Milk Fat Globule Stability

Lipolysis with Special Reference to Automatic Milking Systems

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Abstract

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The implementation of automatic milking systems (AMS) initially caused a lowering of the milk quality regarding free fatty acids (FFA). A high level of FFA increases the risk of rancid flavour in dairy products. The objective of the present thesis was to evaluate different factors in AMS which could potentially cause an increased level of FFA. The stability of the MFG was studied by using model systems.

A correlation (r^2=0.54) was found between the average diameter of the milk fat globule (MFG) and the diurnal fat yield of cows. The activity of a MFG membrane enzyme was found to decline with increasing average MFG size. The results of this work indicate that the MFGs grow larger when the fat synthesis increases, probably because of a limitation in the production of MFG membrane.

This new, obtained knowledge was used to produce milk with various average diameters of MFGs. Three groups of cows were fed concentrates with different fatty acid compositions; one high in saturated fat, another high in unsaturated fat and the last one stimulating high *de novo* synthesis. The feedings resulted in milk with fat contents of 5.0, 3.7 and 4.0%, respectively. The MFGs were significantly larger in the milk with the highest fat content. All three types of milk were pumped at various shear rates and temperatures. Afterwards, measurement of particle size distribution showed that the highest coalescence of MFGs in the milk occurred with the largest MFGs. Moreover, the MFGs were more unstable at a pumping temperature of 31°C compared with lower temperatures. Likewise, an increase was found in the FFA content for milk with the largest MFGs, indicating that milk with a high fat content is more unstable when subjected to mechanical stress. The results recommended to cool raw milk to 5°C before pumping it from the milking unit to the milk bulk tank.

The effect of milking frequency on FFA was studied because cows are more frequently milked in AMS. The level of FFA was significantly higher (1.49 meq./100g fat) in milk from the udder half milked four times daily compared with the milk from the udder half milked twice daily (1.14 meq./100g fat). This is ascribed to the fact that milk from the udder half milked four times daily contained MFGs with a significantly larger average diameter. The results are of great importance for further understanding of the mechanisms behind the increased content of FFA which is frequently observed in AMS.

Keywords: milk fat globule, milk fat globule membrane, fatty acid composition, free fatty acids, pumping, automatic milking systems, γ-glutamyl transpeptidase, xanthine oxidase, milking frequency

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Contents

Appendix

Papers I-IV

I. Wiking, L., Stagsted J., Björck, L. & Nielsen, J.H. (2004). Milk fat globule size is affected by fat production in dairy cows. *International Dairy Journal* 14: 909-913

II. Wiking, L., Björck**,** L. & Nielsen**,** J.H. (2003). The influence of feed on stability of fat globules during pumping of raw milk. *International Dairy Journal* 13: 799-803

III. Wiking, L., Bertram, H.C., Björck, L. & Nielsen. J. H. Evaluation of cooling strategies for pumping of milk - Impact of fatty acid composition on free fatty acid levels *(submitted to Journal of Dairy Research)*

IV. Wiking, L., Nielsen, J.H., Båvius, A-K., Edvardsson, A. & Svennersten-Sjauna, K. Impact of milking frequencies on the level of free fatty acids in milk, fat globule size and fatty acid composition (manuscript to be submitted)

List of abbreviations

ADV	Acid degree value
BDI	Bureau of Dairy Industry
CD 36	Cluster of differentiation 36
FFA	Free fatty acids
FID	Free induction decay
GC	Gas chromatography
LPL	Lipoprotein lipase
MFG	Milk fat globule
MFGM	Milk fat globule membrane
MUC1	Mucin 1
NMR	Nuclear magnetic resonance
PAS	Periodic acid schiff

Background

Automatic milking systems have been commercially available since 1992, and the numbers have grown rapidly during the last years. At present, 400 and 260 dairy farms in use automatic milking systems in Denmark and Sweden, respectively (personal communication, B. Everitt. Swedish Dairy Association). This corresponds approximately to 7 % and 5%, of the total bulk milk in Denmark and Sweden, respectively. In automatic milking systems the cows are milked by assistance of robots. By sensor technology, the robot finds the teats, and clean them before cluster attachment. The cows voluntarily attend the milking unit, and the cows are offered concentrate feed during milking.

This thesis was initiated due to reports in the beginning of implementation of automatic milking systems that the levels of free fatty acids (FFA) were higher in automatic milking systems compared to conventional milking systems (Justesen & Rasmussen, 2000; Klungel, Slaghuis & Hogeveen, 2000). With the steadily growing number of automatic milking systems, the quality of dairy products can be impaired in the future. The focus of this thesis has thus been how changes in the milking technology could affect the formation of FFA in milk.

Compared with conventional milking (i.e. milking twice daily), milk is through the whole day continuously pumped to the milk bulk tank at the farm which involves new cooling strategies. Furthermore, automatic milking often requires harsher mechanical treatment of the milk due to longer distances between milking unit and milk bulk tank and continuous pumping of smaller amounts of milk. Another factor altered from conventional milking is an increased milking frequency due to the fact that cows have free access to the milking unit.

This thesis contains an introduction that reviews present knowledge of the milk fat globule, material and methods and a discussion of the results obtained and finally conclusions. The appendix consists of four papers which together form the basis of this thesis.

Introduction

Milk fat globule

It has been known for more than 300 years that fat exists as globules in milk (Leewenhoeck, 1674). The fat globule size distribution is shown in Figure 1. The diameter of the milk fat globule (MFG) ranges from 0.1-12 µm with an average of around 4.5µm. The size distribution depends on breed as shown in Figure 1.

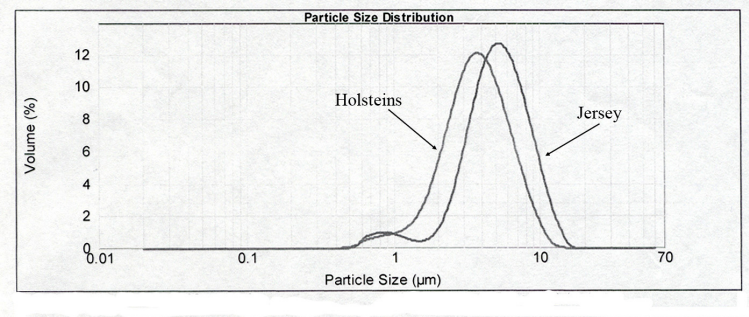

Figure 1. Milk fat globule size distribution from Danish Holsteins and Jersey cows.

Synthesis of the milk fat

The MFG is formed in the secretory cells of the mammary gland. Precursors of milk lipid globules are formed at the endoplasmic reticulum and are transported through the cytosol as small droplets of triglycerides covered by a non-bilayer of polar phospholipids and proteins. During transport the droplets grow in size, apparently due to droplet-droplet fusion (Dylewski *et al*. 1984; Deeney *et al*.1985). At the apical plasma membrane, the droplets are secreted from the epithelial cell. During secretion, the droplets are covered by the plasma membrane and finally pinched off into the lumen of alveolus.

The precursors of milk lipid globules have a group of polypeptides on the surface in common with the membrane of the endoplasmic reticulum (Deeney *et al*., 1985). However, it is still unknown where in the endoplasmic reticulum network the lipid droplets are formed (Mather & Keenan, 1998). Another unknown mechanism is how the lipid droplets are transported to the apical plasma membrane of the cell.

Furthermore, there are two different theories of how the fat droplets are secreted. One theory is that the lipid droplets reach the apical region of the cell, where they are secreted and covered by cellular membranes. The lipid droplets are gradually coated with plasma membrane until a narrow neck of membrane and cytoplasm remains. At the point when the membrane in the neck fuses together, the fat globule is secreted and expelled into the alveolar lumen (Mather & Keenan, 1998). Another possible theory for the secretion of the lipid droplet suggested by

Wooding (1971 & 1973) is that the lipid droplets also associate with secretory vesicles in the apical cytoplasm. Likewise casein should be covered by a secretory vesicle and the content of such may then be released from the apical surface by exocytosis. The first described mechanism is the most accepted (Mather & Keenan, 1998). The hormones prolactin and oxytocin, affect the release of the lipid globules and is thought to affect the final size of the MFG (Ollivier-Bousquet, 2002). The composition of the outer coat of the milk fat globule membrane (MFGM) is to a great extent similar to the apical plasma membrane of the secretory cells.

Composition of the milk fat globule

Many studies and reviews have dealt with the composition of fatty acids in milk (Glass, Jenness & Lohse, 1969; Bitman & Wood, 1990; Jensen, Ferris, Lammi-Keefe, 1991; Bitman *et al*. 1995; Jensen, 2002). Several lipid classes are present in milk as shown in Table 1.

Table 1. Lipid classes in bovine milk. The range covers variations through lactation. Adapted from Bitman & Wood (1990).

Lipid class	Percentage
Triglycerides	95.8-97.4
Phospholipid	0.56-1.11
Cholesterol	0.30-0.53
Diglycerides	1.01-2.25
Monoglycerides	0.03-0.08
Free fatty acids	0.18-0.28
Cholesteryl esters	0.02-0.05

The composition of the fatty acids in milk fat is given in Table 2. The composition of fatty acids in milk is affected by feed and breed. The fatty acids containing from 4 to 14 carbon atoms are synthesized from the acetate and β-hydroxy butrate which are products of the fermentation of carbohydrates in the rumen. This pathway is called *de novo* synthesis. Some of the palmitic acid (C16:0) is also synthesized *de novo*. Long chain fatty acids, *i.e.* those containing 16 or more carbon atoms, are provided to the glands from the blood stream and originate directly from the diet or from the adipose tissue. Palmitic (C16:0) and stearic (C18:0) acids pass through the rumen unchanged while unsaturated fatty acids are subjected to biohydrogenation by the reducing environment caused by the microorganisms in the rumen, resulting mainly in stearic acid together with a smaller amount of oleic acid (C18:1). (Børsting, Hermansen & Weisbjerg, 2003) Furthermore, stearic acid derived from the diet is partly converted to oleic acid by stearoyl-CoA desaturase, in the intestines and the mammary tissue. Unsaturated lipid supplements are often protected/encapsulated to avoid biohydrogenation in the rumen. Moreover, high amounts of unsaturated lipids in the rumen result in incomplete biohydrogenation, so some of the linoleic acid (C18:2) and linolenic acid (C18:3) is transformed into conjugated linoleic acids (CLA). Specific isomers

of CLA together with *trans*-C18:1 in the rumen have a negative effect on the *de novo* fat synthesis resulting in lower fat content of the milk (Bessa *et al*. 2000).

Table 2. Distribution of the major fatty acids in bovine milk fat. Adapted from Jensen (2002).

Fatty acid carbon number	Average range (wt%)
4:0	2-5
6:0	1-5
8:0	1-3
10:0	2-4
12:0	2-5
14:0	8-14
15:0	1-2
16:0	22-35
16:1	1-3
17:0	0.5-1.5
18:0	9-14
18:1	20-30
18:2	1-3
18:3	0.5-2

Jersey cows produce a higher proportion of *de novo* fatty acids C4-16, C18 and lesser C18:1 compared with Holsteins cows (Beaulieu & Palmquist, 1995; DePeters *et al*. 1995; Morales *et al*., 2001; White *et al*. 2001). The lower proportion of C18:1 in Jersey compared to Holstein milk is due to a lower mammary activity of stearoyl-CoA desaturase in Jerseys (Beaulieu & Palmquist, 1995; Drackley *et al.* 2001).

Several studies have been carried out to increase the proportions of unsaturated lipids in milk, in order to obtain "healthier" milk fat and softer butter (Banks, Clapperton & Morag, 1976; Urquhart, Cadden & Jelen, 1984; Grummer, 1991; Goodridge, Ingalls & Crow, 2001; McName, 2002; Gonzalez *et al.* 2003). The main polyunsaturated fatty acids in milk are linolenic acid C18:3 (omega –3 fatty acid) and linoleic acid C18:2 (omega –6 fatty acid). Goodridge, Ingalls & Crow (2001) managed to increase the proportion from 4.8% to 10.3 % by feeding high amounts of protected Linola seed (containing a high level of linoleic acid) and to increase the proportion of linolenic acid from 0.8% to 6.4% by offering a high amount of protected flaxseed. The disadvantage with high levels of unsaturated fatty acid is the susceptibility to oxidation which produces an oxidative rancid flavour in dairy products.

Composition of the milk fat globule membrane

The quantitative composition of the MFGM has been studied by several methods. Therefore, some discrepancies exist between results. Estimates of the composition of MFGM found in the literature are summarised in Table 3. The major proteins found in the MFGM are MUC1, xanthine dehydrogenase/oxidase, PAS III, CD 36, butyrophilin, adipophilin, PAS 6, PAS 7 and fatty-acid binding protein. Three of

the proteins are described in this section. Furthermore, around 25 different enzymes are found associated with the MFGM.

Table 3 Estimates of the major components of the native milk fat globule membrane

Total Protein (mg/g milk fat)	Surface protein coverage of MFG (mg/m^2)	Phospo-lipids (mg/g milk fat)	Surface phospolipids coverage of MFG (mg/ m^2)	Reference
6.4-8.3	1.28-1.85	-	-	Ye *et al*., 2002
18	9	6.5	3.2	Walstra et al, 1999
4.0	-	-	-	Lee & Sherbon, 2002

Xanthine dehydrogenase/oxidase is a redox enzyme containing molybdenum. The M_w of xanthine oxidase is 155 kDa and it accounts for about 8% of the protein in MFGM (Briley & Eisenthal, 1975). The best known function of the enzyme is the oxidation of hypoxanthine to xanthine and xanthine to uric acid. Furthermore, xantine oxidase can reduce NO_3^- to NO_2^-. The latter property is used in cheese manufacturing, where a small amount of nitrate is added because NO_2^- prevents Clostridia from growing. Recently, has been reported that aldehydes, naturally found in milk, can accelerate the oxidation in raw milk through the xanthine oxidase enzyme system (Steffensen, Andersen, Nielsen, 2002). Xanthine oxidase is thought to be a peripheral membrane protein, meaning that it is not a membrane anchor and is thereby easily released from the MFGM. During cooling, xanthine dehydrogenase is thus released into the milk serum where it is activated (Bhavadasan & Ganguli, 1980).

Butyrophilin (PAS 5) comprises over 40% by weight of the MFGM proteins. Its M_w is 67 kDa and contains approx. 5% carbohydrate. Butyrophilin is only expressed on the apical plasma membrane of secretory cells in the mammary tissue, and butyrophilin is a transmembrane protein (Jack & Mather, 1990). The C-terminal of butyrophilin interacts with xanthine oxidase (Figure 2) supported by disulfide bonds between the proteins and thereby stabilises the MFGM (Mather & Keenan, 1998). Mondy and Keenan (1993) reported that butyrophilin and xanthine oxidase are present in the MFGM in constant molar proportions (4:1) through lactation. Later, Ye *et al*. (2002) confirmed the constant ratio except that the ratio was 3:1. Butyrophilin and xanthine oxidase are tightly attached to fatty acids with palmitic, stearic and oleic acids as the predominant protein-bound fatty acids (Keenan & Heid, 1982).

PAS-6 and PAS-7 are abbreviations for Periodic Acid Schiff 6 and 7, respectively. Their M_w ranges from 43 kDa to 53 kDa (Mather, 2000). The amino sequences of PAS 6 and PAS 7 are identical, but vary in glycosylation. The actual glycosylation

site has been determined by Hvarregaard *et al*. (1996). PAS 6 and PAS 7 are loosely associated to MFGM, probably to the phospholipids and can be removed from the membrane by washing with salt solutions (Kanno & Kim, 1990).

When the MFG is expelled through the apical membrane, γ-glutamyl transpeptidase is loosely included in the MFGM. However, in cold-stored milk 74% of the total activity of γ-glutamyl transpeptidase is in the skim milk (Baumrucker, 1979). The enzyme consists of two subunits with M_w of 57 kDa and 25.5 kDa, respectively (Baumrucker, 1980). Furthermore, γ-glutamyl transpeptidase is involved in the amino acid uptake for milk protein synthesis (Johnston *et al*. 2004).

Triglycerides are the major fraction of neutral lipids in the MFGM. However, most of this is believed to originate from contamination (from the core of the MFG) during isolation of the membrane (Walstra 1974 and 1985). Whole milk contains 308 to 606 mg cholesterol /100 g fat (Jensen, 2002). The majority is located in the MFGM. Walstra *et al*. (1999) reported the cholesterol content in the MFGM to be 0,2 mg/m^2. However, the proportion of cholesterol decreases through lactation (Bitman & Wood, 1990). Mono- and diglycerides, FFA and glycospringolipids are also present in the MFGM. The latter of the four consists of neutral glycolipids and gangliosides. The quantity of gangliosides is about 8µg/mg membrane protein and the composition is identical to apical plasma membranes of the secretory cells in the mammary gland (Jensen, 2002).

In bovine milk about 60% of the phospholipids is associated in MFGM while the rest is located in the skim milk phase (Patton & Keenan, 1975). The quantity of phospholipids in milk declines through lactation and the decline is larger than the decrease in total milk fat through lactation (Bitman & Wood, 1990). The most abundant phospholipids are the zwitterionic; phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, while the anionic forms phosphatidylserine and phosphatidylinositol are present in lower amounts. The MGFM phospholipids contain high levels of palmetic and oleic acid, while the short and medium-chain fatty acids are present in very low levels (Mcpherson & Kitchen, 1983). The fatty acid composition in the core milk fat can be changed through the feeding of cows. Simultaneously, the composition of the phospholipids is changed. Smith, Bianco and Dunkley (1977) found that feeding a supplement rich in linoleic acid increases the unsaturation of the phospholipids in outer and inner milk fat globule membranes. However, this unsaturation was less than that of the core lipids. Palmquist and Schanbacher (1991) observed that by feeding palmitic acid to the cow, it is possible to increase the saturation of the lipids in the membrane.

Phospholipids form the basic bilayer in biological membranes in which the nonpolar tails are arranged side-by-side and turn towards the lipids. The polar head groups are orientated towards the aqueous environment. A suggestion of the proposed structure of the MFGM is shown in Figure 2. There is a layer of high-melting triglycerides surrounding the core fat. Xanthine oxidase is assumed to be a peripheral membrane protein since it does not containing a long sequence of

nonpolar amino acids to function as membrane anchor (Mather & Keenan, 1998). However, xanthine oxidase is probably associated with the inner membrane (Mather & Keenan, 1998). Butyrophilin is an integral membrane protein and therefore an important factor in stabilising the MFGM.

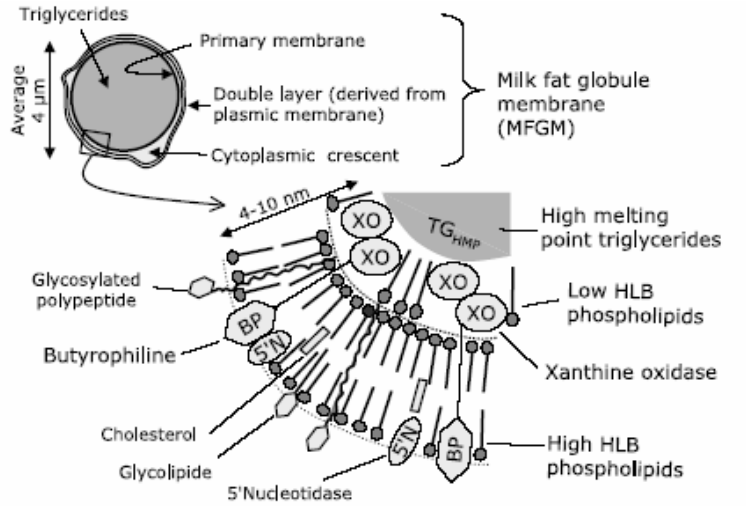

Figure 2. Structure of the milk fat globule membrane. (Michalski *et al*., 2002). Reproduced with permission from Elsevier.

Lipolysis in milk

Lipase

Lipoprotein lipase (LPL) is the enzyme mainly responsible for lipolysis in raw milk. It originates from the mammary gland, where it is involved in the uptake of blood lipids for milk synthesis. The enzyme is active in lipid-water interfaces. Its optimum temperature is 33°C, and pH optimum is about 8.5. It is a relatively heat labile enzyme which is mostly inactivated by a high temperature-short time heat treatment. In milk, LPL is mainly associated with the casein micells (Hohe, Dimick & Kilara. 1985). LPL is brought into contact with the triglycerides when the MFGM is disrupted and casein coats the formed lipid-water interface. The enzyme is activated by apo-lipoprotein CII from the blood which assists LPL to bind onto the fat globule (Bengtsson & Olivecrona, 1982). In spite of the high amount of LPL in milk, lipolysis is limited since milk fat is protected by the membrane and raw milk is normally stored at temperatures far below the optimum temperature of LPL. Furthermore, the products of the hydrolyses of the triglycerides, the FFA, inhibit the enzyme presumably due to that the FFA binding to the LPL. Furthermore, the proteose-peptone component 3 is found to inhibit LPL (Cartier, Chilliard & Paquet, 1990; Girardet et al. 1993).

Several investigations have shown that the activity of LPL in whole milk is not correlated to FFA content in raw milk (Salih & Anderson, 1979; Bachman &

Wilcox, 1990a; Cartier & Chillard, 1990). On the other hand, other studies show that the activity of lipoprotein lipase in the cream fraction is related to the level of lipolysis (Ahrne & Björck, 1985; Bachman & Wilcox, 1990; Cartier & Chillard, 1990). Consequently, the formation of FFA is assumed to be dependent on MFG susceptibility to action of lipases. LPL preferentially hydrolyses fatty acids in position sn-1 and sn-3. The fatty acids placed with high frequencies on position sn-1 and sn-3 are C4, C6, C18 and C18:1 (Walstra & Jennes, 1984; Jensen, 2002). This is in agreement with the recent report by Ouattara et al. (2004) who demonstrated that C4, C6 and C18:1 were the most freqent free fatty acid in a mixture of raw and pasteurised milk stored at 4 °C for 48 hours. Lipases synthesized by bacteria or yeast can also be present in milk. However, if milk is properly stored and has an acceptable hygienic quality, microbial lipases are not an important factor for lipolysis until after several days of storage.

The classic division of lipolysis in milk is between spontaneous and induced lipolysis (Jellema, 1986). The factors affecting spontaneous lipolysis include milking frequencies, udder health and stage of lactation. Induced lipolysis is caused by homogenisation, pumping and temperature fluctuations.

Lipolytic rancid flavour

The rancid flavour developing from lipolysis is often described as gouty or soapy. Several studies have tried to find the relationship between the rancid off-flavour and the level of FFA in milk. As shown in Table 4, the literature based on sensory panel tests indicates no completely clear relationship between the level of FFA and flavour threshold. However, the risk of a rancid off-flavour will always

Table 4. Flavour thresholds for rancidity in pasteurised milk detected by sensory panels.

Threshold - Level of FFA (meq./100g fat)	Country	Reference
2.0	Canada	Pillay, Myhr & Gray (1980).
2.74	USA	Kinter & Day, 1965
1.5	Holland	Tuckey & Stadhouders, 1967
1.25	Denmark	Government Research Inst for Dairy Industry (1962)

increase with an increase in FFA concentration. Shipe, Senyk & Fountain (1980) found that the correlation coefficient between ADV (acid degree value) and rancid flavour score was r^2=0.67. In contrast, Duncan, Christen & Penfield (1991) reported a poor correlation (r^2=0.02) between the level of FFA (similar to acid degree value) and rancidity flavour score. Recently, Gonzalez-Cordova & Vallejo-Cordoba (2004) found a high correlation between short-chain FFA determined quantitatively by solid phase microextraction-GC and sensory scores by using a multiple regression analysis. In addition, they also found a significant correlation (r^2=0.84) between level of FFA and sensory score. The advantages and

disadvantages of the analytical method for determination of the level of FFA are discussed further in the material and method section.

The development of a rancid flavour in milk is greatly affected by the composition of FFA. It is mainly the fatty acids with chain length from 4 to 12, which contribute to the rancid flavour. Duncan & Christen (1991) observed that the flavour threshold in milk for added C4 is 0.20 µmol/ml compared with 0.55 µmol/ml for C18:1. Likewise, Urbach, Stark & Forss (1972) reported that a concentration of 3 ppm added C4 is the flavour threshold in butter, while the threshold for C14 is 100 ppm. Culturing or acidification of the milk increase the appearance of rancid flavour at the same level of FFA, presumably as a result of changing the ratio of fatty acids to fatty acid salt (Tuckey & Stadhouders, 1967). In a Canadian study, rancid off-flavour was the second most appearing off-flavour, after feed-transmitted off-flavour in farm bulk tank milk (Mounchili *et al*. 2004).

Changes induced to milk fat globules during different treatments

Influence of mechanical treatment on MFG stability

It has been suggested that of the final FFA level in pasteurised milk around 60-70% is due to lipolysis occurring during milking and milk transfer to the bulk tank (Anderson, 1983). Mechanical treatments of the milk such as pumping and stirring subject MFG to physical stress. Higher flow velocities during pumping in pipes result in greater friction in the liquid itself and between the liquid and the pipe wall. These relative differences in flow velocity perpendicular to the flow direction are called shear rates. The shear rate depends on the diameter of the pipe and the flow velocity. The presence of air, the temperature of the milk and fat content affect the stability of the MFG during mechanical treatments of milk.

In milking systems, the milk is mixed with air, especially when air is used as a transport medium for the milk. The stability of the MFG is lowered by mixing with air or any other gas during pumping or agitation of the milk. The contact between a MFG and an air bubble results in rupturing of the MFG, since membrane material and part of the core fat will spread over the air/milk plasma interface and will be released into the milk plasma when air bubbles collapse or coalesce (Evers, 2004). Needs, Anderson & Morant (1986) reported that using a claw piece requiring high air bleed instead of a conventional claw increased FFA level by 21 %. Similar results were found by O´Brien, O´Callaghan & Dillon (1998) and Rasmussen et al. (unpublished results, 2005).

Pumping of cream is usually conducted at lower flow rates compared with pumping of milk. Studies have suggested that the stability of the MFG decreases linearly with increasing fat content in milk/cream (Hinrichs & Kessler, 1997; Hinrichs, 1998). This is ascribed to the increased friction between fat globules.

The milk temperature is also a very important factor when milk is exposed to mechanical treatments. Several studies have reported that the maximum

accumulation of FFA upon agitation is at a temperature of ~15 °C and again after ~30°C, with low formation of FFA between 20-30 °C (Fitz-Gerald, 1974; Deeth & Fitz-Gerald, 1977; Bhavadasan, Abraham & Ganguli, 1981; Hisserich & Reuter, 1984). At low temperatures the milk fat is more resistant to mechanical stress. Homogenisation of milk can only be successful at temperatures above 40°C. The effect of temperature on MFG stability is due to crystallization of lipids. One minor factor is that the temperature affects the activity of LPL.

Crystallisation of fat

The most common process during the manufacture of dairy products is cooling. The crystallisation point of milk fat is broad due to a large variety of triglycerides. The crystallisation process of milk fat includes two steps; nucleation and crystal growth.

Crystallisation starts in a supercooled liquid with the formation of submicroscopic crystal nuclei. Nucleation is often heterogenous and takes place at the surface of very small particles. These particles are called catalytic impurities. When a fat crystal has been formed it can act as a catalytic impurity for other triglycerides. In anhydrous fat, it requires a supercooling of only a few degrees centigrade to form enough catalytic impurities to induce crystallisation. In milk, however, it is different since the fat is divided into many globules and in every single globule one nucleus must be formed. The crystallisation rate increases with increasing lipolysis of the fat and it is therefore suggested that crystals of monoglycerides are the predominant catalytic impurities in milk fat (Walstra and van Beresteyn, 1975).The MFG size affects the necessary supercooling to start nucleation, *i.e.* deeper supercooling is required in small globules (Walstra & Beresteyn, 1975)

The growth of crystals in milk is very slow as the many competing molecules try to fit into a vacant site in the crystal network (lattice). Often, incorrect molecules occupy the vacant site for a while before they diffuse out of the lattice, and make place for a proper molecule. The order of the crystal network has to be very systematic and precise.

Triglycerides crystallise in different polymorphic forms (*i.e.* different crystal lattice types), which have different distances between layers of molecules. The three main polymorphic forms of triglycerides are α, β´ and β. All three types are found in milk fat (Walstra and van Beresteyn, 1975; Lopes *et al*. 2001) and 2005). Each polymorphic form is characterized by its own melting point. This contributes to making the overall melting point of milk fat even more complex. The α and β´ forms are metastable. Furthermore, a low melting modification of the β´ forms, is called γ or sub-α form and is reported to have been found in milk (ten Grotenhuis *et al*. 1999 and Lopez *et al*. 2005). By using X-ray diffraction the different polymorphic forms can be monitored. Lopez *et al.* (2001) observed that the nucleation starts in α the polymorphic form at 18 °C by slow cooling (0.15 °C/min) of cream. At 9 °C the formation of the β´ forms begins, and the α + β´ polymorphic forms coexist until the end of cooling (–8 °C). In anhydrous milk fat, nucleation begins in β´ form and thereafter α + β´ polymorphic forms coexist

(Lopez *et al* 2001). By heating cream from –8 to 50°C, the dominant form between -8 to 5 °C is the α form (Lopez *et al.* 2000). Thereafter and up to 17.4 °C the dominant form is coexistence of α + β´ forms. After 15 °C and up to the final melting, the major form is β´. Similar results were found by heating anhydrous milk fat (Lopez *et al.* 2001). The transformation of α crystals into β´ crystals means that once β´ crystals are formed, triglycerides will dissolve from the α crystals and crystallise onto the β´ crystals. The cooling rate has an impact on the onset of crystalisation of milk fat, polymorphic crystal transitions and crystal size (Lopez *et al.* 2005).

Crystallisation of milk fat in MFGs occurs later than in a continuous milk fat phase (Söderberg *et al*. 1989). Buchheim (1970) showed by electron microscopy that crystallisation in MFGs starts predominantly with the high melting triglycerides at the inside of the membrane. In milk and cream the crystal growth is dependent on only a few catalytic impurities being available for starting the nucleation in every single fat globule and the crystals are disturbed by the curvature. Furthermore, the presence of phospholipids in the MFG affects the crystallisation. Vanhoutte *et al*. (2002a & 2002b) reported that the addition of small amounts of phospholipids into anhydrous milk fat delays the onset of crystallisation upon isotermal cooling at 25°C. They suggested that the phospholipid will be absorbed to the initial crystals due to their lower solubility in the melt and the absorbed phospholipids will block further growth of the crystals.

Objective

The implementation of automatic milking systems initially caused a lowering of milk quality regarding total bacterial count, somatic cells count, freezing point of milk, antibiotic residues and FFA. During the recent years quality in terms of the parameters mentioned has generally improved, except FFA. A high level of FFA increases the risk of rancid flavour in dairy products.

The main objective of the present thesis was to evaluate different factors in automatic milking systems which could potentially cause increased level of FFA. The stability of the MFG was described by using model systems.

The specific objectives of this thesis were:

- to describe the factors affecting the size distribution of MFGs (paper I).
- to evaluate the influence of the temperature of the milk and of feed on MFG stability during mechanical stress of raw milk (paper II and III).
- to study the impact of increased milking frequencies of cows on the FFA content in milk (paper IV).

Material and methods

Animals and feed

Milk from Danish Holstein cows was used in paper I, II and III. Swedish Red and White cows were used in paper IV. In paper I milk was randomly collected from cows not administrated any experimental diet. In paper II and III the groups of cows in mid-lactation were offered the diets as shown in Table 5.

Table 5. Composition of feed concentrate offered to the different groups of cows used in paper II and III. Unsaturated and saturated refer to the type of lipid in the concentrate.

Paper	Unsaturated *II*	Saturated *II*	High *de novo* *II & III*	Saturated *III*
Sugar beet pulp	372	555	453	555
Barley	0	0	329	0
Soybean meal	0	312	206	312
Oats	211	0	0	0
Roasted whole soybean	405	0	0	0
Lipid supplement containing 50% palmitic acid and 50% stearic acid	0	121	0	0
Lipid supplement containing 80% palmitic acid and 20% stearic acid	0	0	0	121
Mineral mixture	12	12	12	
Total kg	1000	1000	1000	1000

Pumping experiments

The pumping system (Figure 3) used in paper II and III consisted of 9.5 m pipeline (diameter 2.25 cm), one valve, a balance tank and a centrifugal pump (Alfa-Laval, Sweden). The inlet pipe was fitted at the top of the balance tank. In each treatment, seven litres of raw milk were pumped through the system for 450 seconds. The flow rate was regulated by an frequency converter (ABB ACS 140, Denmark).

Figure 3. Picture of the pumping system used in paper II and III.

Milk fat quality

Determination of size distribution of milk fat globules

Particle size distributions were determined by integrated light scattering using a Mastersizer 2000 (Malvern Instruments Ltd, UK). The refractive indexes according to Michalski, Briard & Michel (2001) were used. The volume-based diameter, $d_{(4,3)} = \frac{\sum N_i d_i^4}{\sum N_i d_i^3}$ was calculated by the integrated software (where N_i is the number of globules in a size class of diameter d_i). The volume-based diameter, $d_{(4,3)}$ was used because it is more sensitive to the presence of coalescence of MFG.

Analysis of free fatty acids in milk

In paper II and III the BDI-method (IDF, 1991) is used to determine the levels of FFA in milk. The advantages of this method are that it is used in many countries and is the most frequently used in combination with sensory tests. The disadvantage is that the recovery of the short-chain fatty acids is poor due to that these are dissolved in the aqueous phase during extraction (Duncan & Christen, 1991; IDF, 1991; Evers, 2003). The method involves an extraction of milk with the BDI reagent (Triton-x-100 and sodium tetraphosphate) to separate the fat. The released fat is dissolved in 2-propanol /ethanol and titrated with ethanolic KOH under nitrogen. Normally, thymol blue is used as indicator, but in the present studies an automatic titration (ABU 96 Tribuette, Radiometer, Denmark) was used. The level of FFA (acid degree value) is expressed as mmol KOH used to neutralize 100g fat.

In paper IV the content of FFA was determined by the Auto-Analyzer II method (Lindqvist, Roos & Fujita, 1975). The advantage of this method is that it is cheap and fast, 40 samples/h. Furthermore, the recovery of short chain fatty acids is higher compared with the BDI-method (IDF, 1991). The method is based on an extraction of the milk sample with a solution containing 2-propanol, heptane and 1 N H_2SO_4. In this method the lipase (is in the aqueous phase) and the lipid are separated, thus the sample can be stored for a week at room temperature without further lipolytic activity. In the auto-analyser, the solution is mixed with the indicator reagent (phenol red, sodium barbital and ethanol) and finally the absorbance is recorded at 560 nm in a colorimeter. These analyses were conducted by STEINS laboratories (Holstebro, Denmark).

Fatty acid composition

The fatty composition was determined by gas chromatography (GC). Prior to GC separation and quantification, milk lipids were trans-esterified to methyl esters in a sodium methylate solution (2 g/l methanol). Analysis of the fatty acid methyl esters was carried out with a GC (6890 series, Hewlett-Packard Co., USA) using an FFAP-column (terephtalic acid modified polyethylene glycol 25m x 200 µm x 0.30 µm) (Hewlett-Packard Co., USA) and helium as carrier gas and a flame ionisation detector. Injection was splitless with an injector temperature of 250 °C. The detector temperature was 300°C. The initial column temperature was 40 °C which was held for 4 min. The temperature was then raised by 10 °C /min to 240°C, and held there for 1 min.

Assays for activity of MFGM enzymes

The activity of xanthine oxidase (paper II) and γ-glutamyl transpeptidase (paper III) in raw milk was determined as markers for disrupted MFGM or amount of MFGM, respectively. These MFGM enzymes were chosen, since the assays are very convenient compared with analysis with immunological methods, *e.g.* butyrophilin.

Analysis of xanthine oxidase activity in the milk serum

Xanthine oxidase activity was determined by the method of Cerbulis and Farrell (1977). The activity was measured just after treatment so the temperature was kept at the exact pumping temperatures. Milk sample (0.2 mL) was mixed with 1 mL 0.05M-disodium hydrogen phosphate buffer (pH 7.4), 0.8 mL H_2O, and 1 mL xanthine solution (20 mg/L H_2O). The mixture was incubated at 25 °C for 5 minutes. The reaction was stopped by addition of 1 mL of 20% trichloroacetic acid followed by centrifugation (2000 x g). The accumulation of uric acid was expressed as the absorbance of the supernatant at 290 nm. The molar extinction coefficient of 1.22×10^4 l mol^{-1} cm^{-1} for uric acid was used. Samples were analysed in triplicate.

Analyses of γ-glutamyl transpeptidase activity in milk

Whole milk (50 µL) was pipetted into 96-well microplates (Nunc, Denmark) and 200 µL substrate mix (0.75M Tris-HCl, pH 6.7, 15mM glycylglycine, 25mM

EDTA, 1 mM γ-glutamyl *p*-nitroanilide) was added. Immediately, absorbance at 412 nm was measured in kinetic mode using an automatic Powerwave microplate reader (Bio-Tek Instruments, USA). Activity of γ-glutamyl transpeptidase in milk was measured as release of *p*-nitroanilide at 412 nm could be completely inhibited by the specific inhibitor acivicin, proving that the activity observed was due to γ-glutamyl transpeptidase.

Milk fat crystallisation

Determination of liquid fat by nuclear magnetic resonance (NMR)

In paper III, the level of liquid fat in milk fat globules was determined by NMR. Cream was produced by centrifugation (1000 x *g*) of the milk for 10 minutes at 4°C. Subsequently the cream samples were incubated at 31 °C for 2 hours before measurements. The amount of liquid fat was determined by NMR using a Maran Benchtop Pulsed NMR Analyzer (Resonance Instruments, UK) with a resonance frequency for protons of 23.2 MHz. The NMR instrument was equipped with an 18 mm variable temperature probe. Approximately 2-3 g cream was placed in sealed NMR tubes and upon temperature equilibration at 31°C the free induction decay (FID) was measured. Subsequently the sample was cooled down to 4°C in ice water, and an FID acquisition at 4°C was carried out immediately and thereafter repeated each 10 min for a total of six times. Liquid fat content was determined as signal amplitude of the FID according to the principles described by Samuelsson & Vikelsøe (1971) and the liquid fat content is expressed in paraffin oil units according to following equation:

$$Liquid\_fat = \frac{signal\_of\_sample \times mass\_of\_standard}{mass\_of\_sample \times signal\_of\_standard}$$

Results and discussion

Influence of feed composition on milk fat globule size

A positive correlation was found between diurnal fat yield of cows and average volume-weighted diameter of MFG (paper I). The effect of this new knowledge was used to produce raw milk with different average MFG sizes based upon various fat contents of the milk (paper II and III), as shown in Figure 4. Three groups of cows were fed diets with different amount and source of lipids. One group was given a diet containing a high level of roasted soybeans which is rich in C18:2 (paper II). The result of this diet was production of milk with an average fat content of only 3.7% as the polyunsaturated fatty acids inhibit the formation of precursors for milk fat in the rumen (Børsting, Hermansen & Weisbjerg, 2003). The fatty acid composition of the produced milk was also affected by the roasted soybeans in the diet.

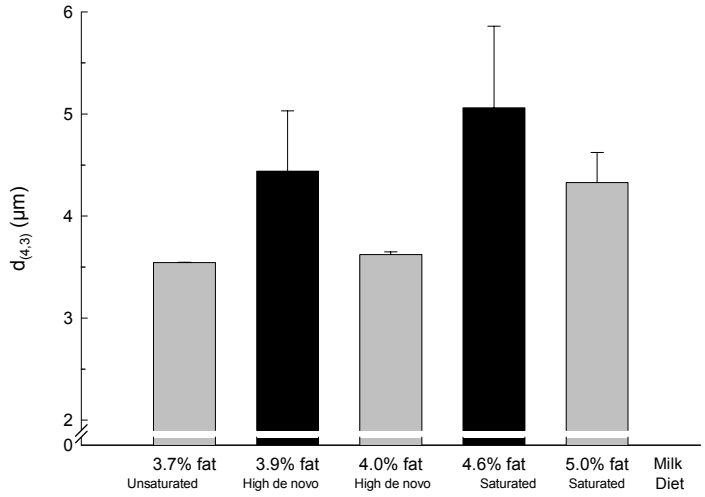

Figure 4. Effect of fat content and diet on average volume-weighted diameter ($d_{(4,3)}$) of MFG. Unsaturated and saturated, refer to the type of lipid in the concentrate. The diets are specified in *Material and methods*. Grey bars: paper II. Black bars: paper III.

The second group of cows was offered a diet which a included large amount of saturated fat supplement. The consequence of this feeding was that the cows produced milk with 5.0% fat. It is well-known that a saturated fat diet supplement increases the fat content in milk (Chilliard, 1993; Shroeder, 2004) but this was a very high fat content for Holsteins cows. The third group of cows was fed a low fat diet that stimulate high *de novo* synthesis of the milk fat, resulting in milk with an average fat content of 4.0%. A similar diet was used in the experiment presented in paper III, where it resulted in an average fat content of 3.9%. The

second diet in this experiment (paper III) contained a large part of saturated fat supplement with a higher proportion of palmitic acid (C16:0) at the expense of stearic acid (C18:0) compared with the experiment presented in paper II. This diet resulted in that the cows produced milk containing on average 4.6% fat. However, in the experiment presented in paper III, the variation in fat content and average diameter of MFG between cows was larger. In both experiments (papers II and III) the milk type with the highest fat content contained larger MFGs than the milk with a lower fat content. These results from the feeding experiments confirmed the correlation between diurnal fat yield of cows and average volume-weighted diameter of MFG, previously described (paper I). Although, the milk yield was not registered (paper II and III), the large increases in fat percentages upon feeding saturated fat supplements also increased diurnal fat yield, since similar studies have shown that the milk yield slightly increases upon this type of supplements to the diet (Chilliard, 1993).

The mechanisms responsible for the increase in average diameter of MFG when the cow produce more fat were studied in the experiment presented in paper I. It was concluded that the increase in average MFG size is due to the limited amount of membrane material during milk fat synthesis. This was indicated by the significantly decreased activity of the MFGM enzyme, γ-glutamyl transpeptidase, in whole milk with increasing MFG size. Furthermore, it was also demonstrated that when the diurnal fat yield increased, the medium size fat globules were transformed into larger fat globules with an average diameter > 8 μm.

The fatty acid composition in the milk was affected by the diets (paper II and III). It was observed that the average diameter of MFGs was positively correlated with the concentration of C16:0, C16:1, C18:0 and C18:1 (paper I). No significant correlation between average diameter of MFGs and C4-14 or C18:2+C18:3 was found. This indicates that the fatty acid in milk originating from the diet C16:0, C16:1, C18:0 and C18:1 affects the size of the MFGs. This was confirmed by the feeding experiments (paper II and III) where diets rich in C16 and C18 resulted in milk with larger MFG diameter. In the study presented in paper II, the milk with the largest diameter of MGF contained higher concentration of C16:0, C16:1 and C18:0 (g fatty acid/100g milk) than the two other milk types. Concerning C18:1, milk from the unsaturated fat diet contained slightly more than milk from the saturated diet *i.e.* 1.17 versus 1.10 g fatty acid/100g milk. In the experiment reported in paper III, the concentrations of C16:0 and C16:1 were highest in the milk with the largest MFG which occurred in milk from the cows fed saturated fat diet compared with the milk coming from the cows administrated the high *de novo* diet. Milk from the high *de novo* diet contained slightly more C18:1 than milk from the saturated diet, 0.69 versus 0.60 g fatty acid/100g milk and marked more C18:0. Recently, Briard *et al*. (2003) found, in agreement with paper I, that large MFGs contain more C18:0 than small MFGs. In contrast they found that small globules contained more C12:0, C14:0 and C16:1. However, in the present study, the average diameter is correlated to the content of fatty acids in the milk (paper I), whereas in Briard *et al.* (2003) the MFG was separated into small and large sizes, and analysed for fatty acid composition (wt%).

The knowledge, obtained in these studies, makes it feasible to design the distribution of MFG through feeding. In the following section it will be showed and discussed that the size of the MFG has an impact on its stability during pumping. Recently, studies of Michalski *et al*. (2002, 2003 & 2004) have shown that the size of native MFG affects the firmness of milk gels, camembert and emmental cheeses. In theses studies, they separated MFGs in size fractions by microfiltration. By designing the distribution of MFG through feeding instead of by microfiltration , processing will appear more careful and products more natural. Furthermore, the awareness of variations in fatty acid composition between small and large MFGs can be applied to development of dairy products with new texture behaviour.

Influence of MFG size and fat content on MFG stability

Pumping is one of the factors inducing lipolysis of milk fat. Already, when the milk is transported from the cows to the farm bulk tank, it is pumped. The majority of FFA in milk is accumulated at farm level (Anderson, 1983). Furthermore, harsh mechanical treatment of milk can result in coalescence of MFGs which means that two or more MFGs share common membrane or fat touches fat, thus they can not be disrupted by shaking (Jennens & Walstra, 1984). This can lead to un-dissolved clumps of milk fat on the surface of unhomogenised drinking milk products which is unwanted by the consumers. Coalescence of MFGs is detected by an increase in the average diameter from the particle size distribution of MFGs.

The highest degree of coalescence of the MFGs during pumping occurred in milk with the highest fat content and MFGs with the largest average diameter (paper II and III). By comparing the milk from the saturated diets in the two studies, the level of coalescence was clearly higher in the experiment presented in paper II than in the other experiment (paper III). Despite, that the inherent average diameter of MFGs was larger in the experiment presented in paper III, the level of coalescence at 31˚C was lower than in the other experiment (paper II). That fact suggests that the fat content in addition with MFG size affect the stability of MFG regarding coalescence, since the fat content was highest in the study presented in paper II.

The accumulation of FFA was dependent on temperature and wall shear rate. However, at temperatures lower than 31˚C, the formation of FFA is highest in the milk with the larges MFGs and the highest fat content (paper II and III), both inherently and after pumping. The level of FFA can not directly be compared in the two experiments (paper II and III) because the milk was stored for 24 and 2 h after pumping, respectively. At 31°C, there was no significant difference after pumping with the highest shear rate between the FFA content in milk with various fat content and diameter of MFG.

The emulsion stability of homogenised cream upon shearing is decreasing with increasing diameter of MFGs (Hinrichs, 1994). Furthermore, Hinrichs (1994) reported that the fat content is crucial for shearing of raw cream (16-45% fat). Likewise, the presents studies demonstrated clearly an impact of fat content in raw

milk, although the studies were performed with raw milk with fat percentages only varying between 3.7-5.0%. Moreover, the fat content and diameter of MFG were regulated trough feeding of the cows. Since pumping of raw milk always takes place in all milk production, the knowledge of the feed-induced MFG stability is very important. The results clearly show that milk producers should be careful by feeding with saturated fat supplement as it increase the risk for lowering the milk quality, especially regarding FFA.

Influence of temperature on milk fat globule stability

The chosen temperatures of milk during pumping was 4-5, 20 and 31 ˚C (paper II and III). These temperatures range from the milk leaving the udder to cool storage temperatures. Milk was cooled direct after milking to the relevant temperature for pumping. Furthermore, the shear rates used in the experiments (paper II and III) were within the ranges occurring in the dairy industry.

Significant coalescence of MFGs only occured at 31 ˚C and only in milk with the highest fat content and largest average MFG diameter (paper II and III). The coalescence of MFG at 31 °C already begins at a shear rate of 365 s^{-1}. At 4-5 ˚C the MFGs were resistant to coalescence upon pumping. It could indicate that a high proportion of the milk fat need to be in liquid phase for initiating coalescence of MFGs. However, it is difficult to explain, why significant coalescence only occurs at 31 °C and only in the milk from the saturated fat diet based on the present work, since the proportion of liquid fat is assumed to vary in the milk from cows offered the saturated fat diet (paper II and III). In the experiment presented in paper III, the content of unsaturated fatty acid (C16:1, C18:1, C18:2 and C18:3) in the milk from cows fed the high *de novo* diet was lower than in the milk from cows fed the saturated fat diet in the experiment presented in paper II. Hence, the larger average diameter of MFG and higher fat content may explain the higher coalescence of MFGs in milk from cows fed the saturated fat diet, but the temperature dependence is not elucidated.

The large difference in the ratio of liquid fat between cream from the high *de novo* and saturated fat diet was demonstrated by NMR studies (paper III). Moreover, the effect of temperature and incubation time on liquid fat in the two milk types was demonstrated. The results of liquid fat in cream were explained by the fatty acid composition of the milk. Mulder & Walstra (1974) reported that partial coalescence only occurs when part of the fat is present as crystals. Our results shows that 14.8 % solid fat in the MFG was sufficient to cause coalescence upon pumping with a shear rate of 565s^{-1}. Whereas levels of solid fat in MFG >26.6 % and at 3.4% did not cause coalescence of MFGs. By using much higher shear rates than in the present study, Hinrichs & Kessler (1997) observed that increased solid fat content in raw cream increased the level of the critical shear rate that caused destabilisation of the MFGs.

The accumulation of FFA in milk upon pumping was highest at 20˚C for milk with the highest fat content and largest average diameter of MFG (paper II). The milk from the unsaturated fat and the high *de novo* diet reached the same level of FFA

at 20 and 31˚C. This is in agreement with other studies reporting that the maximum formation of FFA upon agitation is at a temperature of ~15 °C and again after ~30 °C, (Fitz-Gerald, 1974; Deeth & Fitz-Gerald, 1977; Bhavadasan, Abraham & Ganguli, 1981; Hisserich & Reuter, 1984). The fatty acid composition of milk could be responsible for the high level of FFA at 20˚C for milk with the highest fat content and largest average diameter of MFG. At 5˚C, there was no significant increase in FFA content in milk upon pumping at various shear rates (paper II). However, the formation of FFA significantly increased upon pumping, when milk was cooled to 4˚C followed by 60 min incubation before pumping (paper III) as shown in Figure 5. Further research is needed to understand this observation. Assumptions can be made that the transition of polymorphic crystal forms of milk fat during cooling affect the susceptibility to lipolysis, or the growth of crystal size could have impact on the stability of the MFGM. Likewise, the longer incubation time at 4˚C could be expected to increase the attachment of LPL to the MFG and cause elevated levels of FFA during pumping. Backman & Wilcox (1990b) found that immediate cooling of raw milk after milking increased the level of FFA compared with 1h delayed cooling of the raw milk. However, this observation is only relevant if the raw milk is not subjected to mechanical treatment.

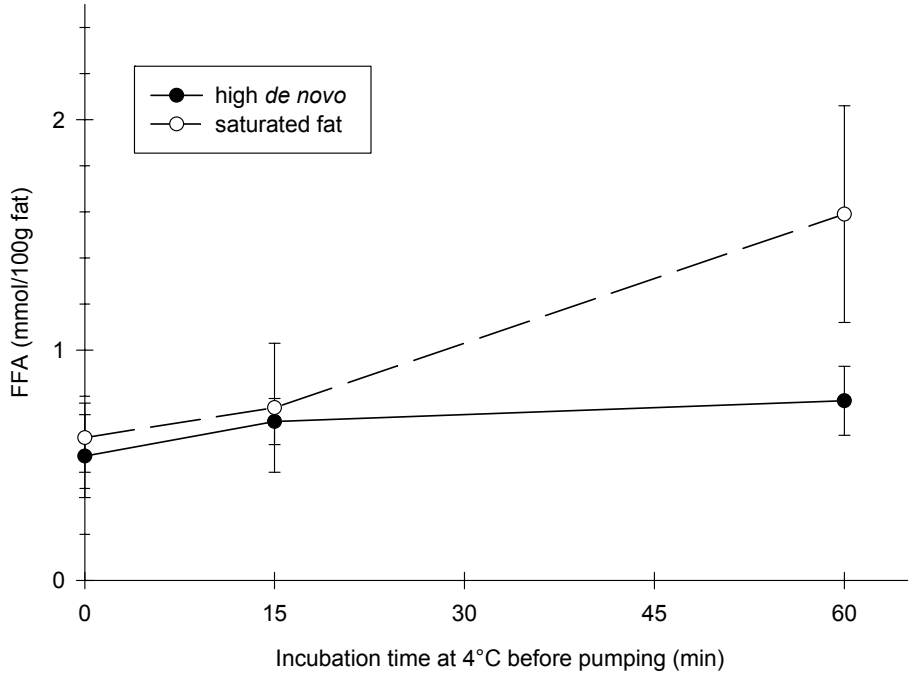

Figure 5. The effect of incubation time before pumping on the level of FFA in raw milk after pumping. The milk was from cows fed the saturated fat diet and the high *de novo* diet, respectively.

The results show that the relationship between coalescence of MFGs and formation of FFA in milk subjected to pumping is complex (paper II and III). At low temperatures (4-5 ˚C), no coalescence of MFGs was detected, whereas the content of FFA significantly increased upon pumping, when milk was stored 60 min at 4˚C before pumping (paper III). Coalescence of MFG was only observed in milk from cows fed the saturated fat supplement diet at 31 ˚C (paper II and III). At the same temperature, the milk from the unsaturated fat and the high *de novo* diet accumulated the highest FFA content upon pumping (paper II), and in these two milk types no coalescence of MFGs was demonstrated at any of the used temperatures. Our results indicate that the formation of FFA begins before coalescence of MFGs occurs. One exception is otherwise when raw milk is subjected to pumping at 20 ˚C.

In order to detect other changes on the MFGM in milk subjected to pumping, the activity of xanthine oxidase in milk serum was determined (paper II). No effect of pumping on xanthine oxidase activity was detected , suggesting that xanthine oxidase is not released from MFGM to the serum phase upon mechanical treatments. In contrast, Back & Reuter (1973) reported that xanthine oxidase is released to milk serum when milk is subjected to shear forces. As expected, cooling of the milk released the xanthine oxidase from the MFGM.

The present results clearly suggest that cooling the milk to 4-5 °C stabilised the MFG upon mechanical treatment, resulting in lower formation of FFA and lower risk of coalescence of MFGs. By transferring the obtained knowledge to milking systems, it suggested that the milk cooling should be placed as close to the udder as possible. Thereby the transportation of warm milk would be reduced, leading to lower levels of FFA.

The effect of increased milking frequency on lipolysis in milk

The introduction of automatic milking systems has made it relevant to study the effects of increased milking frequency *i.e.* milking more than twice daily, since the cows have free access to the milking unit. Studies have reported the average milking frequency in automatic milking systems to be between 2.4-2.6 daily (Svennersten-Sjaunja; Berglund & Petterson, 2000; Hogeveen *et al*., 2001; Petterson & Wiktorsson, 2004). An increase in milking frequency results in higher milk production per cow (Stelwagen, 2001). However, it also affects the milk quality.

In the present study cows were milked 4 times daily on one udder half and twice daily on the opposite udder half (paper IV). The level of FFA was significantly higher (1.49 meq/100 g fat) in milk from the udder half milked four times daily compared with the milk from the udder half milked twice daily (1.14 meq./100 g fat). Similar results have been found by Klei *et al*. 1999 and Slaghuis *et al.* (2004).

In order to study the possible mechanisms behind the increased FFA content in milk upon increased milking frequency, the average diameter of MFG, fatty acid composition and activity of γ-glutamyl transpeptidase in the milk were

determined. Milk from the udder half milked four times per day contained MFGs with a significantly larger average diameter ($d_{(4,3)}$) compared with milk from the udder half milked twice times. Furthermore, the 90% percentiles of MFG size distribution significantly increased upon increased milking frequencies which indicates that transfer to larger globules occur for medium or larger fat globules of the distribution.

In the other experiment (Paper II), it was found that MFGs with the largest average diameter were inherently unstable, resulting in a high level of FFA in milk even without subjecting to pumping. Likewise, the significantly larger average diameter of MFGs in milk upon more frequent milking results in elevated levels of FFA (paper IV). This indicates that MFGs with a large diameter are more susceptible to spontaneous lipolysis. Even though larger globules have a smaller surface area, in total. The surface potential is lower for large MFGs than for small globules, presumably increasing the amount of LPL attaching the MFG.

There was no significant effect on the activity of γ-glutamyl transpeptidase upon milking frequencies (paper IV), indicating that the production of MFGM is sufficient to cover the secreted MFG, when the milking frequency is increased. Furthermore, the results clearly indicate that the *de novo* synthesis of milk fat is not affected by milking frequencies, since the proportion of C4-C14 in milk is invariant between two and four times daily milking (paper IV).

The results clearly demonstrate that increased milking frequency is a factor contributing to the elevated levels of FFA in automatic milking systems. It is also a factor difficult to avoid since more frequent milking increases the milk yield which affects the economic performance of the milk producer. However, other experiments in this thesis (paper II and III) showed that the feeding of cows affected the stability of MFGs during pumping of milk. Similar, spontaneous lipolysis such as caused by increased milking frequency could presumably be lowered trough feeding of cows.

Conclusions

- Rapid cooling of the raw milk before it is pumped from the milking unit in automatic milking systems is to be recommended since accumulation of FFA is minimised in milk at a temperature of 4-5°C during pumping.

- The average diameter of the MFG was positive correlated to the diurnal fat yield. The results clearly indicated that this can be ascribed to a limitation in production of MFG membrane resulting in that the MFGs became larger when the fat synthesis increase. The average diameter of the MFG was also affected by the diets offered and milk from cows fed saturated fat supplements contained MFGs with a large average diameter and had an increased fat content.

- The highest degree of coalescence of MFGs upon pumping of raw milk occurred in milk with the highest fat content and largest average diameter of MFGs. This suggests that the use of saturated fat supplements for dairy cows should be limited, since it will result in milk with a large average diameter of MFGs and a high fat content.

- Milking an udder half four times daily significantly increased the level of FFA in milk compared with milk from the udder half milked twice daily. Furthermore, the average diameter of milk fat globules increased upon increased milking frequency. The results suggest that limiting the milking frequency in automatic milking systems will increase the milk quality in regards to FFA.

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