Plasma transport and mammary uptake of *trans* fatty acids in dairy cows

By

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DECLARATION

I hereby declare that this thesis has been composed by myself, and has not been submitted for any other degree at The University of Nottingham or elsewhere. The work presented herein is my own, and all the work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the design and execution of the experiments contained in this thesis and during its preparation.

Einar VARGAS BELLO PEREZ

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ABSTRACT

In this thesis, aspects of metabolism of lipids in dairy cows were studied, particularly 18:1 *trans* fatty acid (tFA) concentrations in plasma and lipoprotein fractions, and transportation of FA in epithelial mammary gland cell cultures.

Two *in vivo* studies were conducted to elucidate which lipoprotein fractions were involved in bovine plasma transport of tFA by infusing oils that induced different plasma tFA profiles. Fatty acid profiles of plasma and lipoprotein fractions [high (HDL), low (LDL), and very low density (VLDL) lipoprotein fractions] were analysed. Results showed that VLDL fraction was the main transport mechanism of tFA in plasma and was more responsive to supply of tFA.

MAC-T cell cultures were used to study specific aspects of lipid uptake without interference from the cow's digestive system and general metabolism. Treatments consisted of adding long-chain FA solutions to lactogenic medium at varying concentrations. After incubation, cystosolic triacylglycerol (TAG), protein and DNA contents were determined. Results demonstrated that cytosolic TAG accumulation is stimulated by addition of long-chain FA, which affects FA profile of cytosolic free FA.

Overall, results from this thesis enhance knowledge on plasma transport and mammary uptake of 18:1 tFA in dairy cows. The information strengthens understanding of some aspects of milk fat synthesis and metabolism of FA in dairy cows fed with different sources of tFA. Findings from this thesis are important because saturated FA and tFA in milk fat have caused concern among human health researchers and more recently milk fat has gained appreciation as a functional food due to the health-promoting potential of some FA (vaccenic and conjugated linoleic acids) found specifically in ruminant-derived products.

PUBLICATIONS AND PRESENTATIONS

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ABBREVIATIONS

ANOVA	Analysis of variance
BSA	Bovine serum albumin
BW	Body weight
CE	Cholesterol esters
CHD	Coronary heart disease
CLA	Conjugated linoleic acid
CP	Crude protein
d	Day
DM	Dry matter
DNA	Deoxyribonucleic acid
EA	Elaidic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentanoic acid
FA	Fatty acid (s)
FATP	Fatty acid transport protein (s)
FCS	Foetal calf serum
FFA	Free fatty acids
FAME	Fatty acid methyl ester
FO	Fish oil
g	G force
g	Gram
GC	Gas chromatography / gas chromatograph
h	Hour
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
IDL	Intermediate density lipoprotein
IU	International units
kg	Kilogram
L	Litre
LC-PUFA	Long chain polyunsaturated fatty acid (s)
LDL	Low density lipoprotein
LSD	Least significant differences (5% level)
M	Molar
MFD	Milk fat depression
mg	Milligram
min	Minutes
ml	Millilitre
mm	Millimetre
m*M*	Millimolar
mmol	Millimoles
MUFA	Monounsaturated fatty acid (s)
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NEFA	Non-esterified fatty acid (s)
nd	Not detected
NDF	Neutral detergent fibre
NH_3-N	Ammonia nitrogen
PBS	Phosphate buffered saline

pH	Log of reciprocal hydrogen ion concentration
PHVO	Partially hydrogenated vegetable oil
PL	Phospholipids
PUFA	Polyunsaturated fatty acid (s)
rpm	Revolutions per minute
SCD	Stearoyl CoA-desaturase
SFA	Saturated fatty acid (s)
SED	Standard error of difference
SEM	Standard error of the mean
SM	Skimmed milk
SO	Soya oil
TAG	Triacylglycerols
TG	Triglyceride
TC	Total cholesterol
TLC	Thin layer chromatography
tFA	*Trans* fatty acid (s)
UFA	Unsaturated fatty acid (s)
u*M*	Micromolar
v/v	Volume to volume
VA	Vaccenic acid
VFA	Volatile fatty acid (s)
VLDL	Very low density lipoprotein
w/v	Weight to volume
WCOT	Wall coated open tubular

Abbreviations for some of the more common fatty acids

Abbreviation	Systematic name	Common name
C10:0	Decanoic	Capric
C12:0	Dodecanoic	Lauric
C14:0	Tetradecanoic	Myristic
C15:0	Pentadecanoic	Pentadecanoic
C16:0	Hexadecanoic	Palmitic
C16:1	*cis*-9-hexadecanoic	Palmitoleic
C17:0	Heptadecanoic	Margaric
C18:0	Octadecanoic	Stearic
C18:1n *trans* 9	*trans*-9-octadecanoic	Elaidic
C18:1n *trans* 11	*trans*-11-octadecanoic	Vaccenic
C18:1n *cis* 9	*cis*-9-octadecanoic	Oleic
C18:2n *trans* 6	*trans, trans*-9, 12-octadecanoic	Linolelaidic
C18:2n *cis* 9,*trans* 11	9-*cis*-11-*trans*-octadecadienoic acid	Rumenic
C18:2n *cis* 6	*cis, cis*-9, 12-octadecanoic	Linoleic
C18:3n3	All-*cis*-9, 12, 15-octadecatrienoic	α-linolenic
C18:3n6	All-*cis*-6, 9, 12-octadecatrienoic	γ-linoleic
C20:0	Eicosanoic	Arachidic
C20:3n6	8, 11, 14-eicosatrienoic	Dihomo- γ-linoleic
C20:4n6	5, 8, 11, 14-eicosatetranoic	Arachidonic
C20:5n3	5, 8, 11, 14, 17-eicosapentaenoic	EPA
C22:0	Docosanoic	Behenic
C22:1n9	*cis*-13-docosenoic	Erucic
C22:6n3	4, 7, 10, 13, 16, 19-docosahexaenoic	DHA
C24:0	Tetracosanoic	Lignoceric

Chapter 1

Literature review

CHAPTER 1

1. Literature review

1.1 Introduction

Lipids obtained from dairy products are an important part of the human diet in many countries; roughly 75% of the total consumption of fat from ruminant animals comes from bovine milk fat (O'Donnell et al., 1993; Demeyer et al., 1999; Chilliard et al., 2000). Milk fatty acid (FA) composition has numerous effects on milk quality, including its physical properties (e.g. melting point and hardness of butter, crystallization and fractionation of milk fat) and nutritional value (e.g. effects on human health). Different FA (short- and medium-chain saturated, branched, mono- and polyunsaturated, *cis* and *trans*, conjugated FA) present in ruminant milk fat are potentially positive or negative factors for the health of consumers (Williams, 2000; Jensen, 2002; Parodi, 2004; Chilliard et al., 2007). Dairy products provide 25–60% of the overall saturated fat consumption in Europe, which, for decades has made them, a target of concern due to the negative effects of excessive consumption of saturated FA on human health (Givens and Shingfield, 2006; Chilliard et al., 2007). Recently (Elwood et al., 2010) a review from different meta analyses showed that milk intake reduces risk factors for ischemic heart disease, stroke and incident diabetes. However, this does not necessarily apply to full fat dairy products such as butter and cream.

By altering the nutrition of the cow, farmers can markedly and rapidly modulate milk FA composition. The largest changes can be obtained either by altering forages in diets, particularly pasture, or by adding plant or marine lipid supplements to the diet (Chilliard and Ferlay, 2004; Chilliard et al., 2001, 2003, 2007; Lock and Shingfield, 2004; Loor et al., 2005; Dewhurst et al., 2006). Modifying milk fat content by dietary manipulation is not a recent concept (Palmquist and Beaulieu, 1993). In the early 1970s the concern for reducing saturated fat content in the human diet initiated an intense research era (Grummer, 1991). In recent years the interest in reducing fat from dairy products is mainly due to concern about the hypercholesteraemic effects of medium-chain saturated FA (Dewhurst and Lee, 2004). Conversely, in recent decades the dairy industry has increased the fat content of milk by making genetic modifications, principally in the British Friesian breed, with the aim of improving yields of cheese and butter, and milk fat as an energy source in the human diet (Palmquist and Jenkins, 1980; Wu et al., 1991; Dewhurst and Lee, 2004). Given that

economic factors define the future profits for farmers, dietary manipulation of fat content may be the most practical and appropriate approach to change milk FA composition.

Lipids from ruminant milk have been estimated to contain over 400 different FA and this number is mainly due to events that take place within the rumen (Jensen, 2002; Bauman and Griinari, 2003). Diet has major effects on ruminal bacteria population and processes within the rumen. For this reason, diet and nutrition have an important role in determining the fat content and FA composition of milk (Bauman and Griinari, 2003).

Some FA, such as butyric acid, oleic acid, polyunsaturated FA (PUFA) and conjugated linoleic acid (CLA) have potential anticarcinogenic and antiatherogenic effects on human health. Other FA, such as some saturated (lauric, myristic and palmitic acids) and some *trans* FA (tFA), have potential negative effects on human health (Willet et al., 1993; Kinsella et al., 1990; Molkentin, 1999; Parodi, 1999; Chilliard et al., 2000).

Trans fatty acids are produced during biohydrogenation of PUFA in the rumen. They are mixtures of positional and geometric isomers (Griinari and Bauman, 1999; Piperova et al., 2004), which are incorporated into milk fat (Parodi, 1977; Griinari and Bauman, 1999; Loor and Herbein, 2001; Piperova et al., 2002, 2004; Precht et al., 2002). There has been intensive research on tFA, mainly on the negative effect of specific isomers of 18:1 *trans* that can be found in either dairy products or chemically produced partially hydrogenated vegetable oils (PHVOs) (Willet et al., 1993; Wolff, 1995; Chilliard et al., 2000). There is also interest in the role of *trans* isomers of 18:1 mediating the milk fat depression associated with some specific dairy diets (Griinari et al., 1998, 1999; Chilliard et al., 2000).

A number of researchers have highlighted the negative effect of tFA on milk fat concentration. However, the exact mechanisms of this phenomenon remain unclear. Milk fat depression has a great impact on economics, causing important losses to dairy producers. Therefore, more research is needed to understand the exact mechanisms by which tFA cause milk fat depression. Additionally, the influence of tFA on milk

composition is of interest due to their biological effects and potential role in human diseases.

1.2 Lipids

Lipids are a group of heterogeneous chemically substances that are insoluble in water, but soluble in non-polar solvents such as chloroform, hydrocarbons and alcohols (Christie, 1981). Lipids include a wide range of compounds including neutral fats (triacylglycerols), waxes, terpenes and more complex lipids such as glycolipids and phospholipids. Lipids play important biological roles as structural cell membrane components, storage forms of energy, metabolic fuels, emulsifying and insulating agents, steroid hormones, vitamins, prostaglandins and roles in signal transduction (Hajri and Abumrad, 2002; Doege and Stahl, 2006).

1.2.1 Fatty acid nomenclature

The number, geometry, and position of double bonds in FA affect their melting point. Triglycerides high in long-chain saturated FA will easily conglomerate in a crystal lattice and are therefore solid at room temperature (Katan and Zock, 1995). On the other hand, *cis* unsaturated double bonds introduce bends in the molecule that hinders crystal formation, which explains why oils are liquid. The spatial structure of tFA is in between that of saturated FA and *cis* unsaturated FA. As a result, oleic acid melts at 13°C; its *trans* isomer elaidic acid (18:1 *trans* 9) melts at 44°C; and stearic acid (18:0), which is straight and saturated, melts at 72°C (Katan and Zock, 1995).

Different conventions are used to name unsaturated FA according to the number of carbons in the molecule, the position of the centres of unsaturation (double bonds) and the geometrical configuration of the double bonds. The carbon atoms of an acid can be numbered (or lettered) either from the carboxyl group (Δ numbering system) or from the carbon atom furthest from the carboxyl group (n or ω numbering system) (Katan and Zock, 1995). FA names are abbreviated in the Δ nomenclature by listing the number of carbon atoms followed by the number of double bonds: i.e. 16:1 Δ^9. The number after Δ in this classification system is the position of the double bond relative to carboxyl end. For example, in 16:1 Δ^9, the single double bond is nine carbon atoms away from the carboxyl group; meaning that it is between carbon atoms 9 and 10, counting the carboxyl carbon atom as carbon atom number 1. In the ω numbering system, palmitoleic would be referred as 16:1 (n-7). This indicates seven carbon atoms

away from the ω-carbon atom (Katan and Zock, 1995). This system is currently most widely used. Most unsaturated FA are found in the *cis* configuration where the hydrogen atoms are on the same side of the double carbon bond, whereas tFA have hydrogen atoms on opposite sides of the double bond (Figure 1.1) (Food Safety, Authority of Ireland, 2008).

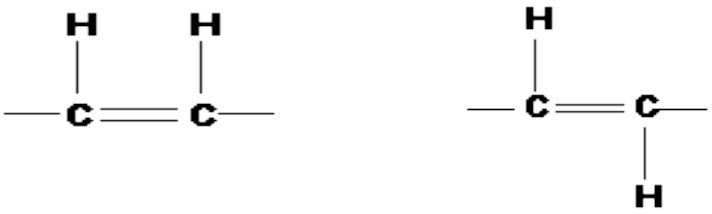


Figure 1.1 *Cis* (left) and *trans* (right) configurations of carbon double bonds in fatty acids (Food Safety, Authority of Ireland, 2008).

1.2.2 Essential fatty acids

Mammals cannot synthesize two PUFA, linoleic and linolenic acids, which, as they and products of their metabolism play essential physiological roles, are therefore essential dietary requirements. Linoleic acid consists of a chain of 18 carbon atoms containing two double bonds separated by one methylene group. In the "n" notation, linoleic is designated n-6 and can be represented as 18:2 (n-6) (AbuGhazaleh et al., 2007). The hydrogen atoms on each side of the double bonds are in the *cis* configuration. Linolenic acid (often denoted as α-linolenic acid to distinguish it from isomeric forms) has three bonds 18:3 (n-3). Linoleic acid has two double bonds at $\Delta^{9,12}$, and α-linolenic has three, $\Delta^{9,12,15}$. These FA cannot be synthesized because animals lack Δ^{12} and Δ^{15} desaturases which prevents introduction of double bonds beyond carbon atom 9 in the chain (Christie, 1982).

The richest sources of linoleic acid are seed oils (i.e. safflower, sunflower, maize and soya bean oils) (AbuGhazaleh et al., 2007). Alpha linolenic acid is found in high amounts in flax (linseed) oil, and in lesser amounts in rapeseed oil, soya bean oil and oats in even lower quantities in cottonseed, corn, palm, safflower and sunflower oils (Shingfield et al., 2010).

1.2.3Conjugated linoleic acid (CLA)

Conjugated linoleic acid refers to the group of isomers of linoleic acid with conjugated double bonds (Bauman et al., 1999; Chichlowski et al., 2005). CLA is

produced during the biohydrogenation of PUFA in the rumen (Figure 1.2) (Griinari and Bauman, 1999; Piperova et al., 2004). CLA is an intermediate in the rumen hydrogenation of linoleic acid, whereas vaccenic acid (18:1 *trans* 11) is a common intermediate in the biohydrogenation of linoleic and α- and γ-linoleic acids (Chilliard et al., 2000). Griinari et al. (2000) demonstrated that endogenous synthesis of CLA *cis* 9, *trans* 11 from vaccenic acid represents the primary source of CLA in milk fat (Shen et al., 2007).

Dairy products and meat from ruminants represent the only significant natural source of CLA (Lawson et al., 2001; Burdge et al., 2005). CLA has been suggested to have numerous beneficial physiological effects such as changes in body composition (Gaullier et al., 2004; Chichlowski et al., 2005) and lower insulin resistance associated with CHD (Aminot-Gilchrist and Anderson, 2004; Chichlowski et al., 2005). Other health effects, such as anti-carcinogenic, anti-atherogenic, anti-obesity, anti-diabetic and immune system enhancement, have been investigated in different animal models, and to a lesser extent in humans (McGuire and McGuire, 2000; Belury, 2002).

An increase in milk CLA, particularly rumenic acid content is associated with an increase in other tFA, particularly 18:1 *trans*-11, which acts as a precursor of rumenic acid via the enzymatic action of Δ^9-desaturase, and to a lesser extent 18:1 *trans*-10 (Williams, 2000; Lock and Garnsworthy, 2002; Lock et al., 2004; Hurtaud and Peyraud, 2007). This was also reported by Chilliard et al. (2000), who found a linear relationship between the content of CLA in milk and 18:1 *trans*. Similarly, Hurtaud and Peyraud (2007) showed strong correlations between milk CLA and 18:1 *trans*.

Dietary starch and fibre levels affects biohydrogenation of FA by ruminal microflora and hence, on the equilibrium of CLA isomers passing to the duodenum (Chilliard et al., 2000; Offer et al., 2001). Supplementation of dairy cow diets with plant oils (soya bean, sunflower, linseed, rapeseed and maize) results in significant increases in milk fat CLA concentrations. Moreover, plant oils high in linoleic acid give the greatest response in cattle. Feeding pasture to dairy cows has been also shown to increase milk fat content of CLA (Stanton et al., 1997; Kelly et al., 1998; Lawless et al., 1998; Chilliard et al., 2000; Boken et al., 2005; Hurtaud and Peyraud, 2007). However, other studies have shown that milk CLA increases with diets low in 18:2, such as those which include fish oil supplements; because of those observations it has been

suggested that CLA could be synthesized by tissues (i.e. adipose tissue) (Chilliard et al., 2000).

Lock and Garnsworthy (2002) demonstrated that dietary lipid manipulation can alter CLA *cis*-9, *trans*-11 content in milk fat when linoleic or linolenic acids are fed to cows. They found similar increases in milk CLA *cis*-9, *trans*-11 content when either dietary linoleic or linolenic acids were increased and higher concentrations were observed when both oils were increased together. They also found that there was considerably more CLA *cis*-9, *trans*-11 (approximately 80%) in milk than that produced in the rumen in agreement with the concept of endogenous synthesis of CLA *cis*-9, *trans*-11 in the mammary gland via the enzymatic action of Δ^9-desaturase.

The concentration of CLA can be increased in pasture grazing cows when their feed is supplemented with oils (Boken et al., 2005; AbuGhazaleh et al., 2007). Other studies (Lawless et al., 1998) have concentrated on supplementation of grazing cows with full-fat soyabeans or full-fat rapeseed to increase milk CLA concentration. Alternatively, fish oil (FO) has been used as an effective means to increase concentration of CLA in milk (AbuGhazaleh et al., 2007). However, there is low efficiency of transfer of dietary (n-3) long-chain PUFA (LCPUFA) to milk (Offer et al., 2001) mainly due to three factors occurring in the rumen:

1. Biohydrogention of these dietary lipids in the rumen (Figure 1.2)
2. Their selective incorporation into certain plasma lipoprotein fractions which are ineffective in delivering FA into the mammary gland
3. A low specificity of the relevant enzymatic systems of the mammary gland for these FA

C18 PUFA

↓

Conjugated C18 PUFA

↓ **one or more reductive steps**

C18:1 *trans*

↓

C18:0

Figure 1.2 General pathway for rumen biohydrogenation of PUFA 18-carbon FA (from Harfoot and Hazlewood, 1988; Bauman and Griinari, 2003)

1.3 Milk fat composition

Lipids in milk normally comprise 60 to 70% saturated fatty acids (SFA; e.g. myristic, palmitic and 18:0 acids) (Yu et al., 1995; Dewhurst and Lee, 2004), 25 to 35% monounsaturated fatty acids (MUFA; e.g. oleic acid) and up to 5% PUFA (e.g. linoleic, CLA, α-linoleic acids) (Jensen, 2002; Corl et al., 2003; Lock et al., 2004; Dewhurst and Lee, 2004). Normally, milk contains low levels of longer-chain PUFA such as eicosapentaenoic acid (EPA; C20:5) and docosaheaenoic acid (DHA; C22:6) (Jacobs et al., 2004; Dewhurst and Lee, 2004). Milk fat contains significantly higher concentrations of short-chain FA and medium-chain FA and relatively lower concentrations of UFA compared with other dietary sources of vegetable and animal fat (Berner, 1993; Kennelly, 1996). Milk fat has been criticized because it contains a less desirable balance of FA than many vegetable or fish oils. Two principal medium-chains FA in butterfat, myristic acid and palmitic acid, have been identified as major dietary factors responsible for raising plasma low density lipoprotein cholesterol (Keys et al., 1965; Hegsted et al., 1965; Grande, et al., 1970; Denke and Grundy, 1992; Nestel et al., 1994). In particular, myristic acid, of which dairy products are a major source, is reputedly more potent than palmitic acid in cholesterol-raising effects (Zock et al., 1994) (Tables 1.1 and 1.2).

Table 1.1 Lipids in milk

Lipid class	% of total lipid
Phospholipid	1.11
Cholesterol	0.46 (15 mg/dl)
Triacylgycerol	95.80
1, 2- Diacylglycerol	2.25
Free fatty acids	0.28
Monoacylglycerol	0.08
Cholesteryl ester	0.02
Fat g/dl	3.25

Adaptated from Jensen, 2002; data was obtained by densitometric analysis of separate milk lipids on TLC plates

Table 1.2 Fat composition of milk in the United Kingdom[1] and composition of the major fatty acids in milk fat[2]

Type of milk	Per 100 g	Fatty acid	Common name
Whole milk			
Fat (g)	3.9	4:0	Butyric
SFA	2.5	6:0	Caproic
MUFA	1.0	8:0	Caprylic
PUFA	0.1	10:0	Capric
tFA	0.1	12:0	Lauric
Semi-skimmed milk		14:0	Myristic
Fat (g)	1.7	15:0	Pentadecanoic
SFA	1.1	16:0	Palmitic
MUFA	0.4	16:1	Palmitoleic
PUFA	Trace	17:0	Margaric
tFA	0.1	18:0	Stearic
Skimmed milk		18:1	Oleic
Fat (g)	0.3	18:2	Linoleic
SFA	0.1	18:3	Linolenic
MUFA	0.1		
PUFA	Trace		
tFA	Trace		

[1]From The Dairy Council, 2010. Data was taken from the 2002 summary edition of The Composition of Foods (Food Standards Agency (2002) McCance and Widdowson's The Composition of Foods, Sixth summary edition. Cambridge: Royal Society of Chemistry); [2] Adaptated from Jensen, 2002

1.3.1 Factors affecting milk fat composition

Ruminant milk FA composition is linked to intrinsic (animal species, breed, genotype, pregnancy and lactation stages) or extrinsic (environmental) factors (Palmquist et al., 1993; Chilliard and Ferlay, 2004; Chilliard et al., 2007). In a given animal species, the

effects linked to breed or genotypes are significant but restricted and they can only be altered over long terms. The effect of lactation stage on milk fat content and FA composition is associated to body fat mobilization in early lactation (Chilliard et al., 1991, 2007), but it only lasts a few weeks each year. Garnsworthy et al. (2006) investigated the variation in citrate with stage of lactation and *de novo* FA synthesis, without confounding dietary effects. They found that cows in late lactation produced milk with a FA profile similar to that of cows in mid lactation. Differences between mid and late lactation in yield of FA were due to differences in milk fat yield, concluding that stage of lactation does not affect the relative incorporation of FA from *de novo* synthesis vs. preformed sources when diet composition is constant.

1.4 Dietary fat in dairy cow diets

Supplementation with lipids has been used to increase the energy concentration of the diet and to avoid negative associative effects on dry matter intake, digestibility, and milk production and composition sometimes experienced with cereal grains (Coppock and Wilks, 1991; Staples et al., 1998; Garnsworthy, 1997). Many sources of supplemental fat have been fed to beef and dairy cattle under experimental conditions. These include blends of animal and vegetable fat, tallow, yellow grease, fishmeal, cottonseeds, soyabeans, rapeseeds, canola seeds, peanut hearts, safflower seeds, sunflower seeds, flaked fat, prilled fat, hydrogenated fat, calcium soaps of fat, medium-chain triglycerides, and free FA (Staples et al., 1998; Funston, 2004). The FA profile of these fat sources varies widely. Coppock and Wilks (1991) reported the FA profile of many of the commonly used fats. The major FA in most feed lipids is linoleic acid whereas linolenic acid predominates in most forage lipids (Staples et al., 1998). Tallow (40.9%) and yellow grease (46.8%) contain different contents of oleic acid (Coppock and Wilks, 1991). Granular fats, such as calcium soaps of palm oil, and prilled fats contain mainly saturated palmitic and stearic acids (Staples et al., 1998).

The fat content of ordinary diets for ruminants is less than 50 g/kg and digestive problems can occur by increasing total fat content of the diet to more than 100 g/kg (Garnsworthy, 1997). Rumen microorganisms cannot utilise large quantities of fat, although limited quantities of FA can be incorporated into microorganisms during cell synthesis. More important than the quantity of FA in the diet is their form, since long-chain UFA have a detergent effect on bacterial cell walls. Under normal circumstances, the ester linkages of tryglycerides are rapidly hydrolysed by bacterial

lipases in the rumen. Once released from the ester combination, unsaturated fatty acids are subsequently hydrogenated to detoxify them (Garnsworthy, 1997). Fibre particles in the rumen can be physically coated by fat and be inaccessible for microbial attack. The magnitude of these effects depends on level, source and type of fat, dietary carbohydrate source and feed intake. Similarly, another problem is that long-chain free FA can form soaps with calcium and magnesium in the rumen. This will detoxify FA, but it can also reduce the availability of minerals (Garnsworthy, 1997).

Theoretically, supplementation of fat could have some of the following advantages:

a. Increase the energy concentration of the diet because fat contains three times more net energy for lactation than protein- and carbohydrate-rich feeds (Palmquist, 1984)

b. Improve the energetic efficiency because reduced loss of energy as heat, methane, and urine may be expected (Palmquist and Jenkins, 1980), and because the dietary fatty FA are incorporated directly into milk fat by the mammary gland (Wu and Huber, 1994; Garnsworthy, 1997)

c. Reduce the risk of rumen acidosis and a decrease in milk fat percent induced by feeding high levels of cereal grains in the diet (Palmquist and Conrad, 1978; Palmquist, 1984)

Lipids can be manipulated by several means in order to avoid adverse effects in the rumen and to retain availability in the small intestine (Palmquist, 1984). These modified fat products are known as “by-pass fats” or “rumen inert fats”, and their protection against rumen microbial action can be natural (i.e., encapsulation in formaldehyde-treated casein or formation of calcium soaps) or physical (i.e., selection of fatty acids with a high melting point and small particle size) (Garnsworthy, 1997). Development of commercial products based on calcium soaps of long-chain fatty acids offers dairy cattle producers a method of increasing energy concentration of rations without disturbing rumen fermentation (Palmquist, 1984; Chalupa et al., 1986; Espinoza et al., 1995).

Fat supplementation has been associated with positive and negative effects on reproduction although (Grummer and Carroll, 1991; Fahey et al., 2002) the amount of

supplemental fat needed to elicit an effect on reproductive function is largely unknown. Some studies indicate that the amount of added plant oil necessary to maximize positive ovarian effects is not less than 4% (Stanko et al., 1997; Thomas, Bao and Williams, 1997). Staples et al. (1998) found that 3% added dietary fat (DM basis) has often positively influenced the reproductive status of the dairy cow. Lower levels of added fat (2%) have also been shown to affect positively reproductive performance (Bellows et al., 1978).

Lipids are rich sources of energy and have important roles in the structure and function of different biological membranes (Cribier et al., 1993; Fouladi-Nashta et al., 2007). Dietary FA are crucial in the reproductive performance of dairy cows due to their influence on the energy balance and other reproductive processes that are not related to energy (Thatcher et al., 2002; Fouladi-Nashta et al., 2007).

Feeding fat to cattle generally improved establishment and maintenance of pregnancy. Potential improvements in fertility of cows caused by fat feeding have generally been associated with enhanced follicle development postpartum, increased diameter of the ovulatory follicle, increased progesterone (PG) concentrations during the luteal phase of the cycle, altered uterine/embryo cross-talk by modulating PG synthesis, and improved oocyte and embryo quality. Some of these effects have been more influenced by the type of FA than by fat feeding *per se*. Differential responses *in vivo* to FA feeding suggest that UFA of the n-6 and n-3 families were most beneficial (Santos et al., 2008).

1.4.1 Saturated fatty acids and oleic acid

There is a huge potential to alter medium-chain SFA (10:0 to 16:0). For example, it has been reported (Roy et al., 2006; Chilliard et al., 2007) that cows fed with hay-based diets can increase these four FA to 56% of milk fat whereas supplementation with linseed oil can decrease them to 29%. Lipid supplements rich in medium-chain FA, can increase these FA; for example, calcium salts of palm oil increased palmitic acid concentration (Chilliard et al., 2007).

On the other hand, it has been shown (Collomb et al., 2004; Loor et al., 2005; Gonthier et al., 2005) that stearic acid in milk can be increased either by dietary 18:0 intake or by supplementation of 18-carbon unsaturated FA, because they are hydrogenated to 18:0 in the rumen (Loor et al., 2004, 2005; Chilliard et al., 2007).

Similarly, secretion of oleic acid can be increased through its direct gut absorption and mammary gland secretion or, perhaps more importantly, from its ruminal biohydrogenation followed by mammary desaturation of 18:0. Also its concentration can be increased by manipulating the distribution of oleamides (amides of oleic acid that resists ruminal biohydrogenation and elevate milk oleic acid concentration when fed to lactating cows) (Jenkins, 1998, 2006; Loor et al., 2002; Chilliard et al., 2007).

1.4.2 Lipid metabolism in the rumen

Unlike monogastrics, in which lipid digestion and absorption take place primarily in the stomach and small intestine, in ruminants the rumen plays an important role. Dietary lipids also affect ruminal fermentation, and ruminal fermentation in turn affects the digestion and utilization of other nutrients. Hence, dietary lipids can have profound effects on ruminant nutrition and animal performance. Grummer (1991) and Mansbridge and Blake (1997) studied ruminal lipids metabolism focusing on manipulation of physicochemical events in the rumen with two purposes:

1) To control the adverse effect of fatty acids on ruminal bacterial growth so that supplementary lipids do not adversely affect ruminal fermentation and nutrient digestion and

2) To regulate ruminal biohydrogenation to alter profiles of fatty acids reaching the small intestine and those secreted in milk.

There are three major factors that influence the transfer of dietary fat to milk fat (Wu et al., 1991; Palmquist et al., 1993):

1) Ruminal biohydrogenation

2) Absorption (digestibility)

3) Deposition in adipose tissue

For decades farmers have fed lipid supplements to modify and improve dairy performance and energy metabolism (Chilliard et al., 1993; Lock and Shingfield, 2004; Chilliard et al., 2007) and milk FA composition (Palmquist et al., 1993; Givens and Shingfield, 2006; Chilliard et al., 2000, 2006, 2007). Both source and presentation form of the lipids influence their effects. Changing the proportion of one category of

FA often has consequences for other FA. Thus, feeding a diet that increases PUFA and/or CLA and decreases in milk SFA results in higher 18:1 *trans* concentration (Lock et al., 2005; Roy et al., 2007; Bauchart et al., 2007; Chilliard et al., 2007).

Dietary lipids are mainly in the form of triacylglycerols, with small amounts in the form of monoacylglycerols, free FA, galactolipids and phospholipids (Mansbridge and Blake, 1997). Most common lipid supplements comprise predominantly of FA (90-95%) with a chain length greater than 14 carbons and typically contain greater than 75% C18 FA. The degree of unsaturation varies considerably (Grummer, 1991). Ingested lipids are subjected to two important microbial transformations in the rumen, lipolysis and biohydrogenation.

1.4.3 Lipolysis

Dietary lipids, if not protected, are rapidly and almost completely hydrolyzed to glycerol and free FA by lipases from different strains of bacteria. This process is referred to as lipolysis. Protozoa are not involved to any great extent in hydrolysis, except for that of phospholipids (Doreau and Ferlay, 1994). According to Bauchart et al. (1990), the extent of ruminal hydrolysis of lipids is high for most unprotected lipids (85-95%). *Anaerovibrio lipolytica,* which is best known for its lipase activity, produces a cell-bound esterase and a lipase (Harfoot, 1978). These lipases hydrolyze acylglycerols completely to free FA and glycerol with little accumulation of mono- or diglycerides (Jenkins, 1993). Glycerol is fermented to volatile or short chain fatty acids, mainly propionate and butyrate (Doreau and Ferlay, 1994), which are absorbed through the ruminal wall, and may eventually be used as precursors for *de novo* synthesis of short- and medium chain FA in the mammary gland (Mansbridge and Blake, 1997). Released FA are subjected to subsequent biohydrogenation and /or used for synthesis of microbial lipids. The dietary short-chain SFA released by microbial lipolysis are also absorbed through the ruminal wall into the blood stream (Doreau and Ferlay, 1994). Fatty acids also are released from plant galactolipids and phospholipids; hydrolysis of those esterified lipids is attributed to a variety of galactosidases and phospholipases produced by ruminal microbes (Jenkins, 1993).

1.4.4 Biohydrogenation

Released UFA are subjected to rapid and extensive biohydrogenation of double bonds by ruminal microbes to saturated products. This process may have evolved to protect microbes from toxic effects of UFA, because UFA are more toxic to ruminal microbes

than SFA (Palmquist and Jenkins, 1980). Biohydrogenation occurs only on free fatty acids with a free carboxyl group adsorbed on feed particles or microbial cells (Harfoot, 1978). The first step in biohydrogenation is an isomerization reaction that converts the *cis*-12 double bond to a *trans*-11 isomer, catalyzed by isomerase. The isomerase is not functional unless the FA has a free carboxyl group. This requirement establishes lipolysis as a prerequisite for biohydrogenation (Kepler et al., 1970; Jenkins, 1993). The extent to which UFA are biohydrogenated depends on several factors, such as forage:concentrate ratio (Kucuk et al., 2001) and type of protective treatment of the dietary fat (Tymchuk, 1998; Whitlock et al., 2002).

Lipolysis and biohydrogenation by rumen bacteria can be reduced by treating lipids chemically (e.g. formaldehyde treatment or calcium salts) or physically (e.g. heat) to resist microbial lipolysis and saturation in the rumen. Intact oilseeds also provide a degree of protection from biohydrogenation by microbial enzymes (Kennelly, 1996; Petit, 2001).

It has been reported (Doreau and Ferlay, 1994; Loor et al., 2005) that the process of biohydrogenation of PUFA in the rumen can be reduced with high concentrate diets causing low conversion of 18:1 *trans* isomers to 18:0. This phenomenon may be provoked by shifts in bacterial populations (Loor et al., 2005) and decreases in pH (Kalscheur et al., 1997; Loor et al., 2005). Loor et al. (2004, 2005) proposed other factors (e.g. dietary starch amount and degradation rate, buffering capacity) that contribute to accumulation of biohydrogenation intermediates in the rumen with high concentrate diets. A shift in biohydrogenation pathways due to diet can have a huge impact if the shift enhances ruminal production of 18:1 *trans* 10 and 18:2 *trans* 10, *cis* 12; which are associated with milk fat depression (Bauman and Griinari, 2003; Loor et al., 2005).

1.4.4.1 Biohydrogenation intermediates

The major tFA intermediates produced from the ruminal metabolism of linoleic acid are 18:1 *trans* 11 and *cis* 9, CLA *trans* 11. However, the dynamics of the ruminal biohydrogenation pathways allow production of a wide range of positional and geometrical FA isomers as well as modified FA such as hydroxyl and keto derivates (Jenkins et al., 2008; Harvatine et al., 2008). These compounds are absorbed and incorporated into milk fat at varying concentrations. However, production of high

concentrations of these FA can be induced only under experimental conditions involving atypical diets (Lock and Bauman, 2004; Shingfield and Griinari, 2007; Harvatine et al., 2008).

1.4.5 Effects of dietary lipids on rumen fermentation

Lipids added to ruminant diets can disrupt ruminal fermentation, causing reduced digestibility of other nutrients, especially those of non-lipid energy sources. It has been shown that ruminal digestion of structural carbohydrate can be reduced 50% or more by less than 10% added fat (Knight et al., 1978). This is accompanied by the reduced production of methane, hydrogen and volatile FA, which are the products of ruminal fermentation. Moreover, decreased ratio of acetate to propionate was observed (Ikwuegbu and Sutton, 1982). This negative effect of added fat on fermentation of structural carbohydrate can be alleviated to some extent by hindgut fermentation, but increased fibre excretion in faeces often still occurs (Palmquist and Jenkins 1980). Several mechanisms have been proposed to explain how dietary lipids interfere with ruminal fermentation. The generally accepted theories are:

- Close physical attachment of microbial matter to feed particles is necessary for cellular digestion in rumen, and physical coating of the fibre with dietary fat prevents microbial attack.
- Direct antimicrobial effects of lipids on certain microorganisms (Jenkins, 1993).

A depression in milk protein content is frequently observed with dietary lipid supplementation (Doreau and Chilliard, 1997). Wu and Hubert (1994) showed that the decrease of milk protein was attributed to a lack of increase in amino acids available to the mammary gland for protein synthesis as milk yield increased during fat supplementation. However, daily protein production may be unchanged as supplemental fat has a positive effect on milk production (Kennelly, 1996). In some cases, high dietary fat can cause decreases in both milk protein percentage and yield. This is possible because dietary fat adversely affects microbial fermentation and microbial protein yield, thereby decreasing the supply of amino acids available for absorption by the animal (Palmquist and Jenkins, 1980). However, previously Wu and

Hubert (1994) proposed four possible mechanisms to explain how dietary fat reduces milk protein concentration:

1. Glucose deficiency
2. Insulin resistance
3. Increased energetic efficiency of milk production
4. Somatothrophin deficiency

Glucose deficiency appears to be the explanation for milk protein depression observed with dietary fat supplementation. In order to maintain adequate protein supplies when fats are included in a diet, dietary protein content should be increased and consideration given to increasing the supply of fermentable carbohydrates. Even where protein supply is sufficient, glucose status may be marginal with high fat diets, due to insulin resistance and absorption requirements (Garnsworthy, 1997).

Compared with fibre and protein, ruminal digestibility of non-structure carbohydrate is less affected by dietary fat. Several studies showed normal starch digestion in the rumen of cattle that were fed additional fat even though dry matter or fibre digestibility was depressed (Jenkins, 1993). Similarly, feeding high fat diets often depresses feed and energy intakes. When cows are fed large amounts of fats which cannot be metabolized, feedback satiety signals may be generated to prevent further intake of food (Choi and Palmquist, 1996).

Detrimental effects of supplemental lipids on ruminal fermentation are affected by several factors, including the degree of unsaturation, amount and type of fat fed. Studies have demonstrated that UFA inhibit fermentation more than SFA (Jenkins, 1993; Doreau and Chilliard, 1997). The mechanism behind this phenomenon is not clear**.** Unsaturated free fatty acids (FFA) concentration in the rumen is affected by the amount and type of lipid fed and also by the rates of lipolysis, biohydrogenation, and formation of carboxylate salts. A free carboxyl group seems important for inhibition of ruminal fermentation. This is because FA derivatives, such as Ca salts of LCFA (Enjalbert, 1994), fatty alcohols, fatty acyl amides and triglycerides inhibit fermentation less than do FFA. Relevant knowledge has been used to produce ruminal inert FA products for energy supply without adversely affecting ruminal fermentation and nutrients utilization.

Compared with single fat sources, blended fat sources may have less or even no adverse effects on ruminal fermentation and utilization of nutrients. Commercial blends of animal fat and vegetable oil sometimes have little effect on fermentation, and more resemble ruminally inert fats, despite their relatively high degree of unsaturation (Jenkins, 1993).

Inclusion of FO, in the diet of ruminants, enhances the concentrations of 20:5n-3 and 22:6n-3 in milk (Offer et al., 1999; Shingfield et al., 2003; Loor et al., 2005a) and muscle (Scollan et al., 2001; Kook et al., 2002). FO is also known to inhibit the complete biohydrogenation of C18 PUFA to C18:0 resulting in an accumulation of 18:1 *trans* 11 (Shingfield et al., 2003, Lee et al., 2005, Loor et al., 2005b). This particular FA is consequently available for endogenous conversion to CLA *cis* 9, *trans* 11 via the action of stearoyl-CoA desaturase in the mammary gland (Griinari et al., 2000; Palmquist et al., 2005) and explains FO's effect in enhancing CLA *cis*-9, *trans*-11 concentration in milk (Offer et al., 1999; Chilliard et al., 2000; Shingfield et al., 2003). Enhancement of CLA in ruminant products (meat and dairy) through dietary manipulation is an important contributor towards the recommended CLA intake of 0.31 g/100 FA (Roche et al., 2001) as part of a complete diet approach (Lee et al., 2008).

1.5 Milk fat synthesis

Fatty acids incorporated into milk triglycerides are derived from two sources, uptake of preformed FA from peripheral circulation and FA synthesis in mammary secretory cells. Depending on breed, stage of lactation and diet, FA synthesis *de novo* in the bovine contributes to proportionately 0.60 on a molar basis or 0.40 by weight to total FA secretion in milk (Shingfield et al., 2010).

Mammary epithelial cells synthesize short- and medium chain FA using acetate and 3-hydroxy-butyrate as substrates. Fatty acid synthesis *de novo* accounts for all 4:0 to 12:0, most of the 14:0 (ca. 95%) and about 50% of 16:0 secreted in milk, whereas all 18 carbon and longer chain FA are thought to be derived from circulating plasma lipids (Chilliard et al., 2000). *De novo* FA synthesis has an absolute requirement for acetyl-CoA, the presence of two key enzymes, acetyl-CoA carboxylase and FA synthase and a supply of NADPH reducing equivalents (Barber et al., 1997). Both 3-hydroxy-butyrate and acetate contribute equally to the initial four carbon unit. Acetate

is converted to acetyl CoA in the cytosol and used to extend the chain length of synthesized FA via the malonyl-CoA pathway, whereas 3-hydroxy-butyrate is incorporated directly following activation to butyryl CoA. Conversion of acetate to malonyl-CoA, catalyzed by ACC, is considered to be the rate limiting step (Bauman and Davis, 1974).

1.5.1 Milk fat depression (MFD)

Decreases in milk fat synthesis are common in cows fed diets containing: high proportions of concentrates, FO and marine algal lipids or ionophores (Bauman and Griinari, 2001 and 2003). Even though certain attributes of a diet are important in the establishment of MFD, there is considerable interaction between the composition of the basal diet, amount and source of dietary lipid supplement and feeding frequency (Chilliard et al., 2007; Shingfield and Griinari, 2007).

A number of theories have been proposed to explain diet induced MFD. The major theories have attributed the decreases in milk fat synthesis during diet-induced MFD to:

1. Reductions in the supply of acetate and 3-hydroxy-butyrate for *de novo* FA synthesis in the mammary gland
2. Elevated insulin secretion stimulating the preferential partitioning of FA towards adipose tissue at the expense of the mammary gland
3. Direct inhibition of mammary lipogenesis by tFA formed during the biohydrogenation of dietary UFA in the rumen (Shingfield et al., 2010).

Of all the hypotheses developed to explain diet-induced MFD, the biohydrogenation theory appears to be the most robust and offers a more convincing explanation for MFD over a wider range of diets (Shingfield et al., 2010). However, increases in ruminal outflow of CLA *trans*-10, *cis*-12, do not explain the decreases in milk fat synthesis in all cases of diet-induced MFD, with the implication that additional biohydrogenation intermediates and/or mechanisms must be involved (Shingfield and Griinari, 2007). Milk fat depressing diets (high concentrate, high starch, PUFA, and pasture sometimes) also alter ruminal environment (decrease in pH with high concentrate) and fermentation products (increased propionate production). MFD diets,

thus, lead to a simultaneous increase of numerous FA isomers and other energetic nutrients, complicating the elucidation of related mechanisms (Glasser et al., 2010).

1.6 Lipid transport

1.6.1 Plasma lipoproteins

The plasma lipoproteins are soluble complexes of lipids (triacylglycerols-TAG, phospholipids, cholesterol esters and cholesterol) with specialized proteins (apolipoproteins) (Vance and Vance, 1991). The main function of these lipoproteins is to transport water insoluble lipids throughout the body. Lipoproteins deliver lipid to cells efficiently. This efficiency is due to the fact that dispersion of lipid by exchange or diffusion is very low. The delivery of lipid by lipoproteins is also very specific when required (Vance and Vance, 1991). The main fluid space through which the lipoproteins circulate is the blood plasma, but many lipoproteins can filter through the vascular wall to interact with parenchymal cells. Liver and intestine are the major sites of lipoprotein synthesis and the major flow of lipids is from tissues capable of lipid synthesis to tissues which make little of their own lipid. There are also important reverse transport systems in which lipid from peripheral tissues is returned to the liver for catabolism and recirculation.

1.6.2 Lipoprotein structure

The major lipoprotein particles are (Small and Shipley, 1974). The composition of these lipoprotein types are summarized in Table 1.3:

- Chylomicrons
- Very low density lipoprotein (VLDL)
- Intermediate density lipoprotein (IDL)
- Low density lipoprotein (LDL)
- High density lipoprotein (HDL)

Table 1.3 Composition of blood lipoproteins

Lipoprotein class[a]	Density (g/ml)	Composition (%)			
		Protein	Triglyceride	Phospholipid	Cholesterol
Chylomicrons	<0.94	1-2	85-95	3-6	3-7
VLDL (β-lipoprotein)	0.94-1.006	6-10	50-65	15-20	20-30
LDL (β-lipoprotein)	1.006-1.063	18-22	4-8	18-24	51-58
HDL (α-lipoprotein)	1.063-1.21	45-55	2-7	26-32	18-25

[a]VLDL denotes very-low-density lipoprotein, LDL low-density lipoprotein, and HDL high-density lipoprotein (From: Church and Pond, 1988).

1.6.3 Chylomicrons

Dietary lipid, consisting mainly of TAG and phospholipids, are hydrolysed in the intestinal lumen, absorbed by the intestinal enterocyte, re-esterified into TAG and package into TAG-rich chylomicron particles. Chylomicrons principally, transport exogenous FA from the intestine to peripheral tissues and the liver but they also transport both dietary and biliary cholesterol (Colowick and Kaplan, 1986).

1.6.4 Very low density lipoprotein

VLDL primarily transports endogenous TAG. The liver produces and secretes TAG-rich VLDL particles that contain apoB_{100}, apoC_I, C_{II} and C_{III}, and apoE. Plasma VLDL particles also receive additional cholesterol esters from HDL by the action of cholesterol ester transfer proteins which acts to transfer cholesterol esters to LDL. VLDL-TAG can also undergo hydrolysis by endothelial cell lipoprotein lipase to produce a VLDL remnant. This remnant particle, termed IDL, can undergo further loss of TAG and apolipoproteins to form LDL which can be taken up via its apoB_{100} fraction (Fielding and Fielding, 1991).

1.6.5 Low density lipoprotein

Lipolysis of VLDL by lipoprotein lipase produces a range of particles of intermediate density (IDL) with different amounts of TAG. These particles are rich in apoE and act

at an important point in lipoprotein metabolism because they may be cleared from the circulation by the liver or they may be further processed to become LDL (Jackson et al., 1976).

1.6.6 High density lipoprotein

HDL is formed in the circulation from apolipoproteins produced by the liver and intestine. $apoA_I$ combines with unesterified cholesterol and phospholipid to form disk shaped particles before undergoing modification to become HDL3 and the HDL2 as they acquire cholesterol ester and other apolipoproteins including $apoA_{II}$, apoE and apoCs (Tall, 1990). The hydrolysis of chylomicrons and VLDL by lipoprotein lipases results in a further net transfer of phospholipids and unesterified cholesterol to HDL (Tall, 1990).

1.6.7 Lipoprotein isolation

Different methods can be used for lipoprotein isolation including; gel filtration, precipitation or antibody affinity columns. The most frequent technique used is the sequential flotation ultracentrifugation because plasma lipoproteins have lower hydrated densities relative to the other plasma proteins (Ray et al., 1954). Sequential centrifugation permits the process of large volumes or many small samples. However, during lipoprotein separation by this method, centrifugation times are often 18 h and greater, and consequently exchanges or transfers including losses of apolipoproteins from the lipoprotein surface may occur (Ray et al., 1954).

1.7 Fatty acids and human health effects

1.7.1 Saturated fatty acids (SFA) in human foodstuffs

A SFA is an aliphatic carboxylic acid with no double bonds between any of its carbon atoms. Table 1.4, provides systemic names, shorthand notations, trivial names and dietary sources of most common and nutritionally important SFA. According to the Food Standards Agency (FSA, 2010) SFA can be found in dairy products, meat, pastries, coconut and palm oils and most British people consume 20% more than the recommended maximum amount (20-30 g/d) of SFA.

Table 1.4 Typical saturated fatty acids in foods

Systemic name	Shorthand notation	Trivial name	Major sources
Tetranoic	4:0	Butyric	Butter
Hexanoic	6:0	Caproic	Butter
Octanoic	8:0	Caprylic	Coconut
Decanoic	10:0	Capric	Most milk fats
Dodecanoic	12:0	Lauric	Palm kernel, coconut
Tetradecanoic	14:0	Myristic	Palm kernel, coconut
Hexadecanoic	16:0	Palmitic	Palm
Octadecanoic	18:0	Stearic	Most animal fats, cocoa
Eicosanoic	20:0	Arachidic	Peanut
Docosanoic	22:0	Behenic	Seeds
Tetracosanoic	24:0	Lignoceric	Peanut

From: Perkins, 1991; Food and Drug Administration, US, 2009; Food Standards Agency, UK, 2010.

1.7.2 Unsaturated fatty acids (UFA) in human foodstuffs

Naturally occurring UFA are mostly in the *cis* configuration. Table 1.5, provides information for common UFA available in food sources. According to the FSA (2010) foods rich in UFA include fish oil (e.g., from mackerel, salmon and trout), sardines, avocados, nuts and seeds, and sunflower, rapeseed, and olive oils.

Table 1.5 Unsaturated fatty acids in foods

Systemic name	Shorthand notation	Trivial name	Major sources
Monounsaturated			
Cis			
9-Tetradecanoic	14:1n5	Myristoleic	Butter
9-Hexadecanoic	16:1n7	Palmitoleic	Seafood, beef
9-Octadecanoic	18:1n9	Oleic	Olive, Canola
11-Octadecanoic	18:1n7	Cis-Vaccenic	Seafood
13-Docosenoic	22:1n9	Eruic	Rapeseed
Trans			
9-Octadecanoic	*Trans*-18:1n9	Elaidic	Hydrogenated fats
11-Octadecanoic	*Trans*-18:1n7	Vaccenic	Hydrogenated fats, Butter
Polyunsaturated			
All Cis			
9,12-Octadienoic	18:2n6	Linoleic	Sunflower, safflower
6,9,12-Octadecatrienoic	18:3n6	γ-Linolenic	Primrose
8,11,14-Eicosatrienoic	20:3n6	dihomo-γ-linoleic	Shark liver
5,8,11,14-Eicosatetraenoic	20:4n6	Arachidonic	Eggs, most animal fats
9,12,15-Octatrienoic	18:3n3	Linolenic	Soybean, canola
5,8,11,14,17-Eicosapentaenoic	20:5n3	Timnodonic	Seafood
7,10,13,16,19-Docosapentaenoic	22:5n3	Clupadonic	Seafood
4,7,10,13,16,19-Docosahexaenoic	22:6n3	Cervonic	Seafood

From: Perkins, 1991; Food and Drug Administration, US, 2009; Food Standards Agency, UK, 2010.

1.7.3 *Trans* fatty acids in human foodstuffs

The most important sources of tFA in the human diet are PHVO, and ruminant milk and meat products (Salter et al., 2006). *Trans* fats can be found in many of the same foods as saturated fat, such as vegetable shortenings, some margarines, crackers, candies, cookies, snack foods, fried foods, baked goods, and other processed foods made with partially hydrogenated vegetable oils (FDA, 2006). According to Hulshof et al., 1999, in many European countries about 50% of tFA come from dairy fat. Ruminant derived lipids often contain 1 to 8% of total FA as tFA (Craig-Schmidt

1998; Salter et al., 2006), and those are predominantly 18:1 *trans* (Emken, 1995; Salter et al., 2006). The most common 18:1 *trans* in ruminant fat is vaccenic acid, accounting for 60% to 80% of total tFA (Emken, 1995; Craig-Schmidt 1998; Salter et al., 2006). tFA from ruminant tFA (vaccenic acid) can be converted to CLA *cis* 9, *trans* 11 (rumenic acid; RA), through the action of stearoyl coenzymeA desaturase. In European countries such as Denmark and Switzerland, legislation limited the use of industrially-produced oils to no more than 2% of fat in human food products (Salter et al., 2006). In 2007, the Food Standards Agency (2010), along with the Scientific Advisory Committee on Nutrition (SACN) recommended that average tFA intake should not exceed 2% of food energy. However, average intakes of tFA in the UK are half the recommended maximum of 2 of total food energy and therefore are not a cause for concern (FSA, 2010).

1.7.4 Dietary fatty acids and blood lipoprotein and cholesterol levels

Absorption of fat after a meal is associated with a large increase in lipid concentration of the blood, referred to as lipidaemia (Helfant and Banka, 1978). Blood lipids consist of dietary lipids absorbed from the intestine, as well as lipids mobilized from depot stores and from synthesis in body tissues, especially the liver and adipose tissues. Diet is the primary source of cholesterol to the body. Cholesterol is also synthesized in many tissues of the body including the liver (Helfant and Banka, 1978). According to the National Health Service (NHS) in England, two out of three adults have a total cholesterol level of 5mmol/l or above. On average, men have a cholesterol level of 5.5 mmol/l and women have a level of 5.6 mmol/l. The UK Government recommends that cholesterol levels should not exceed 5 mmol/l (NHS, 2009).

Diets rich in SFA are believed to increase plasma cholesterol levels (Spady et al., 1993) by increasing blood concentration of LDL which contains about 51 to 58% cholesterol (Church and Pond, 1988). This condition is known as hypercholesterolemia. For example, a meta-analysis of human dietary studies (Mensink et al., 2003) suggested that lauric (12:0), myristic (14:0) and palmitic (16:0) acids each increased HDL fraction more than UFA did but the effect on the LDL fraction was greater. Numerous controlled feeding studies of the effects of different dietary FA on serum cholesterol levels have been summarized in meta-analyses from which predictive equations have been developed (Keys et al., 1966; Yu et al., 1995).

Those studies confirmed that SFA increase and PUFA decrease in the LDL fraction. All three classes of FA (SFA, MUFA and PUFA) elevate HDL fraction when they replace carbohydrates in the diet, and this effect is slightly greater with SFA (Hu and Willet, 2002). Also, TAG levels increase when dietary FA are replaced by carbohydrates. Replacement of saturated fat with carbohydrates proportionally reduces both LDL and HDL fractions; this change in diet would be expected to have minimal benefit on coronary heart disease risk. When MUFA or PUFA replace saturated fat, LDL fraction decreases and HDL fraction changes only slightly (Hu and Willet, 2002).

1.7.4.1 Effects of individual fatty acids on blood cholesterol level

There is agreement that certain, but not all, SFA raise plasma levels of LDL-cholesterol. Short chain fatty acids (butyric acid, caproic acid, caprylic acid, and capric acid), present in a small number of food sources, do not appear to raise LDL-cholesterol levels (Berner, 1993). Medium-chain SFA, (lauric acid, myristic acid, and palmitic acid) are generally considered to raise serum total and LDL-cholesterol (Keys et al., 1965; Hegsted et al., 1965). These three FA account for approximately 41% of milk fat (Berner, 1993). It has been suggested that 14:0 is more hypercholesterolemic than 16:0 (Zock et. al., 1994). The other 59% of milk FA are not hypercholesterolemic compared with medium-chain FA. These FA are short-chain FA, stearic acid (18:0), MUFA, PUFA, and traces of others (Berner, 1993).

1.7.5 Health effects related to milk *trans* fatty acids

Although different studies have reported negative effects of tFA on human health, it is important to take into account that the main sources (PHVOs and milk/meat products from ruminant animals) of dietary tFA differ substantially in the isomer distribution of 18:1 *trans*. Also, there is reasonable evidence that tFA associated with PHVO increase LDL-cholesterol, possibly decrease HDL-cholesterol and increase risk of cardiovascular disease (Salter et al., 2006). The impact of ruminant tFA is much less certain and it may actually be beneficial as it is mainly vaccenic acid (18:1 *trans*-11) that can be converted to CLA (Salter et al., 2006).

In the early 1990s the adverse effects of consuming tFA on blood lipid levels (Mensink and Katan, 1990; Ascherio et al., 1999) and coronary heart disease (Willet and Ascherio, 1994; Ascherio et al., 1999) were identified. There is also evidence that

tFA are also implicated in increasing risk of cardiovascular disease to an extent equal to or greater than the hypercholesterolemic saturated FA (Judd et al., 2002; Baer et al., 2004). In 1994, the UK Department of Health recommended to reduce total fat consumption to 30% of total energy intake, with 10% of energy intake coming from saturated FA (Wood et al., 2003). At the same time it was also advised to increase the intake of PUFA. Besides the health benefits from PUFA for human health, CLA isomers have received much attention due to their health promoting effects (Jahreis et al., 2000; Connor, 2000; Pariza et al., 2000, 2001; Roche et al., 2001; Williams, 2000; Raes et al., 2004).

Despite the fact that tFA have been regarded and implicated as a causal factor of heart diseases, Ackman (2000) questioned this theory and explained that humans have consumed tFA in milk fat throughout history since ruminants were domesticated and their products consumed, and hence human metabolism may have adapted positively to their presence (Ackman, 2000; Jensen, 2002). Nevertheless, potential negative effects on animal health have been observed (Teter et al., 1990; Jensen, 2002). Consumption of tFA by humans (Table 1.6) was estimated by Craig-Schmidt (1998) and Allison et al. (1999); however, Jensen (2002) observed that in both investigations consumption was underestimated when based on data obtained only with capillary GLC columns. Additionally, Jensen (2002) criticized the research conducted by Katan (2000) in which tFA were implicated as a cause of cardiovascular disease; Jensen (2002) mentioned that milk fat is a minor source of tFA and hence it should not be excluded from human diets for this reason.

Table 1.6 Quantities of trans-18:1 in butter and milk fat samples determined by GLC with capillary columns and corrected by analyses with the argentation (Ag)-TLC/GLC method

Source of lipids	Method of analysis n	Capillary only	Ag-TLC/GLC	Correction factors	Reference
France[1]	60	2.00	3.30	1.65	Wolff et al. (1995)
France[2]	24	1.82	3.22	1.77	Wolff (1994)
France[3]	24	2.78	4.28	1.54	Wolff (1994)
Germany[4]	1756	2.47	3.83	1.55	Precht and Molkentin (1996)
Germany[5]	927	1.53	2.65	1.73	Precht and Molkentin (1997)
Germany[6]	236	2.42	3.80	1.57	Precht and Molkentin (1997)
Germany[7]	593	3.53	5.08	1.44	Precht and Molkentin (1997)
Germany[8]	58	2.92	4.35	1.49	Precht and Molkentin (1997)

Adaptated from Jensen, 2002
[1]Butter samples obtained throughout year, [2]autumn butter, [3]spring butter, [4]milk from different seasons, [5]milk from barn fed cows (winter), [6]milk from winter to summer, [7]milk from pasture fed cows (summer), [8]slight energy deficit.

1.7.6 Blood cholesterol level and atherosclerosis

Atherosclerosis is a generalized, chronic, inflammatory vessel disorder and leading to cardiovascular diseases like myocardial infarction and stroke (Nordestgaard and Zacho, 2009). The inflammation is initiated by entrance of cholesterol-rich lipoproteins into in the vessel wall of arteries, and afterwards macrophages absorb these and are transformed into foam cells (Nordestgaard and Zacho, 2009).

Atherosclerosis is the condition of thickening and losing elasticity of the arterial wall, and narrowing of the arterial lumen due to building up of cholesterol and other lipids on the arterial wall. Cholesterol released into blood in the form of LDL can infiltrate arterial walls. At the sites where LDL-cholesterol infiltrated the arterial wall, abnormal smooth muscle cells are multiplied and connective tissue components are increased (Cancel and Tarbell, 2010). Cholesterol accumulates in cells and in extra-cellular spaces of the wall endothelial lining. Calcium salts are deposited on top of the

lipids, and a fibrous net is formed over the mass. This atherosclerotic plaque projects into the arterial lumen (Cancel and Tarbell, 2010).

Dietary tFA have been linked to atherosclerosis but the mechanisms by which they cause the disease remains unclear. Chen et al. (2010) found that dietary tFA cause atherosclerosis by the increase deposition of cholesterol into cellular plasma membranes in vascular tissue as in hypercholesterolemia. This is in agreement with previous research (Lichtenstein et al., 2003; Vega-López et al., 2009) in which the major risk factor of cardiovascular disease caused by dietary tFA is LDL cholesterol levels and total cholesterol/HDL ratios in plasma.

1.7.7 Blood cholesterol level and stroke development

Increased blood cholesterol levels are related to an increased risk of coronary heart disease (CHD) (Imamura et al., 2009). In contrast, the relation between total cholesterol levels and the risk of stroke remains unclear because of conflicting results reported in the literature. The inconsistent results may be due to several reasons. First, because stroke is a heterogeneous syndrome of different etiologic origins, lipid levels may be important for some subtypes of stroke but not for others. An inverse association has been observed between total cholesterol and haemorrhagic stroke, and there is a positive association between total cholesterol and ischemic stroke (Imamura et al., 2009). Furthermore, the association may be different for ischemic stroke subtypes. Second, lipoprotein subfractions are considered to exert varying influence on stroke risk. It is possible that the protective effect of HDL cholesterol against stroke weakens the positive association between total cholesterol and stroke in instances where lipoprotein sub fractions are counted together (Imamura et al., 2009).

The stroke-cholesterol relation is complex and contains several paradoxes. Whereas total cholesterol is a well-documented risk factor for CHD, evidence of a relation between cholesterol and risk of stroke is inconsistent (Olsen et al., 2007). In search of an explanation for this paradox, it has been noted that most large-scale studies on cholesterol and stroke risk have not differentiated between ischemic and hemorrhagic stroke, nor did they differentiate among various subtypes of ischemic stroke. It has been suggested that cholesterol may increase the risk of only certain types of infarcts and that LDL predisposes to hemorrhagic stroke, thus attenuating an association between cholesterol and all stroke (Olsen et al., 2007).

1.7.8 Blood cholesterol level and coronary heart disease

Over the past decades, numerous population-based and intervention studies have identified LDL cholesterol as a key risk factor for CHD. However, although many trials have documented the benefits of lowering plasma LDL levels for primary and secondary prevention of CHD, studies have shown that individuals reaching their LDL target may still be at increased CHD risk if they have detrimental levels of other parameters of the lipoprotein-lipid profile. In this regard, it has been proposed (Kastelein et al., 2008) that other lipid parameters such as cholesterol levels in lipoproteins other than HDL (i.e., non–HDL cholesterol levels), TAG levels, or the total cholesterol (TC) to HDL- cholesterol ratio could better predict cardiovascular outcomes in patients on LDL- cholesterol lowering therapy. However, there is a lack of epidemiological data to suggest that the aforementioned parameters of the lipoprotein-lipid profile could better predict CHD risk than LDL- cholesterol in asymptomatic individuals (Arsenault et al., 2010). Death rates of CHD in both the United Kingdom and the Unites States have declined over the last 40 years, particularly in men. The reasons for these rates are not fully understood. However, they appear to be a consequence of a combination of improved treatment of individuals and changes in risk factor within the whole population. In England and Wales, it has been estimated that 42% of the decrease seen between 1981 and 2000 was due to improved treatment whereas the 58% remaining was due to risk factor conditions within the population (Unal et al., 2004; Salter et al., 2007). Of the risk factors, by far the biggest impact (48% of overall deaths) was from reduction in smoking, with lesser effects of reduced blood pressure (9.5%) and reduced cholesterol (9.6%) (Salter et al., 2007).

For prevention and treatment of CHD, vigorous dietary intervention is needed to lower serum cholesterol level by at least 6% (Temple, 1996). For this purpose, the foods of animal origin should be eaten sparingly, and more prominence should be given to the foods rich in carbohydrate and fibre, especially fruit and vegetables. If acceptability of the diet becomes a problem and people desire more fat in their diet, then the UFA rich oils such as olive or canola could be incorporated in the diet (Temple, 1996). Similarly, Connor and Connor (1997) suggested a prominent therapeutic role for FO in prevention and treatment of coronary artery disease. The n-3 FA of fish and FO have great potential for prevention and treatment of patients with

coronary artery disease. That is because n-3 FA promote synthesis of beneficial nitric oxide in the endothelium (Connor and Connor, 1997). FO has a profound hypolipidemic effect in humans, especially lowering of plasma triacylglycerol. Both very-low-density lipoprotein production and apolipoprotein B synthesis are inhibited by FO. Finally, FO has a mild blood pressure-lowering effect in both normal and mildly hypertensive individuals.

On the other hand, Hu and Willet (2002), reported that there is substantial evidence indicating that diets using non hydrogenated UFA as the predominant form of dietary fat, whole grains as the main form of carbohydrates, an abundance of fruits and vegetables, and adequate omega-3 fatty acids can offer significant protection against CHD.

1.8 Using mammary gland cell lines to study fatty acid uptake

Lipids in milk are present in fat globules; they are formed mainly by triacygycerols and are enclosed within a membrane derived from the secretory epithelial mammary cell (Jensen, 2002; Heid and Keenan, 2005). Although the general principles of lipid droplet formation, growth, movement, and secretion are known, practically few studies have been performed on lipid absorption and secretion by mammary gland cells (Ernens et al., 2007).

Milk fat, normally produced, is assembled in the mammary alveolar cells from FA derived partly by *de novo* synthesis from acetate within these specialized cells, and partly by absorption from the blood. Fatty acids synthesised within alveolar cells are mostly saturated and contain from four to sixteen carbon atoms per molecule (Kinsella et al., 1968). Lipids produced by the lactating bovine mammary cell are distinctive, which facilitates the assessment of the biochemical fidelity of these cells when used *in vitro* (Kinsella et al., 1968).

1.8.1 Immortalized bovine mammary epithelial cell line: MAC-T

Mac-T cells are an established cloned cell line produced from primary bovine epithelial cells by transfection with SV-40 large-T-antigen designated (MAC-T). Differentiation was induced (Turner et al., 1993) by increasing cell to cell interactions on a floating extra cellular matrix in the presence of prolactin. MAC-T cells have normal physiological responses in that it produces milk constituents which comprises

α- and β- casein and lactose. These cells provide a method of studying *in vitro* lactation due to their normal physiological responses. They also provide a method of *in vitro* screening for gene expression in transgenic cows prior to gene transfer when the mammary gland is the target organ of expression and hence reduce the cost of genetic engineering (Zavizion et al., 1995). This cell line has been deposited at the American Type Culture Collection (ATTC) under the accession number CRL 10274. The MAC-T cells were reported to have >150 passages without senescence or replication problems (Turner et al., 1993), which represents continuous culture for more than one year. These cells have a "cobblestone" morphological characteristic when they reach 90-100% confluence. They are also heterogeneous in morphology, growth, and cytogenetic characteristics (Zavizion et al., 1995).

MAC-T cells have been used for gene expression studies (Keating et al., 2008; Kadegowda et al., 2009; Thering et al., 2009) that evaluated *in vitro* effects of certain FA on mammary epithelial cell growth, morphological changes and apoptosis, as well as effects of hormones (insulin and prolactin) on metabolic regulation of TAG biosynthesis (Morand et al., 1998). Johnson et al., (2010) reported changes in TAG, cholesterol and phospholipids distribution in MAC-T cells stimulated with 10 ng / ml of growth hormone. These cells have also been studied for their response to bacterial virulent factors produced by mastitis pathogens. Matthews et al. (1994) reported that MAC-T cells proliferation was inhibited by different concentrations of *Escherichia coli* endotoxin.

Peterson et al. (2004) used this cell line to study the effects of CLA *trans* 10, *cis* 12 on milk fat depression and abundance of mRNA for lipogenic enzymes. The study reported that lipogenesis was inhibited by 56% when cells were incubated with 75 μmol / l of CLA *trans* 10, *cis* 12. Keating et al. (2008) reported that increasing concentrations of CLA isomers *trans* 10, *cis* 12 and *cis* 9, *trans* 11 have negative impacts on cell growth inducing apoptosis of the mammary cells. They observed that at higher doses of CLA (35 μM and above) cell growth was inhibited or in some cases cells were no longer available. Those findings are in agreement with the negative effects seen on milk yield and composition when cows are abomasally infused with high doses of CLA (Bell and Kennelly, 2003). Sorensen et al., (2008) treated MAC-T cells with CLA and other C18-UFA to study effects on lipogenic enzymes. They

concluded that MFD caused by CLA *trans* 10, *cis* 12 could affect a number of lipogenic enzymes including diacylglycerol acyltransferase. Jayan and Herbein (2000) studied the effects of oleic and vaccenic acids on the activity of acetyl-CoA carboxylase-ACC, fatty acid synthetase –FAS and stearoyl-CoA desaturase- SCD. Their data suggested that vaccenic acid inhibited activity of ACC and FAS, which are responsible for synthesis of SFA secreted into milk, and simultaneously enhanced SCD activity.

Other studies used (Yonezawa et al., 2004a, 2004b 2009) cloned mammary gland epithelial cells (bMEC) and worked on the effects of FA on the expression of certain enzymes involved in lipid synthesis. Yonezawa et al., (2004a) measured the effect of LCFA on TAG accumulation on bMEC cells treated with 400 μM palmitate, stearate, oleate and linolate for 24 h and TAG accumulation on bMEC cells treated with the same FA at different concentrations. Their results suggested that exogenous LCFA could enhance accumulation of cytosolic TAG and expression of CD 36 (a FA traslocase) which plays an important role in FA translocation in various cells and plays a positive role in uptake of LCFA in bMEC (Figure 1.3). Yonezawa et al. (2004b) evaluated the effect of 10 mM of acetate, butyrate and octanoate on bMEC for 1, 3, 5 and 7 d and various concentrations of octanoate for 7 d. Octanoate induced the accumulation of cytosolic TAG and formation of lipid droplets in bMEC (Figure 1.4).

Most of the studies using Mac-T cells focus on gene expression and enzymatic responses; hence few data are available on FA uptake and secretion.

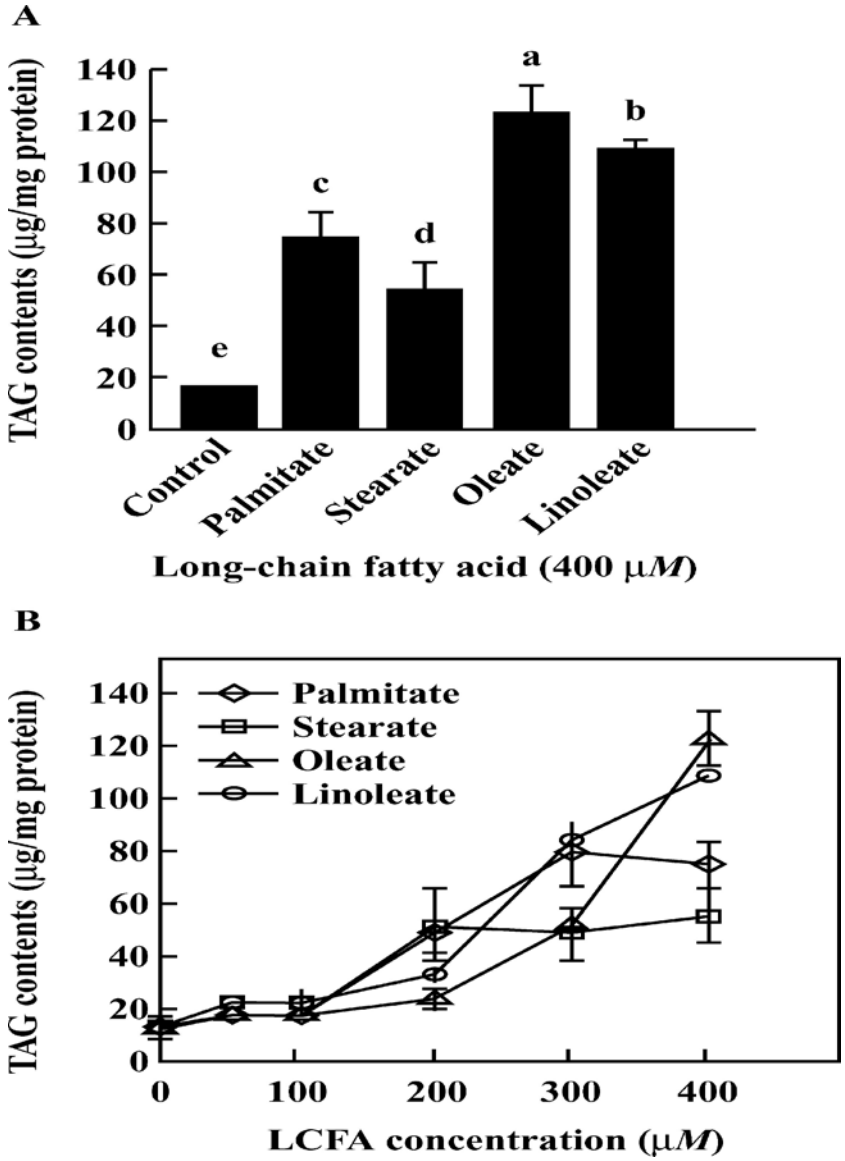


Figure 1.3 A) The bMEC were treated with 400 μM palmitate, stearate, oleate, or linoleate for 24 h. Values with different letters are statistically different ($P < 0.05$). B) The bMEC were treated with various concentrations of palmitate, stearate, oleate, or linoleate complex for 24 h. The data are expressed as means ± SEM (n = 3) (From Yonezawa et al., 2004a).

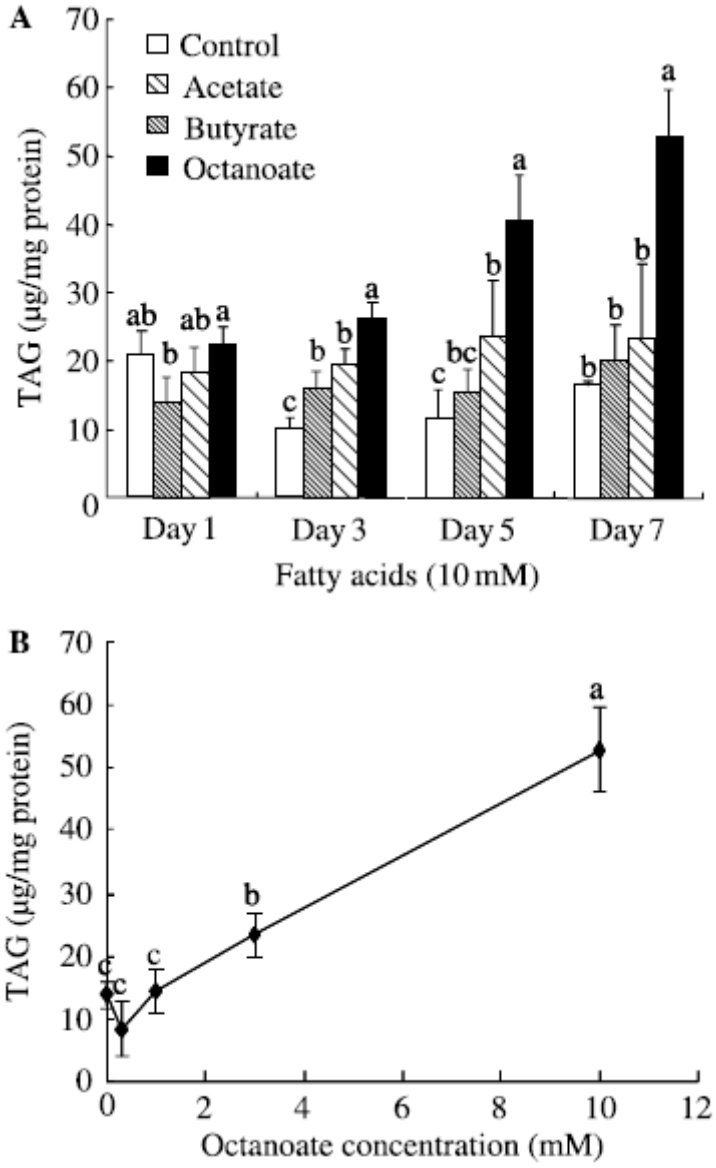


Figure 1.4 A) The bMEC were treated with DMEM containing 10% FCS plus 10 mM-acetate, butyrate, or octanoate for 1, 3, 5 or 7 d, respectively. Values with different letters are statistically different among the columns on the same day ($P < 0.05$). B) bMEC were treated with various concentrations of octanoate for 7 d. Results are expressed as means ± SEM of triplicate dishes (From Yonezawa et al., 2004b).

1.8.2 Fatty acid transport proteins

Fatty acid transport proteins (FATP) are a family of proteins involved in fatty acid uptake and activation. They were identified through their ability to increase fatty acid uptake when over expressed, and all mammalian FATP have been reported to increase FA uptake upon overexpression (Stahl, 2004; Doege and Stahl, 2006; Gimeno, 2007). Studies *in vitro* showed that FATP have both FA uptake and enzymatic activity with distinct subcellular localization, substrate specificities and kinetic properties for individual FATP family members (Gimeno, 2007).

1.8.3 Fatty acid transport across cellular membranes

The mechanism by which FA enter cells has been a matter of debate. Fatty acid must be extracted from the internal face of the plasma membrane and move into the interior of the cell; without extraction, FA would remain available in the membrane to move back to the outer face of the plasma membrane and be recaptured by albumin present in the interstitial fluid (Mashek and Coleman et al., 2006). There are different approaches (Abumrad et al., 1998; Kleinfeld, 2000) that provide evidence that there are two processes involved in LCFA uptake:

1. Passive diffusion through the lipid bilayer
2. Protein facilitated transport

The relative contribution of each component depends on concentrations and molar ratios of FA and albumin in the circulation (Hajri and Abumrad, 2002).

1.8.4 Determinants of fatty acid transport

In the extracellular medium and in the circulation, FA are carried bound to albumin. In the absence of albumin or other FA binding protein (FABP), the concentration of LCFA that can be used is very low and is depleted by cells automatically, meaning the linear portion of the uptake time course would be too short to measure (Hajri and Abumrad, 2002). When mixtures of FA and albumin are used, uptake follows the unbound FA, which is determined by the molar ratio of FA to albumin. Within the cell, one or more types of abundant cytosolic FABP buffer intracellular FA. There is an important role for cytosolic FABP in shuttling FA between plasma membrane and intracellular membranes or metabolic compartments (Hajri and Abumrad, 2002).

1.9 Conclusions

This review has summarised the background of some aspects of lipid structure, milk fat composition and FA metabolism in dairy cows. The review has also studied the role of dietary FA on human health.

However, we do not know how tFA are transported in the blood and partitioned into lipoprotein fractions of dairy cows. Moreover, we do not know how dietary lipids affect lipoprotein fractions in dairy cows and whether those fractions lead to differences in tFA concentrations. We also do not know how certain FA concentrations perform within the bovine mammary gland cells.

All of these questions will help us to understand the regulation of milk fat synthesis that is central to the development of nutritional strategies to enhance the nutritional value of milk in ruminants.

1.10 General objectives

In summary, this thesis aims to:

- Compare plasma cholesterol fractions and FA profiles from cows subjected to different treatments
- Examine the effects of dietary lipids on the transportation of tFA among the plasma cholesterol fractions
- Study LCFA uptake in epithelial mammary gland cell cultures on MAC-T cells line
- Evaluate the effects of lipids on the transportation of FA in epithelial mammary gland cell cultures (MAC-T cells)

C h a p t e r 2

Ruminal pulses of fatty acids in dairy cows: 1. Soya oil and partially hydrogenated vegetable oil

CHAPTER 2

2. Ruminal pulses of fatty acids in dairy cows: 1. Soya oil and partially hydrogenated vegetable oil

2.1 Introduction

In ruminants, tFA are produced naturally during biohydrogenation of PUFA in the rumen and are incorporated into carcass fat or milk fat (Katz and Keeney, 1966; Griinari and Bauman, 1999; Piperova et al., 2004). The predominant tFA in ruminant fat is vaccenic acid (VA; C18:1 *trans*-11), accounting for 60% to 80% of total tFA, although other tFA (e.g. C18:1 *trans-10*) occur when high-fat or high-concentrate diets are fed to ruminants. PHVO contains a mixture of C18:1 *trans* isomers, the most predominant being elaidic acid (EA; C18:1 *trans-9*) (Meijer et al., 2001). Vaccenic acid may not have the same deleterious health effects in humans as tFA from PHVO (Salter et al., 2006).

Cholesterol is important for the transport and clearance of fatty acids in all mammals, and also plays vital roles in cell membrane structure and steroid hormone synthesis. Although proportions of low- and high- density lipoproteins (LDL and HDL) have been studied extensively in humans, they have received little attention in ruminants. The mammary gland appears to have differential uptake of lipids from lipoprotein fractions and affect the efficiency of transfer LCFA from diet to milk (Offer et al., 2001). No *in vivo* study has been published in which effects of different tFA on lipoprotein fractions or their FA profile have been compared in ruminants. We were particularly interested to see if there are different effects of VA (naturally produced in the rumen) and EA (from PHVO).

The main hypothesis being tested in this experiment was that FA profiles of plasma and lipoprotein fractions would be different depending on source of the dietary lipids, which would lead to differences in tFA concentrations. The objective of the present study was to elucidate which lipoprotein fractions are involved in tFA transport and whether there is a difference between 18:1 tFA isomers. This objective was achieved by comparing plasma lipoprotein fractions in cows subjected to ruminal pulses of soya oil (SO; to induce high VA production and absorption) or PHVO with high concentrations of a range of 18:1 tFA isomers.

2.2 Materials and methods

2.2.1 Animals and treatments

Three non-lactating Holstein nuliparous cows [BW 773 ± 63 kg (average ± SD)], each with a rumen cannula were utilized in a 3 x 3 Latin square design with 3 d treatment periods (adapted and modified from Kadegowda et al., 2008 and Castaneda-Gutierrez et al., 2007) followed by 4 d washout interval between treatments to minimize carryover effects (Loor et al., 2005) (Figure 2.1). Cows were treated with ruminal pulses of: 1) skim milk (SM; 500 ml/d) as control; 2) SO (250 g/d in 500 ml/d of SM) and 3) PHVO (250 g/d in 500 ml/d of SM). PHVO was supplied by the manufacturers (Aarhus Karlshamn, Ltd., Hull, UK) and was based on hydrogenated rapeseed oil. Quantities of fat and SM in ruminal pulses were calculated according to previous studies (Ferlay and Doreau, 1992).

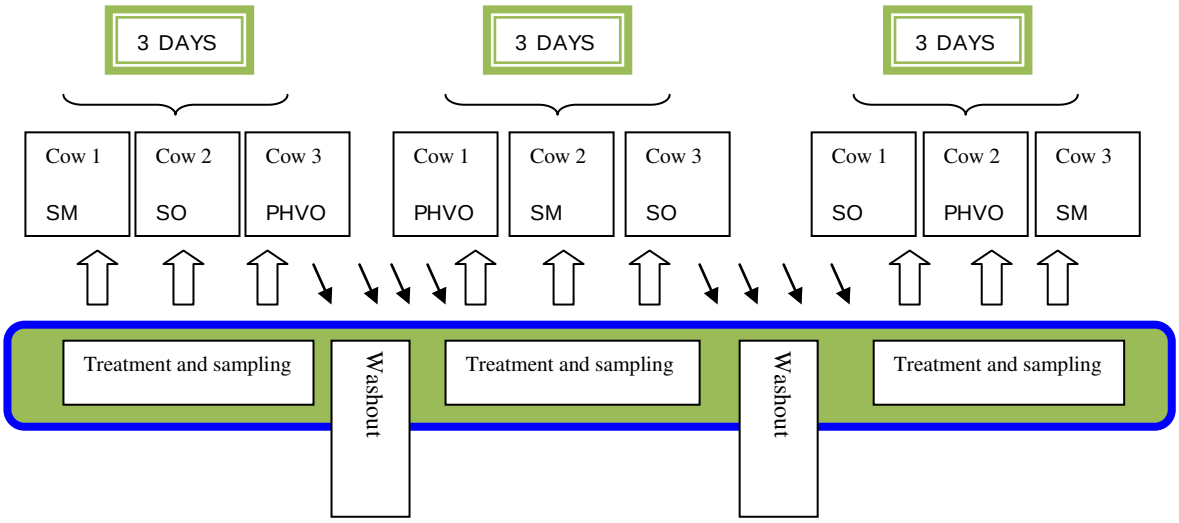


Figure 2.1: The experiment had three treatment periods, each followed by a 4-d washout interval. Cows were given each treatment in a 3 x 3 Latin square design.

Emulsions were prepared the day before each pulse. To provide a homogenised release of pulses, skim milk was used (Loor and Herbein, 2003) (500 ml/d) to emulsify the oils (SO and PHVO) using a laboratory mixer emulsifier (L2R Silverson, Machines Ltd., Waterside, Chesham, Bucks). Before each pulse, emulsions were heated for 30 min at 60 ºC in water bath (Grant Y28, Cambridge Ltd., England) to provide easier diffusion of PHVO. During pulses and sampling periods, cows were housed in tie stalls and had free access to water. Cows were fed a maintenance diet which comprised meadow hay (*ad libitum*) and a concentrate based on barley (25-40 g/kg), sugar beet pulp (8 g/kg) and wheat (8 g/kg) and cane molasses (<5 g/kg),

limestone flour (<5 g/kg), vitamins (<5 g/kg) and minerals (<5 g/kg) [1-2 kg/day at 12:00; Manor Farm Feeds (Owston) Ltd., Oakham].

2.2.2 Sampling, measurements and analyses

Samples of ruminal fluid (50 ml) were collected from various parts of the rumen using a syringe screwed to a stainless tube. Ruminal fluid samples were collected prior to ruminal pulse (0 h) and at 1, 2, 3, and 6 h post pulsing during the 3 d collection periods. Immediately after fluid collection, 10 ml of ruminal fluid was used to determine pH by using a pH meter (Piccolo Plus, HI 1295, Hanna Instruments, Portugal). Following pH determination, ruminal fluid was centrifuged for 10 min at 3,000 x *g* (Centaur 2 MSE, Milton Keynes); 10 ml of ruminal fluid was preserved by adding 1 ml of 25% metaphosphoric acid for VFA determination, and another 10 ml of untreated ruminal fluid was kept for NH_3-N analysis. Samples were frozen (-20ºC) for later analysis. Samples of ruminal fluid were analyzed for VFA using a gas chromatograph (GC; Agilent, GC 6890 series) equipped with a CP-Sil 88 fused-silica capillary column (100 m x 0.25 mm i.d., with 0.2 µm film thickness; Varian Inc., Oxford). A flame ionisation detector was used with an oven initial temperature of 140ºC and maximum temperature of 250ºC with an equilibrium time of 0.50 min. The inlet temperature was 300°C, the split ratio was 15:1 and a 1 µl injection volume was used. The detector temperature was 250ºC, hydrogen carrier gas flow to the detector was 40.0 ml/min, airflow was 450 ml/min and the flow of nitrogen makeup gas was 45.0 ml/min. A standard was included in the GC assay containing acetic, propionic, butyric, isobutyric, valeric and isovaleric acids (Table 2.1).

Table 2.1 Volatile fatty acid concentrations of standard used in GC analysis

	Volume (ml)	Density	Molecular weight	Concentration (m*M*)
Acetic	0.101	1.049	60.05	17.52
Propionic	0.101	0.993	74.08	13.46
Isobutyric	0.101	0.950	88.11	10.79
n-butyric	0.101	0.959	88.11	10.89
Isovaleric	0.101	0.931	102.14	9.10
Valeric	0.101	0.939	102.14	9.20

Individual VFA concentrations for each sample were determined by comparing chromatogram peak areas (ChemStation for GC A.09.01; Agilent Technologies UK Ltd.) as follows:

$$\text{Sample (mmol / l)} = \frac{\text{Sample area}}{\text{Standard area}} \times \text{concentration of standard}$$

Molar concentrations for individual acids were then calculated by dividing the concentration of each VFA by the total concentration of all six VFA in the sample.

Ruminal NH_3-N was determined by a photometric test with a Clinical Chemistry Autoanalyzer using an enzymatic ultraviolet method (Rx Imola; Randox Laboratories Ltd., Cat. No. AM3979). Ruminal NH_3-N samples were diluted with distilled water (1:10; 100µl of ruminal fluid + 900 µl distilled water) before analysis.

Reagents used for Ruminal NH_3-N:

R1a. Reagent: NADH and α-oxoglutarate
R1b. Buffer: Triethanolamine
R2. Glutamate dehydrogenase (GLDH)
CAL. Standard (Ammonia/Ethanol controls; Randox Laboratories Ltd. EA1366, EA1367)

Calculation:
A_{BLANK} = Blank A_1 – Blank A_2
A_{SAMPLE} = Sample A_1 – Sample A_2

Blank A_1: Read initial absorbance of sample and blank
Blank A_2: Read final absorbance of sample and blank

$$\text{Concentration of ammonia (µmol/l)} = \frac{A_{sample} - A_{blank}}{A_{standard} - A_{blank}} \times \text{Standard concentration}$$

Blood samples (10 ml) were obtained via jugular catheter prior to the pulse (0 h) and 1, 2, 3 and 6 h after pulsing during each 3 d collection period for lipoprotein fractionation. Blood was transferred to tubes containing EDTA and immediately

centrifuged for 10 min at 3,000 x *g* (Centaur 2 MSE, Milton Keynes) for harvesting plasma.

Based on their density, lipoprotein fractions were separated sequentially by preparative ultracentrifugation in a Beckman XL-70 Preparative centrifuge (Beckman Coulter UK Ltd., Buckinghamshire, UK). Potassium bromide (KBr) was added to the plasma to obtain the required density calculated by use of the Radding-Steinberg (1960) formula:

$$X = \frac{[(V\ (d_f - d_i)]}{[1 - (0.312 \times d_f)]}$$

X= grams of KBr
d_i= initial density
d_f= final density
V= volume of serum (ml)
0.312= partial specific volume of KBr

The density ranges selected for isolation of the major lipoprotein fractions of the bovine were those normally adopted for human lipoprotein separation, i.e., LDL, d< 1.063 g/ml (this fraction also contains chylomicrons/VLDL fractions) and HDL, d> 1.063 g/ml (Chapman, 1980). The chylomicrons/VLDL fraction was not isolated due to its low concentration. KBr was added to the plasma and mixed until the salt was dissolved. Density was adjusted in order to obtain the LDL fraction. The plasma and salt solution were added to Beckman Quick-Seal tubes. Plasma was then separated into lipoprotein fractions (LDL and HDL fractions) by ultracentrifugation at 39,000 x *g* for 20 h at 12ºC. Tubes were carefully removed after ultracentrifugation and each tube was cut using a Kontron bench tube slicer to retrieve the top layer which was predominantly LDL fraction. Density of lipoprotein and KBr solution was re-adjusted to obtain the HDL fraction and tubes were ultracentrifuged at 39,000 x *g* for 40 h at 12ºC. Tubes were carefully removed from the centrifuge and cut as previously.

Another 10 ml blood sample was collected at each sampling time and stored at -20ºC for analysis of FA profile and quantification of lipoprotein fractions. Lipids from plasma, HDL and LDL fractions were extracted by adaptation of the method by Bligh

and Dyer (1959) and methylated according to the method of Christie (1982) with modifications by Chouinard et al. (1999). All chemicals and solvents used for this method were of analytical grade. For analysis of FA in plasma and lipoprotein fractions, a GC system (Agilent, GC 6890 series) equipped with a CP-Sil 88 fused-silica capillary column (100 m x 0.25 mm i.d., with 0.2 µm film thickness; Varian Inc., Oxford) was used. The GC conditions were as follows: the oven temperature was initially set at 110°C for 4 min after injection, and then increased to 240°C with equilibration time of 2 min. The inlet and flame-ionization detector temperatures were 260°C, the split ratio was 15:1 and a 2 µl injection volume was used. The hydrogen carrier gas flow to the detector was 25 ml/min, airflow was 400 ml/min, and the flow of nitrogen makeup gas was 40 ml/min. Fatty acid peaks were identified by using a fatty acid methyl ester standard (FAME; Supelco 37 Component FAME mix, Bellefonte, PA) and a tFA reference standard (*trans* 11- C18:1, methyl ester, Supelco, Bellefonte, PA).

Concentrations of lipoprotein fractions in plasma (LDL and HDL fractions) were determined using a Clinical Chemistry Autoanalyzer (Rx Imola; Randox Laboratories, Ltd.).

HDL/LDL Calibrator - Cat. No. CH2673
Performance data for HDL cholesterol (Cat. No. CH3811):

Range:	0.04 - 3.73 mmol (1.43 - 144 mg/dl)
Sample type:	EDTA plasma
Intra-assay precision:	1.20% at 0.96 mmol/l (37.07 mg/dl)
	1.53% at 1.5 mmol/l (57.93 mg/dl)
Inter-assay precision:	0.93% at 0.97 mmol/l (37.7 mg/dl)
	0.88% at 1.5 mmol/l (58.1 mg/dl)

Performance data for LDL cholesterol (Cat. No. CH3841):

Range	0.19 - 22.2 mmol (7.35 - 860 mg/dl)
Sample type	EDTA plasma
Intra-assay precision	0.58% at 2.0 mmol/l (77.5 mg/dl)
	0.50% at 4.95 mmol/l (191.4 mg/dl)
Inter-assay precision	1.61% at 1.27 mmol/l (49.0 mg/dl)
	1.78% at 2.78 mmol/l (107.5 mg/dl)

Cholesteryl esters, triglycerides, and phospholipids were separated by thin layer chromatography using the solvent system light petroleum ether (b.p. 40-60):diethyl

ether:acetic acid in ratios of 90:30:1ratio (by volume) (Brown et al., 1980). Lipid subgroup standards were included on each TLC plate to identify the position of the lipids of interest. The bands were then visualized with iodine vapour and scraped to re-extract the lipids (Hara and Radin, 1978; Brow et al., 1980). FA in each band were determined by capillary column GC described for plasma and lipoprotein fractions. The concentration of each FA (g/100g) was calculated for 16:0 to 22:6n3 FA with concentrations higher than 0.3 g/100g.

2.2.3 Statistical analysis

Data were analysed using the GenStat 12th statistical package (VSN International Ltd., Oxford) as a Latin square 3 x 3 design with fixed effects of treatments, periods and sampling times; cow was included as a random effect to account for repeated measures. Fatty acid composition of lipid subgroups within lipoproteins was analysed using a Factorial design based on treatments (3), fractions (2) and lipids (3). Diurnal variations were analysed as POLYANOVA based on days (3) and sampling times (5) factorial. Data are reported as least square means ± SED across all sampling times.

2.3 Results

2.3.1 Composition of treatments

PHVO+SM contained higher concentrations of saturated (18 g/100g), monounsaturated (57 g/100g) and *trans* (51 g/100g) fatty acids and lower concentrations of unsaturated (59 g/100gr) and polyunsaturated (2 g/100g) fatty acids than SO+SM. VA (8 g/100g) and EA (8 g/100g) concentrations were higher in the PHVO+SM mix (Table 2.2).

2.3.2 Ruminal fermentation parameters

Ruminal pH was not affected by treatment. Compared with control, SO and PHVO resulted in lower ($P < 0.05$) concentrations of ruminal NH_3-N and total VFA concentrations. Individual molar concentrations of VFA were not affected by treatment (Table 2.3).

2.3.3 Plasma samples and treatment effects

There was no difference between treatments in concentrations of SFA and PUFA in plasma or lipoprotein fractions (Table 2.4). Compared with control, SO and PHVO resulted in higher ($P < 0.05$) concentrations of MUFA in plasma and the LDL+VLDL fraction, higher ($P < 0.05$) concentrations of *trans* fatty acids in plasma and both lipoprotein fractions, and higher ($P < 0.05$) concentrations of UFA (MUFA+PUFA) and LCFA in the LDL+VLDL fraction (Table 2.4).

Treatments influenced individual FA concentrations of some 18:1 *trans* isomers in plasma samples. SO resulted in higher concentrations of 18:1 *trans* 10, *trans* 11, and 20:1, and lower concentrations of 12:0, 15:0, 18:1 *trans* 5, 20:3n6 and 23:0 compared with control and PHVO (Table 2.5).

Table 2.2 Fatty acid composition of oils and skimmed milk emulsions used for ruminal pulses

	Control	Oils		Treatments[4]	
Fatty acid, g/100 g	SM[1]	SO[2]	PHVO[3]	SO+SM	PHVO+SM
10:0	2.51	-	-	-	-
12:0	2.14	-	0.23	-	0.24
14:0	7.89	0.07	0.18	0.08	0.30
14:1	0.50	-	-	-	-
15:0	0.98	-	-	-	-
16:0	27.02	10.74	7.82	10.84	7.80
16:1	1.54	-	-	-	0.13
17:0	0.63	-	-	0.08	0.07
18:0	11.69	4.28	8.35	4.30	8.15
18:1 *trans*-4	-	-	0.59	-	0.48
18:1 *trans*-5	-	-	1.48	-	1.38
18:1 *trans*-6-8	-	-	17.60	-	16.67
18:1 *trans*-9	-	-	8.39	-	8.33
18:1 *trans*-10	-	-	9.28	-	8.96
18:1 *trans*-11	1.70	-	7.67	-	7.54
18:1 *trans*-12	-	-	7.35	-	6.97
18:1 *cis*-9	21.08	22.42	6.06	22.33	6.27
18:2 n-*trans* 6	-	-	1.13	-	0.84
18:2 n-*cis* 6	3.39	53.03	0.42	52.90	0.46
20:0	-	0.34	0.76	0.34	0.73
18:3 n-6	-	-	0.42	-	0.58
20:1	-	0.20	-	0.18	0.15
18:3 n-3	0.61	6.78	0.06	6.75	0.14
18:2 *cis*-9, *trans*-11	1.03	-	-	-	-
20:2	-	0.05	0.05	-	-
22:0	-	0.38	0.44	0.37	0.42
24:0	-	0.15	0.15	0.14	0.18
Saturated	53.89	15.96	19.93	16.15	17.89
Monounsaturated	24.82	22.62	58.42	22.51	56.88
Polyunsaturated	4	59.86	2.08	59.65	2.02
Unsaturated (Mono+PUFA)	28.82	82.48	60.50	82.16	58.90
Trans	1.7	-	53.49	-	51.17
Unidentified[5]	17.29	1.56	21.57	1.69	23.21

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Single administration per day, [5]Fatty acids < 0.03 g / 100g.

Table 2.3 Effect of treatments on ruminal fermentation parameters

	Control	Treatments[4]			
Parameters	SM[1]	SO[2]	PHVO[3]	SED[5]	*P*-value[6]
pH	6.7	6.5	6.5	0.03	0.111
NH_3-N, m*M*	15[a]	8[b]	13[a]	1.2	0.001
Total VFA, m*M*	90[a]	72[b]	72[b]	5.6	0.003
Acetate	61.9	61.3	61.7	0.96	0.843
Propionate	23.3	23.6	23.5	0.31	0.652
Butyrate	11.2	11.7	11.2	0.58	0.637
Isobutyrate	1.1	1.0	1.1	0.04	0.065
Valerate	0.9	1.0	1.0	0.07	0.523
Isovalerate	1.2	1.1	1.2	0.09	0.131

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Single administration per day; [5]Standard errors of differences between means; [6]*P*-value represents the probability of a treatment effect; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

Table 2.4 Treatment effects on major fatty acid classes in plasma and lipoprotein fractions

		Control	Treatments[4]			
Fatty acid, g/100 g	Fraction	SM[1]	SO[2]	PHVO[3]	SED[5]	*P*-value[6]
Saturated	Plasma	41.5	40.9	41.1	0.61	0.643
	HDL	35.9	34.6	35.4	0.63	0.113
	LDL+VLDL	45.5	45.8	43.8	0.94	0.080
Monounsaturated	Plasma	20.3[a]	22.4[b]	22.6[b]	0.42	<0.001
	HDL	21.2	22.3	22.1	0.52	0.067
	LDL+VLDL	20.0[a]	22.9[b]	23.9[b]	0.74	<0.001
Polyunsaturated	Plasma	21.8	21.0	21.2	0.41	0.131
	HDL	26.0	24.8	25.5	0.72	0.271
	LDL+VLDL	14.1	12.9	13.9	0.73	0.197
Unsaturated	Plasma	42.2	43.4	43.9	0.71	0.050
(Mono+PUFA)	HDL	47.2	47.2	47.6	1.09	0.914
	LDL+VLDL	34.1[a]	35.8[b]	37.8[b]	1.01	0.002
Trans	Plasma	2.3[c]	3.4[a]	3.1[b]	0.15	<0.001
	HDL	1.8[b]	2.2[a]	2.1[a]	0.08	<0.001
	LDL+VLDL	2.8[a]	5.7[b]	5.8[b]	0.66	<0.001
Long chain fatty acids	Plasma	10.5	9.7	10.2	0.65	0.441
	HDL	12.9	13.0	12.6	0.86	0.872
	LDL+VLDL	13.0[a]	11.6[b]	12.9[a]	0.57	0.026
Unidentified[7]	Plasma	16.2	15.5	14.9	0.79	0.220
	HDL	16.7	18.0	16.9	1.14	0.449
	LDL+VLDL	20.2[a]	18.3[b]	18.2[b]	0.78	0.016

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Single administration per day; [5]Standard errors of differences of means; [6]*P*-value represents the probability of a treatment effect; [7]Fatty acids < 0.03 g / 100g; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

Table 2.5 Fatty acid distribution in plasma

	Control	Treatments [4]			
Fatty acid, g/100 g	SM[1]	SO[2]	PHVO[3]	SED[5]	*P*-value[6]
10:0	4.3	4.6	3.6	0.74	0.412
12:0	0.1	0.1	0.1	0.01	0.018
13:0	0.1	0.1	0.1	0.01	0.534
14:0	0.5	0.4	0.4	0.02	0.298
14:1	0.2	0.2	0.2	0.06	0.828
15:0	1.2[a]	1.0[b]	1.1[a]	0.08	0.048
15:1	0.1	0.1	0.1	0.01	0.818
16:0	13.8	13.7	14.0	0.20	0.268
16:1	1.5	1.4	1.5	0.06	0.072
17:0	1.0[a]	0.9[b]	0.9[b]	0.02	<0.001
17:1	0.3	0.2	0.3	0.09	0.124
18:0	19.5	19.7	19.6	0.29	0.911
18:1 *trans*-4	0.0[c]	0.1[b]	0.2[a]	0.02	<0.001
18:1 *trans*-5	0.2[a]	0.1[b]	0.2[a]	0.02	0.008
18:1 *trans*-9	0.0[c]	0.1[b]	0.2[a]	0.02	<0.001
18:1 *trans*-10	0.1[b]	0.2[a]	0.1[b]	0.01	<0.001
18:1 *trans*-11	0.8[b]	1.5[a]	0.9[b]	0.18	<0.001
18:1 *trans*-12	0.2[b]	0.3[a]	0.3[a]	0.02	0.008
18:1 *cis*-9	15.6[b]	16.3[a]	16.6[a]	0.30	0.002
18:1 *cis*-13	0.1[c]	0.1[b]	0.1[a]	0.01	<0.001
18:2n-*trans* 6	0.1	0.1	0.1	0.01	0.116
18:2n-*cis* 6	12.0	11.9	11.9	0.25	0.893
20:0	0.1	0.1	0.1	0.01	0.327
18:3n6	0.2[a]	0.1[b]	0.1[b]	0.01	<0.001
18:3n3	1.9	1.9	1.9	0.07	0.999
18:2 *cis*-9, *trans*-11	0.2	0.3	0.2	0.02	0.302
20:2	0.0	0.1	0.1	0.01	0.442
22:0	0.1	0.1	0.1	0.01	0.331
20:3n6	2.2[a]	1.8[b]	1.9[b]	0.04	<0.001
20:4n6	4.3	4.1	4.1	0.10	0.110
23:0	0.1[b]	-	0.2[a]	0.09	0.027
22:2	0.3	0.3	0.2	0.04	0.099
24:0	0.6	0.6	0.5	0.05	0.240
20:5n3	0.2	0.2	0.3	0.05	0.379
24:1	0.5	0.4	0.4	0.06	0.109
22:6n3	0.3	0.4	0.4	0.07	0.485

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Single administration per day; [5]Standard errors of differences of means; [6]*P*-value represents the probability of a treatment effect; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

2.3.4 Lipoprotein fractions and treatment effects

There was no difference between treatments in plasma concentration of lipoprotein cholesterol fractions (Table 2.6). Compared with control and SO, PHVO resulted in higher concentrations of EA, but lower concentrations of 18:1 *trans* 10 in the HDL fraction (Table 2.7). Compared with control and SO, PHVO, resulted in higher concentrations of 18:1 *trans* 5, *trans* 6-8, and EA, and *trans* 10 in the LDL+VLDL fraction (Table 2.8). Compared with control and PHVO, SO resulted in higher concentrations of VA in both lipoprotein fractions.

Significant interactions ($P < 0.05$) between lipoprotein fractions and treatments were observed. Compared with control and SO, PHVO resulted in higher concentration of 18:1 *trans* 5, *trans* 9 and 18:2n-*trans* 6 in the HDL fraction. On the other hand, compared with control and SO, PHVO resulted in higher concentrations of 18:1 *trans* 5, *trans* 9, *trans* 10 and *trans* 12 in the LDL+VLDL fraction (Table 2.9).

Table 2.6 Concentrations of HDL and LDL+VLDL fractions in plasma

	Control	Treatments[4]			
Parameters (mg/l)	SM[1]	SO[2]	PHVO[3]	SED[5]	*P*-value[6]
HDL	0.4	0.5	0.4	0.05	0.261
LDL+VLDL	0.1	0.1	0.1	0.02	0.519

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Single administration per day; [5]Standard errors of differences of means; [6]*P*-value represents the probability of a treatment effect.

Table 2.7 Fatty acid distribution in the HDL fraction

	Control	Treatments[4]			
Fatty acid, g/100 g	SM[1]	SO[2]	PHVO[3]	SED[5]	*P*-value[6]
10:0	2.4	2.0	1.5	0.72	0.475
12:0	0.1	0.1	0.1	0.02	0.207
13:0	0.1	0.1	0.08	0.01	0.100
14:0	0.1	0.1	0.1	0.02	0.722
14:1	0.1	0.05	0.04	0.01	0.273
15:0	0.8	0.9	0.8	0.12	0.688
15:1	0.1	0.08	0.08	0.02	0.987
16:0	12.1	11.8	12.3	0.63	0.758
16:1	1.1	1.1	1.2	0.06	0.138
17:0	0.9	0.8	0.9	0.07	0.716
17:1	0.2	0.3	0.3	0.05	0.894
18:0	19.2	18.4	18.6	0.61	0.430
18:1 *trans*-4	0.1	0.1	0.1	0.02	0.483
18:1 *trans*-5	-	0.03	0.04	0.01	0.058
18:1 *trans*-9	0.02[a]	0.07[b]	0.09[b]	0.01	<0.001
18:1 *trans*-10	-	0.02	-	0.01	0.059
18:1 *trans*-11	0.5[a]	0.7[b]	0.5[a]	0.04	<0.001
18:1 *trans*-12	0.1[a]	0.2[b]	0.1[a]	0.01	0.004
18:1 *cis*-9	16.7	17.5	17.2	0.94	0.747
18:2n-*trans* 6	0.02	0.03	0.05	0.01	0.051
18:2n-*cis* 6	13.6	13.2	13.8	0.50	0.489
18:3n6	0.1	0.1	0.1	0.02	0.855
20:1	0.2	-	0.1	0.09	0.084
18:3n3	1.8	1.9	1.9	0.17	0.776
20:2	0.07	0.06	0.06	0.01	0.713
20:3n6	2.6	2.2	2.3	0.19	0.105
22:1n9	0.06	0.04	0.03	0.04	0.679
20:4n6	5.9	5.6	5.5	0.43	0.636
23:0	-	-	0.07	0.03	0.172
22:2	0.4	0.3	0.3	0.06	0.330
20:5n3	0.9	0.8	0.8	0.06	0.079
24:1	0.6	0.6	0.6	0.17	0.915
22:6n3	0.4	0.3	0.3	0.04	0.083

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Single administration per day; [5]Standard errors of differences of means; [6]*P*-value represents the probability of a treatment effect; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

Table 2.8 Fatty acid distribution in the LDL+VLDL fraction

	Control	Treatments[4]			
Fatty acid, g/100 g	SM[1]	SO[2]	PHVO[3]	SED[5]	*P*-value[6]
10:0	1.1	0.6	0.5	0.51	0.444
12:0	0.08	0.08	0.08	0.01	0.711
13:0	0.05	0.05	0.04	0.01	0.323
14:0	1.0	1.0	1.0	0.08	0.865
14:1	0.04	0.04	0.04	0.00	0.887
15:0	1.8	1.4	1.4	0.22	0.137
16:0	17.2	16.9	16.8	0.44	0.670
16:1	2.0	1.8	1.9	0.09	0.256
17:0	1.2[a]	1.1[b]	1.1[b]	0.03	<0.001
17:1	0.3	0.3	0.3	0.08	0.879
18:0	21.5[b]	23.1[a]	21.5[b]	0.49	0.002
18:1 *trans*-4	0.08	0.1	0.1	0.03	0.188
18:1 *trans*-5	0.04[a]	0.09[a]	0.2[b]	0.06	0.015
18:1 *trans*-6-8	0.01[a]	0.2[a]	0.4[b]	0.12	0.001
18:1 *trans*-9	0.09[a]	0.2[b]	0.5[c]	0.07	<0.001
18:1 *trans*-10	0.08[a]	0.1[a]	0.3[b]	0.08	0.024
18:1 *trans*-11	1.1[a]	2.6[b]	1.3[b]	0.17	<0.001
18:1 *trans*-12	0.2[a]	0.40[a]	0.5[b]	0.07	<0.001
18:1 *cis*-9	13.9	13.5	13.4	1.14	0.913
18:2n-*trans* 6	0.06	0.04	0.05	0.01	0.308
18:2n-*cis* 6	7.6	7.3	7.8	0.76	0.786
20:0	0.4	0.4	0.4	0.04	0.580
18:3n6	0.1	0.09	0.1	0.02	0.197
20:1	0.06	0.03	0.02	0.03	0.332
18:3n3	1.4	1.4	1.5	0.06	0.278
18:2 *cis*-9, *trans*-11	0.1	0.1	0.09	0.02	0.170
22:0	0.2	0.2	0.2	0.03	0.445
20:3n6	1.3[a]	0.9[c]	1.1[b]	0.13	0.026
20:4n6	2.3	1.9	2.2	0.28	0.466
23:0	0.3	0.2	0.2	0.04	0.268
22:2	0.2	0.1	0.3	0.16	0.402
24:0	0.1	0.1	0.2	0.05	0.362
20:5n3	0.6	0.5	0.4	0.06	0.053
24:1	0.2	0.2	0.2	0.06	0.717
22:6n3	0.2	0.2	0.2	0.02	0.225

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Single administration per day; [5]Standard errors of differences of means; [6]*P*-value represents the probability of a treatment effect; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

Table 2.9 Interactions between fractions and treatments for concentrations of 18:1 *trans* isomers

		Control	Treatments[4]			
Fatty acid, g/100 g	Fraction	SM[1]	SO[2]	PHVO[3]	SED[5]	*P*-value[6]
18:1 *trans*-5	HDL	-	0.03[a]	0.04[a]	0.035	0.043
	LDL+VLDL	0.04[b]	0.10[a]	0.23[a]		
18:1 *trans*-6-8	HDL	-	-	-	0.030	<0.001
	LDL+VLDL	0.49[a]	0.01[c]	0.20[b]		
18:1 *trans*-9	HDL	0.03[c]	0.07[b]	0.09[a]	0.057	<0.001
	LDL+VLDL	0.10[c]	0.27[b]	0.53[a]		
18:1 *trans*-10	HDL	-	0.02[a]	-	0.056	<0.001
	LDL+VLDL	0.08[a]	0.15[a]	0.31		
18:1 *trans*-11	HDL	0.53[a]	0.72[a]	0.57[a]	0.149	<0.001
	LDL+VLDL	1.16[b]	2.70[a]	1.35[b]		
18:1 *trans*-12	HDL	0.17[c]	0.23[a]	0.19[b]	0.039	<0.001
	LDL+VLDL	0.21[c]	0.40[b]	0.52[a]		
18:2n-*trans* 6	HDL	0.02[b]	0.03[b]	0.05[a]	0.010	0.042
	LDL+VLDL	0.06[a]	0.05[a]	0.05[a]		

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Single administration per day; [5]Standard errors of differences of means; [6]*P*-value represents the probability of a fraction-treatment effect; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

2.3.5 Total amount of *trans* fatty acids in lipoprotein fractions

There was no difference between treatments in total amount of tFA of lipoprotein fractions (Table 2.10).

Table 2.10 Treatment effects on *trans* fatty acids in lipoprotein fractions

		Control	Treatments[4]			
Fatty acid	Fraction	SM[1]	SO[2]	PHVO[3]	SED[5]	*P*-value[6]
mg/l	HDL	21	29	28	0.87	0.668
	LDL+VLDL	112	140	184	31.7	0.100

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Single administration per day; [5]Standard errors of differences of means; [6]*P*-value represents the probability of a treatment effect

2.3.6 Fatty acid composition of lipid subgroups within lipoproteins

Total lipid concentration (mg/l) was not affected by treatments. Total lipid concentration was increased in LDL+VLDL-TG. 16:0 and 18:0 were not affected by treatments but were increased in LDL+VLDL-TG. Compared with control, SO and PHVO increased EA. EA was increased in LDL+VLDL-CE. Compared with control and SO, PHVO increased VA. VA was higher in CE and TG compared with PL subgroup. 18:1 *cis* 9 was not affected by treatments but was higher in LDL+VLDL-PL (Table 2.11a).

18:2 *trans* 6 was not affected by treatments but was increased in HDL-TG. 18:2 *cis* 6 was not affected by treatments but was higher in CE than PL and TG subgroups. 18:3n6 was not affected by treatments but higher in HDL-TG. 18:3n3 was not affected by treatments but was higher in CE than TG and PL subgroups. 20:3n6 was not affected by treatments but was was higher in HDL-PL. Compared with SO, PHVO and control increased 20:5n3. 22:6n3 was not affected by treatments but was higher in TG than CE and PL subgroups (Table 2.11b).

Table 2.11a Lipid concentration and fatty acid content (g / 100 g) in each lipid subgroup of lipoprotein fractions in plasma from cows subjected to ruminal pulses

					SED	*P*-value
Total lipid	Treatment	SM	SO	PHVO		
(mg / ml)		3004	2684	2587	209.1	0.117
	Fraction	HDL	LDL			
		1536[b]	3980[a]		170.7	<0.001
	Lipid	CE	PL	TG		
		2739[b]	1921[c]	3615[a]	209.1	<0.001
16:0	Treatment	SM	SO	PHVO		
		25.9	25.3	24.9	0.95	0.607
	Fraction	HDL	LDL			
		23.7[b]	27.0[a]		0.77	<0.001
	Lipid	CE	PL	TG		
		19.4[b]	27.6[a]	29.0[a]	0.95	<0.001
18:0	Treatment	SM	SO	PHVO		
		26.3	26.2	26.6	0.97	0.935
	Fraction	HDL	LDL			
		25.2[b]	27.5[a]		0.97	0.006
	Lipid	CE	PL	TG		
		18.5[c]	28.3[b]	32.3[a]	0.97	<0.001
18:1 *trans* 9	Treatment	SM	SO	PHVO		
		1.7[b]	2.0[a]	2.4[a]	0.11	<0.001
	Fraction	HDL	LDL			
		1.5[b]	2.6[a]		0.09	<0.001
	Lipid	CE	PL	TG		
		3.4[a]	0.9[c]	1.8[b]	0.11	<0.001
18:1 *trans* 11	Treatment	SM	SO	PHVO		
		1.3[b]	1.2[c]	1.5[a]	0.09	0.024
	Fraction	HDL	LDL			
		1.3	1.4		0.07	0.161
	Lipid	CE	PL	TG		
		1.6[a]	0.7[b]	1.7[a]	0.09	<0.001
18:1 *cis* 9	Treatment	SM	SO	PHVO		
		6.1	6.8	7.3	0.54	0.126
	Fraction	HDL	LDL			
		5.4[b]	8.1[a]		0.44	<0.001
	Lipid	CE	PL	TG		
		5.8[b]	9.1[a]	5.3[b]	0.54	<0.001

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; HDL = High density lipoprotein; LDL = Low density lipoprotein. CE = Cholesteryl ester; TC = triglyceride; PL = Phospholipid; [a,b,c] Means down column with different superscripts are different (P<0.05).

Table 2.11b Fatty acid content (g /100 g) in each lipid subgroup of lipoprotein fractions in plasma from cows subjected to ruminal pulses

					SED	*P*-value
18:2 *trans* 6	Treatment	SM	SO	PHVO		
		1.6	1.5	1.4	0.14	0.470
	Fraction	HDL	LDL			
		1.9[a]	1.1[b]		0.12	<0.001
	Lipid	CE	PL	TG		
		1.6[b]	0.9[c]	2.0[a]	0.14	<0.001
18:2 *cis* 6	Treatment	SM	SO	PHVO		
		9.0	8.9	8.9	1.09	0.993
	Fraction	HDL	LDL			
		9.1	8.8		0.89	0.679
	Lipid	CE	PL	TG		
		18.7[a]	5.7[b]	2.5[c]	1.09	<0.001
18:3n6	Treatment	SM	SO	PHVO		
		1.5	1.3	1.5	0.09	0.081
	Fraction	HDL	LDL			
		1.9[a]	1.1[b]		0.08	<0.001
	Lipid	CE	PL	TG		
		1.9[a]	0.9[b]	1.7[a]	0.09	<0.001
18:3n3	Treatment	SM	SO	PHVO		
		2.1	2.3	2.3	0.23	0.785
	Fraction	HDL	LDL			
		2.4	2.0		0.19	0.051
	Lipid	CE	PL	TG		
		4.8[a]	0.7[c]	1.2[b]	0.23	<0.001
20:3n6	Treatment	SM	SO	PHVO		
		1.0	1.1	1.0	0.09	0.730
	Fraction	HDL	LDL			
		1.3[a]	0.8[b]		0.07	<0.001
	Lipid	CE	PL	TG		
		0.7[c]	1.3[a]	1.1[b]	0.09	<0.001
20:5n3	Treatment	SM	SO	PHVO		
		0.7[a]	0.5[b]	0.7[a]	0.01	<0.001
	Fraction	HDL	LDL			
		0.6	0.6		0.01	0.993
	Lipid	CE	PL	TG		
		0.6	0.6	0.6	0.01	0.619
22:6n3	Treatment	SM	SO	PHVO		
		6.6	6.6	6.9	0.40	0.662
	Fraction	HDL	LDL			
		6.6	6.8		0.33	0.450
	Lipid	CE	PL	TG		
		6.8[b]	3.3[c]	10.0[a]	0.40	<0.001

[1]SM = Skim milk; [2]SO = Soya oil; [3]PHVO = Partially hydrogenated vegetable oil; HDL = High density lipoprotein; LDL = Low density lipoprotein. CE = Cholesteryl ester; TC = triglyceride; PL = Phospholipid; [a,b,c] Means down column with different superscripts are different (P<0.05).

2.3.7 Diurnal and daily variations in plasma and lipoprotein fractions on *trans* fatty acids

Generally, sampling day affected tFA of plasma and lipoprotein fractions after cows were ruminally pulsed. Plasma concentrations of all 18:1 *trans* isomers linearly increased, and were higher on Day 3 than on Day 1 and 2 but plasma concentrations of 18:2 n-*trans* 6 did not differ between days (Table 2.12). Plasma concentrations of 18:1 *trans*-4 were greater at 10:00 than other times of day; concentrations of 18:1 *trans*-5 were greater at 11:00 and 15:00 than at other times of day; concentrations of the remaining 18:1 *trans* isomers were not affected by time of sampling.

Table 2.12 Day and sampling time (h) effects in plasma *trans* fatty acids

Fatty acid (g/100 g)							SED[3]	*P*-value[4]	(Lin)[5]	(Quad)[6]
18:1	D[1]	1	2	3						
trans-4		0.07[c]	0.17[b]	0.24[a]			0.022	<0.001	<0.001	0.673
	T[2]	09:00	10:00	11:00	12:00	15:00				
		0.09[c]	0.24[a]	0.15[c]	0.16[b]	0.15[c]	0.029	<0.001	0.910	0.058
18:1	D	1	2	3						
trans-5		0.04[c]	0.18[b]	0.30[a]			0.029	0.002	0.009	0.879
	T	09:00	10:00	11:00	12:00	15:00				
		0.18[b]	0.07[c]	0.25[a]	0.13[b]	0.25[a]	0.037	0.033	0.142	0.471
18:1	D	1	2	3						
trans-9		0.10[c]	0.16[b]	0.23[a]			0.025	<0.001	<0.001	0.992
	T	09:00	10:00	11:00	12:00	15:00				
		0.15	0.19	0.16	0.18	0.14	0.033	0.568	0.395	0.465
18:1	D	1	2	3						
trans-10		0.08[c]	0.18[b]	0.25[a]			0.015	<0.001	<0.001	0.698
	T	09:00	10:00	11:00	12:00	15:00				
		0.17	0.17	0.18	0.18	0.15	0.019	0.526	0.499	0.343
18:1	D	1	2	3						
trans-11		0.80	1.15	1.35			0.094	<0.001	<0.001	0.329
	T	09:00	10:00	11:00	12:00	15:00				
		1.05	1.13	1.04	1.04	1.20	0.121	0.595	0.262	0.380
18:1	D	1	2	3						
trans-12		0.19[a]	0.28[b]	0.40[a]			0.028	<0.001	<0.001	0.624
	T	09:00	10:00	11:00	12:00	15:00				
		0.27[c]	0.36[a]	0.26[c]	0.26[c]	0.30[b]	0.036	0.034	0.898	0.340
18:2	D	1	2	3						
n-*trans* 6		11.70	12.19	12.07			0.254	0.131	0.347	0.028
	T	09:00	10:00	11:00	12:00	15:00				
		11.64	12.27	12.01	11.61	12.40	0.327	0.053	0.201	0.038

[1]Day; [2]Time; [3]Standard errors of differences of means; [4]*P*-value represents the probability of a treatment effect; [5]Linear effect; [6]Quadratic effect; Samples were taken during the 3 d collection period

HDL fraction concentrations of 18:1 *trans*-9, *trans*-11, and *trans*-12 linearly increased and were higher on Day 3 than on Day 1 and 2. HDL fraction concentrations of 18:1 *trans*-4 and 18:2 n-*trans* 6 had quadratic effect. HDL fraction concentrations of 18:1 *trans*-4 were greater at 11:00 than at other times of day; concentrations of 18:1 *trans*-9 were greater at 12:00 and 15:00 than at other times of day; concentrations of 18:2 n-*trans* 6 had quadratic effect; concentrations of the remaining 18:1 *trans* isomers were not affected by time of sampling (Table 2.13).

Table 2.13 Day and sampling time (h) effects in the HDL fraction *trans* fatty acids

Fatty acid, g/100 g							SED[3]	*P*-value[4]	(Lin)[5]	(Quad)[6]
18:1	D[1]	1	2	3						
trans-4		0.07[b]	0.10[a]	0.08[b]			0.010	0.029	0.818	0.008
	T[2]	09:00	10:00	11:00	12:00	15:00				
		0.09[b]	0.09[b]	0.11[a]	0.04[c]	0.09[b]	0.013	0.016	0.599	0.229
18:1	D	1	2	3						
trans-9		0.045[c]	0.062[b]	0.088a			0.010	0.017	0.005	0.752
	T	09:00	10:00	11:00	12:00	15:00				
		0.05[b]	0.04[b]	0.05[b]	0.08[a]	0.09[a]	0.013	0.029	0.005	0.857
18:1	D	1	2	3						
trans-11		0.54[b]	0.57[b]	0.70[a]			0.023	<0.001	<0.001	0.128
	T	09:00	10:00	11:00	12:00	15:00				
		0.58	0.59	0.59	0.63	0.65	0.030	0.450	0.078	0.923
18:1	D	1	2	3						
trans-12		0.16[c]	0.19[b]	0.23[a]			0.007	<0.001	<0.001	0.972
	T	09:00	10:00	11:00	12:00	15:00				
		0.19	0.19	0.18	0.20	0.21	0.010	0.460	0.134	0.700
18:2	D	1	2	3						
n-*trans* 6		0.03[a]	0.02[b]	0.03[a]			0.003	0.022	0.261	0.011
	T	09:00	10:00	11:00	12:00	15:00				
		0.03[b]	0.02[b]	0.03[b]	0.04[a]	0.03[b]	0.004	0.029	0.044	0.480

[1]Day; [2]Time; [3]Standard errors of differences of means; [4]*P*-value represents the probability of a treatment effect; [5]Linear effect; [6]Quadratic effect; Samples were taken during the 3 d collection period

LDL+VLDL concentrations of 18:1 *trans*-9, *trans*-10, *trans*11, and *trans*-12 linearly increased and were higher on Day 3 than on Days 1 and 2, but LDL+VLDL fraction concentrations of 18:1 *trans*-4 and *trans*-5 and 18:2 n-*trans* 6 did not differ between days. LDL+VLDL fraction concentrations of 18:1 *trans* isomers were not affected by time of sampling (Table 2.14).

Table 2.14 Day and sampling time (h) effects in the LDL+VLDL fraction *trans* fatty acids

Fatty acid, g/100 g							SED[3]	*P*-value[4]	(Lin)[5]	(Quad)[6]
18:1 *trans*-4	D[1]	1	2	3						
		0.11	0.09	0.13			0.016	0.326	0.361	0.236
	T[2]	09:00	10:00	11:00	12:00	15:00				
		0.09	0.08	0.09	0.14	0.14	0.020	0.121	0.009	0.717
18:1 *trans*-5	D	1	2	3						
		0.16	0.15	0.16			0.026	0.985	0.963	0.868
	T	09:00	10:00	11:00	12:00	15:00				
		0.17	0.12	0.12	0.23	0.14	0.034	0.113	0.906	0.457
18:1 *trans*-9	D	1	2	3						
		0.13[c]	0.37[b]	0.45[a]			0.049	<0.001	<0.001	0.176
	T	09:00	10:00	11:00	12:00	15:00				
		0.31	0.35	0.28	0.31	0.34	0.064	0.958	0.838	0.802
18:1 *trans*-10	D	1	2	3						
		0.24[c]	0.47[b]	0.64[a]			0.026	<0.001	<0.001	0.343
	T	09:00	10:00	11:00	12:00	15:00				
		0.50	0.41	0.44	0.45	0.44	0.034	0.424	0.485	0.441
18:1 *trans*-11	D	1	2	3						
		1.11[c]	1.83[b]	2.27[a]			0.125	<0.001	<0.001	0.360
	T	09:00	10:00	11:00	12:00	15:00				
		1.73	1.76	1.57	1.93	1.67	0.161	0.622	0.905	0.661
18:1 *trans*-12	D	1	2	3						
		0.29[c]	0.37[b]	0.49[a]			0.032	<0.001	<0.001	0.548
	T	09:00	10:00	11:00	12:00	15:00				
		0.35	0.38	0.35	0.40	0.46	0.041	0.344	0.061	0.589
18:2 n-*trans* 6	D	1	2	3						
		0.07	0.05	0.06			0.005	0.207	0.141	0.319
	T	09:00	10:00	11:00	12:00	15:00				
		0.06	0.07	0.06	0.07	0.06	0.007	0.950	0.948	0.661

[1]Day; [2]Time; [3]Standard errors of differences of means; [4]*P*-value represents the probability of a treatment effect; [5]Linear effect; [6]Quadratic effect; Samples were taken during the 3 d collection period

2.3.8 Diurnal and daily variations in ruminal fermentation parameters

Ruminal pH and NH_3-N did not differ by between days. Ruminal pH linearly decreased and was lower at 15:00 than at other times of day. Ruminal NH_3-N had quadratic effect and was greater at 12:00 than at other times of day. Total volatile FA were not affected by day and time of sampling. Concentrations of acetate, propionate, butyrate, valerate and isovalerate did not differ between days. Concentration of acetate linearly decreased and was lower at 12:00 than at other times of day; molar concentration of propionate linearly increased and was greater at 12:00 than at other times of day; molar concentrations of butyrate and isovalerate had quadratic effect and were greater at 10:00 than at other times of day, but molar concentration of valerate was not affected by time of sampling (Table 2.15).

Table 2.15 Day and time (h) effects on ruminal fermentation parameters

Parameters							SED[3]	*P*-value[4]	(Lin)[5]	(Quad)[6]
pH	D[1]	1	2	3						
		6.49	6.50	6.47			0.028	0.435	0.430	0.308
	T[2]	09:00	10:00	11:00	12:00	15:00				
		6.62[a]	6.51[b]	6.51[b]	6.52[b]	6.27[c]	0.036	<0.001	<0.001	0.152
NH_3-N, m*M*	D	1	2	3						
		13.9	10.1	12.1			2.81	0.100	0.296	0.060
	T	09:00	10:00	12:00						
		11.0[c]	15.8[b]	17.4[a]			2.81	0.002	0.550	<0.001
Total VFA, m*M*	D	1	2	3						
		78.0	77.8	77.9			4.71	0.998	0.976	0.962
	T	09:00	10:00	12:00						
		80.8	76.3	76.7			4.71	0.568	0.458	0.448
Acetate	D	1	2	3						
		62.0	62.1	60.8			0.94	0.310	0.191	0.427
	T	09:00	10:00	12:00						
		63.4[a]	60.8[b]	60.7[b]			0.94	0.010	0.017	0.052
Propionate	D	1	2	3						
		23.3	23.3	23.8			0.31	0.234	0.142	0.387
	T	09:00	10:00	12:00						
		23.1[c]	23.4[b]	24.0[a]			0.31	0.023	0.006	0.936
Butyrate	D	1	2	3						
		11.0	11.2	11.8			0.56	0.347	0.161	0.714
	T	09:00	10:00	12:00						
		10.3[c]	12.0[a]	11.8[b]			0.56	0.007	0.029	0.021
Isobutyrate	D	1	2	3						
		1.1	1.0	1.1			0.04	0.677	0.890	0.384
	T	09:00	10:00	12:00						
		1.0	1.1	1.0			0.04	0.085	0.757	0.028
Valerate	D	1	2	3						
		1.0	1.0	1.0			0.07	0.547	0.864	0.280
	T	09:00	10:00	12:00						
		0.8	1.1	1.1			0.07	<0.001	<0.001	0.001
Isovalerate	D	1	2	3						
		1.2	1.1	1.2			0.09	0.209	0.735	0.083
	T	09:00	10:00	12:00						
		1.1[b]	1.3[a]	1.0[c]			0.09	0.014	0.138	0.011

[1]Day; [2]Time; [3]Standard errors of differences of means; [4]*P*-value represents the probability of a treatment effect; [5]Linear effect; [6]Quadratic effect; Samples were taken during the 3 d collection period

2.4 Discussion

Overall, the results demonstrated that FA profile of plasma and lipoprotein fractions reflected the dietary FA source; ruminal parameters were affected by oil pulses and concentrations of tFA were different in plasma and both lipoprotein fractions. LDL+VLDL fraction was the main fraction involved in transport of tFA.

The design of the experiment enabled examination of effects of different dietary lipids on FA profiles of plasma and lipoprotein fractions. In the current experiment, 250 g/d of SO or PHVO was ruminally pulsed accompanied by skimmed milk as a vehicle (Loor and Herbein, 2001). The aim was to increase tFA supply to the duodenum either as products of ruminal biohydrogenation (SO) or as preformed tFA (PHVO). The ruminal pulse amounts were of a magnitude similar to those used in other studies (Rogers et al., 1979; Ferlay and Doureau, 1992). Ferlay and Doreau (1992) compared two treatments, one in which rapeseed oil was continuously infused into the rumen at 1000 g/d and another with a single administration of 250 g of rapeseed oil. However, they did not use any emulsifier to permit a homogenised release of the oil. They reported that the effects of lipid supplementation on ruminal digestion are less strong with a continuous supply than with a single administration.

This experiment is the first to investigate concentrations of individual 18:1 tFA isomers in lipoprotein fractions of bovine plasma. The current experiment differs from previous research (Baumgard et al., 2002; Mosley et al., 2006; Tyburczy et al., 2008; Lock et al., 2008) mainly in the use of oils rather than using individual FA (i.e. CLA *trans*-10, *cis*-12; oleic acid, VA and EA), enriched oils (i.e. fish oil high in oleic acid; Amorocho et al., 2009) and combination of oils (i.e. fish oil+sunflower oil; Cruz-Hernandez et al., 2007; Belenguer et al., 2010) that might be absorbed and transported differently to the mammary gland. Also, in this experiment, ruminal pulses were used, whereas, previous studies (Loor and Herbein, 2001; Baumgard et al., 2002; Mosley et al., 2006; Tyburczy et al., 2008) used abomasal infusion to study effects of dietary lipids on plasma and lipoprotein fractions mainly because the authors aimed to avoid ruminal biohydrogenation. Thus, differences in FA output in milk between abomasal infusion and oral treatments can be attributed to ruminal metabolism and intestinal bioavailability, or both (Castaneda-Gutierrez et al., 2007). The present experiment also differs from studies (Chilliard et al., 1991, DePeters et al., 2001) that used

duodenal infusions to investigate effects of dietary oils on milk fatty acid profile. The profile of FA absorbed in the duodenum can alter the FA profile of dairy and other animal products, especially modifying FA saturation and CLA concentration (Grummer, 1991; Mansbridge and Blake, 1997; Harvatine and Allen, 2006).

The use of PHVO as ruminal pulse is another difference between other studies (Mosley et al., 2006; Tyburczy et al., 2008). Abomasal infusion of PHVO to dairy cows' results in milk fat depression and this has been attributed to the high levels of 18:1 *trans* isomers (Selner and Schultz, 1980; Gaynor et al., 1994; Romo et al., 1996). PHVO also contains a Gaussian distribution of 18:1 *trans* isomers (approximately 40 to 60% of total FA), that undergo the hydrogenation process resulting in the formation of several conjugated and other unique FA isomers that are present in PHVO, and some of these could regulate milk fat synthesis (Lock et al., 2007; Bauman and Griinari, 2003; Shingfield and Griinari, 2007; Tyburczy et al., 2008).

The concentration of EA in PHVO was higher than that in SO because EA is the major *trans* isomer found in PHVO (Meijer et al., 2001). On the other hand, while VA was not detectable in SO, after biohydrogenation high concentrations of VA were found in plasma and lipoprotein fractions following SO treatment. This is consistent with observations that inclusion of vegetable oils high in PUFA into concentrate diets raises VA and 18:2 cis-9, *trans*-11 content in milk fat (Bauman et al., 2000; Shingfield et al., 2006). Other studies have shown that diets with a higher forage-to-concentrate ratio of 60:40 (Bell et al., 2006) or 73:27 (Roy et al., 2006) and containing safflower oil (6%) or linseed oil (5%), respectively, also increased high levels of VA in milk. AbuGhazaleh et al. (2002) examined the effect of feeding fish oil, extruded soya beans, or their blend on the milk fat concentration of CLA *cis*-9, *trans*-11 and found that the concentration of VA in milk fat was increased 129% by the fish oil diet compared with the control diet, despite the fact that fish oil had low concentration (< 3%) of both linoleic and linolenic acids, the precursors of VA produced by ruminal biohydrogenation. Milk VA concentration was also increased by 136% when extruded soya beans were fed, compared with the control diet. In the current experiment, linoleic acid and oleic acid were higher in the SO treatment; however, linoleic acid has been shown to be more effective than oleic acid in promoting high VA

concentrations in the milk (AbuGhazaleh et al., 2003) and in the rumen (AbuGhazaleh et al., 2003, 2005, 2007).

CLA 18:2 *cis*-9, *trans*-11 was detected in skimmed milk (control) but not in SO nor PHVO. It is known that food products from ruminants, particularly dairy products, are the major dietary source of CLA for humans. Griinari et al., (1998) demonstrated that cows can synthesize CLA from VA. The major CLA isomer in food products from ruminants is 18:2 *cis*-9, *trans*-11, although other CLA isomers are present and these may vary under different rumen conditions (Griinari et al. 1997). CLA levels in food products derived from ruminants can be altered by affecting ruminal production of CLA or VA, or by dietary supplement with these fatty acids (Chouinard et al., 1999).

Usually, supplementing cows with hydrogenated vegetable oils can decrease milk fat content due to their high content of 18:1 *trans* isomers and could raise the concentration of blood cholesterol. Selner and Schultz (1980) found that dairy cows supplemented with hydrogenated vegetable oil containing 13% tFA at 454 g per cow per day decreased slightly milk fat concentration and elevated plasma cholesterol from 190 to 245 mg/100 ml and 18:1 tFA in milk fat from 4.2 to 6.2 g/100g. Hydrogenated vegetable oil containing 49% 18:1 tFA fed at 454 g/d decreased milk fat concentration from 3.9 to 3.1%. Short chain FA in milk fat triglycerides decreased and there were increases in 18:1 *trans* isomers (from 2.6 to 11.2 g/100g), 18:1 *cis* (from 22.9 to 29.0 g/100g), and 18:2 *trans* (from 0.2 to 1.8 g/100g). In milk phospholipids 18:1 *trans* isomers increased from 0.3 to 3.1 g/100g in and 18:1 *cis* isomers increased from 20.5 to 31.4 g/100g. Blood cholesterol esters were increased from 152 to 195 mg/100 ml.

In the current experiment, SO contained high concentrations of UFA and PUFA. If dairy cows were fed with this oil, it could reduce milk fat yield and inhibit *de novo* FA synthesis and FA desaturation, although, in some experiments, oil supplementation has the potential to overcome reductions in *de novo* synthesis caused by PUFA found in vegetable oils (Loor et al., 2002). The origin of short-chain (4:0 to 8:0) and medium-chain FA (10:0 to 14:0) in ruminant milk fat is from *de novo* synthesis, while long-chain FA (> C:18) are derived from precursors in the blood (Bauman and Griinari, 2003). Storry (1972) proposed that long-chain FA taken up by the mammary gland inhibit *de novo* synthesis of short- and medium-chain FA, which was supported

by work with rats and humans (Barber et al., 1997). Neville and Picciano (1997) discussed and summarized studies to support the concept that FA play an integral role in regulating *de novo* FA synthesis in the lactating mammary gland, probably via actions on acetyl-CoA carboxylase (Carroll et al., 2006).

Likewise, Hervas et al. (2008) reported that dietary supplementation with sunflower oil led to an increase in 18:0 and 18:1 isomers in milk of dairy ewes. That response had been reported previously (Chilliard and Ferlay, 2004) in milk from cows and goats and has been also observed in milk fat from ewes supplemented with SO (Mele et al., 2006; Gomez-Cortes et al., 2008). In the current experiment, the increased of 18:1 *trans* isomers found in plasma and lipoprotein fractions following SO pulse can be attributed to a shift in the biohydrogenation pathways, due to alterations in the rumen environmental conditions as a results of the ruminal pulsing.

Kadegowda et al. (2008) found that dry matter intake was affected by abomasal infusion of highly UFA and to a less extend saturated LCFA. Their infusion mixtures contained both SFA and UFA and the maximum amount of FA infused was limited to 400 g/d to avoid possible adverse effects on dry matter intake.

2.4.1 Fatty acid composition of plasma and lipoprotein fractions

The main hypothesis of this experiment was that concentrations of 18:1 tFA in plasma and lipoprotein fractions would be influenced by type of lipid pulsed. This hypothesis was supported by the differences in concentrations of 18:1 tFA in plasma and lipoprotein fractions observed between SO and PHVO.

With a single administration, concentrations of treatments are higher during the first few hours after pulse and lower in subsequent hours. Generally, sampling day had linear effect on 18:1 *trans* isomers in plasma, HDL and LDL+VLDL. Normally, dietary lipids cause a decreased in ruminal degradation (Ferlay and Doreau, 1992). In this study, effects of treatments on tFA profile of plasma and lipoprotein fractions were observed within the first three hours post pulse. Sampling day affected tFA of plasma and lipoprotein fractions. The diurnal variation of tFA in the plasma and cholesterol fractions found in this study may indicate that fluctuations vary according to metabolic status (preprandial and postprandial status) (Diven et al., 1958). The

amounts and composition of lipids circulating in the blood of dairy cattle are dependent upon a number of physiological variables. The nature of the diet, time since feeding, age, breed, pregnancy, and stage of lactation may all affect lipid content and composition (Christie, 1981). In this study, diurnal variations of plasma, lipoprotein fractions and ruminal fermentation parameters and showed that there may be an adaptation of ruminal microflora to the treatments and also reflected a cycle control mechanism balancing rate of lipid entry and rate of removal in the circulatory system. This is possibly related to regulation of lipid supply (diet, production, synthesis) and utilization by peripheral tissues (Bitman et al., 1990).

Differences in tFA concentrations due to type of oil infused led to major changes in the profiles of most 18:1 *trans* isomers in plasma and lipoprotein fractions, in agreement with observations in plasma by Loor and Herbein (2001) in which ruminal infusions of CLA mixtures were used. In the current experiment, SO treatment was higher in 18:1 *cis*-9 and 18:2 n-*cis* 6. Previous *in vivo* and *in vitro* observations indicated that ruminal biohydrogenation of 18:1 *cis*-9 and 18:2 n-6 give rise to geometrical isomers of 18:1 with double bonds at positions 6 to 16 of the carbon chain (Bickerstaffe et al., 1972; Kemp et al., 1975; Loor et al., 2002). Ruminal isomerisation of oleic acid is also associated with production of several 18:1 *trans* isomers but primarily 18:1 *trans*-6, 7, 8, 18:1 *trans*-9, and 18:1 *trans*-10 (Loor et al., 2002; Mosley et al., 2002). Under normal conditions, however, VA is by far the predominant 18:1 *trans* isomer, resulting from hydrogenation of 18:2n-6 in the rumen (Loor et al., 2002). In the current experiment, SO treatment was higher in 18:0 which availability for endogenous 18:1 *cis*-9 production is crucial for milk fat synthesis (Donovan et al., 2000; Chilliard et al., 2003; Loor et al., 2005).

There are several possible processes for production of 18:1 *trans* isomers from 18:1 *cis*-9. One could be that the rumen bacteria possess a multitude of *cis* / *trans* isomerases. It is accepted that there is an isomerase that converts the *cis*- 12 bond of linoleic and linolenic acids to a *trans*-11 bond. Another possibility could be that the 18:1 *trans* isomers are formed as a result of simple chemical double bond migration (Griinari and Bauman, 1999). Ward et al. (1964) suggested that the accumulation of *trans* monoenes from biohydrogenation of ^{14}C-labeled linoleic acid was due to double bond migration. This conclusion was based on the observation that the chemical hydrogenation of linoleic acid with a metal catalyst resulted in migration of the double

bond and formation of tFA. However, enzymatic and metal catalyzed hydrogenations are not parallel processes (Mosley et al., 2002).

Compared with HDL fraction, concentrations of tFA were increased almost 3-fold in LDL+VLDL fraction. These results may indicate which lipoprotein fraction is involved in the transportation of tFA. The increase in *trans* isomers across plasma and lipoprotein fractions with SO and PHVO reflects modification of rumen microbial population and pathways of biohydrogenation usually caused by supplementation with dietary PUFA. Those changes promote formation and accumulation of biohydrogenation intermediates, some of which are potent inhibitors of milk fat synthesis (Bauman and Griinari, 2003).

VA was significantly higher following ruminal pulse of SO compared with control and PHVO in plasma and lipoprotein fractions. EA was significantly higher following ruminal pulse of PHVO compared with control and SO treatments in plasma and lipoprotein fractions. This result is in agreement with observations in lipoprotein fractions by Meijer et al. (2001) in which EA and VA were supplemented in the diet of hamsters. In the current experiment, concentrations of tFA in plasma and lipoprotein fractions were significantly higher following ruminal pulse of SO compared with control and PHVO. This effect may be explained by the composition of the oils where SO had lower concentrations of VA and EA and the interaction effects of ruminal biohydrogenation.

In the current experiment CLA 18:2 *cis*-9, *trans*-11 was too low to be detected in the HDL fraction, however, 18:2 *cis*-9, *trans*-11 was identified in plasma and LDL+VLDL but no difference was observed between control and treatments. The most predominant CLA isomer found in foods of ruminant origin is 18:2 *cis*-9, *trans*-11. This CLA isomer is an intermediate in ruminal biohydrogenation of linoleic acid (Corl et al., 2002).

2.4.2 Fatty acid composition of the lipoprotein lipids

Ruminant plasma lipoprotein fractions are characterized by low concentrations of chylomicrons, VLDL and total TG, whereas more than 90% of plasma lipids are carried by HDL, mainly in the CE and PL lipid sub groups (Offer et al., 2001). In the current experiment concentrations of LDL+VLDL-TG appeared to be the major lipid

subgroups carrying plasma lipids. Because the LDL fraction contains chylomicrons/VLDL fractions, it is possible that TG found in the LDL fraction are associated with chylomicrons which contain dietary TG.

In the current experiment, palmitic acid was increased in LDL+VLDL-TG compared with HDL. Palmitic acid is an important FA for the synthesis of triacylglycerol in the mammary gland (Hansen and Knudsen, 1987). Initiation of acylation of the sn-1 position is a prerequisite for triacylglycerols synthesis, and palmitic acid is the most preferred substrate for the initial acylation of L-α glycerolphosphate by acyltransferase to form sn-1-lysophosphatidic acid (Kinsella and Gross, 1973). It has been shown (Kinsella and Gross, 1973) that myristyl, stearyl, and oleyl coenzyme-A were rapidly acylated when sn-1-lysophosphatidic acid was used as a substrate, but were poorly acylated without the latter indicating that these FA are taken up mostly in the second step of triacylglycerol synthesis (Kadegowda et al., 2008).

To study the transport of FA in bovine plasma, Offer et al. (2001) fed cows with dietary fish oil finding progressive milk depression from those cows supplemented with fish oil. They separated HDL, LDL and VLDL fractions and found that total lipids in the VLDL fraction were increased, although this did not reach statistical significance. The current experiment is different from Offer et al. (2001) because they used lactating cows, stage of lactation has an important effect on FA utilization needed to satisfy specific energy requirements and this may be reflected in the FA profile of lipoprotein lipid subgroups found in our experiment (non-lactating cows). Also, the current experiment involved release of treatments as ruminal pulses rather than supplementing oils in the diet. Despite the fact that the results are different from those of Offer et al. (2001), in the current experiment TG had the larger content of FA than CE and PL. That has implications for the theory that the ruminant mammary gland obtains FA required for assembly of milk lipids by the action of mammary lipoprotein lipase on the TG fraction of chylomicrons and VLDL.

The difference between abomasal and ruminal pulses is probably another factor that may explain the variation in the concentrations of certain FA within each lipid sub group (Ashes et al., 2000; Offer et al., 2001). For example, DePeters et al. (2001) attributed changes in carbon number for TG structure to high concentrations of LCFA

and medium chain FA associated with canola oil infused either in the rumen or abomasum.

In this experiment, PHVO pulse tended to increased VA in LDL+VLDL-TG, however, it did not reach significance. Because the mammary gland extracts FA primarily from TG fractions of plasma, FA in these LDL+VLDL lipid subgroups may possibly be available for milk synthesis in cows supplemented with PHVO. Increased in VA in LDL+VLDL-TG by PHVO might also be explained by the fact that concentrations of dietary or ruminally derived fatty acids in the major plasma lipid fractions are directly proportional to the amounts of fatty acids absorbed from the small intestine (Loor et al., 2002).

2.4.3 Ruminal fermentation

Sampling time had linear effect and decreased ruminal pH and acetate and linearly increased propionate. On the other hand, sampling time had quadratic effect on NH_3-N, butyrate, isobutyrate, valerate and isovalerate. Those changes may indicate that dietary lipids had an impact on ruminal microflora affecting fermentation parameters. Dietary oil supplementation has been shown to affect ruminal fermentation in ruminants (Harfoot & Hazelwood, 1997). Yang et al. (2009) found that supplementation of soybean and linseed oils reduced the concentration of total VFA and increased ruminal NH_3-N. Relatively few data exist relating to the effects of PHVO or SO on ruminal fermentation. In the current experiment, ruminal pulses of both SO and PHVO had quadratic effect on ruminal NH_3-N and total VFA concentrations, which suggests that dietary lipids inhibited microbial activity. Cieslak et al. (2009); supplemented linoleic acid on two rumen ciliate cultures (*Entodinium caudatum* and *Diploplastron affine*) and reported a decreased in the concentration of ruminal NH_3-N and no effects on total VFA concentration, methane production or concentration of acetate and butyrate in the *Diploplastron affine* culture. On the other hand, NH_3-N was reduced in the *Entodinium caudatum* culture. Likewise, Hervas et al. (2008) found no difference in VFA and NH_3-N in an *in vitro* study in which sunflower oil was incubated with ruminal fluid from dairy ewes.

In agreement with the current experiment, Ivan et al. (2001) reported that NH_3-N and total VFA concentrations were reduced in ruminal fluid of sheep supplemented with dietary sunflower seed oil compared with an unsupplemented diet. In the current

experiment, ruminal pulses of PHVO and SO affected VFA concentrations. It has been shown that dietary oils rich in saturated and unsaturated FA may reduce the number of ruminal protozoa both *in vivo* and *in vitro*, moreover, unsaturated C18 FA exert toxic effects on protozoan populations.

Defaunation is known to decrease ruminal NH_3-N which is related to decreased N recycling between bacteria, protozoa and ammonia pools resulting from engulfment and digestion of bacteria by protozoa. Another reason for the reduction of ruminal NH_3-N is that there is an increase in demand for ammonia by ruminal bacteria that use it as substrate for cell synthesis (Dayani et al., 2007).

Possibly a ruminal ecology (i.e., ruminal bacteria, fungi and protozoa) study would be a promising approach to truly understand how 18:1 *trans* isomers affect ruminal fermentation and bacterial populations.

2.5 Conclusion

The results of this experiment demonstrated that 18:1 tFA concentrations of plasma and lipoprotein fractions can be changed by altering dietary lipids. LDL+VLDL appears to be the main lipoprotein fraction involved in transportation of 18:1 *trans* isomers in bovine plasma. Overall, the current experiment demonstrates that dietary lipids rich in either PUFA or tFA can alter the rumen environment, and that both types of FA result in increased plasma and lipoprotein-fraction concentrations of tFA. The results therefore show how plasma FA profiles change when supplementary oils do not bypass the rumen and hence the lipids had extensive effects on rumen microorganisms, as indicated by concentrations of total VFA and ruminal NH_3-N, and on the biohydrogenation process, as indicated by plasma and lipoprotein 18:1 *trans* isomers. Treatment effects are, therefore, in agreement with expectations from dietary supplementation of oils, but are independent of any effects of oil on palatability and feed intake data.

2.6 Implications

Although the results from this experiment suggests that the LDL+VLDL fraction is more responsive to supply of tFA and responsible for the transport of CLA isomers, further fractionation is required to distinguish between true LDL-fraction, chylomicrons and VLDL that are also present in this fraction.

In the current experiment concentrations of tFA were increased in both LDL+VLDL and HDL fractions suggesting that feeding cows with oils rich in UFA and tFA might produce milk with high concentrations of FA undesirable for human consumption and reduce intake of digestible energy (Harvatine and Allen, 2006), milk fat content and yield compared with typical diets (Griinari et al., 1998; Piperova et al., 2000; Peterson et al., 2003; Loor et al., 2005). On the other hand, recently, Brouwer et al. (2010) reported that all FA with a double bond in the *trans* configuration raise the ratio of plasma LDL to HDL regardless of FA origin (tFA industrially produced or from biohydrogenation in the rumen). However, findings from this experiment, shows that altering the supply of tFA from the rumen modifies the tFA content of LDL+VLDL fraction much more than HDL fraction. Thus if it were to alter tFA delivery to the mammary gland it is likely that it is through this fraction.

Results from tFA content in plasma and lipoprotein fractions demonstrated that there are clear differences in their plasma transport that may exert an effect on mammary uptake and utilization of 18-carbon FA, and these results might be related to the location, orientation, and number of double bonds (Tyburczy et al., 2008).

C h a p t e r 3

Ruminal pulses of fatty acids in dairy cows: 2. Fish oil and partially hydrogenated vegetable oil

CHAPTER 3

3. Ruminal pulses of fatty acids in dairy cows: 2. Fish oil and partially hydrogenated vegetable oil

3.1 Introduction

Recently, nutritional recommendations for humans have emphasized the need to decrease intake of SFA and increase intakes of beneficial PUFA, in particular α-linolenic acid (18:3 n-3) and the LC-PUFA (20:5 n-3 and 22:6 n-3), in the diet (Lee et al., 2008). Ruminant products make an important contribution to the human diet but have caused concern due to their high SFA content. This is largely due to microbial biohydrogenation of dietary unsaturated fatty acids in the rumen, although some intermediates of biohydrogenation such as conjugated linolenic acid (CLA *cis*-9, *trans*-11) and 18:1 *trans*-11 (VA) could be important in human health (Lock and Bauman, 2004).

It has been shown that fish oil (FO) either alone or in combination with vegetable oils results in increased concentrations of *trans* fatty acids (tFA) and CLA in duodenal fluid and milk fat tFA and CLA are intermediates in the rumen hydrogenation of PUFA (Lock and Bauman, 2004; Kim et al., 2008). Moreover, inclusion of fish oil in lactating cow diets has been shown to increase CLA, VA and omega-3 fatty acids in milk fat (Abu-Ghazaleh et al., 2002). Fish oil has also been shown to interrupt the complete biohydrogenation of C18 PUFA, resulting in increased production of VA, the precursor for CLA (*cis*-9, *trans*-11) in the mammary gland (Piperova et al., 2002).

The experiment reported in Chapter 2 demonstrated that pulses of partially hydrogenated vegetable oils (PHVO) or soya oil increased the tFA content of both HDL and LDL lipoprotein fractions, but responses varied according to fatty acid source. Because FO contains higher concentrations of long chain PUFA, compared with vegetable oils (Duckett and Gillis, 2010), it is hypothesized that differences in FA concentrations would be observed, particularly in C18:1 *trans* isomers and 18:2 *cis*-9, *trans*-11, which would lead to differences in tFA concentrations of plasma and lipoprotein fractions.

The objective of this experiment was to determine the effects of pulsing FO either alone or in combination with PHVO on plasma FA composition and to determine

which lipoprotein fractions are involved in tFA transport and whether there is a difference between tFA. This objective was achieved by comparing fatty acid profiles of plasma lipoprotein fractions in cows subjected to ruminal pulses of FO (to induce high concentrations of 18:1 tFA) or FO+PHVO (to induce high concentration of tFA).

3.2 Materials and methods

3.2.1 Animals and treatments

Two non-lactating Holstein nuliparous cows, each with a rumen cannula were used in a 2 x 3 Crossover design with 3 d pulsing periods (adapted and modified from Kadegowda et al., 2008 and Castaneda-Gutierrez et al., 2007) followed by 4 d washout interval between treatments to minimize carryover effects (Loor et al., 2005) (Figure 3.1).

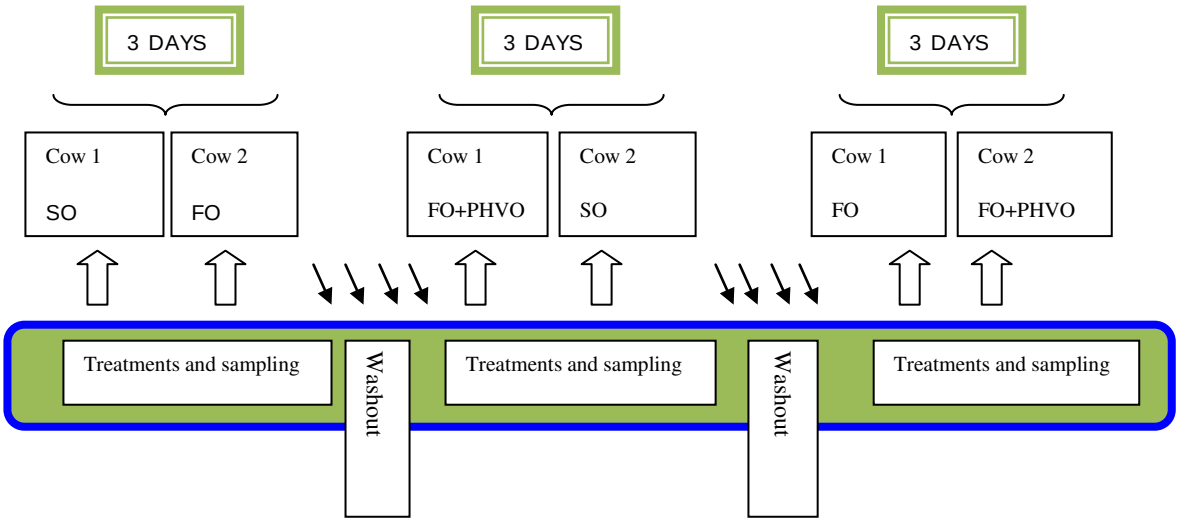


Figure 3.1: The experiment had three treatment periods, each followed by a 4-d washout interval. Cows were grouped in a 2 x 3 crossover design.

Cows were treated with ruminal pulses of: 1) soya oil (SO; 250 ml/d in 500 ml/d of skimmed milk; SM) as control; 2) FO (250 g/d in 500 ml/d of SM) and 3) FO + PHVO (125 g/d FO + 125 g/d PHVO in 500 ml/d of SM). Fish oil was supplied by manufacturers (Rossyew Scotish Ltd.) and was based on salmon oil. Emulsions were prepared the day before each ruminal pulse (Ferlay and Doreau, 1992). To provide a homogenised release of treatments, skim milk was used (500 ml/d) to emulsify the oils (FO and FO+PHVO) using a laboratory mixer emulsifier (L2R Silverson, Machines Ltd., Waterside, Chesham, Bucks). Before each pulsing time, emulsions were heated for 30 min at 60ºC using a water bath (Grant Y28, Cambridge Ltd., England). During

pulsing and sampling periods, cows were housed in tie stalls and had free access to water. Cows were fed a maintenance diet which comprised meadow hay (*ad libitum*) and a concentrate based on barley (25-40 g/kg), sugar beet pulp (8 g/kg) and wheat (8 g/kg) and cane molasses (<5 g/kg), limestone flour (<5 g/kg), vitamins (<5 g/kg) and minerals (<5 g/kg) [1-2 kg/day at 12:00; Manor Farm Feeds (Owston) Ltd., Oakham].

3.2.2 Sampling, measurements and analyses

Samples of ruminal fluid (50 ml) were collected from various parts of the rumen using a syringe screwed to a stainless tube. Ruminal fluid samples were collected prior to pulses (0 h) and at 1, and 3 h post pulsing during the 3 d collection periods. Immediately after fluid collection, 10 ml of ruminal fluid were used to determine pH by using a pH meter (Piccolo Plus, HI 1295, Hanna Instruments, Portugal). Samples were frozen (-20ºC) for later analysis. See Chapter 2 for ruminal VFA and NH_3-N sample preparation and calculations.

Blood samples (20 ml) were obtained via jugular catheter prior to the pulse (0 h), 1, and 3 h after pulse during the last day of each collection period for lipoprotein fractionation. Blood was transferred to tubes containing EDTA and immediately centrifuged for 10 min at 3,000 x *g* (Centaur 2 MSE, Milton Keynes, England) for harvesting plasma. Plasma samples for lipoprotein fractionation were obtained during the last day of each collection period. Plasma was ultracentrifuged at 39,000 x *g* for 16h at a density of 1.006 g/ml (to remove VLDL fraction) and 20 h at a density of 1.063 g/ml (to separate LDL and HDL fractions) at 12ºC using a Beckman XL-70 ultracentrifuge. Another 20 ml plasma sample were collected and stored at -20ºC for analysis of FA profile. See Chapter 2 for more details on the sequential separation of lipoprotein fractions. Concentrations of lipoprotein fractions in plasma (LDL and HDL fractions) were determined using a Clinical Chemistry Autoanalyzer (Rx Imola; Randox Laboratories, Ltd.).

Lipids from plasma, HDL, LDL, and VLDL fractions were extracted by adaptation of the method by Bligh and Dyer (1959) and methylated according to the method of Christie (1982) with modifications by Chouinard et al. (1999). For more details see Chapter 2. The lipid classes (triacylglycerol, phospholipid, and cholesteryl ester) of the lipoprotein fractions were isolated by thin layer chromatography, and their

constituent FA were analysed by capillary column GC as described previously for plasma and lipoprotein fractions. See Chapter 2 for methodology of TLC.

3.2.3. Statistical analysis

Using the GenStat 12th statistical package (VSN International Ltd., Oxford), data were analyzed as a 2 x 3 crossover design with fixed effects of treatments, periods and sampling times; cow was included as a random effect to allow for repeated measures. Fatty acid composition of lipid subgroups within lipoproteins was analysed using a Factorial design based on treatments (3), fractions (3) and lipids (3). Diurnal variations were analysed as Factorial design using POLYANOVA based on days (3) and sampling times (3). Data are reported as least square mean ± SED.

3.3 Results

3.3.1 Treatment composition

The FO/PHVO+SM blend contained higher concentrations of saturated (19 g/100g), MUFA (43 g/100g), UFA (MUFA+PUFA) (48 g/100g) and *trans* (19 g/100g) fatty acids and lower concentrations of polyunsaturated (5 g/100g) and long chain (8 g/100g) fatty acids than FO+SM. Concentrations of VA (3 g/100g) and EA (9 g/100g) were higher in FO/PHVO+SM compared with FO+SM (Table 3.1).

3.3.2 Ruminal fermentation parameters

There was no difference between treatments in ruminal NH_3-N and total VFA concentrations. Compared with control, FO and FO+PHVO resulted in higher concentrations of propionate (Table 3.2).

3.3.3 Plasma samples and treatment effect

Compared with control, all treatments resulted in lower concentration of SFA in plasma. Compared with control and FO, FO+PHVO resulted in higher concentrations of SFA in VLDL fraction; higher concentrations of MUFA in plasma and lipoprotein fractions; higher concentrations of UFA in plasma, LDL and VLDL fractions; and higher concentrations of tFA in plasma and VLDL fraction. Compared with control and FO+PHVO, FO resulted in higher concentrations of PUFA in plasma and higher concentrations of LCFA in plasma, HDL and VLDL fractions (Table 3.3).

Treatment significantly affected concentrations of some 18:1 *trans* isomers in plasma. Compared with control and FO, FO+PHVO resulted in higher concentrations of 18:1 *trans* 5, *trans* 6-8, *trans* 9, *trans* 10, *trans* 11, *trans* 12, *cis* 9, *trans* 15 and 18:2n *trans* 6 (Table 3.4).

Table 3.1 Fatty acid composition of oils and skimmed milk emulsions used for ruminal pulses

	Control	Oils		Treatments [4]		
Fatty acid (g/100 g)	SO+SM[1]	FO[2]	SO	PHVO[3]	FO+SM	FO/PHVO+SM
10:0	-	0.05	0.05	-	-	-
11:0	-	0.12	-	-	0.06	-
12:0	-	0.09	-	0.42	0.06	0.25
14:0	0.11	2.75	0.27	0.29	3.57	2.74
14:1	-	0.07	-	-	0.06	-
15:0	-	0.22	-	-	0.37	0.21
16:0	9.84	6.81	11.55	8.24	8.43	10.15
16:1	0.00	3.59	-	-	4.05	3.25
17:0	0.17	0.20	-	-	0.21	0.18
17:1	-	1.28	-	-	0.72	0.12
18:0	3.65	1.61	4.18	8.11	1.99	5.18
18:1 *trans*-4	-	-	-	0.86	-	0.13
18:1 *trans*-5	-	-	-	1.43	-	0.34
18:1 *trans*-6-8	-	-	-	-	-	0.58
18:1 *trans*-9	-	-	-	22.66	-	8.97
18:1 *trans*-10	-	-	-	-	-	3.09
18:1 *trans*-11	-	0.35	-	15.12	0.44	3.18
18:1 *trans*-12	-	0.42	-	7.25	0.50	2.92
18:1 *cis*-9	19.54	11.14	20.69	15.07	13.87	18.63
18:2 n-*trans* 6	-	-	-	0.17	-	-
18:2 n-*cis* 6	48.73	3.68	52.12	0.55	4.85	3.75
20:0	0.32	0.12	0.45	0.87	0.20	0.46
18:3 n-6	-	-	-	-	0.11	0.09
20:1	-	1.48	-	0.19	1.81	2.18
18:3 n-3	6.22	2.31	6.84	-	2.73	1.45
18:2 *cis*-9, *trans*-11	-	1.08	-	-	1.42	0.94
20:2	-	0.39	-	-	0.43	0.36
22:0	0.35	-	0.41	0.55	-	0.27
20.5 n-3	-	7.34	-	-	10.32	5.34
24:0	-	0.14	-	0.14	0.19	-
22:6 n-3	-	8.48	0.21	-	9.21	8.05
Saturated	14.45	12.11	16.86	18.61	15.08	19.45
Monounsaturated	19.54	18.32	21.00	62.59	21.44	43.39
Polyunsaturated	54.95	21.81	59.17	0.72	17.90	5.29
Unsaturated (Mono+PUFA)	74.49	40.13	80.17	63.31	29.13	48.67
Trans	-	0.76	-	47.50	0.94	19.22
Long chain fatty acids	0.63	35.04	0.63	4.02	22.86	8.25
Unidentified[5]	11.06	11.96	2.34	18.07	21.78	4.40

[1]SO+SM = Soya oil + skim milk; [2]FO = Fish oil; [3]PHVO = Partially hydrogenated vegetable oil;
[4]Single administration per day; [5]Fatty acids < 0.03 g / 100g.

Table 3.2 Effects of treatments on ruminal fermentation parameters

	Control	Treatments[6]			
Parameters	SO[1]	FO[2]	FO/PHVO[3]	SED[4]	*P*-value[5]
pH	6.3[b]	6.5[a]	6.3[b]	0.05	0.002
NH_3-N, m*M*	19	12	14	2.8	0.074
VFA, m*M*	84	81	73	9.4	0.508
Acetate	66	65	65	1.0	0.250
Propionate	29.3[b]	30.8[a]	31.6[a]	0.54	<0.001
Butyrate	16.3	17.3	16.3	0.56	0.117
Isobutyrate	1.7	1.8	1.8	0.08	0.545
Valerate	1.6	1.7	1.5	0.14	0.507
Isovalerate	2.3	2.5	2.2	0.17	0.173

[1]SO = Soya oil; [2]FO = Fish oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Standard errors of differences of means; [5]*P*-value represents the probability of a treatment effect; [6]Single administration per day; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

Table 3.3 Treatment effects on major fatty acid classes in plasma and lipoprotein fractions

		Control	Treatments[6]			
Fatty acid, g/100 g	Fraction	SO[1]	FO[2]	FO/PHVO[3]	SED[4]	*P*-value[5]
Saturated	Plasma	40.6[a]	37.6[b]	38.2[b]	0.61	<0.001
	HDL	38.5	36.7	37.	3.10	0.839
	LDL	46.6	35.9	37.3	6.82	0.287
	VLDL	44.4[a]	40.0[b]	44.6[a]	1.41	0.020
Monounsaturated	Plasma	20.8[a]	19.4[b]	24.7[a]	0.83	<0.001
	HDL	23.8[b]	20.7[c]	26.5[a]	0.75	<0.001
	LDL	16.2[b]	8.2[c]	21.1[a]	2.04	<0.001
	VLDL	17.4[c]	22.4[b]	26.8[a]	1.24	<0.001
Polyunsaturated	Plasma	24.2[b]	27.2[a]	23.9[b]	0.74	<0.001
	HDL	24.5	26.7	24.0	1.56	0.247
	LDL	14.4	10.2	12.4	2.96	0.416
	VLDL	11.5	14.5	11.5	1.50	0.141
Unsaturated	Plasma	45.1[b]	46.6[b]	48.6[a]	0.77	<0.001
(Mono+PUFA)	HDL	48.3	47.4	50.5	1.16	0.067
	LDL	30.7[a]	18.5[b]	33.5[a]	3.58	0.007
	VLDL	29.0[b]	36.9[a]	38.4[a]	2.32	0.008
Trans	Plasma	3.5[b]	3.8[b]	4.9[a]	0.57	0.046
	HDL	4.4	5.3	5.7	1.02	0.489
	LDL	3.7	2.0	6.0	1.68	0.114
	VLDL	4.8[b]	9.2[a]	10.4[a]	0.85	<0.001
Long chain fatty acids	Plasma	0.8[b]	1.1[a]	0.8[b]	0.08	<0.001
	HDL	0.7[b]	1.4[a]	0.9[b]	0.22	0.045
	LDL	2.5	3.8	1.1	1.93	0.431
	VLDL	1.3[a]	1.6[a]	0.9[b]	0.21	0.029
Unidentified	Plasma	12.3	12.6	12.0	0.59	0.349
	HDL	13.1	15.8	12.1	4.23	0.674
	LDL	7.0	7.6	13.6	8.54	0.277
	VLDL	26.4[a]	22.5[a]	16.6[b]	2.77	0.022

[1]SO = Soya oil; [2]FO = Fish oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Standard errors of differences of means; [5]*P*-value represents the probability of a treatment effect; [6]Single administration per day; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

Table 3.4 Fatty acid distribution in plasma

	Control	Treatments[6]			
Fatty acid, g/100 g	SO[1]	FO[2]	FO/PHVO[3]	SED[4]	*P*-value[5]
10:0	2.3[a]	0.8[b]	0.1[b]	0.54	<0.001
12:0	0.2	0.3	0.08	0.21	0.450
13:0	0.2	0.2	0.08	0.08	0.096
14:0	0.4	0.4	0.3	0.08	0.328
14:1	0.3[a]	0.2[b]	0.3[a]	0.06	0.041
15:0	0.7[b]	0.7[b]	0.8[a]	0.04	0.014
15:1	0.1[b]	0.1[a]	0.09[b]	0.02	0.014
16:0	14.4[a]	13.1[b]	13.9[a]	0.36	0.002
16:1	1.1[b]	1.1[b]	1.3[a]	0.07	<0.001
17:0	1.0[b]	1.2[a]	1.2[a]	0.05	0.004
17:1	0.4[a]	0.3[b]	0.5[a]	0.04	0.023
18:0	20.1	19.9	20.7	0.61	0.410
18:1 *trans*-5	0.1	0.1	0.1	0.04	0.669
18:1 *trans*-6-8	0.1[b]	0.1[b]	0.2[a]	0.01	<0.001
18:1 *trans*-9	0.3[b]	0.1[b]	0.5[a]	0.09	<0.001
18:1 *trans*-10	0.2[b]	0.2[b]	0.4[a]	0.05	<0.001
18:1 *trans*-11	1.7	1.5	1.9	0.26	0.412
18:1 *trans*-12	0.3[c]	0.4[b]	0.6[a]	0.09	0.007
18:1 *cis*-9	13.8[b]	12.4[c]	15.8[a]	0.41	<0.001
18:1 *trans*-15	1.1[b]	1.1[b]	1.2[a]	0.05	0.027
18:1 *cis*-11	0.3[b]	0.2[b]	0.4[a]	0.05	0.003
18:1 *cis*-12	0.1	0.1	0.2	0.03	0.871
18:1 *cis*-13	0.1	0.1	0.1	0.02	0.589
18:1 *cis*-14+ *trans*-15	0.2[b]	0.3[a]	0.2[b]	0.03	0.006
18:2n-*trans* 6	0.1[b]	0.1[a]	0.1[a]	0.02	0.013
18:2n-*cis* 6	11.0	11.5	10.8	0.53	0.455
20:0	0.1	0.09	0.1	0.03	0.043
18:3n6	0.07	0.1	0.1	0.01	0.004
20:1	0.05[b]	0.1[a]	0.1[a]	0.02	<0.001
18:3n3	1.7	1.7	1.7	0.07	0.595
18:2 *cis*-9, *trans*-11	0.3[a]	0.2[c]	0.3[b]	0.01	<0.001
20:2	0.1	0.2	0.2	0.04	0.421
22:0	0.1	0.1	0.1	0.02	0.094
20:3n6	2.4[a]	2.7[a]	2.1[b]	0.15	<0.001
22:1n9	0.08[b]	0.09[b]	0.1[a]	0.02	0.011
20:3n3	0.00	0.02	0.04	0.01	0.175
20:4n6	6.0[b]	6.6[a]	5.9[b]	0.18	0.001
23:0	0.2	0.1	0.2	0.07	0.807
22:2	0.2	0.2	0.1	0.09	0.302
24:0	0.00[b]	0.06[a]	0.05[a]	0.01	0.008
20:5n3	1.5[b]	2.6[a]	1.6[b]	0.18	<0.001
24:1	0.4	0.4	0.3	0.05	0.168
22:6n3	0.8[b]	1.1[a]	0.8[b]	0.08	<0.001

[1]SO = Soya oil; [2]FO = Fish oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Standard errors of differences of means; [5]*P*-value represents the probability of a treatment effect; [6]Single administration per day; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

3.3.4 Lipoprotein fractions and treatments effects

No effect of treatments was observed on plasma concentrations of lipoprotein fractions (Table 3.5). Compared with control and FO, FO+PHVO resulted in higher ($P < 0.05$) concentrations of 18:1 *trans* 9 and *trans* 12 in the HDL fraction (Table 3.6). Compared with control and FO, FO+PHVO resulted in higher ($P < 0.05$) concentrations of EA in the LDL fraction (Table 3.7). Compared with control and FO, FO+PHVO resulted in higher ($P < 0.05$) concentrations of 18:1 *trans* 5, *trans* 9 and *trans* 12 in the VLDL fraction (Table 3.8).

Significant interactions ($P < 0.05$) between treatments and lipoprotein fractions were observed in concentrations of 18:1 *trans* 4 and *trans* 11. Compared with control and FO+PHVO, FO resulted in higher concentrations of 18:1 *trans* 4 in the HDL and LDL fractions. Compared with control and FO+PHVO, FO resulted in higher concentrations of 18:1 *trans* 11 in the HDL and VLDL fractions (Table 3.9).

Table 3.5 Concentrations of HDL and LDL+VLDL fractions in plasma

	Control	Treatments[6]			
Parameters (mg/l)	SO[1]	FO[2]	FO/PHVO[3]	SED[4]	*P*-value[5]
HDL	0.9	0.9	1.0	0.06	0.230
LDL+VLDL	0.3	0.2	0.2	0.02	0.107

[1]SO = Soya oil; [2]FO = Fish oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Standard errors of differences of means; [5]*P*-value represents the probability of a treatment effect; [6]Single administration per day.

Table 3.6 Fatty acid distribution in the HDL fraction

	Control	Treatments[6]			
Fatty acid, g/100 g	SO[1]	FO[2]	FO/PHVO[3]	SED[4]	*P*-value[5]
10:0	0.1	0.1	0.1	0.02	0.554
11:0	0.09	0.1	0.09	0.00	0.171
12:0	0.1	0.1	0.1	0.03	0.781
13:0	0.1	0.1	0.1	0.03	0.241
14:0	0.2	0.1	0.1	0.02	0.503
14:1	0.3	0.2	0.3	0.20	0.996
15:0	0.9	0.9	0.9	0.08	0.880
15:1	0.5	0.4	0.5	0.39	0.988
16:0	13.7	12.9	13.3	1.12	0.771
16:1	0.9	1.0	1.1	0.16	0.398
17:0	0.9	1.2	1.0	0.24	0.387
17:1	0.4	0.3	0.2	0.10	0.346
18:0	20.7	19.1	19.8	1.88	0.719
18:1 *trans*-4	0.2	0.6	0.2	0.27	0.287
18:1 *trans*-5	0.8	0.5	0.8	0.80	0.939
18:1 *trans*-9	0.2[b]	0.2[b]	0.5[a]	0.04	<0.001
18:1 *trans*-11	1.5	1.8	1.5	0.20	0.201
18:1 *trans*-12	0.3[b]	0.5[a]	0.6[a]	0.10	0.017
18:1 *cis*-9	15.7[a]	11.7[b]	17.1[a]	1.62	0.026
18:1 *trans*-15	1.1	1.3	1.3	0.14	0.388
18:1 *cis*-11	0.4[a]	0.2[b]	0.5[a]	0.06	0.004
18:1 *cis*-12	0.4	0.3	0.3	0.13	0.730
18:1 *cis*-13	-	0.2	0.2	0.02	0.074
18:2n-*trans* 6	0.02	0.07	0.06	0.05	0.543
18:2n-*cis* 6	10.9	9.3	9.3	0.83	0.164
20:0	0.02	0.02	0.06	0.04	0.554
18:3n6	0.7	0.3	0.5	0.73	0.831
20:1	0.1	0.1	0.04	0.06	0.103
18:3n3	1.3	1.2	1.3	0.11	0.417
18:2 *cis*-9, *trans*-11	0.4[a]	0.2[b]	0.5[a]	0.06	0.013
20:2	0.2[b]	0.5[a]	0.4[b]	0.09	0.013
22:0	0.1[a]	0.1[a]	0.1[b]	0.01	0.010
20:3n6	2.6	3.0	2.3	0.25	0.079
22:1n9	0.3	0.4	0.1	0.19	0.361
20:3n3	-	0.03	0.2	0.18	0.438
20:4n6	6.1	6.4	5.8	0.55	0.621
23:0	0.9	1.3	0.7	0.35	0.367
22:2	0.6	1.1	1.1	0.44	0.398
24:0	0.03	0.04	0.03	0.03	0.924
20:5n3	1.3[b]	3.0[a]	1.7[b]	0.35	0.004
24:1	0.2	0.2	0.2	0.11	0.983
22:6n3	0.7[b]	1.4[a]	0.9[b]	0.21	0.045

[1]SO = Soya oil; [2]FO = Fish oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Standard errors of differences of means; [5]*P*-value represents the probability of a treatment effect; [6]Single administration per day; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

Table 3.7 Fatty acid distribution in the LDL fraction

	Control	Treatments[6]			
Fatty acid, g/100 g	SO[1]	FO[2]	FO/PHVO[3]	SED[4]	*P*-value[5]
10:0	2.3[b]	4.2[a]	2.0[b]	0.60	0.004
13:0	2.0	2.6	0.7	1.76	0.634
14:0	1.3	1.3	0.3	1.22	0.682
15:0	9.6	13.0	5.5	3.55	0.168
16:0	12.3[a]	6.9[b]	12.3[a]	1.65	0.017
16:1	0.5[a]	0.00[b]	0.8[a]	0.22	0.015
17:0	0.3	0.1	0.7	0.27	0.117
18:0	19.7[a]	8.0[b]	16.9[a]	2.77	0.007
18:1 *trans*-4	0.1	1.3	0.4	0.24	0.066
18:1 *trans*-9	0.1[b]	0.1[b]	0.2[a]	0.18	0.004
18:1 *trans*-11	3.1	1.0	2.4	1.17	0.235
18:1 *trans*-12	0.5	0.8	0.9	0.06	0.052
18:1 *cis*-9	9.1[a]	3.1[b]	11.1[a]	1.32	<0.001
18:1 *trans*-15	0.9	0.4	0.9	0.11	0.053
18:1 *cis*-11	0.5	0.5	0.5	0.21	0.753
18:2n-*cis* 6	6.5[a]	1.7[c]	5.0[b]	0.98	0.003
18:3n6	1.7	1.5	0.5	0.85	0.571
20:1	-	-	0.1	0.07	0.250
18:3n3	0.8[a]	0.00[b]	0.6[a]	0.15	<0.001
20:2	0.1	0.2	0.2	0.17	0.971
22:0	-	-	0.1	0.08	0.169
20:3n6	0.7[b]	0.1[b]	1.1[a]	0.30	0.037
22:1n9	0.4	0.7	0.2	0.37	0.510
20:3n3	-	-	0.3	0.23	0.246
20:4n6	2.5	2.1	2.2	1.07	0.926
23:0	0.4	0.8	0.1	0.49	0.428
22:2	0.1	0.6	0.4	0.34	0.415
24:0	0.02	0.0	0.03	0.05	0.853
20:5n3	0.3	0.4	0.9	0.39	0.379
24:1	2.4	2.4	2.0	0.84	0.910
22:6n3	2.5	3.8	1.1	1.94	0.431

[1]SO = Soya oil; [2]FO = Fish oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Standard errors of differences of means; [5]*P*-value represents the probability of a treatment effect; [6]Single administration per day; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

Table 3.8 Fatty acid distribution in the VLDL fraction

	Control	Treatments[6]			
Fatty acid, g/100 g	SO[1]	FO[2]	FO/PHVO[3]	SED[4]	*P*-value[5]
10:0	0.7	0.5	0.3	0.16	0.116
11:0	0.7	0.3	0.4	0.13	0.077
12:0	1.2[a]	0.6[b]	0.6[b]	0.20	0.018
13:0	0.6[a]	0.2[b]	0.3[b]	0.11	0.037
14:0	1.5	1.4	1.4	0.19	0.965
15:0	6.0[a]	3.4[b]	3.6[b]	0.81	0.022
15:1	0.5	0.5	0.7	0.15	0.600
16:0	14.0	15.3	16.7	0.80	0.032
16:1	1.6	1.6	1.7	0.22	0.870
17:0	0.3[b]	1.0[a]	0.9[a]	0.22	0.038
17:1	0.07	0.1	0.2	0.11	0.241
18:0	16.2	14.5	17.1	1.44	0.254
18:1 *trans*-4	1.8[a]	0.9[b]	0.9[b]	0.31	0.030
18:1 *trans*-5	0.3[b]	0.3[b]	1.4[a]	0.15	0.027
18:1 *trans*-9	0.1[b]	0.7[b]	1.3[a]	0.33	0.028
18:1 *trans*-11	2.7[b]	4.6[a]	3.9[a]	0.55	0.020
18:1 *trans*-12	0.7[c]	1.1[b]	1.4[a]	0.10	0.002
18:1 *cis*-9	7.7[b]	8.5[b]	11.9[a]	0.91	0.004
18:1 *trans*-15	1.5	1.4	1.4	0.04	0.055
18:1 *cis*-11	0.3	0.3	0.4	0.07	0.488
18:1 *cis*-12	0.1	0.3	0.4	0.03	0.129
18:2n-*cis* 6	4.3	4.6	4.2	0.63	0.838
20:0	0.7	0.6	1.1	0.62	0.670
18:3n6	1.7[a]	0.7[b]	0.8[b]	0.32	0.035
20:1	0.01[c]	0.7[a]	0.3[b]	0.12	0.001
18:3n3	0.5[b]	1.2[a]	1.0[a]	0.19	0.018
20:2	0.2	0.3	0.08	0.13	0.224
22:0	0.06	0.2	0.3	0.09	0.081
20:3n6	1.0	1.0	0.7	0.53	0.752
22:1n9	1.5[a]	0.7[b]	0.4[b]	0.30	0.014
20:3n3	0.1[b]	0.4[a]	0.5[a]	0.13	0.031
20:4n6	1.9	2.3	1.9	0.40	0.535
23:0	0.8	0.7	0.9	0.48	0.883
22:2	0.1	0.5	0.2	0.15	0.106
24:0	-	1.0	0.02	0.03	0.059
20:5n3	0.3[b]	2.0[a]	1.1[b]	0.37	0.005
24:1	1.2[a]	0.6[b]	0.4[b]	0.25	0.027
22:6n3	1.3[a]	1.6[a]	0.9[b]	0.21	0.029

[1]SO = Soya oil; [2]FO = Fish oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Standard errors of differences of means; [5]*P*-value represents the probability of a treatment effect; [6]Single administration per day; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

Table 3.9 Interactions between treatments and lipoprotein fractions for concentrations of 18:1 *trans* isomers

		Control	Treatments[6]			
Fatty acid g/100 g	Fraction	SO[1]	FO[2]	FO/PHVO[3]	SED[4]	*P*-value[5]
18:1 *trans*-4	HDL	0.2[b]	0.6[a]	0.2[b]	0.30	0.049
	LDL	0.7[b]	1.1[a]	0.4[b]		
	VLDL	1.8[a]	0.9[b]	0.9[b]		
18:1 *trans*-5	HDL	0.8	0.5	0.8	0.64	0.254
	LDL	-	-	0.8		
	VLDL	-	0.2	1.5		
18:1 *trans*-9	HDL	0.2	0.2	0.5	0.21	0.089
	LDL	0.08	0.08	0.8		
	VLDL	0.1	0.7	1.3		
18:1 *trans*-11	HDL	1.5[b]	1.8[a]	1.5[b]	0.75	0.014
	LDL	3.1[a]	1.0[b]	2.4[b]		
	VLDL	2.7[b]	4.6[a]	3.9[a]		
18:1 *trans*-12	HDL	0.3	0.5	0.6	0.15	0.270
	LDL	0.5	0.5	0.9		
	VLDL	0.7	1.1	1.4		
18:1 *trans*-15	HDL	1.1	1.3	1.3	0.13	0.407
	LDL	0.8	0.8	0.9		
	VLDL	1.4	1.4	1.4		
18:2n-*trans* 6	HDL	0.01	0.07	0.06	0.05	0.228
	LDL	0.1	0.2	0.2		
	VLDL	0.3	0.3	0.4		

[1]SO = Soya oil; [2]FO = Fish oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Standard errors of differences of means; [5]*P*-value represents the probability of fraction-treatment effect; [6]Single administration per day; [a,b,c] Means in the same row with different superscripts are different ($P<0.05$).

3.3.5 Total amount of *trans* fatty acids in lipoprotein fractions

Compared with control and FO, FO+PHVO resulted in higher ($P < 0.05$) total amount (mg/l) of *trans* fatty acids in the VLDL fraction (Table 3.10).

Table 3.10 Treatment effects on *trans* fatty acids in lipoprotein fractions

Fatty acid	Fraction	SO[1]	FO[2]	FO/PHVO[3]	SED[4]	*P*-value[5]
mg/l	HDL	26	31	33	1.1	0.315
	LDL	7	3	14	1.0	0.110
	VLDL	9[c]	29[b]	33[a]	1.8	0.032

[1]SO = Soya oil; [2]FO = Fish oil; [3]PHVO = Partially hydrogenated vegetable oil; [4]Standard errors of differences of means; [5]*P*-value represents the probability of a treatment effect; [6]Single administration per day.

3.3.6 Fatty acid composition of lipid subgroups within lipoproteins

Concentrations (mg/l) of total lipid were not affected by treatment but were higher in HDL-TG. 16:0 was not affected by treatments but higher in VLDL-CE. 18:0 was higher in LDL than HDL and VLDL fractions. 18:1 *trans*-9 was not affected by treatments but higher in LDL-CE. 18:1 *trans*-11 was not affected by treatments but higher in TG than CE and PL subgroups. Compared with control and FO, FO+PHVO increased the concentration of oleic acid in HDL-PL (Table 3.11a).

18:2 *trans*-6 was not affected by treatments but higher in CE than TG and PL subgroups. 18:2 *cis*-6 was not affected by treatments but higher in HDL-TG. 18:3n6 was not affected by treatments but higher in CE than in TG and PL subgroups. 18:3n3 was not affected by treatments but higher in HDL-TG.Compared with control and FO+PHVO, FO increased 20:3n6 in LDL-CE and increased 20:5n3 in LDL-TG. Compared with FO and FO+PHVO, SO increased 22:6n3 in VLDL-CE (Table 3.11b).

Table 3.11a Lipid concentration and fatty acid content (g/100g) in lipid subgroups of each lipoprotein fraction of plasma from cows subjected to ruminal pulses

					SED	*P*-value
Total lipid	Treatment	SO	FO	FO+PHVO		
(mg / ml)		573	675	683	50.6	0.056
	Fraction	HDL	LDL	VLDL		
		714[a]	586[b]	631[b]	50.6	0.041
	Lipid	CE	PL	TG		
		658[b]	406[c]	867[a]	50.6	<0.001
16:0	Treatment	SO	FO	FO+PHVO		
		20.3	20.9	20.3	0.69	0.604
	Fraction	HDL	LDL	VLDL		
		20.1[b]	19.9[c]	21.5[a]	0.69	0.038
	Lipid	CE	PL	TG		
		22.3[a]	19.9[b]	19.3[b]	0.69	<0.001
18:0	Treatment	SO	FO	FO+PHVO		
		24.6[a]	23.0[b]	23.0[b]	0.66	0.022
	Fraction	HDL	LDL	VLDL		
		21.3[c]	26.0[a]	23.2[b]	0.66	<0.001
	Lipid	CE	PL	TG		
		24.3	23.4	22.9	0.66	0.098
18:1	Treatment	SO	FO	FO+PHVO		
trans 9		1.7	2.0	1.7	0.18	0.260
	Fraction	HDL	LDL	VLDL		
		1.9[b]	2.2[a]	1.3[c]	0.18	<0.001
	Lipid	CE	PL	TG		
		2.6[a]	1.0[c]	1.9[b]	0.18	<0.001
18:1	Treatment	SO	FO	FO+PHVO		
trans 11		2.3	2.3	1.9	0.19	0.172
	Fraction	HDL	LDL	VLDL		
		2.1	2.1	2.3	0.19	0.614
	Lipid	CE	PL	TG		
		2.4[b]	0.9[c]	3.1[a]	0.19	<0.001
18:1 *cis* 9	Treatment	SO	FO	FO+PHVO		
		5.7[b]	5.4[b]	6.7[a]	0.35	<0.001
	Fraction	HDL	LDL	VLDL		
		7.1[a]	6.4[b]	4.4[c]	0.35	<0.001
	Lipid	CE	PL	TG		
		2.8[c]	10.0[a]	5.1[b]	0.35	<0.001

[1]SO = Soya oil; [2]FO = Fish oil; [3]FO+PHVO = Partially hydrogenated vegetable oil; HDL = High density lipoprotein; LDL = Low density lipoprotein; VLDL = Very low density lipoprotein. CE = Cholesteryl ester; TC = triglyceride; PL = Phospholipid; [a,b,c] Means down column with different superscripts are different (P<0.05).

Table 3.11b Fatty acid content (g/100g) in lipid subgroups of each lipoprotein fraction of plasma from cows subjected to ruminal pulses

					SED	*P*-value
18:2	Treatment	SO	FO	FO+PHVO		
trans 6		2.0	1.9	1.9	0.14	0.530
	Fraction	HDL	LDL	VLDL		
		1.9	2.0	2.0	0.14	0.438
	Lipid	CE	PL	TG		
		2.7[a]	1.7[b]	1.5[c]	0.14	<0.001
18:2 *cis* 6	Treatment	SO	FO	FO+PHVO		
		4.4	4.8	5.1	0.45	0.348
	Fraction	HDL	LDL	VLDL		
		9.7[a]	4.8[b]	0.1[c]	0.45	<0.001
	Lipid	CE	PL	TG		
		0.8[c]	5.4[b]	8.1[a]	0.45	<0.001
18:3n6	Treatment	SO	FO	FO+PHVO		
		2.7	3.0	2.4	0.43	0.292
	Fraction	HDL	LDL	VLDL		
		2.6	3.2	2.3	0.43	0.075
	Lipid	CE	PL	TG		
		4.0[a]	1.4[c]	2.6[b]	0.43	<0.001
18:3n3	Treatment	SO	FO	FO+PHVO		
		2.0	2.1	2.1	0.09	0.688
	Fraction	HDL	LDL	VLDL		
		3.2[a]	1.8[b]	1.2[c]	0.09	<0.001
	Lipid	CE	PL	TG		
		2.3[b]	0.8[c]	3.2a	0.09	<0.001
20:3n6	Treatment	SO	FO	FO+PHVO		
		1.6[b]	1.7[a]	1.4[c]	0.08	0.010
	Fraction	HDL	LDL	VLDL		
		1.6[b]	1.9[a]	1.2[c]	0.08	<0.001
	Lipid	CE	PL	TG		
		2.1[a]	1.3[c]	1.4[b]	0.08	<0.001
20:5n3	Treatment	SO	FO	FO+PHVO		
		1.1[c]	1.7[a]	1.1[b]	0.07	<0.001
	Fraction	HDL	LDL	VLDL		
		1.5[a]	1.5[a]	0.8[b]	0.07	<0.001
	Lipid	CE	PL	TG		
		1.3[b]	1.1[c]	1.4[a]	0.07	<0.001
22:6n3	Treatment	SO	FO	FO+PHVO		
		4.9[a]	3.0[b]	3.9[b]	0.48	0.001
	Fraction	HDL	LDL	VLDL		
		1.3[c]	3.2[b]	7.3[a]	0.48	<0.001
	Lipid	CE	PL	TG		
		4.3[a]	4.0[b]	3.4[b]	0.48	0.145

[1]SO = Soya oil; [2]FO = Fish oil; [3]FO+PHVO = Partially hydrogenated vegetable oil; HDL = High density lipoprotein; LDL = Low density lipoprotein; VLDL = Very low density lipoprotein. CE = Cholesteryl ester; TC = triglyceride; PL = Phospholipid; [a,b,c] Means down column with different superscripts are different (P<0.05).

3.3.7 Diurnal and daily variations in plasma and lipoprotein fractions on *trans* fatty acids

In general, sampling day affected tFA of plasma and lipoprotein fractions after cows were ruminally pulsed. Plasma concentrations of all 18:1 *trans* isomers linearly increased and were higher on Day 3 than on Day 1 and 2 except for 18:1 *trans*-9. Plasma concentrations of all 18:1 *trans* isomers were not affected by time of sampling (Table 3.12). HDL concentrations of all 18:1 *trans* isomers were not affected by time of sampling (Table 3.13). LDL concentrations of all 18:1 *trans* isomers were not affected by time of sampling except for 18:1 *trans*-9 (Table 3.14). VLDL concentrations of all 18:1 *trans* isomers were not affected by time of sampling except for 18:1 *trans*-12 (Table 3.15).

Table 3.12 Day and sampling time (h) effects in plasma *trans* fatty acids

Fatty acid, g/100 g					SED[3]	*P*-value[4]	(Lin)[5]	(Quad)[6]
18:1 *trans*-9	D[1]	1	2	3				
		0.2[c]	0.2[b]	0.3[a]	0.03	0.049	0.028	0.247
	T[2]	09:00	10:00	12:00				
		0.2	0.3	0.2	0.03	0.321	0.302	0.271
18:1 *trans*-10	D	1	2	3				
		0.2[c]	0.2[b]	0.3[a]	0.03	0.012	0.004	0.620
	T	09:00	10:00	12:00				
		0.2	0.2	0.3	0.03	0.621	0.358	0.757
18:1 *trans*-11	D	1	2	3				
		1.1[c]	1.7[b]	2.2[a]	0.09	<0.001	<0.001	0.602
	T	09:00	10:00	12:00				
		1.6	1.7	1.7	0.09	0.774	0.528	0.742
18:1 *trans*-12	D	1	2	3				
		0.3[c]	0.4[b]	0.6[a]	0.03	<0.001	<0.001	0.765
	T	1	2	3				
		0.4	0.4	0.4	0.03	0.971	0.855	0.876
18:2 n-*trans* 6	D	1	2	3				
		0.06[c]	0.08[b]	0.13[a]	0.01	0.004	0.001	0.340
	T	09:00	10:00	12:00				
		0.08	0.09	0.12	0.01	0.273	0.113	0.805

[1]Day; [2]Time; [3]Standard errors of differences of means; [4]*P*-value represents the probability of a treatment effect; [5]Linear effect; [6]Quadratic effect; Samples were taken during the 3 d collection period

Table 3.13 Sampling time (h) effect in the HDL fraction *trans* fatty acids

Fatty acid, g/100 g				SED[1]	*P*-value[2]	(Lin)[5]	(Quad)[6]
18:1 *trans*-4	09:00	10:00	12:00				
	0.2	0.3	0.6	0.20	0.441	0.233	0.708
18:1 *trans*-5	09:00	10:00	12:00				
	0.8	0.1	1.1	0.79	0.497	0.522	0.330
18:1 *trans*-9	09:00	10:00	12:00				
	0.3	0.3	0.3	0.03	0.896	0.702	0.801
18:1 *trans*-11	09:00	10:00	12:00				
	1.6	1.7	1.6	0.16	0.817	0.871	0.548
18:1 *trans*-12	09:00	10:00	12:00				
	0.4	0.5	0.5	0.06	0.677	0.413	0.778
18:2 n-*trans* 6	09:00	10:00	12:00				
	0.06	0.02	0.06	0.042	0.606	0.866	0.336

[1]Standard errors of differences of means; [2]*P*-value represents the probability of a treatment effect; [5]Linear effect; [6]Quadratic effect; Samples were taken at the last day of each collection period

Table 3.14 Sampling time (h) effect in the LDL fraction *trans* fatty acids

Fatty acid, g/100 g				SED[1]	*P*-value[2]	(Lin)[5]	(Quad)[6]
18:1 *trans*-4	09:00	10:00	12:00				
	0.7	0.7	0.4	0.15	0.472	0.307	0.553
18:1 *trans*-9	09:00	10:00	12:00				
	0.3[b]	0.1[c]	0.5[a]	0.08	0.041	0.061	0.060
18:1 *trans*-11	09:00	10:00	12:00				
	2.1	1.8	2.6	0.43	0.447	0.365	0.379
18:1 *trans*-12	09:00	10:00	12:00				
	0.7	0.5	0.9	0.06	0.110	0.117	0.094

[1]Standard errors of differences of means; [2]*P*-value represents the probability of a treatment effect; [5]Linear effect; [6]Quadratic effect; Samples were taken at the last day of each collection period

Table 3.15 Sampling time (h) effect in the VLDL fraction *trans* fatty acids

Fatty acid, g/100 g				SED[1]	*P*-value[2]	(Lin)[5]	(Quad)[6]
18:1 *trans*-4	09:00	10:00	12:00				
	1.2	1.1	1.3	0.23	0.894	0.739	0.747
18:1 *trans*-5	09:00	10:00	12:00				
	0.9	0.8	0.7	0.12	0.585	0.375	0.746
18:1 *trans*-9	09:00	10:00	12:00				
	0.6	0.8	0.6	0.12	0.583	0.814	0.323
18:1 *trans*-11	09:00	10:00	12:00				
	3.7	3.9	3.7	0.45	0.946	0.922	0.757
18:1 *trans*-12	09:00	10:00	12:00				
	1.1[b]	1.1[a]	0.8[c]	0.04	0.004	0.002	0.077

[1]Standard errors of differences of means; [2]*P*-value represents the probability of a treatment effect; [5]Linear effect; [6]Quadratic effect; Samples were taken at the last day of each collection period

3.3.8 Diurnal and daily variations in ruminal fermentation parameters

Ruminal pH, NH_3-N and total VFA were not affected by time of sampling. Molar concentration of propionate linearly increased and was greater at 12:00 than at other times of day; molar concentration of butyrate and valerate linearly decreased and were greater at 09:00 than at other times of day; molar concentration of isobutyrate had quadratic effect and was higher at 09:00 than at other times of day; but molar concentration of acetate and isovalerate were not affected by time of sampling (Table 3.16).

Table 3.16 Day and time (h) effects on ruminal fermentation parameters

Parameters					SED[3]	*P*-value[4]	(Lin)[5]	(Quad)[6]
pH	T[1]	09:00	10:00	12:00				
		6.45	6.40	6.40	0.054	0.529	0.425	0.505
NH_3-N, m*M*	D[2]	1	2	3				
		15.2	13.5	15.5	2.81	0.742	0.930	0.445
	T	09:00	10:00	12:00				
		11.00	15.80	16.40	2.81	0.072	0.040	0.292
Total VFA, m*M*	T	09:00	10:00	12:00				
		76.2	85.8	76.7	8.62	0.461	0.854	0.221
Acetate	T	09:00	10:00	12:00				
		65.7	65.8	64.7	0.68	0.482	0.274	0.613
Propionate	T	09:00	10:00	12:00				
		29.2[c]	30.7[b]	31.9[a]	0.39	<0.001	<0.001	0.258
Butyrate	T	09:00	10:00	12:00				
		17.6[a]	16.7[b]	15.5[c]	0.33	<0.001	<0.001	0.697
Isobutyrate	T	09:00	10:00	12:00				
		1.9[a]	1.6[c]	1.7[b]	0.06	0.005	0.023	0.017
Valerate	T	09:00	10:00	12:00				
		1.8[a]	1.6[b]	1.5[c]	0.09	0.032	0.015	0.322
Isovalerate	T	09:00	10:00	12:00				
		2.4	2.2	2.2	0.12	0.447	0.300	0.465

[1]Time; [2]Day; [3]Standard errors of differences of means; [4]*P*-value represents the probability of a treatment effect; [5]Linear effect; [6]Quadratic effect; Samples analyzed for pH and VFA samples were taken at the last day of each collection period

3.4 Discussion

The current experiment focused on the transport of tFA by bovine plasma VLDL fraction (that also contained chylomicrons - lipoprotein derived from the intestine) because the previous experiment demonstrated that pulses of PHVO and SO increased tFA content in both HDL and LDL fractions. Further fractionation was required to distinguish between true LDL and VLDL fractions that are present in the LDL fraction. The approach was similar to the previous experiment; hence ruminal pulses were used to study effects of dietary lipids on plasma and lipoprotein fractions. As in Chapter 2, the results may differ from other studies because the treatments did not bypass the rumen and hence the biohydrogenation process had an extensive effect on concentrations of FA observed in plasma and lipoprotein fractions. The main hypothesis was that differences in FA concentrations would be observed, particularly in 18:1 *trans* isomers and 18:2 *cis*-9, *trans*-11. This hypothesis was supported by the differences in concentrations 18:1 tFA isomers and 18:2 *cis*-9, *trans*-11 in plasma and lipoprotein fractions, particularly in the VLDL fraction.

This experiment was the first to compare the response to ruminal pulses of PHVO, FO and their blend on concentrations of individual tFA isomers in lipoprotein fractions of bovine plasma. The use of PHVO was discussed in Chapter 2; however, FO was used in this experiment to increase the formation of 18:1 *trans* isomers in the rumen. FO is also interesting because it is often included in ruminant diets to increase the content of long chain n-3 PUFA (22:6n-3 and 20:5n-3; DHA and EPA) for human health reasons (vascular and heart diseases, immune and inflammatory responses and in early development) (Offer et al, 2001; Loor et al., 2005).

In the current experiment, differences in 18:1 *trans* isomers in plasma and lipoprotein fractions are in agreement with the previous experiment and with observations by Loor and Herbein (2001) in which a mixture of CLA (41% 18:2 *cis*-9, *trans*-11 and 44% 18:2 *trans*-10,*cis*-12) was ruminally infused causing alterations in plasma and milk fatty acid profiles. Concentrations of CLA are higher in milk fat from cows offered fresh compared with conserved forages, and can also be increased using whole oilseeds (e.g. SO) or oil supplements (e.g. FO and PHVO) (Griinari and Bauman, 1999), but larger responses have been obtained using FO (Offer et al., 1999). The response mechanism remains unclear, but FO has been shown to increase 18:1 *trans*

isomers in milk fat and the flow of 18:1 tFA entering the duodenum (Wonsil et al., 1994), suggesting that FO modifies biohydrogenation of dietary PUFA in the rumen (Shingfield et al., 2003).

In the current experiment, compared with PHVO, FO increased VA in plasma and lipoprotein fractions. That response is in agreement with AbuGhazaleh et al. (2007) who compared the effect of FO and sunflower oil supplementation on milk CLA *cis*-9, *trans*-11 when dairy cows were managed on pasture or in confinement. They found that FO and sunflower oil supplementation resulted in higher milk concentrations and yields of VA, CLA *cis*-9, *trans*-11 and lower 18:1 *trans*-10 in pasture-fed cows compared with confinement-fed cows. Likewise, Cruz-Hernandez et al. (2007) evaluated different levels of inclusion of sunflower oil (15, 30 and 45 g/kg of DM) and a constant level of FO (5 g/kg of DM) to increase VA and RA in milk and examine the content and composition of CLA and 18:1 *trans* isomers. They found that supplementation with sunflower oil and FO reduced milk fat but milk protein and lactose levels were not affected. There was a linear decrease in all short- and medium-chain SFA in milk fat after 10 d and a corresponding linear increase in total 18:1 *trans* isomers and total CLA.

In this experiment, 18:2 *cis*-9, *trans*-11 was detected in FO and FO+PHVO, earlier studies revealed that CLA content in milk fat is highest when diets are supplemented with a blend of FO and linoleic acid-rich oils. Cows on pasture diets have been shown to have higher concentrations of CLA (Kelly et al., 1998; Lawless et al., 1998; White et al., 2001; Boken et al., 2005) in their milk than those consuming conserved forages. It has also been shown that milk CLA concentration can be enhanced in pasture grazing cows when their feed is supplemented with oils (Lawless et al., 1998; Boken et al., 2005; AbuGhazaleh and Holmes, 2007). Inclusion of FO in total mix ratios is an effective means of increasing milk CLA *cis*-9, *trans*-11 content (Donovan et al., 2000; Whitlock et al., 2002). This appears to occur because of the inhibitory effect of docosahexaenoic acid (DHA) in FO on VA reduction to 18:0 (AbuGhazaleh and Jenkins, 2004). Milk CLA *cis*-9, *trans*-11 response to FO is further enhanced when given in combination with linoleic or linolenic acid-rich oil sources (AbuGhazaleh et al., 2002, 2003). AbuGhazaleh et al. (2003) concluded that supplementing dairy cow

diets with a combination of FO and linoleic acid-rich oils is the most effective dietary regime to increase milk CLA *cis*- 9, *trans*-11 content.

Castaneda-Gutierrez et al. (2005) demonstrated that when Ca salts of FO are fed through a rumen fistula, the negative effects of FO on DM intake and lactation performance were decreased compared to feeding the free oil. In that study, infusion of FO into the rumen clearly suppressed DM intake and milk fat yield compared with abomasal infusion of FO or feeding of Ca salts of FO. Their data indicated that Ca salts of FO minimized the negative effects of FO on rumen fermentation. When FO was supplemented unprotected, at 145 g/day (Castaneda-Gutierrez et al., 2005), 206 g/day (Petit et al., 2002) or 290 to 612 g/day (Donovan et al., 2000), milk fat content and yield were depressed. Donovan et al. (2000) demonstrated a linear negative effect of FO (added at 0, 10, 20 and 30 g/kg of diet DM) on milk fat content and yield. FO supplementation greater than 10 g/kg of DM (290 g/day) decreased DM intake and milk production, although cows fed 10 g/kg FO produced more milk than cows not supplemented with FO. Moreover, Juchem et al., (2008) reported that negative effects of FO on DM intake and yields of milk and milk fat can be modulated by two factors. Firstly, when FO is protected; this is likely to minimize its effects on the rumen microflora (Castaneda-Gutierrez et al., 2005). Secondly, the amount of FO supplementation; in their experiments, cows were fed FO at approximately 80 g/day (3.2 g/kg diet DM), so the amount of FO consumed by cows was significantly less than that demonstrated to affect performance (Donovan et al., 2000). It has been also reported that along with CLA *cis*-9, *trans*-11 the concentration of VA, a substrate for endogenous synthesis of CLA *cis*-9, *trans*-11 in human tissues (Pariza et al., 2001), also increased in milk fat in response to dietary FO (Chilliard et al., 2001; Loor et al., 2005).

Studies have identified that availability of 18:0 for endogenous 18:1 *cis*-9 production seems to be crucial for milk fat synthesis (Donovan et al., 2000; Chilliard et al., 2003; Loor et al., 2005). Furthermore, the reduction in 18:0 availability seems to be the strongest association among all previous studies dealing with milk fat depression induced by FO supplementation (Chilliard et al., 2001; Donovan et al., 2000; Shingfield et al., 2003; Loor et al., 2005; Gama et al., 2008). Inhibition of the reduction of 18:1 *trans* isomers to 18:0 by FO could be related to greater ruminal

protozoa numbers or toxic effects of DHA and eicosapentaenoic acid (EPA), which can in turn lower concentrations of ruminal bacteria associated not only with the final reduction step but also with the overall process of biohydrogenation (Loor et al., 2005). Moreover, at a given dose of incorporation, marine oils or algae rather than unsaturated vegetable oils in dairy diets seem to be more effective for increasing milk CLA *cis*-9, *trans*-11 concentration (Chilliard et al., 2000).

3.4.1 Fatty acid profile of plasma and lipoprotein fractions

As discussed in Chapter 2, sampling day and time affected 18:1 tFA of plasma. Generally, sampling day had linear effect on 18:1 *trans* isomers on plasma. On the other hand, only 18:1 *trans*-12 from VLDL fraction had linear effect on sampling time. With a single administration, concentrations of treatments are higher during the first few hours after pulse and lower in subsequent hours (Ferlay and Doreau, 1992). In this study, effects of treatments on tFA profile of plasma and lipoprotein fractions were observed within the first three hours post pulse. The diurnal variation of tFA in the plasma and cholesterol fractions found in this study may indicate that fluctuations vary according to metabolic status (preprandial and postprandial status) (Diven et al., 1958). The amounts and composition of lipids circulating in the blood of dairy cattle are dependent upon a number of physiological variables. The nature of the diet, time since feeding, age, breed, pregnancy, and stage of lactation may all affect lipid content and composition (Christie, 1981). In this study, diurnal variations of plasma, lipoprotein fractions and ruminal fermentation parameters and showed that there may be an adaptation of ruminal microflora to the treatments and also reflected a cycle control mechanism balancing rate of lipid entry and rate of removal in the circulatory system. This is possibly related to regulation of lipid supply (diet, production, synthesis) and utilization by peripheral tissues (Bitman et al., 1990).

Concentrations (g/100g) of tFA were almost 2 times greater in VLDL than HDL and LDL fractions. These results may support the suggestion from the previous experiment that further fractionation was necessary to distinguish true LDL fraction from chylomicrons and VLDL that are also present in this fraction. However, such comparisons in bovine lipoprotein fractions have not been evaluated in previous studies (Loor and Herbein, 2001; Mosley et al., 2006; Tyburczy et al., 2008). In this

experiment, compared with control, FO and FO/PHVO increased 18:1 *trans* isomers in plasma and VLDL fraction, that may be attributed to an inhibition of the biohydrogentation reduction steps of 18:1 *trans* isomers to 18:0 by LC-PUFA present in FO (Shingfield et al., 2003; AbuGhazaleh and Jenkins, 2004; Lee et al., 2005; Loor et al., 2005; Or-Rashid et al., 2008).

In the current experiment, 18:2 *cis*-9, *trans*-11 was increased by SO and FO+PHVO in plasma and HDL fraction but was not detected in LDL and VLDL fractions. Vegetable or marine oils elevate the concentration and yield of CLA and tFA isomers in milk fat (Chilliard et al., 2000). In this experiment, SO and FO+PHVO increased 18:2 *cis*-9, *trans*-11 in plasma and HDL fraction probably because its concentration of 18:2 n-*cis* 6 was enough to inhibit hydrogenation of 18:1 isomers to 18:0 (Loor and Herbein, 2001).

In the current experiment, the presence of plasma CLA 18:2 *cis*-9, *trans*-11 in HDL probably reflects the predominance of HDL fraction lipid in cattle plasma. Normally, 70-90% of bovine plasma lipids are in the HDL fraction; thus it is expected that the preponderance of 18:2 *cis*-9, *trans*-11 would also be in this fraction (Herdt et al., 1995).

Capper et al. (2007) evaluated the effects of supplementing pregnant and lactating ewes with FO and vitamin E upon the performance of lactating ewes and sucking lambs. They found that the total FA concentration in sucking lamb plasma was reduced by FO supplementation of the ewe. Maternal FO supplementation reduced plasma concentrations of 16:0, 18:0, 18:1 cis-9, 18:2n-6 and 20:4n-6 in sucking lambs. By contrast, increases in 18:1 *trans* isomers; CLA *cis*-9, *trans*-11, 18:3n-3, EPA and DHA were obtained by supplementing ewes with FO.

Addition of FO to the ruminant diet has been shown to increase plasma concentrations of both 18:1 *trans* isomers and CLA produced by incomplete ruminal biohydrogenation of PUFA (Baumgard et al., 2000; Wachira et al., 2000; Chikunya et al., 2004), results that agree with the increase in 18:1 tFA conferred by FO and FO/PHVO treatments within the current experiment. Increases in n-3 PUFA conferred by FO supplementation are often accompanied by a corresponding increase in plasma

20:4n-6 concentration resulting from increased dietary supply (Kitessa et al., 2001). In the current experiment, increased 20:4n-6, EPA and DHA are in agreement with data presented by Capper et al. (2007). They found that significant increase in plasma EPA and DHA in ewes offered diets containing FO were a direct result of increased dietary supply of preformed long-chain PUFA. However, Wachira et al. (2002) and Chikunya et al. (2004) reported the presence of EPA and DHA within muscle and plasma FA of sheep fed diets in which these FA were not detected.

Alternatively, proliferation of bacteria capable of conversion of 18:3 and 18:2 through to 18:0 might be inhibited by the LCPUFA in FO, resulting in increased VA. A study by Wasowska et al. (2006) showed that addition of EPA or DHA (50 mg/l) to pure cultures inhibited growth and isomerase activity of *B. fibrisolvens*, whereas FO, in which the FA were present in triacylglycerols, had no effect. Other reports (Kim et al., 2008) suggested that accumulation of 18:2n-6 in the rumen (by feeding more 18:2n-6 to the ruminant) causes incomplete biohydrogenation.

3.4.2 Fatty acid composition in the lipoprotein lipids

In the current experiment, HDL-CE had higher concentrations (g/100g) of VA, EA and 18:2 *trans*6 in bovine plasma. This high concentration of plasma tFA in HDL-CE probably reflects the predominance of HDL fraction lipid in cattle plasma. Normally, 70-90% of bovine plasma lipids are in the HDL fraction; thus it is expected that the preponderance of tFA would also be in this fraction (Herdt et al., 1995). However, it also depends on whether tFA are coming directly from the intestine (in which case tFA will be present in the VLDL fraction) or whether tFA get incorporated into tissues and then released again (in which case tFA can be found in LDL and/or HDL fractions).

Ruminant plasma lipoprotein profile is characterized by low concentrations of chylomicrons, VLDL and total TG, whereas approximately 90% of plasma lipid is carried by HDL, mainly in the form of CE and PL (Offer et al., 2001). Offer et al. (2001) studied the selective incorporation of dietary FA into plasma lipoprotein classes in cows supplemented with FO (300 g/d). They found that VLDL-TG was not altered by FO supplementation. In the current experiment concentration of VLDL-TG

appeared to be the major lipid sub group carrying plasma FA. These results are different from previous studies (Ashes et al., 2000; Offer et al., 2001) probably due to the fact that FA treatments were given as single ruminal pulses and were exposed to direct ruminal biohydrogenation rather than being supplemented in the diet. Also, because non-lactating cows were used, FA profiles reported may be different from lactating cows because milk fat composition is affected by stage of lactation. More importantly, FA metabolism is affected by lactation, which greatly increases metabolic demand for FA and precursors (Belyea et al., 1990; Palmquist et al., 1993).

The effect of FO and FO/PHVO as ruminal pulses is another factor that may explain variation in concentrations of certain FA within each lipid sub group. For example, DePeters et al. (2001) attributed changes in carbon number for TG structure to high proportions of LCFA and medium chain FA associated with canola oil infused either into the rumen or abomasum. Also in the current experiment non-lactating cows were used, whereas Offer et al. (2001) used mid-lactation cows and hence that may have an effect on FA utilization to satisfy different energy requirements.

There is evidence (Palmquist, 1976; Moore and Christie, 1979; Christie et al., 1986; Scow and Chernick, 1987; Barber et al., 1997) that the ruminant mammary gland obtains FA required for assembly of milk lipids by the action of mammary lipoprotein lipase on the TG fraction of chylomicrons and VLDL. Studies on lactating cows and goats, utilizing arterio-venous difference measurements, mammary gland perfusions and infusions of radioactively labelled lipids, all indicated that the TG component of chylomicrons and VLDL is the main source of milk lipid. Although much larger amounts of CE and PL are present, mainly in HDL, these are poor substrates for lipoprotein lipase and thus make only modest contributions to the formation of milk fat (Offer et al., 2001). The dynamics of lipid transport cannot be inferred simply from the analysis of plasma lipoprotein concentrations. The high fractionation removal rate (~ 0.5 per min) of VLDL from lactating cow plasma (Palmquist and Mattos, 1978) and the large increase in plasma VLDL concentration which takes place at the end of lactation (Palmquist, 1976), supports the high state of flux of this lipoprotein, with influx being balanced by mammary uptake. The efficient delivery of any dietary FA from the small intestine to the mammary gland depends on partitioning of FA into the TG fraction during assembly of chylomicrons / VLDL in the intestinal epithelium. If

FA are instead incorporated only into the PL and CE fractions of lipoproteins, then they will be unavailable to the mammary lipoprotein lipase system (Offer et al., 2001).

Another consideration is that high-oil feed ingredients increase availability of UFA and rumen-derived 18:1 *trans* isomers in blood for uptake and incorporation into milk fat. VA and 18:2 *cis*-9, *trans*-11 are the major intermediates of dietary 18:2n-6 hydrogenation. In contrast, 18:1 *trans*-6/7/8 and *trans*-9 are the major intermediates produced during isomerization of 18:1 *cis*-9. This is in agreement with the decreased of EA in LDL-PL and VLDL-FFA caused by FO in the current experiment. Despite its ruminal origin, the majority of 18:2 *cis*-9, *trans*-11 in milk fat is synthesized within the mammary gland from VA via Δ^9 desaturase. Transfer of dietary 18:2 *trans*-10, *cis*-12 into milk fat may be low due to its preferential incorporation into plasma phospholipids (Loor et al., 2003).

3.4.3 Ruminal fermentation

Ruminal NH_3-N and propionate linearly increased and butyrate and valerate linearly decreased across sampling times. Conversely, Shingfield et al. (2010) studied the effects of incremental amounts (0, 8, 16 and 24 g/kg of DM) of FO as a source of EPA and DHA on ruminal lipid metabolism and the flow of FA at the duodenum in steers fed maize silage-based diets, and reported that the inclusion of FO in the diet had no effect on rumen pH and NH_3-N concentrations. They also observed that supplementing FO increased linearly molar concentration of propionate and reduced linearly butyrate, with a trend towards a linear decrease in molar proportions of acetate in rumen VFA. Likewise, Toral et al. (2009), supplemented ewes with a diet that included sunflower oil (20 g/kg of DM) and FO (10 g/kg of DM) to investigate the impact of the addition of oils on ruminal fermentation parameters; they found no effect on ruminal pH, NH_3-N, lactate and VFA concentrations and concluded that its use to improve the nutritional value of ruminant sub products cannot be precluded.

The effect of FO on ruminal fermentation parameters may be due to the level of inclusion in diets, for example, Lee et al. (2008), examined the effects of incremental amounts (0, 10, 20 and 30 g/kg DM) of FO on biohydrogenation in steers fed sole forage diets based on ensiled grass or red clover. They found that FO in the diets had no effect on rumen pH but decreased rumen NH_3-N concentrations when added at 30

g/kg diet DM. Regardless of the forage fed, FO supplements had no effect on total VFA concentrations or VFA molar proportions.

Amorocho et al. (2009) evaluated catfish oil as a dietary ingredient for dairy cows at different levels (0, 15 and 30 g/kg); they found that total VFA concentrations in ruminal fluid were similar across levels of inclusion; molar proportion of acetate decreased with increasing concentrations of catfish oil, whereas propionate concentration increased by adding catfish oil in the diet. Doreau and Chilliard (1997) also reported a decrease in molar proportion of acetate and an increase in propionate in ruminal fluid of cows fed diets supplemented with 370 g/d of menhaden fish oil. Onetti et al. (2001) and Lewis et al. (1999) reported similar results when tallow was the source of fat. A possible explanation for such effects is that UFA can alter the ruminal ecosystem by suppression of methanogenic (and to a lesser extent cellulolytic) bacteria and protozoa (Van Soest, 1994). A decrease in methane production alters rumen fermentation, leading to an increase in propionate production to maintain the ruminal fermentation balance (Amorocho et al., 2009).

As described before, the effect of lipid supplementation on ruminal fermentation relies mainly on three factors: first, the type of oil (Wachira et al., 2000), second, the level of oil inclusion in the diet (Shingfield et al., 2008) and third, the dietary forage:concentrate ratio, since animals fed a concentrate-rich diet might be more likely to be adversely affected by oil supplementation (Ueda et al., 2003). In lactating cows, a high-forage diet supplemented with a combination of sunflower and FO only induced a transient reduction in total VFA concentration at 2 h post feeding, but no effect was observed at 6 h (Palmquist and Griinari, 2006).

It would be interesting to study further the effect of FO and its blend with PHVO to understand alterations to ruminal ecosystem. As previously reported (AbuGhazaleh and Jenkins, 2004; Boeckaert et al., 2007, 2008; Scollan et al., 2001) marine products, such as FO and algae, proved highly effective at inhibiting rumen biohydrogenation of UFA because LC-PUFA (EPA and/or DHA) are active compounds in this process. Supplementation with LC-PUFA can reduce 18:0 production, resulting in accumulation of various hydrogenation intermediates, predominantly VA and 18:1 *trans*-10 (Boeckaert et al., 2008).

3.5 Conclusion

Differences in FA concentrations between plasma and lipoprotein fractions were observed, particularly in 18:1 *trans* isomers. This experiment reinforces findings reported in Chapter 2 and helps to understand which lipoprotein is involved in the transport of tFA. In this experiment, VLDL appeared to be the main transport mechanism of 18:1 *trans* isomers in bovine plasma. Overall, results from Chapters 2 and 3 demonstrates that dietary lipids rich in PUFA can alter the rumen environment and result in increased plasma and lipoprotein-fraction concentrations of tFA. The results therefore show how plasma FA profiles change when supplementary oils do not bypass the rumen and hence the lipids had extensive effects on the biohydrogenation process, as indicated by plasma and lipoprotein 18:1 *trans* isomers.

3.6 Implications

In this experiment, VLDL fraction appeared to be the major fraction involved in the transportation of tFA. However, further research is needed to understand the metabolism of FA at the cellular level within the mammary gland.

The phenomenon of milk fat depression induced by PUFA supplementation in lactating ruminants has been reviewed in detail (Peterson et al., 2003; Bauman et al., 2006) and it is clear that specific FA produced as intermediates during ruminal biohydrogenation of 18:2n-6 play a significant role in reducing milk fat synthesis through inhibitory effects on mammary gene expression (Peterson et al., 2003). This mechanism would explain the decrease in milk fat concentration conferred by FO supplementation, in combination with effects of diet upon milk yield and milk fat yield (Capper et al., 2008). Hence, use of dietary FO in the dairy diet has to be carefully balanced to avoid milk fat depression and increase the nutritional value of milk fat.

It is becoming clear that the VA and rumenic acid contents in milk fat depend not only on dietary forage-to-concentrate ratio and carbohydrate sources, but also on amounts and types of supplementary oils, with FO being most effective in small quantities. Efforts should be made to reduce the level of other 18:1 *trans* and CLA isomers because no beneficial effect has so far been attributed to these isomers (Cruz-Hernandez et al., 2007).

This experiment helps to understand the basis of the beneficial effect of adding oils to the ruminant diet on FA composition plasma and lipoprotein fractions. However, a better understanding of how ruminal biohydrogenation is modified via differential toxicity to ruminal bacteria of different PUFA including the FO fatty acids is needed. If we can understand how selective FA toxicity, or indeed other factors, affects the physiology of biohydrogenating bacteria in the rumen, we may be able to suggest new, rational dietary modifications that will eventually lead to ruminant products that are healthier for human consumption (Maia et al., 2010).

C h a p t e r 4

Using a bovine mammary epithelial cell line (MAC- T) to study fatty acid metabolism

CHAPTER 4

4. Using a bovine mammary epithelial cell line (MAC- T) to study fatty acid metabolism

4.1 Introduction

Lipids in milk are present in fat globules formed chiefly of triacylglycerols enclosed within a membrane derived from the secretory epithelial mammary cell (Jensen, 2002; Heid and Keenan, 2005). Although the general outlines of lipid droplet formation, growth, movement, and secretion are known, few studies have been performed on lipid absorption and secretion in mammary gland cells (Ernens et al., 2007).

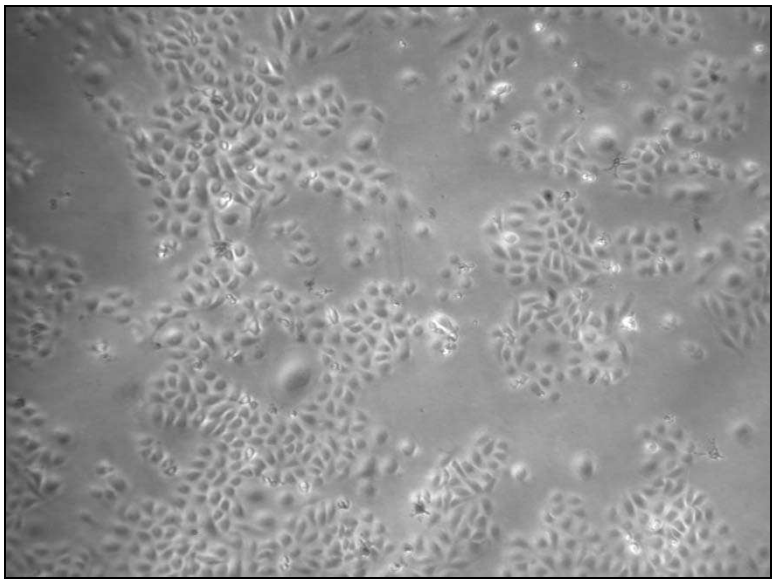
Selective uptake of lipoprotein fractions or specific FA could affect milk fat composition by determining the substrates available for TAG synthesis (Offer et al., 2001). The use of cell cultures allows studying specific aspects of lipid uptake without the interference from the cow's digestive system and general metabolism. The main hypothesis being tested in this experiment was that LCFA and tFA might influence TAG accumulation and FA profile in cytosol of mammary epithelial cells. The objective was to elucidate how cytosolic TAG are accumulated and whether this is influenced by tFA, SFA and UFA. This objective was achieved by adding different FA to a FA-free medium used to culture MAC-T cells.

4.2 Material and methods

4.2.1 Cell culture

Bovine mammary epithelial cells (MAC-T; provided by Juan J. Loor, University of Illinois, USA) were grown in HyQ RPMI-1640 media (Hyclone) supplemented with foetal bovine serum (10%, F7524, Sigma) and ABAM (Antibiotic-antimycotic solution; 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml; A5955, Sigma) in sterile 75 cm^2 flasks coated with Poly-D-Lysine or Collagen I (to ensure cell attachment and facilitate growth) (156472-Nunclon, Fisher Scientific).

Cultures were maintained in a water-jacket incubator at 37°C in the presence of 5% CO_2. Culture medium was changed every 48 h and cells were sub-cultured to 70 to 80% confluence (Figure 4.1) by rinsing once with 3 ml of 0.25% trypsin, and incubated at 37°C until there was evidence of cell detachment. Trypsin activity was inhibited by addition of 25 ml of fresh culture media at 37°C (Kadegowda et al. 2009).



MAC-T cells at ~40% confluence

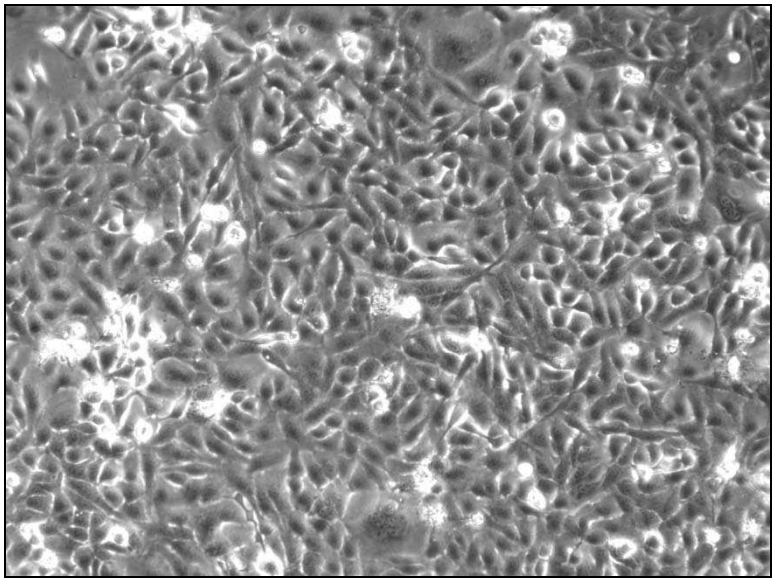


Figure 4.1 MAC-T cells at ~100% ("cobblestone" morphology; Huynh et al. 1991)

4.2.2 Basal medium

Approximately 48 h prior to the last subculture before initializing the experiment, cells were allowed to grow in a basal medium similar to that of Peterson et al. (2004), with modifications. The basal medium was composed of Minimum Essential Medium/Earle's Balanced Salts HyQ (MEM/EBSS, HyClone) with insulin (5 mg/l, I6634, Sigma, St. Louis, MO), hydrocortisone (1 mg/l, H0888, Sigma), transferrin (5 mg/l, T1428, Sigma), ascorbic acid (5 μ*M*/l, A4544, Sigma), sodium acetate (5 m*M*/l, S5636, Sigma), and ABAM (10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml; A5955, Sigma). The basal medium was supplemented with

foetal bovine serum (10%, F7524, Sigma) and growth-promoting hormones (1 mg/l of progesterone, P8783, Sigma; 0.05% lactalbumin, L5385, Sigma; 0.05% α-lactose, 47287-U, Sigma). All basal medium components were filtered before use (sterile syringe filter; pore size: 0.20 µm).

Cells were seeded at a concentration of 1.0 x 10^4 cells / well (Yonezawa et al., 2004; Keating et al., 2008) in sterile 12-well (well diameter: 22.1 mm) cell culture clusters with flat bottom (Costar 3513, Corning Incorporated, USA).

4.2.3 Lactogenic medium

Approximately 24 h before applying treatments (approximately 90% confluence), cells were cultured in a lactogenic medium as reported by Kadegowda et al. (2009), with modifications. The lactogenic medium was prepared as the basal medium, except that high-glucose Dulbecco's modified Eagle's medium (HG-DMEM, HyClone) was used. The lactogenic medium was devoid of foetal bovine serum and was supplemented with BSA (Bovine serum albumin; 1 g/l) and prolactin (2.5 mg/l). All lactogenic medium components were filtered before use (sterile syringe filter; pore size: 0.20 µm).

4.2.4 Preparation of LCFA solutions

Stock solutions of 30 m*M* LCFA were prepared in 13 x 100 mm Pyrex glass tubes with screw caps at 42°C with an equimolar solution of NaOH in water. Sodium salts of the individual FA were suspended in ethanol to obtain a final solution of 95% ethanol. After preparation, the LCFA stock solutions were stored at -20°C until use (Olofsson et al., 2004; Kadegowda et al., 2009) (Tables 4.1 and 4.2).

4.2.5 Treatment of MAC-T cell with exogenous fatty acids

Treatments consisted of adding LCFA (palmitate, stearate, oleate, linoleate, CLA, elaidate and vaccenate) solutions to the lactogenic medium at varying concentrations and culturing cells for a 24-hr incubation period. Ethanol was used as a control. Concentrations of individual FA in the lactogenic medium were 100, 200, 300 and 400 m*M*. At the end of each incubation, lipids from media and cell lysates were extracted *in situ* using hexane:isopropanol (3:2 v/v).

Table 4.1 Preparation of stock LCFA

Fatty acid	Molecular weight (g/mol)	mg of FA	µl of 1*M* NaOH	ml of absolute ethanol[8]
Palmitic[1]	256.43	25.7	112.2	3.34
Stearic[2]	284.40	21.1	83.10	2.47
Linoleic[3]	280.46	26.6	94.80	3.16
Oleic[4]	282.47	28.5	112.89	3.36
Conjugated linoleic acid[5]	280.45	25.4	101.36	3.01
Vaccenic[6]	282.46	28.8	114.24	3.40
Elaidic[7]	282.46	23.5	92.96	2.76

[1]P0500-SIGMA ≥99%; [2]S4751-SIGMA ≥99% (GC); [3]L1376-SIGMA ≥99%; [4]SIGMA O1008 ≥99% (GC); [5]16413-SIGMA Conjugated (9Z, 11E) linoleic acid analytical standard ≥96%; [6]≥95% donated by INRA, France; [7]Elaidic donated by Nestle; [8]Absolute ethanol from Fishers Scientific E/0650DF/P17.

Table 4.2 Concentration of stock[1] LCFA - 30 m*M* (30nmoles / µl)

Concentration (μM)	Amount (µl / ml)[2]
100	3.3
200	6.6
300	9.9
400	13.2

[1]Stock LCFA is not sterile; [2] Amount required per ml of media; LCFA were filtered (sterile syringe filter; pore size: 0.20 µm) along with the lactogenic medium.

4.2.6 Measurement of the TAG, DNA and protein content in cytosol

Cultured cells on 12-well plates (COSTAR 3513) were washed once with ice-cold PBS (phosphate buffered saline) + albumin (to remove FA traces) and twice with PBS, scraped off into 200 µl of 0.05*M* trisodium citrate (Fishers S/3320/60) for DNA and protein quantification and stored at -20°C until analysis. Cells were sonicated (Soniprep 150, MSE, UK, Ltd.) at 5 microns for 10 seconds to disrupt cell membranes and release cellular contents.

DNA quantification was performed by the Hoechst fluorometric method. Hoechst 33258 Dye was used (this binds to the thymine-adenine rich regions of double stranded DNA and exhibits enhanced fluorescence under high ionic strength conditions) for DNA quantification. For this measurement, sensitivity of the Hoechst 33258 Dye assay was approximately 10 ng/ml. The linear dynamic range extended over 3 orders of magnitude from 10 ng/ml to 1 µg/ml DNA.

Protein was determined by the Lowry et al. (1951) protein assay. This method is based on the reactivity of peptide nitrogen with copper ions under alkaline conditions and

the subsequent reduction of the Folin-Ciocalteay phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. Concentration of the reduced Folin reagent was measured by absorbance at 655 nm. Optical density was read using a microplate reader (Microplate Manager Bio-Rad Laboratories, Inc.).

TAG in the cell lysates was extracted using hexane:isopropanol (3:2 v/v) and quantified using a Triglyceride reagent (Infinity Triglyceride, Thermo Scientific, TR22421). The linear range was 0 to 10 mmol/l (885 mg/dl). Optical density was read using a microplate reader (Microplate Manager Bio-Rad Laboratories, Inc.). The measurement wavelength was 550 nm and 655 nm for reference wavelength. Each assay was performed in triplicate and repeated at least 3 times.

4.2.7 Fatty acid analysis from cells and media

Lipids from cells and media were extracted using an adaptation of the method of Bligh and Dyer (1959) and methylated according to the method of Christie (1982) with modifications by Chouinard et al. (1999). All chemicals and solvents used for these methods were of analytical grade. For analysis of FA in cells and media, a GC system (Agilent, GC 6890 series) equipped with a CP-Sil 88 fused-silica capillary column (100 m x 0.25 mm i.d., with 0.2 µm film thickness; Varian Inc., Oxford) was used. The GC conditions were as follows: the oven temperature was initially set at 110°C for 4 min after injection, and then increased to 240°C with equilibration time of 2 min. The inlet and flame-ionization detector temperatures were 260°C, the split ratio was 15:1 and a 2 µl injection volume was used. The hydrogen carrier gas flow to the detector was 25 ml/min, airflow was 400 ml/min, and the flow of nitrogen makeup gas was 40 ml/min. Fatty acid peaks were identified by using a fatty acid methyl ester standard (FAME; Supelco 37 Component FAME mix, Bellefonte, PA) and a tFA reference standard (*trans* 11- C18:1, methyl ester, Supelco, Bellefonte, PA). The concentration of each FA (g/100g) was calculated for 16:0 to 22:6n3 FA with concentrations higher than 0.3 g/100g.

4.3 Statistical analysis and design

Using the GenStat 12th statistical package (VSN International Ltd., Oxford); cytosolic TAG, protein and DNA data was analyzed as Linear Mixed Models with fixed effects of LCFA and concentration; the random effects were run, plate, strips and wells. In the current experiment different runs (of which there were 8) were carried out to obtain replication for each of the measurements. To avoid biased results, treatments were added randomly to different wells (of which there were 408), strips (of which there were 3 in each 12-well plate) and plates (of which there were 35) for each run. Cytosolic fatty acid profile from cell and media data was analyzed as a Linear Mixed Models with fixed effects of LCFA and concentration; the random effect was plate. Data are reported as least square means ± SED across different concentrations (100, 200, 300 and 400 μM).

4.4 Results

4.4.1 Accumulation of cytosolic triacylglycerol

Addition of stearate, oleate, elaidate, vaccenate, linolate and CLA increased cytosolic TAG (from 100 to 400 μ*M*). Compared with control, cytosolic TAG was increased by palmitate and elaidate. Compared with 100 μ*M* (initial concentration), cytosolic TAG content was increased by 300 and 400 μ*M*. Concentration effect linearly increased and had quadratic effect (Table 4.3 and Figure 4.2).

Table 4.3 Cytosolic triacylglycerol content (mmol / well)

	Concentration (μ*M*)							
Fatty acid	100	200	300	400	SED[2]	*P*-value		
Control	0.39	0.35	0.37	0.44	0.062	<0.001[3]		
Palmitate	0.46	0.43	0.43	0.57				
Stearate	0.25	0.26	0.27	0.36				
Oleate	0.38	0.45	0.64	0.71				
Elaidate	0.35	0.35	0.50	0.66				
Vaccenate	0.38	0.41	0.57	0.70				
Linoleate	0.47	0.50	0.66	0.84				
CLA[1]	0.28	0.52	0.68	0.97				
	Fatty acid effect							
	Control		0.39		0.036	<0.001[4]		
	Palmitate		0.47					
	Stearate		0.28					
	Oleate		0.54					
	Elaidate		0.47					
	Vaccenate		0.51					
	Linoleate		0.61					
	CLA		0.61					
	Concentration effect						(Lin)[6]	(Quad)[7]
Concentration (μ*M*)	100		0.37		0.020	<0.001[5]	<0.001	<0.001
	200		0.41					
	300		0.51					
	400		0.65					

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; [4]*P*-value represents the probability of a treatment effect; [5]*P*-value represents the probability of a concentration effect; [6]Linear effect; [7]Quadratic effect.

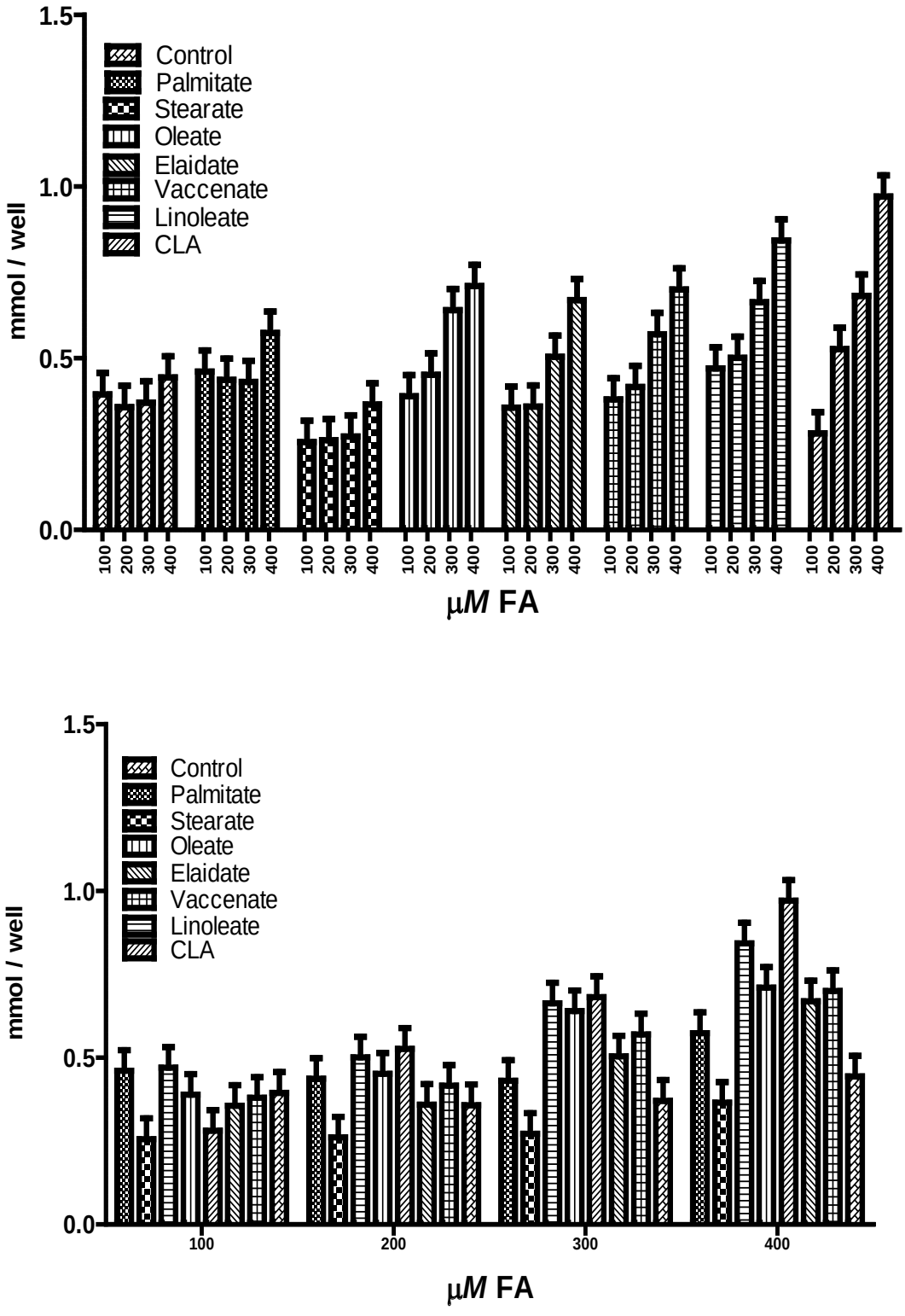


Figure 4.2 Cytosolic triacylglycerol content (mmol / well). Fatty acids (top) and concentration (bottom) effects.

4.4.2 Cytosolic protein content

Compared with control, cytosolic protein content was increased by oleate, vaccenate and linoleate. Cytosolic protein content was not affected by concentration or treatment-concentration interaction (Table 4.4).

Table 4.4 Cytosolic protein content (mg / ml)

	Concentration (μ*M*)							
Fatty acid	100	200	300	400	SED[2]	*P*-value		
Control	1.42	1.48	1.47	1.32	0.311	0.947[3]		
Palmitate	1.84	1.63	1.40	1.25				
Stearate	1.12	1.31	1.31	1.25				
Oleate	2.33	2.47	2.53	2.55				
Elaidate	1.57	1.57	1.64	1.56				
Vaccenate	2.03	2.02	2.11	2.00				
Linoleate	2.72	2.80	2.46	2.21				
CLA[1]	1.36	1.29	1.48	1.35				
	Fatty acid effect							
	Control		1.42		0.176	<0.001[4]		
	Palmitate		1.53					
	Stearate		1.25					
	Oleate		2.47					
	Elaidate		1.59					
	Vaccenate		2.04					
	Linoleate		2.55					
	CLA		1.37					
	Concentration effect						(Lin)[6]	(Quad)[7]
Concentration (μ*M*)	100		1.80		0.105	0.298[5]	0.045	0.398
	200		1.82					
	300		1.80					
	400		1.69					

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; [4]*P*-value represents the probability of a treatment effect; [5]*P*-value represents the probability of a concentration effect; [6]Linear effect; [7]Quadratic effect.

4.4.3 Cytosolic DNA content

Compared with control, cytosolic DNA content was increased by vaccenate and decreased by palmitate. Cytosolic DNA content was not affected by concentration and there was no treatment-concentration interaction (Table 4.5).

Table 4.5 Cytosolic DNA content (µg / ml)

	Concentration (µ*M*)							
Fatty acid	100	200	300	400	SED[2]	*P*-value		
Control	3.36	3.38	2.73	2.65	0.403	0.542[3]		
Palmitate	2.87	2.37	2.32	1.86				
Stearate	2.63	2.79	2.95	2.95				
Oleate	3.08	2.77	3.26	2.83				
Elaidate	2.80	2.89	2.92	2.94				
Vaccenate	3.52	3.84	3.75	3.70				
Linoleate	2.92	3.38	2.65	2.59				
CLA[1]	2.70	2.82	2.80	2.78				
	Fatty acid effect							
	Control		3.03		0.219	<0.001[4]		
	Palmitate		2.36					
	Stearate		2.83					
	Oleate		2.98					
	Elaidate		2.88					
	Vaccenate		3.70					
	Linoleate		2.89					
	CLA		2.78					
	Concentration effect						(Lin)[6]	(Quad)[7]
Concentration (µ*M*)	100		2.99		0.138	0.187[5]	0.044	0.303
	200		3.03					
	300		2.92					
	400		2.79					

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; [4]*P*-value represents the probability of a treatment effect; [5]*P*-value represents the probability of a concentration effect; [6]Linear effect; [7]Quadratic effect.

4.4.4 Accumulation of cytosolic triacylglycerol normalized for protein content

Addition of palmitate, elaidate, vaccenate and linolate increased (from 100 to 400 μ*M*) cytosolic TAG content corrected for protein (μg of TAG / mg protein). Compared with control, cytosolic TAG was increased by palmitate, vaccenate, linoleate and CLA. Concentration effect linearly increased and had quadratic effect. Compared with 100 μ*M* (initial concentration), cytosolic TAG content was increased by 300 and 400 μ*M* (Table 4.6 and Figure 4.3).

Table 4.6 Accumulation of triacylglycerol contents (µg / mg protein)

	Concentration (µ*M*)							
Fatty acid	100	200	300	400	SED[2]	*P*-value		
Control	138.1	200.1	157.5	235.2	120.0	0.002[3]		
Palmitate	352.3	392.7	590.3	817.3				
Stearate	314.2	343.1	209.5	292.1				
Oleate	189.1	145.6	338.6	511.9				
Elaidate	227.8	322.6	340.3	394.3				
Vaccenate	284.7	365.2	427.0	508.0				
Linoleate	221.9	275.0	510.3	919.4				
CLA[1]	294.1	281.2	499.5	583.1				
	Fatty acid effect							
	Control		182.7		71.96	<0.001[4]		
	Palmitate		538.1					
	Stearate		289.7					
	Oleate		296.3					
	Elaidate		321.3					
	Vaccenate		396.2					
	Linoleate		481.7					
	CLA		414.5					
	Concentration effect						(Lin)[6]	(Quad)[7]
Concentration (µ*M*)	100		252.8		39.58	<0.001[5]	<0.001	0.020
	200		290.7					
	300		384.1					
	400		532.7					

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; [4]*P*-value represents the probability of a treatment effect; [5]*P*-value represents the probability of a concentration effect; [6]Linear effect; [7]Quadratic effect.

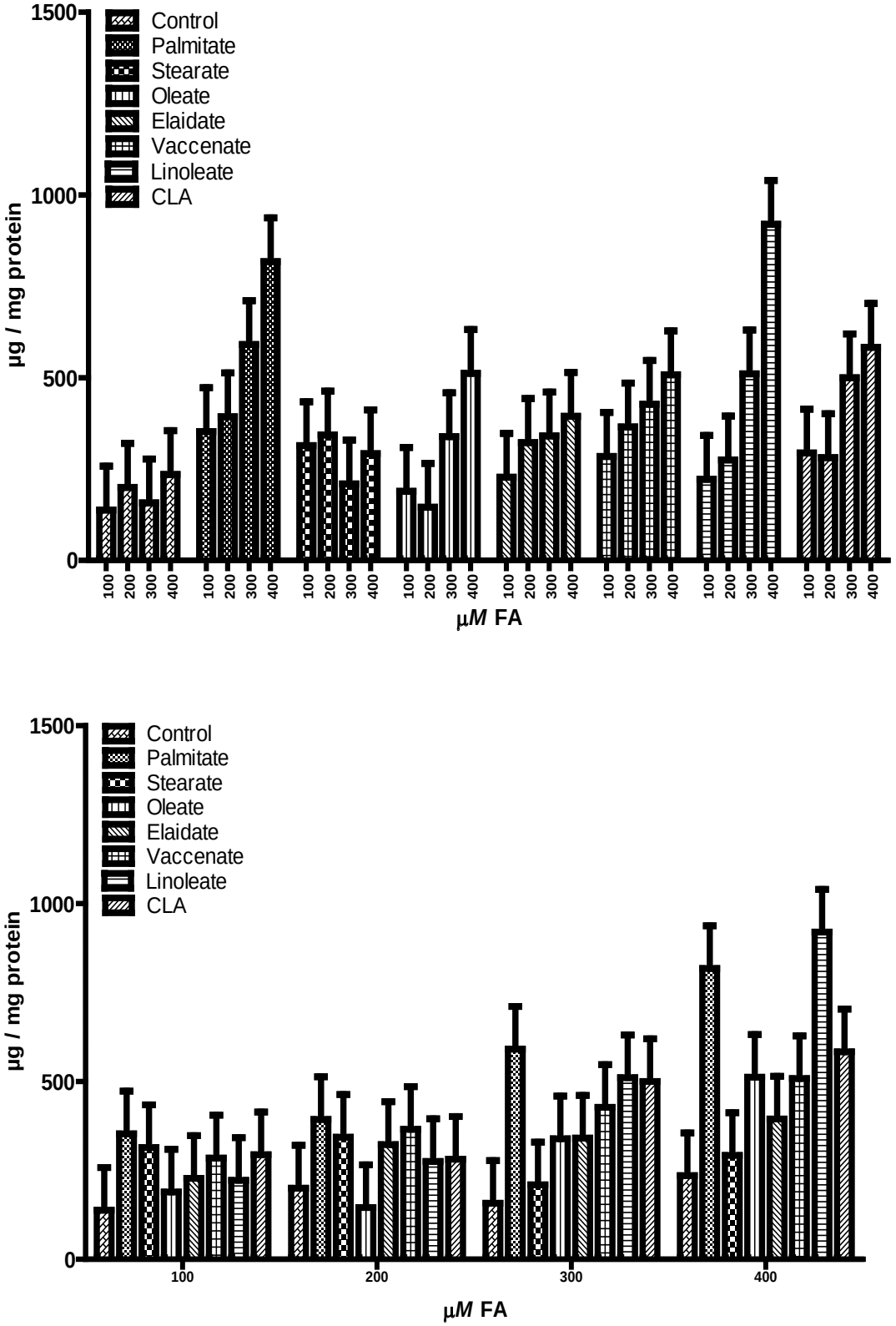


Figure 4.3 Accumulation of triacylglycerol contents (µg / mg protein). Fatty acids (top) and concentration (bottom) effects.

4.4.5 Accumulation of cytosolic triacylglycerol normalized DNA content

Addition of palmitate, oleate, vaccenate and CLA increased (from 100 to 400 μ*M*) cytosolic TAG content (μg of TAG / μg of DNA). Compared with control, cytosolic TAG content was increased by palmitate and CLA. Concentration effect linearly increased and had quadratic effect. Compared with 100 μ*M* (initial concentration), cytosolic TAG content was increased by 300 and 400 μ*M* (Table 4.7 and Figure 4.4).

Table 4.7 Accumulation of triacylglycerol contents (μg of TAG / μg of DNA)

	Concentration (μ*M*)							
Fatty acid	100	200	300	400	SED[2]	*P*-value		
Control	137.8	126.2	139.9	183.8	28.59	0.026[3]		
Palmitate	145.9	158.0	167.4	284.0				
Stearate	126.6	115.5	115.0	140.1				
Oleate	112.5	112.7	148.5	164.9				
Elaidate	131.9	128.6	162.8	225.7				
Vaccenate	105.7	109.8	129.9	165.2				
Linoleate	126.9	114.8	175.1	235.8				
CLA[1]	122.3	189.7	223.0	298.5				
	Fatty acid effect							
	Control		146.9		15.60	<0.001[4]		
	Palmitate		188.8					
	Stearate		124.3					
	Oleate		134.7					
	Elaidate		162.3					
	Vaccenate		127.6					
	Linoleate		163.2					
	CLA		208.4					
	Concentration effect						(Lin)[6]	(Quad)[7]
Concentration (μ*M*)	100		126.2		9.81	<0.001[5]	<0.001	<0.001
	200		131.9					
	300		157.7					
	400		212.3					

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; [4]*P*-value represents the probability of a treatment effect; [5]*P*-value represents the probability of a concentration effect; [6]Linear effect; [7]Quadratic effect.

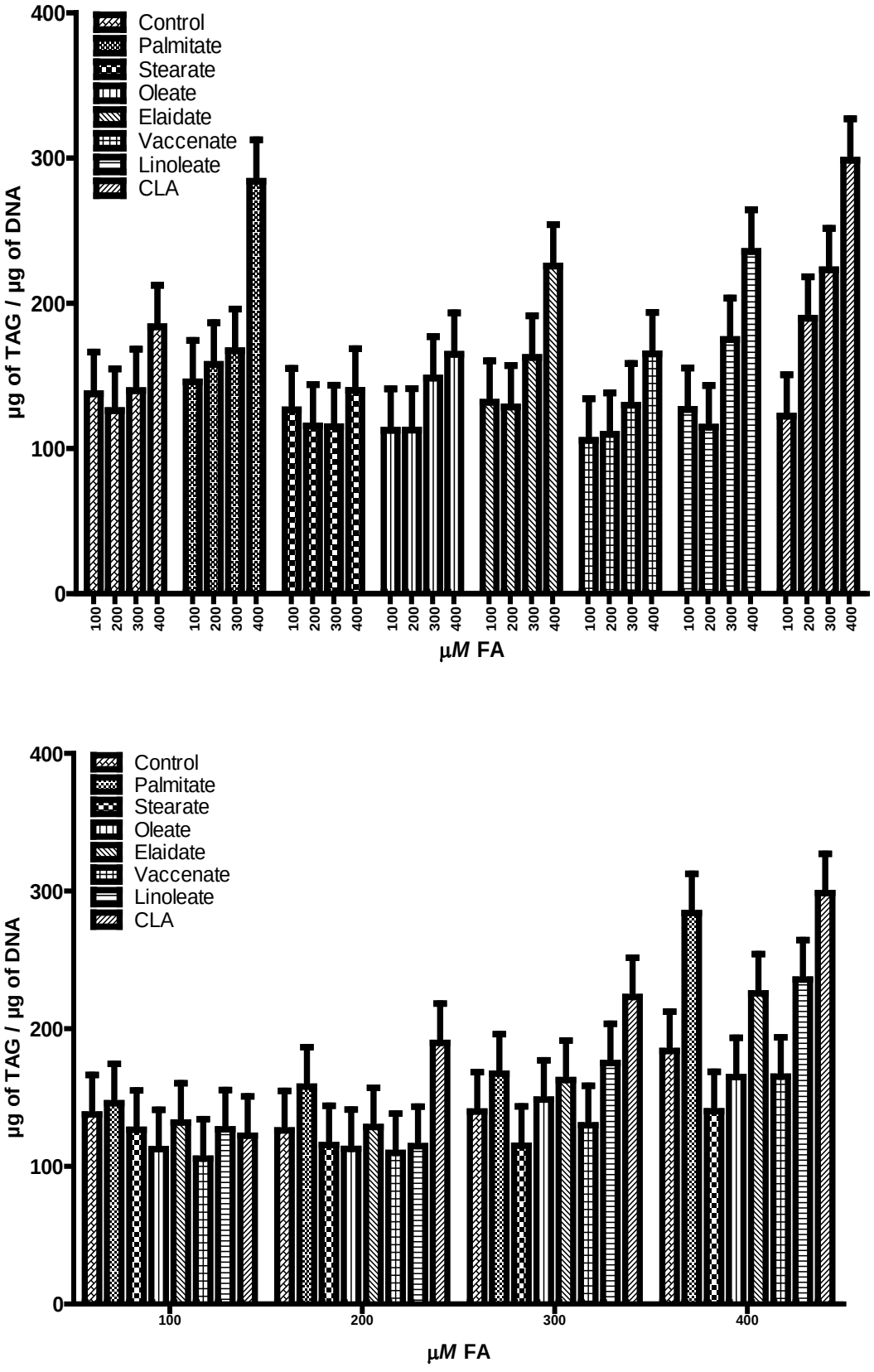


Figure 4.4 Accumulation of triacylglycerol contents (µg of TAG / µg of DNA). Fatty acids (top) and concentration (bottom) effects.

4.4.6 Cytosolic fatty acid profile

4.4.6.1 Saturated fatty acids

- Cytosolic 16:0 concentration was decreased by linoleate (from 100 to 400 μM) and CLA (from 100 to 400 μM).
- Cytosolic 18:0 concentration was decreased by CLA (from 100 to 400 μM).
- Cytosolic 20:0 concentration was increased by linoleate (from 100 to 400 μM), but for other fatty acid treatments 20:0 remained below 1.5 g / 100g (from 100 to 400 μM) of fatty acid (See appendix 3).

4.4.6.2 Monounsaturated fatty acids and *trans* fatty acids

- Cytosolic 16:1 concentration was decreased by CLA (from 100 to 400 μM).
- Cytosolic 18:1 *trans*-9 concentration was increased by elaidate (from 200 to 400 μM); for other fatty acid treatments 18:1 *trans*-9 remained around 10 g / 100g from 100 to 400 μM of fatty acid or not detectable.
- Cytosolic 18:1 *cis*-9 concentration was increased by oleate (from 100 to 300 μM).
- Cytosolic 24:1 concentration was increased by palmitate (from 100 to 400 μM); for all other fatty acid treatments 24:1remained below 2.5 g / 100g from 100 to 400 μM of fatty acid (See appendix 3).

4.4.6.3 Polyunsaturated fatty acids

- Cytosolic 18:2n-*cis* 6 concentration was increased by linoleate (from 100 to 400 μM) and decreased by palmitate (from 100 to 400 μM); for the other fatty acid treatments 18:2n-*cis* 6 remained below 3.5 g / 100g from 100 to 400 μM of fatty acid.
- Cytosolic CLA concentration was increased by CLA (from 100 to 400 μM); for all other fatty acid treatments CLA remained below 5g / 100g from 100 to 400 μM of fatty acid or not detectable.
- Cytosolic 20:4n6 concentration was decreased by oleate, vaccenate, and linoleate (from 100 to 400 μM); for all other fatty acid treatments 20:4n6 remained below 1.7g / 100g from 100 to 400 μM of fatty acid or not detectable (See appendix 3).

4.4.6.4 Major fatty acid classes in cytosol

- MUFA concentration was increased by oleate (from 100 to 300 μM) and elaidate (from 200 to 400 μM).
- PUFA concentration was increased by CLA (from 100 to 400 μM).
- SFA concentration was increased by linoleate (from 100 to 400 μM) and decreased by CLA (from 100 to 400 μM).
- *Trans* fatty acid concentration was increased by vaccenate (from 100 to 300 μM) and elaidate (from 200 to 400 μM).
- UFA concentration was decreased by palmitate (from 100 to 300 μM), and increased by oleate and CLA (from 100 to 400 μM) (See appendix 3).

4.4.7 Media fatty acid profile

4.4.7.1 Saturated fatty acids

- Media 18:0 concentration was decreased by palmitate (from 100 to 400 μM), vaccenate (from 200 to 400 μM) and CLA (from 200 to 400 μM) and increased by stearate (from 100 to 300 μM).
- Media 21:0 concentration was increased by stearate (from 100 to 300 μM) (See appendix 3).

4.4.7.2 Monounsaturated fatty acids

- Media 18:1 *cis*-9 concentration was increased by oleate (from 200 to 400 μM) and palmitate (from 100 to 300 μM) (See appendix 3).

4.4.7.3 Polyunsaturated fatty acids

- Media 18:2n-*cis* 6 concentration was increased by linoleate (from 100 to 400 μM); for the other fatty acid treatments 18:2n-*cis* 6 remained below 2.5 g / 100g from 100 to 400 μM of fatty acid.
- Media CLA concentration was increased by CLA (from 100 to 400 μM); for all other fatty acid treatments CLA was not detectable (See appendix 3).

4.4.7.4 Major fatty acid classes in the media

- Media *trans* fatty acids concentrations were increased by elaidate and vaccenate (from 100 to 400 μM) (See appendix 3).

4.5 Discussion

This experiment is the first to study effects of FA (SFA, UFA, PUFA and tFA) at increasing concentrations on cytosolic TAG, protein and DNA contents, and FA profiles in mammary gland epithelial cells. The design of the experiment allowed analysis of cytosolic and media FA profiles whereas previous studies (Jayan and Herbein, 2000; Peterson et al., 2004; Yonezawa et al., 2004; Keating et al., 2008; Sorensen et al., 2008; Kadegowda et al., 2009; Thering et al., 2009; Kadegowda et al., 2009) focussed on different aspects of mammalian physiology (i.e., enzymatic activity and gene expression). This experiment contributes to knowledge on lipid metabolism in bovine mammary gland cells and its biological significance in milk production because it allowed the study of lipid uptake without the interference from the cow's digestive system and general metabolism.

Milk fat is assembled in the mammary alveolar cells from FA derived partly by *de novo* synthesis from acetate within these specialized cells, and partly by absorption from the blood. Lipids produced by lactating bovine mammary cells are distinctive, which facilitates assessment of the biochemical fidelity of these cells when used *in vitro* (Kinsella et al., 1968). MAC-T cells have normal physiological responses to prolactin in that they produce milk constituents including α- and β- casein and lactose. These cells thus provide a method of studying aspects of milk production *in vitro* (Turner et al., 1993).

The design of the current experiment enabled examination of effects of different LCFA including tFA on cytosolic TAG accumulation and protein and DNA concentration. In the current experiment, control was used to show if there was any effect of increasing amounts of ethanol. Concentrations of the individual FA were 100, 200, 300 and 400 μ*M*. These concentrations were different from Jayan and Herbein (2000; 25, 50 and 100 μ*M* of FA) and Keating et al. (2008; 15, 20, 30, 35, 37.5 and 150 μ*M* of FA) but similar to those used by Yonezawa et al. (2004, 2008; 50, 100, 200, 300 and 400 μ*M* of FA). In the current study, lower concentrations (< 100 μ*M* of FA) were tested in a pilot experiment; however, cytosolic TAG accumulation was not enough to be measured. In experiments of Keating et al. (2008) and Yonezawa et al. (2004, 2008), FA were bound to bovine serum albumin. Albumin is the most abundant

serum protein and one of its main physiological functions is to bind and transport LCFA (Spector, 1986). Based on previous studies (Olofsson et al., 2004; Kadegowda et al., 2009; Thering et al., 2009), in the current experiment, ethanol was used to dissolve sodium salts of FA to avoid additional limitations on LCFA uptake by mammary epithelial cells (FA albumin binding process is selective due to varying degrees of configurational adaptability to its binding sites as FA increase in length; Ashbrook et al., 1975; Spector, 1975).

4.5.1 Cytosolic TAG accumulation (mmol / well)

Mammary epithelial cells accumulate TAG in their cytosol; in these cells, esterified FA such as TAG are derived either from plasma lipids or by *de novo* synthesis from acetate and butyrate (Clegg et al., 2001; Heid and Keenan, 2005). After lipoprotein lipase hydrolysis all FA are absorbed as free FA. The main hypothesis of the current experiment was that cytosolic TAG accumulation (mmol / well) may be stimulated by addition of LCFA at different concentrations and that LCFA may affect the FA profile of cytosolic FFA. In the current experiment palmitate and elaidate increased accumulation of cytosolic TAG content. UFA treatments induced greater cytosolic TAG accumulation than SFA treatments suggesting that SFA are subjected to another metabolic pathway (or not absorbed by cells), and not either TAG synthesis or lipid droplet formation (Yonezawa et al., 2004). In this experiment, treatment concentrations linearly increased cytosolic TAG accumulation and had quadratic effect; this agrees in part with the increase in a concentration dependent manner in cytosolic TAG accumulation observed in primary cultured bMEC cells by Yonezawa et al., (2004).

4.5.2 Cytosolic TAG accumulation normalized for protein (µg / mg protein) and DNA (µg of TAG / µg of DNA) contents

In the current experiment, cytosolic TAG accumulation was normalized for protein (µg / mg protein) and DNA contents (µg of TAG / µg of DNA). Usually in cell cultures, protein content is used as cell proliferation indicator to eliminate variances from cell cultures and allow data comparisons with greater confidence. Normalization is a process by which data are corrected to remove sample-to-sample variability caused by factors other than those being tested in the experiment. These factors included variabilities in cell plating and pipetting inconsistencies (Schagat et al.,

2007). Because MAC-T cells synthesise protein for inclusion in milk, cytosolic TAG accumulation normalized for protein may not be appropriate for this experiment. However, each cell has a fixed amount of DNA, so DNA content per well reflects the number of cells per well. Hence, when cytosolic TAG is corrected for DNA a measure of TAG per cell can be obtained and this is why it is more accurate than normalising for protein content.

In the current experiment, when cytosolic TAG was normalized for protein content (µg / mg protein), palmitate, vaccenate, linoleate and CLA increased cytosolic TAG content. This is in agreement with Yonezawa et al. (2004), who reported that palmitate, and linoleate increased cytosolic TAG (µg / mg protein) in primary culture bovine mammary epithelial cells isolated from the mammary gland of a 102-d-pregnant Holstein heifer. However, in this experiment, cytosolic TAG accumulation (from 221.9 to 919.4 µg / mg protein) achieved by MAC-T cells was greater than Yonezawa et al. (2004; from 54.8 to 122.6 µg / mg protein). In this experiment, treatment concentrations linearly increased cytosolic TAG (µg / mg protein) accumulation and had quadratic effect; this agrees in part with the increase in a concentration dependent manner in cytosolic TAG accumulation observed in primary cultured bMEC cells by Yonezawa et al., (2004).

On the other hand, in the current experiment, palmitate and CLA increased cytosolic TAG adjusted for DNA content (µg of TAG / µg of DNA) and treatment concentrations linearly increased cytosolic TAG and had quadratic effect. Previous studies (Jayan and Herbein, 2000; Yonezawa et al., 2004, 2008) did not quantify cytosolic DNA contents.

As Yonezawa et al. (2004, 2008), in this experiment, MAC-T cells were left for a 24 h incubation period. In the current experiment, sodium salts of LCFA were prepared using ≥99% (GC) purity, and suspended in absolute ethanol whereas Yonezawa et al. (2004, 2008) prepared their treatments (palmitate, stearate, oleate and linoleate) coupled with 0.5% FA free bovine serum albumin. In the study of Jayan and Herbein (2000), FA (stearic, oleic and vaccenic acids) were bound to bovine serum albumin, all media contained a basal concentration of 100 µ*M* stearic acid and the cells were

left for a 3-d incubation period. In those studies, the chemical nature of the FA was not described.

4.5.3 Cytosolic and media fatty acid profiles

Results from cytosolic FA profiles were consistent with most of the FA treatments added to MAC-T cells. Oleate increased oleic acid (from 100 to 300 μM of FA), elaidate increased EA (from 200 to 400 μM of FA), linoleate increased linoleic acid (from 100 to 400 μM of FA), and CLA increased CLA (from 100 to 400 μM of FA). In the current experiment, effects on cytosolic FA concentrations were not consistent within FA classes (tFA, SFA and UFA):

- SFA treatments: palmitate decreased UFA
- *Trans* FA treatments: elaidate increased cytosolic MUFA and tFA
- UFA treatments: oleate increased MUFA and UFA; linolate increased SFA; CLA increased PUFA and UFA and decreased SFA

In the current experiment, stearate increased stearic acid (from 100 to 300 μM of FA), oleate increased oleic acid (from 200 to 400 μM of FA), linoleate increased linoleic acid (from 100 to 400 μM of FA) and CLA increased α-linoleic acid (from 100 to 400 μM of FA), in media samples. Only tFA class was affected by treatments. Media tFA concentrations were increased by elaidate and vaccenate (from 100 to 400 μM of FA). Those results may be explained due to the media enrichment of each individual FA added and perhaps the FA extraction method (Bligh and Dyer, 1959) included some of the FFA added as well as TAG.

Perhaps the different effects of individual FA found in the current experiment may be due to specific activity that each FA has on enzymes (acetyl-CoA carboxylase; ACC and FA synthetase; FAS) involved in the biosynthesis of SFA and SCD responsible for catalyzing biosynthesis of UFA from SFA precursors in MAC-T cells (Jayan and Herbein, 2000). The ability for PUFA to down regulate the expression of genes for enzymes involved in FA synthesis, including ACC, FAS and SCD, has been recognised on membrane lipid composition (Salter and Tarling, 2007).

4.5.4 Cytosolic versus media fatty acid profiles

Cytosolic stearate ranged from 34 to 46 g/100g whereas media ranged from 24 to 30 g/100g at 100 to 400 μM of FA. Cytosolic oleate ranged from 18 to 37 g/100g whereas media ranged from 5 to 14 g/100g at 100 to 400 μM of FA. Cytosolic linoleate ranged from 14 to 34 g/100g whereas media ranged from 2 to 11 g/100g at 100 to 400 μM of FA. Cytosolic CLA ranged from 6 to 38 g/100g whereas media ranged from 2 to 12 g/100g at 100 to 400 μM of FA. The tFA concentrations were decreased (from 0.1 to 13 g/100g) in media compared with cytosolic FA profiles (from 2 to 50 g/100g).

Results from media FA profiles showed disappearance of some FA and that may indicate which FA were not incorporated and absorbed into cytosol of MAC-T cells. Only media FA profiles of stearate, oleate, linoleate and CLA were detected. Results found in the currents experiment may be attributed to different roles of LCFA in cellular metabolism. LCFA are taken up by cells and used for a large number of biological functions such as energy generation and storage, LCFA also contribute to phospholipid synthesis which are necessary for the structure, integrity and function of plasma membranes. FA modulate the function of enzymes and regulate the expression of multiple genes involved in FA metabolism (Hajri and Abumrad, 2002).

The cellular uptake of LCFA may be due to passive diffusion through the lipid bilayer and protein-facilitated transport. The relative contribution of each component depends on the concentrations and molar ratios of FA and albumin in the circulation (Hajri and Abumrad, 2002). In the circulation and extracellular medium, FA are carried quantitatively bound to albumin. Complexes of FA and albumin are used in uptake assays *in vitro* because FA adsorption to assay tubes and pipette walls and FA aggregation may have complications. In the absence of albumin or other FA binding protein, the concentration of LCFA that can be used is very low and is depleted by cells instantaneously; meaning that linear portion of the uptake time course would be too short to measure (Hajri and Abumrad, 2002). In the current experiment, ethanol used to dissolve sodium salts of FA probably affected FA uptake of LCFA (palmitic, stearic, oleic, linoleic and conjugated linoleic acids) and tFA (elaidic and vaccenic acids).

4.5.5 Cytotoxicity (lipotoxicity)

In the current experiment, LCFA did not decrease cytosolic protein (mg / ml) or DNA (µg / ml) contents. However, it is well documented that accumulation of excess lipids in tissues leads to cell dysfunction or cell death (Shaffer, 2003). When cells accumulate more FFA than are required for anabolic or catabolic processes, excess lipid is esterified and stored as TAG in lipid droplets. Although TAG accumulation is essentially a condition of lipid overload, cellular TAG accumulation may initially serve a protective role. Accumulation of excess FFA in TAG pools diverts these molecules from pathways that lead to cytotoxicity and may thus serve as a buffer against lipotoxicity (Shaffer, 2003).

4.5.6 Importance of FA groups on lipid metabolism

LCFA have numerous roles in biological functions of cells, not only as an energy substrate but also as substrates for cell membrane synthesis and as precursors for intracellular signalling molecules (McArthur et al., 1999). Excess LCFA are stored as TAG in lipogenic tissues such as the adipose, liver, testis, ovary and the mammary gland. In the lead up to-, and during lactation, energy metabolism of cells is dramatically modified in order to enable synthesis of milk components (Barber et al., 1997). Animals accordingly diminish the storage of nutrients, particularly of TAG, in adipose tissue. The lactating mammary gland thus uses LCFA which are derived from dietary fats and TAG released from the adipose tissue. Lactating mammary epithelial cells are highly active in synthesis of FA *de novo* (Clegg et al., 2001). Bovine mammary epithelial cells have the ability to produce a remarkable amount of lipid; hence, this lipogenic capacity probably affected results from the current experiment and influenced the transfer of some FA from cytosol to media by decreasing TAG accumulation in cytosol.

In the current experiment, MAC-T cells incubated with SFA (palmitic and stearic acids), UFA (oleic acid), PUFA (linoleic and conjugated linoleic acids), tFA (elaidic and vaccenic acids), increased cytosolic TAG content; however, when TAG content was normalised for cytosolic DNA content SFA (palmitic acid) and PUFA (CLA) noticeably increased the amount of cytosolic TAG content. Hence, these findings may indicate that mammary epithelial cells accumulate lipids depending on chain length of the FA, degree of unsaturation and configuration.

Although activity of lipogenetic enzymes in mammary gland cells was not evaluated, the findings (cytosolic and media FA profiles) from the present study may complement previous studies. For example, Jayan and Herbein (2000) studied exogenous factors that were related to the synthesis of SFA and UFA in MAC-T cells. They analyzed effects of oleic and vaccenic acids on activities of ACC, FAS and SCD. ACC and FAS are major enzymes involved in biosynthesis of SFA in eukaryotic cells and SCD is responsible for biosynthesis of UFA from their saturated precursors. Jayan and Herbein (2000) reported that VA depressed activity of ACC and FAS, along with simultaneous enhancement of mammary desaturase activity and explained that the effects of exogenous FA on ACC and FAS in MAC-T cells were related to the biological function of FA in cells and tissues. For example, FA are components of cell membranes, and FA provided in the media of cultured mammary epithelial cells are primarily incorporated into cell membrane PL. Inhibition of lipogenesis in various tissues by LC-UFA has been reported; in rats, oleic acid and VA inhibited hepatic FAS activity and inhibited the conversion of glucose to lipids (Clarke et al., 1990).

Fatty acids are components of cell membranes, and FA provided in the media of cultured mammary epithelial cells are primarily incorporated into cell membrane phospholipids (Baughman, 1995). The relative amounts of SFA and UFA, as well as the chain length of FA that constitute membranes, are important factors that determine membrane fluidity. In general, fluidity has an inverse relationship to chain length and a direct relationship to degree of unsaturation of FA. Jayan and Herbein (2000) noted that excess incorporation of unsaturated oleic acid and VA into cell membranes, especially at the expense of the corresponding saturated isomer, would increase membrane fluidity above normal limits. SC-SFA have the same effect on membrane fluidity as LC-UFA, meaning that both these groups enhance membrane fluidity when compared with LC-SFA. Therefore inhibition of ACC and FAS activities by the monounsaturated isomers could be a cellular response to reduce synthesis of SC-SFA.

4.5.7 *Trans* fatty acids in lipid metabolism

In the present study, at concentrations of 100, 200, 300 and 400 μ*M*, EA and VA increased cytosolic TAG content (mmol / well). Jayan and Herbein (2000) found a concentration-dependent uptake of VA complexed to FA-free bovine serum albumin

at concentrations of 25, 50 and 100 μ*M*; however, this is the first experiment using EA on MAC-T cells.

Jayan and Herbein (2000) found that the position and geometry of the double bond is a factor deciding the influence of UFA on lipogenic enzyme activity. When compared with *cis* double bonds, *trans* double bonds have a more rigid structure and are less fluid in nature. It is possible that VA may compete with oleic acid for incorporation into cell membranes (oleic acid will be produced in bovine mammary epithelial cells by desaturation of stearic acid). Excess incorporation of VA at the expense of oleic acid would reduce fluidity of cell membranes and therefore enhanced synthesis of *cis*-UFA from available substrates or precursors is prefered.

4.6 Conclusions

Overall, the results of this experiment demonstrate that cytosolic TAG accumulation is stimulated by addition of palmitate and EA at different concentrations, which affects FA profile of cytosolic FFA. Effects on cytosolic TAG accumulation are not consistent within FA classes (tFA, SFA and UFA) but depend on individual FA structure (chain length of the FA, degree of saturation and configuration, and number and orientation of FA double bonds). At concentrations of 100, 200, 300 and 400 μ*M*, LCFA (palmitic, stearic, oleic, linoleic, elaidic, vaccenic and conjugated linoleic acids) did not have detrimental effects on MAC-T cells as determined by cytosolic protein and DNA content, presumably reflecting their role in lipid accumulation and secretion.

4.7 Implications

In this experiment, EA resulted to stimulate cytosolic TAG accumulation and based on protein and DNA content it did not have deleterious effect on mammary gland cells. This finding is important to dairy industry because it is known that MFD is caused by direct inhibition of mammary lipogenesis by tFA formed during the biohydrogenation of dietary UFA in the rumen.

Data reported in the current experiment enhances knowledge on the regulation of milk fat synthesis which is vital to the development of nutritional strategies to improve the nutritional value of milk, decrease milk energy secretion and improve the energy balance of lactating ruminants. FA treatments used in the current experiment included

different lipid classes because in normal conditions dairy cow diets are supplied with lipids containing different FA classes, however, TAG accumulation and uptake into the mammary gland cell will depend on their individual structure.

Understanding the regulation of milk fat synthesis is vital to the development of nutritional strategies to enhance the nutritional value of milk, decrease milk energy secretion and improve the energy balance of lactating ruminants. The current experiment did not assess enzymatic activity; however, it may be necessary to evaluate activity of lipogenic enzymes in mammary gland cells due to its importance on ruminal lipid metabolism and mammary specific regulation of cellular processes (Shingfield et al., 2010).

C h a p t e r 5

General discussion and conclusions

CHAPTER 5

5. General discussion and conclusions

5.1 General discussion

Supplementation of ruminant diets with FA exerts a broad range of effects on milk composition, especially in the milk fat profile. Of particular interest to the current study were plasma transport and mammary uptake of tFA in dairy cows. Effects of dietary source of FA on the FA composition of plasma and lipoprotein fractions were studied, as well as effects of dietary lipids on tFA transportation within lipoprotein fractions and uptake of LCFA in epithelial mammary gland cell cultures (MAC-T cells). Characterization of the mechanisms by which dietary FA are transported and transferred to milk should improve current understanding of effects of dietary FA effects on milk FA profile and MFD.

Ruminant diets typically contain a low percentage of fat, although a significant intake of PUFA is generated from forages and oilseeds and in some cases from fat supplements. Dietary FA are metabolized by ruminal microbes resulting in a large difference between dietary profile and FA profile absorbed from the small intestine (Doreau and Chilliard, 1997; Chilliard et al., 2000). Most dietary FA are esterified, and are almost completely hydrolyzed to free FA in the rumen. Unsaturated free FA are then isomerized (double-bond position and/or orientation changed) and reduced (saturation of double bond; Wallace et al., 2007). The resulting SFA and some of the biohydrogenation intermediates escape the rumen and are subsequently absorbed (Harvatine et al., 2009) (Figure 5.1). *Trans* FA isomers originate from incomplete ruminal biohydrogenation of UFA and might contribute to the development of MFD. Previous studies (Bauman and Griinari, 2001 and 2003; Shingfield and Griinari, 2007) have demonstrated a clear relationship between tFA and MFD. Although feeding unsaturated oils induced MFD, feeding completely hydrogenated (SFA) oils had minimal effects on milk fat yield.

The aim of this thesis was to extend the knowledge on lipid metabolism of dairy cows. The experiments in this thesis studied the changes that dietary FA undergo in the rumen (during biohydrogenation), blood transport (plasma and cholesterol fractions) (Chapters 2 and 3) and mammary gland cell uptake (Chapter 4) (Figure 5.2).

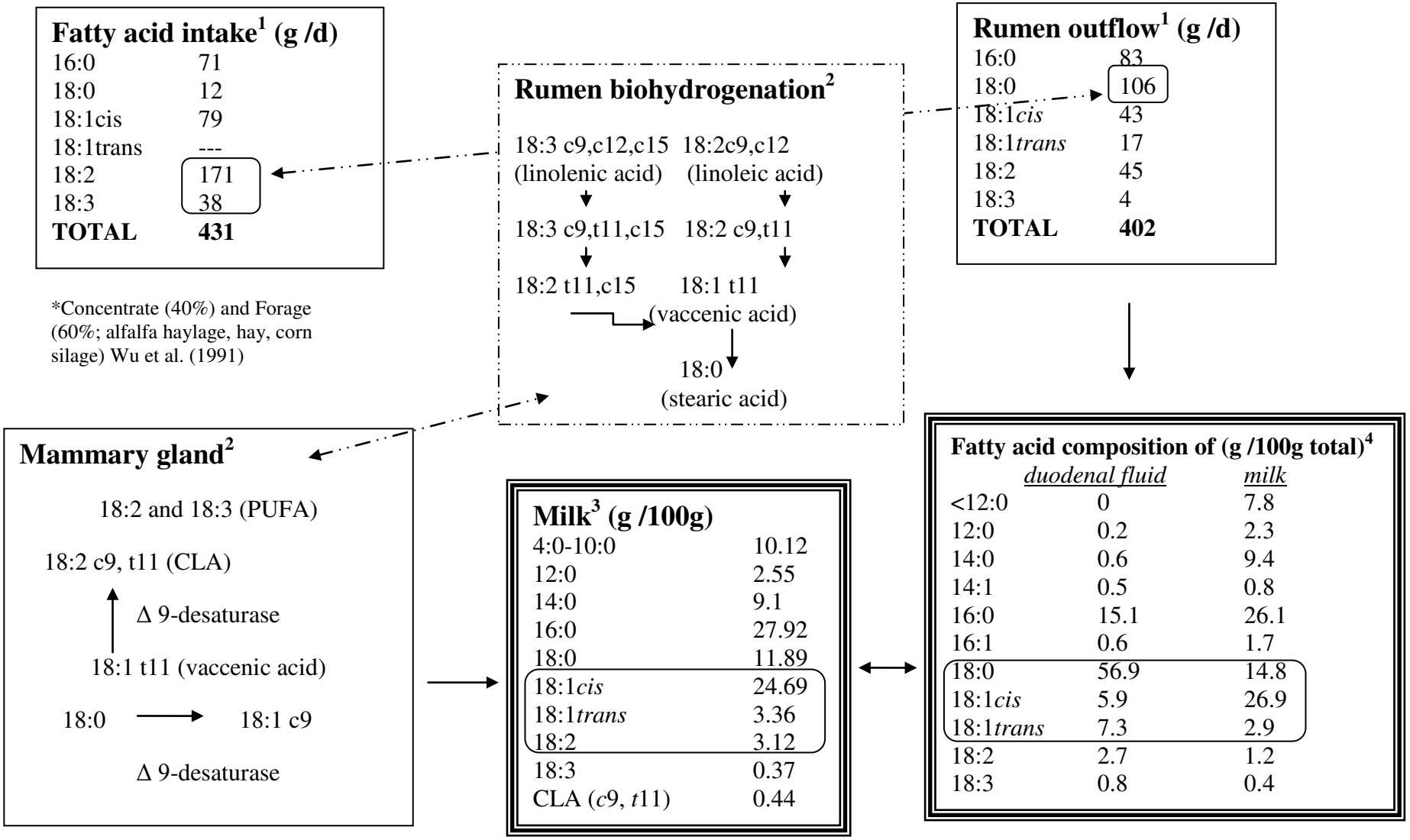


Figure 5.1 Biochemical pathways for ruminal biohydrogenation and endogenous synthesis of FA in dairy cows ([1]Wu et al., 1991; Harfoot and Hazlewood, 1988[2]; Bauman and Griinari, 2003[2]; Garnsworthy et al., 1997[3]; Salter et al., 2006[4]; Lock et al., 2005[4])

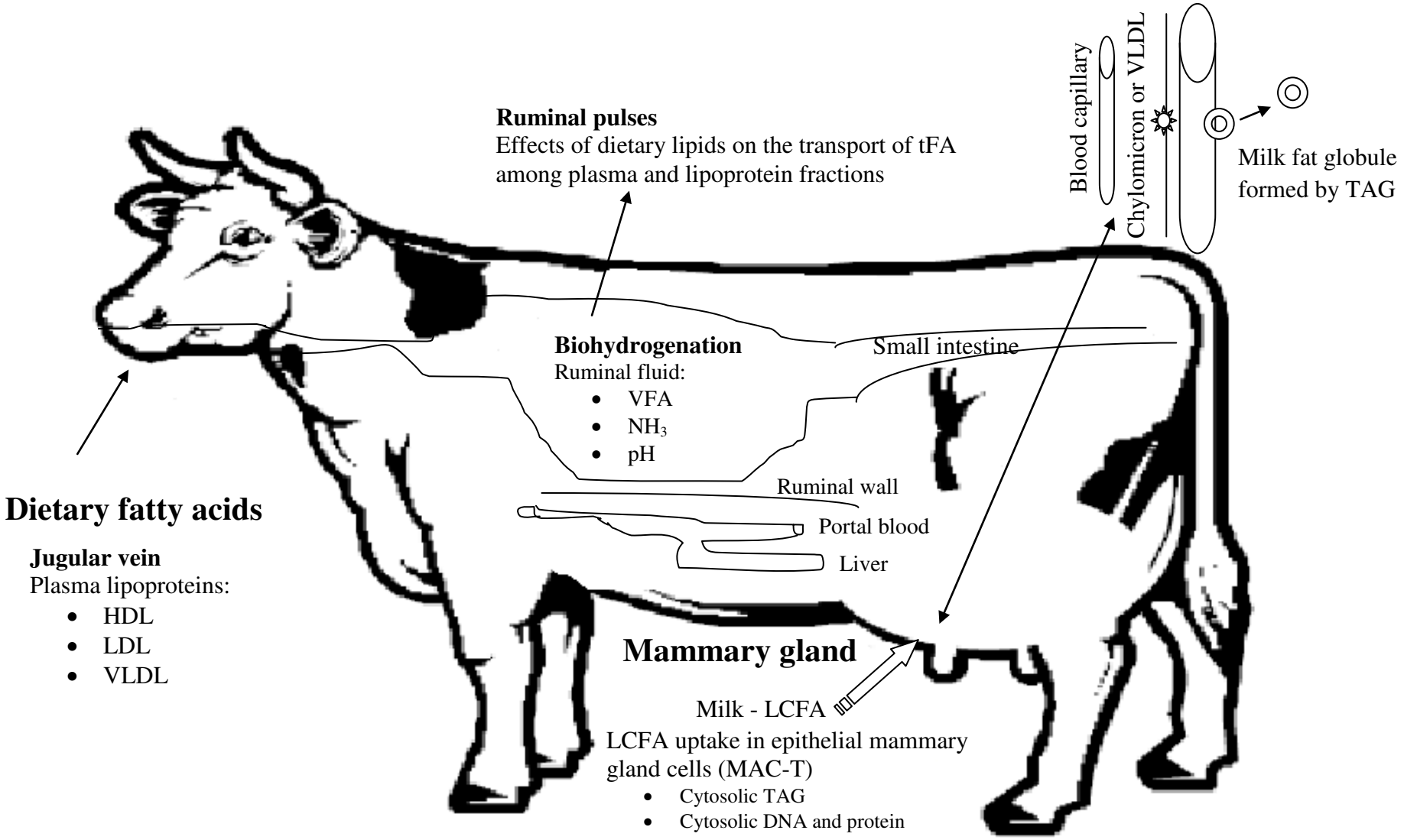


Figure 5.2 The experiments in this thesis studied some of the changes that occur in dietary FA from mouth to mammary gland

5.2 Ruminal pulses of dietary oils

Ruminal pulses were used in the experiments described in Chapters 2 and 3 in order to include the impact of the biohydrogenation process on different 18:1 *trans* isomers. Previous studies used abomasal (Loor and Herbein, 2001; Mosley et al., 2006; Tyburczy et al., 2008) and duodenal (Chilliard et al., 1991, DePeters et al., 2001) infusions to specifically avoid biohydrogenation effects on dietary treatments. Supplementary oils did not bypass the rumen in these experiments and hence lipids had extensive effects on rumen microorganisms, as indicated reflected by changes in fermentation parameters, and on the biohydrogenation process, as indicated by plasma and lipoprotein FA profiles.

Addition of lipids to the diet generally induces a decrease in ruminal degradation, especially of fibre. There are several factors responsible for variations in ruminal digestion (Doreau and Ferlay, 1992):

1) Nature of FA supplied in the diet

2) Percentage of lipids added to the diet, type of fat and any protection process

3) Nature of the basal diet (ruminal digestive disturbances are greater with corn silage-based diets than with hay-based diets)

Numerous experiments with oil supplements have been carried out in recent years; however, the effects of lipids are not completely explained by the factors just mentioned. Therefore, ruminal pulses were chosen to test if FA profiles (particularly tFA concentrations) of plasma and lipoprotein fractions are different depending on source of the dietary lipids.

Fatty acids obtained after ruminal pulses resulted from extensive biohydrogenation of unsaturated FA (Doreau and Ferlay, 1994; Glasser et al., 2008). Rumen pulses do not bypass biohydrogenation hence FA supply to the duodenum will be more similar to when oilseeds or oils are included in the diet. Therefore rumen pulses are more representative of FA normally available to the animal. Post-ruminal infusions can be used to test hypotheses about intestinal uptake, but they only mimic rumen protected

lipids (Glasser et al., 2008). In real production conditions, there is a loss of FA from the rumen either by absorption across the ruminal epithelium or by catabolism to VFA or $C0_2$. In addition, microbes synthesize FA *de novo* from carbohydrate precursors. Therefore, lipids reaching the duodenum consist of FA from both dietary and microbial origins (Jenkins, 1993).

The relative short treatment (single administration per day), sampling (3 d) and washout periods (4 d) in Chapters 2 and 3 were selected to include the extensive biohydrogenation effects on oil pulses that are often observed during the first few hours after administration (Doreau et al., 1989). Oil administration method can affect how long the oil remains in the rumen. With continuous infusion, oil concentration in the rumen varied slightly throughout the day and night. With single administration, oil concentration is higher during the first few hours after administration and lower during the subsequent hours (Ferlay and Doreau, 1992). Because cows were ruminally pulsed with less than 300 g/d of oil, a 3 d sampling period was chosen (adapted and modified from Loor and Herbein, 2001; Kadegowda et al., 2008 and Castaneda-Gutierrez et al., 2007) with a 4 d whashout period.

5.3 Ruminal fermentation

Dietary lipids rich in either PUFA or tFA alter the rumen environment. The data in Chapters 2 and 3 indicated that dietary oil supplementation affects ruminal fermentation in ruminants. In the first experiment, ruminal pulses of both SO (high in PUFA) and PHVO (high in preformed tFA) reduced ruminal NH_3-N and total VFA concentrations, which suggests that dietary lipids inhibited microbial activity. On the other hand in Chapter 3, FO (used to induce high concentrations of tFA, CLA and LC-PUFA) resulted in higher concentrations of propionate than FO/PHVO (used to induce high concentration of tFA) and there was no differential effect between them on ruminal NH_3-N and total VFA concentrations. The lack of FA treatment effects on ruminal NH_3-N and total VFA concentrations is in agreement with the findings of Shingfield et al. (2010) who studied the effects of incremental amounts (0, 8, 16 and 24 g/kg of DM) of FO as a source of EPA and DHA on ruminal lipid metabolism and duodenal flow of FA in steers fed maize silage-based diets. They reported that inclusion of FO in the diet had no effect on rumen pH and NH_3-N concentrations but as in these experiments, tended to decreased total rumen VFA concentrations. The

effect of lipid supplementation on ruminal fermentation relies mainly on three factors: firstly, the type of oil (Wachira et al., 2000), secondly, the level of oil inclusion in the diet (Shingfield et al., 2008) and thirdly, the dietary forage:concentrate ratio, since animals fed a concentrate-rich diet might be more likely to be adversely affected by oil supplementation (Ueda et al., 2003). Dietary factors that affect ruminal fermentation (e.g. high carbohydrate fermentability, high oil) modify ruminal FA metabolism through complex associative effects that result in altered ruminal microbial populations, altered pathways of PUFA biohydrogenation and ruminal outflow of a wide range of biohydrogenation intermediates and this is reflected in the milk FA composition. The predominant metabolic pathways and the microbial capacity for isomerization and biohydrogenation depend on the microbial population and the ruminal environment (Allen, 2000; Palmquist et al., 2005; Jenkins et al., 2008). 18:1 *trans* isomers from ruminal biohydrogenation serve as precursors for the synthesis of SFA in the rumen and of CLA within the tissues of the animal. For example, VA is reduced in the rumen to form 18:0 or is desaturated by Δ^9-desaturase in the mammary tissue, producing 18:2 *cis*-9, *trans*-11, the most abundant CLA isomer in milk (Griinari and Bauman, 1999). This isomer has been shown to have antioxidant and anticarcinogenic properties (McDonald, 2000). Another CLA isomer, 18:2 *trans*-10, *cis*-12, which can be produced in the rumen, has been linked to decreased body fat deposition in animals and humans (Blankson et al., 2000; Gavino et al., 2000). This isomer has also been shown to be responsible for decreased milk fat synthesis (Baumgard et al., 2000). Furthermore, the concentration of 18:1 *trans*-10 in milk fat is directly correlated with milk fat depression (Griinari et al., 1998).

5.4 Plasma fatty acid profile

The biohydrogenation process had an extensive effect on concentrations of FA observed in plasma and lipoprotein fractions. The main hypothesis being tested in those experiments was that FA profiles of plasma and lipoprotein fractions would be different depending on source of the dietary lipids (SO, PHVO and FO), which would lead to differences in 18:1 *trans* isomers (Chapter 2 and 3). This hypothesis was supported by the differences in the tFA isomers concentrations of plasma and lipoprotein fractions, particularly in the LDL+VLDL (Chapter 2) and VLDL (Chapter 3) fractions.

Results from the *in vivo* experiments confirmed that treatments had the desired effect on tFA concentrations of plasma and lipoproteins, for example, in Chapter 2 concentration of EA in PHVO was higher than SO because EA is the major *trans* isomer found in PHVO (Meijer et al., 2001). On the other hand, undetectable VA concentrations were observed in SO, but biohydrogenation resulted in high concentrations of VA in plasma and lipoprotein fractions. Inclusion of vegetable oils high in PUFA into concentrate diets raises VA and 18:2 *cis*-9, *trans*-11 content in milk fat (Bauman et al., 2000; Shingfield et al., 2006). Compared with HDL fraction, concentrations of tFA were increased almost 3-fold in LDL fraction. These results indicate which lipoprotein fraction is involved in transportation of tFA. SO and PHVO increased plasma concentrations of tFA. The increase in *trans* isomers across plasma and lipoprotein fractions with SO and PHVO reflects modification of rumen microbial population and pathways of biohydrogenation usually caused by supplementation with dietary PUFA. Those changes promote formation and accumulation of biohydrogenation intermediates, some of which are potent inhibitors of milk fat synthesis (Bauman and Griinari, 2003).

In Chapter 3, compared with PHVO, FO increased VA in plasma and lipoprotein fractions which is in agreement with AbuGhazaleh et al. (2007) who compared the effect of FO and sunflower oil supplementation on milk CLA *cis*-9, *trans*-11 when dairy cows were managed on pasture or in confinement. They found that FO and sunflower oil supplementation resulted in higher milk VA, CLA *cis*-9, *trans*-11 and lower 18:1 *trans*-10 concentrations and yields in pasture-fed cows compared with confinement-fed cows. Likewise, Cruz-Hernandez et al. (2007) evaluated different levels of inclusion of sunflower oil (1.5, 3 and 4.5 % of DM) and a constant level of FO (0.5 % of DM) to increase VA and RA in milk and examine the content and composition of CLA and 18:1 *trans* isomers, and found that supplementation of sunflower oil and FO reduced milk fat but milk protein and lactose levels were not affected.

Changes found in tFA isomers of plasma and lipoprotein fractions are a result of biohydrogenation effects and type of oil pulsed. *Trans* FA and CLA isomers are the predominant intermediates produced from ruminal metabolism of linoleic acid (Harfoot and Hazelwood, 1988); however, ruminal biohydrogenation pathways are

dynamic, allowing the production of a wide range of positional and geometric isomers as well as modified FA such as hydroxy and keto derivatives (Palmquist et al., 2005; Jenkins et al., 2008). These isomers are absorbed and incorporated into milk fat, thereby allowing the milk FA profile to be used as a proxy for changes occurring in the rumen. Although linoleic acid give raise to different FA intermediates in the biohydrogenation process, other PUFA including the long-chain n-3 FA are also biohydrogenated and likely produce unique FA intermediates (Harvatine et al., 2009). Milk fat depression induced by PUFA supplementation in lactating ruminants (Peterson et al., 2003; Bauman et al., 2006) is caused by 18:1 *trans* isomers produced as intermediates during ruminal biohydrogenation of 18:2n-6 which affect milk yield and milk fat yield (Capper et al., 2008). Hence, supplementing oils in dairy cow diets has to be carefully balanced to avoid milk fat depression and increase the nutritional value of milk fat for human consumption.

5.5 Lipoprotein fractions

In Chapters 2 and 3, differences in tFA concentrations due to type of oil pulsed led to major changes in most 18:1 *trans* isomers in plasma and lipoprotein fractions, this is in agreement with observations in plasma by Loor and Herbein (2001) in which ruminal infusions of CLA mixtures were used.

In Chapter 2, SO and PHVO increased 18:1 *trans* isomers in HDL and LDL+VLDL fractions whereas FO and FO/PHVO increased 18:1 *trans* isomers in the VLDL fraction in Chapter 3, that may be attributed to an inhibition of the biohydrogentation reduction steps of 18:1 *trans* isomers to 18:0 by LC-PUFA present in SO, FO and PHVO (Shingfield et al., 2003; AbuGhazaleh and Jenkins, 2004; Lee et al., 2005; Loor et al., 2005; Or-Rashid et al., 2008).

In Chapter 2, concentrations of tFA were 2 times greater in LDL+VLDL than HDL fraction; however, concentrations of tFA were 2 times greater in VLDL than HDL and LDL fractions in Chapter 3. These results supported findings from Chapter 2 in which further fractionation was suggested to distinguish between true LDL fraction and chylomicrons, and VLDL that are also present in this fraction.

Overall, results from Chapters 2 and 3 showed that VLDL fraction is the main tFA transport mechanism. Most of the FA is associated with TG (and perhaps

phospholipids). Normally, 70-90% of bovine plasma cholesterol is in the HDL fraction; thus it is expected that the preponderance of tFA would also be in this fraction (Herdt et al., 1995). However, if tFA are coming directly from the intestine, they will be present in the chylomicron/VLDL fraction, but if they are incorporated into tissues and then released again, they will be found in LDL (which is formed from VLDL) and/or HDL fractions.

5.6 Fatty acid composition of the lipoprotein lipids

Results from Chapter 2 and 3 differ from those reported by Offer et al. (2001) because treatments were administrated as ruminal pulses and were exposed to a direct ruminal biohydrogenation rather than being supplemented in the diet. The differences between FA profile of oils supplied as ruminal pulses is another factor that may explain the variation in concentrations of certain FA within each lipid sub group (Ashes et al., 2000; Offer et al., 2001). For example, DePeters et al. (2001) attributed changes in carbon number for TG structure to high proportions of LCFA and medium chain FA associated with canola oil infused either in the rumen or abomasum. Also, stage of lactation has an important effect on FA utilization needed to satisfy specific energy requirements and this may be reflected in the FA profile of lipoprotein lipid subgroups found in our *in vivo* experiments (non-lactating cows) compared with those reported by Offer et al. (2001) (mid-lactational cows). The results presented make an important contribution to our understanding of the transfer mechanisms of long-chain PUFA from diet to milk due to selective incorporation of FA into certain plasma lipoprotein classes (i.e., cholesteryl esters, triglycerides, and phospholipids) which may be ineffective in delivering FA to the mammary gland.

In Chapter 2, concentrations of LDL+VLDL-TG appeared to be the major lipid subgroups carrying plasma lipids and tFA, however after further lipoprotein separation (Chapter 3), concentrations of HDL-TG appeared to be the major lipid subgroups carrying plasma lipids and LDL-CE the main lipid sub group involved in the transportation of some 18:1 *trans* isomers (VA, EA and 18:2 *trans*6) in bovine plasma. Approximately, more than 90% of plasma FA are carried by HDL fraction, mainly in the CE and PL lipid subgroups (Offer et al., 2001), conversely, the mammary gland appears to take up TAG from chylomicrons, VLDL and LDL

fractions, but not from HDL fraction, and it does not use esterified FA from LDL or HDL fraction (Christie, 1981).

5.7 Cellular fatty acid metabolism

Findings from Chapter 4 enhance knowledge of lipid metabolism in bovine mammary gland cells and its biological significance in milk production because they allowed the study of lipid uptake without interference from the cow's digestive system and general metabolism. Chapter 4 reported effects of different FA groups (SFA, UFA, PUFA and tFA) on concentrations on cytosolic TAG, protein and DNA contents to explore whether there was any significant discrimination on absorption of exogenous FA by mammary gland epithelial cells. The design of the experiment allowed analysis of cytosolic and media FA profiles whereas previous studies (Jayan and Herbein, 2000; Peterson et al., 2004; Yonezawa et al., 2004; Keating et al., 2008; Sorensen et al., 2008; Kadegowda et al., 2009; Thering et al., 2009; Kadegowda et al., 2009) focused on enzymatic activity and gene expression.

Although albumin complexes carry FA derived from stored TAG in adipose tissue (Spector, 1986), based on previous studies (Olofsson et al., 2004; Kadegowda et al., 2009; Thering et al., 2009), ethanol was used to dissolve sodium salts of FA to avoid additional limitations (The FA-albumin binding process is selective due to varying degrees of configurational adaptability to albumin binding sites as FA increases in length; Ashbrook et al., 1975; Spector, 1975) for LCFA uptake by epithelial mammary cells.

Information is also provided on LCFA metabolism from bovine mammary gland cells. LCFA have many roles in the biological function of cells, not only as energy substrates but also as substrates for cell membrane synthesis and as precursors for intracellular signalling molecules. Excess LCFA are stored as TAG in lipogenic tissues such as adipose, liver, testis, ovary or mammary gland. During the periparturient period, the energy metabolism of cells is dramatically modified in order to enable synthesis of milk components. Animals accordingly diminish storage of nutrients, particularly TAG, in adipose tissue. The lactating mammary gland thus utilizes LCFA which are derived from dietary fats and TAG released from adipose tissue. Lactating mammary epithelial cells are also highly active in *de novo* FA

synthesis. Each gram of mammary gland tissue is known to produce 1 to 2 ml of milk per day, and 2 to 600 g fat/l milk depending on the animal species. Thus, mammary epithelial cells have the ability to produce a remarkable amount of lipid (Yonezawa et al., 2008). When cells accumulate FFA for anabolic or catabolic processes, lipids are esterified and stored as TAG in lipid droplets (Schaffer, 2003). Although it is known that accumulation of excess lipids in many tissues leads to cell dysfunction or cell death, LCFA and tFA treatments did not appear to have detrimental effects on MAC-T cells as determined by cytosolic protein (mg / ml) and DNA (µg / ml) content, presumably reflecting their role in lipid accumulation and secretion.

Palmitate and elaidate increased accumulation of cytosolic TAG (mmol/well) content. However, Yonezawa et al. (2004) reported that palmitate, stearate, oleate and linoleate increased cytosolic TAG (µg / mg protein) in primary culture bovine mammary epithelial cells. UFA treatments induced greater cytosolic TAG accumulation than SFA treatments suggesting that SFA are subjected to another metabolic pathway (or not absorbed by cells), and not either TAG synthesis or lipid droplet formation (Yonezawa et al., 2004).

Palmitate and CLA increased cytosolic TAG adjusted for DNA content (µg of TAG / µg of DNA). However, previous studies (Jayan and Herbein, 2000; Yonezawa et al., 2004, 2008) did not quantify cytosolic DNA content. Cytosolic TAG accumulation was normalized for protein (µg / mg protein) and DNA content (µg of TAG / µg of DNA). However, as each cell has a fixed amount of DNA, changes in DNA content reflects the number of cells present. Hence, when cytosolic TAG is corrected for DNA a measure of TAG per cell can be obtained and this is why it is probably more accurate than normalising for protein content. Moreover, because MAC-T cells synthesise protein for inclusion in milk, cytosolic TAG accumulation normalization for protein may not be appropriate for this experiment.

When cytosolic and media FA profiles were compared, it was found that tFA concentrations were decreased (from 0.1 to 13 g/100g) in media compared with cytosolic FA profiles (from 2 to 50 g/100g). Compared with cytosolic FA profiles, only stearate, oleate, linoleate and CLA were detected in media. These results are attributed to different roles of LCFA in cellular metabolism. LCFA are taken up by

cells and used for a large number of biological functions such as energy generation and storage, LCFA also contribute to phospholipid synthesis which is necessary for the structure, integrity and function of plasma membranes. FA modulate the function of enzymes and regulate the expression of multiple genes involved in FA metabolism (Hajri and Abumrad, 2002).

The design of the current experiment was different to previous studies (Jayan and Herbein, 2000; Yonezawa et al., 2004, 2008) in the following ways:

- Use of MAC-T cells
- Use of ethanol to couple FA treatments
- Individual FA concentrations
- Quantification of DNA (to normalise cytosolic TAG content)

The effects of FA treatments on cytosolic TAG accumulation and FA profiles found in cytosol and media may be due to:

- Specific activity that each FA has on enzymes involved in FA biosynthesis
- Competition between different FA for uptake
- Chain length of the FA, degree of saturation and configuration
- Number and orientation of FA double bond
- FA extraction might include some of the FFA added as well as TAG

5.8 Importance of the results shown in this thesis

Overall, results reported in this thesis are important in that they extend our knowledge of lipid metabolism of dairy cows. In this thesis, the design of the experiments aided understanding of how FA (especially tFA isomers) are transported in blood. On the other hand, accumulation of TAG was studied in mammary epithelial cells to comprehend how FA are metabolised and absorbed in mammary epithelial cells, therefore findings from this thesis cover some aspects that help to understand synthesis of milk FA. This is important because milk fat plays a central role in dairy products and farm efficiency, fat is a major contributor to the energy concentration of whole milk and is essential to many of the physical properties, manufacturing qualities and organoleptic characteristics of dairy products.

From the producers' perspective, milk fat represents a major component of the value of milk, but it is also a significant portion of the energy cost of lactation. Fat is the most variable component of milk and is affected by many factors including genetics, physiological state and environment. However, milk fat is especially responsive to nutrition, providing a practical tool to alter its yield and composition. Diet induced MFD, or low-fat milk syndrome, is characterized by a decrease in milk fat yield of up to 50%, with no change in milk yield or in the yield of other milk components (Bauman and Griinari, 2001). MFD is classically observed in ruminants fed highly fermentable diets or in diets that contain plant or FO supplements. MFD is also a useful variable for evaluating herd management; in many cases onset of diet induced MFD is an indication of modified ruminal fermentation and in more pronounced cases this can be associated with ruminal acidosis and reduced ruminal efficiency (Bauman and Griinari, 2001). *Trans* 18:1 isomers have received much less attention due to the technical challenges of making purified preparations, however, in this thesis, oils high in EA and VA were fed *in vivo* whereas purified EA and VA were used in cultured mammary cell experiments.

In this thesis, VLDL fraction was found to be the lipoprotein responsible for the transport of the majority of tFA. Traditionally, *trans* and saturated FA in milk fat have caused concern among human health experts, although more recently milk fat has garnered appreciation as a functional food due to the health-promoting potential of some FA found specifically in ruminant-derived products (Bauman et al., 2006). During recent decades, increasing emphasis has been placed on the contribution of ruminant derived foods on the development or prevention of chronic human diseases including cancer, cardiovascular disease and insulin resistance. Milk and dairy products are the main source of 12:0 and 14:0 in the human diet and also provide a major contribution to total 16:0 and tFA consumption in industrialized countries (Givens and Shingfield, 2006). Production of ruminant milk with a FA profile more in line with public health recommendations has the potential to improve long-term human health without requiring substantial changes in consumer eating habits (Shingfield et al., 2010). Findings reported in this thesis showed how tFA are transported and accumulated into mammary gland epithelial cells hence these data can be used to predict the amount of FA secreted in milk with positive human health effects such as VA and CLA when cows are supplied with SO and FO.

Understanding the role of diet on milk fat synthesis and the underlying mechanisms regulating mammary lipogenesis in ruminants is central to the formulation of diets and/or supplements for strategic changes in milk fat content and composition. Numerous reviews have considered the effect of nutrition on milk fat synthesis in ruminants (Bauman and Griinari, 2003; Chilliard et al., 2007; Shingfield and Griinari, 2007), whereas several appraisals have considered possible molecular mechanisms underlying diet-induced changes in mammary lipogenesis and fat secretion (Bauman et al., 2008; Bernard et al., 2008; Harvatine et al., 2009). Recent evidence on factors regulating mammary lipogenic enzyme activity and gene expression in ruminants is also considered within the context of highlighting areas for further investigation and providing an insight into the possible causes for variable milk fat secretion responses to changes in diet composition between ruminant species (Shingfield et al., 2010).

5.9 Limitations

There are several factors affecting milk fat composition that were not studied in the experiments described. Ruminant milk FA composition is linked to intrinsic (animal species, breed, genotype, pregnancy and lactation stages) or extrinsic (environmental) factors (Palmquist et al., 1993; Chilliard and Ferlay, 2004; Chilliard et al., 2007). In a given animal species, the effects linked to breed or genotypes are significant but restricted and they can only be achieved over long terms. For example, the effect of lactation stage on milk fat content and FA composition is perceptible and mainly associated to body fat mobilization in early lactation (Chilliard et al., 1991, 2007), but it only lasts a few weeks each year.

Because non-lactating cows were used in Chapters 2 and 3; caution is needed when data are extrapolated to real dairy production conditions. FA profiles reported in those chapters may be different from lactating cows because milk fat composition is affected by stage of lactation. More importantly, FA metabolism is affected by lactation, which greatly increases metabolic demand for FA and precursors. At initiation of lactation, cows are in negative energy balance, causing mobilization of adipose FA and incorporation of these LCFA into milk fat. However, high uptake of LCFA inhibits *de novo* synthesis of short chain FA by mammary tissue (Belyea et al., 1990; Palmquist et al., 1993). For example, Auldist et al. (1998) found that milk from

early lactation (30 d) contained less short chain FA (4:0 to 12:0) than milk from middle (120 d), and late (210 d) lactation periods in New Zealand cows. Such effects were attributed to the physiological inability of dairy cows in early lactation to consume enough dry matter to meet their energy requirements. Synthesis of 4:0 to 12:0 in the mammary gland increased during early lactation then decreased whereas mobilization of FA from adipose tissue was increased (Palmquist et al., 1993; Jensen, 2002). Garnsworthy et al. (2006) reported variations in the concentration of milk citrate (constituent of milk that affects milk processing characteristics) with regard to stage of lactation. This fluctuation was associated to *de novo* synthesis of FA and independent to diet and milk yield.

For any dietary strategy to be useful, it must not compromise rumen fermentation and, concomitantly, dry matter intake and animal production and/or performance (Lourenço et al., 2010). As in the present experiments, others have often report the effect of different nutritional strategies on lipid metabolism, but no further information is given on the impact of a particular strategy on the whole ruminal function and adaptation processes. It may be possible that the 3-day pulsing periods used in Chapters 2 and 3 were not enough to evaluate the extensive effects of oils (SO, FO and PHVO) on ruminal microbiota given that in real production conditions the ruminal microbial ecosystems can adapt to different diets and hence FA profiles reported for plasma and lipoproteins may be underestimated (Fernando et al., 2010). Moreover, extended pulsing periods could reflect time-dependent ruminal adaptations to supplied lipids, which would lead to alterations in the formation of specific biohydrogenation intermediates (tFA and CLA isomers) (Shingfield et al., 2006) and might also affect the animal's general metabolism and feed intake.

Despite the fact that ruminal pulses were used to study the effect of SO, FO and PHVO on the biohydrogenation process, results may be more useful for dairy production systems if cows were fed a total mix ration including those oils. Practically, supplying dairy cow diets with supplements that increase VA production in the rumen can improve the ratio of unsaturated to saturated FA in milk fat (Jayan and Herbein, 2000). In Chapter 4, EA and VA were used along with other purified FA treatments, however in real dairy production conditions; supplementing cows with specific FA is

not possible because it would be too expensive. In dairy farms, feeding grass and linseed is commonly used to increase VA production in the rumen.

Because in Chapter 4 ethanol was used to dissolve sodium salts of FA allowing FA uptake into the cytosol of epithelial cells, cytosolic TAG, DNA and protein contents may be different if FA treatments were bound to albumin.

5.10 Implications

Data reported in Chapters 2 and 3, showed that different sources of tFA (SO, FO and PHVO) augmented tFA contents in HDL, LDL and VLDL fractions. Such findings suggest that feeding cows with oils rich in UFA and tFA might produce milk with high concentrations of FA undesirable for human consumption and reduce intake of digestible energy (Harvatine and Allen, 2006), milk fat content and yield compared with typical diets (Griinari et al., 1998; Piperova et al., 2000; Peterson et al., 2003; Loor et al., 2005).

Milk fat depression induced by PUFA supplementation in lactating ruminants has been reviewed in detail (Peterson et al., 2003; Bauman et al., 2006) and it is clear that specific FA produced as intermediates during ruminal biohydrogenation of 18:2n-6 play a significant role in reducing milk fat synthesis (Peterson et al., 2003). This mechanism would explain the decrease in milk fat concentration conferred by FO supplementation, in combination with effects of diet upon milk yield and milk fat yield (Capper et al., 2008). Hence, use of dietary SO and PHVO (Chapter 2) or FO (Chapter 3) in the dairy diet has to be carefully balanced to avoid milk fat depression and increase the nutritional value of milk fat.

Data reported in Chapter 4 enhances knowledge on the regulation of milk fat synthesis which is vital to the development of nutritional strategies to improve the nutritional value of milk, decrease milk energy secretion and improve the energy balance of lactating ruminants. FA treatments used in Chapter 4 included different lipid classes because in normal conditions dairy cow diets are supplied with lipids containing different FA classes. Results from Chapter 4 showed that FA uptake of mammary gland cells depends on FA individual chemical structures (chain length of the FA, degree of saturation and configuration, and number and orientation of FA double bonds). Likewise, results from tFA content in plasma and lipoprotein fractions in

Chapter 2 and 3 demonstrated that there are clear differences in their plasma transport that may exert an effect on mammary uptake and utilization of 18-carbon FA, and these results might be related to the location, orientation, and number of double bonds (Tyburczy et al., 2008).

5.11 Future work

Overall, data presented in this thesis are helpful in understanding transport and mammary uptake of tFA. However, further studies are needed to see if the same responses are found in lactating cows and with different sources of FA.

Further studies are required to explore mechanisms by which PUFA exert their toxic effects. If we can understand how selective FA toxicity, or indeed other factors, affects the physiology of biohydrogenating bacteria in the rumen, we may be able to suggest new, rational dietary modifications that will eventually lead to ruminant products that are healthier for human consumption (Maia et al., 2010).

There is a growing concern about environmental impact of animal production systems, for that reason, more research would be desirable to understand how dietary tFA interact with methanogenic *Archae*. Manipulating metabolism of lipids in dairy cows may be helpful to reduce environmental impact of dairy production systems. Plant oils and oilseeds in ruminant diets decrease methanogenesis in ruminants (Machmüller et al., 2003; Martin et al., 2008; Beauchemin et al., 2009). Due to the interrelationship between diet composition, rumen metabolism and mammary lipogenesis, on-farm feeding regimens for decreasing greenhouse gas emissions or altering milk FA composition can also be expected to influence milk fat content (Chilliard et al., 2009) depending on diet composition and ruminant species.

Understanding the regulation of milk fat synthesis is vital to the development of nutritional strategies to enhance the nutritional value of milk, decrease milk energy secretion and improve the energy balance of lactating ruminants. Molecular based techniques have provided an insight into the molecular mechanisms underlying the nutritional regulation of mammary lipogenesis in ruminants. Future experiments examining changes in the mammary transcriptome should provide a more comprehensive insight into the role of specific metabolites on milk fat synthesis in ruminant species.

5.12 Conclusions

This thesis has studied some aspects of metabolism of FA in dairy cows, particularly tFA concentrations in plasma and lipoprotein fractions. Comparison of plasma lipoprotein fractions and FA profiles from cows subjected to ruminally pulsed treatments was assessed to understand the effects of dietary lipids on transportation of tFA among the plasma cholesterol fractions. LCFA uptake in epithelial mammary gland cell cultures on MAC-T cells line was carried out to evaluate the effects of lipids on the transportation of FA in epithelial mammary gland cell cultures.

The current study demonstrates that:

I. The LDL fraction is the main transport mechanism of tFA and is more responsive to supply of tFA and PUFA and hence, tFA concentrations of plasma and lipoprotein fractions can be changed by dietary lipids.

II. Dietary lipids rich in either PUFA or tFA can alter ruminal VFA and NH_3-N.

III. SO (induced high VA production in plasma lipoproteins), PHVO (induced high concentrations of tFA in plasma lipoprotein fractions) and FO (interrupted biohydrogenation of C18 PUFA, resulting in increased production of VA) can increase plasma and lipoprotein-fraction concentrations of tFA.

IV. Cytosolic TAG accumulation is stimulated by the addition of palmitate and elaidate at different concentrations affecting FA profile of cytosolic FFA. Cytosolic TAG accumulation depends on individual chemistry of FA (chain length of the FA, degree of saturation and configuration, and number and orientation of FA double bonds).

Overall, the information from this thesis, strengths understanding of some aspects of milk fat synthesis (Chapter 4) and metabolism of FA in dairy cows and also, data from this thesis can be used to predict milk FA profiles from cows fed with different sources of tFA (SO and FO). Findings from this thesis are important because tFA in milk fat have caused concern among human health researchers and more recently milk fat has gained appreciation as a functional food due to the health-promoting potential of some FA (VA and CLA) found specifically in ruminant-derived products

A p p e n d i x 1

Methods associated with *in vivo* experiments

- **Plasma and lipoprotein fatty acid analysis**
- **Plasma lipoprotein fractions**
- **Dialysis of lipoproteins**

A.1.1 Fatty acid analysis of plasma and lipoprotein fractions

Preparation:

Make reagents up just before experiment is carried out and keep cool.

1. Rinse clean tubes with hexane and leave to dry.
2. Adequately label tubes.

Extraction:

3. Pipette 1ml of plasma directly into a labelled tube with screw cap.
4. Add known amount of pentadecanoic acid to tube (200µl).
5. Add 3.75ml of cold, freshly made chloroform: methanol (1:2) mix and vortex for 1 min, 18ml chroloform + 36ml methanol (for 12 samples).
6. Add 1.25ml cold chloroform.
7. Add 1.25ml of HPLC water and mix (giving final extraction ratio of chloroform: methanol: water as 10:9:9 v/v. Vortex for 1 min.
8. Centrifuge at 3400rpm at 15ºC for 15min.
9. Aspirate water above protein layer in test tube.
10. Collect lower organic chloroform phase, containing lipids into labelled tube using a long form Pasteur pipette.
11. Dry down under a steady stream of nitrogen gas at 40ºC.
12. *Rinse walls of test tube with 1ml chloroform (extracted lipids at this time can be stored in -20ºC).

Methylation:

13. Take tubes out of -20ºC and bring to room temperature, evaporate chloroform to dryness.
14. Heat oxalic acid at 95ºC (in dessicator) for 30mins in oven.
15. Weigh out 0.2g of oxalic acid and add 6ml diethyl ether, this is the termination reagent.
16. To make up 5ml methylation reagent: Use 900µl 30%NaOMe and 4.1ml HPLC methanol. 450µl of NaOMe + 2.05ml Methanol (for 12 samples)
17. Add 2ml hexane to dried sample.
18. Add 40µl methyl acetate to each tube.

19. Add 40µl methylation reagent to each tube.
20. Close tube and vortex for 1min, let stand and leave to react for 10mins.
21. Add 60µl termination reagent.
22. Add ~200mg Calcium Chloride and vortex for 1 min, let stand for 1 h.
23. Centrifuge samples at 3400rpm (2400 x g) for 5mins at 15ºC.
24. Transfer top layer to GC vial and store at -20ºC (if injecting within 24hrs) or -80ºC (longer-term storage) for injection into GC or analyse immediately.

A.1.2 Extraction of lipid from plasma and lipoprotein samples for thin layer chromatography

1. Prepare 15ml Falcon tubes containing 5.4ml of hexane:isopronaponol (3:2, v/v) and 3.6 of sodium sulphate (1g/15ml water).
2. Add 1ml of sample to appropriate labelled 15ml falcon tube.
3. Vortex for 30 sec to 1 min.
4. Centrifuge at 2000 rpm for 5 min at room temperature.
5. Transfer the top layer into a solvent-resistant LP tube.
6. Dry under nitrogen on a heating block until all the solvent has evaporated.
7. Pipette 1ml of hexane around the sides of the tube to wash them and then store at -20°C until needed.

Thin layer chromatography (TLC)

1. Prepare TLC plate by lightly drawing a line across the plate, 2cm up from the bottom. On this line, also mark 2cm in from each side of the plate. The samples should not be loaded in these parts as the samples can run off the sides if the plate does not run straight. Divide the line up into as many sections as you need, leaving at least 1cm between each sample.
2. Prepare TLC tank by adding a solvent mix consisting of petroleum ether bp40-60:diethyl ether:acetic acid (90:30:1) to a TLC tank and place lid on with a weight on top. Let the solvent mix to react for 30 min before placing TLC plates.
3. Load 50 µl of standard along a sample section using a positive displacement pipette. Let sample dry between applications.
4. Load 50 µl to 100 µl of sample (depending on the amount of lipid in the sample) onto the TLC plate and put into tank to run.
5. When the solvent had run to within 2cm of the top of the plate, remove and allow drying before placing in a tank, containing iodine vapour, for staining. The iodine vapour reacts with the unsaturated bonds in the fatty acids staining them brown. Once stained, the bands have to be marked in the plate and left to distain until all colours had disappeared. Only stain the standard and some of the samples to localize the needed bands. For bovine plasma and lipoprotein

fractions iodine vapour has a negative effect and fatty acids are lost in the staining process.

Extraction of lipids from TLC plates

Types of lipid and solvents to use;

Cholesterol ester: 2ml hexane:isopropanol (3:2, v/v) twice

Triacylglycerol: 2ml chloroform:methanol (2:1, v/v) twice

Phospholipid: 2ml chloroform:methanol (1:2, v/v) and,

then 2ml chloroform:methanol (1:5, v/v)

1. Scrape TLC plates into labelled vials fro extraction
2. Add required solvent mix and leave for 30 min agitating frequently but gently
3. Remove solvent to a labelled solvent resistant LP4 tube
4. Add another 2ml of solvent mix and leave for another 30 min as before
5. Remove solvent to tube containing previous solvent
6. Cap tube and centrifuge at 2000 rpm for 10 min at 4°C
7. Carefully remove solvent to another labelled methylation tube without disturbing the silica in the bottom of the tube
8. Dry under nitrogen on a heating block until all the solvent has evaporated

Combine base-acid methylation of bovine plasma and lipoprotein fractions

1. Add 1ml toluene to dissolve lipid, and then add 2ml 0.5 M sodium methoxide/methanol.

Place a Teflon-screw cap on the tube and thoroughly mix

0.5M = 2.7g sodium methoxide in 100ml methanol

2. Incubate at 60°C for 20 min with gentle shaking.

3. After 10 min, add 2ml 14% boron-tri-fluoride (BF_3) in methanol and recommence incubation for a further 20 min with gentle shaking.

4. After 10 min, allow samples to cool to room temperature before adding 5ml distilled water.

5. Mix the samples well and add 2ml hexane. Leave samples until two well-defined phases are seen (to aid separation centrifuge at 2000 rpm for 5 min at room temperature).

6. Transfer the top fatty acid methyl ester (FAME) containing hexane layer into a second methylation tube using a Pasteur pipette.

7. Dry combined hexane samples under nitrogen and re-dissolve in an appropriate volume of hexane. Pipette into a gas chromatography vial and store at -20°C until analysis.

A.1.3 Volatile fatty acid analysis

Preparation of metaphosphoric acid 25%:

- Mix 100ml Distilled water with 54.3g metaphosphoric acid.

Preparation of ruminal fluid for GC analysis:

- Centrifuge 10ml of ruminal fluid in15ml conical tubes @ 4000 rpm for 15mins.
- Mix 1ml of supernatant with 200µl 25% Metaphosphoric acid in an epindorf tube (5:1 ratio).
- Incubate at room temperature for 30 minutes.
- Centrifuge epindorfs @ 12000 rpm for 10 minutes.
- Add to tightly capped GC vials for analysis (1µl).
- GC should have an oven temp of 200 ºC and a detector temperature of 250ºC.

A.1.4 Separation of lipoproteins from bovine plasma by Preparative Sequential Ultracentrifugation

1- Invert each tube of bovine plasma samples to maximise homogeneity
2- Work out how much KBr needs to be added to serum sample to obtain required density:

$$\frac{\text{Volume of sample x (required density – initial density)}}{\text{1 – (0.312 x required density)}}$$

$$= \frac{\text{50mls (volume of serum that we had) x (1.06 – 1.006)}}{\text{1 – (0.312 x 1.06)}}$$

$$= \frac{2.7}{1\text{-}0.3307} = \frac{2.7}{0.6993} = 4.03\text{g KBr}$$

Calculations are based on the Radding-Steinberg Formula (1960)

3- Add KBr to plasma and gently mix (prevent bubbles where possible) to dissolve salt.
4- Add solution to Beckman Quick-Seal® Tubes up to neck of tube:
5- Seal with the bench top tube sealer (ensure that you put metal caps on top of tubes first)
6- Spin the tubes at 39000 rpm for 20 h (or 45000 rpm for 15 h but it will take longer to slow down) under vacuum, at 12ºC using 70.1 Ti Rotor (Beckman Optima Ultracentrifuge XL-70 – No brake).
7- After ultracentrifugation, carefully remove from centrifuge and carry upstairs.
8- Using the Kontron bench top tube slicer carefully cut each tube. Top layer is mostly the LDL, VLDL and CM layer. Place tube into slicer with indentation pointing towards the blade (make sure that the blade is fully back). Ensure the tube is just sticking up a little and place your left thumb lightly over the top. With your right hand turn the handle towards you and the blade will move towards the tube. Once you have started cutting the tube, continue until you have cut the tube completely.

9- Using forceps and the side of the tube holder lever the lid off. The fraction of interest will be trapped in the chamber above the blade. Add contents of chamber to collection tube. Rinse the top of the tube and the surface of the tube slicer blade with a small amount of KBr solution (1.063g/ ml) and re-pipette into collection tube, labelled LDL #.

10- Dry the blade with clean tissue paper and draw it back to expose the bottom section of the tube. Using a different plastic Pasteur pipette, pipette some of the lower tube into other collection tube. Very gently tease tube out of holder and agitate serum proteins etc. *Very sticky so be careful not to block end of pipette*. Rinse tube with KBr solution. Once all tubes are done, make solution up to 40 ml with 1.24g/ ml KBr solution.

Adjust the density to 1.24 using calculation below:

$$\frac{40 \times (1.21 - 1.06)}{1 - (0.312 \times 1.21)} = \frac{40 \times 0.15}{1 - (0.377)} = \frac{6}{0.623} = 9.6\text{g}$$

11- Add salt to tubes and gently mix to minimise bubbles but making sure that KBr dissolves.

12- Syringe each sample into labelled (HDL#) Beckman Quick-Seal® ultracentrifuge tubes (polyallomer), using 5ml syringe and 19G x 1½". Use one syringe for each group. Top the tubes up with 1.24 KBr and heat seal as before.

13- Spin the tubes in ultracentrifuge as before (39000rpm) but for double time (40 h or 45000rpm for 30 h). Ultracentrifuge takes about 4 hours to stop spinning as no brake is used because it will disturb fractions.

14- Carefully cut the tubes as in stage 8.

15- Using a plastic Pasteur pipette (one for each group), pipette the top HDL fraction into a labelled tube; rinse the top of the tube and the surface of the tube slicer with a small amount of 1.24 KBr solution and re-pipette into collection tube.

16- Dry the blade with clean tissue paper and draw it back to expose the bottom section of the tube. Pipette some of the contents into another labelled

(lipoprotein free#) and carefully take tube out of holder, agitate and pipette into collection tube.

17- All samples will require dialysis against 0.15M NaCl 3 x 2L using a magnetic stirrer (change solution after one hour of dialysis, again after the 2nd hour and then leave overnight in the cold room).

A.1.5 Salt solutions for ultracentrifugation of lipoproteins

Potassium bromide (KBr) solutions (pH 7.4*) for lipoprotein separation

For 100ml solution:

Density of KBr solution (g/ml)	KBr (g/100ml)	EDTA (mg/100ml)
1.006	0.874	37.2
1.019	2.786	37.2
1.063	9.426	37.2
1.24	39.144	37.2

For 250ml solution:

Density of KBr solution (g/ml)	KBr (g/100ml)	EDTA (mg/100ml)
1.006	2.186	93
1.019	6.964	93
1.063	23.566	93
1.24	97.860	93

For 500ml solution:

Density of KBr solution (g/ml)	KBr (g/100ml)	EDTA (mg/100ml)
1.006	4.372	186
1.019	13.928	186
1.063	47.131	186
1.24	195.720	186

* All KBr solutions should be adjusted to pH 7.4 by using 0.5M NaOH or 0.5M HCl (it is normally too acidic). Check the density of the prepared solutions by weighing 1ml of each solution and calculate the density: d = wt (g)/ vol (ml).

A p p e n d i x 2

Methods associated with cell cultures

- ***In vitro*** **treatments preparation**
- **Cytosolic fatty acid analysis**
- **Cytosolic protein, DNA and TAG quantification**

A.2.1 MAC-T cell preparation for treatments

Growth medium

It is the media used for growth and maintenance of MAC-T cells

To prepare approximately 100 ml:

- 90 ml RPMI 1640 (comprised of amino acids and vitamins; medium was developed by Moore et al., at Roswell Park Memorial Institute; RPMI) (GIBCO/invitrogen 61870-044)
- 10 ml Foetal bovine serum (FBS)
- 1 ml ABAM (antibiotic-antimycotic solution; 10,000 units of penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml)

Basal medium

Prepared approximately 48 h prior to the last subculture before initializing the experiment

To prepare approximately 25 ml:

22.5 ml Minimum essential medium (MEM) (HYCLONE)

62.5 µl Insulin (Sigma I6634-100 mg)

2.5 µl Hydrocortisone (Sigma H0888-1 g)

25 µl Transferrin (Sigma T1428-50 mg)

25 µl Ascorbic acid (Sigma A4544-25 g)

25 µl Sodium acetate (Sigma S5636-500 mg)

250 µl ABAM (Sigma A5955)

2.5 ml FBS (Sigma F7524)

2.5 µl Progesterone (Sigma P8783-1g)

12.5 mg Lactoalbumin (Sigma L5385-25 mg)

12.5 mg α-lactose (Sigma 47287-U)

Lactogenic medium

Prepared at approximately 24 h before applying cell treatments (approximately 90% confluence)

To prepare approximately 100 ml:

100 ml Dulbecco's modified Eagle's medium (DMEM) (Sigma D6429-500 ml)
250 µl Insulin
10 µl Hydrocortisone
100 µl Transferrin
100 µl Ascorbic acid
100 µl Sodium acetate
1 ml ABAM
100 mg Bovine serum albumin (BSA) (Sigma A4919-5 g)
10 µl Progesterone
50 mg Lactoalbumin
50 mg α-lactose
250 µl Prolactin (Sigma L6520-250 IU)

A.2.2 Preparation of LCFA solutions for cell cultures (example)

1. Prepare a 1 M solution of NaOH (e.g., 4 g in 100 mL PBS or water since NaOH MW is 40)

2. Weigh an amount of 16:0 between 20 and 30 mg in a glass tube. Record precisely the amount of the FA (eg., 26.1 mg, which divided by the MW (256.42) = 101.8 μMoles)

3. Add to the glass tube a 112% μMoles of NaOH 1 M solution (e.g., 101.8 μMoles * 1.12 = 114 μMoles of NaOH = 114 μL of 1M NaOH solution) and mix by swirling in a water bath at 42°C

4. Then add an amount of absolute ethanol to obtain a final solution of 30 mM of 16:0 stock solution (e.g., the 101.8 μMoles in 1 L = 101.8 μM, in 1 mL = 101.8 mM, since 30 mM is needed, then 101.8/30 = 3.39 mL – 0.114 mL (the NaOH solution) = 3.28 mL) has to be added. Mix well in the 42°C bath until all the palmitate is dissolved before storing the stock at -20°C.

A.2.3 Fatty acid analysis of MAC-T cells

Extraction of lipid

1) From Media
    - Collect media (1ml)
    - In a solvent resistant tube add 0.8ml of media to 5.4ml of freshly prepared hexane isopropanol (3:2 v/v) –mix well
    - Add 3.6ml of 6.6% anhydrous sodium sulphate (w/v in water) – mix well
    - Centrifuge briefly at room temperature to separate phases
    - Collect top phase and dry down
    - Take up in 1000µl of hexane and carry on with the procedure and preparation of samples for GC analysis

2) From Cells

    - After removing media wash cells with 3 x 1ml phosphate buffered saline (or something similar)
    - Add 1ml of freshly prepared hexane: isopropanol to each well and leave at room temperature for 30 minutes
    - Collect hexane: isopropanol into a solvent resistant tube
    - Add a further 1ml of hexane:isopropanol to each well –mix and combine with original 1ml
    - Dry down and take up in 1000µl of hexane and carry on with the procedure and preparation GC analysis
    - Washed once with ice-cold PBS+albumin (to remove FA traces) and twice with PBS
    - Scrapped off into 200 µl of 0.05*M* trisodium citrate for DNA and protein quantification and stored at -20°C until analysis After 10 min scrape the plate with to dissolve remaining cell debris in the NaoH

A.2.4 Protein assay by method of Lowry et al. (1951)

BSA standards

Make BSA stock at 5 mg/ml

Protein (µg per 50 µl in well)	Concentration for standard (mg/ml)	Volume BSA (5mg/ml)	Volume H_2O
10	0.2	0.2	4.8
20	0.4	0.4	4.6
30	0.6	0.6	4.4
40	0.8	0.8	4.2
50	1.0	1.0	4.0
60	1.2	1.2	3.8

Reagents (make up fresh using stocks)

1. Prepare reagent 1 using:
    a. 5.0 ml 2% Na_2CO_3 in 0.1 M NaOH
    b. 0.5 ml 1% $CuSO_4$
    c. 0.5 ml 2% KNa tartrate
2. Prepare reagent 2 using:
    a. 0.5 ml Folins Ciocaltuae's phenol reagent
    b. 5.0 ml 0.1M NaOH
3. Prepare BSA standards into plate (0-50 µg in 50 µl)
4. Pipette approximate volume of sample (10-50 µl) per well (make up to 50 µl by addition of 40 µl distilled H_2O
5. Add 150 µl 0.1M NaOH to all wells to make total volume of 200 µl per well
6. Add 50 µl of reagent 1 to all wells and pipette up and won to mix. Leave at room temperature for 5 min
7. Add 50 µl of reagent 2 and leave to stand for 20 min at room temperature
8. Read absorbance at 655 nm

A.2.5 DNA quantification – Hoechst fluorimetric method

Stock solutions

- 10x TNE buffer stock solution

  12.11 g Tris base [Tris (hydroxymethyl) aminomethane], MW = 121.14

  3.72 g EDTA, disodium salt, dihydrate, MW = 372.20

  116.89 g NaCl, MW = 58.44

- 1mg/ml Hoechst dye solution (bisBenzimide H33258; SIGMA B2883-25 mg)
- 20 µg/ml calf thymus DNA (SIGMA D4522 1mg) in distilled water

Sample preparation

1. Scrapped off into 200 µl of 0.05*M* trisodium citrate (Fishers S/3320/60)
2. Centrifuge 2500 rpm at 4°C form 10 min
3. Store at -80°C for later analysis

Working solutions

- 2x TNE
- 2 µg/ml dye in 2x TNE

Add 30 µl of 1 mg/ml stock dye solution to 15 ml of 2x TNE and cover with foil to protect from light

Protocol

1. Prepare serial dilutions of stock standard to range from 20 to 0.0312 µg/ml
2. Add 100 µl of distilled water (blank), standard, or sample (10 µl sample diluted to 100 µl in distilled water) to each well
3. Add 100 µl dye solution to each well
4. Scan on fluorescent plate reader to assay amount of DNA

A.2.6 Triacylglyceride (TAG) quantification

The TAG in the cell lysate was extracted using hexane:isopropanol (3:2 v/v) and quantified using a Triglyceride reagent (Infinity Triglyceride, Thermo Scientific, TR22421).

Protocol

1. After cell lysate is extracted, dry down under nitrogen
2. Add to each tube 50µl of isopropanol and mix
3. Load 10 µl of each standard (duplicate)
4. Load10 µl of each sample (duplicate)
5. Add 250 µl of TAG reagent
6. Incubate at 37°C form 15 min
7. Place it into the platereader

TAG standard concentrations (TAG standards varies depending on the sensitivity needed for each assay)

Standard	mmol/l
1	0
2	0.226
3	0.452
4	0.904
5	1.356
6	1.808
7	2.26

Standard preparations from trace DMA precipitating kit (Thermo) 200 mg/dl

Standard	mmol/l	µl of standard	µl of H_2O
1	0	0	1000
2	0.226	100	900
3	0.452	200	800
4	0.904	400	600
5	1.356	600	400
6	1.808	800	200
7	2.26	1000	0

$$\frac{\text{µl standard}}{\text{µl total}} \times \text{mmol/l of standard}$$

A.2.7 Cryopreservation method for MAC-T cells (example)

1. 4 x 80 cm^2 flasks confluent with a few floating cells.
2. Trypsin/EDTA was filtered through 0.2µ syringe filter before use.

Growth medium

RPMI-1640 with FCS (10%) and ABAM (1%) filtered as above before use.

Cryopreservation medium

RPMI-1640 (70%): FCS (20%): DMSO (10%). RPMI-1640 (35 ml) and FCS (10 ml) filtered then 5ml DMSO was added [Method from Wellnitz, O and Kerr, DE. (2004) Veterinary Immunology and Immunopathology, 101, 191-202 and Keys, JE, Guidry, AJ, and Cifran, E. (1997) *In vitro* Cellular Developmental Biology – Animal. 33, 201-205].

3. 1 flask was trypsinized by pipetting off the medium, washing the cells with 3 ml Trypsin/EDTA, pipetting that off and adding a further 3 ml Trypsin/EDTA. The cells were transferred to 2 X 175 cm^2 flasks with 40 ml Growth Medium per flask.
4. Three flasks were prepared separately for cryopreservation. The cells were trypsinized as above and transferred to 15 ml conical centrifuge tubes. The flasks were rinsed with 4ml RPMI-1640 and this was added to the relevant tube. A further 2ml RPMI-1640 was added to each tube to further dilute out the trypsin.
5. The tubes were centrifuged at 700 rpm for 10 minutes in the MSE Centaur centrifuge.
6. Supernatants were removed by pipette. The cells were resuspended in cryopreservation medium (5 ml per tube). Some clumps persisted after repeated gentle pipetting up and down.

Cell counts:

Cells were diluted 1 in 10 with cryopreservation medium (20µl cell suspension in 180µl medium) and counted in a haemocytometer chamber. Five large squares from each of two grids were counted for each preparation (a total of 10 squares).

Flask (1) 212 cells in 10 squares

21.2 cells in 1 square x 10 (dilution factor) x 10000 = 2 120 000 cells per ml

X 5 (volume in tube) = 10.6 x 10^6 cells per flask.

5 cryovials prepared

Flask (2) 263 cells in 10 squares

26.3 cells in 1 square x 10 (dilution factor) x 10000 = 2 630 000 cells per ml

X 5 (volume in tube) = 13.2 x 10^6 cells per flask. Volume made up to 6.7 ml.

7 cryovials prepared

Flask (3) 185 cells in 10 squares

18.5 cells in 1 square x 10 (dilution factor) x 10000 = 1 850 000 cells per ml

X 5 (volume in tube) = 9.25 x 10^6 cells per flask.

5 cryovials prepared

Cells per flask (mean $^{+/-}$ SEM) 11.0 +/- 1.2 x 10^6 cells per flask.

Vials put into a small plastic bag to keep them upright, wrapped in paper towel and placed in a polystyrene box at -80˚C overnight. Transfer to liquid nitrogen for long term storage.

Appendix 3

Cytosolic and media fatty acid profiles

A.3.1 Cytosolic fatty acid profile

A.3.1.2 Saturated fatty acids

Cytosolic 16:0 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	26.31	24.20	26.27	28.78	3.526	<0.001
Palmitate	10.79	19.59	25.62	22.42		
Stearate	26.92	24.32	22.99	25.79		
Oleate	10.81	5.91	4.62	4.91		
Elaidate	12.27	17.48	11.14	9.15		
Vaccenate	17.81	13.32	9.69	9.78		
Linoleate	9.27	6.50	6.23	4.54		
CLA[1]	20.47	16.95	11.18	8.44		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

Cytosolic 18:0 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	30.09	29.20	31.65	38.23	6.081	0.020
Palmitate	6.67	8.47	8.16	6.59		
Stearate	34.56	35.88	42.21	45.78		
Oleate	8.06	5.01	3.94	4.08		
Elaidate	10.23	17.55	9.14	8.20		
Vaccenate	19.89	13.54	8.47	22.64		
Linoleate	8.37	5.91	5.86	5.13		
CLA[1]	24.31	20.08	11.02	8.58		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

Cytosolic 20:0 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	1.170	1.034	1.300	1.314	1.202	<0.001
Palmitate	0.843	0.901	0.520	0.287		
Stearate	1.266	1.102	1.239	1.263		
Oleate	0.924	0.704	0.699	0.574		
Elaidate	0.774	1.001	0.702	0.547		
Vaccenate	0.910	0.609	0.439	1.230		
Linoleate	9.662	16.938	19.236	25.327		
CLA[1]	0.773	0.716	0.625	0.629		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

A.3.1.3 Monounsaturated fatty acids and *trans* fatty acids

Cytosolic 16:1 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	1.514	0.862	1.472	*	0.3383	0.008
Palmitate	2.339	3.300	2.545	1.675		
Stearate	1.098	*	0.778	*		
Oleate	0.841	0.601	0.539	1.203		
Elaidate	1.306	1.104	1.176	0.888		
Vaccenate	1.103	0.782	0.856	0.627		
Linoleate	0.778	0.582	0.484	*		
CLA[1]	1.193	0.981	0.879	0.772		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; *not detectable.

Cytosolic 18:1 *trans*-9 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	3.685	2.205	0.988	1.052	5.694	<0.001
Palmitate	3.308	4.174	2.645	3.213		
Stearate	*	1.944	0.496	1.707		
Oleate	3.805	2.329	2.209	1.975		
Elaidate	15.495	12.867	32.519	39.963		
Vaccenate	*	*	6.801	*		
Linoleate	3.385	2.733	1.889	1.923		
CLA[1]	1.850	*	*	*		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; *not detectable.

Cytosolic 18:1 *cis*-9 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	7.19	5.16	5.78	2.96	3.544	0.002
Palmitate	11.73	9.95	7.98	5.30		
Stearate	14.66	3.09	8.07	5.13		
Oleate	18.63	28.96	37.45	37.09		
Elaidate	11.34	9.95	15.12	11.62		
Vaccenate	7.37	6.98	7.79	5.19		
Linoleate	7.12	5.26	4.22	3.28		
CLA[1]	7.63	6.35	6.99	6.23		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

Cytosolic 24:1 concentration (g/100g)

	Concentration (µ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	0.683	1.045	0.874	0.744	0.9042	0.026
Palmitate	1.198	2.493	3.483	4.563		
Stearate	0.827	1.091	1.708	1.000		
Oleate	1.935	1.425	0.990	2.004		
Elaidate	0.861	0.812	0.674	0.501		
Vaccenate	0.933	0.755	0.577	1.828		
Linoleate	1.141	2.409	1.605	1.237		
CLA[1]	1.043	1.594	0.808	0.659		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

A.3.1.4 Polyunsaturated fatty acids

Cytosolic 18:2n-*cis* 6 concentration (g/100g)

	Concentration (µ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	2.567	1.538	2.766	1.507	1.213	<0.001
Palmitate	1.151	1.068	0.855	0.817		
Stearate	3.367	1.075	1.675	1.551		
Oleate	1.463	1.077	0.852	0.837		
Elaidate	1.844	1.598	2.110	1.243		
Vaccenate	1.684	1.380	1.343	1.249		
Linoleate	14.166	22.961	25.234	33.523		
CLA[1]	1.833	1.701	1.525	1.369		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

Cytosolic CLA concentration (g/100g)

	Concentration (µ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	*	*	*	*	5.383	<0.001
Palmitate	*	*	*	*		
Stearate	2.638	*	0.967	1.135		
Oleate	0.823	1.091	1.195	1.134		
Elaidate	4.835	*	0.761	0.166		
Vaccenate	4.521	3.771	4.774	4.140		
Linoleate	*	*	*	0.648		
CLA[1]	6.709	13.277	28.503	38.538		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; *not detectable.

Cytosolic 20:4n6 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	*	*	*	*	0.2598	0.038
Palmitate	1.148	1.183	0.944	0.767		
Stearate	*	0.540	*	*		
Oleate	1.639	1.233	1.047	0.775		
Elaidate	1.125	*	*	*		
Vaccenate	1.777	1.126	0.957	*		
Linoleate	1.153	0.844	0.644	0.543		
CLA[1]	1.668	1.463	1.661	1.597		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; *not detectable.

A.3.1.5 Major fatty acid classes in cytosol

Cytosolic MUFA concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	14.47	14.32	11.97	7.35	7.199	<0.001
Palmitate	34.53	30.70	23.30	22.50		
Stearate	17.67	9.75	13.31	9.52		
Oleate	43.22	57.42	70.59	69.59		
Elaidate	36.48	36.28	61.61	65.72		
Vaccenate	20.55	18.93	20.99	20.70		
Linoleate	21.87	16.42	13.73	10.16		
CLA[1]	19.79	15.56	16.98	14.10		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

Cytosolic PUFA concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	13.38	14.31	18.45	13.42	4.021	<0.001
Palmitate	19.88	16.11	14.41	22.07		
Stearate	14.10	15.69	12.61	10.22		
Oleate	16.88	15.66	10.60	11.71		
Elaidate	10.40	10.37	8.09	6.86		
Vaccenate	17.89	13.98	11.44	12.39		
Linoleate	30.86	39.10	38.88	43.65		
CLA[1]	18.58	27.21	38.37	49.29		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

Cytosolic SFA concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	61.86	61.61	63.32	72.31	8.952	<0.001
Palmitate	22.55	32.61	37.56	35.21		
Stearate	65.65	66.81	69.16	76.15		
Oleate	22.47	13.30	9.44	10.65		
Elaidate	24.64	40.08	21.89	19.18		
Vaccenate	43.40	31.79	20.62	32.67		
Linoleate	31.64	33.11	36.60	37.50		
CLA[1]	49.60	42.99	28.83	22.17		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

Cytosolic tFA concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	4.26	5.22	2.27	1.56	4.589	<0.001
Palmitate	9.47	8.51	5.87	7.50		
Stearate	1.62	4.63	3.13	2.88		
Oleate	7.12	7.00	4.16	4.72		
Elaidate	36.67	20.49	42.75	50.88		
Vaccenate	12.09	28.72	43.01	33.93		
Linoleate	6.86	5.25	5.53	4.56		
CLA[1]	6.26	4.37	3.60	4.34		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

Cytosolic UFA concentration (Mono + PUFA; g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	27.85	28.64	30.43	20.77	7.462	<0.001
Palmitate	54.42	46.81	37.70	44.57		
Stearate	31.77	25.44	25.92	19.75		
Oleate	60.10	73.08	81.18	81.30		
Elaidate	46.89	46.64	69.71	72.58		
Vaccenate	38.44	32.91	32.44	33.09		
Linoleate	52.73	55.52	52.62	53.81		
CLA[1]	38.37	42.77	55.34	63.39		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

A.3.2 Media fatty acid profile

A.3.2.1 Saturated fatty acids

Media 18:0 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	26.17	23.14	23.69	23.26	3.075	0.014
Palmitate	37.57	33.28	28.84	28.78		
Stearate	24.13	31.51	32.98	30.61		
Oleate	34.66	33.50	34.73	27.76		
Elaidate	35.99	30.52	32.97	28.90		
Vaccenate	33.32	33.39	32.10	29.40		
Linoleate	35.26	35.56	32.77	33.10		
CLA[1]	37.74	39.93	30.29	29.98		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

Media 21:0 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	0.668	1.148	1.402	1.448	0.5798	0.012
Palmitate	0.939	0.742	*	1.511		
Stearate	0.777	1.572	3.409	1.586		
Oleate	1.243	1.583	0.827	1.175		
Elaidate	0.785	1.251	0.843	1.196		
Vaccenate	1.523	1.541	0.534	1.175		
Linoleate	0.987	1.147	0.880	0.956		
CLA[1]	1.231	1.132	0.906	3.116		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; *not detectable.

A.3.2.2 Monounsaturated fatty acids

Media 18:1 *cis*-9 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	1.010	0.966	0.857	1.055	1.448	0.004
Palmitate	2.715	3.014	3.737	3.559		
Stearate	0.919	1.159	1.447	1.126		
Oleate	5.732	5.291	9.062	14.852		
Elaidate	2.101	2.572	2.886	3.127		
Vaccenate	1.807	2.002	2.152	1.910		
Linoleate	2.719	2.390	2.897	2.450		
CLA[1]	1.896	1.903	1.812	2.632		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale);
[3]*P*-value represents the probability of a treatment-concentration interaction.

A.3.2.3 Polyunsaturated fatty acids

Media 18:2n-*cis* 6 concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	0.515	0.502	0.473	0.474	0.3145	<0.001
Palmitate	0.852	0.815	1.061	0.926		
Stearate	0.447	0.497	0.475	0.428		
Oleate	2.060	0.667	0.856	0.914		
Elaidate	0.912	0.934	0.752	0.780		
Vaccenate	0.685	0.883	0.742	0.764		
Linoleate	1.697	4.705	8.092	11.476		
CLA[1]	0.807	0.739	0.760	0.849		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale);
[3]*P*-value represents the probability of a treatment-concentration interaction.

Media CLA concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	*	*	*	*	2.608	0.034
Palmitate	*	*	*	*		
Stearate	*	*	*	*		
Oleate	*	*	*	*		
Elaidate	*	*	*	*		
Vaccenate	*	*	0.637	0.509		
Linoleate	*	*	*	*		
CLA[1]	2.613	2.678	2.966	12.772		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction; *not detectable.

A.3.2.4 Major fatty acid classes in the media

Media tFA concentration (g/100g)

	Concentration (μ*M*)					
Fatty acid	100	200	300	400	SED[2]	*P*-value[3]
Control	0.835	1.090	0.854	1.801	1.529	0.006
Palmitate	1.095	0.652	0.431	1.445		
Stearate	0.673	1.553	2.131	4.127		
Oleate	1.124	2.218	0.247	1.688		
Elaidate	2.767	6.155	6.640	11.483		
Vaccenate	5.487	6.254	7.959	12.825		
Linoleate	1.248	1.283	1.364	2.281		
CLA[1]	2.244	0.181	0.599	0.000		

[1]Conjugated linoleic acid; [2]Standard errors of differences between means (calculated on variance scale); [3]*P*-value represents the probability of a treatment-concentration interaction.

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