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Fakulteten för veterinärmedicin och husdjursvetenskap

Swedish University of Agricultural Sciences
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Effects of palmitic and stearic acids supplementation on milk yield, composition and milk lipolysis in dairy cows

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Abstract

When facing the fact that high yielding dairy cows has an increasing energy requirements, the practice of adding lipid supplements such as concentrates rich in palm oil and/or palm oil industry by-products become more common. Palm oil containing high levels of C16:0 causes high levels of free fatty acids (FFA) which are linked to larger milk fat globules (MFG) which affect the reaction of lipolysis resulting in rancid flavour and processing problems. Instead of using palm oil, rapeseed oil rich in C18:1 C18:2 can be used. In order to evaluate the effect of added C18:0 and C16:0 on milk lipolysis, 30 Swedish Red lactating dairy cows including 15 multiparous (MP) and 15 primiparous (PP) cows were randomly divided in three groups resulting in 3 10 cow-groups (5 PP and 5 MP cows per group) and fed one out of three concentrate mixtures: control (C), a standard concentrate mixture with no fat added; palmitate (P) as C plus the addition of palmitate methyl ester (10 % in DM basis); and stearate (St) as C plus the addition of stearate methyl ester (10 % in DM basis). Milk yield was registered, and milk samples were collected and analysed for milk composition, FFA content and size of MFG. In addition, feed intake and in vivo digestibility were estimated. Results from the experiment showed no treatment effect on feed intake or *in vivo* digestibility. Changes in milk yield and composition were more evident in MP than in PP cows. The inclusion of C16:0 and C18:0 caused an increase in milk yield and milk fat content and yield, with changes being greater for cows supplemented with C18:0. The high fat treatments resulted in higher levels of FFA in milk associated with an increased size of the milk fat globules. While the results of the present study would support the concept that C16:0 may be replaced by C18:0 in treatment for dairy cows without any detrimental effect on milk yield and composition, further conclusions cannot be drawn without the data on FA profile in milk.

Sammanfattning

För att tillgodose högavkastande mjölkkors höga energibehov har det idag blivit vanligt att tillsätta fodertillskott som fettsyror i kraftfoder i foderstaten. Det är vanligt att de fettsyror som tillsätts innehåller stora mängder av palmolja och/eller biprodukter från palmolje-industrin för att minska kostnaderna. Detta till trots framställningen av palmoljans negativa inverkan på miljön. Palmolja innehåller höga mängder av C16:0 vilket kan orsaka höga halter av fria fettsyror i mjölken och som är associeras med större fettkulor i mjölk som kan påverka risken för lipolys. Den kemiska processen lipolys orsakar smakfel i mjölk och påverkar mjölkens processbarhet på mejeri. Genom att ersätta palmolja med rapsolja som produceras i Sverige och som innehåller höga halter av C18:1 Och C18:2 kan risken för lipolys minskas. För att undersöka effekten av tillsatt C18:0 och C16:0 i kraftfoder påverkan på lipolys i mjölk och ifall C18:0 kan ersätta C16:0 valdes 30 SRB (15 förstakalvare och 15 flergångskalvare) ut och delades slumpmässigt in i tre grupper med 10 kor per grupp (5 förstakalvare och 5 flergångskalvare). Kor från varje grupp utfodrades en kraftfoderblandning: kontroll, en standardblandning utan tillsatt fett; palmitat innehållande samma standardblandning som kontroll med tillsatt palmitat metylester (10 % ts); och stearat med samma standardblandning som kontroll med tillsatt stearat metylester (10 % ts). Mjölkprover som togs analyserades för mjölkavkastning, komposition och näringsinnehåll. Vidare analyserades även halten av fria fettsyror och storleken på mjölkfettkulor samt foderintag och smältbarhet. Resultat från detta experiment visade ingen effekt på foderintag eller smältbarhet. Mjölkavkastning och näringsinnehåll i mjölk påverkades i större utsträckning av de fettrika kraftfodren hos flergångskalvare i jämförelse med förstagångskalvare. Inblandning av palmitat och stearate ökade mjölkavkastningen samt fetthalt och fettinnehåll i mjölken med en större effekt i mjölk från kor utfodrade stearat. De fettrika kraftfodren gav upphov till högre nivåer av fria fettsyror i mjölken vilket även associeras med större mjölkfettkulor. Resultatet i denna studie förstärker teorin om att C16:0 kan ersättas av C18:0 i foder till mjölkkor utan att orsaka några skadliga effekter på varken mjölkavkastning eller komposition, men fortsatta slutsatser kan inte tas utan mer data från fettsyraprofilen i mjölk.

Introduction

In order to meet the increasing energy requirements of high yielding dairy cows the practice of adding lipid supplements in the diet has become more common (Jenkins & McGuire, 2006; Pantoja *et al*., 1994; Weisbjerg *et al*., 2008). Most of the lipid supplements contain 90-95 % long chain fatty acids (C14 or longer) with different proportions of saturated and unsaturated fatty acids (Grummer, 1991).

In order to keep the costs for feed production low, concentrates manufactured by feed companies are commonly rich in palm oil and/or palm oil industry by-products (Mosley *et al*., 2007). However, palm oil production may represent a threat to the environment due to the changes in land use associated to palm oil cultivation (deforestation and loss of carbon-sequestering forest land), as well as to the required long range transportation. In addition, it has been shown that feeding palm oil to dairy cows may increase the level of free fatty acids in milk, an effect probably due to an increase in the size of the milk fat globules (Astrup *et al*., 1980a; Hedegaard *et al.*, 2006; Wiking *et al.*, 2005).

Increasing levels of free fatty acids in milk increase the risk for off flavours in milk. The result can be detected as rancid flavour and the milk cannot be consumed resulting in economic losses for the farmer (Ashes *et al*., 1997; Shipe *et al.,* 1978).

Rapeseed oil, on the other hand, is produced in Sweden and it may represent an alternative for replacing palm oil in dairy cows diets. While palm oil is rich in palmitic (C16:0) and oleic (C18:1) acids, rapeseed oil is rich in oleic (C18:1) and linoleic (C18:2) acids. After rumen biohydrogenation, palm oil would provide the host with a mixture of palmitic and stearic (C18:0), while rapeseed oil would be a source of stearic acid.

Palmitic and stearic acids may have a different and independent effect on the structure of the milk fat globules and, therefore, in milk lipolysis. Stearic acid can be readily desaturated to oleic acid in the mammary gland which in turn may affect the structure of the fat globules in a different manner than palmitic acid. Changes in the structure of the milk fat globules would affect their stability and hence their sensitivity to lipolysis.

Aim and hypothesis

The aim of this study was to investigate the effect of feeding lactating dairy cows three different concentrates -namely, Control (C): no fat added; Palmitic (P): as C + 10 % inclusion of palmitic acid methyl ester; and Stearic (S): as Control + 10 % inclusion of stearic acid methyl ester- on milk yield and composition, milk fat fatty acid profile*, milk lipolysis (free fatty acids level in milk) and milk fat globule size.

Hypothesis: Dairy cows diets rich in stearic acid will result in milk with a smaller –more stable- fat globule size and lower levels of free fatty acids (lower milk lipolysis) as compared to palmitic acid rich diets.

(*) The fatty acid composition of the milk fat will not be included in the present report.

Literature review

General

The following literature review covers different factors that may affect milk fat fatty acid composition and milk lipolysis.

Increasing fatty acids in feed

Generally, diets for ruminant animals are characterised by having low lipid content - around 5 % on dry matter (DM) basis in forage and forage-cereal grain based diets. There are several important properties of the lipid supplement to consider before feeding in order to predict the effect of the supplement. Features such as; how well fatty acids (FA) are protected from alterations in the rumen, type and origin of lipid and fatty acid, digestibility, transfer of FA to milk and the effect of milk synthesis in the rumen are of importance (Ashes *et al.*, 1997; Palmquist *et al.*, 1993; Sutton, 1989). Table 1 summarizes the different properties mentioned. Further on, mammary uptake and rate of lipogenesis in the mammary gland are also important features to consider (Enjalbert *el al.*, 1998).

Table 1. *Results from feeding fat supplement, modified from Ashes et al. (1997).*

Properties	***Effects***
Composition of lipids	Levels of triglycerides and FA influence biohydrogenation in the rumen
FA	*Trans* C18 isomers influence biohydrogenation
Digestibility	Increased level of unsaturated FA increases absorption of long chained fatty acids (LCFA)
Transfer to milk	50-60 % of supplemented FA can be found in milk*
Milk fat synthesis	Supplement of C16:0 and C18:0 decreases *de novo* synthesis. LCFA may inhibit Δ-*9 desaturase* activity.

**Depends on factors such as quantity, composition, protection in rumen and digestibility.*

The FA profile of the feed supplement is of great importance and research has shown that fat supplementation may have a negative impact on the rumen fermentation, especially when supplementing unsaturated fatty acids (UFA) (Jenkins, 2003; Jenkins & Jenny, 1989). Instead, feeding with saturated fatty acids (SFA) and protects those from microbial alterations in rumen is more effective when feeding additional fat (Jenkins & McGuire, 2006). SFA have lower digestibility than UFA (Harvatine & Allen, 2006; Jenkins & Jenny, 1989). UFA can decrease digestibility of the total amount of FA (Harvatine & Allen, 2006). Research

performed by Weisbjerg et al. (1991) showed that the digestibility for palmitic acid is higher in compare to stearic acid; similar results have also been obtained by Enjalbert et al. (2000). Moet et al. (2008) concluded rate of milk fat lipogenesis is also related to absorb FA from intestines (g/day). Transfer of absorbed FA in the intestines to milk estimated to be approximately: 42 % for C16:0, 9.5 % for C18:0, without any specific feed treatment. The low absorption ration for C18:0 is because the FA is transferred to the mammary gland and desaturated by specific enzymes. Moate et al. (2008) performed calculations on absorption ratio of C16:0 which resulted in for 1 g of absorbed C16:0, 0.401 g was transferred into milk.

Rumen biohydrogenation of dietary lipids

In the rumen, dietary unsaturated lipids undergo hydrogenation. This process comprises two steps. The first step includes hydrolysis of ester bonds by microbial lipases resulting in the release of FA. Extend of the hydrolysis is 85 % of the lipids entering the rumen (Lock & Bauman, 2004). The second step is conversion of UFA through biochemical progress into SFA by microbial activity (Jenkins, 1993; Lourenço *et al.*, 2010). Figure 1 shows a diagram of the biohydrogenation of unsaturated C18 in the rumen. Microorganisms convert oleic (C18:1), linoleic (C18:2) and linolenic acid (C18:3) -the most predominant FA in ruminant diets- into stearic acid (C18:0) (Kellens *et al.*, 1986; Lock & Bauman, 2004). The biohydrogenation progresses with isomerization of UFA, including conversion of the isomer *cis*- to a *trans*-stereotype. For C18:2 it is the *cis*-12 double bond which by isomerization converts into cis-9, *trans*-11 C18:2 by the enzyme *isomerase*. After forming the *trans*-11 isomer further hydrogenation of the *cis*-9 bond in C18:2 takes place by the enzyme *reductase* and a result trans-11 C18:1 is formed. Further hydrogenation of trans-11 C18:1 into C18:0 can also occur (Jenkins, 1993). However, the rate of this step depends on the rumen environment and type of microorganisms (Kellens *et al.*, 1986). The level of conversion for C18:2 is approximate 75-90 % and for C18:3 85-100 % (Harfoot & Hazlewood, 1997). Other factors affecting the biohydrogenation are: feed composition, feeding level and frequency, dietary FA level and composition (Harfoot & Hazlewood, 1997; Jenkins, 1993).

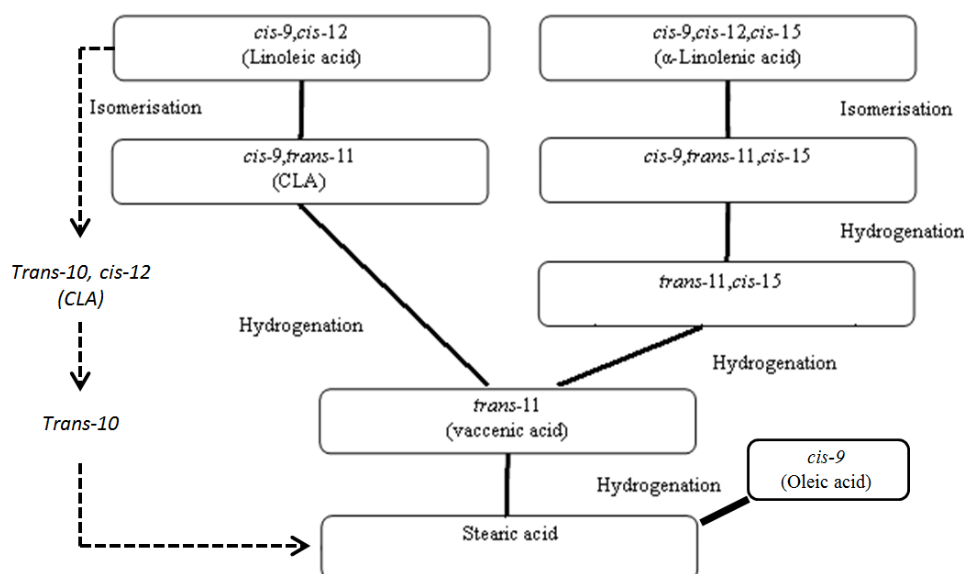

Figure 1. *Overview of biohydrogenation in the rumen* (Bauman *et al.,* 2006).

One consequence of the biohydrogenation is the production of conjugated fatty acids (CLA). These CLA are produced through the process of biohydrogenation of polyunsaturated fatty acids (PUFA) such as linoleic acid (Fig. 1; Bauman *et al.*, 2006). CLA can be present in different isomeric forms, although the most common is *cis*-9, *trans*-11 C18:2 (Lorenço *et al.*, 2010). After rumen biohydrogenation, LCFA reaching the small intestine, are readily absorbed and transported into peripheral circulation through the lymphatic system as chylomicrons (Bauchart, 1992). Some proportion of the CLA synthetized in the rumen may rich the small intestine and, after digestion and absorption, be incorporated into the milk fat. While CLA are considered to be "healthier" for human consumption due to their anti-obesity effects, prevention of cardiovascular diseases, cancer preventing and increased immune response (Allison & Park, 2012; Cook *et al.*, 1993; Ip *et al.*, 1994; Park *et al.*, 1999). Some CLA may impair the synthesis of milk fat (Bauman *et al.*, 2006).

Milk lipids

The total content of fat in cow's milk is approximately 3-5 % (Bauman & Lock, 2010). This level is not constant and depends on several factors such as: breed, parity, stage of lactation, diet and nutritional and health status of the cows. Milk fat is the energy richest component in milk and the different properties of milk fat determinate the different characteristics and physical appearance of milk and milk products (Bauman & Griinari, 2003). Over 95 % of the milk fat is in the form of triglycerides (TAG) (Ashes *et al.*, 1997; Bauman & Griinari, 2003; Walsta *et al.*, 1999). TAG consists of one molecule of glycerol and three FA esterified to the glycerol molecule (Huppertz *et al.*, 2009; Walsta *et al.*, 1999). Remaining lipids are present as phospholipids, cholesterol, cholesterol esters, monoglycerides, diglycerides, and free fatty acids (FFA) (Walsta *et al.*, 1999). Composition of fat in milk is shown in Table 2.

Table 2. *Composition of different lipid classes in milk. Trace elements are not included. Modified from Walsta et al., 1999.*

Lipid class	***Percentage in milk fat (%)***
TAG	98.3
Diglycerides	0.30
Monoglycerides	0.03
FFA	0.10
Phopholipids	0.80
Cholesterol	0.30
Cholesterol esters	0.02

Fat in milk forms milk fat globules (MFG) which acts as oil-in-water emulsion droplets (Walsta *et al.*, 1999). The outer membrane of the MFG consists of different components; primary: phospholipids, lipoproteins, glycolipids and enzymes. The inner membrane or the core consists of different fats; primary of TAG (Wiking *et al.*, 2004). The size of MFG varies from 0.1 to 15 μm depending on types of FA present inside the globules. A smaller MFG is more stable in comparison to a larger. Not only does the size between the MFG vary but also the composition of the membrane surrounding the MFG, called milk fat globule membrane (MFGM). When intact, the MFGM protects the inner layer of TAG from damage and

processes such as lipolysis (Walsta *et al*., 1999).

FA in milk

*A*ccording to Bauman & Griinari (2003) and Jensen (2002) there are over 400 different FA present in milk. However, this number is not exact and further studies are needed to establish an exact number. The profile of the FA varies in isomeric form, saturation and length. The isomeric form depends on if the configuration of the FA is in *cis* or *trans* position (Walsta *et al*., 1999). The most common isomeric form is the *cis* configuration where more than 95 % of the UFA occurs in this position (Huppertz *et al*., 2009). Number of double bonds is an indication on the level of saturation or unsaturation of an FA, where present of a double bond causes the FA to be unsaturated and non present to be saturated (Walsta *et al*., 1999). The position of the double bond on the FA determinate if the FA is conjugated or not conjugated. For a conjugated FA the double bonds are separated by a single bond in the carbon chain, where in a non-conjugated FA the double bonds are separated by a methylene group (Huppertz *et al*., 2009). FA is divided into different categories regarding the length of the carbon chain. LCFA consist of >16 carbons, medium-chained fatty acids (MCFA) of 10-14 carbon and SCFA of 4 to 8 carbons (Bauman & Griinari, 2003).

The composition of FA in milk consists of, approximately, 5 % PUFA, 25 % of monosaturated fatty acids (MUFA) and 70 % of SFA (Grummer, 1991). As shown in Table 3, the most common FA in milk are LCFA such as palmitic (C16:0), myristic (C14:0) and stearic (C18:0) acids. The most occurring MUFA is oleic acid (C18:1). In milk the level of PUFA is only 4 g/100 g milk fat, with linoleic acid (C18:2) and linolenic acid (C18:3) as the most important ones (Mansbridge & Blake, 1997). One of the characteristic of bovine milk is the relatively high presence of short chained fatty acids (SCFA) such as C4:0 and C6:0 (Dils, 1986).

Table 3. Fatty acid presence and level in milk. Modified from Jensen (2002).

Carbon number	***Fatty acid name***	***Average percent range (%)***
4:0	Butyric	2–5
6:0	Caproic	1–5
8:0	Caprlylic	1–3
10:0	Capric	2–4
12:0	Lauric	2–5
14:0	Myristic	8–14
15:0	Pentadecanoic	1–2
16:0	Palmitic	22–35
16:1	Palmitoleic	1–3
17:0	Margaric	0.5–1.5
18:0	Stearic	9–14
18:1	Oleic	20–30
18:2	Linoleic	1–3
18:3	Linolenic	0.5–2

The level of FA varies depending on several factors (Beaulieu & Palmquist, 1995). Differences between breeds such as Jersey and Holstein cows have been registered, stage of lactation, genetics and seasonal effects have impact on the fatty acid profile in milk (Beaulieu & Palmquist, 1995; Palmquist et *al*., 1993). Although, nutrition is still considered to be the factor with greatest impact on milk fat composition (Ashes *et al*., 1997; Bauman *et al*., 2006 Palmquist *et al*., 1993).

Milk fat synthesis

Uptake of plasma lipids by the mammary gland can't take place until chylomicrons are broken down by the enzyme *lipoprotein lipase* present in blood. The end products are FA, glycerol and mono- and di-glycerides, which in turn are used for milk synthesis (Deeth & Fitz-Gerald, 2006; Sjaastad *et al*., 2003). The amount of lipids transferred from the diet into milk fat depends on several factors among others: rates of digestion and absorption of FA, and FA uptake by the adipose tissue (Palmquist *et al*., 1993).

The milk fat synthesis takes place in the epithelial cells of the mammary gland through two different pathways: *de novo* synthesis and synthesis from preformed FA from the blood.

De novo synthesis

Practically all SCFA and MCFA (C4:0-C14:0), as well as half of C16:0 present in milk are *de novo* synthesised in the mammary gland (Grummer, 1991). The synthesis takes place in the cytosol of the epithelial cells in the mammary gland (Bauman & Griinari, 2003; Dils. 1986). Main substrates utilised for fat synthesis in ruminants are glucose –as a source of energy, reducing equivalents and glycerol-, and acetate and β-hydroxybutyrate –as a source of energy

and carbon atoms for fatty acid synthesis. In the process of *de novo* fatty acid synthesis, research performed by Palmquist et al. (1969) illustrated that the first four carbons of the FA are equally provided by β-hydroxybutyrate and acetate. Further in the elongation process only acetate is used. The origin substrate for fatty acid synthesis is acetyl-CoA converted from acetate by the enzyme *acetyl-CoA synthetase* (McDonald *et al*., 2002). Acetyl-CoA is then converted into malonyl-CoA by *acetyl-CoA carboxylase*. Malonyl-CoA is in turn used for the elongation of the fatty acid chain by the action of the enzyme *fatty acid-synthetase* (Dils, 1986; McDonald *et al*., 2002; Weihrauch, 1988). The reducing equivalents required as a hydrogen donors-nicotinamide adenine dinucleotide phosphate (NADPH) - and derived from biochemical pathways such as pentose-phosphate pathway and the isocitrate cycle from glucose (Bauman & Griinari, 2003; Dils, 1986). The final product is C16:0 which is separated by enzymatic activity from the enzyme *thioesterase I* (Smith, 1980). However, this enzyme can release FA from different stages in the synthesis and thus create different length of the FA. FA synthesized in the mammary gland are saturated although the epithelial cells in the mammary gland can produce desaturase enzymes which convert SFA into UFA and thus increasing the level of UFA in the milk (Chilliard *et al*., 2003; Grummer, 1991; Sjaastad *et al*., 2003). Further on the FA undergo esterification with glycerol to synthesize TAG to be secreted into milk.

Preformed FA

Preformed LCFA is the result of the mammary gland uptake of FA from the blood. Lipids in blood can derive from endogenous and exogenous sources. Body fat mobilisation (lipolysis in the adipose tissue) is an endogenous source of FA which is transport in blood as non-esterified fatty acids (NEFA) (Bauman & Griinari, 2003; Dils, 1986). Dietary FA (exogenous source) normally represents the largest source of LCFA in comparison to FA from the adipose tissue (Palmquist & Mattos, 1977). While the uptake of plasma lipids (mostly as chylomicrons) in the mammary gland depends on enzymatic activity of *lipoprotein lipase* found in the mammary cell wall, the uptake of NEFA is proportional to their concentration in plasma.

Preformed SFA can be desaturated in the mammary by the action of Δ-*9 desaturase* which regulate almost all CLA and endogenous produced FA, particulate monosaturated FA (Soyeurt *et al*., 2008). The enzyme Δ-*9 desaturase* is also called *stearoyl-CoA desaturase* and has great importance in the mammary gland due to its ability to regulate the ratio between monosaturated and SFA (Chilliard *et al*., 2003). By regulating the conversion of C18:1 *trans*-11 into C18:2 *cis*-9, *cis*-12 Δ-*9 desaturase* control the assembly of different isomers which represents CLA. The enzyme is responsible for conversions of C10:0 into C10:1 *cis*-9, C12:0 into C12:1 *cis*-9 and more substantial the conversion of C14:0 into C14:1 *cis*-9, C16:0 into C16:1 *cis*-9 and C18:0 into C18:1 *cis*-9 (Miyazaki & Ntambi, 2003; Soyeurt *et al*., 2008). Miyazaki & Ntambi (2003) suggested C16:0 and C18:0 to be the favorite substrates for Δ-*9 desaturase*. Supporting Miyazaki & Ntambi (2003) suggestion; an average of 40 % of the C18:0 transported to the mammary gland are desaturated into C18:1 *cis*-9, which indicates the importance of Δ-*9 desaturase* (Soyeurt *et al*., 2008). The activity of Δ-*9 desaturase* can be altered by different feeding regimes. Chouinard et al. (1999) registered an increasing activity of Δ-*9 desaturase* on C14:0, C16:0, C18:0 when supplementing CLA. Weisbjerg et al. (2008) estimated an increase in Δ-*9 desaturase* activity when feeding fat supplements with high values of palmitic acid. The activity of Δ-*9 desaturase* can regulate the composition of UFA in milk and are therefore an important factor when investigation feeding trials. Glasser et al. (2008) registered the activity of Δ-*9 desaturase* be proportional to the uptake of substrates in

the mammary gland. Additionally, Glasser et al. (2008) estimated a desaturation of added C18:0 to be approximately 54 %.

Inhibition of milk fat synthesis

Productions of *trans*-fatty acids derived from diet or from incomplete biohydrogenation have suggested functioning as an inhibitor of milk fat synthesis. Bauman & Griinari (2003) proposed that alteration of biohydrogenation of linoleic acid influence the syndrome milk fat depression which causes very low lipid levels in milk. The formation of *trans*-10 C18:1 instead of C18:0 is considered to inhibit the milk fat synthesis in the epithelial cells in the mammary gland. Perfield *et al*. (2007), after abomasal infusion of CLA mixtures in dairy cows, reported that *trans*-9, *cis*-11 and *trans*-10, *cis*-12 C18:2 CLA also reduce milk fat synthesis.

Supplementing FA

During normal condition; increased LCFA in the diet inhibits *de novo* synthesised FA (Ashes *et al*., 1997; Palmquist *et al*., 1998). The level of LCFA increases in milk and the composition reflect the composition in the feed (Grummer, 1991; Hermansen, 1995). Several hypothesis have been suggested why *de novo* synthesized FA decreases when adding LCFA as a fat supplement. One of them is supplement of lipids may affect rumen biohydrogenation by reducing the substrates acetate for *de novo* synthesis of FA (Chalupa *et al*., 1984; Grummer, 1991; Hermansen, 1995). A direct alteration on the *de novo* synthesis in the mammary gland has also been suggested meaning a decrease in the activity of the enzyme *acetyl-coenzyme A carboxylase* which is an important enzyme and regulating step incorporated in *de novo* synthesis (Grummer, 1991).

When predicting the outcome milk composition when supplementing FA such as palmitic and stearic acid, the proportion of the added fat and total concentration is of great importance (Hermansen, 1995). Gummer (1991) suggested that proportion of palmitic:stearic acid in feed is similar to proportion in milk which suggests a possible way of predicting milk composition of lipids. However, prediction of some specific FA such as stearic acid is poor, primary due to the mammary gland ability to desaturase stearic acid to oleic acid by the activity of desaturase enzyme as described above (Hermansen, 1995).

Enjalbert et al. (2000) used duodenal infusions of FA in order to investigate their impact on the fatty acid profile in milk. The treatments were a control and 500 g of C16:0, C18:0 or *cis*-C18:1. Results from the experiment reported a decrease in C14:0, 18:0, 18:1.in milk. Levels of C6:0, C8:0 and C12:0 were unaffected suggesting the present study did not affect *de novo* synthesis. When infusing C16:0 the level of C16:0 in milk increased by 30 % although infusions with C18:0 only resulted in a small increase of C18:0 in milk. The milk yield were unaffected by all the different treatments. Earlier investigations performed by Enjalbert et al. (1998) duodenal infusions of 500 g C18:0 significant altered the fatty acid composition in milk, although not in the same rate as 500 g C16:0 in the same experiment. Added C18:0 did not alter milk fat content in a great extend (45 g/l) as C16:0 which caused an increase of 49.9 g/l in contrast to control: 41.1 g/l. The milk yield in this experiment was also unaffected. For both treatments the added FA could be detected in higher values in arterial blood detected through blood samples and in milk. Mammary uptake of FA was higher for the C16:0 infusions, C18:0 displayed no significant results. Production of C4:0 in the mammary gland were positively correlated to the infusion treatments suggesting a positive correlation between

de novo synthesised FA and elevated levels of LCFA. There were no significant effects on mammary desaturation activity for any of the feed treatments.

Mosley et al. (2007) investigated the optimum level of supplemented palmitic acid in concentrates regarding DMI, milk yield and FA profiles. The four different feed levels were: control with no FA added, 500 g/day, 1,000 g/day, and 1,500 g/day. DMI intakes were at highest level when feeding 500 g/day and higher intake caused the opposite effect. The results of fat percentage in milk showed an increased between control (3.44 %) and the different treatments (3.95 %) but no significant different between the treatments could be detected. The milk yield increased between control (30.9 kg/d) to approximately 34 kg/d for the other treatments. The increase of C16:0 in feed caused a 50 % increase of C16:0 in milk. Furthermore, the fatty acid composition in milk was also altered with increasing levels of SFA in compare to decreasing levels of MUFA and PUFA.

Noble et al. (1969) detected a decrease in MCFA when supplementing with C16:0 and C18:0. An inclusion of 10 % C16:0 resulted in an increase of C4:0 and an inclusion of either 5 or 10 % of C18:0 caused an increase in the level of C18:0 and C18:1. This effect was similar for inclusion of C16:0 and the increased level of C16:0 in milk. Storry et al. (1968) registered an increase in milk yield and a linear increase in fat content in milk when adding increasing levels of red palm oil into the diet. The treatments were: 2 %, 4 %, 8 % inclusions of palm oil. The treatments caused an alteration in the fatty acid profile in milk. The level of C16:0 in milk increased, the same as for C4:0 in contrast to C12:0 and C14:0.

Weisbjerg et al. (2008) investigated if supplements of FA, primary C16:0, had any effects on: DMI, milk yield, fat composition and size of MFG in milk. Treatments in the experiment were: control (17 g FA/kg DM), 29 g FA/kg DM, 40 g FA/kg DM and 50 g FA/kg DM. The fat supplemented originated either from palm fatty acid distillate and contained high levels of C16:0 (38.5 %), C18:1 and C18:2. Results regarding the DMI included a decrease when increasing fat levels. Milk yield increased with 1.1 kg ECM when FA concentration increased with 10 g/kg DM. The milk composition was altered with a decrease in SCFA and MCFA could be detected. An increase in the level of C16:0 were registered suggested to be a reaction of the increasing level in feed.

Table 4. Summary of results when supplementing FA.

Authors	***Fat supplements***	***Effect on milk yield***	***Increased FA***	***Decreased FA***
Enjalbert et al. (2000)	Duodenal infusions of control (no fat added), C16:0 infulsions, C18:0 infusions or cis-C18:1 infusions.	No effect	C16:0, C18:0	C14:0, 18:0, 18:1
Mosley et al.(2007)	PALM (86.6 % C16:0, 4.2 % C14:0, 4.1 % C18:1, 2.8 % C18:0, 2.3 % C12:0), 0, 500, 1000 and 1500 g/d	Increased (kg/d)	C16:0	C6:0, 8:0, 10:0, 12:0,14:0, 18:0, 18:1, 18:2, CLA
Noble et al. (1969)	C18:0 (5 % or 10 %)	-	C18:0-18:1	C10:0, 12:0, 14:0, 14:1, 16:1
	C16:0 (5 % or 10 %)	-	C4:0, 16:0-16.1,	C6:0, 8:0, 10:0, 12:0, 14:0, 14:1, 18:0, 18:2
Storry et al. (1968)	Red palm oil	Increased (kg/d)	C4:0, 16:0, 18:0, 18:1, 18:2	C4:0, C10:0, 12:0
Weisbjerg et al. (2008)	Palm Fatty Acid destillate (PFAD), 0, 29, 40 and 52 g/kg DM	Increased (ECM)	C16:0, 18:1	C4-14

Table 4 summarizes results from experiment mentioned above. Only Weisbjerg et al. (2008) registered an increase in desaturase activity in the mammary gland in contrast to Mosley et al. (2007). Although Mosley et al. (2007), Storry et al. (1968) and Weisbjerg et al. (2008) concluded that saturated LCFA inhibit *de novo* synthesis in the mammary gland which Enjalbert et al. (2000) could not find any evidence of. The results from Mosley et al. (2007), Storry et al. (1968) and Weisbjerg et al. (2008) are consistent to results obtained by Hansen & Knudsen (1987) who performed in vitro research on *de novo* synthesis in cells from mammary glands when adding palmitic acid. C18:0 possessed an inhibitory effect on *de novo* synthesized FA except from C4:0. A suggestion for these results is a competition between *de novo* synthesized FA and LCFA for esterfication positions (sn1-, 2-, or 3- position) by the enzymes on glycerol.

Glasser et al. (2008) investigated C18:0 effects on milk composition by comparing different results from research regarding FA supplements and concluded availability and absorption of C18:0 is correlated to the yield of C18:0 in milk. This result is similar to those obtained by Hermansen (1995) that levels of C18:0 in milk were related to absorb C18:0. Levels of C18:0 also displayed a tendency of positively connection to levels of C4-16 in milk; however this result had no significant effect (Glasser et al. 2008). Level of C18:0 in milk has found to be

proportional to DMI, which is not unexpected due to DMI is usually related to milk synthesis (Glasser et al, 2008; Moet et al. 2008). When comparing experiments conducted on added C16:0 and C18:0 it is a common trend when C16:0 had significant effects on milk yield, lipid composition and level of fat in milk where added C18:0 had it not (Astrup *et al*., 1980a; Enjalbert *et al*., 1998; Enjalbert *et al*., 2000).

Fat supplements impact on MFG

Weisbjerg et al. (2008) registered no effect of feeding SFA on the level of FFA in milk and the risk of spontaneously lipolysis. The explanation is suggested to be related to the low increase in MFG (0.092 μm). In contrast, research performed by Wiking et al. (2003) resulted in an increase in diameter of MFG when feeding SFA. Furthermore, larger MFG is related to higher levels of FFA in milk (Wiking *et al*., 2003b; Wiking, 2005). Larger MFG is suggested to be less stable and resistant to mechanical stress which can occur during manufacturing of milk and therefore release a higher level of FFA. Larger MFG is more likely to be related to lipolysis in compare to smaller MFG (Wiking *et al*., 2003b). The level of fat in milk is correlated to the size of the MFG, were increasing FA chain length increases the size of MFG (Weisbjerg *et al*., 2008). Size of MFG can be correlated to the concentrations of the FA C16:0-1 and C18:0-1 where MFG containing most C18:0 is the largest. A correlation between the size of MFG and milk fat synthesis has also been registered where increasing milk fat synthesis increases the size of the MFG (Wiking *et al*., 2003b). Similar results have been obtained from Timmen & Patton (1988) where high levels of C18:0 is correlated with larger diameter of the MFG. The results suggest FA composition in feed can influence the size of MFG in milk and by affecting the size of the MFG trough feeding milk may be more resistant against lipolysis.

Milk lipolysis

The process of lipolysis in milk occurs through hydrolysis of ester bonds in TAG by lipases such as *lipoprotein lipase* (LPL) resulting in the release of FFA, and di- or monoglycerides (Deeth & Fitz-Gerald, 2006; Jensen, 1964; Weihrauch, 1988). Lipases prefer to cleavage *sn*-1 and *sn*-3 positions before than *sn*-2 position (Palmquist *et al*., 1993). After hydrolysis, free, SCFA and MCFA is the main responsible for the occurrence of off flavour in milk (Weihrauch, 1988; Woo & Lindsay, 1983). Lipolysis has negative impacts on both the quality and the shelf life of milk and represents therefore a problem for producers and consumers (Ma *et al*., 2000). During normal conditions most MFG are resistant against lipases and provides protection for TAG storage inside the MFG. By alter conditions such as temperature; it will affect LPLs binding to casein micelles to dissolve and attack the TAG (Sundheim & Bengtsson-Olivecrona, 1987). Other alterations include the stability of MFG which can be affected by agitation causing damage to MFGM and make binding sites for LPL more available (Bhavadasan *et al*., 1982).

Classifications of lipolysis

Lipolysis can be classified in two different categories: induced or spontaneous lipolysis. It is complicate to separate the two different classes apart due to the fact that both, induced and spontaneous lipolysis, can take place at the same time (Weihrauch, 1988).

Induced lipolysis occurs due to physical factors such as agitation which affect the MFGM and makes TAG more available for lipases. Agitation can also cause foaming of the milk and the rate of induced lipolysis would depend upon the type of agitation -such as stirring, pumping or incorporation of air- (Weihrauch, 1988). Temperature has a major influence (Chilliard *et al.*, 2003). Induced lipolysis can occur not only on the farm but also during transport and processing. More risk factors for induced lipolysis include: homogenization, mixing and freezing (Deeth & Fitz-Gerald, 2006).

Spontaneously lipolysis takes place trough activations systems present in the milk and occurs before processing, normally already on the farm. The rate of spontaneously lipolysis depends on three factors: amount and activity of LPL, if the MFGM is intact and presence of activators or inhibitors (Cartier & Chilliard, 1990; Deeth & Fitz-Gerald, 2006; Sundheim, 1988). There are several risk factors affecting spontaneously lipolysis where; mastitis, stage of lactation, season, milk production and nutrition are the one of most importance (Deeth & Fitz-Gerald, 2006). Spontaneously lipolysis is most likely to occur when the cows feed are not sufficient to their nutritional need and the cow is in negative energy balance (Urbach, 1989). Research performed by Ma et al. (2000) found a connection between high somatic cell counts (SCC) and level of lipolysis indication that mastitis can affect lipolysis. Similar results were obtained by Murphy et al. (1989) whit high levels of lipolysis in milk from cows diagnosed with mastitis. Milk containing high SCC values was also agreed by a taste panel to possess a rancid flavour in fewer days of storage in compare to milk containing low SCC values (Santos *et al.*, 2003b). Research have detected that cows in later lactation have higher levels of lipolysis in milk in compare to cows in early lactation (Ahrné & Björk, 1985; Bachman & Wilcox, 1990b; Jensen, 1959). Bachman et al. (1988) detected stage of gestation may influence lipolysis. Milk yield is a factor influencing lipolysis where low yielding cows possesses a higher risk of producing lipolysis than high yielding cows (Deeth & Fitz-Gerald, 2006). Abeni et al. (2005) registered an increase of spontaneously lipolysis whit shorter milking intervals in an AMS system. Two explanations were suggested for this result including increased *de novo* synthesis due to increasing enzymatic activity of a*cetyl coenzyme A carboxylase* and therefore supplementing more favorable substrate (TAG containing SCFA) for LPL, this hypothesis has also been suggested by Klei et al. (1997). The other explanation considered an inhibitor present in the milk such as suggested by Cartier et al. (1990).

Lipases acting on lipolysis

Enzymes related to lipolysis origin from two different sources: from microbes and already present in milk. Lipases originate from microbes are extremely heat resistant and can survive pasteurization and other treatments long after the microbes are killed. Most common microbe producing lipases is the psychrotrophic bacteria *Pseudomonas* (Weihrauch, 1988). Lipolysis due to microbial lipases is mostly occurring in dairy products stored for as long time (Deeth & Fitz-Gerald, 2006). Differences between the lipases origin from microbes and already present in milk are presented in Table 5.

Table 5. *Properties of lipases affecting lipolysis. Modified from Deeth,& Fitz-Gerald, 2006.*

Properties	***Lipases in milk***	***Lipases from bacteria***
Resistant against high temperatures and pasteurization	No	Yes
MFGM work as a protector	Yes	No
Activates from lipoprotein lipases	Yes	No
Active during manufacturing of cheese/butter	Yes	No
Substrate	Fresh milk and cream	Stored dairy products
Levels in raw milk	High	Trace elements only

For enzymes present in milk the LPL is the major one and are identically to *lipoprotein lipase* present in blood (Deeth & Fitz-Gerald, 2006). LPL are synthesised in the secretory cells in the mammary gland and are transported through the capillary where it hydrolyses TAG. LPL presence in milk is explained by a leakage across membranes, from blood to milk (Jensen & Pitas, 1978). In milk LPL are mainly found in the skim milk fraction where 90 % are bound to casein micelles and hence inhibits the enzyme-fat globules interactions (Chilliard *et al.*, 2003; Santos *et al.*, 2003a; Weihrauch, 1988). The binding between caseins and LPL occurs due to different electrostatic charges, where the casein micelles is negatively- and LPL positive charged (Deeth & Fitz-Gerald, 2006). The levels of LPL vary through the stage of lactation with low levels at the beginning and then a rapid increase to a more stable level until the rest of the lactation. Approximately, milk contains 1-2 mg LPL per liter (Weihrauch, 1988). LPL is most active in an alkaline environment with a pH 8-9 and possesses the ability to catalyse the hydrolysis of TAG up to 2 mol TAG/ml/min (Egelrud & Olivecrona, 1972; Jensen & Pitas, 1978).

LPL activates by action from the binding protein apolipoprotein CII (apo CII) (Bengtsson & Olivecrona, 1982; Shen *et al.*, 2010; Tajima *et al.*, 1984). The activation procedure has not yet been fully describe in detail, nonetheless facts such as which residues in LPL apoCII binds to have been discovered by Shen et al. (2010) where the C-terminal is of great importance. ApoCII work as a cofactor to LPL and facilitates the binding to MFG in milk suggesting apoCII to be a limiting chemical step (Bengtsson & Olivecrona, 1982; Shen *et al.*, 2010). However, these results depend on the condition of substrate, hence the MFG and TAG presence. If the MFG are intact, there is nearly non-binding activity of LPL without apoCII (Bengtsson & Olivecrona, 1982). Relation between the activity of LPL, level of FFA and rate of lipolysis in milk has been described briefly by Bachman & Wilcox (1990a).

LPL inhibits by the proteose-peptone component 3 which is present in milk. Proteose-peptone 3 can be divided into two different fractions: polypeptides derived from proteolysis of caseins and glycoproteins which form proteose-peptone component 3. This component is origin from MFGM, primary the glycoprotein fraction-1 (Kester & Brunner, 1982). The progress of how proteose-peptone component 3 inhibits lipolysis is not fully understood, but research suggest proteose-peptone component 3 to possess protection abilities of the MFG and thus inhibiting LPL binding (Girardet *et al.*, 1993).

Thresholds for off-flavours

The relationship between rancid flavour in milk due to lipolysis and increasing levels of FFA in milk have been documented in several experiments (Astrup *et al.*, 1980a; Astrup *et al.*, 1980b; Kinter & Day, 1965). Threshold values due to elevating levels of FFA and rancidity in milk are summarised in Table 6. Other taste description associated with lipolysis except rancid, can be defined as bitter, "goaty" and butyric (Shipe *et al.*, 1978). Results from taste panel tests obtained by Woo & Lindsay (1983) indicated that when levels of butyric acid occurred at the highest level, a rancid flavour could be detected clearly. For C6:0-10:0 the taste could be described as goaty and butyric. When C12:0 occurred in highest concentration the taste was connected to a soapy and metallic flavour.

Table 6. *Threshold values for off flavours in milk. Adapted from Wiking, 2005.*

Threshold value of FFA causing off-flavors (meq./100 g fat)	***Reference***
0.32-0.351 meq./kg fat	Santos et al. (2003)
1,5	Tuckey & Stadhouders, 1967
2	Jensen, 1964
2,74	Kintner & Day, 1964
1,46-3,62	Duncan *et al.* (1991)

Research performed by Santos et al. (2003) found a wide range between panel's members and thresholds when off flavour was detected. When milk contained 2 % fat and $\geq$ 0.35 meq FFA/kg milk 63 % of the panel members could detect off flavour. When FFA concentration were between 0.17-0.20 meq FFA/ kg milk only 13 % of panellist could detect off flavour and when FFA levels where at the highest level with 0,25 meq FFA/kg milk, 34 % could detect off flavour.

FFA impact on lipolysis

Kinter & Day (1964) detected a relation between high levels of milk fat and high levels of FFA, suggesting a connection between rancid flavors in milk when level of FFA were elevated. It is the FFA which is responsible for the rancid flavor and is thus considered to be unwanted in milk (Deeth, 2006). Research performed Astrup et al. (1980a) conducted that adding SFA in feed increases the level of FFA and rate of lipolysis. The opposite effect was detected when feeding UFA. Astrup et al. (1980a) registered that an increase of 6 % C16:0 in commercial concentrate mixture caused an increase in the level of milk fat (from 4.1 % in control to 4.5 % in treatement). The level of FFA also increased and off flavor could be detected (FFA were 1.79 mm/l in control and increased to 2.99 mm/l). Milk yield was unaffected. Changes in the milk composition were registered with increases in C4:0 and decreases in C10:0, C12:0 and C14:0. Level of C16:0 in milk were increasing when the level increased in feed as expected. Further research by Astrup et al. (1980a) whit feeding

concentrates added 600 g C16:0 the level of C16:0 and FFA increased (FFA level when feeding the control diet was 0.45 and increased to 1.49 mm/l) and off flavour could be detected. An increase of 600 g of C14:0 in feed had the same effect (0.58 mm/l FFA), but not in the same rate as C16:0. Repeated investigations with added 600 g C18:0 had no effects on lipolysis (0.44 mm/l FFA) and rancid flavor. The level of C18:0 in milk increased of the level in milk although the milk yield was unaffected.

Research has been performed whether restricted feed intake may influence lipolysis (Astrup *et al*., 1980b). A short termed decreased energy intake increases the FFA level rapidly in milk when a short term decrease in energy intake seems to have a long term increase if FFA in milk.. The increasing levels of FFA caused rancid flavor in milk. Increases in the level of C4:0 were registered to be 20 %, which may also explain the rancid flavor. The milk yield decreased with lower energy intake although the content of milk fat was increased. Suggestions regarding the elevating levels of FFA when restricted feeding may be a response to an increased activity and transport of LPL from blood to milk. This is suggested to be due to mobilizations within tissues and therefore increase substrates for LPL such as TAG (Astrup *et al*., 1980b). However, this suggestion was opposed by Pamquist et al. (1993) who suggested that LPL has favor specific positions for cleavage, primary on the sn-1 and *sn*-3 before *sn*-2 position on the TAG. C16:0 is main esterfied on *sn*-1 or *sn*-2 position in contrast to C4-C10 which are esterfied at *sn*-3 position. As a result, the LPL prefers SCFA before LCFA and the release of FFA increases with decreasing chain length of the FA.

The mechanism regarding the impact of C16:0 on off flavor is not fully understood although its present seems to be an important factor regarding lipolysis and off flavor in milk. C16:0 possesses the ability to change milk fat composition by increasing FFA which can cause lipolysis.

Material and methods

Animals, housing, feeding and management

The experiment was conducted at the Swedish Livestock Research Centre (SLRC) Lövsta, Uppsala, during the spring 2012 (May 10th-June 7th). The study lasted a total of 30 d, comprising 5 d of adaption period and 25 d (day 1 to 25) of experimental/sampling period. The experiment protocol –animal housing, management and sample collection- was approved by the Uppsala Local Ethics Committee.

A total of thirty (30) Swedish Red lactating dairy cows, selected from the SLRC herd based on parity and days in milk, were used. Cows were in mid lactation. Within parity groups -15 multiparous (MP) and 15 primiparous (PP) cows-, animals were randomly assigned to one of three groups, resulting in 3 10 cow-groups (5 PP and 5 MP cows per group). Throughout the experiment, cows from each group were fed one out of three concentrate mixtures: control (C), a standard concentrate mixture with no fat added; palmitate (P) as C plus the addition of palmitate methyl ester (10 % in DM basis); and stearate (St) as C plus the addition of stearate methyl ester (10 % in DM basis). FA methyl esters (purity= 99% minimum) were purchased from Nanjing Xinxu Industry & Trade Co. Ltd., China. Concentrates mixtures were manufactured by Lantmännen, Sweden.

Table 7. *Feed ingredients included in the different concentrate mixtures.*

Ingredients *(kg/100 kg)*	***Control***	***Palmitate***	***Stearate***
Methyl stearate			6.8
Methyl palmitate		6.8	
Beet fibre & wheat middlings		26.2	26.2
Palm kernel expeller		2.5	
Wheat middlings	25		
Heat-treated rapeseed meal	17	0.43	8.1
Maize	16.0	15.8	15.0
Barley	10.5	9.3	10.8
Beet fibre	8.2	6.0	5.0
Oats	7.7		
Oat hulls	7.0	7.0	7.0
Heat-treated soybean meal	4.7	11.0	8.9
Malt culms pellets		10.0	8.4
Potato protein		1.38	
Limestone, salt, vitamins and trace elements	3.93	3.60	3.86

Concentrate ingredients and chemical composition of the concentrate mixtures, as reported by the manufacturer, are listed in Tables 7 and 8, respectively. Concentrate were offered, 4 times a day, according to the cow's calculated energy requirements (Spörndly, 2003). Besides the concentrate mixtures, cows had *ad libitum* access to grass silage. In an attempt to provide cows the same energy intake, concentrate allowance was increased by 10 % to the cows on treatment C. The cows were housed in a tie stall barn and milked twice a day, at 05.30 am and 17.30 pm with a DeLaval milking system. Milk yield and feed intake were registered on a

daily basis.

Table 8. *Chemical composition (dry matter basis) of the different concentrate mixtures as reported by the feed manufacturer.*

Item	Control	Palmitate	Stearate
ME (MJ/kg DM)	12.2	13.8[1]	13.8[1]
Crude protein (%)	18,7	17,5	17,6
Crude fat (%)	4,0	10,7	10,7
Ash (%)	6,7	6,5	6,7
NDF (%)	30,7	29,2	28,2
Starch (%)	27,6	27,0	27
Calcium (Ca; g/kg)	8,5	7,9	8,4
Phosphorous (P; g/kg)	5,9	4,5	5,1
Magnesium (Mg; g/kg)	4,6	4,5	4,5
Potassium (K; g/kg))	8,5	8,4	8,7
Selenium (Se; mg/kg)	0,53	0,51	0,51
AAT (g/kg)	120,9	113,7	113,0
PBV (g/kg)	-1,10	-1,45	0,01
Vit A (Int. Units)	4550	4504	4503
Vit D3 (Int. Units)	2275	2252	2252
Vit E (mg/kg)	46	45	45

[1] Values were recalculated based on a methyl esters inclusion of 6.8%, GE content of 39.4 and 40.1 MJ/kg for M-palmitate and M-sterate, respectively, and a true digestibility of 0.9.

Experimental design, sample collection and analysis

The experiment was conducted as a continuous treatment design (*n*=10). Individual feed intake was registered on daily basis from day 1 to day 25. However, only data from days 19 to 23 (*in vivo* digestibility measurement) were used for the calculations of feed and nutrient intakes and digestibility. The *in vivo* apparent digestibility of the treatment and treatment components was estimated based on feed and faeces sampling during 5 consecutive days (days 19 to 23). Faecal grab samples (around 100 g on fresh basis per cow and sampling time) were collected from all cows at, circa, 8:30 am and 16:00 pm and kept at - 20ºC until pooled by animal. Before to be analysed pooled faeces samples were freeze dried (Scanvac Cool Safe 95/55-80, LaboGene ApS, Lynge, Denmark). Feed samples, silage and concentrates, were collected on days 16, 20 and 23 and stored at - 20ºC until analysis. Faeces and feed samples were analysed for DM, crude protein (Nordic Committee on Food Analysis, 1976), neutral detergent fibre (NDF; Chai & Udén, 1998), ether extract (EE; by using a 1047 Hydrolyzing Unit and a Soxtec System HT 1043 Extraction Unit, FOSS Analytical A/S Foss, Hillerød, Denmark; Official Journal of the European Communities: Determination of crude oils and fat, 1984), ash (by incineration at 550 ºC), and acid insoluble ash (AIA; Van Keulen & Young, 1977).

Milk samples were collected on two different occasions (two non-consecutive days): days 22 (pm milking) and 23 (am milking); and days 24 (pm milking) and 25 (am milking) and kept a 4 ºC. Within sampling occasion, milk samples were pooled according to milk yield, resulting

in 2 composite samples per cow. Milk samples 1 and 2 (days 22-23 and 24-25, respectively) were analysed for milk composition (MilkoScan FT120, Foss, Hillerød, Denmark) and FFA (Deeth *et al*., 1975). In addition, on samples number 2, milk fat globule size distribution was determined as described by Wiking et al. (2003a) at Foulum, Århus University, Denmark.

Statistical analysis

Data was statistically analysed, according to a continuous treatment design, by analysis of variance (ANOVA) using the PROC GLM in SAS software (Version 9.3, SAS Institute Inc., Cary NC USA).

Two different models were used according to the classes included. For analysing data within parity (n=5) the model used was: y= treat (were y= dependent variable; and treat= 3 different treatments); and when data from all 30 cows (n=10) were analysed the model used was: y= treat + parity + treat*parity (were parity= primiparous or multiparous cows). The level significance was set at 5 % ($P < 0.05$). In both models, significant differences between treatment means were evaluated by the PDIFF subroutine option in SAS.

Results and Discussion

Cows appeared to adapt well to management –tie stall barn- and none of them showed any sign of discomfort or health problems throughout the experiment. They adapted to the concentrate mixtures within a period of 4 days, leaving no refusals by day 1 of the experimental period.

In order to minimise the effect that a negative energy balance –early lactation- may have on lipids whole body metabolism, as well on milk fat synthesis and composition, cows used in the present experiment were in mid lactation. Days in milk were 152 ± 28, 148 ± 22, and 149 ± 21 for C, P and St treatments, respectively.

Diet composition

Chemical composition of the grass silage and the different concentrates mixtures is presented in Table 9. As planned, the P and St treatment had a fat level of around 10%. The inclusion of the FA methyl esters in the high fat treatment, resulted in a small decrease in the content of crude protein (CP) (1 point per cent) and NDF (1-2 points per cent) as compared to the C concentrate mixture.

Table 9. *Chemical composition of the different components of the treatments.*

Item	Silage[1]	Concentrates[1]		
		Control	Palmitate	Stearate
Dry matter (DM; %)	24.4 (± 1.6)	88.3 (± 0.3)	85.0 (± 0.4)	86.1 (± 0.1)
Crude protein (% DM)	19.8 (± 0.4)	18.4 (± 0.2)	17.4 (± 0.5)	17.5 (± 0.4)
Fibre (NDF; % DM)	42.2 (± 1.1)	25.0 (± 1.0)	22.8 (± 0.6)	23.8 (± 0.6)
Fat (EE; % DM)	4.45 (± 0.2)	4.4 (± 0.1)	9.9 (± 0.3)	9.6 (± 0.8)
Ash (% DM)	9.3 (± 0.4)	6.9 (± 0.1)	6.4 (± 0.1)	6.8 (± 0.1)

[1] Mean values ± standard deviation; *n*= 3.

Feed and nutrients intake and in vivo digestibility

Feed and nutrient intake data are presented in Table 10. While silage intake was not affected by the treatments, concentrate intake was significantly (P<0.001) higher in cows fed C in compared to P or St treatments as a result of the feeding management. Since the concentrate mixtures were not isoenergetic, with P and St having and estimated ME content 12 % higher than C (Table 8), and in order to minimise differences in energy intake, cows on treatment C were offered 10 % more concentrate than the estimated amount based in their energy requirements.

For MP cows, though not for PP, concentrate intake differed (P<0.001) among all treatments, with C showing the highest and St the lowest intakes. However, the significant difference observed between P and St treatment was numerically small (5%) and it did not have a significant effect on neither the concentrate intake across parity (all cows) nor on the total DMI.

In line with the differences observed in concentrate intake between C and high fat treatments (P and S), total DMI showed a tendency (*P*=0.080) for being lower in P and St. However, and as stated above, the apparent differences in DMI were the result of a feeding management decision. It appeared that in the present study, in contrast to other studies which indicated that adding SFA to the diet may decrease the DMI (Harvatine & Allen, 2005; Pantoja *et al*., 1994), the inclusion of FA did not negatively affect the DMI.

Table 10. *Feed and nutrient intake in lactating dairy cows supplemented with different FA.*

Item	Parity[1]	Treatment				*P=*		
		Control	Palmitate	Stearate	SEM [2]	Treat	Parity	Treat*Parity
Feed intake (kg DM/d)								
Silage	1	8.5	8.8	8.6	1.11	*0.980*		
	≥ 2	9.7	8.8	10.2	0.95	*0.609*		
	All cows	9.1	8.8	9.4	0.73	*0.852*	*0.278*	*0.745*
Concentrate	1	13.2[a]	11.4[b]	11.4[b]	0.26	*<0.001*		
	≥ 2	16.9[a]	14.8[b]	14.1[c]	0.20	*<0.001*		
	All cows	15.0[a]	13.1[b]	12.7[b]	0.16	*<0.001*	*<0.001*	*0.130*
Total intake	1	21.7	20.2	20.0	1.04	*0.485*		
	≥ 2	26.5	23.6	24.3	0.99	*0.136*		
	All cows	24.1	21.9	22.1	0.71	*0.080*	*<0.001*	*0.778*
Nutrient intake (kg/d)								
Organic matter	1	20.0	18.7	18.4	0.98	*0.476*		
	≥ 2	24.5	21.9	22.4	0.90	*0.136*		
	All cows	22.2	20.3	20.4	0.65	*0.078*	*<0.001*	*0.782*
Crude protein	1	4.10	3.72	3.69	0.205	*0.323*		
	≥ 2	5.02	4.32	4.47	0.193	*0.059*		
	All cows	4.56[a]	4.02[b]	4.08[b]	0.141	*0.024*	*<0.001*	*0.729*
Fibre (NDF)	1	6.87	6.31	6.33	0.449	*0.619*		
	≥ 2	8.30	7.10	7.36	0.406	*0.157*		
	All cows	7.58	6.71	6.98	0.303	*0.133*	*0.003*	*0.738*
Fat (EE)	1	0.96[b]	1.52[a]	1.48[a]	0.046	*<0.001*		
	≥ 2	1.17[b]	1.86[a]	1.82[a]	0.048	*<0.001*		
	All cows	1.07 [b]	1.69 [a]	1.65 [a]	0.033	*<0.001*	*<0.001*	*0.343*

[1] Parity: 1, *n*= 5; ≥ 2, *n*= 5; all cows, *n*= 10.

[2] SEM: standard error of the mean.

[a, b] Means in the same row with different superscripts differ at $P<0.05$ (treatment effect).

With regard to parity, while silage DMI was not affected by parity (*P*=0.278), overall and due their higher energy requirements and concentrate allowance, MP cows had a higher (*P*<0.001) intake of concentrate (on average, around 28%), and therefore a higher total DMI than PP cows.

The calculated intakes of organic matter (OM) followed the pattern as DMI, with a tendency (*P*= 0.078) for a higher intake in C than in P and St treatments. While there was no effect of treatment, neither within nor across parity groups, in fibre (NDF) intake, CP intake was lower (*P*=0.024) in P and St treatments than in C. Due to the fact that there were no differences in silage intake, lower CP intakes in the high fat treatments were the result of a lower CP content in the concentrate mixtures P and St (Table 9) together with lower concentrate intakes (Table 10).

As expected and both within and across parity groups, cows fed the high fat treatments (P and St) had a higher (*P*<0.001) intake of fat (EE; on average 56 % when all cows were considered) than those fed the control.

Interestingly, the increased fat level in the treatments and fat intake did not affect neither the DMI nor the apparent *in vivo* digestibility of any of the dietary components considered (DM, OM, CP, NDF and fat; Table 11). Briefly, lipid content higher than 5% on DM basis in ruminants diets may impair the activity of rumen microorganisms, and decrease the digestibility of the different components of the diet, in particular fibre, as well as feed intake (McDonald *et al.*, 2002). However, a more detrimental effect may be expected from unsaturated than from SFA as those used in the present experiment (Jenkins, 2003; Jenkins & Jenny, 1989). Also, in contrast to the findings reported by Weisbjerg *et al.* (1991) and Enjalbert *et al.* (2000), who showed that palmitic acid had a higher digestibility than stearic acid, the AIVD for fat in treatment P and St in the present experiment, were not significantly different. Nonetheless, in the MP cows –who had a higher fat intake than PP cows- the AIVD of fat was numerically higher (on average 74.4 vs 69.9%; *P*=0.150) when fed the P treatment in comparison to C and St treatment (Table 11), which would support the statement that fatty acid absorption decreases with an increase in chain length (Van Soest, 1982), and the findings reported by others (Enjalbert *et al*., 2000; Moet *et al*., 2008; Weisbjerg *et al*., 1991).

Parity did not affect the AIVD of any of the items considered but fibre (Table 11). The AIVD of the NDF fraction was significantly (*P*=0.050) higher in PP than in MP cows (on average, 57.7 and 55.4%, respectively). This effect may have been the result of a lower feed intake with a higher forage:concentrate ratio in PP than in MP cows, which in turn may have decreased the rate of passage –longer retention time in the rumen-, and the occurrence of negative associative effects, respectively (Van Soest, 1982).

In general, and due to the lack of effect of the dietary treatments on AIVD, the intake of apparent digestible nutrients (Table 11) followed a similar pattern than the observed for nutrient intake (Table 10). Thus, the intake of digestible OM and CP were higher, and that of digestible fat was lower in cows fed the C treatment than in cows fed P or St treatments. For MP cows, as a result of the higher AIVD of fat in P than in St treatments, the intake of digestible fat was significantly (*P*<0.001) different among all 3 treatments (P > St > C). Since PP cows had a lower DMI, the intakes of digestible nutrients were lower than in MP cows.

Table 11. *Apparent in vivo digestibility and digestible nutrient intake in lactating dairy cows supplemented with different FA.*

Item	Parity[1]	Treatment			SEM [2]	P=		
		Control	Palmitate	Stearate		Treat	Parity	Treat*Parity
***In vivo* digestibility** (%)								
Dry matter	1	66.2	65.6	66.7	1.65	*0.904*		
	≥ 2	65.7	65.6	65.8	0.81	*0.980*		
	All cows	65.9	65.6	66.3	0.92	*0.887*	*0.663*	*0.950*
Organic matter	1	68.1	67.7	68.4	1.58	*0.956*		
	≥ 2	67.4	67.6	67.7	0.79	*0.978*		
	All cows	67.7	67.6	68.0	0.88	*0.953*	*0.648*	*0.968*
Crude protein	1	67.4	67.3	69.4	1.62	*0.610*		
	≥ 2	67.4	65.2	67.6	0.82	*0.116*		
	All cows	67.4	66.3	68.5	0.91	*0.248*	*0.229*	*0.682*
Fibre (NDF)	1	57.5	57.4	58.3	1.65	*0.918*		
	≥ 2	53.5	55.4	57.0	1.27	*0.187*		
	All cows	55.5	56.4	57.7	1.04	*0.358*	*0.050*	*0.630*
Fat (EE)	1	67.8	69.2	67.4	4.09	*0.945*		
	≥ 2	70.0	74.4	69.8	1.76	*0.150*		
	All cows	68.9	71.8	68.6	2.23	*0.529*	*0.215*	*0.869*
Digestible nutrient intake (kg/d)								
Organic matter	1	13.5	12.6	12.6	0.53	*0.364*		
	≥ 2	16.5	14.8	15.1	0.54	*0.097*		
	All cows	15.0^{a}	13.7^{b}	13.9^{b}	0.38	*0.039*	*<0.001*	*0.787*
Crude protein	1	2.75	2.50	2.56	0.114	*0.297*		
	≥ 2	3.38^{a}	2.81^{b}	2.61^{b}	0.115	*0.002*		
	All cows	3.06^{a}	2.65^{b}	2.59^{b}	0.081	*<0.001*	*0.002*	*0.065*
Fibre (NDF)	1	3.95	3.62	3.70	0.293	*0.721*		
	≥ 2	4.43	3.93	4.36	0.236	*0.302*		
	All cows	4.19	3.78	4.03	0.188	*0.311*	*0.037*	*0.799*
Fat (EE)	1	0.59^{b}	1.05^{a}	1.00^{a}	0.063	*<0.001*		
	≥ 2	0.82^{c}	1.38^{a}	1.27^{b}	0.038	*<0.001*		
	All cows	0.71^{b}	1.22^{a}	1.13^{a}	0.037	*<0.001*	*<0.001*	*0.617*

[1] Parity: 1, n= 5; ≥ 2, n= 5; all cows, n= 10.

[a, b] Means in the same row with different superscripts differ at P<0.05 (treatment effect).

[a, b, C] Means in the same row with different superscripts differ at P<0.05 (treatment effect).

Milk yield and composition

Results for milk yield, milk composition and milk components yield are presented in Table 12. As expected, parity had a significant (*P*<0.001) effect on milk yield, expressed both as actual milk yield (kg/d) and as energy corrected milk (ECM), with higher milk yields in MP than in PP cows. Also, milk from PP cows had higher contents of protein, lactose, total solids,

and, though numerically, fat.

It is worth to mention that there was none treatment effect on milk yield, milk composition or milk components yield in PP cows. This lack of effect could have been due to a lower intake of concentrate, associated to a lower milk yield, in PP than in MP cows.

On the other hand, MP cows fed the St treatment had a tendency (P=0.062) for a higher milk yield and a significantly higher (P=0.004) ECM yield than cows fed C or P treatment. While there is not an apparent reason for the difference in milk yield between the high fat treatments, differences in ECM may be the result of milk yield and milk composition. Though small and no significantly different, fat, protein and lactose contents were numerically higher in milk from MP cows fed the St treatment compare to MP cows fed with P treatment.

Across parity, milk fat content tended (P=0.086) to be higher in treatments P and St than in C mostly due to a significant treatment effect in MP cows. Feeding MP cows the high fat treatments (P and St) resulted in an increased (P=0.010) milk fat content as compared to those fed the C treatment. These results are in apparent contrast to those reported by Enjalbert *et al.* (1998; 2000), who observed, after duodenal infusion of pure FA in lactating dairy cows, that palmitic though no stearic acid increased the milk fat content. However, Astrup *et al.* (1980a) reported an increased, though not significant, in milk fat content when dairy cows were dietary supplemented with either palmitic or stearic acid. While differences among studies may be due to the composition of the control treatment, type and level of FA supply, FA transfer between duodenal digesta and mammary gland, etc., more conclusions could certainly be drawn when the FA profile of the milk fat can be analysed.

Changes in milk yield and milk fat resulted, for MP as well as for all cows, in higher milk fat yields in treatment P and St compared with C. Moreover, and due to the differences in milk yield, in MP cows the milk fat yield was higher in St than in P treatment. It is well known that an increased supply of LCFA to the mammary gland inhibits the process of *de novo* synthesis of FA (all FA with a chain length ≤ C14 and 60 % of C16:0; Ashes *et al.*, 1997; Garnsworthy *et al.*, 2006; Mosley *et al.*, 2007; Noble *et al.*, 1969; Palmquist *et al.*, 1998; Storry *et al.*, 1968; Weisbjerg *et al.*, 2008). In the present experiment, cows fed the high fat treatments certainty had an increased supply of LCFA compared to cows fed the C treatment. Moreover, the inhibition of *de novo* synthesis of FA into the mammary gland, related to a larger supply of LCFA in treatment P and St, can be supported by the content of citric acid in milk. Though not presented in the present report, the contents of citric acid in milk from cows fed the high fat treatments were significantly higher (P<0.001) than that in milk from C treatment. Briefly, citric acid provides the reducing equivalents required for *de novo* synthesis of FA, therefore if this process decreases the level of citric acid increases (Garnsworthy *et al*., 2006). However, and base on the fact that both fat content and milk fat yield were higher in P and St treatments, it appears than in the present study the uptake of LCFA by the mammary gland more than compensated any possible drop in *de novo* synthesis.

In line with the observed differences in CP intake (Tables 10 & 11), milk protein content in milk from MP cows was lower (P=0.008) in P and St than in C treatment. However, due to different milk yields the milk protein yield was numerically the highest in MP cows fed St treatment followed –though not significantly different- by C treatment; with P treatment showing the lowest (P=0.015) milk protein yield.

Table 12. *Milk yield and composition in dairy cows supplemented with different FA.*

Item	Parity[1]	Treatment			SEM [2]	*P=*		
		Control	Palmitate	Stearate		Treat	Parity	Treat*Parity
Milk yield (kg/d)								
Actual	1	29.53	28.85	28.09	2.086	*0.888*		
	≥ 2	34.84	34.64	40.79	1.830	*0.062*		
	All cows	32.18	31.74	34.39	1.387	*0.368*	*<0.001*	*0.137*
ECM[3]	1	30.67	29.84	29.68	1.934	*0.928*		
	≥ 2	33.47[b]	34.91[b]	41.24[a]	1.391	*0.004*		
	All cows	32.07	32.38	35.46	1.192	*0.105*	*<0.001*	*0.042*
Milk composition (%)								
Fat	1	4.35	4.48	4.56	0.244	*0.832*		
	≥ 2	3.78[b]	4.40[a]	4.42[a]	0.139	*0.010*		
	All cows	4.07	4.44	4.48	0.141	*0.086*	*0.117*	*0.413*
Protein	1	3.62	3.43	3.49	0.083	*0.281*		
	≥ 2	3.57[a]	3.14[b]	3.20[b]	0.104	*0.028*		
	All cows	3.59[a]	3.28[b]	3.35[b]	0.067	*0.008*	*0.012*	*0.380*
Lactose	1	4.90	4.77	4.78	0.041	*0.099*		
	≥ 2	4.71	4.71	4.78	0.050	*0.496*		
	All cows	4.79	4.74	4.79	0.033	*0.554*	*0.027*	*0.068*
Total solids	1	13.61	13.38	13.60	0.303	*0.839*		
	≥ 2	12.70	12.94	13.14	0.254	*0.495*		
	All cows	13.16	13.16	13.36	0.198	*0.713*	*0.015*	*0.628*
Milk components yield (kg/d)								
Fat	1	1.23	1.23	1.24	0.082	*0.996*		
	≥ 2	1.27[c]	1.48[b]	1.73[a]	0.061	*<0.001*		
	All cows	1.25[b]	1.36[a]	1.49[a]	0.051	*0.012*	*<0.001*	*0.017*
Protein	1	1.03	0.96	0.95	0.069	*0.674*		
	≥ 2	1.20[a]	1.05[b]	1.36[a]	0.043	*0.015*		
	All cows	1.12	1.00	1.11	0.041	*0.121*	*<0.001*	*0.205*
Lactose yield	1	1.40	1.34	1.30	0.103	*0.801*		
	≥ 2	1.58[b]	1.59[b]	1.89[a]	0.079	*0.026*		
	All cows	1.49	1.46	1.60	0.065	*0.325*	*<0.001*	*0.079*

[1] Parity: 1, *n*= 5; ≥ 2, *n*= 5; all cows, *n*= 10.

[2] SEM: standard error of the mean.

[3] ECM= 0.01 * kg milk yield + 12.2 * kg fat + 7.7 * kg protein + 5.3 * kg lactose (Sjaunja *et al.*, 1990)

[a, , , b, C] Means in the same row with different superscripts differ at $P<0.05$ (treatment effect).

FFA in milk and MFG size

In general terms, milk from cows fed the high fat treatments (P and St) had numerically higher contents of FFA, either expressed as μ equiv/ml of milk or μ equiv/g of milk fat, than milk from cows fed the C treatment. However, significant differences (P<0.05) were detected only in milk from PP cows, as well as when data from all 30 cows were statistically analysed. For PP cows, only milk from cows fed St treatment showed to have a higher content FFA than milk from C treatment, whilst milk from cows fed P treatment had a FFA content that was no different from those of St and C treatments. With regard of data from all cows, milk from cows fed P and St had a higher (P=0.005) content of FFA than C. The effect of treatment on milk FFA tended (P<0.10) to be the same, also, when data were expressed and analysed as μ equiv/g of milk fat. Increased FFA concentration in milk as a result of an increased intake of FA has been reported by others (Astrup *et al.*, 1980a; Kinter & Day, 1964). However, the present results are in contrast to those reported by and Astrup et al. (1980a) and Kinter & Day (1964), who observed that palmitic affected the levels of FFA in milk in a greater extent than stearic acid.

On the other hand, in the present study milk from cows fed the high fat treatments had FFA levels which appeared to be high enough to be associated to the occurrence of off flavours. According to Santos *et al.* (2003), milk with FFA contents higher than 35 μ equiv/g of milk fat were found to have off flavours by more than 60 % of the panel members. However, while the presence of off flavours in milk from the cows fed the high fats treatments cannot be ruled out, this parameter was not evaluated and it should kept in mind that the occurrence of off flavours in milk is also dependent of the milk fatty acid composition.

Table 13. *FFA content and MFG size in milk from dairy cows supplemented with different FA.*

Item	Parity[1]	Treatment			SEM [2]	*P=*		
		Control	Palmitate	Stearate		Treat	Parity	Treat*Parity
Free FA								
μ equiv./ml	1	0.126[b]	0.153[ab]	0.193[a]	0.013	*0.016*		
	≥ 2	0.120	0.156	0.148	0.012	*0.147*		
	All cows	0.123[b]	0.155[a]	0.171[a]	0.010	*0.005*	*0.144*	*0.175*
μ equiv./g of milk fat	1	29.72	34.43	43.60	4.111	*0.091*		
	≥ 2	31.33	35.90	33.53	2.313	*0.539*		
	All cows	30.53	35.16	38.57	2.496	*0.094*	*0.428*	*0.186*
Milk fat globule size (μm)								
	1	4.05	4.32	4.51	0.157	*0.206*		
	≥ 2	4.01	4.45	4.19	0.138	*0.118*		
	All cows	4.03	4.39	4.35	0.110	*0.059*	*0.549*	*0.377*

[1] Parity: 1, n= 5; ≥ 2, n= 5; all cows, n= 10.

[2] SEM: standard error of the mean.

[a, b] Means in the same row with different superscripts differ at P<0.05 (treatment effect).

While increased levels of FFA in milk were associated to increased milk fat contents (Kinter & Day, 1964; present study); it has been also shown that size of the MFG plays an important

role in milk lipolysis. Larger MFG are expected to be less stable and therefore more susceptible to both spontaneous and induced lipolysis than smaller ones (Wiking *et al*., 2003b). Moreover, larger MFG were related to higher levels of FFA in milk (Wiking *et al*., 2003b; Wiking, 2005). MFG size may increase as result of an increased fat intake, increased intake of saturated rather than UFA, and with an increased in the chain length of the FA supplied to the mammary gland (Timmen & Patton,1988; Weisbjerg *et al*., 2008; Wiking *et al*., 2003b). In line with these statements, whilst no significant differences were detected within parity groups, in the present study milk from cows fed treatment P and St showed a strong tendency (*P*=0.059) for having larger MFG size than milk from cows fed the C treatment (Table 13).

Conclusions

The inclusion of palmitic and stearic acid methyl esters in concentrates fed to mid lactation dairy cows had no effect on feed intake and apparent *in vivo* digestibility. Changes in milk yield and composition were more evident in MP rather than in PP cows. The yields of milk and energy corrected milk, as well as the milk fat content and milk fat yield were increased by the inclusion of palmitic and stearic acid in the treatments. It appeared that these changes were, numerically, of a greater magnitude in cows supplemented with stearic acid than with palmitic acid. Also, feeding the cows the high fat treatments resulted in higher levels of FFA in milk associated with an increased size of the MFG. While the results of the present study would support the concept that palmitic acid may be replaced by stearic acid in treatments for cows without any detrimental effect on milk yield and composition, further conclusions cannot be drawn without the data on FA profile in milk.

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