ABSTRACT

Title of Dissertation:	THE EFFECT OF FEEDING MIXED TOCOPHEROL OIL ON BODY ACCUMULATION AND IMMUNE CELL FUNCTIONS IN LACTATING HOLSTEIN DAIRY COWS
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Non-α-tocopherol (i.e. β, γ, and δ-tocopherol) supplements are as important as α-tocopherol with regard to maintain lactating dairy cow health. However, information on non-α-tocopherol bioaccumulation and its effects on immune cell function is not available. A series of experiments were conducted to investigate the effect of mixed tocopherol oil supplement (Tmix; i.e. α, β, γ, and δ-tocopherol) on body accumulation and immune cell functions of the lactating Holstein dairy cow. Tissue, tissue mitochondria, blood and milk were collected from lactating dairy cows to measure the concentration of all four tocopherol isoforms via Tmix supplementation. In addition, polymorphonuclear leukocyte (PMN) were isolated to investigate the effect of Tmix on its function and immune gene expression. In the first experiment, Tmix increased γ-

tocopherol concentrations but did not increase the α-tocopherol concentration in three different types of tissues (i.e. liver, mammary gland and muscle) and liver mitochondria. Within those three different types of tissues, liver showed the highest ability to store tocopherol isoforms (i.e. α- and γ-tocopherol) compared to the other two tissues. Also, amounts of α- and γ-tocopherol were detected in liver mitochondria, but limited amounts were detected in mammary gland mitochondria. In the following experiment, Tmix increased γ-tocopherol concentration in milk and blood as determined via every 8 h and daily measurements. Compared to γ-tocopherol, α-tocopherol showed the highest concentration of the tocopherol isoforms in milk and blood. Limited quantities of β- and δ-tocopherol were detected in milk and blood via Tmix supplementation. In the last experiment, Tmix increased PMN chemotaxis function and did not impair the whole blood respiratory burst response of dairy cows, which might be associated with non-α-tocopherol existing in Tmix. Even though Tmix increased the expression of pro-inflammatory genes in PMN, those are needed during the initial immune activation. Overall, the results of the experiments demonstrated that short-term supplementation with Tmix could compensate γ-tocopherol without altering α-tocopherol in dairy cows. The liver showed the highest capability of accumulating tocopherol isoforms compared to the mammary gland and muscle. In addition, Tmix did not harm immune functions or have any apparent effects on animal health in lactating Holstein dairy cows.

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ACCUMULATION AND IMMUNE CELL FUNCTIONS IN LACTATING
HOLSTEIN DAIRY COWS

by

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LIST OF ABBREVIATIONS

α-TTP: α-tocopherol transfer protein
α-CEHC: 2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman
γ-CEHC: 2,7,8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman
ABC: ATP-binding cassette
ABCA1: ATP-binding cassette transporter A1
AEBSF: 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
AIC: Akaike information criterion
ApoB: Apolipoprotein B-100
ATP: Adenosine triphosphate
BCS: Body condition score
BW: Body weight
BLT-1: Block lipid transport
BHBA: Beta-hydroxybutyric acid
CM: Chylomicrons
CMR: Chylomicron remnants
Ca: Calcium
Cl: Chloride
CaCl$_2$: Calcium dichloride
CO$_3^-$: Carbonate
Complex I: NADH-Q oxidoreductase
Complex III: Q-cytochrome c oxidoreductase
Complex IV: cytochrome c oxidase
COX-4: cytochrome c oxidase
CK: Creatine kinase
CYP4F2: Hepatic cytochrome P450 family 4 subunit 2
CYP3A: Cytochrome P450 family 3
Cyt c: cytochrome c
CXCR: Chemokine receptors
DMI: Dry matter intake
DIM: Days in milk
DM: Dry matter
DNA: Deoxyribonucleic acid
DHA: Docosahexaenoic
DHAR: Dehydroascorbate reductase
dL: deciliter
E$^-$: Vitamin E radical
Eq: Equivalent
FADH$_2$: Flavin adenine dinucleotide
GGT: Gamma-glutamyl transpeptidase
GPX: Glutathione peroxidase
GR: Glutathione reductase
GSH: Glutathione
GSSG: Glutathione disulfide
H$^+$: Protons
HCO$_3^-$: bicarbonate

HClO: Hypochlorous acid
HDL: High density lipoprotein
H_2O_2: Hydrogen peroxide
HPLC: High-performance liquid chromatography
HRP: Horseradish peroxidase
IDL: Intermediate density lipoprotein
IFN-β: Interferon beta-1
IL-6: Interleukin 6
IL-8: Interleukin 8
IgG: Immunoglobulin G
IRAK-1: Interleukin-1 receptor associated kinase 1
IRAK-4: Interleukin-4 associated kinase 4
IM: Intramuscular injection
IV: Intravenous infusion
IU: International units
JAK-2: Janus kinase 2
K: Potassium
KDa: Kilodalton
Kg: Kilogram
L: Liter
LCAT: Lecithin:cholesterol acyltransferase
LDA: Left displaced abomasum
LDL: Low density lipoprotein
LH: Lipid hydroperoxide
LPS: Lipopolysaccharide
LO_2: Peroxyl radical
LOOH: lipid hydroperoxide
LSM: Least square means
M: Mol
μ*M*: Micromolar
Mg: Magnesium
MyD-88: Myeloid differentiation primary response protein 88
NO_2: Nitrogen dioxide
μEq: Microequivalent
mg: Milligram
μg: Microgram
mL: Milliliter
ng: Nanogram
nm: Nanometer
n*M*: Nanomolar
μL: Microliter
NADPH: Reduced nicotinamide adenine dinucleotide phosphate
NEFA: Non-esterified fatty acids
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NGT: Nitrated γ-tocopherol
NPC1L1: Niemann-pick C1-like 1
NRC: National research council
·NO: Nitric oxide

O₂·⁻: Superoxide
·OH: Hydroxyl radical
ONOO·: Peroxynitrite
P: Phosphorus
pg: Picogram
PAMA: pathogen-associated molecular pattern
PLTP: Phospholipid transfer protein
PMA: Phorbol 12-myristate 12-acetate
PMN: Polymorphonuclear leukocytes
PPR: Pattern recognition receptors
PUFA: Poly-unsaturated fatty acids
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SAA: Serum amyloid A
SCC: Somatic cell count
SOD: Superoxide dismutase
SRB-1: Scavenger receptor B-1
SC: Subcutaneous injection
TAG: Triglyceride
THF: Tetrahydrofuran
TLR: Toll-like receptor
TMR: Total mixed ration
Tmix: Mixed tocopherol oil supplement
TNF-α: Tumor necrosis factor alpha
TRAM: TRIF-related adaptor molecule
TRAF-6: TNF receptor-associated factor 6
TRIF: TLR-domain-containing adaptor protein
TTPA: α-tocopherol transfer protein
VLDL: Very low density lipoprotein

CHAPTER 1: LITERATURE REVIEW

Animals will encounter physiological stress from time to time. For dairy cows, the high-risk period for disease and/or disorders is during the transition period, ± 3 weeks relative to parturition (Drackley, 1999). At this time, dairy cows may suffer from other metabolic disorders (e.g. fatty liver and retained placenta) (Grummer, 1993) or infectious diseases (e.g. mastitis and metritis,) (Eckel and Ametaj, 2016). These diseases are not only detrimental to the producer (i.e. economic loss), but also to animal health (Weiss, 1998). Within those two (metabolic disorders and infectious diseases), metabolic disorders are considered as a predisposing factor to infectious diseases (Bobe et al., 2004). Therefore, disrupted normal metabolic pathways need to be reversed to mitigate transition period issues (i.e. high diseases frequency) in dairy cows. How can we minimize the negative consequences of disease at this time? Before we can answer this question, we have to understand what could be the potential reasons causing the metabolic disorders and where we should focus in dairy cows during the stressful transition period.

A recent review paper has shown us that liver is the particular place where disrupted metabolic processes happen, which can lead to clinical diseases in dairy cows (Bobe et al., 2004). The disrupted metabolic process is partly explained by interrupted cell signal transduction pathways. For example, insulin resistance could be partly explained by the disruption of insulin-sensing pathways in dairy cows during the transition period (Sah et al., 2016). Standard insulin receptor (i.e. receptor tyrosine kinase) activation requires the auto-phosphorylation of various tyrosine residues after binding with insulin that can lead to insulin-sensing pathway activation (Bergan-Roller et

al., 2017). What could interrupt the above pathway resulting in insulin sensing fails?
Recent study has shown that the Janus kinase 2 (i.e. non-receptor tyrosine kinase), which
is activated by phosphorylation in the production of insulin-like growth factor 1 signal
transduction pathways, could be attacked at phosphorylation activation sites, and lose
activity by binding reactive nitrogen species (RNS; e.g. peroxynitrate): common stress
mediator sensed by cells during infection and during periods associated with high stress
(Elsasser et al., 2013). In beef cattle, this event is called tyrosine nitration during
inflammatory stress (Elsasser et al., 2012). Therefore, we suggest that RNS could also
attack the normal metabolic molecules (e.g. insulin receptor) during inflammatory stress,
which is common in the transition period in dairy cows (Drackely, 1999). Besides the
insulin receptor signal transduction pathway, number of other key enzymes involved
metabolic signal transduction pathways (e.g. mitogen-activated protein kinase, protein
kinase B, etc.) are disrupted during metabolic diseases that might result from tyrosine
nitration by RNS (Li et al., 2007). Therefore, we propose that we can reestablish the
normal cell signal transduction pathways or lessen the disrupted level by applying
nutrient supplement strategies that target the reduction of reactive nitrogen molecules.
Then the next question we would like to ask is 'what nutrient compound could prevent
the tyrosine nitration?' A potential solution is to supplement γ-tocopherol, an isoform of
vitamin E. Recent study has shown that γ-tocopherol could bind with RNS before those
species react with tyrosine in metabolic molecules involved with tyrosine kinase enzymes
(Hoglen et al., 1997). Alpha-tocopherol shows very limited ability to react with RNS due
to the three methyl groups located on its phenol rings (Conney et al., 1993). Therefore,

supplementing dietary γ-tocopherol to dairy cows may help animals pass through the stressful transition period.

There is limited information for dairy cows regarding the bioaccumulation and concentration of γ- or other non-α-tocopherol (i.e. β- and δ-). It is important to know how much of the different isoforms are distributed in tissues, blood or milk. If a cow's body does not absorb the γ- or other non-α-tocopherol past the liver and into blood or milk, then there is little point of focusing on the liver to mitigating the metabolic perturbations by using non-α-tocopherol. Therefore, this project investigated the effects of Tmix (i.e. mixed tocopherol oil supplement; 9 % α-, 1 % β-, 24 % δ-, and 62 % γ-tocopherol) on body accumulation and immune health in dairy cows. There were three objectives of this study 1) to investigate the accumulation and distribution levels of all four tocopherol isoforms in tissue and mitochondria; 2) to investigate the change of all four tocopherol isoforms (α-, β-, γ- , and δ-tocopherol) in blood and milk over time; and 3) to investigate the effects of all four tocopherol isoforms on bovine polymorphonuclear leukocytes (PMN) function and immunometabolic-related gene expression via Tmix supplementation in dairy cows.

Chemical Isoforms of Vitamin E

Vitamin E is a group of compounds named tocochromanols (Traber and Atkinson, 2007). Tocochromanols consist of a chromanol ring system called the head region, and a hydrophobic prenyl side chain called the tail region. Tocochromanols are divided into two groups, tocopherols and tocotrienols, which differ in the saturation of their 12-carbon lipid tails (saturated in tocopherols versus three double bonds in tocotrienols; Figure 1.1). Tocopherols and tocotrienols can exist in four different forms, α-, β-, γ-, and δ-, which

differ in the number and position of methyl groups on the chromanol head group (Figure 1.1). Compared to tocotrienols, tocopherol has been used more commonly in the animal industry as a vitamin supplement. There are two different types of tocopherols available commercially which are natural tocopherol (i.e. d-tocopherol) and synthetic tocopherol (i.e. dl-tocopherol). For natural tocopherol, it only contains the RRR stereoisomer based on the position of the methyl group in C-2' of the heterocyclic ring and C-4' and C-8' in the carbon tail. For synthetic tocopherol, it includes all eight stereoisomers (i.e. RRR, RSR, RSS, RRS, SRR, SSR, SRS, and SSS) based on the position of methyl groups (i.e. either left "S" or right "R") in C-2' of the heterocyclic ring and C-4' and C-8' in the carbon tail and is called *all-rac*-tocopherol or *dl*-tocopherol (Traber, 2004). Acetate and succinate derivatives of the natural tocopherol have vitamin activity, which are called esterified tocopherols, and the purpose of the derivatives was to extend shelf-life (Traber, 2004). Overall, vitamin E supplementation can be classified as either tocopherols or tocotrienols, and each one of them includes the α-, β-, γ-, or δ- isoform. The resources and requirements of the different tocopherols will be discussed in later sections.

The Roles of Vitamin E in Animals

Vitamin E as an Antioxidant

Vitamin E has been used in feed supplements for livestock for decades with dietary α-tocopherol acetate or succinate as the synthetic isoform most commonly employed in National Research Council (NRC, 2001). Previous studies in dairy cows have shown that 7 μM of vitamin E (i.e. α-tocopherol) is the average cut-off value in serum, inadequate vitamin E (i.e. α-tocopherol) concentration in serum (<7 μM) may partly explain the increased risk of metabolic disorders or infectious diseases in dairy

cows shortly after calving (Mudron et al., 1997; Weiss et al., 1997). Recent studies also

have shown that serum vitamin E (i.e. α-tocopherol) depletion (<7 μ*M*) during the

transition from later pregnancy to early lactation might contribute to the development of

metabolic disorders such as left displaced abomasum and retained placenta (Qu et al.,

2013; Qu et al., 2014) Vitamin E is considered a natural antioxidant via its role in

controlling damage to host cell membranes during lipid peroxidation (Niki and Traber,

2012). Lipid peroxidation (Figure 1.2) refers to when free radicals (e.g. hydroxyl radical

[$^\bullet$OH]) oxidize lipid membranes (e.g. poly-unsaturated fatty acids [PUFAs]) thereby

producing lipid radicals (L$^\bullet$) and H_2O. In an aerobic environment, L$^\bullet$ can oxidize one free

oxygen molecule, giving rise to a lipid peroxyl radical (LOO$^\bullet$). Due to the high reactive

properties, LOO$^\bullet$ can continue to oxidize PUFAs to produce lipid hydroperoxide (LOOH)

and an additional L$^\bullet$. This lipid peroxidation chain reaction occurs continuously causing

damage to the cell membrane and can lead to cell dysfunction (Serreli et al., 2017).

Vitamin E is a molecular antioxidant that can inhibit the lipid peroxidation chain

reaction by the following mechanism (Figure 1.3): vitamin E (i.e. α-tocopherol) donates

the hydrogen from the hydroxyl group of the phenolic ring to LOO$^\bullet$ producing a vitamin

E free radical (E·) and an LOOH. In addition to vitamin E, enzymes (i.e. glutathione

peroxidase [GPX]) also play essential roles in scavenging reactive oxygen species (ROS;

e.g. $O_2^\bullet$ and H_2O_2) in cells (Figure 1.4; (Miller et al., 1993). Superoxide dismutase (SOD)

is the enzyme involved in catalyzing the dismutation of $O_2^\bullet$ to H_2O_2. Hydrogen peroxide

could be reduced to H_2O in the presence of GPX via the oxidation of glutathione (GSH)

to glutathione disulfide (GSSG). Finally, oxidized GSSG can be reduced to GSH in the

presence of nicotinamide adenine dinucleotide phosphate reduced form (NADPH).

Although highly unstable, ROS are required by phagocytic cells for the killing of invading microorganisms during the immune response via respiratory burst (Paape et al., 2003) which will be discussed in more detail below. However, ROS can also cause damage to host cells via the following potential mechanisms: 1) lipid peroxidation of the cell membrane (Figure 1.2); 2) oxidation of proteins, an important mechanism of O_2^- toxicity that is the direct oxidation and inactivation of iron-sulfur proteins, such as aconitases, and the associated release of iron (Fridovich, 1997), and 3) damage of deoxyribonucleic acid (DNA) molecules. Reactive oxygen species could damage DNA molecules causing modification of the purine and pyrimidine base, the deoxyribose backbone, or single and double strand breaks (Gurler et al., 2016). Therefore, the role of vitamin E as an antioxidant is essential for host cell viability and to help control host cell damage by ROS.

Besides ROS, another common type of harmful free radical produced in animals is RNS (Rivera-Mancia et al., 2017). One common form of RNS is the peroxynitrate$^-$ anion, which is a short-lived oxidant species that is produced by the reaction of nitric oxide ($^.$NO) and O_2^- at diffusion-controlled rates at $\sim 1 \times 10^{10} M^{-1} s^{-1}$ (Figure 1.5). When standard physiology systems are exposed to peroxynitrate or $^.$NO, multiple biological effects can happen, with reversible effects on the viability and function of cells (Szabo et al., 2007). Previous study has shown that productions of RNS are associated with diseases/disorders associated with e.g. inflammation, parturition, and immune suppression (Fubini and Hubbard, 2003). Once RNS are produced, they could: 1) trigger cell death via mitochondria; 2) nitrate tyrosine residue in metabolic tyrosine kinase to disrupt the normal cell signal pathway; or 3) potentially disrupt membrane integrity as

well as cytosolic and nuclear receptors (Trachootham et al., 2008). Among the above

potential reversible effects via RNS, the nitration of protein tyrosine should be considered

because many tyrosine residue kinases are involved in cell signal transduction pathways

of normal physiological functions, like the growth hormone pathway and insulin receptor

pathway (Schlessinger, 2000).

Previous study using the peptide mapping method (Elsasser et al., 2012) have

shown that one or two tyrosine residues in a protein become preferentially nitrated

(Figure 1.6). Once the nitration of tyrosine residues occurs, the site nitrated with RNS

will not be available for natural cell signal transduction steps like phosphorylation needed

in tyrosine kinase activation (Radi, 2013). Besides the disruption of cell signal

transduction via tyrosine nitration, the nitrated tyrosine could also initiate the

polyubiquitination of nitrated proteins as a result of increased proteasome degradation or

initiate the autoimmune process (Elsasser et al., 2012). Therefore, compared to oxidative

stress, the nitration of protein tyrosine uncovers the disruption of nitric oxide (NO)

signaling and metabolism towards the pro-oxidant process, referred to as nitroxidative

stress in animals (Roberts et al., 2009). Compared to α-tocopherol, γ-tocopherol shows

more reactive ability with RNS due to its non-fully saturated phenol ring (Figure 1.1)

(Hoglen et al., 1997). Previous study has shown that γ-tocopherol could be nitrated by

peroxynitrate at C-5 of the heterocyclic ring (Figure 1.7) in tocopherol to form nitrated γ-

tocopherol (NGT) (Patel et al., 2007). Gamma-tocopherol could trap RNS before the

nitration of tyrosine occurs to serve as an antioxidant. Therefore, besides α-tocopherol,

γ-tocopherol may serve as an antioxidant supplement to manage nitroxidative stress in

dairy cows.

Vitamin E as an Immune-Enhancer

Besides a commonly used antioxidant, adequate vitamin E status plays a major role in immune function (Chew, 1995). Cells involved in the immune system are rich in PUFAs and utilize oxygen radicals to combat pathogens (Sanchez Perez et al., 2006). Cows with depleted vitamin E status have depressed PMN function (Willshire and Payne, 2011). There are several potential mechanisms by which depleted vitamin E may indirectly impair immune function: a) increased lipid peroxidation decreases function and survival of immune cells, b) increased lipid peroxidation induces proinflammatory gene expression, c) increased lipid peroxidation modulates signal transduction; and d) low vitamin E modulates cell membrane structure and, thereby, alters cell signaling and cell-to-cell communication (Chew, 1995). The relationship between vitamin E and immune cells, mainly focused on PMN, will be discussed in later sections (pp. 17–21)

Absorption and Transport of Vitamin E

Absorption of Vitamin E

There is limited information about the mechanism of vitamin E absorption in dairy cattle (Baldi, 2005; Bontempo et al., 2000). In humans, the absorption efficiency for vitamin E is low (15-45 %) and decreases further in the absence of adequate pancreatic function, bile secretion, triglyceride (TAG) and cholesterol (Traber and Arai, 1999). In lactating cows, the bioavailability for oil-based *all-rac* α-tocopherol is estimated to be 47 % (Bontempo et al., 2000). Figure 1.8 displays the steps of vitamin E absorption, starting with hydrolysis of the ester bond of lipids, including vitamin E esters, in the rumen and by pancreatic esterase in the duodenum (Frank, 2005; Toutain et al., 1995). As ruminants lack a duodenal oil phase, bile acids, adapted to the lower pH in

ruminants by a greater taurine content, act as detergent to emulsify vitamin E from the insoluble particulate phase. Next, vitamin E is incorporated into mixed micelles containing lipids and bile acids, which are then transported to the brush border membrane of the enterocytes for uptake (Goncalves et al., 2015).

Tocopherol is absorbed in the proximal small intestine in all species (Traber, 2014). The mechanism of tocopherol absorption is not completely understood. No intestinal tocopherol transfer proteins have been discovered. Thus, it was assumed that tocopherol is absorbed by passive diffusion. In *in vitro* intestinal model systems (Takada and Suzuki, 2010), the Niemann-Pick C1-Like 1 (NPC1L1) protein facilitates uptake of tocopherol and cholesterol (Narushima et al., 2008) by murine enterocytes. Also, the scavenger receptor B-1 (SRB-1) promotes tocopherol and cholesterol efflux by murine enterocytes (Reboul et al., 2006).

Vitamin E in Circulation

After being absorbed by enterocytes, tocopherols are either bound to SRB-1 and packaged into high density lipoprotein (HDL) complexes that are secreted into the blood (Anwar et al., 2007) or, in the presence of sufficient TAG and apolipoprotein B-100 (ApoB), tocopherols are packaged into chylomicrons and secreted into the lymph system (Frank, 2005; Hidiroglou et al., 1994). In circulation, vitamin E is transported with the lipoprotein fraction (Fairus et al., 2012); no particular tocopherol transport protein has been identified). This explains why dietary vitamin E concentrations are adjusted for serum lipids, specifically cholesterol levels. Lactating dairy cows have lower plasma/serum concentrations of α-tocopherol than humans (10-15 μM versus 20-40 μM). Most of the circulating lipids, including vitamin E, are in the HDL fraction (70-80 %) in

ruminants (Traber, 2013). To my knowledge, the half-life of vitamin E in circulation in lactating dairy cows has not been determined. The time until maximum vitamin E concentrations were obtained in blood is 57.5 h in dairy cows (Baldi, 2005). In humans, the half-life of *RRR*-α-tocopherol in circulation is between 48 h (Traber, 2004) and 60 h (Bruno et al., 2006). In contrast, *SRR*-α-tocopherol (Traber et al., 1994) and γ-tocopherol (Leonard et al., 2005) have a shorter half-life of 15 h. In the endothelial capillaries, lipoprotein lipase hydrolyzes lipid esters. Vitamin E is either a) taken up by target tissues through low density lipoprotein (LDL) receptors, b) taken up by tissue membranes, c) remains in chylomicron remnants, which are taken up by the liver, or d) is bound to HDL, which readily transfer vitamin E to other lipoproteins utilizing phospholipid transfer protein (PLTP) (Traber, 2013).

Tissue Distribution of Vitamin E

Knowledge about the movement of vitamin E within tissues is limited. Vitamin E remains in the lipid soluble fraction of the tissue. In the liver, to move α-tocopherol through the hydrophilic cytosol, α-tocopherol transfer protein (α-TTP), a 30 to 35 kDa, highly conserved, cytosolic, hydrophobic ligand binding protein is required, which moves α-tocopherol from liposomes to microsomes (Liu et al., 2014). The α-TTP is primarily expressed in the liver but also at lower levels in brain, lung, kidney, and spleen (Traber, 2013). An absence of α-TTP in humans results in vitamin E deficiency and tissue damage (Niki and Traber, 2012). Binding to α-TTP is highly specific to α-tocopherol, as α-TTP requires for binding three methyl groups in the chromanol ring, a phytyl tail, and the R-configuration at C-2 where the side chain is attached to the chromanol ring (Traber and Atkinson, 2007). Other vitamin E forms bind at much lower rates than α-tocopherol

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to α-TTP, which explains the much lower half-life and biological activity of other vitamin E forms. Liver, specifically hepatic parenchymal cells, are the primary storage tissues for vitamin E, while the adrenal glands, spleen, kidney, and heart muscle also store vitamin E in rats (Table 1.1) (Uchida et al., 2012). In general, body tissues have limited storage capacity for α-tocopherol. The α-tocopherol exchange among tissues is relatively rapid; specifically among liver, erythrocytes, spleen, and serum/plasma (Hidiroglou et al., 1994). Thus, serum/plasma concentrations provide an acceptable indicator of whole-body α-tocopherol status. Heart, muscle, and spinal cord have a slower α-tocopherol exchange, while brain α-tocopherol transfer is slowest (Hidiroglou and Ivan, 1992). Limited information of non-α-tocopherol concentration in bovine tissues is available. Therefore, one objective of this project was to investigate the accumulation and distribution levels of all four tocopherol isoforms in bovine tissues.

Transport of Tocopherols from Liver and other Tissues

Tocopherols stored in the liver are subsequently transferred to very-low-density lipoproteins (VLDL) to circulate in the blood for biological processes. The cytosolic 30-kDa α-TTP allows the incorporation of α-tocopherol into nascent VLDLs, which are released from the liver. Alpha-tocopherol transfer protein belongs to the CRAL-TRIO family of lipid binding proteins (Panagabko et al., 2003) which specifically binds to α-tocopherol. Deletion of the α-TTP gene has been shown to contribute to vitamin E deficiency in mice (Terasawa et al., 2000; Yokota et al., 2001) indicating that α-TTP is essential for vitamin E transport and tissue distribution. Once α-tocopherol is secreted from the liver, it is either exchanged to other lipoproteins or taken up by tissues via the LDL receptor. Another important protein involved in α-tocopherol transport from the

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liver is ATP-binding cassette transporter A1 (ABCA1), an ATP-binding cassette (ABC) transport protein, which facilitates α-tocopherol secretion mediated by α-TTP when Apo-A1 is the acceptor protein (Traber, 2013). Besides α-tocopherol, ABCA1 also facilitates transport of cholesterol and phospholipids out of cells into HDL (Reboul et al., 2006).

Metabolism of Vitamin E

Vitamin E does not accumulate to toxic levels in the liver. As a consequence, efficient metabolism and excretion are crucial to maintaining vitamin E concentration (Traber and Atkinson, 2007). So far, the only known site of vitamin E metabolism is the liver. The first step of vitamin E metabolism is ω-hydroxylation of the lipid tail, followed by β-oxidation. Hepatic cytochrome P450 family 4 subunit 2 (CYP4F2) and cytochrome P450 family 3 (CYP3A) are involved in the tail shortening reaction, which occurs in the mitochondria (Traber, 2014). High α-tocopherol concentrations promote the tail-shortening reaction of α-tocopherol as well as γ-tocopherol. The primary end products of the tail shortening reaction are α-CEHC (2,5,7,8-tetramethyl-2-(2ˊ-carboxyethyl)-6-hydroxychroman) for α-tocopherol and γ-CEHC (2,7,8-trimethyl-2-(2ˊ-carboxyethyl)-6-hydroxychroman) for γ-tocopherol, which are both found in plasma and urine (Birringer et al., 2002; Lodge et al., 2001).

Excretion of Vitamin E

To increase water solubility, CEHCs are sulfated or, to a smaller extent, glucuronidated (Pope et al., 2002) before being excreted into urine or bile (Brigelius-Flohe and Traber, 1999; Traber and Arai, 1999). In the biliary excretion, multidrug resistance gene product 1 and 2 are involved (Traber, 2014). In addition, because of the low absorption of vitamin E from intestines, non-metabolized vitamin E will be excreted

in small amounts in feces (Frank, 2005; Hidiroglou et al., 1994). Compared to α-tocopherol, limited information is available on body accumulation of γ-tocopherol and its interactions with RNS in dairy cows.

Dietary Sources of Vitamin E in Dairy Cows

The main dietary vitamin E supplements are α- and γ-tocopherol (Table 1.2). Alpha-tocopherol is the most biologically active tocopherol isoform in the body (Traber and Atkinson, 2007). Main dietary sources of α-tocopherol are oils and forages. In the absence of fresh forages, cows become rapidly vitamin E depleted (McDowell et al., 1996). The vitamin E content in cow feed (i.e. forage) is highly variable (Table 1.3) and differs depending on plant species, stage of maturity, environmental conditions, time of cutting, time cutting to dehydration, processing and storing conditions (Jukola et al., 1996; McDowell et al., 1996). Rapid losses of vitamin E in the diet might occur under oxidative conditions, such high temperature, moisture, oxygen, and pelleting (McDowell et al., 1996). Due to the high chances of losing vitamin E in stored forages (Table 1.3), the vitamin E requirement of dairy cows cannot be reached by only providing forages for non-pasture-fed dairy cattle. Therefore, vitamin E supplements are needed to maintain animal health.

Vitamin E Requirements in Dairy Cows

Current NRC recommendations for α-tocopherol in dairy cows are 2.6 IU/kg of body weight (BW), which includes vitamin E from feed-stuffs and supplements (NRC, 2001). One marker to assess vitamin E adequacy is to measure serum/plasma concentrations of α-tocopherol. Serum vitamin E concentrations below 3 μg/mL are considered deficient (McDowell et al., 1996). Deficiency symptoms, such as lesions of

white muscle disease, can be observed at concentrations below 1.5 μg /mL (McDowell et al., 1996). Concentrations of 1.5 or 2-3 μg/mL (equivalent to 7 μ*M* in serum/plasma) are considered marginal, whereas higher α-tocopherol of at least 3 or 4 μg/mL is required for adequacy (McDowell et al., 1996; NRC, 2001). To reach adequate blood values, current NRC recommendations for supplemental α-tocopherol in dairy cows are 1.6 IU/kg BW (approximately 80 IU/kg of dry matter intake [DMI]) during the dry period and 0.8 IU/kg BW (approximately 20 IU/kg DMI) during lactation (NRC, 2001). The following recommendations may be insufficient during times of low feed intake or increased vitamin E excretion, such as in the first weeks postpartum. In addition, greater amounts of vitamin E supplements are needed when vitamin E-deficient forages, concentrates, or total mixed rations (TMR) are fed, when forages of poor quality are fed, when harvesting, drying or storage conditions have decreased vitamin E content, when other feed components require higher vitamin E doses (e.g., PUFA, high nitrates in water), or when cows have greater requirements for production, feed efficiency, or to combat stress or disease (McDowell et al., 1996). In contrast, if fresh forages are fed or cows are on pasture, vitamin E supplementation needs are reduced. Feeding extra vitamin E may also increase the proportion of PUFAs in the milk of pasture-fed cattle and reduce oxidized flavors (Johansson et al., 2014). Thus, some experts recommend injectable vitamin E for cows two weeks before calving (McDowell et al., 1996; Weiss et al., 1998; Baldi, 2005; Politis, 2012). Compared to α-tocopherol, the required amounts for supplementing other tocopherol isoforms (e.g. γ-tocopherol, which was needed to protect against RNS) are unknown.

Vitamin E and Dairy Cow Health

The most natural stress period for dairy cows is the transition period, defined as ± 3 weeks relative to parturition (Drackley, 1999). During this time, dramatic physiological changes occur which might lead to altered immune and inflammatory responses in dairy cows (Sordillo and Raphael, 2013). The sudden physiological changes and altered immune functions contribute to the increased incidence of production diseases (e.g. metabolic disorders or infectious diseases) (Esposito et al., 2014). The severe infectious disease of mastitis will be discussed in a later section (pp.18). Another factor may be free radical stress (i.e. oxidative [via ROS] or nitroxidative stress [via RNS]) (Gaal et al., 2006). The free radical stress occurs when there is an imbalance between the production of free radical molecules and host antioxidant (e.g. vitamin E depletion) capabilities (Bobe et al., 2017). The potential over production of free radical molecules might be associated with sudden physiological changes (i.e. preparation for the ensuing lactation) in transition cows (Sundrum, 2015) or due to the proinflammatory immune response (Mordak and Anthony, 2015). Reduced host antioxidant capabilities may occur with reduced feed intake in dairy cows during the transition period because the dietary source is the primary way of giving vitamin supplements to dairy cows (Schirmann et al., 2016). For these two free radical stresses, oxidative stress has drawn more attention than nitroxidative stress, and α-tocopherol has been used as the essential antioxidant supplement in dairy cows compared to γ-tocopherol. However, nitroxidative stress is detrimental to dairy cattle health as well, especially related to disrupted metabolic pathways (Li et al., 2007). Previous studies have shown that nitrated tyrosine kinases (e.g. JAK-2) happen via the production of peroxynitrite (a RNS) (Elsasser et al., 2012). The overproduction of nitrated tyrosine kinases will block standard

phosphorylation reactions involved in regular cell signal transduction pathways (e.g. insulin receptor, or growth hormone receptor etc.) (Kong et al., 1996). Those perturbations of cell signal transduction pathways contribute to severe metabolic issues (e.g. insulin resistance) in dairy cows during the transition period (Boneh, 2015). Therefore, besides α-tocopherol, the more reactive tocopherol isoforms, γ-tocopherol, to protect against RNS is needed as an extra vitamin E supplement in dairy cows.

Vitamin E and Mitochondrial Health

Mitochondria are one of the essential cell organelles within a cell (Chan, 2006). The main function of mitochondria is the production of adenosine triphosphate (ATP) for energy utilization via oxidative phosphorylation and O_2 consumption to regulate cell metabolism (Orrenius et al., 2007). Oxidative phosphorylation refers to the process that ATP is formed as a result of the transfer of electrons from nicotinamide adenine dinucleotide (reduced form) NADH or flavin adenine dinucleotide (reduced form) $FADH_2$ (NADH or $FADH_2$ from citric acid cycle) to reduce O_2 to H_2O through a series of electron carriers (Orrenius et al., 2007; Figure 1.9).

When electrons (derived from NADH or $FADH_2$) are transported through the electron transport chain via NADH-Q oxidoreductase (complex I), Q-cytochrome *c* oxidoreductase (complex III), and cytochrome *c* oxidase (complex IV), protons (H^+) are pumped from the matrix of the mitochondria to the cytosolic side of the inner mitochondrial membrane. The resulting unequal distribution of protons generates a proton gradient; ATP is synthesized when protons flow back to the mitochondrial matrix through ATP synthase. However, during electron transport in the inner membrane, $O_2^{\bullet}$ can be produced which is not only considered as the precursor of most ROS but can also

be to the precursor of peroxynitrite in the presence of NO (Murphy, 2009). Peroxynitrite can reduce to bicarbonate (HCO_3^-), which leads to the formation of carbonate ($CO_3^{\bullet-}$) and nitrogen dioxide radicals ($^{\bullet}NO_2$). Nitrogen dioxide radicals can play the same roles as $LOO^{\bullet}$, leading to lipid peroxidation (Szabo et al., 2007). Both ROS and RNS can contribute to free radical stress and result in cell damage via lipid peroxidation (Szabo et al., 2007; Valko et al., 2007).

A previous study has shown that mitochondria are essential cell organelles related to cell death during oxidative stress (Elsasser et al., 2012). Increased production of ROS or RNS will contribute to mitochondrial dysfunction, leading to cellular apoptosis (Orrenius et al., 2007). One proposed mechanism of action is shown in Figure 1.10. In the cytosol, ROS or RNS mediates cardiolipin peroxidation (lipid peroxidation) exclusively in the inner mitochondrial membrane. This then initiates the release of cytochrome *c* (Cyt *c*) into the cytosol where Cyt *c* activates capase-9 leading to cellular apoptosis (Fariss et al., 2005).

Tocopherols can control cardiolipin peroxidation via scavenging of ROS as shown in Figure 1.2. Also, vitamin E isoforms (e.g. γ-tocopherol) can scavenge reactive nitrogen species (i.e. peroxynitrite, NO_2) through γ-tocopherol nitration, which, like ROS, can damage proteins, lipids, and DNA (Traber and Nikki, 2012). However, the absorption of tocopherol isoforms (α-, β-, γ- and δ-tocopherol) in mitochondria (mainly in lipid membranes) in tissues from dairy cows is still unclear. This project will determine the bioaccumulation of a dietary tocopherol mix containing α-, β-, γ- and δ-tocopherol in mitochondria within primary peripheral tissue types (i.e. liver and mammary gland) of lactating dairy cows.

Vitamin E Might Help to Improve Polymorphonuclear leukocytes (PMN) Functions during Inflammation

<u>*Mastitis*</u>

Mastitis is defined as an inflammation of the mammary gland. Bovine mastitis is a disease of high economic importance. Financial losses associated with mastitis at the farm-level are mainly due to treatment costs, loss of milk yield, and lower milk quality (Blum et al., 2014). In dairy cows, mastitis is usually associated with the invasion of microorganisms, primarily bacteria. Mastitis-causing pathogens can be mainly classified into two categories: environmental and contagious (Zhao and Lacasse, 2008). Contagious mastitis pathogens within the mammary gland of infected cows are the primary source of infectious pathogens in a dairy herd because the contagious organism is well adapted to survival and growth in the mammary gland lasting weeks, month or years (Fox and Gay, 1993). Transmission of contagious pathogens to uninfected quarters and cows usually occurs during the milking process. Milk from infected quarters can contaminate milking machines, hands of milkers, and towels, which act as reservoirs of infection. The major contagious pathogens are *Staphylococcus aureus* and *Streptococcus agalactiae*. These bacteria can be effectively controlled by procedures who prevent spread of bacteria at milking time through good udder hygiene, proper milking procedures, and post-milking teat disinfection. Use of dry cow antibiotic therapy can help eliminate existing infections and prevent new infections during the early and late dry period (Sol et al., 1994).

Mycoplasmas are another important contagious mastitis pathogen (Fox and Gay, 1993). *Mycoplasma* mastitis may be introduced to the herd by purchased animals and

can be spread during milking. There is no effective treatment for mycoplasma mastitis, but the disease can be controlled by identifying infected animals, followed by segregating and/or culling the infected animals (Fox and Gay, 1993).

In addition to contagious pathogens, environmental pathogens also cause mastitis. The primary source of environmental mastitis pathogens is the habitat of the cow (soil, plant material, manure, bedding, and contaminated water) (Anderson et al., 1982). The most frequently isolated environmental pathogens are several species of streptococci (gram-positive bacteria) and coliforms (gram-negative bacteria).

The endotoxin of gram-negative bacteria (e.g. *Escherichia coli*; *E. coli*) inducing the inflammatory response is lipopolysaccharide (LPS) on the cell wall, which is the pathogen-associated molecular pattern (PAMP). The PAMP will be recognized by pattern recognition receptors (PPR; e.g. toll-like receptors [TLR] and chemokine receptors) which are expressed on the surface of immune cells (e.g. macrophage, PMN, B-lymphocytes) or other cell types (e.g. epithelial cells). The binding of the PAMP to the PPR can activate the inflammatory response through MyD88 (myeloid differentiation primary response protein 88)-dependent pathway that leads to the production of inflammatory cytokines (Figure 1.11). When PPR are activated by PAMP, TLR hetero- (e.g. TLR-2 and TLR-6) or homo-dimerize (e.g. TLR-4), inducing the recruitment of adaptor proteins via the cytoplasmic TLR domain. Adaptor proteins include the TLR-domain-containing proteins: MyD88 TIRAP (TLR-associated protein), Mal (MyD-88 adaptor–like protein), TRIF (TLR-domain-containing adaptor protein inducing IFN-β (Interferon beta-1), and TRAM (TRIF-related adaptor molecule). After recruitment of MyD88, for instance, interleukin (IL)-1 receptor associated kinase 1 (IRAK-1) and IL-4

associated kinase 4 (IRAK-4) are activated. IRAK-4 subsequently activates IRAK-1 by phosphorylation. Both IRAK1 and IRAK-4 leave the MyD88-TLR complex and associate temporarily with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF-6) leading to its ubiquitination (Sun, 2004). Following ubiquitination, TRAF-6 forms a complex with TAB-2/TAB-3/TAK-1 inducing TAK-1 (transforming growth factor-β-activated protein kinase 1) activation (Kanayama, 2004). TAK1 then couples to the IκB kinase (IKK) complex, which includes the scaffold protein leading to the phosphorylation of IκB and the subsequent nuclear localization of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). Activation of NF-κB can trigger the production of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-12 (Kanayama, 2004).

Bovine Blood PMN and Vitamin E during Inflammatory Response

Sensing the pathogens by initiating inflammation is crucial to the recruitment of PMN in mammary tissues of dairy cows (Rainard and Riollet, 2006). Bovine mammary epithelial cells and milk macrophages could first detect pathogens and initiate an inflammatory response following the mechanisms discussed above. During the inflammatory response, circulating neutrophil infiltrate from the blood into the mammary gland through a process called chemotaxis (Figure 1.12**)** and serve as the primary defense mechanism of the innate immune system (i.e. non-specific immunity) against invading microorganisms. The PMN play an important role during the innate immune response. During an infection, PMN migrate from blood to the tissue in response to chemoattractants secreted from either resident macrophages or the epithelial cells (Goldsby et al., 2000). Some of the primary chemoattractants are C5a and cytokines such

as IL-1, IL-2, and IL-8 (Furie, 2014). Cytokines activate the endothelial cells to express

E-selectin and P-selectin which bind tightly to circulating PMN (Radi et al., 2001). This

binding enhances expression and adhesiveness of another PMN adhesion molecule, Mac-

1 (also known as CD11b/ CD18), which is a member of the β_2-integrin family of

leukocyte adhesion molecules. At the same time that Mac-1 expression increases, L-

selectin is proteolytically shed from the PMN surface (Kishimoto et al., 1989). The Mac-

1 molecule allows PMN to bind tightly to activate endothelium via another endothelial

adhesion molecule, ICAM-1 (intercellular adhesion molecule 1). This binding allows

PMN to migrate from the endothelial surface into the infected tissue via a process called

diapedesis along a concentration gradient of chemoattractants, the most important being

complement components C5a and IL-8 (Ranard and Riollet, 2006). Bovine PMN

expresses chemokine receptors (i.e. CXCR-1 and CXCR-2), which can recognize CXC

chemokines (e.g. C5a). These chemokine receptors have been used as potential genetic

markers for PMN function (Yongerman et al., 2004). Once within the infected tissue,

PMN phagocytose and kill invading microorganisms via respiratory burst. Phagocytosis

is the process of ingesting of microorganisms by phagocytic cells (e.g. macrophages,

PMN) (Paape et al., 1979) (Figure 1.13). The first step of phagocytosis requires the

adherence or opsonization of phagocytic cells to the cell wall of bacterial (e.g. endotoxin

LPS from gram-negative bacteria). Phagocytic cells receptors (e.g. TLR) are specific for

certain opsonins, such as the component of complement, boost adherence and

phagocytosis (Goldsby, 2000). After the microorganism is surrounded and enclosed by

pseudopodia from the phagocytic cells, it then enters the cytosol with a membrane

structure called a phagosome. The phagosome then fuses with a lysosome to become a

phagolysosome. During this fusion, a burst of oxidative metabolism called respiratory burst occurs in activated phagocytes. During this respiratory burst, PMN generate ROS (i.e. O_2, H_2O_2) to damage the cell membrane of engulfed microorganisms. Nicotinamide-adenine-dinucleotide-phosphate linked oxidase can catalyze the reduction of O_2 to $O_2^{\cdot-}$. Superoxide anion is extremely toxic to ingested bacteria, can generate H_2O_2 via SOD. In addition to SOD, myeloperoxidase activity produces HClO (hypochlorous acid) from H_2O_2, which is also toxic to ingested microorganisms. However, these ROS can also damage the host cell membranes through lipid peroxidation (as demonstrated in Figure 1.1), oxidation of proteins, or damage of DNA molecules (Halliwell and Aruoma, 1991). Therefore, understanding how to improve PMN function and prevent host cell damage from respiratory burst activity is an essential step to the development of new strategies to control mastitis and improve the health of dairy cows.

Previous studies have shown that α-tocopherol supplements besides antioxidant enzymes (e.g. SOD and GPX) can improve bovine PMN phagocytosis and chemotaxis (Ibeagha et al., 2009; Politis, et al., 2012) as an immune enhancer *in vitro*. At the same time, previous study also has shown that tocopherols (e.g. α-, γ-, δ-tocopherol) and tocopherol metabolites (water-soluble 2, 2'-carboxyethyl hydroxychroman metabolites) can control the PMN oxidative burst to produce ROS or RNS during PMN stimulation from human blood *in vitro* (Varga et al., 2008). However, the effects of β-, γ- and δ-tocopherol on PMN function are still unknown in bovines. Therefore, this project will determine the effects of feeding a tocopherol mix (four tocopherol isoforms- α-, β-, γ- and δ-tocopherol) on bovine PMN function and the expression of genes involved in innate immune functions in bovine PMN *in vitro.*

Overall, vitamin E supplements are necessary to maintain dairy cow health during the transition period when vitamin E deficiency occurs (Johansson et al., 2014). Among those four vitamin E isoforms, non-α-tocopherols (e.g. γ-tocopherol) are needed as importantly as α-tocopherol to protect against RNS (Shi et al., 2013). However, basic information on bioaccumulation and distribution of mixed isoforms (i.e. highly enriched with γ-tocopherol) of vitamin E and their effects on animal health (i.e. specifically immune cell function) in dairy cows are needed. For instance, will the non-α-tocopherol isoforms be accumulated at the high level equal to α-tocopherol? Will the mixed isoforms tocopherol alter immune cell functions? We must understand those two basic and important questions before applying mixed isoforms of vitamin E supplements in dairy cow management.

REFERENCES

Anderson, K. L., A. R. Smith, B. K. Gustafsson, S. L. Spahr, and H. L. Whitmore. 1982. Diagnosis and treatment of acute mastitis in a large dairy herd. J. Am. Vet. Med. Assoc. 181:690-693.

Anwar, K., J. Iqbal, and M. M. Hussain. 2007. Mechanisms involved in vitamin E tranposrt by primary enterocytes and in vivo absorption. J. Lipid Res 48:2028-2038. Baldi, A. 2005. Vitamin E in dairy cows. Livest. Prod. Sci. 98:117-122.

Bergan-Roller, H. E., A. T. Ickstadt, J. D. Kittilson, and M. A. Sheridan. 2017. Insulin and insulin-like growth factor-1 modulate the lipolytic action of growth hormone by altering signal pathway linkages. Gen. Comp. Endocrinol. 248:40-80.

Birringer, M., P. Pfluger, D. Kluth, N. Landes, and R. Brigelius-Flohe. 2002. Identities and idfferences in the metabolim of tocotrienols and tocopherols in HepG2 cells. J. Nutr. 132:3113-3118.

Bobe, G., J. W. Young, and D. C. Beitz. 2004. Invited Review: Pathology, etiology, prevention, and treatment of fatty liver in dairy cows. J. Dairy Sci. 87:3105-3124.

Bobe, G., T. J. Cobb, S. W. Leonard, S. Aponso, C. B. Bahro, D. Koley, E. Mah, R. S. Bruno, and M. G. Traber. 2017. Increased static and decreased capacity oxidation-

reduction potentials in plasma are predictive of metabolic syndrome. Redox Biol. 12:121-128.

Boneh, A. 2015. Signal transduction in inherited metabolic disorders: a model for a possible pathogenetic mechanism. J. Inherit. Metab. Dis. 38:729-740.

Bontempo, V., A. Baldi, F. Cheli, F. Fantuz, I. Politis, S. Carli, and V. Dell'Orto. 2000. Kinetic behaviour of three preparations of alpha-tocopherol afer oral adminstration to postpubertal heifers. Am. J. Vet. Res. 61:589-593.

Blum, S. E., E. D. Heller, and G. Leitner. 2014. Long term effects of *Escherichia coli* mastitis. Vet. J. 201:72-77.

Brigelius-Flohe, R. and M. G. Traber. 1999. Vitamin E: function and metabolism. FASEB J. 13:1145-1155.

Bruno, R. S., S. W. Leonard, J. Atkinson, T. J. Montine, R. Ramakrishnan, T. M. Bray, and M. G. Traber. 2006. Faster plasma vitamin E disappearance in smokers is normalized by vitamin C supplementation. Free Radic. Biol. Med. 40:689-697.

Chan, D. C. Mitochondria: Dynamic organelles in disease, aging, and development. 2006. Cell. 7: 1241-1252.

Chew, B. 1995. Antioxidant vitamins affect food animal immunity and health. J. Nutr. 125:1804-1808.

Conney, R. V., A. A. Franke, P. J. Harwood, V. Hatch-Pigott, L. J. Custer, and L. J. Mordan. 1993. Gamma-tocopherol detoxification of nitrogen dioxide:superiority to alpha-tocopherol. Proc. Natl. Acad. Sci. U.S.A. 90:1171-1175.

Cort, W., T. Vicente, E. Waysek, and B. Williams. 1983. Vitamin E content of feedstuffs determined by high-performance liquid-chromatographic fluorescence. J. Agric. Food Chem. 31: 1330–1333.

Drackley, J. K. 1999. Biology of dairy cows during the transition period: the final frontier? J. Dairy Sci. 82:2259-2273.

Eckel, E. F. and B. N. Ametaj. 2016. Invited review: role of bacterial endotoxins in the etiopathogenesis of periparturient diseases of transition dairy cows. J. Dairy Sci. 99:5967-5990.

Elsasser, T. H., S. Kahl, A. V. Capuco, and W. Schmidt. 2012. Effects of stress on endocrine and metabolic processes and redirection: cross talk between subcellular compartments. Domest. Anim. Endocrinol. 43:132-145.

Elsasser, T.H., S. Kahl, K.M. Lebold, M.G. Traber, J. Shaffer, C. Li, and S. Block. 2013. Short-term alpha-or gamma-delta-enriched tocopherol oil supplementation differentially effects the expression of proinflammatory mediators: selective impacts on characteristics of protein tyrosine nitration in vivo. Vet Sci. Develop. 3: 20-38.

Esposito, G., P. C. Irons, E. C. Webb, and A. Chapwanya. 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Anim. Reprod. Sci. 144:60-71.

Fairus, S., M. N. Rosnah, M. C. Hwee, and K. Sundram. 2012. Alpha-tocopherol is the most abundant tocotrienol isomer circulated in plasma and lipoproteins after postprandial tocotrienol-rich vitamin E supplementation. Nutr J. 11: 5-16.

Fariss, M. W., C. B. Chan, M. Patel, B. V. Houten, and S. Orrenius. 2005. Role of mitochondria in toxic oxidative stress. Mol. Interv. 5:94-111.

Fox, L. K., and J. M. Gay. 1993. Contagious mastitis. Vet. Clin. North Am. Food Anim. Pract. 9: 475-487.

Frank, J. 2005. Beyond vitamin E supplementation: an alternative strategy to improve vitamin E status. J. Plant Physiol. 162:834-843.

Fridovich, I. 1997. Superoxide anion radical,superoxide dismutases, and realted matters. Animal. 2009:1037-1043.

Fubini, B. and A. Hubbard. 2003. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. Free Radic. Biol. Med. 34:1507-1516.

Furie, M. B. 2014. Recruitment of leukocytes: adhesion molecules and chemoattractants A2 - mcManus, Linda M. PP. 275-288 in pathobiology of human disease. R. N. Mitchell ed. Academic Press, San Diego.

Gaal, T., P. Ribiczeyne-Szao, K. Stadler, J. Jakus, J. Reiczigel, P. Kover, M. Mezes, and L. Sumeghy. 2006. Free radicals, lipid peroxidation and the antioxidant system in the blood of cows and newborn calves around calving. Comparative Biochemistry and Physiology Part B: Biochem Mol Biol J. 143:391-396.

Goldsby, R. A., T. J. Kindt, and B. A. Osborne. 2000. Kuby immunology. 4th edn ed. W.H.Freeman and Company, New York.

Goncalves, A., S. Roi, M. Nowicki, A. Dhaussy, A. Huertas, M. J. Amiot, and E. Reboul. 2015. Fat-soluble vitamin intestinal absorption: Absorption sites in the intestine and interactions for absorption. Food Chem. 172:155-160.

Grummer, R. R. 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. J. Dairy Sci. 76:3882-3896.

Gurler, H., E. Malama, M. Heppelmann, O. Calisici, C. Leiding, J. P. Kastelic, and H. Bollwein. 2016. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. Theriogenology. 86:562-571.

Halliwell, B. and O. I. Aruoma. 1991. DNA damage by oxygen-derived species Its mechanism and measurement in mammalian systems. FEBS L. 281:9-19.

Hidiroglou, M. and M. Ivan. 1992. Biokinetics and biliary excretion of radiotocopherol administered orally to sheep. J. Anim Sci. 70:1220-1226.

Hidiroglou, M., M. Ivan, and P. L. Toutain. 1994. Metabolism of tritiated d-alpha-tocopherol and d-alpha-tocopheyl succinate in intraruminally dosed sheep. J. Anim Sci. 72:2124-2130.

Hoglen, N. C., S. C. Waller, I. Gleen Spies, and D. C. Liebler. 1997. Reactions of peroxynitrite with gamma-tocopherol. Chem. Res. Toxicol. 10:401-407.

Ibeagha, A. E., E.M. Ibeagha-Awemu, J.Mehrzad, B.Baurhoo,P. Kgwatalala, and X. Zhao. 2009. The effect of selenium sources and supplementation on neutrophil functions in dairy cows. Animal. 3.07: 1037-1043.

Jia, Z., H. Zhu, J. LI, X. Wang, H. Misra, and Y. Li. 2012. Oxidative stress in spinal cord injury and antioxidant-based intervention. Spinal Cord. 50: 264-274.

Johansson, B., K. W. Persson, SK. Jensen, H. Lindqvist, and E. Nadeau. 2014. Status of vitamins E and A and beta-carotene and health in organic dairy cows fed a diet without synthetic vitamins. J. Dairy Sci. 97: 1682-1692.

Jukola, E., J. Hakkarainen, H. Saloniemi, and S. Sankari. 1996. Blood selenium, vitamin E, vitamin A, and beta-varotene voncentrations and udder health, fertility treatments, and fertility. J. Dairy Sci. 79:838-845.

Kanayama, A., R. B. Seth, L. Sun, C. Ea, M. Hong, A. Shaito, Y. Chiu, L. Deng and Z. J. Chen. 2004. TAB2 and TAB3 activate the NF-B pathway through binding to polyubiquitin chains. Mol. Cell. 15: 535-548.

Kawai, T., and S. Akira. TLR signaling. 2006. Cell Death Differ. 13: 816-825.

Kong, S. K., M. B. Yim, E. R. Stadtman, and P. B. Chock. 1996. Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism: Lymphocyte-specific tyrosine kinses fail to phosphorylate nitrated cdc2(6-20) NH2 peptide. Proc. Natl. Acad. Sci. U.S.A. 93:3377-3382.

Kishimoto, T K., M. A. Jutila, E. L. Berg and E. C. Butcher. 1989. Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. Science, 245:4923-1238. Academic OneFile, Accessed 25 Apr. 2017.

Lampi, A. M. 2011. Analysis of tocopherols and tocotrienols by HPLC. http://lipidlibrary.aocs.org/Analysis/content.cfm/ItemNumber=40389

Leonard, S. W., E. Paterson, J. K. Atkinson, R. Ramakrishnan, C. E. Cross, and M. G. Traber. 2005. Studies in humans using deuterium-labeled alpha- and gamma-tocopherols demonstrate faster plasma gamma-tocopherol disappearance and greater gamma-metabolite production. Free Radic. Biol. Med. 38:857-866.

Li, C. J., S. Kahl, D. Carbaugh, and T. H. Elsasser. 2007. Temporal response of liver signal transduction elements during in vivo endotoxin challenge in cattle: Effects of growth hormone treatment. Domest. Anim. Endocrinol. 32:79-92.

Liu, K., H. L. Luo, Z. Y. Zuo, H. N. Jia, Y. W. Zhang, Y. F. Chang, and L. J. Jiao. 2014. Regulation of sheep alpha-TTP by dietary vitamin E and preparation of monoclonal antibody for sheep alpha-TTP. Gene. 540: 110-116.

Lodge, J. K., J. Ridlington, S. Leonard, H. Vaule, and M. G. Traber. 2001. Alpha-and gamma-tocotrienols are metabolized to carboxyethyl-hydroxychroman derivatives and excreted in human urine. Lipids. 36:43-48.

McDowell, L. R., S. N. Williams, N. Hidiroglou, C. A. Njeru, G. M. Hill, L. Ochoa, and N. S. Wilkinson. 1996. Vitamin E supplementation for the ruminant. Anim. Feed Sci. Technol. 60:273-296.

Miller, J., E. Brezeinska-Slebodzinska, and F. Madsen. 1993. Oxidative stress,antioxidants and animal function. J. Dairy Sci. 76:2812-2823.

Mogensen, L., T. Kristensen, K. Soegaard, S. K. Jensen, and J. Sehested. 2012. Alpha-tocopherol and beta-carotene in roughages and milk in organic dairy herds. Livest. Sci. 145: 44–54.

Mordak, R. and S. P. Anthony. 2015. Periparturient stress and immune suppression as a potential cause of retained palcenta in highly productive dairy cows: examples of prevention. Acta. Vet. Scand. 57:84.

Mudron, P., J. Rehage, P. Sallmann, M. Mertens, H. Scholz, and G. Kovac. 1997. Plasma and liver alpha-tocopherol in dairy cows with left abomasal displacement and fatty liver. J. Vet. Med. Ser. 44:91-97.

Muller, C. E., J. Moller, S. K. Jensen, and P. Uden. 2007. Tocopherol and carotenoid levels in baled silage and haylage in relation to horse requirements. Anim. Feed Sci. Technol. 137: 182–197.

Murphy, M. P. How mitochondria produce reactive oxygen species. 2009. Biochem. J. 417: 1-13.

National Research Council. 2001. Nutrient requirements of dairy cattle. 7th rev.ed. National Academy of Science ed. Washington, DC, National Academy Press.

Narushima, K., T. Takada, Y. Yamanashi, and H. Suzuki. 2008. Niemann-pick C1-like 1 mediates alpha-tocopherol transport. Mol. Pharmacol. 74:42-49.

Niki, E. and M. G. Traber. 2012. A history of vitamin E. Nutr. Metab. 61:207-212.

Orrenius, S., V. Gogvadze, and B. Zhivotovsky. Mitochondrial oxidative stress: Implications for cell death. 2007. Annu. Rev. Pharmacol. Toxicol. 47: 143-83.

Paape, M. J., W. P. Wergin, and A. J. Guidry. and R. E. Pearson. Leukocytes-second line of defense against invading mastitis pathogens. J. Dairy Sci 62.1-4 (1979): 135.

Paape, M. J., D. D. Bannerman, X. Zhao, and J. W. Lee. 2003. The bovine neutrophil:structure and function in blood and milk. Vet. Res. 34:597-627.

Panagabko, C., S. Morley, M. Hernandez, P. Cassolato, H. Gordon, R. Parsons, D. Manor, and J. Atkinson. 2003. Ligand specificity in the CRAL-TRIO protein family. Biochemistry. 42:6467-6474.

Patel, A., F. Libner, T. Netscher, K. Mereiter, and T. Rosenau. 2007. Vitamin E chemistry. Nitration of non-alpha-tocopherols: products and mechanistic considerations. J. Org. Chem. 72:6504-6512.

Packer, L. and J. Fuchs. 1993. Analysis and distribution of vitamin E in vegetable oils and food. Marcel Dekker,Inc., New York.

Politis, I. 2012. Reevaluation of vitamin E supplementation of dairy cows: bioavailability, animal health and milk quality. Animal. 6:1427-1434.

Pope, S. A. S., G. E. Burtin, P. T. Clayton, D. J. Madge, and D. P. R. Muller. 2002. Synthesis and analysis of conjugates of the major vitamin E metabolite, alpha-CEHC. Free Radic. Biol. Med. 33:807-817.

Qu, Y., A. N. Fadden, M. G. Traber, and G. Bobe. 2014. Potential risk indicators of retained placenta and other diseases in multiparous cows. J. Dairy Sci. 97:4151-4165.

Qu, Y., K. Lytle, M. G. Traber, and G. Bobe. 2013. Depleted serum vitamin E concentrations precede left displaced abomasum in early-lactation dairy cows. J. Dairy Sci. 96:3012-3022.

Radi, Z. A., M. E. Kehrli Jr, and M. R. Ackermann. 2001. Cell adhesion molecules, leukocyte trafficking, and strategies to reduce leukocyte infiltration. J. Vet. Intern. Med. 15:516-529.

Radi, R. 2013. Protein tyrosine nitration: biochemical mechanisms and structural basis of its functional effects. Acc. Chem. Res. 46:550-559.

Rainard, P. and C. Riollet. 2006. Innate immunity of the bovine mammary gland. Vet. Res. 37: 369-400.

Reboul, E., A. Klein, F. Bietrix, B. Gleize, C. Malezet-Desmoulins, M. Schneider, A. Margotat, L. Lagrost, X. Collet, and P. Borel. 2006. Scavenger receptor class B type 1 (SR-BI) is involved in vitamin E transport across the enterocyte. J. Biol. Chem. 281:4739-4745.

Rivera-Mancia, S., L. A. Tristan-Lopez, and S. Montes. 2017. Chapter 37 - Reactive nitrogen and oxygen species in hepatic encephalopathy A2 - Muriel, Pablo. PP. 485-500 in Liver Pathophysiology. Academic Press, Boston.

Roberts, R. A., D. L. Laskin, C. V. Smith, F. M. Robertson, E. M. G. Allen, J. A. Doorn, and W. Slikker. 2009. Nitrative and oxidative stress in toxicology and disease. Toxicol. Sci. 112: 4-16.

Sah, S. P., B. Singh, S. Choudhary, and A. Kumar. 2016. Animal models of insulin resistance: A review. Pharmacol. Rep. 68:1165-1177.

Sanchez Perez, M. J., E. Gonzalez-reimers, F. Santolariz-Fernandez, M. J. De La Vega-Prieto, A. Martinez-Riera, P. Abreu Gonzalez, E. Rodriguez-Rodriguez, and M. C. Druan-Castellon. 2006. Lipid peroxidation and serum cytokines in acute alcoholic hepatitis. Alcohol. 41:593-597.

Schirmann, K., D. M. Weary, W. Heuwieser, N. Chapinal, R. L. A. Cerri, and M. A. G. von Keyserlingk. 2016. Short communication: Rumination and feeding behaviors differ between healthy and sick dairy cows during the transition period. J. Dairy Sci. 99:9917-9924.

Schlessinger, J. 2000. Cell signaling by receptor tyrosine kinases. Cell. 103:211-225.

Sordillo, L. M. and W. Raphael. 2013. Significance of metabolic stress, lipid mobilization, and inflammation on transition cow disorders. Vet. Clin. North Am. Food Anim. Pract. 29:267-278.

Sereli, G., A. Lncani, A. Atzeri, A. Angioni, M. Campus, E. Cauli, R. Zurru, and M. Deiana. 2017. Antioxidant effect of natural table olives phenolic extract against oxidative stress and membrane damage in enterocyte-like cells. J. Food Sci. 82: 380-385.

Shi, J. D., T. Golden, C. J. Guo, S. P. Tu, P. Scott, C. S. Yang, and A. J. Gow. 2013. Tocopherol supplementation reduces NO production and pulmonary inflammatory response to bleomycin. Nicric Oixde. 34: 27-36.

Sol, J., O. C. Sampimon, J. J. Snoep, and Y. H. Schukken. 1994. Factors associated with bacteriological cure after dry cow treatment of subclinical staphylococcal mastitis with antibiotics. J. Dairy Sci. 77: 75-79.

Sun, J., F. Li, J. Chen, and J. Xu. 2004. Effect of ketamine on NF-kappa B activity and TNF-alpha production in endotoxin-treated Rats. Ann. Clin. Lab Sci. 34. 2.

Sundrum, A. 2015. Metabolic disorders in the transition period indicate that the dairy cows' ability to adapt is overstressed. Animals. 5:978-1020.

Szabo, C., H. Ischiropoulos, and R. Radi. 2007. Peroxynitrite: biochemistry,pathophysiology and development of therapeutics. Nat. Rev. Drug Discov. 6:662-680.

Takada, T. and H. Suzuki. 2010. Molecular mechanisms of membrane transport of vitamin E. Mol. Nutr. Food Res. 54:616-622.

Terasawa, Y., Z. Ladha, S. W. Leonard, J. D. Morrow, D. Newland, D. Sanan, L. Packer, M. G. Traber, and R. V. J. Farese. 2000. Increased atherosclerosis in hyperlipidemic mice deficient in alpha-tocopherol transfer protein and vitamin E. Proc. Natl. Acad. Sci. U.S.A. 97:13830-13834.

Toutain, P. L., M. Hidiroglou, and E. Charmley. 1995. Pharmacokinetics and tissue uptake of D-alpha-tocopherol in sheep following a single intraperitoneal injection1. J. Dairy Sci. 78:1561-1566.

Traber, M. G. 2004. Vitamin E. PP. 293-304 in Vitamin E. B. Caballero, R. J. Cousins, K. L. Tucker, and T. R. Ziegler eds. Lippincott Williams & Wilkins, Baltimore.

Traber, M. G. 2013. Mechanisms for the prevention of vitamin E excess. J. Lipid Res. 54: 2295-2306.

Traber, M. G. 2014. Vitamin E inadequacy in humans: casues and consequences. Adv. Nutr. 5:503-514.

Traber, M. G. and H. Arai. 1999. Molecular mechanisms of vitamin E transport. Annu. Rev. Nutr.19:343-355.

Traber, M. G., R. Ramakrishnan, and H. J. Kayden. 1994. Human plasma vitamin E kinetics demonstrate rapid recycling of plasma RRR-alpha-tocopherol. Proc. Natl. Acad. Sci. U.S.A. 91:10005-10008.

Traber, M. G. and J. Atkinson. 2007. Vitamin E, antioxidant and nothing more. Free Radic. Biol. Med. 43:4-15.

Trachootham, D., W. Lu, M. A. Ogasawara, N. Rivera-Del Valle, and P. Huang. 2008. Redox regulation of cell survival. Antioxid. Redox Signal. 10:1343-1374.

Uchida, T., C. Abe, S. Nomura, T. Ichikawa, and S. Ikeda. 2012. Tissue distribution of alpha- and gamma-tocotrienol and gamma-tocopherol in rats and interference with their accumulation by alpha-tocopherol. Lipids. 47:129-139.

Valko, M., M. Izakovic, M. Mazur, C. J. Rhodes, and J. Telser. 2004. Role of oxygen radicals in DNA damage and cancer incidence. Int. J. Mol. Cell Biochem. 266: 37-56.

Valko, M., D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, J. Telser. 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39: 44-48.

Varga, Z., E. Kosaras, E. Komodi, M. Katko, I. Karpati, J. Balla, G. Paragh, M. C. Aisa, and F. Galli. 2008. Effects of tocopherols and 2,2-carboxyethyl hydroxychromans on phorbol-ester-stimulated neutrophils. J. Nutr. Biochem. 19:320-327.

Weiss, W. P. 1998. Requirements of fat-soluble vitamins for dairy cows: A review. J. Dairy Sci. 81:2493-2501.

Weiss, W. P., J. S. Hogan, D. A. Todhunter, and K. L. Smith. 1997. Effect of vitamin E supplementation in diets with a low concentration of selenium on mammary gland health of dairy cows. J. Dairy Sci. 80:1728-1737.

Willshire, J. A. and J. H. Payne. 2011. Selenium and vitamin E in dairy cows a review. Cattle Pract. 19:22-30.

Youngerman, S. M., A. M. Saxton, S. P. Oliver, and G. M. Pighetti. 2004. Association of CXCR2 polymorphisms with subclinical and clinical mastitis in dairy cattle. J. Dairy Sci. 87:2442-2448.

Yokota, T., K. Igarashi, T. Uchihara, K. Jishage, H. Tomita, A. Inaba, Y. Li, M. Arita, H. Suzuki, H. Mizusawa, and H. Arai. 2001. Delayed-onset ataxin in mice lacking alpha-tocopherol transfer protein: model for neuronal degeneration caused by chronic oxidative stress. Proc. Natl. Acad. Sci. U.S.A. 98:15185-15190.

Zhao, X., and P. Lacasse. 2008. Mammary tissue damage during bovine mastitis: causes and control. J. Anim. Sci. 86: 57-65.

TABLES:

Table 1.1: Tissue α-tocopherol concentration (μ*g/g* fresh tissue) in Holstein steers after a single gastric dose of 50 IU α-tocopherol/kg of body weight (Eicher et al., 1997).

Tissues	α-tocopherol
Spleen	17.5
Liver	30.8
Adipose	2.5
Muscle	7.4
Gut	6.7
Kidney	24
Heart	14

Table 1.2: Vitamin E content of oils, cereal grains and seeds (per kg edible portion) (adapted from Packer and Fuchs, 1993).

Product	Tocopherol (mg)				Tocotrienol (mg)	
	α	β	γ	δ	α	β
Oils						
Canola	210	1	42	0.4	0.4	-
Corn	112	50	602	18	-	-
Cottonseed	389	-	387	-	-	-
Olive	119	-	7	-	-	-
Palm	256	-	316	70	146	32
Peanut	130	-	214	21	-	-
Soybean	75	15	797	266	2	1
Grains						
Barley	2	0.4	0.3	0.1	11	3
Corn	6	-	45	-	3	-
Oats	5	1	-	-	11	2
Seeds						
Sesame	-	-	227	-	-	-
Sunflower	495	27	-	-	-	-

Table 1.3: Vitamin E content of feed in mg/kg (adapted from Cort et al., 1983; Muller et al., 2007; Mogensen et al., 2012).

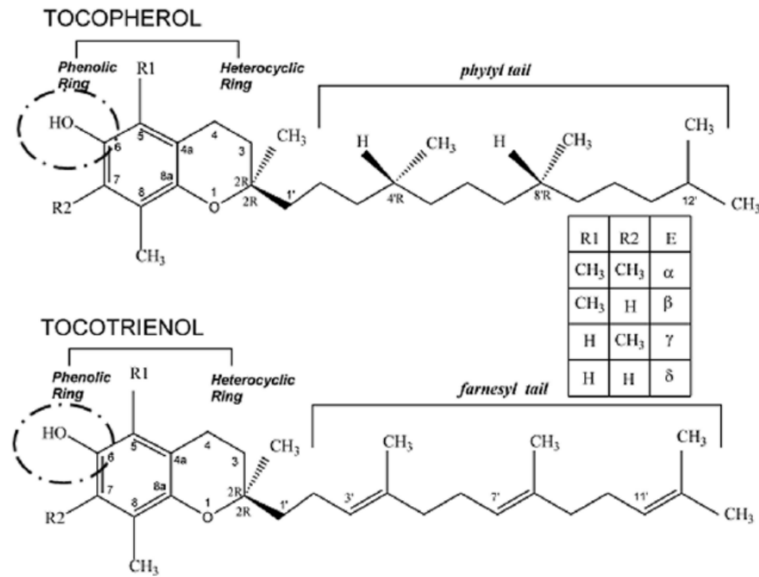| Product	Tocopherol (mg/lbs)				Tocotrienol (mg/lbs)	
	α	β	γ	δ	α	β
Alfalfa, fresh	255.74	-	11.02	-	-	-
Alfalfa,hay	68.34	-	4.41	-	-	-
Fresh grass	116.84	-	-	8.81	-	-
Grass silage	74.96	-	-	11.68	-	-
Grass hay	52.91	-	-	3.53	-	-
Fresh grass silage	69.67	-	-	-	-	-
Stored grass silage	65.48	-	-	-	-	-
Fresh corn silage	62.83	-	-	-	-	-
Stored corn silage	28.00	-	-	-	-	-
Fresh grain silage	112.44	-	-	-	-	-
Stored grain silage	61.95	-	-	-	-	-

FIGURES:

Figure 1.1: Chemical structures of tocopherols and tocotrienols (Lampi, 2011)
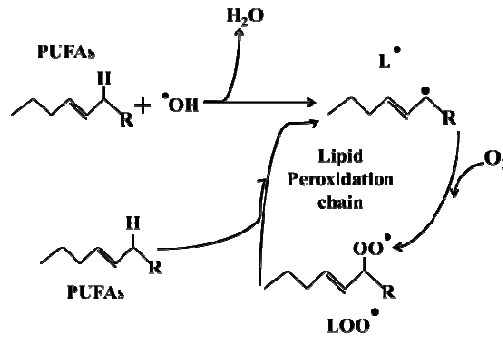
Figure 1.2: Lipid peroxidation chain reaction (adapted from Valko et al., 2004)
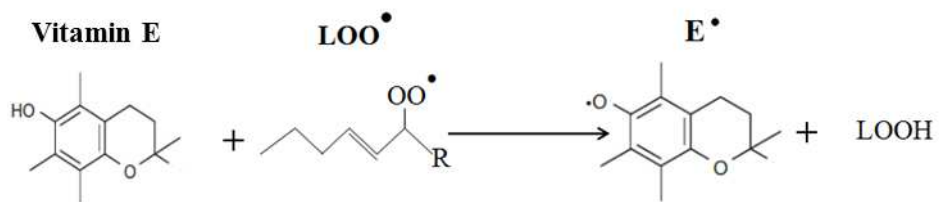
Figure 1.3: Vitamin E (α-tocopherol) can stop the lipid peroxidation chain reaction (adapted from Valko et al., 2004).
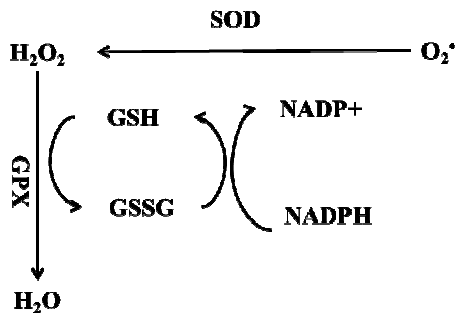
Figure 1.4: The roles of antioxidant enzymes during respiratory burst. GPX = glutathione peroxidase; GSH = glutathione; GSSG = glutathione disulfide (adapted from Jia et al., 2012).
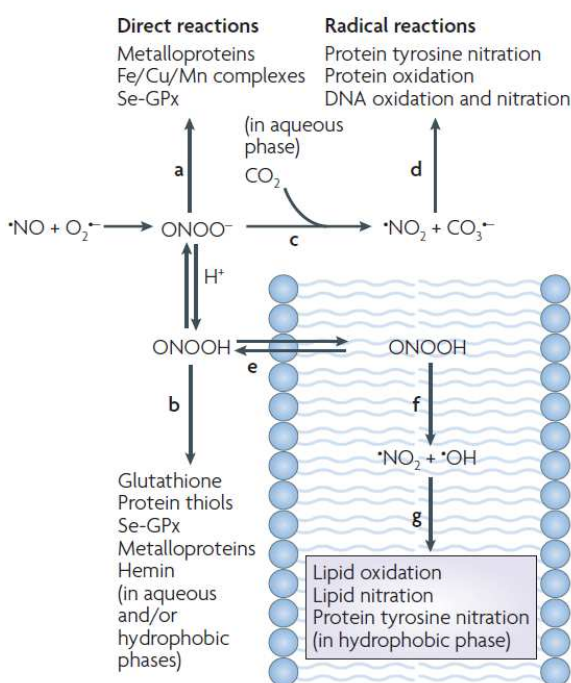
Figure 1.5: Biochemistry of peroxynitrite: Peroxynitrite anion (ONOO⁻) is in equilibrium with peroxynitrous acid (ONNOOH;pK_a=6.8) and either one can undergo direct reactions with biolecules as indicated (a and b), ONNO⁻ react with CO_2 (c), which leads to the formation of carbonate (CO_3^{-}) and nitrogen dioxide ($^.NO_2$) (d), Alternatively, ONNOH could diffuse into lipid bilayer (e), which yields $^.NO_2$ and $^.OH$ (f), which could initiate lipid peroxidation, and protein nitration process (Szabo et al., 2007).
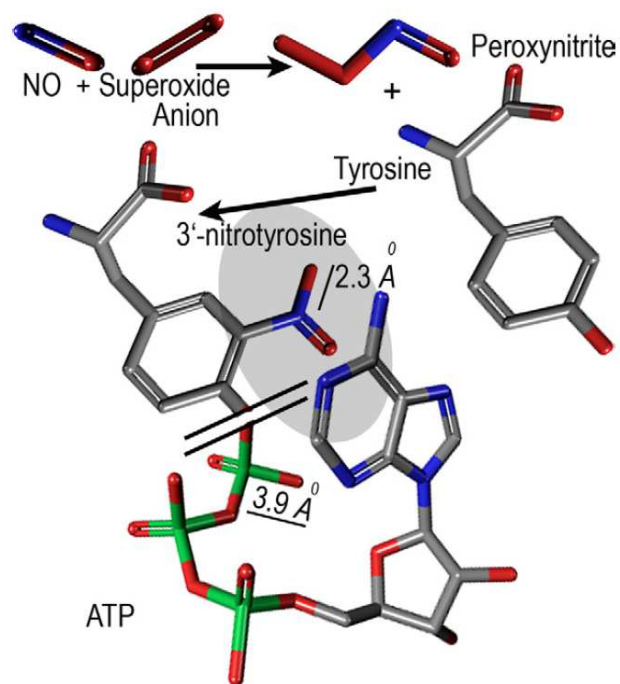
Figure 1.6: Molecular model of the reaction between ONNO⁻ and tyrosine residual, which reacts spontaneously with favorable targets such as the C-3 of phenolic ring of tyrosine. Then significantly interfere with the ability of ATP to access tyrosine on kinases such as Janus Kinase-2 (Elsasser et al., 2012)
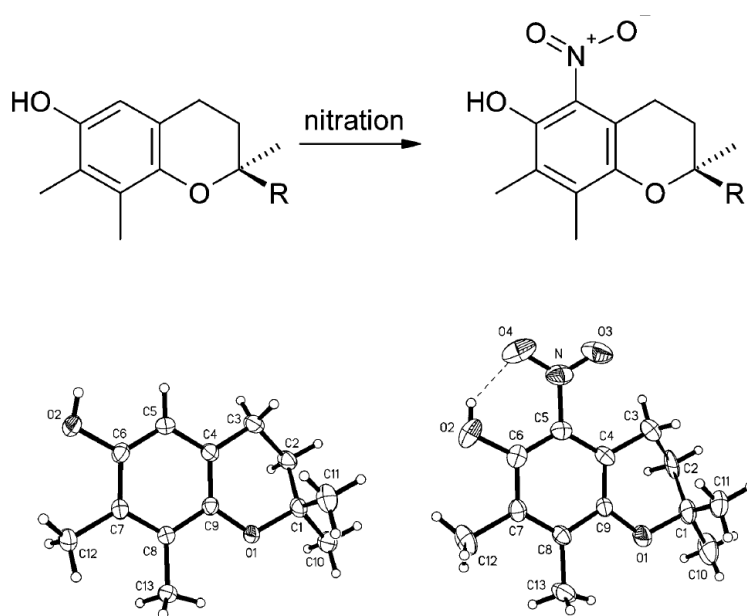
Figure 1.7: Nitration of γ-tocopherol (Patel et al., 2007)
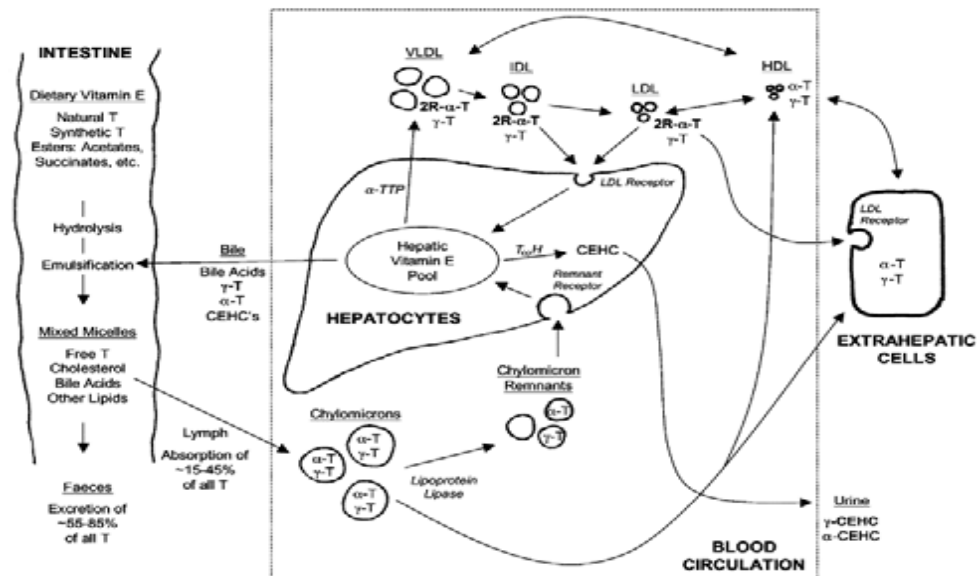
Figure 1.8: Absorption and transport of tocopherols (Frank, 2005)
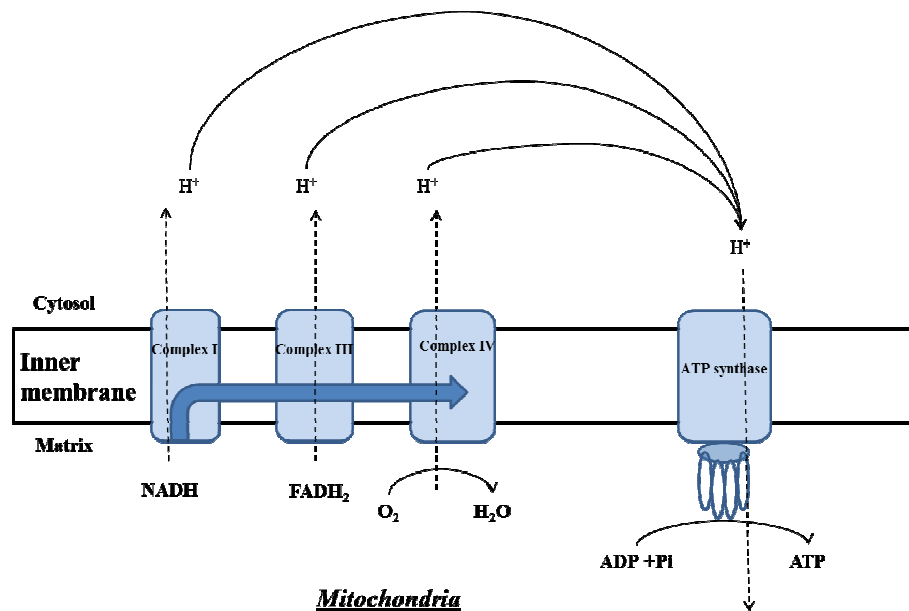
Figure 1.9: The donation of electrons for the generation of ATP during oxidative phosphorylation. Complex I: NADH-Q oxidoreductase, Complex III: Q-cytochrome *c* oxidoreductase, Complex IV: cytochrome *c* oxidase. NADH= Nicotinamide adenine dinucleotide (reduced form): FADH$_2$=Flavin adenine dinucleotide (reduced form): ATP = Adenosine triphosphate
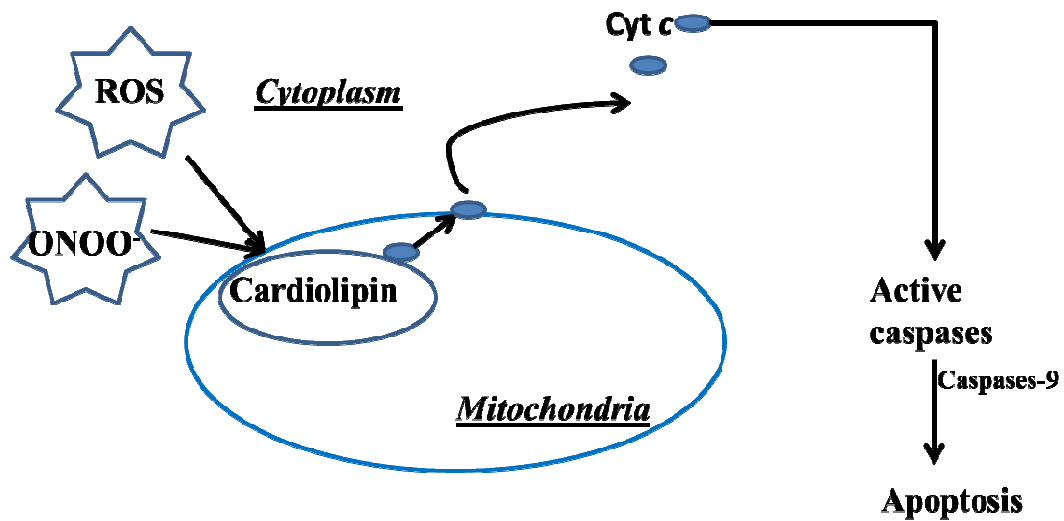
Figure 1.10: Mitochondrial cardiolipin-mediated cell apoptosis.
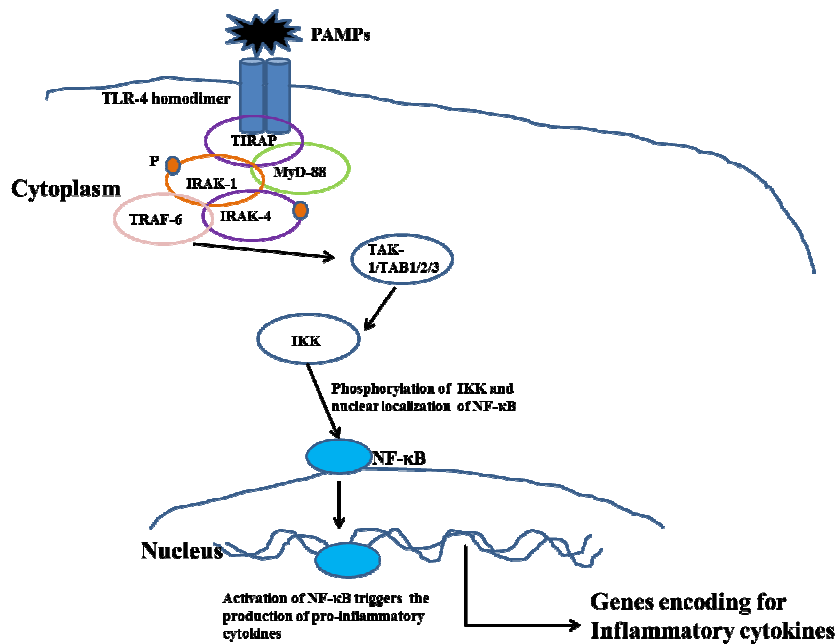
Figure 1.11: Toll-like receptor signaling pathway. PAMPs= Pathogen associated molecular pattern, TIRAP=Toll like receptor associated protein, MyD-88= Myeloid differentiation primary response 88, IRAK-1= Inerleukin-1 receptor associated kinase 1, IRAK4= Interleukin-4 associated kinase 4, TRAF-6= TNF receptor associated factor 6, TAK-1/TAB/2/3= transforming growth factor-β-activated protein kinase 1 complex, IKK= IκB kinase complex, P=Phosphorylation. (adapted from Kawai and Akira, 2006).

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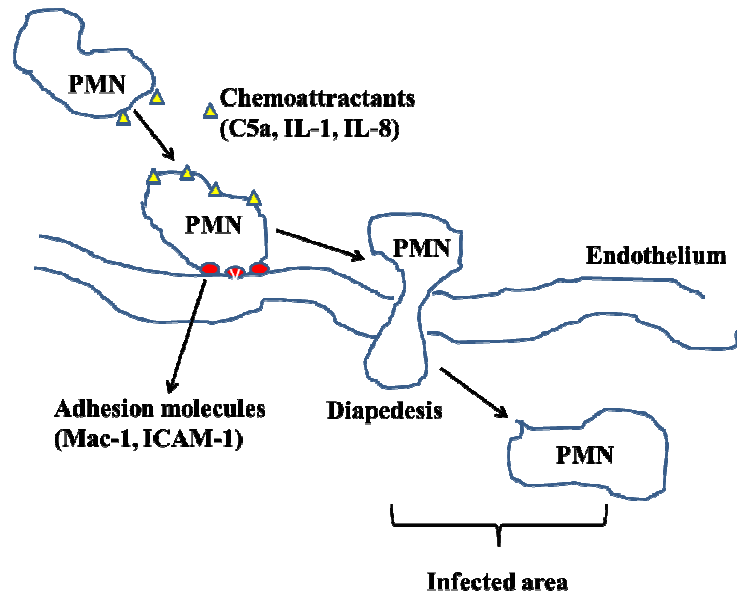
Figure 1.12: Chemotaxis in bovine PMN. PMN= Polymorphonuclear leukocytes. Mac-1= adhesion molecules, ICAM-1= Intercellular adhesion molecule 1, C5a= Complement component 5a, IL-1= Interleukin 1, IL-8= Interleukin 8
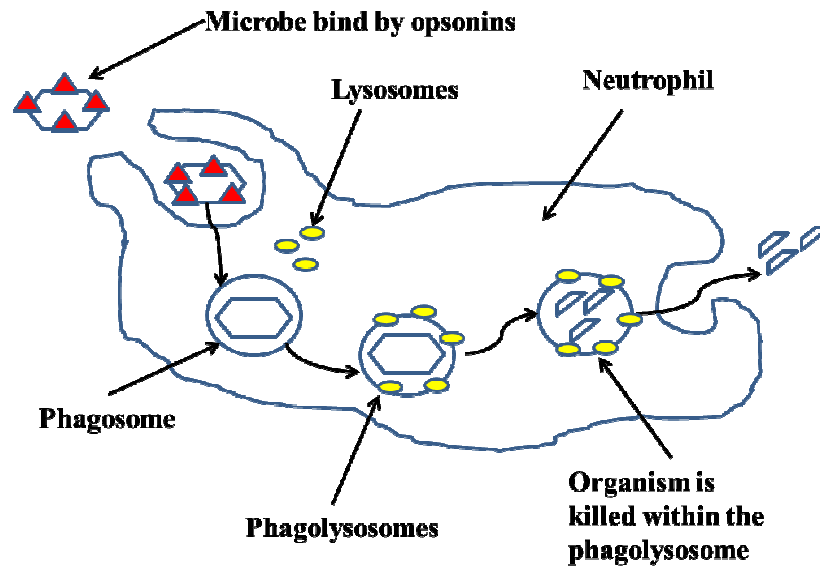
Figure 1.13: Phagocytosis in bovine PMN.

CHAPTER 2:THE DISTRIBUTION OF TOCOPHEROL ISOFORMS IN BOVINE TISSUES AND TISSUE MITOCHONDRIA VIA SHORT-TERM MIXED TOCOPHEROL OIL SUPPLEMMENTATION

ABSTRACT

Vitamin E supplements are needed to protect tissue and mitochondrial integrity. However, limited information of non-α-tocopherol distribution is available in dairy cows. The objective of this study was to determine the pattern of change in the concentrations of 4 isoforms of tocopherol (α-, β-, γ-, and δ-tocopherol) in tissue and mitochondrial fractions from bovine liver and mammary gland after short-term feeding of a vegetable-derived oil particularly enriched with γ- and δ-tocopherols (9 % α-, 1 % β-, 24 % δ-, and 62 % γ-tocopherol). Nine healthy, multiparous Holstein cows (> 90 days in milk) were assigned to dietary control (Control; n = 4) or tocopherol-fed treatment groups (n = 5; mixed tocopherol oil supplement [Tmix]; ~260 g Tmix/cow·d^{-1}, top-dressed) and fed for 9 consecutive days. On d 10 of feeding, tissues were harvested at slaughter and mitochondria were isolated. Tocopherol isoform concentrations were determined by HPLC (high-performance liquid chromatography) and data were analyzed as a complete randomized design. Non-detectable values were obtained for the concentrations of β- and δ-isoforms in tissues and mitochondria. Concentrations of γ-tocopherol increased (0.008 vs.0.03 µg/mg) in liver mitochondria from Tmix cows compared to Control cows. In tissues and mitochondria, the α-isoform concentrations were higher than the γ-isoform. The accumulated portions of the α-isoform to the γ-isoform were similar for mitochondria and tissues regardless of tissue source. Regardless of tocopherol isoform or sample source (i.e., tissue vs. mitochondria), the liver accumulated higher total

tocopherol concentrations compared to the mammary gland (8.2 vs.2.7 µg/g, respectively). In conclusion, the liver had higher tocopherol (α- and γ-isoforms) concentrations than the mammary gland suggesting that the liver may be preferred over the mammary gland for tocopherol accumulation. The α-isoform accumulated at higher concentrations than the γ-isoform in liver perhaps due to a higher affinity of tocopherol transport and binding proteins for α-tocopherol than the γ-isoform.

INTRODUCTION

Vitamin E is one of the most critical and abundant lipid-soluble antioxidant agents in circulating blood and cells of dairy cattle (Rigotti, 2007). Vitamin E divided into two main groups, tocopherols or tocotrienols, based on differences in the degree of saturation of carbon tails (Blatt et al., 2001). In the agricultural animal industry, tocopherols are the more predominate vitamin E supplement compared to tocotrienols (Brigelius-Flohe and Traber, 1999). Within tocopherols, there are four different isoforms (α-, β-, δ-, or γ-) based on the differences in the number and position of methyl groups on the chromanol head group (Jarvinen and Erkkila, 2016).

The distribution of tocopherols (α-, β-, γ- and δ) in major metabolic tissues (e.g. liver and mammary gland) in dairy cattle remains unclear. After being absorbed by enterocytes, tocopherols have two fates where they are either bound to scavenger receptor class B member-1 and packaged into high-density lipoprotein complexes that are secreted into the blood circulation (Anwar et al., 2007), or are packaged into chylomicrons and secreted into the lymph system, in the presence of sufficient TAG and ApoB (Hidiroglou et al., 1990). After either fate, tocopherols are primarily taken up by

the liver via the portal vein due to the expression of the (SEC14L2) and the α-TTP (Meier et al., 2003).

Among the tocopherol isoforms, α-isoform has been studied the most and displays preferential affinity to α-TTP and SEC14L2 compared to other isoforms (Dutta-Roy et al., 1994; Kaempf-Rotzoll et al., 2003). This affinity may partly explain the high α-tocopherol accumulation level in the liver and the high bioavailability in animals (Traber, 2007). In the liver, tocopherols will be reassembled with lipoproteins, cholesterol, and TAG then secreted into the blood for uptake into to other peripheral tissues (Mustacich et al., 2007). In dairy cattle, the liver plays a vital role with regard to tocopherol transportation and storage. However, the information regarding the distribution levels of other tocopherol isoforms (i.e. β-, δ-, and γ-) in peripheral tissues (i.e. mammary gland and muscle) remains to be elucidated in dairy cattle.

In the mitochondria, tocopherol accumulation is needed to protect mitochondrial health (Lauridsen and Jensen, 2012). Mitochondria are vulnerable to oxidative stress and nitrative stress during diseases/disorders or during periods when the metabolic stress is high (Kagan et al., 2007; Yapici et al., 2015). Previous studies have shown that α-tocopherol can be accumulated in mitochondria at high levels after feeding, which is based on the assumption that α-tocopherol is mainly co-localized with docosahexaenoic (DHA) in the non-lipid raft membrane fraction of cells (Atkinson et al., 2008; Li and May, 2003). Previous study has shown that mitochondria contain 35-40 % of the α-tocopherol present in hepatocytes in rodents, which indicates that the mitochondria is the main site to accumulate tocopherol in cells (Li and May, 2003). In addition, Mustacich et al. (2010) showed that α-tocopherol can accumulate and be metabolized in liver

mitochondria via the β-oxidation process in rodents to avoid potential toxicity. Similar to tissues described above, the information on the accumulation level of non-α-tocopherols (i.e. β-, δ-, and γ-isoforms) in mitochondria is unknown. Therefore, the objective of this study was to investigate the accumulation and distribution level of all four tocopherol isoforms (i.e. α-, β-, δ-, and γ-) in tissues (i.e. liver, mammary gland and muscle) and mitochondria (i.e. liver and mammary gland) via short-term mixed tocopherol oil supplementation (Tmix). The hypotheses are that Tmix will increase non-α-tocopherol isoforms (i.e. β-, γ-, and δ-) in tissue and tissue mitochondrial fraction after 9 d feeding.

MATERIALS AND METHODS

All procedures involving the use of live animals were approved in accordance with the regulations and guidelines set forth by the U.S. Department of Agriculture, Beltsville (# 15-009), Animal Care and Use Committee and were approved by the Institutional Animal Care and Use Committee at the University of Maryland, College Park (# 626721-1).

Animals and Treatments

Nine multiparous Holstein cows in mid-lactation (DIM = 179 ± 17) were used for this study. Dairy cows were fed either a mixed tocopherol oil supplement (Tmix) or no supplement (Control) for 9 d consecutively. Cows were housed and fed in tie-stalls each with its own feed bunk, had free access to water and were milked twice daily at 0600 h and 1800 h. Cows were fed on average 22.7 ± 2 kg DM (dry matter) divided into two equal portions with the first allotment put into the feed bunk at 0700 and the second at 1400 h. The diet fed was a TMR formulated to meet NRC requirements (2001) characterized as having 1.5 Mcal/kg of DM for net energy for lactation (NE_L) with

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17.03 % CP (crude protein)/kg DM (Table 2.1) for lactating dairy cows averaging 90 DIM and producing 40 kg of milk/d with a mean BW and body condition score of 598 kg and 3.0 on a 5-point scale (Ferguson et al., 1994), respectively. The supplementation of the daily TMR with the four isoforms of tocopherols was accomplished by adding a concentrated mixed tocopherol oil (Cargill Innovation Center, Velddriel, Netherlands; isoform composition: 9 % α-, 1 % β-, 24 % δ-, and 62 % γ-tocopherol) to a commercial molasses-sweetened calf grower diet (Farmers Cooperative Association Inc., Frederick, MD) as the carrier. To prepare this, 300 g of the concentrated mixed tocopherol oil was blended into 22.68 kg of calf grower diet to generate the Tmix. Based on a previous study (Elsasser et al, 2013) the Tmix was added at ~620 g/cow·d^{-1} into a small pocket formed in the top of the TMR at the morning feed. From the Tmix supplements, each individual cow received 740 mg α-, 80 mg β-, 5080 mg γ- and 1970 mg δ-tocopherol. Besides the Tmix supplements, each cow received 1442 ± 86 mg α-, 0 (i.e. non-detectable) mg β-, 355 ± 5 mg γ- and 89 ± 21mg δ-tocopherol from the basal diet. Cows in the tocopherol-supplemented group (n = 5) were provided the Tmix for 9 d consecutively (i.e. Tmix group). Cows in the Control group (n = 4) were fed the standard TMR for lactating dairy cows (Table 2.1; Control group). For the Control group, cows received tocopherol isoforms only as contained in the basal TMR. After 9 d of feeding, all cows were sacrificed and liver, mammary tissue, and triceps brachii muscle tissue were collected and stored at -80 ºC until further analysis.

Tissue Tocopherol Isoform Analysis

Approximately 20 g of tissue was shipped to the CTI Nutrition Laboratory (Craft Technologies, Inc., Wilson, NC) for tocopherol isoform concentration analysis via high-performance liquid chromatography (HPLC) following the protocol described by Franke

et al. (2007). Briefly, tissue samples (i.e. liver, and triceps brachii muscle) were homogenized with a polytron, and mammary tissues were homogenized with dry ice. After homogenization, tissue samples: liver (~1.4 g), muscle (~2.8 g) and mammary tissue (~2.5 g) were saponified by heating with 0.5-0.6 mL of 10 % pyrogallol in ethanol, and 2-2.5 mL 40 % KOH in methanol at 70 °C for 60 min, then neutralized with HCl. After saponification, samples were extracted 3 times with 5 mL of 80:20 (hexane:THF [tetrahydrofuran]) then washed 3 more times with water and then dried. Twenty μL of sample were injected into the HPLC system. For HPLC, the separation was performed using the ThermoSeparations Products HPLC system (Thermo-Fisher Scientific Inc., Pittsburgh, PA) with a Chromegabond Diol column (ES Industries, 3 μm, 150 x 4 mm), equipped with an FL3000 fluorescence detector and a UV2000 programmable UV/visible detector. The mobile phase was 4 % dioxane in hexane pumped at 1.0 mL/min and a run time of 20 min. The UV detector was set at 269 nm and the fluorescence detector was set at 290 nm excitations and 340 nm emissions.

Tissue Mitochondrial Isolation

Liver and mammary tissue mitochondria were isolated via the commercially available mitochondrial isolation kit from Abcam (Abcam Biotechnology company, Cambridge, UK). The protocol was adjusted to maximize the number of mitochondria isolated. Briefly, ~2 g of tissue were washed 2 times with washing buffer. Tissues were then transferred to the Potter-Elvehjem tissue grinder with addition of 8 mL of isolation buffer for tissue homogenization. The purpose of using Potter-Elvehjem grinder was to protect the integrity of the mitochondria. After grinding, homogenized tissues were transferred to a 15 mL conical tube and then centrifuged at 1,000 × *g* for 10 min at 4 °C.

After centrifugation, the supernatant was collected and transferred to a high-speed

centrifuge tube (Beckman Coulter Life Sciences, Indianapolis, IN) for 12,000 × g

centrifugation for 15 min at 4 °C. After the first centrifugation, the pellet (i.e. isolated

mitochondria) was washed with isolation buffer 2 times and then resuspended in isolation

buffer with a protease inhibitor cocktail (i.e. AEBSF, bestatin, E-64, pepstatin A and

phosphoramidon) to prevent protein degradation (Thermo-Fisher Scientific Inc.). The

pellet was then stored at -80 °C until further analysis. Protein concentration (Western

blot; Bio-Rad Laboratories Inc., Hercules, CA) and mitochondrial identification

(bicinchoninic acid [BCA] protein assay) were assessed (Supplemental Table S2.1). The

purpose of the BCA is to adjust the tocopherol concentration in the isolated

mitochondrial fraction. Mitochondrial integrity identification was performed using a

primary antibody COX-4 (Novus Biological Co., Littleton, CO) and a secondary antibody

anti-rabbit IgG HRP (horseradish peroxidase)-linked (Cell Signaling Technology, Inc.,

Danvers, MA). The protein concentration is presented in Supplemental Table S2.1.

Confirmation of Plasma Membrane and Mitochondrial Membrane Separation

The assumption that tocopherol is mainly located in the cell membrane (e.g.

plasma membrane or mitochondrial membrane) due to its chemical structure (Li and May,

2003). To confirm that the measured tocopherol isoforms are only from mitochondrial

membrane and not from plasma membrane, the following experiment was developed

(Figure 2.1). Briefly, from the tissue homogenate, 3 centrifugation products were

collected: 1) total pellet 12,000 × g (i.e. cell plasma membrane + isolated mitochondria),

2) 1,000 × g pellet (i.e. cell plasma membrane) and 3) 12,000 × g pellet (i.e. isolated

mitochondria). Primary antibody sodium potassium ATPase Alpha-1 (i.e. antibody for

cell plasma membrane) and COX-4 (antibody for isolated mitochondria) were selected. The secondary antibody anti-rabbit IgG HRP-linked and anti-mouse IgG HRP-linked were then used. Western-blot was then run on the 3 centrifugation products to identify the separation between cell plasma membrane and mitochondrial membrane for the mitochondrial isolation procedure.

Isolated Mitochondrial Tocopherol Isoform Analysis

Isolated mitochondrial fraction solutions were transferred to a 15-mL glass tube with Teflon-lined screw caps. Samples were cold-saponified overnight at 4 °C with 0.4 mL of 10 % pyrogallol in ethanol and 0.8 mL of 40 % potassium hydroxide in methanol. Samples were neutralized with HCl and then extracted 3 times with 5 mL of 80:20 (hexane:THF [tetrahydrofuran]) Samples were then washed 2 times with water, then dried the in a speed-vacuum. The dried pellet was then dissolved in 0.25 mL of hexane containing tocopherols (α-, β-, δ-, and γ-isoforms; i.e. internal control), then 20 μL of resuspended sample were injected into the HPLC system as described above.

RNA Isolation and cDNA Synthesis

Approximately 30 mg of tissue (i.e. liver, mammary gland and muscle) were used for the RNA isolation process. Total RNA was extracted using the Trizol (Thermo-Fisher Scientific Inc.) and OMEGA-E.Z.N.A. Total RNA kit (Omega Bio-tek, Inc., Norcross, GA) according to the manufacturer's instructions. Genomic DNA was removed with a DNAase I enzyme. The RNA concentration was measured using the NanoVue Plus (GE Healthcare, Piscataway, NJ). The purity of extracted RNA was calculated by absorbance ratio of 260/280 measured using a NanoVue Plus (GE Healthcare) and averaged 2.0 of cDNA synthesized with 100 ng of RNA per reaction (20

μL) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc.) and following the manufacturer's protocol. Integrity of RNA was measured with a Experion bioanalyzer (Bio-Rad Laboratories Inc.) average RNA quality indicator number (RQI) was 7.20 ± 1.50. The reaction contained 4 μL of 5X reaction mix (blend of oligo [dT] and random hexamer primers) optimized for production of targets <1 kb in length, 1 μL of reverse transcriptase (RNase H+), and variable amounts of RNA template and nuclease-free water in a 20-μL reaction volume. The reaction was performed in a T100 Thermal cycler (Bio-Rad Laboratories Inc.) with a program of 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min.

Primer Selection and Evaluation

Primer selection and efficiency curves were made following Garcia et al. (2015). Briefly, primers were selected from previous publications (Supplemental Table S2.2). Candidate primers were screened to verify proper sequence with the most updated FASTA sequence for corresponding accession number for *Bos taurus* with prefix NM_ (messenger RNA) preferred. Lyophilized primers (~25 nM DNA oligo, with a standard desalting purification method) were obtained from Integrated DNA Technologies (Coralville, IA). Primers were reconstituted with molecular grade water (G-Biosciences, St. Louis, MO). Specificity of primers was verified with a melting curve step included in the qPCR assay as described below. The efficiency of primers was verified by a 6-point serial dilution curve (1:4). Efficiency values ranged from 0.88 to 1.08 with an average value of 0.98 and an average R^2 of 0.99 (Supplemental Table S2.3).

Quantitative Polymerase Chain Reaction Analysis

Quantitative PCR was performed using SYBR green dye (PerfeCta SYBR Green fast mix) from Quanta Biosciences Inc. (Gaithersburg, MD).   Briefly, 4 μL of cDNA template (500 ρg of RNA per reaction) was combined with 5 μL of SYBR green dye and 0.5 μL each of forward and reverse primers (500 nM of each primer per reaction) in a skirted 96-well plate (VWR International, Philadelphia, PA). All samples were run in triplicate and were performed in a single plate per a given gene. The polymerase chain reaction (PCR) was performed in a CFX96 optical reaction module (Bio-Rad Laboratories Inc.) using the following cycling protocol: initial denaturation at 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 60 °C for 30 s data collection, annealing and extension, and a final step at 95 °C for 10 s before the melting curve. The melting curve step was included to verify the formation of primer dimers and of a single PCR product using an incremental temperature of 0.5 °C from 65 °C to 95 °C for 5 s. A signal melting curve was expected to indicate the purity of DNA products. The qPCR results, given as Cq values for each transcript, were analyzed using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Briefly, the Cq of each target gene was calculated and normalized by the geometric mean of the 2 selected reference genes (*LRP10* and *HPCAL1*) (Garcia et al., 2015) to generate ΔCq values. The ΔCq values without transformation were used for statistical analysis.

Statistical Analysis

The PROC MIXED procedure of SAS 9.4 (2017) was used for the statistical analysis. The data were analyzed as a complete randomized design. For the analysis of tocopherol accumulation in tissue types (i.e. liver, mammary tissue and triceps brachii muscle), tocopherol accumulation in mitochondrial fraction and the gene expression, the

model included the fixed effect of Tmix supplementation (i.e. Tmix or Control).

Separation of LSM (least squares mean) and individual comparisons for significant

effects were performed using PDIFF statement with a TUKEY adjustment in SAS. The

model was as follows:

$$Y_i = \mu + T_i + \varepsilon_i$$

where Y_i is the dependent variable; μ is overall mean; T_i is the fixed effect of Tmix at

level i (Tmix or Control) and ε_i is the residual error.

Within Tmix fed cows only (n = 5), tocopherol accumulation for different

isoforms was compared across different tissue types after 9 d of feeding, and the data

were analyzed using a 2 × 3 factorial arrangement with factors including 2 types of

tocopherol isoforms (i.e. α- or γ-tocopherol) and 3 types of tissues (i.e. liver, mammary

tissue or muscle). The fixed variables included tocopherol isoforms and tissue types.

Separation of LSM and individual comparisons for significant effects were performed

using the PDIFF statement with a TUKEY adjustment in SAS. The model was as follows:

$$Y_{ij} = \mu + T_i + W_j + TW_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable; μ is overall mean; T_i is the fixed effect of tocopherol

isoform at level i (α-, β-, γ-, or δ-tocopherol); W_j is the fixed effect of tissue type at level

j (liver, mammary or muscle); TW_{ij} is the interaction of tocopherol isoform × tissue type,

and ε_{ii} is the residual error.

Within Tmix fed cows only (n = 5), the tocopherol accumulation for different

isoforms was compared across different types of tissue mitochondria after 9 d of feeding,

and the data were analyzed using a 2 × 2 factorial arrangement with factors including 2

types of tocopherol isoforms (i.e. α- or γ-tocopherol) and 2 types of tissue mitochondria

(i.e. liver mitochondria or mammary tissue mitochondria). The fixed variables included

tocopherol isoform and tissue mitochondrial type. Separation of LSM and individual

comparisons for significant effects were performed using the PDIFF statement with a

TUKEY adjustment in SAS. The model was as follows:

$$Y_{ij} = \mu + T_i + M_j + TM_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable; μ is overall mean; T_i is the fixed effect of tocopherol

isoform at level i (α-, β-, γ-, or δ-tocopherol); M_j is the fixed effect of mitochondria type

at level j (liver, mammary or muscle); TM_{ij} is the interaction of tocopherol isoform $\times$

mitochondria type, and ε_{ij} is the residual error.

Within Tmix fed cows only (n = 5), the accumulation ratio of α- to γ-tocopherol

was compared across different sample source (i.e. tissue vs. mitochondrial fraction) and

tissue type (i.e. liver vs. mammary tissue), the ratio of the concentration of α- to γ-

tocopherol were calculated for liver and mammary tissues, and liver and mammary tissue

mitochondria. The calculation was as follows:

Ratio = (α-tocopherol concentration) / (γ-tocopherol concentration)

Then the calculated ratios were analyzed using a 2 $\times$ 2 factorial arrangement with

factors including 2 sources of the sample (i.e. tissue or mitochondrial fraction) and 2

types of tissue (i.e. liver or mammary tissue). The fixed variables included sample

sources and tissue types. Separation of LSM and individual comparisons for significant

effects were performed using the PDIFF statement with a TUKEY adjustment in SAS.

The model was as follows:

$$Y_{ij} = \mu + T_i + M_j + TM_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable; μ is overall mean; T_i is the fixed effect of sample

source at level i (tissue or mitochondria); M_j is the fixed effect of tissue type at level j

(liver, mammary or muscle); TM_{ij} is the interaction of sample source × tissue type, and ε_{ij}

is the residual error.

Within Tmix fed cows only (n = 5), the accumulation ratio of tocopherol in

mitochondrial fraction was compared to tocopherol in tissue across different tissue type

and tocopherol isoform, the ratio of the concentration of tocopherol in mitochondria to

tissue was calculated for α- and γ-tocopherol isoforms in liver and mammary tissue types

respectively. The calculation was as follows:

Ratio = (mitochondrial tocopherol concentration) / (tissue tocopherol concentration)

Then the calculated ratios were analyzed using a 2 × 2 factorial arrangement with

factors including 2 types of tocopherol isoforms (i.e. α- or γ-tocopherol) and 2 types of

tissue (i.e. liver or mammary tissue). The fixed variables included tissue type and tissue

tocopherol isoform. Separation of LSM and individual comparisons for significant

effects were performed using the PDIFF statement with a TUKEY adjustment in SAS.

The model was as follows:

$$Y_{ij} = \mu + T_i + S_j + TS_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable; μ is overall mean; T_i is the fixed effect of tocopherol

isoform at level i (α-, β-, γ-, or δ-tocopherol); S_j is the fixed effect of tissue type at level j

(liver, mammary or muscle); TS_{ij} is the interaction of tocopherol isoform × tissue type,

and ε_{ij} is the residual error.

For all models, the degrees of freedom were estimated with the Kenward-Roger

specification in the model statement. The data were presented as LSM and largest

standard error of the mean (SEM). Statistical differences were declared as significant and highly significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. Trends towards significance are discussed at $0.05 < P < 0.10$. For gene analysis, fold-change values were calculated according to (Livak and Schmittgen, 2001). Briefly, LSM (ΔCq) of a factor of interest (Tmix) was subtracted from the LSM (ΔCq) of other referential factor (Control) to generate the $\Delta\Delta$Cq value, which was linearized to a fold-change (FC) value with the formula $2^{-\Delta\Delta Cq}$.

RESULTS

Tocopherol Isoforms Accumulation in Bovine Tissues

In liver, compared with the Control group, concentrations of α-tocopherol were decreased (18.92 vs. 13.72 µg/g tissue; P=0.03; Figure 2.2A) and concentrations of γ-tocopherol were increased (0.84 vs. 2.82 µg/g tissues; P<0.01; Figure 2.2B) after 9 d of Tmix feeding. In mammary tissue, compared with the Control group, concentrations of α-tocopherol were not altered (3.57 vs. 4.05 µg/g tissues; P=0.79; Figure 2.2C) and concentrations of γ-tocopherol were increased (0.22 vs. 0.97 µg/g tissues; P=0.04; Figure 2.1D) after 9 d of Tmix feeding. In muscle, compared to the Control group, concentrations of α-tocopherol tended to decrease (5.95 vs. 5.10 µg/g tissue; P=0.09; Figure 2.2E) and concentrations of γ-tocopherol increased (0.40 vs. 0.67 µg/g tissue; P<0.01; Figure 2.2F) after Tmix feeding. Limited β- and δ-tocopherol (<0.1 µg/g tissue) concentrations were detected in the liver, mammary gland, and triceps brachii muscle in the Control and Tmix groups after 9 d of feeding.

Separation between Plasma Membrane and Mitochondrial Membrane

In the liver (Figure 2.3A), the total pellet fraction showed strong Na/K ATPase and COX-4 protein bands by Western blotting. The $1,000 \times g$ centrifugation fraction showed strong Na/K ATPase and weak COX-4 protein bands. The mitochondrial fraction showed moderate Na/K ATPase and strong COX-4 protein bands. In the mammary tissue (Figure 2.3B), the total pellet fraction showed moderate Na/K ATPase and strong COX-4 protein bands. The $1,000 \times g$ centrifugation fraction showed moderate Na/K ATPase and COX-4 protein bands. The mitochondrial fraction showed moderate Na/K ATPase and strong COX-4 protein bands.

Tocopherol Isoforms Accumulation in Bovine Tissue Mitochondria

Compared to the Control group, concentrations of α-tocopherol were not altered (0.12 vs. 0.13 µg/mg protein; P=0.78; Figure 2.4A) and concentrations of γ-tocopherol were increased (0.008 vs. 0.03 µg/mg protein; P=0.01; Figure 2.4B) in liver mitochondria of Tmix-fed cows. Very low and stable (<0.01 µg/mg protein) amounts of β- and δ-tocopherol were detected in the liver of the Control and Tmix groups after 9 d of feeding.

Accumulation Patterns of Tocopherol Isoforms in Bovine Tissues and Mitochondrial Fraction

Within the Tmix group only, the accumulation ratio of α-tocopherol to γ-tocopherol was lower in the mitochondrial fraction compared to tissue (3.73 vs. 4.69; P<0.01; Figure 2.5A). Regardless of sample source (i.e. tissue and mitochondrial fraction), this ratio was not different in liver vs. the mammary tissue (4.18 vs. 3.86; P=0.45; Figure 2.5B). The ratio of tocopherol in the mitochondrial fraction to tocopherol in tissues were not statistically different for α- and γ-tocopherol (10.86 vs. 16.04; P=0.15; Figure 2.6A) in the liver and mammary gland (12.35 vs. 14.55; P=0.53; Figure 2.6B).

The interaction of tocopherol isoform × tissue was detected ($P <0.01$). Alpha-tocopherol was higher compared with γ-tocopherol in three different types of tissue (Figure 2.7). Liver accumulated α- and γ-tocopherol more compared to muscle and mammary tissues (Figure 2.7). Mammary tissue and muscle accumulated tocopherol at similar levels after Tmix feeding. In the mitochondria, the liver accumulated more tocopherol compared with the mammary tissue (0.08 vs. 0.04 µg/mg protein; $P=0.02$; Figure 2.8A). Alpha-tocopherol accumulated at a higher level in mitochondria compared with γ-tocopherol (0.10 vs. 0.02 µg/mg protein, respectively; $P<0.01$; Figure 2.8B) after Tmix feeding.

The Effect of Tmix on Gene Expression in Bovine Tissues

The effects of Tmix on gene expression in bovine tissues are shown in Table 2.2. In the liver, Tmix increased the expression of *TTPA* (1.96 FC, $P<0.05$) coding for α –TTP in dairy cattle (Supplemental Table S2.4). Tmix decreased the expression of *NR1I3* (0.71 FC, $P<0.05$) coding for a member of transcription factors involved in tocopherol metabolism. In addition, Tmix tended to decrease the expression of *RXRα* (0.71 FC, $P=0.09$) and decreased the expression of *RXRβ* (0.68 FC, $P=0.01$) coding for transcription factors involved in tocopherol metabolism. Tmix did not alter the expression of *CYP4F2* coding for an enzyme involved in tocopherol metabolism. Tmix did not alter the expression of *NR1I2* coding for a transcription factor involved in tocopherol metabolism with RXRα and RXRβ. Tmix did not alter the expression of *NFKB1* coding for inflammatory reaction or *SEC14L2* coding for a tocopherol binding protein involved in tocopherol incorporation.

In the mammary tissue, Tmix did not alter the expression of genes (*PPARG, RXRα, RXRβ, RARα,* and *SCARB1*) coding for proteins involved in tocopherol transport,

incorporation and metabolism. Tmix did not alter the expression of *NFKB1* coding for inflammatory reaction. In muscle tissue, Tmix did not alter the expression of genes *RXRα, RXRβ*, or *NFKB1*.

DISCUSSION

Results showed that Tmix increased the γ-tocopherol concentration in bovine tissues (i.e. liver, mammary gland and triceps brachii muscle) and in liver mitochondria. Regardless of sample source (i.e. tissues or mitochondria), the concentrations of α-tocopherol were higher than γ-tocopherol after 9 d of Tmix feeding in dairy cattle. Regardless of tocopherol isoforms (i.e. mainly α- or γ-tocopherol), the liver showed the highest capacity for storing tocopherol compared to other tissues (i.e. mammary tissue or muscle) in dairy cattle. Very limited β- and δ-tocopherol were detected in tissues or tissue mitochondria in dairy cattle after Tmix supplementation. Tocopherol concentrations were more marked in the mitochondrial fraction compared to tissues. Furthermore, Tmix altered the expression of genes involved in tocopherol transport and metabolism in the liver, but not in the mammary gland and muscle.

Tmix Increased the Concentration of γ-but not α-Tocopherol in Bovine Tissues and Tissue Mitochondria

Tmix increased the concentration of γ-tocopherol in bovine tissues (i.e. liver, mammary tissue, and muscle) after a short-term Tmix supplementation. Previous study demonstrated that pure α-tocopherol supplementation increased the concentration of α-tocopherol in plasma and tissues in dairy cattle (Politis, 2012). However, very limited data are available regarding the concentration of non-α-tocopherol isoforms in dairy cattle. Alpha-tocopherol has been studied more than other tocopherol isoforms for it is

considered to have the highest bioavailability in the body due to its preferred

incorporation in the liver and stability in blood when compared with other isoforms

(Brigelius-Flohe and Traber, 1999). However, as an antioxidant, non-α-tocopherol

isoforms (i.e. β-, γ- or δ-) play a more predominate role against nitrative stress than α-

tocopherol (Wolf, 1997). In addition, γ-tocopherol is the most enriched tocopherol

isoform in plants compared to other isoforms (Jiang et al., 2001). Therefore, non-α-

tocopherol should receive as much attention as α-tocopherol regarding bioaccumulation

in animals. This study investigated the accumulation of all four tocopherol isoforms (i.e.

α- , β-, γ- or δ) after supplementing Tmix in multiple bovine tissues (i.e. liver, mammary

gland and muscle), while previous studies mainly focused on α-tocopherol in liver tissue

(Charmley et al., 1992; Debbie et al., 2010).

Compared to γ-tocopherol, Tmix did not alter the concentration of α-tocopherol in

bovine tissues, while it decreased the concentration of α-tocopherol in the liver after 9 d

of feeding. The partial explanations of these results include the Tmix contained very low

amounts of α-tocopherol isoforms and/or animals could have a potential variation of α-

tocopherol concentration in the liver. Regardless of the reduced α-tocopherol

concentrations after feeding compared to the increased concentrations in liver in previous

studies, the present study showed similar results regarding the levels of α-tocopherol in

the liver (~13-18 µg/g tissues) (Charmley et al., 1992; Eicher et al., 1997). The lack of a

response in α-tocopherol to Tmix in the mammary gland and muscle tissues is similar to

the results of others (Eicher et al., 1997). Regardless of tissue differences, α-tocopherol

showed higher concentrations compared with γ-tocopherol. Reasons could be the high

amounts of α-tocopherol in the basal diet and/or the preferred tocopherol transfer and

binding protein reacting with α-tocopherol compared with other non-α-tocopherols (Stocker and Azzi, 2000). Even though Tmix contained a limited amount of α-tocopherol compared to other tocopherol isoforms (e.g. γ-tocopherol), the basal diet still contained high amounts of α-tocopherol compared to the Tmix which contributed the major source of α-isoform in the tissues. Tocopherol will be absorbed first by the liver after small intestinal absorption (Rigotti, 2007). In the liver, TTPA and SEC14L2 play important roles in the incorporation of tocopherol. Among the four isoforms (i.e. α-, β-, γ- and δ-), the binding proteins showed higher affinity for α-isoform compared with other non-α-tocopherol isoforms, which might partly explain the high concentration of α-tocopherol in dairy cattle.

Mitochondria are cell organelles that are vulnerable to stress and may be damaged either by reactive oxygen or nitrogen species during the stress response (Finkel and Holbrook, 2000). An optimal amount of tocopherol in the mitochondria is needed to fight against stresses. The present results showed that Tmix increased the concentration of γ-tocopherol, but did not alter the concentration of α-tocopherol in liver mitochondria. Previous study has shown that pure α-tocopherol supplementation increased the concentration of the α-isoform in liver mitochondria (Lauridsen and Jensen, 2012). Based on the results of the separation of plasma membrane and mitochondrial membrane in the liver, one might conclude that the detected α- and γ-tocopherols were most likely associated with the mitochondrial membrane, based on the assumption that tocopherol located in membrane structure is due to its lipid-soluble property (Wang and Quinn, 1999). The tocopherol concentration was also measured in the isolated mammary gland mitochondria and the results were presented in the Appendices (Supplemental Figure

S2.1). Due to the limited amounts of α- and γ-tocopherol in the mammary gland in the

Control group, no statistical comparison of isoforms between Control and Tmix could be

made. Within the Tmix group, very low α- and γ-tocopherol concentrations were

detected in mammary gland mitochondria. This suggests that tocopherols could be

incorporated more readily into the liver than the mammary gland mitochondrial fraction.

Tocopherol might be detectable in mammary gland tissues as supported by the

tissue data. Beta- and δ-tocopherol isoforms were detected in tissues and mitochondria at

very low concentrations indicating that the liver or small intestine might have very

limited capability to absorb these two isoforms. Another possible explanation could be

β- and δ-tocopherol were metabolized too rapidly to be detected in the samples. Gut

samples will need to be collected to investigate the fate of β- and δ-tocopherol in the

future.   Overall, the liver mitochondria showed higher tocopherol accumulation than the

mammary gland mitochondria. Alpha-tocopherol accumulated more than γ-tocopherol in

mitochondrial fractions (i.e. liver and mammary tissues).

The ratio of Tocopherol Isoforms (α- and γ-Isoform) Accumulated in Tissue and

Mitochondria

The ratio of α-: γ-tocopherol was lower in the mitochondrial fraction compared to

tissues, which might indicate γ-tocopherol accumulated more into the mitochondrial

fraction than into tissues compared with α-tocopherol. Previous studies have

demonstrated the concentration of α-tocopherol in the mitochondrial fraction (Li and May,

2003; Traber et al., 2007). However, no available data showed the ratio of different

tocopherol isoforms in tissue and mitochondrial fractions. For γ-tocopherol, the ratio of

tocopherol in mitochondria:tissue was numerically higher when compared with α-

tocopherol, indicating that γ-tocopherol tended to accumulate more in the mitochondrial fraction than in tissues.

Regardless of tocopherol isoform, the liver and mammary gland showed similar ratios of tocopherol accumulation in the mitochondrial fraction relative to total tissue accumulation (Figures 2.5B and 2.6B). The ratio of tocopherol in mitochondrial fraction:tissues is about 10 -15 µg/mg (Figures 2.6A and 2.6B), suggesting that more tocopherol was accumulated and detected in the mitochondrial fraction than in tissues.

Tmix Altered the Expression of Genes Involved in Tocopherol Transport and Metabolism in the Liver but not in the Mammary Gland and Muscle Tissues.

Tmix increased the mRNA expression of *TTPA* (Table 2.2) in the liver which is consistent with previous studies in humans (Traber, 1996; Leonard et al., 2002). The α-TTP has been identified as the first protein involved in tocopherol transportation in animals and is mainly expressed in the liver (Arita et al., 1997). It recognizes all four tocopherol isoforms, however, it has preferred affinity to α- than other non-α-tocopherols (Yunsook, et al., 2007). This protein is also involved in maintaining tocopherol levels in plasma in animals by absorbing tocopherol into the liver. Once in the liver, tocopherol is assembled with lipoproteins (e.g. HDL or other lipids) and then secreted back into circulation system as lipid proteins complex (Drevon, 1991). Therefore, one should expect the increased expression of *TTPA* after tocopherol feeding, which is consistent with the present data. Compared to the increase in *TTPA*, the expression of *SEC14L2* was not altered by Tmix feeding. The gene of *SEC14L2* encodes for the α-tocopherol associated binding protein, which can aid in tocopherol absorption in the liver. The lack

of change in expression of *SEC14L2* might be partly associated with other non-α-tocopherols (e.g. β-, δ-, and γ-tocopherol) that exist in the liver.

Contrary to previous study, Tmix decreased the expression of *NR1I3* (i.e. *CAR*), *RXRα* and *RXRβ*, coding for nuclear receptors involved in tocopherol metabolism. Tmix did not alter the expression of *NR1I2* (i.e. *PXR*) in the liver. A previous study demonstrated that tocopherols are able to bind with PXR or CAR where they then bind with RXRα or RXRβ to initiate the metabolism of tocopherol to carboxyethyl-hydroxychromans (Traber, 2004). The current results contradict those of Traber (2004), and it may be partly attributed to the mixed tocopherol isoform supplementation or the sample collection immediately following the feeding trial. In this study, Tmix contains all four isoforms instead of examining only pure α- or γ-tocopherol as in Azzi et al. (2004). The interaction of β- or δ-tocopherol with α- or γ-tocopherol might contribute to the observed results. In addition, samples were collected immediately after the completion of the Tmix feeding trial period. The interval of time was limited so that the metabolism (i.e. the expression of genes in tocopherol metabolism) might not be detectable in liver. Decreased expression of genes associated with tocopherol metabolism (e.g. *NR1I3*, *RXRα* or *RXRβ*) might enable animals to maintain tocopherol levels. Tmix did not alter the expression of *CYP4F2*, coding for cytochrome P450 protein involved in tocopherol metabolism phase I. This might be associated with the decreased mRNA expression of *PXR*, *NR1I3* and *RXR* (upstream regulators of *CYP4F2*). Besides the liver, Tmix showed minimal effects on the expression of genes involved in tocopherol metabolism in the mammary tissue and muscle in dairy cattle.

CONCLUSIONS

This study was the first to measure the distribution of four tocopherol isoforms (i.e. α-, β-, δ-, and γ-isoform) in multiple tissues (i.e. liver, mammary gland and muscle) and tissue sources (i.e. mitochondria in the liver and mammary gland) in dairy cattle. The results indicated that short-term (~9 d) Tmix supplementation increased γ-tocopherol concentrations but did not increase the α-tocopherol concentration in three different types of tissues (i.e. liver, mammary gland and muscle) and liver mitochondria. Beta- and δ-tocopherol were detected in tissues and mitochondria at very low concentrations indicating that the liver or the small intestine might have very limited capability to absorb these two isoforms. In dairy cattle, γ-tocopherol tended to accumulate more into the mitochondrial fraction compared with tissue. Because limited amounts of β- and δ-tocopherol were detected, α- and γ-tocopherol might play predominate roles in dairy cattle as vitamin supplements. In the end, the present data provide additional information regarding the pattern of tocopherol isoforms in dairy cattle in the major tissues (i.e. liver) and mitochondria for the development of management tools regarding tocopherol supplementation in agricultural animal species.

REFERENCES

Anwar, K., J. Iqbal, and M. M. Hussain. 2007. Mechanisms involved in vitamin E transport by primary enterocytes and in vivo absorption. J. Lipid Res. 48: 2028-2038.

Azzi, A., R. Gysin, P. Kempna, A. Munteanu, Y. Negis, L. Villacorta, T. Visarius, and J.-M. Zingg. 2004. Vitamin E mediates cell signaling and regulation of gene expression. Ann. N. Y. Acad. Sci. 1031:86-95.

Arita, M., K. Nomura, H. Arai, and K. Inoue. 1997. Alpha-tocopherol transfer protein stimulates the secretion of alpha-tocopherol from a cultured liver cell line through a brefeldin A-insensitive pathway. Proc. Natl. Acad. Sci. U.S.A. 94:12437-12441.

Atkinson, J., R. F. Epand, and R. M. Epand. 2008. Tocopherols and tocotrienols in membranes: A critical review. Free Radic. Biol. Med. 44:739-764.

Blatt, D. H., S. W. Leonard, and M. G. Traber. 2001. Vitamin e kinetics and the function of tocopherol regulatory proteins. Nutrition. 17:799-805.

Brigelius-Flohe, R. and M. G. Traber. 1999. Vitamin E: function and metabolism. The FASEB J. 13:1145-1155.

Charmley, E., N. Hidiroglou, L. Ochoa, L. R. McDowell, and M. Hidiroglou. 1992. Plasma and hepatic alpha-tocopherol in cattle following oral or intramuscular supplementation. J. Dairy Sci. 75:804-810.

Drevon, C. 1991. Absorption, transport and metabolism of vitamin E. Free Radic. Res. Commun. 14:229-46.

Dutta-Roy, A. K., M. J. Gordon, F. M. Campbell, G. G. Duthie, and W. P. T. James. 1994. Vitamin E requirements, transport, and metabolism: Role of alpha-tocopherol-binding proteins. J. Nutr. Biochem. 5:562-570.

Eicher, S. D., J. L. Morrill, and J. Velazco. 1997. Bioavailability of alpha-tocopherol fed with retinol and relative bioavailability of D-alpha-Tocopherol or DL-alpha-tocopherol acetate. J. Dairy Sci. 80:393-399.

Elsasser, T.H., S. Kahl, K.M. Lebold, M.G. Traber, J. Shaffer, C. Li, and S. Block. 2013. Short-term alpha-or gamma-delta-enriched tocopherol oil supplementation differentially effects the expression of proinflammatory mediators: selective impacts on characteristics of protein tyrosine nitration in vivo. Vet. Sci. Develop. 3: 20-38.

Franke, A. A., S. P. Murphy, R. Lacey, and L. J. Custer. 2007. Tocopherol and tocotrienol levels of foods consumed in Hawaii. J. Agric. Food Chem .55:769-778.

Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. J. Dairy Sci.77:2695-2703.

Finkel, T. and N. J. Holbrook. 2000. Oxidants, oxidative stress and the biology of ageing. Nature. 408:239-247.

Garcia, M., T. H. Elsasser, Y. Qu, X. Zhu, and K. M. Moyes. 2015. Glucose supplementation has minimal effects on blood neutrophil function and gene expression in vitro. J. Dairy Sci. 98:6139-6150.

Gruber, J., S. Fong, C. B. Chen, S. Yoong, G. Pastorin, S. Schaffer, I. Cheah, and B. Halliwell. 2013. Mitochondria-targeted antioxidants and metabolic modulators as pharmacological interventions to slow ageing. Biotechnol. Adv. 31:563-592.

Hidiroglou, M., M. Ivan, and N. Hidiroglou. 1990. Kinetics of intravenously administered vitamin E in sheep and its biliary excretion. Ann. Rech. Vet. 8:228-232.

Jarvinen, R. and A. T. Erkkila. 2016. Tocopherols: physiology and health effects. PP. 303-308 in Encyclopedia of Food and Health. Academic Press, Oxford.

Jiang, Q., S. Christen, M. K. Shigenage, and B. N. Ames. 2001. Gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention. Am. J. Clin. Nutr. 74:714-722.

Kaempf-Rotzoll, D. E., M. G. Traber, and H. Arai. 2003. Vitamin E and transfer proteins. Lipidology. 14:249-254.

Kagan, V. E., J. Jiang, H. Bayir, and D. A. Stoyanovsky. 2007. Targeting nitroxides to mitochondria: location, location, location, and concentration: highlight commentary on mitochondria superoxide dismutase mimetic inhibits peroxide-induced oxidative damage and apoptosis: Role of mitochondrial superoxide. Free Radic. Biol. Med. 43:348-350.

Lauridsen, C. and S. K. Jensen. 2012. Alpha-Tocopherol incorporation in mitochondria and microsomes upon supranutritional vitamin E supplementation. Genes Nutr. 7:475-482.

Leonard, S. W., Y. Terasawa, R. V. Farese Jr, and M. G. Traber. 2002. Incorporation of deuterated RRR- or all-rac-alpha-tocopherol in plasma and tissues of alpha tocopherol transfer protein-null mice. Am. J. Clin. Nutr. 75:555-560.

Li, X. and J. M. May. 2003. Location and recycling of mitochondrial alpha-tocopherol. Mitochondrion. 3:29-38.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2deltadeltaCT method. Methods. 25:402-408.

Meier, R., T. Tomizaki, C. Schulze-Briese, U. Baumann, and A. Stocker. 2003. The molecular basis of vitamin E retention: structure of human alpha-tocopherol transfer protein. J. Mol. Biol. 331:725-734.

Mustacich, D. J., S. W. Leonard, N. K. Patel, and M. G. Traber. 2010. Alpha-tocopherol beta-oxidation localized to rat liver mitochondria. Free Radic. Biol. Med. 48:73-81.

Mustacich, D. J., A. T. Vo, V. D. Elias, K. Payne, L. Sullivan, S. W. Leonard, and M. G. Traber. 2007. Regulatory mechanisms to control tissue alpha-tocopherol. Free Radic. Biol. Med. 43:610-618.

National Research Council. 2001. Nutrient requirements of dairy cattle. 7th rev.ed. National Academy of Science ed. Washington, DC, National Academy Press.

Politis, I. 2012. Reevaluation of vitamin E supplementation of dairy cows: bioavailability, animal health and milk quality. Animal. 6:1427-1434.

Rigotti, A. 2007. Absorption, transport, and tissue delivery of vitamin E. Mol. Aspects Med. 28:423-436.

Stocker, A. and A. Azzi. 2000. Tocopherol-binding proteins: their function and physiological significance. Antioxid. Redox Signal. 2:397-404.

Traber, M. G. H. S. 1996. Vitamin E in humans: demand and delivery. Ann. Rev. Nutr. 16: 321-347.

Traber, M. G. 2004. Vitamin E, nuclear receptors and xenobiotic metabolism. Arch. Biochem. Biophys. 423:6-11.

Traber, M. G. 2007. Vitamin E regulatory mechanisms. Ann. Rev. Nutr. 27: 347-62.

Wang, X. and P. J. Quinn. 1999. Vitamin E and its function in membranes. Prog. Lipid Res. 38:309-336.

Wolf, G. 1997. Gamma-tocopherol: an efficient protector of lipids against nitric oxide-initiated peroxidative damage. Nutr. Rev. 55:376-378.

Yapici, N. B., S. Mandalapu, K. Michael Gibson, and L. Bi. 2015. Targeted fluorescent probes for detection of oxidative stress in the mitochondria. Bioorg. Med. Chem. Lett. 25:3476-3480.

Yunsook, L., and M. G. Traber. 2007. Alpha-tocopherol transfer protein: insights from alpha-tocopherol transfer protein knockout mice. Nutr. Res. Pract. 4: 247-253.

SAS User's Guide: Statistics. [9.4]. 2017. Cary,NC, SAS Inst.,Inc.

TABLES:

Table 2.1. Ingredient and nutrient composition (DM basis) of the total mixed ration[1] and Isoform concentrations in the experimental diets fed for 9 days.

Item	Amount, % of dietary DM
Ingredient	
Corn silage	31.0
Alfalfa-wheat silage	8.0
Cottonseed, whole	4.5
Citrus pulp, dehydrated	4.5
Sugar blend[2]	3.9
Alfalfa hay	3.6
Grass hay	3.6
Grain mix[3]	39.1
Nutrient content	
DM	52.2
NDF	28.1
ADF	18.4
CP	17.0
Ether extract	6.0
NFC	43.4
Ash	
Ca	0.9
P	0.3
Mg	0.5
K	1.2
ME, Mcal/kg of DM	2.5
VitaminA, kIU/kg of DM	19.7
VitaminD, kIU/kg of DM	4.9
VitaminE, IU/kg of DM	16.4

[1]The total mixed ration (52.2 % DM) with the following ingredients: corn silage (31.0 %), alfalfa: wheat silage (8.0 %), alfalfa hay (3.6 %), grass hay (3.6 %), whole cottonseed (4.5 %), dehydrated citrus pulp (4.5), sugar blend (3.9 %), and lactation grain mix (39.1 %). On a DM basis, the ration contained17.0% crude protein (CP), 18.4 % acid detergent fiber (ADF), 28.1 % neutral detergent fiber (NDF), 3.2% lignin, 6.0 % ether extract, 0.9 % calcium (Ca), 0.3 % phosphorus (P), 1.2 % potassium (K), 0.5 % magnesium (Mg), and 2.49 megacalorie (Mcal) of ME/kg..

[2]The sugar blend (3.9 % DM) with the following ingredients: crude protein (1.0 %), crude fat (0.1 %), crude fiber (0.1 %), acid detergent fiber (ADF) (0.1 %), calcium (0.75 %), phosphorus (0.1%), cobalt (8ppm), copper (138ppm), manganese (345ppm), zinc (690ppm), selenium (8.27ppm), vitamin A (3800IU/lb), vitamin D_3 (6250IU/lb), vitamin E (125IU/lb), TSI (total sugar as invert) (33.0%), DM (60.0%) and moisture (40%).

[3]The grain mix (39.1 % DM) with the following ingredients: Corn ground, finely ground (92.0 %), AminoPlus (1.24 %), Soybean meal 48 % (1.24 %), Soybean, whole, roasted (1.55 %), Soybean hulls (1.17 %), Megalac (0.75 %), LysAAmet (0.39 %), Optigen (0.42 %), Limstone (0.34 %), Salt-white (0.21 %), Magnesium oxide (0.17 %), USDA custom dairy min#4 (0.15 %), Potassium-magnesium sulfate (0.11%), Sodium bicarbonate (0.08 %), Urea 45 % (0.04 %), MFP Novus (0.04 %), Mepron M85 (0.02 %), Rumensin 90 (0.003 %), Rovimis H-2 (Biotin) (0.001 %), and Zinpro-4-Plex-C (0.001 %).

Table 2.2. Effect of Mixed tocopherol oil supplement (Tmix[1]) supplementation on the expression of genes in bovine tissues: Control (n = 4) or Tmix (n = 5). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method and presented as Fold-Change (Tmix relative to Control).

	Tmix vs. Control	
	FC	*P*-value
Liver		
CYP4F2	0.96	0.84
NFKB1	0.81	0.28
NR1I2	0.81	0.49
NR1I3	0.71	<0.05
RXRα	0.71	0.09
RXRβ	0.68	0.01
RARα	1.02	0.85
SEC14L2	0.80	0.16
TTPA	1.96	<0.05
Mammary		
NFKB1	0.99	0.90
PPARG	1.33	0.23
RXRα	0.95	0.72
RXRβ	1.17	0.16
RARα	1.15	0.77
SCARB1	0.51	0.25
Muscle		
NFKB1	0.94	0.84
RXRα	0.63	0.14
RXRβ	0.82	0.77

[1]Miexed tocopherol oil supplement (Tmix): 300 g of (9 % α-, 1 % β-, 24 % δ-, and 62 % γ-tocopherol) pure tocopherol mixed with 22.68 kg of calf grower (Farmers Cooperative Association Inc.), then fed at ~620 g/d for 9 consecutive days.

FIGURES:
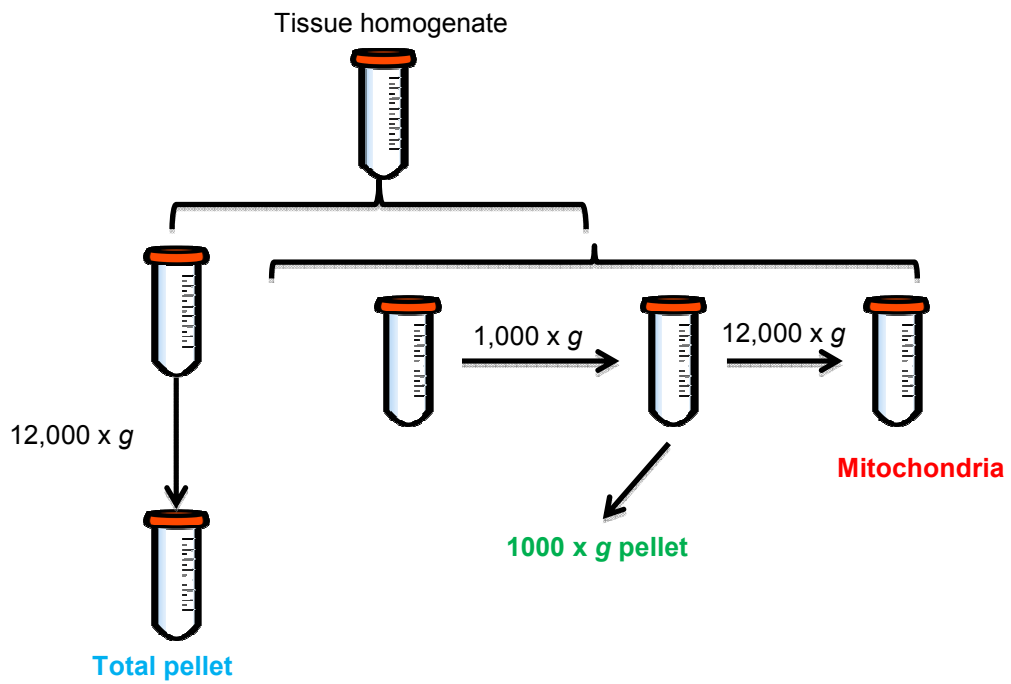
Figure 2.1. Confirmation of plasma membrane and mitochondrial membrane separation in mitochondrial fraction isolation process.
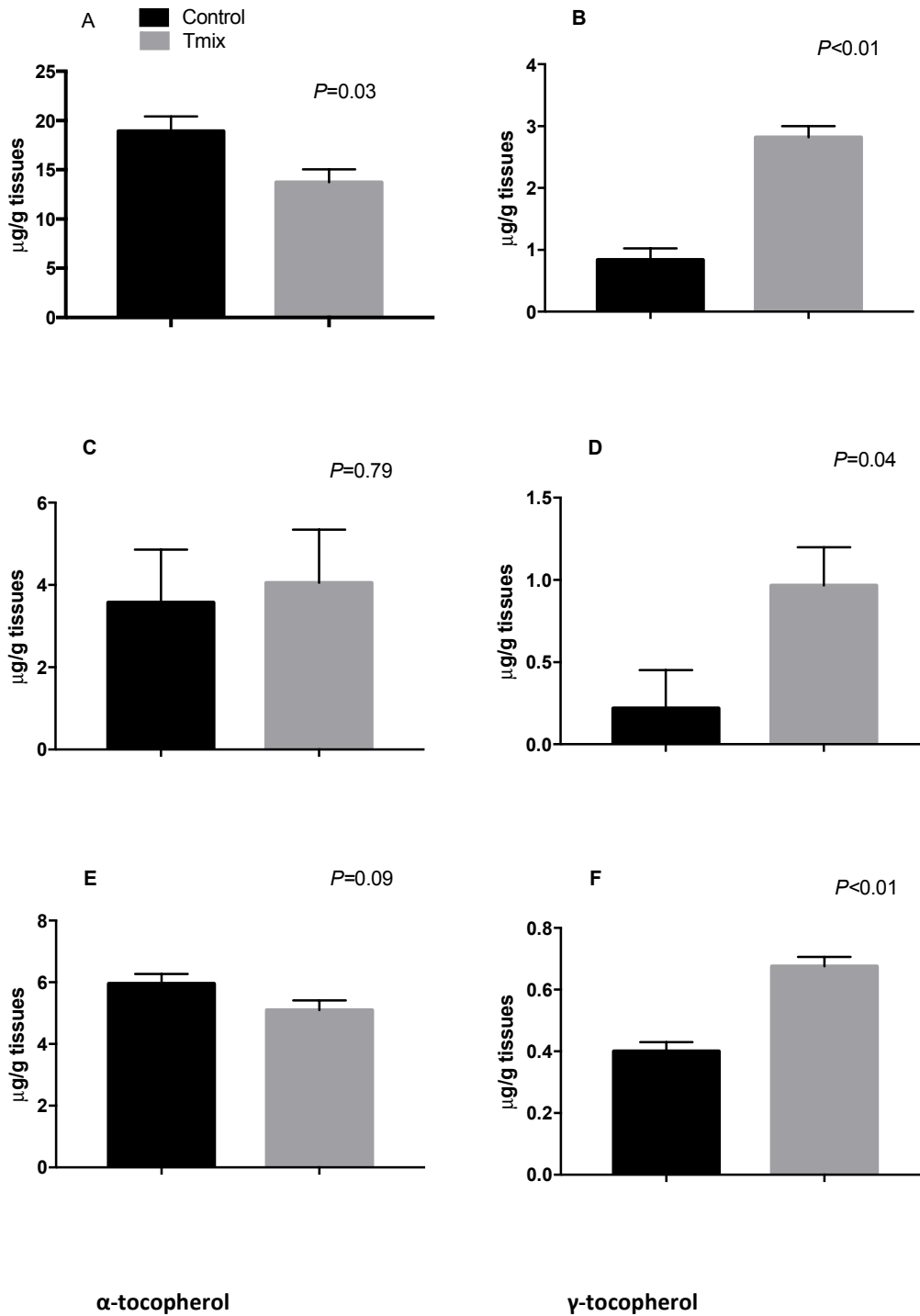
Figure 2.2. Concentrations of α-tocopherol (A) and γ-tocopherol (B) in liver, concentrations of α-tocopherol (C) and γ-tocopherol (D) in the mammary tissue, and concentrations of α-tocopherol (E) and γ-tocopherol (F) in muscle in Control vs. Tmix (mixed tocopherol oil supplement) feeding groups.
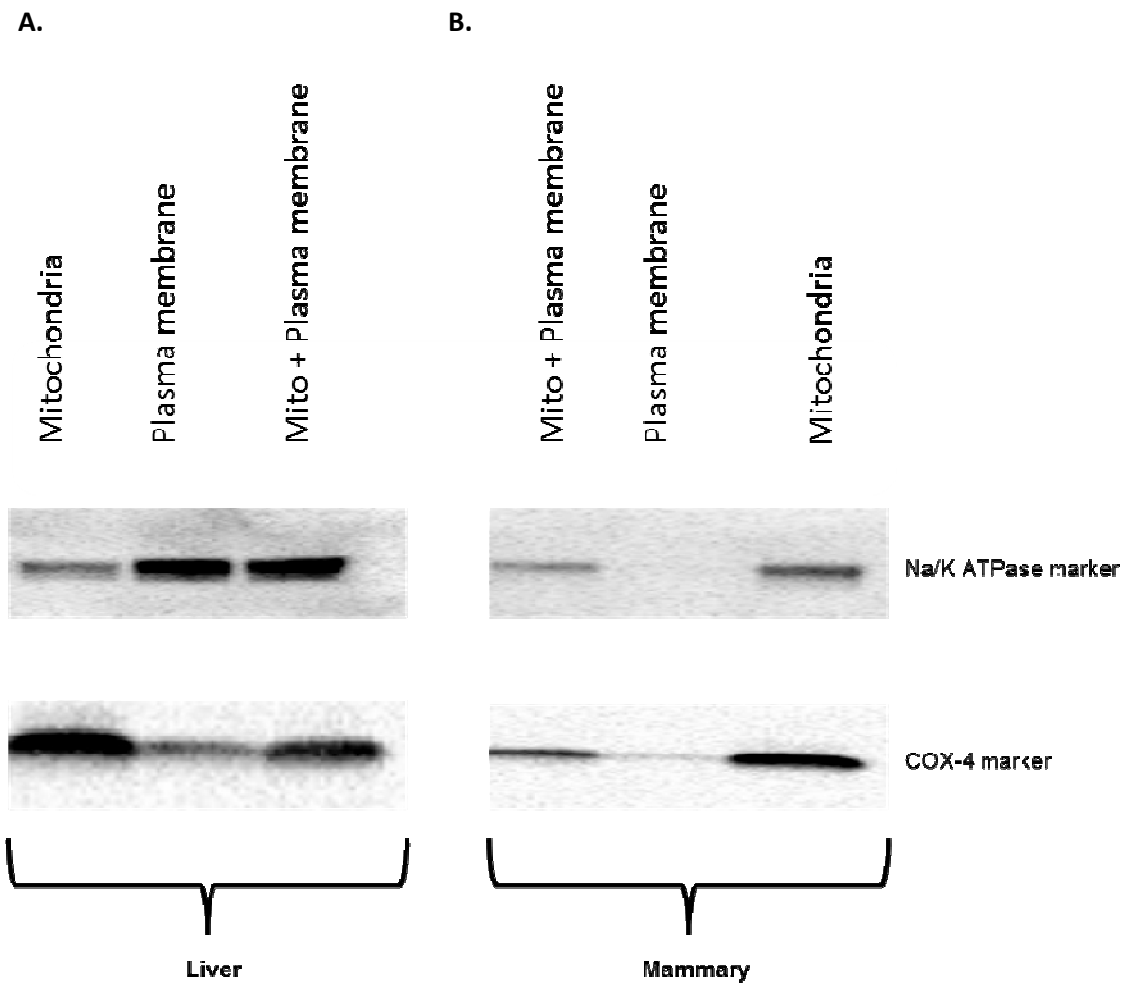
Figure 2.3. Validation of plasma membrane and mitochondrial fraction membrane separation.
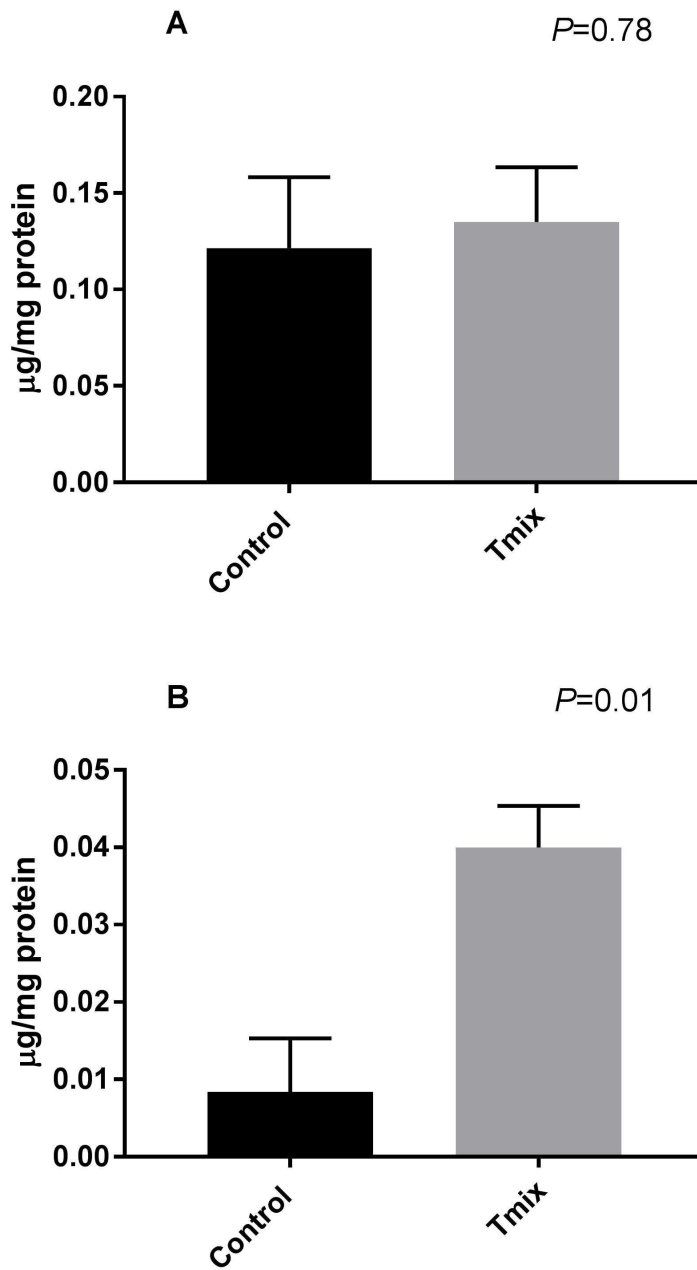
Figure 2.4. Tocopherol isoform concentration in bovine liver mitochondria: Control vs. Tmix (mixed tocopherol oil supplement) feeding groups for either α-tocopherol (A) or γ-tocopherol (B).
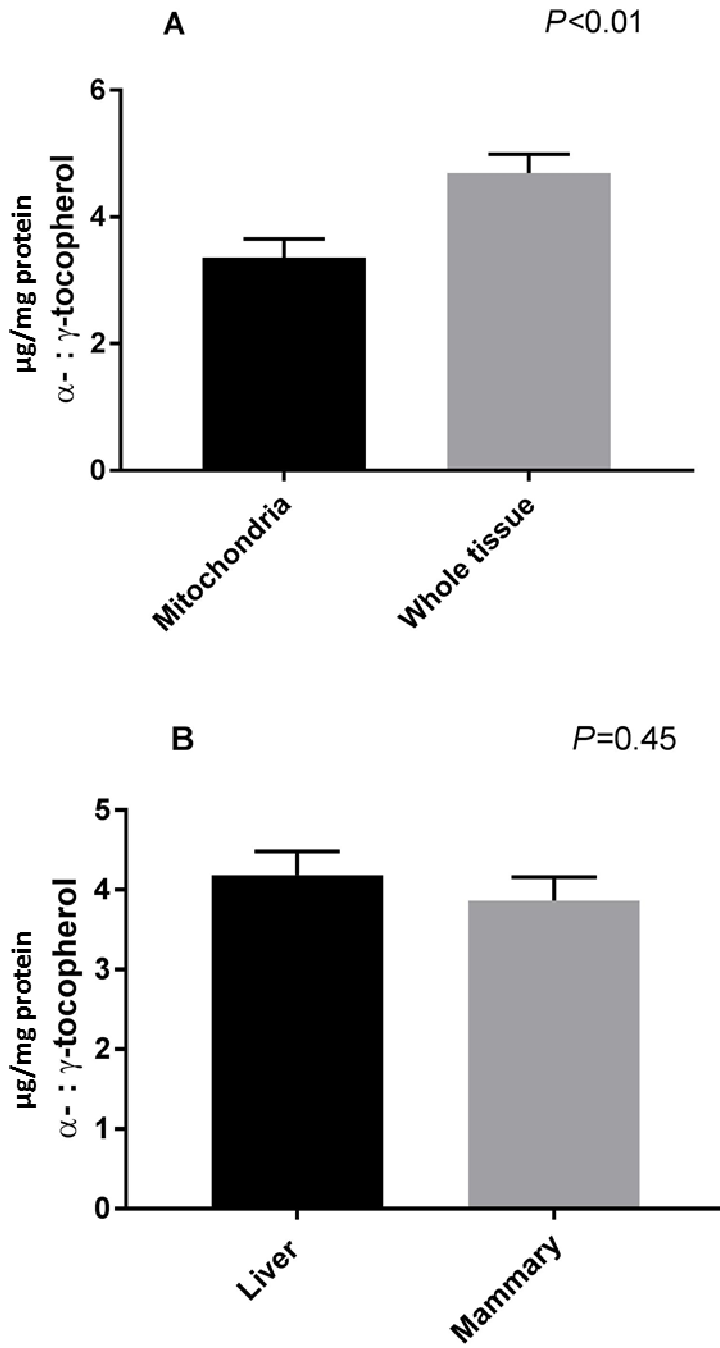
Figure 2.5. The ratio of α:γ-tocopherol in either mitochondrial fraction vs. tissue (A) or liver vs. mammary tissue (B) after 9 d of Tmix (mixed tocopherol oil supplement) feeding.
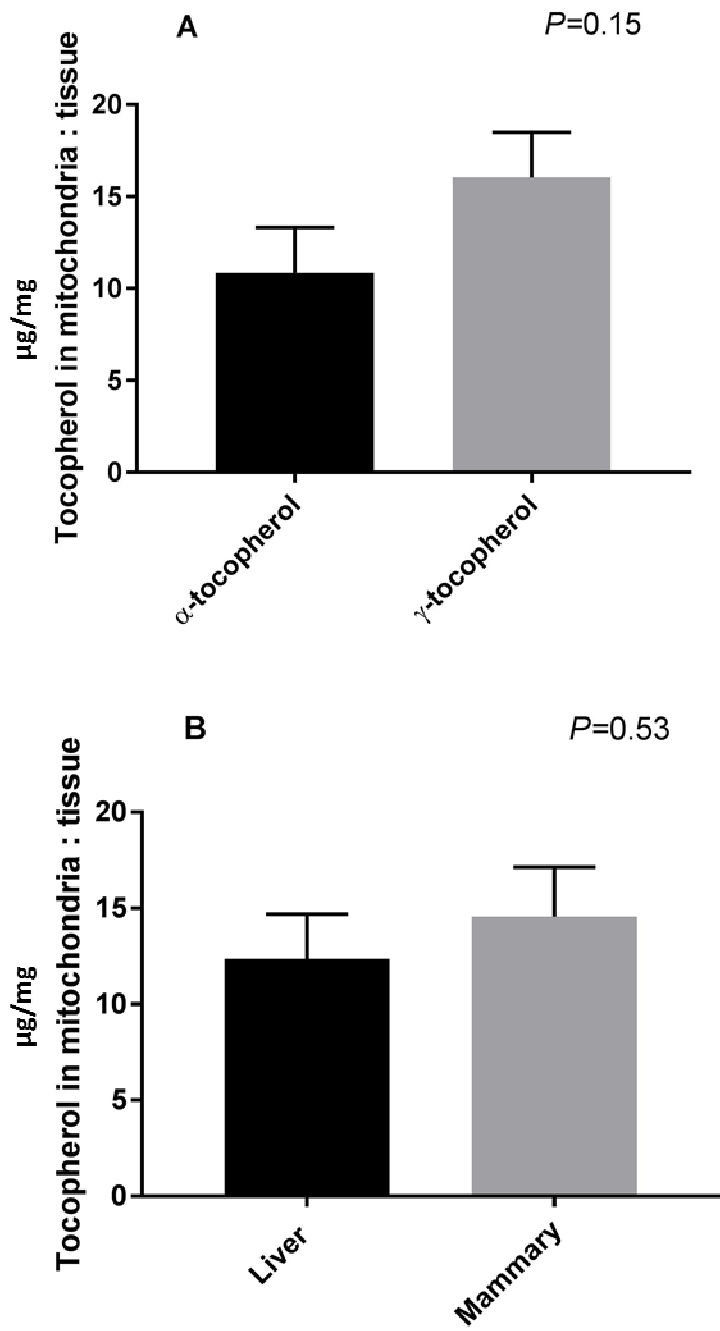
Figure 2.6. The ratio of the concentration of tocopherol in mitochondria : tocopherol in tissues for α-tocopherol vs. γ-tocopherol (A) or the liver vs. the mammary tissue (B) after 9 d of Tmix (mixed tocopherol oil supplement) feeding.
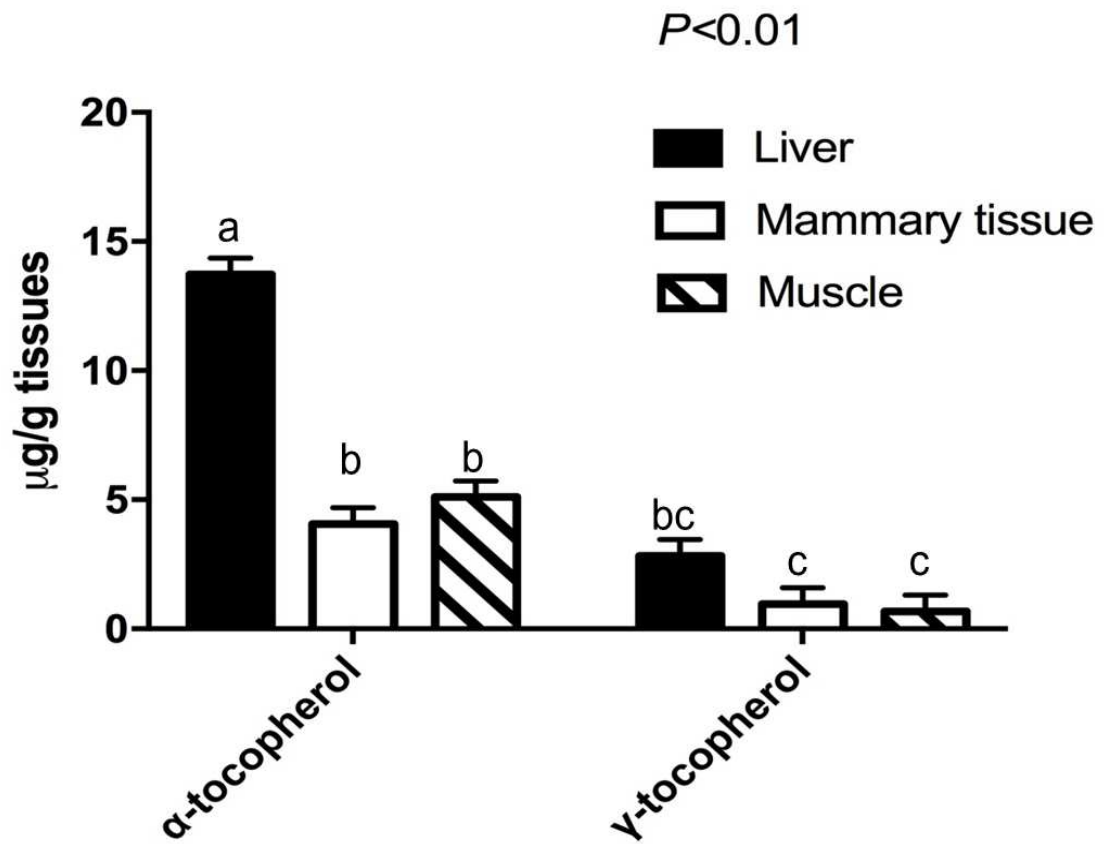
Figure 2.7. The pattern of tocopherol isoforms across different tissue types after 9 d of Tmix (mixed tocopherol oil supplement) feeding. Differing letters indicate significant differences ($P \leq 0.05$) in means comparison.

A

P=0.02

B

P<0.01
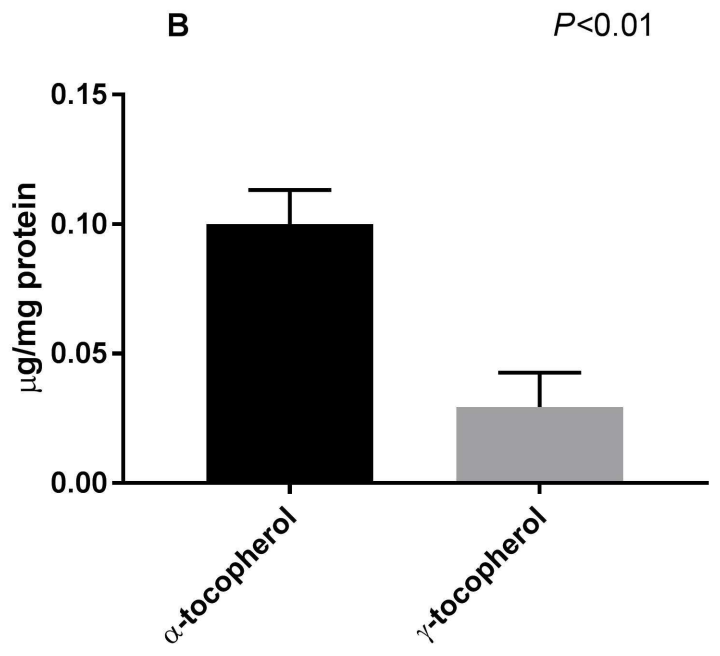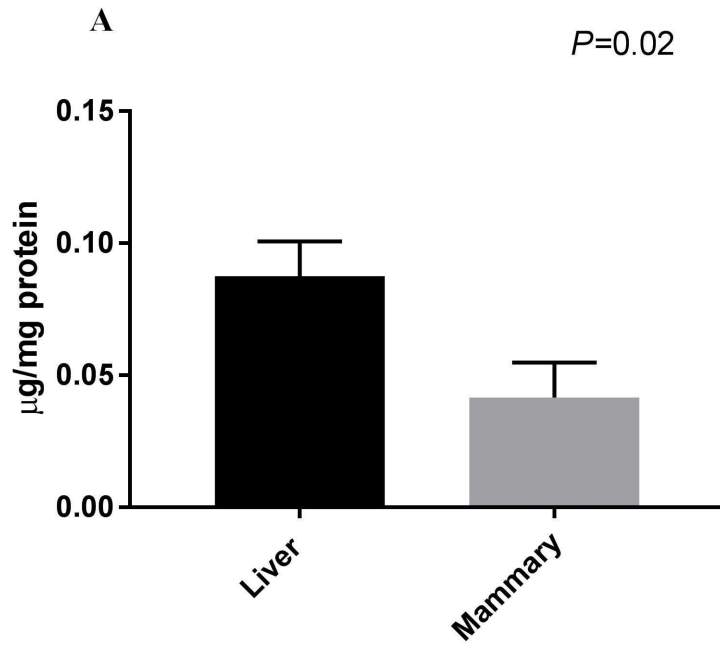
Figure 2.8. The concentration of total tocopherol in the liver or mammary tissue mitochondria (A) or the concentration of α- or γ-tocopherol in tissue mitochondrial fraction (B) after 9 d of Tmix feeding.

CHAPTER 3: THE DISTRIBUTION OF TOCOPHEROL ISOFORMS IN BOVINE MILK AND BLOOD VIA SHORT-TERM MIXED TOCOPHEROL OIL SUPPLEMENTATION

ABSTRACT

Fed over several weeks, diets supplemented with α-tocopherol will increase α-tocopherol concentrations in blood and milk. With new attention being paid to other non-α isoforms of vitamin E, how supplementation of other tocopherol isoforms affects subsequent milk and blood concentrations is poorly understood. The objective of this study was to determine the effect of feeding a mixed tocopherol oil supplement (Tmix) and the frequent pattern of change of 4 isoforms (α-, β-, γ-, and δ) of tocopherol in blood and milk from lactating dairy cows. Twelve multiparous Holstein cows in mid-lactation were used to evaluate the effect of Tmix feeding (i.e. d 0 v d 7 of feeding). A subset (n = 4) were used for the daily measurement. Furthermore, a subset (n = 2) were used for more frequent measurements (every 8 h from d 2 to 5 of Tmix feeding). Composite milk (~25 mL) and whole blood (~15 mL) samples were collected throughout the study. Tocopherol isoform concentrations were determined by high-pressure liquid chromatography. Data were analyzed in a complete randomized design with repeated measures. Significance was declared at $P \leq 0.05$. Gamma and α-tocopherols in blood increased by d 1 and d 2, respectively, after feeding with peak concentrations achieved by d 5 (3.6 ± 0.2 µg /mL for γ- and 14.1± 0.5 µg/mL forα-tocopherol) when compared to d 1 (0.59 ± 0.2 µg/mL for γ- and 10.0 ± 0.5 µg/mL for α-tocopherol). In milk, γ- and α-tocopherol concentrations were elevated by d 2 (0.26 ± 0.04 µg/g) and d 3 (0.72 ± 0.05 µg/g), respectively, compared to d 1 (0.06 ± 0.04 µg/g and 0.52 ± 0.05 µg/g, respectively).

No significant change in blood or milk was detected for the more frequent sampling (i.e. every 8 hour for 52 h). Non-detectable values were obtained for β- and δ-tocopherol in blood and milk, respectively. The data illustrate that ~5 d of Tmix feeding at the level used may be sufficient to reach a higher stabilized range of concentrations of the two measurable isoforms in milk and blood in the lactating Holstein. The data established that this experimental design is adequate towards the further refinement of experiments that will be valuable towards characterizing the kinetics and biological value of α- and γ-tocopherols. Additional sampling at the gut level may be informative in determining the fate of β- and δ-tocopherol.

INTRODUCTION

Vitamin E has been used as a feed supplement for dairy cattle for decades (Baldi, 2005). Dietary α-tocopherol acetate or succinate are the synthetic isoforms most commonly used in the industry by the National Research Council (NRC, 2001). Previous studies in dairy cattle have shown that 7 μM (i.e. ~3 μg/mL) of vitamin E (α-tocopherol) is the average cut-off value in blood, and inadequate vitamin E (α-tocopherol) concentration in blood (< 7 μM: ~3 μg/mL) may partially explain the increased risk of metabolic and infectious diseases/disorders in dairy cows shortly after calving (Mudron et al., 1997; Weiss et al., 1997). Recent studies also have shown that serum vitamin E (i.e. α-tocopherol) depletion (< 7 μM) during the transition from later pregnancy to early lactation might contribute to the development of metabolic disorders such as left displaced abomasum and retained placenta (Qu et al., 2013; Qu et al., 2014). Therefore, it is essential to maintain the adequate vitamin E level in the blood to support animal health. However, among those four tocopherol isoforms of vitamin E, very limited

information of non-α-tocopherol (i.e. β-, δ-, and γ-tocopherol) in blood is available

compared to the α-isoform in dairy cattle. Non-α-tocopherols differ from α-tocopherol

by the number and position of methyl groups on the chromanol ring (Combs and

McClung, 2017). The various isoforms play different roles in maintaining animal health.

Alpha-tocopherol is mainly protective against oxidative stress, while non-α-tocopherols

are primarily protective against nitrative stress (Cobb and Cole, 2015). In addition, γ-

tocopherol is the major form of vitamin E in many plant seeds but has drawn little

attention compared to α-tocopherol (Jiang et al., 2001). The possible reasons for this lack

of attention could be the high and persistent concentration of the α-isoform detected in

animals, and that non-α-tocopherols are metalized too quickly to be detectable in blood

(Traber et al., 1999). However, to our current knowledge, no available data show the

hourly and daily changes of non-α-tocopherol concentrations in blood after feeding

tocopherol supplements to indicate their availability.

Achieving a sufficient concentration of tocopherol (i.e. α-, β-, δ-, and γ-tocopherol)

in milk is also desirable for dairy cattle production. Previous study has shown that

increasing the concentration of α-tocopherol in milk could help to improve the oxidative

stability of milk (Weiss and Wyatt, 2003). In addition, previous study has shown that

giving dietary α-tocopherol increases its concentration in milk. Besides keeping

oxidative stability, adequate tocopherol (i.e. α-, β-, δ-, and γ-tocopherol) concentration is

also essential to maintain high quality of colostrum (Parrish et al., 1947). Previous study

has demonstrated that dietary α-tocopherol helped to protect against mastitis and improve

milk quality by lowering the somatic cells count (SCC) in bulk tank milk (Hogan et al.,

1993). Similar to blood data, the major isoform of tocopherol evaluated in milk was α-

tocopherol. Limited information is available regarding non-α-tocopherol (i.e. β-, δ-, and γ-tocopherol) in milk. Therefore, the main objective of this study was to investigate the effect of Tmix and the more frequent patterns of changes of all four tocopherol isoform concentrations in blood and milk in response to short-term Tmix supplementation. The second objective of this study was to combine the data in blood and milk with the data in tissue and mitochondrial fractions (Chapter 2) to construct the dynamic pattern change of all four tocopherol isoform concentrations in dairy cattle and provide feeding strategies for vitamin E supplementation in agricultural animal species. The hypotheses are that Tmix will increase non-α-tocopherol isoforms (i.e. β-, γ-, and δ-) in blood and milk after 7 d feeding.

MATERIALS AND METHODS

All procedures involving the use of live animals were approved in accordance to the regulations and guidelines set forth by the U.S. Department of Agriculture, Beltsville (# 15-009), Animal Care and Use Committee and were approved by the Institutional Animal Care and Use Committee at the University of Maryland, College Park (# 626721-1).

Animals and Treatments

Twelve multiparous Holstein cows in mid-lactation (179 ± 17 DIM) were used to evaluate the effect of Tmix feeding (i.e. d 0 v 7 d of feeding). A subset (n = 4) were used for the daily measurement. Furthermore, a subset (n = 2) were used for a more frequent measurement (i.e. every 8 h from 2 to 5 d of Tmix feeding). None of the cows displayed any clinical signs of disease and collectively had an average composite milk SCC of 25,000 ± 16,000 cells/mL before enrollment. Cows were housed and fed in tie-stalls each

with its own feed bunk, had free access to water and were milked twice daily at 0600 h and 1800 h. Cows were fed a TMR ad libitum (Table 2.1). Cows were fed on average 22.7 ± 2 kg DM divided into two equal portions with the first allotment put into the feed bunk at 0700 and the second at 1400 h. All cows were fed the Tmix for 7 consecutive days.

Blood and Milk Sample Collection

Jugular blood was collected from all cows after the morning milking and prior to the morning feeding into 15-mL EDTA tubes (Becton Dickinson Vacutainer System, Franklin Lakes, NJ). Plasma was collected from EDTA tubes after centrifugation at 1300 $\times g$ for 15 min at 4 °C and stored in 1.5-mL tubes at -20 °C for future tocopherol isoform analysis. Milk samples (~20 mL) were collected into 25-mL conical tubes (VWR International, Philadelphia, PA). Milk samples were stored at 4 ºC for future tocopherol isoform analysis.

Blood and Milk Tocopherol Isoform Analysis

For blood samples, 300 µL of plasma was mixed with 300 µL of water containing EDTA (10 mg/L) and ascorbic acid (100 mg/L). Samples were precipitated with 600 µL of ethanol containing tocopherol (i.e. α-, β-, δ-, and γ-isoform) as the internal standard and extracted 2 times with 2 mL of hexane. The combined hexane extracts were vacuum dried and reconstituted in 100 µL of hexane. Twenty µL of prepared solutions were injected into the HPLC system. For milk samples, ~ 2 g of milk sample was saponified: 0.4 mL pyrogallol in ethanol, 1 mL 40 % KOH in methanol at 70 ºC for 45 min. Samples were extracted 3 times with 5 mL of (80 hexane: 20 THF [tetrahydrofuran]), then washed 1time in 20 % methanol. The hexane extracts were vacuum dried and reconstituted with

0.5 mL of hexane containing tocopherol (i.e. α-, β-, δ-, and γ-isoform) as the internal

standard. Twenty μL of prepared solutions were injected into the HPLC system.

Separation was performed using the ThermoSeparations Products HPLC system

(Thermo-Fisher Scientific Inc., Pittsburgh, PA) with a Chromegabond Diol column (ES

Industries, west Berlin, NJ, 3μm, 150 x 4mm), equipped with an FL3000 fluorescence

detector and a UV2000 programmable UV/visible detector. The mobile phase was 4 %

dioxane in hexane pumped at 1.0 mL/min and a run time of 20 min. The UV detector was

set at 269 nm and the fluorescence detector was set at 290 nm excitations and 340 nm

emissions.

Statistical Analysis

For the effect of Tmix (i.e. 0 and 7 d), the class variable included cow and Tmix

supplementation. Cow was used as a random term. The model included the fixed effect

of Tmix supplementation as follows:

$$Y_i = \mu + T_i + \varepsilon_i$$

where Y_i is the dependent variable; μ is overall mean; T_i is the fixed effect of

Tmix supplementation at level i (α-, β-, γ-, or δ-tocopherol), and ε_i is the residual error.

For daily and every 8 h measurements, data were analyzed as repeated-measures-in-time

ANOVA using PROC MIXED of SAS 9.4 (2017). The variance-covariance structure of

repeated measures was modeled using the heterogeneous first-order autoregressive

variance-covariance matrix based on smallest AIC (akaike information criterion) value.

For daily measurements only, d 0 was used as a covariate. The model included the fixed

effect of sampling time. The model was as follows:

$$Y_i = \mu + S_i + \varepsilon_i$$

where Y_i is the dependent variable; μ is overall mean; S_i is the fixed effect of time at level i (daily: 0, 1, 2, 3, 4, 5, 6, or 7; hour: 0, 8, 16, 24, 32, 40, 48 or 52h) and ε_i is the residual error.

For all models, the degrees of freedom were estimated with the Kenward-Roger specification in the model statement. The data were presented as LSM and largest SEM. Statistical differences were declared as significant and highly significant at $P \leq 0.05$ and $P < 0.01$, respectively. Trends towards significance are discussed at $0.05 < P < 0.10$.

RESULTS

The Effect of Tmix Feeding

Tmix increased the concentration of α-tocopherol (11.19 vs. 14.89 µg/mL; Figure 3.1 A), the concentration of γ-tocopherol (0.63 vs. 3.05 µg/mL; Figure 3.1 B), and the concentration of total-tocopherol (11.83 vs. 18.01 µg/mL; Figure 3.1 D) after 7 d of Tmix feeding in blood plasma. Even though a very low concentration of β-tocopherol was measured, Tmix increased its concentration (0.02 vs. 0.05 µg/mL; Figure 3.1 C) in blood after 7 d of feeding. Feeding of Tmix increased the concentrations of α-tocopherol (0.61 vs. 0.95 µg/mL; Figure 3.2 A), γ-tocopherol (0.23 vs. 0.56 µg/mL; Figure 3.2 B) and total-tocopherol (0.81 vs. 1.53 µg/mL; Figure 3.2 C) in milk after 7 d.

Daily Measurements of Tocopherol Isoforms

The concentration of α-tocopherol in blood plasma was increased after Tmix feeding and reached peak concentration at d 5 (14.10 µg/mL; Figure 3.3 A) compared to d 1 and kept consistent until d 7. The concentration of γ-tocopherol in blood plasma was increased after Tmix feeding and reached to the peak concentration at d 5 (3.60 µg/mL; Figure 3.3 B) compared to d 1, then dropped at d 6 (3.00 µg/mL; Figure 3.3 B) and kept

constant until d 7. The concentration of total-tocopherol (i.e. α- and γ-tocopherol) in blood plasma was increased after Tmix feeding and reached peak concentration at d 5 (17.80 µg/mL; Figure 3.3 C) compared to d 1 and kept constant until d 7. Limited β- and δ-tocopherol were detected in blood plasma. The concentration of α-tocopherol in milk was increased after Tmix feeding and reached peak concentration around d 4 (0.84 µg/mL; Figure 3.4 A) compared to d 1 and kept constant until d 7. The concentration of γ-tocopherol in milk was increased after Tmix feeding and reached peak concentration around d 4 (0.37 µg/mL; Figure 3.4 B) compared to d 1. At d 7, γ-tocopherol in milk showed the numerically highest concentration (0.48 µg/mL; Figure 3.4 B). The concentration of total-tocopherol (i.e. α- and γ-tocopherol) in milk was increased after Tmix feeding and reached peak concentration around d 4 (1.21 µg/mL; Figure 3.4 C) compared to d 1. At d 7, total-tocopherol in milk showed the numerically highest concentration (1.47 µg/mL; Figure 3.4 C).

Frequent (every 8h) Measurements of Tocopherol Isoforms

After 2 d of Tmix feeding, the concentration of α-tocopherol in blood plasma was not altered through the 8 sampling time points (Figure 3.5 A). After 2 d of Tmix feeding, the concentration of γ-tocopherol in blood plasma was not altered through the first 3 sampling time points (i.e. 0, 8 and 16 h), dropped between 16 and 24 h (Figure 3.5 B), then remained constant. Very low β-tocopherol concentration was detected after 2 d of Tmix feeding (Figure 3.5 C) and the concentration was not altered through the 8 sampling time points. The concentration of total tocopherol (i.e. α-, β- and γ-tocopherol) (Figure 3.5 D) in blood plasma was not altered through the 8 sampling time points.

After 2 d of Tmix feeding, the concentration of α-tocopherol in milk was not altered through the 5 sampling time points (Figure 3.6 A). After 2 of Tmix feeding, the concentration of γ-tocopherol in milk was not altered through the first 4 sampling time points, and the peak concentration was detected at 48 h (0.85 µg/mL; Figure 3.6 B). After 2 d of Tmix feeding, the concentration of total-tocopherol in milk was not altered through the 5 sampling time points (Figure 3.6 C).

DISCUSSION

Tmix Increased α- and γ-Tocopherol Concentrations

Tmix increased α- and γ-tocopherol concentration after 7 d of Tmix supplementation in milk and blood. Very low and stable concentrations of β-tocopherol were detected before and after Tmix feeding. Non-detectable amounts of δ-tocopherol were observed. Tocopherols (e.g. α- or γ-tocopherol) were detected more in blood than milk, which is consistent with previous studies (Charmley et al., 1993; St-Laurent et al., 1990; Weiss and Wyatt, 2003). Even though tocopherol concentrations were low in milk, the tocopherol concentrations (0.2 ~ 0.95 µg/g milk) were within normal ranges from previous studies (0.2 ~ 1.10 µg/g milk) (Weiss and Wyatt, 2003; Meglia et al., 2006). This might indicate that Tmix could compensate of γ-tocopherol in milk and blood, also reach the animal's vitamin E supplements requirement, which supported by the daily measurements data. A previous study also measured the concentration of cholesterol in the blood to indicate α-tocopherol concentration change in blood (Traber and Jialal, 2000). Because cholesterol is primarily in the high density lipoprotein fraction, which is need to transport tocopherols (Herdt and Smith, 1996). In this study, no differences were detected regarding the total cholesterol concentration in blood (refer to Chapter 4), which

might indicate Tmix did not alter α-tocopherol concentration due to the existence of other tocopherol isoforms.

Tmix Increased the Daily α- and γ-Tocopherol Concentrations

Previous study has demonstrated that increasing the amount of dietary α-tocopherol increased its concentration in blood and milk (Lindqvist et al., 2011). An increasing pattern between amount of tocopherol fed and tocopherol concentration in blood and milk has been tested (Weiss and Wyatt, 2003). However, the information of other tocopherol isoforms (e.g. γ-tocopherol) and the time pattern changes of tocopherol concentrations in milk and blood are still unknown. The data showed daily increases of α- and γ-tocopherol concentration in blood plasma and milk via Tmix supplements. The increasing pattern of γ-tocopherol was more marked compared to α-tocopherol in blood especially within the first 2 d of Tmix supplementation. This difference between α and γ might demonstrate that Tmix contributes more γ- than α-tocopherol into the blood. Prior to Tmix feeding, α-tocopherol concentrations were higher compared to γ-tocopherol which demonstrates that there was a high level of α-tocopherol accumulation in blood before Tmix feeding but not much of γ-tocopherol. Tmix could significantly increase γ-tocopherol in blood plasma.

Combined with the liver data (Chapter 2) one might conclude that during the first couple of days (i.e. d 1 – d 4), the peak concentration in blood for tocopherol (i.e. α- and γ-tocopherol) concentration was not reached. This may be partly explained by the rapid liver absorption of tocopherol (i.e. α- and γ-tocopherol) initially until peak concentration in liver was reached (13.72 and 2.82 µg/g tissues) hence reflecting concentrations in blood. This observation might support the proposed tocopherol accumulation mechanism

(i.e. starting accumulation from liver to blood and other peripheral tissues) and the importance of liver in tocopherol transportation in animals (Jañrvinen and Erkkila, 2016). Limited quantities of β- and δ-tocopherol were detected in blood and milk in dairy cows.

Frequent (every 8 h) Measurements did not Alter the α- and γ-Tocopherol Concentrations.

Limited changes of tocopherol isoforms (i.e. α-, β- and γ) were detected in blood and milk every 8 h. The purpose of the every 8 h measurements after 2 d of Tmix feeding was to investigate any changes of tocopherol isoforms in milk and blood during tocopherol within daily accumulation. More importantly, I originally expected to detect β- or δ-tocopherol concentrations in milk and blood within 1 d. However, no detectable concentration of β- and δ-tocopherol were detected in blood and milk even though δ-tocopherol was the second highest enrichment tocopherol isoform in the Tmix. Combined with tissue data, one might conclude that very limited amounts of β- or δ-tocopherol passed through the small intestines, the initial site of tocopherol absorption in animals or even though it is assumed that amounts of β- and δ-tocopherol passed through the small intestines. These two tocopherols metabolism processes were too rapid to be detected by more frequent sampling (every 8 h starting at 2 d post-feeding). Therefore, additional gut level sampling and short time post feeding sampling might be needed to determine the rate of β- or δ-tocopherol in animals. Besides β- or δ-tocopherol, limited changes of α- and γ-tocopherol were detected. The concentrations of α- and γ-tocopherol were consistent with daily measurements to indicate the tocopherol accumulation process in milk and blood. Even though a decreased concentration of γ-tocopherol between 16

and 24 h was observed in blood, this difference might partly be associated with animal variation instead of a Tmix effect.

CONCLUSIONS

Tocopherol isoforms (i.e. α- and γ-tocopherol) start to accumulate after Tmix supplementation (i.e. 11.1 vs. 14.8 µg/mL: 0.6 vs. 0.9 µg/g and 0.6 vs. 3.0 µg/mL; 0.2 vs. 0.5 µg/g) compared to before feeding. Especially for γ-tocopherol, the Tmix compensated for the low concentration of γ-isoform in blood and milk observed prior to feeding. The results indicate that even though Tmix might not mainly contribute α-isoform , α-tocopherol was elevated in animals via standard ration feeding, which partially explained by its absorption preference in liver compared to γ-tocopherol. Also, the data illustrate that ~5 d of Tmix feeding may be sufficient to reach a higher stabilized range of concentrations of the two measurable isoforms (i.e. α- and γ-tocopherol) in milk and blood in the lactating Holstein dairy cow. This study establishes that this experimental design is adequate towards the further refinement of experiments that will be valuable towards characterizing the kinetics and biological value of α- and γ-tocopherols. Additional sampling at the gut level may be informative in determining the fate of other isoforms (i.e. β- and δ-tocopherol).

REFERENCES

Baldi, A. 2005. Vitamin E in dairy cows. Livest. Prod. Sci. 98:117-122.

Charmley, E., J. W. G. Nicholson, and J. A. Zee. 1993. Effect of supplemental vitamin E and selenium in the diet on vitamin E and selenium levels and control of oxidized flavor in milk from Holstein cows. Can. J. Anim. Sci. 73:453-457.

Cobb, C. A. and M. P. Cole. 2015. Oxidative and nitrative stress in neurodegeneration. Neurobiol. Dis. 84:4-21.

Combs, J. and J. P. McClung. 2017. Chapter 8 - Vitamin E. PP. 207-242 in The Vitamins (Fifth Edition). Academic Press.

Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. J. Dairy Sci. 77:2695-2703.

Herdt, T. H., and J. C. Smith. 1996. Blood-lipid and lactation stage factors affecting serum vitamin E concentrations and vitamin E cholesterol ratios in diary cattle. J. Vet. Diagn. Invest. 8:228-232.

Hogan, J. S., W. P. Weiss, and K. L. Smith. 1993. Role of Vitamin E and selenium in host defense against mastitis. J. Dairy Sci. 76:2795-2803.

Jañrvinen, R. and A. T. Erkkila. 2016. Tocopherols: Physiology and Health Effects. PP. 303-308 in Encyclopedia of Food and Health.Academic Press, Oxford.

Jiang, Q., S. Christen, M. K. Shigenage, and B. N. Ames. 2001. Gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention. Am. J. Clin. Nutr. 74:714-722.

Meglia, G. E., S. K. Jensen, C. Lauridsen, W. K. Persson. 2006. Alpha-tocopherol concentration and stereoisomer composition in plasma and milk from dairy cows fed natural or synthetic vitamin E around calving. J. Dairy Sci. 73: 227-234.

Mudron, P., J. Rehage, H. P. Sallmann, M. Mertens, H. Scholz, and G. Kovac. 1997. Plasma and liver alpha-tocopherol in dairy cows with left abomasal displacement and fatty liver. Transbound Emerg. Dis. 44:91-97.

National Research Council. 2001. Nutrient requirements of dairy cattle. 7th rev.ed. National Academy of Science ed. Washington, DC, National Academy Press.

Parrish, D. B., G. H. Wise, and J. S. Hughes. 1947. Properties of the colostrum of the dairy cow tocopherol levels in the colostrum and in the early milk. J. Dairy Sci. 30:849-860.

Qu, Y., A. N. Fadden, M. G. Traber, and G. Bobe. 2014. Potential risk indicators of retained placenta and other diseases in multiparous cows. J. Dairy Sci. 97:4151-4165.

Qu, Y., K. Lytle, M. G. Traber, and G. Bobe. 2013. Depleted serum vitamin E concentrations precede left displaced abomasum in early-lactation dairy cows. J. Dairy Sci. 96:3012-3022.

SAS User's Guide: Statistics. [9.4]. 2017. Cary, NC, SAS Inst.,Inc.

St-Laurent, A. M., M. Hidiroglou, M. Snoddon, and J. W. G. Nicholson. 1990. Effect of alpha-tocopherol supplementation to dairy cows on milk and plasma alpha-tocopherol

concentrations and on spontaneous oxidized falvor in milk. Can. J. Anim. Sci. 70:561-570.

Traber, M. G., and I. Jialal. 2000. Vitamin E, antioxidant and nothing more. Free Radic. Biol. Med. 43: 4-15.

Traber, M. G., E. A. Serbinova, and L. Packer. 1999. 4 - Biological activities of tocotrienols and tocopherols. PP. 55-71 in Antioxidant Food Supplements in Human Health.Academic Press, San Diego.

Weiss, W. P., J. S. Hogan, D. A. Todhunter, and K. L. Smith. 1997. Effect of Vitamin E supplementation in diets with a low concentration of selenium on mammary gland health of dairy cows. J. Dairy Sci. 80:1728-1737.

Weiss, W. P. 1998. Requirements of fat-soluble vitamins for dairy cows: A Review. J. Dairy Sci. 81:2493-2501.

Weiss, W. P. and D. J. Wyatt. 2003. Effect of dietary fat and vitamin E on alpha-Tocopherol in milk from dairy cows. J. Dairy Sci. 86:3582-3591.

Tocopherol Mix and Bovine Milk, Blood Chapter Figures:

FIGURES:

Days of feeding   **Days of feeding**
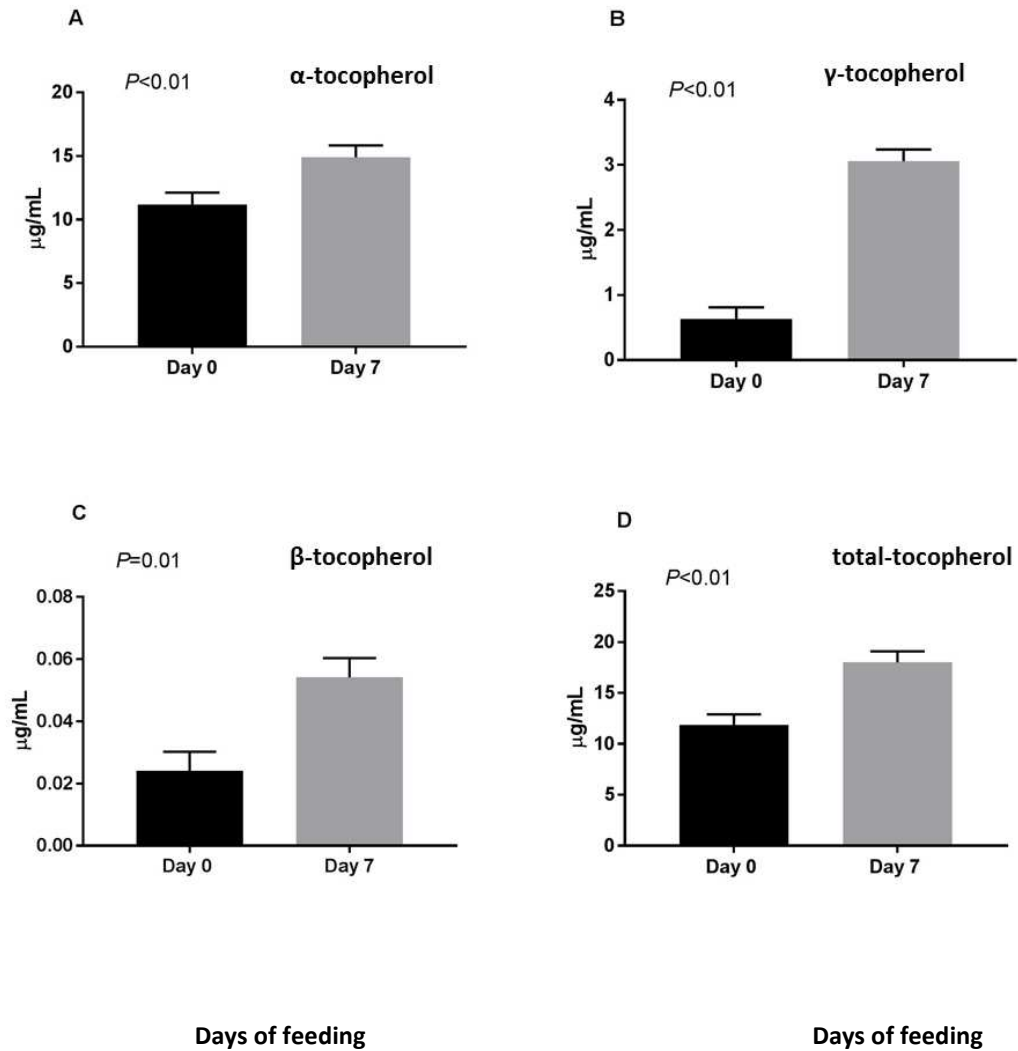
Figure 3.1. Tocopherol isoforms measurements of d 0 pre-feeding vs. d 7 post-feeding Tmix (mixed tocopherol oil supplement) in bovine blood: (A) α-tocopherol, (B) γ-tocopherol, (C) β-tocopherol and (D) total-tocopherol (i.e.α-, γ- and β-tocopherol).

Days of feeding
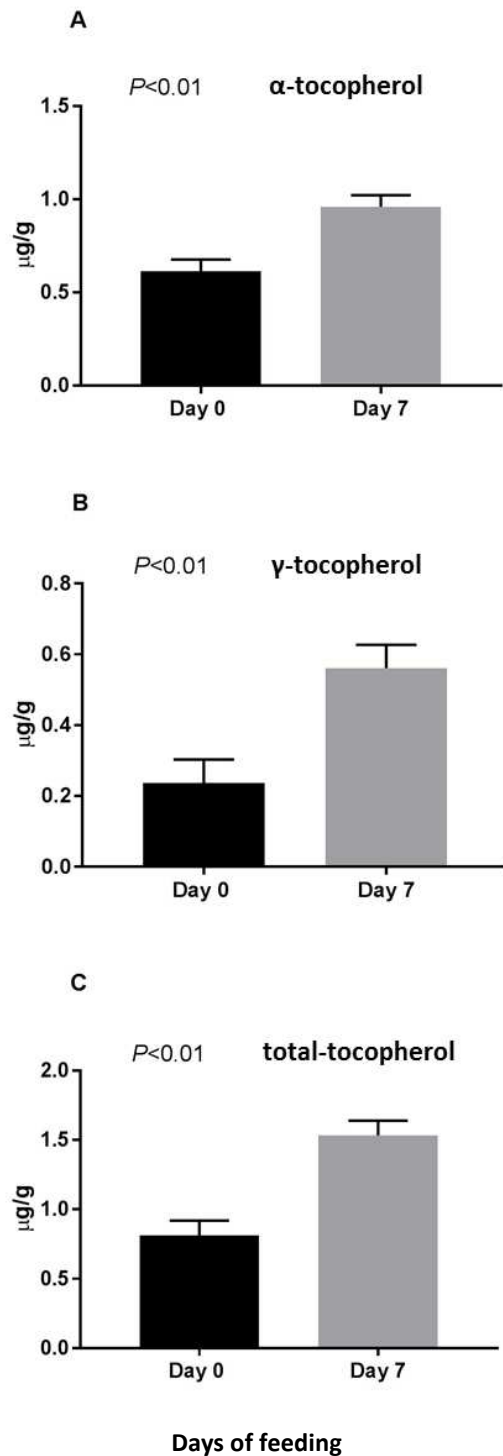
Figure 3.2. Tocopherol isoforms measurements of d 0 pre-feeding vs. d 7 post-feeding Tmix (mixed tocopherol oil supplement) in bovine milk: (A) α-tocopherol, (B) γ-tocopherol and (C) total-tocopherol (i.e. α- and γ-tocopherol).
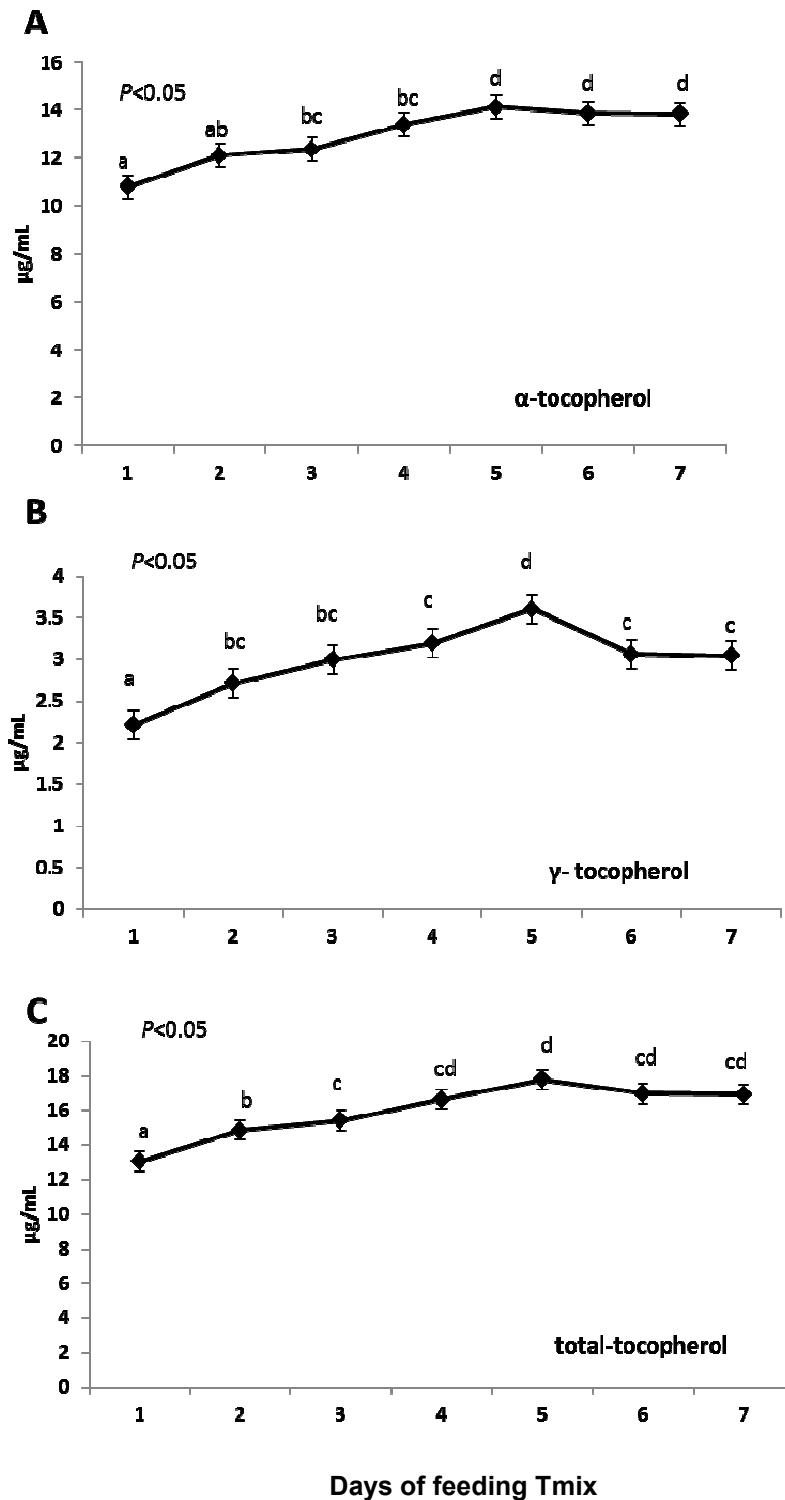
Figure 3.3. Daily tocopherol isoforms measurements in bovine blood (n=4): (A) α-tocopherol, (B) γ-tocopherol and (C) total-tocopherol (i.e. α- and γ-tocopherol) via Tmix (mixed tocopherol oil supplement) feeding. Differing letters indicate significant differences ($P \leq 0.05$).
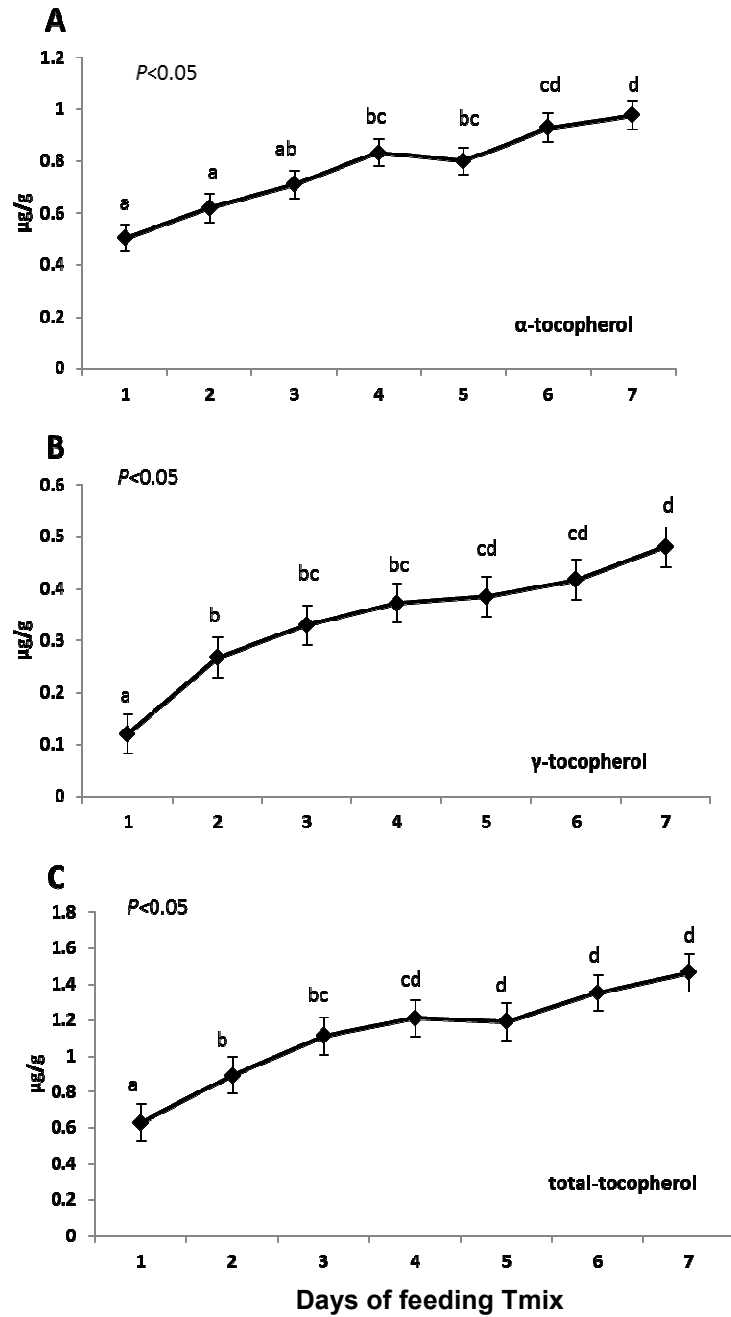
Figure 3.4. Daily tocopherol isoforms measurements in bovine milk (n=4): (A) α-tocopherol, (B) γ-tocopherol and (C) total-tocopherol (i.e. α- and γ-tocopherol) via Tmix (mixed tocopherol oil supplement) feeding. Differing letters indicate significant differences ($P \leq 0.05$).
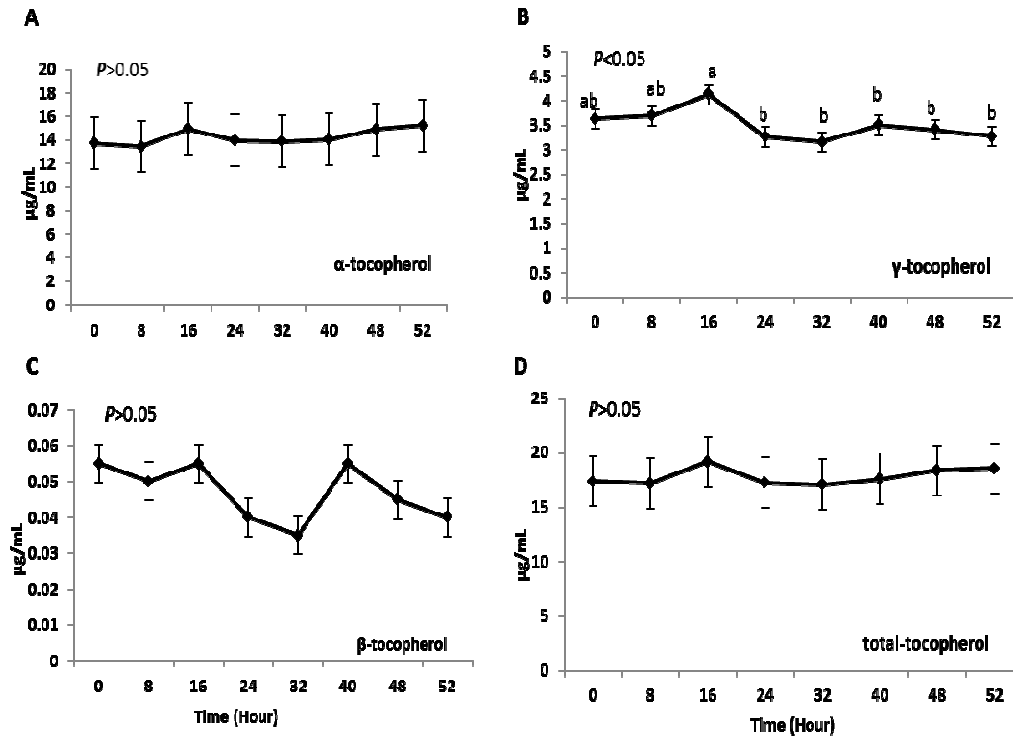
Figure 3.5. Frequent pattern of tocopherol isoform measurements in bovine blood (n=2): (A) α-tocopherol, (B) γ-tocopherol, (C) β-tocopherol and (D) total-tocopherol (i.e. α-, γ- and β-tocopherol) starting at 2 days of Tmix (mixed tocopherol oil supplement) feeding. Differing letters indicate significant differences ($P \leq 0.05$).
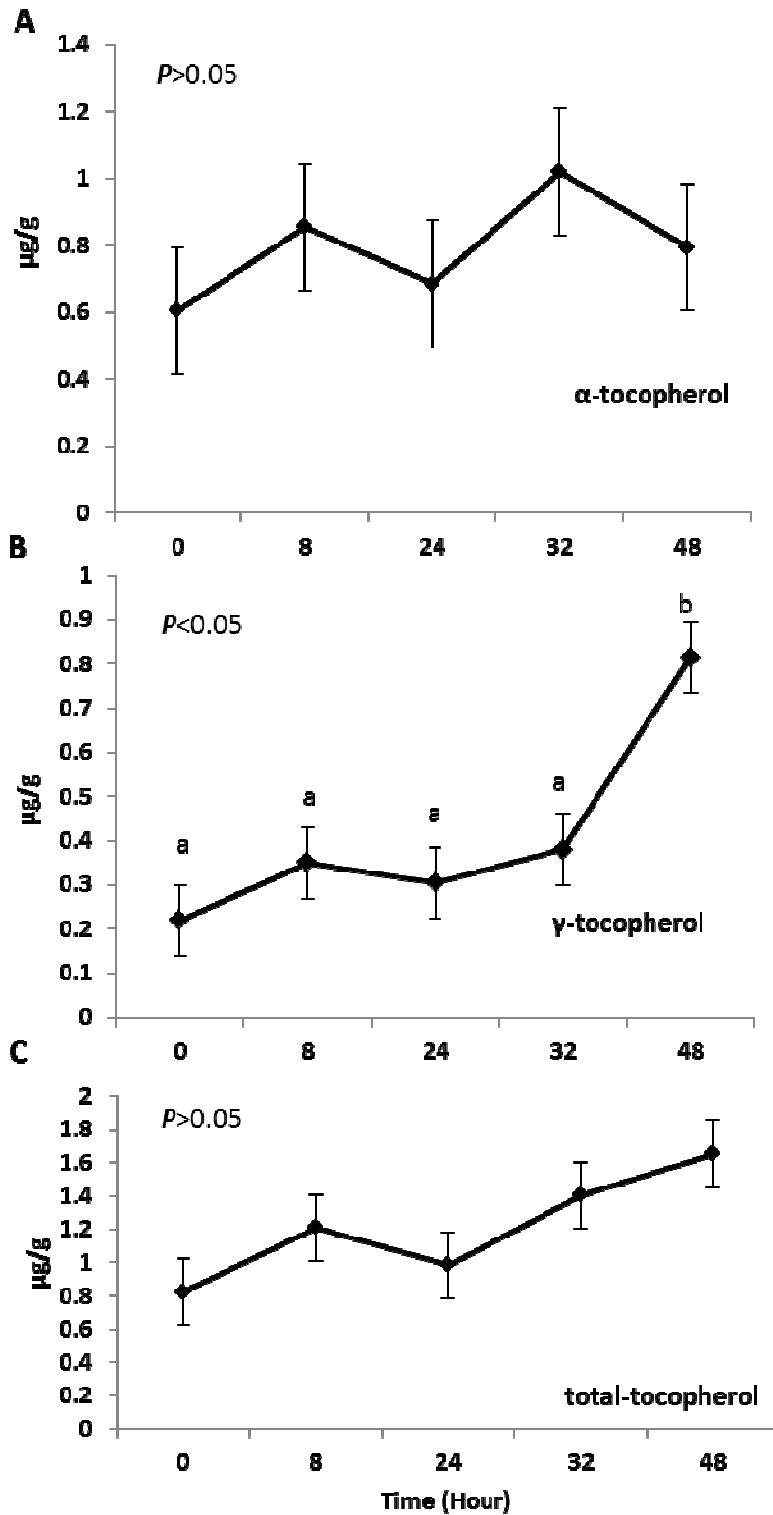
Figure 3.6. Frequent pattern of tocopherol isoforms measurements in bovine milk (n=2): (A) α-tocopherol, (B) γ-tocopherol and (C) total-tocopherol (i.e. α- and γ-tocopherol) after 2 days of Tmix (mixed tocopherol oil supplement) feeding. Differing letters indicate significant differences ($P \leq 0.05$).

CHAPTER 4: THE EFFECTS OF FEEDING OF MIXED TOCOPHEROL OIL ON BLOOD NEUTROPHIL FUNCTION AND IMMUNOMETABOLIC-RELATED GENE EXPRESSION IN LACTATING DAIRY COWS

ABSTRACT

Alpha-tocopherol has been well-studied regarding improving polymorphonuclear leukocytes (PMN) function, especially its involvement in respiratory burst. However, no studies have identified the effect of feeding a tocopherol mix, which contains additional isoforms, on immune cell function. The objective of this study was to investigate how short-term feeding of tocopherol mix alters bovine blood PMN function and immunometabolic-related gene expression. Twelve healthy, multiparous Holstein cows (days in milk [DIM]: 179 ± 17 d) were fed a vegetable-derived mixed tocopherol oil supplement (Tmix) enriched with γ- and δ-isoforms (9 % α-, 1 % β-, 24 % δ-, and 62 % γ-tocopherol) at ~620 g Tmix/cow·d^{-1} top dressed for 7 consecutive days. Jugular blood (~200 mL) was collected from all cows on d 0 prior to feeding and on d 7 post-feeding of Tmix. Whole blood was then used to measure, hematology, metabolites and respiratory burst response via chemiluminescence analysis. Isolated PMN (3×10^6 cells/mL) were used to evaluate chemotaxis function and immunometabolic-regulated gene expression by quantitative real-time PCR. For gene expression analysis, cells were incubated with lipopolysaccharide (LPS) at a final concentration either of 0.0 or 1.5 μg/mL for 2 h. Data were analyzed as a complete randomized design. Significance was declared at $P \leq 0.05$. With regard to function, Tmix improved ($P = 0.04$) PMN chemotaxis function but did not alter ($P = 0.90$) the respiratory burst response in whole blood. For gene expression analysis, LPS challenge increased the expression of pro-

inflammatory genes coding for tumor necrosis factor-α and interleukin-6. However, Tmix did not alter the expression of genes associated with the immune or metabolic response. In addition, Tmix did not alter plasma metabolites and hematology profile except for monocytes. In conclusion, short-term feeding of Tmix did not impair PMN function of respiratory burst but improved PMN chemotaxis. In addition, Tmix did not alter the immunometabolic response of genes in PMN. Additional evaluations of the effect of individual tocopherol isoforms will offer valuable information regarding their specific roles on bovine immune cell function and gene expression.

INTRODUCTION

Bovine mastitis (i.e. an inflammation of the mammary gland) is usually associated with the invasion of a pathogen (Gruet et al., 2001). During mastitis, circulating PMN are recruited and play an essential role in controlling the duration and severity of mastitis (Paape et al., 2003). Optimal PMN function during mastitis is necessary for controlling the inflammatory response (Lauzon et al., 2005). However, PMN can have detrimental effects on the mammary tissue during the inflammatory response. Reasons could be damage associated with the over production of pro-inflammatory cytokines (e.g. TNF-α; (Persson et al., 1993) and/or ROS (Sordillo et al., 2009). This could result in subsequent disorders (i.e. ketosis and milk fever) and diseases (i.e. mastitis and metritis) that frequently occur in dairy cattle (Sordillo, 2016).

Nutritional supplements of vitamin E have been studied to alleviate the negative consequences associated with the inflammatory responses (Barrett et al., 1997; Higuchi et al., 2013; Singh et al., 2013). Potential mechanisms of vitamin E benefits during inflammation are 1) helping to control lipid peroxidation of host tissues associated with

infiltrating PMN; 2) controlling lipid peroxidation-induced inflammation, and 3) modulating the membrane structure of PMN (Chew, 1995). Alpha-tocopherol (i.e. the most biologically active isoform of vitamin E) has been studied for its ability to improve PMN chemotaxis (Luostarinen et al., 1991) and enhance the expression of plasminogen activator urokinase receptor gene (i.e. *PLAUR*) (Pinotti et al., 2003). In addition, α-tocopherol has been studied to improve phagocytosis of PMN in cattle (Hogan et al., 1992) and modulate the expression of pro-inflammatory genes in PMN by controlling lipid peroxidation (i.e. oxidative stress) responses (Sordillo et al., 2009). Most vitamin E research has been conducted during the transition period (Drackely et al., 1999; Pilotto et al., 2016; Qu et al., 2013), a high-risk period, when most cows experience a natural depletion of vitamin E (i.e. α-tocopherol) which can increase susceptibility to disease/disorders (Pinotti et al., 2003; Qu et al., 2013; Qu et al., 2014). Therefore, sufficient vitamin E supplementation is needed to optimize animal health.

Among the eight different isoforms of vitamin E, α-tocopherol has been studied the most in dairy cattle when compared to other forms, such as β-, γ- and δ-tocopherol (Politis et al., 2012; Spears and Weiss, 2008; Weiss and St-Pierre, 2009). In addition, the common commercially available of α-tocopherol supplement is generally the more expensive tocopherol whereas extract mix (i.e. α-, -β-, -γ-, -δ-tocopherol) is more economical and can help to reduce feed costs to the producer. However, the effect of Tmix on animal health has not been fully elucidated. Therefore, the objective of this study was to evaluate the effect of feeding of Tmix on PMN function, as measured by chemotaxis and respiratory burst activity, and immunometabolic-related gene expression in dairy cows. The hypotheses are that a) Tmix will improve chemotaxis function, but

will not impair respiratory burst status, and b) Tmix will mediate the expression of pro-inflammatory genes in circulating PMN.

MATERIALS AND METHODS

All procedures involving the use of live animals were approved in accordance with the regulations and guidelines set forth by the U.S. Department of Agriculture, Beltsville (# 15-009), Animal Care and Use Committee and were approved by the Institutional Animal Care and Use Committee at the University of Maryland, College Park (# 626721-1).

Animals and Treatments

Twelve multiparous Holstein cows in mid-lactation (DIM = 179 ± 17 d) were used for this study.   Cows did not display clinical signs of disease and had an average composite SCC of 25,000 ± 16,000 cells/mL before enrollment. Cows were housed and fed in tie-stalls each with its own feed bunk, had free access to water and were milked twice daily at 0600 h and 1800 h. Cows were fed a standard TMR ad libitum (Table 2.1). Cows were fed twice per day at 0700 h and 1400 h. All cows were top-dressed with the Tmix for 7 d consecutively. Jugular blood (~200 mL) was collected from each cow after the morning milking and prior to the morning feeding on d 0 and d 7 into 15-mL tubes containing acid-citrate dextrose (Thermo-Fisher Scientific Inc.), inverted to mix and placed on ice. Blood samples were processed within 1 h after collection. In addition, blood (~40 mL) was collected into EDTA tubes (Becton Dickinson Vacutainer System**,** Franklin Lakes, NJ) for whole blood chemiluminescence, whole blood hematology, plasma metabolite analysis and plasma tocopherol concentration analysis. Plasma was

collected after centrifugation at $1300 \times g$ for 15 min at 4°C and stored in 1.5-mL tube at -20°C for future analysis.

Whole Blood Respiratory Burst by Chemiluminescence

Reagent Preparation

Respiratory burst was measured via chemiluminesence. A final $CaCl_2$ concentration of 0.5 m*M* as the assay buffer was made with 500 µL of 1 *M* of $CaCl_2$ (Sigma Aldrich Inc., St. Louis, MO) and 500 mL of Hank's Balanced Salt Solution (HBSS; Sigma Aldrich Inc.) The working luminol solution (5 m*M*; Sigma Aldrich Inc.) was prepared fresh each day in a minimal assay buffer solution with 0.1-*M* NaOH (Sigma-Aldrich Inc.). Each tube was vortexed until luminol was in solution and protected from light. For the stimulant, a working solution of phorbol 12-myristate 13-acetate (PMA); 40 ng/µL; Sigma-Aldrich Inc.) was prepared with phosphate buffered saline (PBS; Sigma-Aldrich Inc.) and kept on ice.

Chemiluminescence Assay

Procedures followed those as previously described (Mehrzad et al., 2002) with minor modifications. Briefly, for each sample test, a set of 2 tubes was filled with 500 µL of whole blood and pre-incubated for 10 min at 37 °C in an aluminum heating block. Then, 20 µL of working solution of PMA (i.e. stimulant) or water (i.e. non-simulant) was added to achieve a final concentration of 0.8 or 0.0 µg/mL. After incubation for 15 min at 37 °C, 40-µL aliquots of blood were mixed with 1 mL of minimal assay buffer containing luminol (250 µ*M*). All assay tubes were incubated for an additional 10 min at 37 °C and transferred to the chemiluminometer (Autolumat LB 953; E. G. & G. Berthold, Wildbad, Germany). Chemiluminescence generated in each tube was estimated continuously for 20 min with measurements of light emission were recorded every 60 s (n

= 20 reads/tube). For each sample, mean values of the 2 tubes were used for further data processing. The respiratory burst activity was expressed in relative chemiluminescence units.

Hematology Analysis

Ten mL of whole blood was used for the hematology analysis (IDEXX ProCyte Dx® Hematology Analyzer, Westbrook, ME) to indicate the blood profile of immune cells (e.g. total white cells, neutrophils, monocytes, lymphocytes) as previously described (Garcia et al., 2017).

Isolation of Bovine PMN

Blood PMN were isolated following the previous procedure described by Garcia et al. (2015a) with minor modifications. Briefly, blood (~200 mL) was transferred to 15-mL conical tubes (Thermo-Fisher Scientific Inc.) and centrifuged (Model 5810R, Eppendorf, Hauppauge, NY) for 20 min at $1000 \times g$ at 4 °C. After centrifugation, the plasma, buffy coat, and 1/3 of the red blood cells were discarded. The remaining cells were transferred to a 50-mL tube (Thermo-Fisher Scientific Inc.) and the cell suspension was lysed with 18 mL of ice-cold deionized water. The cell suspension was gently inverted for no longer than 45 s and isotonicity was restored by addition of 2 mL of $10 \times$ PBS (pH 7.4; Sigma-Aldrich Inc.). The solution was then centrifuged for 10 min ($200 \times g$ at 4 °C) and the supernatant was decanted. The pellet was washed with 20 mL of PBS and centrifuged for 5 min ($850 \times g$ at 4 °C). A total of 3 washes were performed. After the final wash, the cell pellet was resuspended in 1 mL of RPMI 1640 (Sigma-Aldrich Inc.). Isolated PMN were counted using the TC20 cell counter (Bio-Rad Laboratories Inc.).

Cell Viability and Differential Analysis

Cell viability was determined using the trypan blue (0.1 %, Bio-Rad Laboratories Inc.) exclusion method (Freshney, 1987). The average viability was 96 % ± 1.34%. Ddifferential cell counts of isolated cells were determined microscopically on cytospin preparations using a commercially available hematology staining kit (Hema-Fast™ 3-Step Hematology Staining Kit; Thermo-Fisher, Scientific Inc.). The average cell differential was 91 % ± 1.10 %).

Treatments and Incubation

Cell suspensions were diluted to desired concentrations of 3×10^6 cells/mL (chemotaxis; final volume 2.0 mL), 4×10^6 cells/mL (gene expression; final volume 2.0 mL) using RPMI 1640 without glutamine (Sigma-Aldrich Inc.) containing 5 % heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich Inc.). For gene expression analysis, 200 µL of PBS containing 1.5 µg/mL LPS (LPS treatment; *Escherichia coli* 055:B5; Sigma-Aldrich Inc.) or without LPS (control treatment) were mixed with 800 µL of RPMI containing 4×10^6 cells/mL isolated PMN cell suspension in 24-well cell incubation plates. The cell culture plates were incubated for 120 min at 37 °C, 95 % relative humidity and 5 % CO_2. For gene expression, following incubation, 1.0 mL of cell suspension was transferred to 2.0-mL RNase-free tubes (Thermo-Fisher Scientific Inc.) and centrifuged ($1000 \times g$ at 4 °C for 2 min). One mL of Trizol (Thermo-Fisher Scientific Inc.) was added to the cell pellet, and vigorously pipetted up and down to lyse the cells completely for the qPCR analysis described below. For chemotaxis, chemoattractants human IL-8 (Sigma-Aldrich Inc.; 100 ng/mL in Hank's Balanced Salt Solution; HBSS) and recombinant human complement 5a (C5a; Sigma-Aldrich Inc.; 83

ng/mL in HBSS) were added to the bottom of a 24-well plate in triplicate. Filters were added (Pore Size 5.0 µm; 6.5 mm diameter; 0.33 cm^2 growth area; Thermo-Fisher Scientific Inc.) to the top of each well. The cell suspension (100 µL) adjusted to 3×10^6 PMN/mL with RPMI-1640 containing 5% FBS was added to the top of the filter. Plates were incubated for 24 h at 37 °C, 5.0 % CO_2, and 95 % relative humidity. Following incubation, the filter was washed 2 times with 200 µL of HBSS and the filter was discarded. The total number of PMN migrating was counted using a TC-20 automated cell counter (Bio-Rad Laboratories Inc.).

RNA Isolation, cDNA Synthesis, and qPCR

Total RNA (n=6/treatment; 4 treatments total) was extracted using the Trizol (Thermo-Fisher Scientific Inc.) and EGA-E.Z.N.A. total RNA kit (Omega Bio-tek, Inc, Norcross, GA) according the manufacturer's instructions. Genomic DNA was removed with DNAase I enzyme (Bio-Rad Laboratories Inc.). The RNA concentration was measured with the NanoVue Plus (GE Healthcare, Piscataway, NJ). The purity of extracted RNA was calculated by absorbance ratio of 260/280 measured using the NanoVue Plus (GE Healthcare) with an average of 1.74 ± 0.39. The integrity of RNA was measured by the RQI with an average of 8.59 ± 1.20, using an Experion Bioanalyzer (Bio-Rad Laboratories Inc.). Complementary DNA (cDNA) was synthesized with 100 ng of RNA per reaction (20 µL) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc.). Reactions contained 4 µL of 5X reaction mix (blend of dTand random hexamer primers) optimized for production of targets <1-kb in length, 1 µL of reverse transcriptase (RNase H+), and variable amounts of RNA templates and nuclease-free water. The reaction was performed in a T100 Thermal Cycler (Bio-Rad Laboratories

Inc.) with a temperature program of 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min.

Primer Selection and Evaluation

Primer selection and efficiency curves were made following procedures described by (Garcia et al., 2016) and the GenBank accession number, gene symbol, hybridization position, sequence, amplicon size, and references for primers used can be found in Supplemental Table S4.1. Briefly, primers were selected from previous publications, except that for *NCF1* and *NCF2* which were designed using Primer Express 3.0.1 with an intended minimum amplicon size of 80 bp (amplicons of 100-120 bp were of priority, if possible) and limited 3' G + C percentage. Candidate primers were screened to verify proper sequence with the most updated FASTA sequence for corresponding accession number for *Bos taurus* with prefix NM_ (messenger RNA), preferentially. Lyophilized primers (~25 nmole DNA oligo, with a standard desalting purification method) were obtained from Integrated DNA Technologies (Coralville, IA). Primers were reconstituted with molecular grade water (G-Biosciences, St. Louis, MO) to a concentration of 100 μM, then diluted to a working primer concentration of 10 μM. The amplification efficiency of primers was verified by a 6-point serial dilution (1:4). Efficiency values ranged from 0.70 to 1.18 with an average value of 0.99 ± 0.09 and an average R^2 of 0.99 ± 0.001 (Supplemental Table S4.3*).*

qPCR Analysis

Quantitative PCR reaction was performed using SYBR green dye (PerfeCta SYBR Green fast mix) from Quanta Biosciences Inc. (Gaithersburg, MD). Gene symbol, gene name, and description of the main biological functions and processes of targets

analyzed in circulating bovine PMN are described in Supplemental Table S4.2. Briefly, 4 µL of cDNA template (500 pg of RNA per reaction) was combined with 5 µL of SYBR Green fast mix , 0.5 µL each of forward and reverse primers (500 nM of each primer per reaction), and 3.4 µL of molecular grade DNase/RNase free water in a skirted 96-well plate (VWR International, Philadelphia, PA). All samples were run in triplicate and were performed on a single plate per a given gene. The PCR reaction was performed in a CFX96 optical reaction module (Bio-Rad Laboratories Inc.) using the following cycling protocol: initial denaturation at 95 °C for 30 s, 40 PCR cycles of 95 °C for 5 s and 60 °C for 30 s data collection, annealing and extension, and a final step at 95 °C for 10 s before the melting curve.   The melting curve step was included to verify the absence of formation of primer dimers and formation of single PCR products using an incremental temperature of 0.5 °C from 65 °C to 95 °C for 5 s. A signal melting curve was expected to indicate the purity of DNA products. The qPCR results, given as Cq values for each transcript, were analyzed using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Briefly, the Cq of each target gene was calculated and normalized by the geometric mean of the 2 selected reference genes (*RPS9, OSBPL2*) (Garcia et al., 2016) to generate ΔCq values. The ΔCq values without transformation were used for statistical analysis. The efficiency of dilution curves and median Cq values for all tested samples can be found in Supplemental Table S4.3.

Analysis of Metabolites in Plasma

Non-sterified fatty acid (NEFA), Cholesterol, Glucose, and Insulin Assays

      All plasma metabolites were run on a single 96-well plate following the manufacturer's instructions. Plasma NEFA was analyzed using a commercially available

kit (NEFA-HR kit; Wako Diagnostics Inc., Richmond, VA). The intra-assay CV was 4.6 %. The plasma cholesterol assay was analyzed using the Liquicolor method from a commercially available kit (Stanbio Laboratories, Boerne, TX). The intra-assay CV was 4.6 % and inter-assay CV was 6.4 %. Plasma glucose was analyzed by a glucose oxidase methodology using a commercially available kit (Stanbio Laboratories). The intra-assay CV was 2.4 %. Plasma insulin was analyzed using an ELISA kit (Thermo-Fisher Scientific Inc.). The intra-assay CV was 1.8 %.

Statistical Analysis

The PROC MIXED procedure of SAS 9.4 (2017) was used for the statistical analysis. The data were analyzed as a complete randomized design. For analysis of hematology profile, metabolites in plasma, whole blood respiratory burst, and PMN chemotaxis, the class variable included cow and Tmix. Cow was used as a random term. The model included the fixed effect of Tmix supplementation (i.e. d 7 or d 0). For chemotaxis, to achieve the normal distribution, cell numbers were natural log-transformed for analysis and then back-transformed for presentation in figures and tables. For respiratory burst via chemiluminescence, the number of PMN in whole blood (as assessed via hemocytology) was added in the model as a covariate factor. Separation of LSM and individual comparisons for significant effects were performed using the PDIFF statement in SAS. The model was as follows:

$$Y_i = \mu + T_i + \varepsilon_i$$

where Y_i is the dependent variable; μ is overall mean; T_i is the fixed effect of Tmix supplementation at level i and ε_i is the residual error.

For gene expression analysis, and the data were analyzed using a 2 × 2 factorial arrangement with factors including 2 levels of Tmix supplementation (i.e. d 7 or d 0) and 2 doses of LPS level (i.e. 0 or 1 µg/mL). The class variable included cow, Tmix supplementation, LPS. Cow was used as a random term. The model included fixed effect of Tmix supplementation (0 and 7 d) and dose of LPS (LPS and control). Separation of LSM and individual comparisons for significant effects were performed using the PDIFF statement in SAS. The model was as follows:

$$Y_{ij} = \mu + T_i + L_j + TL_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable; μ is overall mean; T_i is the fixed effect of Tmix supplementation at level i; L_j is the fixed effect of LPS dose at level j; TL_{ij} is the interaction of Tmix × LPS challenge, and ε_{ij} is the residual error. For all models, the degrees of freedom were estimated with the Kenward-Roger specification in the model statement. The data were presented as LSM and largest SEM. Statistical differences were declared as significant and highly significant at $P \leq 0.05$ and $P < 0.01$, respectively. Trends towards significance are discussed at $0.05 < P < 0.10$. Fold-change values were calculated according to (Livak and Schmittgen, 2001). Briefly, LSM (ΔCq) of a factor of interest (LPS) was subtracted from the LSM (ΔCq) of other referential factor (No LPS) to generate the $\Delta\Delta$Cq value, which was linearized to a fold-change value with the formula $2^{-\Delta\Delta Cq}$.

RESULTS

Plasma Metabolites

Concentrations of plasma glucose were not altered ($P = 0.79$) for cows before (68.92 mg/dL) and after 7 d (68.27 mg/dL) of feeding Tmix. Similarity, concentrations

of plasma insulin were not changed (P = 0.21) for cows on d 0 before (0.23 ng/mL) and d 7 after (0.31 ng/mL) Tmix feeding. In addition, a trend (P = 0.09) was observed for concentrations of NEFA where plasma NEFA concentrations decreased from d 0 before (0.42 mEq/L) and d 7 after (0.27 mEq/L) Tmix feeding. The concentrations of plasma cholesterol did not significantly change (P = 0.53) for cows on d 0 before (392.03 mg/dL) or d 7 after (315.63 mg/dL) Tmix feeding.

Hematology Profiles

Whole blood hematology profiles of immune cells before (i.e. d 0) and after (i.e. d 7) Tmix feeding are shown in Table 4.1. Concentrations of white blood cells were not changed (P = 0.15) after 7 d of Tmix feeding relative to baseline measurements at d 0. Among those white blood cells, concentrations of PMN, lymphocytes, basophils, and eosinophils were not changed ($P \geq 0.17$) on d 7 after Tmix feeding relative to baseline. However, concentrations of monocytes were increased (P = 0.04) relative to baseline after 7 d of Tmix feeding (Table 4.1).

Whole Blood Respiratory Burst by Chemiluminescence

Feeding Tmix did not affect (P = 0.90) the respiratory burst of whole blood by d 7 of Tmix supplementation as determined by relative chemiluminescence response (Figure 4.1).

Chemotaxis of Bovine PMN

Feeding cows Tmix increased (P = 0.03; Figure 4.2A) the number of PMN migrating in response to IL-8 chemoattractant and tended to increase (P = 0.10; Figure 4.2B) the number of PMN migrating in response to C5a chemoattractant after 7 d of feeding Tmix.

PMN Gene Expression

The effects of Tmix supplementation and LPS challenge on PMN gene expression are shown in Table 4.2. There was no significant interaction ($P > 0.05$) between LPS challenge and Tmix supplementation. Tmix did not affect ($P > 0.05$) the expression of genes coding for the inflammatory response of bovine PMN (i.e. *IL6, IL8, IL10, NFKB1, PTGS-2*), except for *MYD88* (1.58 FC, $P < 0.05$) and *TNFA* (1.69 FC, $P < 0.05$). Tmix supplementation did not affect the expression of genes coding for the receptors on the surface of bovine PMN involved in inflammatory signaling pathways (i.e. *TLR2* and *TLR4*) nor genes coding for adhesion/receptors (i.e. *SELL* and *ITGB2*). In addition, Tmix did not affect the expression of *PLAUR* coding for receptors specifically reacting with tocopherol to improve PMN chemotaxis function. Tmix did not affect the expression of *CASP* and *mTOR* coding for proteins involved in cell signaling pathways. Tmix did not affect the expression of (*PPARα, RXRα,* or *RXRβ*), but increased expression of *RARα* (1.28FC, $P = 0.03$)**.**

There was no significant interaction of LPS and Tmix for any of the genes analyzed. The LPS challenge increased the expression of inflammatory genes: *TNFA* (3.92 FC, $P < 0.01$), *NFKB1* (1.06 FC, $P < 0.01$), *IL6* (2.71 FC, $P < 0.01$), *IL10* (1.97 FC, $P < 0.01$) and *PTGS-2* (4.46 FC, $P < 0.01$), but not the expression of other genes. LPS challenge intended to increase the expression of *TLR2* (1.35 FC P=0.09, Table 4.2) coding for the cellular receptor molecule involved in cell interactions of PMN. However, it did not affect the expression of other adhesion/receptors (*SELL, ITGB2* and *PLAUR*) of PMN. LPS challenge increased the expression of *NOS2* (2.12 FC, $P < 0.01$) whereas LPS did not affect the expression of other genes coding for superoxide enzymes of PMN.

LPS challenge did not affect the expression of *CASP3* or *mTOR* coding for proteins involved in cell signaling pathways. LPS challenge did not affect the expression of *PPARα*, *RXRα*, *RXRβ* or *RARα* coding for transcription factors involved in tocopherol-related metabolism in bovine PMN.

DISCUSSION

This study demonstrates that supplementing Tmix may not alter health of the cow in mid-lactation because 1) metabolic profiles (i.e. plasma glucose, insulin and cholesterol) were not altered by Tmix supplementation; 2) Tmix did not alter the hematology profile of whole blood (i.e. red and white blood cell concentrations); 3) Tmix did not impair the whole blood oxidative burst responses;4) Tmix increased the PMN chemotaxis ability; and 5) Tmix showed minimal effects on the immunometabolic gene expression of isolated PMN with or without LPS stimulation.

Tmix did not Alter Blood Metabolic or Hematology Profiles

Metabolic profiles such as glucose and insulin showed similar results to previous studies (Garcia et al., 2016; Qu et al., 2013), and tocopherol mix oil supplementation decreased the NEFA concentration to a normal level of ~0.25 mEq/L (Bionaz et al., 2007). In the current study, mean plasma cholesterol was greater (~300 mg/dL) than previous reports in the literature (~200 mg/dL) (Kessler et al., 2014; Qu et al., 2013). Diet and BW could contribute to this difference (Faye et al., 2015). Tmix feeding did not change the level of plasma cholesterol (*P*=0.53). With regard to the hematology profile, Tmix did increase the monocytes population. This may indicate that Tmix is beneficial to monocyte half-life time and maintains monocytes at stable levels in blood, which is

critical as macrophages (converted from monocytes) serve as the first immune cells to encounter pathogens during disease (Yang et al., 2014).

Tmix Improved PMN Chemotaxis Function but did not Limit Oxidative Burst Responses

Tocopherol mix oil supplementation for 7 d increased PMN chemotaxis function. Chemotaxis by PMN is needed during inflammation and serves as the primary defense mechanism of the innate immune system (Paape et al., 2003). The chemoattractants IL-8 and C5a, are chemokines that activate PMN during the chemotaxis process (Kobayashi, 2008). A previous study in bovine milk PMN reported that supplementing α-tocopherol increases PMN chemotaxis function and the mechanism could be associated with the increased expression of *PLAUR* (plasminogen activator, urokinase receptor) (Politis et al., 2003). Another possible mechanism could be the interaction between tocopherol and cell membrane futility during PMN movement due to the tocopherol accumulated mainly in the cell membrane structure, which has not been clarified (Paape et al., 2003). In this study, in order to understand the possible mechanism of Tmix increasing chemotaxis function, the expression of *PLAUR* was tested (see below for discussion).

Tocopherol mix oil did not alter the whole blood oxidative burst response, as measured by the chemiluminescence assay. Reasons could be associated with using whole blood instead of isolated PMN and/or the effect of tocopherol on respiratory burst may be primarily associated with the α-isoform of vitamin E (Cachia et al., 1998). *In vitro* studies in humans have shown that both α-tocopherol and γ-tocopherol serve as antioxidants and inhibit the superoxide molecules in PMA-stimulated PMN via regulating protein kinase C (PKC) enzyme activity (Chan et al., 2001; Varga et al., 2008).

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However, the present data did not show that Tmix inhibited oxidative burst responses in whole blood. Phorbol 12-myristate 13-acetate was chosen for incubation with blood instead of LPS to mimic the production of superoxide molecules by PMN during phagocytosis (Tian et al., 2005) because unlike LPS, PMA does not require a cell surface receptor (e.g. TLR-4) or LPS binding protein to stimulate PMN oxidative burst (Akira and Hemmi, 2003). Further studies need to be conducted to investigate the effects of Tmix on PMN respiratory burst and the effect of individual tocopherol isoforms on both whole blood and PMN respiratory burst responses. Overall, feeding Tmix did not impair the whole blood oxidative burst response but improved the PMN chemotaxis function of cows in mid-lactation.

LPS Induced a Pro-Inflammatory Response Regarding the Immunometabolic Gene Expression of PMN

In vitro LPS challenge was used to mimic the pro-inflammatory response of PMN using a dose similar to previous study reported for cows in mid-lactation (Sohn et al., 2007). Lipopolysaccharide challenge increased the expression of genes coding for pro-inflammatory mediators (e.g. cytokines). Lipopolysaccharide challenge increased expression of *NFKB,* coding for the inflammatory transcription factor NF-κB, coupled with an increased expression of pro-inflammatory genes (e.g. *IL6* and *TNFA*) coding for pro-inflammatory cytokines. Lipopolysaccharide challenge increased the mRNA expression of the anti-inflammatory cytokine *IL10*. Lipopolysccharide challenge tended to increase the expression of *TLR2* coding for TLR-2 observed in LPS-stimulated PMN (Yang et al., 1998). Results of the current study were consistent with a previous study involving *in vitro* LPS challenge of PMN (Garcia et al., 2015a). Lipopolysccharide

challenge increased the expression of *NOS2*, coding for the nitric oxide synthase induced by LPS stimulation in PMN (Okamoto et al., 2004). Even though the concentration of TNF-α in media was not tested in the current study, previous studies using a similar LPS dose showed increased pro-inflammatory cytokine concentrations (i.e. TNF-α and IL-8) in cell culture media

Tmix Showed Minimal Effects on the Immunometabolic Gene Expression of PMN

Feeding Tmix increased the expression of *TNFA* and increased the expression of *NFKB1,* coding for TNF-α and NF-κB respectively. In addition, Tmix increased the expression of *MYD88,* coding for an adapter protein important during LPS challenge. The increased expression of *MYD88* might be more associated with the increased expression of *NFKB1* than *TLR* in the current study. Contrary to the present data, a previous study showed that giving α-tocopherol decreased the expression of *NFKB1* in rat immune cell lines during inflammatory responses via either LPS or TNF-α incubation (Li-Weber et al., 2002). The possible mechanism could be the antioxidant ability of α-tocopherol, which reduces the production of superoxide anions that can induce the proinflammatory responses via NF-κB signaling pathways (Conner and Grisham, 1996). Besides α-tocopherol, γ-displays anti-inflammatory capabilities by serving as an antioxidant to scavenge ˙NO$_2$˙thereby reducing the expression of cyclooxygenase-2 (*COX2*) and/or inducing the transcription factor PPAR-γ via decreased production of prostaglandins by COX2 reduction (Campbell et al., 2003). In the current study, Tmix (highly enriched with γ-tocopherol) did not alter the expression of *COX2,* an enzyme responsible for formation of prostanoids during inflammation. It is possible that tocopherol metabolized compound (i.e. CEHC) showed more direct inhibition effects *in vitro* compared to

tocopherol (Egger et al., 2003; Grammas et al., 2004). Ultimately, compared to previous studies, the present results showed different effects of tocopherol on the expression of pro-inflammatory genes (i.e. *TNFA, NFKB1* and *MYD88*). Reasons could be associated with the fact that Tmix being comprised all four isoforms wherein β- or δ-tocopherol may have interacted with α- or γ-tocopherol in pro-inflammatory pathways or the tocopherol treatment was performed *in vivo* through feeding, whereas previous studies were completed entirely *in vitro* (Maziere et al., 1996; Erl et al., 1997; La et al., 2002). Further studies need to be conducted to compare the effects of Tmix to individual α- or γ-tocopherol on the PMN expression of pro-inflammatory genes.

Tocopherol mix oil did not alter the expression of *PLAUR* coding for plasminogen activator urokinase receptor, a potential mechanism involved in PMN chemotaxis. A previous study demonstrated supplementation of α-tocopherol increased the expression of *PLAUR* and chemotaxis function (Pinotti et al., 2003) in milk PMN. In addition, Tmix did not alter the expression of *ITGB2* and *SELL* coding for CD18 and L-selectin, respectively, which are adhesion molecules involved in PMN movement along blood vessel walls during chemotaxis (Paape et al., 2003). The improved chemotaxis function results in the current study might not be associated with PMN surface receptors/adhesion mechanisms, but with affecting PMN membrane futility during its movement, considering the assumption of tocopherol being mainly located in plasma membrane due to its chemical propensities (Li and May, 2003). Further studies need to be conducted to identify the mechanisms associated with improving PMN chemotaxis via tocopherol supplementation. Tocopherol mix oil did not alter the expression of *TLR4* and *TLR2* coding for TLR-4 and TLR-2, respectively, which were both considered as the receptor to

LPS challenge to induce pro-inflammatory responses in PMN (Aderem and Ulevitch, 2000). It was expected that Tmix would depress the expression of *TLR4* or *TLR2* to inhibit the pro-inflammatory responses via LPS challenge. The results did not show changes in the expression of these two genes which might indicate that tocopherol did not interact with PMN surface receptors even though tocopherol is located mainly in the cell membrane during LPS challenge. This needs to be confirmed via an *in vitro* incubation experiment.

Tocopherol mix oil increased the expression of *SOD2* coding for superoxide dismutase 2 as an enzyme to scavenge superoxide molecules when PMN are activated via LPS challenge, similar to previous study that showed α- or γ-tocopherol increasing the expression of *SOD2* in rats (Saldeen et al., 1999). However, the present data showed that Tmix increased the expression of *NCF1* coding for the subunit of NADPH oxidase to produce superoxide molecules in activated PMN. This might partly explain the above chemiluminescence data wherein Tmix did not alter the oxidative burst responses of whole blood possibly due to interactions with the other tocopherol isoforms (i.e. β- and δ-tocopherol). In addition, Tmix showed no effect on the expression of *CASP3* coding mediators involved in cell apoptosis pathways and on the expression of *mTOR* coding central regulator involved in mammalian metabolism.

Furthermore, Tmix did not alter the expression of *PPARα* coding for nuclear peroxisome proliferator-activated receptors and the expression of *RXRα* and *RXRβ* both coding for the retinoid X receptor, which are involved in tocopherol metabolism. According to previous studies, tocopherol served as a ligand to bind with *PPARα* paired with *RXR* to initiate tocopherol metabolism pathways in mammals (Campell et al., 2003;

Traber, 2004). Tmix increased the expression of *RARα*, coding for the retinoic acid receptor involved in regulation of retinoic acid (Chen, 2013). In current study, the increased *RARα* expression might be attributed to the negative interaction of retinoic acid and tocopherol (Schelling et al., 1995). Overall, the current data did not demonstrate any major changes in PMN gene expression associated with tocopherol metabolic functions despite their roles in phagocytosis, chemotaxis etc. In other cell types, such as liver, tocopherol supplementation altered expression of genes associated with metabolic functions (Traber, 2013).

CONCLUSIONS

In summary, the present study measured for the first time the effect of Tmix supplementation on PMN functions and gene expression changes in dairy cows in mid-lactation. The current results demonstrate that Tmix supplementation could be used like the pure α-tocopherol supplement (i.e. common vitamin E in cows) in the dairy industry because Tmix did not show any adverse effects on the health of cows in mid-lactation. The blood, milk and tissue results showed that tocopherol concentrations were able to accumulate at a desirable level to meet the cow's dietary requirement via short-term feeding of Tmix. In addition, this work was the first to investigate the effect of Tmix on a number of immunometabolic genes in bovine PMN following a LPS challenge. Tmix showed limited effects on PMN responses to LPS challenge. Even though Tmix increased the expression of pro-inflammatory genes, it does not indicate that high expression of those genes is harmful to PMN. The increased expression of pro-inflammatory genes (i.e. *TNFA, IL6*) might be necessary during the initiation of innate immune functions and later activation of adaptive immune functions. The mechanisms

by which Tmix increased the PMN chemotaxis functions and aided the whole blood

oxidative burst response remains unclear. Future studies are warranted to compare and

understand the mechanism of Tmix and individual tocopherol isoform effects on PMN

function and oxidative burst response.

REFERENCES

Aderem, A. and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. Nature. 406:782-787.

Akira, S. and H. Hemmi. 2003. Recognition of pathogen-associated molecular patterns by TLR family. Immunol. Lett. 85:85-95.

Barrett, J. J., J. S. Hogan, W. P. Weiss, K. L. Smith, and L. M. Sordillo. 1997. Concentrations of alpha-Tocopherol after intramammary infusion of Escherichia coli or lipopolysaccharide. J. Dairy Sci. 80:2826-2832.

Bionaz, M., E. Trevisi, L. Calamari, F. Librandi, A. Ferrari, and G. Bertoni. 2007. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. J. Dairy Sci. 90:1740-1750.

Cachia, O., J. El Benna, E. Pedruzzi, B. Descomps, M. A. Gougerot-Pocidalo, and C. L. Leger. 1998. α-tocopherol inhibits the respiratory burst in human monocytes attenuation of p47 phox membrane translocation and phosphorylation. J. Biol. Chem. 273:32801-32805.

Campbell, S. E., W. L. Stone, S. G. Whaley, M. Qui, and K. Krishnan. 2003. Gamma (gamma) tocopherol upregulates peroxisome proliferator activated receptor (PPAR) gamma (gamma) expression in SW 480 human colon cancer cell lines. BMC. Cancer 3:25.

Chan, S. S., H. P. Monteiro, F. Schindler, A. Stern, and V. B. Junqueira. 2001. Alpha-tocopherol modulates tyrosine phosphorylation in human neutrophils by inhibition of protein kinase C activity and activation of tyrosine phosphatases. Free Radic. Res. 35: 843-856.

Chen, G. X. 2013. Roles of vitamin A metabolims in the development of hepatic insulin resistance. ISRN Hepatol. PP. 21.

Chew, B. P. 1995. Antioxidant vitamins affect food animal immunity and health. J. Nutr 125:1804-1808.

Conner, E. M. and M. B. Grisham. 1996. Inflammation, free radicals, and antioxidants. Nutrition. 12:274-277.

Drackley, J. K. 1999. Biology of dairy cows during the transition period: the final frontier? J. Dairy Sci. 82:2259-2273.

Egger, T., R. Schuligoi, A. Wintersperger, R. Amann, E. Malle, and W. Sattler. 2003. Vitamin E (alpha-tocopherol) attenuates cyclo-oxygenase 2 transcription and synthesis in immortalized murine BV-2 microglia. Biochem. J. 370:459-467.

Erl, W., C. Weber, C. Wardemann, and P. C. Weber. 1997. Alpha-tocopherol succinate inhibits monocytic cell adhesion to endothelial cells by suppressing NF-kappa B moblization. Am. J. Physiol. 273: 634-640.

Faye, B., M. Bengoumi, A. Al-Masaud, and G. Konuspayeva. 2015. Comparative milk and serum cholesterol content in dairy cow and camel. J. King Saud. Sci. 27:168-175.

Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. J. Dairy Sci. 77:2695-2703.

Garcia, M., T. H. Elsasser, D. Biswas, and K. M. Moyes. 2015a. The effect of citrus-derived oil on bovine blood neutrophil function and gene expression in vitro. J. Dairy Sci. 98:918-926.

Garcia, M., T. H. Elsasser, L. Juengst, Y. Qu, B. J. Bequette, and K. M. Moyes. 2016. Short communication: Amino acid supplementation and stage of lactation alter apparent utilization of nutrients by blood neutrophils from lactating dairy cows in vitro. J. Dairy Sci. 99:3777-3783.

Garcia, M., T. H. Elsasser, Y. Qu, X. Zhu, and K. M. Moyes. 2015b. Glucose supplementation has minimal effects on blood neutrophil function and gene expression in vitro. J. Dairy Sci. 98:6139-6150.

Grammas, P., L. Hamdheydari, E. J. Benaksas, S. Mou, Q. N. Pye, W. J. Wechter, R. A. Floyd, C. Stewart, and K. Hensley. 2004. Anti-inflammatory effects of tocopherol metabolites. Biochem. Biophys. Res. Commun. 319:1047-1052.

Gruet, P., P. Maincent, X. Berthelot, and V. Kaltsatos. 2001. Bovine mastitis and intramammary drug delivery: review and perspectives. Adv. Drug Deliv. Rev. 50:245-259.

Higuchi, H., E. Ito, H. Iwano, S. Oikawa, and H. Nagahata. 2013. Effects of vitamin E supplementation on cellular alpha-tocopherol concentrations of neutrophils in Holstein calves. Can. J. Vet. Res. 77:120-125.

Hogan, J. S., W. P. Weiss, D. A. Todhunter, K. L. Smith, and P. S. Schoenberger. 1992. Bovine neutrophil responses to parenteral vitamin E. J. Dairy Sci. 75:399-405.

Kessler, E. C., J. J. Gross, R. M. Bruckmaier, and C. Albrecht. 2014. Cholesterol metabolism, transport, and hepatic regulation in dairy cows during transition and early lactation. J. Dairy Sci. 97:5481-5490.

Kobayashi, Y. 2008. The role of chemokines in neutrophil biology. Front Biosci. 13:2400-2407.

Lal, M. A., H. Brismar, A. C. Eklof, and A. Aperia. 2002. Role of oxidative stress in advanced glycation end product-induced mesangial cell activation. Kideny Int. 61: 2006-2014.

Lauzon, K., X. Zhao, A. Bouetard, L. Delbecchi, B. Paquette, and P. Lacasse. 2005. Antioxidants to prevent bovine neutrophil-induced mammary epithelial cell damage. J. Dairy Sci. 88:4295-4303.

Li, X. and J. M. May. 2003. Location and recycling of mitochondrial alpha-tocopherol. Mitochondrion. 3:29-38.

Li-Weber, M., M. Giaisi, M. K. Treiber, and P. H. Krammer. 2002. Vitamin E inhibits IL-4 gene expression in peripheral blood T cells. Eur. J. Immunol. 32:2401-2408.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) Method. Methods. 25:402-408.

Luostarinen, R., A. Siegbahn, and T. Saldeen. 1991. Effects of dietary supplementation with vitamin E on human neutrophil chemotaxis and generation of LTB4. Ups. J. Med. Sci. 96:103-111.

Maziere, C., M. Auclair, M. Djavaheri-Mergny, L. Packer, and J. C. Maziere. 1996. Oxidized low density lipoprotein induces activation of the transcription factor NF kappa B in fibroblasts, endothelial and smooth muscle cells. Int. J. Biochem. Mol. Biol. 39: 1201-1207.

Mehrzad, J., L. Duchateau, S. Pyorala, and C. Burvenich. 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. J. Dairy Sci. 85:3268-3276.

National Research Council. 2001. Nutrient requirements of dairy cattle. 7th rev.ed. National Academy of Science ed. Washington, DC, National Academy Press.

Okamoto, T., K. Gohil, E. I. Finkelstein, P. Bove, T. Akaike, and v. d. Vliet A. 2004. Multiple contributing roles for NOS2 in LPS-induced acute airway inflammation in mice. Am. J. Physiol. Lung Cell Mol. Physiol. 286: 198-209.

Paape, M. J., D. D. Bannerman, X. Zhao, and J. W. Lee. 2003. The bovine neutrophil: Structure and function in blood and milk. Vet. Res. 34:597-627.

Persson, K., I. Larsson, and S. C. Hallen. 1993. Effects of certain inflammatory mediators on bovine neutrophil migration in vivo and in vitro. Vet. Immunol. Immunopathol. 37:99-112.

Pilotto, A., G. Savoini, A. Baldi, G. Invernizzi, V. C. De, G. Theodorou, P. Koutsouli, and I. Politis. 2016. Short communication: Associations between blood fatty acids, beta-hydroxybutyrate, and alpha-tocopherol in the periparturient period in dairy cows: An observational study. J. Dairy Sci. 99:8121-8126.

Pinotti, L., R. Rebucci, E. Fusi, L. Rossi, and A. Baldi. 2003. Milk choline, alpha-tocopherol and neutrophil chemotaxis in the periparturient dairy cow. Vet. Res. Commun. 27. Suppl 1:265-268.

Politis, I., G. Theodorou, A. D. Lampidonis, A. Kominakis, and A. Baldi. 2012. Short communication: Oxidative status and incidence of mastitis relative to blood alpha-tocopherol concentrations in the postpartum period in dairy cows. J. Dairy Sci. 95:7331-7335.

Politis, I., A. Voudouri, I. Bizelis, and G. Zervas. 2003. The effect of various vitamin E derivatives on the urokinase-plasminogen activator system of ovine macrophages and neutrophils. Br. J. Nutr. 89:259-265.

Qu, Y., A. N. Fadden, M. G. Traber, and G. Bobe. 2014. Potential risk indicators of retained placenta and other diseases in multiparous cows. J. Dairy Sci. 97:4151-4165.

Qu, Y., K. Lytle, M. G. Traber, and G. Bobe. 2013. Depleted serum vitamin E concentrations precede left displaced abomasum in early-lactation dairy cows. J. Dairy Sci. 96:3012-3022.

Saldeen, T., D. Li, and J. L. Mehta. 1999. Differential effects of alpha- and gamma-tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis. J. Am. Coll. Cardiol. 34:1208-1215.

SAS User's Guide: Statistics. [9.4]. 2017. Cary, NC, SAS Inst.,Inc.

Schelling, G. T., R. A. Roeder, M. J. Garber, and W. M. Pumfrey. 1995. Bioavailability and interaction of vitamin A and vitamin E in ruminants. J. Nutr. 125: 1799-1800.

Singh, A. K., S. Pandita, Z. A. Pampoori, R. Huozha, and M. Ashutosh. 2013. Effect of pre-partum supplementation of vitamin E to Murrah buffaloes on immune functions and viability of calves. J. Anim Physiol Anim Nutr (Berl). 97:255-262.

Sohn, E. J., M. J. Paape, E. E. Connor, D. D. Bannerman, R. H. Fetterer, and R. R. Peters. 2007. Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-alpha, IL-1beta, IL-12 and IFN-gamma. Vet. Res. 38:809-818.

Sordillo, L. M. 2016. Nutritional strategies to optimize dairy cattle immunity. J. Dairy Sci. 99:4967-4982.

Sordillo, L. M., G. A. Contreras, and S. L. Aitken. 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Anim. Health Res. Rev. 10:53-63.

Spears, J. W. and W. P. Weiss. 2008. Role of antioxidants and trace elements in health and immunity of transition dairy cows. Vet. J. 176:70-76.

Tian, S. Z., C. J. Chang, C. C. Chiang, H. C. Peh, M. C. Huang, J. W. Lee, and X. Zhao. 2005. Comparison of morphology, viability, and function between blood and milk neutrophils from peak lactating goats. Can. J. Vet. Res. 69:39-45.

Traber, M. G. 2004. Vitamin E, nuclear receptors and xenobiotic metabolism. Arch. Biochem. Biophys. 423:6-11.

Traber, M. G. 2013. Mechanisms for the prevention of vitamin E excess. J. Lipid Res. 54:2295-2306.

Varga, Z., E. Kosaras, E. Komodi, M. Katko, I. Karpati, J. Balla, G. Paragh, M. C. Aisa, and F. Galli. 2008. Effects of tocopherols and 2,2'-carboxyethyl hydroxychromans on phorbol-ester-stimulated neutrophils. J. Nutr. Biochem. 19:320-327.

Weiss, W. P. and N. R. St-Pierre. 2009. A method to quantify changes in supply of metabolizable methionine to dairy cows using concentrations of selenium in milk. J. Dairy Sci. 92:2835-2842.

Yang, J., L. Zhang, C. Yu, X. F. Yang, and H. Wang. 2014. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark. Res. 2:1.

Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature. 395:284-288.

Tocopherol mix and Bovine neutrophils Chapter Tables and Figures:

TABLES:

Table 4.1. Whole blood hematology profiles of immune cells before (i.e. 0 d) and after (i.e. 7 d) feeding a tocopherol supplement[1] to 12 Holstein cows during mid-lactation.

Cell population	Tmix feeding		SEM	*P*-value
	Day 0	Day 7		
Basophils, $\times 10^3/\mu L$	0.01	0.01	0.01	0.30
Eosinophils, $\times 10^3/\mu L$	0.35	0.36	0.08	0.80
Lymphocytes, $\times 10^3/\mu L$	4.00	4.10	0.23	0.18
Monocytes, $\times 10^3/\mu L$	0.80	0.90	0.05	0.04
Neutrophils, $\times 10^3/\mu L$	2.80	3.20	0.20	0.17
White blood cells, $\times 10^3/\mu L$	8.00	8.70	0.40	0.15

[1]Mixed tocopherol oil supplement (Tmix): 300g of (9 % α-, 1 % β-, 24 % δ-, and 62 % γ-tocopherol) pure tocopherol mixed with 22.68 kg of calf grower (Farmers Cooperative Association Inc.), then fed to all cows at ~620 g/d for 7 consecutive days.

Table 4.2. Effect of feeding a tocopherol supplement[1] and LPS challenge[2] on the expression of genes from bovine neutrophils from Holstein cows during mid-lactation (n=6/treatment). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method and presented as fold-change (FC; 7/ 0 d).

	D 7 vs. D 0		+LPS vs. -LPS	
	FC	*P*-value	FC	*P*-value
Pro/anti-inflammatory genes				
COX2(PTGS-2)	1.35	0.32	4.46	<0.01
IL6	1.08	0.71	2.71	<0.01
IL10	1.21	0.36	1.97	<0.01
MYD88	1.58	0.02	1.05	0.89
NFKB1	1.38	0.05	1.06	<0.01
TNFA	1.69	0.04	3.92	<0.01
Adhesion/receptor genes				
ITGB2	1.01	0.65	1.00	0.98
PLAUR	0.99	0.96	1.35	0.15
SELL	1.20	0.28	0.90	0.57
TLR2	1.97	0.30	1.35	0.09
TLR4	1.03	0.88	1.29	0.22
Superoxide enzyme genes				
NCF1	1.47	<0.01	0.97	0.82
NCF2	1.16	0.50	0.84	0.45
NOS2	0.98	0.93	2.12	<0.01
SOD2	1.47	0.03	1.29	0.15
Cell apoptosis genes				
CASP3	1.12	0.46	1.06	0.71
Cell signaling genes				
mTOR	0.96	0.90	0.75	0.43
Transcription factor genes				
PPARα	1.03	0.86	0.96	0.83
RARα	1.28	0.03	0.97	0.77
RXRα	1.14	0.50	0.96	0.82
RXRβ	1.04	0.70	1.05	0.62

[1]Mixed tocopherol oil supplement (Tmix): 300g of (9 % α-, 1 % β-, 24 % δ-, and 62 % γ-tocopherol) pure tocopherol mixed with 22.68 kg of calf grower (Farmers Cooperative Association Inc.), then fed to all cows at ~620 g/d for 7 consecutive days.

[2]On 0 d and 7 d,bovine neutrophils were isolated from dairy cows fed mixed tocopherol oil supplement (Tmix) and incubated with or without lipopolysaccharide (+/- LPS) (1.5 µg/mL) for 2 h *in vitro*.

FIGURES:
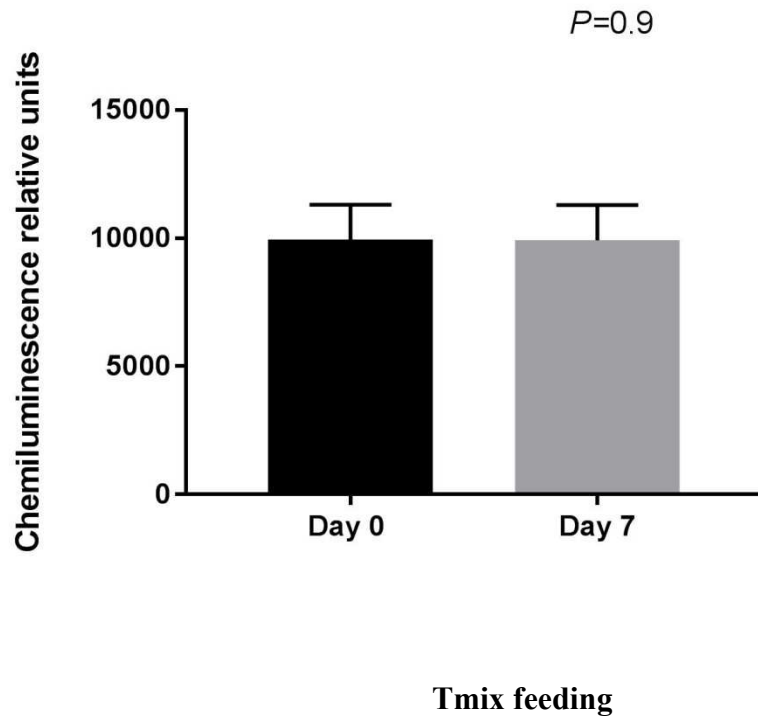
Figure 4.1. The effect of mixed tocopherol oil supplement (Tmix) on whole blood respiratory burst response as measured by chemiluminescence on 0 d before and 7 d after feeding of Tmix to 12 Holstein cows during mid-lactation.
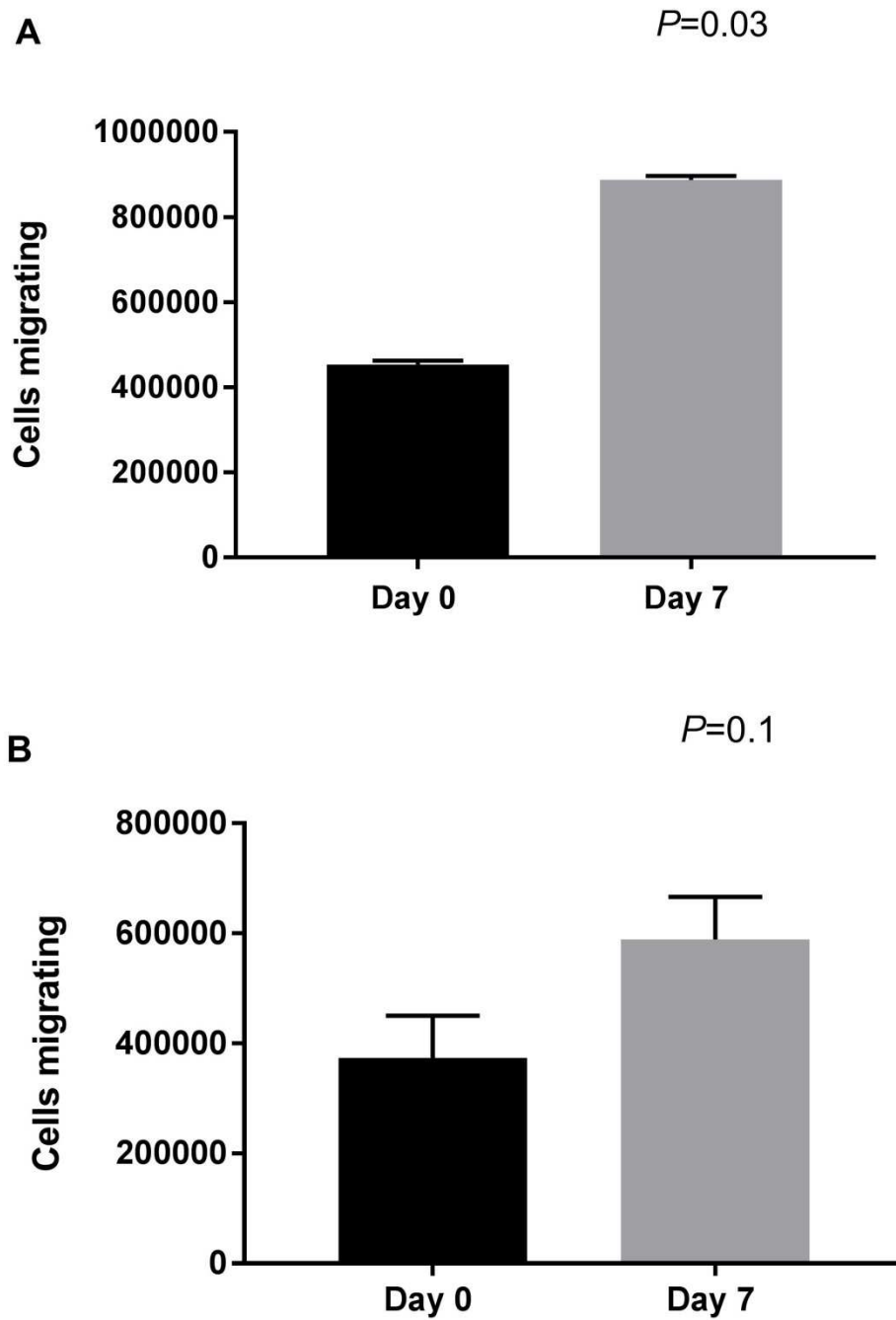
Figure 4.2. The effect of mixed tocopherol oil supplement (Tmix) on bovine neutrophil chemotactic response to (A) bovine interleukin-8 and (B) bovine complement component 5a on 0 d before and 7 d of feeding of Tmix to 12 Holstein cows during mid-lactation.

Tmix feeding

CHAPTER 5: SUMMARY AND CONCLUSIONS

The three objectives of this study were: 1) to investigate the accumulation and distribution level of all four tocopherol isoforms in bovine tissue and mitochondria; 2) to investigate the pattern change of all four tocopherol isoforms (α-, β-, γ-, and δ-tocopherol in bovine blood and milk; and 3) to investigate the effect of all four tocopherol isoforms on bovine PMN function and immune-metabolic-related gene expression via Tmix supplementation of dairy cows.

In **Chapter 2**, the objective was to investigate the accumulation and distribution level of all four tocopherol isoforms in bovine tissue and mitochondria via Tmix supplementation. The results indicated that Tmix supplementation increased γ-tocopherol concentrations, but did not increase the α-tocopherol concentration in three different types of tissues (i.e. liver, mammary gland and muscle) and liver mitochondria. Within those three different types of tissues, liver showed the highest ability to store tocopherol isoforms (i.e. α- and γ-tocopherol) compared to the other tissues. Also, amounts of α- and γ-tocopherol were detected in liver mitochondria, but limited amounts were detected in mammary gland mitochondria. It is concluded that the majority of α- and γ-tocopherol was absorbed into the liver as compared to the mammary gland in both gross tissue and mitochondria. The high concentration of tocopherol isoform accumulation in liver supports the proposed Tmix supplementation strategies of targeting liver to mitigate disrupted metabolic issues using vitamin E supplements during the transition period in dairy cows.

In **Chapter 3**, the objective was to investigate the pattern change of all four tocopherol isoforms (α-, β-, γ-, and δ-tocopherol in bovine blood and milk via Tmix

supplementation. It was demonstrated that supplementation of Tmix could increase γ-tocopherol concentration in milk and blood and via frequent measurements. Even though daily data showed that the α isoform concentrations were highest, Tmix supplementation increased γ concentrations in milk and blood as compared to α-tocopherol (Figures 3.1 and 3.2). Non-detectable of β- and δ-tocopherol were observed in milk and blood via Tmix supplementation. Tmix provided adequate γ-tocopherol concentration (i.e. ~3.2 μg/mL) in blood and exceeded the required value (~3 μg/mL) in dairy cows (Weiss, 1998). In addition, daily measurement results showed that Tmix could provide the high and stable concentrations of both necessary α- and γ-tocopherol isoforms in milk and blood via supplementation for 5 consecutive days. This result could provide a new aspect of vitamin E supplement strategies to meet necessary vitamin E requirements (i.e. α- and γ-tocopherol) in dairy cows, especially when stored vitamin E is depleted during the transition period in dairy cows (Qu et al., 2014). The results support the hypothesis that Tmix could enrich α- and γ-tocopherol isoform concentrations in blood and milk.

In **Chapter 4**, the objective was to investigate the effect of all four tocopherol isoforms on bovine PMN function and immune-metabolic-related gene expression via Tmix supplementation**.** The results indicated that Tmix increased PMN chemotaxis function and did not impair the whole blood respiratory burst response, which might be associated with non-α-tocopherol isoforms present in the Tmix. The increased expression of pro-inflammatory genes (e.g. *TNFA* and *IL6*) might be necessary for the initiation of innate immune functions and further activation of adaptive immune functions. Tmix did not alter hematology and plasma metabolite profiles in dairy cows.

Therefore, it might be concluded that Tmix supplementation will not harm PMN functions.

Overall, it was demonstrated that Tmix supplementation for 5 consecutive days could increase γ-tocopherol without altering α-tocopherol in dairy cows. The liver showed the highest capability of accumulating tocopherol isoforms compared to the mammary gland and muscle. The liver could be the potential target of mitigating metabolic issues using Tmix (highly enriched with γ-tocopherol) in dairy cows during the transition period. Besides, Tmix did not harm immune functions nor alter animal health in dairy cows as measured by PMN chemotaxis, respiratory burst response, hematology profiles and plasma metabolites. Therefore, the potential Tmix supplementation strategies could be developed to focus on mitigating liver metabolic issues caused by biological stress (i.e. oxidative stress and nitroxidative stress) and improving animal health during the transition period.

Supplemental Tables:

Supplemental Table S2.1. Protein concentration in isolated mitochondrial fraction from liver and mammary gland tissue.

Mitochondrial source	Feeding group	Protein concentration (mg/mL)
Liver	Tmix[1]	6.08
Liver	Tmix	6.34
Liver	Tmix	6.56
Liver	Tmix	6.51
Liver	Tmix	5.38
Liver	Control[2]	6.20
Liver	Control	8.09
Liver	Control	8.65
Mammary gland	Tmix	2.23
Mammary gland	Tmix	1.72
Mammary gland	Tmix	1.23
Mammary gland	Tmix	2.68
Mammary gland	Tmix	3.07
Mammary gland	Control	2.77
Mammary gland	Control	1.43
Mammary gland	Control	1.55

[1]Mixed tocopherol oil supplement (Tmix; n=5): 300 g of (9 % α-, 1 % β-, 24 % δ-, and 62 % γ-tocopherol) pure tocopherol mixed with 22.68 kg of calf grower (Farmers Cooperative Association Inc.), then fed to all cows at ~620 g/d for 9 consecutive days.
[2]Control group (n=3).

Supplemental Table S2.2: GenBank accession number, gene symbol, hybridization position, sequence, amplicon size, and references for *Bos taurus* primers used to analyze gene expression by qPCR in bovine tissues.

Accession #	Gene Symbol	Primer	Primer Sequence (5' à 3')	Amplicon size (bp)	References
NM_001035042.1	*CYP4F2*	F.15	ACAAGCCGCTCTTGACAGAA	196	6
		R.191	GAGGCGACGAGAGTTGTTGT		
NM_001098964	*HPCAL*	F.635	CCATCGACTTCAGGGAGTTC	99	7
		R.733	CGTCGAGGTCATACATGCTG		
BC149232.1	*LRP10*	F.2487	CCAGAGGATGAGGACGATGT	139	7
		R.262	CCAGAGGATGAGGACGATGT		
NM_001076409.1	*NFKB1*	F.172	TTCAACCGGAGATGCCACTAC	95	3
		R.266	ACACACGTAACGGAAACGAAATC		
NM001103226.1	*NR1I2*	F.1332	TGAAGGCCTACATCGAGTTCAAC	68	5
		R.1379	GGCCATGATCTTCAGGAACAA		
NM_001079768.2	*NR1I3*	F.312	GAAGGACATGATCCTATCGACAGA	63	1
		R.358	CGTCGCTGGGCCTGTCT		
NM_181024.2	*PPARG*	F.494	AATCAAAGTGGAGCCTGTATC	343	4
		R.787	CCTGATGGCATTATGAGACA		
NM_001304343.1	*RXRα*	F.408	CGCTCCTCAGGCAAGCA	121	8
		R.506	TGTCAATCAGGCAGTCCTTGTT		
NM_001083640.1	*RXRβ*	F.1232	CGTGGGAGCCATCTTCGA	60	8
		R.1273	CTCATGTCCCGCATTTTGG		
NM_174597.2	*SCARB1*	F.1241	GGAATCCCCATGAACTG	366	2
		R.1588	CTTGGGAGCTGATGTCATC		
NM_177943.2	*SEC14L2*	F.1145	GCAGCGATCCTGGCATCTAT	109	6
		R.1233	TCTGAGGCTTTGTCTGGAAGC		
NM_001206676.1	*TTPA*	F.1329	GCCACCAAGCTAGTTCACCT	113	6
		R.1422	CCTAGAGACCTGATGCAGCG		

[1]Cantiello, M., M. Giantin, M. Carletti, R. M. Lopparelli, F. Capolongo, F. Lasserre, E. Bollo, C. Nebbia, P. G. P. Martin, T. Pineau, and M. Dacasto. 2009. Effects of dexamethasone, administered for growth promoting purposes, upon the hepatic cytochrome P450 3A expression in the veal calf. Biochem. Pharmacol. 77:451-463.

[2]Giantin, Me., R. M. Lopperalli, V. Zancanella, P. G. Martin, A. Polizz, G. Gallina, F. Gottardo, C. Montesissa, L. Ravarotto, T. Pineau, and M. Dacasto. 2010. Effect of illicit dexamethasone upon hepatic drug metabolizing enzymes and related transcription factors mRNAs and their potential use as biomarkers in cattle. J. Agric. Food Chem. 58: 1342-1349.

[3]Janovick-Guretzky, N. A., H. M. Dann, D. B. Carlson, M. R. Murphy, J. J. Loor, and J. K. Drackley. 2007. Housekeeping gene expression in bovine liver is affected by physiological state, feed intake, and dietary treatment. J. Dairy Sci. 90:2246-2252.

[4]Liu, X., G. Liu, X. Tan, H. Zhao, H. Cheng, F. Wan, N. Wu, and E. Song. 2014. Gene expression profiling of *SIRT1*, *FoxO1*, and *PPARγ* in backfat tissues and subcutaneous adipocytes of Lilu bulls. Meat Sci. 96:704-711.

[5]Rohrer, L., P. M. Ohnsorg, M. Lehner, F. Landolt, F. Rinninger, and A. V. Eskardstein. 2009. High-density lipoprotein transport through aortic endothelial cells involves scavenger receptor BI and ATP-binding cassette transporter G1. Mol. Med. 104:1142-1150.

[6]Sadri, H., S. Dänicke, U. Meyer, J. Rehage, J. Frank, and H. Sauerwein. 2015. Tocopherols and tocotrienols in serum and liver of dairy cows receiving conjugated linoleic acids or a control fat supplement during early lactation. J. Dairy Sci. 98:7034-7043.

[7]Saremi, B., H. Sauerwein, S. Dänicke, and M. Mielenz. 2012. Technical note: Identification of reference genes for gene expression studies in different bovine tissues focusing on different fat depots. J. Dairy Sci. 95:3131–3138.

[8]Schmitt, E., M. A. Ballou, M. N. Correa, E. J. DePeters, J. K. Drackley, and J. J. Loor. 2011. Dietary lipid during the transition period to manipulate subcutaneous adipose tissue peroxisome proliferator-activated receptor-γ co-regulator and target gene expression. J. Dairy Sci. 94:5913-5925.

Supplemental Table S2.3: Efficiency of dilution curves and median Cq values for all tested samples.

Tissue/Gene	Slope[3]	R[2][4]	Efficiency[5]	Lower value	Higher value	Median Cq
	Dilution curve[1]			**Cq Values across all samples[2]**		
Liver						
CYP4F2	-3.28	0.99	1.02	27.83	30.29	29.01
HPCAL1	-3.11	0.99	1.09	28.36	30.67	30.14
LRP10	-3.15	0.98	1.08	25.19	27.85	26.78
NFKB1	-3.65	0.99	0.96	27.55	30.05	29.59
NR1I2	-3.44	0.99	0.95	26.08	29.42	27.78
NR1I3	-3.17	0.99	1.07	25.32	27.46	26.45
RXRα	-3.59	0.99	0.90	23.99	26.31	24.99
RXRβ	-3.22	0.99	1.04	27.70	29.71	28.90
SEC14L2	-3.57	0.99	0.90	27.16	25.02	25.91
TTPA	-3.51	0.99	0.93	23.12	25.90	24.58
Mammary						
HPCAL1	-3.31	0.99	1.00	28.75	30.33	29.70
LRP10	-3.17	0.99	1.07	25.08	27.55	26.50
NFKB1	-3.26	0.98	1.02	27.67	30.84	29.31
PPARG	-3.46	0.98	0.94	28.44	30.74	30.07
RXRα	-3.18	0.99	1.06	26.96	28.54	27.55
RXRβ	-3.34	0.99	0.99	27.85	29.75	28.93
SCARB1	-2.97	0.99	1.17	29.64	33.77	31.34
Muscle						
LRP10	-3.47	0.99	0.94	27.55	31.06	29.61
HPCAL1	-3.26	0.99	1.02	30.89	34.42	32.87
NFKB1	-3.62	0.99	0.89	30.15	34.17	31.98
RXRα	-3.51	0.99	0.93	26.68	30.74	28.23
RXRβ	-3.33	0.99	0.99	29.40	31.45	30.58

[1] Efficiency was tested with 6 serial dilutions (1:4).
[2] Cq values corresponding to all analyzed samples for each corresponding gene using 4 μL of cDNA. template per reaction generated from 500 ρg of RNA.
[3] Slope of the dilution curve.
[4] Coefficient of determination of the dilution curve.
[5] Efficiency of the dilution curve was calculated as $[(10^{(-1 / Slope)})-1]$.

Supplemental Table S2.4. Gene symbol, gene name, and description of the main biological functions and processes of targets analyzed in bovine tissues.

Symbol	Name	Summary description form GENECARDS (www.genecards.org)
CYP4F2	Cytochrome P450 Family4 Subfamily F Member2	This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids.
HPCAL1	Hippocalcin Like 1	The protein encoded by this gene is a member of neuron-specific calcium-binding proteins family found in the retina and brain. It serves as housekeeping genes in this study.
LRP10	LDL Receptor Related Protein 10	This gene encodes a low density lipoprotein receptor family protein. It serves as housekeeping genes in this study.
NFKB1	Nuclear Factor Kappa B Subunit 1	This gene encodes for a DNA binding subunit of the NF-kappa-B (NFKB) protein complex. NFKB is a transcription regulator that is activated by various intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, ultraviolet irradiation, and bacterial or viral products.
NR1I2(PXR)	Nuclear Receptor Subfamily 1 Group Member 2	This gene product belongs to the nuclear receptor superfamily, members of which are transcription factors characterized by a ligand-binding domain and a DNA-binding domain. The encoded protein is a transcriptional regulator of the cytochrome P450 gene CYP3A4, binding to the response element of the CYP3A4 promoter as a heterodimer with the 9-cis retinoic acid receptor (RXR).
NR1I3(CAR)	Nuclear Receptor Subfamily 1 Group Member 3	This gene encodes a member of the nuclear receptor superfamily, and is a key regulator of xenobiotic and endobiotic metabolism. The protein binds to DNA as a monomer or a heterodimer with the retinoid X receptor and regulates the transcription of target genes involved in drug metabolism and bilirubin clearance, such as cytochrome P450 family members.
PPARG	Peroxisome Proliferator Activated Receptor Gamma	This gene encodes a member of the peroxisome proliferator-activated receptor (PPAR) subfamily of nuclear receptors. PPARs form heterodimers with retinoid X receptors (RXRs) and these heterodimers regulate transcription of various metabolic function genes.
RXRα	Retinoid X Receptor Alpha	Retinoid X receptors (RXRs) and retinoic acid receptors (RARs) are nuclear receptors that mediate the biological effects of retinoids by their involvement in retinoic acid-mediated gene activation.
RXRβ	Retinoid X Receptor Beta	This gene encodes a member of the RXR family of nuclear receptors which are involved in mediating the effects of retinoids.
SCARB1	Scavenger Receptor Class B Member1	The protein encoded by this gene is a plasma membrane receptor for high density lipoprotein cholesterol (HDL). The encoded protein mediates cholesterol transfer to and from HDL.
SEC14L2	SEC14 Like Lipid Binding 2	This gene encodes a cytosolic protein which belongs to a family of lipid-binding proteins including alpha-tocopherol transfer protein and cellular retinol-binding protein.
TTPA	Alpha Tocopherol Transfer Protein	This gene encodes a soluble protein that binds alpha-trocopherol, a form of vitamin E, with high selectivity and affinity. This protein plays an important role in regulating vitamin E levels in the body by

transporting vitamin E between membrane vesicles and facilitating the
secretion of vitamin E from hepatocytes to circulating lipoproteins.

Supplemental Table S4.1: GenBank accession number, gene symbol, hybridization position, sequence, amplicon size, and references for *Bos taurus* primers used to analyze gene expression by qPCR in bovine PMN.

Accession #	Gene Symbol	Primer	Primer Sequence (5' → 3')	Amplicon size (bp)	Reference
NM_001077840.1	*CASP3*	F.454	AAGCCATGGTGAAGAAGGAA	112	4
		R.618	CCTCAGCACCACTGTCTGTC		
AF004944.1	*COX2 (PTGS2)*	F.92	CCAGAGCTCTTCCTCCTGTG	161	1
		R.252	GGCAAAGAATGCAAACATCA		
NM_001034034.2	*GAPDH*	F.908	TTGTCTCCTGCGACTTCAACA	103	5
		R.1010	TCGTACCAGGAAATGAGCTTGAC		
NM_173923.2	*IL6*	F.190	CCAGAGAAAACCGAAGCTCTCAT	100	9
		R.289	CCTTGCTGCTTTCACACTCATC		
NM_174088.1	*IL10*	F.429	GAGAGTCTTCAGTGAGCTCCAAGAG	101	7
		R.529	GCATCTTCGTTGTCATGTAGGTTT		
NM_175781.1	*ITGB2*	F.1230	GACACCCTGAAAGTCACCTACGA	65	12
		R.1317	GAAGGTGATCGGGACGTTGAT		
NM_001014382.2	*MYD88*	F.366	GGAGGACTGCCAAAAGTATATTCTG	104	7
		R.447	GCCATGTCATTTATCCGAGTTATG		
XM_002694043.3	*mTOR*	F.3452	CCCCGATCGTGAAGTTATTTG	141	10
		R.3592	GTGTGCGTACAATCGGATGAA		
NM_174119.4	*NCF1*	F.369	TCCTCAACTTCTTCAAGGTGCG	108	This paper
		R.455	CAGCGTTGTTCTTGCCATCTTT		
NM_174120.2	*NCF2*	F.1302	GCCCCTTTCAGAATTCAGCAT	107	This paper
		R.1341	GCGTCAGGCAGTAGTTTTTCACT		
NM_001076409.1	*NFKB1*	F.172	TTCAACCGGAGATGCCACTAC	95	9
		R.266	ACACACGTAACGGAAACGAAATC		
NM_001076799.1	*NOS2*	F.124	TTGAGATCAACGTCGCTGTG	56	13
		R.130	CATGATGGTCACGTTCTGCT		
BT021611.1	*OSBPL2*	F.198	TGCCGTCACAGGCTTTGAC	100	8
		R.297	CCATTACTTGCTGGTGTCCACAT		
NM_174423.3	*PLAUR*	F.174	GTCCTGAGTGTGTGGGAAGG	140	6
		R.332	CAGTAGCATCTCGACCAGGG		
NM_001034036.1	*PPARα*	F.727	CATAACGCGATTCGTTTTGGA	62	7
		R.811	CGCGGT TTCGGAATCTTCT		
NM_001014942.3	*RARα*	F.794	CAAGACAAATCCTCCGGCTA	220	3
		R.991	TGTTCCGGTCATTTCTCACA		
NM_001101152.2	*RPS9*	F.128	CCTCGACCAAGAGCTGAAG	64	8
		R.191	CCTCCAGACCTCACGTTTGTTC		
NM_001304343.1	*RXRα*	F.408	CGCTCCTCAGGCAAGCA	121	11
		R.506	TGTCAATCAGGCAGTCCTTGTT		
NM_001083640.1	*RXRβ*	F.1232	CGTGGGAGCCATCTTCGA	60	11
		R.1273	CTCATGTCCCGCATTTTGG		

NM_201527.2	*SOD2*	F.272	GAGAAGGGTGATGTTACAGCTCAGA	100	2
		R.371	GGCTCAGATTTGTCCAGAAGATG		
NM_174197.2	*TLR2*	F.88	TGGGTGGAGAACCTCATGGT	100	7
		R.187	CGATAATCCACTTGCCAGGAA		
NM_174198.6	*TLR4*	F.85	TGCGTACAGGTTGTTCCTAACATT	110	7
		R.194	TAGTTAAAGCTCAGGTCCAGCATCT		
NM_173966.3	*TNFA*	F.174	CCAGAGGGAAGAGCAGTCCC	114	7
		R.287	TCGGCTACAACGTGGGCTAC		

[1]Coyne, G. S., D. A. Kenny, S. Childs, J. M. Sreenan, and S. M. Waters. 2008. Dietary n-3 polyunsaturated fatty acids alter the expression of genes involved in prostaglandin biosynthesis in the bovine uterus. Theriogenology 70:772–782.

[2]Garcia, M., T. Elsasser, D. Biswas, and K. M. Moyes. 2014. The effect of citrus-derived oil on bovine blood neutrophil function and gene expression in vitro. J. Dairy Sci. 98:918-926.

[3]Greger, D. L., C. Philipona, and J. W. Blum. 2006. Ontogeny of mRNA abundance of nuclear receptors and nuclear receptor target genes in young cattle. Domest. Anim. Endocrinol. 31:76–87.

[4]Herzog, K., Strüve, K., Kastelic, J.P., Piechotta, M., Ulbrich, S.E., Pfarrer, C., Shirasuna, K., Shimizu, T., Miyamoto, A. and Bollwein, H., 2012. *Escherichia coli* lipopolysaccharide administration transiently suppresses luteal structure and function in diestrous cows. Reproduction. 144 :467-476.

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

[6]Krania, F., Dovolou E, Rekkas CA, Heras S, Pappas I, Soom AV, Amiridis GS. 2015. Urokinase-type plasminogen activator does not affect in vitro bovine embryo development and quality. Acta. Vet. Hungarica. 63:243-54.

[7]Moyes, K. M., J. K. Drackley, D. E. Morin, M. Bionaz, S. L. Rodriguez-Zas, R. E. Everts, H. A. Lewin, and J. J. Loor. 2009. Gene network and pathway analysis of bovine mammary tissue challenged with *Streptococcus uberis* reveals induction of cell proliferation and inhibition of PPAR-gamma signaling as potential mechanism for the negative relationships between immune response and lipid metabolism. BMC. Genomics 10:542.

[8]Moyes, K. M., J. K. Drackley, D. E. Morin, and J. J. Loor. 2010. Greater expression of *TLR2, TLR4*, and *IL6* due to negative energy balance is associated with lower expression of *HLA-DRA* and *HLA-A* in bovine blood neutrophils after intramammary mastitis challenge with *Streptococcus uberis*. Funct. Integr. BMC. Genomics. 10:53–61.

[9]Moyes, K. M., D. E. Graugnard, M. J. Khan, M. Mukesh, and J. J. Loor. 2014. Postpartal immunometabolic gene network expression and function in blood neutrophils are altered in response to prepartal energy intake and postpartal intramammary inflammatory challenge. J. Dairy Sci. 97:2165-2177.

[10]Naeem, A., J. K. Drackley, J. Stamey, and J. J. Loor. 2012. Role of metabolic and cellular proliferation genes in ruminal development is response to enhanced plane of nutrition in neonatal Holstein calves. J. Dairy Sci. 95:1807–1820.

[11]Schmitt, E., M. A. Ballou, M. N. Correa, E. J. DePeters, J. K. Drackley, and J. J. Loor. 2011. Dietary lipid during the transition period to manipulate subcutaneous adipose tissue peroxisome proliferator-activated receptor,co-regulator and target gene expression. J. Dairy Sci. 94:5913–5925.

[12]Seo, J., J.S. Osorio, J.J. Loor. 2013. Purinergic signaling gene network expression in bovine polymorphonuclear neutrophils during the peripartal period. J. Dairy Sci., 96: 7675-7683

[13]Shu, D., A. Heiser, D. N. Wedlock, D. Luo, G. W. de Lisle GW, and B. M. Buddle. 2014. Comparison of gene expression of immune mediators in lung and pulmonary lymph node granulomas from cattle experimentally infected with *Mycobacterium bovi*s. Vet. Immunol. Immunopathol. 160:81-89.

Supplemental Table S4.2. Gene symbol, gene name, and description of the main biological functions and processes of targets analyzed in circulating bovine neutrophils.

Symbol	Name	Summary description from GENECARDS (www.genecards.org)
CASP3	Caspase 3	This gene encodes a protein which is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis.
COX2(PTGS2)	Postaglandin-endoperoxide-synthase 2	Prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase, is the key enzyme in prostaglandin biosynthesis. This gene encodes the inducible isozyme. It is regulated by specific stimulatory events, suggesting that it is responsible for the prostanoid biosynthesis involved in inflammation.
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	This gene encodes a member of the glyceraldehyde-3-phosphate dehydrogenase protein family. It is expressed stable in PMN, therefore served as one of the housekeeping genes in PMN
IL6	Interleukin 6	This gene encodes a cytokine that functions in inflammation and the maturation of immune cells. In addition, the encoded protein has been shown to be an endogenous pyrogen capable of inducing fever in animals with autoimmune diseases or infections
IL10	Interleukin 10	The protein encoded by this gene is produced by neutrophils). IL-10 can block NF-kappa B activity as the role of anti-inflammatory cytokines.
ITGB2	Integrin Subunit Beta 2	Integrins are integral cell-surface proteins that participate in cell adhesion as well as cell-surface mediated signaling. The encoded protein plays an important role in the immune response.
mTOR	Mechanistic target of rapamycin	The protein encoded by this gene belongs to a family of phosphatidylinositol kinase-related kinases.
MYD88	Myeloid differentiation primary response 88	This gene encodes a cytosolic adapter protein that plays a central role in the innate and adaptive immune response. This protein functions as an essential signal transducer in the interleukin-1 and Toll-like receptor signaling pathways.
NCF1	Neutrophil cytosolic factor 1	The protein encoded by this gene is a 47 kDa cytosolic subunit of neutrophil NADPH oxidase. This oxidase is a multicomponent enzyme that is activated to produce superoxide anion.

NCF2	Neutrophil cytosolic factor 2	This gene encodes neutrophil cytosolic factor 2, the 67-kDa cytosolic subunit of the multi-protein NADPH oxidase complex found in neutrophils. This oxidase produces a burst of superoxide.
NFKB1	Nuclear factor kappa B subunit 1	This gene encodes for a DNA binding subunit of the NF-kappa-B (NFKB) protein complex. NFKB is a transcription regulator that is activated by various intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, ultraviolet irradiation, and bacterial or viral products.
NOS2	Nitric oxide synthase 2	This gene encodes a nitric oxide synthase which is expressed in neutrophil and is inducible by a combination of LPS and certain cytokines.
PLAUR	Plasminogen Activator, Urokinase Receptor	This gene encodes the receptor for urokinase plasminogen activator and, given its role in localizing and promoting plasmin formation, likely influences many normal and pathological processes related to cell-surface plasminogen activation and localized degradation of the extracellular matrix.
PPARα	Peroxisome proliferator activated receptor alpha	The action of peroxisome proliferators is thought to be mediated via specific receptors, called PPARs, which belong to the steroid hormone receptor superfamily. PPARs affect the expression of target genes involved in cell proliferation, cell differentiation and in immune responses. This gene encodes the sub-type PPARα, which is a nuclear transcription factor.
RARα	Retinoic acid receptor alpha	This gene represents a nuclear retinoic acid receptor. The encoded protein, retinoic acid receptor alpha, regulates the transcription in a ligand-dependent manner.
RPS9	Ribosomal Protein S9	This gene encodes a ribosomal protein that is a component of the 40S subunit. It served as one of the housekeeping genes in PMN
RXRα	Retinoid X receptor alpha	Retinoid X receptors (RXRs) and retinoic acid receptors (RARs) are nuclear receptors that mediate the biological effects of retinoids by their involvement in retinoic acid-mediated gene activation.
RXRβ	Retinoid X receptor beta	This gene encodes a member of the RXR family of nuclear receptors which are involved in mediating the effects of retinoids .
SELL	L-Selectin	Belongs to a family of adhesion/homing receptors. The gene product is required for binding and subsequent rolling

		of leukocytes on endothelial cells, facilitating their migration into secondary lymphoid organs and inflammation sites.
SOD2	Superoxide dismutase 2, mitochondrial	Member of the superoxide dismutase family; binds to the superoxide by-products of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen.
TLR2	Toll-like receptor 2	They recognize pathogen-associated molecular patterns (PAMP) that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. This gene is expressed most abundantly in peripheral blood leukocytes, and mediates host response to Gram-positive and negative bacteria and yeast via stimulation of NFKB.
TLR4	Toll-like receptor 4	This receptor has been implicated in signal transduction events (innate immunofunctions) induced by bacterial lipopolysaccharide (LPS) a PAMP found in most gram-negative bacteria.
TNFA	Tumor necrosis factor alpha	This gene encodes for the multifunctional proinflammatory cytokine mainly secreted by macrophages; involved in cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer.

*Supplemental Table S4.3***:** Efficiency of dilution curves and median Cq values for all tested samples.

| Gene | Dilution curve[1] | | | Cq Values across all samples[5] | | |
	Slop[2]	R[2][3]	Efficiency[4]	Lower value	Higher value	Median Cq
CASP3	-3.228	0.996	1.04	28.28	31.92	29.52
COX2(PTGS2)	-3.328	0.999	1.00	23.70	28.49	26.37
GAPDH	-3.417	0.998	0.96	23.84	28.08	24.99
IL6	-3.464	0.999	0.94	25.58	28.73	26.97
IL10	-3.150	1.000	1.08	26.32	29.60	27.57
ITGB2	-3.525	0.995	0.92	27.09	30.85	28.30
mTOR	-4.345	0.999	0.70	30.42	35.37	32.35
MYD88	-3.148	0.999	1.00	26.44	30.41	34.48
NCF1	-3.175	0.995	1.07	23.50	27.90	24.37
NCF2	-3.102	0.998	1.10	27.34	31.59	29.04
NFKB1	-3.410	0.998	0.96	24.69	28.27	26.20
NOS2	-3.128	0.998	1.09	27.98	31.61	29.86
OSBPL2	-3.398	1.000	0.97	27.38	31.28	28.44
PLAUR	-3.480	0.999	0.94	24.26	27.91	25.76
PPARα	-3.583	0.998	0.90	32.22	36.39	34.48
RARα	-3.454	0.999	0.95	25.39	29.58	26.71
RPS9	-3.481	0.997	0.94	23.54	28.37	25.66
RXRα	-2.952	0.996	1.18	26.88	30.21	28.18
RXRβ	-3.188	0.999	1.06	27.99	32.84	29.76
SELL	-3.294	0.999	1.01	24.29	28.36	25.66
SOD2	-3.345	0.998	0.99	21.46	25.81	23.06
TLR2	-3.544	0.997	0.91	24.77	28.58	26.80
TLR4	-3.218	0.997	1.05	25.77	29.73	26.79
TNFA	-3.167	0.995	1.07	24.12	29.21	26.17

[1] Efficiency was tested with 6 serial dilutions (1:4)
[2] Slope of the dilution curve
[3] Coefficient of determination of the dilution curve
[4] Efficiency of the dilution curve was calculated as $[(10^{(-1 / Slope)})-1]$
[5] Cq values corresponding to all analyzed samples for each corresponding gene using 4 µL of cDNA template per reaction generated from 500 pg of RNA.

Supplemental Figures:

A

B
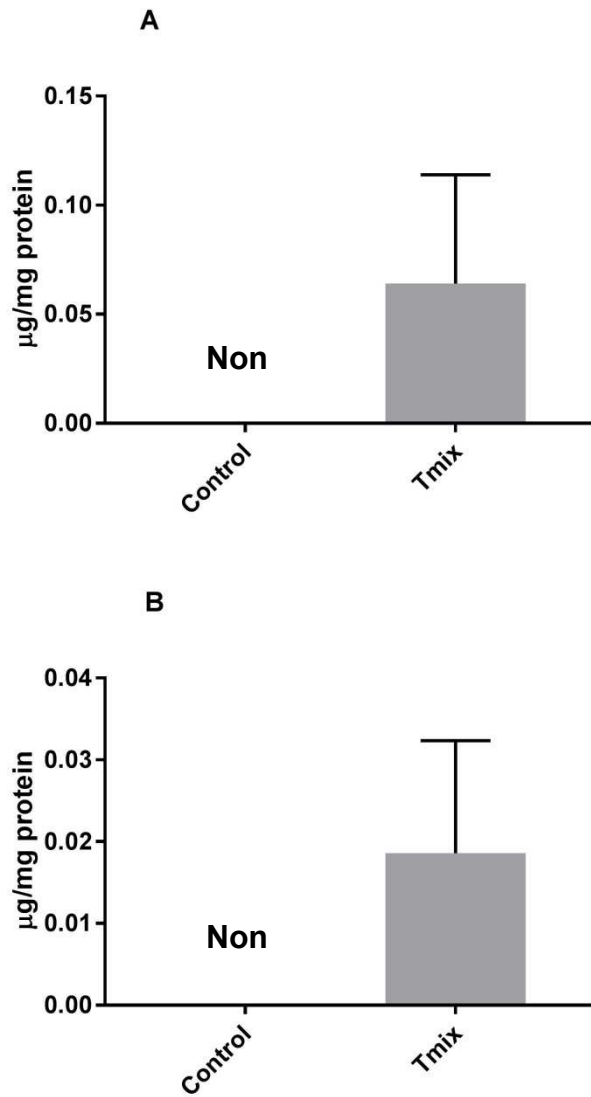
Supplemental Figure S2.1 Tocopherol isoform concentration in bovine mammary mitochondria: Control vs. Tmix (mixed tocopherol oil supplement) feeding groups after 9 d feeding for either α-tocopherol (A) or γ-tocopherol (B).

REFERENCES

Aderem, A. and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. Nature. 406:782-787.

Akira, S. and H. Hemmi. 2003. Recognition of pathogen-associated molecular patterns by TLR family. Immunol. Lett. 85:85-95.

Barrett, J. J., J. S. Hogan, W. P. Weiss, K. L. Smith, and L. M. Sordillo. 1997. Concentrations of alpha-Tocopherol after intramammary infusion of Escherichia coli or lipopolysaccharide. J. Dairy Sci. 80:2826-2832.

Bionaz, M., E. Trevisi, L. Calamari, F. Librandi, A. Ferrari, and G. Bertoni. 2007. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. J. Dairy Sci. 90:1740-1750.

Cachia, O., J. El Benna, E. Pedruzzi, B. Descomps, M. A. Gougerot-Pocidalo, and C. L. Leger. 1998. α-tocopherol inhibits the respiratory burst in human monocytes attenuation of p47 phox membrane translocation and phosphorylation. J. Biol. Chem. 273:32801-32805.

Campbell, S. E., W. L. Stone, S. G. Whaley, M. Qui, and K. Krishnan. 2003. Gamma (gamma) tocopherol upregulates peroxisome proliferator activated receptor (PPAR) gamma (gamma) expression in SW 480 human colon cancer cell lines. BMC. Cancer 3:25.

Chan, S. S., H. P. Monteiro, F. Schindler, A. Stern, and V. B. Junqueira. 2001. Alpha-tocopherol modulates tyrosine phosphorylation in human neutrophils by inhibition of protein kinase C activity and activation of tyrosine phosphatases. Free Radic. Res. 35: 843-856.

Chen, G. X. 2013. Roles of vitamin A metabolims in the development of hepatic insulin resistance. ISRN Hepatol. PP. 21.

Chew, B. P. 1995. Antioxidant vitamins affect food animal immunity and health. J. Nutr 125:1804-1808.

Conner, E. M. and M. B. Grisham. 1996. Inflammation, free radicals, and antioxidants. Nutrition. 12:274-277.

Drackley, J. K. 1999. Biology of dairy cows during the transition period: the final frontier? J. Dairy Sci. 82:2259-2273.

Egger, T., R. Schuligoi, A. Wintersperger, R. Amann, E. Malle, and W. Sattler. 2003. Vitamin E (alpha-tocopherol) attenuates cyclo-oxygenase 2 transcription and synthesis in immortalized murine BV-2 microglia. Biochem. J. 370:459-467.

Erl, W., C. Weber, C. Wardemann, and P. C. Weber. 1997. Alpha-tocopherol succinate inhibits monocytic cell adhesion to endothelial cells by suppressing NF-kappa B moblization. Am. J. Physiol. 273: 634-640.

Faye, B., M. Bengoumi, A. Al-Masaud, and G. Konuspayeva. 2015. Comparative milk and serum cholesterol content in dairy cow and camel. J. King Saud. Sci. 27:168-175.

Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. J. Dairy Sci. 77:2695-2703.

Garcia, M., T. H. Elsasser, D. Biswas, and K. M. Moyes. 2015a. The effect of citrus-derived oil on bovine blood neutrophil function and gene expression in vitro. J. Dairy Sci. 98:918-926.

Garcia, M., T. H. Elsasser, L. Juengst, Y. Qu, B. J. Bequette, and K. M. Moyes. 2016. Short communication: Amino acid supplementation and stage of lactation alter apparent utilization of nutrients by blood neutrophils from lactating dairy cows in vitro. J. Dairy Sci. 99:3777-3783.

Garcia, M., T. H. Elsasser, Y. Qu, X. Zhu, and K. M. Moyes. 2015b. Glucose supplementation has minimal effects on blood neutrophil function and gene expression in vitro. J. Dairy Sci. 98:6139-6150.

Grammas, P., L. Hamdheydari, E. J. Benaksas, S. Mou, Q. N. Pye, W. J. Wechter, R. A. Floyd, C. Stewart, and K. Hensley. 2004. Anti-inflammatory effects of tocopherol metabolites. Biochem. Biophys. Res. Commun. 319:1047-1052.

Gruet, P., P. Maincent, X. Berthelot, and V. Kaltsatos. 2001. Bovine mastitis and intramammary drug delivery: review and perspectives. Adv. Drug Deliv. Rev. 50:245-259.

Higuchi, H., E. Ito, H. Iwano, S. Oikawa, and H. Nagahata. 2013. Effects of vitamin E supplementation on cellular alpha-tocopherol concentrations of neutrophils in Holstein calves. Can. J. Vet. Res. 77:120-125.

Hogan, J. S., W. P. Weiss, D. A. Todhunter, K. L. Smith, and P. S. Schoenberger. 1992. Bovine neutrophil responses to parenteral vitamin E. J. Dairy Sci. 75:399-405.

Kessler, E. C., J. J. Gross, R. M. Bruckmaier, and C. Albrecht. 2014. Cholesterol metabolism, transport, and hepatic regulation in dairy cows during transition and early lactation. J. Dairy Sci. 97:5481-5490.

Kobayashi, Y. 2008. The role of chemokines in neutrophil biology. Front Biosci. 13:2400-2407.

Lal, M. A., H. Brismar, A. C. Eklof, and A. Aperia. 2002. Role of oxidative stress in advanced glycation end product-induced mesangial cell activation. Kideny Int. 61: 2006-2014.

Lauzon, K., X. Zhao, A. Bouetard, L. Delbecchi, B. Paquette, and P. Lacasse. 2005. Antioxidants to prevent bovine neutrophil-induced mammary epithelial cell damage. J. Dairy Sci. 88:4295-4303.

Li, X. and J. M. May. 2003. Location and recycling of mitochondrial alpha-tocopherol. Mitochondrion. 3:29-38.

Li-Weber, M., M. Giaisi, M. K. Treiber, and P. H. Krammer. 2002. Vitamin E inhibits IL-4 gene expression in peripheral blood T cells. Eur. J. Immunol. 32:2401-2408.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) Method. Methods. 25:402-408.

Luostarinen, R., A. Siegbahn, and T. Saldeen. 1991. Effects of dietary supplementation with vitamin E on human neutrophil chemotaxis and generation of LTB4. Ups. J. Med. Sci. 96:103-111.

Maziere, C., M. Auclair, M. Djavaheri-Mergny, L. Packer, and J. C. Maziere. 1996. Oxidized low density lipoprotein induces activation of the transcription factor NF kappa B in fibroblasts, endothelial and smooth muscle cells. Int. J. Biochem. Mol. Biol. 39: 1201-1207.

Mehrzad, J., L. Duchateau, S. Pyorala, and C. Burvenich. 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. J. Dairy Sci. 85:3268-3276.

National Research Council. 2001. Nutrient requirements of dairy cattle. 7th rev.ed. National Academy of Science ed. Washington, DC, National Academy Press.

Okamoto, T., K. Gohil, E. I. Finkelstein, P. Bove, T. Akaike, and v. d. Vliet A. 2004. Multiple contributing roles for NOS2 in LPS-induced acute airway inflammation in mice. Am. J. Physiol. Lung Cell Mol. Physiol. 286: 198-209.

Paape, M. J., D. D. Bannerman, X. Zhao, and J. W. Lee. 2003. The bovine neutrophil: Structure and function in blood and milk. Vet. Res. 34:597-627.

Persson, K., I. Larsson, and S. C. Hallen. 1993. Effects of certain inflammatory mediators on bovine neutrophil migration in vivo and in vitro. Vet. Immunol. Immunopathol. 37:99-112.

Pilotto, A., G. Savoini, A. Baldi, G. Invernizzi, V. C. De, G. Theodorou, P. Koutsouli, and I. Politis. 2016. Short communication: Associations between blood fatty acids, beta-

hydroxybutyrate, and alpha-tocopherol in the periparturient period in dairy cows: An observational study. J. Dairy Sci. 99:8121-8126.

Pinotti, L., R. Rebucci, E. Fusi, L. Rossi, and A. Baldi. 2003. Milk choline, alpha-tocopherol and neutrophil chemotaxis in the periparturient dairy cow. Vet. Res. Commun. 27. Suppl 1:265-268.

Politis, I., G. Theodorou, A. D. Lampidonis, A. Kominakis, and A. Baldi. 2012. Short communication: Oxidative status and incidence of mastitis relative to blood alpha-tocopherol concentrations in the postpartum period in dairy cows. J. Dairy Sci. 95:7331-7335.

Politis, I., A. Voudouri, I. Bizelis, and G. Zervas. 2003. The effect of various vitamin E derivatives on the urokinase-plasminogen activator system of ovine macrophages and neutrophils. Br. J. Nutr. 89:259-265.

Qu, Y., A. N. Fadden, M. G. Traber, and G. Bobe. 2014. Potential risk indicators of retained placenta and other diseases in multiparous cows. J. Dairy Sci. 97:4151-4165.

Qu, Y., K. Lytle, M. G. Traber, and G. Bobe. 2013. Depleted serum vitamin E concentrations precede left displaced abomasum in early-lactation dairy cows. J. Dairy Sci. 96:3012-3022.

Saldeen, T., D. Li, and J. L. Mehta. 1999. Differential effects of alpha- and gamma-tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis. J. Am. Coll. Cardiol. 34:1208-1215.

SAS User's Guide: Statistics. [9.4]. 2017. Cary, NC, SAS Inst.,Inc.

Schelling, G. T., R. A. Roeder, M. J. Garber, and W. M. Pumfrey. 1995. Bioavailability and interaction of vitamin A and vitamin E in ruminants. J. Nutr. 125: 1799-1800.

Singh, A. K., S. Pandita, Z. A. Pampoori, R. Huozha, and M. Ashutosh. 2013. Effect of pre-partum supplementation of vitamin E to Murrah buffaloes on immune functions and viability of calves. J. Anim Physiol Anim Nutr (Berl). 97:255-262.

Sohn, E. J., M. J. Paape, E. E. Connor, D. D. Bannerman, R. H. Fetterer, and R. R. Peters. 2007. Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-alpha, IL-1beta, IL-12 and IFN-gamma. Vet. Res. 38:809-818.

Sordillo, L. M. 2016. Nutritional strategies to optimize dairy cattle immunity. J. Dairy Sci. 99:4967-4982.

Sordillo, L. M., G. A. Contreras, and S. L. Aitken. 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Anim. Health Res. Rev. 10:53-63.

Spears, J. W. and W. P. Weiss. 2008. Role of antioxidants and trace elements in health and immunity of transition dairy cows. Vet. J. 176:70-76.

Tian, S. Z., C. J. Chang, C. C. Chiang, H. C. Peh, M. C. Huang, J. W. Lee, and X. Zhao. 2005. Comparison of morphology, viability, and function between blood and milk neutrophils from peak lactating goats. Can. J. Vet. Res. 69:39-45.

Traber, M. G. 2004. Vitamin E, nuclear receptors and xenobiotic metabolism. Arch. Biochem. Biophys. 423:6-11.

Traber, M. G. 2013. Mechanisms for the prevention of vitamin E excess. J. Lipid Res. 54:2295-2306.

Varga, Z., E. Kosaras, E. Komodi, M. Katko, I. Karpati, J. Balla, G. Paragh, M. C. Aisa, and F. Galli. 2008. Effects of tocopherols and 2,2'-carboxyethyl hydroxychromans on phorbol-ester-stimulated neutrophils. J. Nutr. Biochem. 19:320-327.

Weiss, W. P. and N. R. St-Pierre. 2009. A method to quantify changes in supply of metabolizable methionine to dairy cows using concentrations of selenium in milk. J. Dairy Sci. 92:2835-2842.

Yang, J., L. Zhang, C. Yu, X. F. Yang, and H. Wang. 2014. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark. Res. 2:1.

Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature. 395:284-288.