Nutritional regulation of stearoyl-CoA desaturase in the bovine mammary gland

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Thesis

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Abstract

Increasing the proportion of unsaturated fatty acids (UFA) in milk is believed to be beneficial in terms of human health, thereby increasing the nutritional quality of milk. The proportion of UFA in milk is mainly dependent on the proportion of UFA in the diet, the degree of biohydrogenation of UFA in the rumen, and on activity of the stearoyl-CoA desaturase (SCD) enzyme in the mammary gland. This thesis focuses on SCD in the mammary gland of dairy cows, and how SCD can be influenced by nutrition. In the first study it was shown that supplementing the diet of dairy cows with soybean oil significantly decreases mammary SCD1 expression compared with rapeseed oil or linseed oil and this was partly reflected by the lower desaturase indices in milk. In contrast, mammary SCD5 expression was much lower ($<10^3$) than that of SCD1 and was not affected by dietary plant oil supplementation. To study the changes in genome-wide expression of genes in response to dietary UFA supplementation, mammary tissue samples of the same experiment were used for micro-array analysis. It was found that 972 genes were significantly affected through UFA supplementation, indicating that large transcriptional adaptations occurred in the mammary gland when diets of dairy cows were supplemented with unprotected dietary UFA. Since biopsy of the mammary gland is an invasive and costly method which presents a risk of udder infection, the use of milk somatic cells as a non-invasive, alternative source of mRNA was investigated in the second experiment. Since there was a significant relationship between SCD1 expression in milk somatic cells and mammary tissue, it can be concluded that milk somatic cells can be used as a source of mRNA to study SCD1 expression in dairy cows. To study the effects of acetate (Ac) and β-hydroxybutyrate (BHBA) as well as various long-chain fatty acids (LCFA) on mammary SCD expression, a bovine mammary epithelial cell line (MAC-T) was used in the third experiment. This study showed that Ac up-regulates expression of SCD1 and acetyl-CoA carboxylase in MAC-T cells, which indicates that Ac may increase desaturation and *de novo* synthesis of fatty acids in the bovine mammary gland. In addition it was shown that expression of sterol regulatory binding protein 1 (SREBP-1) and insulin-induced gene 1 protein (INSIG-1) was related to the expression of several lipogenic genes, thereby strengthening the support for the role of SREBP-1 and INSIG-1 as central regulators of lipogenesis in the bovine mammary gland. Overall, it can be concluded that saturated LCFA have little or no effect on SCD1 expression in the bovine mammary gland, whereas unsaturated LCFA inhibit mammary SCD1 expression. The regulation of SCD1 in the bovine mammary gland by LCFA appears to be, at least partly, regulated by the transcription factors SREBP-1 and INSIG-1. Based on the *in vitro* research it appears that short-chain fatty acids, in particular Ac, upregulate mammary SCD1 expression, although further research is needed to verify if short-chain fatty acids induce SCD1 expression in the bovine mammary gland. The recently discovered isoform SCD5 is expressed in bovine mammary tissue, although contribution to Δ9-desaturation of fatty acids appears to be quite low.

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Chapter 1

General Introduction

General Introduction

Milk fat is one of the most important sources of dietary fatty acids (FA) in the Western human diet. In general, about 65-75% of the FA in milk are saturated (Jensen, 2002; Heck et al., 2009), mainly due to the extensive biohydrogenation of dietary unsaturated fatty acids (UFA) occurring in the rumen of dairy cows (Lock & Bauman, 2004). It is generally believed that regular consumption of diets rich in saturated fatty acids (SFA), in particular C14:0 and C16:0, raise serum total and low-density lipoprotein cholesterol levels, thereby increasing the risk of cardiovascular disease and the development of the metabolic syndrome (Mensink et al., 2003; Astrup et al., 2011). In addition, high intake of SFA is related to reduced insulin sensitivity and subsequently to increased type 2 diabetes (Parillo & Riccardi, 2004). Decreasing the proportion of SFA in milk fat may therefore be beneficial for human health. In the Netherlands however, the content of even-chain SFA in milk fat of raw bovine milk was higher in 2005 compared with 1992, which was related to changes in composition of diets fed to dairy cows (Heck et al., 2009). Furthermore, milk and meat of ruminants contains a wide range of conjugated linoleic acids (CLA) which are a mixture of positional and geometric isomers of linoleic acid with a conjugated double-bond system. These CLA originate from the rumen as a result of the biohydrogenation process by rumen microbes. However, the majority of *cis*-9, *trans*-11 CLA, the major CLA isomer found in milk, is endogenously synthesised in the mammary gland by desaturation of C18:1 *trans*-11 (Palmquist et al., 2005). The *cis*-9, *trans*-11 CLA isomer has been associated with numerous health benefits for consumers including prevention of atherosclerosis, different types of cancer and hypertension (Wahle et al., 2004; Bhattacharya et al., 2006).

The proportion of UFA in milk is mainly dependent on the proportion of UFA in the diet, the degree of biohydrogenation in the rumen, and on the extent of desaturation of UFA in the mammary gland (Figure 1). To reduce the proportion of SFA in milk fat, two major strategies can be adopted. One strategy is to increase the supply of UFA to the mammary gland, by reducing the extent of biohydrogenation of UFA in the rumen. To this end, various rumen lipid protection technologies have been developed and evaluated. Such rumen lipid protection technologies involve either modifications of FA structure to resist the action of microbial enzymes or encapsulation of UFA inside a microbial-resistant shell (Jenkins and Bridges, 2007). The efficacy of these protection technologies to reduce biohydrogenation varies widely (Sterk et al., 2010). The second strategy to reduce the proportion of SFA in milk fat is to stimulate the desaturation of SFA within the mammary gland. The present thesis focuses on the latter.

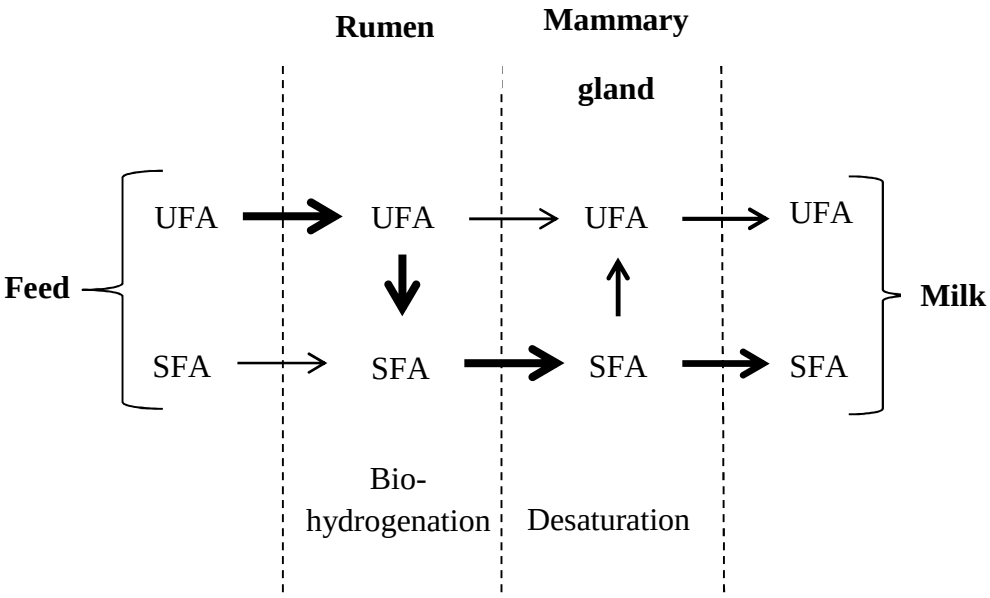

Figure 1. Simplified schematic overview of the flow of saturated fatty acids (SFA) and unsaturated fatty acids (UFA) from feed to milk.

Stearoyl-CoA Desaturase

Fatty acids can be desaturated by the Δ9-desaturase enzyme, also known as stearoyl-CoA desaturase (SCD; EC 1.14.19.1) present in various tissues of animals. Stearoyl-CoA desaturase is a membrane-bound, endoplasmic enzyme which introduces a *cis*-double bond between carbons 9 and 10 in a wide range of FA. The preferred substrates of SCD are C18:0 and, to a lesser extent, C16:0, which are converted to C18:1 *cis*-9 and C16:1 *cis*-9, respectively (Ntambi & Miyazaki, 2004). The oxidative reaction catalysed by SCD requires the electron acceptor cytochrome b5, NAD(P)-cytochrome b5 reductase and molecular oxygen. The electrons flow from NAD(P)H via cytochrome b5 reductase, to cytochrome b5, to SCD, and finally to O_2, which is reduced to H_2O (Figure 2; Paton & Ntambi, 2009). SCD catalyses the critical committed step in the biosynthesis of monounsaturated fatty acids (MUFA), predominately C18:1 *cis*-9. These MUFA serve as the main substrates for the synthesis of membrane phospholipids, triglycerides, cholesterol esters, wax esters and alkyldiacylglycerols (Paton & Ntambi, 2009). In addition, MUFA are thought to serve as mediators in signal transduction as well as cellular differentiation (including neuronal differentiation) and MUFA may also influence apoptosis and mutagenesis in some tumours. Since endogenous synthesised C18:1 *cis*-9 is crucial for these processes, SCD plays a vital role in metabolism and its activity is expected to influence cellular differentiation, insulin sensitivity, metabolic rate, adiposity, atherosclerosis, cancer and obesity (Paton & Ntambi, 2009).

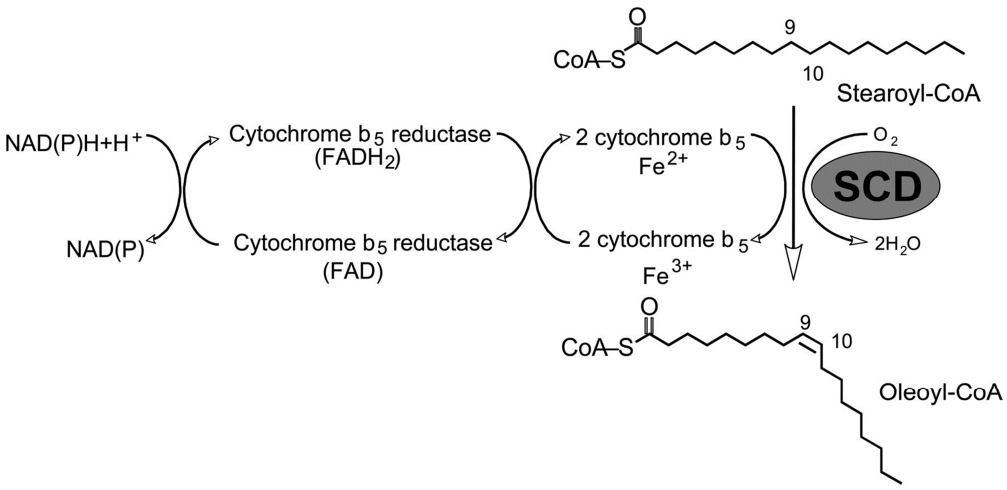

Figure 2. The pathway of electron transfer in the desaturation of fatty acids by stearoyl-CoA desaturase (SCD; Paton & Ntambi, 2009).

Regulation of SCD in Rodents

Most of the research on SCD has been conducted using rodents. In mice, four different isoforms of SCD have been characterized, i.e. SCD1, SCD2, SCD3 and SCD4, which are expressed in different types of tissue. Mouse SCD1 is predominantly expressed in lipogenic tissues including adipose tissue and liver, whereas SCD2 is mainly expressed in brain and at much lower levels in adipose tissue and liver. The SCD3 isoform is primarily expressed in skin, whereas SCD4 is mainly expressed in the heart (Popeijus et al., 2008). In addition to the tissue-specific expression, the SCD isoforms also differ in substrate preference. Whereas SCD1, SCD2 and SCD4 prefer C18:0, and to a lesser extent C16:0, SCD3 appears to only utilize C16:0 as a substrate (Miyazaki et al., 2006). In the mammary gland, all four isoforms of SCD are expressed, although relative mRNA abundance is highest for SCD1 (± 70%) and SCD2 (± 30%), whereas SCD3 and SCD4 are barely detected (<0.01%) (Han et al., 2010). This latter indicates that SCD1 and, to a lesser extent, SCD2 are the most important isoforms of SCD in the mammary gland of mice.

SCD1 is a highly regulated enzyme, at both the transcription and protein level, with a relative short half-life time of approximately 2 to 4 h, as the N-terminus of SCD1 contains a sequence responsible for rapid degradation of the SCD1 protein (Flowers & Ntambi, 2008; Popeijus et al., 2008). Mice with a targeted disruption of SCD1 are deficient in triglycerides, cholesterol esters, wax esters and alkyldiacylglycerols. In addition, these mice have reduced body adiposity, increased insulin sensitivity and are resistant to diet-induced obesity (Ntambi & Miyazaki, 2003).

The expression of SCD1 is affected by numerous dietary, hormonal and environmental factors. Nutrients that have been shown to upregulate SCD1 in rodents include: glucose, fructose and cholesterol, whereas polyunsaturated fatty acids (PUFA) downregulate SCD1 (Ntambi & Miyazaki, 2003). Moreover, insulin (Waters & Ntambi, 1994; Frick et al., 2002) and growth hormone are examples of hormones that upregulate SCD1, whereas leptin (Cohen et al., 2002) has been shown to downregulate SCD1. Several transcription factors are involved in the regulation of SCD1. Liver X receptor (LXR), a member of the nuclear receptor family of transcription factors, upregulates SCD1 by binding to the LXR response element in the SCD1 promoter, as well as LXR-mediated activation of sterol regulatory-element binding protein-1 (SREBP-1) transcription, since SCD1 expression is also induced by the nuclear fragment of SREBP-1 via the sterol regulatory element in its promoter (Flowers & Ntambi, 2008). There are three different isoforms of SREBP, i.e. SREBP-1a, SREBP-1c and SREBP-2, which have specific roles in lipid and cholesterol metabolism. The SREBP-1c isoform is involved in FA synthesis, whereas SREBP-2 is mainly associated with cholesterol synthesis and SREBP-1a appears to be involved in both pathways (Eberlé et al., 2004). Inactivated full length SREBP is bound to the endoplasmic reticulum coupled with SREBP cleavage-activating protein (SCAP) and associated with insulin-induced gene 1 (INSIG-1) or insulin-induced gene 2 (INSIG-2). When activated, the SREBP/SCAP complex dissociates from INSIG and translocates to the Golgi apparatus where proteases release a nuclear fragment of SREBP (nSREBP). Once translocated to the nucleus, nSREBP binds to the sterol regulatory element in the promoter region of target genes (including fatty acid synthase (FAS), SCD1 as well as SREBP-1 itself), thereby stimulating their expression (Eberlé et al., 2004). After feeding a high carbohydrate meal, SCD1 expression is rapidly induced (40-fold over fasting), which is thought to be due to insulin-mediated increases in SREBP-1c activation and subsequent activation of the SCD1 promoter (Paton & Ntambi, 2009).

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors that function as ligand-activated transcription factors regulating the expression of genes involved in metabolism, cellular differentiation and development (Michalik et al., 2006) and can interact with LXR (Paton & Ntambi, 2009). Three isoforms of PPAR are known, i.e. PPARα, PPARδ and PPARγ. The PPARα isoform is highly expressed in liver and plays a key role in FA oxidation (Pyper et al., 2010), whereas PPARδ is mainly involved in FA catabolism in skeletal muscle and inflammatory responses (Grimaldi, 2010). The PPARγ isoform is mainly expressed in adipose tissue where it regulates adipose cell differentiation (Tontonoz & Spiegelman, 2008). When re-feeding mice with a targeted deletion of PPARα after fasting, the normal SCD1 induction is completely attenuated, which is likely due to a loss of LXR/PPARα dimerization (Paton & Ntambi, 2009). The PPARγ agonist rosiglitazone upregulates SCD1 (Way et al., 2001), indicating that SCD1 is a target of PPARγ.

Regulation of SCD in Dairy Cows

In bovine, two isoforms of SCD have been characterized, i.e. SCD1 and SCD5. In lactating ruminants, SCD1 is abundantly expressed in the mammary gland and plays an important role in the production of milk fat (McDonald & Kinsella, 1973; Bernard et al., 2008; Bionaz & Loor, 2008b). The recently discovered SCD5 appears to be predominantly expressed in brain and pancreas (Lengi & Corl, 2007). Contrary to rodents, not much is known about the nutritional regulation of bovine SCD. Feeding cows diets high in concentrate and low in fibre, which causes milk fat depression (MFD), has been shown to inhibit expression of key lipogenic genes, including SCD1, in the mammary gland (Peterson et al., 2003; Harvatine & Bauman, 2006). In addition, abomasal infusion of *trans*-10, *cis*-12 CLA, which is the main biohydrogenation intermediate responsible for the inhibition of milk fat synthesis, also inhibits lipogenic gene expression, including SCD1 (Baumgard et al., 2002; Bauman & Harvatine, 2006). This coordinated inhibition of genes involved in lipogenesis, has been associated with reduced expression of SREBP-1 and proteins involved in the activation and distribution of SREBP-1 towards the nucleus (Harvatine & Bauman, 2006), which is a mechanism clearly demonstrated in rodents (Jump et al., 2005). However, information regarding SCD and its regulation in the bovine mammary gland remains scarce.

Aim and Outline of This Thesis

The main focus of the research described in this thesis was on the SCD enzyme in the mammary gland of dairy cows, and how SCD activity can be influenced by nutrition. More specifically, the effect of FA on mRNA expression of SCD was investigated in the bovine mammary gland. In addition, a non-invasive alternative to mammary tissue for measuring SCD expression in the mammary gland of dairy cows was examined. The overall purpose of this research was to explore nutritional strategies that could increase the activity of SCD in the mammary gland of dairy cows. Increasing SCD activity in the mammary gland could lead to increased MUFA as well as *cis*-9, *trans*-11 CLA content, thereby improving the nutritional quality of milk. In addition, understanding the regulation of SCD in the mammary gland could facilitate this process and lead to an improved FA profile of milk. This thesis describes various experiments aimed at improved understanding of nutritional regulation of SCD in the bovine mammary gland.

Chapter 2 and **3** describe the effect of supplementing the diet of dairy cows with various plant oils differing in FA composition, on gene expression in the mammary gland.
Chapter 2 focuses on the effects on mammary SCD expression and milk FA composition while **Chapter 3** describes the effects on overall mammary gene expression using micro-array analysis.

Chapter 4 describes the use of milk somatic cells as a non-invasive, alternative source of RNA to mammary tissue for measuring SCD expression in the mammary gland of dairy cows.

Chapter 5 describes the effect of acetate and β-hydroxybutyric acid on SCD expression in a bovine mammary epithelial cell line (MAC-T). In addition, the effect of several long-chain fatty acids on SCD expression was investigated.

Chapter 6 contains a general discussion of the results of the various experiments carried out in this thesis. This chapter also contains general conclusions and describes areas recommended for further research to improve our understanding and optimize mammary SCD expression.

Chapter 2

Effects of Feeding Rapeseed Oil, Soybean Oil or Linseed Oil on Stearoyl-CoA Desaturase Expression in the Mammary Gland of Dairy Cows

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Abstract

Stearoyl-CoA desaturase (SCD) is an important enzyme in the bovine mammary gland, which introduces a double bond at the Δ9 location of primarily myristoyl-, palmitoyl-, and stearoyl-CoA. The main objective of this study was to compare the effects of various fatty acids (FA) typically present in dairy cow rations, on the expression of SCD1 and SCD5 in the mammary gland of dairy cows. Twenty-eight Holstein-Friesian cows were randomly assigned to one of the four dietary treatments. The dietary treatments were a basal diet supplemented (DM basis) with either 2.7% rapeseed oil as a source of C18:1 *cis*-9, 2.7% soybean oil as a source of C18:2 *cis*-9,12, 2.7% linseed oil as a source of C18:3 *cis*-9,12,15 or 2.7% of a 1:1:1 mixture of the three oils. The oil supplements were included in the concentrate, which was fed together with maize silage and grass silage. In addition, cows were grazing on pasture, consisting mainly of perennial ryegrass, during daytime. Biopsies from the mammary gland were taken and analysed for mRNA expression of SCD1 and SCD5 by using quantitative RT-PCR. Milk yield as well as milk protein and fat content did not differ between the four dietary treatments. Dietary supplementation with rapeseed oil and linseed oil increased proportions of C18:1 *cis*-9 and C18:3 *cis*-9,12,15 in blood plasma, respectively, compared with the other treatments. Supplementation with soybean oil and linseed oil increased milk FA proportions of C18:2 *cis*-9,12 and C18:3 *cis*-9,12,15, respectively, but supplementation with rapeseed oil did not increase C18:1 *cis*-9 in milk. Mammary SCD1 expression was reduced by supplementation of soybean oil compared with rapeseed oil and linseed oil. In contrast, SCD5 expression did not differ amongst the four treatments. The C16 and C18 desaturation indices, representing proxies for SCD activity, were lower for the soybean oil diet, compared with the diet supplemented with a mixture of the three oils. In conclusion, our study shows that mammary SCD1 expression is significantly down-regulated in dairy cows by feeding unprotected soybean oil compared with rapeseed oil or linseed oil, and this is partially reflected by the lower desaturase indices in the milk. Furthermore, mammary SCD5 expression appears to be differently regulated than that of SCD1.

Introduction

The relationship between dietary fat type and various chronic diseases in humans, including cardiovascular disease, has been extensively investigated. Although various *trans* fatty acids (TFA) have an unfavourable impact on the serum lipoprotein profile, conjugated linoleic acids (CLA) have anti-carcinogenic and anti-atherogenic effects (Lock & Bauman, 2004). Saturated fatty acids (SFA) generally raise serum total and low-density lipoprotein cholesterol levels and increase the risk of cardiovascular disease (Mensink et al., 2003). Moreover, high intake of SFA is related to reduced insulin sensitivity and subsequently to increased type 2 diabetes (Parillo & Riccardi, 2004). Decreasing the proportion of SFA in milk fat may therefore be beneficial for human health. In the Netherlands though, the content of even-chain SFA in milk fat of raw bovine milk was higher in 2005 compared with 1992, which was related to changes in composition of diets fed to dairy cattle (Heck et al., 2009).

Stearoyl-CoA desaturase (SCD) converts SFA into monounsaturated fatty acids (MUFA) by introducing a double bond between carbon atoms 9 and 10 in the saturated carbon chain, but it can also catalyse the desaturation of a wide spectrum of monounsaturated fatty acyl-CoA substrates, including C18:1 *trans*-11 to generate *cis*-9, *trans*-11 CLA (Ntambi & Miyazaki, 2004). Increasing SCD activity is therefore of interest to increase the content of various beneficial fatty acids (FA) in milk. In lactating ruminants, stearoyl-CoA desaturase 1 (SCD1) is abundantly expressed in the mammary gland and plays an important role in the production of milk fat (McDonald & Kinsella, 1973; Bernard et al., 2008; Bionaz & Loor, 2008b). Recently, a novel isoform of SCD, designated stearoyl-CoA desaturase 5 (SCD5), has been identified which appears to be expressed predominantly in brain and pancreas (Wang et al., 2005; Lengi & Corl, 2007). SCD5 is also expressed in the bovine mammary gland (Gervais et al., 2009), but the contribution of SCD5 to mammary SCD activity during lactation and whether its expression can be influenced by dietary FA remains obscure.

Nutritional regulation of SCD1 expression has been extensively studied in rodents, primarily in liver and adipose tissue (Ntambi, 1999), and to a smaller degree in the mammary gland (Lin et al., 2004; Singh et al., 2004). It is well established that polyunsaturated FA (PUFA) supplementation, both n-3 and n-6, results in severe down-regulation of SCD1 in both liver and adipose tissue of rodents, whereas SFA and MUFA have little effect (Ntambi, 1999). In dairy cows, only a few studies have examined the effect of nutritional factors on regulation of SCD in the mammary gland (Bernard et al., 2008). Therefore, knowledge regarding the effect of nutrition on milk fat synthesis in the bovine mammary gland and especially the underlying mechanisms, such as regulation of key lipogenic genes, is still limited. Most of the studies that examined the effect of nutritional factors on mammary SCD in dairy cows, utilized diets that cause milk fat depression (MFD). Harvatine & Bauman (2006) and Peterson et al. (2003) both reported a tendency towards reduction of mammary SCD1 expression in dairy cows with diet-induced

MFD. Addition of fish oil to the diet of dairy cows, which also causes MFD, resulted in a significant reduction of SCD1 mRNA in the mammary gland (Ahnadi et al., 2002). Nevertheless, the effects of the major unsaturated FA C18:1 *cis*-9, C18:2 *cis*-9,12 or C18:3 *cis*-9,12,15, which are typically present in dairy cow rations, on mammary SCD1 and SCD5 expression is still not clear. Consequently, in the present study we investigated the effects of dietary FA supplementation of C18:1 *cis*-9, C18:2 *cis*-9,12 or C18:3 *cis*-9,12,15, by feeding unprotected rapeseed oil, soybean oil or linseed oil, respectively, or its mixture, on both SCD1 and SCD5 expression in the mammary gland of dairy cows and evaluated the effects of these FA on milk desaturation indices, as indicators of mammary SCD activity.

Material and Methods

Animals and Diets

This experiment was conducted at the Cranendonck Research Farm, the Netherlands, between 26 September and 14 November 2007. Twenty-eight Holstein-Friesian cows were distributed in order to ensure uniform distribution among seven blocks based on parity (2.4 ± 0.63), DIM (153 ± 32.8 days), milk yield (25.7 ± 3.08 kg/d) and milk fat content (4.31 ± 0.12 %) (values expressed as mean ± SEM). Cows within each block were randomly assigned to one of the four dietary treatments (n=7 per dietary treatment). The dietary treatments were a basal diet supplemented (DM basis) with either: 2.7% rapeseed oil (RO) as a source of C18:1 *cis*-9, 2.7% soybean oil (SO) as a source of C18:2 *cis*-9,12, 2.7% linseed oil (LO) as a source of C18:3 *cis*-9,12,15 or 2.7% of a 1:1:1 mixture of the three oils (MIX). Oil supplements were not protected against biohydrogenation in the rumen. The oil supplements were included in the concentrate, which was fed, together with maize silage and grass silage, as a mixed ration (MR). Maize silage, grass silage and concentrates comprised 52, 12 and 36% of the MR, respectively, on a DM basis. In addition to the MR, the cows received approximately 1 kg of a commercial standard concentrate per day, through automatic feeding stations. Cows were milked twice daily at 06:00h and 18:00h and were grazing on pasture from 08:00h until 16:00h. At other times, cows were inside a free-stall barn and had ad libitum access to the MR. The pasture used for grazing was established in September 2006 and consisted primarily of ryegrass (*Lolium perenne* L.) with approximately 20% white clover (*Trifolium repens* L.). The paddocks were approximately 5 hectare in size and the total stocking density was 16 cows/ha. The treatment period lasted 23 days and after the treatments ended, cows were fed a post-trial diet (POST), without oil supplementation, for a period of 28 days as control. In the post-trial period there was no concentrate in the MR as all the concentrate was provided through automatic feeding stations. Ingredient and chemical composition of the different concentrates are presented in Table 1 and chemical and FA composition of the pasture and of the different MRs used in this experiment are presented in Table 2. Individual milk production and MR intake per treatment group were recorded daily.

Table 1. Ingredient and chemical composition of the concentrate fed post-trail (POST) and the concentrates supplemented with either: rapeseed oil (RO), soybean oil (SO), linseed oil (LO) or a mixture of these oils (MIX).

Item	POST	RO	SO	LO	MIX
Ingredient, g/kg					
Triticale	-[1]	333.6	333.6	333.6	333.6
Rapeseed meal, formaldehyde treated	-	195.0	195.0	195.0	195.0
Soybean meal	-	102.0	102.0	102.0	102.0
Dried brewers grain	-	79.0	79.0	79.0	79.0
Rapeseed oil	-	75.0	-	-	25.0
Soybean oil	-	-	75.0	-	25.0
Linseed oil	-	-	-	75.0	25.0
Rapeseed meal	108.1	68.0	68.0	68.0	68.0
Citrus pulp	370.0	60.0	60.0	60.0	60.0
Soybean meal, formaldehyde treated	-	47.0	47.0	47.0	47.0
Palm kernel expeller	227.3	-	-	-	-
Sugar beet molasses	101.0	-	-	-	-
Coconut expeller	64.8	-	-	-	-
Wheat gluten feed	45.0	-	-	-	-
Wheat middlings	31.0	-	-	-	-
Soybean hulls	29.3	-	-	-	-
Protapec[2]	13.7	-	-	-	-
Calcium carbonate	2.0	16.3	16.3	16.3	16.3
Sodium chloride	2.4	10.5	10.5	10.5	10.5
Magnesium oxide 90%	3.4	4.8	4.8	4.8	4.8
Urea	-	4.5	4.5	4.5	4.5
Monocalcium phosphate	-	2.3	2.3	2.3	2.3
Vitamin mineral premix	2.0	2.0	2.0	2.0	2.0
Chemical composition, g/kg DM					
DM, g/kg	911.7	891.1	900.5	904.1	901.0
Ash	87.6	81.2	79.3	86.1	84.9
CP	161.2	246.5	296.5	252.3	242.4
Crude fat	55.6	98.0	89.6	93.8	93.9
NDF	326.3	179.8	148.4	196.3	185.2
ADF	249.0	116.8	110.4	122.9	119.3
Starch	35.6	219.8	151.2	172.4	211.7
Sugars	120.0	73.0	105.8	87.1	72.3

[1] Not included; [2] Potato fruit-juice (concentrated) mixed with soybean hulls (Cehave Landbouwbelang, Veghel, the Netherlands).

Table 2. Chemical composition, fatty acid composition and dry matter intake (DMI) of the pasture used for grazing, the mixed ration used as post-trial diet (POST) and the four mixed rations supplemented with either: rapeseed oil (RO), soybean oil (SO), linseed oil (LO), or a mixture of these three oils (MIX).

Item		Mixed rations including the various concentrates[1]				
	Pasture	POST	RO	SO	LO	MIX
Chemical composition, g/kg DM						
DM, g/kg	191.7	560.9	589.4	574.5	573.1	593.6
Ash	90.9	66.1	66.2	64.3	66.5	67.4
CP	229.7	132.2	162.8	180.0	164.9	161.4
Crude fat	55.9	40.5	55.8	52.9	54.3	54.4
NDF	340.4	356.4	305.9	294.0	310.3	307.7
ADF	191.3	227.7	187.0	181.3	185.1	188.0
Starch	-[2]	182.4	227.4	214.7	223.4	224.2
Sugars	206.4	57.4	48.5	55.7	48.7	48.5
Fatty acid composition, g/100g fatty acids						
C8:0	ND[3]	1.4	0.3	0.2	0.2	0.3
C10:0	ND	1.5	0.3	0.2	0.2	0.3
C12:0	ND	16.4	3.5	1.9	1.9	3.7
C14:0	ND	6.0	1.6	0.8	0.8	1.5
C16:0	13.4	13.2	16.2	13.7	10.2	13.0
C16:1 *cis*(c)-9	ND	0.2	1.1	0.1	0.2	0.5
C18:0	1.9	2.6	3.9	3.3	3.1	3.4
C18:1 c9	5.3	16.8	29.2	19.9	20.1	23.3
C18:1 c11	ND	0.8	2.1	1.2	1.6	1.8
C18:2 c9c12	15.0	29.8	31.2	46.4	28.8	34.5
C18:3 c9c12c15	57.2	7.5	6.4	9.1	30.2	14.5
C20:0	ND	0.4	0.4	0.4	0.3	0.4
C22:0	0.7	0.5	0.4	0.5	0.4	0.5
C24:0	0.7	0.4	0.3	0.3	0.3	0.3
Others	0.7	0.6	0.8	0.5	0.6	0.6
Unidentified	5.1	1.9	2.2	1.3	1.3	1.4
DMI, kg/d	5.5[4]	12.8	15.1	14.4	14.5	14.7

[1] Including concentrate which was supplied through the automatic feeding stations.

[2] Not determined.

[3] ND = not detected.

[4] DMI of pasture was 5.5 kg/d during the four dietary treatments and was 4.8 kg/d during POST.

Pasture intake was estimated from the difference between grass height measurements made before and after grazing, assuming grass growth during the period of grazing is a negligible proportion of total grass consumption in view of the short grazing period length and stocking density (Penning, 2004). Sward surface height was measured as described in Abrahamse et al. (2008a) using 40 sward surface height measurements per averaged height observation.

Sampling and Analysis

A representative sample of individual feedstuffs, including pasture, and of the MR was taken during the last 3 days of the treatment and post-trial period. All feed samples were freeze-dried, ground to pass a 1-mm screen and analysed for: DM, ash, CP, crude fat, NDF, ADF, starch, sugars and FA composition. The DM content was determined by drying at 103°C (ISO 6496, ISO, 1999a) and ash content was determined by combustion at 550°C (ISO 5984, ISO, 2002). Crude protein (6.25 x N) was determined using the Kjeldahl method (ISO 5983, ISO, 2005) and the Berntrop method with acid hydrolysis (ISO 6492, ISO, 1999b) was used to determine crude fat. Neutral detergent fibre was determined according to a modified method of Van Soest et al. (1991) with additional incubations in alpha amylase and protease as described by Goelema et al. (1998). Acid detergent fibre was determined according to Van Soest (1973). Starch was analysed using enzymatic hydrolysis (ISO 15914; ISO, 2004) and sugar analysis was carried out using a 40% ethanol solution as described by Abrahamse et al. (2008b).

For FA analysis of the feedstuffs, fat from freeze-dried samples was extracted with chloroform-methanol (2:1 v/v), according to Folch et al. (1957) with minor modifications as described by Khan et al. (2009), except no internal standard was added. Methylation of FA was done as follows: extracted fat was dissolved in 2 ml hexane and 100 mg of anhydrous sodium sulphate was added. Next, 50 µl of a 30% sodium methanolate solution in methanol was added to transesterify the glycerides to their corresponding methyl esters by vortexing for 2 min. at room temperature. Subsequently, 1 ml hydrochloric acid in methanol solution (1.25*M*) was added to esterify the free FA, followed by heating the mixture for 20 min. at 85ºC under constant shaking. The mixture was then cooled down to room temperature under a flow of cold water and shaken vigorously and 1 ml of the upper layer, which contains the FA methyl esters (FAME), was transferred to a 1.5 ml vial and used for gas chromatography (GC) analysis.

On the last day of the treatment period and of the post-trial period, two consecutive milk samples (a.m. and p.m. milking) were obtained, and pooled (1:1 ratio) for analysis. One aliquot was stored at 4°C pending fat, protein and lactose analysis using mid infrared spectrometry (ISO 9622, ISO, 1999c; Qlip NV, Zutphen, the Netherlands). Another aliquot was stored at -20°C pending FA analysis. Milk FA extraction and methylation were performed as described by van Knegsel et al. (2007) with some minor adjustments. First, the milk samples were heated to 45°C and a representative milk sample of 20 ml was centrifuged at 3,000 x g for 10 min. at 4°C. The upper fat layer was then collected with a

spatula, dried on filter paper and transferred to 1.5 ml tube, which was stored overnight at -20°C. Next, the milk fat samples were heated for 10 min. at 60°C, followed by centrifugation at 20,000 x g for 5 min. at 20°C, and the clear fat fraction was transferred to a new 1.5 ml tube containing a small amount of anhydrous sodium sulphate. Afterwards, the milk fat samples were stored again at -20°C until analysis. Before analysis, the frozen samples were heated for 10 min. at 60°C, followed by centrifugation at 20,000 x g for 5 min. at 20°C. Subsequently, 50 µl of milk fat was added to 5 ml of hexane and the glycerol-bound FA were transesterified to methyl esters by vortexing with 100 µl sodium methanolate in methanol (30%). The solution was neutralized with 1 g sodium hydrogen sulphate and dried with anhydrous sodium sulphate. This solution was then transferred to a 1.5 ml vial and used for GC analysis.

For plasma FA analysis, blood samples (10 ml) were collected from the jugular vein in heparin-containing tubes at the last day of the treatment and the post-trial period. The blood samples were centrifuged at 3,000 x g for 15 min. to harvest plasma, which was stored at -20°C pending FA analysis. Fat was extracted using chloroform:methanol (2:1, v/v) as described by Folch et al. (1957). The extracted fat was saponified using methanolic sodium hydroxide and the constituent FA were converted into their methyl esters using boron trifluoride in methanol according to Metcalfe et al. (1966) with minor modifications as described by Khan et al. (2009).

Methylated FA obtained from feed samples, milk samples and blood plasma samples were quantified using a gas chromatograph (TRACE GC Ultra™, Thermo Electron Corporation, Waltham MA, USA) equipped with a flame-ionization detector and auto-sampler. Methylated FA were separated using a fused silica capillary column (100 m x 0.25 mm and 0.2 µm film thickness; Restek RT-2560, Restek, Bellefonte PA, USA) using helium as a carrier gas at a constant flow of 1.5 ml/min. Methylated FA samples of 1 µl were injected into the GC with a split ratio of 1:50. The following program was used for the GC: starting temperature 140ºC for 4 min., followed by an increase at a rate of 4ºC per min. until 240ºC and left for 20 min. at 240ºC. Temperature of the flame-ionization detector was 280ºC. Peaks were identified by comparing the retention times with those of the corresponding FAME standards (S37, Supelco, Poole, Dorset, UK) and, when no commercial standard was available, by using published chromatograms obtained via comparable analytic conditions (Loor et al., 2004; Shingfield et al., 2006; Cruz-Hernandez et al., 2007). Under these conditions, several C18:1-*trans* isomers were not completely resolved and are therefore listed together. Fatty acid desaturation indices in milk, as proxies for SCD activity in the mammary gland, were calculated as the ratio between the product and the sum of the product and substrate FA (Kelsey et al., 2003).

Biopsy Procedure

On the last day of the treatment and the post-trial period, approximately 0.7 to 1.0 g of mammary tissue was obtained from each cow by surgical biopsy from the midpoint section of a rear quarter, according to the method of Farr et al. (1996). Collected tissues were

immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Following biopsy, a single intramammary injection of antibiotics (Avuloxil®; active ingredients: amoxicillin and clavulic acid; Pfizer Animal Health, Capelle a/d IJssel, the Netherlands) was applied in the affected rear quarter. In addition, cows received an intramuscular injection of antibiotics (Excenel®; active ingredient: ceftiofur hydrochloride; Pfizer Animal Health, Capelle a/d IJssel, the Netherlands). Cows were subsequently machine milked as normal and hand-stripped as needed to remove all intramammary blood clots. No mammary infections were observed after biopsy. The biopsy procedure was approved by the Animal Care and Ethics Committee of Wageningen UR Livestock Research, Lelystad, the Netherlands.

RNA Isolation and Real Time PCR

Total RNA was extracted from mammary gland biopsies with Trizol® reagent (Invitrogen, Breda, the Netherlands). First-strand cDNA synthesis was performed from 1 µg of total RNA reaction using Superscript III reverse transcriptase (200 units; Invitrogen), dNTPs (0.5 mM; Roche Diagnostics, Almere, the Netherlands) and random hexamer primers (250 ng; Roche) in a volume of 20-µl at 50 ºC for 1 h. Quantitative RT-PCR was performed on a LightCycler 1.2 by using FastStart DNA Master SYBR Green I reagents (Roche), according to the manufacturer's protocol. The following gene-specific primers were used: for SCD1, forward primer 5'-GGCGTTCCAGAATGACGTTT-3' and reverse primer 5'-AAAGCCACGTCGGGAATTG -3'; for SCD5, 5'-GGCACCGGCAGGACATC-3' (forward) and 5'-GAGCAGTCAGGAGGAAGCAGAA-3' (reverse). 18S RNA and beta-actin (ACTB) were measured to correct for the input of cDNA. For 18S RNA, we used the forward primer 5'-AGAAACGGCTACCACATCCAA-3' and reverse primer 5'-GGGTCGGGAGTGGGTAATTT-3'. For ACTB, 5'-GCCCTGAGGCTCTCTTCCA-3' (forward) and 5'-CGGATGTCGACGTCACACTT-3' (reverse). All primer pairs, except for 18S RNA, were designed in such a way that they span an intron of their corresponding genomic sequence or that its sense or reverse primer anneals on an exon-intron junction. Templates were amplified after a preincubation for 10 min at 95 ºC, followed by amplification for 40 cycles (10 s at 95 ºC, 5 s at 60 ºC, 5 s at 72 ºC). PCR efficiencies were established to be 100% for SCD1 and SCD5, and 95% for the two reference (18S RNA and ACTB) genes. All reactions revealed a single product as determined by melting curve analysis. Specificity of the primer sets were verified by sequencing of the generated amplicons. Expression levels of SCD1 and SCD5 were normalized using the geometric mean of 18S and ACTB. The relative RNA expression levels were calculated using the modified comparative CT method (Pfaffl, 2001). All measurements were performed in duplicate.

Statistical Analysis

Statistical analyses were carried out by ANOVA using the PROC MIXED procedure of SAS (version 9.1, SAS Institute Inc.) for a randomized block design. Covariate analysis

using the post-trial data did not improve the ability to detect differences due to treatment and therefore, post-trial data was not included in the statistical analysis. Treatment effects on animal performance, milk FA composition, blood plasma FA composition and mRNA abundance of the different genes were analysed and considered significant at a probability of $P < 0.05$, and as a trend at a probability of $0.05 < P < 0.10$. To test pair wise comparisons, post hoc analyses were carried out on the least square means adjusted for multiple comparisons using the Tukey-Kramer test. The regression procedure (PROC REG) of SAS was used to analyse correlations between relative abundance of mammary SCD mRNA and the various desaturase indices calculated from milk FA during the treatment period, and to analyse correlations between milk *trans*-10, *cis*-12 CLA and milk fat content as well as short- and medium-chain fatty acids (SMCFA) in milk.

Results

Diet Composition, Intake and Milk Yield

The formulation of the different diets resulted in expected high concentrations of C18:1 *cis*-9, C18:2 *cis*-9,12 and C18:3 *cis*-9,12,15 in the RO, SO and LO diet, respectively, with the MIX diet in between (Table 2). Total DMI was 20.6, 19.9, 20.0 and 20.2 kg/d for diets RO, SO, LO and MIX, respectively, but could not be statistically evaluated since intake was not determined per individual animal. Average milk yield was 28.8 (SEM 3.0) kg/d and did not differ ($P = 0.99$) among the four dietary treatments. In addition, the four treatments did not differ in concentration as well as yield of milk fat ($P = 0.19$ and $P = 0.42$, respectively), protein ($P = 0.36$ and $P = 0.88$, respectively) and lactose ($P = 0.17$ and $P = 0.96$, respectively). Mean milk fat, protein and lactose contents were 3.59 (SEM 0.26), 3.51 (SEM 0.13) and 4.40 (SEM 0.07) %, respectively. Calculated energy balance was positive during days of milk sampling.

Fatty Acid Composition of Blood Plasma

The main FA identified in blood plasma were C18:2 *cis*-9,12 (mean 29.4 g/100g FA), C18:0 (mean 15.9 g/100g FA), C18:1 *cis*-9 (mean 9.0 g/100g FA), C16:0 (mean 9.0 g/100g FA) and C18:3 *cis*-9,12,15 (mean 5.6 g/100g FA). Feeding the RO diet resulted in an elevated blood plasma fraction of C16:0, C16:1 *cis*-9 and C18:1 *cis*-9 (Table 3). Cows fed the LO diet had higher plasma proportions of C18:3 *cis*-9,12,15 compared with the other diets, and higher plasma proportions of C18:2 *trans*-11, *cis*-15 compared with RO and SO.

Mammary SCD1 and SCD5 Expression

Figure 1 shows the results from analysis of SCD1 (a) and SCD5 (b) mRNA expression in the mammary gland using quantitative RT-PCR. Cows fed the SO diet showed a significantly reduced mammary SCD1 expression, expressed as fold change compared to the corresponding control (0.45), compared with the RO (1.00) and LO diet (1.12; SEM = 0.14; $P = 0.01$). The SCD1 expression of cows fed the MIX diet (0.70) was in between the

Table 3. Blood plasma fatty acid composition (g/100 g fatty acids) of cows fed the post-trial diet (POST) or the treatment diets supplemented with either: rapeseed oil (RO), soybean oil (SO), linseed oil (LO), or a mixture of these three oils (MIX). For the four dietary treatments n = 7, and for POST n = 28.

		Dietary treatments					
Fatty acid	POST[1]	RO	SO	LO	MIX	SEM	*P*-value[2]
C4-C12	ND[3]	ND	ND	ND	ND	-	-
C13:0	0.03	0.04	0.04	0.04	0.05	0.008	0.83
Iso-C13:0	ND	ND	ND	ND	ND	-	-
Anteiso-C13:0	0.13	0.07	0.09	0.08	0.05	0.013	0.21
C14:0	0.68	0.61	0.51	0.57	0.56	0.041	0.29
Iso-C14:0	0.09	0.09	0.10	0.12	0.12	0.018	0.66
C14:1 *cis*(c)-9	ND	ND	ND	ND	ND	-	-
C15:0	0.49	0.52	0.49	0.53	0.43	0.050	0.52
Iso-C15:0	0.35	0.33	0.35	0.37	0.33	0.031	0.67
Anteiso-C15:0	0.59	0.52^{a}	0.40^{ab}	0.52^{a}	0.37^{b}	0.037	0.01
C16:0	9.38	9.83^{a}	9.04^{b}	8.44^{b}	8.90^{b}	0.188	<0.001
Iso-C16:0	0.22	0.16	0.20	0.20	0.18	0.013	0.11
C16:1 c9	1.08	1.29^{a}	0.91^{b}	1.03^{b}	0.98^{b}	0.060	<0.001
C17:0	0.63	0.67	0.63	0.62	0.64	0.026	0.45
Iso-C17:0	0.43	0.39^{a}	0.28^{b}	0.31^{ab}	0.25^{b}	0.024	0.002
Anteiso-C17:0	0.15	0.17	0.12	0.13	0.18	0.022	0.15
C17:1 c9	0.25	0.27	0.19	0.20	0.20	0.023	0.07
C18:0	15.36	15.69	16.02	16.00	16.24	0.470	0.86
C18:1 c9	8.09	11.28^{a}	7.74^{b}	8.69^{b}	8.29^{b}	0.418	<0.001
C18:1 c11	0.42	0.71^{a}	0.49^{b}	0.54^{ab}	0.66^{ab}	0.053	0.02
C18:1 c12	0.86	0.71^{b}	1.07^{a}	1.10^{a}	0.86^{ab}	0.086	0.01
C18:1 c13	0.06	0.07	0.04	0.06	0.09	0.012	0.11
C18:1 c15	0.17	0.16	0.17	0.20	0.21	0.018	0.24
C18:1 *trans*(t)-4	0.04	0.04	0.05	0.04	0.03	0.009	0.47
C18:1 t5	0.02	0.03	0.04	0.03	0.03	0.005	0.31
C18:1 t6-8	0.09	0.15	0.15	0.14	0.15	0.019	0.93
C18:1 t9	0.11	0.18^{ab}	0.17^{ab}	0.16^{b}	0.23^{a}	0.016	0.05
C18:1 t10+t11	0.63	1.14	1.27	1.16	1.64	0.214	0.33
C18:1 t12	0.20	0.25	0.30	0.31	0.35	0.032	0.19
C18:1 t13+t14	0.88	0.88	0.77	1.05	1.01	0.084	0.08
C18:1 t16+c14	0.29	0.24^{c}	0.26^{bc}	0.35^{a}	0.32^{ab}	0.019	<0.001
C18:2 c9c12	30.30	28.37	32.59	27.09	29.06	1.667	0.11
C18:2 t11c15	0.08	0.09^{b}	0.08^{b}	0.20^{a}	0.15^{ab}	0.032	0.03
C18:2 t9t12	0.03	0.04	0.04	0.03	0.04	0.005	0.31
C18:2 c9t11 CLA	0.11	0.15	0.14	0.15	0.18	0.019	0.51

Table 3. Continued.

Fatty acid	POST[1]	Dietary treatments				SEM	*P*-value[2]
		RO	SO	LO	MIX		
C18:2 t10c12 CLA	0.07	0.50	0.49	0.59	0.77	0.162	0.60
C18:3 c9c12c15	7.59	4.68^{b}	4.55^{b}	8.08^{a}	5.24^{b}	0.376	<0.001
C18:3 c6c9c12	0.78	0.59	0.56	0.46	0.37	0.060	0.05
C20:0	0.06	0.07	0.07	0.06	0.07	0.008	0.58
C20:1 c11	0.07	0.07^{b}	0.07^{b}	0.14^{a}	0.09^{b}	0.009	<0.001
C20:2 c11c14	0.09	0.11	0.10	0.09	0.10	0.009	0.43
C20:3 n-3	0.03	0.04	0.05	0.05	0.05	0.007	0.65
C20:3 n-6	2.10	1.83	1.63	1.67	1.55	0.135	0.48
C20:4 n-6	1.94	1.87	1.73	1.54	1.56	0.131	0.24
C20:5 n-3	0.99	0.72	0.72	0.85	0.72	0.050	0.20
C22:0	0.05	0.07	0.06	0.07	0.05	0.015	0.64
C22:1 c13	0.04	0.03	0.04	0.03	0.03	0.006	0.90
C22:2 c13c16	0.03	0.03	0.03	0.03	0.03	0.004	0.48
C22:4 n-6	0.87	1.59	1.87	1.77	1.77	0.326	0.93
C22:5 n-3	0.88	0.88	0.79	0.79	0.89	0.076	0.62
C22:6 n-3	0.59	1.00	1.13	1.12	1.05	0.239	0.97
C23:0	0.05	0.06	0.06	0.06	0.06	0.010	0.97
C24:0	0.06	0.06	0.07	0.07	0.06	0.008	0.52
C24:1 c15	0.03	0.06	0.05	0.04	0.05	0.011	0.76
Unidentified	11.46	10.62	11.18	12.05	12.72	1.382	0.70
SFA[4]	28.76	29.35	28.55	28.17	28.53	0.457	0.30
MUFA[5]	13.31	17.54^{a}	13.80^{b}	15.28^{ab}	15.22^{ab}	0.641	0.002
PUFA[6]	46.47	42.49	46.48	44.50	43.53	1.500	0.22
OBCFA[7]	3.37	3.23	2.91	3.13	2.80	0.125	0.07
TFA[8]	2.53	3.69	3.77	4.21	4.90	0.369	0.10

[a,b,c,d] Means within a row without common superscript differ ($P < 0.05$).

[1] Values are shown for comparison only and were not included in the statistical analysis.

[2] Effect of treatment.

[3] ND = not detected.

[4] Saturated fatty acids ∑ (C13:0, *iso*-C13:0, *anteiso*-C13:0, C14:0, *iso*-C14:0, C15:0, *iso*-C15:0, *anteiso*-C15:0, C16:0, *iso*-C16:0, C17:0, *iso*-C17:0, *anteiso*-C17:0, C18:0, C20:0, C22:0, C23:0, C24:0).

[5] Mono-unsaturated fatty acids ∑ (C16:1 c9, C17:1 c9, C18:1 c9, C18:1 c11, C18:1 c12, C18:1 c13, C18:1 c15, C18:1 t4, C18:1 t5, C18:1 t6-8, C18:1 t9, C18:1 t10+t11, C18:1 t12, C18:1 t13+t14, C18:1 t16+c14, C20:1 c11, C22:1 c13, C24:1 c15).

Table 3. Continued.

[6] Poly-unsaturated fatty acids ∑ (C18:2 c9c12, C18:2 t11c15, C18:2 t9t12, C18:2 c9t11 CLA, C18:2 t10c12 CLA, C18:3 c9c12c15, C18:3 c6c9c12, C20:2 c11c14, C20:3 n-3, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:2 c13c16, C22:4 n-6, C22:5 n-3, C22:6 n-3).

[7] Odd- and branched-chain fatty acids ∑ (C13:0, *iso*-C13:0, *anteiso*-C13:0, *iso*-C14:0, C15:0, *iso*-C15:0, *anteiso*-C15:0, *iso*-C16:0, C17:0, *iso*-C17:0, *anteiso*-C17:0, C17:1 c9).

[8] Trans fatty acids ∑ (C18:1 t4, C18:1 t5, C18:1 t6-8, C18:1 t9, C18:1 t10+t11, C18:1 t12, C18:1 t13+14, C18:1 t16+c14, C18:2 t11c15, C18:2 t9t12, C18:2 c9t11 CLA, C18:2 t10c12 CLA).

other diets and did not differ (P = 0.44) from those diets. Mammary SCD5 expression did not differ (P = 0.12) among the four dietary treatments. The average mRNA abundance of SCD1 (1686 ± 174 pg/µl) was substantially higher than that of SCD5 (0.766 ± 0.088 pg/µl).

Fatty Acid Composition of Milk

The effects of diet on milk FA composition are presented in Table 4. Compared with LO, cows fed the MIX diet had lower proportions of C6:0 in milk fat. The C16:0 fraction in milk fat was higher for RO compared with LO and MIX. Compared with the RO and MIX diet, cows fed the SO diet had a higher proportion of C18:0 in milk fat. The RO diet did not result in a significantly higher proportion of C18:1 *cis*-9. The SO treatment did result in increased proportions of C18:2 *cis*-9,12 in milk fat compared with RO and LO. Compared with the other treatments, C18:3 *cis*-9,12,15 was increased for the LO diet. In addition, C18:2 *trans*-11, *cis*-15 was increased for LO compared with RO and SO.

The proportion of long-chain FA (LCFA) in milk fat was higher for MIX, compared with RO. The SO treatment resulted in less odd- and branched-chain fatty acids (OBCFA) in the milk fat compared with RO, primarily due to the lower proportion of C13:0 and C15:0 for SO. Total SFA was significantly lower for MIX compared with RO and SO, and total PUFA was higher for LO and MIX compared with RO. Total TFA tended to be higher for MIX compared with RO (P = 0.09).

The four calculated desaturation indices from milk FA all showed the same tendency, with numerically the lowest values for SO and the highest values for MIX. For the desaturation indices of C16 and C18, the values for SO were significantly lower compared with the MIX diet. The r^2 between the different desaturation indices ranged from 0.66 to 0.82.

Correlation between Desaturation Indices and SCD1 and SCD5 Expression

Figure 2a shows the correlation between the C14 desaturation index, calculated from milk FA, and mammary SCD1 expression of the cows during the treatment period (C14 index = 0.086 (± 0.007) + 0.058 (± 0.017) x SCD1 expression; r^2 = 0.35; P = 0.002).

Table 4. Milk fatty acid composition (g/100 g fatty acids) of cows fed the post-trial diet (POST) or the treatment diets supplemented with either: rapeseed oil (RO), soybean oil (SO), linseed oil (LO), or a mixture of these three oils (MIX). For the four dietary treatments n = 7, and for POST n = 28.

		Dietary treatments					
Fatty acid	POST[1]	RO	SO	LO	MIX	SEM	*P*-value[2]
C4:0	2.72	2.52	2.57	2.48	1.94	0.264	0.32
C6:0	2.13	1.80^{ab}	1.86^{ab}	1.93^{a}	1.33^{b}	0.152	0.04
C8:0	1.42	1.14	1.12	1.22	0.86	0.103	0.10
C10:0	3.16	2.49	2.36	2.65	1.96	0.219	0.18
C11:0	0.45	0.35	0.28	0.35	0.26	0.030	0.08
C12:0	3.91	3.17	2.84	3.27	2.88	0.213	0.42
C13:0	0.22	0.20^{a}	0.13^{b}	0.18^{ab}	0.17^{ab}	0.014	0.01
Iso-C13:0	0.03	0.02^{b}	0.03^{ab}	0.05^{a}	0.02^{ab}	0.007	0.02
Anteiso-C13:0	0.12	0.09	0.07	0.10	0.09	0.008	0.06
C14:0	11.43	10.46	10.20	10.54	9.67	0.368	0.36
Iso-C14:0	0.08	0.06^{b}	0.09^{a}	0.09^{ab}	0.06^{b}	0.007	0.01
C14:1 *cis*(c)-9	1.33	1.37	1.02	1.20	1.43	0.124	0.11
C15:0	1.08	1.15^{a}	0.82^{b}	0.95^{b}	0.95^{b}	0.049	<0.001
Iso-C15:0	0.22	0.20^{b}	0.23^{a}	0.22^{ab}	0.19^{b}	0.009	0.01
Anteiso-C15:0	0.50	0.48	0.44	0.47	0.47	0.016	0.32
C16:0	29.36	27.58^{a}	26.56^{ab}	23.84^{c}	25.11^{bc}	0.584	<0.001
Iso-C16:0	0.19	0.15^{b}	0.18^{a}	0.16^{ab}	0.18^{a}	0.009	0.01
C16:1 c9	1.90	2.12	1.64	1.81	2.24	0.183	0.11
C17:0	0.62	0.61	0.54	0.60	0.57	0.017	0.05
Iso-C17:0	0.17	0.16	0.15	0.15	0.17	0.012	0.65
Anteiso-C17:0	0.24	0.08	0.12	0.12	0.10	0.015	0.42
C17:1 c9	0.19	0.22	0.17	0.19	0.22	0.018	0.13
C18:0	9.10	8.75^{b}	10.91^{a}	9.71^{ab}	8.90^{b}	0.506	0.02
C18:1 c9	19.12	21.31	21.21	21.30	22.65	0.951	0.67
C18:1 c11	0.33	0.71^{ab}	0.46^{c}	0.56^{bc}	0.76^{a}	0.050	0.001
C18:1 c12	0.36	0.32^{b}	0.62^{a}	0.48^{ab}	0.48^{ab}	0.051	0.004
C18:1 c13	0.08	0.11	0.09	0.11	0.13	0.009	0.05
C18:1 c15	0.26	0.25^{b}	0.24^{b}	0.50^{a}	0.35^{b}	0.037	<0.001
C18:1 *trans*(t)-4	0.01	0.03	0.04	0.04	0.03	0.004	0.64
C18:1 t5	0.01	0.03	0.03	0.03	0.03	0.007	0.97
C18:1 t6-8	0.30	0.57	0.49	0.53	0.64	0.042	0.10
C18:1 t9	0.18	0.40	0.35	0.36	0.47	0.031	0.06
C18:1 t10+t11	1.62	3.34	3.54	3.36	4.64	0.491	0.22
C18:1 t12	0.44	0.53^{b}	0.59^{ab}	0.64^{a}	0.65^{a}	0.025	0.01
C18:1 t13+t14	0.35	0.95	0.97	1.39	1.35	0.207	0.29

Table 4. Continued.

Fatty acid	POST[1]	Dietary treatments				SEM	*P*-value[2]
		RO	SO	LO	MIX		
C18:1 t16+c14	0.54	0.46^{c}	0.52^{c}	0.68^{a}	0.60^{b}	0.016	<0.001
C18:2 c9c12	1.49	1.49^{b}	1.90^{a}	1.47^{b}	1.89^{a}	0.073	<0.001
C18:2 t11c15	0.21	0.21^{b}	0.20^{b}	0.66^{a}	0.43^{ab}	0.074	<0.001
C18:2 t9t12	0.01	0.01	0.01	0.04	0.04	0.010	0.11
C18:2 c9t11 CLA	0.63	0.91	1.07	1.05	1.15	0.095	0.36
C18:2 t10c12 CLA	0.01	0.02	0.02	0.02	0.03	0.004	0.38
C18:3 c9c12c15	0.55	0.35^{c}	0.45^{bc}	0.66^{a}	0.54^{b}	0.026	<0.001
C18:3 c6c9c12	0.02	0.01	0.02	0.02	0.02	0.004	0.45
C20:0	0.12	0.12	0.14	0.11	0.12	0.007	0.09
C20:1 c11	0.04	0.06^{ab}	0.04^{b}	0.06^{ab}	0.07^{a}	0.006	0.03
C20:2 c11c14	0.02	0.02	0.02	0.03	0.02	0.006	0.30
C20:3 n-3	0.01	0.01	0.01	0.02	0.01	0.005	0.14
C20:3 n-6	0.07	0.05	0.06	0.06	0.05	0.005	0.79
C20:4 n-6	0.10	0.09	0.09	0.08	0.08	0.006	0.60
C20:5 n-3	0.05	0.03	0.04	0.04	0.04	0.005	0.56
C22:0	0.05	0.05	0.06	0.05	0.05	0.003	0.12
C22:1 c13	0.01	0.01	0.01	0.02	0.01	0.004	0.49
C22:2 c13c16	0.05	0.02^{b}	0.03^{ab}	0.04^{a}	0.03^{b}	0.004	0.01
C22:4 n-6	0.02	0.02	0.02	0.04	0.01	0.006	0.05
C22:5 n-3	0.08	0.07^{ab}	0.07^{ab}	0.08^{a}	0.06^{b}	0.006	0.04
C22:6 n-3	0.01	0.01	0.01	0.02	0.01	0.006	0.29
C23:0	0.02	0.01	0.02	0.03	0.02	0.005	0.16
C24:0	0.04	0.03^{b}	0.04^{ab}	0.06^{a}	0.03^{b}	0.005	0.001
C24:1 c15	0.01	0.01	0.01	0.02	0.01	0.005	0.30
Unidentified	2.24	2.23^{b}	2.17^{b}	3.06^{a}	2.74^{a}	0.101	<0.001
SMCFA[3]	26.09	22.95	21.99	23.28	20.07	1.081	0.18
C16 FA[4]	31.26	29.70^{a}	28.20^{a}	25.65^{b}	27.35^{ab}	0.651	0.002
LCFA[5]	36.32	41.35^{b}	44.39^{ab}	44.38^{ab}	46.37^{a}	1.122	0.03
OBCFA[6]	4.08	3.78^{a}	3.26^{b}	3.62^{ab}	3.47^{ab}	0.105	0.01
SFA[7]	67.35	61.68^{a}	61.78^{a}	59.31^{ab}	56.11^{b}	1.493	0.04
MUFA[8]	27.09	32.78	32.04	33.29	36.75	1.295	0.08
PUFA[9]	3.33	3.31^{b}	4.01^{ab}	4.34^{a}	4.40^{a}	0.201	0.003
TFA[10]	4.31	7.45	7.82	8.80	10.04	0.735	0.09

Table 4. Continued.

Fatty acid	POST[1]	Dietary treatments				SEM	*P*-value[2]
		RO	SO	LO	MIX		
Δ9-desaturase indices[11]							
C14:1 c9	0.11	0.12	0.09	0.10	0.13	0.012	0.11
C16:1 c9	0.07	0.07^{ab}	0.05^{b}	0.07^{ab}	0.08^{a}	0.006	0.02
C17:1 c9	0.25	0.26	0.23	0.24	0.28	0.016	0.18
C18:1 c9	0.69	0.71^{ab}	0.66^{b}	0.69^{ab}	0.72^{a}	0.014	0.04

[a,b,c,d] Means within a row without common superscript differ ($P < 0.05$).

[1] Values are shown for comparison only and were not included in the statistical analysis.

[2] Effect of treatment.

[3] Short- and medium-chain fatty acids ∑ (C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C14:1 c9).

[4] C16 fatty acids ∑ (C16:0, C16:1 c9).

[5] Long-chain fatty acids ∑ (fatty acids with 18 carbons or more).

[6] Odd- and branched-chain fatty acids ∑ (C11:0, C13:0, *iso*-C13:0, *anteiso*-C13:0, *iso*-C14:0, C15:0, *iso*-C15:0, *anteiso*-C15:0, *iso*-C16:0, C17:0, *iso*-C17:0, *anteiso*-C17:0, C17:1 c9).

[7] Saturated fatty acids ∑ (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, *iso*-C13:0, *anteiso*-C13:0, C14:0, *iso*-C14:0, C15:0, *iso*-C15:0, *anteiso*-C15:0, C16:0, *iso*-C16:0, C17:0, *iso*-C17:0, *anteiso*-C17:0, C18:0, C20:0, C22:0, C23:0, C24:0).

[8] Mono-unsaturated fatty acids ∑ (C14:1 c9, C16:1 c9, C17:1 c9, C18:1 c9, C18:1 c11, C18:1 c12, C18:1 c13, C18:1 c15, C18:1 t4, C18:1 t5, C18:1 t6-8, C18:1 t9, C18:1 t10+t11, C18:1 t12, C18:1 t13+t14, C18:1 t16+c14, C20:1 c11, C22:1 c13, C24:1 c15).

[9] Poly-unsaturated fatty acids ∑ (C18:2 c9c12, C18:2 t11c15, C18:2 t9t12, C18:2 c9t11 CLA, C18:2 t10c12 CLA, C18:3 c9c12c15, C18:3 c6c9c12, C20:2 c11c14, C20:3 n-3, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:2 c13c16, C22:4 n-6, C22:5 n-3, C22:6 n-3).

[10] Trans fatty acids ∑ (C18:1 t4, C18:1 t5, C18:1 t6-8, C18:1 t9, C18:1 t10+t11, C18:1 t12, C18:1 t13+14, C18:1 t16+c14, C18:2 t11c15, C18:2 t9t12, C18:2 c9t11 CLA, C18:2 t10c12 CLA).

[11] Δ9-desaturase indices are calculated as: Δ9-desaturase product divided by the sum of the Δ9-desaturase product and substrate.

The r^2 values of the correlation between mammary SCD1 expression and the other three desaturation indices were: 0.35 ($P = 0.002$), 0.25 ($P = 0.01$) and 0.39 ($P = 0.001$) for the C16, C17 and C18 index, respectively. Figure 2b shows the correlation between the C14 desaturation index and mammary SCD5 expression of the cows during the treatment period (C14 index = 0.102 (± 0.009) + 0.016 (± 0.028) x SCD5 expression; $r^2 = 0.02$; $P = 0.57$). The r^2 values of the correlation between mammary SCD5 expression and the other three desaturation indices were: 0.01 ($P = 0.69$), 0.02 ($P = 0.56$) and 0.01 ($P = 0.57$) for the C16, C17 and C18 index, respectively.

Discussion

The aim of this study was to compare the effects of different unprotected dietary unsaturated FA on the expression of SCD1 and SCD5 in the mammary gland of dairy cows. Therefore, we supplemented the diets of lactating cows with either rapeseed oil, soybean oil or linseed oil for a period of 3 weeks in order to increase the flow of C18:1 *cis*-9, C18:2 *cis*-9,12 and C18:3 *cis*-9,12,15 to the mammary gland, respectively. The blood plasma proportion of these three FA was indeed primarily related to their supply with the diet. Addition of unprotected rapeseed oil and linseed oil resulted in a significant higher blood plasma proportion of C18:1 *cis*-9 and C18:3 *cis*-9,12,15, respectively. Supplementation with unprotected soybean oil did not significantly increase the proportion of C18:2 *cis*-9,12 in blood plasma. This is probably due to the fact that C18:2 *cis*-9,12 is the most abundant FA in blood plasma (approximately 30% of total FA) and shows considerable variation. Although the proportion of C18:2 *cis*-9,12 in blood plasma for SO was not significantly different, the proportion of C18:2 *cis*-9,12 in milk fat was significantly higher for SO compared with RO and LO. This indicates that the uptake of C18:2 *cis*-9,12 by the mammary gland was higher for SO compared with RO and LO. Differential uptake of FA by the mammary gland could be the result of changes in the FA composition of the different lipid fractions present in blood plasma. In this study, the total lipid fraction in blood plasma was analysed, which includes cholesterol esters, phospholipids, triacylglycerols and non-esterified FA. Since C18:2 *cis*-9,12 and C18:3 *cis*-9,12,15 are preferably incorporated in plasma cholesterol esters and phospholipids whereas the bovine mammary gland primarily extracts FA from the triacylglycerols and non-esterified FA fractions in blood plasma (Loor et al., 2002), the transfer efficiency of these PUFAs from diet to milk is generally low in dairy cows.

Since the different unsaturated FA sources were added to the diet as unprotected oils, there was most likely an increase in ruminal biohydrogenation intermediates, as indicated by the rather high TFA isomer levels in milk. Some of these TFA can affect expression of several genes involved in lipid metabolism in the mammary gland. The *trans*-10, *cis*-12 CLA isomer has been identified as one of the major ruminal biohydrogenation intermediates, responsible for the inhibition of milk fat synthesis in dairy cows (Baumgard et al., 2002). Studies on the mechanisms involved in the inhibition of milk fat synthesis revealed a coordinated down-regulation of mammary gene expression of rate-limiting lipogenic enzymes, including lipoprotein lipase (LPL), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and SCD, following abomasal *trans*-10, *cis*-12 CLA infusion (Baumgard et al., 2002; Harvatine & Bauman, 2006) or diet-induced MFD (Peterson et al., 2003; Harvatine & Bauman, 2006). This inhibitory effect has been linked to a reduced expression of transcription factor SREBP-1 and proteins involved in the activation and distribution of SREBP-1 towards the nucleus (Harvatine & Bauman, 2006). Indeed, milk *trans*-10, *cis*-12 CLA in our study was negatively correlated with milk fat content (r^2 = 0.54; $P < 0.001$) and proportion of milk SMCFA (r^2 = 0.43; $P < 0.001$). However, the

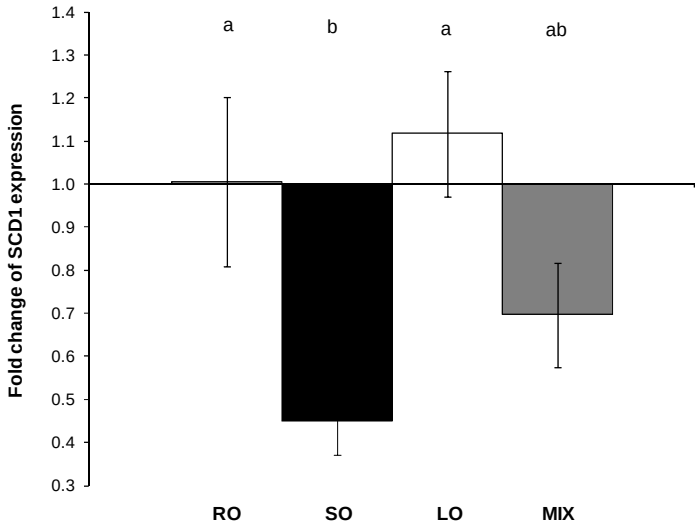
amount of *trans*-10, *cis*-12 CLA in milk fat did not correlate with SCD1 mRNA expression (r^2 = 0.001; *P* = 0.88) or with that of SCD5 (r^2 = 0.009; *P* = 0.48). Abomasal infusion with high doses of *trans*-10, *cis*-12 CLA, causing severe MFD, generally reduces desaturation indices serving as a proxy for SCD activity (Baumgard et al., 2001) and SCD expression (Baumgard et al., 2002). However, lower doses of *trans*-10, *cis*-12 CLA, causing mild MFD, typically do not affect the proxies for SCD activity (Peterson et al., 2002a; de Veth et al., 2004; Kay et al., 2007). Taken together, it is likely that in our study the unprotected dietary oil supplements did increase *trans*-10, *cis*-12 CLA production in the rumen which probably inhibited milk fat synthesis to some extent, but this amount was not sufficient to significantly affect SCD expression.

The higher blood plasma proportion of C16:0 and C16:1 *cis*-9 for the RO diet, most likely reflected the higher concentrations of these FA in the RO diet. The higher proportion of C18:2 *trans*-11, *cis*-15 in blood plasma observed in cows fed the LO diet is in agreement with previous studies, demonstrating that C18:2 *trans*-11, *cis*-15 is the primary biohydrogenation intermediate produced in the rumen from C18:3 *cis*-9,12,15 (Harfoot & Hazlewood, 1988; Loor et al., 2005).

In our study, cows on the SO diet showed a significant lower SCD1 expression (55% reduction) in the mammary gland, compared to the cows that received the RO or LO diet. In rodents, PUFA supplementation, especially of the n-3 and n-6 series, results in suppression of hepatic SCD1 expression, while SFA and MUFA have no or little effect (Ntambi, 1999). Moreover, Singh et al. (2004) found that dietary supplementation of safflower oil, high in C18:2 *cis*-9,12, decreased both SCD1 mRNA levels and total SCD activity in the mammary gland of lactating mice. In goats however, Bernard et al. (2005b) reported that dietary soybean supplementation (± 23 g C18:2 *cis*-9,12 /kg DM) decreased SCD1 expression in subcutaneous adipose tissue, but not in the mammary gland, indicating that the mammary gland might be less sensitive to dietary manipulation by PUFA compared to subcutaneous adipose tissue. In addition, it was shown by Murrieta et al. (2006) that feeding high-linoleate safflower seeds (± 18 g C18:2 *cis*-9,12 /kg DM) to lactating beef cows did not affect SCD1 mRNA abundance in the mammary gland.

Contrary to SO, we found no differences in mammary SCD1 mRNA levels between cows receiving the RO, LO or MIX diet. This is in agreement with Delbecchi et al. (2001), who found no differences in mammary SCD1 expression in dairy cows in response to dietary supplementation of protected or unprotected canola oil (± 38 g C18:1 *cis*-9 /kg DM). Moreover, data from Enjalbert et al. (1998) showed no significant effect on mammary gland desaturation rate of C18:0 in lactating Holstein cows, when C18:1 *cis*-9 was duodenally infused. In goats however, dietary supplementation with oleic sunflower oil or formaldehyde-treated linseed both reduced SCD1 expression in the mammary gland (*P* = 0.07 and *P* < 0.05 respectively; Bernard et al., 2005a). These inconsistent results could be due to species-specific differences in the regulation of SCD1 gene expression, or because we used a lower level of FA supplementation (12 g C18:1 *cis*-9 /kg DM or 12 g C18:3 *cis*-9,12,15 /kg DM in our study versus 30 g C18:1 *cis*-9 /kg DM or 25 g C18:3 *cis*-9,12,15 /kg DM in the study of Bernard et al. (2005a)).

a)



b)

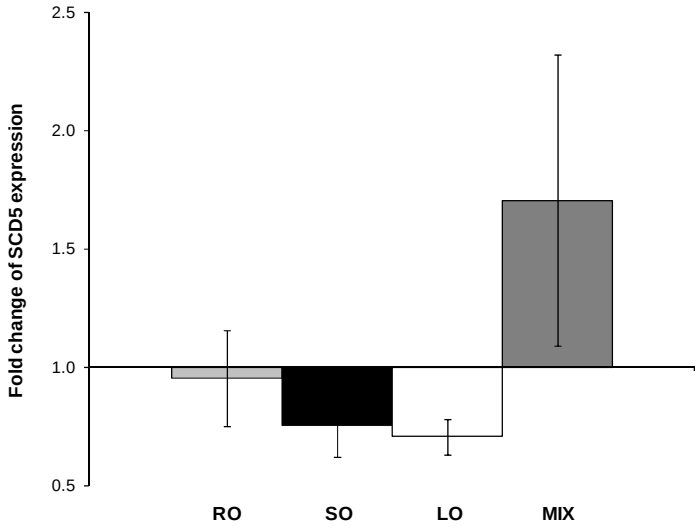
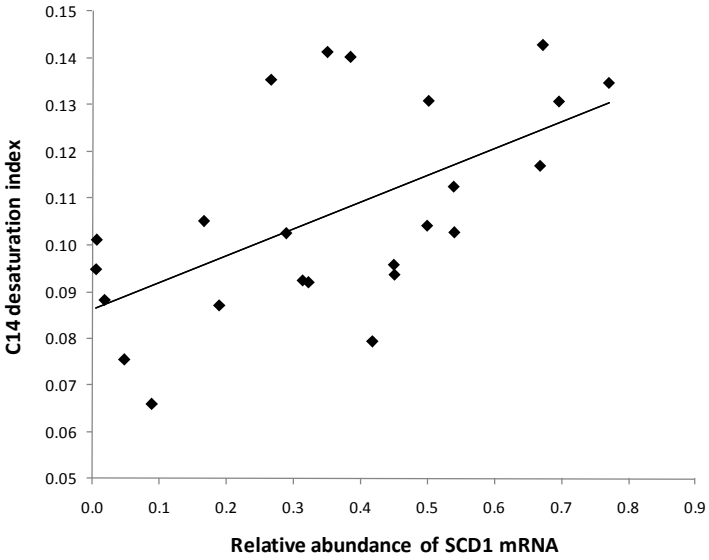


Figure 1. Patterns of mammary SCD1 **(a)** as well as SCD5 **(b)** mRNA expression in cows fed diets supplemented with either: rapeseed oil (RO), soybean oil (SO), linseed oil (LO), or a mixture of these three oils (MIX). Mammary gene expression was determined with Real Time PCR and expressed as fold change compared to the corresponding control values. Vertical lines represent the standard error of the mean and treatments without common superscript differ ($P < 0.05$). For SO and LO n=7, and for RO and MIX n=6.

a)



b)

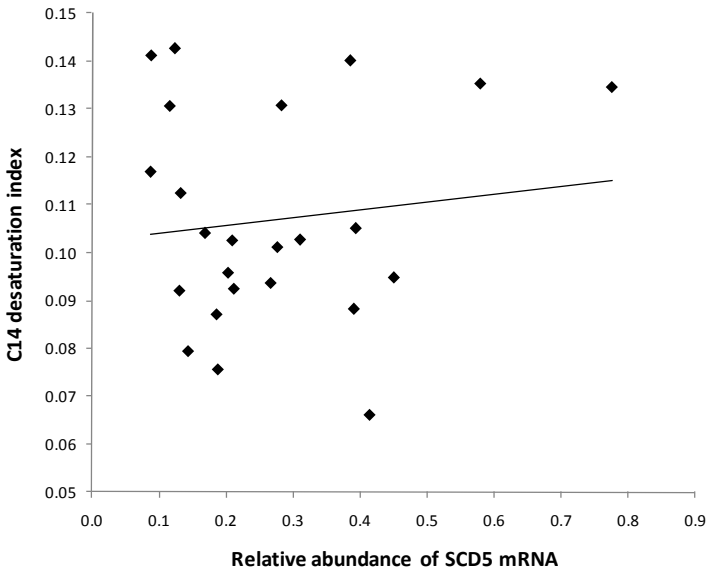


Figure 2. Correlation between the C14 desaturation index and the relative abundance of mammary SCD1 mRNA **(a)** or SCD5 mRNA **(b)** of cows in the treatment period (n=24). The desaturation indices were calculated from milk fatty acids as: C14:1 *cis*-9 / (C14:1 *cis*-9 + C14:0). For SCD1: the C14 index = 0.086 (± 0.007) + 0.058 (± 0.017) x SCD1 expression; $r^2 = 0.35$; $P = 0.002$ and for SDC5 the C14 index = 0.102 (± 0.009) + 0.016 (± 0.028) x SCD5 expression; $r^2 = 0.02$; $P = 0.57$.

Mammary SCD5 expression did not differ between the four dietary treatments, indicating that SCD5 and SCD1 are differently regulated. In line with this, it was demonstrated recently that mammary SCD1 mRNA expression tended to be reduced in dairy cows by intravenous infusion of *trans*-10, *cis*-12 CLA, while there was no effect on the expression of mammary SCD5 (Gervais et al., 2009). The knowledge that SCD5 lacks N-terminal PEST sequences typically found in SCD1 (Lengi & Corl, 2007), which are considered to be a signal for protein degradation (Rechsteiner & Rogers, 1996), may suggest that SCD5 exhibits a higher protein stability than SCD1, and that SCD5 might be less sensitive to nutritional regulation at the mRNA level than SCD1. This is in accordance with our observation that the mRNA abundance for SCD1 was much higher ($>10^3$) than that for SCD5 in the mammary tissue biopsies. In addition, the fact that SCD5 is abundantly expressed in brain, a tissue enriched in PUFA, may suggest that the expression of transcripts for SCD5 is less sensitive to PUFA than that for SCD1 (Lengi & Corl, 2007). Further studies are needed to provide compelling evidence that SCD1 and SCD5 are differentially regulated at their transcriptional and/or protein level in the bovine mammary gland.

The higher proportion of C16:0 in milk fat for the RO diet was most likely caused by the somewhat higher proportion of C16:0 in the RO diet. In contrast to our expectations, the proportion of C18:1 *cis*-9 in milk fat was not higher for the RO diet, although the proportion of C18:1 *cis*-9 in blood plasma was significantly higher for RO compared with the other diets. A lower SCD activity is not supported by our findings, because mRNA levels of SCD1 and SCD5 as well as desaturation indices in milk for the RO diet did not differ compared with LO and MIX. Therefore, we suggest that the uptake of C18:1 *cis*-9 by the mammary gland is somewhat low or that the absorbed C18:1 *cis*-9 is utilized for processes other than milk fat synthesis.

Several pairs of FA that represent a product/substrate relationship for SCD are present in milk fat, and are frequently used to estimate *in vivo* SCD activity within the mammary gland. The desaturase indices which are typically used are the C14, C16, C17 and C18 indices and the *cis*-9, *trans*-11 CLA index. The C14 index is considered the best indicator of SCD activity, since generally most of the C14:0 present in milk originates from *de novo* FA synthesis in the mammary gland and desaturation of C14:0 is the major source of C14:1 *cis*-9 (Peterson et al., 2002b; Lock & Garnsworthy, 2003; Bernard et al., 2008). Indeed, blood plasma levels of C14:0 were low (0.6 ± 0.04 g/100 g FA) and C14:1 *cis*-9 was not detected. In contrast, the absorption of various C16 and C18 FA from the duodenum and from mobilized adipose tissue can be significant (Glasser et al., 2008), and proportions of C16:1 *cis*-9, C17:1 *cis*-9, and in particular C18:1 *cis*-9 in blood plasma FA were substantial. Since no distinction between C18:1 *trans*-10 and *trans*-11 could be made in our study, the *cis*-9, *trans*-11 CLA index was not calculated. The SO diet in our study, which caused a down-regulation of SCD1 mRNA compared with the other unprotected oil supplements, indeed resulted in the lowest values for the C16 and C18 desaturation indices. This suggests that the lower SCD1 expression for the SO diet caused a reduction in SCD1

activity in the mammary gland. There were positive, although not strong, relationships between SCD1 mRNA levels and the four different desaturase indices. Feng et al. (2007) reported a significant relationship between the C14 desaturase index and relative abundance of SCD mRNA in milk somatic cells from dairy cows, indicating that both produce similar estimates of relative SCD activity in the mammary gland. Also in goats, moderate relationships were found among SCD mRNA levels, *in vitro* SCD activity and milk proxy ratios for SCD activity (Bernard et al., 2005a). However, there are also some indications that desaturase indices do not always reflect actual SCD activity in bovine adipose tissue (Archibeque et al., 2005) or in the bovine mammary gland (Bionaz & Loor, 2008b). Differences between the mammary SCD mRNA levels and the desaturase indices calculated from milk FA could be due to effects at the protein level of SCD, or because SCD1 has additional physiological functions like the synthesis of phospholipids (Scaglia & Igal, 2005).

Contrary to SCD1, we found no relationship between SCD5 mRNA abundance and the four desaturase indices. Together with the relative low mRNA levels of SCD5, our data suggest that the contribution of mammary SCD5 to generate milk Δ9 unsaturated FA is relatively small. However, further research on SCD5 is necessary to determine its role in Δ9 desaturation of milk FA in the bovine mammary gland.

Conclusions

This study showed that mammary SCD1 mRNA expression was significantly down-regulated in dairy cows by feeding unprotected soybean oil compared with rapeseed oil or linseed oil. In addition, SCD5 expression was not significantly affected by the four dietary treatments. This inhibitory effect on mammary SCD1 expression by feeding unprotected soybean oil was partially reflected by the lower desaturase indices calculated from milk FA. Mammary expression of SCD1 transcripts appears to be more sensitive to dietary supply of C18:2 *cis*-9,12 than to C18:1 *cis*-9 or C18:3 *cis*-9,12,15. Our data also support the idea that SCD1 and SCD5 expression in the bovine mammary gland respond differentially to dietary FA.

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Chapter 3

Alteration of Gene Expression in Mammary Gland Tissue of Dairy Cows in Response to Dietary Unsaturated Fatty Acids

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Abstract

The aim of this study was to determine the effects of supplementing unprotected dietary unsaturated fatty acids (UFAs) from different plant oils on gene expression in the mammary gland of grazing dairy cows. A total of 28 Holstein–Friesian dairy cows in mid-lactation were blocked according to parity, days in milk, milk yield and fat percentage. The cows were then randomly assigned to four UFA sources based on rapeseed, soybean, linseed or a mixture of the three oils for 23 days, after which, all 28 cows were switched to a control diet for an additional 28 days. On the last day of both periods, mammary gland biopsies were taken to study genome-wide differences in gene expression on Affymetrix GeneChip® Bovine Genome Arrays (no. 900493) by ServiceXS (Leiden, the Netherlands). Supplementation with UFAs resulted in increased milk yield but decreased milk fat and protein percentages. Furthermore, the proportion of *de novo* fatty acids (FAs) in the milk was reduced, whereas that of long-chain FAs increased. Applying a statistical cut-off of false discovery rate of q-values < 0.05 together with an absolute fold change of 1.3, a total of 972 genes were found to be significantly affected through UFA supplementation, indicating that large transcriptional adaptations occurred in the mammary gland when grazing dairy cows were supplemented with unprotected dietary UFA. Gene sets related to cell development and remodelling, apoptosis, nutrient metabolic process, as well as immune system response were predominantly downregulated during UFA supplementation. Such molecular knowledge on the physiology of the mammary gland might provide the basis for further functional research on dairy cows.

Introduction

The growing awareness over the last decade of the association between diet and health has led nutritional quality to become a relevant factor in consumers' food choices. A major development has been the recognition that certain lipids in dairy milk, such as oleic acid, some isomers of the conjugated linoleic acid (CLA) and a-linolenic acid (ALA), can improve human health status and prevent diseases (Bauman et al., 2006). Supplementing the diet of lactating dairy cows with different unsaturated fatty acids (UFA) is a significant attempt to improve milk fat composition for human consumption (Mansbridge & Blake, 1997; Harvatine & Bauman, 2006). Recently functional genomics studies, based on quantitative real-time polymerase chain reaction (qRT-PCR), have described the effects of dietary UFA on the expression of a number of candidate genes in the mammary gland involved in lipid metabolism (Harvatine & Bauman, 2006; Bauman et al., 2008; Bionaz & Loor, 2008a and 2008b; Harvatine et al., 2009; Kadegowda et al., 2009b). There is significant evidence that feeding cows with UFA-rich diets reduces the mammary mRNA abundance of acetylcoenzyme A (CoA) carboxylase (ACACA), fatty acid synthase (FASN), and stearoyl-CoA desaturase 1 (SCD1), as well as the transcription factor sterol regulatory element binding factor 1 (SREBP1) and peroxisome proliferator-activated receptor-g (PPARG; Bauman et al., 2006; Thering et al., 2009). However, information on the genome-wide expression of genes in the mammary gland tissue of dairy cows supplemented with different dietary unprotected UFA is still lacking. Therefore, it is not known yet whether dietary unprotected UFA supplementation also affects processes not related to lipid metabolism and the expression of less obvious genes.

Although qRT-PCR is a powerful approach to obtain a quantitative and highly precise estimates of gene expression; interest in microarray technologies for measuring gene expression has exploded in recent years (Sinicropi et al., 2007). The biggest advantage of microarray technology is its unbiased approach and the large number of transcripts that can be quantified in a single experiment. Microarray studies provide the ability to monitor the genome-wide expression of genes and to discover target genes that would not have been detected by other more focussed methods (Sinicropi et al., 2007). In addition, they provide a holistic view of the molecular events that occur when the mammary gland adapts to changes in the supply of dietary lipids, and consequent changes in the milk yield and composition through feeding strategies. Therefore, the recent development of microarray platforms for bovines in combination with bioinformatics has gained much attention in dairy science to discover genes and molecular pathways related to changes in the environment and/or phenotypic characteristics. Here, these developments have been used to determine the effects of unprotected dietary UFA from different plant oils on the global expression pattern of genes in the mammary gland tissue of grazing dairy cows in order to improve our understanding of mammary gland physiology.

Material and Methods

Animals and Diets

A total of 28 Holstein-Friesian dairy cows in mid-lactation were blocked according to parity (2.4 ± 0.63 births), days in milk (153 ± 32.8 days), milk yield (25.7 ± 1.08 kg/day) and milk fat percentage (4.3% ± 0.12%). Cows were then randomly assigned to treatment groups based on one of the four dietary treatments (n = 7 per dietary treatment). The dietary treatments consisted of a basal diet supplemented with either 2.7% of rapeseed oil, 2.7% of soybean oil, 2.7% of linseed oil or 2.7% of a proportional mixture of the three oils on a dry matter (DM) basis (Table 1). Rapeseed oil was chosen because it is an oil rich in c9-18:1 (oleic acid), whereas soybean oil was chosen as an oil rich in c9,c12-18:2 (linoleic acid) and the linseed oil as an oil rich in ALA. Overall, the concentration and the different oil sources used in the experiment are typically present in dairy cows rations. The oil supplements were included in the concentrate, which was fed, together with maize silage and grass silage, as a mixed ration (MR; Table 1). Maize silage, grass silage and concentrates represented 52%, 12% and 36% of the MR (DM basis). In addition to the MR, each cow received 1 kg of a commercial standard concentrate per day through automatic feeders in the milking parlor. Cows were fed the MR indoors at night, and were grazed on pasture composed of ryegrass (*Lolium perenne* L.), with approximately 20% white clover (*Trifolium repens* L.) during the day (from 0800 h to 1600 h). The average paddock size was 5 ha and the stocking rate was 16 cows/ha. Cows were fed the MR at a level of about 14.5 kg of DM/cow per day, and grazed at a daily herbage intake of approximately 5.5 kg of DM/cow. After the first 23 days (experimental period I), all cows were switched to a control diet without oil supplementation for an additional 28 days (experimental period II; Tables 1 and 2). This design, in which, each cow got both of the intervention in sequence, was chosen because it reduces variability of gene expression between cows due to heterogeneity in genetic background, and increases the statistical power to detect differential gene expression to a specified Type I error rate. A disadvantage of this design is that the effects attributed to UFA supplementation may be confounded with effects due to difference (of 28 days) in lactation stage (see Discussion). Cows were milked twice a day in the milking parlor at the facility. Each of the four groups of cows was kept indoors in separated pens. Individual milk production and MR intake per treatment group were recorded daily during both experimental periods. The pasture intake estimation was limited by the variation in the sward heights before grazing (at 0800 h) and after grazing (1600 h) on the last 3 days of each period, using a rising plate meter (weight: 350 g, diameter: 0.5 m, standing pressure ca. 17.5 N/m^2; Eijkelkamp, Giesbeek, the Netherlands). Samples of individual feedstuffs, including pasture and MR were taken during the last 3 days of the experimental periods, and were then analysed for nutrient composition and fatty acid (FA) profile. Ingredients of the concentrates and MR are presented in Table 1, whereas chemical composition of concentrates and the chemical and FA composition of the MR are presented in Table 2. The chemical and FA composition of the pasture are presented in Table S1.

Table 1. Ingredients of the experimental diets and the concentrates offered in the mixed ration.

Item	Control treatment	UFA-enriched treatment			
		Rapeseed oil	Soybean oil	Linseed oil	Mixture 1:1:1
Ingredients in the mixed rations, g/kg					
Maize silage	520.0	520.0	520.0	520.0	520.0
Grass silage	120.0	120.0	120.0	120.0	120.0
Concentrate[1]	360.0	360.0	360.0	360.0	360.0
Ingredients in the concentrates, g/kg					
Triticale	-	333.6	333.6	333.6	333.6
Rapeseed meal, formaldehyde treated	-	195.0	195.0	195.0	195.0
Soybean meal	-	102.0	102.0	102.0	120.0
Dried brewers grain	-	79.0	79.0	79.0	79.0
Rapeseed oil	-	75.0	-	-	25.0
Soybean oil	-	-	75.0	-	25.0
Linseed oil	-	-	-	75.0	25.0
Rapeseed meal	108.1	68.0	68.0	68.0	68.0
Citrus pulp	370.0	60.0	60.0	60.0	60.0
Soybean meal, formaldehyde treated	-	47.0	47.0	47.0	47.0
Palm kernel expeller	227.3	-	-	-	-
Sugar beet molasses	101.0	-	-	-	-
Coconut expeller	64.8	-	-	-	-
Wheat gluten feed	45.0	-	-	-	-
Wheat middlings	31.0	-	-	-	-
Soybean hulls	29.3	-	-	-	-
Protapec[2]	13.7	-	-	-	-
Calcium carbonate	2.0	16.3	16.3	16.3	16.3
Sodium chloride	2.4	10.5	10.5	10.5	10.5
Magnesium oxide 90%	3.4	4.8	4.8	4.8	4.8
Urea	-	4.5	4.5	4.5	4.5
Monocalcium phosphate	-	2.3	2.3	2.3	2.3
Vitamin mineral premix	2.0	2.0	2.0	2.0	2.0

UFA = unsaturated fatty acids.

[1] Including the concentrate supplied through the automatic feeding station.

[2] Concentrated potato fruit juice mixed with soybean hulls (Cehave Landbouwbelang, Veghel, the Netherlands).

Table 2. Chemical composition of the concentrates and chemical and FA composition of the mixed rations (MR).

Item	Control treatment	UFA-enriched treatment			
		Rapeseed oil	Soybean oil	Linseed oil	Mixture 1:1:1
Chemical composition of concentrates (g/kg DM)					
DM (g/kg)	911.7	891.1	900.5	904.1	901.0
Ash	87.6	81.2	79.3	86.1	84.9
CP	161.2	246.5	296.5	252.3	242.4
Crude fat	55.6	98.0	89.6	93.8	93.9
NDF	326.3	179.8	148.4	196.3	185.2
ADF	249.0	116.8	110.4	122.9	119.3
Starch	35.6	219.8	151.2	172.4	211.7
Sugars	120.0	73.0	105.8	87.1	72.3
Chemical composition of MR (g/kg DM)					
DM (g/kg)	560.9	589.4	574.5	573.1	593.6
Ash	66.1	66.2	64.3	66.5	67.4
CP	132.2	162.8	180.0	164.9	161.4
Crude fat	40.5	55.8	52.9	54.3	54.4
NDF	356.4	305.9	294.0	310.3	307.7
ADF	227.7	187.0	181.3	185.1	188.0
Starch	182.4	227.4	214.7	223.4	224.2
Sugars	57.4	48.5	55.7	48.7	48.5
FA composition of MR (g/100g FA)					
C8:0	1.5	0.3	0.2	0.2	0.3
C10:0	1.5	0.3	0.2	0.2	0.3
C12:0	16.8	3.6	2.0	1.9	3.8
C14:0	6.1	1.7	0.8	0.8	1.5
C16:0	13.5	16.7	13.9	10.3	13.3
C16:1 *cis*(c)-9	0.2	1.1	0.2	0.2	0.5
C18:0	2.7	4.0	3.3	3.2	3.5
C18:1 c9	17.3	30.1	20.3	20.5	23.8
C18:1 c11	0.9	2.2	1.3	1.6	1.8
C18:2 c9c12	30.6	32.2	47.2	29.3	35.2
C18:3 c9c12c15	7.7	6.6	9.3	30.7	14.8
C20:0	0.5	0.4	0.5	0.3	0.4
C22:0	0.5	0.5	0.5	0.4	0.5
C24:0	0.4	0.3	0.3	0.3	0.3

FA = fatty acid; UFA = unsaturated fatty acids; DM = dry matter.

In addition, on the last day of experimental periods I and II, two consecutive milk samples (0700 h and 1700 h milking) were obtained and pooled (0.6 : 0.4 ratio). One aliquot was stored at 4°C until analysis of fat, protein and lactose percentage, and another aliquot was frozen at -20°C until analysis for FA composition by gas chromatography. Fat, protein and lactose percentage was determined using mid-infrared spectrometry (International Standard Organisation (ISO) 9622, 1999c; Qlip NV, Zutphen, the Netherlands).

On the last day of both experimental periods, biopsies were carried out before the afternoon milking. A core of secretory tissue (750 to 1000 mg) of mammary tissue from each cow was obtained by surgical biopsy from the midpoint section of a rear quarter according to the method of Farr et al. (1996). The capsular end of the core was deleted to reduce the gene expression heterogeneity as a result of its greater amount of connective tissue (Farr et al., 1996). Tissue biopsies were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

To prevent local infection, a single intramammary injection of amoxicillin and clavulic acid (Avuloxil®; Pfizer Animal Health, Capelle a/d IJssel, the Netherlands) was applied in the affected rear quarter. Furthermore, a single intramuscular dose of ceftiofur hydrochloride (Excenel®; Pfizer Animal Health, Capelle a/d IJssel, the Netherlands) was given immediately after the biopsy. Within 2 h of the biopsy, cows were machine-milked and hand-stripped as needed to remove all intramammary blood clots, according to the method of Farr et al. (1996). This experiment was conducted at the Cranendonck Research Farm, the Netherlands, between September 26 and November 14, 2007, and procedures were approved by the Animal Care and Ethics Committee of Wageningen UR Livestock Research, Lelystad, the Netherlands.

Chemical Analysis

Composite samples of feeds from the last 3 days of each experimental period were analysed for DM content (ISO 6496, 1999a), ash (ISO 5984, 2002), Kjeldahl N (ISO 5983, 2005), ADF (Van Soest, 1973) and NDF according to a modified method of Van Soest et al. (1991) with additional incubations in a-amylase and protease as described by Goelema et al. (1998). Crude fat was determined by Berntrop method with acid hydrolysis (ISO 6492, 1999b).

Fatty Acid Analysis

The FA of milk and feedstuffs were extracted with chloroform–methanol (2 : 1, v/v) and transesterified to fatty acid methyl esters (FAME) by vortexing with sodium methanolate in methanol (30%). Then, FAME were used for gas chromatography analysis (Trace GC Ultra,Waltham, MA, USA). Specific details with regard to the analysis of FA in milk and feedstuffs are presented in the Supplementary materials. The proportion of de novo synthesis of FA was estimated based on the assumption that all FAs from 4-carbon to 14-carbon and 50% of 16-carbon FAs were synthesised by the mammary gland (Delamaire & Guinard-Flament, 2006). In addition, the proportion of long-chain fatty acid (LCFA) was

calculated by the sum of 50% 16-carbon and all the 18- to 24-carbon FA (Delamaire & Guinard-Flament, 2006).

RNA Isolation, Processing and Microarray Analysis

Total RNA from mammary gland tissue (50 to 100 mg) was isolated using TRIzol® reagent (Invitrogen, Breda, the Netherlands), following the manufacturer's instructions. The RNA purity and concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, the Netherlands), and the RNA quality was assessed using the BioAnalyzer 2100 (Agilent Technologies, Amsterdam, the Netherlands). The RNA was judged as suitable for array hybridization because they showed intact bands corresponding to the 18S and 28S ribosomal RNA subunits, displayed no RNA degradation products, and presented an average RNA integrity number of 8.32 ± 0.05. The RNA of each biopsy was amplified, biotinlabeled and hybridized to single dye Affymetrix GeneChip® Bovine Genome Array (no. 900493) by ServiceXS (Leiden, the Netherlands), as described in the users' manual (Affymetrix GeneChip® Expression Analysis Technical Manual, Santa Clara, CA, USA). As the factorial design and analysis of the microarray experiment is a reliable method to identify the influence of multiple factors on the expression profiles of the probe sets in the microarray (Xu & Faisal, 2010), a total of 56 one-color arrays were prepared, one array per RNA sample. Briefly, total RNA (2 mg per sample) was reverse transcribed to cDNA using a T7-oligo(dT) primer. Following second strand cDNA synthesis, the double-stranded cDNA was purified as a template for the subsequent in vitro transcription reaction. Linearly amplified biotin-labeled cRNA was synthesised in the presence of a biotin-labeled nucleotide analog/RNA mix. The labeled cRNA was purified, fragmented and hybridized to the arrays at 45°C for 16 h with constant rotational mixing at 60 r.p.m. Washing and staining of the arrays was carried out using the Affymetrix GeneChip® Fluidics Station 450 (Santa Clara, CA, USA). The arrays were scanned using an Affymetrix GeneChip® Scanner 7G (Santa Clara, CA, USA) and Affymetrix GeneChip® Operating Software version 1.4, following the GeneChip's specifications. After scanning, the Affymetrix GeneChip Command Console Software automatically acquired and analysed image data and computed an intensity value for each probe cell. A number of quality control parameters associated with assay and hybridization performance were closely monitored. Specific details with regard to these quality control parameters are presented in the Supplementary materials.

Validation of Differential Gene Expression by qRT-PCR

In order to validate microarray analysis, the following four genes measured by microarray analysis were confirmed by qRT-PCR: FASN, fatty acid desaturase 1 (FADS1), fatty acid desaturase 3 (FADS3) and SCD1. Briefly, reverse transcription of 1 mg of the isolated total RNA (see section 'RNA isolation, processing and microarray analysis') was performed in a 20-ml reaction using Superscript III reverse transcriptase (Invitrogen, Breda, the Netherlands), deoxynucleosides (Roche Diagnostics, Almere, the Netherlands) and

random hexamer primers (Roche Diagnostics, Almere, the Netherlands) for 1 h at 50°C according to the manufacturer's protocol (Invitrogen, Breda, the Netherlands). Templates were amplified after a preincubation for 10 min at 95°C, followed by amplification for 40 cycles (10 s at 95°C, 5 s at 60°C and 5s at 72°C) on a LightCycler 1.2 Real-Time PCR System by using FastStart DNA Master SYBR Green I reagents (Roche Diagnostics, Almere, the Netherlands). All reactions revealed a single product as determined by melting curve analysis. Quantitative measurement was taken by establishing a linear amplification curve from serial dilutions of cDNAs for corresponding genes, and efficiencies of the used sets of primers were calculated to be at least 95%. Values were calculated according to the comparative threshold cycle method using 18SRNA as the endogenous reference gene.

Microarray Data Analysis

All microarray analysis including preprocessing, normalization and statistical analysis was carried out using Bioconductor packages (version 2.5) in R programming language (version 2.11). Data were quality assessed before and after normalization using a number of built-in quality control methods implemented in the Bioconductor affycoretools and associated packages to identify eventual irregularities of array hybridization, RNA degradation and data normalization.

Arrays were considered to be of sufficient quality when they showed not more than 10% of specks in Bioconductor's Fitting Probe Level Model (fitPLM) images, were not deviating in RNA degradation and density plots, and were not significantly deviating in Normalized Unscaled Standard Errors Plot and in Relative Log Expression plots (data not shown). Upon rigorous examination of the resulting diagnostic plots, all 56 microarrays were included in further analysis. Affymetrix GeneChip® uses a set of 11 to 20 oligonucleotide probes, each 25 bases long, to represent a single gene (Gautier et al., 2004). The expression level for a single gene is the summary of the data from the entire probe set (Gautier et al., 2004). In this study, the expression levels of probe sets were summarized using the library GeneChip content-corrected robust multichip average algorithm (Wu et al., 2004), employing the empirical Bayes approach for background correction followed by quantile normalization. As many of the original annotations for the Affymetrix GeneChip® Bovine have been found to be erroneous (Gautier et al., 2004), a custom chip definition file (CDF; Bovine_Bt_REFSEQ version 12.0.0), available at http://brainarray.mbni.med.umich.edu/Brainarray/Database/ CustomCDF/12.0.0/refseq.asp was used to re-annotate the probes to new probe sets. Therefore, the original probe set definitions were discarded and all probes were recomposed into new probe sets by mapping each probe via their sequence to unique genes available in Refseq genomics resources. As these custom CDFs are based on the latest genomic knowledge, the newly defined probe sets perform better, and allow for more reliable comparison of gene expression. In addition, as genes are uniquely represented in a custom CDF, bias toward genes represented by multiple probe sets is avoided in gene-set enrichment (de Leeuw et al., 2008). This resulted in gene expression values for 11,495 known genes with unique identifiers from 24,128

transcripts. All microarray experiment data are MIAME (Minimum Information About a Microarray Experiment) compliant and has been deposited in gene expression omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/; accession numbers GSE20909).

Statistical Analyses

Milk yield and composition, as well as FA composition, were analysed using a mixed-effects ANOVA (release 9.1; Statistical Analysis Software Institute Inc. Cary, NC, USA). The model included UFA sources, UFA supplementation (experimental periods), and the interaction between UFA sources and UFA supplementation (experimental periods), as fixed effects and cow within pen as a random effect. When differences between UFA sources were significant ($P < 0.05$), Tukey's test was used to compare means. The same model was used to analyse gene expression. The mixed-effects ANOVA was chosen because when there are more than two conditions to compare, the application of ANOVA F-test is much powerful than a t-test (Cui & Churchill, 2003). ANOVA considers the variability of the expression levels within and among treatments. If the variability of the expression of a gene among treatments is substantially greater than the variability within treatments, this indicates that the gene is differentially expressed. Lastly, the mixed-effects ANOVA allows to treat the cow as a random effect, which captures variability between individual cows within the same condition (Churchill, 2004). Ollier et al. (2009) also analysed genome-wide expression in the mammary gland of goats fed with diets differing in forage-to-concentrate ratio supplemented or not with lipids by ANOVA, including diet, period and animal group effects. However, in our study, the expression of genes in mammary gland was not significantly affected by the different dietary unprotected UFA sources. Therefore, this statistically insignificant variable and the interaction between UFA sources and UFA supplementation (experimental periods) were removed from the model. The final statistical model included UFA supplementation (experimental periods) as fixed effect and cow as a random effect. The P-values were corrected for multiple testing using a false discovery rate (FDR) method, which provides an estimate of the fraction of false discoveries among the significant terms (Bunger et al., 2007). The list of differentially expressed genes was generated using a FDR < 5% (q-value < 0.05) together with an absolute fold change (FC) threshold of 1.3. In addition, three complementary methods were applied to relate changes in gene expression to functional changes. One method, provided via the ErmineJ software program, was based on overrepresentation of gene ontology (GO) terms (Lee et al., 2005). Another approach was the gene-set enrichment analysis (GSEA). The GSEA derives its power by focusing on gene sets, that is, groups of genes that share common biological functions (e.g. biochemical, metabolic or signal transduction routes), chromosomal location or regulation. The GSEA method first calculates an enrichment score (ES) that reflects the degree to which a set of genes is overrepresented at the extremes (top or bottom) of the entire list of genes. Then after, it estimates the significance level of ES by using an empirical phenotype-based permutation test procedure that preserves the complex correlation structure of the gene expression data, and lastly there is an adjustment for

multiple hypothesis testing (Subramanian et al., 2005). Gene set size filter considered a minimum of 15 and a maximum of 500 genes, and the number of permutation was set to 1000. Gene sets were considered significantly enriched at a FDR < 5%. Normalized enriched scores of significantly enriched pathways were calculated. Both applied methods have the advantage that it is unbiased, because no gene selection step is used, and a score is computed based on all genes in a GO term or gene set. The last method used the Ingenuity Pathways Analysis (IPA; version 5.5, Ingenuity Systems, Redwood City, CA, USA) to identify the relevant molecular and cellular functions, canonical pathways, biological functions and the biological interaction networks among significant genes. For IPA analysis, the data set containing gene identifiers and corresponding to an absolute threshold of FC of 1.3 and FDR q-values < 0.05 was uploaded into the application. Each identifier was mapped to its corresponding gene object in the Ingenuity knowledge base. To study the biological interaction networks, genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these genes were then algorithmically generated based on their connectivity. Network analysis returns a score that ranks networks according to their degree of relevance to the network eligible molecules in the data set (Calvano et al., 2005). The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact test. The score is the negative log of this *P*-value. A score of 1.3 thus indicates a *P*-value of 0.05 and is considered statistically significant. Only those molecules that show relationships to other genes, proteins or endogenous chemicals were integrated into the analysis.

Results

DM Intake, Milk Production and Composition

Total estimated MR intake was 12.5 ± 1.50 kg/day when cows were fed with control diet and 14.7 ± 1.50 kg/day when cows were supplemented with UFA. Total DM intake (DMI) was 15.1, 14.4, 14.5 and 14.7 kg/day for diets enriched with rapeseed oil, soybean oil, linseed oil and a proportional mix of them all, respectively, but the differences in DMI could not be statistically evaluated as intake was not determined per individual animal. Average pasture intake was 5.80 ± 0.5 kg/day for cows fed on control diet and 5.52 ± 0.5 kg/day for cows fed on UFA-enriched diet. The milk yield was 15% greater ($P < 0.05$) when supplementing dairy cows with UFA (27.6 ± 1.26 kg/day) relative to the same cows fed the control diet (23.4 ± 1.26 kg/day), irrespective of the UFA source ($P < 0.10$). Milk fat and protein yield did not differ between treatments (1.00 ± 0.05 and 0.91 ± 0.05 kg/day, respectively). Although lactose yield was greater ($P < 0.01$) for cows fed on UFA-enriched diet (1.21 ± 0.04 kg/day) compared with cows fed on control diet (1.02 ± 0.04 kg/day), the total milk fat percentage was lower by over 20% (3.59% v. 4.30% ± 0.60%; $P < 0.05$) and protein percentage was lower by over 6% (3.51% v. 3.75% ± 0.41%; $P < 0.05$) for the same comparison. Lactose content was not different among treatments (4.37% ± 0.05%).

Table 3. FA profile of milk when comparing dairy cows supplemented with UFA relative to the same cows fed a control diet.

Item (n = 28 cows)	Control diet	UFA-enriched diet	s.e	*P*-value[1]
De novo FAs (%)[2]	41.2	35.6	1.03	***
LCFA (%)[3]	50.0	56.1	0.98	***
Total trans–FAs (%)	3.33	7.53	0.56	***
c9,t11-CLA	0.57	0.99	0.093	***
t10,c12-CLA	0.01	0.02	0.003	***

FA = fatty acid; UFA = unsaturated fatty acids; LCFA = Long Chain Fatty Acid; CLA = Conjugated Linoleic Acid.

[1] *P*-value = effect of UFA supplementation; *** levels of significance indicate $P < 0.001$.

[2] *De novo* FA include all FA from C4 to C14 and 50% of C16 FA.

[3] Long Chain Fatty Acids include all FA with 18 carbon atoms or more.

In addition, throughout supplementation of dietary UFA, the proportion of *de novo* FA decreased ($P < 0.001$), and the proportion of LCFAs and *trans*-18 FA in the milk increased ($P < 0.001$; Table 3). Further information of dietary effects on milk FA profiles are given in the Supplementary materials (Table S2 and Table S3).

Differential Expression of Genes in the Mammary Gland

We identified a total of 972 genes differentially expressed in the mammary gland tissue of cows fed on a diet supplemented with UFA compared with the same cows receiving the control diet. The list of differentially expressed genes was generated using a cut-off of FDR q-values < 0.05 together with an absolute FC threshold of 1.3 and further refined by selecting those probe sets mapping to unique Entrez Gene identifiers. The gene identification, symbol and description of these genes are shown in Supplementary material (Table S4). Within these 972 genes, 312 upregulated and 660 downregulated genes were found when cows were supplemented with UFA compared with when cows were fed the control diet. As gene expression was not significantly affected in mammary gland of cows fed on diets differing in the sources of dietary unprotected UFA sources, we focused on the effect of UFA supplementation (experimental period) on gene expression in the mammary gland tissue, instead of the effect of different UFA sources. To validate the microarray gene expression data, mammary gland tissue RNA samples were analysed by qRT-PCR for the genes FASN, FADS1, FADS3 and SCD1. The qRT-PCR results confirmed the microarray expression levels for the selected genes (Supplementary material, Table S5). In addition, correlations between qRT-PCR and microarray gene expressions was consistently high, with most genes having r^2 values > 0.70.

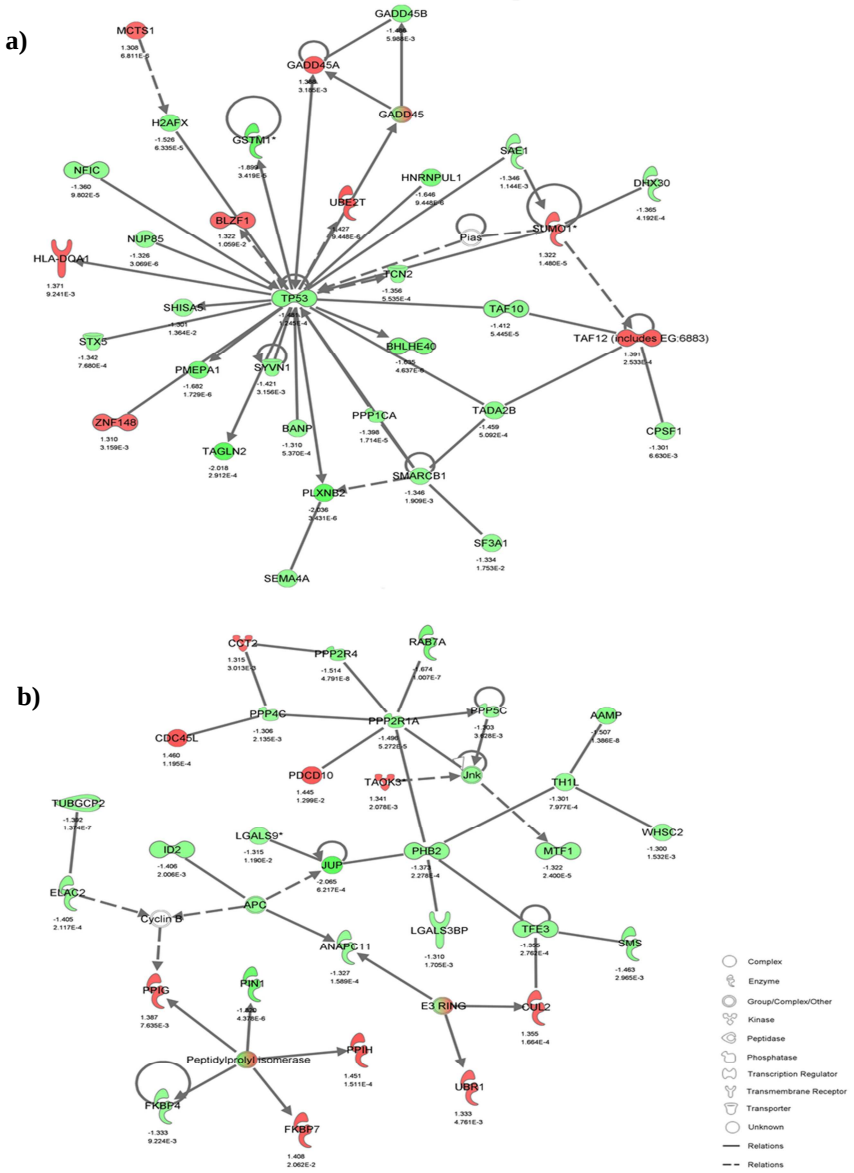

Figure 1. Ingenuity Pathways Analysis (IPA) networks detected when comparing dairy cows supplemented with unsaturated fatty acid relative to the same cows fed a control diet. **(a)** IPA network 1 included genes involved in cell cycle, cancer, cellular assembly and organization and presented a score of 46 and 33 focus genes; **(b)** IPA network 2 included genes involved in cell-mediated immune response, cellular development and amino acid metabolism, with a score of 43 and 30 focus genes. The network displayed graphically as nodes (gene/gene products) and edges (the biological relationship between nodes). The node colour intensity indicates the expression of genes: red upregulated, green downregulated in animals supplemented with UFA relative to the same cows fed a control diet. The shapes of nodes indicate the functional class of the gene product. The fold value and false discovery rate q-values are indicated under each node.

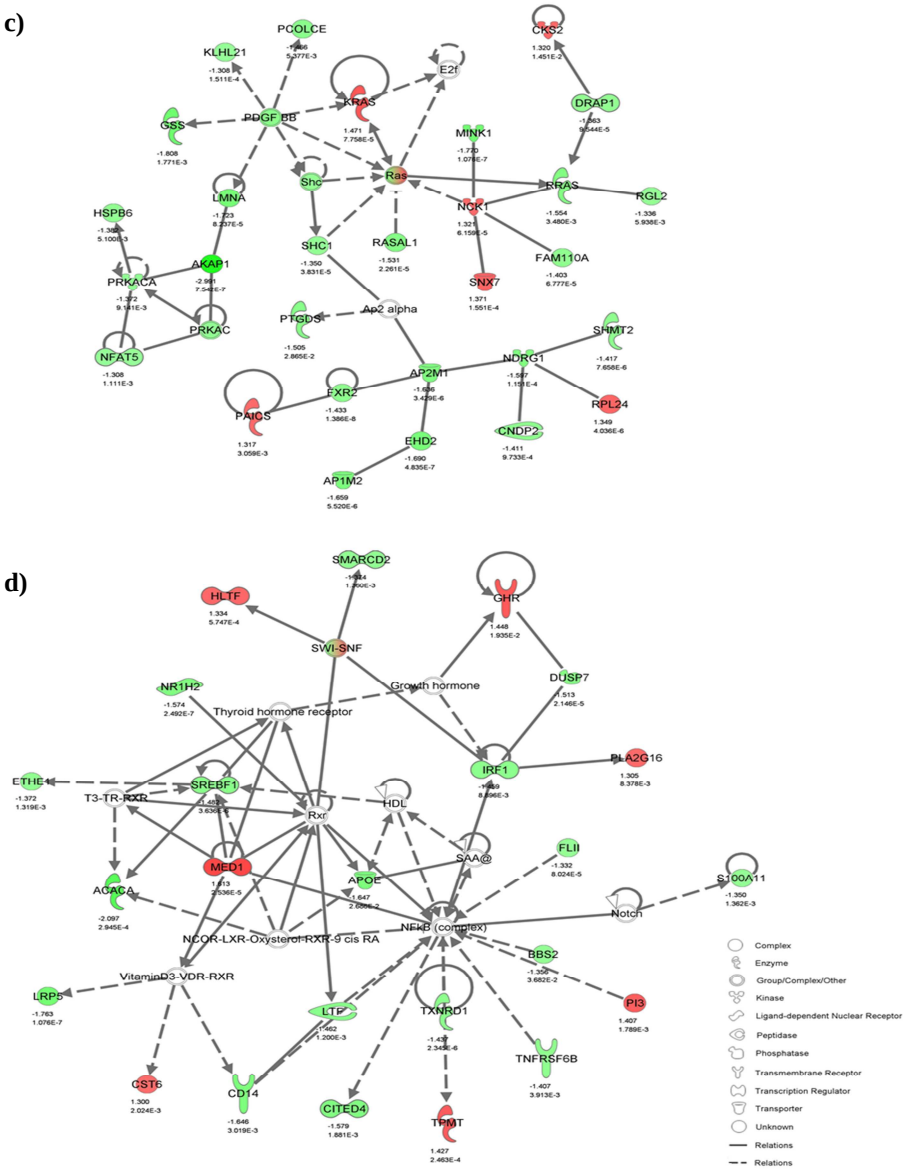

Figure 1. Ingenuity Pathways Analysis (IPA) networks detected when comparing dairy cows supplemented with unsaturated fatty acid relative to the same cows fed a control diet. **(c)** IPA network 3 included genes involved in skeletal and muscular disorders, cell death, dermatological disease, with a score of 40 and 28 focus genes; and **(d)** IPA network 4 included genes involved in connective tissue development and function, as well as tissue morphology and antigen presentation, with a score of 35 and 24 focus genes. The network displayed graphically as nodes (gene/gene products) and edges (the biological relationship between nodes). The node colour intensity indicates the expression of genes: red upregulated, green downregulated in animals supplemented with UFA relative to the same cows fed a control diet. The shapes of nodes indicate the functional class of the gene product. The fold value and false discovery rate q-values are indicated under each node.

Functional Clustering of Differential Expressed Genes in the Mammary Gland

To gain insight into the mammary gland tissue processes that were altered during UFA supplementation, we tested the list of differentially expressed genes using GO term enrichment analysis (Supplementary material; Table S6), GSEA (Supplementary material; Table S7) and IPA (Figure 1). One major finding of all these approaches was that UFA supplementation mainly reduces expressions of genes involved in cellular growth and proliferation, cytoskeleton organization and cellular homeostasis, apoptosis, nutrient metabolism, as well as molecular transport and defence response (Figure 2a). Most of the genes included in these molecular and cellular functions were downregulated during UFA supplementation (Figure 2b). A specific examination of the lipid metabolism IPA molecular and cellular function revealed that the SREBP-1 was downregulated during UFA supplementation (Table 4). Consequently, the expression of gene sets regulated by SREBP-1 were also downregulated (Table 4). Interestingly, the canonical signalling pathways significantly modulated in the mammary gland tissue of dairy cows fed on diets supplemented with UFA relative to the same cows fed on control diet were mainly involved in cellular growth, proliferation and development or immune system response (Supplementary material; Figure S1). Those pathways associated with cellular growth, proliferation and development, included the mammalian target rapamycin (mTOR) signalling pathways, the Janus kinases and signal transfers and activators of transcription (JAK/STAT) signalling pathways, as well as the granulocyte-macrophage colony-stimulating factor (GM-CSF) pathway (Supplementary material; Figure S2a). Most of the genes included in these canonical pathways were downregulated during UFA supplementation (Supplementary material; Figure S2b). In addition, the main canonical pathways involved in immune response were related to interleukin (IL) IL-2, IL-3, IL-8 and IL-6 signalling, as well as natural killer cell signalling (Supplementary material; Figure S3a). Remarkably, most of these canonical pathways included downregulated genes when cows were supplemented with UFA compared with when cows were fed with control diet (Supplementary material; Figure S3b). The IPA networks with the highest significance score (network score >35) are represented in Figure 1a to d. The first network (Figure 1a) presented a score of 46 and 33 focus genes. The major node that was identified during UFA supplementation: tumor protein P53 (P53), is a key transcription factor associated with mammary development in ruminants (Piantoni et al., 2008). The second network (Figure 1b), having a score of 43 and 30 focus genes, indicated gene clusters centred on the serine/threonine-protein phosphatase 2A, and peptidylprolylisomerase. This network presents functions related to immune response, cellular development and amino acid metabolism. The third IPA network (Figure 1c), having a score of 40 and 28 focus genes, centred on Ras. Members of the Ras family of small GTPases function downstream of mitogenic growth factor receptors and interact with a number of effectors to regulate cell proliferation and survival (Swarbrick et al., 2008). The last network (Figure 1d) involved genes associated with connective tissue development and function, as well as tissue

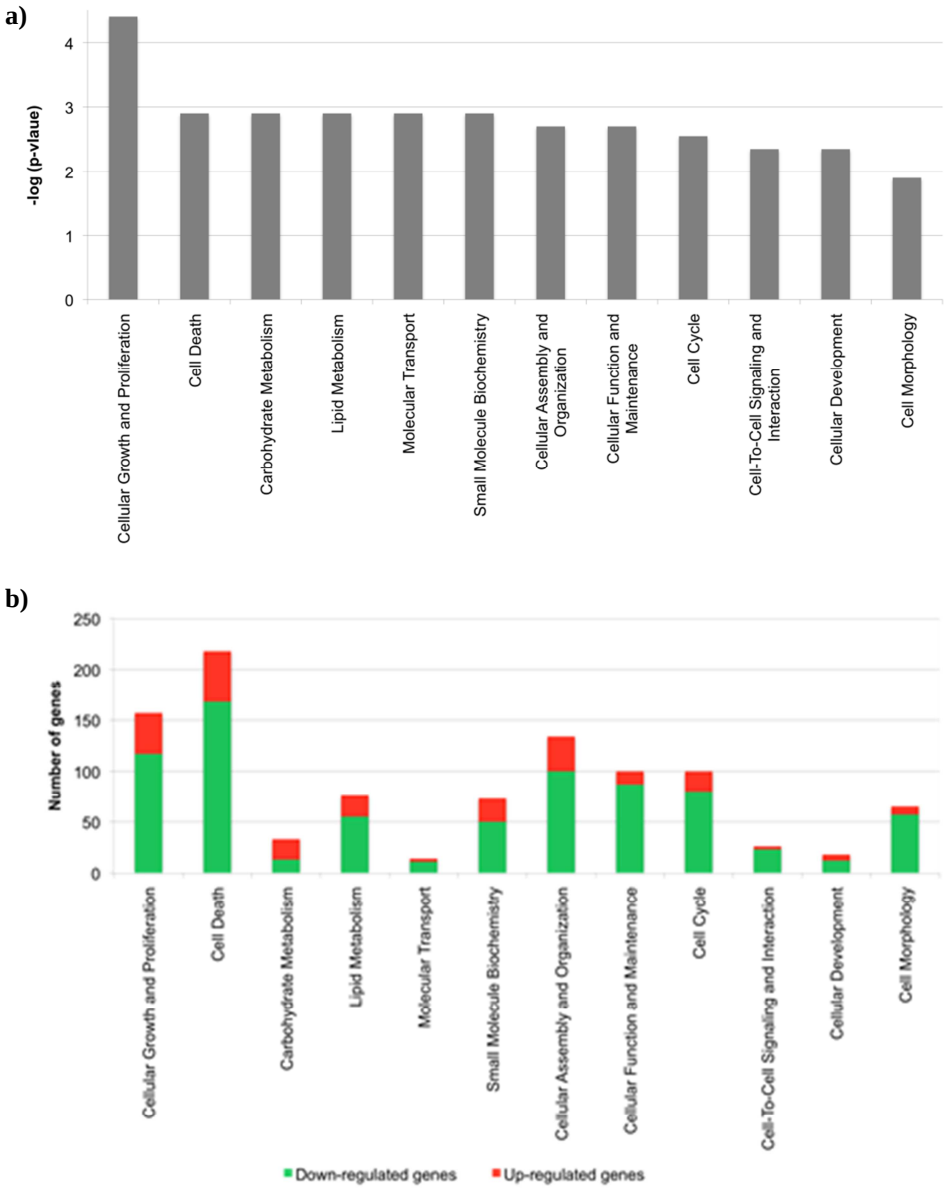

Figure 2. **(a)** Molecular and cellular functions significantly modulated in the mammary gland tissue when comparing dairy cows supplemented with unprotected unsaturated fatty acids (UFAs) relative to the same cows fed control diet. Statistical significance of pathway modulation was calculated via a right-tailed Fisher's Exact test in Ingenuity Pathway and represented as –log (P-value): 2log values exceeding 1.30 were significant false discovery rate q-values < 0.05. **(b)** The downregulated and upregulated genes for each molecular pathway are presented. The colour intensity indicates the expression of genes: red upregulated, green downregulated in animals supplemented with UFA relative to the same cows fed a control diet.

Table 4. Lipid metabolism genes in mammary gland when comparing dairy cows supplemented with UFA relative to the same cows fed a control diet. Lipid metabolism genes were identified by Ingenuity Pathways Analysis and they presented an absolute FC threshold of 1.3 and a FDR < 5%.

Enrez ID	Symbol	Gene title	FC[1]	FDR *q*-value[2]
NM_001046190	*ABCD1*	ATP-binding cassette, sub-family D, member 1	-1.43	***
NM_001034319	*ACAA1*	acetyl-Coenzyme A acyltransferase 1	-1.31	***
NM_174224	*ACACA*	acetyl-coenzyme A carboxylase alpha	-2.10	***
XM_590080	*ACOT4*	acyl-CoA thioesterase	1.32	***
XM_613318	*ACSM3*	acyl-CoA synthetase medium-chain family member 3	1.62	**
NM_174746	*ACSS1*	acyl-CoA synthetase short-chain family member 1	-1.37	***
NM_001105339	*ACSS2*	acyl-CoA synthetase short-chain family member 2	-1.48	**
NM_001034055	*ADIPOR1*	Adiponectin receptor 1	-1.36	***
NM_177518	*AGPAT1*	1-acylglycerol-3-phosphate O-acyltransferase 1	-1.96	***
NM_174233	*AGTR1*	angiotensin II receptor, type 1	1.36	***
NM_173986	*AKT1*	v-akt murine thymoma viral oncogene homolog 1	-1.99	***
NM_001076293	*ALOX5AP*	5-lipoxygenase activating protein	1.45	***
NM_001034523	*AP2M1*	adaptor-related protein complex 2, mu 1 subunit	-1.64	***
NM_174242	*APOA1*	apolipoprotein A-I	1.37	***
NM_173991	*APOE*	apolipoprotein E	-1.65	**
NM_001040469	*C3*	Complement 3	-1.72	***
NM_174008	*CD14*	CD14	-1.65	***
NM_001001601	*CDH5*	cadherin 5, type 2	-1.37	***
NM_173902	*CLU*	Clusterin	-1.49	**
XM_876020	*CNTFR*	cystic fibrosis transmembrane conductance regulator	-1.78	***
NM_174035	*CYBB*	cytochrome b-245, beta polypeptid	1.39	***
NM_001014927	*DHCR7*	7-dehydrocholesterol reductase	-1.36	***
NM_174308	*EDNRA*	endothelin receptor type A	1.40	***
NM_174537	*FCER1G*	Fc fragment of IgE, high affinity I	-1.49	***
NM_001076014	*FIG4*	SAC1 lipid phosphatase domain	1.45	***
NM_001034322	*FKBP4*	FK506 binding protein 4	-1.33	***
NM_176608	*GHR*	Growth hormone	1.45	***

Table 4. Continued.

Enrez ID	Symbol	Gene title	FC[1]	FDR *q*-value[2]
NM_001034627	*GSN*	Gelsolin (amyloidosis, finnish type)	-1.45	***
NM_174087	*IGF2*	insulin-like growth factor 2	-1.31	**
XM_869739	*IL18BP*	interleukin [IL]–18–binding protein	-1.39	**
NM_001077909	*INSIG1*	insulin induced gene 1	-1.49	***
NM_175782	*LGALS1*	lectin, galactoside-binding, soluble, 1	1.36	***
NM_001034768	*LGALS4*	lectin, galactoside-binding, soluble, 4	-1.62	***
NM_001103323	*LIPA*	Lipase A	1.31	**
NM_174103_at	*LIPF*	lipase, gastric	1.31	***
XM_586851	*LIPG*	Lipase G	-1.79	***
XM_865119	*LPIN1*	Lipin	1.59	***
XM_614220	*LRP5*	low density lipoprotein receptor-related protein 5	-1.76	***
NM_180998	*LTF*	lactotransferrin	-1.46	***
NM_001080362	*LYPLA2*	lysophospholipase II	-1.35	***
NM_175793	*MAPK1*	Mitogen-activated protein kinase 1	-1.59	***
XM_001255254	*MAPKAPK2*	mitogen-activated protein kinase-activated protein kinase 2	-1.33	***
NM_001033608	*MIF*	Macrophage migration inhibiting factor	-3.61	***
NM_001081605	*MTMR3*	Homo sapiens myotubularin related protein 3	-1.31	***
NM_174119	*NCF1*	Neutrophil Cytosol Factor 1	-1.33	***
NM_001014883	*NR1H2*	nuclear receptor subfamily 1, group H, member 2	-1.57	***
NM_001083509	*PCCA*	propionyl Coenzyme A carboxylase,	1.35	***
NM_001017953	*PDGFB*	platelet-derived growth factor beta polypeptide	-1.48	***
NM_174577	*PI4KA*	phosphatidylinositol 4-kinase	-1.53	***
NM_174783	*PI4KB*	phosphatidylinositol 4-kinase	-1.31	***
NM_174560_at	*PLA2G15*	phospholipase A2, group XV	1.31	***
NM_001035390	*POR*	P50 cytochrome oxidoreductase	-1.36	***
NM_001046005	*PNPLA2*	patatin-like phospholipase domain containing protein 2	-1.52	***
NM_174161	*PSAP*	sphingolipid activator protein-1	-1.63	***
NM_174791	*PTGDS*	prostaglandin D2 synthase	-1.51	**
NM_174443	*PTGES*	prostaglandin E synthase	-1.67	***
NM_001034310	*PTTG1*	pituitary tumor-transforming 1	1.35	***
NM_001100348	*PXMP3*	peroxisomal membrane protein 3	1.37	***
NM_001035081	*RAB7A*	member RAS oncogene family	-1.67	***

Table 4. Continued.

Enrez ID	Symbol	Gene title	FC[1]	FDR *q*-value[2]
NM_174161	*PSAP*	sphingolipid activator protein-1	-1.63	***
NM_174791	*PTGDS*	prostaglandin D2 synthase	-1.51	**
NM_174443	*PTGES*	prostaglandin E synthase	-1.67	***
NM_001034310	*PTTG1*	pituitary tumor-transforming 1	1.35	***
NM_001100348	*PXMP3*	peroxisomal membrane protein 3	1.37	***
NM_001035081	*RAB7A*	member RAS oncogene family	-1.67	***
NM_001076945	*SCD5*	stearoyl-CoA desaturase 5	1.30	***
NM_174598	*SCNN1A*	Socidum channel	-2.84	***
NM_173882	*SERPINA1*	serpin peptidase inhibitor, clade A	-1.63	***
NM_174821	*SERPING1*	serpin peptidase inhibitor, clade G	-1.51	***
NM_001082443	*SIGIRR*	single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	-1.54	***
NM_174782	*SLC12A2*	solute carrier family 12	1.35	***
NM_001034041	*SNCA*	synuclein, alpha	1.52	***
XM_870939	*SPHK1*	sphingosine Kinase	-1.35	***
NM_001099137	*SRD5A1*	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	-1.48	***
NM_001113302	*SREBP1*	sterol regulatory element binding transcription factor 1	-1.48	***
NM_173960	*SST*	somatostatin	1.38	***
NM_174617	*STAT5B*	signal transducer and activator of transcription 5	-1.58	***
NM_174674	*TNFRSF1A*	tumor necrosis factor receptor superfamily, member 1A	-1.52	***
XM_583785	*TNFSF10*	tumor necrosis factor (ligand) superfamily, member 10	-1.82	***
NM_174703	*TNXB*	tenascin XB	-1.41	**
NM_174201	*TP53*	tumor protein p53	-1.48	***
NM_175776	*TSPO*	translocator protein	-1.38	***

UFA = unsaturated fatty acid; FC = fold change; FDR = false discovery rate; CoA = coenzyme A.

[1] FCs were calculated considering gene expression when cows were fed with UFA-enriched diet compared with the same cows fed control diet.

[2] FDR *q*-value = effect of UFA supplementation.

, * levels of significance indicate $P < 0.01$ and $P < 0.001$, respectively.

morphology and antigen presentation, with a score of 35 and 24 focus genes. Figure 1d shows how UFA supplementation could be related to genes that modulate the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), which is a transcription regulator of genes encoding cytokines, cytokine receptors and cell adhesion molecules that drive immune and inflammatory responses (Sigal, 2006).

Discussion

Milk Production and Composition

Our study suggests that supplementing grazing dairy cows with different unprotected UFA sources increases the milk yield by 15%. This is in agreement with the study of Bu et al. (2007), who reported that supplementing basal diet with either 4.0% soybean oil, 4.0% linseed oil or 2.0% soybean oil and 2.0% linseed oil, resulted in a milk yield increase of 16.7% compared with the control treatment. The greater energy density, protein content and starch content in the enriched-UFA diet could have increased the availability of glucose precursors for lactose synthesis in the mammary gland. This, followed by the reduced NDF content, probably stimulated milk production. However, because the control period was conducted 28 days after UFA supplementation, the effects attributed to diet supplemented with UFA may be confounded with effects due to a difference of 28 days in lactation stage. Therefore, isolation of the specific effects of UFA supplementation on milk production and composition may be complex and challenging to draw clear conclusions.

The reduction in milk fat and protein percentage in cows fed with UFA supplementation most likely resulted from a dilution effect. However, the decreased milk fat and protein contents, without modifications in lactose content, were coupled with the decreased expression of genes associated with the transport processes of nutrients, and with the reduction of fat, and protein metabolism (see section 'Effects of UFA supplementation on nutrient metabolism'). Therefore, these results suggested that the modification of milk components cannot be only accounted for the increase in milk production but also for the decreased activities per cell. Interestingly, the performance of cows fed on different plant oil treatments were the same, suggesting that oils rich in oleic (rapeseed oil), linoleic (soybean oil), ALA (linseed oil) affected the performance of animals in a similar way. Furthermore, cows fed on diets supplemented with different unprotected UFA sources had increased proportion of LCFA and c9,t11-CLA in milk but reduced *de novo* FA synthesis, which in turn, improved the nutrition quality aspects of their milk (Table 3, S2 and S3). It is well established that feeding dairy cows with plant oils results in a reduction in the *de novo* FA, and increases LCFA (Bauman & Griinari, 2003; Bernard et al., 2008; Thering et al., 2009). Altered fermentation of these plant oils results in rumen outflow of unique biohydrogenation intermediates, some of which reduce lipid synthesis in the mammary gland (Bauman & Griinari, 2003; Bauman et al., 2008). In particular, the *trans*-18:2 FA has emerged as an important factor associated with the inhibition of *de novo* FA synthesis (Bauman et al., 2006; Harvatine et al., 2009).This study found similar results. Therefore, it

is likely that in our study the different unprotected UFA sources had undergone ruminal biohydrogenation, increasing the *trans*-FA and CLA isomers reaching the mammary gland, which could be considered as an important factor in the inhibition of the milk synthesis of *de novo* FA proportion in milk. These observations, together with several gene expression effects (see section 'Effects of UFA supplementation on nutrient metabolism') are the major factors in leading us to believe that the dietary UFA supplementation, together with the higher energy and protein content, was indeed the main factor affecting milk yield and composition, rather than the lactation stage.

Differential Gene Expression of Genes in the Mammary Gland

A total of 972 genes were differentially expressed in the mammary gland tissue when supplementing grazing dairy cows with UFA compared with when cows were fed with a control diet, suggesting a large degree of transcriptomic adaptation to the dietary UFAs. Similar to the milk production and composition variables, we acknowledge that the effects of UFA supplementation on gene expression might be confounded by lactation stage, but also by the different amount of dietary protein and energy that was utilized by the cow. There are no studies that report the effects of varying dietary protein and energy levels on the genome-wide expression in the mammary gland of dairy cows. Further, there are no studies that describe the genome-wide expression in the mammary gland of mid-lactation cows. However, Bionaz & Loor (2008b) observed that the expression of 45 genes associated with lipid synthesis and with well-defined roles in mammary lipid metabolism peaked at 60 days post-partum, and thereafter, their mRNA abundance decreased following the lactation curve. As the mRNA expression of most of the genes in our study presented different pattern from the so-called lactation curve, it may be assumed that there is an effect of enriched-UFA diet on their expression. Surprisingly, expressions of genes in the mammary gland were not significantly different between UFA sources. A possible explanation for these results might be that the variability in the FA profile among the dietary treatments was not sufficient because the unprotected UFA sources had undergone extensive biohydrogenation by rumen microorganisms (Chilliard et al., 2007); and therefore, the contrast in absorbed FA composition between supplements was probably too small to cause large differences in mammary gland tissue gene expression. In agreement, Ollier et al. (2009) supplementing mid-lactation multiparous goats with oil from whole intact rapeseed or sunflower did not find significant changes in the expression of 8,382 genes in the mammary gland, despite changes in milk composition. However, the lack of differently expressed genes between UFA sources could also be the result of a high variability in the expression levels of genes in the mammary gland within groups of treatments, as biological variation is intrinsic to all organisms (Churchill, 2002).

Functional Clustering of Differential Expressed Genes in the Mammary Gland

The functional clustering of differentially expressed genes by GO analysis, GSEA and IPA showed that supplementation of UFA leads to downregulation of hundreds of genes that modulate cellular growth proliferation and development cell death, connections between cells and morphology (cytoskeleton organization), apoptosis, cell cycle, nutrient metabolism, as well as immune system response.

Effects of UFA Supplementation on Cellular Growth Proliferation and Development, Cellular Death, Cytoskeleton Organization and Apoptosis

The downregulation of the expression of key genes (P53, PPP2R1A and Ras) associated with cellular growth, cell cycle, remodelling and apoptosis, as well as canonical pathways such as mTOR and JAK/STAT signalling, suggested changes in mammary gland tissue integrity and cell adhesion when cows were supplemented with UFA-enriched diets. The mTOR controls cellular metabolism, growth and proliferation (Panasyuk et al., 2009), and the JAK/STAT pathway is the principal signalling mechanism for a wide array of cytokines and growth factors resulting in cell proliferation, differentiation, cell migration and apoptosis (Rawlings et al., 2004). These cellular events are critical to mammary gland lactation (Rawlings et al., 2004). The information with regard to the effect of UFA supplementation on regulation of genes functioning in remodelling of the mammary gland in dairy cows is lacking. However, Connor et al. (2008) studying the specific mechanisms controlling the increase in milk production in dairy cows during the first few weeks of lactation, reported a downregulation of genes functioning in remodelling of the mammary gland. Therefore, it can be suggested that inhibition related to cell proliferation and remodelling could be mainly occurring in response to UFA-enriched diet that promoted an increase in milk synthesis.

Effects of UFA Supplementation on Immune System Response

We present some of the first data in the bovine that reveal changes in the expression of defence, inflammatory and immune-related genes in response to UFA supplementation. Cows fed with UFA-enriched diet revealed downregulation of many key genes known to be involved in cellular and humoral immune responses, as well as pathogen-induced signalling and cellular stress and injury (Supplementary,material; Figure S3). It featured a number of genes involved in cytokine and IL signalling, which exert potent chemokinetic and chemotactic activity on leukocytes and enhance the bactericidal activity of phagocytes in dairy cows (Pfaffl et al., 2003), as well as T and B cell receptors, natural killer cell signalling, GM-CSF signalling, C-C chemokine receptor type 3 (CCR3) signalling in eosinophils, CXC chemokine receptor 4 (CXCR4) signalling and integrin signalling. Lessard et al. (2003) suggested that cellular immunity of the dairy cows was affected by dietary supplementation of UFA. They observed that 5 days after calving, the lymphocyte proliferative response of cows allocated to linseed treatment was reduced. Connor et al.

(2008) reported that increasing milk yield through milking frequency resulted in a downregulation of several genes that function in innate immune response and inflammation. Furthermore, one major finding of our study was the downregulation of genes associated with NFkB response after UFA supplementation (Figure 1d). In agreement, Lessard et al. (2003) reported that dietary UFA can affect the regulation of cytokine gene expression by modulating the activation of transcription nuclear factors such as NFkB. Though little is known about the expression of defence, inflammatory and immune-related genes in response to dietary UFA supplementation in dairy cows, the results presented here suggest that enriched-UFA diets may affect immune functions of the mammary gland and thus may modify the susceptibility to mastitis in lactating cows and the resulting quality of milk. However, experiments specifically designed to test these hypothesis are warranted to verify the roles of UFA on genes involved in immune system response pathways and networks, together with cell cycle, cell growth and certain apoptotic pathways.

Effects of UFA Supplementation on Nutrient Metabolism

Our microarray data provide insight into the nutrient metabolism adaptations in the mammary gland as a result of UFA supplementation. Our finding suggested that through feeding UFA-enriched diets, the mammary gland reduced overall fat and protein metabolic activity, but increased carbohydrate metabolism. Most of the transcripts involved with biological process related to carbohydrate metabolism (glycolysis and gluconeogenesis, and pentose phosphate pathway) were upregulated (Figure 2b). Glucose is the major precursor for synthesis of lactose, which controls milk volume by maintaining the osmolarity of milk (Finucane et al., 2008). Consistent to increased expression of genes associated with carbohydrate metabolism, lactose and milk yield of cows fed with enriched-UFA diet was greater relative to cows fed with control diet. Under the conditions of this experiment, increasing the fermentable energy content of the diet, by reducing NDF and increasing starch was also likely to stimulate the carbohydrate metabolism. In contrast, supplementing basal diet of dairy cows with unprotected UFA was characterized by substantial downregulation of the mRNA expression of genes in the mammary gland involved in protein synthesis, protein trafficking, protein folding and the regulatory pathways controlling these processes, as well as lipid, and transport processes of nutrients. These changes may explain the reduction of fat and protein percentages in milk of these dairy cows. The most prominent functional characteristic of lipid metabolism category was the downregulation of the transcription factor SREBP-1, when cows were supplemented with dietary unprotected UFA. Therefore, these results suggested that increasing the LCFA and *trans*-FA reaching mammary gland from blood may affect expression of key transcription regulator genes and their response genes. Together with the downregulation of SREBP-1, ACACA, which catalyses the carboxylation of acetyl-CoA to produce malonyl-CoA (Bernard et al., 2008), was found to be downregulated during supplementation of UFA (Table 4). These results support the hypothesis that regulation of genes involved in *de novo*

synthesis of FA is under control of SREBP-1 (Bionaz & Loor, 2008b). However, the observed downregulation of de novo lipid biosynthesis in bovine mammary gland could also be influenced by the downregulation of the insulin-induced gene (INSIG-1; Raghow et al., 2008). When cells have sufficient sterol levels, INSIG-1 retains the SREBP-1 cleavage-activating protein (SCAP)-SREBP-1 in the endoplasmic reticulum and consequently inhibits SREBP-1-mediated gene expression. On the basis of the above observations, it is tempting to speculate that UFA reaching the mammary gland addresses the expression of both SREBP-1 and INSIG-1 to inhibit SREBP-1-mediated gene expression and consequently, at least partially, reduce lipogenic activity in the mammary gland. This is in agreement with Harvatine & Bauman (2006), who reported that dietary treatments causing milk fat depression decreased expression of SREBP-1 and the INSIG-1, consistent with decreased abundance of active SREBP-1. Similarly, our findings underscore that supplementation of dietary UFA decreased the expression of gene sets regulated by PPARG, including those associated with FA import (e.g. acetyl-CoA acyltransferase 1 (ACAA1)), activation and intracellular channeling of FA (e.g. acyl-CoA synthetase short-chain family member 1 and 2 (ACSS1 and ACSS2)) and *de novo* FA synthesis (e.g. ACACA). Furthermore, the genes related to the formation of triglyceride (TG) such as the acylglycerol phosphate acyl-transferase (AGPAT1), thought to be involved in catalyzing the initial step in the synthesis of TG, were downregulated (Table 4). But on the contrary, our data indicated that supplementation of dietary unprotected UFA upregulated the expression of stearoyl-CoA desaturase 5 (SCD5), an isoform of the Δ-9 desaturase family (Lengi & Corl, 2007). The role of SCD5 in the mammary tissue remains elusive, although Gervais et al. (2009) reported important differences between SCD1 and SCD5 regulation and physiological roles when Holstein cows were infused with a lipid emulsion enriched with t10,c12-CLA. However, no effects on the expression of SCD1 were found in this study (Supplementary material; Table S8). This is in agreement with Delbecchi et al. (2001), who reported no differences in the expression levels of SCD1 in the mammary gland when mid-lactation Holstein cows were fed a total MR supplemented with either 4.8% canola meal, 3.3% unprotected canola seeds plus 1.5% canola meal or 4.8% formaldehyde-protected canola seeds. Furthermore, Murrieta et al. (2006) also did not report differences on SCD1 mRNA expression in the mammary gland of crossbred beef cows supplemented with cracked safflower seed supplements. However, most of the studies that examined the effect of milk fat depressing diets on SCD1 expression in the mammary gland, reported a tendency toward reduction of mammary expression of SCD1 (Harvatine & Bauman, 2006; Gervais et al., 2009).

Conclusions

The results of our study suggest that supplementing the diets of grazing dairy cows with different unprotected UFAs decreases milk fat and protein percentage, and increases milk yield. Due to the UFA supplementation, the proportion of LCFAs in milk increases, whereas *de novo* FA synthesis decreases, which in turn, improves the nutrition quality aspects of dairy milk. The UFA supplementation led to robust transcriptional adaptations with 972 genes affected, suggesting a strong impact on metabolism and other cellular functions in the mammary gland. In particular, the functional analysis on these genes indicated that inclusion of dietary UFAs not only reduces the expression of genes associated with lipid and protein metabolism, but unexpectedly also of genes involved in cell–cell interactions, cells morphology (cytoskeleton organization), cell death and immune response. The large-scale transcriptional adaptations occurring in mammary tissue in response to dietary lipids might provide the basis for more detailed functional studies for future research.

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Supplementary Material

Fatty Acid Analysis

For fatty acid (FA) analysis of the feedstuffs, fat from freeze dried samples was extracted with chloroform-methanol (2:1 v/v), according to Folch et al. (1957). The homogenized extracts were filtered (595½, 125 mm diameter, Whatman Sleicher and Schuell, Dassel, Germany), and water was added for a clear separation. The upper phase was completely removed by repeated washing (3 times) with a solution containing 30 mL chloroform, 480 mL methanol and 470 mL sodium chloride solution (7.3 g/L water). Approximately 3 mL of the lower fat containing phase was collected and solvents were evaporated by vacuum centrifugation. The residual fat was dissolved in 2 mL hexane and 100 mg of anhydrous sodium sulphate was added. Fatty acids were transesterified using both acid and base catalysed methods. For basic methylation, 50 µL sodium methanolate in absolute methanol (2 mol L^{-1}) was added. Subsequently, 1 mL hydrochloric acid methanolic solution was added for acid methylation and the mixture was heated for 20 minutes at 85ºC under constant shaking. The mixture was then cooled down to room temperature under a flow of cold water and shaken vigorously. After that, 1 mL of the upper layer, which contains the fatty acid methyl esters (FAME), was transferred to a 1.5 mL vial and used for gas chromatography analysis. For milk FA analysis, milk samples were heated to 45ºC and directly centrifuged at 3,000 x g for 10 min at 4°C. The upper layer (fat and cream) was collected, filtered on folded filter paper, and stored overnight at -20ºC. Then, the mixture was heated for 10 min at 60ºC. The oil substance was centrifuged twice at room temperature (5 min at 20,000 x g), and the fat fraction was transferred to a tube containing a small amount of anhydrous sodium sulphate. Subsequently, 50 µL of milk fat was added to 5 mL of hexane and the glycerol bounds FA were transesterified to FAME by vortexing for 1 min with 100 µL of sodium methanolate in absolute methanol (2 mol L^{-1}). The solution was neutralized with 1 g of sodium hydrogen sulphate and dried with anhydrous sodium sulphate. The FAME from feedstuff and milk were injected into a gas chromatograph (TRACE GC ULTRA, Thermo Electron Corporation, Waltham, MA, USA) equipped with a flame-ionization detector and auto-sampler. The carrier gas was helium. Samples (1 µL) were injected by split injection (split ratio 1:50). Separation of FAME was performed with a fused silica capillary column (100 m x 0.25 mm x 0.2 µm film thickness; Restek RT-2560, Restek, Bellefonte, PA, USA). The oven temperature was programmed from 140ºC for 4 min, followed by an increase of 4ºC per min to 240ºC, and held for 20 min. The FAME concentrations were measured by using the Supelco FAME standards (S37, Supelco, Poole, Dorset, UK).

Quality Control Analysis of Affymetrix GeneChip® Bovine Genome Array

All array images were inspected for the presence of artefacts visible without magnification of the image (i.e., high/low intensity spots, scratches, high regional, or overall background, etc.). Subsequently, the boundaries of the probe area were checked for

fluorescent presence of B2 oligo, which was spiked into each hybridization cocktail. The B2 Oligo serves as a positive hybridization control and is used to place a grid over the image. Furthermore, Noise Values were compared between chips. Noise (Raw Q) was a measure of the pixel-to-pixel variation of probe cells on a GeneChip array. Additionally, Poly-A RNA controls were used to monitor the entire target labelling process. Further, to monitor the hybridization process, Affymetrix 20x Eukaryotic Hybridization Controls were used, composed of a mixture of biotin-labelled cRNA transcripts of *E.coli* genes prepared in staggered concentrations (*Bio*B, *bio*C and *bio*D) and *cre*, the recombinase gene from P1 bacteriophage. The hybridization controls were spiked into the hybridisation cocktail, independent of RNA sample preparation, and were thus used to evaluate sample hybridization efficiency on eukaryotic gene expression arrays. Lastly, the β-actin and GAPDH were used to assess RNA sample and assay quality. The Signal values of the 3' probe sets for actin and GAPDH were compared to the Signal values of the corresponding 5' probe sets. The ratio of the 3' probe set to the 5' probe set were generally no more than 3.

References

Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509.

Table S1. Chemical analyses and fatty acid composition of the pasture.

Item	Pasture
Chemical composition, (g/kg DM)	
DM (g/kg)	191.7
Ash	90.9
CP	229.7
Crude fat	55.9
NDF	340.4
ADF	191.3
Sugars	206.4
FA composition, (g/100g FA)	
C16:0	13.4
C18:0	1.9
C18:1 c9	5.3
C18:2 c9c12	15.0
C18:3 c9c12c15	57.2
C22:0	0.7
C24:0	0.7

Table S2. Fatty acid profile of milk when comparing dairy cows fed with unprotected unsaturated fatty acid (UFA) relative to the same cows fed a control diet.

Fatty acid[3,4,5]	Control diet				UFA-enriched diet							
	UFA sources[1]									*P*- value[2]		
	A	B	C	D	A	B	C	D	s.e.	UFAS	UFAL	UFAS x UFAL
	g/100 g total fatty acids											
De novo FA[6]	41.57	41.53	41.41	40.42	37.34	35.87	35.78	33.06	1.03	0.13	***	0.48
LCFA[7]	49.46	50.38	49.66	50.57	54.07	56.70	55.32	58.14	0.98	0.09	***	0.46
18:0	8.65^{b}	9.83^{a}	9.13^{ab}	8.74^{b}	8.74^{b}	10.90^{a}	9.71^{ab}	8.90^{b}	0.576	*	0.26	0.80
c9-18:1	18.56	18.98	19.19	19.75	21.3	21.21	21.3	22.64	0.863	0.46	***	0.96
c9,c12-18:2	1.35^{B}	1.38^{A}	1.33^{B}	1.47^{A}	1.36^{B}	1.78^{A}	1.35^{B}	1.81^{A}	0.075	***	***	**
18:3n3	0.51^{C}	$0.5b^{C}$	0.50^{A}	0.56^{B}	0.31^{C}	0.42^{BC}	0.63^{A}	0.52^{B}	0.027	***	0.004	***
Total *trans*-FA	3.05	2.88	3.43	4.05	6.31	6.75	7.74	9.32	0.56	0.12	***	0.38
c9,t11 CLA[8]	0.58	0.49	0.55	0.68	0.85	1.02	0.99	1.11	0.09	0.148	***	0.43
t10,c12 CLA	0.008	0.001	0.01	0.01	0.01	0.01	0.02	0.02	0.003	0.09	***	0.77

[1] Within UFA-enriched diet, A = rapeseed oil; B = soybean oil; C = linseed oil; and D = proportional mix of them all. Within control diet, group A, B, C and D were fed with the same control diet.

[2] UFAS = effect of UFA-sources, UFAL = effect of UFA level; *, **, *** levels of significance indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[3] Included n = 28 cows; [4] [a,b,c] Within rows, mean values not bearing a common superscript differ ($P < 0.05$), and [A,B,C] within rows, mean values not bearing a common superscript differ ($P < 0.01$); [5] Only the most relevant fatty acids (FA) are presented. Information related to the complete milk FA profile can be found in Chapter 2.

[6] *De novo* FA include all FA from C4 to C14 and 50% of C16 FA.

[7] Long Chain Fatty Acids include all FA with 18 carbon atoms or more.

[8] CLA = Conjugated Linoleic Acid.

Table S3. Fatty acids concentration of milk (mg/L) when comparing dairy cows fed with unprotected unsaturated fatty acid (UFA) relative to the same cows fed a control diet.

Item[2]	Control diet	UFA-enriched diet	s.e.	*P*- value[1]
De novo FA[3], mg/L	11,343	8,035	418.5	***
C16, mg/L	13,200	9,780	416.7	***
LCFA[4], mg/L	15,282	15,412	467.9	0.84
Unsaturated Fatty Acids, mg/L	12,023	12,397	349.6	0.45
Saturated Fatty Acids, mg/L	28,393	21,160	938.6	***
Polyunsaturated Fatty Acids, mg/L	1,393	1,388	43.1	0.93
n-3 Fatty Acids, mg/L	269.3	201.7	9.28	***
n-6 Fatty Acids, mg/L	721.1	652.46	20.01	**
Trans-octadecenoic Fatty Acids, mg/L	1,798	2,900	72.5	***
cis-9, *trans*-11-CLA[5], mg/L	263.9	368.8	18.80	***
trans-10,*cis*-12-CLA, mg/L	3.56	6.4	0.46	***

[1] Effect of UFA level; *, **, *** levels of significance indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[2] Included n = 28 cows.

[3] *De novo* FA include all FA from C4 to C14 and 50% of C16 FA.

[4] Long Chain Fatty Acids include all FA with 18 carbon atoms or more.

[5] CLA = Conjugated Linoleic Acid.

Table S4. Gene identification, symbol, description, and fold change of the 972 genes differentially expressed in the mammary gland tissue when supplementing grazing dairy cows with unprotected unsaturated fatty acid (UFA) compared with when cows were fed with a control diet. The differentially expressed genes were identified applying a statistical cut-off of false discovery rate (FDR) q-values <0.05 together with an absolute Fold Change (FC) of 1.3.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001034271	*ARPC3*	actin related protein 2/3 complex, subunit 3, 21kDa	2.70	***
NM_001100373	*LOC515994*	hypothetical LOC515994	2.03	**
NM_001075370	*CCBL2*	cysteine conjugate-beta lyase 2	1.89	***
XM_001250172	*SFRS12IP1*	SFRS12-interacting protein 1	1.80	***
NM_205786	*LOC404103*	spleen trypsin inhibitor	1.79	***
NM_001076109	*TIA1*	TIA1 cytotoxic granule-associated RNA binding protein	1.75	***
XM_864367	*SFRS12IP1*	SFRS12-interacting protein 1	1.74	***
NM_001014386	*RNASE1*	ribonuclease	1.74	**
NM_001098875	*MUTED*	muted homolog (mouse)	1.72	***
NM_001034488	*BDH2*	3-hydroxybutyrate dehydrogenase, type 2	1.71	***
NM_001046164	*FBP2*	fructose-1,6-bisphosphatase 2	1.64	***
NM_001102165	*IYD*	iodotyrosine deiodinase	1.63	***
NM_001075225	*NTRK2*	neurotrophic tyrosine kinase, receptor, type 2	1.63	**
NM_001101265	*SHISA2*	shisa homolog 2 (Xenopus laevis)	1.63	*
XM_613318	*ACSM3*	acyl-CoA synthetase medium-chain family member 3	1.62	**
XM_584098	*FIGNL1*	fidgetin-like 1	1.62	***
XM_598984	*MED1*	mediator complex subunit 1	1.61	***
NM_001102017	*CCDC88C*	coiled-coil domain containing 88C	1.61	***
NM_001033619	*RPL18A*	ribosomal protein L18a	1.61	***
NM_001024499	*POLR3D*	polymerase (RNA) III (DNA directed) polypeptide D, 44kDa	1.61	***
XM_865119	*LPIN1*	lipin 1	1.59	**
NM_173936	*MIA*	melanoma inhibitory activity	1.59	**
XR_028430	*LOC524776*	similar to chromodomain helicase DNA binding protein 6	1.57	***
NM_001109962	*DCLK1*	doublecortin-like kinase 1	1.57	**
NM_175801	*FST*	follistatin	1.56	*
NM_001024500	*MRPS17*	mitochondrial ribosomal protein S17	1.55	**
NM_001003902	*MRPS14*	mitochondrial ribosomal protein S14	1.54	***
XM_592516	*CLDN23*	claudin 23	1.54	**
XM_588956	*NA*	NA	1.54	***
NM_176649	*ATP5G1*	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C1 (subunit 9)	1.54	***
XM_001252404	*NA*	NA	1.53	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001105485	*NT5DC1*	5'-nucleotidase domain containing 1	1.53	***
XR_027421	*NA*	NA	1.52	***
NM_174079	*HAS2*	hyaluronan synthase 2	1.52	***
XM_867376	*ENPP1*	ectonucleotide pyrophosphatase/phosphodiesterase 1	1.52	**
NM_001034041	*SNCA*	synuclein, alpha (non A4 component of amyloid precursor)	1.52	***
NM_001075992	*TSC1*	tuberous sclerosis 1	1.52	***
NM_001076133	*PDE12*	phosphodiesterase 12	1.51	***
NM_001038054	*HMG20B*	high-mobility group 20B	1.51	***
NM_175800	*NDUFS4*	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase)	1.51	***
AFFX-r2-Ec-bioB-3	*NA*	NA	1.50	**
NM_001040472	*CD3G*	CD3g molecule, gamma (CD3-TCR complex)	1.50	**
NM_001046120	*C2H1orf144*	chromosome 1 open reading frame 144 ortholog	1.50	***
NM_001114608	*C2H1orf144*	chromosome 1 open reading frame 144 ortholog	1.50	***
XM_586239	*LOC509304*	hypothetical LOC509304	1.50	***
NM_001038192	*THEX1*	three prime histone mRNA exonuclease 1	1.49	**
NM_001045995	*MCEE*	methylmalonyl CoA epimerase	1.49	***
XM_001256926	*MCEE*	methylmalonyl CoA epimerase	1.49	***
NM_001079798	*PHYHIPL*	phytanoyl-CoA 2-hydroxylase interacting protein-like	1.49	*
NM_001076092	*DZIP3*	DAZ interacting protein 3, zinc finger	1.49	**
XM_582415	*NA*	NA	1.48	**
XM_001251905	*LOC783266*	similar to holocarboxylase synthetase	1.48	***
XR_027629	*NA*	NA	1.48	***
NM_001099139	*MGC157372*	hypothetical LOC614796	1.48	***
XM_593333	*MYO9A*	myosin IXA	1.48	***
NM_001110001	*KRAS*	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.47	***
NM_001099030	*KLHL28*	kelch-like 28 (Drosophila)	1.47	***
NM_001034596	*PSMB3*	proteasome (prosome, macropain) subunit, beta type, 3	1.47	***
XM_583018	*PB1*	polybromo 1	1.47	***
NM_174672	*SLC25A16*	solute carrier family 25 (mitochondrial carrier /// Graves disease autoantigen), member 16	1.47	***
XM_593524	*LRP10*	low density lipoprotein receptor-related protein 10	1.46	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001024490	*CDC45L*	CDC45 cell division cycle 45-like (S. cerevisiae)	1.46	***
NM_173985	*AIF1*	allograft inflammatory factor 1	1.46	**
NM_001079801	*GGPS1*	geranylgeranyl diphosphate synthase 1	1.46	***
NM_001098024	*AKAP10*	A kinase (PRKA) anchor protein 10	1.46	***
XM_612699	*LOC533324*	similar to Protein FAM126B	1.45	***
NM_001075618	*PPIH*	peptidylprolyl isomerase H (cyclophilin H)	1.45	***
NM_174635	*CSNK2A1*	casein kinase 2, alpha 1 polypeptide	1.45	**
XM_001256068	*LOC789682*	similar to Mast cell antigen 32 precursor (Mast cell Ag-32) (MCA-32)	1.45	***
NM_001076014	*FIG4*	FIG4 homolog (S. cerevisiae)	1.45	***
NM_176608	*GHR*	growth hormone receptor	1.45	**
NM_001076293	*ALOX5AP*	arachidonate 5-lipoxygenase-activating protein	1.45	**
NM_001001156	*ADAM12*	ADAM metallopeptidase domain 12	1.45	***
NM_001075259	*PDCD10*	programmed cell death 10	1.44	**
NM_001046333	*CAMK2D*	calcium/calmodulin-dependent protein kinase II delta	1.44	***
NM_001079774	*LOC514330*	MMP37-like protein, mitochondrial	1.44	***
XR_028714	*LOC788997*	similar to LOC785621 protein	1.44	**
XM_868564	*LOC616529*	similar to TRD@ protein	1.44	***
NM_001076878	*SLC39A12*	solute carrier family 39 (zinc transporter), member 12	1.44	**
XM_581573	*NA*	NA	1.43	**
NM_001076997	*FMC1*	formation of mitochondrial complexes 1 homolog (S. cerevisiae)	1.43	***
XM_001249856	*RNASE12*	ribonuclease, RNase A family, 12 (non-active)	1.43	*
XM_001251339	*NA*	NA	1.43	*
XR_028227	*LOC785233*	similar to postmeiotic segregation 1	1.43	***
XM_591634	*SRBD1*	S1 RNA binding domain 1	1.43	***
NM_001037469	*H2B*	histone H2B-like	1.43	*
NM_001046257	*NOSTRIN*	nitric oxide synthase trafficker	1.43	*
NM_001038034	*UBE2T*	ubiquitin-conjugating enzyme E2T (putative)	1.43	***
NM_001075531	*TPMT*	thiopurine S-methyltransferase	1.43	***
XR_027447	*LOC782348*	similar to Frizzled-3 precursor (Fz-3) (hFz3)	1.43	***
XM_601152	*LOC522864*	similar to KIAA1370	1.42	***
NM_176659	*NDUFA3*	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3, 9kDa	1.42	***
NM_001076107	*C13H10ORF97*	chromosome 10 open reading frame 97 ortholog	1.42	***
NM_001079582	*SLC15A2*	solute carrier family 15 (H+/peptide transporter), member 2	1.42	**

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001081535	*ENPP4*	ectonucleotide pyrophosphatase/phosphodiesterase 4 (putative function)	1.42	**
NM_001104975	*LOC512150*	similar to Myeloid-associated differentiation marker	1.42	**
NM_001034453	*CCDC104*	coiled-coil domain containing 104	1.41	**
NM_175816	*ATPIF1*	ATPase inhibitory factor 1	1.41	***
XM_001252335	*BLOC1S2*	biogenesis of lysosomal organelles complex-1, subunit 2	1.41	***
XM_597385	*BLOC1S2*	biogenesis of lysosomal organelles complex-1, subunit 2	1.41	***
NM_001100330	*FKBP7*	FK506 binding protein 7	1.41	*
XM_580552	*MERTK*	c-mer proto-oncogene tyrosine kinase	1.41	***
NM_001098079	*RPE*	ribulose-5-phosphate-3-epimerase	1.41	***
NM_001080353	*PI3*	peptidase inhibitor 3, skin-derived (SKALP)	1.41	**
NM_001037600	*FAM82B*	family with sequence similarity 82, member B	1.41	***
NM_001113723	*UCRC*	ubiquinol-cytochrome c reductase complex 7.2 kDa protein	1.41	***
XM_001251797	*LOC783161*	similar to LOC152217 protein	1.40	***
NM_001037597	*NT5C3*	5'-nucleotidase, cytosolic III	1.40	**
XM_591447	*NA*	NA	1.40	**
XM_867971	*LOC616011*	similar to zinc finger, CCHC domain containing 17	1.40	**
NM_174308	*EDNRA*	endothelin receptor type A	1.40	**
XR_028112	*LOC784517*	similar to cationic amino acid transporter 5	1.40	**
NM_001024573	*PPAPDC2*	phosphatidic acid phosphatase type 2 domain containing 2	1.40	**
NM_001101161	*CNOT6*	CCR4-NOT transcription complex, subunit 6	1.40	***
XR_027516	*LOC782395*	similar to malignant fibrous histiocytoma amplified sequence 1	1.40	***
XR_028193	*NA*	NA	1.39	***
NM_001038056	*TM2D2*	TM2 domain containing 2	1.39	***
NM_001077834	*SNW1*	SNW domain containing 1	1.39	***
XM_001252075	*NA*	NA	1.39	***
NM_001034754	*TAF12*	TAF12 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 20kDa	1.39	***
NM_001101858	*FOLH1*	folate hydrolase (prostate-specific membrane antigen) 1	1.39	**
NM_001034247	*GADD45A*	growth arrest and DNA-damage-inducible, alpha	1.39	**
NM_174035	*CYBB*	cytochrome b-245, beta polypeptide	1.39	**
NM_001109807	*PPIG*	peptidylprolyl isomerase G (cyclophilin G)	1.39	**

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001080313	*CCDC53*	coiled-coil domain containing 53	1.39	***
NM_173960	*SST*	somatostatin	1.38	*
NM_177502	*GOT1*	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	1.38	***
NM_001077002	*GPR171*	G protein-coupled receptor 171	1.38	**
NM_001101934	*PDK3*	pyruvate dehydrogenase kinase, isozyme 3	1.38	**
XM_614941	*ATP8B1*	ATPase, class I, type 8B, member 1	1.38	**
XM_603355	*TRD@*	T-cell receptor delta chain	1.38	*
XM_593269	*LOC515280*	similar to hepatocellular carcinoma antigen gene 520	1.38	**
NM_001046266	*GLT8D2*	glycosyltransferase 8 domain containing 2	1.38	**
XM_581642	*WFDC1*	WAP four-disulfide core domain 1	1.38	**
XM_603252	*PIBF1*	progesterone immunomodulatory binding factor 1	1.38	***
XM_001256260	*NA*	NA	1.38	***
NM_001076113	*RIMKLB*	ribosomal modification protein rimK-like family member B	1.38	**
NM_001075566	*FRG1*	FSHD region gene 1	1.38	***
XM_583699	*LOC507141*	CE5 protein-like	1.37	**
XM_001249876	*MRPL23*	mitochondrial ribosomal protein L23	1.37	**
NM_174242	*APOA1*	apolipoprotein A-I	1.37	**
NM_001025350	*SF3B5*	splicing factor 3b, subunit 5, 10kDa	1.37	***
NM_001013601	*BOLA-DQA1*	histocompatibility complex, class II, DQ alpha, type 1	1.37	**
XM_595995	*SNX7*	sorting nexin 7	1.37	***
XM_865687	*NA*	NA	1.37	***
XM_001251264	*SETD1A*	SET domain containing 1A	1.37	***
XM_595033	*IPCEF1*	interaction protein for cytohesin exchange factors 1	1.37	**
NM_001077924	*RPP14*	ribonuclease P/MRP 14kDa subunit	1.37	**
NM_001100348	*PXMP3*	peroxisomal membrane protein 3, 35kDa	1.37	***
XM_001250900	*NA*	NA	1.37	**
XM_586922	*NA*	NA	1.37	**
NM_001077981	*PMS1*	PMS1 postmeiotic segregation increased 1 (S. cerevisiae)	1.37	***
XM_001250072	*NA*	NA	1.37	***
NM_001101887	*ADHFE1*	alcohol dehydrogenase, iron containing, 1	1.37	***
XM_587832	*SRRM2*	serine/arginine repetitive matrix 2	1.37	**
XM_581525	*LOC505265*	similar to tripartite motif protein 5 alpha	1.36	**
XM_583884	*HEATR5A*	HEAT repeat containing 5A	1.36	***
NM_174233	*AGTR1*	angiotensin II receptor, type 1	1.36	**
XM_581489	*FGD4*	FYVE, RhoGEF and PH domain containing 4	1.36	**
XM_001249764	*NA*	NA	1.36	***
XM_001249812	*NA*	NA	1.36	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
XM_614337	*BRWD3*	bromodomain and WD repeat domain containing 3	1.36	***
NM_001046563	*MRPL48*	mitochondrial ribosomal protein L48	1.36	***
XM_606442	*CRLF3*	cytokine receptor-like factor 3	1.36	**
NM_001040514	*THOC7*	THO complex 7 homolog (Drosophila)	1.36	***
XM_001249315	*LOC780994*	hypothetical LOC780994	1.36	***
XM_866139	*FAM96B*	family with sequence similarity 96, member B	1.36	***
XR_028190	*LOC514474*	similar to tumor protein p53 binding protein, 2	1.36	***
XM_868233	*NA*	NA	1.36	***
NM_001034615	*TPD52L2*	tumor protein D52-like 2	1.36	***
XM_001251369	*NA*	NA	1.36	***
NM_001034517	*HBXIP*	hepatitis B virus x interacting protein	1.36	***
NM_001034805	*MRPL13*	mitochondrial ribosomal protein L13	1.36	**
NM_001080317	*MRPL3*	mitochondrial ribosomal protein L3	1.36	***
NM_175782	*LGALS1*	lectin, galactoside-binding, soluble, 1	1.36	**
NM_001034338	*CNIH4*	cornichon homolog 4 (Drosophila)	1.36	***
XM_863865	*LOC613274*	hypothetical protein LOC613274	1.36	***
NM_001038515	*CETN2*	centrin, EF-hand protein, 2	1.36	**
NM_001101256	*LOC616371*	hypothetical LOC616371	1.36	**
NM_001082448	*COPG2*	coatomer protein complex, subunit gamma 2	1.36	**
XM_866738	*NA*	NA	1.36	**
XM_866635	*EML4*	echinoderm microtubule associated protein like 4	1.36	**
NM_001076213	*ZNF45*	zinc finger protein 45	1.36	***
NM_001102365	*HSD17B1*	hydroxysteroid (17-beta) dehydrogenase 1	1.36	***
XM_001253407	*LOC785989*	similar to Hydroxysteroid (17-beta) dehydrogenase 1	1.36	***
NM_001076909	*CUL2*	cullin 2	1.36	***
NM_001076103	*TRIB3*	tribbles homolog 3 (Drosophila)	1.35	**
XM_001252263	*NA*	NA	1.35	***
XM_603768	*LOC525415*	similar to C-C motif chemokine 3-like 1 precursor (Small-inducible cytokine A3-like 1) (Tonsillar lymphocyte LD78 beta protein) (LD78-beta(1-70)) (G0/G1 switch regulatory protein 19-2) (G0S19-2 protein) (PAT 464.2)	1.35	***
NM_001083509	*PCCA*	propionyl Coenzyme A carboxylase, alpha polypeptide	1.35	***
XM_586889	*CCDC112*	coiled-coil domain containing 112	1.35	**
XM_581267	*NA*	NA	1.35	**
XM_582278	*C9H6ORF115*	chromosome 6 open reading frame 115 ortholog	1.35	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
XM_001255988	*C29H11orf 10*	chromosome 11 open reading frame 10 ortholog	1.35	***
NM_174455	*RPL24*	ribosomal protein L24	1.35	***
XM_610489	*FAM86A*	hypothetical protein LOC531984	1.35	**
NM_174320	*FXYD2*	FXYD domain containing ion transport regulator 2	1.35	***
NM_001034310	*PTTG1*	pituitary tumor-transforming 1	1.35	**
XM_584118	*NA*	NA	1.35	***
XM_001253146	*NA*	NA	1.35	**
NM_174782	*SLC12A2*	solute carrier family 12 (sodium/potassium/chloride transporters), member 2	1.35	**
XM_001251104	*PARP14*	poly (ADP-ribose) polymerase family, member 14	1.35	**
XM_613140	*NA*	NA	1.35	**
NM_001101845	*THEM2*	thioesterase superfamily member 2	1.34	***
NM_001034582	*WARS2*	tryptophanyl tRNA synthetase 2, mitochondrial	1.34	***
NM_001076849	*OBFC1*	oligonucleotide/oligosaccharide-binding fold containing 1	1.34	***
NM_001034218	*FETUB*	fetuin B	1.34	***
XM_001250957	*BNIP3L*	BCL2/adenovirus E1B 19kDa interacting protein 3-like	1.34	***
XM_001252613	*NA*	NA	1.34	**
XM_001252642	*TAOK3*	TAO kinase 3	1.34	**
XM_614451	*TAOK3*	TAO kinase 3	1.34	**
NM_001076048	*C11H9ORF 78*	chromosome 9 open reading frame 78 ortholog	1.34	***
XM_616395	*MTHFD2L*	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	1.34	**
NM_001014931	*ORMDL2*	ORM1-like 2 (S. cerevisiae)	1.34	***
NM_001103173	*NBEAL1*	neurobeachin-like 1	1.34	**
XR_028137	*NA*	NA	1.34	***
XM_001256659	*MEGF8*	multiple EGF-like-domains 8	1.34	***
XR_028792	*LOC615688*	similar to multiple EGF-like-domains 8	1.34	***
NM_174594	*RNASE6*	ribonuclease, RNase A family, k6	1.34	*
NM_174323	*GNA14*	guanine nucleotide binding protein (G protein), alpha 14	1.33	***
NM_001014875	*DUSP11*	dual specificity phosphatase 11	1.33	***
NM_001034273	*GTSF1*	gametocyte specific factor 1	1.33	**
XM_588490	*HLTF*	helicase-like transcription factor	1.33	***
XM_600747	*UBR1*	ubiquitin protein ligase E3 component n-recognin 1	1.33	**
NM_001003905	*C16orf80*	chromosome 16 open reading frame 80	1.33	**
XM_584692	*NA*	NA	1.33	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
XR_027545	*LOC781969*	similar to BCL2/adenovirus E1B 19kDa interacting protein 3-like	1.33	***
XM_864087	*NA*	NA	1.33	***
XM_588295	*ELP2*	elongation protein 2 homolog (S. cerevisiae)	1.33	***
NM_001076015	*MRPS31*	mitochondrial ribosomal protein S31	1.33	**
NM_001080903	*RFC2*	replication factor C (activator 1) 2, 40kDa	1.33	***
NM_001035055	*CLDN11*	claudin 11	1.33	*
NM_001075447	*SP140*	SP140 nuclear body protein	1.33	**
NM_001034801	*CEBPG*	CCAAT/enhancer binding protein (C/EBP), gamma	1.33	***
NM_001015656	*NME7*	non-metastatic cells 7, protein expressed in (nucleoside-diphosphate kinase)	1.32	*
XM_001251303	*MGC127989*	hypothetical protein MGC127989	1.32	***
NM_001075525	*C5H12ORF57*	chromosome 12 open reading frame 57 ortholog	1.32	***
XM_584232	*LOC539014*	hypothetical LOC539014	1.32	***
NM_176626	*PAG18*	pregnancy-associated glycoprotein 18	1.32	***
NM_001035439	*METTL7A*	methyltransferase like 7A	1.32	***
XM_001249762	*METTL7A*	methyltransferase like 7A	1.32	***
XM_001249805	*NA*	NA	1.32	***
NM_001034529	*BXDC2*	brix domain containing 2	1.32	**
XM_590080	*ACOT4*	acyl-CoA thioesterase 4	1.32	***
NM_001034764	*POLB*	polymerase (DNA directed), beta	1.32	*
NM_001035458	*SUMO1*	SMT3 suppressor of mif two 3 homolog 1 (S. cerevisiae)	1.32	***
XM_001253763	*SUMO1*	SMT3 suppressor of mif two 3 homolog 1 (S. cerevisiae)	1.32	***
NM_001045899	*BLZF1*	basic leucine zipper nuclear factor 1	1.32	*
NM_001076072	*NCK1*	NCK adaptor protein 1	1.32	***
XM_870116	*NA*	NA	1.32	*
NM_177508	*UMPS*	uridine monophosphate synthetase	1.32	**
NM_001077936	*SLC25A20*	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	1.32	***
NM_001015620	*TYW3*	tRNA-yW synthesizing protein 3 homolog (S. cerevisiae)	1.32	**
NM_001113319	*CKS2*	CDC28 protein kinase regulatory subunit 2	1.32	**
NM_001046361	*PAPD4*	PAP associated domain containing 4	1.32	***
NM_001075964	*ZADH2*	zinc binding alcohol dehydrogenase domain containing 2	1.32	***
XR_027926	*NA*	NA	1.32	**
NM_001098867	*NARS2*	asparaginyl-tRNA synthetase 2, mitochondrial (putative)	1.32	**

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001075162	*FMO2*	flavin containing monooxygenase 2 (non-functional)	1.32	***
NM_205813	*PAICS*	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	1.32	**
XM_001250433	*NA*	NA	1.32	**
NM_001102137	*ACSS3*	acyl-CoA synthetase short-chain family member 3	1.32	**
NM_001046321	*MRPL44*	mitochondrial ribosomal protein L44	1.32	**
NM_001034239	*CCT2*	chaperonin containing TCP1, subunit 2 (beta)	1.32	**
NM_175798	*CD38*	CD38 molecule	1.32	**
XR_027415	*LOC781730*	similar to ecto-NAD+ glycohydrolase	1.32	**
NM_001102358	*PIR*	pirin (iron-binding nuclear protein)	1.31	**
XM_001250844	*SOX6*	SRY (sex determining region Y)-box 6	1.31	***
XM_614052	*SOX6*	SRY (sex determining region Y)-box 6	1.31	***
XM_611708	*DEPDC7*	DEP domain containing 7	1.31	**
XM_001253587	*NA*	NA	1.31	***
XM_865849	*IL23R*	interleukin 23 receptor	1.31	**
NM_001076154	*CFL2*	cofilin 2 (muscle)	1.31	**
XM_001251110	*RC3H2*	ring finger and CCCH-type zinc finger domains 2	1.31	**
XM_582844	*NA*	NA	1.31	**
NM_001102153	*SCARB2*	scavenger receptor class B, member 2	1.31	***
NM_001076038	*STXBP6*	syntaxin binding protein 6 (amisyn)	1.31	***
NM_001081615	*XRCC4*	X-ray repair complementing defective repair in Chinese hamster cells 4	1.31	***
NM_001038558	*CADM1*	cell adhesion molecule 1	1.31	**
NM_001035033	*OMA1*	OMA1 homolog, zinc metallopeptidase (S. cerevisiae)	1.31	***
XM_594106	*LOC540449*	similar to Uncharacterized protein C14orf152	1.31	**
NM_001034476	*SNRPD2*	small nuclear ribonucleoprotein D2 polypeptide 16.5kDa	1.31	***
NM_001033121	*ZNF148*	zinc finger protein 148	1.31	**
NM_001014965	*ANKRD49*	ankyrin repeat domain 49	1.31	***
NM_001079618	*MED30*	mediator complex subunit 30	1.31	**
XM_001251745	*NA*	NA	1.31	**
XM_001251778	*NA*	NA	1.31	**
NM_001083474	*EIF2B3*	eukaryotic translation initiation factor 2B, subunit 3 gamma, 58kDa	1.31	***
XM_001252878	*MTERF*	mitochondrial transcription termination factor	1.31	***
NM_001046003	*MCTS1*	malignant T cell amplified sequence 1	1.31	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
XM_001252845	*NA*	NA	1.31	**
XM_867336	*CCDC88A*	coiled-coil domain containing 88A	1.31	***
NM_001083389	*CCDC58*	coiled-coil domain containing 58	1.31	***
NM_001034641	*MRPL16*	mitochondrial ribosomal protein L16	1.31	***
NM_001034722	*BTG3*	BTG family, member 3	1.31	**
NM_001046364	*BUD31*	BUD31 homolog (S. cerevisiae)	1.31	***
XM_586184	*RNASEH2B*	ribonuclease H2, subunit B	1.31	***
NM_001034341	*FHL1*	four and a half LIM domains 1	1.31	*
NM_001076210	*COQ3*	coenzyme Q3 homolog, methyltransferase (S. cerevisiae)	1.31	***
NM_001015626	*NHP2*	NHP2 ribonucleoprotein homolog (yeast)	1.31	**
NM_001034258	*OCIAD2*	OCIA domain containing 2	1.30	**
NM_001075280	*PLA2G16*	phospholipase A2, group XVI	1.30	**
XM_597427	*PIGV*	phosphatidylinositol glycan anchor biosynthesis, class V	1.30	*
NM_001075389	*C4H7ORF11*	chromosome 7 open reading frame 11 ortholog	1.30	***
NM_001075563	*ERGIC2*	ERGIC and golgi 2	1.30	***
NM_177519	*GALNT1*	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)	1.30	*
NM_001046286	*HSD17B11*	hydroxysteroid (17-beta) dehydrogenase 11	1.30	*
NM_001098120	*DTWD1*	DTW domain containing 1	1.30	*
NM_001038546	*SNUPN*	snurportin 1	1.30	***
NM_001046148	*PSMD11*	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	1.30	***
NM_001012764	*CST6*	cystatin E/M	1.30	**
XM_592333	*ATRX*	alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae)	1.30	**
NM_001045885	*TAPBP*	TAP binding protein (tapasin)	-1.30	***
NM_001077925	*SMCR7*	Smith-Magenis syndrome chromosome region, candidate 7	-1.30	***
XM_001249838	*NA*	NA	-1.30	***
NM_001102353	*WHSC2*	Wolf-Hirschhorn syndrome candidate 2	-1.30	**
NM_001035486	*SHISA5*	shisa homolog 5 (Xenopus laevis)	-1.30	*
NM_001113763	*NA*	NA	-1.30	***
NM_174720	*CPSF1*	cleavage and polyadenylation specific factor 1, 160kDa	-1.30	**
XM_592881	*TH1L*	TH1-like (Drosophila)	-1.30	***
XM_001250861	*TLN1*	talin 1	-1.30	**
NM_001035411	*GBL*	G protein beta subunit-like	-1.30	***
XM_582266	*PRKCQ*	protein kinase C, theta	-1.30	*
XM_587369	*PPP5C*	protein phosphatase 5, catalytic subunit	-1.30	**

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001075577	*ADAL*	adenosine deaminase-like	-1.30	**
NM_001034703	*ELOVL1*	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1	-1.30	**
XM_587853	*BSPRY*	B-box and SPRY domain containing	-1.30	***
NM_001045971	*SPINT2*	serine peptidase inhibitor, Kunitz type, 2	-1.30	**
XM_608202	*SPATA2*	spermatogenesis associated 2	-1.30	**
NM_001038634	*DNASE1L1*	deoxyribonuclease I-like 1	-1.30	**
NM_001098465	*THAP7*	THAP domain containing 7	-1.30	**
NM_001046147	*PDDC1*	Parkinson disease 7 domain containing 1	-1.31	***
XM_582280	*SRM*	spermidine synthase	-1.31	**
NM_001102074	*QSOX1*	quiescin Q6 sulfhydryl oxidase 1	-1.31	***
NM_001076046	*SNRNP70*	small nuclear ribonucleoprotein 70kDa (U1)	-1.31	**
XR_027508	*LOC504986*	similar to polycystin 1	-1.31	***
NM_001099108	*PPP4C*	protein phosphatase 4 (formerly X), catalytic subunit	-1.31	**
NM_001035307	*EDF1*	endothelial differentiation-related factor 1	-1.31	***
NM_001110180	*MRPL52*	mitochondrial ribosomal protein L52	-1.31	**
NM_001077046	*M6PRBP1*	mannose-6-phosphate receptor binding protein 1	-1.31	**
NM_001077883	*DHFR*	dihydrofolate reductase	-1.31	**
XM_001250637	*NA*	NA	-1.31	**
XM_587493	*AGFG2*	ArfGAP with FG repeats 2	-1.31	***
NM_001081605	*MTMR3*	myotubularin related protein 3	-1.31	***
NM_001035497	*UBE2V1*	ubiquitin-conjugating enzyme E2 variant 1	-1.31	***
NM_001035028	*EIF4B*	eukaryotic translation initiation factor 4B	-1.31	***
NM_001037607	*ARFRP1*	ADP-ribosylation factor related protein 1	-1.31	***
NM_001076811	*KLHL21*	kelch-like 21 (Drosophila)	-1.31	***
XM_877976	*NFAT5*	nuclear factor of activated T-cells 5, tonicity-responsive	-1.31	**
NM_001034518	*CYB5R1*	cytochrome b5 reductase 1	-1.31	**
NM_174218	*WARS*	tryptophanyl-tRNA synthetase	-1.31	*
NM_001075620	*BANP*	BTG3 associated nuclear protein	-1.31	***
XM_599314	*ANKRD52*	ankyrin repeat domain 52	-1.31	*
NM_001076445	*RABL4*	RAB, member of RAS oncogene family-like 4	-1.31	***
NM_001046316	*LGALS3BP*	lectin, galactoside-binding, soluble, 3 binding protein	-1.31	**
XM_865144	*DYM*	dymeclin	-1.31	**
NM_001098909	*ELF3*	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	-1.31	***
NM_001035320	*CDK2AP2*	cyclin-dependent kinase 2 associated protein 2	-1.31	***
NM_001075784	*CLCC1*	chloride channel CLIC-like 1	-1.31	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
XM_001250371	*LOC785188*	similar to transcriptional adaptor 2 (ADA2 homolog, yeast)-beta	-1.31	***
XM_616212	*PTPRM*	protein tyrosine phosphatase, receptor type, M	-1.31	***
XM_869282	*LOC617094*	similar to phosphoglucomutase 5	-1.31	*
NM_001035459	*BAD*	BCL2-associated agonist of cell death	-1.31	***
NM_174783	*PI4KB*	phosphatidylinositol 4-kinase, catalytic, beta	-1.31	***
NM_001015578	*EHD1*	EH-domain containing 1	-1.31	***
NM_001076919	*KIAA1949*	KIAA1949	-1.31	**
NM_001102477	*UBA1*	ubiquitin-like modifier activating enzyme 1	-1.31	***
NM_001034319	*ACAA1*	acetyl-Coenzyme A acyltransferase 1	-1.31	***
NM_001104994	*THAP11*	THAP domain containing 11	-1.31	*
NM_001046324	*HSD17B8*	hydroxysteroid (17-beta) dehydrogenase 8	-1.31	***
XM_592945	*LOC515009*	similar to I-1 receptor candidate protein	-1.31	**
NM_001040475	*RBP4*	retinol binding protein 4, plasma	-1.31	*
NM_001046346	*WDR1*	WD repeat domain 1	-1.31	*
NM_001101210	*SLC35C1*	solute carrier family 35, member C1	-1.32	**
NM_001082608	*HNRNPR*	heterogeneous nuclear ribonucleoprotein R	-1.32	***
NM_001100355	*SLC38A7*	solute carrier family 38, member 7	-1.32	**
NM_001015570	*LGALS9*	lectin, galactoside-binding, soluble, 9	-1.32	*
NM_001039177	*LGALS9*	lectin, galactoside-binding, soluble, 9	-1.32	*
XR_027939	*LOC783548*	similar to tankyrase 1-binding protein 1	-1.32	*
XM_608640	*RNF215*	ring finger protein 215	-1.32	***
NM_001040583	*BRI3*	brain protein I3	-1.32	**
NM_001075752	*EIF4H*	eukaryotic translation initiation factor 4H	-1.32	**
NM_001015555	*AUP1*	ancient ubiquitous protein 1	-1.32	**
NM_001024562	*EMP3*	epithelial membrane protein 3	-1.32	***
NM_001104969	*LOC509540*	hypothetical LOC509540	-1.32	**
XM_879421	*ACTR1A*	ARP1 actin-related protein 1 homolog A, centractin alpha (yeast)	-1.32	***
NM_001046620	*VISA*	virus-induced signaling adapter	-1.32	**
XM_001253451	*CD320*	CD320 molecule	-1.32	*
XR_027898	*LOC784355*	similar to KLC4 protein	-1.32	***
NM_001034491	*PDLIM4*	PDZ and LIM domain 4	-1.32	*
NM_001075371	*BSG*	basigin	-1.32	***
XM_877864	*MPPE1*	metallophosphoesterase 1	-1.32	***
NM_174246	*AVPR2*	arginine vasopressin receptor 2	-1.32	***
XM_001250147	*IER5L*	immediate early response 5-like	-1.32	**
NM_001035080	*MTF1*	metal-regulatory transcription factor 1	-1.32	***
XR_027575	*LOC515924*	similar to ret proto-oncogene	-1.32	***
NM_001103183	*POLDIP3*	polymerase (DNA-directed), delta interacting protein 3	-1.32	**
NM_174487	*VEGFB*	vascular endothelial growth factor B	-1.32	**
XM_001254185	*NA*	NA	-1.32	**

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001100315	*TMEM189*	transmembrane protein 189	-1.32	***
NM_001040568	*N-PAC*	cytokine-like nuclear factor n-pac	-1.32	***
NM_001076889	*NRBP1*	nuclear receptor binding protein 1	-1.32	**
NM_174777	*NDST2*	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 2	-1.32	***
XM_001254956	*NA*	NA	-1.32	***
XR_028724	*CHD3*	chromodomain helicase DNA binding protein 3	-1.33	***
NM_001076924	*C19H17ORF28*	chromosome 17 open reading frame 28 ortholog	-1.33	**
NM_001034387	*NUP85*	nucleoporin 85kDa	-1.33	***
XM_585307	*NPLOC4*	nuclear protein localization 4 homolog (S. cerevisiae)	-1.33	**
NM_001103224	*ANXA6*	annexin A6	-1.33	**
NM_001113724	*ANAPC11*	anaphase promoting complex subunit 11	-1.33	***
NM_001098953	*MAPK12*	mitogen-activated protein kinase 12	-1.33	***
NM_001076931	*PRSS8*	protease, serine, 8	-1.33	***
NM_001099192	*ADSSL1*	adenylosuccinate synthase like 1	-1.33	***
XM_001257227	*NA*	NA	-1.33	***
NM_001075850	*WDR13*	WD repeat domain 13	-1.33	***
XM_589271	*FBLN2*	fibulin 2	-1.33	**
XM_001255254	*MAPKAPK2*	mitogen-activated protein kinase-activated protein kinase 2	-1.33	**
NM_001075211	*SNF8*	SNF8, ESCRT-II complex subunit, homolog (S. cerevisiae)	-1.33	***
NM_001102268	*CCNY*	cyclin Y	-1.33	**
XM_599356	*LOC521099*	similar to family with sequence similarity 20, member A	-1.33	***
NM_001035026	*BSDC1*	BSD domain containing 1	-1.33	***
XM_592304	*FLII*	flightless I homolog (Drosophila)	-1.33	***
NM_174834	*MYH10*	myosin, heavy chain 10, non-muscle	-1.33	***
NM_001034322	*FKBP4*	FK506 binding protein 4, 59kDa	-1.33	**
NM_174119	*NCF1*	neutrophil cytosolic factor 1	-1.33	**
NM_001081510	*SF3A1*	splicing factor 3a, subunit 1, 120kDa	-1.33	**
NM_001076538	*ST14*	suppression of tumorigenicity 14 (colon carcinoma)	-1.33	**
NM_001046133	*PUF60*	poly-U binding splicing factor 60KDa	-1.34	***
NM_205788	*CEACAM8*	carcinoembryonic antigen-related cell adhesion molecule 8	-1.34	***
NM_001075154	*RGL2*	ral guanine nucleotide dissociation stimulator-like 2	-1.34	**
NM_001105480	*BLES03*	basophilic leukemia expressed protein BLES03	-1.34	***
NM_001034315	*AHCY*	S-adenosylhomocysteine hydrolase	-1.34	**
XM_585239	*EIF4GI*	eIF4GI protein	-1.34	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001035375	*SDF4*	stromal cell derived factor 4	-1.34	***
NM_001014901	*RBM14*	RNA binding motif protein 14	-1.34	***
XM_869552	*YIF1B*	Yip1 interacting factor homolog B (S. cerevisiae)	-1.34	***
NM_001038523	*CCDC124*	coiled-coil domain containing 124	-1.34	***
NM_001038030	*SGTA*	small glutamine-rich tetratricopeptide repeat (TPR)-containing, alpha	-1.34	**
XM_870523	*SDC3*	syndecan 3	-1.34	***
XM_001249718	*LOC783159*	similar to v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65	-1.34	***
NM_001076372	*CCND2*	cyclin D2	-1.34	**
AFFX-Bt_GST_5	*NA*	NA	-1.34	**
NM_001035489	*MRPL38*	mitochondrial ribosomal protein L38	-1.34	***
NM_001098003	*TMCO3*	transmembrane and coiled-coil domains 3	-1.34	***
NM_001075444	*STX5*	syntaxin 5	-1.34	***
NM_001098967	*TMEM138*	transmembrane protein 138	-1.34	***
XM_880923	*NA*	NA	-1.34	***
NM_001076487	*H1F0*	H1 histone family, member 0	-1.34	*
XM_867588	*INTS3*	integrator complex subunit 3	-1.34	***
NM_001081545	*RAB40C*	RAB40C, member RAS oncogene family	-1.34	***
NM_001046048	*SELENBP1*	selenium binding protein 1	-1.34	*
NM_001102505	*RAF1*	v-raf-1 murine leukemia viral oncogene homolog 1	-1.34	**
NM_001046548	*C7H19orf22*	chromosome 19 open reading frame 22 ortholog	-1.35	***
NM_001081711	*SAE1*	SUMO1 activating enzyme subunit 1	-1.35	**
XM_001251013	*AQP5*	aquaporin 5	-1.35	**
NM_001040557	*SMARCB1*	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	-1.35	**
XM_870269	*CUL4A*	cullin 4A	-1.35	***
NM_001075218	*S100A16*	S100 calcium binding protein A16	-1.35	***
NM_001035426	*SCAMP3*	secretory carrier membrane protein 3	-1.35	***
NM_001080362	*LYPLA2*	lysophospholipase II	-1.35	**
NM_001098856	*S100A11*	S100 calcium binding protein A11 (calgizzarin)	-1.35	**
NM_001035035	*PDXP*	pyridoxal (pyridoxine, vitamin B6) phosphatase	-1.35	***
NM_001101852	*TSPAN4*	tetraspanin 4	-1.35	***
NM_001075305	*SHC1*	SHC (Src homology 2 domain containing) transforming protein 1	-1.35	***
XM_590012	*GRK4*	G protein-coupled receptor kinase 4	-1.35	**

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
XM_001251271	*NA*	NA	-1.35	**
NM_001077922	*RHOB*	ras homolog gene family, member B	-1.35	***
XM_001251998	*NA*	NA	-1.35	***
NM_174509	*CACNB3*	calcium channel, voltage-dependent, beta 3 subunit	-1.35	***
NM_001098093	*ST5*	suppression of tumorigenicity 5	-1.35	***
NM_001046470	*TMEM109*	transmembrane protein 109	-1.35	***
XM_870939	*SPHK1*	sphingosine kinase 1	-1.35	***
NM_001075649	*NAT15*	N-acetyltransferase 15 (GCN5-related, putative)	-1.35	***
NM_001024546	*LPCAT3*	lysophosphatidylcholine acyltransferase 3	-1.35	**
NM_001075811	*TFE3*	transcription factor binding to IGHM enhancer 3	-1.35	***
NM_001014927	*DHCR7*	7-dehydrocholesterol reductase	-1.36	**
NM_001077094	*CYTH2*	cytohesin 2	-1.36	**
NM_001038160	*BBS2*	Bardet-Biedl syndrome 2	-1.36	*
NM_174195	*TCN2*	transcobalamin II /// macrocytic anemia	-1.36	***
XM_591526	*FMNL3*	formin-like 3	-1.36	***
XM_594391	*GPSM3*	G-protein signaling modulator 3 (AGS3-like, C. elegans)	-1.36	**
XR_028354	*LOC614048*	similar to bromodomain-containing protein 4	-1.36	**
XM_864659	*TMEM195*	transmembrane protein 195	-1.36	**
NM_174660	*SLC25A6*	solute carrier family 25 (mitochondrial carrier /// adenine nucleotide translocator), member 6	-1.36	**
XM_593945	*LOC540422*	similar to tigger transposable element derived 5	-1.36	***
XM_864245	*GOLGA1*	golgi autoantigen, golgin subfamily a, 1	-1.36	***
NM_001015589	*BSCL2*	Bernardinelli-Seip congenital lipodystrophy 2 (seipin)	-1.36	***
NM_001024574	*NFIC*	nuclear factor I/C (CCAAT-binding transcription factor)	-1.36	***
NM_001083388	*COL18A1*	collagen, type XVIII, alpha 1	-1.36	**
NM_001083703	*RNF185*	ring finger protein 185	-1.36	***
NM_001035429	*TPST2*	tyrosylprotein sulfotransferase 2	-1.36	*
NM_001034055	*ADIPOR1*	adiponectin receptor 1	-1.36	***
NM_001076214	*DRAP1*	DR1-associated protein 1 (negative cofactor 2 alpha)	-1.36	***
NM_001038585	*PCID2*	PCI domain containing 2	-1.36	***
XM_584231	*DBNDD2*	dysbindin (dystrobrevin binding protein 1) domain containing 2	-1.36	***
NM_001076535	*DHX30*	DEAH (Asp-Glu-Ala-His) box polypeptide 30	-1.37	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_176788	*CEBPB*	CCAAT/enhancer binding protein (C/EBP), beta	-1.37	*
XM_602222	*TXNDC15*	thioredoxin domain containing 15	-1.37	***
XM_001252640	*LOC512397*	similar to Uncharacterized protein KIAA0552	-1.37	***
XM_589910	*NA*	NA	-1.37	***
NM_001076151	*WDR45*	WD repeat domain 45	-1.37	***
NM_001046511	*ARD1A*	ARD1 homolog A, N-acetyltransferase	-1.37	***
NM_001035390	*POR*	cytochrome P450 reductase	-1.37	*
NM_001101204	*DGCR8*	DiGeorge syndrome critical region gene 8	-1.37	***
XM_865427	*OSBPL11*	oxysterol binding protein-like 11	-1.37	**
NM_001101949	*AKR7A2*	aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	-1.37	**
NM_001083774	*CHCHD10*	coiled-coil-helix-coiled-coil-helix domain containing 10	-1.37	***
NM_174789	*SERPINB6*	serpin peptidase inhibitor, clade B (ovalbumin), member 6	-1.37	**
NM_001001601	*CDH5*	cadherin 5, type 2 (vascular endothelium)	-1.37	***
NM_001076383	*MID1IP1*	MID1 interacting protein 1 (gastrulation specific G12 homolog (zebrafish))	-1.37	***
NM_001046123	*MRPS5*	mitochondrial ribosomal protein S5	-1.37	*
NM_001034344	*ETHE1*	ethylmalonic encephalopathy 1	-1.37	**
NM_174584	*PRKACA*	protein kinase, cAMP-dependent, catalytic, alpha	-1.37	**
NM_174746	*ACSS1*	acyl-CoA synthetase short-chain family member 1	-1.37	**
NM_001075380	*C7H19orf25*	chromosome 19 open reading frame 25 ortholog	-1.37	***
NM_001075440	*SEMA4A*	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4A	-1.37	***
XM_586037	*ZBED1*	zinc finger, BED-type containing 1	-1.37	**
NM_001046198	*PHB2*	prohibitin 2	-1.37	***
XM_001256027	*NA*	NA	-1.37	**
XM_001256033	*SMARCD2*	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2	-1.37	**
NM_001035338	*CDC37*	cell division cycle 37 homolog (S. cerevisiae)	-1.38	**
NM_175776	*TSPO*	translocator protein (18kDa)	-1.38	**
NM_001102149	*MGC159954*	hypothetical LOC533041	-1.38	**
XM_865548	*LOC614166*	similar to Uncharacterized protein C8orf42 homolog	-1.38	***
NM_174505	*ATP6V0D1*	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1	-1.38	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001035099	*CD81*	CD81 molecule	-1.38	***
XM_615408	*TUBB1*	tubulin, beta 1	-1.38	***
NM_001046089	*HGS*	hepatocyte growth factor-regulated tyrosine kinase substrate	-1.38	***
NM_001037589	*331879680 0*	membrane-associated ring finger (C3HC4) 2	-1.38	***
NM_174552	*IFNAR1*	interferon, alpha /// receptor	-1.38	**
NM_001083436	*GUSB*	glucuronidase, beta	-1.38	***
XM_582199	*AACS*	acetoacetyl-CoA synthetase	-1.38	***
NM_001075592	*MANSC1*	MANSC domain containing 1	-1.38	**
NM_174220	*YARS*	tyrosyl-tRNA synthetase	-1.38	***
XM_589879	*ANO9*	anoctamin 9	-1.38	***
NM_001035409	*STIM1*	stromal interaction molecule 1	-1.38	*
NM_001076951	*MGC14281 1*	hypothetical protein LOC618672	-1.38	***
NM_001075799	*TRIM27*	tripartite motif-containing 27	-1.38	***
NM_001075922	*DDIT4*	DNA-damage-inducible transcript 4	-1.38	**
NM_001075124	*LAMP1*	lysosomal-associated membrane protein 1	-1.38	***
NM_001076027	*HSPB6*	heat shock protein, alpha-crystallin-related, B6	-1.38	**
NM_001080244	*PFKL*	phosphofructokinase, liver	-1.38	***
NM_001075997	*NUDCD3*	NudC domain containing 3	-1.38	***
XM_868381	*GRAMD1A*	GRAM domain containing 1A	-1.38	***
XM_001256238	*NA*	NA	-1.38	*
NM_001038169	*TEX264*	testis expressed 264	-1.38	***
XM_001252095	*NA*	NA	-1.38	***
XM_001252121	*TEX264*	testis expressed 264	-1.38	***
NM_001102093	*FFR*	Protein fat-free homolog	-1.38	***
NM_001075415	*SLC25A39*	solute carrier family 25, member 39	-1.38	***
NM_174801	*PPM1G*	protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	-1.39	***
XM_594051	*NA*	NA	-1.39	***
XR_028564	*LOC787736*	similar to Selenoprotein M precursor (Protein SelM)	-1.39	**
NM_001075832	*EFHD1*	EF-hand domain family, member D1	-1.39	***
XM_868906	*SUPT6H*	suppressor of Ty 6 homolog (S. cerevisiae)	-1.39	***
XM_589440	*GNAI2*	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	-1.39	***
NM_174428	*POLD2*	polymerase (DNA directed), delta 2, regulatory subunit 50kDa	-1.39	***
NM_001014964	*ARAF*	v-raf murine sarcoma 3611 viral oncogene homolog	-1.39	***
NM_001040537	*GTF2H5*	general transcription factor IIH, polypeptide 5	-1.39	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001037456	*GIYD*	GIY-YIG domain containing	-1.39	***
XR_027423	*LOC539690*	similar to Complement component C1q receptor precursor (Complement component 1 q subcomponent receptor 1) (C1qR) (C1qRp) (C1qR(p)) (C1q/MBL/SPA receptor) (Matrix-remodeling-associated protein 4) (CD93 antigen) (CDw93)	-1.39	**
NM_001079600	*TMEM120A*	transmembrane protein 120A	-1.39	***
XM_001252004	*NA*	NA	-1.39	***
NM_001098149	*TUBGCP2*	tubulin, gamma complex associated protein 2	-1.39	***
NM_001102136	*NXN*	nucleoredoxin	-1.39	***
XM_613708	*CRTAP*	cartilage associated protein	-1.39	**
NM_001034522	*AMBRA1*	autophagy/beclin-1 regulator 1	-1.39	***
NM_001046191	*CRELD2*	cysteine-rich with EGF-like domains 2	-1.39	**
XM_591339	*RFWD3*	ring finger and WD repeat domain 3	-1.40	**
NM_001040602	*RHOF*	ras homolog gene family, member F (in filopodia)	-1.40	***
NM_001012287	*BREH1*	retinyl ester hydrolase type 1	-1.40	***
XM_580317	*COL4A1*	collagen, type IV, alpha 1	-1.40	**
NM_174511	*CCL3*	chemokine (C-C motif) ligand 3	-1.40	***
NM_001046140	*SDCCAG3*	serologically defined colon cancer antigen 3	-1.40	***
NM_001035316	*PPP1CA*	protein phosphatase 1, catalytic subunit, alpha isoform	-1.40	***
NM_001038156	*LRRC28*	leucine rich repeat containing 28	-1.40	***
NM_205798	*TMBIM1*	transmembrane BAX inhibitor motif containing 1	-1.40	***
XM_001257081	*NA*	NA	-1.40	***
XR_028890	*NA*	NA	-1.40	***
NM_001046478	*RHBDD2*	rhomboid domain containing 2	-1.40	***
XR_028604	*LOC508226*	similar to CDC42-binding protein kinase beta	-1.40	***
NM_001038110	*VASP*	vasodilator-stimulated phosphoprotein	-1.40	***
NM_001014961	*FAM110A*	family with sequence similarity 110, member A	-1.40	***
NM_201528	*SLC2A8*	solute carrier family 2 (facilitated glucose transporter), member 8	-1.40	***
XR_028087	*LOC516155*	similar to Mannosyl-oligosaccharide glucosidase (Processing A-glucosidase I)	-1.40	***
NM_001035326	*CTSA*	cathepsin A	-1.40	***
XM_867034	*ELAC2*	elaC homolog 2 (E. coli)	-1.40	***
NM_001078079	*AIF1L*	allograft inflammatory factor 1-like	-1.41	***
XM_001252568	*NA*	NA	-1.41	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
XM_614021	*DHX57*	DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57	-1.41	**
NM_001034231	*ID2*	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	-1.41	**
NM_001101306	*TNFRSF6B*	tumor necrosis factor receptor superfamily, member 6b, decoy	-1.41	**
NM_001076053	*FAM73B*	hypothetical protein LOC535315	-1.41	***
NM_001076219	*YIPF3*	Yip1 domain family, member 3	-1.41	**
NM_001035108	*CNDP2*	CNDP dipeptidase 2 (metallopeptidase M20 family)	-1.41	**
NM_001075656	*JUNB*	jun B proto-oncogene	-1.41	**
NM_001083439	*SUPT5H*	suppressor of Ty 5 homolog (S. cerevisiae)	-1.41	***
NM_001098968	*TAF10*	TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 30kDa	-1.41	***
NM_001076237	*SLC39A7*	solute carrier family 39 (zinc transporter), member 7	-1.41	***
NM_174703	*TNXB*	tenascin XB	-1.41	**
NM_001038029	*TBC1D20*	TBC1 domain family, member 20	-1.41	***
XM_590408	*GPIHBP1*	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1	-1.41	***
NM_001015592	*PFN1*	profilin 1	-1.41	**
XM_580667	*GAS6*	growth arrest-specific 6	-1.42	**
NM_001034282	*SHMT2*	serine hydroxymethyltransferase 2 (mitochondrial)	-1.42	***
NM_001075340	*MFSD5*	major facilitator superfamily domain containing 5	-1.42	***
NM_001045879	*PDIA4*	protein disulfide isomerase family A, member 4	-1.42	**
NM_001103245	*EFHD2*	EF-hand domain family, member D2	-1.42	***
NM_001076272	*ZFPL1*	zinc finger protein-like 1	-1.42	***
NM_001046615	*VPS4A*	vacuolar protein sorting 4 homolog A (S. cerevisiae)	-1.42	***
NM_001076405	*RNF167*	ring finger protein 167	-1.42	***
NM_001101899	*SYVN1*	synovial apoptosis inhibitor 1, synoviolin	-1.42	**
XM_614120	*ATP13A1*	ATPase type 13A1	-1.42	***
NM_205801	*CLDN3*	claudin 3	-1.42	***
NM_001040486	*SLC38A3*	solute carrier family 38, member 3	-1.42	**
NM_001077943	*FAM62A*	family with sequence similarity 62 (C2 domain containing), member A	-1.42	***
XM_001253241	*NA*	NA	-1.42	***
NM_001097575	*RTEL*	regulator of telomere elongation helicase 1	-1.43	***
NM_001101915	*ALDOA*	aldolase A, fructose-bisphosphate	-1.43	***
NM_001105489	*NPDC1*	neural proliferation, differentiation and control, 1	-1.43	**

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
XM_588361	*TSEN54*	tRNA splicing endonuclease 54 homolog (S. cerevisiae)	-1.43	***
NM_001014939	*TBCB*	tubulin folding cofactor B	-1.43	***
NM_001046008	*PXMP2*	peroxisomal membrane protein 2, 22kDa	-1.43	**
NM_001046190	*ABCD1*	ATP-binding cassette, sub-family D (ALD), member 1	-1.43	***
NM_001102006	*SSH3*	slingshot homolog 3 (Drosophila)	-1.43	***
XM_870452	*FXR2*	fragile X mental retardation, autosomal homolog 2	-1.43	***
NM_001082602	*DDX41*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41	-1.44	***
NM_001034473	*ZNF205*	zinc finger protein 205	-1.44	***
NM_174625	*TXNRD1*	thioredoxin reductase 1	-1.44	***
XM_612111	*NA*	NA	-1.44	**
XM_581459	*EHD4*	EH-domain containing 4	-1.44	***
XR_027523	*LOC506239*	similar to Spectrin beta chain, brain 2 (Spectrin, non-erythroid beta chain 2) (Beta-III spectrin)	-1.44	***
NM_001102356	*EIF3B*	eukaryotic translation initiation factor 3, subunit B	-1.44	***
NM_174216	*VEGFA*	vascular endothelial growth factor A	-1.44	**
NM_001076496	*SCN1B*	sodium channel, voltage-gated, type I, beta	-1.44	***
NM_001034246	*STAP2*	signal transducing adaptor family member 2	-1.44	***
NM_001046340	*H2AFY*	H2A histone family, member Y	-1.44	***
XM_589863	*FKBP8*	FK506 binding protein 8, 38kDa	-1.44	**
NM_176670	*ATP5D*	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	-1.45	***
NM_001079612	*SLC35A4*	solute carrier family 35, member A4	-1.45	***
XM_001250904	*NA*	NA	-1.45	***
NM_001110446	*DGCR6L*	DiGeorge syndrome critical region gene 6-like	-1.45	***
NM_001034627	*GSN*	gelsolin (amyloidosis, Finnish type)	-1.45	**
NM_001113284	*GSN*	gelsolin (amyloidosis, Finnish type)	-1.45	**
XM_582618	*EVL*	Enah/Vasp-like	-1.45	***
NM_173885	*ACK1*	activated p21cdc42Hs kinase	-1.45	***
NM_001078101	*ZYX*	zyxin	-1.46	**
NM_001046402	*ALKBH7*	alkB, alkylation repair homolog 7 (E. coli)	-1.46	***
XM_606600	*NA*	NA	-1.46	***
NM_001075600	*EXOC3*	exocyst complex component 3	-1.46	**
NM_001076184	*PAK4*	p21 protein (Cdc42/Rac)-activated kinase 4	-1.46	***
XM_605214	*LOC526838*	similar to transcriptional adaptor 2 (ADA2 homolog, yeast)-beta	-1.46	***
NM_177432	*IRF1*	interferon regulatory factor 1	-1.46	**
XM_001253779	*NA*	NA	-1.46	***
XM_599078	*NA*	NA	-1.46	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_174383	*LOXL1*	lysyl oxidase-like 1	-1.46	*
NR_003958	*H19*	H19, imprinted maternally expressed untranslated mRNA	-1.46	*
XM_611412	*NA*	NA	-1.46	*
XR_028883	*NA*	NA	-1.46	**
NM_180998	*LTF*	lactotransferrin	-1.46	**
NM_001035471	*SMS*	spermine synthase	-1.46	**
XM_001255494	*AKR7A2*	aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	-1.46	**
NM_174078	*GUK1*	guanylate kinase 1	-1.46	***
NM_173892	*ASS1*	argininosuccinate synthetase 1	-1.46	**
NM_001038507	*GLTSCR2*	glioma tumor suppressor candidate region gene 2	-1.46	***
NM_001034775	*BCL7B*	B-cell CLL/lymphoma 7B	-1.46	***
NM_001079587	*ZFYVE21*	zinc finger, FYVE domain containing 21	-1.47	***
XM_001255790	*NA*	NA	-1.47	***
NM_001045888	*PCOLCE*	procollagen C-endopeptidase enhancer	-1.47	**
NM_174277	*CLTLB*	clathrin, light polypeptide B (light chain B)	-1.47	***
XM_610785	*NA*	NA	-1.47	**
XM_873294	*SYT17*	synaptotagmin XVII	-1.47	**
NM_174837	*CYB561*	cytochrome b-561	-1.47	***
NM_001024485	*POMGNT1*	protein O-linked mannose beta1,2-N-acetylglucosaminyltransferase	-1.47	***
XM_589552	*MAP7D1*	MAP7 domain containing 1	-1.47	***
NM_001075718	*TPP1*	tripeptidyl peptidase I	-1.47	***
NM_001046435	*CALCOCO 1*	calcium binding and coiled-coil domain 1	-1.47	***
NM_001046411	*KRT7*	keratin 7	-1.48	**
AFFX-Bt_GST_3	*NA*	NA	-1.48	***
NM_001035354	*GPD1*	glycerol-3-phosphate dehydrogenase 1 (soluble)	-1.48	*
NM_174077	*GPX3*	glutathione peroxidase 3 (plasma)	-1.48	*
NM_001101841	*RNPS1*	RNA binding protein S1, serine-rich domain	-1.48	***
XM_865331	*SNRNP200*	small nuclear ribonucleoprotein 200kDa (U5)	-1.48	***
NM_001105339	*ACSS2*	acyl-CoA synthetase short-chain family member 2	-1.48	*
NM_001099137	*SRD5A1*	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	-1.48	*
AFFX-Bt_GST_M	*NA*	NA	-1.48	***
XM_867318	*STX10*	syntaxin 10	-1.48	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_174201	*TP53*	tumor protein p53	-1.48	***
NM_001098029	*FBLN1*	fibulin 1	-1.48	*
NM_001034763	*SH3BGRL3*	SH3 domain binding glutamic acid-rich protein like 3	-1.48	***
NM_001113302	*SREBF1*	sterol regulatory element binding transcription factor 1	-1.48	***
NM_174741	*BCAM*	basal cell adhesion molecule (Lutheran blood group)	-1.48	**
NM_001034477	*ARFIP2*	ADP-ribosylation factor interacting protein 2	-1.48	**
NM_182988	*BANF1*	barrier to autointegration factor 1	-1.48	***
XM_001250905	*NA*	NA	-1.48	***
XM_001250955	*NA*	NA	-1.48	***
NM_001017953	*PDGFB*	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	-1.48	***
XR_028669	*LOC788643*	similar to platelet-derived growth factor beta	-1.48	***
NM_001040604	*GADD45B*	growth arrest and DNA-damage-inducible, beta	-1.49	**
NM_001035460	*MGC128049*	hypothetical protein LOC615081	-1.49	***
NM_001075789	*SOX18*	SRY (sex determining region Y)-box 18	-1.49	***
XM_863839	*ZMIZ2*	zinc finger, MIZ-type containing 2	-1.49	***
NM_174537	*FCER1G*	Fc fragment of IgE, high affinity I, receptor for /// gamma polypeptide	-1.49	**
NM_001017936	*RAB25*	RAB25, member RAS oncogene family	-1.49	**
XM_614378	*SPRYD3*	SPRY domain containing 3	-1.49	***
NM_001077909	*INSIG1*	insulin induced gene 1	-1.49	**
XM_001249363	*NA*	NA	-1.49	**
XM_001249423	*NA*	NA	-1.49	**
XM_001251214	*LOC782581*	similar to DEXI	-1.49	***
NM_001076337	*MRPL14*	mitochondrial ribosomal protein L14	-1.49	***
XM_001252338	*LOC784449*	similar to KIAA1324	-1.49	*
NM_001046020	*RARRES2*	retinoic acid receptor responder (tazarotene induced) 2	-1.49	**
NM_001078116	*ABHD12*	abhydrolase domain containing 12	-1.49	**
XM_866852	*EIF4ENIF1*	eukaryotic translation initiation factor 4E nuclear import factor 1	-1.50	***
NM_001037477	*PPP2R1A*	protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform	-1.50	***
NM_001105376	*IPO9*	importin 9	-1.50	***
NM_001105390	*IPO13*	importin 13	-1.50	***
NM_174521	*CORO1A*	coronin, actin binding protein, 1A	-1.50	*
NM_001083401	*PRKCDBP*	protein kinase C, delta binding protein	-1.50	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
XM_001250004	*PFN1*	profilin 1	-1.50	**
XM_588401	*IMPA2*	inositol(myo)-1(or 4)-monophosphatase 2	-1.50	***
NM_174791	*PTGDS*	prostaglandin D2 synthase 21kDa (brain)	-1.51	*
NM_001100300	*C11H9ORF 142*	chromosome 9 open reading frame 142 ortholog	-1.51	***
NM_174821	*SERPING1*	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	-1.51	**
NM_001076995	*AAMP*	angio-associated, migratory cell protein	-1.51	***
NM_001046209	*RAB26*	RAB26, member RAS oncogene family	-1.51	**
NM_001034424	*MAF1*	MAF1 homolog (S. cerevisiae)	-1.51	***
NM_001101294	*DUSP7*	dual specificity phosphatase 7	-1.51	***
NM_001105491	*TMEM9*	transmembrane protein 9	-1.51	***
NM_001046173	*PPP2R4*	protein phosphatase 2A activator, regulatory subunit 4	-1.51	***
NM_174261	*CAPNS1*	calpain, small subunit 1	-1.52	***
XR_028390	*NA*	NA	-1.52	*
NM_001034810	*SEPX1*	selenoprotein X, 1	-1.52	***
NM_174674	*TNFRSF1A*	tumor necrosis factor receptor superfamily, member 1A	-1.52	***
XM_599833	*LOC521568*	similar to ATP-binding cassette, sub-family C, member 4	-1.52	**
XM_590721	*NUMA1*	nuclear mitotic apparatus protein 1	-1.52	**
NM_001045951	*CARHSP1*	calcium regulated heat stable protein 1, 24kDa	-1.52	**
NM_001046005	*PNPLA2*	patatin-like phospholipase domain containing 2	-1.52	**
NM_001038071	*MAP2K2*	mitogen-activated protein kinase kinase 2	-1.52	***
NM_001098862	*LZTS2*	leucine zipper, putative tumor suppressor 2	-1.52	***
NM_001046130	*SH3GL1*	SH3-domain GRB2-like 1	-1.53	***
NM_001079780	*H2AFX*	H2A histone family, member X	-1.53	***
NM_001046479	*ERI3*	exoribonuclease 3	-1.53	***
NM_174577	*PI4KA*	phosphatidylinositol 4-kinase, catalytic, alpha	-1.53	***
NM_001082445	*MED25*	mediator complex subunit 25	-1.53	***
XM_592305	*CLSTN1*	calsyntenin 1	-1.53	***
NM_001034445	*NADK*	NAD kinase	-1.53	***
XM_001256815	*NA*	NA	-1.53	***
XM_590469	*RASAL1*	RAS protein activator like 1 (GAP1 like)	-1.53	***
XM_587533	*PRSS22*	protease, serine, 22	-1.53	*
NM_001113243	*CORO1B*	coronin, actin binding protein, 1B	-1.53	***
XR_028308	*LOC787905*	similar to Lectin, galactoside-binding, soluble, 4 (galectin 4)	-1.53	***
NM_001035353	*PLVAP*	plasmalemma vesicle associated protein	-1.53	***
NM_001076030	*OGDH*	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	-1.53	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001014941	*BCAP31*	B-cell receptor-associated protein 31	-1.54	***
NM_001013597	*MOCS1*	molybdenum cofactor synthesis 1	-1.54	***
NM_001076419	*NENF*	neuron derived neurotrophic factor	-1.54	**
NM_001097561	*GNB2*	guanine nucleotide binding protein (G protein), beta polypeptide 2	-1.54	***
NM_001035313	*TGFB1I1*	transforming growth factor beta 1 induced transcript 1	-1.54	***
NM_001046026	*DULLARD*	dullard homolog (Xenopus laevis)	-1.54	***
XM_587457	*ATP2C2*	ATPase, Ca++ transporting, type 2C, member 2	-1.54	*
NM_001077926	*ABO*	ABO blood group (transferase A, alpha 1-3-N-acetylgalactosaminyltransferase /// transferase B, alpha 1-3-galactosyltransferase)	-1.54	**
NM_001035500	*MRPS34*	mitochondrial ribosomal protein S34	-1.54	***
NM_001034225	*OS9*	osteosarcoma amplified 9, endoplasmic reticulum associated protein	-1.54	**
NM_001082443	*SIGIRR*	single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	-1.54	***
NM_001075166	*STUB1*	STIP1 homology and U-box containing protein 1	-1.55	***
NM_001102242	*PYGO2*	pygopus homolog 2 (Drosophila)	-1.55	***
XM_001256288	*NA*	NA	-1.55	***
NM_001076418	*TPRG1L*	tumor protein p63 regulated 1-like	-1.55	***
NM_001083655	*PCDH1*	protocadherin 1	-1.55	***
NM_001075246	*METTL2B*	methyltransferase like 2B	-1.55	***
NM_001038688	*RRAS*	related RAS viral (r-ras) oncogene homolog	-1.55	**
XM_583844	*SERPINB1*	serpin peptidase inhibitor, clade B (ovalbumin), member 1	-1.55	**
NM_001034325	*MGC127874*	hypothetical LOC508617	-1.56	***
NM_001034748	*GPSN2*	glycoprotein, synaptic 2	-1.56	***
NM_001077132	*MGC137894*	hypothetical protein MGC137894	-1.56	***
NM_174329	*GPR68*	G protein-coupled receptor 68	-1.56	***
XM_001252015	*LOC783399*	similar to Equ c1	-1.56	*
NM_183082	*AIP*	aryl hydrocarbon receptor interacting protein	-1.56	***
NM_001083432	*TMEM214*	transmembrane protein 214	-1.56	**
NM_001046139	*TSTA3*	tissue specific transplantation antigen P35B	-1.56	**
NM_001040607	*ASCL2*	achaete-scute complex homolog 2 (Drosophila)	-1.56	**
NM_001110018	*MAPK3*	mitogen-activated protein kinase 3	-1.57	**
NM_001082471	*BAG3*	BCL2-associated athanogene 3	-1.57	**
NM_001001162	*USF2*	upstream transcription factor 2, c-fos interacting	-1.57	**

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001079511	*C25H16orf 14*	chromosome 16 open reading frame 14 ortholog	-1.57	**
XM_001252534	*NA*	NA	-1.57	**
NM_001098139	*SLC44A2*	solute carrier family 44, member 2	-1.57	***
NM_001024510	*CORO1B*	coronin, actin binding protein, 1B	-1.57	***
NM_001014883	*NR1H2*	nuclear receptor subfamily 1, group H, member 2	-1.57	***
NM_174617	*STAT5B*	signal transducer and activator of transcription 5B	-1.58	***
XM_001256223	*LOC789476*	similar to Signal transducer and activator of transcription 5B	-1.58	***
XM_610839	*FGFRL1*	fibroblast growth factor receptor-like 1	-1.58	***
NM_001098864	*ASNA1*	arsA arsenite transporter, ATP-binding, homolog 1 (bacterial)	-1.58	***
NM_001080726	*CITED4*	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	-1.58	**
NM_001045884	*AP1M1*	adaptor-related protein complex 1, mu 1 subunit	-1.58	***
NM_001102480	*TMEM43*	transmembrane protein 43	-1.58	***
NM_001077012	*IFRD2*	interferon-related developmental regulator 2	-1.58	***
NM_174202	*TPSB1*	tryptase beta 1	-1.58	***
NM_001081607	*VIPR1*	vasoactive intestinal peptide receptor 1	-1.58	***
XR_028159	*NA*	NA	-1.58	***
NM_175793	*MAPK1*	mitogen-activated protein kinase 1	-1.59	***
NM_001075960	*CMTM7*	CKLF-like MARVEL transmembrane domain containing 7	-1.59	***
NM_001075190	*LMF1*	lipase maturation factor 1	-1.59	***
NM_001035009	*NDRG1*	N-myc downstream regulated 1	-1.60	***
NM_001105322	*KRT17*	keratin 17	-1.60	***
XM_001249839	*NA*	NA	-1.60	***
XM_001249888	*NA*	NA	-1.60	***
NM_001076352	*LMO2*	LIM domain only 2 (rhombotin-like 1)	-1.60	***
XM_001256510	*HDLBP*	high density lipoprotein binding protein	-1.60	***
XM_869177	*CLK2*	CDC-like kinase 2	-1.60	***
NM_001034353	*ERGIC3*	ERGIC and golgi 3	-1.60	**
NM_001078066	*ESAM*	endothelial cell adhesion molecule	-1.60	***
XM_001255460	*CENPB*	centromere protein B, 80kDa	-1.60	***
NM_174611	*SLC6A8*	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	-1.60	***
NM_174064	*GDI1*	GDP dissociation inhibitor 1	-1.61	***
XM_866231	*TMEM157*	similar to transmembrane protein 157	-1.61	***
XM_591419	*SPEN*	spen homolog, transcriptional regulator (Drosophila)	-1.61	***
NM_001083434	*AKT1S1*	AKT1 substrate 1 (proline-rich)	-1.61	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001113242	*ERI3*	exoribonuclease 3	-1.61	***
NM_001034768	*LGALS4*	lectin, galactoside-binding, soluble, 4	-1.62	***
XM_001251425	*NA*	NA	-1.62	*
NM_001101908	*TMEM120B*	transmembrane protein 120B	-1.62	*
NM_001046317	*MPHOSPH 10*	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)	-1.62	***
NM_001077065	*SLC9A3R2*	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2	-1.62	***
NM_175825	*GSTM1*	glutathione S-transferase M1	-1.62	***
XM_867316	*GALE*	UDP-galactose-4-epimerase	-1.63	***
NM_001034398	*SLC29A1*	solute carrier family 29 (nucleoside transporters), member 1	-1.63	***
NM_173882	*SERPINA1*	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	-1.63	**
NM_001102509	*SURF4*	surfeit 4	-1.63	***
NM_001014951	*TMEM171*	transmembrane protein 171	-1.63	***
NM_174161	*PSAP*	prosaposin	-1.63	***
NM_001024929	*BHLHB2*	basic helix-loop-helix domain containing, class B, 2	-1.63	***
NM_001101305	*LOC788925*	hypothetical protein LOC788925	-1.64	***
NM_001034523	*AP2M1*	adaptor-related protein complex 2, mu 1 subunit	-1.64	***
XR_028269	*LOC785503*	similar to adaptor-related protein complex 2, mu 1 subunit	-1.64	***
NM_174396	*MYO1C*	myosin IC	-1.64	***
XM_001256191	*LOC789436*	similar to Vigilin (High density lipoprotein-binding protein) (HDL-binding protein)	-1.64	***
XM_593707	*NA*	NA	-1.65	***
NM_001083466	*HNRNPUL 1*	heterogeneous nuclear ribonucleoprotein U-like 1	-1.65	***
NM_174442	*PTBP1*	polypyrimidine tract binding protein 1	-1.65	***
NM_174008	*CD14*	CD14 molecule	-1.65	**
NM_173991	*APOE*	apolipoprotein E	-1.65	*
NM_001038534	*RNASEH2A*	ribonuclease H2, subunit A	-1.65	***
XM_610753	*RFNG*	RFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	-1.66	***
XR_028440	*NA*	NA	-1.66	***
NM_001034511	*AP1M2*	adaptor-related protein complex 1, mu 2 subunit	-1.66	***
NM_001034629	*EPHX1*	epoxide hydrolase 1, microsomal (xenobiotic)	-1.66	***
NM_001097984	*ALDOC*	aldolase C, fructose-bisphosphate	-1.66	***
XR_027442	*LOC533435*	similar to BTB (POZ) domain containing 2	-1.66	***
NM_001034456	*MLF2*	myeloid leukemia factor 2	-1.66	***
NM_001076812	*LOC507340*	hypothetical LOC507340	-1.67	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_174629	*UQCRC1*	UQCRC1 protein	-1.67	***
NM_001102121	*TAPBPL*	TAP binding protein-like	-1.67	***
NM_174443	*PTGES*	prostaglandin E synthase	-1.67	***
NM_001035081	*RAB7A*	RAB7A, member RAS oncogene family	-1.67	***
NM_001078078	*PMEPA1*	prostate transmembrane protein, androgen induced 1	-1.68	***
NM_001101271	*LOC618094*	hypothetical LOC618094	-1.68	***
NM_001046387	*MRI1*	methylthioribose-1-phosphate isomerase homolog (S. cerevisiae)	-1.68	***
NM_001046138	*RHOC*	ras homolog gene family, member C	-1.69	***
NM_001046431	*EHD2*	EH-domain containing 2	-1.69	***
NM_001075651	*PDXK*	pyridoxal (pyridoxine, vitamin B6) kinase	-1.69	**
NM_001037483	*POLR2J*	polymerase (RNA) II (DNA directed) polypeptide J, 13.3kDa	-1.70	***
NM_001105481	*RAB8A*	RAB8A, member RAS oncogene family	-1.71	***
NM_001035420	*CNN2*	calponin 2	-1.71	***
NM_001105441	*ABHD14B*	abhydrolase domain containing 14B	-1.72	***
NM_001040469	*C3*	complement component 3	-1.72	***
NM_001099053	*LOXL2*	lysyl oxidase-like 2	-1.72	***
NM_001034053	*LMNA*	lamin A/C	-1.72	***
NM_001034054	*PGAM1*	phosphoglycerate mutase 1 (brain)	-1.73	***
NM_001075122	*SRPR*	signal recognition particle receptor (docking protein)	-1.74	***
NM_001079649	*CRIP2*	cysteine-rich protein 2	-1.74	***
NM_001103250	*CYB5R3*	cytochrome b5 reductase 3	-1.74	***
XM_001256604	*LOC790007*	similar to EH domain-containing protein 4 (Hepatocellular carcinoma-associated protein 10/11)	-1.74	***
NM_001046124	*HM13*	histocompatibility (minor) 13	-1.75	***
NM_001040554	*HLA-A*	major histocompatibility complex, class I, A	-1.75	***
NM_174194	*TBCD*	tubulin folding cofactor D	-1.75	***
XM_614220	*LRP5*	low density lipoprotein receptor-related protein 5	-1.76	***
XR_027372	*LOC534416*	similar to WW domain-binding protein 11 (WBP-11) (SH3 domain-binding protein SNP70) (Npw38-binding protein) (NpwBP)	-1.76	***
NM_001077852	*SLC9A3R1*	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	-1.77	***
XM_001254012	*NA*	NA	-1.77	***
NM_001099376	*MINK1*	misshapen-like kinase 1 (zebrafish)	-1.77	***
NM_001083804	*SCLY*	selenocysteine lyase	-1.77	***
XR_028267	*NA*	NA	-1.77	***
XR_027440	*NA*	NA	-1.77	***
NM_176662	*PRKCSH*	protein kinase C substrate 80K-H	-1.77	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001075452	*ROGDI*	rogdi homolog (Drosophila)	-1.78	***
XM_876020	*CNTFR*	ciliary neurotrophic factor receptor	-1.78	***
XM_586851	*LIPG*	lipase, endothelial	-1.79	***
NM_001015571	*ARF3*	ADP-ribosylation factor 3	-1.80	***
NM_001034802	*RAB24*	RAB24, member RAS oncogene family	-1.80	**
XM_001254705	*IGK*	Ig kappa chain	-1.80	**
NM_001099705	*PCDHGA8*	protocadherin gamma subfamily A, 8	-1.80	***
NM_001102513	*PCDHGB4*	protocadherin gamma subfamily B, 4	-1.80	***
NM_001103334	*PCDHGC3*	protocadherin gamma subfamily C, 3	-1.80	***
NM_001114080	*PCDHGC3*	protocadherin gamma subfamily C, 3	-1.80	***
XR_028187	*NA*	NA	-1.80	***
XM_001250641	*SAMD1*	sterile alpha motif domain containing 1	-1.80	***
NM_001099706	*ECM1*	extracellular matrix protein 1	-1.80	***
NM_001015630	*GSS*	glutathione synthetase	-1.81	**
NM_001046129	*WIPI2*	WD repeat domain, phosphoinositide interacting 2	-1.81	***
XM_863846	*SULF2*	sulfatase 2	-1.81	***
XM_614520	*LOC534672*	similar to family with sequence similarity 20, member C	-1.81	***
XM_882308	*TINAGL1*	tubulointerstitial nephritis antigen-like 1	-1.82	***
XM_583785	*TNFSF10*	tumor necrosis factor (ligand) superfamily, member 10	-1.82	***
NM_001075473	*LOC510860*	C4b-binding protein alpha-like	-1.82	***
NM_001034632	*PIN1*	peptidylprolyl cis/trans isomerase, NIMA-interacting 1	-1.82	***
NM_001046367	*SPINT1*	serine peptidase inhibitor, Kunitz type 1	-1.83	***
NM_001099138	*C1QTNF5*	C1q and tumor necrosis factor related protein 5	-1.83	***
NM_001034039	*COL1A1*	collagen, type I, alpha 1	-1.84	***
NM_001034452	*TRABD*	TraB domain containing	-1.84	***
NM_001114855	*BoLA*	major histocompatibility complex, class I, A	-1.85	***
XM_001251326	*NA*	NA	-1.85	***
NM_001014957	*PYCR1*	pyrroline-5-carboxylate reductase 1	-1.85	***
XM_001254365	*NA*	NA	-1.85	***
XR_028526	*NA*	NA	-1.86	***
NM_001046613	*CLPTM1*	cleft lip and palate associated transmembrane protein 1	-1.86	***
NM_001099002	*ORAI1*	ORAI calcium release-activated calcium modulator 1	-1.87	***
XM_603601	*ATXN7L3*	ataxin 7-like 3	-1.88	***
NM_001046509	*MACROD1*	MACRO domain containing 1	-1.88	***
XM_867430	*GGT1*	gamma-glutamyltransferase 1	-1.88	***
XM_881439	*GSTM2*	glutathione S-transferase M2	-1.90	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001034735	*CD74*	CD74 molecule, major histocompatibility complex, class II invariant chain	-1.91	***
NM_001078041	*PLD3*	phospholipase D family, member 3	-1.91	***
XM_001253549	*DUSP28*	dual specificity phosphatase 28	-1.91	***
NM_001046358	*CMTM8*	CKLF-like MARVEL transmembrane domain containing 8	-1.92	***
NM_001105051	*RING1*	ring finger protein 1	-1.93	***
XM_001249906	*NA*	NA	-1.95	***
NM_177518	*AGPAT1*	1-acylglycerol-3-phosphate O-acyltransferase 1 (lysophosphatidic acid acyltransferase, alpha)	-1.96	***
NM_001080246	*PKN1*	protein kinase N1	-1.97	***
NM_173986	*AKT1*	v-akt murine thymoma viral oncogene homolog 1	-1.99	***
NM_001101056	*CSNK1G2*	casein kinase 1, gamma 2	-1.99	***
NM_174710	*ADRBK1*	adrenergic, beta, receptor kinase 1	-2.00	***
NM_001035319	*RNH1*	ribonuclease/angiogenin inhibitor 1	-2.00	***
XM_001249950	*NA*	NA	-2.01	***
NM_001013599	*TAGLN2*	transgelin 2	-2.02	***
NM_001077125	*KLHL12*	kelch-like 12 (Drosophila)	-2.02	***
XM_584919	*PLXNB2*	plexin B2	-2.04	***
NM_001035087	*HMOX2*	heme oxygenase (decycling) 2	-2.04	***
NM_001076205	*RBM42*	RNA binding motif protein 42	-2.05	***
NM_001076026	*LIN37*	lin-37 homolog (C. elegans)	-2.05	***
NM_001004024	*JUP*	junction plakoglobin	-2.06	***
NM_174493	*ZFP36*	zinc finger protein 36, C3H type, homolog (mouse)	-2.07	***
NM_176650	*ARHGDIA*	Rho GDP dissociation inhibitor (GDI) alpha	-2.08	***
XM_001249809	*LOC784095*	similar to Rho GDP-dissociation inhibitor 1 (Rho GDI 1) (Rho-GDI alpha) (GDI-1)	-2.08	***
NM_001040480	*PSMB8*	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	-2.08	***
NM_174224	*ACACA*	acetyl-coenzyme A carboxylase alpha	-2.10	***
XM_001255074	*LOC787813*	similar to acetyl-CoA-carboxylase	-2.10	***
NM_001077871	*SLC25A29*	solute carrier family 25, member 29	-2.12	***
XM_001250445	*NA*	NA	-2.12	***
XM_001253132	*NA*	NA	-2.12	***
NM_001083371	*C7H19ORF43*	chromosome 19 open reading frame 43 ortholog	-2.12	***
NM_001015655	*CFL1*	cofilin 1 (non-muscle)	-2.15	***
XM_001249857	*NA*	NA	-2.15	***
NM_001035285	*SERINC2*	serine incorporator 2	-2.16	***
XM_592497	*TGFB1*	transforming growth factor, beta 1	-2.17	***

Table S4. Continued.

Unigene ID	Symbol	Gene title	FC[1]	FDR *q*value[2]
NM_001075210	*PVRL2*	poliovirus receptor-related 2 (herpesvirus entry mediator B)	-2.20	***
NM_001075495	*CDC42EP1*	CDC42 effector protein (Rho GTPase binding) 1	-2.23	***
NM_174561	*MAN2B1*	mannosidase, alpha, class 2B, member 1	-2.24	***
NM_001083800	*IGLL1*	immunoglobulin lambda-like polypeptide 1	-2.25	***
NM_001080241	*HPN*	hepsin	-2.28	***
XM_001254640	*LOC787164*	similar to hepsin (transmembrane protease, serine 1)	-2.28	***
XM_590109	*PKM2*	pyruvate kinase, muscle	-2.31	***
XR_028808	*LOC519422*	similar to Transcription intermediary factor 1-beta (TIF1-beta) (Tripartite motif-containing protein 28) (Nuclear corepressor KAP-1) (KRAB-associated protein 1) (KAP-1) (KRAB-interacting protein 1) (KRIP-1) (RING finger protein 96)	-2.35	***
NM_174656	*SLC25A1*	solute carrier family 25 (mitochondrial carrier /// citrate transporter), member 1	-2.35	***
NM_001076397	*ENG*	endoglin	-2.83	***
NM_174598	*SCNN1A*	sodium channel, nonvoltage-gated 1 alpha	-2.84	***
XM_610582	*AKAP1*	A kinase (PRKA) anchor protein 1	-2.99	***
XR_028125	*NA*	NA	-2.99	***
NM_001033608	*MIF*	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	-3.61	***
XM_871314	*LOC404062*	immunoglobin light chain VJ region	-5.93	***

[1] FC = Fold change were calculated considering gene expression when cows were fed with UFA-enriched-diet compared with the same cows fed control diet.

[2] FDR q-value = effect of UFA supplementation; *, **, *** levels of significance indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table S5. Results for qPCR assays and correlation with microarray data when comparing dairy cows supplemented with unprotected unsaturated fatty acids (UFA) relative to the same cows fed control diet.

Gene Symbol	qPCR Fold Change	Array Fold Change	Correlation	*P*-value of correlation[1]
FASN	-1.16	-1.17	0.70	*
FADS3	-1.27	-1.27	0.71	*
FADS1	1.37	1.39	0.73	**
SCD1	-1.27	-1.28	0.71	**

[1] *P*-value = *, **, *** levels of significance indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table S6. Global transcriptional profiles based on cellular component, molecular functions and biological processes by Gene Ontology (GO) annotation determined in Erminj analysis in the mammary gland when comparing dairy cows supplemented with unprotected unsaturated fatty acids (UFA) relative to the same cows fed control diet.

Name	ID	Probes	Genes	Raw Score	FDR q-value[1]
Cellular component					
Ribosome	GO:0005840	122	121	2.26	***
Respiratory chain	GO:0070469	45	45	3.06	***
Mitochondrial membrane part	GO:0044455	60	60	3.05	***
Proton-transporting ATP synthase complex	GO:0045259	17	17	3.52	**
Lysosome	GO:0005764	66	65	2.13	**
Mitochondrial matrix	GO:0005759	80	79	2.00	**
Mitochondrial lumen	GO:0031980	80	78	2.02	**
Golgi apparatus part	GO:0044431	112	107	1.82	**
Cell projection	GO:0042995	121	118	1.78	**
Proton-transporting two-sector ATPase complex	GO:0016469	38	37	2.43	**
Pigment granule	GO:0048770	49	47	2.24	**
Vacuole	GO:0005773	75	74	1.97	**
Golgi membrane	GO:0000139	99	94	1.85	**
Ribosomal subunit	GO:0033279	30	30	2.59	**
Microtubule cytoskeleton	GO:0015630	121	119	1.67	*
Endosome	GO:0005768	67	67	1.93	*
Proteasome complex	GO:0000502	22	22	2.86	*
Nuclear periphery	GO:0034399	12	12	3.68	*
Cell fraction	GO:0000267	126	125	1.65	*
Chromosome	GO:0005694	89	88	1.79	*
Mitochondrial ribosome	GO:0005761	15	15	2.89	*
Coated membrane	GO:0048475	37	36	2.19	*
External side of plasma membrane	GO:0009897	40	40	2.11	*
Organellar large ribosomal subunit	GO:0000315	11	11	3.20	*
Chromosomal part	GO:0044427	73	72	1.80	*
Cytosolic part	GO:0044445	27	27	2.25	*
Cell surface	GO:0009986	56	56	1.88	*
Nuclear matrix	GO:0016363	10	10	3.59	*
Cell junction	GO:0030054	123	122	1.61	*
Large ribosomal subunit	GO:0015934	18	18	2.74	*
Anchoring junction	GO:0070161	25	25	2.29	*
Nuclear envelope	GO:0005635	55	54	1.86	*
Nucleolus	GO:0005730	64	63	1.77	*
Nuclear membrane	GO:0031965	24	23	2.28	*

Table S6. Continued.

Name	ID	Probes	Genes	Raw Score	FDR q-value[1]
Basolateral plasma membrane	GO:0016323	38	37	1.96	*
Insoluble fraction	GO:0005626	117	116	1.59	*
Clathrin coat	GO:0030118	19	18	2.58	*
Focal adhesion	GO:0005925	16	16	2.71	*
Late endosome	GO:0005770	21	21	2.28	*
Membrane fraction	GO:0005624	112	111	1.55	*
Coated pit	GO:0005905	14	13	2.66	*
Mitochondrial large ribosomal subunit	GO:0005762	12	12	2.98	*
Proton-transporting two-sector ATPase complex	GO:0033177	18	18	2.55	*
DNA-directed RNA polymerase II, holoenzyme	GO:0016591	12	11	2.77	*
Molecular functions					
Structural constituent of ribosome	GO:0003735	97	96	2.29	***
Phosphatase activity	GO:0016791	95	92	2.12	***
Phosphoric ester hydrolase activity	GO:0042578	124	121	2.02	***
Inorganic cation transmembrane transporter activity	GO:0022890	69	68	2.31	**
Hydrogen ion transmembrane transporter activity	GO:0015078	57	56	2.51	**
Monovalent inorganic cation transmembrane transporter activity	GO:0015077	60	59	2.44	**
Oxidoreductase activity, acting on NADH or NADPH	GO:0016651	33	33	2.79	**
Protein dimerization activity	GO:0046983	113	109	1.89	**
Protein serine/threonine kinase activity	GO:0004674	123	121	1.75	*
Cofactor binding	GO:0048037	116	116	1.79	*
Enzyme activator activity	GO:0008047	62	61	1.96	*
Threonine-type endopeptidase activity	GO:0004298	16	16	3.30	*
Phosphoprotein phosphatase activity	GO:0004721	55	52	2.10	*
Vitamin binding	GO:0019842	62	62	2.00	*
Active transmembrane transporter activity	GO:0022804	121	117	1.68	*
NADH dehydrogenase activity	GO:0003954	15	15	3.05	*
Magnesium ion binding	GO:0000287	117	114	1.70	*
Actin binding	GO:0003779	88	85	1.79	*
Transferase activity, transferring acyl groups	GO:0016746	83	80	1.84	*
Ligase activity, forming carbon-nitrogen bonds	GO:0016879	75	71	1.87	*
Enzyme binding	GO:0019899	66	65	1.91	*
Nucleoside-triphosphatase regulator activity	GO:0060589	109	106	1.73	*

Table S6. Continued.

Name	ID	Probes	Genes	Raw Score	FDR q-value[1]
NADH dehydrogenase (ubiquinone) activity	GO:0008137	13	13	3.16	*
Antioxidant activity	GO:0016209	29	29	2.35	*
Oxidoreductase activity, acting on sulfur group of donors	GO:0016667	22	19	2.70	*
Acyltransferase activity	GO:0008415	72	70	1.86	*
Protein homodimerization activity	GO:0042803	61	58	1.94	*
Isomerase activity	GO:0016853	63	62	1.93	*
Lyase activity	GO:0016829	83	81	1.75	*
GTPase regulator activity	GO:0030695	104	101	1.68	*
Small GTPase regulator activity	GO:0005083	70	68	1.85	*
Transferase activity, transferring acyl groups other than amino-acyl groups	GO:0016747	74	72	1.81	*
Electron carrier activity	GO:0009055	95	93	1.68	*
GTPase activator activity	GO:0005096	51	50	1.98	*
Acid-amino acid ligase activity	GO:0016881	59	55	1.87	*
ATPase activity	GO:0016887	107	105	1.62	*
G-protein coupled receptor activity	GO:0004930	115	114	1.64	*
Peptidase inhibitor activity	GO:0030414	65	62	1.83	*
Sodium ion binding	GO:0031402	25	25	2.30	*
Small conjugating protein ligase activity	GO:0019787	41	39	2.01	*
Metallopeptidase activity	GO:0008237	78	70	1.78	*
Endopeptidase inhibitor activity	GO:0004866	62	59	1.80	*
Biological Processes					
Electron transport chain	GO:0022900	67	67	2.78	***
Intracellular protein transport	GO:0006886	127	123	1.98	**
Oxidative phosphorylation	GO:0006119	45	44	2.78	**
Negative regulation of metabolic process	GO:0009892	123	117	1.91	**
Negative regulation of cellular metabolic process	GO:0031324	114	109	1.96	**
Lipid biosynthetic process	GO:0008610	104	103	2.03	**
Small GTPase mediated signal transduction	GO:0007264	87	85	2.06	**
Energy derivation by oxidation of organic compounds	GO:0015980	46	45	2.48	**
Regulation of protein metabolic process	GO:0051246	126	123	1.81	**
Cell redox homeostasis	GO:0045454	33	33	2.67	**
Monovalent inorganic cation transport	GO:0015672	101	99	1.91	**
Cytoskeleton organization	GO:0007010	76	75	2.11	**
Mitochondrial ATP synthesis coupled electron transport	GO:0042775	8	8	5.69	**

Table S6. Continued.

Name	ID	Probes	Genes	Raw Score	FDR q-value[1]
Cellular amine metabolic process	GO:0044106	126	125	1.77	**
Negative regulation of macromolecule metabolic proces	GO:0010605	114	109	1.86	**
Nucleotide biosynthetic process	GO:0009165	109	103	1.89	**
Purine nucleotide metabolic process	GO:0006163	91	87	1.97	**
ATP synthesis coupled electron transport	GO:0042773	10	10	5.02	**
Ribonucleotide metabolic process	GO:0009259	79	76	2.05	**
Cellular homeostasis	GO:0019725	114	111	1.81	**
Purine ribonucleotide metabolic process	GO:0009150	75	72	2.05	**
Respiratory electron transport chain	GO:0022904	12	12	4.33	**
Ribonucleotide biosynthetic process	GO:0009260	76	73	1.99	*
Purine nucleotide biosynthetic process	GO:0006164	82	79	1.95	*
Purine ribonucleotide biosynthetic process	GO:0009152	72	69	2.00	*
Regulation of cellular component organization	GO:0051128	89	84	1.94	*
Cellular respiration	GO:0045333	31	30	2.70	*
Organophosphate metabolic process	GO:0019637	59	57	2.07	*
Defense response	GO:0006952	136	125	1.70	*
Actin cytoskeleton organization	GO:0030036	45	44	2.31	*
Death	GO:0016265	121	120	1.74	*
Phospholipid metabolic process	GO:0006644	54	52	2.14	*
Actin filament-based process	GO:0030029	45	44	2.25	*
Negative regulation of biosynthetic process	GO:0009890	84	79	1.86	*
Nucleoside triphosphate metabolic process	GO:0009141	70	67	1.93	*
Cellular amino acid metabolic process	GO:0006520	100	99	1.76	*
Response to DNA damage stimulus	GO:0006974	78	75	1.88	*
Negative regulation of macromolecule biosynthetic process	GO:0010558	79	75	1.88	*
Negative regulation of cellular biosynthetic process	GO:0031327	81	77	1.88	*
Regulation of cellular protein metabolic process	GO:0032268	100	98	1.77	*
Purine ribonucleoside triphosphate metabolic process	GO:0009205	66	63	1.94	*
Cell death	GO:0008219	117	116	1.72	*
Regulation of leukocyte activation	GO:0002694	41	41	2.16	*
Negative regulation of developmental process	GO:0051093	110	110	1.72	*
Regulation of hydrolase activity	GO:0051336	75	75	1.84	*
Membrane organization	GO:0016044	77	74	1.89	*

Table S6. Continued.

Name	ID	Probes	Genes	Raw Score	FDR q-value[1]
Hexose metabolic process	GO:0019318	71	70	1.92	*
Monosaccharide metabolic process	GO:0005996	77	76	1.85	*
Purine nucleoside triphosphate metabolic process	GO:0009144	67	64	1.94	*
Regulation of cell activation	GO:0050865	42	42	2.16	*
Protein amino acid dephosphorylation	GO:0006470	45	42	2.18	*
Glucose metabolic process	GO:0006006	54	54	2.01	*
Regulation of organelle organization	GO:0033043	46	45	2.06	*
ATP metabolic process	GO:0046034	57	56	2.00	*
Negative regulation of apoptosis	GO:0043066	75	75	1.82	*
Negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	GO:0045934	67	64	1.92	*
Negative regulation of protein metabolic process	GO:0051248	37	35	2.20	*
Negative regulation of nitrogen compound metabolic process	GO:0051172	69	66	1.88	*
Regulation of cellular component biogenesis	GO:0044087	34	33	2.22	*
Proton transport	GO:0015992	39	38	2.19	*
Protein folding	GO:0006457	69	68	1.85	*
Glycerophospholipid metabolic process	GO:0006650	30	29	2.32	*
Nucleoside triphosphate biosynthetic process	GO:0009142	64	61	1.87	*
Sodium ion transport	GO:0006814	36	36	2.24	*
DNA repair	GO:0006281	59	57	1.90	*
Protein targeting	GO:0006605	45	44	2.07	*
ATP biosynthetic process	GO:0006754	54	53	1.92	*
Glucose metabolic process	GO:0006006	54	54	2.01	*
Regulation of organelle organization	GO:0033043	46	45	2.06	*

[1] FDR q-value = effect of UFA supplementation; *, **, *** levels of significance indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table S7. Gene Set Enrichment Analysis (GSEA) for gene expression in the mammary gland when comparing dairy cows supplemented with unprotected unsaturated fatty acids (UFA) relative to the same cows fed control diet.

Name	Size	NES	FDR q-value[1]
Oxidative phosphorilation	92	2.25	***
Ribosome	28	2.18	***
Electron transport chain	87	2.11	***
Sig IL4 Receptor in B Lymphocytes	16	-2.08	*
St B cell antigen receptor	28	-2.07	*
ERK1 ERK2 MAPK Signaling pathway	19	-2.05	*
SIG CD40 Pathway map	28	-2.01	*
HS Focal Adhesion Kegg	113	-1.94	*
HS IL-6 NET pathway 18	76	-1.93	*
MAPKINASE signaling pathway	50	-1.93	*
ST FAS signaling pathway	45	-1.93	*
FMLP induced chemokine gene expresión in HMC-1 cells	19	-1.92	*
HS Integrin-mediated cell adhesión	68	-1.92	*
Links between PYK2 and MAP Kinase	16	-1.90	*
HS IL-9 NET pathway 20	16	-1.89	*
SIG BCR signaling pathway	32	-1.89	*
ST Integrin signaling pathway	49	-1.89	*
ST GAQ pathway	19	-1.85	*
HS S1P signaling	19	-1.85	*
VEGF signaling pathway	47	-1.81	*
Ceramide signaling pathway	15	-1.80	*
NRF2-Regulated genes combined	18	-1.80	*
HS MAPK cascade	24	-1.80	*
HS P38 MAPK signaling pathway	26	-1.77	*
Bioactive peptide induced signaling pathway	19	-1.76	*
HS IL-7 NET pathway19	40	-1.75	*
Phospholipids as signalling intermediaries	15	-1.74	*
HS MAPK signaling pathway	120	-1.74	*
Apoptosis	52	-1.72	*
Angiotensin II mediated activation of JNK pathway via PYK2 dependent signaling	16	-1.71	*
HS Regulation of actin cytoskeleton	95	-1.71	*

[1] FDR q-value = effect of UFA supplementation; *, **, *** levels of significance indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table S8. Expression (fluorescence units) of SCD1 and SCD5 in the mammary gland tissue when comparing dairy cows supplemented with unprotected unsaturated fatty acids (UFA) relative to the same cows fed control diet.

Item	Control diet	UFA-enriched	s.e.	*P*- value[1]
SCD1, fluorescence units	6058	5896	348.5	0.74
SCD5, fluorescence units	59.38	84.53	3.03	***

[1] *P*-value = effect of UFA supplementation; *, **, *** levels of significance indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[2] Included n = 28 cows.

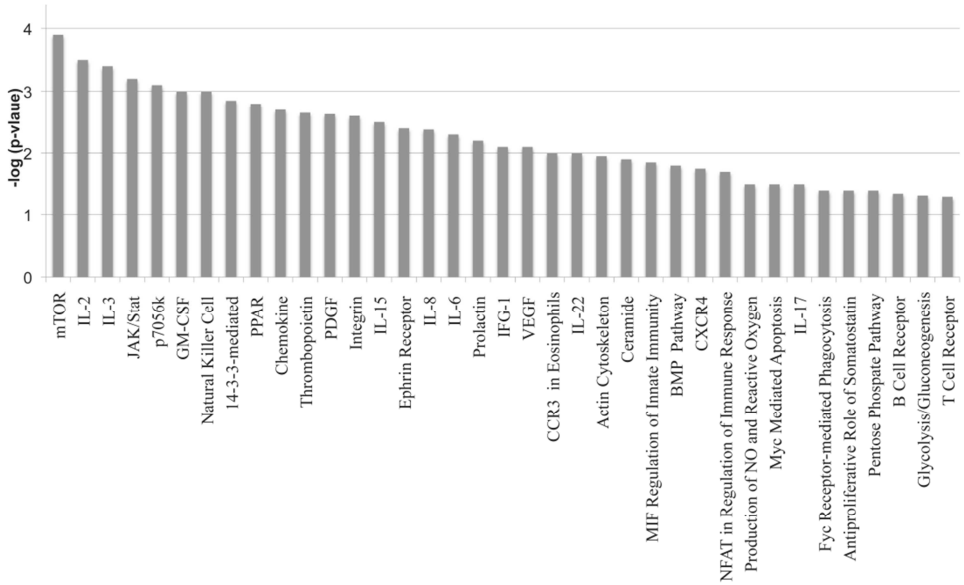

Figure S1. Canonical signalling pathways significantly modulated in the mammary gland tissue when comparing dairy cows supplemented with unprotected unsaturated fatty acids (UFA) relative to the same cows fed control diet. Statistical significance of pathway modulation was calculated via a right tailed Fisher's Exact test in Ingenuity Pathway and represented as –log (*P*-value): -log values exceeding 1.30 were significant false discovery rate (FDR) q-values < 0.05).

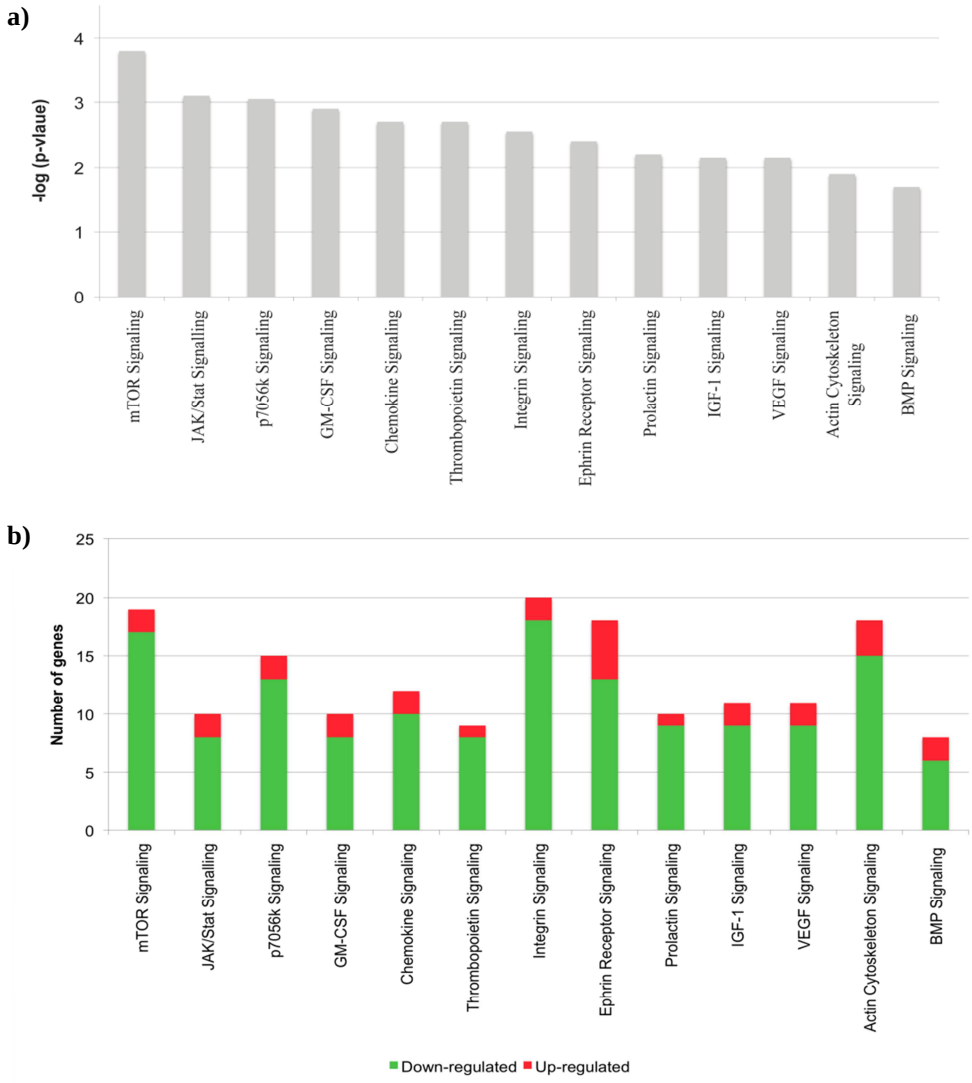

Figure S2. a) Canonical pathways involved in cellular growth, proliferation and development, and significantly modulated in the mammary gland tissue when comparing dairy cows supplemented with unprotected unsaturated fatty acids (UFA) relative to the same cows fed control diet. Statistical significance of pathway modulation was calculated via a right-tailed Fisher's Exact test in Ingenuity Pathway and represented as –log (*P* value): -log values exceeding 1.30 were significant false discovery rate (FDR) q-values < 0.05); **b)** The down-regulated and up-regulated genes for each molecular pathway are presented. The colour intensity indicates the expression of genes: red up-regulated, green down-regulated in animals supplemented with UFA relative to the same cows fed a control diet.

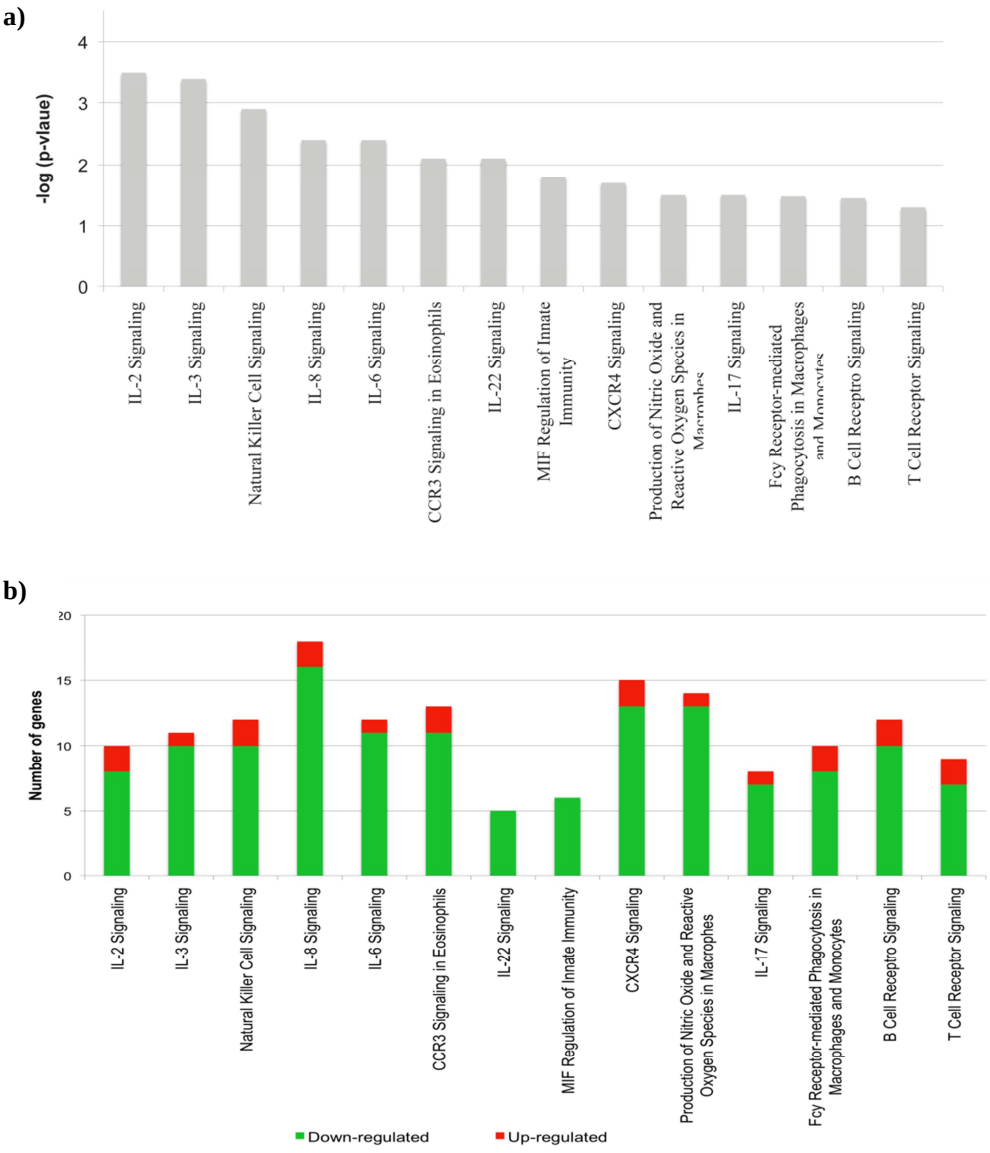

Figure S3. a) Canonical pathways involved in immune system response significantly modulated in the mammary gland tissue when comparing dairy cows supplemented with unsaturated fatty acids (UFA) relative to the same cows fed control diet. Statistical significance of pathway modulation was calculated via a right-tailed Fisher's Exact test in Ingenuity Pathway and represented as –log (*P* value): -log values exceeding 1.30 were significant false discovery rate (FDR) q-values < 0.05); **b)** The down-regulated and up-regulated genes for each molecular pathway are presented. The colour intensity indicates the expression of genes: red up-regulated, green down-regulated in animals supplemented with UFA relative to the same cows fed a control diet.

Chapter 4

Comparison Between Stearoyl-CoA Desaturase Expression in Milk Somatic Cells and in Mammary Tissue of Lactating Dairy Cows

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Submitted

Abstract

Stearoyl-CoA desaturase (SCD) is an important enzyme in the bovine mammary gland, where it inserts a *cis*-double bond at the Δ9 position in a wide range of fatty acids. Investigating SCD expression in the bovine mammary gland generally requires invasive biopsy to obtain mammary tissue. The aim of this study was to evaluate the use of milk somatic cells as a non-invasive alternative to biopsy for measuring mammary SCD expression in dairy cows. Both milk somatic cells and mammary tissue were collected from fourteen Holstein-Friesian cows and used for analysis of SCD expression by qRT-PCR. The SCD5 mRNA levels in mammary tissue compared with SCD1 were low, and for several milk somatic cell samples, SCD5 expression was even below the limit of detection. A significant relationship was found between SCD1 expression in milk somatic cells and in mammary tissue. In addition, SCD1 expression in milk somatic cells was significantly related to Δ9-desaturase indices in milk, which are commonly used as an indicator of SCD1 activity within the mammary gland. Our study showed that milk somatic cells can be used as a source of mRNA to study SCD1 expression in dairy cows, offering a non-invasive alternative to mammary tissue samples obtained by biopsy.

Introduction

Various studies in human subjects indicate that saturated fatty acids (SFA) increase the risk of cardiovascular disease and are involved in the development of the metabolic syndrome (Astrup et al., 2011). Altering the fatty acid (FA) composition of milk fat to be more in line with public health recommendations, would reduce SFA intake through milk. Reducing the SFA proportion of milk fat can be achieved by increasing the activity of stearoyl-CoA desaturase (SCD) in the mammary gland of dairy cows. Stearoyl-CoA desaturase is an enzyme that inserts a *cis*-double bond at the Δ9 position in a wide range of FA. The preferred substrates for SCD are stearic acid (C18:0) and palmitic acid (C16:0), generating oleic acid (C18:1 *cis*-9) and palmitoleic acid (C16:1 *cis*-9) respectively, but SCD can also convert other unsaturated FA, e.g. vaccenic acid (C18:1 *trans*-11) into rumenic acid (C18:2 *cis*-9, *trans*-11 CLA; Ntambi & Miyazaki, 2004). Several health benefits have been attributed to rumenic acid, including anticarcinogenic and antiatherogenic effects (Pariza et al., 2001; Wahle et al., 2004). In bovine, two isoforms of SCD, designated stearoyl-CoA desaturase 1 (SCD1) and stearoyl-CoA desaturase 5 (SCD5), have been identified (Lengi & Corl, 2007). Stearoyl-CoA desaturase 1 is abundantly expressed in the mammary gland of lactating dairy cows and has a key role in milk fat synthesis (McDonald & Kinsella, 1973; Bernard et al., 2008; Bionaz & Loor, 2008b). Conversely, the importance of the recently discovered isoform SCD5 in milk fat synthesis remains unclear. Contrary to SCD1, no relationship was observed between the relative low SCD5 mRNA abundance in bovine mammary tissue and Δ9-desaturation indices for milk, suggesting that SCD5 plays a minor role in the desaturation of milk FA (Jacobs et al., 2011).

To assess SCD expression in the bovine mammary gland, mammary tissue is usually obtained by biopsy. However, this technique is costly and invasive and can potentially lead to infection of the mammary gland, with detrimental effects on animal welfare. In addition, this invasive method can influence the results of the experiment. Potentially, isolated milk somatic cells can be used as a non-invasive alternative, since these cells contain exfoliated secretory epithelial cells of the mammary gland. Previous studies have shown that milk provides a convenient and non-invasive source of viable mammary epithelial cells which can be used for gene expression studies in various species (Boutinaud & Jammes, 2002). In goats, Boutinoud et al. (2002) reported that the relative mRNA expression of α-S1, κ-casein and α-lactalbumin was identical in both milk somatic cells and mammary tissue. A similar effect was found for the expression of lipogenic genes (including acetyl-CoA carboxylase, fatty acid synthase and SCD1) in milk somatic cells and mammary tissue of beef cows (Murrieta et al., 2006). Two studies have examined mammary gene expression in dairy cows using milk somatic cells (Feng et al., 2007; Boutinaud et al., 2008). However, both studies did not compare the expression level of genes in milk somatic cells with those in the native mammary gland. Since milk somatic cells contain mainly leukocytes and only a small proportion of secretory epithelial cells, the quantification of mammary transcripts

could be imprecise. Therefore, the aim of this study was to evaluate the use of milk somatic cells as a non-invasive alternative to biopsy for measuring SCD expression in the mammary gland of dairy cows. To this end, we measured the expression levels of SCD in isolated milk somatic cells by quantitative RT-PCR and compared these with the levels in mammary tissue obtained by biopsy. In addition, SCD expression levels were compared with Δ9-desaturation indices calculated from milk FA, which are commonly used as an indicator of SCD activity in the mammary gland.

Material and Methods

Animals and Treatments

The samples utilized in this study were collected from two separate experiments involving Holstein-Friesian cows, that evaluated the effects of linseed supplementation on performance and FA profile of milk. These two studies were chosen because it was anticipated that linseed supplementation would inhibit mammary SCD expression (Bernard et al., 2005a), thereby creating more variation in SCD expression. Samples from Experiment 1 were used to compare SCD expression in milk somatic cells and mammary tissue as well as to study the relationship between SCD expression and the Δ9-desaturation indices in milk. Samples from Experiment 2 were used to further investigate the relationship between SCD expression in milk somatic cells and the desaturation indices in milk. Experiment 1 was approved by the Institutional Animal Care and Ethics Committee of Wageningen UR Livestock Research (Lelystad, the Netherlands) and Experiment 2 by the Institutional Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands).

Experiment 1

Experiment 1 was conducted to study the effect of linseed supplementation on health and production of periparturient dairy cows. Fourteen Holstein-Friesian cows were paired according to parity, expected date of calving and milk performance in the previous lactation. Cows within each pair were randomly assigned to one of two treatment groups: 1) “Linseed”; or 2) “Control”. Cows in the Linseed group received a basal diet supplemented with a concentrate-mixture including linseed (4.8% of total diet on dry matter basis), whereas cows in the Control group received a basal diet supplemented with a concentrate mixture without linseed (Table 1). All cows received the basal diet *ad libitum*, which was a forage mixture that consisted of maize silage, wilted grass silage, grass seed straw, solvent extracted soybean meal and formaldehyde treated soybean meal (Mervobest™, Agrifirm, Apeldoorn, the Netherlands). This basal diet was supplemented with a concentrate mixture that was fed individually through automatic concentrate dispensers. Cows were group-housed in a cubicle shed with continuous access to water and were milked twice daily at 0600 and 1700h. Experimental treatments started 3 weeks before the expected calving date and lasted until 6 weeks after calving (Mach et al., unpublished).

Table 1. Ingredient composition of the treatment diets fed in Experiment 1 and 2.

	Treatments					
	Experiment 1		Experiment 2[1]			
Ingredient, % DM	Control	Linseed	CL	EL	FL	DL
Grass silage	30.2	32.6	31.2	31.0	31.2	31.2
Maize silage	19.6	21.2	29.4	29.2	29.4	29.4
Grass seed straw	4.3	4.7	-	-	-	-
Maize	13.8	12.9	5.2	4.5	6.0	5.4
Wheat	7.7	7.3	6.8	6.4	7.3	7.7
Soybean meal	6.9	3.7	4.9	4.7	5.0	4.9
Rapeseed meal	-	-	6.5	6.1	6.9	6.9
Soybean meal treated	4.5	5.3	-	-	-	0.2
Linseed	-	4.8	-	-	-	-
Crushed linseed	-	-	6.5	-	-	-
Extruded linseed	-	-	-	9.9	-	-
Spray-dried linseed oil	-	-	-	-	3.3	-
Linseed oil	-	-	-	-	-	2.1
DHA Gold[2]	-	-	-	-	-	0.4
Others (inclusion <5%)	13.0	7.5	9.5	8.2	10.9	11.8

[1] CL = crushed linseed; EL = extruded whole linseed; FL = formaldehyde treated spray-dried linseed oil; DL = microalgae high in docosahexaenoic acid (DHA Gold®) in combination with linseed oil.

[2] DHA Gold®, Martek Biosciences Corp., Columbia, MD.

For the isolation of somatic cells, 1 L of milk was sampled from each cow on the last day of the experimental period (week 6) during the AM milking. This milk sample was kept on ice until somatic cell isolation, which was completed within 2 hours after milking. Simultaneously, individual milk samples were taken and stored at -20°C until FA analysis. Within 2 hours after milking, a mammary biopsy was taken from each cow. Approximately 0.7 to 1.0 g of mammary tissue was obtained by surgical biopsy from the midpoint section of a rear quarter, according to the method of Farr et al. (1996) as described by Jacobs et al. (2011). Collected tissue was immediately snap-frozen in liquid nitrogen and stored at -80°C until total RNA extraction.

Experiment 2

Experiment 2 was conducted to study the effect of different sources of linseed on performance and milk FA profile of dairy cows. Four multiparous Holstein-Friesian dairy cows in early lactation (mean days in milk: 52 ± 22) fitted with a ruminal cannula (10 cm i.d.; Bar Diamond Inc., Parma, ID) were fed diets with differently processed linseed according to a 4 x 4 Latin square design. Experimental periods were 21 d in length. Cows were housed in individual tie-stalls with continuous access to water and milked twice daily at 0630 and 1700h. Dietary treatments consisted of a basal diet (a mixture of grass silage and maize silage) and concentrates, which were supplemented with either 1) crushed linseed (CL), 2) extruded whole linseed (EL), 3) formaldehyde-treated spray dried linseed oil (FL), and 4) microalgae high in docosahexaenoic acid (DHA Gold®; Martek Biosciences Corp., Columbia, MD) in combination with linseed oil (DL; Table 1) and were designed to provide equal amounts of C18:3n3. Treatments were chosen based on an *in vitro* study aimed at decreasing ruminal biohydrogenation of C18:3n3 applying several chemically or technologically treated linseed products (Sterk et al. 2010). The basal diet, concentrates and linseed products were thoroughly mixed immediately before feeding. Diets were offered as two equal meals just before milking. After day 7, diets were fed at 95% of *ad libitum* intake to prevent variation in feed intake and C18:3n3 intake between treatments. Detailed information about this experiment has been described by Sterk (2011).

For the isolation of somatic cells, 1 L of milk was sampled from each cow on the last day of each experimental period during the AM milking, including the week before the experiment (as control). This milk sample was kept on ice until somatic cell isolation, which was completed within 2 hours after milk sampling. In addition, individual milk samples were taken and stored at -20°C until FA analysis.

Fatty Acid Analysis

Fatty acid composition of the milk samples of Experiment 1 was analysed as described by Jacobs et al. (2011) and those of Experiment 2 were analysed as follows. Total lipids were extracted from the milk samples with diethyl ether and petroleum ether according to the Rose-Gottlieb method (AOAC, 1990). Fatty acids from milk lipids were methylated with 2.0 *N* of methanolic $NaOCH_3$, neutralized with $NaHSO_4$ and dried with Na_2SO_4. Fatty acid methyl esters (FAME) were recovered in 1 mL of hexane.

The FAME were quantified using gas chromatography (Trace GC Ultra™, Thermo Fisher Scientific, Waltham, MA, USA) with a fused silica capillary column (100 m x 0.250 mm and 0.2 μm film thickness; Supelco; SP2560, Bellefonte PA, USA). The carrier gas was helium at a constant flow of 1.5 mL/min. The flame ionization detector was set at 280ºC. The time-temperature program used, started with an initial temperature of 70ºC for 4 min, increased with 1ºC/min to 165ºC for 20 min, increased with 2ºC/min to 170ºC for 10 min, and increased with 4ºC/min to a final temperature of 215ºC for 20 min. The FAME were identified using external standards (S37, Supelco, Bellefonte PA, USA; odd and branched chain fatty acids, *trans*-11-C18:1, *cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2,

Larodan Fine Chemicals AB, Malmö, Sweden). Fatty acids *trans*-6+7+8-C18:1, *trans*-10-C18:1, *trans*-12-C18:1, *trans*-13+14-C18:1, *cis*-12-C18:1, *cis*-13-C18:1, *cis*-14+trans-16-C18:1, *cis*-15-C18:1, *trans*-11,*cis*-15-C18:2 were identified according to the elution sequence reported by Loor et al. (2004) and Shingfield et al. (2006).

Somatic Cell Isolation

Milk somatic cells were isolated according to the method described by Feng et al. (2007), with some minor modifications. Fresh milk samples of 1 L were transferred to 10 sterile 100-mL conical tubes and, to limit formation of casein micelles, 100 µL of 0.5 *M* ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) was added to each tube. Somatic cells were pelleted by centrifugation at 2,700 x *g* for 10 min at 4°C. Then, the cream layer was removed with a spatula and skim milk was discarded. The cell pellet was washed twice in 25 mL of ice-cold PBS (pH = 7.2) containing 0.5 m*M* EDTA, followed by centrifugation at 2,700 x *g* for 15 min at 4°C. The supernatant was discarded and the remaining cell pellet was resuspend in 1 mL of Trizol® reagent (Invitrogen, Breda, the Netherlands) and stored at -80°C until total RNA isolation.

RNA Isolation and Real-Time PCR

Frozen mammary tissue samples were homogenized in liquid nitrogen using a mortar and pestle. Total RNA from milk somatic cells and mammary tissue was isolated using Trizol® reagent (Invitrogen, Breda, the Netherlands), according to the manufacturer's instructions. Total RNA was treated with DNAse I and column purified using the PureLink™ Micro-to-Midi Total RNA Purification System kit (Invitrogen, Breda, the Netherlands). To assess RNA quality, samples were analysed on a RNA 6000 NanoChip® (Agilent Technologies, Amstelveen, the Netherlands) using the Agilent 2100 bioanalyzer® (Agilent Technologies, Amstelveen, the Netherlands) according to the manufacturer's instructions. The Agilent 2100 expert software was used to calculate the RNA Integrity Number (RIN), as a measure for the state of RNA intactness (Schroeder et al., 2006). First-strand cDNA synthesis and quantitative real-time PCR was performed as described previously (Jacobs et al., 2011). The following gene-specific primers were used: for SCD1, forward primer 5'-GGCGTTCCAGAATGACGTTT-3' and reverse primer 5'-AAAGCCACGTCGGGAATTG -3'; for SCD5, 5'-GGCACCGGCAGGACATC-3' (forward) and 5'-GAGCAGTCAGGAGGAAGCAGAA-3' (reverse). Cytokeratin 8 (KRT8) or beta-actin (ACTB) was used as internal standard. For KRT8, we used the forward primer 5'-ATTTGCCTCCTTCATCGACAA-3' and reverse primer 5'-GCTCCGGGCAGTCTTCTG-3'. For ACTB, 5'-GCCCTGAGGCTCTCTTCCA-3' (forward) and 5'-CGGATGTCGACGTCACACTT-3' (reverse). Templates were amplified after a preincubation for 10 min at 95 ºC, followed by amplification for 40 cycles (10 s at 95 ºC, 5 s at 60 ºC, 5 s at 72 ºC). All reactions revealed a single product as determined by melting curve analysis. Specificity of the primer sets were verified by sequencing of the generated amplicons. PCR efficiencies for the genes were established to be at least 86%.

Relative mRNA expression for each gene of interest (I) was calculated using the formula: $(1 + E[I])^{-Ct[I]} / (1 + E[H])^{-Ct[H]}$, where E = amplification efficiency and H = housekeeping gene.

Statistical Analysis

The regression procedure (PROC REG) of SAS (version 9.2, SAS Institute Inc.) was used to analyse relationships between relative abundance of SCD mRNA in milk somatic cells and mammary tissue, as well as the various desaturase indices calculated from milk FA. The various desaturase indices (i.e., C14, C16, C17 and C18 index) were calculated from milk FA as the ratio between the Δ9-desaturase product and the sum of the product and the substrate FA of SCD, e.g. C14:1 *cis*-9 / (C14:1 *cis*-9 + C14:0). To evaluate the effect of dietary linseed supplementation on the relative abundance of SCD mRNA and the various desaturation indices, analyses were carried out by ANOVA using the PROC MIXED procedure of SAS with treatment as fixed effect. Differences were considered significant at a probability of $P < 0.05$, and as a trend at a probability of $0.05 < P < 0.10$.

Results

Total RNA Yield and Quality

Total RNA yield was 181.4 µg (SEM = 13.1 µg) per mammary tissue sample of 0.15 to 0.20 g obtained from biopsy (Experiment 1). Total RNA yield from milk somatic cells was 6.0 µg per sample (SEM = 1.9 µg) in Experiment 1 and 4.2 µg per sample (SEM = 0.6 µg) in Experiment 2, obtained with 1 L milk samples. After DNAse treatment and column purification, the A_{260}/A_{280} ratio was > 1.90 in all samples, indicating that protein contamination was negligible. Several milk somatic cell samples did not yield sufficient RNA (< 2 µg per sample) and therefore, four samples of Experiment 1 (n = 10 remaining) and five samples of Experiment 2 (n=15 remaining) were excluded from further analysis. The average RNA integrity number (RIN) was 7.1 (SEM = 0.30) for mammary tissue samples and 7.6 (SEM = 0.21) for milk somatic cell samples.

Relationship Between SCD Expression in Milk Somatic Cells and in Mammary Tissue

In Experiment 1, both SCD1 and SCD5 expression were measured by qRT-PCR analysis in milk somatic cells as well as mammary tissue. For four out of ten milk somatic cell samples SCD5 mRNA level was below the limit of detection and therefore, only the results for SCD1 expression are presented (Figure 1). As internal controls we used either ACTB, known to be expressed in virtually all mammalian cells, or KRT8, which is characteristic of mammary luminal cells (Moll et al., 1982; Abd El-Rehim et al., 2004).

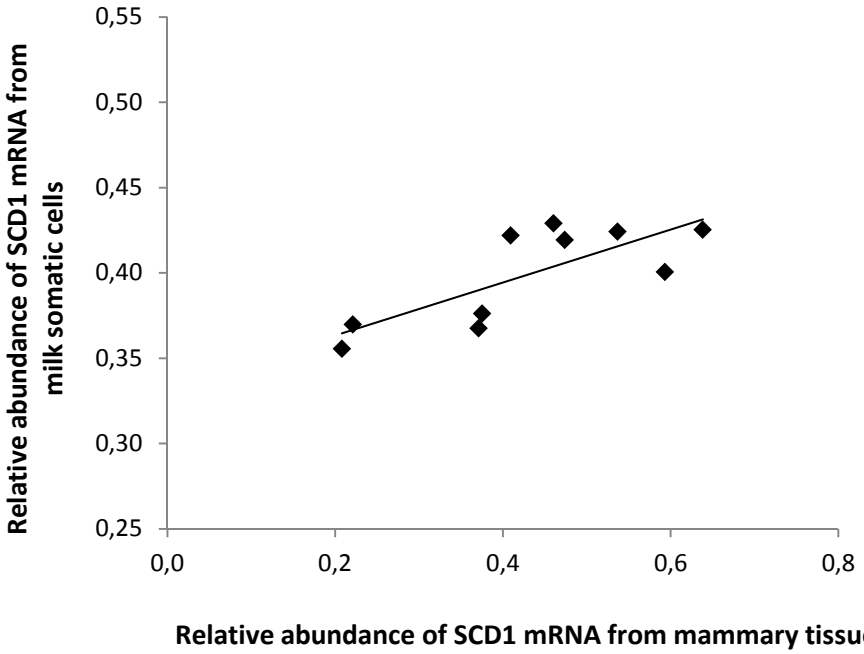

Figure 1. Relationship between relative abundance of SCD1 mRNA in mammary tissue and milk somatic cells (Experiment 1; n = 10). SCD1 expression in milk somatic cells = 0.332 (± 0.020) + 0.155 (± 0.0450) x SCD1 expression in mammary tissue; $r^2 = 0.60$; $P < 0.01$.

When KRT8 was used as endogenous control gene instead of ACTB, relationship between SCD1 expression in mammary tissue and milk somatic cells was significantly improved ($r^2 = 0.60$; $P < 0.01$ for KRT8 (Figure 1) versus $r^2 = 0.26$; $P = 0.17$ for ACTB). Therefore, KRT8 was used as endogenous control gene in the results presented.

Relationship between SCD1 expression and Δ9-desaturase indices

Experiment 1

Figure 2a shows the relationship between SCD1 expression in mammary tissue and the C14 desaturation index ($r^2 = 0.34$; $P = 0.08$). The r^2 values of the relationship between mammary SCD1 expression and the C16, C17 and C18 indices were 0.36 ($P = 0.04$), 0.38 ($P = 0.02$) and 0.22 ($P = 0.14$), respectively. Figure 2b shows the relationship between SCD1 expression in milk somatic cells and the C14 desaturation index ($r^2 = 0.52$; $P = 0.02$). The r^2 values of the relationship between SCD1 expression in milk somatic cells and the C16, C17 and C18 indices were 0.45 ($P = 0.03$), 0.47 ($P = 0.09$) and 0.32 ($P = 0.18$), respectively.

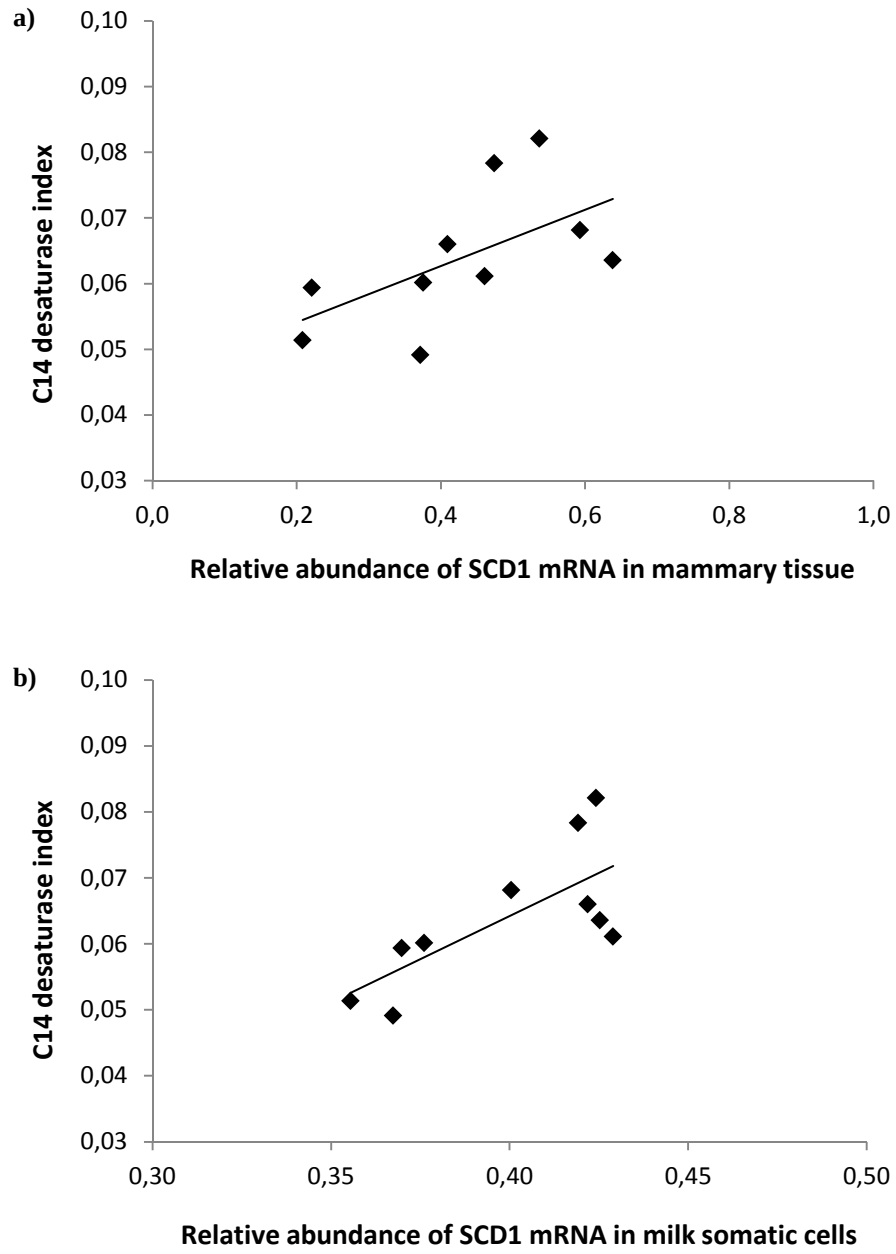

Figure 2. Relationship between the C14 desaturase index and the relative abundance of SCD1 mRNA in mammary tissue **(a)** as well as milk somatic cells **(b)** of the same cows (Experiment 1; n=10). For mammary tissue: C14 index = 0.046 (± 0.009) + 0.043 (± 0.021) x SCD1 mRNA; r^2 = 0.34; *P* = 0.08 and for milk somatic cells: C14 index = -0.040 (± 0.036) + 0.261 (± 0.089) x SCD1 mRNA; r^2 = 0.52; *P* = 0.02.

Experiment 2

A significant relationship was observed between relative abundance of SCD1 expression in milk somatic cells and the C14 desaturation index (C14 index = -0.032 (± 0.021) + 0.247 (± 0.047) x SCD1 expression milk somatic cells; $r^2 = 0.70$; $P < 0.01$). The r^2 values of the relationship between SCD1 expression in milk somatic cells and the C16, C17 and C18 indices were 0.77 ($P < 0.001$), 0.65 ($P < 0.001$) and 0.44 ($P < 0.01$), respectively.

Figure 3a shows the relationship between SCD1 expression in milk somatic cells and the C14 desaturation index of experiment 1 and 2 combined ($r^2 = 0.70$; $P < 0.001$). Figure 3b shows the relationship between SCD1 expression in milk somatic cells and the C16 desaturation index of experiment 1 and 2 combined ($r^2 = 0.63$; $P < 0.001$). When the highest value was removed, the r^2 values of the C14 and C16 index changed to 0.32 and 0.34, respectively. The r^2 values of the relationship between SCD1 expression in milk somatic cells and the C17 and C18 indices were 0.37 ($P < 0.01$) and 0.35 ($P < 0.01$), respectively.

Effect of linseed supplementation on SCD1 expression

In experiment 1, relative expression of SCD1 in milk somatic cells was not different ($P = 0.12$) between cows on the control diet (0.44 ± 0.021) and cows on the linseed supplemented diet (0.40 ± 0.018). Similarly, relative expression of SCD1 in mammary tissue was not different ($P = 0.26$) between cows on the control diet (0.49 ± 0.070) and cows on the linseed supplemented diet (0.39 ± 0.057). In addition, the C14 desaturation index was not affected ($P = 0.19$) by linseed supplementation and was 0.07 ± 0.005 and 0.06 ± 0.004 for cows on the control and linseed supplemented diet, respectively.

In experiment 2, relative expression of SCD1 in milk somatic cells was not affected ($P = 0.84$) by the different linseed treatments and was 0.45 ± 0.13, 0.41 ± 0.02, 0.42 ± 0.05, 0.39 ± 0.02 and 0.47 ± 0.19 for the control, CL, EL, FL and DL treatment, respectively. In addition, the C14 desaturation index was not affected ($P = 0.37$) by the different linseed treatments and was 0.07 ± 0.02, 0.06 ± 0.01, 0.08 ± 0.02, 0.06 ± 0.01 and 0.10 ± 0.06 for the control, CL, EL, FL and DL treatment, respectively.

Discussion

The aim of this study was to evaluate the use of milk somatic cells for quantitative analysis of SCD mRNA, as a non-invasive alternative to mammary biopsy. Milk somatic cells have been previously used to study gene expression levels in humans (Lindquist et al., 1994; Alcorn et al., 2002), goats (Boutinaud et al., 2002) beef cows (Murrieta et al., 2006) and dairy cows (Feng et al., 2007; Boutinaud et al., 2008). Whether the use of bovine milk somatic cells as an alternative source of SCD mRNA is valid, depends on several criteria. We used the following three criteria: 1) harvest of sufficient quantity of mRNA from milk

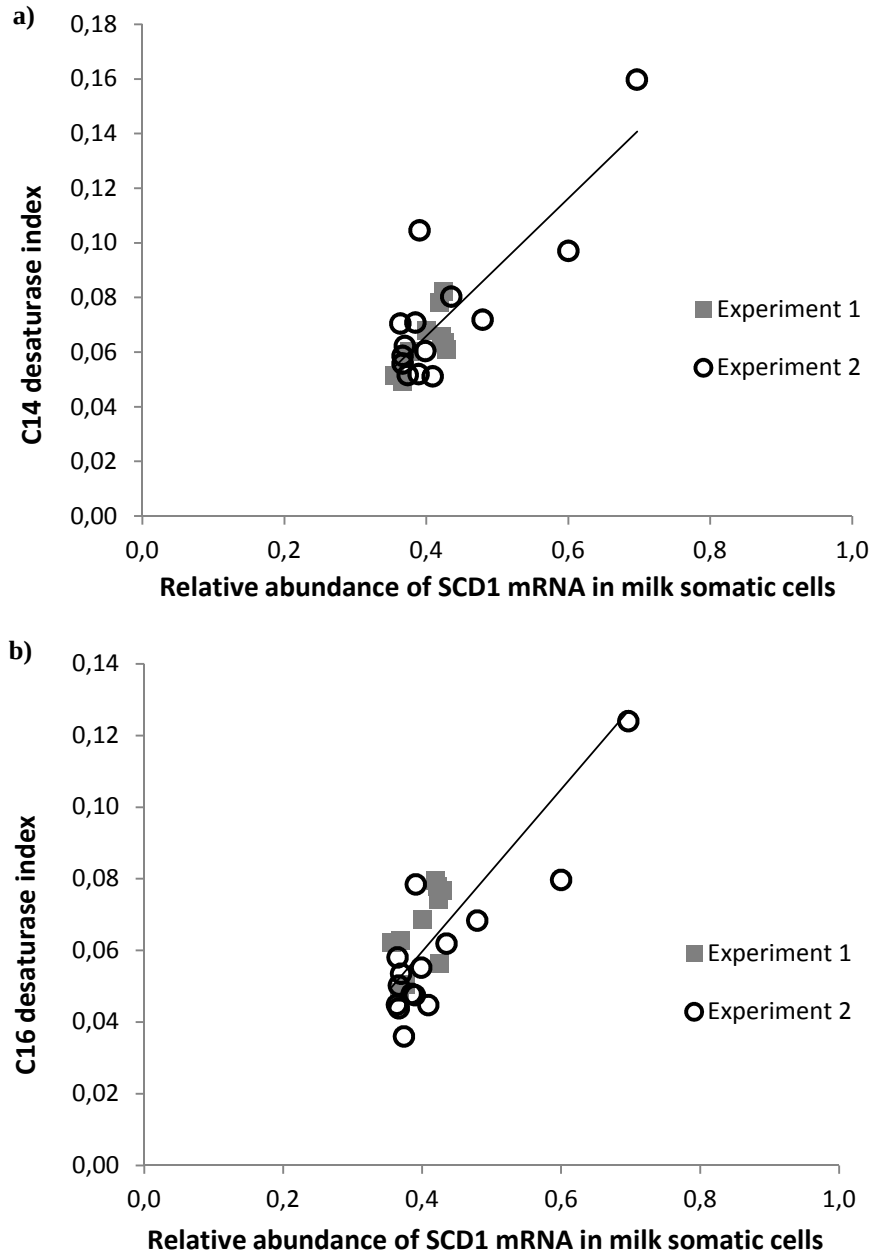

Figure 3. Relationship between the relative abundance of SCD1 mRNA in milk somatic cells and the C14 desaturase index **(a)** as well as the C16 desaturase index **(b)** of cows in Experiment 1 (n = 10) and Experiment 2 (n = 15). C14 index = -0.035 (± 0.015) + 0.252 (± 0.035) x SCD1 mRNA; $r^2 = 0.70$; $P < 0.001$ and C16 index = -0.016 (± 0.013) + 0.188 (± 0.030) x SCD1 mRNA; $r^2 = 0.63$; $P < 0.001$.

somatic cells; 2) satisfactory relationship between SCD expression in mammary tissue and in somatic cells; 3) relationship between SCD expression in somatic cells and Δ9-desaturation indices to be at least as good as the relationship between SCD expression in mammary tissue and desaturation indices.

Total RNA Yield and Quality

In order for milk somatic cells to be considered a suitable alternative to mammary tissue, it is essential that each sample yields sufficient RNA of good quality. Total RNA extracted from milk somatic cells was low (range: 1.6 to 14.3 µg per 1 L milk sample) compared with the study of Feng et al. (2007) (range: 3.3 to 26.9 µg per 1 L milk sample). Feng et al. (2007) showed that total RNA yield is directly related to the amount of viable somatic cells. In our study, several milk samples did not yield sufficient RNA and were therefore not used in subsequent analysis. Collecting a larger quantity of milk would obviously increase the amount of somatic cells, thereby increasing RNA yield. In addition, time of milk sampling can influence total somatic cell count as well as the proportion of mammary epithelial cells. In goats, total and epithelial cell count were substantially higher in post-milk samples compared with samples taken during milking (Boutinaud et al., 2002). Another issue, which can hamper RNA isolation from milk somatic cells, is the formation of casein micelles. In the present study, EDTA was used to prevent the formation of casein micelles, but this prevention may have been incomplete. Techniques that limit the formation of casein micelles could therefore increase RNA yield from milk somatic cells. The RIN values in our study show that the quality of the RNA isolated from milk somatic cells was comparable to that of mammary tissue.

Relationship Between SCD Expression in Milk Somatic Cells and in Mammary Tissue

The bovine mammary gland expresses both SCD1 and SCD5 (Gervais et al., 2009; Jacobs et al., 2011), although the mRNA abundance of SCD5 is substantially lower than that of SCD1 (Jacobs et al., 2011). Correspondingly, in this study we found low SCD5 expression levels in mammary tissue compared with SCD1 as determined by qRT-PCR. For several milk somatic cell samples, SCD5 expression was even below the limit of detection and therefore, SCD5 results are not presented. This was most likely caused by the low and variable proportion of mammary epithelial cells of total somatic cells in bovine milk and indicates that the use of milk somatic cells might not be suitable for measuring genes with low levels of mRNA.

There are substantial differences in amount and cell type distribution of somatic cells between species, due to differences in milk secretion, milk volume and immune defence mechanisms (Boutinaud and Jammes, 2002). The major cell type in somatic cells from bovine milk is leukocytes, including macrophages, lymphocytes and polymorphonuclear neutrophils (Boutinaud and Jammes, 2002). Consequently, mammary epithelial cells only represent a small proportion (± 2%) of total somatic cells in bovine milk, and variation is

substantial (Feng et al., 2007; Boutinaud et al., 2008). This low and variable amount of epithelial cells is a concern for quantification of mammary transcripts by using a housekeeping gene that is expressed in leukocytes as well, since the expression of such a housekeeping gene does not reflect the proportion of mammary epithelial cells (Boutinaud et al., 2008). Besides the ubiquitously expressed ACTB, we tested whether KRT8, which is characteristic to mammary epithelial cells (Moll et al., 1982; Abd El-Rehim et al., 2004), could be used as endogenous control gene to correct for the differences in proportion of epithelial cells in total milk somatic cells. Cytokeratin 8, which is expressed as cytoplasmic as well as cell-surface protein, has been successfully used to isolate living mammary epithelial cells from somatic milk cells by immunopurification (Boutinaud et al., 2008). In addition, epithelial keratins are relatively abundant and stable (Hudson, 2002), which is essential for housekeeping genes. Compared with ACTB, the use of KRT8 as endogenous control gene significantly improved the relationship between SCD1 expression in mammary tissue and milk somatic cells, ($r^2 = 0.60$; $P < 0.01$ for KRT8 versus $r^2 = 0.26$; $P = 0.17$ for ACTB).

Parallel to our results, Boutinaud et al. (2002) found that in goats, the relative amount of milk protein mRNA was identical in both milk somatic cells and mammary tissue samples. Also, in beef cows a positive correlation was found between expression of lipogenic genes in milk somatic cells and mammary tissue (Murrieta et al., 2006). Taken together, this indicates that milk somatic cells can be used as a representative source of mRNA to analyse mammary SCD1 expression, since results are comparable to those obtained from mammary tissue.

Relationship Between SCD1 Expression and Δ9-desaturase Indices

Substrate-product relationships from several pairs of milk FA are commonly used as an indicator of Δ9-desaturase activity within the mammary gland. The C14 desaturase index is considered the best indicator of SCD activity, since generally almost all C14:0 and C14:1 *cis*-9 originate from *de novo* synthesis in the mammary gland (Bernard et al., 2008; Heck et al., 2009; Jacobs et al., 2011). In this study, a positive relationship between SCD1 mRNA expression in mammary tissue and the C14 desaturase index was found ($r^2 = 0.34$; $P = 0.08$), which was comparable to a previous study ($r^2 = 0.35$; $P = 0.002$; Jacobs et al., 2011). Similarly, Bernard et al. (2005a) reported positive, although again not strong, relationships between mRNA levels of SCD and milk proxy ratios.

We found positive relationships between SCD1 expression in milk somatic cells and the C14 index in both experiments, indicating that SCD1 expression in milk somatic cells provides a reasonable estimation of SCD activity in the mammary gland. A similar conclusion was drawn by Feng et al. (2007) who reported a significant relationship between the C14 desaturase index and relative abundance of SCD1 mRNA in milk somatic cells. Interestingly, the relationship between the various desaturation indices and SCD1 expression in milk somatic cells was somewhat better compared to the relationship between these indices and SCD1 expression in mammary tissue. This suggests that SCD1 mRNA

expression in milk somatic cells provides a better reflection of SCD1 activity in the mammary gland, compared with the expression measured in mammary tissue obtained from biopsy. This could be due to the fact that mammary tissue provides a "snapshot" of SCD1 expression, while milk somatic cells are accumulated over a period of time, thereby averaging out possible diurnal variation in SCD1 expression.

Conclusions

Our results show that using milk somatic cells as a source of mRNA to examine SCD1 expression in dairy cows yields results comparable with mammary tissue. In addition, mRNA abundance of SCD1 measured in milk somatic cells was significantly related to Δ9-desaturase indices in milk. The mRNA expression of SCD5 in both mammary tissue and in milk somatic cells was low. The yield of total RNA from milk of dairy cows is rather low and further investigation is needed to improve the yield of RNA from milk. This study showed that milk somatic cells can be used as a source of mRNA to study SCD1 expression in dairy cows, offering a non-invasive alternative to mammary tissue samples obtained by biopsy.

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Chapter 5

Effects of Short- and Long-Chain Fatty Acids on Stearoyl-CoA Desaturase Expression in Bovine Mammary Epithelial Cells

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Submitted

Abstract

Stearoyl-CoA desaturase (SCD) is an important enzyme in the bovine mammary gland since it introduces a *cis*-double bond at the Δ9 position in a wide range of fatty acids. Several long-chain polyunsaturated fatty acids inhibit expression of SCD, but information on the effect of short-chain fatty acids on mammary SCD expression is scarce. We used a bovine mammary cell line (MAC-T) to assess the effect of acetic acid (Ac) and β-hydroxybutyric acid (BHBA) on the mRNA expression of SCD via qRT-PCR, and compared this to the effect of various long-chain fatty acids on SCD expression as well as expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). In addition, expression of sterol regulatory binding protein 1 (SREBP-1), insulin-induced gene 1 protein (INSIG-1) and peroxisome proliferator-activated receptors (PPARs) were measured to examine if these transcription factors are involved in the regulation of SCD expression in bovine mammary epithelial cells. MAC-T cells were treated for 12 h without fatty acid additions (CON) or with either 5 m*M* Ac, 5 m*M* BHBA, a combination of 5 m*M* Ac + 5 m*M* BHBA, 100 µ*M* palmitic acid (PA), 100 µ*M* stearic acid (SA), 100 µ*M* oleic acid (OA), 100 µ*M* *trans*-vaccenic acid (TVA), 100 µ*M* linoleic acid (LA) or 100 µ*M* α-linolenic acid (ALA). Treatment incubations were performed in triplicate. In comparison with CON, expression of SCD1 was increased by Ac (+61%) and reduced by OA (-61%), LA (-84%) and ALA (-88%). Contrary to SCD1, MAC-T cells did not express SCD5 mRNA. Expression of ACC was also increased by Ac (+44%) and reduced by LA (-48%) and ALA (-49%). Compared with control, FAS expression was not significantly affected by the treatments. The mRNA level of SREBP-1 was not affected by Ac or BHBA, but was reduced by OA (-44%), TVA (-42%), LA (-62%) and ALA (-68%) compared with control. Expression of INSIG-1 was down-regulated by SA (-37%), OA (-63%), TVA (-53%), LA (-81%) and ALA (-91%). Both PPARα and PPARδ expression was not significantly affected by the treatments. These results show that acetate up-regulates expression of SCD1 and ACC in MAC-T cells, which indicates that acetate may increase desaturation and *de novo* synthesis of fatty acids in the bovine mammary gland. Furthermore, the results strengthen the support for the role of SREBP-1 and INSIG-1 as central regulators of lipogenesis in the bovine mammary gland.

Introduction

Stearoyl-CoA desaturase (SCD) is a key enzyme in mammary lipid metabolism since it introduces a *cis*-double bond at the Δ9 position in a wide range of fatty acids (FA). The preferred substrates of SCD are C18:0 and, to a lesser extent, C16:0, which are converted to C18:1 *cis*-9 and C16:1 *cis*-9, respectively (Ntambi & Miyazaki, 2004). Since C18:1 *cis*-9 has a considerable lower melting point than C18:0, SCD plays a critical role in maintaining fluidity of cell membranes as well as milk fat. In addition, SCD is responsible for the conversion of C18:1 *trans*-11 into C18:2 cis-9, *trans*-11 (CLA), which has been associated with several health benefits, including anticarcinogenic and antiatherogenic effects (Bhattacharya et al, 2006; Reynolds & Roche, 2010).

It is well-known that in rodents, polyunsaturated fatty acids (PUFA) inhibit SCD expression in both liver and adipose tissue, whereas saturated fatty acids and monounsaturated fatty acids have little effect (Ntambi, 1999). In addition, it was shown that feeding mice a diet high in C18:1 *cis*-9 or C18:2 *cis*-9,12 inhibits both mRNA expression and activity of SCD in the mammary gland (Singh et al., 2004). Conversely, in ruminants only a few studies investigated the effect of FA on mammary SCD expression. Kadegowda et al. (2009a) reported that addition of C16:0, but not C18:0, increased expression of SCD in a bovine mammary cell line (MAC-T), whereas C18:1 *cis*-9, C18:1 *trans*-10, C18:2 *trans*-10, cis-12 and C20:5 all decreased expression of SCD. In addition, it was shown in the same cell line that promoter activity of bovine SCD could be inhibited by C18:1 *cis*-9, whereas C18:0, C18:1 *trans*-11, C18:2 and C18:3 had no effect (Keating et al., 2006).

Signalling mechanisms involved in the regulation of lipogenic genes, including SCD, have been comprehensively described in rodent liver and adipose tissue, but relatively little is known about these signalling mechanisms in the mammary gland of ruminants (Bernard et al., 2008). In rodents, transcriptional mechanisms are responsible for changes in mRNA expression of various lipogenic genes (Foufelle & Ferre, 2002) and critical transcription factors involved in the regulation of SCD include sterol regulatory binding protein 1 (SREBP-1) and peroxisome proliferator-activated receptors (PPARs) (Paton & Ntambi, 2009; Han et al., 2010). The SREBP family consist of transcription factors controlling the expression of a range of enzymes required for endogenous cholesterol, FA, triacylglycerol and phospholipid synthesis (Eberlé et al., 2004). In addition, insulin-induced gene 1 protein (INSIG-1) mediates negative feedback control of SREBP-1 (Horton et al., 2002; Dong & Tang, 2010). Similarly to rodents, it has been reported that SREBP-1 plays a pivotal role in FA synthesis in the bovine mammary gland (Peterson et al., 2004; Harvatine & Bauman, 2006; Bauman et al., 2011). Peroxisome proliferator-activated receptors are a group of nuclear receptor proteins that function as ligand-activated transcription factors regulating the expression of genes involved in metabolism, cellular differentiation and development (Michalik et al., 2006). Three isoforms of PPAR are known, designated PPARα, PPARδ and PPARγ, and these isoforms are involved in regulating SCD expression in rodents

(Paton & Ntambi, 2009). Correspondingly, it has been suggested that PPARγ activation regulates milk fat synthesis in bovine mammary epithelial cells (Kadegowda et al., 2009a).

Contrary to long-chain fatty acids (LCFA), no information is available on the effect of short-chain fatty acids (SCFA) on mammary SCD expression. Acetic acid (Ac) and β-hydroxybutyric acid (BHBA), which originate from ruminal fermentation, are the main precursors for de novo synthesis of FA in the bovine mammary gland (Chilliard et al., 2000). The two main enzymes involved in *de novo* FA synthesis are acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Chilliard et al., 2000). It is hypothesized that both Ac and BHBA could alter the mRNA expression of genes involved in mammary lipid metabolism, including SCD. In this study we used MAC-T bovine mammary epithelial cells as a model to investigate the effect of the SCFA Ac and BHBA and various LCFA including C16:0, C18:1 *cis*-9 and C18:2 *cis*-9,12 on the mRNA expression of SCD as well as ACC and FAS in the bovine mammary gland. In addition, expression of SREBP-1, INSIG-1 and PPARs were measured to examine if these transcription factors are involved in the regulation of SCD expression in bovine mammary epithelial cells. Moreover, FA composition of the MAC-T cells was measured to examine whether this was affected by the different treatments.

Material and Methods

Reagents

Sodium acetic acid (S5636), sodium DL-β-hydroxybutyric acid (H6501), bovine serum albumin (BSA, fatty acid free; A8806), bovine insulin (I0516), bovine apo-transferrin (T1428), hydrocortisone (H0888), progesterone (P8783) and phosphate buffered saline (PBS; pH = 7.4; P3813) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The LCFA palmitic acid (C16:0, N-16A), stearic acid (C18:0, N-18A), oleic acid (C18:1 cis-9, U-46A), *trans*-vaccenic acid (C18:1 *trans*-11, U-49A), linoleic acid (C18:2 cis-9,12, U-59A) and linolenic acid (C18:3 cis-9,12,15, U-62A) were obtained from Nu-Chek Prep Inc. (Elysian, MN, USA). Dulbecco's modified eagle's medium F-12 (DMEM/F-12), fetal bovine serum (FBS, 10091-148), penicillin/streptomycin (15070-063) and TRIzol® reagent (15596-026) were purchased from Invitrogen (Carlsbad, CA, USA). Bovine prolactin (AFP710E) was obtained from the National Hormone & Peptide Program (NHPP), NIDDK, and Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA, USA).

Cell Culture and Treatments

This study was performed using an established clonal cell line produced from primary bovine mammary alveolar cells (MAC-T) by stable transfection with SV-40 large T-antigen (Huynh et al., 1991). Cells were cultured in DMEM/F-12 supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin (5,000 units of penicillin and 5,000 µg of streptomycin per mL) at 37ºC in a humidified atmosphere of 5% CO2. The MAC-T cells were seeded in vented 75 cm2 flasks (Corning Inc., Corning, NY, USA) and grown to

Table 1. Primer sequences of genes selected for quantitative polymerase chain reaction analysis.

Gene[1]	Primer sequence
SCD1	F 5'-GGCGTTCCAGAATGACGTTT-3'
	R 5'-AAAGCCACGTCGGGAATTG -3'
ACC	F 5'-CATCTTGTCCGAAACGTCGAT-3'
	R 5'-CCCTTCGAACATACACCTCCA-3'
FAS	F 5'-ACCTCGTGAAGGCTGTGACTCA-3'
	R 5'-TGAGTCGAGGCCAAGGTCTGAA-3'
18S	F 5'-AGAAACGGCTACCACATCCAA-3'
	R 5'-GGGTCGGGAGTGGGTAATTT-3'
ACTB	F 5'-GCCCTGAGGCTCTCTTCCA-3'
	R 5'-CGGATGTCGACGTCACACTT-3'
MRPL39	F 5'-AGGTTCTCTTTTGTTGGCATCC-3'
	R 5'-TTGGTCAGAGCCCCAGAAGT-3'
SREBP-1	F 5'-GGTTTCCAGAGGGACCTGAGT-3'
	R 5'-TGGCCCCTGCCATCAGT-3'
INSIG-1	F 5'-GCATCGACAGTCACCTTGGA-3'
	R 5'-TGTCAAGGAGAGCTGAACGTTATT-3'
PPARα	F 5'-GGATGTCCCATAACGCGATT-3'
	R 5'-GGTCATGCTCACACGTAAGGATT-3'
PPARδ	F 5'-TGTGGCAGCCTCAATATGGA-3'
	R 5'-GACGGAAGAAGCCCTTGCA-3'

[1] SCD1 = stearoyl-coenzyme A desaturase 1; ACC = acetyl coenzyme A carboxylase; FAS = fatty acid synthase; 18S = 18S ribosomal RNA; ACTB = β-actin; MRPL39 = mitochondrial ribosomal protein L39; SREBP-1 = sterol regulatory element-binding protein 1; INSIG-1 = insulin induced gene 1; PPARα = peroxisome proliferator-activated receptor alpha; PPARδ = peroxisome proliferator-activated receptor delta.

approximately 90% confluency in DMEM/F-12 supplemented with 10% FBS and 1% penicillin/streptomycin for 24 h. Subsequently, the cell monolayer was rinsed twice with 10 mL PBS and incubated in a lactogenic media (adapted from Kadegowda et al., 2009a) comprised of DMEM/F-12 supplemented with 1% penicillin/streptomycin, 1 g/L BSA, 5 mg/L bovine insulin, 5 mg/L bovine apo-transferrin, 2.5 mg/L bovine prolactin, 1 mg/L hydrocortisone, and 1 mg/L progesterone. This lactogenic media was refreshed after 24 h and cells were cultured for 48 h at 37ºC in the lactogenic media before treatment with FA. Before addition to the MAC-T cells, the LCFA were complexed with BSA as sodium salts as described by Sørensen et al. (2008) with modifications. First, 0.1 mmol FA was dissolved in 1 ml hexane:isopropanol (3:2) followed by addition of 10 mL 0.1 N sodium hydroxide. This solution was then mixed and the hexane:isopropanol layer was evaporated using nitrogen gas. Subsequently, 1 mL of this solution was slowly added to 2 ml of 5% (w/v) BSA, and the resulting FA-BSA solution was stored overnight at 4 ºC, followed by storage at -20 ºC until use.

Treatments were performed in the presence of lactogenic media at 37 ºC. For LCFA treatment, cells were treated with 100 µ*M* FA-BSA solution and for SCFA treatment with either 5 m*M* Ac, 5 m*M* BHBA or a mixture of Ac and BHBA (5 m*M* each). In order to keep the amount of BSA similar among all treatment groups, BSA was applied to the control, Ac and BHBA treatments as well. Treatments were performed in triplicate and cells were harvested after 12 h of incubation for subsequent analysis of gene expression and FA composition.

RNA Extraction and Real Time PCR

Total RNA was isolated using ice-cold TRIzol® reagent, according to the manufacturer's instructions, as described previously (Jacobs et al., 2011). SYBR® Green qRT-PCR was performed with a ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) utilizing the SYBR® Green PCR Master Mix (Applied Biosystems). PCR conditions were as follows: denaturation at 95°C for 10 min followed by amplification of 40 cycles of 15s at 95°C and 1 min at 60°C. Oligonucleotide primers used are presented in Table 1. PCR efficiencies for the genes were established to be at least 91%. Melting curve analysis was carried out to determine primer specificity. Relative mRNA expression for each gene of interest (I) was calculated using the formula: $(1 + E[I])^{-Ct[I]} / (1 + E[GMH])^{-Ct[GMH]}$, where E = amplification efficiency and GMH = geometric mean of the three housekeeping genes (ACTB, 18S and MRPL39). Each sample was run in duplicate.

Lipid Extraction and FA Analysis

The MAC-T cells were collected, pelleted by centrifugation (1000 x g for 10 min. at 4°C), and washed twice with PBS. Total lipids were extracted using methanol and chloroform according to Bligh & Dyer (1959). Extracted lipids were trans-esterified using potassium hydroxide in methanol (0.5 N) followed by boron trifluoride in methanol (10% w/w) at 100°C for ten minutes each. Fatty acid methyl esters (FAME) were dissolved in n-hexanes and quantified using a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) using a CP-Sil 88 WCOT fused silica column (100 m x 0.25 mm i.d. x 0.2 µm film thickness; Varian Inc., Lake Forest, CA, USA) according to our previous published methods (Caldari-Torres et al., 2011). The FAME were identified by comparison of retention times with known FAME standards (Caldari-Torres et al., 2011).

Statistical Analysis

Statistical analyses were carried out by ANOVA using the PROC MIXED procedure of SAS (version 9.2, SAS Institute Inc.) to evaluate the effects of the treatments on mRNA abundance of genes and FA composition of the cells. The model included treatment as fixed effect and to test pair-wise comparisons, post hoc analyses were carried out on the least square means adjusted for multiple comparisons using the Tukey-Kramer test. Differences were considered significant at a probability of $P < 0.05$, and as a trend at a probability of $0.05 < P < 0.10$. The regression procedure (PROC REG) of SAS was used

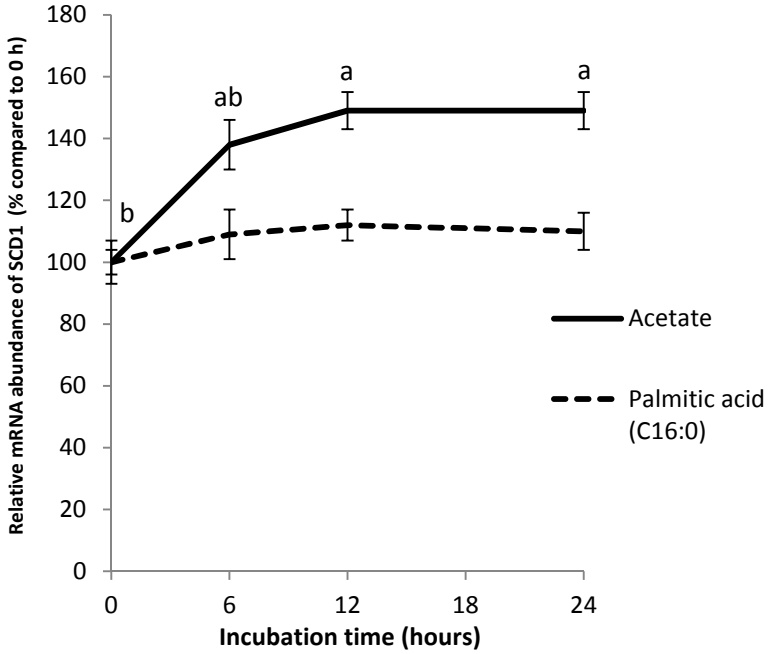

Figure 1. Relative mRNA abundance of SCD1 in MAC-T cells treated with either 5 m*M* acetate or 100 µ*M* palmitic acid (C16:0) after increasing incubation time. Vertical lines represent the standard error of the mean, and time points within treatment without a common letter differ ($P < 0.05$).

to analyse relationships between relative abundance of the different genes as well as relationships between mRNA abundance of SCD1 and the desaturase indices. Relationships between SCD1 mRNA abundance and the desaturase indices were calculated without the treatments that involved substrates and products of that particular desaturase index. Results presented are expressed as mean ± SEM.

Results

Expression of Lipogenic Genes

A preliminary time response study showed that the maximal effect of Ac and C16:0 on SCD1 expression was reached after 12 h of incubation (Figure 1). Therefore, MAC-T bovine mammary epithelial cells were treated for 12 h with Ac, BHBA or various LCFA, after which the mRNA expression of the lipogenic enzymes SCD1, ACC and FAS was determined by qRT-PCR. As shown in Figure 2, the transcript of SCD1 was increased by

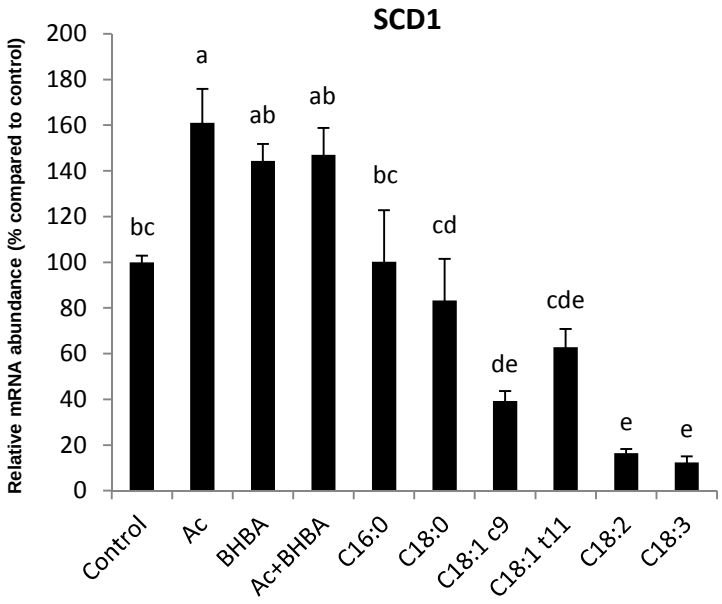

Figure 2. Relative mRNA abundance of SCD1 in MAC-T cells after treatment (12h) with either: 5 m*M* acetate (Ac), 5 m*M* β-hydroxybutyrate (BHBA), combination of Ac and BHBA (Ac+BHBA), or 100 µ*M* of the following LCFA: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 c9), *trans*-vaccenic acid (C18:1 t11), linoleic acid (C18:2) or linolenic acid (C18:3). Vertical lines represent the standard error of the mean, and treatments without a common letter differ ($P < 0.05$).

Ac (+61%) and reduced by C18:1 *cis*-9 (-61%), C18:2 *cis*-9,12 (-84%) and C18:3 *cis*-9,12,15 (-88%) compared with control cells. Contrary to SCD1, MAC-T cells did not express SCD5 mRNA (Figure 3). Expression of ACC was also increased by Ac (+44%) and reduced by C18:2 *cis*-9,12 (-48%) and C18:3 *cis*-9,12,15 (-49%) compared with control cells (Figure 4a). Compared with control, FAS expression was not significantly affected by the treatments (Figure 4b). However, FAS expression was lower for the C18:2 *cis*-9,12 and C18:3 *cis*-9,12,15 treatment compared with Ac or BHBA and for the C18:1 *cis*-9 treatment compared with BHBA.

Expression of Transcription Factors

The mRNA level of SREBP-1 was reduced by C18:1 *cis*-9 (-44%), C18:1 *trans*-11 (-42%), C18:2 *cis*-9,12 (-62%) and C18:3 *cis*-9,12,15 (-68%) compared with control (Figure 5a). Expression of INSIG-1 was down-regulated by C18:0 (-37%), C18:1 *cis*-9 (-63%), C18:1 *trans*-11 (-53%), C18:2 *cis*-9,12 (-81%) and C18:3 *cis*-9,12,15 (-91%) compared with control (Figure 5b). Both PPARα (Figure 6a) and PPARδ (Figure 6b) expression were not significantly affected by the treatments, and PPARγ was not expressed in the MAC-T

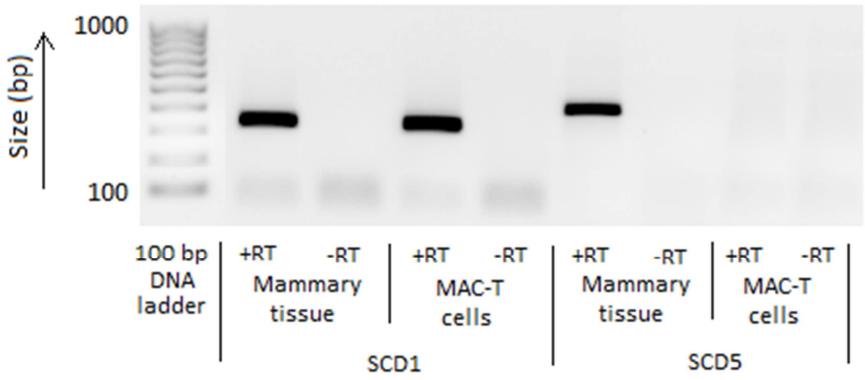

Figure 3. Detection of SCD1 and SCD5 in native and immortalized (MAC-T) bovine mammary epithelial cells (0.8% agarose gel stained with ethidium bromide). Native mammary tissue was collected via biopsy. Total RNA was converted to cDNA in the presence of reverse transcriptase (+RT) or absence of reverse transcriptase (-RT; negative control). PCR conditions were as follows: denaturation at 94°C for 2 min followed by amplification of 40 cycles of 30s at 94°C, 45s at 30°C and 2 min at 72°C, followed by 8 min at 72°C. Primers used for SCD1 were: CTACACAACCACCACCACCA (forward), CAGGGCACCCATCAGATAGT (reverse) and for SCD5: CTTCCTCCTGACTGCTCTGG (forward) and GTGGGGACTACGAAGCACAT (reverse).

cells (data not shown). Correlations between the relative mRNA abundance of the lipogenic genes SCD1, ACC and FAS and the transcription factors SREBP-1, INSIG-1, PPARα and PPARδ are shown in Table 2.

Fatty Acid Composition

Table 3 shows the FA composition of total lipids extracted from the MAC-T cells. As expected, application of the various LCFA resulted in an increased proportion of the corresponding FA in the cells. C18:1 *trans*-11 as well as C18:2 *cis*-9, *trans*-11 CLA were only detected in cells treated with C18:1 *trans*-11. The C16 and C18 desaturase indices were significantly affected by the different treatments. As predicted, the C18 index was significantly higher upon addition of C18:1 *cis*-9, which is the desaturase product itself. There was a significant relationship between the C18 desaturase index and relative SCD1 mRNA abundance (r^2 = 0.42; P < 0.001). However there was no significant relationship between the C16 desaturase index and relative SCD1 mRNA abundance (r^2 = 0.05; P = 0.28).

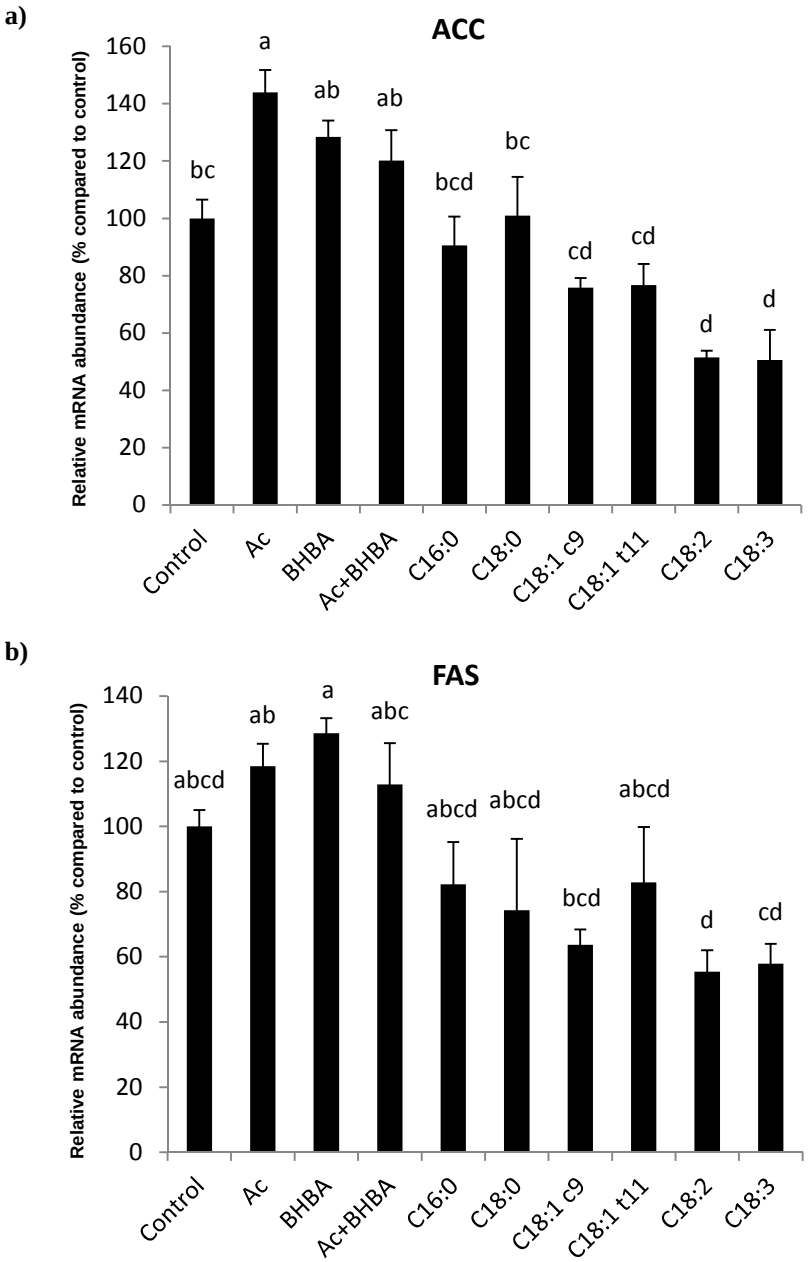

Figure 4. Relative mRNA abundance of ACC **(a)** and FAS **(b)** in MAC-T cells after treatment (12h) with either: 5 m*M* acetate (Ac), 5 m*M* β-hydroxybutyrate (BHBA), combination of Ac and BHBA (Ac+BHBA), or 100 µ*M* of the following LCFA: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 c9), *trans*-vaccenic acid (C18:1 t11), linoleic acid (C18:2) or linolenic acid (C18:3). Vertical lines represent the standard error of the mean, and treatments without a common letter differ ($P < 0.05$).

Table 2. Correlation (r^2 values) between the relative mRNA abundance of the lipogenic genes: stearoyl-coenzyme A desaturase 1 (SCD1), acetyl coenzyme A carboxylase (ACC) and fatty acid synthase (FAS) and the transcription factors: sterol regulatory element-binding protein 1 (SREBP-1), insulin induced gene 1 (INSIG-1), peroxisome proliferator-activated receptor alpha (PPARα) and peroxisome proliferator-activated receptor delta (PPARδ).

	SCD1	ACC	FAS	SREBP-1	INSIG-1	PPARα	PPARδ
SCD1	-	0.81**	0.72**	0.68**	0.86**	0.04	0.16#
ACC	0.81**	-	0.76**	0.65**	0.72**	0.05	0.12
FAS	0.72**	0.76**	-	0.49**	0.56**	0.08	0.16#
SREBP-1	0.68**	0.65**	0.49**	-	0.85**	0.10	0.20#
INSIG-1	0.86**	0.72**	0.56**	0.85**	-	0.08	0.18#
PPARα	0.04	0.05	0.08	0.10	0.08	-	0.30*
PPARδ	0.16#	0.12	0.16#	0.20#	0.18#	0.30*	-

= $P < 0.05$
* = $P < 0.01$
** = $P < 0.001$

Discussion

The aim of this study was to examine the effects of the SCFA Ac and BHBA as well as various LCFA on expression of several lipogenic genes and transcriptional regulators in bovine mammary epithelial cells. In addition, FA composition of the cells was analysed to investigate whether this was affected by the different treatments. The data on the FA composition of the cells demonstrate that the LCFA were taken up by the cells, rather than incomplete washing of the cells, since *cis*-9, *trans*-11 CLA was only detected in cells treated with C18:1 *trans*-11. Apparent desaturation of C18:1 *trans*-11 to *cis*-9, *trans*-11 CLA was 26.5% of total C18:1 *trans*-11 taken up by the cells. This is in close agreement with the 25.7% reported by Mosley et al. (2006) in dairy cows using ^{13}C labelled FA.

The upregulation of ACC and SCD1 by Ac indicates that Ac may increase *de novo* synthesis and desaturation of FA in bovine mammary epithelial cells. In contrast with our results, Yonezawa et al. (2004) found that addition of Ac decreased activity of ACC in primary cultured bovine mammary epithelial cells. This discrepancy could be due to temporal differences since Yonezawa et al. (2004) treated the cells for 7 d opposed to 12 h

Table 3. Fatty acid composition of total lipids extracted from MAC-T cells after treatment (12h) with either: 5 m*M* acetate (Ac), 5 m*M* β-hydroxybutyrate (BHBA), combination of Ac and BHBA (Ac+BHBA), or 100 µ*M* of the following LCFA: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 c9), *trans*-vaccenic acid (C18:1 t11), linoleic acid (C18:2) or linolenic acid (C18:3).

Fatty acid (g/100g FA)	Control	Treatments									SEM	*P*-value[1]
		Ac	BHBA	Ac + BHBA	C16:0	C18:0	C18:1 c9	C18:1 t11	C18:2	C18:3		
C16:0	19.45^{b}	19.55^{b}	20.29^{b}	19.62^{b}	23.41^{a}	16.81^{c}	16.54^{c}	16.03^{c}	16.05^{c}	15.93^{c}	0.26	<0.001
C16:1 c9	3.03^{bc}	2.76^{bcd}	3.18^{b}	2.55^{bcd}	4.95^{a}	2.49^{bcd}	2.33^{cd}	2.35^{cd}	2.07^{d}	2.41^{cd}	0.15	<0.001
C18:0	5.74^{bc}	5.92^{bc}	6.21^{b}	5.94^{bc}	5.50^{bc}	10.42^{a}	5.11^{cd}	4.58^{d}	5.68^{bc}	5.57^{bc}	0.18	<0.001
C18:1 c9	34.44^{b}	35.69^{b}	34.09^{b}	35.26^{b}	30.37^{c}	36.79^{b}	40.83^{a}	30.91^{c}	25.46^{d}	26.28^{d}	0.62	<0.001
C18:1 c11	3.46^{ab}	3.44^{ab}	3.64^{a}	3.38^{abc}	3.93^{a}	2.84^{cd}	3.00^{bcd}	2.97^{bcd}	2.48^{d}	2.46^{d}	0.12	<0.001
C18:1 t11	-[2]	-	-	-	-	-	-	8.12	-	-	-	-
CLA c9, t11	-	-	-	-	-	-	-	2.89	-	-	-	-
C18:2	18.94^{b}	17.17^{bc}	16.77^{bc}	18.28^{bc}	17.99^{bc}	17.26^{bc}	16.84^{bc}	14.78^{c}	32.54^{a}	17.08^{bc}	0.83	<0.001
C18:3	0.46^{b}	0.39^{b}	0.43^{b}	0.42^{b}	0.36^{b}	0.40^{b}	0.42^{b}	0.45^{b}	0.48^{b}	16.10^{a}	0.18	<0.001
Others[3]	11.83^{ab}	12.02^{ab}	12.16^{ab}	11.21^{ab}	10.29^{b}	10.11^{b}	12.14^{ab}	13.13^{a}	12.02^{ab}	10.77^{ab}	0.57	0.03
Unidentified	2.66	3.06	3.25	3.33	3.21	2.87	2.78	3.79	3.21	3.39	0.29	0.31
Δ9-desaturation indices[4]												
C16:1 c9	0.13^{b}	0.12^{b}	0.14^{b}	0.12^{b}	0.17^{a}	0.13^{b}	0.12^{b}	0.13^{b}	0.11^{b}	0.13^{b}	0.01	<0.001
C18:1 c9	0.86^{b}	0.86^{b}	0.85^{bc}	0.86^{b}	0.85^{bc}	0.78^{e}	0.89^{a}	0.87^{ab}	0.82^{d}	0.83^{cd}	0.01	<0.001

[a-f] Means within a row without a common superscript differ ($P < 0.05$).

Table 3. Continued.

[1] Effect of treatment.

[2] Not detected.

[3] Sum of minor fatty acids: ∑ (C14:0, C14:1 c9, C15:0, C16:1 t9, C16:1 c7/c8, C16:1 c11, C17:0, C17:1 c9, C17:1 c10, C18:1 t6-8, C18:1 t9, C18:1 t10, C18:1 t12, C18:1 c12, C18:1 c13, C18:1 c14/t16, C20:0, C20:1 c8, C20:1 c9, C20:1 c11, CLA t10c12, C20:2 (n-6), C20:3 (n-6), C20:3 (n-3), C20:4 (n-6), C20:5 (n-3), C22:0, C24:0, C24:1 c15, C22:4 (n-6), C22:5 (n-3), C22:6 (n-3)).

[4] Δ9-desaturase indices are calculated as: Δ9-desaturase product divided by the sum of the Δ9-desaturase product and substrate.

in our study. In addition, possible post-translational effects could result in differences between mRNA expression and activity of ACC. However, it has been shown that intraruminal infusion of either Ac or BHBA increases the amount of SCFA in the milk of dairy cows (Storry & Rook, 1965). This suggest that an increased availability of substrate, increases *de novo* fatty acid synthesis and/or decreases the incorporation of preformed LCFA in the bovine mammary gland. Although Ac upregulated ACC in our study, addition of Ac did not result in an increase of C16:0 in total lipids extracted from the cells. It has been speculated that mammary cells have a requirement for LCFA for lipid droplet formation, since upregulation of ACC and FAS, together with Ac availability, did not increase lipid droplet formation in MAC-T cells (Kadegowda et al., 2009a). In addition, the incubation of 12 h in our study, although appropriate for mRNA expression, might not have been sufficient to fully detect differences in FA composition of the cells. There was no significant effect of BHBA or the combination of Ac and BHBA on any of the genes measured, compared to the control treatment, although numerically these additions resulted in a similar response. This suggest that Ac has a more pronounced effect on lipogenic gene expression in bovine mammary epithelial cells compared to BHBA.

C16:0 has been shown to upregulate SCD1 expression in MAC-T cells (Kadegowda et al., 2009a). However, we did not find a significant effect of C16:0 treatment on SCD1 expression in MAC-T cells. This contradiction could be due to the fact that our MAC-T cells did not express PPARγ, since Kadegowda et al. (2009a) suggested that the effect of C16:0 on SCD1 expression was partly mediated through PPARγ. Addition of C18:0 to the MAC-T cells did also not affect SCD1 expression. This is in line with Kadegowda et al. (2009a) who also reported that C18:0 did not affect SCD1 expression in MAC-T cells. Moreover, Jayan & Herbein (2000) reported that SCD activity was not affected when C18:0 was added to MAC-T cells.

The main product of SCD is C18:1 *cis*-9 arising from desaturation of C18:0 (Ntambi, 1999). Addition of this product to the MAC-T cells resulted in down-regulation of SCD1. This suggests that C18:1 *cis*-9 inhibits SCD1 expression in a negative feedback loop. This is in agreement with Keating et al. (2006), who showed that the SCD promoter activity was

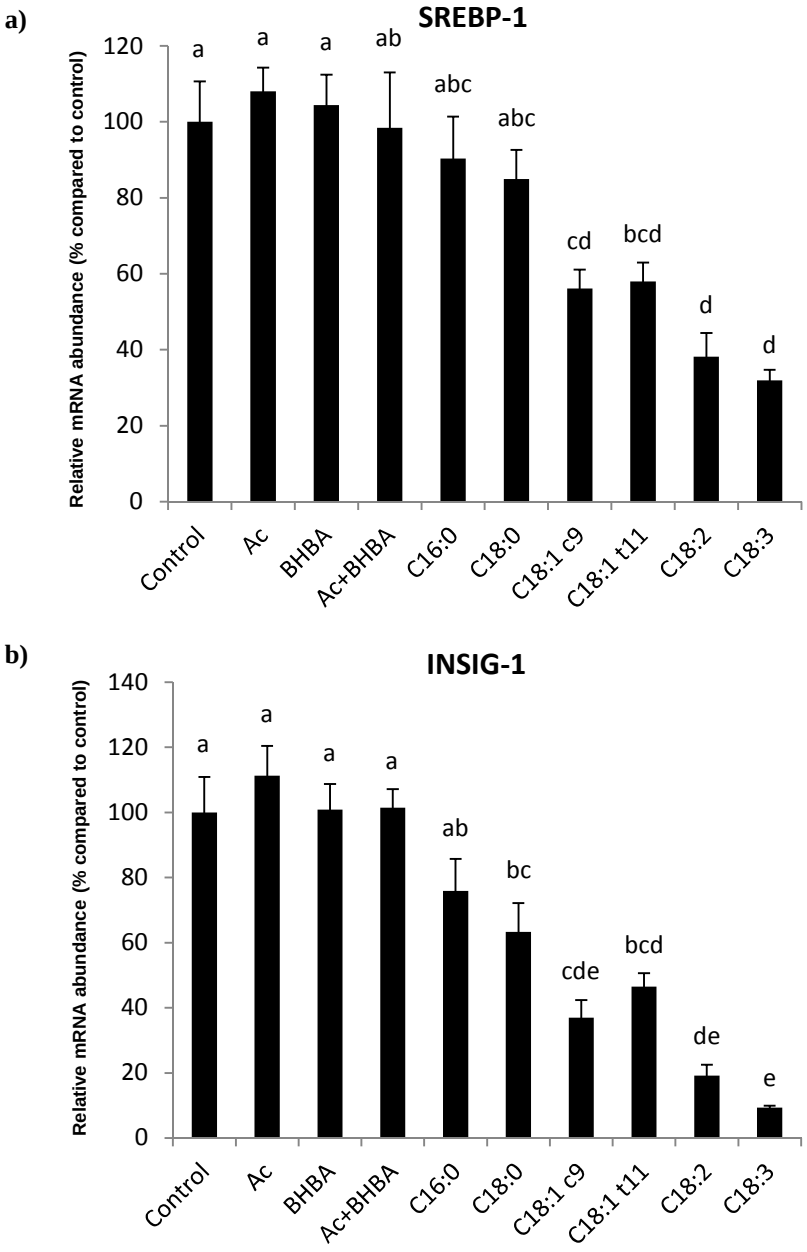

Figure 5. Relative mRNA abundance of SREBP-1 **(a)** and INSIG-1 **(b)** in MAC-T cells after treatment (12h) with either: 5 m*M* acetate (Ac), 5 m*M* β-hydroxybutyrate (BHBA), combination of Ac and BHBA (Ac+BHBA), or 100 µ*M* of the following LCFA: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 c9), *trans*-vaccenic acid (C18:1 t11), linoleic acid (C18:2) or linolenic acid (C18:3). Vertical lines represent the standard error of the mean, and treatments without a common letter differ ($P < 0.05$).

downregulated by C18:1 *cis*-9 in MAC-T cells. Similarly, it has been reported that C18:1 *cis*-9 inhibits both SCD1 expression (Kadegowda et al., 2009a) and SCD1 activity (Jayan & Herbein, 2000) in MAC-T cells. These results are in line with the role of SCD as key regulator of cell membrane fluidity as well as milk fat fluidity, by maintaining a rather constant C18:0 / C18:1 *cis*-9 ratio.

C18:1 *trans*-11 can be converted by SCD to *cis*-9, *trans*-11 CLA. When C18:1 *trans*-11 was added to the MAC-T cells, we found no significant effect on expression of SCD1. Correspondingly, it was shown that the promoter activity of SCD1 was not altered in MAC-T cells treated with C18:1 *trans*-11 (Keating et al., 2006). However, Jayan & Herbein (2000) reported that SCD activity was increased in MAC-T cells following the addition of C18:1 *trans*-11. This could suggest that C18:1 *trans*-11 has a direct effect on SCD activity without influencing the mRNA expression of SCD1.

It is well-known that n-3 and n-6 PUFA inhibit hepatic SCD1 expression in rodents (Ntambi, 1999). Also it was shown that C18:2 *cis*-9,12 decreases SCD1 expression in mammary gland of mice (Singh et al., 2004). In our study SCD1 expression was decreased with both the C18:2 *cis*-9,12 and C18:3 *cis*-9,12,15 treatments. These results show that the PUFAs C18:2 *cis*-9,12 and C18:3 *cis*-9,12,15 downregulate SCD1 expression in bovine mammary epithelial cells, similarly to rodents. Keating et al. (2006) found no differences in promoter activity of SCD1 when MAC-T cells were treated with C18:2 *cis*-9,12 or C18:3 *cis*-9,12,15, but they used a lower dose of FA (30 µ*M* versus 100 µ*M* in our study). We found recently that supplementing the diet of dairy cows with soybean oil (mainly C18:2 *cis*-9,12) decreased SCD1 expression in the mammary gland compared with rapeseed oil (mainly C18:1 *cis*-9) or linseed oil (mainly C18:3 *cis*-9,12,15) (Jacobs et al., 2011).

The C16 desaturase index was only affected by the C16:0 treatment, most likely due to the high addition of C16:0 to the cells. The C16 desaturase index was not related to the relative mRNA abundance of SCD1 (r^2 = 0.05; P = 0.28). However, we observed a significant relationship between the C18 desaturase index and relative SCD1 mRNA abundance (r^2 = 0.42; $P < 0.001$). This indicates that the C18 desaturase index is a better indicator of SCD activity compared to the C16 desaturase index, when calculated from total lipids extracted from bovine mammary epithelial cells. In biopsies of the bovine mammary gland, Jacobs et al. (2011) reported moderate correlations between SCD1 and C16 or C18 index (r^2 of 0.35 and 0.39, respectively), whereas Bionaz & Loor (2008b) and Invernizzi et al. (2010) concluded that desaturation indices were poor predictors of SCD activity.

Similarly to SCD1, ACC expression was downregulated with both C18:2 *cis*-9,12 and C18:3 *cis*-9,12,15 addition. FAS expression displayed a similar trend as ACC but FAS expression was only numerically lower with addition of C18:2 *cis*-9,12 or C18:3 *cis*-9,12,15 compared to control. In rodents it is known that PUFA supplementation inhibits expression of genes involved in de novo lipid synthesis, including ACC and FAS (Jump & Clarke, 1999).

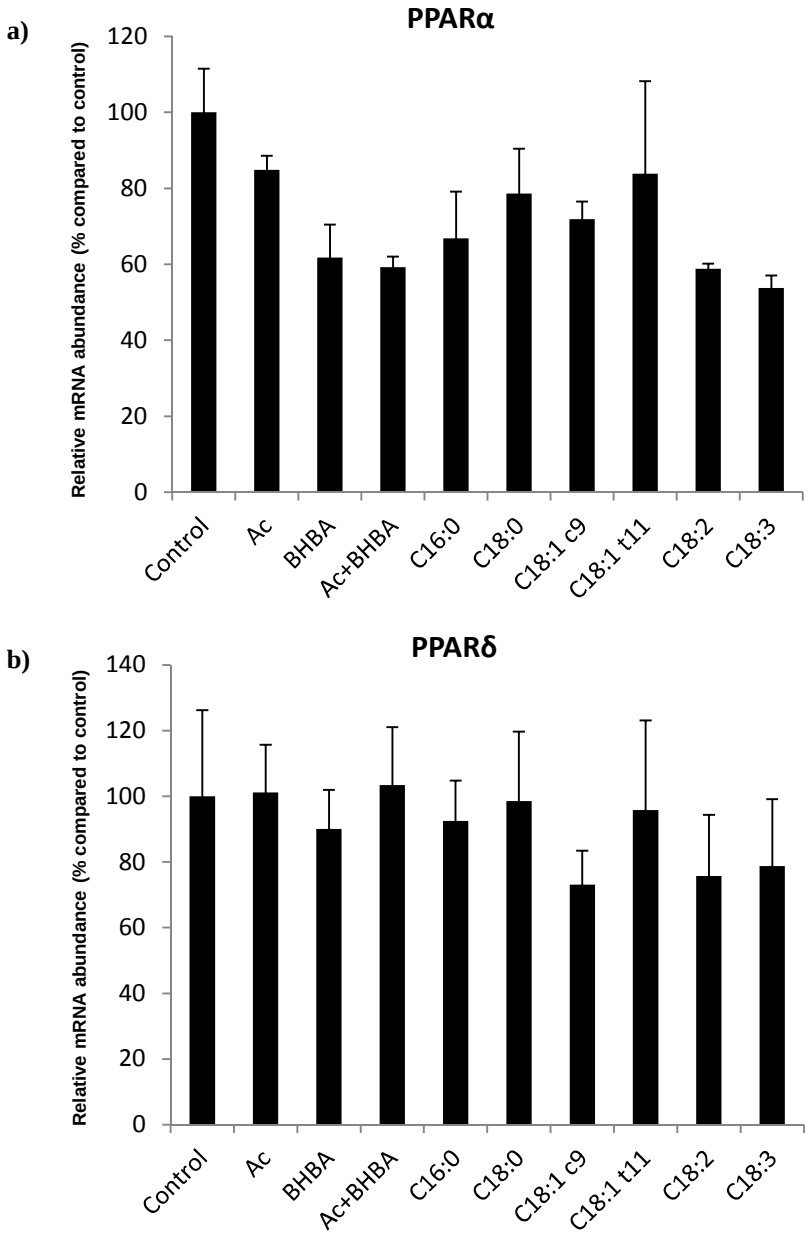

Figure 6. Relative mRNA abundance of PPARα **(a)** and PPARδ **(b)** in MAC-T cells after treatment (12h) with either: 5 m*M* acetate (Ac), 5 m*M* β-hydroxybutyrate (BHBA), combination of Ac and BHBA (Ac+BHBA), or 100 µ*M* of the following LCFA: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 c9), *trans*-vaccenic acid (C18:1 t11), linoleic acid (C18:2) or linolenic acid (C18:3). Vertical lines represent the standard error of the mean. No statistical differences ($P < 0.05$) between treatments in both PPARα and PPARδ expression were found.

The expression patterns across all treatments for ACC, FAS and SCD1 showed a quite similar trend. Furthermore, correlations between relative expression of the different lipogenic genes were rather high (range r^2: 0.72 - 0.81; $P < 0.001$), which supports the idea that these lipogenic genes are regulated by common transcription factors. The SREBP family consist of several transcription factors that act as master regulators of lipid and cholesterol metabolism by controlling the expression of a range of enzymes required for endogenous cholesterol, FA, triacylglycerol and phospholipid synthesis (Eberlé et al., 2004). It has been shown that *trans*-10, *cis*-12 CLA downregulates both SREBP-1 and INSIG-1 in MAC-T cells (Peterson et al., 2004) as well as in vivo in the bovine mammary gland (Harvatine & Bauman, 2006) providing strong support for SREBP-1 and INSIG-1 as a central signalling pathway regulating FA synthesis in the bovine mammary gland. In our study, SREBP-1 and INSIG-1 were expressed parallel to those of the lipogenic genes (SCD1, ACC and FAS) and correlations between both SREBP-1 and INSIG-1 and the lipogenic genes were moderate to high (range r^2: 0.49 - 0.86; $P < 0.001$). This indicates that effect of FA on expression of SCD1, ACC and FAS in our study was, at least partly, mediated through SREBP-1 and INSIG-1. This strengthens the support for the role of SREBP-1 and INSIG-1 as central regulators of lipogenesis in the bovine mammary gland.

In our study, PPARα and PPARδ were not affected by the treatments whereas PPARγ was not expressed by the MAC-T cells. It has been suggested that PPARγ activation regulates milk fat synthesis in bovine mammary epithelial cells (Kadegowda et al., 2009a). In our study, the addition of PUFA to the cells resulted in downregulation of ACC and SCD1 as expected, despite the fact that PPARγ was not expressed. This suggest that PPARγ expression is not a prerequisite for the inhibitory effect of PUFA on ACC and SCD1 expression.

Conclusions

This study showed that Ac upregulates both SCD1 and ACC expression in bovine mammary epithelial cells, which indicates that Ac may increase *de novo* synthesis and desaturation of FA in the bovine mammary gland. In addition, both the PUFAs C18:2 *cis*-9,12 and C18:3 *cis*-9,12,15 downregulate SCD1 and ACC expression. The effects of FA on the expression of the lipogenic genes SCD1, ACC and FAS appears to be, at least partly, regulated by the transcription factor SREBP-1 and INSIG-1, which strengthens the support for the role of SREBP-1 and INSIG-1 as central regulators of lipogenesis in the bovine mammary gland.

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Chapter 6

General Discussion

General Discussion

Increasing the proportion of unsaturated fatty acids (UFA) in milk is believed to be beneficial in terms of human health, thereby increasing the nutritional quality of milk. In the Netherlands however, the content of even-chain, saturated fatty acids (SFA) in milk fat of raw bovine milk was higher in 2005 compared with 1992, which was related to changes in the composition of diets fed to dairy cows (Heck et al., 2009). The proportion of UFA in milk is mainly dependent on the proportion of UFA in the diet, the degree of biohydrogenation in the rumen, and on the activity of stearoyl-CoA desaturase (SCD) in the mammary gland. This thesis focuses on SCD in the mammary gland of dairy cows, and how SCD can be influenced by nutrition. More specifically, the effect of short- and long-chain fatty acids (FA) on mRNA expression of SCD was investigated in the bovine mammary gland. In addition, a non-invasive alternative to mammary tissue for measuring SCD expression in the mammary gland of dairy cows was examined.

Supplementing the diet of dairy cows with soybean oil (high in C18:2 *cis*-9,12) reduced stearoyl-CoA desaturase 1 (SCD1) expression in the mammary gland compared to rapeseed oil (high in C18:1 *cis*-9) or linseed oil (high in C18:3 *cis*-9,12,15), whereas stearoyl-CoA desaturase 5 (SCD5) expression was much smaller than SCD1 expression and did not differ between treatments (**Chapter 2**). Mammary tissue of cows in this study were also used for micro-array analysis to investigate the changes in genome-wide expression of genes in response to dietary UFA supplementation (**Chapter 3**). This analysis showed that supplementing the diet of dairy cows with UFA results in predominant downregulation of gene sets related to cell development and remodelling, apoptosis, nutrient metabolic process and immune system response in the mammary gland. A non-invasive, alternative source of mRNA was investigated (**Chapter 4**) in order to allow more routine evaluation of nutritional effects on mammary SCD expression. Results showed that using milk somatic cells as a source of mRNA to examine SCD1 expression in dairy cows, yields results comparable with mammary tissue and therefore, milk somatic cells can provide a non-invasive alternative to mammary tissue samples obtained by biopsy to study effects on SCD1 expression. To further examine the effects of individual FA, in particular short-chain fatty acids (SCFA) on SCD expression, a bovine mammary epithelial cell line (MAC-T) was used (**Chapter 5**). Results of this study revealed that acetate (Ac) upregulates expression of both acetyl-CoA carboxylase (ACC) and SCD1 in MAC-T cells, indicating that Ac may increase *de novo* synthesis and desaturation of FA in the bovine mammary gland. Furthermore, expression of both sterol regulatory binding protein 1 (SREBP-1) and insulin-induced gene 1 (INSIG-1) was significantly related to the expression of the lipogenic genes, supporting the role of SREBP-1 and INSIG-1 as central regulators of lipogenesis in the bovine mammary gland. In this general discussion, SCD1 and its regulation, the role of SCD5 compared with SCD1, the use of desaturation indices as proxies of SCD activity, and non-nutritional factors affecting SCD activity are discussed.

Stearoyl-CoA Desaturase 1

Until recently it was thought that only one isoform of SCD (i.e. SCD1) was present in bovine species and is therefore commonly referred to as just simply SCD. In 2007, Lengi & Corl (2007) identified a novel SCD isoform in bovine, designated SCD5 (see paragraph 6.3) Consequently, most of the research and available literature on SCD in dairy cows involves the SCD1 isoform. In lactating ruminants, the highest activity of SCD is found within the mammary gland (McDonald & Kinsella, 1972). During lactation, SCD1 is markedly upregulated and SCD1 is one of the most abundantly expressed genes in the lactating mammary gland (Bionaz & Loor, 2008b). Furthermore, it has been proposed that SCD plays a central role in milk fat synthesis by providing endogenous C18:1 *cis*-9 for mammary triacylglyceride synthesis (Bionaz & Loor, 2008b). Based on arterio-venous differences in the SCD products and substrates, desaturation of C18:0 to C18:1 *cis*-9 in dairy cows was estimated to be 52% (Enjalbert et al., 1998). Using ^{13}C-labelled FA, Mosley & McGuire (2007) reported that desaturation of C14:0, C16:0 and C18:0 in the mammary gland of dairy cows was estimated to be 7.0%, 2.5% and 48.7% respectively, resulting in 92%, 56% and 43% of C14:1 *cis*-9, C16:1 *cis*-9 and C18:1 *cis*-9 in milk originating from substrate desaturation. In addition, it has been shown that desaturation of C18:1 *trans*-11 is the major source of *cis*-9, *trans*-11 CLA in milk of dairy cows. Studies using sterculic oil to inhibit SCD activity have shown that 78 to 91% of total *cis*-9, *trans*-11 CLA in milk originates from desaturation of C18: *trans*-11 by SCD (Corl et al., 2001; Kay et al., 2004). From the study reported in Chapter 5, it was calculated that 26.5% of the C18:1 *trans*-11 taken up by the mammary epithelial cells (MAC-T) was desaturated to *cis*-9, *trans*-11 CLA. This is in close agreement with the 25.7% reported by Mosley et al. (2006) in dairy cows using ^{13}C labelled FA.

Regulation of Stearoyl-CoA Desaturase 1

Knowledge about the nutritional regulation of bovine SCD is limited compared to rodents. The nuclear transcription factor SREBP-1 is known to be an important regulator of SCD in rodents (Bernard et al., 2008). The SREBP family consists of several transcription factors that act as master regulators of lipid and cholesterol metabolism by controlling the expression of a range of enzymes required for endogenous cholesterol, FA, triacylglycerol and phospholipid synthesis (Eberlé et al., 2004). Results from the study reported in Chapter 5 showed that the expression of the lipogenic genes ACC, FAS and SCD1 was significantly correlated with the expression of SREBP-1 as well as INSIG-1 in bovine mammary epithelial cells (Figure 1). This indicates that the expression of these lipogenic genes are, at least partially, coordinated through the SREBP-1 mechanism. Similar results have been presented by other *in vitro* studies investigating lipogenic gene expression in bovine mammary epithelial cells. Peterson et al. (2004) reported that supplementing *trans*-10, *cis*-12 CLA to bovine mammary epithelial cells (MAC-T), downregulated ACC, FAS and SCD1 expression and this coincided with a reduction in proteolytic activation of SREBP-1. In addition, SREBP-1 expression was downregulated by *trans*-10, *cis*-12 CLA in

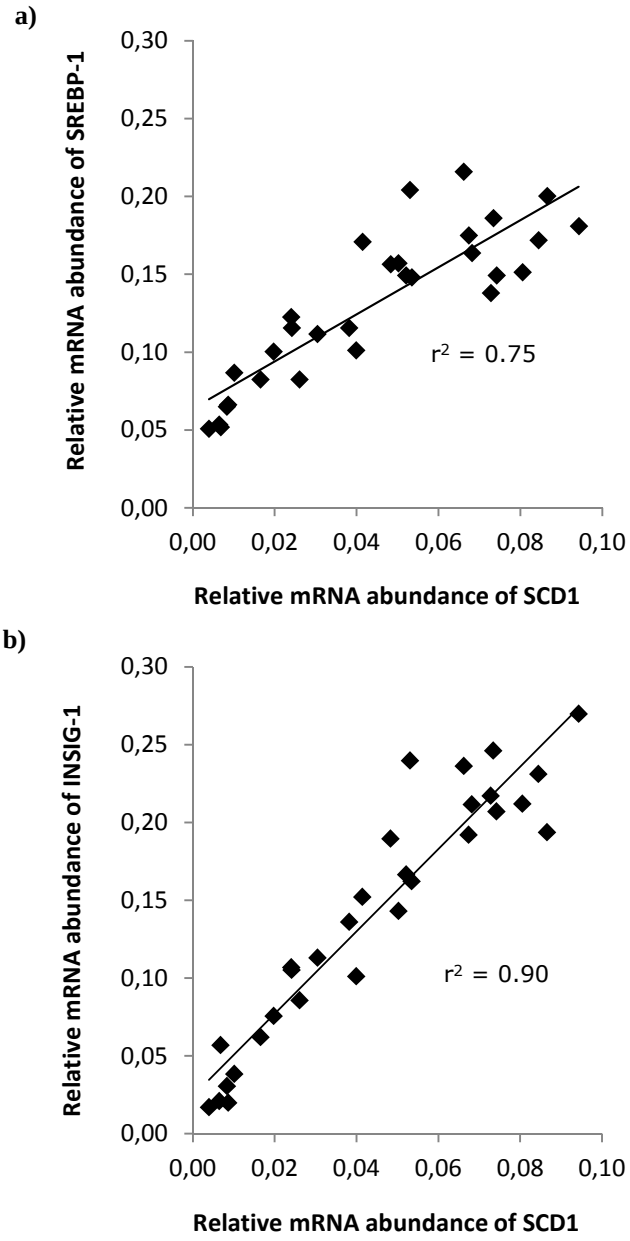

Figure 1. Relationship between relative mRNA abundance of SCD1 and SREBP-1 **(a)** as well as SCD1 and INSIG-1 **(b)** in MAC-T cells treated with various FA (n = 30; Chapter 5). SREBP-1 expression = 0.064 (±0.009) + 1.51 (±0.163) x SCD1 expression; $r^2 = 0.75$, $P < 0.001$. INSIG-1 expression = 0.024 (±0.009) + 2.64 (±0.171) x SCD1 expression; $r^2 = 0.90$, $P < 0.001$.

MAC-T cells parallel to the downregulation of lipogenic genes, including ACC, FAS and SCD1 (Kadegowda et al., 2009a). These results are supported by the fact that the promoter of SCD1 contains a region designated as SCD transcriptional enhancer element (STE) to which SREBP is predicted to bind (Keating et al., 2006).

Micro-array analysis of mammary tissue in the study reported in Chapter 3 showed that UFA supplementation downregulated expression of numerous genes involved in lipid metabolism, including ACC, which concurred with reduction of SREBP-1 as well as INSIG-1 expression. This is analogous with the *in vitro* results of Chapter 5 where C18:2 *cis*-9,12 and C18:3 *cis*,9,12,15 caused a reduction in ACC and SCD1 expression coupled with a downregulation of SREPB-1 and INSIG-1. These results are also in line with other studies examining expression of genes involved in lipid metabolism in the bovine mammary gland. It has been shown that either a low forage/high concentrate diet or abomasal infusion of *trans*-10, *cis*-12 CLA caused an inhibition of lipogenic gene expression, including ACC and FAS, which coincided with a downregulation of SREBP-1 as well as INSIG-1 in the mammary gland of dairy cows (Harvatine & Bauman, 2006; Gervais et al., 2009). Overall, the results from Chapter 3 and 5 strengthen the support for the central role of SREBP-1 in the regulation of lipogenesis in the bovine mammary gland.

The peroxisome proliferator-activated receptor (PPAR) family consists of three subtypes (PPARα, PPARδ and PPARγ) which are nuclear receptors that function as ligand-activated transcription factors regulating the expression of genes involved in metabolism, cellular differentiation and development (Michalik et al., 2006). In rodents, the PPARγ agonist rosiglitazone upregulates SCD1 (Way et al., 2001), indicating that SCD1 is a target of PPARγ. Correspondingly, Kadegowda et al. (2009a) reported that several lipogenic genes, including ACC, FAS, DGAT1, SREBP-1 and INSIG-1, are putative PPARγ target genes in bovine mammary epithelial cells. In addition, it has been shown that PPARγ and its target genes were markedly upregulated in mammary tissue of dairy cows during onset of lactation, suggesting a role of this nuclear receptor in milk fat synthesis (Bionaz & Loor, 2008b). Neither PPARα or PPARδ expression was affected by FA treatments in MAC-T cells (Chapter 5). Remarkably, PPARγ was not expressed by the MAC-T cells used. Nevertheless, the addition of the poly unsaturated fatty acid (PUFA) C18:2 *cis*-9,12 or C18:3 *cis*-9,12,15 to the MAC-T cells resulted in downregulation of ACC and SCD1 as expected, indicating that PPARγ expression is not a prerequisite for the inhibitory effect of PUFA on ACC and SCD1 expression in bovine mammary epithelial cells.

Stearoyl-CoA Desaturase 5

Recently, a novel bovine isoform of SCD was identified, designated SCD5, which appears to be primarily expressed in brain (Lengi & Corl, 2007). This new bovine desaturase gene seems to be an ortholog of the recently described human SCD5 gene, rather than a homolog of bovine SCD1 or any of the described murine SCD isoforms (Lengi & Corl, 2007). One of the reasons for the presence of multiple isoforms of SCD might be tissue-specific expression and/or substrate preference of the different isoforms. Although

SCD5 appears to be predominantly expressed in brain and pancreas (Wang et al., 2005; Lengi & Corl, 2007), it is also expressed in bovine mammary tissue (Gervais et al., 2009). In contrast to SCD1, mammary expression of SCD5 was not affected by supplementing the diet of dairy cows with various plant oils (Chapter 2). In addition, mRNA abundance of SCD5 in the mammary gland was much lower ($<10^3$) than that of SCD1. Similarly, in the study reported in Chapter 4, mRNA abundance of SCD5 was substantially lower than that of SCD1, indicating that SCD5 is less imperative regarding Δ9-desaturation of FA compared with SCD1. This is supported by the fact that the desaturation indices calculated from milk FA, which are frequently used to estimate mammary SCD activity, showed no relationship with relative mRNA abundance of SCD5 (e.g., C14 index: $r^2 = 0.02$; $P = 0.57$), while there was a significant relationship with SCD1 ($r^2 = 0.35$; $P = 0.002$; Chapter 2). However, SCD5 lacks N-terminal PEST sequences typically found in SCD1 (Lengi & Corl, 2007), which are considered to be a signal for protein degradation (Reichsteiner & Rogers, 1996), suggesting that protein stability is higher for SCD5 compared with SCD1. The results reported in Chapter 3 indicate that expression of SCD5 is less sensitive to changes in FA supply compared with SCD1 and that regulation of SCD5 differs from that of SCD1. One other study investigated SCD5 expression in the bovine mammary gland (Gervais et al., 2009), showing that intravenous infusion of *trans*-10, *cis*-12 CLA tended to reduce SCD1 expression in the mammary gland, whereas SCD5 expression was not affected. This again indicates that regulation of SCD5 differs from that of SCD1. Nevertheless, further research is necessary to determine the significance of SCD5 regarding Δ9-desaturation of FA in the bovine mammary gland.

Desaturase Indices

Several ratios of milk FA, referred to as desaturase indices, are frequently used as proxies to estimate SCD activity within the mammary gland. These desaturase indices are calculated as the ratio between the product and the sum of the product and substrate FA, e.g. C14:1 *cis*-9 / (C14:1 *cis*-9 + C14:0). Various desaturase indices are used, though the C14 index is considered the best indicator of SCD activity, since virtually all C14:0 and C14:1 *cis*-9 originate from *de novo* FA synthesis within the mammary gland (Lock & Garnsworthy, 2003; Bernard et al., 2008). In Chapter 2, the C14 desaturase index was positively, although not strongly, related to mRNA levels of SCD1 ($r^2 = 0.35$; $P = 0.002$), indicating that this desaturase index can be used to estimate SCD activity in the mammary gland. Furthermore, the relationships between the different desaturase indices were high (range r^2: 0.66 – 0.87; $P < 0.001$; Figure 2). Two observations were somewhat higher compared to the other data points and when these values were removed from the analysis, the r^2 changed from 0.87 and 0.75 to 0.75 and 0.63 for the C14 index compared with the C16 index or the C18 index, respectively. Correspondingly, a similar positive relationship was found between the C14 desaturase index and expression of SCD1 in mammary tissue ($r^2 = 0.34$; $P = 0.08$) as well as milk somatic cells ($r^2 = 0.52$; $P = 0.02$) in Chapter 3.

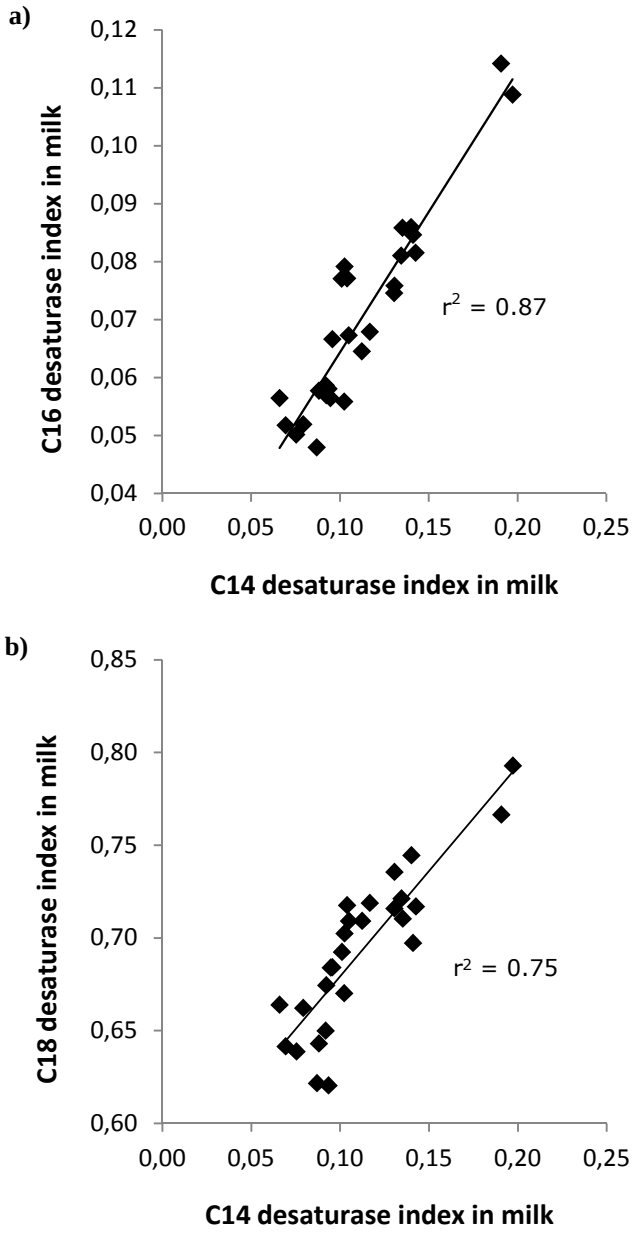

Figure 2. Relationship between the C14 and C16 desaturase index **(a)** as well as the C14 and C18 desaturase index **(b)** (n = 28; Chapter 2). C16 index = 0.016 (±0.004) + 0.48 (±0.038) x C14 index; r^2 = 0.87, P < 0.001. C18 index = 0.57 (±0.015) + 1.14 (±0.13) x C14 index; r^2 = 0.75, P < 0.001.

Other studies also found moderate relationships between desaturase indices and mRNA levels of SCD1 in dairy cows (Feng et al., 2007) and goats (Bernard et al., 2005a).

Overall these results indicate that desaturase indices can be used as a convenient tool to estimate SCD activity within the mammary gland. However, it has been reported that desaturase indices do not always reflect actual SCD activity in bovine adipose tissue (Archibeque et al., 2005) or in the bovine mammary gland (Bionaz & Loor, 2008b; Invernizzi et al., 2010). Particularly, when the entire lactation cycle is considered, desaturase indices appear to be poorly associated with SCD1 mRNA levels in the mammary gland (Bionaz & Loor, 2008b). The lack of correlation between the various desaturase indices in the latter study, especially in the beginning of lactation, indicates that additional factors influence the amount of milk FA used for the calculation of these indices. Possible factors include the varying contribution of mobilized FA from body reserves, selective uptake of FA by the mammary gland, and varying contribution of *de novo* synthesised FA to total milk FA. In addition, substrate preference of SCD1 and selective use of FA for milk triglyceride synthesis could influence the various desaturase indices as well.

Milk Fat Depression and SCD

Assessing the effect of dietary FA on mammary gene expression in ruminants is complex since microbes in the rumen can alter the dietary FA. This so-called biohydrogenation results in saturation of FA, i.e. removal of double bonds, and in isomerization of the double bonds, i.e. shift from *cis* to *trans* configuration. Fatty acid analysis of blood plasma as well as milk in the study reported in Chapter 2, revealed that dietary oil supplementation most likely increased the amount of *trans* FA produced in the rumen. It has been shown that *trans* FA can affect expression of lipogenic genes, including SCD1, in the mammary gland. In particular *trans*-10, *cis*-12 CLA has been identified as one of the key ruminal biohydrogenation intermediates responsible for the inhibition of milk fat synthesis, often referred to as milk fat depression (MFD) (Shingfield & Griinari, 2007). Indeed, *trans*-10, *cis*-12 CLA in milk was positively correlated with milk fat % in Chapter 2 (Figure 3). Diets that cause MFD include highly fermentable diets (low forage / high concentrate) and diets supplemented with plant or fish oil (high PUFA; Bauman & Griinari, 2003). Feeding cows diets that cause MFD has been shown to inhibit expression of key lipogenic genes, including SCD1, in the mammary gland (Peterson et al., 2003; Harvatine & Bauman, 2006). In addition, abomasal infusion of *trans*-10, *cis*-12 CLA also inhibits lipogenic gene expression, including SCD1 (Baumgard et al., 2002; Harvatine & Bauman, 2006).

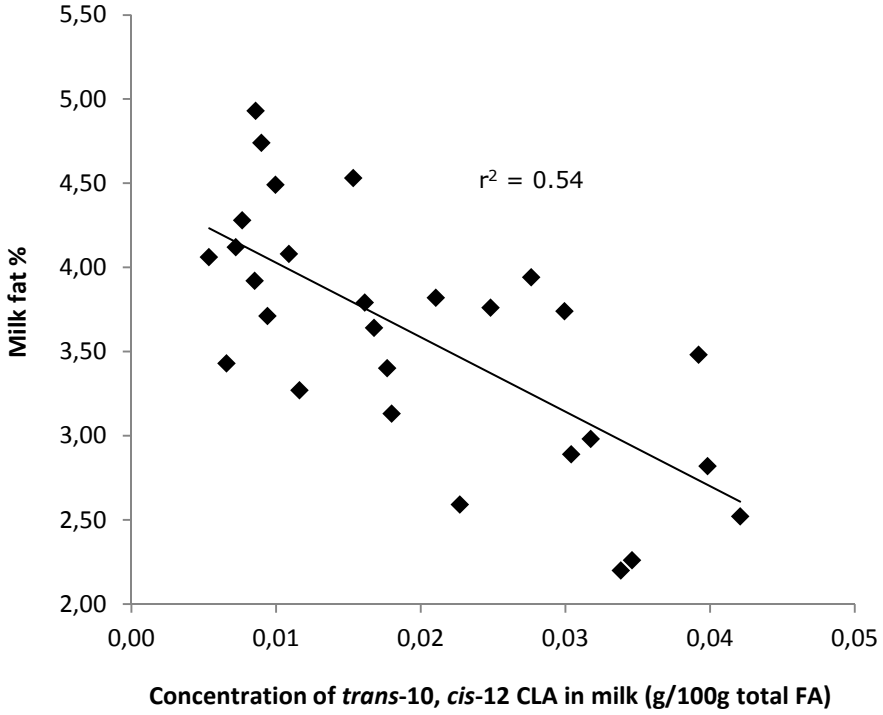

Figure 3. Relationship between concentration of *trans*-10, *cis*-12 CLA in milk and milk fat % (n = 28; Chapter 2). Milk fat % = 4.48 (±0.20) + -44.4 (±8.64) x t10c12 CLA; $r^2 = 0.54$, $P < 0.001$.

Milk Fat Fluidity and SCD

Milk fat fluidity is an important prerequisite for the secretion of milk by the mammary gland. Since the milk fat globule needs to be liquid, most of the FA have to be esterified to triacylglycerols in combinations that have a melting point at or below 39°C, the body temperature of the cow (Timmen & Patton, 1988). Since both C16:0 and C18:0 are solid at body temperature, there is a physiological need to convert a portion of each to C16:1 *cis*-9 and C18:1 *cis*-9, respectively, that are liquid (Garnsworthy et al., 2010). It is believed that incorporating FA with a relative low melting point, i.e. C18:1 *cis*-9 or C4:0 to C10:0, in the final step of triglyceride synthesis is the main mechanism by which milk fluidity is regulated (Timmen & Patton, 1988). Since SCD1 controls the synthesis of C18:1 *cis*-9, SCD1 is thought to be essential in regulating milk fluidity. Dietary supplementation of fish oil or marine algae rich in C20:5n-3 or C22:6n-3 results in MFD and a pronounced decrease in C18:0 supply to the mammary gland since especially the last step in the biohydrogenation process in the rumen is inhibited and *trans*-10 rather than *trans*-11 intermediates are formed (Boeckaert et al., 2008; Sterk et al., 2010). It has been suggested that this decreased amount of C18:0 available for conversion to C18:1 *cis*-9 by SCD1 could

reduce the ability of the mammary gland to maintain the required milk fluidity and therefore inhibit milk fat synthesis (Loor et al., 2005; Gama et al., 2008). However, abomasal infusion of sterculic oil, which is a source of cyclopropene FA that strongly inhibit the activity of the SCD1 enzyme, does not inhibit milk fat synthesis despite severely reduced SCD1 activity in the mammary gland (Griinari et al., 2000; Corl et al., 2001). This indicates that the mammary gland has a remarkable ability to maintain milk fat secretion over a substantial range in FA profile (Harvatine et al., 2009).

Other Factors Influencing SCD Activity

Several studies have demonstrated a substantial variation in desaturase indices in milk of dairy cows on a similar diet (Lock & Garnsworthy 2002; Kelsey et al., 2003; Lock & Garnsworthy 2003). Moreover, when switched between diets, the ranking of cows for desaturase indices remains consistent (Peterson et al., 2002b; Lock & Garnsworthy 2003), suggesting that genetic variation in SCD activity could play a role. Several studies used desaturase indices to estimate heritability of SCD in dairy cows. Moderate heritabilities for the C14 desaturase index were reported by Soyeurt et al. (2008; $h^2 = 0.20$), Mele et al. (2009; $h^2 = 0.27$), Stoop et al. (2009; $h^2 = 0.45$) and Garnsworthy et al. (2010; $h^2 = 0.38$). In addition to genetic variation within breed, variation in desaturase indices between different breeds has also been reported (Morales et al., 2000; Kelsey et al., 2003; Soyeurt et al., 2006), suggesting genetic variation between breeds. However, the variation in heritability of SCD between the different studies is rather high, which might be partly related to differences in phenotypic variation between studies (i.e., low phenotypic variation related to high heritability and vice versa). For example, the highest heritability of SCD was obtained in a study in which all animals were heifers and in which all animals received a winter ration based on silage in February or March. This could mean that in practice (i.e., large phenotypic variation), heritability of SCD activity might be relatively low. Negative genetic correlations between desaturation indices and milk fat percentage have been reported (Schennink et al., 2008; Soyeurt et al., 2008), indicating that genetic selection on desaturation indices could have negative effects on milk fat percentage. In addition, it was shown that the SCD1 polymorphism A293V has a significant effect on individual desaturation indices, but not on the overall desaturation index, suggesting an effect of this polymorphism on substrate specificity of SCD (Schennink et al., 2008).

Seasonal variation in desaturation indices has also been reported (Lock & Garnsworthy, 2003; Heck et al., 2009). In the study of Heck et al. (2009), all desaturation indices showed the lowest values in spring (March - June) and the highest values in autumn (October), indicating lower SCD activity in spring compared with autumn. However, these data are not in line with results of Lock & Garnsworthy (2003), who showed that desaturation indices were highest in May/June (C14 index) and July (C16 and CLA index). It seems that differences in diet throughout the season (i.e., fresh grass in summer, more silage and concentrate in winter) affect the desaturation indices by changes in the supply of FA to the

mammary gland. Therefore, differences in feeding strategies between the two studies could play a role in the contradictory results of apparent SCD activity throughout the season.

The effect of production variables (e.g., stage of lactation, milk yield and milk fat content) on desaturation indices (i.e., apparent SCD activity) has been examined (Kelsey et al., 2003; Lock et al., 2005). Kelsey et al. (2003) reported that *cis*-9, *trans*-11 CLA content in milk as well as the CLA index varied over 3-fold among individual cows on the same diet. Small or no relationships between the CLA index and parity or days in milk were observed, indicating little effect of these variables on SCD activity and suggesting genetic variation in rumen outflow of C18:1 *trans*-11 and/or tissue SCD activity. In addition, *cis*-9, *trans*-11 CLA content in milk fat and the CLA desaturase index were essentially independent of milk yield, milk fat percentage, and milk fat yield (Kelsey et al., 2003). These results are in agreement with Lock et al. (2005) who found that under normal conditions, the *cis*-9, *trans*-11 CLA content of milk and SCD activity in the mammary gland are independent of stage of lactation, milk yield, milk fat content, and milk fat yield.

Implications and Future Research

The main objective of the research described in this thesis was to investigate the effect of FA on SCD expression in the mammary gland of dairy cows. The purpose of this investigation was to find nutritional strategies that increase mammary SCD activity, thereby increasing MUFA as well as *cis*-9, *trans*-11 CLA content in milk, which would improve the nutritional quality of milk. In addition, a non-invasive alternative to biopsy for measuring SCD expression in the mammary gland of dairy cows was examined.

Overall, it can be concluded that saturated LCFA have little or no effect on SCD1 expression in the bovine mammary gland, whereas unsaturated LCFA inhibit mammary SCD1 expression. The inhibitory effect of unsaturated LCFA on mammary SCD1 expression appears to increase proportionally with the amount of double bonds in the LCFA (i.e., more double bonds results in higher inhibition of SCD1 expression). Therefore, it seems difficult to enhance SCD1 expression in the mammary gland by supply of LCFA. In order to limit inhibition of mammary SCD1 expression, supply of PUFA to the mammary gland should be restricted. The regulation of SCD1 in the bovine mammary gland by LCFA appears to be, at least partly, regulated by the transcription factors SREBP-1 and INSIG-1. Further research is needed to elucidate the role of these transcription factors and to identify possible additional genes that are involved in the regulation of SCD1 in the bovine mammary gland. Understanding the regulation of SCD1 in the bovine mammary gland could facilitate the search for nutritional strategies that could increase the activity of SCD in the mammary gland. Further research on the effect of SCFA on mammary SCD expression is needed to verify if SCFA can induce SCD1 expression in the bovine mammary gland. The recently discovered isoform SCD5 is expressed in bovine mammary tissue, although contribution to Δ9-desaturation of FA appears to be quite low. Additional research is required to elucidate the role of SCD5 in the mammary gland and examine its contribution to Δ9-desaturation of FA. Milk somatic cells can be used as a source of mRNA

to examine SCD1 expression in dairy cows, as a non-invasive alternative to mammary tissue samples obtained by biopsy. However, the rather low yield of RNA from milk somatic cells requires further research for improvement.

References

Abd El-Rehim, D. M., S. E. Pinder, C. E. Paish, J. Bell, R. W. Blamey, J. F. Robertson, R. I. Nicholson, and I. O. Ellis. 2004. Expression of luminal and basal cytokeratins in human breast carcinoma. J. Pathol. 203: 661-671.

Abrahamse, P. A., J. Dijkstra, B. Vlaeminck, and S. Tamminga. 2008a. Frequent allocation of rotationally grazed dairy cows changes grazing behavior and improves productivity. J. Dairy Sci. 91: 2033-2045.

Abrahamse, P. A., B. Vlaeminck, S. Tamminga, and J. Dijkstra. 2008b. The effect of silage and concentrate type on intake behavior, rumen function, and milk production in dairy cows in early and late lactation. J. Dairy Sci. 91: 4778-4792.

Ahnadi, C. E., N. Beswick, L. Delbecchi, J. J. Kennelly, and P. Lacasse. 2002. Addition of fish oil to diets for dairy cows. II: Effects on milk fat and gene expression of mammary lipogenic enzymes. J. Dairy Res. 69: 521-531.

Alcorn, J., X. Lu, J. A. Moscow, and P. J. McNamara. 2002. Transporter gene expression in lactating and non-lactating human mammary epithelial cells using real-time reverse transcription-polymerase chain reaction. J. Pharmacol. Exp. Ther. 303: 487-496.

Archibeque, S. L., D. K. Lunt, C. D. Gilbert, R. K. Tume, and S. B. Smith. 2005. Fatty acid indices of stearoyl-CoA desaturase do not reflect actual stearoyl-CoA desaturase enzyme activities in adipose tissues of beef steers finished with corn-, flaxseed-, or sorghum-based diets. J. Anim. Sci. 83: 1153-1166.

Astrup, A., J. Dyerberg, P. Elwood, K. Hermansen, F. B. Hu, M. Uhre Jakobsen, F. J. Kok, R. M. Krauss, J. M. Lecerf, P. Legrand, P. Nestel, U. Risérus, T. Sanders, A. Sinclair, S. Stender, T. Tholstrup, and W. C. Willett. 2011. The role of reducing intakes of saturated fat in the prevention of cardiovascular disease: where does the evidence stand in 2010? Am. J. Clin. Nutr. 93: 684-688.

Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Annu. Rev. Nutr. 23: 203-227.

Bauman, D. E., I. H. Mather, R. J. Wall, and A. L. Lock. 2006. Major advances associated with the biosynthesis of milk. J. Dairy Sci. 89: 1235-1243.

Bauman, D. E., J. W. Perfield, 2nd, K. J. Harvatine, and L. H. Baumgard. 2008. Regulation of fat synthesis by conjugated linoleic acid: Lactation and the ruminant model. J. Nutr. 138: 403-409.

Bauman, D. E., K. J. Harvatine, and A. L. Lock. 2011. Nutrigenomics, rumen-derived bioactive fatty acids, and the regulation of milk fat synthesis. Ann. Rev. Nutr. 31: 299-319.

Baumgard, L. H., J. K. Sangster, and D. E. Bauman. 2001. Milk fat synthesis in dairy cows is progressively reduced by increasing supplemental amounts of *trans*-10, *cis*-12 conjugated linoleic acid (CLA). J. Nutr. 131: 1764-1769.

Baumgard, L. H., E. Matitashvili, B. A. Corl, D. A. Dwyer, D. E. Bauman. 2002. *Trans*-10, *cis*-12 conjugated linoleic acid decreases lipogenic rates and expression of genes involved in milk lipid synthesis in dairy cows. J. Dairy Sci. 85: 2155-2163.

Bernard, L., J. Rouel, C. Leroux, A. Ferlay, Y. Faulconnier, P. Legrand, and Y. Chilliard. 2005a. Mammary lipid metabolism and milk fatty acid secretion in alpine goats fed vegetable lipids. J. Dairy Sci. 88: 1478-1489.

Bernard, L., C. Leroux, M. Bonnet, J. Rouel, P. Martin, and Y. Chilliard. 2005b. Expression and nutritional regulation of lipogenic genes in mammary gland and adipose tissues of lactating goats. J. Dairy Res. 72: 250-255.

Bernard, L., C. Leroux, and Y. Chilliard. 2008. Expression and nutritional regulation of lipogenic genes in the ruminant lactating mammary gland. Adv. Exp. Med. Biol. 606: 67-108.

Bhattacharya, A., J. Banu, M. Rahman, J. Causey, and G. Fernandes. 2006. Biological effects of conjugated linoleic acids in health and disease. J. Nutr. Biochem. 17: 789-810.

Bionaz, M., and J. J. Loor. 2008a. Acsl1, agpat6, fabp3, lpin1, and slc27a6 are the most abundant isoforms in bovine mammary tissue and their expression is affected by stage of lactation. J. Nutr. 138: 1019-1024.

Bionaz, M., and J. J. Loor. 2008b. Gene networks driving bovine milk fat synthesis during the lactation cycle. BMC Genomics 9: 366.

Boeckaert, C., B. Vlaeminck, J. Dijkstra, A. Issa-Zacharia, T. van Nespen, W. van Straalen, and V. Fievez. 2008. Effect of dietary starch or micro algae supplementation on rumen fermentation and milk fatty acid composition of dairy cows. J. Dairy Sci. 91: 4714-4727.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Boutinaud, M., and H. Jammes. 2002. Potential uses of milk epithelial cells: A review. Repr. Nutr. Dev. 42: 133-147.

Boutinaud, M., H. Rulquin, D. H. Keisler, J. Djiane, and H. Jammes. 2002. Use of somatic cells from goat milk for dynamic studies of gene expression in the mammary gland. J. Animal Sci. 80: 1258-1269.

Boutinaud, M., M. H. Ben Chedly, E. Delamaire, and J. Guinard-Flament. 2008. Milking and feed restriction regulate transcripts of mammary epithelial cells purified from milk. J. Dairy Sci. 91: 988-998.

Bu, D. P., J. Q. Wang, T. R. Dhiman, and S. J. Liu. 2007. Effectiveness of oils rich in linoleic and linolenic acids to enhance conjugated linoleic acid in milk from dairy cows. J. Dairy Sci. 90: 998-1007.

Bunger, M., H. M. van den Bosch, J. van der Meijde, S. Kersten, G. J. E. J. Hooiveld, and M. Muller. 2007. Genome-wide analysis of PPAR alpha activation in murine small intestine. Physiol. Gen. 30: 192-204.

Caldari-Torres, C., A. L. Lock, C. R. Staples, and L. Badinga. 2011. Performance, metabolic, and endocrine responses of periparturient Holstein cows fed 3 sources of fat. J. Dairy Sci. 94: 1500-1510.

Calvano, S. E., W. Xiao, D. R. Richards, R. M. Felciano, H. V. Baker, R. J. Cho, R. O. Chen, B. H. Brownstein, J. P. Cobb, S. K. Tschoeke, C. Miller-Graziano, L. L. Moldawer, M. N. Mindrinos, R. W. Davis, R. G. Tompkins, and S. F. Lowry. 2005. A network-based analysis of systemic inflammation in humans. Nature 437: 1032-1037.

Chilliard, Y., A. Ferlay, R. M. Mansbridge, and M. Doreau. 2000. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. Ann. Zootech. 49: 181-205.

Chilliard, Y., F. Glasser, A. Ferlay, L. Bernard, J. Rouel, and M. Doreau. 2007. Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. Eur. J. Lipid Sci. Technol. 109: 828-855.

Churchill, G. A. 2002. Fundamentals of experimental design for cDNA microarrays. Nature Gen. 32: 490-495.

Churchill, G. A. 2004. Using ANOVA to analyze microarray data. Biotechniques 37: 173-177.

Cohen, P., M. Miyazaki, N. D. Socci, A. Hagge-Greenberg, W. Liedtke, A. A. Soukas, R. Sharma, L. C. Hudgins, J. M. Ntambi, and J. M. Friedman. 2002. Role for stearoyl-CoA desaturase-1 in leptin-mediated weight loss. Science 297: 240-243.

Connor, E. E., S. Siferd, T. H. Elsasser, C. M. Evock-Clover, C. P. Van Tassell, T. S. Sonstegard, V. M. Fernandes, and A. V. Capuco. 2008. Effects of increased milking frequency on gene expression in the bovine mammary gland. BMC Genomics 9: 362.

Corl, B. A., L. H. Baumgard, D. A. Dwyer, J. M. Griinari, B. S. Phillips, and D. E. Bauman. 2001. The role of Δ9-desaturase in the production of cis-9, trans-11 CLA. J. Nutr. Biochem. 12: 622-630.

Cruz-Hernandez, C., J. K. G. Kramer, J. J. Kennelly, D. R. Glimm, B. M. Sorensen, E. K. Okine, L. A. Goonewardene, and R. J. Weselake. 2007. Evaluating the conjugated linoleic acid and *trans*-18:1 isomers in milk fat of dairy cows fed increasing amounts of sunflower oil and a constant level of fish oil. J. Dairy Sci. 90: 3786-3801.

Cui, X. Q., and G. A. Churchill. 2003. Statistical tests for differential expression in cDNA microarray experiments. Gen. Biol. 4: 210-220.

De Leeuw, W. C., H. Rauwerda, M. J. Jonker, and T. M. Breit. 2008. Salvaging Affymetrix probes after probe-level re-annotation. BMC Research Notes 1: 66-71.

Delamaire, E., and J. Guinard-Flament. 2006. Increasing milking intervals decreases the mammary blood flow and mammary uptake of nutrients in dairy cows. J. Dairy Sci. 89: 3439-3446.

Delbecchi, L., C. E. Ahnadi, J. J. Kennelly, and P. Lacasse. 2001. Milk fatty acid composition and mammary lipid metabolism in Holstein cows fed protected or unprotected canola seeds. J. Dairy Sci. 84: 1375-1381.

deVeth, M. J., J. M. Griinari, A. M. Pfeiffer, and D. E. Bauman. 2004. Effect of CLA on milk fat synthesis in dairy cows: Comparison of inhibition by methyl esters and free fatty acids, and relationships among studies. Lipids 39: 365-372.

Dong, X. Y., and S. Q. Tang. 2010. Insulin-induced gene: A new regulator in lipid metabolism. Peptides 31: 2145-2150.

Eberlé, D., B. Hegarty, P. Bossard, P. Ferre, and P. Foufelle. 2004. SREBP transcription factors: master regulators of lipid homeostasis. Biochimie 86: 839-848.

Enjalbert, F., M.-C. Nicot, C. Bayourthe, and R. Moncoulon. 1998. Duodenal infusions of palmitic, stearic or oleic acids differently affect mammary gland metabolism of fatty acids in lactating dairy cows. J. Nutr. 128: 1525-1532.

Farr, V. C., K. Stelwagen, L. R. Cate, A. J. Molenaar, T. B. McFadden, and S. R. Davis. 1996. An improved method for the routine biopsy of bovine mammary tissue. J. Dairy Sci. 79: 543-549.

Feng, S., A. M. Salter, T. Parr, and P. C. Garnsworthy. 2007. Extraction and quantitative analysis of stearoyl-CoA desaturase mRNA from dairy cow milk somatic cells. J. Dairy Sci. 90: 4128-4136.

Finucane, K. A., T. B. McFadden, J. P. Bond, J. J. Kennelly, and F. Q. Zhao. 2008. Onset of lactation in the bovine mammary gland: gene expression profiling indicates a strong inhibition of gene expression in cell proliferation. Funct. Integr. Gen. 8: 251-264.

Flowers M. T., and J. M. Ntambi. 2008. Role of stearoyl-coenzyme A desaturase in regulating lipid metabolism. Curr. Opin. Lipidol. 19: 248-256.

Folch, J., M. Lees, and H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509.

Foufelle, F., and P. Ferre. 2002. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. Biochem. J. 366: 377-391.

Frick, F., D. Linden, C. Ameen, S. Eden, A. Mode, and J. Oscarsson. 2002. Interaction between growth hormone and insulin in the regulation of lipoprotein metabolism in the rat. Am. J. Physiol. Endocrinol. Metab. 283: E1023-E1031.

Gama, M. A. S., P. C. Garnsworthy, J. M. Griinari, P. R. Leme, P. H. M. Rodrigues, L. W. O. Souza, and D. P. D. Lanna. 2008. Diet-induced milk fat depression: Association with changes in milk fatty acid composition and fluidity of milk fat. Livest. Sci. 115: 319-331.

Garnsworthy, P. C., S. Feng, A. L. Lock, and M. D. Royal. 2010. Heritability of milk fatty acid composition and stearoyl-CoA desaturase indices in dairy cows. J. Dairy Sci. 93: 1743-1748.

Gautier, L., L. Cope, B. M. Bolstad, and R. Irizarry. 2004. Affy-analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20: 307-315.

Gervais, R., J. W. McFadden, A. J. Lengi, B. A. Corl, and P. Y. Chouinard. 2009. Effects of intravenous infusion of *trans*-10, *cis*-12 18:2 on mammary lipid metabolism in lactating dairy cows. J. Dairy Sci. 92: 5167-5177.

Glasser, F., A. Ferlay, M. Doreau, P. Schmidely, D. Sauvant and Y. Chilliard. 2008. Long-chain fatty acid metabolism in dairy cows: a meta-analysis of milk fatty acid yield in relation to duodenal flows and de novo synthesis. J. Dairy Sci. 91: 2771-2785.

Goelema, J. O., M. A. M. Spreeuwenberg, G. Hof, A. F. B. van der Poel, and S. Tamminga. 1998. Effect of pressure toasting on the rumen degradability and intestinal digestibility of whole and broken peas, lupins and faba beans and a mixture of these feedstuffs. Anim. Feed Sci. Technol. 76: 35-50.

Griinari, J. M., B. A. Corl, S. H. Lacy, P. Y. Chouinard, K. V. V. Nurmela, and D. E. Bauman. 2000. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Δ9-desaturase. J. Nutr. 130: 2285-2291.

Grimaldi, P. A. 2010. Metabolic and nonmetabolic regulatory functions of peroxisome proliferator-activated receptor beta. Curr. Opin. Lipidol. 21: 186-191.

Han, L. Q., H. J. Li, Y. Y. Wang, H. S. Zhu, L. F. Wang, Y. J. Guo, W. F. Lu, Y. L. Wang, and G. Y. Yang. 2010. mRNA abundance and expression of SLC27A, ACC, SCD, FADS, LPIN, INSIG, and PPARGC1 gene isoforms in mouse mammary glands during the lactation cycle. Genet. Mol. Res. 9: 1250-1257.

Harfoot, C. and G. Hazlewood. 1988. Lipid metabolism in the rumen. Pages 285-322. In: Hobson, P.N. (Ed.), *The Rumen Microbial Ecosystem*. Elsevier, London, UK.

Harvatine, K. J. and D. E. Bauman. 2006. SREBP1 and thyroid hormone responsive spot 14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA. J. Nutr. 136: 2468-2474.

Harvatine, K. J., J. W. Perfield II, and D. E. Bauman. 2009. Expression of enzymes and key regulators of lipid synthesis is upregulated in adipose tissue during CLA-induced milk fat depression in dairy cows. J. Nutr. 139: 849-854.

Heck, J. L. M., H. J. F. van Valenberg, J. Dijkstra, and A. C. M. van Hooijdonk. 2009. Seasonal variation in the Dutch bovine raw milk composition. J. Dairy Sci. 92: 4745-4755.

Horton, J. D., J. L. Goldstein, and M. S. Brown. 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J. Clin. Invest. 109: 1125-1131.

Hudson, D. L. 2002. Keratins as markers of epithelial cells. In: Wise C, editor. Epithelial cell culture protocols, methods in molecular biology. Humana Press,157-168.

Huynh, H. T., G. Robitaille, and J. D. Turner. 1991. Establishment of bovine mammary epithelial cells (MAC-T): An in vitro model for bovine lactation. Exp. Cell Res. 197: 191-199.

Invernizzi, G., B. J. Thering, M. A. McGuire, G. Savoini, and J. J. Loor. 2010. Sustained upregulation of stearoyl-CoA desaturase in bovine mammary tissue with contrasting changes in milk fat synthesis and lipogenic gene networks caused by lipid supplements. Funct. Integ. Gen. 10: 561-575.

ISO 1999a. Animal Feeding Stuffs - Determination of moisture and other volatile matter content. 6496:1999. International Organization for Standardization, Geneva, Switzerland.

ISO 1999b. Animal Feeding Stuffs - Determination of fat content. 6492:1999. International Organization for Standardization, Geneva, Switzerland.

ISO 1999c. Animal Feeding Stuffs - Whole milk, determination of milk fat, protein and lactose content Guidance on the operation of mid-infrared instruments. 9622:1999. International Organization for Standardization, Geneva, Switzerland.

ISO 2002. Animal Feeding Stuffs - Determination of crude ash. 5984:2002. International Organization for Standardization, Geneva, Switzerland.

ISO 2004. Animal Feeding Stuffs - Enzymatic determination of total starch content. 15914:2004. International Organization for Standardization, Geneva, Switzerland.

ISO 2005. Animal Feeding Stuffs - Determination of nitrogen content and calculation of crude protein content. 5983:2005. International Organization for Standardization, Geneva, Switzerland.

Jacobs, A. A. A., J. van Baal, M. A. Smits, H. Z. H. Taweel, W. H. Hendriks, A. M. van Vuuren, and J. Dijkstra. 2011. Effects of feeding rapeseed oil, soybean oil, or linseed oil on stearoyl-CoA desaturase expression in the mammary gland of dairy cows. J. Dairy Sci.94: 874-887.

Jayan, G., and J. Herbein. 2000. "Healthier" dairy fat using trans-vaccenic acid. Nutr. Food Sci. 30: 304-309.

Jenkins, T. C., and W. C. Bridges. 2007. Protection of fatty acids against ruminal biohydrogenation in cattle. Eur. J. Lipid Sci. Technol. 9: 778-789.

Jensen, R. G. 2002. The composition of bovine milk lipids: January 1995 to December 2000. J. Dairy Sci. 85: 295-350.

Jump, D. B., D. Botolin, Y. Wang, J. Xu, B. Christian, and O. Demeure. 2005. Fatty acid regulation of gene transcription. J. Nutr. 135: 2503-2506.

Kadegowda, A. K. G., M. Bionaz, L. S. Piperova, R. A. Erdman, and J. J. Loor. 2009a. Peroxisome proliferator-activated receptor-γ activation and long-chain fatty acids alter lipogenic gene networks in bovine mammary epithelial cells to various extents. J. Dairy Sci. 92: 4276-4289.

Kadegowda, A. K. G., M. Bionaz, B. Thering, L. S. Piperova, R. A. Erdman, and J. J. Loor. 2009b. Identification of internal control genes for quantitative polymerase chain reaction in mammary tissue of lactating cows receiving lipid supplements. J. Dairy Sci. 92: 2007-2019.

Kay, J. K., T. R. Mackle, M. J. Auldist, N. A. Thomson, and D. E. Bauman. 2004. Endogenous synthesis of cis-9, trans-11 conjugated linoleic acid in dairy cows fed fresh pasture. J. Dairy Sci. 87: 369-378.

Kay, J. K., T. R. Mackle, D. E. Bauman, N. A. Thomson, and L. H. Baumgard. 2007. Effects of a supplement containing *trans*-10, *cis*-12 conjugated linoleic acid on bioenergetic and milk production parameters in grazing dairy cows offered ad libitum or restricted pasture. J. Dairy Sci. 90: 721-730.

Keating, A. F., J. J. Kennelly, and F. Q. Zhao. 2006. Characterization and regulation of the bovine Stearoyl-CoA desaturase gene promoter. Biochem. Biophys. Res. Commun. 344: 233-240.

Kelsey, J. A., B. A. Corl, R. J. Collier, and D. E. Bauman. 2003. The effect of breed, parity, and stage of lactation on conjugated linoleic acid (CLA) in milk fat from dairy cows. J. Dairy Sci. 86: 2588-2597.

Khan, N. A., J. W. Cone, and W. H. Hendriks. 2009. Stability of fatty acids in grass and maize silages after exposure to air during the feed out period. Anim. Feed Sci. Technol. 154: 183-192.

Lee, H. K., W. Braynen, K. Keshav, and P. Pavlidis. 2005. ErmineJ: tool for functional analysis of gene expression data sets. BMC Bioinformatics 6: 269.

Lengi, A. J., and B. A. Corl. 2007. Identification and characterization of a novel bovine stearoyl-CoA desaturase isoform with homology to human SCD5. Lipids 42: 499-508.

Lessard, M., N. Gagnon, and H. V. Petit. 2003. Immune response of postpartum dairy cows fed flaxseed. J. Dairy Sci. 86: 2647-2657.

Lin, X., J. J. Loor, and J. H. Herbein. 2004. *Trans*-10, *cis*-12 18:2 is a more potent inhibitor of de novo fatty acid synthesis and desaturation than *cis*-9, *trans*-11 18:2 in the mammary gland of lactating mice. J. Nutr. 134: 1362-1368.

Lindquist, S., L. Hansson, O. Hernell, B. Lonnerdal, J. Normark, M. Stromquist, and S. Bergstrom. 1994. Isolation of mRNA and genomic DNA from epithelial cells in human milk and amplification by PCR. Biotechniques 17: 692-696.

Lock, A. L., and P. C. Garnsworthy. 2002. Independent effects of dietary linoleic and linoleic fatty acids on the conjugated linoleic acid content of cows' milk. Anim. Sci. 74: 163-176.

Lock, A. L. and P. C. Garnsworthy. 2003. Seasonal variation in milk conjugated linoleic acid and Δ9-desaturase activity in dairy cows. Livest. Prod. Sci. 79: 47-59.

Lock, A. L. and D. E. Bauman. 2004. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids 39: 1197-1206.

Lock, A. L., D. E. Bauman, and P. C. Garnsworthy. 2005. Effect of production variables on the cis-9, trans-11 conjugated linoleic acid content of cows' milk. J. Dairy Sci. 88: 2714-2717.

Loor, J. J., J. H. Herbein, and C. E. Polan. 2002. *Trans* 18:1 and 18:2 isomers in blood plasma and milk fat of grazing cows fed a grain supplement containing solvent-extracted or mechanically extracted soybean meal. J. Dairy Sci. 85: 1197-1207.

Loor, J. J., K. Ueda, A. Ferlay, Y. Chilliard, and M. Doreau. 2004. Biohydrogenation, duodenal flow, and intestinal digestibility of *trans* fatty acids and conjugated linoleic acids in response to dietary forage:concentrate ratio and linseed oil in dairy cows. J. Dairy Sci. 87: 2472-2485.

Loor, J. J., K. Ueda, A. Ferlay, Y. Chilliard, and M. Doreau. 2005. Intestinal flow and digestibility of trans fatty acids and conjugated linoleic acids (CLA) in dairy cows fed a high-concentrate diet supplemented with fish oil, linseed oil, or sunflower oil. Anim. Feed Sci. Technol. 119: 203-225.

Mansbridge, R. J., and J. S. Blake. 1997. Nutritional factors affecting the fatty acid composition of bovine milk. Br. J. Nutr. 78 Suppl 1: S37-47.

McDonald, T. M., and J. E. Kinsella. 1973. Stearoyl-CoA desaturase of bovine mammary microsomes. Arch. Biochem. Biophys. 156: 223-231.

Mele, M., R. Dal Zotto, M. Cassandro, G. Conte, A. Serra, A. Buccioni, G. Bittante, and P. Secchiari. 2009. Genetic parameters for conjugated linoleic acid, selected milk fatty acids, and milk fatty acid unsaturation of Italian Holstein-Friesian cows. J. Dairy Sci. 92: 392-400.

Mensink, R. P., P. L. Zock, A. D. M. Kester, and M. B. Katan. 2003. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. Am. J. Clin. Nutr. 77: 1146-1155.

Metcalfe, L. D., A. A. Schimitz, and J. R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38: 514-515.

Michalik, L., J. Auwerx, J. P. Berger, V. K. Chatterjee, C. K. Glass, F. J. Gonzalez, P. A. Grimaldi, T. Kadowaki, M. A. Lazar, S. O'Rahilly, C. N. A. Palmer, J. Plutzky, J. K. Reddy, B. M. Spiegelman, B. Staels, and W. Wahli. 2006. International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. Pharmacol. Rev. 58: 726-741.

Miyazaki, M., S. M. Bruggink, and J. M. Ntambi. 2006. Identification of mouse palmitoyl-CoA delta 9 desaturase. J. Lipid Res. 47: 700-704.

Moll, R., W. W. Franke, D. L. Schiller, B. Geiger, and R. Krepler. 1982. The catalog of human keratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 31: 11-24.

Morales, M. S., D. L. Palmquist, and W. P. Weiss. 2000. Effects of fat source and copper on unsaturation of blood and milk triacylglycerol fatty acids in Holstein and Jersey cows. J. Dairy Sci. 83: 2105-2111.

Mosley, E. E., B. Shafii, P. J. Moate, and M. A. McGuire. 2006. *Cis*-9, *trans*-11 conjugated linoleic acid is synthesized directly from vaccenic acid in lactating dairy cattle. J. Nutr. 136: 570-575.

Mosley, E. E., and M. A. McGuire. 2007. Methodology for the *in vivo* measurement of the Δ9-desaturation of myristic, palmitic, and stearic acids in lactating dairy cattle. Lipids 42: 939-945.

Murrieta, C. M., B. W. Hess, E. J. Scholljegerdes, T. E. Engle, K. L. Hossner, G. E. Moss, and D. C. Rule. 2006. Evaluation of milk somatic cells as a source of mRNA for study of lipogenesis in the mammary gland of lactating beef cows supplemented with dietary high-linoleate safflower seeds. J. Anim. Sci. 84: 2399-2405.

Ntambi, J. M. 1999. Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. J. Lipid Res. 40: 1549-1558.

Ntambi, J. M., and M. Miyazaki. 2003. Recent insights into stearoyl-CoA desaturase-1. Curr. Opin. Lipidol. 14: 255-261.

Ntambi, J. M., and M. Miyazaki. 2004. Regulation of stearoyl-CoA desaturases and role in metabolism. Prog. Lipid Res. 43: 91-104.

Ollier, S., C. Leroux, A. de la Foye, L. Bernard, J. Rouel, and Y. Chilliard. 2009. Whole intact rapeseeds or sunflower oil in high-forage or high-concentrate diets affects milk yield, milk composition, and mammary gene expression profile in goats. J. Dairy Sci. 92: 5544-5560.

Palmquist, D. L., A. L. Lock, K. J. Shingfield, and D. E. Bauman. 2005. Biosynthesis of conjugated linoleic acid in ruminants and humans. In: Advances in Food and Nutrition Research (S. L. Taylor, ed.), Vol. 50 Elsevier Inc., San Diego, CA. pp. 179-217.

Panasyuk, G., I. Nemazanyy, A. Zhyvoloup, V. Filonenko, D. Davies, M. Robson, R. B. Pedley, M. Waterfield, and I. Gout. 2009. mTORbeta splicing isoform promotes cell proliferation and tumorigenesis. J. Biol. Chem. 284: 30807-30814.

Parillo, M. and G. Riccardi. 2004. Diet composition and the risk of type 2 diabetes: epidemiological and clinical evidence. Br. J. Nutr. 92: 7-19.

Pariza, M. W., Y. Park, and M. E. Cook. 2001. The biologically active isomers of conjugated linoleic acid. Prog. Lipid Res. 40: 283-298.

Paton, C. M., and J. M. Ntambi. 2009. Biochemical and physiological function of stearoyl-CoA desaturase. Am. J. Physiol. Endocrinol. Metab. 297: E28-E37.

Penning, P. D. 2004. Herbage intake handbook, 2nd edn. The British Grassland Society, Reading, UK, 191 p.

Peterson, D. G., L. H. Baumgard, and D. E. Bauman. 2002a. Short communication: Milk fat response to low doses of *trans*-10, *cis*-12 conjugated linoleic acid (CLA). J. Dairy Sci. 85: 1764-1766.

Peterson, D. G., J. A. Kelsey, and D. E. Bauman. 2002b. Analysis of variation in *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in milk fat of dairy cows. J. Dairy Sci. 85: 2164-2172.

Peterson, D. G., E. A. Matitashvili, and D. E. Bauman. 2003. Diet-induced milk fat depression in dairy cows results in increased *trans*-10, *cis*-12 CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis. J. Nutr. 133: 3098-3102.

Peterson, D. G., E. A. Matitashvili, and D. E. Bauman. 2004. The inhibitory effect of trans-10, cis-12 CLA on lipid synthesis in bovine mammary epithelial cells involves reduced proteolytic activation of the transcription factor SREBP-1. J. Nutr. 134: 2523-2527.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic. Acids Res. 29: 2002-2007.

Piantoni, P., M. Bionaz, D. E. Graugnard, K. M. Daniels, R. M. Akers, and J. J. Loor. 2008. Gene expression ratio stability evaluation in prepubertal bovine mammary tissue from calves fed different milk replacers reveals novel internal controls for quantitative polymerase chain reaction. J. Nutr. 138: 1158-1164.

Popeijus, H. E., W. H. Saris, and R. P. Mensink. 2008. Role of stearoyl-CoA desaturases in obesity and the metabolic syndrome. Int. J. Obes. 32: 1076-1082.

Pyper, S., N. Viswakarma, S. Yu, and J. K. Reddy. 2010. PPARα: energy combustion, hyperlipidaemia, inflammation and cancer. Nucl. Recept. Signal. 8: e002.

Raghow, R., C. Yellaturu, X. Deng, E. A. Park, and M. B. Elam. 2008. SREBPs: The crossroads of physiological and pathological lipid homeostasis. Trends Endocrin. Metab. 19: 65-73.

Rawlings, J. S., K. M. Rosler, and D. A. Harrison. 2004. The JAK/STAT signaling pathway. J. Cell Sci. 117: 1281-1283.

Rechsteiner, M. and S. W. Rogers. 1996. PEST sequences and regulation by proteolysis. Trends Biochem. Sci. 21: 267-271.

Reynolds, C., M., and H. M. Roche. 2010. Conjugated linoleic acid and inflammatory cell signaling. Prostaglandins Leukot. Essent. Fatty Acids 82: 199-204.

Scaglia, N. and R. A. Igal. 2005. Stearoyl-CoA desaturase is involved in the control of proliferation, anchorage-independent growth, and survival in human transformed cells. J. Biol. Chem. 280: 25339-25349.

Schennink, A., J. M. L. Heck, H. Bovenhuis, M. H. P. W. Visker, H. J. F. van Valenberg, and J. A. M. van Arendonk. 2008. Milk-fatty acid unsaturation: Genetic parameters and effects of stearoyl-CoA desaturase (SCD1) and acyl CoA:diacylglycerol acyltransferase 1 (DGAT1). J. Dairy Sci. 91: 2135-2143.

Schroeder, A., O. Mueller, S. Stocker, R. Salowsky, M. Leiber, M. Gassmann, S. Lightfoot, W. Menzel, M. Granzow, and T. Ragg. 2006. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Molecular Biology 7: 3.

Shingfield, K. J., C. K. Reynolds, G. Hervas, J. M. Griinari, A. S. Grandison, and D. E. Beever. 2006. Examination of the persistency of milk fatty acid composition responses to fish oil and sunflower oil in the diet of dairy cows. J. Dairy Sci. 89: 714-732.

Shingfield, K. J., and J. M. Griinari. 2007. Role of biohydrogenation intermediates in milk fat depression. Eur. J. Lipid Sci. Technol. 109: 799-816.

Sigal, L. H. 2006. Basic science for the clinician 39: NF-kappaB-function, activation, control, and consequences. J. Clin. Rheumat. 12: 207-211.

Singh, K., D. G. Hartley, T. B. McFadden, and D. D. Mackenzie. 2004. Dietary fat regulates mammary stearoyl-CoA desaturase expression and activity in lactating mice. J. Dairy Res. 71: 1-6.

Sinicropi, D., M. Cronin, and M. Liu. 2007. Gene expression profiling utilizing microarray technology and RT-PCR. BioMEMS Biom. Nanotech. 1: 23-46.

Sørensen, B. M., E. Chris Kazala, G. K. Murdoch, A. F. Keating, C. Cruz-Hernandez, J. Wegner, J. J. Kennelly, E. K. Okine, and R. J. Weselake. 2008. Effect of CLA and other C18 unsaturated fatty acids on DGAT in bovine milk fat biosynthetic systems. Lipids 43: 903-912.

Soyeurt, H., P. Dardenne, A. Gillion, C. Croquet, S. Vanderick, P. Mayeres, C. Bertozzi, and N. Gengler. 2006. Variation in fatty acid contents of milk and milk fat within and across breeds. J. Dairy Sci. 89: 4858-4865.

Soyeurt, H., F. Dehareng, P. Mayeres, C. Bertozzi, and N. Gengler. 2008. Variation of Δ9-desaturase activity in dairy cattle. J. Dairy Sci. 91: 3211-3224.

Sterk, A., R. Hovenier, B. Vlaeminck, A. M. van Vuuren, W. H. Hendriks, and J. Dijkstra. 2010. Effects of chemically or technologically treated linseed products and docosahexaenoic acid addition to linseed oil on biohydrogenation of C18:3n3 *in vitro*. J. Dairy Sci. 93: 5286-5299.

Sterk, A. 2011. Ruminal fatty acid metabolism – Altering rumen biohydrogenation to improve milk fatty acid profile of dairy cows. PhD thesis, Wageningen University, the Netherlands; Chapter 5.

Stoop, W. M., A. Schennink, M. H. P. W. Visker, E. Mullaart, J. A. M. van Arendonk, and H. Bovenhuis. 2009. Genome-wide scan for bovine milk-fat composition. I. Quantitative trait loci for short- and medium-chain fatty acids. J. Dairy Sci. 92: 4664-4675.

Storry, J. E., and J. A. Rook. 1965. Effect in the cow of intraruminal infusions of volatile fatty acids and of lactic acid on the secretion of the component fatty acids of the milk fat and on the composition of blood. Biochem J. 96: 210-217.

Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander, and J. P. Mesirov. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genomewide expression profiles. Proc. Nat. Acad. Sci. USA 102: 15545-15550.

Swarbrick, A., E. Roy, T. Allen, and J. M. Bishop. 2008. Id1 cooperates with oncogenic Ras to induce metastatic mammary carcinoma by subversion of the cellular senescence response. Proc. Nat. Acad. Sci. USA 105: 5402-5407.

Thering, B. J., D. E. Graugnard, P. Piantoni, and J. J. Loor. 2009. Adipose tissue lipogenic gene networks due to lipid feeding and milk fat depression in lactating cows. J. Dairy Sci. 92: 4290-4300.

Timmen, H., and S. Patton. 1988. Milk fat globules: fatty acid composition, size and *in vivo* regulation of fatty liquidity. Lipids 23: 685-689.

Tontonoz, P., and B. M. Spiegelman. 2008. Fat and beyond: the diverse biology of PPARgamma. Ann. Rev. Biochem. 77: 289-312.

Van Knegsel, A. T. M., H. van den Brand, J. Dijkstra, W. M. van Straalen, M. J. W. Heetkamp, S. Tamminga, and B. Kemp. 2007. Dietary energy source in dairy cows in early lactation: Energy partitioning and milk composition. J. Dairy Sci. 90: 1467-1476.

Van Soest, P. J. 1973. Collaborative study of acid-detergent fibre and lignin. J. Assoc. Off. Anal. Chem. 56: 781-784.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74: 3583-3597.

Wahle, K. W., S. D. Heys, and D. Rotondo. 2004. Conjugated linoleic acids: are they beneficial or detrimental to health? Prog. Lipid Res. 43: 553-587.

Wang, J., L. Yu, R. E. Schmidt, C. Su, X. Huang, K. Gould, G. Cao. 2005. Characterization of HSCD5, a novel human stearoyl-CoA desaturase unique to primates. Biochem. Biophys. Res. Commun. 332: 735-42.

Waters, K. M., and J. M. Ntambi. 1994. Insulin and dietary fructose induce stearoyl-CoA desaturase 1 gene expression of diabetic mice. J. Biol. Chem. 269: 27773-27777.

Way, J. M., W. W. Harrington, K. K. Brown, W. K. Gottschalk, S. S. Sundseth, T. A. Mansfield, R. K. Ramachandran, T. M. Willson, and S. A. Kliewer. 2001. Comprehensive messenger ribonucleic acid profiling reveals that peroxisome proliferator-activated receptor γ activation has coordinate effects on gene expression in multiple insulin-sensitive tissues. Endocrinology 142: 1269-1277.

Wu, Z., R. Irizarry, R. Gentleman, F. Martinez-Murillo, and F. Spencer. 2004. A modelbased background adjustment for oligonucleotide expression arrays. J. Am. Stat. Assoc. 99: 909-917.

Xu, W., and M. Faisal. 2010. Factorial microarray analysis of zebra mussel (Dreissena polymorpha: Dreissenidae, Bivalvia) adhesion. BMC Genomics 11: 341-358.

Yonezawa, T., S. Yonekura, M. Sanosaka, A. Hagino, K. Katoh, and Y. Obara. 2004. Octanoate stimulates cytosolic triacylglycerol accumulation and CD36 mRNA expression but inhibits acetyl coenzyme A carboxylase activity in primary cultured bovine mammary epithelial cells. J. Dairy Res. 71: 398-404.

Summary

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Samenvatting

Summary

Increasing the proportion of unsaturated fatty acids (UFA) in milk is believed to be beneficial in terms of human health, thereby increasing the nutritional quality of milk. In the Netherlands however, the proportion of UFA in milk decreased in the last decade, which is most likely related to changes in composition of diets fed to dairy cows. These changes include a lower proportion of fresh grass, reduced crude protein and fat content of grass and grass silage, and increased proportion of maize silage. The proportion of UFA in milk is mainly dependent on the proportion of UFA in the diet, the degree of biohydrogenation of UFA in the rumen, and on activity of the stearoyl-CoA desaturase (SCD) enzyme in the mammary gland. The SCD enzyme creates a double bond at the Δ9 position in a wide range of fatty acids (FA), thereby making these FA unsaturated. This thesis focuses on SCD in the mammary gland of dairy cows, and how SCD can be influenced by nutrition. More specifically, the effect of short- and long-chain fatty acids on mRNA expression of SCD was investigated in the bovine mammary gland. The purpose of this research was to explore nutritional strategies that could increase the activity of SCD in the mammary gland of dairy cows, thereby improving the FA profile of milk. In addition, a non-invasive alternative to mammary tissue for measuring SCD expression in the mammary gland of dairy cows was examined.

The objective of the first experiment (**Chapter 2**) was to compare the effects of various FA typically present in dairy cow rations, on the expression of both SCD1 and SCD5 (the two known bovine isoforms of SCD) in the mammary gland of dairy cows. Twenty-eight Holstein-Friesian cows were randomly assigned to one of the four dietary treatments being a basal diet supplemented (DM basis) with either 2.7% rapeseed oil as a source of C18:1 *cis*-9, 2.7% soybean oil as a source of C18:2 *cis*-9,12, 2.7% linseed oil as a source of C18:3 *cis*-9,12,15 or 2.7% of a 1:1:1 mixture of the three oils. After the treatment period of 23 days, all cows were switched to a control diet for an additional 28 days. At the end of both the treatment period and the control period, tissue from the mammary gland was taken by biopsy and analysed for mRNA expression of SCD1 and SCD5 by using quantitative real-time polymerase chain reaction (qRT-PCR). Milk yield as well as milk protein and fat content did not differ between the four dietary treatments. Mammary SCD1 expression was significantly down-regulated in dairy cows by feeding soybean oil compared with rapeseed oil or linseed oil, and this was partially reflected by the lower desaturase indices in the milk, which are frequently used as proxies for mammary SCD activity. In contrast, SCD5 expression in the mammary gland was much lower ($<10^3$) than that of SCD1 and did not differ amongst the four treatments, indicating that mammary expression of SCD5 is less sensitive to changes in FA supply compared with SCD1.

To study the changes in genome-wide expression of genes in response to dietary UFA supplementation, mammary tissue samples of experiment 1 were also used for micro-array analysis (**Chapter 3**). In this study, expression of the entire genome was compared between the four UFA diets and the control (no UFA supplementation). Compared to control, milk yield was higher but concentrations of milk fat and protein were lower when UFA were included in the diet. Furthermore, the proportion of *de novo* synthesised FA in milk was reduced, whereas that of long-chain fatty acids (LCFA) increased. Applying a statistical cut-off of false discovery rate of q-values < 0.05 together with an absolute fold change of 1.3, a total of 972 genes were found to be significantly affected through UFA supplementation, indicating that large transcriptional adaptations occurred in the mammary gland when diets of dairy cows were supplemented with unprotected dietary UFA. Gene sets related to cell development and remodelling, apoptosis, nutrient metabolic process, as well as immune system response were predominantly downregulated during UFA supplementation.

Since biopsy of the mammary gland is an invasive and costly method which presents a risk of udder infection, the use of milk somatic cells as a non-invasive, alternative source of mRNA was investigated in experiment 2 (**Chapter 4**). Both milk somatic cells and mammary tissue were collected from fourteen Holstein-Friesian cows fed diets with or without linseed and used for analysis of SCD expression by qRT-PCR. Expression of SCD5 in mammary tissue was low compared with SCD1. A significant relationship ($r^2 = 0.60$; $P < 0.01$) was found between SCD1 expression in milk somatic cells and in mammary tissue. In addition, SCD1 expression in milk somatic cells was significantly related to Δ9-desaturase indices in milk (r^2 between 0.32 and 0.52), which are commonly used as an indicator of SCD activity within the mammary gland. This relationship was better than the relationship between SCD1 expression in mammary tissue and Δ9-desaturase indices in milk (r^2 between 0.22 and 0.38). The yield of total RNA from milk of dairy cows was rather low, and further investigation is needed to improve the yield of RNA from milk. In conclusion, this study showed that milk somatic cells can be used as a source of mRNA to study SCD1 expression in dairy cows, offering a non-invasive alternative to mammary tissue samples obtained by biopsy.

Acetic acid (Ac) and β-hydroxybutyric acid (BHBA) are important precursors for *de novo* FA synthesis in the bovine mammary gland. However, contrary to LCFA, information on the effect of these short-chain FA on mammary SCD expression is scarce. Therefore, in experiment 3 (**Chapter 5**), a bovine mammary cell line (MAC-T) was used to assess the effect of Ac and BHBA on the mRNA expression of SCD via qRT-PCR, and to compare this to the effect of various LCFA on SCD expression, as well as expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). In addition, expression of sterol regulatory binding protein 1 (SREBP-1), insulin-induced gene 1 protein (INSIG-1) and peroxisome proliferator-activated receptors (PPARs) were measured to examine if these transcription factors are involved in the regulation of SCD expression in bovine mammary epithelial cells. MAC-T cells were treated for 12 h without FA additions (CON) or with

either 5 m*M* Ac, 5 m*M* BHBA, a combination of 5 m*M* Ac + 5 m*M* BHBA, 100 µ*M* palmitic acid (PA), 100 µ*M* stearic acid (SA), 100 µ*M* oleic acid (OA), 100 µ*M* *trans*-vaccenic acid (TVA), 100 µ*M* linoleic acid (LA) or 100 µ*M* α-linolenic acid (ALA). In comparison with CON, expression of SCD1 was increased by Ac (+61%) and reduced by OA (-61%), LA (-84%) and ALA (-88%). Contrary to SCD1, MAC-T cells did not express SCD5 mRNA. Expression of ACC was also increased by Ac (+44%) and reduced by LA (-48%) and ALA (-49%). Compared with CON, FAS expression was not significantly affected by the treatments. The mRNA level of SREBP-1 was not affected by Ac or BHBA, but was reduced by OA (-44%), TVA (-42%), LA (-62%) and ALA (-68%) compared with CON. Expression of INSIG-1 was down-regulated by SA (-37%), OA (-63%), TVA (-53%), LA (-81%) and ALA (-91%). Both PPARα and PPARδ expression was not significantly affected by the treatments. These results show that Ac up-regulates expression of SCD1 and ACC in MAC-T cells, which indicates that Ac may increase desaturation and *de novo* synthesis of FA in the bovine mammary gland. Furthermore, the results strengthen the support for the role of SREBP-1 and INSIG-1 as central regulators of lipogenesis in the bovine mammary gland.

Overall, it can be concluded that saturated LCFA have little or no effect on SCD1 expression in the bovine mammary gland, whereas unsaturated LCFA inhibit mammary SCD1 expression. The inhibitory effect of unsaturated LCFA on mammary SCD1 expression appears to increase proportionally with the amount of double bonds in the LCFA (i.e., more double bonds results in higher inhibition of SCD1 expression). Therefore, it seems difficult to enhance SCD1 expression in the mammary gland by supply of LCFA. In order to limit inhibition of mammary SCD1 expression, supply of poly unsaturated fatty acids (PUFA) to the mammary gland should be restricted. The regulation of SCD1 in the bovine mammary gland by LCFA appears to be, at least partly, regulated by the transcription factors SREBP-1 and INSIG-1. Based on the *in vitro* research it appears that short-chain FA, in particular Ac, upregulate mammary SCD1 expression, although further research is needed to verify if short-chain FA induce SCD1 expression in the bovine mammary gland. The recently discovered isoform SCD5 is expressed in bovine mammary tissue, although contribution to Δ9-desaturation of FA appears to be quite low.

Samenvatting

Het verhogen van het aandeel onverzadigde vetzuren (UFA) in melk wordt over het algemeen als positief gezien wat betreft de gezondheid van de mens, en draagt daarmee bij aan het verhogen van de voedingskwaliteit van melk. In Nederland daarentegen, is het aandeel UFA in melk het laatste decennium juist afgenomen, wat waarschijnlijk gerelateerd is aan veranderingen in het rantsoen van melkkoeien. Deze veranderingen zijn o.a. een lager aandeel vers gras, verminderd ruw eiwit en vet gehalte van gras en graskuil, en een verhoogd aandeel maïskuil. Het aandeel UFA in melk wordt voornamelijk bepaald door het aandeel UFA in het rantsoen, de mate van biohydrogenatie van UFA in de pens, en door de activiteit van het stearoyl-CoA desaturase (SCD) enzym in het uier. Het SCD enzym creëert een dubbele binding op de Δ9 positie van uiteenlopende vetzuren (FA) en maakt deze daarbij dus onverzadigd. Dit proefschrift richt zich op SCD in het uier van melkkoeien en hoe SCD in het uier beïnvloed wordt door voeding van de melkkoe. In het bijzonder is het effect van kort- en langketen FA op mRNA expressie van SCD in het uier van melkkoeien onderzocht. Het doel van dit onderzoek was het verkennen van voedingsstrategieën die de activiteit van SCD in het uier kunnen verhogen, en zodoende leiden tot een verbeterd vetzuurprofiel van melk. Ook is onderzoek uitgevoerd naar een niet-invasieve methode om SCD expressie te meten in het uier van melkkoeien, als alternatief voor biopsie van het uier.

Het doel van het eerste experiment (**Hoofdstuk 2**) was het onderzoeken van de effecten van verschillende FA op expressie van zowel SCD1 als SCD5 (de twee isovormen van SCD in het rund) in het uier van melkkoeien. In dit experiment werden 28 Holstein-Friesian koeien willekeurig toegewezen aan één van de vier rantsoenen bestaande uit een basis rantsoen gesupplementeerd (droge stof basis) met hetzij 2.7% raapzaadolie als een bron van C18:1 *cis*-9, of 2.7% sojaolie als een bron van C18:2 *cis*-9,12, of 2.7% lijnzaadolie als een bron van C18:3 *cis*-9,12,15, of 2.7% van een 1:1:1 mengsel van de drie oliën. Na de experimentele periode van 23 dagen werden alle koeien op een controle rantsoen gezet gedurende 28 dagen. Aan het einde van zowel de experimentele als de controle periode, werd uierweefsel verzameld door middel van biopsie waarin mRNA expressie van SCD1 en SCD5 werd geanalyseerd met behulp van kwantitatieve real-time polymerase chain reaction (qRT-PCR). Melkproductie alsmede de gehalten aan melkvet, -eiwit en -lactose waren niet verschillend tussen de vier experimentele behandelingen. Expressie van SCD1 in het uier was significant down-gereguleerd in melkkoeien in de sojaolie groep vergeleken met de raapzaadolie en lijnzaadolie groep. Dit was gedeeltelijk terug te vinden in de lagere Δ9-desaturase indices in melk, die veelvuldig gebruikt worden als indicatoren voor SCD activiteit in het uier. De SCD5 expressie in het uier was echter veel lager ($<10^3$) dan de expressie van SCD1 en verschilde ook niet tussen de vier behandelingen, wat suggereert dat SCD5 expressie in het uier minder gevoelig is voor veranderingen in FA aanbod vergeleken met SCD1.

Om de veranderingen in genexpressie van het totale genoom als gevolg van UFA supplementatie van het rantsoen te bestuderen, is het uierweefsel van experiment 1 ook gebruikt voor micro-array analyse (**Hoofdstuk 3**). In deze studie werd expressie van het totale genoom vergeleken tussen de vier UFA rantsoenen en het controle rantsoen (geen UFA toevoeging). Vergeleken met de controle was de melkproductie hoger, maar waren de gehalten aan melkvet en -eiwit lager voor de koeien op het UFA rantsoen. Verder was het aandeel FA in melk afkomstig van *de novo* synthese gereduceerd, terwijl het aandeel langketen vetzuren (LCFA) toegenomen was. Het toepassen van een statistische limiet voor "false discovery rate" van q-waarde < 0.05 samen met een absolute "fold change" van 1.3, resulteerde in 972 genen waarvan de expressie significant veranderde als gevolg van UFA supplementatie. Dit suggereert dat aanzienlijke transcriptionele adaptatie plaatsvindt in het uier van melkkoeien wanneer onbeschermde UFA worden toegevoegd aan het rantsoen. Groepen van genen gerelateerd aan cel ontwikkeling en reorganisatie, apoptose, metabolische processen van nutriënten, en immuunsysteem respons waren voornamelijk down-gereguleerd als gevolg van UFA supplementatie.

Aangezien biopsie van het uier een invasieve en dure methode is die bovendien een risico vormt voor uierinfectie, is in experiment 2 (**Hoofdstuk 4**) onderzocht of somatische cellen in melk gebruikt kunnen worden als niet-invasieve, alternatieve bron van mRNA voor het onderzoeken van SCD expressie in het uier. Hiervoor zijn zowel somatische cellen uit melk als uierweefsel verzameld van 14 Holstein-Friesian koeien, op een rantsoen met of zonder lijnzaad, en geanalyseerd op SCD expressie met behulp van qRT-PCR. Expressie van SCD5 in uierweefsel was laag vergeleken met SCD1. Er was een significante relatie ($r^2 = 0.60$; $P < 0.01$) tussen SCD1 expressie in somatische cellen uit melk en in uierweefsel. Bovendien was SCD1 expressie in somatische cellen uit melk significant gerelateerd aan Δ9-desaturase indices in melk (r^2 tussen 0.32 en 0.52), die veelvuldig gebruikt worden als indicatoren voor SCD activiteit in het uier. Deze relatie was beter dan de relatie tussen SCD1 expressie in uierweefsel en Δ9-desaturase indices in melk (r^2 tussen 0.22 en 0.38). De hoeveelheid totaal RNA geïsoleerd uit melk van koeien was laag en verder onderzoek is vereist om de RNA opbrengst uit melk te verhogen. Concluderend laat deze studie zien dat somatische cellen uit melk gebruikt kunnen worden als alternatieve bron van mRNA om SCD1 expressie te onderzoeken in melkkoeien, en zo een niet-invasief alternatief bieden voor uierweefsel via biopsie.

Azijnzuur (Ac) en β-hydroxyboterzuur (BHBA) zijn belangrijke precursoren voor *de novo* synthese van FA in het uier van de melkkoe. Echter, in vergelijking met LCFA, is de informatie over de effecten van deze kortketen FA op de expressie van SCD en andere belangrijke genen voor de FA synthese in het uier schaars. Derhalve is in experiment 3 (**Hoofdstuk 5**) een boviene uiercellijn (MAC-T) gebruikt om de effecten van Ac en BHBA op mRNA expressie van SCD te onderzoeken via qRT-PCR, en deze te vergelijken met het effect van verschillende LCFA op SCD expressie, evenals expressie van acetyl-CoA carboxylase (ACC) en fatty acid synthase (FAS). Bovendien werd de expressie van sterol regulatory binding protein 1 (SREBP-1), insulin-induced gene 1 protein (INSIG-1) en peroxisome proliferator-activated receptors (PPARs) gemeten om te onderzoeken of deze

transcriptiefactoren betrokken zijn bij de regulatie van SCD expressie in boviene uier epitheelcellen. MAC-T cellen werden gedurende 12 uur geïncubeerd zonder FA toevoeging (CON), of met toevoeging van 5 m*M* Ac, 5 m*M* BHBA, een combinatie van 5 m*M* Ac + 5 m*M* BHBA, 100 µ*M* palmitinezuur (PA), 100 µ*M* stearinezuur (SA), 100 µ*M* oliezuur (OA), 100 µ*M* *trans*-vacceenzuur (TVA), 100 µ*M* linolzuur (LA) of 100 µ*M* α-linoleenzuur (ALA). In vergelijking met CON nam de expressie van SCD1 toe met Ac (+61%) en daalde deze met OA (-61%), LA (-84%) en ALA (-88%). In tegenstelling tot SCD1 werd geen SCD5 expressie waargenomen in de MAC-T cellen. De expressie van ACC nam toe met Ac (+44%) en daalde met LA (-48%) en ALA (-49%). Vergeleken met CON was FAS expressie niet significant beïnvloed door de verschillende behandelingen. Het mRNA niveau van SREBP-1 werd niet beïnvloed door Ac of BHBA, maar nam af met OA (-44%), TVA (-42%), LA (-62%) en ALA (-68%) vergeleken met CON. De expressie van INSIG-1 was down-gereguleerd met SA (-37%), OA (-63%), TVA (-53%), LA (-81%) en ALA (-91%). Zowel PPARα als PPARδ expressie was niet significant beïnvloed door de verschillende behandelingen. Deze resultaten laten zien dat expressie van SCD1 en ACC wordt up-gereguleerd door Ac in MAC-T cellen, wat suggereert dat Ac de desaturatie en *de novo* synthese van FA kan verhogen in het uier van melkkoeien. Bovendien versterken deze resultaten de rol van SREBP-1 en INSIG-1 als centrale regulatoren van lipogenese in het uier van melkkoeien.

Allesomvattende kan geconcludeerd worden dat verzadigde LCFA weinig of geen effect hebben op SCD1 expressie in het uier van melkkoeien, terwijl onverzadigde LCFA de SCD1 expressie verminderen. Het remmende effect van onverzadigde LCFA op SCD1 expressie in het uier lijkt proportioneel toe te nemen met het aantal dubbele bindingen in de onverzadigde LCFA (d.w.z. meer dubbele bindingen resulteert in een hogere remming van SCD1 expressie). Daardoor lijkt het moeilijk om SCD1 expressie in het uier significant te verhogen door het aanbieden van LCFA. Om remming van SCD1 expressie in het uier te beperken, zou het aanbod van meervoudig onverzadigde vetzuren (PUFA) naar het uier beperkt moeten worden. De regulatie van SCD1 in het uier van melkkoeien door LCFA lijkt, ten minste gedeeltelijk, gereguleerd door de transcriptiefactoren SREBP-1 en INSIG-1. Gebaseerd op het *in vitro* onderzoek lijken kortketen FA, in het bijzonder Ac, SCD1 expressie te up-reguleren, hoewel verder onderzoek noodzakelijk is om te verifiëren of kortketen FA de SCD1 expressie kunnen verhogen in het uier van melkkoeien. De recent ontdekte isovorm SCD5 komt tot expressie in het uier van melkkoeien, maar de bijdrage aan Δ9-desaturatie van FA lijkt vrij laag.

Dankwoord

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Acknowledgments

Dankwoord

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Curriculum Vitae

About the Author

Antonius Adrianus Arnoldus (Antoon) Jacobs werd geboren op 27 september 1981 te Eindhoven en groeide op in Eersel, op het melkveebedrijf van zijn ouders. In 1998 behaalde hij zijn HAVO-diploma aan het Rythovius College te Eersel. In datzelfde jaar begon hij aan de studie Veehouderij aan de Hogere Agrarische School te 's-Hertogenbosch (HAS Den Bosch). Deze opleiding rondde hij in 2003 succesvol af met Rundveehouderij als specialisatie. In september 2004 begon hij aan de MSc-opleiding Dierwetenschappen aan de Wageningen Universiteit met als specialisatie Diervoeding. Deze studie werd in november 2006 succesvol afgerond met afstudeervakken over aminozuur-synchronisatie bij vleeskalveren (leerstoelgroep Diervoeding) en over treksterkte van tropische grassen (leerstoelgroep Gewas- en Onkruidecologie) waarvoor hij 6 maanden bij de "Sustainable Ecosystems" groep van CSIRO in Townsville, Australië verbleef. Na een periode van 8 maanden als onderzoeker bij de leerstoelgroep Diervoeding van de Wageningen Universiteit, begon hij in augustus 2007 als promovendus bij diezelfde leerstoelgroep. Tijdens zijn promotieonderzoek verbleef hij 8 maanden bij de "Department of Animal Science" van de Michigan State University in East Lansing, Verenigde Staten. Het resultaat van dit promotieonderzoek is beschreven in dit proefschrift.

Antonius Adrianus Arnoldus (Antoon) Jacobs was born on September 27, 1981 in Eindhoven, the Netherlands and grew up in Eersel, on the dairy farm of his parents. In 1998 he graduated from the secondary school "Rythovius College" in Eersel. In that same year he started the BSc study Animal Husbandry at the "Hogere Agrarisch School" in 's-Hertogenbosch. This study was successfully completed in 2003 with cattle husbandry as specialisation. In September 2004 he started the MSc study Animal Sciences at the Wageningen University with a specialisation in Animal Nutrition. In November 2006, this study was successfully completed with theses concerning amino acid synchrony in veal calves (Animal Nutrition group) and concerning fracture properties of tropical grasses (Crop and Weed Ecology group) for which he stayed 6 months at the Sustainable Ecosystems group of CSIRO in Townsville, Australia. After a period of 8 months as researcher at the Animal Nutrition group of the Wageningen University, he started with a PhD project in August 2007 within the same group. During his PhD project, he stayed for 8 months at the department of Animal Science of the Michigan State University in East Lansing, United States. The results of this PhD project are described in this thesis.

Publications

Refereed Scientific Publications

Jacobs, A.A.A., J. van Baal, M.A. Smits, H.Z.H. Taweel, W.H. Hendriks, A.M. van Vuuren, and J. Dijkstra. 2011. Effects of feeding rapeseed oil, soybean oil, or linseed oil on stearoyl-CoA desaturase expression in the mammary gland of dairy cows. J. Dairy Sci. 94: 874-887.

Mach, N., A.A.A. Jacobs, L. Kruijt, J. van Baal, and M.A. Smits. 2011. Alteration of gene expression in mammary gland tissue of dairy cows in response to dietary unsaturated fatty acids. Animal 5 (08): 1217-1230.

Jacobs, A.A.A., J.A. Scheper, M.A. Benvenutti, I.J. Gordon, D.P. Poppi, and A. Elgersma. 2011. Tensile fracture properties of seven tropical grasses at different phenological stages. Grass Forage Sci. doi: 10.1111/j.1365-2494.2011.00812.x, published online by Blackwell Publishing Ltd., UK, on July 18, 2011.

Jacobs, A.A.A., J. Dijkstra, W.H. Hendriks, J. van Baal, and A.M. van Vuuren. 2011. Comparison between stearoyl-CoA desaturase expression in milk somatic cells and in mammary tissue of lactating dairy cows. Submitted.

Jacobs, A.A.A., J. Dijkstra, J.S. Liesman, M.J. VandeHaar, A.L. Lock, A.M. van Vuuren, W.H. Hendriks, and J. van Baal. 2011. Effects of short- and long-chain fatty acids on stearoyl-CoA desaturase expression in bovine mammary epithelial cells. Submitted.

Contributions to Conferences and Symposia

Jacobs, A.A.A., J.S. Liesman, M.J. VandeHaar, J. Dijkstra, A.M. van Vuuren and J. van Baal. 2011. Effects of short- and long-chain fatty acids on expression of lipogenic genes in bovine mammary epithelial cells. J. Dairy Sci. Vol. 94, E-Suppl. 1: p.750. From: ADSA-ASAS Joint Annual Meeting, New Orleans, LA, USA, July 10-14, 2011.

Mach, N., J. van Baal, L. Kruijt, A.A.A. Jacobs, and M.A. Smits. 2011. Dietary unsaturated fatty acids affect the mammary gland integrity and health in lactating dairy cows. In: BMC Proceedings 2011, 5 (Suppl.4): p.S35. From: International Symposium on Animal Genomics for Animal Health (AGAH 2010) Paris, France, May 31 - June 02, 2010.

Mach, N., A.A.A. Jacobs, L. Kruijt, J. van Baal, and M.A. Smits. 2010. Alteration of gene expression in mammary gland tissue of dairy cows in response to dietary unsaturated fatty acids. In: Programme and Abstract book 32nd Conference for the International Society for Animal Genetics, Edingburgh, Scotland, July 26-30, 2010.

Jacobs, A.A.A., A.M. van Vuuren, J. van Baal, D. van de Hengel, and J. Dijkstra. 2009. Effects of feeding rapeseed oil, soybean oil or linseed oil on stearoyl-CoA desaturase expression in the mammary gland of dairy cows. In: Ruminant Physiology: Digestion, Metabolism, and Effect of Nutrition on Reproduction and Welfare. Proceedings of the XIth International Symposium on Ruminant Physiology, Clermont-Ferrand, France, September 6-9, 2009. Y. Chilliard, F. Glasser, Y. Faulconnier, F. Bocquier, I. Veissier, and M. Doreau, eds., p.544-545, Wageningen Academic Publishers, Wageningen, the Netherlands.

Jacobs, A.A.A., J.J.G.C. van den Borne, S.J.J. Alferink, and W.J.J. Gerrits. 2008. The effect of synchronizing the availability of individual amino acids on protein and energy metabolism in preruminant calves. In: Proceedings of the 33rd congress of Dutch Speaking Nutrition Researchers (NVO), Wageningen, the Netherlands, April 25, 2008, p.73-74.

Jacobs, A.A.A., J. van Baal, A.C.M. van Hooijdonk, W.H. Hendriks, A.M. van Vuuren, and J. Dijkstra. 2008. Increasing desaturase activity in the mammary gland of dairy cows through nutrition to improve the fatty acid profile of milk. In: Proceedings of the 33rd congress of Dutch Speaking Nutrition Researchers (NVO), Wageningen, the Netherlands, April 25, 2008, p.27-28.

Alferink, S.J.J., J.J.G.C. van den Borne, A. Habets, A.A.A. Jacobs, and W.J.J. Gerrits. 2007. Intravenous administration of lysine and threonine to a deficient diet results in low nitrogen utilization in preruminant calves. In: Energy and protein metabolism and nutrition, EAAP publication No. 124, Vichy, France, September 9-13, 2007, p.473-474. Wageningen Academic Publishers, Wageningen, the Netherlands.

Training and Supervision Plan

Name	Antoon Jacobs
Group	Animal Nutrition Group
Daily supervisors	Dr. ir. J. Dijkstra
	Dr. J. van Baal
Supervisor	Prof. dr. ir. W.H. Hendriks

The Basic Package	**Year**	**Credits***
Philosophy of Science and Ethics	2007	1.5
WIAS Introduction Course	2008	1.5
International Conferences		
6th International Symposium on Ruminant Physiology (ISRP), Clermont-Ferrand, France	2009	1.2
Tri-state Dairy Nutrition Conference, Fort Wayne, IN, USA	2010	0.6
ADSA-ASAS Joint Annual Meeting, New Orleans, LA, USA	2011	1.5
Seminars and Workshops		
WIAS Science Day, Wageningen, the Netherlands (4x)	2008-2011	1.2
WIAS Seminar: "Strategies to improve health and fertility in dairy cows", Wageningen, the Netherlands	2008	0.2
33rd ANR Forum (former NVO), Wageningen, the Netherlands	2008	0.3
34th ANR Forum (former NVO), Melle, Belgium	2009	0.3
International Symposium: "Nutritional strategies to manage the challenges of today's dairy cows, Wageningen, the Netherlands	2009	0.3
WIAS Seminar: "Genetics of milk quality", Wageningen, the Netherlands	2009	0.3
5th Annual Animal Science Graduate Research Forum (ASGRF), East Lansing, MI, USA	2010	0.3
36th ANR Forum (former NVO), Leuven, Belgium	2011	0.3
Presentations		
33rd ANR Forum, Wageningen, the Netherlands (oral)	2008	1.0
33rd ANR Forum, Wageningen, the Netherlands (poster)	2008	1.0
Advances in Feed Evaluation Science, Wageningen, the Netherlands (oral)	2009	1.0

6th International Symposium on Ruminant Physiology (ISRP), Clermont-Ferrand, France (poster)	2009	1.0
ADSA-ASAS Joint Annual Meeting, New Orleans, LA, USA (oral)	2011	1.0
In-Depth Studies		
Science Meet Society: Biomass use: Food, Feed or Fuel?	2007	1.5
Nutrition in the Omics Era	2008	1.0
Design of Animal Experiments	2008	1.0
Statistics for the Life Sciences	2008	2.0
Nutrient Density of Milk	2009	0.8
Advances in Feed Evaluation Science	2009	0.3
Professional Skills Support Courses		
Supervising MSc Thesis Work	2008	1.0
PhD Competence Assessment	2008	0.3
Scientific Writing	2009	1.8
Techniques for Writing and Presenting a Scientific Paper	2010	1.2
Research Skills Training		
External period (Feb-Sep 2010) at Michigan State University, East Lansing, MI, USA	2010	2.0
Didactic Skills Training		
Inleiding Dierwetenschappen (praktijkproject) (3x)	2007-2009	1.5
Toegepaste Dierbiologie (4x)	2008-2011	1.4
Animal Nutrition and Physiology	2009	0.3
Management Skills Training		
Organisation 33rd ANR Forum, Wageningen, the Netherlands	2008	1.0
Total		**32**

* one ECTS credit equals a study load of approximately 28 hours

Colophon

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