The Effect of Dietary Fat on Fatty Acid Composition, Gene Expression and Vitamin Status in Pre-Ruminant Calves

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Chia-Yu Tsai

Major Professor: Pedram Rezamand, Ph.D.

Committee Members: Matthew E. Doumit, Ph.D.; Philip F. Mixter, Ph.D.

Department Administrator: Amin Ahmadzadeh, Ph.D.

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Authorization to Submit Thesis

This thesis of Chia-Yu Tsai, submitted for the degree of Master of Science with a Major in Animal Science and titled "The Effect of Dietary Fat on Fatty Acid Composition, Gene Expression and Vitamin Status in Pre-ruminant Calves," has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: ______________________________ Date: ______________
Pedram Rezamand, Ph.D.

Committee Members: ______________________________ Date: ______________
Matthew E. Doumit, Ph.D.

______________________________ Date: ______________
Philip F. Mixter, Ph.D.

Department Administrator: ______________________________ Date: ______________
Amin Ahmadzadeh, Ph.D.

Abstract

Dietary saturated (SFA) and unsaturated fatty acids (UFA) alter fatty acid (FA) composition of various tissues, serum and lipid-soluble vitamins. The objective of this study was to examine the effect of dietary SFA and UFA on adipose, liver, serum, polymorphonuclear (PMN) and peripheral blood mononuclear cells' (PBMC) FA profiles, selected gene expression of inflammatory mediators, and their relation with vitamin content in pre-ruminant calves. Twelve Holstein male calves were randomly assigned to two treatments. Starting 3 d of age, 6 calves on SFA received 120 mL palm oil/d, and 6 calves on UFA received 80 mL flaxseed oil plus 40 mL conjugated linoleic acid per day. After 50 d, all animals were euthanized and samples were obtained. Gas-chromatography was used to analyze FA composition. High-performance liquid chromatography was used to analyze α-tocopherol and retinol in liver tissues as well as α-tocopherol, retinol and β-carotene in serum. Liver and adipose tissue were analyzed for relative gene expression of interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12, interferon-γ, peroxisome proliferator-activated receptor-γ, TNF-α, retinol binding protein-4 and NF-κB. The PBMC were examined for gene expression of IL-1β, IL-6, TNF-α and intercellular adhesion molecule-1; PMN were analyzed for expression of caspase-1, IL-8 receptor and L-selectin (L-SEL). Data were analyzed using the Proc TTEST of SAS with significance declared at $P \leq 0.05$. The UFA had greater α-linolenic acid compared to SFA calves in all three [non-esterified fatty acid (NEFA), neutral lipid (NL) and phospholipids (PL)] fractions of liver, adipose and serum as well as PBMC and PMN. The higher content of α-LA in calves fed UFA resulted in greater eicosapentaenoic acid (EPA) in all three lipid fractions of serum as well as NL and PL fractions of adipose tissue. In addition, PBMC and PMN had greater EPA in UFA calves. The UFA group however, had lower γ-linolenic acid compared with SFA calves in all three fractions of liver as well as NL and PL fractions of serum. Dietary UFA also increased total PUFA in three lipid fractions of serum and adipose. The lipid-soluble vitamins content in serum was reduced by dietary UFA. Moreover, L-SEL expression was upregulated in calves receiving UFA. This may indicate that UFA supplementation elevated the substrate of PUFA biosynthesis, but possibly degraded the lipid soluble vitamins to protect these FA from oxidation. Greater PUFA content may influence the migration of PMN from the blood to tissues, affecting overall inflammatory responses.

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Dedication

I would like to dedicate this thesis to my dear family.

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Literature Review

Introduction

Reducing calfhood diseases is a worldwide goal for the commercial dairy industry. Calfhood diseases increase the cost of production including veterinary, mortality, reduced growth, and delay the age of first calving (Windeyer *et al*., 2014). Because of risk factors, such as management of calving, pathogens of colostrums, environmental stressors and feeding (Windeyer *et al*., 2014), newborn calves mainly suffer from digestive and respiratory problems during the pre-weaned period. According to The National Animal Health Monitoring System (NAHMS) reported during 2006, digestive and respiratory problems caused 56.5% and 22.5% of mortality of the total death pre-weanning heifer, respectively (USDA, 2007). These two major diseases cause annually $33.5 and $14.7 losses per pre-weaned calf, respectively (Kaneene and Hurd 1990). Calves have been demonstrated to receive active immunity by vaccination of their pregnant dams against respiratory pathogens during less than 1 month old. The immunity can be affected however by the development of immune system, stress, and infections (Dudek *et al*., 2014). Therefore, further attempts to maintain newborn calves' health and reduce disease morbidity through enhancing physiological pathways via dietary treatments are critical.

The preruminant calf is fed whole milk or milk replacer with the high content of fat (200-230g/kg DM; Graulet et al., 2000). Fat fed has nutritional and economical advantages to satisfy energy requirement and replace carbohydrates with inexpensive lipid sources. In addition, dietary polyunsaturated fatty acid (PUFA) can modulate immune system to improve growth performance and health. Ballou et al. (2008) showed that milk replacer containing fish oil weakens acute phase responses, and the effect was linear in the range of 5 to 10% of the lipid replaced as the fatty acid (FA) from fish oil. This was beneficial to calves' health and provided a better balance between a necessary versus an excessive acute phase response. Furthermore, dietary fish oil tended to down-regulate pro-inflammatory cytokine such as tumor necrosis factor-α (TNF-α) gene expression after lipopolysaccharide (LPS) stimulation (Karcher et al., 2014). Conjugated linoleic acid suppressed

pro-inflammatory cytokines such as TNF-α, interleukin (IL)-1β, IL-6 expression in peripheral blood mononuclear cell of weaned pigs (Changhua et al., 2005). In addition, flaxseed oil can decrease the expression of IL-1β and IL-6 in dietary treatment of ewes (Caroprese et al., 2015). Overall, these studies showed that dietary FA can adjust immune response to achieve proper immunity. For this reason, dietary FA may decrease calfhood diseases and treatment cost while increasing the growth performance.

Lipids

Lipids are a component found both in plants and animal tissues; they can be dissolved in organic solvents but not in water. The lipids of fruits and seeds contain predominantly triacylglycerol (TG), which is an ester form composed of a glycerol and three fatty acids (FA). The common FA in the lipid profiles are α-linolenic (C18:3 n3), oleic acid (C18:1 *Cis*) and palmitic acid (C16:0). In animals, lipids are a main source of energy, stored as fat. Energy yield from glycogen and fats are 17 and 39MJ / kg DM, respectively. Therefore, fats have high energy yield as a source of energy. (McDonald *et al*., 2002)

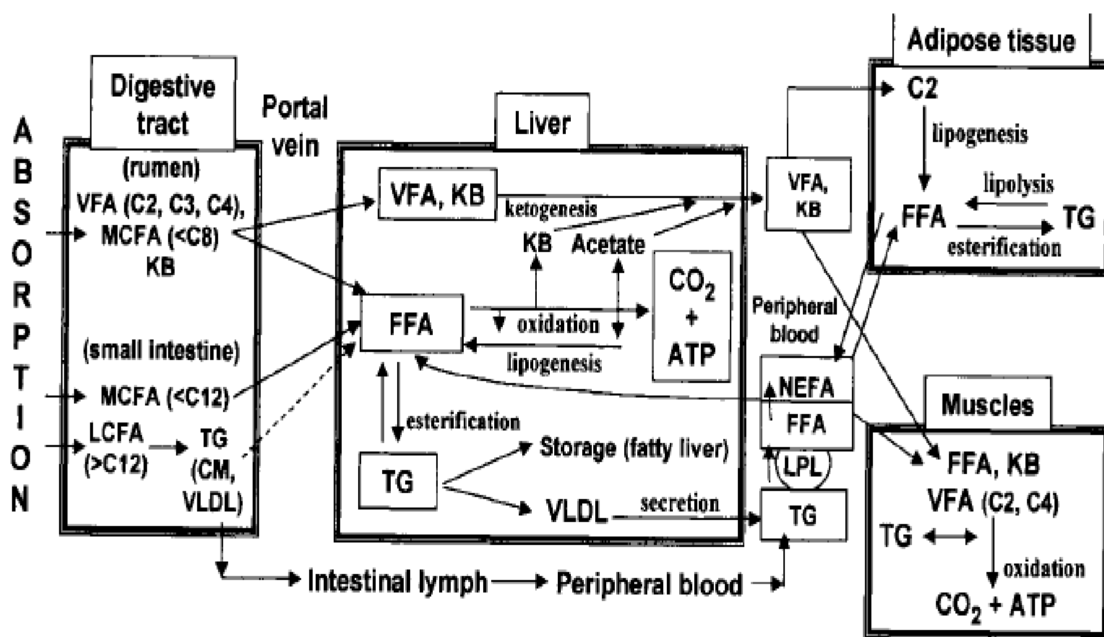
Fatty acids are composed of a single carboxyl group and an unbranched carbon chain. They can be saturated (no double bonds), monounsaturated (one double bond) or polyunsaturated fatty acids (more than one double bond; PUFA). Depending on the spatial arrangement, the fatty acid structure can be divided to *cis* form (hydrogen atoms lie on the same side) and *trans* form (hydrogen atoms lie on the opposite side). The omega (ω) or n is presented as carbon position from methyl end of the FA chemistry structure. The PUFA may be grouped as ω3, ω6 and ω9 families referring to their first double bond nearest to the methyl end carbon, such as α-linolenic (18:3 n3), linoleic (18:2 n6) oleic acid (18:1 n9) (McDonald *et al*., 2002).

Lipid Metabolism in Preruminant and Ruminant

In ruminants, ruminal microorganisms can bio-hydrogenate to de novo synthesize or lipolysize dietary fat (Jenkins 1993). Volatile fatty acid (VFAs; C2-C6) and some medium-chain fatty acid (MCFA; < C8) can be absorbed by rumen wall, in contrast to monogastrics and preruminants that digest and absorb lipid mainly at the small intestine. Newborn calves during milk sucking period are without rumen function until four months old, when rumen is fully developed. The nutrients ingested by-pass the calf's rumen through the reflex of esophageal groove closure to abomasum. At this period, pre-ruminants are similar with monogastrics to absorb lipids. Bile acid and pancreatic lipase are capable of emulsification and decomposition to process dietary fat, and then free fatty acids will be

transferred to micelle phase and absorbed in intestinal tract of enterocytes (5% in duodenum, 20% in upper jejunum, 25% in mid and lower jejunum, and 50% in ileum; Leat and Harrison, 1975). The free fatty acids in enterocytes will be repacked as lipoproteins to transport through the lymphatic system. Lipoproteins contain different amounts of cholesterol, triglyceride, phospholipid and proteins to compose hydrophilic surface and hydrophobic inner. Lipoproteins can carry lipid to liver and peripheral tissues for an energy source or for storage. Chylomicrons are the largest and the least dense of lipoprotein to transfer triglycerides with apolipoproteins (Apo) to process exogenous lipids (Bauchart, 1993). When lipoprotein lipase (LPL) hydrolyzes triglycerides, non-esterified fatty acid (NEFA) are released to peripheral tissues or liver for oxidation or storage, and remnant chylomicrons are taken by liver (Hocquette and Bauchart, 1999; Lusis et al., 2004). Chylomicrons are primary lipoprotein to process exogenous lipid; preruminants receiving lipids from diets, can increase chylomicron synthesis and secretion from the intestines (Laplaud et al., 1990). In sheep, dietary PUFA has been demonstrated to induce chylomicron secretion compared with saturated fatty acid (SFA; Harrison et al., 1974). In addition, very low density lipoproteins are the other endogenous triglyceride transporter formed in liver to assist hydrophobic triglyceride transfer in blood (Hocquette and Bauchart, 1999; Laplaud et al., 1990; Lusis et al., 2004). Compare with human, ruminants have less liver VLDL synthesis (Bauchart et al., 1989 and Kleppe et al., 1998). Dietary high lipid levels have influence on chylomicrons and VLDL synthesis in preruminant calves (Bauchart and Levueux 1985). When VLDL transfer to peripheral tissues, LPL can hydrolyze VLDL to increase levels of NEFA for adipose and muscle tissue energy or esterified as triglycerides to storage. Moreover, LPL hydrolyzed VLDL also produce intermediate density lipoproteins, which are the intermediates between VLDL and LDL (Bauchart 1993). The calves' liver and peripheral tissues capture IDL rapidly and can cause the lower concentration of IDL in plasma and intestinal lymph (Laplaud et al., 1990; Bauchart et al., 1989). Low density lipoproteins are the intravascular end products of derivative from IDL, which are degraded from VLDL, and are involved in cholesterol distribution to tissue (Bauchart, 1993). High density lipoproteins occupy more than 80% of total plasma lipoproteins in pre-ruminants (Bauchart et

al., 1989; Bauchart and Levueux 1985) and ruminants (Stead and Welch, 1975; Jonas 1975). HDL precursors (nascent HDL) interact with ATP binding cassette transporter A-1 (ABCA1; Oram, 2003) on peripheral tissues to take free cholesterol and phospholipids; after the free cholesterol uptake by nascent HDL, the cholesterol can be esterified to cholesteryl ester by lecithin cholesterol acyl transferase (LCAT; Lusis et al., 2004) forming mature HDL. The mature HDL can transport to liver through the scavenger receptor class B type I (SR-B1; Valacchi et al., 2011) for further bile secretion and VLDL re-synthesis (Burchart, 1993). Furthermore, all of lipoproteins can switch between different categorization by cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP; Lusis et al., 2004) to transfer the content of cholesteryl and phospholipid and adjust particle size. Overall, lipid hydrophobic characteristics require lipoproteins to assist transportation in lymphatic system and body circulation.



(Hocquette and Bauchart, 1999)

Immune Response

The immune system defends the host against pathogens and it includes innate or adaptive immune reactions. Immune cell progenitors are originally derived from hematopoietic stem cells in

bone marrow and mature in different lymphoid organs and tissues (Parham, 2009). Lymphocytes are main components of immune system, and mammals' lymphocytes functional are regulated in bone marrow and thymus (Wilkie 1974). In comparative study, immunology composition of mammals is similar (Davis and Hamilton, 1998). The major innate immune cells in the blood stream are peripheral blood mononuclear cells (PBMC) and polymorphonuclear neutrophils (PMN). PBMC and PMN have specific receptors to recognize components of invading pathogen, and respond to secrete cytokines and chemokines such as interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor (TNF)-α that recruit more immune cells to an inflammation site. Initially immune cells migrate from the blood stream to infection site using an adhesion molecule, either selectins (SEL), integrins and intercellular adhesion molecules (ICAM-1). These assist the cell adhesion on the vascular endothelium then the cells cross the endothelium toward the site of infection. In addition, PBMC and PMN can engulf recognized pathogens into phagosomes and fuse with a mediator of lysosomes forming phagolysosome to destroy the pathogens. Pathogen peptides can be presented on major histocompatibility complex (MHC) to T cells and activate adaptive immunity, which includes memory cells to improve next rapid immune reaction against the invading pathogens (Parham, 2009).

Although the immune system is the main defense against invading pathogens for the host, an inappropriate immune activity can cause reactions such as autoimmune, allergic, inflammatory bowel, chronic inflammatory diseases (Calder 2008). The FA supply has been demonstrated to alter the immune cells function because FA alter the composition of the cells membrane phospholipid, which relates to the membrane lipid fluidity, signal transcription factors, and bioactive synthesis of lipid mediator (Calder 2008; 2012; 2013). The FA composition of phospholipid in cell membranes may alter structure of proteins associated with the membranes, such as ion channel or receptors affecting enzymatic function (de Pablo and Alvarez de Cienfuegos, 2000). In addition, arachidonic acid (AA), which is released by phsopholipase A2 from phospholipid in plasma membrane, can be converted by cyclooxygenase (COX) and lipoxygenase (LOX) to lipid mediators eicosanoids, such as prostaglandin

(PG), thromboxanes (TX) and leukotrienes (LT; Marion-Letellier et al., 2015). The eicosanoids are involved in immune responses cytokines production (Rappaport and Dodge 1981; Rola-Pleszczynski and Stankova 1992), lymphocyte proliferation (Shapiro et al., 1993) and cytotoxicity (Bary and Brahmi 1986; Calder, 2013; de Pablo and Alvarez de Cienfuegos, 2000). Furthermore, the FA composition can alter signal transcription factors such as peroxisome proliferator activated receptors (PPAR) and nuclear factor-kappa B (NF-κB; Chinetti et al., 2000). The PPARs interact with ligands of intracellular metabolites, which are derived from phospholipid of plasma membrane forming heterodimerization with 9-cis retinoic acid receptor known as retinoic-X-receptor (RXR; Calder 2012). NF-κB is a key in encoding protein relates to inflammation such as cytokines, adhesion molecules and COX2 (Kumar et al., 2004; Sigal 2006). Inactive NF-κB is a trimer located in the cytosol, and is inhibited by subunit IκB. When inflammatory stimuli signal phosphorylation of IκB, NF-κB dimmers translocate to the nucleus, increasing pro-inflammatory cytokines, adhesion molecules and COX2 (Perkins 2007; Calder 2013). Peroxisome proliferator activated receptors however, have anti-inflammatory function by preventing NF-κB activate gene transcription and pro-inflammatory proteins release (Marx et al., 1999; Calder 2000). All together, the immune system is the host defense system for preventing pathogens invasion, but must avoid inappropriate immune response causing host physiological damage. The supplementary n-3 PUFA may alter the phospholipid FA profiles that alter lipid fluidity, transcription factors and lipid mediators of inflammation, therefore altering this balance

Carotenoids and Vitamin E with PUFA

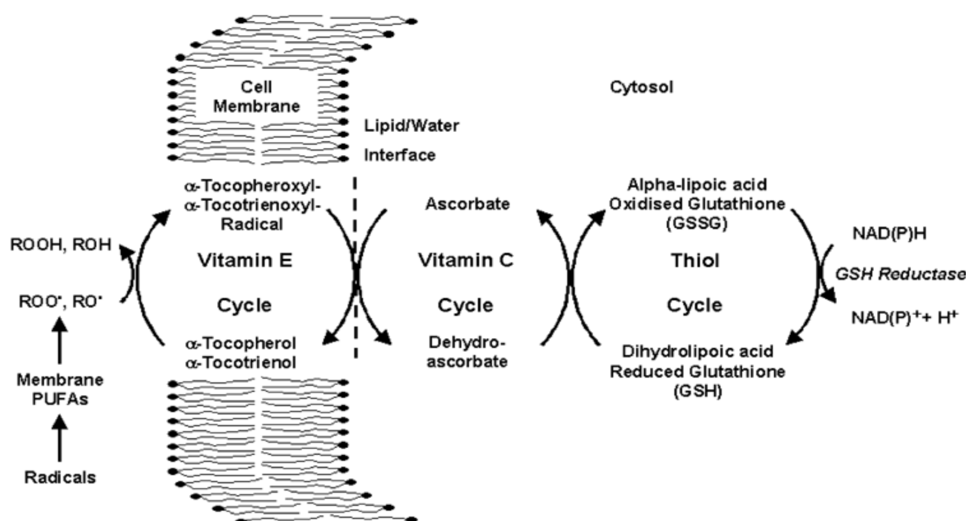
Vitamins are organic components that support animals’ growth and health. They can be categorized into lipid soluble and water soluble vitamins. Lipid soluble vitamin A (retinol), E (α-tocopherol) and β-carotene (precursor of retinol) are part of antioxidants that assist removing free radicals (Fiedor and Byrda 2014; Herrera and Barbas 2001). PUFA’s structure contains two or more double bonds that make them sensitive to oxidation and may cause lipid peroxidation, releasing oxidants and free radicals. Free radicals contain unpaired one or more electrons in outer orbit, and

their high reaction capacity may induce damage to cell function, development and health (Aslani and Ghobadi, 2016; Raederstorff et al., 2015). When there is an increase of n-3 PUFA ingestion, it may cause oxidative stress as well as increase in the antioxidants requirements such as vitamin E (Nair et al., 1993).

Vitamin E

Vitamin E has eight different isoforms, which can be categorized to tocopherol isoforms and tocotrienol isoforms, depending on the saturated or unsaturated side chain on the chromanol ring; each category has α-, β-, γ-, or δ- form. All of the vitamin E isoforms have some antioxidant ability; however, α-tocopherol has the highest bioactivity because α-tocopherol transfer protein (α-TTP) protects rapid degradation of α-tocopherol in the cell. In addition, α-TTP can bind with ATP-binding cassette transporter to assist α-tocopherol transport from hepatocyte to peripheral tissues (Raederstorff et al., 2015). Atkinson et al. (2010) concluded that α-tocopherol may stabilize PUFA rich region in membrane bilayer. When ingesting the high amount of n-3 PUFA, the unstable double bonds of FA increase the oxidative stress in phospholipids of the membrane. Free radical ($R^•$) in membrane of phospholipids can snatch a hydrogen atom (H) from PUFA, and convert it to free radical of PUFA ($PUFA^•$), which can react with oxygen, becoming peroxyl free radical of PUFA ($PUFA\text{-}OO^•$). Alpha-tocopherol (TocH) is considered an antioxidant because it can donate a H atom to $PUFA\text{-}OO^•$ forming hydroperoxide PUFA (PUFA-OOH), and scavenge the $R^•$ from the PUFA. The α-tocopherol radical ($Toc^•$) can regenerate to TocH via vitamin C and the reduction form of glutathione or ubiquinol system (Herrera and Barbas, 2001). The lipid soluble vitamins antioxidants can be degraded. The concentration of α-tocopherol has been shown significantly reduced in adipose, liver, muscle and plasma when the piglets received 3% of linseed oil and safflower oil compared to 3% of coconut oil (Prévéraud et al., 2014). This may indicate that lipid soluble vitamins as antioxidants were degraded to protect the oxidable PUFA and cause lower concentration in the specimens tested. Because dietary PUFA can increase oxidative stress and free radicals production, vitamin E, as an antioxidant,

participates in free radical scavenging. The free radical may indirectly affect other micronutrients such as retinol and β-carotene status.



(van Meeteren et al., 2005)

Carotenoids

Carotenoids (Crts) are 40-carbon base lipophilic pigments, and can be found in plants, algae, fungi and phototropic bacteria and cyanobacteria (Fiedor and Burda 2014). Mammals however, cannot synthesize carotenoids, so their carotenoids have received through dietary intake. Carotenoids can be categorized as carotenes and xanthophylls groups, and xanthophylls' chemical structure has additional oxygen at ending group comparing with carotenes (Hammond and Renzi, 2013). Most of the xanthophylls cannot be converted to vitamin A, but carotenes, such as β-carotene are the main sources of pro-vitamin A and often as a supplement in livestock (McDonald et al., 2002). Beta-c 15,15'oxygenase can convert β-carotene to retinal in the intestinal mucosa, and the retinal can undergo reduction by retinal reductase to retinol (Harrison, 2012). Human and rats have around 50% conversion rate for β-carotene to retinol; however, in ruminants only 15% of β-carotene is converted to retinol (McDonald et al., 2002). Carotenoids with conjugated polyene structure allow electrons easily delocalize in single or double covalent bonds and cause a lower ground state of a molecule. The structure and lipophilic characteristic cause carotenoids easily into the cell membrane to reduce lipid

peroxidation, and stabilize membrane structure (Hammond and Renzi, 2013). Carotenoids are known as quenchers of singlet oxygen ($^1O_2$) and reactive oxygen species (ROS) scavenger via physical and chemical quenching to stabilize unpaired electrons (Fiedor and Burda 2014; Kaulmann and Bohn 2014; Skibsted, 2012). When scavenge $^1O_2$ unpaired electron, carotenoids physically adopt the electron, and convert excited energy to heat, or process undergoing oxidation and oxygenation as chemical quencher (Fiedor and Burda 2014; Kaulmann and Bohn 2014). In addition, carotenoids can accept or donate an electron to ROS and other free radicals ($R^{\bullet}$) becoming carotenoids radical anion ($Crt^{\bullet -}$), carotenoids radical cation ($Crt^{\bullet +}$), radical adduct formation ($RCrt^{\bullet}$), or transfer hydrogen causing carotenoids radical ($Crt^{\bullet}$) (Fiedor and Burda 2014). The purpose of scavenging the radicals is to can protect the damage from oxidation.

When increasing the dietary PUFA, the oxidative stress and lipid peroxidation can increase. Reports have shown α-tocopherol and β-carotene can act as radical-trapping antioxidants to avoid the damage to the cell membrane. Palozza and Krinsky (1992) have used NADPH/ADP/Fe^{3+} or 2,2'-azobis(2-amidinopropane) (AAPH)[2] to induce rat liver microsomes lipid peroxidation, and detected malondialdehyde (MDA) as lipid peroxidation products. The results showed that when AAPH or NADPH induced lipid peroxidation take place, β-carotene with α-tocopherol can inhibit MDA production compared with each antioxidant individually. In addition, when lipid peroxidation occurred, β-carotene and α-tocopherol will be used as antioxidants; however, the consumption of α-tocopherol was faster than β-carotene. This may indicate the lower concentration of serum β-carotene and α-tocopherol when dietary PUFA intake is increased to protect oxidable FA.

PUFA and lipid soluble vitamins bioavailability

Dietary β-carotene and α-tocopherol may be consumed as antioxidants which can reduce those lipid-soluble vitamin concentrations in body; however, the dietary bioavailability, absorption and transport may affect the concentration of these antioxidants too. The lipid soluble vitamins absorption

and transport are similar with those of dietary lipids. In small intestine, the lipid soluble vitamins incorporated into lipid micelles in lumen of small intestine, and the micelles diffuse monoglycerides and free FA into mucosa of intestinal. The monoglycerides and free FA are repackaged into chylomicron and released into lymphatic system. Dietary PUFA however, may negatively affect the lipid soluble vitamins bioavailability. Xanthophylls have higher bioaccessibility with SFA present in butter or palm oil compared with monounsaturated FA (MUFA) or PUFA (Gleize et al., 2013). The same group also showed that lutein and zeaxanthin were higher in bioavailability when they were presented with peanut or coconut oil compared with in rapeseed oil (Huo et al., 2007; O'Connell et al., 2008). In addition, feeding dietary vitamin E with coconut oil resulted in a greater α-tocopherol concentration in piglets' plasma, liver and adipose tissue than control diet (Prévéraud et al., 2014). In the previous result, lipid soluble vitamins bioavailability associated with dietary FA composition. Gleize et al. (2013) suggest fish oil FA can lead bigger physical size of micelle and reduce less xanthophylls solubilization in micelle. Furthermore, oxidative fish oil can induce xanthophylls degradation to stabilize oxidable FA fractions (Gleize et al., 2013). All in all, dietary PUFA can cause the oxidative stress elevation and lower bioavailability of lipid soluble vitamins, further causing the lower concentration of lipid soluble vitamins in circulation.

Dietary Fatty Acid

Conjugated linoleic acid (CLA)

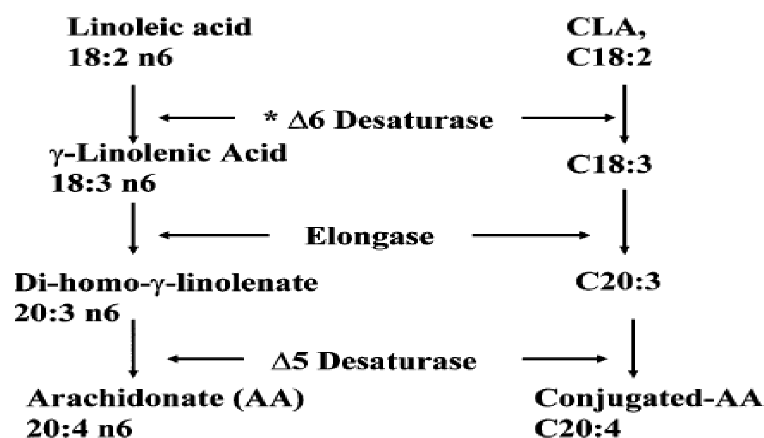
CLA is a group of derivatives from linoleic acid (LA; C18:2 *c*9, *c*12) with two conjugated double bonds. The CLA double bond is present at carbon position 9 and 11 or 10 and 12 causing eight theoretically possible isomers (*c*9, *c*11; *c*9, *t*11; *t*9, *c*11; *t*9, *t*11; *c*10, *c*12; *c*10, *t*12; *t*10, *c*12; and *t*10, *t*12; O'Quinn *et al*., 2000). However, the *c*9, *t*11 CLA and *t*10, *c*12 CLA isomers are most abundant in natural and synthetic blends (Piras, 2014). About 80 to 90 percent *c*9, *t*11 isomer of total CLA and 3 to 5 percent *t*10, *c*12 of total CLA occurs in ruminants' products (Parodi, 2003). Conjugated linoleic acid

can be found in various meats and dairy products from cows and sheep because of the actions ruminal anaerobic bacterium through *Butyrivibrio fibrisolvens* bio-hydrogenation (Kepler *et al*., 1966). During microbial biohydrogenation, CLA is the intermediate product between LA and vaccenic acid (VA, C18:1 *t*11). The linoleic acid isomerase transfer double bond at carbon-12 position of LA to carbon-11 position forming *c*9, *t*11 CLA, which goes rapidly through hydrogenation of *c*9 carbon reserving VA. (Khanal and Dhiman, 2004). The VA in the rumen can be further hydrogenated into stearic acid (C18:0); however, the limited biohydrogenation C18 unsaturated fatty acids cause the VA to accumulate in the rumen (Keeny, 1970). Other C18 PUFA such as α-linolenic acid (*c*9, *c*12, *c*15, C18:3) and γ-linolenic (*c*6, *c*9, *c*12, C18:3) also can be hydrogenated and transferred double bonds position, forming VA by reductase and isomerase enzymes (Piras, 2014). While the VA is absorbed in intestine and transported into tissues; it is processed by Δ9-desaturase forming *c*9, *t*11 CLA isomer (Hauswirth et al., 2004; Piras, 2014). This biochemical conversion represents the endogenous synthesis of *c*9, *t*11 CLA as the primary milk CLA source in lactating cows (Piras, 2014; Griinari et al., 2000).

Conjugate Linoleic Acid Metabolism

Dietary *c*9, *t*11 CLA has been identified preferentially in rat neutral lipid fraction of the liver due to CLA particular conjugated diene (CD) double bond structure that can be superimposed on oleic acid (Banni *et al*., 2001). Similarly, CLA in mammary and adipose tissues has much higher content in neutral lipid (NL) fraction compared with that of liver and plasma (Carta et al., 2002; Banni, 2002). An isomer of *c*9, *t*11 CLA has same carbons numbers and double bonds with LA; and they also shared same enzymes of desaturase and elongase to form 20 carbons end products, but CLA preserve CD structure (Carta et al., 2002; Banni, 2002). The Δ6 desaturase introduces a double bond at position 6 forming the conjugated linolenic acid (CD 18:3), and the elongase adding two carbon atoms as conjugated eicosatrienoic (CD 20:3); further the Δ5 desaturase removes two hydrogen atoms from conjugated eicosatrienoic forming conjugated arachidonic acid (CD 20:4; CAA). The pathway of LA

competes same desaturase and elongase with CAA formation; LA goes through desaturation, elongation forming linolenic acid (C18:3), γ-linolenic acid (C20:3), and arachidonic acid (C20:4; AA; Belury, 2002; Piras, 2014). CD 20:4 was found in higher content in the phospholipid (PL) fraction; this might be the CD20:4 with two cis configurations of non-conjugated double bonds, which cause the structure more similar with PUFA and have higher affinity into PL (Carta et al., 2002; Banni et al., 2001). In addition, CD 18:3 and CD 20:3 are preferentially found in NL fraction, which allows them to be stored in mammary and adipose tissues; this cause advantage inhibition of AA and its precursors into PL. The stored CLA and its derivatives in mammary and adipose tissues can continually support CAA biosynthesis in those tissues (Carta et al., 2002; Banni, 2002; Banni et al., 2001). As a result, dietary CLA can reduce LA metabolism in mammary up to 1%, also significantly reduce by 50% of AA, which is the precursor of prostaglandin and leukotriene metabolized by cyclooxygenase (COX) and lipoxygenase (LOX; Banni et al., 1999)



(Belury 2002)

Conjugate Linoleic Acid Immunomodulator Features.

Supplementary CLA can compete with AA to modify cells membrane of phospholipid composition in lipid bilayer. The limitation of AA caused by CLA may reduce the pro-inflammatory derivatives production such as PGE2 and COX2; further affecting the transcription factor or receptor of inflammation NF-κB or PPAR-γ, respectively (Stachowska et al., 2007). CLA can increase

PPAR-γ expression in macrophages (Yu et al., 2002), and negative feedback meditates COX2 expression through NF-κB pathway in macrophage (Inoue et al., 2000), preadipocytes (Evans et al., 2000), and porcine muscle cells (Meadus et al., 2002). CLA however, has affected on both pro- or anti-inflammatory cytokine secretions (Kim et al., 2011; Bhattacharya et al., 2006). CLA treatment can inactivate NF-κB (Cheng et al., 2004) and limit COX2 expression (Flowers and Thompson, 2009), and inhibit PGE2 synthesis to enhance macrophage phagocytosis.

After treatment with IFN- γ to stimulate RAW 264.7 macrophage cells, CLA can reduce PGE_2, TNF-α, IL-1β, IL-6 and COX2 mRNA gene expression, and can induce PPAR-γ (Yu et al., 2002). Similar findings were also showed in the PBMC isolated from the weaned pigs stimulated lipopolysaccharide (LPS). LPS is outer membrane component of Gram-negative bacteria that causes immune responses. CLA suppressed the TNF-α, IL-1β and IL-6 production and gene expression, and enhanced PPAR-γ expression in weaned pigs' PBMC stimulated by LPS (Changhua et al., 2005). Bovine PBMC stimulated by LPS induced TNF-α, however; the CLA and PPAR-γ lower the pro-inflammatory cytokine production caused by LPS (Perdomo et al., 2011). Moreover, comparing human PBMC incubated with or without CLA after LPS stimulation, CLA reduced COX2, PGE_2 and NF-κB gene expression and activation, but increased PPAR-γ expression and macrophage phagocytosis (Stachowska et al., 2007). In addition, the mouse bone marrow-derived immature dendritic cells (DC) treated with *c*9, *t*11 CLA after LPS stimulation showed greater IL-10 gene expression and decreased IL-12. This can indicate that CLA induces humoral immunity to release antibodies when LPS activated the DC defense system toward T cell help 2 (T_H2) pathway. In addition, the IL-10 can inhibit IL-12 toward T cell help 1 (T_H1) pathway, which is related to cellular immunity and macrophage activation (Loscher et al., 2005). In vivo study in broiler chicks fed CLA for six weeks elevated antibody production in response to sheep red blood cells (Zhang et al., 2005). Kelley et al. (2000; 2001) and Albers et al. (2003) found dietary CLA did not alter PGE_2, IFN- γ, TNF-α, IL-1β and IL-6 production in stimulated PBMC or TNF-α in monocytes (Kelley et al., 2001). Furthermore,

human ingestion of 3 g/day of mixed CLA can induce plasma level of IgA, IgM and IL-10, and decrease TNF-α and IL-1β. The result indicates that CLA may have both pro-inflammatory to inhibit PGE2 and activate macrophage phagocytosis, and anti-inflammation to elevate IL-10 production toward humoral immunity.

Conjugate Linoleic Acid and Fatty Acid Profile

Dietary fatty acids may affect the composition of fatty acid in the body. Dietary CLA has been shown to increase the content of CLA in Holstein calves' *m. longissimus dorsi*, liver and perirenal fat, which was associated with a decrease in AA concentration in *m. longissimus dorsi* (Marounek et al., 2008). In addition, dietary CLA may alter fat depots profiles. Gillis et al. (2004) demonstrated that Angus × Hereford heifers fed rumen-protected CLA increased intramuscular adipose tissue PUFA content, and decreased oleic acid and total MUFA concentration in adipose tissues (intramuscular, subcutaneous and perianal). The results also showed that in Holstein bulls fed CLA and linseed, a higher level of CLA, α-linoleic acid and a decreased n6/n3 ratio in the intramuscular and subcutaneous fat was observed (Gómeza et al., 2015). When CLA supplied in dairy cows, the PBMC fatty acid profiles also changed. Dietary 50 and 100 g/day of CLA induced the t10, c12 CLA of total fatty acid in the PBMC; however, the CLA did not affect c9, t10 isomer composition (Renner et al., 2012). CLA supplement showed negative impact on the meat of broiler chickens; it significantly induced SFA profiles and decreased unsaturated FA concentration (Cho et al., 2013).

Conjugate Linoleic Acid and Lipid Soluble Vitamins

CLA can limit the lipid uptake by inhibiting lipoprotein lipase, which is the main enzyme to assist chylomicrons and VLDL uptake into tissues. Furthermore, CLA inhibits lipid synthesis in muscle, adipose and liver (Ringseis et al., 2004; Tous et al., 2012), and may affect lipid soluble vitamins concentration. Recent data demonstrated that rumen-protected CLA supplement in lactating Holstein cows can improve milk level of retinol (+34%) and α-tocopherol (+44%) than CLA-free

control group, but it did not change concentration in plasma (Gessner et al., 2015). This has been shown in lactating ewes; rumen-protected CLA supplement in lactating ewes induced tocopherol (+40%) and retinol (+32%) concentration in milk, but CLA caused milk fat reduction by 23% (Zeitz et al., 2014). In addition, PPAR-α and RXR heterodimer has directly regulation retinoid of intracellular concentration, and dietary CLA in mice can induce retinol and retinyl esters status in liver compared with fed PPAR-α agonist (Carta et al., 2014). All in all, dietary CLA did not show a negative effect on lipid soluble vitamins concentration in milk, serum, and liver samples; even through CLA can cause lower uptake of lipid into tissues and milk fat content: CLA still can promote vitamins concentration.

Flaxseed Oil

Flaxseed oil contains high α-linolenic acid, which is an n3 essential FA (Petit and Côrtes, 2010). Alpha-linolenic acid, similar to other PUFA, assists lipid transport, lipoprotein enzymes and form parts of membranes (McDonald *et al*., 2002). It is also a precursor of eicosapentaenoic acid (EPA; C20:5 n3) and docosahexaenoic acid (DHA; C22:6 n3) via undergoing desaturases and elongases processing that can alter immune functions.

Flaxseed Oil Immunomodulatory Features

Dietary flaxseed may alter immune responses. Caroprese et al. (2015) supplied whole flaxseed in ewes' diet and demonstrated that it reduced IL-6 and IL-1β from d14 to d42 postpartum, but the IL-10 significantly increased by flaxseed treatment on d14. Whole flaxseed supplement in postpartum dairy cows did affect the PGE2 concentration in serum; however, IFN-γ and PGE2 in PBMC did not change by dietary treatments (Lessard et al., 2003). In addition, supplemental flaxseed oil did not change jejunum gene expression of main IL-12p35, IFN-γ and IL-10 after the piglets were challenged with *Escherichia coli*; but the chemokine IL-8 was significantly elevated. The IL-8 elevation did not affect monocytes and neutrophils phagocytic activity (Chytilová et al., 2013). Furthermore, feeding ground

flaxseed induced 2.7-fold increased PPAR-γ gene expression in Angus steers muscle tissues. This can indicate the flaxseed regulated lipid metabolism (Kronberg et al., 2006).

Flaxseed Oil and Fatty Acid Profiles

The fatty acid profile can be altered with supplemental flaxseed in the daily ration. Petit and Côrtes (2010) examined flaxseed supplement effect on milk fatty acid composition. The milk fatty acid profile was altered; flaxseed significant induced α-linolenic and n3 PUFA concentration, and reduced SFA and n6/n3 ratio. Petit (2015) recent study also examined increasing level of whole flaxseed in ration, the results showed linear increase in α-linolenic and n3 PUFA, and decreased in AA, n-6 PUFA and SFA concentration in milk when whole flaxseed was fed compared to no flaxseed. Feeding α-linolenic acid-rich flaxseed during early lactation also has shown significant increases in α-linolenic acid, EPA and DHA, and reduction in n6/n3 ratio in the plasma fatty acid profile compared to control group (Matras et al., 2014). During the transition period, dietary whole flaxseed increased α-linolenic acid concentration of liver at week 4 ($P < 0.05$), and plasma at week 2 and 4 ($P < 0.01$) postpartum (Petit et al., 2007). The placenta passage in ruminants however, is limited. Moallem and Zachut (2012) fed flaxseed oil to late pregnant dairy cows and observed elevated α-linolenic acid and total n3 PUFA profile in the cows' plasma, but the newborn calf plasma did not correlate with dam plasma concentration. This is likely related to low passage of α-linolenic acid through bovine placenta with low permeability.

Flaxseed and Lipid Soluble Vitamins

Polyunsaturated fatty acid is highly oxidable FA that may increase the requirement of antioxidants (Nair et al., 1993). Flaxseed oil has rich content of α-linolenic acid and antioxidants nutrients such as tocopherols and flavonoids (Pouzo et al., 2016). Angus steers received 0.7% corn grain (FLAX-0), 0.7% corn grain plus whole flaxseed at 0.125% (FLAX-1) or corn plus whole flaxseed at 0.250% (FLAX-2) of BW. When the steers received FLAX-1 had highest α-tocopherol

content (2.37 μg/mL) than FLAX-0 (1.96 μg/mL) and FLAX-2 (2.37 μg/mL) in plasma circulation. Beta-carotene content trended higher in FLAX-1 (8.99 μg/mL) compared to FLAX-0 (6.32 μg/mL) and FLAX-2 (6.94 μg/mL; P = 0.07); and there was no statistical significant in retinol. In addition, *longissimus thoracis* α-tocopherol content was higher in FLAX-1 (1.65 μg/g) compared with FLAX-0 (1.15 μg/g) and FLAX-2 (1.34 μg/g). Beta-carotene and retinol was not altered by dietary treatments. The only low level of flaxseed supplement showed adequate antioxidants capacity to protect dietary PUFA away from oxidation in muscle (Pouzo et al., 2016). This may also indicate that oxidative stress increased when the higher content of PUFA caused antioxidants concentration to reduce in the plasma and muscle. Ponnampalam et al. (2016) fed vitamin E-rich flaxseed (10.7%) to meat of lamb, it did not show a decreased of vitamin E status in muscle. In addition, the vitamin-E rich flaxseed decreased lipid oxidation of the meat at day 4. Flaxseed has natural micronutrients as antioxidants. If fed flaxseed at low dosage, endogenous antioxidants can assist to remove radicals of PUFA oxidation, however fed over dosage, PUFA may require exogenous antioxidants to remove free radicals.

Palm Oil

Palm oil contains mainly SFA (i.e., palmitic acid; C16:0) at approximately 40 % of total FA, and other UFAs are oleic acid (C18:1) and linoleic acid (C18:2) around 50% (Bianchi et al., 2014). Palm oil gives high SFA content, which has been identified to increase risk of cardiovascular diseases, atherosclerosis, and coronary heart disease. If consumption of high palm oil is consumed it can increase the risk of cardiovascular disease by reducing good HDL cholesterol, and elevation of LDL cholesterol concentration (EDEM, 2002). In contrast, Mukherjee and Mitra (2009) discussed palm oil as healthy diet, and concluded that palm oil can assist in “synthesis of protective cholesterol HDL and remove LDL cholesterol”. In addition, the plasma cholesterol only elevates when there is dietary excess cholesterol. Although, “2015-2020 Dietary Guidelines for Americans” still recommend decrease SFA ingestion.

Palm Oil and ImmunomodulatoryFeatures

Dietary palm oil may have a negative effect on the immune system due to the highly content of SFA in the total FA of palm oil (Bianchi et al., 2014; EDEM, 2002). Dietary 4% and 6% of palm oil added to feed for 60 and 120 days caused pro-inflammatory cytokines TNF-α, IL-1 and IL-6 rising compared to control diet in dairy sheep serum (Bianchi et al., 2014). In addition, serum immunoglobulin (Ig) level of IgG, IgM and IgE were elevated by dietary palm oil at day 60 and 120 (Bianchi et al., 2014). Mice fed 200g of palm oil for five months induced auto reactive T-cell, IgG and PGE2 levels compared to dietary fish oil, but cytokines production of IFN-γ, IL-10 from spleen cells did not significantly differ (Wu et al., 2001). In contrast, Nanji et al. (1999) examined palm, fish or corn oil between ethanol inducing alcoholic fatty liver or dextrose as a control group in rodents. The alcoholic fatty liver induced lipid peroxidation and plasma endotoxin in three of FA dietary treatments compared to the control group; however, dietary palm oil did mitigate lipid peroxidation and plasma endotoxin compared with fish and corn oil treatments (Nanji et al., 1999). In addition, the palm oil inhibited NF-κB activation and IκBα degradation and pro-inflammatory cytokines of TNF-α, IL-1β, IFN-γ and IL-12 p40 gene expression were be inhibited too, but anti-inflammatory cytokines IL-4 and IL-10 induced the highest gene expression by palm oil in the fatty liver rat. In comparison to human monocytic cells line cultured with a tocotrienol-rich fraction of palm oil after LPS stimulation, the NF-κB, TNF-α and IL-8 were also inhibited (Wu et al., 2008). The effect of palm oil supplementation on the response of pro- or anti- inflammatory cytokines may change by inactivation of the transcription factor. This effect may be related to the content of antioxidants such as tocopherol that may increase the tolerance of oxidation and ability of anti-inflammatory responses.

Palm Oil and Fatty Acid Profile

Dietary palm oil can alter fatty acid composition in meat and milk (Partida et al., 2007). Palm oil predominantly contains palmitic, oleic and linoleic acids. Karami et al. (2013) examined how dietary

palm oil can maintain kid goats' concentration of palmitic acid in plasma and kidney tissues compared with dietary canola treatments; also in liver and *Longissimus lumborum* have higher AA and n6/n3 ratio were observed. However, α-linolenic was at lower concentration in plasma, liver, kidney and muscle compared to canola oil treatment. In young bulls fed palm oil significantly higher palmitic acid and stearic (C18:0) were observed compared with lard-tallow mix (Partida et al., 2007). In addition, supplementing 70g/day calcium soap of palm oil in lactation ewes resulted in higher palmitic acid, AA and the n6/n3 ratio of total fatty acid in milk compare with 128 g/day of extruding linseed. The passive of fatty acid from the dietary different fatty acid treatments ewes of milk to sucking lamb also showed altered intramuscular and subcutaneous fatty acid profiles. The lambs had higher palmitic acid and n6/n3 ratio in both tissues (Gómez-Cortés et al., 2014). Dietary palm oil can increase palmitic acid and increase n6/n3 ratio that may increase the SFA accumulation and decrease the n3 profile of total fat in circulation or in the tissues.

Palm Oil and Vitamin

Palm oil enrich the content of vitamin E both tocopherols and tocotrienols isoforms (Kamat et al., 1995; 1997). Feeding 30 % palm oil to male rats can induce α-tocopherols concentration in plasma and reduce plasma and erythrocytes lipid peroxidation compared with that of feeding 30 % of perilla oil (Nanjo et al., 1993). The tocotrienol-rich fraction (TRF) of palm oil has consisted of 26% α-tocopherols, 26% α-tocotrienol, 36% γ- tocotrienol and 12% δ- tocotrienol (Kamat et al., 1995; 1997); dietary TRF has inhibited lipid peroxidation and protein oxidation in rat brain mitochondria and liver microsomes compared with vitamin E (16.5% of α-tocopherols; Kamat et al., 1997). In comparison, some palm oil showed a rich content of both α-tocopherols and β-carotene, and when fed, it elevated plasma and hepatic content of retinol and β-carotene in rats (Aquino et al., 2015). Palm oil rich in lipid-soluble vitamins as antioxidants can inhibit lipid peroxidation in various tissues.

Summary

Dietary n3 PUFA-rich grass and flaxseed can increase the α-LA, EPA and total PUFA content in ruminants' meat, milk and plasma. High content of n3 PUFA may reduce arachidonic acid biosynthesis, and reduce pro-inflammatory responses. Thus, fed α-LA-rich and CLA diet can induce PPAR-γ and anti-inflammatory cytokines, and reduce NF-κB and pro-inflammatory mediators. Dietary high PUFA however, may induce lipid-soluble vitamins requirement for stabilizing lipid peroxidation.

Hypothesis

Dietary UFA can increase total PUFA, n3 PUFA and α-LA as well as reduce arachidonic acid and pro-inflammatory cytokines. This will lead to a greater use of α-tocopherol, β-carotene and retinol to prevent lipid peroxidation.

Objective

The objective of this study was to examine in the effect of dietary SFA and UFA on FA profile of adipose, liver, serum, PMN and PBMC, gene expression of inflammatory markers, as well as vitamin content in pre-ruminant calves.

Methods and Materials

Animals, Treatments and Experimental Design

Twelve 3-d old Holstein male calves were assigned to two treatments of either saturated or unsaturated fat sources in a completely randomized design. Dietary fatty acids (FA) were started at 3 day of calves' age, and the initial body weight was 94.6 ± 2.6 Kg. All calves were raised in individual cages (1.47 × 0.84 m) for 50 days. Six calves were fed 4 L/day of whole milk containing 120 mL Palm Fruit Oil- Organic (Jedwards International, Braintree, MA) as saturated group (SFA) and the other group was fed 4 L/day of whole milk containing 80 mL Flax Seed Oil - Virgin (α-linolenic acid C18:3n-3, ω-3; Jedwards International, Braintree, MA) and 40 mL Lutalin® (CLA) oil (> 56% of conjugated linoleic acid contains isomers of cis-9, trans-11 and trans-10, cis-12) (BASF, New Jersey, USA) as unsaturated group (UFA). Purina® Calf Starter 18 (Purina, Missouri, USA) was fed 2 lbs/day starter from day 25 to 50. The calves were fed twice a day at 06:00 h and 18:00 h. After the calves received the dietary treatment for 50 days, all animals were euthanized, and the samples were obtained. The average body weight at this time was 146.8 ± 2.0 Kg. All animal procedures were approved by the University of Idaho Animal Care and Use Committee (# 2013-77).

Sample Collection

Blood, liver, and perirenal adipose tissues were collected after the calves were euthanized. Blood samples were collected into 50 mL conical polypropylene centrifuge tubes with 200 µL of 50 mg/mL heparin (Sigma Aldrich, St. Louis, MO). Peripheral blood mononucleocytes cells (PBMC) and polymorphoneuclear cells (PMN) were isolated from 50 mL of blood by gradient centrifugation using Histopaque 1077 and 1119 (Sigma Aldrich, St. Louis, MO). After centrifugation at 800 × *g* for 10 min at 4℃, serum was collected and stored at -80℃. The buffy coat was collected and washed with Hank's Balanced Salt Solution (HBSS) and the isolated PBMC and PMN were re-suspended in HBSS,

separated into two 50 mL conical polypropylene centrifuge tube, and stored at -80℃ for RNA extraction and FA analysis, respectively. The liver and perirenal adipose tissue samples were collected into 2 mL micro tube, frozen in liquid nitrogen immediately, and stored at -80℃ for RNA extraction.

Lipid Analysis

Lipids of serum, PBMC, PMN, liver and adipose tissues were extracted by chloroform: methanol (2:1) as described (Clark et al., 1982). Adipose, liver and serum lipids were fractionated by Sep-Pak aminopropyl cartridges (Waters, Milford, MA) as described (Watts et al., 2013). The fractionation columns were conditioned with hexane, the neutral lipid (NL) was eluted by chloroform: propanol (2:1), non esterifed FA (NEFA) was eluted by 2% acetic acid of ether, and phospholipids (PL) eluted by methanol. The adipose, liver and serum lipid fractions, and lipids of PBMC and PMN were processed with sodium methoxide for methylation (Christie, 1982). Lipids were dissolved in 2 mL sodium methoxide, and kept in water bath at 80℃ for 10 min with 5% methanolic hydrochloric acid. Methylated lipid samples were analyzed by Agilent 7890A gas-chromatography system (Agilent Technologies, Santa Clara, CA) equipped with an autosampler, a flame-ionization and an Agilent HP-88 column (100m x 0.25mm with a 0.20-μm film thickness, Agilent Technologies). After the sample injection, oven temperature was 120℃ for 1 minute, increased to 175℃ at a rate of 10℃/ min and held for 10 min, increased to 210℃ and held for 5 min, increased to 230℃ at a rate of 5℃/ min and held for 5 min and increased 5℃/ min to 240 ℃ and held for 5 min. Fatty acids were identified using a Supelco 37 Component fatty acid methyl esters mix.

High-Performance liquid chromatography (HPLC) analysis

Serum samples are analyzed for α-tocopherol, β-carotene and retinol, and liver tissues were analyzed for α-tocopherol and retinol f. The serum and liver tissues were acidified by 20 μL 2 N acetic acid, denature by adding 420 μL acetonitrile, and extracted by 1.5 mL organic solvents mixture

(hexane: 2-propanol, 6.5: 1.5; v/v). After one minute vortex and centrifugation at 1000 × *g* for 3 minutes, the organic layer was transferred to another tubes, and dried by light nitrogen stream then reconstituted in mobile phase (78.2 % Acetonitrile, 13.0 % Dichloromethane, 8.7% Methanol and 0.1% n-butanol) for injection. The HPLC instrument included Separation Module 2695 with a Symmetry C_{18} separation column (4.6 × 150 mm, 3.5 μm particle size) and Photodiode Array Detector (PDA) 2998. Waters 2659 and PDA 2998 were controlled by Empower 3 software (Waters®, Milford, MA). The α-tocopherol, β-carotene and retinol were detected at wavelengths of 290nm, 450nm and 325nm, respectively at retention time of 3.10, 5.56 and 1.57 minutes. The temperature of auto sampler room set was 4℃, and the column heat was 50℃ maximum. Mobile phase flow rate was maintained at 1.5mL/ min for a total of 6.5 minutes for detection.

Gene Expression Analysis

Total RNA was collected from liver, adipose, PBMC and PMN using NucleoSpin® RNA and RNA XS (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. The RNA concentration was determined by NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE) spectrophotometer. The complimentary DNA (cDNA) was synthesized by using Applied Biosystems High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) from total RNA. One μL of single strand cDNA was reacted with 10 μL per reaction of TagMan® Universal Master Mix II with Uracil-N-Glycosylase (UNG; Applied Biosystem) and 1 μL TagMan® 20X assay buffer, the volume was adjusted to 20 μL per reaction by addition of RNase-free water in 96 well plates, and analyzed in 7500 Fast Real-Time PCR instrument (Applied Biosystem). Liver and adipose tissue cDNA were analyzed for interleukin-1β (IL-1β), IL-6, IL-8, IL-10, IL-12 p40, interferon-γ (IFN-γ), peroxisome proliferator activated receptor-γ (PPAR-γ), tumor-necrosis factor-α (TNF-α), retinol binding protein-4 (RBP-4) and nuclear factor of kappa light polypeptide gene enhancer in B-cell 1 (NF-κB) gene expression. Peripheral blood mononuclear cells (PBMC) were analyzed for gene expression of IL-1β, IL-6, TNF-α and intercellular adhesion molecule 1 (ICAM-1); PMN cells were

analyzed for gene expression of Caspase-1, interleukin-8 receptor (IL-8R) and L-selectin. The data obtained from sample cycle threshold values (Ct) were adjusted by endogenous control (RPS9 and GAPDH). Bovine IL-1β, IL-6, IL-8, TNF-α, PPAR-γ, RBP-4, NF-κB, ICAM-1, L-Selectin, RPS9, and GAPDH primer/probes were developed by Applied Biosystems (Table 7). IFN-γ (Accession number NM_174086.1, Forward Primer CTCTGAGAAACTGGAGGACTTCAAA, Reverse Primer GGCTTTGCGCTGGATCTG, Probe ATCCACCGGAATTTG), IL-8R primer/probe (Accession number DQ389113, Forward Primer ATGCGGGTCATCTTTGCTG, Reverse Primer ATGAGGGTGTCCGCGATC, Probe CTCGTCTTCCTGCTCTGCTGGCT), IL-10 (Accession number NM_174088, Forward Primer CAAGGAGCACGTGAACTCACT, Reverse Primer CCGCCTTGCTCTTGTTTTCG, Probe CTGTCATCGCTTTCTGC), and IL-12 P40 (Accession number NM_174356.1, Forward Primer AGCTGGGAGTACCCTGACA, Reverse Primer AACACAAAACGTCAGGGAGAAGT, Probe CACCCCGCATTCCT) was generated by Primer Express (Version 1.5, Applied Biosystems) to set for PCR amplification. Caspase-1 (Accession Number XM_002692921) was designed by Custom Plus TaqMan RNA Assay (Applied Biosystems). Polymorphism chain reaction followed the TagMan® Universal Master Mix II instruction. 96 well plates with samples in a 7500 Fast Real-Time PCR were held at 50℃ for 2 minutes UNG incubation, 95℃ for 10 minutes polymerase activation; the temperature gradient was 40 cycles of 95℃ for 15 second denaturing and 60℃ for 1 min annealing/extension.

Statistical Analysis

The parameters obtained from the calves' fatty acid composition, gene expression and vitamins content in two dietary treatments were analysis by Proc TTEST in SAS (Statistical Analysis System; V. 9.4 SAS Inst. Inc., Cary, NC). The statistical model was: $Y_{ij} = \mu + R_i + e_{ij}$ where the μ = overall mean, R_i = fixed effect of diet (i = 1, 2), and e_{ij} = residual error term. (Maamouri et al., 2014).

Fatty acid composition data were presented least square means (LSM) and the largest standard error of the mean (SEm) between two treatments. Vitamin statuses and gene expression were presented LSM with SEm in each group. Gene expression data were obtained using target gene Ct values to normalize with the endogenous control gene (RPS9 and GAPDH). For graphic illustration, gene data are shown in fold change ($2^{-\Delta\Delta Ct}$ method). All of the significant effects were declared at $P \leq 0.05$.

Results and Discussion

Fatty Acid Analysis

Perirenal Adipose Predominant FA in both treatments in calves' included primarily C16:0 (palmitic acid), C18:0 (stearic acid) and C18:1 cis (oleic acid) content in NEFA, NL, PL fractions (Table 2.1, 2.2 and 2.3). Comparing SFA and UFA treatments, dietary UFA increased C18:3 n3 (α-linolenic acid; α-LA; Figure 1) in the three fractions as well as eicosapentaenoic acid (EPA) in NL and PL fraction (Table 2.1, 2.2 and 2.3; Figure 3). Dietary UFA increase total n3 PUFA in perirenal adipose of three fractions (Table 2.4, 2.5 and 2.6). In addition, dietary UFA reduced pro-inflammatory C20:4 (arachidonic acid) in NEFA (0.17 vs. 0.06 g/100 g fatty acid methyl esters; FAME) and PL (2.50 vs. 1.02 g/100 g FAME) fractions, respectively (Table 2.1 and 2.3). Dietary UFA induced total n3 PUFA in NEFA (3.61 vs. 14.33 g/100 g FAME), NL (2.64 vs. 11.63 g/100 g FAME) and PL (14.98 vs. 26.02 g/100 g FAME) fractions. Furthermore, UFA reduced n6:n3 ratio in NEFA (12.74 vs. 0.56 g/100 g FAME), NL (8.76 vs. 0.87 g/100 g FAME) and PL (8.10 vs. 0.69 g/100 g FAME) fractions. Dietary rich content of α-LA such as flaxseed and flaxseed oil can increase level of α-LA and EPA in meat of animals (Garcia, 2012). On the other hand, calves fed with CLA oil did not show greater α-LA, EPA and DHA content compared with control diet in perirenal fat (Marounek et al., 2008). Dietary CLA fed to Holstein bull did not change α-LA, EPA and total of n3 PUFA content, but when same diet was supplemented with α-LA-rich linseed (10% diet DM), the intramuscular fat content of α-LA, EPA and total of n3 PUFA significantly increased compared with control diet and CLA group (Gómez et al., 2015). This may indicate that dietary α-LA-rich ingredients can increase content of α-LA, EPA and n3 PUFA, as well as decrease arachidonic acid content in animal tissues. However, CLA alone may not modulate the α-LA, EPA and n3 PUFA content in perirenal fat and intramuscular tissue of Holstein bulls.

Hepatic Tissue Predominant FAs in NEFA, NL and PL fractions of liver were primarily of palmitic acid and stearic acid (Table 3.1, 3.2 and 3.3). Dietary SFA had higher stearic acid content in the NEFA fraction (26.05 vs. 23.21 g/100 g FAME) compared with UFA (Table 3.1). In addition, dietary UFA induced α-LA in the NEFA (0.48 vs.11.28 g/100 g FAME), PL (0.59 vs. 12.06 g/100 g FAME) and NL (0.43 vs. 9.09 g/100 g FAME) fractions (Table 3.1, 3.2 and 3.3; Figure 1). Similar results have been reported regarding perirenal adipose tissues. Dairy cow during transition period, dietary whole flaxseed increased α-linolenic acid concentration of liver at week 4 postpartum (Petit et al., 2007). Dietary UFA however reduced n6 fatty acid of γ-linolenic (C18:3 n6; Figure 6) in NEFA (0.27 vs. 0 g/100 g FAME; Table 3.1), NL (0.21 vs. 0.04 g/100 g FAME; Table 3.2) and PL (0.21 vs. 0.04 g/100 g FAME; Table 3.3). Dietary UFA also reduced arachidonic acid in PL fraction (5.99 vs. 0.07 g/100 g FAME; Table 3.2). Dietary SFA elevated docosahexaenoic acid (DHA) content in NEFA (2.09 vs. 0.49 g/100 g FAME; Table 3.1), NL (2.71 vs. 0.77 g/100 g FAME; Table 3.2) and PL (3.77 vs. 1.04 g/100 g FAME; Table 3.3) fractions of liver. Dietary α-LA increased EPA, but may limit DHA synthesis. DHA biosynthesis limitation might be caused by lower affinity and limited elongation enzymes. Alpha-LA has two to three times greater affinity to Δ 6 desaturase compared to linoleic acid, and the same enzyme will be used on 24 carbon fatty acids to synthesis DHA and 22:5 n6 (Portolesi et al., 2007; Gibson et al., 2013). Devarshi et al. (2013) reported that dietary flaxseed oil up-regulated 1.7 fold of hepaticΔ 6 desaturase than control animals. In addition, Δ 5 desaturase was up-regulated 10.1 fold change expression than that in diabetic rats. This may indicate that α-LA-rich flaxseed oil had higher bio-conversion rate to EPA but this may have limited the amount of Δ 6 desaturase for DHA biosynthesis.

Serum lipids Dietary UFA increased α-LA (Figure 1) content in serum NEFA (2.67 vs. 17.45 g/100 g FAME; Table 4.1), NL (1.65 vs. 29.57 g/100 g FAME; Table 4.2) and PL (0.51 vs. 11.80 g/100 g FAME; Table 4.3) fractions, as well as total PUFA (Figure 5) in NEFA (20.08 vs. 37.14 g/100 g FAME; Table 4.4), NL (48.49 vs. 68.62 g/100 g FAME; Table 4.5) and PL (39.63 vs. 46.33 g/100 g

FAME; Table 4.6) fractions. Moallem and Zachut (2012) fed flaxseed oil to late pregnant dairy cows and observed elevated α-LA and total n3 PUFA profile in plasma. In addition, dietary UFA increased EPA content (Figure 3) in serum NEFA (0.01 vs. 0.39 g/100 g FAME; Table 4.1), NL (0.20 vs. 0.57 g/100 g FAME; Table 4.2) and PL (0.16 vs. 0.63 g/100 g FAME; Table 4.3) fractions. Increase dietary α-LA can increase EPA biosynthesis in mammals; however, it may limit DHA in plasma and tissues (Portolesi et al., 2007; Gibson et al., 2013). Compared with Portolesi et al. (2007) and Gibson et al. (2013), dietary UFA lowered DHA in serum PL (0.57 vs. 0.24 g/100 g FAME; Table 4.3). Moreover, dietary UFA reduced γ-linolenic acid (Figure 6) in NL (0.55 vs. 0.12 g/100 g FAME; Table 4.2) and PL (0.16 vs. 0.03 g/100 g FAME; Table 4.3) as well as, arachidonic acid (Figure 7) in NL (1.18 vs. 0.52 g/100 g FAME; Table 4.2) and PL (3.07 vs. 1.37 g/100 g FAME; Table 4.3). Dietary flaxseed can increase α-LA, EPA and PUFA but also decrease γ-linolenic acid and arachidonic acid (Petit and Côrtes, 2010; Petit, 2015; Matras et al., 2014). In addition, supplementary flaxseed oil in human diet increased plasma α-LA and EPA content (Mantzioris et al., 1994). Thus, dietary UFA can increase n3 PUFA and reduce n6 PUFA in serum lipid fractions; this may be a benefit to animals' health as well.

Immune cell lipids Fatty acid in immune cells, which included PBMC and PMN cells were predominantly palmitic and, stearic acid, oleic acid and linolenic acid, but there were no detectable differences in those four main fatty acids by treatments (Table 5.1 and 6.1). Dietary UFA increased α-LA (0.99 vs. 3.97 g/100 g FAME) and EPA (0.25 vs. 1.21 g/100 g FAME; Table 5.1) in PBMC, as well as α-LA (0.41 vs. 6.59 g/100 g FAME) and EPA (0.35 vs. 1.23 g/100 g FAME; Table 6.1; Figure 2 and 4) in PMN. Fatty acids of immune cells can regulate immune responses. Increased α-LA ingestion can induce mononuclear cells content of EPA; this can reduce pro-inflammatory eicosanoids production, and regulate immune responses (Calder 2008). Lipid composition in the membrane of immune cells can affect membrane lipid bilayer fluidity, receptor formation and signaling pathways. This further affects the production of different eicosanoids (Alexander, 1998). In addition, human dietary α-LA in meals for 126 day, α-LA delayed PBMC hypersensitivity and proliferation compared

with control (Kelley et al., 1991). Mantzioris et al. (1994) showed that human ingestion of flaxseed oil resulted in an increased α-LA and EPA concentration in neutrophils. Our data indicated that dietary UFA can increase n3 PUFA in immune cells, which may down-regulate pro-inflammatory gene expressions.

Gene expression

Target genes including IL-6, IL-1β, TNF-α, and ICAM-1 were detected in PBMC (Table 8). The analysis did not show any detectable significance differenct between SFA and UFA. In addition, target genes including CASP-1, IL-8R and L-SEL were detected in PMN cells (Table 9). The L-SEL had 1.3 fold change increase by dietary UFA (Figure 8). On the other hand, the CASP-1 and IL-8R were not statistically different between treatments. In the present study, dietary flaxseed in postpartum dairy cows did not affect pro-inflammatory cytokine IFN-γ (Lessard et al., 2003). Fedding 6% α-LA-enriched of Camelina meal to lactation dairy cows reduced expression of of IL-1β, IL-8 and TNF-α in PBMC (Rezamand et al., 2016). Compared to our results; we fed 2% of flaxseed oil and 1% of CLA to the calves on a daily basis, this lower feeding rate may have caused lack of any significant difference in expression of pro-inflammatory cytokines of PBMC. In addition, supplemental palm oil at 1.5% or safflower oil at 1.5% to dairy cow at 35 or 85 days postpartum (Silvestre et al., 2011). Dietary safflower oil significantly induced TNF-α in neutrophils at 35 days of postpartum, as well as increase in TNF-α and IL-1β production after LPS stimulation. The dietary intervention did not show an effect in TNF-α and IL-1β induced at 85 days of postpartum. The safflower oil is a linoleic acid-rich ingredient, and dietary safflower oil greater L-SEL (CD 62L) expression on neutrophils at days 4 and 7 relative to parturition (Silvestre et al., 2011). Compared with our dietary ingredients (Table 1), sum of total linoleic acid from Lutalin® , flaxseed oil and Purina® starter is greater content than that of palm fruit oil. This may explain in part why dietary UFA had induced L-SEL in PMN. In other species, ingestion of linoleic acid had induced L-SEL expression on neutrophil than control group (Rodrigues et al., 2010). In both Silvestre et al. (2011) and Rodrigues et al. (2010), dietary

linoleic acid decreased the threshold of activation of pro-inflammatory response; this may alter innate inflammatory response. In contrast, in our study, chemokine receptor and cytokines did not change by dietary FA. Cell culture of immune cells with exogenous stimulator such as LPS may show different dietary effects. All in all, dietary UFA in calves induced L-SEL adhesion molecular in PMN, and may decrease threshold of activation of innate immune response, but this did not change other immune related mediators in our study.

Hepatic and perirenal adipose tissues were analyzed for target genes IL-1β, IL-6, IL-8, IL-10, IL-12, IFN-γ, PPAR-γ, TNF-α, RBP4 and NF-κB. No significant differences were detected between SFA and UFA groups (Table 10 and 11). Dietary flaxseed oil significantly reduced hepatic TNF-α, IL-6, IFN-γ and NF-κB in diabetes rats, but did not have an effect in control group that received flaxseed oil (Jangale et al., 2013 and). In addition, dietary flaxseed oil induced PPAR-α and down-regulated IL-6 and TNF-α in diabetes rats. Dietary flaxseed oil in healthy rats reduced PPAR-α than control group, but the diet did not affect IL-6 and TNF-α (Devarshi et al., 2013). This shows that dietary flaxseed oil may induce PPAR-α and down-regulate TNF-α, IL-6, IFN-γ and NF-κB in rats with diabetes, but when rats were in healthy status, dietary flaxseed reduced PPAR-α, but did not affect TNF-α, IL-6. In contrast, when mice were fed flaxseed, had colonic injury and inflammation by dextran sodium sulfate (DSS) in drinking water, they showed elevated serum and colon IL-6 and IL-1β as well as serum IFN-γ compared with healthy mice plus DSS. Genes related to NF-κB were induced by dietary flaxseed vs. DSS (Zarepoor et al., 2014). Dietary flaxseed exacerbated colonic injury and inflammation during DSS exposure. When those results are compared with our data, the dietary intervention did not alter hepatic gene expression; the reason might be related to the fact that our calves did not receive exogenous stimulation that would induce their immune responses activation.

On the other hand, dietary flaxseed oil in obese rats did not affect cytokines such as IL-10 and IL-6 in perirenal adipose compared with obese control group. This however caused significant reduction in TNF-α in perirenal adipose tissue (Baranowski et al., 2012). In addition, dietary t10,

c12-CLA or c9, t11-CLA in obese rats did not alter concentration of IL-6, IL-10 and TNF-α; it also did not affect NF-κB and its related members (DeClercq et al., 2010). In contrast, the t10, c12-CLA isomer has been demonstrated to induce inflammatory responses in human and mouse (Belda et al., 2012). Dietary t10, c12-CLA rapidly induced pro-inflammatory cytokines such as IL-6 and TNF-α in mice adipose tissue. In vitro study of CLA-induced adipocytes IL-6 secretion regulated by NF-κB pathway was demonstrated (Poirier et al., 2006). In our experiment with peripheral adipose tissue, pro-inflammatory, anti-inflammatory cytokines as well as transcription factors did not show any significant dietary effect. Our study did not have a negative control group and we had a mixture of dietary CLA and flaxseed oil. Moreover, our Lutalin® has minimum 56% of CLA and 50% of the two isomers each. This may affect how genes of inflammatory mediators are expressed in our study.

Expression of genes results did not show treatment effect. This might be related to lack of exogenous stimulation, negative control group and having fed a lipids mixture diet. For this reason, our further study should focus on the different dietary fats with exogenous stimulators in various tissues and immune cells to better understand the effect of dietary fat on immune response during infection.

Lipid Soluble Vitamin

Hepatic α-tocopherol and retinol were detected to compare effects of different dietary treatments. Dietary UFA trended to increase α-tocopherol content in hepatic tissue (1817 ± 246 vs. 1281 ± 191 ng/mL, $P = 0.09$; Table 13). No significant dietary effect was detected for retinol content of hepatic tissue.

Serum α-tocopherol, β-carotene and retinol content were detected in serum to compare effects of different dietary treatments. Dietary SFA caused greater α-tocopherol (1964 ± 72 vs. 1552 ± 100 ng/mL, $P < 0.01$), retinol (147 ± 8 vs. 106 ± 7 ng/mL, $P < 0.01$) and β-carotene (89 ± 5 vs. 63 ± 3 ng/mL, $P < 0.01$) in serum (Table 14; Figure 9).

Polyunsaturated fatty acids are highly oxidable that may increase the requirement of antioxidants (Nair et al., 1993). Angus steers that received 0.125% body weight (BW) of flaxseed had greater α-tocopherol in plasma and *longissimus thoracis* compared with dietary 0.250% BW of flaxseed and control. In addition, 0.125% flaxseed of BW trended to have a higher content of β-carotene compared to 0.250% of BW flaxseed and control, but did not affect retinol (Pouzo et al., 2016). Higher flaxseed in diet induced higher requirement of antioxidants. This may have indicated that requirement of α-tocopherol and β-carotene increased when the dietary higher content of PUFA reduced the antioxidant concentration in the plasma and muscle. Raederstorff et al. (2015) showed that dietary PUFA increased requirement of vitamin E in reducing oxidative stress. Vitamin E can accept cell membrane electrons from oxidable PUFA and stabilize the membrane structure (Atkinson et al., 2010). Vitamin E can trap free radicals and donate hydrogen to protect lipids and phospholipids of membrane (Zhao et al., 2016). Antioxidants such as α-tocopherol, β-carotene and retinol can stabilize PUFA from oxidation; this can cause lower concentration in plasma and storage. Intake of α-tocopherol has positive correlation with plasma α-tocopherol status (Zhao et al., 2016). Our dietary UFA had high content of α-tocopherol in flaxseed oil and high retinol in Lutalin® oil (Table 12). Dietary UFA however significantly decreased concentration of α-tocopherol, retinol and β-carotene in serum. This result is in line with the notion that dietary PUFA increases the requirement of lipid-soluble vitamins and reduces concentration of those vitamins.

On the other hand, α-tocopherol has α-tocopherol transfer protein to protect its rapid degradation and assist the transport from hepatocyte to peripheral tissues (Raederstorff et al., 2015). Dietary saturated FA-rich coconut oil fed to growing pigs lead to greater α-tocopherol in liver and plasma (Prévéraud et al., 2014). In addition, dietary n6 PUFA-rich safflower oil and MUFA-rich olive oil can increase α-tocopherol in hepatic tissue. Dietary n3 PUFA-rich linseed oil however decreased α-tocopherol in liver, adipose tissue and blood (Prévéraud et al., 2014). Compared with our results, liver storage of α-tocopherol trended to be greater by dietary UFA. This may have been caused by

MUFA-rich Lutalin® and n6 PUFA-rich Purina® Starter (Table 1.2). Prévéraud et al. (2014) and Gleize et al. (2013) suggested two possible pathways of dietary PUFA reduce lipid-soluble vitamins bioaccessibility. One is plasma vitamin E degraded to protect PUFA from oxidation; the other is radius size and zeta potential of mixed micelles was smaller with ingestion of SFA type fat.

Overall, dietary UFA can reduce concentration of serum α-tocopherol, β-carotene and retinol; however MUFA, n6 PUFA, and α-tocopherol transfer protein may assist the storage of α-tocopherol in hepatic tissues to reduce α-tocopherol degradation.

Conclusion

Dietary UFA can increase α-LA in all lipid fractions of adipose, liver and serum as well as PBMC and PMN. Alpha-linolenic is a precursor of EPA; we found higher content of α-LA in adipose tissues and serum, which correlated with greater EPA content in three lipid fractions of serum as well as NL and PL fractions of adipose tissue. Dietary UFA had higher α-LA and EPA content in PMN and PBMC. In addition, dietary UFA had increased sum of PUFA in all lipid fractions of adipose and serum. In contrast, dietary UFA reduced n6 fatty acids γ-linolenic acid and it derivative arachidonic acid. Dietary UFA reduced γ-linolenic content in liver three lipid fractions as well as serum NL and PL fractions. The arachidonic acid was decreased in NEFA, PL of adipose, PL of liver, and NL, PL of serum. Thus, dietary UFA can increase n3 fatty acid content and reduced n6 fatty acid. These may regulate inflammatory response.

Dietary UFA did not affect inflammatory mediators at gene level in adipose, liver and PBMC. Although adhesion molecular L-SEL of PMN had 1.3 fold higher expression than dietary SFA group. Dietary linoleic acid can decrease the threshold of activation for a pro-inflammatory response. Sum of total linoleic acid from our treatment of Lutalin® , flaxseed oil and Purina® starter showed a greater linoleic acid content than palm fruit oil; this may explain that dietary UFA had induced L-SEL upregulation in PMN.

Dietary UFA resulted in a decrease in α-tocopherol, β-carotene and retinol in serum as well as a trend for increase in α-tocopherol in hepatic tissue. Lipid-soluble vitamins can be used to protect PUFA from oxidation. In addition, dietary PUFA may increase mixed micelles' size and decrease lipid-soluble vitamins absorption in enterocytes and serum circulation. Mono-unsaturated FA, n6 PUFA, and α-tocopherol transfer protein however may assist storage of α-tocopherol in hepatic tissues to reduce its degradation.

Overall, dietary UFA can increase n3 PUFA, total PUFA, and decrease n6 PUFA and lipid-soluble vitamins in serum as well as upregulate gene expression. Increased dietary UFA can alter n3 PUFA profile in various tissues, serum and PMN in calves; this can improve the health of new born calves. In addition, dietary UFA can increase L-SEL that may improve PMN migration to defend against exogenous pathogens. However, increase PUFA ingestion has to include supplemental lipid-soluble vitamins to protect PUFA from oxidation.

Tables

Table 1.1. Fatty acid (g/100g of fatty acid methyl ester) composition of dietary treatments (n=3 per treatment).

Fatty acid (g/100g)	Treatment				
	Palm Fruit	Lutalin®	Flax Seed	Purina®	SEm[1]
C14:0	1.02	0.11	0.04	0.93	0.04
C14:1	-	-	-	0.18	0.02
C15:0	0.07	0.02	0.02	0.15	0.01
C15:1	-	0.004	0.02	-	0.001
C16:0	53.87	9.30	5.3	17.67	0.90
C16:1	0.10	0.09	0.05	0.70	0.02
C17:0	0.15	0.02	0.07	0.37	0.02
C17:1	0.02	0.04	0.05	0.34	0.02
C18:0	4.97	5.59	4.31	5.63	0.27
C18:1 TRANS	0.32	-	0.05	-	0.05
C18:1 CIS	32.89	38.39	23.25	26.19	0.81
C18:2 TRANS	-	-	-	0.02	0.00
C18:2 CIS	5.71	2.23	15.04	43.78	0.84
C20:0	0.33	-	0.19	-	0.001
C18:3 N3	0.10	38.71	50.80	2.79	0.59
C20:1	0.14	0.38	0.53	0.41	0.04
C21:0	0.03	0.70	0.01	0.07	0.09
C20:2	-	2.79	0.04	0.04	0.12
C20:3 N6	0.07	1.02	0.02	0.19	0.01
C20:3 N3	-	-	0.02	0.01	0.003
C22:1	-	-	0.03	0.01	0.004
C20:4	-	0.28	0.01	0.05	0.003
C23:0	-	-	-	0.04	0.00
C20:5	-	-	-	0.05	0.01
C22:2	0.01	0.03	0.02	0.06	0.01
C24:0	0.07	0.26	0.10	0.16	0.01
C24:1	-	0.02	0.01	0.03	0.01
C22:6	-	-	-	0.09	0.03

[1] SEm presents the largest value from the treatment

Table 1.2. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of dietary treatments. (n=3 per treatment).

Fatty acid	Treatment				
(g/100g)	Palm Fruit	Lutalin®	Flax Seed	Purina®	SEm[1]
Σ MUFA	33.47	38.93	23.95	27.86	0.81
Σ N6	5.79	6.35	15.13	44.14	0.85
Σ N3	0.10	38.7	50.82	2.95	0.59
N6:N3	59.05	0.16	0.30	14.98	7.91
Σ PUFA	5.89	45.06	65.95	47.09	0.85
De novo	1.16	0.12	0.05	0.96	0.05
Σ Unsaturated	39.35	83.99	89.93	74.94	0.97
Σ Saturated	60.65	16.01	10.07	25.06	0.97
Unsat/Sat Ratio	0.65	5.25	8.93	2.99	0.17

[1] SEm presents the largest value from the treatment

Table 2.1. Fatty acid (g/100 g of fatty acid methyl ester) composition of non-esterified fatty acid fraction of the adipose tissue (perirenal) obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
C14:0	3.73	3.45	0.20	0.30
C14:1	0.63	0.52	0.06	0.15
C15:0	0.26	0.27	0.02	0.66
C16:0	31.72	27.42	0.59	<0.01
C16:1	3.05	2.77	0.45	0.63
C17:0	0.49	0.55	0.03	0.17
C17:1	0.22	0.29	0.16	0.70
C18:0	17.81	16.36	0.87	0.21
C18:1 TRANS	0.71	0.71	0.71	0.23
C18:1 CIS	36.46	30.51	0.44	<0.01
C18:2 TRANS	-	0.04	0.03	0.18
C18:2 CIS	3.06	4.80	0.31	<0.01
C18:3 N6	0.01	-	0.01	0.36
C20:0	0.14	0.23	0.01	<0.01
C18:3 N3	0.25	9.15	0.65	<0.01
C20:1	0.34	1.99	0.16	<0.01
C21:0	0.05	0.21	0.02	<0.01
C20:2	0.05	0.19	0.02	<0.01
C22:0	0.06	0.06	0.01	0.75
C20:3 N6	0.05	0.05	0.05	0.77
C20:3 N3	0.02	0.02	0.02	0.77
C22:1	-	0.05	0.02	0.02
C20:4	0.17	0.06	0.03	<0.01
C23:0	-	0.05	0.01	0.01
C20:5	-	0.02	0.01	0.16
C24:0	0.01	0.01	0.01	0.93
C24:1	0.01	0.02	0.01	0.62
C22:6	0.01	-	0.01	0.36

Table 2.2. Fatty acid (g/100 g of fatty acid methyl ester) composition of neutral lipid fraction of the adipose tissue (perirenal) obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
C14:0	3.94	3.41	0.61	0.44
C14:1	0.63	0.55	0.05	0.27
C15:0	0.22	0.20	0.04	0.56
C15:1	3.31	0.03	3.31	0.37
C16:0	29.01	24.19	3.64	0.25
C16:1	3.96	2.83	1.83	0.58
C17:0	0.53	0.34	0.07	0.06
C17:1	0.29	0.24	0.02	0.54
C18:0	9.22	8.09	3.81	0.82
C18:1 TRANS	6.21	10.64	6.14	0.53
C18:1 CIS	39.24	39.24	6.18	0.23
C18:2 TRANS	0.06	0.04	0.02	0.60
C18:2 CIS	2.16	4.33	0.90	0.09
C18:3 N6	0.06	0.05	0.03	0.81
C20:0	0.12	4.54	2.86	0.18
C18:3 N3	0.22	6.76	1.42	<0.01
C20:1	0.21	2.07	0.13	<0.01
C21:0	0.02	0.07	0.02	0.02
C20:2	0.04	0.21	0.03	<0.01
C22:0	0.06	0.08	0.02	0.58
C20:3 N6	0.01	0.04	0.02	0.23
C20:3 N3	0.04	0.13	0.03	0.04
C22:1	0.02	0.02	0.01	0.92
C20:4	0.04	0.05	0.01	0.76
C23:0	0.01	0.05	0.01	<0.01
C22:2	-	0.01	0.01	0.37
C20:5	-	0.02	0.01	0.05
C24:0	0.01	0.01	0.01	0.97
C24:1	0.03	0.03	0.01	0.90
C22:6	0.01	0.01	0.01	0.21

Table 2.3. Fatty acid (g/100 g of fatty acid methyl ester) composition of phospholipid fraction of the adipose tissue (perirenal) obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
C12:0	1.04	1.12	0.26	0.78
C13:0	2.97	2.27	0.42	0.20
C14:0	1.84	1.69	0.26	0.64
C14:1	0.27	0.15	0.17	0.61
C15:0	-	0.09	0.22	0.36
C16:0	25.00	20.68	1.56	0.04
C16:1	1.26	1.08	0.29	0.64
C17:0	0.19	0.34	0.16	0.47
C17:1	0.71	1.59	0.56	0.18
C18:0	24.41	25.60	1.47	0.55
C18:1 TRANS	1.89	2.10	0.55	0.73
C18:1 CIS	24.38	15.34	1.07	<0.01
C18:2 CIS	9.02	8.37	0.91	0.57
C18:3 N3	0.12	13.44	1.68	<0.01
C20:1	-	1.52	0.44	<0.01
C21:0	-.	0.22	0.22	0.36
C20:3 N6	1.80	0.92	0.12	<0.01
C20:3 N3	1.27	0.83	0.19	0.13
C22:1	0.99	0.51	0.12	<0.01
C20:4	2.50	1.02	0.30	<0.01
C22:2	-	0.15	0.15	0.36
C20:5	0.27	1.30	0.19	<0.01
C24:1	0.09	0.07	0.09	0.91

Table 2.4. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of non-esterified fatty acid fraction of the adipose tissue (perirenal) obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
Σ MUFA	41.42	36.43	0.89	<0.01
Σ N6	3.32	5.14	0.32	<0.01
Σ N3	0.27	9.19	0.65	<0.01
N6:N3	12.74	0.56	1.57	<0.01
Σ PUFA	3.61	14.33	0.86	<0.01
De novo	4.37	4.08	0.26	0.43
Σ Unsaturated	45.03	50.76	0.84	<0.01
Σ Saturated	54.98	49.24	0.84	<0.01
Unsat/Sat Ratio	0.82	1.03	0.03	<0.01

Table 2.5. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of neutral lipid fraction of the adipose tissue (perirenal) obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P*
	Saturated	Unsaturated		
Σ MUFA	53.89	47.12	6.22	0.36
Σ N6	2.38	4.72	0.88	0.07
Σ N3	0.26	6.91	1.40	<0.01
N6:N3	8.76	0.87	2.75	0.03
Σ PUFA	2.64	11.63	1.98	<0.01
De novo	4.26	3.69	0.65	0.45
Σ Unsaturated	56.53	58.74	5.72	0.76
Σ Saturated	43.47	41.26	5.72	0.76
Unsat/Sat Ratio	1.61	1.57	0.49	0.94

Table 2.6. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of phospholipid fraction of the adipose tissue (perirenal) obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
Σ MUFA	29.58	22.36	1.18	<0.01
Σ N6	13.32	10.46	1.16	0.07
Σ N3	1.66	15.56	1.92	<0.01
N6:N3	8.10	0.69	2.12	0.02
Σ PUFA	14.98	26.02	2.97	0.01
De novo	2.87	2.81	0.41	0.88
Σ Unsaturated	44.56	48.38	2.61	0.24
Σ Saturated	55.44	51.62	2.61	0.24
Unsat/Sat Ratio	0.81	0.96	0.09	0.21

Table 3.1. Fatty acid (g/100 g of fatty acid methyl ester) composition of non-esterified fatty acid fraction of the liver tissue obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
C14:0	1.74	2.71	0.52	0.11
C14:1	0.25	0.50	0.11	0.07
C15:0	0.33	0.71	0.19	0.10
C16:0	22.56	23.50	1.45	0.58
C16:1	0.58	1.50	0.43	0.08
C17:0	0.65	0.77	0.03	0.02
C17:1	0.31	1.03	0.36	0.11
C18:0	26.05	23.21	0.77	0.01
C18:1 TRANS	0.16	0.17	0.04	0.79
C18:1 CIS	12.87	11.62	0.77	0.17
C18:2 TRANS	0.01	-	0.01	0.36
C18:2 CIS	13.47	12.73	1.07	0.56
C18:3 N6	0.27	-	0.02	<0.01
C20:0	0.17	0.32	0.07	0.08
C18:3 N3	0.48	11.28	1.79	<0.01
C20:1	0.46	1.44	0.93	<0.01
C21:0	0.05	0.31	0.04	<0.01
C20:2	0.35	0.57	0.07	0.02
C22:0	3.06	0.40	1.00	0.05
C20:3 N6	1.70	0.37	0.79	0.15
C20:3 N3	7.76	0.37	1.62	0.01
C22:1	0.04	0.27	0.25	0.39
C20:4	1.96	2.25	1.68	0.88
C23:0	0.33	0.81	0.14	0.02
C20:5	0.76	1.22	0.31	0.19
C24:0	0.15	0.30	0.07	0.09
C24:1	0.79	0.06	0.04	<0.01
C22:6	2.09	0.49	0.17	<0.01

Table 3.2. Fatty acid (g/100 g of fatty acid methyl ester) composition of neutral lipid fraction of the liver tissue obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
C14:0	1.32	1.77	0.39	0.35
C14:1	0.18	0.17	0.04	0.88
C15:0	0.29	0.32	0.03	0.39
C15:1	-	0.02	0.01	0.06
C16:0	21.01	20.31	1.72	0.73
C16:1	0.32	0.18	0.13	0.37
C17:0	0.62	0.79	0.10	0.16
C17:1	0.09	0.28	0.11	0.15
C18:0	24.13	22.48	2.33	0.52
C18:1 TRANS	4.97	3.10	2.97	0.58
C18:1 CIS	10.69	12.17	3.35	0.73
C18:2 TRANS	0.11	0.12	0.04	0.91
C18 :2 CIS	14.89	15.65	1.12	0.58
C18:3 N6	0.21	0.04	0.05	0.02
C20:0	0.20	0.30	0.08	0.26
C18:3 N3	0.59	12.06	2.40	<0.01
C20:1	0.39	1.52	0.36	0.03
C21:0	0.10	0.20	0.04	0.08
C20:2	0.37	0.56	0.10	0.13
C22:0	5.27	0.77	0.36	<0.01
C20:3 N6	0.02	0.09	0.03	0.04
C20:3 N3	6.10	3.28	2.02	0.22
C22:1	0.01	0.07	0.02	0.04
C20:4	3.20	0.06	1.99	0.18
C23:0	0.17	0.38	0.19	0.34
C22:2	0.70	1.22	0.36	0.21
C20:5	0.02	0.26	0.13	0.12
C24:0	0.11	0.62	0.19	0.04
C24:1	1.05	0.17	0.87	<0.01
C22:6	2.71	0.77	0.27	<0.01

Table 3.3. Fatty acid (g/100 g of fatty acid methyl ester) composition of phospholipid fraction of the liver tissue obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
C14:0	0.40	0.28	0.08	0.26
C14:1	0.13	0.08	0.01	0.01
C15:0	0.15	0.18	0.04	0.63
C16:0	15.44	15.95	1.46	0.81
C16:1	0.25	0.33	0.09	0.42
C17:0	0.67	1.00	0.15	0.07
C17:1	0.23	0.38	0.11	0.26
C18:0	34.15	29.79	1.90	0.10
C18:1 TRANS	0.37	1.46	0.34	0.02
C18:1 CIS	12.24	10.79	0.70	0.13
C18:2 TRANS	0.12	0.23	0.13	0.48
C18:2 CIS	12.76	15.64	2.65	0.34
C18:3 N6	0.21	0.04	0.02	<0.01
C20:0	0.08	0.33	0.03	<0.01
C18:3 N3	0.43	9.09	1.18	<0.01
C20:1	0.56	1.54	0.12	<0.01
C21:0	0.03	0.25	0.02	<0.01
C20:2	0.36	0.63	0.05	<0.01
C22:0	4.48	0.91	1.04	0.01
C20:3 N6	0.98	0.09	0.94	0.39
C20:3 N3	2.76	6.21	2.75	0.30
C22:1	0.04	0.17	0.14	0.42
C20:4	5.99	0.07	0.39	<0.01
C23:0	0.30	0.70	0.19	0.08
C22:2	0.77	1.94	0.44	0.03
C20:5	0.19	0.18	0.18	0.94
C24:0	0.04	0.44	0.14	0.03
C24:1	1.60	0.23	0.15	<0.01
C22:6	3.77	1.04	0.24	<0.01

Table 3.4. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of non-esterified fatty acid fraction of the liver tissue obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
Σ MUFA	15.47	16.58	1.51	0.50
Σ N6	17.77	15.92	1.86	0.47
Σ N3	11.09	13.36	2.26	0.44
N6:N3	2.39	1.27	1.08	0.35
Σ PUFA	28.85	29.27	3.82	0.92
De novo	2.17	3.63	0.72	0.10
Σ Unsaturated	44.32	45.86	2.37	0.58
Σ Saturated	55.68	54.15	2.37	0.58
Unsat/Sat Ratio	0.80	0.87	0.08	0.51

Table 3.5. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of neutral lipid fraction of the liver tissue obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
Σ MUFA	17.70	17.66	1.06	0.98
Σ N6	19.49	17.73	2.67	0.58
Σ N3	9.41	16.38	3.14	0.09
N6:N3	3.43	1.26	1.36	0.17
Σ PUFA	28.90	34.10	4.54	0.32
De novo	1.43	1.97	0.45	0.33
Σ Unsaturated	46.60	51.76	3.60	0.21
Σ Saturated	53.40	48.24	3.60	0.21
Unsat/Sat Ratio	0.87	1.13	0.15	0.16

Table 3.6. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of phospholipid fraction of the liver tissue obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
Σ MUFA	15.44	14.98	1.01	0.69
Σ N6	21.19	18.61	3.08	0.46
Σ N3	7.14	16.53	2.81	0.03
N6:N3	4.10	1.32	0.94	0.03
Σ PUFA	28.33	35.14	3.95	0.25
De novo	0.48	0.32	0.12	0.29
Σ Unsaturated	43.77	50.12	4.11	0.24
Σ Saturated	56.23	49.88	4.11	0.24
Unsat/Sat Ratio	0.82	1.04	0.12	0.22

Table 4.1. Fatty acid (g/100 g of fatty acid methyl ester) composition of non-esterified fatty acid fraction of the serum obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
C14:0	3.68	2.54	0.84	0.25
C14:1	0.40	0.40	0.11	0.99
C15:0	0.43	0.52	0.11	0.47
C15:1	0.19	0.10	0.09	0.45
C16:0	27.72	19.24	2.09	<0.01
C16:1	0.91	0.68	0.18	0.25
C17:0	0.86	0.69	0.29	0.61
C17:1	0.71	0.35	0.19	0.12
C18:0	22.47	18.42	1.33	0.06
C18:1 TRANS	0.80	0.60	0.30	0.61
C18:1 CIS	16.13	11.17	1.78	0.04
C18:2 TRANS	3.65	1.40	2.39	0.41
C18:2 CIS	9.82	14.19	1.42	0.05
C18:3 N6	0.26	0.10	0.15	0.33
C20:0	0.24	0.22	0.18	0.92
C18:3 N3	2.67	17.45	1.54	<0.01
C20:1	1.04	3.41	1.06	0.09
C21:0	0.02	0.26	0.09	0.04
C20:2	0.10	0.26	0.12	0.27
C22:0	0.41	0.12	0.14	0.10
C20:3 N6	2.15	1.66	0.49	0.42
C20:3 N3	0.35	0.43	0.12	0.64
C22:1	0.25	0.34	0.15	0.62
C20:4	0.93	0.90	0.21	0.91
C23:0	1.13	0.59	0.71	0.50
C22:2	0.10	0.18	0.10	0.50
C20:5	0.01	0.39	0.06	<0.01
C24:0	0.32	0.50	0.20	0.52
C24:1	0.43	0.76	0.29	0.35
C22:6	0.05	0.20	0.15	0.39

Table 4.2. Fatty acid (g/100 g of fatty acid methyl ester) composition of neutral lipid fraction of the serum obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
C14:0	3.43	1.71	0.55	0.02
C14:1	0.17	0.10	0.04	0.12
C15:0	0.59	0.38	0.08	0.04
C15:1	0.04	0.03	0.02	0.71
C16:0	19.53	11.42	0.85	<0.01
C16:1	1.11	0.30	0.13	<0.01
C17:0	0.19	0.21	0.05	0.68
C17:1	0.12	0.06	0.03	0.09
C18:0	7.65	6.63	0.63	0.20
C18:1 TRANS	0.29	0.16	0.11	0.27
C18:1 CIS	15.17	7.36	1.57	<0.01
C18:2 TRANS	0.14	0.07	0.14	0.66
C18:2 CIS	43.12	36.28	1.60	<0.01
C18:3 N6	0.55	0.12	0.06	<0.01
C20:0	0.20	0.17	0.06	0.72
C18:3 N3	1.65	29.57	0.92	<0.01
C20:1	0.34	1.69	0.18	<0.01
C21:0	-	0.08	0.01	<0.01
C20:2	0.02	0.26	0.04	<0.01
C22:0	0.79	0.13	0.22	0.03
C20:3 N6	0.36	0.18	0.10	0.15
C20:3 N3	1.00	0.53	0.07	<0.01
C22:1	0.17	0.01	0.37	0.34
C20:4	1.18	0.52	0.07	<0.01
C23:0	0.08	0.23	0.04	<0.01
C22:2	0.10	0.35	0.08	0.03
C20:5	0.20	0.57	0.09	<0.01
C24:0	0.02	0.04	0.01	0.23
C24:1	0.45	0.31	0.10	0.32
C22:6	0.18	0.17	0.06	0.92

Table 4.3. Fatty acid (g/100 g of fatty acid methyl ester) composition of phospholipid fraction of the serum obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
C14:0	0.36	0.19	0.08	0.07
C14:1	0.04	0.05	0.03	0.76
C15:0	0.29	0.32	0.12	0.82
C15:1	0.02	0.02	0.02	0.86
C16:0	19.35	18.00	1.02	0.27
C16:1	0.25	0.30	0.05	0.47
C17:0	0.56	0.62	0.11	0.64
C17:1	0.23	0.18	0.06	0.58
C18:0	21.41	22.15	1.03	0.62
C18:1 TRANS	0.12	0.35	0.10	0.07
C18:1 CIS	13.77	8.05	0.35	<0.01
C18:2 TRANS	0.91	0.21	0.66	0.35
C18:2 CIS	30.41	29.84	1.39	0.73
C18:3 N6	0.16	0.03	0.04	<0.01
C20:0	0.58	0.16	0.48	0.42
C18:3 N3	0.51	11.80	0.44	<0.01
C20:1	0.27	1.08	0.07	<0.01
C21:0	0.02	0.19	0.02	<0.01
C20:2	0.09	0.40	0.04	<0.01
C22:0	1.88	0.52	0.43	0.02
C20:3 N6	2.29	0.71	1.50	0.36
C20:3 N3	1.31	0.77	0.29	0.17
C22:1	0.04	0.06	0.03	0.51
C20:4	3.07	1.37	0.65	0.05
C23:0	0.33	0.50	0.20	0.53
C22:2	0.17	0.33	0.09	0.14
C20:5	0.16	0.63	0.09	<0.01
C24:0	0.08	0.19	0.07	0.20
C24:1	0.21	0.11	0.07	0.37
C22:6	0.57	0.24	0.11	0.02

Table 4.4. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of non-esterified fatty acid fraction of the serum obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
Σ MUFA	20.83	17.82	1.18	0.09
Σ N6	17.01	18.68	2.04	0.55
Σ N3	3.08	18.46	1.57	<0.01
N6:N3	11.74	1.01	3.16	0.02
Σ PUFA	20.08	37.14	3.12	<0.01
De novo	4.34	3.55	0.76	0.40
Σ Unsaturated	40.92	54.96	3.25	<0.01
Σ Saturated	59.08	45.05	3.25	<0.01
Unsat/Sat Ratio	0.72	1.25	0.11	<0.01

Table 4.5. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of neutral lipid fraction of the serum obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
Σ MUFA	17.85	10.01	3.33	<0.01
Σ N6	45.47	37.79	1.55	<0.01
Σ N3	3.02	30.84	0.99	<0.01
N6:N3	15.14	1.23	0.50	<0.01
Σ PUFA	48.49	68.62	1.73	<0.01
De novo	4.30	1.87	0.89	0.04
Σ Unsaturated	66.34	78.64	1.16	<0.01
Σ Saturated	33.66	21.36	1.17	<0.01
Unsat/Sat Ratio	1.98	3.75	0.26	<0.01

Table 4.6. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of phospholipid fraction of the serum obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
Σ MUFA	14.95	10.20	0.37	<0.01
Σ N6	37.09	32.89	1.24	0.02
Σ N3	2.55	13.43	0.55	<0.01
N6:N3	17.24	2.46	4.44	0.02
Σ PUFA	39.63	46.33	1.31	<0.01
De novo	0.38	0.38	0.12	0.99
Σ Unsaturated	54.58	56.53	1.03	0.20
Σ Saturated	45.42	43.47	1.03	0.20
Unsat/Sat Ratio	1.21	1.31	0.05	0.18

Table 5.1. Fatty acid (g/100 g of fatty acid methyl ester) composition of the peripheral blood mononuclear cells obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-vlaue*
	Saturated	Unsaturated		
C14:0	3.41	4.03	1.41	0.71
C14:1	0.54	1.29	0.42	0.14
C15:0	0.48	0.60	0.07	0.20
C15:1	0.29	1.75	0.75	0.11
C16:0	29.21	28.92	2.96	0.94
C16:1	0.32	0.52	0.23	0.45
C17:0	0.46	0.61	0.12	0.31
C17:1	1.52	0.63	0.61	0.21
C18:0	18.58	19.83	1.19	0.45
C18:1 TRANS	0.66	1.24	0.53	0.35
C18:1 CIS	16.60	13.46	1.78	0.19
C18:2 TRANS	0.13	0.15	0.09	0.82
C18:2 CIS	9.54	8.30	2.21	0.61
C18:3 N6	0.07	0.27	0.16	0.28
C20:0	0.24	0.48	0.17	0.21
C18:3 N3	0.99	3.97	0.65	<0.01
C20:1	0.57	0.92	0.13	0.13
C21:0	0.21	0.18	0.06	0.67
C20:2	0.42	0.50	0.13	0.67
C22:0	0.97	0.48	0.32	0.20
C20:3 N6	0.26	0.45	0.12	0.19
C20:3 N3	5.49	2.70	3.21	0.44
C22:1	0.16	0.20	0.07	0.65
C20:4	4.94	1.49	2.58	0.25
C23:0	0.11	0.25	0.15	0.42
C22:2	0.12	0.05	0.05	0.27
C20:5	0.25	1.21	0.25	0.01
C24:0	0.09	1.39	1.25	0.35
C24:1	1.16	1.73	3.44	0.71
C22:6	0.65	0.42	0.19	0.35

Table 5.2. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of the peripheral blood mononuclear cells obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-vlaue*
	Saturated	Unsaturated		
Σ MUFA	21.83	21.74	2.41	0.97
Σ N6	15.48	11.20	3.52	0.29
Σ N3	7.37	8.31	3.23	0.80
N6:N3	4.48	1.58	1.34	0.08
Σ PUFA	22.85	19.51	5.29	0.57
De novo	3.75	4.69	1.62	0.60
Σ Unsaturated	44.68	41.25	4.21	0.50
Σ Saturated	55.32	58.75	4.21	0.50
Unsat/Sat Ratio	0.86	0.72	0.13	0.39

Table 6.1. Fatty acid (g/100 g of fatty acid methyl ester) composition of the polymorphonuclear neutrophil obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
C14:0	0.95	1.26	0.47	0.59
C14:1	0.51	0.24	0.25	0.35
C15:0	0.36	0.30	0.08	0.49
C15:1	0.57	0.16	0.50	0.45
C16:0	24.85	22.51	1.65	0.31
C16:1	0.32	0.53	0.11	0.13
C17:0	0.40	0.44	0.04	0.43
C17:1	0.51	0.49	0.0	0.72
C18:0	20.83	21.16	1.18	0.85
C18:1 TRANS	0.98	0.81	0.18	0.50
C18:1 CIS	12.88	11.26	1.06	0.25
C18:2 TRANS	0.07	0.19	0.06	0.13
C18:2 CIS	16.50	16.81	2.90	0.93
C18:3 N6	0.03	0.02	0.03	0.85
C20:0	0.25	0.28	0.02	0.18
C18:3 N3	0.41	6.59	0.73	<0.01
C20:1	0.53	1.19	0.11	<0.01
C21:0	0.03	0.36	0.23	0.21
C20:2	0.40	1.19	0.51	0.18
C22:0	1.45	0.73	0.33	0.08
C20:3 N6	0.65	0.50	0.32	0.70
C20:3 N3	10.58	4.93	2.14	0.05
C22:1	0.14	0.11	0.05	0.75
C20:4	2.07	1.38	2.01	0.76
C23:0	0.07	0.31	0.09	0.03
C22:2	-	0.38	0.38	0.36
C20:5	0.35	1.23	0.32	0.04
C24:0	0.09	1.25	1.10	0.34
C24:1	1.23	0.83	0.53	0.50
C22:6	0.74	0.63	0.16	0.55

Table 6.2. Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of the polymorphonuclear neutrophil obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated) or flax seed oil mixed with conjugated linoleic acid (Unsaturated) for 50 days, starting at 3 days of age (n = 6 per treatment).

Fatty acid (g/100 g)	Treatment		SEM	*P-value*
	Saturated	Unsaturated		
Σ MUFA	17.66	15.63	0.93	0.13
Σ N6	17.70	20.46	2.27	0.40
Σ N3	14.10	13.37	1.98	0.73
N6:N3	1.26	1.70	0.30	0.23
Σ PUFA	31.80	33.83	3.40	0.63
De novo	1.04	1.95	0.91	0.37
Σ Unsaturated	49.46	49.46	3.17	1.00
Σ Saturated	50.54	50.54	3.17	1.00
Unsat/Sat Ratio	1.01	1.01	0.11	0.97

Table 7 Taqman® gene expression assay bovine primer/probe sets used for real-time polymerase chain reactions.

Gene[1]	Assay Identification	Reference Sequence
IL-6	Bt03211905_m1	NM_173923.2
IL-1β	Bt03212745_m1	NM_174093.1
TNF-α	Bt03259156_m1	NM_173966.3
ICAM-1	Bt03213910_g1	NM_174348.2
IL-8	Bt03211906_m1	NM_173925.2
RBP4	Bt03252073_m1	NM_001040475.2
L-selectin	Bt03223211_m1	NM_174182.1
NF-κB	Bt03243457_m1	NM_001076409.1
PPAR-γ	Bt03217547_m1	NM_181024.2
RPS9	Bt03272017_m1	NM_001101152.2
GAPDH	Bt03210913_g1	NM_001034034.2

[1] IL-6: interleukin-6, IL-1β: interleukin-1β, TNF-α: tumor necrosis factor-α, ICAM-1: intercellular adhesion molecule-1, IL-8: interleukin-8, RBP-4: retinol binding protein-4, NF-κB: nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, PPAR-γ: peroxisome proliferator activated receptor-γ, RPS9: Ribosomal protein S9, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Table 8. Delta Ct value ($Ct_{target\ gene}$-$Ct_{housekeeping\ gene}$) of interleukin-6 (IL-6), IL-1β, tumor necrosis factor-α (TNF-α) and intracellular adhesion molecule (ICAM) of the peripheral blood mononuclear cells obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated; SFA) or flax seed oil mixed with conjugated linoleic acid (Unsaturated; UFA) for 50 days, starting at 3 days of age (n = 6 per treatment).

Gene	Treatment		*P-value*
	SFA	UFA	
IL-6	15.51 ± 0.50	15.00 ± 0.61	0.52
IL-1β	6.60 ± 0.43	6.80 ± 0.58	0.81
TNF-α	5.77 ± 0.16	5.51 ± 0.17	0.26
ICAM-1	7.66 ± 0.20	7.94 ± 0.27	0.42

Table 9. Delta Ct value ($Ct_{target\ gene}$-$Ct_{housekeeping\ gene}$) of caspase-1 (CASP), interleukin-8 receptor (IL-8R) and L-selectin (SEL) of the polymorphonuclear neutrophil obtained from Holstein dairy calves fed milk containing either palm fruit oil (saturated; SFA) or flax seed oil mixed with conjugated linoleic acid (Unsaturated; UFA) for 50 days, starting at 3 days of age (n = 6 per treatment).

Gene	Treatment		*P-value*
	SFA	UFA	
CASP-1	7.60 ± 0.33	7.12 ± 0.15	0.20
IL-8R	1.33 ± 0.27	1.97 ± 0.61	0.35
L-SEL	2.49 ± 0.18	2.06 ± 0.06	0.04

Table 10. Delta Ct value (Ct $_{\text{target gene}}$-Ct $_{\text{housekeeping gene}}$) of interleukin-1β (IL-1β), IL-6, IL-8, IL-10, IL-12, interferon-γ (IFN-γ), peroxisome proliferator activated receptor-γ (PPAR-γ), tumor necrosis factor-α (TNF-α), retinol binding protein-4 (RBP-4), and nuclear factor-κB (NF-κB) of the liver tissue obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated; SFA) or flax seed oil mixed with conjugated linoleic acid (Unsaturated; UFA) for 50 days, starting at 3 days of age (n = 6 per treatment).

Gene	Treatment		*P-value*
	SFA	UFA	
IL-1β	8.43 ± 0.16	8.30 ± 0.70	0.86
IL-6	17.74 ± 0.55	17.27 ± 0.28	0.43
IL-8	13.48 ± 0.22	13.50 ± 0.38	0.96
IL-10	9.13 ± 0.56	8.59 ± 0.61	0.53
IL-12	4.66 ± 0.74	5.68 ± 1.25	0.50
IFN-γ	13.01 ± 0.39	13.59 ± 0.32	0.27
PPAR-γ	9.17 ± 0.27	8.84 ± 0.27	0.40
TNF-α	8.47 ± 0.23	8.72 ± 0.39	0.60
RBP4	-4.00 ± 0.24	-3.71 ± 0.38	0.52
NF-κB	5.69 ± 0.18	6.20 ± 0.58	0.44

Table 11. Delta Ct value (Ct $_{\text{target gene}}$-Ct $_{\text{housekeeping gene}}$) of interleukin-1β (IL-1β), IL-6, IL-8, IL-10, IL-12, interferon-γ (IFN-γ), peroxisome proliferator activated receptor-γ (PPAR-γ), tumor necrosis factor-α (TNF-α), retinol binding protein-4 (RBP-4), and nuclear factor-κB (NF-κB) of the adipose tissue obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated; SFA) or flax seed oil mixed with conjugated linoleic acid (Unsaturated; UFA) for 50 days, starting at 3 days of age (n = 6 per treatment).

Gene	Treatment		*P-value*
	SFA	UFA	
IL-1β	12.88 ± 0.32	12.60 ± 0.28	0.53
IL-6	16.77 ± 0.58	15.76 ± 0.39	0.20
IL-8	15.03 ± 0.34	15.29 ± 0.74	0.75
IL-10	11.07 ± 0.38	11.06 ± 0.34	0.99
IL-12	8.14 ± 1.03	7.87 ± 0.89	0.85
IFN-γ	12.50 ± 1.54	12.97 ± 0.94	0.61
PPAR-γ	3.27 ± 0.66	2.94 ± 0.57	0.68
TNF-α	11.83 ± 2.75	10.33 ± 0.57	0.25
RBP4	0.94 ± 0.65	-0.23 ± 0.34	0.17
NF-κB	7.36 ± 1.51	6.75 ± 0.17	0.38

Table 12. Means (± SEm) of α-tocopherol, retinol and β-carotene content of the palm fruit oil, flax seed oil, conjugated linoleic acid and starter meal.

(ng/mL)	Treatment			
	Palm	Flax seed	Lutalin®	Purina® Starter[1]
α-Tocopherol	398.4 ± 23.2	9320.6 ± 24.8	N.D.	4910.1 ± 775.4
Retinol	6.1 ± 1.7	64.6 ± 3.3	300.3 ± 4.9	2105.2 ± 302.4
β-carotene	N.D	20.2 ± 0.6	N.D.	1222.5 ± 113.0

N.D. : not detected

[1] Content presented in ng/g of DM

Table 13. Concentration of α-tocopherol and retinol (ng/mL) of the liver tissue obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated; SFA) or flax seed oil mixed with conjugated linoleic acid (Unsaturated; UFA) for 50 days, starting at 3 days of age (n = 6 per treatment).

(ng/mL)	Treatment		*P-value*
	SFA	UFA	
α-Tocopherol	1816.8 ± 245.7	1280.6 ± 191.4	0.09
Retinol	411.3 ± 30.1	339.6 ± 41.1	0.17

Table 14. Concentration of α-Tocopherol, retinol and β-carotene (ng/mL) of the serum obtained from Holstein dairy calves fed milk containing either palm fruit oil (Saturated; SFA) or flax seed oil mixed with conjugated linoleic acid (Unsaturated; UFA) for 50 days, starting at 3 days of age (n = 6 per treatment).

(ng/mL)	Treatment		*P-value*
	SFA	UFA	
α-Tocopherol	1964.3 ±72.0	1552.1 ±99.7	<0.01
Retinol	147.1 ±7.6	105.7±6.9	<0.01
β-carotene	89.1±4.6	62.6±2.9	<0.01

Figures

Figure 1. The effects of dietary fat saturated (closed bar) vs. unsaturated (opened bar) consumed by Holstein dairy calves (3 d old) for a 50 day period on α-linolenic acid content of various lipid fractions (non-esterified fatty acid: NEFA, neutral lipid: NL and phospholipid: PL) of adipose and hepatic tissue, and serum (** represents $P < 0.01$)

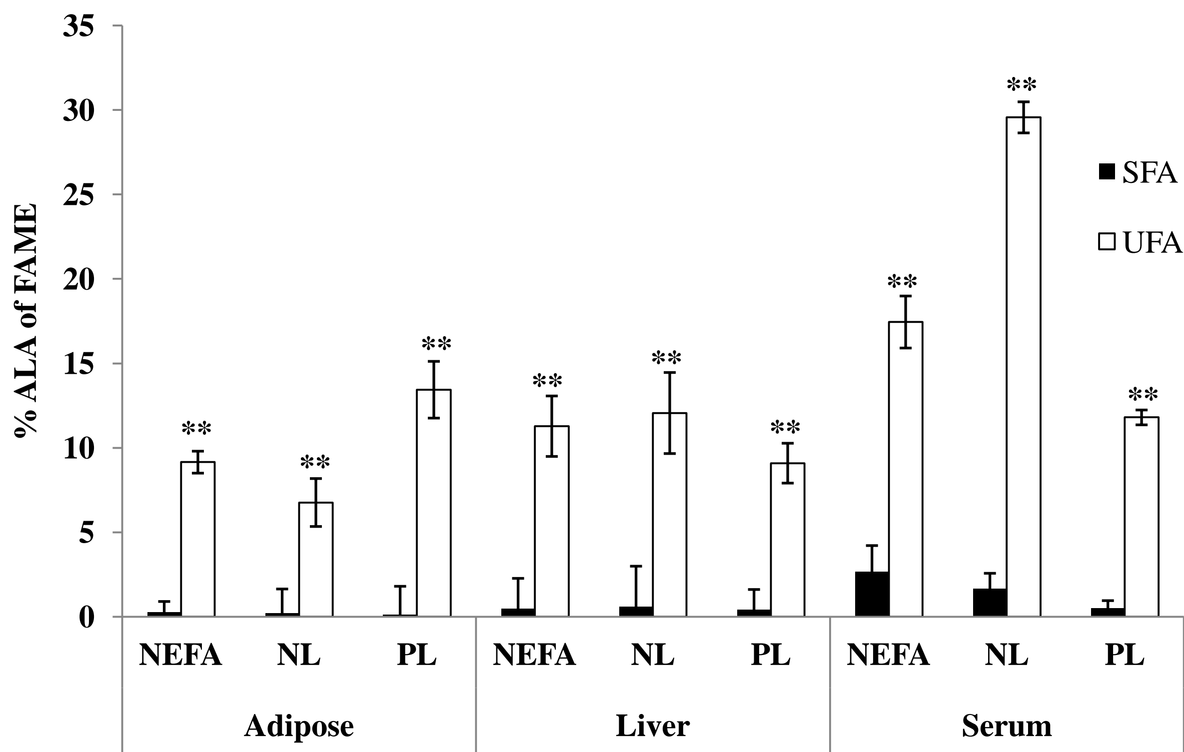


Figure 2. The effects of dietary fat saturated (closed bar) vs. unsaturated (opened bar) consumed by Holstein dairy calves (3 d old) for a 50 day period on α-linolenic acid content of peripheral blood mononuclear cells and polymorphonuclear neutrophil (** represents $P < 0.01$)

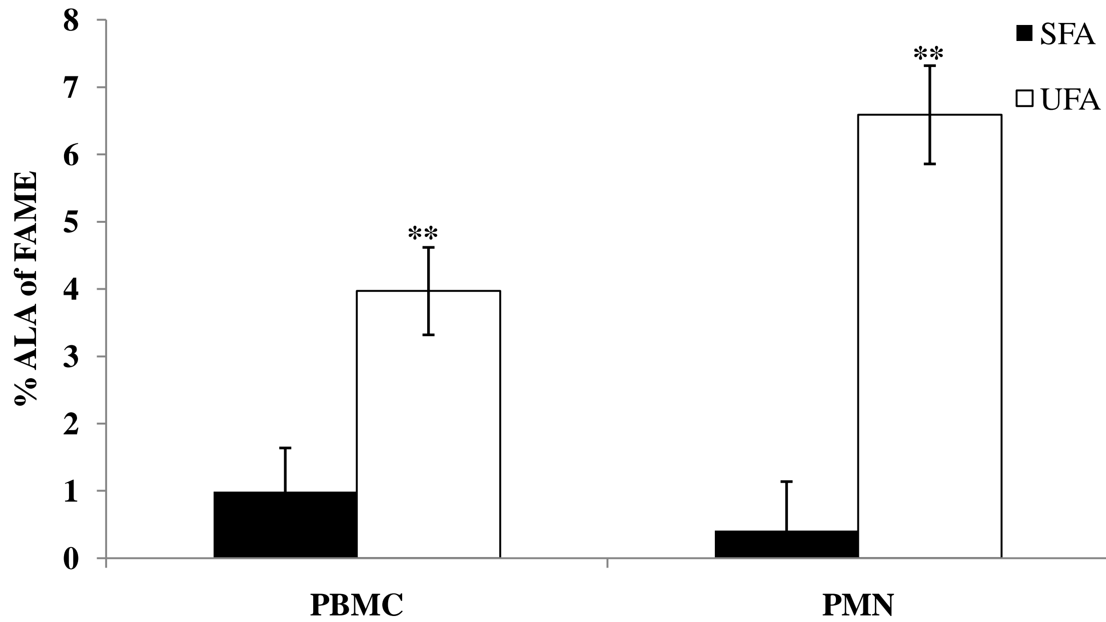


Figure 3. The effects of dietary fat saturated (closed bar) vs. unsaturated (opened bar) consumed by Holstein dairy calves (3 d old) for a 50 day period on eicosapentaenoic acid content of various lipid fractions (non-esterified fatty acid: NEFA, neutral lipid: NL and phospholipid: PL) of adipose tissue and serum (** represents $P < 0.01$; * represents $P < 0.05$)

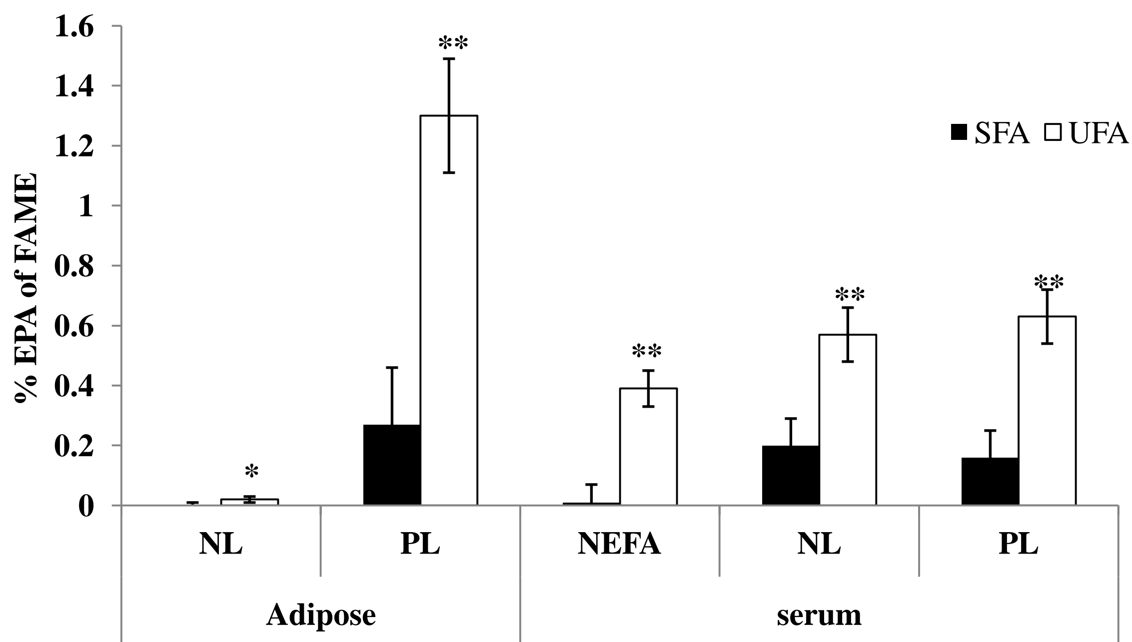


Figure 4. The effects of dietary fat saturated (closed bar) vs. unsaturated (opened bar) consumed by Holstein dairy calves (3 d old) for a 50 day period on eicosapentaenoic acid content of peripheral blood mononuclear cells and polymorphonuclear neutrophils. (* represents $P < 0.05$)

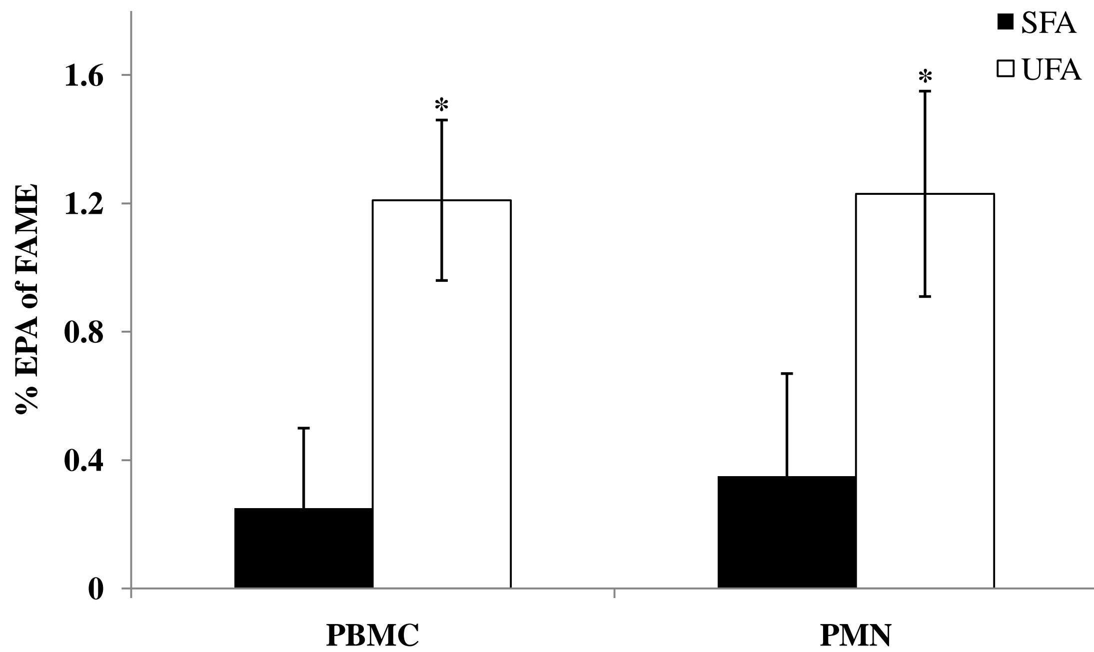


Figure 5. The effects of dietary fat saturated (closed bar) vs. unsaturated (opened bar) consumed by Holstein dairy calves (3 d old) for a 50 day period on polyunsaturated fatty acid content of various lipid fractions (non-esterified fatty acid: NEFA, neutral lipid: NL and phospholipid: PL) of adipose tissue and serum (** represents $P < 0.01$)

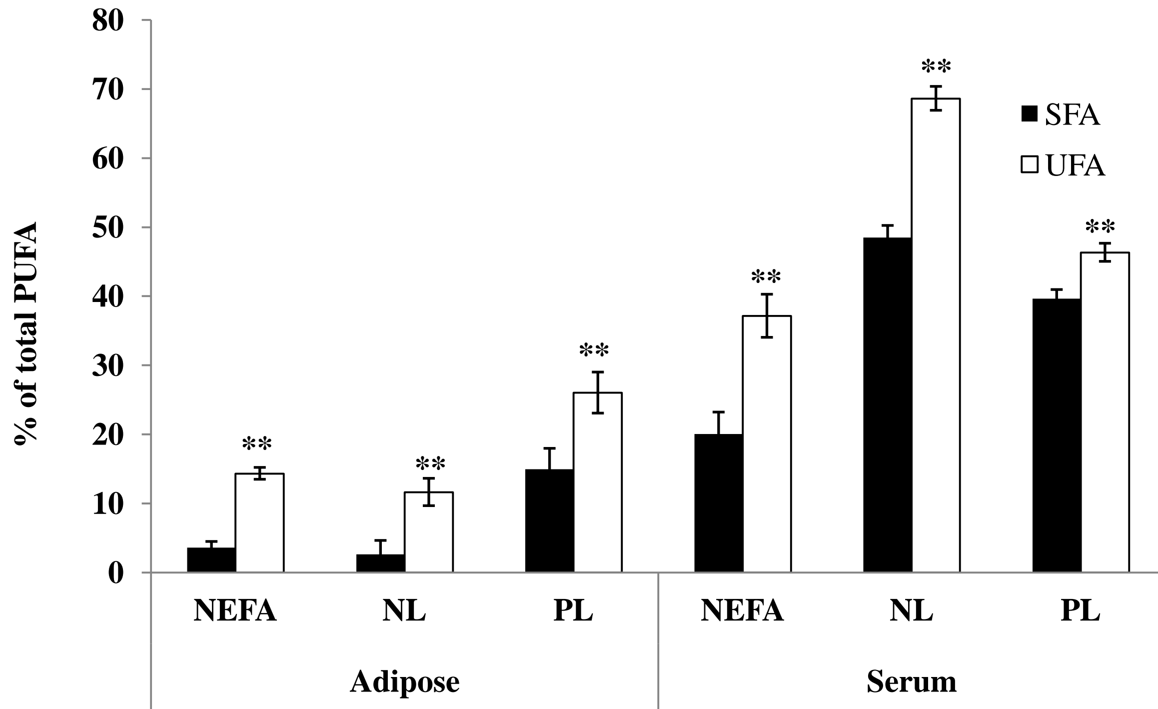


Figure 6. The effects of dietary fat saturated (closed bar) vs. unsaturated (opened bar) consumed by Holstein dairy calves (3 d old) for a 50 day period on γ-linolenic acid content of various lipid fractions (non-esterified fatty acid: NEFA, neutral lipid: NL and phospholipid: PL) of hepatic tissue and serum (** represents $P < 0.01$; * represents $P < 0.05$)

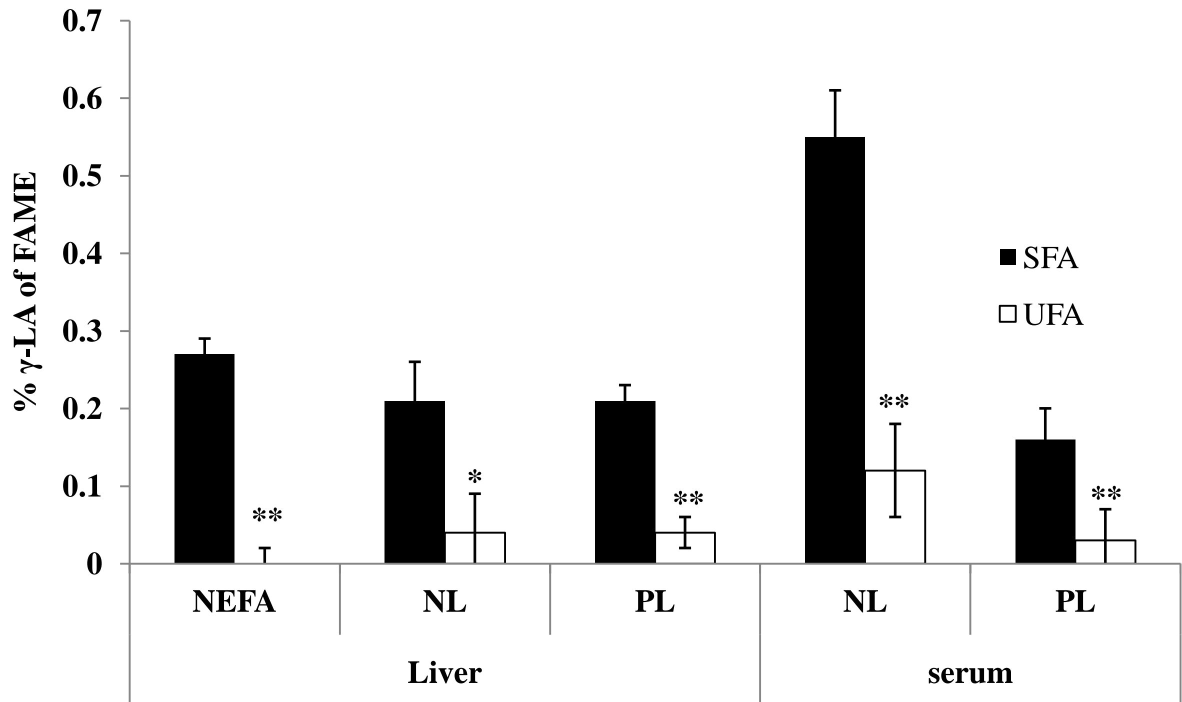


Figure 7. The effects of dietary fat saturated (closed bar) vs. unsaturated (opened bar) consumed by Holstein dairy calves (3 d old) for a 50 day period on arachidonic acid content of various lipid fractions (non-esterified fatty acid: NEFA, neutral lipid: NL and phospholipid: PL) of adipose and hepatic tissue, and serum (** represents $P < 0.01$; * represents $P < 0.05$)

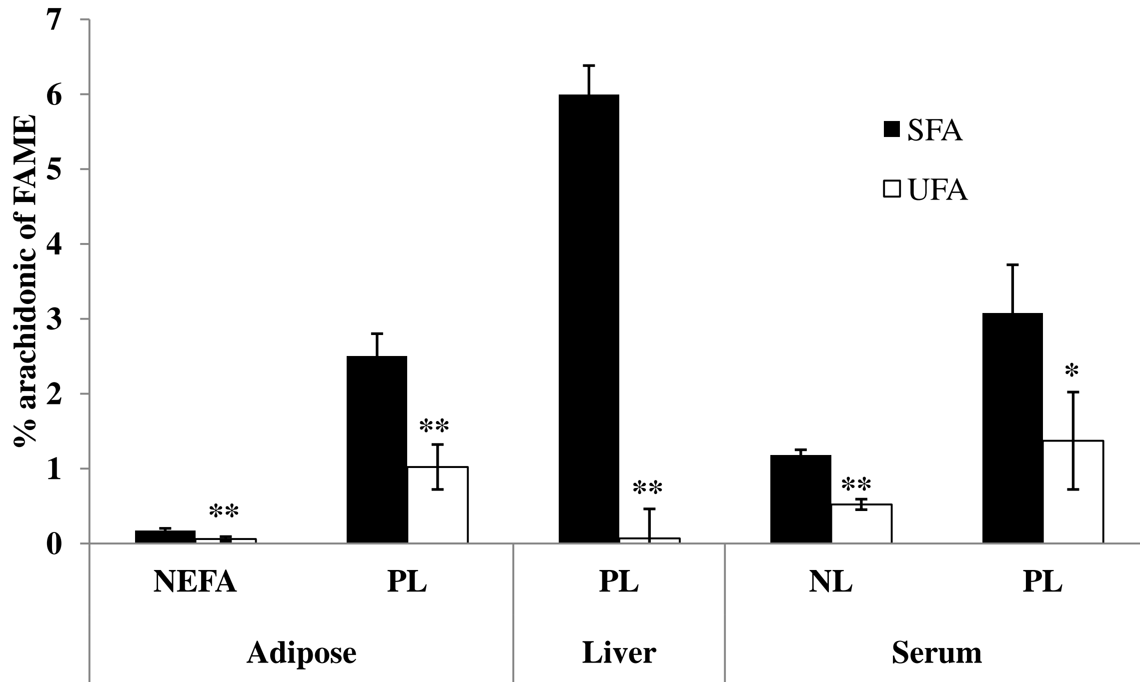


Figure 8. The Fold change ($2^{-\Delta\Delta Ct}$ method) of L-selectin (SEL) in the polymorphonuclear neutrophil obtained from Holstein dairy calves dietary fat saturated (closed bar) vs. unsaturated (opened bar) consumed by Holstein dairy calves (3 d old) for a 50 day period (n = 6 per treatment)

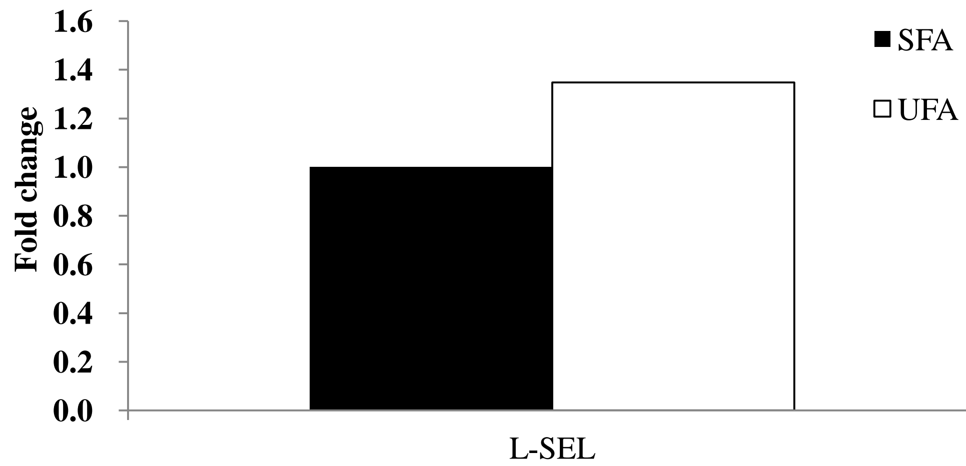
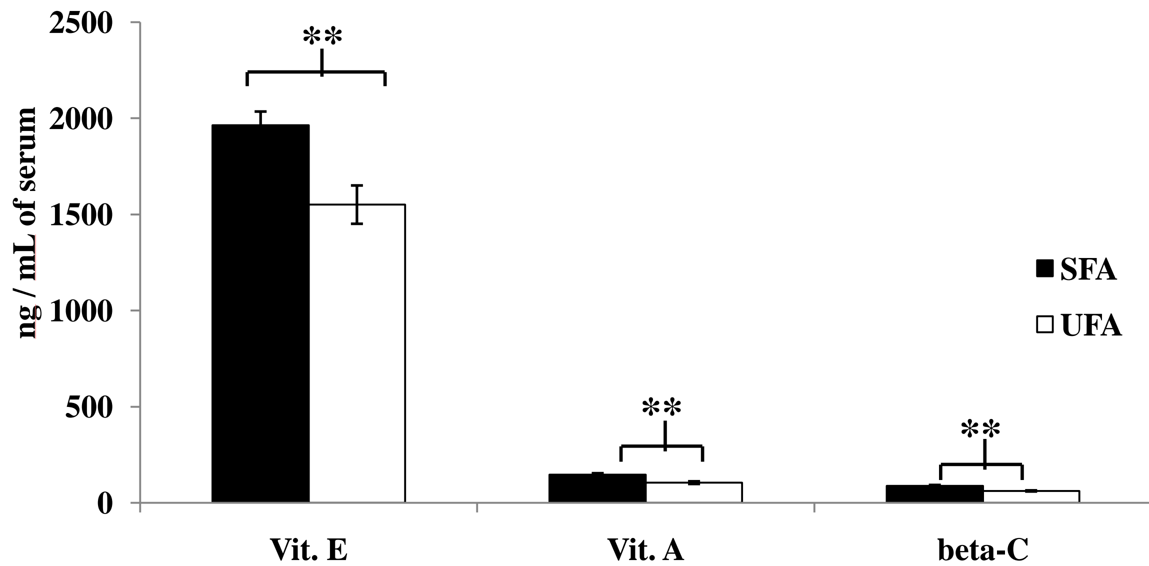


Figure 9. Concentration of α-tocopherol, β-carotene and retinol (ng/mL) of the serum obtained from Holstein dairy calves dietary fat saturated (closed bar) vs. unsaturated (opened bar) consumed by Holstein dairy calves (3 d old) for a 50 day period (n = 6 per treatment) (** represents $P < 0.01$)



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Appendix

University of Idaho
Institutional Animal Care and Use Committee

Date: Thursday, September 12, 2013
To: Matthew Doumit
From: University of Idaho
Institutional Animal Care and Use Committee
Re: Protocol 2013-77
Will feeding supplemental polyunsaturated fatty acids (PUFAs) to pre-ruminating calves to preferentially stimulate development of marbling?

Your requested amendment to the animal care and use protocol shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Thursday, September 12, 2013.

This protocol was originally submitted for review on: Tuesday, June 11, 2013
The original approval date for this protocol is: Tuesday, July 16, 2013
This approval will remain in affect until: Friday, September 12, 2014
The protocol may be continued by annual updates until: Saturday, July 16, 2016

Comments

Your request to provide tissues from dead calves to other investigators (Drewnoski, Rezamand, Williams, Wilmore) has been approved.

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Brad Williams, DVM
Campus Veterinarian
University of Idaho
208-885-8958