Identifying Gene Expression Differences Induced by Diets that Lead to Higher Omega-3 Fatty-Acid Deposition in Beef Cows

by

Aidin Foroutannaddafi

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Department of Agricultural, Food and Nutritional Science
University of Alberta

ABSTRACT

A strong emphasis on the type of fatty acids (FAs) consumed in the human diet has emerged in recent years. In this context, the various health benefits of consuming omega-3 (n-3) polyunsaturated fatty acids (PUFAs) have been widely reported. Supplementation of beef cattle diets with flaxseed, a rich source of alpha-linolenic acid (ALA), has been shown to increase n-3 FAs content of muscle and fat tissues. Towards the goal of enhancing beef FA composition, 64 crossbred cull cows (~30 months of age) with similar breed composition were randomized by weight/body condition, and fed one of four 50:50 forage:concentrate diets on a DM basis (16 cows/treatment), containing ground barley grain with either hay or silage, supplemented with 0 or 15% ground flaxseed (DM basis). Cows were slaughtered after spending 140 days on the treatment diets. For the initial transcriptional analysis, five cows from each of the four diets (20 in total), were selected using an index equation (Index=0.40(18:3n-3) + 0.60(Long Chain:3n)) which ranked cows based on the amount of alpha-linolenic acid (18:3n-3) and long chain omega-3 (3n) FAs in kidney fat collected at slaughter. Cattle scoring the highest indexes were selected from diets which included flax and those scoring the lowest indexes were selected from diets which did not contain flax. RNA was isolated from *Longissimus thoracis* (LT) muscle, subcutaneous fat (SC), and kidney fat (KF) tissues of each of the 20 cows, and hybridized in duplicate to BOMC 24K 70-mer microarrays. Differentially expressed (DE) genes (contrast: Flax-high index vs. No-Flax-low index) were identified using linear modeling and the empirical Bayes approach within Limma to produce moderated t-statistics and associated p-values (Limma package, Biocoductor), incorporating the BH significance correction for multiple tests. Sixty-eight and 166 transcripts with p-value < 0.05 and < 0.10 respectively, were found to be DE in the two tissues, LT muscle and SC fat combined (no DE genes were found in KF), between Flax-

high index, and No-Flax-low index groups. DE genes (p-value < 0.1) were imported to DAVID and IPA software, and resulted in identifying eight DE genes associated with FA metabolism. Fidelity of microarray results were tested for the eight DE genes by traditional real-time PCR. Subsequently 40 genes related to FA metabolism were selected for high-throughput real-time PCR gene expression analysis in all LT and SC samples collected from cattle in the feeding trial (31 and 61 samples for LT and SC, respectively) to identify expression differences in the flax-supplemented cattle versus non-supplemented. Out of 40 genes, eight were those DE associated with FA metabolism (p-value < 0.1) identified from the analysis of microarray data, while the remaining 32 genes were selected from a combination of the results of the microarray experiment (genes with p-value ≥ 0.1), literature, and in consultation with an Alberta Livestock and Meat Agency (ALMA) funded project 2010R038R - Identifying DNA markers for enhancing beneficial fatty acids in beef. Correlation analysis was performed between 40 genes and FA profiles of LT and SC tissues and resulted in identifying strong candidate genes for selection of cattle with a healthier FA profile in meat.

Dedication

This work is dedicated to my parents, Dr. Abdolreza Foroutannaddafi and Mrs. Vida Jahanara for their love, sacrifice, and tolerance. It is also dedicated to my beloved wife, Azadeh Yasari, for her support in every single moment of my life.

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LIST OF ABBREVIATIONS

AII	Angiotensin II
AA	Arachidonic acid
ALA	Alpha-linolenic acid
AP-O	Aminopeptidase O
aRNA	Amino-modified RNA
ATP	Adenosine triphosphate
BOMC	Bovine oligo microarray consortium
BTA19	*Bos taurus* autosome 19
BTA26	*Bos taurus* autosome 26
C/EBPα	CCAAT/enhancer-binding protein-α
CACT	Carnitine-acylcarnitine translocase
Calibrated rConc	Calibrated relative concentration
CHD	Coronary heart disease
CLA	Conjugated linoleic acid
CLNA	Conjugated linolenic acid
CNS	Central nervous system
CoA	Coenzyme A
Coenzyme Q	Ubiquinone
CPE	Ceramide phosphoethanolamine
CT	Cycle threshold
CVD	Cardiovascular disease
DAG	Diacylglycerol

DE	Differentially expressed
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FA	Fatty acid
FAME	Fatty acid methyl ester
GAL	GenePix Array List
GLA	Gamma-linolenic acid
GO	Gene ontology
GPR	GenePix results files
GPR120	G protein-coupled receptor 120
GPS	GenePix Settings files
GTP	Guanosine triphosphate
HB	Hay:Ground Barley
HBF	Hay:Ground Barley with Ground Flaxseed
HUFA	Highly unsaturated fatty acid
IDL	Intermediate-density lipoprotein
IKB	Ingenuity knowledge base
Inr	Pyrimidine-rich initiator
IP3	Inositol trisphosphate

IPA	Ingenuity pathway analysis
KF	Kidney fat
LA	Linoleic acid
LCFA	Long-chain fatty acid
LDL	Low-density lipoprotein
LT	*Longissimus thoracis*
MUFA	Monounsaturated fatty acid
n-3	Omega-3
n-6	Omega-6
OA	Oleic acid
OAA	Oxaloacetate
PA	Palmitic acid
PBMC	Peripheral blood mononuclear cell
PE	Phosphatidylethanolamine
PEP	Phosphoenolpyruvate
PUFA	Polyunsaturated fatty acid
QTL	Quantitative trait loci
RIN	RNA integrity number
RT	Reverse transcription
RXR	Retinoid X receptor
SB	Silage:Ground Barley
SBF	Silage:Ground Barley with Ground Flaxseed
SC	Subcutaneous fat

SFA	Saturated fatty acid
SNP	Single nucleotide polymorphism
SRE-1	Sterol regulatory element 1
ST	Semitendinosus muscle
TAG	Triglyceride or triacylglycerol
TCA cycle	Tricarboxylic acid cycle or citric acid cycle
TE	Thioesterase
TLR	Toll-like receptor
TM	Melting temperature
UFA	Unsaturated fatty acid
VA	Vaccenic acid
VLDL	Very low-density lipoprotein

CHAPTER 1: GENERAL INTRODUCTION

1.1. INTRODUCTION

Since the late 1950s, the American Heart Association has recommended that dietary cholesterol, saturated fat, and total fat should be reduced for the prevention of cardiovascular disease and stroke, which suggests to reduce consumption of fat and meat primarily from red meat (Lichtenstein et al., 2006, AHA, 2008); subsequently, several studies have investigated meat intake and cancer risk (Alaejos et al., 2008; Cross et al., 2007; Huxley et al., 2009). Components of meat linked to chronic disease risk include fat content and dietary cholesterol (Hu et al., 1999, Lichtenstein et al., 2006). Dietary suggestions have ranged from excluding fats completely from diets to moderate fat intake because of the role fat plays in the body (Laaksonen et al., 2005; Ohlund et al., 2008), as well as its effect on the eating quality of meat (Uemoto et al., 2011). Health problems are typically associated with saturated fatty acid (SFA) content of fat and the imbalance between the ratio of omega-6 (n-6) and n-3 PUFAs in the diet (Gutierrez-Gil et al., 2010; Simopoulos, 2008). Studies have shown that the consumption of PUFAs can have beneficial health effects for human well-being including reduction of cardiovascular diseases (Pan et al. 2012), inflammatory (Cabre et al., 2012), and neurological disorders (Kalmijn et al., 1997), as well as cancer prevention (Kim et al., 2009).

It has been reported that Western diets have a ratio of n-6:n-3 higher than 10:1, and the healthy effects are associated with ratios lower than 4:1 (Bartram et al. 1995; Broughton et al., 1997; Simopoulos, 2002; Simopoulos, 2008). Emphasis has now shifted from fat quantity to fat quality (Laaksonen et al. 2005; Ohlund et al., 2008) which suggests that the SFA fraction of diet should be reduced while the share of PUFA should be elevated. FA composition of beef contributes to quality from a fat-related, health standpoint.

Studies have shown that supplementation of flax (n-6:n-3 ratio in flax oil is 1:3) in the diet of cattle increases the n-3 content of beef and decreases the n-6:n-3 ratio (He et al., 2011; LaBrune et al., 2008; Maddock et al., 2006). Therefore, the inclusion of flax or flax oil in the diet of beef cattle can positively change the FA profile of meat (He et al., 2011). In the present study, the effects of supplementing flax in the diet of mature cull cows, and subsequent changes in FA profile of *Longissimus thoracis* muscle, subcutaneous fat, and kidney fat, in relation to changes in gene expression, were investigated. The objective of this project was to find those genes whose expression is associated with higher levels of long chain n-3 FAs, as well as those genes whose expression is associated with a lower content of SFAs. This was achieved by the use of a 24K spot microarray which was used to compare gene expression between Flax-high index and No-Flax-low index cattle. Once gene expression results were obtained, subsequent bioinformatic biological pathway analysis of the differentially expressed genes was performed to find those genes associated with metabolism of FA. Following that, genes for the high-throughput gene expression analysis were chosen partially from the results of microarray experiment that contrasted gene expression between Flax-high index and No-Flax-low index cattle (p-value $<$ 0.1), and the remaining were selected from a combination of the results of the microarray experiment (genes with p-value $\geq$ 0.1), literature, and in conjunction with an ALMA funded project 2010R038R (Identifying DNA markers for enhancing beneficial fatty acids in beef). Then, associations between gene expression values and FA measurements were investigated. Significant correlations between genes and FAs would prioritize candidate genes which can be used as a screening tool to select cattle that have the potential to produce healthier FA profiles in their tissue. This can be achieved by investigation for the presence of single nucleotide polymorphisms (SNPs) used for genetic selection.

1.2. THESIS OBJECTIVES

The aims of this project were: firstly to identify functional candidate genes that influence or are associated with FA metabolism in *Longissimus thoracis* muscle, subcutaneous fat, and kidney fat of beef cattle. This was accomplished by selecting a subset of animal tissues which would theoretically represent extremes of FA profiles, and the use of a 24,000 spot microarray, which at the time provided the best chance to capture a holistic view of the genes and pathways altered by n-3 supplementation. The second aim was to conduct a biological pathway analysis of DE genes identified from the microarray to interrogate genes and pathways affected by flax supplementation that could be related to FA content of muscle and fat. The third aim was to measure gene expression from DE genes identified by the microarrays, as well as genes identified from biological pathway analysis and from literature, on the entire cohort of tissue samples to understand the biological variation present in a larger population, and obtain a more precise measurement of gene expression that could be correlated to FA profiles of muscle and fat. Gene expression identified as significantly correlated to FA profile would indicate that these genes are candidates for the control of FA profiles in beef meat and meat products.

1.3. THESIS HYPOTHESES

The specific hypotheses of this study are as follows:

- n-3 PUFA supplementation (in the form of flax) will induce differences in host gene expression pattern and help to identify those genes and biological pathways related to FA metabolism and deposition in muscle and fat tissues.
- Association analysis between the identified genes and individual or groups of FAs will help to prioritize those genes related to FA metabolism as candidate genes to be used for selection to improve FA profile of meat.

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CHAPTER 2: LITERATURE REVIEW

2.1. INTRODUCTION

The relationship between diet, health and lifestyle is now important for consumers and researchers alike as we witness an increase in obesity and the rise of diet related diseases. Meeting consumer requirements for healthier meat will lead to adoption of new strategies by the beef industry to enhance the nutritional status of their products. In beef, dietary cholesterol and fat, especially saturated fats, are linked to chronic diseases especially cardiovascular disease (Lichtenstein et al., 2006; AHA, 2008) and cancer risk (Cross et al., 2007; Huxley et al., 2009). Improving the fat content of meat via dietary manipulation, or other means such as genetic selection, will lead to healthier meat products ultimately benefiting human health. In this context, the various health benefits of consuming n-3 PUFAs have been widely reported, particularly the evidence that n-3 exert a preventive effect for cardiovascular diseases, cancer, inflammatory, and neurological disorders (Berbert et al., 2005; Pan et al. 2012; Kim et al., 2009; Satoh et al., 2009; Kalmijn et al., 1997; Volker et al., 2000). In this chapter, we review the importance of essential fatty acids (EFAs) including n-3 and n-6, as well as how the amount of n-3 FAs is increased in the meat via the dietary sources to improve the FA profile of the meat. Moreover, the fate of FAs ingested by a ruminant animal from the rumen until their incorporation into muscle and fat tissues is described. In addition, the function of some key genes associated with FA metabolism, how diets containing n-3 and n-6 PUFAs affect expression level of these genes, and how genetic selection can be performed to improve FA content in meat, are reviewed.

2.2. ESSENTIAL FATTY ACIDS

Fatty acids are building blocks of lipids and different lipids have different functions in the animal body. Some have structural functions such as phospholipids and cholesterol (cholesterol also serves as a precursor for the synthesis of steroid hormones, bile acids, and vitamin D), and some

as a storage source of energy in the form of triglycerides (TAGs) (Dowhan et al., 2008; Lehninger et al., 2004). n-6 and n-3 PUFAs, well known as essential fatty acids, are important structural components of phospholipids which affect cell membrane properties, such as fluidity and permeability (Stillwell and Wassall, 2003; Raphael and Sordillo, 2013). EFAs have received attention for their preventive effect on cardiovascular diseases, cancer, inflammatory, and neurological disorders (Berbert et al., 2005; Pan et al. 2012; Kim et al., 2009; Satoh et al., 2009; Kalmijn et al., 1997; Volker et al., 2000). EFAs are a group of FAs that are required in the diet of mammals since they cannot synthesize them, and because they are essential for normal growth and development (Russo, 2009). Structures of different types of FAs are depicted in Figure 2.1. SFAs are those FAs which do not contain any double-bond in their structure, while unsaturated FAs (UFAs) are those having at least one double-bond in their structure. The UFAs containing one double-bond are known as monounsaturated FAs (MUFAs) while those have more than one double-bond are called PUFAs. A double-bond can have two configurations: if both hydrogen atoms of a double-bond are located at the same side this configuration is called *Cis*, and if they are located on the opposite sides this configuration is called *trans*. EFAs are sub classified as n-6 and n-3 FAs (Food and Nutrition Board, Institute of Medicine, 2002). In all n-6 FAs, the first double-bond is located between carbons six and seven from the omega end (the methyl end of FA chain length) of the FA (n-6), whereas in all n-3 FAs, the first double-bond is located between carbons three and four from the omega end of the FA (n-3). In an n-3 FA like ALA, 18:3 notation shows that ALA has 18 carbon atoms with three double-bonds in its structure and n-3 indicates that the first double-bond is in the omega-3 position, which defines it as an n-3 FA (Figure 2.1). For an n-6 FA such as linoleic acid (LA; 18:2n-6), 18:2 shows LA is an 18-carbon FA with two double-bonds and n-6 means that the first double-bond is in the omega-6 position.

Mammals can synthesize long-chain n-6 FAs (20 carbons or more), such as dihomo-gamma-linolenic acid (DGLA; 20:3n-6) and arachidonic acid (AA; 20:4n-6), from LA and long-chain n-3 FAs, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), from ALA (Food and Nutrition Board, Institute of Medicine, 2002).

2.2.1. Dietary sources of omega-3 and omega-6 fatty acids

n-3 FAs may be derived from both plant and animal sources. ALA is the major n-3 FA found in plant tissue and seeds. Oils high in ALA are perilla, flaxseed (also known as linseed), canola, echium, and chia (Abedi and Sahari, 2014). Algae contain significant amounts of n-3 FAs (Abedi and Sahari, 2014). Fish feeding on marine vegetation accumulate significant amounts of long-chain n-3s such as EPA and DHA in their tissues (Sanders, 1985 and 1987).

n-6 FAs are consumed in adequate quantities given widespread availability by most plant oil sources such as corn, soybean, sunflower, safflower, sesame, grains, and nuts (Barnard, 1998; Russo, 2009). Gamma-linolenic acid (GLA) is a less common n-6 FA which has anti-inflammatory effects (Kapoor and Huang, 2006), and can be found in plants like borage, hemp, and black currant (Barnard, 1998).

2.2.2. Omega-6:Omega-3 ratio

Anthropological and epidemiological studies have reported that human evolved on a diet with an n-6:n-3 FA ratio of 1:1 (Eaton and Konner, 1985; Leaf and Weber, 1987; Simopoulos, 1990) while in today's Western diets this ratio is between 10:1 and 20:1 (Simopoulos, 2008), indicating that compared to the diet on which humans evolved and their genetic patterns were established the n-3 FAs content of Western diets is low. Consuming a diet with n-6:n-3 ratio of 5:1 in patients suffering from asthma showed beneficial effect, while a ratio of 10:1 depicted adverse effects (Broughton, 1997). In patients with colorectal cancer, a diet with n-6:n-3 ratio of 2.5:1

decreased rectal cell proliferation, whereas that of with ratio of 4:1 showed no effect (Bartram et al. 1993, 1995). A high n-6:n-3 ratio promotes the pathogenesis of many diseases including cardiovascular disease, cancer, inflammatory, and autoimmune diseases, while a lower n-6:n-3 ratio (increased levels of n-3 FAs) exert suppressive effects (Berbert et al., 2005; Kim et al., 2009; Pan et al. 2012; Satoh et al., 2009; Simopoulos, 2008; Volker et al., 2000). n-6 compete with n-3 FAs for use in the body for the same elongation and desaturation enzymes, however both Δ-4 and Δ-6 desaturases prefer n-3 to n-6 FAs (Hague and Christoffersen, 1984 and 1986; Nassu et al., 2011). French and coworkers (2000) reported that the n-6:n-3 ratio in the intramuscular fat of grazing steers was less than those fed grain based diets. It also has been reported that supplementation of flax in the diet of cattle (the n-6:n-3 ratio in flax oil is 1:3), raises the n-3 content of beef and reduces its n-6:n-3 ratio (Kronberg et al., 2006; LaBrune et al., 2008; Maddock et al., 2006).

Eicosanoids such as prostaglandins, thromboxanes, and leukotrienes are metabolic products of AA and EPA (Weber et al., 1986; Lewis et al., 1986), and in Western diets increased amounts of n-6 FA fuels the production of these metabolic products from AA rather than EPA (Simopoulos, 1991). These metabolic products of AA are biologically active in very small quantities and if their amounts increase decidedly, they can cause atherosclerosis (accumulation of white blood cells and remnants of dead cells, including FAs, cholesterol and TAGs in the artery wall), allergic and inflammatory disorders (Simopoulos, 2008). It has been reported that LA enriched diets raise the amount of LA in low-density lipoprotein (LDL) and LDL oxidation (Reaven et al, 1991 and 1994) which is associated with severity of coronary atherosclerosis (Regnstrom et al., 1992). In addition, the American Heart Association (AHA, 2009) has reported that high intake of AA is associated with coronary heart disease (CHD), therefore decreasing consumption of LA

declines AA content of tissue, and as a result, lowers the inflammatory potential of AA and the risk of CHD. Therefore high amounts of n-6 FAs and a high n-6:n-3 ratio in human diet increase the pathogenesis of diseases such as cardiovascular disease, allergic and inflammatory disorders, on the other hand, reducing n-6:n-3 ratio (high levels of n-3s) have repressing effects.

2.2.3. Implications of omega-3 and omega-6 fatty acids for health

The potential benefits of n-3 and n-6 PUFAs have been widely reported in several areas, including cardiovascular diseases (Pan et al. 2012), cancer (Kim et al., 2009), inflammatory (Berbert et al., 2005; Satoh et al., 2009; Volker et al., 2000), and neurological disorders (Kalmijn et al., 1997). The mechanisms underlying why n-3 FAs can be associated with decreased rate of diseases have not been identified clearly but it can be partly related to n-6:n-3 ratio in the membrane phospholipids, inhibition of activation of the pro-inflammatory transcription factor, nuclear factor kappa B (NF-κB) and therefore reducing expression of inflammatory genes, and activation of the anti-inflammatory transcription factors such as peroxisome proliferator activated receptor γ (PPARγ) (Calder, 2013; Raphael and Sordillo, 2013; Stanley et al., 2012). Phospholipids consist of a glycerol molecule esterified at carbon 1 (sn-1) and carbon 2 (sn-2) positions to 2 FAs (in a cell membrane n-3 and n-6 PUFAs such as AA, EPA, and DHA are incorporated in the sn-2 position of phospholipids) and at carbon 3 (sn-3) position to a phosphorylated head group (Figure 2.2) (Raphael and Sordillo, 2013). It has been reported that n-3 FAs can increase membrane fluidity (presence of SFAs in phospholipids make them less fluid and more susceptible to rupturing) and changes in the structure and fluidity of cell membranes can change function of neurotransmitters, receptors, and ion channels (Song 2007). They also reported that substituting n-6s with n-3s, conferring less inflammatory properties than n-6s, should decrease the inflammatory impacts associated with inflammatory neurodegenerative

diseases such as Alzheimers and Parkinsons (Song 2007). Heller et al. (2003) also reviewed that during inflammatory stimulation of rabbit lungs by calcium ionophore A23187 (which triggers AA metabolism) which caused increased lung weight, dietary fish oil decreased inflammation and therefore reduced the increased lung weight by 50% compared to controls which received n-6 FAs. Therefore, ingestion of n-3 dietary sources, especially fish oil which contains EPA and DHA, is associated with higher incorporation of these FAs in the membrane phospholipids resulted in reduced inflammation in persons suffering from inflammatory diseases.

Research has shown that obtaining about 5-10% of total caloric intake from n-6 PUFA can decline risk of CHD relative to lower intakes (Kris-Etherton et al., 2010). Cholesterol is transported in the blood plasma within protein particles, lipoproteins, and accumulation of the lipoproteins other than high-density lipoprotein (HDL; high levels of HDL carries cholesterol back to the liver for excretion or re-utilization and associated with reduced CHD risk) in the artery wall can cause atherosclerosis and therefore increase the risk of CHD (Kontush and Chapman, 2006; Simopoulos, 2008). Mensink and Katan (1992) reported LA is the most potent FA for lowering serum total and LDL cholesterol when substituted for dietary SFA. Increased expression of LDL receptor and redistribution of LDL cholesterol from plasma to tissue, increased bile acid production and cholesterol catabolism, and decreased conversion of very low-density lipoprotein (VLDL) to LDL, are the mechanisms by which LA lowers blood cholesterol (Fernandez and West, 2005).

Pan et al. (2012) reported that higher dietary consumption of ALA is associated with lower risk of cardiovascular disease (CVD). Other studies also showed the anti-inflammatory, anti-thrombotic and anti-arrhythmic effects of n-3 FAs especially EPA and DHA which can be

helpful for prevention of atherosclerosis (Moreno et al., 2003) and CHD (Mozaffarian et al., 2005).

Similar to cholesterol, TAG is transported in the blood plasma within lipoproteins and accumulation of the lipoproteins in the artery wall can cause atherosclerosis and therefore increase the risk of cardiovascular diseases and stroke (Kontush and Chapman, 2006; Simopoulos, 2008). Many controlled clinical experiments have depicted that high EPA and DHA consumption can significantly lower serum TAG concentrations (as reviewed by Jacobson et al., 2012). A 2004 study showed that fish oil supplements, rich in EPA and DHA, caused a net decrease in TAG to about 10-33% (Balk et al., 2004). One possible mechanism to explain how these n-3 FAs reduce TAG is that EPA and DHA increase lipoprotein lipase activity which leads to an increase in chylomicron TAG clearance (Harris et al., 2008; Khan et al., 2002; Park and Harris, 2003).

DHA and AA are found at high concentrations in the phospholipids of the brain's gray matter suggesting that they are important for the function of central nervous system (CNS) (Innis, 2008). Studies showed the positive relationship between high fish intake and lower risks of; dementia (Kalmijn et al., 1997), impaired cognitive function (Kalmijn et al., 2004), and Alzheimer's disease (Kalmijn et al., 1997). Mitigation of inflammation, improved cerebral blood flow, and reduced amyloid aggregation are those mechanisms which have been proposed for the protective effect of n-3 FAs in the brain and vascular system (Fotuhi et al., 2009).

Several studies revealed that a n-3 FA enriched diet or high n-3 FA content in red blood cell (erythrocyte) membranes were negatively associated with the development of breast cancer (Kuriki et al., 2007; Kim et al., 2009) and colorectal cancer (Kimura et al., 2007; Theodoratou et

al., 2007). On the other hand, a high animal fat or n-6 containing diet is positively correlated to the increased risk of breast (Larsson et al., 2004) and colorectal cancers (Giovunncci and Willett, 1994; Ames et al., 1995). In a 2007 study, significant reductions in colorectal cancer were observed when high amounts of n-3 FAs were consumed, and also when EPA or DHA were taken individually (Theodoratou et al., 2007).

Inflammatory diseases such as rheumatoid arthritis (a chronic inflammatory autoimmune disease of the joints and bones) and metabolic syndrome (individuals with this syndrome are potent to cardiovascular disease and diabetes mellitus, type 2) may also be reduced by the dietary sources of n-3 FAs (Berbert et al., 2005; Satoh et al., 2009; Volker et al., 2000). Volker et al. (2000) reported improvements in clinical status in patients with rheumatoid arthritis when dietary fish oil was consumed by patients. Similarly, Berbert et al. (2005) reported higher reduction of rheumatoid arthritis symptoms when fish oil supplements were used compare to soy oil. A study conducted by Satoh et al. (2009) showed reduction in the frequency of cardiovascular disease development in patients having metabolic syndrome when the diet of patients were supplemented with EPA compared to the control group.

Therefore, both n-3 and n-6 PUFAs are important components of cell membranes and n-3 FAs are associated with reduced risk of cardiovascular diseases, cancer, inflammatory, and neurological disorders, on the other hand, high levels of n-6 FAs can have an adverse effect.

2.2.4. Nutritional supplements to improve the omega 3 content of products

Limiting fish intake due to seasonal availability, and low ability and preference of customers to buy fish excludes this main source of n-3 FAs in many areas of the world. ALA is a more stable form of n-3 PUFA available in plant sources including green leafy vegetables and commonly

consumed oils such as flaxseed, soybean, and canola oils, and walnuts (Mozaffarian, 2005; Mozaffarian et al., 2005; Williams and Burdge, 2006). However, dietary EPA and DHA provided from marine sources have been shown to have more beneficial effects for human health as compared to ALA, since conversion of ALA to EPA and DHA in the human body is limited (about 8% for EPA and < 0.1% for DHA) (Williams and Burdge, 2006).

Several studies have been carried out to improve n-3 FA content of pork (Coates et al., 2009), beef (Medeiros et al., 2007) plus both chicken egg and meat (Leskanich and Noble, 1997). The results of the study conducted by Coates et al. (2009) showed that compared to the control diet, regular consumption of n-3 enriched pork improved cardiovascular risk factors. In this experiment, the n-3 enriched pork was obtained by including 15% PorcOmega (a fortified tuna fishmeal product) in the diet of the pigs.

Inclusion of marine oils or meals in chicken diet can enhance n-3 FA content of both poultry meat and eggs (Hargisa and Van Elswyk, 1993). Plant sources are also effective in enriching meat and egg products with ALA (Leskanich and Noble, 1997). Flaxseed or linseed (*Linum usitatissimum*) is well established as a rich source of ALA (Kronberg et al., 2006; Zachut et al., 2010). The form in which flaxseed is fed affects the amount of n-3s deposited into the egg, for example inclusion of 10% whole flaxseed into the diet of chickens yielded approximately 13.5 mg ALA per gram of yolk whereas 10% milled flaxseed yielded 16.2mg ALA/g yolk (Van Elswyk 1997). These differences suggested that possibly ground flaxseeds are better digested than whole seeds, which causes more release of n-3 FAs incorporated into the yolk.

In ruminant animals, rumen microorganisms, hydrogenate up to 95% of dietary PUFA, and also produce a unique group of FAs, conjugated linolenic acid (CLNA) and conjugated linoleic acid

(CLA), which are reported to be beneficial for human health (Destaillats et al., 2005). CLA and CLNA are hydrogenation intermediates that are deposited into meat and milk, before becoming completely converted to stearic acid (18:0) (Destaillats et al., 2005; Lee and Jenkins, 2011). Rumenic acid (RA) (Cis-9, trans-11-18:2) is the most significant isomer of CLA which is partly produced from incomplete hydrogenation of LA and ALA in the rumen, but most of that is created from the desaturation of vaccenic acid (VA), another intermediate of rumen hydrogenation, in milk fat and body fat of lactating cows (Griinari, 2000). Both the cow's genetics and the type of the diet fed to the cows impact the composition of milk fat, although diet currently plays a much larger role (Dewhurst et al., 2006). Compared with other ingredients in dairy diets, fresh forage contains high amounts of ALA, and a high intake of fresh growing forage increases ALA and LA which partly passes unchanged into the milk or are converted to VA, RA and other conjugated isomers of LA and ALA (Butler et al., 2011).

In beef cattle, many experiments have shown that supplementing flax in the diet can raise the n-3 and decrease the n-6:n-3 ratio content of beef (Kronberg et al., 2006; LaBrune et al., 2008; Maddock et al., 2006). In beef cattle, grazing can increase the levels of ALA as compared to a concentrate diet. French et al. (2000) reported that more ALA was incorporated in adipose tissue of steers when the proportion of grass in the diet was increased. Moreover, duration of consuming forages may also impact n-3 FA concentration in *Longissimus* muscle. It was reported that increasing the duration of grazing resulted in increased level of ALA [muscle: from 1.03 to 1.29 mg/100 g of muscle, $P < 0.05$; subcutaneous fat: from 0.52 to 0.88 mg/g of subcutaneous fat, $P < 0.05$] and decreased ratio of n-6:n-3 in both muscle [from 2.21 to 1.46 ($P < 0.05$)] and subcutaneous fat [from 2.64 to 1.65 ($P < 0.05$)] tissues (Noci et al., 2005). Scollan et al. (2001) also reported an elevated level of ALA in *Longissimus* muscle when duration of forage

feeding increased. In terms of potential downstream health benefits for consumers of n-3 enriched health products, the result of an experiment done by Medeiros and coworkers (2007) revealed that feeding enriched n-3 FA beef (cattle raised on a 10% flaxseed diet that had high amounts of ALA in their muscle tissue compared to cattle fed a control diet of corn) to rats increased n-3 PUFA content of heart and liver membranes and decreased serum cholesterol levels. Similarly, McAfee et al. (2011) compared the effect of red meat (beef and lamb) consumption from grass-fed animals compared to concentrate-fed animals on consumer blood concentrations of n-3 PUFAs. The results showed that due to the higher availability of n-3 FAs in grass-fed animals compared to cereal-fed animals, individuals consuming grass-fed meat had significantly higher levels of n-3s in plasma and platelets, compared with those eating concentrate-fed meat.

Therefore, even if n-3 FAs are not be provided from marine sources for human consumption, pork, beef, plus both chicken egg and meat enriched with n-3 FAs from the animals' diet are viable sources of n-3 in food that can be added to human diet to improve the dietary FA profile required for human well-being.

2.3. METABOLISM OF LIPIDS IN RUMINANT ANIMALS

The two fats of greatest interest in beef are intramuscular fat and subcutaneous fat which can be manipulated to benefit consumer health (Scollan et al., 2006). The intramuscular fat is composed of phospholipids, which forms the membranes of cells and organelles, and TAG deposited in adipose cells adjacent to muscle as a condensed source of energy, also referred as marbling (Scollan et al., 2006). The fat profile of muscle is associated with the extent of the marbling and its composition which is the fraction that can be altered through genetics and diet intervention,

since the fraction of muscle phospholipids is fairly consistent and contains around 50% PUFA (Abbott et al., 2012; Dannenberger et al., 2004). However, amounts of SFAs and MUFAs especially palmitic acid (PA, C16:0) and oleic acid (OA, cis-9-18:1) in subcutaneous fat are high, while PUFA content of subcutaneous fat is approximately 2–3 % of total FA (Wood et al., 2008). The concentrations of n-3 and n-6 PUFAs are high in lean beef but these high amounts can be diluted with SFA and MUFA when carcass fatness increases, especially if they will be incorporated into the muscle as marbling (Kamihiro, 2013).

In ruminant animals, the net deposition of lipids is coming from three sources including *de novo* lipogenesis (lipid synthesis) in the cytosol of the fat cell, direct deposition from the diet, and microbial synthesis (Laliotis et al., 2010). When dietary fats reach the rumen they are transformed before absorption in the intestine; therefore the amount and types of fats leaving the rumen is different from what is ingested by the animal. Dietary FAs are broken down in the rumen by enzymes (lipases, galactosidases and phospholipases) produced firstly from different bacteria (i.e. *Anaerovibrio lipolytica*) and secondly from protozoa (Harfoot and Hazlewood, 1988). These enzymes can break the bonds between the FAs and the glycerol to release free FAs and glycerol. Glycerol is fermented rapidly into volatile FAs and some FAs are used by bacteria for the synthesis of phospholipids, the building blocks of cell membranes. Following hydrolysis, biohydrogenation occurs by rumen microorganisms (Wattiaux and Grummer, 2004, Noble, 1981). When a high grain diet is ingested, the fraction of lipolytic and biohydrogenating microbes is lower than that of a high forage diet, allowing greater escape of unbroken fats (Byers and Schelling, 1993). As well, the extent of lypolysis is associated to the type of fat fed. For example, plant oils such as flaxseed oil are hydrolyzed more completely (about 90%) compared

to fish oils (less than 50%) (Byers and Schelling, 1993). Limits to hydrolysis results in decreased fat modification by the rumen microbes (Byers and Schelling, 1993).

During hydrogenation, hydrogen atoms are added to the double-bonds of FAs. Almost all UFAs in plants have a *cis* form between the double-bounds and consequently depot fat in non-ruminants reflects the diet and most FAs present will be in the *cis* configuration (Byers and Schelling, 1993). In ruminant animals rumen microorganisms are capable of producing different *trans* isomers of FAs, and can change the chain length and position of double-bonds, therefore creating odd- and branched-chain FAs. As a consequence, all of these changes make depot fat of ruminants noticeably different from the fat provided by the diet. The rumen protozoa and bacteria perform *de novo* synthesis of long-chain FAs (LCFAs) (Byers and Schelling, 1993). The precursors used for FA synthesis include odd-, even-, and branched-chain substrates which causes production of odd-, even-, and branched-chain FAs, respectively. For instance, from the odd-carbon substrates such as propionic and valeric acid, odd-chain FAs will be produced, whereas butyric and caproic acids, the two even-chain substrates, result in even-carbon FAs, and iso-acids (isobutyric and isovaleric) result in odd-, even-, and branched-chain FAs (Byers and Schelling, 1993). Rumen microorganisms can also change the length of FAs through α- and β-oxidation (Byers and Schelling, 1993). Stearic acid (C18:0) is the final product of hydrogenation of the FAs containing 18 carbons in their structure. However, if a high concentration of LA is provided, hydrogenation is halted before this last step, resulting in different *cis* and *trans* isomers of C18:1 FA (Harfoot et al. 1973).

Microbial phospholipids compromise between 10-15% of the fats reaching the ruminant small intestine and the remaining are free (non-esterified) SFAs, mostly PA (C16:0) and stearic acids

bound to feed and microbial particles (Wattiaux and Grummer, 2004). Limited buffering of pancreatic secretions (have low levels of bicarbonate which is alkaline) lead to low pH of the ingesta leaving the abomasum and also this pH remains low through the upper half of the small intestine. Therefore, protonation of FAs takes place at this pH and FA soaps which were insoluble in the rumen are solubilised resulting in elevated FA and mineral absorption (Byers and Schelling, 1993). FAs need to be transferred from the insoluble form to the soluble phase and emulsified into the micellar form for digestion and both pancreatic juice and bile (secreted from the liver) are required for normal FA digestion (Byers and Schelling, 1993; Noble, 1981). Both the bile and pancreatic ducts join together at the beginning of duodenum but the amount of bile coming from the liver is higher than pancreatic juice in ruminant animals (Moore and Christie, 1984). Overall, bile acid and pancreatic juice are mixed with the contents of the small intestine and form water miscible particles, micelles, which can enter the enterocytes (intestinal cells) (Bauchart, 1993). Several steps are involved in absorption. In summary, FAs are absorbed into enterocytes through the microvilli, re-esterified (which occurs in the smooth endoplasmic reticulum (ER) membranes) with glycerol (coming from blood glucose) to form TAGs, and then TAGs are coated with phospholipids, apoproteins (or apolipoproteins which are produced in the rough endoplasmic reticulum) and cholesterol to form the lipoprotein particles, chylomicrons, in the Golgi apparatus, which will be released into the intercellular space by exocytosis. They exit through the cells to the lymph system and then plasma (Byers and Schelling, 1993; Moore and Christie, 1984). FAs having less than 14 carbons in their chain enter the blood directly, and are then transferred to the liver where rapid oxidation of FAs takes place (Byers and Schelling, 1993; Moore and Christie, 1984). In ruminants, the absorbed FAs are mostly embedded into VLDL as compared to chylomicrons, while in non-ruminants FAs are predominately incorporated in

chylomicrons (Moore and Christie, 1984). The reason is that in ruminants, surface film (phospholipids, cholesterol and apoproteins) synthesis coincides with synthesis of core material (TAGs), and results in formation of small VLDL particles, while in non-ruminants synthesis of TAGs is faster than surface film synthesis, and large chylomicrons particles result (Moore and Christie, 1984). It is important to note that chylomicron particles are synthesized from the dietary fat while VLDL particles are synthesized in the liver. In ruminants, when the chylomicrons and VLDL enter the plasma, they acquire apoproteins, apo-C and apo-E, from plasma HDL which was synthesized in the liver. Apo-C inhibits catabolism of chylomicron and VLDL in the liver and allows their diversion to other tissues such as muscle, adipose, and mammary tissues. Once chylomicron and VLDL particles reach the tissues, one of the apo-C components (apo-C_{II}) activates lipoprotein lipase (LPL) in the lining capillaries in skeletal muscle, fat depots and the mammary gland. LPL lipolysis the core material, TAGs, of the plasma chylomicron and VLDL to non-esterified FAs (NEFAs) and glycerides which are then taken up by cells and used for energy (FAs will be oxidized to release energy), or will be esterified to form new TAGs in adipose tissues, muscle, and mammary glands (Noble, 1981; Moore and Christie, 1984). About 80% of core TAGs from chylomicron and VLDL particles are removed by LPL which converts these lipoprotein entities to the smaller particles, LDL or intermediate-density lipoprotein (IDL), which contain less TAGs compared to chylomicron and VLDL. LDL and IDL are then taken up and metabolized by the liver and then liver releases TAG as VLDL. Upon the act of LPL, most part of the surface film (cholesterol, phospholipids, and apo-C) is also removed from chylomicron and VLDL and transferred to HDL to circulate in plasma (Noble, 1981; Moore and Christie, 1984). Regarding the adipose tissue, these FAs may be released into the plasma to be delivered to other tissues, and in the liver they will be re-esterified within the enoplasmic

reticulum to make TAGs which will be secreted as VLDL. Skeletal and heart muscle (and other oxidative tissues) are the only pure consumers which use these FAs (taken up either from lipoprotein-TAG or from the plasma NEFA pool) for oxidation (Frayn et al., 2006).

2.3.1. Fate of fatty acids in different tissues

Flow of FAs between tissues is related to nutritional state. In the fed state, excess nutrients are stored due to the action of insulin, which is released in response to the high blood glucose, which causes up-regulation of LPL in adipose tissue and suppresses fat mobilization while down-regulating muscle LPL (Frayn et al., 2006). Muscle LPL is more active during a fasting state or exercise conditions when higher amounts of FAs are required for muscle (Frayn et al., 2006). Due to the LPL activity in adipose tissue, lipoprotein particles will be hydrolysed, part of the released FAs will be taken up by the adipocyte (FAs are esterified to form new TAG in the adipocyte) and the remaining will be released as NEFAs into the plasma which are then taken up by other tissues such as muscle and liver (Frayn et al., 2006). Insulin increases the amount of LPL-derived FAs taken up by the adipose tissue but even after a meal about 50% may be released as NEFA into the plasma (Frayn et al., 2006). Most of the TAG in adipose tissue fat stores comes from the TAG fraction of plasma lipoproteins (from the diet) and with a small contribution of *de novo* lipogenesis (the synthesis of FAs from glucose and other non-lipid precursors) (Frayn et al., 2006). During starvation, adipose tissue fat mobilization takes place when the stored TAGs will be hydrolysed by hormone-sensitive lipase (HSL or LIPE), which is capable of removing two FAs of TAG but cannot hydrolyse monoacylglycerols, and there is a separate monoacylglycerol lipase (that is constitutively expressed in adipose tissue) which catalyses the last step in TAG lipolysis. Subsequently, FAs are released as NEFAs into the plasma and are bound to the serum albumin and transported to other tissues including skeletal

muscle and heart, then FAs dissociate from albumin and enter the myocyte (muscle cell), and are oxidized to release ATP which fuels muscle contraction and other energy requiring metabolisms in the muscle cell (Frayn et al., 2006; Lehninger et al., 2004). Most FAs taken up by the liver are converted to TAG and returned to adipose tissue.

The liver obtains plasma FAs as NEFA, from lipolysis of circulating TAG and from the uptake of remnant lipoprotein particles (Frayn et al., 2006; Lehninger et al., 2004). FAs in the hepatocytes (in outer mitochondrial membrane) are esterified to CoA by acyl-CoA synthetases (ACSSs) to form fatty acyl-CoA which may then enter the mitochondria and be oxidized for ATP production or be used in the endoplasmic reticulum and mitochondria as a substrate to synthesize phospholipids, or recycled as new TAGs in the endoplasmic reticulum where VLDL is produced (Frayn et al., 2006; Lehninger et al., 2004). CPT1 (carnitine palmitoyltransferase 1) is a key regulatory enzyme that determines the transfer of acyl-CoA from the cytosol to the mitochondria (Frayn et al., 2006; Lehninger et al., 2004). Activity of CPT1 is regulated by the cytosolic malonyl-CoA (the first intermediate in the cytosolic synthesis of long chain FAs which is made from the acetyl-CoA) concentration, which is an inhibitor of CPT1 (Frayn et al., 2006; Lehninger et al., 2004). In a fed state when a high carbohydrate food is consumed, the blood glucose level is increased which triggers secretion of insulin. Then insulin-dependent protein phosphatise dephosphorylates acetyl-CoA carboxylase (ACC, the first enzyme in the FA synthesis) to activate it and as a result of that ACC catalyzes the formation of malonyl-CoA from acetyl-CoA. High concentration of malonyl-CoA causes FA synthesis rather than its oxidation, while during starvation or between meals when blood glucose is low and thereby insulin level is low (glucagon level is high), FA oxidation dominates (Frayn et al., 2006; Lehninger et al., 2004). During the oxidation of FAs, acetyl-CoA formed in the liver (during the mitochondrial beta-

oxidation of FAs, acyl-CoA is converted to acetyl-CoA) may either enter the tricarboxylic acid (TCA) cycle to produce energy or may undergo synthesis of ketone bodies including acetone, acetoacetate and D-beta-hydroxybutyrate (Frayn et al., 2006; Lehninger et al., 2004).

Muscle receives FAs from the plasma albumin-bound NEFAs or from the FA pool produced by the hydrolysis of circulating TAGs by LPL (Frayn et al., 2006). In muscle, due to the hydrolysis of TAGs (most likely HSL is the key enzyme controlling this process), FAs are released, enter into the mitochondria to be used as a substrate for beta-oxidation to provide energy, or they can be used for esterification to produce TAGs (Frayn et al., 2006; Lehninger et al., 2004). TAGs are stored in muscle mostly in two parts: some in adipose cells located between the muscle fibres (marbling in a cut of meat), and those stored within the muscle fibre itself (Frayn et al., 2006). Therefore the tissues, adipose, muscle and liver, cooperate together to coordinate FA metabolism and a good example of that is mobilization of fats from adipose tissue to the skeletal muscle to prepare its demands during physical activity (Frayn et al., 2006; Lehninger et al., 2004).

2.4. Genes associated with fatty acid metabolism

There are many genes identified in the metabolism of FAs, but few that we have found a direct connection between genetic differences and FA levels. Genes such as Stearoyl-CoA desaturase [*SCD*] and Diacylglycerol O-acyltransferase 1 [*DGAT1*] are the two well-known examples of these genes in which researchers have found polymorphisms associated with FA composition in beef and dairy cattle (Avilés et al. 2013; Kgwatalala et al., 2009; Taniguchi et al. 2004; Oh et al., 2011). Several studies have identified quantitative trait loci (QTL) and single nucleotide polymorphisms (SNPs) associated with FA composition in beef cattle. Some of these SNPs have been found in genes which directly play important roles in FA metabolism in cattle (Kgwatalala

et al., 2007; Oh et al., 2011) and others in gene regulatory elements which can control the expression levels of these genes (Hoashi et al., 2007; Sampath and Ntambi, 2006).

SCD (a delta-9-desaturase) is an endoplasmic reticulum resident enzyme that catalyzes desaturation of SFAs to MUFAs (Ohsaki et al., 2007), as well as trans-vaccenic acid (18:1 trans-11) into an n-7 FA, rumenic acid (cis-9, trans-11 CLA) in the bovine (Griinari et al. 2000). Bovine *SCD* gene has been mapped on *Bos taurus* autosome 26 (BTA26) (Campbell et al., 2001). Polymorphisms within *SCD* have been found to affect the FA composition in both meat and milk of cattle (Taniguchi et al., 2004; Kgwatalala et al., 2009). Li et al. (2012) surveyed the associations a non-synonymous coding SNPs of bovine *SCD* (c.878C>T) with FA profile of brisket adipose tissue of 223 Canadian commercial cross-bred beef steers. The results showed that the 'CC' SNP was significantly correlated to higher amounts of MUFAs and PUFAs and lower concentrations of SFAs. The results suggested that *SCD* is a strong candidate gene influencing FA composition in beef cattle and selection based on 'CC' SNPs can lead to higher deposition of MUFAs and PUFAs in beef adipose tissue.

Fatty acid synthase [FASN] and Sterol regulatory element-binding transcription factor 1 (or Sterol Regulatory Element-Binding Protein 1, SREBP1) [SREBF1] are other key enzymes in metabolism of FAs. The enzyme encoded by *FASN* gene is a multifunctional protein that regulates *de novo* biosynthesis of long chain SFAs (Roy et al., 2001). Its main function is to catalyze the synthesis of palmitic acid from acetyl-CoA and malonyl-CoA, in the presence of NADPH. Among SFAs, lauric acid (C12:0), myristic acid (C14:0) and PA (C16:0) are associated with the most harmful cardiovascular effects which raise serum cholesterol (Keys et al. 1974). The location of bovine *FASN* is on *Bos taurus* autosome 19 (BTA19) where several QTL affecting beef FA composition (Zhang et al., 2008), adipose fat and milk fat content (Morris et

al., 2007; Roy et al., 2006) were found. Bovine *FASN* expression was higher in brain, testis and adipose tissue than in liver and heart (Roy et al., 2005). In the *FASN* complex four exons (39 to 42 located at the 3′-end of *FASN*) encode for the thioesterase (TE) domain are responsible for synthesis of FA. Zhang et al. (2008) found three SNPs, AF285607: g.17924A>G, g.18663T>C and g.18727C>T, in the TE domain of the bovine *FASN* gene in Angus bulls, out of which g.17924A>G and g.18663T>C were significantly associated with FA profile of *Longissimus dorsi* muscle. With the SNP 17924A>G, those animals with g.17924GG genotype had lower myristic acid, PA, and total SFAs, and higher oleic acid (C18:1), total MUFAs, and health index in the total lipid and triacylglycerol fraction compared to the animals with SNP g.17924AA. A similar association was also found in animals with SNP g.18663CC as compared to cattle with g.18663TT genotype. The results suggests that g.17924A>G and g.18663T>C SNPs can be used in breeding programs to produce beef that have a healthier FA composition.

It has been demonstrated that SREBF transcription factors play key roles in the regulation of *FASN* and *SCD* (Hoashi et al., 2007; Shimano et al., 1999). *SREBF1* is a transcription factor which binds to the Sterol regulatory element 1 (SRE-1), a decamer in 5′ flanking of the genes encoding the low density lipoprotein (LDL) receptor, which enhances transcription when cells are depleted of sterols, and is silenced by accumulation of sterols (Yokoyama et al., 1993). *SREBF1* regulates FA and cholesterol (which is derived from plasma LDL) synthesis, therefore it controls lipid homeostasis. When the sterol level decreases, *SREBF1* binds to SRE-1 and activates transcription to maintain FA homeostasis, or activates cholesterogenic genes to maintain cholesterol homeostasis (Eberlé et al., 2004). Similar to *FASN*, *SREBF1* is also located on the *Bos taurus* autosome 19 (BTA19). Since *SREBF1* regulates the expression of the *SCD* gene, Hoashi et al. (2007) surveyed the presence of polymorphisms in *SREBF1* in the bovine.

They identified an 84 base deletion (Short type: S) and an insertion (Long type: L) in intron 5 of *SREBF1* in Japanese Black cattle. Results showed that the S type was associated to a higher MUFA proportion and a lower melting point in intramuscular fat and suggested that this polymorphism may serve as a potential genetic marker to improve FA profile of beef.

Fatty acid desaturase 1 (D5D, delta-5 desaturase) [FADS1] and fatty acid desaturase 2 (D6D, delta-6 desaturase) [FADS2] are the key enzymes for the synthesis of highly UFAs (HUFAs), such as AA and DHA, that are incorporated in phospholipids and perform essential physiological functions (Tanaka et al., 2009). It has been reported that two DNA polymorphisms in the promoter region of *FADS1*, deletion/insertion of GTG in the case of rs133053720 and SNP rs42187276 [A/G], were significantly associated with concentrations of some FAs in brisket adipose tissue of Canadian crossbred beef steers (Han et al., 2013). Animals with DD (deletion/deletion) genotype of 133053720 and SNP GG in rs42187276 had greater C17:0, total branched chain FAs, 12t C18:1, 13t/14t C18:1, and 15t C18:1, but lower 13c C18:1 concentration in brisket adipose tissue. These polymorphisms in *FADS1* may also serve as potential genetic markers in breeding programs to improve FA profile of beef that are of benefit to human health.

2.5. Concluding remarks

During the past few decades consumers have started seeking animal products of low fat content, and are especially conscious of SFAs which are harmful for human health. Depot fat in non-ruminants reflects the diet, whereas in ruminant animals, irrespective of a diet type, approximately 90% of lipids reaching to the small intestine are SFAs which is due to the biohydorgenation of lipids by the rumen microbes. However, research has shown that inclusion of a source of n-3 PUFAs into the diet of cattle can increase the amount of these healthy FAs in

both muscle and fat tissues of the animal. The objectives of this chapter were: firstly to show the importance of n-3 and n-6 PUFAs for health, and how to improve the FA profile of meat via the dietary sources of n-3 FAs. The second aim was to explain the fate of FAs ingested by ruminants from the rumen until their incorporation into muscle and fat tissues. The third aim was to show some important genes associated with FA metabolism in the meat of beef cattle as well as their polymorphisms related to the increased healthier FAs or decreased harmful FAs in meat. Therefore, we can use these polymorphisms in breeding programs to produce beef that have a healthier FA composition, without phenotyping them.

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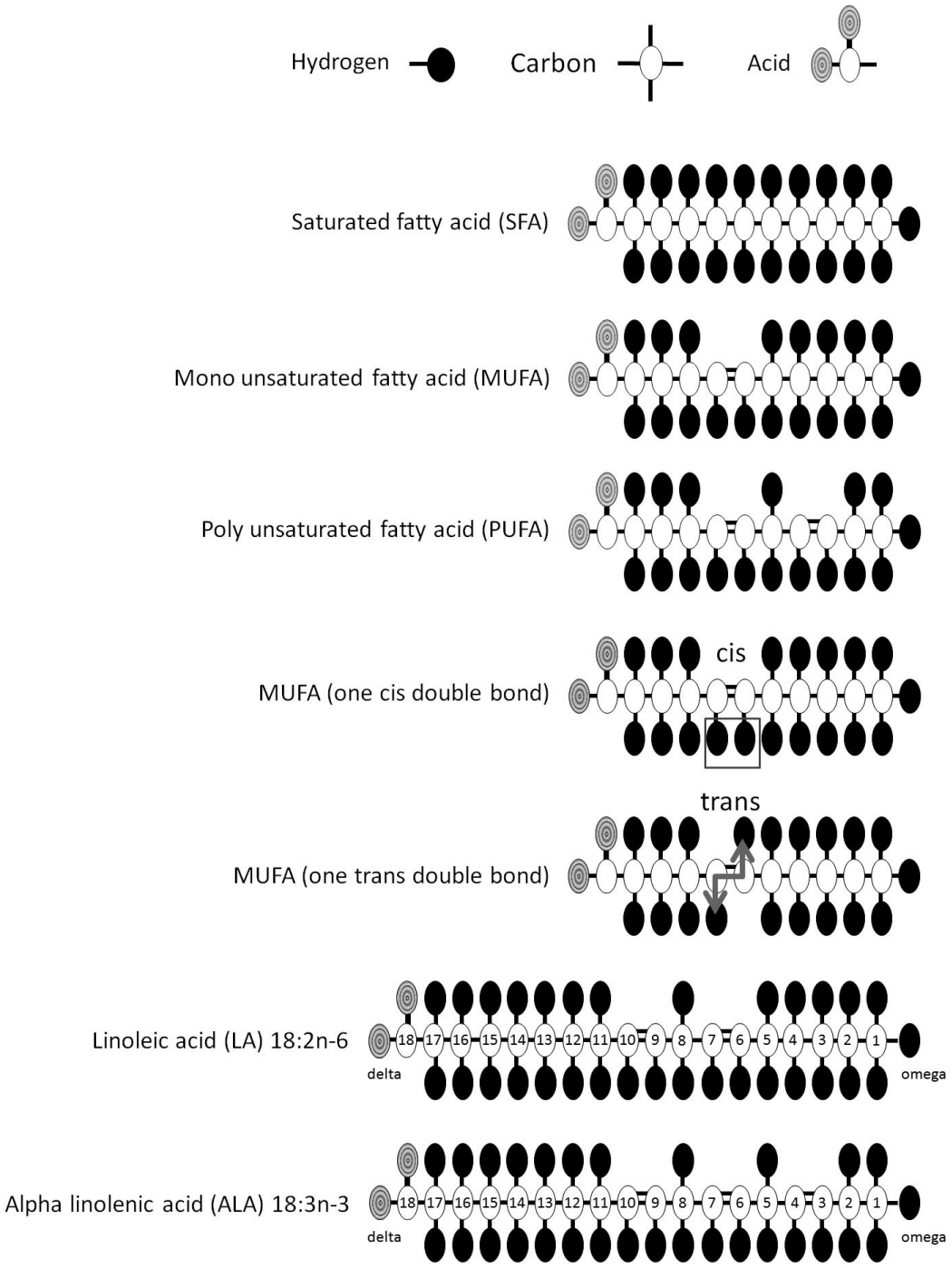

Figure 2.1. Structure of different types of fatty acids

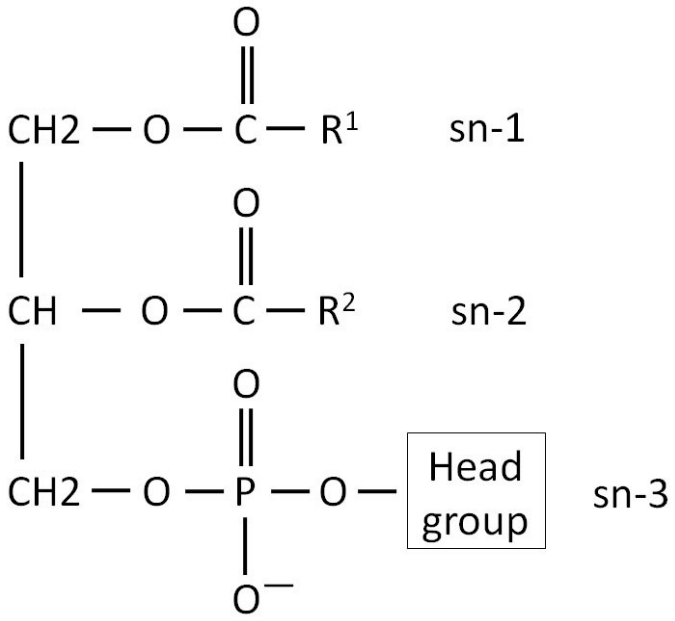

Figure 2.2. Structure of phospholipids

CHAPTER 3. TRANSCRIPTIONAL ANALYSIS IN *LONGISSIMUS THORACIS* MUSCLE, SUBCUTANEOUS AND KIDNEY FAT OF CATTLE FED DIETS WITH SUPPLEMENTAL FLAXSEED

3.1. INTRODUCTION

Type of FAs in the human diet is of importance to health-conscious consumers. The potential benefits of n-3 and n-6 PUFAs have been widely reported related to cardiovascular diseases, cancer, inflammatory, and neurological disorders (Cabre et al., 2012; Kalmijn et al., 1997; Kim et al., 2009; Pan et al. 2012). Feeding flaxseed, a dietary source of ALA, has shown to increase levels of n-3 FAs in pork, poultry, dairy products (Legrand et al., 2010). As well, n-3 FAs provided by flax in the diet of cattle has been shown to raise the n-3 FA content and reduce the n-6:n-3 ratio in intramuscular fat of beef steers (He et al., 2011; LaBrune et al., 2008; Maddock et al., 2006). Hence, supplementing the diet of beef cattle with flax or flax oil has the potential to beneficially change the FA profile of the meat, and therefore make it more appealing to health-conscious consumers (He et al., 2011).

Flaxseed supplementation has shown to change the FA profile and gene expression patterns in the rumen, musle, and adipose tissue of dairy and beef cattle as well as goats (Ebrahimi et al., 2013; Kronberg et al., 2006; Li et al., 2012). cDNA microarrays have become a widely used technology which allows the monitoring of expression levels of thousands of genes simultaneously (Duggan et al., 1999). For example, Lehnert and coworkers (2007) used cDNA microarrays to identify differentially expressed genes in developing bovine *Longissimus* muscle from two different beef cattle breeds (Wagyu and Piedmontese). Similarly, Wang et al. (2009) studied gene expression patterns during intramuscular fat development (from 3 to 25 months) in beef cattle via cDNA microarray. Lee et al. (2010) used bovine cDNA microarrays to identify

biological pathways for beef marbling in Hanwoo cattle. Therefore, bovine cDNA microarrays provide opportunities to identify patterns of gene expression regulating different molecular pathways in beef cattle.

Some pathway analysis tools have been developed for the analysis, integration, and interpretation of the biological data derived from 'omics experiments, such as microarrays and RNAseq experiments. The two widely used pathway analysis tools are the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov; Huang da et al., 2009) which can identify clusters of related gene ontology (GO) terms that are over-represented in the list of genes that are differentially expressed, and Ingenuity Pathway Analysis software (IPA; Ingenuity Systems; Redwood City, CA; www.ingenuity.com) which characterizes biological functions that are over-represented in the DE list, and determines over-represented signaling and metabolic canonical pathways. Therefore, these tools will help to understand biological meaning behind large list of genes.

The objective of the present study is to detect gene expression differences between Flax-high index and No-Flax-low index cows. This information may identify underlying functional candidate genes that influence, or are associated with FA metabolism in *Longissimus thoracis* muscle, subcutaneous fat, and kidney fat of beef cattle. Selection for gene expression differences, or polymorphisms that affected the function of these candidate genes, may help to improve the FA profiles of muscle and fat in beef cattle.

3.2. MATERIALS AND METHODS

3.2.1. Animals and treatments

The cows used for tissue collection belonged to the feeding experiment conducted by He *et al.* (2012), and Nassu et al. (2011). The study was conducted in the Lethbridge Research Centre

feedlot of Agriculture and Agri-Food Canada (Lethbridge, AB, Canada) and was approved by the center's Animal Care Committee under the auspices of the Canadian Council of Animal Care (CCAC, 1993). In brief, 64 crossbred cull cows (~30 months of age) with similar breed composition, were randomized by weight/body condition (average BW of 620±62 kg (mean±SD)), and fed 50:50 forage:concentrate diets on a DM basis (16 cows/treatment) in a 2x2 factorial design. The 4 diets consisted of hay:ground barley (HB), hay:ground barley with ground flaxseed (HBF), silage:ground barley (SB), and silage:ground barley with ground flaxseed (SBF). Flaxseed was substituted for barley grain at 15% of the ration DM, resulting in a dietary lipid content of ~6% of DM. With the addition of a vitamin and mineral supplement, all diets fully met or exceeded the nutrient requirements of cull cows, as recommended by NRC (1996).

3.2.2. Tissue collection and RNA isolation

All cows were slaughtered after 140 days on the diet. Three tissues including *Longissimus thoracis* (LT) muscle, subcutaneous fat (SC) and kidney fat (KF) were collected for RNA isolation: *Longissimus thoracis* muscle was collected from the medial side of the carcass at the 10^{th} rib; subcutaneous fat from backfat situated on the right side of the animal close to the 10^{th} rib; and kidney fat from fat surrounding the kidneys. The tissue was immediately frozen in liquid nitrogen and then stored at – 80°C until RNA isolation. The frozen tissue was ground by mortar and pestle in the presence of liquid nitrogen. Total RNA was isolated by the TRIzol® procedure (Invitrogen, Carlsbad, USA), using 1 ml TRIzol® to every 50 mg of tissue. Fifty mg of tissue was transferred to a 2 ml metal hard tissue grinding beaded tube (Bertin Technologies, Montigny le Bretonneux, France) and 1 ml TRIzol reagent was added to the tube in order to perform the lysis and homogenization steps of tissue in TRIzol with Cryolys® and Precellys® tissue homogenization system (Bertin Technologies, Montigny le Bretonneux, France).

Homogenization was performed at 5500 rpm, 2 times for 30 s (10 s pause between two cycles) at 4 ^{o}C, then the tubes were centrifuged at 12,000 g, 4 ^{o}C for 10 min, and following that supernatant was transferred to a new tube. Afterwards, RNA isolation steps were completed according to the TRIzol® procedure (Invitrogen, Carlsbad, USA). After the isolation procedure, the RNA was quantified on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA), and tested for integrity on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). All RNA samples used for the microarray experiment had an RNA integrity number (RIN) score of ≥ 7.

3.2.3. Bovine 24K cDNA microarray

For the microarray experiment, five cows from each of the four diets (20 in total) were selected based on the FA profiles of the kidney fat collected at slaughter. The FA composition was determined by Nassu et al. (2011) and He et al. (2012) in the Lethbridge Research Centre feedlot of Agriculture and Agri-Food Canada (Lethbridge, AB, Canada). In brief, 50 mg sample was collected and lipids were extracted using procedures as described by Radin (1981) and Kronberg et al. (2006). FAs were methylated using a combined base/acid methylation procedure (Lock and Garnsworthy, 2002). Then, fatty acid methyl esters (FAME) were quantified using a gas chromatograph (Hewlett Packard GC System 6890, Mississauga, Ontario, Canada) equipped with a flame ionization detector and SP-2560 fused silica capillary column (75 m × 0.18 mm × 0.14 μm; Supelco Inc., Oakville, Ontario, Canada). An index equation was designed to rank cows based on the alpha-linolenic acid (18:3n-3) and long chain n-3 (3n) FAs [sum of 20:3n-3, 20:5n-3 and 22:5n-3] that accumulated in the fat: *Index=0.40(18:3n-3) + 0.60(Long Chain:3n)*. Cattle scoring the highest indexes were selected from diets which included flax, and cattle scoring the lowest indexes were selected from diets which did not contain flax.

Gene expression of selected RNAs was measured by hybridization of fluorescent-labeled amino-modified RNA (aRNA) to Bovine Oligo Microarray Consortium (BOMC) 70mer oligo microarray slides. These arrays contain 24,000 bovine oligonucleotide probes, which represent 16,846 unique gene loci designed from BOMC genes that were homologous to Ensembl vertebrate proteins (http://www.bovineoligo.org/). The oligos were spotted onto GAPS II Coated Slides (Corning Incorporated, Corning, USA) at the BIO5 Institute of the University of Arizona (Tucson, AZ, USA). Before use in the hybridization experiments, immobilization, which enhances binding of cDNA to the GAPS coated surface, was performed as follows: the microarray slides were rehydrated with 50°C sterile ddH2O for 10s and then dried on heating block for 5s (this step was repeated for 4-6 times to make sure all spots were rehydrated). Oligonucleotides were crosslinked to the slide surface by exposing the DNA side of the microarray slide to 180 mJ of UV irradiation via Stratagene Stratalinker 1800 UV cross-linker (Stratagene, La Jolla, USA). Slides were subsequently washed in 1% SDS dissolved in sterile ddH2O for 5 min at room temperature, and then were rinsed by plunging into sterile ddH2O for 10 times followed by a rinse in 100% ethanol for 3 min. Slides were spin-dried by centrifuging for 2 min at 1000 rpm, following that they were stored in a light proof box under cool, dry, conditions before use in the hybridization experiment. It is important to note that for each tissue a total of 20 microarray slides were used in duplicate for each individual RNA sample.

3.2.4. Fluorescent-labeled amino-modified RNA

To prepare the RNA for hybridization, 1 μg of total RNA was reverse-transcribed, converted to cDNA, then in-vitro transcribed to produce amino modified RNA, which can then be coupled with a Cy5 fluorescent dye, using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Carlsbad, USA) according to the instructions provided by the manufacturer. Briefly,

first strand cDNA was synthesized in a 20 µl reaction and contained 10 µl of RNA (100 ng/µl) plus 10 µl of reverse transcription master mix which consisted of 1 µl nuclease-free water, 1µl T7 oligo (dT) primer, 2 µl 10x first strand buffer, 4 µl dNTP Mix, 1 µl RNase inhibitor, and 1 µl ArrayScript. Amplification was performed in a thermal cycler at 42°C for 2 hours with 50°C in the lid. Then the second strand cDNA was synthesized by adding 80 µl second strand master mix (63 µl nuclease free water, 10 µl 10× second strand buffer, 4 µl dNTP mix, 2 µl DNA polymerase, and 1 µl RNase H) to each sample and incubating the reaction for 2 h at 16°C. cDNA purification was performed as follows: 250 µl of cDNA binding buffer was added to each sample, the mixture was passed through a cDNA filter cartridge and centrifuged for 1 min at 10000 ×g, the cDNA filter cartridge was washed with 500 µl wash buffer and centrifuged for 1 min at 10000 ×g, the cDNA filter cartridge was transferred to a cDNA elution tube and eluted by adding 18µl of preheated (55°C) nuclease free water to the cartridge, leaving the reaction at room temp for 2 min and then centrifuging it for 1 min at 10000 ×g. Then in vitro transcription to synthesize Amino Allyl-Modified aRNA was performed by adding 26ul of IVT (In Vitro Transcription) master mix (3 µl aaUTP (50mM), 12 µl ATP, CTP, and GTP Mix, 3 µl UTP solution (50mM), 4 µl T7 10× reaction buffer, and 4 µl T7 enzyme mix) to the eluted cDNA, incubating the mixture in a thermal cycler for 14 h at 37°C, and stopping the reaction by adding 58 µl nuclease free water to the aRNA sample. Then aRNA purification was completed as follows: 350 µl of aRNA binding buffer and 250 µl 100% ethanol were added to the aRNA sample respectively, and the mixture was passed through an aRNA filter cartridge and centrifuged for 1 min at 10000 ×g. Subsequently 650 µl wash buffer was added to the aRNA filter cartridge and centrifuged for 1 min at 10000 ×g, the aRNA filter cartridge was then transferred to an aRNA collection tube, and 200 µl of 55°C nuclease free water was added to the

aRNA filter cartridge. The aRNA collection tube was incubated at 55°C on heat block for 10 min, and then the tube was centrifuged for 90 s at 10000 ×g until all water is passed the filter. Quantity of aRNA was measured by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA) and all aRNA samples were diluted to 5 µg/µl. The aRNA size was also tested by 1% agarose gel electrophoresis and a smear from 250 to 5000 nt (nucleotide) was observed in which the average size of amino allyl aRNA was around 1400 nt.

Then aRNA:dye coupling reaction and dye labeled aRNA purification were performed. Briefly, 10 µg of amino allyl modified aRNA was transferred to a nuclease free microcentrifuge tube and vacuum dried until no liquid remained. The dried aRNA was resuspended with 9 µl of preheated (50°C) coupling buffer. Then 11 µl of Cy5 and Cy3 dyes were added to each individual aRNA sample, and a common reference aRNA, respectively, and incubated for 45 min at room temp in a dark drawer. The common reference RNA was a mixture of each individual sample RNA from all 3 tissues so that the reference contained as many different expression transcripts as possible. The purpose of the mixed reference RNA was to increase the chance of obtaining comparative measurements of genes with low expression in the experimental samples, or that were turned on or off by the experimental treatment. To stop the reaction, 4.5 µL of 4M Hydroxylamine was added to each labeled aRNA and incubated for 15 min at room temp in a dark drawer. Then 5.5 µl of nuclease free water was added to bring each sample to 30 µl. Dye labeled aRNA purification was performed as follows: 105 µl aRNA binding buffer and 75 µl 100% ethanol were added to each sample respectively, the mixture was passed through a labeled aRNA filter cartridge and centrifuged for 1 min at 10000 ×g. The labeled aRNA filter cartridge was transferred into a labeled collection tube, 500 µl wash buffer was added to each labeled aRNA filter cartridge and centrifuged for 1 min at 10000 ×g, and the labeled aRNA filter cartridge was

transferred to a labeled elution tube and eluted 2 times by adding 10 µl of preheated (55°C) nuclease free water, leaving the reaction at room temp for 2 min and then centrifuging for 90 s at 10000 ×g. The purified labeled aRNA was stored over night at -20°C in the dark (it can be stored for long term at -80°C). Number of dye molecules incorporated per 1000 nt of labeled aRNA was obtained by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA), and incorporation was between 30-50 nt per 1000 nt of labeled aRNA for all samples (best hybridization results are obtained with aRNA having between 20-50 labeled nucleotides per thousand of labeled aRNA).

Before hybridization, the slides were pre-hybridized in 50% pre-hybridization buffer (25 ml formamide, 12.5 ml RNase free water, 12.5 ml of 20% saline sodium citrate [SSC] (175.3 g NaCl and 88.2 g Sodium Citrate were dissolved in 1 L of ddH_2O, pH=7), 0.75 ml of 10% sodium dodecyl sulfate [SDS], and 0.5 g BSA) for 45 min at 42°C. The pre-hybridized slides were transferred into a new holder and washed twice in purified (Millipore) water for 5 min at 110 rpm. The slides were removed from the water and dipped into isopropanol 2-3 times and then were spin-dried. Then the slides were prewarmed (45-50°C) on heat block and cover slips were added to cover the microarray spots. The labeled aRNA was vacuum-dried and resuspended in 9 µl nuclease free water. One µl fragmentation buffer was added to the labeled aRNA and then the solution was incubated at 70°C for 15 min, after which 1 µl of stop solution was added and the fragmented, labeled aRNA was placed on ice until use. Twenty-seven µl of the 30% formamide hybridization buffer (22.5 µl of deionized formamide, 18.75 of µl 20x SSC, 0.75 µl of 10% SDS, 6.0 µl of Yeast tRNA (10mg/ml), and 6 µl of Mouse cot 1 DNA), was added to each of the fragmented labeled aRNA sample. Each fragmented labeled aRNA (Cy3= common reference RNA, Cy5= each individual RNA sample) sample was mixed individually and then mixed

together. Following that, the mixture was added slowly to one side of coverslip to ensure even distribution over the microarray surface, then the slides were placed in a hybridization chamber (Genetix, New Milton, UK) for 16 h at 42°C. After hybridization the slides were washed 3 times for 5 min with the pre-warmed (42°C) low-stringency wash buffer (2X SSC and 0.5% SDS) at 110 rpm on a shaker, followed by 3 times for 5 min with high-stringency wash buffer (0.5X SSC and 0.2% SDS) at 110 rpm on a shaker, then 3 times for 2 min with a final wash solution (0.05X SSC) at 110 rpm on a shaker, and then the slides were spin-dried.

Hybridized slides were scanned at 5 μm resolution using GenePix 4200 AL microarray scanner (Axon Instruments Inc., Union city, USA) and GenePix® Pro 6.0 software (Molecular Devices, Corp., Sunnyvale, USA), at approximately 320/280 V PMT (photo multiplier tube) for the 635 and 532 nm wavelengths, respectively. The GenePix Array List (GAL) file Bovine_v1.1.Xb, specific for the BOMC 24K 60-mer microarray was used to align spots and assign an ID for each spot on the array. Slides were then normalized within GenePix to determine the normalization factors for each wavelength channel, as a quality control measure. If these factors were not between 0.8 and 1.2, the slide was rescanned with a more suitable PMT setting. Individual slide scan information was saved as GenePix Settings files (.gps), and all intensity data required for downstream analysis were saved as GenePix Results files (.gpr). All subsequent data analysis was performed using R software (Version 3.0.2).

3.2.5. Analysis of differential gene expression

All raw microarray data has been submitted to the ArrayExpress (https://www.ebi.ac.uk/arrayexpress) database (accession: E-MTAB-3371). Differentially expressed (DE) genes (Contrast: Flax-high index vs. No-Flax-low index) were identified using linear modeling and the empirical Bayes approach within Limma in R software (Version 3.0.2)

to produce moderated t-statistics and associated p-values (Limma package, Biocoductor), incorporating the BH significance correction for multiple tests (Smyth, 2004; Smyth and Speed, 2003; Smyth et al., 2005). Then those DE transcripts with p-value < 0.1 were selected for pathway analysis. The annotations for the probes on the array BOMC were updated using a custom pipeline written in R software (Version 3.0.2) which was performed by Dr. Jason Grant at Livestock Gentec Center of the University of Alberta (Edmonton, AB, Canada). Probe sequences were aligned using BLAST-like alignment tool (BLAT, https://genome.ucsc.edu/cgi-bin/hgBlat) and an alignment cutoff of 95% to multiple sources of gene sequence data: Ensembl cDNA (Version 69), NCBI UniGene (Build 101) and Ensembl Ab Initio Predictions (Version 69). If no hits were found, the probe sequences were also aligned to the *Bos taurus* genome (UMD 3.1). Probes were assigned a human ortholog if it aligned to gene with a known human ortholog. Otherwise the aligned gene sequence was queried against the human protein database using blastx (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and an E-value cutoff of 0.001 was used to find the best match. In the case of probes that only aligned to the genome, the genomic sequence from the hit including 200 bp upstream and downstream was used in the blastx query. List of DE genes containing the gene symbol and log2 ratio (log2 fold change) for each gene, was imported into Ingenuity Pathway Analysis software (IPA; Ingenuity Systems; Redwood City, CA; www.ingenuity.com) for gene function analysis and network generation. Core analysis was performed to map DE genes to the Ingenuity Knowledge Base (IKB), then create molecular networks, identify biological functions that were over-represented in the DE lists, and to determine over represented signaling and metabolic canonical pathways using a Fisher's exact test with the cut-off for p-value < 0.05. All genes that were associated with a biological term in the Ingenuity Knowledge Base (IKB) were used in the analysis. The DE gene list was also

uploaded to the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov; Huang da et al., 2009), and the list of all the genes in Bovine 24K cDNA microarray slide was imported as the background list. Functional annotation clustering was performed to identify clusters of related gene ontology (GO) terms that were significantly enriched among DE compared with the entire set of annotated bovine genes using a modified Fisher's exact test, which is called EASE Score Threshold, (p-value < 0.1) to calculate a more conservative probability. All the aforementioned pathway analysis aided us to understand the associations between DE genes and FA metabolism, and to help identify more genes that might play important roles in these pathways, and therefore be good candidates for the control of the FA profile of meat.

3.2.6. Designing and testing of real-time PCR primers for verification of DE genes

For designing of forward and reverse primers of each gene, the approved gene name of each transcript was identified by Hugo gene nomenclature committee (HGNC, Hinxton, UK) software which is available online at http://www.genenames.org/. The reference sequence of all of the isomers of each gene for *Bos taurus* were aligned with all of the *Homo sapiens* and *Mus musculus* isoforms of that gene using Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) to select the longest isoform of *Bos taurus* which covers most areas of that gene. The target and excluded regions of mRNA of the selected isoform was identified by BLAT Search Genome program (https://genome.ucsc.edu/cgi-bin/hgBlat). After which all required primer quality information (target regions, excluded regions, product size range between 90-200 bp (base pair), number, primer size between 18-22 bp, primer Tm [melting temperature] between 59-61^{o}C, primer GC% between 40-65, and CG clamp equal to 1) for the selected isoform was submitted into Primer3 software (Version 0.4.0)

(http://bioinfo.ut.ee/primer3-0.4.0/). Those primers with minimum probability of having hairpin, self-dimer, and hetero-dimer were selected by an oligoanalyzer tool (http://www.idtdna.com/). Then both primers of each gene were aligned to the genome of *Bos taurus* and other organisms to ensure the primers were specific for that gene. Finally, primer amplification was tested on a serial dilution of a cDNA reference sample (the serial dilution for each tissue was tissue-specific), with reference to the original input RNA concentration (20, 10, 5, 2.5, 1.25, 0.625, and 0.2 ng), plus control samples which consisted of the reference RNA without conversion to cDNA by reverse transcriptase (no RT control), and water instead of template cDNA (no template control). Inclusion of the controls was used to confirm product identity and interrogate interference from primer-dimer or other non-specific amplification products for each primer-pair by real-time PCR. For each primer pair test amplification, if any of following conditions existed; poor agreement between duplicates, improper amplification curve, multiple peaks in melt curve, or a reaction efficiency of lower than 90% or higher than 110%, those primers were rejected and new primer-sets were designed. For all the primer-pairs used in the final analyses, the product identity was confirmed by sequencing of PCR products, Tm was set at 60 ^{o}C, and the reaction efficiency was between 90 and 110%.

3.2.7. Verification of differential expression

A subset of significant and interesting DE genes associated with the metabolism of FAs, which were highlighted by pathway analysis of the results of microarray experiment, were chosen for SYBRgreen real-time PCR verification of their expression level in the original RNAs used for the experiment. The expression level of these genes was verified in both the 20 animals used for the microarrays, and the remaining animals in the entire experiment which added 11 and 41 animals for LT and SC tissues, respectively. Total RNA was isolated as described earlier. cDNA

synthesis was performed using the High Capacity cDNA Reverse Transcription Kit and the standard protocol (Applied Biosystems, Foster City, USA). Briefly, each tissue's reference RNA was created by pooling 1 µL of each individual sample RNA for that tissue that contained 150ng. Fifteen hundred nanograms of each individual total RNA and each reference RNA were used for individual and reference cDNA synthesis, respectively. Ten microliters of reverse transcription (RT) reaction mix per reaction was prepared by mixing 2 µl of 10X RT buffer, 0.8 µl of 25X dNTP Mix (100 mM), 2 µl of 10X RT Random Primer, 1 µl of RNase inhibitor (20 U/µl), 1 µl of MultiScribe™ Reverse Transcriptase (50 U/µl), and 3.2 µl of RNase free water. The RT mix was added to 10 µl of RNA (150 ng/µl), gently mixed, and the entire reaction was then incubated at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. The cDNA was placed on ice, aliquoted in appropriate volumes to be used in the real-time PCR mixes, and then frozen at –20°C until ready for use.

For each tissue a standard curve was created by serial dilution of a cDNA reference sample, with reference to the original input RNA concentration (20, 10, 5, 2.5, 1.25, 0.625, and 0.2 ng). The serial dilution for each tissue was tissue-specific and was made freshly from the aliquoted cDNA before each real-time PCR reaction. Individual sample cDNAs for each tissue were diluted to a final concentration equal to 1 ng input RNA for the real-time PCR reaction. Real-time PCR was carried out in 96-well optical reaction plates (Applied Biosystems, Foster City, USA) using the Applied Biosystems Step-One-Plus real-time PCR system (Applied Biosystems, Foster City, USA). The total volume of each reaction was 20 µL and contained 4 µl of cDNA template (1 ng/µl), 0.5 µl of each forward and reverse primer (10 µM), 10 µl of the 2× KAPA SYBR® FAST qPCR Master Mix ABI Prism™ (Kapa Biosystems, Boston, USA), and 5 µl of RNase free water. Control samples in each run consisted of the reference RNA without conversion to cDNA by

reverse transcriptase (no RT control), and water instead of template cDNA (no template control). All samples, standard curves, and controls were run in duplicate on each plate. Amplification conditions consisted of: [1] hold stage for 30 s at 95°C for enzyme activation; [2] cDNA amplification stage for 40 cycles of 3 s at 95°C and then 30 s at 60°C; and [3] a final melt curve stage which consisted of 15 s at 95°C, 1 min at 60°C, and then heating (melt) of the reaction with a ramp rate of +0.3°C per 15 s from 60°C to 95°C to obtain fluorescence measurements that reflect the Tm of the main product while minimizing interference from primer-dimer or other non-specific amplification products. The cycle threshold values (CTs) were calculated by plotting normalized fluorescence (ΔRn) in relation to cycle number with the assistance of the StepOne Software v2.3 (Applied Biosystems, Foster City, USA). Product identity was confirmed via visual inspection of the melt curve as well as sequence of the PCR products from a few samples. In addition, for each primer pair we observed an agreement between duplicates in the standard curve, a good amplification plot, a single peak in the melt curve, plus the reaction efficiency was between 90-110%.

The results were exported to an Excel file by the StepOne Software v2.3 (Applied Biosystems, Foster City, USA) and the quantity mean values were used for subsequent data analysis. The quantity mean values of all samples for all of the endogenous genes were used to find the best gene or best combination of two genes (the geometric mean value of the best two genes) using NormFinder software, version 20, (Andersen et al., 2004) in order to normalize the target gene data. Ribosomal protein L19 [*RPL19*], actin, beta [*ACTB*], peptidylprolyl isomerase A (cyclophilin A) [*PPIA*], eukaryotic translation elongation factor 1 alpha 2 [*EEF1A2*], glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*], hydroxymethylbilane synthase [*HMBS*], tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta [*YWHAZ*] were

those endogenous genes used in this experiment, amongst them *RPL19* and *PPIA* were selected as the best endogenous genes to normalize data for LT and SC tissues, respectively. For normalization, the quantity mean value of each sample for each target gene was divided by the quantity mean value of the best endogenous gene selected by NormFinder.

3.2.8. Statistical analysis for real-time PCR

To analyze the real-time PCR data, SAS Ver. 9.2 was used and normal and non-normal data were analyzed by the PROC TTEST and NPAR1WAY, respectively. The normality of data was tested by PROC UNIVARIATE. If the data was normal PROC TTEST (two-tailed) was used, and if the variance of a gene for Flax-high index vs. No-Flax-low index was equal the p-value for the method "Pooled" was selected, otherwise the p-value for the method "Sattherthwaite" was selected. The fold change was also obtained by dividing the mean value of Flax-high index to No-Flax-low index. And if the data was not normal NPAR1WAY was used for analysis and the p-value of Kruskal-Wallis Test was selected. The fold change for the non-normal data was also obtained by dividing the mean value of Flax-high index to No-Flax-low index group from the results of PROC TTEST. For both normal and non-normal data the cut of p-value of a trend was < 0.1, and for significance < 0.05.

3.3. RESULTS

3.3.1. Differential gene expression between groups Flax-high index and No-Flax-low index groups

In total, 53 genes plus 15 EST with p-value < 0.05 and 135 genes plus 31 EST with p-value < 0.1 were found to be differentially expressed in LT and SC tissues combined of cattle from the Flax-high index group compared to No-Flax-low index (Appendix 1). This total consisted of 48 genes and 15 EST (p-value < 0.05), and 119 genes and 29 EST (p-value < 0.1) found to be DE in LT tissue. The number of DE transcripts was lower in SC as compared with LT and included 5 genes

and 0 EST with p-value < 0.05, and 16 genes and 2 EST with p-value < 0.1. From 166 transcripts (LT= 148, SC= 18) with p-value < 0.1, 118 (71%) exhibited decreased expression in tissue samples from Flax-high index cows. No differentially expressed transcripts were found in KF tissue.

The list of 166 DE genes from LT and SC was then submitted to the Ingenuity Pathway Analysis (IPA) software for functional annotation and mapping to canonical metabolic and regulatory pathways. A summary of the IPA Biological Functions was presented in Table 3.1. The significant (p-value < 0.05) subcategories of major interest were "Carbohydrate Metabolism" (11 genes), "Molecular Transport" (16 genes), and "Small Molecule Biochemistry" (16 genes).

Functional analysis of the same data set by DAVID identified 25 significant (p-value < 0.1) clusters. Prominent among the top clusters were terms associated with development of muscle tissue. Cluster 1 contained the GO (Gene ontology, http://www.geneontology.org) terms muscle organ development (GO:0007517), striated muscle tissue development (GO:0014706), muscle tissue development (GO:0060537), cardiac muscle tissue development (GO:0048738), and heart development (GO:0007507). Cluster 4 contained the GO terms related to oxidative phosphorylation process which included hydrogen ion transmembrane transporter activity (GO:0015078), monovalent inorganic cation transmembrane transporter activity (GO:0015077), and inorganic cation transmembrane transporter activity (GO:0022890). Cluster 6 was related to carbohydrate metabolism and included GO terms like glycolysis (GO:0006096), glucose catabolic process (GO:0006007), hexose catabolic process (GO:0019320), and monosaccharide catabolic process (GO:0046365).

After the IPA and DAVID analyses, as well as a background literature search, 8 DE transcripts from the microarray were selected to have a high potential to be associated with FA metabolism

(Table 3.2), out of which six (75%) exhibited elevated expression in tissues from Flax-high index cattle. Out of the 8 DE genes, 1 was DE in SC fat while the remaining seven were DE in LT muscle.

3.3.2. Pathway analysis of DE genes

The list of the eight DE genes was then imported into IPA for gene function analysis and network generation. Table 3.3 presents the major category of interest which is Molecular and Cellular Functions with the top subcategories including "Lipid Metabolism" (5 genes), "Small Molecule Biochemistry" (7 genes), "Cellular Function and Maintenance" (1 genes), "Carbohydrate Metabolism" (3 genes), and "Molecular Transport" (2 genes). Prominent among the top subcategories was Lipid Metabolism and from the five genes in this subcategory, sterile alpha motif domain containing 8 [*SAMD8*], succinate dehydrogenase complex, subunit A, flavoprotein (Fp) [*SDHA*], and sphingomyelin synthase 1 [*SGMS1*] have functions related to FA metabolism, 2-hydroxyacyl-CoA lyase 1 [*HACL1*] has a role in alpha-oxidation of FAs, and phospholipase C, beta 4 [*PLCB4*] which is a phospholipase enzyme, plays a role in hydrolysis of phosphatidylinositol. The gene interaction network of the 8 DE genes associated with FA metabolism is depicted in Figure 3.1. This network contains 35 molecules among them *SAMD8*, *SDHA*, *SGMS1*, *HACL1*, *PLCB4*, aconitase 2, mitochondrial [*ACO2*], chromosome 8 open reading frame, human C9orf3 [*C8H9orf3*], and upstream transcription factor 2 [*USF2*] were those DE associated with FA metabolism. Log2 ratio (Log2 fold change) of the 8 DE (red = up-regulated, green = down-regulated) was in agreement with the results of microarray and the IPA also predicted relationships (orange = predicted activation, blue = predicted inhibition) among some of the genes in the network.

3.3.3. Validation of microarray expression data by real-time PCR

The 8 significantly DE genes (*C8H9orf3*, *SGMS1*, *PLCB4*, *ACO2*, *SDHA*, *SAMD8*, *USF2*, and *HACL1*) that were identified by microarray analysis as being related to FA metabolism were chosen for SYBRgreen real-time PCR verification. Real-time PCR was performed on the 20 samples used on the microarrays, plus the rest of the tissues collected from the experiment (an additional 11 samples for LT and 41 for SC). Table 3.2 displays a comparison in the expression changes determined in these genes by microarray versus real-time PCR. Most of the genes tested agreed in the direction of change between Flax-high index and No-Flax-low index samples except for *SGMS1*. This may be due to non-specific hybridization on this spot on the microarray or type I error (the incorrect rejection of a true null hypothesis (a "false positive")). In addition, table 3.2 displays that some genes are non-significant in their expression between the Flax-high index and No-Flax-low index samples when measured by real-time PCR, but those that are significant using 20 samples in general continue to be significant when more biological samples were added.

3.4. DISCUSSION

In LD muscle, 148 DE transcripts (p-value < 0.1) spots on our cDNA array were associated with a significant differential expression between Flax-high index and No-Flax-low index animals. Furthermore, 18 DE transcripts were also found in SC tissue, whereas no significant DE transcripts detected in KF. The difference in the number of DE genes amongst three tissues is thought to be related to the tissues' inherent biological activity. Muscle is thought to be more metabolically active than fat tissue since it contains a higher proportion of amino acids as opposed to FAs, and the rate of protein turn-over is higher than fat turn-over in cattle (Byers and Schelling, 1993). As well, the ratio of PUFA/SFA is higher in phospholipids and unlike adipose tissue, in muscle the ratio of phospholipids to neutral lipids (lipid droplets are composed of a

neutral lipid core, consisting mainly of triacylglycerols (TAGs), which is surrounded by a surface film containing phospholipids, cholesterol and apoproteins) is higher (Raes et al., 2004). Nassu and coworkers (2011), who studied the FA profiles of LT and SC from the cows used in the present study, reported that approximately three fold higher n-3 and n-6 FAs were deposited in LT compared to SC and also incorporation of n-3 FAs into both muscle and fat tissues was 2.2 fold higher when flax was supplemented into the diet of cattle. It has been reported that dietary EPA and DHA induce activation of the mTOR-p70s6k signaling pathway which promotes skeletal muscle protein synthesis in human (Smith et al., 2011). Similarly, Gingras et al. (2007) showed that infusion of n-3 FAs into bovine abomasum up-regulated protein synthesis in skeletal muscle. Therefore presence of more DE genes in muscle compared to fat tissue when comparing Flax-high index to No-Flax-low index groups was expected. We are not sure why no DE genes were seen in KF as compared to SC tissue considering that it appeared that diet influenced n-3 levels in kidney fat to a greater extent than subcutaneous backfat (Dugan, personal communication). It might be due to the technical difficulty in obtaining RNA from KF since it has large lipid globules and therefore less nucleic acid per gram of tissue compared to LT or SC tissues. As well, we know that kidney fat, a capsule of adipose tissue surrounding the kidney to help protect it from external forces and to help prevent it from moving around in abdominal cavity, normally has a higher concentration of SFAs (myristic acid (C14:0), PA, and stearic acid (C18:0)) compared with any of the other fat depots (Aksu, 2009). Hence, although in kidney fat the amount of n-3 FAs was higher in Flax-high index group compared to No-Flax-low index (unpublished data), the results of microarray studies showed that this increase in the n-3 content of kidney fat did not lead to much change in the gene expression pattern in this tissue. Tchkonia and coworkers (2005) reported the presence of 2 subtypes of preadipocytes (switching can take

place between subtypes) within the different major fat depots in human, one was rapidly replicated, differentiated, and had higher expression of CCAAT/enhancer-binding protein-α [*CEBPA*] (an adipogenic transcription factor) and was found more in subcutaneous fat while the other subtype was more abundant in omental fat. If these two subtypes are also present in the same proportions in these two adipose tissue in cattle then it could imply that SC fat is biologically more active (rapidly replicating, differentiating, and has higher expression of adipogenic transcription factor) than KF, which could be one of the underlying causes in the difference of the number if DE genes found in SC as opposed to KF due to diet changes.

As n-3 FAs play an important role in immune function (Kiecolt-Glaser et al., 2014), it was expected that a significant number of genes related to immune response would show variations in expression level between Flax-high index and No-Flax-low index animals. Nine DE genes associated with immune response function were detected including toll-like receptor 2 [*TLR2*], complement component (3d/Epstein Barr virus) receptor 2 [*CR2*], Kirsten rat sarcoma viral oncogene homolog [*KRAS*], T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3 [*TCIRG1*], crystallin, alpha B [*CRYAB*], Tax1 (human T-cell leukemia virus type I) binding protein 1 [*TAX1BP1*], basic leucine zipper transcription factor, ATF-like 3 [*BATF3*], mucin 1, cell surface associated [*MUC1*], and Spi-B transcription factor (Spi-1/PU.1 related) [*SPIB*]. Six of those genes were down-regulated, including *TLR2*, *KRAS*, *TCIRG1*, *BATF3*, *MUC1*, and *SPIB*, while *CR2*, *CRYAB*, and *TAX1BP1* were up-regulated. The key gene among the mentioned genes was TLR2. The protein encoded by TLR2 is a member of the Toll-like receptor (TLR) family which plays a fundamental role in recognition of pathogens and activation of innate immunity (Rock et al. 1998). Results of a study conducted by Yi et al. (2011) on the effect of n-3 PUFA on toll-like receptors in patients with severe multiple trauma showed that the

expression of *TLR2* and *TLR4* in peripheral blood mononuclear cells (PBMCs) was significantly lower in n-3 PUFA group than in control group. Similarly, Oh et al. (2010) reported that G protein-coupled receptor 120 [*GPR120*] acts as an n-3 FA receptor/sensor. They compared the anti-inflammatory effect of EPA (C20:5n-3) and DHA (C22:6n-3) in wild type and *GPR120* knockout mice and found that n-3 FAs mediate the anti-inflammatory effect by signalling *GPR120* to inhibit TLR inflammatory signalling pathway. Therefore, down-regulation of the *TLR2* in the Flax-high index group suggests that n-3 PUFA may suppress the inflammatory response mediated by the TLR signaling pathways.

In the present study, amongst the 166 DE spots, 8 DE genes were found to be associated with FA metabolism. Among the 8 DE, *HACL1* was identified in SC fat and the remaining seven, *C8H9orf3*, *SGMS1*, *PLCB4*, *ACO2*, *SDHA*, *SAMD8*, and *USF2*, were detected in LT muscle. Most of the genes tested by real-time PCR agreed in the direction of change between Flax-high index and No-Flax-low index samples except for *SGMS1* so reaching to a conclusion of whether *SGMS1* was up-regulated or down-regulated in the Flax-high index group compared to No-Flax-low index one is difficult. *SGMS1* synthesizes sphingomyelin, which is a unique form of a phospholipid called sphingophospholipid, by transferring phosphatidylcholine (the phosphatidyl head group) on to the primary hydroxyl of ceramide (a ceramide is composed of sphingosine and a fatty acid). This reaction is bidirectional which depends on the respective levels of ceramide and the sphingolipid (Huitema et al., 2004; Vacaru et al., 2009). There might be several possible reasons why our PCR verification was failed for this transcript. These include experimental errors in the production of the cDNA arrays, non-specific hybridization on this spot on the microarray, or type I error.

HACL1 was the DE gene (p-value < 0.1) identified in SC tissue that showed elevated expression in the Flax-high index group compared to No-Flax-low index. This gene encodes a peroxisomal (in peroxisomes oxidation of very long chain FAs and branched chain FAs takes place) enzyme catalyzes alpha-oxidation of 3-methyl-branched FAs, i.e. phytanic acid, in which HACL catalyzes a carbon-carbon cleavage in 2-hydroxy-3-methylacyl-CoA to produce formyl-CoA and a 2-methyl-branched fatty aldehyde (Foulon et al., 1999; Mannaerts et al., 2000). Therefore, the higher availability of LCFAs in the Flax-high index cows, in agreement with observations of He et al (2012), and Nassu et al. (2011), and in SC fat could lead to increased expression of this gene which can oxidize LCFAs in peroxisomes of SC tissue.

ACO2 and *SDHA* were those DE genes (p-value < 0.1) identified in LT tissue that showed elevated expression in the Flax-high index group compared to No-Flax-low index. Both genes function within the mitochondria which are the energy-producing centers in cells. ACO2 is a mitochondrial enzyme that catalyzes the interconversion of citrate to isocitrate via cis-aconitate in the TCA cycle, which is a series of chemical reactions that result in producing energy through oxidation of acetate, in the form of acetyl-CoA, derived from fats (obtained from conversion of acyl-CoA to acetyl-CoA), carbohydrates, and proteins (Beinert and Kennedy, 1993). SDHA is also a mitochondrial enzyme which converts succinate to fumarate as part of the TCA Cycle (Hirawake et al., 1994). Hence, up-regulation of *ACO2* and *SDHA* in LT muscle does infer that feeding flax alters energy metabolism within the muscle tissue, and also that there is a higher presence of LCFAs reaching the muscle (TCA substrate) from the digestive tract, in agreement with observations of He et al (2012), and Nassu et al. (2011). In addition, Ahmed et al. (2014) reported that diets high in n-3 FAs induced up-regulation of the genes involved in the TCA cycle including malate dehydrogenase and GTP-specific succinyl CoA synthase, suggesting that

feeding flax to the cow can increase the availability of n-3 FAs and their ruminal intermediates to tissue and trigger the TCA cycle to oxidize FAs.

In the present study *USF2*, a member of the evolutionary conserved basic-Helix-Loop-Helix-Leucine Zipper family which functions as a cellular transcription factor, represented higher expression in the Flax-high index animals compared to No-Flax-low index ones. The protein encoded by *USF2* can activate transcription through pyrimidine-rich initiator (Inr) elements and E-box motifs. This gene has also been involved as a key regulator of a wide number of gene regulation networks including stress and immune response, cell cycle and proliferation (Corre and Galibert, 2006). Moreover, USF transcription factor can bind to FAS insulin-responsive element 3 (FIRE3) in the promoter of the fatty acid synthase (*FASN*) gene and decrease its expression, which then ultimately reduces the catalyzation of the formation of LCFAs from acetyl-CoA and malonyl-CoA (Roder et al., 1999). Berger et al. (2006) reported that dietary fish oil (source of n-3 FAs) decreases expression of *FASN* and reduces FA synthesis in mouse liver probably due to binding of *USF2* to *FASN*. Therefore, increased circulating LCFAs in the Flax-high index cattle and in LT muscle could induce expression of *USF2* to bind to *FASN* and potentially reduce FA synthesis in LT.

Another gene which was up-regulated in the Flax-high index group was *C8H9orf3* (or aminopeptidase O (AP-O)) which is an aminopeptidase that catalyzes the removal of an amino acid from the amino terminus of a protein or peptide (Diaz-Perales et al., 2005). This enzyme is most closely related in sequence to leukotriene A4 hydrolase (LTA4H), an enzyme which participates in arachidonic acid (AA) metabolism (Shimizu et al., 1984). Leukotrienes are a family of eicosanoid inflammatory mediators produced in leukocytes (white blood cells) and other immune cells by the oxidation of AA via the enzyme arachidonate 5-lipoxygenase (Funk et

al., 1989; Hammarstrom, 1983). Strasser et al. (1985) reported that dietary EPA increased formation of leukotriene B5 (LTB5) and LTB5/LTB4 ratio (corresponded to the ratio of EPA/AA; LTB5 is 10 to 30 times less potent than LTB4 to mediate some symptoms associated with inflammatory reactions) in phospholipids of human neutrophils (a type of white blood cells). Similarly, Ikehata et al. (1992) reported that dietary EPA or fish oil increased the amount of LTB5 and LTB5/LTB4 ratio in patients with active Crohn's disease. Therefore, it can be presumed that the increase in the n-3 content of muscle through supplementation of flax into the diet of cow elevated the expression of *C8H9orf3* in the Flax-high index animals which might increase the amount of the less inflammatory leukotriene, LTB5, in phospholipids of neutrophils.

PLCB4 was the other DE gene which showed elevated expression in Flax-high index animals. Phospholipases are a group of enzymes that hydrolyze phospholipids into FAs and other lipophilic molecules. The protein encoded by *PLCB4* catalyzes the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol from phosphatidylinositol 4,5-bisphosphate, a phospholipid that is located in the plasma membrane (Alvarez et al., 1996). Locher et al. (1989) surveyed the effect of either fish oil or EPA on LDL and angiotensin II (AII), both of them induce synthesis of IP3, and found that n-3 FAs decreased synthesis of IP3 stimulated by LDL or AII in cultured rat vascular muscle cells. Similar to that, Sperling et al. (1993) reported that dietary n-3 PUFAs (EPA and DHA) decreased formation of IP3 through inhibition of *PLCB4* in neutrophils of human. This finding would appear in contrast to the results of the present study in which *PLCB4* was up-regulated in Flax-high index cattle, although we do not know the levels of LDL or AII in our study. As well, the results may differ because the aforementioned experiments were not conducted in ruminants (considering the affect of rumen microbes on FAs), and it could

be that an upstream regulatory pathway could cause the elevated expression of *PLCB4* in LT muscle.

In the present study *SAMD8* showed a decreased expression in the Flax-high index animals compare to No-Flax-low index. It is an endoplasmic reticulum transferase which has no sphingomyelin synthase activity but catalyzes conversion of phosphatidylethanolamine (PE) and ceramide to ceramide phosphoethanolamine (CPE), the sphingomyelin analogue of phosphatidylethanolamine, although with low product yield. It appears to operate as a ceramide sensor to control ceramide homeostasis in the endoplasmic reticulum rather than a converter of ceramides (Vacaru et al., 2009). We are unsure why the expression of *SAMD8* decreased in the Flax-high index group and this observation is new therefore further studies are needed to validate these results. However, our speculation is that since n-3 and n-6 FAs are important structural components of phospholipids, where the ratio of PUFA/SFA is higher (Raes et al., 2004), possibly increased circulating LCFAs in the Flax-high index cattle and in LT muscle reduced the need for synthesis of ceramide phosphoethanolamine by SMAD8 and increased hydrolysis of phospholipids by *PLCB4* in LT muscle.

3.5. CONCLUSIONS

It has been reported that meat, including beef, can be a major source of long chain n-3 FAs for the people who do not consume a lot of fish (Howe et al. 2006), therefore improving the n-3 content of beef can benefit those people who enjoy eating beef or do not have access to desirable fish products. Among the Canadian beef grades, cull cows have been shown to have higher amounts of CLA and n-3 FAs compare to beef consuming high concentrate diets (Dugan et al., 2008), so improving their FA profile could increase their economic value. In this study, the pathway analysis helped to identify many important DE genes, such as those associated with

immune response function, oxidative phosphorylation process, carobohydrate metabolism, and muscle tissue development in muscle and fat tissues of the Flax-high index cows versus the No-Flax-low index. As well, eight DE genes associated with FA metabolism were found in muscle and fat tissues, out of which seven, one, and zero were identified in LT, SC, and KF tissues, respectively. The present study identified eight DE genes which possibly involved in pathways to increase oxidation of FAs (*HACL1*, *ACO2*, and *SDHA*), decrease FA synthesis (*USF2*), reduce phospholipids synthesis or increase their hydrolysis (*SAMD8* and *PLCB4*), and increase the amount of LTB5 in phospholipids of neutrophils (*C8H9orf3*). The expression results of the eight DE genes associated with FA metabolism outlined in this project can be used to do correlation analysis between these genes with individual or groups of FAs. Discovery of SNPs linked to FA profiles and subsequently predicting the breeding values of an individual from SNP data can give us the opportunity to genetically select animals that inherently have improved FA profiles in their muscle and fat tissues (Meuwissen et al., 2001; Saatchi et al., 2011).

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Table 3.1. Top biological functions of DE genes obtained by Ingenuity Pathway Analysis (IPA)

Diseases and Disorders	p-value Range[a]	# Molecules
Cancer	3.37E-05 - 2.93E-02	81
Gastrointestinal Disease	3.37E-05 - 2.93E-02	52
Tumor Morphology	5.86E-05 - 2.52E-02	9
Endocrine System Disorders	1.40E-03 - 2.55E-02	4
Organismal Injury and Abnormalities	2.09E-03 - 2.81E-02	31
Molecular and Cellular Functions		
Cell Death and Survival	3.86E-04 - 2.52E-02	19
Carbohydrate Metabolism	5.93E-04 - 2.52E-02	11
Molecular Transport	5.93E-04 - 2.52E-02	16
Small Molecule Biochemistry	5.93E-04 - 2.52E-02	16
Gene Expression	6.85E-04 - 2.66E-02	32
Physiological System Development and Function		
Tissue Morphology	9.73E-05 - 2.93E-02	18
Cardiovascular System Development and Function	4.44E-04 - 2.55E-02	14
Organ Morphology	2.89E-03 - 2.80E-02	19
Organismal Development	2.89E-03 - 2.95E-02	28
Embryonic Development	2.90E-03 - 2.77E-02	22

[a] Range of FDR-corrected p-values for all significant ($P < 0.05$) individual biological function terms grouped under each category, calculated by Fisher's exact test.

Table 3.2. List of differentially expressed genes associated with fatty acid metabolism

Gene Symbol	*Gene Name*	*Gene Function*	*Tissue*	*Microarray (20 samples)*			*Real-time PCR (20 samples)*			*Real-time PCR (LT n=31, SC n=61)*		
				Expression	*p-value*	*Fold Change*[e]	*Expression*	*p-value*	*Fold Change*[c]	*Expression*	*p-value*	*Fold Change*[c]
C8H9orf3	chromosome 8 open reading frame, human C9orf3	Fatty acid biosynthethic process	LT[a]	Flax[c] > No-Flax[d]	0.02**	1.73	Flax > No-Flax	0.19	1.22	Flax > No-Flax	0.47	1.04
SGMS1	sphingomyelin synthase 1	Synthesis of sphingomyelin	LT	Flax < No-Flax	0.02**	0.65	Flax > No-Flax	0.12	1.23	Flax > No-Flax	0.16	1.14
PLCB4	phospholipase C, beta 4	Hydrolysis of phosphatidylinositol	LT	Flax > No-Flax	0.04**	1.31	Flax > No-Flax	0.17	1.16	Flax > No-Flax	0.22	1.11
ACO2	aconitase 2, mitochondrial	TCA cycle	LT	Flax > No-Flax	0.05*	1.25	Flax > No-Flax	0.01**	1.55	Flax > No-Flax	0.01**	1.32
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	TCA cycle	LT	Flax > No-Flax	0.06*	1.23	Flax > No-Flax	0.01**	1.45	Flax > No-Flax	0.01**	1.24
SAMD8	sterile alpha motif domain containing 8	Phospholipid metabolic process	LT	Flax < No-Flax	0.06*	0.71	Flax < No-Flax	0.83	0.98	Flax < No-Flax	0.12	0.86
USF2	upstream transcription factor 2	Fatty acid biosynthethic process	LT	Flax > No-Flax	0.08*	1.7	Flax > No-Flax	0.01**	1.24	Flax > No-Flax	0.06*	1.09
HACL1	2-hydroxyacyl-CoA lyase 1	Alpha oxidation of fatty acids	SC[b]	Flax > No-Flax	0.07*	1.33	Flax > No-Flax	0.32	1.18	Flax > No-Flax	0.04**	1.17

[a] *Longissimus thoracis* muscle
[b] Subcutaneous fat
[c] Flax-high index
[d] No-Flax-low index
[e] A value ≥ 1 indicates that expression is higher in the Flax-high index animals
** p-value < 0.05
* p-value < 0.1

Table 3.3. Top biological functions of the 8 DE genes associated with fatty acid metabolism

Diseases and Disorders	p-value Range[a]	# Molecules	Genes
Cancer	4.17E-04 - 2.15E-02	1	*SDHA*
Connective Tissue Disorders	4.17E-04 - 4.17E-04	1	*PLCB4*
Developmental Disorder	4.17E-04 - 8.35E-04	2	*PLCB4*, *SDHA*
Endocrine System Disorders	4.17E-04 - 2.15E-02	1	*SDHA*
Hereditary Disorder	4.17E-04 - 1.16E-02	3	*PLCB4*, *ACO2*, *SDHA*
Molecular and Cellular Functions			
Lipid Metabolism	3.13E-04 - 3.82E-02	5	*SAMD8*, *SGMS1*, *HACL1*, *SDHA*, *PLCB4*
Small Molecule Biochemistry	3.13E-04 - 3.82E-02	7	*SAMD8*, *SGMS1*, *USF2*, *HACL1*, *SDHA*, *ACO2*, *PLCB4*
Cellular Function and Maintenance	8.35E-04 - 8.35E-04	1	*USF2*
Carbohydrate Metabolism	1.25E-03 - 3.82E-02	3	*USF2*, *ACO2*, *PLCB4*
Molecular Transport	1.25E-03 - 3.45E-02	2	*USF2*, *SGMS1*
Physiological System Development and Function			
Organ Morphology	4.17E-04 - 2.72E-02	3	*USF2*, *PLCB4*, *SGMS1*
Reproductive System Development and Function	4.17E-04 - 3.13E-02	1	*USF2*
Tissue Morphology	4.17E-04 - 3.86E-02	3	*USF2*, *PLCB4*, *SGMS1*
Hair and Skin Development and Function	1.67E-03 - 1.67E-03	1	*USF2*
Nervous System Development and Function	3.75E-03 - 2.23E-02	1	*PLCB4*

[a] Range of FDR-corrected p-values for all significant ($P < 0.05$) individual biological function terms grouped under each category, calculated by Fisher's exact

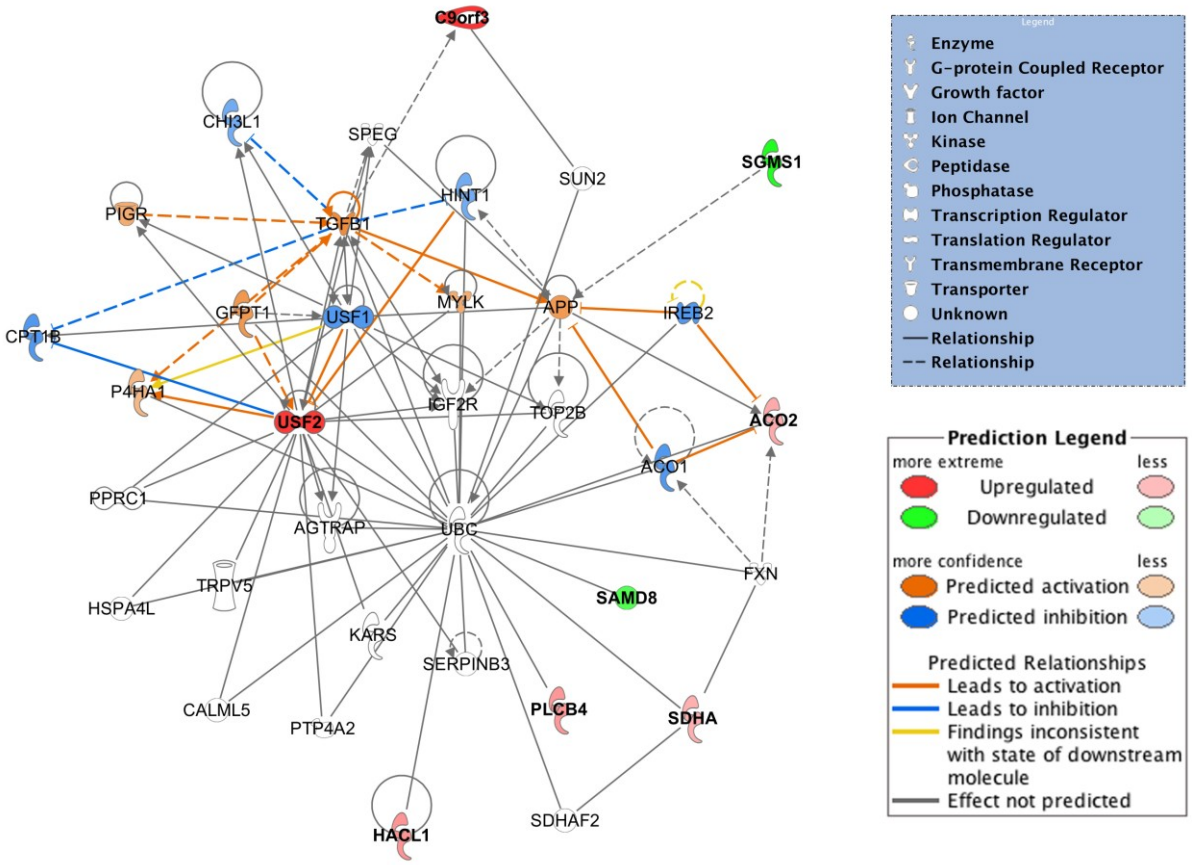

Figure 3.1. Gene interaction network between the eight differentially expressed genes (bolded in this network) related to metabolism of fatty acids and other genes in Ingenuity Knowledge Base (IKB)

CHAPTER 4. ASSOCIATIONS BETWEEN GENE EXPRESSION IN MEAT AND FAT TISSUES IN BEEF CATTLE AND TISSUE FATTY ACID PROFILES

4.1. INTRODUCTION

Investigating the expression of genes within muscle and fat tissue of the bovine, in relation to FA profiles within those tissues, provides an opportunity to understand the biological control of FA profiles in meat. Significant correlations between individual genes and/or pathways and FA levels can be exploited through selection for gene expression, or discovery of a polymorphism within the gene that could also be used in selection, which could increase beneficial FAs, or decrease harmful FAs. In terms of associations between gene SNPs and FAs in cattle, many studies have been performed on well-known SNPs, for example Li et al. (2012) reported that within the 878 C>T SNP in *SCD*, cattle with 878 CC genotype had higher concentrations of MUFAs and PUFAs and lower amounts of SFAs in brisket adipose tissue. Goszczynski et al. (2014) reported that cattle with rs109759779 SNP in *LIPE* gene had higher MUFAs concentration in muscle tissue. Zhang et al. (2008) reported that in the SNP 17924A>G of *FASN*, animals with g.17924GG genotype had lower SFAs and higher MUFAs in the total lipid and triacylglycerol fraction compared to the animals with SNP g.17924AA. However, a limited number of association studies have been performed between genes and FAs, and of those that were performed most were genes well-known to be involved in FA metabolism. Therefore, identifying significant correlations between new genes and FA profiles will add to the basic cohort of genes know to influence the levels of FAs in meat, ultimately helping geneticists explain more variation in FA profiles and also aid them to develop molecular tests that will select cattle that are genetically capable of producing beneficial FA profiles in their muscle and fat tissues.

Real-time PCR is a standard method for quantification of gene expression and has been widely employed as a validation method for microarray studies. However, it is a relatively low throughput and high cost technique typically performed in a 96 or 384 well plate format. A high-throughput gene expression instrument, the BioMark HD real-time PCR platform (Fluidigm Corporation, San Francisco, USA), is a new technology which allows the monitoring of expression levels of hundreds of genes simultaneously (Spurgeon et al., 2008). It is a microfluidic technology uses the integrated fluidic circuits (IFC) which contain hundreds of microfluidic controlled valves and interconnected channels to move molecules of biological samples and reagents in a variety of patterns (Melin and Quake, 2007). With this technology, it is possible to measure the expression of up to 96 genes in up to 96 samples in one run and the sample and reagent usage is 50 to 100 times lower compare to the conventional real-time PCR (Jang et al., 2011; Spurgeon et al., 2008). Therefore, this new real-time PCR technology is well-established when small amounts of sample are available, and when whole genes related to a specific pathway are screened.

This study investigated the effects of flax supplementation in the diet of mature cull cows, and subsequent changes in FA profile of *Longissimus thoracis* muscle and subcutaneous fat, in the context of related changes in gene expression. In this study, 40 genes were selected for high-throughput BioMark HD real-time PCR gene expression analysis. These genes were partially chosen from results of the previous study in which the analysis of a microarray experiment (Contrast: Flax-high index vs. No-Flax-low index) resulted in identifying eight DE genes associated with FA metabolism (p-value < 0.1), whilst the remaining 32 genes were selected from a combination of the results of the microarray experiment (genes with p-value ≥ 0.1), literature, and in consultation with an ALMA funded project 2010R038R (Identifying DNA

markers for enhancing beneficial fatty acids in beef). The objectives of the present study are to identify expression differences in the flax-supplemented cattle versus non-supplemented, and to investigate the correlation between gene expression values and individual or group of FAs found in the meat and fat tissues of the animals. Then genes associated with FA levels can be directed into genomic studies as identification of candidate genes for selection to improve the FA profile of meat.

4.2. MATERIALS AND METHODS

4.2.1. Animals and treatments

The cows used for tissue collection belonged to the feeding experiment conducted by He et al. (2012), and Nassu et al. (2011). The study was conducted in the Lethbridge Research Centre feedlot of Agriculture and Agri-Food Canada (Lethbridge, Canada), and was approved by the center's Animal Care Committee under the auspices of the Canadian Council of Animal Care (CCAC, 1993). In brief, 64 crossbred cull cows (~30 months of age) with similar breed composition were randomized by weight/body condition (average BW of 620±62 kg (mean±SD)), and fed 50:50 forage:concentrate diets on a DM basis (16 cows/treatment) in a 2x2 factorial design. The 4 diets consisted of hay:ground barley (HB), hay:ground barley with ground flaxseed (HBF), silage:ground barley (SB), and silage:ground barley with ground flaxseed (SBF). Flaxseed was substituted for barley grain at 15% of the ration DM, resulting in a dietary lipid content of ~6% of DM. With the addition of a vitamin and mineral supplement, all diets fully met or exceeded the nutrient requirements of cull cows, as recommended by NRC (1996).

4.2.2. Tissue collection and RNA isolation

All cows were slaughtered after 140 days on the diet. Two tissues including *Longissimus thoracis* muscle and subcutaneous fat were collected for RNA isolation: *Longissimus thoracis*

muscle was collected from the medial side of the carcass at the 10th rib, and subcutaneous fat from backfat situated on the right side of the animal close to the 10th rib. In total, 31 and 61 samples were collected from LT and SC tissues respectively. The tissue was immediately frozen in liquid nitrogen and then stored at – 80°C until RNA isolation. The frozen tissue was ground by mortar and pestle in the presence of liquid nitrogen. Total RNA was isolated by the TRIzol® procedure (Invitrogen, Carlsbad, USA), using 1 ml TRIzol® to every 50 mg of tissue. Fifty mg of tissue was transferred to a 2 ml metal hard tissue grinding beaded tube (Bertin Technologies, Montigny le Bretonneux, France) and 1 ml TRIzol reagent was added to the tube in order to perform the lysis and homogenization steps of tissue in TRIzol with Cryolys® and Precellys® tissue homogenization system (Bertin Technologies, Montigny le Bretonneux, France). Homogenization was performed at 5500 rpm, 2 times for 30 s (10 s pause between two cycles) at 4 °C, then the tubes were centrifuged at 12,000 g, 4 °C for 10 min, and following that supernatant was transferred to a new tube. Afterwards, RNA isolation steps were completed according to the TRIzol® procedure (Invitrogen, Carlsbad, USA). The RNA pellet was resuspended in 30 µl RNase free water. After the pellet had dissolved completely, the RNA was quantified on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA), and tested for integrity on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). All RNA samples had RIN score of ≥ 7 and if any sample had a RIN score of less than 7, that sample was re-extracted to get a RIN of ≥ 7. Individual RNAs were diluted to 150 ng/µl with RNase-free water prior to cDNA systhesis and stored at -80 °C.

4.2.3. Reverse transcription

cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit and the standard protocol (Applied Biosystems, Foster City, USA). Briefly, each tissue's reference

RNA was created by pooling 1 µL of each individual sample RNA (150 ng/µl). For each of the individual RNAs and the reference pool, 10 µl of reverse transcription (RT) reaction mix per reaction was prepared by mixing 2 µl of 10X RT buffer, 0.8 µl of 25X dNTP Mix (100 mM), 2 µl of 10X RT Random Primer, 1 µl of RNase inhibitor (20 U/µl), 1 µl of MultiScribe™ Reverse Transcriptase (50 U/µl), and 3.2 µl of RNase free water. The RT mix was added to 10 µl of each RNA and the reference pool (150 ng/µl), gently mixed, and the entire reaction was then incubated at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. The resulting cDNA was placed on ice, aliquoted in appropriate volumes to be used in the real-time PCR mixes, and then frozen at –20°C until ready for use.

4.2.4. Selection of genes

In total, eight endogenous or "housekeeping" genes, plus 40 target genes associated with metabolism of FAs, were selected for each tissue (Table 4.1). Apart from a few differences, the gene list for *Longissimus thoracis* muscle was fairly similar to subcutaneous fat. Out of the selected 40 gene of interest targets, 8 were those DE genes identified through the analysis of microarray data with p-value of less than 0.1, as discussed in chapter 3 of this thesis. The remaining 32 were selected partly from the results of the microarray experiment (genes with a p-value ≥ 0.1), and, the rest of the genes were chosen from literature and in consultation with an ALMA funded project (2010R038R - Identifying DNA markers for enhancing beneficial fatty acids in beef) which investigated the association of genomic SNPs in bovine with FA profile in meat.

4.2.5. Designing and testing of real-time PCR primers

After genes of interest had been identified, forward and reverse primers of each gene were designed using the following procedure: the approved gene name of each transcript was

identified by Hugo gene nomenclature committee (HGNC, Hinxton, UK) software which is available online at http://www.genenames.org/. Then reference sequence of all of the isomers of each gene for *Bos taurus* were aligned with all of the *Homo sapiens* and *Mus musculus* isoforms of that gene using Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) to select the longest isoform of *Bos taurus* gene transcript which covers most areas of that gene. The target and excluded (corresponding to introns) regions of mRNA of the selected isoform were identified by BLAT Search Genome program (https://genome.ucsc.edu/cgi-bin/hgBlat?command=start). After which all required primer quality information (target regions, excluded regions, product size range between 90-200 bp (base pair), number, primer size between 18-22 bp, primer Tm (melting temperature) between 59-61^{o}C, primer GC% between 40-65, and CG clamp equal to 1) for the selected isoform was submitted into Primer3 software (Version 0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/). Primer pairs were then tested with the IDT oligoanalyzer tool (http://www.idtdna.com/) to find those with minimum probability of allowing hairpins, self- and hetero-dimers. Then both primers of each gene were aligned to the genome of *Bos taurus* and other organisms to ensure the primers were specific for that gene/amplicon. To test primer amplification parameters and efficiency, a serial dilution of a cDNA reference sample (the serial dilution for each tissue was tissue-specific), with reference to the original input RNA concentration (20, 10, 5, 2.5, 1.25, 0.625, and 0.2 ng) was amplified. This was carried out in 96-well optical reaction plates (Applied Biosystems, Foster City, USA) using the Applied Biosystems Step-One-Plus real-time PCR system (Applied Biosystems, Foster City, USA). The total volume of each reaction was 20 µL and contained 4 µl of each reference cDNA template (20, 10, 5, 2.5, 1.25, 0.625, and 0.2 ng/µl), 0.5 µl of each forward and reverse primer (10 µM), 10 µl of the 2× KAPA SYBR® FAST qPCR

Master Mix ABI Prism™ (Kapa Biosystems, Boston, USA), and 5 µl of RNase free water. Control samples in each run consisted of the reference RNA without conversion to cDNA by reverse transcriptase (no RT control), and water instead of template cDNA (no template control). All standard curves and controls were run in duplicate on each plate. Amplification conditions consisted of: [1] hold stage for 30 s at 95°C for enzyme activation; [2] cDNA amplification stage of 3 s at 95°C and then 30 s at 60°C for 40 cycles; and [3] a final melt curve stage which consisted of 15 s at 95°C, 1 min at 60°C, and then heating (melt) of the reaction with a ramp rate of +0.3°C per 15 s from 60°C to 95°C to obtain fluorescence measurements that reflect the Tm of the main product while minimizing interference from primer-dimer or other non-specific amplification products. For each primer pair, if any of the following conditions was detected, including no agreement between duplicates in the standard curve, improper amplification plot, two or more peaks in melt curve, or a reaction efficiency of lower than 90% or higher than 110%, those primers were rejected and new primer-sets were designed. For all the primer-pairs the product identity was confirmed via visual inspection of the melt curve as well as sequence of the PCR products from a few samples. Tm for all subsequent reactions was at 60^{o}C, and the reaction efficiencies were between 90 and 110%.

4.2.6. Pre-PCR amplification

Before real-time PCR can be conducted on the 96.96 Dynamic Array (Fluidigm microfluidic array), all individual samples must be pre-amplified. For pre-amplification of cDNA, all gene assay primers were pooled into one tube (500 nM concentration for each gene assay (forward plus reverse primer)). Both reference cDNA and individual sample cDNAs of each tissue were diluted to 10 ng/µl with reference to the original input RNA concentration. One pre-amplification reaction was performed per individual cDNA sample (31 for LT and 61 for SC).

The reference cDNA for each tissue (separately) was also pre-amplified. The pre-PCR amplification reaction was performed in a total volume of 5 µl containing 2.5 µl of 2X TaqMan PreAmp Master Mix (Applied Biosystems, Foster City, USA), 0.5 µl of 500 nM pooled primer mixture, 1 µl of cDNA (10 ng/µl), and 1 µl of RNase free water. Two control samples which consisted of no template control (water instead of template cDNA) and no RT control (the reference RNA without conversion to cDNA by reverse transcriptase) were also used. The pre-amplification PCR program consisted of 1 cycle 95°C for 10 min, and then 10 cycles of 95°C for 15 s and 60°C for 4 min. After pre-amplification PCR, the products were cleaned up with Exonuclease I to remove unincorporated primers: 2 µl of diluted exonuclease I (4U/µl) (containing 0.2 µl of Exonuclease I reaction buffer and 0.4 µl of Exonuclease I (20 U/µl) (New England BioLabs, Ipswich, USA)), plus 1.4 µl of RNase free water, was added to each 5 µl pre-amplification reaction; incubated at 37°C for 30 min and then 80°C for 15 min to stop the reaction. The reaction was then diluted 5-fold by adding 18 µl of TE buffer (10mM Tris-HCI, 1.0 mM EDTA; TEKnova, Hollister, USA) and stored at -20°C until further use. Before performing the formal fluidigm experiment, all pre-amplifications were tested with 2X SsoFast EvaGreen Supermix with Low ROX (Bio-Rad, Hercules, USA) via ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, USA) to confirm that specific products could be amplified from the pre-amplified samples. The PCR thermal and reaction conditions were the same as the section 2.5 (Designing and testing of real-time PCR primers) which was described above, but in the reaction 2X SsoFast EvaGreen Supermix with Low ROX was substituted for SYBR green. For all primer-sets, the product identity was confirmed via interrogation of the melt curve, Tm was 60^{o}C, and the reaction efficiency was between 90 and 110%.

4.2.7. Gene expression analysis using 96.96 dynamic array

For each tissue a standard curve was created by serial dilution of a pre-amplified reference cDNA which had an initial concentration of 0.4 ng/µl with reference to the original input RNA concentration (standard curve concentrations: 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125, and 0.004 ng/µl). The serial dilution for each tissue was tissue-specific and was always made fresh from the aliquoted pre-amplified reference cDNA before each real-time PCR reaction. Individual sample pre-amplified cDNAs for each tissue were diluted to a final concentration equal to 0.1 ng/µl input RNA for the real-time PCR reaction. In a 96.96 dynamic array chip, each serial dilution included 4 replicates while individual samples were run in duplicate. Real-time PCR was carried out using the 96.96 Dynamic Array IFC (Fluidigm Corporation, San Francisco, USA). Specifically, a 5 µl sample mixture was prepared for each sample which contained 2.5 µl of 2X SsoFast EvaGreen Supermix with Low ROX (Bio-Rad, Hercules, USA), 0.25 µl of 20X DNA Binding Dye Sample Loading Reagent (Fluidigm Corporation, San Francisco, USA), and 2.25 µl of each of diluted pre-amplified cDNA. Five µl of assay mix was also prepared for each gene which contained 2.5 µl of 2X Assay Loading Reagent (Fluidigm Corporation, San Francisco, USA), 2.25 µl of 1X DNA Suspension Buffer (Fluidigm Corporation, San Francisco, USA), and 0.25 µl of 100 µM each of forward and reverse primers, mixed together. The assay mix was also prepared for control samples in each run which consisted of the no pre-amp control (no RT control (the reference RNA without conversion to cDNA by reverse transcriptase) without conversion to pre-amplified cDNA by 2X TaqMan PreAmp Master Mix), and two no template controls (one was pre-amplified reaction which contained water for template, and the other was solely water). The IFC Controller HX (Fluidigm Corporation, San Francisco, USA) for the 96.96 Dynamic Array IFC was used to prime the fluidics array (chip) with control line fluid (as per standard protocol). After priming, samples and assay mixes were pipetted in the appropriate

inlets then loaded into the chip using the IFC Controller HX (as per standard protocol). Once the samples and assay mixes were loaded onto the chip it was placed in the BioMark HD Instrument (Fluidigm Corporation, San Francisco, USA) for real-time PCR amplification. The program consisted of 70°C for 40 min, 60°C for 30 s, 95°C for 60 s followed by 30 cycles at 96°C for 5 s and 60°C for 20 s. After completion of the run, a final melt curve was performed which consisted of 3 s at 60°C and then heating (melt) of the reaction with a ramp rate of 1°C per 3 s from 60°C to 95°C. The melt was performed to obtain fluorescence measurements that reflect the Tm of the main product to confirm that a single specific product was amplified. All gene assays passed this quality control.

4.2.8. Data preprocessing and analysis

Amplification fluorescence data was collected using BioMark Data Collection Software, version 4.1.3 (Fluidigm Corporation, San Francisco, USA), and the cycle of threshold (Ct) was calculated by plotting normalized fluorescence (ΔRn) in relation to cycle number via Real-time PCR Analysis Software, version 4.1.3 (Fluidigm Corporation, San Francisco, USA). Real-time PCR Analysis Software was also used to determine the valid PCR reactions; for each primer pair we observed an agreement between duplicates in the standard curve, proper amplification plot, a single peak in the melt curve, as well as a reaction efficiency of between 90-110%. Reactions that did not meet these criteria were labeled invalid reactions, and were not used for later analyses and treated as missing data. The results were exported to an Excel file and the mean values of calibrated relative concentration (Calibrated rConc) were used for the later analysis. Calibrated rConc values are essentially a concentration of original starting RNA in a sample, assigned relative to where the sample falls on the standard curve of input RNA concentrations versus Ct (same as for traditional real-time PCR). The Calibrated rConc mean of the sample

duplicate values of all samples for all endogenous genes were used to find the best gene or combination of normalization genes using NormFinder software (version 20) (Andersen et al., 2004), in order to normalize the target gene data. Peptidylprolyl isomerase A [*PPIA*] was selected as the best endogenous gene to normalize data for LT muscle, while for SC a combination of glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*] and ribosomal protein L19 [*RPL19*] (the geometric mean of the gene expression value for each of the two genes) was used. For normalization, the Calibrated rConc mean value of each sample for each target gene was divided by the Calibrated rConc mean value of the best endogenous gene(s) selected by NormFinder. The resulting normalized gene expression values were used in downstream statistical analysis. Due to the poor amplification (invalid reactions as described above) results for some genes (for LT: RNA, 18S ribosomal [*RNA18S*], *ACTB*, *EEF1A2*, *GAPDH*, acyl-CoA dehydrogenase, C-2 to C-3 short chain [*ACADS*], *FADS1*, patatin-like phospholipase domain containing 2 [*PNPLA*2 or *ATGL*], protein kinase, AMP-activated, alpha 2 catalytic subunit [*PRKAA2*], and stearoyl-CoA desaturase 5 [*SCD5*]; and for SC: *ACTB*, *HMBS*, ribosomal protein, large, P0 [*RPLP0*], *YWHAZ*, *DGAT1*, fatty acid binding protein 4, adipocyte [*FABP4*], phospholipase A2, group VI (cytosolic, calcium-independent) [*PLA2G6*], *SAMD8*, and sterol regulatory element binding transcription factor 2 [*SREBF2*]), were removed for further data analysis. From the mentioned genes, *RNA18S*, *ACTB*, *EEF1A2*, *GAPDH*, *HMBS*, *RPLP0*, *YWHAZ* were endogenous genes and the others were target genes.

4.2.9. Statistical analysis of the genes

To analyze the real-time PCR data SAS Ver. 9.2 was used. The contrasts in gene expression measured between flax vs. no-flax were analyzed by PROC TTEST and NPAR1WAY for normal and non-normal data, respectively. Normality of gene expression data was tested by

PROC UNIVARIATE and if it was normal PROC TTEST (two-tailed) was used for analysis. Within the PROC TTEST results if variance of a gene was equal (between the 2 treatment groups) the p-value for the method "Pooled" was selected, otherwise the p-value for the method "Sattherthwaite" was selected. Fold change in gene expression between groups was also obtained by dividing the mean value of flax to no-flax group. If the gene expression data was not normal NPAR1WAY was used for analysis and the p-value of Kruskal-Wallis Test was selected; fold change for the non-normal data was also obtained by dividing the mean value of flax to no-flax group from the results of PROC TTEST. For both normal and non-normal data the cut-off p-value of a trend was < 0.1, and for significance < 0.05.

4.2.10. Correlations between fatty acid measurements and gene expression data

SAS Ver. 9.2 was used to perform the correlation analysis between gene expression and FA amounts and concentrations in LT and SC tissues. Data for FA measurements in both LT and SC were provided by the previous experiments performed by He *et al.* (2012) and Nassu et al. (2011). FA profiles were expressed as percent fatty acid methyl ester (%FAME) for LT muscle and SC, as well as mg FAME/g tissue for LT (Tables 4.3, 4.4, and 4.5). FAMEs are esters of FAs which are derived by trans-esterification of fats with methanol. The %FAME of each FA for each sample was calculated as follows: *[(mg FAME/g tissue of each FA)/(mg FAME/g tissue of sum of all the FA profile)] ×100*. Normality of the data for all genes and FAs were tested and the normal data was used for later analysis. If the data for some genes or FAs were not normal the Box-Cox transformation within PROC TRANSREG was used to find the best model for data transformation. All of the genes became normal while few FAs could not be normalized: % FAME of SC (PUFA-with trans-non-conjugated, n-3, C18:3n-3, C22:5n-3, n-6/n-3 [omega-6/omega-3], c12-18:1, c14-18:1, c15-18:1, Total-t-18:1, and Index (40:60) [Index [0.40(18:3n) +

0.60(Long Chain:3n)])]; % FAME of LT (PUFA-with trans-non-conjugated, Tot-CLA [Total-Conjugated Linoleic Acid], Tot-c,t-CLA, c14-18:1, c15-18:1, Tot-t-18:1, and C17:0); and mg FAME/g tissue of LT (n-3, C18:3n-3, C22:5n-3, and n-6/n-3). For these FAs the transformation or raw data that was closest to normal was used for the correlation analysis. There was no data available for C20:5n-3, Tot-CLNA [Total- conjugated α-linolenic acid], c9,t11,t15-18:3, and c9,t11,c15-18:3 of %FAME of SC tissue therefore these FAs were removed from the correlation analysis. Pearson correlation coefficients (r) were calculated using the PROC CORR procedure in SAS (Ver. 9.2) to analyze the association between gene expression and many individual FAs and important combinations of FAs such as SFA, MUFA, PUFA, n-3, n-6, and the n-6:n-3 ratio, as well the index equation used to rank cows for selection for the original microarrays, within each tissue. As described in chapter 3, an index equation was designed to rank cows based on the alpha-linolenic acid (18:3n-3) and long chain n-3 (3n) FAs [sum of 20:3n-3, 20:5n-3 and 22:5n-3] that accumulated in the fat: *Index=0.40(18:3n-3) + 0.60(Long Chain:3n)*. Hence, we also calculated the coefficient between all genes and this index. P-values of < 0.05 indicated a significant correlation between gene expression and FA abundance.

4.3. RESULTS

4.3.1. Gene expression analysis using 96.96 dynamic array

Genes that were significantly differentially expressed (p-value < 0.05), and those that displayed a trend for significance (p-value < 0.1) in DE between flax-supplemented and no-flax-supplemented cows are found in Table 4.2. In LT tissue, only three genes including *ACO2*, carnitine palmitoyltransferase 2 [*CPT2*], and solute carrier family 25 (carnitine/acylcarnitine translocase), member 20 [*SLC25A20*] could be considered as DE. All of these 3 genes exhibited elevated expression in the flax-fed group compared to no-flax. In SC tissue, the identified DE

genes were *ACO2*, *HACL1*, phosphoenolpyruvate carboxykinase 2 (mitochondrial) [*PCK2*], and ubiquitin C [*UBC*]. Three of these genes, *ACO2*, *HACL1*, and *PCK2*, were more highly expressed in the flax group, whereas the expression of *UBC* was lower in the flax group.

4.3.2. Association between genes and fatty acids

Results of the significant (p-value < 0.05) correlation analysis between gene expression and mg FAME/g tissue, and %FAME tissue in LT, are available in Tables 4.3 and 4.4, respectively. Significant correlations between gene expression and FA abundance were obtained between 14 genes and FA profiles in mg FAME/g LT tissue, while 19 genes and FA profiles were correlated to each other in %FAME LT tissue. Pearson correlation coefficients (r) ranged between -0.48 and 0.58, and -0.62 and 0.58 mg FAME/g tissue and %FAME tissue, respectively, in LT. Since mg FAME/g tissue and %FAME tissue in LT were related to each other, correlation results with gene expression are very similar. For instance, *ACO2* which has a role in the TCA cycle displays a positive correlation between its expression and PUFA-with trans-non-conjugated, and Tot-t-18:1 in both mg FAME/g and %FAME, as well as a negative correlation between *ACO2* and n-6 FAs.

Results from correlation analysis between gene expression and % of FAME in SC tissue are found in Table 4.5. A significant (p-value < 0.05) correlation was obtained between the expression of 19 genes and FA profiles in SC, and the pearson correlation coefficient (r) ranged between -0.45 and 0.42. There were both similarities and differences in the significantly correlated gene lists for SC as compared to LT tissue. For example, significant correlations between FAs and acyl-CoA oxidase 1, palmitoyl [*ACOX1*] and *CPT1B* genes were only present in LT tissue. In addition, correlations between FAs and Lipase, hormone-sensitive [*LIPE*], Leptin [*LEP*], 3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble) [*HMGCS1* or HMG-CoA

synthase], and *PCK2* were only observed for SC tissue. Similar patterns were observed for expression of *ACO2* within SC and LT muscle and respective tissue FA profiles; a positive correlation was found between this gene and PUFA-with trans-non-conjugated, and Tot-t-18:1, plus a negative correlation between *ACO2* and n-6/n-3 ratio was seen in both LT and SC tissues.

4.4. DISCUSSION

The FA composition was determined by Nassu et al. (2011) and He et al. (2012) in the Lethbridge Research Centre feedlot of Agriculture and Agri-Food Canada (Lethbridge, AB, Canada). If gene expression is significantly correlated to FA profiles there exists an opportunity to select for gene expression, or search for a polymorphism within the gene, that might increase beneficial FAs, or decrease harmful FAs. For simplicity, significant correlations between gene expression and non-normal FA data will not be discussed, although these are presented and noted as non-normal data in Tables 4.3-4.5. We will begin our discussion with the association analysis between gene expression and single or groups of FAs (within both SC and LT tissues), followed by genes that were significantly DE (p-value < 0.05), as well as those that displayed a trend for DE (p-value < 0.1), between flax vs. no-flax treated animals.

Free FAs (provided from the diet or synthesized in the cells) should undergo enzymatic reactions which are catalyzed by acyl-CoA synthases [ACSs], such as acyl-CoA synthetase short-chain family member 1 [*ACSS1*] and acyl-CoA synthetase short-chain family member 2 [*ACSS2*], in the cytosolic side of the mitochondrial membrane to be esterified to fatty acyl-CoAs which can either be used in the surface of smooth endoplasmic reticulum and the mitochondrial inner membrane to synthesize the phospholipid component of membranes, or be transferred into the mitochondria to undergo oxidation (Lehninger et al., 2004). Conversion of free FAs with CoA to their acyl-CoA esters is composed of two sequential bidirectional reactions and therefore the

composition of a reacting system (a mixture of chemical reactants and products) tends to continue changing until equilibrium is reached, where the rates of the forward and reverse reactions are exactly equal and no further net change occurs in the system, however formation of acyl-CoA is more favourable reaction compared to its hydrolysis (Lehninger et al., 2004). The fatty acyl-CoA produced by *ACSS1* is utilized exclusively for the synthesis of cellular lipids while the acyl-CoA generated by *ACSS2* is specifically degraded through beta-oxidation (Wakil et al, 1983). In our study in LT muscle, *ACSS1* was moderately negatively correlated to PUFAs (CLAs and CLNAs) and C18:1, which could indicate that the conversion of these FAs to their acyl-CoA esters was not needed when their acyl-CoAs were abundant. *ACSS2* was moderately negatively correlated to PUFAs while moderately positively correlated to MUFAs and SFAs in LT muscle, which can indicate that there was a need for conversion of MUFAs and SFAs to their acyl-CoA esters when their acyl-CoAs were available in low amounts, whereas when there were ample amounts of PUFA acyl-CoAs the need for this conversion was removed. However, in SC tissue *ACSS2* was weakly negatively correlated with C14:0 (Myristic acid).

After conversion of a FA to its fatty acyl-CoA ester in the cytosol, fatty acyl-CoA can undergo oxidation. In animal cells, beta-oxidation of FAs takes place in the mitochondria and FAs with chain length ≤ 12 can directly enter the mitochondrial membrane without the help of membrane transporters while those having ≥ 14 carbons (most of the FAs obtained from the diet or liberated from the adipose tissue) cannot directly enter the mitochondria and should firstly undergo the enzymatic reactions of the carnitine cycle (Figure 4.1) (Lehninger et al., 2004). *CPT1*, *SLC25A20*, and *CPT2* encode the enzymes of the carnitine cycle. In this cycle, from the available acyl-CoA and carnitine in the cytosol, *CPT1* makes acyl-carnitine (removes CoA) which is transported via the carnitine-acylcarnitine translocase (CACT), the enzymes encoded by

SLC25A20, into the mitochondrial matrix and converted to acyl-CoA + carnitine by *CPT2*. Carnitine will be returned into the cytosol by the CACT but acyl-CoA is converted via beta-oxidation in the mitochondria to acetyl-CoA which can either be oxidized by the TCA cycle in the mitochondria to produce energy or be used for FA synthesis in the cytosol (Britton et al. 1995; Strijbis et al., 2010). The carnitine-mediated entry of fatty acyl-CoA is the rate-limiting step for beta-oxidation of FAs in the mitochondria which is governed partly by substrate availability (Lehninger et al., 2004). On the other hand, beta-oxidation of dietary and endogenous LCFAs is tightly regulated through allosteric inhibition of *CPT1* by malonyl-CoA, the first intermediate in the synthesis of FAs, so any changes to *CPT1* can affect the whole carnitine shuttle (Lehninger et al., 2004). *CPT1* (both *CPT1A* and *CPT1B*) was moderately positively correlated to the PUFAs while moderately negatively correlated with most of MUFAs (except c7-16:1 and c12-18:1) and SFAs in LT muscle. *SLC25A20* was also moderately positively correlated to PUFAs (except C20:4n-6 which is arachidonic acid, AA) and Tot-t-18:1 in LT muscle. Similarly, *CPT2* was also showed a moderate positive correlation with PUFAs and c12-18:1 in LT muscle. Therefore, all the three genes involved in carnitine shuttle were directly related to each other and were positively correlated to PUFAs, thus *CPT1* and *CPT2* were also positively correlated to c12-18:1, which showed that the expression of these genes increased in response to the increased PUFAs which is in agreement with the study reviewed by Buckley and Howe (2010) where dietary long-chain n-3 FAs increased oxidation of FAs (via increased expression of *CPT1*) and energy expenditure, and decreased fat deposition. In SC tissue, *CPT1A* also showed a moderate negative correlation with MUFAs but it had a moderate positive correlation with C14:0, and *SLC25A20* also showed a positive correlation with C15:0 which could indicate that the expression of *CPT1A* and *SLC25A20* increased in response to the

increased SFA in SC fat. Hessvik et al. (2010) reported that treating human abdominal muscle cells with ALA, 20:5n-3 (EPA), and OA up-regulated the expression of *SLC25A20*. The reason for the negative correlation between n-6 FA (AA) and *SLC25A20* could be due to lower deposition of n-6 compared to n-3 FAs in both LT and SC tissues which is likely related to the competitive inhibition of elongation and desaturation of LA by ALA (Nassu et al., 2011). Lemas et al. (2012) performed a study on 1141 Eskimo individuals fed 20:5n-3 (EPA) and 22:6n-3 (DHA) (EPA and DHA are capable to modulate gene expression to favor increased FA oxidation and reduced fat deposition) to understand which polymorphisms in *CPT1* gene are associated with oxidation of FAs since it was previously reviewed by Buckley and Howe (2010) that dietary long-chain n-3 PUFAs raised the expression of *CPT1* resulted in increased oxidation of FAs and energy expenditure. The results showed that L479 allele of the P479L SNP in *CPT1* gene is associated with decreased risk of cardiovascular disease (through increased HDL-cholesterol) and reduced obesity. Hence, due to the fact that *CPT1*, *SLC25A20*, and *CPT2* were positively related to healthier FAs, especially n-3s, they could possibly be used as screening genes for bovine genetic selection with improved FA profile. *CPT2* and *SLC25A20* were also those DE genes (p-value < 0.1) identified in LT tissue showed elevated expression in the flax-fed group compared to no-flax. This does infer that feeding flax changes the energy metabolism within the muscle tissue, and that there is a higher presence of LCFAs reaching the muscle from the digestive tract, in agreement with observations of He et al. (2012), and Nassu et al. (2011).

Once acyl-CoA is transferred from the cytosol to the mitochondrial matrix by the carnitine-shuttle it should undergo beta-oxidation in the mitochondria, which is oxidative removal of successive two-carbons in the form of acetyl-CoA (starting from the carboxyl end of the fatty acyl chain) (Lehninger et al., 2004). Acyl-CoA dehydrogenase, very long chain [*ACADVL*]

encodes an enzyme which catalyzes the first step in beta-oxidation of long- and very-long-chain FAs in the mitochondrial matrix (Aoyama et al., 1995). Beta-oxidation is an irreversible process and is regulated by substrate availability, provided by the carnitine shuttle (Lehninger et al., 2004). In SC fat, *ACADVL* showed a moderate negative correlation with MUFAs and since MUFAs also had a negative correlation with *CPT1*, this negative association could be expected.

The mitochondrial matrix is the major site of oxidation of FAs but peroxisomes are other organelles of animals which are able to oxidize fatty acyl-CoA to acetyl-CoA (Lehninger et al., 2004). One of the differences between mitochondrial and peroxisomal oxidation is in the first oxidative step of FAs where the energy is released as ATP in the mitochondrial matrix while in the peroxisome it is released as heat (Lehninger et al., 2004). Another difference is in the specificity for fatty acyl-CoAs, peroxisomes are more active on very-long-chain FAs and branched-chain FAs, such as phytanic acid, (these less common dietary FAs are obtained from the fat of ruminant animals, dairy products, meat, and fish) and catalyze them to shorter-chain fatty acyl-CoAs which are exported to the mitochondrial matrix to be oxidized in the TCA cycle (Lehninger et al., 2004). In terms of branched-chain acyl-CoAs which have methyl group on their beta carbon, beta-oxidation is not possible and they should undergo alpha-oxidation which removes two carbons from the carboxyl end of fatty acyl-CoA so the obtained fatty acyl-CoA does not have methyl group on its beta carbon, which then can be oxidized further by beta-oxidation (Lehninger et al., 2004). The key genes we investigated that relate to the functioning of the alpha- and beta-oxidation in peroxisomes are *HACL1* and *ACOX1* respectively (Varanasi et al., 1994; Foulon et al., 1999; Mannaerts et al., 2000). *ACOX1* is a peroxisomal enzyme which catalyzes the first step in beta-oxidation of FAs (desaturation of acyl-CoAs to 2-trans-enoyl-CoAs) (Varanasi et al., 1994) and since beta-oxidation is an irreversible process so it is regulated

by the amount of substrate (Lehninger et al., 2004). In LT muscle, *ACOX1* was moderately negatively associated with ≤ 18 carbons n-6s, MUFAs, and SFAs which showed that possibly there was no need for oxidation of these FAs in the peroxisomes since their oxidation usually occurs in the mitochondrial matrix. *HACL1* encodes a peroxisomal enzyme catalyzes alpha-oxidation of 3-methyl-branched FAs, i.e. phytanic acid, in which *HACL1* catalyzes a carbon-carbon cleavage in 2-hydroxy-3-methylacyl-CoA to produce formyl-CoA and a 2-methyl-branched fatty aldehyde (Foulon et al., 1999; Mannaerts et al., 2000). Similar to *ACOX1*, *HACL1* was moderately negatively correlated to C16:0 (PA) in LT tissue and in SC fat it was weakly negatively correlated to 20:3n-6 (Dihomo gamma linoleic acid, DGLA) and these negative correlations could be related to the fact that in peroxisomes oxidation of very-long-chain FAs (> 22C) and branched-chain FAs takes and *HACL1* usually catalyzes alpha-oxidation of branched-chain FAs. In SC tissue, *HACL1* also showed higher expression in flax-fed cows which could be due to the higher availability of LCFAs in the fat tissue of flax-fed group compared to non-flax-fed animals as mentioned previously (He et al., 2012; Nassu et al., 2011).

After beta-oxidation of acyl-CoA to acetyl-CoA in the mitochondria, acetyl-CoA then enters the TCA cycle, which is a series of chemical reactions that result in producing energy through oxidation of acetate, in the form of acetyl-CoA, derived from fats, carbohydrates, and proteins (Lehninger et al., 2004). Therefore any change in availability of FAs for beta-oxidation will ultimately affect the functioning of the TCA cycle as it is governed partly by substrate availability (Lehninger et al., 2004). The key genes we investigated that relate to the functioning of the TCA cycle are *ACO2* and *SDHA*, encode the mitochondrial enzymes aconitase and succinate dehydrogenase respectively (Beinert and Kennedy, 1993; Hirawake et al., 1994). *ACO2* and *SDHA* function directly inside the formal TCA cycle, converting citrate to isocitrate,

and succinate to fumarate, respectively (Beinert and Kennedy, 1993; Hirawake et al., 1994). These two steps in the TCA cycle are tightly regulated through allosteric inhibition of aconitase by fluorocitrate (Swanson and Graham, 1994) and inhibition of succinate dehydrogenase by malonate (an analog of succinate not normally present in a cell) (Koeppen and Riley, 1987) as well as their regulation by the substrate availability (Lehninger et al., 2004). *ACO2* is closer to the beginning of the TCA cycle and acetyl-CoA, therefore its action could be more related to the amount of FAs coming from the carnitine shuttle and beta-oxidation of FAs (Lehninger et al., 2004). In LT muscle, *ACO2* was positively correlated to PUFAs (except n-6 FAs) and MUFAs with ≥ 18 carbons while negatively correlated with < 17 carbons MUFAs and SFAs, and in SC tissue it was positively correlated to ≥ 18 carbons PUFAs. Similarly, *SDHA* was negatively correlated with < 17 carbons MUFAs and SFAs is LT, and it was negatively correlated with n-6 FAs in SC. Therefore the results for both *ACO2* and *SDHA* are in agreement with the most of the correlations between the genes of carnitine shuttle and beta-oxidation pathways. *ACO2* is also one of those DE genes (p-value < 0.1) identified in both LT and SC tissues which showed elevated expression in the flax-fed group compared to no-flax. Hence, its positive association with bio-hydrogenated intermediates of n-3 FAs from the flax diet would indicate that these are used as fuel for the TCA cycle as opposed to n-6s when they are abundant, or possibly related to the competitive inhibition of elongation and desaturation of LA by ALA (Nassu et al., 2011), and therefore less LA was used as a substrate for the TCA cycle. Therefore, the elevated expression of *ACO2* in n-3 supplemented cows, its negative association with harmful SFAs, and its positive association with healthy n-3s and CLAs as well as the negative association of *SDHA* with harmful SFAs, make these genes as strong candidates to genetically select animals having beneficial FA profile in their muscle and fat tissues.

Phosphoenolpyruvate carboxykinase 1 [PCK1] and PCK2 are cytosolic and mitochondrial (respectively) enzymes that play a key role in gluconeogenesis and can regulate glucose homeostasis (Stark et al., 2014). In the presence of guanosine triphosphate (GTP), these genes convert oxaloacetate (OAA) to phosphoenolpyruvate (PEP), the rate-limiting step in the metabolic pathway that produces glucose from lactate and other precursors derived from the TCA cycle (Stark et al., 2014). Expression of these genes is normally regulated by circulating hormones to match systemic glucose demand, however their expression is associated tightly with TCA cycle activity, therefore these genes should be partly regulated by the amount of substrate coming from the TCA cycle (Burgess et al., 2007; Stark et al., 2014). In LT muscle, *PCK1* was moderately positively associated with ≥ 18 carbons MUFAs and SFAs and it was weakly positively associated with CLAs in SC fat. These results are similar to associations between the same FAs with *ACO2* and *SDHA*, key genes of the TCA cycle. All of these results were also in agreement with observations of Davis and Gibson (1967), Collins et al. (2006), and Navarro et al. (2010) which suggest that MUFAs, PUFAs, and SFAs with ≥ 18 carbons induce up-regulation of PCK genes. In this study, *PCK2* showed elevated expression in flax-fed group than non-flax-fed group in SC fat, which could be due to the increased metabolism of LCFAs and higher TCA cycle activity of the long-chain acetyl-CoA precursors, especially n-3s, which came from the flax diet, in the mitochondrial matrix.

When the diet provides higher than enough fuel to fulfill a cell or organism's energy requirements, oxidation of FAs to provide the needed energy is not essential and is therefore down-regulated, so the excess (lipids, proteins, and carbohydrates) is converted to FAs (acetyl-CoAs) (Lehninger et al., 2004). The key genes regulating oxidation or synthesis of FAs are acetyl-CoA carboxylases (ACCs) including acetyl-CoA carboxylase alpha [*ACACA*] and acetyl-

CoA carboxylase beta [*ACACB*] (Lehninger et al., 2004; Wakil and Abu-Elheiga, 2009). ACCs encode enzymes which catalyze the irreversible carboxylation of acetyl-CoA at the cytosolic side of mitochondrial membrane to form malonyl-CoA, which functions as inhibitor of *CPT1* activity so the transfer of the fatty acyl group to the mitochondrial matrix for beta-oxidation will not happen (Lehninger et al., 2004; Wakil and Abu-Elheiga, 2009). Therefore by the act of ACCs, FA oxidation is reduced while FA synthesis is increased. The malonyl-CoA produced by *ACACA* is used by *FASN* for the synthesis of FAs in the cytosol, whereas the one generated by *ACACB* functions as inhibitor of *CPT1* activity (Wakil and Abu-Elheiga, 2008). Since ACCs inhibit *CPT1* activity, those FAs which had a positive correlation with *CPT1* likely have negative correlation with ACCs and vice versa. In LT muscle, *ACACA* depicted a moderate negative correlation with PUFAs and a moderate positive correlation with c15-18:1 in LT. It was also weakly negatively correlated with C14:0 in SC tissue which is in contrast to the positive correlation between this FA and *CPT1*. In addition, the obtained correlation results are in agreement with the previous study reviewed by Wakil et al. (1983) where LCFAs inhibit activity of Acetyl-CoA Carboxylases. However, *ACACB* depicted a moderate positive correlation to 22:5n-3 (Docosapentaenoic acid, DPA) in LT tissue which is in contrast to what was expected and the study reviewed by Wakil et al. (1983). This suggests that unlike other LCFAs, DPA possibly has a direct relationship with the activity of *ACACB*, the higher the availability of DPA in the muscle, the more expression of *ACACB* therefore less beta-oxidation occurs. Matsumoto et al. (2012) reported many SNPs in the *ACACA* gene associated with different FAs in dairy cows. For instance, cows with T/T type of the SNP in the coding sequence of *ACACA* showed higher % of C18:0 compared to T/C type in the milk, and cows with CC/GT type of SNP in the promoter of *ACACA* showed a higher amounts of C18:2 in the meat compared to those having GT/GT.

Zhang et al. (2009) reported that beef cattle with g.2203GG genotype in *ACACA* had higher amounts of TAG, SFAs, MUFAs compared with those with g.2203GT genotype in *Longissimus dorsi* muscle.

Thyroid hormone responsive [*THRSP* or *SPOT14*] encodes an enzyme which decreases the availability of malonyl-CoA (Knobloch et al., 2013). It showed a moderate positive correlation to MUFAs and SFAs in LT tissue but moderate negative correlation to PUFAs. We are unsure in terms of association of *THRSP* with different FAs since its precise function is not known (Kuemmerle and Kinlaw, 2011). However, Oh et al. (2014) reported that cattle with GG genotype of g.78 G>A and CC genotype of g.184 C>T in *THRSP* gene had higher PUFAs and MUFAs while lower SFA content in *Longissimus dorsi* muscle. Therefore, selection for these genotypes of *THRSP* is possibly associated with healthier FA profile of meat.

Following the production of malonyl-CoA, FAs synthesis is performed via *FASN* in the cytosol to produce the C16:0 (PA), the principal product of *FASN* in animal cells (Lehninger et al., 2004). PA then will be transferred to the smooth endoplasmic reticulum which then may be lengthened to form C18:0 (Stearic acid, SA) or even longer via elongation enzymes such as ELOVL fatty acid elongase 5 [*ELOVL5*], or desaturated through desaturases like *SCD* (Lehninger et al., 2004). Therefore, in theory the association results between elongases and desaturases with FAs would likely be in agreement with what was obsereved between *ACACA* and FAs. Mammals cannot convert C18:1 to LA or ALA so these FAs should be provided from the diet (Lehninger et al., 2004). *ELOVL5* catalyzes the elongation of several LC-PUFA substrates with the higher activity towards 18:3n-6 (GLA) which is converted into C20:3n-6 (DGLA) (Leonard et al., 2000). On the one hand, it was unexpected that this gene was moderately positively correlated with MUFAs and SFAs but highly negatively and moderately

negatively correlated to n-6s and n-3s, respectively, in LT tissue, since its main role of action is elongating PUFAs. On the other hand, these correlations are in agreement with that was observed for the correlation between *ACACA* and FAs. Moreover, Leonard et al. (2000) reported that besides elongating n-3 and n-6 FAs, *ELOVL5* can elongate MUFAs such as 16:1 and 18:1. In SC fat, *ELOVL5* showed a moderate negative correlation with C14:0 which was expected since *ACACA* was also negatively correlated to C14:0. SCD is an endoplasmic reticulum resident enzyme catalyzes desaturation of SFAs to MUFAs. It specifically catalyzes insertion of the first double bond in the cis-Δ9 position of saturated fatty acyl-CoA substrates including palmitoyl-CoA and stearoyl-CoA to produce palmitoleyl- and oleyl-CoA (Scaglia and Igal, 2005), as well as 18:1 trans-11 (trans-vaccenic acid) into cis-9, trans-11 CLA (Rumenic acid) in the bovine (Griinari et al. 2000). Therefore as SFAs and MUFAs increase in LT tissue, *SCD* expression is increased as these FAs are its substrates. Indeed, *SCD* showed a positive association with MUFAs and SFAs in this study. On the other hand, in LT muscle, the negative correlation between PUFA and *SCD* expression would suggest that as the amount of PUFA increases in tissue, expression of *SCD* decreases which is likely mediated through down-regulating the expression of *SREBF*, which regulates the expression of *SCD*, and which also had a negative correlation with PUFAs in LT. Moreover, this negative association is also in agreement with the observation of Waters et al. (2009) where dietary PUFA reduced expression of both *SREBF* and *SCD* genes. In SC fat, *SCD* was weakly positively correlated to CLA, which is in contrast to what was expected since *SCD* catalyzes conversion MUFA to CLA and not CLA to other PUFAs. As well, we have not seen a significant correlation between *SREBF* and CLA in SC tissue to verify this positive association. Li et al. (2012) reported that in the 878 C>T SNP in *SCD* (this SNP causes an amino acid change from alanine (A) to valine (V)), the 'CC' genotype

was significantly correlated to higher amounts of MUFAs and PUFAs and lower concentrations of SFAs in brisket adipose tissue. Barton et al. (2010) also reported that in the SNP 878 C>T of *SCD* gene, animals with AA or AV genotype significantly depicted low amounts of SFAs, high amounts of MUFAs, and high MUFA/SFA ratio compared to those with VV genotype in both muscle fat and subcutaneous fat of Fleckvieh bulls. Therefore, these results suggests that *SCD* is a strong candidate gene influencing FA composition in beef cattle since it was positively associated with harmful SFAs and selection based on 'CC' SNPs can lead to higher deposition of MUFAs and PUFAs in beef adipose tissue and less deposition of harmful SFAs in fat depots of beef cattle.

If most of the FAs (synthesized FAs or those ingested by the animal) do not undergo the oxidation process, they will have one of two fates; they can be stored as TAGs or incorporated into the phospholipids (Lehninger et al., 2004). Sphingomyelin is a unique form of a phospholipid called sphingophospholipid which is synthesized via *SGMS1*, by transferring phosphatidylcholine (the phosphatidyl head group) on to the primary hydroxyl of ceramide (a ceramide is composed of sphingosine and a fatty acid). This reaction is bidirectional which depends on the respective levels of ceramide and the sphingolipid (Huitema et al., 2004; Vacaru et al., 2009). In LT muscle, *SGMS1* showed a moderate positive correlation to PUFAs (n-3 and n-6) and a moderate negative correlation to MUFAs and SFAs. n-6 and n-3 PUFAs are important structural components of phospholipids (Stillwell and Wassall, 2003; Raphael and Sordillo, 2013) and in phospholipids, the ratio of PUFA/SFA is higher (Raes et al., 2004). Therefore, these correlations might be expected since PUFAs are substrates of *SGMS1* and suggest that PUFAs up-regulates expression of *SGMS1*, or that high concentration of SFAs and MUFAs decrease expression of *SGMS1*. SAMD8 is an endoplasmic reticulum transferase which has no

sphingomyelin synthase activity but catalyzes conversion of phosphatidylethanolamine (PE) and ceramide to ceramide phosphoethanolamine (CPE), the sphingomyelin analogue of phosphatidylethanolamine, although with low product yield (Vacaru et al., 2009). Since *SAMD8* has a direct role in the synthesis of the sphingomyelin analogue ceramide phosphoethanolamine, therefore similar to *SGMS1*, *SAMD8* showed a moderate negative correlation to SFAs in LT muscle which suggest that SFAs induce down-regulation of *SAMD8*. Since *SGMS1* and *SAMD8* are negatively associated with SFAs and positively associated with PUFA, therefore selection for these genes might increase healthier PUFAs of membranes in muscle tissue.

As it was mentioned earlier the other fate of free FAs is incorporation into TAGs. *DGAT1* catalyzes the terminal and only committed step in TAG synthesis by using diacylglycerol (DAG) and fatty acyl-CoA as substrates and it is essential for the formation of adipose tissue and for survival (Cases et al., 1998). This gene was moderately positively and moderately negatively associated with C18:0 and c7-16:1 respectively, in LT muscle. This result is in semi-agreement with the study of Schennink et al. (2008) who reported that in the bovine *DGAT1* K232A polymorphism the *DGAT1* A allele is associated with lower undesirable FAs (10:0, 12:0, 14:0, and 16:0), and with higher 18:0 and CLAs. Therefore, *DGAT1* is a key gene associated with healthier FAs and selective breeding can make a significant contribution to change the FA composition of cattle's meat.

When the diet does not provide enough energy for the cells, the stored TAG is hydrolyzed to free FAs in adipocytes and through the blood free FAs go to the site of action (i.e. liver to be oxidized for providing energy; or muscle to be incorporated into phospholipid membranes or to be oxidized to provided the energy which fuels muscle contraction) (Lehninger et al., 2004). PNPLA2 (or adipose triglyceride lipase [ATGL]), catalyzes the initial step in the hydrolysis of

TAGs in adipose tissue of mammals (Zimmermann et al., 2004). The expression of *PNPLA2* was positively correlated to the presence of CLAs which is somewhat in agreement with the studies of Park et al. (1999) and Hargrave et al. (2002) in which CLAs induce body fat loss. LIPE is another enzyme which hydrolyzes stored TAGs, diacylglycerols, monoacylglycerols, cholesteryl esters, and other lipid and water soluble substrates; therefore its main function is to mobilize the stored fats (Holm et al., 1988). In SC tissue, this gene was positively correlated to SFAs while negatively correlated with c9-18:1, in agreement with observations of Yonezawa et al. (2008), suggests that its expression is increases with the presence of SFAs. A positive correlation between *LIPE* and c9-16:1 was also obsereved which is in agreement with the observations of Bolsoni-Lopes et al. (2013) where treatment of mice adipocytes with c9-16:1 elevated expression of *LIPE*. Goszczynski et al. (2014) reported that rs109759779 SNP in *LIPE* is associated with higher 18:1 and total-MUFA concentration in the meat of cattle, which also makes it an ideal candidate for selection to improve FA profiles of meat.

LEP encodes a hormone (leptin which is known as satiety hormone), secreted by white adipocytes, which regulates body weight or actually regulates the amount of fat stored in the body. It acts as part of a signaling pathway which inhibits food intake and/or regulate energy expenditure (if leptin is secreted, energy expenditure increases) to maintain constancy of the adipose mass (Friedman, 2000; Soukas et al., 2000). In SC tissue, the negative association of *LEP* with 20:4n-6 (AA) is in agreement with the observations of Pérez-Matute et al. (2003) where treating the mice adipocytes with this FA reduced expression of *LEP*.

Cholesterol, similar to long-chain FAs, is synthesized from acetyl-CoA (Lehninger et al., 2004). *SREBF1*, *SREBF2*, *HMGCS1*, and 3-hydroxy-3-methylglutaryl-CoA reductase [or HMG-CoA reductase; *HMGCR*] are key genes which regulate cholesterol synthesis (Hua et al., 1993;

Yokoyama et al., 1993). When cholesterol levels are low, *SREBF* is bound to SRE-1 which is a decamer in 5′ flanking of *HMGCR* activating *HMGCR* transcription, which has a key role in the synthesis of cholesterol (Eberlé et al., 2004; Lehninger et al., 2004). In LT muscle, *SREBF2* showed a moderate negative correlation to CLNA (c9,t11,t15-18:3), CLAs, c9-16:1, and 14:0 while showed a moderate positive association to c9-18:1. The observed correlations are in agreement with the studies of Kim et al. (1999), Roche et al. (2002), Brandebourg and Hu (2005), and Nakatani et al. (2005) where the dietary PUFAs (n-3, n-6, CLA, CLNA) decrease the expression of SREBF gene. Ou et al. (2001) have also demonstrated that presence of PUFA reduce *SREBF1* expression, and Dobrosotskaya et al. (2002) have shown that SFAs down-regulated the expression of *SREBF* gene. The reason for the positive correlation of c9-18:1 with *SREBF2* is unclear and it is in contrast to some studies (Hannah et al., 2001; Hsu and Huang 2006) in which c9-18:1 reduced expression of *SREBF1*. Similar to *SREBF2*, it is unusual that Tot-MUFA was moderately positively correlated to *SREBF1* in LT muscle which is in contrary with what has been reported (Hannah et al., 2001; Hsu and Huang 2006) where c9-18:1 reduces expression of *SREBF1*. Nafikov et al. (2013) reported that haplotype H1 of *SREBF1* significantly decreased undesirable SFAs such as 12:0 and 14:0 in the milk of dairy cows. Hoashi et al. (2007) surveyed the presence of polymorphism in *SREBF1* and identified an 84 base deletion (Short type: S) and an insertion (Long type: L) in intron 5 of *SREBF1* in Japanese Black cattle. Results showed that the S type was associated to a higher MUFA proportion in intramuscular fat. As well, Barton et al. (2010) reported that in the 84-bp Ins/Del polymorphism, animals with LS genotype significantly depicted higher amounts of C14:1 cis-9 compared to those with LL genotype in subcutaneous fat of Fleckvieh bulls which suggest that this polymorphism may serve as a potential genetic marker to improve FA profile of beef. *HMGCS1* encodes an enzyme which

binds acetyl-CoA with acetoacetyl-CoA to form 3-Hydroxy-3-Methylglutaryl-CoA [HMG-CoA], which is the substrate for *HMGCR*. HMG-CoA reductase is a rate limiting enzyme in synthesis of cholesterol. In the present study, both *HMGCS1* and *HMGCR* expression was positively correlated to the presence of c9-18:1 and negatively correlated to C14:0. Therefore, these associations were expected since *SREBF* genes is a transcription factor which binds to the promoter region of HMG-CoA reductase to increase its expression level and in this study *SREBF2* was positively correlated to c9-18:1 and negatively correlated to 14:0.

UBC encodes a polyubiquitin protein which can be conjugated as a precursor to other proteins and then depending on the residues to which ubiquitin is conjugated, can lead to various effects within a cell such as protein degradation, cell-cycle regulation, DNA repair, and regulation of other cell signaling pathways (Radici et al., 2013). In SC tissue, *UBC* was weakly positively correlated to the total amount of all SFAs determined in this study. Qi et al. (2006) reported that *UBC* represses FA synthesis in adipose tissue by degrading acetyl-coenzyme A carboxylase (ACC). As well it has been shown that 16:0 inhibits the activity of ACC (16:0 high, ACC low, beta-oxidation allowed to increase) (Brun et al., 1997). Our study also showed lower expression of *UBC* in flax-fed animals (contains n-3 FAs), in agreement with this function as a higher abundance of circulating LCFAs would reduce the need for FA synthesis (He et al., 2012; Nassu et al. 2011).

4.5. CONCLUSIONS

Several studies have identified polymorphisms associated with FA composition in beef and dairy cattle (Avilés et al. 2013; Hoashi et al. 2007; Kgwatalala et al., 2009; Li et al., 2012; Oh et al., 2011; Schennink et al., 2008; Taniguchi et al. 2004). Many of the previous studies were performed to identify SNPs in large groups of animals but most of them did not consider the

effect of diet on FA profile of muscle and fat. In this study, analyzing genes that are expressed when the n-3 FA content of the tissue of beef cattle rises via dietary supplementation of flaxseed provided an excellent opportunity to identify and comprehend more of the genes that control this process. In the present study, the correlations between genes and FA profiles in LT and SC tissues of a group of cattle fed flax vs. non-flax-fed group identified several candidate genes including *CPT1A*, *CPT1B*, *SLC25A20*, *CPT2*, *ACOX1*, *ACO2*, *SDHA*, *ACACA*, *ACACB*, *THRSP*, *SREBF1*, *SREBF2*, *HMGCS1*, *HMGCR*, *ELOVL5*, *SCD*, *DGAT1*, *PNPLA2*, *LIPE*, *LEP*, *SGMS1*, and *SAMD8*, all of them were associated with improved FA profile in muscle and fat tissues of beef cows. Amongst the mentioned genes, the top candidates are *SCD* (associated with reduced amount of harmful SFAs through desaturating them), *SGMS1* (associated with higher amounts of PUFAs and lower amounts of SFAs incorporating in sphingolipids), *SAMD8* (associated with less amounts of SFA in ceramide phosphoethanolamine (CPE), the sphingomyelin analogue of phosphatidylethanolamine), and *LIPE* (associated with higher hydrolysis of TAGs having SFAs). Therefore, these genes can be used as a screening tool to select cattle that have the potential to produce healthier FAs, especially n-3s, in their tissue. As well, they can be used as new gene targets to identify SNPs on a genomic scale.

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Table 4.1. List of the selected genes used in Biomark HD real-time PCR for both *Longissimus thoracis* muscle and subcutaneous fat tissues

***Longissimus thoracis* muscle and subcutaneous fat**			***Longissimus thoracis* muscle and subcutaneous fat**		
Gene symbol	***Gene name***	***Type***	***Gene symbol***	***Gene name***	***Type***
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Endo	*FABP4* [b]	fatty acid binding protein 4, adipocyte	Target
PPIA	peptidylprolyl isomerase A (cyclophilin A)	Endo	*SREBF1* [b]	sterol regulatory element binding transcription factor 1	Target
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	Endo	*SREBF2* [b]	sterol regulatory element binding transcription factor 2	Target
ACTB	actin, beta	Endo	*ACACA* [b]	acetyl-CoA carboxylase alpha	Target
RPL19	ribosomal protein L19	Endo	*PCK1* [b]	phosphoenolpyruvate carboxykinase 1 (soluble)	Target
HMBS	hydroxymethylbilane synthase	Endo	*PRKAA2* [b]	protein kinase, AMP-activated, alpha 2 catalytic subunit	Target
RNA18S	RNA, 18S ribosomal	Endo	*HMGCR* [b]	3-hydroxy-3-methylglutaryl-CoA reductase	Target
PNPLA2 [b]	patatin-like phospholipase domain containing 2	Target	*CPT1A* [b]	carnitine palmitoyltransferase 1A (liver)	Target
C8H9orf3 [a]	chromosome 8 open reading frame, human C9orf3	Target	***Longissimus thoracis* muscle**		
SGMS1 [a]	sphingomyelin synthase 1	Target	***Gene symbol***	***Gene name***	***Type***
PLCB4 [a]	phospholipase C, beta 4	Target	*EEF1A2*	eukaryotic translation elongation factor 1 alpha 2	Endo
ACO2 [a]	aconitase 2, mitochondrial	Target	*FADS1* [b]	fatty acid desaturase 1	Target
SDHA [a]	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Target	*UCP2* [b]	uncoupling protein 2 (mitochondrial, proton carrier)	Target
SAMD8 [a]	sterile alpha motif domain containing 8	Target	*ACADS* [b]	acyl-CoA dehydrogenase, C-2 to C-3 short chain	Target
USF2 [a]	pstream transcription factor 2, c-fos interacting	Target	*ACACB* [b]	acetyl-CoA carboxylase beta	Target
HACL1 [a]	2-hydroxyacyl-CoA lyase 1	Target	*ACOX1* [b]	acyl-CoA oxidase 1, palmitoyl	Target
SCD [b]	stearoyl-CoA desaturase (delta-9-desaturase)	Target	*ACOX2* [b]	acyl-CoA oxidase 2, branched chain	Target
ACSS1 [b]	acyl-CoA synthetase short-chain family member 1	Target	*SCD5* [b]	stearoyl-CoA desaturase 5	Target
UBC [b]	ubiquitin C	Target	*CPT1B* [b]	carnitine palmitoyltransferase 1B (muscle)	Target
ACADVL [b]	acyl-CoA dehydrogenase, very long chain	Target	**Subcutaneous fat**		
NR1H3 [b]	nuclear receptor subfamily 1, group H, member 3	Target	***Gene symbol***	***Gene name***	***Type***
ELOVL5 [b]	ELOVL fatty acid elongase 5	Target	*RPLP0*	ribosomal protein, large, P0	Endo
PLA2G6 [b]	phospholipase A2, group VI (cytosolic, calcium-independent)	Target	*HMGCS1* [b]	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	Target
SLC25A20 [b]	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	Target	*FASN* [b]	fatty acid synthase	Target
DGAT1 [b]	diacylglycerol O-acyltransferase 1	Target	*LEP* [b]	leptin	Target
PPARA [b]	peroxisome proliferator-activated receptor alpha	Target	*apoD* [b]	apolipoprotein D	Target
AHSA1 [b]	AHA1, activator of heat shock 90kDa protein ATPase homolog 1 (yeast)	Target	*apoE* [b]	apolipoprotein E	Target
THRSP [b]	thyroid hormone responsive	Target	*PPARG* [b]	peroxisome proliferator-activated receptor gamma	Target
CPT2 [b]	carnitine palmitoyltransferase 2	Target	*PCK2* [b]	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	Target
ACSS2 [b]	acyl-CoA synthetase short-chain family member 2	Target	*LIPE* [b]	lipase, hormone-sensitive	Target
LPL [b]	lipoprotein lipase	Target			

[a] Eight differentially expressed genes identified through the analysis of microarray data with p-value < 0.1

[b] Thirty-two target genes selected from the results of the microarray experiment (which had a p-value ≥ 0.1), literature, and in consultation with an ALMA funded project (2010R038R)

Table 4.2. Genes that are significant DE (p-value < 0.05), as well as those that display a trend for DE (p-value < 0.1), between flax-fed versus no-flax-fed bovine tissues

Gene Symbol	Gene Name	Gene Function	Tissue	Expression	Fold Change[c]	P-Value
ACO2	aconitase 2, mitochondrial	TCA cycle	LT[a]	Flax > No-Flax	1.22	0.02
CPT2	carnitine palmitoyltransferase 2	Beta-oxidation of long-chain fatty acids	LT	Flax > No-Flax	1.14	0.04
SLC25A20	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	Beta-oxidation of long-chain fatty acids	LT	Flax > No-Flax	1.13	0.08
ACO2	aconitase 2, mitochondrial	TCA cycle	SC[b]	Flax > No-Flax	1.15	0.08
HACL1	2-hydroxyacyl-CoA lyase 1	Alpha oxidation of fatty acids	SC	Flax > No-Flax	1.39	0.04
PCK2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	Regulation of gluconeogenesis	SC	Flax > No-Flax	1.13	0.06
UBC	ubiquitin C	Regulation of different cell signaling pathways	SC	Flax < No-Flax	0.85	0.05

[a] *Longissimus thoracis* muscle
[b] Subcutaneous fat
[c] A value ≥ 1 indicates that expression is higher in the flax-supplemented animal

Table 4.3. Significant ($p < 0.05$) correlations between gene expression and FA or group of FAs in *Longissimus thoracis* muscle (mg FAME/g tissue)

Gene: Gene function	Fatty acid	Pearson correlation coefficients (r)
ACO2: TCA cycle	PUFA[a]-with trans-non-conjugated	0.41
	C20:3n-6	-0.47
	C20:4n-6	-0.45
	n-6[b]/n-3[c] *	-0.35
	c11-18:1	0.58
	c14-18:1	0.38
	Tot[d]-t-18:1	0.43
ACOX1: Beta-oxidation of fatty acids	C18:2n-6	-0.36
	c9-14:1	-0.37
	c9-16:1	-0.37
	SAT	-0.35
	C16:0	-0.39
CPT1A: Beta-oxidation of long-chain fatty acids	C20:5n-3	0.49
	C22:5n-3 *	0.38
	Tot-MUFA[e]	-0.39
	cis-MUFA	-0.43
	c9-16:1	-0.37
	c9-18:1	-0.45
	c15-18:1	-0.42
	SAT	-0.38
	C15:0	-0.37
	C16:0	-0.41
	C17:0	-0.36
	C18:0	-0.37
CPT1B: Beta-oxidation of long-chain fatty acids	cis-MUFA	-0.36
	c9-18:1	-0.37
	C16:0	-0.37
SLC25A20: Beta-oxidation of long-chain fatty acids	PUFA-with trans-non-conjugated	0.39
	C20:4n-6	-0.36
	n-3 *	0.4
	C18:3n-3 *	0.37
	n-6/n-3 *	-0.44
	Tot-t-18:1	0.37
ACSS1: Synthesis of fatty acyl-CoA	Tot-CLNA[f]	-0.39
	c9,t11,t15-18:3	-0.35
	c9,t11,c15-18:3	-0.4
	Tot-CLA[g]	-0.41
	Tot-t,t-CLA	-0.35
	Tot-c,t-CLA	-0.42
	c11-18:1	-0.39
	c14-18:1	-0.41
	Tot-t-18:1	-0.37
ACSS2: Synthesis of fatty acyl-CoA	C20:4n-6	-0.36
	Tot-MUFA	0.44
	cis-MUFA	0.46
	c7-16:1	0.35
	c9-16:1	0.43
	c9-18:1	0.46
	c15-18:1	0.49
	SAT	0.4
	C14:0	0.35
	C15:0	0.38

	C16:0	0.38
	C17:0	0.39
	C18:0	0.43
THRSP: Regulation of lipogenesis	Tot-MUFA	0.42
	cis-MUFA	0.42
	c7-16:1	0.38
	c9-18:1	0.44
	SAT	0.39
	C15:0	0.38
	C16:0	0.41
	C17:0	0.4
	C18:0	0.45
ACACA: Synthesis of fatty acids	c15-18:1	0.38
ELOVL5: Elongation of long-chain polyunsaturated fatty acids	Tot-MUFA	0.46
	cis-MUFA	0.47
	c9-14:1	0.36
	c7-16:1	0.36
	c9-16:1	0.46
	c9-18:1	0.47
	c15-18:1	0.47
	SAT	0.46
	C14:0	0.44
	C15:0	0.36
	C16:0	0.47
	C17:0	0.38
	C18:0	0.48
SCD: Desaturation of fatty acids	Tot-MUFA	0.55
	cis-MUFA	0.57
	c9-14:1	0.36
	c7-16:1	0.45
	c9-16:1	0.52
	c9-18:1	0.57
	c13-18:1	0.41
	c15-18:1	0.56
	SAT	0.52
	C14:0	0.47
	C15:0	0.44
	C16:0	0.53
	C17:0	0.5
	C18:0	0.56
SREBF2: Regulation of cholesterol homeostasis	c9,t11,t15-18:3	-0.38
	Tot-CLA	-0.42
	Tot-t,t-CLA	-0.48
	Tot-c,t-CLA	-0.4
	c9-16:1	-0.38
	C14:0	-0.41
PCK1: Regulation of gluconeogenesis	Tot-MUFA	0.36
	cis-MUFA	0.35
	c9-18:1	0.36
	C18:0	0.36
SGMS1: Synthesize the sphingolipid, sphingomyelin	Tot-MUFA	-0.36
	cis-MUFA	-0.36
	c9-18:1	-0.37
	C15:0	-0.39

[*] Non-normal data
[a] Polyunsaturated fatty acids
[b] Omega-6
[c] Omega-3
[d] Total

[e] Monounsaturated fatty acid
[f] Conjugated α-linolenic acid
[g] Conjugated linoleic acid
n-3= sum of C18:3n-3, C20:5n-3 and C22:5n-3; n-6= sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6 and C22:4n-6; PUFA-with trans-non-conjugated= sum of t11,t15-18:3, c9t13-/t8c13-18:2, t8c13-18:2, c9t12-18:2/c16-18:1, t9c12-18:2 and t11c15-18:2; Tot-CLNA= sum of c9,t11,t15-18:3 and c9,t11,c15-18:3; Tot-CLA= sum of Tot-c,t-CLA and Tot-t,t-CLA; cis-MUFA= sum of c9-14:1, c9-15:1, c7-16:1, c9-16:1, c10-16.1, c11-16:1, c12-16:1, c13-16:1, c5-17:1, c7-17:1, c9-17:1, c10-17:1, c9-18:1, c11-18:1, c12-18:1, c13-18:1, c14-18:1, c15-18:1, c9-20:1, c11-20:1 and c13-22:1; Tot-MUFA= sum of Tot-t-18:1 and cis-MUFA; Tot-t-18:1= sum of t6-t8-18:1, t9-18:1, t10-18:1, t11-18:1, t12-18:1, t13-t14-18:1, t15-18:1 and t16-18:1; SAT= sum of C14:0, C15:0, C16:0, C17:0, C18:0, C19:0, C20:0, C21:0, C22:0, C24:0 and Tot-Branched [sum of C14:0iso, C15:0iso, C15:0ai, C16:0iso, 17:0iso, C17:0ai and C18:0iso]

Table 4.4. Significant ($p < 0.05$) correlations between gene expression and FA or group of FAs in *Longissimus thoracis* muscle (%FAME)

Gene: Gene function	Fatty acid	Pearson correlation coefficients (r)
ACO2: TCA cycle	PUFA[a]-with trans-non-conjugated *	0.36
	n-6[b]	-0.39
	C18:2n-6	-0.35
	C20:3n-6	-0.46
	C20:4n-6	-0.38
	n-6/n-3[c]	-0.35
	Tot[d]-MUFA[e]	0.37
	c7-16:1	-0.41
	c11-18:1	0.53
	Tot-t-18:1 *	0.37
	C16:0	-0.37
SDHA: TCA cycle	c9-16:1	-0.43
	C16:0	-0.4
	C17:0 *	0.35
CPT1A: Beta-oxidation of long-chain fatty acids	PUFA-all cis-non conjugated	0.57
	n-3	0.58
	C18:3n-3	0.45
	C20:5n-3	0.57
	C22:5n-3	0.51
	Tot-CLNA[f]	0.37
	c9,t11,t15-18:3	0.43
	Tot-CLA[g] *	0.4
	Tot-c,t-CLA *	0.41
	cis-MUFA	-0.44
	c7-16:1	0.36
	c9-18:1	-0.46
	c12-18:1	0.44
	Index (40:60)	0.38
CPT1B: Beta-oxidation of long-chain fatty acids	PUFA-all cis-non conjugated	0.4
	n-3	0.37
	C20:5n-3	0.4
	C22:5n-3	0.46
	Tot-CLNA	0.37
	SAT	-0.32
	C14:0	-0.3
CPT2: Beta-oxidation of long-chain fatty acids	PUFA-with trans-non-conjugated *	0.41
	C18:3n-3	0.39
	n-6/n-3	-0.37
	c9,t11,t15-18:3	0.45
	Tot-CLA *	0.41
	Tot-t,t-CLA	0.4
	Tot-c,t-CLA *	0.39
	c12-18:1	0.36
	Tot-t-18:1 *	0.42
	Index (40:60)	0.36
SLC25A20: Beta-oxidation of long-chain fatty acids	PUFA-with trans-non-conjugated *	0.4
	n-6/n-3	-0.44
	Tot-t-18:1 *	0.37
HACL1: Alpha oxidation of fatty acids	C16:0	-0.37
ACACB: Synthesis of fatty acids	C22:5n-3	0.38
ACACA: Synthesis of fatty acids	PUFA-all cis-non conjugated	-0.38
	C20:5n-3	-0.37
ACSS2: Synthesis of fatty acyl-CoA	PUFA-all cis-non conjugated	-0.49
	n-6	-0.45

	C18:2n-6	-0.44
	C20:4n-6	-0.46
	C20:5n-3	-0.42
	C22:5n-3	-0.38
	c9,t11,c15-18:3	-0.43
	Tot-MUFA	0.38
	cis-MUFA	0.37
THRSP: Regulation of lipogenesis	PUFA-all cis-non conjugated	-0.5
	n-6	-0.48
	C18:2n-6	-0.46
	C20:4n-6	-0.45
	C20:5n-3	-0.4
	C22:5n-3	-0.45
DGAT1: Synthesis of triacylglycerol	c7-16:1	-0.37
	C18:0	0.37
ELOVL5: Elongation of long-chain polyunsaturated fatty acids	PUFA-all cis-non conjugated	-0.56
	n-6	-0.5
	C18:2n-6	-0.51
	C20:4n-6	-0.44
	n-3	-0.41
	C20:5n-3	-0.45
	C22:5n-3	-0.365
	c7-16:1	-0.43
SCD: Desaturation of fatty acids	PUFA-all cis-non conjugated	-0.62
	n-6	-0.56
	C18:2n-6	-0.56
	C20:3n-6	-0.35
	C20:4n-6	-0.53
	n-3	-0.44
	C20:5n-3	-0.59
	C22:5n-3	-0.54
	c9,t11,c15-18:3	-0.46
	c7-16:1	-0.44
SREBF1: Regulation of cholesterol homeostasis	Tot-MUFA	0.36
SREBF2: Regulation of cholesterol homeostasis	Tot-t,t-CLA	-0.43
	c9-16:1	-0.38
	c9-18:1	0.36
	C14:0	-0.43
	C17:0 *	0.39
SGMS1: Synthesize the sphingolipid, sphingomyelin	PUFA-all cis-non conjugated	0.42
	n-6	0.44
	C18:2n-6	0.45
	C20:4n-6	0.37
	C22:5n-3	0.37
	c7-16:1	0.43
	C15:0	-0.38
SAMD8: Convert phosphatidylethanolamine (PE) and ceramide to the sphingolipid, ceramide phosphoethanolamine (CPE)	SAT	-0.43
	C14:0	-0.4
	C16:0	-0.38

* Non-normal data
[a] Polyunsaturated fatty acids
[b] Omega-6
[c] Omega-3
[d] Total
[e] Monounsaturated fatty acid
[f] Conjugated α-linolenic acid
[g] Conjugated linoleic acid
n-3= sum of C18:3n-3, C20:5n-3 and C22:5n-3; n-6= sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6 and C22:4n-6; PUFA-all cis-non conjugated= sum of n-3, n-6, c9c15-18:2, and c12,c15-18:2; PUFA-with trans-non-

conjugated= sum of t11,t15-18:3, c9t13-/t8c13-18:2, t8c13-18:2, c9t12-18:2/c16-18:1, t9c12-18:2 and t11c15-18:2; Tot-CLNA= sum of c9,t11,t15-18:3 and c9,t11,c15-18:3; Tot-CLA= sum of Tot-c,t-CLA and Tot-t,t-CLA; cis-MUFA= sum of c9-14:1, c9-15:1, c7-16:1, c9-16:1, c10-16.1, c11-16:1, c12-16:1, c13-16:1, c5-17:1, c7-17:1, c9-17:1, c10-17:1, c9-18:1, c11-18:1, c12-18:1, c13-18:1, c14-18:1, c15-18:1, c9-20:1, c11-20:1 and c13-22:1; Tot-MUFA= sum of Tot-t-18:1 and cis-MUFA; Tot-t-18:1= sum of t6-t8-18:1, t9-18:1, t10-18:1, t11-18:1, t12-18:1, t13-t14-18:1, t15-18:1 and t16-18:1; SAT= sum of C14:0, C15:0, C16:0, C17:0, C18:0, C19:0, C20:0, C21:0, C22:0, C24:0 and Tot-Branched [sum of C14:0iso, C15:0iso, C15:0ai, C16:0iso, 17:0iso, C17:0ai and C18:0iso]; Index (40:60)= 0.40(18:3n-3) + 0.60(Long Chain:3n [sum of 20:3n-3, 20:5n-3 and 22:5n-3])

Table 4.5. Significant ($p < 0.05$) correlations between gene expression and FA or group of FAs in subcutaneous fat tissue (%FAME)

Gene: Gene function	Fatty acid	Pearson correlation coefficients (r)
ACO2: TCA cycle	PUFA[a]-with trans-non-conjugated *	0.3
	n-3 [b*]	0.27
	C18:3n-3 *	0.27
	n-6[c]/n-3 *	-0.28
	Tot[d]-CLA[e]	0.41
	Tot-t,t-CLA	0.3
	Tot-c,t-CLA	0.42
	Tot-t-18:1 *	0.3
	Index (40:60) *	0.25
SDHA: TCA cycle	C20:4n-6	-0.26
CPT1A: Beta-oxidation of long-chain fatty acids	Tot-MUFA[f]	-0.28
	c9-18:1	-0.31
	c13-18:1	-0.35
	C14:0	0.34
SLC25A20: Beta-oxidation of long-chain fatty acids	C15:0	0.28
ACADVL: Beta-oxidation of long-chain fatty acids	Tot-MUFA	-0.25
	c13-18:1	-0.25
HACL1: Alpha-oxidation of fatty acids	C20:3n-6	-0.28
	c15-18:1 *	0.26
	Index (40:60) *	0.25
ACACA: Synthesis of fatty acids	C14:0	-0.23
ACSS2: Synthesis of fatty acyl-CoA	C14:0	-0.25
ELOVL5: Elongation of long-chain polyunsaturated fatty acids	C14:0	-0.35
SCD: Desaturation of fatty acids	Tot-CLA	0.26
	Tot-c,t-CLA	0.27
PNPLA2: Hydrolysis of triglycerides in adipose tissue	Tot-CLA	0.26
	Tot-c,t-CLA	0.27
LIPE: Hydrolyzes stored triglycerides to free fatty acids in adipose tissue and heart and in steroidogenic tissues such as testis, it converts cholesteryl esters to free cholesterol for steroid hormone production	c9-16:1	0.27
	c9-18:1	-0.32
	C14:0	0.29
	C15:0	0.36
	C16:0	0.31
LEP: Regulation of body weight	C20:4n-6	-0.35
HMGCS1: Synthesis of cholesterol	c9-18:1	0.32
	C14:0	-0.45
HMGCR: Synthesis of cholesterol	c9-18:1	0.32
	C14:0	-0.41
NR1H3: Regulation of cholesterol homeostasis	C22:5n-3 *	-0.26
PCK1: Regulation of gluconeogenesis	Tot-CLA	0.26
	Tot-c,t-CLA	0.26
PCK2: Regulation of gluconeogenesis	PUFA-with trans-non-conjugated *	0.26
UBC: Regulation of different cell signaling pathways	C22:5n-3 *	-0.39

	SAT	0.26

* Non-normal data
[a] Polyunsaturated fatty acids
[b] Omega-3
[c] Omega-6
[d] Total
[e] Conjugated linoleic acid
[f] Monounsaturated fatty acid

n-3= sum of C18:3n-3, C20:5n-3 and C22:5n-3; n-6= sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6 and C22:4n-6; PUFA-with trans-non-conjugated= sum of t11,t15-18:3, c9t13-/t8c13-18:2, t8c13-18:2, c9t12-18:2/c16-18:1, t9c12-18:2 and t11c15-18:2; Tot-CLA= sum of Tot-c,t-CLA and Tot-t,t-CLA; Tot-MUFA= sum of Tot-t-18:1 and cis-MUFA [sum of c9-14:1, c9-15:1, c7-16:1, c9-16:1, c10-16.1, c11-16:1, c12-16:1, c13-16:1, c5-17:1, c7-17:1, c9-17:1, c10-17:1, c9-18:1, c11-18:1, c12-18:1, c13-18:1, c14-18:1, c15-18:1, c9-20:1, c11-20:1 and c13-22:1]; Tot-t-18:1= sum of t6-t8-18:1, t9-18:1, t10-18:1, t11-18:1, t12-18:1, t13-t14-18:1, t15-18:1 and t16-18:1; SAT= sum of C14:0, C15:0, C16:0, C17:0, C18:0, C19:0, C20:0, C21:0, C22:0, C24:0 and Tot-Branched [sum of C14:0iso, C15:0iso, C15:0ai, C16:0iso, 17:0iso, C17:0ai and C18:0iso]; Index 40:60= 0.40(18:3n-3) + 0.60(Long Chain:3n [sum of 20:3n-3, 20:5n-3 and 22:5n-3])

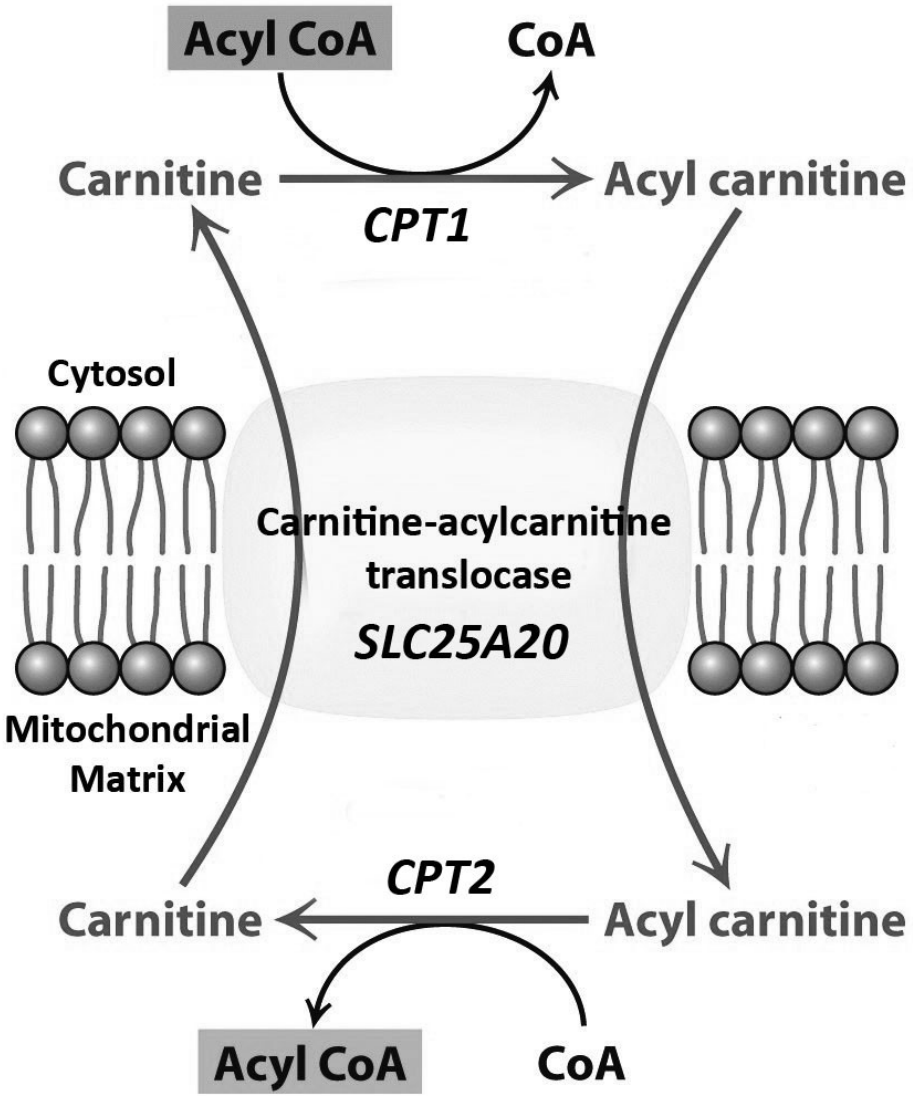

Figure 4.1. Schematic view of carnitine cycle (Berg et al., 2006)

CHAPTER 5. GENERAL CONCLUSION

In the previous studies conducted bu Nassu et al. (2011) and He et al. (2012), which measured FA concentrations in muscle and fat tissues of the animals used in this project, it was reported that flaxseed significantly increased the amount of n-3 FAs, especially ALA, in both *Longissimus thoracis* muscle and subcutaneous fat of cull cows. Therefore, it was expected that n-3 PUFA supplementation (in the form of flax) will induce changes in host gene expression pattern and helps to identify those genes and biological pathways related to FA metabolism and deposition in muscle and fat tissues, which were observed in the first study (Chapter 3). A high-throughput microarray analysis of about 24K bovine transcripts was performed on *Longissimus thoracis* muscle, subcutaneous fat, and kidney fat tissues of each cow (20 samples per tissue per cow), and revealed 116 transcripts that showed differential expression pattern between Flax-high index and No-Flax-low index cows. Following that, pathway analysis tools and literature review investigations identified eight DE genes (LT: *C8H9orf3*, *SGMS1*, *PLCB4*, *ACO2*, *SDHA*, *SAMD8*, and *USF2*; SC: *HACL1*) associated with FA metabolism. Fidelity of microarray results was tested for the eight DE genes by real-time PCR and most of the genes tested agreed in the direction of change between Flax-high index and No-Flax-low index cows, although some were insignificant DE. This highlights the need to perform more precise gene expression measurements on results obtained from microarrays. The eight DE genes can be used for selection purposes to select cows having more n-3 FAs in their tissues but it should be done with caution since there were a limited number of animals in this study, therefore it is recommended to measure both expression of these genes and the amount of FAs in muscle and fat tissues of a larger population and if the same results are observed, these genes can be used to estimate long-chain n-3 FAs in cows before slaughter. As well, SNPs of these genes can be identified.

Discovery of SNPs linked to FA profiles and subsequently predicting the breeding values of an individual from SNP data can give us the opportunity to genetically select animals that inherently have improved FA profiles in their meat (Meuwissen et al., 2001; Saatchi et al., 2011).

Then correlations between the tissue FA measurements and the expression data for 40 selected genes associated with FA metabolism in a larger group of animals (LT=31 and SC=61) were performed in the second study (Chapter 4) to identify key genes which can be funneled into genomic studies for selection to improve FA content of beef. Correlation analysis resulted in identifying several significantly associated genes including *CPT1*, *SLC25A20*, *CPT2*, *ACO2*, *SDHA*, *ACACA*, *ACACB*, *THRSP*, *SREBF1*, *SREBF2*, *HMGCS1*, *HMGCR*, *SCD*, *DGAT1*, *PNPLA2*, *LIPE*, *LEP*, *SGMS1*, and *SAMD8*. Changes in gene expression of these genes are either associated with an increase in percentage of desirable FAs like CLAs and n-3s, or with the amount of harmful SFAs. These results incidcate that these genes can be used as a screening tool to select cattle that have the potential to produce higher beneficial FAs, especially CLAs and n-3s, in their tissues inherently. This will be achieved by identifying the SNPs from these genes. Currently a few studies are underway to identify SNPs that are associated with desirable FA profile in beef cattle. For example within *THRSP* gene, cattle with GG genotype of g.78 G>A SNP and CC genotype of g.184 C>T SNP had higher amounts of PUFAs and MUFAs but lower SFAs content in *Longissimus dorsi* muscle (Oh et al., 2014). As well, cattle with g.2203GG genotype in *ACACA* had higher amounts of SFAs and MUFAs compared with those with g.2203GT genotype in *Longissimus dorsi* muscle (Zhang et al, 2009). A gene expression study in beef cows such as the one performed here will highly complement a large-scale SNP analysis. ASReml statistical package (Gilmour et al., 2009) can be used to see the associations of all the identified SNP markers with some individual and groups of FAs affecting meat quality. Once the

SNPs that influence the FA composition in fat and muscle tissues are identified and verified, they can be used to evaluate an animal's breeding value (genomic breeding value) or genetic potential of beneficial FAs without phenotyping them. Therefore, the use of the SNP markers identified from this project can help to breed for healthier beef which not only increases the profitability and competitiveness of beef industry, but also improves the health and well-being of people who consume beef and beef related products.

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Appendix 1. List of DE genes obtained from the comparison between Flax-high index and No-Flax-low index cows

***Longissimus thoracis* muscle**

Spot Annotation	*Fold Change*[a]	*p-Value*
Lysine (K)-specific demethylase 6A (*KDM6A*)	0.61	0.01
Strawberry notch homolog 1 (Drosophila) (*SBNO1*)	0.62	0.01
Squamous cell carcinoma antigen recognized by T cells (*SART1*)	0.76	0.01
Signal sequence receptor, delta (*SSR4*)	0.76	0.01
Regulatory solute carrier protein, family 1, member 1 (*RSC1A1*)	0.73	0.01
Protein tyrosine phosphatase, receptor type, K (*PTPRK*)	0.61	0.01
Mitogen-activated protein kinase 9 (*MAPK9*)	0.78	0.01
Scavenger receptor cysteine rich domain containing, group B (4 domains) (*SRCRB4D*)	0.83	0.01
KIAA2026 ortholog (*KIAA2026*)	0.8	0.01
Ankyrin repeat domain 26 (*ANKRD26*)	0.73	0.01
X (inactive)-specific transcript (*XIST*)	0.72	0.02
Nuclear casein kinase and cyclin-dependent kinase substrate 1 (*NUCKS1*)	0.68	0.02
Alpha thalassemia/mental retardation syndrome X-linked (*ATRX*)	0.65	0.02
Chromosome 8 open reading frame, human C9orf3 (*C8H9orf3*)	**1.73**	**0.02**
Sphingomyelin synthase 1 (*SGMS1*)	**0.65**	**0.02**
T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3 (*TCIRG1*)	0.68	0.02
Ubiquitin-conjugating enzyme E2D 3 (*UBE2D3*)	0.73	0.02
Phosphoglycerate mutase 2 (muscle) (*PGAM2*)	0.84	0.02
Kirsten rat sarcoma viral oncogene homolog (*KRAS*)	0.63	0.02
Anaphase promoting complex subunit 5 (*ANAPC5*)	0.76	0.02
Selenoprotein X, 1 (*SEPX1*)	1.46	0.02
Glycoprotein IX (platelet) (*GP9*)	0.76	0.02
Nuclear factor (erythroid-derived 2)-like 1 (*NFE2L1*)	1.37	0.02
Muscleblind-like splicing regulator 1 (*MBNL1*)	0.75	0.03
TEA domain family member 1 (SV40 transcriptional enhancer factor) (*TEAD1*)	1.35	0.03
Rho GTPase activating protein 5 (*ARHGAP5*)	0.76	0.03
Ankyrin repeat domain 17 (*ANKRD17*)	0.66	0.03
Exocyst complex component 8 (*EXOC8*)	0.68	0.03
E74-like factor 2 (ets domain transcription factor) (*ELF2*)	0.78	0.03
Tuberous sclerosis 1 (*TSC1*)	0.77	0.03
cAMP-regulated phosphoprotein, 19kDa (*ARPP19*)	1.5	0.03
Complement component 4 binding protein, beta (*C4BPB*)	0.62	0.03
Ribosomal protein L39 (*RPL39*)	0.71	0.03
Ribosomal protein S24 (*RPS24*)	0.72	0.03
Hepatic and glial cell adhesion molecule (*HEPACAM*)	0.8	0.03
Mucin 1, cell surface associated (*MUC1*)	0.53	0.03
Ubiquitin specific peptidase 12 (*USP12*)	1.32	0.03
Oxysterol binding protein-like 2 (*OSBPL2*)	0.68	0.03
Nuclear factor (erythroid-derived 2)-like 1 (*NFE2L1*)	1.41	0.03
Glucose-6-phosphate isomerase (*GPI*)	0.85	0.03
PDZ domain containing 8 (*PDZD8*)	0.8	0.03
DEAH (Asp-Glu-Ala-His) box polypeptide 36 (*DHX36*)	0.75	0.04
Phospholipase C, beta 4 (*PLCB4*)	**1.31**	**0.04**
Zinc finger protein 187 (*ZNF187*)	0.77	0.04
C-type lectin domain family 2, member D (*CLEC2D*)	0.71	0.04
Phospholysine phosphohistidine inorganic pyrophosphate phosphatase (*LHPP*)	1.25	0.04
Rho GTPase activating protein 6 (*ARHGAP6*)	0.18	0.04
Ubiquitin specific peptidase 13 (isopeptidase T-3) (*USP13*)	0.68	0.04
G protein-coupled receptor 141 (*GPR141*)	0.72	0.05
egl-9 family hypoxia-inducible factor 1 (*EGLN1*)	1.56	0.05
O-sialoglycoprotein endopeptidase (*OSGEP*)	0.86	0.05
Golgi reassembly stacking protein 2, 55kDa (*GORASP2*)	1.54	0.05
Copper metabolism (Murr1) domain containing 1 (*COMMD1*)	0.69	0.05
NEDD8 activating enzyme E1 subunit 1 (*NAE1*)	0.78	0.05
Aconitase 2, mitochondrial (*ACO2*)	1.25	0.05
Striatin, calmodulin binding protein (*STRN*)	1.32	0.06
Ubiquitin specific peptidase 19 (*USP19*)	1.31	0.06
CAP-GLY domain containing linker protein 1 (*CLIP1*)	1.44	0.06
Guanylate kinase 1 (*GUK1*)	1.44	0.06
General transcription factor IIH, polypeptide 5 (*GTF2H5*)	0.72	0.06
Ribosomal protein L38 (*RPL38*)	0.63	0.06
Homeobox containing 1 (*HMBOX1*)	0.71	0.06
Family with sequence similarity 210, member B (*FAM210B*)	0.82	0.06
Uncharacterized LOC788038 (MGC157082)	0.75	0.06
ADP-ribosylation factor-like 5A (*ARL5A*)	0.78	0.06
TSC22 domain family, member 1 (*TSC22D1*)	1.32	0.06
Collagen, type I, alpha 2 (*COL1A2*)	1.5	0.06
Clathrin, light chain A (*CLTA*)	0.52	0.06
Chromosome 8 open reading frame, human C9orf3 (*C8H9orf3*)	1.32	0.06

***Longissimus thoracis* muscle**

Spot Annotation	*Fold Change*	*p-Value*
Transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C) (*TCEB1*)	1.31	0.06
Myeloma overexpressed 2 (*MYEOV2*)	1.35	0.06
Zinc finger, CCHC domain containing 14 (*ZCCHC14*)	0.77	0.06
Tubulin polymerization-promoting protein family member 3 (*TPPP3*)	1.43	0.06
Fructosamine 3 kinase related protein (*FN3KRP*)	4.42	0.06
Fem-1 homolog a (C. elegans) (*FEM1A*)	1.29	0.06
Solute carrier family 38, member 2 (*SLC38A2*)	1.23	0.06
NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1, 6kDa (*NDUFC1*)	1.33	0.06
Actinin, alpha 4 (*ACTN4*)	0.73	0.06
Septin 6 (*SEPT6*)	1.78	0.06
G protein-coupled receptor 171 (*GPR171*)	0.82	0.06
Cell cycle progression 1 (*CCPG1*)	1.36	0.06
Sterile alpha motif domain containing 8 (*SAMD8*)	**0.71**	**0.06**
Canopy 4 homolog (zebrafish) (*CNPY4*)	0.62	0.06
Teratocarcinoma-derived growth factor 1 (*TDGF1*)	0.79	0.06
Forkhead box N3 (*FOXN3*)	0.79	0.06
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*SDHA*)	**1.23**	**0.06**
Ribosomal protein S11 (*RPS11*)	0.78	0.06
Ubiquitin protein ligase E3 component n-recognin 3 (putative) (*UBR3*)	1.4	0.06
Phospholamban (*PLN*)	1.5	0.06
Guanylate binding protein family, member 6 (*GBP6*)	0.79	0.07
anti-Mullerian hormone receptor, type II (*AMHR2*)	0.72	0.07
Chromodomain helicase DNA binding protein 1 (*CHD1*)	0.55	0.07
Basic leucine zipper transcription factor, ATF-like 3 (*BATF3*)	0.68	0.07
Dickkopf-like protein 1 (*DKK1*)	0.86	0.07
odz, odd Oz/ten-m homolog 3 (Drosophila) (*ODZ3*)	0.36	0.07
Eukaryotic translation initiation factor 2C, 2 (*EIF2C2*)	0.63	0.07
Far upstream element (FUSE) binding protein 1 (*FUBP1*)	0.76	0.07
Mitochondrially encoded cytochrome c oxidase I (*MT-CO1*)	1.57	0.07
Basic leucine zipper and W2 domains 1 (*BZW1*)	1.49	0.07
Histidine triad nucleotide binding protein 2 (*HINT2*)	0.8	0.07
Dystrobrevin binding protein 1 (*DTNBP1*)	0.82	0.07
PHD finger protein 3 (*PHF3*)	0.74	0.07
Ribosomal protein L28 (*RPL28*)	0.8	0.07
family with sequence similarity 214, member A (*FAM214A*)	1.43	0.07
Coiled-coil domain containing 97 (*CCDC97*)	0.79	0.07
Lamin B receptor (*LBR*)	0.83	0.07
Upstream transcription factor 2, c-fos interacting (*USF2*)	**1.7**	**0.08**
RAD50 interactor 1 (*RINT1*)	0.81	0.08
Ribosomal protein S11 (*RPS11*)	0.77	0.08
Nuclear casein kinase and cyclin-dependent kinase substrate 1 (*NUCKS1*)	0.74	0.08
Tax1 (human T-cell leukemia virus type I) binding protein 1 (*TAX1BP1*)	1.2	0.08
Zinc finger protein 772 (*ZNF772*)	0.75	0.08
Family with sequence similarity 63, member B (*FAM63B*)	0.81	0.08
FCH domain only 2 (*FCHO2*)	0.78	0.08
Zinc finger protein 638 (*ZNF638*)	0.67	0.09
Xin actin-binding repeat containing 2 (*XIRP2*)	1.85	0.09
Transcription factor Dp-2 (E2F dimerization partner 2) (*TFDP2*)	0.69	0.09
Toll-like receptor 2 (*TLR2*)	0.72	0.09
Spi-B transcription factor (Spi-1/PU.1 related) (*SPIB*)	0.79	0.09

Subcutaneous fat

Spot Annotation	*Fold Change*	*P-Value*
FAST kinase domains 1 (*FASTKD1*)	0.76	0.04
Sorbin and SH3 domain containing 2 (*SORBS2*)	0.54	0.04
U2 small nuclear RNA auxiliary factor 1 (*U2AF1*)	0.8	0.04
Crystallin, alpha B (*CRYAB*)	1.61	0.04
Zinc ribbon domain containing 1 (*ZNRD1*)	0.76	0.04
AKT interacting protein (*AKTIP*)	1.42	0.06
Family with sequence similarity 149, member A (*FAM149A*)	0.65	0.07
Low density lipoprotein receptor-related protein 11 (*LRP11*).	1.61	0.07
2-hydroxyacyl-CoA lyase 1 (*HACL1*)	**1.33**	**0.07**
Phosphoglycerate mutase 1 (brain) (*PGAM1*)	1.44	0.07
SRY (sex determining region Y)-box 5 (*SOX5*)	0.84	0.07
Complement component (3d/Epstein Barr virus) receptor 2 (*CR2*)	1.38	0.07
Selenoprotein X, 1 (*SEPX1*)	1.3	0.08
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide (*YWHAH*)	1.41	0.08
TOR signaling pathway regulator (*TIPRL*)	1.16	0.09
ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F6 (*ATP5J*)	0.86	0.09

[a] A value ≥ 1 indicates that expression is higher in the Flax-high index animals