ROLE OF FORAGE SPECIES AND CONSERVATION METHOD IN RUMINAL LIPID METABOLISM, MAMMARY LIPOGENESIS AND MILK FATTY ACID COMPOSITION IN LACTATING COWS

DOCTORAL THESIS

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ACADEMIC DISSERTATION

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DEPARTMENT OF AGRICULTURAL SCIENCES I **PUBLICATIONS** I 21

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LIST OF ORIGINAL PUBLICATIONS

This thesis consists of a general discussion and the following original publications subsequently referred to in the text by their Roman numerals:

I Halmemies-Beauchet-Filleau, A., Kairenius, P., Ahvenjärvi, S., Crosley, L.K., Muetzel, S., Huhtanen, P., Vanhatalo, A., Toivonen, V., Wallace, R.J., and Shingfield, K.J. 2013. Effect of forage conservation method on ruminal lipid metabolism and microbial ecology in lactating cows fed diets containing a 60:40 forage-to-concentrate ratio. J. Dairy Sci. 96:2428–2447.

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Experiments were conducted in the metabolism unit, MTT Agrifood Research Finland, Jokioinen, Finland (Publications I-IV), or at the Viikki research farm, University of Helsinki, Finland (Publication V).

CONTRIBUTIONS

The contributions of all authors to the original articles of this thesis are described in the following table (initials of authors are listed in alphabetical order).

	I	II	III	IV	V
Planning the experiment	AV KS PH PK SA	AV KS PH PK SA	AH AV KS TH	AH AV KS TH	AH AV KS TK
Conducting the experiment	AH KS PK SA	AH KS PK SA	AH TH	AH TH	AH AV TK
Sample analysis	AH KC SM PK VT	AH PK VT	AH ML KS VT	AH VT	AH AL VT TK
Data analysis	AH KS PK	AH KS PK	AH KS	AH KS	AH TK
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ABSTRACT

The objective of the research described in this thesis was to develop forage-rich diets for dairy cows for altering milk fatty acid composition, with the potential to improve human health. Emphasis was placed on the potential to decrease milk fat medium chain saturated fatty acids (SFA; 12:0, 14:0 and 16:0) and increase *cis*-9 18:1 and polyunsaturated fatty acids (PUFA) concentrations, 18:3n-3 in particular. Experiments encompassed detailed investigations of ruminal and mammary lipid metabolism, in order to understand the mechanisms underlying diet-induced changes in bovine milk fatty acid composition.

Experiments documented in publications I and II involved a detailed physiological study in which, the effects of forage conservation method on fatty acid intake, ruminal lipid metabolism, plasma fatty acid profile, mammary lipid metabolism and milk fat composition of lactating cows were investigated. Drying of grass resulted in substantial decreases in forage fatty acid content, 18:2n-6 and 18:3n-3 in particular, whereas fatty acid losses due to ensiling were rather small. Compared with fresh grass or grass silage (GS), feeding diets based on grass hay lowered the extent of lipolysis and biohydrogenation of dietary unsaturated fatty acids in the rumen, while the extent of silage fermentation had minor effects, leading to similar flows of 18:2n-6 and 18:3n-3 at the omasum across all diets. On all diets, triacylglycerol in circulating lipids was the primary source of fatty acids taken up by mammary gland. In cows fed fresh grass, non-esterified fatty acids (NEFA) also served as a source of fatty acids for milk fat synthesis. A higher uptake of preformed fatty acids along with decreases in fatty acid synthesis *de novo* accounted for higher *cis*-9 18:1, *trans*-11 18:1, *cis*-9,*trans*-11 CLA, 18:2n-6 and 18:3n-3, and lower 16:0 and total SFA in milk fat of cows fed fresh grass relative to dried grass. In cows fed diets based on conserved grasses, differencies in the composition of circulating lipids, mammary uptake of fatty acids, mammary *de novo* synthesis and milk fatty acid composition were marginal.

The impact of replacing GS with red clover silage (RCS) on fatty acid intake, ruminal lipid metabolism and milk fat composition is reported in publications III and IV. Replacing GS with RCS linearly decreased proteolysis and the extent of lipolysis and biohydrogenation of dietary unsaturates in the rumen. The proportion of RCS in the diet had no effect on the amounts or on the relative proportions of major lipid classes at the omasum. On average, NEFA, polar lipids, triacylglycerols, diacylglycerols and monoacylglycerols accounted for 80, 12, 4, 2 and 1% of total fatty acids in omasal digesta, respectively. Increases in the flow of 18:2n-6 and 18:3n-3 at the omasum accounted for the higher secretion of these fatty acids in milk from diets containing RCS, with no evidence of impaired PUFA bioavailability in contrast to nitrogen or decreases in mammary *de novo* synthesis of SFA up to a chain length of 16-carbon. Furthermore, forage species had no effect on the flow of bound phenols at the omasum that are formed as a consequence of polyphenol oxidase (PPO) activity. Current data suggest that the changes in ruminal lipid metabolism when RCS replaces GS, are not directly related to PPO activity and/or the formation of protective protein matrices, but are more likely to be related to the role of forage species on rumen digestion kinetics and ruminal microbial populations.

The potential to mimic the changes in milk fat composition due to forage conservation method or species by supplementing diets based on RCS with moderate amounts of plant oils or camelina expeller was also examined (V). Moderate lipid supplementation did not impair silage dry matter intake or milk production, but altered milk fatty acid composition. Changes in milk fat composition to plant lipids were characterised by decreases in SFA synthesized *de novo* and increases in the concentrations of unsaturated fatty acids, including *trans*-11 18:1, *cis*-9,*trans*-11 CLA and those inherent in specific lipid supplements (*cis*-9 18:1, 18:2n-6 and 18:3 for rape, sunflower and camelina lipids, respectively). The increase in milk *trans* fatty acids due to lipid supplementation was rather small, except when camelina expeller was fed.

Results demonstrated potentially beneficial effects of fresh grass and RCS on milk fatty acids. In particular, fresh grass has potential to decrease milk fat medium chain SFA and to increase *cis*-9 18 concentrations, whereas RCS has potential to increase 18:3n-3. Moderate inclusion of plant lipid supplements on RCS based diet can be used to alter milk fatty acid composition yet further, through additional decreases in medium chain SFA and increases in unsaturated fatty acids inherent to lipid supplements.

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ABBREVIATIONS

ACC	acetyl-CoA carboxylase
BHBA	β-hydroxy butyric acid
CE	cholesterol ester
CEX	camelina-seed expeller
CLA	conjugated linoleic acid
CO	camelina-seed oil
DAG	diacylglycerol
DM	dry matter
DMI	dry matter intake
ECM	energy corrected milk
EFA	esterified fatty acids
FAME	fatty acid methyl ester
FAS	formic acid treated silage
FM	fresh matter
GS	grass silage
MAG	monoacylglycerol
MUFA	monounsaturated fatty acid
NADPH	nicotinamide adenine dinucleotide phosphate
NAN	non-ammonia nitrogen
NEFA	non-esterified fatty acids
NDF	neutral detergent fibre
OBCFA	odd- and branched-chain fatty acids
OM	organic matter
PCR	polymerase chain reaction
PL	phospholipid
POL	polar lipid
PPO	polyphenol oxidase
PUFA	polyunsaturated fatty acid
RCS	red clover silage
RO	rapeseed oil
SFA	saturated fatty acid
SFO	sunflower-seed oil
TAG	triacylglycerol
TMR	total mixed ration
UTS	untreated silage
VFA	volatile fatty acids

Keywords: dairy cow, forage conservation, red clover, rumen, lipolysis, biohydrogenation, plasma, mammary lipogenesis, milk fatty acid composition, 18:3n-3, conjugated linoleic acid, saturated fatty acid

1 INTRODUCTION

Lipids are an important component of milk, affecting the nutritional value, aroma, flavour, texture and storage characteristics of dairy products, as well as, the price at retail. However, ruminant meat and milk are characterised by a high concentration of saturated fatty acids (SFA) and a low content of polyunsaturated fatty acids (PUFA), due in part, to extensive biohydrogenation of dietary unsaturates in the rumen (Kim et al., 2009; Shingfield et al., 2010). In humans, consumption of excessive amounts of SFA, the medium chain 12:0, 14:0 and 16:0 in particular, is associated with an increase in cardiovascular disease risk and development of insulin resistance and dyslipidaemia (WHO, 2003; Shingfield et al., 2008b). Milk and dairy products are typically a major source of medium-chain and total SFA in the Western diet (Hulshof et al., 1999; Kris-Etherton et al., 2000; National Public Health Institute of Finland, 2008). Therefore, there is considerable interest in lowering the amounts of medium chain SFA and increasing *cis*-9 18:1, 18:2n-6, conjugated linoleic acid (CLA) and 18:3n-3 in milk as a strategy to improve long-term human health, and thereby lower the social and economic burden of chronic disease (Gebauer et al., 2006; Shingfield et al., 2008b).

Replacing SFA and *trans* fatty acids with *cis* containing monounsaturated fatty acids (MUFA) and PUFA is thought to be more effective in the prevention of coronary heart disease than lowering overall fat intake from the human diet (Hu et al., 1997). It has been predicted that replacing 2.2 g of SFA consumption equivalent to 7.6% of average daily SFA intake in European populations with MUFA and PUFA would decrease total blood cholesterol by 0.06 mmol/l and thereby result in 3,000 (-0.77%) and 9,800 (-1.67%) fewer deaths due to stroke and coronary heart disease in the EU-15 member states (Lloyd-Williams et al., 2008). Recent predictions of the possible impact of decreasing milk fat SFA content from 70 to 55% (-8.8% in daily SFA intake) and increasing milk *cis* MUFA concentrations from 20 to 32%, suggest that implementation across the whole food chain may lower deaths from coronary heart disease in Europe, by between 2.0 and 3.9% (Givens, 2008).

Forage plants have the special ability to synthesize 18:3n-3 *de novo*, and therefore, represent a natural, environmentally-sustainable and relatively inexpensive source of essential fatty acids and other nutrients for ruminants (Dewhurst et al., 2003c). Depending on the production system, fresh or conserved forages typically contribute to between 25 to 100% of the energy requirements of lactating cows, but little is known about the effect of forage conservation on ruminant lipid metabolism and milk fat composition (Dewhurst et al., 2006). In Finland, fresh grass, hay or straw and silage contribute, on average, to 6, 1 and 46% of total dry matter intake (DMI), respectively, based on records from herds participating in the

National milk recording scheme in 2012 (Huhtamäki, 2013). During hay-making, exposure to solar radiation and extensive wilting result in oxidative losses of unsaturated fatty acids from cut herbage (Dewhurst and King, 1998; Boufaïed et al., 2003a). However, direct ensiling of herbage has only minor effects on forage fatty acid content and composition, provided no secondary fermentation takes place (Boufaïed et al., 2003a; Dewhurst et al., 2006).

Lipolysis is a prerequisite for ruminal biohydrogenation, but studies *in vivo* reporting the distribution of esterified and non-esterified fatty acid (NEFA) fractions in the diet and digesta of lactating cows are scarce (Fievez et al., 2007). The rate of 18:3n-3 disappearance and 18:0 formation during *in vitro* incubations with mixed rumen bacteria was shown to be higher for fresh grass and silage compared with hay prepared from the same grass (Boufaïed et al., 2003b), suggesting that the extent of ruminal metabolism of forage lipid is lower for dried grass compared with fresh or ensiled grass.

Milk fat from grazing cows is known to contain lower proportions of SFA and higher *cis*-9 18:1, *cis*-9,*trans*-11 CLA and 18:3n-3 concentrations compared with diets containing conserved forages (Dewhurst et al., 2006; Chilliard et al., 2007; Ferlay et al., 2011), responses that may be explained by the inhibitory effects of increases in long chain fatty acid supply on acetyl-CoA carboxylase (ACC) activity, and the synthesis of SFA *de novo* in mammary secretory cells (Shingfield et al., 2010). Nevertheless, available data suggest that the impact of forage conservation on milk fat composition is not explained solely in terms of differences in dietary fatty acid intake (Lock and Garnsworthy, 2003; Mohammed et al., 2009). Compared with silage, milk from hay-based diets result in higher concentrations of 18:2n-6 and 18:3n-3, despite much lower intakes of these PUFA (Shingfield et al., 2005). Furthermore, restricting silage fermentation has often resulted in a higher lipogenic to glucogenic ratio of VFA in the rumen (Huhtanen, 1998; Shingfield et al., 2002b; Jaakkola et al., 2006a) that may result in a higher milk fat yield (Shingfield et al., 2002a).

In addition to conservation, forage species also influences milk fat composition (Dewhurst et al., 2006). Milk fat from cows fed red clover silage (RCS; *Trifolium pratense*) contains higher concentrations of 18:3n-3 compared with grass silage (GS; Dewhurst et al., 2003b, Al-Mabruk et al., 2004; Vanhatalo et al., 2007; Moorby et al., 2009). Studies in lactating cows and growing steers have demonstrated that the benefits of red clover on milk or meat PUFA concentration are related to higher postruminal flows of 18:2n-6 and 18:3n-3 (Dewhurst et al., 2003a; Lee et al., 2003; 2006), but the reasons underlying these differences remain unclear. Red clover has a higher polyphenol oxidase (PPO) activity compared with grasses (Lourenço et al., 2008; Koivunen et al., 2012), that has been suggested to inhibit proteolysis and lipolysis in the rumen, due to the formation of protected protein-phenol matrices that may entrap and thereby

protect forage lipids from metabolism in the rumen (Kim et al., 2009; Van Ranst et al., 2010).

To meet the nutrient and energy requirements of high-yielding dairy cows, forages are typically supplemented with concentrates. Even though the intake of fatty acids can be increased and the fatty acid composition of milk modified by lipid supplements (Dewhurst et al., 2006; Glasser et al., 2008a; Shingfield et al., 2008b), forage DMI is often decreased at high rates of plant or marine lipid inclusion (Lock and Shingfield, 2004; Huhtanen et al., 2008; Shingfield et al., 2012). However, a moderate amount of supplementary plant lipids in forage-rich diets (concentrate fat content less than 60 g/kg DM with the average consumption of concentrate and forage-to-concentrate ratio of 8.7 kg DM/d and 52:48, respectively), may avoid adverse effects on DMI (Huhtanen et al., 2007; 2008) and therefore maintain the beneficial effects associated with lipid from forages.

There are various potential plant lipids such as those in rapeseed, sunflower-seed, linseed and camelina-seed available for supplementary use. Except for camelina, most of these lipid supplements are well-known. Camelina (*Camelina sativa* L.) is an ancient, low-input oilseed crop in the family *Brassicaceae.* It grows in the temperate climate zone in Europe, Asia and North America as summer or winter annual crop (Zubr, 2003; CFIA, 2012). Besides containing significant amounts of essential fatty acids 18:2n-6 and 18:3n-3, camelina seeds are relatively abundant in essential amino acids (Zubr, 2003) indicating the potential of this oilseed as both a high quality protein and lipid supplement for ruminants.

The basal forage in the diet is also an important determinant of milk fatty acid composition responses to plant lipids (Lock and Shingfield, 2004; Roy et al., 2006; Chilliard et al., 2007). Inclusion of 18-carbon rich plant oils in GS or maize silage based diets typically lower the proportion of medium-chain SFA and increase 18:0, *cis*-9 18:1, CLA and *trans* fatty acid concentrations in bovine milk fat (Givens and Shingfield, 2006; Chilliard et al., 2007; Shingfield et al., 2008b), but there is limited data on the impact of lipid supplements on milk fat composition in cows fed diets based on RCS.

2 OBJECTIVES OF THE STUDY

The ultimate goal of the research reported in this thesis was to develop forage-rich diets for dairy cows that alter milk fatty acid composition, in a way thought to be beneficial for human health. The changes targeted included a decrease in medium chain SFA and an increase in *cis*-9 18:1 and 18:3n-3 concentrations. For individual experiments, the objectives were to examine the effect of forage conservation method (I, II), forage species (III, IV) and supplementary plant oils or camelina expeller (CEX; V) on ruminal (I, III) and mammary lipid metabolism (II), and milk fatty acid composition (II, IV, V). Ruminal and mammary lipid metabolism, and the factors that influence them, were investigated in detail to understand the mechanisms underlying the diet-induced changes in bovine milk fatty acid composition (Figure 1).

The main hypotheses tested in this research were:

- feeding fresh grass relative to hay decreases the concentration of SFA and increases PUFA concentration in milk fat due to changes in the supply of long chain fatty acids, and as a result, alterations in mammary lipogenesis (I, II)
- conservation of grass as hay relative to silage increases milk fat PUFA concentration due to a decrease in the extent of ruminal lipid metabolism (I, II)
- restricting silage fermentation increases *de novo* synthesis of short and medium chain SFA in the mammary gland due to a higher supply of lipogenic precursors, in the absence of substantial changes in the secretion of preformed long chain fatty acids (I, II)
- the effect of replacing GS with RCS on milk fat composition is related to lower lipolysis and biohydrogenation of dietary unsaturated fatty acids in the rumen through the action of PPO (III, IV)
- moderate inclusion of plant lipid as oil or CEX in diets based on RCS do not decrease forage DMI, but lower SFA and increase unsaturated fatty acid concentrations (*cis*-9 18:1, 18:2n-6, and 18:3n-3 for rape, sunflower and camelina lipid supplementation, respectively) in milk, with the changes in unsaturates being related to the composition of a given lipid supplement (V).

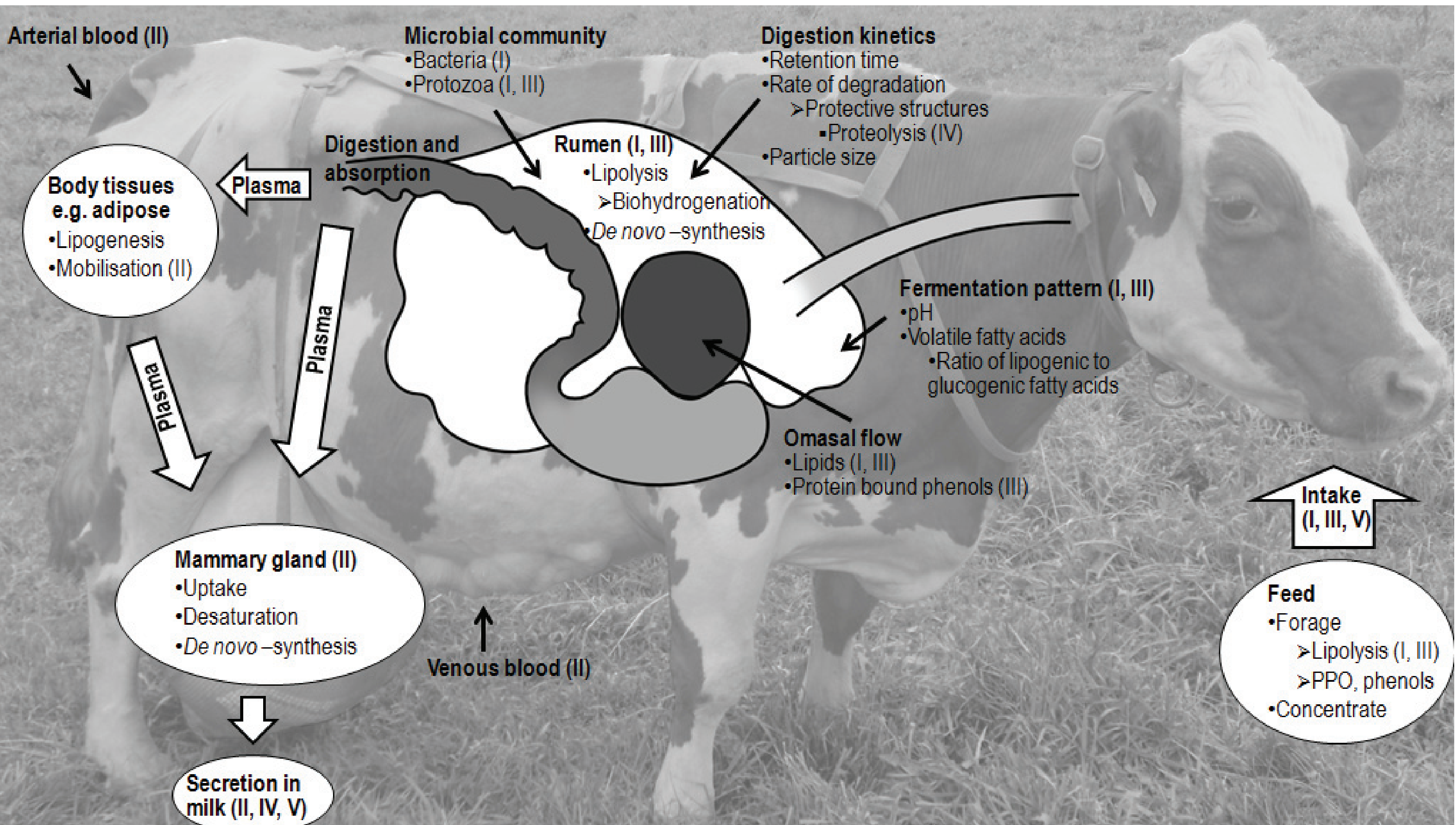

Figure 1 The main factors influencing the fatty acid composition of milk from lactating cows and the measurements made in publications I to V. PPO=polyphenol oxidase

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS AND PROCEDURES

The studies documented in publications I-V were conducted as four separate experiments (Table 1). All experiments were performed with multiparous Finnish Ayrshire dairy cows in early or in mid-lactation fitted with rumen cannulae, except for experiment 4, in which intact cows were used. Experiments were conducted as a replicated 3 x 3 (expt. 2; with 5 cows, 3 treatments, and 3 periods), 4 x 4 (expt. 3) or 5 x 5 (expt. 4) Latin square, except for experiment 1 that was carried out according to a much simpler design where all cows were fed fresh grass during the first experimental period followed by dried hay prepared from the same grass swards during the second period (Figure 2). In experiment 1, fresh grass was harvested each morning, treated with a small amount of a formic acid-based additive (0.5 L/t grass; 760 g of formic acid and 55 g of ammonium formate) and stored in batches of 10 to 15 kg fresh matter (FM) in a freezer at 4°C before feeding out, in order to minimize potential oxidative deterioration. Experimental periods lasted for 14 d for experiments 1 and 2. The experimental design used in experiment 1 may be criticized due to the confounding effects of treatment with time. However, minimising variations in herbage maturity and composition was considered more important to understanding the biological impact of forage conservation, than time related effects associated with short experimental periods. This approach was considered justified, given that several experiments have demonstrated that for cows in mid-lactation, the time dependent changes in milk production and milk fat composition are marginal (Roy et al., 2006; Shingfield et al., 2006), compared with the differences expected between fresh and conserved forages (Chilliard et al., 2001; Dewhurst et al., 2006; Mohammed et al., 2009). For experiments 3 and 4, each experimental period lasted for 21 d.

The experimental procedures used are described in detail within individual publications, and therefore only a brief outline is provided presently. Feed intake was determined as the difference between the amount of feeds offered and the amount of refused feeds. Rumen fermentation was measured by sampling rumen fluid through rumen cannulae at regular intervals (I, III). Rumen bacterial populations capable of biohydrogenation were determined by quantitative polymerase chain reaction (PCR) analysis of ruminal digesta (I). In publications I, III and IV, the omasal sampling technique in combination with a triple marker system were used to assess nutrient flow entering the omasal canal (Ahvenjärvi et al., 2000). Bacterial samples were collected manually from reticular digesta and rumen microbial protein synthesis was assessed using ^{15}N as a microbial marker (I, IV). Diet digestibility was measured by total faecal

collection (I, IV) or using acid insoluble ash as a marker (V). Blood samples taken from epigastric (mammary) vein were considered to represent venous blood (II) and that from coccygeal vessels (tail vein) to represent arterial blood (II, V). Lipid in arterial and venous plasma was extracted in duplicate using a mixture of hexane and isopropanol 3:2 (v:v). Extracts were separated into several lipid classes [NEFA, triacylglycerol (TAG), phospholipid (PL) and cholesterol ester (CE)] by solid phase extraction using Bond Elut® NH_2 aminopropyl bonded silica cartidges (500 mg, Varian Inc., Lake Forest, CA) and a solvent system (II). Mammary plasma flow was estimated according to the Fick principle using phenylalanine and tyrosine as markers (II). Daily milk yields of all experimental cows were recorded throughout each experiment. Samples for the analysis of milk composition were collected and composited according to yield over four consecutive milkings. Mammary metabolism of nutrients was studied by the measurement of arterio-venous differences and the secretion of fatty acids in milk. For all experiments, cows were housed in individual stalls with continous acces to water and milked twice daily.

After adjusting the pH to 2.0 by hydrochloric acid, lipid in freeze-dried feeds and omasal digesta were extracted in duplicate by sonication using a 3:2 (v:v) mixture of hexane and isopropanol as a solvent. Extracts were separated into several lipid classes by solid phase extraction using Bond Elut® NH_2 aminopropyl bonded silica cartidges (500 mg, Varian Inc., Lake Forest, CA) and a solvent system (I), or by thin layer chromatography using preparative silica plates (1.13895.0001, Merck, Darmstadt, Germany) developed using a 70:30:2 (v:v) mixture of hexane, diethylether and acetic acid (III). Lipid in milk samples was extracted using a mixture of ammonia, ethanol, diethylether and hexane (0.2:1:2.5:2.5, v/v; II, IV, V). Esterified lipids were methylated using methanolic sodium methoxide as a catalyst, whereas methanolic sulphuric acid was used for the transesterification of NEFA (I-V). Fatty acid methyl esters (FAME) of feeds in experiment 4 and concentrate in experiments 1 and 2 were prepared in a one-step extraction-transesterification procedure (Shingfield et al., 2003). The FAME recovered were analysed by gas- and liquid-chromatography (I-V).

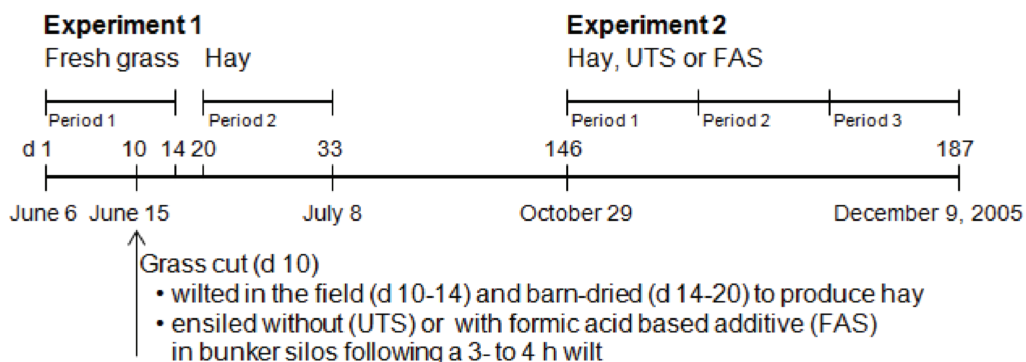

Figure 2 Schematic describing the scheduling of ensiling or drying of grass swards relative to the feeding of dietary treatments to cows in experiments 1 and 2 (Publications I and II).

3.2 EXPERIMENTAL TREATMENTS

Experiment 1 was conducted to evaluate the impact of forage conservation by drying on ruminal lipid metabolism, plasma lipids, mammary lipogenesis and milk fatty acid composition. Dietary treatments comprised fresh chopped grass or barn-dried grass hay fed *ad libitum* and supplemented with 7 kg/d of standard concentrate.

In experiment 2 the effect of different forage conservation methods on ruminal lipid metabolism, plasma lipids, mammary lipogenesis and milk fatty acid composition were compared. Dietary treatments comprised grass hay, extensively fermented GS (untreated, UTS) or restrictively fermented GS prepared with formic acid based additive (FAS). Forages were fed *ad libitum* and supplemented with 9 kg/d of the same standard concentrate as in experiment 1. All forages in experiments 1 and 2 were prepared from the same primary growths of timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*). Conservation of forages coincided with the collection of samples from cows offered fresh grass (experiment 1, period 1, day 10) to minimize differences in herbage maturity between experimental treatments (Figure 2).

Experiment 3 investigated the effect of incremental replacement of GS with RCS in the diet on ruminal lipid metabolism and milk fatty acid composition. Experimental treatments consisted of total mixed rations (TMR) containing 600 g forage/kg diet dry matter (DM) with RCS replacing GS in the ratio of 0, 1/3, 2/3 and 1 on a DM basis. Forages were supplemented with a standard concentrate and TMR were offered *ad libitum*. Grass silage was prepared from primary growths of timothy and meadow fescue and RCS from secondary growths. Both silages were ensiled with formic acid based additive.

Experiment 4 examined the impact of various plant oils that differed in fatty acid composition and CEX on DMI, milk production and milk fatty acid composition in cows fed diets based on RCS. The purpose of this experiment was to examine the possibility to mimic the changes in milk fat composition due to forage conservation method or forage species by supplementing diets based on RCS with moderate amounts of plant lipids. Furthermore, the potential of plant lipids to futher extend the beneficial effects of RCS on milk fatty acid composition was investigated. Experimental treatments consisted of five concentrates (12 kg/d) containing no additional lipid (control), or 29 kg lipid/kg concentrate FM from rapeseed oil (RO), sunflower-seed oil (SFO), camelina-seed oil (CO) or CEX as sources of *cis*-9 18:1, 18:2n-6 and 18:3n-3, respectively. Red clover silage was prepared from secondary growths, ensiled with formic acid based additive and fed *ad libitum.*

Table 1 Summary of experiments

Publ.	Exp.	Design and animals	Dietary ingredients	Treatments	Objective
I, II	1	Simple design: all cows zero-grazed Grass 1st period followed by Hay during 2nd period 5 rumen fistulated dairy cows 229 DIM	Fresh grass (Grass) Grass hay (Hay) Untreated grass silage (UTS) Formic acid treated grass silage (FAS) Standard concentrate (C)	Grass *ad lib* Hay *ad lib* + C 7 kg/d	Effect of forage conservation method on • ruminal lipid metabolism • rumen fermentation characteristics • rumen microbial communities • plasma lipids • mammary lipogenesis • milk production • milk fatty acid composition
	2	3 x 3 Latin Square 5 rumen fistulated dairy cows 53 DIM		Hay *ad lib* UTS *ad lib* FAS *ad lib* + C 9 kg/d	
III, IV	3	4 x 4 Latin Square 4 rumen fistulated dairy cows 108 DIM	Grass silage (GS) Red clover silage (RCS) Standard concentrate (C)	Total mixed rations *ad lib* • 60%of DM from forage, DM ratio between RCS and GS 0, 1/3, 2/3, 1 • 40% of DM from C	Effect of replacing grass silage with red clover silage on • ruminal lipid and N metabolism • rumen fermentation characteristics • milk production • milk fatty acid composition
V	4	5 x 5 Latin Square 5 intact dairy cows 115 DIM	Red clover silage (RCS) Standard concentrate • no additional lipid (CON) • 2.9% lipid in FM from rapeseed oil (RO), sunflower oil (SFO), camelina oil (CO), or camelina expeller (CEX)	RCS *ad lib* + Concentrate 12 kg/d • CON • RO • SFO • CO • CEX	Effect of moderate amounts of plant lipids in the RCS based diet on • DM intake • milk production • milk fatty acid composition

DIM = days in milk, DM = dry matter, FM = fresh matter

4 RESULTS AND DISCUSSION

4.1 FATTY ACIDS IN FEEDS

In temperate climates, common forage plants typically contain 10-45 g fatty acids/kg DM (Table 2). Most of the lipid is in the form of glycolipid and PL located within thylakoid membranes of chloroplasts in leaves. The thylakoid membrane contains about 400 g of lipids/kg DM (Buccioni et al., 2012). Irrespective of forage species, 18:3n-3 is the major fatty acid (on average 46-64% of fatty acids), followed by 16:0 (14-19%) and 18:2n-6 (11-22%; Table 2). The fatty acid content and composition of forages varies due to many factors, including plant species, cultivar within species (Boufaïed et al., 2003a; Elgersma et al., 2005, III), number of harvests (Morand-Fehr and Tran, 2001), temperature, light intensity (Hawke, 1973), N fertilization of grass (Boufaïed et al., 2003a), stage of maturity (Elgesma et al., 2003; Vanhatalo et al., 2007; Koivunen et al., 2012) and conservation method (Boufaïed et al., 2003a; Koivunen et al., 2012; I).

Concentrates, cereals and oilseeds, in particular, typically contain relatively high concentrations of 16:0, *cis*-9 18:1 and 18:2n-6 in the form of TAG (Harfoot and Hazlewood 1988; Morand-Fehr and Tran, 2001; I, III, V).

4.1.1 Effect of forage conservation

Most of the lipid (89%) in fresh grass was esterified (I) confirming earlier estimates of 85-98% (Elgersma et al., 2005; Vanhatalo et al., 2007). Consistent with previous reports (Table 2), 18:3n-3 represented more than 50% of total fatty acids (I). Fresh chopped grass offered during zero-grazing closely resembled that of parent herbage with respect to both fatty acid content and composition (I; Figure 3).

Hydrolysis of esterified lipid in grasses was extensive during hay-making, which was found to continue during storage (Bath and Hill, 1967; I; Figures 2 and 3). Relative to parent herbage, drying of grass decreased forage fatty acid content by 65%, due in the most part, to losses of 18:2n-6 (57%) and 18:3n-3 (70%; I). Previous investigations on grass species have reported more moderate losses of 12-29%, 17-29% and 11-33% for total fatty acids, 18:2n-6 and 18:3n-3, respectively, as a result of drying (Boufaïed et al., 2003a; Ferlay et al., 2006). Oxidative losses of PUFA during field wilting are associated with the activity of the lipoxygenase system initiated during damage of plant tissues. Plant lipases release fatty acids from damaged membranes that are converted to hydroperoxy PUFA, which are further oxidised into a range of volatile compounds, including aldehydes and alcohols (Dewhurst et al., 2006). The use of mower-conditioner and an extensive wilt in the field in the present work (I) may

also, at least in part, explain the losses of fatty acids in grasses during drying. In addition, leaf shatter may also contribute to fatty acid losses during hay-making (Dewhurst et al., 2006).

Succesful ensiling has little effect on fatty acid concentration and composition of the resulting silage (Elgersma et al., 2005), but causes major changes in the relative proportions of esterified and non-esterified lipid (Vanhatalo et al., 2007; Van Ranst et al., 2010, I, Figure 3). Ensiling is associated with a substantial decrease in the amount of fatty acids present in polar membrane lipids (POL), which is accompanied by an increase in NEFA and neutral fractions [TAG, diacylglycerol (DAG), monoacylglycerol MAG)] *in silo*, due to the action of plant and microbial enzymes (Lee et al., 2004; Van Ranst et al., 2009a; 2010). Consistent with earlier observations, NEFA accounted for 56 and 71% of total fatty acids in UTS and FAS compared with 11% in parent grass in experiments 1 and 2 (I) and, on average, 74% of total fatty acids in GS and RCS in experiment 3 (III).

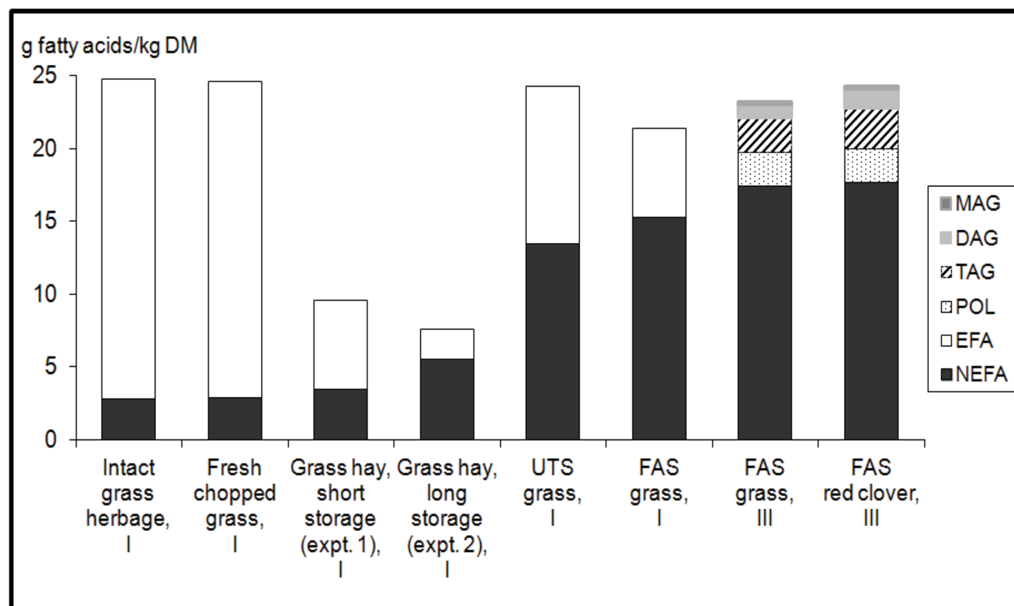

Figure 3 Distribution of fatty acids in fresh herbage, hay, untreated silage (UTS) or formic acid treated silage (FAS). MAG = monoacylglycerols, DAG = diacylglycerols, TAG = triacylglycerols, POL = polar lipids, EFA = esterified fatty acids, NEFA = non-esterified fatty acids.

4.1.2 Effect of forage species

Even though common forage plants differ in fatty acid content and composition (Table 2), the differences among species are rather small compared with that within species and between experiments (see 4.1 for common sources of variation). Much of this variation is due to differences in forage maturity (Elgersma et al., 2005; Vanhatalo et al., 2007, Koivunen et al., 2012). In general, clovers have higher total fatty acid concentrations than grasses or lucerne (*Medicago sativa*; Table 2). Among grasses and legumes, ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) often have the highest 18:3n-3 content, whereas lucerne has the lowest (Table 2). In addition, maize silage is rich in 18:2n-6 relative to grasses and legumes (Morand-Fehr and Tran, 2001; Ferlay et al., 2006).

In the present work, RCS and GS were prepared at a rather early growth stage (RCS in leaf stage to late bud stage, GS in heading stage; III). Silages had similar fatty acid content, but the fatty acid profile differed. Red clover silage contained more 18:2n-6, and less 18:3n-3, in DM than GS. Both silages were prepared using a rather high application rate (5 L/tn fresh herbage) of a formic acid based additive and were well preserved. Recently Koivunen et al. (2012) showed that using formic acid during ensiling increased the 18:3n-3 content of GS, but decreased 18:3n-3 abundance in RCS. In publication V, the 18:3n-3 content of RCS was particularly low (34% of fatty acids), possibly due to some secondary fermentation, as indicated by the relatively high pH, and rather high volatile fatty acid (VFA) and ammonium-N concentrations. Secondary fermentation is known to accelerate 18:3n-3 losses *in silo* (Chilliard et al., 2001). In addition, the red clover herbage used to prepare silage was at a rather late growth stage (mid-flowering – late flowering; V), while advances in forage maturity are known to lower forage 18:3n-3 content (Elgersma et al., 2005; Vanhatalo et al., 2007; Koivunen et al., 2012).

Despite the inherently higher activity of PPO in red clover (Lourenço et al., 2008; Koivunen et al., 2012), there were no differences in the proportion of total fatty acids as NEFA in RCS and GS in the present work (III). Nevertheless, the proportion of soluble N in total N was lower in RCS relative to GS (Dewhurst et al., 2003a; Vanhatalo et al., 2006; 2009; IV). The lack of an effect of forage species on differences in the relative abundance of lipid fractions between silages may be attributed, at least in part, to the rather long ensiling time in the present work (22 and 34 weeks at the start of experiment 3 for RCS and GS, respectively). Previously, activating latent PPO, by damaging red clover to different degrees, decreased lipolysis during the first days of ensiling, but the lipid protecting role of protein-bound phenols was not evident after 60 d of storage, probably as a result of increasing microbial lipase activity under anaerobic ensiling conditions, where PPO is no longer active (Van Ranst et al., 2010). Besides forage species and the duration of silage storage, other factors including cultivar, growth stage, DM content at ensiling and the

extent of fermentation *in silo* may also contribute to the extent of lipolysis during ensiling (Vanhatalo et al., 2007; Van Ranst et al., 2009a,b; Koivunen, 2010).

Table 2 Mean fatty acid composition (g/100g fatty acids) and total fatty acid content (g fatty acids/kg dry matter) in fresh herbage for some common forage grasses and legumes

	16:0	18:0	*cis*-9 18:1	18:2n-6	18:3n-3	∑fatty acids	Reference
Grasses							
Ryegrass (*Lolium perenne*) n=13							
Mean	16	2.0	2.6	11	64	22	Dewhurst et al., 2001
SD	3.1	1.43	1.71	1.3	7.6	2.0	Elgersma et al., 2005
Min	13	0.8	1.5	8.7	46	19	Van Ranst et al., 2009a,b
Max	24	5.0	6.8	13	71	24	
Meadow Fescue (*Festuca pratensis*) n=6							
Mean	19	2.7	6.8	13	55	23	Dewhurst et al., 2001
SD	1.9	1.58	1.13	3.5	4.0	2.0	Boufaïed et al., 2003a
Min	16	1.3	5.0	6.6	51	21	
Max	22	4.8	8.0	16	62	26	
Timothy (*Phleum pratense*) n=7							
Mean	19	3.0	5.4	19	49	20	Dewhurst et al., 2001
SD	1.1	1.30	1.32	2.8	3.9	2.4	Boufaïed et al., 2003a
Min	17	2.0	2.7	15	45	16	
Max	20	5.1	6.6	22	57	23	
Meadow Fescue x Timothy (*Festuca pratensis* x *Phleum pratense*) n=6							
Mean	12	1.3	3.7	16	60	21	Vanhatalo et al., 2007
SD	3.0	0.80	1.60	7.1	15.3	9.0	Koivunen, 2010
Min	9	0.6	2.3	11	38	10	l
Max	15	2.3	6.5	29	74	33	
Legumes							
White clover (*Trifolium repens*) n=12							
Mean	14	2.0	3.1	16	61	33	Boufaïed et al., 2003a
SD	2.2	0.48	1.14	2.3	4.8	5.5	Van Ranst et al., 2009a,b
Min	11	1.5	1.6	12	55	28	
Max	18	2.9	5.1	20	68	42	
Red clover (*Trifolium pratense*) n=19							
Mean	15	2.6	3.7	18	56	27	Boufaïed et al., 2003a
SD	3.7	1.47	2.47	3.1	11.3	7.3	Vanhatalo et al., 2007
Min	9	1.1	1.5	13	36	13	Van Ranst et al., 2009a,b
Max	22	7.1	8.9	24	72	40	Koivunen, 2010
Lucerne (*Medicago sativa*) n=6							
Mean	23	4.2	5.0	21	41	17	Boufaïed et al., 2003a
SD	1.9	0.57	1.66	2.7	4.0	3.0	Whiting et al., 2004
Min	21	3.4	2.7	17	38	14	Ribeiro et al., 2005
Max	25	4.7	6.6	23	49	23	

4.1.3 Concentrate supplements

Concentrate supplements fed in studies reported in the current thesis were comprised of rolled barley, solvent extracted rapeseed meal and molassed sugarbeet pulp (experiments 1-3) in which lipid is abundant in 18:2n-6, followed by 16:0 and *cis*-9 18:1 (I, III) primarily as TAG (Harfoot and Hazlewood, 1988; III). In experiment 4, concentrates also contained wheat, cereal bran, sugar-beet molasses and various plant oils or CEX.

Modifying milk fat through concentrate and lipid supplements has been extensively investigated (Chilliard et al., 2001; Dewhurst et al., 2006). Palm oil rich in 16:0 has often been used as a cheap source of dietary energy for lactating cows, whereas olive oil and RO are rich in *cis*-9 18:1, with SFO, safflower and soya oil as a source of 18:2n-6, and with linseed (Dewhurst et al., 2006; Glasser et al., 2008a; Shingfield et al., 2008b; Table 3) and CO predominanting in 18:3n-3 (Hurtaud and Peyraud, 2007; Table 3; V). In the present work RO, SFO and sources of camelina lipid were used as sources of *cis*-9 18:1, 18:2n-6 and 18:3n-3, respectively (V).

Table 3 Fatty acid composition (g/100g fatty acids) of selected oilseeds

	16:0	18:0	*cis*-9 18:1	18:2n-6	18:3n-3	*cis*-11 20:1	Reference
Camelina	5.3	2.7	13	15	38	15	Zubr, 2003
Linseed	6.1	3.4	18	16	54		Glasser et al., 2008a
Olive	11	4.0	77	6.0	0.7		Manso et al., 2011
Palm	50-55	3.0-6.0	30-35	8.0-10			Manso et al., 2006
Rape	4.8	2.1	61	21	9.2		Glasser et al., 2008a
Safflower	6.7	2.3	15	76			Bell et al., 2006
Soya	11	4.1	22	54	7		Glasser et al., 2008a
Sunflower	5.1	4.3	22	67	0.2		Glasser et al., 2008a

4.2 FATE OF DIETARY LIPID IN THE RUMEN

Esterified lipids in ingested forage and concentrate are rapidly and extensively hydrolysed in the rumen by the action of lipases produced by a small number of bacteria (Doreau and Ferlay, 1994; Demeyer and Doreau, 1999). Accordingly, most fatty acids (71-85%) at the omasum were present as NEFA at present work (I, III) confirming much earlier estimates of 62-93% measured in sheep and goats (Bath and Hill, 1967; Lennox et al., 1968; Bickerstaffe et al., 1972) and more recent reports in sheep (80-90%; Atkinson et al., 2006). The lipase produced by *Anaerovibrio lipolytica* can hydrolyse TAG and DAG, whereas galactolipase and phospholipase activity is associated with certain strains of *Butyrivibrio* (Harfoot and Hazlewood, 1988).

Low rumen pH and dietary factors such as increases in forage maturity (Palmquist et al., 2005) and high PPO activity may decrease lipolysis in the rumen (Lee et al., 2007). Furthermore, the extent of ruminal lipolysis is thought to be higher for unprotected plant lipids than for forages (Doreau and Ferlay, 1994), due to the need to rupture the cell wall by mastification and/or microbial digestion before the structural lipids of forages can be hydrolysed (Palmquist et al., 2005). However, in the present work ruminal lipolysis was extensive (80-93%) for all diets based on either fresh or conserved grass (I, III). These estimates are similar to previous values (82-97%) for unprotected plant lipids (Miller and Cramer, 1969; Bauchart et al., 1990), that may be associated, at least in part, with the high digestibility of experimental diets [whole tract digestibility of organic matter (OM) averaged 76-80%]. Nevertheless, replacing GS with RCS in the diet decreased ruminal lipolysis of dietary esterified lipids from 85% to 70% (III).

Fatty acids liberated during lipolysis are absorbed onto feed particles, where unsaturated NEFA are extensively hydrogenated (Doreau and Ferlay, 1994; Glasser et al., 2008b; Table 4; I; III). Consist with these considerations, the NEFA fraction in omasal digesta was rich in 18-carbon biohydrogenation intermediates including *trans*-18:1 and CLA, 18:0 and longer-chain (more than 18-carbon) SFA, whereas the majority of 18:2n-6 and 18:3n-3 escaped the rumen as esterified lipid (I, Figure 4). This suggests that 18:2n-6 and 18:3n-3 bypass the rumen primarily within chloroplasts (Kim et al., 2009). *Cis*-9 18:1 and 16:0 were present as esterified lipid and NEFA in almost equal amounts (I, III), highlighting the contribution of both diet and microbial lipid to ruminal outflow. These findings are consistent with earlier investigations reporting the distribution of fatty acids within lipid fractions in duodenal digesta of small ruminants (Bickerstaffe et al., 1972; Atkinson et al., 2006).

Due to extensive metabolism of unsaturated fatty acids in the rumen (Table 4), 18:0 accounted for on average 44-53% of total fatty acids leaving the rumen (I, III), an estimate in agreement with previous reports (Lee et al., 2003; Loor et al., 2004; Lee et al., 2006). *Cis*-9,*trans*-11 CLA

was the most abundant CLA isomer in omasal digesta accounting for 48 to 83% of total CLA flow, while *trans*-11 18:1 accounted for, on average, 40-56% of *trans* 18:1. The omasal flow of *trans*-11 18:1 was many times higher compared with *cis*-9,*trans*-11 CLA or *trans*-11,*cis*-15 18:2 (I, III) consistent with previous investigations (Dewhurst et al., 2003a; Loor et al., 2004; Shingfield et al., 2008a). These findings would tend to indicate that the metabolism of *cis*-9,*trans*-11 CLA and *trans*-11,*cis*-15 18:2 to *trans*-11 18:1 occurs at a faster rate than the final reduction of *trans*-11 18:1 to 18:0, suggestions that have been demonstrated *in vitro* (Ribeiro et al., 2007; Khiaosa-Ard et al., 2009; Honkanen et al., 2012). Both lipolysis and the reduction of *trans*-18:1 to 18:0 are considered rate-limiting for biohydrogenation (Bauman et al., 1999; Palmquist et al., 2005; Kim et al., 2009). For dietary 18-carbon unsaturates, there are numerous biohydrogenation pathways operating in the rumen (Harfoot and Hazlewood 1988; Chilliard et al., 2007; Shingfield et al., 2010), that are dependent on the ruminal microbial ecosystem (Chilliard et al., 2000b; McKain et al., 2010), with *Butyrivibrio fibrisovens* being the most studied bacterium capable of biohydrogenation (Harfoot and Hazlewood, 1988).

Rumen bacteria and protozoa can incorporate dietary and/or synthesize *de novo* fatty acids of different chain lengths (Doreau and Ferlay, 1994). Flow of total fatty acids at the omasum exceeded dietary intake (10-182 g/d) on all diets in the current series of experiments (I; III), confirming a net synthesis of fatty acids in the rumen of cows fed diets containing relatively low amounts of lipid (Doreau and Ferlay, 1994; Sauvant and Bas, 2001). Higher flows at the omasum were due in the main to a net synthesis of odd- and branched-chain fatty acids (OBCFA) in the rumen, but on certain diets the synthesis of 16:0 and 18:0 was also substantial (I, III), confirming previous findings *in vitro* (Patton et al., 1970) and *in vivo* (Bock et al., 1991; Ferlay et al., 1993).

4.2.1 Effects of forage conservation

Rumen protozoal counts or specific *Butyrivibrio* populations known to be capable of biohydrogenation were not substantially affected by forage conservation method in the present work (I), and therefore, do not in isolation, explain the observed differences in ruminal lipid metabolism across diets.

Fresh Grass vs. Grass Hay. Despite a lower proportion of fatty acids in fresh grass as NEFA, replacing hay with fresh grass was associated with more extensive apparent lipolysis of dietary esterified lipid in the rumen (I in Table 4), consistent with much earlier investigations *in vitro* (Faruque et al., 1974). It has been suggested that the decrease in lipid metabolism associated with extensive wilting during hay production may be related to slower degradation of DM in the rumen (Boufaïed et al., 2003b). Even though the kinetics of digestion were not determined in the present work, the differences in the extent of ruminal OM and neutral detergent fibre (NDF) digestion due to drying of grass were neglible, such that differences in ruminal degradation of forage particles are not a likely explanation for the 7.1 percentage unit increase in ruminal lipolysis when cows were fed fresh grass (I). Previous investigations have also given rise to the suggestion that ruminal lipolysis and biohydrogenation are higher on diets based on fresh herbage compared with conserved forages due to the action of endogenous plant lipases (Doreau et al., 2005). Plant lipases have been reported to stay active for at least 5 h in the rumen, with higher activities reported in rumen fluid of cows at pasture compared with those fed hay (Faruque et al., 1974). Alternatively, it is possible that chloroplasts are entrapped by surrounding cell walls during drying introducing a physical barrier to microbial lipases.

In agreement with more extensive ruminal lipolysis, the apparent biohydrogenation of 18:2n-6 and 18:3n-3 in the rumen was higher (3.4 and 6.4%-units, respectively) in cows fed fresh grass than hay diet, leading to similar omasal flows of 18:2n-6 and 18:3n-3, despite of a higher PUFA intake during zero-grazing (114 and 148 vs. 89 and 58 g/d for 18:2n-6 and 18:3n-3 intakes, respectively; I). More extensive biohydrogenation on fresh grass relative to hay diet has also been demonstrated previously *in vitro* (Boufaïed et al., 2003b) and in sheep *in vivo* (Doreau et al., 2005 in Table 4).

The omasal flow of the initial intermediates of 18:2n-6 and 18:3n-3 biohydrogenation, namely *cis*-9,*trans*-11 CLA and *cis*-9,*trans*-11,*cis*-15 18:3, were lower for fresh grass than hay diet, whereas the flows of secondary or penultimate intermediates, including *cis*-18:1 (Δ11,15), *trans*-18:1 (Δ6-9, 13-14), *trans*-11,*cis*-15 18:2 and geometric isomers of Δ11,13 CLA were higher (I; refer to Shingfield et al., 2010 for details of intermediate formation). These findings suggest significant differences in the rate of NEFA released in the rumen and in the rate of ruminal biohydrogenation between diets. A higher rate of lipolysis and the initial

steps of biohydrogenation on fresh grass diet compared with hay is further supported by the higher amount of 18-carbon unsaturates recovered as 18:0 and *trans*-11 18:1 in cows fed fresh grass (113 vs. 77%; I).

Omasal flow of *trans*-11 18:1 was doubled by zero-grazing compared with hay (I) consistent with previous findings (Mohammed et al., 2009; Coppa et al., 2011), that can be attributed to the higher consumption of PUFA during zero-grazing. However, even greater ruminal outflows of *trans*-11 18:1 could be expected in cows at pasture relative to zero-grazing due to the selective consumption of leaves enriched in chloroplasts during grazing (Mohammed et al., 2009).

Grass Hay vs. Grass Silages. Compared with grass hay, feeding silage increased lipolysis and the biohydrogenation of 18-carbon PUFA in the rumen (I in Table 4), that is in agreement with earlier studies *in vitro* (Boufaïed et al., 2003b). Hydrolysis of lipid after storage of equal length was rather similar for hay and silages. However, on average ca. 35% of fatty acids ingested on silage diets were in the form of NEFA, whereas this fraction represented only 19% of fatty acids on the hay diet due to the lower fatty acid content of dried grass. Thus, more unsaturated fatty acids in silages compared with hay were readily subjected to ruminal biohydrogenation, a process that represents a survival mechanism for rumen microbes against the toxic effects of dietary unsaturated NEFA on bacterial growth (Lourenço et al., 2010).

Ruminal digestibilities of DM and NDF were similar between diets in the present study (I). However, earlier reports *in situ* have indicated that the rate of DM degradability in the rumen is lower for hay than silage when conserved forages are prepared simultanously from the same herbage (Thiago et al., 1991; Petit and Flipot, 1992; Crushnahan and Gordon, 1995). These findings have not been confirmed *in vivo* based on rumen evacuation (Huhtanen and Jaakkola, 1993). The integrity of cell structures may be preserved during hay-making, whereas ensiled material has been subjected to fermentation processes that may result in the weakening of leaf structures such as hemicellulose (Thiago et al., 1991; Wattiaux et al., 1992). This may predispose the lipid in silages to a more rapid and extensive metabolism in the rumen relative to that in hay, which may offer an explanation for the more extensive ruminal lipid metabolism in cows fed silage compared with hay.

Despite higher intakes of *cis*-9 18:1, 18:2n-6 and 18:3n-3 on silage based diets compared with hay (on average 62, 121 and 55 vs. 69, 151 and 181 g/d, respectively), flows of *cis*-9 18:1 and 18:2n-6 were the same, while 18:3n-3 at the omasum was only marginally higher in cows fed silage (I). Previous studies in sheep have also reported similar flows of 18:2n-6 and 18:3n-3 at the duodenum on GS relative to grass hay (Doreau et al., 2003). Consistent with a higher intake of unsaturated fatty acids and more extensive metabolism of lipids in the rumen, feeding silage increased the omasal flow of 18:0 and several 18-carbon unsaturated biohydrogenation intermediates, including, *cis*-18:1 (Δ12-16), *trans*-18:1 (Δ4-9, 11-16),

Δ11,15 18:2, Δ12,15 18:2, Δ11,13 CLA, Δ13,15 CLA and *cis*-9,*trans*-11,*cis*-15 18:3 relative to hay (I; Doreau et al., 2003; Chilliard et al., 2007; Shingfield et al., 2010). More extensive and rapid lipolysis and hydrogenation of lipid on silage diets compared with hay is also supported by the higher proportion of biohydrogenated 18-carbon unsaturates recovered as 18:0 and *trans*-11 18:1 (103 vs. 82% for silage and hay based diets; respectively I).

***Extent of Silage Fermentation*.** Restricting silage fermentation using a formic acid based additive had no effect on the omasal flow of 18:0, *cis*-9 18:1, 18:2n-6 and 18:3n-3, but increased, or tended to increase, the flow of specific 18-carbon biohydrogenation intermediates, including *trans*-10 18:1, *trans*-10,*cis*-12 CLA and Δ11,13 CLA (I), that may be attributed to numerically higher intake of 18:2n-6 and 18:3n-3 on FAS compared with UTS. In addition, the hydrolysis of grass lipids *in silo* was more extensive in FAS than UTS (71 vs. 56% of fatty acids as NEFA). Therefore, marginally higher amounts of unsaturated fatty acids were readily subjected to biohydrogenation upon ingestion in cows fed the FAS diet. Consistent with these observations, Boufaïed et al. (2003b) reported higher 18:3n-3 rates of biohydrogenation on FAS relative to UTS *in vitro*. The extent of biohydrogenation of dietary 18-carbon unsaturates were, however, similar between different silage diets in the present study (I).

4.2.2 Grass silage vs. red clover silage

Lipid Intake. The linear decrease in 18:3n-3 (from 160 to 133 g/d) and increase in 18:2n-6 (from 108 to 120 g/d) intake when RCS replaced GS in the diet reflected in the main differences in the fatty acid composition of lipid classes among forage species in the present work (III). Nevertheless, the differencies in fatty acid intake were numerically rather small (III). Previously, Vanhatalo et al. (2007) reported similar 18-carbon PUFA intakes between diets based on RCS and GS, whereas Lee et al. (2003; 2006) reported increases, and Dewhurst et al. (2003a) and Moorby et al. (2009) noted decreases in 18:3n-3 intake for diets based on GS than RCS. Inconsistencies between studies can be explained by several factors including differences in fatty acid profile between forage species and cultivars, herbage maturity, silage preparation (wilting in particular) and extent of fermentation *in silo*, DMI responses and the amount and composition of concentrate in the diet (Boufaïed et al., 2003a; Dewhurst et al., 2006; Vanhatalo et al., 2007).

Ruminal Lipolysis and Biohydrogenation. Replacing GS with RCS decreased the lipolysis of dietary esterified lipids and the biohydrogenation of 18:3n-3 in the rumen, which resulted in linear increases in the flow of 18:3n-3 in all lipid fractions at the omasum (III; Figure 4). These findings are consistent with previous investigations *in vitro* (Lee et al., 2004; 2007; Van Ranst et al., 2010) and measurements of total fatty acids at the

duodenum in lactating cows (Dewhurst et al., 2003a) and growing cattle (Lee et al., 2003; 2006). The lower protective effect of RCS against ruminal biohydrogenation of *cis*-9 18:1 and 18:2n-6 compared with 18:3n-3 can be attributed to the lower proportion of *cis*-9 18:1 and 18:2n-6 intake originating from POL in forages relative to TAG in concentrate supplements (III). Irrespective of forage species, 18:3n-3 was the predominant fatty acid in POL (on average 76% of fatty acids), whereas the abundance of 18:2n-6 and *cis*-9 18:1 in POL were marginal (9 and 1% of fatty acids, respectively; III).

Possible Role of Protein Bound Phenols. It has been proposed that the inherently higher PPO activity in red clover relative to grass is responsible for less extensive metabolism of forage lipids in the rumen (Kim et al., 2009). The PPO enzyme is a stress activated copper metalloprotein that catalyses the oxidation of endogenous phenols to quinones in the presence of oxygen. Quinones formed during the action of PPO are highly reactive, electrophilic molecules which may covalently modify and crosslink a variety of nucleophilic cellular constituents, such as sulpho amino acids (Igarashi and Yasui, 1985). This results in the formation of cross-linked protein polymers (Kim et al., 2009) that may offer a physical barrier for entrapped lipids against plant and bacterial lipases.

Polyphenol oxidase exists in either an active or a latent state (Van Ranst et al., 2011). Latent red clover PPO can be activated by its endogenous substrates such as phaselic acid and clovamide. Substrate activation can result in almost total conversion of latent PPO to the active form within 10 min (Lee et al., 2009c). In healthy tissues this is prevented by the separate subcellular compartmentation of the PPO enzyme (chloroplast) and its substrates (vacuole; Van Ranst et al., 2011). In the present work (III-IV), grass and red clover swards were cut using a mower conditioner, chopped and harvested with a precision forage harvester after a short wilt (approximately 3-4 h and 1-2 h, respectively). Chopped forage was well compacted during the filling of bunker silos. It is plausible that the harvesting strategy used allowed for the effective activation of PPO, as would be expected by extensive rupture of cell structures in the presence of molecular oxygen. Recently, Lee et al. (2013) highlighted the importance of non-PPO induced oxidation of endogenous phenols during extended wilting (2–24 h) of damaged red clover herbage. This suggests that a more extensive wilt of red clover, that was not feasible in the present work due to prolonged suboptimal weather conditions, could allow for a greater formation of protective structures.

A lower degradability of amino acids *in silo* and in the rumen [including sulpho amino acids cysteine and methionine (from 64 to 48 and from 63 to 52%, respectively)], together with the decrease in N availability for absorption when RCS replaced GS (whole tract N digestibility decreased from 67 to 61% and the proportion of N intake excreted in faeces increased from 34 to 39%; IV), would tend to support the formation of protective protein complexes in RCS. In addition, Huhtanen et al. (2013)

recently suggested that a higher faecal N output on diets containing RCS is not related to greater amounts of particle-associated crude protein, but is more probably explained by the incomplete digestion of quinone-protein complexes compared with GS. Against expectations, the flow of bound phenols at the omasum was similar among diets in the present study (III). Due to the lack of determination of endogenous phenols (free and protein bound) of parent herbage, PPO activity (active and latent) of the cut herbage at ensiling and phenol content (free and protein bound) of resultant silages it is, however, impossible to draw definitive conclusions on the possible role of PPO on the observed differences between forage species on ruminal metabolism of nitrogen. In addition, the procedure used to determine protein bound phenols may not be quantitative in all situations due to lowered protein solubility as a result of extensive binding of phenols (underestimation), and the presence of high concentrations of free phenols (overestimation; Lee et al., 2013).

Despite of the apparent differences in the susceptibility of forage proteins to degradation between GS and RCS (IV), the lipolysis of forage lipids *in silo* was equally high and extensive (on average 74% of fatty acids as NEFA) for GS and RCS (III). It seems unlikely that a significant amount of NEFA could have been encapsulated by possible formation of a protective phenol-protein layer during wilting and ensiling because of timing difference between the availability of oxygen and the accumulation of NEFA in forages. Firstly, the formation of protein bound phenols through PPO or auto-oxidation requires oxygen, and therefore takes primarily place before ensiling. Indeed, the aerobic phase *in silo* is short and generally lasts only a few hours due to the respiration of the plant material and aerobic and facultative aerobic microorganisms (Pitt et al., 1985; Oude Elferink et al., 2000). At a similar DM content of plant material at ensiling (24%), as was the case in the present study (23 and 19% for grass and red clover, respectively, IV), it has been shown that 90% of the oxygen is lost within 15 minutes and that less than 0.5% remains after 30 minutes (Sprague, 1974). Secondly, the level of NEFA in parent and drying herbage is rather low. Irrespective of forage species (rye grass, red clover, white clover) both parent and wilted (cut, crushed and wilted for 8 h) herbage contained less than 10% of fatty acids as NEFA (Van Ranst et al., 2009b). The slow accumulation of NEFA during wilting may be related to the extensive conversion of NEFA released to volatile compounds (see chapter 4.1.1 for more details). Even though the accumulation of NEFA *in silo* is rather rapid during the first days of ensiling, which becomes slower thereafter (Van Ranst et al., 2010), the anaerobic conditions attained render the formation of bound phenols and possible further protective encapsulation of NEFA unlikely also at this stage.

A similar transfer of 18-carbon PUFA from omasal digesta into milk across GS and RCS diets (IV) indicates that the protective mechanism responsible for lowered ruminal metabolism of dietary PUFA in RCS diets does not impair the bioavailability of dietary fatty acids for absorption. This

together with no differences in the susceptibility of forage lipids to lipolysis *in silo*, would tend to suggest that in contrast to N, the possible products of PPO activity were not a major part of the mechanism protecting dietary lipids in RCS from ruminal metabolism in the current experiment.

Possible Role of Ruminal Digestion Kinetics. It is possible that changes in ruminal digestion kinetics may also contribute to higher ruminal escape of dietary PUFA in lactating cows fed diets based on RCS relative to GS. The rate of DM and potentially digestible NDF outflow from the rumen has been reported to be higher for RCS containing diets compared with GS (Dewhurst et al., 2003a; Kuoppala et al., 2009) that may promote PUFA escape. There is also some evidence of smaller particle size of forage material in the rumen of cows fed diets containing RCS compared with GS (Bertilsson and Murphy, 2003; Huhtanen et al., 2013). In contrast, Dewhurst et al. (2003a) reported no difference in rumen particle size distribution in cows offered GS and RCS, but direct comparisons are confounded with between-experiment differences in the range and distribution of particle sizes determined. Besides promoting ruminal escape, decreases in forage particle size may also inhibit the adherence of ruminal bacteria (Buccioni et al., 2012). Feed particles are the most active site for biohydrogenation in the rumen (Harfoot and Hazlewood, 1988). Nevertheless, biohydrogenation of unsaturated fatty acids has been demonstrated to be consistently lower for RCS than GS during batch incubations with ruminal fluid *in vitro* (Lee et al., 2007; Van Ranst et al., 2010), in which variations in outflow rate are controlled.

Possible Role of Ruminal Microbiota. Ultimately, the differences in the rumen fermentation pattern (III) and decrease in microbial non-ammonia nitrogen (NAN) flow in response to RCS in the diet (IV) could also be interpreted as reflecting changes in the predominant bacterial populations and their growth in the rumen. However, microbial NAN flow has often been higher (Dewhurst et al., 2003a; Vanhatalo et al., 2006) or similar (Vanhatalo et al., 2009) on diets based on RCS relative to GS. It is possible that energy rather than amino acids available restricted microbial protein synthesis in the rumen of cows fed RCS containing diets in the present study, since ruminal net degradation of amino acids was more extensive as the proportion of RCS in the diet increased in the present study (ruminal balance of total amino acids calculated as the difference between omasal flow and intake of amino acids increased gradually from -237 to -493 g/d; IV). Higher lactic acid content of RCS relative to GS and the lowest DMI for the diet containing solely RCS (IV) may, at least in part, explain lowered energy supply.

Recently, Huws et al. (2010) reported substantial changes in rumen microbial communities of steers fed RCS compared with GS, including a decrease in the abundance of *Anaerovibrio lipolytica*. In the present work, feeding RCS increased the flow of 17:0 and decreased that of *iso* 15:0 and *iso* 17:0 at the omasum (III). Previous investigations have reported similar changes in OBCFA at the duodenum (Lee et al., 2008) and milk fat

(Vanhatalo et al., 2007) during comparisons of RCS and GS in the diet. Based on inherent differences in the fatty acid composition of rumen bacteria, Vlaeminck et al. (2006) proposed the use of *iso* 17:0 as a marker of the abundance of cellololytic bacteria *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens*, the latter also exhibiting phospholipatic activity and thought responsible for biohydrogenation in the rumen (Lourenço et al., 2010).

Flow of Fatty Acids at the Omasum. The influence of RCS on ruminal metabolism of 18-carbon unsaturated fatty acids seems to be related to a decrease in the rates of the first few stages of biohydrogenation (III). Besides increasing 18:3n-3 flow at the omasum, feeding RCS relative to GS increased ruminal outflow of 18:2n-6 as NEFA and DAG (Figure 4), and that of *cis*-9 18:1 as NEFA (III), in agreement with previous findings reporting total fatty acid flows at the duodenum in lactating cows (Dewhurst et al., 2003a) or steers (Lee et al., 2008). Furthermore, feeding RCS compared with GS increased linearly the omasal flow of the first intermediate of the major 18:2n-6 and 18:3n-3 biohydrogenation pathways *cis*-9,*trans*-11 CLA and *cis*-9,t*rans*-11,*cis*-15 18:3, respectively (III; see Shingfield et al., 2010 for common pathways of biohydrogenation). In contrast, the flow of the end-product, 18:0 and several secondary and penultimate biohydrogenation intermediates, including *trans*-18:1 (Δ5-8, 11) and *trans*-11,*cis*-15 18:2, at the omasum were higher when RCS and GS were fed together rather than offered separately (III), that probably reflects higher DMI when mixtures of forages were fed (IV). These observations also indicate that there was no additional protection of fatty acids from ruminal metabolism on RCS diets once the biohydrogenation process was initiated.

Replacing GS with RCS in the diet increased linearly the flow of *cis*-18:1 (Δ11,12) and *trans*-18:1 (Δ12-16) as NEFA at the omasum, with concominant decreases in *trans*-10,*cis*-12, *trans*-11,*cis*-13 and *trans*-9,*trans*-11 CLA (III), that could indicate changes in the relative importance of minor pathways of biohydrogenation (Shingfield et al., 2010), and therefore supports changes in microbial population (Huws et al., 2010) and/or activity in the rumen due to forage species. However, the changes in the flows of these fatty acids at the omasum were numerically very small.

The concentration of S3,R7,R11,15-*tetramethyl*-16:0 in forage and net synthesis of S3,R7,R11,15-*tetramethyl*-16:0 in the rumen were both lower for RCS relative to GS. The decrease in S3,R7,R11,15-*tetramethyl*-16:0 flow at the omasum in response to RCS in the diet is consistent with previous findings in steers (Lee et al., 2006). Appearance of 3,7,11,15-*tetramethyl*-16:0 in ruminal digesta arises from bacterial hydrogenation of the phytol moiety of chlorophyll a and b (Patton and Benson, 1966). Typically, RCS and GS contain similar amounts of chlorophyll (Lee et al., 2006), suggesting that one or more attributes of red clover may protect chlorophyll contained in chloroplasts from degradation in the rumen.

Rumen protozoa are relatively rich in PUFA mainly due to their ingestion of chloroplasts (Kim et al., 2009) thereby redirecting dietary PUFA from lipolysis and biohydrogenation in the rumen. In the present study, rumen protozoal counts were similar across treatments (III), but it is possible that the smaller forage particle size on RCS containing diets, relative to GS (Bertilsson and Murphy, 2003; Huhtanen et al., 2013), promotes the ingestion of chloroplasts by protozoa. However, the selective retention of protozoa in the rumen, particularly on high forage diets (Huws et al., 2012) suggests that other possible protective mechanisms play a more important role.

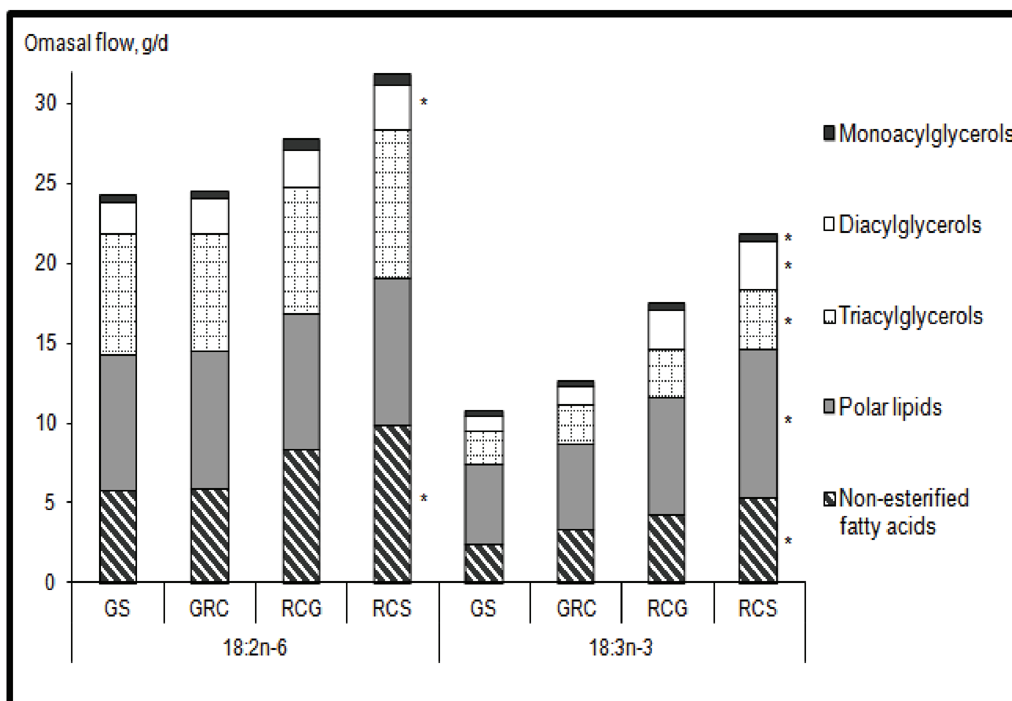

Figure 4 The effect of forage species on the flow of 18:2n-6 and 18:3n-3 at the omasum (III). Refers to total mixed rations comprised (600 g/kg DM) grass silage (GS), mixtures (on a DM basis) of grass and red clover silage of 2:1 (GRC) or 1:2 (RCG) or red clover silage (RCS). * Indicates linear changes (P<0.05) within lipid classes across treatments.

Table 4 Ruminal lipolysis and biohydrogenation of 18-carbon unsaturates *in vivo* in ruminants fed various basal diets containing no additional lipid

Basal forage	Animal	DM intake kg/d[1]	Forage to concentrate ratio	Lipolysis %	Biohydrogenation %			Reference
					cis-9 18:1	18:2 n-6	18:3 n-3	
Fresh								
L	wether					61	91	Doreau and Poncet, 2000
G	cow	17	64:36	93	70	84	93	I
G	wether	1.2-1.3				83-91	94-98	Doreau et al., 2005
RC	wether	1.3				88	97	Doreau et al., 2005
Hay								
L	wether					76	85	Doreau & Poncet, 2000
G	cow	18-20	60:40-67:33	80-86	66-75	81-85	87	I
G	wether	1.3	100:0		70	86	91	Doreau et al., 2003
G	wether	1.2-1.3				76-86	81-91	Doreau et al., 2005
G	cow	20-21	35:65-65:35		58-60	75-78	84-90	Loor et al., 2004
Silage								
L	wether					74	90	Doreau and Poncet, 2000
L	cow	19	64:36			88	90	Dewhurst et al., 2003a
G	cow	20-22	61:39-64:36	92-93	76-77	88	96	I
G	cow	20	60:40	85	59	78	93	III
G	cow	17	69:31-70:30		71	88	96	Adler et al., 2013
G	cow	17	60:40			86	95	Dewhurst et al., 2003a
G	wether	1.3	100:0		64	86	92	Doreau et al., 2003
G	wether	1.2-1.3				86-94	92-98	Doreau et al., 2005
G	steer	4.2	100:0		45	86	92	Lee et al., 2003
G	steer	3.6-4.3	100:0		11-54	89-92	94-95	Lee et al., 2006
G	cow	15	58:42		80	92	98	Shingfield et al., 2008a
G:RC, 2:1	cow	20	60:40	81	59	78	91	III
G:RC, 6:4	steer	7.0	100:0		33	83	79	Lee et al., 2003
G:RC, 5:4	cow	18	70:30		65	86	93	Adler et al., 2013
G:RC, 1:1	steer	4.5-4.7	100:0		17-22	89	90	Lee et al., 2006
G:RC, 1:1	cow	21	67:33			88	93	Dewhurst et al., 2003a
G:RC, 1:2	cow	20	60:40	78	58	77	89	III
RC	cow	18	60:40	70	53	74	85	III
RC	cow	19	64:36			87	89	Dewhurst et al., 2003a
RC	wether	1.3				90	95	Doreau et al., 2005
RC	steer	6.4	100:0		66	84	84	Lee et al., 2003
RC	steer	4.8	100:0		11	88	85	Lee et al., 2006
G:WC, 6:4	steer	8.5	100:0		35	80	85	Lee et al., 2003
G:WC, 1:1	cow	22	68:32			87	95	Dewhurst et al., 2003a
WC	steer	8.4	100:0		52	83	88	Lee et al., 2003
WC	cow	21	66:44			83	93	Dewhurst et al., 2003a
M	cow	28	50:50		56	84	86	Onetti et al., 2004
M:L, 1:1	cow	27	50:50		60	79	82	Onetti et al., 2004[2]
M: L hay, 6:1	cow	11	70:30		79	91	95	Doreau et al., 2009
M:L hay, 1:1	cow	27	50:50		60-61	82	84-85	Onetti et al., 2004[2]

G = grass, L = lucerne, RC = red clover, WC = white clover, M = maize
[1] Dry matter intake not reported in all studies [2] = 2% of diet DM as tallow

4.3 LIPIDS IN RUMINANT PLASMA

Circulating CE and PL were the major fatty acid repositories in blood, whereas the concentrations of fatty acids in TAG and NEFA were many times lower (II in Figure 5), confirming earlier reports (Christie, 1981a:, Offer et al., 2001; Tyburczy et al., 2008). The fatty acid profile of different plasma lipid classes is known to vary according to the need to meet the requirements of different tissues (Christie 1981b). Consistent with previous reports, TAG and NEFA were relatively abundant in 16:0, 18:0 and *cis*-9 18:1, whereas 18:2n-6 and 18:3n-3 were preferentially incorporated into plasma CE and PL (II in Figure 5; Offer et al., 2001; Loor et al., 2002; Tyburczy et al., 2008). Furthermore, the majority of *trans*-11 18:1 was transported in PL and TAG fractions, whereas CE was the major repository for *cis*-9, *trans*-11 CLA (II in Figure 5). However, during periods of increased supply both 18:2n-6 and 18:3n-3 may be transported in blood within TAG and NEFA (Lacount et al., 1994; Loor et al., 2002).

Across grass based diets, 18:2n-6 and 18:3n-3 represented on average 39 and 11 g/100 g of fatty acids in arterial blood (II in Figure 5), respectively, but only 3.8 and 1.7 g/100 g of fatty acids at the omasum (I). There were no significant associations between 18:2n-6 and 18:3n-3 at the omasum and enrichment in arterial lipids, other than the amount of 18:3n-3 in TAG. These findings reflect both the limited influence of forage conservation method on the postruminal supply of 18:2n-6 and 18:3n-3 (I), as well as the sparing of essential fatty acids for vital biological functions and possible remodeling of fatty acids in the liver and other tissues (Noble, 1984; Christie et al., 1986; Chilliard et al., 2007).

Differences in the amount of *trans*-11 18:1 and *cis*-9, *trans*-11 CLA in arterial lipid fractions were closely associated with changes in the flow at the omasum, consistent with previous reports examining the relation between fatty acid supply and plasma lipid concentrations in lactating cows (Glasser et al., 2007; Doreau et al., 2009). Concentrations of 16:0, 18:0 and *cis*-9 18:1 in arterial blood were only weakly associated with changes in the flows at the omasum with significant positive intercepts indicating that postruminal supply is not the sole determinant of these fatty acids in arterial blood of lactating cows in positive calculated energy balance. Other factors including *de novo* synthesis, elongation, desaturation and mobilization of these fatty acids in several tissues including adipocytes and enterocytes (Chilliard et al., 2007; Glasser et al., 2007; Shingfield et al., 2010), as well as differences in the relative entry and removal rate from the circulation may also contribute.

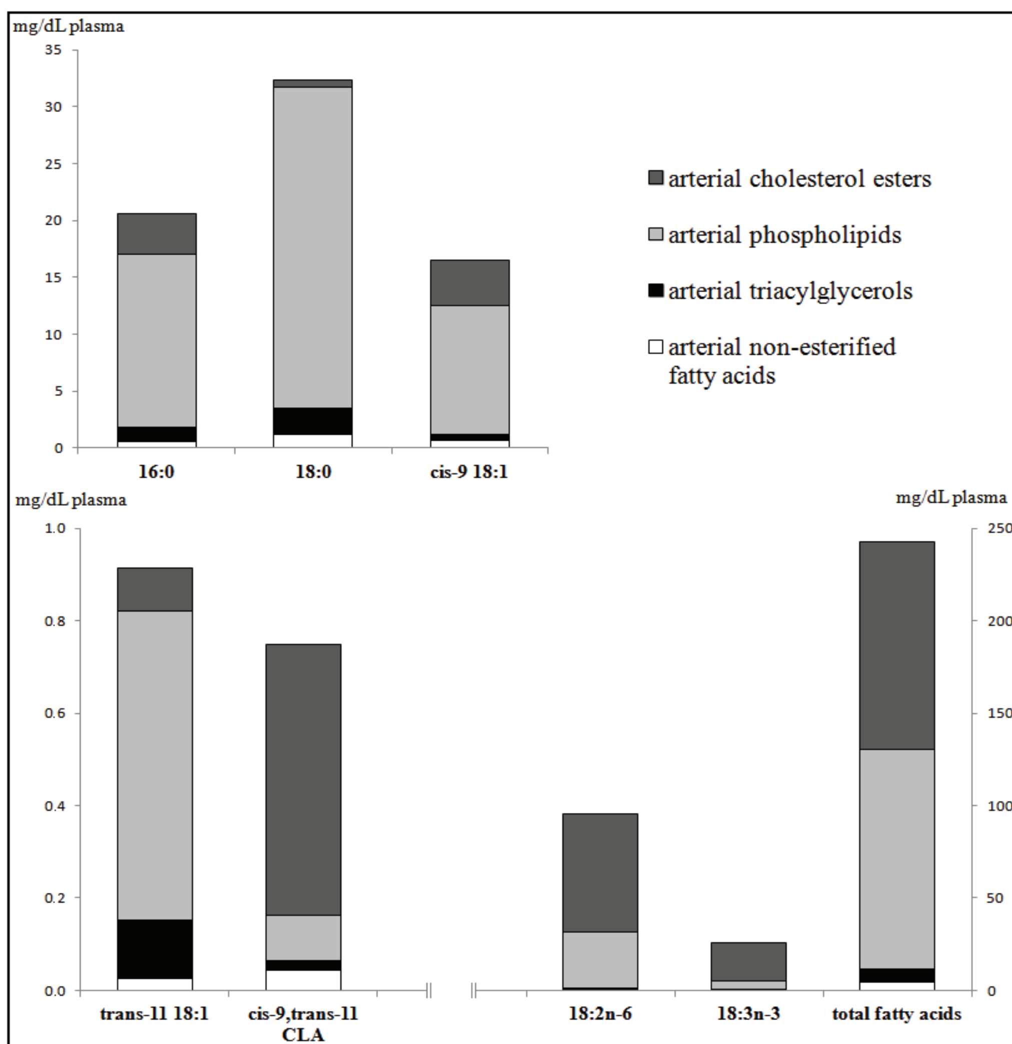

Figure 5 Distribution of specific and total fatty acids between lipid fractions in arterial blood of lactating cows (II).

Fresh Grass vs. Grass Hay. Zero-grazing increased arterial concentrations of fatty acids in PL and TAG by 12 and 17%, respectively compared with hay (II) consistent with a numerical increase in the flow of fatty acids at the omasum (437 and 365 g/d; I). The concentrations of plasma lipid classes (PL, CE, TAG and NEFA) have been reported to increase in direct relation to fatty acid supply (MacLeod et al., 1972; Christie, 1981b, Loor et al., 2002). It is also possible that the digestibility of fatty acids was higher from fresh grass compared with hay (Doreau et al., 2005). The higher whole tract OM digestibility and improved transfer efficiency of 18:2n-6 and 18:3n-3 from omasal digesta into milk (84 vs. 74% and 68 vs. 64% respectively) on fresh grass diet relative to hay is consistent with this suggestion (I, II). Fresh grass resulted in higher circulating NEFA concentrations in both arterial and venous blood compared with hay, changes that were not accompanied by differences in plasma glucose (II). Previous studies have reported that plasma NEFA

concentrations are higher in grazing cows compared with cows fed diets based on conserved forages, responses that have been attributed to lower circulating insulin (Agenäs et al., 2002; Boken et al., 2005; Kay et al., 2005), even in the absence of changes in glucose concentrations (Agenäs et al., 2002). In addition, plasma NEFA concentration generally increases during the nutrient restriction of lactating cows reflecting enhanced lipolysis and decreased lipogenesis in the adipose tissue (Chilliard et al., 2000a).

Concentrations of 18:3n-3 in plasma TAG and CE were 1.7- and 3.7-times higher, respectively, in cows fed fresh grass diet relative to hay (II) confirming earlier comparisons of plasma total fatty acids between cows at pasture and offered diets based on conserved forages (Kay et al., 2005; Fincham et al., 2009; Mohammed et al., 2009; LaTerra et al., 2010). The higher supply of 18:2n-6 on fresh grass diet relative to hay (I) resulted in a 190% increase in the relative abundance of 18:2n-6 in plasma TAG, with minimal or no changes in the proportions of this fatty acid in other plasma lipid classes. Given that fresh grass markedly increased the concentration of NEFA in plasma, the absolute amount of 18:2n-6 transported as NEFA was also higher in cows fed diets containing fresh grass compared with hay (II). Plasma TAG and NEFA fractions respond rapidly to the changes in the supply of 18:2n-6, whereas the response in PL and further in CE is much slower, but persists for much longer (Christie, 1981b).

Despite of the higher flow at the omasum on fresh grass diet (I), the relative concentration of *trans*-11 18:1 in plasma TAG and NEFA was similar in cows fed fresh grass and hay. Nevertheless, the absolute amount of *trans*-11 18:1 circulating in arterial blood in these fractions was markedly higher during zero-grazing relative to feeding hay (II) consistent with earlier data comparing total fatty acids in plasma of cattle fed fresh forages or TMR (Kay et al., 2005; Fincham et al., 2009; LaTerra et al., 2010) or cows at pasture, zero-grazed or offered GS (Mohammed et al., 2009).

The proportion of *cis*-9 18:1 and 16:0 in NEFA were 2.6- and 1.4-times higher in cows fed fresh grass compared with hay, that along with the overall increase in NEFA in arterial blood (169%; II), could be considered evidence of a significant contribution of adipose to the supply of fatty acids in the peripheral circulation (Chilliard et al., 2000a,b).

Grass Hay vs. Grass Silages. Compared with drying, grass conservation by ensiling enhanced omasal total fatty acid flow (on average 422, 551 and 587 g/d for hay, UTS and FAS, respectively; I) and consequently the concentration of fatty acids transported in PL and TAG fractions (II) confirming the positive relation between fatty acid supply and the concentration of lipid classes in the plasma (MacLeod et al., 1972; Christie, 1981b; Loor et al., 2002). In addition, several studies have reported a higher digestibility of fatty acids from GS compared with hay (Steele, 1983; Doreau et al., 2005).

Comparisons between hay, FAS and UTS indicated few changes in the fatty acid composition of plasma lipid fractions (II), which is in agreement with the rather similar postruminal flows of fatty acids across

diets based on conserved grasses (I). Feeding silage compared with hay has been demonstrated to induce relatively minor changes in plasma fatty acid composition of cows (He et al., 2011) and sheep (Steele, 1980). It is possible that longer experimental periods would have been required to detect differences in blood lipids on diets based on conserved forages, given that the changes in fatty acid supply may be relatively small. Even though all plasma lipid classes responded to increased supply of fatty acids within days (Moore et al., 1969; Christie, 1981b), plasma PL and CE concentrations were shown to be increased up to 60 d in response to dietary oilseed supplements or duodenal infusions of plant oils (Scislowski et al., 2005).

Extent of Silage Fermentation. Feeding FAS relative to UTS had no significant effect on plasma concentration or composition of fatty acids (II) consistent with similar fatty acids flows at the omasum (I).

4.4 LIPID METABOLISM IN THE MAMMARY GLAND

4.4.1 Uptake

Effect of Forage Conservation. Measurements of arterio-venous differences and uptake of fatty acids from different lipid fractions across the mammary gland confirmed that, irrespective of forage conservation method, TAG is the major source of preformed fatty acids for milk fat synthesis in the bovine mammary glands (II; Hartmann and Lacelles, 1964; Glascock et al., 1966; Loor et al., 2005a, Shingfield et al., 2010). The extraction rate of TAG was similar across diets and averaged 50% (II). In cows fed fresh grass NEFA also served as a source of fatty acids for milk fat synthesis (II). The mammary glands take up TAG from circulating very low-density lipoproteins and chylomicrons via the action of lipoprotein lipase, whereas NEFA circulates in plasma bound to albumin (Shingfield et al., 2010).

Since mammary extraction of NEFA was negligible and plasma NEFA levels low on all diets except for cows fed fresh grass, the calculations of mammary fatty acid uptake reported in this thesis are based on the sum of TAG and NEFA extraction (fresh grass diet) or TAG only (diets based on hay, FAS and UTS; II). Consistent with previous reports (Gagliostro et al., 1991; Enjalbert et al., 1998; Chilliard et al., 2000b), there was a close relationship between arterial concentrations of TAG and TAG + NEFA with the corresponding arterio-venous differences across the mammary glands (II).

Plasma PL and CE are generally not considered as a major source of fatty acids for the mammary glands, although there is some evidence of a modest uptake of fatty acids from these lipid fractions (Glascock et al., 1966; Yang et al., 1978; Gagliostro et al., 1991; II). The concentrations of

PUFA in CE and PL in plasma are typically several orders of magnitude higher than those of TAG and NEFA and therefore even low rates of extraction may contribute significantly to the supply of PUFA available for milk fat synthesis. However, measuring mammary uptake of fatty acids from CE and PL may be below the sensitivity of the arterio-venous technique. Nevertheless, mammary uptake of PUFA from plasma TAG + NEFA or only TAG explained the differences in milk fatty acid composition and yield of 18:3n-3 between treatments, but could only account for 70 and 84% of 18:2n-6 output in milk for cows fed fresh grass and conserved grass forages, respectively (II). Besides extraction of PL and/or CE, it is possible that limited amounts of 18:2n-6 could be synthesized endogenously in the bovine mammary glands based on evidence that *trans*-12 18:1 is desaturated to 18:2n-6 during incubations with microsomes prepared from the liver of rats (Mahfouz et al., 1980). It is possible that endogenous synthesis may, at least in part, explain the discrepancy between mammary uptake and secretion of 18:2n-6 in this and previous studies (Enjalbert et al., 1998; II).

Studies reporting mammary uptakes of individual fatty acids are scarce. In the present work, mammary uptake of 16:0, 18:0 and *cis*-9 18:1 from plasma accounted for on average 19-23, 34-47 and 7-22% of total fatty acid uptake in cows fed diets based on fresh or conserved grass, respectively (II), values that are in agreement with earlier reports (Thompson and Christie, 1991; Enjalbert et al., 1998).

Mammary uptake of fatty acids was 70% higher in the same cows during zero-grazing compared with diets based on grass hay. Relative to hay, zero-grazing increased markedly mammary uptake of 18:3n-3 from plasma, with the uptake of 18:2n-6 being also numerically higher (II). This is in broad agreement with earlier investigations reporting higher concentrations of 18:3n-3 in the milk from grazing cows, compared with dried or ensiled forage (Dewhurst et al., 2006; Chilliard et al., 2007; Mohammed et al., 2009; Ferlay et al., 2011), and increased secretion of 18:2n-6 and 18:3n-3 in milk in the present study (II).

Direct comparisons of 16:0 + *cis*-9 16:1, 18:0 + *cis*-9 18:1 and total fatty acids across the mammary glands relative to the flow at the omasum (70, 146 and 199 vs. 24, -1 and 6 g/d, for fresh grass and hay, respectively) suggests that part of medium- and long chain fatty acids used for the synthesis of milk fat originated from body tissues, with the apparent contribution being much greater during zero-grazing compared with feeding hay (II). More extensive mobilization of adipose is also supported by higher NEFA content in plasma of cows fed fresh grass compared with hay and subtantially lower, albeit still slightly positive calculated energy balance on the diet based on fresh grass (II).

Feeding hay or silages had no significant effect on mammary uptake of fatty acids, but feeding silage tended to increase that of 18:0 and *trans*-11 18:1 relative to hay, consistent with the higher secretion of 18:0, *trans*-11 18:1 and *cis*-9,*trans*-11 CLA in milk (II). In addition, restricting silage

fermentation was associated with higher mammary uptake of 18:3n-3 and higher 18:3n-3 output in milk (II).

4.4.2 *De novo* synthesis

Short- and medium chain SFA are synthesised *de novo* from acetate and β-hydroxy butyric acid (BHBA) in mammary epithelial cells in the presence of two key enzymes, ACC and fatty acid synthetase and a supply of nicotinamide adenine dinucleotide phosphate (NADPH) reducing equivalents (Shingfield et al., 2010). Long chain fatty acids are known to exert inhibitory effects on ACC activity with the effects being more potent when the number of carbon atoms and/or the degree of unsaturation, the number of *trans* double bonds in particular, increases (Chilliard et al., 2000b).

Effect of Forage Conservation. Mammary *de novo* synthesis accounted for practically all 12:0 and 14:0 secreted in milk (II) consistent with previous reports (Table 5) with no significant variation across treatments. In contrast, forage conservation had a substantial influence on mammary 16:0 synthesis *de novo*. In cows fed hay or silage, *de novo* synthesis accounted for ca. 79% of 16:0 secreted in milk, a contribution that was much lower in zero-grazed cows (53%). Most accounts indicate that between 39 and 81% (Table 5) of 16:0 in bovine milk fat is synthesised in the mammary gland. However, differences in animal genetics (Åkerlind et al., 1999; Ferlay et al., 2011), diet composition (Palmquist et al., 1967; Enjalbert et al., 1998; Chilliard et al., 2007) and analytical methods may all contribute to this variability.

The decreased mammary *de novo* synthesis of SFA on fresh grass diet relative to hay diet can be explained, at least in part, by the higher uptake of 18-, 20- and 22-carbon unsaturated fatty acids across the mammary glands as indicated by the higher secretion of these fatty acids in milk (II, Chilliard et al., 2000b). Furthermore, restricting silage fermentation by formic acid increased mammary *de novo* synthesis of SFA (4:0-10:0, 14:0) (II) that can be attributed to higher lipogenic to glucogenic ratio of VFA in the rumen of cows fed FAS relative to UTS (I), a typical response for feeding restrictively fermented GS (Huhtanen, 1998; Shingfield et al., 2002b).

Table 5 Relative contribution of *de novo* synthesis in the bovine and caprine mammary glands to the secretion of 12:0, 14:0 and 16:0 in milk

Basal diet	Animal	DMI, kg/d	F:C	Method	De novo % 12:0	14:0	16:0	Reference
Hay*	nanny goat			^{14}C-acetate	94	71	24	Annison et al., 1967
Lucerne hay**								
High forage	cow			^{14}C-acetate	105	72	59	Palmquist et al., 1967
Low forage					108	97	66	
Hay (species not specified)								
Before infusion	nanny	1.6	32:68	AV			43	Bickerstaffe and
Intravenous infusion sterculic acid	goat	1.7	29:71				31	Johnson, 1972
After infusion		1.7	29:71				42	
Hay (species not specified)	cow	15	42:58	AV	100	100	64	Bickerstaffe et al., 1974
Meadow hay	cow	16	50:50	^{3}H-palmitic acid			55	Glascock and Welsh, 1974
Hay (species not specified)	nanny goat	1.6	50:50	^{14}C-acetate ^{14}C-BHBA	102	75	45	Smith et al., 1974
Hay (species not specified)***	cow			AV			65	Peeters et al., 1979
Mixture of maize silage and grass hay								
Control	cow		65:34	AV		92	81	Enjalbert et al., 1998
Infusion duodenum 16:0			65:34			84	39	
Infusion duodenum 18:0			65:34			88	65	
Infusion duodenum *cis*-9 18:1			65:34			74	50	
Grass								
Fresh	cow	17	64:36	AV	98	94	54	II
Hay		18	67:33		98	94	78	
Hay		20	60:40		99	95	79	
Untreated silage		20	61:39		97	96	79	
Formic acid treated silage		22	64:36		99	96	80	

DMI= dry matter intake, AV=arterio-venous technique, F:C=forage-to-concentrate ratio, BHBA=β-hydroxy butyric acid
*Hay *ad libitum* and 1 kg concentrate per day
**High forage: Hay *ad libitum* (about 10 kg/d) and 1 kg concentrate per 2.5 kg milk
Low forage: Hay 2.3 kg/d and concentrate *ad libitum* (12-18 kg/d)
***Supplemented with commercial concentrate

4.4.3 Desaturation

Effect of Forage Conservation. Measurements of 18:0 desaturation in the mammary gland in the present study (44-67%; II) are comparable with previous estimates (47-78%) based on infusions of labeled substrates (Bickerstaffe et al., 1974; Mosley et al., 2007) or arterio-venous differences (Enjalbert et al., 1998). The formation of *cis*-9 18:1 accounted for, on average, 71% of total desaturation of fatty acids in the mammary glands, followed by *cis*-9 16:1 (11%) and *cis*-9 14:1 (10%), whereas the relative contribution of *cis*-9,*trans*-11 CLA was marginal (2%; II). Nevertheless, about 79% of milk fat *cis*-9,*trans*-11 CLA secreted in milk originated from endogenous synthesis in the mammary glands, with little variation among grass based diets (II). This value is in good agreement with earlier estimates based on infusions of sterculic oil to inhibit stearoyl-CoA desaturase (Griinari et al., 2000; Corl et al., 2001), infusions of *trans*-11 18:1 (Shingfield et al., 2007; Tyburczy et al., 2008) or administration of labeled *trans*-11 18:1 (Mosley et al., 2006).

4.5 FATTY ACIDS IN MILK

In 2012, milk fat content and yearly fat output of herds participating in the Finnish milk recording scheme averaged 4.15% and 368 kg, respectively (Table 6). Jersey and Finncattle cow breeds had the highest and Holstein had the lowest milk fat content, Finnish Ayrshire being an intermediate (Table 6). Breeds with inherently high milk fat content have typically higher concentrations of *de novo* synthesised short and medium-chain SFA in milk than those with lower milk fat content (DePeters et al., 1997; Sol Morales et al., 2000; Drackley et al., 2001; Ferlay et al., 2006).

Table 6 Milk production traits of different cow breeds in Finland in 2012 (ProAgria, 2013)

Cow breed	Distribution of breeds, %	Milk yield, kg/yr	Milk fat content, %	Milk fat yield, kg/yr
Finnish Ayrshire	61	8,571	4.28	367
Holstein	38	9,434	3.95	373
Finncattle	1	6,109	4.40	269
Jersey	0.1	7,750	4.69	363
Mean		8,865	4.15	368

Milk fat is essentially comprised of TAG (96 to 98%) and small amounts of DAG, MAG, NEFA, PL and retinol esters (Jensen, 2002; Shingfield et al., 2010). Milk fat is reported to contain more than 500 individual fatty acids (Demeyer and Doreau, 1999), the quantitatively most abundant being the SFA of 4 to 18-carbons (16:0 in particular), *cis*-9 18:1, *trans*-18:1, 18:2n-6 and 18:3n-3 (Table 7). The potential to decrease milk fat 16:0 content by changes in diet composition is high (Dewhurst et al., 2006; Roy et al., 2006; Glasser et al., 2008a; Shingfield et al., 2008b; Rego et al., 2009; II, V).

Table 7 Average composition of the major fatty acids in bovine milk (derived from Jensen, 2002)

Fatty acid	Average range (g/100g fatty acids)
4:0	2–5
6:0	1–5
8:0	1–3
10:0	2–4
12:0	2–5
14:0	8–14
15:0	1–2
16:0	22–35
cis-9 16:1	1–3
17:0	0.5–1.5
18:0	9–14
cis-9 18:1[1]	20–30
18:2n-6	1–3
18:3n-3	0.5–2

[1] Contains about 3% of total fatty acids *trans* 18:1 with double bonds 4 to 16.

Besides by rumen microbes, 15:0 and 17:0 can be synthesized *de novo* using propionyl-CoA as primer in adipose tissue and the mammary glands (Vlaeminck et al., 2006; Dewhurst et al., 2007). This is confirmed by higher (on average 77%) secretion of 15:0 and 17:0 in milk relative to omasal flows on all experimental diets in the present studies (Table 8). In addition, the secretion of *iso* 17:0 exceeded omasal flow on average by 30% (Table 8) confirming substantial postruminal synthesis of this fatty acid as reported previously (Dewhurst et al., 2007). Milk OBCFA has been proposed as markers for specific rumen bacteria due to the inherent differences in their fatty acid profile (Vlaeminck et al., 2006), but based on the findings of the present work, the potential of measurements of OBCFA in milk to describe changes in the concentrations of these fatty acids in the rumen appears limited.

Secretion in milk of several biohydrogenation intermediates of 18-carbon unsaturated fatty acids (Chilliard et al., 2007; Shingfield et al., 2010), including *cis*-18:1 (Δ11, 13, 15) and *trans*-18:1 (Δ6-9, 15), exceeded the flow at the omasum in the present work (Table 8). Though limited in number, the secretion of several *cis*-18:1 (Δ11, 13, 15) and *trans*-18:1 (Δ9, 10) in milk has exceeded postruminal flows also in previous investigations (Table 8). This suggests that limited amounts of these fatty acids may be formed postruminally in tissues from 18-carbon unsaturated fatty acids, by oxidation of longer carbon chain fatty acids and/or by elongation of shorter carbon chain fatty acids. Indeed, it has recently been demonstrated *in vitro* that *trans*-9 16:1 can be elongated to *trans*-11 18:1 in bovine adipocytes (Kadegowda et al., 2013). These findings highlight the importance and contribution of lipid metabolism in tissues to milk fatty acid composition.

Table 8 Transfer efficiency (%) of selected odd- and branched-chain fatty acids and 18:1 isomers excluding *cis*-9 from the gut into milk (I-IV)

	I-IV					Kalcheur et al., 1997, Piperova et al., 2002[1]				Loor et al., 2004, 2005a[2]				Loor et al., 2005b,c[3]			Kairenius et al., 2006, Shingfield et al., 2012[4]			
Fatty acid	Mean	SD	Min	Max	n	HF-NB	HF-B	LF-NB	LF-B	LC	HC	LCO	HCO	FO	LO	SFO	C	FO75	FO150	FO300
Odd- and branced-chain																				
15:0	177	25.7	132	207	9					101	130	66	66	101	79	68				
iso 15:0	90	15.4	73	112	9					23	13	24	7	45	35	29				
anteiso 15:0	76	7.6	62	85	9					49	33	41	21	44	31	28				
17:0	177	32.9	123	240	9					134	132	90	60	92	74	68				
iso 17:0	130	66.7	72	262	9					172[5]	188[5]	155[5]	164[5]							
18:1					9															
cis-11	123	45.3	77	212	9					92	58	115	32	71	50	48	143	139	94	66
cis-12	84	23.3	64	136	9					86	53	74	28	37	45	52	70	70	125	90
cis-13	239	133.6	160	577	9					140	114	285	37	87	57	67	125	113	113	80
cis-15	98	11.3	88	113	4					75	54	215	16	44	38	44				
cis-16	66	18.5	36	103	9															
trans-4	76	24.1	57	135	9					27	22	34	12	39	20	32	43	45	36	38
trans-5	72	13.5	58	101	9					5	9	8	7	47	32	41	51	51	191	75
trans-6+7+8	96	20.3	70	127	9	18	63	38	8	73	42	66	22	47	38	44	60	64	56	49
trans-9	174	97.2	91	359	9	54	95	65	53	70	48	62	22	67	55	57	101	85	71	59
trans-10	63	9.1	51	72	9	72	68	57	46	137	51	67	26	46	32	37	58	62	62	49
trans-11	45	7.2	35	57	9	53	55	43	48	37	32	44	15	49	30	31	57	49	49	47
trans-12	60	11.6	50	88	9	69	68	54	65	75	56	64	31	54	38	41	64	69	66	59
trans-13+14	57	15.5	38	80	9	53	52	41	50	58	37	70	29	41	31	35	43	41	44	44
trans-15	117	74.6	54	281	9	42	49	35	39	40	24	53	12	33	28	31	75	72	63	56
trans-16+*cis*-14	58	11.9	46	85	9	52	61	37	56	50	35	51	22	40	33	42	56	62	79	69

[1]HF=60% of diet DM a 60:40 DM mixture of maize silage and alfalfa haylage, LF=25% of diet DM a 60:40 DM mixture of maize silage and alfalfa haylage, NB=no buffer, B=buffer, DM intake was 21, 22, 24 and 24 g/d for HF-NB, HF-B, LF-NB and LF-B, respectively.
[2]LC=65% of diet DM grass hay, HC=35% of diet DM grass hay, O=3% of DM linseed oil, DM intake 20 kg/d for all diets.
[3]35% of diet DM grass hay, FO=2.5% of diet DM fish oil, LO=5% of diet DM linseed oil, SFO=5% diet DM sunflower oil, DM intake was 17, 17 and 19 kg/d for FO, LO and SFO, respectively.
[4]50-59% of diet DM grass silage, C=no additional lipid, FO75=75 g/d fish oil, FO150=150 g/d fish oil, FO300=300 g/d fish oil. DM intake was 19, 19, 18, 16 kg/d, respectively.
[5]Contains *trans*-9 16:1.

4.5.1 Effect of forage conservation

Fresh Grass vs. Grass Hay. Feeding fresh chopped grass or barn-dried grass hay had no effect on milk yield or ME intake, but fresh grass diet was associated with increased milk fat output and energy corrected milk (ECM) production, primarily due to higher mammary uptake of preformed fatty acids from arterial blood (II).

16:0. Feeding fresh grass decreased milk fat 16:0 content and yield relative to dried hay (from 37 to 27 g/100g fatty acids and from 344 to 283 g/d, respectively) primarily due to a 1.78-times lower *de novo* synthesis in the mammary glands, consistent with increases in the supply of preformed fatty acids inhibiting synthesis of 16:0 *de novo* (Enjalbert et al., 1998 in Table 5; Shingfield et al, 2010). Earlier studies have also demonstrated that milk from pasture contains markedly lower concentrations of 16:0 compared with diets based on conserved forages (typical range in milk from fresh or conserved forages of between 23 and 38 g/100g fatty acids; Dewhurst et al., 2006; Chilliard et al., 2007; Table 9).

18:2n-6 and 18:3n-3. Feeding fresh grass increased milk fat 18:2n-6 and 18:3n-3 concentrations (from 1.3 to 1.7 and from 0.6 to 0.7 g/100g fatty acids, respectively) and yields (from 13 to 15 and from 5.0 to 7.1 g/d, respectively) compared with hay diet consistent with an increase in the abundance of these fatty acids in plasma TAG and greater extraction across the mammary glands (II). Pasture feeding has often increased milk fat 18:3n-3 content compared with conserved forages (typical range in milk from fresh or conserved forages of between 0.3 and 2.0 g/100g fatty acids; Table 9), whereas the effect on 18:2n-6 is much more variable (Dewhurst et al., 2006; Chilliard et al., 2007; Table 9). However, direct comparisons of conservation method on milk fat PUFA are, in most studies, confounded by the differences in the forage-to-concentrate ratio of the diet (Ferlay et al., 2006; Dewhurst et al., 2006), forage species (Kelly et al., 1998; Leiber et al., 2005; Couvreur et al., 2006) or forage maturity (Mohammed et al., 2009). Compared with feeding hay, zero-grazing decreased the transfer efficiency of 18:3n-3 (from 8.5 to 4.8%) and 18:2n-6 (from 14.4 to 13.7%) from feed to milk that was associated with higher lipolysis and biohydrogenation of dietary lipid in the rumen during zero-grazing (II, Figure 6). In general, zero-grazing results in a similar transfer efficiency of 18:3n-3 from feed into milk relative to grazing, whereas the effect of grass drying is variable (Figure 6). The variability in 18:3n-3 transfer efficiency from the diet into milk in cows on hay based diets may be related to differences in forage-to-concentrate ratio, grass species and 18:3n-3 intake between studies. However, the transfer efficiency of PUFA from omasal digesta into milk was higher during zero-grazing relative to feeding hay (refer to chapter 4.3). It is possible that the higher efficiency of transfer is related to a higher digestibility of fatty acids in the small intestine in cows fed fresh grass compared with hay, a suggestion that is supported by the higher whole tract OM digestibility (I) and/or implied greater mobilization of

tissue adipose to support milk production during zero-grazing (refer to chapter 4.4.1).

Cis-9 18:1. Fresh grass resulted in higher concentration and secretion of *cis*-9 18:1 in milk fat than hay that may arise from higher uptake of *cis*-9 18:1 from arterial plasma across the mammary glands (II). It is probable that mobilization of adipose also contributed to these differences as discussed previously (refer to chapter 4.4.1).

18-carbon Biohydrogenation Intermediates. Feeding fresh grass enhanced milk fat of *cis* 18:1 (Δ10-16), *trans* 18:1 (Δ6-16), Δ11,13 CLA, Δ13,15 CLA, Δ9,12 18:2, Δ11,15 18:2 and *cis*-9,*trans*-11,*cis*-15 18:3 concentrations compared with hay (II) reflecting the higher unsaturated fatty acid intake and more extensive accumulation of 18-carbon biohydrogenation intermediates in the rumen (Shingfield et al., 2010; I). Milk fat *trans*-11 18:1 and *cis*-9,*trans*-11 CLA concentrations and yields were enhanced by feeding fresh chopped grass compared with hay (II) that is in agreement with earlier studies (Dewhurst et al., 2006; Chilliard et al., 2007; Table 9). The higher enrichment in milk was explained by a higher uptake of *trans*-11 18:1 from plasma across the mammary glands increasing substrate supply for endogenous *cis*-9,*trans*-11 CLA synthesis, while the relative proportion of *trans*-11 18:1 desaturated in the mammary glands was unchanged (II). Based on recent evidence the concentrations of *trans*-11 18:1 and *cis*-9,*trans*-11 CLA in milk fat could be expected to be higher in grazing cows compared with zero-grazing due to the selective consumption of leafy material rich in 18:3n-3 at pasture (Mohammed et al., 2009).

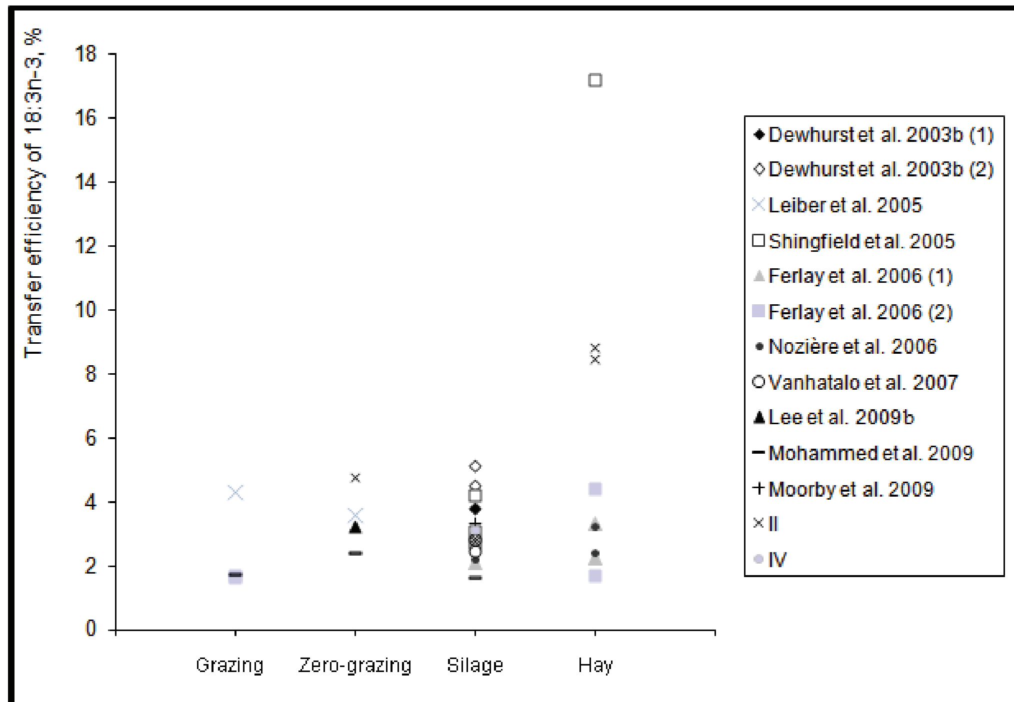

Figure 6 Effect of conservation method of grass on the efficiency of 18:3n-3 transfer from the diet into milk in cows fed diets containing no additional lipid

Table 9 Influence of forage conservation method on milk fatty acid composition of cows offered diets containing no additional lipid

Forage	DM intake, kg/d	Forage to concentrate ratio	Fat yield, g/d	g/100g milk fatty acids									Reference
				12:0	14:0	16:0	18:0	*c*9 18:1	*t*11 18:1	*c*9*t*11 CLA	18:2n-6	18:3n-3	
LH:(G+RC)P, 2:3	16	46:54	860	2.6	9.4	25	15	31[1]		0.9	4.3	0.8	Dhiman et al., 1999
LH:(G+RC)P, 1:3	15	80:20	640	2.2	8.9	24	15	33[1]		1.4	2.7	1.5	
(G+ RC)P	14	100:0	490	2.3	9.1	25	12	33[1]		2.2	1.4	2.0	
LS	19	100:0	620		11	36	6.6	17[1]			1.1	0.8	Whiting et al., 2004
LZG	18	100:0	640		10	30	8.9	22[1]			1.6	1.1	
MS:LH, 5:2	23	34:66	1300	3.3	11	29	12	19	2.7[2]	0.6	4.3	0.7	Boken et al., 2005
GP	12*		1200	2.4	8.9	26	16	21	3.9[2]	0.7	3.7	0.8	
MS:GS:GH, 7:6:1.5	20	73:27	707	1.7	9.7	32	11	23	1.6	0.7	1.7	0.3	Kay et al., 2005
GP	16	100:0	562	1.5	10	28	12	22	3.9	1.8	0.7	1.0	
H:GS:MS 1:6:3	18			3.1	11	34	10	19	0.9[3]	0.5	1.4	0.5	Leiber et al., 2005
GP	16	100:0		3.9	12	25	10	18	4.0[3]	1.7	0.9	0.7	
GZG	17	100:0		4.0	12	29	9.3	16	3.3[3]	1.4	0.8	0.6	
MS	19	83:17	990	3.7	12	31	10	19[4]	0.9	0.5	1.6	0.2	Couvreur et al., 2006
MS:(G+WC)ZG, 2:1	18	82:18	1030	3.8	12	28	11	20[4]	1.5	0.5	1.3	0.4	
MS:(G+WC)ZG, 1:2	19	82:18	1030	3.9	12	27	11	19[4]	3.1	1.2	1.4	0.6	
(G+WC)ZG	18	82:18	1010	3.4	11	24	11	21[4]	4.7	1.7	1.3	0.7	
NH (week 3)	15	87:13	550	20[5]		28	10	18	1.3	0.6	1.0	1.7	Ferlay et al., 2006
NH (week 6)	16	87:13	520	19[5]		32	8.9	18	1.3	0.6	1.4	0.8	
NP (week 3)	16	97:3	610	15[5]		23	11	24	3.7	1.7	1.1	1.0	
NP (week 6)	14	97:3	490	16[5]		30	9.2	22	1.8	0.8	1.2	0.7	
GZG	19	89:11	1270	3.1	9.0	29	12	23	4.3[2]	1.0[6]	1.3	0.8	Lee et al., 2009b
RCZG	20	90:10	1310	3.0	12	27	11	25	5.1[2]	1.1[6]	2.4	1.5	
GS	16	83:17	580	3.3	11	38	7.3	13[7]	1.0	0.5	0.8	0.3	Mohammed et al., 2009
GZG	18	85:15	790	3.2	10	26	11	17[7]	3.5	1.4	1.0	0.8	
GP	19	86:14	930	3.0	10	24	10	18[7]	4.7	2.1	1.1	0.7	
(G+WC)P	5*		820	2.8	10	24	14	22	2.7	1.2	1.1	0.6	Rego et al., 2009
GH	18	67:33	1010	4.2	13	37	6.2	15	0.6	0.3	1.3	0.6	II
GZG	17	64:36	1100	3.3	11	27	10	23	1.1	0.6	1.5	0.7	

G = grass, L = lucerne, M = maize, N=natural mountain herbage RC = red clover, WC = white clover

H=hay, P=pasture, S=silage, ZG=zero-grazing

*Concentrate intake, kg/d

[1]18:1 isomers not specified, [2]contain all isomers of *trans* 18:1, [3]contain *trans*-10 18:1 [4]contain *trans*-13 18:1, [5]contain 10:0, [6]contain all isomers of CLA, [7]contain *cis*-10 18:1

Grass Hay vs. Grass Silages. Compared with hay based diets, feeding silage increased ME intake, milk yield and milk fat output (II), consistent with previous findings (Shingfield et al., 2002a), the latter due to higher synthesis of short and medium chain SFA *de novo* (4:0-8:0, 14:0, 16:0; II) and numerically higher mammary uptakes of preformed medium- and long chain fatty acids from arterial blood. Consistent with increased mammary SFA synthesis, the concentration of BHBA in arterial blood was also higher in cows fed silage relative to hay (II; Shingfield et al., 2002b; Ferlay et al., 2006), primarily as a result of higher molar proportions of butyric acid in the rumen VFA (I).

Conservation of grass by ensiling resulted in marginally lower milk fat 18:2n-6 and 18:3n-3 concentrations compared with drying (II) consistent with slight decreases or no changes reported previously (Table 10). In contrast, the reverse was true during the conservation of lucerne (Onetti et al., 2004). However, the output of 18:2n-6 and 18:3n-3 in milk did not differ between hay, FAS and UTS treatments (II).

Owing to a higher intake of 18-carbon PUFA and more extensive accumulation of biohydrogenation intermediates (I) ensiling rather than drying resulted in higher milk fat concentrations of 18:0, *trans* 18:1 (Δ12-16), *trans*-11,*cis*-15 18:2, Δ9,12 18:2, Δ12,15 18:2, Δ13,15 CLA and *cis*-9,*trans*-11,*cis*-15 18:3. A higher output of *cis*-9,*trans*-11 CLA in milk on silage diets relative to hay can be explained by increased uptake of *trans*-11 18:1 across the mammary glands (II). Even though feeding GS did not affect the relative abundance of *trans*-11 18:1 in plasma TAG relative to grass hay, the absolute concentration of TAG was higher (II). Previously, forage conservation by ensiling compared with drying has been reported to increase (Onetti et al., 2004), decrease (Ferlay et al., 2006 in Table 10) or have no effect (Nozière et al., 2006 in Table 10) on milk fat *trans*-11 18:1 and *cis*-9,*trans*-11 CLA content. The inconsistencies in milk fat responses between studies may be explained by the large differencies in treatment effects on 18-carbon PUFA intake. It is also possible that differences in forage-to-concentrate ratio (Table 10) and other dietary ingredients (25% of diet DM as maize silage rich in starch in the study of Onetti et al., 2004) between experiments also influenced lipid metabolism in the rumen and consequently milk fat responses (Loor et al., 2004; Chilliard et al., 2007).

Extent of Silage Fermentation. Feeding FAS compared with UTS increased milk fat yield as a result of increased mammary synthesis of SFA, that can be explained by both a higher ME intake and higher arterial BHBA content (II), in accordance with a relatively high molar ratio of lipogenic to glucogenic VFA in cows fed the FAS diet (I; Huhtanen, 1998; Shingfield et al., 2002b; Jaakkola et al., 2006a,b).

Restricting silage fermentation using a formic acid based additive had relatively minor effects on milk fat composition relative to UTS (II). Ensiling method had no effect on milk fat 18:2n-6 and 18:3n-3 concentrations, but the secretion was 13 and 9.8% higher, respectively, for FAS than UTS. These observations are in accordance with the numerical increase in the intake and mammary uptake of these PUFA (II). Milk fat outputs of *trans*-11 18:1 and *cis*-9,*trans*-11 CLA were also increased on FAS compared with UTS diet (II), consistent with previous reports (Shingfield et al., 2005 in Table 10).

Table 10 Comparison of dried and ensiled forages on milk fatty acid composition of cows offered diets containing no additional lipid

Forage	DM intake, kg/d	Forage to concentrate ratio	Fat yield g/d	g/100g milk fatty acids									Reference
				12:0	14:0	16:0	18:0	*c*9 18:1	*t*11 18:1	*c*9*t*11 CLA	18:2n-6	18:3n-3	
GH	20	61:39	1180	4.0	13	35	9.2	15	3.8[1]	0.4[2]	1.2	0.5	Shingfield et al., 2005
GS (untreated)	20	63:37	1220	3.8	13	35	9.8	15	3.6[1]	0.4[2]	1.0	0.5	
GS (inoculant)	20	62:38	1240	3.9	13	34	10	15	3.7[1]	0.4[2]	1.0	0.4	
GS (formic acid)	21	63:37	1280	4.0	13	34	10	15	4.3[1]	0.5[2]	0.9	0.3	
GH	15	90:10	590	20[3]		30	8.2	15	1.9	0.9	1.0	1.0	Ferlay et al., 2006
GS	13	87:13	450	19[3]		32	7.9	16	0.9	0.5	1.1	0.9	
NH (energy demand)	20	53:47	870	22[3]		35	6.3	14	0.5	0.3	1.8	0.4	Nozière et al., 2006
NH (low energy)	17	64:36	780	22[3]		36	7.0	13	0.5	0.4	1.4	0.4	
GS (energy demand)	17	69:31	860	20[3]		38	6.7	13	0.5	0.2	1.2	0.5	
GS (low energy)	13	88:12	680	20[3]		36	6.8	12	0.5	0.4	1.0	0.5	
GH	20	60:40	1130	3.9	13	37	8.3	12	0.7	0.3	1.3	0.5	II
GS (untreated)	20	61:39	1200	3.7	13	37	9.1	12	0.8	0.3	1.1	0.4	
GS (formic acid)	22	64:36	1320	3.6	12	35	9.8	12	0.9	0.3	1.1	0.4	

G = grass, N=natural mountain herbage
H=hay, S=silage
[1] sum of all isomers of *trans* 18:1 with a double bond from 4 to 16, [2] sum of all isomers of CLA,[3] also includes 10:0

4.5.2 Grass silage vs. red clover silage

Animal Performance. Feeding GS and RCS as a mixture increased milk yield reflecting changes in DMI (IV). A higher DMI when diets contained both GS and RCS compared with either forage alone has also been reported earlier (Tuori et al., 2002; Bertilsson and Murphy, 2003; Dewhurst et al., 2003a; Kuoppala et al., 2009). In other studies the intake of RCS has been shown to be higher compared with a mixture of GS and RCS or GS alone (Dewhurst et al., 2003b; Moorby et al., 2009). In the current work, diets based on RCS diet resulted in the lowest DMI (IV). Discrepancies in intake responses to forage species between studies may reflect differences in herbage composition and maturity at harvesting, extent of fermentation *in silo*, nutrient digestibility and the forage-to-concentrate ratio of the diet, factors known to influence the intake potential of diets in lactating cows (Huhtanen et al., 2007; Kuoppala et al., 2009). Even though the ME intake on RCS diet was 20 MJ/d lower than that of GS diet, cows on the RCS treatment produced 0.6 kg/d more ECM (IV) that supports an improved apparent nutrient utilization for milk production on early harvested RCS diets compared with GS (6.8 vs. 7.7 MJ ME consumed/kg ECM produced, respectively in IV; Vanhatalo et al., 2009). However, it is possible that cows fed RCS used more body reserves to support lactational performance contributing to the overall improvement in energy utilization on RCS diet. This is supported by a loss of body condition score (-0.06 per 21 d on pure RCS in a scale of 1 to 5) and a more negative calculated ME balance (4.73, -2.57, -1.22 and -18.3 MJ/d for GS, GRC, RCG and RCS, respectively) in cows fed diets based on RCS. Moorby et al. (2009) also reported decreases in the body weight and body condition score as the proportion of RCS in the diet increased.

In the current work, forage species had no effect on milk fat yield (IV) consistent with the findings of earlier studies (Steinshamn, 2010). A lower milk fat concentration that has often been reported for diets based on RCS compared with GS (Steinshamn, 2010) seems to be a consequence of dilution, although decreased mammary synthesis of SFA *de novo* has also been postulated (Steinshamn, 2010) arising from an increase in the supply of preformed long chain fatty acids (Vanhatalo et al., 2007).

18:2n-6 and 18:3n-3. In general, bovine milk fat 18:3n-3 content ranges from 0.4 to 0.7 and from 0.7 to 1.5 g/100 fatty acids in diets based on GS and RCS, respectively (Table 11; II; IV; V). The increases in milk fat PUFA content to RCS (IV; Table 11) is not explained by differences in fatty acid intake (III). In the present investigation incremental replacement of GS with RCS in the diet decreased the 18:3n-3 intake. (III).

Overall, the composition of milk fat (IV) reflected changes in ruminal lipid metabolism and the flow of fatty acids at the omasum due to forage species (III). Milk fat 18:2n-6, 18:3n-3 and total PUFA concentration increased linearly in response to higher proportions of RCS in the diet (IV) consistent with corresponding increases in the postruminal flow of PUFA

available for absorption (Dewhurst et al., 2003b; Lee et al., 2006; III). Previous studies have demonstrated that RCS increases the PUFA content of milk (Table 11) and muscle (Scollan et al., 2006; Lee et al., 2009a). The higher transfer of 18:2n-6 and 18:3n-3 from the diet into milk in response to RCS in the diet (Figures 7 and 8) can be attributed to lowered metabolism of dietary lipids in the rumen (Dewhurst et al. 2003a; Lee et al., 2003; 2006; III). Relative to grass and other legumes, the efficiency of 18:3n-3 transfer from the diet into milk also appears to be higher for red clover, but similar to that for maize silage based diets (Figure 7) and increases in direct relation to the amount of red clover in the diet (Figure 8). However, the milk fat 18:3n-3 content on diets based on maize silage is typically very low (0.2-0.3 g/100g; Couvreur et al., 2006 in Table 9, Chilliard et al., 2001 in Table 11) compared with diets based on RCS (Table 11, IV, V).

Conjugated Linoleic Acid. Studies reporting the effect of RCS on the distribution of CLA isomers in milk are limited. Replacing GS with RCS in the diet had no effect on milk fat *cis*-9,*trans*-11 CLA concentration consistent with earlier findings (Table 11). In contrast, milk fat concentrations and yields (all data not shown) of *trans*-7,*cis*-9 CLA, *trans*-10,*cis*-12 CLA and *trans*-11,*trans*-13 CLA were linearly increased in response to RCS in the diet (IV) in agreement with an earlier report (Vanhatalo et al., 2007), but the magnitude of these changes was numerically extremely small. Despite an inverse relation between inclusion of RCS in the diet on the flow of *trans*-10,*cis*-12 CLA at the omasum, RCS increased linearly the secretion of this CLA isomer in milk (from 7.3 to 14 mg/d). It is possible that this reflects differences in the absorption of minor fatty acids. However, the apparent intestinal digestibility of *trans*-10,*cis*-12 CLA has been high (>86%) under various dietary conditions (Loor et al., 2004; Loor et al., 2005c). These findings could also be interpreted as evidence for part of *trans*-10,*cis*-12 CLA secreted in milk being synthesized postruminally, even though the estimates reported are subject to error given the rather small amounts at the omasum and in milk fat.

18:0 and 18:1 Isomers. Forage species had no effect on milk fat 18:0 concentration (IV) consistent with earlier findings (Table 11) and the rather small differences in 18:0 flow at the omasum across treatments (III). While RCS in the diet enhanced the concentration of several *cis*-18:1 (Δ11-16) and *trans*-18:1 (Δ6-10, 12-16) in milk fat (IV) attributed to metabolism of 18-carbon unsaturates (Chilliard et al., 2007; Shingfield et al., 2010), the increases remained numerically small that is in agreement with earlier observations (Vanhatalo et al., 2007). The proportion of *cis*-9 18:1 in milk fat was similar across treatments in the present study (IV), while others have reported increases in response to RCS (Vanhatalo et al., 2007 and Moorby et al., 2009 in Table 11).

Saturated Fatty Acids. Red clover silage in the diet decreased linearly the proportions of several short and medium chain SFA in milk fat (4:0-8:0, 14:0, 16:0; IV). Several studies have reported similar findings (Dewhurst et al., 2003b, experiment 2; Vanhatalo et al., 2007; Moorby et

al., 2009), whereas others reported no differences in the levels of 14:0 and 16:0 in milk fat between these forage species (Dewhurst et al., 2003b experiment 1; Al-Mabruk et al., 2004; Steinshamn, 2010; see Table 11). Since the daily yields of SFA up to chain length of 16-carbon were unaffected by RCS in the diet in the present work (IV), it is probable that the slightly decreased milk fat concentration of short and medium chain SFA on RCS diets relative to GS was primarily due to dilution by increases in the secretion of long chain fatty acids rather than a decrease in the mammary synthesis of SFA *de novo*.

Odd- and Branched-Chain Fatty Acids. Forage species markedly influenced milk fat OBCFA composition (IV). Increasing level of RCS in the diet enhanced linearly milk fat 15:0 and 17:0, whereas that of *iso* 15:0 and *iso* 17:0 were decreased (IV) consistent with earlier findings (Dewhurst et al., 2003b; Tuori et al., 2004; Vanhatalo et al., 2007; Vanhatalo et al., 2008; Moorby et al., 2009) and equivalent changes in postruminal flows (Lee et al., 2008; III). While a significant part of OBCFA secreted in milk may be synthesized postruminally (Dewhurst et al., 2007; Table 8), the similar changes in milk OBCFA profile on diets containing RCS across numerous studies tends to point towards shifts in the abundance of predominant rumen microbes, when RCS replaces GS in the diet.

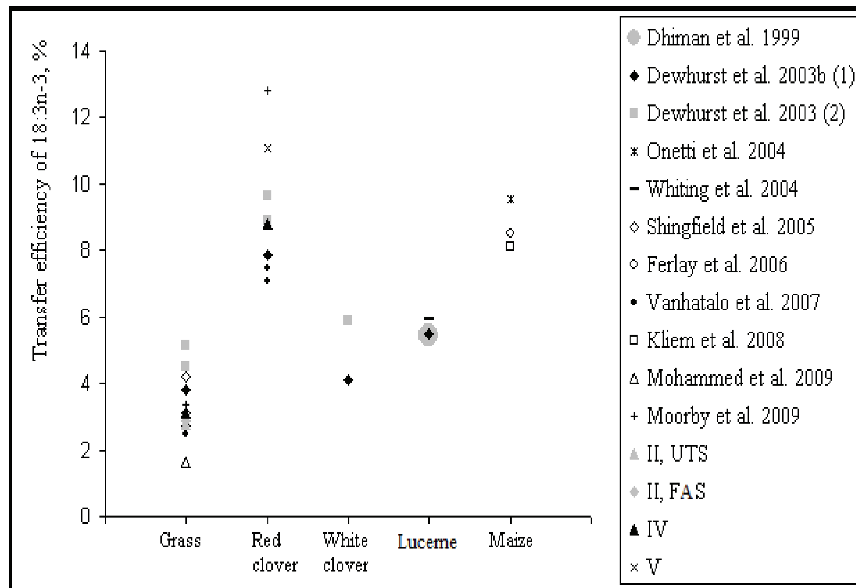

Figure 7 Effect of forage species offered in ensiled form on the efficiency of 18:3n-3 transfer from the diet into milk on diets not containing additional lipid

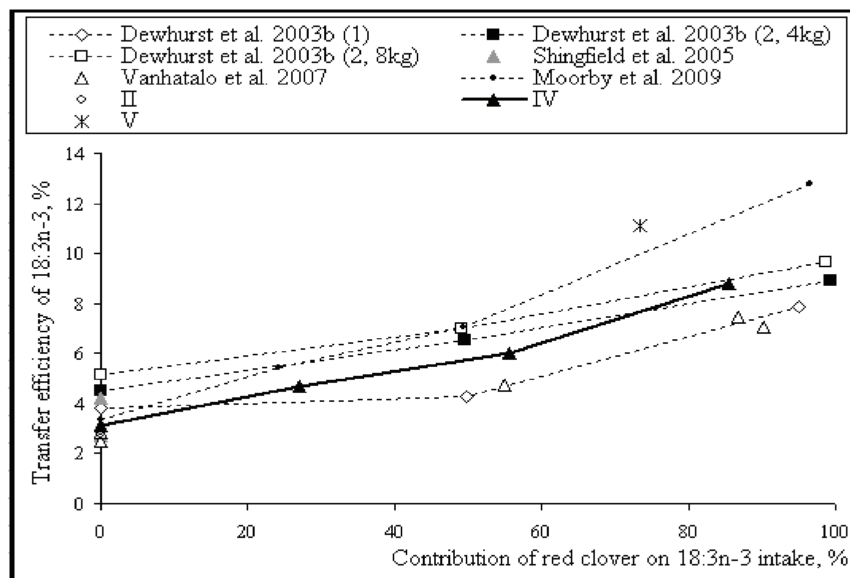

Figure 8 Effect of incremental replacement of grass silage by red clover silage on the efficiency of 18:3n-3 transfer from the diet into milk

Table 11 Effect of forage species on milk fatty acid composition of cows offered diets containing no additional lipid

Forage	DM intake, kg/d	Forage to concentrate ratio	Fat yield g/d	g/100g milk fatty acids									Reference
				12:0	14:0	16:0	18:0	*c*9 18:1	*t*11 18:1	*c*9*t*11 CLA	18:2n-6	18:3n-3	
MS, 68% DM intake		68:32		8.2[1]	12	33	8.1	20[2]			2.2	0.3	Chilliard et al. ,2001
GS	18	63:37	1100	4.3	12	32	11	25[3]			1.4	0.4	Dewhurst et al., 2003b
GS:RCS, 1:1	20	65:35	1300	4.3	12	30	10	24[3]			1.7	0.5	(experiment 1)
RCS	20	66:34	1290	4.3	12	31	10	24[3]			1.8	0.8	
GS:WCS, 1:1	20	66:34	1300	4.5	12	31	10	23[3]			1.5	0.5	
WCS	20	65:35	1400	4.7	13	30	10	23[3]			1.8	0.9	
LS	20	67:33	1200	4.3	12	29	10	23[3]			1.6	0.5	
GS	17	80:20	870	3.3	12	34	11	20[3]	0.5	0.4	0.9	0.5	Dewhurst et al., 2003b
GS	19	65:45	1100	3.5	12	33	11	21[3]	0.4	0.4	1.1	0.4	(experiment 2)
GS:RCS, 1:1	18	81:19	890	3.0	11	34	10	20[3]	0.8	0.5	1.1	0.8	
GS:RCS, 1:1	20	67:33	1090	3.7	12	33	11	19[3]	0.6	0.4	1.2	0.6	
RCS	20	83:17	990	2.9	11	32	12	20[3]	1.5	0.4	1.5	1.5	
RCS	22	69:31	1120	3.3	11	31	12	20[3]	1.3	0.4	1.6	1.3	
WCS	23	70:30	1170	4.2	13	33	10	18[3]	1.0	0.3	1.5	1.0	
GS	16	58:42	840	3.1	11	31	13	23[4]	1.3	0.5	1.2	0.5	Al-Mabruk et al., 2004
RCS	13	65:35	920	3.3	11	31	12	24[4]	1.2	0.4	1.5	0.9	
RCS	20	54:46	1270	3.4	12	31	11	19	1.2	0.5	2.0	1.0	Tuori et al., 2004
FGS	20	55:45	1290	3.6	12	32	10	19	1.1	0.5	1.8	0.7	
GS	19	53:47	1280	3.2	12	31	11	19	1.3	0.6	1.7	0.6	
GS:RCS, 1:1	20	55:45	1300	3.3	12	31	11	19	1.4	0.6	1.9	0.8	
GS:FGS, 1:1	20	55:45	1300	3.3	12	31	11	20	1.3	0.6	1.7	0.7	
RCS (early, E), I cut	19	56:44	1240	3.3	11	27	12	22	1.2	0.6	2.4	0.9	Pursiainen et al., 2006,
RCS (late, L), I cut	17	52:48	1270	3.3	11	28	11	22	1.1	0.6	2.2	0.7	Vanhatalo et al., 2008
RCS (E), II cut	21	60:40	1210	3.7	12	28	11	20	1.1	0.5	2.4	0.9	
RCS (L), II cut	20	58:42	1230	3.5	11	28	12	21	1.1	0.6	2.3	0.9	
GS (E), I cut	23	62:38	1250	4.0	12	30	11	19	1.0	0.5	1.9	0.4	
GS (L), I cut	17	49:51	1120	3.4	11	28	12	22	1.0	0.5	2.1	0.5	
GS (E)	21	62:38	1090	3.6	12	29	10	16	1.0	0.4	1.2	0.4	Vanhatalo et al., 2007 ;
GS (L)	20	60:40	1030	3.5	12	28	11	17	1.0	0.4	1.3	0.4	2009
GS (L):RCS (E), 1:1	22	65:35	1060	3.4	11	28	11	17	1.0	0.4	1.5	0.9	
RCS (E)	19	60:40	1000	3.0	10	26	11	19	0.9	0.4	1.8	1.3	
RCS (L)	20	60:40	1050	3.1	10	27	10	18	1.0	0.4	1.7	0.9	

Table continues

Table continues

Forage	DM intake, kg/d	Forage to concentrate ratio	Fat yield g/d	g/100g milk fatty acids									Reference
				12:0	14:0	16:0	18:0	*c*9 18:1	*t*11 18:1	*c*9*t*11 CLA	18:2n-6	18:3n-3	
GS	17	79:21	960	3.7	12	39	8.9	18	2.2	0.5	1.0	0.6	Moorby et al., 2009
GS:RCS, 2:1	18	80:20	950	3.5	12	38	9.0	18	2.4	0.5	1.1	0.8	
GS:RCS, 1:2	18	81:19	940	3.4	12	37	9.3	19	2.5	0.4	1.3	1.0	
RCS	19	82:18	920	3.1	11	37	8.7	20	2.3	0.4	1.6	1.5	
GS	17	67:33	800	4.8	15	36	8.5	16	1.1[5]	0.3	1.5	0.7	Adler et al., 2013
GS	17	69:31	770	4.6	15	38	8.6	15	1.1[5]	0.2	1.4	0.5	
GS:RCS 5:4	18	70:30	790	4.8	15	35	7.9	16	2.0[5]	0.5	1.9	0.9	
GS (untreated)	20	61:39	1200	3.7	13	37	9.1	12	0.8	0.3	1.1	0.4	II
GS (formic acid)	22	64:36	1320	3.6	12	35	9.8	12	0.9	0.3	1.1	0.4	
GS	20	60:40	1000	3.6	13	34	8.9	15	1.1	0.5	1.2	0.5	IV
GS:RCS, 2:1	20	60:40	1050	2.6	13	34	8.3	15	1.1	0.6	1.4	0.7	
GS:RCS, 1:2	20	60:40	990	3.6	12	33	7.9	15	1.1	0.6	1.6	0.9	
RCS	18	60:40	1030	3.6	12	33	8.2	15	1.2	0.6	1.8	1.2	
RCS	23	55:45	1230	3.9	13	32	7.6	13	1.0	0.4	2.1	1.1	V

FG = fodder galega (*Galega orientalis* Lam.), G = grass, L = lucerne, M = maize, RC = red clover, WC = white clover
H=hay, S=silage
[1] contain also 10:0, [2] 18:1 isomers not specified, [3] contain also *cis*-11 18:1, [4] contain all isomers of 18:1 except *trans*-11 18:1, [5] contain all isomers of *trans* 18:1

4.5.3 Lipid supplements for forage-rich diets based on red clover silage

The intake of RCS and total DM was not altered by moderate amounts of plant lipids in concentrate supplements that comprised approximately 46% of total diet DMI in the present work (V). These findings are in accordance with responses predicted by models of intake developed using published data (Huhtanen et al., 2007; 2008). At higher inclusion rates, plant oils or oilseeds may induce negative effects on DMI (Huhtanen et al., 2008; Roy et al., 2006). In this experiment, inclusion of camelina as an expeller rather than oil in concentrate supplements tended to decrease silage DMI, consistent with previous findings (Hurtaud and Peyraud, 2007), a response that can not be explained solely on the basis of dietary fatty acid intake.

The effects of lipid supplements on milk yield and milk fat are variable and known to be dependent on inclusion rate, degree of unsaturation, physical form and basal diet composition (Lock and Shingfield, 2004; Roy et al., 2006; Shingfield et al., 2010). In the current work, inclusion of plant lipids in concentrate supplements had no effect on milk yield or milk fat secretion in cows fed RCS based diets, possibly due to the intake of DM and ME being rather similar across treatments.

For all treatments, milk fat 18:3n-3 content was relatively high (V) due to the use of RCS as the basal forage (Dewhurst et al., 2006; Table 11). Supplementing red clover based diets with CO increased milk fat 18:3n-3 concentrations on average by 0.18%-units compared with SFO to a final concentration of 1.17 (g/100g fatty acids) a response that was marginal relative to the control containing RCS alone (Table 11).

Plant oils and CEX in the diet decreased the relative proportions of 6- to 14-carbon fatty acids and 16:0 in milk fat on average by 2.8 and 5.4%-units, respectively. Earlier studies have shown that plant oils and oilseeds in the diet typically lower the concentration of short and medium chain SFA in bovine milk (Dewhurst et al., 2006; Givens and Shingfield, 2006; Glasser et al., 2008a) responses that can be explained by the inhibitory effects of long chain fatty acids on ACC activity and the synthesis of SFA *de novo* in mammary secretory cells (Shingfield et al., 2010, II). Comparisons between lipid treatments indicated that the composition or form of lipid in the diet had no substantive effects on the secretion of fatty acids synthesized *de novo*, but altered the relative abundance of long chain fatty acids in milk fat, including *trans*-9,*cis*-11 CLA and *trans*-10,*cis*-12 CLA with potential to inhibit mammary lipogenesis (Shingfield and Griinari, 2007).

Plant lipids in the diet enriched the content of unsaturated fatty acids inherent to lipid supplements in milk fat. Rapeseed oil in the diet increased milk fat *cis*-9 18:1 (+4.1%-unit compared with control diet), *trans*-18:1 (Δ4, 6-9) and *trans*-7,*cis*-9 CLA content in agreement with previous reports (Shingfield et al., 2008b; Givens et al., 2009; Rego et al., 2009; V). Supplement of SFO specifically enriched 18:2n-6, *trans*-8,*trans*-10 CLA and Δ10,12 CLA in milk (Chilliard et al., 2007; Shingfield et al., 2008a;

Rego et al., 2009; V). The concentrations of almost all the other 18:2 fatty acids, Δ11,15 18:2, Δ12,15 18:2, Δ11,13 CLA, Δ12,14 CLA and Δ13,15 CLA in particular, were higher in milk from cows fed CO relative to SFO (V) that is in agreement with the changes in milk fatty acid profile in cows fed linseed oil supplements as a source of 18:3n-3 in the diet (Collomb et al., 2004; Shingfield et al., 2008b; Rego et al., 2009).

The form of camelina in the diet had a major impact on the concentration of 18-carbon fatty acids in milk fat, but the concentrations of the short and medium chain SFA (sum of 4- to 14-carbon SFA, 16:0) were unaltered. Milk 18:3n-3 content was lower on CEX than CO, presumably as a result of a lower 18:3n-3 intake since the efficiency of transfer of 18:3n-3 from the diet into milk was comparable between CEX and CO (approximately 7.9%). Concentrations of *cis*-12 18:1, *cis*-15 18:1, *trans* 18:1 (Δ4, 6-15), *cis*-9,*trans*-11 CLA and Δ11,15 18:2 were higher for CEX compared with CO, but those of 18:0 and *cis*-9 18:1 were lower (-2.5 and -3.0 %-units, respectively) and similar to the control diet, suggesting that complete biohydrogenation of lipid was lower for the CEX than CO. It is possible that the presence of a physically disrupted seed coat offers some protection for unsaturated fatty acids against metabolism in the rumen (Doreau et al., 2009) or that other components in camelina oilseeds exert inhibitory effects on ruminal biohydrogenation. Previous investigations have reported similar changes in milk fat 18-carbon fatty acids during comparisons of CEX with rapeseed or linseed expeller in the diet of lactating cow (Mihhejev et al., 2007) or replacement of rapeseed meal with CEX in the diet of sheep (Szumacher-Strabel et al., 2011). Further studies are required to substantiate these considerations and identify potential compounds in camelina oilseeds with potential bioactivity in the rumen.

5 CONCLUSIONS

Fatty Acids in Feed and in Postruminal Digesta

1. Irrespective of species and conservation method, 18:3n-3 was the major fatty acid in forages (34-55% of fatty acids), followed by 18:2n-6 (15-22%) and 16:0 (15-21%). Drying of grass resulted in substantial decreases in fatty acid content, 18:2n-6 and 18:3n-3 in particular, whereas losses of fatty acids were neglible due to ensiling. Drying and ensiling were both associated with extensive lipolysis during storage (lasting more than 19 weeks).
2. The majority of 18:2n-6 and 18:3n-3 at the omasum originated from ruminal escape of esterified (polar and neutral) lipid, whereas 18-carbon biohydrogenation intermediates and 18:0 were almost exclusively in the form of NEFA.

Effect of Forage Conservation Method

1. Compared with fresh chopped grass or GS, feeding diets based on grass hay lowered the extent of lipolysis and biohydrogenation of dietary unsaturated fatty acids in the rumen. Effects of grass drying on ruminal lipid metabolism explained the higher efficiency of 18:3n-3 transfer from the diet into milk. Differences in the extent of fermentation *in silo* had minimal effects on ruminal lipid metabolism.
2. Changes in the ruminal lipid metabolism due to grass conservation method were not associated with changes in rumen protozoal numbers or specific populations of ruminal bacteria known to be capable of biohydrogenation.
3. Zero-grazing increased circulating NEFA and TAG concentrations relative to diets based on conserved grasses. On all diets, TAG was the major source of fatty acids taken up by the bovine mammary gland. During zero-grazing, circulating NEFA also served as a source of fatty acids for milk fat synthesis, with evidence to suggest a substantial contribution from the mobilization of adipose.
4. In zero-grazed cows, a higher uptake of preformed medium and long fatty acids across the mammary glands was associated with substantial decrease in the synthesis of 16:0 *de novo*. *De novo* synthesis accounted for 53% of 16:0 secreted in milk in zero-grazed cows, a contribution that was much higher in cows fed hay and silage (ca. 79%).
5. Compared with feeding hay, zero-grazing resulted in substantial decreases in milk fat 10:0-16:0 (-9.6 %-unit for 16:0) and higher *cis*-9 18:1 (+8.6 %-unit), *trans*-11 18:1, 18:2n-6, *cis*-9,*trans*-11 CLA and 18:3n-3 (+0.2 %-unit) concentrations. Several factors contributed to these differences including, changes in the composition and concentration of circulating lipids, differences in mammary extraction of NEFA and TAG from arterial blood and synthesis of fatty acids *de novo*, with no evidence of altered desaturation of fatty acids in the mammary glands.
6. Conservation of grass by ensiling rather than drying or restricting fermentation *in silo* using formic acid based additive, increased mammary *de novo* synthesis of SFA (4- to 8-carbon, 14:0), and as a result milk fat secretion. Increases in mammary SFA synthesis were

attributable to shifts towards butyrate production in the rumen. Nevertheless, differences in milk fatty acid composition across diets based on hay, UTS or FAS were rather small.

Effect of Forage Species

1. Forage species had a major impact on ruminal lipid metabolism. Replacing GS with RCS in the diet decreased linearly the lipolysis and ruminal biohydrogenation of dietary unsaturated fatty acids. The proportion of RCS in the diet had no effect on the relative distribution of fatty acids present in different lipid fractions in the rumen. However, replacing GS with RCS increased linearly the flow of 18:3n-3 in all lipid fractions, and those of *cis*-9 18:1 and 18:2n-6 as NEFA at the omasum.
2. Forage species had no influence on the extent of lipolysis *in silo*. Furthermore, the bioavailability of PUFA for absorption was not impaired, in contrast to that of N, when RCS replaced GS in the diet. These findings offer no support to PPO activity and/or protective protein networks as a major factor contributing to the protection of lipid in RCS from metabolism in the rumen, suggesting that differences in rumen digestion kinetics and rumen microbial communities may be more important.
3. Milk fat 18:2n-6 and 18:3n-3 contents (from 1.2 to 1.8 and from 0.5 to 1.2 g/100g fatty acids, respectively) and yields increased linearly in response to higher proportions of RCS in the diet, while mammary synthesis of short and medium chain SFA *de novo* remained constant, as inferred from the similar secretion of these fatty acids in milk.

Effect of Moderate Lipid Supplementation on Forage-rich Diet

Moderate amounts of plant lipids in diets based on RCS had no adverse effects on silage DMI or milk fat secretion, but modified milk fat profile in terms of lower 12:0, 14:0 and 16:0 content (-0.7, -1.3 and -5.5 %-unit, respectively) and increases in the concentration of unsaturated fatty acids inherent to lipid supplements. However, the potential of moderate amounts of camelina lipids to enhance milk fat 18:3n-3 content was limited. The increase in milk *trans* fatty acids due to lipid supplementation was relatively minor, except when CEX was fed.

Implications

1. Results demonstrated the beneficial effects of fresh grass and RCS on milk fat profile. In particular, fresh grass has potential to decrease milk fat medium chain SFA and RCS increase 18:3n-3 concentrations. Moderate inclusion of plant lipid in forage-rich diet may be used to further alter milk fat composition, principally through additional decreases in medium chain SFA.
2. Decreases in milk fat SFA content in cows fed fresh grass compared with hay or silage were similar to that when diets based on RCS were supplemented with moderate amounts of plant oils or CEX. However, the beneficial effects of fresh grass on milk fat *cis*-9 18:1 and RCS on 18:3n-3 concentrations were higher than could be achived by moderate amounts of RO or camelina lipid supplementation, respectively.

6 FUTURE RESEARCH

Present research demonstrated that a significant part of medium- and long chain fatty acids used for the synthesis of milk fat originated from body tissues, presumably adipose, in cows fed fresh grass. The experimental periods used were rather short (14 d) due to the need to minimize differences in herbage maturity between experimental treatments. Therefore, there is a necessity to investigate longer-term effects of feeding fresh grass compared with conserved forages on the coordination of lipid metabolism in the mammary glands and adipose. Present findings raises the question besides lipolysis can grazing promote fatty acid transformations and synthesis *de novo* in tissue adipose to support milk fat secretion in the long-term? Biopsies of adipose tissue and subsequent gene expression (quantitative PCR of candidate genes, microarrays) and protein expression analysis could be used as tools to compare the lipid metabolism in adipose tissue of cows fed fresh or conserved forages in more detail. Besides NEFA, measurements of arterial concentrations of hormones influencing whole body energy metabolism such as insulin and leptin could also deepen the understanding of possible mechanisms underlying the ‘fresh grass effect’.

It was speculated that ruminal lipolysis and biohydrogenation could be higher on diets based on fresh herbage, compared with conserved forages, due to the action of endogenous plant lipases. To study this in more depth, lipase activities (galacto- and phospholipases in particular) in the rumen content of cows fed fresh grass or conserved forages could be determined. In addition, plant meditated lipolysis could be investigated *in vitro* by incubating crushed forage with physiological buffer solution (lipolysis by plant lipases) or heat-treated filtrate of rumen liquid (plant lipases), filtrate of rumen liquid (plant and microbial lipases) and by measuring lipase activity and lipid fractions at several timepoints over a 24h period.

The findings of this thesis offered no support to PPO activity and/or protective protein networks as a major contributor to dietary lipid protection in cows fed RCS relatively to GS. However, due to the lack of analysis of bound phenols in silages and possible biases in the quantitative determination of bound phenols in omasal digesta definitive conclusions on the role of PPO and bound phenols in ruminal protection of lipids and proteins in RCS cannot be made. Comparing ruminal lipid and N metabolism in cows fed RCS prepared from genetically high phenol content and PPO activity to that with low phenol content and PPO activity, following either direct cut or extensive wilting to allow maximal oxidation of phenols, using grass or other forage species as a reference would be needed to obtain a more definite answer. Analysing phenol content quantitatively (free and bound) and PPO activity (active and latent) from intact parent herbages and wilted herbage, as well as phenols (free and

bound) of resultant silages and postruminal digesta would be crucial. These analysis combined with the determination of digestion kinetics and fatty acid composition of forage particles of different sizes, as well as rumen microbial ecology, would also be needed to provide a deeper insight into the contribution of different mechanisms proposed to explain the ‘red clover effect’.

Even though the diet of dairy cows naturally contain significant amounts of 18:2n-6 and 18:3n-3, postruminal flow of these essential fatty acids is limited due to extensive biohydrogenation of unsaturated fatty acids in the rumen. Besides possible health effects in humans when incorporated in milk or meat, increased absorption of essential fatty acids may promote also animal health and fertility. However, little is known about the nutritional adequacy of essential fatty acids in high-yielding dairy cows and this deserves more research effort. In addition, development of commercially applicable, efficient and safe methods to protect essential fatty acids from ruminal biohydrogenation without impairing their bioavailability for absorption could be considered a research priority.

It seems plausible that camelina oilseed contain compound(s) with potential to effectively inhibit the last step of biohydrogenation of dietary unsaturated fatty acids in the rumen and further studies are required to identify these substances. Camelina oilseed is reported to contain antinutritive compounds including glucosinolates with 9-methyl-sulfinyl-nonyl-glucosinolate, 10-methyl-sulfinyl-decyl-glucosinolate and 11-methyl-sulfinyl-undecyl-glucosinolate as main compounds, sinapine and condensed tannins (Russo et al., 2012). *In vitro* studies of PUFA biohydrogenation (see e.g. Honkanen et al., 2012 for details) in the presence of these and other potential extracts from camelina oilseed could be used to investigate their possible bioactivity in the rumen. However, *in vivo* studies combining ruminal lipid metabolism and microbial ecology (abundance of biohydrogenating bacteria responsible for the final reduction of 18:1 to 18:0 in particular) are required to validate the efficacy of promising compound(s) and to explore the possible mode of action.

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