University of Alberta

Analytical Considerations and Biology of Milk Conjugated Linoleic Acid Synthesis in the Bovine

by

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Abstract

Biosynthesis of milk conjugated linoleic acid (CLA), a component of milk fat with demonstrated health benefits, requires a dietary source of PUFA. Even with PUFA supplementation, milk CLA is highly variable. Therefore, this study was aimed at identifying factors responsible for the variations in rumen CLA precursors and milk CLA.

Study 1 evaluated the efficiency of CLA production by grazing cows compared to those fed grass silage or fresh grass. Grazing cows were more efficient than those fed grass silage or fresh grass in milk CLA production. About 75% of the variability in milk CLA was related to the differences in PUFA (l8:2n-6 + 18:3n-3) intake and the remainder was related to factors regulating the extent of PUFA biohydrogenation in the rumen. This study demonstrated that PUFA intake is important but it is not the only factor responsible for the observed variation in milk CLA production.

Study 2 evaluated the effect of diets differing in rate of starch degradation on rumen PUFA biohydrogenation and milk CLA. Concentrations of ruminal *t*11-18:1 and milk CLA were greater for barley-based diets than corn-based diets and were not different between rolling and grinding, indicating that factors inherent in the source of starch were responsible for the observed differences and these factors could not be modified by rolling or grinding the grain.

Study 3 examined the effect of stage of lactation on persistency of milk *t*10-18:1, *t*11-18:1 and CLA for control and test (supplemented with PUFA and monensin) diets from calving to 270 days in milk. Milk concentrations of *t*11-18:1 and RA remained similar across the lactation length and were greater for the test diet compared to the control. Changes in milk *t*10-18:1 concentration during lactation appeared to reflect an effect of the degree of rumen fermentation on PUFA biohydrogenating bacteria.

Although PUFA intake is important for milk CLA production, only those diets that give rise to increased ruminal *t*11-18:1 result in greater milk CLA. Concentrations of rumen *t*11-18:1 is influenced by the amount of PUFA consumed, degree of shift to *t*10-18:1 and the extent of PUFA biohydrogenation in the rumen.

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List of Abbreviations

Only those abbreviations that are used throughout this thesis are listed below. Author-derived abbreviations are shown in bold type at first use in every chapter.

ACC = acetyl-CoA carboxylase

ADF = acid detergent fiber

BHBA = beta hydroxy butyric acid

CLA = conjugated linoleic acid

CP = crude protein

CV = coefficient of variation

DAG = diacylglycerol

DGAT = diacylglycerol acyltransferase

DHA = docosahexaenoic acid (22:6n-3)

DIM = days in milk

DM = dry matter

DMI = dry matter intake

DPA = docosapenatenoic acid (22:5n-3)

EE = ether extract

EPA = eicosapentaenoic acid (20:5n-3)

FAME = fatty acid methyl esters

FATP = fatty acid transport protein

FAS = fatty acid synthetase

F: C = forage:concentrate

FCM = fat corrected milk

GC/GLC = gas chromatography

GPAT = glycerol phosphate acyltransferase

HPLC = high-performance liquid chromatography

LA = linoleic acid

LDL = low density lipoproteins

LN = α-linolenic acid

LPL = lipoprotein lipase

MAG = monoacyl glycerol

MFD = milk fat depression

MUFA = monounsaturated fatty acids

MUN = milk urea nitrogen

NEFA = non-esterified fatty acid

NDF = neutral detergent fiber

PUFA = polyunsaturated fatty acids

RA = rumenic acid (*c*9*t*11-CLA)

SCD = stearoyl-CoA desaturase

SEM = standard error of the mean

SNP = single nucleotide polymorphism

TAG = triacylglycerol

TLC = thin layer chromatography

TMR = total mixed ration

VA = vaccenic acid (*t*11-18:1)

VFA = volatile fatty acids

VLDL = very low density lipoproteins

Introduction

1.1 General introduction

Bovine milk is an important component of human diet. The nutritive value of cow's milk as a health promoting drink is evident from several health beneficial effects: anti-hypertensive activity (Lopez-Fandino et al., 2006); anti-viral activity (Pan et al., 2006); antibacterial activity (Exposito and Recio, 2006); anti-oxidative property (Pihlanto, 2006) of milk peptides; bone health promoting effect of milk minerals (Cashman, 2006); and anti-cancer effects of conjugated linoleic acid (*c*9*t*11-CLA or simply **CLA**), a component of milk fat (Pariza and Hargreaves, 1985). However milk fat has often been criticized for having a relatively high proportion of saturated fatty acids and a low proportion of polyunsaturated fatty acids (**PUFA**) (Berner, 1993). The relatively high concentrations of 12:0 and 14:0 have been identified as major risk factors for coronary heart disease as these fatty acids were reported to increase low density lipoprotein (LDL) cholesterol in human blood.

Researchers have been interested in modifying the composition of milk fat for decades. Much of the earlier work focused on increasing the n-6 and n-3 PUFA in milk. This required prevention of PUFA biohydrogenation in the rumen so that more PUFA is incorporated into milk fat. Researchers utilized several ways to prevent PUFA biohydrogenation in the rumen such as formaldehyde protected lipids and calcium salts of fatty acids (Jenkins and Bridges, 2007). The discovery of the potential beneficial effects of CLA on health (anti-cancer, anti-obesity, anti-diabetic) has resulted in hundreds of studies targeted at increasing milk CLA concentration. Over the last decade, considerable effort has been devoted to evaluating feeding strategies that enhance milk concentrations of CLA. As much as a 10-fold increase in milk CLA was achieved by supplementing diets with a source of PUFA substrate (Bell et al., 2006; Shingfield et al., 2006). However, it was found that diets resulting in an increase in milk CLA are often

accompanied by a depression in milk fat yield (Piperova et al., 2000; Roy et al., 2006). Also, considerable variability in milk CLA has been reported (Peterson et al., 2002). More recent studies have focused on understanding the mechanisms for the possible variations in milk CLA and the fat depressing effects of diets formulated to enhance milk CLA. This involved looking into the effects of diet on rumen PUFA biohydrogenation by analyzing rumen lipids; flow of fatty acid post-rumen by analyzing omasal or duodenal lipid; flow of fatty acids to the mammary tissue by analyzing plasma lipids and finally relating these to the end products in milk.

Rumen ingesta, plasma and milk lipids are complex matrices and present many challenges in terms of resolving and identifying several fatty acids present in these lipids. The degree of complexity of milk fatty acids can be visualized from the amount of overlapping between *cis* (***c***) and *trans* (***t***) isomers of 16:1, 18:1, 20:1 and CLA (Kramer et al., 2008). Because many of the *t*-18:1 and CLA isomers have been shown to have different biological effects, an extensive analysis of the isomeric composition is required for correct interpretation of the results. A good starting point to understand PUFA metabolism in dairy cows is the methods required to isolate, derivatize and analyze the fatty acid profile. Because milk fat has an extremely diverse range of fatty acids (Jensen, 2002), this review starts with a brief introduction to the structure of fatty acids followed by lipid extraction, derivatization and analytical methods followed by PUFA metabolism in rumen, mammary lipid synthesis and the various factors influencing milk CLA concentrations. The focus of this review is to gain knowledge of the methods that result in improved resolution of *trans* and CLA isomers in milk and to understand the various factors influencing milk CLA isomers.

Lipids

The term Lipid has traditionally been used to describe a variety of natural products including fatty acids and their derivatives, steroids, terpenes, carotenoids, and bile acids, which are readily soluble in organic solvents such as diethyl ether, hexane, benzene or chloroform. The principal lipid classes consist of fatty acid moieties (carboxyl group – COOH) linked by an ester bond to an alcohol (mainly glycerol). Lipids can be divided into two broad classes – 'simple' and 'complex' based on the number of hydrolysis products produced per mole. Simple lipids (example, triacylglycerols and diacylglycerols) on hydrolysis yields only fatty acid and alcohol components (glycerol). Unesterified or free fatty acids, which occur in small amounts in most animal and plant tissues, and free sterols (example Cholesterol) are also classified as simple lipids. Complex lipids on hydrolysis yield glycerol, fatty acids, and inorganic phosphate or organic base or polyhydroxy compounds. Examples of complex lipids include: glycerophospholipids, glycolipids and sphingolipids.

Structure of fatty acids

The common fatty acids of plant and animal origin contain even numbers of carbon atoms in straight chains and may be fully saturated or contain one, two, or more double bonds, which generally have a *cis*-configuration. Saturated fatty acids do not have double bonds [example myristic acid (14:0), palmitic acid (16:0)]. Fatty acids with a single double bond are described as monoenoic fatty acids or monounsaturated fatty acids (**MUFA**) and the double bond could be present in *cis* (eg. oleic acid or *c*9-18:1) or *trans*-configuration (eg. elaidic acid *t*9-18:1). Fatty acids with two or more double bonds are described as PUFA. Long chain n3-PUFA such as eicosapentaenoic acid (**EPA**), docosapentaenoic acid (**DPA**) and docosahexaenoic acid (**DHA**) are formed from 18:3n-3 (α-linolenic acid or **LN**) through a series of reactions involving desaturation and chain elongation

while the long chain n6-PUFA such as arachidonic acid (20:4n-6) and 22:5n-6 are formed from 18:2n-6 (linoleic acid or **LA**) (Figure 1-1).

Polyunsaturated fatty acids with two or more cis-double bonds separated by a single methylene group (methylene-interrupted unsaturation) are described as non-conjugated PUFA while the PUFA with double bonds not separated by methylene group (methylene-uninterrupted unsaturation) are described as conjugated PUFA. Some examples of conjugated and non-conjugated PUFA are listed below (Table 1-1). To gain a better understanding of the lipids, structures of some of the fatty acids are shown from Fig. 1-2 to 1-5.

Table 1-1. List of non-conjugated and conjugated PUFA

Non-conjugated PUFA	Conjugated PUFA
*c*9*c*12-octadecadienoic (18:2n-6)	*c*9*t*11-CLA
*c*6*c*9*c*12-octadecatrienoic (18:3n-6)	*t*7*c*9-CLA
*c*9*c*12 *c*15-octadecatrienoic (18:3n-3)	*t*8*c*10-CLA
*c*8 *c*11*c*14-eicosatrienoic (20:3n-6)	*t*10*c*12-CLA
*c*5*c*8*c*11*c*14-eicosatetraenoic (20:4n-6)	*t*11*c*13-CLA
*c*4*c*7*c*10*c*13-*c*16-docosapentaenoic (22:5n-6)	*t*9*t*11- CLA
*c*5*c*8*c*11*c*14*c*17-eicosapentaenoic (20:5n-3)	*t*11*t*13- CLA
*c*4*c*7*c*10*c*13-*c*16*c*19-docosahexaenoic (22:6n-3)	*t*12*t*14-CLA

Note: CLA = conjugated linoleic acid; PUFA = polyunsaturated fatty acids
c = *cis*; *t* = *trans*

Branched chain fatty acids are common constituents of bacterial lipids, but can enter the food chain and appear in animal tissues. Those found frequently have a single methyl group on the penultimate (*iso*) or antepenultimate (*anteiso*) carbon atoms (Fig. 1-6) Phytanic acid (3,7,11,15-tetramethyl-hexadecanoic acid) a metabolite of phytol, is present in trace amounts in animal adipose tissue lipids (Lough, 1977).

1.2 Lipid analysis

Lipids include a wide range of compounds such as phospholipids, glycolipids, sphingolipids, sulfolipids, glycerides, cholesterol esters, and wax

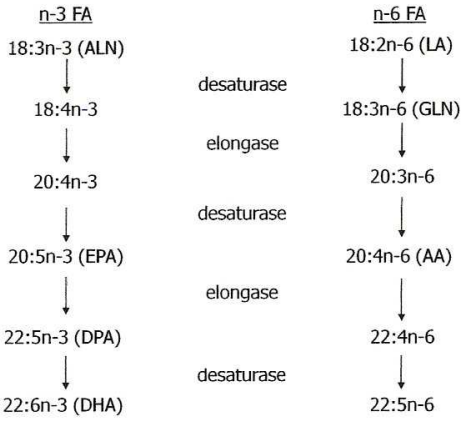

Figure 1-1. Synthesis of long-chain PUFA in mammals. AA – Arachidonic acid; ALN – Alpha linolenic acid; DHA – Docosahexaenoic acid; DPA – Docosapentaenoic acid; GLN – Gamma linolenic acid; EPA – Eicosapentaenoic acid; FA – fatty acid; PUFA – polyunsaturated fatty acid; Adapted from Bloeden and Szapary, 2004

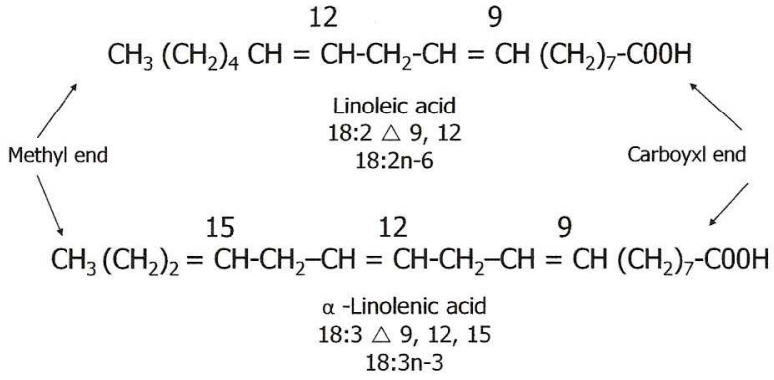

Figure 1-2. Structure of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3). The nomenclature using 'n' indicates the number of the first carbon bearing double bond by counting from the methyl end while the nomenclature using 'Δ' describes the positions of the double bonds by counting from the carboxyl end of the fatty acid.

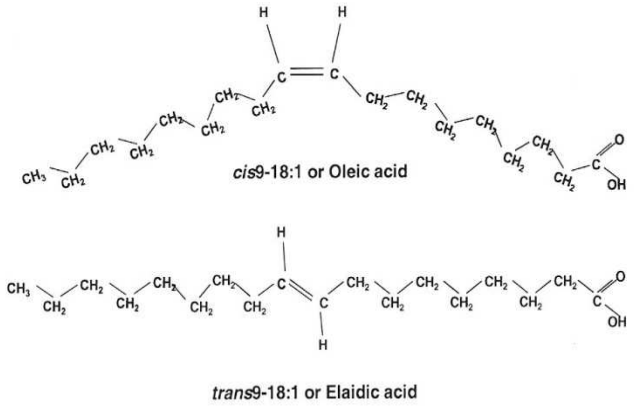

Figure 1-3. Geometric isomers of 18:1 Δ 9. Note the difference in the orientation of hydrogen bonds between cis and trans isomers of 18:1 Δ9.

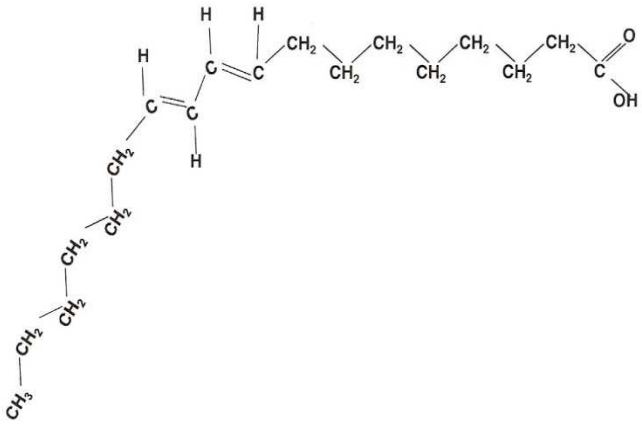

Figure 1-4. Structure of *cis*-9, *trans*-11 CLA. Note the difference in the orientation of hydrogen bonds at carbon number 9 and 11 counting from the carboxyl end (-COOH).

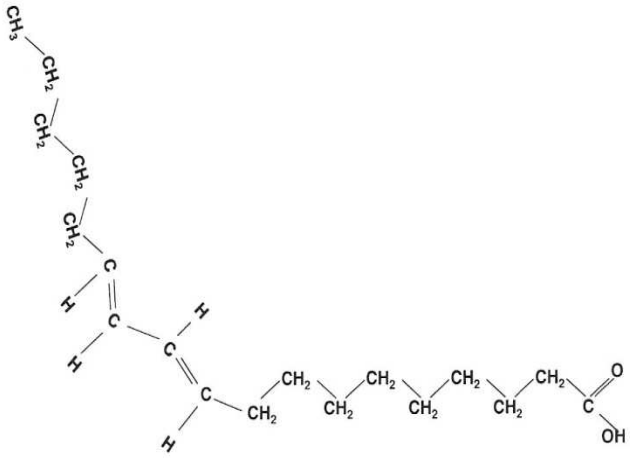

Figure 1-5. Structure of *trans*-10, *cis*-12 CLA. Note the difference in the orientation of hydrogen bonds at carbon number 10 and 12 counting from the carboxyl end (-COOH).

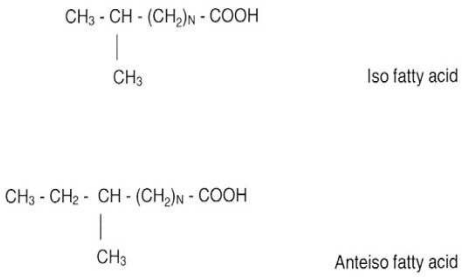

Figure 1-6. Structure of *iso* and *anteiso* fatty acids. Iso fatty acids have a methyl group at the penultimate carbon while the anteiso fatty acids have methyl group at the antepenultimate carbon.

esters that are linked to the long-chain alkyl chains by ester, ether, alk-1-enyl ether, or N-acyl linkages in the structures (Cruz-Hernandez et al., 2006).

Extraction of lipids

In nutritional experiments, it is often necessary to isolate and identify the fatty acid components and to determine the overall fatty acid composition of the total lipids. The first step in any lipid analysis is the isolation of lipids from tissues by extraction with organic solvents, and the removal of non-lipid contaminants from these extracts. For some of the matrices like plant lipids or bacterial lipids it may be necessary to disrupt the cells before extracting with a solvent. The ideal solvent or solvent mixture for extracting lipids from tissues should be sufficiently polar to remove all lipids from their association with cell membranes or with lipoproteins, but should not react chemically with those lipids. At the same time, it should not be so polar that triacylglycerols and other non-polar simple lipids (neutral lipids) do not dissolve. The extracting solvent must also inhibit lipase activity to minimize oxidation of lipids. Lipids which contain no markedly polar groups (eg. triacylglycerols or cholesterol esters) are highly soluble in less polar hydrocarbon solvents such as hexane, benzene or cyclohexane and also in slightly more polar solvents such as chloroform or diethyl ether; they are rather insoluble, however, in polar solvents such as methanol. It is important to recognize that the water in tissues or that used to wash the lipid extracts can alter markedly the properties of organic solvents.

The more common simple and complex lipids are in general readily extracted with chloroform-methanol mixtures but there are exceptions, for example, polyphosphoinositides or lysophospholipids. In spite of the few limitations, most workers in the area of lipid research generally accept that a mixture of chloroform and methanol in the ratio of 2:1 (v/v) will extract lipid more exhaustively from animal, plant or bacterial tissues than most other simple

solvent systems. The two established methods of lipid extraction are Folch et al. (1957) and Bligh and Dyer (1959) or modifications thereof. These methods use chloroform/methanol/water mixtures in different ratios and are useful for extraction of both neutral (simple) and polar (complex) lipids. The method by Hara and Radin (1978) using the less toxic solvents of hexane and isopropanol is less commonly used.

Removal of non-lipid contaminants

Most of the contaminating compounds can be removed from chloroform-methanol mixtures simply by shaking the combined solvents with one quarter their total volume of water, or even better with a quarter of the total volume of a dilute salt solution (0.88% KCl or NaCl). These solvents partition into two phases with the lower phase consisting of chloroform-methanol-water in the ratio 86:14:1 (v/v/v) and an upper phase in which the proportions are 3:48:47 (v/v/v) respectively. The lower phase contains the purified lipid and the upper phase contains the non-lipid contaminants together with any gangliosides which may have been present (Christie, 1982).

Derivatization

Methyl, propyl and butyl derivatives of different lipid classes have been described. However methoxy derivatives are preferred because of their greater volatility and superior resolution by gas chromatography (Ulberth et al., 1999). The methoxy products from *O*-acyl (esters, glycosides) and *N*-acyl side chains (sphingomyelins) are called fatty acid methyl esters (**FAME**) while alkyl-1enyl ethers (plasmalogenic lipids) yields dimethylacetals (**DMA**). Methoxy derivatives of the shorter-chain (4:0 to 10:0) FAME in milk requires appropriate correction factors (Wolff et al., 1994; Wolff et al., 1995b) to account for the lower flame ionization detection response in gas chromatography. To eliminate the use of correction factors, isopropyl and butyl esters have been used for the analysis of

short-chain fatty acids. However, this requires merging the results of the isopropyl (or butyl) esters with FAME.

The suitability of various catalysts for the methylation of specific lipid classes and CLA containing fatty acids have been described in detail by Cruz-Hernandez et al. (2004) and Cruz-Hernandez et al. (2006) (Table 1-2). Methyoxy

Table 1-2. The Suitability of Catalysts for the Methylation of Specific Lipid Classes, and for Treatment of CLA Containing Fatty Acids

Lipid class	Structures	Catalyst[a]				
		HCl	BF_3	TMG	$NaOCH_3$	DAM
Esters	RCH2-CO-OR1	Y	Y	Y	Y	No
Free Fatty Acids	RCH2-COOH	Y	Y	Y	No	Y
Amides	RCH2-CO-NHR1	Y, L	Y, L	No	No	No
Alk-1-enyl ethers	RCH2-O-CH=CHR'	Y	Y	?	No	No
Phospholipid Esters	R(PO4X)(OOCR1)	Y	Y	?	Y	No
Cholesterol Esters	RCOO-cholesteryl	Y,L	Y, L	?	No	No
Ethers	RCH2-O-CH2R1	No	No	No	No	No
Conjugated Bonds[b]	RCH=CH-CH=CHR1	Isom	Isom	Stable	Stable	Stable

[a]TMG, tetramethylguanidine; DAM, diazomethane (or TMS-DAM, trimethylsilyl diazomethane); L, longer reaction times and generally higher temperatures are required; No, catalyst not suitable; Y, suitable catalyst; ?, reaction unknown.

[b] Fatty acids containing conjugated double bonds may be present as an ester, amide or ether; see suitability of respective lipid type above. The conjugated double bond system in the alkyl chain of CLA may be isomerized (Isom) or is stable (Stable) using the methylation conditions. Reproduced with permission from Cruz-Hernandez et al. (2004)

derivatives are prepared by reaction of the lipid matrix with methanol in the presence of catalytic amounts of acid or base. Acid-catalyzed methylating reagents include 5% solution (w/v) of methanolic HCl, H_2SO_4 in dry methanol, acetyl chloride in dry methanol or BF_3/methanol. Of these, methanolic HCl is more commonly used and is applicable for the methylation of all common lipid structures, including free fatty acids, esters, glycosides, sphingomyelins and plasmalogenic lipids excepting ethers. Methylation with methanolic HCl is complete when the reaction is carried at 80^{o}C for 1h. The disadvantage with acid

methylation procedures is that they isomerize *c*/*t* CLA isomers to *t*-*t* CLA and produce methoxy artifacts (Kramer et al. 1997). Methylation with methanolic HCl under milder conditions (lower temperature; Park et al. 2001) has been reported to decrease CLA isomerization. However, acid methylation at lower temperature may not be complete (Kramer et al. 1997).

Methylation of the extracted lipid in the presence of an alkali is described as base-catalyzed methylation. A reaction of the lipid extract with sodium methoxide (0.5N methanolic base #33080, Supelco Inc., Bellefonte, PA) for 15 min at 50^{o}C is used for methylation of the matrices containing CLA because this does not isomerize *c*/*t* CLA isomers to *t*-*t* isomers unlike methanolic HCl. However base-catalyzed methylation does not methylate free-fatty acids, sphingomyelins and plasmalogenic lipids. Milk lipids are best methylated using base-catalyzed methylation because 98% of the milk lipids are neutral lipids. However other matrices containing CLA and having both neutral and polar lipids (for instance, blood, meat) require both acid and base methylation procedures. The former methylates all the lipid classes except ethers while the later prevents isomerization of CLA isomers. A detailed description of the two separate methylation procedures (base and acid) can be found in Cruz-Hernandez et al. (2006) and Kraft et al. (2008).

Chormatography methods used in lipid analysis

Thin layer chromatographic (TLC) separation of lipid classes: The separation of lipid classes depends on the objectives of the experiment. One-directional TLC procedures are preferred for rapid group separations of neutral lipids (Malins and Mangold, 1960; Freeman and West, 1966) while two-directional TLC provides good resolution of polar lipids. Thin layer chromatography of complex lipids was described in greater detail by Chrsitie (1982) and Kramer et al. (1983). A number of developing solvents are used in the separation of different lipid

classes using TLC. Table 1-3 provides details of the common developing solvent systems used in TLC (reproduced with permission from Cruz-Hernandez et al. (2006).

About 98% of the milk lipids are triacylglycerols. A TLC separation of milk lipids is often not required if the objective is to investigate the effect of diet on milk fatty acid composition. However, if the objective is to investigate the effect of diet on sphingolipids in milk, it does require a TLC separation. An assessment of lipid classes of a matrix by TLC may be helpful in making a decision as to what derivatization methods to use to get a complete fatty acid profile of the matrix.

Table 1-3. Common Developing Solvent Systems Used in the Separation of Different Lipid Classes Using Thin Layer Chromatography (TLC)

#	Plates	Solvent	Ratio	Separation of
1	G	Hex/E/HAc	85:15:1	CE, FAME, TAG, FFA, Ch
2	G	Hex/E/HAc	70:30:1	MAG, (DAG plus Ch), FFA, TAG
3	G	1,2-Dichloroethane	100	FAME from dimethylacetals
4	G. BA	Benzene	100	sn 2-MAG from sn 1- and sn 3-MAG; sn 1,3-DAG from sn 1,2- and sn 2,3-DAG
5	G, Ag+	Hex/E	90:10	FAME of saturates, and *cis* and *trans* Monoenes
6	G, Ag+	Hex/E	70:30	FAME of *cis* and *trans* di- and triunsaturates
7	H	C/M/H2O	65:25:4	TAG, DPG, PE, (PS plus PI), PC, SM, LPC
8	H	C/M/HAc/H2O	65:43:1:3	TAG, Ch, DPG, PE, (PS plus PI), PC, SM, LPC
9	H	1: C/A/M/HAc/H2O 2: C/A/M/HAc/H2O 3: Hex/E/HAc	65:25:4 50:20:10:15:5 85:15:1	PC, PE, PS, PI, DPG, PA, SM, LPC, CER, FFA Ch, TAG, FAME, CE

Abbreviations: A, acetone; Ag+, silver ion impregnated TLC plates; BA, boric acid impregnated TLC plates; C, chloroform; Ch, cholesterol; CE, cholesteryl esters; CER, cerebrosides; DAG, diacylglycerol; DPG, diphosphatidylglycerol (cardiolipin); E, diethyl ether; FAME, fatty acid methyl esters; FFA, free fatty acids; G, silica gel G plates; H, silica gel H plates; Hex, hexane; HAc, acetic acid; H2O, water; LPC, lyso-phosphatidylcholine; M, methanol; MAG, monoacylglycerol; NH_4OH, concentrated ammonium hydroxide; PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SP, sphingomyelin; TAG, triacylglycerol; TLC, thin layer chromatography. Reproduced with permission from Cruz-Hernandez et al. (2006)

Gas Chromatography: Gas-liquid chromatography is a form of partition chromatography in which the compounds to be separated are volatilized and

passed in a stream of inert gas (mobile phase) through a column in which a high boiling-point liquid (stationary phase) is coated onto a solid supporting material. Substances in the column are separated according to their partition coefficients, depending on their volatilities and on their relative solubilities in the liquid phase. They finally emerge from the column as peaks of concentration, exhibiting a Poisson distribution. These peaks are detected by flame ionization which converts the concentration of the component in the gas phase into an electrical signal. This signal is amplified and passed through a recorder to obtain a tracking of the peaks for each component as it is eluted. The areas under the peaks are directly related to the mass of the components present. The concept of the number of theoretical plates, originally derived from distillation columns is used to determine the efficiency of a column. If columns are too loosely packed, the separations are poor; if too tightly packed, they may block the injection needles.

High-performance liquid chromatography (HPLC): A HPLC instrument is equipped with a quaternary pump, an autosampler, a diode array detector and software to analyze the peaks. Use of Ag^{+}-HPLC column (ChromSpher 5 Lipids column; Varian Inc., made by Chrompack Inc., Middleburg, The Netherlands) for resolving CLA isomers was first reported by Sehat et al.(1998). Later, three such columns were used in series for improved separation of CLA isomers (Sehat et al., 1999). The mobile phase was hexane containing 0.1% acetonitrile and 0.5% diethyl ether (or tertiary butyl methyl ether) and the pump was operated isocratically at a flow rate of 1.0 ml/min. The mobile phase was continuously mixed using a magnetic stirring bar to maintain acetonitrile in solution. The diode array detector can be set at different wavelengths for identifying various isomers. Conjugated linoleic acid methyl esters are typically identified at 233nm, non-conjugated FAME at 205 nm and conjugated trienes at 268 nm. The elution order of the CLA isomers is *trans-trans* followed by *cis-trans* which is then followed by *cis-cis* isomers.

Standards

The most commonly used reference FAME mixture # 463 (Nu-Chek Prep, Elysian, MN), is very useful to identify fatty acids ranging from 4:0 to 24:0 including some *trans*-MUFA isomers (*t*916:1, *t*918:1, *t*11-18:1) and *cis*-MUFA (*c*9-12:1, *c*9-14:1, *c*9-16:1, *c*9-18:1, *c*11-18:1, *c*5-20:1, *c*8-20:1, *c*11-20:1, *c*13-22:1 and *c*15-24:1). However, it does not have conjugated 18:2 isomers, non-conjugated 18:2 isomers (except *t*9*t*12-18:2) and some long-chain saturated fatty acids (**SFA**). As such, the standard # 463 can be spiked with the CLA mixture, UC-59M (has *t*8*c*10-, *c*9*t*11-, *t*10*c*12-, *c*11*t*13-CLA and their corresponding *c*/*c* and *t*/*t* CLA isomers) and some of the SFA (21:0, 23:0, and 26:0) which are present in milk in lower concentrations. Alternatively, the CLA mixture and a mixture of other SFA not found in # 463 standard can be analyzed separately on GC. Several pure CLA isomers (c9t11-, t9c11-, c11t13-, t10c12 and t9t11-CLA) are available from Matreya Inc. (Pleasant Gap, PA). Specific *cis*- and *trans*-18:1 isomers are available from Sigma. Most of the *cis* and *trans* isomers of 16:1, 18:1 and 20:1 can be obtained from milk fat by Ag^+-TLC or by using Ag^+-SPE (solid phase extraction) cartridges available from Supelco Inc. (Bellafonte, PA). The Ag^+-TLC method was described in detail by Cruz-Hernandez et al.(2006) and the methods for obtaining the *cis* and *trans* MUFA from milk fat by Ag^+-SPE cartridges were described by Kramer et al.(2008). Once all the fatty acids in milk are identified using the various standards described above, milk itself can serve as the best standard because of the diverse range of fatty acids that are found in milk.

1.3 Factors influencing the separation of *t*-18:1 and CLA isomers

The various factors influencing the separation of *t*-18:1 and CLA isomers are discussed in the following headings:

***Column length and type*:** Although in theory, the longer the columns, the better the resolution that can be achieved, this is not always so and very long columns are not always practical. The use of a capillary column as long as 200m (chemically bonded) has been reported (Cruz-Hernandez et al., 2006). However, the authors reported that such a long column requires higher head pressures and the joint between the two 100m columns is prone to leak. The desired separations are therefore obtained by varying the nature of the stationary phase. Columns with numerous stationary phases including certain polyesters, polyethylene glycol, silicone elastomers and the highly polar cyanoalkyl polysiloxane are available. Fused silica columns coated with cyanoalkyl polysiloxane stationary phase available in 100 m length are marketed under names like CP Sil 88, SP2560, SP2380 and BPX-70. These columns were extensively studied and have been recommended for the separation of complex mixtures of geometric and positional isomers of MUFA (Wolff and Bayard, 1995a; Mossoba et al., 1997; Precht and Molkentin, 1999; Precht and Molkentin, 2000) as in milk fats. The 100 m CP Sil 88 or SP2560 columns have also been shown to improve the separation of 16:1 isomers (Precht and Molkentin, 2000; Kramer et al., 2008), 18:2 isomers (Precht and Molkentin, 2003; Kramer et al., 2008), 20:1 to 18:3 (Precht and Molkentin, 1999; Kramer et al., 2008) and the CLA region (Cruz-Hernandez et al., 2004 and 2006; Shingfield et al., 2006). Fused silica columns coated with CARBOWAX (polyethylene glycol) type stationary phase marketed under the names SUPELCOWAX 10 and DB-WAX were also used for the analyses of milk fats (Chouinard et al., 2001; Dhiman et al., 1999) although they are not as polar as the cyanoalkyl polysiloxane type. Kramer et al. (2002) evaluated the 60 m SUPELCOWAX 10 and the 100 m CP Sil 88 for the analysis of milk fat and concluded that the 100 m highly polar CP Sil 88 column is necessary to obtain full spectrum of *trans*- and CLA isomers in milk fat. They suggested that the 60m SUPELCOWAX 10 column at best serves as a complementary GC to provide different separations in certain regions based on

its intermediate polarity. Several other columns of slightly lower polarity, such as HP 88 (Agilent Technologies), TC 70 (GL Sciences Inc.; Shirasawa et al., 2007) and BPX-70 (SGE Inc.) are also available. However the performance of these columns is still to be established. Based on the improved separation of most of the trans isomers discussed above, there is general agreement that the 100m long capillary column coated with the highly polar (100% cyanoalkyl polysiloxane) stationary phase is necessary for the improved resolution of trans fatty acids from all matrices.

***Carrier gas*:** Carrier gas acts as the mobile phase for the movement of the volatilized compounds through the column. Generally helium or hydrogen has been used as the carrier gas in gas chromatography. However, hydrogen has been shown to provide better resolution of most of the trans fatty acids (Kramer et al., 2002; Ratnayake et al., 2006) and this was attributed to its higher thermal conductivity (Grob and Biedermann, 2002).

***Sample load*:** The temperature program at 175^{o}C provides fairly good separation of *trans* and *cis* isomers in milk fat. Even then, overloading a capillary column can markedly affect the resolution of the closely eluting peaks particularly the *c*/*t* isomers of 16:1, 18:1 and 20:1 respectively. Dilutions of the sample sometimes help to lower the concentration of the sample to be injected into the GC column and benefit separation of *t*10-18:1 from *t*11-18:1. However, this will compromise the PUFA region. A concentration of 1mg of fat per ml of hexane can be used as a starting sample load. However, appropriate sample load needs to be worked out for a particular GC to ensure that the MUFA are well separated and PUFA still remain detectable.

***Temperature programming*:** Selection of column and temperature programming depends on the objectives of the experiment. Because the objective of this

review is to gain a better understanding of the various methods for improved resolution of *trans*- and CLA-isomers in milk fat, the focus is on the different temperature programs used with the 100 m long capillary columns coated with the highly polar cyanoalkyl polysiloxane stationary phase.

In general, if the sample to be analyzed contains components that differ widely in volatility, it is preferable to start programming the column temperature at a lower setting so that the more volatile components elute first. After allowing sufficient time for the highly volatile components to elute, the column temperature can then be programmed to increase at a fixed reproducible rate so that the less volatile components are eluted in a reasonable time. Because the temperature program used affects the relative elution order of saturated, monounsaturated and polyunsaturated FAME, changes in column temperature is a rapid and inexpensive way to improve the resolution of the *t*- and *c*-18:1 isomers (Kramer et al., 2008).

There is no single temperature program that can separate all the isomers of interest. Often temperature conditions are programmed based on the type of sample and the objectives of the study. For example, the 175^{o}C program (45^{o}C to 215^{o}C) which consists of 2 temperature increases: 45^{o}C (held for 4 min), 13^{o}C/min to 175^{o}C (held for 27 min), 4^{o}C/min to 215^{o}C (held for 35 min) has been reported in a number of studies for the analysis of milk fat (Cruz-Hernandez et al., 2004; Cruz-Hernandez et al., 2007; Mutsvangwa et al., 2003; Mohammed et al., 2009) as well as partially hydrogenated vegetable oils (Cruz-Hernandez et al., 2006). It should be noted that the 175^{o}C program for partially hydrogenated vegetable oils provides a near baseline resolution of peaks for *t*13-/*t*14-18:1 and *c*9-18:1 with the elution of the *t*15-18:1 shortly after *c*9-18:1. Such a separation, however, is not possible with milk fat using the same temperature program because of larger differences in the concentration of adjacent *t*-18:1 isomers.

Milk fat analyzed using the 175^{o}C program provides fairly good separation of most of the saturated FAME and PUFA. However, the MUFA regions particularly the 16:1, 18:1 and 20:1 regions show considerable overlapping (discussed in detail in the next section) with the 175^{o}C program. Another temperature program (163^{o}C program) described and compared with the 175^{o}C program by Kramer et al. (2008) is very similar to the 175^{o}C program in the separation of *c*- and *t*-18:1 isomers except for the partial separation of *t*15-18:1 from *c*9-18:1 (Fig.1-7). This approach was used to resolve most of the milk fat FAME using one GC run followed by a second isothermal GC analysis at temperatures ranging from 160-180^{o}C (Griinari et al., 1998; Shingfield et al., 2006). The 150^{o}C program consisting of 2 temperature increases: 45^{o}C (held for 4 min), 13^{o}C/min to 150^{o}C (held for 47 min), 4^{o}C/min to 215^{o}C (held for 35 min) has been reported to be useful in analyzing most of the geometric and positional isomer of 16:1, 18:1 and 20:1 isomers without prior silver ion separation when combined with the 175^{o}C program (Kramer et al., 2008). However, the CLA isomers could not be resolved completely even with the combination of the two GC methods (175^{o}C and the 150^{o}C program; Kramer et al., 2008) and requires a complimentary Ag^{+}-HPLC separation. It should be noted that the *t*13-/*t*14-18:1, *t*6-/*t*8-18:1 and the *c*6-/*c*8-18:1 remains inseparable by the three temperature programs described above. A prior separation of geometric isomers using silver ion-thin layer chromatography (Ag^{+}-TLC) followed by a GC analysis of the fractions at a isothermal temperature of 120^{o}C resolved all the 18:1 isomers (*t*4- to *t*16-18:1) except *t*6-*t*8-18:1 and *c*6-*c*8-18:1 (Fig. 1-8) (Cruz-Hernandez et al., 2006; Precht and Molkentin, 1997; Cruz-Hernandez et al., 2007). Under these conditions, the elution of *c*- and *t*-18:1 isomers could be achieved in approximately 3.5 h.

***Overlapping peaks*:** There are 4 main regions in the chromatogram of milk FAME that has considerable overlapping. These include the regions from: a) 16:0 to 18:0 b) 18:0 to 18:2n-6 c) 20:0 to 18:3n-3 and d) the CLA region.

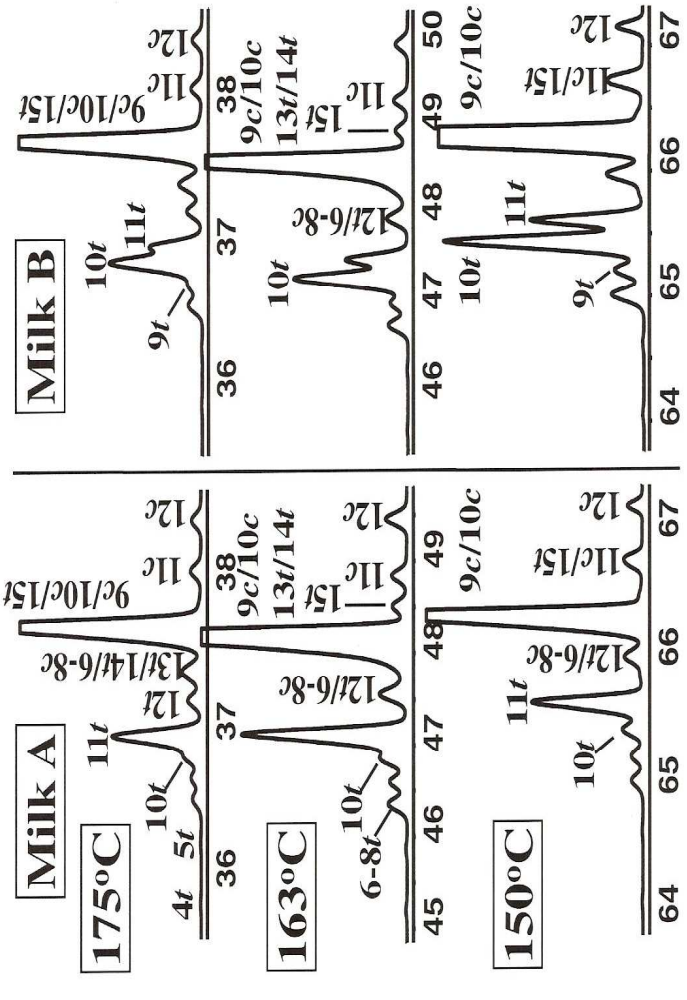

Figure 1-7. Partial GC chromatogram of two milk fats at different temperature programs shown from 4t to 12c-18:1. *t* = trans; *c*= cis. Note: Milk A and B differ in the proportions of *t*10- and *t*11-18:1. Reproduced and modified with permission from Kramer et al. (2008).

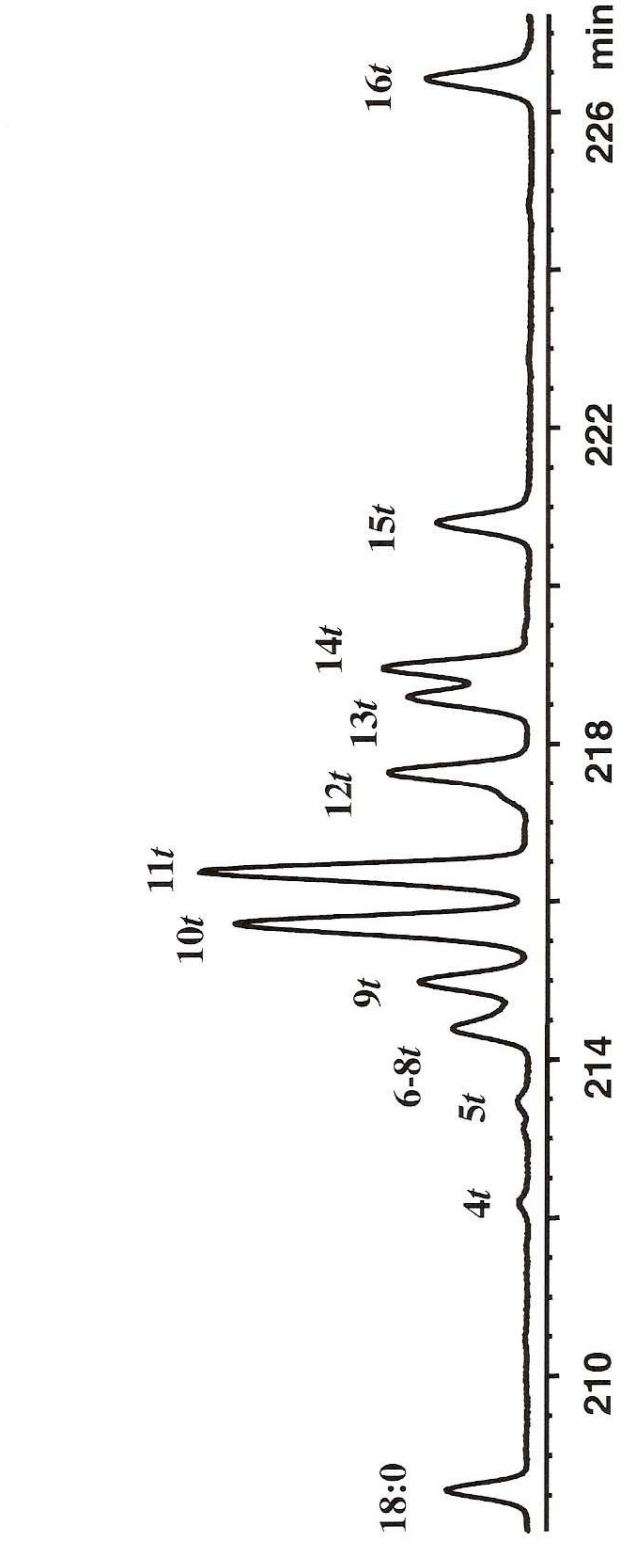

Figure 1- 8. Partial GC chromatogram of the 18:1 region from *trans* (*t*) fraction isolated using Ag+-Solid Phase Extraction (SPE) chromatography and analyzed isothermally at 120°C. Reproduced and modified with permission from Kramer et al. (2008).

a) 16:0 to 18:0 region: This region has overlapping branched-chain (*iso* and *anteiso*) fatty acids of 17:0 and *c*/*t* isomers of 16:1. It can be observed from the chromatogram at 175°C (Fig 1-9) that the peak 9*t*-16:1 eluting to the right of 17:0 *iso* is free of any interferring peaks compared to the chromatogram at 150°C (Fig. 1-10), where 9*t*-16:1 co-elutes with 17:0 *iso* and remains as the left shoulder of 17:0 *iso*. The peaks 6*t*/7*t*- and 8*t*-16:1 in the chromatogram at 175°C (Fig. 1-9) are co-eluted with 17:0 *iso* compared to the chromatogram at 150°C (Fig. 1-10), where 6*t*/7*t*- and 8*t*-16:1 are devoid of any coeluting peaks. In the chromatogram at 175°C, the peak 17:0 *anteiso* is co-eluted with 7*c*- and 13*t*-16:1 while the peaks 11*t*/12*t*- and 9*c*-16:1 are free of interferring peaks compared to the chromatogram at 150°C, where 17:0 *anteiso* is co-eluted with only 9*c*-16:1 while the peak 7*c*-16:1 co-eluted with 11*t*/12*t*-16:1. The peak 17:0 is free of interferring peaks in the chromatogram at 150°C while at 175°C, 17:0 is co-eluted with *c*12-16:1. These differences at the two temperatures are advantageous in the separation of c/t isomers of 16:1 from the branched chain fatty acid of 17:0.

b) 18:0 to 18:2n-6 region: This region comprises the overlapping *c*/*t* isomers of 18:1 and 18:2n-6 isomers. The predominant *c*-18:1 isomer is *c*9-18:1 while the predominant *t*-18:1 depends on the diet and could be *t*10-18:1 or *t*11-18:1. It can be observed that the peaks *t*6- to *t*11-18:1 are free of interfering peaks in the chromatograms at 175°C (Fig. 1-11) or at 150°C (Fig. 1-12). The resolution of the isomers *t*10- and *t*11-18:1 at 175°C depends on the concentrations of the total *t*-18:1 and is determined by the diet. Milk fat from cows offered diets supplemented with PUFA have higher concentrations of *t*11-18:1 when the dietary forage content is moderate to high. Greater concentrations of *t*10-18:1 has been observed in milk when the forage content is low (Griinari et al., 1998). The peaks *t*10- and *t*11-18:1 resolve well at 175°C program for diets not supplemented with PUFA (also

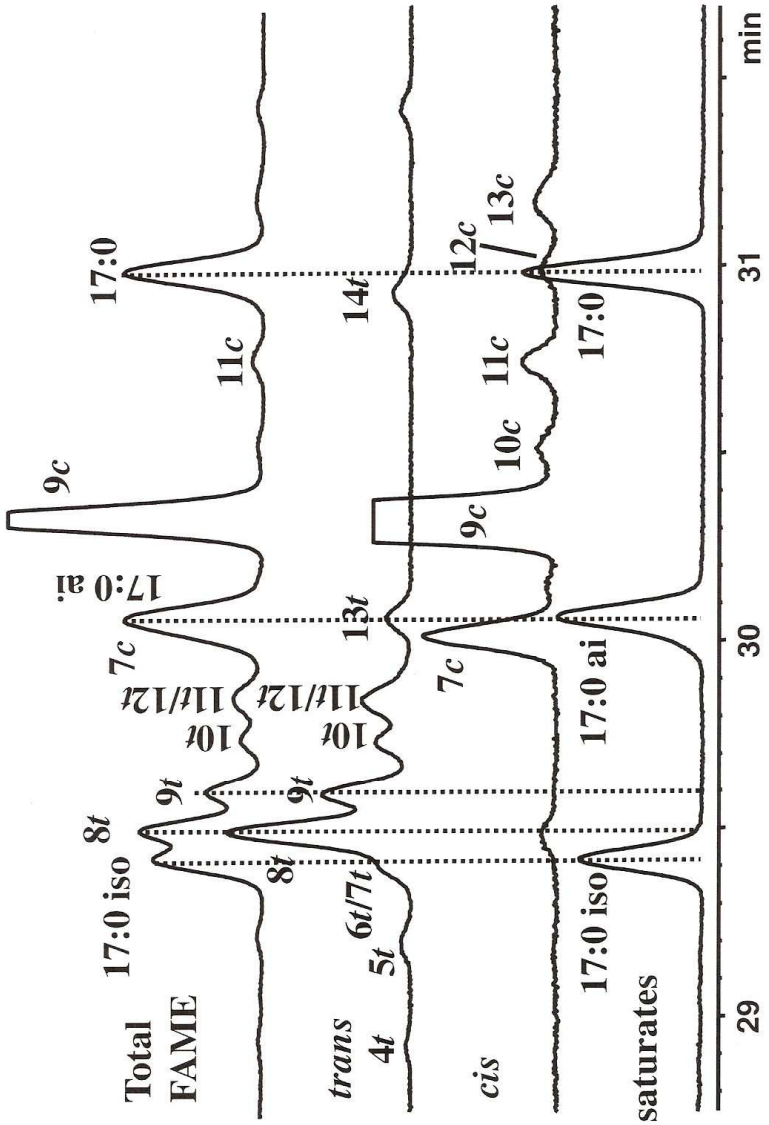

Figure 1- 9. Partial GC chromatogram of milk fat showing the 16:1 region (175°C program). The total milk fatty acid methyl esters (FAME) were compared to the *trans* (*t*), *cis* (*c*) and saturated fatty acid fractions isolated from total milk fat FAME using Ag^+-Solid Phase Extraction (SPE) columns. Adapted from Kramer et al. (2008).

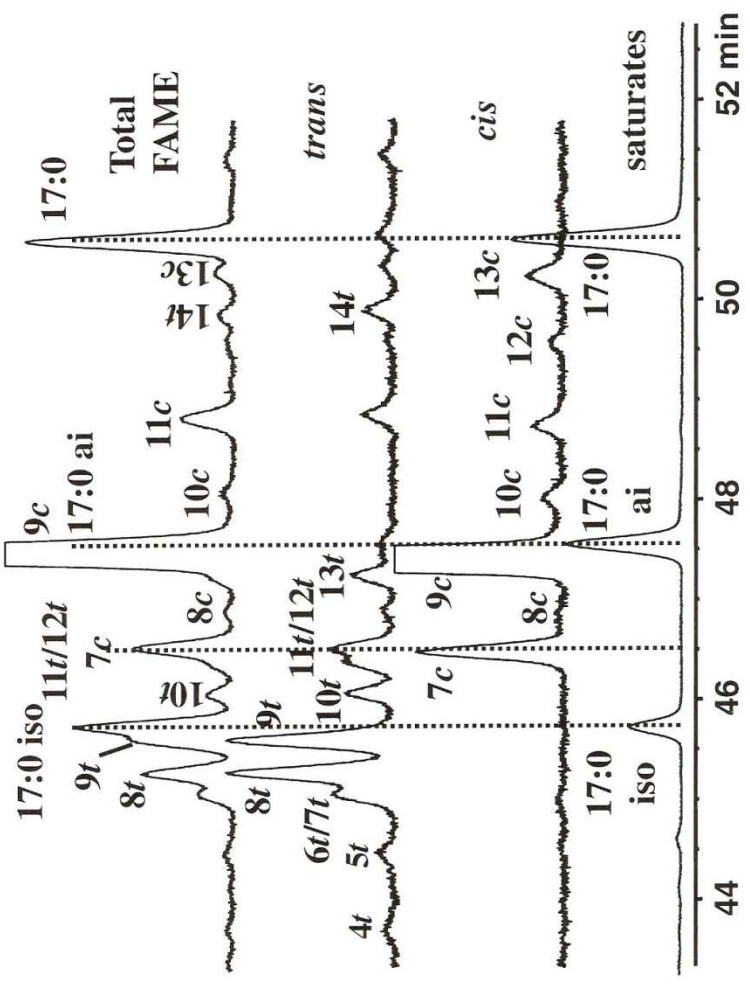

Figure 1- 10. Partial GC chromatogram of milk fat showing the 16:1 region (150°C program). The total milk fatty acid methyl esters (FAME) were compared to the *trans* (*t*), *cis* (*c*) and saturated fatty acid fractions isolated from total milk fat FAME using Ag^{+}-Solid Phase Extraction (SPE) columns. Adapted from Kramer et al. (2008).

depends on the concentration of hexane extract injected). However, they do not resolve well at 175°C program if milk contains greater concentrations of either *t*11-18:1 or *t*10-18:1. Under these circumstances, it is preferable to use the temperature program at 150°C which better resolves *t*10-18:1 from *t*11-18:1 compared to the program at 175°C. At 175°C program, the peak *t*12-18:1 is free of interfering peaks (Fig. 1-11), while at 150°C program, it is co-eluted with *c*6-8-18:1 (Fig. 1-12). On the other hand, the isomer *t*13-/*t*14-18:1 co-eluted with *c*6-8-18:1 at 175°C and with *c*9-18:1 at 150°C. The isomer *t*15-18:1 co-eluted with *c*9-18:1 at 175°C and with *c*11-18:1 at 150°C. There was a reversal in the elution order of *t*16-18:1 and *c*14-18:1; at 175°C *t*16-18:1 eluted before and at 150°C slightly after *c*14-18:1.

The small component of *c*6-8-18:1 can be determined by difference from either *t*13/*t*14-18:1 peak at 175°C or *t*12-18:1 peak at 150°C. The predominant isomer *c*9-18:1 contained the minor isomer *c*10- and *t*15-18:1 at 175°C and *c*10- plus *t*13-/*t*14-18:1 at 150°C. Because the concentration of *c*9-18:1 in milk is so high, the minor peak *c*10-18:1 cannot be resolved from *c*9-18:1 under any of the temperature conditions (150°C or 175°C); and even at an isothermal condition of 120°C. The isomers *c*11- and *c*12-18:1 are free of interfering peaks at 175°C. Representative calculations of the 18:1 isomers were explained in detail by Kramer et al. (2008).

The region between *c*13-18:1 and 18:2n-6 consist of overlapping peaks of *t*,*t*-18:2, *c*/*t*-18:2 and *c*-18:1 isomers. The *t*,*t*-18:2 isomers usually elutes before the *c*/*t*-18:2 isomers (Fig. 1-11). The isomer *t*9*t*12-18:2 can be identified using the 463 standard, however the structure of other *t*,*t* 18:2 isomers is not known. The *c*-18:1 isomers in this region include *c*13-, *c*14, *c*15- and *c*16-18:1 and can be separated from *c*/*t* 18:2 isomers by using the two temperature programs 175°C and 150°C as described by Kramer et al. (2008). The isomers *c*13-, *c*15 and *c*16-

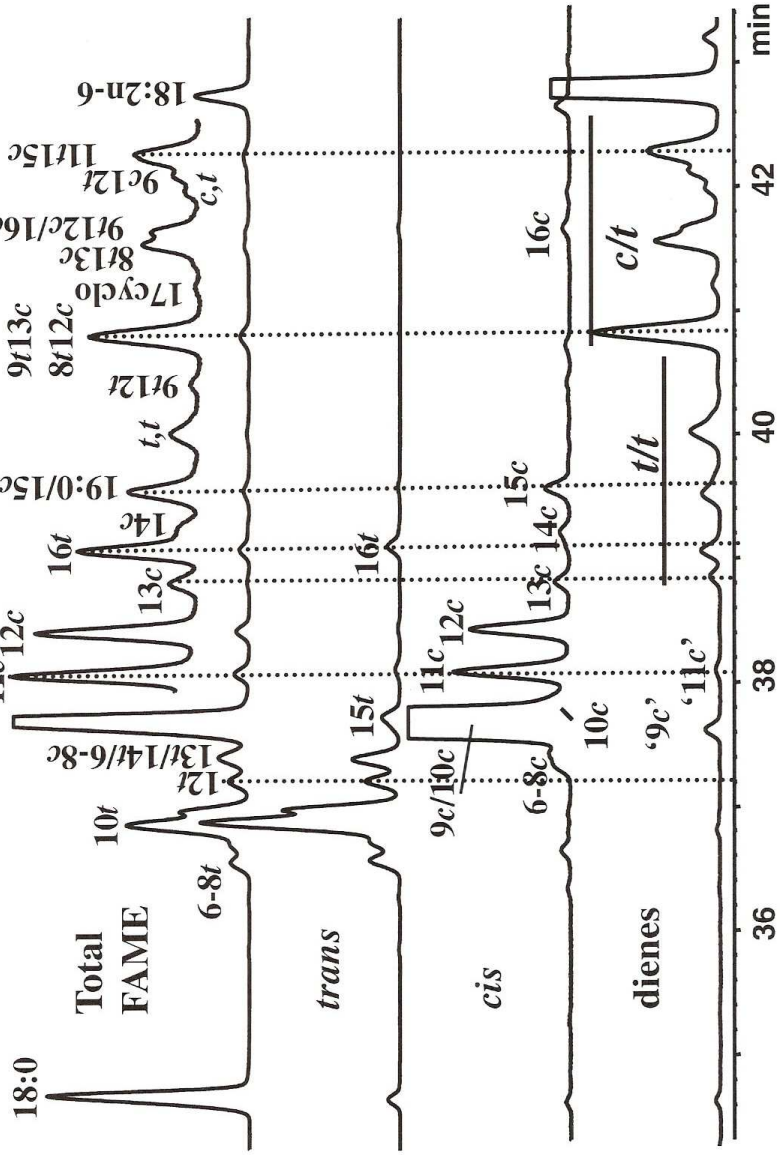

Figure 1-11. Partial GC chromatogram of milk fat showing the 18:0 to 18:2n-6 region (175°C program). An enlargement of the *c*11-18:1 to *t*11*c*15-18:1 is inserted for clarity. The *trans* (*t*), *cis* (*c*) and diene fractions isolated from total milk fat FAME using Ag^{+}-Solid Phase Extraction (SPE) columns are compared to the total milk fatty acid methyl esters (FAME). Adapted from Kramer et al. (2008).

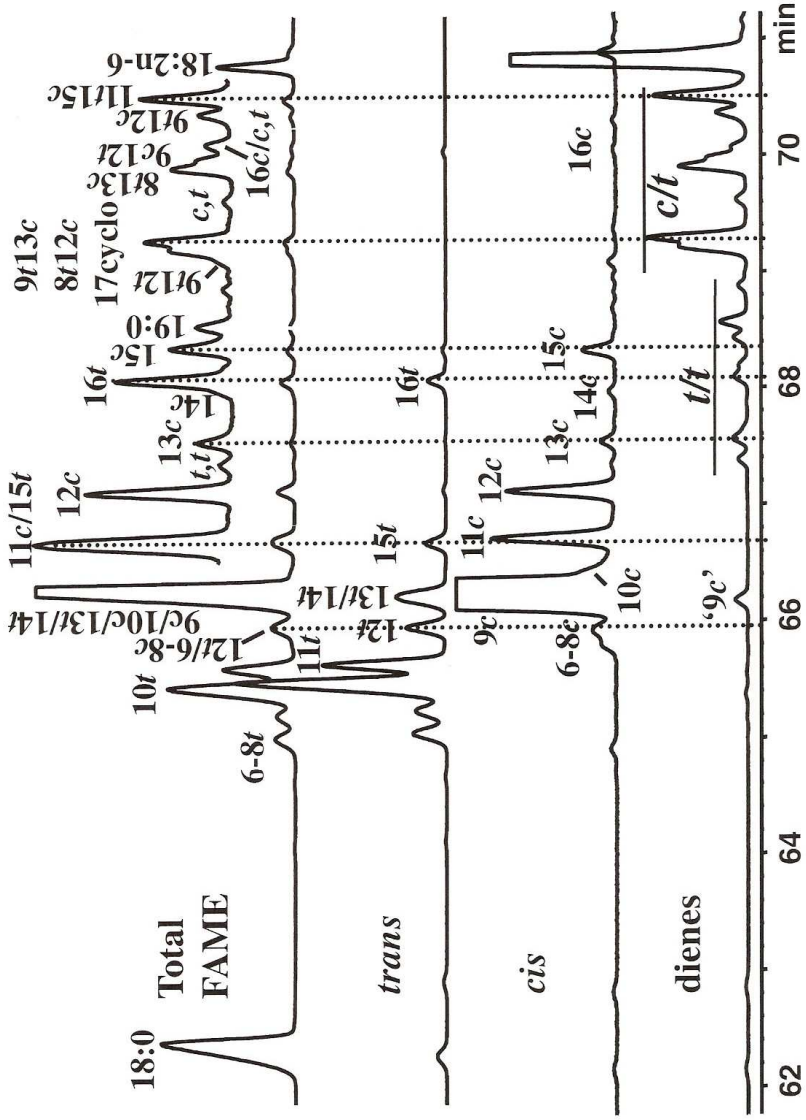

Figure 1-12. Partial GC chromatogram of milk fat showing the 18:0 to 18:2n-6 region (150°C program). An enlargement of the *c*11-18:1 to *t*11*c*15-18:1 is inserted for clarity. The *trans* (*t*), *cis* (*c*) and diene fractions isolated from total milk fat FAME using Ag+-Solid Phase Extraction (SPE) columns are compared to the total milk fatty acid methyl esters (FAME). Adapted from Kramer et al. (2008).

18:1 co-eluted with *t,t*-18:2 or *c*/*t* 18:2 isomers under both temperature conditions. It was observed that 19:0 co-elutes with *c*14/*t*16-18:1 for one column and with *c*15-18:1 for a different column of the same manufacturer (personal communication). This suggests that two different columns manufactured by the same company can sometimes be different in polarity leading to differences in elution order as described above. Similar differences were also observed for the peak 21:0 (discussed later).

The identification of *c*/*t*-18:2 isomers is important because they are intermediates of PUFA metabolism. The different *c*/*t*-18:2 isomers elute in the order *t*9*c*13- plus *t*8*c*12-18:2, unknown *c*/*t*-18:2, *t*8*c*13- plus *c*9*t*12-, *t*9*c*12- and *t*11*c*15-18:2. Among the *c*/*t*-18:2 isomers, the predominant one is *t*9*c*13- plus *t*8*c*12-18:2 followed usually by *t*8*c*13- plus *c*9*t*12-18:2. The isomer *c*16-18:1 often co-elutes with the latter and could only be partially resolved at both temperature programs. The isomer *t*11*c*15-18:2 is a metabolite of 18:3n-3 and could be found in greater concentrations if diet offered has greater concentrations of 18:3n-3.

c) 20:0 to 18:3n-3 region: The region from 20:0 to 18:3n-3 is occupied by the overlapping *c*/*t* isomers of 20:1 and geometric and positional isomers of 18:3n-3 (Fig 1-13). The predominant cis isomers of 20:1 include *c*9- and *c*11-20:1 which can be identified using the 463 standard. This standard has *c*5, *c*8 and *c*11-20:1 isomers. However, in milk fat, *c*8-20:1 is never observed. The isomer *c*9-20:1 is biological and elutes after *c*8-20:1 in the standard. Kramer et al. (2008) reported several *t*-20:1 isomers in milk samples obtained from cows fed 50:50 forage: concentrate diet supplemented with sunflower oil. They reported that *t*-20:1 isomers (*t*6-11-, *t*12-, *t*13-, *t*14-, *t*15/*t*16- and *t*17-20:1) could be resolved using the temperature programs at 175^{o}C (Fig. 1-13) and 150^{o}C (Fig. 1-14). From the figure, it could be observed that there is no interference of other peaks in the

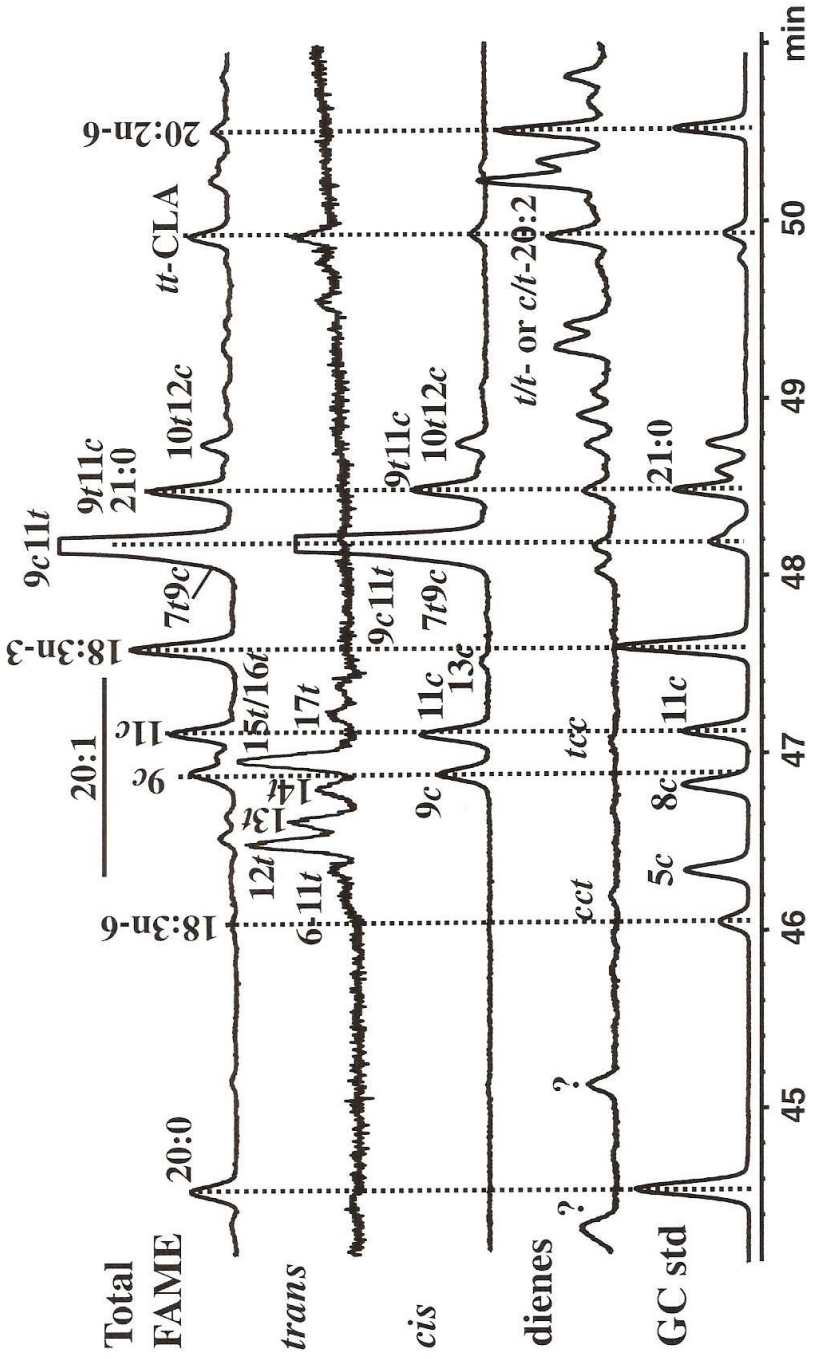

Figure 1- 13. Partial GC chromatogram of milk fat showing the 20:0 to 20:2 region (175°C program). The total milk fatty acid methyl esters (FAME) are compared to the *trans* (*t*), *cis* (*c*) and diene fractions isolated from total milk FAME using Ag^{+}-Solid Phase Extraction (SPE) columns and the GC standard # 463 spiked with 21:0 and conjugated linoleic acid (CLA) isomers. Adapted from Kramer et al. (2008).

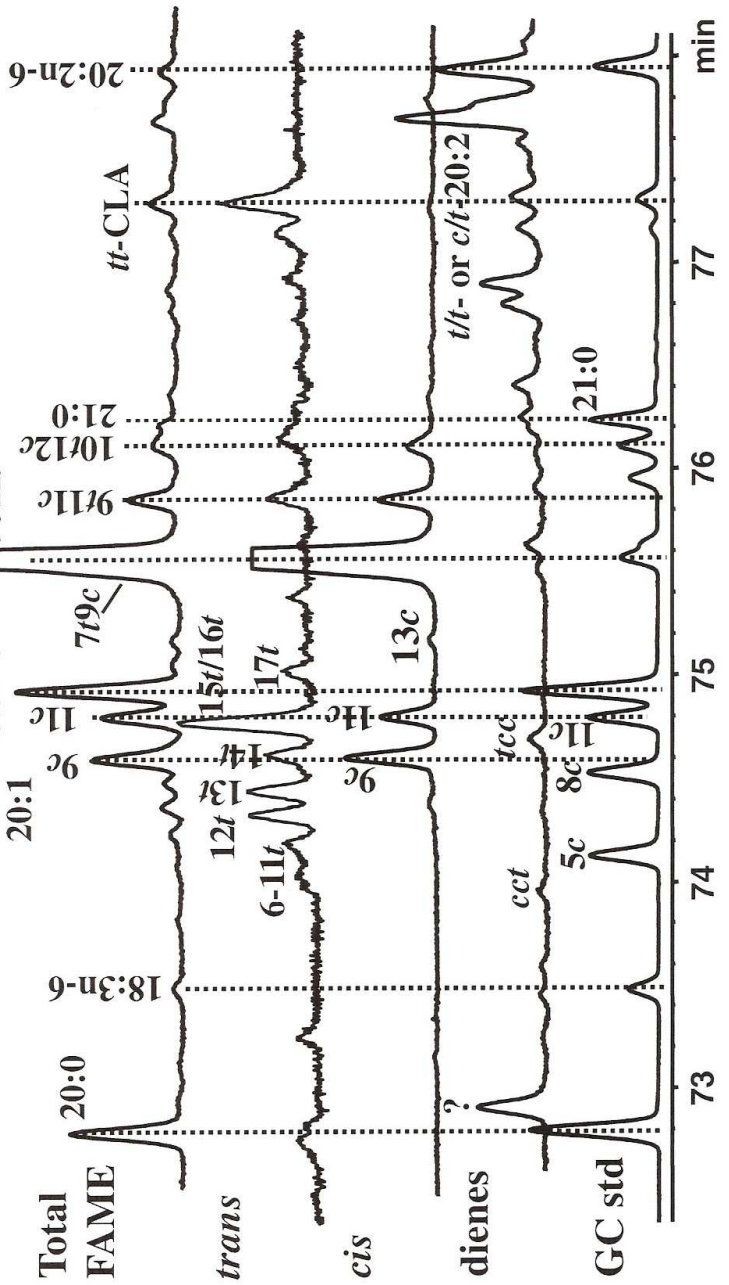

Figure 1-14. Partial GC chromatogram of milk fat showing the 20:0 to 20:2 region (150°C program). The total milk fatty acid methyl esters (FAME) are compared to the *trans* (*t*), *cis* (*c*) and diene fractions isolated from total milk fat FAME using Ag^{+}-Solid Phase Extraction (SPE) columns and the GC standard # 463 spiked with 21:0 and conjugated linoleic acid (CLA) isomers. Adapted from Kramer et al. (2008)

region from *t*6- to *t*13-20:1 which eluted before *c*9-20:1 at both temperature programs. The FAME pair *t*15-/*t*16-20:1 could be resolved from the *c*-20:1 isomers only at 175^{o}C (Fig. 1-13) while *c*13-20:1 could be separated at 150^{o}C (Fig. 1-14).

The major 18:3 isomers in milk fat are *c*9*c*12*t*15-18:3 (*cct*-18:3) and *t*9*c*12*c*15-18:3 (*tcc*-18:3) with only trace amounts of *c*9*t*12*c*15-18:3 (*ctc*-18:3). The *cct*-18:3 isomer elutes shortly after 18:3n-6. The *tcc*-18:3 isomer co-elutes with *c*11-20:1 at 175^{o}C (Fig. 1-13). However, at 150^{o}C this isomers gets resolved from *c*11-20:1 isomers and could be identified as the peak between *c*9-20:1 and *c*11-20:1 (Fig. 1-14).

d) CLA region: The peak *c*9*t*11-CLA is often co-eluted with *t*7*c*9- and *t*8*c*10-CLA and cannot be separated using either of the two temperature programs (Fig 1-13 and 1-14). This suggests that a complimentary Ag^{+}-HPLC is a must for the complete resolution of CLA isomers. Because *t*7*c*9-CLA is the second most-abundant CLA isomer, identification of *c*9*t*11-CLA without separating *t*7*c*9-CLA often overestimates its concentration. This region has major interferences with 21:0 and 20:2 isomers (Fig. 1-13 and 1-14). The peak 21:0 co-elutes with *t*9*c*11-CLA at 175^{o}C (Fig. 1-13) but free of co-eluting peaks at 150^{o}C (Fig. 1-14). However, one should exercise caution in the identification of 21:0 because this peak has been reported to shift either before or after *t*9*c*11-CLA at 175^{o}C with different columns of the same manufacturer (Fig. 1-15). Therefore for correct identification of 21:0, it is recommended to spike 463 standard with 21:0 FAME. The isomer *t*10*c*12-CLA often co-elutes with *t*/*t* or *c*/*t*-20:2 isomers (Roach et al., 2000) at 175^{o}C and could be misleading. This isomer can be best resolved by Ag^{+}-HPLC.

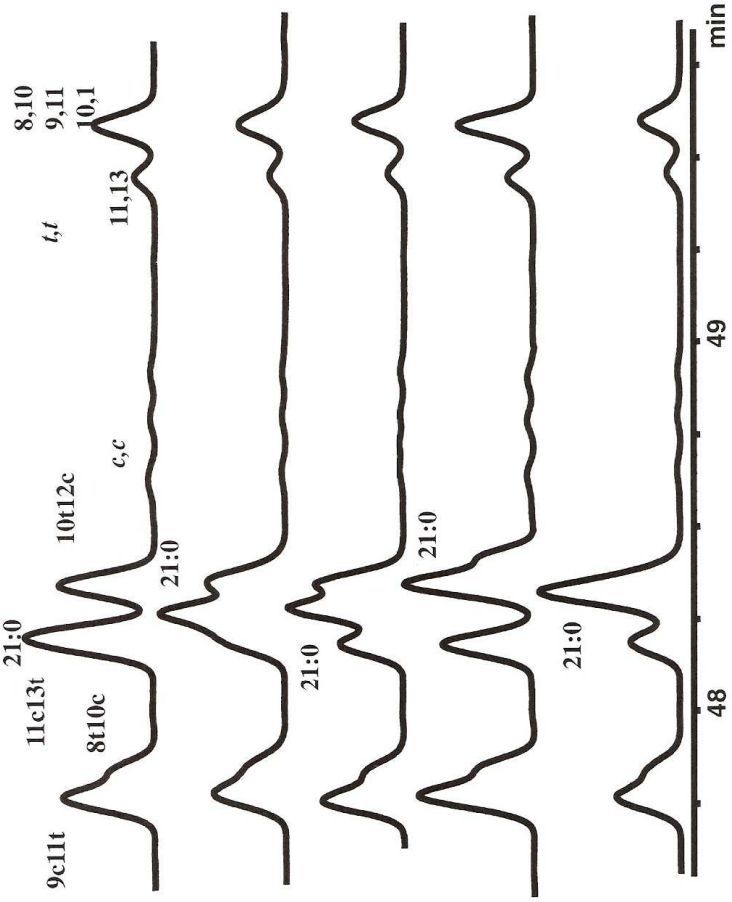

Figure 1-15. Interference of conjugated linoleic acid (CLA) region with 21:0 peak and its shift from column to column. Note: *c = cis; t= trans*. CLA region starts from 9c11*t*-CLA to *t,t*-CLA. Reproduced with permission from Cruz-Hernandez et al (2006).

1.4 Conjugated Linoleic Acid

Conjugated linoleic acid is a collective term that describes the positional and geometrical isomers of LA (*c*9*c*12-18:2) having two conjugated double bonds. Theoretically, a number of conjugated linoleic acid isomers are possible that differ in the position of double bond pairs (eg. 6-8, 7-9, 8-10, 9-11, 10-12, 11-13, 12-14). Additional differences can exist in the configuration of the double bond so that *cis-trans*, *trans-cis*, *cis-cis* or *trans-trans* configurations are all possible. The major isomer of conjugated linoleic acid in milk fat is *c*9*t*11-CLA and it can represent 80-90% of total conjugated linoleic acid isomers under many feeding conditions when cows are fed low to moderate levels of PUFA (Parodi, 1977). Other conjugated linoleic acid isomers found in milk include: *t*7*c*9-CLA, *t*9*c*11-CLA, *t*10*c*12-CLA, *t*11*c*13-CLA, *c*10*c*12-CLA, *c*11*c*13CLA and *trans-trans* CLA (*t*7*t*9-, *t*9*t*11-, *t*10*t*12- and *t*11*t*13-CLA). Throughout this review, linoleic acid (*c*9*c*12-18:2) is referred to as LA**,** α-linolenic acid (*c*9*c*12*c*15-18:3) as LN and *c*9*t*11-CLA as simply CLA or RA (rumenic acid) unless specified.

Biosynthesis of CLA

Conjugated linoleic acid is formed in the rumen as an intermediate product of microbial biohydrogenation of dietary LA. It is also synthesized endogenously in the mammary epithelial cells by desaturation of *t*11-18:1 (formed as an intermediate of both LA and LN biohydrogenation in rumen and supplied to mammary tissue) under the catalytic influence of Δ^9-desaturase which adds a double bond at the 9^{th} carbon converting *t*11-18:1 to *c*9*t*11-18:2 (CLA) (Bauman et al., 1999). Endogenous synthesis of CLA from *t*11-18:1 has been proposed to be the major pathway for CLA synthesis accounting for an estimated 78% of the CLA in milk fat of dairy cows (Corl et al., 2001). Stearic acid (18:0) and palmitic acid (16:0) are the major substrates for stearoyl-*CoA desaturase* and the fatty acid products of this reaction are components of milk fat that help maintain membrane fluidity. *Trans*7 *cis*9-CLA which is the second

largest CLA isomer in milk fat has been reported to be endogenously synthesized from *t*7-18:1 as the substrate (Corl et al., 2002). The enzyme *stearoyl-CoA* desaturase has been reported to be expressed in different tissues depending on the species. In rodents, its activity was reported to be maximum in liver (Ntambi et al., 1995). In lactating ruminants, mammary gland is the major site of endogenous synthesis of CLA while in growing ruminants, adipose tissue is the major site (Bickerstaffe and Annison, 1970; Kinsella, 1972). The predominant pathways of biohydrogenation of LA and LN are described in detail in the next section.

1.5 Rumen lipid metabolism: Lipolysis and PUFA biohydrogenation

The nature of dietary lipids depends on the ingredients in the diet. Generally in a TMR based diet, forages are mixed with grain and fed. The nature of lipid in grain is mostly triglyceride while the forage lipid comprises phospholipids, glycolipids and free fatty acids. It is well known that dietary lipids are hydrolyzed first by the action of bacterial lipases resulting in the formation of unesterified fatty acids and glycerol. Unsaturated fatty acids are then biohydrogenated resulting in the formation of saturated fatty acids. A free carboxyl group is required for biohydrogenation to occur and therefore it cannot proceed without prior lipolysis of dietary lipid.

Lipolysis occurs under the catalytic action of lipase released by lipolytic organisms (eg. *Anaerovibrio lipolytica*, *Butyrivibrio fibrisolvens* etc). Lipolysis was low at pH < 6.0 and was further inhibited with decreasing pH (Beam et al., 2000). Hobson (1965) reported that the growth of *Anaerovibrio lipolytica* was slow at pH 5.7 and was completed inhibited at pH 5.3. Russell and Dombrowski (1980) reported that that growth yield of *Butyrivibrio fibrisolvens* was 75% of its maximum yield when pH was 5.75, while at pH 5.5 the organism was washed out in continuous cultures. Henderson et al. (1969) from their studies in batch

culture reported that lipase produced by *A. lipolytica* was severely inhibited at pH 5.8. In a different study with olive oil as substrate, lipase activity was observed to be most active at pH 7.4, while at pH 6.6 only 50% of the maximum activity was recorded (Henderson, 1971). Therefore, inhibitory effect of pH on lipolysis is due to the effect of pH on the growth of lipolytic organisms as well as interference with lipase activity. Increasing the amount of PUFA from 40 mg soybean oil to 80 mg soybean oil while keeping the culture pH similar has been reported to inhibit lipolysis (Van Nevel and Demeyer, 1996). Beam et al. (2000) also reported a decline in the rate of lipolysis from 44%/h to 30%/h with an increase in soybean oil from 2 to 10% in the culture. Based on the above discussion, it can be concluded that lipolysis is inhibited by rumen pH as well as by the amount of PUFA, however, it was reported to be more sensitive to pH than to PUFA content (Beam et al., 2000).

Following lipolysis, dietary unsaturated fatty acids are biohydrogenated to saturated fatty acid through a series of reactions catalyzed by isomerases and reductases. The major biohydrogenation pathways of LA and LN which are well studied are described below (Harfoot and Hazlewood, 1988) (Fig. 1-16). Dietary LA is converted to *c*9*t*11-CLA under the catalytic action of linoleate isomerase. This is followed by the formation of *t*11-18:1 and 18:0 under the action of reductases. On the other hand, LN (*c*9*c*12*c*15-18:3) is isomerized to *c*9*t*11*c*15-18:3 followed by reduction reactions resulting in the formation of *t*11*c*15-18:2, *t*11-18:1 and 18:0. Griinari and Bauman (1999) presented a putative pathway where *t*10-18:1 is formed from the reduction of *t*10*c*12-CLA in the rumen as an alternative way of biohydrogenation of linoleic acid. Its formation from biohydrogenation of LA is well supported with evidence from in vitro studies (Kepler et al., 1966; Kim et al., 2000). However, when rumen contents are analyzed from a cow offered diet supplemented with a source of LA, a range of *t*18:1 isomers (ranging from *t*4 to *t*16), conjugated 18:2 isomers and non-

conjugated 18:2 isomers could be observed. Although, there is no *in vivo* evidence demonstrating the origin of 18:1 biohydrogenation intermediates, Shingfield et al. (2008), based on the flow of 18:2 fatty acids at the omasum together with the changes in the 18:1 fatty acids profile in omasal digesta following sunflower oil supplementation, proposed putative pathways of LA metabolism in the rumen showing possible origin of several 18:1 isomers and CLA isomers in the rumen (Fig. 1-17).

Having discussed the products of PUFA metabolism, some of the factors influencing PUFA biohydrogenation in the rumen are now discussed. Harfoot et al. (1973) reported from an *in vitro* study that concentrations of LA exceeding 1.0 mg/ml of culture contents interfered with the second reduction leading to the accumulation of *t*11-18:1 at the expense of 18:0. The concentration of 1mg/ml of LA is equivalent to 1g/L. Assuming an average of 100L of rumen contents for an adult cow, the amount of LA that could be inhibitory is equivalent to 100g. A cow offered a diet containing 50:50 F: C (35% barley silage and 15% alfalfa hay and about 38% barley grain) and consuming 20 kg DM/d would consume about 150g of LA/d. This suggests that cows offered a control diet (as described above) without any added lipid and containing an ether extract (EE) of 2% also has enough LA to inhibit complete biohydrogenation of PUFA leading to greater *t*11-18:1. Consistent with this, Beam et al. (2000) reported that amount of PUFA supplemented was the major factor influencing rates of LA biohydrogenation. They observed an overall biohydrogenation rate of 14.3%/h for LA from soybean oil which declined at the rate of 1.2%/h for unit percentage increase in LA. They also observed that biohydrogenation was also influenced by the number of double bonds in the fatty acid with greater rates of biohydrogenation observed for 18:2 fatty acid than for 18:1 fatty acid. Consistent with this, ruminal rates of disappearance of 18:1, 18:2 and 18:3 fatty acids were 78, 83, and 94%

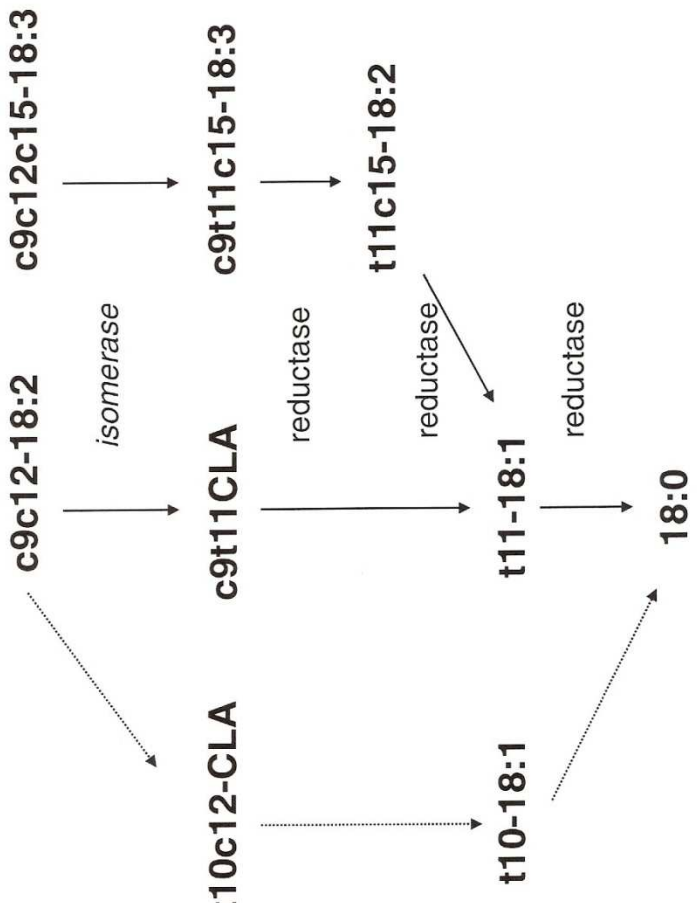

Figure 1-16. Major PUFA biohydrogenation pathways. c= cis; t= trans; CLA = conjugated linoleic acid

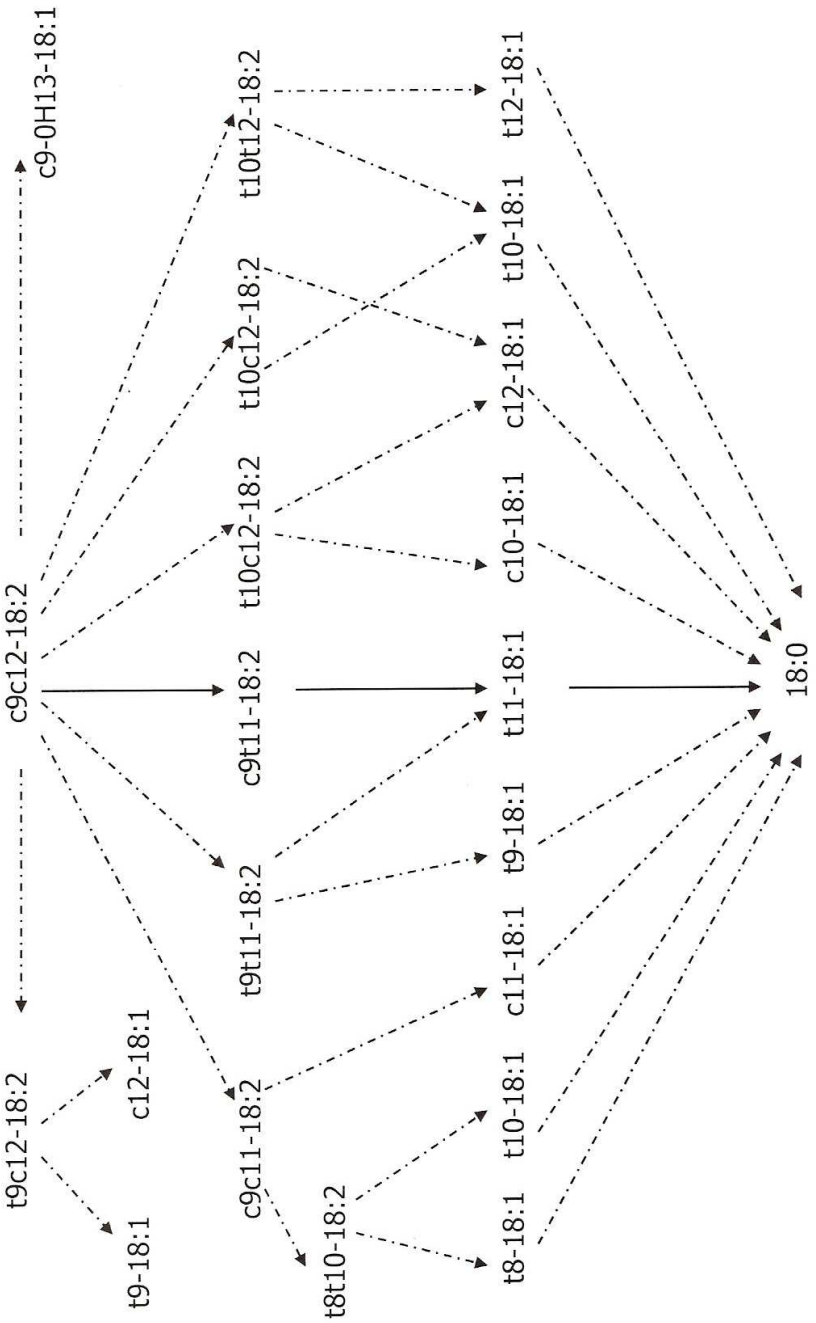

Figure 1-17. Putative pathways showing origins of conjugated 18:2 isomers and *trans*(*t*)-18:1 isomers in rumen. c = cis; dotted arrows denote putative pathways; solid arrows denote the established pathways. Adapted from Shingfield et al. (2008).

respectively in cows fed animal-vegetable fat (Pantoja et al., 1996). When fish oil was supplemented as the lipid source, LA biohydrogenation was reported to be only 9.6%/h (Palmquist and Kinsey, 1994). Fatty acid loss by ruminal absorption or catabolism is generally considered minor. Therefore, disappearance of fatty acids in the rumen can be mainly attributed to the loss due to biohydrogenation.

Besides concentration and source of PUFA, rumen pH was also reported to inhibit PUFA biohydrogenation (Martin and Jenkins, 2002; Kalscheur et al., 1997b), however LA biohydrogenation was only partially inhibited at pH 5.2. Furthermore, no free LN was detected in culture tubes even at the lowest pH (Van Nevel and Demeyer, 1996). The above discussion suggests that unlike lipolysis, PUFA biohydrogenation is more sensitive to the amount and source of PUFA than to pH.

1.6 Mammary lipid synthesis

Ruminant milk fat comprises short chain fatty acids, odd and branch-chain fatty acids, a relatively high proportion of saturated fatty acids, a low proportion of polyunsaturated fatty acids, and *trans* fatty acids, including conjugated linoleic acid. The fatty acids in milk arise from two sources, *de novo* synthesis within the mammary epithelial cells and uptake from circulation (Figure 1-18). Short and medium chain fatty acids (4:0 – 14:0) and a portion of the 16:0 fatty acids are almost exclusively *de novo* synthesized from acetate and β-hydroxy butyrate formed in the rumen as a result of carbohydrate fermentation and represent 40-50% of the fatty acids secreted in milk.

Preformed fatty acids account for the remaining 16:0 and all of the long-chain fatty acids (17:0 and above) and are derived from triglycerides circulating in very low-density lipoprotein (**VLDL**) or chylomicrons or from circulating free fatty acids (**NEFA**). These long-chain fatty acids originate mainly from absorption

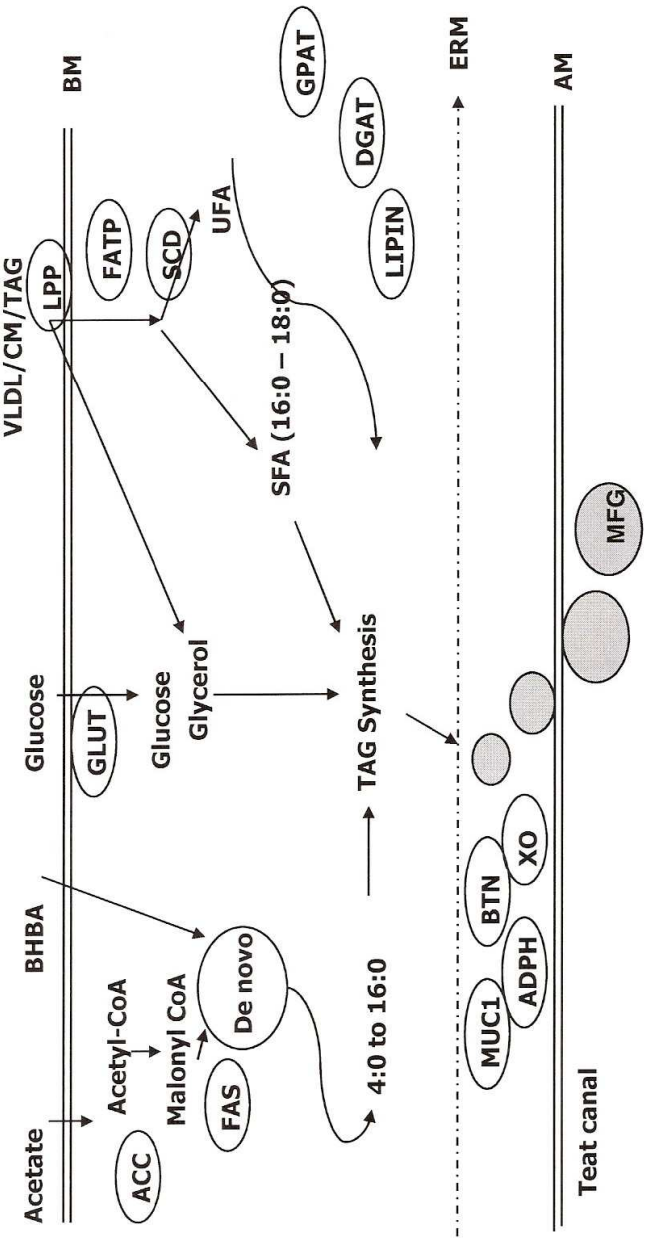

Figure 1-18. Schematic representation of milk fat synthesis. Key proteins are shown in ovals: ACC = acetyl-CoA carboxylase; ADPH = adipophilin; AM = apical membrane; BHBA = beta hydroxy butyric acid; BM = basal membrane; BTN = butyrophilinCM = chylomicrons; DGAT = diacylglycerol acyltransferase; ERM = endoplasmic reticulum; FAS = fatty acid synthetase; FATP = fatty acid transport protein; GLUT = glucose transporters GPAT = glycerol phosphate acyltransferase; LPP = lipoprotein lipase; MFG = milk fat globule; MUC = mucin; SCD = stearoyl-CoA desaturase; TAG = triacylglycerol; UFA = unsaturated fatty acid; VLDL = very low density lipoprotein; XO = xanthine oxidoreductase. Adapted from Harvatine et al., 2009.

of dietary lipid from the gut and from mobilization of body fat. The latter accounts for less than 10% of milk fatty acids and its proportion increases in the early lactation when cows are in negative energy balance in proportion to the energy deficit (Bauman and Griinari, 2001). Unlike other ruminant tissues, mammary tissue cannot convert 16:0 to 18:0 by chain elongation (Moore and Christie, 1981). However, mammary epithelial cells express a high Δ^9-desaturase activity, which converts 18:0 to *c*9-18:1 (Kinsella, 1972).

Molecular mechanisms

De novo synthesis of fatty acids occurs in the cytoplasm of mammary epithelial cells and involves co-ordinated activity of the enzymes *acetyl-CoA carboxylase* (**ACC**) and *fatty acid synthase* (**FAS**). The former catalyses the formation of malonyl-CoA from acetate and the latter catalyses condensation cycles of malonyl-CoA with either acetyl-CoA or hydroxy butyrate (Barber et al., 1997). The uptake of circulating triglycerides is catalyzed by *lipoprotein lipases* (**LPP**) resulting in the formation of free fatty acids and glycerol. The transport of fatty acids into the mammary epithelial cells is catalyzed by fatty acid transport protein (**FATP**). Some of the fatty acids (for instance 18:0, *t*11-18:1) could be desaturated by *stearoyl-CoA desaturase* (**SCD**) resulting in the formation of *c*9-unsaturated fatty acid (*c*9-18:1, *c*9*t*11-CLA). The resulting pool of free fatty acids (*de novo* and preformed fatty acids) is then esterified to glycerol sequentially via *glycerol-3 phosphate acyl transferase* (**GPAT**), *acyl glycerol phosphate acyl transferase* (**AGPAT**), and *diacyl glycerol acyl transferase* (**DGAT**) (Bernard et al., 2008). Synthesis of triglycerides occurs on the endoplasmic reticulum and is secreted from the apical membrane of the cell as milk fat globule under the influence of certain proteins (butyrophilin, Ogg et al., 2004; xanthine oxidoreductase, McManaman et al., 2002; mucin 1 and adipophilin (Reinhardt and Lippolis, 2006; Cavaletto et al., 2008). Changes in the abundance of gene

transcripts for the lipogenic enzymes described above represent potential points of regulation and are reported to be transcriptionally regulated via sterol response element binding protein (**SREBP**1) which is described as a central signaling pathway in the regulation of mammary lipogenesis (Harvatine etal., 2009; Fig. 1-19).

1.7 Factors influencing milk CLA content

Conjugated linoleic acid content in milk can be influenced by **animal factors** (breed, age, parity, stage of lactation, desaturase activity) **dietary factors** (pasture, TMR diets, type and level of oil supplement, basal diet composition, use of marine lipids, and feed additives like ionophores and plant extracts) and **rumen factors** (F: C ratio, dietary starch and fiber, rumen pH, rumen fill and passage rate). Although forage: concentrate ratio, starch and fiber are actually dietary factors, they are described under rumen factors as they affect the rumen fermentation and hence influence the rumen environment. Among the three factors described above, diet plays a major role and strongly influences the CLA content in milk. Excellent reviews on dietary factors have been published in the last decade (Chilliard et al., 2001; Dhiman et al., 2005; and Collomb et al., 2006).

Animal factors

In this section, the effect of breed, parity, stage of lactation and animal to animal variation on milk CLA content are discussed. Although, animal factors influence milk CLA significantly, their contribution to the variation in milk CLA is less compared to dietary factors. Kelsey et al. (2003) reported that breed, parity and days in milk account for < 0.1, < 0.3 and < 2.0% of total variation in milk CLA content respectively. On pasture, Montbeliardes had higher concentrations of CLA (18.5 mg/g fat) compared to Normandes (16.4 mg/g) or Holstein Friesian (average of Irish and Dutch HF=16.6 mg/g) (Lawless et al., 1999). However, they

reported wide variations among individual cows within the breeds ranging from 4.8 mg/g to 35.6 mg/g fat. Milk fat concentrations of CLA were 7.2 mg/g and 5.9

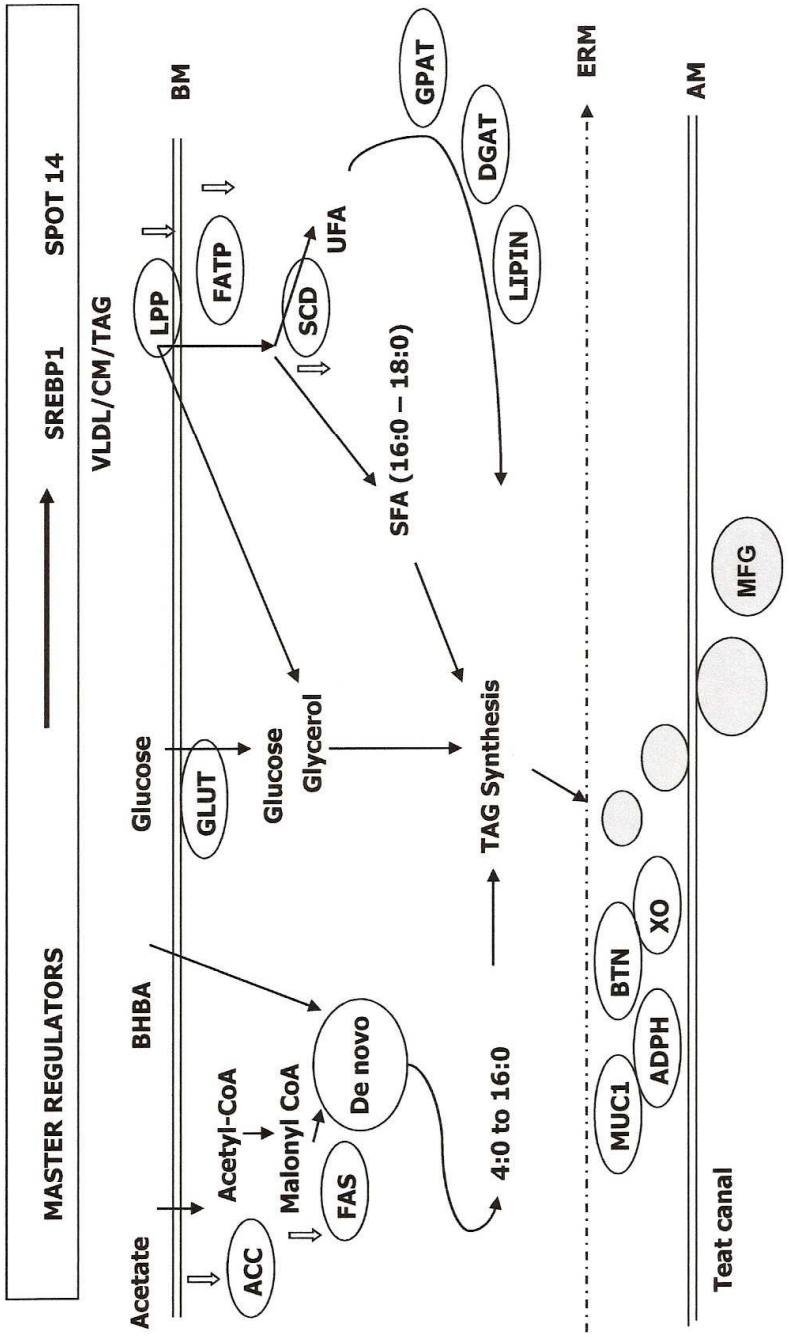

Figure 1-19. Regulation of mammary lipogenesis. Open arrows next to the ovals represent potential point of regulation of mammary lipogenesis. Key proteins are shown in ovals: ACC = acetyl-CoA carboxylase; ADPH = adipophilin; AM = apical membrane; BHBA = beta hydroxy butyric acid; BM = basal membrane; BTN = butyrophilinCM = chylomicrons; DGAT = diacylglycerol acyltransferase; ERM = endoplasmic reticulum; FAS = fatty acid synthetase; FATP = fatty acid transport protein; GLUT = glucose transporters GPAT = glycerol phosphate acyltransferase; LPP = lipoprotein lipase; MFG = milk fat globule; MUC = mucin; SCD = stearoyl-CoA desaturase; SREBP = sterol response element binding protein TAG = triacylglycerol; UFA = unsaturated fatty acid; VLDL = very low density lipoprotein; XO = xanthine oxidoreductase. Adapted from Harvatine et al., 2009.

mg/g on pasture and 4.1 mg/g and 3.2 mg/g on TMR ration for Holstein and Jersey cows respectively (White et al., 2001). They reported a significant breed and treatment effect on milk CLA content. The proportions of milk CLA were reported to be relatively constant across weeks for grazing cows compared to that for total mixed ration (**TMR**) fed cows. Whitelock et al. (2002) reported that Brown Swiss milk responded less to dietary manipulations compared to Holstein milk. However, the number of animals used in this study was limited (four Brown Swiss and eight Holsteins). Medrano et al. (1999) in their study identifying polymorphisms in bovine *scd* gene reported that Brows Swiss, Holstein and Jersey breeds differed in mammary *scd* activity. These differences could be confirmed from the study of Beaulieu and Palmquist (1995) and White et al (2001) wherein they reported that Holsteins produced significantly higher total 18:1 isomers than Jersey cows on the same diet. From the above discussion, it appears that breed effect on milk CLA, although less compared to diet effect, is significant.

There are limited data on the effect of parity number on milk CLA content. Stanton et al. (1997) reported that cows in the 5^{th} lactation or higher had more CLA in milk (0.59%) compared to cows in 2^{nd} to 4^{th} lactation (0.41%). However, they did not notice any relationship between lactation number and milk CLA content in a different study in which full fat rapeseed was fed to cows. Studies comparing milk CLA content between different stages of lactation are also limited. Kelsey et al. (2003) reported that stage of lactation had little effect on milk CLA or desaturase index. However, Audlist et al. (1998) reported a small increase going from 7.9 mg/g in early lactation to 9.7 mg/g in late lactation in their study with pasture fed cows at early (30 DIM), mid (120 DIM) and late (210 DIM) lactation. It is hard to conclude the effect of parity and stage of lactation on milk CLA considering the limited number of studies in this direction.

Milk CLA content can vary substantially from animal to animal even on the same diet. Up to 2-3 fold variation in milk CLA has been reported among individual cows on the same diet (Kelly et al., 1998; Peterson et al., 2002; Kelsey et al., 2003; Lock and Garnsworthy, 2004; Bell et al., 2006). These differences among individual cows were related to the rumen biohydrogenation intermediates and mammary desaturase activity (Peterson et al., 2002). Although treatment differences in the concentrations of rumen biohydrogenation intermediates (*t*10-18:1 and *t*11-18:1) were well discussed in most studies, individual animal variations in its concentrations are not reported. Mammary desaturase activity in most of the studies evaluating dietary effects on milk fatty acid composition has been based on ratios of specific fatty acid pairs (*c*12:1/12:0; *c*14:1/14:0; *c*16:1/16:0; *c*18:1/18:0 and *c*9*t*11-18:2/*t*11-18:1) described as desaturase indices (Bauman et al., 2001; Peterson et al., 2002). Of these, the ratio *c*9-14:1/14:0 has been reported to give a more accurate measure of mammary desaturase activity because 14:0 is synthesized *de novo* by the mammary tissue (Corl et al., 2002; Lock and Garnsworthy, 2002). These ratios have been reported to be influenced by breed (Kelsey et al., 2003), season (Lock and Garnsworthy, 2003) and lipid supplementation (Chouinard et al., 1999).

Recently, there has been an increase in the number of studies correlating milk fatty acid composition with animal genetics in an attempt to explore individual animal differences in milk CLA (Soyeurt et al., 2006; 2007; Milanesi et al., 2008; Kgwatalala et al., 2009a,b). Taniguchi et al. (2004) identified 8 single-nucleotide polymorphisms (SNP) in their study involving stearoyl-CoA desaturase (SCD) from 20 Japanese Black steers. They reported significant correlations between Ala293Val SCD1 genotypes and the fatty acid composition of intramuscular fat. Mele et al. (2007) in their study investigating SCD gene polymorphisms in Italian Holsteins reported that the relative frequencies of bovine SCD genotypes AA, AV and VV were 27%, 60% and 13% respectively. They

found that the A allele was associated with higher values for the fatty acid pair 14:1/14:0 and for the total MUFA percentage. However, they did not find any difference between SCD genotypes and milk CLA content. A significant effect of genotype (AA, AV and VV) on C10-, C12-and C14-index was reported in Canadian Jersey cows (Kgwatalala et al., 2009a) and in Canadian Holstein cows (Kgwatalala et al., 2009b). In both studies the genotypes AA and AV were significantly greater in the respective indices compared to the genotype VV. However, the C16-, C18- and the CLA index were not influenced by the SCD genotypes studied. The above findings suggest that the C16-, C18- and CLA index are not reliable indicators of mammary desaturase activity. It could be best interpreted from the C10-, C12- and C14 indices.

Dietary factors

The various dietary approaches described in the literature and their effect on milk CLA production will be discussed under the following headings: a) Effect of forage type and method of feeding; b) Oilseed and oil supplements; c) Fish oil/Marine lipid supplements; d) Ionophores; and e) Plant extracts.

Effect of forage type and method of feeding: Fresh grass is a rich source of LN. The concentration of LN in grass varies with the age of the grass, being higher when young and lower as the plant matures (Dewhurst et al., 2001). Further the concentration of LN is higher in leaves compared to stem portions. With the beginning of Spring when grass is growing, the leaves to stem ratio is greater which could account for the higher milk CLA in summer compared to other seasons. Further the fatty acid profile of grass species has been reported to be largely influenced by environmental factors, such as light, cutting interval, season and fertilizer regime (Dewhurst and King, 1998). Collomb et al. (2002) reported that CLA concentrations were 7.8, 14.1 and 21.3 mg/g fat from lowland to mountains and highlands respectively. They attributed the differences in milk

CLA with altitude to the decreasing proportion of grasses and a corresponding increase in dicotyledonous species.

Hay making reduces LN concentration considerably compared to silage making (Boufaied et al., 2003). Ensiling also reduces the fatty acid content of forage and the extent of PUFA loss during ensiling depends on factors such as duration of wilt and oxidative losses of unsaturated fatty acids from cut grass at the time of harvest (Steele and Noble, 1983; Dewhurst and King, 1998; Dewhurst et al., 2002). Forage conservation with the application of formic acid as additive has been shown to retain LN compared to those conserved without additives (Shingfield et al., 2005b). In general, milk CLA response varies among the forages, with fresh grass > hay > maize silage > grass silage (Chilliard and Ferlay, 2004).

Kelly et al. (1998) reported that milk CLA concentrations were doubled as cows on TMR were switched to pasture feeding (4.6 mg/g vs. 10.9 mg/g milk fat). In this study, no lipid supplements were added to the TMR diet. The reasons for the lower milk CLA observed for TMR diet compared to pasture in their study could be due to differences in the nature of lipids, availability of PUFA and amount of total PUFA between the two diets. These differences in the source and availability of PUFA could have resulted in greater levels of *t*11 C18:1 in rumen for pasture cows compared to TMR fed cows resulting in greater milk CLA for grazing cows following its desaturation to *c*9*t*11-CLA in the mammary gland.

Vegetable oils/oilseed feeding: Most of the oil supplements differ in the amounts of LA and LN they provide and hence influence milk fat CLA differently (Kennelly et al., 1996). For example, canola oil is a good source of *c*9-18:1 and LA; sunflower oil is a good source of LA and flax oil is a good source of LN. For enhancing milk CLA concentrations, it is necessary to supplement diets with a

source of LA or LN. Depending on the type and level of oil/oilseed supplemented, CLA increments in milk fat ranging from 6.3 to 41.2 mg/g have been reported with vegetable oils. Bell et al. (2006) reported milk CLA concentrations of 41.2 mg/g with safflower oil supplementation (6% DM and 60:40 F: C ratio) and 51.5 mg/g fat with a combination of safflower oil (6% DM) and monensin (24 ppm). Milk CLA concentrations as high as 53.7 mg/g (Shingfield et al., 2006) has been reported with the use of combinations of sunflower oil (3%) and fish oil (1.5%).

Dhiman et al. (2000) observed a dose-dependent increase in milk CLA with soybean oil (7.5, 7.6, 14.5 and 20.8 mg/g CLA with 0.5%, 1%, 2% and 4% soybean oil respectively) indicating the importance of dietary substrate. In the same study, Dhiman et al. (2000) also compared linseed oil with soybean oil and demonstrated that soybean oil was more effective in increasing milk CLA compared to linseed oil (21 mg/g and 16.3 mg/g of CLA with 3.6% soybean oil and 4.4% linseed oil respectively). Collomb et al. (2004) reported 107% increase in milk CLA content with 33% increase in the daily intake of LA (from 1 kg to 1.4 kg sunflower seed). From the above data, it could be concluded that oil supplements rich in LA and LN influence milk CLA concentrations differently which could be attributed to the differences in the biohydrogenation pathways for LA and LN. To identify the independent effects of LA and LN on milk CLA, Lock and Garnsworthy (2002) supplemented a unique mixture of oils in the concentrate to formulate 4 diets differing in LA and LN. The diets used were LL (low in LA and LN), LH-(low in LA but high in LN), HL- (high in LA and low in LN) and HH-(high in LA and LN). They reported milk CLA yields of 5, 7, 7, and 8 g/d with LL, LH, HL and HH diets respectively. According to Lock and Garnsworthy (2002), there was no difference in milk CLA response when either LA or LN was high but was synergistic when both were high. Bu et al. (2007) in a study comparing diets containing 4% soybean oil, 4% flax oil and a combination of 2% flax and 2% soybean oil reported that the effects of mixing sources of LA and LN

(50:50) on *t*11-18:1 and milk CLA were additive but not greater than when fed separately. The difference in response among the treatments for the two studies discussed above may be due to difference in total substrate supplied (LA + LN). In the study of Lock and Garnsworthy (2002), the synergistic effect of HH treatment was due to higher intake of total substrate for HH (596 g) compared to HL (477 g) and LH (467 g) treatments. In the study of Bu et al. (2007) the amount of total substrate was not very different among the treatments which could be the reason for the lack of synergistic effect with the combination of soybean oil and flax oil. This suggests that intake of total substrate accounts for maximum variation in milk CLA response.

Oil seeds have been used either whole or processed. Gonthier et al. (2005) reported milk CLA concentrations of 14, 14, and 19 mg/g fat by supplementing diets with 12.6% DM of raw, micronized and extruded flax seed respectively. Milk CLA concentrations were 3.1 mg/g and 19.9 mg/g with raw ground and extruded soybeans respectively when supplemented at 17.5% on DM basis (Chouinard et al., 2001). Collomb et al. (2004) in a study comparing ground rape seed, ground sunflower seed and ground linseed recorded milk CLA concentrations of 8.2 mg/g and 17.1 mg/g with 1 kg and 1.4 kg of ground sunflower seed respectively; 5.3 and 8.2 mg/g with 1kg and 1.4 kg of ground linseed respectively; and 6.2 mg/g with 1kg of ground rape seed. The above data suggest that processing increases lipid access to the rumen microbes compared to the raw seed, resulting in better lipid digestion and flow of biohydrogenation intermediates. In general, milk CLA response varies with the method of presentation of oil/oilseed, with oils > extruded seeds > raw seeds.

Interaction between dietary lipid source and forage type: Milk CLA response also depends on the nature of interaction between the oil supplements and the forage type in the diet. The increase in milk fat CLA in response to diets

supplemented with oils rich in LA and containing high concentrates has been shown to be transient (Bauman et al., 2000). Dhiman et al. (2000) reported a decline in the concentration of *t*11-18:1 and CLA for diets based on Lucerne and maize silage after 1-2 weeks of feeding. Similar findings were reported by Roy et al. (2006) for maize silage based diets containing higher proportion of concentrate. However, the increase in milk CLA concentrations with hay based diets supplemented with linseed oil in the same study was reported to be more persistent. They attributed the decrease in milk fat *t*11-18:1 and CLA over time with maize silage and high concentrate based diets to possible shifts in microbial populations leading to changes in biohydrogenation resulting in *t*10-18:1 replacing *t*11-18:1 as the predominant *t*-18:1 isomer in the rumen. Similar changes were reported for corn silage based diets supplemented with fish oil and sunflower oil in a 1:2 ratio (Shingfield et al., 2006) and for maize silage based diets supplemented with fish oil and sunflower oil in a 2:3 ratio in the high concentrate treatment (Shingfield et al., 2005a). Contrary to the above findings, Bell et al. (2006) in a study comparing barley silage based diets (F:C ratio = 50:50) supplemented safflower oil or linseed oil at 6% DM reported increases in milk CLA and *t*11-18:1 concentrations that were maintained for the 8 week duration of the study. The study of Bell et al. (2006) differed from the ones discussed above in basal diet composition and in the type and amount of oil supplemented. They used barley silage based diets and there was no difference in fiber content between the diets. However, information on rumen pH or dietary starch was lacking. The lack of time-dependent changes in *t*11-18:1 and CLA in their study could be due to difference in basal diet composition. However, *t*10-18:1 concentrations were significantly higher for safflower diets compared to flax diet indicating that there was a greater shift in microbial populations for safflower diet compared to flax diet. Roy et al. (2006) also reported higher *t*10-18:1 for diets supplemented with a source of LA (sunflower oil) compared with

those supplemented with a source of LN (linseed oil) suggesting that a shift to *t*10-18:1 is more likely to occur for diets containing greater concentrations of LA.

A number of factors are believed to play a role in inducing shifts in microbial populations resulting in greater concentration of *t*10-18:1. High starch and low fiber together with possible interaction between fiber level and PUFAs have been tested. Griinari and Bauman (2003) reported that shifts in ruminal biohydrogenation pathways are more pronounced when starch replaces fiber in the diet. Starch intake and ratio of starch to fiber in the diet has been shown to be positively associated with increases in milk fat *t*10-18:1 for diets containing sunflower oil and fish oil (Shingfield et al., 2006). High starch diets could influence biohydrogenation pathways favoring *t*10-18:1 formation by a reduction in ruminal pH and associated changes in the rumen bacterial populations. Chilliard and Ferlay (2004) demonstrated significant effects of basal diet on milk fat *t*10-18:1 content in goats. They reported that milk fat *t*10-18:1 was significantly lower with hay based diets compared to maize silage based diets. They also reported that there was significant interaction between F: C ratio and PUFA (linseed oil at 4.4% DM) on milk fat *t*10-18:1 in goats. The concentrations of *t*10-18:1 in goats was significantly lower with high forage (70% alfalfa hay, 8% starch) diets and significantly higher with low forage (46% alfalfa hay and 29% starch) diets. In the same study, the effect of F: C ratio on *t*11-18:1 and CLA was however, not significant.

Marine lipids: Fish oils are rich sources of EPA and DHA. Dietary fish oil supplementation has been reported to increase milk CLA concentrations from 0.2-0.6% for control diets to 1.5-2.7% for diets supplemented with a source of PUFA (Chilliard et al., 2001). AbuGhazaleh et al. (2004) and Wasowska et al. (2006) demonstrated *in vitro* that fish oils interfere with the biohydrogenation of LA and LN from basal diet, specifically inhibiting conversion of *t*11-18:1 to 18:0.

This results in increased ruminal production of trans-octadecenoic acids and a decrease in the concentrations of 18:0. Fish oils are low in 18 carbon PUFA, hence, feeding fish oil in combination with a source of LA/LN would be expected to increase milk CLA content much more than would be achieved with a PUFA source or fish oil alone (Whitlock et al., 2002). Shingfield et al. (2003) in a study using 1.6 % fish oil recorded 94 mg/g and 16.6 mg/g of *t*11-18:1 and CLA respectively in milk. In another study (Shingfield et al., 2006) with 1.5 % fish oil and 3% sunflower oil in a corn silage based diet (F: C=65:35), they reported as high as 53.7 mg/g CLA by day 5. However, they reported a fall in CLA to 23.5 mg/g by day 15.

Shingfield et al. (2005a) in their study evaluating the effect of proportion of concentrate and forage type on fatty acid composition of milk in cows fed sunflower oil and fish oil in the ratio of 2:3 (1.2 % fish oil and 1.8 % sunflower oil) reported that the concentrations of *t*11-18:1, total CLA and c9*t*11-CLA were independent of the forage type. However, high concentrate diets tended to decrease total CLA, *c*9*t*11-CLA and *t*11-18:1 and increased the concentrations of *t*10*c*12-CLA and *t*9*c*11-CLA.

In conventional dairy cow diets, EPA and DHA are present at minimal concentrations of < 0.1 % of total fatty acids. With the use of fish oils and marine algae in CLA research, only marginal increases of EPA and DHA in milk fat (0.2-0.4 %) were recorded (Chilliard et al., 2001). The extent to which marine lipid supplements enhance milk fat secretion of long chain PUFAs (n-3) is referred to as 'transfer efficiency'. Chilliard et al. (2001) from a review of several studies with fish oil supplementation reported that typical transfer efficiency of EPA and DHA were 2.6 % and 4.1 % respectively. Shingfield et al. (2006) reported transfer efficiencies of 2.0 % and 1.8 % for EPA and DHA respectively with 1.5 % FO and 3.0 % sunflower oil supplemented in a corn silage based diet (F:C =65:35).

However, Cant et al. (1997) reported transfer efficiencies as high as 9.3 % and 16.2 % for EPA and DHA respectively at an inclusion level of 2 % fish oil. The wide variation in the transfer efficiencies between Cant et al. (1997) and other studies reported are not obvious. There were, however, differences in the sampling time and source of fish oil between the studies. Unlike EPA and DHA, transfer efficiency of DPA was reported to be much higher than for EPA and DHA (20%-Shingfield et al., 2003; 30%-Offer et al., 1999). Higher transfer efficiency of DPA could be probably due to its lower ruminal biohydrogenation and to a relatively higher use by the mammary gland (Chilliard et al., 2000). When fish oil was administered postruminally (@270g/d), transfer efficiencies of EPA, DPA and DHA were reported to be as high as 20%, 71% and 18% respectively (Chilliard and Doreau, 1997) reflecting the impact of ruminal biohydrogenation on transfer efficiency.

Two hypotheses were proposed as a basis for the low transfer efficiencies of EPA and DHA into milk (Lock and Bauman, 2004). One hypothesis suggests the low transfer efficiency because of extensive biohydrogenation of these FA in the rumen. Second hypothesis proposes that low transfer of the two FA could be the result of their partitioning into plasma lipid fractions that are less available to the mammary gland. Lipid uptake by mammary gland is most efficient when the plasma lipid fraction exists in triglyceride form. However, recent studies have shown that supplements of EPA and DHA occur more in the cholesterol ester and phospholipid fractions compared to the triglyceride fraction of plasma lipids (AbuGhazaleh et al., 2003).

Several studies reported lower milk fat concentrations with fish oil diets compared to control diets (Loor et al., 2005b-2.5 % fish oil; Loor et al., 2005c-1.5 % fish oil and Shingfield et al., 2006-1.5 % fish oil). Similar findings were observed in a study comparing diets supplemented with 0.5 % fish oil along with 1.5 %, or

3 % or 4.5 % sunflower oil (Cruz-Hernandez et al., 2007). However Shingfield et al. (2005a) in a study comparing fish oil-1.2 % and sunflower oil-1.8 % in maize silage and corn silage based diets differing in F: C ratio did not observe any difference in milk fat concentration or yield when compared to control diet. This was possibly because the cows in their study were in late lactation. Chilliard et al. (2001) in their review mentioned that the decrease in milk fat content is poorly related to the amount of fish oil in the diet (r^2 = 0.38). They reported that milk fat content in cows fed fish oil is better related to the amount of EPA (r^2 = 0.75) or EPA + DHA (r^2 = 0.68).

Ionophores: Ionophores are lipophilic compounds introduced into cattle rations as feed additives. There are about seven different ionophores approved for use in livestock in the United States (McGuffey et al., 2001) of which monensin and lasalocid are commonly used. Monensin is utilized in cattle rations for its increased efficiency of energy metabolism, nitrogen metabolism and in the control of abnormal rumen fermentation (Bergen and Bates, 1984). The above beneficial effects of monensin are derived mainly by altering rumen fermentation.

Monensin has been reported to cause a shift in bacterial population from Gram positive to Gram negative (Moss et al*.,* 2000). Cellulolytic ruminococci and *Butyrivibrio fibrisolvens* were reported to be particularly sensitive to ionophores, but *Bacteroides (Fibrobacter) succinogenes* was able to grow even in the presence of 2.5 µg of monensin per ml (Chen and Wolin, 1979). *Streptococcus bovis,* which proliferates when starch is abundant and produce lactate, has been reported to be sensitive to monensin (Russell, 1987). However *Megasphaera elsedenii* (lactate utilizing species) and *Selenomonas ruminantium* (a less active lactate utilizer) are resistant to monensin (Nagaraja and Taylor, 1987). Bacteria that reduce succinate to propionate are resistant to ionophores resulting in an

increase in propionate production (McGuffey et al., 2001). Several of the Gram positive bacteria are involved in rumen biohydrogenation including *Butyrivibrio fibrisolvens*. Ionophores have been reported to inhibit linoleic acid biohydrogenation, resulting in a decrease in 18:0 and increase in *t*-18:1 concentration in ruminal contents (Fellner et al., 1997). Bell et al. (2006) reported an increase in *t*-18:1 isomers in milk when monensin was supplemented at 24 mg/kg DM. Odongo et al. (2007) observed that *t*11-18:1 was greater for monensin supplemented diets (@ 24 mg/kg DM) compared to the control diet.

However, the effect of ionophores in increasing milk *t*11-18:1 and CLA have been reported to be transient (Guan et al., 2006; Johnson and Johnson, 1995; Sauer et al., 1998), possibly because of the ability of rumen microbes to adapt to the ionophore supplemented. Guan et al. (2006) observed that the duration of suppression of methane production was longer when animals were on a low-concentrate diet than when on a high concentrate diet. This was in agreement with Johnson et al. (1997) and Odongo et al. (2007) who suggested that the adaptation response for ionophores might be related to the composition of the diet fed in terms of available energy. Another strategy to overcome the response of adaptation to ionophores by rumen microbes is to rotate feeding different ionophore combinations. However, definitive effects of such ionophore combinations and its effects on adaptations are lacking.

Monensin supplementation has also been reported to decrease milk fat yield. McGuffey et al. (2001) compiled reports from 11 publications and reported that monensin produced significantly higher milk yields and lower milk fat contents compared to control diets. Bell et al. (2006) observed no difference in milk fat compared to the control when monensin alone was supplemented (@24 mg/kg DM) for diets containing 60:40 F: C ratio. However they recorded milk fat

depression (**MFD**) when monensin was supplemented along with a source of PUFA (safflower oil at 6% DM). Odongo et al. (2007) observed a fall in milk fat yield when monensin alone was supplemented at 24 mg/kg DM. However, the MFD was reported to be greater when a source of PUFA was also supplemented (Alzahal et al., 2008). Phipps et al. (2000) also observed a depression in milk fat when monensin was supplemented at 7.5, 15 and 24 mg/kg DM compared to the control. Jenkins et al. (2003) in an *in vitro* study reported a significant interaction between monensin and fat. The above findings suggest that MFD for monensin supplemented diets is greater when a source of PUFA is added. Duffield (2003) reported that the negative association between monensin and milk fat is due to an interaction between monensin and dietary factors. However, Bell et al. (2006) observed no effect on milk fat content or yield in cows receiving monensin. Effects are variable which could be due to differences in ruminal adaptations in which ionophore resistant species replace ionophore sensitive bacteria for ruminal biohydrogenation.

Plant extracts: In recent years, interest in the use of plant extracts as modifiers of rumen fermentation has increased considerably because of the reduced social acceptance in the use of antibiotics in animal feeds. Plant extracts contain a variety of organic compounds like saponins, tannins and essential oils. The effects of saponins (Cheeke, 1999), tannins (Min et al., 2003) and essential oils (Calsamiglia et al., 2007) on rumen microbial fermentation has been extensively reviewed. These substances have been shown to exhibit antimicrobial activity against a variety of bacteria, yeasts and molds (Gershenzon and Croteau, 1991). Some of the studies that evaluated the role of these substances on rumen fermentation have also investigated their effects on milk fatty acid composition. Benchaar et al. (2006) in their study comparing the effect of monensin and essential oils on milk fatty acid composition did not find any difference in the concentrations of milk CLA. Similar findings were reported in another study

comparing the effect of essential oils and silage type (alfalfa vs corn silage) on milk CLA (Benchaar et al., 2007). Benchaar and Chouinard (2009) reported no differences in milk CLA, *t*10-18:1 *and t*11-18:1 for cows offered cinnamaldehye (major component of cinnamon oil), condensed tannins from quebracho trees and saponins from *Yucca schidigera* compared to the control. However, in an *in vitro* study, inclusion of cinnamaldehye was reported to induce a *t*10-18:1 shift (Lourenço et al., 2008). Vasta et al. (2009) in their study investigating the effect of tannins (from carob, acacia leaves and quebracho) on rumen biohydrogenation of linoleic acid *in vitro,* reported that vaccenic acid was accumulated while stearic acid was reduced. They concluded that tannins reduce ruminal biohydrogenation of linoleic acid by inhibiting the proliferation of ruminal micro-organisms. More recently, a similar study investigating the effect of condensed tannins on biohydrogenation of α-linolenic acid *in vitro* reported that *t*11-18:1 was higher and 18:0 lower compared to the control (Khiaosa-Ard et al., 2009). Inconsistencies among the above studies could be due to the differences in the environment for the growth of rumen bacteria (*in vitro* vs *in vivo*).

Rumen factors

Apart from diets being different in the amount and source of substrate, diets can also influence the rumen environment and in turn modify the microbial activity associated with PUFA biohydrogenation in the rumen. Various factors or combinations of factors in diets could be responsible for its effects on microbial activity. Diets differing in forage to concentrate ratio can influence rumen biohydrogenation and milk CLA concentrations due to differences in

a) the amounts of total substrate (LA + LN)
b) the amount of total starch
c) the amount of fermentable starch
d) rumen pH and in turn shifts in microbial populations

e) interactions between forage and oil depending on the source of forage.

It is well known that unsaturated fatty acids are extensively biohydrogenated in the rumen. However the degree of biohydrogenation differs with the type of the diet. Dietary lipids are first hydrolyzed by bacterial lipases in the rumen. The extent of lipolysis has been reported to be high (85-95%) with unprotected lipids compared to those fed in a protected form. Further lipolysis is reported to be higher with conventional oils as compared to the lipids in cellular structures (Bauchart et al., 1990). Gerson et al. (1985) have shown that lipase activity was higher for diets rich in fiber than for diets rich in starch. On average, biohydrogenation of linoleic and linolenic acids (based on measurement of duodenal flows) has been reported to be 80 and 92%, respectively (Doreau and Ferlay, 1994). Biohydrogenation of unsaturated fatty acids is shown to be reduced with the increase in percentage of concentrates in the diet. Van Nevel and Demeyer (1996) demonstrated that biohydrogenation of linoleic and linolenic acid (based on LA or LN disappearance) was 50 and 65% respectively when 70% concentrates were used in the diet. They attributed the lower percentage of PUFA hydrogenation to the inhibitory effect of low pH associated with high concentrate feeding on lipolysis and hydrogenation. Kalscheur et al. (1997b) demonstrated the effect of low rumen pH on biohydrogenation inhibition and increased outflow of *t*-18:1 intermediates from the rumen. Jurjanz et al. (2004) attributed the reduction in rumen pH and increased levels of *t*-18:1 isomers in milk to the differences in the rate of degradation of starch in the rumen.

Martin and Jenkins (2002) in their study on factors affecting CLA and vaccenic acid (**VA**) production by mixed ruminal bacteria reported that culture pH could have major influence on the production of CLA. Qiu et al. (2004) determined the effects of pH in continuous culture on flows of CLA and TVA and

reported that a lower pH of 5.8 produced more CLA *in vitro* compared to another culture at a pH 6.5.

Animal to animal variations in milk CLA response were also attributed to differences in intake characteristics, for instance, amount of time spent chewing, ruminating and saliva production which in turn could influence rumen pH and microbial populations and biohydrogenation of PUFAs (Offer, 2002; Elgersma et al., 2003). From the above discussion, it appears that rumen pH plays a significant role in the increased outflow of *t*-18:1 intermediates from the rumen.

Rumen fill and passage rate: Rumen fill and passage rate can influence milk CLA content as digesta residence time in the rumen influence degree of biohydrogenation of unsaturated fatty acids in the rumen. In general, smaller and heavier feed particles like grain and fresh forage have more rapid transit times in the rumen compared to lighter feed particles like hay and silage. Higher passage rates in grazing animals compared to animals fed conserved forage based rations could be one of the reasons that would limit PUFA biohydrogenation. Chaves and Boudon (2005) in their study to test the influence of rumen fill on intake and milk production in dairy cows fed perennial rye grass, reported a negative relationship between rumen fill and DMI. Measuring passage rate requires the use of markers. The information on passage rate will be valuable for evaluating diets in terms of its response on milk CLA. It should be noted that CoEDTA and a triple marker system based on yetterbium acetate (YbAc), CoEDTA and chromium-mordanted straw has been reported to reduce the concentrations of milk fatty acids containing a *cis*-9 double bond (Shingfield et al., 2008). They further reported that a triple marker system based on CrEDTA, YbAc and indigestible neutral detergent fiber can be used to estimate nutrient flow without altering milk fat composition in lactating cows.

1.8 Milk fat depression

The low-fat milk syndrome (referred to as MFD) is a challenging biological problem involving interrelationships between digestive processes and tissue metabolism. Milk fat depression was first recognized in 1885 when a reduction in milk fat yield was observed in dairy cows fed beets (Griinari and Bauman, 2001). Milk fat depression is defined as a decreased mammary capacity for lipid synthesis due to a co-ordinated transcriptional down-regulation of enzymes and proteins involved in milk fat synthesis (Harvatine et al., 2009). Milk fat content and composition can be markedly affected by diet and has been extensively reviewed (Sutton, 1989; Grummer, 1991; Palmquist et al., 1993; Doreau et al., 1999; Jensen, 2002; Jenkins and McGuire, 2006).

According to Davis and Brown (1970), diets that induce MFD can be classified into 2 broad groups. One group involved the high grain/low-fiber diets that provided large amounts of readily fermentable carbohydrates. Diets where the fiber content is adequate but the fiber source is pelleted or ground were also reported to fall into this group. Certain diets based on corn silage may appear to contain an adequate level of fiber, however its effectiveness in maintaining normal rumen function and hence normal milk fat is less than the fiber in grass silage based diets. Even a high proportion of grain in corn silage can reduce the effectiveness of fiber in the diet. Because the effectiveness of dietary fiber is the main determinant of the ability of the forages to buffer the rumen and support normal milk fat, they used the term low-fiber or more appropriately low effective fiber diets as the description for this group of diets (Davis and Brown, 1970). The second group of diets involved diets supplemented with a source of PUFA (vegetable oilseeds or oils and marine oils). The presence of PUFA was considered a prerequisite for MFD to occur with low fiber diets (Griinari et al., 1998). Diets supplemented with plant oils but containing sufficient fiber to maintain normal rumen function did not record MFD (Brown et al., 1962;

Kalscheur et al., 1997a). Therefore, diets supplemented with plant oil or oilseed but containing adequate effective fiber do not fall into this group. In contrast to the diets supplemented with plant oils, diets supplemented with fish oil or oils from marine mammals and marine algae were reported to induce MFD, regardless of the level of starch or fiber in the diet (Chilliard et al., 2001; Ahnadi et al., 2002).

Several theories have been proposed to explain MFD, with the basis for all these theories being an alteration in rumen fermentation (Bauman and Griinari, 2001, 2003). The first theory proposed that MFD was caused by a limitation in the supply of precursors - acetate and butyrate (Doreau and Chilliard, 1997). According to the second theory (glucogenic-insulin theory of MFD), increased ruminal production of propionate and increased hepatic rate of gluconeogenesis increased insulin secretion, resulting in an inhibition of adipose tissue lipolysis and increased uptake of lipogenic precursors by extra-mammary tissues which are insulin sensitive (McClymont and Vallance, 1962; Annison et al., 1974).

Davis and Brown (1970) were the first to introduce the '*Trans* Fatty Acid' theory of MFD which suggested a possible relationship between *trans* octadecenoic acid and MFD. Later Griinari et al. (1998) found that MFD was associated with a specific increase in *t*10-18:1, rather than *t*18:1 isomers in general. Griinari and Bauman (1999) presented a putative pathway where *t*10-18:1 was formed from the reduction of *t*10*c*12-CLA in the rumen as an alternative way of biohydrogenation of linoleic acid. The possibility for the formation of *t*10-18:1 in the rumen was demonstrated as early as 1966 when Kepler et al. (1966) showed that *Butyrivibrio fibrisolvens* was able to hydrogenate *t*10*c*12-CLA to *t*10-18:1. More recently *Megasphaera elsdenii* YJ-4 has also been shown to produce *t*10*c*12-CLA (Kim et al., 2000). To accommodate

for the limitations in the *trans* fatty acid theory, Bauman and Griinari (2001) proposed the 'biohydrogenation theory' of MFD. This theory is based on the concept that unique fatty acid intermediates produced in rumen biohydrogenation inhibit the ability of the mammary gland to synthesize milk fat.

Later, several feeding studies (Offer et al., 2001a; Piperova et al., 2000; Loor et al., 2002) supported the biohydrogenation theory of MFD, based on stronger correlations between milk fat yield and *t*10-18:1. Because *t*10-18:1 was not available in pure form at that time, infusion studies were confined to the use of *t*10*c*12-CLA. Baumgard et al.(2000) and later Loor and Herbein (2003) in their infusion trials using pure-CLA isomers demonstrated that *t*10*c*12-CLA inhibited milk fat synthesis. Baumgard et al. (2000) reported 48% reduction in milk fat yield with the infusion of 13.6 g/d of *t*10*c*12-CLA which was accompanied by a dramatic reduction (> 35%) of mRNA abundance of lipogenic enzymes (ACC, FAS, LPL, FABP, SCD, GPAT and AGPAT). However, with feeding studies, the concentrations of *t*10*c*12-CLA in the rumen, duodenal fluid or milk always remained very low compared to the concentrations recorded in infusion studies (Piperova et al., 2000; Bauman and Griinari, 2003; Loor et al., 2004a,b; 2005a,b). Besides, in cows fed marine oil supplemented diet for which MFD was observed, only traces of milk *t*10*c*12-CLA was recorded, but substantial increases in *t*10-18:1 were observed (Offer et al., 2001b; Loor et al., 2005c). More recently Lock et al. (2007) using chemically synthesized *t*10-18:1 infused postruminally at 42.6 g/d/cow did not observe any effect on milk fat yield. The authors speculated that when rumen environment is altered, there could be several other biohydrogenation intermediates which would act synergistically to induce milk fat depression rather than *t*10-18:1 alone depressing milk fat.

Several isomers have been proposed as potential inhibitors of milk fat based on negative correlations between milk fat yield and concentrations of

specific isomer in milk. Some of these include *t*9*c*11-CLA (Roy et al., 2006; Shingfield et al., 2005a; 2006) *t*7*c*9-CLA (Kadegowda et al., 2009 using principal component analysis), *c*9*t*13-18:2, *c*9*t*12-18:2, *t*11*c*13-CLA, *t*11*c*15-18:2 (Loor et al., 2005a). Several *c*- and *t*-18:1 isomers and CLA isomers tested in cows using infusion studies are tabulated in Table 1-4. From this, it could be observed that no *c*- or *t*-18:1 isomers is inhibitory to milk fat yield, however 3 CLA isomers [*t*10*c*12-CLA (11 studies), *c*10*t*12-CLA (1 study) and *t*9*c*11-CLA (1 study)] have been reported to be inhibitory to milk fat yield.

Table 1-4. List of trans- and CLA isomers tested in infusion studies in cows

	Isomer(s) investigated	Effect on milk fat yield	Reference
1	*c*9-18:1	Promotes	Enjalbert et al., 1998
2	*c*11-18:1	Neutral	Shingfield et al., 2007a
3	*c*12-18:1	Neutral	Shingfield et al., 2007a
4	*t*9-18:1	Neutral	Rindsig and Schultz (1974)
5	*t*10-18:1	Neutral	Lock et al., 2007
6	*c*9-18:1	Promotes	Enjalbert et al., 1998
7	*t*11-18:1	Neutral	Rindsig and Schultz (1974) Shingfield et al., 2007a
8	*t*12-18:1	Neutral	Rindsig and Schultz (1974) Shingfield et al., 2007a
9	*c*9*t*11-CLA	Neutral	Baumgard et al., 2000; 2002 Loor and Herbein (2003) Shingfield et al., 2006
10	*t*9*c*11-CLA	Inhibitory	Perfield et al., 2007
11	*t*10*c*12-CLA	Inhibitory	Bell et al., 2003 Perfield et al., 2004; 2006; 2007 Baumgard et al., 2000; 2001;02 Loor and Herbein (2003) De Veth et al., 2004 Peterson et al., 2002 Saebo et al., 2005
12	*c*10*t*12-CLA	Inhibitory	Saebo et al., 2005
13	*t*8*c*10-CLA	Neutral	Perfield et al., 2004
14	*c*11*t*13-CLA	Neutral	Perfield et al., 2004
15	*t*9*t*11-CLA	Neutral	Perfield et al., 2007
16	*t*10*t*12-CLA	Neutral	Saebo et al., 2005 Perfield et al., 2006

Note: CLA = conjugated linoleic acid; *c* = *cis*; *t* = *trans*

A decrease in *scd* activity with a subsequent decrease in *c*9-18:1 has been proposed to cause diet induced MFD (Loor and Herbein, 2003; Loor et al., 2005a). Recently, Shingfield and Griinari (2007b) proposed this as an extension of the biohydrogenation theory of MFD, suggesting that specific inhibition of *scd* over extended periods of time would induce MFD through changes in the mammary supply of 18:0 and *c*9-18:1. However, Harvatine et al. (2009) reported that decreased activity of *scd* is not a prerequisite for MFD owing to the lack of direct evidence supporting inhibition of *scd* as a causative factor for diet-induced MFD. More recently Granlund et al. (2005) based on an *in vitro* study looking into the effects of structural changes in fatty acids on lipid accumulation in adipocytes and hepatocytes in mice reported that a change in the carbon chain (desaturation) between the carboxyl group and the *c*/*t* 10, 12 double bond structure will reduce the anti-lipogenic effects of *t*10*c*12CLA. This was consistent with the abolition of the inhibitory effects of *t*10*c*12-CLA on milk fat following the action of Δ^6-desaturase (Saebo et al., 2005) which introduces a double bond between 6^{th} and 7^{th} carbon atoms leading to the formation of *c*6*t*10*c*12-18:3. From the above finding and the information in Table 1-4, it could be observed that a small difference in structure of fatty acids could result in striking differences in its potency and action on lipid accumulation. Considering the significance of the structure of the fatty acid or isomer in its mode of action, Shingfield and Griinari (2007b) reported that future attempts towards the elucidation of the structure and function of additional minor biohydrogenation intermediates would require more sensitive analytical techniques. They recommended that the carboxyl group has to be derivatized with a nitrogen-containing agent because nitrogen is preferable to alkyl chain to minimize ionization and migration of double bonds during mass spectroscopy.

1.9 Conclusions and implications

In summary, diet plays a major role in influencing milk CLA production. Although substrate supply (LA + LN) in the diet determines the amount of milk CLA content, a favourable rumen environment is also important to help favor greater *t*11-18:1 production in the rumen. Interplay of a number of factors such as basal diet composition, F: C ratio, dietary starch, fiber content and possible interactions between forage and lipid source play an important role in determining the rumen environment. Most of the studies conducted world-wide have tested each of these parameters separately to determine what favors greater *t*11-18:1 production in the rumen; however diets used in the various studies differed in the basal diet composition which does not allow direct comparisons to be made between the studies. Fish oil appears to be a promising dietary component in enhancing CLA content and n-3 PUFA in milk. Further, the lipid lowering and energy sparing effect of fish oil could be better utilized in transition cows to help recover from the negative energy balance and still maintain milk CLA production. Effect of ionophores on milk CLA appears to be variable. Ruminal concentrations of *t*11-18:1 appears to determine milk CLA content. Therefore it is important to minimize shifts to *t*10-18:1 production in the rumen so that *t*11-18:1 in rumen can be maximized. The key to this is to understand the nature of dietary interactions on PUFA biohydrogenation.

1.10 General objectives

The general objective of this research was to gain an overall understanding of the biology of milk CLA synthesis. In this regard, several experiments were carried out with the following goals:

- To evaluate the efficiency of grazing cows in comparison to zero-grazing and grass silage feeding aimed at identifying factors that make grazing cows so efficient in milk CLA production.

- To investigate the effect of rate of starch fermentation on rumen PUFA biohydrogenation and milk CLA aimed at understanding the influence of rumen fermentation pattern on the end-products of PUFA biohydrogenation.

- To study the effect of stage of lactation on milk fatty acid composition aimed at examining the persistency of milk *t*10-18:1, *t*11-18:1 and CLA for full lactation length.

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Grazing cows are more efficient than zero-grazing and grass silage feeding on milk rumenic acid production

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2.1 Introduction

Rumenic acid (**RA**) is a component of milk fat with demonstrated health benefits. Biosynthesis of milk RA requires a source of polyunsaturated fatty acid (**PUFA**) substrate such as linoleic acid (**LA**) and linolenic acid (**LN**) in the feed that is biohydrogenated in the rumen supplying a combination of precursor, vaccenic acid (**VA**) and small amounts of product (RA) to the mammary gland (Bauman et al., 1999). The extent of ruminal biohydrogenation of PUFA in turn has been reported to be influenced by the initial concentration of dietary LA (Harfoot et al., 1973), passage rate and rumen pH (Martin and Jenkins, 2002; Troegeler-Meynadier et al., 2003; Qiu et al., 2004). In the mammary tissue, most of the RA is formed from VA by the action of $\triangle^9$-desaturase (Griinari et al., 2000). Thus biosynthesis of milk RA is dependent on the amount of substrate in feed, extent of biohydrogenation in the rumen and the desaturase activity of the mammary tissue. Although the significance of the above three factors on milk RA concentrations is well known, the relative contribution of each has not been established.

Fresh grass is a rich source of LN. However its concentration varies with the growth stage and the form in which it is fed. Chilliard and Ferlay, (2004) reported that milk RA response varies among forages, with fresh grass > hay > maize silage > grass silage. Further pasture (grazing) and barn (zero-grazing) feeding, which is basically feeding fresh grass outdoor and indoors, respectively, has been reported to influence milk RA response differently (Leiber et al., 2005). Offer (2002) and Elgersma et al. (2003) found that beneficial fatty acids (**FA**) including RA were greater in milk for grazed animals than for zero-grazed grass or grass silage.

The present study was designed to investigate possible mechanisms underpinning the differences in milk RA concentrations and yields among cows

grazing (**G**), zero-grazing (**ZG**) or fed grass silage (**GS**) with the following objectives: i) to determine the supply of substrate from the different diets; ii) to investigate if offered and consumed grass by grazing cows differed in substrate content; iii) to determine the effect of the diets on rumen environment; iv) to gain an understanding of the biosynthesis of milk RA by evaluating the FA composition of feed, rumen ingesta, plasma and milk with specific interest in VA and RA; v) to investigate if there were treatment differences in the efficiency of milk RA production; vi) to identify the most important variables contributing to the differences in milk RA.

2.2 Materials and methods

Experimental design and treatments

All procedures were carried out under license in accordance with the European Community Directive 86-609 – EC. Six spring-calving rumen-cannulated pluriparous (lactation number 2 to 5) Holstein Friesian dairy cows in early lactation (average milk yield 21.9 ± 4.8 L/d; average days in milk 76 ± 18 days; average body weight 542 ± 34 kg) were balanced for milk yield and days in milk and assigned to two squares. Within a square the animals were randomly assigned to 3 treatments: grazed grass, zero grazed grass and grass silage. In a 3×3 Latin square design with three 21-day periods. Cows received 3 kg/day per cow of concentrate supplement. The experiment was undertaken between June and August on a perennial ryegrass sward at the Dairy Production Research Centre, Moorepark, Ireland (55°10′ N, 8°16′ W). All the experimental animals were grazing a perennial rye grass sward before the feeding experiment. Silage was harvested from the same perennial rye grass sward after 5 weeks growth, field wilted for two hours and baled in bags in May.

Management of grazing cows

A grazing area of 1.7 ha consisted of two main grazing blocks. These were sub-divided into two equal halves to offer grass for G or ZG animals. Paddock area utilized for the study was divided width-wise in such a way that the G and ZG cows received the same quality of grass throughout the experiment. Grass cuts (**GC**) were taken twice weekly (at 4 cm from the ground) to determine the herbage mass by cutting two strips (1.2 x 10 m) with a motor Agria (Etesia UK Ltd., Warwick, UK). Ten grass height measurements were recorded before and after harvesting on each cut strip using an electronic plate meter (Urban and Caudal, 1990) with a plastic plate (30 x 30 cm and 4.5 kg/m^2) [Agro systemes, Choiselle, France]. This determined the sampled height precisely and allowed the calculation of the sward density [dry matter per hectare divided by the pre-cutting height minus the post-cutting height (kg DM/cm per ha)]. All mown herbage from each strip was collected, weighed and a representative sample (300g) retained. A 100 g sub-sample of the herbage was dried overnight at 90^{o}C in an oven for DM determination. The remaining 200 g of the herbage collected from the two strips was bulked, sub-sampled (~100 g) and stored at -20^{o}C. Pre-grazing and post-grazing sward heights were recorded daily by taking measurements across the diagonals of the grazed strip. The measured pre-grazing sward height, multiplied by the mean sward density was used to calculate the daily herbage allowance of the grazing animals. Grazing cows were strip-grazed through each half of the two blocks to offer 20 kg dry matter/cow per day. Electric fences were used for strip-grazing and were moved once daily with a back fence so that the cows did not have access to the area already grazed. A concentrate containing rolled barley (300 g/kg), citrus pulp (460 g/kg) soybean meal (180 g/kg), soybean oil (30 g/kg) and mineral/vitamin mix (30 g/kg) was offered to all cows at a rate of 3 kg/d before the evening milking.

Individual intakes for the grazing cows were measured using a controlled release n-alkane marker (Alkane CRC, Captec (NZ) Ltd, Auckland, New Zealand) in each period. For each animal, one alkane bullet was introduced into the rumen through the canula on day 7. Herbage snip (**HS**) samples (approximately 500 g in polythene bags were collected by following the cow at several points in the paddock and cutting a representative sample of grass with a clipper), which closely represented the herbage that the cows were selecting were collected daily from the 7th day following the introduction of the bullet for a period of 6 days and stored at -20^{o}C. Fecal grab samples (approximately 250 g) were collected twice daily for 7 days from the 8th day following the introduction of the bullet and stored at -20^{o}C until analysis. The HS were freeze dried, milled and analyzed individually. The fecal grab samples of each grazing cow from the total collection period were bulked to obtain one sample per cow per period. This was dried for 48 h at 40^{o}C, milled and analyzed. The n-alkane concentration in the HS and feces was determined following the method described by Mayes et al. (1986).

Management of indoor cows

Cows were housed in tie-stalls with water available at all times. Indoor cows were offered *ad-libitum* grass or silage. The intakes were adjusted to have at least 5-10 % of weigh-backs. Grass offered to the ZG animals was cut twice daily at 4 cm height from the ground with a motor Agria. Offered and refused grass and silage samples were collected twice daily from the indoor fed cows during the experimental period and DM intakes calculated. Concentrate feeding was the same as for the G cows.

Sampling and chemical analyses

Samples of grass offered to the grazing cows were collected bi-weekly from GC (4 cm from the ground) and combined into one sample per week.

Forage samples for the indoor cows (ZG and GS) were collected daily (AM and PM). Both morning and evening samples offered to ZG and GS were bulked by treatment on a weekly basis to obtain three samples per treatment per period. All the forage samples collected were freeze-dried and ground through a 1-mm screen using a Wiley mill (Thomas-Wiley Philadelphia, PA). Samples were analyzed for DM content (at 135^{o}C for two hours), ash (overnight at 550^{o}C), ADF, NDF [Ankom filter bag technique with the reagents sodium sulphite and alpha-amylase; Undersander et al. (1993)], and nitrogen content (multiplied by 6.25 for determination of CP using Leco nitrogen combustion analyzer, Leco Corporation, St. Joseph MI, USA). For the grazing cows, HS were collected (4 cm from the ground) in the 3rd week of each period for 6 consecutive days. These samples were freeze-dried, milled and combined to obtain one sample per period representing consumed grass for the grazing cows. Herbage snips were collected by following each cow at different points within the paddock and collected grass samples (4 cm from ground) using a hand-held grass cutter. The concentrate fed was sampled once per period. The combined forage samples from week 3 of each period and the concentrate from the respective period were utilized for FA analysis. Total lipids from the forage and concentrate samples were extracted with chloroform/methanol (1:1) along with an internal standard (23:0) and methylated with methanolic HCl. The fatty acid methyl esters (**FAME**) obtained were purified by thin layer chromatography using hexane/diethyl ether/acetic acid (85:15:1) and then analyzed by GLC. The FA contents of forage and concentrate were determined from the quantity of the sample analyzed relative to the internal standard. The product of forage DM intake and the forage FA content gave the forage FA intake. Likewise the FA intake from the concentrate supplement was also calculated. Total FA intake of individual animals was determined from the sum of the forage and concentrate FA intake. LA and LN intakes were estimated from the product of the proportions of the respective FA

from the total DMI with the total FA intake. Total substrate intake was determined by summing LA and LN intake.

Rumen sampling was done both AM (before feeding) and PM (six hours after morning feed) on days 18 and 19. Rumen samples were collected from dorsal, ventral, cranial and caudal regions of the rumen in two sterilized 250 mL containers covered with air tight lids. One of the containers was stored at -20^{o}C for FA determination and the contents from the other was passed through 4 layers of cheese-cloth and stored frozen in six 14 mL Falcon tubes. Two tubes were utilized for recording pH immediately after collection using a Radiometer pH meter fitted with a glass electrode (Radiometer, Copenhagen) and the other four tubes were stored at -20^{o}C until utilized for VFA and ammonia nitrogen analysis following the procedures of Ranfft et al. (1973) and Short (1954) respectively. Wet rumen fill was recorded on the final day (day 21) of each period by evacuating and weighing the total rumen contents. Dry matter content of a representative sample was measured and used to calculate rumen DM fill. After collecting the samples, the whole contents were placed back into the animal's rumen.

Rumen samples for FA determination were freeze-dried and milled. The samples collected on days 18 and 19 were pooled and about 0.5 g of the pooled samples was utilized for FA analysis following the protocol described by Or-Rashid et al. (2008). The hexane extract obtained following methylation [with TMS-diazomethane (TCI America, Portland, OR)] was condensed to about 100 µL under a stream of N_2 and applied to a Silica Gel-G plate (Fisher Scientific, Ottawa, ON Canada). The thin layer chromatography (**TLC**) plate was developed using the solvent - hexane/diethyl ether/acetic acid (in the ratio 85:15:1 v/v/v) and visualized with 2', 7'-dichlorofluorescein under UV light. The FAME band was scraped off the TLC plate and extracted using hexane for analysis by GLC.

Blood was collected from the coccygeal vessels in heparinized vacutainers at the same time as the rumen was sampled both AM and PM on days 18 and 19 of each period. Collected blood samples were placed on ice immediately and centrifuged within an hour of collection at 4^{o}C for 30 min at 3000 *g*. Plasma was harvested and stored at -20^{o}C until analysis. The two AM and two PM samples were pooled animal wise and utilized for lipid extraction. Plasma lipids were extracted using a modification of the Bligh and Dyer procedure as described by Cruz-Hernandez et al. (2004). Total lipids extracted from 1mL of plasma were divided into two aliquots for acid-catalyzed and base-catalyzed methylation procedures respectively as described by Kraft et al. (2008). In brief, base-catalyzed methylation was performed using 0.5 mL of 0.5*N* $NaOCH_3$/methanol (Supelco Inc., Bellefonte, PA) for 30 min at 50^{o}C and the acid-catalyzed methylation was performed using 0.5 mL of 5% HCl gas in anhydrous methanol (w/v) for 1 h at 80^{o}C. The results obtained from both methylation procedures were used in combination (Kraft et al., 2008; Kramer et al., 2008) to overcome the limitations of each methylation procedure. The acid-catalyzed methylation procedure facilitated the identification of dimethyl acetals (**DMA**) in addition to the FAME. The methylation products obtained were purified by TLC on silica gel G plates using n-hexane/diethyl ether/acetic acid (85:15:1) as developing agent. The TLC band containing FAME and DMA that coelute were identified using a TLC standard containing FAME, triacylglycerol, free cholesterol, cholesteryl ester, and free FA (Nu-Chek prep Inc., Elysian, MN) after spraying the plates with 2,7'-dichlorofluorescin (0.01% w/v) in methanol and visualizing under UV light at 254 nm. The FAME/DMA band identified was scraped off the TLC plate and extracted using 5 mL of chloroform, dried under N_2 and resuspended in n-hexane for GLC analysis. The derivatization products were analyzed using two different temperature programs as described by Kramer et al. (2008).

Milk yield was recorded daily. Milk sampling was done twice (AM and PM) on d 18 and d 19 of each period. The AM and PM samples were pooled for each cow on each sampling day in proportion to the morning and evening milk yields. Milk samples from the two consecutive days were again pooled in proportion to the daily yields and divided into two aliquots. One aliquot was preserved with potassium dichromate and analyzed for protein, fat, and lactose using a Milkoscan 203 (Foss Electric DK-3400, Hillerod, Denmark). The second aliquot was stored at -20^{o}C until analyzed for milk FA composition using GLC. Milk lipids were extracted using a chloroform/methanol/water (2:2:1.8) mixture at a 20:1 solvent to sample volume ratio and methylated using $NaOCH_3$/methanol (Cruz-Hernandez et al., 2004). The FAME were analyzed using a Hewlett Packard Model 5890 Series II GLC equipped with a flame ionization detector, an autosampler (HP Model 7673), a 100 m CP-Sil 88 fused capillary column (Varian Inc., Mississauga, ON), software program (Agilent ChemStation, Version A.10) and operated in a splitless mode that was flushed 0.3 min after injection. Injector and detector temperatures were kept at 250^{o}C. Hydrogen was used as carrier gas at a flow rate of 1 mL/min and for the flame ionization detector at 40 mL/min. Other gases used were purified air at 250 mL/min and N_2 makeup gas at 25 mL/min. The injection volume was 1 µL of FAME mixtures containing about 1 µg/µL. The FAME obtained from each milk sample were analyzed using two temperature programs (175^{o}C and 150^{o}C) as described by Kramer et al. (2008). The FAME were identified by comparison with a GLC reference standard (#463) spiked with a mixture of four positional conjugated linoleic acid (**CLA**) isomers (#UC-59M), 21:0, 23:0 and 26:0 obtained from Nu-Chek Prep Inc. (Elysian, MN). Identification of 16:1, 18:1 and 20:1 isomers was based on available isomers in the GLC reference standard (#463), comparison with published reports and based on principles of silver-ion separation. Confirmation of 18:1 isomers was made using Ag^{+}-solid phase extraction

separated fractions as described by Kramer et al. (2008). Individual FAME were reported as a percentage of total FAME.

Statistical analysis

Feed FA data were analyzed using the GLM procedure of SAS (version 9.1.3, SAS Institute Inc.). Dry matter intakes, LA and LN intake, efficiency of milk RA production, milk yield, composition, rumen pH, VFA, ammonia concentrations and FA data from rumen, plasma and milk were analysed using the MIXED procedure of SAS (version 9.1, SAS Institute, Cary, NC) with treatment as the fixed effect; square, period and cow (square) as random effects. Differences among least square means were declared significant when the *P* value was < 0.05. Trends were discussed when $0.05 \geq P < 0.1$. The model used for the analysis was: $Y_{ijkl} = \mu + T_i + P_j + S_k + C(S)_l + e_{ijkl}$ where Y_{ijkl} is the observation, μ is the overall mean, T_i is the fixed effect of treatment; P_j is the random effect of period; S_k is the random effect of square; $C(S)_l$ is the random effect of cow within square and e_{ijkl} is the residual error. The relationship between milk RA yield and source of substrate (GC or HS) for G cows was investigated using REG procedure of SAS. Step-wise regression analysis was used to identify the variables that most influenced milk RA yield with multiple factors using STEP WISE procedure of SAS. The threshold value to keep a term in the model was $P \leq 0.09$.

2.3 Results and Discussion

Grass silage is rarely offered as the sole feed to lactating dairy cows because the DM intakes are generally too low to provide adequate nutrients. However, in this study the cows were past peak milk production and the silage was supplemented with 3 kg of concentrate per day. In practice, cows offered grass at this stage of lactation would not normally receive concentrate supplement, but to avoid any confounding effects, cows on G and ZG also received the same supplement at 3 kg/day.

Dry matter intakes, milk yield and composition

Nutrient composition of the forages is presented in Table 2-1 and the values are typical for these forages in Ireland. The lesser DM intake for GS in comparison to G (Table 2-2) was anticipated because previous studies have shown that voluntary intake of forage DM when conserved as silage is lower than that of the herbage from which it was produced (Demarquilly, 1973; Harris et al., 1966). Silage in the current study was harvested after 5 weeks of growth while cows on G and ZG were offered grass that was about 3 weeks old. The differences in the growth-stage and the higher fiber content in GS compared to G and ZG, indicative of a lower digestibility, could have also contributed to the lower intake of GS.

Table 2-1. Chemical composition of the experimental diets and concentrate supplement

	Diets			Concentrate
	Grazed grass[1]	Zero grazed grass	Grass silage	Supplement[2]
DM %	17.9	18.2	22.9	88.6
CP %	24.1	19.8	12.1	21.8
Total fat %[3]	4.1	3.3	1.9	1.9
NDF %	41.5	45.4	50.8	21.6
ADF%	ND[4]	25.5	30.1	9.2
Ash%	8.7	9.4	7.4	8.7
NE_L Mcal/lb	ND[4]	0.7	0.7	ND[4]

[1]Chemical composition of the herbage snips (consumed grass for grazing cows). The DM, CP, total fat, NDF and ash content in grass cuts (representative of offered grass for grazing cows) were 18.6%, 20.4%, 3.1%, 46.4% and 12.9% respectively.
[2]Concentrate supplement containing rolled barley-30%; citrus pulp-46%; soybean meal-18%; soybean oil-3%; and mineral/vitamin mix- 3% was offered to all cows @ 3 kg/d.
[3]Determined by GLC and expressed as triglycerides.
[4]Not determined

Table 2-2. Dry matter intake, milk yield, milk composition and rumenic acid yield

	Diets[1]				
	G	ZG	GS	*P* value	SEM
Forage DMI (kg/d)	16.1[a]	15.3[a]	13.0[b]	0.02	0.60
Total DMI (kg/d)	18.7[a]	17.9[a]	15.6[b]	0.02	0.60
Total forage fatty acid intake (kg/d)	0.6[a]	0.5[b]	0.2[c]	< 0.01	0.02
Total fatty acid intake (kg/d)	0.7[a]	0.5[b]	0.3[c]	< 0.01	0.02
Milk yield (L/d)	24.6[a]	20.1[b]	16.1[c]	< 0.01	1.33
Fat (%)	3.8[ab]	4.0[a]	3.5[b]	0.04	0.15
Protein (%)	3.4[a]	3.2[a]	2.8[b]	< 0.01	0.17
Lactose (%)	4.6	4.5	4.2	NS	0.15
Fat yield (kg/d)	0.93[a]	0.79[b]	0.58[c]	< 0.01	0.05
Protein yield (kg/d)	0.82[a]	0.64[b]	0.47[c]	< 0.01	0.04
Lactose yield (Kg/d)	1.13[a]	0.90[b]	0.70[c]	< 0.01	0.06
RA[2] yield (g/d)	19.0[a]	10.8[b]	2.8[c]	< 0.01	1.13

[abc] values in the same row not sharing a common superscript are significantly different ($P < 0.05$).
[1]Diets were grazing (G), zero-grazing (ZG) and grass silage (GS) plus 3 kg of concentrate to all the diets.
[2]RA = rumenic acid.

Milk yield was greater for G than for ZG by 22% which was in turn greater than for GS by 25%. The difference in the average milk yield between G and GS reflects the difference in total DMI between these treatments. Milk yield was greater for G than ZG, but forage DMI and total DMI were not different between G and ZG. The higher milk yields for G animals compared to ZG could be partly due to the opportunity for selective grazing for G cows. Grazing cows can more readily reject unsuitable forage and preferentially consume the leafy and more easily digestible portions of the grass compared to ZG cows. Consistent with this, higher organic matter digestibility (Morgan et al., 1989) was observed in G (HS or consumed grass – 83.9%; GC or offered grass – 79.1%) compared to ZG (80.3%). Similar differences in the milk yields between G and ZG were reported in studies by Runcie (1960) and Paetzold and Stottmeister (1966).

The lower milk fat percentage for GS compared to G and ZG could possibly be due to diet-induced differences in rumen fermentation pattern evidenced by the differences in the odd and branched chain fatty acids (**OBCFA**)

in milk (discussed under milk FA composition). Milk protein percentage was lower for GS with no difference between G and ZG. Lower milk protein percentage has been reported previously for silage compared to grass based diets (Keady et al. 1995; Younge et al. 2004) and this may be due to the lower flow of microbial nitrogen and total amino acid nitrogen for silage compared to grass based diets (Younge et al. 2004). Milk lactose percentage was not different among the treatments.

Forage fatty acid composition

The FA composition of grazed grass was determined from offered grass (GC) as well as consumed grass (HS). The FA composition of the consumed grass along with ZG and GS is tabulated in Table 2-3 and the important FA in the offered grass is reported as a foot note. The relevance of HS as equivalent to the consumed grass is discussed in the section 'substrate intake' below. Linolenic acid (18:3n-3) represented the major FA in the forage accounting for 47% in grass silage and about 56% in fresh grass (Table 2-3). The concentrations of n-6 PUFA were greater for grass silage compared to grazed grass and zero grazed grass while the total n-3 PUFA content was greater for grazed grass and zero grazed grass than for grass silage. There was no period effect on the composition of FA except for total *cis*(***c***)-18:1 indicating that the grass was almost similar across the periods.

Table 2-3. Fatty acid composition of of the experimental diets and concentrate supplement

Fatty acids[2]	Diets[1]			*P* value	SEM	Period effect	Concentrate Supplement[4]
	G[3]	ZG	GS			*P* value	
12:0	0.18	0.26	0.32	NS	0.04	NS	0.06
13:0	0.03	0.05	0.04	NS	0.01	NS	0.01
14:0	0.40[c]	0.52[bc]	0.79[a]	< 0.01	0.03	NS	0.26
15:0	0.11	0.15	0.12	NS	0.03	NS	0.11
16:0	14.91[b]	15.48[b]	17.16[a]	< 0.01	0.34	NS	24.47
17:0	0.13	0.14	0.18	NS	0.01	NS	0.17
18:0	1.72	1.80	2.39	NS	0.26	NS	4.43
∑*t*-18:1	0.56	0.58	0.50	NS	0.08	NS	0.62
∑*c*-18:1	2.06[c]	2.38[b]	3.76[a]	< 0.01	0.09	0.04	25.86
*c*9/c10-18:1	1.53b	1.70[b]	2.96[a]	< 0.01	0.09	NS	23.97
19:0	0.03	0.04	0.04	NS	0.01	NS	0.04
20:0	0.41[c]	0.46[bc]	0.58[a]	< 0.01	0.02	NS	0.50
21:0	0.05[b]	0.05[b]	0.08[a]	0.03	0.01	NS	0.07
22:0	0.98	1.05	1.19	NS	0.05	NS	0.60
24:0	0.77[b]	0.85[ab]	1.02[a]	0.04	0.05	NS	0.47
26:0	0.68[b]	0.77[ab]	0.79[a]	0.03	0.03	NS	0.13
18:2n-6	9.96[c]	10.92[b]	14.42[a]	< 0.01	0.15	NS	35.11
18:3n-3	57.70[a]	54.56[a]	47.11[c]	< 0.01	0.71	NS	2.65
20:2n-6	0.04[b]	0.05[b]	0.08[a]	< 0.01	0.00	NS	0.05
20:4n-6	0.03	0.03	0.02	NS	0.01	NS	0.00
20:5n-3	0.01[b]	0.02[b]	0.07[a]	< 0.01	0.00	NS	0.02
22:2n-6	0.03[b]	0.03[b]	0.08[a]	< 0.01	0.01	NS	0.04
∑ SFA[5]	21.42[b]	22.89[b]	26.07[a]	< 0.01	0.61	NS	31.47
∑ n-6 PUFA[6]	10.06[c]	11.03[b]	14.60[a]	< 0.01	0.15	NS	35.20
∑ n-3 PUFA[6]	57.76[a]	54.63[a]	47.19[c]	< 0.01	0.71	NS	2.67
∑*trans* fatty acid	0.59	0.61	0.53	NS	0.08	NS	0.64

[abc]Values within rows with different superscript are significantly different ($P < 0.05$).

[1]Diets: G = grazing; ZG = zero-grazing; GS = grass silage

[2] Fatty acids are expressed as a percentage of total fatty acid methyl esters; *c* = *cis*; *t* = *trans*.

[3]G = Fatty acid composition for G cows estimated from herbage snips or consumed grass. The concentrations of 16:0, *c*9/c10-18:1, 18:2n-6 and 18:3n-3 was 16.97, 1.88, 10.96 and 51.39 respectively in G diet when estimated from grass cuts or offered grass

[4]Concentrate supplement containing rolled barley-30%; citrus pulp-46%; soybean meal-18%; soybean oil-3%; and mineral/vitamin mix- 3% was offered to all cows @ 3 kg/d.

[5]SFA = saturated fatty acid (12:0 to 26:0).

[6]PUFA = polyunsaturated fatty acid.

Substrate intake

It is well established that cows tend to graze selectively and the degree of selection is influenced by the amount of forage available (Hardison et al., 1954; Lesperance et al., 1960). Since cows on diet G were offered 20 kg/d of DM

(which is on the higher range of the daily herbage allowance normally offered to cows at this stage of lactation) a high probability of selective grazing would be expected with these cows.

Collection of herbage snips (HS) as representative of the grass actually consumed by grazing cows has been used in several studies (Kennedy et al., 2005; Kennedy et al., 2006; McEvoy et al., 2008). Further the composition of the whole herbage offered (grass cuts) to grazing cows has been reported as an unreliable estimate of what cows actually consume when there is an opportunity for selective grazing (Hardison et al., 1954; Fontenot and Blaser, 1965; Johnstone-Wallace, 1937; Johnstone-Wallace and Kennedy, 1944). Thus, basing substrate intake on the composition of grass cuts (GC or offered herbage) would not reflect what the cows were actually consuming. Therefore LA and LN intakes calculated from the herbage snips (consumed grass) was taken as being representative of the substrate consumed from grass by the cows on diet G. Intakes of LA and LN estimated from offered grass (GC) was also tabulated for a discussion of the differences between offered and consumed grass for G cows (Table 2-4). It was assumed that there was no difference in the concentrations of LA and LN between what was offered and consumed by the cows on ZG and GS as the form in which these diets were fed prevented the animals from being selective.

Table 2-4. Substrate intake (from total DMI)

	Diets[1]				*P* value	SEM
	G		ZG	GS		
Name of the variable	Consumed[2]	Offered[3]				
LA (18:2n-6) intake[4] g/d	91.80[a]	75.46[b]	76.71[b]	50.54[c]	< 0.01	2.90
LN (18:3n-3) intake[5] g/d	341.49[a]	235.16[b]	249.91[b]	113.32[c]	< 0.01	17.24
Total substrate intake[6] g/d	433.28[a]	310.61[b]	326.62[b]	163.86[c]	< 0.01	17.89

[abc] values in the same row not sharing a common superscript are significantly different ($P < 0.05$).
[1]G = grazing; ZG = zero-grazed grass; GS = grass silage plus 3 kg of concentrate to all the three diets.
[2]Consumed = substrate intake estimated for G cows from herbage snips.
[3]Offered = substrate intake estimated for G cows from grass cuts.
[4]LA = linoleic acid.
[5]LN = linolenic acid.
[6]Total substrate = sum of 18:2n-6 and 18:3n-3 in diet.

There were no significant differences in LA and LN intake per day between G and ZG when the respective values for G estimated from offered grass were compared to ZG (Table 2-4). However the intakes of LA and LN were greater for G compared to ZG when the respective values for G were quantified from consumed grass (HS). Lesser intakes of LA and LN for GS compared to other treatments could be explained by the lower DMI as well as the lower proportion of LN in grass silage. Lower proportions of LN for grass silage relative to grazed grass and zero grazed grass could be due to oxidation of PUFA during wilting and ensiling. Losses of FA during forage conservation and storage is well documented (Lough and Anderson, 1973; Steele and Noble, 1983) and is mediated by plant lipases releasing LN and LA from the cut surface of the plant tissue (Thomas, 1986).

Rumen factors

In the previous section, it was observed that there were differences in LA and LN intakes as well as total substrate intake among the treatments. Apart from treatments being different in the amount of substrate they supplied, diets could also influence the rumen environment differently and in turn modify the microbial activity associated with biohydrogenation of PUFA in the rumen. Some

of the factors reported to influence rumen biohydrogenation of PUFA based on *in vitro* and *in vivo* studies include rumen pH (Van Nevel and Demeyer, 1996; Martin and Jenkins, 2002), rumen fill/passage rate (Qiu et al., 2004), amount and source of lipid (Bell et al., 2006; Cruz-Hernandez et al., 2007), possible interactions between forage and lipid source (Chilliard and Ferlay, 2004), and/ or inhibition of rumen biohydrogenation by components in specific botanical species of the forage (Leiber et al., 2005). Discussion here will be confined to the differences in rumen pH, fermentation end products, rumen fill and its possible influence on lipolysis and biohydrogenation of PUFA in the rumen. The rumen variables measured are presented in Table 2-5.

Table 2-5. Rumen pH, volatile fatty acids and ammonia nitrogen (NH_3-N)

Rumen factors	Diets[1]			*P* value	SEM
	G	ZG	GS		
Rumen pH	6.32[b]	6.79[a]	6.71[a]	< 0.01	0.08
Total VFAs (µmoles/mL)	85.91	86.80	78.61	NS	5.94
Acetate	49.49	53.27	47.27	NS	3.18
Propionate	16.63	15.29	14.61	NS	1.02
Butyrate	11.51	10.63	10.61	NS	0.79
Acetate: propionate	2.98[b]	3.48[a]	3.23[b]	< 0.01	0.10
NH_3-N (µg/mL)	24.88	25.65	16.06	NS	5.92
Wet rumen fill (kg)	88.75[b]	102.50[a]	103.30[a]	< 0.01	9.32
Dry rumen fill (kg)	9.65	10.73	10.09	NS	1.08

[abc] values in the same row not sharing a common superscript are significantly different.
[1]Diets: G = grazing; ZG = zero-grazing; GS = grass silage plus 3 kg of concentrate to all the diets.

Rumen pH: Rumen pH was lower for G with no significant difference between ZG and GS. Rumen pH is determined by various factors, but mainly by structural properties of the feed. This in turn influences the chewing activity and flow of saliva to the rumen. Lower pH for G compared to GS could be explained by the lower NDF values recorded in grazed grass. Higher fiber content promotes chewing and increases salivation to buffer the acids produced in the rumen. In the present study, the mean pH of silage juice was 4.1 but the rumen pH for GS compared to G was not lower, suggesting an efficient buffering system in the

animals to compensate for the lower pH of the silage diet. The difference in rumen pH between G and ZG could be due to difference in digestibility of the grass. Theoretically, the offered grass for grazing and zero-grazing animals was the same, but there was greater opportunity for grazing animals to choose the more digestible leafy portions and reduce the intake of more fibrous stem portions of the grass. Consistent with this, higher NDF values were recorded for zero grazed grass compared to grazed grass (45.4 vs 41.5). Fresh grass is generally higher in soluble carbohydrates compared to grass silage. Although not analyzed, differences in water soluble carbohydrates (besides fiber) could have also contributed to the differences in rumen pH between G and GS.

Volatile fatty acids and ammonia nitrogen in the rumen: There was no significant difference in the mean VFA (total as well as individual) and ammonia nitrogen concentrations in the rumen among the treatments. We also attempted to investigate whether or not there was a difference in rumen fermentation pattern among the treatments using milk OBCFA**,** and milk fat percentage as alternatives. There is growing evidence to support the use of milk OBCFA to predict differences in rumen fermentation pattern (Vlaeminck et al., 2006b) and in general as a diagnostic tool for rumen function in relation to dietary effects (Valeminck et al., 2006a). The sum of *iso* FA (13:0 *iso*, 14:0 *iso*, 15:0 *iso*, 16:0 *iso* and 17:0 *iso*) and *anteiso* FA (13:0 *anteiso*, 15:0 *anteiso* and 17:0 *anteiso*) in milk were significantly greater for G and ZG than for GS with no difference between G and ZG (Table 2-8) indicating that the fermentation pattern was possibly different for GS compared to G and ZG. Milk fat percentage was significantly less for GS with comparable values for G and ZG. Since a shift in rumen fermentation pattern is determined by the relative proportions of cellulolytic and amylolytic bacteria in the rumen, the results from OBCFA pattern in milk reflects a possible shift in rumen bacterial population between the treatments (particularly GS from G and ZG), but the difference may not have been large enough to produce a

significant difference in indicators of rumen fermentation pattern (VFA and ammonia nitrogen) or this was not detected due to infrequent sampling.

Rumen fill and passage rate: Rumen fill and passage rate can influence milk RA content because these factors can influence the degree of biohydrogenation of unsaturated FA. Chaves and Boudon (2005) in a study designed to test the influence of rumen fill on intake and milk production in dairy cows fed perennial rye grass, reported a negative relationship between rumen fill and DMI. Based on this, it was anticipated that less rumen fill would be associated with increased DMI, which in turn would increase digesta passage rate resulting in greater accumulation of biohydrogenation intermediates for G than for GS. Consistent with this, rumen fill (wet) was less for G compared to GS (Table 2-5) and total DMI was greater for G than for GS (Table 2-2). However, the difference in wet rumen fill between G and ZG with similar DMI cannot be explained on this basis. Dry rumen fill was not different across the treatments and hence cannot explain the negative relationship between rumen fill and DMI as reported by Chaves and Boudon (2005). Regression analysis of rumen fill (both wet and dry) and DMI (forage DMI and total DMI) was not significant (data not presented) suggesting that the difference in rumen fill and total DMI in the current study were not related.

Rumen and plasma fatty acid composition

The FA composition of the rumen contents for the different treatments is shown in Table 2-6. The FA composition of the rumen reflects both feed lipids as well as metabolic products of dietary PUFA by the rumen microbes. The most abundant ruminal FA was 18:0 (ranging from 45-47%) that showed no treatment effect, followed by 16:0 (11-14%) and *t*11-18:1 (4-12%). Ruminal concentration of 16:0 was greater for GS than for G and ZG. Vaccenic acid was the 2nd most abundant FA for G and the 3rd most abundant FA for ZG and GS diets. Total CLA

isomers in rumen ranged from 0.45 to 0.57% and were not different among the treatments. Ruminal lipids were high in total SFA, which included long-chain SFA up to 26:0. Phytanic acid, the isoprenoid SFA derived from the phytol side-chain of chlorophyll, was present at greater concentrations for GS with no difference between G and ZG. This is consistent with the finding that phytanic acid occurs in greater concentrations in plasma lipids for cows offered grass silage (Lough, 1977)

Ruminal concentrations of LA (18:2n-6) and LN (18:2n-3) were greater for GS than for G, but this did not translate into correspondingly greater levels of VA for the GS group suggesting that there were differences in lipolysis, degree of PUFA biohydrogenation and possibly rumen bacterial populations across the treatments. It is well established that ingested lipids are mainly hydrolyzed by rumen bacterial lipases and to a lesser extent by plant lipases (see review by Jenkins et al., 2008). Faruque et al. (1974) reported that plant lipases in the leaves of pasture plants remain active for at least 5 h in the presence of metabolizing rumen micro-organisms indicating that lipolysis of ruminal lipids for G and ZG diets could be influenced by both plant lipases and bacterial lipases, while for GS diets lipolysis was influenced mainly by bacterial lipases (Boufaïed et al., 2003a,b). Thus, the reduced ruminal concentrations of LA and LN for G and ZG could be due to a more active biohydrogenation compared to that for GS.

Table 2-6. Effect of treatments on rumen fatty acid composition

Fatty acids[2]	Diets[1]			*P* value	SEM
	G	ZG	GS		
14:0	0.67[b]	0.73[b]	0.90[a]	0.02	0.06
15 *iso*	0.84[ab]	1.05[a]	0.77[b]	0.07	0.09
15 *anteiso*	1.12	1.17	1.23	NS	0.08
*c*11-14:1	0.91	0.77	0.98	NS	0.10
15:0	1.30[b]	1.38[b]	1.95[a]	< 0.01	0.09
16 *iso*	0.21[c]	0.27[ab]	0.29[a]	< 0.01	0.02
16:0	11.37[c]	13.13[b]	14.56[a]	< 0.01	0.27
*c7*16:1	0.63[b]	0.64[b]	2.05[a]	< 0.01	0.14
c9 16:1	0.04[c]	0.05[ab]	0.06[a]	0.02	0.01
c11 16:1	0.10[b]	0.09[b]	0.16[a]	< 0.01	0.01
17 *iso*	0.24[c]	0.30[ab]	0.33[a]	< 0.01	0.01
17 *anteiso*	0.58[b]	0.78[a]	0.73[ab]	0.07	0.07
Phytanic acid	0.54[b]	0.64[b]	1.91[a]	< 0.01	0.09
17:0	0.49[c]	0.53b	0.66[a]	< 0.01	0.03
c5 17:1	0.06	0.06	0.07	NS	0.02
c7 17:1	0.05	0.02	0.03	NS	0.03
*c*10-17:1	0.08	0.08	0.07	NS	0.02
18 *iso*	0.04[c]	0.05[b]	0.07[a]	< 0.01	0.00
18:0	47.43	45.88	45.34	NS	1.25
∑*t*-18:1	16.84[a]	13.48[b]	7.80[c]	< 0.01	0.59
∑*c*-18:1	3.41	3.21	3.68	NS	0.18
*c*9/c10/*t*15-18:1	1.78[b]	1.72[b]	2.14[a]	0.03	0.09
*c*11-18:1	0.36[a]	0.37[a]	0.31[b]	0.04	0.02
*c*12-18:1	0.28	0.28	0.33	NS	0.04
*c*13-18:1	0.08[b]	0.08[b]	0.12[a]	< 0.01	0.01
*c*15-18:1	1.30[b]	1.38[b]	1.95[a]	< 0.01	0.09
19:0	0.16	0.16	0.16	NS	0.01
*t*9*t*12-18:2	0.02	0.03	0.01	NS	0.01
*t*9*c*12-18:2	0.15[a]	0.12[b]	0.07[c]	< 0.01	0.01
*c*9*t*13/*t*8*c*12-18:2	0.12	0.09	0.10	NS	0.02
*t*11*c*15-18:2	1.05	1.02	1.23	NS	0.12
18:2n-6	0.99[c]	1.57[ab]	2.03[a]	< 0.01	0.20
20:0	0.59[c]	0.66[b]	0.88[a]	< 0.01	0.02
c/c/t-18:3	0.03[b]	0.05[a]	0.05[a]	0.02	0.01
c/t/c-18:3	0.01	0.01	0.02	NS	0.00
*c*8-20:1	0.01[b]	0.03[a]	0.03[a]	0.05	0.01
*c*11-20:1	0.04[b]	0.04[b]	0.06[a]	0.05	0.01
18:3n-3	0.70[b]	2.43[a]	2.12[a]	0.02	0.40
∑-CLA	0.45	0.45	0.57	NS	0.06
20:2n-6	0.01[b]	0.02[a]	0.02[a]	< 0.01	0.00
21:0	0.13	0.13	0.08	NS	0.03
22:0	0.86[b]	1.03[a]	1.12[a]	< 0.01	0.04
20:3n-6	0.03	0.03	0.02	NS	0.00
20:3n-3	0.004	0.003	0.010	NS	0.00
20:4n-6	0.01	0.01	0.02	NS	0.01
20:5n-3	0.002	0.003	0.017	NS	0.01
23:0	3.64	5.03	3.53	NS	1.51
22:2n-6	0.02[b]	0.02[b]	0.05[a]	< 0.01	0.00
24:0	0.59	0.82	0.80	NS	0.14

*c*13-24:1	0.004	0.001	0.009	NS	0.01
*c*15-24:1	0.09[b]	0.10[b]	0.24[a]	< 0.01	0.01
25:0	0.48	0.50	0.34	NS	0.05
26:0	1.35[b]	1.38[b]	2.43[a]	< 0.01	0.08
∑ SFA	72.70	75.84	76.94	NS	1.73
∑ trans MUFA	4.33[a]	3.35[b]	2.38[c]	< 0.01	0.29
∑ cis MUFA	13.93[b]	12.85[b]	20.15[a]	< 0.01	0.85
***t*18:1 isomers**					
*t*4-18:1	0.08	0.08	0.08	NS	0.01
*t*5-18:1	0.06[a]	0.05[ab]	0.03[c]	< 0.01	0.00
*t*6 to 8-18:1	0.53[a]	0.47[ab]	0.35[c]	< 0.01	0.04
*t*9-18:1	0.23	0.28	0.23	NS	0.04
*t*10-18:1	0.85[ab]	0.94[a]	0.67[b]	0.08	0.25
*t*11-18:1	12.30[a]	9.31[b]	4.21[c]	< 0.01	0.66
*t*10: *t*11-18:1	0.08[b]	0.11[b]	0.17[a]	< 0.01	0.02
*t*12-18:1	0.76[a]	0.60[b]	0.54[b]	< 0.01	0.04
*t*13/14/*c*6-8-18:1	2.10	1.71	1.65	NS	0.28
CLA isomers[3]					
*c*9*t*11-CLA/RA	0.09[b]	0.09[b]	0.13[a]	0.04	0.03
*t*10*c*12-CLA	0.01[b]	0.01[b]	0.02[a]	0.05	0.00
*c*9*c*11-CLA/ *t*11*c*13-CLA	0.12[b]	0.11[b]	0.18[a]	0.02	0.02
11*t*13*t*-CLA	0.17	0.18	0.13	NS	0.02
*t*12*t*14-CLA	0.06	0.06	0.08	NS	0.02

[abc] values in the same row not sharing a common superscript are significantly different.
[1]Diets: G = grazing; ZG = zero-grazing; GS = grass silage plus 3 kg of concentrate to all the diets.
[2]*c* = *cis*; *t* = *trans*; CLA = conjugated linoleic acid; SFA = saturated fatty acid (14:0 to 26:0 including branched-chain fatty acids); MUFA = monounsaturated fatty acid; RA = rumenic acid; Fatty acids are expressed as a percentage of total fatty acid methyl esters.
[3] CLA isomers were only resolved using GLC, not additional Ag^{+}-HPLC.

The plasma FA composition presented in Table 2-7 was obtained by combining the results of both acid and base methylation procedures (Kramer et al., 2008). The derivatization products obtained from methylation of *O*-acyl and *N*-acyl side-chains yielded FAME while those from alk-1-enyl ethers yielded DMA. Total DMAs ranged from 2.4 to 2.7% of the total FAME and were not different among the treatments. Plasma concentrations of the two most abundant FA, LA and 18:0, were not different among treatments. Plasma concentrations of LN were lesser for GS than for G and ZG while the concentrations of *c*9-/*c*10-18:1 was greater for GS than for G and ZG. Total CLA isomers in plasma ranged from 0.27 to 0.38% with lower values for GS compared to G. Phytanic acid which co-eluted with 17:0 *anteiso* using the 175°C program could be resolved using the

150^{o}C program. Plasma phytanic acid concentrations showed the same treatment differences as were found in the ruminal lipids; they were significantly higher for GS with no difference between G and ZG.

Vaccenic acid and trans10-18:1 in rumen and plasma: There were significant differences in ruminal total *t*-18:1 and VA (Table 2-6) among the treatments with greater values recorded for G than for ZG and GS. This could be attributed to the differences in LA and LN intake, degree of biohydrogenation, rumen pH or digesta kinetics across the treatments. In the current study, there were diet-induced differences in rumen pH, which could have inhibited biohydrogenation of PUFA resulting in the differences in VA among the treatments. Consistent with this, Kalscheur et al. (1997) demonstrated the effect of low rumen pH on biohydrogenation inhibition and increased outflow of *t*-18:1 intermediates from the rumen. More recently, FA oxidation products, which are released from fresh pastures when damaged through mastication or mechanical cutting, have also been reported to influence microbial metabolism of PUFA in the rumen owing to their anti-microbial properties (Lee et al., 2007).

Table 2-7. Effect of treatments on plasma fatty acid composition

Fatty acids[2]	Diets[1]			*P* value	SEM
	G	ZG	GS		
14:0	0.51	0.51	0.56	NS	0.04
15 *iso*	0.54	0.56	0.52	NS	0.04
15 *anteiso*	0.89	0.83	0.89	NS	0.07
*c*9-14:1	0.003	0.040	0.004	NS	0.02
15:0	0.68^{b}	0.72^{b}	0.84^{a}	0.03	0.03
16 *iso*	0.28	0.28	0.27	NS	0.02
16:0	10.04	10.17	10.20	NS	0.43
∑*t*-16:1	0.58^{a}	0.47^{b}	0.46^{b}	0.01	0.04
∑*c*-16:1	1.69	1.62	1.94	NS	0.12
*t*5-16:1	0.09^{ab}	0.07^{b}	0.13^{a}	0.03	0.02
*t*6-8-16:1	0.15	0.15	0.18	NS	0.01
*t*9-16:1	0.32^{a}	0.26^{b}	0.15^{c}	< 0.01	0.03
c7/t11 16:1	0.59	0.65	0.73	NS	0.05
c9 16:1	0.91	0.81	1.02	NS	0.09
c11 16:1	0.07	0.07	0.07	NS	0.01
c13 16:1	0.13^{b}	0.15^{ab}	0.18^{a}	0.06	0.02
17 *iso*	0.26	0.27	0.23	NS	0.02
17 *anteiso*	1.45	1.48	1.95	NS	0.27
Phytanic acid	1.72^{b}	2.01^{b}	5.02^{a}	< 0.01	0.43
17:0	0.80^{b}	0.82^{b}	0.99^{a}	< 0.01	0.05
c5 17:1/18:1 DMA[3]	0.12^{b}	0.13^{b}	0.19^{a}	< 0.01	0.01
c7 17:1/18:1 DMA	0.28^{b}	0.31^{b}	0.49^{a}	< 0.01	0.02
*c*9-17:1	0.40^{b}	0.35^{b}	0.57^{a}	< 0.01	0.02
*c*11-17:1	0.03	0.03	0.03	NS	0.01
18 *iso*	0.06^{ab}	0.06^{b}	0.07^{a}	0.05	0.00
18:0	14.25	13.80	13.92	NS	0.91
∑*t*-18:1	3.79^{a}	2.79^{b}	1.84^{c}	< 0.01	0.27
∑*c*-18:1	9.97^{b}	8.63^{b}	12.42^{a}	< 0.01	0.51
*c*6-8-18:1	0.27^{b}	0.29^{b}	0.46^{a}	0.01	0.04
*c*9/c10-18:1	8.73^{b}	7.46^{b}	10.99^{a}	< 0.01	0.48
*c*11-18:1	0.36	0.34	0.32	NS	0.03
*c*12-18:1	0.42^{b}	0.37^{b}	0.49^{a}	0.01	0.05
*c*13-18:1	0.08	0.06	0.06	NS	0.01
*c*15-18:1	0.11	0.11	0.11	NS	0.01
19:0	0.09^{b}	0.09^{b}	0.11^{a}	0.08	0.01
*t*9*t*12-18:2	0.07^{a}	0.05^{b}	0.04^{b}	0.02	0.01
*t*9*c*12-18:2	0.06	0.06	0.06	NS	0.01
*c*9*t*12-18:2/16c	0.15	0.13	0.15	NS	0.01
*t*8*c*13-18:2	0.28^{b}	0.27^{b}	0.38^{a}	0.03	0.03
*c*9*t*13/*t*8*c*12-18:2	0.19	0.17	0.19	NS	0.02
*t*11*c*15-18:2	0.20^{a}	0.14^{b}	0.11^{c}	< 0.01	0.02
*c*9*c*15-18:2	0.27	0.25	0.29	NS	0.05
18:2n-6	26.38	26.41	25.31	NS	0.92
20:0	0.09^{b}	0.11^{b}	0.13^{a}	< 0.01	0.00
18:3n-6	0.71^{b}	0.77^{ab}	0.89^{a}	0.08	0.12
c/c/t-18:3	0.17^{a}	0.19^{a}	0.09^{b}	< 0.01	0.01
c/t/t-18:3	0.12^{a}	0.09^{b}	0.12^{a}	< 0.01	0.01
*c*7-20:1/*t,c,c*-18:3	0.20^{a}	0.17^{b}	0.09^{c}	< 0.01	0.01
*c*9-20:1	0.02	0.03	0.03	NS	0.00

*c*10-20:1	0.07[ab]	0.07[b]	0.08[a]	0.09	0.00
*c*11-20:1	0.03	0.02	0.04	NS	0.01
18:3n-3	12.01[a]	14.00[a]	7.32[b]	< 0.01	0.85
18:4n-3	0.44[a]	0.46[a]	0.30[b]	0.05	0.10
∑-CLA	0.38[a]	0.33[ab]	0.27[b]	0.01	0.02
20:2n-6	0.07	0.08	0.08	NS	0.00
21:0	0.01	0.01	0.02	NS	0.01
22:0	0.08	0.06	0.13	NS	0.03
20:3n-6	1.07	1.06	1.19	NS	0.15
20:3n-3	0.01[b]	0.02[a]	0.01[ab]	0.05	0.00
20:4n-6	0.97	1.10	1.33	NS	0.20
20:4n-3	0.85[a]	0.91[a]	0.59[b]	0.03	0.12
23:0	0.47	0.50	0.49	NS	0.03
20:5n-3	1.15	1.17	1.23	NS	0.37
24:0	0.32[b]	0.34[b]	0.42[a]	0.02	0.02
*c*15-24:1	0.21[b]	0.24[b]	0.35[a]	< 0.01	0.02
26:0	0.05[b]	0.05[b]	0.06[a]	0.04	0.00
22:4n-6	0.08	0.09	0.08	NS	0.01
22:5n-6	0.03	0.04	0.02	NS	0.01
22:5n-3	0.55	0.55	0.59	NS	0.11
22:6n-3	0.06	0.07	0.06	NS	0.01
Desaturase index					
*c*9 14:1 / 14:0	0.01	0.07	0.01	NS	0.03
*c*9 16:1 / 16:0	0.09[ab]	0.08[b]	0.1[a]	0.03	0.01
*c*9 18:1 / 18:0	0.61[b]	0.55[b]	0.79[a]	< 0.01	0.07
*c*9*t*11CLA / *t*11 18:1	0.08[b]	0.09[b]	0.18[a]	< 0.01	0.02
∑ DMAs[3]	2.56	2.41	2.69	NS	0.17
***t*-18:1 isomers**					
*t*4-18:1	0.08[ab]	0.06[b]	0.09[a]	0.03	0.01
*t*5-18:1	0.04	0.03	0.02	NS	0.01
*t*6 to 8-18:1	0.17	0.15	0.16	NS	0.01
*t*9-18:1	0.14[a]	0.12[b]	0.13[ab]	0.04	0.01
*t*10-18:1	0.18	0.15	0.14	NS	0.02
*t*11-18:1	2.18[a]	1.47[b]	0.66[c]	< 0.01	0.20
*t*10: *t*11-18:1	0.11[b]	0.12[b]	0.22[a]	< 0.01	0.02
*t*12-18:1	0.25[a]	0.20[b]	0.16[bc]	< 0.01	0.02
*t*13/14-18:1	0.44	0.38	0.27	NS	0.05
*t*15-18:1	0.08	0.06	0.05	NS	0.01
*t*16-18:1/14c	0.24[a]	0.19[b]	0.16[bc]	0.02	0.02
CLA isomers[4]					
*t*7*c*9-CLA	0.01	0.01	0.01	NS	0.00
*t*8*c*10-CLA	0.02[a]	0.01[ab]	0.01[b]	0.04	0.00
*c*9*t*11-CLA/RA	0.17[a]	0.14[ab]	0.11[b]	0.01	0.01
*t*10*c*12-CLA	0.003	0.002	0.002	NS	0.00
*t*11*c*13-CLA	0.02[ab]	0.02[a]	0.01[b]	0.07	0.00
*c*12*t*12-CLA	0.003	0.003	0.004	NS	0.00
*t*12*t*14-CLA	0.01[a]	0.01[a]	0.01[b]	0.02	0.00
*t*11*t*13-CLA	0.04[a]	0.04[a]	0.02[b]	0.02	0.00
*t*10*t*12-CLA	0.01	0.01	0.00	NS	0.00
*t*9*t*11-CLA	0.04	0.03	0.03	NS	0.01
*t*8*t*10-CLA	0.02	0.02	0.02	NS	0.00
*t*7*t*9-CLA	0.02	0.01	0.02	NS	0.00

[abc] values in the same row not sharing a common superscript are significantly different.

[1]Diets: G = grazing; ZG = zero-grazing; GS = grass silage plus 3 kg of concentrate to all the diets.
[2]*c* = *cis*; *t* = *trans*; CLA = conjugated linoleic acid; RA = rumenic acid; Fatty acids are expressed as a percentage of total fatty acid methyl esters.
[3]Dimethyl acetal.
[4]CLA isomers were resolved using GLC and Ag^{+}-HPLC.

The relative concentration of *t*10-18:1 in all treatments, although less than 1%, was of interest since it has been suggested as an indicator of a shift in rumen bacterial populations (Cruz-Hernandez et al., 2006). Based on the greater *t*10- to *t*11-18:1 ratio there appears to be a shift in rumen bacterial profile for GS compared to G and ZG (Figure 2-1).

The concentrations of total *t*-18:1 and VA in plasma (Table 2-7) followed a similar trend as that in rumen, but the relative amounts of these FA were 4- to 6-times lower in plasma lipids. Plasma concentrations of *t*10-18:1 was not different among treatments, but the *t*10- to *t*11-18:1 ratio was greater for GS than for G and ZG (Figure 2-1) similar to that observed in rumen. Plasma concentrations of *t*11*c*15-18:2, an intermediate of ruminal biohydrogenation of LN (Griinari and Bauman, 1999), was significantly different across the treatments with greater values for G than for ZG and GS indicating that there were differences in the extent of biohydrogenation of LN in the rumen.

Rumenic acid and trans10cis12-CLA in rumen and plasma: Rumenic acid in the rumen was greater for GS with no difference between G and ZG (Table 2-6). Since RA is an intermediate of LA biohydrogenation and is not part of the classical biohydrogenation pathway of LN, it would be expected that the concentration of RA in the rumen would be proportional to the intake of LA in the diet (Table 2-4). But on the contrary, the concentration of RA in the rumen was highest for GS, the diet with the least amount of intake of LA. An explanation is not readily apparent, other than possible components in the silage

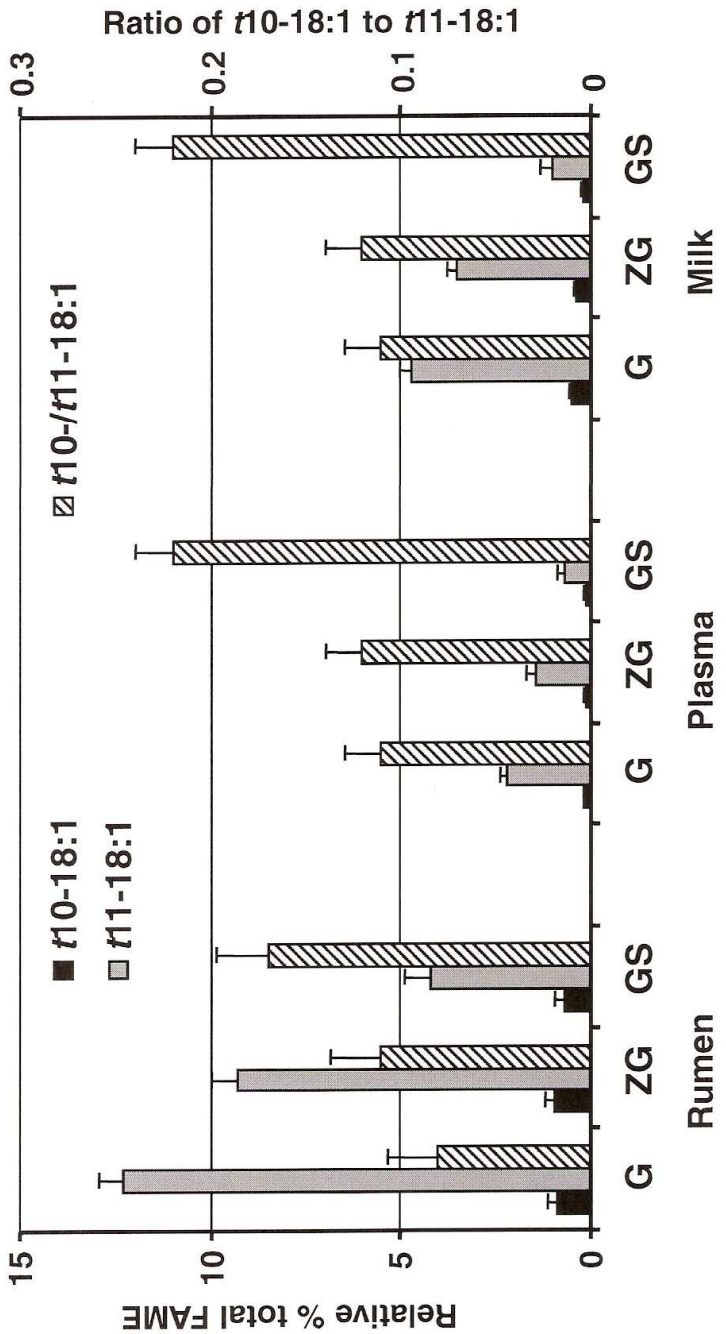

Figure 2-1. Ratio of *t*10-18:1:*t*11-18:1 in rumen, plasma and milk. The isomers t10-18:1 and t11-18:1 are expressed as relative percentage of total fatty acid methyl esters (FAME) on the left y-axis; the ratio of t10-18:1 to t11-18:1 is shown on the right y-axis. Treatments G = grazing; ZG = zero-grazing and GS = grass silage.

interfering with the biohydrogenation of RA to VA. However, it should be noted that the concentrations of RA in the rumen have little to do with the final concentrations of RA in milk. More than 80% of RA in milk has been shown to be derived from vaccenic acid via Δ^9-desaturation (Griinari et al., 2000), and the concentrations of VA in the rumen were indeed proportional to the intake of LA in the diet.

In contrast to the rumen concentrations of RA, plasma RA concentrations (Table 2-7) were greater for G than for GS indicating some degree of intestinal or hepatic desaturase activity had occurred with greater proportions of VA being converted to RA for G and ZG. Plasma RA concentrations were generally low (< 0.2%). The other CLA isomers were only present in trace amounts including *t*7*c9*-CLA (0.01%) and *t*10*c*12-CLA (0.002%).

Milk fatty acid composition

Milk FA composition is presented in Table 2-8. Higher concentrations of total SFA were recorded for GS and ZG than for G and could be accounted for mainly by the difference in the concentrations of 16:0 and 14:0 and to a lesser extent by a difference in the concentrations of minor SFA such as *iso* and *anteiso*, 13:0 and 15:0 and 19:0 and 20:0. There was no significant difference in the concentrations of shorter chain FA from 4:0 to 15:0 (except 6:0 and 8:0) and some of the longer chain SFA (22:0, 24:0) among the treatments. The sum of the branched chain FA (*iso* + *anteiso*) was less for GS than for G and ZG which were similar. Phytanic acid was present only in trace amounts in milk lipids indicating some kind of 'barrier' in the transfer of this isoprenoid FA into milk lipids. The total concentration of both the n-6 and n-3 PUFA ranged from 1.01 to 1.25 and 0.53 to 0.91 respectively, with lesser concentrations for GS than for G or ZG (Table 2-8).

Greater concentrations of total *t*-18:1, total *trans*-mono unsaturated fatty acids (**MUFA**) and total *cis*-MUFA were recorded in milk lipids for G and ZG than for GS. The concentrations of *c*9-/*c*10-18:1 accounted for about 95% of the total *c*-18:1 isomers across all treatments, and it was significantly different across the treatments with greater values recorded for G than for GS.

Table 2-8. Effect of treatments on milk fatty acid composition

Fatty acids[2]	Diets[1]			*P* value	SEM
	G	ZG	GS		
4:0	3.63	3.87	3.94	NS	0.20
6:0	2.34[b]	2.57[a]	2.57[a]	0.03	0.12
7:0	0.32	0.40	0.31	NS	0.04
8:0	1.34[b]	1.45[a]	1.39[ab]	0.08	0.09
9:0	0.03	0.03	0.03	NS	0.01
10:0	2.76	3.02	2.95	NS	0.28
11:0	0.37	0.37	0.43	NS	0.02
12:0	2.96	3.16	3.29	NS	0.29
*c*9-12:1	0.08	0.08	0.10	NS	0.01
c11-12:1	0.04	0.03	0.01	NS	0.01
13:0 *iso*	0.05 [a]	0.06[a]	0.04[b]	< 0.01	0.00
13:0 *anteiso*	0.09	0.09	0.11	NS	0.01
13:0	0.08[b]	0.08[b]	0.10[a]	0.06	0.01
14:0 *iso*	0.12[a]	0.13[a]	0.09[b]	< 0.01	0.01
14:0	9.79[b]	10.39[ab]	11.16[a]	0.02	0.37
15:0 *iso*	0.31[b]	0.35[a]	0.22[c]	< 0.01	0.02
15:0 *anteiso*	0.61[a]	0.59[a]	0.42[b]	< 0.01	0.03
*c*9-14:1	0.94[ab]	0.89[b]	1.16[a]	0.09	0.11
15:0	1.11[b]	1.10[b]	1.34[a]	< 0.01	0.03
*c*9-15:1	0.02	0.03	0.02	NS	0.00
16:0 *iso*	0.24[a]	0.24[a]	0.15[b]	< 0.01	0.01
16:0	23.65[c]	26.31[b]	37.90[a]	< 0.01	1.15
∑*t*-16:1	0.44[a]	0.35[b]	0.20[c]	< 0.01	0.05
∑*c*-16:1	1.51[b]	1.42[b]	1.94[a]	< 0.01	0.13
t8-16:1	0.06	0.06	0.07	NS	0.00
*t*9-16:1	0.32[a]	0.23[b]	0.08[c]	< 0.01	0.04
t10-16:1	0.03	0.03	0.03	NS	0.00
*c*7/*t*11 16:1	0.26[a]	0.22[ab]	0.16[c]	< 0.01	0.02
*c*8/*t*12 16:1	0.03	0.04	0.02	NS	0.01
c9 16:1	1.11[b]	1.04[b]	1.62[a]	< 0.01	0.12
c11 16:1	0.03	0.04	0.03	NS	0.00
c13 16:1	0.11[b]	0.13[ab]	0.15[a]	0.04	0.01
17:0 *iso*	0.47[a]	0.44[a]	0.29[b]	< 0.01	0.03
17:0 *anteiso*	0.82[a]	0.74[a]	0.54[b]	< 0.01	0.04
17:0	0.57	0.55	0.56	NS	0.03
c5 17:1	0.02[bc]	0.03[ab]	0.04[a]	0.02	0.00
c7 17:1	0.10	0.10	0.10	NS	0.00
*c*9-17:1	0.22[ab]	0.19[b]	0.24[a]	0.05	0.01
18:0 *iso*	0.05	0.05	0.06	NS	0.00
18:0	10.12[a]	10.58[a]	7.26[b]	< 0.01	0.42
∑*t*-18:1	7.83[a]	6.18[b]	2.67[c]	< 0.01	0.50
∑*c*-18:1	19.53[a]	17.55[b]	13.92[c]	< 0.01	1.16
*c*6-8-18:1	0.17[a]	0.15[ab]	0.12[b]	0.08	0.02
*c*9/c10-18:1	18.38[a]	16.50[b]	13.18[c]	< 0.01	1.09
*c*11-18:1	0.32[a]	0.26[b]	0.16[c]	< 0.01	0.02
*c*12-18:1	0.16[a]	0.15[ab]	0.13[b]	0.09	0.03
*c*13-18:1	0.08[a]	0.07[a]	0.04[b]	0.01	0.01
*c*14-18:1	0.06[a]	0.06[a]	0.02[b]	< 0.01	0.01
*c*15-18:1	0.21[a]	0.20[a]	0.12[b]	< 0.01	0.02

*c*16-18:1	0.09[a]	0.09[a]	0.05[b]	0.02	0.01
*c*17-18:1	0.08	0.07	0.07	NS	0.01
19:0	0.19[a]	0.19[a]	0.11[b]	< 0.01	0.01
17cyclo	0.08	0.07	0.08	NS	0.01
*t*9*c*12-18:2	0.09[a]	0.06[b]	0.02[c]	< 0.01	0.01
*c*9*t*13/*t*8*c*12-18:2	0.38[a]	0.30[ab]	0.18[b]	0.03	0.04
*t*11*c*15-18:2	0.70[a]	0.54[b]	0.28[c]	< 0.01	0.07
*c*9*c*15-18:2	0.03	0.04	0.04	NS	0.00
18:2n-6	1.08[a]	1.03[a]	0.82[b]	< 0.01	0.16
20:0	0.12[b]	0.14[a]	0.14[a]	0.03	0.01
18:3n-6	0.03	0.03	0.03	NS	0.00
c/c/t-18:3	0.02[a]	0.02[a]	0.01[b]	0.03	0.00
*c*7-20:1/*t,c,c*-18:3	0.02[a]	0.02[a]	0.01[b]	0.01	0.00
*c*9-20:1	0.09[b]	0.08[b]	0.10[a]	0.01	0.00
*c*11-20:1	0.03	0.02	0.03	NS	0.00
18:3n-3	0.68[b]	0.82[a]	0.34[c]	< 0.01	0.13
∑-CLA	2.39[a]	1.66[b]	0.69[c]	< 0.01	0.19
20:2n-6	0.02	0.02	0.02	NS	0.00
22:0	0.08	0.08	0.07	NS	0.01
20:3n-6	0.04	0.04	0.04	NS	0.00
20:3n-3	0.01[b]	0.02[a]	0.01[b]	0.01	0.00
20:4n-6	0.04[c]	0.05[b]	0.06[a]	< 0.01	0.01
23:0	0.02[b]	0.03[a]	0.02[b]	0.05	0.00
20:5n-3	0.05[b]	0.07[a]	0.05[b]	< 0.01	0.01
24:0	0.04	0.04	0.04	NS	0.00
26:0	0.03[b]	0.03[b]	0.04[a]	0.08	0.00
22:4n-6	0.01	0.01	0.01	NS	0.00
22:5n-6	0.02	0.02	0.01	NS	0.00
22:5n-3	0.07	0.08	0.09	NS	0.01
22:6n-3	0.02	0.02	0.02	NS	0.00
Summaries					
∑ SFA	62.40[c]	67.13[b]	75.82[a]	< 0.01	2.01
∑ SCFA (4:0-15:0)	26.98[b]	28.65[ab]	29.67[a]	0.06	1.35
∑ *iso + anteiso* FA	2.76[a]	2.70[a]	1.91[b]	< 0.01	0.12
∑ trans MUFA	8.27[a]	6.53[b]	2.87[c]	< 0.01	0.53
∑ cis MUFA	22.60[a]	20.46[b]	17.66[c]	< 0.01	1.06
∑ n-6 PUFA	1.25[a]	1.22[a]	1.01[b]	0.01	0.17
∑ n-3 PUFA	0.91[b]	1.07[a]	0.53[c]	< 0.01	0.14
Desaturase index					
*c*9-14:1 / 14:0	0.10	0.09	0.10	NS	0.01
*c*9-16:1 / 16:0	0.05[a]	0.03[b]	0.04[ab]	0.08	0.00
*c*9-18:1 / 18:0	1.84[a]	1.56[b]	1.80[ab]	0.08	0.11
*c*9*t*11-CLA / *t*11-18:1	0.44b	0.40b	0.52a	0.02	0.04

[abc] Values in the same row not sharing a common superscript are significantly different ($P < 0.05$).

[1] Diets: G = grazing; ZG = zero-grazing; GS = grass silage plus 3 kg of concentrate to all the diets.

[2] *c = cis*; *t = trans*; CLA = conjugated linoleic acid; SFA = saturated fatty acid (4:0 to 26:0 including branched chain fatty acids); SCFA = shorter-chain fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; Fatty acids were expressed as a percentage of total fatty acid methyl esters.

Milk vaccenic acid, trans10-18:1, rumenic acid and desaturase index: Among the *t*-18:1 isomers, VA (*t*11-18:1) was most abundant followed by *t*13/14-18:1 in all the treatments (Table 2-9). There was a significant difference in the concentration of milk VA among the treatments with concentrations being over 4-fold and 3-fold greater for G and ZG compared to GS. This is the net result of the differences in substrate intake and the extent of ruminal biohydrogenation reflected in the concentrations of ruminal and plasma VA. The greater concentrations of VA for G than for ZG could be explained by the high intake of LA and LN for G due to selective grazing of grass rich in these FA.

The concentration of the *t*10-18:1 isomer was less for GS than for G and ZG with no differences between the latter two. Furthermore, the *t*10- to *t*11-18:1 ratio was greater for GS than for G and ZG (Figure 2-1; Table 2-9). In the current study, there was a decrease in milk fat yield among the treatments from G to ZG and GS (Table 2-2) that did not appear to be related to known milk fat depressing fatty acids such as *t*10*c*12-CLA (Griinari et al., 2000), *t*9*c*11-CLA (Perfield II et al., 2007), or *t*10-18:1 (Griinari et al., 1999). Therefore, the relationships between milk fat yield (dependent variable) and milk concentrations of *t*10-18:1, *t*10*c*12-CLA, *t*7*c*9-CLA and *t*9*c*11-CLA (independent variables) was investigated using step-wise regression analysis. None of the independent variables entered the model even at $P < 0.2$. However, repeating the regression analysis using the *t*10: *t*11-18:1 ratios in milk, plasma and rumen revealed that 61% of the variability of milk fat yield was determined by the differences in milk *t*10: *t*11-18:1 ratio ($P < 0.01$).

Table 2-9. Effect of treatments on the composition of *trans*-18:1 and CLA isomers in milk fat

Isomer[2]	Diets[1]			*P* value	SEM
	G	ZG	GS		
Trans 18:1 isomers					
*t*4-18:1	0.03[a]	0.04[a]	0.02[b]	0.02	0.00
*t*5-18:1	0.03[a]	0.03[a]	0.02[b]	< 0.01	0.00
*t*6 to *t*8-18:1	0.34[a]	0.31[b]	0.20[c]	< 0.01	0.03
*t*9-18:1	0.30[a]	0.26[b]	0.17[c]	< 0.01	0.03
*t*10-18:1	0.51[a]	0.39[a]	0.20[b]	< 0.01	0.06
*t*11-18:1	4.73[a]	3.49[b]	0.99[c]	< 0.01	0.30
*t*10: *t*11-18:1	0.11[b]	0.12[b]	0.22[a]	< 0.01	0.02
*t*12-18:1	0.41[a]	0.34[b]	0.20[c]	< 0.01	0.04
*t*13/*t*14-18:1	0.85[a]	0.76[a]	0.44[b]	0.02	0.10
*t*15-18:1	0.23[a]	0.22[a]	0.13[b]	0.02	0.03
*t*16-18:1	0.39[a]	0.35[a]	0.22[b]	< 0.01	0.04
CLA isomers					
*t*7*c*9-CLA	0.04[a]	0.04[a]	0.02[b]	< 0.01	0.00
*t*8*c*10-CLA	0.01	0.01	0.01	NS	0.00
*c*9*t*11-CLA/RA	2.07[a]	1.38[b]	0.54[c]	< 0.01	0.17
*t*9*c*11-CLA	0.02[a]	0.01[b]	0.01[b]	< 0.01	0.00
*t*10*c*12-CLA	0.002	0.004	0.002	NS	0.00
*t*11*c*13-CLA	0.09[a]	0.08[a]	0.05[b]	0.01	0.01
*t*12*t*14-CLA	0.04[a]	0.03[b]	0.02[c]	< 0.01	0.00
*t*11*t*13-CLA	0.08[a]	0.07[a]	0.03[b]	< 0.01	0.01
*t*10*t*12-CLA	0.01	0.01	0.01	NS	0.00
*t*9*t*11-CLA	0.02[a]	0.02[a]	0.01[b]	< 0.01	0.00
*t*8*t*10-CLA	0.002	0.003	0.003	NS	0.00
*t*7*t*9-CLA	0.007	0.005	0.004	NS	0.00

[abc] values in the same row not sharing a common superscript are significantly different ($P < 0.05$).
[1]Diets: G = grazing; ZG = zero-grazing; GS = grass silage plus 3 kg of concentrate to all the diets.
[2] *t* = *trans;* *c* = *cis;* CLA = conjugated linoleic acid; RA = rumenic acid; Fatty acids were expressed as a percentage of total fatty acid methyl esters.

Higher concentrations of total CLA and RA were observed for G than for GS (Tables 2-8 and 2-9). The concentrations of these isomers were less for ZG than for G. Among the total CLA isomers RA was the predominant one accounting for 86%, 83% and 77% for treatments G, ZG and GS, respectively. Other CLA isomers recorded in appreciable concentrations were *t*12*t*14-, *t*11*c*13, *t*11*t*13- and *t*7*c*9-18:2 (Table 2-9). Considering the small proportion of LA compared to LN in forage and the finding that biohydrogenation of LN does not produce RA in the rumen, most of the milk RA yield in this study probably

resulted from the differences in the supply of VA to the mammary tissue and subsequent mammary desaturase activity.

Although desaturase activity can be determined using ratios of 4 different pairs of FA (Peterson et al., 2002), the ratio *c*9-14:1/14:0 has been reported to give a more accurate measure of mammary desaturase activity since 14:0 is a *de novo* synthesized FA by the mammary tissue (Corl et al., 2002; Griinari et al., 2000; Lock and Garnsworthy, 2002). Desaturase index calculated based on ratio of milk *c*9-14:1/14:0 was not different across the treatments (Table 2-8). Further milk desaturase index did not enter into the model when step-wise regression analysis of different variables influencing milk RA was done indicating that mammary desaturase activity was not influenced by the differences in milk VA observed here. Although the present results do not support desaturase activity to be influenced by the treatments, there appears to be difference in desaturase activity among individual animals. The desaturase index of individual cows (based on the ratio *c*9-14:1/14:0) ranged from 0.06 to 0.16 with a CV of 28% suggesting high variation among the cows within and between treatments. Part of this variation may be due to genetic differences among the cows (Mele et al., 2007).

Since there were no treatment effects on mammary desaturase index, the difference in milk RA response among the treatments can be related to the differences in LA and LN intakes and the influence of diets on rumen environment. A lower concentration of milk RA for GS was due to lower DMI and lower intakes of LA and LN than for G or ZG. The higher response in milk RA observed for G relative to ZG could be partly due to the selective grazing (HS) by the grazing cows, which resulted in a higher intake of LA and LN compared to that found in the offered grass (GC). Consistent with this, strong correlations were observed between milk RA yield (g/d) and intakes of LA and LN quantified

from HS when compared to those quantified from GC (Table 2-10). Part of the differences in milk RA response between G and ZG could also be explained by the differences in rumen environment favouring greater ruminal VA concentrations.

Table 2-10. Predictive equations for milk rumenic acid yield (g/d) with substrate (18:2n-6 or 18:3n-3) intake variables from two sources – Grass cuts (GC)[1] and Herbage snips (HS)[2]

Equation	R^2	*P*
RA yield g/d = 0.35 intake 18:2n-6 HS – 14.62	0.78	< 0.01
RA yield g/d = 0.06 intake 18:3n-3 HS – 4.11	0.79	< 0.01
RA yield g/d = 0.05 total substrate[3] intake HS – 5.74	0.79	< 0.01
RA yield g/d = 0.40 intake 18:2n-6 GC – 15.93	0.52	< 0.01
RA yield g/d = 0.08 intake 18:3n-3 GC – 4.27	0.49	< 0.01
RA yield g/d = 0.06 total substrate[3] intake GC – 6.17	0.49	< 0.01

[1]Represents animal wise substrate intake from all treatments (n = 17) with values for grazing cows estimated from grass cuts (offered grass).
[2]Represents animal wise substrate intake from all treatments (n = 17) with values for grazing cows estimated from herbage snips (consumed grass).
[3]Total substrate represents sum of 18:2n-6 + 18:3n-3.

Efficiency of milk rumenic acid production

Evaluation of the milk RA response based on the relative percentages of milk RA does not give a true picture of the efficiency of RA production when there are differences in the DMI, milk yield, fat yield and LA and LN intakes across the treatments. Therefore we calculated RA yield per 100 g of total substrate (LA + LN) intake (efficiency) to investigate if there were treatment differences on the efficiency of milk RA production. Efficiency of milk rumenic acid production was significantly different (*P* < 0.01; SEM – 0.17) across the treatments with higher values recorded for G (2.23) and ZG (1.50) than for GS (0.62) indicating that G cows were more efficient than ZG or GS cows because they were able to produce more RA yield per 100 g of total substrate intake. These differences in the efficiency of milk RA production could be due to a combination of factors regulating ruminal VA production and its supply to the mammary tissue.

It was of interest to note that the efficiency of ruminal VA production followed the same trend as that of milk RA production. Rumen VA pool size, calculated as the product of rumen lipid content and rumen VA content, was greater ($P < 0.01$; SEM – 2.26) for G (45.3 g) than for ZG (28.5 g) or GS (7.7 g). Rumen VA yield/100g total substrate intake (efficiency) was greater ($P < 0.01$; SEM – 0.74) for G (10.3 g) than for ZG (8.9 g) or GS (4.8 g) indicating that there was some influence of diet on rumen environment favouring greater production of VA by G cows.

Dietary influence on rumen environment and milk RA yield is clearly evident when data from individual animals were considered (Figure 2-2). For instance, cow 'A' produced 26 g RA/d with a total substrate (LA + LN) intake of 378 g/d on treatment G, and only 13 g RA/d with a total substrate intake of 325 g/d on treatment ZG; a 50% drop in RA yield for a 15% drop in substrate intake compared to G. When this cow was on treatment GS, she produced only 3.5 g/d of RA by consuming 167 g/d total substrate; a 90% drop in RA yield for a 55% drop in substrate intake compared to G. On the other hand, cow 'E' produced 17 g RA/d by consuming 425 g/d of total substrate on treatment G, and only 9 g RA/d with a total substrate intake of 355 g/d on treatment ZG; a 50% drop in RA yield for a 16% drop in intake. On treatment GS, cow 'E' produced 3 g RA/d with a total substrate intake of 158 g/d resulting in a 80% drop in RA yield for a 60% drop in substrate intake compared to G. These results clearly demonstrated that the intake of dietary substrates was not the only factor controlling milk RA production.

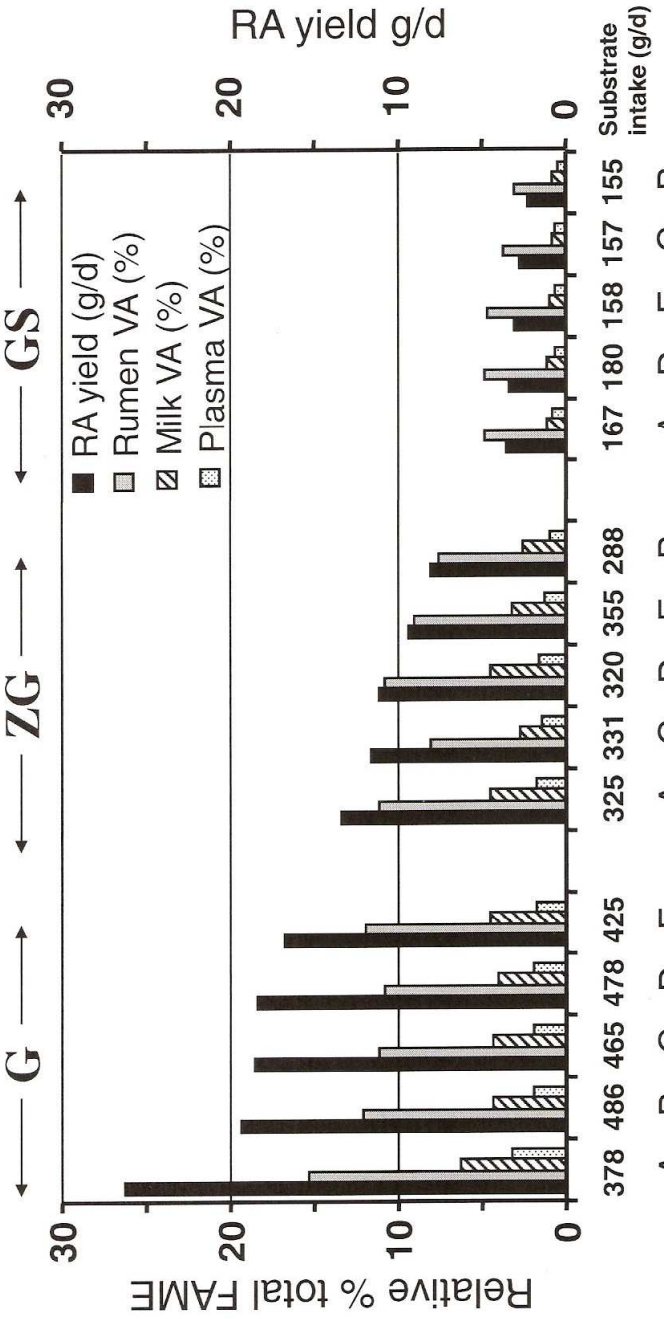

Figure 2-2. Animal- and treatment-wise RA yield, and ruminal, milk and plasma VA concentrations. The relative concentration of vaccenic acid (VA) is presented on the left y-axis, and rumenic acid (RA) yield is shown on the right y-axis. Substrate intake is the sum of 18:2n-6 and 18:3n-3. FAME = fatty acid methyl esters. Treatments G = grazing; ZG = zero-grazing and GS = grass silage.

A step-wise regression analysis was used to identify the variables that most influenced milk RA yield. The relationship between milk RA yield and source of substrate [GC (offered) or HS (consumed)] for G cows was first investigated to find out which of these two are strongly related to milk RA yield using REG procedure of SAS. Evaluation of the equations for the prediction of RA yield with single factors [LA intake (from HS), LN intake (from HS), total substrate intake (LA + LN from HS), LA intake (from GC), LN intake (from GC) and total substrate intake (LA + LN from GC)] revealed that the substrate intake quantified from HS was strongly correlated with milk RA yield compared to the substrate intake estimated from GC (Table 2-10). Subsequently the substrate intake variables LA and LN estimated from HS for G cows together with LA and LN from ZG and GS were utilized in a step-wise regression analysis with multiple factors using STEP WISE procedure of SAS. The threshold value to keep a term in the model was $P \leq 0.09$.

Step-wise regression analysis using the variables LA intake, LN intake, forage DMI, total DMI, milk yield, milk fat yield and milk desaturase index revealed that 79.4% of the variability in milk RA yield was determined by the differences in intake of LN ($P < 0.01$), and only about 6.7% of the variation was influenced by forage DMI ($P = 0.02$). These results are in agreement with those of Bargo et al. (2006) who reported that LN intake contributed the most (41%) to the variability in milk CLA. When the variables, VA and RA from rumen and plasma, milk VA, rumen fill (wet and dry), and rumen pH were also added to the model, 95.3% of variability in milk RA yield was determined by the differences in plasma VA concentrations (Table 2-11). The variables subsequently selected in the model include LN intake and LA intake, although their contribution in improving the model R^2 was very small. Milk desaturase index did not enter into

Table 2-11. Step-wise regression analysis of a group of variables[1] with milk rumenic acid (RA) yield (g/d) (n = 17; $P \leq 0.09$)

Variable	Partial R^2	Model R^2	F value	*P* value
Plasma VA (a)	0.9533	0.9533	265.63	< 0.01
Intake 18:3n-3 (b)	0.0319	0.9853	25.97	< 0.01
Intake 18:2n-6 (c)	0.0063	0.9915	8.17	0.02
Equation	RA yield g/d = 15.77 + 7.38a + 0.14b – 0.67c			

[1]Independent variables considered for the model include – intake 18:2n-6, 18:3n-3, rumen vaccenic acid (VA), plasma VA, wet rumen fill, dry rumen fill, rumen pH and milk desaturase index.

the model even at a significance level of 0.2 indicating that it was not influenced by the differences in milk VA in this study. Regression analysis using plasma VA as dependent variable and total DMI, forage DMI, LA intake, LN intake, total substrate (LA + LN) intake, ruminal LA, ruminal LN, ruminal VA, rumen pH, and rumen fill (wet and dry) at $P \leq 0.09$ revealed that 93% of the variability in plasma VA was determined by ruminal VA ($P < 0.01$). Wet rumen fill subsequently entered in the model, however, it improved the model R^2 by an additional 2% only ($P = 0.08$). Repeating the analysis with the same independent variables listed above but with ruminal VA as dependent variable revealed that 75% of the variability in ruminal VA was determined by total substrate (LA + LN) intake ($P < 0.01$); 7% by forage DMI ($P = 0.05$); 5.7% by LN intake ($P = 0.04$); and dry rumen fill by an additional 3.8% ($P = 0.05$).

2.4 Conclusions

The overall findings suggest that grazing cows are more efficient than zero-grazing and grass silage fed cows in milk RA production. Approximately 75% of the variability in milk RA yield in the current study can be explained by the variation in total substrate intake. The rest of the variability is due to differences in efficiency of milk RA production that is influenced by the interplay of various factors regulating ruminal VA production and its supply to the mammary tissue. These factors include (but not limited to): differences in DMI, rumen fill, rumen pH, rumen bacterial population's, intake characteristics (pattern of chewing,

rumination, saliva production etc), and digesta kinetics favoring increased ruminal VA production or biohydrogenation inhibition and differences in assimilation and flow of biohydrogenation intermediates to the mammary tissue. Further studies are required to investigate the role of digesta kinetics (operating at the rumen as well as the small intestine), animal genetics, rumen bacterial populations and other factors influencing absorption of the biohydrogenation intermediates and contributing to the differences in plasma VA and in turn to the variability in milk RA production.

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Effect of grain type and processing method on rumen fermentation and milk rumenic acid concentration

3.1 Introduction

The importance of diet in increasing milk rumenic acid (**RA**) concentrations has been well demonstrated (Chilliard et al., 2001; Bell et al., 2006). Polyunsaturated fatty acid (**PUFA**) substrates such as linoleic acid (**LA**) and α-linolenic acid (**LN**) in the diet undergo biohydrogenation in the rumen resulting in formation of major intermediates like *trans*(***t***)11-18:1 (vaccenic acid or **VA**) and *t*10-18:1. These intermediates are the products of two different pathways of PUFA metabolism and the abundance of either intermediate is determined by the diet and its influence on the rumen environment (Kramer et al., 2004). The concentrations of the intermediates in the rumen depends on the extent to which PUFA substrates are metabolized and are influenced by the initial concentration of dietary LA (Harfoot et al., 1973), passage rate, and rumen pH (Troegeler-Meynadier et al., 2003; Qiu et al., 2004). Altered rumen function resulting from a low rumen pH has been demonstrated to reduce the extent of PUFA biohydrogenation both *in vitro* (Martin and Jenkins, 2002) and *in vivo* (Kalscheur et al., 1997). Because rumen pH is mainly influenced by the fermentable carbohydrate in the diet, it is possible that the amount of starch and/or the rate of ruminal degradation of starch could play a role in altering rumen pH, which in turn could affect biohydrogenation intermediates in the rumen.

It is well established that barley starch is more rapidly and completely degraded in the rumen compared with corn starch (Ørskov, 1986; Huntington, 1997). Furthermore, rumen availability and degradability of grain starch can be increased by processing grain (Hale, 1973; Theurer, 1986). Previous studies have shown that the feeding of grain-based diets decreases rumen pH (Yang and Beauchemin, 2007; Penner et al., 2009). We investigated whether such a change in the rumen pH due to grain source and processing method would affect the metabolism of PUFA as measured by the resultant end products. For this

purpose a detailed fatty acid analysis was conducted on the rumen, plasma and milk fat of these cows and related to changes in rumen pH and to the profile of *t*-18:1 and conjugated linoleic acid (**CLA**) isomers.

In most studies, the mean rumen pH calculated from the pH measured twice a day (Abughazaleh et al., 2003; Mohammed et al., 2009) or six times a day (Kalscheur et al., 1997; Boeckaert et al., 2008) has been reported. Mean rumen pH often does not reflect the minimum pH experienced by the cows during the day, which may explain why differences in rumen *t*11-18:1 between zero-grazed and grass silage fed cows were not related to mean pH (Mohammed et al., 2009). Therefore, we utilized a continuous rumen pH monitoring system (Lethbridge Research Centre Rumen pH Measurement System, Dascor, Escondido, CA; Penner et al., 2006) in the current study so we could more accurately describe the rumen pH changes and relate them to the PUFA intermediates in the rumen.

It was hypothesized that a rapidly fermentable starch source (barley) would inhibit PUFA biohydrogenation to a greater extent and result in greater concentrations of *t*11-18:1 in rumen and milk RA compared to a less fermentable starch source (corn). A reduction in grain particle size by grinding results in an increase in starch availability and degradability for ground diets compared to rolled diets. Our second hypothesis therefore was that ground diets (barley or corn) would inhibit PUFA biohydrogenation to a greater extent than rolled diets resulting in greater concentrations of ruminal *t*11-18:1 and milk RA for ground diets. Because of the differences in particle size between rolled and ground diets and the expected differences in rates of starch fermentation between barley and corn, we further hypothesized that milk from cows fed barley-based diets (**BBD**) and ground diets (corn or barley) would have higher concentrations of *t*10-18:1 and lower milk fat yield compared to the milk from cows fed corn-based diets

(**CBD**) and rolled diets (barley or corn). To test these hypotheses an experiment was designed with the following objectives: i) to characterize the changes in ruminal pH as a result of feeding two grain sources known to differ in rates of starch fermentation; ii) to determine whether differences in rates of starch degradation achieved through processing, and the resultant changes in ruminal pH, would affect the metabolism of PUFA as measured by the resultant end products, specifically the *t*-18:1 and CLA isomers; and iii) to determine the variability in milk RA and milk fat yield.

3.2 Materials and methods

Experimental design and treatments

All procedures were carried out according to the guidelines of the Canadian Council on Animal Care (Institutional Animal Use Approval Number: 2006-46). Eight Holstein cows were used in a replicated 4 × 4 Latin square design with a 2 x 2 factorial arrangement of treatments with 21-d periods. Treatments were 2 grain sources (barley vs corn) and 2 processing methods (rolled vs ground). The cows were rumen-cannulated, multiparous (lactation number 2 to 5) and in mid-lactation (average milk yield, 38.0 ± 6.1 kg/d; average days in milk, 154.1 ± 57.3; average body weight, 721 ± 41 kg). The cows were assigned to two squares such that milk yield and days in milk were similar for both squares. Within a square, the cows were randomly assigned to four dietary treatments: dry rolled barley (RB), ground barley (GB), dry rolled corn (RC) and ground corn (GC).

The grains (barley or corn) used in the study were obtained from single lots. Each grain was either dry rolled or ground in a single batch. Grinding was done in a hammer mill (Stirdon, USA) using a 2.38-mm screen. Ingredient composition of diets is given in Table 3-1. All diets contained whole sunflower seed, which contained 64% linoleic acid and 0.13% linolenic acid. Prior to ration

formulation, all the ingredients were screened for starch content and LA and LN contents, and all dietary treatments were formulated to contain similar amounts of starch and total substrate (LA + LN). Experimental diets were offered once daily (0900 h) at 110% of expected intake. All cows were housed in tie stalls with continuous access to water. Cows were milked in their stalls twice daily (0500 and 1700 h).

Table 3-1. Ingredient, chemical and fatty acid composition of the experimental diets

	Treatments[1]			
	Barley		Corn	
	RB	GB	RC	GC
Ingredient composition (g/kg DM)				
Alfalfa silage	33.11	33.11	33.11	33.11
Alfalfa hay	9.07	9.07	9.07	9.07
Barley grain	39.58	39.58	0.00	0.00
Corn grain	0.00	0.00	26.08	26.08
Canola meal	5.90	5.90	20.45	20.45
Corn gluten meal	2.59	2.59	2.27	2.27
Sunflower seed	8.62	8.62	7.89	7.89
Premix[2]	0.91	0.91	0.91	0.91
Salt	0.23	0.23	0.23	0.23
Chemical composition (%)				
DM	66.1	66.3	66.4	67.3
CP	20.2	20.4	22.7	22.2
EE	4.5	4.2	4.4	4.3
Ash	7.2	6.8	7.3	7.1
Total carbohydrates[3]	68.0	68.5	65.6	65.7
NFC[4]	41.2	43.2	40.4	41.5
Starch	36.1	37.1	35.5	35.2
NDF	26.9	25.3	25.2	24.2
ADF	15.6	14.4	15.9	15.2
Fatty acid composition (% of FAME)				
14:0	0.39	0.38	0.30	0.25
16:0	15.57	16.95	11.88	12.44
*c*9-16:1	0.19	0.11	0.26	0.25
18:0	3.98	3.88	3.60	3.33
*c*9-18:1	13.21	13.09	18.04	19.33
18:2n-6	45.24	45.06	44.02	43.27
18:3n-3	8.11	8.05	7.19	7.77
18:2n-6 + 18:3n-3	51.21	51.04	53.35	53.11

[1]Diets were rolled barley (RB), ground barley (GB), rolled corn (RC) and ground corn (GC).

[2]Premix contained (minimum %) Ca – 0.1, P – 0.6, Na – 11.5, Mg – 0.3; (mg/kg) Fl – 10, I – 80, Zn – 5000, Mn – 3100, Cu – 1170, Co – 6.2; Vitamin A – 1265 kIU/kg, Vit. D – 142 kIU/kg and Vit E – 3800 IU/kg
[3]Total carbohydrate = 100 – (CP + EE + Ash)
[4]NFC (nonfibrous carbohydrate) = 100 – (NDF + CP + EE + Ash)

In situ degradation

Representative samples of rolled barley, rolled corn, ground barley and ground corn were obtained. Nitrogen free polyester bags (part # R 510, Ankom Technology, Macedon, NY) with the manufacturer specified pore size of 50 ± 15 µm were sealed with a sample size to obtain approximately 12.5 mg/cm^2 surface area. Quadruplicate sets of bags were prepared for each incubation time and inserted with weights in the ventral sac of the rumen. Rumen incubation times were 0, 1, 2, 4, 8, 12, 24, 48 and 72 h. One of the rumen-cannulated cows offered the rolled barley diet was used for the in situ degradation study after completion of the main experiment. Bags were soaked in warm tap water prior to ruminal incubation for 15 min to remove the soluble fraction. The percent residue remaining in the bag after the pre-ruminal soak was used as the zero time value and this represented the potentially insoluble but digestible material. At the end of each incubation time, bags were rinsed in cold water until the running water was clear. To minimize contamination of the grain DM with microbial DM, the bags were left overnight at -20^{o}C, thawed and rinsed again in cold water, drained and allowed to dry at 55^{o}C for 48 h. Samples were ground through a 1-mm screen and starch was determined (Karkalas, 1985). Rate of degradation for the degradable fraction (b) was determined by fitting the data to the exponential equation $R_t = R_0 (e^{kt})$, where k = degradation rate for the degradable fraction; R_t = degradation at time t; and R_0 = degradation at time zero.

Sampling and chemical analyses

Samples of TMR and orts were collected every day in the last week of each period and representative samples of total mixed rations (**TMR**) and orts

from each period were prepared and stored at -20°C. The DM content of alfalfa hay and alfalfa silage was determined weekly to adjust dietary allocation of forages to maintain a consistent forage:concentrate ratio. The orts samples were pooled by animal, and one sample per period was stored at -20°C. The TMR and orts were dried at 55°C for 72 h and ground through a 1-mm screen using a Wiley mill (Thomas-Wiley, Philadelphia, PA). Samples were analyzed for DM content (at 135°C for 2 h), ash (overnight at 550°C), ADF, NDF (Ankom filter bag technique with the reagents sodium sulphite and alpha-amylase; Van Soest et al., 1991), nitrogen content (determination of CP using a Leco nitrogen combustion analyzer, Leco Corporation, St. Joseph MI, USA) and ether extract (Goldfisch extraction apparatus). Total carbohydrates in TMR were determined by subtracting the sum of the percentage of CP, ether extract and ash from 100 while the non structural carbohydrates (NSC) were calculated by substracting the sum of the percentage of CP, NDF, ash and ether extract from 100 (Beauchemin et al., 1997). Starch content was determined following the enzymatic method described by Karkalas (1985). Absorbance was determined at 450 nm using a plate reader (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA).

Feed fatty acid (**FA**) composition was determined using 30 mg of TMR samples (by period) by direct methylation using 1 ml of 5% anhydrous HCl/methanolic (w/v) along with an internal standard ($C_{23:0}$ @ 10% of the dietary fat). Methylation was done for 1 h at 80°C. Following methylation the fatty acid methyl esters (**FAME)** were extracted using hexane, dried down to 200 µL and applied onto thin layer chromatography (**TLC**) plate and developed in hexane: diethyl ether: acetic acid solvent (85:15:1). Fatty acid methyl esters were then scraped off the TLC plate and extracted with chloroform. After removal of chloroform the FAME were taken up in 1 ml hexane and analyzed by GLC using a 100m CP Sil capillary column (Varian Inc., Mississauga, ON) at the 175°C

temperature program as described by Kramer et al. (2008). Fiber intake and total substrate intake was estimated from their contents in the TMR offered and orts.

Rumen pH was recorded using the Lethbridge Research Centre Rumen pH Measurement System (LRCpH; Dascor, Escondido, CA) as described by Penner et al. (2006). This system recorded rumen pH every 30 s continuously for the last three days of each period. The pH readings were averaged for each minute and presented as mean daily pH, minimum daily pH, maximum daily pH, duration (h/d) for which rumen pH remained < 5.8, < 5.5 and < 5.2. Similar pH thresholds (5.8, 5.5 and 5.2) were used by Maekawa et al. (2002) and Beauchemin and Yang (2005) to describe the severity of ruminal acidosis. The number of daily episodes of rumen pH < 5.8 was also recorded. A daily episode was defined to begin when rumen pH was below 5.8 and to end when pH reached or exceeded 5.8.

Rumen samples for volatile fatty acids (**VFA**) and NH_3 analysis were collected from 4 different locations in the rumen [cranial aspect (reticulum), dorsal sac (fiber mat), ventral sac, and caudal aspect], composited and squeezed through 4 layers of cheese cloth at 0600, 1200 and 1800 h on day 19, at 0900 and 1500 h on day 20 and at 2100 and 2400 h on day 21 of each period. Five milliliters of filtrate was preserved by adding 1 ml of 25% (wt/vol.) HPO_3 to determine VFA concentrations (Ranfft et al., 1973) and another 5 ml of filtrate was preserved by adding 1 ml of 1% H_2SO_4 to determine NH_3 concentration (Fawcett and Scott, 1960). The samples were stored at -20^{o}C until analysis. Rumen samples for FA analysis consisted of both fluid and solids and were collected at the same time as described for VFA and NH_3 analyses and stored at -20^{o}C. For FA analysis the samples were freeze-dried and milled, and 0.5 g of the sample pooled by cow each period was utilized for FA analysis following the protocol described by Mohammed et al. (2009).

Blood samples were collected from each cow at 4 h intervals, on the last 3 d of each period; day 19 at 0700 and 1500 h; day 20 at 1100 and 1900 h; day 21 at 2300 and 0300 h. Plasma concentrations of glucose, insulin and non-esterified fatty acids (**NEFA**) were determined for each sample, and the values were averaged by period before statistical analysis. Glucose concentrations were determined using the glucose oxidase/peroxidase enzyme (No. P7119, Sigma, St. Louis, MO) and dianisidine dihydrochloride (No. F5803, Sigma) (Karkalas, 1985). Absorbance was determined at 450 nm with a plate reader (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA). Insulin concentrations were determined following the kits instructions (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) NEFA concentrations were determined with NEFA-C-kit (Wako Chemicals USA, Richmond, VA) using the modified procedure described by Johnson and Peters (1993). Fatty acid composition was determined from plasma samples pooled by cow each period. Plasma lipids were extracted, methylated and analyzed by GLC as described by Mohammed et al. (2009).

Milk was sampled AM and PM on the last two days of each period. The AM and PM samples were pooled for each cow on each sampling day in proportion to the respective milk yields. Milk samples from the two days were again pooled in proportion to the daily yields and divided into two aliquots. One aliquot was preserved with potassium dichromate and analyzed for crude protein, fat, lactose and milk urea nitrogen using mid-infrared spectroscopy at the central milk testing laboratory Edmonton, Alberta, Canada (MilkoScan FT 6000, Foss Electric, Hillerod, Denmark) and the other aliquot was stored at –20°C for fatty acid analysis using GLC. Milk lipids were extracted, methylated and analyzed by GLC as described by Mohammed et al. (2009). The FAME were analyzed using a Hewlett Packard Model 5890 Series II GLC equipped with a flame ionization detector, an autosampler (HP Model 7673), a 100 m CP-Sil 88 fused capillary column (Varian Inc., Mississauga, ON), software program (Agilent

ChemStation, Version A.10) and operated in a splitless mode that was flushed 0.3 min after injection. Injector and detector temperatures were kept at 250^{o}C. Hydrogen was used as carrier gas at a flow rate of 1 mL/min and for the flame ionization detector at 40 mL/min. Other gases used were purified air at 250 mL/min and N_2 makeup gas at 25 mL/min. The injection volume was 1 µL of FAME mixtures containing about 1 µg/µL. The FAME obtained from each milk sample were analyzed using two temperature programs (175^{o}C and 150^{o}C) as described by Kramer et al. (2008). The FAME were identified by comparison with a GLC reference standard (#463) spiked with a mixture of four positional conjugated linoleic acid (**CLA**) isomers (#UC-59M), 21:0, 23:0 and 26:0 obtained from Nu-Chek Prep Inc. (Elysian, MN). Identification of 16:1, 18:1 and 20:1 isomers was based on available isomers in the GLC reference standard (#463), comparison with published reports and based on principles of silver-ion separation. Confirmation of 18:1 isomers was made using Ag^{+}-solid phase extraction separated fractions as described by Kramer et al. (2008). Individual FAME were reported as a percentage of total FAME (g/100g) and the short-chain FAME were corrected for their flame ionization detector deficiency based on theoretical considerations (Wolff et al., 1995).

Statistical analysis

Data for intakes, production, rumen fermentation and fatty acid composition of lipids in rumen, plasma and milk were analyzed by the MIXED procedure of SAS (version 9.1, SAS Institute, Cary, NC) with treatments as the fixed effect; square, period and cow (square) were random effects. The model included the main effects (grain type and processing) and the interaction. Least square means for the grain type, method of processing and their interaction were calculated for each variable and the differences among the least square means were declared significant at $P < 0.05$. Data for the percentage grain retained on each sieve was analyzed by the GLM procedure of SAS. Step-wise

regression analysis was used to identify the variables that influenced the concentrations of *t*10-18:1 and *t*11-18:1 and RA in rumen contents and milk. The threshold value to keep a term in the model was $P < 0.05$.

3.3 Results

Grain particle size distribution and in situ degradation

The proportion of particles on the various sieves was similar for ground barley and ground corn indicating that grinding achieved a similar particle size for both grains (Table 3-2). Rolled barley had a greater proportion of large particles (> 4 mm) and a lower proportion of small particles (< 1 mm) than rolled corn, despite using the same roller settings. Differences in the resulting particle size distribution due to rolling reflected inherent characteristics of the grains. *In situ* degradation rates (% h^{-1}) for rolled corn, ground corn, rolled barley and ground barley were 5.4, 8.9, 17.0 and 19.4, respectively, for DM and 6.3, 10.8, 25.3 and 43.8, respectively, for starch. These data indicate a faster rate of degradation for barley grain than corn grain, with grinding increasing the degradation rate of both grains.

Table 3-2. Percentage grain retained on sieve

	Grain type[1]				SEM	*P* value
	Barley		Corn			
Particle category[2]	RB	GB	RC	GC		
Above 4 mm	21.0[a]	0.0[c]	8.6[b]	0.0[c]	1.68	< 0.01
> 2 mm and < 4 mm	60.0[a]	4.6[b]	61.9[a]	3.6[b]	1.67	< 0.01
> 1 mm and < 2 mm	16.2[b]	29.6[a]	15.3[b]	27.3[a]	1.92	< 0.01
> 425 µm and < 1 mm	1.9[c]	37.7[a]	8.6[b]	40.0[a]	1.71	< 0.01
< 425 µm	1.0[c]	28.0[a]	5.7[b]	29.1[a]	1.29	< 0.01

[a-c] Means in a same row not bearing a common superscript are significantly different ($P < 0.05$).
[1]Rolled barley (RB), ground barley (GB), rolled corn (RC) and ground corn (GC).
[2]Canadian standard sieve series: numbers 5, 10, 18 and 40 with a mesh size 4 mm, 2 mm, 1 mm and 425 µm respectively.

Intakes and milk production

Dry matter intake was greater for cows fed CBD than BBD with no effects of grain processing (Table 3-3). Fiber (NDF) intakes and starch intakes were not influenced by the grain type or method of processing. Although diets were formulated to contain similar amounts of LA and total substrate (LA + LN), there was an interaction between grain type and method of processing for LA intake (P = 0.02) and total substrate intake (P = 0.03). Linolenic acid intakes were greater for CBD than BBD with no effects of grain processing. Despite differences in DMI, milk yields and 4% FCM were not affected by treatments. Milk fat content was greater for CBD than for BBD with lower values observed for grinding compared to rolling (Table 3-3). Milk fat yield was also greater for CBD than for BBD, however, it was not influenced by the method of grain processing. The content and yield of milk protein and lactose were not influenced by the grain type or the method of processing. Milk urea nitrogen was greater for CBD than for BBD, with no effects of processing, reflecting the higher dietary protein in CBD.

Table 3-3. Effect of grain type and method of processing on intakes and milk production

	Treatments[1]					Significance[2]		
	Barley		Corn					
Variables	RB	GB	RC	GC	SEM	Gr	Pr	Gr*Pr
Intake								
DM (kg/d)	25.1	25.4	27.2	26.9	0.81	0.01	NS	NS
NDF (kg/d)	6.7	6.4	6.9	6.5	0.25	NS	NS	NS
Starch (kg/d)	9.1	9.4	9.6	9.5	0.29	NS	NS	NS
Linoleic acid (18:2n-6) (g/d)	231.1	276.4	337.2	297.4	32.32	< 0.01	NS	0.02
Linolenic acid (18:2n-3) (g/d)	41.5	51.3	54.6	53.9	3.98	0.01	NS	NS
Total substrate	272.6	327.7	391.8	351.3	35.88	< 0.01	NS	0.03
Production								
Milk yield (kg/d)	34.8	34.0	33.1	34.0	3.32	NS	NS	NS
4 % FCM (kg/d)	28.66	25.66	29.27	28.85	3.07	NS	NS	NS
Fat (%)	2.77	2.45	3.28	2.94	0.21	< 0.01	0.03	NS
Protein (%)	3.28	3.31	3.35	3.39	0.09	NS	NS	NS
Lactose (%)	4.50	4.51	4.46	4.56	0.06	NS	NS	NS
Fat yield (kg/d)	0.98	0.81	1.08	1.01	0.13	0.03	NS	NS
Protein yield (kg/d)	1.15	1.12	1.08	1.14	0.10	NS	NS	NS
Lactose yield (Kg/d)	1.58	1.54	1.47	1.57	0.16	NS	NS	NS
MUN (mg/dL)	16.12	15.21	19.27	17.60	1.21	< 0.01	NS	NS

[1]Diets were rolled barley (RB), ground barley (GB), rolled corn (RC) and ground corn (GC).
[2]Gr = grain type; Pr = processing; Gr*Pr = interaction between grain type and method of processing.
NS = not significant ($P \geq 0.05$)

Rumen fermentation

Daily minimum rumen pH was lower for BBD than for CBD (Table 3-4). Cows fed BBD experienced longer duration below the pH thresholds of 5.8, 5.5 and 5.2 than those fed CBD. However, none of the rumen pH variables measured was influenced by the method of processing. Daily mean pH and maximum pH were not influenced by grain type or method of processing. Standard deviation of the daily rumen pH (1440 data points recorded per day) was greater for BBD than for CBD indicating greater pH fluctuations. Rumen pH averaged on an hourly basis and plotted (Fig. 3-1) for 72 h resembled a biphasic curve reaching its maximum in the morning before feeding (Figure 3-1). It started to fall with the onset of feeding (marked by a downward arrow), reached its lowest level by the end of the day (8-10 hours after the onset of feeding) followed by a gradual rise overnight reaching its maximum by the next morning. It could be observed that

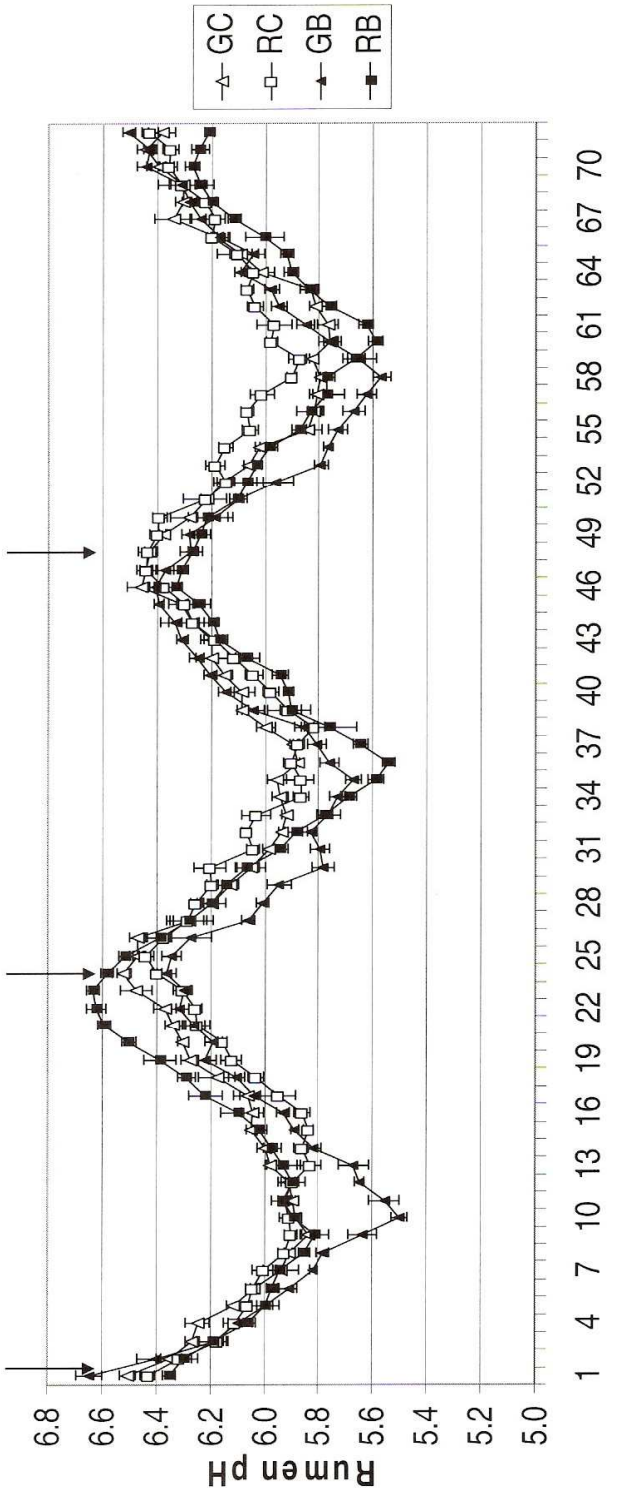

Figure 3-1. Effect of treatments on rumen pH. Diets were rolled barley (RB), ground barley (GB), rolled corn (RC) and ground corn (GC). Rumen pH recorded every 30 s for 72 hours using the LRCpH system was averaged for each hour and plotted. The arrows denotes the time of feeding for 3 consecutive days.

the lowest points of the curve were data from cows fed BBD while data points for cows fed CBD were at a slightly higher level. Furthermore, the standard error bars were longer for BBD than for CBD. The number of daily episodes when rumen pH remained < 5.8 was not influenced by the grain type or method of processing. However, the average duration per episode (pH < 5.8) was greater for BBD than for CBD, but was not different between processing methods.

Table 3-4. Effect of grain type and method of processing on rumen fermentation

	Treatments[1]					Significance[2]		
	Barley		Corn					
	RB	GB	RC	GC	SEM	Gr	Pr	Gr*Pr
Daily minimum pH	5.34	5.31	5.51	5.55	0.09	0.01	NS	NS
Daily mean pH	6.06	6.01	6.12	6.12	0.10	NS	NS	NS
Daily maximum pH	6.74	6.73	6.72	6.73	0.08	NS	NS	NS
Standard deviation	0.34	0.33	0.25	0.26	0.03	0.01	NS	NS
Duration pH<5.8 (h/d)	6.53	7.49	4.08	4.55	2.07	0.03	NS	NS
Duration pH<5.5 (h/d)	2.39	3.07	0.85	0.91	0.94	0.01	NS	NS
Duration pH<5.2 (h/d)	0.78	0.77	0.03	0.13	0.35	0.04	NS	NS
Daily episodes pH < 5.8	11.4	10.9	10.6	13.7	3.13	NS	NS	NS
Average duration per Episode pH < 5.8 (min/d)	44.1	41.3	20.1	18.3	13.56	0.02	NS	NS
Total VFA (mmol/L)	115.2	118.9	115.9	114.7	6.34	NS	NS	NS
VFA molar proportions (mmol/100mol)								
Acetic acid	46.1	44.9	48.9	48.0	0.90	< 0.01	0.02	NS
Propionic acid	27.8	28.8	21.7	23.7	1.20	< 0.01	NS	NS
Butyric acid	9.1	9.4	11.0	11.1	0.42	< 0.01	NS	NS
Isobutyric acid	4.4	3.9	5.4	4.8	0.25	< 0.01	< 0.01	NS
Valeric acid	4.4	4.3	4.1	4.2	0.21	NS	NS	NS
Isovaleric acid	3.7	3.2	4.8	4.3	0.19	< 0.01	< 0.01	NS
Caproic acid	4.3	5.5	4.2	3.9	0.58	NS	NS	NS
Acetate: Propionate	1.7	1.6	2.3	2.1	0.12	< 0.01	<0.01	NS
Ammonia N (mg/dL)	24.0	23.0	34.3	29.1	1.99	< 0.01	0.04	NS

[1]Diets were rolled barley (RB), ground barley (GB), rolled corn (RC) and ground corn (GC).
[2]Gr = grain type; Pr = processing; Gr*Pr = interaction between grain type and method of processing. NS = not significant ($P \geq 0.05$).

Concentrations of rumen acetate and butyrate were greater for CBD than for BBD (Table 3-4) while that of propionate was greater for BBD than for CBD. Rumen acetate was greater for rolling than for grinding while those of propionate and butyrate were not different between grinding and rolling. The

ratio of acetate to propionate was lower for BBD than for CBD, and a lower ratio was observed for grinding compared to rolling. The concentrations of isobutyrate and isovalerate were lower for BBD than for CBD, and for grinding compared to rolling. Total VFA however were not influenced by treatments. Rumen NH_3 concentrations were greater for CBD than for BBD with greater values observed for rolling than for grinding.

Rumen fatty acid composition

The sum of the saturated fatty acid (**SFA**) was greater for CBD than for BBD with greater concentrations observed for rolling than for grinding. There was an interaction between the main effects for the concentrations of 14:0 and 16:0 in the rumen (Table 3-5). Concentrations of 16:0 were greater for BBD than for CBD with greater values observed for rolling compared to grinding. A significant interaction between the main effects was also observed for the sum of the iso and anteiso FA. Concentrations of 15:0 *iso* were influenced by both main effects and those of 16:0 *iso* by the grain type only. There was an interaction between the main effects for the sum of the n6-PUFA with greater values observed for BBD than for CBD and without any effects of grain processing. The sum of the n6-PUFA was represented mainly by the differences in the concentrations of 18:2n-6. Sum of the n3-PUFA were greater for grinding than rolling and represented mainly by the differences in the concentrations of 18:3n-3 with no effects of the grain type. The sum of the *c/t*-18:2 isomers were greater for BBD than for CBD with no effect of processing.

Individual 16:1 isomers were not influenced by the grain type or method of processing. However, the sum of the *t*-16:1 isomers were greater for grinding than for rolling (more so for CBD) with no difference between the grain types. The sum of the *c*-16:1 isomers was greater for CBD than for BBD and represented mainly by the differences in *c*9-16:1 and *c*13-16:1 with no difference between

rolling and grinding. The sum of the *t*-18:1 isomers in rumen contents were greater for BBD than for CBD and were represented mainly by the difference in the concentrations of *t*10-18:1 and *t*11-18:1. The sum of the *c*-18:1 was greater for CBD than for BBD. However, sums of the *t*-18:1 and *c*-18:1 isomers were not different between rolling and grinding. Concentrations of *t*10-18:1 and *t*11-18:1 were greater for BBD than for CBD with no difference between rolling and grinding. The ratio of *t*10:*t*11-18:1 was also greater for BBD than for CBD with no effect of processing. Concentrations of *c*11-20:1 and *c*13-22:1 were greater for BBD than for CBD with no difference between rolling and grinding. The sum of the *trans*-monounsaturated fatty acids (*t*-**MUFA**) was greater for BBD than for CBD while those of *cis* (***c***)-MUFA were lower for BBD than for CBD. The sum of the CLA isomers in the rumen was not different between the treatments and ranged from 0.21 to 0.29%. Concentration of *c*9*t*11-CLA, which was the predominant isomer, was not different between the grain types but was greater for grinding compared to rolling. Concentrations of *t*10*c*12 CLA, however, were not different across the treatments.

Table 3-5. Effect of grain type and method of processing on rumen fatty acid composition

Variable[3]	Treatments[1]				SEM	Significance[2]		
	Barley		Corn					
	RB	GB	RC	GC		Gr	Pr	Gr*Pr
14:0	0.48	0.42	0.37	0.43	0.04	< 0.01	NS	< 0.01
15:0 *iso*	0.35	0.27	0.24	0.23	0.03	< 0.01	0.03	NS
15:0 *anteiso*	0.32	0.30	0.29	0.29	0.02	NS	NS	NS
15:0	0.38	0.32	0.37	0.36	0.03	NS	0.02	0.05
16:0 *iso*	0.07	0.07	0.13	0.12	0.02	< 0.01	NS	NS
16:0	10.74	10.19	9.69	9.81	0.19	< 0.01	< 0.01	< 0.01
17:0 *iso*	0.04	0.04	0.04	0.03	0.004	NS	NS	NS
17:0 *anteiso*	0.26	0.19	0.23	0.24	0.03	NS	NS	NS
17:0	0.19	0.18	0.20	0.19	0.01	NS	NS	NS
18:0 *iso*	0.01	0.01	0.01	0.01	0.001	NS	NS	NS
18:0	25.86	21.59	32.16	31.19	2.28	< 0.01	0.04	NS
19:0	0.06	0.05	0.06	0.06	0.01	NS	NS	NS
20:0	0.51	0.49	0.55	0.56	0.01	< 0.01	NS	NS
21:0	0.01	0.01	0.004	0.003	0.003	NS	NS	NS
22:0	0.78	0.79	0.74	0.74	0.01	< 0.01	NS	NS
24:0	0.40	0.39	0.40	0.42	0.01	NS	NS	NS
26:0	0.10	0.08	0.10	0.11	0.008	0.02	NS	0.05
∑ SFA	**49.73**	**44.41**	**53.54**	**53.28**	**2.89**	**< 0.01**	**0.03**	**NS**
∑ *iso* + *anteiso* FA	**1.05**	**0.87**	**0.94**	**0.93**	**0.05**	**NS**	**< 0.01**	**0.01**
*t*4-16:1	0.01	0.01	0.01	0.01	0.002	NS	NS	NS
*t*9-16:1	0.01	0.01	0.01	0.01	0.001	NS	NS	NS
*t*10-16:1	0.001	0.001	0.001	0.001	0.001	NS	NS	NS
*t*11-16:1	0.05	0.06	0.05	0.06	0.01	NS	NS	NS
*t*12-16:1	0.02	0.02	0.02	0.02	0.003	NS	NS	NS
*c*7-16:1	0.05	0.05	0.05	0.06	0.01	NS	NS	NS
*c*9-16:1	0.07	0.07	0.10	0.11	0.006	< 0.01	NS	NS
*c*11-16:1	0.01	0.01	0.01	0.10	0.001	NS	NS	NS
*c*13-16:1	0.02	0.02	0.03	0.03	0.003	< 0.01	NS	NS
∑*trans*-16:1	**0.10**	**0.10**	**0.07**	**0.10**	**0.007**	**NS**	**0.04**	**NS**
∑*cis*-16:1	**0.15**	**0.14**	**0.18**	**0.20**	**0.01**	**< 0.01**	**NS**	**NS**
*c*7 17:1	0.01	0.01	0.01	0.01	0.002	NS	NS	NS
*c*9-17:1	0.04	0.04	0.05	0.04	0.01	NS	NS	NS
*t*4-18:1	0.04	0.03	0.05	0.04	0.004	< 0.01	0.01	NS
*t*5-18:1	0.03	0.03	0.03	0.03	0.004	NS	NS	NS
*t*6 to *t*8-18:1	0.59	0.66	0.51	0.52	0.06	0.03	NS	NS
*t*9-18:1	0.48	0.56	0.35	0.37	0.08	0.03	NS	NS
*t*10-18:1	3.10	3.81	1.16	1.36	0.78	< 0.01	NS	NS
*t*11-18:1	2.80	3.53	1.82	2.12	0.50	0.01	NS	NS
*t*12-18:1	0.83	0.77	0.84	0.86	0.07	NS	NS	NS
*t*13/*t*14/*c*6-8-18:1	1.99	1.85	1.91	2.02	0.16	NS	NS	NS
*c*9/c10/15t-18:1	7.57	8.05	8.42	8.23	0.41	< 0.01	NS	0.03
*c*11-18:1	0.81	0.86	0.97	1.12	0.04	< 0.01	< 0.01	NS
*c*12-18:1	1.08	1.08	0.79	0.97	0.12	NS	NS	NS
*c*13-18:1	0.05	0.05	0.04	0.05	0.004	NS	NS	NS

*c*14/*t*16-18:1	0.57	0.45	0.67	0.64	0.06	< 0.01	NS	NS
*c*15-18:1	0.13	0.11	0.09	0.12	0.01	NS	NS	0.04
*c*16-18:1	0.05	0.07	0.03	0.04	0.01	0.01	NS	NS
∑ *trans*-18:1	**9.88**	**11.25**	**6.68**	**7.32**	**1.14**	**< 0.01**	**NS**	**NS**
∑ *cis*-18:1	**10.25**	**10.66**	**11.02**	**11.18**	**0.41**	**0.02**	**NS**	**NS**
***t*10: *t*11-18:1**	**1.09**	**1.04**	**0.65**	**0.64**	**0.21**	**0.03**	**NS**	**NS**
*c*5-20:1/*t,c,c*-18:3	0.001	0.003	0.002	0.002	0.001	NS	NS	NS
*c*9-20:1	0.01	0.01	0.01	0.01	0.001	NS	NS	NS
*c*11-20:1	0.14	0.14	0.09	0.09	0.01	< 0.01	NS	NS
13*c*-22:1	0.02	0.01	0.007	0.008	0.001	< 0.01	NS	NS
*c*15-24:1	0.01	0.03	0.01	0.01	0.01	NS	NS	NS
∑ *trans* MUFA	**9.98**	**11.35**	**6.76**	**7.42**	**1.14**	**< 0.01**	**NS**	**NS**
∑ *cis* MUFA	**10.64**	**11.03**	**11.38**	**11.56**	**0.41**	**0.02**	**NS**	**NS**
*c*9*t*11-CLA/RA	0.15	0.21	0.16	0.19	0.03	NS	0.02	NS
*t*10c12-CLA	0.01	0.02	0.01	0.01	0.01	NS	NS	NS
*c*9*c*11-CLA	0.002	0.004	0.002	0.003	0.002	NS	NS	NS
c11*c*13-CLA	0.02	0.01	0.01	0.01	0.003	NS	NS	NS
*t*12*t*14-CLA	0.01	0.02	0.02	0.01	0.01	NS	NS	NS
*t*11*t*13-CLA	0.002	0.002	0.007	0.003	0.002	0.03	NS	NS
tt-CLA	0.01	0.02	0.02	0.01	0.01	NS	NS	NS
∑-CLA	**0.21**	**0.29**	**0.22**	**0.24**	**0.04**	**NS**	**NS**	**NS**
*t*9*t*12-18:2	0.04	0.05	0.04	0.05	0.01	NS	NS	NS
*t*9*c*13/*t*8*c*12-18:2	0.02	0.03	0.02	0.02	0.002	< 0.01	0.03	NS
*c*9*t*12-18:2	0.15	0.14	0.15	0.15	0.01	NS	NS	NS
*t*8*c*13-18:2	0.12	0.12	0.09	0.10	0.01	< 0.01	NS	NS
*t*9*c*12-18:2	0.27	0.30	0.15	0.19	0.05	0.02	NS	NS
*t*11*c*15-18:2	0.03	0.03	0.01	0.02	0.004	0.02	NS	NS
18:2n-6	29.58	33.37	27.89	26.87	2.57	< 0.01	NS	0.01
18:3n-6	0.01	0.01	0.01	0.01	0.002	NS	NS	NS
c/c/t-18:3	0.01	0.001	0.00	0.01	0.002	NS	NS	< 0.01
18:3n-3	1.28	1.43	1.11	1.39	0.11	NS	0.01	NS
c/t 20:2	0.31	0.29	0.25	0.27	0.05	NS	NS	NS
20:2n-6	0.02	0.02	0.03	0.03	0.01	NS	NS	NS
20:3n-6	0.01	0.01	0.006	0.008	0.002	NS	NS	NS
20:5n-3	0.01	0.01	0.01	0.01	0.001	NS	NS	NS
22:2n-6	0.004	0.005	0.003	0.005	0.001	NS	NS	NS
22:4n-6	0.03	0.03	0.03	0.01	0.01	NS	NS	NS
22:5n-3	0.004	0.008	0.001	0.00	0.003	0.03	NS	NS
22:6n-3	0.04	0.03	0.03	0.04	0.01	NS	NS	NS
∑ n-6 PUFA	29.63	33.41	27.93	26.92	2.57	< 0.01	NS	0.01
∑ n-3 PUFA	1.51	1.69	1.34	1.61	0.12	NS	0.01	NS
n-6/n-3	19.84	19.82	21.52	17.66	2.11	NS	NS	NS
∑ *c/t* -18:2	0.65	0.68	0.48	0.55	0.05	< 0.01	NS	NS

[1]Diets were rolled barley (RB), ground barley (GB), rolled corn (RC) and ground corn (GC).

[2]Gr = grain type; Pr = processing; Gr*Pr = interaction between grain type and method of processing.

[3]FAME = fatty acid methyl esters (expressed as a percentage of total FAME; *c = cis*; *t = trans*; CLA = conjugated linoleic acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; RA = rumenic acid. NS = not significant ($P \geq 0.05$)

Plasma metabolites and fatty acid composition

There were no main effects or interactions for the concentrations of plasma metabolites, including glucose, insulin and NEFA (data not presented). The sum of the SFA in plasma was greater for CBD than for BBD with no effect of processing (Table 3-6). Concentration of 18:0 was greater for CBD than for BBD while that of 16:0 was greater for BBD than for CBD. Concentration of 14:0 and 17:0 was greater for BBD than for CBD. The sum of the *iso* and *anteiso* FA was not influenced by the treatments. However, concentration of 15:0 *iso* was greater for rolling than for grinding, while that of 17:0 *iso* was greater for BBD than for CBD. The sum of the n6-PUFA was not influenced by the treatments. The sum of the n3-PUFA was greater for CBD than for BBD with no effects of processing. The n3-PUFA in plasma was represented mainly by the concentrations of 18:3n-3, 22:5n-3 and 20:5n-3. Concentration of 18:3n-3 was greater for CBD than for BBD with greater values observed for rolling than for grinding. The ratio of n6:n3 PUFA and the sum of *c/t*-18:2 isomers were greater for BBD than for CBD with no difference between rolling and grinding.

The sum of the *t*-16:1 isomers was greater for BBD than for CBD. The *c*-16:1 isomers were represented mainly by *c*9-16:1 followed by *c*13-16:1; both of which did not differ among treatments. Concentration of *c*10-16:1 was greater for BBD than for CBD, while that of *c*11-16:1 showed an interaction between the main effects. The sum of the *t*-18:1 isomers was greater for BBD than for CBD while the sum of *c*-18:1 isomers was greater for CBD than for BBD. Neither was affected by processing method. The *t*-18:1 isomers, represented mainly by *t*10-18:1 and *t*11-18:1, were greater for BBD than for CBD with no difference between rolling and grinding. The ratio of *t*10:*t*11-18:1 was also greater for BBD than for CBD, without any difference between rolling and grinding, similar to that observed in rumen contents. Concentration of *c*-18:1 isomers, represented mainly by *c*9-/*c*10-18:1, was greater for CBD than for BBD with higher values

Table 3-6. Effect of grain type and method of processing on plasma fatty acid composition

Variable[3]	Treatments[1]				SEM	Significance[2]		
	Barley		Corn					
	RB	GB	RC	GC		Gr	Pr	Gr*Pr
14:0	0.28	0.29	0.24	0.25	0.02	< 0.01	NS	NS
15:0 *iso*	0.06	0.04	0.06	0.05	0.01	NS	< 0.01	NS
15:0 *anteiso*	0.09	0.10	0.10	0.09	0.01	NS	NS	NS
15:0	0.42	0.43	0.38	0.38	0.02	0.01	NS	NS
16:0 *iso*	0.11	0.11	0.15	0.13	0.01	0.01	NS	NS
16:0	11.92	11.96	11.36	11.58	0.12	< 0.01	NS	NS
17:0 *iso*	0.25	0.31	0.18	0.19	0.06	0.02	NS	NS
17:0 *anteiso*	1.40	1.38	1.46	1.45	0.56	NS	NS	NS
17:0	0.93	1.08	0.84	0.87	0.04	< 0.01	0.02	NS
18:0 *iso*	0.03	0.04	0.03	0.03	0.01	NS	NS	NS
18:0	19.23	18.38	21.45	20.91	0.66	< 0.01	NS	NS
19:0	0.09	0.10	0.09	0.05	0.02	NS	NS	NS
20:0	0.07	0.05	0.07	0.06	0.01	NS	NS	NS
21:0	0.02	0.01	0.02	0.01	0.004	NS	NS	NS
22:0	0.31	0.37	0.26	0.32	0.03	NS	0.03	NS
23:0	0.46	0.53	0.50	0.52	0.06	NS	NS	NS
24:0	0.31	0.26	0.26	0.31	0.04	NS	NS	NS
∑ SFA	**35.94**	**35.43**	**37.44**	**37.18**	**0.89**	**0.03**	**NS**	**NS**
∑ *iso* FA	**0.89**	**0.88**	**1.02**	**0.95**	**0.08**	**NS**	**NS**	**NS**
∑ *anteiso* FA	**1.54**	**1.54**	**1.61**	**1.59**	**0.58**	**NS**	**NS**	**NS**
*t*5-16:1	0.02	0.03	0.01	0.01	0.005	0.01	NS	NS
*t*9-16:1	0.15	0.11	0.10	0.13	0.04	NS	NS	NS
*t*10-16:1	0.03	0.02	0.02	0.02	0.01	NS	NS	NS
*t*11/*t*12-16:1	0.07	0.06	0.04	0.06	0.01	NS	NS	NS
*c*7-16:1	0.07	0.03	0.06	0.08	0.02	NS	NS	NS
*c*9-16:1	1.76	1.74	1.21	1.35	0.51	NS	NS	NS
*c*10-16:1	0.04	0.04	0.03	0.02	0.01	0.04	NS	NS
*c*11-16:1	0.02	0.03	0.03	0.01	0.01	NS	NS	0.03
*c*13-16:1	0.12	0.12	0.12	0.12	0.01	NS	NS	NS
∑*trans*-16:1	**0.28**	**0.24**	**0.17**	**0.22**	**0.05**	**0.04**	**NS**	**NS**
∑*cis*-16:1	**2.01**	**1.95**	**1.44**	**1.58**	**0.50**	**NS**	**NS**	**NS**
c7 17:1	0.11	0.09	0.10	0.09	0.01	NS	NS	NS
*c*9-17:1	0.11	0.09	0.07	0.08	0.01	NS	NS	NS
*t*4-18:1	0.08	0.09	0.09	0.08	0.01	NS	NS	NS
*t*5-18:1	0.03	0.04	0.04	0.03	0.01	NS	NS	NS
*t*6 to *t*8-18:1	0.12	0.16	0.07	0.07	0.02	< 0.01	NS	NS
*t*9-18:1	0.17	0.22	0.11	0.13	0.03	0.02	NS	NS
*t*10-18:1	1.02	1.42	0.17	0.23	0.28	< 0.01	NS	NS
*t*11-18:1	0.92	1.01	0.50	0.66	0.20	0.01	NS	NS
*t*12-18:1	0.48	0.51	0.41	0.42	0.04	< 0.01	NS	NS
*t*13/*t*14/*c*6-8-18:1	0.94	0.99	0.86	0.83	0.05	< 0.01	NS	NS
*c*9/c10/15t-18:1	4.92	4.15	5.93	5.80	0.18	< 0.01	< 0.01	0.02
*c*11-18:1	0.57	0.58	0.54	0.60	0.04	NS	NS	NS
*c*12-18:1	1.28	1.26	0.99	1.07	0.14	NS	NS	NS

*c*13-18:1	0.04	0.04	0.03	0.03	0.003	NS	NS	NS
*c*14/*t*16-18:1	0.20	0.21	0.23	0.23	0.02	NS	NS	NS
*c*15-18:1	0.08	0.07	0.06	0.10	0.02	NS	NS	NS
*c*16-18:1	0.12	0.14	0.08	0.08	0.04	NS	NS	NS
∑ *cis*-18:1	**7.21**	**6.45**	**7.86**	**7.92**	**0.26**	**< 0.01**	**NS**	**NS**
∑ *trans*-18:1	**3.75**	**4.46**	**2.26**	**2.45**	**0.54**	**< 0.01**	**NS**	**NS**
***t*10: *t*11-18:1**	**1.20**	**1.78**	**0.34**	**0.34**	**0.23**	**< 0.01**	**NS**	**NS**
*c*5-20:1/*t,c,c*-18:3	0.01	0.01	0.01	0.00	0.01	NS	NS	NS
*c*11-20:1	0.03	0.02	0.02	0.02	0.01	NS	NS	NS
*c*15-24:1	0.17	0.22	0.16	0.16	0.03	NS	NS	NS
∑ *trans* MUFA	**4.03**	**4.71**	**2.43**	**2.68**	**0.55**	**< 0.01**	**NS**	**NS**
∑ *cis* MUFA	**11.01**	**10.41**	**10.74**	**11.07**	**0.56**	**NS**	**NS**	**NS**
*c*9*t*11-CLA/RA	0.20	0.25	0.14	0.15	0.03	< 0.01	NS	NS
*t*10c12-CLA	0.04	0.05	0.02	0.02	0.01	0.03	NS	NS
*c*9*c*11-CLA	0.01	0.01	0.01	0.01	0.003	NS	NS	NS
*c*11*c*13-CLA	0.01	0.00	0.01	0.01	0.004	0.01	NS	NS
*t*12*t*14-CLA	0.003	0.01	0.01	0.01	0.003	NS	NS	NS
*t*11*t*13-CLA	0.01	0.01	0.02	0.01	0.01	NS	NS	NS
tt-CLA	0.08	0.08	0.06	0.06	0.01	NS	NS	NS
∑ CLA	**0.38**	**0.42**	**0.30**	**0.31**	**0.05**	**0.02**	**NS**	**NS**
*t*9*t*12-18:2	0.02	0.11	0.03	0.02	0.03	NS	NS	NS
*t*9*c*13/*t*8*c*12-18:2	0.10	0.09	0.08	0.07	0.01	0.02	NS	NS
*c*9*t*12-18:2	0.14	0.16	0.12	0.09	0.04	NS	NS	NS
*t*9*c*12-18:2	0.19	0.19	0.18	0.15	0.02	NS	NS	NS
*t*11*c*15-18:2	0.06	0.07	0.08	0.06	0.02	NS	NS	NS
18:2n-6	34.51	35.00	34.21	34.41	0.70	NS	NS	NS
18:3n-6	0.30	0.30	0.34	0.29	0.03	NS	NS	NS
c/c/t-18:3	0.02	0.01	0.03	0.03	0.004	< 0.01	NS	NS
18:3n-3	1.78	1.62	2.06	1.89	0.06	< 0.01	< 0.01	NS
20:2n-6	0.12	0.13	0.11	0.12	0.01	NS	NS	NS
20:3n-6	4.01	3.94	4.29	3.95	0.18	NS	NS	NS
20:3n-3	0.01	0.02	0.01	0.02	0.01	NS	NS	NS
20:4n-6	2.16	2.09	2.12	2.07	0.13	NS	NS	NS
20:5n-3	0.29	0.32	0.33	0.31	0.04	NS	NS	NS
22:4n-6	0.37	0.38	0.33	0.35	0.05	NS	NS	NS
22:5n-3	0.82	0.90	0.69	0.83	0.09	0.02	0.01	NS
22:6n-3	0.06	0.06	0.05	0.06	0.01	NS	NS	NS
∑ n-6 PUFA	**41.45**	**41.82**	**41.40**	**41.18**	0.73	NS	NS	NS
∑ n-3 PUFA	**2.97**	**2.92**	**3.15**	**3.11**	0.09	< 0.01	NS	NS
n-6/n-3	**14.1**	**14.5**	**13.2**	**13.3**	0.58	< 0.01	NS	NS
∑ *c/t* -18:2	**0.80**	**0.92**	**0.69**	**.57**	0.07	< 0.01	NS	NS
Desaturase index								
*c*9-16:1 / 16:0	0.15	0.14	0.11	0.12	0.04	NS	NS	NS
*c*9-18:1 / 18:0	0.26	0.23	0.28	0.28	0.01	< 0.01	NS	NS
*c*9*t*11-CLA / *t*11-18:1	0.30	0.50	0.28	0.24	0.14	NS	NS	NS

[1]Diets were rolled barley (RB), ground barley (GB), rolled corn (RC) and ground corn (GC).
[2]Gr = grain type; Pr = processing; Gr*Pr = interaction between grain type and method of processing.

[3]FAME = fatty acid methyl esters (expressed as a percentage of total FAME; *c = cis*; *t = trans*; CLA = conjugated linoleic acid; MUFA = monounsaturated fatty acid; RA = rumenic acid.
NS = not significant ($P > 0.05$)

observed for rolling than for grinding. The sum of the *t*-MUFA was greater for BBD than for CBD with no difference between rolling and grinding. The sum of the *c*-MUFA, however, was not influenced by the treatments. Total plasma CLA isomers ranged from 0.30 to 0.42 with greater values observed for BBD than for CBD. Rumenic acid, the most abundant CLA isomer, was greater for BBD than for CBD with no difference between rolling and grinding. Concentration of *t*10*c*12 CLA was also greater for BBD than for CBD.

Milk fatty acid composition

Total SFA and total short-chain fatty acid (**SCFA**) were greater for CBD than for BBD (Table 3-7) and were not affected by processing. The most abundant SFA in milk was 16:0 followed by 14:0 and 18:0. Concentrations of 16:0 and 14:0 were not different among the treatments while that of 18:0 was greater for CBD than for BBD with no difference between rolling and grinding. The sum of the *iso* FA was not different among the treatments. However, concentrations of 13:0 *iso*, 14:0 *iso* and 15:0 *iso* were greater for CBD than for BBD while that of 17:0 *iso* was greater for BBD than for CBD. The sum of the *anteiso* FA was greater for BBD than for CBD. Concentrations of 17:0 *anteiso* were also greater for BBD than for CBD with no difference between rolling and grinding. The sum of the n6-PUFA in milk was greater for BBD than for CBD with greater values observed for grinding than for rolling. The most abundant n6-PUFA in milk was 18:2n-6, which showed a similar trend as that of total n6-PUFA. Sum of the n3-PUFA were not different between the grains; however, the concentrations were greater for grinding than for rolling. The most abundant n3-PUFA in milk, 18:3n-3, showed a similar trend as that of total n3-PUFA. The ratio

of n6:n3 PUFA and sum of the *c/t*-18:2 isomers were greater for BBD than for CBD with no difference between the methods of processing.

The sum of *t*-16:1 and *c*-16:1 isomers in milk were greater for BBD than for CBD with no difference between rolled and ground diets. Concentrations of *t*9-16:1 and *t*6-8-16:1 were greater for BBD than for CBD. The sum of the *c*-18:1 isomers was greater for CBD than for BBD with no difference between rolling and grinding. Concentrations of *c*9-/*c*10-18:1 was greater for CBD than for BBD while that of *c*15-18:1 was greater for BBD than for CBD. The sum of *t*-18:1 isomers was greater for BBD than for CBD with no difference between rolling and grinding. Among the *t*18:1 isomers, *t*6-8-, *t*9-, *t*10- and *t*11-18:1 were greater for BBD than for CBD. The ratio of *t*10:*t*11-18:1, however, was not influenced by the treatments. Among the 20:1 isomers, *c*5-20:1 was greater for CBD than for BBD while that of *c*11-20:1 was greater for BBD than for CBD. The sum of the *t*-MUFA was greater for BBD than for CBD while the sum of *c*-MUFA was greater for CBD than for BBD. Mammary desaturase index (based on 14:1/14:0; 16:1/16:0 and 18:1/18:0 ratios) was significantly greater for BBD than for CBD (Table 3-7) suggesting that the conversion of *t*11-18:1 to RA was greater for BBD compared to CBD. Desaturase indices were, however, not different between rolling and grinding.

Table 3-7. Effect of grain type and method of processing on milk fatty acid composition

	Treatments[1]					Significance[2]		
	Barley		Corn					
Variable[3]	RB	GB	RC	GC	SEM	Gr	Pr	Gr*Pr
4:0	3.01	2.88	3.53	3.31	0.24	NS	NS	NS
5:0	0.16	0.14	0.12	0.15	0.02	NS	NS	NS
6:0	1.87	1.79	2.25	2.10	0.17	0.01	NS	NS
7:0	0.05	0.05	0.04	0.04	0.01	NS	NS	NS
8:0	1.14	1.11	1.38	1.28	0.12	0.01	NS	NS
9:0	0.06	0.06	0.05	0.05	0.009	NS	NS	NS
10:0	2.58	2.56	3.07	2.87	0.29	0.02	NS	NS
11:0	0.32	0.35	0.37	0.35	0.04	NS	NS	NS
12:0	3.06	3.05	3.36	3.23	0.27	NS	NS	NS
13:0 *iso*	0.01	0.01	0.02	0.02	0.00	<0.01	NS	NS
13:0 *anteiso*	0.08	0.09	0.09	0.08	0.01	NS	NS	NS
13:0	0.14	0.15	0.11	0.12	0.01	<0.01	NS	NS
14:0 *iso*	0.07	0.07	0.10	0.10	0.009	<0.01	NS	NS
14:0	9.72	9.44	10.10	9.83	0.47	NS	NS	NS
15:0 *iso*	0.12	0.11	0.14	0.13	0.009	0.01	NS	NS
15:0 *anteiso*	0.35	0.36	0.34	0.33	0.02	NS	NS	NS
15:0	1.18	1.24	0.99	1.03	0.08	<0.01	NS	NS
16:0 *iso*	0.19	0.18	0.22	0.18	0.01	NS	0.04	NS
16:0	19.70	19.60	19.72	19.05	0.50	NS	NS	NS
17:0 *iso*	0.24	0.25	0.20	0.21	0.01	< 0.01	0.01	NS
17:0 *anteiso*	0.32	0.34	0.27	0.27	0.02	< 0.01	NS	NS
17:0	0.59	0.58	0.48	0.50	0.04	< 0.01	NS	NS
18:0 *iso*	0.04	0.04	0.04	0.04	0.00	NS	NS	NS
18:0	8.73	7.97	11.09	10.75	0.74	< 0.01	NS	NS
Cyclo 17	0.02	0.02	0.02	0.02	0.01	NS	NS	NS
19:0	0.10	0.09	0.10	0.09	0.01	NS	NS	NS
20:0	0.14	0.14	0.16	0.16	0.01	< 0.01	NS	NS
21:0	0.05	0.04	0.03	0.03	0.004	< 0.01	0.04	NS
22:0	0.07	0.07	0.08	0.08	0.005	< 0.01	NS	NS
23:0	0.01	0.02	0.02	0.02	0.003	NS	NS	0.01
24:0	0.02	0.02	0.03	0.02	0.003	NS	NS	NS
∑ SFA	**54.18**	**52.85**	**58.51**	**56.43**	**1.91**	**0.01**	**NS**	**NS**
∑ SCFA (4:0-10:0)	**8.87**	**8.59**	**10.43**	**9.80**	**0.79**	**0.02**	**NS**	**NS**
∑ *iso* FA	**0.67**	**0.68**	**0.72**	**0.68**	**0.03**	**NS**	**NS**	**NS**
∑ *anteiso* FA	**0.76**	**0.79**	**0.70**	**0.68**	**0.03**	**< 0.01**	**NS**	**NS**
*c*9-12:1	0.09	0.09	0.09	0.09	0.01	NS	NS	NS
*c*9-14:1	1.06	1.12	0.93	0.94	0.11	<0.01	NS	NS
*t*6-8-16:1	0.16	0.18	0.06	0.06	0.04	0.02	NS	NS
*t*9-16:1	0.17	0.16	0.10	0.11	0.02	< 0.01	NS	NS
t10-16:1	0.04	0.04	0.03	0.04	0.00	NS	NS	NS
*t*11-16:1	0.17	0.17	0.15	0.15	0.01	NS	NS	NS
*t*13-16:1	0.02	0.02	0.02	0.02	0.003	NS	NS	NS
*c*7-16:1	0.19	0.18	0.17	0.21	0.02	NS	NS	NS
*c*10-16:1	0.06	0.06	0.04	0.05	0.006	< 0.01	NS	NS
*c*11-16:1	0.04	0.04	0.03	0.03	0.005	0.01	NS	NS
*c*12-16:1	0.03	0.03	0.03	0.03	0.004	NS	NS	NS
*c*13-16:1	0.10	0.11	0.11	0.11	0.01	NS	NS	NS

∑*trans*-16:1	**0.56**	**0.58**	**0.36**	**0.39**	**0.06**	**< 0.01**	**NS**	**NS**
∑*cis*-16:1	**1.65**	**1.82**	**1.43**	**1.49**	**0.14**	**< 0.01**	**NS**	**NS**
c5 17:1	0.01	0.01	0.008	0.001	0.003	0.03	NS	NS
c7 17:1	0.03	0.03	0.02	0.03	0.004	NS	NS	NS
*c*9-17:1	0.20	0.23	0.14	0.16	0.016	< 0.01	NS	NS
*t*4-18:1	0.03	0.04	0.04	0.04	0.004	NS	NS	NS
*t*5-18:1	0.03	0.04	0.04	0.03	0.004	NS	NS	NS
*t*6 to *t*8-18:1	0.59	0.66	0.47	0.48	0.06	< 0.01	NS	NS
*t*9-18:1	0.60	0.59	0.56	0.56	0.04	0.04	NS	NS
*t*10-18:1	3.47	4.15	0.92	1.12	1.28	0.03	NS	NS
*t*11-18:1	2.63	2.64	1.54	1.86	0.34	< 0.01	NS	NS
*t*12-18:1	0.90	0.96	0.88	0.89	0.08	NS	NS	NS
*t*13/*t*14-18:1	1.54	1.67	1.53	1.57	0.12	NS	NS	NS
*t*15-18:1	0.44	0.49	0.52	0.53	0.05	NS	NS	NS
∑ *trans*-18:1	**12.89**	**14.18**	**8.82**	**9.41**	**1.70**	**0.01**	**NS**	**NS**
***t*10: *t*11-18:1**	**1.17**	**1.66**	**0.62**	**0.62**	**0.51**	**NS**	**NS**	**NS**
*c*6-8-18:1	0.12	0.16	0.09	0.09	0.03	0.03	NS	NS
*c*9/c10-18:1	18.85	17.88	21.16	21.37	1.10	< 0.01	NS	NS
*c*11-18:1	0.32	0.35	0.37	0.35	0.04	NS	NS	NS
*c*12-18:1	3.06	3.05	3.36	3.23	0.27	NS	NS	NS
*c*13-18:1	0.14	0.15	0.11	0.12	0.02	< 0.01	NS	NS
*c*14/*t*16-18:1	0.52	0.52	0.58	0.57	0.04	NS	NS	NS
*c*15-18:1	1.18	1.24	0.99	1.03	0.08	< 0.01	NS	NS
*c*16-18:1	0.28	0.25	0.28	0.27	0.03	NS	NS	NS
∑ *cis*-18:1	**22.15**	**21.35**	**24.03**	**24.54**	**1.17**	**< 0.01**	**NS**	**NS**
*c*7-20:1	0.01	0.01	0.004	0.01	0.002	< 0.01	NS	NS
*c*9-20:1	0.12	0.12	0.13	0.13	0.01	NS	NS	NS
*c*11-20:1	0.06	0.07	0.04	0.05	0.01	0.02	NS	NS
∑ *trans* MUFA	**13.46**	**14.77**	**9.19**	**9.79**	**1.76**	**< 0.01**	**NS**	**NS**
∑ *cis* MUFA	**25.38**	**24.87**	**26.83**	**27.44**	**1.20**	**< 0.01**	**NS**	**NS**
t/t -18:2	0.11	0.10	0.06	0.06	0.01	< 0.01	NS	NS
*t*9*t*12-18:2	0.04	0.05	0.03	0.03	0.01	NS	NS	NS
*t*9*c*13/*t*8*c*12-18:2	0.58	0.64	0.52	0.53	0.06	0.02	NS	NS
*c*9*t*12-18:2	0.14	0.13	0.12	0.14	0.02	NS	NS	NS
*t*9*c*12-18:2	0.22	0.18	0.09	0.11	0.04	0.01	NS	NS
*t*11*c*15-18:2	0.04	0.05	0.04	0.04	0.01	NS	NS	NS
18:2n-6	3.81	4.35	3.27	3.55	0.19	< 0.01	0.02	NS
18:3n-6	0.04	0.04	0.04	0.04	0.002	NS	NS	NS
18:3n-3	0.52	0.59	0.54	0.56	0.02	NS	0.01	NS
20:2n-6	0.03	0.03	0.03	0.03	0.001	NS	NS	NS
20:3n-6	0.15	0.16	0.15	0.14	0.01	0.03	NS	0.04
20:3n-3	0.01	0.01	0.01	0.01	0.002	NS	NS	NS
20:4n-6	0.11	0.12	0.12	0.11	0.01	NS	NS	NS
20:5n-3	0.03	0.03	0.03	0.03	0.002	NS	NS	NS
22:2n-6	0.003	0.01	0.003	0.01	0.003	NS	NS	NS
22:3n-3	0.02	0.02	0.01	0.01	0.01	NS	NS	NS
22:4n-6	0.03	0.02	0.02	0.02	0.002	NS	NS	NS
22:5n-3	0.06	0.06	0.06	0.06	0.01	NS	NS	NS

∑ n-6 PUFA	4.17	4.73	3.63	3.90	0.19	< 0.01	0.02	NS
∑ n-3 PUFA	0.63	0.71	0.64	0.67	0.03	NS	0.01	NS
n-6/n-3	6.65	6.67	5.65	5.85	0.19	< 0.01	NS	NS
∑ *c/t* -18:2	1.21	1.25	0.92	0.98	0.08	< 0.01	NS	NS
Desaturase index								
*c*9-14:1 / 14:0	0.11	0.12	0.09	0.09	0.01	< 0.01	NS	NS
*c*9-16:1 / 16:0	0.06	0.07	0.05	0.06	0.01	0.01	NS	NS
*c*9-18:1 / 18:0	2.19	2.30	1.94	2.02	0.11	< 0.01	NS	NS
*c*9*t*11-CLA / *t*11-18:1	0.56	0.55	0.53	0.53	0.03	NS	NS	NS

[1]Diets were rolled barley (RB), ground barley (GB), rolled corn (RC) and ground corn (GC).
[2]Gr = grain type; Pr = processing; Gr*Pr = interaction between grain type and method of processing.
[3]FAME = fatty acid methyl esters (expressed as a percentage of total FAME; *c* = *cis*; *t* = *trans*; CLA = conjugated linoleic acid; SFA = saturated fatty acid (4:0 to 26:0 including branched chain fatty acids); SCFA = shorter-chain fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
NS = not significant ($P > 0.05$)

The sum of the CLA isomers in milk ranged from 0.96 to 1.69% among the treatments with greater concentrations observed for BBD than for CBD and without any difference between rolling and grinding. Concentrations of the first most abundant CLA isomer, *c*9*t*11-CLA (RA), and the second most abundant CLA isomer, *t*7*c*9-CLA, were greater for BBD than for CBD while those of *t*10*c*12-CLA were not influenced by the treatments (Table 3-8). The RA yield per 100 g total substrate intake was significantly different ($P < 0.01$) with greater values observed for BBD than for CBD (rolled barley – 4.5 ± 0.47; ground barley – 3.6 ± 0.47; rolled corn – 2.4 ± 0.47; ground corn – 2.7 ± 0.47) suggesting that cows offered BBD were more efficient in milk RA production.

Table 3-8. Effect of grain type and method of processing on CLA isomers in milk fat

	Treatments[1]					Significance[2]		
	Barley		Corn					
Variable[3]	RB	GB	RC	GC	SEM	Gr	Pr	Gr*Pr
CLA isomers								
*t*7*c*9-CLA	0.12	0.14	0.09	0.10	0.016	0.01	NS	NS
*t*8*c*10-CLA	0.001	0.002	0.001	0.001	0.001	NS	NS	NS
*c*9*t*11-CLA/RA	1.46	1.45	0.81	0.98	0.18	< 0.01	NS	NS
*t*10*c*12-CLA	0.001	0.001	0.000	0.001	0.001	NS	NS	NS
*c*11*t*13-CLA	0.001	0.000	0.001	0.001	0.001	NS	NS	NS
*t*11*c*13-CLA	0.002	0.01	0.001	0.005	0.006	NS	NS	NS
*t*12*t*14-CLA	0.007	0.007	0.007	0.006	0.001	NS	NS	NS
*t*11*t*13-CLA	0.01	0.01	0.009	0.01	0.001	0.04	NS	NS
*t*10*t*12-CLA	0.01	0.01	0.02	0.01	0.002	NS	NS	NS
*t*9*t*11-CLA	0.01	0.02	0.01	0.01	0.002	< 0.01	NS	NS
*t*8*t*10-CLA	0.005	0.005	0.004	0.003	0.001	0.01	NS	NS
*t*7*t*9-CLA	0.006	0.005	0.005	0.004	0.001	NS	NS	NS
*t*6*t*8-CLA	0.006	0.006	0.004	0.004	0.001	0.02	NS	NS
∑ CLA	**1.67**	**1.69**	**0.96**	**1.14**	**0.19**	**< 0.01**	**NS**	**NS**

[1]Diets were rolled barley (RB), ground barley (GB), rolled corn (RC) and ground corn (GC).
[2]Gr = grain type; Pr = processing; Gr*Pr = interaction between grain type and method of processing.
[3] *t* = *trans;* *c* = *cis;* CLA = conjugated linoleic acid ; RA = rumenic acid.
NS = not significant ($P > 0.05$)

3.4 Discussion

The primary objective of this study was to determine the effect of starch degradability on rumen biohydrogenation of PUFA and milk RA content. Starch degradability was varied using two sources of starch (barley and corn) and by method of processing (rolling and grinding). The diets were formulated to contain similar starch and total substrate (LA + LN) content. Compared to BBD, CBD required a lesser proportion of grain (40 vs 26%) to achieve similar dietary starch concentrations because of the higher starch content in corn compared to barley (76 vs 53%).

Intakes and production

The lower DMI for cows offered BBD than for those offered CBD was expected and could be attributed to greater concentrations of ruminal propionate for BBD than for CBD because propionic acid has been reported to

induce hypophagia (Allen, 2000). Similar differences in intakes for corn and barley diets were reported by McCarthy et al. (1989), Casper et al. (1990) and Silveira et al. (2007). Greater rate of *in situ* starch digestion and lower minimum daily rumen pH for BBD compared to CBD provides evidence of greater rumen degradability of barley starch than corn starch.

Grain processing by grinding has been reported to lower DMI compared to rolling for both BBD (Mathison, 1996) and CBD (Owens et al., 1996). Contrary to this, DMI in the current study was not influenced by the method of processing. *In situ* rate of starch digestion was however greater for ground diets compared to the rolled diets. Increased rates of ingesta passage from the rumen for ground diets compared to the rolled diets may have counteracted the effects of increased rates of rumen fermentation resulting in a lack of difference in DMI between rolling and grinding. Alternatively grinding may not have had as much impact on degradability as was expected. Studies comparing corn and barley diets that recorded greater DMI for corn than barley also observed greater milk yields for corn than for barley (McCarthy et al., 1989 and Silveira et al., 2007) which could be attributed to the greater DMI. However, in the current study, milk yield was not different between BBD and CBD in spite of the greater DMI for CBD. The dietary CP contents were slightly higher for CBD than for BBD while the diets in the study of McCarthy et al. (1989) and Silveira et al. (2007) were iso-nitrogenous. The lack of difference in milk yield between the treatments in spite of the greater DMI recorded for CBD could be possibly due to the higher dietary CP content of CBD compared to BBD which could have reduced ME allowable milk due to additional energy required for the conversion of excess protein into urea. Lack of differences in milk yield due to processing could be attributed to the lack of difference in DMI between rolled and ground diets. The greater intakes of LA, LN and total substrate intake for CBD compared to BBD, in spite of

the similar amounts of total substrate in the offered diets, could be attributed to the greater DMI for CBD than for BBD.

Milk fat depression

Milk fat depression (**MFD**) is normally observed in cows fed low fiber diets (Griinari et al., 1998). Several theories have been proposed to explain the causes of MFD (Bauman and Griinari, 2003). However, more recent reviews (Shingfield and Griinari, 2007) support the biohydrogenation theory of MFD according to which diet-induced MFD alters rumen lipid metabolism resulting in the formation of specific biohydrogenation intermediates in the rumen which inhibit milk fat synthesis. The lower milk fat content and yield for BBD compared to CBD was in agreement with our hypothesis and could be attributed to the greater concentrations of *t*10-18:1 for BBD than for CBD. Although milk fat content was lower for the ground diets compared to the rolled diets, which was consistent with our hypothesis, milk concentrations of *t*10-18:1 were not different between rolled and ground diets. This could be attributed to the lack of processing effect on the minimum rumen pH or on duration pH remained < 5.8. Several studies have associated milk fat depression with increases in milk concentrations of *t*10-18:1 (Baumgard et al., 2000; 2001; Peterson et al. 2002). However, Lock et al. (2007) reported that *t*10-18:1 has no role in MFD based on an infusion study. Alternatively, it is also possible that *t*10-18:1 is not the only isomer responsible for MFD. Several other biohydrogenation intermediates have been associated with MFD. For example, *t*10*c*12-CLA has been reported to induce MFD (Sæbø et al., 2005). However, in our study milk concentration of *t*10*c*12-CLA was not influenced by the treatments. Lack of difference in milk *t*10*c*12-CLA despite the milk fat depression observed for cows fed BBD was surprising. Pure isomers of *t*10*c*12-CLA have been reported to induce milk fat depression (Sæbø et al., 2005; deVeth et al., 2004). However, milk *t*10*c*12-CLA concentrations have been reported to be far less with diet-induced milk fat

depression compared to that induced with post-ruminal *t*10*c*12-CLA infusions (Bauman and Griinari, 2003).

Recently *t*6-*t*8-18:1 (Precht et al., 2002; Loor et al., 2005) and *t*7*c*9-CLA (Pottier et al., 2006; Kadegowda et al., 2008) have also been associated with MFD. The higher concentrations of *t*6-8-18:1 and *t*7*c*9-CLA for BBD compared to CBD could be related to the observed milk fat depression. We first investigated the relationships using single factors. Milk fat yield was negatively related to milk concentrations of *t*7*c*9-CLA with a r^2 value of 0.21 (P = 0.01; n = 32) and to milk t6-8-18:1 with a r^2 value of only 0.17 (P = 0.02). Subsequently, step-wise regression analysis was done using milk fat yield as the dependent variable and dietary factors (fiber intake, starch intake), rumen factors [rumen pH (minimum and mean) individual and total rumen VFA, acetate:propionate ratio, rumen NH_3], and concentrations of total *t*-18:1, *t*4-18:1 to *t*16-18:1 and *t*10:*t*11-18:1 ratio in rumen and milk, and milk concentration of *c*9*t*11-CLA, *t*7*c*9-CLA, *t*10*c*12-CLA as independent variables. The model tested was significant (P < 0.01; R^2 = 0.55). Surprisingly none of the expected variables [*t*10*c*12-CLA (Griinari et al., 2000); *t*10-18:1 (Griinari and Bauman, 1999); *t*7*c*9-CLA (Kadegowda et al., 2008)] entered the model. Starch intake contributed 45% of the variability to milk fat yield with an additional 10% contributed by milk concentrations of *c*9*t*11-CLA. Stronger positive correlations between total starch intake and milk t10-18:1 concentration have been reported (Shingfield et al., 2005; Nielson et al., 2006) and dietary sources high in starch have been related to MFD (Bauman and Griinari, 2003; Nielson et al., 2006). In the current study, total starch intake was similar among the treatments; however, milk fat content (%) depressions were greater for BBD than CBD (2.77 vs 3.28) and for ground diets compared to rolled diets (2.45 vs 2.77 for barley and 2.94 vs 3.28 for corn, respectively). The above differences between barley and corn diets indicate that starch degradability may be more important in MFD than total starch intake.

Rumen fermentation

It is well established that barley starch is more rapidly and completely degraded in the rumen compared to corn starch and the differences in degradability between the two has been explained by the differences in the physical and chemical characteristics of grains (Ørskov, 1986; Huntington, 1997). The greater rate of *in situ* starch degradation for rolled barley compared to rolled corn (25.3 vs 6.3 % h^{-1}) supports the above established principle. Grinding physically reduces grain particle size thereby increasing surface area and access of starch granules to rumen microbes resulting in an increase in ruminal starch digestion (Gaylean et al., 1981; Theurer, 1986). As such, the rate of *in situ* starch digestion for the ground diets was greater than the rolled diets.

The type of VFA produced has been related to the composition of the diet (Murphy, 1984). In general, fermentation of structural carbohydrates yields greater amounts of acetic acid and lower amounts of propionic acid compared to the fermentation of starch which yield greater amounts of propionic acid. In the current study, the greater proportion of propionate for BBD than for CBD could be attributed to greater starch fermentation for BBD evidenced by the greater rate of *in situ* starch digestion for BBD than for CBD. Similar findings were reported by Casper et al. (1990). Greater concentrations of rumen acetate and butyrate for CBD than for BBD could be attributed to the slower rate of starch digestion for CBD evidenced by the slower rate of *in situ* starch digestion for CBD than for BBD. Similar changes with rumen butyrate (Khorasani et al., 2001 and Yang et al., 1997) and acetate concentrations (McCarthy et al., 1989) have been reported in their studies comparing CBD and BBD. Greater concentrations of acetate for rolling than for grinding and greater concentrations of propionate for grinding than for rolling could be attributed to the inverse relationship between acetate and propionate, which reflect the relative proportions of cellulolytic and amylolytic bacteria in the rumen. A reduction in the grain particle size with

grinding increases its fermentability in the rumen resulting in an increase in propionate and a decrease in acetate. Similar findings were reported by Knowlton et al. (1996) in their study comparing cracked corn and ground corn. Greater concentration of the branched-chain FA (isobutyric and isovaleric acid) in rumen for CBD than for BBD could be due to greater protein fermentation in the rumen as CBD was slightly higher in CP content than BBD. Similarly the greater concentrations of rumen NH_3 for CBD than for BBD were attributed to the higher CP content of the CBD compared to the BBD. Rumen bacteria can utilize more NH_3 nitrogen for protein synthesis in the presence of readily available energy (NRC, 2001). Because grinding increases fermentability of grain starch, the utilization of NH_3 nitrogen for microbial protein synthesis could have been higher in ground diets compared to rolled diets resulting in lower NH_3 nitrogen in ground diets than in rolled diets (Meyer et al., 1967; Shabi et al., 1999). The shift in the end products of fermentation observed in our study highlights the fact that grain source and processing influence rumen availability of carbohydrates and protein which in turn affects the end products of digestion (Dijkstra, 1984).

The role of rumen pH in inhibiting biohydrogenation has been well documented (Martin and Jenkins, 2004; Kalscheur et al., 1997). To our knowledge, this is the first study to continuously monitor rumen pH as a means of investigating effects of rumen fermentation on the products of PUFA metabolism. A low rumen pH for BBD compared to CBD was in agreement with our hypothesis and could be attributed to greater degradability of barley starch than corn starch, evidenced by the greater *in situ* rate of DM and starch degradation for BBD than CBD. However, a lack of difference in the minimum rumen pH or duration pH < 5.8 between rolled and ground diets was not anticipated. This was contrary to what we hypothesized and to the findings from the *in situ* rate of DM and starch degradation. Processing may not have had as

much impact on degradability as was expected suggesting that factors inherent in the source of starch were responsible for the observed differences and could not be modified by processing to an extent so as to produce a significant processing effect. Alternatively, it is possible that passage rate of starch from the rumen was faster for ground diets compared to the rolled diets (Shellenberger and Kesler, 1961; Ewing et al., 1986) which would have decreased the accumulation of VFA in the rumen thereby minimizing pH depressions. This could also explain the differences observed between the *in situ* and *in vivo* findings.

Rumen pH is a reflection of the production and neutralization of fermentation acids in the rumen. To investigate the factors influencing rumen pH, the relationship between minimum pH (dependent variable) and dietary factors (starch intake, fiber intake) and rumen factors (individual VFA, total VFA, acetate: propionate ratio, and rumen NH_3) (independent variables) was determined using step-wise regression analysis (n = 32; P = 0.05). The model tested was significant ($P < 0.01$; $R^2 = 0.65$) with 38% of the variability in minimum pH contributed by the concentrations of propionic acid. The variables subsequently entered into the model were valeric acid and starch intake contributing an additional 19 and 7% to the variability, respectively. Repeating the regression analysis using mean pH as the dependent variable and the other variables described above as independent variables revealed that 39% of the variability in mean pH was attributable to propionic acid concentrations in the rumen with an additional 12 and 9% attributable to valeric acid and NH_3 concentrations, respectively. The above findings suggest that propionic acid and valeric acid were the important variables regulating pH, and starch intake and NH_3 had additional contributions to the minimum pH and mean pH, respectively.

PUFA metabolism

Substrate intake (LA and LN intake) and the concentrations of substrate (LA and LN) and 18:0 in the rumen give an indication of the extent of PUFA biohydrogenation. Greater ruminal concentration of LA and lesser concentration of 18:0 for BBD than CBD despite greater LA intake for CBD suggest that inhibition of biohydrogenation of PUFA was greater for BBD than for CBD which was in agreement with our hypothesis. Greater inhibition of PUFA biohydrogenation for BBD than for CBD could be possibly due to the inhibitory effect of rumen pH depression with BBD on lipolysis and to the conversion of *t*11-18:1 to 18:0 resulting in an increase in ruminal LA, *t*11-18:1 and a decrease in 18:0. The effect of grain processing on the extent of PUFA biohydrogenation was milder, evidenced only by the lower concentration of 18:0 for ground diets than for rolled diets. This could be attributed to the lack of difference in rumen pH between rolled and ground diets. Concentrations of 18:0 in plasma and milk were also greater for CBD than for BBD supporting greater PUFA inhibition for BBD than for CBD. Greater concentrations of LA in milk for the ground diet compared to the rolled diet also supports the greater inhibition of PUFA biohydrogenation for the ground diet than for rolled diet.

The isomers *t*10-18:1 and *t*11-18:1 in the rumen are intermediary products of two different pathways of PUFA metabolism. Differences in concentrations of these isomers among the treatments reflect a possible shift in bacterial populations responsible for PUFA biohydrogenation (Cruz-Hernandez et al., 2006). The higher concentrations of *t*11-18:1 in rumen contents for BBD than for CBD (Table 3-5) was attributed to the greater inhibition of PUFA biohydrogenation evidenced by the greater LA and lower 18:0 concentrations. The greater concentrations of rumen *t*10-18:1 for BBD compared to CBD was in agreement with our hypothesis and could be attributed to the greater rumen degradability of starch and to the lower rumen pH and longer duration pH < 5.8

for BBD than CBD. Low rumen pH can cause a shift in the bacterial population triggering the *t*10-18:1 pathway of PUFA metabolism. The greater ratio of *t*10:*t*11-18:1 for BBD than for CBD provides evidence of a shift in the bacterial population. The ratio *t*10:*t*11-18:1 in plasma was also greater for BBD than for CBD similar to those observed for rumen fatty acids supporting a greater shift in microbial populations for BBD. Similar differences in the ratio *t*10:*t*11-18:1 confirming a shift in bacterial population for a grazing study was reported by Mohammed et al. (2009).

We hypothesized that ruminal *t*10-18:1 would also be greater for ground diets compared to rolled diets. Contrary to our hypothesis, *t*10-18:1 was not influenced by the method of processing. *In situ* rates of starch degradation indicated that ground diets had greater rates of starch digestion than rolled diets. However, *in vivo* data did not support these differences. Processing may not have had as much impact on starch degradation as was expected, suggesting that factors inherent in the source of starch were responsible for the observed differences and the degree of processing in the current study could not modify starch digestion to the extent as to produce a significant processing effect. Alternatively, increased rate of ingesta passage from the rumen for ground diets compared to the rolled diets may have counteracted the effects of increased rate of rumen starch degradability resulting in a lack of difference between rolling and grinding. Concentrations of *t*10- and *t*11-18:1 in milk also showed a similar trend as that in rumen and plasma. However the ratio of *t*10:t11-18:1 in milk was not different.

A lower rumen pH has been implicated as being responsible for the inhibition of biohydrogenating bacteria (Martin and Jenkins, 2002) leading to the greater concentrations of *t*11-18:1 in rumen as well as for the shift of biohydrogenation to *t*10-18:1 leading to greater concentrations of *t*10-18:1 in

the rumen (Griinari and Bauman, 1999). However there is no clear demarcation as to what range of rumen pH is responsible for the shift to *t*10-18:1. A shift to *t*10-18:1 for diets containing lower fiber has been reported (Griinari and Bauman, 1999). In the current study, both CBD and BBD had forage:concentrate ratio of 42:58 and hence relatively low fiber content. Furthermore the intake of fiber (NDF) was similar for cows offered CBD and BBD. Therefore elevated *t*10-18:1 would be expected for both BBD and CBD. However, the concentrations of *t*10-18:1 were greater than the concentrations of *t*11-18:1 for BBD and vice-versa for CBD in all the matrices analyzed. The greater *t*10-18:1 for BBD could be explained by the greater fermentability of barley starch compared to corn starch. Based on the above discussion, it appears that a lower rumen pH is required for both inhibition of PUFA biohydrogenation and for a shift of biohydrogenation to *t*10-18:1 pathway. However a shift to *t*10-18:1 could be greater when the rumen pH falls below a certain threshold. It is difficult to specify a threshold rumen pH at which a shift to *t*10-18:1 could occur because it may not be just rumen pH being below a certain threshold but possibly duration that the pH remained below the threshold. Greater concentrations of *t*10-18:1 for BBD than for CBD can therefore be explained not only by the greater degradability of barley starch but also by the duration that rumen pH remained below the thresholds 5.8, 5.5 and 5.2 compared to CBD.

To investigate the possible factors determining the concentrations of *t*10-18:1, step-wise regression analysis was used with concentrations of *t*10-18:1 as dependent variable and dietary factors (intake of LA, LN, total substrate (LA + LN), starch intake, fiber intake, starch:fiber ratio, starch:substrate ratio), rumen factors (min pH, mean pH, max pH, duration pH < 5.8, 5.5, 5.2, concentrations of individual VFA, total VFA, acetate:propionate ratio, rumen NH_3 and sum of the *iso* and *anteiso* FA in rumen) as independent variables (n = 32). Duration pH < 5.2 ($P < 0.01$; $R^2 = 0.75$) explained 54% of the variability in *t*10-18:1 concentration.

Proportions of caproic acid and butyric acid in rumen fluid which subsequently entered into the model explained an additional variation of 10% and 9% respectively. This is in agreement with Griinari et al. (1998) who reported that diets low in fiber and containing unsaturated FA reduced rumen pH resulting in increased proportions of *t*10-18:1 in milk. Findings from the regression analysis discussed above indicate that a shift in microbial populations favoring greater *t*10-18:1 concentration occurred when the duration pH < 5.2 increased.

CLA isomers

Greater concentrations of milk RA for BBD despite greater intakes of LA for CBD could be attributed to the differences in the extent of rumen biohydrogenation of PUFA evidenced by the greater concentrations of VA and greater desaturase index for BBD compared to CBD. Although *t*10-18:1 was greater for BBD than for CBD in the rumen and plasma, this did not translate into greater *t*10*c*12-CLA for BBD than for CBD in milk because animals lack Δ^{12}-desaturase (Saeki et al., 2004). Presence of greater concentrations of *t*10*c*12-CLA in rumen and plasma with negligible amounts in milk can be explained by the fact that *t*10*c*12-CLA peaks co-elute with isomers of 20:2 FAME and are difficult to separate unless a 20:2 standard is also used in the identification or HPLC is used to separate the CLA isomers (Roach et al., 2000). In milk *t*10*c*12-CLA was observed in the GC results but when the CLA region from the GC was corrected with the HPLC separation of CLA isomers, we could hardly detect any *t*10*c*12-CLA isomer in milk. It could be observed that *t*7*c*9-CLA was recorded in milk but not in rumen and plasma. This is because milk CLA isomers were separated by both GC and HPLC while those in rumen and plasma were separated only by GC.

The RA yield per 100 g total substrate intake showed a significant grain effect with greater values observed for BBD than for CBD suggesting that cows offered BBD were more efficient in milk RA production. Despite lower intake of

substrate, cows fed BBD were more efficient in milk RA production which could be attributed mainly to the greater inhibition of PUFA biohydrogenation and a greater mammary desaturase index for BBD than for CBD. However, we do not know the extent to which the variability in milk RA could be attributed to the above factors. Step-wise regression analysis with concentrations of milk RA (dependent variable) and dietary factors [LA intake, LN intake, total substrate (LA + LN) intake], rumen factors [rumen pH (minimum, mean, maximum), pH duration (< 5.8, 5.5, 5.2), individual and total VFA, rumen NH_3], concentrations of *t*10-18:1 and *t*11-18:1 in rumen, plasma and milk, and milk desaturase indices (14:1/14:0; 16:1/16:0 and 18:1/18:0) revealed that the model was significant ($P < 0.01$; $R^2 = 0.99$) with 94% of the variability determined by the concentrations of *t*11-18:1 in milk, with an additional 4% variability contributed by the desaturase index (RA/VA). Repeating the regression analysis with milk *t*11-18:1 as dependent variable and the variables described above (except *t*11-18:1) as independent variables revealed that the model was significant ($P < 0.01$; $R^2 = 0.96$) with 72% of the variability in milk *t*11-18:1 concentrations contributed by plasma *t*11-18:1 and an additional 9% by rumen *t*11-18:1. Other variables subsequently entered into the model (variability contributed in parenthesis) were duration pH < 5.8 (5%), caproic acid proportion (4%), butyric acid proportion (2%), rumen *t*10-18:1 concentration (2%), and starch:fiber ratio (1%). The above findings suggest that concentrations of milk *t*11-18:1 and plasma *t*11-18:1, which are the net result of the substrate consumed and extent of biohydrogenation in the rumen, were the important determinants of milk RA.

3.5 Conclusions

Comparison of milk rumenic acid from cows fed diets containing similar starch and total PUFA substrate but differing in the source of starch and method of grain processing revealed that barley-based diets increased the concentrations of rumen *t*11-18:1 and milk RA relative to corn-based diets. Grain

processed by grinding did influence some of the rumen fermentation end-products and reduced milk fat content compared to rolling, however, rumen *t*11-18:1 and milk RA were not influenced by the method of processing. Milk profiles of *t*-18:1 and CLA isomers were more strongly influenced by the source of grain starch than by the method of grain processing indicating that factors inherent in the starch source were responsible for the observed differences and these factors could not be modified by the processing methods used in this study.

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Persistency of milk *trans*-18:1 isomers and rumenic acid in Holstein cows over a full lactation

4.1 Introduction

Rumenic acid (**RA**) is a component of milk fat shown to possess several health benefits (anti-cancer, anti-obesity and anti-diabetic) in animal models based on biomedical studies (Belury, 2002). Diets supplemented with a source of polyunsaturated fatty acid (**PUFA**) substrate such as linoleic acid (**LA**) and α-linolenic acid (**LN**) has been shown to increase milk concentrations of RA precursor, vaccenic acid (*t*11-18:1) and milk RA (Bell et al. 2006; Cruz-Hernandez et al. 2007). Diets also exert variable influence on PUFA biohydrogenation in the rumen resulting in the formation of either *t*11-18:1 or *t*10-18:1 as the major end product (Griinari et al. 1998). It is desirable to have greater concentrations of *t*11-18:1 or RA in milk because human intestine can utilize RA directly and can also convert *t*11-18:1 to RA (Salminen et al., 1998). However, diets supplemented with a source of LA have been reported to induce a shift to *t*10-18:1 over time and the initial increase in *t*11-18:1 can be transient (Bauman et al. 2000; Roy et al. 2006; Shingfield et al. 2006). Bell et al. (2006) in a study comparing barley silage based diets (forage:concentrate ratio = 50:50) supplemented with safflower oil or linseed oil at 6% DM also reported an increase in *t*10-18:1 but the increases in milk RA and *t*11-18:1 concentrations in their study were maintained for the 8 weeks duration of the study. The study by Bell et al. (2006) was different from the aforementioned studies in the basal composition of the diet.

Studies that examined the persistency of milk RA for an entire lactation are limited and the findings are not consistent. Auldist et al. (1998) in a study involving pasture-fed dairy cows (n = 80), calved in 4 different seasons, analyzed 3 milk samples per cow (d 30, d 120 and d 210 of lactation) and reported a significant season and stage of lactation (SOL) effect on total CLA in milk. A significant week of lactation effect on milk RA was reported in cows fed corn silage and alfalfa hay based diet for a period of 16 weeks (Kay et al. 2005). In

their study, milk for FA composition was sampled at 3 instances only (wk 1, 8 and 16). Kelsey et al. (2003) reported that SOL had little effect on milk RA from 113 TMR fed Holstein cows that were sampled on 1 calendar day. Dunshea et al. (2008) in a grazing study analyzed milk for RA collected from 24 farms at 4 week intervals for the entire lactation. They reported that the variation in milk RA response over the lactation was more related to pasture quality than to the season of calving or stage of lactation. The number of times milk was sampled per cow in the above studies ranged from once to 8 times over the entire lactation.

The present study was designed to compare barley silage based diets differing in PUFA and an ionophore, monensin (control vs test diet) from calving until 270 days in milk (**DIM**) by sampling milk every other week (18 times in 270 DIM). It was hypothesized that milk concentrations of *t*11-18:1 and RA are not significantly altered within lactation. The objectives of this study were to examine: i) the persistency of milk concentrations of RA, *t*10-18:1 and *t*11-18:1 for the entire lactation; ii) the effect of diets and stage of lactation on milk fatty acid composition.

4.2 Materials and methods

Experimental design and treatments

All procedures were carried out according to the guidelines of the Canadian Council on Animal Care (Institutional Animal Use Approval Number: 2005-20). Twenty-four Holstein cows from the Dairy Research and Technology Centre, University of Alberta were used. Cows were allotted to two groups based on their previous year's milk yield so that both group's average milk yield was similar. Each group was allotted to one of the two treatments: control and test diet. The experimental diets had forage: concentrate ratio of 50:50. In the test diet, sunflower seed (11%), fish oil (0.5%) and monensin (22 mg/kg DM) were

supplemented by replacing an equivalent amount of barley grain from the control diet. Ingredient, chemical and fatty acid composition of experimental diets is given in Table 1. The cows were put on the experimental diets from 2 weeks before calving until they were 300 DIM. Experimental diets were offered once daily (0900 h) at 110% of expected intake. All cows were housed in tie stalls with continuous access to water. Cows were milked in their stalls twice daily (0500 and 1700 h).

Sampling and chemical analyses

Representative samples of TMR were collected every fortnight during the study period and stored at -20^{o}C. The DM content of alfalfa hay and barley silage was determined weekly to adjust dietary allocation of forages to maintain a constant forage:concentrate ratio. The TMR were dried at 55^{o}C for 72 h and ground through a 1-mm screen using a Wiley mill (Thomas-Wiley, Philadelphia, PA). Samples were analyzed for DM content (at 135^{o}C for 2 h), ash (overnight at 550^{o}C), ADF, NDF (Ankom filter bag technique with the reagents sodium sulphite and alpha-amylase; Van Soest et al., 1991), nitrogen content (determination of CP using a Leco nitrogen combustion analyzer, Leco Corporation, St. Joseph MI, USA) and ether extract (Goldfisch extraction apparatus). Feed fatty acid (**FA**) composition was determined using 30 mg of TMR samples (by period) by direct methylation using 1 ml of 5% anhydrous HCl/methanolic (w/v) along with an internal standard ($C_{23:0}$ @ 10% of the dietary fat). Methylation was done for 1 h at 80^{o}C. Following methylation the fatty acid methyl esters (**FAME)** were extracted using hexane, dried down to 200 µL and applied onto thin layer chromatography (**TLC**) plate and developed in hexane: diethyl ether: acetic acid solvent (85:15:1). Fatty acid methyl esters were then scraped off the TLC plate and extracted with chloroform. After removal of chloroform the FAME were taken up in 1 ml hexane and analyzed by GLC. Intake of each cow's dietary LA, LN

and total substrate (LA + LN) was estimated from its DMI and concentration of respective PUFA substrates in diet.

Table 4-1. Ingredient, chemical and fatty acid composition of the experimental diets

	Treatments[1]	
	Control	Test
Ingredient composition (% DM)		
Alfalfa hay	15.0	15.0
Barley silage	35.0	35.0
Supplement A5[2]	11.6	11.6
Barley grain	38.4	24.72
Sunflower seed	-	11.2
Monensin[3]	-	1.98
Fish oil[4]	-	0.5
Chemical composition (%)		
DM	53.7	54.4
CP	17.3	17.3
EE	2.1	4.9
Starch	39.8	31.7
NDF	34.7	35.4
ADF	17.7	18.9
Ash	7.2	6.8
Fatty acid composition (% of FAME)[5]		
14:0	1.46	1.32
16:0	22.46	15.07
18:0	1.75	3.36
*c*9-18:1	14.30	15.56
18:2n-6	34.23	47.30
18:3n-3	10.53	5.30
EPA	0.24	0.84
DPA	0.02	0.08
DHA	0.26	0.38

[1]Diets were control and test diet; test diet is control diet supplemented with sunflower seed, fish oil (0.5%) and monensin.
[2]Supplement contained 31.7% ground corn, 5.4% canola meal, 30.3% corn gluten meal, 9.7% fish meal, 5% dairy premix, 1.6% Magnesium oxide, 4.3% limestone, 3.2% sodium biocarbonate, 4.3% molasses and 4.5% biofos.
[3]Contained 4,400 mg of Rumensin (Elanco Animal Health, ON, Canada)/kg.
[4]Fish oil was included in the ration prior to feeding and contained 16.1% eicosapentaenoic acid (EPA) and 8.1% docosahexaenoic acid (DHA)
[5]FAME = fatty acid methyl esters ; *c* = cis ; DPA = docosapentaenoic acid.

Milk was sampled AM and PM every fortnight during the study period. The AM and PM samples were pooled for each cow on each sampling day in proportion to the yields. One aliquot was preserved with potassium dichromate and analyzed for protein, fat, and lactose using infrared spectroscopy at the central milk testing laboratory Edmonton, Alberta, Canada (MilkoScan 605, Foss Electric, Hillerod, Denmark) and the other aliquot was stored at –20°C for fatty acid analysis using GLC. Milk lipids were extracted, methylated and analyzed by GLC as described by Mohammed et al. (2009). The FAME were analyzed using a Hewlett Packard Model 5890 Series II GLC equipped with a flame ionization detector, an autosampler (HP Model 7673), a 100 m CP-Sil 88 fused capillary column (Varian Inc., Mississauga, ON), software program (Agilent ChemStation, Version A.10) and operated in a splitless mode that was flushed 0.3 min after injection. Injector and detector temperatures were kept at 250^{o}C. Hydrogen was used as carrier gas at a flow rate of 1 mL/min and for the flame ionization detector at 40 mL/min. Other gases used were purified air at 250 mL/min and N_2 makeup gas at 25 mL/min. The injection volume was 1 µL of FAME mixtures containing about 1 µg/µL. The FAME obtained from each milk sample were analyzed using two temperature programs (175^{o}C and 150^{o}C) as described by Kramer et al. (2008). The FAME were identified by comparison with a GLC reference standard (#463) spiked with a mixture of four positional conjugated linoleic acid (**CLA**) isomers (#UC-59M), 21:0, 23:0 and 26:0 obtained from Nu-Chek Prep Inc. (Elysian, MN). Identification of 16:1, 18:1 and 20:1 isomers was based on available isomers in the GLC reference standard (#463), comparison with published reports, and based on principles of silver-ion separation. Confirmation of 18:1 isomers was made using Ag^{+}-solid phase extraction separated fractions as described by Kramer et al. (2008). Individual FAME were reported as a percentage of total FAME and the short-chain FAME were

corrected for their flame ionization detector deficiency based on theoretical considerations (Wolff et al., 1995).

Statistical analysis

Data obtained from 11 cows per treatment were included in the analysis because one cow from each treatment did not complete the study. Data were analyzed by the MIXED procedure of SAS (version 9.1, SAS Institute, Cary, NC). Cow within treatment was the experimental unit, with treatment as the fixed effect and period as the repeated measure. The variance-covariance matrix structure for each variable was chosen based on the Schwarz's Bayesian criterion. Data obtained for each variable at the different time points was averaged for every 90 days in milk starting from the first sampling week resulting in 3 data sets per cow per treatment. This corresponds to three stages of lactation [early-lactation (**EL**), mid-lactation (**ML**) and late-lactation (**LL**)]. Least square means for the treatment, period and treatment and period interaction were calculated for each variable and the differences among the least square means were declared significant at $P < 0.05$. Step-wise regression analysis was used to identify the variables that influenced the concentrations of fat yield, *t*11-18:1 and *c*9*t*11-CLA in milk. The threshold value to keep a term in the model was $P < 0.05$.

4.3 Results

Ingredient, chemical and fatty acid composition of experimental diets is presented in Table 1. It should be noted that dietary starch was lower for test diet compared to the control because test diet was formulated by replacing 13.7% of barley grain from control diet with sunflower seed (11.2%), monensin (1.98%) and fish oil (0.5%). The concentrations of LA, eicosapentaenoic acid (**EPA**), docosapentaenoic acid (**DPA**) and docosahexaenoic acid (**DHA**) were greater in the test diet compared to the control because of supplementation

with sunflower seed and fish oil. However, concentration of LN was greater for the control diet than the test diet and could be attributed to the greater concentration of LN in barley grain (data not presented).

Dry matter intakes were not different between the treatments but were greater during ML than during EL and LL (Table 2; Fig. 4-1). Milk yield was also not different between the treatments. However, 4% fat corrected milk (**FCM**) was greater for control diet than for the test diet. Milk yield was greater in the EL and ML than during LL (Table 2). Milk yield reached maximum during EL, reached a plateau in ML period and started to decline during the LL period (Fig. 4-1). Four % FCM decreased with increasing DIM. Protein and lactose content in milk showed an interaction between treatments and SOL with no difference between the treatments; however, they were different among the stages of lactation. Protein content increased from EL to LL while lactose content decreased from EL to LL. Milk protein yield was greater for control than for the test diet and was greater during EL and ML than during LL. Lactose yield followed the same trend as lactose content. There was an interaction between treatments and SOL for milk fat content and yield. Milk fat content and yield were lower for the test diet compared to the control diet with greater concentrations observed for fat content during EL and LL compared to the ML period (Table 2; Fig. 4-2a). Milk fat yield, however, decreased from EL to LL. Concentrations of the total *de novo* synthesized FA (4:0 – 15:1) and total preformed FA showed an interaction between treatments and SOL. Total *de novo* synthesized FA was lower for test diet compared to the control diet with lower values observed in the EL compared to the ML and LL period. Total preformed FA (17:0 and above) was greater for the test diet than control diet and decreased from EL to LL. There was an interaction between treatments and SOL for the intake of LA, LN and total substrate (LA + LN). Intake of dietary LA was greater for the test diet than for

control diet and increased from EL to LL (Table 2). Intake of dietary LN and total substrate (LA + LN) showed similar trend as dietary LA.

Table 4-2. Effect of stage of lactation and treatments[1] on intakes and milk production

Variable[2]	Early lactation (EL)		Mid lactation (ML)		Late lactation (LL)		SEM	Tr x SOL[3]	Contrasts			
	Ctrl	Test	Ctrl	Test	Ctrl	Test			Ctrl vs Test	EL vs ML	EL vs LL	ML vs LL
DMI	19.9	18.1	20.9	20.7	19.3	18.7	0.82	NS	NS	< 0.01	NS	< 0.01
Milk yield	38.5	34.9	36.0	32.4	28.1	24.9	1.69	NS	NS	0.01	< 0.01	< 0.01
4% FCM	35.1	27.7	30.6	24.2	24.4	20.6	1.41	NS	< 0.01	< 0.01	< 0.01	< 0.01
Fat %	3.4	2.6	3.0	2.3	3.1	2.8	0.13	0.03	< 0.01	< 0.01	NS	< 0.01
Protein %	2.9	2.9	3.2	3.3	3.4	3.3	0.06	< 0.01	NS	< 0.01	< 0.01	< 0.01
Lactose %	4.5	4.6	4.5	4.4	4.4	4.3	0.07	0.01	NS	< 0.01	< 0.01	0.01
Fat yield	1.3	0.9	1.1	0.7	0.9	0.7	0.06	0.01	< 0.01	< 0.01	< 0.01	0.01
Protein yield	1.1	1.0	1.1	1.0	0.9	0.8	0.04	NS	0.03	NS	< 0.01	< 0.01
Lactose yield	1.7	1.6	1.6	1.4	1.2	1.1	0.08	NS	NS	NS	< 0.01	< 0.01
RA yield g/d	6.8	16.0	8.1	11.7	5.5	13.2	1.15	< 0.01	< 0.01	NS	NS	NS
Fatty acids origin												
De novo (4:0 – 15:1)	25.5	16.7	30.8	18.9	30.2	18.1	0.62	< 0.01	< 0.01	< 0.01	< 0.01	NS
16:0 and 16:1	31.9	25.9	33.0	25.6	34.1	25.0	0.50	0.01	< 0.01	NS	NS	NS
Preformed (17:0 and above)	38.5	52.2	32.4	50.2	32.2	51.5	0.87	< 0.01	< 0.01	< 0.01	< 0.01	NS
Dietary LA intake	116.8	415.7	141.6	502.6	167.9	510.8	18.04	< 0.01	< 0.01	< 0.01	< 0.01	NS
Dietary LN intake	41.1	44.6	42.6	60.1	49.9	53.2	2.16	< 0.01	0.01	< 0.01	< 0.01	NS
Total (LA + LN) intake	157.8	460.3	184.2	562.7	217.8	563.9	20.10	< 0.04	< 0.01	< 0.01	< 0.01	NS

[1]Treatments were control and test diet; test diet is control diet supplemented with sunflower seed, fish oil (0.5%) and monensin.
[2]LA = linoleic acid ; LN = linolenic acid ; FCM = fat corrected milk.
[3]Tr x SOL = interaction between treatment and stage of lactation.
NS = not significant ($P > 0.05$)

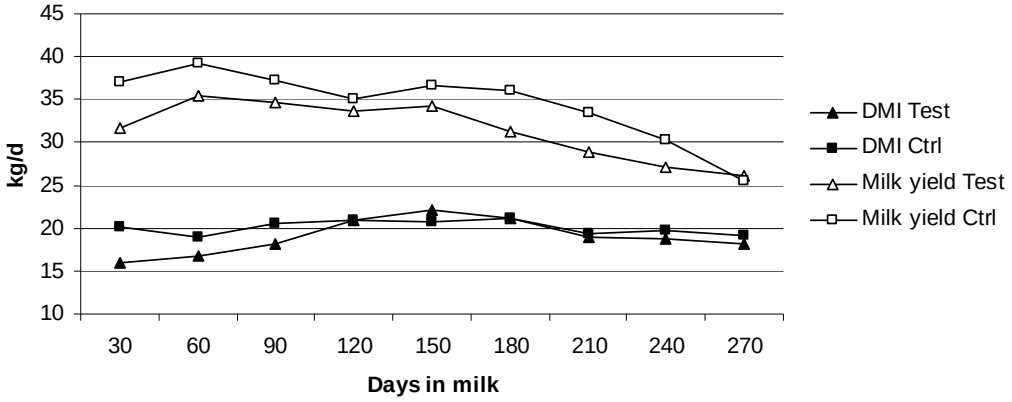

Figure 4-1. Effect of control and test diets on DMI and milk yield from calving to 270 days in milk. Test diet is control diet supplemented with sunflower seed (11.2% DM), fish oil (0.5% DM) and monensin (22mg/kg DM).

The sum of the saturated fatty acids (**SFA**) in milk was greater for the control diet than the test diet and was not influenced by the SOL (Table 3). Total short chain fatty acid (SCFA) was also greater for control than test diet; however, its concentrations decreased with increasing DIM. A decline in SCFA from EL through LL was consistent with the decline in milk fat yield from EL to LL. Concentrations of 12:0 and 14:0 increased from EL through LL and are consistent with *de novo* fatty acid synthesis (Fig. 4-2b). There was an interaction between treatments and SOL for milk 16:0 with greater values observed for control diet than the test diet. However, milk concentrations of 16:0 were not influenced by the SOL. The sum of the *iso* FA was not different between treatments; however, its concentrations were greater during EL and LL than during ML. The sum of the *anteiso* FA was greater for control than for test diet with no difference from EL to LL. The ratio of *iso*:*anteiso* FA was greater for test diet than control diet with greater concentrations observed during EL and LL than during ML.

Table 4-3. Effect of stage of lactation and treatments[1] on milk fatty acid composition

Variable[2]	Early lactation (EL)		Mid lactation (ML)		Late lactation (LL)		SEM	Tr x SOL[3]	Contrasts			
	Ctrl	Test	Ctrl	Test	Ctrl	Test			Ctrl vs Test	EL vs ML	EL vs LL	ML vs LL
4:0	1.46	1.82	0.20	0.72	0.10	0.54	0.09	NS	<0.01	<0.01	<0.01	NS
5:0	0.05	0.06	0.03	0.07	0.03	0.06	0.004	<0.01	<0.01	NS	<0.01	<0.01
6:0	0.50	0.56	0.35	0.65	0.28	0.50	0.03	<0.01	<0.01	NS	<0.01	<0.01
7:0	0.46	0.60	0.42	0.82	0.33	0.65	0.04	<0.01	<0.01	0.01	NS	<0.01
8:0	1.38	0.74	1.34	0.71	1.34	0.76	0.06	NS	<0.01	NS	NS	NS
9:0	0.07	0.03	0.15	0.03	0.13	0.03	0.01	<0.01	<0.01	<0.01	<0.01	0.02
10:0	3.12	1.43	3.90	1.66	3.86	1.75	0.15	0.05	<0.01	<0.01	<0.01	NS
11:0	0.38	0.14	0.68	0.20	0.61	0.19	0.02	<0.01	<0.01	<0.01	<0.01	0.01
12:0	3.54	1.76	5.02	2.27	4.97	2.25	0.16	<0.01	<0.01	<0.01	<0.01	NS
13:0	0.15	0.06	0.38	0.09	0.31	0.08	0.02	<0.01	< 0.01	<0.01	<0.01	<0.01
13:0 *iso*	0.03	0.03	0.02	0.03	0.02	0.03	0.002	NS	0.02	NS	NS	NS
13:0 *anteiso*	0.08	0.04	0.14	0.07	0.13	0.07	0.004	<0.01	<0.01	<0.01	<0.01	NS
14:0	11.17	7.04	13.25	8.59	13.43	8.25	0.26	NS	<0.01	<0.01	<0.01	NS
14:0 *iso*	0.06	0.06	0.06	0.06	0.08	0.07	0.006	NS	NS	NS	0.05	<0.01
15:0 *iso*	0.18	0.15	0.14	0.13	0.16	0.14	0.007	NS	0.04	<0.01	0.04	0.01
15:0 *anteiso*	0.41	0.34	0.46	0.40	0.44	0.38	0.01	NS	<0.01	<0.01	0.01	NS
15:0	1.33	0.89	2.46	1.08	2.21	0.98	0.10	<0.01	0.01	<0.01	<0.01	0.01
16:0 *iso*	0.21	0.21	0.18	0.21	0.22	0.25	0.02	0.02	NS	0.03	NS	0.01
16:0	29.80	23.26	30.54	22.72	31.73	22.27	0.51	0.01	<0.01	NS	NS	NS
17:0 *iso*	0.34	0.42	0.31	0.42	0.32	0.39	0.01	NS	<0.01	NS	NS	NS
17:0 *anteiso*	0.70	0.64	0.64	0.58	0.70	0.57	0.03	NS	NS	<0.01	NS	NS
17:0	0.70	0.66	0.75	0.54	0.69	0.53	0.03	<0.01	<0.01	NS	0.02	0.05
18:0 *iso*	0.08	0.08	0.06	0.06	0.06	0.06	0.004	NS	NS	<0.01	<0.01	NS
18:0	8.90	8.95	5.60	8.57	6.17	10.21	0.36	<0.01	<0.01	<0.01	0.05	<0.01
17:0 cyclo	0.12	0.20	0.19	0.26	0.18	0.26	0.01	NS	<0.01	<0.01	<0.01	NS
19:0	0.10	0.11	0.08	0.11	0.08	0.11	0.004	0.04	<0.01	<0.01	0.02	NS
20:0	0.20	0.18	0.15	0.20	0.16	0.21	0.01	<0.01	0.02	0.01	NS	0.03
22:0	0.09	0.08	0.06	0.09	0.06	0.09	0.004	<0.01	<0.01	0.01	NS	NS
23:0	0.02	0.02	0.02	0.02	0.02	0.02	0.001	0.01	NS	NS	NS	NS
24:0	0.03	0.03	0.02	0.03	0.02	0.03	0.002	0.03	0.01	<0.01	<0.01	NS
26:0	0.02	0.02	0.02	0.02	0.02	0.02	0.002	NS	NS	NS	NS	NS
∑ SFA	65.59	50.45	67.48	51.17	68.68	51.49	0.99	NS	<0.01	NS	NS	NS
∑ SCFA	7.05	5.25	6.40	4.66	6.06	4.29	0.21	NS	<0.01	<0.01	<0.01	NS
∑ *iso* FA	0.57	0.54	0.48	0.51	0.54	0.55	0.03	0.04	NS	<0.01	NS	<0.01
∑ *anteiso* FA	1.19	1.03	1.24	1.05	1.27	1.01	0.04	NS	<0.01	NS	NS	NS
***Iso*:*anteiso* FA**	0.47	0.52	0.38	0.48	0.43	0.54	0.02	NS	<0.01	<0.01	NS	0.01

*c*9-12:1	0.08	0.03	0.16	0.06	0.15	0.06	0.01	<0.01	<0.01	<0.01	<0.01	NS
*c*9-14:1	0.78	0.64	1.40	1.11	1.37	1.06	0.06	NS	<0.01	<0.01	<0.01	NS
*t*5-16:1	0.05	0.06	0.05	0.05	0.05	0.05	0.004	NS	NS	NS	NS	NS
*t*6*t*8-16:1	0.06	0.18	0.10	0.24	0.08	0.17	0.02	0.01	<0.01	<0.01	NS	<0.01
*t*9-16:1	0.03	0.12	0.07	0.18	0.06	0.12	0.01	0.02	<0.01	<0.01	NS	<0.01
*t*10-16:1	0.06	0.31	0.07	0.28	0.06	0.33	0.02	0.01	<0.01	NS	NS	NS
*t*11*t*12-16:1	0.04	0.20	0.05	0.21	0.05	0.20	0.01	NS	<0.01	NS	NS	NS
∑ *t*-16:1	0.24	0.88	0.35	0.97	0.29	0.87	0.04	NS	<0.01	0.03	NS	<0.01
*c*7*t*13-16:1	0.13	0.14	0.09	0.10	0.09	0.11	0.01	NS	NS	<0.01	<0.01	NS
*c*9-16:1	1.51	1.54	1.70	1.65	1.60	1.62	0.14	NS	NS	NS	NS	NS
*c*10-16:1	0.02	0.03	0.03	0.04	0.03	0.04	0.03	NS	<0.01	<0.01	<0.01	NS
*c*11-16:1	0.06	0.06	0.06	0.04	0.10	0.04	0.02	NS	NS	NS	NS	NS
*c*13-16:1	0.13	0.06	0.24	0.10	0.23	0.10	0.01	<0.01	<0.01	<0.01	<0.01	NS
∑ *c*-16:1	1.85	1.84	2.12	1.92	2.06	1.89	0.14	NS	NS	NS	NS	NS
*c*7-17:1	0.05	0.05	0.04	0.04	0.03	0.03	0.002	NS	NS	<0.01	<0.01	0.01
*c*9-17:1	0.31	0.28	0.33	0.21	0.31	0.20	0.02	0.04	<0.01	NS	0.03	NS
*t*4-18:1	0.03	0.04	0.03	0.04	0.02	0.04	0.002	NS	<0.01	NS	NS	0.02
*t*5-18:1	0.03	0.04	0.03	0.04	0.02	0.04	0.002	NS	<0.01	NS	0.05	0.03
*t*6-8-18:1	0.21	0.80	0.35	1.01	0.28	0.81	0.05	NS	<0.01	<0.01	NS	<0.01
*t*9-18:1	0.31	0.81	0.38	0.91	0.32	0.77	0.04	NS	<0.01	0.01	NS	<0.01
*t*10-18:1	0.58	4.18	2.09	5.48	1.28	3.48	0.51	NS	<0.01	<0.01	NS	<0.01
*t*11-18:1	1.08	5.64	1.19	4.48	0.90	4.92	0.37	0.04	<0.01	NS	NS	NS
*t*12-18:1	0.30	0.86	0.35	0.96	0.34	0.98	0.03	NS	<0.01	0.02	0.01	NS
*t*13*t*14-18:1	0.56	1.09	0.62	1.23	0.62	1.33	0.04	NS	<0.01	0.01	<0.01	NS
*t*15-18:1	0.19	0.32	0.21	0.35	0.22	0.40	0.02	NS	<0.01	NS	0.01	<0.01
∑ *t*-18:1	3.56	14.12	5.53	14.87	4.29	13.20	0.71	NS	<0.01	0.01	NS	0.01
t10:t11-18:1	0.48	0.80	1.55	1.36	1.43	1.00	0.26	NS	NS	<0.01	0.05	NS
*c*6-8-18:1	0.15	0.28	0.19	0.25	0.14	0.15	0.02	<0.01	0.01	NS	<0.01	<0.01
*c*9*c*10-18:1	19.43	21.00	13.84	18.18	14.58	19.90	0.65	NS	<0.01	<0.01	<0.01	<0.01
*c*11-18:1	0.62	0.77	0.82	0.68	0.78	0.56	0.04	<0.01	NS	NS	NS	0.01
*c*12-18:1	0.22	0.45	0.27	0.61	0.27	0.62	0.04	NS	<0.01	<0.01	0.01	NS
*c*13-18:1	0.11	0.17	0.10	0.13	0.10	0.12	0.01	NS	<0.01	<0.01	0.02	NS
*c*14*t*16-18:1	0.27	0.32	0.25	0.37	0.26	0.43	0.02	<0.01	<0.01	NS	<0.01	0.01
*c*15-18:1	0.12	0.27	0.19	0.34	0.20	0.33	0.03	NS	<0.01	<0.01	0.02	NS
*c*16-18:1	0.09	0.13	0.11	0.17	0.12	0.18	0.007	NS	<0.01	<0.01	<0.01	NS
∑ *c*-18:1	20.73	23.06	15.52	20.36	16.18	21.86	0.65	NS	<0.01	<0.01	<0.01	<0.01
*c*5-20:1	0.02	0.02	0.02	0.02	0.02	0.03	0.002	NS	0.01	NS	NS	NS
*c*9-20:1	0.18	0.22	0.21	0.27	0.20	0.28	0.01	0.02	<0.01	<0.01	<0.01	NS
*c*11-20:1	0.09	0.17	0.11	0.17	0.08	0.14	0.01	NS	<0.01	NS	0.04	<0.01
*c*13-22:1	0.02	0.03	0.03	0.04	0.02	0.04	0.002	NS	<0.01	<0.01	0.01	NS
*c*15-24:1	0.01	0.02	0.01	0.02	0.01	0.02	0.002	NS	<0.01	NS	NS	NS

∑ *c*-MUFA	24.14	26.37	19.96	24.23	20.45	25.63	0.70	NS	<0.01	<0.01	0.01	0.02
∑ *t*-MUFA	3.80	15.00	5.88	15.84	4.59	14.07	0.75	NS	<0.01	0.01	NS	0.01
tt-18:2	0.03	0.08	0.07	0.08	0.05	0.07	0.01	<0.01	<0.01	<0.01	NS	NS
*t*9*t*12-18:2	0.02	0.04	0.04	0.04	0.02	0.03	0.004	NS	0.03	NS	NS	<0.01
*t*8*c*13*c*9*t*12 -18:2	0.09	0.13	0.09	0.15	0.09	0.17	0.005	<0.01	<0.01	<0.01	<0.01	<0.01
*t*9*c*13*t*8*c*12 -18:2	0.02	0.03	0.02	0.03	0.02	0.02	0.002	0.02	<0.01	0.04	<0.01	NS
*t*9*c*12-18:1	0.03	0.06	0.04	0.09	0.05	0.06	0.005	<0.01	<0.01	<0.01	NS	<0.01
*t*11*c*15 -18:2	0.18	0.45	0.21	0.40	0.18	0.32	0.03	NS	<0.01	NS	0.04	0.05
18:2n-6	2.15	2.34	2.36	2.75	2.33	2.75	0.09	NS	0.01	<0.01	<0.01	NS
18:3n-6	0.03	0.02	0.03	0.02	0.03	0.02	0.002	NS	<0.01	NS	NS	NS
c/*t*/*t*-18:3	0.01	0.01	0.004	0.01	0.008	0.009	0.001	0.01	0.03	NS	NS	NS
c/*c*/*t*-18:3	0.01	0.01	0.01	0.02	0.01	0.01	0.001	NS	NS	NS	NS	0.01
20:4n-6	0.11	0.09	0.11	0.05	0.12	0.05	0.005	<0.01	<0.01	<0.01	<0.01	NS
20:3n-6	0.07	0.06	0.08	0.05	0.09	0.05	0.004	0.02	<0.01	NS	NS	NS
18:3n-3	0.37	0.31	0.33	0.27	0.33	0.29	0.02	NS	0.01	<0.01	0.02	NS
20:2n-6	0.02	0.03	0.03	0.04	0.03	0.04	0.002	NS	<0.01	0.02	0.04	NS
20:5n-3	0.05	0.06	0.04	0.06	0.04	0.06	0.003	NS	<0.01	NS	NS	NS
t/*c*/*c*-18:3	0.02	0.03	0.02	0.03	0.01	0.03	0.003	NS	<0.01	NS	NS	NS
22:2n-6	0.03	0.03	0.02	0.02	0.02	0.02	0.002	NS	NS	<0.02	<0.02	NS
22:4n-6	0.02	0.01	0.02	0.01	0.02	0.01	0.002	<0.01	<0.01	NS	NS	NS
22:5n-3	0.06	0.07	0.06	0.08	0.06	0.08	0.004	NS	<0.01	NS	NS	NS
22:6n-3	0.05	0.04	0.06	0.06	0.06	0.06	0.003	0.01	NS	<0.01	<0.01	NS
∑ n6-PUFA	2.43	2.59	2.67	2.94	2.65	2.94	0.10	NS	0.04	<0.01	<0.01	NS
∑n3-PUFA	0.55	0.53	0.51	0.51	0.51	0.52	0.02	NS	NS	NS	NS	NS
n6:n3-PUFA	4.41	4.97	5.24	5.76	5.20	5.75	0.15	NS	0.01	<0.01	<0.01	NS
Desaturase index												
*c*914:1/14:0	0.07	0.09	0.11	0.13	0.10	0.13	0.01	NS	0.01	<0.01	<0.01	NS
*c*916:1/16:0	0.05	0.06	0.06	0.07	0.05	0.07	0.01	NS	0.01	NS	NS	NS
*c*918:1/18:0	2.21	2.35	2.55	2.13	2.43	1.97	0.11	0.02	0.04	NS	NS	0.01
*c*9*t*11-CLA/ *t*11-18:1	0.48	0.32	0.65	0.37	0.72	0.37	0.02	<0.01	<0.01	<0.01	<0.01	0.02

[1]Treatments were control and test diet; test diet is control diet supplemented with sunflower seed, fish oil (0.5%) and monensin.
[2]FAME = fatty acid methyl esters (expressed as a percentage of total FAME; *c* = *cis*; *t* = *trans*; CLA = conjugated linoleic acid; SFA = saturated fatty acid (4:0 to 26:0 including branched chain fatty acids); SCFA = shorter-chain fatty acid (4:0-10:0); MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
NS = not significant ($P > 0.05$)
[3]T x SOL = interaction between treatment and stage of lactation.

Total *t*-MUFA was greater for the test diet than for control diet with greater concentrations observed during ML than during EL and LL. A similar trend was observed for the sum of the *t*-16:1 and *t*-18:1 isomers. Total *t*-18:1 isomers (4-15) was greater for the test diet than for control diet. Concentrations of *t*11-

18:1 showed an interaction between treatments and SOL with greater values observed for the test diet compared to the control. Besides *t*11-18:1, concentrations of *t*10-18:1 was also greater for the test diet compared to the control indicating a shift in biohydrogenation pathway towards the formation of *t*10-18:1. Concentrations of *t*10-18:1 was greater during ML than during EL and LL, while that of *t*11-18:1 was not influenced by the SOL. Examination of the pattern of *t*10-18:1 and *t*11-18:1 for the test diet from the start to the end of the lactation (Fig. 4-2c) revealed that concentrations of *t*11-18:1 increased from 30 – 90 DIM followed by a fall in its concentration from 91 – 150 DIM and increased thereafter until the end of lactation. Concentrations of *t*10-18:1 however increased from 30 – 180 DIM (excepting from 61-90 DIM when there was a fall) and declined thereafter until the end of lactation. The ratio of *t*10:*t*11-18:1 was not different between the treatments but was greater during ML than during EL. Greater concentrations of *t*-16:1, *t*-18:1 and *t*10-18:1 and lesser concentrations of 18:0 during ML than during EL and LL (Fig. 4-2d) suggest that PUFA biohydrogenation was inhibited to a greater extent during ML than during EL and LL.

The sum of the *cis* (***c***)-MUFA was greater for the test diet than for control diet with greater concentrations observed in EL and LL than during ML. A similar trend was noticed for the sum of the *c*-18:1 isomers. Concentrations of all the *c*-18:1 isomers (except *c*11-18:1) were greater for the test diet than control diet. Concentrations of *c*9-/*c*10-18:1 was different among the stages of lactation and followed the same trend as that of milk fat content and 18:0 supporting the fact that it was derived from 18:0 by mammary desaturase activity (Fig. 4-3a).

The total n6-PUFA was greater for the test diet than control diet with greater values observed during ML and LL than during EL. Total n3-PUFA was not influenced by the treatment or SOL. The ratio of n6:n3-PUFA was greater for the test diet than for control diet with greater values observed during ML and LL

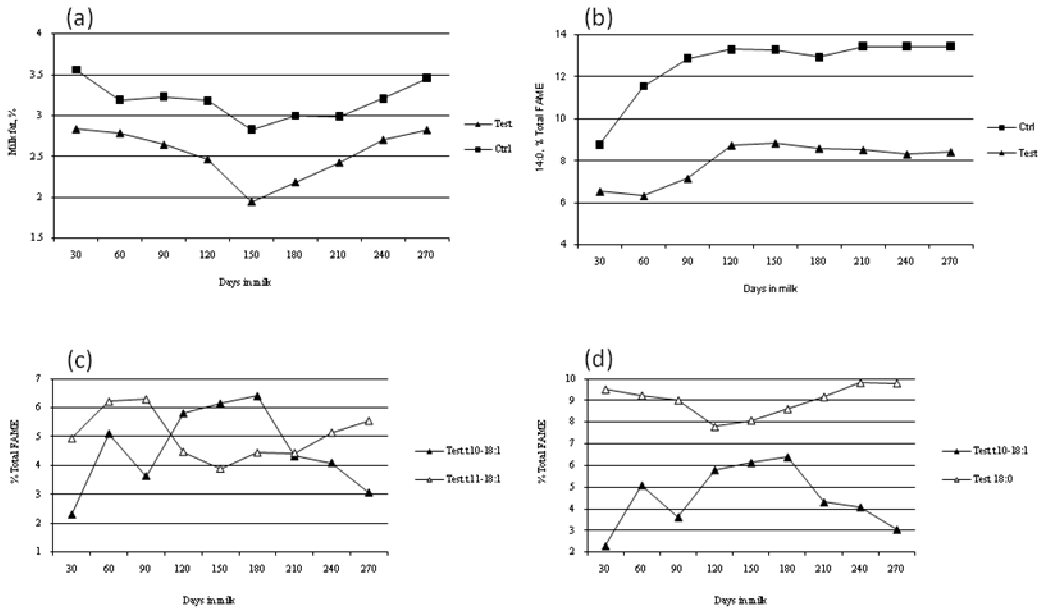

Figure 4-2. Effect of control and test diets on milk fat content (a), milk fat 14:0 concentration (b) and pattern of t10-18:1 and t11-18:1 (c) and 18:0 and t10-18:1 (d) for the test diets from calving to 270 days in milk. Test diet is control diet supplemented with sunflower seed (11.2% DM), fish oil (0.5% DM) and monensin (22mg/kg DM). FAME = fatty acid methyl esters

than during EL. Milk concentrations of LA were greater for the test diet than for control diet and could be attributed to the supplementation of sunflower seed for the test diet. Concentrations of milk LA increased from EL through LL consistent with the intakes of dietary LA. However, concentrations of milk LN were greater for control diet than for test diet and its concentrations were not influenced by the SOL. Concentrations of 20:5n-3 and 22:5n-3 were greater for test diet than for control diet and could be attributed to the supplementation with fish oil for the test diet. The concentrations of these isomers were however not influenced by the SOL. Desaturase index based on ratios *c*9-14:1/14:0 and *c*9-16:1/16:0 was greater for the test diet compared to the control diet with increasing values observed for *c*9-14:1/14:0 from EL to LL. Consistent with this, the ratio *c*9-14:1/14:0 (Fig. 4-3b) showed an increasing trend with increasing DIM. The fatty acid pairs *c*9-18:1/18:0 and *c*9t11-CLA/*t*11-18:1 showed an interaction between treatments and SOL with greater values observed for control diet compared to the test diet. The ratio *c*9*t*11-CLA/*t*11-18:1 increased with increasing DIM similar to that for *c*914:1/14:0.

The sum of the CLA isomers in milk fat ranged from 0.60 to 2.46% with greater concentrations observed for the test diet than control diet and were not influenced by the SOL (Table 4). There was an interaction between treatments and SOL for milk RA with greater concentrations observed for test diet than control diet. The lack of SOL effect on milk RA was consistent with the hypothesis that concentrations of milk RA are determined by the diet and not by the SOL. It should be noted that milk concentrations of *t*11-18:1 were also not different with the SOL and showed similar trends (Fig. 4-3c and 4-3d). Concentrations of *t*10*c*12-CLA and *t*7*c*9-CLA showed an interaction between treatments and SOL with greater values observed for test diet than for control and its concentrations increased with increasing DIM.

Table 4-4. Effect of stage of lactation and treatments[1] on CLA isomers in milk fat

Variable[2]	Early lactation (EL)		Mid lactation (ML)		Late lactation (LL)		SEM	Tr x SOL[3]	Contrasts			
	Ctrl	Test	Ctrl	Test	Ctrl	Test			Ctrl vs Test	EL vs ML	EL vs LL	ML vs LL
*t*11*t*13-CLA	0.01	0.02	0.004	0.01	0.01	0.02	0.002	NS	< 0.01	NS	NS	NS
*t*10*t*12-CLA	0.002	0.01	0.002	0.01	0.004	0.01	0.001	NS	< 0.01	NS	NS	NS
*t*9*t*11-CLA	0.004	0.01	0.01	0.02	0.01	0.03	0.002	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
*t*8*t*10-CLA	0.002	0.01	0.001	0.01	0.005	0.02	0.001	< 0.01	< 0.01	< 0.01	< 0.01	0.02
*c*12*t*12-CLA	0.01	0.01	0.01	0.02	0.01	0.02	0.002	< 0.01	< 0.01	0.01	0.02	NS
*t*11*c*13-CLA	0.006	0.01	0.001	0.003	0.002	0.002	0.001	0.01	< 0.01	< 0.01	< 0.01	NS
*c*11*t*13-CLA	0.01	0.004	0.004	0.001	0.002	0.01	0.001	< 0.01	NS	< 0.01	NS	NS
*t*10*c*12-CLA	0.002	0.02	0.007	0.07	0.002	0.09	0.004	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
*c*9*t*11-CLA[4]	0.52	1.79	0.77	1.58	0.64	1.71	0.11	0.02	< 0.01	NS	NS	NS
*t*8*c*10-CLA	0.008	0.008	0.003	0.008	0.001	0.01	0.001	< 0.01	< 0.01	< 0.01	< 0.01	NS
*t*7*c*9-CLA	0.04	0.25	0.08	0.45	0.06	0.51	0.02	< 0.01	< 0.01	< 0.01	< 0.01	NS
∑-CLA	0.60	2.15	0.90	2.23	0.75	2.46	0.14	NS	< 0.01	NS	NS	NS

[1]Treatments were control and test diet; test diet is control diet supplemented with sunflower seed, fish oil (0.5%) and monensin.
[2] *t* = *trans;* *c* = *cis;* CLA = conjugated linoleic acid.
[3]T x SOL = interaction between treatment and stage of lactation.
[4]*c*9*t*11-CLA is also called Rumenic acid or RA
NS = not significant ($P > 0.05$)

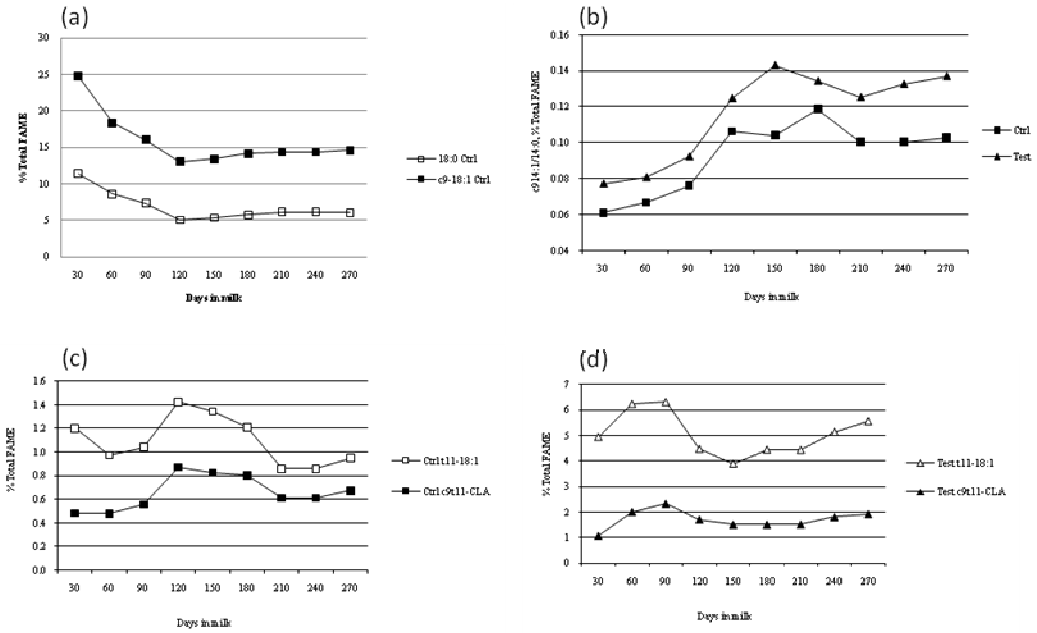

Figure 4-3. Effect of diets (control/test) on milk fat concentrations of 18:0 and c9-18:1 (a), c9-14:1/14:0 (b) and pattern of changes on milk fat concentrations of t11-18:1 and c9t11-CLA for control diet (c) and test diet (d) from calving to 270 days in milk. Test diet is control diet supplemented with sunflower seed (11.2% DM), fish oil (0.5% DM) and monensin (22mg/kg DM). FAME = fatty acid methyl esters, CLA = conjugated linoleic acid.

4.4 Discussion

Stage of lactation

The lower DMI during EL was expected because cows in the early lactation do not reach peak intake until 9-13 weeks of lactation (Kertz et al. 1991). The lower DMI in the transition period has been explained by animal (breed, parity), diet and management factors (Grummer et al. 2004). In the current study, cows were on a traditional (56 days) dry-cow management system with the first 28 days (far-off dry period) on a high-fiber low-energy diet and the second 28 days (close-up dry period) on a low-fiber high-energy diet. A gradual switch to treatment diets was made starting from 2 weeks prior to calving.

Greater milk yield in the EL could be attributed to the peak milk production recorded in the 8th week following calving (Fig. 4-1). Greater fat content in the EL compared to the ML period in spite of the lower *de novo*

synthesized fatty acids in the EL period suggest that mobilization of body fat was an important contributor to milk fat. An increase in the concentrations of the total *de novo* synthesized fatty acids and a decrease in the concentrations of the total preformed FA from EL to LL provides evidence that there was greater mobilization of body fat in the EL period. These observations are consistent with the findings of Palmquist et al. (1993) and Kay et al. (2005).

The pattern of *t*10- and *t*11-18:1 from EL to LL appeared to be more related to the DMI (possibly more specifically to the carbohydrate intake). During EL, the DMI were lower compared to that in the ML. As the intakes increased from EL to ML, the concentrations of *t*11-18:1 declined and the concentrations of *t*10-18:1 increased. Later as the intakes decreased from ML to LL, the concentrations of *t*11-18:1 increased and the concentrations of *t*10-18:1 decreased. This is consistent with the finding that restricted feeding compared to unrestricted feeding resulted in greater concentrations of milk *t*11-18:1 (Jiang et al. 1996). Greater intakes are often accompanied by greater fermentation of organic matter in the rumen resulting in a decrease in rumen pH. Although diets in the current study were not low in fiber compared to the lower fiber diets for which a shift to *t*10-18:1 is expected, a shift to *t*10-18:1 was still observed in this study and could be because the basal diet in this study was barley-based which is readily fermented in the rumen.

The lack of SOL effect on milk *t*11-18:1 was consistent with our hypothesis. Dhiman et al. (2000) reported a decline in the concentration of *t*11-18:1 and RA for diets based on Lucerne and maize silage after 1-2 weeks of feeding treatment diets. Roy et al. (2006) also reported that the increase in milk concentrations of *t*11-18:1 was only transient for maize silage based diets containing higher proportion of concentrate. Similar changes were reported for corn silage based diets supplemented with fish oil and sunflower oil in 1:2 ratio

(Shingfield et al. 2006) and for maize silage based diets supplemented with fish oil and sunflower oil in 2:3 ratio in the high concentrate treatment (Shingfield et al. 2005a). However, the increase in milk *t*11-18:1 and RA concentrations in the same study with hay based diets supplemented with linseed oil was reported to be more persistent. The concentration of *t*11-18:1 in the current study was relatively stable and remained similar throughout lactation except during 91-150 DIM. Differences in persistency of *t*11-18:1 in the current study compared to other studies could be due to differences in the composition of basal diet. Majority of the studies that reported a transient (within 2 weeks) increase for milk *t*11-18:1 had corn-silage based diet while the diets in the current study were barley silage based. Consistent with this, Bell et al. (2006) in a study comparing diets based on barley-silage and supplemented with safflower oil or flax oil reported an increase in *t*11-18:1 and RA that were maintained for the 8 week duration of the study.

The lack of SOL effect for milk RA was also consistent with our hypothesis that milk RA may not be influenced by the SOL. Similar findings were reported by Stanton et al. (1997) and Kelsey et al. (2003). Auldist et al. (2002) reported a decrease in RA with increasing DIM for grazing cows. They attributed the pattern of milk RA for grazing cows to the substrate (LN) content in the grass suggesting that milk RA was influenced by the dietary substrate and not by the SOL. In the current study, total substrate intakes increased from EL to LL but this did not translate into an increase in milk RA from EL to LL. To arrive at an explanation for this, we determined the efficiency of milk RA production (RA yield per 100 g total substrate intake; Mohammed et al. 2009). Efficiency of milk RA decreased from EL to LL ($P < 0.01$; 3.89 ± 0.24; 3.30 ± 0.24 and 2.51 ± 0.24 for EL, ML and LL, respectively) in spite of the increase in substrate intake from EL to LL suggesting that substrate intake is not the only factor determining milk RA production. It is possible that diets offered in the ML and LL did not favor greater production of

*t*11-18:1 in the rumen as evidenced by the lack of SOL effect on milk *t*11-18:1. Kay et al. (2005) comparing milk samples collected at wk 1, 8 and 16 reported a week of lactation effect for milk RA. They explained that the increase in milk RA from wk 1 to wk 16 was a week of lactation effect and not the result of dietary changes because all cows consumed the same TMR throughout their study. However they did not report substrate intakes.

Dietary Treatments

A lower milk fat for the test diet compared to the control was expected and could be attributed to the inhibitory effect of PUFA (from sunflower seeds and fish oil) on *de novo* synthesis evidenced by the lower concentrations of the sum of *de novo* synthesized fatty acids (4:0 to 15:0) for the test diet compared to the control diet. A depression in milk fat has been reported for diets supplemented with a source of LA (AlZahal et al. 2008; Bell et al. 2006; Roy et al. 2006) or with fish oil (AbuGhazaleh et al. 2003; Shingfield et al. 2003). Effect of monensin on milk fat response is variable with some reporting a depression (Phipps et al. 2000; Odongo et al. 2007) while others observed no change (Bell et al. 2006). Effect of monensin on milk fat depression was reported to be greater when diets contained a source of PUFA (AlZahal et al. 2008) or when the grain source was barley (Jenkins et al. 2003). Feeding supplemental fat is often accompanied by a decrease in milk protein content (Jenkins and McGuire 2006). Mean milk protein content was not influenced by the treatments. However, milk protein yield was lower for the test diet compared to the control diet.

Diets supplemented with PUFA have been reported to increase PUFA content of milk at the expense of lowering the concentrations of SFA, particularly 16:0, 14:0 and 12:0. Consistent with this, concentrations of total SFA, 16:0, 14:0 and 12:0 were significantly lower for the test diet compared to the control diet. Greater concentrations of LA, EPA and DPA for the test diet compared to the

control could be attributed to the supplementation with sunflower seeds and fish oil.

Greater concentrations of total *t*-18:1, *t*11-18:1 and *t*10-18:1 for the test diet compared to the control diet suggests that the extent of PUFA biohydrogenation was greater for the control diet than for the test diet. The conversion of *t*11- or *t*10-18:1 to 18:0 in the rumen has been reported to be inhibited by LA (Beam et al. 2000) as well as by fish oil (AbuGhazaleh and Jenkins 2004). Consistent with this, the greater concentrations of PUFA and fish oil in the test diet compared to the control could have inhibited the extent of PUFA biohydrogenation in the rumen resulting in an increase in milk *t*10- and *t*11-18:1.

A shift to *t*10-18:1 has been reported to occur for diets low in fiber and containing a source of PUFA (Griinari et al. 1998) as well as for diets containing fish oil (AbuGhazaleh et al. 2003; Shingfield et al. 2003, 2005). However, we have not come across studies that reported greater *t*10-18:1 in milk for diets supplemented with monensin alone. In the current study, the F: C ratio was 50: 50 with similar fiber content (NDF) between the control and the test diets. These diets cannot be classified as low fiber diets. However they differed in PUFA and total starch with the former being relatively higher for the test diet and the latter higher for the control diet. Although milk concentrations of *t*10-18:1 for the test diet was greater than the control diet, its concentration relative to the concentration of *t*11-18:1 was less for the test diet. But for the control diet, the concentration of *t*10-18:1 was greater relative to the concentration of *t*11-18:1 indicating a greater shift to *t*10-18:1 for the control diet. Proportions of *t*10-and *t*11-18:1 similar to that of the control and test diets in this study were also observed for barley- and corn-based diets respectively in a different study (unpublished) investigating the rate of starch degradability on *t*-18:1 isomers in milk suggesting that greater proportions of barley starch in control diet and

hence greater fermentability compared to the test diet could be one of the reasons for the increased *t*10-18:1 shift in control diet. From the above discussion, it appears that PUFA content, fish oil and increased fermentability of starch could be responsible for the shift in the biohydrogenation pathway.

Lactation average milk RA concentration for the control diet was 0.64 % FAME or 6.4 mg/g. Lactation average milk RA concentrations from grazing studies (Auldist et al. 1998) were slightly greater (8.0 mg/g) than the values recorded in the current study and could be attributed to the greater opportunities for selective feeding and to the efficiency of grazing system (Mohammed et al. 2009) compared to TMR based diets (Kelly et al. 1998). Greater concentrations of *c*9*t*11-CLA for test diet compared to the control diet were due to greater intakes of total substrate (LA + LN) for the test diet than control diet. However, it should be noted that the efficiency of milk RA production was greater for the control diet compared to the test diet ($P < 0.01$; 3.79 ± 0.25 vs 2.69 ± 0.25) indicating that control diet resulted in an environment that was more favorable for greater *t*11-18:1 production in the rumen.

Step-wise regression analysis using milk fat RA (dependent variable) and a group of independent variables (intake of LA, LNA, LA + LNA, *t*10-18:1, *t*11-18:1, ratio of *t*10:*t*11-18:1 and desaturase indices) revealed that the model was significant ($P < 0.01$; $R^2 = 0.97$) with 94% of the variability contributed by the concentrations of *t*11-18:1 and an additional variability of 1.9% and 1.0% contributed by 14:1/14:0 and *c*9*t*11-CLA/*t*11-18:1 respectively. Similar values were reported by Mohammed et al. (2009) in a grazing study. Because milk concentrations of *t*11-18:1 is derived from rumen *t*11-18:1, diets that result in greater ruminal concentrations of *t*11-18:1 would produce greater milk concentrations of CLA.

Milk fat yield followed a trend that was exactly opposite to that observed for *t*10*c*12- and *t*7*c*9-CLA isomers and therefore could be inversely related. Step-wise regression analysis with milk fat yield as the dependent variable and a group of variables (*t*10-18:1, *t*11-18:1, ratio *t*10: *t*11-18:1, *t*10*c*12-CLA, *t*7*c*9-CLA and sum of the SCFA) as independent variable revealed that the model was significant ($P < 0.01$; $R^2 = 0.54$) with 47% of the variability in fat yield contributed by the sum of the SCFA and an additional 6.6% of the variability contributed by the ratio *t*10: *t*11-18:1. Repeating the regression model with the same variables described above except the sum of the SCFA revealed that 42% of the variability in milk fat yield was related to *t*10-18:1. Although *t*10-18:1 was believed to play a role in milk fat depression (**MFD**) when Baumgard et al. (2000; 2001) demonstrated that *t*10*c*12-CLA inhibited milk fat synthesis, recent study by Lock et al. 2007 did not support *t*10-18:1 as the isomer responsible for MFD based on an infusion trial using synthetic *t*10-18:1. Kadegowda et al. (2008) based on principal component analysis of a group of studies reported that *t*10-18:1 was related to MFD only when its concentrations in milk reached a threshold level. It appears that MFD is an outcome of the interaction among several *t*-18:1 isomers, CLA isomers or both. Under these circumstances, infusion studies with pure isomers alone may not be enough to understand the complexity of the interactions. Biological infusions of mixtures of possible isomers (*t*10-18:1, *t*10*c*12-CLA, *t*7*c*9-CLA and *t*9*c*11-CLA) in different combinations could be of help in understanding the interactions among isomers responsible for MFD.

4.5 Conclusions

Diets supplemented or not with a source of PUFA (from sunflower seed and fish oil) and monensin (test vs control) were evaluated for persistency of milk RA from calving to 270 days in milk. Results revealed that milk concentrations of *t*11-18:1 and RA remained similar throughout lactation and were greater for the test diet compared to the control. *De novo* synthesized fatty

acids increased and preformed fatty acids decreased with increasing DIM. Changes in the concentrations of *t*10-18:1 in milk throughout lactation appeared to reflect the pattern of DMI indicating that the concentration of *t*10-18:1 is determined by an interaction between the degree of rumen fermentation and PUFA biohydrogenation.

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Discussion and conclusions

5.1 Introduction

Conjugated linoleic acid (*c*9*t*11-CLA or **CLA**) is a component of milk fat shown to have potential health benefits (anti-cancer, anti-obesity, anti-diabetic and immune modulatory effects) in animal models. Since the discovery of anti-cancer effects of CLA (Pariza and Hargreaves, 1985) several studies were conducted by animal nutritionists in an attempt to identify dietary regimens that enhance milk concentrations of CLA. From the literature, it can be observed that diets supplemented with a source of polyunsaturated fatty acids (**PUFA**), particularly a source of linoleic acid (**LA**) has been reported to result in as much as a 10-fold increase in milk concentrations of CLA (Bell et al., 2006; Shingfield et al., 2006). But dietary strategies that resulted in enrichment of milk CLA also caused milk fat depression (**MFD**) (Bell et al., 2006; Piperova et al., 2000; Roy et al., 2006). Since then, researchers have attempted to identify various factors that influence milk CLA and possible isomers that could be responsible for MFD.

Dhiman et al. (2005) in a review on factors influencing milk CLA concluded that among the various factors, diet plays a significant role in influencing milk CLA content. More specifically, it is the substrate content in the diet that is strongly related to milk CLA (Mohammed et al., 2009). However, very few studies have reported substrate intakes (AbuGhazaleh et al., 2003; Mohammed et al., 2009; Shingfield et al., 2003). Ingestion of LA and LN in cows is highly variable. It depends on the nature of forage (fresh or conserved), type of silage (barley silage/corn silage/maize silage/grass silage), type of fresh grass (rye grass/legume), age of the forage when harvested or ensiled, type of grain (corn, barley) amount of grain, source of lipid and amount of lipid supplemented (Dewhurst and King, 1998; Dewhurst et al., 2001, Dewhurst et al., 2002, Mohammed et al., 2009). It is possible to predict substrate intakes based on the diets offered to the cows. However, every ingredient in the ration is variable in the amount of LA and LN and is largely influenced by the age of the crop,

fertilizer regime and other environmental factors existing at the time of harvest (Dewhurst et al., 2001, 2002). Besides differences in the substrate content of the diets offered, cows eating behaviour can also determine differences in the amount of substrate consumed (Mohammed et al., 2009). Therefore a calculation of individual animal's substrate intake is important and can explain much of the animal to animal variation in milk CLA. Estimating the amount of substrate consumed and CLA precursors in the rumen, helps to gain a better understanding of the extent of PUFA biohydrogenation in the rumen and the impact of diet induced changes on rumen environment. The primary goal of the research presented in this thesis was to gain an overall understanding of the biology of milk CLA synthesis aimed at identifying some of the factors responsible for the variations in milk CLA among the experimental diets. Because a detailed separation of *trans*- and CLA-isomers is important in making correct interpretation of the findings, the significance of improved separation of the isomers of interest is also discussed.

5.2 Significance of improved separation of *trans*-18:1 and CLA isomers and certain considerations to avoid overestimation of isomer concentrations

The amount of detail required in any analysis depends on the objectives of the study. As such, researchers will choose the methods that will address their objectives. Discussion here is confined to milk fatty acid analysis. Milk fatty acid analysis can be done using a 30m, 60m or 100m column. The number of peaks that elute using these 3 three columns differ considerably. For instance, a chromatogram obtained from a 60m column will show all *t*-18:1 isomers as a single peak compared to that obtained from a 100m column which shows *t*-18:1 isomers ranging from *t*4- to *t*16-18:1. Similarly all *t*-16:1 isomers elute as a single peak when analyzed on a 60m columns compared to the range of peaks (*t*5- to *t*13-16:1) that elute on a 100m column. The details on separating the 16:1, 18:1, 20:1 and CLA isomers in milk fat were discussed in section 1.1 (Chapter 1).

We have seen that a 100 m column considerably improves the quality of separation of several monounsaturated fatty acids. However, when it comes to the point of identification of these isomers, there is no single standard available in the market that can be used to identify all the fatty acids in milk. For instance, the standard # 463 (NuChek) which is commonly used for identifying milk fatty acids, will show 52 different peaks when analyzed with the 175^{o}C temperature program. However, when a hexane extract of milk fat is analyzed on GC at the same temperature program, one can see an average of 130 different peaks. The majority of the remaining peaks in milk not present in the standard can be identified using various methods described in sections 1.3d and 1.3e (Chapter 1). Certain experiments require reporting only the major fatty acids. Under these circumstances, the analyst should still integrate all the peaks including the unknowns and use the area occupied by the unknowns in the total area for the calculation of the % fatty acid methyl esters (**FAME**) of the fatty acids of interest. Failure to do this will result in an overestimation of the % FAME of the fatty acids of interest.

To calculate the percent overestimation of FAME when unknowns are ignored, two different chromatograms from one study in which all FAME were identified with the unknowns included in the total were selected. The same chromatograms were again integrated based only on the peaks that can be identified by the 463 standard (NuChek). There were several unknowns and the total area occupied by these unknowns was not included in the total to work out the relative differences in concentrations of various FAME between the two methods. Results revealed that ignoring the unknowns overestimated all the FAME by 7 % relative to the chromatograms for which unknowns were considered in the total FAME. This was for a comparison between chromatograms analyzed on a 100 m column at 175^{o}C temperature program. Besides ignoring unknowns, reporting a peak, which is often coeluted with other

peaks, as a single peak leads to overestimation of its concentration. For example, consider the CLA peak (*c*9*t*11-CLA). It is well known that *c*9*t*11-CLA represents about 90 % of the CLA isomers found in milk. However, it should be noted that *t*7*c*9-CLA, the 2nd largest CLA isomer and *t*8*c*10-CLA coelute with *c*9*t*11-CLA. The concentrations of *t*7*c*9-CLA can be as high as 10% of the total CLA isomers depending on the diet. It is not possible to separate these isomers without a complimentary Ag^{+}-HPLC. Readers should therefore make a cautious judgment of the results from various studies based on the column length used, methods used to separate the co-eluting peaks and whether or not unknowns were considered in the total.

5.3 Substrate (LA + LN) intake

It is well demonstrated that diet strongly influences milk CLA content. This was demonstrated in the studies where a 10-fold increase in milk CLA was recorded for diets supplemented with a source of PUFA compared to the control (Bell et al., 2006; Shingfield et al., 2006). There are various sources of PUFA that can be added to the basal diet. Sunflower, safflower and soybean (seed or oil) are rich sources of LA while flax seed or oil is a rich source of α-linolenic acid (**LN**) (Kennelly et al., 1996). Fish oil or other products of marine origin (algae) form a rich source of longer-chain PUFA (20:5n-3, 22:5n-3, 22:6n-3). Fresh grass is rich in LN. Ruminal CLA formed from LA biohydrogenation contributes very little to the milk CLA. However, biohydrogenation of both LA and LN in the rumen results in the formation of *t*11-18:1 which is desaturated to CLA in the mammary tissue under the influence of Δ^{9}-desaturase. The amount of CLA formed in milk therefore depends on the amount of *t*11-18:1 supplied from the rumen to the mammary tissue and by the activity of mammary desaturase. The contribution of mammary desaturase activity to the variability in milk CLA ranged from 0 to 5% from the findings of study 1 and 2. The amount of *t*11-18:1 formed in the rumen is largely determined by the diet. From studies 1 and 2, we have seen that the

variability in milk CLA is strongly related to the amount of *t*11-18:1 in plasma which in turn is strongly related to the amount of *t*11-18:1 in the rumen. The concentrations of *t*11-18:1 in the rumen is related to the intake of PUFA. Therefore the importance of substrate intake on milk CLA is clearly evident when the sum of the intakes of LA and LN are compared among diets within an experiment or between studies. For example, a grazing cow with 18 kg DM/d (without any concentrate supplements) consumed a total substrate intake of 403 g/d (calculated from study 1) compared to a TMR fed (without additional lipid supplementation) cow with 20 kg DM/d which consumed only 186 g/d (calculated from study 3). The milk CLA yield for the grazing cow was 19g for 403 g of total substrate intake per day compared to only 6.8 g for 186 g of total substrate intake for the TMR fed cow. The efficiency of milk CLA yield (CLA yield per 100 g total substrate intake) was 4.7 for grazing compared to 3.6 for the TMR fed cows indicating that grazing cows are more efficient than the TMR fed cows. From the above discussion, we have seen that grazing cows are efficient in CLA production with LN as the major source of PUFA in the diet. Contrary to this, LN in TMR based diets does not appear to be as efficient as LA in milk CLA production. For instance, Bell et al. (2006) comparing safflower oil and flax seed oil at 6% DM reported greater concentrations of milk CLA for cows fed safflower oil than for cows fed flax seed oil. AbuGhazaleh et al. (2003) in a study comparing diets supplemented with 1% fish oil plus 2% fat source high in 18:0 or *c*9-18:1 (sunflower seed) or LA (sunflower seed) or LN (flax seed) reported greater concentrations of milk CLA for diets containing high LA followed by LN, *c*9-18:1 and 18:0. Total substrate intakes (LA + LN in g/d) for the diets high in 18:0, *c*9-18:1, LA and LN were 223, 259, 459 and 472 respectively. Compared to the cows fed high LA diet, cows offered with the high LN diet had lower milk CLA inspite of greater intakes of total substrate suggesting that LN is not as efficient as LA in milk CLA synthesis in TMR based diets. These differences in efficiency of utilization of substrate for CLA precursor formation between TMR and grazing

could be attributed to the differences in the nature of lipid (glycolipids and phospholipid in fresh forage lipid compared to triglycerides in grain lipid), possible differences in lipolysis (fresh forage lipid lipolyzed by bacterial and plant lipases compared to grain lipids lipolyzed by mainly bacterial lipases) and rumen environment (rumen bacteria, rumen pH etc).

Longer chain PUFA like eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) cannot act as substrate for the formation of *t*11-18:1 or CLA. However, their presence in the diet has been reported to inhibit the final step in the biohydrogenation of LA or LN (AbuGhazaleh and Jenkins, 2004). This was evidenced by an increase in the concentrations of *t*11-18:1 and a decrease in 18:0 following incubation of rumen contents with EPA and DHA *in vitro* (AbuGhazaleh and Jenkins, 2004). Therefore supplementation of fish oil together with a source of LA or LN results in greater ruminal production of *t*11-18:1.

5.4 Lipolysis and PUFA biohydrogenation

Lipolysis of rumen lipids was reported to be more sensitive to rumen pH than to the concentration of PUFA (Beam et al., 2000) and inhibited when rumen pH falls below 5.5 (Hobson, 1965). In study 2, we observed that the minimum daily rumen pH was as low as 5.3 for barley-based diets (**BBD**) and 5.5 for corn-based diets (**CBD**). Under these circumstances lipolysis should be completely inhibited for BBD and should not have any intermediary products of PUFA biohydrogenation because biohydrogenation of PUFA cannot proceed in the absence of a free carboxyl group. The greater ruminal concentrations of *t*11-18:1 or *t*10-18:1 for the cows offered barley based diet, does not support the theory that lipolysis was inhibited. This discrepancy in the *in vivo* observations compared to the *in vitro* findings could be because a cow's rumen is a continuous culture where there is continuous flow of ingesta and change in dilution rates unlike a batch culture. The number of daily rumen pH episodes

that were less than 5.8 ranged from 10-13 per day with the duration of each such episode reached an average maximum of 42 min/d for barley diets and 20 min/d for the corn-based diets. Considering these fluctuations in rumen pH over a day, the net effect of rumen pH depressions over the day in inhibiting lipolysis may not be substantial.

In the section on rumen lipid metabolism, we discussed that PUFA biohydrogenation is more sensitive to the amount and source of PUFA and is less likely to be inhibited by a lower pH. It was also evident that even a control diet containing no added lipid has enough PUFA to inhibit the conversion of *t*11-18:1 to 18:0. Therefore, we should expect greater *t*11-18:1 in all the experiments (study 1-3). However it can be observed from the *t*-18:1 profile of rumen (study 1 and 2) and milk (study 1-3) that there are differences in the proportions of *t*10-18:1 and *t*11-18:1 among diets with some diets favoring an increase in *t*11-18:1 while others favoring an increase in *t*10-18:1. The isomers *t*11-18:1 and *t*10-18:1 are products of two different pathways in the biohydrogenation of LA with the former representing a product of the major biohydrogenation pathway and the latter representing a product of the alternative biohydrogenation pathway. The presence of these two products reflects the activity of two different groups of LA biohydrogenating bacteria. An increase in the concentrations of *t*10-18:1 among experimental diets is described as a shift or deviation from the major LA biohydrogenation pathway (forming *t*11-18:1) to an alternative biohydrogenation pathway that results in the formation of *t*10-18:1. What is causing the *t*10-18:1 shift? Is it the source of PUFA or a lower rumen pH that is responsible for the *t*10-18:1 shift? It has been reported that *t*10-18:1 is formed in the rumen from the reduction of *t*10*c*12-CLA as an alternative way of biohydrogenation of linoleic acid (Griinari and Bauman, 1999) when low-fiber diets are fed. Greater concentrations of *t*11-18:1 in rumen and milk for grazing cows compared to grass-silage fed cows can therefore be attributed to the

source of major PUFA in the diet which is LN. A very small fraction of *t*10-18:1 was observed in both rumen and milk and it was greater for grazing cows compared to silage fed cows. Because *t*10-18:1 is not formed as part of the LN biohydrogenation pathway, it could have been part of the LA biohydrogenation and the differences in its concentrations between fresh grass and silage could be due to the differences in rumen pH between fresh grass and silage. It should be noted that the rumen pH recorded in study 1 is the mean value of two recordings per day and as such the differences in rumen pH among the treatments is not very great.

In study 2, both CBD and BBD had forage:concentrate ratio of 42:58 and hence lower fiber content. Furthermore the intake of fiber (NDF) was also similar for cows offered CBD and BBD. Therefore *t*10-18:1 should have been higher than t11-18:1 for both BBD and CBD. However it can be observed that the concentrations of *t*10-18:1 was greater than the concentrations of *t*11-18:1 for BBD and vice-versa for CBD in all the matrices analyzed. These differences in the shift to *t*10-18:1 for BBD could be explained by the greater fermentability of barley starch compared to corn starch. So a greater *t*10-18:1 shift can be expected for diets containing rapidly fermentable sugars (for instance, fresh grass, TMR with a basal diet containing barley grain or barley silage, maize silage) and supplemented with a source of PUFA containing predominantly LA.

From the above discussion, it is clear that conditions responsible for *t*10-18:1 shift are well demonstrated. However, identification of bacteria responsible for the shift to *t*10-18:1 pathway is very challenging and progress in this direction has been limited. In general, *Butyrivibrio* species are predominant on high-roughage diets, while selenomonads, peptostreptococci, streptococci and lactobacilli predominate when high-concentrate diets are fed (Dehority and Orpin, 2003). Mackie and Gilchrist (1979) reported that the percentages of

amylolytic and lactate-utilizing bacteria in sheep increased from 1.6% and 0.2% on the low concentrate (10%) diet to 21.2% and 22.3% respectively on the high concentrate (71%) diet. *Lactobacillus*, *Butyrivibrio* and *Eubacterium* were the principal genera of amylolytic bacteria with the high-concentrate diet, while *Anaerovibrio* and *Propionibacterium* were the predominant lactate utilizers. In a similar study, Roxas (1980) reported that the percentages of amylolytic and lactate utilizing bacteria were 50% and 25% during roughage feeding and 85% and 32% when feeding a high concentrate diet. The predominant amylolytic bacteria isolated when feeding a high concentrate diet were *Butyrivibrio*, *Selenomonas*, *Bacteroides* and *Streptococcus*, while *Selenomonas*, *Propionibacterium* and *Anaerovibrio* were the predominant lactate utilizers. Kepler (1966) demonstrated that *Butyrivibrio fibrisolvens* biohydrogenates LA to *t*11-18:1. More recently Maia et al. (2007) demonstrated the formation of *t*10*c*12-CLA from LA by pure cultures of *Propionibacterium acnes*, which is further reduced to *t*10-18:1 in the rumen. Based on the diet-dependent shifts in rumen bacteria discussed above, it is speculated that a *t*10-18:1 shift for high concentrate diets (particularly those containing rapidly fermentable carbohydrates) could be due to formation of greater concentrations of lactic acid which lowers the rumen pH. In response to the greater concentrations of lactic acid, the growth of *Propionibacterium*, a lactate utilizer is favoured which possibly biohydrogenates LA to *t*10-18:1 resulting in a greater *t*10-18:1 shift.

Real-time PCR techniques made it possible to verify the shifts in rumen bacterial populations with a change in proportion of dietary concentrate. Kleive et al. (2003) established the population changes of *Megasphaera elsdenii* YE34 and *Butyrivibrio fibrisolves* YE 44 in the rumen of cattle fed high grain diets. Kim et al. (2008) showed major changes in the bacterial community within steers fed fish oil based on 16S rRNA gene polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis of total eubacterial population and

Butyrivibrio group. Tajima et al. (2000) monitored the quantitative changes of up to ten different rumen bacteria using real-time PCR technique, when cows were switched from hay to grain diet. Boeckaert et al. (2008) showed that algae feeding changed the rumen fatty acid profile and these changes were correlated with changes in the bacterial community determined by cluster analysis of DGGE bands. Thus, progress in identifying more biohydrogenating bacteria and the shift with various diets is only possible with the newer molecular techniques based on total genomic DNA analysis, considering the limitations in culturing rumen bacteria.

5.5 Individual animal variation

Individual animal variation can influence the mean milk CLA considerably. This can be a problem in a commercial setting if value added milk enriched with CLA is to be produced. There was considerable variation among animals (study 1, 2 and 3) on a particular diet in the ability to produce milk CLA. This could be attributed to several factors. Even if we provide a very well mixed diet of similar chemical composition to a group of cows, no two cows would have identical intake of nutrients. This is because cows sort feeds and it depends on the particle size distribution of forages and grain (DeVries et al., 2007). This is where a researcher has limited control in making the cow eat what is offered to her. Eating behavior again depends on the constituents in the bolus. A cow would chew longer if she has more fibrous material in her bite. The amount of DM and fiber in the bite in turn determines the rate of salivary secretion (Meyer et al., 1964). This suggests that a group of cows feeding on a particular diet can have differences in the amount of saliva they secrete. Rumen pH is a reflection of the production and neutralization of fermentation acids in the rumen. Penner et al. (2009) reported that there is considerable individual variation in the rates of VFA clearance in the rumen. This together with the eating behavior and variations in the rates of salivary secretion can cause considerable variations in the rumen pH

among the cows offered the same diet. A lower rumen pH has been reported to cause *t*10-18:1 shift (Griinari and Bauman, 1999). Part of the differences could also be due to differences in the kinetics of absorption and tissue supply of the biohydrogenation intermediates.

It is well known that most of the milk CLA is derived from *t*11-18:1 under the influence of mammary Δ^9-desaturase (Griinari et al., 2000). However, diet does not seem to induce substantial differences in mammary desaturase activity. Diet induced differences in mammary desaturase index (from study 1 and 2) was responsible for only 4-5% of the variability in milk CLA. About 95% of the variability in milk CLA was determined by the differences in rumen *t*11-18:1. Therefore diets resulting in greater concentrations of rumen *t*11-18:1 and its flow to the mammary tissue appear to determine the concentrations of CLA in milk.

In study 1 (Fig 2-2), we have observed that cows were more efficient in milk CLA production when grazing compared to zero-grazing and grass silage feeding. When examined within a particular treatment, it can be observed that a cow could produce greater CLA yield with less substrate intake compared to another cow which produced less CLA yield even with greater substrate intake than the previous cow. This can be attributed to possible differences in the concentrations of PUFA biohydrogenating bacteria that they harbor. In conclusion, individual animal differences in milk CLA could be attributed to the differences in the animal's genetics, eating behavior, intake characteristics, possible differences in VFA clearance rates, desaturase activity and types and concentrations of PUFA biohydrogenating bacteria in the rumen.

5.6 Milk fat depression

Two approaches have been described in the literature for identifying the isomers responsible for MFD. One approach involves infusing the isomer under investigation into the abomasum and studying its effect on milk fat yield (biological MFD). The second approach involves feeding cows the diet under investigation and finding correlations between the concentrations of specific isomers in the rumen or milk and milk fat yield (diet-induced MFD). Both approaches have pros and cons. The advantage with the first approach is that it is biological and not mathematical unlike the second approach and the effects of the isomer under investigation can be confidently related to the observed effect on milk fat yield. The disadvantage with the biological approach is that the isomer under investigation is infused into the abomasum and hence it does not exert any changes in the rumen fermentation patterns unlike what is expected in a normal feeding study. So the observed changes in milk fat are the net effect of the isomer infused into the abomasum together with the existing rumen fermentation pattern induced by the diet at the time of infusion. With the biological approach, the specific effect of the isomer under investigation can be known but its interactions with other isomers produced as part of the normal fermentation process cannot be known. In a feeding study however, the observed changes in milk fat yield are the net effect of the original conditions in the rumen induced by the diet. Here the observed effect can be confidently related to the diet induced changes in the rumen, however it is difficult to specifically relate the changes in any specific isomer in the rumen to milk fat yield. Correlation approach relates two or more variables based on their pattern of changes. However the relationship between the variables identified by this approach need not necessarily be a cause and effect relationship. Readers therefore should make a cautious judgment of the findings based on the approach used together with their own observations from their studies.

Diet induced MFD has been described for diets low in effective fiber, high in readily fermentable carbohydrates (Piperova et al., 2000; Roy et al., 2006), and those supplemented with a source of PUFA (Griinari et al., 1998; Bell et al., 2006), fish oil (AbuGhazaleh et al., 2003) or monensin (Phipps et al., 2000; Odongo et al., 2007). In study 1, diets were different in the amount of total substrate offered and milk fat yield in this study was strongly related to the ratio *t*10:*t*11-18:1. In study 2, diets offered had similar total PUFA and total starch but differed in the fermentability of starch. Milk fat yield in this study was strongly related to total starch intake (45%). In study 3, milk fat depression was strongly related (42%) to the concentrations of *t*10-18:1 in milk. In this study, the test diet was different from the control diet in that it had fish oil (0.5%), monensin (24mg/kg DM) and additional substrate from sunflower seed. From the above findings, it could be concluded that when diets were different in total PUFA, MFD was more related to the intermediates of PUFA metabolism and when diets were similar in PUFA but different in fermentable carbohydrate, MFD was more related to the amount of fermentable carbohydrate ingested. Although milk fat depressing effects of monensin and fish oil have been reported, the supplementation of monensin or fish oil in this study could not be related to MFD because this study did not have diets exclusively supplemented with monensin or fish oil.

The effect of monensin on milk fat is variable with some studies reporting a depression (Phipps et al., 2000) while others observed no difference (Bell et al.,2006; Odongo et al., 2007). The effects of monensin on milk fat appears to be influenced by interactions between monensin and dietary factors (effective fiber and dietary NSC) as reported by Duffield et al. (2003). The effect of fish oil on milk fat also appears to be variable. Shingfield et al. (2003) reported a mild but significant depression in milk fat accompanied by an increase in milk *t*10-18:1 for cows offered diets containing 60:40 forage:concentrate and supplemented with

fish oil (1.6% DM basis) compared to the control. AbuGhazaleh et al. (2003) supplemented diets containing 50:50 F:C with 1% fish oil and 2% PUFA source containing either high 18:0, *c*9-18:1, LA or LN, and observed greater MFD for diets supplemented with LA accompanied by an increase in milk *t*10-18:1. In study 3, MFD was observed for diets containing 0.5% fish oil supplemented with sunflower seed and monensin which was also accompanied by an increase in milk *t*10-18:1. However, other studies (Shingfield et al., 2005; Cruz-Hernandez et al., 2007) observed an increase in milk *t*10-18:1 without significant reduction in milk fat yield. Studies in which diets are supplemented with monensin or fish oil alone are limited. Most studies found in the literature have some source of PUFA in combination with monensin or fish oil which makes it difficult to relate the milk fat depressing effect to either monensin or fish oil. The role of monensin or fish oil in inhibiting PUFA biohydrogenation leading to accumulation of *t*11-18:1 has been demonstrated. However, studies where monensin alone was shown to cause *t*10-18:1 shift has not been reported, while fish oil alone has been shown to increase in milk *t*10-18:1 (Shingfield et al., 2003).

It should be noted that an increase in milk CLA in a TMR based diet supplemented with a source of PUFA (particularly LA) often lowers the milk fat yield and the severity of this depends on the source and amount of PUFA together with the source and amount of fermentable carbohydrate in the diet. However, in a grazing situation, increase in milk CLA is not often accompanied by a depression in milk fat. Clearly, the above two diets differ in the major biohydrogenation pathway with the TMR based diet (supplemented with LA) undergoing predominantly LA biohydrogenation while grazing cows undergo mainly LN biohydrogenation. This indicates that the lack of MFD for grazing cows could be because formation of *t*10-18:1 is not likely to occur in LN biohydrogenation. As such, the amount of *t*10-18:1 formed in grazing cows is less compared to a TMR based diet. This supports *t*10-18:1 as the isomer mainly

responsible for MFD. However abomasal infusion of *t*10-18:1 has not been reported to induce MFD (Lock et al., 2007). The isomer *t*10*c*12-CLA has been strongly considered to be responsible for MFD based on infusion studies (Baumgard et al., 2000; Baumgard et al., 2001; Shingfield et al., 2007; Harvatine et al., 2009). However, the concentration of this isomer in milk barely exceeded 0.01% of the total FAME in study 2 and 3 in which MFD was clearly evident. It should be noted that *t*10*c*12-CLA identified based only on GC is likely to be misidentified unless a standard containing 21:0 and 20:2 FAME is used because these peaks elute in the same region where *t*10*c*12-CLA often elutes (Roach et al., 2000). However, in studies where a complimentary Ag^{+}-HPLC is used for identification of CLA isomers, *t*10*c*12-CLA is not likely to be misidentified. Therefore cautious judgement of the findings should be made based on the methods used. It should be noted that MFD was greater in studies 2 and 3 in which *t*7*c*9-CLA was also considerably higher and could be a potential isomer responsible for MFD. In both studies, *t*7*c*9-CLA was strongly related to milk fat yield when analyzed by a simple regression model. However step-wise regression analysis (study 3) revealed that milk fat yield was strongly related to *t*10-18:1. Inconsistencies in the relationship between MFD and individual *t*-18:1 or CLA isomers could be possibly because MFD may be an outcome of an interaction among several *t*-18:1 isomers or CLA isomers or both and not due to a single isomer.

5.7 Concluding remarks

The ration of a dairy cow is generally formulated to meet its maintenance and production requirements which vary with the days in milk and milk yield at that time. Therefore its energy, fiber and protein requirements keep changing with the stage of lactation and production. Besides, the dry matter content and nutrients in the forages do not remain constant throughout the year. The products of PUFA biohydrogenation formed in the rumen of a dairy cow are

determined by the net effect of the interactions between dietary carbohydrate (starch, fiber) and PUFA. Therefore the variations in milk CLA in cows within lactation could be attributed to the variations in PUFA intake, differences in dietary nutrients and their interactions on rumen PUFA biohydrogenation. A consistent production of milk CLA requires that a cow should be able to produce consistently similar amounts of *t*11-18:1 in the rumen. The key to minimize the variations in the production of *t*11-18:1 in rumen is to understand the complexity of the interactions among dietary nutrients on PUFA biohydrogenation.

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Comparison of rumen bacterial diversity with grazing, zero-grazing and grass silage feeding as determined by 16S rRNA gene sequence analysis

Introduction

Rumenic acid (**RA**) is a component of milk fat shown to have multiple health benefits in animal models. Milk fat content of RA is mainly determined by amount of dietary substrate (linoleic acid and linolenic acid content) consumed and the extent of polyunsaturated fatty acid (PUFA) biohydrogenation in the rumen (Mohammed et al., 2009). Ruminal PUFA biohydrogenation has been reported to be influenced by rumen pH (Martin and Jenkins, 2002; Kalscheur et al., 1997), forage: concentrate (F:C) ratio and dietary starch content (Griinari et al., 1998; Shingfield et al., 2005) and shifts in microbial populations. Shifts in rumen bacterial populations have been well studied for diets differing in F: C ratio (Tajima et al., 2000; Klieve et al., 2003). However, there are very few studies that reported shifts in rumen bacterial populations for diets used to increase milk RA concentrations (Paillard et al., 2007; Boeckaert et al., 2008). These studies used quantitative PCR (real-time PCR or qPCR) using specific primers to quantify the numbers of the specific bacterium under investigation. In this study, we used universal bacterial primer to amplify the variable region of the 16S rRNA gene and constructed 16S rRNA gene libraries from cows Grazing (G), Zero-grazing (ZG) or fed Grass silage (GS). It was hypothesized that there will be a shift in the rumen bacterial population among the treatments. The objective of this study was to compare the phylogenetic distribution of rumen bacteria among the treatments.

Materials and methods

Experimental design

Six spring-calving rumen cannulated pluriparous Holstein Friesian dairy cows in early lactation were randomly assigned to 3 treatments – G, ZG and GS in a 3×3 Latin square design with three 21 day periods. The experiment was carried out on a perennial ryegrass sward at Dairy Production Research Centre, Moorepark, Ireland. All the experimental animals were grazing a perennial rye

grass sward before the feeding experiment. Silage was harvested from the same perennial rye grass sward in May 2005. The nutrient composition of the experimental diets, intakes, milk yield and milk fatty acid composition can be found in Mohammed et al. (2009) (Chapter 2).

Sampling

Sampling was done in the third week (D18) of each period in the morning before feeding. Rumen samples were collected aseptically from dorsal, ventral, cranial and caudal aspects of the rumen in sterilized 250ml containers covered with air tight lids and transferred to anaerobic chamber on ice. In the anaerobic chamber the sample was mixed and squeezed through two layers of cheese cloth to separate the rumen liquid from the solids. One milliliter of rumen liquid was dispensed in 1.5 ml eppendorf tubes and centrifuged at 10000 rpm for 10min. The supernatant was discarded and the pellet obtained was resuspended in 1.4 ml of ASL buffer (Qiagen) and stored at -20C until extraction of DNA.

DNA Extraction, amplification of 16s rRNA gene and DGGE analysis

Genomic DNA was extracted from 0.25 ml of the rumen samples by the repeated bead beating + column (RBB+C) method described by Yu and Morrison (2004). DNA extracted from each animal was mixed treatment wise on an equi-molar basis to obtain 3 DNA samples representing the three treatments G, ZG and GS. Full 16S rRNA gene was amplified using the primers 27f (5'-AGATTGATCMTGGCTAGGGA-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). PCR reaction (50µL) was set up in 0.2ml tubes using a PCR Express thermal cycler (Hybaid, Teddington, United Kingdom). Each 50µl reaction buffer contained a final concentration of 20mM Tris-HCl; 50mM KCl; 1.5mM MgCl2; 0.2mM of each deoxynucleotide triphosphate; 0.4µM of each primer and 2.5U of Expand High Fidelity PCR system (Boehringer Mannheim, Germany). The number of cycles in the PCR reaction was restricted to 15. To increase the specificity of the

amplification, hot start PCR was used by withholding the DNA polymerase in the initial denaturation step. The amplification program used was: 94^{o}C for 10 minutes, 30 cycles consisting of 94^{o}C for 30s, 56^{o}C for 30s and 72^{o}C for 45s, and a final extension of 72^{o}C for 7 minutes.

Gel extraction, cloning and sequencing of PCR products

PCR products were run in a 1.5% low melting point agarose gel. The band containing the PCR product was excised from the gel and stored in 1.5ml eppendorf tubes at -20C until extraction. The band DNA was extracted using Qiaex II extraction kit as per the manufacturer's instructions. The extracted DNA was then cloned using TOPO TA cloning kit (Invitrogen). The cloning reaction was plated treatment wise and the three plates were sent to Lark Technologies Inc., Essex, United Kingdom to sequence 100 clones per treatment using M13f, M13r and 1100r (5'-GGGTTGCGCTCGTTG-3') primers.

Sequence analysis

The sequence fragments were assembled using contig express (Vector NTI Advance 10, Invitrogen). The consensus sequence obtained was compared with the Genbank Ribosomal database for similarity to the rumen 16S rRNA gene clones. Chimeric sequences were identified and removed and the remaining sequences were phylogenetically analyzed.

Results

A total of 174 clones from the three treatments were phylogenetically analyzed. The clonal distribution is tabulated in Table 5-1. The phylogenetic distribution of the clones using Libshuff was not significant indicating that there was no shift in the bacterial population among the treatments.

Table 5-1. Overall distribution of clones from 16S rRNA libraries generated from grazing, zero-grazing and grass silage fed cows

Phylum	%	Genus	Number of clones
Cyanobacteria	0.6% (1)	Prevotella	52
Proteobacteria	0.6% (2)	Fibrobacter	5
Fibrobacteres	2.8% (5)	Butyrivibrio	5
Firmicutes	45.6% (81)	Succiniclasticum	3
Bacteroidetes	48.3% (85)	Other genus	26
		Unclassified	83

Discussion and conclusions

The ratio of milk *t*10-18:1: *t*11-18:1 was significantly greater for GS compared to G and ZG with no difference between the latter two treatments indicating that there was a greater t10-18:1 shift for GS compared to G and ZG (Mohammed et al., 2009) (Table 2-9). Further milk RA (*c*9*t*11-CLA) was significantly lower for GS compared to G and ZG (Table 2-9). Based on the above findings, we anticipated that there will be a shift in the rumen bacterial population among the treatments. In the absence of a shift in rumen bacterial populations, the significant differences in milk RA among the treatments could have been due to quantitative differences in CLA producers among the treatments apart from substrate intake differences. Findings from the current study indicate that comparing rumen bacterial diversity by amplification of rumen bacterial 16S rRNA gene with a universal primer may not be sensitive enough to reveal a shift in the PUFA biohydrogenating bacteria. This suggests that a qPCR approach quantifiying specific PUFA producing bacterium can better explain the differences in milk RA.

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