COLOSTRUM SUPPLEMENTATION WITH OMEGA-3 FATTY ACIDS MAY DECREASE OXIDATIVE STRESS BUT DOES NOT IMPROVE GROWTH IN CALVES

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ABSTRACT

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Our objective was to determine if supplementing colostrum with oils rich in omega-3 fatty acids (n-3 FA) would give calves a health and performance advantage in early life. Three experiments encompassed this objective. The first utilized 16 Holstein calves (n=16; 8 bulls and 8 heifers). Control calves (CON) received no supplement in colostrum whereas calves with treatment (FFE) received 60 mL 1:1 ratio fish and flaxseed oil with 200 mg vitamin E in the first feeding of colostrum. FFE elevated plasma N-3 FA and decreased oxidant status index (OSi) during wk 1. 24 Holstein calves (n=24; 12 bulls and 12 heifers) were studied in the second experiment with the main objective of analyzing the dose response of our colostrum supplement. Treatments included CON and fish and flaxseed oil in amounts of 30, 60, and 120 mL supplemented to colostrum (FF30, FF60, and FF120, respectively). FF treatments increased n-3 FA and some n-3 FA-derived oxylipids but failed to reduce OSi. The third experiment aimed to observe health and growth of supplemented calves (n=180; all heifers) to determine if molecular changes observed in previous experiments could have biologically relevant effects on early life performance. The three treatments were CON, FF (60 mL 1:1 fish and flaxseed oil), and FFE (60 mL 1:1 fish and flaxseed oil with 200 mg vitamin E). We found that FF and FFE did not alter health or growth. In conclusion, though n-3 FA supplemented in colostrum was a viable strategy to enhance n-3 FA content in plasma during wk 1 of life and decrease indicators of oxidative stress, a one-time dose in colostrum did not improve calf health or growth in early life.

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KEY TO ABBREVIATIONS

ALA α-linolenic acid

AOP antioxidant potential

ARA arachidonic acid

AU arbitrary unit

cDNA complimentary deoxy-ribose nucleic acid

COX cyclooxygenase

CYP cytochrome

DHA docosahexaenoic acid

DiHDoHE dihydroxy-docosahexaenoic acid

DiHDPE dihydroxy-docosapentaenoic acid

DiHEPE dihydroxy-eicosapentaenoic acid

DiHETE dihydroxyl-tetraenoic acid

DiHETrE dihydroxy-eicosatrienoic acid

EPA eicosapentaenoic acid

FA fatty acids

FPT failur of passive transfer

HdoHE hydroxyl-docosahexaenoic acid

HpDoHE hydroperoxy-docosahexaenoic acid

HETE hydroxyl-eicosatetraenoic acid

HEPE hydroxyl-eicosapentaenoic acid

IgG immunoglobulin

IL-1β interleukin 1β

IL-4 interleukin 4

IL-6 interleukin 6

IL-8 interleukin 8

IL-10 interleukin 10

LC-MS liquid chromatography-mass spectrometry

LOX lipoxygenase

LT leukotrienes

LTB_4 leukotriene B_4

LTB_5 leukotriene B_5

LTC_4 leukotriene C_4

LTD_4 leukotriene D_4

MR milk replacer

mtROS mitochondrial ROS

mtDNA mitochondrial deoxy-ribose nucleic acid

n-3 FA omega-3 fatty acids

n-6 FA omega-6 fatty acids

NFκB nuclear factor κ B

Nitric oxide NO

NSAID non-steroidal anti-inflammatory drug

OSi oxidant status index

P protectin

PCR polymerase chain reaction

PG prostaglandins

PGE_2 prostaglandin E_2

PGE_3 prostaglandin E_3

$PGF_{2\alpha}$ prostaglandin $F_{2\,\alpha}$

PGI_2 prostaglandin I_2

PGG_2 prostaglandin G_2

PGH_2 prostaglandin H_2

PGH_3 prostaglandin H_3

PUFA polyunsaturated fatty acids

RNA ribose nucleic acid

RFU relative fluorescent units

RONS reactive oxygen and nitrogen species

Rv resolvins

TE trolox equivalents

TGF-β transforming growth factor β

TNF-α tumor necrosis factor α

TX thromboxanes

WBC white blood cells

CHAPTER 1.

REVIEW OF LITERATURE: NEONATAL CALF DISEASE SUSCEPTIBILITY AND THE ROLE OF PUFA*

*A version of this manuscript will be submitted to the *Journal of Dairy Science*

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ABSTRACT

Calves may experience physiological stress in early life that increases susceptibility to disease by causing damage to tissues. Strategies to prevent and alleviate tissue damage should be considered. This review highlights current knowledge of the roles oxidative stress and inflammation play in instigating tissue damage to increase disease susceptibility. Prevention of disease with nutrition is becoming a popular area of interest and may provide solutions by decreasing oxidative stress and inflammation. I further discuss how the competing metabolic substrates omega-6 fatty acids (n-6 FA) and omega-3 fatty acids (n-3 FA) influence this narrative. During metabolism by the same enzymes, n-6 FA produce more pro-inflammatory cell signaling molecules while n-3 FA produce more anti-inflammatory molecules. Previous research found omega-3 fatty acids (n-3 FA) attenuate inflammation and reduce oxidative damage in rodents. Calf diets are not formulated with significant n-3 FA and therefore contain a higher ratio of n-6:n-3 FA content in their tissues. Past research observed potential benefits to growth, symptoms of disease, and inflammatory gene expression in calves fed milk replacer with

increased n-3 FA content. Future research in calves should examine if increasing dietary n-3 FA may reduce oxidative damage and inflammatory markers when supplemented directly after the stressful event of birth. Furthermore, the relationship between oxidative stress, inflammatory tissue damage, and disease susceptibility should be greater elucidated in neonates.

INTRODUCTION

According to NAHMS (2014), 5% of heifers die before weaning in the United States. Scours and pneumonia are the most common diseases calves are susceptible to. Calves that experience disease are more likely to face delayed age at first calving. Culling and premature death are two outcomes more likely to occur in heifers treated for scours or pneumonia during their first 3 months of life (Waltner-Toews, 1986). Additionally, milk quality may be affected. Sick calves produce lower milk, protein, and fat yields during their first lactation (Heinrichs and Heinrichs, 2011). Therefore, strategies to prevent disease in pre-weaned calves may enhance vitality and growth to favorably shape future production performance.

Several events occur in early life that increase disease susceptibility in the newborn. At birth, factors such as dystocia (Murray and Leslie, 2013) or maternal stress in utero (Ling et al., 2018) can exacerbate physiological stress and increase reactive oxygen species (ROS) production. Neonates must also adapt to the extrauterine environment and alter their metabolic processes (Hillman et al., 2012). Thus, at birth and after, cellular damage by ROS, called oxidative stress (Abuelo et al., 2019), may result. Both diarrhea (Ranjan et al., 2006) and pneumonia (Al-Qudah, 2009) are associated with increased oxidative stress in calves. Indicators of oxidative stress such as lipid peroxides increased and antioxidant defenses such as glutathione, superoxide dismutase, ant catalase were reduced in pneumonic calves (Al-Qudah, 2009).

Similarly, diarrheic calves have greater lipid peroxides and lower antioxidant minerals copper and zinc (Ranjan et al., 2006). Oxidative stress may lead to inflammatory dysfunction through the activation of pro-inflammatory transcription factors. Likewise, inflammation can lead to oxidative stress from phagocytic oxidative burst (Abuelo et al., 2019). Calves detected with elevated inflammatory markers within 1 wk after birth have greater morbidity incidence in the first 4 mo of age (Murray et al., 2014). To protect calves from disease, methods that alleviate oxidative stress and inflammation after parturition should be further explored.

Dietary interventions may be one solution. Nutritional supplementation of omega-3 FA (n-3 FA) to milk replacer reduces symptoms of disease and improves calf performance (Ballou and DePeters, 2008), which may be attributed to n-3 FA anti-inflammatory regulation of inflammatory gene expression (Karcher et al., 2014). In contrast to omega-6 fatty acids (n-6 FA), n-3 FA supply more anti-inflammatory products which can downregulate pro-inflammatory cytokine gene expression, initiate tissue repair, and decrease reactive oxygen species (ROS) production (Gabbs et al., 2015). Therefore, supplementing n-3 FA may aid calves by modulating inflammation and oxidative stress to prevent disease.

This review discusses the physiological stress calves may encounter following parturition that may increase disease susceptibility. Dietary manipulation of n-3 FA may provide solutions by attenuating oxidative stress and inflammation. Moreover, potential strategies to prevent calf disease are proposed for future research opportunities.

OXIDATIVE STRESS

Free radicals are highly reactive molecules due to their unpaired valence electron (Sordillo and Aitken, 2009) and are produced during normal processes of metabolism or immune

response (Abuelo et al., 2019). There are 9 mitochondrial enzymes known to instigate ROS production during cellular respiration (Andreyev et al., 2005). To maintain homeostasis, cells have defensive mechanisms to neutralize free radicals. Enzymatic and non-enzymatic processes detoxify ROS such as catalase and α-tocopherol (Andreyev et al., 2005). Thus, cells achieve stability where ROS production is balanced with ROS detoxification.

Likewise, the immune response normally produces free radicals. For example, many phagocytes release ROS during the innate immune response via oxidative burst (Winterbourn et al., 2016). The enzyme NADPH oxidase in phagosomes produce superoxide to become ROS. Phagosomes then release free radicals to destroy ingested pathogens in the cell. To maintain function in high ROS and inflammatory environments, macrophages are equipped with DNA repair systems such as base excision and DNA double-strand break repair mechanisms (Ponath and Kaina, 2017). Free radical scavengers such as glutathione peroxidase in cells also neutralize ROS to prevent cellular damage (Andreyev et al., 2005).

Excessive ROS attack on macromolecules when protective mechanisms are lacking causes damage and dysfunction to cells called oxidative stress (Mavangira and Sordillo, 2018). Due to free radical instability, they may appropriate an electron from a variety of cellular components such as lipids, proteins, and DNA to achieve electron balance if not neutralized by antioxidants. Halliwell (2007) describes the progression of oxidative stress beginning with 1) low ROS concentrations stimulate cell proliferation; protein phosphatase enzymes are disabled by ROS, causing increased protein phosphorylation; 2) Due to stimulated proliferation, intracellular Ca^{2+}, a transition ion, increases and increases free radical formation (Sordillo and Aitken, 2009); mild oxidative stress ensues to damage mitochondria and enhance cellular damage (Halliwell, 2007); 3) In response, the cell will increase antioxidant concentrations, repair

DNA damage, and activate certain transcription factors to control damage; 4) If cellular defense mechanisms are unsuccessful, cell apoptosis commences. However, severe oxidative stress inactivates caspases and stops apoptosis. Necrosis may result and further spread oxidative damage by releasing toxic and oxidized cellular contents into the surrounding tissue. Inflammation may be activated (Halliwell, 2007).

Physiological changes may cause an imbalance of the normal processes of ROS production and antioxidant availability, and, in turn, cause oxidative stress. For example, dairy cattle during late gestation may experience a rise in ROS production due to increased metabolic demands (Ling et al., 2018) where increased cellular respiration may increase free radical production. Concurrently, cows may eat less and thus consume less antioxidants. An increase in ROS production coupled with a decrease in antioxidants can cause oxidative stress (Abuelo et al., 2019).

OXIDATIVE STRESS IN CALVES

Calves may experience elevated oxidative stress in early life that increase disease susceptibility. Several series of events occur during the periparturient period of the calf that cumulatively mediate oxidative stress. First, maternal oxidative stress affects the developing fetus. Neonatal calves from dams experiencing oxidative stress have decreased birth weights, increased ratio of ROS to antioxidants after birth, and a prolonged pro-inflammatory response (Ling et al., 2018); these alterations may negatively affect calf viability.

The placenta may also contribute to fetal oxidative stress during development. The placenta produces copious amounts of ROS during certain developmental stages or in cases of

pathological pregnancies (Myatt and Cui, 2004). The severity of oxidative stress the fetus may experience is contingent on antioxidant mechanisms and amount of ROS insult in the placenta.

Additional ROS production occurs after birth in the neonate. The in utero environment is relatively hypoxic in nature where oxygen is tightly regulated by the placenta (Myatt and Cui, 2004). Thus, the neonate is not yet accustomed to increased oxygen levels occurring in the extrauterine environment. Oxygen overwhelms neonatal tissues after birth and oxygen radical production is naturally increased from an abrupt increase in cellular respiration (Abuelo et al., 2019). In evidence of this, Vento et al. (2001) observed increased oxidative stress up to the first month of life in human neonates resuscitated with pure oxygen at birth.

Dystocia-induced stress can decrease neonatal concentrations of some vitamins involved in antioxidant defenses such as vitamin C and β-carotene (Civelek et al., 2008). Additionally, reduced catalase activity and increased oxidative damage was reported in calves delivered via cesarean section (Erisir et al., 2013). Decreased antioxidants may be due to increased cortisol (Civelek et al., 2008). Thus, calves from difficult births are more susceptible to oxidative stress.

Following birth, colostrum is the first milk produced from the dam and modulates immunological, physiological, and metabolic processes of the neonate (Chae et al., 2017). The balance of pro-oxidants and antioxidants in colostrum may regulate oxidative stress at the critical window after birth (Abuelo et al., 2013). Additionally, the neonate must adapt to biological processes outside the womb (Aydogdu et al., 2018). By the first 30 d of age, heart rate has gradually decreased and respiration rates have become less irregular (Piccione et al., 2007). Increased metabolic demands and thermoregulation are also critical during this time frame (Hillman et al., 2012). Oxidative stress may occur as metabolic and immunologic processes mature.

INTERACTIONS BETWEEN OXIDATIVE STRESS AND INFLAMMATION

Oxidative stress may be exacerbated when activating the inflammatory response. Inflammation is a natural first response to tissue injury or invading foreign stimuli to eliminate microbial invaders. The five cardinal signs of inflammation are redness, heat, swelling, pain, and loss of tissue function (Bradford et al., 2015). Mechanistically, damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) bind to recognition receptors on various cells or proteins throughout the body. Activated receptors initiate pathways that produce antimicrobial or pro-inflammatory responses to pathogens or injury (Abbas et al., 2018). Complexes such as inflammasomes are stimulated to produce pro-inflammatory cytokines such as IL-1β and IL-18 (Abbas et al., 2018) which recruit leukocytes among other functions (Bradford et al., 2015).

The mitochondria play key roles in triggering inflammation in response to DAMPs. Under oxidative stress, mitochondrial membrane integrity declines due to free radical attack. Release of organelle contents such as mitochondrial DNA (mtDNA) and mitochondrial ROS (mtROS) ensues. Both are considered DAMPs and are associated with inflammasome activation. Exact mechanisms of how mtROS activate of inflammasomes are not yet well understood. However, increased mitochondrial antioxidant levels are associated with decreased inflammasome activation, and DAMPs such as mtDNA can be oxidized by mtROS to expedite further cellular damage (West, 2017). Following necrosis of damaged cells, DAMPs such as mitochondrial components are released into the surrounding tissue to further activate inflammatory responses (Davidovich et al., 2014).

One example of how oxidative stress and inflammation cause tissue damage is through increasing permeability of endothelial cells. Inflammatory cells utilize ROS as a weapon to

injure foreign molecules, but their excessive release can disrupt the body's own tissues such as endothelial barriers (Ushio-Fukai, 2008). Microbial calf diarrhea and pneumonia are inflammatory diseases of the gut (Barigye et al., 2008) and lung (Caverly, 2003), respectively. Excessive ROS production modulates ion channels, kinases, and phosphatases sensitive to redox balance which causes endothelial permeability (Ushio-Fukai, 2008). Permeability of the endothelial barrier may also occur from loss of adhesion between cells. Hydrogen peroxide can increase intracellular uptake of Ca^{2+}, drawing Ca^{2+} away from important roles in tight junction function (Mittal et al., 2014). Dysfunction of endothelial tight junctions compromises the body's physical barrier against pathogens and can lead to additional pathogen insult. Furthermore, an influx of microorganisms from reduced endothelial barrier integrity can readily enter the bloodstream due to increased vascular permeability during inflammation to instigate sepsis (Ushio-Fukai, 2008). Thus, inflammation and oxidative stress are actively involved in the pathogenesis of a variety of disorders in the lung and gut. Finding strategies that attenuate excessive inflammation and oxidative stress to maintain endothelial barriers may be important to protect calves from infectious disease.

PUFA: ROLES IN INFLAMMATION AND OXIDATIVE STRESS

Polyunsaturated fatty acids (PUFA) linoleic and α-linolenic acid are considered essential in the diet. The n-6 FA linoleic acid and arachidonic acid (ARA) and the n-3 FA α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexanoic acid (DHA) enter oxidative pathways to produce oxylipids (Mavangira and Sordillo, 2018). Oxylipids are further diversified as metabolism progresses from cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) enzymes (Gabbs et al., 2015). Additionally, prostaglandin-like compounds called

isoprostanes are formed by free radical damage. Oxylipids and isoprostanes act as signaling molecules by autocrine or paracrine actions to mediate inflammation. Of greatest interest to this review are functions in inflammation and oxidative stress. The PUFA content in the tissue of calves consists primarily of n-6 FA and fewer n-3 FA (Noble, 1981). Thus, many n-6 FA-derived metabolites are produced.

Oxylipids

Linoleic acid and ARA follow a series of diverse enzymatic pathways leading to pro-inflammatory products and some anti-inflammatory products. Oxylipids from LOX metabolism of linoleic acid have a variety of known functions. Oxo-octadecadienoic acids have functions in activating pro-inflammatory transcription in monocytes or anti-inflammatory processes in endothelial cells. Similarly, hydroxy-octadecadienoic acids present diverse actions on cell proliferation and inflammation based on cell type or condition (Gabbs et al., 2015). Dihydroxy-octadecenoic acids and epoxy-octadecenoic acids are linoleic acid derived products from CYP, and several isomers are involved in instigating mitochondrial dysfunction to result in cell death (Gabbs et al., 2015).

Many more ARA derived oxylipids are known. Weissmann et al. (1980) reported the chemotactic nature of ARA itself due to the potency strength of metabolites. The production of 2-series prostaglandins (PG) from ARA is undertaken by the COX enzymatic pathway. PG synthetases then diversify the array of prostaglandins and thromboxanes formed. Perhaps most notable of their functions, prostaglandins are especially influential vasodilators. Their functions are necessary as initiators of the inflammatory response. Known to contribute to all five cardinal signs of inflammation, their concentration coincides with higher ROS production: the reaction by

hydroperoxidase that converts PGG_2 to PGH_2 causes free radical formation, for example (Belch, 1988). Therefore, inflammation and oxidative stress may be affected by ARA metabolism. 4-series leukotrienes (LT) are oxylipid derivatives of ARA from the enzymatic action of LOX that also mediate inflammation. In bovines, for example, LTB_4 can instigate neutrophil chemotaxis (Gabbs et al., 2015). However, most non-steroidal anti-inflammatory drugs (NSAIDs) target inhibition of COX over LOX, indicating a greater focus on decreasing PG synthesis to reduce inflammation (Belch, 1988). Alternatively, lipoxin A_4 and B_4 have anti-inflammatory roles. Lipoxin A_4 functions to inhibit granulocyte recruitment (Levy et al., 2002) and enhances epithelial cell healing (Gronert et al., 2005). Lipoxin B_4 is a regulator of neutrophil activation (Lee et al., 1989), and even causes inflammatory resolving processes in mucosal tissue. Hydroxy-eicosatetraenoic acids, epoxy-eicosatrienoic acids, and dihydroxy-eicosatrienoic acids are some oxylipids formed from CYP oxidation of ARA. Predominant functions include vasodilation. However, there are some exceptions such as 20-HETE that stimulates production of cytokines but also vasoconstricts arteries (Gabbs et al., 2015).

Initiation and resolution of inflammation is a necessary process, and the dietary essential n-6 FA must be balanced with n-3 FA to foster homeostatic inflammation. Belch (1988) concluded that inhibiting the synthesis of ARA derived 2-series PG and 4-series LT may attenuate inflammatory symptoms. One way to combat elevated levels of n-6 FA metabolism is through diverting enzymatic activity from n-6 FA to alternative n-3 FA substrates.

N-3 FA also hold a variety of roles within cell membranes and share the same metabolic enzymes as n-6 FA. Commonly found in n-3 FA enriched diets, ALA is further metabolized to become eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). Recently, EPA and DHA have been a hot topic of interest due to their ability to resolve inflammation, benefit endothelial

cell function, reduce pro-inflammatory cytokine production, and more (Wiktorowska-Owczarek, 2015).

In contrast with n-6 FA, n-3 FA largely enact anti-inflammatory functions and some pro-inflammatory functions. Many n-3 FA-derived oxylipids regulate cytokines and pro-inflammatory gene expression (Schmitz and Ecker, 2008). Due to their direct competition of enzymatic metabolism, more n-3 FA can decrease n-6 FA metabolism and the production of pro-inflammatory oxylipids that follow. COX and LOX produce resolvins and protectins from EPA and DHA that down-regulate the inflammatory response (Serhan et al., 2000). Williams-Bey et al., 2014, found DHA treatment suppressed NFκB expression. Additionally, EPA and DHA decrease leukocyte chemotaxis and expression of adhesion (Calder, 2010). Oxidative stress may also be affected by n-3 FA. Resolvin D1 and protectin inhibit RONS production, for example (Gabbs et al., 2015). EPA forms some oxylipids that are pro-inflammatory as well. Three-series PG and thromboxanes stimulate inflammation and mitogenic behaviors but are much less potent than n-6 FA-derived 2-series counterparts. PGE_3 and 5-series LT are less efficient in cell signaling compared to PGE_2 and 4-series LT from ARA, for example (Gabbs et al., 2015).

Alternatively, resolvins are anti-inflammatory in nature and are some of the most notable n-3 FA derived oxylipids from CYP. Production of E-series resolvins derived from EPA are associated with increased EPA supplementation (Calder, 2008). Resolvin E1 diminishes the amount of polymorphonuclear tissue that builds up during migration (Serhan et al., 2000). As a continuation of their influence, resolvins decrease free radical production from immune cells – consequently influencing oxidative stress. Forms of D-resolvins derived from DHA decrease inflammation and tissue damage caused by neutrophils (Bohr et al., 2013), and increase phagocyte ability to destroy antigens (Chiang et al., 2012). D-series protectins from DHA

promote tissue healing (Gronert et al., 2005) and prevent oxidative damage (Mukherjee et al., 2004). Additionally, CYP products protectin DX can decrease COX enzymatic activity and ROS production (Gabbs et al., 2015) and maresins decrease oxidative stress (Sun et al., 2017), restrict inflammatory processes, and aid in tissue reparation (Serhan et al., 2015). Lastly, among a host of other CYP products, di-hydroxy-eicosatetraenoic acids and di-hydroxy-docosahexaenoic acids mainly suppress platelet aggregation (Gabbs et al., 2015).

Isoprostanes

As a subspecies of oxylipids, isoprostanes are reliable biomarkers of oxidative stress because their concentration directly reflects oxidative damage. Isoprostane concentrations in neonates are much greater than in adults (Belik et al., 2010). High isoprostane concentrations are related to an array of health disorders (Weinberger et al., 2006). Isoprostanes may play important roles in early life as post-natal calves undergo the adaptive process from fetus to neonate but decreasing their concentrations may protect against early life diseases (Belik et al., 2010). Although isoprostanes are associated with increased oxidative stress, they are also bioactive molecules able to mediate cell functions.

Isoprostanes from ARA are known predominantly for their vasoconstricting actions. Montuschi et al. (2004) reported F_2-isoprostanes are associated with platelet activation, bronchoconstriction, and hypoxic-ischemic encephalopathies. In contrast, F_3 isoprostanes from EPA are considered anti-inflammatory because their increase results in a decrease of F_2 isoprostanes (Gao et al., 2006). Furthermore, n-3 FA-derived isoprostanes can function to decrease production of free radicals and inflammatory activators and may aid cell injury response to oxidative damage (Joumard-Cubizolles et al., 2017).

DIETARY SOURCES OF PUFA

Modern dairy cattle production relies to some extent on a total mixed ration diet containing both forage-based and grain-based ingredients. Traditionally, forages were the predominant source of nutrients. The ratio of n-6:n-3 FA is decreased in milk from cows fed fresh grasses compared to those fed silage or hay incorporated in a common cow diet (Nguyen et al., 2019).

Realizing the important roles of n-3 FA, there are multiple strategies producers can utilize to enhance n-3 FA intake of cows fed total mixed rations. The essential n-3 FA, ALA, is provided through vegetable oilseeds such as linseed, flaxseed, or canola (Nguyen et al., 2018). Dietary ALA may be enzymatically converted to the more active n-3 FA EPA and DHA, though the rate of conversion is relatively minimal (Whelan and Rust, 2006). Thus, to render the greatest anti-inflammatory effects of n-3 FA, providing direct dietary sources of EPA and DHA is of great interest. Marine oils from fish or algal sources are rich in EPA and DHA and can increase n-3 FA content in tissues. Animal agriculture has utilized dietary supplementation of these products in various species of livestock production to produce n-3 FA-enriched eggs, meat, and milk (Woods and Fearon, 2009).

Intake of dietary n-3 FA is limited in ruminants compared to n-6 FA, and biohydrogenation in the rumen provides an additional challenge. Modification of PUFA are often required to pass through the rumen unaltered; one such strategy is feeding Ca salts of PUFA (Garcia et al., 2014). However, in pre-ruminant neonatal calves, the rumen is essentially considered inert and underdeveloped where liquid feed bypasses the reticulo-rumen compartment via the esophageal groove. This provides a unique opportunity in early life to easily supply calves with essential PUFA for the animal's benefit.

Supplementing vitamin E protects n-3 FA from oxidation. Vitamin E exacerbates the anti-inflammatory and thus antioxidant effects from n-3 FA when additionally supplemented together. (Bo et al., 2016). For example, a study comparing rats supplemented with n-3 FA, vitamin E, or both observed higher glutathione levels in various tissues from those given both. The two nutrients synergistically improve glutathione peroxidase and superoxide dismutase activity better than n-3 FA or vitamin E alone (Narayanankutty et al., 2017). Thus, dietary supplementation of n-3 FA may consider additionally including vitamin E.

OMEGA-3 FA SUPPLEMENTATION AND CALF VITALITY

Calder, (2008) suggested that manipulation of n-6 to n-3 FA ratios could alter immune cell function. Elevating n-3 FA concentrations in neonatal offspring was previously attempted through supplementing dams in late gestation. Reyes-Hernandez et al., (2018) discovered that, upon supplementing gestational murines with ARA and DHA, ARA was favored by the placenta and DHA concentrations did not increase. Likewise, Moallem and Zachut, (2012) observed an increase in DHA in newborn calf plasma born from dams supplemented with fish oil, but total n-6 FA concentrations also tended to increase. There is clear placental selectivity of FA, and fetal plasma FA do not always mirror their dams' plasma FA content. Thus, the PUFA content vastly differs from mother to fetus in ruminants. Newborn ruminant plasma contains vastly decreased concentrations of both linoleic and linolenic acid compared to their dams (Leat, 1966), indicating dietary manipulation in the bovine dam to supply essential FA may not always affect the fetus.

Prior research reflects largely beneficial results upon daily supplementation of n-3 FA in calf milk replacer. The current literature emphasized measuring growth rates, health, and

markers of inflammation. Little research on oxidative stress in bovine calves following n-3 FA supplementation has been reported.

During an LPS challenge in jersey calves, those fed n-3 FA in milk replacer expressed attenuated signs of infection, suggesting that n-3 FA may curb hyperinflammatory responses in calves experiencing endotoxic infections (Ballou and DePeters, 2008). A milk replacer supplement including flaxseed oil improved calf feed efficiency, antibody response, average daily gain, and signs of disease in calves (Hill et al, 2011). Cytokine gene expression has also been assessed following n-3 FA fish and flaxseed supplementation (Karcher et al., 2014) where fish oil decreased TNF-α expression after LPS stimulation of whole blood. Conversely, flaxseed oil decreased expression of interleukin 8 (IL-8) and interleukin 4 (IL-4), the latter of which may cause negative effects on adaptive immune function if decreased. Overall, however, flaxseed oil increased feed efficiency during the pre-weaning period (Karcher et al., 2014).

A beneficial effect on immune function was reported with docosahexaenoic acid (DHA) enriched algae supplemented to milk replacer (Flaga et al., 2019). However, algae reduced growth rates due to decreased palatability and feed intake. Therefore, enriched oils may be a more viable form of n-3 FA supplementation to pre-weaned calves.

FUTURE INSIGHTS AND CONCLUSION

Methods to prevent early life disease should effectively be considered and supplementing via nutritional means is one strategy. Providing n-3 FA may provide a protective and healing advantage against oxidative stress or inflammatory dysfunction. Newborn calves are susceptible to oxidative stress in due to *in-utero* stress, birth events, and radical shifts in metabolism. PUFA have the potential to mediate or alleviate inflammatory and oxidative stress. Previously, n-3 FA

supplementation to milk replacer has benefited calf immune function and performance during the pre-weaning period. However, several questions have yet to be answered. Does early life oxidative or inflammatory stress decrease growth and hinder lifetime potential? Could altering the dietary ratio of n-6 : n-3 FA directly after birth provide key anti-inflammatory molecules to attenuate oxidative stress? Could supplementation of colostrum with n-3 FA provide a quick source of n-3 FA to decrease oxidative stress?

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CHAPTER 2.

COLOSTRUM SUPPLEMENTATION WITH OMEGA-3 FATTY ACIDS AND A-TOCOPHEROL ALTERS PLASMA PUFA AND DECREASES AN INDICATOR OF OXIDATIVE STRESS IN NEWBORN CALVES*

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ABSTRACT

Our objective was to characterize the effects of supplementing newborn calves with omega-3 fatty acids (n-3 FA) and α-tocopherol on blood lipid profiles and oxidant status in early life. Sixteen calves received 0 or 60 mL of 1:1 fish and flaxseed oil with 200 mg α-tocopherol in 2.8 L colostrum within 6 h after birth. Colostrum was >22% on the Brix scale. Blood was sampled on days 1, 2, 4, 7, 14, and 21 after birth for assessment of plasma PUFA, α-tocopherol, total serum protein, and oxidant status index, an indirect indicator of oxidative stress that examines the balance between the concentration of reactive oxygen and nitrogen species and antioxidant capacity in serum. Health was observed daily. Weight and hip height were recorded at birth, 3 wk, and 8 wk. Data were analyzed with a mixed procedure using SAS 9.4. Treatment did not alter concentration of total protein in blood serum, prevalence of diarrhea or other signs

of disease, or rate of growth. Feeding n-3 FA and α-tocopherol increased plasma concentrations of the n-3 FA including α-linolenic, eicosapentaenoic, and docosahexaenoic acids with a concomitant decrease in oxidant status index during the first week of life. Concentrations of α-tocopherol decreased with supplementation, but all calves maintained adequate concentrations. Oxidant status index of treated calves returned to the level of control calves by day 14. We conclude a colostrum supplement of n-3 FA and α-tocopherol is safe to administer to newborn calves, reduces oxidant status in the first week of life, and may improve health and performance.

INTRODUCTION

Calves are especially vulnerable in the days following birth; this is due in part to physiological stress at birth and suboptimal immunocompetence (Murray and Leslie, 2013). Maternal stress in late gestation, onset of oxygen respiration, and dystocia can plague newborn calves by instigating oxidative stress and a prolonged state of inflammation (Saugstad, 2003; Hulbert and Moisá, 2016; Ling et al., 2018). In fact, this major stress period has been called the "birth critical window" (Hulbert and Moisá, 2016). Our long-term goal is to improve health of calves in the first 2 wk of life without the use of antibiotics to enhance feed efficiency and growth.

In cattle, daily dietary supplements of omega-3 fatty acids (n-3 FA) have been recognized for their influence on health (Moallem, 2018), inflammation, and oxidative stress (Mavangira and Sordillo, 2018). Previous studies indicate n-3 FA supplementation to milk replacer improved growth, health, or indicators of immune function in calves (Ballou et al., 2008; Ballou and DePeters, 2008; Karcher et al., 2014). This is accomplished, in part, by production of anti-inflammatory n-3 FA metabolites coupled with antioxidant properties (Mavangira and Sordillo,

2018). Effects of feeding diets that enhance growth rates during the pre-weaning period impacts age of first service, conception, and first lactation milk yield (Brown et al., 2005; Davis-Rincker et al., 2011). All pre-weaned calf studies involving n-3 FA supplementation to date have relied on milk replacer or calf starter as the vehicle, but none to our knowledge supplemented these FA to colostrum in a one-time dose. Such a burst in supplemental n-3 FA could boost blood n-3 FA and mitigate oxidative stress experienced in the first 2 wk of life.

Increasing plasma PUFA concentrations in young calves was previously attempted through supplementing prepartum dams, but results indicate placental selectivity of FA is regulated beyond additional dietary modifications (Garcia et al., 2014). Mixed results are reported upon the ability of maternal colostrum to mirror an increase in prepartum PUFA dietary intake (Leiber et al., 2011; Jolazadeh et al., 2019). Thus, we chose to supplement maternal colostrum directly with n-3 FA enriched oils instead of relying on late-gestation dam supplementation.

Our hypothesis was that supplementing maternal colostrum directly with a 60-mL blend of fish and flaxseed oils rich in n-3 FA and α-tocopherol, often included with PUFA supplementation, would increase plasma concentrations of n-3 FA and will decrease oxidant status index (an indicator of oxidative stress in the days following birth) to potentially improve calf health and growth during the first 3 wk of life. However, if added n-3 FA hinder absorption of nutrients or immunoglobulins in colostrum, mortality and morbidity rates may increase. Our specific aims were to conduct a study focused on monitoring health, passive transfer, indicators of oxidative stress, and n-3 FA plasma concentrations following colostrum n-3 FA supplementation during the first week of life.

MATERIALS AND METHODS

Experiment design

The study was conducted during the months of March to May of 2018 at the Michigan State University Dairy Teaching & Research Center and was approved by the Institutional Care and Use Committee (approval #03/18-035-00). Outside temperature averaged 12.5 °C. Throughout the course of the study, the maximum temperature reported was 30 °C and the minimum was -10 °C.

Sixteen calves (8 bulls and 8 heifers) were blocked by gender and randomly assigned to control or treatment groups at birth. The ratio of calves born from multiparous to primiparous cows were the same Control (CON) calves received 2.8 L maternal colostrum within 6 hr after birth without any additional supplementation. Treatment (FFE) calves received colostrum supplemented with a 60-mL 1:1 mixture of fish and flaxseed oil and 200 mg (134 IU) α-tocopherol oil. Additionally, the supplement included 12-mL tween-80 to function as an emulsifier by incorporating the hydrophobic n-3 enriched oils into the colostrum suspension and to facilitate FA absorption. Colostrum was not pooled and CON calves received no placebo supplementation, so treatments were not isocaloric.

All colostrum was from dams at the MSU dairy that had met quality standards (> 50 g/L immunoglobulins). The pouches were stored frozen and retrieved at random as calves were born, thawed, and sampled for fatty acid profile analysis (Table 1). Calves were bottle-fed unless esophageal tube feeding was necessary. After colostrum feeding, calves received a bovine rotavirus and coronavirus prevention vaccine and intramuscular injections of 1 mL vitamins A and D, 200 mg α-tocopherol, and 2 mg selenium. All calves at 12-18 h after birth received a second feeding of 1.9 L colostrum without supplementation.

Calves were housed outside following their first d of life in individual calf hutches bedded with straw and had no physical contact with other calves. They were provided with access to water and calf starter grain *ad libitum* and fed with milk replacer at 900, 1600, and 2100 hr. Land O' Lakes Cow's Match Warm Front (Arden Hills, MI) milk replacer composition from manufacturer's specifications is given in Table 2. Feeding schedule and amounts are listed in Table 3. Calves were then followed for 3 wk after birth for assessment of health indicators and growth.

Weights and hip heights were measured d 1, 7 (+/- 1 d), 14 (+/- 1 d), and 21 (+/- 1 d) of age. Prior to weaning at 8 wk, calves were again weighed to monitor average daily gain during the pre-weaning period.

Calves were observed daily before the morning milk replacer feeding to visually score feces, ocular, nasal, and ear to assess general health. Health scoring was based on University of Wisconsin-Madison's Veterinary Medicine Calf Health Scoring Chart (https://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf).

Sample collection

Blood was collected from the jugular vein of calves on d 1 (24 +/- 4 hr), 2 (48 +/- 4 hr), 4 (+/- 1d), 7 (+/- 1d), 14 (+/- 2d), and 21 (+/- 2d) after birth in 2 tubes containing serum separator or EDTA. Serum and plasma were collected after centrifugation at 1700 *g* for 15 min at 4℃. Plasma collected from EDTA tubes after centrifugation were stored at -20 ℃ before FA analysis. Serum aliquots designated for oxidant status assessment were flash frozen immediately in liquid nitrogen and transported in dry ice before storing at -80 ℃. Remaining serum was tested with a digital brix refractometer for serum total protein concentrations and stored at -20 ℃. Colostrum

was sampled from each calf's first feeding and stored at -20 ℃. Frozen serum and collected colostrum samples were shipped to Saskatoon Colostrum Company (Saskatoon, SK) for further analysis of immunoglobulin concentrations with radial immunodiffusion. Colostrum was also assessed for PUFA composition using liquid chromatography mass spectrometry (LC-MS) quantification after hydrolysis and FA solid phase extraction. Plasma concentrations of α-tocopherol were analyzed with ultra-performance liquid chromatography by the Michigan State University Veterinary Diagnostics Laboratory (East Lansing, MI).

Colostrum polyunsaturated fatty acid analysis

An antioxidant-reducing agent of 50% methanol, 25% ethanol, and 25% water with 0.9 m*M* of BHT, 0.54 m*M* EDTA, 3.2 m*M* TPP, and 5.6 m*M* indomethacin, as described in Kuhn et al. (2019) was added at 20 µL to 125 µL of thawed colostrum. Samples underwent lipid hydrolysis via the addition of 178 µL of KOH and incubating for 45 min at 45 ℃. Once samples cooled to room temperature, they were centrifuged at 4800 *g* for 10 min at 4 ℃. HCl at 6 *M* was added to the removed supernatant in increments of 10 µL until the supernatant pH was decreased to 4 or less. An internal standard mixture of 15µL was added before undergoing solid phase extraction with Oasis HLB 12 cc LP extraction columns (Waters, Milford, MA) via a Biotage ExtraHera (Biotage, Charlotte, NC), further described in Kuhn et al., 2019. Samples were then dried in a Savant SpeedVac and reconstituted in a 1.5:1 methanol:HPLC water. After filtration, samples were placed in glass vials with inserts and stored at -20 ℃ until LC/MS analysis.

Plasma polyunsaturated fatty acid analysis

Extraction and analysis of plasma PUFA followed methods modified from Mavangira et al. (2015). In brief, 1 mL of plasma was thawed on ice and 1 mL of 4% formic acid and 4 µL/ml of an antioxidant reducing agent to protect samples from lipid peroxidation during processing (O'Donnell et al., 2008) was added to plasma. A mixture of internal standards of 15 µL was added to each sample mixture as well, consisting of 0.25 µ*M* 5(S)-HETE-d_8, 0.25 µ*M* 15(S)-HETE-d_8, 0.5 µ*M* 8(9)-EET-d_{11}, 0.5 µ*M* PGE_2-d_9, and 0.25 µ*M* 8,9-DHET-d_{11}. Waters (Waters, Melford, MA) Oasis Prime HLB 3cc solid phase extraction columns were used for solid phase extraction performed by Biotage ExtraHera (Biotage, Charlotte, NC). After loading samples onto columns and excess infranatant was discarded with nitrogen, columns were washed with 3 mL 5% methanol and then 2.5 mL of 90:10 acetonitrile:methanol eluted samples. Leftover solvents were evaporated with a Savant SpeedVac. A mixture of 1.5:1 methanol:HPLC water brought the PUFA back into solution before filtering and dispensing into glass chromatography vials with inserts. Vials were stored at -20 ℃ until analysis with LC/MS. A 6-point standard curve was utilized with the internal standards previously mentioned for quantification of PUFA concentrations.

Oxidant status index

Oxidant status, an indirect indicator of oxidative stress, was assessed with 2 separate assays and calculated as a ratio of reactive oxygen and nitrogen species (RONS) to antioxidant capacity. The concentrative balance of RONS and antioxidants together may impact the potential for oxidative stress to occur, and as such, oxidant status requires the measurement of both effectors concurrently.

RONS concentrations in serum were measured by counting the relative fluorescent units per µL (RFU/µL) (using the Cell Biolabs, Inc. Oxiselect in vitro ROS/RNS assay kit (Cell Biolabs, Inc., San Diego, CA). Briefly, a fluorogenic probe is added to samples where free radicals react to form 2',7'-dichlorodihydrofluorescein diacetate (DCF), and the fluorescence intensity of DCF can be related to the concentration of free radicals in the sample. Samples were then measured at 530 nm excitation and 480 nm emission where blank sample fluorescence was subtracted by sample values (Ling et al., 2018). Antioxidant potential (AOP) of the same samples was quantified through the units of Trolox equivalents (TE), a synthetic analog of α-tocopherol, where values of AOP were compared with a photometric plate reader. 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid was used as a radical cation to be reduced by antioxidants. The reduction potential of each sample, compared as Trolox equivalents, was compared by a photometric plate reader. This decolorization assay is further described in Re et al., 1999. Both assays used to assess oxidant status are detailed further by Putman et al. (2018).

STATISTICAL ANALYSIS

Data were analyzed with a mixed effects model using SAS 9.4 (SAS Institute Inc., Cary, NC) to assess with a mixed effects model. Fixed effects were gender, treatment, and repeated measure of day. Random effects were block within gender and calf within block, gender, and treatment. Differences in pre-weaning daily gains, health observations, and colostrum IgG count were analyzed with the T-test procedure in SAS 9.4 to compare treatment means. Normality was assumed if $P > 0.05$ with the general linear model procedure's Bartlett test for homogeneity of variance. If a dataset was not considered normal, the data was log-transformed and least squares means (LSM) were back-transformed to original units for interpretation of tables and figures.

Standard errors (SE) of log-transformed data were calculated as: positive SE = $10^{(\text{transformed LSM+ transformed SE})}$ – back-transformed LSM and negative SE = back-transformed LSM – $10^{(\text{transformed LSM – transformed SE})}$. Differences in main effects were significant if $P \leq 0.05$ and tendencies if $0.05 < P \leq 0.10$. Differences in interactions were significant if $P \leq 0.10$ and tendencies reported if $0.10 < P \leq 0.15$.

We tested for effects of ambient temperature and temperature swing during the first 3 d of age for each calf, and no differences were observed between CON and FFE ($P = 0.56$). Temperature swing did not influence analysis of variables, so the effect of temperature was not included in the mixed model as a random factor. The percentage of calves from primiparous dams was similar for CON and FFE ($P = 0.64$); likewise, parity was not considered necessary to include in analysis.

RESULTS

Health and growth

FFE did not alter number of diarrheal medications given per calf during the first 3 wk of life (1.3 and 2.3 for CON and FFE; $P = 0.18$). Calves maintained generally healthy dispositions across treatment groups, with CON averaging 1.4 diarrheal treatment per calf and FFE averaging 1.0 diarrheal remedy per calf ($P = 0.59$) in the first 3 wk of life. By 3 wk of age, the average amount of refusals, or incomplete milk replacer meals, per calf for each treatment group was 1.3 and 2.3 refusals for CON and FFE, respectively ($P = 0.18$).

Overall, calves were healthy with little incidence of disease in the first 3 wk of life. Health scores did not differ between CON and FFE. On a scale of 0-3 (with 0 = normal and 3 =

most unhealthy) FFE nasal scores tended to be lower ($P = 0.07$) than CON: 0.1 and 0.4 nasal score, respectively.

Across all calf first feedings, IgG concentrations in colostrum were greater than 50 g/L, indicating all calves received colostrum of acceptable antibody quality. Calf serum IgG concentrations during the first week of life were similar for CON and FFE (36 and 31 g/L, respectively). Apparent efficiency of IgG absorption was not different in CON and FFE as calculated using the Penn State colostrum calculator spreadsheet (Jones and Heinrichs, 2016). Colostrum and calf serum brix readings were similar in feeding groups.

FFE calves tended to have higher birth weights (46 kg) compared to CON calves (42 kg) ($P = 0.09$). Daily gains during individual wk 1, 2, and 3 of age did not differ ($P \geq 0.92$). Overall, average daily gain from birth to 3 wk was not different ($P = 0.55$; 0.44 kg/d for CON and 0.39 kg/d for FFE), and growth was not different during the pre-weaning period from birth to 8 wk (0.63 and 0.55 kg / d for CON and FFE; $P = 0.35$).

During the first week of life, all calves maintained average serum protein concentrations above 52 g/L. One calf had serum concentrations below 52 g/L and experienced acute infection of the hindgut during the second week of life. Removal of this calf from the study did not influence results.

Polyunsaturated fatty acid profile and α-tocopherol in plasma

Omega-3 fatty acids. The concentration of non-esterified docosahexaenoic acid (DHA) in plasma was increased ($P = 0.01$) during the first week after supplementation. Concentrations of eicosapentaeonic acid (EPA) and α-linolenic acid (ALA) increased with FFE ($P < 0.001$) during the first week of life (Figure 1). EPA was exacerbated ($P \leq 0.01$) by the differences of treatment

by day effect on d 1, 2, and 4 after birth. On d 7 after birth, FFE also tended ($P = 0.09$) to exhibit increased free EPA concentrations compared with the control.

Omega-6 fatty acids. Flaxseed oil contains 16.5% linoleic acid, which is a precursor to pro-inflammatory arachidonic acid (ARA). FFE did not increase linoleic acid in plasma of FFE compared to CON ($P = 0.18$). FFE increased ARA and dihomo-γ-linolenic acid during the first week of life ($P = 0.03$ and $P = 0.02$, respectively), both of which result from downstream metabolism of linoleic acid. The ratio of n-6 : n-3 FA over the same time period was decreased by FFE in the first week ($P < 0.0001$). This was especially true on sample d 1 and 2 of age with a strong treatment by day interaction ($P < 0.002$). Values are given in Table 4.

A-tocopherol. Concentrations of plasma α-tocopherol during the first week of life were lower in FFE than CON ($P = 0.02$). All calves maintained similar α-tocopherol concentrations at 1 d of age, but FFE exhibited lower concentrations the following days in wk 1. This change corresponded with increased n-3 FA concentrations during wk 1 (Figure 1).

Oxidant status index

Oxidant status index (OSi) was decreased by FFE during the first week of life (Figure 2). Because OSi is determined by combining two separate assays, values are considered arbitrary units (AU). CON increased OSi values from 49 to 73 AU from 1 to 2 d of age. The difference between CON OSi values on 4 and 7 d of age was even more evident, with an increase from 67 to 108 AU. The main effect of day in the model varied greatly ($P < 0.001$) as OSi increased over time. After the first week of life, OSi decreased to 76 AU at 14 d and increased again to 92 AU at 21 d.

The OSi values for FFE generally exhibited greater stability with less fluctuation between d. Contrasting to CON, FFE decreased OSi values from 41 to 32 AU at 1 to 2 d. FFE OSi gradually increased from 32 to 79 AU on 2 to 7 d, but this increase was lower and gradual compared with CON. Furthermore, FFE OSi remained relatively constant from d 7 to 21 of age with daily OSi means varying by only 8 AU. Overall, treatment by day effect was not different from CON and FFE ($P = 0.45$).

The decreased OSi in FFE was not due to AOP in serum ($P = 0.14$). The average of treatments during the first week of life for CON and FFE were 6.5 and 7.1 TE, respectively. The concentration of RONS tended to decrease ($P = 0.08$) in FFE calves (452 and 344 RFU/μL in CON and FFE, respectively).

DISCUSSION

This is the first study to show that a dietary supplement in colostrum containing n-3 FA and α-tocopherol improves oxidant status and reduces the accumulation of RONS that is prevalent in newborn calves. The mechanism for this may be due to the altered ratio of free plasma n-6 : n-3 FA of 23 to 9 with FFE.

Newborns experience high levels of RONS production at birth through maternal oxidative stress in late gestation (Ling et al., 2018), placental preference for pro-RONS biomolecules (Braekke et al., 2006), and abrupt increase in oxygen exposure at birth (Frank, 1985). These contributing factors may lead to oxidative stress around birth when the calves' antioxidant capacity is inundated (Saugstad, 2003). Abuelo et al. (2014) showed that OSi increased from birth to 6 d of age and suggested this increase was possibly due to metabolic demands adapting to the extrauterine environment. Our results observed RONS concentrations in

both CON and FFE calves ($P = 0.02$) and antioxidant capacity tended to decrease over the same time frame ($P = 0.06$), which confirmed OSi increased during the first wk of life. Increase in OSi occurred at 2 d, but FFE delayed this increase.

Though FFE improved OSi during the first wk of life compared to CON, OSi increased over the first wk of life in all calves. Abuelo et al. (2014) found that OSi remained elevated compared to the first d of life at a consistent level from 6 to 29 d of age in calves. The scope of the current study only sampled to 21 d of age, but our data were consistent with past results. OSi has not been measured in previous studies supplementing milk replacer with n-3 FA. Therefore, a future experiment observing effects of OSi from n-3 FA supplementation additionally in milk replacer may be of interest.

The mechanisms by which FFE decreased OSi resulted more from decreased RONS production ($P = 0.08$) than a large increase in antioxidant capacity ($P = 0.14$); however, in both cases, FFE averaged lower RONS and higher antioxidant capacity than CON, yielding a decreased OSi ($P < 0.01$). One possible mechanism is through increased n-3 FA, which compete for the same enzymatic reactions n-6 FA utilize to produce a large variety of known pro-inflammatory metabolites (Sordillo, 2018). Cytochrome enzymes, for example, use both n-3 and n-6 FA as substrates, but with increasing concentrations of n-3 FA, will sometimes favor EPA over ARA as a substrate (Arnold et al., 2010). Notably, EPA and DHA produce oxylipids with anti-inflammatory properties and oxylipids with lower pro-inflammatory potential than the competing ARA substrate and subsequently greater metabolism of EPA will result in decreased RONS production. Thus, a prolonged response in decreased oxidant status such as what was observed may have been assisted by n-3 FA catabolism. Some metabolites such as resolvins and protectins act as anti-inflammatory signaling molecules and are not only effective at decreasing

inflammation - they also protect against hyperinflammatory conditions (Calder, 2010). A definitive association of downregulated inflammation with decreased RONS production has been observed after supplementation with n-3 FA (Takahashi et al., 2002). A future study should examine what products of n-3 FA metabolism may impact the oxidant balance after a one-time dose in colostrum as reported in the current study.

Our observation of decreased RONS production by the n-3 FA in FFE may have further improved with the addition of 200 mg (334 IU) α-tocopherol, which was included to protect n-3 FA from peroxidation (Wang et al., 2010). In our study, we observed decreased α-tocopherol concentrations in FFE calves during wk 1. Supplementation of PUFA is known to decrease α-tocopherol concentrations in blood (Raederstorff et al., 2015), and we speculate the depressed α-tocopherol concentrations observed in FFE calves were due to depletion by n-3 FA to protect from peroxidation. Incorporating 200 mg α-tocopherol in the FFE treatment, which is greater than the minimum recommendation of 0.6 mg α-tocopherol per g PUFA (Harris and Embree, 1963), was not enough to sustain plasma α-tocopherol concentrations of FFE calves at the same concentrations as in CON; nevertheless, FFE calves did not show visible signs of α-tocopherol deficiency and plasma concentrations remained within recommended levels (Reddy et al., 1986).

We cannot discern if n-3 FA alone, α-tocopherol alone, or their combination was responsible for the decreased oxidant status of the FFE treatment. However, all calves received an intramuscular injection of 100 mg of α-tocopherol and 2 mg of selenium at birth, and this supplement alone was unable to attenuate oxidative stress as much as the addition of FFE. The possibility that calves would benefit from additional antioxidants above 100 mg of a-tocopherol and 2 mg selenium should be explored.

Transfer of IgG from colostrum to calf is a primary mediator of calf disease resistance and resilience. Failure of passive transfer (< 52 g protein per L serum) puts the calf at risk for poor vitality and immune function. To our knowledge, no one has published effects of a lipid supplement to colostrum. We found FFE did not alter normal absorption of IgG. Serum protein concentrations remained similar across CON (65 g/L) and FFE (64 g/L) where $P = 0.35$. Kamada et al. (2007) observed increased IgG concentrations in calves fed selenium-supplemented colostrum. Based on this finding, one might expect the presence of α-tocopherol in our supplement might also increase IgG concentrations, but we did not observe this.

CONCLUSION

A colostrum supplement of 60-mL of a 1:1 fish:flaxseed oil blend with 200 mg α-tocopherol is safe to feed calves with no negative effects on passive transfer, growth, or indicators of health. The supplement increased plasma concentrations of ALA, EPA, and DHA, decreased the n-6 : n-3 FA ratio in plasma, and decreased oxidant status index during the first wk of life. We speculate this change in n-6 : n-3 FA ratio and decreased oxidant status may potentially improve health and growth.

APPENDIX

Table 2.1. Least squares means of treatments CON and FFE PUFA concentrations (n*M*) in randomly assigned maternal colostrum of first feeding before FFE supplementation.

	Treatment[1]		
Colostrum PUFA, n*M*	CON	FFE	SEM
C18:2n-6	352	261	57.1
C18:3n-3	22.9	20.9	3.53
C20:3n-6	41.9	26.6	27.9
C20:4n-6	110	72.6	22.1
C20:5n-3	17.5	17.7	2.94
C22:4n-6	32.3	17.2	8.60
C22:5n-3	111	93.5	12.8
C22:6n-3	1.68	2.27	0.396

[1]Treatments: CON = control, no supplement added to colostrum; FFE = 60 mL 1:1 ratio fish:flaxseed oil blend with 200 mg α-tocopherol added to colostrum

Table 2.2. Composition of Cow's Match Warm Front milk replacer (Land O' Lakes, Arden Hills, MI) fed to calves during the pre-weaning period according to manufacturer's specifications.

Milk Replacer Composition, air-dry basis	
Crude Protein, not less than %	28
Crude Fat, not less than %	10
Crude Fiber, not more than %	0.20
Calcium, not less than or more than %	0.75 - 1.25
Sodium, not more than %	1.20
Phosphorous, not less than %	0.70
Vitamin A, not less than IU / kg	9,072
Vitamin D_3, not less than IU / kg	2,268
Vitamin E, not less than IU / kg	68

Table 2.3. Feeding schedule of all calves on study starting at day 1 of age. Each liter contained 0.13 kg of milk replacer powder.

	Milk Replacer Feeding Schedule, L per feeding		
Age, wk	Morning, 900 hr	Afternoon, 1600 hr	Evening, 2100 hr
0-1	2.4	2.4	2.8
1-6	2.8	2.8	3.8
6-8	3.8	0	0

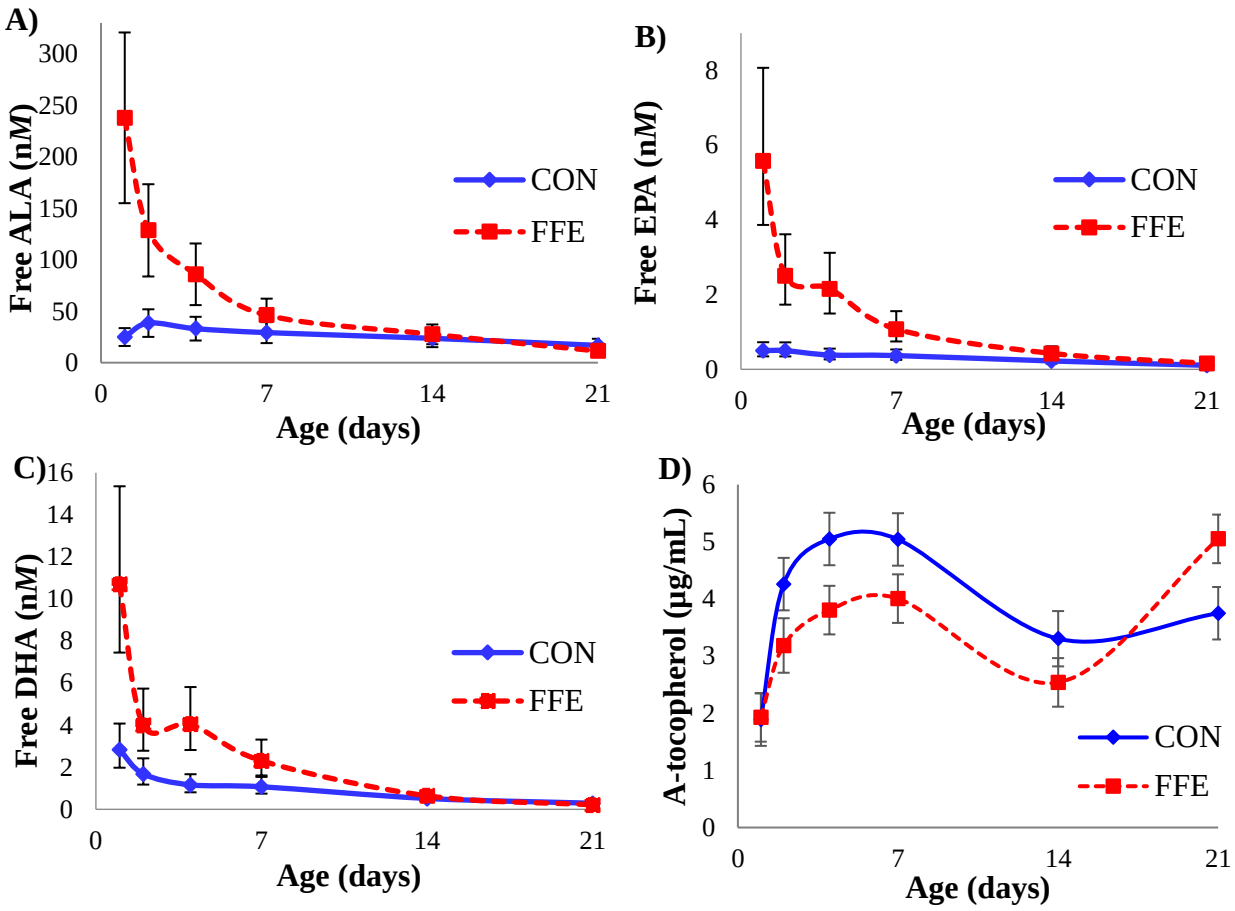
Table 2.4. Least squares means of treatments CON and FFE plasma PUFA concentrations d 1, 2, 4, and 7 of age.

	Treatment[1]			
Plasma PUFA (n*M*)	CON	FFE	CI (95%) CON	CI (95%) FFE
C18:2n-6	1220	1500	930 – 1500	1190 - 1760
C18:3n-3 ***	30.9	96.5	23.2 – 41.1	72.5 – 129
C20:3n-6 *	0.259	0.405	0.163 – 1.43	0.309 – 1.65
C20:4n-6 *	3.97	5.75	2.62 – 5.33	4.40 – 7.10
C20:5n-3 ***	0.434	2.39	0.222 – 0.847	1.22 – 4.66
C22:4n-6	0.0235	0.0263	0.015 – 0.037	0.017 - 0.041
C22:5n-3 †	1.46	1.98	0.914 – 2.01	1.43 – 2.53
C22:6n-3 **	1.8	4.5	0.977 – 2.50	2.79 – 7.16
Σ n-6 FA : n-3 FA ***	20.5	8.85	15.7 – 26.8	6.80 – 11.5

†$P \leq 0.10$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ difference between CON and FFE

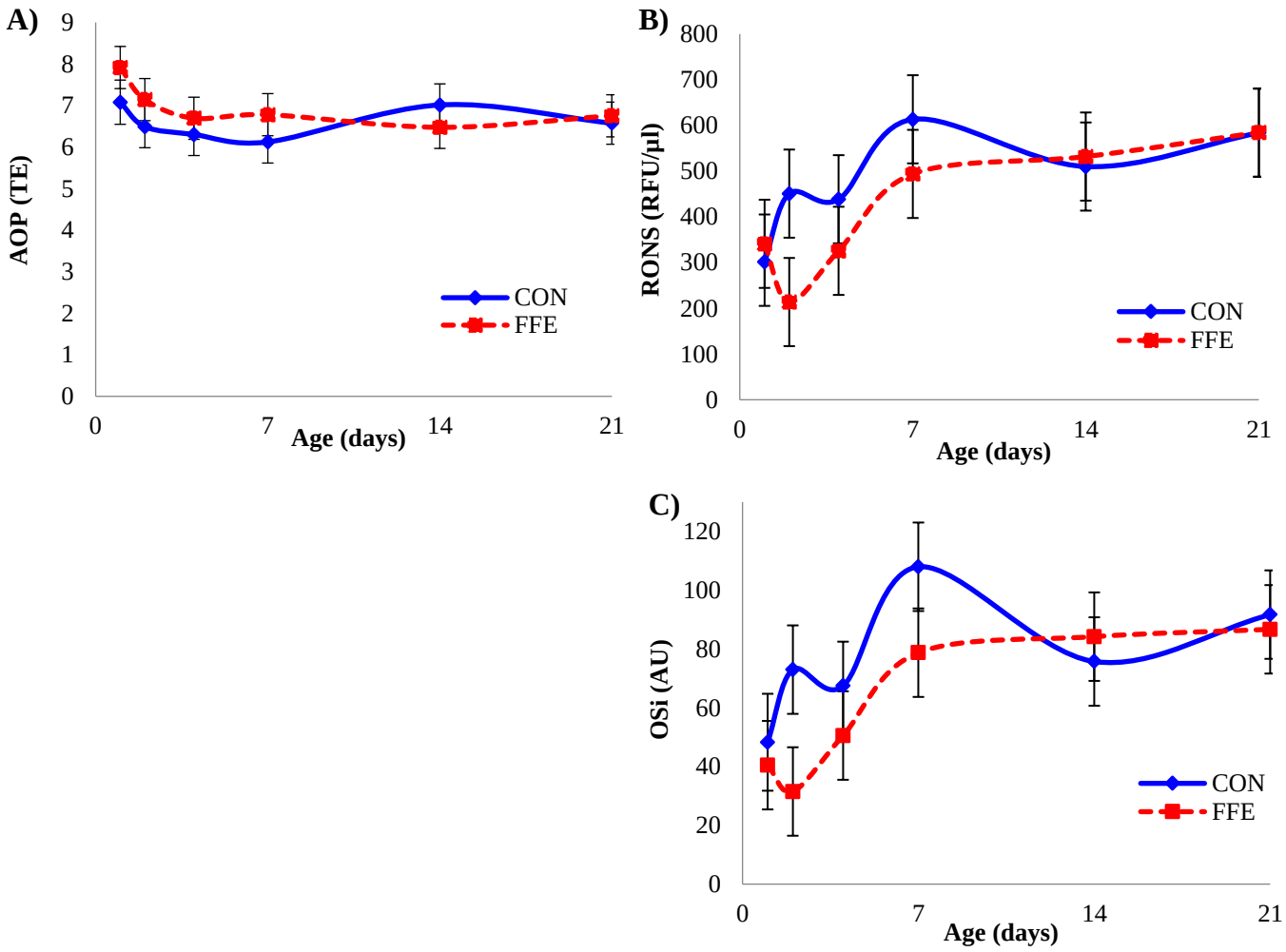
[1]Treatments: CON = control, no supplement added to colostrum; FFE = 60 mL 1:1 ratio fish:flaxseed oil blend with 200 mg α-tocopherol added to colostrum

Figure 2.1. ALA, EPA, DHA, and α-tocopherol in plasma



Changes in plasma n*M* concentrations of α-linolenic acid (ALA) (Panel A), eicosapentaenoic acid (EPA) (Panel B), docosahexaenoic acid (DHA) (Panel C), and plasma α-tocopherol µg/mL (Panel D) as age increases of CON and FFE groups. FA data are shown as LSM back-transformed from the model ± standard error bars. Calves given FFE increased plasma n-3 FA concentrations ($P \leq 0.01$) and decreased α-tocopherol concentrations ($P = 0.02$) during the first week of age.

Figure 2.2. AOP, RONS, and OSi



Changes in reactive oxygen and nitrogen species (RONS) (Panel A), antioxidant potential (AOP) (Panel B), and oxidant status index (OSi) (Panel C) as age in days increases of CON and FFE groups by relative fluorescent units per µL (RFU/µL), Trolox equivalents (TE), and arbitrary units (AU), respectively. Data shown are represented as LSM ± standard errors predicted from the model. During the first week of age, FFE did not alter antioxidant potential (P = 0.14), and tended to decrease RONS (P = 0.08). Overall, FFE decreased OSi wk 1 (P = 0.01)

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CHAPTER 3.

COLOSTRUM SUPPLEMENTATION WITH OMEGA-3 FATTY ACIDS ALTERS PLASMA PUFA AND INFLAMMATORY MEDIATORS IN NEWBORN CALVES*

*A version of this manuscript will be submitted to the *Journal of Dairy Science*

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ABSTRACT

Our objective was to determine the dose response to fish and flaxseed oil when supplemented in colostrum on concentrations of plasma FA, FA metabolites, and index of oxidative stress during the critical first week of life in calves. We hypothesize n-3 FA supplemented in colostrum in a linear dose-dependent fashion will associate with increased plasma n-3 FA concentrations, increased plasma n-3 FA concentrations, and decreased oxidative stress. Twenty-four male and female calves were randomly assigned to receive 0, 30, 60, or 120 mL of a 1:1 fish to flaxseed oil supplement in colostrum. All calves received randomly chosen maternal colostrum (≥ 22% Brix) with their respective treatment within 6 hr after birth. Blood was sampled before first feeding after birth and on d 1, 2, 4, 7, and 14 d of age to assess oxidant status and plasma NEFA, phospholipid FA, and oxylipid concentrations. Health indicators were observed daily. Indicators of general health and growth were unaffected by treatment. Supplemented calves exhibited greater concentrations of n-3 FA in plasma as free and

phospholipid FA and some n-3 and n-6 FA derived oxylipids. Fish and flaxseed oil treatments did not alter oxidant status, but they decreased isoprostane concentrations in plasma, indicating oxidative stress was decreased. Such responses cumulatively indicate several modes of action by which dietary n-3 FA may have supported an anti-inflammatory state. In conclusion, supplement of colostrum with n-3 FA administered in volumes of 30, 60, and 120 mL exhibited linear effects on FA metabolite concentrations and plasma n-3 FA concentrations as plasma free FA and phospholipid FA membranes, decreased biomarkers of oxidative stress, and did not alter oxidant status or impact health or growth. Our findings suggest neonatal calves may benefit from n-3 FA supplementation in colostrum to encourage a greater anti-inflammatory state.

INTRODUCTION

The transition period is physiologically stressful on the dam and calf at parturition (Ling et al., 2018), contributing to an increased risk of immune dysfunction and oxidative stress. Though acute inflammation is necessary to attain normal birth function, unresolved inflammation and oxidative stress can lead to increased disease incidence (Bradford et al., 2015) and decreased calf vitality (Abuelo et al., 2013), taking energy away from contributing to more favorable outputs, such as growth, to maintain homeostasis (Wolowezuk et al., 2008). Phospholipids are the primary location where omega-3 fatty acids (n-3 FA) are stored before they undergo enzymatic oxidation to produce oxylipids functioning as cell mediators that can help resolve and repair inflammatory and oxidative tissue damage (Raphael and Sordillo, 2013).

The predominant n-3 FA available for feeding are α-linolenic acid (ALA); C18:3, eicosapentaenoic acid (EPA); C20:5, and docosahexaenoic acid (DHA); C22:6. Fish and flaxseed oil, abundant sources of n-3 FA, when daily supplemented to milk replacer, have shown

to affect inflammatory gene expression, health, and growth with outcomes generally seen as beneficial (Ballou and DePeters, 2008; Karcher et al., 2014).

However, reactive oxygen species overproduction occurs after birth in the neonate as oxygen concentrations entering tissues increase at relatively high proportions in comparison to *in utero* fetal amounts (Frank and Sosenko, 1987). High oxygen concentrations may prove toxic to newborn tissues, and free radical production is a mechanism by which such toxicity is believed to be mediated (Buonocore et al., 2001). Dystocia can likewise decrease neonatal cocnentrations of vitamins with antioxidant capabilities such as vitamins A, C, and B-carotene with a concomitant increase in cortisol (Civelek et al., 2008), further affecting the redox balance. Because oxidative stress may be magnified at birth (Buonocore et al., 2001), our focus was to supplement fish and flaxseed oil to colostrum, rather than a daily supplement in milk replacer. We specifically aimed to provide this as a one-time dose to observe its effects on calf health and metabolism during the first week of life which might, via the diet, alleviate post-birth physiological stress during the adaptive process transition of fetus to neonate.

The purpose of this study was to answer 2 main objectives. First, we wanted to determine the effects of oxidative stress, oxylipid production, health, growth, and plasma FA composition to determine a dose response of n-3 FA supplementation. Second, we wanted to determine if n-3 FA could impact the concentration of inflammatory mediators in serum. In a previous experiment (Opgenorth et al., 2019), we observed decreased oxidant status during the first week of life in calves calves supplemented with fish : flaxseed oil and α-tocopherol in colostrum. A-tocopherol is a formidable antioxidant and was not included in treatments of the present study to examine the effects of n-3 FA alone on inflammatory and oxidative stress mediator concentrations.

We hypothesize that n-3 FA will increase plasma n-3 FA concentrations, leading to an increase in the biosynthesis of anti-inflammatory n-3 derived oxylipids which will decrease oxidative stress in a linear dose dependent manner.

MATERIALS AND METHODS

The study was conducted during the months of June to July of 2018 at the Michigan State University Dairy Teaching and Research Center, and was approved by the Michigan State University Animal Care and Use Committee. Calves were born between June 3 and July 17. Outside temperature averaged 21 °C. Throughout the course of the study, the maximum temperature reported was 34 °C and the minimum was 6 °C.

Experimental design

Twenty-four Holstein (12 male, 12 female) calves were assigned one of four treatment groups in a randomized block design by gender. Treatments followed a pattern of varying amounts of a 1:1 mixture of fish:flaxseed oil, which also included polysorbate-80 at 0.2 mL / mL of oils to emulsify the oils and enhance absorption.

Treatments were assigned as follows: treatment 1, CON: no supplement in colostrum; treatment 2, FF30: 30 mL fish : flaxseed oil blend in colostrum; treatment 3, FF60: 60 mL fish:flax oil blend in colostrum; treatment 4, FF120, 120 mL fish : flaxseed oil blend in colostrum.

Each calf received a randomly assigned pouch of maternal colostrum not from its dam that achieved ≥ 22% (≥ 50 g/L immunoglobulins) on the Brix scale to ensure quality (Bielmann et al., 2010). The pouches were thawed and sampled for immunoglobulin concentration and FA

profile analysis. The supplement for treatment calves was then distributed to 2.8 L colostrum, but control calves did not receive any extra supplement. Calves were bottle fed within 6 hours after birth unless tube feeding was necessary. After birth, all calves were removed from the dam immediately and received a bovine rotavirus and coronavirus vaccine and intramuscular supplements of 1 mL of vitamin A and D VetOne equivalent to 500,000 IU vitamin A and 75,000 IU vitamin D_3 (MWI Animal Health, Boise, ID) and 2 mL BO-SE (Merck Animal Health, Madison, NJ) which contains 50 mg vitamin E and 1 mg selenium per mL. A second colostrum feeding of 1.9 L was given 12-18 hr after birth, but no supplement was added to the second feeding for any calves.

Calves were housed outside after their first day of life in individual calf hutches bedded with sand and were isolated in such a way to prevent physical contact with other calves. They were provided with unlimited access to water and calf starter grain at all times and fed daily with milk replacer at approximately 900, 1600, and 2100 hr. Milk replacer composition is listed in Table 2. Milk replacer feeding schedule and quantity of meals throughout their pre-weaning period are given in Table 3. Health was scored for 2 weeks after birth. Medication, milk replacer refusal feedings, and disease incidence were recorded throughout the pre-weaning period, and body measurements of weight, wither height, and heart girth were recorded at birth, 2 wk of age, and weaning.

Calves were observed daily by trained researchers blind by treatment before the AM milk replacer feeding to assign feces, eyes, nose, and ear scores to assess general health for 2 wk after birth. Health scoring was based off methods by McGuirk, University of Wisconsin (https://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf) on a 0 to 3 scale where scoring was described as fecal; 0 = normal to 3 = watery, discolored, or bloody,

eyes; 0 = no discharge to 3 = excessive discharge, nose; 0 = normal discharge to 3 = excessive discharge, ear; 0 = normal ear alertness to 3 = tilted head or blatant ear droop.

Colostrum polyunsaturated fatty acid analysis

An antioxidant-reducing agent of 50% methanol, 25% ethanol, and 25% water with 0.9 mM of BHT, 0.54 mM EDTA, 3.2 mM TPP, and 5.6 mM indomethacin, as described in Kuhn et al. (2018) was added at 20 µL to 125 µL of thawed colostrum. Samples underwent lipid hydrolysis via the addition of 177.5 µL KOH and incubating for 45 min at 45 ℃. Once samples cooled to room temperature, they were centrifuged at 4800 *g* for 10 min at 4 ℃. HCl at 6 M was added to the removed supernatant in increments of 10 µL until the supernatant pH was decreased to 4 or less. A mixture of internal standards of 15 µL was added to each sample mixture as well, consisting of 0.25 µM 15(S)-HETE-d_8, 0.5 µM 8(9)-EET-d_{11}, 0.5 µM PGE_2-d_9, and 0.25 µM 8,9-DHET-d_{11}. Samples underwent solid phase extraction with Oasis HLB 12 cc LP extraction columns (Waters, Milford, MA) via a Biotage ExtraHera (Biotage, Charlotte, NC), further described in Kuhn et al. (2018). Samples were then dried in a Savant SpeedVac and reconstituted in a 1.5:1 methanol:HPLC water. After filtration, samples were placed in glass vials with inserts and stored at -20 ℃ until LC/MS analysis.

Blood collection and analysis

Blood was collected from the jugular vein of calves on d 0 (before first colostrum feeding), 1 (24 +/- 4hrs), 2 (48 +/- 4hrs), 4 (+/- 1d), 7 (+/- 1d), and 14 (+/- 2d) after birth. Analysis of 0 d samples were used as a covariate in the statistical model, but if not significant, were not included in results. Serum was harvested for Brix serum total protein score. Serum was

harvested and immediately flash frozen in liquid nitrogen and placed in dry ice during transit to a -80 ˚C freezer for later analysis of oxidant status index. Plasma was harvested immediately and stored at -20 ˚C until analysis of FA phospholipid profiles. Another aliquot of plasma was immediately flash frozen with liquid nitrogen and stored at -80 ˚C for future analysis of oxylipid and free PUFA concentrations.

Oxidant status index

Oxidant status, an indicator of oxidative stress, was assessed with two separate assays and calculated as a ratio of RONS to antioxidative capacity. The concentrative balance of RONS and antioxidants together impact the potential for oxidative stress to occur, and as such, oxidant status requires the measurement of both effectors concurrently.

Reactive oxygen and nitrogen species (RONS) and antioxidant potential (using the Cell Biolabs, Inc. Oxiselect in vitro ROS/RNS assay kit (Cell Biolabs, Inc., San Diego, CA). Free radicals in samples convert an added reagent to a fluorescing product that can be measured to relate to total free radical concentration as described previously (Putman et al., 2018).

Antioxidant potential (AOP) of the same samples was quantified through the units of Trolox equivalence, a synthetic analog of vitamin E, where values of AOP were compared with a photometric plate reader. 2,2’-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid was used as a radical cation to be reduced by antioxidants as described previously (Putman et al., 2018). The reduction potential of each sample, compared as Trolox equivalence, was compared by a photometric plate reader. This decolorization assay is further described in Re et al. (1999).

Plasma free polyunsaturated fatty acids, oxylipids, and isoprostanes

Sample preparation. Extraction and analysis of plasma for analysis of free PUFA, oxylipid, and isoprostane concentrations followed methods modified from Mavangira et al. (2015). In brief, 1 mL of plasma was thawed on ice and 1 mL 4% formic acid and 4 µL/mL of an antioxidant reducing agent to protect samples from lipid peroxidation during processing (O'Donnell et al., 2008) was added to plasma. The antioxidant-reducing agent was 50% methanol, 25% ethanol, and 25% water with 0.9 m*M* of BHT, 0.54 m*M* EDTA, 3.2 m*M* TPP, and 5.6 m*M* indomethacin, as described in Kuhn et al., 2018. A mixture of internal standards of 15 µL was added to each sample mixture as well, consisting of 0.25 µ*M* 5(S)-HETE-d_8, 0.25 µ*M* 15(S)-HETE-d_8, 0.5 µ*M* 8(9)-EET-d_{11}, 0.5 µ*M* PGE_2-d_9, and 0.25 µ*M* 8,9-DHET-d_{11}. Waters (Waters, Melford, MA) Oasis Prime HLB 3cc solid phase extraction columns were used for solid phase extraction performed by Biotage ExtraHera (Biotage, Charlotte, NC). After loading samples onto columns and excess infranatant was discarded with nitrogen, columns were washed with 3 mL 5% methanol and then 2.5 mL of 90:10 acetonitrile:methanol eluted samples. Leftover solvents were evaporated with a Savant SpeedVac. A mixture of 1.5:1 methanol:HPLC water brought the FA back into solution before filtering and dispensing into glass chromatography vials with inserts.

Quantification of oxylipids. In short, the quantification of metabolites was accomplished on a Waters Xevo-TQ-S tandem quadrupole mass spectrometer using multiple reaction monitoring. Chromatography separation was performed with an Ascentis Express C18 HPLC column, held at 50 ^{o}C and autosampler held at 10 ^{o}C. Mobile phase bottle A was water containing 0.1% formic acid and mobile phase bottle B was acetonitrile, the flow rate was 0.3 mL/min. Liquid chromatography separation took 15 min per sample with linear gradient steps

programmed as follows (A:3B ratio): time 0 to 0.5 min (99:1), to (60:40) at 2.0 min; to (20:80) at 8.0 min; to (1:99) at 9.0 min; 0.5 min held at (1:99) until min 13.0; then return to (99:1) at 13.01 min, and held at this condition until 15.0 min. All oxylipids were detected using electrospray ionization in negative-ion mode. Cone voltages and collision voltages were optimized for each analyte using Waters QuanOptimize software and data analysis was carried out with Waters MassLynx software.

Quantification of isoprostanes. Quantification of isoprostanes was accomplished with a Waters Xevo TQ-S tandem quadrupole mass spectrometer using multiple reaction monitoring. Chromatography separation was performed with a Waters Acquity UPLC utilizing a BEH C18 1.7 μ*M* (2.1 × 150 mm) column, held at 50 ºC and auto sampler held at 10 ºC. Mobile phase bottle A was 0.1% acetic acid and mobile phase bottle B was acetonitrile, mobile phase bottle C was methanol, the flow rate was 0.3 mL/min. The gradient initial phase A:B, 80:20 to 1 minute changing to A:B:C, 50:30:20, to 7 minutes changing to A:B:C, 1:80:19, to 7.01 changing back to initial phase and holding until 10 minutes. All oxylipids were detected using electrospray ionization in negative-ion mode. Cone voltages and collision voltages were optimized for each analyte using Waters QuanOptimize software and data analysis was carried out with Waters MassLynx software.

Quantification of free polyunsaturated fatty acids. Briefly, reverse-phase LC/MS on a Waters Acquity UPLC utilizing a BEH C18 1.7 μ*M* (2.1 × 100 mm) column with a flow rate of 0.6 mL/min at 50°C. The quadrupole MS was in electrospray negative ionization mode and voltage was −3 kV with the turbo ion spray source temperature at 450°C. The gradient mobile phase was programmed in the following manner (A/B/D ratio): time 0 to 0.5 min (30/5/65), to (65/5/30) at 1.0 min, to (85/10/5) at 5.50 min, to (89/10/1) at 7.0 min, and held until 11.5 min,

then return to (30/5/65) at 11.01 min, and held at this condition until 15.0 min. In this gradient mobile phase A = Acetonitrile, B = Methanol, and D = 0.1% Formic Acid. Fatty acids were quantified by matching mass-1 and retention time with corresponding deuterated internal standard abundance and calibrated to a linear 7-point standard curve ($R^2 > 0.99$) using Waters Empower 3 software (Waters).

Plasma phospholipid fatty acid analysis

Phospholipids were analyzed using methods adapted from Folch et al. (1957) and Kramer et al. (1997). In brief, total lipids were extracted from an aliquot of 5 mL plasma by shaking samples in 6 mL methanol and 12 mL chloroform. A solution of 2 % sodium chloride in water was then added. The chloroform layer with lipids was filtered out, dried, and weighed for total lipid content. Next, the phospholipid fractions were isolated using solid phase extraction. Columns were initially washed with 1.2 mL 7:1 acetone:water and eluted with 4 mL hexane before loading lipid samples dissolved in 0.8 mL hexane:methyl tert-butyl ether:acetic acid (100:3:0.3). Cholesterol esters were first eluted with 14 mL hexane, and free FA eluted with 8 mL hexane:chloroform:ethyl acetate (100:5:5). Columns were washed with 6 mL 2:1 chloroform:isopropanol. Next, the triglyceride fraction was collected via 8 mL chloroform:methanol:acetic acid (100:2:2), and phospholipids eluted via 10 mL methanol:chloroform:water (10:5:4). The phospholipid fraction was then washed with 3 mL 5% sodium chloride in water and phospholipids in the chloroform layer were collected. Only the phospholipid fraction was analyzed in the interest of relevance to experiment objectives.

After phospholipid content was calculated, 0.5 mL of the internal standard, C17:1 10-heptadecenoic acid in toluene, was added.

Phospholipids were then methylated into fatty acid methyl esters (FAME) as previously described (Lock et al., 2013). Hexane reconstituted FAME in solvent to produce a 1% solution for gas-liquid chromatography analysis. Plasma phospholipid FA concentrations were calculated as a percentage of total phospholipids recovered from plasma in g / 100 g.

STATISTICAL ANALYSIS

Data were analyzed using SAS version 9.4 with a mixed procedure. Fixed effects were gender, treatment, and day. Random effects were block within gender and calf within block, gender, and treatment. When analyzing statistics for body weight and average daily gain up to weaning, the variable week replaced day. Average daily gain of the first two weeks of life, health scores, and d 1, 2, 4, and 7 of blood variables were analyzed with the mixed model. Blood variables from d 14 were included in figures but not in statistical analysis, as we expected most variables to return to baseline by d 14. All *P*-values or data expressed in tables are results from analyzing d 1, 2, 4, and 7 after birth only. After birth and before the first colostrum feeding, blood was sampled and analyzed to use as a potential covariate in the model. If the covariate was not significant and did not correlate with d, it was not included in the model.

Assays were conducted by block. The order on the well plate of the RONS concentration assay significantly impacted resulting values, and therefore, was included in the model for oxidant status index analysis as order within block. Effect of block was confounded with order. Treatment was not confounded with order, as treatments were random with respect to order of wells. Pre-weaning average daily gain, general health observations, and colostrum immunoglobulin count, brix measures, and apparent efficiency of absorption were analyzed with the general linear model ANOVA procedure.

Normality was assumed if a variable's general linear model procedure's Bartlett homogeneity of variance test indicated $P > 0.05$. Data were log-transformed if $P \leq 0.05$ and transformed least squares means were back-transformed from the model for interpretation in tables and figures.

Differences were considered significant if main effect $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. Differences were considered significant if interaction $P \leq 0.10$ and a tendency if $0.10 < P \leq 0.15$.

RESULTS

Health and growth

During the first 2 wk of life, 14 calves were treated for diarrhea. Calves typically showed initial symptoms of diarrhea 10-12 d after birth. Across treatment groups, the average number of medication doses for diarrhea per calf was 1.4 during the first 2 wk of life, and differences between treatments were not significant ($P = 0.71$). If a calf did not complete a meal of milk replacer, it was recorded as one refusal. The average number of refusals per calf was 0.5 during the first 2 wk and was similar across treatment groups ($P = 0.64$). One calf died at 25 d of age, presumably from heat stress; all available data for this calf were included in the dataset. Average health scores were 1.18, 0.17, 0.09, and 0.02 for fecal, eye, nasal, and ear scores, respectively, and were not altered by treatments ($P > 0.20$).

Concentrations of immunoglobulins and total protein in serum in calves in the first week was ≥ 10 g / L and ≥ 52 g / L, respectively, indicating all calves received colostrum of acceptable quality regarding antibody absorption in the bloodstream (Calloway et al., 2002; Godden, 2008). Treatments did not alter serum total protein or immunoglobulin concentrations

during the first wk of life (P = 0.96 and 0.98, respectively). Colostrum fatty acid concentrations were averaged for each treatment group (Table 1). Adrenic acid in colostrum varied among calves given different treatments (P=0.01), but was the only fatty acid that significantly differed in concentrations relative to average colostrum concentrations given to calves.

Treatment did not alter growth rates in the first 2 wk of life or during the pre-weaning period (P > 0.20). Average wither height, heart girth, and body weight gain at 2 wk was 0.87 cm / d, 0.72 cm / d, and 0.60 kg / d. All calves maintained similar growth (0.51, 0.64, 0.78, and 0.60 kg / d for CON, FF30, FF60, and FF120, respectively) during the pre-weaning period (P=0.31), indicating FF treatment did not interfere with calf health and growth.

Plasma free polyunsaturated fatty acids

FF treatments increased free concentrations of the n-3 FA (ALA, EPA, DHA, and docosapentaenoic acid), in plasma on d 1, 2, 4, and 7 after birth in a linear fashion (P < 0.001). This corresponded well with the FA found in fish and flaxseed oil. DHA was significantly increased in plasma free PUFA concentrations with FF treatments where CON, FF30, FF60, and FF120 averaged 8, 10, 14, and 29 nM DHA (P<0.001) during the first week after supplementation. Likewise, EPA also increased with increasing FF supplement volume (0.6, 0.8, 2.7, and 6.0 nM, respectively; P < 0.001) ALA significantly differed in concentration among CON and FF calves during the first week of life (24, 46, 73, 140 nM, respectively; P<0.001) as well. All three primary n-3 FA concentrations were predominantly increased by FF treatments (P ≤ 0.01) on d 1, 2, and 4 after birth. By d 7 after birth, concentrations either returned to normal or tended to be elevated (P ≥ 0.09) with FF, and by d 14, n-3 FA concentrations all returned to baseline (Figure 1).

Linoleic acid, 15% of the flaxseed oil, is a precursor to arachidonic acid (AA). Calves given FF supplementation at varying amounts had plasma concentrations of 2800-2900 nM free linoleic acid compared to CON (2000 nM), but this difference was not significant ($P = 0.27$). AA was also not significantly different with concentrations of 17, 14, 16, and 22 nM free AA for CON, FF30, FF60, and FF120, respectively ($P = 0.75$). The concentration of n-6 FA remained constant for all calves while primary n-3 FA increased in FF treatments. Thus, during the first week of life, free n-6 : n-3 FA ratios in plasma were decreased with FF supplementation in a linear fashion (P=0.01), where CON calves averaged an n-6 : n-3 FA ratio of 28 and with increasing FF treamtents, decreased to 16, 13, and 8 n-6 : n-3 FA, respectively. All plasma free PUFA concentrations analyzed are given with polynomial contrasts in Table 4.

Plasma phospholipid fatty acids

The linear trend of increased free n-3 FA and decreased free n-6 : n-3 FA ratio was also associated with similar changes in the phospholipid FA fraction. As expected, ALA, EPA, and DHA increased in the phospholipid with increasing FF supplementation in a linear fashion ($P < 0.001$) (Figure 2). Linoleic and AA did not increase with treatment ($P = 0.86$, $P = 0.98$, respectively). Total n-3 FA and total PUFA increased in a linear fashion ($P < 0.001$). Total n-3 FA concentrations were 4, 4, 6, and 7 g / 100 g for CON, FF30, FF60, and FF120, respectively. Total PUFA concentrations followed a similar trend of 23, 25, 26, and 26 g / 100 g for respective treatments ($P < 0.001$). However, total n-6 FA did not significantly change with treatments (27 g / 100 g; $P = 0.98$). The n-6:n-3 plasma phospholipid FA ratio decreased with FF calves (8, 6, 5, and 4 n-6:n-3 FA, respectively; $P < 0.001$). FF treatments decreased mono-unsaturated plasma phospholipid FA and increased saturated fatty acids ($P = 0.02$, $P = 0.02$, respectively). For all

PUFA with significant treatment effects, the effect of d was also significant ($P \leq 0.02$). All least squares means of plasma phospholipid PUFA quantifications by treatment and contrasts are listed in Table 5.

Oxidant status index, oxylipids, and isoprostanes

Concentrations of 5 oxylipids and 1 isoprostane in plasma were altered by FF treatments (Figure 3). FF treatments increased EPA derived 5,6-dihydroxy-eicosatetraenoic acid (5,6-DiHETE) by 35, 70, and 197 % of CON, respectively ($P < 0.01$), 14,15-DiHETE by 67, 106, and 116 percent of CON, respectively ($P < 0.001$), and 17,18-DiHETE by 117, 169, and 159 percent of CON, respectively ($P = 0.01$). FF treatments increased DHA derived 19,20-dihydroxy-docosapentaenoic acid (19,20-DiHDPA) by 57, 53, and 55 percent of CON, respectively ($P = 0.01$). N-6 derived leukotriene B_4 (LTB_4) increased with FF treatments 100, 166, and 128 percent of CON ($P = 0.02$), and 8-iso-prostaglandin-A_2 decreased by 59, 32, and 33 percent of CON (8-iso-PGA-2) ($P = 0.01$). 5,6-lipoxin A_4 (LXA_4) tended to increase ($P = 0.06$) with FF treatments by 106, 104, and 206 percent of CON. 14,15-DiHETE, 19,20-DiHDPA, 17,18-DiHETE, 5,6-DiHETE, and LTB_4 increased ($P < 0.02$) in a linear fashion with increasing FF treatment.

Cytochrome P450 epoxygenase (CYP) and lipoxygenase (LOX) products were primarily impacted by supplementation. No oxylipids analyzed that derived from cyclooxygenase (COX) pathways were altered in concentration by FF treatments. Average concentrations of oxylipids and isoprostanes for each treatment during wk 1 are reported in Table 6 and Table 7, respectively.

FF treatment did not decrease oxidant status index (OSi) during the first week of life ($P = 0.35$). RONS concentrations and AOP remained constant across treatment groups ($P = 0.71$ and

P = 0.40, respectively). Least square means of indicators of oxidative stress, including 8-iso-PGA-2, are shown as treatment by sampling day in Figure 4.

DISCUSSION

Increasing doses of 30, 60, and 120 mL 1:1 fish:flaxseed oil blend supplemented in colostrum did not impact health or growth in calves, decreased phospholipid n-6 FA : n-3 FA profile, increased free and phospholipid n-3 FA concentrations, and increased several oxylipids deriving from n-3 FA during the first week of life. Though OSi was unaltered by FF treatments, concentrations of an n-6 FA derived isoprostane, 8-iso-PGA_2, were reduced, indicating oxidative stress was decreased in FF calves the first week of life.

To become non-esterified, most plasma free FA must first be hydrolyzed from adipose tissue triglycerides during lipolysis, and there is often a direct association between plasma free FA and adipose FA composition (Hellmuth et al., 2013). Thus, observing an increase in free n-3 FA in our experiment is indicative of increased n-3 FA composition of adipose tissue. Dietary n-3 FA supplementation in murine research found that n-3 FA diminish macrophage infiltration of adipose tissues (Todorčević and Hodson, 2015). Macrophage infiltration is associated with a pro-inflammatory state in transition cows (Contreras et al., 2017), so in the current study, we postulate increased free n-3 FA may usher a response in adipose tissue from the diet that may be anti-inflammatory in nature. Our previous experiment which supplemented 60 mL 1:1 ratio fish : flaxseed oil blend with an additional 200 mg α-tocopherol (Opgenorth et al., 2019) observed increased free ALA, EPA, and DHA in plasma similar to the current study.

We also analyzed phospholipid FA content, which is of particular interest because, once incorporated in phospholipid membranes, phospholipid PUFA and esterified forms cleaved by

phospholipase A2 become available for enzymatic oxidation, resulting in the production of cell signaling molecules able to mediate inflammation and oxidative stress (Raphael and Sordillo, 2013). With an increase in the n-3 FA in the plasma phospholipid of FF calves, we observed some markedly increased n-3 FA derived oxylipid concentrations. Raphael et al., 2014, proposed that manipulation of oxylipids may be feasible via dietary PUFA, and we found that with one n-3 FA supplementation, several oxylipid concentrations were altered. Though we observed oxylipids in plasma, Contreras et al., 2012 similarly found that increasing n-3 FA in the phospholipid profile of endothelial cells leads to increased beneficial n-3 FA derived oxylipid concentrations. They also observed a decrease in reactive oxygen species (Contreras et al., 2012), though we did not find a corresponding RONS decrease in plasma in our experiment.

Oxylipids are products of PUFA substrates of enzymes such as LOX, COX, and CYP. They are cell signaling molecules able to mediate inflammation and its resolution through a variety of mechanisms. Oxylipids that increased in concentration by FF30, FF60, and FF120 were primarily end products of metabolism of EPA and DHA. 14,15 Di-HETE, 17,18-DiHETE, and 5,6-Di-HETE from EPA and 19,20-DiHDPA from DHA increased linearly with increased n-3 FA supplementation. Of the aforementioned oxylipids, all are end products of CYP enzymatic activity. N-3 FA are more favorable substrates to LOX and CYP enzymes when compared to n-6 FA substrates (Zhang et al., 2014), but COX favors n-6 FA (Wada et al., 2007). This may explain why no oxylipids formed from COX pathways were altered by increasing n-3 FA supplementation. Dietary supplementation of linoleic acid causes an observed increase in COX activity (Marchix et al., 2015), and DHA decreases COX expression (Massaro et al., 2006). Though we did not analyze gene expression, it is possible FF treatments may decrease COX expression due to an increase in DHA concentrations and a decrease in the ratio of n-6 : n-3 FA.

Many pain-relieving drugs such as aspirin or meloxicam function to inhibit COX activity (Brune and Patrignani, 2015), which is the very reason they are considered anti-inflammatory. The only COX-produced oxylipid that linearly decreased with increasing FF supplementation was thromboxane-2, though no overall difference ($P = 0.21$) in concentration among treatments were observed. Thus, further analysis of COX gene expression may be warranted to provide further evidence of the anti-inflammatory effects of FF treatments on neonatal calves.

Deriving from the n-6 FA, AA, LTB4 and LXA4 also increased with FF supplementation. Interestingly, AA, the source of these oxylipids, did not increase as plasma free PUFA or phospholipid PUFA in FF supplemented calves compared to CON. LXA4 is anti-inflammatory and LTB4 has some known pro-inflammatory functions. LXA4 functions to enhance epithelial cell wound healing in rodents (Gronert et al., 2005) and initiate remodeling of phospholipids in humans (Nigam et al., 1990) among many more actions (Gabbs et al., 2015). Contrastingly, LTB_4 helps instigate neutrophil chemotaxis in bovines (Heidel et al., 1989).

This study is perhaps the first to describe oxylipid and isoprostane concentrations during the first week of life of any neonate mammalian species. Oxylipid concentrations differ among cows in varying stages of lactation, and concentrations are not always necessarily indicative of physiological changes (Kuhn et al., 2017; Putman et al., 2019). Though calf oxylipid concentrations were found to be much smaller than adults (n*M* vs. µ*M*), the periparturient stage of the dairy cow can yield some interesting parallels to neonatal calves. Kuhn et al., 2017 suggested LXA4 is preferentially produced in periparturient cows, perhaps as a mechanism to reduce the systemic inflammatory state observed after parturition. We found supplementing calves with n-3 FA in their first meal tends to increase LXA4 concentrations, which is curious considering LXA4 is derived from AA. FF treatments contained 7.7% linoleic acid; however,

little differences in plasma n-6 FA content were observed, and the n-6:n-3 phospholipid FA ratio decreased. Traditionally, LXA4 decreases in concentration with n-3 FA supplementation (Poulsen et al., 2008). Nevertheless, LXA4 concentrations increased, perhaps displaying the same partiality observed in periparturient cows with a similar fundamental concept of reducing systemic inflammation.

Isoprostanes are direct biomarkers of oxidative stress because their production indicates RONS damage to the phospholipid membrane and thus cellular components (Montuschi et al., 2004). A decrease in concentration indicates a decrease in oxidative damage and physiological stress; therefore, FF treatments decreased oxidative stress due to a decrease in 8-iso-PGA2 in plasma, even though OSi, the concentration of RONS to AOP, remained unchanged.

Some evidence suggests neonate immune processes gravitate towards a pro-inflammatory state at birth. The placenta favors pro-oxidant isoprostanes derived from n-6 FA pathways. In humans, 8-iso-prostaglandin-$F_{2\alpha}$, a prominent and well-studied isoprostane known to indicate increased free radical concentrations, is highly concentrated in the umbilical vein (Braekke et al., 2006). Placental production of this biomarker of oxidative stress may be regulated by hormones, though explanations as to why this phenomenon occurs are still unclear (Hermenegildo et al., 2002). The placenta may require a certain degree of inflammation in order to detach normally (Boro et al., 2014), and inflammation plays important and necessary roles in the calving process (Bradford et al., 2015). However, oxidative stress can impact calves well beyond the acute response at birth (Abuelo et al., 2013), and the additional supplementation of antioxidant and anti-inflammatory nutrients after birth may be beneficial for resolving these pathways quickly to avoid decreased calf viability and a disadvantaged immune system.

CONCLUSION

A positive linear effect of fish:flaxseed oil supplemental volume in colostrum did not alter oxidant status, but did decrease the isoprostane 8-iso-PGA2, a direct biomarker of lipid peroxidation during oxidative stress. Fish and flaxseed supplemented in colostrum impacted several PUFA metabolite concentrations and plasma n-3 FA concentrations as non-esterified FA and phospholipid membrane components. No negative consequences on health or growth of n-3 FA supplemented calves compared to control calves were observed, regardless of n-3 FA dose level. The data indicate supplementing n-3 FA in colostrum may promote an anti-inflammatory state after birth which could enhance calf performance in early life.

APPENDIX

Table 3.1. Least squares means of treatments PUFA content in randomly assigned maternal colostrum of first feeding before supplementation.

	Treatment[1]				
Colostrum FA (n*M*)	CON	FF30	FF60	FF120	SEM
C18:2n-6	422	362	255	441	68.6
C18:3n-3	78.4	26.0	59.2	40.5	29.9
C20:3n-6	47.1	52.5	40.7	57.6	10.8
C20:4n-6	151	144	122	194	32.1
C20:5n-3	59.9	32.5	38.6	64.1	16.7
C22:4n-6 **	24.2	36.5	34.1	61.6	7.06
C22:5n-3	152	148	97	222	33.7
C22:6n-3	12.0	5.60	7.51	15.4	4.58

† $0.05 < P \leq 0.10$
** $P \leq 0.01$
[1]Treatments: CON = control, no supplement added to colostrum; FF30 = 30 mL 1:1 ratio fish and flaxseed oil blend added to colostrum; FF60 = 60 mL 1:1 ratio fish and flaxseed oil blend added to colostrum; FF120 = 120 mL 1:1 ratio fish and flaxseed oil blend added to colostrum.

Table 3.2. Composition of Land O Lakes Warm Front (Arden Hills, MI) milk replacer fed to calves during the pre-weaning period.

Milk Replacer Composition, air-dry basis	
Crude Protein, not less than %	27
Crude Fat, not less than %	10
Crude Fiber, not more than %	0.15
Calcium, not less than or more than %	0.75 - 1.25
Sodium, not more than %	1.20
Phosphorous, not less than %	0.70
Vitamin A, not less than IU / kg	9,072
Vitamin D_3, not less than IU / kg	2,268
Vitamin E, not less than IU / kg	68

Table 3.3. Feeding schedule of all calves on study starting at d 1 of age. Each liter contained 0.13 kg of milk replacer powder.

	Milk Replacer Feeding Schedule, L per feeding		
Age, wk	Morning, 900 hr	Afternoon, 1600 hr	Evening, 2100 hr
0-1	2.4	2.4	2.8
1-6	2.8	2.8	3.8
6-8	3.8	0	0

Table 3.4. Least squares means of plasma free PUFA concentrations.

Plasma PUFA (n*M*)	Treatment[1] CON (±CI 95%)	FF30 (±CI 95%)	FF60 (±CI 95%)	FF120 (±CI 95%)	L[2]	Q[3]	C[4]	*P*[5]
C18:2n-6	925 (608-1410)	907 (572-1437)	1120 (750-1670)	1300 (854-1980)	0.20	0.93	0.64	0.58
C18:3n-3***	24.3 (16-38)	46.1 (29-73)	73 (46-115)	138 (86-223)	<0.001	0.43	0.93	0.004
C20:3n-6	1.11 (0.82-1.49)	1.88 (1.39-2.53)	1.48 (1.09-1.99)	1.75 (1.30-2.36)	0.07	0.74	0.53	0.26
C20:4n-6	16.9 (8.3-34)	14.2 (6.6-30)	16.4 (8-34)	22.2 (11-46)	0.56	0.65	0.80	0.89
C20:5n-3***	0.641 (0.36-1.15)	0.832 (0.47-1.49)	2.69 (1.5-4.8)	6.01 (3.3-11)	<0.001	0.69	0.14	<0.001
C22:5n-3*	0.292 (0.2-0.4)	0.428 (0.3-0.6)	0.358 (0.3-0.5)	0.888 (0.6-1.3)	0.02	0.45	0.30	0.05
C22:6n-3**	8.00 (5.6-12)	10.3 (7.2-15)	13.5 (9.4-19)	29.1 (20-42)	<0.001	0.57	0.93	0.001
Σ n-6 FA : n-3 FA*	28.4 (18-36)	16.0 (10-20)	12.7 (9.5-19)	7.59 (6-15)	0.004	0.56	0.22	0.02

† $0.05 < P \leq 0.10$
* $P \leq 0.05$
** $P \leq 0.01$
*** $P \leq 0.0001$
[1]Treatments: CON = control, no supplement added to colostrum; FF30 = 30 mL 1:1 ratio fish and flaxseed oil blend added to colostrum; FF60 = 60 mL 1:1 ratio fish and flaxseed oil blend added to colostrum; FF120 = 120 mL 1:1 ratio fish and flaxseed oil blend added to colostrum.
[2]L=linear polynomial contrast.
[3]Q=quadratic polynomial contrast.
[4]*C*=cubic polynomial contrast.
[5]*P*=treatment effect *P*-value.

Table 3.5. Least squares means of plasma phospholipid FA concentrations averaged from samples d 1, 2, 4, 7, and 14.

Plasma Phospholipid FA (g / 100 g)	Treatment[1]								
	CON	FF30	FF60	FF120	SEM	L[2]	Q[3]	C[4]	*P*[5]
C12:0	0.02	0.02	0.02	0.02	0.002	0.21	0.90	0.25	0.41
C16:0	19.9	20.1	20.8	20.2	0.24	0.52	0.16	0.36	0.36
C16:1 7c/8c	0.30	0.30	0.30	0.29	0.008	0.34	0.82	0.83	0.78
C16:1 9c †	0.73	0.70	0.65	0.60	0.02	0.01	0.82	0.76	0.07
C16:1 10c/13t	0.06	0.06	0.06	0.06	0.004	0.28	0.40	0.26	0.39
C16:1 11c †	0.05	0.05	0.05	0.04	0.004	0.02	0.97	0.92	0.10
C17:0	0.49	0.50	0.48	0.50	0.01	0.60	0.64	0.39	0.73
C18:0	21.7	22.7	22.1	22.0	0.21	0.94	0.08	0.06	0.10
C18:1 6-8t**	0.07	0.06	0.06	0.05	0.002	0.002	0.51	0.47	0.01
C18:1 9t	0.07	0.08	0.08	0.08	0.003	0.07	0.44	0.93	0.22
C18:1 10t	0.03	0.04	0.03	0.03	0.005	0.73	0.56	0.18	0.50
C18:1 11t	0.19	0.18	0.18	0.18	0.01	0.44	0.81	0.67	0.81
C18:1 12t	0.19	0.18	0.18	0.18	0.01	0.90	0.71	0.94	0.98
C18:1 6-8c/13/14t	0.14	0.13	0.13	0.13	0.01	0.31	0.60	0.67	0.59
C18:1 9c**	19.5	17.4	16.9	16.6	0.43	0.002	0.04	0.44	0.005
C18:1 11c	2.57	2.62	2.49	2.50	0.09	0.44	0.92	0.39	0.70
C18:1 12c	0.12	0.12	0.11	0.10	0.01	0.03	0.72	0.81	0.13
C18:1 13c	0.20	0.20	0.17	0.18	0.01	0.14	0.31	0.35	0.25
C18:1 14c/16t	0.08	0.07	0.07	0.07	0.003	0.61	0.54	0.76	0.83
C19:0	0.08	0.08	0.09	0.08	0.005	0.81	0.71	0.37	0.79
C18:2 (n-6)	20.7	21.1	20.6	20.2	0.77	0.53	0.75	0.62	0.86
C18:3 (n-3)***	0.41	0.93	1.10	1.11	0.05	<0.001	<0.001	0.13	<0.001
CLA 9c, 11t †	0.09	0.10	0.08	0.08	0.01	0.04	0.90	0.10	0.08
C20:0	0.06	0.07	0.06	0.06	0.01	0.17	0.38	0.56	0.39
C20:2 (n-6)	0.29	0.29	0.30	0.27	0.02	0.31	0.43	0.81	0.62
C20:3 (n-9)	0.20	0.19	0.16	0.15	0.03	0.03	0.64	0.39	0.11

Table 3.5 (cont'd)

C20:3 (n-6)	1.65	1.56	1.45	1.41	0.10	0.19	0.59	0.82	0.51
C22:0	0.04	0.04	0.04	0.04	0.003	0.78	0.79	0.51	0.89
C20:4 (n-6)	4.65	4.51	4.44	4.41	0.47	0.71	0.85	0.98	0.98
C23:0	0.10	0.11	0.11	0.11	0.01	0.46	0.37	0.42	0.52
C20:5 (n-3)***	0.36	0.60	1.04	1.33	0.09	<0.001	0.02	0.47	<0.001
C24:0	0.04	0.04	0.04	0.04	0.004	0.51	0.30	0.40	0.51
C22:4 (n-6)	0.35	0.34	0.33	0.30	0.05	0.32	0.90	0.88	0.77
C22:5 (n-6)	0.11	0.11	0.11	0.08	0.02	0.05	0.19	0.41	0.11
C22:5 (n-3)*	1.35	1.38	1.49	1.67	0.07	0.005	0.71	0.73	0.03
C22:6 (n-3)***	1.18	1.58	1.84	2.16	0.09	<0.001	0.11	0.84	<0.001
Σ SFA*	35.5	36.0	36.8	36.4	0.27	0.3	0.03	0.28	0.02
Σ MUFA*	28.0	26.2	24.2	25.2	0.55	0.02	0.02	0.45	0.02
Σ PUFA***	22.9	24.8	26.0	25.9	0.43	<0.001	0.006	0.97	<0.001
Σ n-3 FA***	3.50	4.39	5.72	6.57	0.17	<0.001	0.02	0.36	<0.001
Σ n-6 FA	26.9	27.3	27.3	27.2	0.59	0.80	0.72	0.95	0.98
Σ n-6 FA:n-3 FA***	7.64	5.65	4.65	3.81	0.23	<0.001	0.01	0.81	<0.001

† $0.05 < P \leq 0.10$
* $P \leq 0.05$
** $P \leq 0.01$
*** $P \leq 0.001$
[1]Treatments: CON = control, no supplement added to colostrum; FF30 = 30 mL 1:1 ratio fish and flaxseed oil blend added to colostrum; FF60 = 60 mL 1:1 ratio fish and flaxseed oil blend added to colostrum; FF120 = 120 mL 1:1 ratio fish and flaxseed oil blend added to colostrum.
[2]L=linear polynomial contrast.
[3]Q=quadratic polynomial contrast.
[4]C=cubic polynomial contrast.
[5]*P*=treatment effect *P*-value.

Table 3.6. Least squares means of plasma oxylipid concentrations during the first week of life.

			Treatments[1]							
Oxylipid[2] Plasma Concentrations (n*M*)	PUFA Substrate[3]	Oxidative Pathway[4]	CON	FF30	FF60	FF120	L[5]	Q[6]	C[7]	*P*[8]
11,12-DHET	AA	CYP	50.6 (43-60)	64.7 (54-77)	59.2 (50-70)	49.9 (42-59)	0.59	0.13	0.37	0.39
12,13-EpOME	LA	CYP	2.78 (1.9-4.1)	4.93 (3.4-7.1)	4.36 (3.0-6.4)	3.82 (2.6-5.7)	0.51	0.1	0.29	0.23
17,18-DiHETE**	EPA	CYP	2460 (1600-3800)	5340 (3400-8300)	6620 (4300-10000)	6370 (4100-9900)	0.008	0.02	0.56	0.01
12,13-DiHOME	LA	CYP	1.98 (1.5-2.7)	2.21 (1.7-2.8)	2.42 (1.9-3.1)	2.12 (1.7-2.7)	0.74	0.3	0.84	0.74
13-HODE	LA	LOX	110 (96-130)	137 (120-160)	140 (120-160)	129 (110-150)	0.37	0.1	0.65	0.29
14,15-DiHETE***	EPA	CYP	163 (130-210)	271 (210-350)	335 (260-440)	352 (270-460)	0.004	0.003	0.09	<0.001
12-HHTrte	AA	COX	2.73 (1.9-4.0)	3.03 (2.1-4.5)	3.39 (2.3-5.0)	3.52 (2.4-5.1)	0.35	0.72	0.92	0.78
13-OxODE	LA	LOX	29.2 (27-31)	28 (26-30)	29.3 (27-32)	26.5 (25-28)	0.12	0.49	0.3	0.27
15-HETE	AA	LOX	14.7 (12-18)	16.9 (14-21)	16.4 (13-20)	19.4 (16-24)	0.07	0.99	0.43	0.24
15-OxoETE	AA	LOX	0.147 (0.08-0.28)	0.198 (0.10-0.38)	0.146 (0.07-0.30)	0.138 (0.07-0.28)	0.74	0.75	0.54	0.89
15d-PGJ2	AA	COX	306 (210-450)	256 (180-370)	363 (250-530)	265 (182-387)	0.80	0.55	0.21	0.55
17-HDoHE	DHA	COX/LOX	4.43 (3.2-6.2)	4.77 (3.4-6.7)	4.56 (3.3-6.4)	6.29 (4.4-8.9)	0.17	0.57	0.67	0.49
19,20-DiHDPA**	DHA	CYP	216 (160-290)	340 (260-450)	330 (250-440)	335 (250-440)	0.02	0.02	0.16	0.01
19,20-EpDPE	DHA	CYP	45.4 (33-62)	56.2 (41-76)	64.9 (48-88)	75.4 (55-100)	0.03	0.53	0.96	0.14
20-HETE	AA	CYP	130 (30-570)	167 (38-740)	159 (36-700)	136 (31-600)	0.93	0.29	0.64	0.7

Table 3.6 (cont'd)

5,6-DiHETE***	EPA	CYP	21.4 (18-26)	29.1 (24-35)	36.4 (30-44)	68.3 (56-83)	<0.001	0.85	0.76	<0.001
5,6-LXA4†	AA	LOX	0.507 (0.31-0.83)	1.05 (0.64-1.7)	1.04 (0.63-1.7)	1.56 (0.95-2.6)	0.02	0.36	0.35	0.06
5-HETE	AA	LOX	7.68 (6.1-9.7)	9.26 (7.3-12)	10.5 (8.3-13)	11 (8.7-14)	0.06	0.35	0.98	0.2
5-OxoETE	AA	LOX	1.2 (0.75-1.9)	1.26 (0.77-2.0)	1.25 (0.77-2.0)	1.16 (0.71-1.9)	0.88	0.82	0.97	0.99
8,9-DHET	AA	CYP	12.5 (10-16)	17.6 (14-22)	12.8 (10-16)	13.2 (11-16)	0.68	0.32	0.03	0.14
9,10-DiHOME	LA	CYP	7.8 (6.4-9.4)	9.12 (7.5-11)	10.2 (8.4-12)	9.69 (8.0-12)	0.21	0.24	0.9	0.38
9,10-EpOME	LA	CYP	1.73 (1.3-2.2)	2.27 (1.8-2.9)	2.16 (1.7-2.8)	2.14 (1.7-2.8)	0.43	0.32	0.48	0.54
9-HETE	AA	Non	1.48 (1.1-2.1)	1.93 (1.4-2.7)	1.41 (1.0-2.0)	1.75 (1.3-2.4)	0.8	0.94	0.25	0.68
9-HODE	LA	LOX/Non	51.9 (46-59)	62.6 (55-71)	62.5 (55-72)	62.9 (55-72)	0.17	0.22	0.51	0.28
9-OxoODE	LA	LOX	50.3 (45-56)	53.2 (47-59)	53.9 (48-60)	53.3 (48-60)	0.55	0.52	0.89	0.84
LTB4*	AA	LOX	0.571 (0.40-0.81)	0.941 (0.67-1.3)	1.25 (0.88-1.8)	1.07 (0.77-1.5)	0.03	0.03	0.97	0.03
PGD2	AA	COX	0.94 (0.62-1.4)	0.98 (0.63-1.5)	0.97 (0.64-1.5)	1.01 (0.66-1.5)	0.84	0.97	0.93	1
PGE2	AA	COX	3.86 (2.2-6.7)	8.82 (5.1-15)	5.81 (3.4-10)	8.08 (4.7-14)	0.31	0.54	0.22	0.40
Resolvin D1†	DHA	LOX	0.0893 (0.055-0.15)	0.149 (0.091-0.24)	0.053 (0.035-0.081)	0.072 (0.047-0.11)	0.22	0.75	0.03	0.09
Resolvin D2	DHA	LOX	0.703 (0.52-0.95)	0.719 (0.52-0.99)	0.57 (0.42-0.77)	0.733 (0.54-0.99)	0.99	0.4	0.28	0.58
TXB2	AA	COX	9.53 (7.4-12)	9.18 (6.9-12)	7.45 (5.7-9.7)	6.57 (5.1-8.5)	0.05	0.88	0.56	0.21
11,12-EET	AA	CYP	1.48 (1.1-2.1)	1.07 (0.76-1.5)	1.1 (0.77-1.5)	1.32 (0.93-1.9)	0.85	0.17	0.63	0.5

Table 3.6 (cont'd)
† $0.05 < P \leq 0.10$
* $P \leq 0.05$
** $P \leq 0.01$
*** $P \leq 0.001$
[1]Treatments: CON = control, no supplement added to colostrum; FF30 = 30 mL 1:1 ratio fish and flaxseed oil blend added to colostrum; FF60 = 60 mL 1:1 ratio fish and flaxseed oil blend added to colostrum; FF120 = 120 mL 1:1 ratio fish and flaxseed oil blend added to colostrum.
[2]Oxylipids: dihydroxy-eicosatrienoic acid (DHET); epoxyoctadecenoic acid (EpOME); dihydroxy-eicosatetraenoic acid (DiHETE); dihydroxy-octadecenoic acid (DiHOME); hydroxy-octadecadienoic acid (HODE); hydroxy-heptadecatrienoic acid (HHTrte); oxo-octadecadienoic acid (OxODE); hydroxy-eicosatetraenoic acid (HETE); oxo-eicosatetraenoic acid (OxoETE); prostaglandin J (PGJ); hydroxy-docosahexaenoic acid (HDoHE); dihydroxy-docosapentaenoic acid (DiHDPA); epoxy-docosapentaenoic acid (EpDPE); lipoxin A (LXA); leukotriene B (LTB); prostaglandin D (PGD); prostaglandin E (PGE); thromboxane (TXB); epoxy-eicosatrienoic acid (EET)
[3]Substrates: arachidonic acid (AA); linoleic acid (LA); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA)
[4]Pathways: lipoxygenase (LOX); cyclooxygenase (COX); cytochrome P450 (CYP); non-enzymatic (Non)
[5]L=linear polynomial contrast.
[6]Q=quadratic polynomial contrast.
[7]C=cubic polynomial contrast.
[8]Overall *P*=the overall treatment effect *P*-value of oxylipid

Table 3.7. Least squares means of plasma isoprostane concentrations during the first week of life.

Isoprostane[2] Plasma Concentrations (n*M*)	PUFA Substrate[3]	Oxidative Pathway[4]	Treatment[1]				L[3]	Q[4]	C[5]	*P*[6]
			CON	FF30	FF60	FF120				
6-keto-$PGF_{1\alpha}$	AA	COX	6.68 (5.4-8.2)	6.23 (5.1-7.6)	5.73 (4.7-7.0)	7.09 (5.7-8.8)	0.75	0.37	0.8	0.80
2,3-dinor-8-iso-$PGF_{2\alpha}$	AA	Non	0.221 (0.14-0.36)	0.306 (0.19-0.49)	0.283 (0.18-0.45)	0.332 (0.21-0.52)	0.37	0.71	0.6	0.73
5-$iPF2_{\alpha}$-VI	AA	Non	1.07 (0.83-1.4)	1.09 (0.84-1.4)	1.04 (0.81-1.4)	0.97 (0.75-1.3)	0.56	0.82	0.88	0.93
8-12-iso-$iPF2_{\alpha}$-VI	AA	Non	4.11 (3.7-4.6)	4.3 (3.8-4.8)	4.09 (3.6-4.6)	4.1 (3.7-4.6)	0.86	0.86	0.63	0.96
8-iso-15KETO-PGE_2	AA	Non	2.09 (1.7-2.5)	1.78 (1.5-2.1)	2.13 (1.8-2.6)	1.97 (1.6-2.4)	0.98	0.91	0.27	0.72
8-iso-15R-$PGF_{2\alpha}$	AA	Non	0.345 (0.21-0.56)	0.365 (0.25-0.59)	0.407 (0.25-0.67)	0.591 (0.37-0.94)	0.13	0.72	0.99	0.46
8-iso PGA1	AA	Non	0.144 (0.10-0.21)	0.109 (0.075-0.16)	0.122 (0.081-0.18)	0.168 (0.11-0.25)	0.43	0.25	0.66	0.53
8-iso PGA2**	AA	Non	0.712 (0.49-1.0)	0.289 (0.20-0.42)	0.481 (0.33-0.70)	0.554 (0.38-0.81)	0.98	0.02	0.008	0.01
8-iso-$PGF_{2\alpha}$	AA	Non	2.45 (1.6-3.7)	2.66 (1.8-4.0)	3.77 (2.6-5.6)	2.8 (1.9-4.2)	0.64	0.32	0.49	0.61
$PGF_{2\alpha}$	AA	COX	0.992 (0.67-1.5)	0.646 (0.43-0.97)	0.938 (0.59-1.5)	0.825 (0.55-1.2)	0.86	0.65	0.18	0.51

** $P \leq 0.01$

[1]Treatments: CON = control, no supplement added to colostrum; FF30 = 30 mL 1:1 ratio fish and flaxseed oil blend added to colostrum; FF60 = 60 mL 1:1 ratio fish and flaxseed oil blend added to colostrum; FF120 = 120 mL 1:1 ratio fish and flaxseed oil blend added to colostrum.

[2]Isoprostanes: prostaglandin (PG)

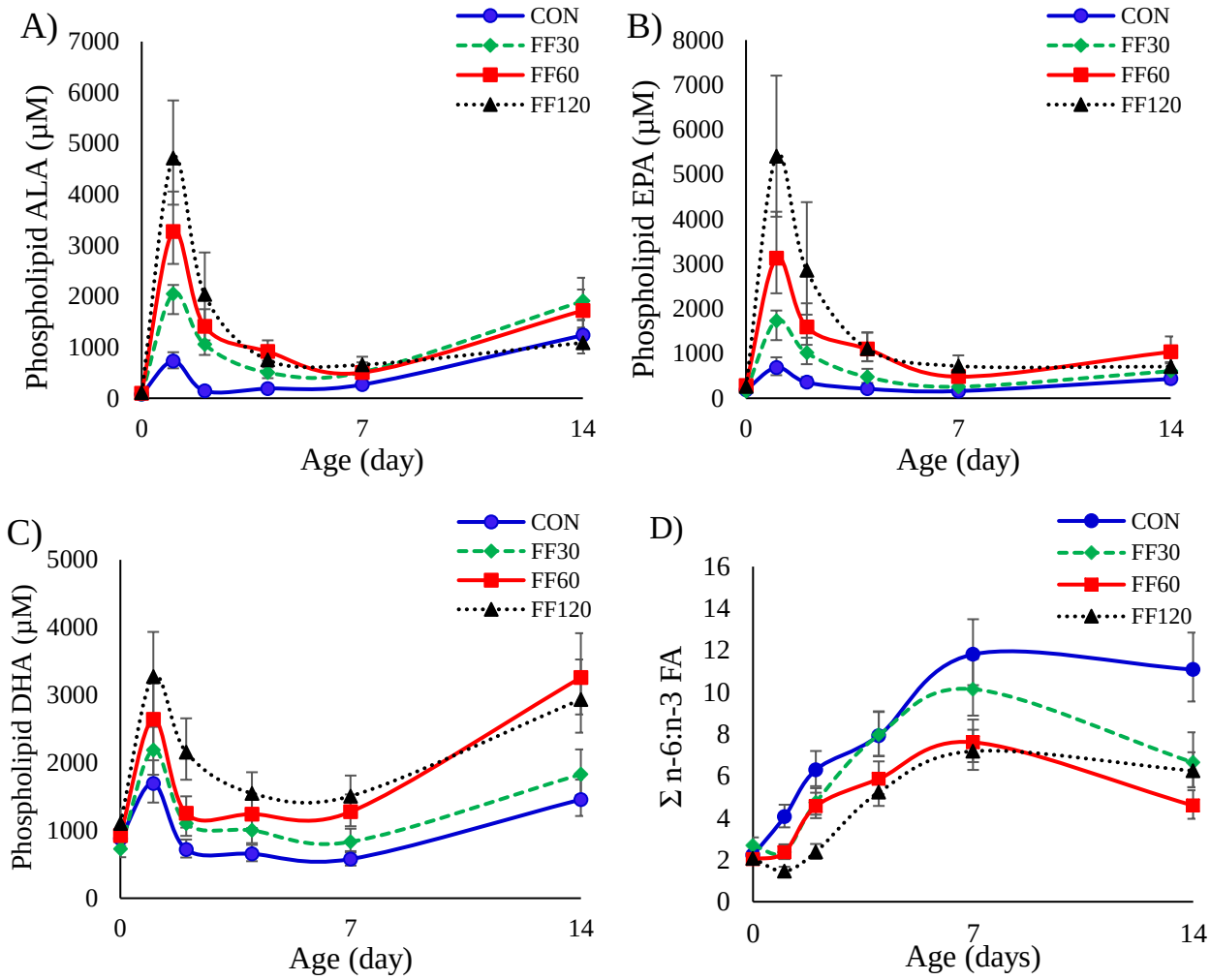
[3]Substrate: arachidonic acid (AA)

[4]Pathway: cyclooxygenase (COX); non-enzymatic (Non)

[5]L=linear polynomial contrast.

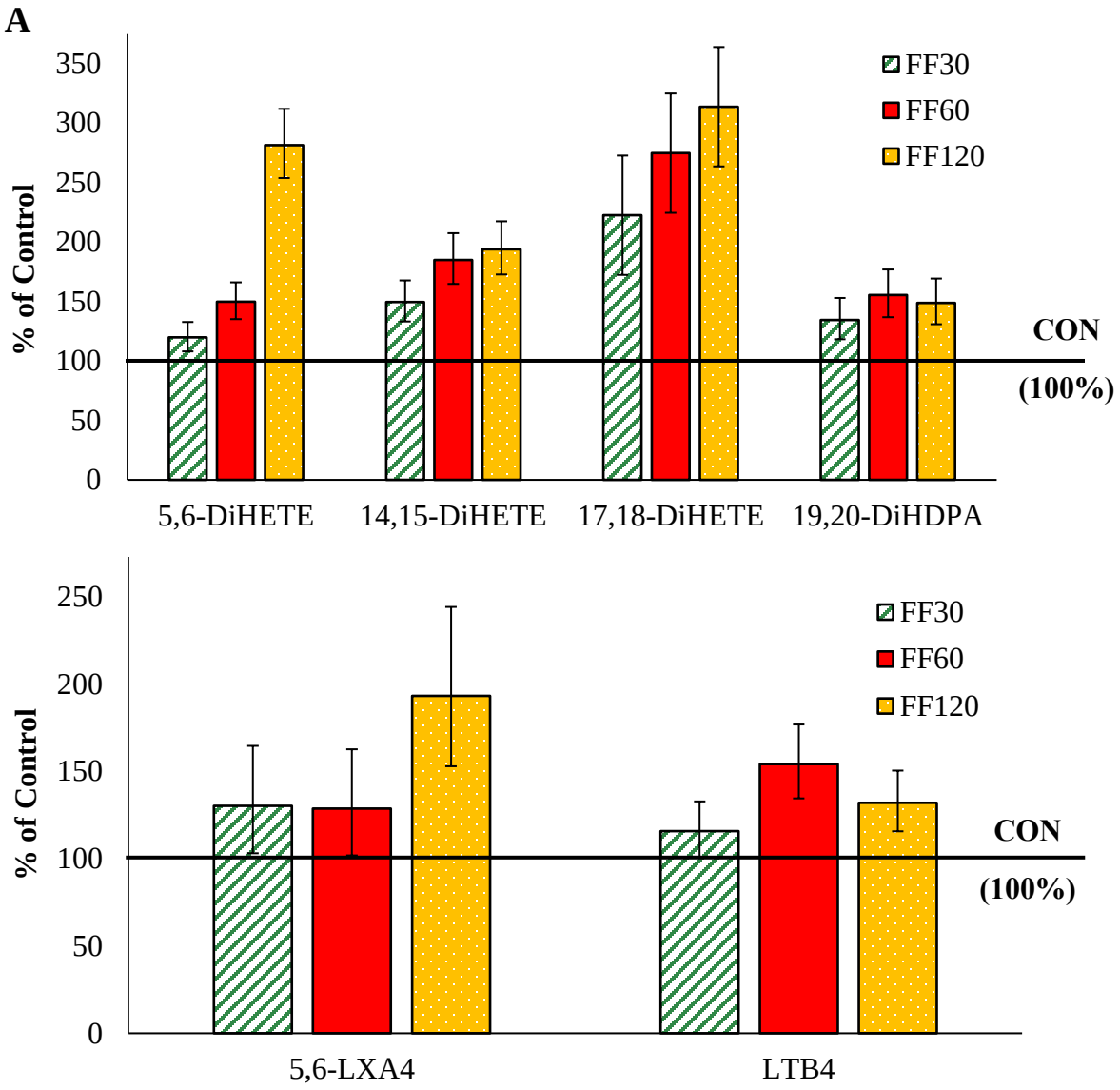
Table 3.7 (cont'd)
[6]Q=quadratic polynomial contrast.
[7]C=cubic polynomial contrast.
[8]Overall *P*=the overall treatment effect *P*-value of isoprostanes

Figure 3.1. Plasma phospholipid FA concentrations and n-6:n-3 FA ratio.



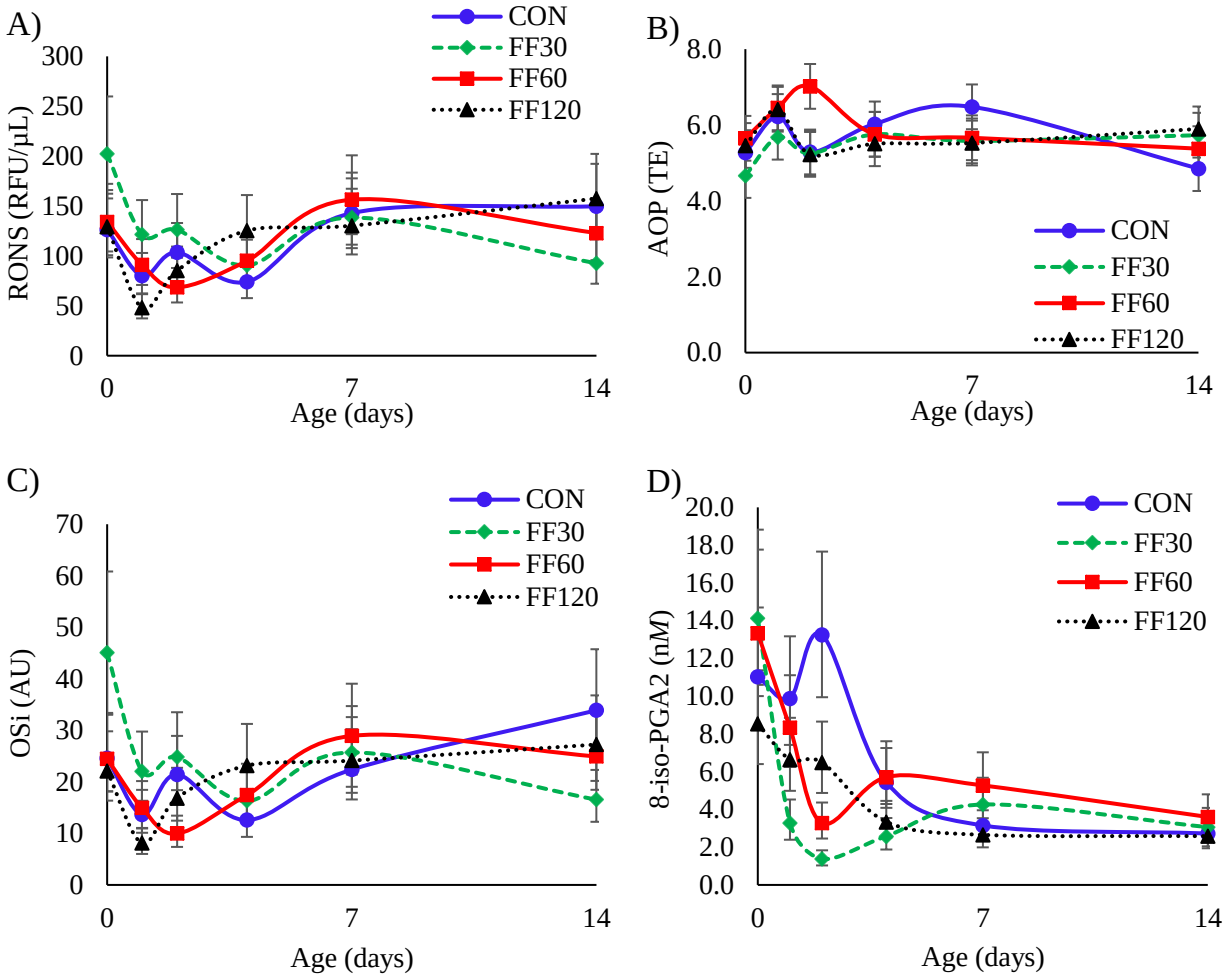
Treatment by day phospholipid FA concentration (µ*M*) least squares means of α-linolenic acid (ALA) (A), eicosapentaenoic acid (EPA) (B), docosahexaenoic acid (DHA) (C), and total n-6 : total n-3 FA ratio (D) predicted from the model. ALA, EPA, and DHA are back-transformed for interpretation with adjusted standard errors. During wk 1, FF treatments increased ALA, EPA, and DHA and decreased the n-6 : n-3 FA ratio ($P < 0.001$)

Figure 3.2. Plasma oxylipids as percent of control.



FF treatments depicted as percent of CON for n-3 FA derived oxylipids (A) and n-6 FA derived oxylipids (B) that differed from CON concentrations during the first week of age. Oxylipids were: dihydroxy-eicosatetraenoic acid (DiHETE); dihydroxy-docosapentaenoic acid (DiHDPA); lipoxin (LX); leukotriene (LT). Overall treatment *P*-values were: 5,6-DiHETE: $P < 0.01$; 14,15-DiHETE: $P < 0.001$; 17,18-DiHETE: $P = 0.01$; 19,20-DiHDPA: $P = 0.01$; 5,6-LXA4: $P = 0.06$; LTB4: $P = 0.02$

Figure 3.3. Indicators of oxidative stress.



Least squares means of treatments during the first two weeks of life of reactive oxygen and nitrogen species (RONS), A; antioxidant potential (AOP), B; oxidant status index (OSi), C; and 8-iso-prostaglandin A2 (8-iso-PGA2), D – all indicators of oxidative stress. RONS, OSi, and 8-iso-PGA2 means are back-transformed from the model for interpretation with adjusted standard errors. FF treatments did not alter RONS, AOP, or OSi, but did decrease 8-iso-PGA2 (P=0.01) during the first week of age.

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CHAPTER 4.

COLOSTRUM SUPPLEMENTATION WITH OMEGA-3 FATTY ACIDS DOES NOT ALTER CALF OUTCOME ON A HEALTHY COMMERCIAL FARM*

*A version of this manuscript will be submitted to the *Journal of Dairy Science*

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ABSTRACT

Our objective was to supplement colostrum with omega-3 fatty acids (n-3 FA) to provide anti-inflammatory mediators that may improve the immune response of neonatal calves. We hypothesized a colostrum supplement containing 60-mL of a 1:1 ratio fish:flaxseed oil with or without 200 mg α-tocopherol might provide an advantageous start to early life by enhancing health and growth. Calves were blocked by birth order and randomly assigned to 1 of 3 treatments: no supplement added to colostrum (CON); 60 mL 1:1 fish : flaxseed oil blend (FF); 60 mL 1:1 fish : flaxseed oil blend with 200 mg α-tocopherol (FFE). After colostrum feeding, all calves were housed in individual hutches and fed milk replacer 3 times per day. In total, 180 heifer calves (n=60 per treatment) were enrolled on a commercial farm. Health was scored 3 times per week until weaning. Weight, wither height, and heart girth were measured after birth, 3 wk, and 8 wk of age to assess pre-weaning growth. A subgroup of 54 calves (18 blocks or 18 calves per treatment) were sampled 2 d (+/- 8 hr) after birth to evaluate oxidant status, serum total protein, and inflammatory gene and cytokine protein expression in blood after an in vitro

LPS challenge as indicators of health and immunity. At 9 wk, calves were transported 18 h to another farm, and medical records were kept as an indicator of disease incidence up to 13 wk of age. Calf mortality was less than 2 percent which is below industry average, and exceptional health was observed throughout the study. Health scores and growth were similar throughout the pre-weaning period regardless of treatment. Serum total protein indicated successful passive transfer in all calves, and oxidant status index was not affected by treatments on d 2 of age. Concentrations of tumor necrosis factor α (TNF-α) increased with LPS stimulation, but the increase was not altered by treatment. Likewise, leukocyte gene expression of TNF-α, interleukins 8 and 10, and cyclooxygenase-2 increased upon LPS stimulation, but the fold change did not differ with treatment. In conclusion, 60 mL 1:1 ratio fish:flaxseed oil colostrum supplement did not enhance pre-weaning calf performance. Supplementing n-3 FA in a one-time meal may not provide the anti-inflammatory benefits observed with continuous feeding.

INTRODUCTION

Calves may experience oxidative stress at birth because production of reactive oxygen and nitrogen species (RONS) is increased (Ling et al., 2018). RONS damage to cellular components may cause inflammation and increase disease susceptibility. One potential way to reduce oxidative stress is supplement calves with n-3 FA, which have previously been shown to produce oxylipids that can decrease oxidative stress and inflammation (Mavangira and Sordillo, 2018). Immune response is modulated with fish or flaxseed oil supplemented to milk replacer (Ballou and DePeters, 2008; Karcher et al., 2014). Flaxseed oil in calf starter enhances growth (Hill et al., 2009). We speculated similar benefits may occur from a substantial one-time dose of fish and flaxseed oils in colostrum.

We supplemented n-3 FA in calves' first colostrum and found altered oxylipid and isoprostane concentrations indicative of decreased oxidative stress and increased n-3 FA profiles (Opgenorth et al., 2019; Opgenorth et al., unpublished data). Our results indicated n-3 FA decrease the n-6:n-3 FA ratio in plasma and may decrease oxidative stress during the first week of life. The inclusion of α-tocopherol may also be important in enhancing the beneficial effects of n-3 FA (Opgenorth et al., 2019). However, general health and growth rates were unaffected. Tao and Dahl, 2013, cautioned against results of health and growth of studies with lower sample sizes such as treatment n=10. Prior research continuously supplemented fish oil and flaxseed oil during the pre-weaning period and saw beneficial results with n=16 to 24 per treatment.

Seeing that the n-3 FA supplements were safe to feed calves with no observed negative effects on passive transfer or general health and growth, we increased calf sample size to 180 calves at a commercial dairy farm where we calculated 92 percent chance of detecting a difference in health scores greater than 0.3 at $P < 0.05$.

A short-term decrease in oxidative stress that benefits the immune response during the first few days of life may benefit calves during the pre-weaning period by advancing health and thus, growth. N-3 FA may alleviate oxidative stress; thus, we hypothesized that supplementing n-3 FA in colostrum will improve pre-weaning health and growth. Our primary objective was to determine if 60 mL fish:flaxseed oil or 60 mL fish:flaxseed oil with additional 200 mg vitamin E supplemented to colostrum would decrease health scores and disease incidence and increase growth. Our secondary objective was to examine the in vitro response of an immune challenge on inflammatory markers.

MATERIALS AND METHODS

The study was conducted during the months of March to May of 2019 at a commercial Michigan dairy farm and was approved by the Michigan State University Animal Care and Use Committee. One hundred and eighty Holstein-Friesian heifer calves were assigned to blocks as they were born and to 1 of 3 treatments within block in a randomized block design. Based on the variation in health scores of 0.5, we used the power procedure of SAS version 9.4 to determine that 60 calves per treatment would provide a 92 percent chance of detecting a difference in health scores greater than 0.3 at $P < 0.05$. Treatments were 1) no supplement added to colostrum (CON); 2) supplement of 60 ml of a 1:1 fish:flaxseed oil blend with 20% polysorbate 80 for emulsification (FF); and 3) supplement of FF with 200 mg α-tocopherol. Sixty calves were enrolled each week within a 4-day window for 3 consecutive weeks.

Colostrum was acquired from freshening cows at the farm. If Brix >22%, the colostrum was pooled and stored in a bulk tank. The bulk tank was emptied twice daily and pooled colostrum was distributed into 2.8 L bottles with 45 mL 50% potassium sorbate solution in water to prevent bacterial growth. Bottles were stored at -20° C and then warmed before feeding. Supplements were added directly to the colostrum bottle and inverted to mix. All calves were fed by esophageal tube within 2 hr after birth. Calves were ear notched to test for bovine leukemia virus and given 4 mL of intramuscular VetOne Vitamin E-AD (MWI Animal Health, Boise, ID) injection which includes 300 IU vitamin E, 100,000 IU vitamin A, and 10,000 IU vitamin D_3 per mL. Calves received a second feeding of 1.9 L colostrum with no supplements by nipple bottle 12 to 18 h after birth. Calves that refused to drink the full 1.9 L were fed with esophageal tube to complete the meal. Only the first colostrum feeding contained treatment supplements.

Calves were placed in a heated pen within 1 h after birth. Within 24 h, they were placed in individual outdoor hutches bedded with straw and sawdust and had no contact with other calves for the rest of the study. At 2 d of age, calves were vaccinated against bovine respiratory syncytial virus, bovine rhinotracheitis, and parainfluenza 3 (Inforce 3, Zoetis, Parsippany, NJ, USA). Dehorning paste was applied within the first week of life.

On d 2, 3, and 4, calves received 2.8 L of milk per feeding at 500 and 1900 h. Milk on these days was a 50:50 blend of unsaleable milk from the farm and milk replacer (11.5% solids, HerdFirst; Cargill, Minneapolis, MN). Milk replacer was blended in varying amounts per feeding depending on the quantity of unsaleable milk available each day. At 3 to 4 d of age, calves received water and calf starter grain ad libitum. Starting on d 5, calves were fed 2.8 L of milk 3 times per day at 500, 1300, and 1900 h. At 6 to 7 wk, the milk per feeding was decreased to 1.9 L. At 7 to 8 wk of age, milk per feeding was decreased to 0.94 L. At 8 to 9 wk of age, calves were fed 0.94 L at the 500 h feeding only. At 9 to 10 wk of age, calves were transported for 18 h to another farm after receiving their final 0.94 L feeding. Calves were weighed within 1 h after birth. Initial heart girth and wither height were measured at 2 to 3 d of age. Calves were weighed and measured similarly at 3 wk of age and just prior to weaning at 8 wk of age. Eye, nasal, fecal, and ear scores were assigned for each calf 3 d per wk by trained researchers blind to treatment using the health scoring system of McGuirk, University of Wisconsin (https://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf). Health scores were averaged by week of age. Records of any medications given to calves were recorded as a measure of disease incidence from birth to 1 month post-transport (until 13 wk of age).

Blood collection and analysis

In each weekly cohort, 6 blocks of calves born within a 16-h time period were selected for blood sampling so that 18 blocks (54 calves) were sampled for the experiment. Blood was sampled at 48 +/- 8 h after birth via jugular venipuncture for 1 serum separator tube (SST) and 2 EDTA plasma tubes using the BD vacutainer system (Becton, Dickinson and Company, Franklin Lakes, NJ). Blood was placed on ice immediately after collection before processing. SST tubes were spun at 1700 *g* at 4° C for 15 minutes, and 2 mL of serum were immediately aliquoted, flash frozen in liquid nitrogen, placed in dry ice, and stored at -80° C upon returning to campus laboratories. Remaining serum was tested for serum total protein with a digital Brix refractometer to assess passive transfer. Calves with total protein greater than or equal to 52 g/L were considered to have successful passive transfer (Calloway et al., 2002).

Within 2 mo of storage, serum was assessed for antioxidant potential (Re et al., 1999) and RONS (OxiSelect™ In Vitro ROS/RNS Assay Kit, Green Fluorescence; Cell Biolabs, inc., San Diego, CA, USA) to calculate the oxidant status index (OSi), which quantifies the balance of RONS and antioxidants and indicates the potential for oxidative stress. Both assays assessing oxidant status are detailed by Putman et al. (2019). Free radicals in samples convert an added reagent to a fluorescing product that can be measured to relate to total free radical concentration as described previously (Putman et al., 2018).

Values of antioxidant potential (AOP) were quantified with a photometric plate reader. 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid was used as a radical cation to be reduced by antioxidants to measure reduction potential of each sample. This assay measuring Trolox equivalents, a synthetic analog of vitamin E, is further described in Re et al. (1999).

Whole blood from the 2 EDTA plasma tubes was transferred to 2 tubes of 5 ml each for LPS

stimulation of immune cells. Methods were adapted from Karcher et al. (2014). One tube was inoculated with LPS (from *Escheria coli* 0111: B4; InvivoGen, San Diego, CA) at 5 µg/ml blood, and the other tube was inoculated with control media. Blood was incubated for 3.5 hours in a 37° C water bath. After incubation, blood was centrifuged at 1300 x g at 4° C for 10 minutes. Plasma was flash frozen in liquid nitrogen, placed on dry ice for transportation, and stored at -80 ℃ until analyzed for TNF-α proteins (Bovine TNF-α ELISA Kit; Thermo Scientific, Frederick, MD, USA). The remaining leukocyte layers were processed with QIAamp RNA Blood Mini Kit (QIAGEN, Germantown, MD, USA) to remove erythrocytes. The remaining white blood cells (WBC) were suspended in 600 µl buffer RLT for RNA isolation, flash frozen in liquid nitrogen, placed on dry ice during transportation, and stored at -80°C until analyzed for gene expression.

We extracted WBC RNA using QIAcube automation (QIAGEN, Germantown, MD, USA) with QIAamp RNA Blood Mini Kit (QIAGEN, Germantown, MD, USA). The RNase-free DNase (Ziagen, Valencia, CA) digested DNA, and all RNA samples were suspended in 40-µl RNase-free water before undergoing cDNA synthesis with a High Capacity cDNA reverse transcriptase kit with RNase inhibitor (Applied Biosystems, Foster City, CA). Afterwards, cDNA was amplified with Taqman PreAmp Kit (Applied Biosystems, Foster City, CA).

We analyzed samples in triplicate for expression of cyclooxygenase-2 (COX-2), interleukin-8 (IL-8), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF- α) using quantitative real-time PCR (ABI QuantStudio 7 Flex PCR system; (Applied Biosystems, Foster City, CA). The reaction mix for each well consisted of 5 µL Taqman Gene Expression PCR Master Mix (Applied Biosystems, Foster City, CA), 2.5 µL amplified cDNA sample, 0.5 µL Taqman Gene Expression Assay Mix (Applied Biosystems, Foster City, CA), and 2 µL RNase-

DNase-free water. We included 3 endogenous controls of B2M, PGK1, and GUSB Taqman Assays for reference and 1 non-template control per PCR plate for contamination detection.

Standard method thermal cycling conditions were: 48 ℃ for 30 min, 95 ℃ for 10 min, and 40 cycles of 95 ℃ for 15 sec followed by 60 ℃ for 1 min. The 3 reference genes of B2M, PGK1, and GUSB relative to the calibrator of CON, non-stimulated LPS samples normalized target gene abundance calculated by the RQ method described by Livak and Schmittgen, (2001).

STATISTICAL ANALYSIS

Data were analyzed using the mixed procedure of SAS 9.4. Fixed effects were treatment and cohort (starting week of study). Random effects were block within cohort and calf within block, cohort, and treatment. Pre-planned contrasts were 1) CON vs FF and FFE; and 2) FF vs FFE. Least square means of fixed effects were adjusted with Tukey-Kramer *P*-values. Birth weights and initial heart girths and hip heights were used as covariates for growth rates. Differences for main effects were considered significant if $P \leq 0.05$ and trends if $0.05 < P \leq 0.10$. Differences for interactions were considered significant if $P \leq 0.10$ and trends if $0.10 < P \leq 0.15$.

RESULTS

Calves were exceptionally healthy throughout the study with an average of 0.43 medications per calf from birth to 13 wk of age. Two calves died during the pre-weaning trial period: one from failure to thrive less than 12 h post-parturition and one from pneumonia. Most medications during the first 8 wk of life were to treat diarrhea. Pneumonia was the primary disease after transport. Treatment did not alter number of medications per calf ($P = 0.89$).

Because calves were healthy, health scores were generally low (Figure 1; Table 1). Overall fecal scores tended to be higher with FF and FFE compared to CON (CON: 0.65; FF: 0.70; FFE: 0.69 fecal score; $P = 0.09$), but fecal scores were not different for any specific week. Average nasal scores were 0.13 and were not altered by treatments ($P = 0.46$). Treatment with FF increased eye scores compared to FFE (0.32 vs. 0.26; $P = 0.03$), but eye scores of FF and FFE compared to CON were not different ($P = 0.81$). Treatments did not alter ear scores ($P = 0.94$) with mean scores of 0.16 for all calves. We estimated 92 percent chance of detecting a difference in health scores greater than 0.3 at $P < 0.05$. However, mean differences of health scores were lower than 0.3, indicating our chances of detecting differences among treatments were markedly reduced.

Body weight gain averaged 0.66 kg/d throughout the 8-wk pre-weaning period and was not altered by treatment ($P = 0.60$; Table 2). Gain in heart girth during the pre-weaning period was not different for CON vs. FF and FFE ($P = 0.12$) or FF vs. FFE ($P = 0.13$). Treatments did not alter ($P = 0.13$) gain in heart girth the first 3 wk of age. However, FFE tended ($P = 0.08$) to have more heart girth growth than FF. Treatments did not alter gain in wither heights during the first 3 wk ($P = 0.82$) or 8 wk of life ($P = 0.26$).

Serum total protein concentration at 2 d of age of the 54 calves out of 180 sampled were well above the threshold for successful passive transfer and were above the benchmark value of 52 g/L indicating successful passive transfer of immunoglobulins. Treatment did not alter OSi, AOP, or ROS (Table 3). Treatment did not alter the LPS-stimulation of TNF-α concentration (Table 3). Treatment did not alter expression of IL-8, TNF-α, COX-2, and IL-10) in control blood. Inoculation with LPS markedly increased ($P < 0.001$) expression of these genes, indicating that LPS stimulation was successful. In LPS-stimulated blood, expression of IL-8 was

not altered by treatment overall, but FF tended to decrease IL-8 expression compared to FFE (P = 0.07). Treatment did not alter expression of other genes in LPS-stimulated samples nor the LPS-induced changes in gene expression (Table 4).

DISCUSSION

Supplementing calves with a 60-mL blend of fish:flaxseed oil in colostrum with or without 200 mg α-tocopherol did not provide any benefits to calf immune function, health, or growth during the first 8 weeks of life on a commercial farm. Pre-weaned calves have an average mortality rate of 8 percent in the United States, excluding stillbirths (USDA, 2007). We conducted our study at a commercial farm where mortality was 1 percent in pre-weaned calves during the study. The average number of medications given to calves displaying signs of disease was 0.43 per calf. Only one third of research calves were given some form of medication from birth to 1 month after weaning. In our previous studies, (Opgenorth et al., 2019; Opgenorth et al., unpublished), average medications per calf were 1.2 and 1.4. In those studies, supplementation seemed promising, but in this study with exceptionally healthy calves, we saw no benefit to n-3 FA supplementation.

We also observed no change in LPS-stimulated expression of inflammatory genes, as has been previously observed with n-3 FA supplementation of calves (Garcia et al., 2016; Karcher et al., 2014). Perhaps one reason changes in gene expression occurred in those studies was that n-3 FA were fed for several weeks before sample analysis, whereas we gave a one-time supplement and collected leukocytes 48 h (+/- 8 h) later.

We previously showed that n-3 FA sometimes decreased oxidative stress in calves supplemented with 60-mL fish:flaxseed oil. Supplementation with 200 mg α-tocopherol

decreased OSi, and fish:flaxseed oil without additional 200 mg α-tocopherol decreased 8-iso-prostaglandin-$F_{2\alpha}$, a biomarker of oxidative stress (Opgenorth et al., 2019; Opgenorth et al., unpublished). We speculate additional 200 mg α-tocopherol may have protected tissues from RONS damage which decreased OSi. No difference in oxidative stress was reported in the current study, but sampling calves over the same period instead of only 48 h of age may have yielded more comprehensive results. Our prior research was conducted at a University farm where calves were bottle-fed colostrum instead of esophageal tube feeding. However, Desjardins-Morrissette et al. (2018) determined there is no difference in the abomasal emptying rates between bottle and tube-fed calves, and we do not speculate feeding method would alter n-3 FA absorption or effectiveness.

Previously, we found a fish:flaxseed oil supplement decreased the n-6 : n-3 FA ratio for at least 2 wk in plasma phospholipid FA (Opgenorth et al., Ch3). However, increased plasma free fatty acid and oxylipid concentrations with fish and flaxseed oil supplementation returned to control levels by d 7 of age in that study, indicating that beneficial effects might be short-term. We speculate short-term benefits may not be able to provide the benefits seen from continuous n-3 Fa supplementation. Long-term supplementation of n-3 FA provides greater benefits than short-term dietary inclusion, and n-3 FA effects are partially dependent on the duration consumed (Hooijmans et al., 2011; Palozza et al., 1996). Therefore, the calf may benefit more from n-3 FA supplementation in colostrum if followed by further supplementation in milk replacer.

When quality standards are achieved, colostrum alone may be substantial enough for calves. Artificial intervention additionally provided may not add further benefits. Colostrum supplements or replacers are only recommended when maternal colostrum is below caliber

(Cabral et al., 2013). Continuous nutritional supplementation seems to provide greater benefits than one-time supplementation to alleviate oxidative stress and further strengthen immunity.

CONCLUSION

In conclusion, supplementing colostrum with 60-mL of 1:1 blend of fish:flaxseed oil with or without 200 mg α-tocopherol did not enhance measures of immune function on day 2 or health or growth of calves before weaning on a commercial farm where calves were exceptionally healthy. Perhaps, this treatment might have been beneficial on a farm with health similar to industry averages. We speculate that supplementing a single dose of n-3 FA at birth does not provide the same benefit for immune function as previously reported with long-term supplementation.

APPENDIX

Table 4.1. Effects of treatments on health scores and medications

	Treatment[1]				*P*-values[2]		
Item[3]	CON	FF	FFE	SEM	CvT	TvT	*P*-Value
Fecal Scores	0.65	0.70	0.68	0.02	0.04	0.55	0.09
Eye Scores	0.30	0.32	0.26	0.02	0.81	0.03	0.10
Nasal Scores	0.12	0.14	0.14	0.01	0.23	0.68	0.45
Ear Scores	0.16	0.16	0.16	0.01	0.03	0.02	0.97
Medications	0.43	0.40	0.50	0.21	0.89	0.42	0.71

[1]Treatments: CON = control, no supplement added to colostrum; FF = 60 mL 1:1 ratio fish:flaxseed oil blend added to colostrum; FFE = 60 mL 1:1 ratio fish:flaxseed oil blend with 200 mg α-tocopherol added to colostrum
[2]*P*-values: CvT: *P*-value contrast of CON vs. FF and FFE; TvT: *P*-value contrast of FF vs. FFE; *P*: overall *P*-value of treatment effect
[3]Item: fecal, eye, nasal, and ear scores were determined on a scale of 0-3.

Table 4.2. Average daily gain in weight, heart girth, and wither height wk 0-3 and wk 0-8 during the pre-weaning period.

	Treatments[1]				
Item	CON	FF	FFE	SEM	*P*-value
Gain in body weight, (kg/d)					
Week 0-3	0.58	0.59	0.60	0.01	0.88
Week 0-8	0.66	0.65	0.67	0.01	0.60
Gain in heart girth, (cm/d)					
Week 0-3	0.32	0.28	0.32	0.01	0.13
Week 0-8	0.38	0.36	0.37	0.01	0.10
Gain in wither height, (cm/d)					
Week 0-3	0.16	0.15	0.15	0.02	0.82
Week 0-8	0.21	0.20	0.21	0.01	0.26

[1]Treatments: CON = control, no supplement added to colostrum; FF = 60 mL 1:1 ratio fish:flaxseed oil blend added to colostrum; FFE = 60 mL 1:1 ratio fish:flaxseed oil blend with 200 mg α-tocopherol added to colostrum

Table 4.3 Effects of treatments on total protein, tumor necrosis factor-α, and oxidant status index 48 h +/- 8 h after birth.

	Treatment[1]						
Item[2]	CON	FF	FFE	SEM	CvT	TvT	*P*-Value
STP, (g/L)	64	65	65	0.12	0.60	0.85	0.85
TNF-α, (Δ ng/mL)	0.12	0.12	0.10	0.02	0.94	0.27	0.73
OSi, (AU)	68	55	65	6.4	0.30	0.28	0.32

[1]Treatments: CON = control, no supplement added to colostrum; FF = 60 mL 1:1 ratio fish:flaxseed oil blend added to colostrum; FFE = 60 mL 1:1 ratio fish:flaxseed oil blend with 200 mg α-tocopherol added to colostrum

[2]Item: serum total protein (STP) in serum; tumor necrosis factor-α (TNF- α) increase from non-stimulated plasma to lipopolysaccharide-stimulated plasma after 3.5 h incubation; oxidant status index (OSi) in serum

Table 4.4 Effect of treatment means on inflammatory gene expression of leukocytes in non-stimulated and LPS-stimulated whole blood.

Target gene, (RQ)	Treatments[1]				*P*-values[2]		
	CON	FF	FFE	SEM	CvT	TvT	*P*
Δ[3]							
IL-8	62	47	71	5.2	-	-	<0.001
IL-10	11	19	12	1.1	-	-	<0.001
TNF-α	8.2	9.3	7.8	0.84	-	-	<0.001
COX-2	29	28	34	0.69	-	-	<0.001
No LPS[4]							
IL-8	3.0	2.1	2.2	1.2	0.15	0.85	0.35
IL-10	0.93	0.71	1.0	0.19	0.71	0.97	0.93
TNF-α	1.7	1.6	1.4	0.32	0.69	0.59	0.80
COX-2	1.8	1.5	1.7	0.39	0.77	0.72	0.90
LPS[5]							
IL-8	68	47	70	9.1	0.36	0.07	0.12
IL-10	13	14	14	1.7	0.56	0.96	0.84
TNF-α	9.8	11	9.0	1.6	0.92	0.39	0.68
COX-2	30	30	35	3.6	0.62	0.30	0.52

[1]Treatments: CON = control, no supplement added to colostrum; FF = 60 mL 1:1 ratio fish:flaxseed oil blend added to colostrum; FFE = 60 mL 1:1 ratio fish:flaxseed oil blend with 200 mg α-tocopherol added to colostrum

[2]*P*-values: CvT: *P*-value contrast of CON vs. FF and FFE; TvT: *P*-value contrast of FF vs. FFE; *P*: overall *P*-value of treatment effect

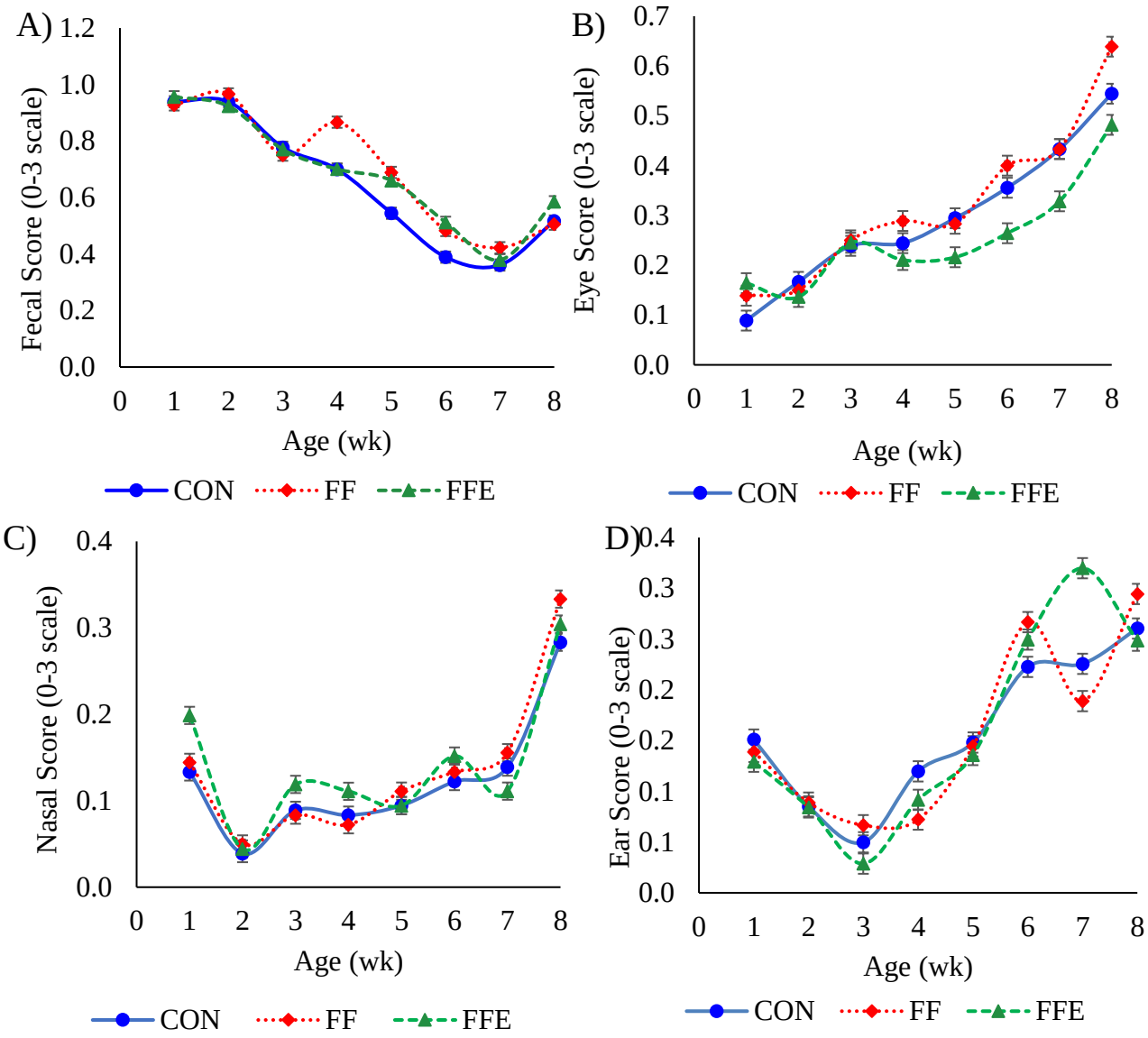
[3]Δ: average paired difference between non-stimulated and LPS-stimulated sample for each calf per treatment.

[4]No LPS: average gene expression with no stimulation of LPS

[5]LPS: average gene expression after LPS stimulation

Interleukin-8 (IL-8); interleukin-10 (IL-10); tumor necrosis factor-α (TNF-α); cyclooxygenase-2 (COX-2).

Figure 4.1. Effects of treatment on health scores.



Changes in average fecal (Panel A), eye (Panel B), nasal (Panel C), and ear (Panel B) scores during the first 8 weeks of life by CON, FF, and FFE. Data shown are represented as least squares means +/- standard errors predicted from the model. Overall treatment *P* = 0.09, 0.10, 0.45, and 0.94, respectively.

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CHAPTER 5.

CONCLUSIONS & FUTURE IMPLICATIONS

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In our studies, we found that a one-time supplement of n-3 FA in colostrum succeeded in decreasing the n-6 : n-3 FA ratio in plasma and indicators of oxidative stress in the first week of life for Holstein calves. Supplementation of 60 mL 1:1 fish:flaxseed oil blend with 200 mg α-tocopherol decreased oxidant status index. Supplementation of the same blend for 30, 60, and 120 mL 1:1 fish:flaxseed oil without α-tocopherol did not alter oxidant status index, but did decrease 8-iso-prostaglandin A2 during the first week of life. Thus, n-3 FA supplements with or without α-tocopherol decreased indicators of oxidative stress. Supplementing 60 or 120 mL was more effective at decreasing n-6:n-3 FA ratios and increasing n-3 FA and n-3 FA derived oxylipids in plasma. However, attenuating oxidative stress during the first week of life did not enhance pre-weaning health or growth. Furthermore, supplementing n-3 FA in colostrum on a well-managed commercial farm did not render any additional benefits to the calf and failed to alter the inflammatory gene expression in leukocytes of whole blood stimulated with LPS. Overall, although n-3 FA supplementation in colostrum may decrease oxidative stress during the first week after birth, there were no observed benefits to health or growth.

The rewards n-3 FA may provide to calf health may be limited to supplementation on farms that have greater health challenges than the commercial farm researched in this thesis. In

this case, proper colostrum management and administration should be a primary area to consider before experimenting with supplements.

Among our results, we observed that supplementing n-3 FA in colostrum is safe to feed calves. Colostrum supplementation may be a viable approach in future research to provide added nutrients to the calf. The physiological opportunity of the open gut may enhance our ability to manipulate the diet after birth.

There are clear benefits of n-3 FA supplementation such as their ability to decrease inflammatory and oxidative stress. Many of the molecular modifications observed were readily activated soon after supplementation, but future research should analyze these effects from continuous n-3 FA supplementation. Therapeutic nutritional strategies may require long-term supplementation to deliver meaningful benefits. Thus, there are two stages of early life to consider: indirect supplementation via dam to fetus during late gestation, or throughout the pre-weaning period. Maternal supplementation of n-3 FA during late gestation must consider several challenges 1) placental selectivity of FA, 2) rumen biohydrogenation, and 3) possible negative influences on the pro-inflammatory processes initiating healthy parturition. Thus, future research in this area should explore potential benefits of calf supplementation of n-3 FA in colostrum and milk replacer throughout the pre-weaning period.