in liver microsomes (Ghosal et al., 1994, Wetterau et al., 1990). PDI was considered to be involved in intracellular transport and secretion of milk fat globule, but we identified this protein in milk serum not in MFGM. The reason why this protein is present in milk serum needs to be investigated. Ghosal et al. (1994) demonstrated that PDI dissociated from lipid droplets before its release from epithelial cells. Its presence in milk serum could also be due to its loose attachment to MFGM. During separation of MFGM and serum from milk, it may be disassociated from MFGM and end up in the serum fraction.

Two proteins we identified play a role in protein folding, peptidyl-prolyl cis-trans isomerase A (PPIA, Table 3.2) and 15 KDa selenoprotein (SEP15, Table 3.2) (Gladyshev et al., 2001, Stamnes et al., 1992), but their specific role in milk protein folding process has not been established yet.

3.4.3 Lactose Synthesis

Glucose transport. Glucose is a primary source not only for lactose synthesis but also synthesis of other milk components (Bionaz and Loor, 2011). Thus, the absorption of glucose from blood to the mammary gland is important. The glucose absorption mainly involves specific transporters. There are two protein families which are related to the glucose transport system: the facilitative GLUT transporters and Na^+-dependent glucose transporters (Isaji et al., 2005). Two out of four Na^+-dependent glucose transporters were identified in the MFGM fraction: SGLT1and SGLT4 (Table 3.3), but none of the GLUT proteins. Interestingly, the major glucose transporters (GLUT1) in the mammary gland, was never identified in milk. This is probably because its location in basolateral membrane of epithelial cell (Shennan and Peaker, 2000), which does not end up in the MFGM fraction. Instead, SGLT1was also found previously in MFGM (Reinhardt and Lippolis, 2008). The membrane subcellular location of SGLT1 may imply that it is secreted together with the milk fat globule. The function of SGLT4 was suggested by Isaji et al. (2005) to be the transporter for four types of sugars, including mannose, glucose, fructose and 1,5-anhydro-D-glucitol. Since mannose is mainly used for protein glycosylation, the function of SGLT4 could be for both lactose synthesis and protein glycosylation.

Carbohydrate metabolism and lactose synthesis. The proteins in the lactose synthase enzyme complex were all identified in bovine milk. It includes beta-1,4-galactosyltransferase (B4GALT1, Table 3.3) and its regulator α-lactalbumin (LALBA, Table 3.3). Lactose synthase binds galactose and glucose to form lactose in the Golgi apparatus. Synthesized lactose is then secreted together with milk proteins through an exocytotic pathway to milk (McManaman and Neville, 2003). B4GALT1 was only identified in the milk serum fraction but not in MFGM. This is consistent with the results of Keenan et al (Keenan, 1974, Keenan and Huang, 1972). But, Powell et al. (1977) reported that B4GALT1 was also present in bovine MFGM. They argued that the absence of B4GALT1 in MFGM was due to the higher temperature than 4°C they used of the wash procedures which may have released the enzyme from MFGM.

Another protein possibly playing a role in lactose synthesis is aldose 1-epimerase (GLAM, Table 3.3). It is an enzyme involved in galactose metabolism pathway. The two main products in the pathway are UDP-galactose and UDP-glucose (Bosch, 2006). These products can then be utilized as an energy source and as precursors for milk constituents in mammary gland (Scott et al., 1976).

Table 3.3. 5 proteins related to lactose synthesis.

Protein name	Gene name	IPI number	Source[1]
Transport			
Na^+/glucose cotransporter 1	SGLT1	IPI00717216	M
Na^+/glucose cotransporter 4	SGLT4	IPI00701822	M
Lactose synthesis			
Aldose 1-epimerase	GLAM	IPI00712164	M
Beta-1,4-galactosyltransferase 1	B4GALT1	IPI00685910	S
Alpha-lactalbumin	LALBA	IPI00717424	M,S

[1]S: milk serum; M: MFGM

3.4.4 Transporters for Minerals and Vitamins

Besides of proteins, lipids and lactose, milk is also an important source of minerals and vitamins. The minerals and vitamins are transported from blood to milk and specific transport proteins are present for their secretion in milk (McManaman and Neville, 2003). Our result shows transporters for vitamins (folate, riboflavin), ions (iron, copper) and phosphates secretion (Table 3.4).

Vitamin transporters. Folate receptor alpha protein, also called folate-binding protein 1 (FOLR1, Table 3.4), was identified in both the MFGM and the milk serum fraction. Folate is thought to be secreted together with FOLR1 as a complex from mammary gland cells to

milk (Selhub et al., 1984). The binding to FOLR1 protects folate from bacterial degradation and also enhances the bioavailability by promoting the folate absorption in the gut of neonates (Colman et al., 1981, Jones et al., 2003).

ATP-binding cassette, sub-family G (ABCG2, Table 3.4) is a protein from the ATP-binding cassette family (ABC). The ABC proteins transport various substrates including metabolites, lipids and drugs across the cellular membranes by the hydrolysis of ATP (Mani et al., 2011). ABCG2-/- knockout mice had 60 times less riboflavin in milk compared to the wild type. It was therefore proposed that this protein helps the secretion of riboflavin to milk. It has also been suggested that ABCG2 is able to transport other vitamins (Van Herwaarden et al., 2007). Bionaz et al. (2008) found a large scale up-regulation of ABCG2 mRNA during lactation. This seems to imply that ABCG2 has other functions in milk synthesis besides riboflavin secretion.

Table 3.4. 7 proteins involved in transport of minerals, vitamins and phosphate intracellular or secretion

Protein name	Gene name	IPI number	Source[1]
Vitamins transport			
Folate receptor alpha	FOLR1	IPI00708447	M,S
ATP-binding cassette, sub-family G	ABCG2	IPI00690408	M,S
Ions transport			
Lactotransferrin	LTF	IPI00710664	M,S
Neutrophil Gelatinase-Associated Lipocalin	LCN2	IPI00685784	M
Serotransferrin	TF	IPI00690534	M,S
Ceruloplasmin	CP	IPI00703491	S
Phosphate transport			
Sodium-dependent phosphate transport protein 2B	SLC34A2	IPI00703813	M,S

[1]S: milk serum; M: MFGM

Mineral transporters. There are three possible iron transporters found in milk: lactoferrin, transferrin and neutrophil gelatinase-associated lipocalin (LCN2, Table 3.4). These proteins all have a high affinity for irons. But their exact role during iron secretion to milk is still poorly understood. Since lactoferrin is only for 5-10% saturated, it still has capacity to bind free iron during secretion (Lönnerdal, 2007). Transferrin has been shown to transport 6% of the iron from plasma to milk in mice (Ollivier-Bousquet, 1998, Sanchez et al., 1992).

Ceruloplasmin, a protein with copper binding capacity, was also detected in milk (Table 3.4). During milk secretion, it is thought that part of the copper in the mammary gland epithelial cells is incorporated together with ceruloplasmin and secreted to milk. Donley found that ceruloplasmin binds 25% of all copper in rat milk (Donley et al., 2002,

Lönnerdal, 2007). The copper bound to ceruloplasmin is easily absorbed by neonates (Donley et al., 2002, Tsymbalenko et al., 2009, Wooten et al., 1996).

Phosphate transporter. Sodium-dependent phosphate transport protein 2B (SLC34A2, Table 3.4) is a Na^+ dependent phosphate transporter, which may be the main phosphate transporter in vivo. SLC34A2 was found to be expressed in the apical membrane of the mammary gland in mice during lactation but not in virgin and early lactation mice. Miyoshi et al. (2001) therefore suggested that it has a secretory function in the mammary gland. This protein was also identified by others in bovine milk MFGM (Reinhardt and Lippolis, 2006, Smolenski et al., 2007). It may be involved in phosphate transport to milk and partially responsible for the colloidal phosphate in milk (Charoenphandhu et al., 2010, Forster et al., 2007). However, the exact function of SLC34A2 in the mammary gland is not clear.

3.4.5 Membrane Trafficking

Membrane trafficking systems allow cells to take up, transport and expel particles (proteins, carbohydrate, and lipids) in vesicular carriers. It consists of transportation between distinct organelles, including the ER, Golgi complex, plasma membrane, and tubulovesicular transport intermediates. It includes endocytosis, exocytosis and transcytosis processes. During membrane trafficking, many proteins are involved to make the trafficking run smoothly (Jin et al., 1996, Starr et al., 2009, Zerial and McBride, 2001). Many of the milk components such as proteins, phosphate, calcium, lactose and citrate are thought to be secreted by exocytosis. The IgG, IgA, part of transferrin, and serum albumin are secreted by transcytosis (McManaman and Neville, 2003). The distinct functions of these membrane trafficking proteins in exocytosis and transcytosis of milk components are, however, still not well established. The detection of these proteins in MFGM suggests that they may be involved in trafficking and secretion of milk components.

In total, 23 proteins related to membrane/protein trafficking, were found in this study such as Rab proteins, SNARE proteins, and SAR proteins.

Twelve of the membrane trafficking proteins are Rab proteins (Table 3.5). These Rab proteins belong to the Ras superfamily of small GTPases. Rab proteins function as central regulators of vesicle budding, motility and fusion (Stenmark and Olkkonen, 2001, Zerial and McBride, 2001). The Rab proteins found in this study are mainly localized in ER, Golgi, endosome and plasma membrane. They play a role in endocytosis, transcytosis and exocytosis processes. An interesting example of membrane trafficking proteins with regard to milk synthesis is Rab 18. Rab 18 was shown to mediate regulation of lipid droplets and intracellular lipid droplets transport from ER to Golgi (Hutagalung and Novick, 2011). Rab

25 has been shown to regulate endocytosis and transcytosis in epithelial cells (Hutagalung and Novick, 2011). Wang et al (2000) also found that Rab 25 regulated the apical membrane recycling and transcytosis of IgA in Madin-Darby Canine Kidney Cells. Although there are not many studies carried out on the specific functions of these Rab proteins in milk synthesis and secretion, the importance of these proteins, especially for protein secretion, can be deduced from the importance of exocytosis and transcytosis for milk protein secretion.

Table 3.5. 23 proteins related to membrane trafficking (endocytosis, transcytosis, and exocytosis)

Protein name	Gene Name	IPI number	Source[1]
GTP-ase/EH domain-containing protein 4	EHD4	IPI00696647	M
Gamma-soluble NSF attachment protein	SNAPG	IPI00711042	M
GTP-binding protein SAR1a	SAR1A	IPI00717465	M
GTP-binding protein SAR1b	SAR1B	IPI00716201	M
Mannose-6-phosphate receptor binding protein 1	M6PRBP1	IPI00700098	M
Polymeric immunoglobulin receptor	PIGR	IPI00696714	M,S
RAB-11A	RAB11A	IPI00695221	M
RAB-13	RAB13	IPI00687642	M,S
RAB-18	RAB18	IPI00691826	M
RAB-1A	RAB1A	IPI00829520	M
RAB-1B	RAB1B	IPI00727050	M
RAB-25	RAB25	IPI00710347	M
RAB-2A	RAB2A	IPI00695881	M
RAB-35	RAB35	IPI00852464	M
RAB-3D	RAB3D	IPI00709014	M
RAB-5B	RAB5B	IPI00694198	M
RAB-7A	RAB7A	IPI00704752	M
RAB-8A	RAB8A	IPI00718035	M
Secretory carrier-associated membrane protein 2	SCAMP2	IPI00867311	M
Synaptobrevin homolog YKT6	YKT6	IPI00708611	M
Synaptosomal-associated protein	SNAP23	IPI00718671	M
Syntaxin 3	STX3	IPI00867107	M
Syntaxin binding protein 2	STXBP2	IPI00734153	M

[1]S: milk serum; M: MFGM

We detected three SNARE proteins (SNAP23, syntaxin 3, synaptobrevin homolog YKT6), one SNAP protein (SNAPG) and syntaxin binding protein 2 (STXBP2) (Table 3.5). STXBP2 is highly associated with SNARE proteins. Different SNARE proteins form a SNARE complex to promote the fusion of vesicle and target membrane during membrane trafficking (Chat et al., 2011). SNAP23 is considered to be a central protein to form different SNARE complexes which are involved in both casein vesicle secretion and lipid

droplets fusion (Boström et al., 2007, Chat et al., 2011). SNAP23, syntaxin 3 and YKT6 were also identified previously in bovine MFGM (Reinhardt and Lippolis, 2006). The exact function of syntaxin 3 and YKT6 during milk secretion is however not clear. To our knowledge, there is hardly any study focusing on these proteins' function during milk secretion.

Another important membrane transporter expressed in milk is polymeric immunoglobulin receptor (PIGR, Table 3.5), which is present in both the MFGM and serum fraction. This protein mediates the intracellular transport and secretion of IgA and IgM (Kulseth et al., 1995). Besides its function as transporter, this protein contains an immunoglobulin domain. It was recently demonstrated to have some inhibitory effect on HIV-1 enzyme (Murgiano et al., 2009, Ng and Ye, 2004). The two different functions of this protein could be the reason that it is detected in both the MFGM and serum fraction in a relatively high amount. PIGR in MFGM could function as transporter in the membrane system, whereas the PIGR in serum could play a role as host defense protein.

3.4.6 General Discussion

As discussed above, many milk synthesis and secretion proteins were detected in milk. These proteins are involved in all the major milk synthesis and secretion pathways (lipid, protein, lactose, minerals and vitamins), and thus provide a possibility to study the synthesis and secretion of milk components directly in milk. A large group of the detected proteins is related to lipid synthesis and secretion. Milk lipid synthesis and secretion in the mammary gland is complex and involves many enzymes, transporters, signaling proteins and regulatory systems. Proteins in different steps of the lipid synthesis and secretion in the mammary gland were identified in this study. The high number of identified lipid synthesis and secretion proteins indicates the possibility to use bovine milk for studying the underlying mechanisms of lipid synthesis and secretion.

Another large group of proteins is membrane trafficking proteins. The membrane trafficking system is complicated and a relation between the membrane trafficking proteins and the milk components has not been studied in much detail. Most of the identified membrane trafficking proteins were present in the MFGM fraction, which can easily be explained by the localization of these proteins in the membrane of epithelial cells causing them to be incorporated in the MFGM during MFG secretion. Their presence may also indicate that these proteins have a specific function in milk fat globule fusion and secretion, but further studies are needed to fully understand the role in the transport and secretion of milk components.

The number of proteins involved in protein synthesis is lower than proteins involved in lipid synthesis. The proteins found in this study are only involved in a few steps of the whole pathway of milk protein synthesis. We can, therefore, learn more about lipid synthesis than about protein synthesis from the proteome in milk. Although, the number of proteins related to protein synthesis was relatively low, a wide range of membrane trafficking proteins was detected. As discussed before, these proteins may be related to the transport of many different milk components, and, since protein is one of the major components that are transported, we may obtain useful information on the secretion of milk proteins.

So far, not many studies focused on the milk synthesis and secretion proteins present in milk. This paper, presents one of the first attempts to elucidate the functions of these proteins and their possible roles in the different steps of the metabolic pathways based on their presence in milk. This will provide new routes and insights for future studies on milk synthesis and secretion.

3.5 Conclusion

Milk synthesis and secretion in the mammary gland is a complex process involving many proteins. Milk proteomics provides an excellent possibility to study lactation by using milk itself. The identification of these proteins in milk offers new opportunities to advance our knowledge of lactation physiology and biology especially the lipid synthesis and secretion pathways.

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Chapter 4

Changes in milk proteome and metabolome associated with DGAT1 K232A polymorphism in lactating cows

Abstract

Acyl CoA: diacylglycerol acyltransferase 1 (DGAT1) is the enzyme that catalyses the synthesis of triglycerides from diglycerides and acyl-CoA. The DGAT1 K232A polymorphism was previously shown to have a significant influence on bovine milk production (milk yield, fat content, protein content and fatty acid composition). However, the mechanism of this influence is not well elucidated. In this study, proteomics (LC-MS/MS) and metabolomics (^{1}H-NMR, GC-FID) were applied to determine the milk fat globule membrane proteome and the polar and lipid composition of milk samples from cows with DGAT1 KK and AA genotype. The milk samples from cows with the DGAT1 KK genotype contained more stomatin, sphingomyelin and long chain fatty acids than milk samples from cows with the DGAT1 AA genotype. The differences between the DGAT1 genotypes may be related to stomatin-sphingomyelin lipid rafts as well as cell structural (cytoskeleton) differences in epithelial cells of the mammary gland. The results also suggest that the K polymorphism of DGAT1 prefers longer chain fatty acids, whereas the A polymorphism of DGAT1 prefers shorter and median chain fatty acids during synthesis of triglycerides. It is remarkable that a single amino acid mutation in the DGAT1 enzyme leads not only to differences in triglyceride composition but also to differences in membrane composition of the epithelial cells of the mammary gland.

4.1 Introduction

Fat is a major constituent in animal milk. It varies from less than 2% in horse milk to more than 50% in fur seal milk (Walstra et al., 2006). Bovine milk contains about 3-5% fat, of which 98% is triglycerides (Walstra et al., 2006). Triglycerides are mainly synthesized from diglycerides in the mammary gland, which is a committed step in the whole triglycerides synthesis process. The enzyme catalysing this step is acyl CoA: diacylglycerol acyltransferase (DGAT). DGAT1 is one of two isoforms (DGAT1 and DGAT2) in cows and other mammals. It was reported that DGAT1 plays a crucial role in milk production, based on the fact that DGAT1-knockout mice are unable to produce milk and there are almost no lipid droplets accumulating in the secretion region of the mammary gland (Smith et al., 2000). In 1998, Cases et al. identified the K232A polymorphism in the bovine DGAT1 gene (Cases et al., 1998). This polymorphism was shown to have a significant effect on bovine milk production and milk characteristics. It was also shown that the DGAT1 polymorphism significantly influenced milk fatty acid composition (Schennink et al., 2007). The DGAT1 K polymorphism was found to associate with higher fat percentage, higher fat yield, higher protein percentage, lower protein yield and lower milk yield (Grisart et al., 2002, Schennink et al., 2007). Many studies have investigated the effect of DGAT1 polymorphism on milk production, with a focus on fat production in different cow breeds (Berry et al., 2010, Cardoso et al., 2011, Komisarek et al., 2011, Molee et al., 2012). However, the mechanism behind the association between DGAT1 polymorphism and milk (fat) synthesis is unclear. In this paper, advanced proteomics and metabolomics techniques were applied to further elucidate this mechanism.

Proteins in buttermilk, which are enriched in milk fat globule membrane (MFGM) proteins, from cows with different DGAT1 polymorphism (KK and AA) were analysed by using advanced proteomics techniques (filter-aided samples preparation coupled with dimethyl labelling and nanoLC-Orbitrap-MS/MS) (Lu et al., 2011). This MFGM is a 3-layer membrane that covers milk fat globules in milk. Proteins in MFGM are considered to be partly representative of epithelial cells because of their specific origin (endoplasmic reticulum membrane, cytoplasm and apical plasma membrane of epithelial cells) (McManaman and Neville, 2003). Many milk synthesis and secretion proteins have been identified in MFGM (Lu et al., 2011). The differences of MFGM proteins could reflect the changes in the epithelial cells. Meanwhile the lipid composition and serum metabolites of the selected milk samples were analysed by ^{1}H-NMR and the fatty acid composition was analysed by gas chromatography. Milk proteome and metabolome analysis were combined to investigate the mechanisms behind the effect of DGAT1 on milk synthesis.

4.2 Materials and Methods

4.2.1 Sample selection

Bovine milk samples were selected according to DGAT1 polymorphisms (KK and AA) and fat content (3-4%, 4-5%, 5-6%) from the Dutch Milk Genomics Initiative (MGI) biobank of 1918 cows. Eight to ten cows were selected for each genotype and milk fat content. In total, milk samples of 55 cows were used for further analysis. As described by Schennink et al (2007), genotyping of DGAT1 polymorphisms was performed using a Taqman allelic discrimination method in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) on DNA isolated from blood samples of selected cows. Fat percentage of each milk sample was measured by using a MilkoScan FT6000 (Foss Electric, Hillerod, Denmark) at COKZ laboratory (Netherlands controlling Authority For Milk and Milk Products, Leusden, the Netherlands).

4.2.2 Proteomics analysis

Protein determination of buttermilk

In this study, the buttermilk samples obtained from the MGI biobank were used to study the MFGM proteome, since it is mainly consists of MFGM proteins (the separation procedure for buttermilk can be found in the supplementary material). Protein concentrations of buttermilk was determined using the Dumas method with a NA 2100 Protein nitrogen analyzer (CE Instruments, Milan, Italy).

Filter aided sample preparation (FASP)-dimethyl labelling-nanoLC-Orbitrap-MS/MS

The method used to prepare buttermilk samples for LC-MS/MS analysis was based on FASP (Wiśniewski et al., 2009) and dimethyl labelling (Boersema et al., 2009). Then, the prepared samples were analyzed using nanoLC-Orbitrap-MS/MS. The detailed procedures were described in Lu et al. (2011).

Data analysis

Each run with all MS/MS spectra was analysed using MaxQuant 1.1.1.36 with Andromeda as peptide search engine (Cox and Mann, 2008, Cox et al., 2011). The database for peptide/protein searches was a concatenated IPI bovine database downloaded from ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/ (27-09-2011) with reverse sequences (for the false discovery rate) generated by MaxQuant. The contaminants database of MaxQuant was also used for peptide/protein searches including sequences of trypsin and human keratins.

Identification and quantification of proteins were simultaneously performed in MaxQuant. Carbamidomethylated cysteines were set as fixed modification; oxidation of methionine, N-terminal acetylation and de-amidation of asparagine or glutamine were set as variable modification for both identification and quantification. Mass deviation of 0.5 Da was set as maximum allowed for MS/MS peaks, and a maximum of two missed cleavages were allowed. Maximum false discovery rates (FDRs) were set to 1% both on peptide and protein levels. Minimum required peptide length was 6 amino acids for both identification and quantification. A minimum of 2 peptides for each protein were required for reliable identification. Dimethyl labelling were set as doublets with dimethLys0 and dimethNter0 as light and dimethLys4 and dimethNter4 as heavy. Razor and unique peptides were used for quantification. Normalized H/L ratio were used for further statistical analysis.

4.2.3 ^{1}H-NMR analysis

Milk lipid composition analysis

For milk lipid composition measurements, 400 µl whole milk was added to 600 µl deuterated chloroform and vortexed thoroughly. The mixture was centrifuged at 20,000g for 30 mins. Then, 200 µl of the deuterated chloroform phase (bottom liquid phase) was used for ^{1}H-NMR analysis (Nuclear magnetic resonance spectrometer Avance III with a 600 MHz/54 mm UltraShielded Plus magnet equipped with a CryoPlatform cryogenic cooling system, a BCU-05 cooling unit, an ATM automatic tuning and matching unit). The samples were measured in 3 mm NMR tubes (Bruker matching system). Measurements were performed at 300K. Identification of the different lipid molecules was based on Tukiainen et al. (2008) and Vinaixa et al. (2010).

Milk serum metabolites analysis

Polar metabolites were obtained from the deuterated chloroform samples (see above) by taking 100 microliter of the upper liquid water phase, mixing with 100 microliter phosphate buffer, transferring into a 3 mm NMR tube and measuring in Nuclear magnetic resonance spectrometer Avance III with a 600 MHz/54 mm UltraShielded Plus magnet equipped with a CryoPlatform cryogenic cooling system, a BCU-05 cooling unit, an ATM automatic tuning and matching unit. Measurements were performed at 300K. Eleven molecules were quantitatively measured (formic acid, adenine, fumaric acid, orotic acid, lactose, phosphocholine, choline, creatinine, citrate, acetyl and acetate). 1D NOESY, CPMG, and JRES spectra were measured. The analysis of the JRES datasets was performed according to Fonville et al. (2010).

4.2.4 GC Ultra chromatograph analysis for fatty acid composition

As described by Schennink et al (2007), milk fat composition was measured at COKZ laboratory (Netherlands controlling Authority For Milk and Milk Products, Leusden, the Netherlands). Fatty acid methyl esters were prepared from fat extract according to ISO Standard 15884 (ISO-IDF, 2002b) followed by analysis using a Trace GC Ultra chromatograph (Thermo Electron Corporation, Waltham, MA) according to ISO standard 15885 (ISO-IDF, 2002a). A Varian Fame Select column (100m × 0.25mm ID, Varian Inc., Palo Alto, CA) was applied in the GC system. The initial temperature was 70°C for 1 min, raised to 225°C at the speed of 3°C /min and kept at 225°C for 5 min. One µl of sample was injected. Peaks were identified and quantified using standard methyl ester samples (Sigma-Aldrich, Zwijndrecht, the Netherlands; Larodan, Malmö, Sweden).

4.3 Results

4.3.1 MFGM proteome of cows with DGAT1 KK and AA polymorphism

In this study, the MFGM proteome of two samples could be compared in a single proteomics analysis due to the use of different isotope labels (heavy and light). In order to obtain the most information of the effect of DGAT1 polymorphisms (KK and AA) on the MFGM proteome, four comparisons were made (Table 4.1).

Table 4.1 Four comparisons performed in proteomics analysis. DGAT1 polymorphism: KK and AA; milk fat content: 3-4%, 4-5% and 5-6%.

influence factors	polymorphisms & fat content	polymorphisms	fat content in KK	fat content in AA
heavy dimethyl label	5-6% KK	4-5% KK	5-6% KK	5-6% AA
vs	vs	vs	vs	vs
light dimethyl label	3-4% AA	4-5% AA	3-4 % KK	3-4% AA

The K polymorphism of DGAT1 is normally associated with higher fat content in milk than the A polymorphism of DGAT1 (Schennink et al., 2007). However, besides DGAT1 polymorphisms, fat content could also influence the MFGM proteome. Thus, milk samples with the same fat content (4-5%) from DGAT1 KK and DGAT1 AA cows were compared to eliminate the influence of fat content on MFGM protein composition. Finally, in order to see the influence of fat content on the MFGM proteome without interference of DGAT1 polymorphisms, a separate comparison in DGAT1 AA and KK cows was done between milk samples with 5-6% fat and 3-4% fat. In each comparison, pooled buttermilk samples were used (4-5 buttermilk samples/pool). All the comparisons were done in both biological

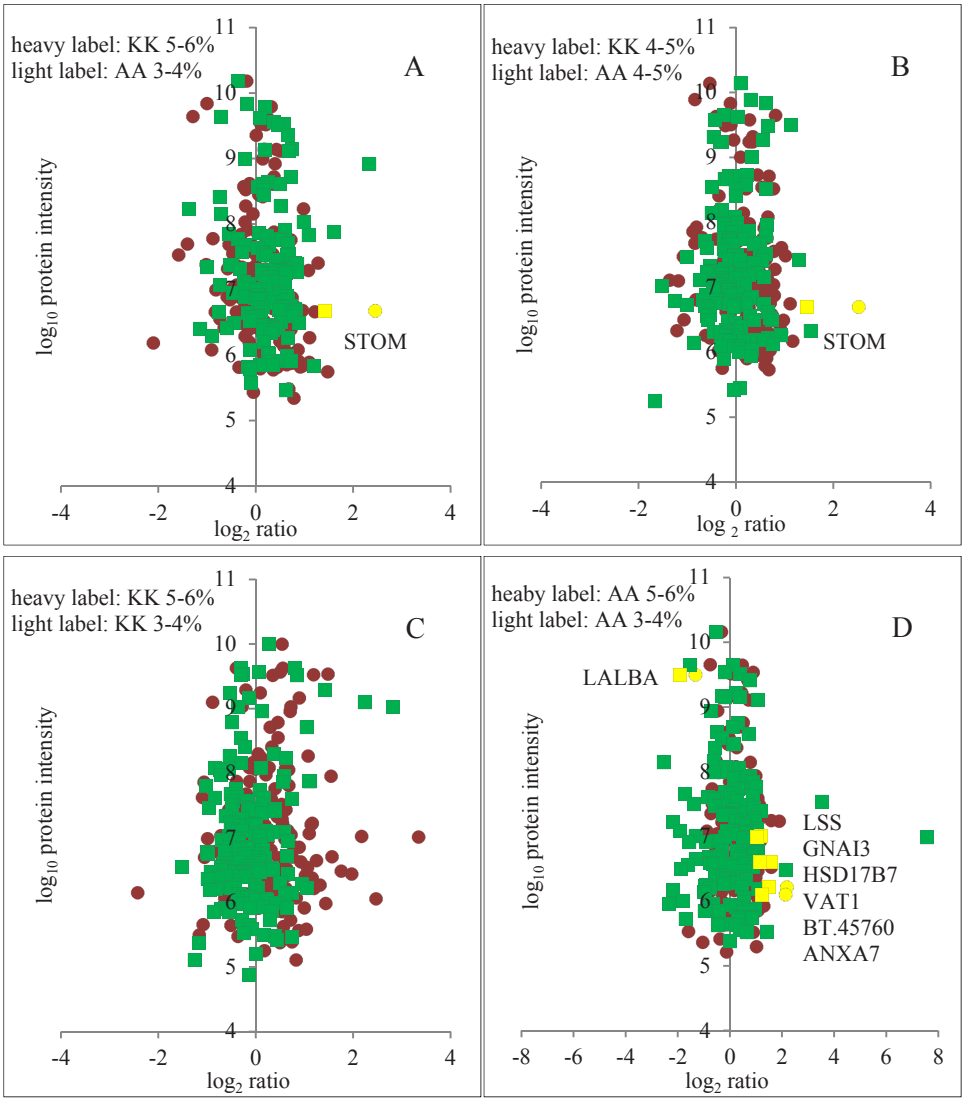

Figure 4.1. Comparison of proteins in buttermilk with different DGAT1 polymorphism and fat content by quantitative proteomics analysis. For each protein, $\log_2$ normalized protein ratios (H/L) are plotted against average protein intensity of biological duplicate in each comparison. Green square: biological replicate 1; red dot: biological replicate 2; yellow dot/square: >2-fold changed proteins with the gene name. STOM, stomatin; LALBA, alpha-lactalbumin; LSS, lanosterol synthase; GNAI3, guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3; HSD17B7, HSD17B7 protein; VAT1, VAT1 protein; ANXA7, annexin A7.

and technical duplicates. The differences in proteome in the different comparisons are shown in Figure 4.1. Two-fold change in protein abundance was used as significant cut-off in the quantitative proteomics analysis (Lu et al., 2011). In total, 249 proteins were identified and quantified in buttermilk. As expected, the major proteins of MFGM (butyrophilin, xanthine dehydrogenase/oxidase, lactadherin, perilipin-2 and CD36) were all identified in buttermilk in a relative high amount compared to the other proteins. Milk samples of DGAT1 KK cows always contained a higher amount of stomatin than milk samples in DGAT1 AA cows (Figure 4.1 A and B). However, the amount of stomatin in MFGM is not influenced by fat content (Figure 4.1 C and D). Thus, stomatin seems to be influenced only by DGAT1 polymorphism but not fat content. Stomatin is a protein that consists of 289 amino acids (Figure 4.2). The number of proteins within the cows with the AA polymorphism which are different dependent on samples with different fat content (7) is larger than that for cows with the KK polymorphism (0) (Figure 4.1, C and D). It thus seems that as milk fat content increases (from 3-4% to 5-6%), the MFGM proteome is more consistent in DGAT1 KK cows than that in DGAT1 AA cows. The same was observed for fatty acid composition, where the milk fatty acid composition in milk samples between DGAT1 KK cows did not differ as much as that between DGAT1 AA cows (data not shown). Furthermore, there is no common protein significantly changed in the comparisons between fat content within the DGAT1 polymorphisms (Figure 4.1 C and D), indicating that the MFGM proteome is not strongly influenced by milk fat content.

MSDKRPAVDTQARRLPDSFKDSPSTGLGVCGWILVAVSFLFTVITFPVSIWMCIKIIK
EYERAIIFRLGRILQGGAKGPGLFFILPCTDSFIKVDMR**TISFDIPPQEILTK**DSVTIS
VDGVVYYR**VQNATLAVANITNADSATR**LLAQTTLRNVLGTKNLSQILSDREEIAH
NMQCTLDDATDDWGIKVERVEIK**DVKLPVQLQRAMAAEAEASR**EAR**AKVIAAE**
GEMNASRALKEASMVITESPAALQLR**YLQTLTTIAAEK**NSTIIFPLPIDMLQAIMGP
KQ

Figure 4.2. Amino acids sequence of stomatin. Peptides in bold are identified and quantified in this study.

4.3.2 Difference in fatty acid composition between DGAT1 polymorphisms

Fatty acids composition of all selected milk samples was analysed by gas chromatography and 27 fatty acids were quantified. Significantly changed fatty acids between DGAT1 KK and AA cows are shown in Table 4.2. Milk from DGAT1 AA cows had increased short and medium chain fatty acids (C4-C14), whereas increased long-chain fatty acids was observed in milk from DGAT1 KK cows. The amount of carbons in milk fat was also calculated

(Table 4.3). Similar to the results presented in Table 4.2, milk fat in DGAT1 AA cows contains a higher amount of carbons in short and median chain fatty acids (C4-C14). In contrast, even though not significant, milk fat in DGAT1 KK cows contains a higher amount of carbon in long chain fatty acids (C16-C20). The total amount of milk fatty acid carbons in milk samples of DGAT1 KK and AA cows was not different.

Table 4.2 Significant different fatty acids in milk samples from DGAT1 KK and AA cows (KK 3-6% fat 28 samples; AA 3-6% fat 27 samples), % of total fat (g/100g)

	C10:0	C12:0	C14:0	C4:0-C14:0	C15:0	C16:1	C17:0	C18:0	C18:1 trans11
KK	2.78	3.68	10.86	24.87	1.19	1.54	0.48	9.56	0.87
AA	3.03	4.18	11.92	26.47	1.08	1.32	0.44	8.73	0.70

Table 4.3 Significant different fatty acids ($p<0.05$) in the amount of carbon of milk samples from DGAT1 KK and AA cows (KK 3-6 % fat 28 samples; AA 3-6% fat 27 samples), mole/mole fat

	C4:0	C10:0	C12:0	C14:0	C15:0	C16:1	C17:0	C18:0	C18:1 trans11	C4-C14	C16-C20[1]	Total amount of carbon[2]
KK	0.41	0.39	0.54	1.62	0.18	0.24	0.07	1.47	0.14	3.72	10.02	13.92
AA	0.39	0.43	0.61	1.77	0.16	0.20	0.06	1.34	0.11	3.92	9.83	13.91

[1] $p=0.08$; [2] $p=0.88$

4.3.3 ^{1}H-NMR analysis of milk lipid composition in DGAT1 KK and AA cows

In total, 31 peaks in ^{1}H-NMR spectra were assigned and integrated according to the available literature and human metabolites database (www.hmdb.ca). The amount of triglycerides (TAG), diglycerides (DAG), monoglycerides (MAG), different fatty acids and phospholipids were compared between milk samples from DGAT1 KK and AA cows. Sphingomyelin and CLA are significantly higher in milk samples from DGAT1 KK cows.

As described above, the amount of TAG and DAG were also determined. DGAT1 is the enzymes that catalyses the step from DAG to TAG during lipid synthesis. However, the relative amount of TAG and DAG in the lipid fraction are not different between milk samples from DGAT1 KK and AA cows. The amount of TAG showed a higher positive correlation with fat content ($r^2=0.99$) in milk. The amount of DAG also showed a positive correlation with milk fat content, but the correlation coefficient is around 0.26. There is also a positive correlation between the amount of TAG and DAG ($r^2=0.61$). The analysis of the polar metabolites by NMR did not indicate significant differences in the DGAT1 KK and DGAT1 AA samples.

4.4 Discussion

In our study we applied proteomics and metabolomics techniques to understand the effect of DGAT1 polymorphism in cows on milk composition. For the proteomics analysis, MFGM proteins were chosen as study material because of their specific origin (ER membrane, cytoplasm and apical membrane of epithelial cells in mammary gland) (McManaman and Neville, 2003). Due to this origin, it is considered to be a good representation for the secretory cells in mammary gland. By using advanced proteomic techniques, 249 proteins were identified and quantified in MFGM-enriched buttermilk including a wide range of low-abundant proteins. This high number of identified proteins enabled a detailed comparison of MFGM protein composition of DGAT1 KK and AA cows, which could be used to interpret the differences between epithelial cells of DGAT1 KK and AA cows.

In the proteomics analyses, stomatin was the only protein that was clearly different between milk samples from DGAT1 KK and AA cows. It was found that in human stomatin, cys-30 (this cysteine amino acid residue also presents in bovine stomatin) was palmitoylated. This palmitoylation of the protein was assumed to lead to the increased affinity of this protein to plasma membrane (Snyers et al., 1999). Because of this post translational modification, the peptides containing cys-30 could not be identified in the current proteomic analysis (Figure 4.2) as the peptide becomes too hydrophobic for easy ionization using electrospray. However, 7 other peptides were observed and quantified in our experiments with at least 2 peptides quantified for each comparison, which ensured a good accuracy of the protein quantification. To our knowledge, there is no research done on the function of stomatin in secretory cells in the mammary gland. Stomatin is a protein that was first identified in the membrane of erythrocytes (Snyers et al., 1999), and was shown to be ubiquitously expressed in vertebrate tissues and different cell lines (Umlauf et al., 2004). It was observed to co-localize with membrane-associated cytoskeleton (Snyers et al., 1997). Stomatin was also shown to be a scaffolding component in lipid rafts (also known as lipid microdomain) and expressed mainly in plasma membrane. It was suggested to play an important structural role in lipid raft formation (Salzer and Prohaska, 2001, Umlauf et al., 2004). In addition, stomatin is suggested to be involved in cytoskeleton formation and ion transport across the cell membrane (Snyers et al., 1997, Snyers et al., 1999).

The amount of sphingomyelin was observed to be significantly higher in milk samples from DGAT1 KK cows. Sphingomyelin is a major component of cell membranes. In milk, sphingomyelin mainly locates in MFGM. Sphingomyelin is, together with phosphatidylcholine, one of the two major phospholipids in MFGM. It accounts for

approximately 25% of phospholipids in bovine milk (Palmquist et al., 2006). Recently, Lopez et al. observed that sphingomyelin was located in lipid rafts, a liquid-ordered domain, of MFGM while other glycerophospholipid were present in liquid-disordered domains (Lopez et al., 2010, Lopez and Menard, 2011).

Both stomatin and sphingomyelin are thus shown to be the important components of lipid rafts in membrane systems and both of them were present in higher concentration in MFGM of DGAT1 KK cows. Thus, we hypothesize that there are more stomatin-sphingomyelin lipid rafts in MFGM of DGAT1 KK cows. Furthermore, only stomatin instead of calveolins and flotillins, which are normally occurring scaffolding proteins in lipid rafts, were observed in bovine MFGM. This could imply that stomatin is the scaffolding protein in MFGM lipid rafts. Since MFGM is derived from the outer leaflet of ER membrane and the double-layer of apical plasma membrane from mammary epithelial cells, this could imply that the epithelial cell membranes of DGAT1 KK cows have more stomatin-structured lipid rafts. Lipid rafts were shown to be involved in many intracellular processes including signal transduction, sorting and trafficking in secretory pathways (Brown and London, 1998). The importance of lipid rafts in membrane budding and fission has also been stressed by Huttner and Zimmerberg (2001). They considered that membrane budding and fission was strongly influenced by raft formation around a nascent bud. McManaman and Neville (2003) suggested that lipid droplets were secreted from epithelial cells in a "budding" process through apical plasma membrane. Thus, stomatin-sphingomyelin lipid rafts may have an essential role in lipid droplet budding from plasma membrane to the alveolar lumen. It was previously shown that the DGAT1 enzyme from cows with the K polymorphism has a significant larger Vmax in producing triglycerides, which leads to a relatively higher fat content in milk (Grisart et al., 2004). The higher amount of stomatin-sphingomyelin lipid rafts could help cope with efficient lipid droplet budding from epithelial cells to milk in DGAT1 KK cows. However, further elucidation of the localisation and function of lipid rafts and its composition in and on epithelial cells is needed.

Similar to our data reported in Table 4.2, Schennink et al. (2007) also observed that DGAT1 A was associated with a higher amount of fatty acids shorter than C16 and a lower amount of C18:0. In addition, as shown in Table 4.3, the total amount of milk fat carbon was not different between milk samples from DGAT1 KK and AA cows. This means that the total energy in milk fat is the same in DGAT1 KK and AA cows. However, there was variation in the origin of this milk fat energy (C4-C14 versus C16-C20). Short and medium chain fatty acids (C4-C14) in bovine milk are known to be mainly synthesized *de novo* in the mammary gland. Long-chain fatty acids (>C16), on the other hand, are mostly derived

from blood (Palmquist, 2006). It seems that cows with DGAT1 KK and AA polymorphism have preference for different fatty acids during TAG synthesis. The same was previously suggested by Schennink et al (2007).

The effect of this DGAT1 polymorphism was only studied on milk parameters, however, the polymorphism of DGAT1 will be the same throughout the whole cow. Thus, if the DGAT1 K and A enzyme have preference for different fatty acids during TAG synthesis, the same effects as shown in milk should also be true in other lipid-metabolizing organs in cows. Therefore, the fatty acid composition of other lipid fractions such as blood and adipose tissue could be different between cows with different DGAT1 polymorphisms.

4.5 Conclusion

The data shown in this paper suggest that stomatin-sphingomyelin lipid rafts, as well as the membrane organization or cell structure of epithelial cells in mammary gland could be different between DGAT1 KK and AA cows. This difference in cell architecture could influence the milk (fat) production. DGAT1 K seems to prefer long chain fatty acids whereas DGAT1 A seems to prefer short and medium chain fatty acids. These observations in the present study, especially with regard to the difference in membrane structure, could provide a new direction for further understanding of the mechanisms by which the DGAT1 polymorphism influences milk synthesis.

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Supplementary material

Separation of buttermilk from whole milk

100 ml milk sample was heated to 27°C in a water bath. Five ml HCL (4M) was added to the milk sample and the sample was shaken for 45 min until the fat was clearly separated. The water phase was discarded and the fat phase was washed with milli-Q water. The fat phase was then heated in the oven at 70°C for 30 min. Milli-Q water was added to the fat phase (1:1, v:v) and the sample was heated in the oven at 70°C for another 15 min. Finally, the sample was centrifuged at 3500 rpm for 15 min. The water phase in the bottom was the buttermilk containing MFGM proteins for further proteomic analysis.

Chapter 5

Membrane protein composition of different sized milk fat globules

Abstract

In bovine milk, milk fat globules (MFG) have a heterogeneous size distribution with diameters varying from 0.1 to 15 μm. Differences in lipid composition (lipid core and milk fat globule membrane (MFGM)) have been studied in different sized MFG fractions, but not the protein composition of MFGM. In this study, two size fractions of MFG (7.6±0.9 μm and 3.3±1.2 μm) were obtained by centrifugation. The protein composition of MFGM in large and small MFG was compared using advanced proteomics techniques (filter aided sample preparation-dimethyl labelling-nanoLC-Orbitrap-MS/MS). Two frequently studied proteins in lipid droplet biogenesis, perilipin-2 and TIP47, increased in large and small MFG, respectively. In large MFG, cytoplasmic vesicle proteins (heat shock proteins, 14-3-3 proteins and Rabs), microfilaments and intermediate filaments related proteins (actin and vimentin) and host defense proteins (cathelicidins) were increased. On the other hand, cholesterol synthesis enzymes (lanosterol synthase and sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating) were increased in small MFG. These results indicate that vesicle proteins and microfilaments and intermediate filaments could play an important role in lipid droplet growth and/or secretion. The presence of cathelicidins and the concentration difference between large and small MFG throw new light on its origin in bovine milk. The observations from this study clearly demonstrated the difference in protein composition of the membranes of small and large MFG. These results will lead to a better understanding of fat globule formation and secretion.

5.1 Introduction

Milk fat is secreted by the mammary gland in the form of globules. These milk fat globules (MFG) consist of a lipid core (mainly triglycerides) covered by a 3-layer membrane structure called the milk fat globule membrane (MFGM). In bovine milk, the diameter of the fat globules varies from around 0.1 to 15 μm. It was previously shown that fatty acid composition and phospholipid composition vary between different sized MFG. For example, smaller MFG contained a higher amount of polyunsaturated fatty acids (Lopez et al., 2011, Mesilati-Stahy et al., 2011). It was also shown that the phospholipid composition of the MFGM varied between different MFG sizes (Mesilati-Stahy et al., 2011). Although the differences in composition of the lipid core and the phospholipids of the MFGM have been studied as a function of MFG size, there is no research carried out on the relation between MFG size and protein composition of the MFGM.

In the last few decades, large improvements in the understanding of milk components synthesis and secretion were achieved (McManaman and Neville, 2003, Neville, 2006). However, some aspects remained unsolved. Electronic micrograph and immunocytochemical data showed that, in mammary gland, milk lipid is synthesized between the layers of the endoplasmic reticulum (ER) membrane and secreted into cytoplasm as micro lipid droplets (LD). Part of these micro LD will fuse with each other to form larger LD, when moving to the apical plasma membrane. Via a special budding process, the LD is released from epithelial cells to milk as MFG. During this MFG synthesis and secretion process, a 3-layer membrane will cover the globules. This 3-layer membrane originates from the outer layer of ER membrane and the double layer of apical plasma membrane (Mather and Keenan, 1998, McManaman and Neville, 2003). The secretion of both smaller and larger LD to milk results in the wide range of MFG sizes. However, the mechanism of the formation of different sized LD in mammary gland is still not clear (Mather and Keenan, 1998). Also, the budding process of different sized LD has not been studied before. During this budding process, it was shown that proteins in MFGM such as butyrophilin (BTN), xanthine dehydrogenase (XDH) and perifilin-2 (ADFP) are involved (Dewettinck et al., 2008, Heid and Keenan, 2005, Mather and Keenan, 1998). The origin of MFGM in combination with these observations that MFGM contains proteins involved in fat secretion make it interesting to study the protein composition of MFGM in different sized fat globules. This could help understand the mechanisms behind the wide range of MFG sizes. In the past, the study of MFGM proteins was limited by limitations in analytical techniques. Only the major proteins in MFGM were analysed by using traditional biochemical methods (Kobylka and Carraway, 1972, Mather and Keenan, 1975). As

proteomics techniques developed, much more proteins (up to 170 proteins) with diverse possible biological functions were identified and quantified in MFGM fraction (Hettinga et al., 2011, Lu et al., 2011, Reinhardt and Lippolis, 2006). Proteomics techniques have been applied, and were shown as a useful technique to help advance our knowledge about lactation (Boehmer et al., 2008, Reinhardt and Lippolis, 2008). Due to the specific synthesis and secretion process of MFG, they contain proteins from epithelial cells, which can be evidenced by similarity of phospholipid and protein composition between MFGM and plasma membrane of epithelial cells in mammary gland (Keenan et al., 1970, Keenan et al., 1989). Proteins with biological functions associated with milk synthesis and secretion also show that it is possible to use MFGM for studying lactation (Lu et al., 2011).

In our study, MFG were separated according to their size resulting in a fraction of small MFG and a fraction of large MFG. The protein composition of MFGM of both fractions was analysed by advanced proteomics techniques (FASP-dimethyl labelling-nanoLC-Orbitrap-MS/MS).

5.2 Materials and Methods

5.2.1 Milk samples

Milk samples were collected from seven healthy Holstein cows which were in mid lactation (100-200 days) between December 2011 and January 2012 from the Wageningen University Farm (Wageningen, The Netherlands). Raw milk samples were obtained immediately after milking in the morning and transported to the lab for the subsequent analyses on the same day. Milk composition was analysed using a MilkoScan 134A/B (Foss Electric, Denmark)

5.2.2 Milk fat globule separation

Milk samples were heated to 40°C in a water bath. The heated milk samples were then centrifuged at a speed of 190 g for 10 min at 15°C to separate cream and skim milk. The top layer of cream was removed to be centrifuged with phosphate buffered saline (PBS; pH 6.8, 0.1M, 1:10, v:v) at 390 g for 10 min at 10°C. The washing solution was discarded after centrifugation. This step was repeated for 3 times. The cream obtained using this procedure was the large MFG fraction. The skim milk was centrifuged at 390 g for 10 min at 10°C. This step was repeated 3 times. The cream after each centrifugation step was discarded except after the last step. The obtained cream was washed 3 times with PBS (pH 6.8, 0.1M, 1:10, v:v) at 390 g for 10 min at 10°C. The cream obtained using this procedure was the small MFG fraction.

5.2.3 Determination of globule diameters

The small and large MFG fractions were diluted with 35mM EDTA/NaOH buffer (pH 7.0, 1:1, v:v) in order to dissociate casein micelles, followed by heating to 40°C. The diameters of globules were analysed using a Mastersizer 2000 (Malvern Instrument Ltd, UK). Refractive index of cream samples was 1.458 at 633 nm. Milli-Q water was used as dispersant and its refractive index was 1.33.

5.2.4 Separation of proteins in milk fat globule membrane

SDS solution (0.4%) was added to small and large MFG fractions (1:1, v:v). After cooling at 4°C for 15 min, the cream solution was sonicated for 3 min (Transsonic 700, D-78224 SINGEN/Htw, Germany). It was then centrifuged at 1500 g for 5 min. The bottom part of the solution contains the MFGM proteins. Protein concentration was determined by using BCA assay (Thermo Scientific Pierce BCA protein assay kit, USA)

5.2.5 Protein composition analysis by using FASP-dimethyl labelling-nanoLC-Orbitrap-MS/MS

MFGM proteins (10 μg) of large MFG and small MFG were prepared and compared by using FASP-dimethyl labelling-nanoLC-Orbitrap-MS/MS. The procedure is described in detail in Lu et al. (2011).

5.2.6 Proteomic data analysis

In total, seven LC-MS/MS raw files were generated (large vs small MFG of milk samples from seven cows). Each run with all MS/MS spectra obtained were analysed by using MaxQuant 1.2.2.5 with Andromeda as peptide search engine (Cox and Mann, 2008, Cox et al., 2011). The database for peptide/protein searches was a concatenated bovine reference database downloaded from Uniprot (www.uniprot.org, 10-05-12) with reverse sequences generated by MaxQuant. The contaminants database of MaxQuant was also used for peptide/protein searches including sequences of trypsin and human keratins. Identification and quantification of proteins were simultaneously performed by MaxQuant. Carbamidomethylated cysteines were set as fixed modification; oxidation of methionine, N-terminal acetylation and de-amidation of asparagine or glutamine were set as variable modification for both identification and quantification. Mass deviation of 0.5 Da was set as maximum allowed for MS/MS peaks, and a maximum of two missed cleavages were allowed. Maximum false discovery rates (FDRs) were set to 1% both on peptide and protein level. Minimum required peptide length was six amino acids for both identification

and quantification. A minimum of 2 peptides for each protein were required for reliable identification. Dimethyl labelling was based on doublets with dimethLys0 and dimethNter0 as light; dimethLys4 and dimethNter4 as heavy. Razor and unique peptides were used for quantification. Normalized H/L ratios were used for further statistics analysis.

5.2.7 Significant analysis and GO enrichment analysis

One sample t-tests were performed on the protein ratio of each protein in seven comparisons (large MFG/ small MFG), comparing the average $\log_2$ ratio to 0. The Gene Ontology (GO) enrichment analysis of significantly changed proteins ($p<0.05$) was done using DAVID bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009). The GO terms shown in 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.

5.3 Results

By using centrifugation, two sizes of MFG were obtained with an average diameter of 3.3±1.2 μm and 7.6±0.9 μm respectively for milk samples from seven individual cows (Table 5.1). Figure 5.1 shows the size distribution of the MFG in the seven individual samples. As expected based on the size differences, small MFG have a larger membrane surface than large MFG (Table 5.1).

Table 5.1. Size parameters of fat globules in whole milk, small milk fat globules (SMFG) and large milk fat globules (LMFG) and milk composition of the whole milk. Data values represents mean±SD of milk samples and separated MFGs from seven cows.

Fraction	D_{43} μm	D_{32} μm	Specific surface area (m^2/g fat)	Fat %	Protein %	Lactose %
Whole milk	3.95±0.26	3.47±0.27	1.89±0.15	3.95±0.56	3.58±0.23	4.53±0.07
SMFG	3.32±1.21	2.93±1.06	2.23±0.81			
LMFG	7.61±0.90	4.31±0.17	1.16±0.05			

Diameters $d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$, $d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$ where n_i is the number of fat globules with diameter d_i ; specific surface area= $6/(d_{32}*\varphi)$ φ=0.92g/L for fat globule (Walstra et al., 2006)

In total, 4692 peptides were identified and quantified resulting in 157 proteins with at least 1 unique peptide and 2 razor and unique peptides in every milk sample for further statistical analysis. There are 49 and 23 proteins significantly increased in large and small MFG, respectively (Table 5.2 and Table 5.3). Next, a functional analysis of significantly changed

proteins was performed based on gene ontology and literature information. In large MFG, significantly increased proteins are mainly associated with protein transport/stabilization, host defense and cytoskeleton organization. These proteins show enrichment in the subcellular locations "cytoplasmic vesicle" and "intracellular non-membrane-bounded organelle", which is mainly cytoskeleton/chromosome (Figure 5.2 and Figure 5.4). Proteins significantly increased in small MFG are mainly associated with lipid metabolic process and cholesterol synthesis and showed "apical membrane" and "integral membrane" as enriched subcellular location (Figure 5.3 and Figure 5.4). Not only the low abundant proteins, but also the major proteins differed between large and small MFG, with an increase of lactadherin (MFGE8), perifilin-2 (ADFP) and glycosylation-defendant cell adhesions moledule 1 (PP3) in large MFG and an increase of platelet glycoprotein 4 (CD36) and mucins (MUC1, MUC15 and MUC16) in small MFG. The largest difference between large and small MFG was seen for the host defense proteins, cathelicidins (CATHL1, CATHL6 and CATHL4), lactoferrin (LTF), complement C3 (C3), lymphocyte cytosolic protein 1 (LCP1) and monocyte differentiation antigen CD14 (CD14), which were much more abundant in large MFG. Enzymes involved in lipid synthesis also showed increase in both large MFG (fatty acid synthase, FASN) and small MFG (acyl-CoA synthetase/ACSLs, lanosterol synthase /LSS, acetyl-CoA carboxylase 1/ACACA, sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating/NSDHL and glycerol-3-phosphate acyltransferase 4/AGPAT6).

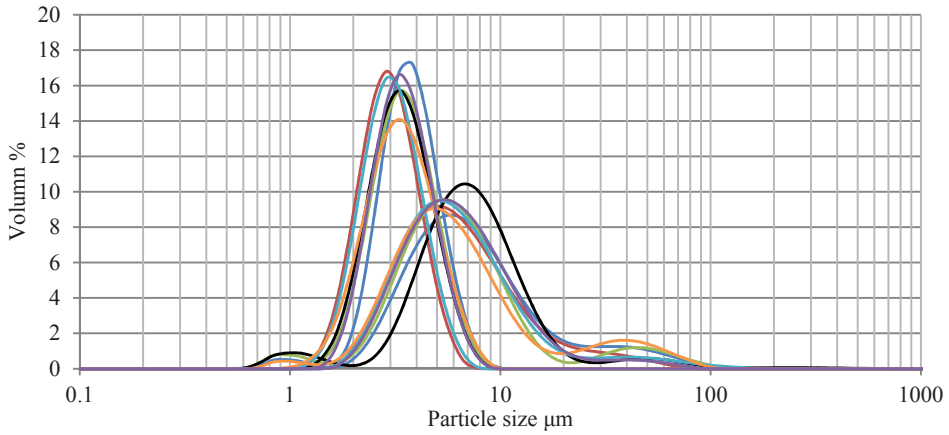

Figure 5.1. Size distribution of small MFG and large MFG determined by laser light scattering using Mastersizer 2000. The MFG separated from the same milk are shown in the same colour.

Table 5.2. Proteins with significantly higher concentration in large MFG and their $\log_2$ protein ratio in seven comparisons (large/small). Proteins were listed in the order of descending protein intensity derived from MaxQuant.

Uniprot entry	Gene name	Protein name	Average ratio	1	2	3	4	5	6	7
Q95114	MFGE8	Lactadherin	0.50	0.36	0.38	0.42	0.55	0.71	0.40	0.72
Q9TUM6	ADFP	Perilipin-2/ Adipose differentiation-related protein	0.12	0.29	-0.08	0.12	0.15	0.07	0.04	0.22
P80195	GLYCAM1 /PP3	Glycosylation-dependent cell adhesion molecule 1	0.65	1.16	0.42	0.53	0.21	1.22	0.51	0.48
F1N726	GP2	Glycoprotein 2 (Zymogen granule membrane)	0.71	2.13	0.03	1.05	0.52	0.08	0.17	0.97
P60712	ACTB	Actin, cytoplasmic 1	2.35	1.89	1.05	4.08	1.16	3.62	2.84	1.85
Q0IIG8	RAB18	Ras-related protein Rab-18	0.33	0.30	-0.07	0.44	0.13	0.54	0.45	0.54
P22226	CATHL1	Cathelicidin-1	4.25	5.22	2.66	5.39	2.47	4.68	3.19	6.17
P62803		Histone H4	4.23	3.78	3.30	5.62	1.87	4.52	3.85	6.68
Q71SP7	FASN	Fatty acid synthase	0.79	1.10	-0.40	1.31	0.28	1.22	1.14	0.86
P46193	ANXA1	Annexin A1	3.11	3.29	2.06	4.09	1.45	3.94	3.59	3.35
Q3T101	IGL@	IGL@	0.78	1.77	0.28	1.12	0.21	0.41	0.71	0.97
P24627	LTF	Lactoferrin	2.20	2.31	-0.07	4.60	0.15	3.90	2.85	1.67
P28782	S100A8	Protein S100-A8	3.55	4.08	3.28	3.98	1.72	3.65	3.21	4.92
F1N2R1	STOM	STOM protein	0.50	0.32	-0.16	0.34	0.43	1.20	1.02	0.38
P48616	VIM	Vimentin	3.55	2.66	3.15	5.69	1.90	4.66	3.60	3.18
P19120	HSPA8	Heat shock cognate 71 kDa protein	0.65	0.30	0.33	1.43	0.10	1.22	0.74	0.42
P54228	CATHL6	Cathelicidin-6	3.51	3.59	2.46	4.53	2.02	3.69	3.20	5.06
A7MAZ5	HIST1H1C	HIST1H1C protein	3.98	3.47	2.77	6.32	2.05	4.60	4.16	4.49
Q8SPP7	PGLYRP1	Peptidoglycan recognition protein 1	4.61	5.35	2.08	6.53	2.64	5.40	3.37	6.93
P0CH28	UBC	Polyubiquitin-C	0.53	1.19	0.20	0.70	0.48	0.45	0.23	0.46
P33046	CATHL4	Cathelicidin-4	4.19	5.40	2.94	5.66	2.25	4.46	3.28	5.33
F1MH40	IGK	IGK protein	1.12	2.02	0.71	2.16	0.35	1.28	0.52	0.82
Q2UVX4	C3	Complement C3	2.51	3.46	2.25	3.05	1.57	1.88	3.11	2.23
P68103	EEF1A1	Elongation factor 1-alpha 1	0.90	0.69	0.07	1.80	0.29	1.43	1.40	0.62
Q05927	NT5E	5-nucleotidase	0.44	1.06	-0.14	0.33	0.34	0.88	0.31	0.29
P62261	YWHAE	14-3-3 protein epsilon	0.67	0.23	0.04	2.00	0.35	0.73	1.09	0.23
Q9XSJ4	ENO1	Enolase	2.39	2.14	0.59	4.28	0.91	4.79	2.88	1.17
P04272	ANXA2	Annexin A2	0.53	0.26	0.39	1.74	0.30	0.52	0.27	0.22
P10096	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	2.85	2.21	1.63	4.54	1.08	4.79	3.50	2.23
Q5E9F7	CFL1	Cofilin 1 (Non-muscle)	1.43	0.46	0.36	3.50	0.07	2.93	2.21	0.45
Q3ZC00	LCP1	Lymphocyte cytosolic	3.24	3.28	1.92	4.72	1.31	4.28	3.75	3.40
F1N514	CD5L	CD5L protein	0.61	0.96	0.52	1.42	-0.20	0.53	0.03	1.01
Q3T149	HSPB1	Heat shock 27kDa protein 1	2.51	2.14	1.42	4.36	1.47	3.70	2.51	1.97
Q95122	CD14	Monocyte differentiation	0.39	0.54	-0.29	0.62	0.38	0.83	0.27	0.42

Table 5.2 Continued.

Uniprot entry	Gene name	Protein name	Average ratio	1	2	3	4	5	6	7
P63103	YWHAZ	14-3-3 protein zeta/delta	1.20	0.93	0.00	2.58	0.66	2.11	1.44	0.64
Q76LV1	HSP90AA1	Heat shock protein HSP 90-alpha	1.38	1.28	0.27	3.04	0.40	1.75	1.77	1.16
F1MIR4	RAB2A	Ras-related protein Rab-2A	0.19	0.11	0.07	0.35	0.11	0.17	0.40	0.11
P62157	CALM	Calmodulin	1.27	0.56	0.33	2.73	0.28	2.94	1.61	0.45
P05307	P4HB	P4HB protein	1.18	0.89	0.74	3.11	0.44	1.31	1.10	0.68
F1MMC6	HSPA5	78 kDa glucose-regulated protein	1.40	0.85	0.91	3.18	0.65	1.78	1.32	1.11
P68250	YWHAB	14-3-3 protein beta/alpha	1.13	0.96	0.47	2.40	0.30	1.91	1.39	0.46
Q5E9B1	LDHB	L-lactate dehydrogenase B chain	1.99	1.20	0.74	3.10	0.95	3.69	2.55	1.69
A1L528	RAB1A	Ras-related protein Rab-1A	0.27	0.32	0.23	0.58	-0.10	0.68	0.16	0.00
Q27965	HSPA1B	Heat shock 70 kDa protein 1B	0.83	0.82	0.35	1.05	0.48	1.89	0.71	0.53
P19803	ARHGDIA	Rho GDP-dissociation inhibitor 1	0.65	0.96	0.32	1.00	0.10	1.17	0.98	0.04
P49951	CLTC	Clathrin heavy chain 1	1.74	1.20	1.45	3.49	0.95	1.84	1.95	1.27
P38657	PDIA3	PDIA3 protein	1.71	1.07	0.99	3.69	0.84	2.57	2.07	0.76
P19483	ATP5A1	ATP synthase subunit alpha	2.08	1.89	1.63	3.77	1.27	2.22	2.38	1.41
Q863B3	SND1	Staphylococcal nuclease domain-containing protein 1	0.55	0.41	0.25	1.21	0.00	0.89	0.84	0.22

Table 5.3. Proteins with significantly higher concentration in small MFG and their $\log_2$ protein ratio in seven comparisons (large/small). Proteins were listed in order of descending protein intensity derived from MaxQuant.

Uniprot entry	Gene name	Protein name	Average ratio	1	2	3	4	5	6	7
Q27960	SLC34A2	Sodium-dependent phosphate transport protein 2B	-0.51	-0.41	-0.57	-0.45	-0.14	-0.84	-0.67	-0.47
P02663	CSN1S2	Alpha-S2-casein	-1.47	-0.77	-2.87	0.48	-1.82	-1.53	-1.96	-1.84
P26201	CD36	Platelet glycoprotein 4	-0.43	-0.18	-0.12	-0.60	-0.32	-0.68	-0.78	-0.35
Q1LZF6	ACSL1	Acyl-CoA synthetase long-chain family member 1	-0.54	-0.36	-0.55	-0.66	-0.33	-0.79	-0.58	-0.49
Q8WML4	MUC1	Mucin-1	-0.31	-0.49	-0.82	-0.58	-0.16	0.03	-0.05	-0.07
Q2KIS4	DHRS1	Dehydrogenase/reductase (SDR family) member 1	-0.42	-0.18	-0.20	-0.82	-0.13	-0.49	-0.82	-0.32
Q8MI01	MUC15	Mucin 15, cell surface associated	-0.61	-0.22	-0.95	-1.02	-0.30	-0.71	-0.63	-0.43
P84466	LSS	Lanosterol synthase	-0.32	-0.15	-0.07	-0.66	-0.12	-0.55	-0.51	-0.21

Table 5.3 Continued.

Uniprot entry	Gene name	Protein name	Average ratio	1	2	3	4	5	6	7
G3N2D8	GGT1	Gamma-glutamyltranspeptidase 1	-0.55	-0.44	-0.69	-0.86	-0.26	-0.68	-0.52	-0.40
Q2T9M8	SNAP23	Synaptosomal-associated protein	-0.20	0.14	-0.10	-0.27	-0.20	-0.36	-0.26	-0.35
Q3SYS6	CHP	Calcium-binding protein p22	-0.39	-0.62	-0.35	-0.21	-0.22	-0.49	-0.41	-0.40
P15396	ENPP3	Ectonucleotide pyrophosphatase/ phosphodiesterase family member 3	-0.51	-0.35	-0.55	-0.53	-0.33	-0.70	-0.61	-0.53
Q9TTS3	ACACA	Acetyl-CoA carboxylase 1	-0.47	-0.26	-0.94	-0.47	-0.42	-0.39	-0.43	-0.40
F1MD24	SGLT1	Na+/glucose cotransporter	-0.18	-0.18	-0.26	-0.03	-0.08	-0.47	-0.17	-0.08
Q3SX32	TIP47 /M6PRBP1	Mannose 6 phosphate receptor binding protein 1/TIP47	-1.19	-0.87	-0.94	-2.06	-0.96	-1.48	-0.97	-1.04
Q3ZBE9	NSDHL	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	-0.48	-0.68	-0.25	-1.18	-0.17	-0.43	-0.56	-0.08
D5HSX1	PRKG2	cGMP-dependant type II protein kinase	-0.10	0.04	-0.15	-0.17	-0.16	-0.09	0.00	-0.19
G3N0S9		Uncharacterized protein	-1.21	-1.88	-0.51	-0.93	-0.86	-2.27	-0.86	-1.18
F1MRA6	MUC16	Mucin-16	-0.45	-0.21	-0.57	-0.75	-0.40	-0.39	-0.54	-0.27
A5PK13	LRRC8C	Leucine-rich repeat-	-0.31	0.04	-0.23	-0.46	-0.34	-0.89	-0.26	-0.01
A4FUD2	HSD17B7	17-beta hydroxysteroid	-0.33	0.02	-0.17	-0.40	-0.47	-0.47	-0.48	-0.35
F1MEX9	ACSL3	Long-chain-fatty-acid--CoA ligase 3	-0.24	-0.40	0.02	-0.30	-0.34	-0.03	-0.27	-0.39
A3FPG8	AGPAT6	Glycerol-3-phosphate	-0.26	-0.38	-0.49	-0.20	-0.30	-0.46	-0.15	0.18

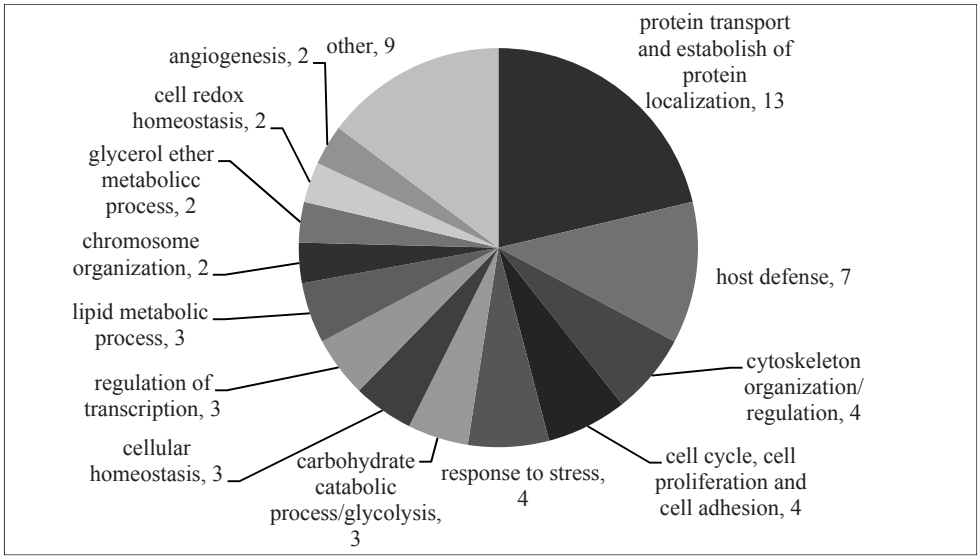

Figure 5.2. Biological functions of proteins with significantly higher concentration in large MFG compared to small MFG. Function was assigned based on gene ontology as well as literature references for each protein. The number of proteins in each function category is labelled in the chart.

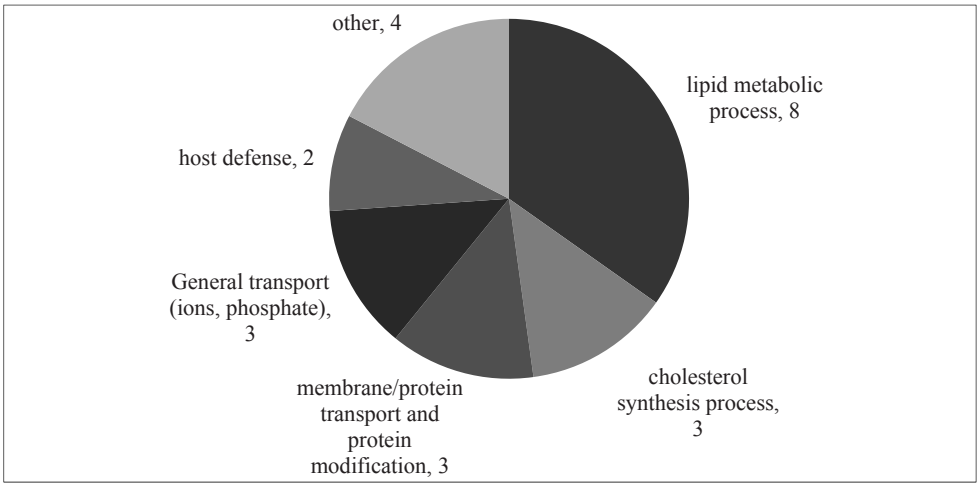

Figure 5.3. Biological functions of proteins with significantly higher concentration in small MFG compared to large MFG. Function was assigned based on gene ontology as well as literatures references for each protein. The number of proteins in each function category is labelled in the chart.

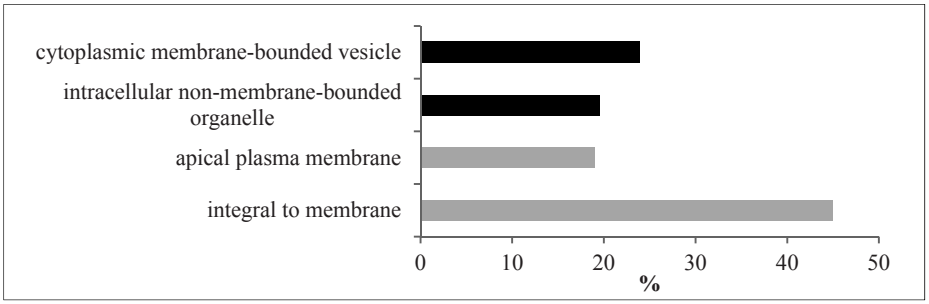

Figure 5.4. Gene ontology (cellular component) enrichment of significantly changed proteins in large and small MFG by using DAVID Bioinformatics Resources 6.7. The GO terms shown in this figure were significantly enriched ($p<0.05$) compared with the GO of all proteins identified in this study. Black: GO term enriched in large MFG. Grey: GO term enriched in small MFG.

5.4 Discussion

To our knowledge, the present study is the first proteome analysis of the membrane from MFG that have been separated according to size. Unlike factors that may influence the size of LD in other cell types, factors determining MFG size have not been extensively studied. That could be due to the complexity of MFGM, being a 3-layer membrane including ER membrane and apical plasma membrane, rather than the monolayer membrane of intracellular LD.

In lipid biogenesis studies, PAT proteins (perifilin/adipophilin/adipose differentiation related proteins/TIP47), which were also one of the most abundant proteins in MFGM, were considered essential (Ducharme and Bickel, 2008, Thiele and Spandl, 2008, Walther and Farese, 2009). In this study, two of these PAT proteins (ADFP and TIP47) were observed in bovine MFGM, with more ADFP in large MFG and more TIP47 in small MFG. This is comparable to a previous study on adipocytes that showed a higher concentration of ADFP in larger LD while more TIP47 in smaller LD (Wolins et al., 2005). The reason for this difference in distribution between large and small LD was, however, not clear. In order to create or maintain large LD, either accumulation of neutral lipid, fusion of small LD, or inhibition of triglycerides lipolysis have been suggested to play an important role (Ducharme and Bickel, 2008, Thiele and Spandl, 2008, Walther and Farese, 2009). ADFP was previously shown to be important for creating large LD by inhibiting lipolysis in mammary epithelial cells, which thereby may explain the higher concentration of ADFP in larger MFG (Russell et al., 2007). In the present study, another protein (GLYCAM1/PP3)

that could inhibit lipolysis (Sorensen et al., 1997) showed a higher concentration in large MFG. However, because of the high level of glycosylation, PP3 has been proposed to be present in the outer bilayer of MFGM, which is originating from apical plasma membrane instead of the intracellular membrane covering LD (Vanderghem et al., 2011). Whether this protein is involved in regulation of LD size before secretion is therefore not clear.

In the present study, a higher concentration of cytoplasmic vesicle proteins in large MFG was observed. This could indicate an association of proteins of cytoplasmic vesicles with the membrane of large MFG/LD. It has been shown previously that larger LD attracted more membrane-bound vesicles on their surfaces because of the long-range London-Van der Waals and electrostatic forces (Wooding, 1971, Wu et al., 2000). However, besides such a physical interaction, also an actively regulated process may play a role. This hypothesis is based on the function of these proteins in LD. In the present study, mainly three groups of cytoplasmic vesicle proteins are present in higher concentration in large MFG including heat shock proteins (HSPA8, HSPB1, HSP90AA1, HSPA5 and HSPA1B), 14-3-3 proteins (YWHAE, YWHAZ and YWHAB) and proteins from the Rab GTPase family (Rab 18, Rab 2A and Rab 6B). These proteins have been previously reported in bovine and human MFGM (Cavaletto et al., 2004, Reinhardt and Lippolis, 2006). Heat-shock proteins are chaperone proteins with main function of sustaining correct structure of proteins and preventing of aggregation. HSP70 (HSPA1B) has been observed in intracellular LD in rat adipocytes where its suggested function was stabilisation of the membrane monolayer, transporting of nascent proteins to LD, or renaturation of denatured proteins in the membrane layer (Jiang et al., 2007). 14-3-3 proteins may also help transport of membrane proteins out from ER to other organelles (Shikano et al., 2006). Rab proteins are a group of proteins that play an important role in almost every step in membrane trafficking, including vesicle formation, vesicle transportation and membrane fusion (Mountjoy et al., 2008). Among the Rab proteins, Rab 18 was observed in the surface of intracellular LD in HepG2 cells where it was involved in the apposition of ER and LD for transport of neutral lipid/fatty acids from ER to LD, which could result in the growth of LD (Ozeki et al., 2005). Based on this existing knowledge of the function of these cytoplasmic vesicle proteins on growth of LD and stability of its membrane, we propose that the increase of these proteins in large MFG is for the intracellular stabilization of the LD membrane and avoidance of lipolysis. However, further studies are needed to elucidate the function of these proteins in MFG biogenesis and stability.

The enrichment of non-membrane bound proteins, mainly cytoskeleton proteins (vimentin, plasmin L and actin) as while as cytoskeleton associated proteins (annexins and cofilin) in large MFG could suggest the involvement of these proteins in the heterogeneity in size of

MFG. The cytoskeleton, mainly microtubule, has previously been proposed to function in LD transport and fusion in different cell types as well as in epithelial cells of the mammary gland (Boström et al., 2005, Ducharme and Bickel, 2008, Wu et al., 2000). However, the presence of actin (microfilaments), vimentin (intermediate filaments) and their associated proteins (plasmin L and cofilin) instead of tubulin (microtubule) in this study could indicate the significance of microfilaments and intermediate filaments in addition to microtubule on the growth or transport of LD in mammary epithelial cells. Immunological evidence showed that actin was concentrated in the apical region of mammary epithelial cells and it was localized along to the cytoplasmic surface of LDs. Actin was suggested to help envelope LD by plasma membrane during budding to milk (Mather and Keenan, 1998). Beta-actin, the isoform also observed in this study, was shown to bind to intracellular LD in rat adrenocortical cells and adipocytes (Fong et al., 2001). Thus, one could assume that in the apical region of the cell, actin can bind to LD before its secretion and thereby regulate LD transport and growth, probably by being involved in LD fusion as microtubule proteins do. Another cytoskeleton protein observed in this study is vimentin. This protein has been identified previously in MFGM using proteomics analysis (Cavaletto et al., 2008). However, the reason of its presence in MFGM is unclear, because in mammary secretory cells, intermediate filaments are thought to be composed of cytokeratin rather than vimentin (Keenan and Dylewski, 1995). In adipocytes, however, vimentin intermediate filaments were observed to form cage-like structures around growing LD indicating its involvement in the growth of LD (Londos et al., 1999). This could possibly explain the higher concentration of vimentin in large MFG in this study.

Unexpectedly, host defense proteins, mainly cathelicidins, were found to differ the most between large and small MFG. Cathelicidins have been thought to mainly originate from neutrophils, which are the major type of somatic cells in milk (Smolenski et al., 2011). Contamination of the large MFG fraction with neutrophils may therefore seem an obvious reason for the high level of cathelicidins in it. This is however unlikely, because myeloperoxidase, the most abundant protein in neutrophils (Haegens et al., 2008), was not identified in MFGM. In addition, the number of neutrophils should be low due to the three washing and centrifugation steps used when isolating the MFG fractions. Because cathelicidin 1 ranked 15th based on the signal intensity of the 157 proteins identified in this study, it indicates that the origin of these cathelicidins might not only be neutrophils but also epithelial cells in the mammary gland. Cathelicidin-related antibacterial polypeptides have also been identified in the murine mammary gland (Murakami et al., 2005). However, to our knowledge, the expression of cathelicidins in bovine mammary gland has not been studied. Moreover, previous research has shown that cathelicidin-like proteins can bind to

plasma membrane lipid rafts of macrophages by selective interaction with phospholipids or cholesterol (Robinson et al., 2012). It has also been shown that the antimicrobial peptide part of the cathelicidin chain can bind with phospholipids in bacterial cell membranes (Nguyen et al., 2011, Sevcsik et al., 2007). The presence of cathelicidins in MFGM may thus be related to an interaction between the cathelicidins and the phospholipids present in the MFGM. The reason for the higher concentration of cathelicidins in large MFG may be related to the different membrane curvatures of large and small MFG (Walther and Farese, 2009) or to the differences in phospholipid composition of the MFGM (Walther and Farese, 2009), as the antimicrobial peptide of cathelicidin has been shown to interact differently with different phospholipids (Sevcsik et al., 2007).

Both of the cholesterol synthesis enzymes (LSS and NSDHL), identified in MFGM in this study, were present in higher concentrations in the small MFG fraction. Cholesterol is known to be an essential component for membrane integrity, permeability, fluidity and further functionality. Both LSS and NSDHL have been found to localize in intracellular LD in yeast and adipocytes (Caldas and Herman, 2003, Goodman, 2009, Mullner et al., 2004, Ohashi et al., 2003). These observations raised the possibility that LD could be the site of lipid/cholesterol synthesis rather than only one central lipid depot (Caldas and Herman, 2003). LD as the site of lipid/cholesterol synthesis could then be used to explain the higher concentration of LSS and NSDHL in small MFG since it requires relatively more membrane material, and therefore more cholesterol, to cover small MFG than large MFG. However, whether these enzymes in LD contribute to the synthesis of lipids is controversial. Ohashi et al (2003) observed that in CHO cells, NSDHL redistributed from LD to ER when depleting fatty acids in culture medium. However, elevating oleic acid increased the localization of NSDHL back to LD but with decreasing conversion of lanosterol to cholesterol. It thus seems that the translocation of enzymes from ER to LD decreased cholesterol synthesis either by inhibiting the enzyme activity or changing reaction environment. Thus LD was suggested to be a regulator of lipid metabolism by sheltering lipid synthesis related enzymes. The heterogeneous distribution of lipid synthesizing enzymes (higher concentration of ACSLs, ACACA, AGPAT6, LSS and NSDHL in small MFG; higher concentration of FASN in large MFG) might be regulated by intracellular lipid homeostasis. Higher demands on intracellular lipids could induce the fusion of small LD to large ones to release the enzymes back to ER or other organelles for more active lipid synthesis and further influence milk synthesis. However, cell biology studies focusing on the proteins discussed in this paper would be needed for further elucidation of the processes underlying the difference in fat globule size.

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Chapter 6

Changes in milk proteome and metabolome associated with energy balance in post parturient dairy cows

Abstract

The early lactation period of dairy cows, which produce high quantities of milk, is characterized by an insufficient energy intake to cover milk production and body maintenance requirements. Mobilization of body reserves occurs to compensate this negative energy balance (NEB) and probably as a consequence there is a higher susceptibility to diseases and metabolic disorders. There are several diagnostic methods to detect NEB, usually involving ketosis related parameters. Due to the easy availability of milk, this is a preferred matrix but simple and robust predictors of NEB level are missing. To better understand the physiological mechanism of NEB, milk of cows in different energy balance status and lactation stage were analysed by untargeted metabolomics and proteomics techniques. Milk of cows in severe NEB showed higher concentrations of acute phase response proteins, unsaturated fatty acids and galactose-1-phosphate. Improved EB resulted in higher concentration of cholesterol, cholesterol synthesis related proteins and stomatin. The presence of stomatin and galactose-1-phosphate in milk was strongly dependent on the EB of the cows.

6.1 Introduction

For high-producing dairy cows, energy intake capacity in early lactation is limited and cannot meet the energy requirements for milk yield and maintenance (de Vries and Veerkamp, 2000). As a consequence, dairy cows in early lactation experience an energy deficit, or negative energy balance (NEB). Cows compensate this energy deficit by mobilizing body reserves, which consist mainly of body fat (Tamminga et al., 1997). During this period of NEB, the immune system may be compromised (Mallard et al., 1998) and cows are known to be more susceptible to diseases and metabolic disorders, such as ketosis, mastitis and fatty liver (Grummer, 1993, van Knegsel et al., 2012). Thus, much attention has been paid to develop an indicator in milk for estimating energy balance (EB) or metabolic status in high-producing dairy cows. Indicators for EB and metabolism vary from total milk fat to protein ratio (Heuer et al., 2001, van der Drift et al., 2012), specific milk fatty acid content, to ketone bodies (Enjalbert et al., 2001, van der Drift et al., 2012). Disease events during NEB in early lactation can affect the total lactation performance of cows with implications for animal management and milk composition (Grummer and Rastani, 2003). Therefore, many studies have focused on nutritional and management strategies to improve the energy balance in order to reduce the incidence and severity of associated metabolic disorders (Dann et al., 2006, Odens et al., 2007, Van Knegsel et al., 2007). Recently, several studies showed that shortening or eliminating the dry period (DP) has potential to improve the EB of dairy cows in early lactation (de Feu et al., 2009, Rastani et al., 2005). This improved EB resulted mainly from a reduction in milk yield and had major consequences for plasma metabolites, including reduced concentration of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) (Schlamberger et al., 2010, Watters et al., 2008). In addition, the incidence of metabolic disorders, like ketosis (Rastani et al., 2005) and liver fattening (Andersen et al., 2005), in the next lactation was also reduced.

In recent years, developments in proteomics techniques led to the identification and characterization of an increasing number of low abundant proteins in milk, especially in the milk fat globule membrane (MFGM) fraction (D'Alessandro et al., 2011, Lu et al., 2011, Reinhardt and Lippolis, 2006). The MFGM can be considered as an ideal source to study proteins in mammary secretory cells (Cebo, 2012). Proteins present in this milk fraction originate from the apical plasma membrane, cytoplasm and endoplasmic reticulum membrane, covering different cellular locations and therefore functions (McManaman and Neville, 2003). These functions include, amongst others, milk synthesis and transport, and immune defense (Hettinga et al., 2011, Lu et al., 2011, Smolenski et al., 2007). Changes in

MFGM proteins may thus reflect changes in the function and metabolism of the mammary secretory cells.

A wide range of studies focused on specific metabolites in milk or plasma of dairy cows as biomarkers or predictors for health or metabolic status of dairy cows (Van Haelst et al., 2008) The use of an untargeted, broad-spectrum technique such as nuclear magnetic resonance (NMR) can give a more detailed view of the milk metabolome in relation to metabolic pathways involved in animal health or energy status. Recently, Klein et al (2012) used NMR to study the milk metabolome and elucidated the interaction of phospholipid and fatty acid metabolism, through possible phospholipid break down as energy provider in early lactation.

To our knowledge, little work has been done on studying a wide range of low abundant proteins and metabolites in milk of dairy cows in relation to EB in early lactation. The aims of the current study were first to quantify both low abundant proteins and metabolites in milk from cows during early lactation using advanced proteomics (FASP-dimethyl labelling-Orbitrap-LC-MS/MS) and metabolomics (^{1}H-NMR) techniques. Second, to relate the amount of low abundant proteins and metabolites in milk to the energy status and lactation stage of dairy cows in early lactation. Differences in energy status between cows were achieved by altering the DP length. To our knowledge, this is the first attempt to use a combination of the milk proteome and metabolome to elucidate mechanisms involved in the effect of NEB on changes in metabolomic pathways in cows and use this knowledge to further develop an indicator for NEB.

6.2 Materials and Methods

6.2.1 Cows and milk samples

Milk samples of 20 (of which 10 cows were used in metabolomics analysis) healthy Holstein cows after two different dry period lengths, 0 (0DP) and 60 days (60DP), were collected. Cows in both dry period regimes were fed the same diet postpartum and only cows in 2nd and 3rd lactation were selected. Milk samples from week 2 and week 14 postpartum of each cow were collected and stored at -20°C. Milk production, milk composition, and energy balance of selected cows were recorded. Fisher's LSD was applied for pairwise comparison of different samples.

6.2.2 Experimental Design

In proteomics analysis, cows from the same DP length (0DP or 60DP) were randomly divided in two groups as biological replicates (5 cows/group). The milk samples in each group were pooled for week 2 and week 14 separately. In metabolomics analysis, samples were analysed per individual cow per time moment. Three types of protein ratios were obtained which are associated with the effect of EB, and/or lactation stage (LS) on milk proteome, the protein ratio of 0DP vs 60DP in week 2 (mainly focusing on EB changes); the protein ratio of week 14 vs week 2 of 60DP (focusing on both EB and LS changes); and the protein ratio of week 14 vs week 2 of 0DP (mainly focusing on LS changes). In metabolomic analysis, data was compared in the same way.

6.2.3 Proteomics analysis

6.2.3.1 Separation of MFGM proteins and FASP-dimethyl labelling-nanoLC-Orbitrap-MS/MS

MFGM protein separation, preparation and analysis were performed in duplicate; and based on the method described by Lu et al. (2011). The pooled milk samples were centrifuged at 1500 g (10 min) to obtain cream (top layer) and skimmed milk (bottom layer). The cream (0.5 ml) was separated, washed with 5 ml of PBS (0.1M, pH 6.8) and centrifuged at 1500 g (10 min). Thereafter, the washing solution was disposed of. This was repeated three times. The washed milk cream was mixed with 0.4% SDS (1:1, v/v), sonicated for 1 min and centrifuged at 1500 g (10 min). Subsequently, the MFGM proteins (bottom layer) were separated from the remaining fat. The protein concentration was determined using the BCA assay (Thermo Scientific Pierce BCA protein assay kit, USA). MFGM proteins (10 μg) were prepared by using filter-aided sample preparation (FASP), and labelled with dimethyl labelling before LC-MS/MS measurements.

6.2.3.2 Data analysis

Protein identification and quantification. In total, 12 LC-MS/MS raw files were obtained (0DP vs 60DP in week 2, week 14 vs week 2 of 60DP, and week 14 vs week 2 of 0DP; with both biological and technical duplicates). All MS/MS spectra obtained in each run were analyzed by MaxQuant 1.2.2.5, with Andromeda as peptide search engine (Cox and Mann, 2008, Cox et al., 2011). The database for peptide/protein searches was a concatenated bovine reference database downloaded from Uniprot (www.uniprot.org) with reverse sequences generated by MaxQuant. The contaminants database of MaxQuant was also used for peptide/protein searches including sequences of trypsin and human keratins.

Identification and quantification of proteins was simultaneously performed in MaxQuant. Carbamidomethylated cysteine was set as fixed modification; oxidation of methionine, N-terminal acetylation and de-amidation of asparagine or glutamine were set as variable modification for both identification and quantification. A mass deviation of 0.5 Da was set as maximum allowed for MS/MS peaks, and a maximum of two missed cleavages were allowed. Maximum false discovery rates (FDRs) were set to 1% both on peptide and protein levels. Minimum required peptide length was six amino acids for both identification and quantification. Minimum of 2 peptides for each protein were required for reliable identification. Dimethyl labelling was adjusted to doublets with dimethLys0 and dimethNter0 as light and dimethLys4 and dimethNter4 as heavy. Razor and unique peptides were used for quantification. Normalized H/L ratio was used for further statistical analysis. A change in this protein ratio of 1.5-2 was used to find possibly changed proteins, whereas a ratio of 2 was set as threshold for significant differences (Lu et al, 2011).

Cluster analysis and GO enrichment analysis. Clusters of proteins were obtained according to the protein ratios described in section 6.2.2, with complete Euclidean as algorithm in Perseus 1.2.0.17 (MaxQuant). The gene ontology (GO) enrichment of clustered proteins was performed by using DAVID bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009). The result section shows the significantly enriched GO terms, compared to the GO of all identified proteins in this study (Fisher Exact test, $p<0.05$).

6.2.4 Metabolomics analysis

6.2.4.1 Sample preparation

Milk samples were thawed at room temperature. In order to isolate milk lipid and milk serum, samples were ultra-centrifuged at 117,500 g and 4ºC (75 min). The protein pellet was discarded, the lipid fraction was kept and the milk serum was further centrifuged at 16,000 g and 20ºC (20 min) for removal of remaining fat. For the measurement of milk serum metabolites, 175 µl of milk serum was thoroughly mixed with 175 µl of phosphate buffer (0.3M, pH 6.0, and 1mM of 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP; Sigma-Aldrich, Germany)).

Hydrophobic metabolites were extracted according to a slightly modified Folch method (Folch et al., 1957). The samples were dissolved in deuterated chloroform.

6.2.4.2 NMR measurements

Nuclear magnetic resonance spectrometer Avance III with a 600 MHz/54 mm UltraShielded Plus magnet equipped with a CryoPlatform cryogenic cooling system, a BCU-05 cooling unit, an ATM automatic tuning and matching unit. Measurements were done at 300K.

Baseline corrections and zero alignment were performed manually for each spectra. NOESY, JRES spectra were obtained for all milk serum samples and calibrated to internal TSP (δ=0.00 ppm). JRES data analysis was done according to Fonville et al. (2010).

6.2.4.3 Metabolite identification

Assignment of milk serum metabolites resonances was performed by comparing with published literature, the Human Metabolome Database version 2.0 online library (http://hmdb.ca/) and internal standards. Assignment of lipid metabolites resonances was performed by comparing with the work of Tukiainen et al. (2008), and Vinaixa et al. (2010).

6.2.4.4 Data Analysis

Data pre-processing and statistical analysis was performed using the free access software MultiExperiment Viewer (MeV), available at http://www.tm4.org/mev/. The data pre-processing included standardization by generating the $\log_2$ for the ratio of concentration over median of each compound. The tools used included: hierarchical clustering, data normalization and visualizations in heat maps, all included in the basic MeV package. Significant differences were calculated by paired Student's t-test, when comparing cows at the same DP in different weeks, or by a un-paired t-test when comparing different cows at the same week postpartum ($p<0.05$).

6.3 Results

6.3.1 Energy balance of the cows

Milk production traits and energy balance of the cows used in the proteomics and metabolomics analyses are shown in Table 6.1. In week 2, cows in 0DP have a less negative energy balance than cows in 60DP. Further, compared to week 2, the energy balance is increased in week 14. The difference in EB is larger in cows with a 60DP (454 $kJ/kg^{0.75}\cdot d$) than in cows with a 0DP (234 $kJ/kg^{0.75}\cdot d$).

Table 6.1. Milk production, milk composition, and energy balance of dairy cows in early lactation (week 2 and week 14 postpartum) after 0DP or 60DP. Twenty cows were used for proteomics analysis and a subset of ten cows were used for metabolomics analysis. Data represent means (±SD).

	Proteomics analysis				Metabolomics analysis			
	0DP 10 cows		60DP 10 cows		0DP 5 cows		60DP 5 cows	
Week postpartum	2	14	2	14	2	14	2	14
Milk yield (kg/d)	32.05±5.94^{a}	35.40±6.98^{ac}	37.95±3.82^{bc}	41.02±3.00^{b}	31.84±5.01^{a}	35.35±6.74^{ab}	38.73±3.69^{b}	40.20±3.49^{b}
Lactose (%)	4.49±0.15^{ab}	4.54±0.10^{ab}	4.44±0.13^{a}	4.55±0.10^{b}	4.55±0.09^{a}	4.55±0.10^{a}	4.39±0.17^{a}	4.56±0.12^{a}
Fat (%)	5.16±0.61^{ab}	4.54±0.75^{ac}	5.11±0.73^{a}	4.14±0.68^{c}	5.30±0.73^{a}	4.75±0.86^{a}	5.10±0.43^{a}	4.50±0.63^{a}
Protein (%)	4.21±0.42^{a}	3.74±0.36^{b}	3.82±0.26^{b}	3.41±0.24^{c}	4.18±0.41^{a}	3.78±0.41^{ab}	3.94±0.27^{a}	3.48±0.24^{b}
EB[1] (kJ/kg$^{0.75}$·d)	-164±139^{a}	71±56^{b}	-462±170^{c}	-8±79^{b}	-162±132^{a}	70±48^{b}	-468±144^{c}	-13.±85^{b}

[1]Energy balance; calculated with the VEM system (Van Es, 1975)

$^{a, b, c}$ values in the same row with different superscripts differ significantly within proteomics analysis and metabolomics analysis (p<0.05)

6.3.2 Proteomics analysis of milk fat globule membrane

Three hundred proteins were identified and quantified in MFGM. Of these, 44% are membrane proteins, including plasma membrane and intracellular membrane of organelles such as endoplasmic reticulum, mitochondria, and Golgi apparatus. 26% of the three hundred proteins are originating from cytoplasm, whereas secreted proteins are the third largest group (22%). The effect of an increase in EB in week 2 due to shortening of DP (0DP vs 60DP in week 2) resulted in 20 proteins different in concentration (Figure 6.1, left circle, supplementary Table S6.1). The effect of an increase of EB and LS in cows with a 60DP (week 14 vs week 2 of 60DP) resulted in the largest change in the milk proteome with 40 proteins changed in concentration (Figure 6.1&6.2, right circle, Table S6.2). The effect of an increase in LS of cows with a 0DP (week 14 vs week 2 in 0DP) resulted in the least differences in the milk proteome with 15 proteins changed in concentration (Figure 6.2, left circle, Table S6.3). Increase of EB due to shortening of DP may include other factors affecting the milk proteome, such as morphological differences in the mammary gland. The same is valid for an increase of EB and LS in cows with a 60DP. Therefore, only proteins that changed significantly in both comparisons were considered to be specifically resulting from an increase in EB. Acyl-CoA synthetase long-chain family member 1 (ACSL1), stomatin (STOM) and NADH-cytochrome b5 reductase 3 (CYB5R3), which are proteins that are related to lipid metabolism, were found to consistently increase

with increasing EB. An increase in EB was also related to a decrease in lactoperoxidase (LPO) and lipopolysaccharide-binding protein (LBP), which are host defense related proteins (Figure 6.1). An increase in LS in 60DP is concomitant with an increase in EB (week 14 vs week 2 of 60DP), whereas in 0DP the energy effect in changes in milk proteome is hampered (week 14 vs week 2 of 0DP). Therefore the changed proteins in the intersection of these two comparisons were considered to be mainly the result of an increase in LS. These proteins are three immunoglobulins (IGJ, IGK and IGL@) and complement C3 (C3). The unchanging of these proteins when assessing the proteome changes due to EB (Figure 6.2), strengthens the idea that the changes of these proteins are due to increased LS.

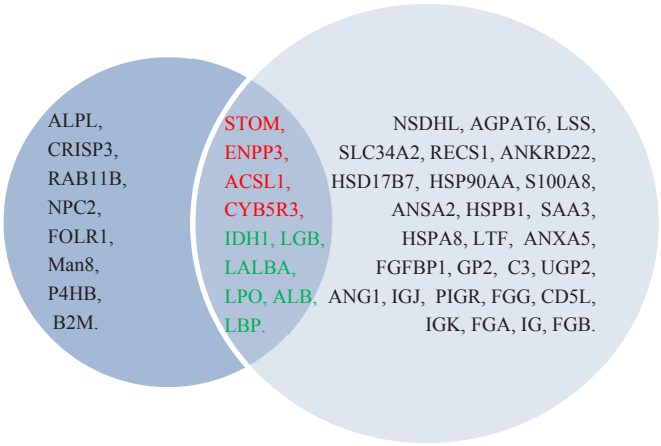

Figure 6.1. Venn diagram of changes in milk proteome according to EB. Circle on the left shows the proteins with a changed ratio (> 1.5 fold) in 0DP vs 60 DP, in week 2. Circle on the right shows the proteins with a changed ratio (>1.5 fold) in week 14 vs week 2 of 60DP. Proteins in the intersection of both circles are considered to be changed due to an increase in EB, because they were consistently changed in both comparisons. Proteins in red increased as EB increased, whereas proteins in green decreased as EB increased.

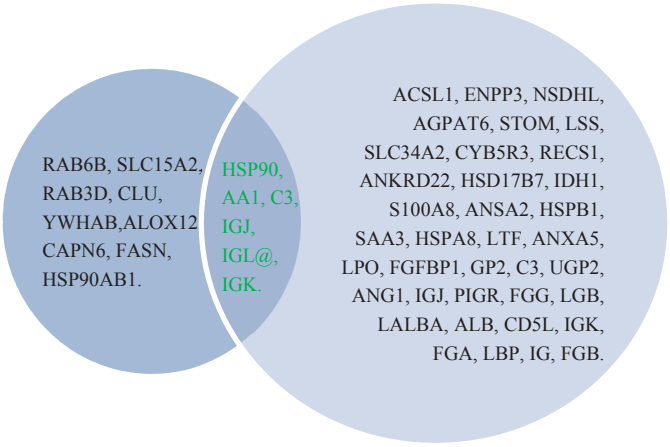

Figure 6.2. Venn diagram of changes in milk proteome according to LS. Circle on the left shows the proteins with a changed ratio (>1.5 fold) in week 14 vs week 2 of 0DP. Circle on the right shows the proteins with a changed ratio (>1.5 fold) in week 14 vs week 2 of 60DP. Proteins in the intersection of both circles are considered to be changed due to an increase in LS, because they were consistently changed in both comparisons. Proteins in green decreased as LS increased.

The results presented in Figures 6.1&6.2 show changes of individual proteins due to increases of EB or LS. However, proteins usually do not act alone, but work together in protein interaction networks. To study this interaction, the biological process associated with changes in EB and LS was identified by performing cluster analysis based on protein ratio and gene ontology (GO) enrichment analysis. Figure 6.3 shows clusters based on protein ratios of 0DP vs 60 DP in week 2; week 14 vs week 2 of 60DP; and week 14 vs week 2 of 0DP. Two clusters delimited by yellow can be seen in the heatmap. Cluster A comprises proteins related to the cholesterol biosynthesis process (Figure 6.4). These proteins are more abundant with an increase in EB and/or LS (Figure 6.3). This is seen in all the three comparisons, but the effect is more obvious when cows transit from week 2 to week 14 after a 60DP. This is also the comparison where the difference in EB between groups is the largest. Cluster B comprises proteins mainly involved in acute phase response (Figure 6.4), and are less abundant with an increase in EB and/or LS (Figure 6.3).

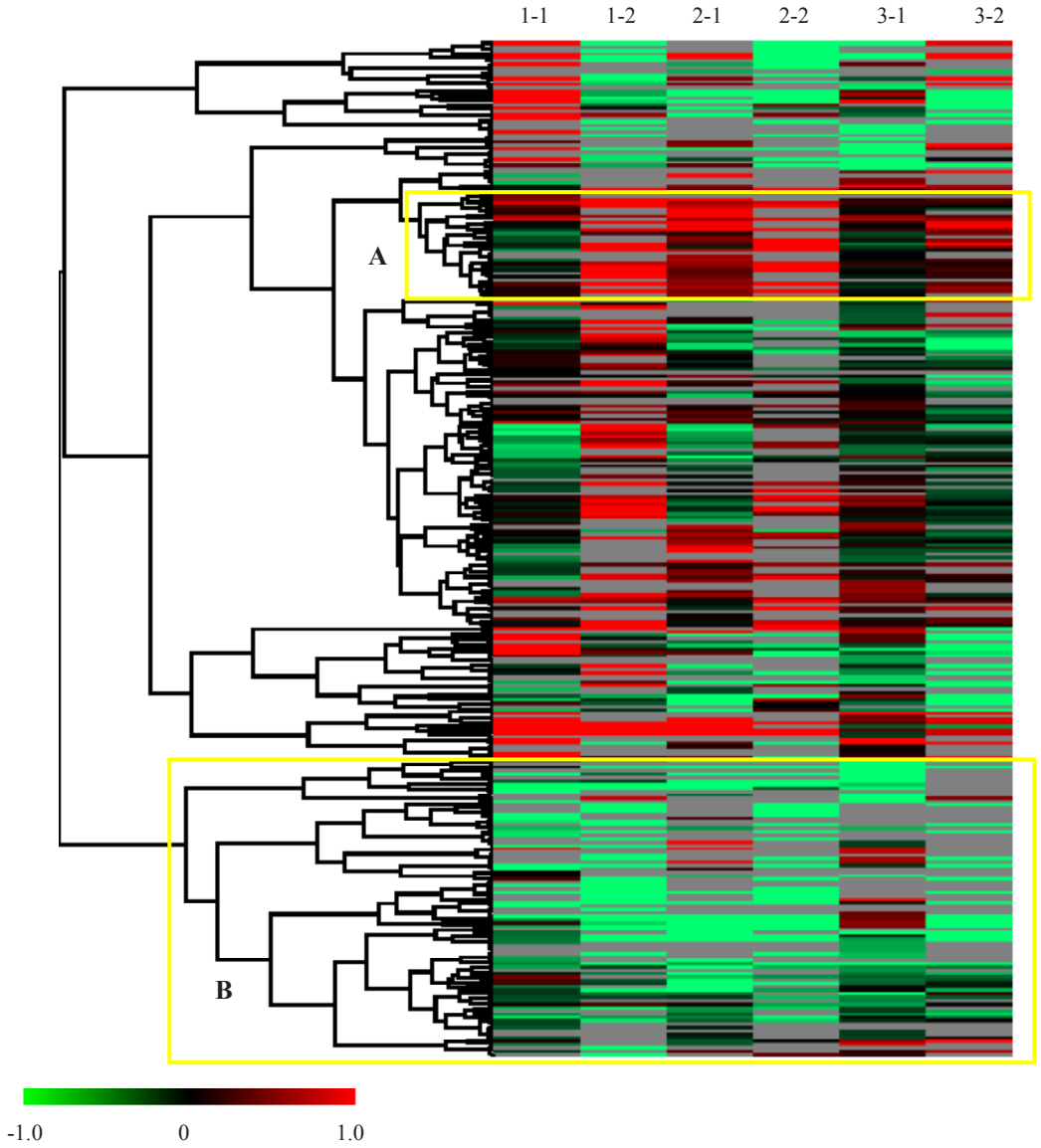

Figure 6.3. Heatmap of log_2 protein ratio with increased EB, 1-1 and 1-2 (biological duplicates of 0 DP vs 60 DP in week 2); increased EB and LS, 2-1 and 2-2 (biological duplicates of week 14 vs week 2 of 60DP) and increase LS 3-1 and 3-2 (biological duplicates of week 14 vs week 2 of 0DP). Two clusters of proteins are delimited by yellow. **A**, cluster of proteins which increased with increased EB and LS. **B**, cluster of proteins which decreased with increased EB and LS. Complete Euclidean algorithm was applied in cluster analysis by using Perseus 1.2.0.17.

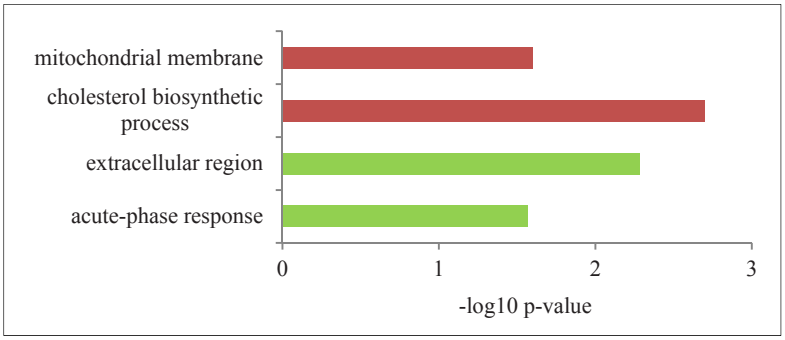

Figure 6.4. GO enrichment for subcellular location and biological processes of proteins in cluster A (red bars) and cluster B (green bars) as shown in Figure 6.3. Comparison was made to GO terms of all identified proteins in the present study (DAVID bioinformatics Resources 6.7) ($p<0.05$).

6.3.3 Metabolomics results

Milk serum and milk lipid spectra of 10 cows in week 2 and week 14 were analyzed and compared for assessing the effect of EB and/or LS on the milk metabolome. Analysis of milk serum spectra resulted in integration of 45 non-redundant peaks. Thirty peaks were assigned either to a compound or a family of compounds, whereas 15 remained unknown. Similar to proteomic data analysis, results were analyzed mainly focusing on the effect of increasing EB (0DP vs 60DP, week 2), increasing EB and LS (week 14 vs week 2, 60DP) and increasing LS (week 14 vs week 2, 0DP), as shown in Figures 6.5 to 6.7.

Increase in EB and LS (transition from week 2 to week 14 in 60 DP cows) resulted in 13 significantly decreased milk serum metabolites. These include acetyl-carnitine, phosphorylcholine, phosphocreatine, galactose-1-phosphate, glutamate, several UDP-hexoses and several N-acetylated sugars. Choline was the only serum metabolite that significantly increased with increased EB and LS (Figure 6.6).

Figure 6.5 shows the effect of increased EB on milk serum metabolome, due to shortening of DP (0DP vs 60 DP, week 2). This resulted in 13 metabolites being significantly changed. Galactose-1-phosphate significantly decreased and acetate, acetoacetate, alanine, beta-hydroxybutyrate, free carnitine, N-acetylated sugars, oxaloacetate, proline, pyruvate and succinate significantly increased. The increase of EB due to shortening of DP may include other factors affecting milk serum metabolome, such as morphological differences. The same is valid for an increase of EB and LS in cows with 60DP. Therefore only metabolites changing significantly in both comparisons were considered to be resulting exclusively

from an increase in EB. In this study, the only consistently changed metabolite was galactose-1-phosphate.

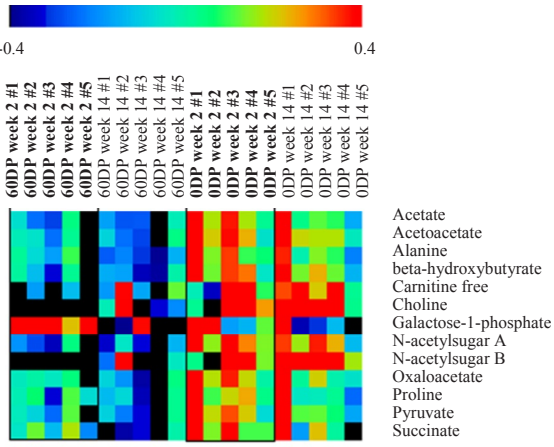

Figure 6.5. Changes in the milk serum metabolome mainly according to increased EB (0DP vs 60DP, week 2). Significant differences were calculated by unpaired Student's t-test.

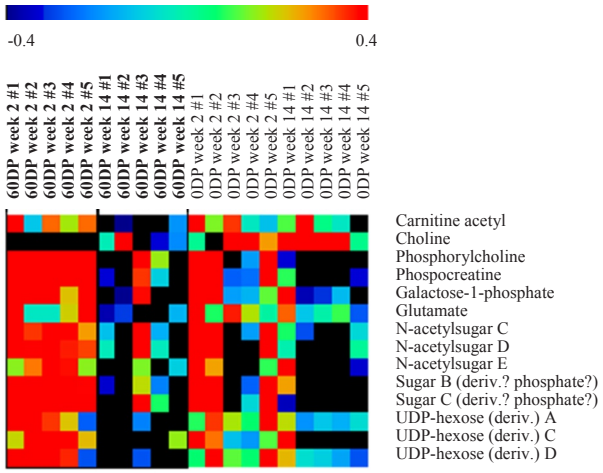

Figure 6.6. Changes in the milk serum metabolome according to an increase in EB and LS (week 2 vs week 14, 60DP). Significant differences were calculated by paired Student's t-test.

The heatmap based on the increase in LS (week 14 vs week 2, 0DP) shows a significant decrease in acetate, acetone, butyrate, beta-hydroxybutyrate, phosphorylcholine, phosphocreatine, glutamate, hippurate, N-acetyl-sugars, oxaloacetate, proline, pyruvate, and UDP-hexose (Figure 6.7).

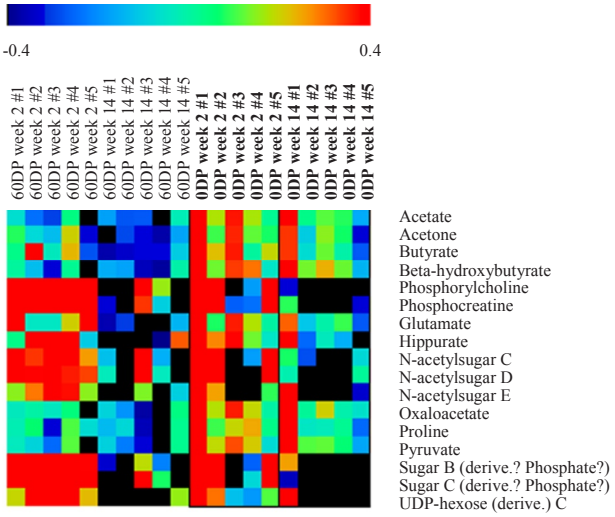

Figure 6.7. Changes in the milk serum metabolome mainly according to increased LS (week 2 vs week 14, 0DP). Significant differences were calculated by paired Student's t-test.

In the spectra of the milk lipid fraction, several lipid components were identified. In the data analysis we have focused on the amount of fatty acids (FA), unsaturated FA (UFA), polyunsaturated FA (PUFA) and cholesterol (TC). The amount of these compounds was calculated, relativize to the amount of triacylglycerols (TAG). The transition from week 2 to week 14 resulted in a significant decrease of UFA. The amount of PUFA and FA in milk fat did not change; TC increased (Figure 6.8), although this was only significant in cows with a 60DP (data not shown). Figure 6.9 depicts the chemical shifts and respective chemical bonds assigned to the above mentioned lipid metabolites.

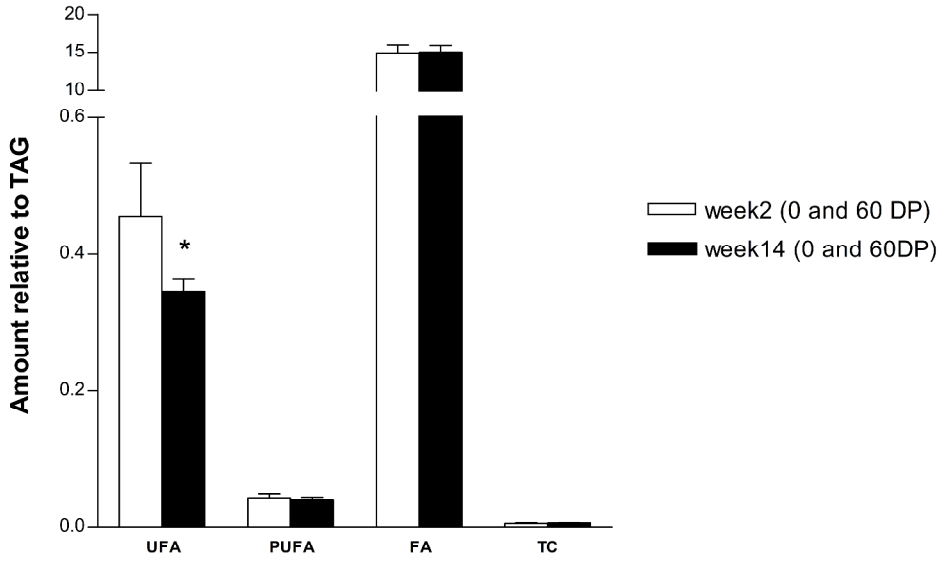

Figure 6.8. Changes in relative amount of lipid metabolites present in milk fat. Bars represent average amount (±SD) of lipid metabolite in week 2 and week 14 postpartum (n=10),*p<0.05

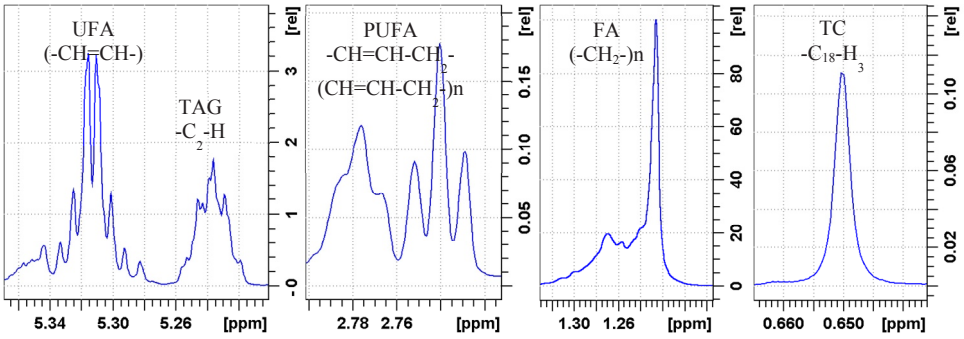

Figure 6.9. Sections of the ^{1}H-NMR spectra of a milk lipid extract, depicting the chemical shifts and chemical bonds of the five metabolites that were quantified in this study.

6.4 Discussion

6.4.1 Indicators for energy balance and metabolic health

A long list of studies is available which present or evaluate a variety of indicators in milk for estimating energy balance or metabolic status in high-producing dairy cows in early

lactation. Indicators for energy balance and metabolism vary from total milk fat to protein ratio (Heuer et al., 2001, van der Drift et al., 2012), specific milk fatty acid content (Van Haelst et al., 2008), to ketone bodies (Enjalbert et al., 2001, van der Drift et al., 2012). Most indicators concern major components in milk or metabolites directly associated with disease, like ketone bodies. It can be expected that alterations in the milk proteome and metabolome not only unravel alterations in biochemical pathways associated with NEB, as will be discussed below, but also can be considered as an indicator for energy status or metabolic health of the cow. In this perspective, Klein et al. (2012) showed that the ratio of glycerophosphocholine to phosphocholine in milk is an early predictor for ketosis in dairy cows. The current study focused on healthy cows and shows that NEB in early lactation is associated with increased concentrations of galactose-1-phophate and decreased concentrations of stomatin pointing to these two compounds as possible indicators for NEB.

6.4.2 Acute phase response during negative energy balance

The periparturient period of dairy cows is associated with a compromised immune system (Mallard et al., 1998). This can be related to major metabolic demands imposed by the start of lactation, which results in a NEB and increased risk for metabolic disorders (Grummer, 1993). The present study (cluster B in Figure 6.3) shows that in healthy cows with lower EB and earlier in lactation (week 2), the MFGM contains more proteins related to acute inflammatory and immune response (alpha-1-acid glycoprotein, alpha-2-HS-glycoprotein, beta-2-microglobulin, complement C3, complement component C9, fibronectin, IGL protein, IGK protein, inter-alpha-trypsin inhibitor heavy chain H4, lipopolysaccharide-binding protein, MHC class I antigen, peroxiredoxin-1, prothrombin, syntaxin binding protein 2 and zinc- alpha-2-glycoprotein, Table S6.5). Due to the specific origin of MFGM, this could imply that these acute phase proteins are present in higher concentrations in or on mammary epithelial cells of cows in NEB and early lactation. This is in line with recent studies, indicating that dairy cows in early lactation have increased plasma concentrations of acute phase proteins like haptoglobulin and ceruloplasmin (Bossaert et al., 2012). Previous studies have shown that high yielding and clinically healthy cows, also have inflammatory responses in liver and uterus, during the transition period and early lactation (Bionaz et al., 2007, Trevisi et al., 2012, Wathes et al., 2009), although the exact mechanisms behind this effect are not completely understood. There are several hypotheses explaining this inflammatory response, including elevated levels of circulating NEFAs.

A first hypothesis concerns the change in FA composition in milk and blood of the periparturient cow. The increase of energy needs at the onset of lactation of periparturient cows is associated with mobilization of body reserves (fat and protein). This period, in

which extended lipolysis of adipose tissue occurs, is responsible for partitioning of NEFAs into the blood stream. This partitioning will influence the FA profile in milk, for example by an increase in UFA concentration, mainly C18:1 (Mather and Keenan, 1998, Rukkwamsuk et al., 2000). The same trend was observed in our results for milk fat composition, which showed a higher concentration of UFA in NEB. In cows, the presence of UFA has been related to pro-inflammatory diseases such as mastitis and metritis (Sordillo et al., 2009). Also in humans, an increased level of circulating NEFAs in blood is associated with increased systemic inflammatory conditions (Wood et al., 2009). The exact mechanisms explaining these conditions are not completely clear. However, studies suggest that NEFAs can interfere with membrane fluidity, lipid rafts, receptor binding and activation of signal transduction cascades, leading to inflammatory responses (De Lima et al., 2007, Wood et al., 2009, Yaqoob and Calder, 2007). A second hypothesis concerns the stress associated with parturition in dairy cows. The stress induced by the rapid increase in milk production can explain the acute phase response in NEB. In epithelial cells, the onset of milk production requires high efficient activities of transport, synthesis and secretion of milk components, challenging the homeostasis of the mammary gland. This can result in an acute stress response that stimulates the transient adaptation when other homeostatic mechanisms (e.g. mammary gland epithelial cell proliferation and differentiation) cannot cope with this high demand for milk production (Medzhitov, 2008). It is also known that physiological stress, and damage or malfunction of tissue can induce an inflammatory response (Medzhitov, 2008). These physiological processes taking place in periparturient cows may thus explain why acute phase proteins are present at higher concentrations in cows in early lactation after a 60DP.

6.4.3 Galactose-1-phosphate in milk serum during negative energy balance

Results of the current study show the presence of galactose-1-phosphate in milk serum of cows in severe NEB (week2 of 60DP) but not, or very low amount, for cows with an improved EB (week 2 of 0DP and week 14). Sugar phosphates are intermediates in lactose synthesis, a process confined to cell cytosol and Golgi apparatus (Kuhn et al., 1980). Consequently, the presence of galactose-1-phosphate in severe NEB suggests that there is a leakage of these components from mammary epithelial cells into milk, possibly through apoptotic cells. It has been previously reported that the apoptotic index in mammary gland of cows in early lactation can be up to 4-fold higher than in later lactation (Capuco et al., 2001). As discussed above, during the onset of lactation, there is excessive metabolic stress and stress related to milk production which can induce apoptosis in a higher percentage of epithelial cells in mammary gland (Kerr et al., 1972, Medzhitov, 2008, Monks et al., 2008,

Potten, 1992). This could then also explain the presence of galactose-1-phosphate in milk serum from cows with NEB, which to our knowledge has never been reported before.

6.4.4 Lack of components in lipid rafts in cellular membrane system during negative energy balance

Results on protein composition of MFGM (cluster A in Figure 6.3) in the current study shows a decrease of enzymes and transporters related to cholesterol synthesis (apolipoprotein A-I, HSD17B7 protein, lanosterol synthase, NADH-cytochrome b5 reductase3 and sterol-4-alpha-carboxylate 3-dehydrogenase, Table S6.4) in cows with severe NEB. Further, ^{1}H-NMR data showed that the concentration of cholesterol in milk fat is lower in the same cows, indicating that in cows in NEB, the synthesis and transport of cholesterol into milk is impaired. Considering that MFGM accounts for the majority of cholesterol present in milk (Long et al., 1980), results of the current study indicate that MFGM, and therefore the membrane of mammary epithelial cells of cows in severe NEB, contains less cholesterol. Stomatin (STOM), which is a protein found in lipid rafts in cell membranes (Snyers et al., 1998), showed the same trend as cholesterol. Lipid rafts are organized subdomains of the plasma membrane that contain high concentrations of cholesterol, combined with specific proteins like STOM (Mairhofer et al., 2009, Pike, 2003). It can therefore be hypothesized that a decrease in cholesterol and STOM in milk of NEB cows is related to a differently organized, or a change in the number of, lipid rafts in MFGM. This can have implications on the organization and therefore function of the membrane on epithelial cells in the mammary gland. The presence of STOM, instead of other usual scaffolding proteins (calveolin and flotillin) in lipid rafts (Umlauf et al., 2004) could indicate that in MFGM the lipid rafts are centred around, and organized by, STOM. To our knowledge, this is the first time the EB of dairy cows is related to organization of the plasma membrane in general or lipid rafts in particular. The exact reasons and consequences of this difference of STOM centred lipid raft for the secretion of milk will need to be determined in detail in future research. It is well known, however that lipid rafts are important for many biological processes of cells, including T-cell signalling, sorting of lipid and proteins (Simons and Vanmeer, 1988), regulation of ion channels (Dart, 2010) and calcium homeostasis (Isshiki and Anderson, 1999).

6.4.5 Lipid and energy metabolism in negative energy balance

Results of the current study indicate that NEB is associated with increased inflammatory response, apoptosis of epithelial cells and changes in lipid metabolism. This suggests that the mammary epithelial cells of cows in NEB, compared with cows in improved EB, are

differently organized and can therefore be thought to work differently. Therefore, it can be hypothesised that the alteration of cellular activity in NEB cows can be related to the underdevelopment of mitochondria, endoplasmic reticulum (ER) and mitochondria-associated ER membrane (MAM) in epithelial cells. Communication between mitochondria, ER and MAM is paramount in signal transduction, bioenergetics, metabolism, cell death, etc. The region where mitochondria, ER and MAM associate is also the centre for lipid synthesis activity. Mitochondria and MAM may provide energy and NADPH for TAG synthesis and biogenesis of other lipids (Walther and Farese, 2009). Cholesterol is synthesized in ER and thereafter transported to other organelles (Van Meer et al., 2008). The physical membrane connection between mitochondria and ER, MAM, contains high levels of cholesterol, which in turn is associated with lipid rafts in the membranes (Hayashi et al., 2009). Furthermore, MAM is involved in compartmentalization of signalling proteins between ER and mitochondria. Some of these are receptors (e.g. IP3) involved in cell viability due to its regulation of calcium homeostasis. Therefore, a lower cholesterol synthesis and concentration, as shown in this study, could affect the organizational properties of MAM and interfere with calcium homeostasis, which in turn is associated with the previously discussed apoptotic cells in NEB. Further, phospholipids and calcium are also transported through MAM to mitochondria, which is important for proper functioning of the citric acid cycle. This is of paramount importance in mammary epithelial cells, since the proper production of ATP and NADPH are important in fatty acid synthesis (Palmquist, 2006). The higher concentration of UFA, probably originating from body fat reserves, combined with a lower concentration of saturated fatty acids, in NEB cows, indicates that the *de novo* synthesis, for which mitochondria are vital, is occurring less efficiently. Concomitant mobilization of fatty acids from adipose tissue occurs, which can induce the acute phase response.

Studies during the lactation cycle show that development of mitochondria and ER in bovine epithelial cells increases only after parturition, until peak production of milk (Qu et al., 2012). This suggests that peak lactation is related to the full development of mitochondria and ER, even though most cows will still be in a NEB. Further, in mice mammary gland, the major phase of mitochondrial development is also after parturition, which is slightly after epithelial cell proliferation (Jones and Rosano, 1972). Due to the paramount role of ER, MAM and mitochondria in lipid metabolism and energy production, the underdevelopment of those organelles in NEB cows may be responsible for the difference in lipid composition of milk, in particular cholesterol.

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Supplementary data

Table S6.1. Proteome changes in MFGM according to an increase in EB in week 2 due to shortening of DP (0DP vs 60DP in week 2). 1 and 2 are biological duplicates. Proteins with 1.5-2-fold change were shown and a 2-fold change was used as significant level (in bold). Ratios shown are $\log_2$ ratio.

Uniprot entry	protein name	Gene name	1	2
P09487	**Alkaline phosphatase, tissue-nonspecific isozyme**	**ALPL**	**3.11**	**3.41**
F1N2R1	**STOM protein**	**STOM**	**3.08**	**2.36**
F6R3I5	**Cysteine-rich secretory protein 2**	**CRISP3**	**1.88**	**1.04**
P15396	**Ectonucleotide pyrophosphatase/phosphodiesterase family member 3**	**ENPP3**	**1.70**	**2.05**
Q1LZF6	**Acyl-CoA synthetase long-chain family member 1**	**ACSL1**	**1.34**	**2.69**
Q3MHP2	Ras-related protein Rab-11B	RAB11B	0.80	0.71
P07514	NADH-cytochrome b5 reductase 3	CYB5R3	0.79	1.61
Q9XSG3	Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1	-0.72	-1.14
P02754	Beta-lactoglobulin	LGB	-0.74	-1.22
P79345	Epididymal secretory protein	NPC2	-0.86	-1.41
P02702	Folate receptor alpha	FOLR1	-0.91	-0.60
P13753	MHC class I antigen	Man8	-0.92	-1.63
Q9TRB9	Enterotoxin-binding glycoprotein PP20K		-0.99	-1.21
P05307	Protein disulfide-isomerase	P4HB	-0.99	-1.55
P00711	**Alpha-lactalbumin**	**LALBA**	**-1.24**	**-1.55**
P01888	**Beta-2-microglobulin**	**B2M**	**-1.39**	**-1.74**
P80025	**Lactoperoxidase**	**LPO**	**-1.51**	**-1.76**
P02769	**Serum albumin**	**ALB**	**-1.69**	**-1.23**
G3N1R1	**Uncharacterized protein**		**-1.86**	**-1.79**
Q2TBI0	**Lipopolysaccharide-binding protein**	**LBP**	**-2.22**	**-2.25**

Table S6.2. Proteome changes in MFGM according to an increase of EB and LS in cows with a 60DP (week 14 vs week 2 of 60DP). 1 and 2 are biological duplicates. Proteins with 1.5-2-fold change were shown and a 2-fold change was used as significant level (in bold). Ratios shown are $\log_2$ ratio.

Uniprot entry	Protein Name	Gene name	1	2
Q1LZF6	**Acyl-CoA synthetase long-chain family member 1**	**ACSL1**	**2.09**	**3.12**
P15396	**Ectonucleotide pyrophosphatase/phosphodiesterase family member 3**	**ENPP3**	**1.92**	**1.08**
Q3ZBE9	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	NSDHL	1.18	0.88
A3FPG8	**Glycerol-3-phosphate acyltransferase 4**	**AGPAT6**	**1.03**	**2.95**
F1N2R1	STOM protein	STOM	0.98	2.91
P84466	Lanosterol synthase	LSS	0.79	1.74
Q27960	Sodium-dependent phosphate transport protein 2B	SLC34A2	0.71	1.72
P07514	NADH-cytochrome b5 reductase 3	CYB5R3	0.65	0.86
Q6QRN7	PP1201 protein	RECS1	0.63	0.68
A2VDV1	Ankyrin repeat domain 22	ANKRD22	0.62	1.38
A4FUD2	17-beta hydroxysteroid dehydrogenase	HSD17B7	0.61	2.16
Q76LV2	Heat shock protein HSP 90-alpha	HSP90AA1	-0.59	-1.68
Q9XSG3	Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1	-0.60	-0.82

Table S6.2 Continued.

Uniprot entry	Protein Name	Gene name	1	2
P28782	Protein S100-A8	S100A8	-0.65	-1.16
P04272	Annexin A2	ANXA2	-0.81	-0.89
Q3T149	Heat shock 27kDa protein 1	HSPB1	-0.83	-1.47
F1MHZ0	Serum amyloid A protein	SAA3	-0.95	-1.65
P19120	Heat shock cognate 71 kDa protein	HSPA8	-1.04	-0.70
P24627	Lactotransferrin	LTF	-1.11	-0.92
P81287	Annexin A5	ANXA5	-1.18	-0.61
P80025	**Lactoperoxidase**	**LPO**	**-1.20**	**-1.58**
Q9MZ06	**Fibroblast growth factor-binding protein 1**	**FGFBP1**	**-1.37**	**-1.07**
F1N726	**Glycoprotein 2 (Zymogen granule membrane)**	**GP2**	**-1.54**	**-1.45**
Q2UVX4	**Complement C3**	**C3**	**-1.58**	**-1.46**
Q07130	**UTP--glucose-1-phosphate uridylyltransferase**	**UGP2**	**-1.75**	**-2.04**
P10152	**Angiogenin-1**	**ANG1**	**-1.81**	**-2.33**
Q3SYR8	**Immunoglobulin J chain**	**IGJ**	**-1.92**	**-2.26**
P02754	**Major allergen beta-lactoglobulin**		**-2.31**	**-2.15**
P81265	**Polymeric immunoglobulin receptor**	**PIGR**	**-2.34**	**-1.27**
Q3T101	**IGL@ protein**	**IGL@**	**-2.40**	**-2.23**
P12799	**Fibrinogen gamma-B chain**	**FGG**	**-2.43**	**-1.20**
P02754	**Beta-lactoglobulin**	**LGB**	**-2.53**	**-1.00**
P00711	**Alpha-lactalbumin**	**LALBA**	**-2.53**	**-2.07**
P02769	**Serum albumin**	**ALB**	**-2.55**	**-2.43**
F1N514	**CD5L protein**	**CD5L**	**-2.88**	**-1.64**
F1MH40	**IGK protein**	**IGK**	**-2.94**	**-1.41**
P02672	**Fibrinogen alpha chain**	**FGA**	**-2.99**	**-2.90**
Q2TBI0	**Lipopolysaccharide-binding protein**	**LBP**	**-3.45**	**-2.16**
A5D7Q2	**IG**	**IG**	**-3.45**	**-2.04**
P02676	**Fibrinogen beta chain**	**FGB**	**-4.15**	**-2.49**

Table S6.3. Proteome changes in MFGM according to an increase in LS of cows with a 0DP (week 14 vs week 2 in 0DP). 1 and 2 are biological duplicates. Proteins with 1.5-2-fold change were shown and a 2-fold change was used as significant level (in bold). Ratios shown are $\log_2$ ratio.

Uniprot entry	Protein name	Gene name	1	2
P62803	**Histone H4**		**1.29**	**1.26**
A1L528	Ras-related protein Rab-6B	RAB6B	1.04	0.64
F1MPK7	**Solute carrier family 15 member 2**	**SLC15A2**	**1.03**	**1.38**
A4FV54	Ras-related protein Rab-8A	RAB8A	0.59	1.10
Q3T101	IGL@ protein	IGL@	-0.61	-2.57
P17697	Clusterin	CLU	-0.63	-1.44
P68250	14-3-3 protein beta/alpha	YWHAB	-0.71	-1.13
P27479	Arachidonate 12-lipoxygenase, 12S-type	ALOX12	-0.82	-0.62
Q3SYR8	Immunoglobulin J chain	IGJ	-0.83	-0.97
E1BJ18	**Calpain-6**	**CAPN6**	**-1.00**	**-1.26**
Q76LV2	Heat shock protein HSP 90-alpha	HSP90AA1	-1.16	-0.97
Q2UVX4	**Complement C3**	**C3**	**-1.40**	**-2.67**

Table S6.3 Continued.

Uniprot entry	Protein name	Gene name	1	2
Q71SP7	**Fatty acid synthase**	**FASN**	**-1.51**	**-1.03**
F1MH40	**IGK protein**	**IGK**	**-1.53**	**-2.73**
Q76LV1	**Heat shock protein HSP 90-beta**	**HSP90AB1**	**-1.76**	**-1.27**

Table S6.4. Proteins and their log_2 protein ratio with increased EB, 1-1 and 1-2 (biological duplicates of 0 DP vs 60 DP in week 2), increased EB and LS, 2-1 and 2-2 (biological duplicates of week 14 vs week 2 of 60DP) and increase LS 3-1 and 3-2 (biological duplicates of week 14 vs week 2 of 0DP) in cluster A of Figure 6.3. NaN, non-detectable or non-quantifiable.

Uniprot entry	Protein name	Gene name	1-1	1-2	2-1	2-2	3-1	3-2
Q3ZBE9	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	NSDHL	0.44	NaN	1.18	0.88	0.08	0.02
P02722	ADP/ATP translocase 3	SLC25A6	0.44	NaN	NaN	NaN	NaN	NaN
Q3SX32	Mannose 6 phosphate receptor binding protein 1	M6PRBP1	0.52	1.88	1.24	NaN	0.16	0.33
P07514	NADH-cytochrome b5 reductase 3	CYB5R3	0.79	1.61	0.65	0.86	0.19	0.18
P84466	Lanosterol synthase	LSS	0.32	1.63	0.79	1.74	0.10	0.02
Q0VCJ8	Methyltransferase-like protein	METTL9	0.20	NaN	0.93	NaN	-0.05	-0.28
P08239	GNAI2 protein	GNAI2	0.15	NaN	1.00	NaN	0.05	NaN
F1MD24	Na+/glucose cotransporter	SGLT1	0.67	0.57	1.24	NaN	0.54	0.34
A4FUD2	HSD17B7 protein	HSD17B7	0.81	NaN	0.61	2.16	0.65	0.53
Q3SYS6	Calcium-binding protein p22	CHP	-0.03	0.93	0.97	NaN	0.20	0.96
Q5GJ77	Glycerol-3-phosphate acyltransferase 1, mitochondrial	GPAM	-0.05	NaN	0.94	NaN	-0.02	0.98
A3FPG8	Glycerol-3-phosphate acyltransferase 4	AGPAT6	-0.20	1.55	1.03	2.95	0.21	0.53
P38409	Guanine nucleotide-binding protein subunit alpha-11	GNA11	-0.54	0.27	0.14	NaN	-0.26	0.46
Q58DG6	F-box/LRR-repeat protein 20	FBXL20	-0.29	NaN	0.46	NaN	-0.20	NaN
A5PKG9	Tyrosine-protein kinase Fyn	FYN	-0.30	NaN	0.45	2.36	-0.09	0.54
P15497	Apolipoprotein A-I	APOA1	-0.12	0.99	0.26	2.08	-0.46	1.03
Q9TUM6	Perilipin-2	PLIN2	-0.16	1.62	-0.11	2.00	-0.21	0.47
Q27960	Sodium-dependent phosphate transport protein 2B	SLC34A2	-0.58	1.56	0.71	1.72	-0.10	-0.01
Q3ZBG1	RAB14 protein	RAB14	NaN	NaN	NaN	NaN	-0.01	NaN
P63097	Guanine nucleotide-binding protein G(i) subunit alpha-1	GNAI1	NaN	NaN	0.63	NaN	0.00	NaN
A5PK13	Leucine-rich repeat-containing protein 8C	LRRC8C	-0.40	NaN	0.67	NaN	-0.03	0.32
Q3SZF2	ADP-ribosylation factor 4	ARF4	0.05	0.79	0.57	1.62	0.21	0.10
P62935	Peptidyl-prolyl cis-trans isomerase A	PPIA	-0.18	1.27	0.54	1.45	-0.11	0.22
P18892	Butyrophilin subfamily 1 member A1	BTN1A1	-0.11	1.27	0.29	1.29	0.05	0.25
Q3SZA1	Rho-related GTP-binding protein RhoF	RHOF	NaN	1.10	0.37	NaN	0.02	0.17
E1BA29	Guanine nucleotide-binding protein G(q) subunit alpha	GNAQ	-0.04	1.04	0.41	NaN	-0.10	0.21
Q0VCI2	Syntaxin-19	STX19	NaN	NaN	0.55	NaN	NaN	NaN

Table S6.4 Continued.

Uniprot entry	Protein name	Gene name	1-1	1-2	2-1	2-2	3-1	3-2
F1MUP9	Synaptic vesicle membrane protein VAT-1 homolog	VAT1	0.33	1.34	0.55	1.52	-0.11	0.38
A5PKL2	RRAS2 protein	RRAS2	0.11	NaN	0.68	NaN	-0.18	0.58
E1BJV0	EH-domain containing 4	EHD4	0.09	1.20	0.44	1.37	-0.04	0.50
A4FV54	Ras-related protein Rab-8A	RAB8A	0.17	NaN	0.55	NaN	-0.01	NaN
A2VDV1	Ankyrin repeat domain 22	ANKRD22	0.09	NaN	0.62	1.38	0.10	NaN

Table S6.5 Proteins and their log_2 protein ratio with increased EB, 1-1 and 1-2 (biological duplicates of 0 DP vs 60 DP in week 2), increased EB and LS, 2-1 and 2-2 (biological duplicates of week 14 vs week 2 of 60DP) and increase LS 3-1 and 3-2 (biological duplicates of week 14 vs week 2 of 0DP) in cluster B of Figure 6.3. NaN, non-detectable or non-quantifiable.

Uniprot entry	Protein name	Gene name	1-1	1-2	2-1	2-2	3-1	3-2
Q2UVX4	Complement C3	C3	-0.22	-0.69	-1.58	-1.46	-1.40	-2.67
Q3ZC00	Lymphocyte cytosolic protein 1 (L-plastin)	PLS3	NaN	NaN	NaN	NaN	-1.41	NaN
F1MH40	IGK protein	IGK	-0.06	-0.96	-2.94	-1.41	-1.53	-2.73
A5PK12	CD177 protein	CD177	NaN	NaN	NaN	NaN	-2.51	NaN
F1N514	CD5L protein	CD5L	-1.05	-0.43	-2.88	-1.64	-2.66	NaN
P60661	Myosin light polypeptide 6	MYL6	NaN	NaN	NaN	NaN	-2.01	NaN
Q07130	UTP--glucose-1-phosphate uridylyltransferase	UGP2	-0.29	-0.19	-1.75	-2.04	-2.00	NaN
Q2TBI0	Lipopolysaccharide-binding protein	LBP	-2.22	-2.25	-3.45	-2.16	-0.79	NaN
P01888	Beta-2-microglobulin	B2M	-1.39	-1.74	-3.98	-0.30	-2.21	NaN
P07589	Fibronectin	FN1	-1.33	NaN	NaN	NaN	NaN	NaN
E1BKT9	desmoplakin	DSP	NaN	0.76	NaN	NaN	-1.13	0.52
Q863B3	Staphylococcal nuclease domain-containing protein 1	SND1	-3.73	NaN	NaN	NaN	-1.29	NaN
P00735	Prothrombin	F2	NaN	-2.07	NaN	-4.38	NaN	-2.12
Q3SZR3	Alpha-1-acid glycoprotein	ORM1	NaN	-2.33	NaN	-3.67	NaN	NaN
Q3T052	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	NaN	-2.43	NaN	-3.31	NaN	NaN
P17690	Beta-2-glycoprotein 1	APOH	-1.39	NaN	NaN	-3.17	NaN	NaN
F1MLZ1	Cytochrome b reductase 1	CYBRD1	-1.41	NaN	0.27	NaN	NaN	NaN
P13753	MHC class I antigen	Man8	-0.92	-1.63	NaN	NaN	NaN	NaN
Q29443	Serotransferrin	TF	NaN	-1.70	NaN	-2.64	NaN	-2.83
E1B7U2	Glutathione S-transferase mu 3 (Brain)	GSTM3	-0.73	NaN	-0.58	NaN	-0.70	NaN
P79345	Epididymal secretory protein E1	NPC2	-0.86	-1.41	NaN	-1.92	NaN	-2.98
Q28107	Coagulation factor V	F5	-0.88	NaN	NaN	NaN	NaN	NaN
P12763	Alpha-2-HS-glycoprotein	AHSG	NaN	-1.56	NaN	-1.9	NaN	NaN
Q9TT94	Stearoyl-CoA desaturase	SCD	NaN	NaN	1.68	NaN	-0.23	NaN
P05307	Protein disulfide-isomerase	P4HB	-0.99	-1.55	NaN	NaN	-0.23	NaN

Table S6.5 Continued.

Uniprot entry	Protein name	Gene name	1-1	1-2	2-1	2-2	3-1	3-2
A6QQT4	Aldehyde dehydrogenase 3B1 (Fragment)	ALDH3B1	1.05	NaN	1.23	NaN	0.77	NaN
F1MIU3	Uncharacterized protein	Bt.43619	NaN	NaN	NaN	NaN	0.77	NaN
P34955	Alpha-1-antiproteinase	SERPINA1	NaN	-1.08	NaN	NaN	NaN	NaN
P15467	Ribonuclease, RNase A family, 4	RNASE4	NaN	-1.1	NaN	-2.55	0.64	-4
E1B749	solute carrier family 5 (sodium/glucose cotransporter), member 9	SLC5A9	NaN	NaN	0.92	NaN	0.64	NaN
Q9TRB9	Enterotoxin-binding glycoprotein PP20K (Fragment)		-0.99	-1.21	NaN	-1.28	-0.18	-4.74
F6RF48	SFT2D2 protein	SFT2D2	-1.03	NaN	0.14	NaN	NaN	NaN
Q0II59	Pyridoxal kinase	PDXK	0.01	NaN	NaN	NaN	NaN	NaN
Q3SX14	Gelsolin	GSN	0.01	NaN	-0.74	NaN	-1.29	NaN
A2I7N1	Serpin A3-5	SERPINA3-5	0.26	-2.08	NaN	-1.51	NaN	-3.57
Q32KV6	Nucleotide exchange factor SIL1	SIL1	NaN	-1.79	NaN	NaN	NaN	NaN
Q3MHN5	Vitamin D-binding protein	GC	NaN	-1.72	NaN	-1.48	NaN	NaN
P80025	Lactoperoxidase	LPO	-1.51	-1.76	-1.2	-1.58	NaN	-3.13
Q3ZCH5	Zinc-alpha-2-glycoprotein	AZGP1	NaN	-1.85	NaN	-1.57	NaN	NaN
E1B6Z6	Neutrophil gelatinase-associated lipocalin	LCN2	NaN	-2.51	NaN	-1.74	1.77	NaN
P00711	Alpha-lactalbumin	LALBA	-1.24	-1.55	-2.53	-2.07	-0.06	-3.60
Q3MHN2	Complement component C9	C9	NaN	-1.25	NaN	NaN	NaN	NaN
Q03763	Desmoglein-1	DSG1	NaN	NaN	NaN	NaN	0.42	NaN
P02769	Serum albumin	ALB	-1.69	-1.23	-2.55	-2.43	0.41	-3.59
A5D7Q2	IG	IG	-0.54	-1.68	-3.45	-2.04	0.45	-2.33
P02754	Beta-lactoglobulin	LGB	-0.28	-1.02	-2.42	-1.58	0.47	-4.52
P10152	Angiogenin-1	ANG1	NaN	-1.21	-1.81	-2.33	NaN	NaN
E1BJ18	Calpain-6	CAPN6	-0.4	NaN	-1.57	NaN	-1.00	-1.26
P81265	Polymeric immunoglobulin receptor	PIGR	-0.43	-1.17	-2.34	-1.27	-0.14	-1.74
Q3T101	IGL@ protein	IGL@	-0.45	-1.35	-2.4	-2.23	-0.61	-2.57
Q5E973	60S ribosomal protein L18	L18	-0.49	NaN	NaN	NaN	-0.66	NaN
F1MM04	protein tyrosine phosphatase, receptor type, J	PTPRJ	NaN	NaN	NaN	NaN	-0.65	NaN
A6QP26	Sirtuin-like protein 2	SIRT2	NaN	NaN	NaN	NaN	-0.65	NaN
P13214	Annexin A4	ANXA4	NaN	-1.21	NaN	-1.48	-0.73	NaN
Q0VCU1	Cytoplasmic aconitate hydratase	ACO1	-0.65	NaN	NaN	NaN	-0.72	NaN
Q2HJD5	Enoyl-CoA hydratase domain-containing protein 1	ECHDC1	-0.61	NaN	-1.22	NaN	NaN	NaN
Q76LV2	Heat shock protein HSP 90-alpha	HSP90AA1	-0.51	-1.05	-0.59	-1.68	-1.16	-0.97
P19120	Heat shock cognate 71 kDa protein	HSPA8	-0.63	-0.26	-1.04	-0.7	-0.55	-0.47

Table S6.5 Continued.

Uniprot entry	Protein name	Gene name	1-1	1-2	2-1	2-2	3-1	3-2
F1MLR4	Ciliary neurotrophic factor receptor subunit alpha	CNTFR	-0.11	-0.48	-1.20	NaN	-0.47	-0.73
O97680	Thioredoxin	TXN	0.41	NaN	-0.75	-0.57	-0.37	-0.39
Q5E947	Peroxiredoxin-1	PRDX1	0.27	-0.32	-1.03	NaN	-0.42	-0.28
F1N726	Glycoprotein 2 (Zymogen granule membrane)	GP2	0.28	-0.25	-1.54	-1.45	-0.39	-0.09
F1MGG1	Multidrug resistance-associated protein 4	ABCC4	-0.48	NaN	-1.47	NaN	-0.05	-0.1
P81287	Annexin A5	ANXA5	-0.22	-0.95	-1.18	-0.61	0.02	-0.65
Q9TTS3	Acetyl-CoA carboxylase 1	ACACA	-0.21	-0.49	-0.45	NaN	-1.03	0.22
P62261	14-3-3 protein epsilon	YWHAE	-0.29	-0.25	-0.2	-0.75	-0.52	-0.34
Q3ZBG9	Phospholipid scramblase 2	PLSCR2	-0.34	NaN	NaN	NaN	-0.5	NaN
Q03247	Apolipoprotein E	APOE	0.04	0.26	0.07	-0.65	-0.62	-0.02
Q9XSG3	Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1	-0.72	-1.14	-0.6	-0.82	-0.24	NaN
Q3SYX0	Protein NDRG1	NDRG1	NaN	NaN	-0.54	NaN	-0.22	-0.68
F1MHC2	Syntaxin binding protein 2	STXBP2	-0.55	NaN	-0.34	NaN	-0.52	-0.82
P02702	Folate receptor alpha	FOLR1	-0.91	-0.6	-0.52	-1.42	-0.02	-0.43
P63103	14-3-3 protein zeta/delta	YWHAZ	-0.25	-0.91	-0.36	-0.81	-0.17	0.04
P17248	Tryptophanyl-tRNA synthetase, cytoplasmic	WARS	-0.25	NaN	NaN	NaN	NaN	NaN
Q5EA55	Golgin subfamily A member 7	GOLGA7	-0.32	NaN	-0.1	NaN	NaN	NaN
F1MHB9	Uncharacterized protein	Bt.43693	NaN	NaN	NaN	NaN	-0.23	NaN
E1BP91	Puromycin-sensitive aminopeptidase	NPEPPS	NaN	NaN	-0.09	NaN	-0.22	NaN
Q17QB7	RAB35 protein	RAB35	-0.29	NaN	-0.16	NaN	-0.27	0.1
A4FV54	Ras-related protein Rab-8A	RAB8A	0.13	NaN	0.11	-0.02	0.29	1.1
P62833	Ras-related protein Rap-1A	RAP1A	-0.45	NaN	-0.35	NaN	0.84	NaN
P06394	Keratin, type I cytoskeletal 10	KRT10	NaN	-1.66	NaN	NaN	NaN	NaN
Q1JQD4	PDZ domain-containing protein GIPC2	GIPC2	-0.23	NaN	0.34	NaN	0.18	0.7
A0JNP2	Secretoglobin family 1D member	SCGB1D	NaN	-1.65	NaN	0.37	0.19	NaN

Chapter 7

General discussion

Two research questions form the foundation of the work described in this thesis. The first research question was how to identify and quantify low-abundant proteins in milk by using proteomic techniques. The second research question was whether these low-abundant proteins could explain the variation in milk composition. The results described in **chapter 3** showed that a large number of proteins (>200/measurement) in bovine milk could be identified and accurately quantified by using filter aided sample preparation (FASP) together with dimethyl labelling in nanoLC-Orbitrap-MS/MS. In **chapters 4 to 6**, the comparison of the milk protein and lipid composition is shown for three variables: 1) DGAT1 genetic variants of cows; KK vs AA, 2) milk fat globules (MFG) size; small vs large, and 3) energy status of cows; negative energy balance vs improved energy balance. Differences in low-abundant protein and lipid composition were found and used to explain the effect of these factors on milk synthesis and secretion pathways.

In this chapter, the applicability of proteomics (FASP-dimethyl labelling-nanoLC-Orbitrap-MS/MS) and metabolomics (^{1}H-NMR) techniques in studying milk synthesis and secretion will be discussed followed by a discussion of the new insights gained from the work described in this thesis. Finally, several suggestions will be given, first, on investigating the function of proteins and lipids that were significantly different in the different experiments, and, second, on investigating the nutritional value of milk proteins, with a focus on the low-abundant ones.

7.1 Non-targeted -omics techniques

7.1.1 The usefulness of non-targeted proteomics techniques

Milk fat globule membrane (MFGM) proteins were used for investigating the milk synthesis and secretion pathways in the studies described in this thesis. The rationale for using MFGM to study these pathways is that MFGM is originating from the endoplasmic reticulum, cytoplasm and apical plasma membrane of the secretory cells in the mammary gland (Mather and Keenan, 1998). The conformation of this rationale is described in **chapter 3**, where it is shown that most of the milk proteins related to milk synthesis and secretion were present in MFGM. To answer the research questions of this thesis, not only the identification but also the quantification of proteins was important. For this reason, as discussed in **chapter 2**, FASP and dimethyl labelling were chosen as the sample preparation and quantification method instead of other often used techniques, such as 2D-gel and 1D-gel. FASP was chosen because it has previously been shown to be better for analysing membrane proteins than other sample preparation methods. Dimethyl labelling

was chosen because it was shown to be a cheap and simple approach for accurate quantification.

The high number of proteins identified and quantified in the studies described in **chapters 4, 5 and 6**, shows the usefulness of this method in milk proteome studies. To our knowledge, this thesis shows one of the most extensive bovine MFGM proteomes. Milk is a complex bio-fluid in which the amount of proteins varies over 8 to 10 orders of magnitude (Picariello et al., 2012). The larger the dynamic range of the proteomics technique used, the larger the number of proteins that can be found. This larger range of identified proteins may lead to the identification of new, lower abundant proteins that show biologically relevant differences between samples. An example is, the 14-3-3 proteins that are 500-1500 times lower in abundance than butyrophilin (the most abundant MFGM protein). These 14-3-3 proteins were present in higher concentrations in larger MFG (**chapter 5**). This finding may lead to new understanding of the importance of this group of low abundant proteins in milk lipid droplet formation. The 14-3-3 proteins would not have been found without the high dynamic range of the proteomics technique used for this study.

Another advantage of using FASP-dimethyl labelling-nanoLC-Orbitrap-MS/MS is its ability to detect small changes (>1.5 fold) in protein abundance (**chapter 2**). For obtaining quantitative data in proteomics experiments, several strategies exist. The two strategies that were tested during the studies were label-free and dimethyl labelling. Label-free quantification, by using peak intensity or spectral count for quantifying protein abundance, avoids complex and time consuming labelling steps. However, the main disadvantage of label-free quantification is that it is the least accurate method for quantitative proteomics (Bantscheff et al., 2007). It was shown previously that label-free quantification was unable to detect 2-fold changes in protein abundance (Hendrickson et al., 2006). Despite the fact that the DGAT1 K232A polymorphism showed a significantly influence on milk fat production, the change of proteins in milk from DGAT1 KK and DGAT1 AA cows was between 0 and 5 fold (**chapter 4**). Such small change could not have been detected using label-free quantification. The use of a labelling method, as applied in this study, made it possible to discover for example stomatin, the only protein that was different (2-5 fold) between milk from cows with DGAT1 KK and AA genotype (**chapter 4**). Also in the other studies described in this thesis, relatively small changes in protein abundance were detected, which could probably not have been detected with label-free quantification. In conclusion, the choice of an adequate quantification strategy for proteomics is decisive to pick up small differences. The labelling technique as used in this study showed to be superior to a label-free technique for the quantification of low-abundant proteins.

7.1.2 The usefulness of combining proteomics and metabolomics

The reason for choosing proteomics as tool for studying milk synthesis and secretion is described in the introduction (**section 1.2**): many proteins involved in these processes were expected in the MFGM. In addition to proteins, the metabolites, both lipids and water-soluble metabolites, formed in these processes may give additional information about the synthesis and secretion pathways. Therefore, to better understand these synthesis and secretion processes, proteomics was, in several of the studies described in this thesis, combined with ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) analysis of the milk serum and milk lipids. Important advantages of ^{1}H-NMR for studying these metabolites are: 1) the relatively easy and fast sample preparation, 2) that ^{1}H-NMR measurements can be used routinely to differentiate the abundance of compounds in different milk samples once the compounds are assigned in the NMR spectra, and 3) the simultaneous qualification and quantification of different metabolite classes obtained from one single NMR measurement of milk lipid or milk serum. These metabolites include e.g. neutral lipids, phospholipids and fatty acids in milk fat. Especially when there are no assumptions which particular metabolites may change, the screening of all lipid or serum metabolites in one single NMR measurement is much easier than using different measurements or instruments. An example of the benefits of combining proteomics with metabolomics can be seen in the study of the effect of DGAT1 K232A polymorphism (**chapter 4**). This experiment showed a change in the stomatin together with the change in sphingomyelin. These components are both major components in membrane lipid rafts. Combining both techniques helped in revealing that the difference in lipid raft structure could be a possible reason for the variation in milk composition between DAGT1 KK and AA cows. Also, in the study of NEB, the hypothesis of the variation in lipid raft structure would not have been achieved without combining proteomics with metabolomics data. The addition of metabolomics analyses to the proteomics analyses in this study, thus helped in formulating hypotheses to explain the findings in the different studies.

7.1.3 Synergy of non-targeted and targeted analyses in biology

An important characteristic of the two main techniques (proteomics and metabolomics) used in this study are their non-targeted nature, which is a common feature of –omics technology. The non-targeted nature of –omics technology allows a holistic evaluation of low abundant compounds (e.g. genes, proteins, metabolites) in a given biological or molecular system (Evans, 2000). When no a priori assumption on changes in low-abundant compounds can be made, a holistic profiling methodology can be useful in generating hypotheses on the underlying mechanisms. In this study, the available knowledge on milk

synthesis and secretion did not lead to a priori assumption (e.g. expected changes in milk proteins or lipids), therefore a non-targeted screening of proteins and metabolites was applied. There are two important advantages of this non-targeted/holistic analysis:
1.The large amount of data obtained on proteins and metabolites can be systematically studied by using appropriate 147ioinformatics tools. An example is e.g. the decrease of acute phase proteins and the increase of cholesterol synthesis proteins in milk when the energy balance (EB) of lactating cows improves (**chapter 6**). This type of result may help to unravel the impact of specific experimental factors on biological processes as a whole, rather than studying individual components in a biological system.
2.The holistic screening of low abundant components generates results that can't be anticipated beforehand based on the available knowledge. In this study, one of the surprising results is the importance of stomatin in milk synthesis and secretion. As discussed in **chapter 4 and 6**, stomatin is a protein first identified in red blood cells, which has never been studied before in epithelial cells of the mammary gland, let alone its involvement in milk synthesis and secretion.

Besides a non-targeted approach, also targeted experiments can be performed, which normally studies the cause-effect relationship between variables (Evans, 2000). These targeted experiments can help to elucidate underlying biological mechanisms. The outcome of non-targeted analyses can be used to select the appropriate targets for this approach. An example of a targeted approach could be an immunofluorescence analysis to see the interaction of cytoplasmic vesicle proteins such as 14-3-3 proteins with lipid droplets to check their role in lipid droplet formation (**chapter 5**). So in conclusion, both non-targeted and targeted approaches have their value. The research described in this thesis uses the non-targeted approach, where the outcome of the study provides valuable input for future targeted analyses.

7.2 Lessons learned about milk synthesis and secretion

7.2.1 It is more about secretion than synthesis

In the study described in **chapter 3**, not only the membrane transporters but also the major enzymes involved in lipid synthesis and secretion were identified in MFGM. In **chapters 4, 5 and 6**, the effect of different factors on the lipid synthesis and secretion pathways from the milk proteome is described. The enzymes (acyl-CoA synthetase, acetyl-CoA carboxylase, fatty acid synthase, glycerol-3-phosphate acyltransferase) catalysing the synthesis of neutral lipids, were generally not changed (**chapter 4 and 6**). Instead, the enzymes involved in cholesterol synthesis (lanosterol synthase, LSS and sterol-4-alpha-

carboxylate 3-dehydrogenase, decarboxylating, NSDHL) were shown to be different when studying the effect of EB of cows and MFG size. As discussed in **chapter 5 and 6**, cholesterol is the major component of MFGM as well as of the intracellular membrane system. The increase of both cholesterol synthesis enzymes and milk cholesterol in cows with improved EB indicated that there are differences in membrane structure that might influence the intracellular milk synthesis and secretion. The emerging importance of membrane structures in milk synthesis and secretion is also shown by the observation of changes in stomatin abundance, which is believed to be a scaffolding protein in membrane lipid rafts, as affected by DGAT1 K232A polymorphism, EB of cows and MFG size. In secretory cells, the membrane system is the place where milk components are transported and secreted (for details, see section 1.2). Overall, these results seem to indicate that the milk secretion/transport process rather than synthesis of milk components itself, is influenced by the factors investigated in this study.

7.2.2 The emerging role of stomatin in milk synthesis and secretion

The only protein significantly changed throughout the study is stomatin. The amount of stomatin was increased in the milk of cows with DGAT1 KK genotype, in large size of MFG and in milk of cows with an improved EB. This implies that stomatin is important in milk synthesis and secretion. As discussed before, stomatin is a protein that was first identified in erythrocytes; patients with overhydrated hereditary stomatocytosis were shown not to express this protein in their red blood cells (Kadurin et al., 2009). The human homolog of stomatin has a hairpin-loop structure causing its tight binding to the membrane bilayers. One proline residue (Pro47) and the palmitoylation of two cysteine residues (Cys30 and Cys87) determine its hairpin-loop structure (Kadurin et al., 2009). These residues are also conserved in the bovine homolog of stomatin (Figure 7.1). It turned out that there is a 93% similarity between human stomatin and bovine stomatin, using blast analysis. The topology of stomatin is expected to be similar between these two species, therefore, it was predicted that bovine stomatin also has a hairpin-loop structure that causes tight binding to membrane bilayers. Because the human stomatin was shown to be located in membrane lipid rafts (Salzer and Prohaska, 2001), it is plausible that bovine stomatin is also located in lipid rafts of the plasma membrane of mammary gland (**chapter 4 and 6**). However, the way stomatin may be involved in milk synthesis and secretion is not clear. Based on the results in this study, two hypotheses on how stomatin could influence milk synthesis and secretion have been devised. First, stomatin and its related lipid rafts could regulate the membrane trafficking/secretion of milk components, because one of the known functions of lipid rafts is the regulation of membrane fusion and fission (Huttner and Zimmerberg, 2001). Second, stomatin could influence the condition and/or activity of

mammary epithelial cells through regulation of ion channels in the cell membrane according to its function in other cell types, including erythrocytes as discussed before (Kadurin et al., 2009, Price et al., 2004, Stewart, 2004).

The function and structure of stomatin have been studied in, amongst others, erythrocytes, sensory neuron cells and UAC epithelial cell lines, but have never been mentioned or studied in epithelial cells in the mammary gland (Price et al., 2004, Snyers et al., 1997, Zhu et al., 1999). The present study helps to elucidate the importance of stomatin in the mammary gland and its function in milk synthesis and secretion. Recently, two other papers also showed the importance of stomatin in milk synthesis and secretion (Cebo et al., 2012, Pisanu et al., 2012). The results presented in this thesis, together with these two recent papers, show that it would be worthwhile to further investigate the function of stomatin in epithelial cells of the mammary gland and its relation to milk synthesis and secretion.

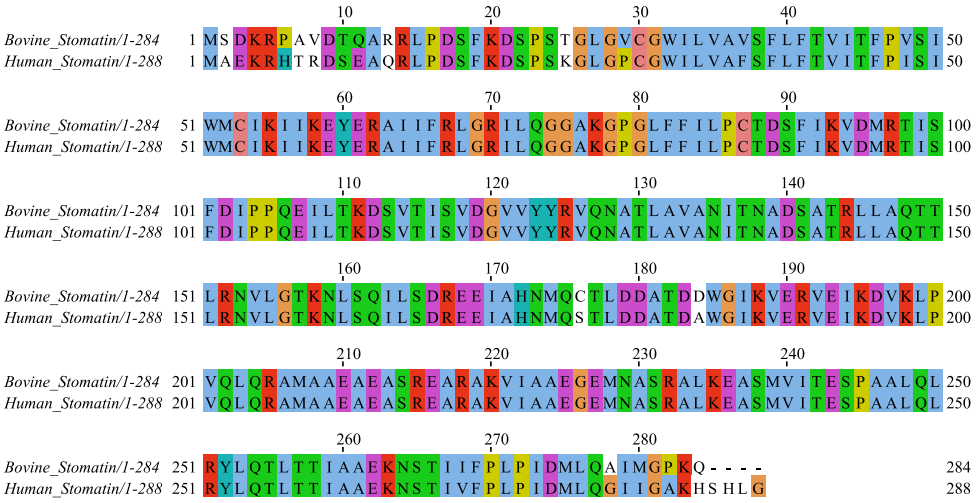

Figure 7.1. Alignment of bovine and human stomatin using Clustal Omega.

7.2.3 The importance of cholesterol in milk synthesis and secretion

As discussed in section 7.2.1, the enzymes involved in neutral lipid synthesis were generally not changed. Instead, the two enzymes involved in cholesterol synthesis (LSS and NSDHL) that were identified in MFGM were significantly changed in two of the three studies performed (different size of MFG and EB of cows). Cows with a less negative or a positive EB are supposed to be healthier than the cows with severe NEB (**chapter 6**). Both cholesterol and cholesterol synthesis related enzymes increased in these healthier cows. This could imply that sufficient supply of cholesterol to the intracellular membrane system

is important for improving the cell function during lactation. In the study described in **chapter 5** it was shown that the amount of cholesterol synthesis enzymes increased when large membrane surfaces were required for covering small MFG. The synthesis or supply of cholesterol to the membrane is important for the appropriate cell function and milk lipid droplet covering during lactation. Thus, it seems that cholesterol is important for the proper functioning of the milk synthesis and secretion process.

7.2.4 The cytoskeleton and cytoplasmic vesicle proteins are important for milk secretion

In order to perceive as complete as possible the blueprint of the MFGM proteome, all the proteins identified in at least three milk samples of the different studies (**chapter 4, 5 and 6**) were combined resulting in 487 non-redundant proteins. All these proteins were then classified according to their gene ontology biological process by using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/), see Figure 7.2. The vesicle trafficking proteins and the cytoskeleton organization proteins in MFGM are the largest groups of proteins after those related to signal transduction, protein/membrane trafficking and host defense (Figure 7.2). Also as described in **chapter 5**, cytoplasmic vesicle proteins and cytoskeleton proteins were shown to be increased in large MFG, which indicates their involvements in lipid droplet transport and formation. Based on the above two observations, it is hypothesized that cytoplasmic vesicle proteins and cytoskeleton proteins are important proteins involved in MFG transport and secretion. The involvement of cytoplasmic vesicles in secretion of MFG has also been shown in several other studies. The theory that cytoplasmic vesicles and MFG could form large vacuoles that are secreted in milk has been raised (Wooding, 1971, 1973, Wu et al., 2000), however, no further evidence underpinning this theory has been published. Also, in the prevailing model of MFG secretion, the cytoplasmic vesicles are not thought to play a role. The same is true for the cytoskeleton proteins. Several observations in the mammary gland revealed the association of cytoskeleton proteins with secretion of MFG (Mather and Keenan, 1998). However, no further investigation has been done to study this in more detail. The observations in the studies described in this thesis bring back the importance of cytoskeleton and the cytoplasmic vesicles in MFG secretion. More targeted experiments are needed to elucidate their role in milk synthesis and secretion.

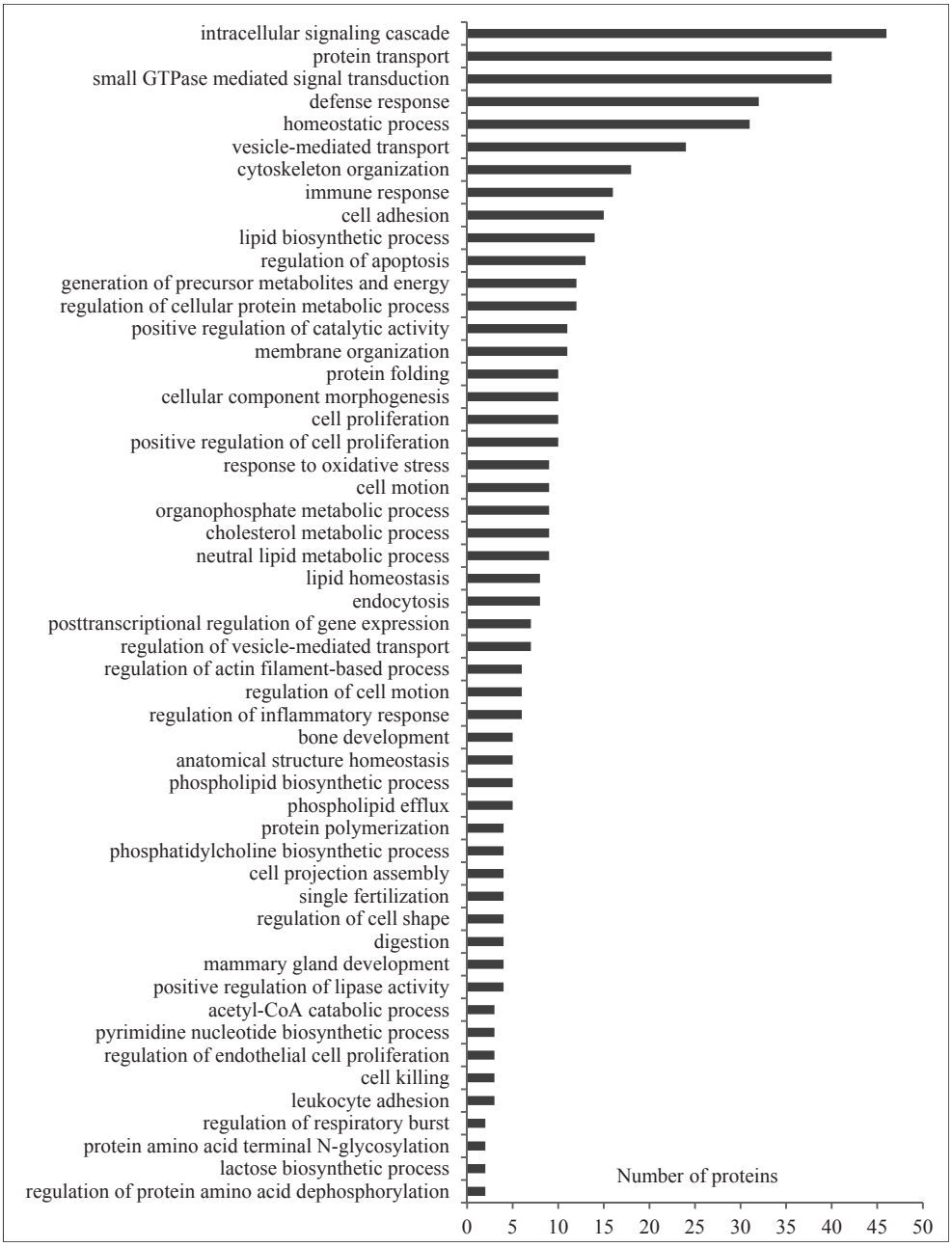

Figure 7.2. Gene ontology biological process of proteins identified in at least three milk samples in the different studies described in this thesis. Proteins were classified using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/).

7.3 Recommendations

In this study, milk is used to gain knowledge on milk synthesis and secretion pathways. The results of the different experiments show that milk itself can be used to study milk synthesis and secretion. Because of the specific secretion pathway of MFG, the proteins in this fraction partly represent the proteins in mammary epithelial cells. To our knowledge, there is no study that compares the MFGM proteome with the epithelial cell proteome. This would be useful because it is much easier to use milk instead of biopsied cells for studying lactation biology. Even though, both MFGM proteomes and mammary gland proteomes have been published, it is difficult to compare the two because of the difference in proteomics methods used. In proteomics analysis, the number of proteins identified is highly dependent on the method used for analysis, including sample preparation, the sensitivity of the mass spectrometry, and the software used for protein identification. Using the exact same proteomics method is thus important for correct comparison of the results obtained. The methods described in this thesis, have shown to be capable to analyse complex mixtures of membrane and cytoplasmic proteins, and are therefore suitable to study the similarity between proteins in MFGM and epithelial cells of the mammary gland.

The present study uses a non-targeted approach of which the outcomes provide the base/targets for further exploration of milk synthesis and secretion. The function of stomatin, cholesterol and cholesterol synthesis enzymes would be worth further examination, using a targeted approach

The function, localization and topology of stomatin have been studied in other cell types but not in epithelial cells of the mammary gland. To better understand the function of stomatin in milk synthesis and secretion, several aspects need to be studied:
1.The localization of stomatin in MFGM can be examined using immunofluorescence microscopy. Additionally, the exact location of stomatin in either the monolayer and/or double layer of MFGM can be determined by using freeze-fracture immunocytochemistry, which can then be used to identify the intracellular origin of stomatin.
2. Stomatin is proposed to be changed together with sphingomyelin or cholesterol as part of lipid rafts (**chapter 4 and 6**). The interaction of stomatin with cholesterol has been observed in oocytes and HEK293T cells (Huber et al., 2006, Kadurin et al., 2009). This interaction was shown to be essential for the regulating function of stomatin-cholesterol lipid rafts on ion channels. It would thus be worthwhile to investigate the co-localization of stomatin with sphingomeylin or cholesterol in MFGM by immunofluorescence microscopy or digitonin precipitation. Subsequently, the effect of these interactions on milk synthesis and secretion can be examined.

3.The function of stomatin in milk synthesis and secretion could be elucidated by using lactating stomatin knockout mice and compare them with lactating wild type mice.

Another group of interesting proteins are the cholesterol synthesis enzymes (LSS and NSDHL), for which the same approach as for stomatin could be used for elucidation of their function. Furthermore, the relation of these enzymes and the secretion of cholesterol to milk would be an interesting topic for further investigation.

The importance of cholesterol in membrane function is beyond doubt, however, the relation of cholesterol and the cholesterol enriched lipid raft with milk synthesis and secretion has hardly been studied. Many studies have investigated the influence of cholesterol depletion on cellular activity. These studies showed that cholesterol influenced diverse signalling pathways, membrane trafficking as well as many other biological processes (Hao et al., 2001, Kabouridis et al., 2000, Subtil et al., 1999). In order to speculate on the function of cholesterol in milk synthesis and secretion, the techniques of cholesterol depletion using e.g. cyclodextrin, could also be applied on the epithelial cell lines of the mammary gland.

The proteins in milk are extraordinarily diverse and more than 400 proteins were identified in milk in this study. The focus was on the proteins with functions related to milk synthesis and secretion. Milk in general, however, is supposed to deliver nutrients and protective components to neonates. Besides protein functions in cows, protein functions in calves and humans who consumed milk are also important. The potential nutritional function of the whole range of MFGM proteins is therefore worth investigating, to understand the role of milk in human and animal nutrition.

The observations described in this thesis, and the further studies as recommended above, increase our understanding of lactation biology and leads to a better understanding of lactation biology and the health benefits of milk.

7.4 Main conclusions

The main conclusions from the work described in this thesis are:

1. FASP-dimethyl labelling-nanoLC-Orbitrap-MS/MS is useful to identify and quantify low-abundant milk proteins.

2. Using non-targeted proteomics analysis of the MFGM aids in finding proteins that are candidates for future targeted biological research.

3. Combining metabolomics and proteomics data leads to a more complete view of the physiology of the mammary gland during milk synthesis and secretion.

4. Stomatin, cholesterol and cholesterol synthesis enzymes were shown to be important for milk synthesis and secretion in dairy cows.

5. Cytoplasmic vesicle and cytoskeleton proteins were shown to be involved in lipid droplet secretion.

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Summary

Large variation in bovine milk composition of Dutch Holstein cows has been observed. The milk fat content ranged, for instance, from below 2% to over 6%. The factors influencing the milk composition include genetic variation, lactation stage, feed and animal health. These factors influence the milk synthesis and secretion process in the mammary gland and the variations in this process lead to the variation in milk composition. The understanding of milk synthesis and secretion was improved during the last few decades, however, there are still unknown aspects, especially with regard to the lipid synthesis and secretion pathways. In this thesis, a proteomics technique was used to identify and quantify low-abundant proteins in milk that are related to milk synthesis and secretion (**chapter 2 and 3**). These low-abundant proteins were then used to elucidate the mechanisms behind the variation in milk composition. By using a combination of proteomics and metabolomics techniques, the variation in the milk/lipid synthesis and secretion processes were shown as affected by DGAT1 polymorphism, fat globule size and energy balance of the cow (**chapter 4, 5 and 6**).

In **chapter 2**, the proteomics technique used in this thesis is described for bovine milk proteins. Proteins in milk can be present in casein micelles, in the serum (the water soluble fraction) or on fat globules. These fat globules have a triple membrane layer, which is called the milk fat globule membrane (MFGM), with proteins being bound to or encapsulated in the membrane layer. Proteins in MFGM are considered to be partly representative of epithelial cells due to their origin (endoplasmic reticulum membrane, cytoplasm and apical membrane of epithelial cells). Therefore, MFGM proteins may be useful for studying milk synthesis and secretion pathways in the mammary gland. Nowadays, proteomics techniques can identify over a thousand proteins in one sample, however, quantification of these proteins is as yet not straightforward. In this chapter, an improved method is described to quantify the low-abundant MFGM proteins. This method is based on filter-aided sample preparation (FASP) combined with dimethyl labelling. This analytical approach made it possible to quantitatively study the detailed protein composition of many milk samples in a relatively short period of time. Thus, these methods were shown to be suitable for further answering of the biological questions in this thesis.

The biological function of all proteins that could be identified in milk is discussed in **chapter 3**. The proteomics technique described in **chapter 2** was applied to study the MFGM and milk serum, resulting in the identification of 246 proteins. Sixty-three proteins, most of which were present in MFGM, were related to milk synthesis and secretion. Within this group, the membrane trafficking proteins and lipid synthesis and secretion proteins are the most prominent proteins. The high number of milk (lipid) synthesis and secretion

proteins present in milk, mainly in MFGM, indicates that it is possible to study milk (especially lipid) synthesis and secretion by using MFGM. In **chapter 4, 5 and 6**, the MFGM proteome was compared in different studies to understand the effect of several factors on milk/lipid synthesis and secretion pathways.

In **chapter 4**, the effect of the DGAT1 K232A polymorphism on the milk proteome and metabolom is described. Acyl CoA: diacylglycerol acyltransferase 1 (DGAT1) is the enzyme that catalyses the synthesis of triglycerides from diglycerides and acyl-CoA. The DGAT1 K232A polymorphism was previously shown to have a significant influence on bovine milk production (milk yield, fat content, protein content and fatty acid composition). However, the mechanism behind this effect is not completely elucidated. In this study, proteomics and metabolomics were applied to determine the MFGM proteome and the polar and lipid composition of milk samples from cows with DGAT1 KK and AA genotype. The milk samples from cows with the DGAT1 KK genotype contained more stomatin, sphingomyelin and long chain fatty acids than milk samples from cows with the DGAT1 AA genotype. These differences between DGAT1 genotypes may indicate that stomatin-sphingomyelin lipid rafts and other membrane or cell structure differences in epithelial cells of the mammary gland influence the milk production. The results also suggest that the K polymorphism of DGAT1 prefers long chain fatty acids, whereas the A polymorphism of DGAT1 prefers short and medium chain fatty acids during synthesis of triglycerides.

In **chapter 5**, the MFGM proteome of different sized milk fat globules (MFG) are compared to investigate the synthesis and secretion pathways of MFG in secretory cells. In bovine milk, MFG have a heterogeneous size distribution with diameters varying from 0.1 to 15 μm. Differences in lipid composition (lipid core and MFGM) have been studied in different sized MFG fractions, but not the protein composition of the MFGM. In this study, two size fractions of MFG (7.6±0.9 μm and 3.3±1.2 μm) were obtained by centrifugation. The protein composition of MFGM in large and small MFG were compared using proteomics techniques. Two frequently studied proteins in lipid droplet biogenesis, perilipin-2 and TIP47, increased in large and small MFG, respectively. In large MFG, cytoplasmic vesicle proteins (heat shock proteins, 14-3-3 proteins and Rabs), microfilaments and intermediate filaments related proteins (actin and vimentin) and host defense proteins (cathelicidins) were increased. On the other hand, cholesterol synthesis enzymes (lanosterol synthase and decarboxylating sterol-4-alpha-carboxylate 3-dehydrogenase) were increased in small MFG. These results indicate that vesicle proteins and microfilaments and intermediate filaments could play an important role in lipid droplet growth and/or secretion. The presence of cathelicidins and the concentration difference between large and small MFG throw new light on its origin in bovine milk. The

observations from this study clearly demonstrate the difference in protein composition of the membranes of small and large MFG, leading to a better understanding of fat globule formation and secretion.

In **chapter 6**, the differences in MFGM proteome and milk metabolites are described for milk of cows with a negative energy balance (NEB) and an increased/positive energy balance (PEB). The early lactation period of high producing dairy cows is characterized by an insufficient energy intake to cover milk production and body maintenance requirements. Body reserve (fat) mobilization occurs to compensate this NEB and concomitantly there is a higher susceptibility to diseases and metabolic disorders. To better understand the physiological mechanism of NEB, milk of cows with different energy balance status and lactation stage were analyzed by ^{1}H-NMR and LC-MS/MS. Milk of cows in severe NEB showed higher concentrations of acute phase response proteins, unsaturated fatty acids and galactose-1-phosphate. Improved EB resulted in higher concentrations of cholesterol, cholesterol synthesis related proteins and stomatin. These results indicate that, compared to cows with improved EB, cows during NEB show an acute phase response, an increased apoptosis of mammary gland cells and differences in membrane structure (stomatin-related lipid rafts) of secretory cells in mammary gland.

In **chapter 7**, three aspects are discussed based on the results described in this thesis. First, the applicability of the techniques used in this thesis are discussed. Proteomics technique (FASP-dimethyl labelling-nanoLC-Orbitrap-MS/MS) and metabolomics technique (^{1}H-NMR) enable us to detect biologically relevant proteins and formulate hypotheses about their origin and function. Moreover, this non-targeted exploratory research provides targets for future research. Second, the new insights gained from this thesis for understanding milk synthesis and secretion process were deliberated upon. Stomatin, cholesterol and cholesterol synthesis enzymes were shown to be important for milk synthesis and secretion in lactating cows in all the different studies. Cytoplasmic vesicle proteins and cytoskeleton proteins were suggested to be important for lipid droplet formation and secretion. Finally, recommendations for future research are given.

Samenvatting

Er is veel variatie waargenomen in melksamenstelling van Nederlandse Holstein koeien. Het melkvetgehalte varieerde bijvoorbeeld van minder dan 2% tot meer dan 6%. De factoren die melksamenstelling beïnvloeden zijn onder andere genetische variatie, lactatiestadium, voer en diergezondheid. Deze factoren beïnvloeden het proces van melksynthese en -secretie in de melkklier en variatie in dit proces veroorzaakt variatie in melksamenstelling. Het begrijpen van melksynthese en -secretie is de afgelopen decennia verbeterd, maar er zijn nog onbekende aspecten, vooral waar het reactiepaden voor vetsynthese en -secretie betreft. In dit proefschrift werden proteomics technieken gebruikt voor het identificeren en kwantificeren van laag-abundante eiwitten in melk die gerelateerd zijn aan melksynthese en -secretie (**hoofdstuk 2 en 3**). Deze laag-abundante eiwitten werden vervolgens gebruikt om de mechanismen achter de variatie in melksamenstelling op te helderen. Door een combinatie van proteomics en metabolomics technieken werd de variatie in de processen van melk/vetsynthese en -secretie bestudeerd, onder invloed van DGAT1 polymorfisme, vetbolgrootte en energiebalans van de koe (**hoofdstuk 4, 5 en 6**).

In **hoofdstuk 2** worden de proteomics technieken die gebruikt zijn in dit proefschrift beschreven voor koemelkeiwitten. Eiwitten in melk kunnen aanwezig zijn in caseïnemicellen, in het serum (de water oplosbare fractie) of op vetbolletjes. Deze vetbolletjes hebben een drielaagsmembraan, dat het melkvetbolmembraan (MFGM, Milk Fat Globule Membrane) genoemd wordt, waar eiwitten aan gebonden of in ingekapseld zijn. Eiwitten in het MFGM worden door hun herkomst geacht representatief te zijn voor epitheelcellen (endoplasmatisch reticulummembraan, cytoplasma en apicale membraan van epitheelcellen). Derhalve kunnen MFGM-eiwitten nuttig zijn voor het bestuderen van reactiepaden voor melksynthese en -secretie in de melkklier. Tegenwoordig kunnen met proteomics technieken meer dan duizend eiwitten in een monster geïdentificeerd worden, echter, kwantificering van deze eiwitten is nog niet eenvoudig. In dit hoofdstuk wordt een verbeterde methode beschreven om de laag-abundante MFGM eiwitten te kwantificeren. Deze methode is gebaseerd op filter-geholpen monstervoorbereiding (FASP, Filter Aided Sample Preparation) in combinatie met dimethyllabeling met formaldehyde. Deze analytische benadering maakt het mogelijk om kwantitatief de gedetailleerde eiwitsamenstelling van vele melkmonsters in een relatief korte tijd te bestuderen. Zo werd aangetoond dat deze werkwijze geschikt is voor verdere beantwoording van de biologische vragen in dit proefschrift.

De biologische functie van alle eiwitten die geïdentificeerd kunnen worden in melk wordt besproken in **hoofdstuk 3**. De proteomics techniek beschreven in hoofdstuk 2 werd toepast op MFGM en melkserum, wat resulteerde in de identificatie van 246 eiwitten. Drieënzestig eiwitten, waarvan de meeste in MFGM, betroffen eiwitten gerelateerd aan melksynthese en

-secretie. Binnen deze groep zijn de membraantransporteiwitten en vetsynthese en -secretie eiwitten de meest prominente eiwitten. Het grote aantal eiwitten betrokken bij melk(vet)synthese en -secretie in melk, en vooral in MFGM, geeft aan dat het mogelijk is om melk(vooral vet)synthese en -secretie te onderzoeken op basis van MFGM. In **hoofdstuk 4, 5 en 6**, is het MFGM-proteoom in verschillende studies vergeleken om het effect van meerdere factoren op reactiepaden voor melk(vet)synthese en -secretie te begrijpen.

In **hoofdstuk 4** wordt het effect van het DGAT1 K232A polymorfisme op het melkproteoom en -metaboloom beschreven. Acyl CoA:diacylglycerol acyltransferase 1 (DGAT1) is het enzym dat de synthese van triglyceriden uit diglyceriden en acyl-CoA katalyseert. Van het DGAT1 K232A polymorfisme werd eerder aangetoond dat het een belangrijke invloed heeft op koemelkproductie (melkproductie, vetgehalte, eiwitgehalte en vetzuursamenstelling). Echter is het mechanisme achter dit effect niet volledig opgehelderd. In deze studie werden proteomics en metabolomics toegepast op het MFGM-proteoom en de polaire lipiden van melkmonsters van koeien met het DGAT1 KK en AA genotype. De melkmonsters van koeien met het DGAT1 KK genotype bevatte meer stomatine, sfingomyeline en lange-keten vetzuren dan melkmonsters van koeien met het DGAT1 AA genotype. Deze verschillen tussen DGAT1 genotypes kunnen erop duiden dat stomatine-sfingomyeline lipiden *rafts* (microdomeinen van het celmembraan) en andere membraan- of celstructuurverschillen in epitheelcellen van de melkklier invloed hebben op de melkproductie. De resultaten suggereren ook dat het K-polymorfisme van DGAT1 voorkeur heeft voor lange-keten vetzuren, terwijl het A-polymorfisme van DGAT1 voorkeur heeft voor korte- en middellange-keten vetzuren, tijdens de synthese van triglyceriden.

In **hoofdstuk 5** wordt het MFGM-proteoom van melkvetbolletjes van verschillende grootte vergeleken om de reactiepaden voor melkvetbolsynthese en -secretie te onderzoeken in secretoire cellen. In koemelk hebben melkvetbolletjes een heterogene grootteverdeling met diameters variërend van 0,1 tot 15 μm. Verschillen in vetsamenstelling (kern en membraan) zijn eerder bestudeerd in melkvetbolfracties van verschillende afmetingen, maar niet de eiwitsamenstelling van het MFGM. In deze studie werden twee melkvetbolfracties van verschillende grootte (7,6 ± 0,9 μm en 3,3 ± 1,2 μm) verkregen door centrifugeren. De eiwitsamenstelling van MFGM in grote en kleine melkvetbolletjes werden vergeleken met behulp van proteomics technieken. Twee vaak bestudeerde eiwitten in vetbolvorming, perilipine-2 en TIP47, namen toe in respectievelijk grote en kleine melkvetbolletjes. In grote melkvetbolletjes waren cytoplasmatische vesikeleiwitten (*heat shock* eiwitten, 14-3-3 eiwitten en Rab-eiwitten), microfilamenten en intermediaire filament-verwante eiwitten

(actine en vimentine) en afweereiwitten (cathelicidinen) verhoogd. Anderzijds waren cholesterolsynthese-enzymen (lanosterol synthase en decarboxylerend sterol-4-alpha-3-carboxylaat dehydrogenase) verhoogd in kleine melkvetbolletjes. Deze resultaten geven aan dat vesikeleiwitten, microfilamenten en intermediaire filamenten een belangrijke rol in vetdruppelgroei en/of -secretie spelen. De aanwezigheid van cathelicidinen en het concentratieverschil erin tussen grote en kleine melkvetbolletjes werpen een nieuw licht op hun oorsprong in koemelk. De observaties uit deze studie tonen duidelijk het verschil in eiwitsamenstelling in de membranen van kleine en grote melkvetbolletjes aan, wat leidt tot een beter begrip van de vorming en secretie van melkvetbolletjes.

In **hoofdstuk 6** worden de verschillen in MFGM-proteoom en melkmetabolieten beschreven voor melk van koeien met een negatieve energiebalans (NEB) en een verhoogde/positieve energiebalans (PEB). De vroege lactatieperiode van hoog producerende melkkoeien wordt gekenmerkt door onvoldoende energie-inname om de melkproductie en het lichaamsonderhoud te dekken. Lichaamsreserve(/vet)mobilisatie treedt op om de NEB te compenseren en tegelijkertijd is er een hogere gevoeligheid voor ziekten en metabole stoornissen. Om het fysiologische mechanisme van NEB beter te begrijpen werd melk van koeien met verschillende energiebalansstatus en lactatiestadium geanalyseerd met ^{1}H-NMR en LC-MS/MS. Melk van koeien met ernstige NEB vertoonden hogere concentraties van acutefase-eiwitten, onverzadigde vetzuren en galactose-1-fosfaat. Een verbeterde energiebalans resulteerde in hogere concentraties van cholesterol, cholesterolsynthese-gerelateerde eiwitten en stomatine. Deze resultaten geven aan dat, in vergelijking tot koeien met verbeterde energiebalans, koeien tijdens NEB een acutefase-reactie, een verhoogde apoptose van melkkliercellen en verschillen in membraanstructuur (stomatine-gerelateerde lipiden *rafts*) van secretoire cellen in melkklieren tonen.

In **hoofdstuk 7** worden drie aspecten besproken aan de hand van de resultaten beschreven in dit proefschrift. Ten eerste wordt de toepasbaarheid van de technieken die gebruikt zijn in dit proefschrift besproken. Proteomics technieken (FASP-dimethyllabelling-nanoLC-Orbitrap-MS/MS) en metabolomics technieken (^{1}H-NMR) stellen ons in staat om biologisch relevante eiwitten te detecteren en hypotheses te formuleren over de herkomst en functie ervan. Bovendien geeft dit niet-gerichte, verkennende onderzoek aanwijzingen voor toekomstig onderzoek. Ten tweede worden de nieuwe inzichten die uit dit proefschrift voortkomen met betrekking tot het begrijpen van het proces van melksynthese en -secretie besproken. Stomatine, cholesterol en cholesterolsynthese enzymen bleken in de verschillende studies belangrijk te zijn voor melksynthese en -secretie in melkgevende koeien. Cytoplasmatische vesikeleiwitten en cytoskeleteiwitten worden geopperd belangrijk

te zijn voor vetdruppelvorming en -secretie. Tot slot worden aanbevelingen voor toekomstig onderzoek gegeven.

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-Jing Lu 芦晶-

Curriculum Vitae

Jing Lu, was born on 12th Jan, 1983 in Acheng, Heilongjiang Province, China. In 2002, she started undergraduate study in Food Science and Engineering in China Agricultural University. After obtaining her BSc as Beijing outstanding graduate in 2006, she was recommended to enter Master programme of agricultural products processing and preservation engineering in China Agricultural University. She did research on the functional properties of whey protein isolate conjugated with saccharides through Maillard Reaction and obtained Master degree in 2008. In the same year, she received scholarship from China scholarship council and enrolled in the PhD project in Wageningen Univeristy at Product Design and Quality Management group. Her research focused on the understanding of the biology of milk synthesis and secretion using proteomics and metabolomics techniques.

List of Publications

Jing Lu, Jeni Anggrek, Sjef Boeren, Toon van Hooijdonk, Jacques Vervoort and Kasper Hettinga. Membrane protein composition of different sized milk fat globules. In preparation.

Jing Lu, Sjef Boeren, Toon Van Hooijdonk, Jacques Vervoort and Kasper Hettinga. Changes in milk proteome and metabolome associated with DGAT1 K232A polymorphism in lactating cows. In preparation.

Jing Lu*, Elsa Antunes Fernandes*, Ana Elizabeth Páez Cano, Jantipa Vinitwatanakhun, Sjef Boeren, Toon van Hooijdonk, Ariette van Knegsel, Jacques Vervoort, Kasper Hettinga. Changes in milk proteome and metabolome associated with energy balance in post parturient dairy cows. Submitted. * shared first authorship.

Jing Lu, Toon van Hooijdonk, Sjef Boeren, Jacques Vervoort and Kasper Hettinga. Identification of lipid synthesis and secretion proteins in bovine milk. Submitted.

Jing Lu, S. Boeren, S.C. de Vries, H.J.F. van Valenberg, J. Vervoort, K. Hettinga. Filter-aided sample preparation with dimethyl labeling to identify and quantify milk fat globule membrane proteins (2011). Journal of Proteomics. (75): 34-43

Guanhao Bua, Yongkang Luo, **Jing Lu** and Ying Zhang (2010). Reduced antigenicity of β-lactoglobulin by conjugation with glucose through controlled Maillard reaction conditions. Food and Agricultural Immunology. (21): 143-156.

Bu, Guanhao, **Lu Jing**, Zheng, Zhe; Luo Yongkang (2009) Influence of Maillard reaction conditions on the antigenicity of bovine α-lactalbumin using response surface methodology. Journal of the Science of Food and Agriculture (89): 2428-2434

Jing Lu, Huixing Shen, Yongkang Luo (2007). Distinguish the species of the Marine Products. Meat Research, (12): 24-27

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Jing Lu, Guanhao Bu, Yongkang Luo.(2007). Studied of forming whey protein-polysaccharides conjugates and it's functional properties. China Dairy Industry,35(5):4-8

Jianhui Zhang, **Jing Lu**, Yongkang Luo, et al. (2006) .Studies of the compound thermal protectants of the bovine milk Immunoglobulin (IgG). China Dairy Industry, 34(3): 5-8

Abstracts and Proceedings

Jing Lu, S. Boeren, J. Vervoort, H. van Valenberg, S. de Vries, J. van Arendonk, T. van Hooijdonk, and K. Hettinga (2012), Identification and quantification of milk synthesis and secretion related proteins in bovine milk using a proteomics approach. 9th International Symposium Milk genomics and Human health (www.milkgenomics.org). Wageningen, The Netherlands.

Jing. Lu, S. Boeren, J. Vervoort, H. van Valenberg, S. de Vries, J. van Arendonk, T. van Hooijdonk, and K. Hettinga (2012). Identification and quantification of milk synthesis and secretion related proteins in bovine milk using a proteomics approach. 2012 Annual Meeting American Dairy Science Association American Society of Animal Science, Pheonix, USA: Lactation Biology p. 303.

Jing Lu, Hein van Valenberg, Sjef Boeren, Jacques Vervoort, Kasper Hettinga (2010). Use of filter-based sample preparation with dimethyl labeling to identify & quantify membrane proteins in milk. 4th EuPA Scientific Meeting A Proteomics Odyssey Towards Next Decades, Estoril, Portugal: p.47-48.

Overview of Completed Training Activities

Discipline specific activities

Courses

Nutrient Density of Milk, VLAG, Wageningen, The Netherlands, 2008
2nd Proteomics course, VLAG&EPS, Wageningen, The Netherlands, 2008
Advanced Food Analysis, VLAG, Wageningen, The Netherlands, 2010
Experimental Design in Proteomics, European Proteomics Association & Portuguese Proteomics Network, Lisbon, Portugal, 2010
Uniprot protein sequence databases: use and pitfalls, Swiss Institute of Bioinformatics, Lausanne, Switzerland , 2011
3rd Proteomics course, VLAG, Wageningen, The Netherlands, 2011
Bioinformatics- A User's Approach, EPS, Wageningen, The Netherlands, 2011
Advanced visualization, integration and biological interpretation of ~omics data, VLAG&WIAS, Wageningen, The Netherlands, 2011
1-Day Master class Nutrient Density of Milk, Milk Genomics and Health Benefits of Dairy, VLAG&NZO, Wageningen, The Netherlands, 2011

Conference & symposium

6th NIZO conference, NIZO, Arnhem, The Netherlands, 2009
Genetics of Milk Quality, WIAS&VLAG, Wageningen, The Netherlands, 2009
4th EuPA meeting (oral & poster presentation), European Proteomics Association & Portuguese Proteomics Network, Lisbon, Portugal, 2010
NPC progress meeting, Netherlands Proteomics Centre, Utrecht, The Netherlands, 2010
Nutrition and fat metabolism in dairy cattle, WIAS, Wageningen, The Netherlands, 2011
ADSA annual meeting (poster presentation), American Dairy Science Association, Phoenix, USA, 2012
9th International Symposium on Milk Genomics and Human Health (oral presentation), International Milk Genomics Consortium, Wageningen, The Netherlands, 2012

General courses

VLAG PhD week, VLAG, Wageningen, The Netherlands, 2009
Competence Assessment, Wageningen University Graduate School, The Netherlands, 2009
English Academic Writing I, Language Services, Wageningen, The Netherlands, 2009

Techniques for Writing and Presenting Scientific Papers, Wageningen University Graduate School, The Netherlands, 2010
Scientific Writing, Language Service, Wageningen, The Netherlands, 2011
Career Assessment, Wageningen University Graduate School, The Netherlands, 2011
Teaching and Supervising Thesis Students, Educational Staff Development of WUR, The Netherlands, 2011
Career Orientation, Wageningen University Graduate School, The Netherlands, 2012
Applied Statistics, VLAG, Wageningen, The Netherlands, 2012

Optional courses and activities

Preparing PhD research proposal, 2008
Excursion to Ghent Universtity, Dairy Science and Technology, Ghent, Belgium, 2009
Advanced Statistics, WUR biometris, The Netherlands, 2009
PhD trip to Australia, PDQ group, Sydney & Melbourne, Australia, 2010

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