Effect of Supplementation with Fish Oil or Microalgae on Milk Fatty Acid Composition and Lipogenic Gene Expression in Cows Managed in Confinement or Pasture Systems

by

Payam Vahmani

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Abstract

Modifying milk fat composition to enhance its content of valuable fatty acids (FA) is required to meet the needs of a society which is becoming better informed about the relationship between diet and health. Manipulating the cow's diet is an effective, natural way to modify the amount and composition of milk fat of cows. The two main factors that affect the cow's diet concern management system (MS; pasture vs. confinement), and supplementation of diets with lipid supplements. Marine oils specifically are fed to enhance milk with n-3 long-chain polyunsaturated FA (n-3 LC-PUFA). The effects of source of marine lipid supplement (LS; fish oil vs. microalgae) in the cow's diet and its interaction with MS on milk fat composition have not been studied. Thus, the main objective was to determine the interaction of MS and LS on milk FA profile and on expression of lipogenic genes in mammary, adipose and liver of lactating dairy cows. Compared with cows in confinement, grazing cows produced milk fat with lower content of unfavorable FA (12:0-16:0), while increasing the levels of beneficial FA including *cis*-9 18:1, 18:3 n-3 and conjugated 18:2. Feeding either fish oil or microalgae improved levels of n-3 LC-PUFA and reduced those of 16:0 in milk fat regardless of MS, but concurrently increased the level of other *trans* 18:1 isomers at the expense of *trans*-11 18:1. The reduced secretion of 12:0-16:0 in milk from grazing compared with confined cows was associated with lower mammary expression of lipogenic genes suggesting that part of the effect of MS on milk FA profile is mediated transcriptionally. The effect of LS on lipogenic gene expression was tissue specific with the greatest response to treatment observed in liver despite its minor role in lipogenesis in cattle relative to the mammary and adipose. Major conclusions were that milk produced in pasture systems has a more healthful FA profile than that of confinement systems, and that MS and LS have tissue specific effects on lipogenic gene expression in dairy cattle which have important effects on cow performance and healthfulness of the milk FA profile.

List of Abbreviations Used

ACACA	acetyl-CoA carboxylase
ACTB	β-Actin
AGPAT	acyl glycerol phosphate acyl transferase
ALA	α-linolenic acid
BCS	body condition score
BHBA	beta-hydroxybutyrate
BUN	blood urea nitrogen
BW	body weight
cDNA	complementary DNA
CLA	conjugated linoleic acid
CO	control
CON	confinement
CVD	cardiovascular disease
DGAT	diacyl glycerol acyl transferase
DHA	docosahexaenoic acid
DIM	days in milk
DM	dry matter
DMI	dry matter intake
EBAL	net energy balance
EIF3K	eukaryotic translation initiation factor 3, subunit K
EPA	eicosapentaenoic acid
FA	fatty acid
FADS	Fatty acid desaturase
FAME	fatty acid methyl esters
FASN	fatty acid synthetase
FCM	fat corrected milk
FFA	free fatty acid
FID	flame ionization detector
FO	fish oil

GC	gas chromatography
glycerol~P	glycerol-3-phosphate
GPAT	glycerol-3 phosphate acyl transferase
ICG	internal control gene
INSIG	insulin-induced gene
LA	linoleic acid
LPL	lipoprotein lipase
LS	lipid supplement
LXR	liver X- activated receptor
MA	microalgae
MCSFA	medium-chain saturated fatty acids
MFD	milk fat depression
mRNA	messenger RNA
MRPL39	mitochondrial ribosomal protein L39
MS	management system
MUFA	monounsaturated fatty acids
n-3 LC-PUFA	n-3 long-chain PUFA
NEFA	non-esterified fatty acids
NE_L	net energy for lactation
NSC	non-structural carbohydrates
OA	oleic acid
OBCFA	odd- and branched-chain fatty acids
PA	palmitic acid
PAS	pasture
PHVO	partially hydrogenated vegetable oils
PPAR	peroxisome proliferator activated receptor
PPIA	peptidylprolyl isomerase A
PUFA	polyunsaturated fatty acids
qRT-PCR	quantitative real-time PCR
RA	rumenic acid
RBH	ruminal biohydrogenation

RPS9	ribosomal protein S9
SA	stearic acid
SCAP	SREBP chaperone
SCD	stearoyl CoA *desaturase*
SCSFA	short-chain saturated fatty acids
SFA	saturated fatty acids
SFA:UFA	saturated to unsaturated fatty acids ratio
SREBP	sterol response element binding protein
SUBQ	subcutaneous adipose
TAG	triacylglyceride
THRSP	thyroid hormone responsive spot14
TMR	total mixed ration
UFA	unsaturated fatty acids
UXT	ubiquitously expressed transcript
VA	vaccenic acid
VFA	volatile fatty acids
VLDL	very low-density lipoproteins

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Chapter 1 Introduction

Large portions of this introduction are published as:

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The evolving discovery of beneficial compounds in food and increasing consumer awareness of the health implications of dietary choices have created a demand for foods containing beneficial components including nutrients that may not have been considered essential in the human diet in the traditional sense. Concurrently, detrimental compounds in foods are being identified. Opportunities to improve the healthfulness of animal food products require research. Animal derived foods may be enriched naturally via the diet of livestock, and animal nutritionists have focused research attention over the last decades on manipulating the diet of animals to cause deposition of beneficial components in the foods (i.e. egg, meat and milk) we derive from them. Interest in modifying the fat content and fatty acid (FA) composition of animal products to improve their healthfulness has been increasing over the last three decades.

Nutrition is known to affect the fat content of bovine milk, while the FA composition of milk has been largely ignored by the industry. Generally the focus of modification of fat composition of milk has been to increase the content of one or more polyunsaturated FA (PUFA). However, ruminal biohydrogenation (RBH) reduces the concentration of PUFA in milk. As a result of RBH, the profile of FA leaving the rumen is very different from that of ingested FA. While linoleic acid (LA; 18:2 n-6) and α-linolenic acid (ALA; 18:3 n-3) are naturally the predominant FA in ruminant diets, stearic acid (SA; 18:0) is the main FA leaving the rumen for absorption in the small intestine. Numerous RBH intermediates (e.g. conjugated 18:2 and *trans* 18:1 isomers) escape complete RBH (Shingfield et al., 2010) and are assimilated by the animal causing a high degree of complexity of milk fat, with more than 400 FA present (Jensen, 2002).

Since the RBH of unprotected unsaturated FA (UFA) exceeds 80% (Doreau and Chilliard, 1997; Jenkins and Bridges, 2007), methods have been developed over the past several decades to protect dietary UFA against RBH and improve their transfer to ruminant food products. The approaches used for ruminal protection of supplementary UFA include encapsulation inside a microbial resistant shell (enrobing with formaldehyde treated protein or saturated lipids), transformation of FA to their salt derivatives (e.g. calcium salts or amides), or feeding whole oilseeds. However, for the majority of dietary UFA, none of these approaches provides complete protection from RBH (Jenkins and Bridges, 2007). Concurrently, attempts have been made to increase ruminal outflow and enrich product content of RBH intermediates, particularly conjugated linoleic acid (CLA) by supplementing unprotected PUFA. The potential to enrich milk with these FA is much greater than that of dietary PUFA (Lock and Bauman, 2004; Chilliard et al., 2007).

Table 1.1 is a compilation of successful dietary manipulation techniques used to modify milk FA composition. Two primary factors that influence milk fat yield and its composition are the cow's management system (pasture or confinement) and the supplementation of diets with oils. Marine oils are fed specifically to enhance milk fat content of n-3 long-chain PUFA (n-3 LC-PUFA), particularly eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3). The effects of source of marine oil (fish or algae) in the cow's diet and its interaction with management system on milk fat have not been studied.

Feeding oil supplements to dairy cows can also reduce the milk fat content of unfavorable saturated FA (SFA) including 12:0, 14:0 and 16:0 (Table 1.1). This effect

has been attributed mainly to down-regulation of mammary lipogenic gene expression by specific RBH intermediates such as *trans*-10, *cis*-12 18:2 (Harvatine et al., 2009; Shingfield et al., 2010). Previous studies (Chilliard et al., 2007; Vahmani et al., 2013) showed that compared to cows fed conserved forages and more grain in confinement, grazing cows produce milk with a higher fat content of beneficial UFA and lower fat content of unfavorable SFA. However, the mechanism of a dairy management effect (e.g. mammary transcriptional regulation) has not yet been studied. Furthermore, it has been established in rodents that PUFA supplementation down-regulates lipogenic gene expression in liver and adipose tissue resulting in decreased lipogenesis in these tissues (Jump, 2002; 1314, Wang and Jones, 2004). However, the effects of PUFA supplementation on lipogenic gene expression in extra-mammary tissues (e.g. liver and adipose) in lactating dairy cattle are scarce. Therefore, the goal of this research was to examine the interaction of dairy farm management system (MS; pasture vs. confinement) and source of marine lipid supplement (LS; fish oil vs. microalgae) on milk FA composition and on expression of genes involved in lipogenesis in mammary, liver and adipose tissue in lactating dairy cows. The current chapter begins with a mechanistic overview of ruminal lipid metabolism, milk fat synthesis and milk fat depression in ruminants, and includes on the current state of knowledge regarding impact of bovine milk FA on human health. Background information on dairy system impacts on milk FA, and the effects of supplemental marine oils on ruminal and mammary responses in dairy cows is also provided. Experiments described in Chapters 2 and 3 detail the effects of MS, LS and their interaction on cow performance, energy balance, milk FA composition

and lipogenic gene expression in dairy cows. The final chapter (Chapter 4) is a general discussion of the major findings of this research, and conclusion.

1.1 Lipid Metabolism in the Rumen

Ruminant diets naturally contain 1-4 % lipid (dry matter; DM basis) (Van Soest, 1994), the source of which is mainly forage and grain. The FA in forages are contained primarily in phospholipids and glycolipids, whereas triacylglyceride (TAG) is the major form of lipid in grains and oil seeds. Generally, a forage-based diet provides ALA as the predominant FA, while in a total mixed ration (TMR) containing a grain-based concentrate, LA predominates.

Following ingestion, dietary lipids are exposed to lipolysis and RBH. During lipolysis, the phospholipids, glycolipids and TAG are hydrolyzed by the lipases of lipolytic bacteria (eg. *Butyrivibrio fibrisolvens and Anaerovibrio lipolytica*) yielding free FA (FFA) in the rumen (Harfoot and Hazlewood, 1988). These FFA are mainly adsorbed onto feed particles and are either directly incorporated into microbial lipids or biohydrogenated. A free carboxyl group on FFA is required for RBH to proceed; therefore, lipolysis is an essential step of ruminal UFA metabolism.

Unsaturated FA are toxic to rumen microorganisms such that RBH is considered to be a detoxification mechanism (Kemp and Lander, 1984). The RBH process involves several steps performed by bacterial *isomerases* and *reductases*. The *isomerases* change the configuration of double bonds in the FA from *cis* to *trans*, and the *reductases* hydrogenate the double bonds, reducing the level of unsaturation of FA (Harfoot and Hazlewood, 1988). Reduction is the rate-limiting step in RBH, whereas the isomerization step is rapid (Harfoot and Hazlewood, 1988).

The major RBH pathways of LA and ALA are well elucidated (Figure 1.1; Harfoot and Hazlewood, 1988; Griinari and Bauman, 1999; Jenkins et al., 2008). During the first step of the RBH of LA and ALA, the *cis*-12 double bond is isomerized to *trans*-11 double bond to form FA with conjugated *cis*/*trans* double bonds (Figure 1.1). These conjugated intermediates are then sequentially reduced to *trans*-11 18:1 (vaccenic acid; VA) which is the predominant RBH intermediate found in the rumen. The final RBH step where VA is reduced to SA is rate limiting (Kemp and Lander, 1984) which allows for the accumulation and subsequent escape of VA from the rumen to the duodenum. On average, the extent of RBH of LA and ALA in the rumen is reported to be 85 and 93% respectively (Doreau and Ferlay, 1994).

Recent *in vitro* and *in vivo* studies (Chilliard et al., 2007 and Jenkins et al., 2008) suggest that besides the major RBH pathways of LA and ALA described above, numerous minor RBH pathways exist in the rumen, resulting in the formation of a wide range of RBH intermediates (Figure 1.2). Although most of these RBH intermediates are likely biohydrogenated to SA, some escape depending on feeding management, and these are absorbed in the small intestine, and incorporated into ruminant meat and milk.

Besides LA and ALA, oleic acid (OA; *cis*-9 18:1) is also found in ruminant diets, coming mainly from oil seeds (e.g. canola and sunflower). However, less information is available about the fate of OA during RBH. Harfoot and Hazlewood (1988) suggested that OA is biohydrogenated directly to SA without the formation of any RBH intermediates. In contrast, recent *in vitro* studies (Figure 1.3; Shingfield et al., 2010) reported that the RBH of OA involves the formation of several *trans* 18:1 isomers

including *trans* 6- *trans* 16 18:1. The extent of RBH of OA is lower than that of LA and ALA, ranging between 58 and 87% (Shingfield et al., 2010).

1.2 Milk Fat Synthesis in Dairy Cows

The fat content of bovine milk typically ranges from 30 to 50 g/kg depending on breed of cow, its stage of lactation and feeding management (Shingfield et al., 2010). Bovine milk fat is mostly composed of TAG (97- 98% of total milk lipids), with the remaining lipid split among: cholesterol (approximately 0.42%), 1,2-diacylglycerides (0.28-0.59 %), phospholipids (0.2-1.0 %), and trace amounts of FFA, monoacylglycerides and cholesterol esters (Jensen, 2002).

Milk fat typically has a high content of SFA (60-70%) followed by monounsaturated FA (MUFA; 25-30%), and has a low content (~ 5%) of PUFA, especially n-3 LC-PUFA (Dewhurst et al., 2005). The milk FA are either synthesized *de novo* in the mammary gland or are taken-up by the mammary as pre-formed FA from blood (Figure 1.4). *De novo* synthesized FA include short (4:0-8:0) and medium (10:0-14:0) chain SFA as well as about 50 % of 16:0 (palmitic acid; PA) (Shingfield et al., 2010). The remaining milk FA (C16-C24) are extracted mainly from circulating very low-density lipoproteins (VLDL) and chylomicra, as well as non-esterified FA (NEFA). These preformed FA originate from the diet and from adipose tissue. The latter usually accounts for 4-8% of milk FA, but its proportion increases during early lactation when cows are in negative energy balance and blood levels of NEFA are relatively high (Bauman and Griinari, 2001).

In ruminants, acetyl CoA and butyryl CoA, formed from the acetate and beta-hydroxybutyrate (BHBA) originating from the ruminal fermentation of carbohydrates, are

the predominant precursors for *de novo* FA synthesis, which occurs mainly in the cytosol of lactating mammary epithelial cells and in the adipocytes of late lactation or non-lactating dairy cows (Moore and Christie, 1979). The *de novo* FA synthesis pathway is controlled by the activity and abundance of two key enzymes, acetyl-CoA carboxylase (ACACA) and fatty acid synthase (FASN), as well as the availability of NADPH. The ACACA catalyzes the synthesis of malonyl-CoA from acetyl CoA, which is considered to be the rate limiting step in *de novo* FA synthesis. The FASN enzyme catalyzes elongation of either a primary malonyl-CoA, or butyryl CoA by the stepwise addition of malonyl-CoA units up to C16 (Barber et al., 1997). The required reducing equivalent is generated in the pentose phosphate pathway as well as by isocitrate dehydrogenase. The contribution of BHBA to *de novo* FA synthesis is limited mostly to the initial four carbon unit, accounting for about 8 % the total milk FA carbons (Palmquist et al., 1969).

Besides *de novo* synthesis, another source of milk FA is circulating TAG contained in lipoproteins which are taken up by the action of mammary lipoprotein lipase (LPL) bound to the capillary endothelial surface (Figure 1.4; Moore and Christie, 1979). The LPL hydrolyzes TAG at the core of the chylomicra and VLDL and releases FA which are transported into the mammary epithelial cell by the action of FA transport proteins (Shingfield et al., 2010).

Due to RBH, the majority of FA absorbed by the mammary secretory cell are saturated. The *de novo* synthesized FA are entirely saturated as well. However, the mammary stearoyl CoA *desaturase* (SCD), also known as Δ-9 *desaturase*, can desaturate the saturated FA (10:0-19:0) to their respective *cis* 9 MUFA. Activity of mammary SCD accounts for approximately 90, 50 and 60% of *cis*-9 14:1, *cis*-9 16:1 and OA respectively

(Mosley and McGuire, 2007), as well as 70–95% of the RA (Griinari et al., 2000; Corl et al., 2001).

The pool of FA resulting from *de novo* synthesis, uptake from blood and Δ-9 *desaturase* activity are then esterified to glycerol-3-phosphate (glycerol~P) in the endoplasmic reticulum (Figure 1.4). About 70% of the glycerol~P originates from the catabolism of glucose in the glycolysis pathway. The remainder is derived during the hydrolysis of TAG (Luick and Kleiber, 1961). In the glycerol~P pathway, the positions sn1 to sn3 of glycerol~P are acylated sequentially via the actions of glycerol-3 phosphate acyl transferase (GPAT), acyl glycerol phosphate acyl transferase (AGPAT), and diacyl glycerol acyl transferase (DGAT), respectively (Moore and Christie, 1979). The positioning of FA on the glycerol backbone is not random. Fatty acids with a relatively low melting point including short-chain SFA (SCSFA; 4:0-8:0) and 18:1 are preferentially esterified at sn-3. Medium-chain SFA (MCSFA; 10:0-14:0) are preferentially esterified at sn-2. Palmitic acid is esterified equally at the sn-1 and sn-2 positions, whereas SA is selectively esterified at the sn-1 position (Jensen, 2002).

1.3 Effects of Milk Fatty Acids on Human Health

Milk and dairy products are major dietary sources of SFA in developed countries (Kliem and Givens, 2011). During the last several decades, association of SFA with cardiovascular disease (CVD) has caused reduced consumption of milk fat in these countries (Lubary et al., 2011). The SFA in bovine milk range from 4 to 24 carbons in length with the major ones (% of milk FA) listed in order of descending concentration consisting of: 16:0 (22-35%), 18:0 (9-14%), 14:0 (8-14%), 12:0 (2-5%), 4:0 (2-5%), 10:0 (2-4%), 6:0 (1-5%) and 8:0 (1-3%) (Jensen, 2002). Three SFA in particular (12:0, 14:0,

16:0) are hypercholesterolemic and increase the risk of CVD (Williams, 2000); however, despite the presence of these FA in milk fat, there is a lack of epidemiological evidence that consuming milk fat increases the risk of CVD (Kliem and Givens, 2011; Givens, 2012). In fact, a number of epidemiological studies suggest that increased consumption of milk and dairy products may reduce the risk of CVD (Kliem and Givens, 2011).

The short chain SFA (4:0-10:0) and SA do not raise blood cholesterol (Williams, 2000). In fact, some of these FA have health benefits. Dairy products are the major dietary source of 4:0 (butyric acid), which has been shown to have anti-carcinogenic properties in animal models and cancer cell lines (Shingfield et al., 2008). Caprylic acid (8:0) and capric acid (10:0) provide a quick energy because of their rapid absorption and consequently are a valuable energy source in clinical nutrition (Gibson, 2011).

Feeding strategies that reduce the milk fat content of 12:0-16:0 could reduce the potential adverse effects of milk fat consumption on vascular health (Shingfield et al., 2008; Givens, 2012). Indeed, consumption of dairy products in which 12:0-16:0 were partially replaced by healthful MUFA and PUFA via changes in feeding management of cows lowered total and LDL-cholesterol in humans (Noakes et al., 1996). The feeding of PA rich fat supplements however, to increase energy intake and milk fat content has become a common practice in today's dairy industry (Lock et al., 2011). Unfortunately, the potential detrimental effects of this practice on the healthfulness of milk fat by increasing milk content of PA have been over looked.

Oleic acid is the dominant UFA in milk and the second major FA (after PA) constituting 20-30 % of total milk FA (Jensen, 2002). Milk and dairy products are considered major dietary sources of this FA (Kliem and Givens, 2011) which has been

shown to improve vascular health. Increased dietary intake of OA at the expense of SFA can reduce the risk of CVD by lowering blood cholesterol (Williams, 2000), and consumption of milk and dairy products with higher content of OA can improve vascular health (Noakes et al., 1996).

After OA, the group of the *trans* 18:1 FA is the second major source of milk MUFA, ranging from 0.88 to 7.65 % of total milk FA (Dewhurst et al., 2006) depending on diet and feeding practices. The *trans* 18:1 isomers in ruminant fat include *trans*-4 18:1 to *trans*-16 18:1 with VA being the dominant isomer (60-80% of the total *trans* 18:1) (Shingfield et al., 2008). Levels of *trans*-9 and *trans*-10 18:1 are minor in milk, but are the major *trans* FA in partially hydrogenated vegetable oils (PHVO) and are considered detrimental to human health (Shingfield et al., 2008). Vaccenic acid is typically the major source of RA in animal tissues via Δ-9 *desaturase*, and thus could be considered to have anti-carcinogenic properties (Corl et al., 2003; Lock et al., 2004). Furthermore, VA has been shown to have anti-atherogenic properties in mice (Bassett et al., 2010). A number of studies have also shown that the consumption of butter naturally enriched with VA and RA improved blood lipid profile and reduced CVD risk in non- ruminant animal models compared to standard butter (reviewed by Shingfield et al., 2008). However, since these effects were not as consistent in humans, more studies are needed to confirm the effects of VA and RA along with reduced levels of SFA on human cardiovascular health.

The content of PUFA in milk fat is very low due primarily to extensive RBH. The main PUFA in bovine milk fat are LA (1-3%) and ALA (0.5-2%) (Jensen, 2002). Milk fat is a unique source of CLA in the human diet contributing about 75% of CLA intake (Lock and Bauman, 2004). The content of CLA in bovine milk ranges from 0.34 to 1.07

% of total fat depending on feeding management (Dhiman et al., 2005). To date, about 20 CLA isomers have been identified in milk fat of which RA constitutes 66-89% (Shingfield et al., 2008). During the last two decades, several health benefits have been attributed to CLA including reductions in the rates of cancer, obesity, atherogenesis, and osteoporosis (Park, 2009). However, the majority of these health claims originate from *in vitro* and animal model studies where high doses of synthetic CLA used may not simulate the typical human intake from milk and dairy products (McCrorie et al., 2011; Dilzer and Park, 2012). The intake of total CLA from natural sources in the US (ruminant milk and meat) is estimated to be 151-212 mg/day (McCrorie et al., 2011). However, an intake of 3 g/day of CLA may be needed for an equivalent anti-carcinogenic effect in human adults (Ip et al., 1994). As well as differences related to amount of CLA used, synthetic preparations of CLA used in animal model studies typically contain almost equal amounts of the two predominant isomers, RA and *trans*-10, *cis*-12 CLA, whereas the concentration of the latter is very low in milk fat (0.01-1.61% of total CLA isomers) (Shingfield et al., 2008). The biological activities reported for CLA are based on the combined effect of these two isomers (McCrorie et al., 2011; Dilzer and Park, 2012). There is limited information on the specific effects of individual CLA isomers including RA and *trans*-10, *cis*-12 CLA which seem to have equivalent anti-carcinogenic properties (Park, 2009). The *trans*-10, *cis*-12 CLA has potent anti-lipogenic properties in rodents (Park, 2009) and causes milk fat depression in dairy cows (Shingfield et al., 2010), whereas RA is more effective in improving the blood lipid profile in animal models and possibly humans (Shingfield et al., 2008). More research on isomer specific health effects of CLA is needed.

The well-known multiple health benefits of EPA and DHA (Ruxton, 2004), have resulted in successful attempts to increase the concentrations of these FA in milk which is naturally a poor source of EPA and DHA (<0.1% of total FA; Lock and Bauman, 2004). Feeding marine oil (fish oil or algae) is the only way to enrich milk with EPA and DHA since feed sources of these FA are typically absent in cattle diets. Fresh forages and oil seeds provide some ALA, the precursor to EPA and DHA but efficiency of conversion of ALA to EPA and DHA in cattle is very low. Feeding linseed oil, a rich source of ALA, to dairy cows did not increase the plasma or milk concentrations of EPA or DHA, despite an increased concentration of ALA in milk and plasma (Loor et al., 2005b; Ponter et al., 2006).

1.4 Milk Fat Depression

Milk fat depression (MFD), or low-fat milk syndrome is a phenomenon in which milk fat yield is reduced by up to 50%, with no change in milk yield or in the yields of protein or lactose (Bauman and Griinari, 2001). Diet induced MFD is typically observed in cows fed diets containing high levels of readily fermentable non-structural carbohydrates (NSC) in high grain/low-fiber diets, or when plant or marine lipid supplements are fed (Bauman and Griinari, 2001). As much as a 43% reduction in milk fat percentage and yield has been reported in cows fed a milk fat-depressing diet containing 25:70 % forage/concentrate, supplemented with 5% soybean oil (Piperova et al., 2000). The occurrence of diet induced MFD is widespread in the dairy industry. Diet induced MFD has been the subject of numerous studies over the past century and several theories have been proposed to explain this phenomenon. The most recent, the "biohydrogenation theory" proposes that pathways of RBH are altered under certain

dietary conditions to produce unique FA that are potent inhibitors of milk fat synthesis (Figure 1.5; Bauman and Griinari, 2001). Several *cis* and *trans*-18:1 isomers and CLA isomers have been tested in post-ruminal infusion studies to identify the specific RBH intermediates causing MFD. Among these, *trans*-10, *cis*-12 CLA has been identified as a potent inhibitor of milk fat synthesis (Baumgard et al., 2000; Baumgard et al., 2001; Loor and Herbein, 2003; Perfield et al., 2004; de Veth et al., 2004; Perfield et al., 2006). Furthermore, several studies have found strong correlations between diet-induced MFD and levels of *trans*-10 18:1 in milk (Griinari et al., 1998; Piperova et al., 2000; Loor et al., 2005a; Kadegowda et al., 2008). Griinari and Bauman (1999) proposed a putative RBH pathway wherein *trans*-10, *cis*-12 CLA is formed from the initial isomerization of LA and is subsequently reduced to *trans*-10 18:1 prior to full hydrogenation to SA. High-grain/low-forage diets are known to result in a shift toward the microbial populations in the rumen that use this alternate RBH pathway resulting in the formation of *trans*-10, *cis*-12 CLA and *trans*-10 18:1 at the expense of RA and VA respectively (Bauman and Griinari, 2001; Loor et al., 2005a; Shingfield and Griinari, 2007).

Several additional RBH intermediates besides those already mentioned may be involved in the etiology of diet induced MFD, based on the negative correlation between their levels in milk fat and milk fat yield. Some of these include *trans*-6, *trans*-7, and *trans*-8 18:1 (Bradford and Allen, 2004; Kadegowda et al., 2008), *trans* 9-, and *trans*-11 18:1 (Bradford and Allen, 2004), *trans*-9, *cis*-11 CLA (Roy et al., 2006; Shingfield et al., 2005; Shingfield et al., 2006) *trans*-7, *cis*-9 CLA (Piperova et al., 2002; Kadegowda et al., 2008), *cis*-9, *trans*-13 18:2, *cis*-9, *trans*-12 18:2, *trans*-11, *cis*-13 CLA, *trans*-11, *cis*-15 18:2 (Loor et al., 2005a). However, among these isomers, only *cis*-10 *trans*-12 CLA

(Saebo et al., 2005) and *trans*-9, *cis*-11 CLA (Perfield et al., 2007) have been tested in post-ruminal infusion studies. Collectively these results suggest of the likelihood that there must be other RBH intermediates besides *trans*-10, *cis*-12 CLA and *trans*-10 18:1 that are involved in diet induced MFD. There is also a possibility that synergism may exist among isomers to induce MFD (Lock et al., 2007).

1.5 Molecular Mechanisms Involved in Milk Fat Depression

A number of studies have reported that reductions in milk fat synthesis in cows fed milk fat depressing diets (Ahnadi et al., 2002; Piperova et al., 2000; Peterson et al., 2003; Loor et al., 2005d) or infused with *trans*-10, *cis*-12 CLA (Baumgard et al., 2000; Baumgard et al., 2002) were accompanied with decreased gene expression of mammary lipogenic enzymes, particularly ACACA, FASN and SCD. Similar results were reported in rats (Ringesis et al., 2004) and mice (Lin et al., 2004) fed *trans*-10, *cis*-12 CLA, or when the bovine mammary cell line MAC-T was incubated with this CLA isomer (Peterson et al., 2004). More recently, research on the etiology of MFD has focused on describing the molecular mechanism(s) by which RBH intermediates regulate lipogenic gene expression in bovine mammary gland. The mammary lipogenic genes are likely regulated through a common pathway, which is mainly mediated by sterol response element binding protein1 (SREBP1; also called sterol response element binding factor1, SREBF1) (Figure 1.5; Peterson et al., 2003; Peterson et al., 2004; Harvatine and Bauman, 2006). Peterson et al. (2004) reported *trans*-10, *cis*-12 CLA decreased abundance of the active form of SREBP1 in MAC-T cells, which coincided with decreased mRNA levels for ACACA, FASN and SCD. Harvatine and Bauman (2006) measured the mammary gene expression of cows fed a milk fat depressing diet or infused intravenously with

trans-10, *cis*-12 CLA. Both treatments decreased abundance of SREBP1 mRNA, consistent with decreased expression of mammary lipogenic genes indicating that SREBP1 is a central regulator of milk fat synthesis in lactating dairy cows.

Sterol response element binding proteins are a family of transcription factors that are considered master regulators of lipid synthesis (Eberle et al., 2004) and exist in three isoforms: SREBP1a, SREBP1c, and SREBP2. Both SREBP1a and c are encoded by the same gene, but differ in their first exons due to different transcription start sites (Osborne, 2000). A different gene encodes for SREBP2. Both SREBP1a and SREBP1c stimulate the transcription of lipogenic genes primarily, whereas SREBP2 is mainly involved in the transcriptional activation of genes coding for cholesterogenic enzymes (Espenshade and Hughes, 2007). While SREBP1a and SREBP2 are expressed ubiquitously at low levels in all tissues, SREBP1c is predominantly expressed in lipogenic tissues (Shimomura et al., 1997).

This family of DNA-binding proteins is inactive as synthesized and is activated after forming a complex with SREBP cleavage activating protein (SCAP; also called SREBP chaperone). The SREBP-SCAP complex is retained in the endoplasmic reticulum (ER) membrane by binding to a third protein called insulin-induced gene (INSIG; Eberle et al., 2004; Goldstein et al., 2006). Activation of SREBP is initiated by the cleavage of INSIG from the SREBP-SCAP complex. The complex is transported to the Golgi apparatus via COPII vesicles. In the Golgi the SREBP nuclear fragment (nSREBP), also called mature SREBP, is released from the complex by the sequential action of Site-1 (S1P) and Site-2 (S2P) proteases. The nSREBP can enter the nucleus, where it activates

the transcription of target genes by binding to sterol-regulatory elements (SRE) in gene promoters (Eberle et al., 2004; Goldstein et al., 2006).

Polyunsaturated fatty acids are known to inhibit SREBP-1 activity through several mechanisms (Jump, 2002). Lee et al. (2008) showed that PUFA promote the retention of immature SREBP1 in the ER membrane by inhibiting the degradation of INSIG1. Polyunsaturated fatty acids can also reduce the mRNA abundance of SREBP1 by both transcriptional and post-transcriptional mechanisms. Xu et al. (2001) reported increased decay of SREBP1 mRNA, a post-transcriptional mechanism, in rat hepatocytes (the main site of lipogenesis in rats) treated with 20:4 n-6 or 20:5 n-3. Transcriptional inhibition of SREBP1 by PUFA is mediated mainly by another transcription factor known as liver X-activated receptor (LXR). Polyunsaturated fatty acids are known to inhibit LXR activation, resulting in reduced transcription of the SREBP1 gene (Horton et al., 2002).

Recently, an important role has been proposed for peroxisome proliferator activated receptor-γ (PPARγ) in the transcriptional regulation of genes involved in milk fat synthesis in lactating dairy cows (Bionaz and Loor, 2008). Peroxisome proliferator activated receptors are members of the nuclear hormone receptor family which are ligand-modulated transcription factors. Activating ligands of PPARs include non-esterified PUFA and related metabolites such as eicosanoids (Desvergne and Wahli, 1999; Berger and Moller, 2002). Besides PPARγ, there are two other isoforms of PPAR (α and β/δ) which are encoded by separate genes. PPARα is predominantly expressed in tissues with high FA oxidation activity (e.g. liver, muscle, heart). Ligand binding of PPARα stimulates the transcription of genes involved in FA oxidation. The function of PPAR β/δ is similar to that of PPARα; however, in contrast to PPARα, it is expressed

ubiquitously in all tissues. PPARγ levels are highest in lipogenic tissues such as adipose and lactating mammary gland. In contrast to PPARα, PPARγ is involved in adipocyte differentiation and lipid synthesis/accumulation. Bionaz and Loor (2008) reported an up-regulation of PPARγ mRNA by more than 3-fold with the onset of lactation in dairy cows, which paralleled a rise in genes expressed in milk fat synthesis. Supporting evidence of the role of PPARγ in regulating bovine milk fat synthesis includes the work of Kadegowda et al. (2009) which showed that treatment of MAC-T cells with rosiglitazone (a PPARγ agonist), resulted in the mRNA up-regulation of ACACA, FASN, SREBP1 and INSIG1. The role of PPARγ in diet induced MFD has not been established. The mammary expression of PPARγ gene was not altered by *trans*-10, *cis*-12 CLA- or diet-induced MFD (Harvatine et al., 2009), or when MAC-T cells were treated with *trans*-10, *cis*-12 CLA (Kadegowda et al., 2009).

Additional transcription factors and other regulatory proteins may be involved in the regulation of milk fat synthesis. A role has been suggested for thyroid hormone responsive spot 14 (THRSP) in the regulation of mammary lipogenesis (Harvatine and Bauman, 2006). Although its exact biochemical function is unclear, THRSP is a nuclear protein assumed to act as a transcriptional co-activator in the regulation of lipogenesis in mammary and adipose tissue. Knockdown of this gene in mice resulted in a significant reduction in *de novo* FA synthesis in the lactating mammary gland (Zhu et al., 2005). Recently, Harvatine and Bauman (2006) reported a reduction in the expression of THRSP in the mammary gland of cows fed milk fat depressing diets or infused intravenously with *trans*-10, *cis*-12 CLA, supporting a potential role for THRSP in the molecular mechanisms behind MFD.

1.6 Dairy System Impacts on Milk Fat Composition Related to Human Health

Due to differences in feeding management, the FA composition of milk fat varies among dairy production systems which range from total confinement, in which a TMR is fed, to those that are pasture-based. The predominant system of confined feeding has developed rapidly over the last few decades without concern for the impact of this change on the nutritional value of milk beyond its fat content. A closer look at how the feeding management system affects the healthfulness of milk is needed.

1.6.1 Dairy Farm Management Systems

Over the last 60 years, the source of forage in dairy systems in temperate North America has shifted from that of grazed pasture to silage. One main driver of this shift has been the favorable milk price to feed cost ratio, particularly between 1940 and 1980 when the focus of the dairy industry switched from production per unit of land to production per cow (Bargo et al., 2003). Pasture-based systems remain important in some countries including Australia, New Zealand and Argentina as well as in Western Europe where longer growing seasons prevail. Emergence of confinement systems has been paralleled by the development of specialized management strategies, particularly higher feeding rate of grain, needed to support the higher milk yield potential of cows which has increased significantly in the US for example, from 3,191 kg/y in 1960 to 8,263 kg/y in 2000 (Bargo et al., 2003). This change has occurred without evaluation by the industry on the effects of the switch in diet of lactating cows on the healthfulness of milk.

Interest in the pasture-based dairy system in temperate regions relates to the increasing feed cost as well as concerns over environmental and animal-welfare issues

associated with confinement of cows (Dillon, 2006). Although higher milk production is achieved by confining cows, the higher profitability and lower environmental impact of pasture-based systems result from reduced use of purchased inputs including grain, as well as less constructed infrastructure (Fontaneli et al., 2005). Rotationally grazed forage also provides the cheapest source of energy for dairy cows. A review of data from several countries (U.S., Denmark, Germany, The Netherlands, UK, France, Ireland, Australia and New Zealand) showed that on average with every 10% increase in the amount of grazed forage in the diet, the cost of milk production declined by 2.5 cents per liter (Dillon, 2006). These data also show that the cost of milk production in Australia and New Zealand in systems where pasture accounts for about 85-90 % of the dairy ration is about one third of the production cost of confinement systems in the U.S. A review of pasture systems in the U.S. showed that milk production increased linearly when cows are fed up to 10 kg concentrate per day, with an average production response of 1 kg milk per kg of concentrate (Bargo et al., 2003). However, high fossil energy prices renew interest of milk producers in reducing concentrate use through grazing. Research of pasture systems in temperate regions has focused on increasing milk production of grazing cows profitably by using rotational grazing and limiting the use of feed supplements. Type of forage and amount of energy supplement in a dairy ration have been related previously to differences in the healthfulness of milk fat (Dewhurst et al., 2006; Chilliard et al., 2007), an opportunity that remains to be exploited, much less understood by the dairy industry.

1.6.2 Dairy Systems and Milk FA Composition

As well as having environmental, cost and welfare benefits, milk from pasture systems has a more healthful FA composition than that of confinement systems in terms

of both the SFA:UFA ratio as well as the nature of these FA (Table 1.2). Several studies have shown that compared to cows fed conserved forages in confinement, grazing cows and cows fed fresh forage produce milk with lower content of 12:0-16:0, and higher content of beneficial UFA (e.g. OA, VA, RA and ALA). This observation is mainly attributed to higher content of PUFA, primarily ALA, in fresh forage (50-75% of total FA; Dewhurst, 2010).

Oxidation of PUFA during the preservation of forage (Dewhurst et al., 2006; Lubary et al., 2011) results in about 10 times less FA after drying as hay or fermenting as silage than that of fresh forage (Table 1.3). As well, corn silage and grain based concentrate, two main components of TMR, have lipid fractions that are relatively low in FA with predominant FA being OA and LA (Table 1.3). Lipids that are typically supplemented to lactating cows include commercial products fed to boost milk and fat production, which are comprised mainly of industrially-saturated plant lipids rich in SA and PA, and oilseeds, which are fed as an energy source, to improve reproductive performance, alter milk FA composition, and suppress enteric methanogenesis. These are typically high in content of OA and LA with the exception of flaxseed, which is rich in ALA (Table 1.3).

The differences in milk FA composition between pasture and confinement production have been attributed mainly to the higher intake of ALA in pasture systems and its ruminal metabolism which results in increased ruminal outflow of ALA and RBH products, particularly VA and SA (Figure 1.6). Since conversion of VA to SA is the last step of RBH and is rate limiting, these FA comprise the main substrates for mammary Δ-9 *desaturase*. Therefore, increased uptake of VA and SA by the mammary gland elevates

the milk concentrations of RA and OA respectively, as well as VA and SA (Figure 1.6). Furthermore, the increased mammary uptake of circulating PUFA (e.g. ALA) and their RBH intermediates reduce milk content of *de novo* synthesized FA, in particular 12:0-16:0, through the inhibition of mammary enzymes involved in *de novo* FA synthesis (Chilliard et al., 2007).

Besides the high intake of ALA in pasture systems, the elevated digestibility of structural carbohydrate in high quality pasture reduces ruminal retention time (Schroeder et al., 2004) and contributes to the differences in milk FA composition compared with that of confined cows. Grazing cows are also typically fed an energy supplement consisting of non-structural carbohydrates in discrete meals which in consort with low intake of effective fiber results in the potential for an episodic reduction in ruminal pH in grazing cows compared to confined cows consuming TMR. These conditions (low ruminal pH and ruminal retention time) can limit both lipolysis (the prerequisite for RBH) as well as RBH of dietary UFA (Figure 1.6). As a result, more dietary UFA (e.g. intact ALA) and RBH intermediates (e.g. VA) exit the rumen before their complete hydrogenation to SFA (Figure 1.6). Furthermore, some metabolites in fresh forages (e.g. saponins, polyphenols and catecholamines) can have inhibitory effects on ruminal lipolysis and biohydrogenation (Kalač and Samková, 2010) resulting in increased ruminal outflow of native UFA and their RBH intermediates in grazing cows (Dewhurst et al., 2006).

1.7 The Effects of Feeding Marine Oils on Dairy Cow Response

The following sections regard the impact of feeding marine oils on ruminal and mammary responses in dairy cows. The comprehensive list of responses includes ruminal

lipid metabolism, dry matter intake (DMI), milk production, milk composition (fat, protein and lactose), and milk FA composition focusing on OA, *trans* 18:1, CLA, EPA and DHA.

1.7.1 Ruminal Lipid Metabolism

Polyunsaturated fatty acids, particularly long chain ones (e.g. EPA and DHA), are known to have detrimental effects on rumen microorganisms. Addition of PUFA to rumen bacterial cultures (AbuGhazaleh and Jenkins, 2004b; Maia et al., 2007; Potu et al., 2011) or supplementation of cow diets with marine or plant oils (Kim et al., 2008; 966, Huws et al., 2010) were observed to induce shifts in rumen bacterial communities with ensuing effects on intake, and rate of formation and amount of fermentation products. Maia et al. (2007) showed that addition of any PUFA (LA, ALA, EPA and DHA) at 50 µg/ml to pure cultures inhibited the growth of two *cellulolytic Ruminococcus* species (*R. flavefaciens and R. albus*), as well as some butyrate-producing bacteria including *Clostridium proteoclasticum, Butyrivibrio hungatei and Eubacterium ruminantium*. The latter three species are known to be involved in the last step of RBH, in which *trans* 18:1 isomers are converted to SA. Maia et al. (2007) ranked PUFA regarding their toxicity to rumen bacteria as: EPA > DHA> ALA> LA. Likewise, Wasowska et al. (2006) reported that the addition of EPA and DHA at 50 µg/ml to the culture medium of *Butyrivibrio fibrisolvens* a major organism involved in RBH, inhibited its growth and *isomerase* activity. Kim et al. (2008) investigated the effect of level of dietary fish oil (0, 1, or 3% of DM) on the composition of ruminal bacterial community and duodenal FA composition in steers fitted with ruminal and duodenal cannula. Their results showed that duodenal flow of SA decreased significantly with increasing dietary level of fish oil (152.7, 115.1

and 58.9 g/d for 0, 1, or 3% fish oil, respectively), while that of *trans* 18:1 increased with level of fish oil (42.5, 73.2 and 83.4 g/d for 0, 1, or 3% fish oil, respectively). Major changes in the rumen bacterial community were observed in steers fed the 3% fish oil diet including *Butyrivibrio* spp. Quantification of ruminal bacterial DNA by real-time PCR showed that the abundance (pg/ng of total DNA) of *Butyrivibrio* bacteria that produce SA declined 19% by feeding 1% (DM basis) fish oil to lactating ewes (Belenguer et al., 2010).

Several studies have shown that feeding fish oil or marine algae inhibits the complete RBH of dietary C18 PUFA, resulting in a reduced rumen concentration of SA, and increased concentrations of *trans* 18:1 and CLA isomers, particularly VA, *trans*-10 *cis*-12 CLA, *trans*-10 18:1 and RA (Loor et al., 2005c; Wasowska et al., 2006; Boeckaert et al., 2008b; Kim et al., 2008; Or-Rashid et al., 2008). It has been proposed that EPA and DHA may inhibit the *reductase* activity of ruminal bacteria resulting in the accumulation of RBH intermediates, particularly *trans* 18:1 fatty acids in the rumen (AbuGhazaleh and Jenkins, 2004a).

1.7.2 Dry Matter Intake

Feeding marine oils has been reported to have either no impact on DMI or to reduce it. A reduction in DMI was observed in cows fed diets containing fish oil at 1-2% of dietary DM (Cant et al., 1997; Donovan et al., 2000; Whitlock et al., 2002; AbuGhazaleh et al., 2002) or DHA-rich microalgae (*Schizochytrium sp*.) at 1% (Boeckaert et al. 2008a) or 4% (Franklin et al., 1999) of dietary DM. Ruminal infusion of fish oil (Castaneda-Gutierrez et al., 2007) or marine microalgae (Boeckaert et al. 2008a) also caused a reduction in DMI. The negative effect of marine oils on DMI has

been attributed to the depressive effect of PUFA on fiber digestion in the rumen as well as to low acceptability of diets containing these lipid supplements (Cant et al., 1997; Donovan et al., 2000). Lipid supplements, particularly unsaturated ones, can have an inhibitory effect on ruminal fiber digestibility. Two main theories have been suggested: 1) inhibitory effects on rumen microbial growth and metabolism, and 2) physical coating of the feed particles in the rumen, making them less available for microbial fermentation (Palmquist and Jenkins, 1980; Jenkins, 1987).

Conversely, some studies did not show any effect on DMI after feeding unprotected fish oil at 2.5% (Loor et al., 2005b) or 3.7% (Ahnadi et al., 2002) of dietary DM. Discrepancies among studies may be explained by differences in the nature (e.g. content of long chain PUFA) of fish oils fed among studies. Ruminal protection of fish oil can diminish the negative effect on DMI. For example, calcium salts of fish oil fed at levels up to 2.3% of dietary DM (Allred et al., 2006; Moussavi et al., 2007; Castaneda-Gutierrez et al., 2007) did not affect DMI, likely due to a reduction in the release rate of PUFA in the rumen (Castaneda-Gutierrez et al., 2007).

1.7.3 Milk Yield, Milk Protein and Lactose

Milk production was decreased in cows fed fish oil at 2% (Whitlock et al., 2002) or 3% (Donovan et al., 2000) of dietary DM, or microalgae (Boeckaert, et al., 2008a) at 1% of dietary DM. However, other studies showed no changes in milk production after feeding 1% (Donovan et al., 2000) or 2% (Cant et al., 1997; AbuGhazaleh et al., 2002) fish oil, or 4% microalgae (Franklin et al., 1999). Other differences in feeds and feeding management cannot be ruled out in explaining discrepancies among studies. Similar

inconsistencies were found in the effects of marine lipid supplements on milk protein level, although milk lactose level is consistently not affected by oil supplementation.

1.7.4 Milk Fat

Feeding PUFA to dairy cows can reduce milk fat content and yield. The extent of reduction in milk fat content is dependent on the amount of oil supplement fed. Milk fat content was decreased linearly (2.99, 2.79, 2.37, and 2.30%) when fish oil was supplemented at 0, 1, 2, and 3% of dietary DM (Donovan et al. 2000). In a study by Cant et al. (1997) the addition of fish oil to the diet of dairy cows at 2% of dietary DM reduced milk fat percentage by 29.8% compared to cows fed a control diet without lipid supplement. In another study, when fish oil was included at 2% of dietary DM, milk fat content fell by 17.4 and 9.4 % compared to cows fed a control diet (without lipid supplement) or a control diet with 2% added lipid from extruded soybeans, respectively (Whitlock et al., 2002). Similarly, supplementation of microalgae at 1% of DMI reduced milk fat content by 53% (Boeckaert, et al., 2008a). Feeding fish oil in the form of a calcium salt had less effect on milk fat compared to unprotected fish oil. Castaneda-Gutierrez et al. (2007) found that milk fat content was higher (3.61% vs. 2.76%) in cows infused ruminally with calcium salts of fish oil compared to those infused with unprotected fish oil provided similar amount of PUFA. Consistent with this result, milk fat was unaffected in cows fed diets containing (DM basis) 2.7% Ca salts of palm and fish oils (Allred et al., 2006) or 2.3% Ca salts of fish oil (Moussavi et al., 2007). However, other methods used to protect PUFA against RBH, including coating the microalgae with xylose (Franklin et al., 1999) and encapsulation of fish oil in a

glutaraldehyde-treated protein (Lacasse et al., 2002) did not diminish the milk fat reducing effect of fish oil.

Several mechanisms have been proposed to explain the depressive effect of marine oils on milk fat content and yield in dairy cows. Early studies related the phenomenon to reduced ruminal production of acetate, the main substrate for *de novo* FA synthesis, due to depressed fiber digestion (Storry et al., 1974; Palmquist and Jenkins, 1980). However, no decreases in the ruminal acetate concentration or acetate to propionate ratio have been reported when cod liver oil was fed (Beitz and Davis, 1964) or infused into the rumen (Pennington and Davis, 1975) despite significant reductions in milk fat content. Furthermore, feeding fish oil at ≤ 1% of dietary DM did not affect ruminal concentration of acetate or the acetate: propionate ratio (Doreau and Chilliard, 1997; Shingfield et al., 2012).

Another suggested mechanism is that feeding marine oils leads to increased ruminal outflow of specific RBH intermediates which subsequently can act as inhibitors of mammary lipogenesis resulting in MFD. Some of these intermediates such as *trans*-10 18:1, *trans*-9 *cis*-11 CLA and *trans*-10, *cis*-12 CLA have been identified as having anti-lipogenic properties (Shingfield et al., 2010). Ahnadi et al. (2002) reported that mammary mRNA abundance of lipogenic enzymes such as ACACA, FASN, SCD was decreased in cows fed 3.7% (DM basis) unprotected fish oil or 3% glutaraldehyde-protected (microcapsules) of fish oil. This effect was similar to that seen during *trans*-10, *cis*-12 CLA -induced milk fat depression (Harvatine and Bauman, 2006). Ahnadi et al. (2002) suggested that the inhibitory effect of fish oil on mammary lipogenic gene expression is mainly caused by *trans* 18:1 or CLA isomers arising from the rumen.

The long chain PUFA, particularly EPA and DHA, may also contribute directly to MFD in cows fed marine oils. The anti-lipogenic effects of these FA have been confirmed in numerous studies using rodent models and cell cultures (Jump et al., 2008). Few studies tested intravascular (Storry et al., 1969) or post-ruminal (Pennington and Davis, 1975; Chilliard and Doreau, 1997) infusions of fish oil to assess the direct post-ruminal effect of PUFA on milk fat in dairy cows. Storry et al. (1969) reported that intravenous infusion of cod liver oil at the daily rate of 400 g reduced milk fat content by 17 %. Pennington and Davis (1975) found that abomasal infusions of cod liver oil (225 g/d) decreased the content of milk fat by 9.6% while ruminal infusion of a similar amount of oil reduced milk fat percentage by 16 %. Chilliard and Doreau (1997) compared ruminal and duodenal infusions of fish oil (300 ml/d) and observed that ruminal infusion caused a 20% higher reduction in milk fat content compared to duodenal infusion (3.54, 3.22 and 2.51% milk fat for control, duodenal and ruminal infusions, respectively). Similarly, Loor et al. (2005e) found that milk fat content was lowest with ruminal infusion of 300 mL/d fish oil (2.5%) compared with duodenal infusion (3.2%) or the control (3.5%). It is apparent from these studies that inhibition of milk fat synthesis is greater with ruminal infusion of fish oil, suggesting that the inhibitory effect of marine oils on mammary lipogenesis is mainly indirect through their effect on RBH (i.e. increased production of *trans*-18:1 and CLA isomers). Furthermore, it has been shown that EPA and DHA are extensively biohydrogenated in the rumen (Doreau and Chilliard, 1997; AbuGhazaleh and Jenkins, 2004a), which reduces the possibility of a direct role of these FA in MFD.

Another mechanism suggested for MFD when feeding marine oils to dairy cows is the reduced supply of SA to mammary for synthesis of OA via Δ-9 *desaturase* (Loor et al, 2005e; Shingfield and Griinari, 2007). The n-3 LC-PUFA in fish oil and algae are known to inhibit complete RBH of C18 PUFA resulting in a decreased outflow of SA from the rumen (Doreau and Chilliard, 1997; Shingfield et al., 2003; Loor et al., 2004; Loor et al., 2005c). Reduced supply of SA for the endogenous production of OA in the mammary could be a factor limiting milk fat synthesis in dairy cows (Loor and Herbein, 2003).

1.7.5 Milk FA Composition

Bovine milk fat is complex containing more than 400 FA (Jensen, 2002) due to the extensive transformation of dietary FA in the rumen. Feeding marine oils to dairy cows is associated with a number of changes in the profile of milk FA, particularly FA with ≥ 18 carbons. However, milk fat concentrations of *de novo* synthesized FA (6:0-14:0) and 16:0 (~50% of which is synthesized *de novo*) are unchanged or slightly reduced by feeding fish oil or microalgae (Donovan et al., 2000; AbuGhazaleh et al., 2002; Lacasse et al., 2002; Allred et al., 2006; Chilliard et al., 2007; Boeckaert, et al., 2008a). In contrast, the concentration of these FA in milk decreased significantly when cows were fed plant oils (e.g. sunflower, rapeseed or linseed) (Loor et al., 2005b; Rego et al., 2009). Several studies have reported a sharp decline in the percentage and yield of both SA and OA in milk when cows were fed fish oil or microalgae. Donovan et al. (2000) reported that milk fat percentages of SA and OA decreased linearly with increasing level of fish oil (% of dietary DM): 9.38 and 16.47 (0% oil), 6.98 and 14.52 (1% oil), 4.43 and 11.37 (2% oil), 4.03 and 10.89 (3% oil). Feeding just 1% microalgae decreased milk fat

content of SA and OA by 65% and 20% respectively (Boeckaert et al., 2008a). The marked reduction in percentages and yields of SA and OA in milk with fish oil or microalgae is mainly due to the reduction in RBH of *trans*-18:1 to SA which results in the reduced availability of SA for desaturation to OA and for its intact secretion into the milk fat (Loor et al., 2005e; Loor et al., 2005b; Chilliard et al., 2007). Feeding marine oils may also lower abundance and/or activity of mammary LPL, thereby decreasing mammary uptake of FA, including SA and OA from blood (Storry et al., 1974; Loor et al., 2005e).

The potential health benefits postulated for VA and RA (Shingfield et al., 2008) have resulted in attempts to increase the concentrations of these FA in milk by feeding marine or plant oils. However, when fed in equal amounts (by weight), marine oils are more effective in increasing milk concentrations of these FA (Chilliard et al., 2007). The incremental inclusion of fish oil in dairy rations (0, 1 and 2 % of dietary DM) increased linearly the concentrations of milk RA (0.60, 1.58 and 2.23 % of total FA, respectively) and VA (1.21%, 3.07% and 6.08% of total FA, respectively); however, no further increase in the concentrations of RA and VA was observed when the level of fish oil was increased to 3% of dietary DM (Donovan et al., 2000). Shingfield et al. (2003) reported that a fish oil supplement of 250 g/d resulted in a 5.2-fold increase in the content of VA (1·80 vs. 9·39 %) and a 4.2-fold increase in the content of RA (0.39 vs. 1.66 %) in milk fat when compared with the control diet. Similarly, ruminal infusion of 300 ml/d fish oil increased the yields of milk VA and RA by 6.2-fold (7.69 vs. 47.8 g/d) and 4.1-fold (3.97 vs. 16.41 g/d), respectively, compared with the cows fed a control diet (Loor et al., 2005e). Boeckaert, et al., 2008a found that the content of VA (3.55 vs. 1.17 %) and RA

(1.00 vs. 0.48 %) in milk fat from cows supplemented with microalgae at 1% of dietary DM was higher than that of cows receiving none (control diet). Although in comparison to plant oils, marine oils are lower in C18 PUFA, the substrates for VA and RA production in the rumen, marine oils stimulate the production of VA and RA from C18 PUFA provided by other feed ingredients (e.g. oil seeds, forages and grains). The EPA and DHA in marine oils interfere with the completeness of RBH, which results in increased concentration of RBH intermediates, particularly VA, at the expense of SA (AbuGhazaleh and Jenkins, 2004a; Chilliard et al., 2007). The increased production rate of VA increases its circulating level and stimulates the endogenous synthesis of RA in the mammary gland. The majority (70–95%) of RA in milk fat is synthesized via the action of mammary Δ-9 *desaturase* on VA (Griinari et al., 2000; Corl et al., 2001).

Beside their major effects on VA and RA levels in milk, feeding plant or marine oils to dairy cows differentially modifies the profile of other *trans* 18:1 and 18:2 isomers (Chilliard et al., 2007). Feeding marine oils has been associated with increased levels of milk *trans*-18:1 isomers including *trans*-6 to *trans*-14 18:1, and *trans* 18:2 isomers including *trans*-11 *cis*-15 18:2 and *trans*-9, *cis*-11 CLA (Shingfield et al., 2003; Chilliard et al., 2007; Boeckaert, et al. 2008a). Some of these isomers, such as *trans*-10 18:1 and *trans*-9, *cis*-11 CLA, have been implicated in MFD in dairy cows (Shingfield et al, 2010). Furthermore, *trans* FA, excepting VA and RA, have been generally associated with negative health effects such as CVD (Mozaffarian et al., 2006; Shingfield et al., 2008). Therefore, developing lipid supplements that increase the milk content of VA and RA without substantial increases in other *trans* FA should be an important objective for the dairy industry.

Concentrations of EPA and DHA in milk and dairy products produced without feeding marine oils to cows are extremely low (<0.1% of total FA) (Lock and Bauman, 2004). Fish oil and more recently microalgae have been fed to dairy cows to increase the concentrations of EPA and DHA in milk; however, the extent of RBH has been reported to be in the range of 78-100 % for EPA and 74-98% for DHA (Lock and Bauman, 2004) resulting in transfer efficiencies from diet to milk of 2.6% for EPA and 4.1% for DHA (Chilliard et al., 2001). Consequently, with supplementation of marine oils, level of EPA+DHA rarely exceeds 0.5% of total milk FA (Chilliard et al., 2007). Transfer efficiencies of EPA and DHA were increased to 18-33% and 16-25% respectively, when fish oil was infused post-ruminally (Chilliard et al., 2001).

Several studies reported improved transfer efficiency of EPA and DHA by using various technologies to protect these FA against RBH. Kitessa et al. (2004) reported transfer efficiencies as high as 32% and 18% for EPA and DHA respectively when dairy cows were fed formaldehyde-protected tuna oil. Similarly, supplementation of a high DHA microalgae coated with xylose resulted in a higher transfer efficiency of DHA (16.7%) into milk compared to the unprotected microalgae (8.4%) (Franklin et al., 1999). However, Castaneda-Gutierrez et al. (2007) observed no improvement in the transfer efficiencies of EPA and DHA into the milk of cows infused ruminally with calcium salts of fish oil compared to those infused with unprotected fish oil. The authors concluded that although calcium salts of fish oil prevent the adverse effects of fish oil on DMI and milk fat yield, they do not protect DHA and EPA against RBH. Similarly, encapsulation of fish oil in a glutaraldehyde-treated protein did not improve the transfer efficiency of EPA and DHA into milk (Lacasse et al., 2002). Discrepancies in the improvement of

transfer efficiency are likely due to differences in the efficacy of the protection methods. Another reason suggested for the low transfer efficiencies of dietary EPA and DHA into milk is that these FA are preferentially packaged into plasma cholesterol ester and phospholipid fractions which are less efficiently taken up by the mammary compared to the FA in TAG and NEFA (AbuGhazaleh et al., 2003; Lock and Bauman, 2004).

Table 1.1 Dietary approaches used to modify milk FA composition in dairy cows[1]

Fatty acid[2]	Fresh forage/pasture	linoleate-rich oils (e.g. sunflower, soybean)	α-linolenate rich oils (e.g. flaxseed)	Marine oils (fish or algae)
12:0-16:0	−	−	−	0/−
*c*9 18:1	++	+	+	−
*c*9, *t*11 CLA	++	++	++	+++
18:3 n-3	+	0	+	0
EPA+DHA	0	0	0	+

[1]Adapted from Chilliard et al., 2007; −, +, 0 = decrease, increase and no change respectively.
[2]c = *cis*, t = *trans*; CLA = conjugated linoleic acid; EPA = eicosapentaenoic acid (20:5 n-3), DHA = docosahexaenoic acid (22:6 n-3).

Table 1.2 The content of selected fatty acids (g/100 g total FA) and SFA:UFA ratio in milk from cows grazing or fed fresh forage vs. cows fed conserved forages in confinement[1]

Fatty acid	Pasture/fresh forages[3]	Conserved forages[3]
Palmitic	25.7±1.6	31.2±3.4
Oleic	22.4±3.6	18.6±2.6
Vaccenic	3.5±0.6	1.3±0.4
α-linolenic	0.9±0.2	0.7±0.2
Rumenic	1.4±0.2	0.5±0.2
SFA:UFA[2]	1.8±0.3	2.7±0.5

[1]Mean and standard deviation of 10 studies using fresh forages or pasture and 12 studies using silages and hay (calculated from the review by Kalac and Samkova, 2010).
[2]Ratio of saturated to unsaturated fatty acids.

Table 1.3 Total ether extractable (EE) lipid, and concentrations of total and selected fatty acids (FA) of fresh and conserved forages, grains, oilseeds and commercial lipid supplements used in dairy rations

	EE	FA	FA composition (g/100 g total FA)				
	(%DM[1])	(%DM)	Palmitic	Stearic	Oleic	Linoleic	ALA[2]
Fresh forage							
Perennial ryegrass[3]	3.6	1.8	12.9	3.0	3.2	14.6	65
Meadow fescue[3]	3.2	1.6	17.7	1.5	4.4	15.9	43.4
Tall fescue[4]	-	2.2-3.9	11-22	1.4-4.9	2-4.	9.1-11.8	55-72
Cocksfoot[3]	3.6	1.9	11.2	2.6	-	76.5	-
Timothy[4]	-	2.0-2.1	19.5	4.3-5.1	5-6	15	50
Red clover[3]	5.1	2.3	14.2	3.7	-	5.6	72
White clover[3]	5.0	2.2	6.5	0.5	6.6	18.5	61
Alfalfa[3]	5.3	2.5	2.7	<0.5	<0.5	1.7	95
Perennial sward[5]	3.6-4.3	-	11.0	1.4	2.0	14.5	46.5
Hay							
Cocksfoot[4]	-	0.2	24	2.8-4.5	3-4	16-17	27-35
Perennial ryegrass[4]	-	0.3	15.8	1.8	2.0	14.0	55.9
Alfalfa[4]	-	0.1	30.0	6.0	8.0	24.4	23.2
Perennial sward[5]	2.4	-	16.9	2.5	4.8	13.3	26.1
Silage							
Corn[4]	-	0.1-0.4	16-17	2.4-2.9	19-24	49	3-11
Perennial ryegrass[4]	-	0.4	21.2	2.0	2.8	13.4	52
Perennial sward[5]	3.7	-	13.1	1.3	4.1	21.2	29.8
Grains							
Corn[3]	3.6-5.0	3.2	16.3	2.6	30.9	47.8	2.3
Barley[3]	1.7-2.9	1.6	27.6	1.5	20.5	43.3	4.3
Oilseeds							
Sunflower[3]	38-45	34.7	5.5	3.6	21.7	68.5	<0.5
Soybeans[3]	16-25	19	10.7	3.9	22.8	50.8	6.8
Flaxseeds[6]	36	-	5.1-7.1	2.8-4.0	16-22	15-18	50-59
Commercial lipid supplements[7]							
Alifet-High energy®	99	-	22.3	31.7	11.5	0.19	0.16
Energy Booster®	100	-	41.9	44.5	5.5	1.7	0.72
Megalac®	85	-	46.0	4.48	35.3	8.2	0.30

[1]Dry matter;[2] α-linolenic acid; [3] Schroeder et al., 2004; [4] Kalac and Samkova, 2010; [5] Vahmani et al., unpublished data; [6] Woods et al., 2009; [7] Carriquiry et al., 2008.

Figure 1.1 Major pathways for the biohydrogenation of linoleic and α-linolenic acids in the rumen (Adopted from Harfoot and Hazlewood, 1988).

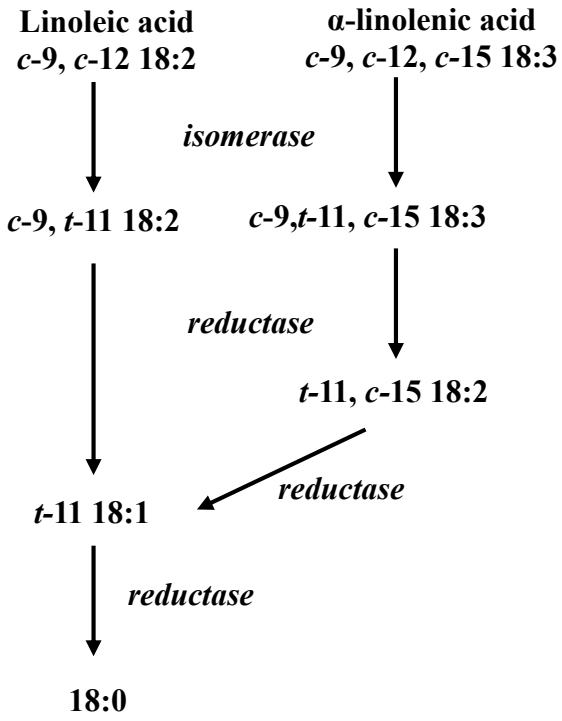


Figure 1.2 The ruminal biohydrogenation pathways of linoleic and α-linolenic acids (Adapted from Shingfield et al., 2010). t = *trans*, c = *cis*; Solid arrows represent the major established pathways; dashed arrows describe the minor putative pathways suspected by the occurrence of the minor RBH intermediates during the ruminal metabolism studies.

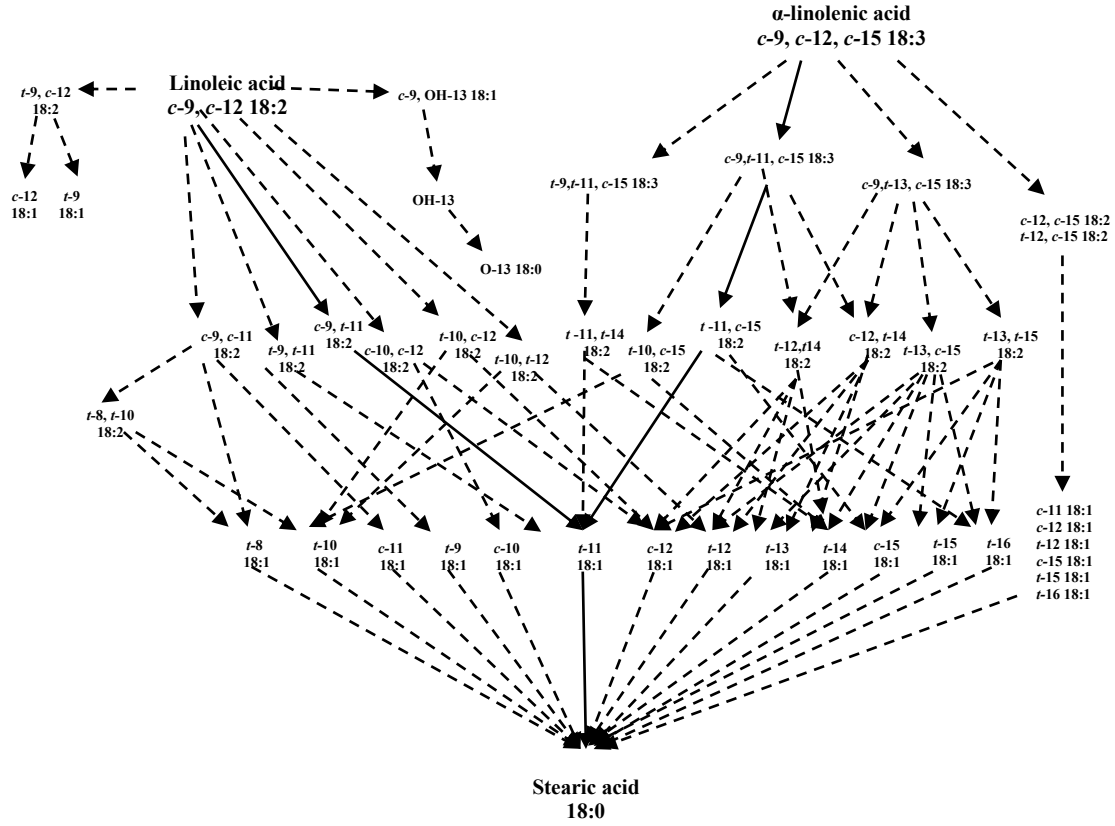


Figure 1.3 Putative biohydrogenation pathways of oleic acid in the rumen (Adapted from Shingfield et al., 2010). Solid arrow represents the major pathway; dashed arrows describe the minor pathways.

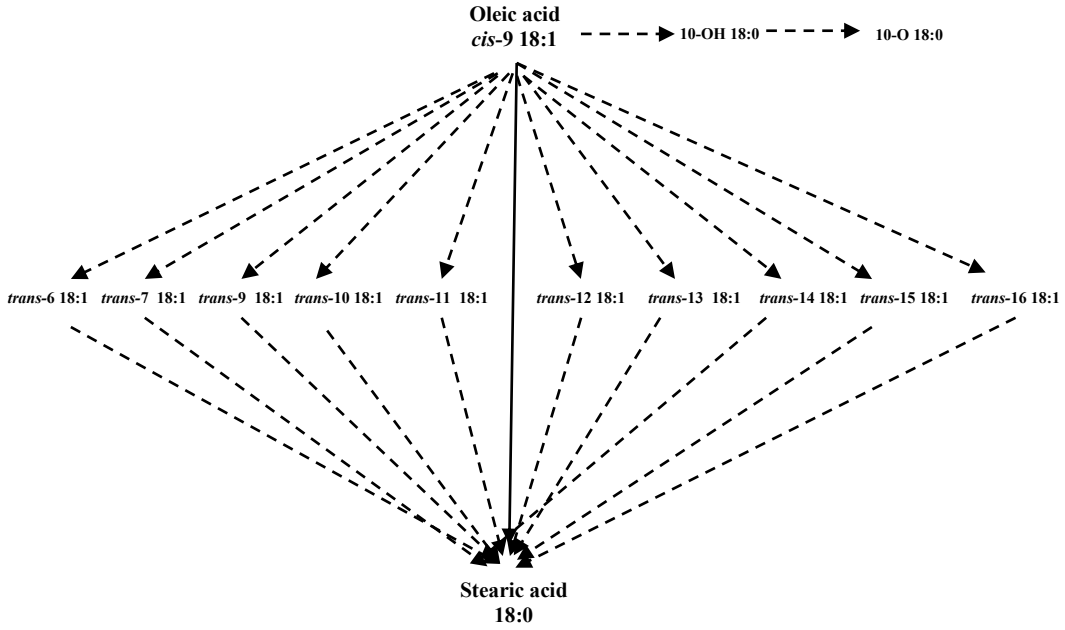


Figure 1.4 Diagram of milk fat synthesis in in the bovine mammary epithelial cell (adapted from Harvatine et al., 2009 and Shingfield et al., 2010). Abbreviations: UFA = unsaturated fatty acids, RBH = ruminal biohydrogenation, SFA = saturated fatty acids, HSL = Hormone sensitive lipase, TAG = triacylglyceride , VLDL = very low density-lipoprotein, CM = chylomicron, FFA = free fatty acids, BHBA = beta-hydroxybutyrate, GLUT = glucose transporters, LPL= lipoprotein lipase, FATP = fatty acid transport proteins, FABP = fatty acid binding protein, Glycerol-3-P = glycerol-3 phosphate, SCD = stearoyl-CoA desaturase, ACACA = acetyl-CoA carboxylase, FASN = fatty acid synthase, FA = fatty acids, GPAT= glycerol-3 phosphate acyl transferase, AGPAT = 1-acyl glycerol 3-phosphate acyl transferase, DGAT = diacylglycerol acyltransferase 1, CLD = cytoplasmic lipid droplet, MFG = milk fat globule.

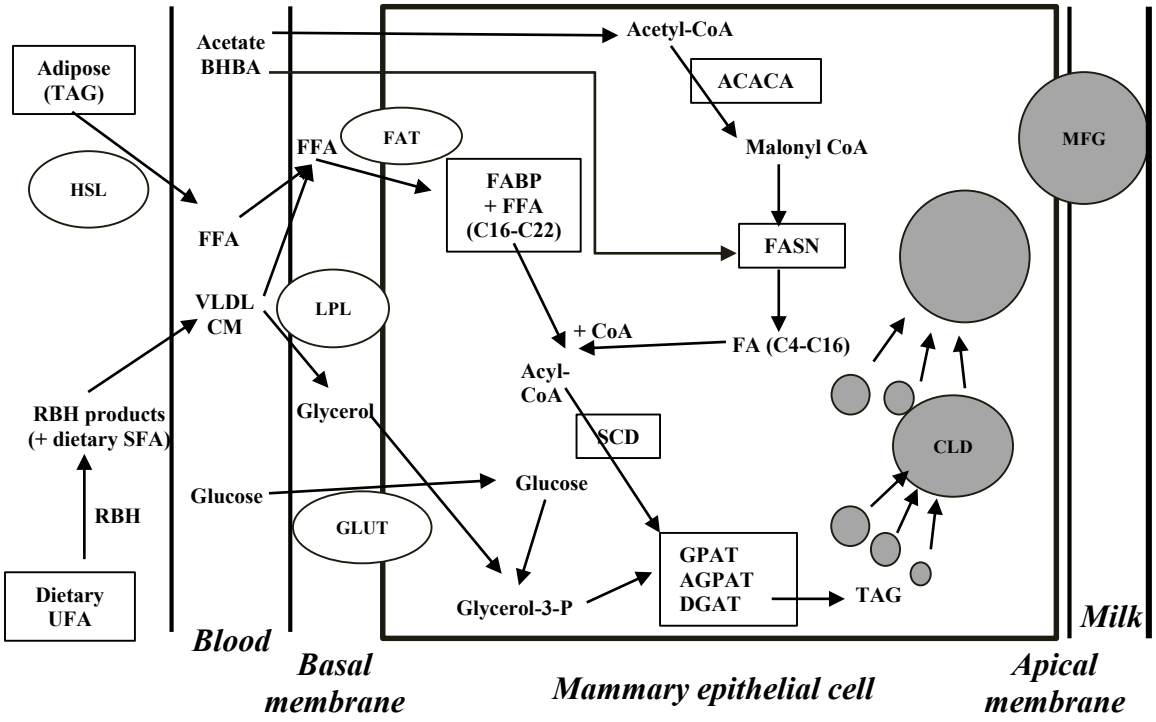


Figure 1.5 Schematic relationships between RBH and mammary lipogenesis (Chilliard et al., 2007; Shingfield et al., 2010). PUFA = polyunsaturated FA, SFA = saturated fatty acids, RBH = ruminal biohydrogenation, SREBP1 = sterol response element binding protein 1, ACACA = acetyl-CoA carboxylase, FASN = fatty acid synthase, LPL= lipoprotein lipase, SCD = stearoyl-CoA desaturase, LCFA = long chain fatty acids, MUFA = monounsaturated fatty acids.

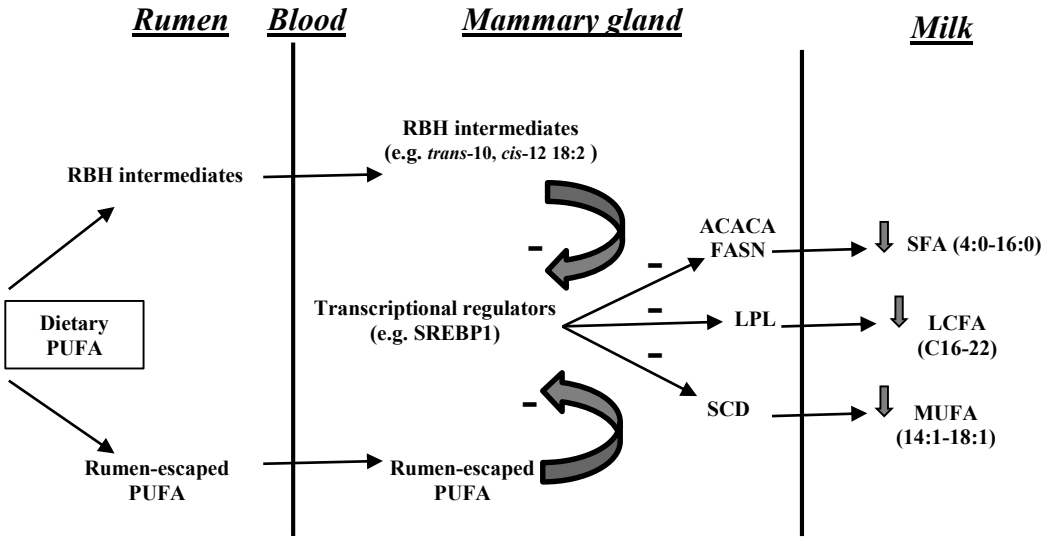
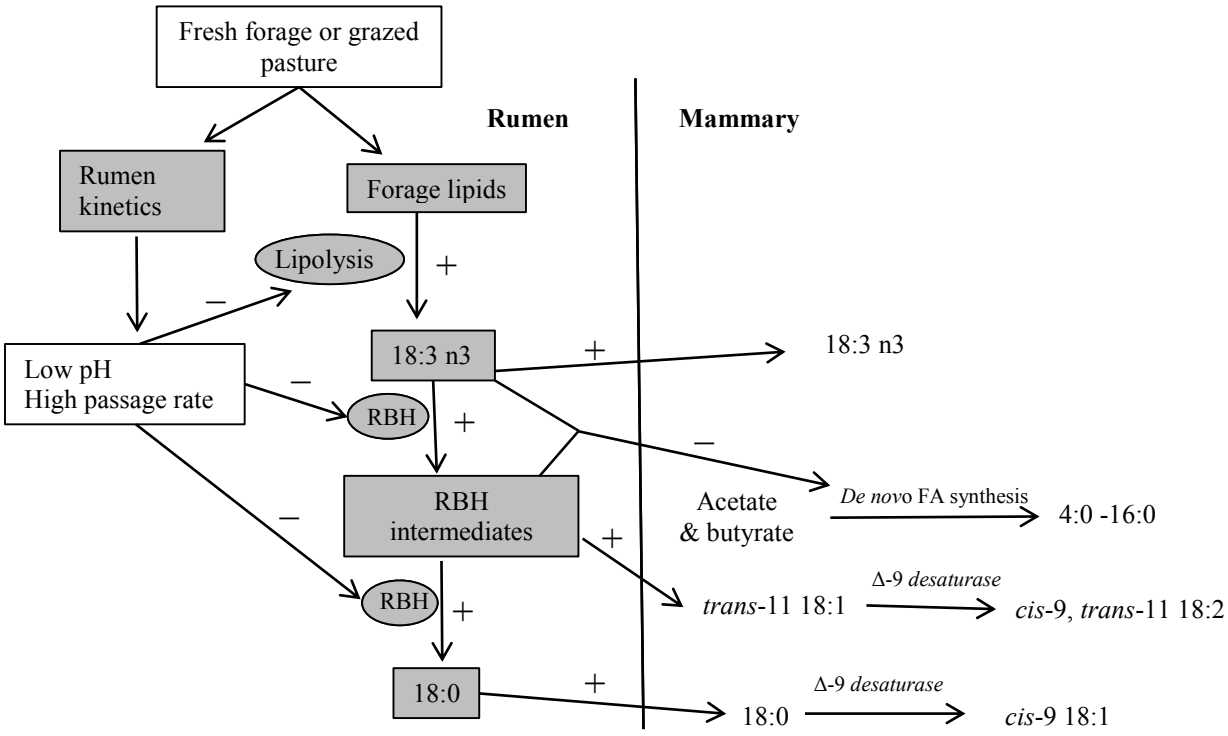


Figure 1.6 The relationship between ruminal lipid metabolism and milk fatty acid composition in grazing cows. 18:3 n3 = α-linolenic acid, RBH = ruminal biohydrogenation, 4:0 = butyric acid, 16:0 = palmitic acid, *trans*-11 18:1 = vaccenic acid, *cis*-9, *trans*-11 18:2 = rumenic acid, *cis*-9 18:1 = oleic acid.



Chapter 2 Effect of Supplementation with Fish Oil or Microalgae on Fatty Acid Composition of Milk from Cows Managed in Confinement or Pasture Systems

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2.1 Abstract

The objective of this study was to examine the interaction between lipid supplement (LS) and management system (MS) on fatty acid (FA) composition of milk that could affect its healthfulness as a human food. Forty-eight pre-partal Holstein cows were blocked by parity and predicted calving date and deployed across pasture (PAS; n = 23) or confinement (CON; n = 25) systems. Cows within each system were assigned randomly to a control (no marine oil supplement) or to one of two isolipidic (200 g/d) marine oil supplements: fish oil (FO) or microalgae (MA) for 125 ± 5 days starting 30 days pre-calving. The experiment was conducted as a split-plot design with MS being the whole plot treatment, and LS as the sub-plot treatment. Cows were housed in a tie-stall barn from −30 until 28 ± 10 days in milk (DIM) and were fed TMR with similar formulations. The PAS group was then adapted to pasture and rotationally grazed on a perennial sward until the end of experiment (95 ± 5 DIM). Milk samples were collected at 60 and 90 DIM for major components and FA analyses. Milk yield (kg/d) was lower in PAS (34.0) compared with CON (40.1). Milk fat percent was reduced with MA compared with FO (3.00 vs. 3.40) and the control (3.56). However, milk fat yield (kg/d) was not affected by lipid supplements. Compared with CON, PAS produced milk fat with a lower content of 12:0 (−38%), 14:0 (−28%) and 16:0 (−17%), and more *cis*-9 18:1 (+32%), 18:3 n-3 (+30%), conjugated linoleic acid (CLA; +70%) and *trans* 18:1 (+34%). Both supplements, regardless of MS, reduced similarly the milk fat content of 16:0 (−12%) and increased CLA (+28%) and n-3 long-chain polyunsaturated FA (n-3 LC-PUFA; +150%). Milk fat content of *trans* 18:1 (*trans*-6 to *trans*-16) was increased with FO or MA, although the effect was greater with MA (+81%) than with FO (+42%). The interaction

between MS and LS was significant only for *trans*-11 18:1 (vaccenic acid; VA) and *cis*-9, *trans*-11 CLA (rumenic acid; RA). In contrast to CON, feeding FO or MA to PAS cows did not increase milk fat content of VA and RA. We concluded that compared to CON, PAS milk had a more healthful FA composition. Feeding either FO or MA improved n-3 LC-PUFA and reduced levels of 16:0 in milk fat regardless of MS, but concurrently increased the *trans* 18:1 isomers other than VA, at the expense of VA, particularly in grazing cows.

2.2 Introduction

Milk in temperate North America is increasingly produced using year round confinement systems and to a lesser and shrinking extent, pasture systems which employ grazing for about half the year. The diets of cows are substantially different between these systems. Grazing cows consume fresh forage with less grain fed typically twice daily at milking, whereas confined cows are fed conserved forages, corn silage and grains in a TMR. Although higher milk production is achieved by confining cows, the higher profitability (Rust et al., 1995) and lower environmental impact (Arsenault et al., 2009) of pasture systems results from reduced use of purchased inputs (e.g. grain) and lower use of farm infrastructure and machinery. As well as having environmental and cost benefits, milk from grazing cows has a more healthful FA profile than that of confined cows (Vahmani et al., 2013). Previous studies (Dewhurst et al., 2006; Chilliard et al., 2007; Kalac and Samkova, 2010) showed that compared to cows fed conserved forages in confinement, grazing cows produce milk with a lower fat content of saturated FA (**SFA)** particularly 12:0, 14:0 and 16:0, and higher content of beneficial unsaturated FA (**UFA**) including *cis*-9 18:1 (oleic acid, **OA**), *trans*-11 18:1 (vaccenic acid; **VA**), conjugated

linoleic acid (**CLA**) particularly *cis*-9, *trans*-11 CLA (rumenic acid; **RA**), and 18:3 n-3 (α-linolenic acid; **ALA**).

Milk fat is naturally almost devoid of n-3 long-chain PUFA (**n-3 LC-PUFA**), specifically eicosapentaenoic acid (**EPA**; 20:5 n-3) and docosahexaenoic acid (**DHA**; 22:6 n-3). Fish oil has been the most common source of n-3 LC-PUFA used in dairy rations to improve the milk fat content of these FA. Microalgae, at the bottom of marine food chains, are the primary producers of n-3 LC-PUFA. Recently, biofermenter technology has been used for production of heterotrophic microalgal biomass (e.g. *Schizochytrium* sp.) which is mainly used as a source of DHA for aquaculture (Sijtsma and de Swaaf, 2004). A few recent studies (Boeckaert, et al., 2008a; AbuGhazaleh et al., 2009; Glover et al., 2012; Stamey et al., 2012) have successfully used microalgae as a ration supplement for the enrichment of milk with DHA.

The majority of consumed PUFA is biohydrogenated in the rumen and not incorporated into milk intact. The transfer efficiencies of EPA and DHA from diet into milk have been estimated to be 2.6% and 4.1% respectively (Chilliard et al., 2001). However, the transfer efficiencies of EPA and DHA were increased several fold when the source is protected from ruminal biohydrogenation (**RBH;** Franklin et al., 1999; Kitessa et al., 2004).

Although microalgae and fish oil are not significant sources of C18 UFA (precursors for ruminal production of VA and RA), they are more effective than plant oils in increasing milk concentrations of VA and RA (Chilliard et al., 2007). The n-3 LC-PUFA may inhibit the final step of RBH where VA is converted to 18:0 (AbuGhazaleh and Jenkins, 2004a). The enhanced ruminal outflow of VA increases the milk fat content

of VA as well as that of RA, because of mammary conversion of VA to RA (Shingfield et al., 2010).

Higher intake of ALA by cows grazing fresh forage in pasture systems and specific ruminal conditions in grazing cows (e.g. low ruminal pH and high ruminal passage rate) play roles in causing differences in milk FA composition compared with that of confined cows (Schroeder et al., 2004; Dewhurst et al., 2006). Depressed ruminal pH combined with high ruminal passage rate in grazing cows can reduce the completeness of RBH and potentially increase transfer rate of dietary UFA and their RBH intermediates to milk compared with cows fed in confinement. There is increasing focus on the healthfulness of food and knowledge regarding the health impacts of the types of fats in our diets. The potential for the dairy farm management system to affect the healthfulness of milk fat requires further study. The objective of this research was to determine the interaction of dairy farm management system (**MS**; pasture vs. confinement) and source of marine lipid supplement (**LS**; fish oil vs. microalgae) on milk FA composition. We hypothesized that response of milk FA composition to LS would be affected by MS. We further hypothesized that the different proportions of EPA and DHA in the n-3 LC-PUFA of supplemented fish oil vs. microalgae would influence differently the milk FA composition including content of CLA and *trans* 18:1 isomers.

2.3 Materials and Methods

2.3.1 Animals, Experimental Design and Treatments

All procedures and protocols involving animals in this study were approved by the Animal Care and Use Committee of the Dalhousie Agricultural Campus, Truro, Nova Scotia. Forty eight pre-partal Holstein cows were blocked by parity and predicted calving

date and deployed randomly within block to pasture (n = 23) or confinement (n = 25) systems. Cows within each system were assigned randomly to a control (no marine oil supplement), fish oil (**FO;** EPAX 5500, Pronova Biocare, Alesund, Norway) or microalgae (**MA;** Algamac-3050, Aquafauna Bio-Marine, Hawthorne, CA). Both FO and MA were encapsulated with a saturated lipid containing 75% 18:0 and 25% 16:0 to provide protection against RBH (Animal Nutrition Laboratory, Faculty of Agriculture, Dalhousie University, Truro, Nova Scotia, Canada), and were stored at −20°C until feeding which began 30 days before calving and continued until 95 ± 5 DIM. Supplements provided similar amounts of total lipid (200 g/d) and LC-PUFA (~ 65 g/d; Table 2.1), but contained different proportions of EPA and DHA. The predominant LC-PUFA in FO was EPA, whereas DHA was the major LC-PUFA in MA (Table 2.1). Lipid supplements were mixed with 1 kg of TMR (as-fed; Table 2.2) and were hand-fed to both pasture and confinement groups. This was the only TMR offered to grazing cows.

Pasture and confinement groups were housed in the same tie-stall facility and fed four different TMR: 1. dry cow (−32 to −22 DIM), 2. close-up (−21 DIM to calving), 3. post-calving (calving to 9 DIM), 4. lactation (10 DIM to either 28 ± 10 DIM or 95 ± 5 DIM for pasture and confinement respectively). All rations were formulated using the CPM-Dairy cattle ration analyzer (Cornell-Penn-Miner Ver. 3.0.8; Miner Institute, Chazy, NY). Pasture group was adapted to pasture at 28 ± 10 DIM by increasing the grazing time from 12 h/d for one week to 21 h/d, which continued until the end of experiment (95 ± 5 DIM). Cows rotationally grazed a perennial sward consisting of bluegrass (*Poa pratensis*; 17-20%), white clover (*Trifolium repens*; 14-18%), timothy (*Phleum pratense*; 8-12%) and other pasture species and forbs (28-30%). The period of

stay was based on target sward heights, which were 20-30 and 5-10 cm for pre- and post-grazing respectively. Stocking rate varied from 23 to 30 cows/ha.

Cows were milked daily at 0600 h and 1600 h and individual milk production was recorded at each milking. Confined cows were fed ad libitum twice daily at 0700 h and 1500 h allowing ~ 10% orts (as-fed basis). Amount of feed offered and refused was recorded daily for the calculation of DMI. Concentrate was fed to the pasture group at the rate of 25% (w/w as fed) of milk yield, to a maximum of 8.0 kg daily. All grazing cows received the maximal level. This concentrate was the same as that used in the lactation TMR for confined cows (Table 2.2) and was fed in 2 equal portions at morning and afternoon milkings. The FA and chemical composition of feeds are presented in Tables 2.1 and 2.2 respectively. Water was provided free choice.

2.3.2 Measurements, Sampling and Analyses

Milk yield and feed intake were recorded daily. Milk samples were collected during four consecutive milkings on 59-60 and 89-90 DIM. Samples were composited by day proportional to milk yield at each milking and then split into two aliquots. One aliquot was analyzed for fat, protein and lactose by infrared spectrophotometry (Fossomatic 4000 Milkoscan Analyzer, Foss North America, Brampton, Ontario). The second aliquot was flushed with nitrogen gas and then stored at −80°C for FA analysis. Body weight and BCS (Wildman et al., 1982) were recorded on 60 and 90 DIM, as well as −31 DIM which were used as covariates. Samples of pasture, TMR and concentrate were collected weekly and stored at −20°C. Frozen samples were later composited by month and analyzed as described by Glover et al. (2012).

Lipids were extracted from milk samples using chloroform/methanol/water (2:1:0.8, by vol) based on a modified Folch et al. (1957) procedure as described by Budge et al. (2006). Extracted lipids were derivatized using 0.5 N $NaOCH_3$/methanol (15 min at 50°C; Supelco Inc., Bellefonte, PA; Cruz-Hernandez et al., 2006). The resulting fatty acid methyl esters (**FAME**) were dissolved to 0.5 mg/mL in hexane for analysis using two gas chromatography (**GC**) methods (modification of Kramer et al., 2008). The temperature program for the first method, GC method A, was 45°C for 4 min, then the temperature was increased to 175°C at a rate of 13°C/min, held for 27 min, and then increased to 215°C at a rate of 4°C/min and held for 35 min. GC method A had a column flow rate of 0.5 mL/min and used a split injection with a ratio of 3:1. The temperature program for GC method B used a plateau of 150°C held for 47 min instead of 175°C for 27 min, but otherwise was the same as GC method A. The column flow rate for GC method B was 0.7 mL/min and split-less injection was used. The gas chromatograph (Varian 3800 GC, Varian, Walnut Creek, CA) was equipped with a Varian 8100 autosampler, a flame ionization detector (**FID**), and a Supelco SP-2560 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness). The injector and the detector were maintained at 250°C, and 270°C respectively. The gas flow to the FID was set as follows: air 300 mL/min, hydrogen 30 mL/min, and helium make-up gas 29 mL/min with a carrier gas flow of either 0.5 or 0.7 mL/min of hydrogen depending on the GC method. Reference standards 463, 481B, 21:0 and CLA isomer mixture UC-59M were purchased from Nu-Chek Prep. Inc. (Elysian, MN) and used for the identification of FAME. Any CLA or *trans* 18:1 isomers not in the above standards was identified by

comparison with previously reported GC analysis of milk FAME (Cruz-Hernandez et al., 2006; Kramer et al., 2008).

2.3.3 Calculations

The intake of pasture forage in grazing cows was calculated using the difference between NE_L output (lactation + maintenance) and concentrate NE_L intake as well as the NE_L content of pasture (Cosgrove and Cooper, 2007). Total DMI of grazing cows was the sum of concentrate DMI and estimated pasture DMI.

Transfer efficiencies of EPA and DHA from diet to milk were calculated for individual cows fed lipid supplement as: [(FA yield (g/d) in milk) − (mean of FA yield (g/d) of control cows)] / [amount of FA (g/d) provided by the lipid supplement]. Milk FA yields were calculated according to Glasser et al. (2007).

2.3.4 Statistical Analysis

Data were analyzed using the Mixed procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC) according to a split-plot model. Management system and LS were considered as fixed factors, and block was treated as a random factor. To limit the confounding effect of seasonal changes in pasture forage composition and number of days on pasture, only milk FA data from DIM 90 was used for statistical analysis. For the remaining variables, DIM was included in the model as a repeated factor. A covariate was added to the model for the analyses of BW and BCS data, for which pre-trial measurements (−31 DIM) were available. Least squares means were separated using the PDIFF statement in SAS. Differences were declared significant at $P \leq 0.05$ and tendencies declared at $0.05 > P \leq 0.10$.

2.4 Results

2.4.1 Animal Performance

No interactions between MS and LS (**MS × LS**) were observed regarding animal performance variables (Table 2.3). Dry matter intake, and yields of uncorrected milk, 3.5% fat corrected milk (3.5% FCM), fat, protein and lactose were lower ($P \leq 0.05$) for pasture compared with confinement. Body condition score tended ($P = 0.10$) to be lower in grazing compared with confined cows. The main effect of LS was significant only for milk fat percent, which was reduced (−16%; $P < 0.01$) with MA compared with either FO or the control. Supplementation with FO did not change milk fat percent compared with the control.

2.4.2 Milk FA Composition

No significant MS × LS were observed for any of the selected FA or FA categories presented in Table 2.4, but the main effects of MS and LS were significant ($P \leq 0.05$) for most FA. Milk concentrations of 12:0, 14:0 and 16:0 were reduced ($P \leq 0.01$) by 38%, 28% and 17% when cows were grazing compared with those in confinement. The concentrations of 18:0 (+22%), OA (+32%) and ALA (+30%) were greater ($P < 0.05$) in milk from cows on pasture compared with those in confinement. Supplementation with MA significantly reduced ($P < 0.05$) milk fat concentrations of 12:0 and 14:0 by 22% and 15% respectively compared with either FO or the control. Supplementation with FO did not change the concentrations of these FA compared with the control. Both lipid supplements reduced ($P < 0.01$) the concentration of 16:0 by 12% compared with the control.

The milk fat concentrations of n-3 LC-PUFA paralleled the concentrations of these FA in marine oil supplements fed (Tables 2.1 and 2.4). Cows fed FO had higher ($P < 0.01$) milk fat concentrations of EPA (+100%) and 22:5 n-3 (+86%) compared with those fed the control or MA. Both supplements increased ($P < 0.01$) the milk fat concentration of DHA compared with the control and the effect was greater ($P < 0.01$) with MA (+900%) than with FO (+600%). Supplementation with MA also increased ($P < 0.01$) the concentration of 22:5 n-6, while this FA was not detectable in milk from cows fed control or FO (Table 2.4). The calculated transfer efficiency of EPA from FO into milk was 3.46 ± 0.98% and 2.97 ± 0.88% in grazing and confined cows respectively. The transfer efficiency of supplemental DHA into milk was similar between FO and MA, and between grazing and confined cows with the overall mean of 5.32 ± 1.05%.

Grazing reduced ($P = 0.01$) milk fat content of SFA by 14% compared with confinement (Table 2.4). Concurrently, the pasture group had higher ($P < 0.05$) milk fat contents of *cis* MUFA (+23%), *trans* MUFA (+35%) and CLA (+70%) compared with the confinement group. The higher milk fat content of *cis* MUFA and *trans* MUFA in pasture group was mainly due to the substantial increase in the content of OA (+32%) and *trans* 18:1 (+34%). Grazing cows tended ($P = 0.09$) to have a higher milk fat content of n-3 PUFA, which was mainly due to the higher content of ALA in pasture (Table 2.1). The milk SFA:UFA ratio was lower ($P = 0.01$) for cows on pasture compared with those in confinement despite having higher content of 18:0. Supplementation with MA reduced ($P < 0.05$) the content of SFA (−9.0%) compared with FO or the control. However, FO did not change the concentration of SFA. The concentration of *trans* 18:1 was increased ($P < 0.01$) with either FO or MA, although the effect was greater ($P < 0.01$) with MA

(+81%) than with FO (+42%). Both lipid supplements increased ($P \leq 0.01$) milk fat content of n-3 PUFA, CLA and n-3 LC-PUFA compared with the control by 40%, 28% and 150% respectively. Supplementation with FO or MA reduced ($P < 0.01$) the n-6:n-3 and SFA:UFA ratios (Table 2.4).

Milk fat concentrations of *trans* 18:1 and CLA isomers are presented in Table 2.5. The main effect of MS was not significant for most of *trans* 18:1 isomers except for VA and *trans*-16 18:1, which were higher for the pasture group. However, the main effect of LS was significant ($P < 0.01$) for almost all isomers except for *trans*-4 and *trans*-5 18:1 ($P > 0.10$). Supplementation with FO or MA increased ($P < 0.01$) the milk fat content of *trans*-6 to *trans*-16 18:1 compared with the control and the effect was always greater ($P < 0.01$) with MA than with FO. The range of increase compared with control was 37-80% and 67-115% for FO and MA respectively. We observed a significant MS × LS ($P = 0.03$) for VA (Table 2.5). As shown in Figure 2.1, supplementation of grazing cows with FO or MA did not significantly increase the milk concentration of VA, whereas both supplements increased ($P < 0.05$) the concentration of VA in confined cows and the effect was greater ($P < 0.05$) with MA (+45%) than with FO (+22%).

The milk fat of grazing cows had greater ($P < 0.01$) concentrations of RA and *trans*-11, *cis*-13 CLA compared with those of cows in confinement (Table 2.5). Both lipid supplements increased ($P < 0.01$) the milk content of *trans*-9, *cis*-11 CLA, *cis*-11, *trans*-13 CLA, and *trans*, *trans* CLA isomers. A significant MS × LS was observed ($P = 0.04$) for RA with a similar response trend to VA (Figure 2.1). The concentrations of *trans*-10, *cis*-12 and *trans*-11, *trans*-13 CLA were not affected by dietary treatments (Table 2.5).

Table 2.6 presents *trans*-18:1 isomer distribution as a percentage of total *trans*-18:1 for all treatment combinations. As expected, VA was the major milk *trans* 18:1 isomer across all dietary treatments. The MS × LS was significant ($P < 0.05$) for the relative concentration of *trans*-11 (VA) to *trans*-16 18:1. The relative concentration of VA for the pasture group was reduced ($P < 0.01$) by feeding FO or MA, which was accompanied by an increase in the relative concentrations of other *trans*-18:1 isomers with the double bonds on carbons 12 to 16 (Table 2.6). In contrast, the relative concentration of VA was not affected when FO or MA were fed to the confinement group. The relative concentrations of *trans*-9 and *trans*-10 18:1 were not affected by MS or LS. However, the relative concentration of *trans*-6 to *trans*-8 18:1 was reduced ($P < 0.01$) with either FO or MA. Relative concentrations of *trans*-4 and *trans*-5 18:1 tended ($P \leq 0.08$) to be higher in confinement compared with pasture, and tended ($P \leq 0.07$) to be reduced by lipid supplementation.

2.5 Discussion

2.5.1 Animal Performance

The lower yields of milk, milk fat, protein and lactose observed in the pasture group compared with those in confinement group are related to the lower estimated DMI in grazing cows (Table 2.3). The tendency ($P = 0.10$) of reduced BCS in grazing cows compared with confined cows is likely due to the greater mobilization of body reserves (Agenas et al., 2002; Vahmani et al., 2011).

Despite the reduced milk fat percent in MA fed cows compared with those fed FO or the control, milk fat yield was not affected by LS ($P = 0.22$; Table 2.3). Feeding fish oil or microalgae to dairy cows has been associated with either reduction (Cant et al.,

1997; Franklin et al., 1999; Ahnadi et al., 2002; Boeckaert, et al., 2008a) or no change (Allred et al. 2006; Stamey et al., 2012) in milk fat yield. The discrepancy could be related to differences in the amount of lipid supplements fed, rumen protection method, length of feeding period, and composition of basal diets.

2.5.2 Milk FA Composition

Compared with confinement, grazing increased milk fat content of several beneficial UFA (ALA, OA, VA and RA) at the expense of 12:0-16:0. These results are consistent with previous studies showing that pasture-based milk has a significantly more healthful FA composition than that of milk from confined cows fed conserved forages and more concentrate (Dewhurst et al., 2006; Kalac and Samkova, 2010). This phenomenon has been related mainly to the higher intake of PUFA in grazing cows, particularly ALA, the predominant FA (50-75% of total FA) in fresh forage (Chilliard et al., 2007). The majority of this FA is lost by ensiling and storing (Dewhurst et al., 2006; Kalac and Samkova, 2010). Consistent with these studies, ALA was the major FA in pasture, whereas in TMR, linoleic acid (**LA**; 18:2 n-6) was the predominant FA, which originated mainly from concentrate (Table 2.1). The lower milk fat content of 12:0-16:0 in grazing cows was likely due to reduced mammary *de novo* FA synthesis (Couvreur et al., 2007). The majority of 12:0 and 14:0, and about 50% of 16:0 are synthesized *de novo* in the mammary gland, a process that is inhibited directly by ALA or indirectly via its RBH intermediates (Chilliard et al., 2007). Consequently, we observed higher milk concentrations of ALA, *trans* 18:1 and CLA in grazing cows compared with confined cows (Table 2.4). Similarly, the lower milk fat content of 16:0 in cows fed FO or MA could be due to the inhibition of mammary *de novo* FA synthesis (Ahnadi et al., 2002).

Fish oil and MA provided 25 and 51 g 16:0 per day respectively (Table 2.1), an insufficient amount apparently to compensate for reduced mammary synthesis of 16:0. The higher milk fat content of OA of grazing cows relates to the increased mammary supply of 18:0 providing more substrate for mammary Δ^9-desaturase. The higher intake of ALA by grazing cows can increase the ruminal outflow of its RBH end product 18:0, as well as numerous RBH intermediates (Chilliard et al., 2007). Greater mobilization of body reserves in grazing cows compared with confined cows could also have contributed to higher mammary supply of 18:0 (Agenas et al., 2002). Mobilization of body fat also releases 16:0 but since the milk fat content of this FA was 17% lower ($P = 0.01$; Table 2.4) in grazing cows, the source of increased levels of 18:0 in milk of grazing cows was likely not adipose.

The relatively lower milk content of ALA of grazing cows in our study (0.57% vs. 0.9 ± 0.2% in the review by Vahmani et al., 2013) was likely due to a lower concentration of ALA in our pasture (48% of total FA; Table 2.1) relative to levels observed in previous studies (50-75% of total FA; Chilliard et al., 2007). The concentration of ALA in pasture can be affected by several factors such as season, species composition and stage of plant growth (Dewhurst et al., 2006). Although there was a tendency ($P = 0.09$) for higher milk fat content of n-3 PUFA, we did not observe any improvement in the n6:n3 ratio of milk from grazing cows which coincided with a concurrent increase in the content of n-6 PUFA, particularly CLA, in milk from grazing cows (Table 2.4).

The estimated transfer efficiency of supplemental DHA into milk in our study is close to the average value (4.1%) for unprotected marine oils (Chilliard et al., 2001).

However, high variation in transfer efficiency for both unprotected and protected fish oil (unprotected: 1.6-16.2% vs. protected: 5.1-18.0%) and microalgae (unprotected: 1.60-8.40% vs. protected: 2.0-16.7%) was found in other studies (Cant et al., 1997; Franklin et al., 1999; Kitessa et al., 2004; Castaneda-Gutierrez et al., 2007; Osborne et al., 2008; Stamey et al., 2012). This variation can be explained by differences in the amount of supplemental lipid fed and its content of DHA, efficacy of ruminal protection, duration of feeding trials and basal diet composition (Chilliard et al., 2001; Chilliard et al., 2007). The low transfer efficiency of EPA and DHA into milk has been attributed to RBH, as well as preferential incorporation into the blood phospholipid and cholesterol ester fractions, which are less efficiently used by the mammary compared to triacylglycerol and NEFA (Chilliard et al., 2001).

Despite the relatively low transfer efficiency, feeding FO or MA increased the milk fat content of n-3 LC-PUFA 1.5 fold compared with the control (Table 2.4). Using the average consumption (~ 700 g/d) of milk equivalent (~ 3.6% milk fat basis) in the U.S. and Canada (Informa Economics, 2010), our enriched milk would provide ~ 59 mg/d more n-3 LC-PUFA than the control milk. The intake of n-3 LC-PUFA in the U.S. and Canada is estimated to be 100-200 mg/d (Kris-Etherton et al., 2000; Denomme et al., 2005), which is considerably lower than the recommended adequate intake of 650 mg/d (Simopoulos et al., 1999).

The higher milk fat concentration of *trans* 18:1 and CLA (Table 2.4) in grazing cows compared with confined cows was related mainly to increased concentrations of VA and RA respectively (Table 2.5). Vaccenic acid is an intermediate in the RBH of OA, LA and ALA, and is the predominant RBH intermediate found in ruminant milk and meat

(Shingfield et al., 2008). Rumenic acid is the predominant CLA isomer found in ruminant fat and can be produced either in the rumen as a RBH intermediate, or via mammary Δ^9-desaturase action on VA (Shingfield et al., 2008; Shingfield et al., 2010). Mammary desaturase activity accounts for 64-97% of milk RA (Shingfield et al., 2010); therefore, dietary treatments that increase ruminal production of VA (e.g. feeding pasture) will increase milk fat content of both VA and RA. Besides higher intake of ALA, a precursor to VA, ruminal conditions in grazing cows (e.g. ruminal microbiome, pH, rumen fill and digesta kinetics) could also contribute to higher ruminal production/outflow of VA (Mohammed et al., 2009). While the exact role of many of these factors remains to be elucidated, the higher ruminal passage rate and lower ruminal pH in grazing cows compared with TMR-fed cows (Schroeder et al., 2004) could lead to a greater outflow of RBH intermediates such as VA from the rumen. Besides RA, the predominate CLA isomer, we observed a higher milk fat concentration of *trans*-11, *cis*-13 CLA in grazing cows compared with confined cows (Table 2.5). This CLA isomer is produced in the rumen as an intermediate in the RBH of ALA and is known as the second major CLA isomer (after RA) found in milk produced on Alpine pastures (Chilliard et al., 2007).

Supplementation of dairy cows with marine oils stimulates ruminal production of VA, increasing milk fat content of VA, as well as its Δ^9- desaturation product, RA (Chilliard et al., 2007). We observed significant improvements in milk fat content of these two FA only when FO or MA were fed to confined cows, but not when these supplements were fed to grazing cows (Figure 2.1). We found that supplementation of grazing cows with FO or MA increased the relative concentration (% of total *trans* 18:1) of several other *trans* 18:1 isomers (*trans*-12 to *trans*-16 18:1) at the expense of VA

(Table 2.6). In contrast to our results, previous studies consistently reported improvements in milk fat content of VA and RA when grazing cows were supplemented with marine oils (AbuGhazaleh et al., 2007; AbuGhazaleh and Holmes, 2007; Brown et al., 2008). However, in these studies, marine oils were fed along with a source of LA and ALA (e.g. sunflower oil). In studies (Rego et al., 2005; Kitessa et al., 2004; Glover et al., 2012) in which marine oils were fed alone (with no plant oils) to grazing cows, *trans* 18:1 and CLA were reported as sums, not as individual isomers. Many factors could be involved in the different responses of confined and grazing cows in terms of milk VA and RA, to feeding FO or MA in our study. Some of these factors may include differences in intake and type of RBH substrates (i.e. LA and ALA), ruminal pH, and ruminal microbiome. The ruminal production of VA may have also reached its maximum in grazing cows; therefore, feeding FO or MA resulted in an increase in the production of alternative RBH intermediates (e.g. *trans*-12 to *trans*-16 18:1; Table 2.6).

Besides VA, which is considered beneficial (Shingfield et al., 2008), feeding FO or MA increased milk fat levels of several other *trans* 18:1 isomers, and the extent of the increase was greater with MA than with FO (Table 2.5). This phenomenon could be due to the higher proportion (about two times) of DHA in MA compared to FO (Table 2.1). AbuGhazaleh and Jenkins (2004a) suggested that compared with EPA, DHA is a stronger inhibitor of RBH, resulting in the accumulation of higher levels of RBH intermediates (e.g. *trans* 18:1 isomers) in the rumen. Generally, *trans* FA have been associated with increased risk of cardiovascular disease (CVD) by increasing blood LDL cholesterol and reducing HDL cholesterol (Gebauer et al., 2007). Ruminant foods contribute about 20% of total *trans* FA intake of North Americans (Shingfield et al., 2008). The major dietary

source of *trans* FA (i.e. *trans* 18:1) however, is partially hydrogenated vegetable oil (**PHVO**; 80% of total *trans* FA intake). In contrast to PHVO *trans* FA, there is no evidence that ruminant *trans* FA are associated with CVD risk (Shingfield et al., 2008) due to the difference in profile of *trans* 18:1 between PHVO and ruminant fat (Gebauer et al., 2007). The predominant *trans* 18:1 in ruminant fat is VA (40-70% of total *trans* 18:1), whereas *trans*-6−8, *trans*-9 and *trans*-10 18:1 are predominant in PHVO, comprising more than 60% of total trans 18:1 (Shingfield et al., 2008). The health effects of individual *trans* 18:1 isomers (other than VA) are negative or unknown. Improving the milk FA profile should target those FA considered beneficial to human health while identifying and lowering those that are potentially harmful.

2.6 Conclusions

Our results confirm previous research showing milk produced in pasture systems has a more healthful FA profile than that of confinement systems. Compared with confined cows, grazing cows produced milk with a higher content of beneficial UFA (OA, VA, RA and ALA) and lower content of 12:0-16:0. The use of pastures in dairy systems in temperate North America could improve the image of milk fat which is currently associated only with high content of SFA and less of beneficial UFA. In addition to the beneficial effects of pasture on milk FA composition, feeding FO or MA could also enhance the milk fat concentration of n-3 LC-PUFA and reduce the content of 16:0 in both pasture and confinement systems without compromising milk fat yield. However, feeding FO or MA interacted with MS in terms of milk fat content of VA and RA. In contrast to confined cows, marine oil supplements did not improve milk fat content of VA and RA in grazing cows. This could be due to differences between grazing

and confinement in terms of intake of RBH substrates and ruminal environment. Supplementing dairy cow diets with FO or MA resulted in increased milk fat levels of several *trans* 18:1 isomers other than VA, the health effects of which are unknown. Thus, more studies are needed on human health consequences of modifying the UFA composition of milk fat by feeding oils to dairy cows.

2.7 Acknowledgments

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Table 2.1 Fatty acid composition, g FA/100 g of total FA (values in parentheses represent g/d of the FA) of feeds and lipid supplements

Fatty acid	Concentrate	Pasture	TMR[1]	MA[2]	FO[3]
14:0	0.15	0.43	0.48	5.31(17.4)	0.40(8.0)
16:0	11.95	10.93	14.53	25.02(51.1)	12.67(25.3)
18:0	2.09	1.21	1.98	32.48(75.1)	38.68(77.4)
18:1 *cis*-9	21.69	2.30	13.46	0.06(0.1)	4.10(8.2)
18:2 *cis*-9, *cis*-12	51.69	13.23	35.46	ND[4]	0.57(1.13)
18:3 *cis*-9, *cis*-12, *cis*-15	2.24	48.14	13.36	ND	1.29(2.58)
20:5 n-3	ND	ND	ND	1.61(3.0)	15.85(31.7)
22:5 n-3	ND	ND	ND	ND	2.01(4.0)
22:5 n-6	ND	ND	ND	9.54(17.7)	ND
22:6 n-3	ND	ND	ND	24.23(45.0)	12.33(24.7)
SFA[5]	15.62	15.81	21.09	62.80(143.6)	51.74(110.7)
MUFA	24.56	7.76	19.44	0.06(0.11)	7.35(14.7)
PUFA	56.77	71.35	56.05	35.51(66.9)	36.33(72.0)
C20-22 PUFA	0.13	0.77	0.36	35.51(65.9)	32.62(65.3)
C20-22 n-3 PUFA	0.04	0.28	0.11	25.84(48.0)	30.18(60.3)

[1]TMR was fed from 10 to 95 ± 5 DIM to the confinement group only.
[2]Rumen-protected microalgae.
[3]Rumen-protected fish oil.
[4]Values < 0.05% were considered not detectable (ND).
[5]Total saturated FA.

Table 2.2 Ingredient and nutritive composition of feeds

Item	TMR[1]	Concentrate	Pasture
Ingredient, % of DM			
Corn silage	22.46		
Grass silage	28.61		
2nd cut hay	6.10		
Barley	10.40	24.30	
Corn distillers, dried	4.30	10.05	
Energy Booster®[2]	0.95	2.21	
Soybean meal, 48% CP	10.64	24.85	
Corn grain	10.40	24.30	
TOP SOY[3]	3.55	8.28	
Limestone	0.95	2.21	
Dicalcium phosphate	0.24	0.55	
Sodium bicarbonate	0.47	1.10	
Magnesium oxide	0.05	0.11	
Yeast Culture[4] (dehydrated)	0.24	0.55	
Iodized salt	0.38	0.88	
Vitamin and mineral mix[5]	0.26	0.61	
Nutrient content, (DM basis)			
DM, % of as fed	53.22	90.65	24.37
CP, %	17.89	25.68	16.59
NE_L, Mcal/kg	1.76	2.06	1.53
NDF, %	31.95	13.93	41.27
ADF, %	18.53	5.93	28.63
Ash, %	8.02	9.67	9.14
Ether extract, %	4.64	6.05	3.94

[1]TMR was fed from 10 to 95 ± 5 DIM to the confinement group only. [2]Contained primarily 16:0 and 18:0 (Energy Booster®, Milk Specialties Co., Dundee, IL).
[3]SHUR-GAIN TOP SOY (bypass soybean meal), Shur-Gain, Guelph, ON, Canada.
[4]*Saccahromyces cerevisiae* culture (Diamond V. Mills, Cedar Rapids, IA).
[5]Contained 0.48% Cu, 0.73% Zn, 0.1% Co, 105 ppm Se, 150,000 IU/kg vitamin A, 1600,000 IU/kg vitamin D and 13,500 IU/kg vitamin E.

Table 2.3 Effect of management system and lipid supplement on milk yield, milk composition, BW, BCS and DMI[1]

	Management system[2]			Lipid supplement[3]				Effect (*P*-value)[4]		
Item	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	MS×LS
Milk yield, kg/d	34.02	40.09	4.14	36.48	35.57	39.11	4.21	0.05	0.15	0.77
3.5%FCM[5], kg/d	32.76	40.45	3.80	37.64	35.09	37.08	3.89	0.04	0.40	0.95
Fat, %	3.18	3.46	0.09	3.56[a]	3.40[a]	3.00[b]	0.11	0.16	<0.01	0.49
Fat, kg/d	1.10	1.41	0.12	1.34	1.21	1.21	0.13	0.05	0.22	0.98
Protein[*], %	2.81	3.04	0.05	2.96	2.97	2.85	0.05	0.08	0.09	0.07
Protein, kg/d	0.95	1.22	0.12	1.09	1.06	1.11	0.12	0.02	0.67	0.75
Lactose, %	4.57	4.62	0.04	4.57	4.64	4.58	0.05	0.42	0.42	0.36
Lactose, kg/d	1.56	1.86	0.19	1.67	1.66	1.80	0.19	0.05	0.24	0.89
BW, kg	610	665	33.2	630	634	648	29.7	0.27	0.59	0.29
BCS[6]	2.51	2.83	0.07	2.63	2.70	2.68	0.08	0.10	0.75	0.98
DMI[*7], kg/d	19.26	23.41	2.02	21.95	20.71	21.34	2.05	0.02	0.23	0.94

[a–b]Values within lipid supplement and in the same row not sharing a common superscript are significantly different ($P \leq 0.05$).
[1]Least squares means are from combined 60 and 90 DIM.
[2]PAS = pasture system; CON = confinement system.
[3]Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA) or control (CO; no marine oil supplement).
[4]Effects of management system (MS), lipid supplement (LS) and their interaction (MS × LS).
[5]3.5% fat corrected milk.
[6]Body condition score (1 = thin to 5 = fat; Wildman et al., 1982).
[7]DMI of grazing cows based on net energy balance (Cosgrove and Cooper, 2007).
[*]Significant DIM × MS ($P \leq 0.05$).

Table 2.4 Effect of management system and lipid supplement on milk fatty acid composition (g/100 g of total FA)[1]

	Management system[2]			Lipid supplement[3]				Effect (*P*-value)[4]		
Fatty acid[5]	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	MS×LS
10:0	2.26	2.69	0.48	2.57	2.51	2.37	0.52	0.39	0.93	0.19
12:0	2.65	4.13	0.26	3.73^{a}	3.59^{a}	2.85^{b}	0.27	0.03	<0.01	0.37
14:0	8.40	11.66	0.41	10.88^{a}	10.29^{a}	8.93^{b}	0.44	0.01	<0.01	0.50
14:1 *cis*-9	0.97	1.36	0.06	1.29	1.17	1.04	0.08	0.05	0.08	0.42
15:0	0.94	1.20	0.04	1.15^{a}	1.07^{ab}	0.98^{b}	0.05	0.02	0.01	0.30
16:0	25.09	30.30	0.50	29.86^{a}	27.19^{b}	26.04^{b}	0.61	0.02	<0.01	0.66
16:1 *cis*-9	1.24	1.26	0.09	1.30	1.17	1.27	0.08	0.88	0.23	0.19
17:0	0.76	0.75	0.01	0.75	0.76	0.75	0.01	0.90	0.77	0.41
18:0	11.16	9.16	0.34	10.19	10.62	9.68	0.40	0.04	0.23	0.51
18:1 *cis*-9[*]	19.95	15.08	0.54	16.91	17.00	18.64	0.64	0.02	0.11	0.93
18:2 n-6	2.02	2.06	0.07	1.98	2.02	2.11	0.08	0.66	0.28	0.55
18:3 n-3	0.57	0.44	0.02	0.49	0.51	0.51	0.02	0.04	0.88	0.48
20:5 n3	0.09	0.09	<0.01	0.06^{a}	0.14^{b}	0.07^{a}	0.01	0.74	<0.01	0.35
22:5 n6	0.02	0.03	<0.01	0.01^{a}	0.01^{a}	0.06^{b}	<0.01	0.34	<0.01	0.12
22:5 n3	0.09	0.08	<0.01	0.06^{a}	0.13^{b}	0.06^{a}	0.01	0.82	<0.01	0.17
22:6 n3	0.13	0.11	0.01	0.02^{a}	0.14^{b}	0.20^{c}	0.01	0.33	<0.01	0.13
SFA	54.51	63.32	1.24	61.72^{a}	59.40^{a}	55.63^{b}	1.32	0.01	<0.01	0.25
OBCFA	3.30	3.40	0.10	3.43	3.39	3.23	0.11	0.39	0.15	0.83
cis-MUFA	23.37	19.01	0.59	20.59	20.90	22.07	0.64	0.03	0.18	0.59
trans-MUFA	6.54	4.85	0.18	4.14^{a}	5.70^{b}	7.24^{c}	0.22	0.02	<0.01	0.90
cis-18:1	20.99	16.28	0.56	17.88	18.15	19.87	0.66	0.02	0.07	0.91
trans-18:1	5.93	4.43	0.17	3.67^{a}	5.22^{b}	6.64^{c}	0.21	0.02	<0.01	0.85
CLA	1.38	0.81	0.04	0.92^{a}	1.12^{b}	1.24^{b}	0.05	0.01	<0.01	0.24
n-6 PUFA	3.70	3.17	0.07	3.24^{a}	3.40^{ab}	3.67^{b}	0.08	0.03	<0.01	0.20
n-3 PUFA	0.94	0.81	0.03	0.69^{a}	0.99^{b}	0.94^{b}	0.04	0.09	<0.01	0.56
n-3 LC-PUFA	0.31	0.30	0.01	0.15^{a}	0.41^{b}	0.35^{b}	0.02	0.70	<0.01	0.84
n-6:n-3	4.09	4.06	0.11	4.77^{a}	3.47^{b}	3.99^{c}	0.13	0.86	<0.01	0.85
SFA:UFA	1.66	2.41	0.07	2.39^{a}	2.00^{b}	1.72^{c}	0.08	0.01	<0.01	0.08

[a–c]Values within lipid supplement and in the same row not sharing a common superscript are significantly different ($P \leq 0.05$).

[1]Least squares means from 90 DIM.

[2]PAS = pasture system; CON = confinement system.

[3]Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA) or control (CO; no marine oil supplement).

[4]Effects of management system (MS), lipid supplement (LS) and their interaction (MS × LS).

[5]SFA = total saturated FA; OBCFA = total odd- and branched-chain FA; CLA = total conjugated linoleic acid isomers; n-3 LC-PUFA = n-3 long-chain (C20-22) PUFA; SFA:UFA = saturated to unsaturated FA ratio.

[*]Co-elutes with *cis*-10 18:1.

Table 2.5 Effect of management system and lipid supplement on *trans* 18:1 and conjugated 18:2 isomers (g/100 g of total FA)[1]

	Management system[2]			Lipid supplement[3]				Effect (*P*-value)[4]		
trans 18:1 isomer	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	MS×LS
trans-4	0.03	0.04	<0.01	0.03	0.03	0.04	<0.01	0.49	0.59	0.46
trans-5	0.03	0.04	<0.01	0.03	0.03	0.04	<0.01	0.16	0.52	0.86
trans-6-*trans*-8	0.37	0.35	0.02	0.27[a]	0.37[b]	0.45[c]	0.02	0.44	<0.01	0.56
trans-9	0.33	0.32	0.01	0.23[a]	0.34[b]	0.42[c]	0.01	0.61	<0.01	0.54
trans-10	0.54	0.50	0.03	0.36[a]	0.53[b]	0.65[c]	0.03	0.49	<0.01	0.92
trans-11[5]	2.79	1.29	0.09	1.61	1.94	2.40	0.11	0.01	<0.01	0.03
trans-12	0.38	0.45	0.04	0.25[a]	0.45[b]	0.54[c]	0.02	0.11	<0.01	0.72
trans-13/*trans*-14	0.72	0.80	0.04	0.46[a]	0.83[b]	0.99[c]	0.04	0.19	<0.01	0.28
trans-15	0.28	0.28	0.01	0.17[a]	0.31[b]	0.37[c]	0.01	0.93	<0.01	0.08
trans-16	0.42	0.37	0.04	0.26[a]	0.40[b]	0.47[c]	0.01	0.06	<0.01	0.08
Conjugated 18:2 isomer										
cis-9, *trans*-11[6]	1.02	0.51	0.03	0.62	0.74	0.87	0.13	0.01	<0.01	0.04
trans-9, *cis*-11	0.03	0.03	0.01	0.02[a]	0.03[b]	0.03[b]	<0.01	0.55	0.03	0.51
cis-11, *trans*-13	0.05	0.04	<0.01	0.03[a]	0.06[b]	0.05[b]	0.01	0.58	<0.01	0.16
trans-10, *cis*-12	0.01	0.01	<0.01	0.01	0.01	0.01	<0.01	0.98	0.57	0.09
trans-11, *cis*-13[7]	0.06	0.04	<0.01	0.04[a]	0.05[b]	0.05[b]	<0.01	0.03	0.02	0.69
trans-11, *trans*-13	0.02	0.01	0.01	0.01	0.02	0.02	0.01	0.12	0.16	0.68
Other *trans*-*trans*[8]	0.14	0.12	0.01	0.11[a]	0.14[b]	0.15[b]	0.01	0.35	0.01	0.73

[a–c]Values within lipid supplement and in the same row not sharing a common superscript are significantly different ($P \leq 0.05$).
[1]Least squares means from 90 DIM.
[2]PAS = pasture system; CON = confinement system.
[3]Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA) or control (CO; no marine oil supplement).
[4]Effects of management system (MS), lipid supplement (LS) and their interaction (MS × LS).
[5]Vaccenic acid.
[6]Rumenic acid; co-elutes with *trans*-7, *cis*-9 and *trans*-8, *cis*-10 18:2.
[7]Co-elutes with *cis*-9, *cis*-11 18:2.
[8]*trans*-7, *trans*-9 + *trans*-8, *trans*-10 + *trans*-9, *trans*-11 + *trans*-10, *trans*-12 18:2.

Table 2.6 The *trans* 18:1 isomer distribution (% of total *trans*-18:1) of milk fat from Holstein cows on pasture or in confinement receiving no marine oil supplement (control; CO), rumen-protected fish oil (FO) or rumen-protected microalgae (MA)[1]

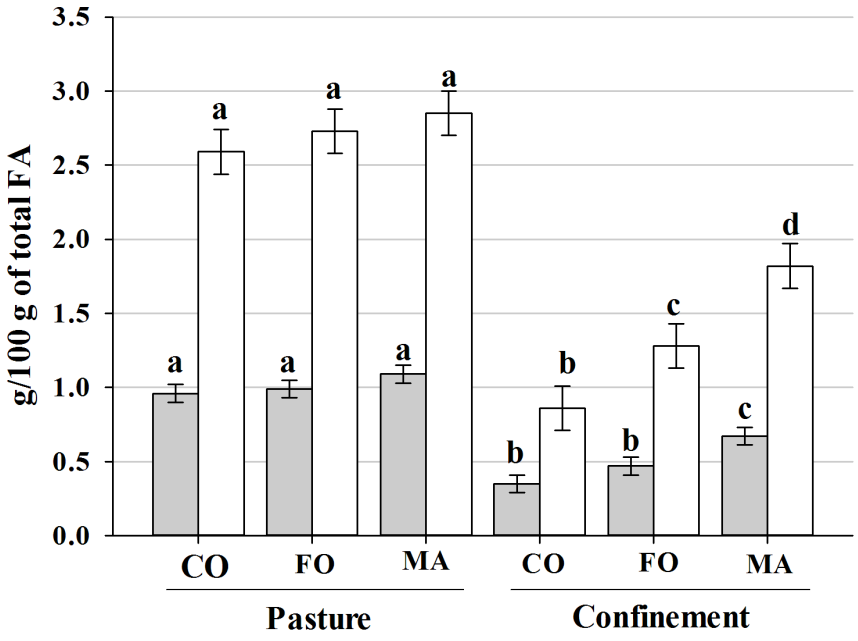
	Pasture			Confinement				Effect (*P*-value)[2]		
trans 18:1 isomer	CO	FO	MA	CO	FO	MA	SEM	MS	LS	MS×LS
trans-4	0.58	0.56	0.44	1.26	0.74	0.88	0.16	0.08	0.06	0.17
trans-5	0.56	0.47	0.42	1.34	0.88	0.91	0.14	0.06	0.07	0.34
*trans*6-*trans*8	7.09	6.61	6.06	9.03	7.59	7.63	0.36	0.08	<0.01	0.09
trans-9	5.89	6.05	5.62	7.56	7.09	7.13	0.44	0.13	0.33	0.35
trans-10	9.81	9.63	10.63	11.10	11.42	11.33	0.93	0.38	0.64	0.66
trans-11	53.2^{a}	45.2^{b}	43.5^{b}	29.0^{c}	28.6^{c}	29.8^{c}	2.05	0.02	<0.01	<0.01
trans-12	5.22^{a}	7.26^{b}	7.55^{b}	9.38^{c}	10.57^{d}	10.19^{cd}	0.40	0.01	<0.01	0.04
*trans*13/*trans*14	8.74^{a}	13.07^{b}	14.08^{b}	17.74^{cd}	19.53^{c}	17.07^{d}	0.51	<0.01	<0.01	<0.01
trans-15	3.68^{a}	5.13^{b}	5.22^{b}	6.38^{c}	7.07^{d}	6.08^{c}	0.22	<0.01	<0.01	<0.01
trans-16	4.51^{a}	6.21^{b}	6.16^{b}	7.24^{c}	7.46^{c}	6.26^{d}	0.22	0.02	<0.01	<0.01

$^{a–d}$Values within each row not sharing a common superscript are significantly different ($P \leq 0.05$); Letter grouping was performed only when MS×LS was significant ($P \leq 0.05$) to show differences among all 6 treatment combinations.

[1]Least squares means from 90 DIM.

[2]Effects of management system (MS), lipid supplement (LS) and their interaction (MS × LS).

Figure 2.1 Least squares means showing management system × lipid supplement interactions for milk fat content of *trans*-11 18:1 (vaccenic acid; white bars) and *cis*-9, *trans*-11 CLA (rumenic acid; gray bars) in Holstein cows on pasture or in confinement receiving no marine oil supplement (control; CO), rumen-protected fish oil (FO) or rumen-protected microalgae (MA); Least squares means within fatty acid type with different letters are significantly different ($P \leq 0.05$). Error bars represent 2 × SEM.



Chapter 3 Effects of Management System (Pasture vs. Confinement) and Marine Oil Supplementation on Lipogenic Gene Expression in Mammary, Liver and Adipose Tissues of Lactating Dairy Cows

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3.1 Abstract

Research was conducted to evaluate the impact of diet on the expression of genes encoding for key proteins involved in lipid metabolism that affect the fatty acid (FA) profile of milk in lactating dairy cows. Effects of management system (MS) and marine lipid supplementation (LS) on the relative mRNA abundance of genes encoding proteins required for FA uptake (LPL), *de novo* FA synthesis (ACACA, FASN), FA desaturation (SCD1, FAD1, FAD2) and transcriptional regulation of lipogenesis (SREBF1, SCAP, INSIG1, THRSP and PPARG) in mammary, liver and subcutaneous adipose (SUBQ) tissues were studied. Forty-eight peripartal Holstein cows were blocked by parity and predicted calving date and assigned to either a pasture (n = 23) or confinement (n = 25) system. Within each system, cows were allocated randomly to a control (no oil supplement) or one of two isolipidic (200 g/d) supplements, fish oil (FO) or microalgae (MA), for 125 ± 5 days starting 30 days pre-calving. Tissues were collected from four cows (100 ± 2 DIM) from each treatment combination (24 cows in total) for gene expression analysis using quantitative real-time PCR. The mammary mRNA abundance of PPARG (−32%) and FASN (−29%) was lower in grazing compared to confined cows which was accompanied by reduced (−43%) secretion of *de novo* synthesized FA in milk. Grazing was associated with reduced expression of ACACA (−48%), FASN (−48%) and THRSP (−53%) in SUBQ which was consistent with the lower BCS (i.e. lower net adipose tissue deposition) in grazing compared to confined cows. No effect of MS was observed regarding lipogenic gene expression in liver. However, hepatic lipogenic gene expression was greatly affected by LS. Feeding either FO or MA down-regulated hepatic expression of lipogenic genes including FASN, SCD1, FASD2 and THRSP. In

mammary, both FO and MA reduced the expression of SREBF1 (−15%) compared with the control. However, the mammary transcription of lipogenic enzymes and milk FA secretion were not affected by LS. Lipid supplementation did not affect the expression of any of the genes in SUBQ. The present data confirm previous research that gene transcription is an important regulatory mechanism for lipogenesis and that MS and LS have tissue specific effects on lipogenic gene expression in dairy cattle which have important influences on cow performance and healthfulness of the milk FA profile.

3.2 Introduction

Dairy milk and its products are major sources of fat in American diets contributing an estimated 19% of total fat and 32% of saturated fat consumed (Weinberg, et al., 2004). In milk fat, certain saturated fatty acids (**SFA)** including 12:0, 14:0 and 16:0 are considered detrimental to human health, while other FA such as 4:0-10:0, *cis*-9 18:1 (oleic acid; **OA**), 18:3 n-3 (α-linolenic acid; **ALA**), *cis*-9, *trans*-11 conjugated linoleic acid (*cis*-9, *trans*-11 **CLA,** common name rumenic acid; **RA**) are considered beneficial (Shingfield et al., 2008). The SFA 4:0-14:0 plus ~50% of 16:0 in milk are synthesized in the mammary gland from acetate and butyrate whereas the remaining milk FA (16-24 carbons in length) originate from the diet (Shingfield et al., 2010). Feeding plant or marine oils to dairy cows has been shown to improve the milk fat content of beneficial unsaturated FA (**UFA**) including OA, n-3 PUFA and RA and reduce the concentration of SFA including 12:0-16:0 (Chilliard et al., 2007). The latter effect has been attributed mainly to down-regulation of mammary lipogenic gene expression by specific ruminal biohydrogenation (**RBH**) intermediates such as *trans*-10, *cis*-12 CLA (Harvatine et al., 2009a; Shingfield et al., 2010).

Previous studies (Chilliard et al., 2007; Vahmani et al., 2013) have shown that compared to cows fed conserved forages and more grain in confinement, grazing cows produce milk fat with a higher content of beneficial UFA and lower content of detrimental SFA. However, the effect of dairy farm management system (**MS**; pasture vs. confinement) or its interaction with PUFA supplementation on lipogenic gene expression have not been studied.

Previous research has established that PUFA supplementation down-regulates lipogenic gene expression in liver and adipose tissue of rodents, resulting in decreased lipogenesis in these tissues (Jump, 2002; Wang and Jones, 2004). However, studies investigating effects of PUFA supplementation on lipogenic gene expression in extra-mammary tissues in dairy cattle are scarce. Recently, Harvatine et al. (2009b) reported that intravenous infusion of *trans*-10, *cis*-12 CLA reduced mammary expression of lipogenic genes in dairy cows causing severe milk fat depression; however, the opposite effect (i.e. increased lipogenic gene expression) was seen in adipose tissue. To evaluate the tissue specific effect of PUFA supplementation in dairy cows, the objective of this study was to determine the effect of **MS** and marine lipid supplementation (**LS**) and their interaction on mRNA abundance of genes encoding proteins required for FA uptake, *de novo* FA synthesis, desaturation and transcriptional regulation of lipogenesis in mammary, liver and subcutaneous adipose (**SUBQ**) in lactating dairy cows. We hypothesized that grazing would reduce mRNA abundance of genes involved in milk fat synthesis. Secondly, we hypothesized that the lipogenic gene expression response in mammary to LS would be different from that in extra-mammary tissues including liver

and SUBQ. We further hypothesized that there would be an interaction between MS and LS on expression of lipogenic genes in these tissues.

3.3 Materials and Methods

3.3.1 Experimental Design, Treatments and Animal Measurements

All procedures performed on cows in this study were according to the Canadian Council for Animal Care guidelines and were approved by the Animal Care and Use Committee at the Faculty of Agriculture of Dalhousie University. Details of the experimental design and animal management have been reported elsewhere (Chapter 2)[1]. Briefly, 48 peripartal Holstein cows were blocked by parity and predicted calving date and assigned within block to either a pasture (n = 23) or confinement (n = 25). Within system cows were allocated randomly to a control (no oil supplement) or to one of two isolipidic (200 g/d) marine oil supplements: fish oil (**FO**) or microalgae (**MA**), for 125 ± 5 days starting 30 days pre-calving. Both supplements provided similar amounts of long chain (C20-C22) PUFA (**LC-PUFA**; ~ 65 g/d), but contained different proportions of EPA and DHA. The predominant LC-PUFA in MA was DHA (24% of total FA), whereas EPA and DHA were equally predominant and comprised about 27% of total FA in FO. Fatty acid composition of lipid supplements is presented in Chapter 2 (Table 2.1)[2]. The experiment was conducted as a split-plot design with MS as the whole plot treatment, and LS as the sub-plot treatment. Both pasture and confinement groups were housed in a tie-stall barn from −30 until 28 ± 10 days in milk (**DIM**) and were fed TMR with similar formulations. The pasture group was then adapted to a pasture and grazed

[1]Referred to as Vahmani et al., 2013 in the submitted manuscript.
[2]Referred to as supplemental Table S1 in the submitted manuscript.

rotationally on a perennial sward until the end of the experiment (95 ± 5 DIM). The confinement group remained in the tie-stall barn and was fed a TMR (forage/concentrate ratio of 56:44, DM basis) *ad libitum* twice daily (0700 and 1500 h). Grazing cows were fed 8.0 kg concentrate/d (as-fed basis) in equal portions at milking (0600 and 1600 h). The concentrate was similar to that used in the TMR for confined cows. The nutrient and FA composition of feeds are available in Chapter 2 (Tables 2.1 and 2.2)[3]. Cows had continuous access to water.

3.3.2 Blood Collection and Analyses

Blood samples were collected from the coccygeal vein immediately before the morning feeding on 60 and 90 DIM. The samples were allowed to clot for 3 h at room temperature and centrifuged (15 min at 3,000 × g). Serum samples were transported on dry ice to the Animal Health Laboratory (Guelph, Ontario) for analysis of BUN, glucose, NEFA and BHBA using commercially available kits and a COBAS analyzer (Roche Cobas 6000- c501, Roche Diagnostics, Indianapolis, IN).

3.3.3 Tissue Collection

At 100 ± 2 DIM, four cows from each treatment combination (24 cows in total) were transferred to a commercial abattoir immediately after morning milking for tissue sampling. Mammary, liver and SUBQ (between the hook and pin bones) were sampled immediately after exsanguination, snap frozen in liquid nitrogen and stored at −80°C until RNA extraction.

3.3.4 RNA Extraction and cDNA Synthesis

[3] Referred to as supplemental Tables S1 and S2 in the submitted manuscript.

Total RNA was isolated from 200 mg of mammary and liver tissues using the RNeasy® Midi Kit, and total RNA in SUBQ was extracted from 400 mg of tissue using the RNeasy® Lipid Tissue Midi Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Concentration of RNA was determined by absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and absorbance was measured to ensure the absorbance ratios of 260:280 and 260:230 were between 1.8 and 2.1 for all RNA samples. Integrity of RNA was confirmed by visualization of the 28s and 18s ribosomal RNA bands after electrophoresis of 500 ng of each RNA sample on a 1% agarose gel stained with ethidium bromide. One μg of total RNA from each sample was reverse-transcribed in a final volume of 20 μL using the Quantitect® Reverse Transcription kit with genomic DNA (gDNA) Wipeout (Qiagen, Valencia, CA) according to the manufacturer's instructions. The cDNA samples were diluted 1:50 in DNase/RNase free water and stored at −30°C until analysis by quantitative real-time PCR (**qRT-PCR**). Serial dilutions (5 point, 5-fold dilution) of pooled cDNA (made from undiluted cDNA samples) were prepared for each tissue type for generation of standard curves for qRT-PCR.

3.3.5 Primer Design and Evaluation, and Quantitative PCR

Primers were either designed using Primer 3.0 software (http://frodo.wi.mit.edu/primer3/input.htm) or drawn from previous publications. Sequences and other details of primers used in this study are presented in Table 3.1. The primers were subjected to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure specificity of the primer and lack of homology with non-target sequence. Primer specificity was verified by agarose gel electrophoresis of amplified cDNA (PCR product)

and by melt-curve analysis following qRT-PCR. Each primer pair produced a single PCR product of expected size when visualized on agarose gel and yielded a single melt curve peak.

Quantitative real-time PCR was performed in a MicroAmp™ Optical 96-well reaction plate (Applied Biosystems) in a final volume of 20 µL per well containing 8.4 µL of diluted cDNA, 10 µL of FastStart Universal SYBR Green Master Mix (ROX; Roche Applied Science) and 0.8 µL each of 10 µM forward and reverse primers. Each plate contained all 24 samples within each tissue type in duplicate for the gene being evaluated, a 5-point standard curve of pooled cDNA in triplicate, as well as non-template and reverse transcription negative controls. The reactions were performed in an ABI Step-One-Plus real-time PCR system (Applied Biosystems) with initial denaturing for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C (denaturation) and 1 min at 57.4°C (annealing + extension). This was followed by a melt curve analysis to ensure specific amplification. Amplification efficiency (E) was calculated using the slope of the standard curve according to the following equation: $E = 10^{(-1/slope)} - 1$ (Step-One Plus™ software version 2.1, Applied Biosystems). Real-time PCR runs with efficiencies between 90 and 110% were considered acceptable and used for data analysis. Relative mRNA levels were calculated from the standard curve on each PCR plate using the Step-One Plus™ Software (version 2.1, Applied Biosystems, CA). Expression of genes involved in FA uptake [lipoprotein lipase (**LPL**)], *de novo* FA synthesis [acetyl- coenzyme A carboxylase (**ACACA**), fatty acid synthase (**FASN**)], FA desaturation [stearoyl-CoA desaturase1 (**SCD1**), fatty acid desaturase 1 (**FADS1**), fatty acid desaturase 2 (**FADS2**)] and transcriptional regulation of lipogenesis [sterol regulatory element binding

transcription factor 1 (**SREBF1**), SREBF chaperone (**SCAP**), insulin induced gene 1 (**INSIG1**), thyroid hormone responsive spot 14 (**THRSP**), peroxisome proliferator activated receptor-γ (**PPARG**)] were measured in mammary, liver and SUBQ tissues.

In addition to the target genes, the relative mRNA levels for six candidate internal control genes (**ICG**) were determined in all samples for each tissue. These genes included mitochondrial ribosomal protein L39 (**MRPL39**), ubiquitously expressed transcript (**UXT**), eukaryotic translation initiation factor 3, subunit K (**EIF3K**), β-Actin (**ACTB**), peptidylprolyl isomerase A (**PPIA**/cyclophilin A) and ribosomal proteins 9 (**RPS9**). Results were analyzed using geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/) to determine gene expression stability (Vandesompele et al., 2002). For liver, UXT, EIF3K and RPS9 were the most stably expressed, while UXT, EIF3K and MRPL39 were the most stable in mammary and SUBQ tissues. The relative mRNA levels of the target genes were normalized using the geometric mean of the three selected ICG.

3.3.6 Fatty Acid Analysis

Milk FA were analyzed by gas liquid chromatography according to the modified method of Kramer et al. (2008) as described in Chapter 2[4]. Yield of FA (g/d) were calculated according to Glasser et al. (2007) and were subsequently converted to molar yield (moles/day).

3.3.7 Statistical Analysis

Data were analyzed as a split design using the MIXED Procedure of SAS (SAS Inst. Inc., Cary, NC) as described in Chapter 2[4]. Normalized mRNA abundance data were

[4] Referred to as Vahmani et al. (2013) in the submitted manuscript.

square-root transformed before statistical analysis. Least squares means were separated using the PDIFF statement of SAS. Statistical significance was declared at $P \leq 0.05$ and tendencies at $0.06 \leq P \leq 0.10$.

3.4 Results

3.4.1 Animal Performance, Energy Balance and Blood Metabolites

Dry matter intake, milk yield and milk composition were determined on 60 and 90 DIM and data have been described in Chapter 2[4]. Briefly, DMI (19.26 vs. 23.40 kg/d), milk production (34.0 vs. 40.1 kg/d), milk fat (1.10 vs. 1.41 kg/d), protein (0.95 vs. 1.22 kg/d) and lactose (1.56 vs. 1.86 kg/d) were lower ($P \leq 0.05$) for pasture compared with confinement. The effect of LS on milk production and milk composition (yields and contents) was significant ($P \leq 0.05$) only for milk fat content which was reduced with MA compared with FO (3.00 vs. 3.40%) and the control (3.56%).

No interactions between MS and LS (**MS × LS**) were observed regarding energy balance variables ($P \geq 0.11$; Table 3.2). Energy intake and plasma content of glucose were lower ($P \leq 0.03$) for grazing cows than that for confined cows. Body condition score and calculated net energy balance (**EBAL**) tended ($P = 0.10$) to be lower for grazing cows compared with those in confinement. Concurrently, grazing cows also tended ($P = 0.06$) to have higher plasma concentrations of NEFA and BHBA. None of the energy balance variables was affected by LS ($P \geq 0.13$). Blood urea nitrogen was unchanged by either MS or LS ($P \geq 0.18$).

3.4.2 Milk Fatty Acid Secretion

No significant MS×LS were observed ($P \geq 0.21$) for molar yields of any of the individual FA or the summations (Table 3.3). Secretion of 12:0, 14:0 and 16:0 and 18:2

n-6 in milk were lower ($P \leq 0.01$) for grazing cows than for confined cows. Concurrently, the secretion of *trans*-11 18:1 (vaccenic acid; **VA**) and RA was higher ($P \leq 0.05$), and the secretion of total CLA tended ($P = 0.07$) to be higher for grazing cows. The output of *de novo* synthesized FA in milk was lower ($P = 0.02$) for grazing cows compared with those in confinement. However, MS did not affect ($P = 0.87$) the output of preformed FA (i.e. FA taken up from blood). The molar and mass yield of total FA tended ($P = 0.07$) to be lower for pasture than that for confinement.

Supplementation with MA increased ($P \leq 0.02$) the molar yield of VA and RA compared with the control (Table 3.3), while feeding FO tended ($P = 0.07$) to increase the yield of these FA relative to the control. Both FO and MA increased ($P \leq 0.01$) the yield of *trans*-10 18:1 relative to the control and the effect was greater with MA (+33%) than with FO (+61%). The yield of *trans*-10, *cis*-12 CLA however, was not affected by LS. Supplementation with either FO or MA increased ($P \leq 0.02$) the secretion of total *trans* 18:1 and total CLA compared with the control. Lipid supplementation did not affect ($P \geq 0.13$) the output of any of the *de novo* synthesized FA in milk. The desaturation indexes were not significantly affected ($P \geq 0.12$) by either FS, LS or their interaction (Table 3.3).

3.4.3 Gene Expression

No significant MS×LS were observed ($P \geq 0.12$) for the expression of any of the genes in mammary, liver and SUBQ (Tables 3.4-3.6)[5]. Tissues responded differently to the treatments in terms of relative mRNA abundance. In mammary, the expression of LPL ($P = 0.08$), FASN ($P = 0.06$), FADS1 ($P = 0.08$) and SCAP ($P = 0.09$) tended to be lower for pasture than that for confinement. The expression of PPARG and FADS2 was

[5]Tables 3.4-3.6 are combined in one table in the submitted manuscript.

significantly ($P \leq 0.04$) lower for cows on pasture compared with those in confinement. Supplementation with either FO or MA reduced ($P = 0.02$) the expression of mammary SREBF1. Concurrently, MA increased ($P \leq 0.04$) the abundance of SCAP mRNA compared with FO and the control.

In SUBQ, expression of ACACA, FASN, INSIG1 and THRSP tended to be lower ($P \leq 0.07$) for pasture than for confinement (Table 3.5). Lipid supplementation did not affect ($P \geq 0.14$) the mRNA expression of any of the genes evaluated in SUBQ.

Management system did not affect ($P \geq 0.20$) the expression of any of the genes evaluated in liver except ACACA whose expression tended ($P = 0.10$) to be higher in grazing cows (Table 3.6). However, compared with mammary and SUBQ, more genes were significantly affected by LS in liver. The expression of FASN, SCD1, FADS2 and THRSP was reduced ($P \leq 0.02$) by both FO and MA. The expression of FADS1 was reduced ($P = 0.01$) with MA compared with the control, and was not significantly ($P = 0.13$) affected with FO. The abundance of SREBF1 mRNA tended ($P = 0.07$) to be reduced with either FO or MA, while the SCAP mRNA levels tended ($P = 0.08$) to be increased by LS.

3.5 Discussion

3.5.1 Energy Balance and Blood Metabolites

The lower EBAL of cows on pasture compared with those in confinement was expected due to lower energy intake and higher activity associated with grazing (Bargo et al., 2002). The lower blood glucose level in grazing cows is consistent with previous studies and is mainly related to the lower intake of non-structural carbohydrate compared with confined cows (Kay et al., 2005). The increased levels of plasma NEFA and BHBA

in grazing cows are consistent with lower EBAL which is associated with greater mobilization of adipose FA compared with confined cows (Agenas et al., 2002; Kay et al., 2005). Consistent with the increased catabolism in adipose tissue, we observed a tendency ($P = 0.10$) for lower BCS in grazing compared with confined cows (Table 3.2).

3.5.2 Milk Fatty Acid Secretion and Mammary Lipogenic Gene Expression

The reduced mammary levels of FASN mRNA (−29%; $P = 0.06$) in grazing compared with confined cows was accompanied by reduced (−43%; $P = 0.02$) secretion of *de novo* synthesized FA in milk. This observation is consistent with the role of FASN in milk fat synthesis which is to produce even-chain FA with 4-16 carbons (Bernard et al., 2008). We are unaware of comparable studies in which effects of MS on mammary gene expression in dairy cows were measured. Studies where cows were fed milk fat depressing diets (i.e. low-forage/high-oil diets) show down-regulation of mammary lipogenic enzyme gene expression (e.g. ACACA, FASN and SCD), which was associated with increased milk *trans*-10 18:1 and *trans*-10, *cis*-12 CLA (Ahnadi et al., 2002; Harvatine and Bauman, 2006; Angulo et al., 2012). Contrary to these studies, we did not observe greater concentrations of either *trans*-10, *cis*-12 CLA or *trans*-10 18:1 in milk from grazing cows despite lower mammary FASN gene expression and depressed secretion of *de novo* synthesized FA. This discrepancy could be due to other differences between pasture and confinement treatments including energy intake, ruminal VFA production, PUFA intake and production of other RBH intermediates. Further investigation is needed to elucidate the role of these factors in regulating milk fat synthesis in dairy cows.

Despite the lower LPL expression (−26%; $P = 0.06$) in mammary tissue of grazing cows, which is responsible for the uptake of preformed FA from circulating chylomicrons and low-density lipoproteins, the yield of preformed FA in milk was similar ($P = 0.87$) between treatments. Consistent with the current study, Angulo et al., 2012 observed that reduced mammary expression of LPL in cows fed milk fat depressing diets was not accompanied by lower secretion of preformed FA in milk, whereas in the same study the reduction in milk *de novo* synthesized FA was accompanied by down-regulation of FASN in mammary. Conversely, increased secretion of C18 FA in milk of goats supplemented with plant oils was not accompanied by increased mammary LPL mRNA abundance or activity (Bernard et al., 2009). The authors related this observation to the fact that other factors such as substrate availability (i.e. availability of circulating preformed FA) might play a more important role than LPL activity in regulating the secretion of preformed FA in ruminants.

The relatively lower mammary expression of FADS1 and FADS2 in grazing cows could be due to the higher intake of ALA on pasture which could directly or indirectly (i.e. through increased ruminal production of RBH intermediates) reduce the expression or activity of these enzymes (Chuang et al., 2004; Zhu et al., 2010). The extent of down-regulation was more pronounced for FADS2 (−35%, $P = 0.03$) than for FADS1 (−19%, $P = 0.08$). Other information on this effect in the cow was not found; however, in mice feeding flaxseed oil, a rich source of ALA, also reduced hepatic expression of FADS2 mRNA but did not affect that of FADS1 (Zhu et al., 2010). Fatty acid *desaturase* 1 and 2 are expressed in many tissues (e.g. brain, liver, heart and placenta) and are essential for the synthesis of highly unsaturated FA (e.g. 20:4 n6, 20:5 n-3 and 22:6 n-3) from 18:2 n-

6 and 18:3 n-3 (Nakamura and Nara, 2003). In the current study, despite the reduced mammary expression of FADS1 and FADS2 in grazing cows, the secretion of 20:5 n-3 and 22:6 n-3 in milk was not different between pasture and confinement. This could be due to the fact that the majority of dietary PUFA including ALA, the precursor for synthesis of EPA and DHA, is biohydrogenated in the rumen and that the conversion of ALA of to 20:5 n-3 and 22:6 n-3 is very limited in mammals (Burdge, 2006), including dairy cows (Loor et al., 2005; Ponter et al., 2006).

We observed a 32% reduction ($P = 0.04$) in for the mammary expression of PPARG, a ligand-activated nuclear receptor that plays a key role in transcriptional regulation of various proteins involved in lipogenesis and adipogenesis (Kersten, 2001) in grazing cows compared with confined cows. In bovine mammary tissue, this transcription factor may be involved in the regulation of milk fat synthesis (Bionaz and Loor, 2008). These authors observed an up-regulation of PPARG transcription at the onset of lactation (~3-fold relative to -15 DIM), which was sustained throughout lactation. Furthermore, ligand activation of PPARG in bovine mammary cells resulted in up-regulation of lipogenic genes including ACACA and FASN (Kadegowda et al., 2009). Our results combined with observations from previous studies suggest that the lower mammary expression of FASN and the ensuing reduced secretion of *de novo* synthesized FA in milk of grazing cows compared with confined cows might be related in part to the down-regulation of mammary PPARG gene expression.

Feeding FO or MA reduced ($P = 0.02$) the expression of SREBF1 in mammary by 15% compared with the control. Sterol regulatory element binding factor 1 is a transcription factor that activates the transcription of key lipogenic enzymes including

those involved in milk fat synthesis (Harvatine et al., 2009a). Down-regulation of mammary lipogenic genes (e.g. ACACA, FASN and SCD) during diet-induced milk fat depression has been related to reductions in the transcription and activation of SREBF1 by specific RBH intermediates particularly *trans*-10 *cis*-12 CLA (Peterson, et al., 2004; Harvatine and Bauman, 2006; Angulo et al., 2012). In the current study milk *trans*-10, *cis*-12 CLA content was not affected by LS; however both FO and MA increased milk fat content of *trans*-10 18:1 (the RBH product of *trans*-10, *cis*-12 CLA) which has also been associated with diet induced milk fat depression in dairy cows (Shingfield et al., 2010). Contrary to previous findings, the reduced mammary expression of SREBF1 in cows fed marine oils in the current study was not accompanied by either reduced gene expression of lipogenic enzymes or reduced milk fat yield. This discrepancy could be due to the higher doses of supplemental lipids used in previous studies (3.1-4.5% vs. 1.0% DM) and the subsequent higher degree of down-regulation of SREBF1 (−15% vs. −27-35%) in the current study. Thus, it is possible that the down-regulation of SREBF1 by FO or MA in the current study was insufficient to alter the gene expression of lipogenic enzymes.

Supplementation with MA resulted in an increase in the expression (+28%; P = 0.04) of SCAP, a SREBF1 regulatory protein required for the proteolytic activation of SREBF1. Although not statistically significant, feeding FO resulted in a numerically greater mRNA expression of SCAP than that of control. This result is in agreement with Invernizzi et al. (2010) who reported a sustained up-regulation of SCAP expression in mammary tissue of cows fed a milk fat depressing diet containing a blend of fish/soybean oil for 21 days. Bionaz and Loor (2008) observed a gradual increase in the mammary mRNA abundance of SCAP of dairy cows throughout the lactation cycle. The increased

mammary expression of SCAP in cows fed oil supplements may be an adaptive response to maintain milk fat synthesis (Invernizzi et al, 2010). Further studies are needed to determine the role of SCAP in milk fat synthesis.

3.5.3 mRNA Expression of Lipogenic Genes in SUBQ

The greater expression of ACACA (+94%; $P = 0.06$) and FASN (+92%; $P = 0.06$) in SUBQ of confined cows compared with that of grazing cows (Table 3.5) is suggestive of increased adipose *de novo* lipogenesis and is consistent with the greater BCS in confined cows (Table 3.2). Dietary energy intake above the amounts needed for maintenance, growth, reproduction and activity is the major determinant of the rate of adipogenesis in animals. Rocco and McNamara (2013) observed that energy-restriction in early lactation reduced the rate of lipogenesis in SUBQ. Conversely, energy overfeeding resulted in up-regulation of most of the lipogenic genes in SUBQ including ACACA, FASN, LPL, SCD1, INSIG1, SCAP and PPARG, in peripartal dairy cows (Ji et al., 2012), and ACACA, FASN and SCD in steers (Duckett et al., 2009). Therefore, the reduced expression of ACACA and FASN in SUBQ of grazing cows was likely due to the lower energy intake and subsequent lower substrate availability for lipogenesis (Tables 3.2 and 3.5). Among the transcriptional regulators evaluated in SUBQ, the only significant effect was for THRSP whose expression was greater (+114%; $P = 0.06$) in confined cows than that in grazing cows. THRSP is a nuclear protein and in the rodent liver and adipose tissue, expression of this protein was induced by lipogenic stimuli such as thyroid hormone (T_3) and dietary carbohydrates, which in turn increased the rate of lipogenesis (Kinlaw et al., 1995; Obregon, 2008). There is little information about the function of THRSP in ruminants particularly in dairy cows. Wang et al., 2009 reported

that THRSP mRNA abundance in longissimus muscle from high-marbling beef heifers was highly correlated with intramuscular fat content. In another beef study (Graugnard et al., 2009), THRSP expression in Longissimus lumborum muscle of growing steers was positively correlated with blood levels of both glucose and insulin, as well as with gene expression of lipogenic enzymes including ACACA, FASN and SCD1. Furthermore, feeding high starch diets resulted in up-regulation of THRSP mRNA in subcutaneous (Duckett et al., 2009) and intramuscular (Graugnard et al., 2009) adipose tissues of steers. Graugnard et al. (2009) suggested that THRSP might play an important role in transcriptional regulation of adipogenesis in cattle. Similarly in the current study, the increased expression of ACACA and FASN in SUBQ of confined cows, which relates positively to their higher energy intake, may be mediated by THRSP. More studies will have to be conducted to determine the role of this transcription factor in the regulation of lipogenesis and adipogenesis in dairy cows.

Feeding milk fat depressing diets containing a blend of fish oil and soybean oil (3.5% of dietary DM) to cows up-regulated the SUBQ expression of lipogenic genes including LPL, SCD1 and THRSP (Thering et al., 2009). They suggested that the greater abundance of lipogenic mRNA in SUBQ is related to the repartitioning of energy from milk fat production to adipogenesis. In the present study, we did not observe any effect of LS on lipogenic gene expression in SUBQ which is probably related to the lack of an LS treatment effect on milk fat yield.

3.5.4 Hepatic Lipogenic Gene Expression

The response of hepatic lipogenic gene expression to LS was similar to what has previously seen in rodents where dietary PUFA suppressed the transcription of several

lipogenic genes in liver including SREBF1, THRSP, FASN, ACACA, SCD1, FAD1 and FAD2 (Jump and Clarke, 1999; Jump, 2002; Matsuzaka et al., 2002). In contrast to humans and rodents where liver is the primary site of lipogenesis, ruminant liver has a very limited lipogenic capacity particularly for FA synthesis (Bergen and Mersmann, 2005). The mRNA abundance of ACACA, FASN and LPL was low in the liver of dairy goats compared to that of adipose and mammary tissues (Bernard et al., 2009). Conversely, SCD1 was found to be highly expressed in the liver of lactating ewes (Ward et al., 1998) and goats (Bernard et al., 2009), implying that liver might play a role in Δ-9 *desaturation* of absorbed FA. Herdmann et al. (2010) observed that the liver of beef cattle had a higher content of LC n-3 PUFA compared to erythrocytes, muscle and adipose tissues, which was further increased by feeding ALA suggesting that elongase and desaturase (i.e. FAD1 and FAD2) enzymes are active in the liver of ruminants.

In rodents and humans, PUFA can reduce the accumulation of triacylglycerol (**TAG**) in liver by enhancing hepatic FA oxidation and suppressing *de novo* FA and TAG synthesis (Jump and Clarke, 1999; Jump, 2002). The effect of PUFA on both FA oxidation and lipogenesis is mediated mainly via transcriptional regulatory mechanisms involving PPARα and SREBF1. Polyunsaturated fatty acids are ligand activators of PPARα, which up-regulates the expression of genes involved in FA oxidation. In contrast, PUFA suppress both transcription and activation of SREBF1 resulting in down-regulation of genes involved in lipid synthesis. Adding flaxseed to diets of peripartal dairy cows reduced hepatic TAG concentrations after calving. The authors suggested that feeding a source of n-3 PUFA to peripartal cows might reduce the risk of developing fatty liver during the transition period (Petit et al., 2007). However, in a comparable study

in which pre-partal cows were supplemented with fish oil (0.9% of dietary DM), hepatic TAG content was not affected after calving despite an increased concentration of LC n-3 PUFA in the liver (Ballou et al., 2009). Although we did not measure the hepatic content of TAG or expression of genes involved in TAG synthesis, the reduced expression of SREBF1 in MA fed cows may have diminished hepatic TAG synthesis. More detailed studies are needed to confirm whether feeding LC n-3 PUFA during the transition period can help prevent fatty liver in dairy cows.

3.6 Conclusions

The reduced secretion of *de novo* synthesized FA in milk from grazing cows compared with confined cows was associated with lower mammary expression of lipogenic genes including FASN and PPARG suggesting that at least part of the difference in milk FA secretion between pasture and confinement systems could be mediated at the level of gene transcription. In contrast to results from MFD studies, the reduced *de novo* synthesized FA in milk of grazing cows in current study was not related to milk *trans*-10 18:1 or *trans*-10 *cis*-12 CLA, implying the importance of other factors (e.g. other RBH intermediates and availability of substrates for lipogenesis) in controlling synthesis of milk FA.

Reduced expression of lipogenic genes (ACACA, FASN and THRSP) in the SUBQ of grazing cows in comparison to confined cows was consistent with a lower BCS (i.e. lower adipose tissue deposition) and was related to the lower energy intake of grazing cows.

The effect of LS on lipogenic gene expression was tissue specific with the greatest response observed in liver despite the minor role of liver in lipid synthesis in

ruminants. Both FO and MA down-regulated hepatic expression of several lipogenic genes which could be related to the preferential deposition of LC n-3 PUFA in liver (Herdmann et al., 2010). Our results confirm previous research that gene transcription is an important regulatory mechanism for lipogenesis and that MS and LS have tissue specific effects on lipogenic gene expression in dairy cattle which have important influences on cow performance and healthfulness of the milk FA profile.

3.7 Acknowledgments

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Table 3.1 Primers used for quantitative real-time PCR

Gene[1]	Accession No.[2]	Direction	Primer sequence (5' to 3')	Source[3]
ACACA	NM_174224	Sense	AGCACGCCAGGTTCTTATTG	5
		Antisense	AAATCGACGTTTCGGACAAG	
ACTB	AY141970	Sense	GCGTGGCTACAGCTTCACC	3
		Antisense	TTGATGTCACGGACGATTTC	
PPIA	BC105173	Sense	CTTTCACAGAATAATTCCGGG	5
		Antisense	CAGTACCATTATGGCGTGTGA	
EIF3K	NM_001034489	Sense	CCAGGCCCACCAAGAAGAA	4
		Antisense	TTATACCTTCCAGGAGGTCCATG	
FADS1	XM_612398	Sense	GTCAGCCTTCAATGACTGGT	5
		Antisense	GGGCTTGGACTGGTACTTTA	
FADS2	AY731088	Sense	AAGACGGCTGAGGACATGAG	5
		Antisense	GCAAGGACAACTGCCGTAAT	
FASN	NM_001012669	Sense	GCTAGGCATGGAGTCCTCTG	5
		Antisense	ATCAGCGCGTAGTAGGCTGT	
INSIG1	XM_589325	Sense	AAAGTTAGCAGTCGCGTCGTC	2
		Antisense	TTGTGTGGCTCTCCAAGGTGA	
LPL	NM_001075120	Sense	TGGACGGTGACAGGAATGTA	5
		Antisense	GTCCCACCAGCTTGGTGTAT	
MRPL39	NM017446	Sense	AGGTTCTCTTTTGTTGGCATCC	4
		Antisense	TTGGTCAGAGCCCCAGAAGT	
PPARG	NM_181024	Sense	CCAAATATCGGTGGGAGTCG	2
		Antisense	ACAGCGAAGGGCTCACTCTC	
RPS9	DT860044	Sense	CCTCGACCAAGAGCTGAAG	1
		Antisense	CCTCCAGACCTCACGTTTGTTC	
SCAP	DV935188	Sense	CCATGTGCACTTCAAGGAGGA	2
		Antisense	ATGTCGATCTTGCGTGTGGAG	
SCD1	AF188710	Sense	ACCATCACAGCACCTCCTTC	5
		Antisense	TACTCAAGCTTGGGCTTTGG	
THRSP	AY656814	Sense	CTACCTTCCTCTGAGCACCAGTTC	2
		Antisense	ACACACTGACCAGGTGACAGACA	
SREBF1	TC263657	Sense	CCAGCTGACAGCTCCATTGA	2
		Antisense	TGCGCGCCACAAGGA	
UXT	NM_001037471	Sense	CAGCTGGCCAAATACCTTCAA	4
		Antisense	GTGTCTGGGACCACTGTGTCAA	

[1]ACACA = acetyl-CoA carboxylase; ACTB = ß-actin; PPIA = peptidylprolyl isomerase A (cyclophilin A); EIF3K= eukaryotic translation initiation factor 3, subunit K; FADS1= Fatty acid desaturase 1 (delta-5 desaturase); FADS2 = fatty acid desaturase 2 (delta-6 desaturase); FASN = fatty acid synthase; INSIG1 = insulin induced gene 1; LPL = lipoprotein lipase; MRPL39 = mitochondrial ribosomal protein L39; PPARG = peroxisome proliferator-activated receptor γ; RPS9 = ribosomal protein S9; SCAP = SREBF chaperone; SCD1 = stearoyl-CoA desaturase 1 (delta-9-desaturase); THRSP = thyroid hormone responsive (SPOT14); SREBF1 = sterol regulatory binding factor-1; UXT = ubiquitously expressed transcript; [2]GeneBank sequences; [3]1 = Bionaz and Loor, 2007; 2 = Bionaz and Loor, 2008; 3 = Harvatine et al, 2009; 4 = Kadegowda et al, 2009; 5 = this paper.

Table 3.2 Effect of management system and lipid supplement on energy intake, energy balance, BW, BCS and blood metabolites[1]

	Management system[2]			Lipid supplement[3]				Effect (*P*-value)[4]		
Item	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	MS×LS
NE_L intake,* Mcal/d	34.20	42.03	3.30	38.52	37.47	38.35	3.34	0.02	0.65	0.97
EBAL[5] Mcal/d	2.25	4.73	0.88	3.44	2.78	3.64	0.99	0.10	0.56	0.11
BW, kg	610	665	33.2	630	634	648	29.7	0.27	0.59	0.29
BCS[6]	2.51	2.83	0.07	2.63	2.70	2.68	0.08	0.10	0.75	0.98
Glucose* mmol/L	2.63	3.10	0.06	2.81	2.91	2.87	0.08	0.03	0.65	0.20
BHBA* umol/L	898	520	99	721	644	735	93	0.06	0.16	0.84
NEFA** mmol/L	0.16	0.12	0.01	0.15	0.13	0.14	0.01	0.06	0.13	0.36
BUN mmol/L	5.48	5.17	0.24	5.58	5.47	4.91	0.28	0.47	0.18	0.81

[a–b]Values within lipid supplement and in the same row not sharing a common superscript are significantly different ($P \leq 0.05$).
[1]Least square means are from combined 60 and 90 DIM.
[2]PAS = pasture system; CON = confinement system.
[3]Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA) or control (CO; no marine oil supplement) for 120 ± 5 days starting −30 DIM.
[4]*P*-values for the effects of management system (MS), lipid supplement (LS) and their interaction (MS × LS).
[5]Calculated net energy balance = net energy intake − (net energy for maintenance + net energy for lactation).
[6]Body condition score (1 = thin to 5 = fat; Wildman et al., 1982).
*Significant DIM × MS ($P \leq 0.05$); **Significant MS × LS × DIM ($P \leq 0.05$).

Table 3.3 Effect of management system and lipid supplement on milk fatty acid yield (moles/day)[1]

	Management system[2]			Lipid supplement[3]				Effect (*P*-value)[4]		
FA[5]	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	MS×LS
10:0	0.135	0.196	0.050	0.164	0.174	0.159	0.052	0.21	0.94	0.33
12:0	0.133	0.260	0.034	0.216	0.202	0.170	0.035	0.02	0.13	0.40
14:0	0.379	0.662	0.070	0.570	0.518	0.474	0.073	0.02	0.17	0.46
14:1 *cis*-9	0.031	0.055	0.006	0.048	0.041	0.041	0.006	0.05	0.55	0.85
15:0	0.040	0.065	0.006	0.059	0.051	0.048	0.007	0.04	0.19	0.35
16:0	1.002	1.523	0.135	1.408	1.200	1.180	0.144	0.03	0.11	0.35
16:1 *cis*-9	0.048	0.065	0.007	0.060	0.051	0.056	0.007	0.10	0.40	0.38
17:0	0.029	0.036	0.003	0.033	0.032	0.031	0.003	0.10	0.73	0.54
18:0	0.420	0.412	0.032	0.434	0.428	0.385	0.036	0.82	0.40	0.97
18:1 *trans*	0.218	0.205	0.024	0.157[a]	0.218[b]	0.259[b]	0.026	0.60	0.00	0.89
18:1 *cis*	0.728	0.738	0.064	0.711	0.734	0.754	0.069	0.87	0.78	0.78
18:1 *trans*-10	0.020	0.024	0.002	0.015[a]	0.022[b]	0.029[c]	0.003	0.14	<0.01	0.47
18:1 *trans*-11	0.101	0.058	0.011	0.061[a]	0.081[ab]	0.098[b]	0.012	0.04	0.01	0.97
18:1 *cis*-9	0.726	0.714	0.063	0.690	0.719	0.752	0.068	0.83	0.60	0.71
18:2 n-6	0.072	0.095	0.011	0.084	0.083	0.083	0.011	0.07	0.97	0.95
18:3 n-3	0.021	0.019	0.003	0.020	0.021	0.020	0.003	0.57	0.91	0.93
Total CLA	0.049	0.037	0.005	0.034[a]	0.045[b]	0.050[b]	0.005	0.07	0.01	0.96
*c*9, *t*11 CLA	0.038	0.024	0.004	0.024[a]	0.031[ab]	0.036[b]	0.004	0.05	0.02	0.97
*t*10, *c*12 CLA	0.002	0.002	0.000	0.002	0.002	0.002	0.000	0.30	0.45	0.43
20:5 n3	0.003	0.004	0.000	0.002[a]	0.005[b]	0.002[a]	0.000	0.11	<0.01	0.31
22:5 n3	0.003	0.003	0.000	0.002	0.004	0.002	0.000	0.13	<0.01	0.89
22:6 n3	0.003	0.004	0.000	0.000[a]	0.005[b]	0.008[c]	0.001	0.47	<0.01	0.69
De novo	0.739	1.299	0.153	1.111	1.023	0.922	0.159	0.02	0.22	0.54
Preformed	1.565	1.584	0.156	1.456	1.637	1.631	0.165	0.87	0.29	0.69
Total FA moles/d	3.607	4.676	0.473	4.318	4.109	3.997	0.495	0.07	0.66	0.67
Total FA g/d	1058.2	1316.0	128.1	1188.8	1217.7	1154.9	130.7	0.07	0.80	0.21
C14 index	0.108	0.109	0.006	0.110	0.102	0.112	0.007	0.89	0.56	0.92
C16 index	0.046	0.040	0.003	0.042	0.041	0.046	0.003	0.28	0.13	0.13
C18 index	0.639	0.621	0.012	0.627	0.615	0.649	0.013	0.33	0.12	0.59

[a–b]Values within lipid supplement and in the same row not sharing a common superscript are significantly different ($P \leq 0.05$); [1]Least square means from 90 DIM; [2]PAS = pasture system; CON = confinement system; [3]Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA) or control (CO; no marine oil supplement) for 125 ± 5 days starting −30 DIM; [4] *P*-values for the effects of management system (MS), lipid supplement (LS) and their interaction (MS × LS). [5] *trans* 18:1 = total *trans* 18:1 isomers (*trans*-4 to *trans*-16 18:1); Total CLA = total conjugated linoleic acid isomers; *c*9, *t*11 CLA = *cis*-9, *trans*-11 CLA; *t*10, *c*12 CLA = *trans*-10, *cis*-12 CLA; *De novo* = Sum of FA with chain lengths from 6 to 14; Preformed = sum of FA with chin lengths from 18 to 24.

[*] C14 Desaturation index = *cis*-9 14:1/(14:0 + *cis*-9 14:1); C16 Desaturation index = *cis*-9 16:1/(16:0 + *cis*-9 16:1); C18 Desaturation index = *cis*-9 18:1/(18:0 + *cis*-9 18:1).

Table 3.4 Effect of management system and lipid supplement on mRNA expression of genes coding for lipogenic enzymes, desaturation enzymes and transcription regulators in mammary tissue of lactating Holstein cows [1]

	Management system[2]			Lipid supplement[3]				Effect (*P*-value)[4]		
Gene[5]	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	MS×LS
LPL	1.540	2.069	0.131	1.870	1.739	1.805	0.142	0.08	0.75	0.92
ACACA	1.594	1.846	0.099	1.669	1.660	1.830	0.111	0.15	0.36	0.49
FASN	1.536	2.156	0.140	1.763	1.807	1.968	0.158	0.06	0.52	0.31
SCD1	1.538	1.938	0.125	1.785	1.714	1.715	0.130	0.15	0.89	0.82
FADS1	1.552	1.918	0.131	1.677	1.705	1.823	0.141	0.08	0.52	0.12
FADS2	1.421	2.177	0.131	1.857	1.749	1.791	0.144	0.03	0.78	0.13
SREBF1	1.687	1.874	0.063	1.980[a]	1.671[b]	1.691[b]	0.077	0.17	0.02	0.34
SCAP	1.442	1.898	0.130	1.518[a]	1.644[a]	1.948[b]	0.146	0.09	0.04	0.24
INSIG1	1.237	1.743	0.207	1.483	1.439	1.548	0.246	0.20	0.95	0.18
THRSP	1.510	1.971	0.114	1.929	1.729	1.564	0.138	0.11	0.21	0.20
PPARG	1.589	2.348	0.193	1.847	1.841	2.216	0.200	0.04	0.09	0.12

[a–b]Values within lipid supplement and in the same row not sharing a common superscript are significantly different ($P \leq 0.05$).

[1]Least squares means for mRNA levels (expressed in arbitrary units) at 100 DIM.

[2]PAS = pasture system; CON = confinement system.

[3]Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA) or control (CO; no marine oil supplement) from −30 to 100 DIM.

[4]*P*-values for the effects of management system (MS), lipid supplement (LS) and MS by LS interaction (MS × LS).

[5]LPL = lipoprotein lipase; ACC = acetyl- coenzyme A carboxylase; FASN = fatty acid synthase; SCD1 = stearoyl-CoA desaturase1 (Δ^9-desaturase); FADS1= Fatty acid desaturase 1 (Δ^5-desaturase); FADS2 = Fatty acid desaturase 2 (Δ^6-desaturase); SREBF1 = Sterol regulatory element binding transcription factor-1 ; SCAP = SREBF chaperone; INSIG1 = Insulin induced gene 1; THRSP = Thyroid hormone responsive (SPOT14); PPARG = peroxisome proliferator activated receptor-γ.

Table 3.5 Effect of management system and lipid supplement on mRNA expression of genes coding for lipogenic enzymes, desaturation enzymes and transcription regulators in subcutaneous adipose tissue of lactating Holstein cows [1]

	Management system[2]			Lipid supplement[3]				Effect (*P*-value) [4]		
Gene[5]	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	MS×LS
LPL	1.201	1.550	0.224	1.182	1.400	1.544	0.228	0.27	0.28	0.94
ACACA	0.975	1.887	0.183	1.164	1.528	1.601	0.216	0.06	0.29	0.85
FASN	0.890	1.707	0.223	1.052	1.389	1.455	0.245	0.06	0.25	0.92
SCD1	0.900	1.802	0.239	1.077	1.400	1.575	0.235	0.12	0.24	0.52
FADS1	1.648	1.470	0.239	1.884	1.516	1.278	0.265	0.53	0.14	0.14
FADS2	1.700	1.449	0.341	1.894	1.431	1.399	0.366	0.47	0.29	0.17
SREBF1	1.486	1.430	0.175	1.625	1.535	1.399	0.263	0.84	0.38	0.82
SCAP	1.436	1.549	0.143	1.433	1.507	1.537	0.160	0.59	0.86	0.77
INSIG1	0.818	1.579	0.146	1.210	1.033	1.353	0.178	0.07	0.47	0.51
THRSP	0.750	1.605	0.159	0.955	1.251	1.327	0.178	0.06	0.29	0.46
PPARG	1.608	1.339	0.134	1.488	1.317	1.616	0.149	0.18	0.22	0.96

[a–b]Values within lipid supplement and in the same row not sharing a common superscript are significantly different ($P \leq 0.05$).

[1]Least squares means for mRNA levels (expressed in arbitrary units) at 100 DIM.

[2]PAS = pasture system; CON = confinement system.

[3]Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA) or control (CO; no marine oil supplement) from −30 to 100 DIM.

[4]*P*-values for the effects of management system (MS), lipid supplement (LS) and MS by LS interaction (MS × LS).

[5]LPL = lipoprotein lipase; ACC = acetyl- coenzyme A carboxylase; FASN = fatty acid synthase; SCD1 = stearoyl-CoA desaturase1 (Δ^9-desaturase); FADS1= Fatty acid desaturase 1 (Δ^5-desaturase); FADS2 = Fatty acid desaturase 2 (Δ^6-desaturase); SREBF1 = Sterol regulatory element binding transcription factor-1 ; SCAP = SREBF chaperone; INSIG1 = Insulin induced gene 1; THRSP = Thyroid hormone responsive (SPOT14); PPARG = peroxisome proliferator activated receptor-γ.

Table 3.6 Effect of management system and lipid supplement on mRNA expression of genes coding for lipogenic enzymes, desaturation enzymes and transcription regulators in liver of lactating Holstein cows[1]

	Management system[2]			Lipid supplement[3]				Effect (*P*-value) [4]		
Gene[5]	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	MS×LS
LPL	1.142	1.209	0.116	1.193	1.214	1.119	0.110	0.73	0.77	0.14
ACACA	1.378	1.012	0.094	1.299	1.189	1.096	0.115	0.10	0.48	0.82
FASN	1.354	1.263	0.090	1.612[a]	1.189[b]	1.124[b]	0.111	0.55	0.02	0.80
SCD1	1.186	1.152	0.101	1.684[a]	1.145[b]	0.678[c]	0.124	0.83	0.00	0.31
FADS1	1.230	1.198	0.124	1.578[a]	1.237[ab]	0.828[b]	0.152	0.87	0.01	0.21
FADS2	1.196	1.233	0.099	1.667[a]	1.055[b]	0.922[b]	0.122	0.82	0.00	0.73
SREBF1	1.179	1.302	0.061	1.369	1.220	1.094	0.075	0.29	0.07	0.85
SCAP	1.273	1.196	0.087	1.123	1.307	1.211	0.081	0.60	0.08	0.40
INSIG1	1.196	1.122	0.177	1.310	1.089	1.079	0.191	0.73	0.46	0.19
THRSP	1.034	1.205	0.094	1.479[a]	1.087[b]	0.792[b]	0.115	0.33	0.00	0.73
PPARG	1.786	2.122	0.158	1.633	2.241	1.987	0.195	0.27	0.12	0.85

[a–b]Values within lipid supplement and in the same row not sharing a common superscript are significantly different ($P \leq 0.05$).

[1]Least squares means for mRNA levels (expressed in arbitrary units) at 100 DIM.

[2]PAS = pasture system; CON = confinement system.

[3]Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA) or control (CO; no marine oil supplement) from −30 to 100 DIM.

[4]*P*-values for the effects of management system (MS), lipid supplement (LS) and MS by LS interaction (MS × LS).

[5]LPL = lipoprotein lipase; ACC = acetyl- coenzyme A carboxylase; FASN = fatty acid synthase; SCD1 = stearoyl-CoA desaturase1 (Δ^9-desaturase); FADS1= Fatty acid desaturase 1 (Δ^5-desaturase); FADS2 = Fatty acid desaturase 2 (Δ^6-desaturase); SREBF1 = Sterol regulatory element binding transcription factor-1 ; SCAP = SREBF chaperone; INSIG1 = Insulin induced gene 1; THRSP = Thyroid hormone responsive (SPOT14); PPARG = peroxisome proliferator activated receptor-γ.

Chapter 4 Conclusions

It is contingent upon the agriculture sector to produce healthful food, while creating profitable opportunities in farming. Dairy products are characterized in part by their fat content, but bovine milk fat is a complex material containing varying amounts of FA of different type which are produced by the extensive ruminal transformation of dietary FA, during which UFA are biohydrogenated, creating some beneficial FA such as VA and RA, while destroying desirable PUFA. The mammary gland contributes most of the OA and RA in milk fat via the action of mammary Δ-9 *desaturase* on SA and VA respectively.

Feed and feeding management differ significantly among dairy production systems with consequences on milk composition. Milk is increasingly produced in the temperate agricultural zones using year round total confinement of cows and to a lesser and shrinking extent, pasture systems which employ grazing for up to half the year. In Chapter 1, I reviewed the effects of the system of production on milk fat composition and implications for human health. On pasture, cows consume fresh forage with grain fed typically twice daily at milking compared to confined cows that are fed conserved forages, corn silage and grains in a total mixed ration. Fresh forage is high in ALA. This PUFA as well as the products of its RBH VA and RA, beneficial FA, are incorporated into milk fat. Depressed ruminal pH combined with high ruminal passage rate in grazing cows can reduce the efficiency of RBH and increase transfer rate of dietary UFA and their RBH intermediates to milk while depressing mammary synthesis of SFA. The results from Chapter 2 show that compared to confinement, milk produced in a pasture system has a FA profile characterized by a lower content of unfavorable SFA (12:0-16:0),

and a higher content of beneficial UFA including ALA, OA, VA and RA. Thus, the use of pasture systems in temperate North America could improve the image of milk fat which is typically known to have a high content of SFA and low content of beneficial UFA. Unfortunately dairy farmers are currently not rewarded for producing nutritionally improved milk fat.

The impacts of marine oil supplementation on ruminal and mammary responses in dairy cows are reviewed in Chapter 1. The milk fat content of n-3 LC-PUFA which is naturally low can be enriched with these beneficial FA by feeding marine oils to dairy cows. Fish oil has been the most common source of EPA and DHA used in dairy rations to improve the milk fat content of these FA. Microalgae, at the bottom of marine food chains, are the primary producers of n-3 LC-PUFA. Cultured microalgae is an environmentally-sound alternative to fish oil and can be used as a ration supplement for the enrichment of milk with n-3 LC-PUFA particularly DHA. The transfer efficiencies of EPA and DHA from diet into milk are very low which is due mainly to their extensive RBH. However, the transfer efficiencies of EPA and DHA may be improved when the source is protected from RBH. Feeding marine lipid supplements has also been shown to increase the concentrations of VA and RA in milk although marine oils do not contain significant amounts of C18 PUFA, the precursors for ruminal production of VA and RA. Feeding marine oils alters the rumen microbial population resulting in incomplete RBH of C18 PUFA present in feed ingredients and accumulation of VA and RA. The results from Chapter 2 indicated that feeding FO or MA could also enhance the milk fat concentration of n-3 LC-PUFA and reduce the content of 16:0 in both pasture and confinement systems without compromising milk fat yield. However, feeding FO or MA

interacted with management system in terms of milk fat content of VA and RA. In contrast to confined cows, marine oil supplements did not improve milk fat content of VA and RA in grazing cows. This could be due to differences between grazing and confinement in terms of intake of RBH substrates and ruminal environment, which deserve further study. Furthermore, supplementing dairy cow diets with FO or MA resulted in increased milk fat levels of several *trans* 18:1 isomers other than VA, the health effects of which are unknown. Thus, more studies are needed on human health consequences of modifying the UFA composition of milk fat by feeding oils to dairy cows. Another consequence of feeding PUFA including marine oils to dairy cows is MFD which is currently thought to be the result of reduced mammary expression of lipogenic genes and a reduced supply of SA to the mammary for synthesis of OA. Molecular mechanisms involved in MFD are reviewed in Chapter 1. Down-regulation of mammary lipogenic genes during MFD has been related mainly to reduced transcriptional activation of lipogenic genes by specific RBH intermediates particularly *trans*-10 *cis*-12 CLA.

Chapter 3 presented the first study to compare mammary lipogenic gene expression in grazing cows vs. confined cows, and to examine the concurrent lipogenic gene expression response to PUFA supplementation in bovine mammary, liver and adipose tissue. This work revealed that at least part of the difference between pasture and confinement in milk FA secretion (esp. *de novo* synthesized FA) could be mediated transcriptionally. In contrast to results from MFD studies, the reduced milk *de novo* synthesized FA in grazing cows was not related to milk *trans*-10 18:1 or *trans*-10 *cis*-12 CLA, implying that other factors (e.g. other RBH intermediates and availability of

nutrients including acetate and glucose) might have played a role in regulating milk fat synthesis. Finally this work illustrates that the effect of LS on lipogenic gene expression is tissue specific with the greatest response observed in liver despite its low lipogenic capacity in cattle.

Future research should focus on simultaneous improvement of FA profile in bovine milk and improved animal performance.

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Appendix A Copyright Permission Letters

A large portion of Chapter 1 is published as a book chapter in: Milk Fat: Composition, Nutritional Value and Health Implications, by Nova Science Publishers Inc., Hauppauge, NY. Chapter 2 has been accepted in June 2013 for publication in the Journal of Dairy Science. Chapter 3 has been submitted to the Journal of Dairy Science in July 2013 and is currently under peer-review.

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Appendix A.2 Copyright Permission for Chapter 2

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Effect of Supplementation with Fish Oil or Microalgae on Fatty Acid Composition of Milk from Cows Managed in Confinement or Pasture Systems

Vahmani, P., A. Fredeen, and K. Glover. 2013. Effect of supplementation with fish oil or microalgae on fatty acid composition of milk from cows managed in confinement or pasture systems. Journal of Dairy Science, Accepted June 2013 (Manuscript ID: JDS-13-6914).

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Name: PETER HARRISON Title: VICE PRESIDENT HEALTH+MEDICAL SCIE

Signature: [redacted] Date: 18 July 2013

Appendix B Feed Analysis Methods

Samples of pasture, TMR and concentrate were collected weekly and stored at −20 ºC. Frozen samples were later composited by month. One sub-sample was dried (55ºC for 72 h) to determine DM content and ground (1 mm sieve; Wiley mill, Philadelphia, PA) for analysis of NDF and ADF (Ankom 200 Fiber Analyzer, Ankom Technology Corp., Fairport, NY), CP (6.25 × N; Leco FP-528, Leco Corporation, St. Joseph, MI), ash (550°C for 12 h) and ether extract (Soxtec Avanti 2050 Automatic Extraction System, Foss, Tecatur, Höganäs, Sweden). Another sub-sample was freeze-dried and ground (1mm screen) and stored at -80ºC for subsequent FA analysis.

Total feed lipid was extracted with chloroform/methanol (2:1) and methylated with Hilditch reagent (0.5 N H_2SO_4 in methanol). The resulting fatty acid methyl esters (FAME) were purified on silica gel thin-layer chromatography (TLC) plates (Sigma-Aldrich, St. Louis, MO, USA) using petroleum ether/diethyl ether/acetic acid (90:10:1) as the running solvent. The FAME were quantified using a Varian 450 gas chromatograph (Varian, Walnut Creek, CA, USA) equipped with a Varian 8400 auto-sampler, and a 30 m DB-23 capillary column (Supelco, Bellefonte, PA, USA). A sample volume of 1 μL (0.5 mg/mL) was injected using split-less injection mode. Temperature of the injector and flame ionization detector (FID) was maintained at 250ºC and 270ºC respectively. The helium carrier gas was set to 20 psi. The oven temperature program was: 50ºC for 1 min, increasing at 45ºC/ min to 153ºC, held at temperature for 2 min, then increased at 2.3ºC/min to 174ºC, held at temperature for 0.2 min, then increased at the rate of 2.5ºC/min to 210ºC maintained for 5 min. Fatty acid methyl ester peaks were identified by comparing their retention times to those obtained with FAME standards (GLC 87, 461 and 463; Nucheck Prep Inc., Elysian, MN, USA).